IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

WILSON WOLF MANUFACTURING CORPORATION, Plaintiff, vs. SAREPTA THERAPEUTICS, INC., Defendant.	Case No.: 19-2316-RGA **JURY TRIAL DEMANDED**

FIRST AMENDED COMPLAINT FOR PATENT INFRINGEMENT

Plaintiff Wilson Wolf Manufacturing Corporation ("Wilson Wolf"), for its First Amended Complaint against Defendant Sarepta Therapeutics, Inc., ("Sarepta"), states and alleges as follows:

THE PARTIES

1. Plaintiff Wilson Wolf is a corporation organized under the laws of the State of Minnesota, with its principal place of business in New Brighton, Minnesota.

2. Upon information and belief, Defendant Sarepta is a corporation organized under the laws of the state of Delaware with its designated registered agent located at 251 Little Falls Drive, Wilmington, Delaware.

JURISDICTION AND VENUE

3. This Court has jurisdiction over the subject matter of this action pursuant to 28 U.S.C. §§ 1331 and 1338(a), as this is an action for patent infringement arising under the patent laws of the United States, 35 U.S.C. § 1 *et seq.*

4. This Court has personal jurisdiction over Sarepta because it is a Delaware corporation and essentially "at home" in this District. Further, Sarepta has caused tortious injury

to Wilson Wolf through its acts of patent infringement, and on information and belief, regularly does or solicits business, or engages in a persistent course of conduct in this District or derives substantial revenue from things used or consumed in this District.

5. Venue is proper in this District under 28 U.S.C. §§ 1391(b)(1) and 1400(b), because Sarepta is incorporated under the laws of Delaware and has its designated registered agent located in this District, and therefore "resides" in this District within the meaning of those statutes.

FACTUAL ALLEGATIONS

I. Wilson Wolf Develops Innovative Devices and Methods to Grow Cells

6. Wilson Wolf is a leader in the design of innovative devices and methods to grow cells in a laboratory environment.

7. The process of growing cells in a laboratory environment is called "culturing" cells. Innovative cell culture technology allows a lab to grow cells in greater volume, to grow cells faster, and to grow cells with lower risks of contamination.

8. Cell culture technology is critical to many fields, including biology and medicine. Cell culture technology is important, for example, when cells are grown for purposes of scientific investigation and research. Scientists grow cells to study how cancer develops and evolves. In contrast, doctors grow cells to diagnose cancer in a particular patient, and to select and calibrate treatment options for that patient. Cell culture technology is also used when cells are grown for commercial production of medications. For example, drug companies grow cells that produce monoclonal antibodies and other proteins that are used to treat diseases. These medications produced by cells are sometimes referred to as "biopharmaceuticals."

9. Cells in culture can also be used to replicate specially engineered "viral vectors" in large quantities. These viral vectors can be introduced into a patient to treat genetic disorders. This is known as "gene therapy."

10. Another rapidly-expanding field of cell culture technology involves the production of cells which can *themselves* be used to treat diseases. Some cells naturally occurring as part of the body's immune system are very good at fighting illnesses. For example, certain lymphocytes naturally infiltrate tumors and attack cancerous cells, while "natural killer" cells help the body fight viral infections. Unfortunately, the patient's body typically does not have enough of these cells to mount an effective immune system response to overcome the illness. Using cell culture techniques, a small quantity of these cells from the patient can be expanded into an "army" of cells that can be reintroduced to the patient to support recovery.

11. Wilson Wolf has developed devices and methods that have revolutionized the process of culturing cells.

12. In order to grow, cells need food and oxygen. To provide food, cells are typically grown in a liquid medium that contains nutrients for the cells. To provide oxygen, many devices rely on the oxygen in the gas residing above the liquid medium. Oxygen enters the liquid medium through the gas-liquid interface and is available to the cells.

13. Prior to Wilson Wolf's innovations, the conventional wisdom was that nutrients do not move very far in the liquid medium. As a result, cells only benefit from liquid medium very close to them; excess medium is wasted, and medium is very expensive. Based on that conventional wisdom, cells were typically being grown in flasks with a very thin (2-3 mm) layer of liquid medium; the vast majority of the flask contained no medium and no cells, wasting a significant amount of space. Also according to conventional wisdom, oxygen could only travel a short way into the liquid medium. If a flask contained more than a very thin layer of liquid medium, the medium would suffocate the cells.

14. The traditionally shallow depth of liquid medium led to inefficient use of space. For example, one manufacturer recommends a working volume of 0.2 mL to 0.3 mL per square centimeter of cell growth surface area in the cell culture flask. For a standard 225 cm^2 flask with 850 mL of total volume, the recommended working volume is 45 mL to 67.5 mL. With a recommended working volume of 45 mL to 67.5 mL, only a small fraction of the space that the flask occupies is being used to grow cells. The remaining space is just gas. This wasted space above the thin layer of liquid medium is often referred to as "head space."

15. The image below illustrates the traditional shallow depth of medium in a cell culture flask. The liquid medium is the thin red/orange layer in the bottom of the flask. The flask is mostly empty. The empty space above the thin layer of liquid medium is the headspace.

16. The traditional limits on the amount of liquid medium per flask meant that one had to culture cells in multiple flasks in order to obtain a given volume of culture. For example, to obtain a 1000 mL volume of culture, one would need to culture cells in 15 to 22 T-225 cm^2 flasks with a working volume of 45 mL to 67.5 mL each. The requirement that 15 to 22 devices be fed and monitored increases labor costs and contamination risks.

17. The inefficient use of space in a cell culture flask is compounded by the fact that cells are typically cultured in an incubator. The incubator provides a controlled temperature and gas environment. Incubator space is limited. And only so many flasks can fit within a given

volume of incubator space. Inefficient use of flask space therefore leads to inefficient use of incubator space. Based on conventional wisdom about medium thickness, decades of cell culture devices and methods made inefficient used of flask and incubator space. As a result, the process of culturing cells was slower, more cumbersome, and more prone to contamination than necessary.

18. Wilson Wolf challenged the conventional wisdom and developed devices and methods that grew more cells, in less space, with less labor and lower risk of contamination. Wilson Wolf challenged the conventional wisdom in at least two related ways. First, instead of having cells "breath" through a thin layer of liquid medium, Wilson Wolf had cells "breath" through a gas permeable membrane. With gas permeable material, instead of relying on the headspace within the device as a source of oxygen, cells can get oxygen from outside the device. This eliminated the need for headspace within the device. Second, Wilson Wolf found that nutrients and oxygen could move further in the medium than the conventional wisdom taught. This eliminated the design constraint imposed by the conventional wisdom that the liquid medium should be confined to a thin layer above the cells.

19. By using these insights, Wilson Wolf pioneered several new device designs and cell culture methods. In one design, a device with a single gas-permeable growth surface could support far more medium than taught by the conventional wisdom, allowing cell growth to proceed for a longer time before replenishing the medium. In another design, multiple growth surfaces could be stacked in a single device filled with medium, increasing the number of cells grown in a given volume of space. Other designs combined multiple growth surfaces with more medium than taught by the conventional wisdom. Wilson Wolf has been awarded several U.S. patents for its innovative cell culture devices and methods, including the patents in suit.

II. Wilson Wolf's Asserted Patents

20. Wilson Wolf owns U.S. Patent No. 9,441,192 ("the '192 Patent"), entitled "Cell culture methods and devices utilizing gas permeable materials," which issued on September 13, 2016. A copy of the '192 Patent is attached as Exhibit A.

21. Independent claim 1 of the '192 Patent is set forth below:

> 1. A method of culturing cells comprising:
>
> adding medium and animal cells into a static cell culture device that is not compartmentalized by a semi-permeable membrane, at least a portion of said cell culture device is comprised at least in part of a non porous gas permeable material, ambient gas is in contact with at least a portion of said gas permeable material, and
>
> placing said cell culture device in a cell culture location that includes ambient gas at a composition suitable for animal cell culture, wherein said cell culture device is oriented in a position such that at least a portion of said cells reside upon at least a portion of said gas permeable material, the uppermost location of said medium is elevated beyond 2.0 cm from the lowermost location of said medium, and said device is in a state of static cell culture.

22. Wilson Wolf owns U.S. Patent No. 8,697,443 ("the '443 Patent"), entitled "Cell culture methods and devices utilizing gas permeable materials," which issued April 15, 2014. A copy of the '443 Patent is attached hereto as Exhibit B.

23. Independent claim 26 of the '443 Patent is set forth below.

> 26. A method of culturing cells in a cell culture device comprised at least in part of a gas permeable material and including at least one access port and including at least two scaffolds, the method comprising:
>
> a) adding cells and a volume of liquid medium into said cell culture device;
>
> b) orienting said cell culture device into an inoculation position such that said scaffolds reside at different elevations within said cell culture device;
>
> c) allowing cells to settle upon said scaffolds;
>
> d) adding enough liquid medium to prevent a unique gas-liquid interface from forming directly above at least one scaffold when the device is oriented in the

inoculation position and to have at least a portion of the liquid medium in contact with at least a portion of said gas permeable material;

e) placing the cell culture device in a cell culture location that includes ambient gas at a composition suitable for cell culture, said ambient gas making contact with said gas permeable material; and

f) not perfusing said liquid medium when said device is in said cell culture location.

III. Sarepta Infringes Wilson Wolf's Patents

24. Sarepta has infringed the patents in suit through its use of cells and/or cell-derived products including viral vectors manufactured using the Corning HYPERStack cell culture device. Such cells and cell-derived products are products made by a process patented in the United States, within the meaning of 35 U.S.C. § 271(g). The HYPERStack is a multiple-shelf device that uses gas-permeable material to oxygenate cells. In use, the device is filled with liquid medium.

25. The processes and methods patented by the '192 and '443 Patents, as well as products, such as the HYPERStack, that enable the use of these patented processes and methods, are research tools that are used in laboratories and manufacturing facilities in the development of cells and cell-derived products.

26. The processes and methods patented by the '192 and '443 Patents are not subject to any regulatory approval process that applies to the cells and cell-derived products that are developed using them.

27. As research tools, the processes and methods patented by the '192 and '443 Patents and the products that enable the use of these patented processes and methods do not constitute "patented inventions" within the meaning of 35 U.S.C. § 271(e)(1).

28. A 2019 Sarepta presentation entitled "A New Era of Medicine is Upon Us," reflects that one or more Sarepta products have been manufactured using the HYPERStack. See Exhibit C. In an earnings call for the third quarter of 2019, Doug Ingraham, Sarepta's president and CEO

stated that Sarepta's SRP-9001 product was made using Corning HYPERStacks. See Exhibit D, at 14 (excerpts from call transcript).

29. On information and belief, although some batches of SRP-9001 were manufactured for use by Sarepta in connection with submissions to the FDA, other batches of SRP-9001 were not manufactured for use by Sarepta for FDA purposes, and were instead used for other business purposes.

30. While some of Sarepta's infringement was strictly to generate infringement for the FDA, some of its infringement was for both FDA filings and other non-FDA purposes, and some of their infringement was solely for non-FDA purposes. For example, Sarepta had some batches of such products manufactured using Wilson Wolf's patented processes and methods for use to develop, improve, and optimize its manufacturing process for commercialization purposes. Sarepta also had some batches of such products manufactured using Wilson Wolf's patented process and methods for use in manufacturing capacity development and yield optimization for purposes of commercialization of the SRP-9001 product.

31. Even while conducting its clinical trials of SRP-9001, Sarepta moved forward in anticipation of commercialization of that product. For example, in 2018 Sarepta entered into a "manufacturing partnership" with Brammer Bio to build manufacturing capacity for the SRP-9001 product. *See* Exhibit F (Sarepta Press Release). The arrangement with Brammer Bio was designed to "integrate process development, clinical production and testing, and commercial manufacturing with the goal of bringing micro-dystrophin gene therapies to the patient community urgently and in sufficient supply." *Id.*

32. In 2019, Sarepta entered into a license agreement with Roche for commercialization of the SRP-9001 product outside of the U.S. that has been described as the single biggest such

license in biopharma history. Roche agreed to pay more than $1.1 billion up front for the commercial rights to SRP-9001 outside of the United States. The manufacture of Sarepta's SRP-9001 product using Wilson Wolf's patented processes and methods supported that commercialization agreement.

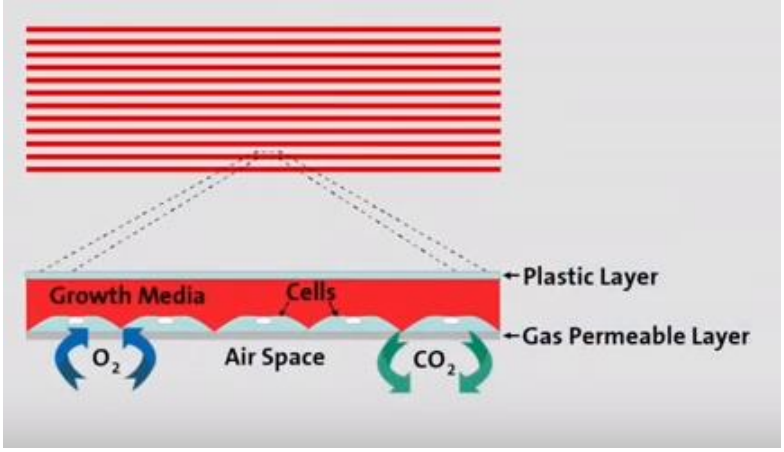
33. The manufacture of some of Sarepta's SRP-9001 product using Wilson Wolf's patented processes and methods was done to assist in commercialization of the product, and was not done to create information for FDA submissions. Sarepta itself stated that it developed its program to "expedite development and commercialization" of its gene therapy products, including SRP-9001. *See* Exhibit G at 9.

34. Because the manufacture of some of Sarepta's SRP-9001 product using Wilson Wolf's patented processes and methods was done to advance and support commercialization of the product, and was not done to create information for FDA submissions, Sarepta's use of that product falls outside of the Safe Harbor of 35 U.S.C. § 271(e)(1). Moreover, even if all of Sarepta's usage were strictly to provide information to the FDA, Wilson Wolf's intellectual property relates to research tools, and research tools are not included in the Safe Harbor.

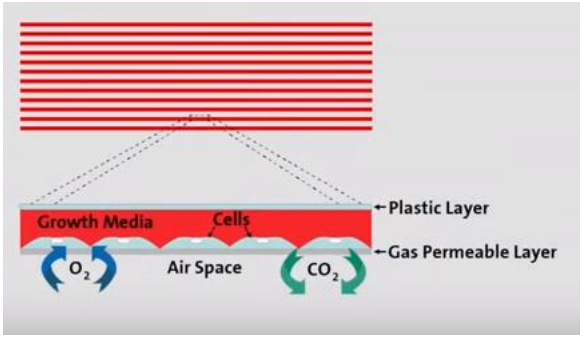
35. Sarepta has infringed at least claim 1 of the '192 Patent through its use of cells and/or cell-derived products including viral vectors manufactured using the HYPERStack, as set forth in the table below. The left side of the table contains the language of claim 1 of the '192 Patent. The right side of the table contains information on the HYPERStack and its use, including quoted text from an article entitled "Closed System Cell Culture Protocol Using HYPERStack Vessels with Gas Permeable Material Technology," authored by six Corning staff members, attached as Exhibit E, and images from Corning video entitled "Filling and Emptying the Corning®

HYPERStack® Cell Culture Vessel," posted on YouTube at https://www.youtube.com/watch?v=6CPcW_qWu_w.

A method of culturing cells comprising:	The HYPERStack is used to culture cells. "The HYPERStack Vessel is a multilayered vessel for . . . culturing of cells. . . ." Exhibit E (Protocol ¶ 1(1)) .
adding medium and animal cells into a static cell culture device	In use, medium and animal cells are added to the HYPERStack. The HYPERStack is a static cell culture device. "Inoculating Media": "Inject the Cell Suspension into the Media Bag and Mix well." "Using the bag stand, raise the media bag to help the cell suspension flow into the vessel." Exhibit E (Protocol ¶¶ 5(2) 6(5)).
that is not compartmentalized by a semi-permeable membrane,	The HYPERStack does not have a semi-permeable membrane.
at least a portion of said cell culture device is comprised at least in part of a nonporous gas permeable material,	"The HYPERStack vessels function via gas permeable material which allows gas exchange to occur. . . ." Exhibit E (Abstract ¶ 1).
ambient gas is in contact with at least a portion of said gas permeable material, and	"Rather than containing this 'headspace' for gas exchange within the vessel, the gas permeable products have air spaces . . . beneath each culture chamber which is open to the atmosphere." Exhibit E (Protocol ¶ 1(2)).
placing said cell culture device in a cell culture location that includes ambient gas at a composition suitable for animal cell culture,	"Move the HYPERStack vessel to the incubator." Exhibit E (Protocol ¶ 7(6)). Incubators used in cell culture contain ambient gas at a composition suitable for cell culture.

wherein said cell culture device is oriented in a position such that at least a portion of said cells reside upon at least a portion of said gas permeable material,	The HYPERStack is placed in the incubator such that at least some of the cells reside on the gas permeable material.  
the uppermost location of said medium is elevated beyond 2.0 cm from the lowermost location of said medium,	The uppermost location of medium is elevated more than 2.0 cm from the lowermost location of said medium, as can be seen in the picture above, from which the dimensions of the device filled with medium can be appreciated.
and said device is in a state of static cell culture.	The HYPERStack is cultured in a static state.

36. Sarepta has infringed at least claim 1 of the '443 Patent through its use of cells and/or cell-derived products including viral vectors manufactured using the HYPERStack, as set forth in the table below. The left side of the table contains the language of claim 1 of the '443 Patent. The right side of the table contains information on the HYPERStack and its use, including information authored by Corning staff, attached as Exhibit E.

A method of culturing cells in a cell culture device comprised at least in part of a gas permeable material	The HYPERStack is a cell culture device comprised at least in part of gas permeable material. See Exhibit E (Protocol ¶ 2(1)) ("The Stackette is the individual cell culture compartment that is made up of the top plate and gas permeable film.").
and including at least one access port and including at least two scaffolds, the method comprising:	The HYPERStack has at least one access port. See Exhibit E (Protocol ¶ 2(5)) ("The Liquid handling tube is connected to the liquid manifold and is used to make all closed system fluid manipulations."). The HYPERStack has at least two scaffolds. See Exhibit E (Protocol ¶¶ 2(1), 2(2)) ("The Stackette is the individual cell culture compartment that is made up of the top plate and gas permeable film. The cells are cultured within this compartment.") ("The Liquid Manifold connects each of the 12 stackette layers together within a HYPERStack module.").
a) adding cells and a volume of liquid medium into said cell culture device;	Cells and media are added into the HYPERStack. See Exhibit E (Protocol ¶ 6(6)) ("Using the bag stand, raise the media bag to help the cell suspension flow into the vessel.").
b) orienting said cell culture device into an inoculation position such that said scaffolds reside at different elevations within said cell culture device;	The device is oriented into a position such that the scaffolds reside one above the other at different elevations in the device as shown below. 
c) allowing cells to settle upon said scaffolds;	Cells settle upon the scaffolds, as shown in the diagram above.

d) adding enough liquid medium to prevent a unique gas-liquid interface from forming directly above at least one scaffold when the device is oriented in the inoculation position and to have at least a portion of the liquid medium in contact with at least a portion of said gas permeable material;	The user adds enough liquid medium to the HYPERStack to prevent a unique gas-liquid interface from forming above at least one scaffold when the device is in the inoculation position. See Exhibit E (Abstract ¶ 1) ("The HYPERStack vessels function via gas permeable material which allows gas exchange to occur, therefore eliminating the need for internal headspace within a vessel. The elimination of headspace allows the compartment where cell growth occurs to be minimized to reduce space, allowing more layers of cell growth surface area with the same volumetric footprint.") This can also be seen in the image below. 
e) placing the cell culture device in a cell culture location that includes ambient gas at a composition suitable for cell culture, said ambient gas making contact with said gas permeable material; and	The HYPERStack is placed in an incubator as shown in the image above. Incubators contain ambient gas at a composition suitable for cell culture. The HYPERStack has "air spaces . . . beneath each culture chamber which is open to the atmosphere." See Exhibit E (Protocol ¶ 1(2)).
f) not perfusing said liquid medium when said device is in said cell culture location.	The liquid medium in the HYPERStack is not perfused when the device is in the incubator.

COUNT I

INFRINGEMENT OF THE '192 PATENT AND THE '443 PATENT

37. Wilson Wolf incorporates by reference the above paragraphs as if stated herein.

38. The '192 Patent and the '443 Patent (collectively "the Patents-in-Suit") are valid and enforceable.

39. Sarepta has directly infringed at least one claim of the '192 Patent, including, without limitation, Claim 1 of the '192 Patent to the harm and detriment of Wilson Wolf, and to the benefit and profit of Sarepta.

40. Sarepta has directly infringed at least one claim of the '443 Patent, including, without limitation, Claim 1 of the '443 Patent to the harm and detriment of Wilson Wolf, and to the benefit and profit of Sarepta.

41. Sarepta's acts of direct infringement include, but are not limited to, its use in the United States of cells and/or cell-derived products including viral vectors manufactured according to Wilson Wolf's patented methods using the HYPERStack cell culture vessel.

42. Sarepta's use of cells and/or cell-derived products including viral vectors manufactured according to Wilson Wolf's patented methods using the HYPERStack cell culture vessel falls outside of the Safe Harbor of 35 U.S.C. § 271(e)(1).

43. Sarepta's infringement is irreparably harming Wilson Wolf.

44. Wilson Wolf is entitled to money damages in an amount to be determined at trial, and to preliminary and permanent injunctive relief.

PRAYER FOR RELIEF

WHEREFORE, Wilson Wolf prays for relief as follows:

1. A judgment that Sarepta has infringed the '192 Patent and the '443 Patent;

2. A judgment awarding Wilson Wolf damages in an amount to be determined at trial, but not less than a reasonable royalty;

3. An order enjoining Sarepta preliminarily, and permanently thereafter, from infringing, inducing infringement, and from contributing to the infringement of the '192 Patent and the '443 Patent;

4. A judgment awarding Wilson Wolf its costs incurred herein, including attorneys' fees for an exceptional case pursuant to 35 U.S.C. § 285; and

5. A judgment awarding Wilson Wolf such other and further relief as the Court may deem just and equitable.

JURY DEMAND

Pursuant to Rule 38 of the Federal Rules of Civil Procedure, Wilson Wolf hereby demands a jury trial as to all issues so triable.

Dated: April 22, 2020

Respectfully submitted,

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EXHIBIT A

US009441192B2

(12) **United States Patent**
Wilson et al.

(10) **Patent No.: US 9,441,192 B2**
(45) **Date of Patent: *Sep. 13, 2016**

(54) **CELL CULTURE METHODS AND DEVICES UTILIZING GAS PERMEABLE MATERIALS**

(71) Applicant: **Wilson Wolf Manufacturing**, New Brighton, MN (US)

(72) Inventors: **John R. Wilson**, New Brighton, MN (US); **Douglas A. Page**, Eden Prairie, MN (US); **Daniel Welch**, Zimmerman, MN (US); **Alison Robeck**, Monticello, MN (US)

(73) Assignee: **Wilson Wolf Manufacturing**, New Brighton, MN (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **14/810,071**

(22) Filed: **Jul. 27, 2015**

(65) **Prior Publication Data**

US 2016/0024465 A1 Jan. 28, 2016

Related U.S. Application Data

(62) Division of application No. 10/961,814, filed on Oct. 8, 2004.

(60) Provisional application No. 60/509,651, filed on Oct. 8, 2003.

(51) **Int. Cl.**

C12M 3/00	(2006.01)
C12M 1/24	(2006.01)
C12N 5/00	(2006.01)

(Continued)

(52) **U.S. Cl.**

CPC ***C12M 23/08*** (2013.01); ***C12M 23/24*** (2013.01); ***C12M 25/06*** (2013.01); ***C12M 25/14*** (2013.01); ***C12N 5/00*** (2013.01); ***C12N 5/0602*** (2013.01); *C12N 2500/02* (2013.01)

(58) **Field of Classification Search**

CPC C12M 23/06; C12M 23/08; C12M 23/24; C12M 27/12; C12M 25/06; C12M 25/14; C12N 5/00; C12N 5/0602; C12N 2500/02

See application file for complete search history.

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Application and File History for U.S. Appl. No. 14/809,484, filed Jul. 27, 2015, inventors Wilson et al.

(Continued)

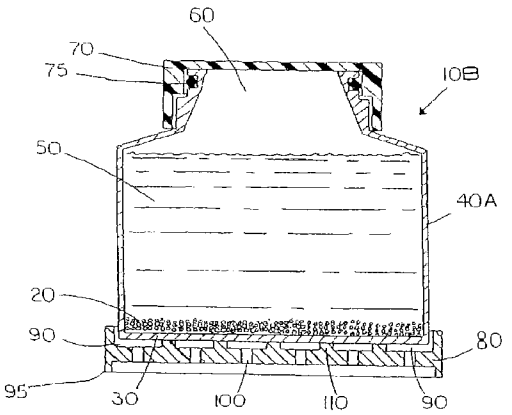
Primary Examiner — William H Beisner

(74) *Attorney, Agent, or Firm* — Patterson Thuente Pedersen, P.A.

(57) **ABSTRACT**

Gas permeable devices and methods are disclosed for cell culture, including cell culture devices and methods that contain medium at heights, and certain gas permeable surface area to medium volume ratios. These devices and methods allow improvements in cell culture efficiency and scale up efficiency.

30 Claims, 16 Drawing Sheets



(51) **Int. Cl.**

C12M 1/12	(2006.01)
C12M 1/04	(2006.01)
C12N 5/071	(2010.01)

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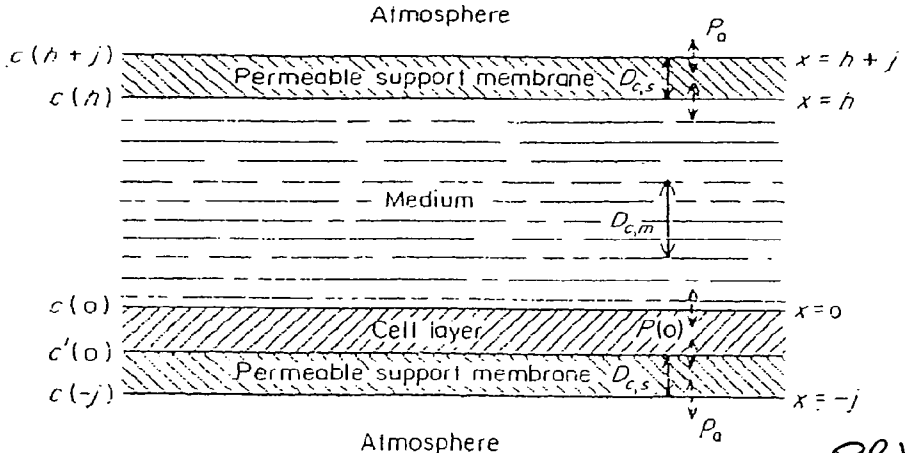
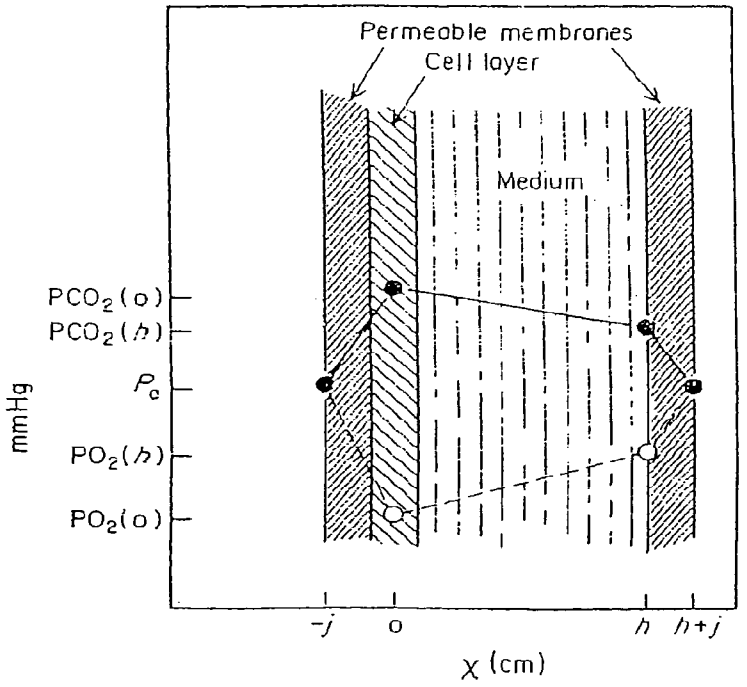
Application and File History for U.S. Appl. No. 12/499,633, filed Jul. 8, 2009, inventor Wilson.

FIG. 1A



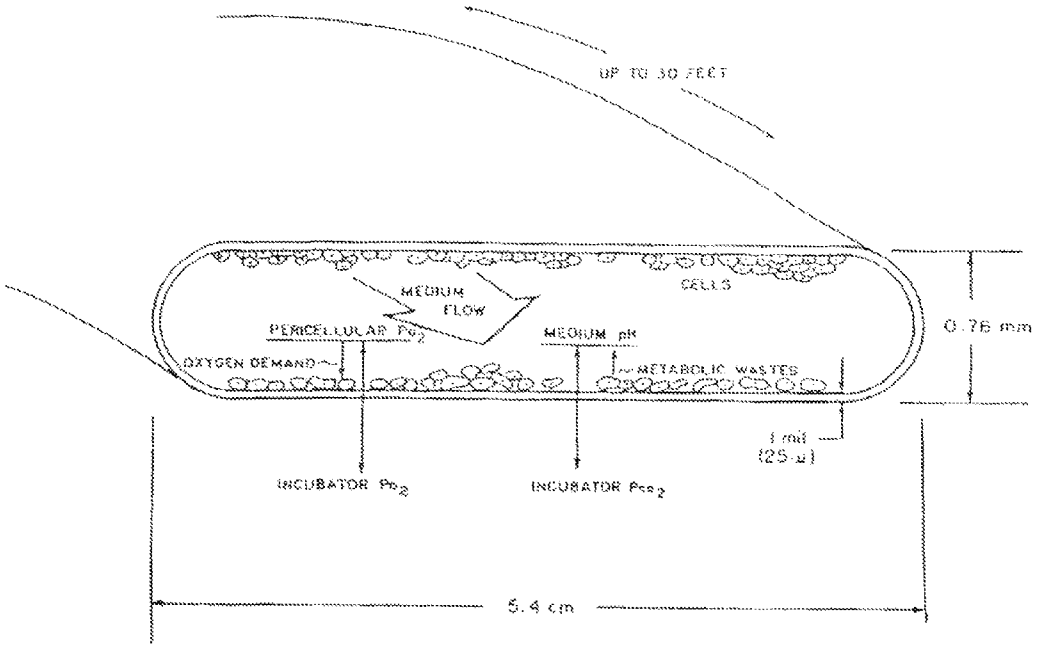
PRIOR ART

FIG. 1B



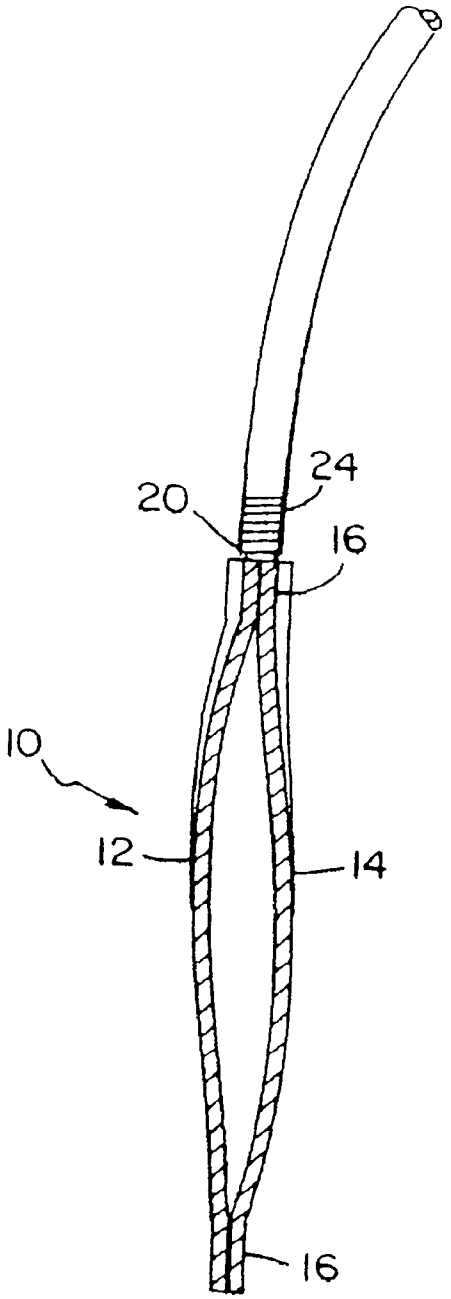
PRIOR ART

FIG. 2



PRIOR ART

FIG. 3



PRIOR ART

FIG. 4A

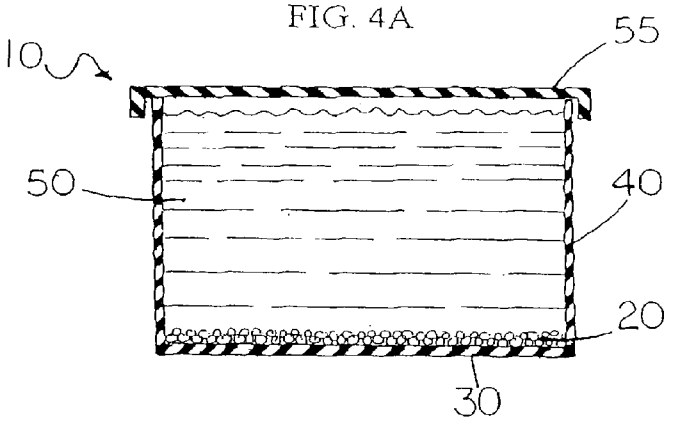


FIG. 4B

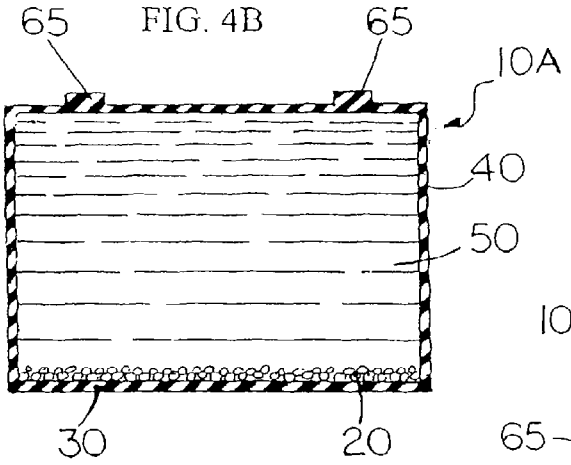


FIG.4C

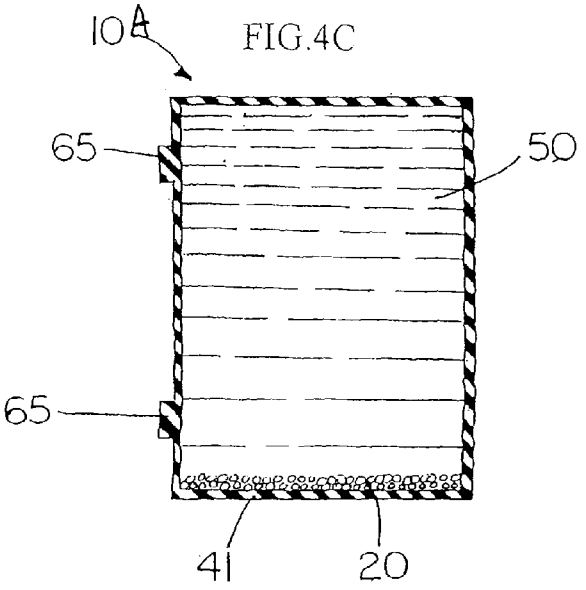


FIG. 5

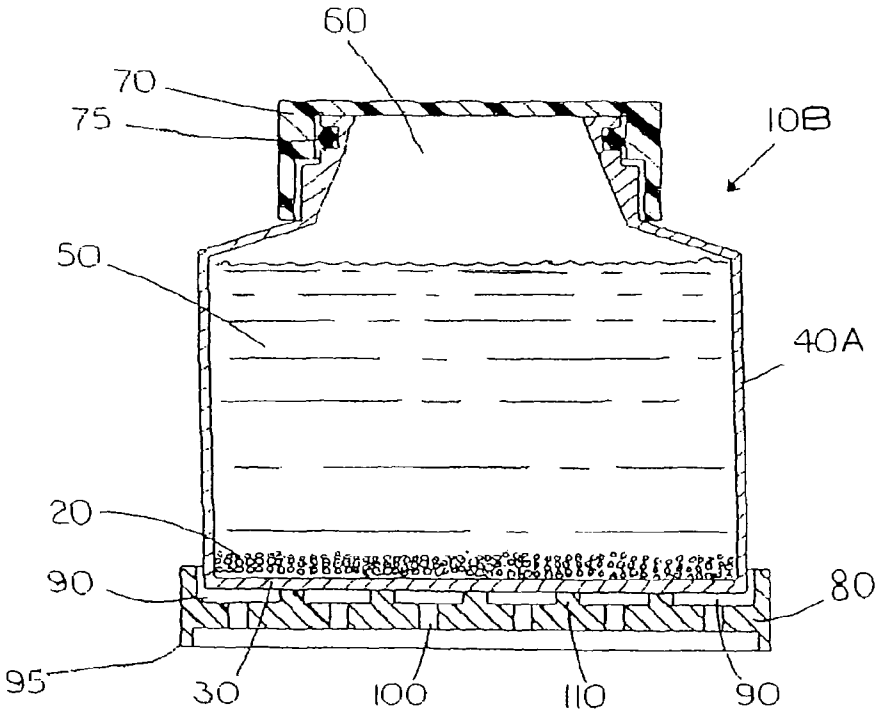

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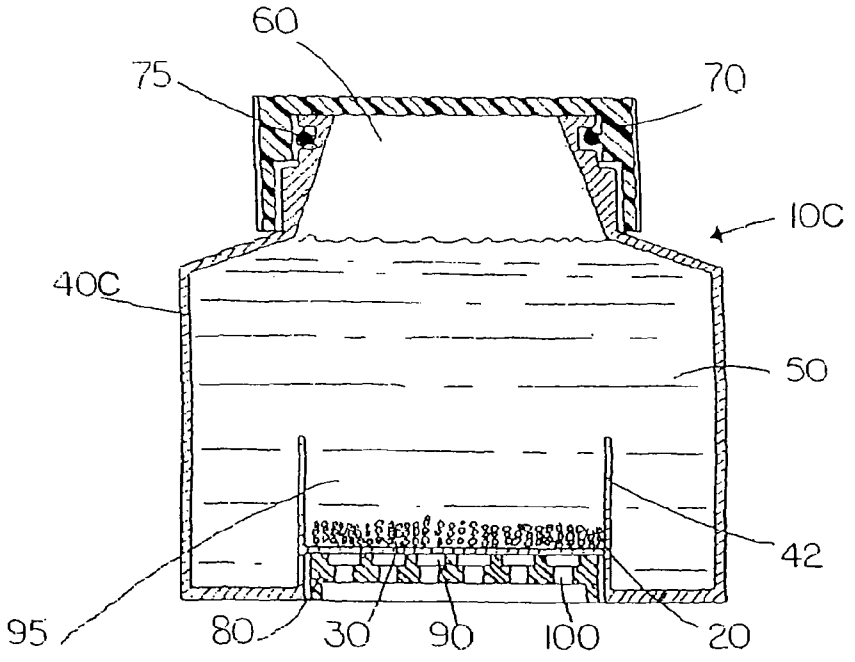
FIG. 6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95
80
30
90
100
20

FIG. 7A

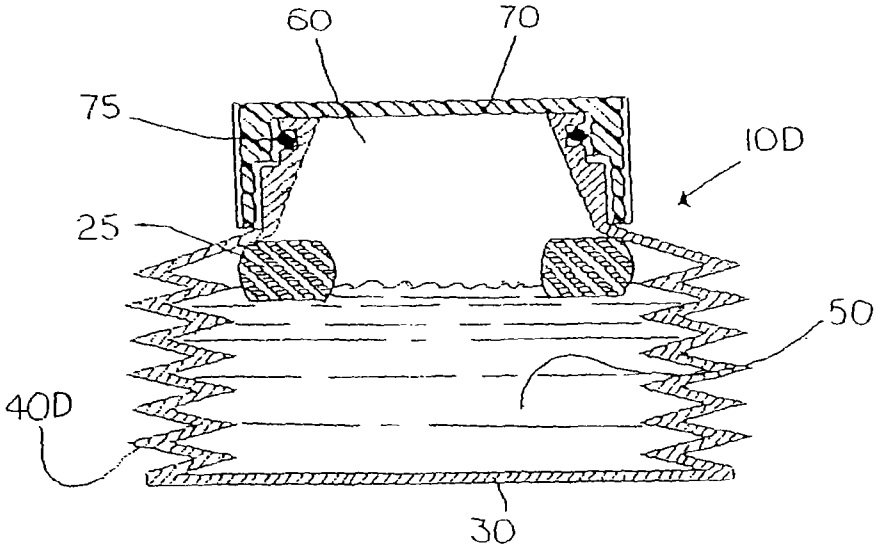

FIG. 7B

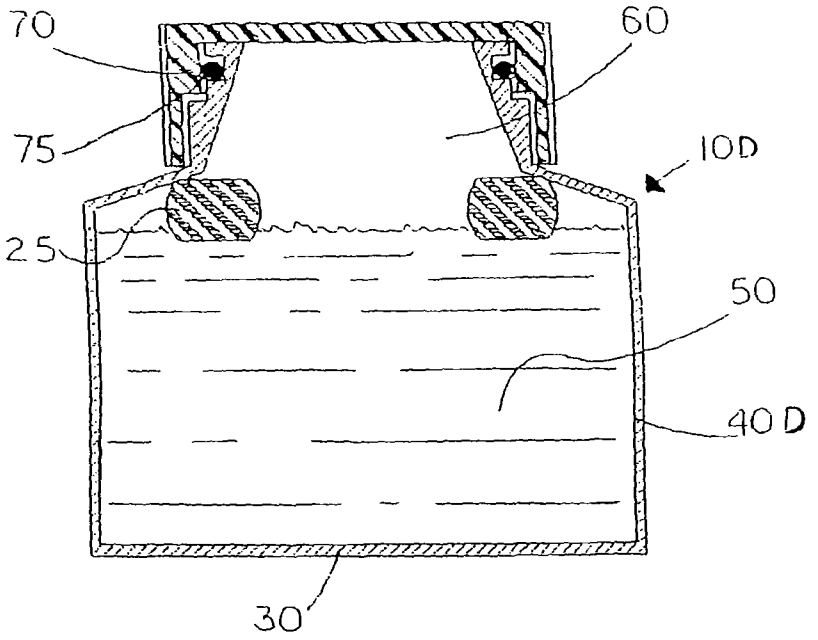

FIG. 8

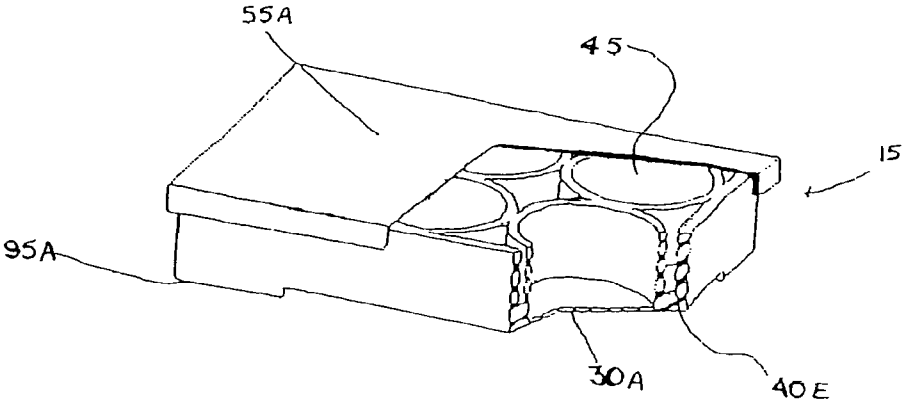

FIG. 9A

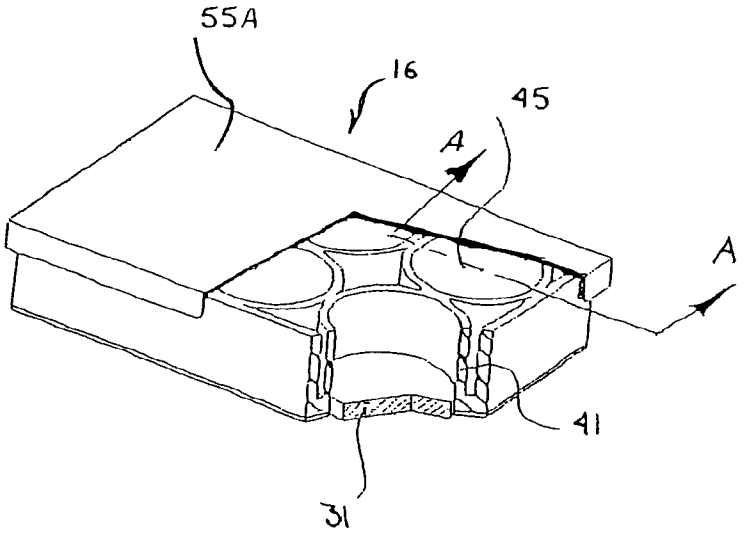

FIG. 9B

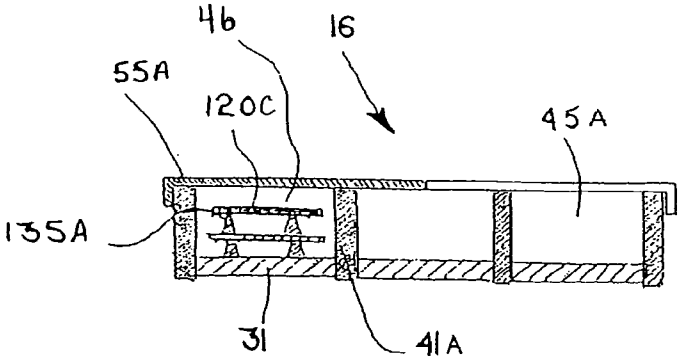

FIG. 10A

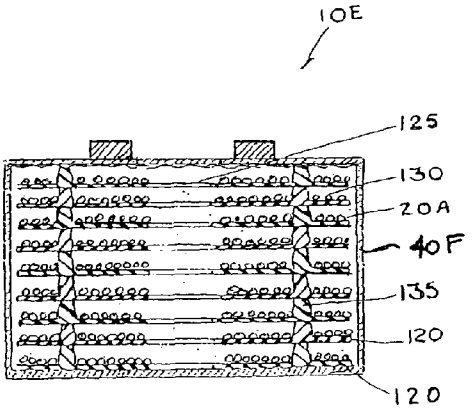

FIG. 10B

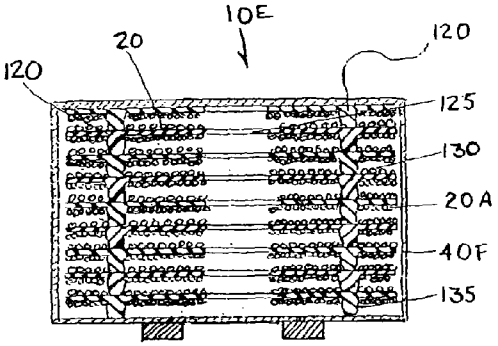

FIG. 11

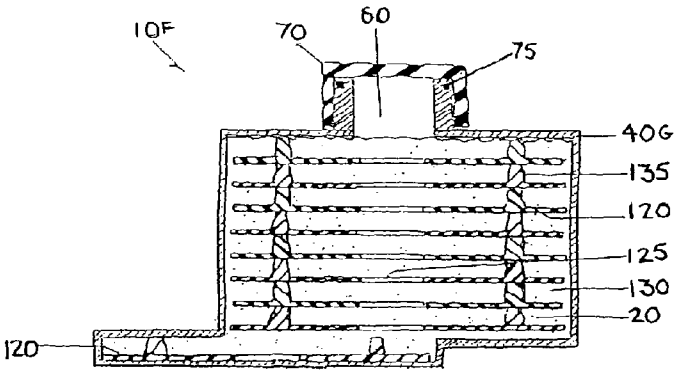

FIG. 12A

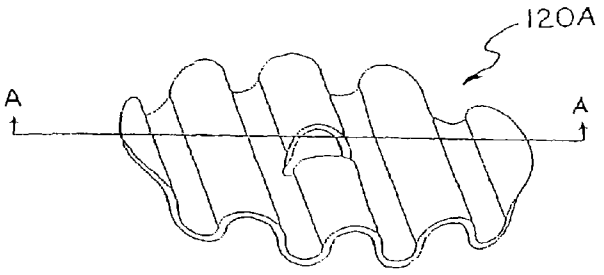

FIG. 12B

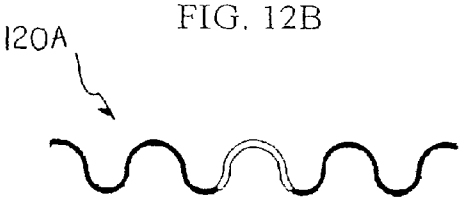

FIG. 12C

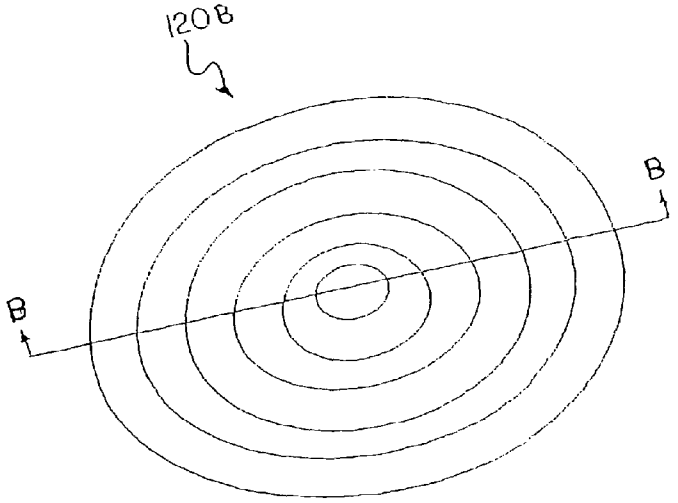

FIG. 12D

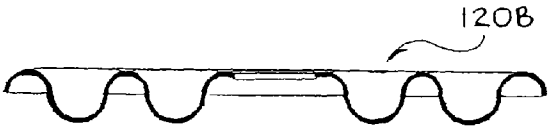

FIG. 13

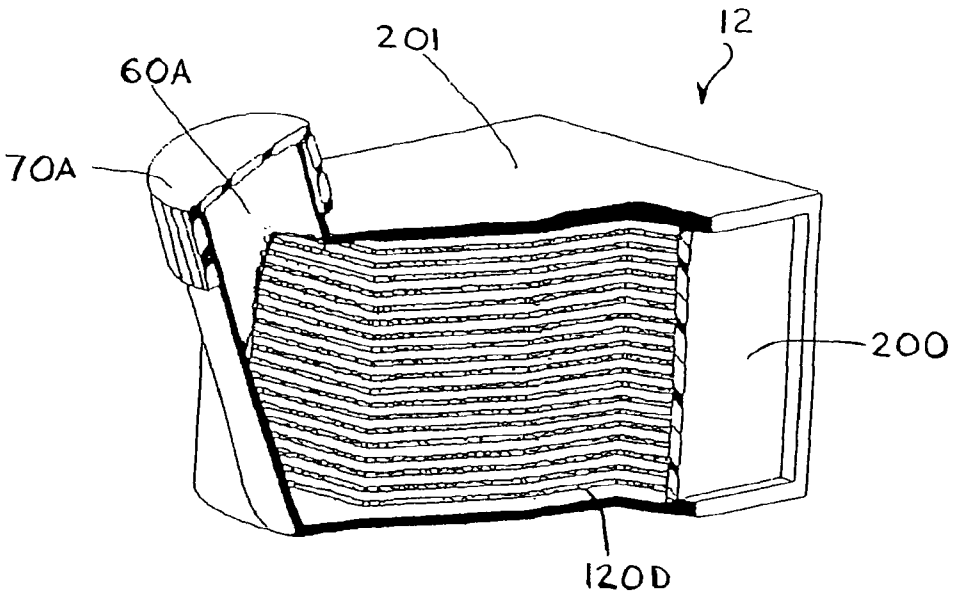

FIG. 14A

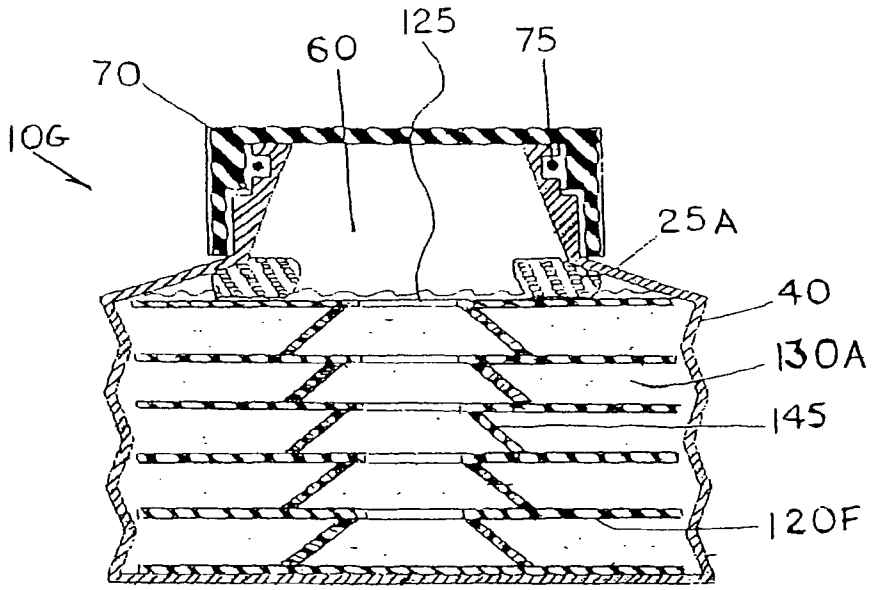

FIG. 14B

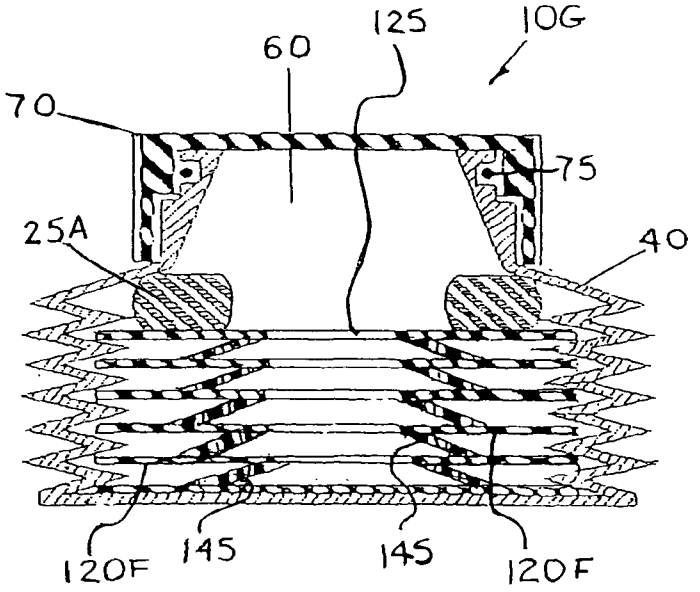

FIG. 14C

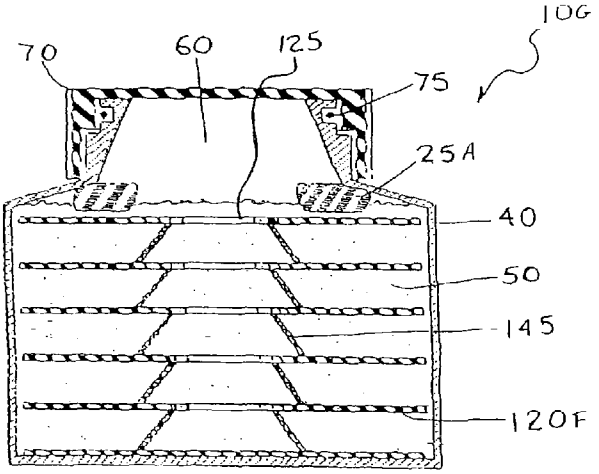

FIG. 14D

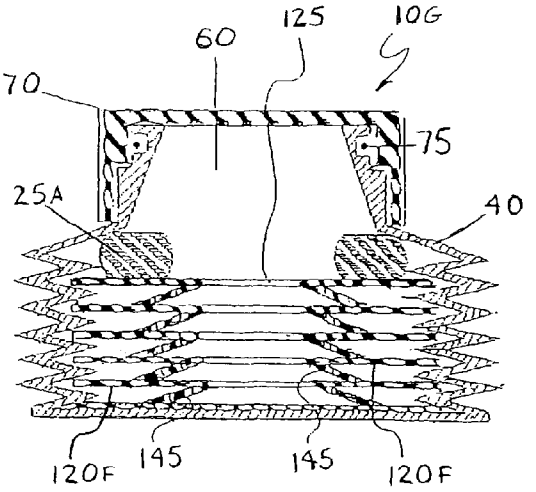

FIG. 14E

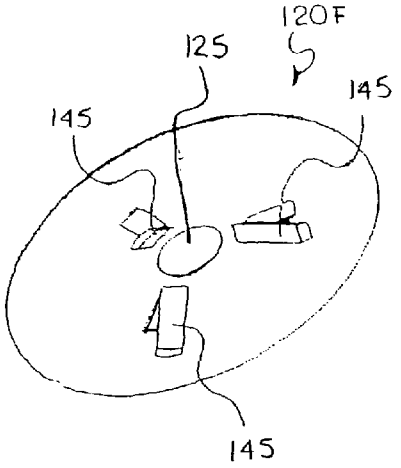

FIG. 15A

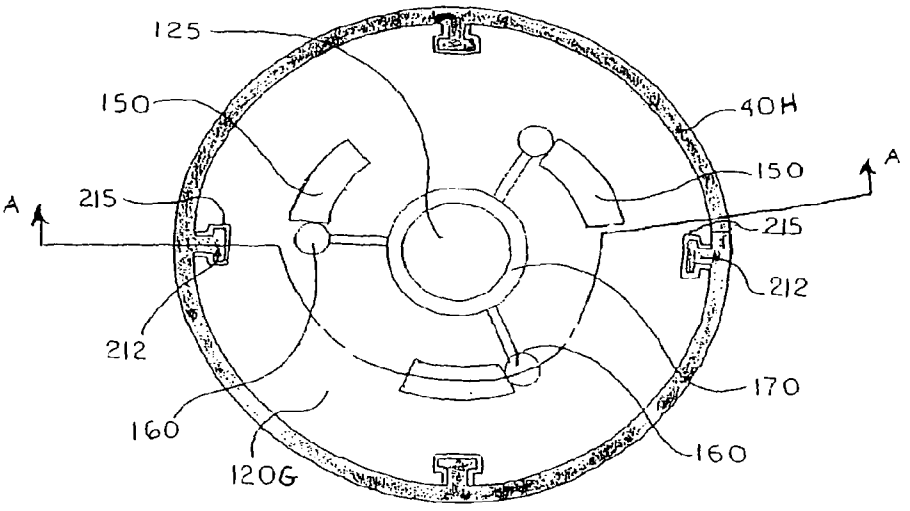

FIG. 15B

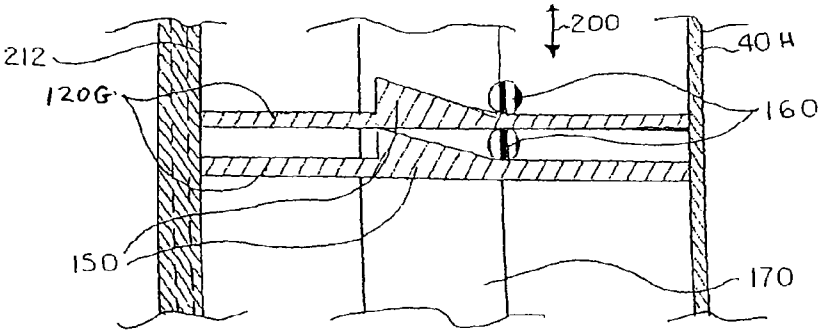

FIG. 15C

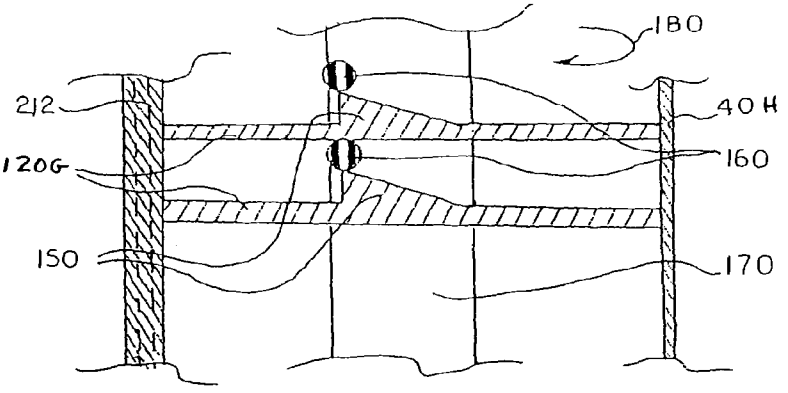

FIG. 16

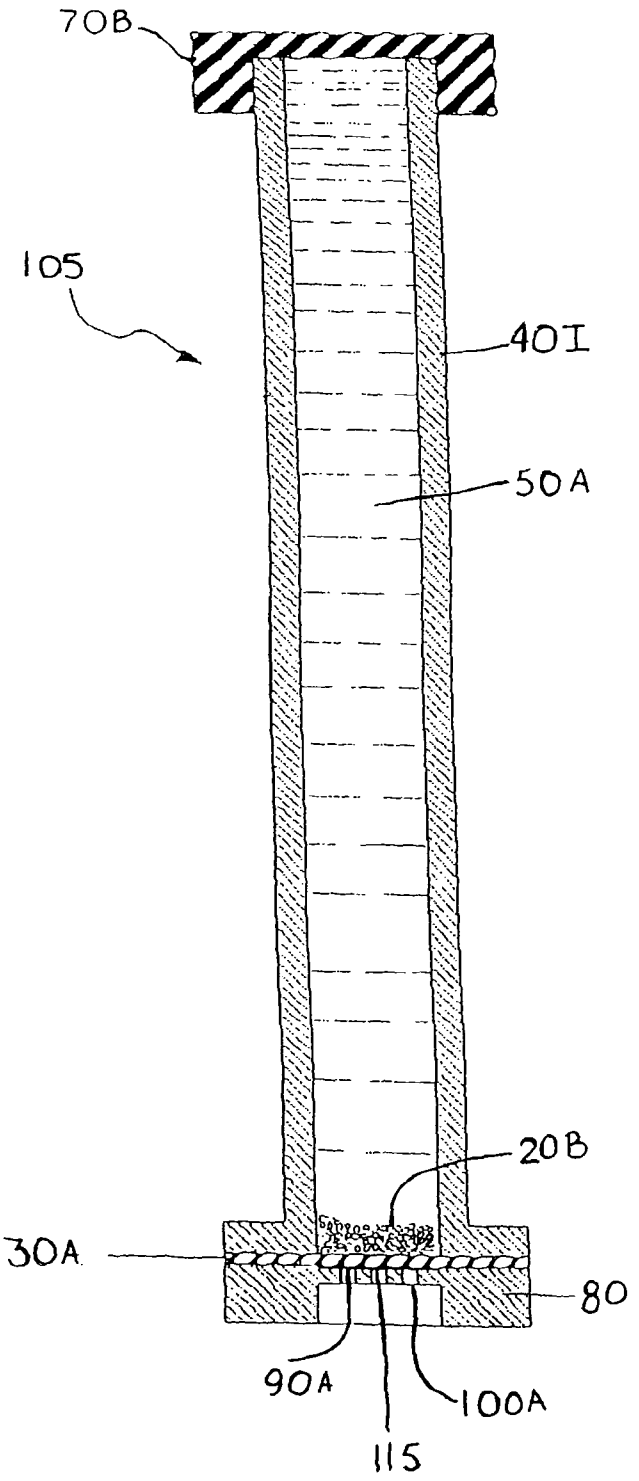

FIG. 17

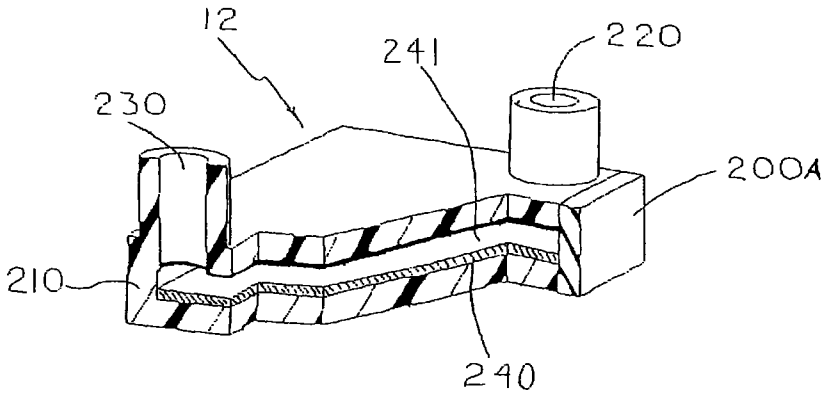
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FIG. 18

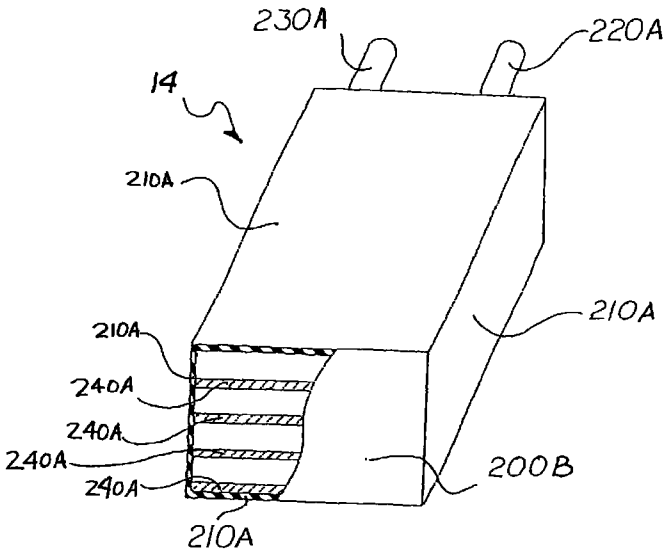
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FIG. 19A

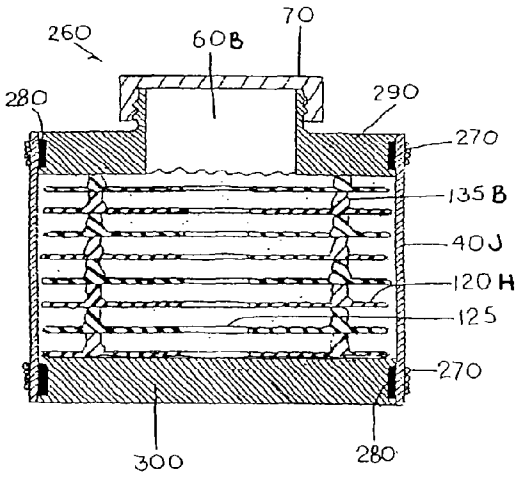


FIG. 19B

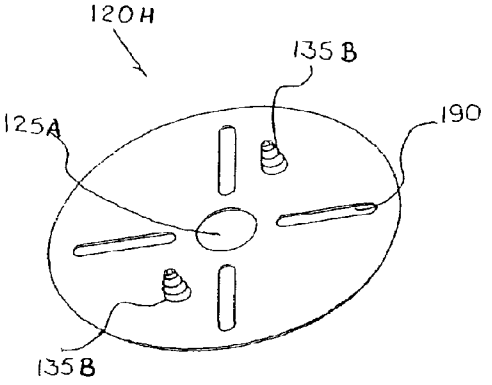
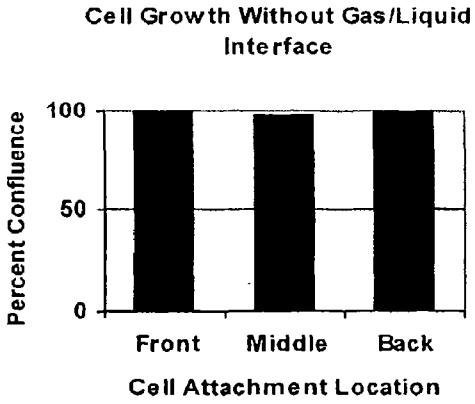


FIG. 20



CELL CULTURE METHODS AND DEVICES UTILIZING GAS PERMEABLE MATERIALS

RELATED APPLICATION

The present application is a divisional of U.S. patent application Ser. No. 10/961,814, filed Oct. 8, 2004 which claims priority to U.S. Provisional Application No. 60/509,651 filed Oct. 8, 2003, both of which are hereby incorporated herein in their entirety by reference.

TECHNICAL FIELD

The technical field of the invention relates to methods and devices that improve cell culture efficiency. They utilize gas permeable materials for gas exchange, allow an increased height of cell culture medium, reduce the ratio of gas permeable device surface area to medium volume capacity, and integrate traditional cell support scaffolds. A variety of benefits accrue, including more efficient use of inventory space, incubator space, disposal space, and labor, as well as reduced contamination risk.

DISCUSSION OF LIMITATIONS OF CONVENTIONAL TECHNOLOGIES DESCRIBED IN RELATED ART

The culture of cells is a critical element of biotechnology. Cells are cultured in small quantities during the research stage, and typically the magnitude of the culture increases as the research moves towards its objective of benefiting human and animal health care. This increase in magnitude is often referred to as scale up. Certain devices and methods have become well established for research stage cell culture because they allow a wide variety of cell types to be cultured, and are therefore useful to the widest audience. These devices include multiple well tissue culture plates, tissue culture flasks, roller bottles, and cell culture bags. Unfortunately, these devices are inefficient and they become even less efficient in terms of labor, contamination risk, and cost during scale up. There is a need to create alternative devices and methods that research and retain scale up improve research and scale up efficiency. This discussion identifies many of the limitations in conventional technologies and points towards solutions that are subsequently described in more detail.

One attribute that is essential for research scale cell culture is a low level of complexity. Devices that minimize complexity do not require ancillary equipment to mix or perfuse the cell culture medium. They are often referred to as static devices. Static devices can be subdivided into two broad categories, 1) those that are not gas permeable and oxygenate the cells by way of a gas/liquid interface and 2) those that are gas permeable and oxygenate the cells by way of gas transfer through the device housing. The traditional petri dish, multiple well tissue culture plate, tissue culture flask, and multiple shelf tissue culture flask are in the first category. The cell culture bag and compartmentalized flasks are in the second category. All of these static devices are inefficient for a variety of reasons, including the limited height at which medium can reside in them.

Medium height is limited in the petri dish, multiple well tissue culture plate, tissue culture flask, and multiple shelf tissue culture flask due to the method of providing gas exchange. To meet cellular demand, oxygen must diffuse from a gas/liquid interface to the lower surface of the device where cells reside. To ensure adequate oxygen supply, the maximum height of cell culture medium recommended for use in these devices is about 3 mm.

Limited culture medium height leads to disadvantages. It creates a small medium volume, which can only support a small quantity of cells. Medium needs to be continually removed and added to sustain cultures, which increases handling frequency, labor, and contamination risk. The only way to culture more cells in a device is to make the footprint of the device larger so that more medium can be present. Creating a device with large footprint is challenging from a manufacturing standpoint, quickly outgrows the limited amount of space available in a typical incubator and flow hood, and makes the device more difficult to handle. Thus, commercially available cell culture devices are small. Scaling up the culture therefore requires using multiple devices or selecting more sophisticated, complex, and costly alternatives.

The tissue culture flask provides a good example of the problems inherent to static devices that rely upon a gas/liquid interface to function. Tissue culture flasks allow cells to reside upon surfaces typically ranging from 25 cm^2 to 225 cm^2 in area. The height of medium that is recommended for tissue culture flasks is between 2 mm and 3 mm. For example, Corning® recommends a 45 ml-67.5 ml working volume for its T-225 cm^2 flask. Thus, a 1000 ml culture requires between 15 and 22 T-225 cm^2 flasks. Not only does this require 15 to 22 devices to be fed, leading to increasing labor and contamination risk, it also makes very inefficient use of space because flasks are designed in a manner that holds about 95% gas and only 5% medium. For example, the body of a typical T-175 flask has a footprint approximately 23 cm long by 11 cm wide, is about 3.7 cm tall, and therefore occupies about 936 cm^3 of space. However, it typically operates with no more than about 50 ml of medium. Thus, the medium present in the body (50 ml), relative to the space occupied by the body (936 cm^3) demonstrates that nearly 95% of the flask's content is merely gas. This inefficient use of space adds shipping, sterilization, storage, and disposal cost, in addition to wasting precious incubator space.

Another commonly used research scale cell culture device is the multiple well tissue culture plate. As with the traditional tissue culture flask, maintaining a gas/liquid interface at a height of only 2 mm to 3 mm above the bottom of each well is standard operating procedure. In order to provide protection against spillage when the plates are moved around the cell culture laboratory, each well of a typical commercially available 96 well tissue culture plate is about 9 mm deep. The depth increases up to about 18 mm for a six well tissue culture plate. In the case of the ninety-six well plate, gas occupies about 75% of each well and medium occupies about 25% of each well. In the case of the six-well plate, gas occupies about 95% of each well and medium occupies about 5% of each well. This inefficient geometry adds cost to device shipping, sterilization, storage, and disposal.

In many applications, the need to frequently feed the culture by removing and replacing the small volume of medium can be problematic. For example, if the purpose of the multiple well tissue culture plate is to perform experiments, manipulating the medium could affect the outcome of those experiments. Also, because the medium volume is so small, a detrimental shift in solute concentration can occur with just a small amount of evaporation. A multiple well tissue culture plate that allowed medium to reside at an increased height without loss of cell culture function would be superior to the traditional plate by minimizing the

manipulations needed to keep the culture alive, and reducing the magnitude of concentration shifts caused by evaporation.

Frequently medium exchange is also time consuming, costly, and leads to elevated contamination risk. Attempts to mitigate the problem by special liquid handling equipment such as multi-channel pipettes do not address the source of the problem, low medium height. The best solution is to allow more medium to reside in each well. Unfortunately, that solution is not possible with traditional plates due to the need for gas exchange by way of the gas/liquid interface.

Better alternatives to traditional devices are needed. If tissue culture devices were available that did not rely solely upon a gas/liquid interface to function, were just as easy to use as traditional flasks and multiple well plates, allowed more cells to be cultured in a device of the same footprint, and were easily and linearly scalable, the efficient gains would translate into reduced costs for those using cells to advance human and animal health care. It will be shown herein how the use of gas permeable materials and novel configurations can achieve this objective.

Cell culture devices that eliminate the gas/liquid interface as the sole source of gas exchange have been proposed, and made their way into the market. This approach relies on the use of a lower gas permeable membrane to bring gas exchange to the bottom of the medium. That, as opposed to sole reliance on gas/liquid interfaces, allows more gas transfer. The proposed and commercially available devices include cell culture bags, compartmentalized gas permeable flasks, gas permeable cartridges, gas permeable petri dishes, gas permeable multiple well plates, and gas permeable roller bottles.

Unfortunately, each of the gas permeable devices has inherent inefficiencies and scale up deficiencies. Primary limitations of cell culture bags, gas permeable cartridges, gas permeable petri dishes, gas permeable multiple well plates, compartmentalized gas permeable flasks, and gas permeable roller bottles include limited medium height, excessive gas permeable surface area to medium volume ratios, and poor geometry for culturing adherent cells. This has the effect of forcing numerous devices to be required for scale up, restricting device design options, and increasing cost and complexity as scale up occurs.

Close examination of prior art surrounding gas permeable devices demonstrates how conventional wisdom, and device design, limits the height of medium and the volume of medium that resides in them. In the 1976 paper entitled Diffusion in Tissue Cultures on Gas-permeable and Impermeable Supports (Jensen et al., J. Theor. Biol. 56, 443-458 (1976)), the theory of operation for a closed container made of gas permeable membrane is analyzed. Jensen et al. describes diffusion as the mode of solute transport in the medium and the paper states that "diffusion proceeds according to Fick's laws." Jensen et al. state "FIG. **2** [of Jensen et al.] shows the diffusional characteristics for cells cultured in a bag made of gas permeable material." FIG. **1**A, herein, shows FIG. **2** of Jensen et al. in which $D_c m$ is the diffusion constant of medium. FIG. **1**B, herein, shows FIG. **3** of Jensen et al. in which the model of steady state values for PO_2 and PCO_2 in a gas permeable container are shown as a linear decay throughout the medium, based on diffusion.

In 1977, Jensen (Jensen, Mona D. "Mass cell culture in a controlled environment", Cell Culture and its Applications, Academic Press 1977) described a "major innovation" by the use of "gas permeable, nonporous plastic film" to form a cell culture device. FIG. **2**, herein, shows FIG. **2** of Jensen. As shown in FIG. **2**, herein, the device created a very low height of medium, only 0.76 mm, and a very high gas permeable surface to medium volume ratio. For scale up, the device gets as long as 30 feet and is perfused using custom equipment.

In 1981, Jensen (Biotechnology and Bioengineering. Vol. XXIII, Pp. 2703-2716 (1981)) specifically stated "culture vessel design must incorporate a small diffusional distance which is fixed and constant for all the cells cultured. The design must be such that scaling-up the culture does not change the diffusion distance." Indeed, the conventional wisdom that medium should not reside at a height very far from the gas permeable membrane continues to this day, as evidenced by the commercial products that utilize gas permeable materials and the patents that are related to them. Furthermore, a high gas permeable surface to medium volume ratio continues.

A variety of gas permeable cell culture devices have entered the market and been proposed since 1981. However, continued reliance on diffusion as a primary design factor appears to be the case based upon review of the patents, device design, device specifications, and operating instructions for gas permeable devices. As design criteria, the model for diffusion limits medium height, leads to high gas permeable surface to medium volume ratios, and contributes to inefficient device geometry.

Commercially available gas permeable cell culture devices in the form of bags are currently a standard device format used for cell culture. As with the configuration of Jensen, these products allow gas exchange through the lower and upper surface of the medium via gas permeable materials. Unlike the device presented by Jensen, perfusion is not required. Typically they are not perfused, and reside in a cell culture incubator. This reduces cost and complexity and has made them an accepted device in the market. However, the limited distance between the gas permeable membranes when cell culture medium resides in them has the effect of making them geometrically unsuitable for efficient scale up. As more medium is needed, bag size must increase proportionally in the horizontal direction. Thus, they are generally unavailable in sizes beyond 2 liters, making numerous devices required for scale up. Furthermore, they are not compatible with the standard liquid handling tools used for traditional devices, adding a level of complexity for those performing research scale culture.

Bags are fabricated by laminating two sheets of gas permeable films together. A typical bag cross-section is shown in FIG. 3 taken from U.S. Pat. No. 5,686,304, which has been commercialized as the Si-Culture™ bag (Medtronic Inc.). A beneficial feature of traditional static cell culture devices is a uniform distribution of medium over the area where cells reside. Those skilled in the art specifically take great care to level incubators for the purpose of ensuring that the medium resides at a constant height throughout the device. By looking at the bag cross-section of FIG. **3**, it can be seen how medium does not reside at a uniform height above the entire lower gas permeable film, no matter how level the incubator is. Since the films mate at the perimeter, medium is forced to reside at a different height near the perimeter than elsewhere in the bag. As medium volume increases, the bag begins to take a cylindrical shape and medium distribution becomes worse. Cells can be subjected to potential nutrient gradients due to the non-uniform shape. If too much medium is in the bag, the lower surface will reside in a non-horizontal state. That also creates problems. Suspension cells residing in the bag will not distribute uniformly. Instead, they will gravitationally settle in the low point, pile up, and die as nutrient and oxygen gradients form within the pile. In the case of adherent cells, they will not

seed uniformly because the amount of inoculum residing in each portion of the bag will vary. In addition to the geometric problems created if bags are overfilled, the weight of medium in excess of 1000 ml can also damage the bag as described in U.S. Pat. No. 5,686,304. Even if the geometric limitations of bags were overcome, instructions and patents related to the bags and other gas permeable devices indicate a limit exists based on the belief that diffusion barriers prevent devices from functioning when medium resides at too great a height.

Cell culture bags are commercially available from OriGen Biomedical Group (OriGen PermaLife™ Bags), Baxter (Lifecell® X-Fold™ related to U.S. Pat. Nos. 4,829,002, 4,937,194, 5,935,847, 6,297,046 B1), Medtronic (Si-Culture™, U.S. Pat. No. 5,686,304), Biovectra (VectraCell™), and American Fluoroseal (VueLife™ Culture Bag System, covered by U.S. Pat. Nos. 4,847,462 and 4,945,203). The specifications, operating instructions, and/or patents dictate the medium height and the gas permeable surface area to medium volume ratio for each product.

Pattillo et al. (U.S. Pat. Nos. 4,829,002 and 4,937,194 assigned to Baxter International Inc.) states that typically bags are "filled to about one quarter to one half of the full capacity, to provide a relatively high ratio of internal surface area of volume of the media and cells, so that abundant oxygen can diffuse into the bag, and carbon dioxide can diffuse out of the bag, to facilitate cell metabolism and growth." In light of Pattillo et al. the best medium height attained for the Baxter Lifecell® X-Fold™ bags is for their 600 cm^2 bag, which yields a medium height of 1.0 cm to 2.0 cm and a gas permeable surface area to medium volume ratio of 2.0 cm^2/ml to 1.0 cm^2/ml.

The product literature for the VectraCell™ bag states "VectraCell 1 L containers can hold up to 500 mL of media. VectraCell 3 L containers can hold up to 1500 mL of media." Thus, as with the Baxter bags, maximum medium capacity is at one half the bags total capacity. Of the various bag sizes offered, the 3 L bag allows the highest medium height, 1.92 cm, and has the lowest gas permeable surface area to medium volume ratio of 1.04 cm^2/ml.

A 1.6 cm medium height is recommended for the Si-Culture™ bag in the product literature and specified in U.S. Pat. No. 5,686,304 when it resides on an orbital shaker that physically mixes the medium. That leads to a gas permeable surface area to medium volume ratio of 1.25 cm^2/ml when used in a mixed environment. Since mixing is generally used to break up diffusional gradients and enhance solute transfer, one skilled in the art would conclude that medium height should be reduced when this bag is not placed on an orbital shaker.

The product literature for the VueLife™ bag specifically recommends filling VueLife™ Culture Bags with media at a height of no more than one centimeter thick, because "additional media might interfere with nutrient or gas diffusion." Thus, diffusional concerns limit medium height in the VueLife™ bags. That leads to a gas permeable surface area to medium volume ratio of 2.0 cm^2/ml at a medium height of 1.0 cm.

The product literature for the OriGen PermaLife™ bags specify nominal volume at a medium height of 1.0 cm, the equivalent height of the VueLife™ bags. Of the various PermaLife™ bags offered, their 120 ml bag offers the lowest gas permeable surface area to medium volume ratio of 1.8 cm^2/ml.

The net result of the limited medium height is that culture scale up using these products is impractical. For example, if the Lifecell X-Fold™ bag were scaled up so that is could contain 10 L of medium at a medium height of 2.0 cm, its footprint would need to be at least 5000 cm^2. Not only is this an unwieldy shape, the footprint can quickly outsize a standard cell culture incubator, leading to the need for custom incubators. Also, the gas transfer area utilized in the bags is larger than necessary because all of these configurations rely upon both the upper and lower surfaces of the bag for gas transfer.

This impractical geometry has restricted the size of commercially available bags. Recommended medium volume for the largest bag from each supplier is 220 ml for the OriGen PermaLife™ bags, 730 ml for the VueLife™ bags, 1000 ml for the Lifecell® X-Fold™ bags, 1500 ml for the VectraCell™ bags, and 2000 ml for the Si-Culture™ bags when shaken. Therefore, scale up requires the use of numerous individual bags, making the process inefficient for a variety of reasons that include increased labor and contamination risk.

Another deficiency with cell culture bags is that they are not as easy to use as traditional flasks. Transport of liquid into and out of them is cumbersome. They are configured with tubing connections adapted to mate with syringes, needles, or pump tubing. This is suitable for closed system operation, but for research scale culture, the use of pipettes is an easier and more common method of liquid handling. The inability to use pipettes is very inconvenient when the desired amount of medium to be added or removed from the bags exceeds the 60 ml volume of a typical large syringe. In that case the syringe must be connected and removed from the tubing for each 60 ml transfer. For example, a bag containing 600 ml would require up to 10 connections and 10 disconnections with a 60 ml syringe, increasing the time to handle the bag and the probability of contamination. To minimize the number of connections, a pump can be used to transfer medium. However, this adds cost and complexity to small-scale cultures. Many hybridoma core laboratories that utilize cell culture bags fill them once upon setup, and do not feed the cells again due to the high risk of contamination caused by these connections and the complexity of pumps.

Matusmiya et al. (U.S. Pat. No. 5,225,346) attempts to correct the problem of liquid transport by integrating the bag with a medium storage room. The culture room and medium storage room are connected and when fresh medium is needed, medium is passed from the medium room to the culture room. While this may help in medium transport, there is no resolution to the limited medium height and high gas permeable surface area to medium volume ratios that limit bag scale up efficiency. The disclosure presents a medium height of 0.37 cm and gas permeable surface area to medium volume ratio of 5.4 cm^2/ml.

Cartridge style gas permeable cell culture devices have been introduced to the market that, unlike cell culture bags, have sidewalls. These types of devices use the sidewall to separate upper and lower gas permeable films. That allows uniform medium height throughout the device. Unfortunately, these devices are even less suitable for scale up than bags because they only contain a small volume of medium. The small medium volume is a result of an attempt to create a high gas permeable surface area to medium volume ratio.

One such product called Opticell® is provided by Bio-Chrystal Ltd. This product is a container, bounded on the upper and lower surfaces by a gas permeable silicone film, each with a surface area of 50 cm^2. The sidewall is comprised of materials not selected for gas transfer, but for providing the rigidity needed to separate the upper and lower gas membranes. Product literature promotes its key feature, "two growth surfaces with a large surface area to volume

ratio." In an article for Genetic Engineering News (Vol. 20 No. 21 Dec. 2000) about this product, patent applicant Barbera-Guillem states "with the footprint of a microtiter plate, the membrane areas have been maximized and the volume minimized, resulting in a space that provides for large growth surfaces with maximum gas interchange." The operating protocol defining how to use this product specifies introduction of only 10 ml of medium, thereby limiting the height at which medium can reside to 0.2 cm. U.S. patent application Ser. No. 10/183,132 (filed Jun. 25, 2002), associated with this device, states a height up to 0.5 inches (1.27 cm) is possible, but more preferred would be a height of about 0.07 to about 0.08 inches (0.18 cm to about 0.2 cm). WO 00/56870, also associated with this device, states a height up to 20 mm is possible, but more preferred would be a height of 4 mm. Even if the greater height of 1.27 cm described in the patent were integrated into the commercial device, that medium height does not exceed that allowed in bags. Furthermore, that would only reduce the gas permeable surface area to medium volume ratio to 1.00 cm^2/ml, which is similar to the bag. U.S. patent application Ser. No. 10/183,132 shows a configuration in which only one side of the device is gas permeable. In that configuration, which was not commercialized, a gas permeable surface area to medium volume ratio of 0.79 cm^2/ml at a medium height of 0.5 inches (1.27 cm) would be attained, which is somewhat lower than that of cell culture bags. Therefore, despite a sidewall, even when the geometry allows the maximum medium height, there is not improved scale up efficiency relative to bags.

Cartridge style gas permeable cell culture devices have also been introduced to the market by Laboratories MABIO-International®, called CLINIcell® Culture Cassettes. Like the Opticell®, neither the product design nor the operating instructions provide for an increase in medium height, or a reduced gas permeable surface area to medium volume ratio, relative to bags. The operating instructions for the CLINIcell® 25 Culture Cassette state that no more than 10 ml of medium should reside above the lower 25 cm^2 gas permeable surface. Since the surface area of the lower gas permeable material is only 25 cm^2, that creates a medium height of only 0.4 cm. Also, since the top and bottom of the device are comprised of gas permeable material, there is a high gas permeable surface area to medium volume ratio of 5.0 cm^2/ml. The operating instructions for the CLINIcell® 250 Culture Cassette state that no more than 160 ml of medium should reside above the lower 250 cm^2 gas permeable surface, leading to a low medium height of 0.64 cm and a high gas permeable surface area to medium volume ratio of 3.125 cm^2/ml.

Cartridge style gas permeable cell culture devices have recently been introduced to the market by Celartis, called Petaka™. Like the Opticell® and CLINIcell® Culture Cassettes, these devices also have a sidewall that functions as a means of separating the upper and lower gas permeable films. Unlike those products, it is compatible with a standard pipettes and syringes, so it improves convenience of liquid handling. Yet, neither the product design nor the operating instructions provide for an increase in medium height, or a reduced gas permeable surface area to medium volume ratio, relative to bags. The operating instructions state that no more than 25 ml of medium should reside between the upper and lower gas permeable surfaces, which comprise a total surface area of 160 cm^2. Product literature specifies "optimized media/surface area" of 0.156 ml/cm^2. Thus, the medium height is only 0.31 cm and the optimized gas permeable surface area to medium volume ratio is 6.4 cm^2/ml.

The limitations of the commercially available cartridge style gas permeable devices for scale up become clear when reviewing the maximum culture volume available for these devices. Opticell® provides up to 10 ml of culture volume, CLINIcell® Culture Cassettes provide up to 160 ml of culture volume, and Petaka™ provides up to 25 ml of culture volume. Therefore, just to perform a 1000 ml culture, it would take 100 Opticell® cartridges, 7 CLINIcell® Culture Cassettes, or 40 Petaka™ cartridges.

Vivascience Sartorius Group has introduced gas permeable petri dishes into the market called petriPERM. The petriPERM 35 and petriPERM 50 are products in the form of traditional 35 mm and 50 mm diameter petri dishes respectively. The bottoms are gas permeable. The walls of the petriPERM 35 mm dish and petriPERM 50 mm dish are 6 mm and 12 mm high respectively. Vivascience product specifications show the petriPERM 35 has a gas permeable membrane area of 9.6 cm^2 and a maximum liquid volume of 3.5 ml, resulting in a maximum medium height of 0.36 cm, and the petriPERM 50 has a gas permeable membrane area of 19.6 cm^2 and a maximum liquid volume of 10 ml, resulting in a maximum medium height of 0.51 cm. The petriPERM products are designed with a cover that allows the upper surface of medium to be in communication with ambient gas, and a lower gas permeable material that allows the lower surface of the medium to be in communication with ambient gas. Thus, the minimum gas permeable surface area to medium volume ratio of the petriPERM 35 is 2.74 cm^2/ml and of the petriPERM 50 is 1.96 cm^2/ml. Like other gas permeable devices, the petriPERM products are also inefficient for scale up. Just to perform a 1000 ml culture, at least 100 devices are needed. Furthermore, these devices are not capable of being operated as a closed system.

Gabridge (U.S. Pat. No. 4,435,508) describes a gas permeable cell culture device configured with a top cover like a petri dish, designed for high resolution microscopy. The depth of the well is based on the "most convenient size for microscopy", 0.25 inch (0.635 cm). At best, the device is capable of holding medium at a height of 0.635 cm.

Vivascience Sartorius Group has also introduced gas permeable multiple well tissue culture plates called Lumox Multiwell into the market. These products are also distributed by Greiner Bio-One. They are available in 24, 96, and 394 well formats. The bottom of the plate is made of a 50 micron gas permeable film with a very low auto-fluorescence. Wall height of each well is 16.5 mm for the 24-well version, 10.9 mm for the 96-well version, and 11.5 mm for the 384-well version. Maximum working medium height for each well are specified to be 1.03 cm for the 24-well version, 0.97 cm for the 96-well version, and 0.91 cm for the 384-well version. Although medium height is improved relative to traditional multiple well plates, it falls within the limits of other static gas permeable devices.

Fuller et al. (WO 01/92462 A1) presents a gas permeable multiple well plate that increases the surface area of the lower gas permeable silicone material by texturing the surface. However, the wall height is limited to merely that of "a standard microtiter plate", thereby failing to allow an increase in medium height relative to traditional plates.

In general, it would be advantageous if static gas permeable cell culture devices could utilize membranes that are thicker than those used in commercially available devices. Conventional wisdom for single compartment static gas permeable cell culture devices that rely upon silicone dic-

tates that proper function requires the gas permeable material to be less than about 0.005 inches in thickness or less, as described in U.S. Pat. No. 5,686,304. The Si-Culture™ bag is composed of di-methyl silicone, approximately 0.0045 inches thick. Barbera-Guillem et al. (U.S. patent application Ser. No. 10/183,132) and Barbera-Guillem (WO 00/56870) state that the thickness of a gas permeable membrane can range from less than about 0.00125 inches to about 0.005 inches when the membranes comprised suitable polymers including polystyrene, polyethylene, polycarbonate, polyolefin, ethylene vinyl acetate, polypropylene, polysulfone, polytetrafluoroethylene, or silicone copolymers. Keeping the films this thin is disadvantageous because the films are prone to puncture, easily get pinholes during fabrication, and are difficult to fabricate by any method other than calendaring which does not allow a profile other than sheet profile. It will be shown herein how an increased thickness of silicone beyond conventional wisdom does not impede cell culture.

Improved static gas permeable devices are needed. If gas permeable devices were capable of scale up in the vertical direction, efficiency would improve because a larger culture could be performed in a device of any given footprint, and more ergonomic design options would be available.

Compartmentalized, static gas permeable devices, are another type of product that provides an alternative to traditional culture devices. However, they also are limited in scale up efficiency by medium height limitations and excessive gas permeable surface area to medium volume ratios. These types of devices are particularly useful for creating high-density culture environments by trapping cells between a gas permeable membrane and a semi-permeable membrane. Although not commercialized, Vogler (U.S. Pat. No. 4,748,124) discloses a compartmentalized device configuration that places cells in proximity of a gas permeable material and contains non-gas permeable sidewalls. The cell compartment is comprised of a lower gas permeable material and is bounded by an upper semi-permeable membrane. A medium compartment resides directly and entirely above the semi-permeable membrane. A gas permeable membrane resides on top of the medium compartment. Medium is constrained to reside entirely above the gas permeable bottom of the device. The patent describes tests with a cell culture compartment comprised of 0.4 cm sidewalls, a medium compartment comprised of 0.8 cm sidewalls, a cell culture volume of 9 ml, a basal medium volume of 18 ml, a lower gas permeable membrane of 22 cm^2, and an upper gas permeable membrane of 22 cm^2. That creates a cell compartment medium height of 0.4 cm and allows medium to reside at a height of 0.8 cm in the medium compartment. Furthermore, there is a high total gas permeable surface area to total medium volume ratio of 1.63 cm^2/ml. In a paper entitled "A Compartmentalized Device for the Culture of Animal Cells" (Biomat., Art. Cells, Art. Org., 17(5), 597-610 (1989)), Vogler presents biological results using the device of U.S. Pat. No. 4,748,124. The paper specifically cites the 1976 Jensen et al. and 1981 Jensen papers as the "theoretical basis of operation." Dimensions for test fixtures describe a 28.7 cm^2 lower and 28.7 cm^2 upper gas permeable membrane, a cell compartment wall height of 0.18 cm allowing 5.1 ml of medium to reside in the cell compartment, and a medium compartment wall height of 0.97 cm allowing 27.8 ml of medium to reside in the medium compartment. Total medium height is limited to 0.18 cm in the cell compartment, 0.97 cm in the medium compartment, with a high total gas permeable surface area to total medium volume ratio of 1.74 cm^2/ml.

Integra Biosciences markets compartmentalized gas permeable products called CELLine™. As with Vogler's device, the cell compartment is bounded by a lower gas permeable membrane and an upper semi-permeable membrane. However, unlike the Vogler geometry, all medium in the device does not need to reside entirely above the gas permeable membrane. Only a portion of the basal medium need reside above the semi-permeable membrane. The patents that cover the Integra Biosciences products, and product literature, describe the need to keep the liquid height in the cell compartment below about 15 mm. A ratio of 5 ml to 10 ml of nutrient medium per square centimeter of gas permeable membrane surface area is described for proper cell support (U.S. Pat. No. 5,693,537 and U.S. Pat. No. 5,707,869). Although the increase in medium volume to cell culture area is advantageous in terms of minimizing the frequency of feeding, in practice the medium height above each centimeter of gas permeable surface area is limited. The commercial design of the devices covered by these patents demonstrates that they, like the other gas permeable devices, limit the amount of medium that can reside above the cells. Over half of the medium volume resides in areas not directly above the semi-permeable membrane in order to reduce the height of medium residing directly above the cells. The non-gas permeable sidewalls of the device are designed so that when the device is operated in accordance with the instructions for use, the height at which medium resides above the semi-permeable membrane in the CELLine™ products is approximately 5.2 cm in the CL1000, 3.5 cm in the CL350, and 1.1 cm in the CL6Well. When operated in accordance with the instructions for use, the height of medium residing in the cell culture compartment is 15 mm for the CL1000, 14 mm for the CL350, and 26 mm for the CL6Well. The patents describe, and the devices integrate, a gas/liquid interface at the upper surface of the medium. Thus, the gas transfer surface area to medium volume ratio is also limited because gas transfer occurs through the bottom of the device and at the top of the medium. The gas transfer surface area to medium volume ratio for each device is approximately 0.31 cm^2/ml for the CL1000, 0.32 cm^2/ml for the CL350, and 1.20 cm^2/ml for the CL6Well.

Bader (U.S. Pat. No. 6,468,792) also introduces a compartmentalized gas permeable device. Absent sidewalls, it is in the form of a bag. It is compartmentalized to separate the cells from nutrients by a microporous membrane. As with the other compartmentalized gas permeable devices, medium height is limited. U.S. Pat. No. 6,468,792 states although medium heights up to 1 to 2 cm can be achieved in the apparatus, actual heights need to be tailored based upon the O2 supply as a function of "medium layer in accordance with Fick's law of diffusion." Since the upper and lower surfaces of the bag are gas permeable, a minimum total gas permeable surface area to total medium volume ratio of 1.0 cm^2/ml is attained when the apparatus is filled to its maximum capacity.

If compartmentalized gas permeable devices were capable of increasing their scale up potential in the vertical direction, they would have a more efficient footprint as the magnitude of the culture increases. A static, compartmentalized, gas permeable device that accommodates vertical scale up is needed.

Gas permeable devices that attempt to improve efficiency relative to static gas permeable devices have been introduced. The devices operate in a similar manner as the traditional roller bottle and attempt to improve mass transfer by medium mixing that comes with the rolling action.

However, efficient scale up is not achieved. One reason is that, like static devices, design specifications constrain the distance that medium can reside from the gas permeable device walls. This limits device medium capacity. Thus, multiple devices are needed for scale up.

Spaulding (U.S. Pat. No. 5,330,908) discloses a roller bottle configured with gas permeable wall that is donut shaped. The inner cylinder wall and the outer cylinder wall are in communication with ambient gas. The gas permeable nature of the walls provides oxygen to cells, which reside in the compartment bounded by the inner and outer cylinder walls. The cell compartment is filled completely with medium, which is advantageous in terms of limiting cell shear. Spaulding states "the oxygen efficiency decreases as a function of the travel distance in the culture media and effectiveness is limited to about one inch or less from the oxygen surface." Thus, the design limits stated by Spaulding include keeping the distance between the inner cylindrical wall and the outer cylindrical wall at 5.01 cm or less in order to provide adequate oxygenation. In that manner, cells cannot reside more than 2.505 cm from a gas permeable wall. That also leads to a gas permeable surface area to medium volume ratio of about 0.79 cm^2/ml. Furthermore, the need to have a hollow gas permeable core wastes space. The device only has an internal volume of 100 ml of medium for every 5 cm in length, as opposed to 500 ml for a traditional bottle of equivalent length. The medium volume limitation makes this device less efficiently scalable than the traditional roller bottle, because more bottles are needed for a culture of equivalent volume. Another problem with the device is the use of etched holes, 90 microns in diameter, for gas transfer. These holes are large enough to allow gas entry, but small enough to prevent liquid from exiting the cell compartment. However, they could allow bacterial penetration of the cell compartment since most sterile filters prevent particles of 0.45 microns, and more commonly 0.2 microns, from passing.

In a patent filed in December 1992, Wolf et al. (U.S. Pat. No. 5,153,131) describes a gas permeable bioreactor configured in a disk shape that is rolled about its axis. The geometry of this device attempts to correct a deficiency with the proposal of Schwarz et al. U.S. Pat. No. 5,026,650. In U.S. Pat. No. 5,026,650, a gas permeable tubular insert resides within a cylindrical roller bottle and the outer housing is not gas permeable. Although it was successful at culturing adherent cells attached to beads, Wolf et al. state that it was not successful at culturing suspension cells. The device is configured with one or both of the flat ends permeable to gas. The disk is limited to a diameter of about 6 inches in order to reduce the effects of centrifugal force. The inventors state "the partial pressure or the partial pressure gradient of the oxygen in the culture media decreases as a function of distance from the permeable membrane", which is the same thought process expressed by Jensen in 1976. They also state "a cell will not grow if it is too far distant from the permeable membrane." Therefore, the width is limited to less than two inches when both ends of the disk are gas permeable. These dimensional limitations mean that the most medium the device can hold is less than 1502 ml. Therefore, more and more devices must be used as the culture is scaled up in size. Also, the gas permeable surface area to medium volume ratio must be at least 0.79 ml/cm^2 and cells must reside less than 1.27 cm from a gas permeable wall. Furthermore, the device does not adapt for use with existing laboratory equipment and requires special rotational equipment and air pumps.

In a patent filed in February 1996, Schwarz (U.S. Pat. No. 5,702,941) describes a disk shaped gas permeable bioreactor with gas permeable ends that rolls in a similar manner as a roller bottle. Unfortunately, as with U.S. Pat. No. 5,153,131, the length of the bioreactor is limited to about 2.54 cm or less. Unless all surfaces of the bioreactor are gas permeable, the distance becomes even smaller. Maximum device diameter is 15.24 cm. Thus, the gas permeable surface area to medium volume ratio must be at least 0.79 ml/cm^2 and cells can never reside more than 1.27 cm from a gas permeable wall. Even with the rolling action, this does not render a substantial reduction in the gas permeable surface area to medium ratio relative to traditional static culture bags, and requires more and more devices to be used as the culture is scaled up in size.

A commercially available product line from Synthecon Incorporated, called the Rotary Cell Culture System™, integrates various aspects of the Spaulding, Schwarz, and Wolf et al. patents. The resulting products are have small medium capacity, from 10 ml to 500 ml, require custom rolling equipment, are not compatible with standard laboratory pipettes, and are very expensive when compared to the cost of traditional devices that hold an equal volume of medium. Thus, they have made little impact in the market because they do not address the need for improved efficiency in a simple device format.

Falkenberg et al. (U.S. Pat. No. 5,449,617 and U.S. Pat. No. 5,576,211) describes a gas permeable roller bottle compartmentalized by a dialysis membrane. The medium volume that can be accommodated by the bottle is 360 ml, of which 60 ml resides in the cell compartment and 300 ml in the nutrient compartment. In one embodiment, the ends of the bottle are gas permeable. U.S. Pat. No. 5,576,211 states the when the end of the bottle is gas permeable, "gas exchange membranes with a surface area of a least 50 cm^2 have been proven to be suitable for cell cultures of 35 ml." Therefore, the minimum gas permeable surface area to volume ratio is 1.43 cm^2/ml. In another embodiment, the body of the bottle is gas permeable, with a surface area of 240 cm^2. That gas permeable surface oxygenates the entire 360 ml volume of medium that resides in the vessel. Therefore, the minimum gas permeable surface area to volume ratio is 0.67 cm^2/ml. The diameter of the bottle is approximately 5 cm, and the length of the bottle is approximately 15 cm. Thus, the bottle is much smaller than a traditional roller bottle, which has a diameter of approximately 11.5 cm and a length up to approximately 33 cm. Although this device is useful for high-density suspension cell culture, its limited medium capacity fails to reduce the number of devices needed for scale up. Furthermore, it is not suitable for adherent culture because it makes no provision for attachment surface area.

Falkenberg et al. (U.S. Pat. No. 5,686,301) describes an improved version of the devices defined in U.S. Pat. No. 5,449,617 and U.S. Pat. No. 5,576,211. A feature in the form of collapsible sheathing that prevents damage by internal pressurization is disclosed. Gas is provided by way of the end of the bottle and can "diffuse into the supply chamber" by way of the gas permeable sheathing. Unfortunately, it fails to reduce the number of devices needed for scale up because the bottle dimensions remain unchanged. Furthermore, it remains unsuitable for adherent culture.

Vivascience Sartorius Group sells a product called the miniPERM that is related to the Falkenberg et al. patents. The maximum cell compartment module is 50 ml and the maximum nutrient module is 400 ml. Thus, the maximum volume of medium that can reside in the commercial device

is 450 ml. The small size of the commercial device, combined with the need for custom rolling equipment, renders it an inefficient solution to the scale up problem.

There exists a need to improve the rolled gas permeable devices so that they can provide more medium per device, thereby reducing the number of devices needed for scale up. That can be achieved if a decreased gas permeable surface area to medium volume ratio is present. Another problem is that non-standard laboratory equipment is needed for operation of the existing devices. The use of standard laboratory equipment would also allow more users to access the technology.

The prior discussion has focused on design deficiencies that limit efficient scale up in existing and proposed cell culture devices. In addition to the previously described limitations, there are additional problems that limit scale up efficiency when adherent cell culture is the objective.

For traditional static devices that rely upon a gas/liquid interface for oxygenation, the adherent cell culture inefficiency is caused by limited attachment surface area per device. For example, only the bottom of the device is suitable for cell attachment with petri dishes, multiple well plates, and tissue culture flasks. The traditional flask provides a good example of the problem. As described previously, a typical T-175 flask occupies about 936 cm^3. Yet, it only provides 175 cm^2 of surface area for adherent cells to attach to. Thus, the ratio of space occupied to growth surface, 5.35 $cm3/cm^2$, is highly inefficient.

Products that attempt to address the surface area deficiency of traditional flasks are available. Multi-shelved tissue culture flasks, such as the NUNC™ Cell Factory (U.S. Pat. No. 5,310,676) and Corning CellStack™ (U.S. Pat. No. 6,569,675), increase surface area is by stacking polystyrene shelves in the vertical direction. The devices are designed to allow medium and gas to reside between the shelves. This reduces the device footprint relative to traditional flasks when increasing the number of cells being cultured. The profile of the multi-shelved flasks is also more space efficient that traditional flasks. For example, the space between shelves of the NUNC™ Cell Factory is about 1.4 cm, as opposed to the 3.7 cm distance between the bottom and top of a typical T-175 flask. The reduced use of space saves money in terms of sterilization, shipping, storage, incubator space, and device disposal. This style of device also reduces the amount of handling during scale up because one multi-shelved device can be fed as opposed to feeding multiple tissue culture flasks. Furthermore, the use of traditional polystyrene is easily accommodated. Unfortunately, the device is still sub-optimal in efficiency since each of its shelves requires a gas/liquid interface to provide oxygen.

CellCube® is an adherent cell culture device available from Corning Life Sciences. It is configured in a similar manner to the multiple shelved tissue culture flasks, but it eliminates the gas/liquid interface. The distance between the vertically stacked cell attachment shelves is therefore reduced because gas is not present. That reduces the amount of space occupied by the device. However, in order to provide gas exchange, continuous perfusion of oxygenated medium is required. That leads to a very high level of cost and complexity relative to the Corning CellStack™, rendering it inferior for research scale culture.

Static gas permeable devices do not provide a superior alternative to the NUNC™ Cell Factory, Corning CellStack™, or CellCube®. Cell culture bags and gas permeable cartridges can provide more attachment area than traditional tissue culture flasks. That is because they could allow cells to be cultured on both the upper and lower device surfaces. However, gas permeable materials that are suitable for cell attachment can be much more expensive than traditional polystyrene. Also, even if both the upper and lower surfaces of a gas permeable device allowed cells to grow, only a two-fold increase in surface area would be obtained relative to a traditional gas/liquid interface style device that occupied the same footprint. Furthermore, the scale up deficiencies that have been described previously remain limiting.

Fuller et al. (IPN WO 01/92462 A1) presents a new bag that textures the surface of the gas permeable material in order to allow more surface area for gas transfer and cell attachment. However, medium height is also limited to that of the commercially available bags. That is because this bag is fabricated in the same manner as the other bags. Gas permeable surface area to medium volume ratio becomes even higher than that of other bags, and non-uniform medium distribution is present.

Basehowski et al. (U.S. Pat. No. 4,939,151) proposes a gas permeable bag that is suitable for adherent culture by making the bottom gas permeable, smooth, and charged for cell attachment. The inner surface of the top of the bag is textured to prevent it from sticking to the lower gas permeable surface. This bag only utilizes the lower surface for cell attachment, rendering it only as efficient in surface area to footprint ratio as a traditional flask.

To date, guidance is inadequate on how to create a device that eliminates the reliance on a gas/liquid interface and can integrate the scaffold of the multiple layer flasks without the need for perfusion. Static gas permeable devices only allow gas transfer through the bottom and top of the device. Thus, if traditional scaffolds are included, such as the styrene shelves provided in the multi-shelved tissue culture flasks, they will have the effect of inhibiting gas exchange at the cell location. Gas permeable materials should be located in a manner in which the attachment scaffold does not prevent adequate gas transfer. How that becomes beneficial will be further described in the detailed description of the invention herein.

The need to provide more efficient cell culture devices during scale up is not limited to static cell culture devices, but also applies to roller bottles. Traditional roller bottles function by use of a gas/liquid interface. The geometry is a clever way of providing more surface area and medium volume while occupying a smaller footprint than flasks and bags. Their universal use provides testimony to the market desire for devices that provide more efficient geometry, since that leads to reductions in the use of inventory space, incubator space, labor, and biohazardous disposal space.

When bottles are used for adherent culture, cells attach to the inner wall of the bottle. Cells obtain nutrients and gas exchange as the rolling bottle moves the attached cells periodically through the medium and gas space. Roller bottle use is not limited to adherent cells. They are also commonly used to culture suspension cells. For example, the culture of murine hybridomas for the production of monoclonal antibody is routinely done in roller bottles. In typical suspension cell culture applications, efficiency improvements related to footprint and size versus flasks can be attained, the handling simplicity of the roller bottle is superior to cell culture bags, and the low cost and level of complexity is superior to spinner flasks. Corning®, the leading supplier of roller bottles recommends medium volume for an 850 cm^2 bottle between 170 ml and 255 ml. The actual capacity of the bottle is about 2200 ml. Therefore, although the roller bottle provides advantages for both adherent and suspension cell culture, it is still very inefficient in geometry because the vast majority of the roller

bottle, about 88%, is comprised of gas during the culture process. Roller bottles also deviate from the simplicity of static devices because ancillary roller mechanisms are required. Furthermore, they subject the cells to shear force. Those shear forces can damage or kill shear sensitive cells, and are not present in the traditional static devices.

McAleer et al. (U.S. Pat. No. 3,839,155) describes a roller bottle device configured to allow cells to attach to both sides of parallel discs oriented down the length of the bottle. Unlike the traditional bottle that rolls in the horizontal position, this device tumbles end over end to bring the discs through medium and then through gas. It does nothing to reduce the volume of gas residing in the bottle. On the contrary, it states "another advantage of the present invention is that extremely low volumes of fluid can be used." It relies entirely upon the presence of a large volume of gas, which must be perfused, in the bottle to function. The excessive volume of gas that hinders the efficient use of space in traditional bottles remains. Also, shear forces are not reduced.

Spielmann (U.S. Pat. No. 5,650,325) describes a roller bottle apparatus for providing an enhanced liquid/gas exchange surface. Trays are arranged in parallel within the bottle. The trays allow an increase of surface area for culture and are designed to allow liquid to flow over them as the bottle rotates. In the case of adherent cells, more surface area is available for attachment. In the case of suspension cells, they are stirred "in contact with gas and liquid phases" by the trays. Shear forces remain present. Although this apparatus provides an improved surface area, it relies entirely upon the presence of gas in the bottle to provide gas exchange. Thus, it does not address the fundamental limitation in space efficiency, which is the excessive volume of gas that must reside in the bottle.

If the roller bottle could be made to allow a vastly improved medium volume to gas ratio, it would provide a more economical option because the number of devices needed for scale up would be reduced. Since the typical medium volume for an 850 cm^2 bottle is 170 ml to 255 ml, but the capacity is 2200 ml, about a 9 to 13 fold increase in nutrient capacity could be made available by filling the bottle with medium. To retain simplicity, a non-complicated method of oxygenating the culture independent of a gas/liquid interface would need to exist. Also, for adherent culture, surface area should increase in proportion to the increase in medium volume. A gas permeable device with these characteristics could lead to a 9-fold to 13-fold reduction in the cost of sterilization, shipping, storage, use of incubator space, labor, and disposal cost. Shear forces on the cells could also be reduced.

For adherent culture, proposed and commercially available rolled gas permeable devices do not provide a superior alternative to traditional bottles because they have not integrated traditional attachment surfaces. Instead they rely upon small sections of attachment area or beads. Beads bring a new set of problems to those performing adherent culture. They are difficult to inoculate uniformly, it is not possible to assess cell confluence or morphology microscopically, and they must be separated from the cells that are attached to them if cell recovery is desired.

Attempts to eliminate the use of beads in gas permeable roller bottles have been made. Nagel et al. (U.S. Pat. No. 5,702,945), attempts to create the ability for the Falkenberg et al. devices to culture adherent cells without beads. One cell attachment matrix is provided in the cell culture compartment at the inner face of the gas membrane. Although adherent culture is possible, the bottle dimensions remain unchanged and, due to its small size, it fails to reduce the number of devices needed for scale up. Also, oxygen must transfer first through the gas permeable membrane and then through the cell attachment matrix to reach the cells. Furthermore, only one layer of cell attachment matrix is available, as opposed to the multiple layers of the NUNC™ Cell Factory and Corning CellStack™. Additionally, microscopic assessment of cell confluence and morphology is not accommodated.

An improved gas permeable roller bottle is needed. It should be capable of being filled with medium, used in standard roller racks, allowing an increase in cell attachment area in direct proportion to the increased medium volume, and retain the ease of use of the traditional bottle. It will be shown herein how this can be achieved.

Singh (U.S. Pat. No. 6,190,913) states that for "all devices that rely on gas-permeable surfaces, scale-up is limited". A bag is disclosed for resolving the scale up deficiencies of gas permeable devices. The non-gas permeable bag integrates medium and gas, in roughly equal proportions. The bag is placed on a rocker plate, and the rocking motion creates a wave in the medium, which enhances gas transfer. This patent covers the commercial product, available from Wave Biotech called the Wave Bioreactor. Unfortunately, custom rocking and temperature control equipment must be purchased for the apparatus to function, and the bag does not substantially alter the capacity to hold medium. As with gas permeable bags, the Wave Bioreactor bags are filled with medium to no more than one half of their carrying capacity. Thus, they limit medium height and inherit similar scale up deficiencies as gas permeable bags.

In summary, a need exists for improved cell culture devices and methods that bring more efficiency to research scale cell culture, and do not lose efficiency during scale up. Traditional devices that rely upon a gas/liquid interface to function are inefficient in terms of labor, sterilization cost, shipping cost, storage cost, use of incubator space, disposal cost, and contamination risk. Those devices include the petri dish, multiple well tissue culture plate, tissue culture flask, multiple shelved tissue culture flask, and roller bottle. Gas permeable devices are also inefficient, and in many cases lose the simplicity of the devices that require a gas/liquid interface to function. The petriPERM and Lumox multiwell plate gas permeable devices are in the form of their traditional counterparts, and inherit the inefficiencies of traditional devices. Gas permeable bags are inefficient due to medium height limitations, non-uniform medium distribution, use of high gas permeable material surface area to medium volume ratios, and the contamination risk present during feeding. Gas permeable cartridges are inefficient because they have a low height of medium, use a high gas permeable surface area to medium volume, house a small volume of medium, and require a very large number of units to be maintained during scale up. Rolled gas permeable devices are inefficient for scale up because they have geometry constraints that limit the distance that the walls can be separated from each other, require a large number of units during scale up due to limited medium volume, and often require custom rolling equipment. When adherent culture is desired, traditional devices have a very inefficient device volume to attachment surface area ratio, wasting space. Static, mixed, and rolled gas permeable devices become even more inefficient for adherent culture for reasons that include limited surface area, the use of beads for increased surface area, lack of traditional sheet styrene surfaces, and inability to perform microscopic evaluations.

Certain embodiments disclosed herein provide more efficient cell culture devices and methods, that overcome the limitations of prior devices and methods, by creating gas permeable devices that can integrate a variety of novel attributes. These various attributes include gas exchange without reliance upon a gas/liquid interface, increased medium height, reduced gas permeable surface area to medium volume ratios, gas exchange through the device side walls, cell support scaffolds that are comprised of traditional materials, and increased gas permeable material thickness.

SUMMARY OF THE INVENTION

It has been discovered that for gas permeable devices comprised of a lower gas permeable material, it can be beneficial to increase medium height beyond that dictated by conventional wisdom or allowed in commercially available devices. It is contemplated by the inventors hereof that convection of substrates within cell culture medium plays a more important role than previously recognized. It would appear that the historic reliance upon diffusion for mass transfer underestimates the contribution that convection makes. That would result in underestimating the rate of travel of substrates such as glucose and lactate in cell culture medium, and a failure to recognize that medium residing farther away from cells than traditionally allowed can be useful to the cells. If the rate of travel of substrates in medium were underestimated, medium residing in areas believed to be too far away from the cells would incorrectly deemed to be wasted. The logical consequence would be to unnecessarily configure the gas permeable device to hold less medium than could be useful to the cells, in order to reduce the space occupied by the device, making it more economically sterilized, shipped, stored, and disposed of.

In any event, and as an example of how medium residing at a distance beyond conventional wisdom can be beneficial, tests were conducted in which medium height was increased far beyond that suggested previously, or even possible in commercially available static gas permeable devices. Evaluations of a common cell culture application, using murine hybridomas, demonstrated that more cells were able to reside in a given footprint of the device by increasing medium height relative to conventional wisdom. This benefit, not previously recognized, allows a variety of cell culture device configurations that provide more efficient cell culture and process scale up to become available.

The inventive apparatus and methods herein demonstrate that the gas/liquid interface is not necessary for adequate gas exchange when a wall of a device is gas permeable, scaffolds are present, and the device is operated in a static mode. This eliminates the need for excess device size that results from the presence of gas in traditional devices, and allows gas permeable devices to integrate traditional scaffolds. This allows a variety of cell culture device configurations that occupy less space than prior devices, and makes them more efficient for scale up. Again, it is contemplated by the inventors that the role of convection may be a contributing factor.

It has also been discovered that geometric configurations for gas permeable roller bottles, that contradict the guidance of conventional wisdom, can successfully culture cells. The new geometry allows the device to contain more medium than previously possible, thereby yielding a geometric shape that improves scale up efficiency. This allows cell culture device configurations to exist that eliminate the wasted space of traditional bottles that contain gas for oxygenation, and are superior to gas permeable bottles in terms of scale up efficiency.

It has also been discovered that cells can be effectively cultured using silicone gas permeable material that is thicker than conventional wisdom advocates.

These discoveries have made it possible to create new devices and methods for culturing cells that can provide dramatic efficiency and scale up improvements over current devices such as the petri dish, multiple well tissue culture plate, tissue culture flask, multiple shelved tissue culture flask, roller bottle, gas permeable petri dish, gas permeable multiple well plate, gas permeable cell culture bag, compartmentalized gas permeable devices, and gas permeable rolled devices.

Certain embodiments disclosed herein provide superior gas permeable cell culture devices, by increasing wall height in order to allow increased medium heights and reduced gas permeable surface area to medium volume ratios.

Certain embodiments disclosed herein provide superior cell culture methods using gas permeable cell culture devices, by increasing medium heights and reducing gas permeable surface area to medium volume ratios.

Certain embodiments disclosed herein provide superior cell culture devices, by allowing gas exchange through a sidewall at least partially comprised of gas permeable material.

Certain embodiments disclosed herein provide superior cell culture methods using gas permeable devices, by allowing gas exchange through a sidewall at least partially comprised of gas permeable material.

Certain embodiments disclosed herein provide a superior alternative to gas permeable multiple well tissue culture plates, by increasing wall height in order to allow increased medium height and reduced gas permeable surface area to medium volume ratios.

Certain embodiments disclosed herein provide a superior alternative to gas permeable petri dishes, by increasing wall height in order to allow increased medium height and reduced gas permeable surface area to medium volume ratios.

Certain embodiments disclosed herein provide a superior alternative to the method of cell culture in gas permeable cell culture bags, by increasing medium height in order to provide more nutrient support and reducing gas permeable surface area to medium volume ratios.

Certain embodiments disclosed herein provide a superior alternative to the gas permeable cartridges, by increasing wall height in order to allow increased medium heights and reduced gas permeable surface area to medium volume ratios.

Certain embodiments disclosed herein provide a superior alternative to the gas permeable roller bottles, by creating a geometry that allows medium to reside at a distance from the gas permeable material beyond that previously possible.

Certain embodiments disclosed herein provide superior gas permeable cell culture devices that can be operated in the horizontal and vertical position.

Certain embodiments disclosed herein provide a superior alternative to the compartmentalized gas permeable devices, by increasing wall height in order to allow increased medium heights and reducing gas permeable surface area to medium volume ratios.

Certain embodiments disclosed herein provide a superior cell culture methods using compartmentalized gas permeable devices, by increasing medium height and reducing gas permeable surface area to medium volume ratios.

Certain embodiments disclosed herein provide superior gas permeable cell culture devices that utilize silicone material for gas exchange, by configuring them with silicone that is greater than 0.005 inches thick.

Certain embodiments disclosed herein provide an improved cell culture bag in which the gas permeable material is silicone that exceeds 0.005 inches thick.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**A and FIG. **1**B are obtained from Jensen et al., "Diffusion in Tissue Cultures on Gas-permeable and Impermeable Supports", J. Theor. Biol. 56, 443-458 (1976), FIG. **1**A shows FIG. **2**, and FIG. **1**B shows FIG. **3**, of this Jensen et al. paper in which $D_c m$ is the diffusion constant of medium and the model for steady state values of PO_2 and PCO_2 are shown in a gas permeable container.

FIG. **2** is a copy of FIG. **2** from Jensen, "Mass cell culture in a controlled environment", Cell Culture and its Applications, Academic Press 1977, showing a gas permeable cell culture device configured with a low medium height capacity.

FIG. **3** is a copy of FIG. 2 of U.S. Pat. No. 5,686,304, which has been commercialized as the Si-Culture™ bag (Medtronic Inc.), showing a typical cell culture bag cross-section.

FIG. **4**A is an embodiment of a cell culture device with a housing comprised of a lower gas permeable material, configured to allow a large volume of medium to reside above its lower gas permeable material. A removable lid protects it from contaminants. FIG. **4**B is an embodiment of a cell culture device with a housing comprised of a lower gas permeable material, configured to allow a large volume of medium to reside above its lower gas permeable material. The container is accessible by septum. FIG. **4**C is an embodiment of a cell culture device with the walls comprised of gas permeable material such that the device can be laid on its side and operated in the non-rolling or rolling position.

FIG. **5** is an embodiment of a gas permeable cell culture device with a lower gas permeable material configured to allow cells to distribute evenly about its lower surface and provide gas to the underside of the lower gas permeable material.

FIG. **6** is an embodiment of a gas permeable cell culture device configured to maintain medium in areas not directly above the cells being cultured, in order to provide additional nutrient support without a further increase in device profile.

FIG. **7**A and FIG. **7**B are two views of an embodiment of a gas permeable cell culture device configured so that it can adjust in height as the volume of medium within it changes, thereby occupying as little space as possible at each stage of the culture process and allowing the capability of being sterilized, shipped, stored, and disposed of in a minimum volume condition which reduces the cost of the process.

FIG. **8** is an embodiment of a gas permeable cell culture device configured in a multiple well format, capable of holding an increased volume of medium per well relative to traditional multiple well tissue culture devices, thereby allowing more efficient research scale culture by increasing the amount of cells present per well, reducing feeding frequency, and allowing better clone selection possibilities.

FIG. **9**A and FIG. **9**B are views of embodiments of a gas permeable cell culture device in a multiple well format, configured with a gas permeable sidewall. The lower surface of each well of the device can be comprised of exactly the same material as traditional tissue culture flasks. Elimination of the gas/liquid interface as a requirement for gas exchange allows for an increased number of cells per well and/or reduced frequency of feeding, better use of incubator space, as well as cost reductions in sterilization, shipping, storage, and disposal.

FIG. **10**A and FIG. **10**B show an embodiment of a gas permeable cell culture device configured with scaffolds for culturing adherent cells without need of a gas/liquid interface. It is linearly scalable in the horizontal and vertical direction creating superior efficiency relative to traditional adherent culture devices. It is capable of culturing cells on either one or both sides of the scaffolds. It can be operated in either the rolled or in the unrolled state.

FIG. **11** is an embodiment of a gas permeable cell culture device configured with scaffolds, at least one of which is suitable for optimal microscopic cell assessment.

FIG. **12**A, FIG. **12**B, FIG. **12**C, and FIG. **12**D show embodiments of scaffolds configured to provide a further increase in surface area, bringing even more efficiency to the gas permeable cell culture device.

FIG. **13** is an embodiment of a gas permeable cell culture device with scaffolds and at least one sidewall comprised of gas permeable material. The need for a gas/liquid interface as a means of gas exchange is eliminated, leading to more efficient use space and the related cost benefits in terms of sterilization, shipping, storage, use of incubator space, and disposal.

FIG. **14**A, FIG. **14**B, FIG. **14**C, and FIG. **14**D show views of an embodiment of a gas permeable cell culture device configured with scaffolds, the location of which can be adjusted for benefits that can include minimizing the use of trypsin, altering the ratio of medium to culture area, and minimizing shipping, inventory, and disposal space. FIG. **14**E shows a scaffold configured to maintain equal distance between it, and its neighboring scaffolds.

FIG. **15**A, FIG. **15**B, and FIG. **15**C show an embodiment of scaffolds configured such that the distance between each can be altered while the body of the device remains at a fixed height. This embodiment can provide benefits that include minimizing the use of trypsin, or altering the ratio of medium to culture area, without need to make the body of the device change shape.

FIG. **16** is a cross-sectional view of a tubular test fixture used to assess the effect of medium height on cell growth and antibody production. Biological evaluations using this test fixture demonstrated the benefit of increasing medium height beyond the limits of conventional wisdom, and the ability to reduce the gas permeable surface area to medium volume ratio of prior devices. These surprising results allow device configurations not previously contemplated to exist.

FIG. **17** is a cross-sectional view of a test fixture used to assess the ability to culture adherent cells in the absence of a gas/liquid interface by allowing gas transfer through a sidewall of the test fixture. Biological evaluations using this test fixture demonstrated the ability to culture cells in the absence of a gas/liquid interface. These surprising results allow device configurations not previously contemplated to exist.

FIG. **18** is a cross-sectional view of a test fixture used to assess the ability to culture adherent cells in the absence of a gas/liquid interface by allowing gas transfer through a sidewall of the test fixture. Multiple scaffolds were integrated into the test fixture. Biological evaluations using this test fixture demonstrated the ability to culture cells in the absence of a gas/liquid interface. These surprising results allow device configurations not previously contemplated to exist.

FIG. **19**A is a cross-sectional view of a test fixture used to assess the ability to seed cells onto the upper and lower surfaces of a scaffold. FIG. **19**B shows one scaffold of the test fixture of FIG. **19**A. Biological evaluations using this test fixture demonstrated the ability to culture cells in the absence of a gas/liquid interface when gas exchange occurred through the sidewall of the device, that a low gas permeable material surface area to attachment surface area is functional, that that a low gas permeable material surface area to medium volume is functional, and that cells can be cultured when the device is in the unrolled position or in the rolled position.

FIG. **20** is a cell distribution pattern, as described in Example 4.

DETAILED DESCRIPTION OF THE INVENTION

By configuring gas permeable devices to be capable of holding medium at a height not contemplated in prior cell culture devices or methods, advantages can accrue including reduced handling frequency, labor, sterilization cost, shipping cost, storage cost, use of incubator space, disposal cost, and contamination risk. Reducing the ratio of gas permeable surface area to medium volume to a ratio not contemplated in prior cell culture devices or methods can also increase culture efficiency. It allows an increase in medium height without a corresponding increase in device length or width. In the preferred embodiments, provisions are made that allow either medium height to increase or the ratio of gas permeable surface area to medium volume to decrease. Provisions can also be made that allow both the medium height to increase and the ratio of gas permeable surface area to medium volume to decrease.

A wide variety of embodiments for gas permeable devices and methods that allow medium to reside at heights beyond conventional wisdom are possible. They can take the form of prior devices, or entirely new forms. If the form is a gas permeable petri dish up to 50 mm in diameter, medium height should preferably exceed 0.36 cm. A preferred wall height is in excess of 6 mm. If the form is a gas permeable petri dish greater than 50 mm in diameter, medium height should preferably exceed 0.51 cm. A preferred wall height is in excess of 12 mm. If the form is a multiple well tissue culture plate with 384 wells or more, medium height should preferably exceed 0.91 cm and preferred well depth is in excess of 11.5 mm; greater than 24 wells to less than 384 wells, medium height should preferably exceed 0.97 cm and preferred well depth is in excess of 10.9 mm; 24 wells or less, medium height should preferably exceed 1.03 cm and preferred well depth is in excess of 16.5 mm. If the form is a gas permeable cartridge, medium height and wall height should preferably be greater than 1.27 cm. If in the form of a cell culture bag, medium height should preferably reside beyond 2.0 cm in height at the highest point. If the form is a compartmentalized device, and all medium in the device resides entirely above the semi-permeable membrane, medium height in the nutrient compartment should preferably reside beyond 1.0 cm in height above the semi-permeable membrane. If the form is a compartmentalized gas permeable device, medium height in the nutrient compartment should preferably reside beyond 5.2 cm in height above the semi-permeable membrane.

If it is the design objective to reduce the gas permeable surface area to medium volume ratio relative to conventional wisdom, a wide variety of embodiments for gas permeable devices and methods are possible. They can take the form of prior devices, or entirely new forms. If the form is a gas permeable petri dish below 50 mm in diameter, the gas permeable surface area to medium volume ratio should preferably be below 2.74 cm^2/ml. If the form is a gas permeable petri dish 50 mm or greater in diameter, the gas permeable surface area to medium volume ratio should preferably be below 1.96 cm^2/ml. If the form is a multiple well tissue culture plate with 384 wells or more, the gas permeable surface area to medium volume ratio should preferably be below 1.10 cm^2/ml; less than 24 wells to less than 384 wells, the gas permeable surface area to medium volume ratio should preferably be below 1.03 cm^2/ml; 24 wells or less, the gas permeable surface area to medium volume ratio should preferably be below 0.97 cm^2/ml. If the form is a gas permeable cartridge in which two sides of the cartridge are gas permeable, the surface area to medium volume ratio should preferably be below 0.79 cm^2/ml. If in the form of a cell culture bag, the gas permeable surface area to medium volume ratio should preferably be below 1.0 cm^2/ml. If the form is a compartmentalized device, and all medium in the device resides entirely above the semi-permeable membrane, the gas permeable surface area to medium volume ratio should preferably be below 1.74 cm^2/ml. If the form is a compartmentalized device, and all medium in the device does not reside entirely above the semi-permeable membrane, the gas permeable surface area to medium volume ratio should preferably be below 0.31 cm^2/ml.

FIG. **4**A shows a cross-sectional view of one embodiment of the invention. Gas permeable cell culture device **10** is configured to allow cells **20** to reside upon lower gas permeable material **30**. Although FIG. **4**A shows gas permeable cell culture device **10** structured in the style of a petri dish, any number of shapes and sizes are possible that allow medium to reside at a height beyond that of conventional wisdom.

Top cover **55** can be removed to allow medium **50** to be conveniently added and removed, by either pouring or pipetting, to and from gas permeable cell culture device **10**. However, access for medium **50** can also be made in any number of ways common to cell culture devices, including by way of caps, septums, and tubes. In the event that a closed system is desired, gas permeable cell culture device **10** can be configured with inlet and outlet tubes that can be connected to medium source and waste bags by way of a sterile tubing connection, using equipment such as that made by Terumo Medical Corp. (Somerset, N.J.). Septum configurations, or any other techniques known to those skilled in the art, can also be used to create a closed container. For example, as shown in FIG. **4**B, gas permeable cell culture device **10** can be alternatively configured as a closed container with septums **65**.

In the event that gas permeable cell culture device **10** is to be completely filled with medium **50**, and cells are intended to settle out of medium **50** by gravity, the profile of the top of gas permeable cell culture device **10** preferably allows medium **50** to reside at a uniform height above gas permeable material **30**. This will allow uniform deposit of cells onto lower gas permeable material **30**, when cells gravitationally settle from suspension within medium **50**. The configuration of FIG. **4**B achieves this purpose.

The lower gas permeable material, e.g., material **30**, can be any membrane, film, or material used for gas permeable cell culture devices, such as silicone, flouroethylenepolypropylene, polyolefin, and ethylene vinyl acetate copolymer. A wide range of sources for learning about gas permeable materials and their use in cell culture can be used for

additional guidance, including co-pending U.S. patent application Ser. No. 10/460,850 incorporated herein in its entirety. The use of the words film and membrane imply a very thin distance across the gas permeable material, and the inventors have found that the embodiments of this invention function when the gas permeable material of the described devices and methods is beyond the thickness associated with films and membranes. Therefore, the portion of the device that contributes to gas exchange of the culture is called a gas permeable material herein.

Those skilled in the art will recognize that the gas permeable material should be selected based on a variety of characteristics including gas permeability, moisture vapor transmission, capacity to be altered for desired cell interaction with cells, optical clarity, physical strength, and the like. A wide variety of information exists that describe the types of gas permeable materials that have been successfully used for cell culture. Silicone is often a good choice. It has excellent oxygen permeability, can allow optical observation, is not easily punctured, typically does not bind the cells to it, and can be easily fabricated into a wide variety of shapes. If silicone is used, it may be less than about 0.2 inches, about 0.1 inches, about 0.05 inches, or about 0.030 inches in the areas where gas transfer is desired. The best selection of material depends on the application. For example, Teflon® may be preferred in applications that will be exposed to cryopreservation. For adherent culture, in which cells are to attach to the gas permeable material, WO 01/92462, U.S. Pat. No. 4,939,151, U.S. Pat. No. 6,297,046, and U.S. patent application Ser. No. 10/183,132 are among the many sources of information that provide guidance.

If silicone is used as a gas permeable material, increasing thickness beyond conventional wisdom may expand the options for design, cost reduce the manufacturing process, and minimize the possibility of puncture. For example, molding a part with a large surface area when the part must be very thin can be difficult because material may not flow into the very small gap between the core and the body of the mold. Thickening the part, which widens that gap, can make the molding process easier. In additional to possible molding advantages, thicker gas permeable materials also are less likely to puncture or exhibit pinholes.

The height of walls, e.g., walls **40**, plays an important role in device scale up efficiency. Prior static gas permeable devices limit medium height. For example, bags provide no walls and instructions limit medium height, while cartridge style devices only provide a very low wall height (e.g. Opticell® cartridges, CLINIcell® Culture Cassettes, and Petaka™ cartridges). An object of this invention is to provide for increased medium height, thereby increasing device efficiency. The height of the walls can dictate how much medium is allowed to reside in the device. Adding medium provides a larger source of substrates, and a larger sink for waste products. By increasing wall height when more medium is needed during scale up, the geometry of the device is more compatible with the shape of incubators, flow hoods, and biohazard disposal bags. Furthermore, the increase in volume relative to the surface area upon which cells reside can allow more medium per cell to be present. That can have the effect of reducing feeding frequency, thereby reducing labor and contamination risk. It can also have the effect of increasing the number of cells residing per square centimeter of device footprint.

Structuring walls to allow an increase in medium volume can also have the beneficial effect of diminishing the effects of medium evaporation. Medium evaporation is a problem in cell culture because it alters the concentration of solutes residing in the medium. Existing gas permeable devices are prone to such an event because they have a high gas permeable surface area to medium volume ratio. Attempts to prevent such an event are described in U.S. patent application Ser. No. 10/216,554 and U.S. Pat. No. 5,693,537 for example. However, simply allowing an increase in the volume of medium in the device can reduce the impact of evaporation. If prior static gas permeable devices allowed an increase in medium volume to gas permeable surface area ratio, the rate of solute concentration change when evaporation is present would be reduced proportionally.

In a preferred embodiment, walls should be capable of allowing medium to reside at a height that exceeds that of devices that rely upon a gas/liquid interface and more preferably exceeds that of typical static gas permeable devices. For example, the height of wall **40** is beyond 3 mm, and more preferably beyond 2.0 cm, and will thus provide advantages. By providing users of the device the option of adding more medium to the device than prior gas permeable devices, many advantages accrue including the ability to house more cells per device, feed the device less frequently, and scale the device up without increasing the footprint. Walls can be comprised of any biocompatible material and should mate to lower gas permeable material in a manner that forms a liquid tight seal. The methods of mating a lower gas permeable material to walls include adhesive bonding, heat sealing, compression squeeze, and any other method commonly used for generating seals between parts. As an option, walls and lower gas permeable material can be formed of the same material and fabricated as a single entity. For example, if silicone is used, walls and the lower gas permeable material could be liquid injection molded, or dip molded, into a single gas permeable piece. That has the advantage of creating a gas permeable surface for cells to reside upon when a gas permeable cell culture device is stood vertically as shown in FIG. **4**B, or laid on its side as shown in FIG. **4**C, which shows gas permeable wall **41** with cells **20** resting thereupon.

Laying certain gas permeable cell culture devices on a side can help make optimal use of incubator space as the profile of the device can be reduced when it is too tall for narrowly spaced incubator shelves. In the case where it is desirable to have the gas permeable cell culture device reside on its side, making the device square or rectangular, instead of circular, will create a flat surface for cells to reside on when on its side. That is advantageous as it prevents localized areas for cells to pile upon each other, potentially causing harmful gradients. In the case where the device depth and width differ in dimension, three alternate surface areas are available for cells to reside upon, and three alternative maximum medium heights exist, depending on the position gas permeable cell culture device is placed in. When the device is structured for operation in these alternate positions, the surface upon which the device resides is preferably comprised of gas permeable material. That allows cells that settle by gravity onto this surface to be at optimal proximity for gas exchange.

Walls are preferably configured with enough structural strength that medium is retained in a relatively symmetrical shape above gas permeable material in order to make most efficient use of lab space, minimize gradient formation within a medium, and allow a uniform deposit of cells upon a lower gas permeable material during inoculation. It is also advantageous if walls allow visual assessment of color changes in medium in order to determine pH or contamination status. Walls may be configured in a manner that allows a gas permeable cell culture device to be easily lifted

by hand. When it is desirable for walls to be gas permeable, and if a separate entity is placed around walls to retain them in a rigid position, it preferably should not block gas contact with the majority of walls.

Gas permeable cell culture devices can be configured to function either in the static or rolled mode. To do so, gas permeable cell culture devices should preferably be cylindrical. A cylindrically shaped body provides more volume than a square or rectangular body when the device is to be placed in a standard roller rack. However, a non-cylindrical body shape can still function on a roller rack by attaching a circular housing around the body. If it is desired to provide users with the option of device functioning in the vertical, horizontal, or rolling position, both the bottom and the sidewalls of the gas permeable cell culture device should be comprised of gas permeable material. If the device is only to be operated in the horizontal, rolled or unrolled, position, it may be more cost effective and minimize surface area for evaporation if the ends of the device are not comprised of gas permeable material.

If a gas permeable cell culture device is configured in a cylindrical shape with a lower gas permeable material, and the walls are comprised of gas permeable material, it can be stood vertically or rolled depending on user preference. It can be advantageous to roll gas permeable cell culture device when maximum mixing will benefit an application, such as can be the case when seeking to decrease antibody production time. If this option is desired, the walls of gas permeable cell culture device should be made gas permeable in the same manner described for lower gas permeable material. Although there are no restrictions on bottle length or diameter, it can be advantageous if the walls conform to the diameter of standard roller bottles so that gas permeable cell culture device can function on a standard roller rack.

If it is desirable to reduce cell shear, filling the device entirely with medium will eliminate gas from the device so that it cannot contribute to cell shear. The ports can be designed in any number of ways that reduce the risk of contamination as medium fills the device entirely. Also, when the device is to be rolled or function on its side, only side surfaces need be comprised of gas permeable material.

The scale up advantages provided by a device that allows medium to reside at a height that exceeds conventional wisdom will become apparent to those skilled in the art, in light of the Examples demonstrating biological outcomes herein. As an example of scale up efficiency, when a gas permeable cell culture device is cylindrical, operated in the vertical position, and the bottom provides for gas exchange, doubling the diameter increases the volume by a factor of four when the height is held constant. For example, a device of approximately 4.5 inches in diameter and about 7.7 inches tall, will house about 2 L of medium. By making the device 9.0 inches in diameter, it will house 8 L of medium. By making the device 18.0 inches in diameter, it will house 32 L of medium. Thus, culture volume can easily be scaled up while holding key parameters constant, such as the medium height and gas permeable surface area to medium volume ratio. By holding these parameters constant, protocols that are developed in a small volume device are likely to remain unchanged as device volume increases.

When a gas permeable cell culture device is operated in the vertical position, and suspension cells are being cultured, it is beneficial if ambient gas can make relatively unobstructed contact with the underside of the lower gas permeable material. For example, in incubators in which the shelves are non perforated, gas transfer in and out of the culture can be limited if the lower gas permeable material makes contact with the incubator shelf. In the embodiment shown in the cross-sectional view of FIG. **5**, lower gas permeable material support **80** acts to ensure that lower gas permeable material **30** is in contact with ambient gas by maintaining a gas compartment **90**. In the preferred embodiment, gas compartment **90** is maintained by allowing lower gas permeable material support **80** to make partial contact with lower gas permeable material **30** in a manner that does not diminish the amount of gas exchange required to support the culture. In addition to allowing exposure to ambient gas, lower gas permeable material support **80** maintains lower gas permeable material **30** in a substantially horizontal state such that cells **20** do not pile up in any low points. That would cause diffusional gradients and limit cell growth relative to a condition in which cells **20** could distribute evenly across lower gas permeable material **30**. Therefore, a design objective for lower gas permeable material support **80** may be to contact lower gas permeable material **30** in as many locations as needed to keep it substantially horizontal while still allowing adequate gas contact with the lower surface of lower gas permeable material **30**. Those skilled in the art will recognize there are many ways to achieve this objective. As shown in FIG. **5**, projections **110** achieve this objective.

A "bed of nails" configuration is one way to maintain lower gas permeable material **30** in a substantially horizontal position while allowing adequate gas exchange. For example, 1 mm×1 mm squares, distributed evenly and projecting 1 mm from the lower gas permeable material support can retain the lower gas permeable material in a substantially horizontal position. When the projections **110** occupied 50% of the surface of lower gas permeable material support **80** as shown in FIG. **5**, this configuration allowed adequate gas exchange to culture about 10 to 15 million murine hybridoma cells per square centimeter on a silicone membrane of about 0.004 inches thick. As also shown in FIG. **5**, lower gas access openings **100** allow gas to enter and exit gas compartment **90** of lower gas permeable material support **80** by passive diffusion. This allows gas permeable cell culture device **10**B to function in ambient conditions without need of ancillary pumping mechanisms. Feet **95** elevate lower gas permeable material support **80**, allowing ambient gas to be available to lower gas access openings **100**. This information also is applicable to maintaining a gas compartment around sidewalls when the device functions as described on its side in either the rolling or non-rolling mode. Other possibilities of allowing adequate gas access to a gas permeable material can be utilized. For example, the CELLine™ products from Integra Biosciences AG utilize open mesh elevated from a lower plastic support by feet to allow gas access to the gas permeable membrane. U.S. Pat. No. 5,693,537 also provides additional guidance for this feature.

In the configuration shown in FIG. **5**, cap **70** covers medium access port **60** to prevent contamination. O-ring **75** ensures that medium **50** will not leak from gas permeable cell culture device **10**B, such as when it is in the horizontal position, completely filled, or accidentally dropped.

In certain embodiments, the medium does not need to reside entirely above the lower gas permeable material. A portion of the medium can reside in areas not directly above a lower gas permeable material in order to reduce the profile of a vertical cell culture device, which may be desirable for use in incubators with limited distance between shelves. The cross-sectional view of FIG. **6** shows an embodiment configured for suspension cell culture in which walls **40**C are offset from lower gas permeable material **30** in order to

decrease the profile of gas permeable cell culture device **10**C when operated in the vertical position. In this configuration, the ratio of medium volume to surface area upon which cells reside can be held constant while the profile of the device is reduced in size by simply increasing the width, or diameter, of gas permeable cell culture device **10**C. Care should be taken to ensure that cells **20** continue to reside above lower gas permeable material **30** during inoculation, feeding, and handling. Interior walls **42** achieve this by allowing gravity to keep cells **20** in the area above lower gas permeable material **30**. In a preferred embodiment, the walls should be capable of allowing medium to reside at a height above lower gas permeable material **30** that exceeds 3 mm.

FIG. **7**A and FIG. **7**B show cross-sectional views of a preferred embodiment for a gas permeable cell culture device that can raise or lower its height in response to the volume of medium residing within it. In FIG. **7**A, medium **50** is added to gas permeable cell culture device **10**D and makes contact with buoyant shoulder **25**. In FIG. **7**B, medium **50** exerts an upward force on buoyant shoulder **25**, causing gas permeable cell culture device **10**D to rise in height in response to the increasing volume of medium **50**. In the configuration shown, walls **40**D are bellows shaped to allow extension and contraction of the height of gas permeable cell culture device **10**D. Buoyant shoulder **25** can be any biocompatible material that is less dense than medium **50**. It can also be an integral part of walls **40**. It should be sized to displace the appropriate volume of medium **50** in order to exert enough force to extend gas permeable cell culture device **10**D upward. In this configuration, gas permeable cell culture device **10**D only occupies as much space as needed to perform the culture and one product can be the optimal size for a variety of applications. For example, the volume of medium suitable for culturing hybridomas may differ from the amount of medium suitable for maintaining pancreatic islets. In that case, gas permeable cell culture device **10**D only need occupy as much space as needed for each application. Also, it allows sterilizing, shipping, storage, incubation, and disposal in the minimum volume condition, thereby reducing the cost of the culture process. Those skilled in the art will recognize that there are many other ways of altering the device profile other than buoyancy, including a wide variety of mechanical mechanisms such as those described in co-pending U.S. patent application Ser. No. 10/460,850.

FIG. **8** shows an embodiment for a gas permeable multiple well plate **15**, in which the bottom of each well is gas permeable. The properties of lower gas permeable material **30**A are the same as those described in the embodiment of FIG. **4**A. Although a six well plate is shown, any number of individual wells **45** can be present, including the traditional formats of six, twenty-four, forty-eight, and ninety-six wells. Walls **40**E are structured to allow medium to reside at a height above lower gas permeable material **30**A that exceeds the wall height of traditional multiple well plates, thereby increasing the number of cells that can reside in each well while reducing the footprint relative to traditional multiple well plates. For example, murine hybridoma cells typically can reside at a density of 1×10^6 cells per ml of medium. When the well has a diameter of 8.6 mm, and 2 mm of medium height, 0.12 ml of medium is present and about 0.12×10^6 cells can reside per well. However, if 1 ml of medium could reside in the well by making the wall taller, enough medium to support nearly five times as many cells (i.e. 1×10^6 cells per ml) could be present per well, provided that number of cells could reside upon a gas permeable material with a surface area of 0.58 cm^2 (i.e. 8.6 mm diameter). Example 1 demonstrates that many more than 1×10^6 murine hybridoma cells can reside on a surface area this size depending on medium volume. Not only can more medium support more cells, it can allow feeding frequency to be reduced, and reduce the rate at which evaporation alters medium composition.

Walls can be comprised of any biocompatible material and should mate to the lower gas permeable material in a manner that forms a liquid tight seal. The methods of mating lower gas permeable material **30**A to walls **40**E are the same as those described for the embodiment of FIG. **4**A. Also, as described in the embodiment of FIG. **4**A, walls **40**E and lower gas permeable material **30**A can be formed of the same material and fabricated as a single entity. Lower gas permeable material **30**A can be supported in a substantially horizontal position as shown in FIG. **5**, where lower gas permeable material support **80** is configured with lower gas access openings **100** in communication with gas compartment **90**. In the event that the span of the bottom of well **45** is small, support may be unnecessary because the physical strength of lower gas permeable material **30**A can retain it in an adequate horizontal position, depending on the thickness and physical properties of the gas permeable material. In this case, feet **95**A can be used to elevate gas permeable multiple well plate **15** so that gas transfer is not a problem in an incubator with non-perforated shelves. Top cover **55**A prevents contamination and minimizes evaporation.

FIG. **9**A shows a cutaway of a perspective view, and well **45**A of FIG. **9**B shows cross-section A-A, of a preferred embodiment for a gas permeable multiple well plate **16**. In this embodiment, the walls of the wells are gas permeable. Although a six well plate is shown, any number of individual wells **45**A can be present, including the traditional formats of six, twenty-four, forty-eight, and ninety-six wells. This configuration may be useful when it is desirable to retain either the microscopic, attachment surface, or light visibility properties of the traditional multiple well tissue culture plate. Yet, by making each well **45**A deeper than the maximum depth of traditional multiple well plates used for cell culture, more medium can be made available for culture and the gas permeable nature of the walls will allow proper gas exchange of the culture, rendering the location of the gas/liquid interface inconsequential. Non-gas permeable bottom **31** mates to gas permeable wall **41** in a liquid tight manner. There are a number of ways to achieve this objective. For example, the diameter of non gas permeable bottom **31** can slightly exceed the diameter of gas permeable wall **41**, causing gas permeable wall **41** to apply a force against non gas permeable bottom **31**, thereby creating a liquid tight seal. Gas permeable wall **41** can have any of the properties as described for the gas permeable material of FIG. **4**A. However, in a preferred embodiment gas permeable wall **41** is comprised of silicone because of its ability to be easily fabricated by liquid injection molding, and its capacity to stretch and provide a liquid tight seal against non-gas permeable bottom **31**. Non-gas permeable bottom **31** can be any plastic commonly used in traditional multiple well tissue culture plates, or any other cell attachment material known to those skilled in the art.

It may be less expensive to fabricate each well of gas permeable multiple well plate **16** out of gas permeable material, including the well bottom, thereby eliminating the seal joint. Then, if adherent culture is desired, a suitable scaffold can be placed at the bottom of the well. Care should be taken to ensure optical clarity if microscopic evaluation is desired. Any cell attachment surface known to those skilled in the art of cell culture can be placed in the wells.

If the cell attachment surface is buoyant, making it a press fit into the well can keep it in the desired position. Many other methods of retaining it in position are also possible.

FIG. **10**A and FIG. **10**B show cross-sectional views of one embodiment of a gas permeable cell culture device that utilizes space more efficiently when culturing adherent cells. Scaffolds **120** reside within gas permeable cell culture device **10**E. Sidewalls **40**F are comprised of a gas permeable material, thereby allowing gas exchange through the sides of the device. In this manner, gas permeable cell culture device **10**E is not limited in height, as scaffolds **120** can be scaled uniformly as height increases. Allowing more cells to be cultured is simply a matter of making the device taller, adding more scaffolds **120**. In the preferred embodiment, the distance between each scaffold **120** is kept constant during scale up. For example, by configuring scaffolds **120** to have spacers **135**, they can be kept an equal distance apart and retained parallel to the bottom of gas permeable cell culture device **10**E, making scale up in the vertical direction linear. Pipette access opening **125** allows pipette access throughout gas permeable cell culture device **10**E and provides an opening to vent gas as medium is added. Although shown in the center, pipette access can be in any location, or can be eliminated entirely in favor of any other form of liquid handling such as needles and septum. In FIG. **10**A, cells **20**A are well suspended in inoculum **130** and will distribute evenly about the upper surface of each scaffold **120**, since the volume of inoculum **130** above each scaffold **120** is equal. If both sides of scaffold **120** are intended to culture adherent cells, inoculation can occur in two steps by inoculating one side of scaffolds **120** first, as shown in FIG. **10**A. After cells have gravitationally deposited and attached onto the surface of scaffolds **120**, gas permeable cell culture device **10**E is then re-inoculated, rotated one hundred eighty degrees to expose the opposite side of scaffolds **120**, and cells **20**A are allowed to settle and attach to the exposed surface of scaffolds **120** as shown in FIG. **10**B.

Post cell attachment, typically less than 24 hours to seed one side of the scaffolds, the device can be operating in any static position that is convenient, such as vertical, inverted, or on its side. If desired, it can be rolled if a user desires a format more similar to a roller bottle. Unlike traditional devices, the device can be filled completely with medium, as gas exchange occurs by way of the gas permeable walls and the need for a gas/liquid interface is not present. In this manner, the device is more efficient in its use of space than traditional devices since gas does not need to be present in the device for gas exchange of the culture. The limiting factors to the number of cells that can be cultured in the device include the amount of scaffold surface area, the volume of medium present, the gas permeability and thickness of the material used for the device walls, the distance the cells reside from the gas permeable walls of the device, and the type of cells being cultured.

Understanding the importance of the medium volume to scaffold area ratio when designing the gas permeable cell culture device can help predict the output of the device. For instance, if the culture has been historically conducted in a roller bottle, the medium volume to surface area of the roller bottle culture can be replicated in the gas permeable cell culture device. For example, if the existing culture had been performed in a traditional 850 cm^2 roller bottle using 150 ml of medium, and the gas permeable cell culture device was to have the same outside shape as the traditional bottle, the medium volume to surface area ratio could be held constant. A gas permeable cell culture device constructed in the shape of the traditional 850 cm^2 roller bottle can hold about 2200 ml of medium. That is a 14.67 fold increase in medium volume relative to the 150 ml medium volume of the traditional roller bottle. Therefore, a 14.67 fold increase in surface area, which is 12,470 cm^2, is needed to keep an equivalent medium to surface area ratio. Thus, when a gas permeable cell culture device contains 2200 ml of medium and has a scaffold surface area of 12,470 cm^2, it can be expected to culture the same number of cells as about fifteen traditional 850 cm^2 roller bottles that normally operate with 150 ml per bottle, and the feeding frequency should be about the same.

The ability to microscopically assess cell confluence is useful for many applications. If the lowest scaffold comprises the bottom of gas permeable cell culture device, it can be used to assess cell confluence. When the volume of medium residing above each scaffold is equal during inoculation, the amount of cells residing upon any of the scaffolds will be relatively equal throughout the culture. Thus, one scaffold can be representative of the others. For some microscopes, the ability to physically move the lowest scaffold into a position that allows microscopic observation by inverted scopes can allow a better assessment of confluence and morphology. The configuration shown in the cross-sectional view of FIG. **11** shows how this can be achieved. If wall 4GH is flexible, as will be the case when it is fabricated out of many gas permeable materials such as silicone, it can be pleated to allow movement of the lowest scaffold **120** relative to gas permeable cell culture device **10**F. Microscopic evaluation can also be made possible by manufacturing gas permeable cell culture device **10**F in the fixed position shown in FIG. **11**, thereby eliminating the need to move the lowest scaffold **120** relative to gas permeable cell culture device **10**F.

Although the scaffolds shown in FIG. **10**A, FIG. **10**B, and FIG. **11** are flat, they can be any geometric shape that allows cells to attach. For example, corrugating the surface can increase surface area relative to a planar surface, thereby increasing the amount of adherent cells that can reside upon a given scaffold. FIG. **12**A shows a perspective view of a round corrugated scaffold **120**A, which is corrugated in a linear direction. FIG. **12**B shows cross-sectional view A-A. FIG. **12**C shows a perspective view of round corrugated scaffold **120**B, which is corrugated in the circular direction, and FIG. **12**D shows cross-sectional view B-B. For some applications in which a high rate of gas transfer is needed to support highly active cells, the configuration of FIG. **12**A may be superior because the channels for gas transfer are unobstructed by the edge of the scaffold, as is the case for the configuration of FIG. **12**C. For other applications in which the gas permeable cell culture device is rolled, the configuration of FIG. **12**C may be superior because the shape will minimize turbulence, which could cause cell shear.

The configurations, methods of microscopically viewing, and methods of increasing scaffold area such as those described in FIG. **10**A, FIG. **11**, and FIG. **12**, can be integrated into a multiple well format. These configurations are completely scalable in size. FIG. **9**B shows high surface area well **46**, configured with multiple scaffolds **120** maintained a predetermined distance apart by spacers **135**. Making them the size of the wells of a typical traditional multiple well tissue culture plate will allow a substantial increase in

the number of adherent cells present per well. The walls **41**A are preferably gas permeable.

FIG. **13** shows a cutaway view of configuration for a gas permeable cell culture device that is useful for culturing cells in a format similar to that of a tissue culture flask. In this embodiment, at least one wall of the device provides gas transfer. This device is beneficial because it allows the gas permeable cell culture device to retain the same attributes as the traditional tissue culture flask while achieving a more compact use of space. The desirable attributes include easy medium delivery and removal by way of pouring or pipetting, microscopic observation capability, the ability to easily see color changes in the medium that may indicate contamination or pH changes, and capability for device stacking to make the most efficient use of shipping, storage, and incubator space. However, it is superior to the tissue culture flask because the gas/liquid interface required for tissue culture flask operation is eliminated and one or more scaffolds can be present. In the embodiment shown, gas permeable cell culture device **12** is comprised of a liquid tight enclosure with at least one gas permeable wall **200**. Medium access port **60**A is covered by cap **70**A. Scaffolds **120**D are oriented parallel to each other, with a gap between them to allow inoculum and medium to reside in between each scaffold **120**D. Preferably, scaffolds **120**D are positioned an equal distance apart to allow an equivalent volume of inoculum or medium to reside above each of them. The gas permeable material of gas permeable wall **200** has the same attributes as those described for lower gas permeable material **30** of the embodiment shown in FIG. **4**A. In the preferred embodiment, scaffolds **120**D have identical material characteristics as those present in traditional tissue culture flasks. Top wall **201** and bottommost scaffold **120**D are clear, allowing visual assessment of medium color as well as microscopic evaluation of the bottom scaffold **120**D. Making the rear or other walls gas permeable can create more gas transfer capacity. That will have the effect of making it possible to further increase the footprint of gas permeable cell culture device **12**. For example, if the gas transfer capacity of gas permeable wall **200** supports cells residing upon scaffolds **120**D of a five inch width, making the opposing side wall gas permeable will allow enough gas transfer capacity when scaffolds **120**D that are ten inches wide. Gas permeable cell culture device **12** is unlimited in scale up capacity in the vertical direction.

FIG. **14**A through FIG. **14**E show another method of utilizing space more efficiently when culturing cells. In this configuration, scaffolds **120**F reside within gas permeable cell culture device **10**G, which is capable of expanding in volume as medium **50** is added. In FIG. **14**A, gas permeable cell culture device **10**G is in a collapsed position under its own weight. That allows efficient use of space for shipping, sterilization, and storage prior to use. Scaffolds **120**F are as close to each other as possible. Each scaffold **120**F is molded with spring arms **145** that exert force on the lower, neighboring scaffold **120**F. Spring arms **145**, in compression, want to distend, but cannot because the weight of the upper portion of gas permeable cell culture device **100** exceeds the spring force. In FIG. **14**B, gas permeable cell culture device **10**G has risen in height in response to the force exerted by the addition of inoculum **130**A against buoyant shoulder **25**A. The displacement of inoculum **130**A by buoyant shoulder **25**A exerts an upward force that, when combined with the spring force of spring arms **145**K, exceeds the weight of the upper portion of gas permeable cell culture device **100**. Scaffolds **120**F separate and maintain an equal distance from each other due to the force exerted by spring arms **145** against their lower, neighboring scaffold **120**F. Maintaining an equal distance from each other is particularly beneficial during inoculation, when the volume of inoculum **130**A residing directly above each of scaffolds **120**F dictates the amount of cells that will be deposited onto each of scaffolds **120**F. By allowing an equal volume of inoculum **130**A to reside above each scaffold **120**F, and equal number of cells can reside upon each scaffold **120**F. In FIG. **14**C, gas permeable cell culture device **10**G has risen in height again relative to FIG. **14**B in response to the addition of medium **50** as the cell population expands and nutrient demand increases. Scaffolds **120**F further separate and maintain an equal distance from each other due to the force exerted by spring arms **145** against their lower, neighboring scaffold **120**F. The constant distance between each of scaffolds **120**F ensures a constant medium **50** volume to surface area ratio at all cell locations, reducing the potential for gradient formation. In FIG. **14**D, gas permeable cell culture device **10**G has collapsed due to the removal of medium **50** and loss of upward force of buoyant shoulder **25**A. It is now at an efficient size for disposal. In the event that adherent cell recovery is needed, allowing gas permeable cell culture device **10**G to collapse is beneficial when removing medium **50** and adding trypsin. In this manner, only a small volume of trypsin is needed to recover cells. Those skilled in the art will recognize that many other methods of altering the height of gas permeable cell culture device **10**G can be applied.

Spring arms **145** can be molded directly into scaffold **120**F, as shown in the perspective view of FIG. **14**E. A spring arm **145**, preferably located in at least three places, ensures that scaffold **120**F remains in plane and parallel to its neighboring scaffold **120**F. Although any material conducive to cell attachment is acceptable, a preferred material for scaffold **120**F is polystyrene, which is quite brittle. Therefore, care should be taken to ensure that spring arms **145** are configured in accordance with good molded part design to prevent cracking under stress. Techniques for low stress part design are well known to those skilled in the art of plastic part design.

Moving the position of the scaffolds independent of the height of the gas permeable cell culture device may be desired. For example, this may be practical when it is more economical to configure the gas permeable cell culture device with non-extending walls, but the application can still benefit by altering the medium volume to surface area ratio above each of the scaffolds during culture. FIG. **15**A through FIG. **15**C show one embodiment for achieving that objective. For clarity, only a portion of the gas permeable cell culture device is shown. In the top view of a portion of a gas permeable cell culture device shown in FIG. **15**A, three elevation posts **160** are positioned to travel up each of three ramps **150** in order to change the distance between the scaffolds.

The method of varying the distance between scaffolds can best be understood by reviewing FIG. **15**B and FIG. **15**C. FIG. **15**B shows cross-section A-A of FIG. **15**A. As shown in FIG. **15**B, two scaffolds **120**G are shown the position in which the distance between them is at a minimum. Ramp **150** emanates from the top of scaffold **120**G and elevation post **160** emanates from scaffold locator screw **170**. Elevation post **160** has not begun travel up ramp **150**. It can be

seen that the minimum distance between scaffolds is dictated by the height of ramp **150**, which makes contact with the underside of the scaffold **120**G that resides above it. Referring to FIG. **15**C, scaffolds **120**G are in the position of maximum distance between them. Scaffold locator screw **170** has been rotated in the direction of rotation arrow **180**, causing elevation post **160** to rise up ramp **150** and elevate the scaffold **120**G residing above it. When elevation post **160** resides at the highest point of ramp **150**L, the maximum distance between scaffolds **120**L is attained as is equal to the height of ramp **150** plus the height of elevation post **160**. Scaffolds **120**G should be prevented from rotating when scaffold locator screw **170** is turned, thereby allowing ramp **150** to remain in a fixed position while elevation post **160** travels up it. This can be achieved by mating scaffolds **120**G to the interior of the gas permeable cell culture device wall by way of a tongue and groove arrangement. As best shown in the top view of a scaffold of FIG. **15**A, tongue **212** emanates from gas permeable wall **40**H and mates to groove **215** in each scaffold **120**G. Not only does this prevent rotation of scaffold **120**G during rotation of locator screw **170**, it also prevents gas permeable wall **40**H from pulling away from scaffold **120**G. In this manner, the shape of the gas permeable cell culture device is retained. Locator screw **170** can be configured to allow a sterile pipette tip to rotate it, thereby preventing contamination of the device and allowing the use of standard laboratory tools to rearrange the distance between scaffolds.

The invention will be further described with reference to the following non-limiting Examples.

EXAMPLES

Example 1

The Effect of Medium Height Upon Cell Growth and Antibody Production

Evaluations were conducted in order to assess the impact of altering medium height upon cell growth and antibody production in a device comprised of a lower gas permeable material. The effect of altering the gas permeable material surface area to medium volume ratio was also assessed. Single compartment test fixtures configured with a lower gas permeable materials and the capacity to hold medium at heights beyond conventional wisdom were compared to single compartment control test fixtures that held medium at a height within the bounds of conventional wisdom. Comparisons were made relative to the 1.6 cm medium height limits specified for the Si-Culture bag (U.S. Pat. No. 5,686,304). Control test fixtures were configured to house medium at a height of 1.6 cm, and the gas permeable material used for of all test fixtures consisted of gas permeable material obtained from actual Si-Culture™ bags.

Tubular test fixtures **105** were constructed as shown in FIG. **16**. Walls **401** were machined out of Ultem 1000 (high temperature polycarbonate) cylindrical stock, resulting in a tube with an inner diameter of 1.00 inch and an outer diameter of 1.50 inch. The thick walls ensured that gas transfer through the walls would not assist the cultures. Lower gas permeable material **30**A was fabricated from 0.0045 inches thick sheets of silicone removed from Si-Culture™ bags and secured in a liquid tight manner to the bottom of the machined tube yielding a 5.07 cm^2 growth area for cells **20**B to reside upon. Lower gas permeable material support **80**M was also machined out of Ultem 1000. Lower gas permeable material **30**A was held in the horizontal position by mesh **115** which maintained gas compartment **90**A. Mesh **115** was comprised of 0.020 inch diameter strands at 16 strands per inch. Lower gas access openings **100**A allowed gaseous communication with the 5% CO_2, 95% R.H., and 37C ambient environment. Comparisons were made for the capacity of the devices to grow cells **20**B when differing amounts of medium **50**A resided within the test fixture. Cap **70**B, secured tightly to walls **401**, protected tubular test fixture **105** from contamination. Tests compared the results when medium **50**A resided at a height of about 1.6 cm, 3.2 cm, 5.6 cm, 10.2 cm, 15.3 cm, and 20.4 cm above the cells. Medium **50**A consisted of Hyclone HyQSFM4MAb-Utility supplemented with 10% Hyclone FBS. Cells **20**B were murine hybridoma cells secreting IgG, inoculated at a seeding density of 0.76×10^6 per cm^2 of lower gas permeable material **30**A. Ambient conditions were 5% CO_2, 95% R.H., and 37 C. Periodic cell counts and monoclonal antibody production measurements by ELISA were taken. TABLE 1 shows the results.

TABLE 1

Medium Height Affect Upon Cell Growth and Antibody Production

Volume of medium (ml)	Height of medium above gas permeable material (cm)	Gas permeable surface area to medium volume ratio (cm^2/ml)	Maximum live cells per device ($\times10^6$)	Maximum live cells per cm^2 of gas permeable material ($\times10^6$)	Mab produced per test fixture (ug)	Time to maximum amount of mab produced (days)	Mab per ml of medium consumed (ug/ml)
8.1	1.60	0.63	29.7	5.85	2742	9	339
16.2	3.20	0.31	51.0	10.05	7395	12	457
25.8	5.09	0.20	59.1	11.65	10673	18	374
51.7	10.20	0.10	61.1	12.05	15252	15	295
77.6	15.31	0.07	67.2	13.25	23044	22	299
103.4	20.39	0.05	86.4	17.04	32881	25	318

Dividing each parameter measured in any given test fixture by the corresponding parameter of the test fixture representing conventional wisdom (i.e. 1.6 cm) clearly shows the advantages of allowing medium to reside at heights beyond conventional wisdom. Gas permeable surface area to medium volume ratio is determined by dividing the ratio of the test fixture by the ratio of the Si-Culture™ bag when it contains medium at a height of 1.6 cm (i.e. 1.25 cm^2/ml). TABLE 2 presents the data of TABLE 1 in this manner.

TABLE 2

Normalized data						
Normalized by height of medium above gas permeable membrane	Normalized by maximum live cells per device	Normalized by gas permeable surface area to medium volume ratio relative to Si-Culture ™ bag	Normalized by Mab produced per test fixture	Normalized by Mab per ml of medium consumed	Normalized by time to attain maximum Mab amount	Normalized by footprint of space occupied
1.00	1.00	50%	1.00	1.00	1.00	1.00
2.00	1.72	25%	2.70	1.35	1.50	0.50
3.18	1.99	16%	3.89	1.11	2.00	0.28
6.38	2.06	8%	5.56	0.87	1.67	0.16
9.57	2.26	6%	8.40	0.88	2.50	0.10
12.75	2.91	4%	11.99	0.94	2.83	0.08

The data of TABLE 2 clearly shows the advantages of altering the geometry of gas permeable cell culture devices to allow more medium to reside above the cells. For example, the last row shows that when the device is allowed to hold medium at a height that is 12.75 times greater than the traditional cell culture bag, it is capable of culturing 2.91 fold more cells per cm^2 of floor space occupied, producing 11.99 times more monoclonal antibody (Mab) with only a 2.83 fold increase in the time to complete production. Also, when the gas permeable material surface area to medium volume ratio is compared to that of the Si-Culture™ bag, dramatically reduced ratios are possible. Cultures were effectively grown even when the ratio was only 4% of that used by the Si-Culture™ bag. That allows a wider variety of device configurations to exist, including allowing the device footprint to remain fixed as medium height is increased. It also minimizes the effects of evaporation, as more medium is present per cm^2 of gas permeable surface area.

Importantly, this data demonstrates that device footprint can remain small as the culture is increased. TABLE 3 shows the surface area of the device footprint needed to house the volume of medium residing in the test fixtures. The first row shows the medium volume in the test fixture. The second row shows the footprint area of the test fixture, which remained fixed as more and more medium was added. The third row shows the footprint surface area that would be required in a typical bag to hold the volume of medium residing in the test fixture. In this case, the footprint is shown for a Si-Culture™ bag when it contains the volume of row one at the manufacturers recommended medium height of 1.6 cm. The fourth row shows the difference in footprint area. For example, when the test fixture contains 103.4 ml of medium, the Si-Culture™ bag when operated according to manufacturers recommendation would have a footprint of 64.6 cm^2, but the test fixture only has a footprint of 5.1 cm^2. Thus, the test fixture that allowed medium to reside at a height of 20.39 cm only needed a footprint of 8% of that needed for a Si-Culture™ bag to produce roughly the same amount of Mab.

TABLE 3

Much more efficient use of floor space.						
	Volume of medium in device (ml)					
	8.1	16.2	25.8	51.7	77.6	103.4
Test fixture footprint (cm^2)	5.1	5.1	5.1	5.1	5.1	5.1
Bag footprint with medium at 1.6 cm high (cm^2)	5.1	10.1	16.1	32.3	48.5	64.6

TABLE 3-continued

Much more efficient use of floor space.						
	Volume of medium in device (ml)					
	8.1	16.2	25.8	51.7	77.6	103.4
Ratio of test fixture footprint to bag footprint (%)	100%	50%	32%	16%	11%	8%

Benefits relative to all of the conventional configurations are numerous. The unwieldy shape of traditional cell culture bags can be avoided allowing a wide variety of benefits to accrue related to more efficient use of incubator space, easier medium delivery and removal, and reduced contamination risk. The small volume of medium present in gas permeable cartridges can be increased substantially by making them taller, and reducing the ratio of gas permeable membrane to medium volume capacity. That has the effect of allowing fewer units to be needed during scale up. For traditional gas permeable formats of the petri dish and multiple well plate, more cells can reside per unit without increasing the footprint of the devices, or the number of devices needed, and the frequency of feeding can be reduced. Minimized evaporative effects can be achieved in all configurations because the gas permeable surface area to medium volume ratio can be significantly reduced.

Example 2

Effect of Thickness of Gas Permeable Silicone on Cell Growth

Conventional wisdom, as dictated by U.S. Pat. No. 5,686,304 and U.S. patent application Ser. No. 10/183,132, and the design of commercially available gas permeable products that use silicone, dictates that silicone thickness of greater than 0.005 inches should not be used. However, increasing the thickness is advantageous from a manufacturing and product reliability standpoint. Therefore, evaluations were conducted to assess the impact of the thickness of a lower silicone gas permeable material on cell growth. The material thickness of conventional wisdom was compared to the same material at increasing thickness.

Tubular test fixtures were constructed as shown in FIG. **16**. Walls were machined out of Ultem 1000 (high temperature polycarbonate) cylindrical stock, resulting in a tube with an inner diameter of 1.00 inch and an outer diameter of 1.50 inch. Four distinct thickness configurations of lower

gas permeable material were created from sheets of silicone removed from Si-Culture™ bags. Lower gas permeable material **30**A was made into double, triple, and quadruple layers, formed by adhering the silicone sheets together using UV curing silicone glue distributed evenly about the face and sheets were laminated together leaving no air gaps between them. Post curing, the laminated sheets and a single sheet control were secured in a liquid tight manner to the bottom of the machined tube yielding a 5.07 cm^2 growth area for cells to reside upon. Tests were conducted in triplicate. Lower gas permeable material **30**A was held in the horizontal position by lower gas permeable material support **80**, configured as described in Example 1. Tests compared the results when medium resided at heights of 20.4 cm above the cells. Medium consisted of Hyclone HyQSFM4MAb-Utility supplemented with 10% Hyclone FBS. Murine hybridoma cells were inoculated at a seeding density of 4.3×10^6 live cells per square cm of lower gas permeable material. Ambient conditions were 5% CO_2, 95% R.H., and 37 C. Periodic cell counts and glucose measurements were taken. TABLE 4 shows the results.

TABLE 4

Effect of Thickness of Gas Permeable Silicone on Cell Growth

Membrane Thickness (in)	Maximum viable cells per cm^2 ($\times10^6$)	Normalized: Membrane Thickness	Normalized: Maximum viable cells per cm^2
0.0045	15.2	1.00	1.00
0.016	15.5	3.56	1.02
0.024	13.49	5.33	0.89
0.033	12.0	7.33	0.79

The data was normalized by referencing it against the data collected for the single 0.0045 inch thick sheet that represents conventional wisdom. It can clearly be seen that the effect of dramatically increasing thickness does not have a significantly negative impact on the capacity to support cell growth. When the material thickness was increased about four-fold, from 0.0045 inch to 0.016 inch, there was no affect upon cell growth. When the silicone membrane thickness was increased 5.33 fold, from 0.0045 inch to 0.024 inch, the growth capacity was diminished by only 11%. Likewise, a 7.33 fold increase in thickness beyond conventional wisdom resulted in growth capacity being diminished by only 21%. In many cell culture applications, such as hybridoma culture for monoclonal antibody production, 79% viability is routinely accepted. For example, in the CELLine™ products, hybridoma viability is commonly at 50%, as described in the operating instructions. Thus, device design can accommodate thicker silicone walls without a dramatic reduction in performance. Fabrication and functional improvements may result from increasing the thickness, such as simplified liquid injection molding or less pinhole potential. In summary, it is possible to design a highly functional cell culture device with thicker walls than previously believed possible.

Example 3

The Ability to Culture Cells at a High Liquid Height in a Rolled and Unrolled Device

Evaluations were conducted to assess the advantages that could be obtained by configuring gas permeable cell culture devices in ways that differ from conventional wisdom. Two general formats were evaluated, 1) unrolled gas permeable devices and 2) rolled gas permeable devices. In the unrolled gas permeable device configuration, medium height was well beyond the limits imposed by conventional wisdom. The ratio of gas permeable surface area to medium volume was reduced far below that of conventional wisdom. In the rolled gas permeable device configuration, medium was allowed to reside farther away from the gas permeable wall, and more medium was allowed to reside per device, than that of the state of the art gas permeable rolled bottles.

The production of monoclonal antibody is a common application in cell culture bags and roller bottles. A traditional 850 cm^2 roller bottle functioned as a control. Test fixtures were constructed in accordance with the embodiments shown in FIG. **4**, and dimensionally configured to have the same dimensions as a traditional 850 cm^2 Corning® roller bottle. The gas permeable material was the same as that of the Si-Culture™ bag, as further defined in U.S. Pat. No. 5,686,304. The gas permeable surface area of non-rolled test fixture was limited to that of the bottom surface of the fixture, and was 98 cm^2. The sidewalls were not gas permeable. The gas permeable surface area of the rolled test fixture was limited to that of the entire cylindrical sidewall surface of the fixture, and was 850 cm^2, and the ends were not gas permeable. Medium consisted of Hyclone SFM4MAb, supplemented with 2.5% Hyclone FBS. Each test fixture was inoculated with a cell density of 0.04×10^6 murine hybridoma cells per ml of medium used. The test fixtures each received 2050 ml of medium. Ambient conditions were 5% CO_2, 95% R.H., and 37 C.

The traditional roller bottle received 255 ml of medium, the maximum amount of medium recommended for use in roller bottles. The presence of antibody was determined by ELISA. TABLE 5 shows the results.

TABLE 5

Effect of rolling versus standing on antibody production time

Test Fixture Style	Maximum amount of antibody produced (mg)	Time to reach maximum production(days)
Unrolled Novel Device	289	16
Rolled Novel Device	302	13
Traditional Roller Bottle	33	13

TABLE 5 shows how the rolled and the non-rolled gas permeable test fixtures, which occupied the same amount of space as the traditional roller bottle control, were able to produce about nine times as much antibody. TABLE 5 also demonstrates how the rolled gas permeable format can be used to decrease the amount of time needed to generate antibody relative to its standing gas permeable counterpart. A 20% reduction in time, three days, was attained. Importantly, both the roller and unrolled formats can create a at least a nine fold improvement in efficient geometry in terms of space, leading to reduced cost of sterilization, shipping, storage, labor, incubator space, and disposal when compared to the traditional roller bottle.

The results also clearly demonstrate the advantage obtained by configuring gas permeable devices in ways that depart from conventional wisdom. The height of medium in the unrolled test fixture was about 20.9 cm, over ten times the highest recommended height of traditional cell culture bags. Had the device been structured with 2.0 cm of medium height, it would have needed a footprint of 1025 cm^2 to

house an equivalent volume of medium, which is over ten times the footprint of the unrolled test fixture.

Benefits of geometry of the rolled gas permeable device were numerous. The rolled test fixture contained a volume of medium nearly eight times the maximum volume of medium recommended for traditional roller bottles (255 ml), over four times the medium volume of Rotary Cell Culture System™ from Synthecon Inc., nearly five times the medium volume of the MiniPERM, and well beyond that allowed in the patent proposals of Spaudling, Schwarz, Wolf et al., and Falkenberg et al. Also, medium resided up to 5.6 cm from any portion of the gas permeable wall of the test fixture, over double the limit specified in the patent proposals of Spaudling, Schwarz, and Wolf et al. The rolled test fixture was able to function on a standard roller rack, as opposed to the commercially available Rotary Cell Culture System™ from Synthecon™ Inc., and the MiniPERM™ from Vivascience Sartorius Group, which all require custom equipment to roll. Thus, the scale up efficiency of the rolled gas permeable device is much superior to other devices and approaches.

Example 4

Ability to Culture Adherent Cells in the Absence of a Gas/Liquid Interface

Evaluations were conducted to assess the ability to culture adherent cells without the presence of a gas/liquid interface by allowing gas exchange to occur via gas permeable walls. A test fixture was constructed in a manner, as shown in FIG. **17**, that eliminated the possibility of gas transfer by way of a gas/liquid interface. Gas permeable wall test fixture **12** consisted of a rectangular liquid tight enclosure **241**, configured with one gas permeable wall **200**A and five non-gas permeable walls **210**. Gas permeable wall **200**A was composed silicone membrane, approximately 0.0045 inches thick, purchased from Medtronic Inc. (Minneapolis). This membrane is used by Medtronic to fabricate the Si-Culture™ bag. Fluid delivery port **220** and fluid removal port **230** allow inoculation and feeding. Bottom attachment scaffold **240** consisted of a section of plastic removed from a Falcon tissue culture flask in order to provide an equivalent attachment surface as the control Falcon™ T-175 tissue culture flask. The inner dimensions of enclosure **241** were 6 cm deep, 10 cm wide, and 0.635 cm high. Thus, gas permeable wall **200**A was 10 cm wide and 0.635 cm high creating a surface area of 6.35 cm^2. Bottom attachment scaffold **240** was 10 cm wide and 6 cm deep, allowing an attachment surface of 60 cm^2. Gas permeable wall test fixture **12** was filled entirely medium during inoculation, thereby eliminating any gas/liquid interface. Thus, gas exchange could only occur by way of diffusion in the direction perpendicular to gas permeable wall **200**A. Inoculum consisted of 60,000 live BHK cells (98% viability) suspended in 38.1 ml of EMEM medium supplemented with 10% Hyclone FBS and 1% L-glutamine. Thus, the seeding density was 10,000 live cells per cm^2 of available attachment scaffold **240** area. The surface area of gas permeable membrane to volume of medium was 0.167 cm^2/ml. The surface are of gas permeable membrane to surface area of attachment scaffold was 0.106 cm^2/cm^2. The control T-175 tissue culture flask was inoculated with the same cells, at equivalent seeding density and viability. Gas permeable wall test fixture **12** and the T-175 control were placed in a standard cell culture incubator at 5% CO_2, 95% R.H., and 37° C.

Cells settled gravitationally onto bottom attachment scaffold **240** and the control T-175 flask, and the cultures were maintained until confluence was reached. Both the test fixture and the control exhibited a confluent monolayer over the entire attachment scaffold. By visual microscopic comparison, the cell density of both gas permeable test fixture **12** and the T-175 control flask appeared nearly identical. The T-175 flask was trypsinized, cells were counted, and it was determined that cells had reached a density of approximately 190,000 cells per cm^2. The test fixture was subjected to Wright Giemsa staining to determine the distribution of cells over bottom attachment scaffold **240**. FIG. **20** shows the distribution pattern, where "Front" is in proximity of gas permeable wall **200**, "Middle" is about midway between gas permeable wall **200** and opposing non-gas permeable wall **210**, and "Back" is in proximity of opposing non-gas permeable wall **210**.

FIG. **20** clearly indicates that cells will grow to confluence upon a scaffold in the absence of a gas/liquid interface, mechanical mixing, or perfusion, when a wall of the device is gas permeable. Thus, gas transfer by way of walls is adequate for cell culture devices of the types described herein including those shown in FIG. **9**A, FIG. **9**B, FIG. **10**A, FIG. **10**B, FIG. **11**, and FIG. **14**A through FIG. **14**E to fully function. Example 4 also indicates that only one of the walls of a gas permeable cell culture device needs to be comprised of gas permeable material, thereby opening up a wide array of device design options. For example, a gas permeable device could be configured in a traditional T-Flask format by making a sidewall gas permeable. In this manner, more medium could be made available for the culture or the device profile could be reduced since no gas/liquid interface is needed.

Example 5

The Ability to Culture Cells on Multiple Attachment Scaffolds in the Absence of a Gas/Liquid Interface

Evaluations were conducted to assess the ability to culture adherent cells on multiple scaffolds without the presence of a gas/liquid interface. Gas exchange occurred via a gas permeable device wall. Gas permeable test fixtures were constructed in a manner, as shown in FIG. **18**, that eliminated the possibility of gas transfer by way of a gas/liquid interface. Multiple scaffold test fixture **14** consisted of a rectangular liquid tight enclosure configured with one gas permeable wall **200**B and five non-gas permeable walls **210**A. Gas permeable wall **200**B was composed of molded silicone material, 0.015 thick. Fluid delivery port **220**A and fluid removal port **230**A allow inoculation and feeding. Attachment scaffolds **240**A consisted of plastic removed from NUNC™ Cell Factory cell culture devices. The inner dimensions of multiple scaffold test fixture **14** were 15.24 cm long, 7.62 cm wide, and 2.54 cm high. Thus, gas permeable wall **200**B was 7.62 cm wide and 2.54 cm high creating a gas permeable material surface area of 19.35 cm^2. Each attachment scaffold **240**A was 6.6 cm wide and 15.03 cm long, creating an attachment surface area of 99 cm^2 per attachment scaffold **240**A.

In one test group of multiple scaffold test fixtures **14**, four attachment scaffolds **240**A were arranged vertically, one above the other, with a 5.08 mm gap between each of them, resulting in a total attachment surface area of 396 cm^2 per device. The volume of medium within this version of multiple scaffold test fixture **14** was 195 ml. The surface area

of gas permeable membrane to volume of medium was 0.099 cm^2/ml. The surface area of gas permeable membrane to total surface area of attachment scaffolds **240**A was 0.049 cm^2/cm^2.

In another test group of multiple scaffold test fixtures **14**, five attachment scaffolds were arranged vertically, one above the other, with a 2.54 mm gap between each of them, resulting in a total attachment surface area of 495 cm^2 per device. The volume of medium within each multiple scaffold test fixture was 170 ml. The surface area of gas permeable membrane to volume of medium was 0.114 cm^2/ml. The surface area of gas permeable membrane to total surface area of attachment scaffolds **240**A was 0.039 cm^2/cm^2.

Multiple scaffold gas permeable test fixtures **14** were filled entirely with medium during inoculation, thereby eliminating any gas/liquid interface. Thus, gas exchange could only occur by way of diffusion in the direction perpendicular to the gas permeable wall. The seeding density was 15,000 live BHK cells per cm^2 of available attachment scaffold area. Medium consisted of Gibco GMEM supplemented with 10% Hyclone FBS and 1% Gibco Penicillin Streptomycin. The control T-175 tissue culture flask was also inoculated with BHK cells, at equivalent seeding density and viability, in 30 ml of the same medium composition. Multiple scaffold gas permeable test fixtures **14** and the T-175 control were placed in a standard cell culture incubator at 5% CO_2, 95% R.H., and 37° C.

Cells settled gravitationally onto each attachment scaffold **240**A and the control T-175 flask, and the cultures were maintained until confluence was reached. Within four days, cultures were terminated. All attachment scaffolds **240**A were removed from multiple scaffold gas permeable test fixture **14**. By visual microscopic comparison, the cell density of both test groups of multiple scaffold gas permeable test fixtures **14** and the T-175 control flask appeared nearly identical, at approximately 95% confluence.

This demonstrates the ability to make much more efficient use of space by eliminating the need to maintain a gas headspace in a culture device. Since the device only holds the medium needed to support the culture, it can be significantly reduced in profile. The novel device is much more compact than the traditional T-flask, NUNC™ Cell Factory, and Corning CellStack™. This results in savings in sterilization, shipping, storage, and disposal cost. Furthermore, incubator space and flow hood space are used more efficiently.

Example 6

Gas Permeable Unrolled Cell Culture Device for Adherent Cell Culture Inoculated in the Vertical Position

A test fixture was constructed to evaluate the capacity of a non-rolled, gas permeable cell culture device configured with more than one scaffold to culture cells relative to traditional flasks. FIG. **19**A shows a cross-section of gas permeable test fixture **260**. Scaffolds **120**H were arranged vertically and a consistent gap was maintained between each scaffold **120**H by spacers **135**B. Wall **40**J was gas permeable, comprised of silicone purchased from Medtronic Inc. (Minneapolis), approximately 0.0045 inches thick. Suture **270** applied force to gas permeable wall **40**J, squeezing it against bulkhead gasket **280** to create a liquid tight seal between gas permeable wall **40**J and upper bulkhead **290** and lower bulkhead **300**. Medium access port **60**B allowed fluid delivery to, and removal from, gas permeable test fixture **260**. Cap **70** prevented contamination and was tightly closed during operation. FIG. **19**B shows a perspective view of scaffold **120**H. It was made of tissue culture treated polystyrene, 0.040 inches thick. Pipette access opening **125**A, with a diameter of 035 inches, allowed pipette access and prevented gas from becoming trapped between scaffolds **120**H. Four vent slots **190** allowed additional area for trapped gas to exit, ensuring that all gas/liquid interfaces were removed. The surface area per side of each scaffold **120**H was about 86 cm^2. The inner diameter of gas permeable test fixture **260** was 4.4 inches and the internal height as measured from the inner surface of lower bulkhead **300** to the inner surface of upper bulkhead **290** was 2.25 inches. Thus, the gas permeable material surface area was 561 cm^2. Eight scaffolds **120**H were stacked vertically with spacers **135**B maintaining a gap of about 0.25 inch between each. The combined surface area of the tops of the eight scaffolds **120**H was 695 cm^2. The internal volume of gas permeable test fixture **260** was approximately 500 ml. Therefore, the gas permeable material to medium volume ratio was 561 cm^2/500 ml, or 1.12 cm^2/ml.

10.425×10^6 BHK cells, suspended in 500 ml Gibco GMEM medium supplemented with 1% Gibco Amino Acids Solution and 10% Hyclone FBS were inoculated into gas permeable test fixture **260**P, creating a seeding density of 15,000 cells per cm^2 of attachment surface area. A control T-175 flask was also seeded with 15,000 cells per cm^2 of attachment surface area in 30 ml of the equivalent medium.

After approximately 96 hours, the cultures were terminated. Gas permeable test fixture **260** was disassembled and each of scaffolds **120**H was microscopically examined, indicating a confluent pattern of cells was present on the upper surface of each of the eight scaffolds **120**H. The control T-175 flask was also confluent as determined by microscopic evaluation. The T-175 flask and gas permeable test fixture **260** were trypsinized and standard cell counting techniques were used to determine the quantity of cells present. TABLE 6 summarizes the findings.

TABLE 6

Gas permeable cell culture device vs. T-flask

Device	Total Cells ($\times10^6$)	Viability (%)	Medium Present (ml)	Height of Medium Above Cells (cm^2)
Gas permeable cell test fixture 260	220.8	98	500	0.72
Control T-flask	26.3	95	30	0.17

TABLE 6 demonstrates that cells were able to proliferate and remain healthy in the novel gas permeable test fixture **260**, despite the absence of a gas/liquid interface.

The volume of space occupied by each device is noteworthy. Gas permeable test fixture **260** had a footprint of 100 cm^2 and a height, including the neck, of 7.6 cm. Thus, the space occupied was about 760 cm^3. The T-175 flask, including the neck, had a footprint approximately 23 cm long by 11 cm wide, and the body was about 3.7 cm tall. Thus, the space occupied was about 936 cm^3. Since gas permeable test fixture **260** cultured about 8.4 times more cells than the T-175 flask, it would take 8.4 T-175 flasks to yield an equivalent amount of cells over the same time period. TABLE 7 shows the difference in space that would be occupied if T-175 flasks were used to produce the same

number of cells cultured by gas permeable test fixture **260**, based on the experimental results of TABLE 6.

TABLE 7

Device	Volume of space occupied per device (cm^3)	Devices to produce 221 × 10^6 cells in 3 days	Volume of space needed (cm^3)
One novel gas permeable cell culture device 260	760	1	760
Control T-flasks	936	8.4	7862

The advantage of eliminating the gas/liquid interface is clear. Over a ten-fold reduction of space is obtained by gas permeable test fixture **260**. This leads to cost savings in sterilization, shipping, storage, use of incubator space, and waste disposal. Furthermore, the number of devices that need to be handled is significantly reduced, leading to a dramatic labor and contamination risk reduction.

Example 7

Gas Permeable Unrolled Cell Culture Device for Adherent Cell Culture Inoculated in the Vertical and Inverted Position

Using the test fixture shown in FIG. **19**A, as previously defined in Example 6, an experiment was conducted to determine if cells would attach to both the top and bottom surfaces of the scaffolds. This could be accomplished by a two-step inoculation. In step one, a first inoculum was placed into the gas permeable test fixture while oriented in the vertical position. Cells were allowed to gravitate onto, and attach to the top surface of, the scaffolds over a 24-hour period. In step two, a second inoculum was placed into the gas permeable test fixture. Gas permeable test fixture was inverted to allow the cells of the second inoculum to gravitate onto, and attach to the bottom surface of, the scaffolds.

This process was undertaken, with each inoculation consisting of enough BHK cells to seed the exposed surfaces of the scaffolds at a density of 15,000 cells per cm^2. Medium composition was the same as that described in EXAMPLE 6. The time interval between the first inoculation and the second inoculation was twenty-four hours. The culture was terminated seventy-two hours after the second inoculation. The device was disassembled and each scaffold was microscopically assessed. Cells were uniformly distributed on both the top and bottom surfaces of each scaffold. Subsequently, the cells were removed using trypsin and a count was performed. The average quantity of live cells per cm^2 of surface area was 144×10^5, with viability greater than 99%.

Cells were thus able to attach and proliferate on the top and bottom of scaffold **120**. Therefore, it is possible for the novel gas permeable cell culture device to be further reduced in size relative to conventional devices. For adherent cell culture, a wide variety of scaffold geometry can exist that have cell attachment area in any plane.

Example 8

Gas Permeable Unrolled Cell Culture Device for Adherent Cell Culture Inoculated in the Vertical and Inverted Position with Limited Distance Between Scaffolds

A test was conducted to determine if inserting more scaffold area into the device could further reduce device size. For additional space savings, the upper and lower surface of each scaffold was used to culture cells. The gas permeable test of Example 7 was fabricated with additional scaffolds. The number of scaffolds and distance between the scaffolds was chosen to create a volume to surface area ratio roughly equivalent to a traditional tissue culture flask. Recommended medium volume for a traditional T-175 flask varies from about 16-32 ml (Invitrogen Life Technologies). This dictates that medium reside about 0.09-0.18 cm from the attachment surface. The test device of this example was to be inoculated in two steps, allowing cells to reside on the upper and lower surfaces of each scaffold. Therefore, in order to get a conservative assessment of the value the gas permeable cell culture device can bring in terms of space and labor savings, 0.34 cm medium height was allowed to reside between each of the scaffolds. In this manner, the medium to surface area ratio was held constant relative to the T-175 flask. In effect, each scaffold surface had access to one half the medium between it, and the scaffold adjacent to it had access to the other half. Thus, the medium available to each side of a scaffold was consistent with the traditional tissue culture flask height of 0.17 cm per square centimeter of growth surface.

Fourteen scaffolds were inserted into the test device and evenly spaced approximately 0.34 cm apart. A T-175 flask, with 30 ml of medium residing at a height of 0.17 cm acted as a control. Inoculation using BHK cells was performed in two steps, as detailed in Example 7. Medium composition was the same as that described in Example 6. Seventy-two hours after the second inoculation, the culture was terminated and the device was disassembled and each scaffold was microscopically assessed for cell distribution upon the upper and lower surface. Each scaffold exhibited a distribution pattern on the upper and lower surface that was approximately equivalent to that of the T-175 flask. TABLE 7 shows an example of how increasing the surface area of the novel gas permeable cell culture device reduces the space needed to culture a given amount of cells when compared to the traditional T-175 flask. For example, when then novel gas permeable cell culture device contains 2432 cm^2 of scaffold surface area, fourteen T-175 flasks would be needed to provide equal surface area. If 1.7 mm of medium is intended to be available for each cm^2 of scaffold surface area, the volume of space occupied by the novel gas permeable cell culture device can be determined. TABLE 8 shows that in this case, the dramatically difference in the volume of space occupied by each type of device.

TABLE 8

Gas permeable device output with increased surface area

Device	Available Surface area for cell attachment (cm^2)	Number of devices needed	Volume of medium needed (cm^3)	Volume of space occupied per device (cm^3)
One novel gas permeable cell culture device	2432	1	420	760
T-175 flask	2432	14	420	12,292

It can be seen that when the gas permeable cell culture device is designed to have the same medium to surface area ratio as the traditional flask, a much more efficient use of space results. The volume of space occupied by the gas permeable cell culture device is only one-sixteenth of that occupied by T-175 flasks when an equivalent amount of cells

are desired. This translates directly into cost reductions for sterilization, shipping, storage, and disposal.

It is to be understood that the invention is not limited to the above embodiments, which are shown for purposes of illustration and described above, but is intended to include any modification or variation thereof falling within the scope of the appended claims.

Example 9

Gas Permeable Rolled Cell Culture Device for Adherent Cell Culture Inoculated in the Vertical Position

Gas permeable test fixture **260** was constructed, as shown in the cross-sectional view of FIG. **19**A and further defined in Example 5, to evaluate the capability of rolling a gas permeable cell culture device configured with more than one scaffold.

With gas permeable test fixture **260** in the vertical, unrolled position, 10.425×10^6 BHK cells, suspended in 500 ml Gibco GMEM medium supplemented with 1% Gibco Amino Acids Solution and 10% Hyclone FBS were inoculated into gas permeable test fixture **260**, creating a seeding density of 15,000 cells per cm^2 of attachment surface area. A control T-175 flask was also seeded with 15,000 cells per cm^2 of attachment surface area in 30 ml of the equivalent medium.

After approximately 24 hours, the gas permeable test fixture was places upon a standard roller rack at rotated at 1 RPM. Three days after the commencement of rolling, gas permeable test fixture was disassembled and each of the scaffolds was microscopically examined, indicating a confluent pattern of cells was present on the upper surface of each of the eight scaffolds. The control T-175 flask was also confluent as determined by microscopic evaluation.

This demonstrates that proliferation of cells is uninhibited by rolling the novel gas permeable cell culture device. Thus, creating a device that can be rolled or unrolled allows users greater options for protocol development.

GUIDE TO REFERENCE CHARACTERS IN DRAWINGS

10 gas permeable cell culture device
12 gas permeable wall test fixture
14 multiple scaffold test fixture
15 gas permeable multiple well plate
16 gas permeable wall multiple well plate
20 cells
25 buoyant shoulder
30 lower gas permeable material
31 non-gas permeable bottom
40 walls
41 gas permeable wall
42 interior walls
45 individual wells
45 high surface area well
46 medium
50 top cover
55 medium access port
65 septum
70 cap
75 o-ring
80 lower gas permeable material support
90 gas compartment
95 feet
100 lower gas access openings
105 tubular test fixtures
110 projections
115 mesh
120 scaffolds
125 pipette access opening
130 inoculum
135 spacer
145 spring arm
150 ramps
160 elevation posts
170 scaffold locator screw
180 rotation arrow
190 vent slots
200 gas permeable wall
201 top wall
210 non-gas permeable wall
212 tongue
215 groove
220 fluid delivery port
230 fluid removal port
240 attachment scaffold
241 enclosure
260 gas permeable test fixture
270 suture
280 bulkhead gasket
290 upper bulkhead
300 lower bulkhead

Those skilled in the art will recognize that numerous modifications can be made to this disclosure without departing from the spirit on the inventions described herein. Therefore, it is not intended to limit the breadth of the invention to the embodiments illustrated and described. Rather, the scope of the invention is to be interpreted by the appended claims and their equivalents. Each publication, patent, patent application, and reference cited herein is hereby incorporated herein by reference.

What is claimed is:

1. A method of culturing cells comprising:

adding medium and animal cells into a static cell culture device that is not compartmentalized by a semi-permeable membrane, at least a portion of said cell culture device is comprised at least in part of a non porous gas permeable material, ambient gas is in contact with at least a portion of said gas permeable material, and

placing said cell culture device in a cell culture location that includes ambient gas at a composition suitable for animal cell culture, wherein said cell culture device is oriented in a position such that at least a portion of said cells reside upon at least a portion of said gas permeable material, the uppermost location of said medium is elevated beyond 2.0 cm from the lowermost location of said medium, and said device is in a state of static cell culture.

2. The method of claim **1** wherein the uppermost location of said medium is elevated at least 3.2 cm from the lowermost location of said medium.

3. The method of claim **1** wherein the uppermost location of said medium is elevated at least 4.0 cm from the lowermost location of said medium.

4. The method of claim **1** wherein the uppermost location of said medium is elevated at least 5.09 cm from the lowermost location of said medium.

5. The method of claim **1** wherein the uppermost location of said medium is elevated at least 6.0 cm from the lowermost location of said medium.

6. The method of claim 1 wherein the uppermost location of said medium is elevated at least 7.0 cm from the lowermost location of said medium.

7. The method of claim 1 wherein the uppermost location of said medium is elevated at least 8.0 cm from the lowermost location of said medium.

8. A method of culturing cells comprising:

adding medium and animal cells into a static cell culture device that is not compartmentalized by a semi-permeable membrane, the bottom of said device is comprised at least in part of a non porous gas permeable material, and

placing said device in a cell culture location that includes ambient gas at a composition suitable for animal cell culture, wherein said non porous gas permeable material is in a horizontal plane, the uppermost location of said medium is more than 2.0 cm above the lowermost location of said medium, at least a portion of said gas permeable material is in contact with ambient gas, and said device is not subjected to mixing or perfusion.

9. The method of claim 8 wherein the uppermost location of said medium is at least 2.5 cm above the lowermost location of said medium.

10. The method of claim 8 wherein the uppermost location of said medium is at least 3.2 cm above the lowermost location of said medium.

11. The method of claim 8 wherein the uppermost location of said medium is at least 4.0 cm above the lowermost location of said medium.

12. The method of claim 8 wherein the uppermost location of said medium is at least 5.09 cm above the lowermost location of said medium.

13. The method of claim 8 wherein the uppermost location of said medium is at least 6.0 cm above the lowermost location of said medium.

14. The method of claim 8 wherein the uppermost location of said medium is at least 7.0 cm above the lowermost location of said medium.

15. The method of claim 8 wherein the uppermost location of said medium is at least 8.0 cm above the lowermost location of said medium.

16. The method of claim 8 wherein the uppermost location of said medium is at least 9.0 cm above the lowermost location of said medium.

17. The method of claim 8 wherein the uppermost location of said medium is at least 10.2 cm above the lowermost location of said medium.

18. A method of culturing cells comprising:

adding medium and animal cells into a static cell culture device that is not compartmentalized by a semi-permeable membrane, said device including one or more cell growth surfaces for the culture of animal cells comprised at least in part of a non porous gas permeable material, at least a portion of said non porous gas permeable material is in contact with ambient gas, and

placing said device in a location that includes ambient gas at a composition suitable for animal cell culture, wherein said medium is in contact with said non porous gas permeable material, and the uppermost location of said medium is more than 2.0 cm above the lowermost location of said medium, and said device is not subjected to mixing or perfusion.

19. The method of claim 18 wherein the uppermost location of said medium is at least 2.5 cm above the lowermost location of said medium.

20. The method of claim 18 wherein the uppermost location of said medium is at least 3.2 cm above the lowermost location of said medium.

21. The method of claim 18 wherein the uppermost location of said medium is at least 4.0 cm above the lowermost location of said medium.

22. The method of claim 18 wherein the uppermost location of said medium is at least 5.09 cm above the lowermost location of said medium.

23. The method of claim 18 wherein the uppermost location of said medium is at least 6.0 cm above the lowermost location of said medium.

24. The method of claim 18 wherein said device includes a gas permeable material support in contact with said gas permeable, liquid impermeable material.

25. A method of culturing cells comprising:

adding medium and animal cells into a static cell culture device that is not compartmentalized by a semi-permeable membrane, said device is comprised at least in part of a non porous gas permeable material, at least a portion of said non porous gas permeable material is in contact with ambient gas and is in contact with a gas permeable material support, and

placing said device in a location that includes ambient gas at a composition suitable for animal cell culture, wherein said non porous gas permeable material is in a horizontal plane, and the uppermost location of said medium is elevated more than 2.0 cm beyond the lowermost location of said medium, and said device is not subjected to mixing or perfusion.

26. The method of claim 25 wherein the uppermost location of said medium is elevated at least 2.5 cm beyond the lowermost location of said medium.

27. The method of claim 25 wherein the uppermost location of said medium is elevated at least 3.2 cm beyond the lowermost location of said medium.

28. The method of claim 25 wherein the uppermost location of said medium is elevated at least 4.0 cm beyond the lowermost location of said medium.

29. The method of claim 25 wherein the uppermost location of said medium is elevated at least 5.09 cm beyond the lowermost location of said medium.

30. The method of claim 25 wherein the uppermost location of said medium is elevated at least 6.0 cm beyond the lowermost location of said medium.

* * * * *

EXHIBIT B

US008697443B2

(12) **United States Patent**
Wilson et al.

(10) **Patent No.: US 8,697,443 B2**
(45) **Date of Patent: Apr. 15, 2014**

(54) **CELL CULTURE METHODS AND DEVICES UTILIZING GAS PERMEABLE MATERIALS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 995 days.

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(65) **Prior Publication Data**

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(62) Division of application No. 10/961,814, filed on Oct. 8, 2004.

(60) Provisional application No. 60/509,651, filed on Oct. 8, 2003.

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C12N 5/00 (2006.01)
C12M 3/00 (2006.01)

(52) **U.S. Cl.**
USPC **435/395**; 435/297.5; 435/299.2; 435/304.2

(58) **Field of Classification Search**
USPC 435/304.2, 304.3, 297.5, 395, 402
See application file for complete search history.

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(57) **ABSTRACT**

Gas permeable devices and methods are disclosed for cell culture, including cell culture devices and methods that contain medium at heights, and certain gas permeable surface area to medium volume ratios. These devices and methods allow improvements in cell culture efficiency and scale up efficiency.

51 Claims, 16 Drawing Sheets

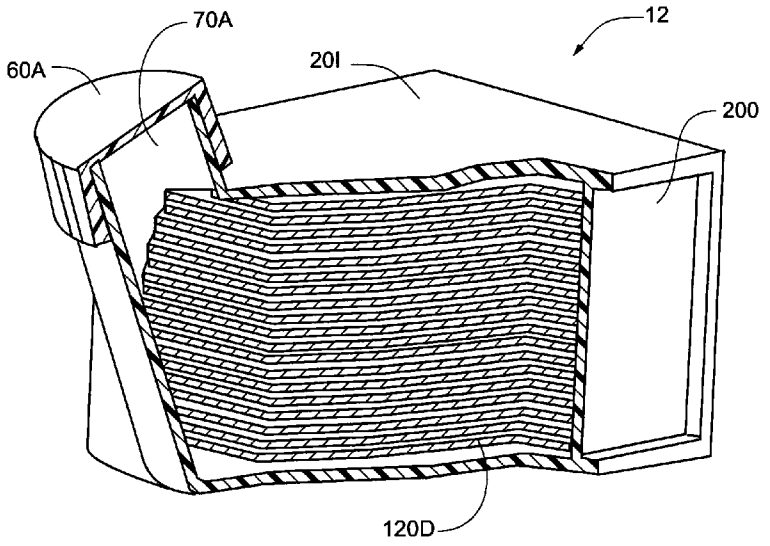

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Fig. 1A

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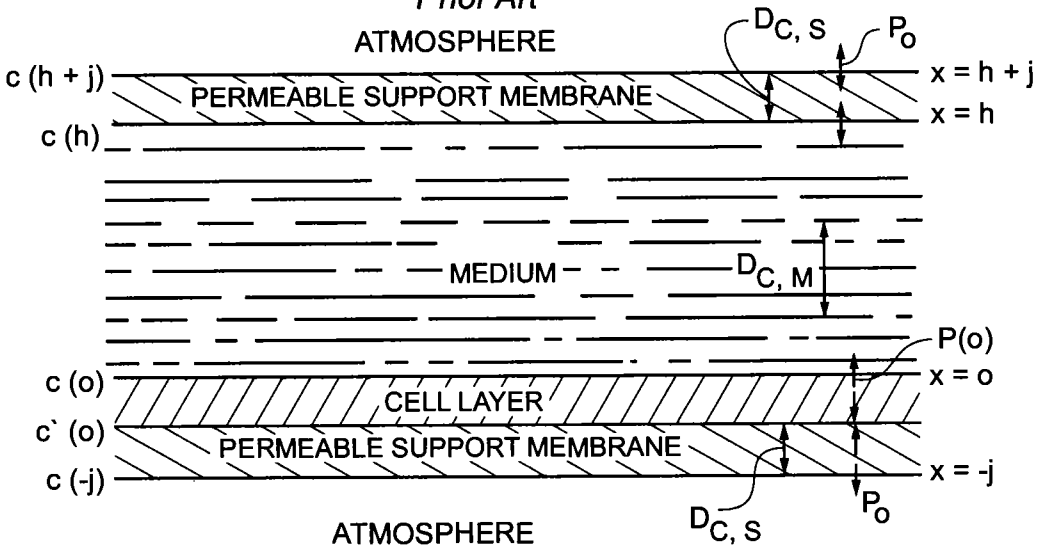
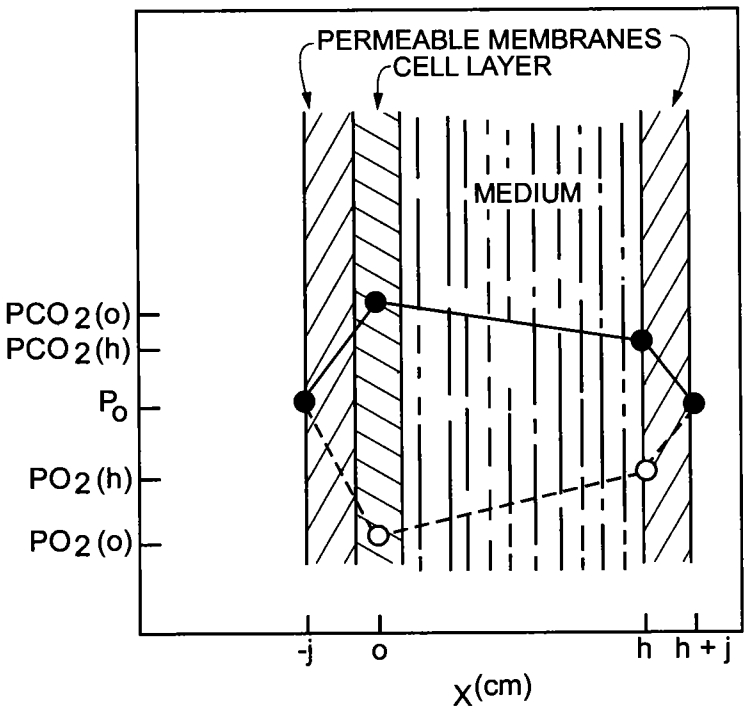


Fig. 1B

Prior Art



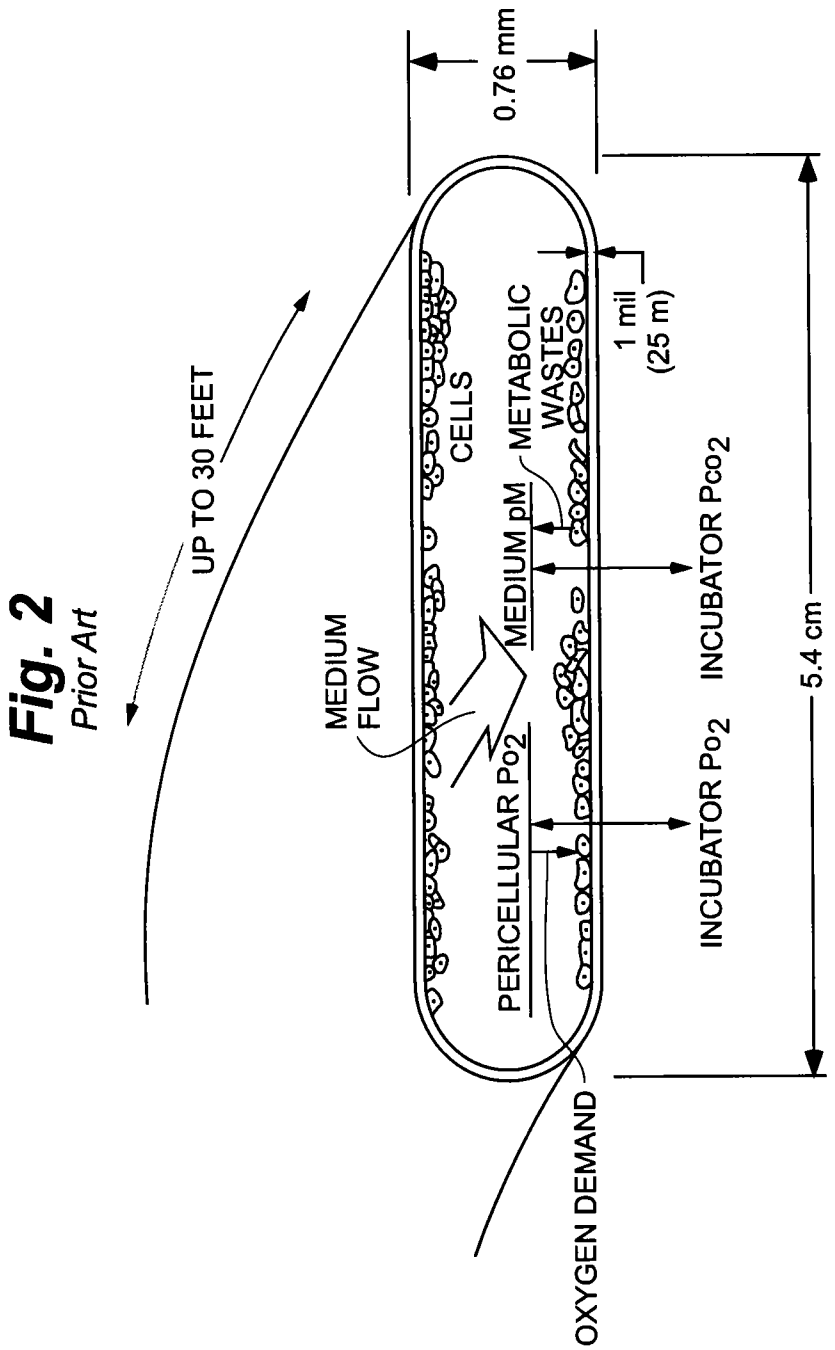


Fig. 2
Prior Art

Fig. 3
Prior Art

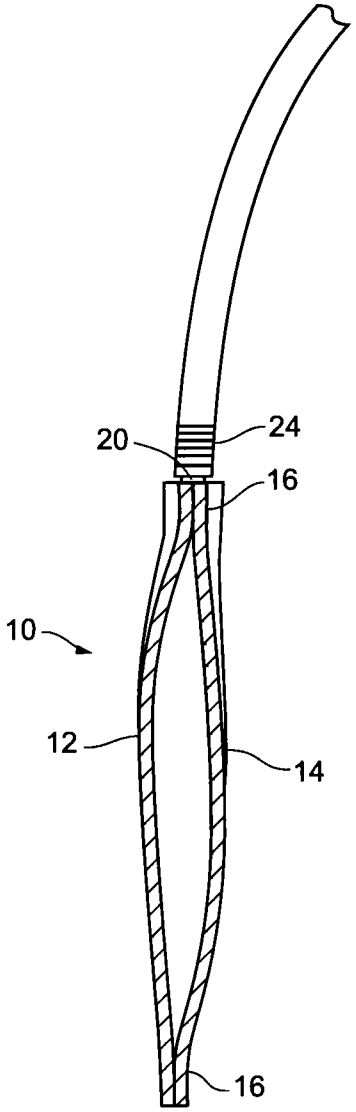
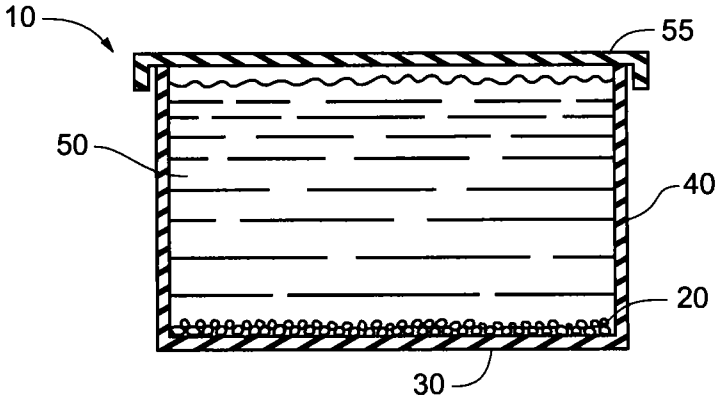


Fig. 4A



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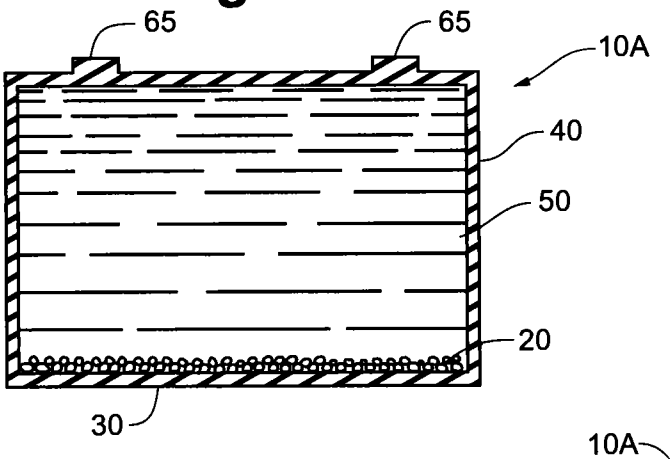
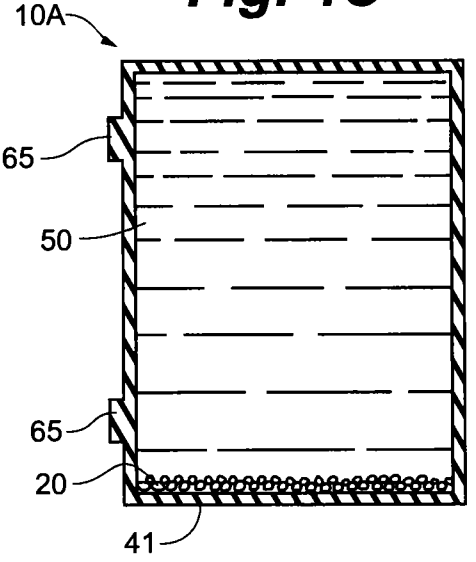


Fig. 4C



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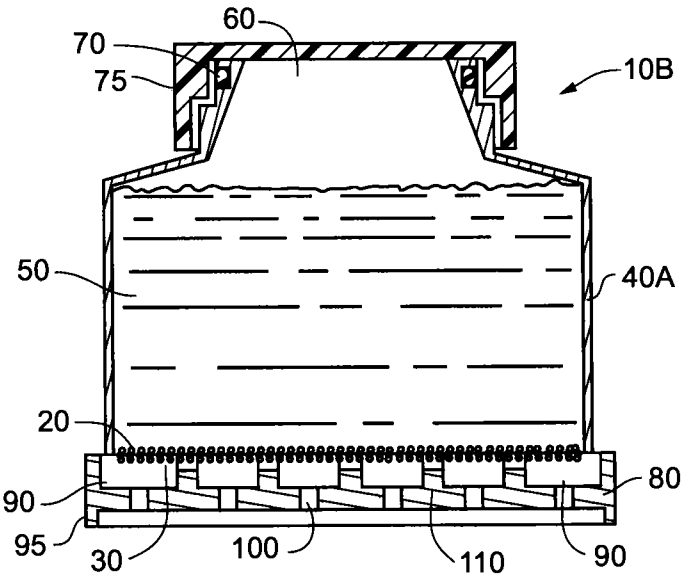
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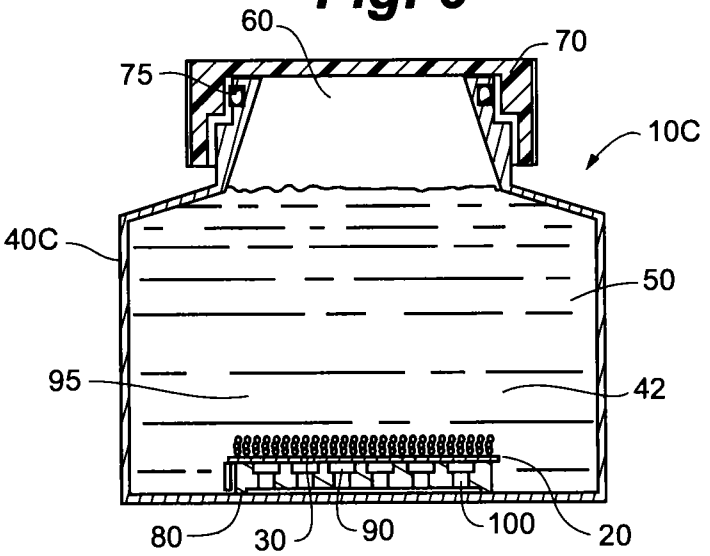
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Fig. 7A

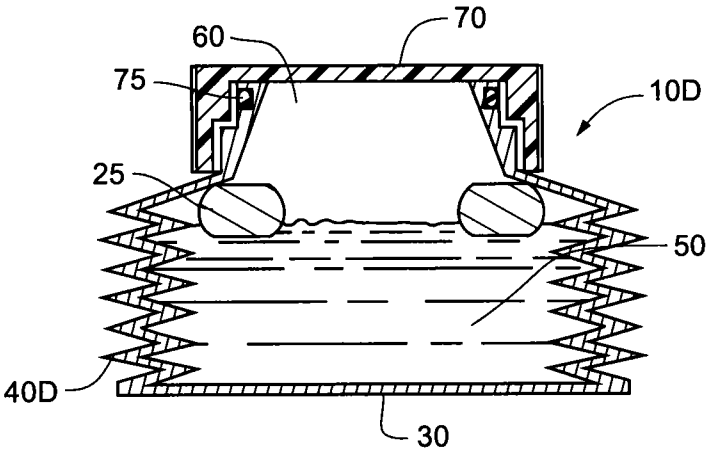


Fig. 7B

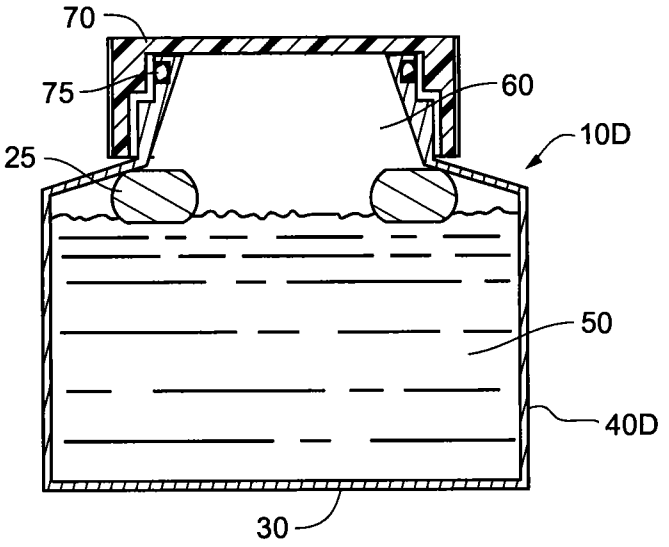
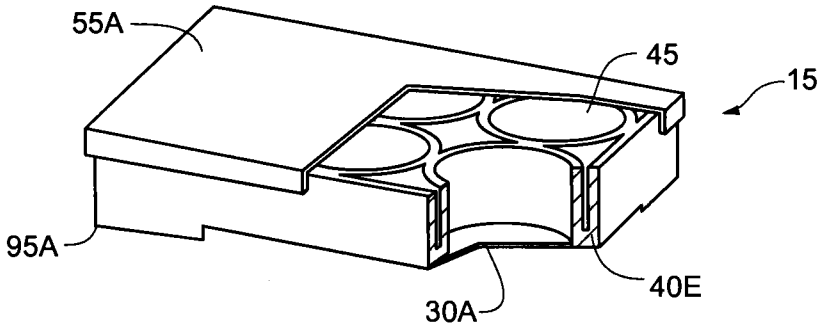


Fig. 8



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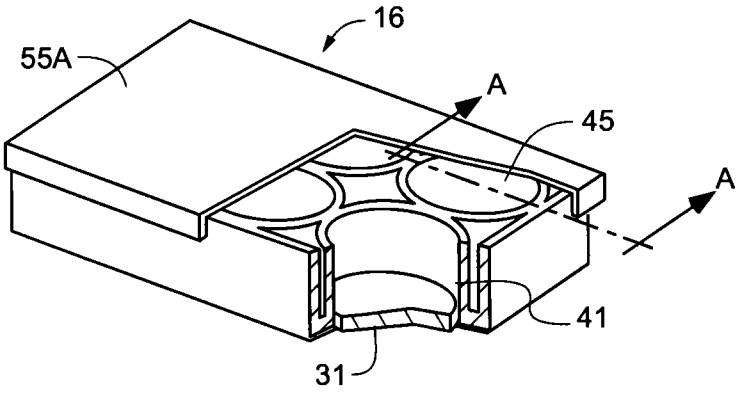
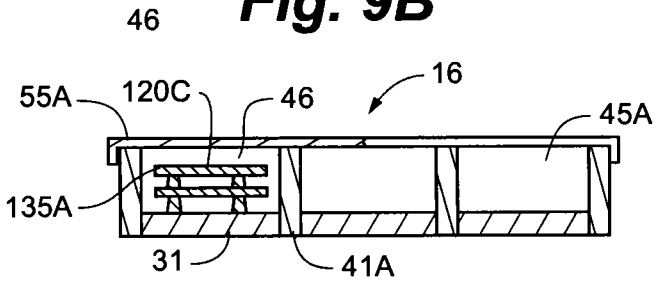


Fig. 9B



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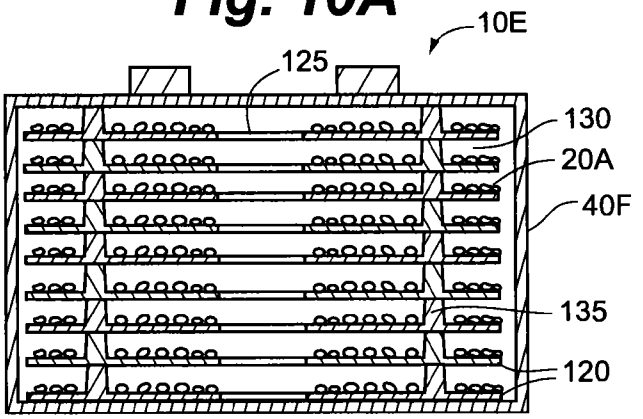

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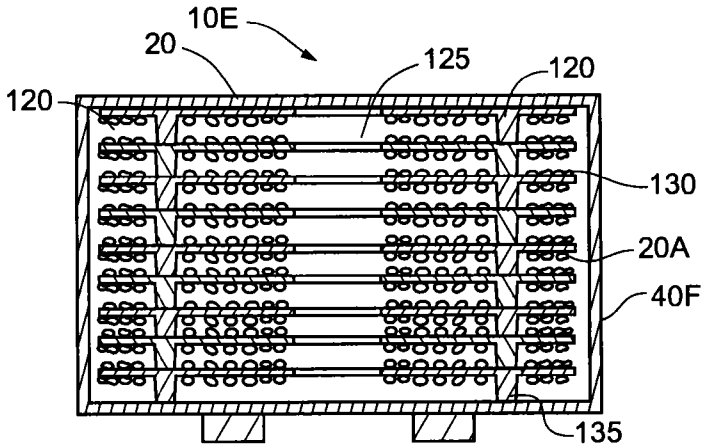


Fig. 11

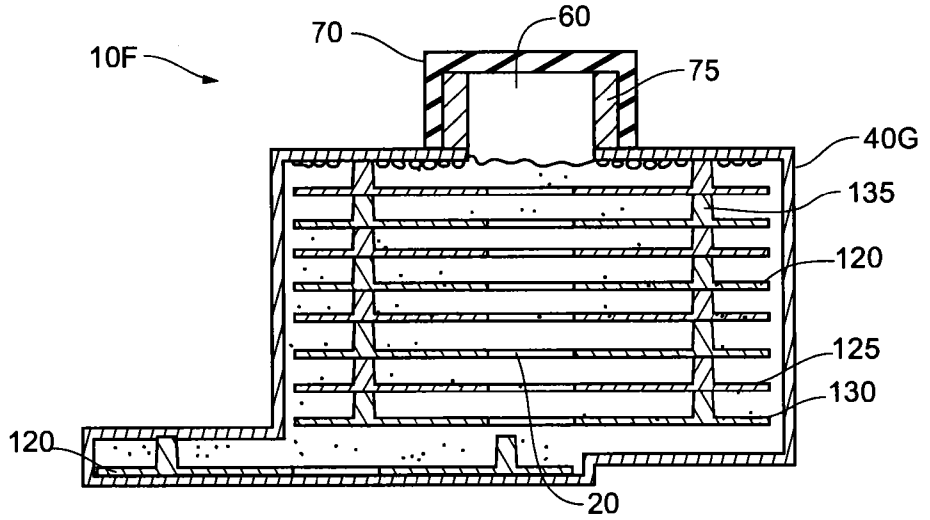
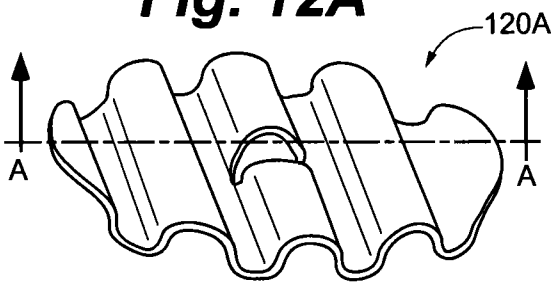


Fig. 12A



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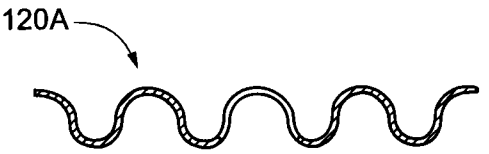

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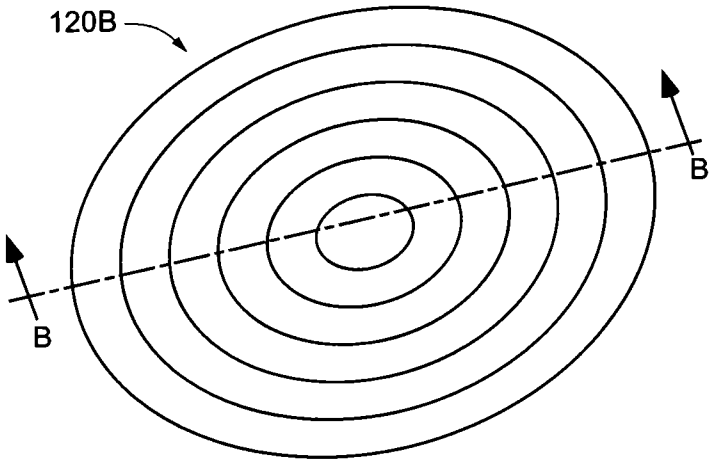
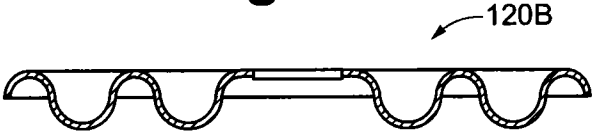


Fig. 12D



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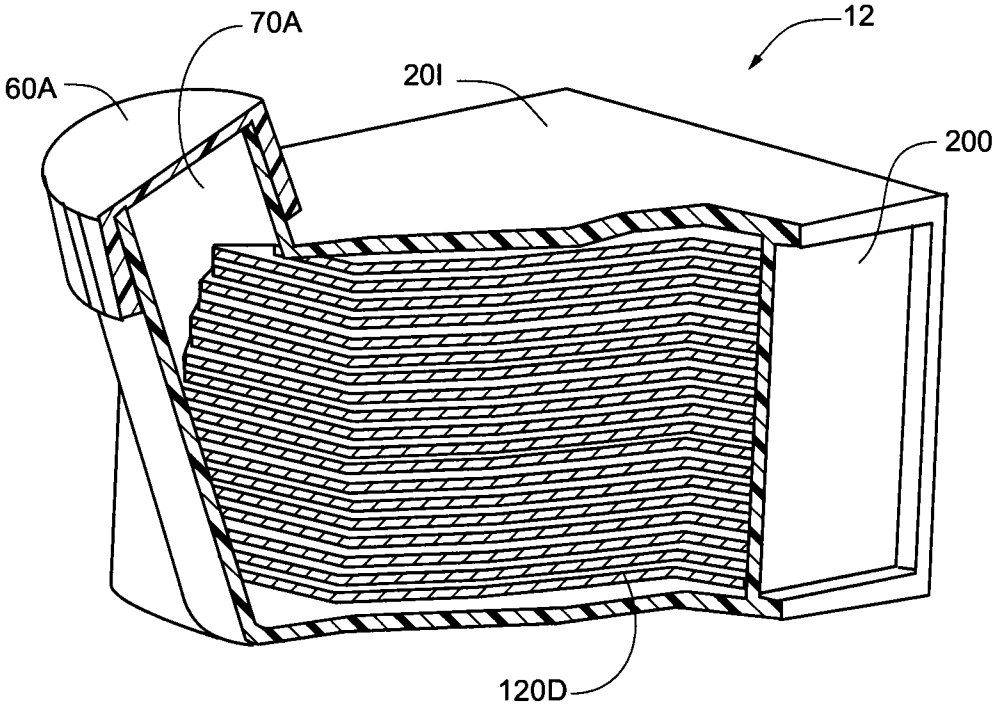

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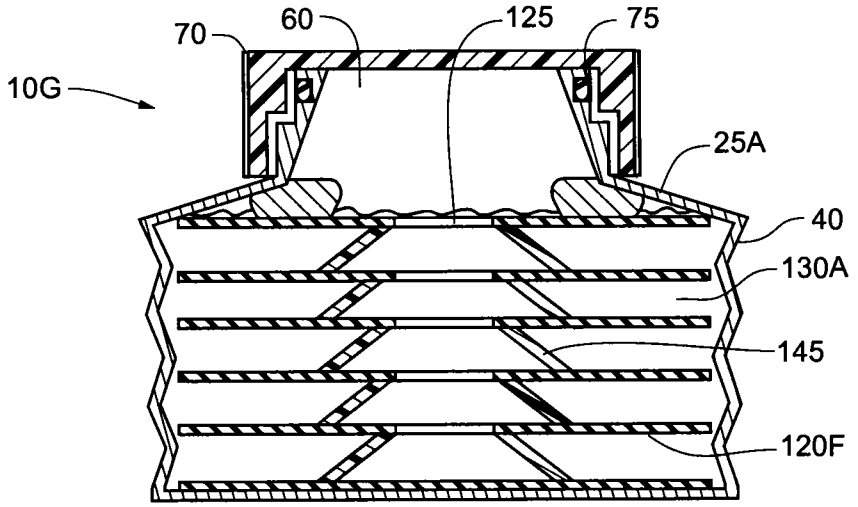

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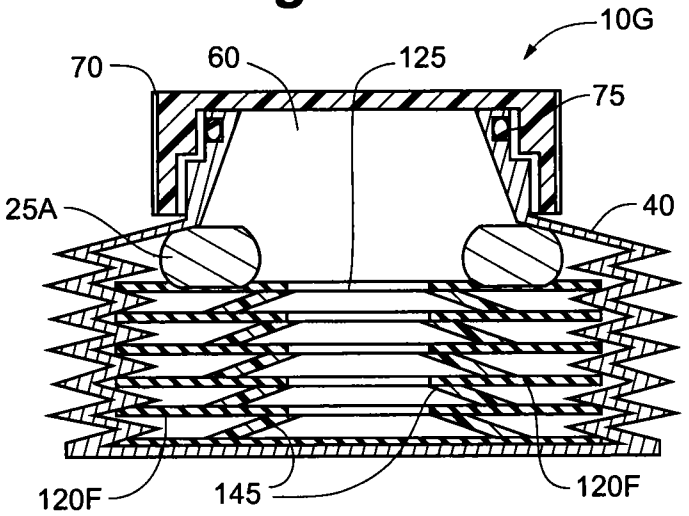
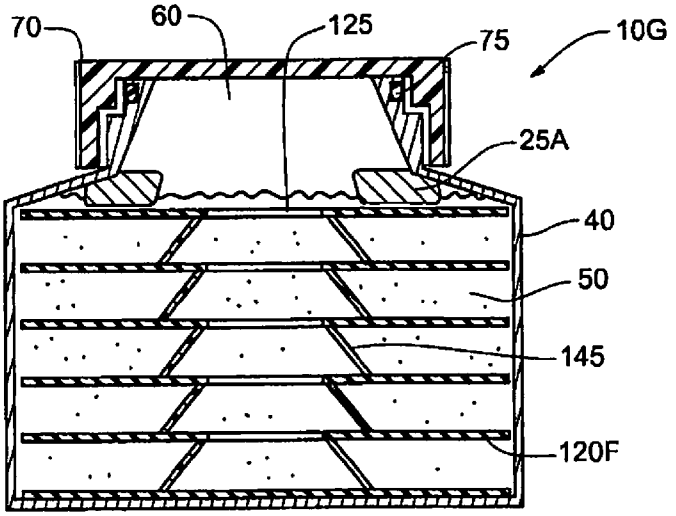


Fig. 14C



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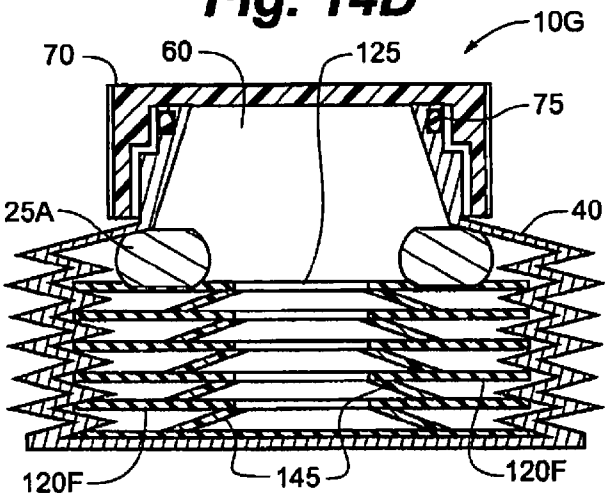
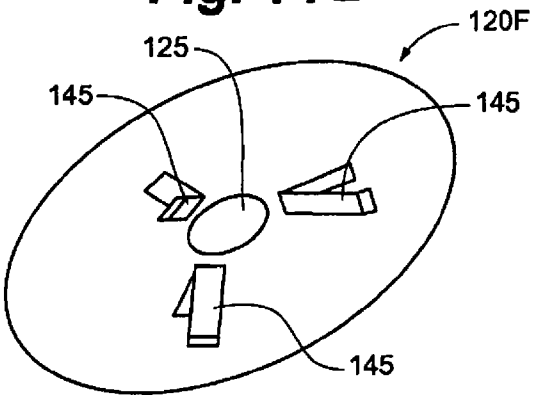


Fig. 14E



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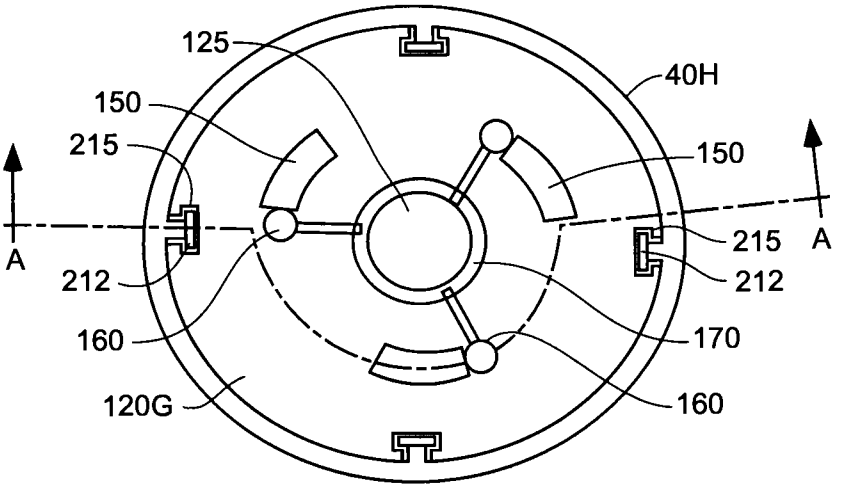

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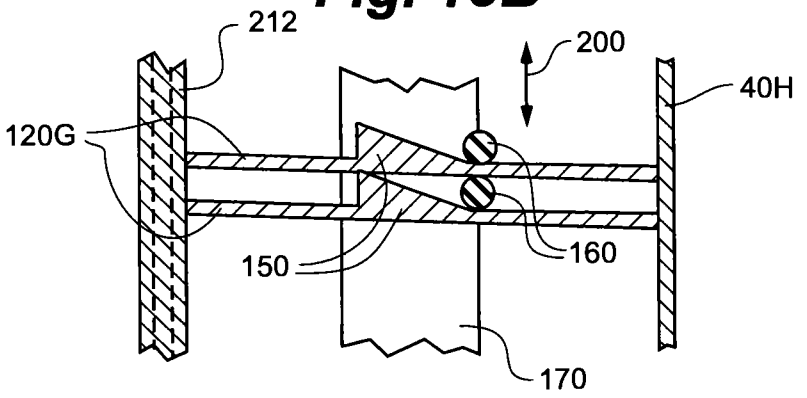

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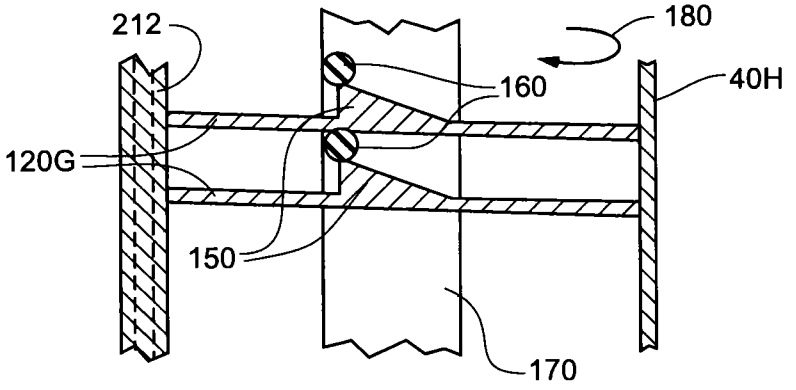

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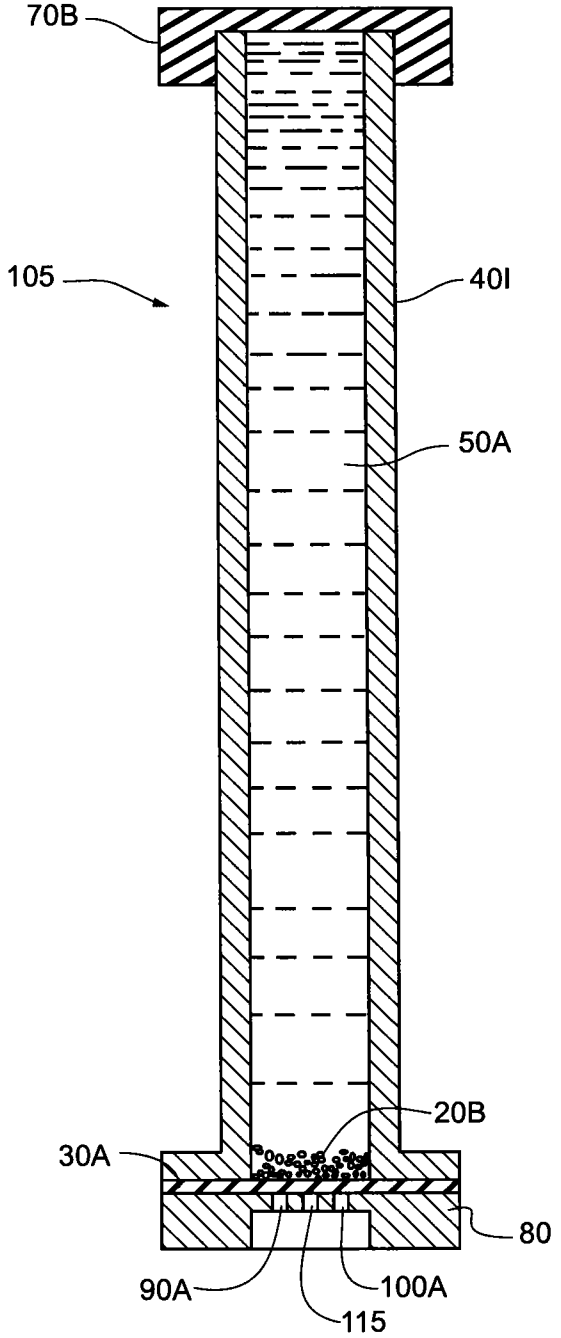

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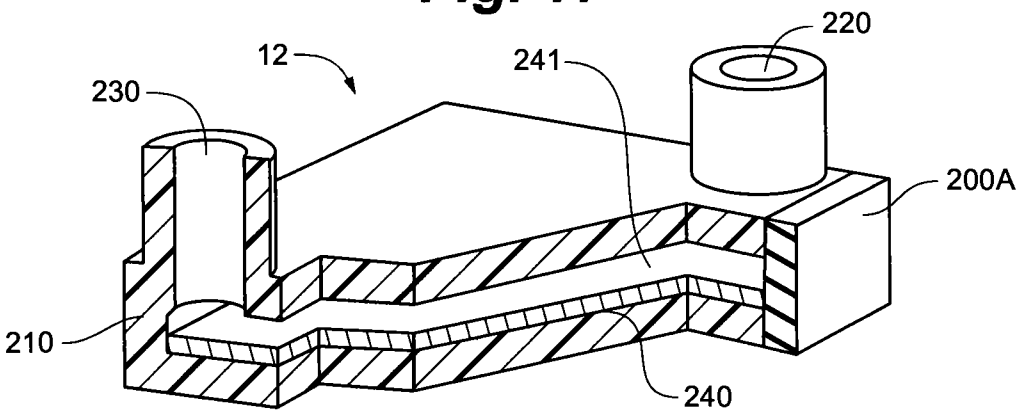
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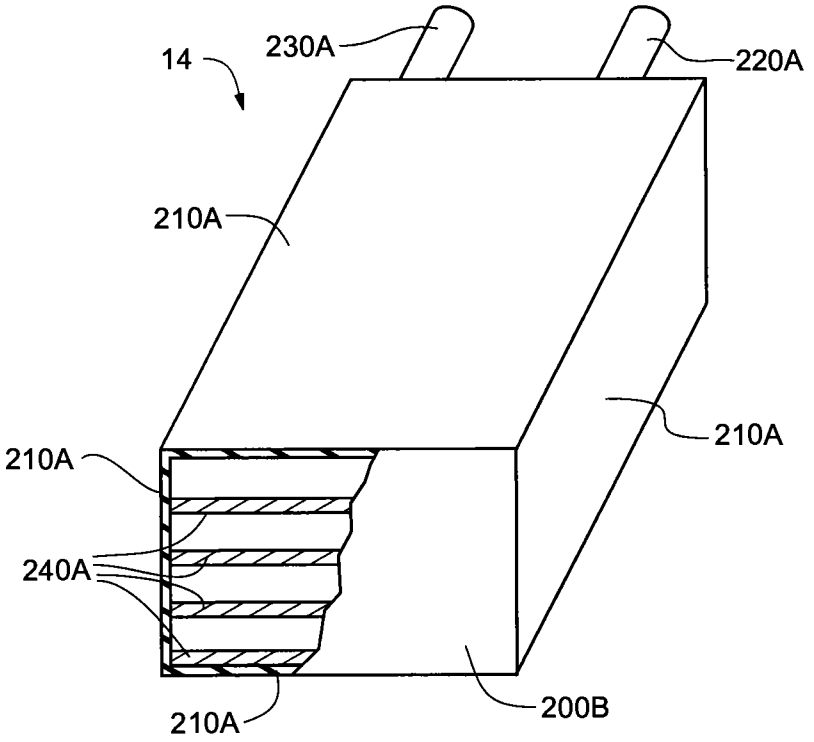
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210A

Fig. 19A

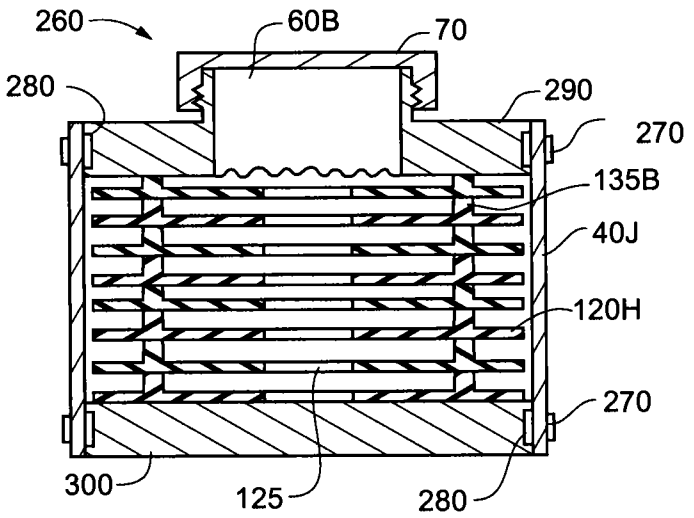


Fig. 19B

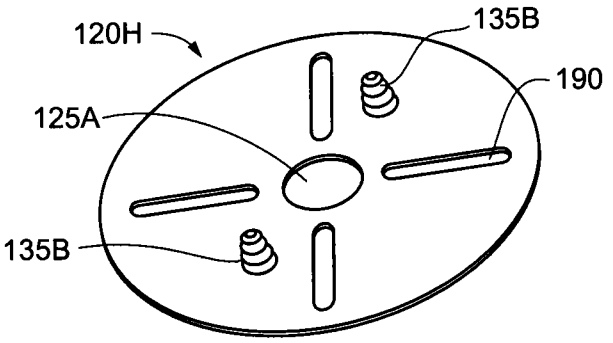
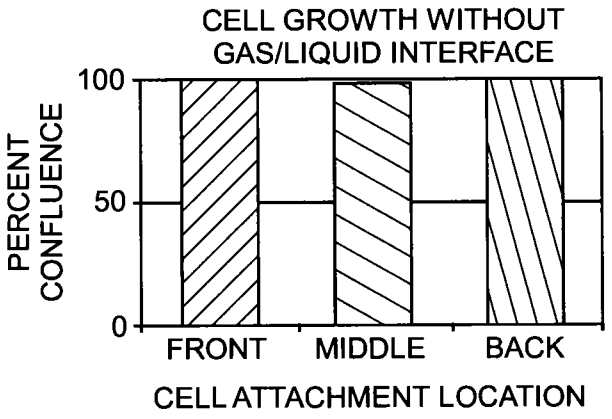


Fig. 20



CELL CULTURE METHODS AND DEVICES UTILIZING GAS PERMEABLE MATERIALS

RELATED APPLICATION

The present application claims priority to U.S. application Ser. No. 10/961,814 filed Oct. 8, 2004 which claims priority to U.S. Provisional Application No. 60/509,651 filed Oct. 8, 2003, which is hereby incorporated herein in its entirety by reference.

TECHNICAL FIELD

The technical field of the invention relates to methods and devices that improve cell culture efficiency. They utilize gas permeable materials for gas exchange, allow an increased height of cell culture medium, reduce the ratio of gas permeable device surface area to medium volume capacity, and integrate traditional cell support scaffolds. A variety of benefits accrue, including more efficient use of inventory space, incubator space, disposal space, and labor, as well as reduced contamination risk.

DISCUSSION OF LIMITATIONS OF CONVENTIONAL TECHNOLOGIES DESCRIBED IN RELATED ART

The culture of cells is a critical element of biotechnology. Cells are cultured in small quantities during the research stage, and typically the magnitude of the culture increases as the research moves towards its objective of benefiting human and animal health care. This increase in magnitude is often referred to as scale up. Certain devices and methods have become well established for research stage cell culture because they allow a wide variety of cell types to be cultured, and are therefore useful to the widest audience. These devices include multiple well tissue culture plates, tissue culture flasks, roller bottles, and cell culture bags. Unfortunately, these devices are inefficient and they become even less efficient in terms of labor, contamination risk, and cost during scale up. There is a need to create alternative devices and methods that research and retain scale up improve research and scale up efficiency. This discussion identifies many of the limitations in conventional technologies and points towards solutions that are subsequently described in more detail.

One attribute that is essential for research scale cell culture is a low level of complexity. Devices that minimize complexity do not require ancillary equipment to mix or perfuse the cell culture medium. They are often referred to as static devices. Static devices can be subdivided into two broad categories, 1) those that are not gas permeable and oxygenate the cells by way of a gas/liquid interface and 2) those that are gas permeable and oxygenate the cells by way of gas transfer through the device housing. The traditional petri dish, multiple well tissue culture plate, tissue culture flask, and multiple shelf tissue culture flask are in the first category. The cell culture bag and compartmentalized flasks are in the second category. All of these static devices are inefficient for a variety of reasons, including the limited height at which medium can reside in them.

Medium height is limited in the petri dish, multiple well tissue culture plate, tissue culture flask, and multiple shelf tissue culture flask due to the method of providing gas exchange. To meet cellular demand, oxygen must diffuse from a gas/liquid interface to the lower surface of the device where cells reside. To ensure adequate oxygen supply, the maximum height of cell culture medium recommended for use in these devices is about 3 mm.

Limited culture medium height leads to disadvantages. It creates a small medium volume, which can only support a small quantity of cells. Medium needs to be continually removed and added to sustain cultures, which increases handling frequency, labor, and contamination risk. The only way to culture more cells in a device is to make the footprint of the device larger so that more medium can be present. Creating a device with large footprint is challenging from a manufacturing standpoint, quickly outgrows the limited amount of space available in a typical incubator and flow hood, and makes the device more difficult to handle. Thus, commercially available cell culture devices are small. Scaling up the culture therefore requires using multiple devices or selecting more sophisticated, complex, and costly alternatives.

The tissue culture flask provides a good example of the problems inherent to static devices that rely upon a gas/liquid interface to function. Tissue culture flasks allow cells to reside upon surfaces typically ranging from 25 cm^2 to 225 cm^2 in area. The height of medium that is recommended for tissue culture flasks is between 2 mm and 3 mm. For example, Corning® recommends a 45 ml-67.5 ml working volume for its T-225 cm^2 flask. Thus, a 1000 ml culture requires between 15 and 22 T-225 cm^2 flasks. Not only does this require 15 to 22 devices to be fed, leading to increasing labor and contamination risk, it also makes very inefficient use of space because flasks are designed in a manner that holds about 95% gas and only 5% medium. For example, the body of a typical T-175 flask has a footprint approximately 23 cm long by 11 cm wide, is about 3.7 cm tall, and therefore occupies about 936 cm^3 of space. However, it typically operates with no more than about 50 ml of medium. Thus, the medium present in the body (50 ml), relative to the space occupied by the body (936 cm^3) demonstrates that nearly 95% of the flask's content is merely gas. This inefficient use of space adds shipping, sterilization, storage, and disposal cost, in addition to wasting precious incubator space.

Another commonly used research scale cell culture device is the multiple well tissue culture plate. As with the traditional tissue culture flask, maintaining a gas/liquid interface at a height of only 2 mm to 3 mm above the bottom of each well is standard operating procedure. In order to provide protection against spillage when the plates are moved around the cell culture laboratory, each well of a typical commercially available 96 well tissue culture plate is about 9 mm deep. The depth increases up to about 18 mm for a six well tissue culture plate. In the case of the ninety-six well plate, gas occupies about 75% of each well and medium occupies about 25% of each well. In the case of the six-well plate, gas occupies about 95% of each well and medium occupies about 5% of each well. This inefficient geometry adds cost to device shipping, sterilization, storage, and disposal.

In many applications, the need to frequently feed the culture by removing and replacing the small volume of medium can be problematic. For example, if the purpose of the multiple well tissue culture plate is to perform experiments, manipulating the medium could affect the outcome of those experiments. Also, because the medium volume is so small, a detrimental shift in solute concentration can occur with just a small amount of evaporation. A multiple well tissue culture plate that allowed medium to reside at an increased height without loss of cell culture function would be superior to the traditional plate by minimizing the manipulations needed to keep the culture alive, and reducing the magnitude of concentration shifts caused by evaporation.

Frequently medium exchange is also time consuming, costly, and leads to elevated contamination risk. Attempts to mitigate the problem by special liquid handling equipment such as multi-channel pipettes do not address the source of the problem, low medium height. The best solution is to allow more medium to reside in each well. Unfortunately, that solution is not possible with traditional plates due to the need for gas exchange by way of the gas/liquid interface.

Better alternatives to traditional devices are needed. If tissue culture devices were available that did not rely solely upon a gas/liquid interface to function, were just as easy to use as traditional flasks and multiple well plates, allowed more cells to be cultured in a device of the same footprint, and were easily and linearly scalable, the efficient gains would translate into reduced costs for those using cells to advance human and animal health care. It will be shown herein how the use of gas permeable materials and novel configurations can achieve this objective.

Cell culture devices that eliminate the gas/liquid interface as the sole source of gas exchange have been proposed, and made their way into the market. This approach relies on the use of a lower gas permeable membrane to bring gas exchange to the bottom of the medium. That, as opposed to sole reliance on gas/liquid interfaces, allows more gas transfer. The proposed and commercially available devices include cell culture bags, compartmentalized gas permeable flasks, gas permeable cartridges, gas permeable petri dishes, gas permeable multiple well plates, and gas permeable roller bottles.

Unfortunately, each of the gas permeable devices has inherent inefficiencies and scale up deficiencies. Primary limitations of cell culture bags, gas permeable cartridges, gas permeable petri dishes, gas permeable multiple well plates, compartmentalized gas permeable flasks, and gas permeable roller bottles include limited medium height, excessive gas permeable surface area to medium volume ratios, and poor geometry for culturing adherent cells. This has the effect of forcing numerous devices to be required for scale up, restricting device design options, and increasing cost and complexity as scale up occurs.

Close examination of prior art surrounding gas permeable devices demonstrates how conventional wisdom, and device design, limits the height of medium and the volume of medium that resides in them. In the 1976 paper entitled Diffusion in Tissue Cultures on Gas-permeable and Impermeable Supports (Jensen et al., J. Theor. Biol. 56, 443-458 (1976)), the theory of operation for a closed container made of gas permeable membrane is analyzed. Jensen et al. describes diffusion as the mode of solute transport in the medium and the paper states that "diffusion proceeds according to Fick's laws." Jensen et al. state "FIG. 2 [of Jensen et al.] shows the diffusional characteristics for cells cultured in a bag made of gas permeable material." FIG. **1**A, herein, shows FIG. 2 of Jensen et al. in which $D_c m$ is the diffusion constant of medium. FIG. **1**B, herein, shows FIG. 3 of Jensen et al. in which the model of steady state values for P_{O_2} and P_{CO_2} in a gas permeable container are shown as a linear decay throughout the medium, based on diffusion.

In 1977, Jensen (Jensen, Mona D. "Mass cell culture in a controlled environment", Cell Culture and its Applications, Academic Press 1977) described a "major innovation" by the use of "gas permeable, nonporous plastic film" to form a cell culture device. FIG. **2**, herein, shows FIG. 2 of Jensen. As shown in FIG. **2**, herein, the device created a very low height of medium, only 0.76 mm, and a very high gas permeable surface to medium volume ratio. For scale up, the device gets as long as 30 feet and is perfused using custom equipment.

In 1981, Jensen (Biotechnology and Bioengineering. Vol. XXIII, Pp. 2703-2716 (1981)) specifically stated "culture vessel design must incorporate a small diffusional distance which is fixed and constant for all the cells cultured. The design must be such that scaling-up the culture does not change the diffusion distance." Indeed, the conventional wisdom that medium should not reside at a height very far from the gas permeable membrane continues to this day, as evidenced by the commercial products that utilize gas permeable materials and the patents that are related to them. Furthermore, a high gas permeable surface to medium volume ratio continues.

A variety of gas permeable cell culture devices have entered the market and been proposed since 1981. However, continued reliance on diffusion as a primary design factor appears to be the case based upon review of the patents, device design, device specifications, and operating instructions for gas permeable devices. As design criteria, the model for diffusion limits medium height, leads to high gas permeable surface to medium volume ratios, and contributes to inefficient device geometry.

Commercially available gas permeable cell culture devices in the form of bags are currently a standard device format used for cell culture. As with the configuration of Jensen, these products allow gas exchange through the lower and upper surface of the medium via gas permeable materials. Unlike the device presented by Jensen, perfusion is not required. Typically they are not perfused, and reside in a cell culture incubator. This reduces cost and complexity and has made them an accepted device in the market. However, the limited distance between the gas permeable membranes when cell culture medium resides in them has the effect of making them geometrically unsuitable for efficient scale up. As more medium is needed, bag size must increase proportionally in the horizontal direction. Thus, they are generally unavailable in sizes beyond 2 liters, making numerous devices required for scale up. Furthermore, they are not compatible with the standard liquid handling tools used for traditional devices, adding a level of complexity for those performing research scale culture.

Bags are fabricated by laminating two sheets of gas permeable films together. A typical bag cross-section is shown in FIG. **3** taken from U.S. Pat. No. 5,686,304, which has been commercialized at the Si-Culture™ bag (Medtronic Inc.). A beneficial feature of traditional static cell culture devices is a uniform distribution of medium over the area where cells reside. Those skilled in the art specifically take great care to level incubators for the purpose of ensuring that the medium resides at a constant height throughout the device. By looking at the bag cross-section of FIG. **3**, it can be seen how medium does not reside at a uniform height above the entire lower gas permeable film, no matter how level the incubator is. Since the films mate at the perimeter, medium is forced to reside at a different height near the perimeter than elsewhere in the bag. As medium volume increases, the bag begins to take a cylindrical shape and medium distribution becomes worse. Cells can be subjected to potential nutrient gradients due to the non-uniform shape. If too much medium is in the bag, the lower surface will reside in a non-horizontal state. That also creates problems. Suspension cells residing in the bag will not distribute uniformly. Instead, they will gravitationally settle in the low point, pile up, and die as nutrient and oxygen gradients form within the pile. In the case of adherent cells, they will not seed uniformly because the amount of inoculum residing in each portion of the bag will vary. In addition to the geometric problems created if bags are overfilled, the weight of medium in excess of 1000 ml can also damage the bag as

described in U.S. Pat. No. 5,686,304. Even if the geometric limitations of bags were overcome, instructions and patents related to the bags and other gas permeable devices indicate a limit exists based on the belief that diffusion barriers prevent devices from functioning when medium resides at too great a height.

Cell culture bags are commercially available from OriGen Biomedical Group (OriGen PermaLife™ Bags), Baxter (Lifecell® X-Fold™ related to U.S. Pat. Nos. 4,829,002, 4,937,194, 5,935,847, 6,297,046 B1), Medtronic (Si-Culture™, U.S. Pat. No. 5,686,304), Biovectra (VectraCell™), and American Fluoroseal (VueLife™ Culture Bag System, covered by U.S. Pat. Nos. 4,847,462 and 4,945,203). The specifications, operating instructions, and/or patents dictate the medium height and the gas permeable surface area to medium volume ratio for each product.

Pattillo et al. (U.S. Pat. Nos. 4,829,002 and 4,937,194 assigned to Baxter International Inc.) states that typically bags are "filled to about one quarter to one half of the full capacity, to provide a relatively high ratio of internal surface area of volume of the media and cells, so that abundant oxygen can diffuse into the bag, and carbon dioxide can diffuse out of the bag, to facilitate cell metabolism and growth." In light of Pattillo et al. the best medium height attained for the Baxter Lifecell® X-Fold™ bags is for their 600 cm^2 bag, which yields a medium height of 1.0 cm to 2.0 cm and a gas permeable surface area to medium volume ratio of 2.0 cm^2/ml to 1.0 cm^2/ml.

The product literature for the VectraCell™ bag states "VectraCell 1 L containers can hold up to 500 mL of media. VectraCell 3 L containers can hold up to 1500 mL of media." Thus, as with the Baxter bags, maximum medium capacity is at one half the bags total capacity. Of the various bag sizes offered, the 3 L bag allows the highest medium height, 1.92 cm, and has the lowest gas permeable surface area to medium volume ratio of 1.04 cm^2/ml.

A 1.6 cm medium height is recommended for the Si-Culture™ bag in the product literature and specified in U.S. Pat. No. 5,686,304 when it resides on an orbital shaker that physically mixes the medium. That leads to a gas permeable surface area to medium volume ratio of 1.25 cm^2/ml when used in a mixed environment. Since mixing is generally used to break up diffusional gradients and enhance solute transfer, one skilled in the art would conclude that medium height should be reduced when this bag is not placed on an orbital shaker.

The product literature for the VueLife™ bag specifically recommends filling VueLife™ Culture Bags with media at a height of no more than one centimeter thick, because "additional media might interfere with nutrient or gas diffusion." Thus, diffusional concerns limit medium height in the VueLife™ bags. That leads to a gas permeable surface area to medium volume ratio of 2.0 cm^2/ml at a medium height of 1.0 cm.

The product literature for the OriGen PermaLife™ bags specify nominal volume at a medium height of 1.0 cm, the equivalent height of the VueLife™ bags. Of the various PermaLife™ bags offered, their 120 ml bag offers the lowest gas permeable surface area to medium volume ratio of 1.8 cm^2/ml.

The net result of the limited medium height is that culture scale up using these products is impractical. For example, if the Lifecell X-Fold™ bag were scaled up so that is could contain 10 L of medium at a medium height of 2.0 cm, its footprint would need to be at least 5000 cm^2. Not only is this an unwieldy shape, the footprint can quickly outsize a standard cell culture incubator, leading to the need for custom incubators. Also, the gas transfer area utilized in the bags is larger than necessary because all of these configurations rely upon both the upper and lower surfaces of the bag for gas transfer.

This impractical geometry has restricted the size of commercially available bags. Recommended medium volume for the largest bag from each supplier is 220 ml for the OriGen PermaLife™ bags, 730 ml for the VueLife™ bags, 1000 ml for the Lifecell® X-Fold™ bags, 1500 ml for the VectraCell™ bags, and 2000 ml for the Si-Culture™ bags when shaken. Therefore, scale up requires the use of numerous individual bags, making the process inefficient for a variety of reasons that include increased labor and contamination risk.

Another deficiency with cell culture bags is that they are not as easy to use as traditional flasks. Transport of liquid into and out of them is cumbersome. They are configured with tubing connections adapted to mate with syringes, needles, or pump tubing. This is suitable for closed system operation, but for research scale culture, the use of pipettes is an easier and more common method of liquid handling. The inability to use pipettes is very inconvenient when the desired amount of medium to be added or removed from the bags exceeds the 60 ml volume of a typical large syringe. In that case the syringe must be connected and removed from the tubing for each 60 ml transfer. For example, a bag containing 600 ml would require up to 10 connections and 10 disconnections with a 60 ml syringe, increasing the time to handle the bag and the probability of contamination. To minimize the number of connections, a pump can be used to transfer medium. However, this adds cost and complexity to small-scale cultures. Many hybridoma core laboratories that utilize cell culture bags fill them once upon setup, and do not feed the cells again due to the high risk of contamination caused by these connections and the complexity of pumps.

Matusmiya et al. (U.S. Pat. No. 5,225,346) attempts to correct the problem of liquid transport by integrating the bag with a medium storage room. The culture room and medium storage room are connected and when fresh medium is needed, medium is passed from the medium room to the culture room. While this may help in medium transport, there is no resolution to the limited medium height and high gas permeable surface area to medium volume ratios that limit bag scale up efficiency. The disclosure presents a medium height of 0.37 cm and gas permeable surface area to medium volume ratio of 5.4 cm^2/ml.

Cartridge style gas permeable cell culture devices have been introduced to the market that, unlike cell culture bags, have sidewalls. These types of devices use the sidewall to separate upper and lower gas permeable films. That allows uniform medium height throughout the device. Unfortunately, these devices are even less suitable for scale up than bags because they only contain a small volume of medium. The small medium volume is a result of an attempt to create a high gas permeable surface area to medium volume ratio.

One such product called Opticell® is provided by Bio-Chrystal Ltd. This product is a container, bounded on the upper and lower surfaces by a gas permeable silicone film, each with a surface area of 50 cm^2. The sidewall is comprised of materials not selected for gas transfer, but for providing the rigidity needed to separate the upper and lower gas membranes. Product literature promotes its key feature, "two growth surfaces with a large surface area to volume ratio." In an article for Genetic Engineering News (Vol. 20 No. 21 Dec. 2000) about this product, patent applicant Barbera-Guillem states "with the footprint of a microtiter plate, the membrane areas have been maximized and the volume minimized, resulting in a space that provides for large growth surfaces

with maximum gas interchange." The operating protocol defining how to use this product specifies introduction of only 10 ml of medium, thereby limiting the height at which medium can reside to 0.2 cm. U.S. patent application Ser. No. 10/183,132 (filed Jun. 25, 2002), associated with this device, states a height up to 0.5 inches (1.27 cm) is possible, but more preferred would be a height of about 0.07 to about 0.08 inches (0.18 cm to about 0.2 cm). WO 00/56870, also associated with this device, states a height up to 20 mm is possible, but more preferred would be a height of 4 mm. Even if the greater height of 1.27 cm described in the patent were integrated into the commercial device, that medium height does not exceed that allowed in bags. Furthermore, that would only reduce the gas permeable surface area to medium volume ratio to 1.00 cm^2/ml, which is similar to the bag. U.S. patent application Ser. No. 10/183,132 shows a configuration in which only one side of the device is gas permeable. In that configuration, which was not commercialized, a gas permeable surface area to medium volume ratio of 0.79 cm^2/ml at a medium height of 0.5 inches (1.27 cm) would be attained, which is somewhat lower than that of cell culture bags. Therefore, despite a sidewall, even when the geometry allows the maximum medium height, there is not improved scale up efficiency relative to bags.

Cartridge style gas permeable cell culture devices have also been introduced to the market by Laboratories MABIO-International®, called CLINIcell® Culture Cassettes. Like the Opticell®, neither the product design nor the operating instructions provide for an increase in medium height, or a reduced gas permeable surface area to medium volume ratio, relative to bags. The operating instructions for the CLINIcell® 25 Culture Cassette state that no more than 10 ml of medium should reside above the lower 25 cm^2 gas permeable surface. Since the surface area of the lower gas permeable material is only 25 cm^2, that creates a medium height of only 0.4 cm. Also, since the top and bottom of the device are comprised of gas permeable material, there is a high gas permeable surface area to medium volume ratio of 5.0 cm^2/ml. The operating instructions for the CLINIcell® 250 Culture Cassette state that no more than 160 ml of medium should reside above the lower 250 cm^2 gas permeable surface, leading to a low medium height of 0.64 cm and a high gas permeable surface area to medium volume ratio of 3.125 cm^2/ml.

Cartridge style gas permeable cell culture devices have recently been introduced to the market by Celartis, called Petaka™. Like the Opticell® and CLINIcell® Culture Cassettes, these devices also have a sidewall that functions as a means of separating the upper and lower gas permeable films. Unlike those products, it is compatible with a standard pipettes and syringes, so it improves convenience of liquid handling. Yet, neither the product design nor the operating instructions provide for an increase in medium height, or a reduced gas permeable surface area to medium volume ratio, relative to bags. The operating instructions state that no more than 25 ml of medium should reside between the upper and lower gas permeable surfaces, which comprise a total surface area of 160 cm^2. Product literature specifies "optimized media/surface area" of 0.156 ml/cm^2. Thus, the medium height is only 0.31 cm and the optimized gas permeable surface area to medium volume ratio is 6.4 cm^2/ml.

The limitations of the commercially available cartridge style gas permeable devices for scale up become clear when reviewing the maximum culture volume available for these devices. Opticell® provides up to 10 ml of culture volume, CLINIcell® Culture Cassettes provide up to 160 ml of culture volume, and Petaka™ provides up to 25 ml of culture volume. Therefore, just to perform a 1000 ml culture, it would take 100 Opticell® cartridges, 7 CLINIcell® Culture Cassettes, or 40 Petaka™ cartridges.

Vivascience Sartorius Group has introduced gas permeable petri dishes into the market called petriPERM. The petriPERM 35 and petriPERM 50 are products in the form of traditional 35 mm and 50 mm diameter petri dishes respectively. The bottoms are gas permeable. The walls of the petriPERM 35 mm dish and petriPERM 50 mm dish are 6 mm and 12 mm high respectively. Vivascience product specifications show the petriPERM 35 has a gas permeable membrane area of 9.6 cm^2 and a maximum liquid volume of 3.5 ml, resulting in a maximum medium height of 0.36 cm., and the petriPERM 50 has a gas permeable membrane area of 19.6 cm^2 and a maximum liquid volume of 10 ml, resulting in a maximum medium height of 0.51 cm. The petriPERM products are designed with a cover that allows the upper surface of medium to be in communication with ambient gas, and a lower gas permeable material that allows the lower surface of the medium to be in communication with ambient gas. Thus, the minimum gas permeable surface area to medium volume ratio of the petriPERM 35 is 2.74 cm^2/ml and of the petriPERM 50 is 1.96 cm^2/ml. Like other gas permeable devices, the petriPERM products are also inefficient for scale up. Just to perform a 1000 ml culture, at least 100 devices are needed. Furthermore, these devices are not capable of being operated as a closed system.

Gabridge (U.S. Pat ent No. 4,435,508) describes a gas permeable cell culture device configured with a top cover like a petri dish, designed for high resolution microscopy. The depth of the well is based on the "most convenient size for microscopy", 0.25 inch (0.635 cm). At best, the device is capable of holding medium at a height of 0.635 cm.

Vivascience Sartorius Group has also introduced gas permeable multiple well tissue culture plates called Lumox Multiwell into the market. These products are also distributed by Greiner Bio-One. They are available in 24, 96, and 394 well formats. The bottom of the plate is made of a 50 micron gas permeable film with a very low auto-fluorescence. Wall height of each well is 16.5 mm for the 24-well version, 10.9 mm for the 96-well version, and 11.5 mm for the 384-well version. Maximum working medium height for each well are specified to be 1.03 cm for the 24-well version, 0.97 cm for the 96-well version, and 0.91 cm for the 384-well version. Although medium height is improved relative to traditional multiple well plates, it falls within the limits of other static gas permeable devices.

Fuller et al. (WO 01/92462 A1) presents a gas permeable multiple well plate that increases the surface area of the lower gas permeable silicone material by texturing the surface. However, the wall height is limited to merely that of "a standard microtiter plate", thereby failing to allow an increase in medium height relative to traditional plates.

In general, it would be advantageous if static gas permeable cell culture devices could utilize membranes that are thicker than those used in commercially available devices. Conventional wisdom for single compartment static gas permeable cell culture devices that rely upon silicone dictates that proper function requires the gas permeable material to be less than about 0.005 inches in thickness or less, as described in U.S. Pat. No. 5,686,304. The Si-Culture™ bag is composed of di-methyl silicone, approximately 0.0045 inches thick. Barbera-Guillem et al. (U.S. patent application Ser. No. 10/183,132) and Barbera-Guillem (WO 00/56870) state that the thickness of a gas permeable membrane can range from less than about 0.00125 inches to about 0.005 inches when the membranes comprised suitable polymers including polysty-

rene, polyethylene, polycarbonate, polyolefin, ethylene vinyl acetate, polypropylene, polysulfone, polytetrafluoroethylene, or silicone copolymers. Keeping the films this thin is disadvantageous because the films are prone to puncture, easily get pinholes during fabrication, and are difficult to fabricate by any method other than calendaring which does not allow a profile other than sheet profile. It will be shown herein how an increased thickness of silicone beyond conventional wisdom does not impede cell culture.

Improved static gas permeable devices are needed. If gas permeable devices were capable of scale up in the vertical direction, efficiency would improve because a larger culture could be performed in a device of any given footprint, and more ergonomic design options would be available.

Compartmentalized, static gas permeable devices, are another type of product that provides an alternative to traditional culture devices. However, they also are limited in scale up efficiency by medium height limitations and excessive gas permeable surface area to medium volume ratios. These types of devices are particularly useful for creating high-density culture environments by trapping cells between a gas permeable membrane and a semi-permeable membrane. Although not commercialized, Vogler (U.S. Pat. No. 4,748,124) discloses a compartmentalized device configuration that places cells in proximity of a gas permeable material and contains non-gas permeable sidewalls. The cell compartment is comprised of a lower gas permeable material and is bounded by an upper semi-permeable membrane. A medium compartment resides directly and entirely above the semi-permeable membrane. A gas permeable membrane resides on top of the medium compartment. Medium is constrained to reside entirely above the gas permeable bottom of the device. The patent describes tests with a cell culture compartment comprised of 0.4 cm sidewalls, a medium compartment comprised of 0.8 cm sidewalls, a cell culture volume of 9 ml, a basal medium volume of 18 ml, a lower gas permeable membrane of 22 cm^2, and an upper gas permeable membrane of 22 cm^2. That creates a cell compartment medium height of 0.4 cm and allows medium to reside at a height of 0.8 cm in the medium compartment. Furthermore, there is a high total gas permeable surface area to total medium volume ratio of 1.76 cm^2/ml. In a paper entitled "A Compartmentalized Device for the Culture of Animal Cells" (Biomat., Art. Cells, Art. Org., 17(5), 597-610 (1989)), Vogler presents biological results using the device of U.S. Pat. No. 4,748,124. The paper specifically cites the 1976 Jensen et al. and 1981 Jensen papers as the "theoretical basis of operation." Dimensions for test fixtures describe a 28.7 cm^2 lower and 28.7 cm^2 upper gas permeable membrane, a cell compartment wall height of 0.18 cm allowing 5.1 ml of medium to reside in the cell compartment, and a medium compartment wall height of 0.97 cm allowing 27.8 ml of medium to reside in the medium compartment. Total medium height is limited to 0.18 cm in the cell compartment, 0.97 cm in the medium compartment, with a high total gas permeable surface area to total medium volume ratio of 1.74 cm^2/ml.

Integra Biosciences markets compartmentalized gas permeable products called CELLine™. As with Vogler's device, the cell compartment is bounded by a lower gas permeable membrane and an upper semi-permeable membrane. However, unlike the Vogler geometry, all medium in the device does not need to reside entirely above the gas permeable membrane. Only a portion of the basal medium need reside above the semi-permeable membrane. The patents that cover the Integra Biosciences products, and product literature, describe the need to keep the liquid height in the cell compartment below about 15 mm. A ratio of 5 ml to 10 ml of nutrient medium per square centimeter of gas permeable membrane surface area is described for proper cell support (U.S. Pat. Nos. 5,693,537 and 5,707,869). Although the increase in medium volume to cell culture area is advantageous in terms of minimizing the frequency of feeding, in practice the medium height above each centimeter of gas permeable surface area is limited. The commercial design of the devices covered by these patents demonstrates that they, like the other gas permeable devices, limit the amount of medium that can reside above the cells. Over half of the medium volume resides in areas not directly above the semi-permeable membrane in order to reduce the height of medium residing directly above the cells. The non-gas permeable sidewalls of the device are designed so that when the device is operated in accordance with the instructions for use, the height at which medium resides above the semi-permeable membrane in the CELLine™ products is approximately 3.8 cm in the CL1000, 2.6 cm in the CL350, and 1.9 cm in the CL6Well. When operated in accordance with the instructions for use, the height of medium residing in the cell culture compartment is 15 mm for the CL1000, 14 mm for the CL350, and 26 mm for the CL6Well. The patents describe, and the devices integrate, a gas/liquid interface at the upper surface of the medium. Thus, the gas transfer surface area to medium volume ratio is also limited because gas transfer occurs through the bottom of the device and at the top of the medium. The gas transfer surface area to medium volume ratio for each device is approximately 0.31 cm^2/ml for the CL1000, 0.32 cm^2/ml for the CL350, and 1.20 cm^2/ml for the CL6Well.

Bader (U.S. Pat. No. 6,468,792) also introduces a compartmentalized gas permeable device. Absent sidewalls, it is in the form of a bag. It is compartmentalized to separate the cells from nutrients by a microporous membrane. As with the other compartmentalized gas permeable devices, medium height is limited. U.S. Pat. No. 6,468,792 states although medium heights up to 1 to 2 cm can be achieved in the apparatus, actual heights need to be tailored based upon the O2 supply as a function of "medium layer in accordance with Fick's law of diffusion." Since the upper and lower surfaces of the bag are gas permeable, a minimum total gas permeable surface area to total medium volume ratio of 1.0 cm^2/ml is attained when the apparatus is filled to its maximum capacity.

If compartmentalized gas permeable devices were capable of increasing their scale up potential in the vertical direction, they would have a more efficient footprint as the magnitude of the culture increases. A static, compartmentalized, gas permeable device that accommodates vertical scale up is needed.

Gas permeable devices that attempt to improve efficiency relative to static gas permeable devices have been introduced. The devices operate in a similar manner as the traditional roller bottle and attempt to improve mass transfer by medium mixing that comes with the rolling action. However, efficient scale up is not achieved. One reason is that, like static devices, design specifications constrain the distance that medium can reside from the gas permeable device walls. This limits device medium capacity. Thus, multiple devices are needed for scale up.

Spaulding (U.S. Pat. No. 5,330,908) discloses a roller bottle configured with gas permeable wall that is donut shaped. The inner cylinder wall and the outer cylinder wall are in communication with ambient gas. The gas permeable nature of the walls provides oxygen to cells, which reside in the compartment bounded by the inner and outer cylinder walls. The cell compartment is filled completely with medium, which is advantageous in terms of limiting cell shear. Spaulding states "the oxygen efficiency decreases as a

function of the travel distance in the culture media and effectiveness is limited to about one inch or less from the oxygen surface." Thus, the design limits stated by Spaulding include keeping the distance between the inner cylindrical wall and the outer cylindrical wall at 5.01 cm or less in order to provide adequate oxygenation. In that manner, cells cannot reside more than 2.505 cm from a gas permeable wall. That also leads to a gas permeable surface area to medium volume ratio of about 0.79 cm^2/ml. Furthermore, the need to have a hollow gas permeable core wastes space. The device only has an internal volume of 100 ml of medium for every 5 cm in length, as opposed to 500 ml for a traditional bottle of equivalent length. The medium volume limitation makes this device less efficiently scalable than the traditional roller bottle, because more bottles are needed for a culture of equivalent volume. Another problem with the device is the use of etched holes, 90 microns in diameter, for gas transfer. These holes are large enough to allow gas entry, but small enough to prevent liquid from exiting the cell compartment. However, they could allow bacterial penetration of the cell compartment since most sterile filters prevent particles of 0.45 microns, and more commonly 0.2 microns, from passing.

In a patent filed in December 1992, Wolf et al. (U.S. Pat. No. 5,153,131) describes a gas permeable bioreactor configured in a disk shape that is rolled about its axis. The geometry of this device attempts to correct a deficiency with the proposal of Schwarz et al. U.S. Pat. No. 5,026,650. In U.S. Pat. No. 5,026,650, a gas permeable tubular insert resides within a cylindrical roller bottle and the outer housing is not gas permeable. Although it was successful at culturing adherent cells attached to beads, Wolf et al. state that it was not successful at culturing suspension cells. The device is configured with one or both of the flat ends permeable to gas. The disk is limited to a diameter of about 6 inches in order to reduce the effects of centrifugal force. The inventors state "the partial pressure or the partial pressure gradient of the oxygen in the culture media decreases as a function of distance from the permeable membrane", which is the same thought process expressed by Jensen in 1976. They also state "a cell will not grow if it is too far distant from the permeable membrane." Therefore, the width is limited to less than two inches when both ends of the disk are gas permeable. These dimensional limitations mean that the most medium the device can hold is less than 1502 ml. Therefore, more and more devices must be used as the culture is scaled up in size. Also, the gas permeable surface area to medium volume ratio must be at least 0.79 ml/cm^2 and cells must reside less than 1.27 cm from a gas permeable wall. Furthermore, the device does not adapt for use with existing laboratory equipment and requires special rotational equipment and air pumps.

In a patent filed in February 1996, Schwarz (U.S. Pat. No. 5,702,941) describes a disk shaped gas permeable bioreactor with gas permeable ends that rolls in a similar manner as a roller bottle. Unfortunately, as with U.S. Pat. No. 5,153,131, the length of the bioreactor is limited to about 2.54 cm or less. Unless all surfaces of the bioreactor are gas permeable, the distance becomes even smaller. Maximum device diameter is 15.24 cm. Thus, the gas permeable surface area to medium volume ratio must be at least 0.79 ml/cm^2 and cells can never reside more than 1.27 cm from a gas permeable wall. Even with the rolling action, this does not render a substantial reduction in the gas permeable surface area to medium ratio relative to traditional static culture bags, and requires more and more devices to be used as the culture is scaled up in size.

A commercially available product line from Synthecon Incorporated, called the Rotary Cell Culture System™, integrates various aspects of the Spaulding, Schwarz, and Wolf et al. patents. The resulting products are have small medium capacity, from 10 ml to 500 ml, require custom rolling equipment, are not compatible with standard laboratory pipettes, and are very expensive when compared to the cost of traditional devices that hold an equal volume of medium. Thus, they have made little impact in the market because they do not address the need for improved efficiency in a simple device format.

Falkenberg et al. (U.S. Pat. Nos. 5,449,617 and 5,576,211) describes a gas permeable roller bottle compartmentalized by a dialysis membrane. The medium volume that can be accommodated by the bottle is 360 ml, of which 60 ml resides in the cell compartment and 300 ml in the nutrient compartment. In one embodiment, the ends of the bottle are gas permeable. U.S. Pat. No. 5,576,211 states the when the end of the bottle is gas permeable, "gas exchange membranes with a surface area of a least 50 cm^2 have been proven to be suitable for cell cultures of 35 ml." Therefore, the minimum gas permeable surface area to volume ratio is 1.43 cm^2/ml. In another embodiment, the body of the bottle is gas permeable, with a surface area of 240 cm^2. That gas permeable surface oxygenates the entire 360 ml volume of medium that resides in the vessel. Therefore, the minimum gas permeable surface area to volume ratio is 0.67 cm^2/ml. The diameter of the bottle is approximately 5 cm, and the length of the bottle is approximately 15 cm. Thus, the bottle is much smaller than a traditional roller bottle, which has a diameter of approximately 11.5 cm and a length up to approximately 33 cm. Although this device is useful for high-density suspension cell culture, its limited medium capacity fails to reduce the number of devices needed for scale up. Furthermore, it is not suitable for adherent culture because it makes no provision for attachment surface area.

Falkenberg et al. (U.S. Pat. No. 5,686,301) describes an improved version of the devices defined in U.S. Pat. Nos. 5,449,617 and 5,576,211. A feature in the form of collapsible sheathing that prevents damage by internal pressurization is disclosed. Gas is provided by way of the end of the bottle and can "diffuse into the supply chamber" by way of the gas permeable sheathing. Unfortunately, it fails to reduce the number of devices needed for scale up because the bottle dimensions remain unchanged. Furthermore, it remains unsuitable for adherent culture.

Vivascience Sartorius Group sells a product called the miniPERM that is related to the Falkenberg et al. patents. The maximum cell compartment module is 50 ml and the maximum nutrient module is 400 ml. Thus, the maximum volume of medium that can reside in the commercial device is 450 ml. The small size of the commercial device, combined with the need for custom rolling equipment, renders it an inefficient solution to the scale up problem.

There exists a need to improve the rolled gas permeable devices so that they can provide more medium per device, thereby reducing the number of devices needed for scale up. That can be achieved if a decreased gas permeable surface area to medium volume ratio is present. Another problem is that non-standard laboratory equipment is needed for operation of the existing devices. The use of standard laboratory equipment would also allow more users to access the technology.

The prior discussion has focused on design deficiencies that limit efficient scale up in existing and proposed cell culture devices. In addition to the previously described limitations, there are additional problems that limit scale up efficiency when adherent cell culture is the objective.

For traditional static devices that rely upon a gas/liquid interface for oxygenation, the adherent cell culture ineffi-

ciency is caused by limited attachment surface area per device. For example, only the bottom of the device is suitable for cell attachment with petri dishes, multiple well plates, and tissue culture flasks. The traditional flask provides a good example of the problem. As described previously, a typical T-175 flask occupies about 936 cm^3. Yet, it only provides 175 cm^2 of surface area for adherent cells to attach to. Thus, the ratio of space occupied to growth surface, 5.35 cm3/cm^2, is highly inefficient.

Products that attempt to address the surface area deficiency of traditional flasks are available. Multi-shelved tissue culture flasks, such as the NUNC™ Cell Factory (U.S. Pat. No. 5,310,676) and Corning CellStack™ (U.S. Pat. No. 6,569,675), increase surface area is by stacking polystyrene shelves in the vertical direction. The devices are designed to allow medium and gas to reside between the shelves. This reduces the device footprint relative to traditional flasks when increasing the number of cells being cultured. The profile of the multi-shelved flasks is also more space efficient that traditional flasks. For example, the space between shelves of the NUNC™ Cell Factory is about 1.4 cm, as opposed to the 3.7 cm distance between the bottom and top of a typical T-175 flask. The reduced use of space saves money in terms of sterilization, shipping, storage, incubator space, and device disposal. This style of device also reduces the amount of handling during scale up because one multi-shelved device can be fed as opposed to feeding multiple tissue culture flasks. Furthermore, the use of traditional polystyrene is easily accommodated. Unfortunately, the device is still sub-optimal in efficiency since each of its shelves requires a gas/liquid interface to provide oxygen.

CeilCube® is an adherent cell culture device available from Corning Life Sciences. It is configured in a similar manner to the multiple shelved tissue culture flasks, but it eliminates the gas/liquid interface. The distance between the vertically stacked cell attachment shelves is therefore reduced because gas is not present. That reduces the amount of space occupied by the device. However, in order to provide gas exchange, continuous perfusion of oxygenated medium is required. That leads to a very high level of cost and complexity relative to the Corning CellStack™, rendering it inferior for research scale culture.

Static gas permeable devices do not provide a superior alternative to the NUNC™ Cell Factory, Corning CellStack™, or CellCube®. Cell culture bags and gas permeable cartridges can provide more attachment area than traditional tissue culture flasks. That is because they could allow cells to be cultured on both the upper and lower device surfaces. However, gas permeable materials that are suitable for cell attachment can be much more expensive than traditional polystyrene. Also, even if both the upper and lower surfaces of a gas permeable device allowed cells to grow, only a two-fold increase in surface area would be obtained relative to a traditional gas/liquid interface style device that occupied the same footprint. Furthermore, the scale up deficiencies that have been described previously remain limiting.

Fuller et al. (IPN WO 01/92462 A1) presents a new bag that textures the surface of the gas permeable material in order to allow more surface area for gas transfer and cell attachment. However, medium height is also limited to that of the commercially available bags. That is because this bag is fabricated in the same manner as the other bags. Gas permeable surface area to medium volume ratio becomes even higher than that of other bags, and non-uniform medium distribution is present.

Basehowski et al. (U.S. Pat. No. 4,939,151) proposes a gas permeable bag that is suitable for adherent culture by making the bottom gas permeable, smooth, and charged for cell attachment. The inner surface of the top of the bag is textured to prevent it from sticking to the lower gas permeable surface. This bag only utilizes the lower surface for cell attachment, rendering it only as efficient in surface area to footprint ratio as a traditional flask.

To date, guidance is inadequate on how to create a device that eliminates the reliance on a gas/liquid interface and can integrate the scaffold of the multiple layer flasks without the need for perfusion. Static gas permeable devices only allow gas transfer through the bottom and top of the device. Thus, if traditional scaffolds are included, such as the styrene shelves provided in the multi-shelved tissue culture flasks, they will have the effect of inhibiting gas exchange at the cell location. Gas permeable materials should be located in a manner in which the attachment scaffold does not prevent adequate gas transfer. How that becomes beneficial will be further described in the detailed description of the invention herein.

The need to provide more efficient cell culture devices during scale up is not limited to static cell culture devices, but also applies to roller bottles. Traditional roller bottles function by use of a gas/liquid interface. The geometry is a clever way of providing more surface area and medium volume while occupying a smaller footprint than flasks and bags. Their universal use provides testimony to the market desire for devices that provide more efficient geometry, since that leads to reductions in the use of inventory space, incubator space, labor, and biohazardous disposal space.

When bottles are used for adherent culture, cells attach to the inner wall of the bottle. Cells obtain nutrients and gas exchange as the rolling bottle moves the attached cells periodically through the medium and gas space. Roller bottle use is not limited to adherent cells. They are also commonly used to culture suspension cells. For example, the culture of murine hybridomas for the production of monoclonal antibody is routinely done in roller bottles. In typical suspension cell culture applications, efficiency improvements related to footprint and size versus flasks can be attained, the handling simplicity of the roller bottle is superior to cell culture bags, and the low cost and level of complexity is superior to spinner flasks. Corning®, the leading supplier of roller bottles recommends medium volume for an 850 cm^2 bottle between 170 ml and 255 ml. The actual capacity of the bottle is about 2200 ml. Therefore, although the roller bottle provides advantages for both adherent and suspension cell culture, it is still very inefficient in geometry because the vast majority of the roller bottle, about 88%, is comprised of gas during the culture process. Roller bottles also deviate from the simplicity of static devices because ancillary roller mechanisms are required. Furthermore, they subject the cells to shear force. Those shear forces can damage or kill shear sensitive cells, and are not present in the traditional static devices.

McAleer et al. (U.S. Pat. No. 3,839,155) describes a roller bottle device configured to allow cells to attach to both sides of parallel discs oriented down the length of the bottle. Unlike the traditional bottle that rolls in the horizontal position, this device tumbles end over end to bring the discs through medium and then through gas. It does nothing to reduce the volume of gas residing in the bottle. On the contrary, it states "another advantage of the present invention is that extremely low volumes of fluid can be used." It relies entirely upon the presence of a large volume of gas, which must be perfused, in the bottle to function. The excessive volume of gas that hinders the efficient use of space in traditional bottles remains. Also, shear forces are not reduced.

Spielmann (U.S. Pat. No. 5,650,325) describes a roller bottle apparatus for providing an enhanced liquid/gas exchange surface. Trays are arranged in parallel within the

bottle. The trays allow an increase of surface area for culture and are designed to allow liquid to flow over them as the bottle rotates. In the case of adherent cells, more surface area is available for attachment. In the case of suspension cells, they are stirred "in contact with gas and liquid phases" by the trays. Shear forces remain present. Although this apparatus provides an improved surface area, it relies entirely upon the presence of gas in the bottle to provide gas exchange. Thus, it does not address the fundamental limitation in space efficiency, which is the excessive volume of gas that must reside in the bottle.

If the roller bottle could be made to allow a vastly improved medium volume to gas ratio, it would provide a more economical option because the number of devices needed for scale up would be reduced. Since the typical medium volume for an 850 cm^2 bottle is 170 ml to 255 ml, but the capacity is 2200 ml, about a 9 to 13 fold increase in nutrient capacity could be made available by filling the bottle with medium. To retain simplicity, a non-complicated method of oxygenating the culture independent of a gas/liquid interface would need to exist. Also, for adherent culture, surface area should increase in proportion to the increase in medium volume. A gas permeable device with these characteristics could lead to a 9-fold to 13-fold reduction in the cost of sterilization, shipping, storage, use of incubator space, labor, and disposal cost. Shear forces on the cells could also be reduced.

For adherent culture, proposed and commercially available rolled gas permeable devices do not provide a superior alternative to traditional bottles because they have not integrated traditional attachment surfaces. Instead they rely upon small sections of attachment area or beads. Beads bring a new set of problems to those performing adherent culture. They are difficult to inoculate uniformly, it is not possible to assess cell confluence or morphology microscopically, and they must be separated from the cells that are attached to them if cell recovery is desired.

Attempts to eliminate the use of beads in gas permeable roller bottles have been made. Nagel et al. (U.S. Pat. No. 5,702,945), attempts to create the ability for the Falkenberg et al. devices to culture adherent cells without beads. One cell attachment matrix is provided in the cell culture compartment at the inner face of the gas membrane. Although adherent culture is possible, the bottle dimensions remain unchanged and, due to its small size, it fails to reduce the number of devices needed for scale up. Also, oxygen must transfer first through the gas permeable membrane and then through the cell attachment matrix to reach the cells. Furthermore, only one layer of cell attachment matrix is available, as opposed to the multiple layers of the NUNC™ Cell Factory and Corning CellStack™. Additionally, microscopic assessment of cell confluence and morphology is not accommodated.

An improved gas permeable roller bottle is needed. It should be capable of being filled with medium, used in standard roller racks, allowing an increase in cell attachment area in direct proportion to the increased medium volume, and retain the ease of use of the traditional bottle. It will be shown herein how this can be achieved.

Singh (U.S. Pat. No. 6,190,913) states that for "all devices that rely on gas-permeable surfaces, scale-up is limited". A bag is disclosed for resolving the scale up deficiencies of gas permeable devices. The non-gas permeable bag integrates medium and gas, in roughly equal proportions. The bag is placed on a rocker plate, and the rocking motion creates a wave in the medium, which enhances gas transfer. This patent covers the commercial product, available from Wave Biotech called the Wave Bioreactor. Unfortunately, custom rocking and temperature control equipment must be purchased for the apparatus to function, and the bag does not substantially alter the capacity to hold medium. As with gas permeable bags, the Wave Bioreactor bags are filled with medium to no more than one half of their carrying capacity. Thus, they limit medium height and inherit similar scale up deficiencies as gas permeable bags.

In summary, a need exists for improved cell culture devices and methods that bring more efficiency to research scale cell culture, and do not lose efficiency during scale up. Traditional devices that rely upon a gas/liquid interface to function are inefficient in terms of labor, sterilization cost, shipping cost, storage cost, use of incubator space, disposal cost, and contamination risk. Those devices include the petri dish, multiple well tissue culture plate, tissue culture flask, multiple shelved tissue culture flask, and roller bottle. Gas permeable devices are also inefficient, and in many cases lose the simplicity of the devices that require a gas/liquid interface to function. The petriPERM and Lumox multiwell plate gas permeable devices are in the form of their traditional counterparts, and inherit the inefficiencies of traditional devices. Gas permeable bags are inefficient due to medium height limitations, non-uniform medium distribution, use of high gas permeable material surface area to medium volume ratios, and the contamination risk present during feeding. Gas permeable cartridges are inefficient because they have a low height of medium, use a high gas permeable surface area to medium volume, house a small volume of medium, and require a very large number of units to be maintained during scale up. Rolled gas permeable devices are inefficient for scale up because they have geometry constraints that limit the distance that the walls can be separated from each other, require a large number of units during scale up due to limited medium volume, and often require custom rolling equipment. When adherent culture is desired, traditional devices have a very inefficient device volume to attachment surface area ratio, wasting space. Static, mixed, and rolled gas permeable devices become even more inefficient for adherent culture for reasons that include limited surface area, the use of beads for increased surface area, lack of traditional sheet styrene surfaces, and inability to perform microscopic evaluations.

Certain embodiments disclosed herein provide more efficient cell culture devices and methods, that overcome the limitations of prior devices and methods, by creating gas permeable devices that can integrate a variety of novel attributes. These various attributes include gas exchange without reliance upon a gas/liquid interface, increased medium height, reduced gas permeable surface area to medium volume ratios, gas exchange through the device side walls, cell support scaffolds that are comprised of traditional materials, and increased gas permeable material thickness.

SUMMARY OF THE INVENTION

It has been discovered that for gas permeable devices comprised of a lower gas permeable material, it can be beneficial to increase medium height beyond that dictated by conventional wisdom or allowed in commercially available devices. It is contemplated by the inventors hereof that convection of substrates within cell culture medium plays a more important role than previously recognized. It would appear that the historic reliance upon diffusion for mass transfer underestimates the contribution that convection makes. That would result in underestimating the rate of travel of substrates such as glucose and lactate in cell culture medium, and a failure to recognize that medium residing farther away from cells than traditionally allowed can be useful to the cells. If the rate of travel of substrates in medium were underestimated, medium

residing in areas believed to be too far away from the cells would incorrectly deemed to be wasted. The logical consequence would be to unnecessarily configure the gas permeable device to hold less medium than could be useful to the cells, in order to reduce the space occupied by the device, making it more economically sterilized, shipped, stored, and disposed of.

In any event, and as an example of how medium residing at a distance beyond conventional wisdom can be beneficial, tests were conducted in which medium height was increased far beyond that suggested previously, or even possible in commercially available static gas permeable devices. Evaluations of a common cell culture application, using murine hybridomas, demonstrated that more cells were able to reside in a given footprint of the device by increasing medium height relative to conventional wisdom. This benefit, not previously recognized, allows a variety of cell culture device configurations that provide more efficient cell culture and process scale up to become available.

The inventive apparatus and methods herein demonstrate that the gas/liquid interface is not necessary for adequate gas exchange when a wall of a device is gas permeable, scaffolds are present, and the device is operated in a static mode. This eliminates the need for excess device size that results from the presence of gas in traditional devices, and allows gas permeable devices to integrate traditional scaffolds. This allows a variety of cell culture device configurations that occupy less space than prior devices, and makes them more efficient for scale up. Again, it is contemplated by the inventors that the role of convection may be a contributing factor.

It has also been discovered that geometric configurations for gas permeable roller bottles, that contradict the guidance of conventional wisdom, can successfully culture cells. The new geometry allows the device to contain more medium than previously possible, thereby yielding a geometric shape that improves scale up efficiency. This allows cell culture device configurations to exist that eliminate the wasted space of traditional bottles that contain gas for oxygenation, and are superior to gas permeable bottles in terms of scale up efficiency.

It has also been discovered that cells can be effectively cultured using silicone gas permeable material that is thicker than conventional wisdom advocates.

These discoveries have made it possible to create new devices and methods for culturing cells that can provide dramatic efficiency and scale up improvements over current devices such as the petri dish, multiple well tissue culture plate, tissue culture flask, multiple shelved tissue culture flask, roller bottle, gas permeable petri dish, gas permeable multiple well plate, gas permeable cell culture bag, compartmentalized gas permeable devices, and gas permeable rolled devices.

Certain embodiments disclosed herein provide superior gas permeable cell culture devices, by increasing wall height in order to allow increased medium heights and reduced gas permeable surface area to medium volume ratios.

Certain embodiments disclosed herein provide superior cell culture methods using gas permeable cell culture devices, by increasing medium heights and reducing gas permeable surface area to medium volume ratios.

Certain embodiments disclosed herein provide superior cell culture devices, by allowing gas exchange through a sidewall at least partially comprised of gas permeable material.

Certain embodiments disclosed herein provide superior cell culture methods using gas permeable devices, by allowing gas exchange through a sidewall at least partially comprised of gas permeable material.

Certain embodiments disclosed herein provide a superior alternative to gas permeable multiple well tissue culture plates, by increasing wall height in order to allow increased medium height and reduced gas permeable surface area to medium volume ratios.

Certain embodiments disclosed herein provide a superior alternative to gas permeable petri dishes, by increasing wall height in order to allow increased medium height and reduced gas permeable surface area to medium volume ratios.

Certain embodiments disclosed herein provide a superior alternative to the method of cell culture in gas permeable cell culture bags, by increasing medium height in order to provide more nutrient support and reducing gas permeable surface area to medium volume ratios.

Certain embodiments disclosed herein provide a superior alternative to the gas permeable cartridges, by increasing wall height in order to allow increased medium heights and reduced gas permeable surface area to medium volume ratios.

Certain embodiments disclosed herein provide a superior alternative to the gas permeable roller bottles, by creating a geometry that allows medium to reside at a distance from the gas permeable material beyond that previously possible.

Certain embodiments disclosed herein provide superior gas permeable cell culture devices that can be operated in the horizontal and vertical position.

Certain embodiments disclosed herein provide a superior alternative to the compartmentalized gas permeable devices, by increasing wall height in order to allow increased medium heights and reducing gas permeable surface area to medium volume ratios.

Certain embodiments disclosed herein provide a superior cell culture methods using compartmentalized gas permeable devices, by increasing medium height and reducing gas permeable surface area to medium volume ratios.

Certain embodiments disclosed herein provide superior gas permeable cell culture devices that utilize silicone material for gas exchange, by configuring them with silicone that is greater than 0.005 inches thick.

Certain embodiments disclosed herein provide an improved cell culture bag in which the gas permeable material is silicone that exceeds 0.005 inches thick.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**A and FIG. **1**B are obtained from Jensen et al., "Diffusion in Tissue Cultures on Gas-permeable and Impermeable Supports", J. Theor. Biol. 56, 443-458 (1976), FIG. **1**A shows FIG. **2**, and FIG. **1**B shows FIG. **3**, of this Jensen et al. paper in which $D_c m$ is the diffusion constant of medium and the model for steady state values of P_{O_2} and P_{CO_2} are shown in a gas permeable container.

FIG. **2** is a copy of FIG. **2** from Jensen, "Mass cell culture in a controlled environment", Cell Culture and its Applications, Academic Press 1977, showing a gas permeable cell culture device configured with a low medium height capacity.

FIG. **3** is a copy of FIG. 2 of U.S. Pat. No. 5,686,304, which has been commercialized as the Si-Culture™ bag (Medtronic Inc.), showing a typical cell culture bag cross-section.

FIG. **4**A is an embodiment of a cell culture device with a housing comprised of a lower gas permeable material, configured to allow a large volume of medium to reside above its lower gas permeable material. A removable lid protects it from contaminants. FIG. **4**B is an embodiment of a cell culture device with a housing comprised of a lower gas permeable material, configured to allow a large volume of medium

to reside above its lower gas permeable material. The container is accessible by septum. FIG. **4**C is an embodiment of a cell culture device with the walls comprised of gas permeable material such that the device can be laid on its side and operated in the non-rolling or rolling position.

FIG. **5** is an embodiment of a gas permeable cell culture device with a lower gas permeable material configured to allow cells to distribute evenly about its lower surface and provide gas to the underside of the lower gas permeable material.

FIG. **6** is an embodiment of a gas permeable cell culture device configured to maintain medium in areas not directly above the cells being cultured, in order to provide additional nutrient support without a further increase in device profile.

FIG. **7**A and FIG. **7**B are two views of an embodiment of a gas permeable cell culture device configured so that it can adjust in height as the volume of medium within it changes, thereby occupying as little space as possible at each stage of the culture process and allowing the capability of being sterilized, shipped, stored, and disposed of in a minimum volume condition which reduces the cost of the process.

FIG. **8** is an embodiment of a gas permeable cell culture device configured in a multiple well format, capable of holding an increased volume of medium per well relative to traditional multiple well tissue culture devices, thereby allowing more efficient research scale culture by increasing the amount of cells present per well, reducing feeding frequency, and allowing better clone selection possibilities.

FIG. **9**A and FIG. **9**B are views of embodiments of a gas permeable cell culture device in a multiple well format, configured with a gas permeable sidewall. The lower surface of each well of the device can be comprised of exactly the same material as traditional tissue culture flasks. Elimination of the gas/liquid interface as a requirement for gas exchange allows for an increased number of cells per well and/or reduced frequency of feeding, better use of incubator space, as well as cost reductions in sterilization, shipping, storage, and disposal.

FIG. **10**A and FIG. **10**B show an embodiment of a gas permeable cell culture device configured with scaffolds for culturing adherent cells without need of a gas/liquid interface. It is linearly scalable in the horizontal and vertical direction creating superior efficiency relative to traditional adherent culture devices. It is capable of culturing cells on either one or both sides of the scaffolds. It can be operated in either the rolled or in the unrolled state.

FIG. **11** is an embodiment of a gas permeable cell culture device configured with scaffolds, at least one of which is suitable for optimal microscopic cell assessment.

FIG. **12**A, FIG. **12**B, FIG. **12**C, and FIG. **12**D show embodiments of scaffolds configured to provide a further increase in surface area, bringing even more efficiency to the gas permeable cell culture device.

FIG. **13** is an embodiment of a gas permeable cell culture device with scaffolds and at least one sidewall comprised of gas permeable material. The need for a gas/liquid interface as a means of gas exchange is eliminated, leading to more efficient use space and the related cost benefits in terms of sterilization, shipping, storage, use of incubator space, and disposal.

FIG. **14**A, FIG. **14**B, FIG. **14**C, and FIG. **14**D show views of an embodiment of a gas permeable cell culture device configured with scaffolds, the location of which can be adjusted for benefits that can include minimizing the use of trypsin, altering the ratio of medium to culture area, and minimizing shipping, inventory, and disposal space. FIG. **14**E shows a scaffold configured to maintain equal distance between it, and its neighboring scaffolds.

FIG. **15**A, FIG. **15**B, and FIG. **15**C show an embodiment of scaffolds configured such that the distance between each can be altered while the body of the device remains at a fixed height. This embodiment can provide benefits that include minimizing the use of trypsin, or altering the ratio of medium to culture area, without need to make the body of the device change shape.

FIG. **16** is a cross-sectional view of a tubular test fixture used to assess the effect of medium height on cell growth and antibody production. Biological evaluations using this test fixture demonstrated the benefit of increasing medium height beyond the limits of conventional wisdom, and the ability to reduce the gas permeable surface area to medium volume ratio of prior devices. These surprising results allow device configurations not previously contemplated to exist.

FIG. **17** is a cross-sectional view of a test fixture used to assess the ability to culture adherent cells in the absence of a gas/liquid interface by allowing gas transfer through a sidewall of the test fixture. Biological evaluations using this test fixture demonstrated the ability to culture cells in the absence of a gas/liquid interface. These surprising results allow device configurations not previously contemplated to exist.

FIG. **18** is a cross-sectional view of a test fixture used to assess the ability to culture adherent cells in the absence of a gas/liquid interface by allowing gas transfer through a sidewall of the test fixture. Multiple scaffolds were integrated into the test fixture. Biological evaluations using this test fixture demonstrated the ability to culture cells in the absence of a gas/liquid interface. These surprising results allow device configurations not previously contemplated to exist.

FIG. **19**A is a cross-sectional view of a test fixture used to assess the ability to seed cells onto the upper and lower surfaces of a scaffold. FIG. **19**B shows one scaffold of the test fixture of FIG. **19**A. Biological evaluations using this test fixture demonstrated the ability to culture cells in the absence of a gas/liquid interface when gas exchange occurred through the sidewall of the device, that a low gas permeable material surface area to attachment surface area is functional, that that a low gas permeable material surface area to medium volume is functional, and that cells can be cultured when the device is in the unrolled position or in the rolled position.

FIG. **20** is a cell distribution pattern, as described in Example 4.

DETAILED DESCRIPTION OF THE INVENTION

By configuring gas permeable devices to be capable of holding medium at a height not contemplated in prior cell culture devices or methods, advantages can accrue including reduced handling frequency, labor, sterilization cost, shipping cost, storage cost, use of incubator space, disposal cost, and contamination risk. Reducing the ratio of gas permeable surface area to medium volume to a ratio not contemplated in prior cell culture devices or methods can also increase culture efficiency. It allows an increase in medium height without a corresponding increase in device length or width. In the preferred embodiments, provisions are made that allow either medium height to increase or the ratio of gas permeable surface area to medium volume to decrease. Provisions can also be made that allow both the medium height to increase and the ratio of gas permeable surface area to medium volume to decrease.

A wide variety of embodiments for gas permeable devices and methods that allow medium to reside at heights beyond conventional wisdom are possible. They can take the form of

prior devices, or entirely new forms. If the form is a gas permeable petri dish up to 50 mm in diameter, medium height should preferably exceed 0.36 cm. A preferred wall height is in excess of 6 mm. If the form is a gas permeable petri dish greater than 50 mm in diameter, medium height should preferably exceed 0.51 cm. A preferred wall height is in excess of 12 mm. If the form is a multiple well tissue culture plate with 384 wells or more, medium height should preferably exceed 0.91 cm and preferred well depth is in excess of 11.5 mm; less than 24 wells to less than 384 wells, medium height should preferably exceed 0.97 cm and preferred well depth is in excess of 10.9 mm; 24 wells or less, medium height should preferably exceed 1.03 cm and preferred well depth is in excess of 16.5 mm. If the form is a gas permeable cartridge, medium height and wall height should preferably be greater than 1.27 cm. If in the form of a cell culture bag, medium height should preferably reside beyond 2.0 cm in height at the highest point. If the form is a compartmentalized device, and all medium in the device resides entirely above the semi-permeable membrane, medium height in the nutrient compartment should preferably reside 1.0 cm in height above the semi-permeable membrane. If the form is a compartmentalized gas permeable device, medium height in the nutrient compartment should preferably reside beyond 3.8 cm in height above the semi-permeable membrane.

If it is the design objective to reduce the gas permeable surface area to medium volume ratio relative to conventional wisdom, a wide variety of embodiments for gas permeable devices and methods are possible. They can take the form of prior devices, or entirely new forms. If the form is a gas permeable petri dish up below 50 mm in diameter, the gas permeable surface area to medium volume ratio should preferably be below 2.74 cm^2/ml. If the form is a gas permeable petri dish 50 mm or greater in diameter, the gas permeable surface area to medium volume ratio should preferably be below 1.96 cm^2/ml. If the form is a multiple well tissue culture plate with 384 wells or more, the gas permeable surface area to medium volume ratio should preferably be below 1.10 cm^2/ml; less than 24 wells to less than 384 wells, the gas permeable surface area to medium volume ratio should preferably be below 1.03 cm^2/ml; 24 wells or less, the gas permeable surface area to medium volume ratio should preferably be below 0.97 cm^2/ml. If the form is a gas permeable cartridge in which two sides of the cartridge are gas permeable, the surface area to medium volume ratio should preferably be below 0.79 cm^2/ml. If in the form of a cell culture bag, the gas permeable surface area to medium volume ratio should preferably be below 1.0 cm^2/ml. If the form is a compartmentalized device, and all medium in the device resides entirely above the semi-permeable membrane, the gas permeable surface area to medium volume ratio should preferably be below 1.74 cm^2/ml. If the form is a compartmentalized device, and all medium in the device does not reside entirely above the semi-permeable membrane, the gas permeable surface area to medium volume ratio should preferably be below 0.31 cm^2/ml.

FIG. **4**A shows a cross-sectional view of one embodiment of the invention. Gas permeable cell culture device **10** is configured to allow cells **20** to reside upon lower gas permeable material **30**. Although FIG. **4**A shows gas permeable cell culture device **10** structured in the style of a petri dish, any number of shapes and sizes are possible that allow medium to reside at a height beyond that of conventional wisdom.

Top cover **55** can be removed to allow medium **50** to be conveniently added and removed, by either pouring or pipetting, to and from gas permeable cell culture device **10**. However, access for medium **50** can also be made in any number of ways common to cell culture devices, including by way of caps, septums, and tubes. In the event that a closed system is desired, gas permeable cell culture device **10** can be configured with inlet and outlet tubes that can be connected to medium source and waste bags by way of a sterile tubing connection, using equipment such as that made by Terumo Medical Corp. (Somerset, N.J.). Septum configurations, or any other techniques known to those skilled in the art, can also be used to create a closed container. For example, as shown in FIG. **4**B, gas permeable cell culture device **10** can be alternatively configured as a closed container with septums **65**.

In the event that gas permeable cell culture device **10** is to be completely filled with medium **50**, and cells are intended to settle out of medium **50** by gravity, the profile of the top of gas permeable cell culture device **10** preferably allows medium **50** to reside at a uniform height above gas permeable material **30**. This will allow uniform deposit of cells onto lower gas permeable material **30**, when cells gravitationally settle from suspension within medium **50**. The configuration of FIG. **4**B achieves this purpose.

The lower gas permeable material, e.g., material **30**, can be any membrane, film, or material used for gas permeable cell culture devices, such as silicone, fluoroethylenepolypropylene, polyolefin, and ethylene vinyl acetate copolymer. A wide range of sources for learning about gas permeable materials and their use in cell culture can be used for additional guidance, including co-pending U.S. patent application Ser. No. 10/460,850 incorporated herein in its entirety. The use of the words film and membrane imply a very thin distance across the gas permeable material, and the inventors have found that the embodiments of this invention function when the gas permeable material of the described devices and methods is beyond the thickness associated with films and membranes. Therefore, the portion of the device that contributes to gas exchange of the culture is called a gas permeable material herein.

Those skilled in the art will recognize that the gas permeable material should be selected based on a variety of characteristics including gas permeability, moisture vapor transmission, capacity to be altered for desired cell interaction with cells, optical clarity, physical strength, and the like. A wide variety of information exists that describe the types of gas permeable materials that have been successfully used for cell culture. Silicone is often a good choice. It has excellent oxygen permeability, can allow optical observation, is not easily punctured, typically does not bind the cells to it, and can be easily fabricated into a wide variety of shapes. If silicone is used, it may be less than about 0.2 inches, about 0.1 inches, about 0.05 inches, or about 0.030 inches in the areas where gas transfer is desired. The best selection of material depends on the application. For example, Teflon® may be preferred in applications that will be exposed to cryopreservation. For adherent culture, in which cells are to attach to the gas permeable material, WO 01/92462, U.S. Pat. Nos. 4,939,151, 6,297,046, and U.S. patent application Ser. No. 10/183,132 are among the many sources of information that provide guidance.

If silicone is used as a gas permeable material, increasing thickness beyond conventional wisdom may expand the options for design, cost reduce the manufacturing process, and minimize the possibility of puncture. For example, molding a part with a large surface area when the part must be very thin can be difficult because material may not flow into the very small gap between the core and the body of the mold. Thickening the part, which widens that gap, can make the molding process easier. In additional to possible molding

advantages, thicker gas permeable materials also are less likely to puncture or exhibit pinholes.

The height of walls, e.g., walls **40**, plays an important role in device scale up efficiency. Prior static gas permeable devices limit medium height. For example, bags provide no walls and instructions limit medium height, while cartridge style devices only provide a very low wall height (e.g. Opticell® cartridges, CLINIcell® Culture Cassettes, and Petaka™ cartridges). An object of this invention is to provide for increased medium height, thereby increasing device efficiency. The height of the walls can dictate how much medium is allowed to reside in the device. Adding medium provides a larger source of substrates, and a larger sink for waste products. By increasing wall height when more medium is needed during scale up, the geometry of the device is more compatible with the shape of incubators, flow hoods, and biohazard disposal bags. Furthermore, the increase in volume relative to the surface area upon which cells reside can allow more medium per cell to be present. That can have the effect of reducing feeding frequency, thereby reducing labor and contamination risk. It can also have the effect of increasing the number of cells residing per square centimeter of device footprint.

Structuring walls to allow an increase in medium volume can also have the beneficial effect of diminishing the effects of medium evaporation. Medium evaporation is a problem in cell culture because it alters the concentration of solutes residing in the medium. Existing gas permeable devices are prone to such an event because they have a high gas permeable surface area to medium volume ratio. Attempts to prevent such an event are described in U.S. patent application Ser. No. 10/216,554 and U.S. Pat. No. 5,693,537 for example. However, simply allowing an increase in the volume of medium in the device can reduce the impact of evaporation. If prior static gas permeable devices allowed an increase in medium volume to gas permeable surface area ratio, the rate of solute concentration change when evaporation is present would be reduced proportionally.

In a preferred embodiment, walls should be capable of allowing medium to reside at a height that exceeds that of devices that rely upon a gas/liquid interface and more preferably exceeds that of typical static gas permeable devices. For example, the height of wall **40** is beyond 3 mm, and more preferably beyond 2.0 cm, and will thus provide advantages. By providing users of the device the option of adding more medium to the device than prior gas permeable devices, many advantages accrue including the ability to house more cells per device, feed the device less frequently, and scale the device up without increasing the footprint. Walls can be comprised of any biocompatible material and should mate to lower gas permeable material in a manner that forms a liquid tight seal. The methods of mating a lower gas permeable material to walls include adhesive bonding, heat sealing, compression squeeze, and any other method commonly used for generating seals between parts. As an option, walls and lower gas permeable material can be formed of the same material and fabricated as a single entity. For example, if silicone is used, walls and the lower gas permeable material could be liquid injection molded, or dip molded, into a single gas permeable piece. That has the advantage of creating a gas permeable surface for cells to reside upon when a gas permeable cell culture device is stood vertically as shown in FIG. **4**B, or laid on its side as shown in FIG. **4**C, which shows gas permeable wall **41** with cells **20** resting thereupon.

Laying certain gas permeable cell culture devices on a side can help make optimal use of incubator space as the profile of the device can be reduced when it is too tall for narrowly spaced incubator shelves. In the case where it is desirable to have the gas permeable cell culture device reside on its side, making the device square or rectangular, instead of circular, will create a flat surface for cells to reside on when on its side. That is advantageous as it prevents localized areas for cells to pile upon each other, potentially causing harmful gradients. In the case where the device depth and width differ in dimension, three alternate surface areas are available for cells to reside upon, and three alternative maximum medium heights exist, depending on the position gas permeable cell culture device is placed in. When the device is structured for operation in these alternate positions, the surface upon which the device resides is preferably comprised of gas permeable material. That allows cells that settle by gravity onto this surface to be at optimal proximity for gas exchange.

Walls are preferably configured with enough structural strength that medium is retained in a relatively symmetrical shape above gas permeable material in order to make most efficient use of lab space, minimize gradient formation within a medium, and allow a uniform deposit of cells upon a lower gas permeable material during inoculation. It is also advantageous if walls allow visual assessment of color changes in medium in order to determine pH or contamination status. Walls may be configured in a manner that allows a gas permeable cell culture device to be easily lifted by hand. When it is desirable for walls to be gas permeable, and if a separate entity is placed around walls to retain them in a rigid position, it preferably should not block gas contact with the majority of walls.

Gas permeable cell culture devices can be configured to function either in the static or rolled mode. To do so, gas permeable cell culture devices should preferably be cylindrical. A cylindrically shaped body provides more volume than a square or rectangular body when the device is to be placed in a standard roller rack. However, a non-cylindrical body shape can still function on a roller rack by attaching a circular housing around the body. If it is desired to provide users with the option of device functioning in the vertical, horizontal, or rolling position, both the bottom and the sidewalls of the gas permeable cell culture device should be comprised of gas permeable material. If the device is only to be operated in the horizontal, rolled or unrolled, position, it may be more cost effective and minimize surface area for evaporation if the ends of the device are not comprised of gas permeable material.

If a gas permeable cell culture device is configured in a cylindrical shape with a lower gas permeable material, and the walls are comprised of gas permeable material, it can be stood vertically or rolled depending on user preference. It can be advantageous to roll gas permeable cell culture device when maximum mixing will benefit an application, such as can be the case when seeking to decrease antibody production time. If this option is desired, the walls of gas permeable cell culture device should be made gas permeable in the same manner described for lower gas permeable material. Although there are no restrictions on bottle length or diameter, it can be advantageous if the walls conform to the diameter of standard roller bottles so that gas permeable cell culture device can function on a standard roller rack.

If it is desirable to reduce cell shear, filling the device entirely with medium will eliminate gas from the device so that it cannot contribute to cell shear. The ports can be designed in any number of ways that reduce the risk of contamination as medium fills the device entirely. Also, when the device is to be rolled or function on its side, only side surfaces need be comprised of gas permeable material.

The scale up advantages provided by a device that allows medium to reside at a height that exceeds conventional wisdom will become apparent to those skilled in the art, in light of the Examples demonstrating biological outcomes herein. As an example of scale up efficiency, when a gas permeable cell culture device is cylindrical, operated in the vertical position, and the bottom provides for gas exchange, doubling the diameter increases the volume by a factor of four when the height is held constant. For example, a device of approximately 4.5 inches in diameter and about 7.7 inches tall, will house about 2 L of medium. By making the device 9.0 inches in diameter, it will house 8 L of medium. By making the device 18.0 inches in diameter, it will house 32 L of medium. Thus, culture volume can easily be scaled up while holding key parameters constant, such as the medium height and gas permeable surface area to medium volume ratio. By holding these parameters constant, protocols that are developed in a small volume device are likely to remain unchanged as device volume increases.

When a gas permeable cell culture device is operated in the vertical position, and suspension cells are being cultured, it is beneficial if ambient gas can make relatively unobstructed contact with the underside of the lower gas permeable material. For example, in incubators in which the shelves are non perforated, gas transfer in and out of the culture can be limited if the lower gas permeable material makes contact with the incubator shelf. In the embodiment shown in the cross-sectional view of FIG. **5**, lower gas permeable material support **80** acts to ensure that lower gas permeable material **30** is in contact with ambient gas by maintaining a gas compartment **90**. In the preferred embodiment, gas compartment **90** is maintained by allowing lower gas permeable material support **80** to make partial contact with lower gas permeable material **30** in a manner that does not diminish the amount of gas exchange required to support the culture. In addition to allowing exposure to ambient gas, lower gas permeable material support **80** maintains lower gas permeable material **30** in a substantially horizontal state such that cells **20** do not pile up in any low points. That would cause diffusional gradients and limit cell growth relative to a condition in which cells **20** could distribute evenly across lower gas permeable material **30**. Therefore, a design objective for lower gas permeable material support **80** may be to contact lower gas permeable material **30** in as many locations as needed to keep it substantially horizontal while still allowing adequate gas contact with the lower surface of lower gas permeable material **30**. Those skilled in the art will recognize there are many ways to achieve this objective. As shown in FIG. **5**, projections **110** achieve this objective.

A "bed of nails" configuration is one way to maintain lower gas permeable material **30** in a substantially horizontal position while allowing adequate gas exchange. For example, 1 mm×1 mm squares, distributed evenly and projecting 1 mm from the lower gas permeable material support can retain the lower gas permeable material in a substantially horizontal position. When the projections **110** occupied 50% of the surface of lower gas permeable material support **80** as shown in FIG. **5**, this configuration allowed adequate gas exchange to culture about 10 to 15 million murine hybridoma cells per square centimeter on a silicone membrane of about 0.004 inches thick. As also shown in FIG. **5**, lower gas access openings **100** allow gas to enter and exit gas compartment **90** of lower gas permeable material support **80** by passive diffusion. This allows gas permeable cell culture device **10**B to function in ambient conditions without need of ancillary pumping mechanisms. Feet **95** elevate lower gas permeable material support **80**, allowing ambient gas to be available to lower gas access openings **100**. This information also is applicable to maintaining a gas compartment around sidewalls when the device functions as described on its side in either the rolling or non-rolling mode. Other possibilities of allowing adequate gas access to a gas permeable material can be utilized. For example, the CELLine™ products from Integra Biosciences AG utilize open mesh elevated from a lower plastic support by feet to allow gas access to the gas permeable membrane. U.S. Pat. No. 5,693,537 also provides additional guidance for this feature.

In the configuration shown in FIG. **5**, cap **70** covers medium access port **60** to prevent contamination. O-ring **75** ensures that medium **50** will not leak from gas permeable cell culture device **10**B, such as when it is in the horizontal position, completely filled, or accidentally dropped.

In certain embodiments, the medium does not need to reside entirely above the lower gas permeable material. A portion of the medium can reside in areas not directly above a lower gas permeable material in order to reduce the profile of a vertical cell culture device, which may be desirable for use in incubators with limited distance between shelves. The cross-sectional view of FIG. **6** shows an embodiment configured for suspension cell culture in which walls **40**C are offset from lower gas permeable material **30** in order to decrease the profile of gas permeable cell culture device **10**C when operated in the vertical position. In this configuration, the ratio of medium volume to surface area upon which cells reside can be held constant while the profile of the device is reduced in size by simply increasing the width, or diameter, of gas permeable cell culture device **10**C. Care should be taken to ensure that cells **20** continue to reside above lower gas permeable material **30** during inoculation, feeding, and handling. Interior walls **42** achieve this by allowing gravity to keep cells **20** in the area above lower gas permeable material **30**. In a preferred embodiment, the walls should be capable of allowing medium to reside at a height above lower gas permeable material **30** that exceeds 3 mm.

FIG. **7**A and FIG. **7**B show cross-sectional views of a preferred embodiment for a gas permeable cell culture device that can raise or lower its height in response to the volume of medium residing within it. In FIG. **7**A, medium **50** is added to gas permeable cell culture device **10**D and makes contact with buoyant shoulder **25**. In FIG. **7**B, medium **50** exerts an upward force on buoyant shoulder **25**, causing gas permeable cell culture device **10**D to rise in height in response to the increasing volume of medium **50**. In the configuration shown, walls **40**D are bellows shaped to allow extension and contraction of the height of gas permeable cell culture device **10**D. Buoyant shoulder **25** can be any biocompatible material that is less dense than medium **50**. It can also be an integral part of walls **40**. It should be sized to displace the appropriate volume of medium **50** in order to exert enough force to extend gas permeable cell culture device **10**D upward. In this configuration, gas permeable cell culture device **10**D only occupies as much space as needed to perform the culture and one product can be the optimal size for a variety of applications. For example, the volume of medium suitable for culturing hybridomas may differ from the amount of medium suitable for maintaining pancreatic islets. In that case, gas permeable cell culture device **10**D only need occupy as much space as needed for each application. Also, it allows sterilizing, shipping, storage, incubation, and disposal in the minimum volume condition, thereby reducing the cost of the culture process. Those skilled in the art will recognize that there are many other ways of altering the device profile other than buoyancy, including a wide variety of mechanical mecha-

nisms such as those described in co-pending U.S. patent application Ser. No. 10/460,850.

FIG. **8** shows an embodiment for a gas permeable multiple well plate **15**, in which the bottom of each well is gas permeable. The properties of lower gas permeable material **30**A are the same as those described in the embodiment of FIG. **4**A. Although a six well plate is shown, any number of individual wells **45** can be present, including the traditional formats of six, twenty-four, forty-eight, and ninety-six wells. Walls **40**E are structured to allow medium to reside at a height above lower gas permeable material **30**A that exceeds the wall height of traditional multiple well plates, thereby increasing the number of cells that can reside in each well while reducing the footprint relative to traditional multiple well plates. For example, murine hybridoma cells typically can reside at a density of 1×10^6 cells per ml of medium. When the well has a diameter of 8.6 mm, and 2 mm of medium height, 0.12 ml of medium is present and about 0.12×10^6 cells can reside per well. However, if 1 ml of medium could reside in the well by making the wall taller, enough medium to support nearly five times as many cells (i.e. 1×10^6 cells per ml) could be present per well, provided that number of cells could reside upon a gas permeable material with a surface area of 0.58 cm^2 (i.e. 8.6 mm diameter). Example 1 demonstrates that many more than 1×10^6 murine hybridoma cells can reside on a surface area this size depending on medium volume. Not only can more medium support more cells, it can allow feeding frequency to be reduced, and reduce the rate at which evaporation alters medium composition.

Walls can be comprised of any biocompatible material and should mate to the lower gas permeable material in a manner that forms a liquid tight seal. The methods of mating lower gas permeable material **30**A to walls **40**E are the same as those described for the embodiment of FIG. **4**A. Also, as described in the embodiment of FIG. **4**A, walls **40**E and lower gas permeable material **30**A can be formed of the same material and fabricated as a single entity. Lower gas permeable material **30**A can be supported in a substantially horizontal position as shown in FIG. **5**, where lower gas permeable material support **80** is configured with lower gas access openings **100** in communication with gas compartment **90**. In the event that the span of the bottom of well **45** is small, support may be unnecessary because the physical strength of lower gas permeable material **30**A can retain it in an adequate horizontal position, depending on the thickness and physical properties of the gas permeable material. In this case, feet **95**A can be used to elevate gas permeable multiple well plate **15** so that gas transfer is not a problem in an incubator with non-perforated shelves. Top cover **55**A prevents contamination and minimizes evaporation.

FIG. **9**A shows a cutaway of a perspective view, and well **45**A of FIG. **9**B shows cross-section A-A, of a preferred embodiment for a gas permeable multiple well plate **16**. In this embodiment, the walls of the wells are gas permeable. Although a six well plate is shown, any number of individual wells **45**A can be present, including the traditional formats of six, twenty-four, forty-eight, and ninety-six wells. This configuration may be useful when it is desirable to retain either the microscopic, attachment surface, or light visibility properties of the traditional multiple well tissue culture plate. Yet, by making each well **45**A deeper than the maximum depth of traditional multiple well plates used for cell culture, more medium can be made available for culture and the gas permeable nature of the walls will allow proper gas exchange of the culture, rendering the location of the gas/liquid interface inconsequential. Non-gas permeable bottom **31** mates to gas permeable wall **41** in a liquid tight manner. There are a number of ways to achieve this objective. For example, the diameter of non gas permeable bottom **31** can slightly exceed the diameter of gas permeable wall **41**, causing gas permeable wall **41** to apply a force against non gas permeable bottom **31**, thereby creating a liquid tight seal. Gas permeable wall **41** can have any of the properties as described for the gas permeable material of FIG. **4**A. However, in a preferred embodiment gas permeable wall **41** is comprised of silicone because of its ability to be easily fabricated by liquid injection molding, and its capacity to stretch and provide a liquid tight seal against non-gas permeable bottom **31**. Non-gas permeable bottom **31** can be any plastic commonly used in traditional multiple well tissue culture plates, or any other cell attachment material known to those skilled in the art.

It may be less expensive to fabricate each well of gas permeable multiple well plate **16** out of gas permeable material, including the well bottom, thereby eliminating the seal joint. Then, if adherent culture is desired, a suitable scaffold can be placed at the bottom of the well. Care should be taken to ensure optical clarity if microscopic evaluation is desired. Any cell attachment surface known to those skilled in the art of cell culture can be placed in the wells. If the cell attachment surface is buoyant, making it a press fit into the well can keep it in the desired position. Many other methods of retaining it in position are also possible.

FIG. **10**A and FIG. **10**B show cross-sectional views of one embodiment of a gas permeable cell culture device that utilizes space more efficiently when culturing adherent cells. Scaffolds **120** reside within gas permeable cell culture device **10**E. Sidewalls **40**F are comprised of a gas permeable material, thereby allowing gas exchange through the sides of the device. In this manner, gas permeable cell culture device **10**E is not limited in height, as scaffolds **120** can be scaled uniformly as height increases. Allowing more cells to be cultured is simply a matter of making the device taller, adding more scaffolds **120**. In the preferred embodiment, the distance between each scaffold **120** is kept constant during scale up. For example, by configuring scaffolds **120** to have spacers **135**, they can be kept an equal distance apart and retained parallel to the bottom of gas permeable cell culture device **10**E, making scale up in the vertical direction linear. Pipette access opening **125** allows pipette access throughout gas permeable cell culture device **10**E and provides an opening to vent gas as medium is added. Although shown in the center, pipette access can be in any location, or can be eliminated entirely in favor of any other form of liquid handling such as needles and septum. In FIG. **10**A, cells **20**A are well suspended in inoculum **130** and will distribute evenly about the upper surface of each scaffold **120**, since the volume of inoculum **130** above each scaffold **120** is equal. If both sides of scaffold **120** are intended to culture adherent cells, inoculation can occur in two steps by inoculating one side of scaffolds **120** first, as shown in FIG. **10**A. After cells have gravitationally deposited and attached onto the surface of scaffolds **120**, gas permeable cell culture device **10**E is then re-inoculated, rotated one hundred eighty degrees to expose the opposite side of scaffolds **120**, and cells **20**A are allowed to settle and attach to the exposed surface of scaffolds **120** as shown in FIG. **10**B.

Post cell attachment, typically less than 24 hours to seed one side of the scaffolds, the device can be operating in any static position that is convenient, such as vertical, inverted, or on its side. If desired, it can be rolled if a user desires a format more similar to a roller bottle. Unlike traditional devices, the device can be filled completely with medium, as gas exchange occurs by way of the gas permeable walls and the need for a gas/liquid interface is not present.

In this manner, the device is more efficient in its use of space than traditional devices since gas does not need to be present in the device for gas exchange of the culture. The limiting factors to the number of cells that can be cultured in the device include the amount of scaffold surface area, the volume of medium present, the gas permeability and thickness of the material used for the device walls, the distance the cells reside from the gas permeable walls of the device, and the type of cells being cultured.

Understanding the importance of the medium volume to scaffold area ratio when designing the gas permeable cell culture device can help predict the output of the device. For instance, if the culture has been historically conducted in a roller bottle, the medium volume to surface area of the roller bottle culture can be replicated in the gas permeable cell culture device. For example, if the existing culture had been performed in a traditional 850 cm^2 roller bottle using 150 ml of medium, and the gas permeable cell culture device was to have the same outside shape as the traditional bottle, the medium volume to surface area ratio could be held constant. A gas permeable cell culture device constructed in the shape of the traditional 850 cm^2 roller bottle can hold about 2200 ml of medium. That is a 14.67 fold increase in medium volume relative to the 150 ml medium volume of the traditional roller bottle. Therefore, a 14.67 fold increase in surface area, which is 12,470 cm^2, is needed to keep an equivalent medium to surface area ratio. Thus, when a gas permeable cell culture device contains 2200 ml of medium and has a scaffold surface area of 12,470 cm^2, it can be expected to culture the same number of cells as about fifteen traditional 850 cm^2 roller bottles that normally operate with 150 ml per bottle, and the feeding frequency should be about the same.

The ability to microscopically assess cell confluence is useful for many applications. If the lowest scaffold comprises the bottom of gas permeable cell culture device, it can be used to assess cell confluence. When the volume of medium residing above each scaffold is equal during inoculation, the amount of cells residing upon any of the scaffolds will be relatively equal throughout the culture. Thus, one scaffold can be representative of the others. For some microscopes, the ability to physically move the lowest scaffold into a position that allows microscopic observation by inverted scopes can allow a better assessment of confluence and morphology. The configuration shown in the cross-sectional view of FIG. **11** shows how this can be achieved. If wall 4 GH is flexible, as will be the case when it is fabricated out of many gas permeable materials such as silicone, it can be pleated to allow movement of the lowest scaffold **120** relative to gas permeable cell culture device **10**F. Microscopic evaluation can also be made possible by manufacturing gas permeable cell culture device **10**F in the fixed position shown in FIG. **11**, thereby eliminating the need to move the lowest scaffold **120** relative to gas permeable cell culture device **10**F.

Although the scaffolds shown in FIG. **10**A, FIG. **10**B, and FIG. **11** are flat, they can be any geometric shape that allows cells to attach. For example, corrugating the surface can increase surface area relative to a planar surface, thereby increasing the amount of adherent cells that can reside upon a given scaffold. FIG. **12**A shows a perspective view of a round corrugated scaffold **120**A, which is corrugated in a linear direction. FIG. **12**B shows cross-sectional view A-A. FIG. **12**C shows a perspective view of round corrugated scaffold **120**B, which is corrugated in the circular direction, and FIG. **12**D shows cross-sectional view B-B. For some applications in which a high rate of gas transfer is needed to support highly active cells, the configuration of FIG. **12**A may be superior because the channels for gas transfer are unobstructed by the edge of the scaffold, as is the case for the configuration of FIG. **12**C. For other applications in which the gas permeable cell culture device is rolled, the configuration of FIG. **12**C may be superior because the shape will minimize turbulence, which could cause cell shear.

The configurations, methods of microscopically viewing, and methods of increasing scaffold area such as those described in FIG. **10**A, FIG. **11**, and FIG. **12**, can be integrated into a multiple well format. These configurations are completely scalable in size. FIG. **9**B shows high surface area well **46**, configured with multiple scaffolds **120** maintained a predetermined distance apart by spacers **135**. Making them the size of the wells of a typical traditional multiple well tissue culture plate will allow a substantial increase in the number of adherent cells present per well. The walls **41**A are preferably gas permeable.

FIG. **13** shows a cutaway view of configuration for a gas permeable cell culture device that is useful for culturing cells in a format similar to that of a tissue culture flask. In this embodiment, at least one wall of the device provides gas transfer. This device is beneficial because it allows the gas permeable cell culture device to retain the same attributes as the traditional tissue culture flask while achieving a more compact use of space. The desirable attributes include easy medium delivery and removal by way of pouring or pipetting, microscopic observation capability, the ability to easily see color changes in the medium that may indicate contamination or pH changes, and capability for device stacking to make the most efficient use of shipping, storage, and incubator space. However, it is superior to the tissue culture flask because the gas/liquid interface required for tissue culture flask operation is eliminated and one or more scaffolds can be present. In the embodiment shown, gas permeable cell culture device **12** is comprised of a liquid tight enclosure with at least one gas permeable wall **200**. Medium access port **60**A is covered by cap **70**A. Scaffolds **120**D are oriented parallel to each other, with a gap between them to allow inoculum and medium to reside in between each scaffold **120**D. Preferably, scaffolds **120**D are positioned an equal distance apart to allow an equivalent volume of inoculum or medium to reside above each of them. The gas permeable material of gas permeable wall **200** has the same attributes as those described for lower gas permeable material **30** of the embodiment shown in FIG. **4**A. In the preferred embodiment, scaffolds **120**D have identical material characteristics as those present in traditional tissue culture flasks. Top wall **201** and bottommost scaffold **120**D are clear, allowing visual assessment of medium color as well as microscopic evaluation of the bottom scaffold **120**D. Making the rear or other walls gas permeable can create more gas transfer capacity. That will have the effect of making it possible to further increase the footprint of gas permeable cell culture device **12**. For example, if the gas transfer capacity of gas permeable wall **200** supports cells residing upon scaffolds **120**D of a five inch width, making the opposing side wall gas permeable will allow enough gas transfer capacity when scaffolds **120**D that are ten inches wide. Gas permeable cell culture device **12** is unlimited in scale up capacity in the vertical direction.

FIG. **14**A through FIG. **14**E show another method of utilizing space more efficiently when culturing cells. In this configuration, scaffolds **120**E reside within gas permeable cell culture device **10**G, which is capable of expanding in volume as medium **50** is added. In FIG. **14**A, gas permeable cell culture device **10**G is in a collapsed position under its own weight. That allows efficient use of space for shipping, sterilization, and storage prior to use. Scaffolds **120**F are as close to each other as possible. Each scaffold **120**F is molded with

spring arms **145** that exert force on the lower, neighboring scaffold **120**F. Spring arms **145**, in compression, want to distend, but cannot because the weight of the upper portion of gas permeable cell culture device **10**G exceeds the spring force. In FIG. **14**B, gas permeable cell culture device **10**G has risen in height in response to the force exerted by the addition of inoculum **130**A against buoyant shoulder **25**A. The displacement of inoculum **130**A by buoyant shoulder **25**A exerts an upward force that, when combined with the spring force of spring arms **145**K, exceeds the weight of the upper portion of gas permeable cell culture device **10**G. Scaffolds **120**F separate and maintain an equal distance from each other due to the force exerted by spring arms **145** against their lower, neighboring scaffold **120**F. Maintaining an equal distance from each other is particularly beneficial during inoculation, when the volume of inoculum **130**A residing directly above each of scaffolds **120**F dictates the amount of cells that will be deposited onto each of scaffolds **120**F. By allowing an equal volume of inoculum **130**A to reside above each scaffold **120**F, and equal number of cells can reside upon each scaffold **120**F. In FIG. **14**C, gas permeable cell culture device **10**G has risen in height again relative to FIG. **14**B in response to the addition of medium **50** as the cell population expands and nutrient demand increases. Scaffolds **120**F further separate and maintain an equal distance from each other due to the force exerted by spring arms **145** against their lower, neighboring scaffold **120**F. The constant distance between each of scaffolds **120**F ensures a constant medium **50** volume to surface area ratio at all cell locations, reducing the potential for gradient formation. In FIG. **14**D, gas permeable cell culture device **10**G has collapsed due to the removal of medium **50** and loss of upward force of buoyant shoulder **25**A. It is now at an efficient size for disposal. In the event that adherent cell recovery is needed, allowing gas permeable cell culture device **10**G to collapse is beneficial when removing medium **50** and adding trypsin. In this manner, only a small volume of trypsin is needed to recover cells. Those skilled in the art will recognize that many other methods of altering the height of gas permeable cell culture device **10**G can be applied.

Spring arms **145** can be molded directly into scaffold **120**F, as shown in the perspective view of FIG. **14**E. A spring arm **145**, preferably located in at least three places, ensures that scaffold **120**F remains in plane and parallel to its neighboring scaffold **120**F. Although any material conducive to cell attachment is acceptable, a preferred material for scaffold **120**F is polystyrene, which is quite brittle. Therefore, care should be taken to ensure that spring arms **145** are configured in accordance with good molded part design to prevent cracking under stress. Techniques for low stress part design are well known to those skilled in the art of plastic part design.

Moving the position of the scaffolds independent of the height of the gas permeable cell culture device may be desired. For example, this may be practical when it is more economical to configure the gas permeable cell culture device with non-extending walls, but the application can still benefit by altering the medium volume to surface area ratio above each of the scaffolds during culture. FIG. **15**A through FIG. **15**C show one embodiment for achieving that objective. For clarity, only a portion of the gas permeable cell culture device is shown. In the top view of a portion of a gas permeable cell culture device shown in FIG. **15**A, three elevation posts **160** are positioned to travel up each of three ramps **150** in order to change the distance between the scaffolds.

The method of varying the distance between scaffolds can best be understood by reviewing FIG. **15**B and FIG. **15**C. FIG. **15**B shows cross-section A-A of FIG. **15**A. As shown in FIG. **15**B, two scaffolds **120**G are shown the position in which the distance between them is at a minimum. Ramp **150** emanates from the top of scaffold **120**G and elevation post **160** emanates from scaffold locator screw **170**. Elevation post **160** has not begun travel up ramp **150**. It can be seen that the minimum distance between scaffolds is dictated by the height of ramp **150**, which makes contact with the underside of the scaffold **120**G that resides above it. Referring to FIG. **15**C, scaffolds **120**G are in the position of maximum distance between them. Scaffold locator screw **170** has been rotated in the direction of rotation arrow **180**, causing elevation post **160** to rise up ramp **150** and elevate the scaffold **120**G residing above it. When elevation post **160** resides at the highest point of ramp **150**L, the maximum distance between scaffolds **120**L is attained as is equal to the height of ramp **150** plus the height of elevation post **160**. Scaffolds **120**G should be prevented from rotating when scaffold locator screw **170** is turned, thereby allowing ramp **150** to remain in a fixed position while elevation post **160** travels up it. This can be achieved by mating scaffolds **120**G to the interior of the gas permeable cell culture device wall by way of a tongue and groove arrangement. As best shown in the top view of a scaffold of FIG. **15**A, tongue **212** emanates from gas permeable wall **40**H and mates to groove **215** in each scaffold **120**G. Not only does this prevent rotation of scaffold **120**G during rotation of locator screw **170**, it also prevents gas permeable wall **40**H from pulling away from scaffold **120**G. In this manner, the shape of the gas permeable cell culture device is retained. Locator screw **170** can be configured to allow a sterile pipette tip to rotate it, thereby preventing contamination of the device and allowing the use of standard laboratory tools to rearrange the distance between scaffolds.

The invention will be further described with reference to the following non-limiting Examples.

EXAMPLES

Example 1

The Effect of Medium Height Upon Cell Growth and Antibody Production

Evaluations were conducted in order to assess the impact of altering medium height upon cell growth and antibody production in a device comprised of a lower gas permeable material. The effect of altering the gas permeable material surface area to medium volume ratio was also assessed. Single compartment test fixtures configured with a lower gas permeable materials and the capacity to hold medium at heights beyond conventional wisdom were compared to single compartment control test fixtures that held medium at a height within the bounds of conventional wisdom. Comparisons were made relative to the 1.6 cm medium height limits specified for the Si-Culture bag (U.S. Pat. No. 5,686,304). Control test fixtures were configured to house medium at a height of 1.6 cm, and the gas permeable material used for of all test fixtures consisted of gas permeable material obtained from actual Si-Culture™ bags.

Tubular test fixtures **105** were constructed as shown in FIG. **16**. Walls **401** were machined out of Ultem 1000 (high temperature polycarbonate) cylindrical stock, resulting in a tube with an inner diameter of 1.00 inch and an outer diameter of 1.50 inch. The thick walls ensured that gas transfer through the walls would not assist the cultures. Lower gas permeable material **30**A was fabricated from 0.045 thick sheets of silicone removed from Si-Culture™ bags and secured in a liquid tight manner to the bottom of the machined tube yielding a 5.07 cm^2 growth area for cells **20**B to reside upon. Lower gas permeable material support **80**M was also machined out of

Ultem 1000. Lower gas permeable material **30**A was held in the horizontal position by mesh **115** which maintained gas compartment **90**A. Mesh **115** was comprised of 0.020 inch diameter strands at 16 strands per inch. Lower gas access openings **100**A allowed gaseous communication with the 5% CO_2, 95% R.H., and 37 C ambient environment. Comparisons were made for the capacity of the devices to grow cells **20**B when differing amounts of medium **50**A resided within the test fixture. Cap **70**B, secured tightly to walls **401**, protected tubular test fixture **105** from contamination. Tests compared the results when medium **50**A resided at a height of about 1.6 cm, 3.2 cm, 5.6 cm, 10.2 cm, 15.3 cm, and 20.4 cm above the cells. Medium **50**A consisted of Hyclone HyQSFM4MAb-Utility supplemented with 10% Hyclone FBS. Cells **20**B were murine hybridoma cells secreting IgG, inoculated at a seeding density of 0.76×10^6 per cm^2 of lower gas permeable material **30**A. Ambient conditions were 5% CO_2, 95% R.H., and 37 C. Periodic cell counts and monoclonal antibody production measurements by ELISA were taken. TABLE 1 shows the results.

TABLE 1

Medium Height Affect Upon Cell Growth and Antibody Production

Volume of medium (ml)	Height of medium above gas permeable material (cm)	Gas permeable surface area to medium volume ratio (cm^2/ml)	Maximum live cells per device ($\times10^6$)	Maximum live cells per cm^2 of gas permeable material ($\times10^6$)	Mab produced per test fixture (ug)	Time to maximum amount of mab produced (days)	Mab per ml of medium consumed (ug/ml)
8.1	1.60	0.63	29.7	5.85	2742	9	339
16.2	3.20	0.31	51.0	10.05	7395	12	457
25.8	5.09	0.20	59.1	11.65	10673	18	374
51.7	10.20	0.10	61.1	12.05	15252	15	295
77.6	15.31	0.07	67.2	13.25	23044	22	299
103.4	20.39	0.05	86.4	17.04	32881	25	318

Dividing each parameter measured in any given test fixture by the corresponding parameter of the test fixture representing conventional wisdom (i.e. 1.6 cm) clearly shows the advantages of allowing medium to reside at heights beyond conventional wisdom. Gas permeable surface area to medium volume ratio is determined by dividing the ratio of the test fixture by the ratio of the Si-Culture™ bag when it contains medium at a height of 1.6 cm (i.e. 1.25 cm^2/ml). TABLE 2 presents the data of TABLE 1 in this manner.

TABLE 2

Normalized data

Normalized by height of medium above gas permeable membrane	Normalized by maximum live cells per device	Normalized by gas permeable surface area to medium volume ratio relative to Si-Culture ™ bag	Normalized by Mab produced per test fixture	Normalized by Mab per ml of medium consumed	Normalized by time to attain maximum Mab amount	Normalized by footprint of space occupied
1.00	1.00	50%	1.00	1.00	1.00	1.00
2.00	1.72	25%	2.70	1.35	1.50	0.50
3.18	1.99	16%	3.89	1.11	2.00	0.28
6.38	2.06	8%	5.56	0.87	1.67	0.16
9.57	2.26	6%	8.40	0.88	2.50	0.10
12.75	2.91	4%	11.99	0.94	2.83	0.08

The data of TABLE 2 clearly shows the advantages of altering the geometry of gas permeable cell culture devices to allow more medium to reside above the cells. For example, the last row shows that when the device is allowed to hold medium at a height that is 12.75 times greater than the traditional cell culture bag, it is capable of culturing 2.91 fold more cells per cm^2 of floor space occupied, producing 11.99 times more monoclonal antibody (Mab) with only a 2.83 fold increase in the time to complete production. Also, when the gas permeable material surface area to medium volume ratio is compared to that of the Si-Culture™ bag, dramatically reduced ratios are possible. Cultures were effectively grown even when the ratio was only 4% of that used by the Si-Culture™ bag. That allows a wider variety of device configurations to exist, including allowing the device footprint to remain fixed as medium height is increased. It also minimizes the effects of evaporation, as more medium is present per cm^2 of gas permeable surface area.

Importantly, this data demonstrates that device footprint can remain small as the culture is increased. TABLE 3 shows the surface area of the device footprint needed to house the volume of medium residing in the test fixtures. The first row shows the medium volume in the test fixture. The second row shows the footprint area of the test fixture, which remained fixed as more and more medium was added. The third row shows the footprint surface area that would be required in a typical bag to hold the volume of medium residing in the test fixture. In this case, the footprint is shown for a Si-Culture™ bag when it contains the volume of row one at the manufacturers recommended medium height of 1.6 cm. The fourth row shows the difference in footprint area. For example, when the test fixture contains 103.4 ml of medium, the Si-Culture™ bag when operated according to manufacturers recommendation would have a footprint of 64.6 cm^2, but the test fixture only has a footprint of 5.1 cm^2. Thus, the test fixture that

allowed medium to reside at a height of 20.39 cm only needed a footprint of 8% of that needed for a Si-Culture™ bag to produce roughly the same amount of Mab.

TABLE 3

Much more efficient use of floor space.

Volume of medium in device (ml)	8.1	16.2	25.8	51.7	77.6	103.4
Test fixture footprint (cm^2)	5.1	5.1	5.1	5.1	5.1	5.1
Bag footprint with medium at 1.6 cm high (cm^2)	5.1	10.1	16.1	32.3	48.5	64.6
Ratio of test fixture footprint to bag footprint (%)	100%	50%	32%	16%	11%	8%

Benefits relative to all of the conventional configurations are numerous. The unwieldy shape of traditional cell culture bags can be avoided allowing a wide variety of benefits to accrue related to more efficient use of incubator space, easier medium delivery and removal, and reduced contamination risk. The small volume of medium present in gas permeable cartridges can be increased substantially by making them taller, and reducing the ratio of gas permeable membrane to medium volume capacity. That has the effect of allowing fewer units to be needed during scale up. For traditional gas permeable formats of the petri dish and multiple well plate, more cells can reside per unit without increasing the footprint of the devices, or the number of devices needed, and the frequency of feeding can be reduced. Minimized evaporative effects can be achieved in all configurations because the gas permeable surface area to medium volume ratio can be significantly reduced.

Example 2

Effect of Thickness of Gas Permeable Silicone on Cell Growth

Conventional wisdom, as dictated by U.S. Pat. No. 5,686,304 and U.S. patent application Ser. No. 10/183,132, and the design of commercially available gas permeable products that use silicone, dictates that silicone thickness of greater than 0.005 inches should not be used. However, increasing the thickness is advantageous from a manufacturing and product reliability standpoint. Therefore, evaluations were conducted to assess the impact of the thickness of a lower silicone gas permeable material on cell growth. The material thickness of conventional wisdom was compared to the same material at increasing thickness.

Tubular test fixtures were constructed as shown in FIG. **16**. Walls were machined out of Ultem 1000 (high temperature polycarbonate) cylindrical stock, resulting in a tube with an inner diameter of 1.00 inch and an outer diameter of 1.50 inch. Four distinct thickness configurations of lower gas permeable material were created from sheets of silicone removed from Si-Culture™ bags. Lower gas permeable material **30**A was made into double, triple, and quadruple layers, formed by adhering the silicone sheets together using UV curing silicone glue distributed evenly about the face and sheets were laminated together leaving no air gaps between them. Post curing, the laminated sheets and a single sheet control were secured in a liquid tight manner to the bottom of the machined tube yielding a 5.07 cm^2 growth area for cells to reside upon. Tests were conducted in triplicate. Lower gas permeable material **30**A was held in the horizontal position by lower gas permeable material support **80**, configured as described in Example 1. Tests compared the results when medium resided at heights of 20.4 cm above the cells. Medium consisted of Hyclone HyQSFM4MAb-Utility supplemented with 10% Hyclone FBS. Murine hybridoma cells were inoculated at a seeding density of 4.3×10^6 live cells per square cm of lower gas permeable material. Ambient conditions were 5% CO_2, 95% R.H., and **37**C. Periodic cell counts and glucose measurements were taken. TABLE 4 shows the results.

TABLE 4

Effect of Thickness of Gas Permeable Silicone on Cell Growth

Membrane Thickness (in)	Maximum viable cells per cm^2 ($\times10^6$)	Normalized: Membrane Thickness	Normalized: Maximum viable cells per cm^2
0.0045	15.2	1.00	1.00
0.016	15.5	3.56	1.02
0.024	13.49	5.33	0.89
0.033	12.0	7.33	0.79

The data was normalized by referencing it against the data collected for the single 0.0045 inch thick sheet that represents conventional wisdom. It can clearly be seen that the effect of dramatically increasing thickness does not have a significantly negative impact on the capacity to support cell growth. When the material thickness was increased about four-fold, from 0.0045 inch to 0.016 inch, there was no affect upon cell growth. When the silicone membrane thickness was increased 5.33 fold, from 0.0045 inch to 0.024 inch, the growth capacity was diminished by only 11%. Likewise, a 7.33 fold increase in thickness beyond conventional wisdom resulted in growth capacity being diminished by only 21%. In many cell culture applications, such as hybridoma culture for monoclonal antibody production, 79% viability is routinely accepted. For example, in the CELLine™ products, hybridoma viability is commonly at 50%, as described in the operating instructions. Thus, device design can accommodate thicker silicone walls without a dramatic reduction in performance. Fabrication and functional improvements may result from increasing the thickness, such as simplified liquid injection molding or less pinhole potential. In summary, it is possible to design a highly functional cell culture device with thicker walls than previously believed possible.

Example 3

The Ability to Culture Cells at a High Liquid Height in a Rolled and Unrolled Device

Evaluations were conducted to assess the advantages that could be obtained by configuring gas permeable cell culture devices in ways that differ from conventional wisdom. Two general formats were evaluated, 1) unrolled gas permeable devices and 2) rolled gas permeable devices. In the unrolled gas permeable device configuration, medium height was well beyond the limits imposed by conventional wisdom. The ratio of gas permeable surface area to medium volume was reduced far below that of conventional wisdom. In the rolled gas permeable device configuration, medium was allowed to reside farther away from the gas permeable wall, and more medium was allowed to reside per device, than that of the state of the art gas permeable rolled bottles.

The production of monoclonal antibody is a common application in cell culture bags and roller bottles. A traditional 850 cm^2 roller bottle functioned as a control. Test fixtures were constructed in accordance with the embodiments shown in FIG. **4**, and dimensionally configured to have the same dimensions as a traditional 850 cm^2 Corning® roller bottle. The gas permeable material was the same as that of the

Si-Culture™ bag, as further defined in U.S. Pat. No. 5,686,304. The gas permeable surface area of non-rolled test fixture was limited to that of the bottom surface of the fixture, and was 98 cm^2. The sidewalls were not gas permeable. The gas permeable surface area of the rolled test fixture was limited to that of the entire cylindrical sidewall surface of the fixture, and was 850 cm^2, and the ends were not gas permeable. Medium consisted of Hyclone SFM4MAb, supplemented with 2.5% Hyclone FBS. Each test fixture was inoculated with a cell density of 0.04×10^6 murine hybridoma cells per ml of medium used. The test fixtures each received 2050 ml of medium. Ambient conditions were 5% CO_2, 95% R.H., and **37**C.

The traditional roller bottle received 255 ml of medium, the maximum amount of medium recommended for use in roller bottles. The presence of antibody was determined by ELISA. TABLE 5 shows the results.

TABLE 5

Effect of rolling versus standing on antibody production time

Test Fixture Style	Maximum amount of antibody produced (mg)	Time to reach maximum production
Unrolled Novel Device	289	16
Rolled Novel Device	302	13
Traditional Roller Bottle	33	13

TABLE 5 shows how the rolled and the non-rolled gas permeable test fixtures, which occupied the same amount of space as the traditional roller bottle control, were able to produce about nine times as much antibody. TABLE 5 also demonstrates how the rolled gas permeable format can be used to decrease the amount of time needed to generate antibody relative to its standing gas permeable counterpart. A 20% reduction in time, three days, was attained. Importantly, both the roller and unrolled formats can create a at least a nine fold improvement in efficient geometry in terms of space, leading to reduced cost of sterilization, shipping, storage, labor, incubator space, and disposal when compared to the traditional roller bottle.

The results also clearly demonstrate the advantage obtained by configuring gas permeable devices in ways that depart from conventional wisdom. The height of medium in the unrolled test fixture was about 20.9 cm, over ten times the highest recommended height of traditional cell culture bags. Had the device been structured with 2.0 cm of medium height, it would have needed a footprint of 1025 cm^2 to house an equivalent volume of medium, which is over ten times the footprint of the unrolled test fixture.

Benefits of geometry of the rolled gas permeable device were numerous. The rolled test fixture contained a volume of medium nearly eight times the maximum volume of medium recommended for traditional roller bottles (255 ml), over four times the medium volume of Rotary Cell Culture System™ from Synthecon Inc., nearly five times the medium volume of the MiniPERM, and well beyond that allowed in the patent proposals of Spaudling, Schwarz, Wolf et al., and Falkenberg et al. Also, medium resided up to 5.6 cm from any portion of the gas permeable wall of the test fixture, over double the limit specified in the patent proposals of Spaudling, Schwarz, and Wolf et al. The rolled test fixture was able to function on a standard roller rack, as opposed to the commercially available Rotary Cell Culture System™ from Synthecon™ Inc., and the MiniPERM™ from Vivascience Sartorius Group, which all require custom equipment to roll. Thus, the scale up efficiency of the rolled gas permeable device is much superior to other devices and approaches.

Example 4

Ability to Culture Adherent Cells in the Absence of a Gas/Liquid Interface

Evaluations were conducted to assess the ability to culture adherent cells without the presence of a gas/liquid interface by allowing gas exchange to occur via gas permeable walls. A test fixture was constructed in a manner, as shown in FIG. **17**, that eliminated the possibility of gas transfer by way of a gas/liquid interface. Gas permeable wall test fixture **12** consisted of a rectangular liquid tight enclosure **241**, configured with one gas permeable wall **200**A and five non-gas permeable walls **210**. Gas permeable wall **200**A was composed silicone membrane, approximately 0.0045 thick, purchased from Medtronic Inc. (Minneapolis). This membrane is used by Medtronic to fabricate the Si-Culture™ bag. Fluid delivery port **220** and fluid removal port **230** allow inoculation and feeding. Bottom attachment scaffold **240** consisted of a section of plastic removed from a Falcon tissue culture flask in order to provide an equivalent attachment surface as the control Falcon™ T-175 tissue culture flask. The inner dimensions of enclosure **241** were 6 cm deep, 10 cm wide, and 0.635 cm high. Thus, gas permeable wall **200**A was 10 cm wide and 0.635 cm high creating a surface area of 6.35 cm^2. Bottom attachment scaffold **240** was 10 cm wide and 6 cm deep, allowing an attachment surface of 60 cm^2. Gas permeable wall test fixture **12** was filled entirely medium during inoculation, thereby eliminating any gas/liquid interface. Thus, gas exchange could only occur by way of diffusion in the direction perpendicular to gas permeable wall **200**A. Inoculum consisted of 60,000 live BHK cells (98% viability) suspended in 38.1 ml of EMEM medium supplemented with 10% Hyclone FBS and 1% L-glutamine. Thus, the seeding density was 10,000 live cells per cm^2 of available attachment scaffold **240** area. The surface area of gas permeable membrane to volume of medium was 0.167 cm^2/ml. The surface are of gas permeable membrane to surface area of attachment scaffold was 0.106 cm^2/cm^2. The control T-175 tissue culture flask was inoculated with the same cells, at equivalent seeding density and viability. Gas permeable wall test fixture **12** and the T-175 control were placed in a standard cell culture incubator at 5% CO_2, 95% R.H., and 37° C.

Cells settled gravitationally onto bottom attachment scaffold **240** and the control T-175 flask, and the cultures were maintained until confluence was reached. Both the test fixture and the control exhibited a confluent monolayer over the entire attachment scaffold. By visual microscopic comparison, the cell density of both gas permeable test fixture **12** and the T-175 control flask appeared nearly identical. The T-175 flask was trypsinized, cells were counted, and it was determined that cells had reached a density of approximately 190,000 cells per cm^2. The test fixture was subjected to Wright Giemsa staining to determine the distribution of cells over bottom attachment scaffold **240**. FIG. **20** shows the distribution pattern, where "Front" is in proximity of gas permeable wall **200**, "Middle" is about midway between gas permeable wall **200** and opposing non-gas permeable wall **210**, and "Back" is in proximity of opposing non-gas permeable wall **210**.

FIG. **20** clearly indicates that cells will grow to confluence upon a scaffold in the absence of a gas/liquid interface, mechanical mixing, or perfusion, when a wall of the device is gas permeable. Thus, gas transfer by way of walls is adequate

for cell culture devices of the types described herein including those shown in FIG. **9**A, FIG. **9**B, FIG. **10**A, FIG. **10**B, FIG. **11**, and FIG. **14**A through FIG. **14**E to fully function. Example 4 also indicates that only one of the walls of a gas permeable cell culture device needs to be comprised of gas permeable material, thereby opening up a wide array of device design options. For example, a gas permeable device could be configured in a traditional T-Flask format by making a sidewall gas permeable. In this manner, more medium could be made available for the culture or the device profile could be reduced since no gas/liquid interface is needed.

Example 5

The Ability to Culture Cells on Multiple Attachment Scaffolds in the Absence of a Gas/Liquid Interface

Evaluations were conducted to assess the ability to culture adherent cells on multiple scaffolds without the presence of a gas/liquid interface. Gas exchange occurred via a gas permeable device wall. Gas permeable test fixtures were constructed in a manner, as shown in FIG. **18**, that eliminated the possibility of gas transfer by way of a gas/liquid interface. Multiple scaffold test fixture **14** consisted of a rectangular liquid tight enclosure configured with one gas permeable wall **200**B and five non-gas permeable walls **210**A. Gas permeable wall **200**B was composed of molded silicone material, 0.015 thick. Fluid delivery port **220**A and fluid removal port **230**A allow inoculation and feeding. Attachment scaffolds **240**A consisted of plastic removed from NUNC™ Cell Factory cell culture devices. The inner dimensions of multiple scaffold test fixture **14** were 15.24 cm long, 7.62 cm wide, and 2.54 cm high. Thus, gas permeable wall **200**B was 7.62 cm wide and 2.54 cm high creating a gas permeable material surface area of 19.35 cm^2. Each attachment scaffold **240**A was 6.6 cm wide and 15.03 cm long, creating an attachment surface area of 99 cm^2 per attachment scaffold **240**A.

In one test group of multiple scaffold test fixtures **14**, four attachment scaffolds **240**A were arranged vertically, one above the other, with a 5.08 mm gap between each of them, resulting in a total attachment surface area of 396 cm^2 per device. The volume of medium within this version of multiple scaffold test fixture **14** was 195 ml. The surface area of gas permeable membrane to volume of medium was 0.099 cm^2/ml. The surface area of gas permeable membrane to total surface area of attachment scaffolds **240**A was 0.049 cm^2/cm^2.

In another test group of multiple scaffold test fixtures **14**, five attachment scaffolds were arranged vertically, one above the other, with a 2.54 mm gap between each of them, resulting in a total attachment surface area of 495 cm^2 per device. The volume of medium within each multiple scaffold test fixture was 170 ml. The surface area of gas permeable membrane to volume of medium was 0.114 cm^2/ml. The surface area of gas permeable membrane to total surface area of attachment scaffolds **240**A was 0.039 cm^2/cm^2.

Multiple scaffold gas permeable test fixtures **14** were filled entirely with medium during inoculation, thereby eliminating any gas/liquid interface. Thus, gas exchange could only occur by way of diffusion in the direction perpendicular to the gas permeable wall. The seeding density was 15,000 live BHK cells per cm^2 of available attachment scaffold area. Medium consisted of Gibco GMEM supplemented with 10% Hyclone FBS and 1% Gibco Penicillin Streptomycin. The control T-175 tissue culture flask was also inoculated with BHK cells, at equivalent seeding density and viability, in 30 ml of the same medium composition. Multiple scaffold gas permeable test fixtures **14** and the T-175 control were placed in a standard cell culture incubator at 5% CO_2, 95% R.H., and 37° C.

Cells settled gravitationally onto each attachment scaffold **240**A and the control T-175 flask, and the cultures were maintained until confluence was reached. Within four days, cultures were terminated. All attachment scaffolds **240**A were removed from multiple scaffold gas permeable test fixture **14**. By visual microscopic comparison, the cell density of both test groups of multiple scaffold gas permeable test fixtures **14** and the T-175 control flask appeared nearly identical, at approximately 95% confluence.

This demonstrates the ability to make much more efficient use of space by eliminating the need to maintain a gas headspace in a culture device. Since the device only holds the medium needed to support the culture, it can be significantly reduced in profile. The novel device is much more compact than the traditional T-flask, NUNC™ Cell Factory, and Corning CellStack™. This results in savings in sterilization, shipping, storage, and disposal cost. Furthermore, incubator space and flow hood space are used more efficiently.

Example 6

Gas Permeable Unrolled Cell Culture Device for Adherent Cell Culture Inoculated in the Vertical Position

A test fixture was constructed to evaluate the capacity of a non-rolled, gas permeable cell culture device configured with more than one scaffold to culture cells relative to traditional flasks. FIG. **19**A shows a cross-section of gas permeable test fixture **260**. Scaffolds **120**H were arranged vertically and a consistent gap was maintained between each scaffold **120**H by spacers **135**B. Wall **40**J was gas permeable, comprised of silicone purchased from Medtronic Inc. (Minneapolis), approximately 0.0045 thick. Suture **270** applied force to gas permeable wall **40**, squeezing it against bulkhead gasket **280** to create a liquid tight seal between gas permeable wall **40** and upper bulkhead **290** and lower bulkhead **300**. Medium access port **60**B allowed fluid delivery to, and removal from, gas permeable test fixture **260**. Cap **70**C prevented contamination and was tightly closed during operation. FIG. **19**B shows a perspective view of scaffold **120**H. It was made of tissue culture treated polystyrene, 0.040 inches thick. Pipette access opening **125**A, with a diameter of 0.75 inches, allowed pipette access and prevented gas from becoming trapped between scaffolds **120**H. Four vent slots **190** allowed additional area for trapped gas to exit, ensuring that all gas/liquid interfaces were removed. The surface area per side of each scaffold **120**H was about 86 cm^2. The inner diameter of gas permeable test fixture **260** was 4.4 inches and the internal height as measured from the inner surface of lower bulkhead **300** to the inner surface of upper bulkhead **290** was 2.25 inches. Thus, the gas permeable material surface area was 561 cm^2. Eight scaffolds **120**H were stacked vertically with spacers **135**B maintaining a gap of about 0.25 inch between each. The combined surface area of the tops of the eight scaffolds **120**H was 695 cm^2. The internal volume of gas permeable test fixture **260** was approximately 500 ml. Therefore, the gas permeable material to medium volume ratio was 561 cm^2/500 ml, or 1.12 cm^2/ml.

10.425×10^6 BHK cells, suspended in 500 ml Gibco GMEM medium supplemented with 1% Gibco Amino Acids Solution and 10% Hyclone FBS were inoculated into gas permeable test fixture **260**P, creating a seeding density of 15,000 cells per cm^2 of attachment surface area. A control T-175 flask was also seeded with 15,000 cells per cm^2 of attachment surface area in 30 ml of the equivalent medium.

After approximately 96 hours, the cultures were terminated. Gas permeable test fixture **260** was disassembled and each of scaffolds **120**H was microscopically examined, indicating a confluent pattern of cells was present on the upper surface of each of the eight scaffolds **120**H. The control T-175 flask was also confluent as determined by microscopic evaluation. The T-175 flask and gas permeable test fixture **260** were trypsinized and standard cell counting techniques were used to determine the quantity of cells present. TABLE 6 summarizes the findings.

TABLE 6

Gas permeable cell culture device vs. T-flask

Device	Total Cells ($\times 10^6$)	Viability (%)	Medium Present (ml)	Height of Medium Above Cells (cm^2)
Gas permeable cell test fixture 260	220.8	98	500	0.72
Control T-flask	26.3	95	30	0.17

TABLE 6 demonstrates that cells were able to proliferate and remain healthy in the novel gas permeable test fixture **260**, despite the absence of a gas/liquid interface.

The volume of space occupied by each device is noteworthy. Gas permeable test fixture **260** had a footprint of 100 cm^2 and a height, including the neck, of 7.6 cm. Thus, the space occupied was about 760 cm^3. The T-175 flask, including the neck, had a footprint approximately 23 cm long by 11 cm wide, and the body was about 3.7 cm tall. Thus, the space occupied was about 936 cm^3. Since gas permeable test fixture **260** cultured about 8.4 times more cells than the T-175 flask, it would take 8.4 T-175 flasks to yield an equivalent amount of cells over the same time period. TABLE 7 shows the difference in space that would be occupied if T-175 flasks were used to produce the same number of cells cultured by gas permeable test fixture **260**, based on the experimental results of TABLE 6.

TABLE 7

Device	Volume of space occupied per device (cm^3)	Devices to produce 221×10^6 cells in 3 days	Volume of space needed (cm^3)
One novel gas permeable cell culture device 260	760	1	760
Control T-flasks	936	8.4	7862

The advantage of eliminating the gas/liquid interface is clear. Over a ten-fold reduction of space is obtained by gas permeable test fixture **260**. This leads to cost savings in sterilization, shipping, storage, use of incubator space, and waste disposal. Furthermore, the number of devices that need to be handled is significantly reduced, leading to a dramatic labor and contamination risk reduction.

Example 7

Gas Permeable Unrolled Cell Culture Device for Adherent Cell Culture Inoculated in the Vertical and Inverted Position

Using the test fixture shown in FIG. **19**A, as previously defined in Example 6, an experiment was conducted to determine if cells would attach to both the top and bottom surfaces of the scaffolds. This could be accomplished by a two-step inoculation. In step one, a first inoculum was placed into the gas permeable test fixture while oriented in the vertical position. Cells were allowed to gravitate onto, and attach to the top surface of, the scaffolds over a 24-hour period. In step two, a second inoculum was placed into the gas permeable test fixture. Gas permeable test fixture was inverted to allow the cells of the second inoculum to gravitate onto, and attach to the bottom surface of, the scaffolds.

This process was undertaken, with each inoculation consisting of enough BHK cells to seed the exposed surfaces of the scaffolds at a density of 15,000 cells per cm^2. Medium composition was the same as that described in EXAMPLE 6. The time interval between the first inoculation and the second inoculation was twenty-four hours. The culture was terminated seventy-two hours after the second inoculation. The device was disassembled and each scaffold was microscopically assessed. Cells were uniformly distributed on both the top and bottom surfaces of each scaffold. Subsequently, the cells were removed using trypsin and a count was performed. The average quantity of live cells per cm^2 of surface area was 144×10^5, with viability greater than 99%.

Cells were thus able to attach and proliferate on the top and bottom of scaffold **120**. Therefore, it is possible for the novel gas permeable cell culture device to be further reduced in size relative to conventional devices. For adherent cell culture, a wide variety of scaffold geometry can exist that have cell attachment area in any plane.

Example 8

Gas Permeable Unrolled Cell Culture Device for Adherent Cell Culture Inoculated in the Vertical and Inverted Position with Limited Distance Between Scaffolds

A test was conducted to determine if inserting more scaffold area into the device could further reduce device size. For additional space savings, the upper and lower surface of each scaffold was used to culture cells. The gas permeable test of Example 7 was fabricated with additional scaffolds. The number of scaffolds and distance between the scaffolds was chosen to create a volume to surface area ratio roughly equivalent to a traditional tissue culture flask. Recommended medium volume for a traditional T-175 flask varies from about 16-32 ml (Invitrogen Life Technologies). This dictates that medium reside about 0.09-0.18 cm from the attachment surface. The test device of this example was to be inoculated in two steps, allowing cells to reside on the upper and lower surfaces of each scaffold. Therefore, in order to get a conservative assessment of the value the gas permeable cell culture device can bring in terms of space and labor savings, 0.34 cm medium height was allowed to reside between each of the scaffolds. In this manner, the medium to surface area ratio was held constant relative to the T-175 flask. In effect, each scaffold surface had access to one half the medium between it, and the scaffold adjacent to it had access to the other half. Thus, the medium available to each side of a scaffold was consistent with the traditional tissue culture flask height of 0.17 cm per square centimeter of growth surface.

Fourteen scaffolds were inserted into the test device and evenly spaced approximately 0.34 cm apart. A T-175 flask, with 30 ml of medium residing at a height of 0.17 cm acted as a control. Inoculation using BHK cells was performed in two steps, as detailed in Example 7. Medium composition was the same as that described in Example 6. Seventy-two hours after the second inoculation, the culture was terminated and the device was disassembled and each scaffold was microscopically assessed for cell distribution upon the upper and lower surface. Each scaffold exhibited a distribution pattern on the upper and lower surface that was approximately equivalent to

that of the T-175 flask. TABLE 7 shows an example of how increasing the surface area of the novel gas permeable cell culture device reduces the space needed to culture a given amount of cells when compared to the traditional T-175 flask. For example, when then novel gas permeable cell culture device contains 2432 cm^2 of scaffold surface area, fourteen T-175 flasks would be needed to provide equal surface area. If 1.7 mm of medium is intended to be available for each cm^2 of scaffold surface area, the volume of space occupied by the novel gas permeable cell culture device can be determined. TABLE 8 shows that in this case, the dramatically difference in the volume of space occupied by each type of device.

TABLE 8

Gas permeable device output with increased surface area

Device	Available Surface area for cell attachment (cm^2)	Number of devices needed	Volume of medium needed (cm^3)	Volume of space occupied per device (cm^3)
One novel gas permeable cell culture device	2432	1	420	760
T-175 flask	2432	14	420	12,292

It can be seen that when the gas permeable cell culture device is designed to have the same medium to surface area ratio as the traditional flask, a much more efficient use of space results. The volume of space occupied by the gas permeable cell culture device is only one-sixteenth of that occupied by T-175 flasks when an equivalent amount of cells are desired. This translates directly into cost reductions for sterilization, shipping, storage, and disposal.

It is to be understood that the invention is not limited to the above embodiments, which are shown for purposes of illustration and described above, but is intended to include any modification or variation thereof falling within the scope of the appended claims.

Example 9

Gas Permeable Rolled Cell Culture Device for Adherent Cell Culture Inoculated in the Vertical Position

Gas permeable test fixture **260** was constructed, as shown in the cross-sectional view of FIG. **19**A and further defined in Example 5, to evaluate the capability of rolling a gas permeable cell culture device configured with more than one scaffold.

With gas permeable test fixture **260** in the vertical, unrolled position, 10.425×10^6 BHK cells, suspended in 500 ml Gibco GMEM medium supplemented with 1% Gibco Amino Acids Solution and 10% Hyclone FBS were inoculated into gas permeable test fixture **260**, creating a seeding density of 15,000 cells per cm^2 of attachment surface area. A control T-175 flask was also seeded with 15,000 cells per cm^2 of attachment surface area in 30 ml of the equivalent medium.

After approximately 24 hours, the gas permeable test fixture was places upon a standard roller rack at rotated at 1 RPM. Three days after the commencement of rolling, gas permeable test fixture was disassembled and each of the scaffolds was microscopically examined, indicating a confluent pattern of cells was present on the upper surface of each of the eight scaffolds. The control T-175 flask was also confluent as determined by microscopic evaluation.

This demonstrates that proliferation of cells is uninhibited by rolling the novel gas permeable cell culture device. Thus, creating a device that can be rolled or unrolled allows users greater options for protocol development.

Guide to Reference Characters in Drawings

10	gas permeable cell culture device
12	gas permeable wall test fixture
14	multiple scaffold test fixture
15	gas permeable multiple well plate
16	gas permeable wall multiple well plate
20	cells
25	buoyant shoulder
30	lower gas permeable material
31	non-gas permeable bottom
40	walls
41	gas permeable wall
42	interior walls
45	individual wells
46	high surface area well
50	medium
55	top cover
60	medium access port
65	septum
70	cap
75	o-ring
80	lower gas permeable material support
90	gas compartment
95	feet
100	lower gas access openings
105	tubular test fixtures
110	projections
115	mesh
120	scaffolds
125	pipette access opening
130	inoculum
135	spacer
145	spring arm
150	ramps
160	elevation posts
170	scaffold locator screw
180	rotation arrow
190	vent slots
200	gas permeable wall
201	top wall
210	non-gas permeable wall
212	tongue
215	groove
220	fluid delivery port
230	fluid removal port
240	attachment scaffold
241	enclosure
260	gas permeable test fixture
270	suture
280	bulkhead gasket
290	upper bulkhead
300	lower bulkhead

Those skilled in the art will recognize that numerous modifications can be made to this disclosure without departing from the spirit on the inventions described herein. Therefore, it is not intended to limit the breadth of the invention to the embodiments illustrated and described. Rather, the scope of the invention is to be interpreted by the appended claims and their equivalents. Each publication, patent, patent application, and reference cited herein is hereby incorporated herein by reference.

What is claimed is:

1. A method of culturing cells in a cell culture device comprised at least in part of a gas permeable material and including at least one access port and including at least two scaffolds, the method comprising:

a) adding cells and a volume of liquid medium into said cell culture device;

b) orienting said cell culture device into an inoculation position such that said scaffolds reside at different elevations within said cell culture device;

c) allowing cells to settle upon said scaffolds;

d) adding enough liquid medium to prevent a unique gas-liquid interface from forming directly above at least one scaffold when the device is oriented in the inoculation position and to have at least a portion of the liquid medium in contact with at least a portion of said gas permeable material;

e) placing the cell culture device in a cell culture location that includes ambient gas at a composition suitable for cell culture, said ambient gas making contact with said gas permeable material; and

f) not perfusing said liquid medium when said device is in said cell culture location.

2. The method of claim **1** wherein said access port is covered by an access port cap.

3. The method of claim **1** wherein said cell culture device has two access ports.

4. The method of claim **1** wherein said cell culture location is within a cell culture incubator.

5. The method of claim **1** wherein said scaffolds are comprised of polystyrene.

6. The method of claim **1** wherein said scaffolds are flat.

7. The method of claim **1** wherein said scaffolds are parallel to each other.

8. The method of claim **1** wherein said scaffolds have a rectangular perimeter.

9. The method of claim **1** wherein said scaffolds have a square perimeter.

10. The method of claim **1** wherein said scaffolds have a circular perimeter.

11. The method of claim **1** wherein each said scaffold is oriented in a horizontal plane and each horizontal plane is at a distinct elevation when said cell culture device resides in said inoculation position.

12. The method of claim **1** wherein each said scaffold is oriented in a horizontal plane and each horizontal plane is at a distinct elevation when said cell culture device resides in said cell culture location.

13. The method of claim **1** wherein said cells are adherent cells and further including the step of waiting for a period of time until said cells adhere to said scaffolds and then re-orienting said cell culture device such that scaffolds are oriented in a vertical plane when said cell culture device resides in said cell culture location.

14. The method of claim **1** wherein the volume of liquid medium provides at least 0.2 ml of liquid medium volume for every 1 cm^2 of scaffold surface area upon which said cells reside.

15. The method of claim **1** wherein said cell culture device is further adapted so that a portion of said liquid medium resides in a portion of said cell culture device other than directly above said scaffolds.

16. The method of claim **1** wherein said cell culture device includes at least one gas permeable material support, at least a portion of which is in contact with said gas permeable material.

17. The method of claim **16** wherein said cell culture device includes a gas compartment bounded at least in part by said gas permeable material support and said gas permeable material and the gas within said cell culture location moves to and from said gas compartment by passive movement through at least one gas access opening in said gas permeable material support.

18. The method of claim **1** wherein the gas permeable material of said cell culture device includes silicone.

19. The method of claim **1** wherein the bottommost scaffold of said culture device, as determined when said culture device is in the inoculation position, is clear.

20. The method of claim **1** wherein said gas of said cell culture location is at a composition of about 95% relative humidity, about 37 degrees Celsius, and about 5% carbon dioxide.

21. The method of claim **1** wherein while in said cell culture location, said liquid medium is not mixed with equipment designed to mix said liquid medium.

22. The method of claim **1** wherein said device is not rolled with a roller mechanism.

23. The method of claim **1** wherein a unique gas-liquid interface does not form directly above more than one scaffold when the device is oriented in the inoculation position.

24. The method of claim **1** wherein a unique gas-liquid interface does not form directly above any but the uppermost scaffold when the device is oriented in the inoculation position.

25. The method of claim **1** wherein a unique gas-liquid interface does not form directly above any scaffold when the device is oriented in the inoculation position.

26. A method of culturing cells in a cell culture device comprised at least in part of a gas permeable material and including at least one access port and including at least two scaffolds, the method comprising:

a) adding cells and a volume of liquid medium into said cell culture device;

b) orienting said cell culture device into an inoculation position such that said scaffolds reside at different elevations within said cell culture device;

c) allowing cells to settle upon said scaffolds,

d) adding enough liquid medium to prevent a unique gas-liquid interface from forming directly above at least one scaffold when the device is oriented in the inoculation position and to have at least a portion of the liquid medium is in contact with at least a portion of said gas permeable material;

e) said device residing in a cell culture location that includes ambient gas at a composition suitable for culturing said cells, said ambient gas making contact with said gas permeable material; and

f) said liquid medium residing in a static state when culturing said cells.

27. The method of claim **26** wherein said liquid medium is not mixed with equipment designed to mix said liquid medium when said device is culturing said cells within said cell culture location.

28. The method of claim **26** wherein said liquid medium is not perfused when said device is culturing said cells within said cell culture location.

29. The method of claim **26** wherein said device is not rolled with roller mechanisms when said device is culturing said cells within said cell culture location.

30. The method of claim **26** wherein a unique gas-liquid interface does not form directly above more than one scaffold when the device is oriented in the inoculation position.

31. The method of claim **26** wherein a unique gas-liquid interface does not form directly above any but the uppermost scaffold when the device is oriented in the inoculation position.

32. The method of claim **26** wherein a unique gas-liquid interface does not form directly above any scaffold when the device is oriented in the inoculation position.

33. The method of claim **26** wherein said access port is covered by an access port cap.

34. The method of claim 26 wherein said cell culture device has two access ports.

35. The method of claim 26 wherein cell culture location is within a cell culture incubator.

36. The method of claim 26 wherein said scaffolds are comprised of polystyrene.

37. The method of claim 26 wherein said scaffolds are flat.

38. The method of claim 26 wherein said scaffolds are parallel to each other.

39. The method of claim 26 wherein said scaffolds have a rectangular perimeter.

40. The method of claim 26 wherein said scaffolds have a square perimeter.

41. The method of claim 26 wherein said scaffolds have a circular perimeter.

42. The method of claim 26 wherein each said scaffold is oriented in a horizontal plane and each horizontal plane is at a distinct elevation when said cell culture device resides in said inoculation position.

43. The method of claim 26 wherein each said scaffold is oriented in a horizontal plane and each horizontal plane is at a distinct elevation when said culture device resides in said cell culture location.

44. The method of claim 26 wherein said cells are adherent cells and further including the step of waiting for a period of time until said cells adhere to said scaffolds and then re-orienting said cell culture device such that scaffolds are oriented in a vertical plane when said cell culture device resides in said cell location.

45. The method of claim 26 wherein the volume of liquid medium provides at least 0.2 ml of liquid medium volume for every 1 cm^2 of scaffold surface area upon which said cells reside.

46. The method of claim 26 wherein said cell culture device is further adapted so that a portion of said liquid medium resides in a portion of said cell culture device other than directly above said scaffolds.

47. The method of claim 26 wherein said cell culture device includes at least one gas permeable material support, at least a portion of which is in contact with said gas permeable material.

48. The method of claim 26 wherein said cell culture device includes a gas compartment bounded at least in part by said gas permeable material support and said gas permeable material and the gas within said cell culture location moves to and from said gas compartment by passive movement through at least one gas access opening in said gas permeable material support.

49. The method of claim 26 wherein the gas permeable material of said cell culture device includes silicone.

50. The method of claim 26 wherein the bottommost scaffold of said cell culture device is clear.

51. The method of claim 26 wherein said gas of said cell culture location is at a composition of about 95% relative humidity, about 37 degrees Celsius, and about 5% carbon dioxide.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 8,697,443 B2
APPLICATION NO. : 12/753573
DATED : April 15, 2014
INVENTOR(S) : Wilson et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the specification

Column 4, Line 44:
Delete "at" and insert --as--.

Column 9, Line 41:
Delete "1.76" and insert --1.63--.

Column 10, Line 17:
Delete "3.8" and insert --5.2--.

Column 10, Line 18:
Delete "2.6" and insert --3.5--. Delete "1.9" and insert --1.1--.

Column 21, Line 9:
Delete "less" and insert --greater--.

Column 21, Line 21:
Insert --beyond-- after the word "reside".

Column 21, Line 24:
Delete "3.8" and insert --5.2--.

Column 21, Line 31:
Delete "up".

Column 30, Line 61:
Delete "120E" and insert --120F--.

Signed and Sealed this
Twelfth Day of April, 2016

Michelle K. Lee

Michelle K. Lee
Director of the United States Patent and Trademark Office

In the specification

Column 32, Line 63:
Delete "0.045" and insert --0.0045 inches--.

Column 38, Line 18:
Insert --inches-- after "0.0045".

Column 40, Line 35:
Insert --inches-- after "0.0045".

Column 40, Line 36:
Delete "40" and insert --40J--.

Column 40, Line 37:
Delete "40" and insert --40J--.

Column 40, Line 40:
Delete "70C" and insert --70--.

Exhibit C

A *NEW ERA* OF MEDICINE IS UPON US

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FORWARD-LOOKING STATEMENTS

This presentation contains "forward-looking statements." Any statements that are not statements of historical fact may be deemed to be forward-looking statements. Words such as "believe," "anticipate," "plan," "expect," "will," "may," "intend," "prepare," "look," "potential," "possible" and similar expressions are intended to identify forward-looking statements. These forward-looking statements include statements relating to our opportunities in the rare disease space; potential solutions and market opportunities with our RNA technologies, gene therapy and gene editing; the potential of gene therapy's applicability across disease; the potential benefits of our technologies and scientific approaches, including the potential of RNA-targeted medicine to increase or decrease production of a protein involved in a disease, the potential of exon skipping to allow for production of an internally truncated but functional dystrophin protein, the potential benefits of PMO and PPMO, including PPMO's potential to greatly increase cell penetration, lead to more efficient dosing and deliver to unique muscle types; the potential benefits of MHCK7, AAVrh74, SR2, SR3 and β-SARCOGLYCAN; the expectation to have 3 RNA therapies on the U.S. market in 2020, serving ~30% of the DMD community; our plans for the future and expected milestones, including developing PPMOs that can serve 43% of DMD, our goal to identify 2 gene therapy product candidates per year; the potential of our LGMD portfolio to generate a steady stream of gene therapy candidates; the potential benefits of *iCELLis* adherent mammalian manufacturing technology; our forecast curve for gene therapy; our sustainable model for transformative gene therapies, including our business model, expected forecast curve, having gene therapy manufacturing capacity in 2020 and an access and reimbursement plan in 2021; our expected development horizon of 3.5 years; the estimated number of patients suffering from DMD, LGMD, CMT, MPS IIIA and pompe; our goal to have 40 programs; and the estimated epidemiology associated with the different diseases.

These forward-looking statements involve risks and uncertainties, many of which are beyond our control and are based on our current beliefs, expectations and assumptions regarding our business. Actual results and financial condition could materially differ from those stated or implied by these forward-looking statements as a result of such risks and uncertainties and could materially and adversely affect our business, results of operations and trading price. Potential known risk factors include, among others, the following: our data for our different programs, including golodirsen, casimersen, micro-dystrophin and LGMD may not be sufficient for obtaining regulatory approval; our product candidates, including those with strategic partners, may not result in viable treatments suitable for commercialization due to a variety of reasons including the results of future research may not be consistent with past positive results or may fail to meet regulatory approval requirements for the safety and efficacy of product candidates; even if our programs result in new commercialized products, we may not achieve any significant revenues from the sale of such products; success in preclinical testing and early clinical trials, especially if based on a small patient sample, does not ensure that later clinical trials will be successful; if the actual number of patients suffering from DMD, LGMD, MPS IIIA, CMT and/or pompe is smaller than estimated, our revenue and ability to achieve profitability may be adversely affected; various factors may decrease the market size of our product and product candidates, including the severity of the disease, patient demographics and the response of patients' immune systems to our product candidates; our dependence on our manufacturers to fulfill our needs for our clinical trials and commercial supply, including any inability on our part to accurately anticipate product demand and timely secure manufacturing capacity to meet product demand, may impair the availability of products to successfully support various programs, including research and development and the potential commercialization of our gene therapy product candidates; we may not be able to successfully scale up manufacturing of our product candidates in sufficient quality and quantity or within sufficient timelines; current reimbursement models may not accommodate the unique factors of our gene therapy product candidates; we may not be able to execute on our business plans and goals, including meeting our expected or planned regulatory milestones and timelines, clinical development plans, and bringing our product candidates to market, for various reasons including possible limitations of our financial and other resources, manufacturing limitations that may not be anticipated or resolved for in a timely manner, and regulatory, court or agency decisions, such as decisions by the United States Patent and Trademark Office; and those risks identified under the heading "Risk Factors" in Sarepta's 2018 Annual Report on Form 10-K or and most recent Quarterly Report on Form 10-Q filed with the Securities and Exchange Commission (SEC) and in its other SEC filings.

For a detailed description of risks and uncertainties Sarepta faces, you are encouraged to review Sarepta's filings with the SEC. We caution investors not to place considerable reliance on the forward-looking statements contained in this presentation. The forward-looking statements in this presentation are made as of the date of this presentation only and, other than as required under applicable law, Sarepta does not undertake any obligation to publicly update its forward-looking statements.

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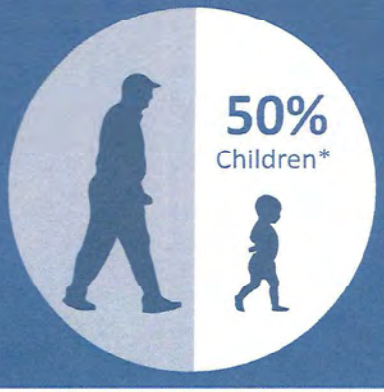
400M

Rare disease patients w/w



Rare diseases
are responsible for

35%

of deaths
in the first year of life*

30%

of children
with a rare disease
will never reach their
5th birthday*

EVERY DAY

Duchenne takes the life of a ***child***

*http://www.fromhopetocures.org/fighting-rare-diseases. Accessed September 2019.

THE TIME IS NOW FOR *SOLUTIONS*

SAREPTA THERAPEUTICS

SAREPTA IS LEADING THIS REVOLUTION

Armed with the most advanced science in genetic medicine, we are in a daily race to rescue lives otherwise stolen by rare disease.

At Sarepta, every day is another 24 hours to stand up for patients, advance technology, challenge convention and ***drag tomorrow into today.***

A MULTI-PLATFORM APPROACH TO THE PROBLEM

RNA

PMO PPMO

Duchenne Muscular Dystrophy (DMD)

GENE THERAPY

Two approaches to DMD; Six forms of limb-girdle muscular dystrophy (LGMD); Charcot-Marie-Tooth (CMT) disease; Mucopolysaccharidosis type IIIA (MPS IIIA)

GENE EDITING

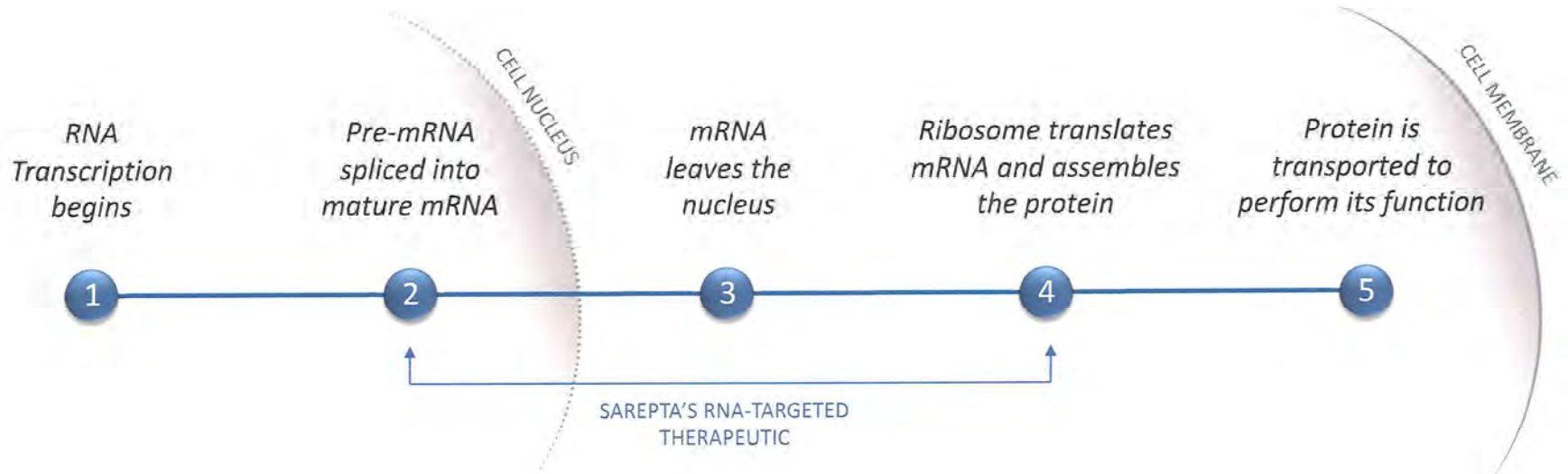
DMD

11	>12	1
PROGRAMS	PROGRAMS	PROGRAM

RNA ENGINE

SAREPTA THERAPEUTICS

AN ELEGANT RNA PRECISION GENETIC MEDICINE APPROACH



RNA-targeted Medicine *- Designed to increase or decrease production of a protein involved in a disease*

A PROPRIETARY AND DIFFERENTIATED APPROACH IN RNA TECHNOLOGY

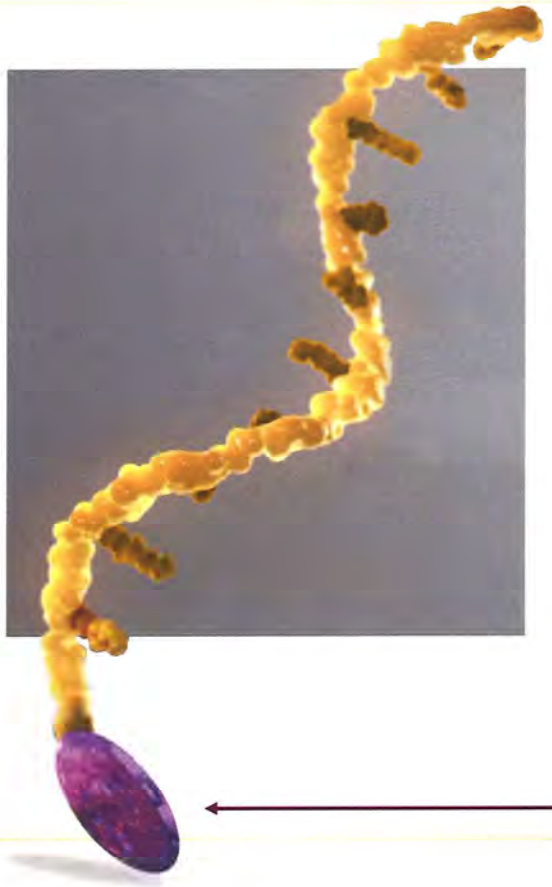
PHOSPHORODIAMIDATE MORPHOLINO OLIGOMER (PMO) CHEMISTRY

Specificity: Enhanced affinity for targeting pre-mRNA for precise binding to the selected RNA target

Stability: Highly resistant to degradation by enzymes

Versatility: Ability to rapidly design and construct drug candidates that are specific for human or pathogen RNA; and target specific tissues

Safety: Built upon a charge-neutral backbone, which may be reflected in tolerability



PEPTIDE PHOSPHORODIAMIDATE MORPHOLINO OLIGOMER (PPMO) CHEMISTRY

Enhances PMO

- Same precision genetic medicine backbone
- Conjugated peptide greatly increases cell penetration
- Could potentially lead to more efficient dosing for patients
- Able to deliver PMOs to unique muscle types (e.g., heart)

PMO TECHNOLOGY FOR DUCHENNE MUSCULAR DYSTROPHY

PROBLEM: *Duchenne is caused by a genetic mutation that prevents the body from producing dystrophin, a protein the muscles need to work properly.*

PRECISION GENETIC MEDICINE: *Phosphorodiamidate Morpholino Oligomers (PMOs) are designed in precise sequence, bind to the target, and direct the body to make a functional form of the dystrophin protein.*

EXON SKIPPING:

Dystrophin Gene

DRIVING OUR RNA ENGINE FORWARD

TARGETS DYSTROPHIN DEFICIENCY, THE UNDERLYING CAUSE OF DMD

If successful, Sarepta will have three RNA-therapies on the U.S. market in 2020, serving ~30% of the DMD community

ETEPLIRSEN*

Approved to treat patients with genetic mutations that are amenable to exon 51 skipping (13% of DMD population).

GOLODIRSEN

SRP-4053

Being developed to treat patients with genetic mutations that are amenable to exon 53 skipping (8% of DMD population).

CASIMERSEN

SRP-4045

Being developed to treat patients with genetic mutations that are amenable to exon 45 skipping (8% of DMD population).

- PPMOs FOR DMD
- OTHER DISEASE TARGETS

Exon skipping is intended to allow for production of an internally truncated but functional dystrophin protein

*Candidate received accelerated approval in the U.S., confirmatory studies required

GENE THERAPY *ENGINE*

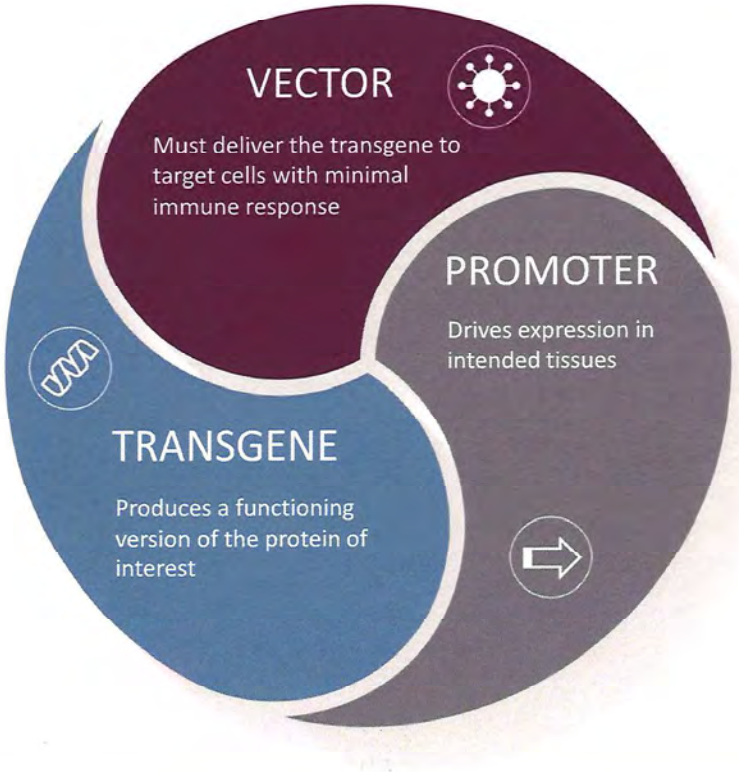
SAREPTA THERAPEUTICS

SAREPTA'S GENE THERAPY ENGINE AT WORK: NOW, AND INTO THE FUTURE

PROGRAM	DISCOVERY	PRECLINICAL	CLINICAL	COMMERCIAL
SRP-9001 Micro-dystrophin				
SRP-9003 (LGMD2E β-sarcoglycan)				
SRP-9004 (LGMD2D α-sarcoglycan)				
SRP-9005 (LGMD2C γ-sarcoglycan)				
MYO-201 (LGMD2B Dysferlin)				
SRP-9006 (LGMD2L Anoctamin 5)				
Calpain 3 (LGMD2A) (Nationwide Children's)				
LYS-SAF302 (MPS IIIA) (Lysogene)				
GALGT2 (Nationwide Children's)				
Micro-dystrophin (Genethon)				
Neurotrophin 3 (CMT1A) (Nationwide Children's)				
Pompe Disease (Lacerta)				
CNS-1 (Lacerta)				
CNS-2 (Lacerta)				

2 TARGETS ***PER YEAR*** - NEW GENE THERAPY CENTER OF EXCELLENCE

CRITICAL COMPONENTS OF A GENE THERAPY CONSTRUCT



SAREPTA'S GENE THERAPY ENGINE AT WORK – DUCHENNE MUSCULAR DYSTROPHY

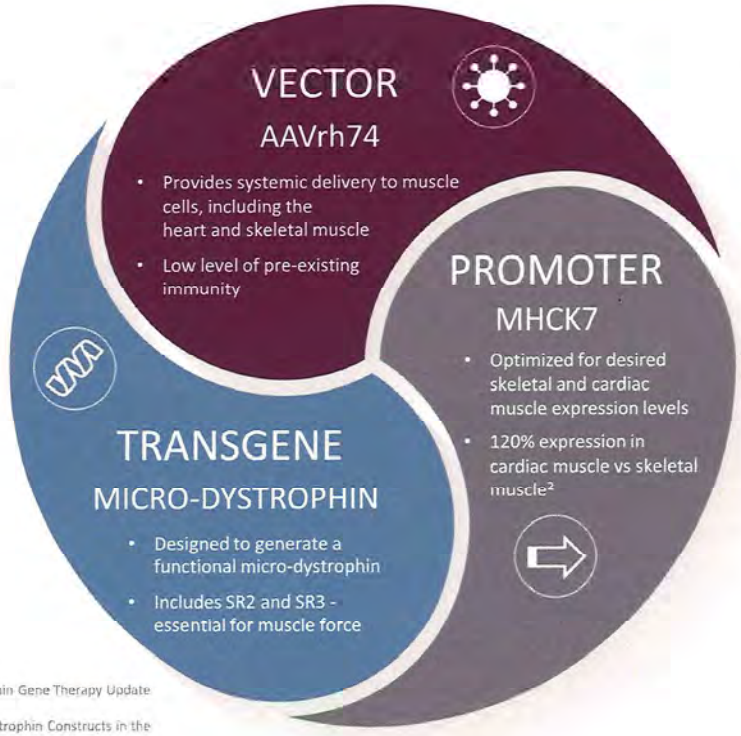
THREE-MONTH EXPRESSION RESULTS - MICRO-DYSTROPHIN GENE THERAPY FOR DMD*

81%
Expression of micro-dystrophin in muscle fibers[1]

96%
Expression of micro-dystrophin measured by signal intensity[1]

96%
Expression of micro-dystrophin measured by Western blot**,[1]

64%
Reduction of creatine kinase[1]



NINE-MONTH CLINICAL RESULTS - MICRO-DYSTROPHIN GENE THERAPY FOR DMD AVERAGE CHANGE FROM BASELINE***

NSAA
6.5
POINT IMPROVEMENT

TIME TO RISE
.8
SECOND IMPROVEMENT

4 STAIRS UP
1.2
SECOND IMPROVEMENT

100 M
7.95
SECOND IMPROVEMENT

1. Clinical Update: Micro-dystrophin Study-101 - March 25, 2019 - Micro-dystrophin Gene Therapy Update Conference Call - Sarepta Therapeutics Data on File; at Day 270.
2. Potter et al. Functional and Histological Improvements Comparing 4 Micro-dystrophin Constructs in the mdx Mouse Model of DMD. ASGCT 2019
2. Potter et al. Functional and Histological Improvements Comparing 4 Micro-dystrophin Constructs in the mdx Mouse Model of DMD. AIM 2019

ClinicalTrials.gov Identifier: NCT03375164
Sarepta Therapeutics Data on File

*Data from the 4 patients dosed in Study NCT03375164
**NCH Western blot method
***North Star Ambulatory Assessment (NSAA), Time to Rise, 4 Stairs Up, and 100M

SAREPTA'S GENE THERAPY ENGINE AT WORK – LGMD2E CONSISTENT RESULTS IN NEW THERAPEUTIC AREA

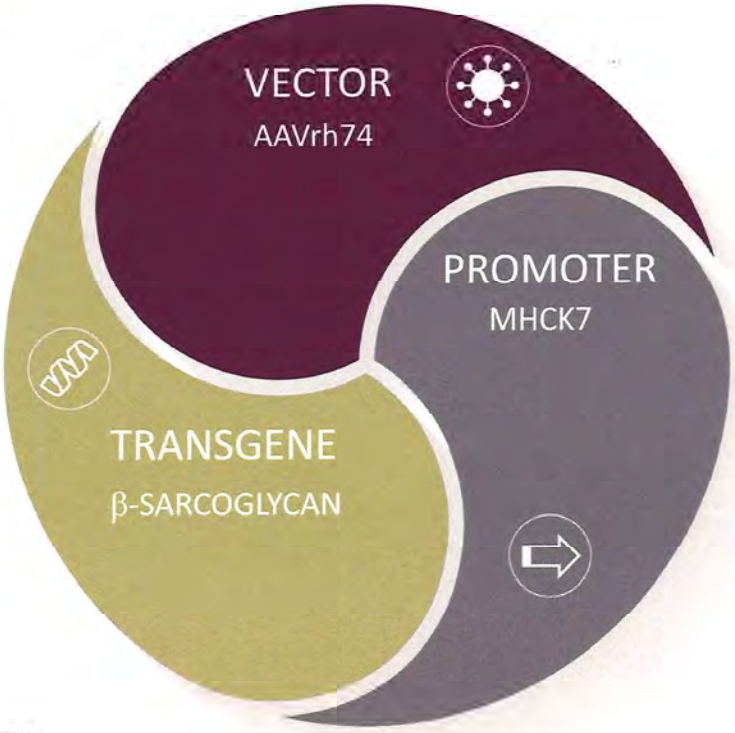
TWO-MONTH EXPRESSION RESULTS - β-SARCOGLYCAN GENE THERAPY FOR LIMB-GIRDLE MUSCULAR DYSTROPHY TYPE 2E*,[1]

51%
Expression of β-sarcoglycan in muscle fibers

47%
Expression of β-sarcoglycan measured by signal intensity

36%
Expression of β-sarcoglycan measured by Western blot**

82%
Reduction of creatine kinase[1]



NINE-MONTH CLINICAL RESULTS - β-SARCOGLYCAN GENE THERAPY FOR LIMB-GIRDLE MUSCULAR DYSTROPHY TYPE 2E*,[2]

All patients **improve in all functional endpoints** at 9 months

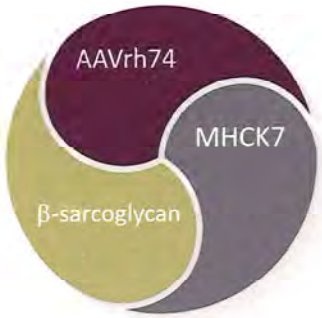
*Data from the 3 patients dosed in Study NCT03652259
**NCH Western blot method
1. http://investorrelations.sarepta.com/news-releases/news-release-details/sarepta-therapeutics-announces-positive-and-robust-expression. At Day 270.

2. http://investorrelations.sarepta.com/news-releases/news-release-details/sarepta-therapeutics-announces-positive-functional-results-srp

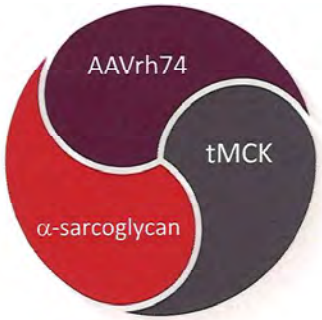
GENE THERAPY ENGINE AT WORK ACROSS LGMD PORTFOLIO

OPPORTUNITY TO GENERATE
A STEADY STREAM OF
GENE THERAPY CANDIDATES

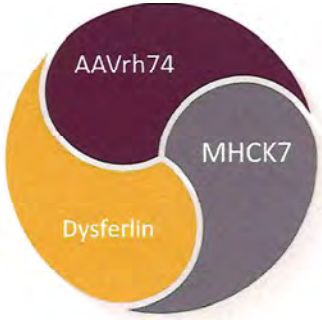
LGMD PORTFOLIO



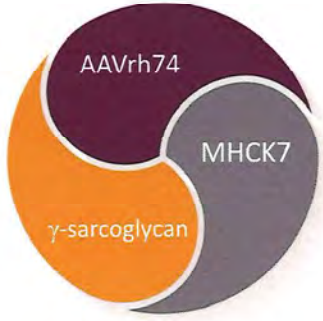
LGMD2E



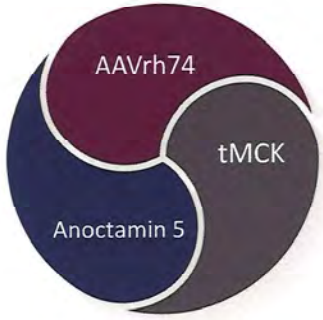
LGMD2D



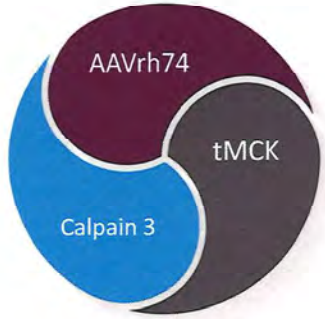
LGMD2B



LGMD2C



LGMD2L



LGMD2A

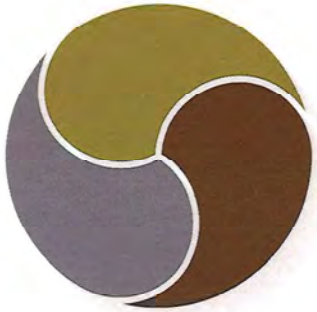
GENE THERAPY ENGINE AT WORK ACROSS PIPELINE PROGRAMS

DUCHENNE
MUSCULAR DYSTROPHY

LIMB-GIRDLE
MUSCULAR DYSTROPHY

MUCOPOLYSACCHARIDOSIS

CHARCOT-MARIE-TOOTH
DISEASE



POMPE DISEASE

SETTING THE GOLD STANDARD IN *GENE THERAPY MANUFACTURING*

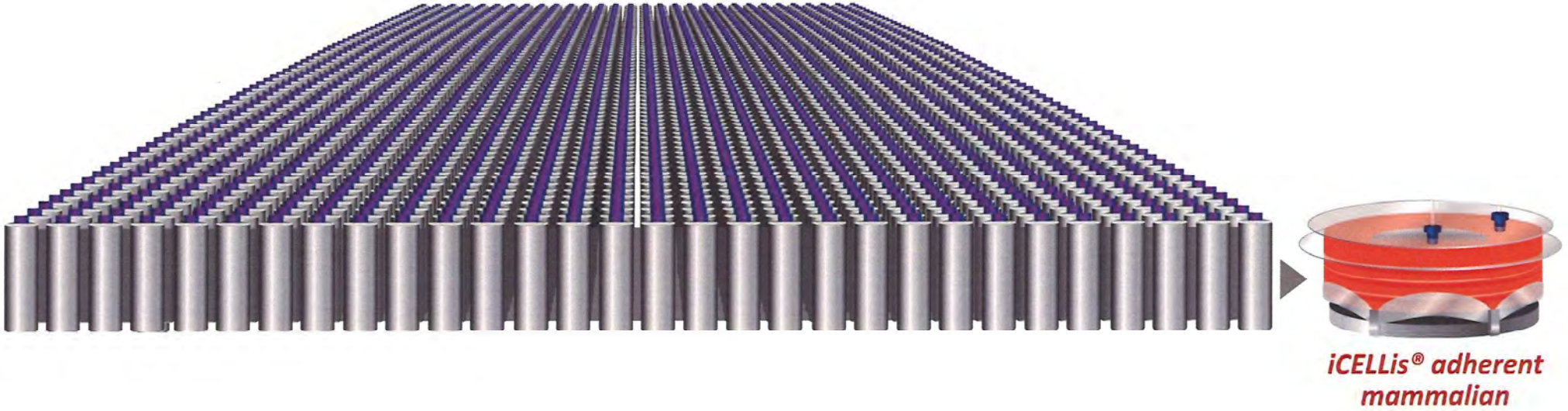
SAREPTA THERAPEUTICS

SAREPTA'S HYBRID STRATEGY - EXTERNAL THOUGHT LEADERS COMPLEMENT OUR INTERNAL EXPERTISE

SAREPTA'S MANUFACTURING EXPERTISE

ADVANCING GENE THERAPY MANUFACTURING

A deliberate and strategic move from HYPERstack adherent mammalian to ***iCELLis® adherent mammalian***



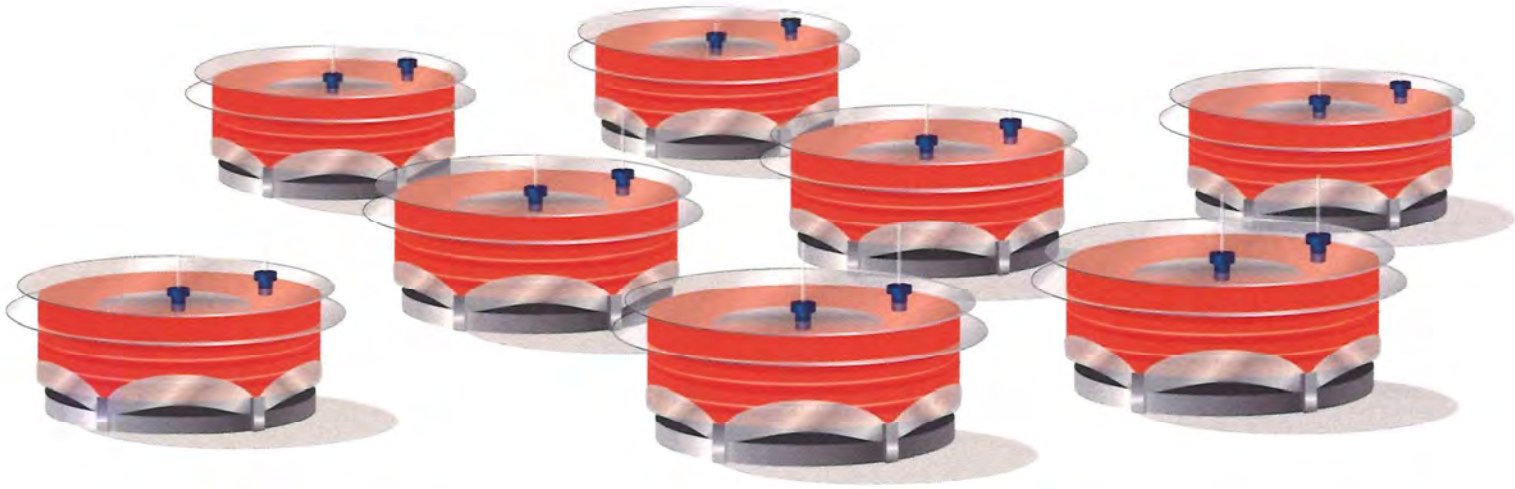
iCELLis® adherent mammalian

ADVANCING GENE THERAPY MANUFACTURING

BENEFITS

- *Mitigated Risk*
- *Increased Speed*
- *Expanded Scale*
- *Improved Cost Efficiency*

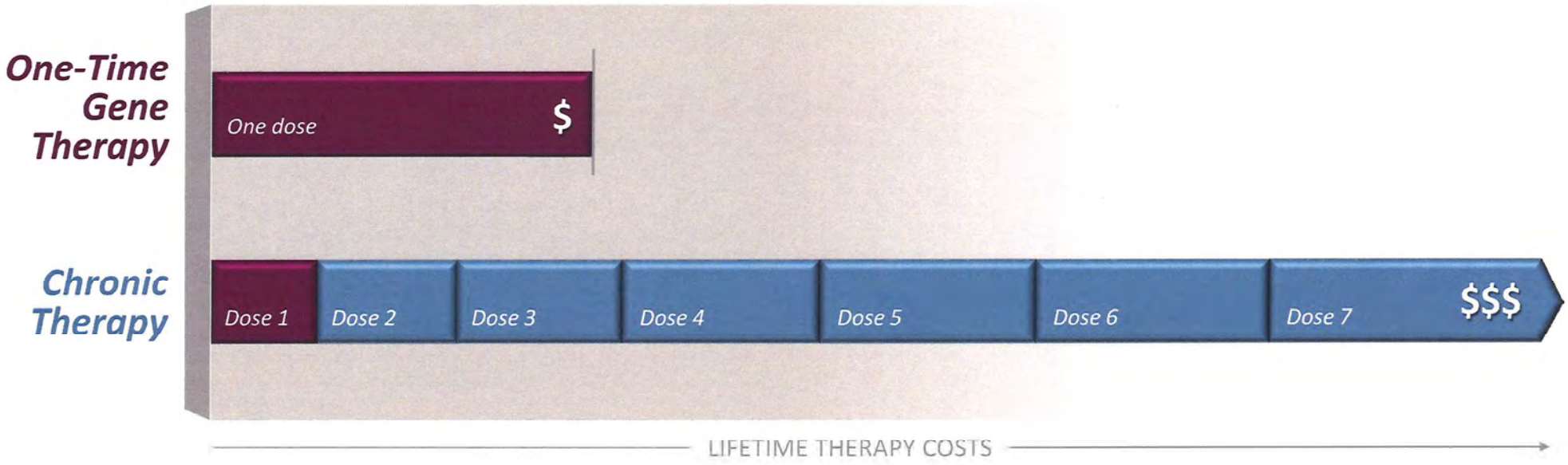
A deliberate and strategic move from HYPERstack adherent mammalian to ***iCELLis® adherent mammalian***



SAREPTA'S SUSTAINABLE MODEL FOR *ONE-TIME TRANSFORMATIONAL THERAPIES*

SAREPTA THERAPEUTICS

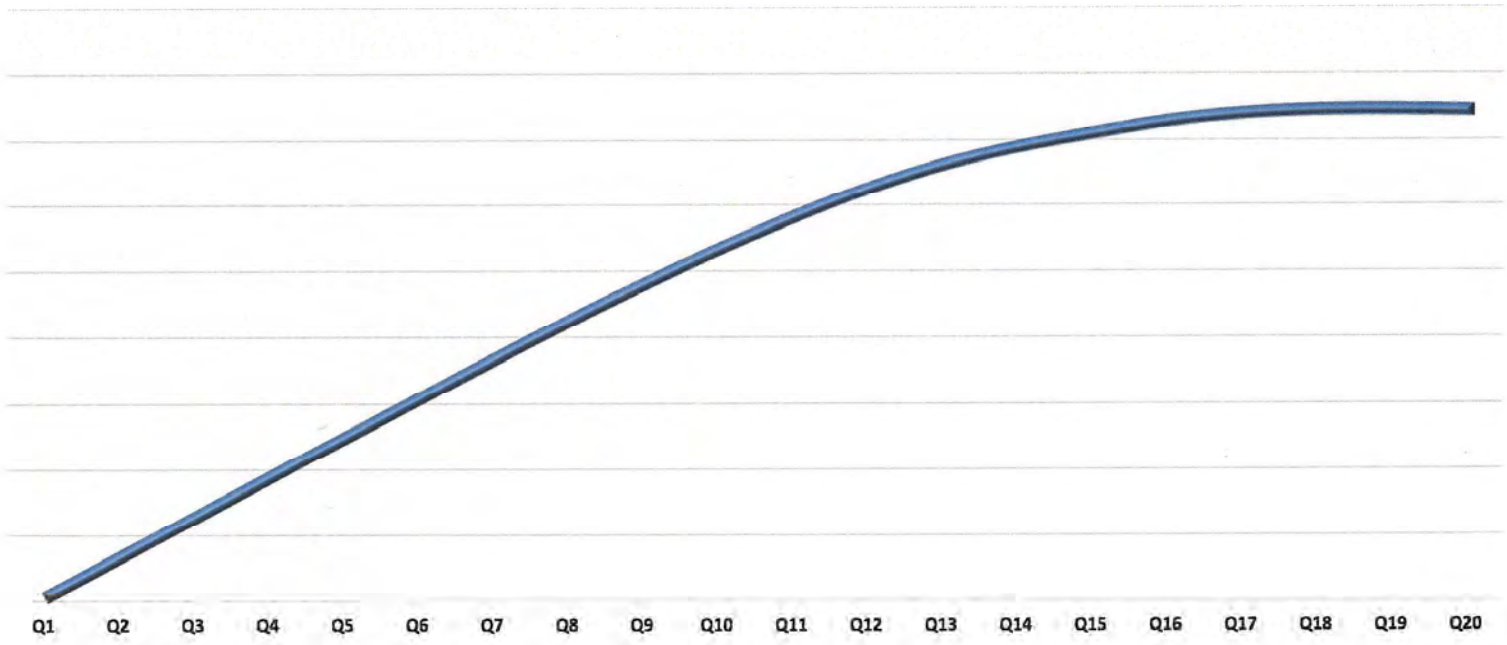
ONE-TIME GENE THERAPY MODEL VS. CHRONIC THERAPY MODEL



For illustrative purposes

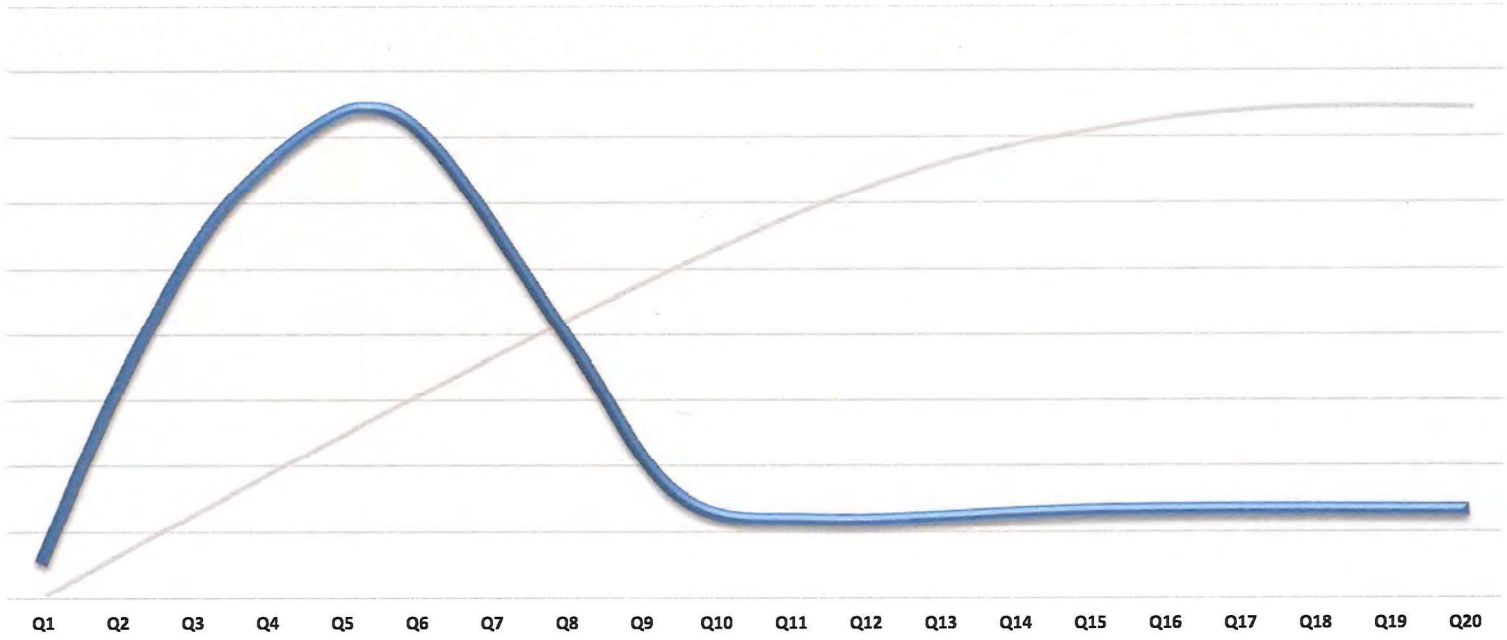
TRADITIONAL DRUG DEVELOPMENT FORECAST CURVE

CHRONIC DOSING MODEL . . .



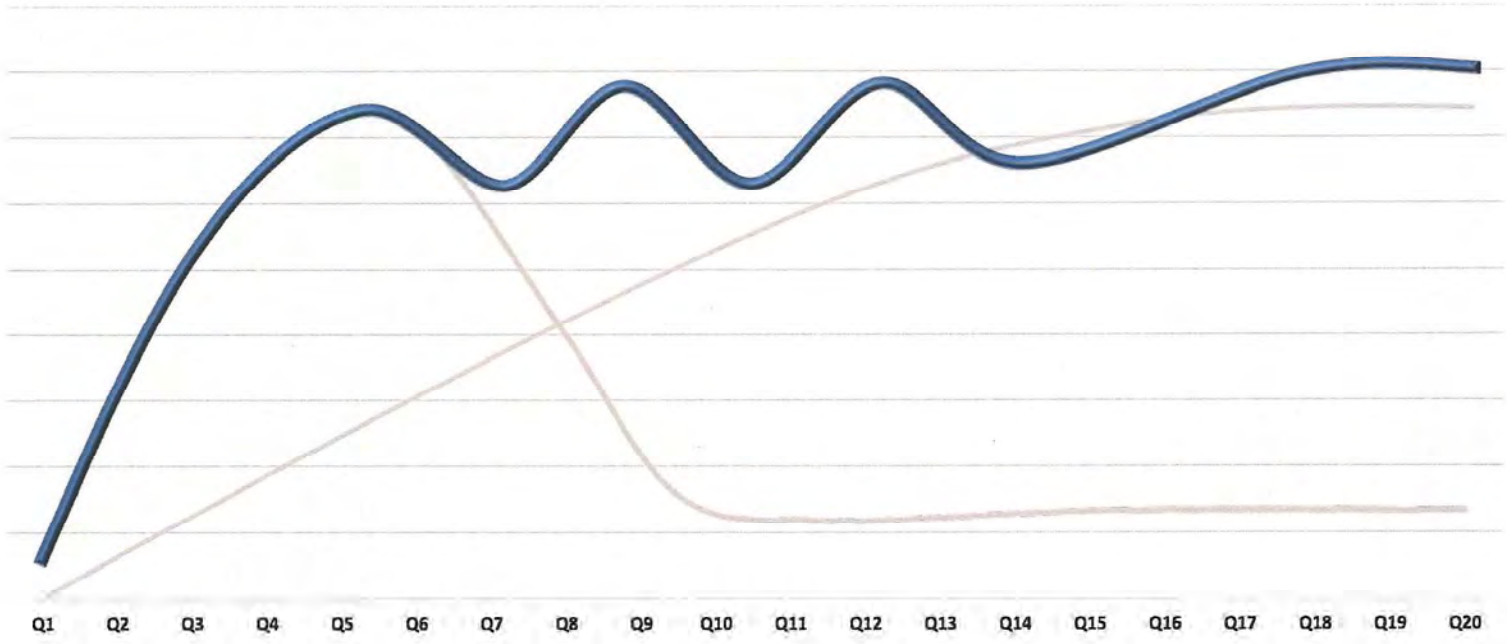
GENE THERAPY FORECAST CURVE

ONE-TIME TREATMENT MODEL . . .



SAREPTA'S FORECAST CURVE

CREATING A VIABLE BUSINESS MODEL FOR GENE THERAPY



SAREPTA'S SUSTAINABLE MODEL FOR TRANSFORMATIVE GENE THERAPIES

DISCOVERY ENGINE

Differentiated technologies unmatched in biotech

STEADY STREAM OF GENE THERAPIES

3.5 YEARS (from discovery)

MANUFACTURING

The most manufacturing capacity globally

FULFILL CLINICAL & COMMERCIAL NEEDS

READY IN 2020

ACCESS AND REIMBURSEMENT

Working with key stakeholders to bring medicines to patients

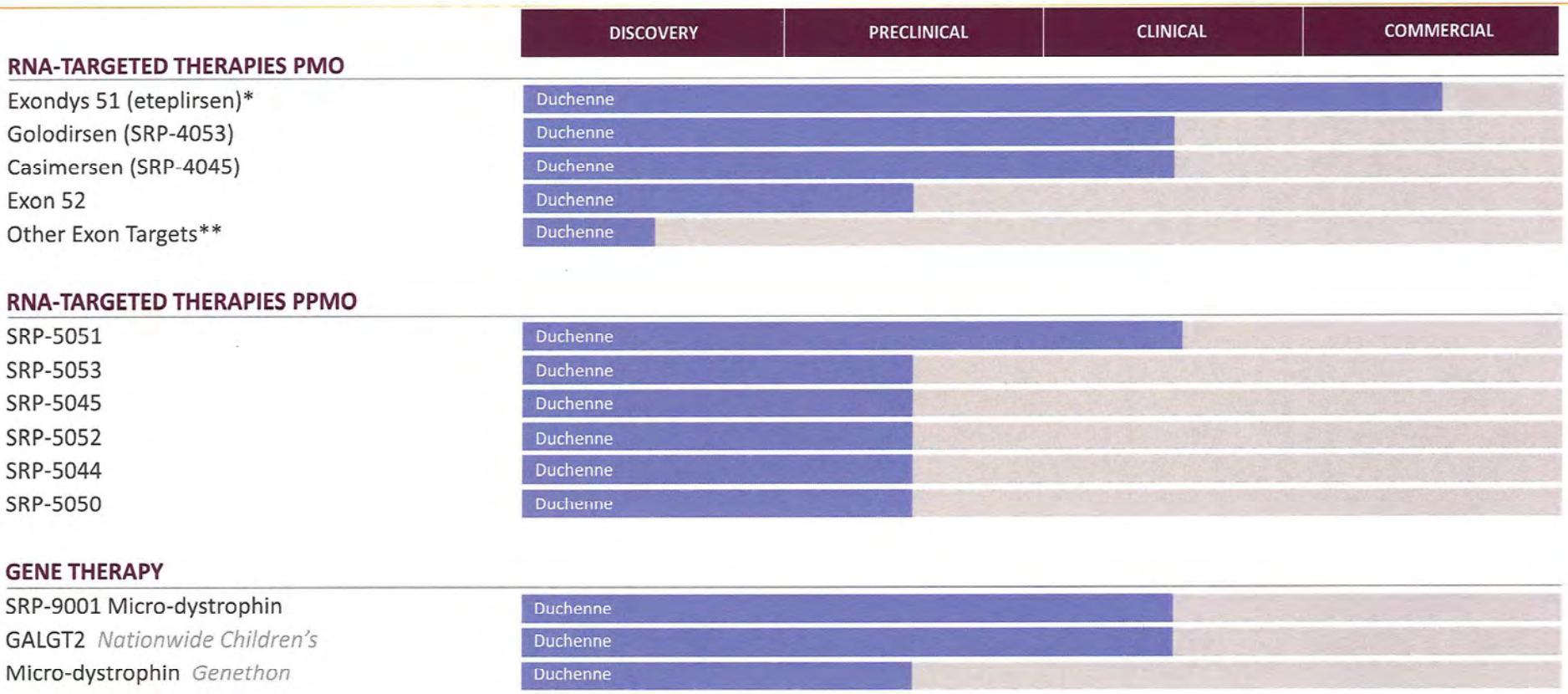
PATIENTS

READY IN 2021

A BURGEONING PIPELINE *TODAY*....

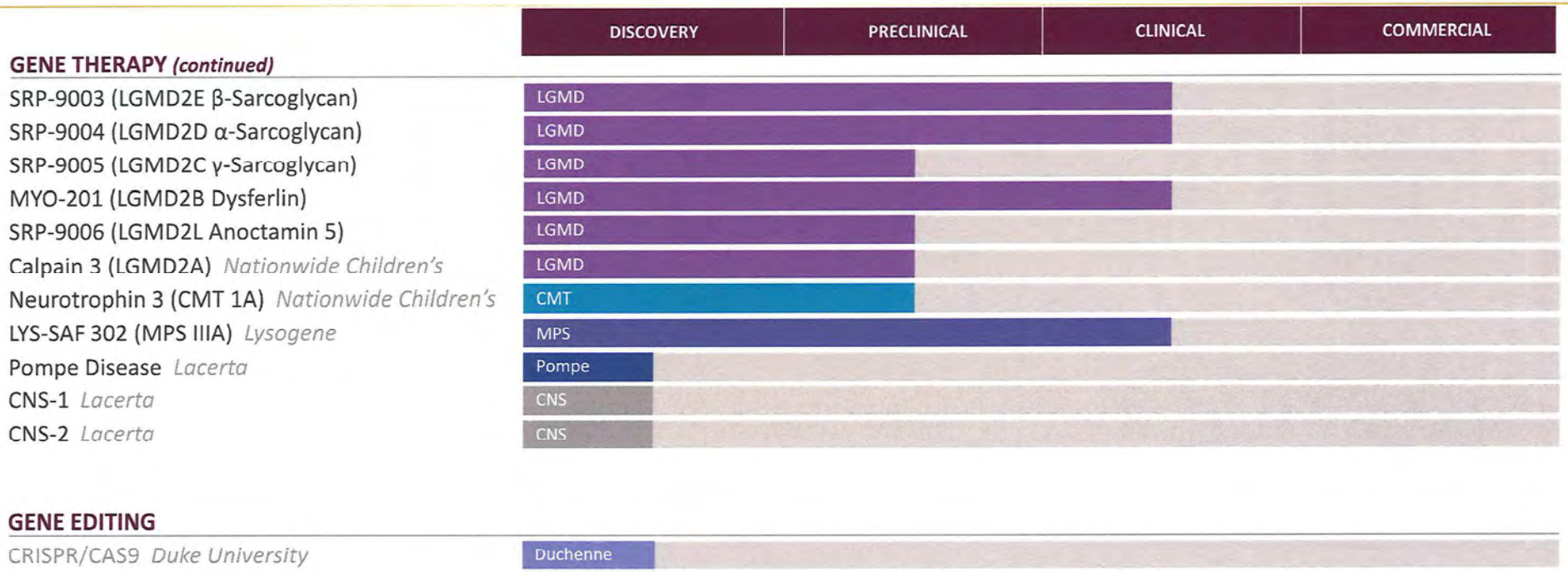
SAREPTA THERAPEUTICS

TODAY'S OPPORTUNITY: ~30 PROGRAMS IN DEVELOPMENT



*Candidate received accelerated approval in the U.S., confirmatory studies required
**Other exon targets in development: 43, 44, 50, and 55

TODAY'S OPPORTUNITY: ~30 PROGRAMS IN DEVELOPMENT



Duchenne – Duchenne muscular dystrophy **LGMD** – Limb-girdle muscular dystrophy **CMT** – Charcot-Marie-Tooth disease **MPS** – Mucopolysaccharidosis **CNS** – Central nervous system

DUCHENNE MUSCULAR DYSTROPHY (DMD)

DMD is a rare, fatal neuromuscular genetic disease

INHERITANCE
DMD is inherited in an X-linked recessive pattern.[1]

SYMPTOMS
Muscle weakness becomes increasingly noticeable by age 3 to 5, and most patients use a wheelchair by the time they are 11. During adolescence, cardiac and respiratory muscle deterioration lead to serious, life-threatening complications.[1,2]

POPULATION
DMD affects approximately 1 in 3,500-5,000 males born worldwide.[3]

TREATMENT
Exon-skipping drugs are available for patients with certain dystrophin mutations, broadly applicable gene therapy in development.

Genetic Root of Disease	One or more mutations in the gene that codes for dystrophin[3]
Missing Protein	Dystrophin[3]
Cellular Alteration	Instability of skeletal and cardiac muscle cell membranes[3]
Tissue Deterioration	Muscle deterioration and replacement with fatty and fibrotic tissue[1]
Function Loss	Typically loss of ambulation in early teens and progressive pulmonary and cardiac complications leading to mortality in late 20s[2]

1. Hoffman EP, Brown RH, et al. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell. 1987;51:919-928.
2. Passamano L, Taglia A, et al. Improvement of survival in Duchenne Muscular Dystrophy: retrospective analysis of 835 patients. Acta Myologica. 2012;31(1): 121-125.
3. National Institutes of Health. Genetics Home Reference. Duchenne and Becker muscular dystrophy. Accessed May 2019.

LIMB-GIRDLE MUSCULAR DYSTROPHY (LGMD)

INHERITANCE
The LGMDs are a group of genetically heterogeneous, autosomal inherited (recessive more common than dominant) muscular dystrophies with childhood to adult onset.[1]

SYMPTOMS
Individuals may first notice a problem when they begin to walk with a "waddling" gait because of weakness of the hip and leg muscles. They may have trouble getting out of chairs, rising from a toilet seat or climbing stairs. As this weakness progresses, the person may require the use of assistive mobility devices.[2]

POPULATION
Approximate global prevalence of LGMDs as a group is 1.63 per 100,000 (Prevalence estimates range from 0.56 to 5.75 per 100,000).[1] Over 30 subtypes exist.[3] Both genders are affected equally.[2]

TREATMENT
No cause-specific treatment is available for any of the LGMD subtypes.

Genetic Root of Disease	Broad range of mutations in various genes responsible for protein production[3]
Missing Proteins	Depends on specific subtype, such as proteins involved in the dystrophin associated protein complex (DAPC), sarcolemma, dystroglycan complex as well as intracellular proteins[3]
Cellular Alteration	These critical proteins are responsible for muscle function, regulation and repair[2]
Affected muscles	Affects skeletal muscle and in some cases affects cardiac and/or diaphragm[2,3]
Function Loss	Progressive weakness and wasting of hip or shoulder girdle muscles[1]; in some cases cardiac abnormalities and respiratory decline[2]

1. Liewluck T, Milone M, et al. Untangling the complexity of limb-girdle muscular dystrophies. Muscle Nerve. 2018;58(2):167-177.
2. Muscular Dystrophy Association. Limb-girdle muscular dystrophy (LGMD). Accessed May 2019.
3. Murphy AP, Straub V. The Classification, Natural History and Treatment of the Limb Girdle Muscular Dystrophies. *J Neuromuscular Diseases*. 2015;2(s2):S7-S19.

MUCOPOLYSACCHARIDOSIS TYPE IIIA (MPS IIIA)

INHERITANCE
Autosomal recessive, neurodegenerative lysosomal storage disease caused by mutations in the N-sulfoglucosamine sulfohydrolase (SGSH) gene, which encodes an enzyme called Heparan-N-sulfamidase necessary for heparan sulfate (HS) recycling in cells.[1,2]

SYMPTOMS
Signs and symptoms usually become apparent in early childhood and include speech problems, developmental delays, challenging behaviors, extreme hyperactivity and poor sleep.[3]

POPULATION
MPS IIIA has a worldwide incidence of up to 1.62 per 100,000 live births.[4]

TREATMENT
No approved product indicated to treat MPS IIIA.[3]

Genetic Root of Disease	Mutation in the *SGSH* gene[5]
Deficient enzyme	Heparan-N-sulfamidase[6]
Cellular Alteration	Build up of HS in cells[6]
Tissue Deterioration	Neurodegeneration[6]
Function Losses	Significant cognitive impairment, loss of speech, death within second decade of life[3]

1. Heron B, Mikaeloff Y, et al. Incidence and Natural History of Mucopolysaccharidosis Type III in France and Comparison with United Kingdom and Greece. American Journal of Medical Genetics Part A. 2011; 155A(1):58-68.
2. National Institutes of Health. Genetics Home Reference. SGSH gene. Accessed May 2019.
3. National Institutes of Health. Genetic and Rare Diseases Information Center. Mucopolysaccharidosis type IIIA. Accessed May 2019.
4. Zelei T, Csetneki K, et al. Epidemiology of Sanfilippo syndrome: results of a systematic literature review. Orphanet J of Rare Dis. 2018;13(1):53.
5. NIH. Genetics Home Reference. Mucopolysaccharidosis type III. Accessed May 2019.
6. Muenzer J. Overview of the mucopolysaccharidoses. *Rheumatology (Oxford)*. 2011;50(suppl 5):v4-12.

CHARCOT-MARIE-TOOTH (CMT) DISEASE
SAREPTA'S LEAD INDICATION - CMT1A

INHERITANCE
CMT1A is an autosomal dominant disease resulting from the duplication of the *PMP22* gene.[1]

SYMPTOMS
CMT1A symptoms involve muscle wasting leading to foot drop, balance difficulties and muscle weakness which can progress to affect motor skills limiting ability to walk and use hands.[2]

POPULATION
CMT is the most common inherited neuromuscular disorder and affects approximately 1 in 3,300 individuals worldwide.[3]

TREATMENT
No approved product indicated to treat CMT1A.

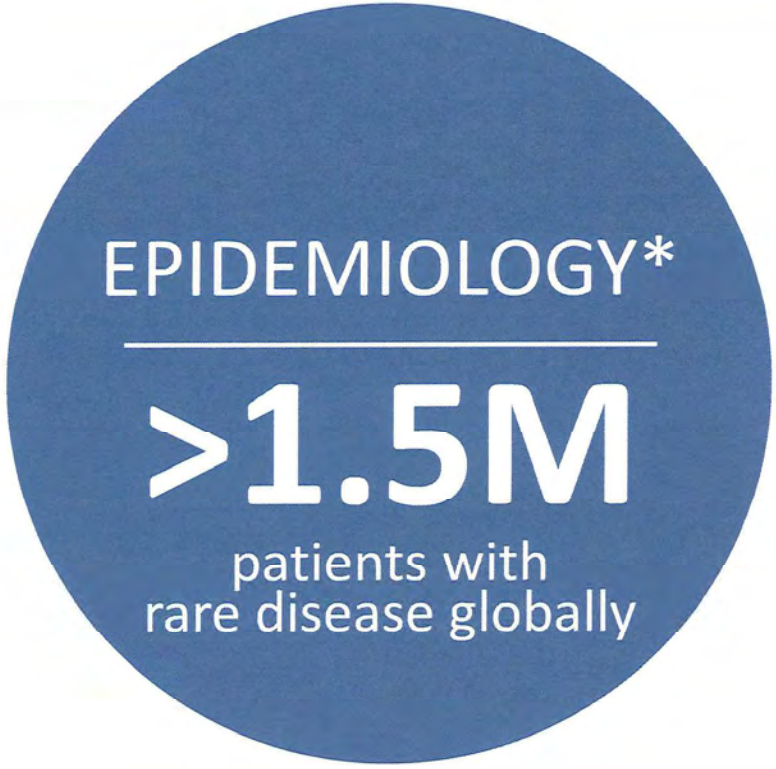
Genetic Root of Disease	Duplication of *PMP22* gene [1]
Protein Accumulation	Aggresomes build up in the Schwann cell impairing its ability to survive [4]
Cellular Alteration	Damage to Schwann cells leads to demyelination [2]
Tissue Deterioration	Lack of myelin coating leads to degeneration of peripheral nerves [2]
Disease Impact	Limited to no ability of the nerves to communicate with their targets (muscles) [2]

1. National Center for Advancing Translational Sciences. Genetic and Rare Disease Information Center. Charcot-Marie-Tooth disease type 1A.
2. National Institute of Neurological Disorders and Stroke. Charcot-Marie-Tooth Disease Fact Sheet. Accessed May 2019.
3. National Institutes of Health. Genetics Home Reference. Charcot-Marie-Tooth disease. Accessed August 2019.
4. Niemann A, Berger P, Suter U. Pathomechanisms of Mutant Proteins in Charcot-Marie-Tooth Disease. *NeuroMol Med*. 2006; 8(1-2):217-242.

THIS IS JUST THE BEGINNING OF *OUR COMMITMENT*

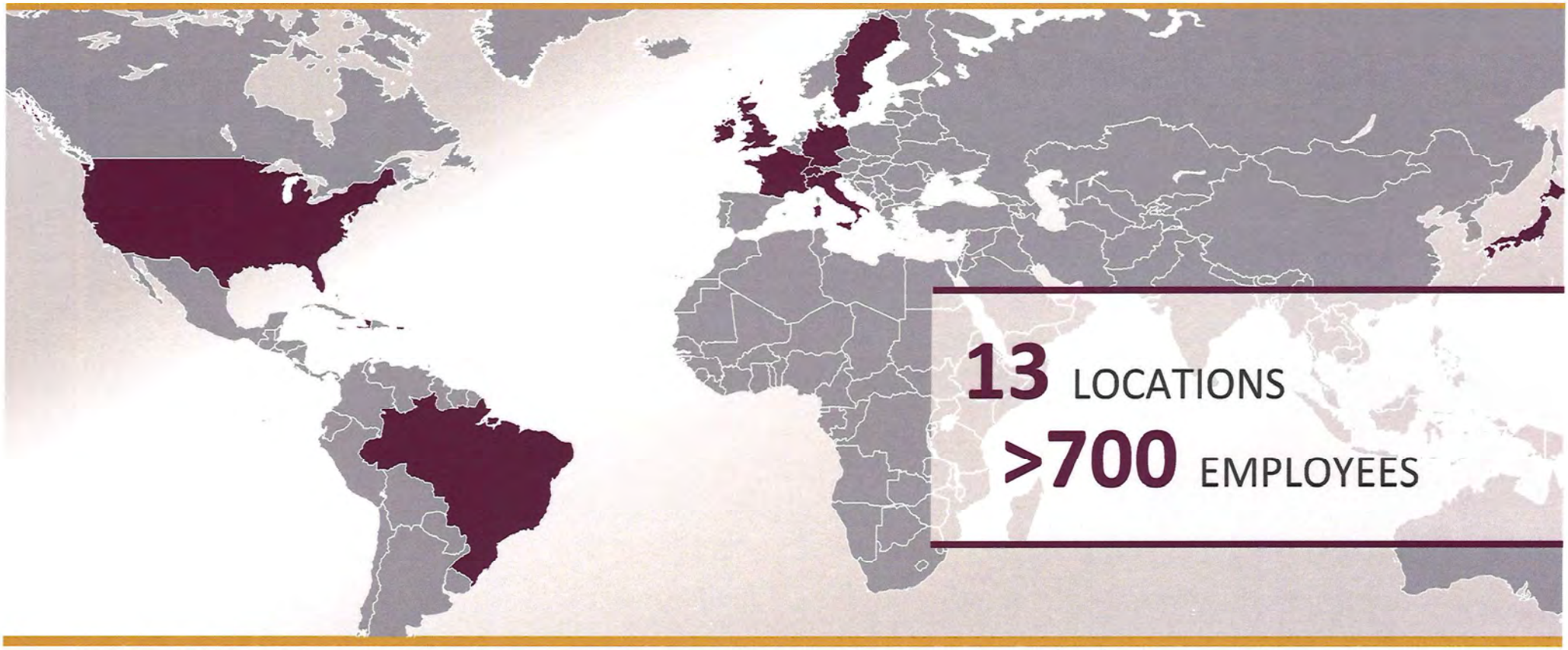
SAREPTA THERAPEUTICS

TOMORROW'S PROMISE: 40 PROGRAMS THAT COULD IMPACT MILLIONS OF PATIENTS AROUND THE GLOBE



*Based on published epidemiology

AN EXPANDING GLOBAL PRESENCE



THE TIME IS NOW TO *DRIVE INNOVATION*

SAREPTA THERAPEUTICS

"We choose to go to the moon. We choose to go to the moon in this decade and do the other things, not because they are easy, but because they are hard, because that goal will serve to organize and measure the best of our energies and skills, ***because that challenge is one that we are willing to accept, one we are unwilling to postpone,*** *and* ***one which we intend to win...*** *"*

PRESIDENT JOHN F. KENNEDY
Address at Rice University
September 12, 1962

PATIENTS CAN'T WAIT *SO NEITHER WILL WE*

SAREPTA THERAPEUTICS

DRAGGING TOMORROW INTO TODAY

#DraggingTomorrowIntoToday

Exhibit D

Sarepta Therapeutics (SRPT) Q3 2019 Earnings Call Transcript

SRPT earnings call for the period ending September 30, 2019.

Motley Fool Transcribing (MFTranscribing)

Nov 8, 2019 at 1:00PM

Sarepta Therapeutics (NASDAQ:SRPT)
Q3 2019 Earnings Call
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IMAGE SOURCE: THE MOTLEY FOOL.

Contents:

Prepared Remarks:

Operator

Good day, ladies and gentlemen, and welcome to the Sarepta Therapeutics third-quarter 2019 earnings call. [Operator instructions] As a reminder, today's call is being recorded. And now I'd like to introduce your host for today's program, Ian Estepan, senior vice president, chief of staff, and corporate affairs.

Ian Estepan -- *Senior Vice President, Chief of Staff, and Corporate Affairs*

Thank you, Michelle, and thank you, all, for joining today's call. Earlier today, we released our financial results for the third quarter of 2019. The press release is available on our website at www.sarepta.com, and our 10-Q was filed with the SEC earlier this afternoon. Joining us on the call today are Doug Ingram, Sandy Mahatme; Bo Cumbo, Dr.

Gilmore O'Neill; and Dr. Rodino-Klapac. After our formal remarks, we'll open up

behalf of the Division of Neurology toward Sarepta, I would unequivocally and emphatically disagree. Let me reiterate that I remain convinced that we were treated very fairly and professionally by the Division of Neurology. Also, I'm very proud of the Sarepta team and how they comported themselves during this review. From my perspective, we have gone a long way in the last two and a half years in forging a positive evidence-based working relationship with the division.

We will work diligently to address the VYONDYS CRL. But with that, I do not intend to provide a prediction on outcome or on timing or to provide interviews during the process. However, I will provide an update to the patient, physician, and investment communities once we have definitive clarity on the outcome of those discussions. Now moving to our positive achievements in the quarter.

We have made some enormous amount of progress in this third quarter. EXONDYS continues to perform well with third-quarter sales above consensus at $99 million. That is a 26% increase over the same quarter last year. Commenting for a moment on a confirmatory trial for EXONDYS, to remind you, this trial comprises three arms: one with EXONDYS at 100 mg per kg and another at 200 mg per kg versus our current dose at 30 mg per kg.

The trial design, which was an FDA requirement, will answer whether higher doses of EXONDYS provide even more benefit than the currently approved dose. Now since the comparator arms involve higher doses than the currently approved dose, we were required to begin our confirmatory trial with a healthy human volunteer study. We have completed this trial, and based on the results, we have initiated the main confirmatory trial. We will begin dosing this quarter.

Staying on our RNA franchise. We have moved to our multi-ascending dose trial for our next-generation RNA platform, the PPMO, and we are dosing trial participants now. We will have safety and dosing insight in 2020. If our PPMO shows encouraging results, in addition to SRP-5051, that's the construct that we're currently in a multi-ascending dose regarding, we have five additional constructs that have already been built, which in total have the potential to treat as much as 43% of the DMD community.

We are also conducting research now on new therapeutic targets that could be served by our PPMO platform. Moving next to our gene therapy platform. As you know, we are spending enormous resource and energy to build out our vision of an enduring gene therapy engine. Between our research and clinical-stage programs, we have more than 14 therapeutic candidates advancing through research and development.

We have made great progress thus far this year and quarter, led by our most

advanced program, SRP-9001, for DMD, which, at least to my knowledge, is the highest-potential late-stage gene therapy program currently in biotech. As you should be aware, our double-blind, placebo-controlled SRP-9001 micro-dystrophin trial, the trial that we call Study 2, was fully dosed by midyear, but we took advantage of the availability of additional study material and previously announced that we had increased the study n from 24 patients to 40 patients, significantly increasing the study power and confidence in this study. In addition to our initial site with Dr. Jerry Mendell at Nationwide Children's Hospital, we have added a second site at UCLA with Dr.

Perry Shieh. And I'm very proud to be associated with that clinician and investigator. Both sites are actively dosing patients, and we remain on target to complete our dosing by year-end. Micro-dystrophin manufacturing is progressing well.

From a capacity perspective, Brammer has now completed the buildout of our single-use micro-dystrophin manufacturing facility in Lexington, Massachusetts. We also have dedicated suites with Paragon in Maryland with actually substantially greater capacity than our dedicated Lexington facility, which means we have robustly secured capacity well in advance of launch. Our analytical development work proceeds well, and we continue to make progress on process development and yield optimization. Given our recent capacity, analytical development and process development progress, we remain on track to commence our next trial, Study 301, with commercial development supply by mid-2020.

Now Study 2 is being conducted with clinical material from Nationwide Children's Hospital. Study 301 will be a multicenter, multi-country, placebo-controlled trial using commercial process material from our hybrid manufacturing model with Brammer and Paragon. The main study will include DMD patients ages four to seven, but we are also planning a separate study for older and non-ambulatory patients, as well. Commenting on a few of our other gene therapy programs.

Following exceptional expression and biomarker results in our first three-patient cohort dosed with our construct for limb-girdle 2E, in October, we announced positive nine-month functional results in that same cohort. Consistent with robust expression of the native beta-sarcoglycan protein, that is the cause of the disease, all patients improved on every functional endpoint by the nine-month time point. Consistent with the protocol, we will treat an additional three-patient cohort with a higher dose, and then in early 2020, we will decide on the dose for

what we hope to be the pivotal trial. These results will help inform dosing not

production levels.

I'm getting a thumbs up from Dr. O'Neill suggesting that I haven't made a mistake there. And we'll have that before the middle of next year. I apologize if there are parts of your question that I was unable to hear and therefore I haven't answered.

Operator

Our next question comes from Brian Abrahams of RBC Capital. Your line is open.

Brian Abrahams -- *RBC Capital Markets -- Analyst*

Hi. Thanks very much for taking my questions. On micro-dystrophin, anything you're seeing so far with the additional cohort of patients to suggest that this new batch might be any different from the batch used to dose that first 24, I guess, 12 patients perhaps with respect to manufacturing composition, the way the second center administers it or stability through transportation out to L.A.? And then secondarily, I recognize you're not giving play-by-play updates on your FDA feedback, but we did see recently that Cedar is going to reorganize the office in neuroscience and other areas of the office of new drugs between now and year-end. And just wondering if that might mean a change in leadership of the group that's responsible for the golo application or any other implications this might have for your ongoing dialogue with the agency on golodirsen, casimersen, and your future gene therapies.

Thanks.

Doug Ingram -- *President and Chief Executive Officer*

Taking the first question regarding micro-dystrophin, please understand this is a blinded placebo-controlled trial, so we'll await those results. But with that said, also understand that the material for this trial is the same process and the same manufacturer, both in our clinical material coming from the same hyper -- GMP hyperstack, the adhering process at Nationwide Children's Hospital. So on the patients there, we have no concerns regarding the material itself. And we're very cognizant of things like the supply chain and the way it's transported, so we're very confident on those regard.

So things are going well. I don't want to -- it is a blinded trial, so we'll see the functional results and other related issues once all the dosing is done, and we unwind that portion at the end of 48 weeks. But we certainly are very confident on that material itself and the process for the trial, both with Dr. Jerry Mendell and also with Dr.

Perry Shieh of UCLA. Talking about the reorganization, the short answer on that

is that we are not going to -- and I'm certainly not going to speculate on what that could mean for golodirsen at all. I will say this, we have had a very productive relationship both with Dr. Billy Dunn and Dr.

Bastings, both of whom we have considerable regard for. So we think this is certainly a good answer for those in neuroscience and neuromuscular across the industry.

Operator

Our next question comes from Debjit Chattopadhyay of H.C. Wainwright. Your line is open.

Debjit Chattopadhyay -- *H.C. Wainwright and Company -- Analyst*

Hey, good afternoon. So given the tools in the toolbox currently, would you consider vectorized exon-skipping approach? Or are you limited by manufacturing capacity as far as your vectorized exon-skipping is concerned? And just a follow-up on the same question. In terms of the comparability between wild-type dystrophin expression and micro-dystrophin, is there a correlation or a conversion, say, for example, if you have 8% wild-type expression, that should have the same kind of functional benefits as, say, a 30% micro-dystrophin expression? Thank you so much.

Doug Ingram -- *President and Chief Executive Officer*

So on the first question, we have significant programs focused on Duchenne muscular dystrophy both from an RNA perspective, as well as a gene therapy perspective. Others have different approaches. We are generally not focused on, nor do we intend to have a focus anytime in the near future on the concept of using gene therapy to deliver exon-skipping modality. We're -- it's not something we're interested in as a gene transfer therapy.

I should also note, however, that as it relates to CRISPR-Cas9, which itself is a form of exon skipping, it is not in the RNA side. It actually is directly at the genome itself. That is an approach that Dr. Charlie Gersbach is taking.

Now he's taking a different approach than others in that regard. His CRISPR/Cas9 approach actually does a fairly significant cut. And if it is successful -- and this is a research program at this point. This is not a development stage program, and we are some ways away from a development-stage program.

But if that works, it would be an approach that could be available perhaps to as many as 50% of patients that have DMD, but that is a research program that is

some ways out. As it relates to the first -- it's really the basic question about the

Exhibit E

Video Article

Closed System Cell Culture Protocol Using HYPERStack Vessels with Gas Permeable Material Technology

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Abstract

Large volume adherent cell culture is currently standardized on stacked plate cell growth products when microcarrier beads are not an optimal choice. HYPERStack vessels allow closed system scale up from the current stacked plate products and delivers>2.5X more cells in the same volumetric footprint. The HYPERStack vessels function via gas permeable material which allows gas exchange to occur, therefore eliminating the need for internal headspace within a vessel. The elimination of headspace allows the compartment where cell growth occurs to be minimized to reduce space, allowing more layers of cell growth surface area within the same volumetric footprint.

For many applications such as cell therapy or vaccine production, a closed system is required for cell growth and harvesting. The HYPERStack vessel allows cell and reagent addition and removal via tubing from media bags or other methods.

This protocol will explain the technology behind the gas permeable material used in the HYPERStack vessels, gas diffusion results to meet the metabolic needs of cells, closed system cell growth protocols, and various harvesting methods.

Video Link

The video component of this article can be found at https://www.jove.com/video/2499/

Protocol

1. HYPERStack Vessel - Gas Permeable Material Technology Background

1. The HYPERStack Vessel is a multilayered vessel for closed system culturing of cells that relies on gas exchange through a 76.2micron gas permeable polymer film for cellular metabolism.
2. The HYPERStack vessel differs from more traditional cell culture vessels in that it does not have a "headspace" above the cells inside the vessel. Rather than containing this "headspace" for gas exchange within the vessel, the gas permeable products have air spaces (referred to as "tracheal" spaces) beneath each culture chamber which is open to the atmosphere.
3. This tracheal space enables the cells growing in each cell culture compartment of the vessel to have equivalent gas exchange.
4. The gas permeable film allows gas exchange to occur while maintaining a sterile environment. All fluid manipulations for each of the multiple layers occur through a single entry port.

2. HYPERStack Vessel Component Summary

1. The Stackette is the individual cell culture compartment that is made up of the top plate and gas permeable film. The cells are cultured within this compartment.
2. The Liquid Manifold connects each of the 12 stackette layers together within a HYPERStack module. Modules are connected with tubing to form vessels in multiples of 12 layers. The manifold allows the user to make one fluid manipulation to the entire vessel.
3. The Air Manifold also connects the stackette layers together, but it used to displace air from the vessel when fluid additions occur. It contains a fill line for use during filling.
4. The Tracheal Space is the open air space between each stackette layer, allowing gas exchange to occur through each layers' gas permeable film.

5. The Liquid handling tubing is connected to the liquid manifold and is used to make all closed system fluid manipulations. This component can be customized.
6. The Vent tubing is connected to the air manifold, contains an air filter, and is used to release excess air while maintaining sterility.
7. The chase tubing has a filter and clamp and are connected to the liquid handling tubing and are used to evacuate the fluid from the liquid handling tubing after filling the vessel.
8. The Corning Stack Manipulator or CSM is a handling device to assist in placing the vessels in the correct positions during use.
9. The Filling Wedge is a stainless steel assist used when manually filling the HYPERStack vessels.

3. Gas Diffusion Results Through Gas Permeable Film Used in the HYPERStack Vessels

1. In traditional cell growth systems with vented headspace, the oxygen in the media is depleted an average of 50% over 3 days in culture. (Figure 1)
2. In the same cell growth system, the oxygen gradient in a 3mm height of media is almost 50% greater at the media to headspace junction than it is at the cell layer. (Figure 2)
3. The diffusion of oxygen through the 76.2micron gas permeable polystyrene film is equal to the diffusion through 2.6mm of media. The gas permeable film used in the HYPERStack vessels allows the gas exchange to occur at the cell layer.
4. The % of oxygen in the tracheal space of the HYPERStack vessels remains constant as cells grow to confluence. (Figure 3)

4. Media Bag Preparation

1. The HYPERStack-12 layer vessel takes 1.3L of media and the HYPERStack-36 layer vessel takes 3.9L of media.
2. Prior to inoculating your bagged media, if serum is to be used, tube weld the serum bag to the media bag and mix thoroughly.
3. Clamp off approximately 300 mL of media in the bag using large bag clamps. This will ensure all inoculated cells are used during filling and none are remaining in the media bag. Place media bag on bag stand.

5. Inoculating Media

1. Fill a syringe with tubing attached with cell suspension. Tube weld 3/16" tubing from a syringe containing cell suspension to the media bag.
2. Inject the Cell Suspension into the Media Bag and Mix well.

6. Fill Procedure

1. Tube weld the inoculated media bag to the HYPERStack vessel's 3/8" liquid handling tubing. (Using Sterile Connects or Multipurpose connects (MPCs), you can also attach the media bag to the vessel).
2. Close the clamps on the liquid handling and chase tubes on the HYPERStack.
3. Place the HYPERStack 36 Layer Vessel into the CSM in the load position; hook the vent filter tube into the holding clamp on the CSM. Tighten the lid and move the CSM to the fill position. By putting the vessel into the correct fill position; the air filter is now in the highest position to prevent wetting during fill operation. The 10° angle permits equilibration of the liquid to the layers during filling. The HYPERStack-12 layer vessel placed on its side on the filling wedge is in the correct position for filling.
4. Clear air from the fill line by positioning the bag at the same level as the HYPERStack vessel (not higher). Keeping the chase tube clamp closed; open the liquid handling tube clamp and the media bag clamp to allow fluid to enter the vessel.
5. Using the bag stand, raise the media bag to help the cell suspension flow into the vessel.
6. Fill the vessel until the all of the inoculated media enters the vessel; the 300 mL of media should still remain in the top portion of the media bag. Remove the clamp from the media bag to continue filling the vessel. As liquid approaches the upper air manifold, slow down the fill rate by lowering the media bag in order to prevent over filling. Slowly bring the liquid level to the fill line and clamp the liquid handling tubing.

7. Isolation Procedure to Lock Liquid Into the Layers

1. Bring the HYPERStack vessel in the CSM to the isolation position so both sets of manifolds are in the highest position and lower the media bag below the height of the vessel. The HYPERStack-12 layer vessel can be lifted into the isolation position by placing a hand on the side of the vessel and using the other to lift the filling wedge.
2. With the chase filter held in an upright position, open the chase tube clamp. This will empty or chase the media in the liquid handling tube back into the media bag. Once the tubing is empty, close the clamp on the media bag tubing.
3. Keeping the chase tube filter in an upright position, open the clamp on the liquid handling tube on the HYPERStack to allow any remaining liquid to enter and equilibrate in the vessel. Wait 1-2 minutes for this to occur.
4. Turn the HYPERStack vessel in the CSM so that the manifolds are on the left. Lower the vessel to the load position on the CSM. Rocking the HYPEStack-12 layer vessel slightly to the left will permit the removal of the filling wedge. Close the clamps on both the chase and liquid handling tubing.
5. The media bag may now be removed from the vessel or it may remain attached for use later in the harvest procedure. To store the attached media bag, roll the empty bag and any remaining media in the bag, and place under the retaining bands in the storage tray of the HYPERStack vessel.
6. Move the HYPERStack vessel to the incubator. When carrying the vessel care should be taken to keep liquid from entering the air vent filter. This is accomplished by tipping the manifold end of the HYPERStack slightly upward.

8. Harvest Protocol

1. To begin the harvest procedure, tube weld the cell dissociation solution and quench bags together to form the harvesting bag assembly. Ensure all tubing clamps are closed. (Use cartoon to demonstrate)
2. Remove HYPERStack vessel from the incubator, release the media bag from under the retaining bands, and hang on the bag stand. Place the vessel in the CSM in the load position and tighten the lid to secure the vessel. Hook the vent tubing into the holding clamp and move the CSM to the fill position.
3. Making sure the media bag on the bag stand is hanging lower than the HYPERStack; open the clamp on the media bag tubing and the vessel's liquid handling tubing to allow media to flow into the attached bag.
4. Once the vessel is about ¾ empty, change the CSM settings to the final empty position. When the HYPERStack vessel and liquid handling tubing are empty, lift the tubing to chase the media past the clamp on the spent media bag tubing, then close both the clamp on the liquid handling tubing and the clamp on the spent media bag tubing.
5. Replace the spent media bag with the harvesting bag assembly by tube welding and raise the height of the cell dissociation bag assembly above the height of the HYPERStack using the bag stand.
6. Place the CSM in the equilibration position. Open the cell dissociation solution tube clamp and transfer the solution into the HYPERStack. When the transfer is complete, close the clamps on the liquid handling tubing and the cell dissociation solution tubing.
7. Bring CSM to the load position. By holding the horizontal positioning lever open, gently rock the vessel side to side using the wheel, distributing the solution over the cell layers. The vessel may occasionally be returned to the equilibration position during rocking to maintain equal distribution of the solution. It may not be visually apparent that the layers are completely coated, but the dissociation solution will be equally distributed across the surface of the cells. Once the solution has been adequately distributed, release the lever on the CSM to lock the HYPERStack in the load position. Leave the HYPERStack in this position during the required dissociation time for your cells.
8. Once the cells have detached; using turbidity as a guide, move the CSM to the equilibration position. Care should be taken not to over digest the cells. Open the quench bag clamp and the HYPERStack liquid handling tubing clamp to allow the quench media to enter the vessel. Close the clamp on the HYPERStack liquid handling tubing once the transfer is complete.
9. Return the CSM to the load position and hold the horizontal positioning lever to rock the vessel side to side.
10. Using the bag stand, lower the position of the harvest bag assembly below the level of the HYPERStack. Adjust the CSM to the empty position, open the vessel liquid handling tube clamp and transfer the cell solution back into the quench bag. Return the CSM to the load position and disconnect the harvest bag assembly from the vessel using a tube sealer. The cell solution is ready for processing.

9. Representative Results

- Harvesting time needs to be optimized when converting from a standard polystyrene surface to the gas permeable polystyrene surface. Cells tend to release faster on the HYPERStack product.
- To view cells under a microscope the vent and liquid handling tubing needs to be clamped off. Place the HYPERStack vessel upside down, with the manifolds pointing down, on the microscope stage.
- Prevention steps can be taken to avoid wetting the filters during use which will cause them to stop functioning. By not over filling the HYPERStack vessel and keeping the vent and chase tubing elevated when the clamps are open, no wetting should occur.
- The pressure of the closed clamps on the tubing causes it to remain closed after the clamps are opened. Pinching the tubing in the opposite direction will open it.
- Media flow time entering and exiting the HYPERStack vessel via gravity feed is based on the media height difference between the top level of media in the HYPERStack vessel and the top level of media in the media container. The greater distance, the faster the flow. The maximum flow is limited by the rating of the filters to 1.5L/min.
- Maintaining proper fill volume in the HYPERStack vessel will prevent air bubble formation in the cell growth chambers. Air bubbles can occur due to sampling or incubating in an unhumidified environment due to evaporative water loss. The sampling amount and frequency as well as incubation conditions will determine if additional fluid should be added by the end user.

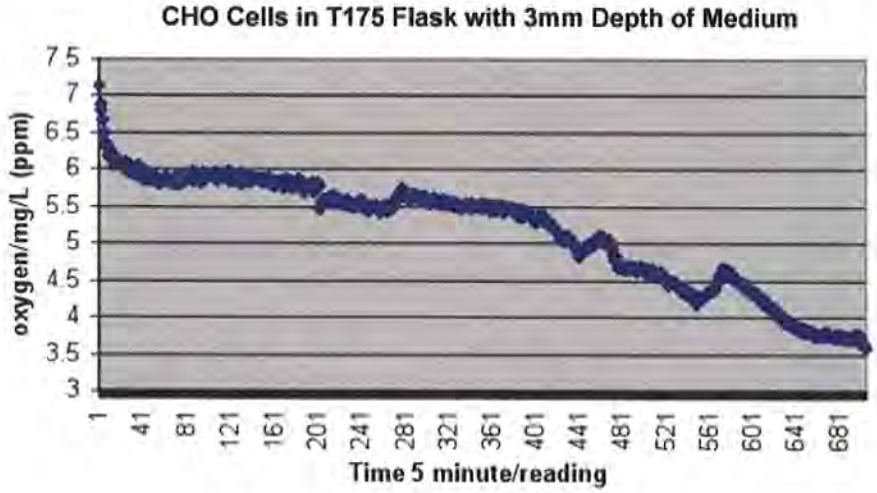

Figure 1. Oxygen depletion of media in a standard cell culture vessel. The figure shows the decrease in mg/L of oxygen at the level of the cells over 3 days.

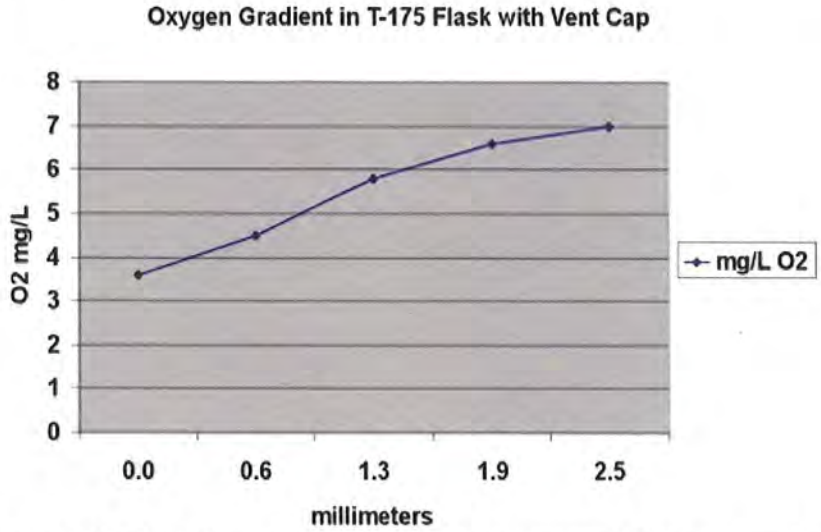

Figure 2. Oxygen gradient in media in a standard cell culture vessel. The figure shows the mg/L of Oxygen is greatest at the media to headspace intersection than at the cell layer.

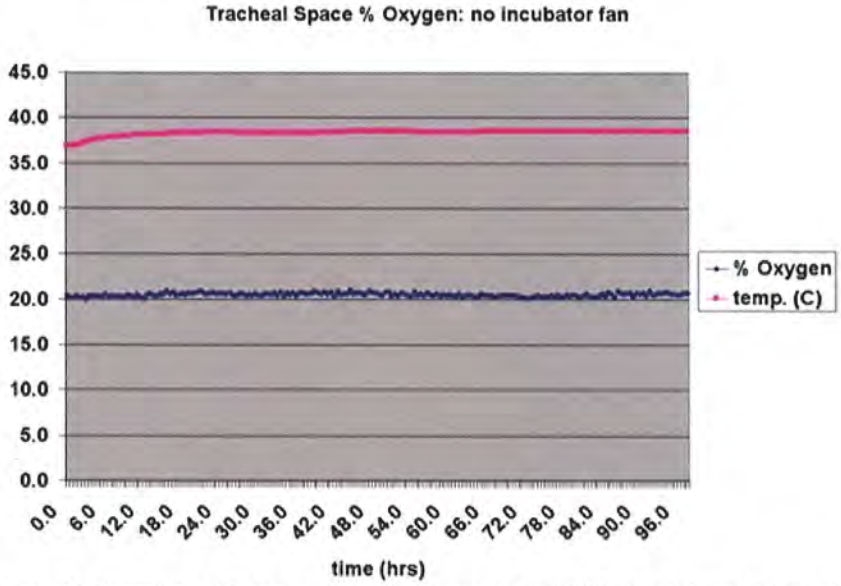

Figure 3. Percentage of Oxygen in the tracheal space of the HYPERStack during cell growth. The figure shows that over 96 hours, the percentage of oxygen between the layers, which supplies each gas permeable film bottom, remains constant. This demonstrates the ability of the cells to access oxygen during growth to confluency.

Discussion

HYPERStack vessel has successfully grown engineered, primary and stem cells. Fluid additions into the vessel have been conducted via aseptic bag connections by gravity feed and also by pumping from a sterile media container. The vessel provides a greater than 2.5X increase in growth area vs. a stacked plate product of the same volumetric footprint. The HYPERStack-12 has 6,000cm^2 growth area, the HYPERStack-36 has 18,000cm^2 growth area, and the HYPERStack-120 has 60,000cm^2 of growth area. By increasing the surface area, users are able to produce more cells from the same cell population or lot and reduce variability. They are also able to save space and/or labor by using fewer vessels to achieve the same cell output, or increase cell output without adding people, cleanroom suites, or incubators. The system is produced with low particulate assembly methods and without the use of adhesives. It is a closed system which is preassembled with tubing sets and filters, triple bagged and provided sterile.

Disclosures

The HYPERStack vessel gas permeable technology is covered under US Patent #: 7745209. The authors are employees of Corning Life Sciences, Inc that produces the instrument used in this article.

Acknowledgements

The authors would like to thank Lonza Walkersville for their assistance with closed system knowledge, bag design, loan of bag sealers and welders, as well as creating the bag to HYPERStack schematics.

EXHIBIT F

Sarepta Therapeutics Enters into Long-term Strategic Manufacturing Partnership with Brammer Bio to Support Gene Therapy Development and Commercial Supply

-- The partnership will provide commercial supply for a potential micro-dystrophin gene therapy product launch and other neuromuscular programs in the pipeline --

-- Hybrid model enables Sarepta to maintain control over process development, while leveraging Brammer Bio's world-class manufacturing capabilities --

CAMBRIDGE, Mass., June 13, 2018 (GLOBE NEWSWIRE) -- Sarepta Therapeutics, Inc. (NASDAQ: SRPT), a commercial-stage biopharmaceutical company focused on the discovery and development of precision genetic medicine to treat rare neuromuscular diseases, announced today that it has entered into a long-term strategic manufacturing partnership with Brammer Bio, which will provide Sarepta access to clinical and commercial manufacturing capacity for its micro-dystrophin Duchenne muscular dystrophy (DMD) gene therapy program and a manufacturing platform for future gene therapy programs, such as Limb girdle muscular dystrophy (LGMD).

Sarepta has adopted a hybrid internal and external development and manufacturing model. Under this model, Sarepta will continue to build internal expertise in all aspects of AAV-based manufacturing while externally Brammer Bio will provide scalable best-in-class manufacturing capabilities. The collaboration model will integrate process development, clinical production and testing, and commercial manufacturing with the goal of bringing micro-dystrophin gene therapies to the patient community urgently and in sufficient supply.

Brammer Bio will partner with Sarepta to design and build dedicated commercial manufacturing capacity within their facility with cutting-edge capabilities. Once complete, the facility is expected to provide robust manufacturing capacity to support the unusually high demands typical for systemic administration of the micro-dystrophin therapy for DMD.

"As we have stated in the past, Sarepta is committed to becoming one of the most meaningful genetic medicine companies in the world over the coming few years. The Brammer Bio partnership and dedicated

gene therapy capacity, once complete, will represent more annual gene therapy supply than any currently existing facility," stated Doug Ingram, Sarepta's president and chief executive officer.

Mr. Ingram added, "At Sarepta, we pride ourselves in partnering with the best and brightest to advance our mission. With that in mind, we are proud to have selected Brammer Bio as our partner because they are among the world's most advanced cGMP gene therapy manufacturers, with expertise that spans all aspects of AAV-based gene therapy development, manufacturing and release. Our hybrid approach enables us to leverage both our internal expertise and capabilities and Brammer Bio's capacity and expertise, allowing for minimal changes to the process to accelerate therapies for patients with DMD and LGMD."

"Brammer is delighted to partner with Sarepta Therapeutics to use our team's deep development, clinical and commercial expertise to support the rapid development of Sarepta's gene therapy products to serve patients in the United States and globally," said Mark Bamforth, Brammer Bio's president and chief executive officer.

Brammer Bio's team of 400+ operates in Massachusetts and Florida. The 74,000 square-foot early clinical campus consists of three buildings in Alachua, Fla., comprised of a process development and analytical development facility, adjacent to its cGMP Phase 1/2 clinical manufacturing operation with a third warehouse and office building. Brammer Bio's cGMP facility has been supporting gene therapy clinical development for 12 years at this location.

Brammer Bio has 165,000 square-feet of facilities in Massachusetts for Phase 3 and commercial cGMP viral vector manufacturing. The facility located in Cambridge was built out late in 2017 and the facility in Lexington will be operational in 2019; both are supported by the warehouse and distribution center in Somerville.

About Sarepta Therapeutics

Sarepta Therapeutics is a commercial-stage biopharmaceutical company focused on the discovery and development of precision genetic medicine to treat rare neuromuscular diseases. The Company is primarily focused on rapidly advancing the development of its potentially disease-modifying Duchenne muscular dystrophy (DMD) drug candidates. For more information, please visit www.sarepta.com.

About Brammer Bio

Brammer Bio provides clinical and commercial supply of viral vectors for in vivo gene and ex vivo modified-cell based therapies, process and analytical development, and regulatory support, enabling large pharma and biotech clients to accelerate the delivery of novel medicines to improve patient health. Brammer is owned by Ampersand Capital Partners, the only institutional investor in the company, and its founders. For more information, please visit www.brammerbio.com.

Forward-Looking Statements

This press release contains "forward-looking statements." Any statements contained in this press release that are not statements of historical fact may be deemed to be forward-looking statements. Words such as "believes," "anticipates," "plans," "expects," "will," "intends," "potential," "possible" and similar expressions are intended to identify forward-looking statements. These forward-looking statements include statements regarding the expectation that the partnership with Brammer Bio will provide Sarepta access to clinical and commercial manufacturing capacity for its micro-dystrophin DMD gene therapy program and a manufacturing platform for future gene therapy programs, such as LGMD; Sarepta's plan to continue to build internal expertise in all aspects of AAV-based manufacturing while Brammer Bio will provide scalable best-in-class manufacturing capabilities; the collaboration model integrating process development, clinical production and testing, and commercial manufacturing with the goal of bringing micro-dystrophin gene therapies to the patient community urgently and in sufficient supply; the expectation that Brammer Bio's facility will provide robust manufacturing capacity to support the unusually high demands typical for systemic administration of the micro-dystrophin therapy for DMD; Sarepta's commitment to becoming one of the most meaningful genetic medicine companies in the world over the coming few years; the expectation that the Brammer Bio partnership and dedicated gene therapy capacity, once complete, will represent more annual gene therapy supply than any currently existing facility; and the hybrid approach allowing Sarepta for minimal changes to the process to accelerate therapies for patients with DMD and LGMD.

These forward-looking statements involve risks and uncertainties, many of which are beyond Sarepta's control. Known risk factors include, among others: the expected benefits and opportunities related to the agreement with Brammer Bio may not be realized or may take longer to realize than expected; Sarepta's dependence on Brammer Bio to produce its product candidates, including any inability on Sarepta's part to accurately anticipate product demand and timely secure manufacturing capacity to meet product demand, may impair the availability of product to successfully support various programs, including research and development and the potential commercialization of Sarepta's gene therapy product candidates; if Brammer Bio were to cease providing quality manufacturing and related services to Sarepta, and

Sarepta is not able to engage appropriate replacements in a timely manner, Sarepta's ability to manufacture its gene therapy product candidates in sufficient quality and quantity would adversely affect Sarepta's various product research, development and commercialization efforts; if Brammer Bio fails to adhere to applicable cGMP and other applicable government regulations, or experiences manufacturing problems, Sarepta will suffer significant consequences, which could significantly delay or negatively impact the success of Sarepta's development efforts for its product candidates; Sarepta may not be able to successfully scale up manufacturing of its product candidates in sufficient quality and quantity or within sufficient timelines, or be able to secure ownership of intellectual property rights developed in this process, which could negatively impact the development of its product candidates and next generation chemistries like gene therapy; Sarepta's gene therapy programs may not result in any viable treatments suitable for clinical research or commercialization due to a variety of reasons, including the results of future research may not be consistent with past positive results or may fail to meet regulatory approval requirements for the safety and efficacy of product candidates or may never become commercialized products due to other various reasons including possible limitations of Company financial and other resources, manufacturing limitations that may not be anticipated or resolved for in a timely manner, and regulatory, court or agency decisions, such as decisions by the United States Patent and Trademark Office with respect to patents that cover our product candidates; and even if Sarepta's gene therapy programs result in new commercialized products, Sarepta may not achieve any significant revenues from the sale of such products; and those risks identified under the heading "Risk Factors" in Sarepta's most recent Annual Report on Form 10-K for the year ended December 31, 2017 and most recent Quarterly Report on Form 10-Q filed with the Securities and Exchange Commission (SEC) as well as other SEC filings made by the Company which you are encouraged to review.

Any of the foregoing risks could materially and adversely affect the Company's business, results of operations and the trading price of Sarepta's common stock. For a detailed description of risks and uncertainties Sarepta faces, you are encouraged to review Sarepta's 2017 Annual Report on Form 10-K and most recent Quarterly Report on Form 10-Q filed with the SEC as well as other SEC filings made by Sarepta. We caution investors not to place considerable reliance on the forward-looking statements contained in this press release. Sarepta does not undertake any obligation to publicly update its forward-looking statements based on events or circumstances after the date hereof.

Internet Posting of Information

We routinely post information that may be important to investors in the 'For Investors' section of our web-site at www.sarepta.com. We encourage investors and potential investors to consult our website regularly for important information about us.

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EXHIBIT G

UNITED STATES
SECURITIES AND EXCHANGE COMMISSION
Washington, D.C. 20549

FORM 10-K

(Mark One)

☒ **ANNUAL REPORT PURSUANT TO SECTION 13 OR 15(d) OF THE SECURITIES EXCHANGE ACT OF 1934**

For the fiscal year ended December 31, 2018

Or

☐ **TRANSITION REPORT PURSUANT TO SECTION 13 OR 15(d) OF THE SECURITIES EXCHANGE ACT OF 1934**

For the transition period from to

Commission file number: 001-14895

Sarepta Therapeutics, Inc.

(Exact name of registrant as specified in its charter)

Delaware	**93-0797222**
(State or other jurisdiction of incorporation or organization)	**(I.R.S. Employer Identification Number)**
215 First Street **Suite 415** **Cambridge, MA**	**02142**
(Address of principal executive offices)	**(Zip Code)**

Registrant's telephone number, including area code: (617) 274-4000

Securities registered pursuant to Section 12(b) of the Act:

Tile of Each Class	**Name of Exchange on Which Registered**
Common Stock, $0.0001 par value	**The NASDAQ Stock Market LLC** **(The NASDAQ Global Select Market)**

Securities registered pursuant to Section 12(g) of the Act:
None

Indicate by check mark if the registrant is a well-known seasoned issuer, as defined in Rule 405 of the Securities Act. Yes ☒ No ☐

Indicate by check mark if the registrant is not required to file reports pursuant to Section 13 or Section 15(d) of the Act. Yes ☐ No ☒

Indicate by check mark whether the registrant (1) has filed all reports required to be filed by Section 13 or 15(d) of the Securities Exchange Act of 1934 during the preceding 12 months (or for such shorter period that the registrant was required to file such reports), and (2) has been subject to such filing requirements for the past 90 days. Yes ☒ No ☐

Indicate by check mark whether the registrant has submitted electronically every Interactive Data File required to be submitted pursuant to Rule 405 of Regulation S-T (§ 232.405 of this chapter) during the preceding 12 months (or for such shorter period that the registrant was required to submit such files). Yes ☒ No ☐

Indicate by check mark if disclosure of delinquent filers pursuant to Item 405 of Regulation S-K (§ 229.405 of this chapter) is not contained herein, and will not be contained, to the best of the registrant's knowledge, in definitive proxy or information statements incorporated by reference in Part III of this Form 10-K or any amendment to this Form 10-K. ☐

Indicate by check mark whether the registrant is a large accelerated filer, an accelerated filer, a non-accelerated filer, a smaller reporting company, or an emerging growth company. See the definitions of "large accelerated filer," "accelerated filer," "smaller reporting company" and "emerging growth company" in Rule 12b-2 of the Exchange Act.

Large accelerated filer	☒	Accelerated filer	☐
Non-accelerated filer	☐	Smaller reporting company	☐
Emerging growth company	☐		

If an emerging growth company, indicate by check mark if the registrant has elected not to use the extended transition period for complying with any new or revised financial accounting standards provided pursuant to Section 13(a) of the Exchange Act. ☐

Indicate by check mark whether the registrant is a shell company (as defined in Rule 12b-2 of the Act). Yes ☐ No ☒

The aggregate market value of the voting and non-voting common equity held by non-affiliates of the registrant as of June 29, 2018 (the

last business day of the registrant's most recently completed second fiscal quarter) based on the closing price of $132.18 as reported on the Nasdaq Global Select Market was approximately $8,769,647,061.

The number of outstanding shares of the registrant's common stock as of the close of business on February 22, 2019 was 71,292,129.

DOCUMENTS INCORPORATED BY REFERENCE

The registrant has incorporated by reference into Part III of this Annual Report on Form 10-K, portions of its definitive Proxy Statement for its 2018 annual meeting to be filed with the Commission no later than 120 days after the end of the fiscal year covered by this Annual Report on Form 10-K.

Sarepta Therapeutics, Inc.
FORM 10-K INDEX

PART I

Item 1. Business.

Overview

We are a commercial-stage biopharmaceutical company focused on helping patients through the discovery and development of unique RNA-targeted therapeutics, gene therapy and other genetic therapeutic modalities for the treatment of rare diseases. Applying our proprietary, highly-differentiated and innovative technologies, and through collaborations with our strategic partners, we are developing potential therapeutic candidates for a broad range of diseases and disorders, including Duchenne muscular dystrophy ("DMD"), Limb-girdle muscular dystrophies ("LGMDs"), Mucopolysaccharidosis type IIIA ("MPS IIIA") and Pompe.

Our first commercial product in the U.S., EXONDYS 51® (eteplirsen) Injection ("EXONDYS 51"), was granted accelerated approval by the FDA on September 19, 2016. EXONDYS 51 is indicated for the treatment of DMD in patients who have a confirmed mutation of the DMD gene that is amenable to exon 51 skipping. EXONDYS 51 uses our phosphorodiamidate morpholino oligomer ("PMO") chemistry and exon-skipping technology to skip exon 51 of the dystrophin gene. Exon skipping is intended to promote the production of an internally truncated but functional dystrophin protein.

The original PMO structure and variations of this structure that are so-called PMO-based (collectively "PMO-based") are central to our proprietary chemistry platform. PMO technologies can be used to selectively up-regulate or down-regulate the production of a target protein through pre-mRNA splice alteration. Thus, PMO-based compounds have the potential to be designed to create more, less, or none of certain proteins, or produce analogues of endogenous proteins. This technology can be used to correct disease-causing genetic errors by inducing the targeted expression of novel proteins.

In addition to our commercial-stage product, we have PMO-based product candidates in clinical development designed to treat those patients with DMD who have genetic mutations amenable to skipping exon 53 of the Duchenne gene (SRP-4053) and exon 45 of the Duchenne gene (SRP-4045) (golodirsen and casimersen, respectively). In December 2018, we completed the submission of our rolling New Drug Application ("NDA") to the U.S. Food and Drug Administration ("FDA") seeking accelerated approval for golodirsen. The FDA accepted the NDA and granted priority review status for golodirsen with a targeted regulatory action date of August 19, 2019. The FDA also indicated that it does not intend to conduct an advisory board for golodirsen. We are currently conducting both a Phase 1/2 clinical trial and a Phase 3 placebo controlled confirmatory clinical trial (ESSENCE) studying casimersen. We anticipate submitting an NDA to the FDA for casimersen in 2019 if we believe that the results of an interim dystrophin analysis in the ESSENCE trial are positive. We also have other product candidates in discovery and preclinical development that are designed to skip other exons.

The PMO chemistry platform is highly adaptable, and we have developed next-generation PMO-based chemistries for advancing RNA-targeted therapeutics. These next-generation chemistries are specifically designed to enhance tissue targeting, intracellular delivery, target selectivity and drug potency. One of these novel technologies is based on cell-penetrating peptide-conjugated PMO ("PPMO"). The PPMO features covalent attachment of a cell-penetrating peptide to a PMO with the goal of enhanced delivery into the cell. Our most advanced PPMO product candidate is SRP-5051, which is designed to treat DMD in patients with genetic mutations amenable to exon 51 skipping. We are currently conducting a first-in-human, single ascending dose, Phase 1 clinical trial for this product candidate, which we expect to complete in 2019.

As part of our multifaceted approach to DMD, we are also exploring gene therapy technologies to treat DMD. In collaboration with Nationwide Children's Hospital ("Nationwide"), we are testing a product candidate, SRP-9001, that aims to express a smaller but still functional version of dystrophin ("micro-dystrophin"). We use a unique adeno-associated virus ("AAV") vector called AAVrh74 to transport the transgene – the genetic material that will make the protein of interest – to the target cells. Micro-dystrophin is used because naturally-occurring dystrophin is too large to fit in an AAV. On October 3, 2018, Nationwide presented positive results from a Phase 1/2a clinical trial testing SRP-9001 in four individuals with DMD enrolled in the trial. In the fourth quarter of 2018, we commenced a placebo-controlled trial with the goal to establish the functional benefits of micro-dystrophin expressions. We plan to conduct a confirmatory trial using commercial supply of SRP-9001 by the end of 2019, pending regulatory feedback.

In 2018, through a number of strategic collaboration and licensing arrangements, we expanded our pipeline to include programs that aim to treat a broad range of rare diseases in addition to DMD, such as LGMDs, Charcot-Marie-Tooth ("CMT"), MPS IIIA and Pompe disease. One of our strategic partners, Myonexus Therapeutics, Inc. ("Myonexus") develops gene therapy programs for various forms of LGMDs. The most advanced of Myonexus' product candidates, MYO-101, is designed to transfer a gene that codes for and restores beta-sarcoglycan protein with the goal of restoring the dystrophin associated protein complex. MYO-101 utilizes the same vector and promoter used in the development of SRP-9001. Myonexus commenced a Phase 1/2a trial of MYO-101 in the fourth quarter of 2018, and on February 27, 2019, we announced positive two-month data from the first three-patient cohort dosed in the MYO-101 trial.

Our pipeline includes 25 programs at various stages of pre-clinical and clinical development, reflecting our aspiration to apply our multifaceted approach and expertise in precision genetic medicine to make a profound difference in the lives of patients suffering from rare diseases.

Objectives and Business Strategy

We believe that our proprietary technology platforms and collaborations can be used to develop novel pharmaceutical products to treat a broad range of diseases and address key currently-unmet medical needs. We intend to leverage our technology platforms, organizational capabilities, collaborations and resources to lead the field of precision genetic medicines, including the treatment of rare, neuromuscular and other diseases, with a diversified portfolio of product candidates. In pursuit of this objective, we intend to focus on the following activities:

- building our gene therapy engine, including developing gene therapy product candidates, operationalizing our manufacturing strategy and establishing our commercial foundation in preparation for potential regulatory approvals;
- advancing the development of additional exon-skipping product candidates (e.g., golodirsen and casimersen), launching potential approved products and supporting marketed products;
- investing in next-generation precision medicine through internal research, strategic partnerships, collaborations and other potential opportunities;
- ensuring we have the appropriate capitalization to fund our business objectives and strategies, including by raising additional capital through licensing, collaborations and offerings of equity and / or debt; and
- nurturing our culture, which is based on bias to action, a self-starter mentality, smart and appropriate risk-taking and high ethics.

Core Therapeutic Areas

DMD: We primarily focus on rapidly advancing the development of our potentially disease-modifying pipeline of exon-skipping, gene therapy and gene editing product candidates targeting DMD. DMD is a rare x-linked recessive genetic disorder affecting children (primarily males) that is characterized by progressive muscle deterioration and weakness. It is the most common type of muscular dystrophy. DMD is caused by an absence of dystrophin, a protein that protects muscle cells. The absence of dystrophin in muscle cells leads to significant cell damage and ultimately causes muscle cell death and fibrotic replacement. In the absence of dystrophin protein, affected individuals generally experience the following symptoms, although disease severity and life expectancy vary:

- muscle damage characterized by inflammation, fibrosis and loss of myofibers beginning at an early age;
- muscle weakness and progressive loss of muscle function beginning in the first few years of life;
- decline of ambulation and respiratory function after the age of seven;
- total loss of ambulation in the pre-teenage or early teenage years;
- progressive loss of upper extremity function during mid- to late-teens; and
- respiratory and/or cardiac failure, resulting in death before the age of 30.

LGMDs are autosomal recessive, monogenic, rare neuromuscular diseases caused by missense and deletion mutations. These diseases affect males and females equally. Some types of LGMDs affect skeletal muscle and cardiac muscle. More severe forms of LGMDs mimic DMD. LGMDs as a class affect an estimated range of approximately 1 in every 14,500 to 1 in every 123,000 individuals. Currently, there are no available treatment options for LGMDs.

MPS IIIA is a rare inherited neurodegenerative lysosomal storage disorder characterized by intractable behavioral problems and developmental regression resulting in early death. It is caused by mutations in the SGSH gene, which encodes an enzyme called Heparan-N-sulfamidase necessary for heparan sulfate (“HS”) recycling in cells. The disrupted lysosomal degradation and resulting storage of HS and glycolipids such as gangliosides leads to severe neurodegeneration. MPS IIIA affects approximately 1 in 100,000 individuals and is inherited in an autosomal recessive pattern. There are currently no treatment options for patients.

CMT is a group of hereditary, degenerative nerve diseases that are caused by mutations in genes that produce proteins involved in the structure and function of either the peripheral nerve axon or the myelin sheath. CMT can cause degeneration of motor skills, resulting in muscle weakness, and limiting patients’ ability to walk or use their hands, and in some cases, can cause degeneration of sensory nerves, resulting in a reduced ability to feel heat, cold, and pain. CMT affects approximately 1 in every 2,500

individuals, while CMT type 1A, which is most often caused by an extra copy of the PMP22 gene, affects approximately 50,000 patients in the U.S. Most patients are diagnosed at infancy, while other patients develop symptoms at adolescence. Currently, there are no available treatment options.

Pompe disease is caused by mutation in the gene that codes for the enzyme acid alpha-glucosidase ("GAA"), which is responsible for metabolizing glycogen in lysosomes. The disease causes buildup of glycogen in the body's cells, which in certain organs and tissues, especially muscles, impairs ability to function normally. Pompe disease is progressive and often debilitating, disables the heart and skeletal muscles with muscle weakness worsening over time. It affects both sexes equally and is often fatal. Pompe disease affects an estimated 1 in approximately every 40,000 individuals.

Our Commercial Product

EXONDYS 51, our first commercial product, approved by the FDA on September 19, 2016, is indicated for the treatment of DMD in patients who have a confirmed mutation of the DMD gene that is amenable to exon 51 skipping EXONDYS 51 uses our PMO chemistry and exon-skipping technology to skip exon 51 of the dystrophin gene. PMO-based compounds are synthetic compounds that bind to complementary sequences of RNA by standard Watson-Crick nucleobase pairing. The two key structural differences between PMO-based compounds and naturally occurring RNA are that the PMO nucleobases are bound to synthetic morpholino rings instead of ribose rings, and the morpholino rings are linked by phosphorodiamidate groups instead of phosphodiester groups. Replacement of the negatively charged phosphodiester in RNA with the uncharged phosphorodiamidate group in PMO eliminates linkage ionization at physiological pH. Due to these modifications, PMO-based compounds are resistant to degradation by plasma and intracellular enzymes. Unlike the RNA-targeted technologies such as siRNAs and DNA gapmers, PMO-based compounds operate by steric blockade rather than by cellular enzymatic degradation to achieve their biological effects. Thus, PMOs use a fundamentally different mechanism from other RNA-targeted technologies.

We are in the process of assessing and conducting various EXONDYS 51 clinical trials, including studies that are required to comply with regulatory NDA and studies we need to conduct to comply with our post-marketing FDA requirements/commitments to verify and describe the clinical benefit of EXONDYS 51.

EXONDYS 51 targets the most frequent series of mutations that cause DMD. Approximately 13% of DMD patients are amenable to exon 51 skipping. For the years ended December 31, 2018, 2017, and 2016, the Company recorded net revenue of $301.0 million, $154.6 million, and $5.4 million, respectively, related to the sale of EXONDYS 51.

Our Pipeline

Golodirsen (SRP-4053) uses our PMO chemistry and exon-skipping technology to skip exon 53 of the DMD gene. Golodirsen is designed to bind to exon 53 of dystrophin pre-mRNA, resulting in exclusion, or "skipping", of this exon during mRNA processing in patients with genetic mutations that are amenable to exon 53 skipping. We are enrolling and dosing patients in ESSENCE (4045-301), our Phase 3 placebo controlled confirmatory trial in patients who have a confirmed mutation of the DMD gene that is amenable to exon 45 or 53 skipping using casimersen and golodirsen, respectively. Golodirsen is also being evaluated in a Phase 1/2 trial having two parts. Part I of the Phase 1/2 trial has been completed, and Part II, an open-label portion of the trial, is expected to be completed in 2019 (Study 4053-101). In September 2017, we announced positive results of an analysis that included biopsies of the bicep muscle at baseline and on-treatment at the Part II, Week 48 time point. The 4053-101 interim trial results demonstrated statistical significance on all primary and secondary biological endpoints. In December 2018, we completed the submission of our rolling NDA to the FDA seeking accelerated approval for golodirsen. The FDA accepted the NDA and granted priority review status for golodirsen with a targeted regulatory action date of August 19, 2019. The FDA also indicated that it does not intend to conduct an advisory board for golodirsen.

Casimersen (SRP-4045) uses our PMO chemistry and exon-skipping technology to skip exon 45 of the DMD gene. Casimersen is designed to bind to exon 45 of dystrophin pre-mRNA, resulting in exclusion, or "skipping", of this exon during mRNA processing in patients with genetic mutations that are amenable to exon 45 skipping. We are enrolling and dosing patients in ESSENCE, further described above. We have completed a dose titration portion (Phase 1) and the open-label portion (Phase 2) of a Sarepta sponsored Phase 1/2 the trial clinical trial studying casimersen (4045-101). We anticipate submitting an NDA to the FDA for casimersen in 2019 if we believe the results of an interim dystrophin analysis in the ESSENCE study are positive.

SRP-5051 uses our next-generation chemistry platform, PPMO, and our exon-skipping technology to skip exon 51 of the dystrophin gene. The PPMO technology features covalent attachment of a cell-penetrating peptide to a PMO with the goal of enhanced delivery into the cell. In pre-clinical research, our proprietary class of PPMO compounds demonstrated an increase in dystrophin production and a more durable response compared to PMO. In addition, PPMO treatment in non-human primates results in high levels of exon-skipping in skeletal, cardiac and smooth muscle tissues. Pre-clinical trials also indicate that PPMOs may require less frequent dosing than PMOs, and that PPMOs could potentially be tailored to reach other organs beyond muscle.

In the fourth quarter of 2017, we received clearance from the FDA and commenced a first-in-human, single ascending dose, trial for the treatment of DMD using SRP-5051 in patients who are amenable to exon 51 skipping. We expect to complete this trial in 2019.

SRP-9001 (micro-dystrophin gene therapy program), in collaboration with Nationwide, aims to express micro-dystrophin – a smaller but still functional version of dystrophin. A unique, engineered micro-dystrophin is used because naturally-occurring dystrophin is too large to fit in an AAV vector. SRP-9001 developed in collaboration with Nationwide employs the AAVrh74 vector, which is designed to be systemically and robustly delivered to skeletal, diaphragm and cardiac muscle without promiscuously crossing the blood brain barrier, which we believe makes it a strong candidate to treat peripheral neuromuscular diseases. An MHCK7 promoter was chosen for its ability to robustly express in the heart, which is critically important for patients with DMD, who typically die from pulmonary or cardiac complications. Lastly, the transgene was designed to maintain spectrin-like repeats 2 and 3, which has been reported to be critical to maintaining muscle force.

In the fourth quarter of 2017, an investigational new drug ("IND") application for the micro-dystrophin gene therapy program, in collaboration with Nationwide, was cleared by the FDA, and a Phase 1/2a clinical trial in individuals with DMD was initiated. On October 3, 2018, Nationwide presented what we believe to be positive updated results from the Phase 1/2a clinical trial in four individuals with DMD enrolled in the trial. In the fourth quarter of 2018, we commenced a placebo-controlled trial with the goal to establish the functional benefits of micro-dystrophin expressions. We plan to conduct a confirmatory trial using commercial supply of SRP-9001 by the end of 2019, pending regulatory feedback.

MYO-101. We are collaborating with Myonexus to develop gene therapy programs for various types of LGMDs. All the Myonexus programs use the AAVrh.74 vector, the same vector used in the micro-dystrophin gene therapy program, to transfect a restorative gene. The most advanced of Myonexus' product candidates, MYO-101, aims to treat LGMD2E, also known as beta-sarcoglycanopathy, a severe and debilitating form of LGMD characterized by progressive muscle fiber loss, inflammation and muscle fiber replacement with fat and fibrotic tissue. MYO-101 is designed to transfect a gene that codes for and restores beta-sarcoglycan protein with the goal of restoring the dystroglycan complex. MYO-101 has generated positive pre-clinical safety and efficacy data utilizing the AAVrh.74 vector. Myonexus commenced a Phase 1/2a trial of MYO-101 in the fourth quarter of 2018, and on February 27, 2019, we announced positive two-month data from the first three-patient cohort dosed in the MYO-101 trial.

GALGT2. An additional gene therapy program for DMD and other muscular dystrophies, also in collaboration with Nationwide, aims to express the enzyme GALGT2 from an AAV vector. We believe that GALGT2 modifies the dystrophin associated protein complex (DAPC) and up-regulates utrophin (a protein significantly homologous to dystrophin) to protect muscle from damage in the absence of dystrophin. We believe that the micro-dystrophin and GALGT2 technologies have the potential to treat all or nearly all DMD patients regardless of mutation.

In the fourth quarter of 2017, an IND application for GALGT2 was cleared by the FDA, and a Phase 1/2a clinical trial testing GALGT2 for the treatment of DMD was initiated.

LYS-SAF 302. We are collaborating with Lysogene S.A. ("Lysogene") to develop a gene therapy, LYS-SAF302, to treat MPS IIIA. LYS-SAF302 is an AAV-mediated gene therapy, the goal of which is to replace the faulty N-sulfoglucosamine sulfohydrolase ("SGSH") gene with a healthy copy of the gene. LYS-SAF302 employs the AAVrh10 virus, chosen for its ability to target the central nervous system ("CNS"). Proof-of-concept was established in MPS IIIA pre-clinical models demonstrating strong expression, broad distribution, and the ability of the compound to correct lysosomal storage defects by producing the missing enzyme. Safety data from an IND-enabling toxicity and a biodistribution Good Laboratory Practice ("GLP") trial showed that, at any dose level evaluated, LYS-SAF302 was not associated with unexpected mortality, change in clinical signs, body weight, behavior or macroscopic findings in the brain.

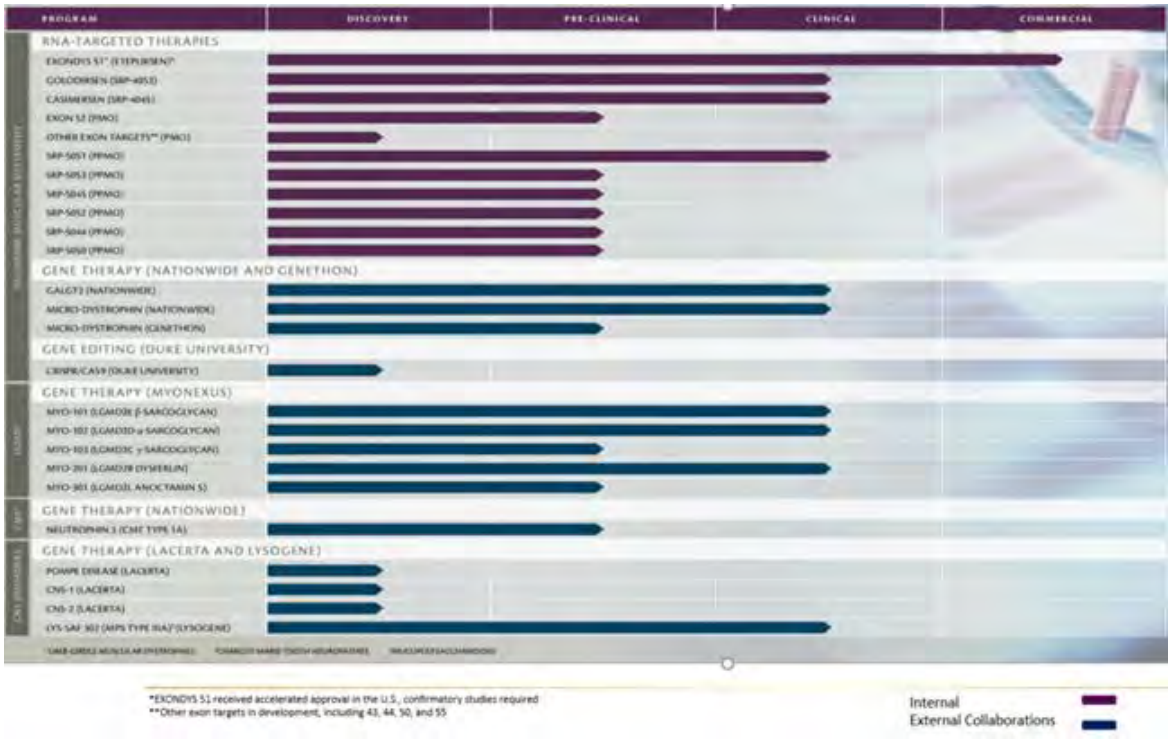
The first patient has been dosed in AAVance, a global Phase 2/3 clinical trial of LYS-SAF302, aiming at evaluating the effectiveness of a one-time delivery of a AAVrh10 virus carrying the N-SGSH gene.

Neutrophin 3 (CMT Type 1A). A gene therapy program in collaboration with Nationwide that aims to express neurotrophin 3 ("NT-3") encoding the NTF3 gene to treat CMT neuropathies, including CMT type 1A. We believe that the delivery of NT-3 may have applicability to other sub-types of CMT in addition to other neuropathies and muscle-wasting diseases. Pre-clinical research has shown the ability of the NT-3 gene construct to regenerate nerves. Further pre-clinical research is under way to explore its potential. A clinical trial to test NT-3 gene therapy is planned to commence dosing in 2019 for CMT type 1A, pending regulatory feedback. We believe that the delivery of NT-3 may have applicability to other sub-types of CMT in addition to other muscle-wasting diseases.

Programs in Collaboration with Lacerta. Our collaboration with Lacerta Therapeutics, Inc. ("Lacerta") utilizes proprietary AAV capsid variants and a scalable vector manufacturing platform to develop treatments for central nervous system and lysosomal storage diseases. The lead candidate, still in discovery phase, is a gene therapy approach with a novel AAV variant for the treatment of Pompe disease.

CRSPR/Cas9. We are exploring, in collaboration with Duke University, the gene-editing technology CRSPR/Cas9 that aims to restore dystrophin expression by removing or "excising" exons directly from the dystrophin gene to correct out-of-frame mutations. CRSPR/Cas9 technology can also potentially be used to fix stop codon mutations in the dystrophin gene so that dystrophin can be translated to a function protein. This program is in the discovery phase.

The chart below summarizes the status of our more advanced programs, including those with our strategic partners:



Manufacturing, Supply and Distribution

We have developed proprietary state-of-the-art Chemistry, Manufacturing and Controls ("CMC") and manufacturing capabilities that allow synthesis and purification of our product candidates to support both clinical development as well as commercialization. Our current main focus in manufacturing is to continue scaling up production of our PMO-based therapies and optimizing manufacturing for PPMO and gene therapy-based product candidates. We have entered into certain manufacturing and supply arrangements with third-party suppliers which will in part utilize these capabilities to support production of certain of our product candidates and their components. In 2017, we opened a facility in Andover, Massachusetts, which significantly enhances our research and development manufacturing capabilities. However, we currently do not have internal large scale Good Manufacturing Practices ("GMP") manufacturing capabilities to produce our product and product candidates for commercial and/or clinical use. For our current and future manufacturing needs, we have entered into supply agreements with specialized contract manufacturing organizations (each a "CMO") to produce custom raw materials, the active pharmaceutical Ingredients ("APIs") and finished goods for our product candidates. All of our CMO partners have extensive technical expertise, GMP experience and experience manufacturing our specific technology.

For our commercial DMD program, we have commenced work with our existing manufacturers to increase product capacity from mid-scale to large-scale. While there are a limited number of companies that can produce raw materials and APIs in the quantities and with the quality and purity that we require for EXONDYS 51, based on our diligence to date, we believe our current network of manufacturing partners are able to fulfill these requirements, and are capable of continuing to expand capacity as needed. Additionally, we have, and will continue to evaluate further relationships with additional suppliers to increase overall capacity as well as further reduce risks associated with reliance on a limited number of suppliers for manufacturing.

EXONDYS 51 is distributed in the U.S. through a limited network of home infusion specialty pharmacy providers that deliver the medication to patients and a specialty distributor that distributes EXONDYS 51 to hospitals and hospital outpatient clinics. With respect to the pre-commercial distribution of eteplirsen to patients outside of the U.S., we have contracted with third party distributors and service providers to distribute eteplirsen in certain countries on a named patient basis and through our ex-U.S. early

access programs (“EAP”). We plan to continue building out our network for commercial distribution in jurisdictions in which eteplirsen is approved.

Our gene therapy manufacturing capabilities have been greatly enhanced through partnerships with Brammer Bio LLC (“Brammer”), Paragon Bioservices, Inc. (“Paragon”) and Aldevron LLC (“Aldevron”). We have adopted a hybrid manufacturing strategy in which we are building internal manufacturing expertise relative to all aspects of AAV-based manufacturing, including gene therapy and gene editing supply, while closely partnering with first-in-class manufacturing partners to expedite development and commercialization of our gene therapy programs. The partnership with Brammer will support our clinical and commercial manufacturing capacity for our micro-dystrophin DMD gene therapy programs and LGMD programs, while also acting as a manufacturing platform for potential future gene therapy programs. The collaboration integrates process development, clinical production and testing, and commercial manufacturing. Our partnership with Paragon will provide us access to additional commercial manufacturing capacity for our micro-dystrophin DMD gene therapy program, as well as a manufacturing platform for future gene therapy programs, such as LGMD. Aldevron will provide GMP-grade plasmid for our micro-dystrophin DMD gene therapy program and LGMD programs, as well as plasmid source material for future gene therapy programs, such as CMT, MPS IIIA, Pompe and other CNS diseases.

Manufacturers and suppliers of product candidates are subject to the FDA’s current GMP (“cGMP”) requirements and other rules and regulations prescribed by foreign regulatory authorities. We depend on our third-party partners for continued compliance with cGMP requirements and applicable foreign standards.

Material Agreements

We believe that our RNA-targeted and gene therapy technologies could be broadly applicable for the potential development of pharmaceutical products in many therapeutic areas. To further exploit our core technologies, we have and may continue to enter into research, development or commercialization alliances with universities, hospitals, independent research centers, non-profit organizations, pharmaceutical and biotechnology companies and other entities for specific molecular targets or selected disease indications. We may also selectively pursue opportunities to access certain intellectual property rights that complement our internal portfolio through license agreements or other arrangements.

Myonexus

On May 3, 2018, we purchased from Myonexus, a privately-held Delaware corporation, a warrant to purchase common stock of Myonexus (the “Warrant”), which, in combination with amendments to the Myonexus certificate of incorporation, provides us with an exclusive option (the “Option”) to acquire Myonexus. In consideration for the Warrant, we made an up-front payment of $60.0 million to Myonexus. On February 26, 2019, we delivered to Myonexus an exercise notice (the “Exercise Notice”) stating our intention to exercise the Option.

Prior to the delivery of the Exercise Notice, on February 26, 2019, we entered into a letter agreement (the “Letter Agreement”) with Myonexus to amend certain terms of the Warrant to (i) reduce the payment price we would be required to make at the closing of the Option exercise from $200.0 million to $165.0 million, subject to certain adjustments (the “Warrant Exercise Price”), and (ii) terminate our obligation to pay any development milestone payments that have yet to be earned under the Warrant and pay Myonexus shareholders an additional amount in recognition of amounts Myonexus expended toward the achievement of those milestones, agreed for this purpose to be $6.0 million, to be paid upon exercise of the Option. Our obligation to make contingent payments to the Myonexus’ former shareholders following the exercise of the Option upon achievement of a threshold amount of net sales of Myonexus products and the receipt and subsequent sale of a priority review voucher with respect to a Myonexus product will remain unchanged.

We retain the right to terminate the Warrant at any time prior to the closing of the Option exercise, which is expected to occur at the end of our first fiscal quarter ending March 31, 2019, subject to the expiration or termination of the waiting period under the Hart-Scott-Rodino Antitrust Improvements Act of 1976, as amended.

BioMarin

License Agreement

On July 17, 2017, Sarepta Therapeutics, Inc. and Sarepta International C.V. (collectively, “Sarepta”) and BioMarin Leiden Holding BV, BioMarin Nederlands BV and BioMarin Technologies BV (collectively, “BioMarin”) executed a License Agreement (the “License Agreement”), pursuant to which BioMarin granted Sarepta a royalty-bearing, worldwide license under patent rights (“Licensed Patents”) and know-how (“Licensed Know-How”) controlled by BioMarin with respect to BioMarin’s DMD program, which are potentially necessary or useful for the treatment of DMD, to practice and exploit the Licensed Patents and Licensed Know-How in all fields of use and for all purposes, including to develop and commercialize antisense oligonucleotide products that target one or more exons of the dystrophin gene to induce exon skipping, including eteplirsen (collectively, the “Products”).

The license granted by BioMarin to Sarepta under the terms of the License Agreement is exclusive, even as to BioMarin, with respect to the Licensed Patents, and is non-exclusive with respect to Licensed Know-How. Under the License Agreement, BioMarin has the option to convert the exclusive license under the Licensed Patents into a co-exclusive license (co-exclusive with BioMarin) ("BioMarin Co-Exclusive Option").

Under the terms of the License Agreement, Sarepta is required to pay BioMarin an up-front payment of $15.0 million, and BioMarin will be eligible to receive up to $20.0 million from Sarepta per dystrophin gene exon (other than exon 51) targeted by one or more Products in specified regulatory milestones, as well as an additional $10.0 million milestone, payable following the regulatory approval of eteplirsen by the European Medicines Agency in the EU ("EMA"). BioMarin will also be eligible to receive $15.0 million from Sarepta upon the achievement of $650 million in sales, as well as royalties segmented by specified geographic markets, in some jurisdictions dependent on the existence of a patent, ranging from four (4) to eight (8) percentages of net sales on a product-by-product and country-by-country basis.

Milestones and royalties are payable with respect to eteplirsen (an exon 51 skipping Product), casimersen (an exon 45 skipping Product), golodirsen (an exon 53 skipping Product) and other Products. For eteplirsen, casimersen and golodirsen, the royalty term will expire upon the end of 2023 in the U.S., upon September 30, 2024 in the European Union ("EU") and no later than September 30, 2024 in other countries provided certain conditions are met. For Products other than exon 45 skipping Products, exon 51 skipping Products and exon 53 skipping Products, the royalty term will end on a country-by country basis upon expiration of granted Licensed Patents covering the applicable Product. The royalties for all Products are subject to reduction upon BioMarin's exercise of the BioMarin Co-Exclusive Option. All royalties are subject to further potential reductions, including for generic competition and, under specified conditions, for a specified portion of payments that Sarepta may become required to pay under third-party license agreements, subject to a maximum royalty reduction.

Unless earlier terminated, the License Agreement will expire upon the expiration of the last-to-expire royalty term. Either party may terminate the License Agreement in the event of the other party's uncured material breach. BioMarin may also terminate the License Agreement on a Licensed Patent-by-Licensed Patent basis under specified circumstances relating to patent challenges by Sarepta.

Settlement Agreement

On July 17, 2017, Sarepta and The University of Western Australia on the one hand, and the BioMarin Parties and Academisch Ziekenhuis Leiden ("AZL") on the other hand (collectively, the "Settlement Parties"), executed a Settlement Agreement pursuant to which all legal actions in the U.S. and certain legal actions in Europe (the "Actions") would be stopped or withdrawn as between the Settlement Parties. Specifically, the terms of the Settlement Agreement require that existing efforts pursuing ongoing litigation and opposition proceedings would be stopped as between the Settlement Parties, and the Settlement Parties would cooperate to withdraw the Actions before the European Patent Office (except for actions involving third parties), the U.S. Patent and Trademark Office, the U.S. Court of Appeals for the Federal Circuit and the High Court of Justice of England and Wales, except for the cross-appeal of the Interlocutory Decision of the Opposition Division dated April 15, 2013 of the European Patent Office of EP 1619249B1 ("EP '249 Appeal") in which Sarepta will withdraw its appeal and BioMarin/AZL will continue with its appeal with Sarepta having oversight of the continued appeal by BioMarin/AZL.

Additionally, under the terms of the Settlement Agreement, the Settlement Parties agree to release each other and the customers, end-users, agents, suppliers, distributors, resellers, contractors, consultants, services and partners of Sarepta or BioMarin (as applicable) from claims and damages related to (i) the patent rights controlled by the releasing party that are involved in the Actions, (ii) with respect to Sarepta and UWA, its patent rights related to the patent rights involved in the Actions, and (iii) with respect to BioMarin and AZL, all of the Licensed Patents and Licensed Know-How.

Under the terms of the Settlement Agreement, Sarepta made an upfront payment of $20.0 million to BioMarin.

University of Western Australia

In April 2013, we entered into an agreement with University of Western Australia ("UWA") under which an existing exclusive license agreement between the two parties was amended and restated (the "Amended and Restated UWA License Agreement"). The Amended and Restated UWA License Agreement grants us specific rights to the treatment of DMD by inducing the skipping of certain exons. EXONDYS 51, golodirsen and casimersen fall under the scope of the license agreement. Under the Amended and Restated UWA License Agreement, we may be required to make payments of up to $6.0 million in aggregate to UWA based on the successful achievement of certain development and regulatory milestones relating to EXONDYS 51 and up to five additional product candidates. As of the date of this Annual Report, $2.0 million of the $6.0 million development and regulatory milestone payments has been made. We may also be obligated to make payments to UWA of up to $20.0 million upon the achievement of certain sales milestones. Additionally, we may be required to pay a low-single-digit percentage royalty on net sales of

products covered by issued patents licensed from UWA during the term of the Amended and Restated UWA License Agreement. However, we have the option to purchase future royalties up-front. Under this option, prior to the First Amendment (defined below), we could be required to make a one-time royalty payment of $30.0 million to UWA.

In June 2016, we entered into the first amendment to the Amended and Restated UWA License Agreement (the "First Amendment") with UWA. Under the First Amendment, we made an up-front payment of $7.0 million to UWA upon execution of the First Amendment. Under the terms of the First Amendment, UWA has waived rights to certain royalties and amended the timing of certain other royalty payments under the Amended and Restated UWA License Agreement, including lowering the one-time royalty payment that is due by us upon exercise of the option to purchase future royalties up-front. Upon exercise of the option to purchase future royalties up-front, we will be obligated to make a $23.0 million payment to UWA. Additionally, we would still be obligated to make up to $20.0 million in payments to UWA upon achievement of certain sales milestones.

Currently, the latest date on which an issued patent covered by our agreement with UWA expires is November 2030 (excluding any patent term extension, supplemental protection certificate or pediatric extensions that may be available); however, patents granted from pending patent applications could result in a later expiration date.

Strategic Alliances

In connection with our multi-front battle against DMD and other rare neuromuscular diseases, we have entered into a number of partnering opportunities. We believe these collaborations, taken along with our own programs, represent a comprehensive approach to treating these rare neuromuscular diseases.

Nationwide Children's Hospital

In December 2015, we entered into an exclusive license agreement with Nationwide to acquire exclusive rights to its GALGT2 gene therapy program. This program explores the potential surrogate gene therapy approach to DMD. In the fourth quarter of 2017, the IND application for the GALGT2 gene therapy program was cleared by the FDA, and a Phase 1/2a clinical trial in individuals with DMD was initiated.

In addition, in December 2016, we entered into an exclusive option agreement with Nationwide to acquire exclusive rights to their micro-dystrophin gene therapy program as well as a sponsored research agreement to conduct pre-IND research and conduct the first clinical trial with the lead micro-dystrophin gene therapy. In October 2018, we exercised our exclusive license option and an option under the sponsored research agreement and entered into an exclusive license agreement with Nationwide to acquire exclusive rights to their micro-dystrophin gene therapy program. On October 3, 2018, Nationwide presented positive updated results from our Phase 1/2a clinical trial testing SRP-9001 in four individuals with DMD enrolled in the trial.

Furthermore, in October 2018, we entered into an exclusive option agreement with Nationwide to acquire exclusive rights to their NT-3 gene therapy program for the treatment of certain CMT neuropathy subtypes, including CMT Type 1A. The option agreement contains pre-determined economic terms for the exclusive license to be entered into upon us exercising our option. The clinical trial to test NT-3 gene therapy is planned to commence dosing in 2019 for CMT type 1A, pending regulatory feedback.

Lysogene

In October 2018, we entered into a license agreement with Lysogene, a gene therapy company focused on the treatment of orphan diseases of the CNS, for the development of a gene therapy, LYS-SAF302, to treat MPS IIIA. Concomitantly, we also entered into an option with Lysogene to acquire an exclusive license to an additional CNS-targeted gene therapy candidate. Lysogene is responsible for completion of the pivotal trial for LYS-SAF302. We have exclusive commercial rights to LYS-SAF302 and exclusive option rights for the additional CNS-targeted gene therapy program in the United States and all territories outside of Europe, and Lysogene will retain exclusive commercial rights to each program in Europe. We will be responsible for global manufacturing of LYS-SAF302 and will supply Lysogene for its territory. If all milestones are met, we may be required to pay up to $130.8 million in development and commercial milestones and tiered royalties upon commercialization.

Lacerta

In August 2018, we entered into a license and option agreement with Lacerta, a gene therapy company using a constellation of proprietary AAV vector technologies to develop treatments for CNS-targeted and lysosomal storage diseases. Under this agreement, we have an exclusive license to Lacerta's gene therapy candidate for Pompe disease and exclusive options to obtain an exclusive license for two additional gene therapy candidates. Lacerta will manage the majority of pre-clinical development for the Pompe candidate while we will lead clinical development and commercialization. We will owe development and sales-based milestones to Lacerta and pay single-digit royalties on net sales.

Duke University

In October 2017, we entered into a sponsored research and exclusive option agreement with Duke University, granting us an exclusive option to an exclusive license to intellectual property and technology related to certain CRISPR/Cas9 technology developed in the laboratory of Charles A. Gersbach, Ph.D. The underlying premise of Dr. Gersbach's approach is to restore dystrophin expression by removing or "excising" exons from the dystrophin gene. This includes a strategy to excise exons potentially enabling treatment for a majority of the DMD patient population.

Genethon

In May 2017, we entered into a gene therapy research collaboration and option agreement with Genethon to jointly develop micro-dystrophin gene therapy products for the treatment of DMD. Under the terms of the collaboration, Genethon is responsible for the early development work, and we have the option to co-develop Genethon's micro-dystrophin program, which includes exclusive U.S. commercial rights.

Charley's Fund Agreement

In October 2007, Charley's Fund, Inc. ("Charley's Fund"), a nonprofit organization that funds drug development and discovery initiatives specific to DMD, awarded us a research grant of approximately $2.5 million and, in May 2009, the grant authorization was increased to a total of $5.0 million. Pursuant to the related sponsored research agreement, the grant was provided to support the development of product candidates related to exon 50 skipping using our proprietary exon-skipping technologies. As of December 31, 2017, Charley's Fund had made payments of approximately $3.4 million to us and no payments have been made to us since this date. Revenue associated with this research and development arrangement is recognized based on the proportional performance method. To date, we have recognized approximately $0.1 million as revenue. We have deferred $3.3 million of previous receipts, which are anticipated to be recognized as revenue upon resolution of outstanding performance obligations.

Previously, we noted unexpected toxicology findings in the kidney as part of our series of pre-clinical trials for AVI-5038, our PMO-based candidate designed for the treatment of individuals with DMD who have an error in the gene coding for dystrophin that can be treated by skipping exon 50. We have conducted additional pre-clinical trials and have not alleviated the toxicity problem. Pursuant to the terms of our agreement with Charley's Fund, the receipt of additional funds is tied to the satisfaction of certain clinical milestones. Because of the toxicity issues with AVI-5038, satisfaction of the additional milestones under the agreement is unlikely and we do not expect to receive any additional funds from Charley's Fund.

Summit

On October 3, 2016, we entered into an exclusive Collaboration and License Agreement (the "Collaboration Agreement") with (Oxford) Ltd. ("Summit"), which grants us the exclusive right to commercialize products in Summit's utrophin modulator pipeline in the EU, Switzerland, Norway, Iceland, Turkey and the Commonwealth of Independent States. On June 27, 2018, Summit announced that it decided to discontinue the development of ezutromid after reviewing the top-line results from its Phase 2 trial.

Patents and Proprietary Rights

Our success depends in part upon our ability to obtain and maintain exclusivity for our product, product candidates and platform technologies. We typically rely on a combination of patent protection and regulatory exclusivity to maintain exclusivity for our product and product candidates, whereas exclusivity for our platform technologies is generally based on patent protection and trade secret protection. In addition to patent protection, regulatory exclusivity, and trade secret protection, we also protect our product, product candidates and platform technologies with copyrights, trademarks, and contractual protections.

We actively seek patent protection for our product candidates and certain of our proprietary technologies by filing patent applications in the U.S. and other countries as appropriate. These patent applications are directed to various inventions, including, but not limited to, active ingredients, pharmaceutical formulations, methods of use, and manufacturing methods. In addition, we actively acquire exclusive rights to third party patents and patent applications to protect our in-licensed product candidates and corresponding platform technologies.

We do not have patents or patent applications in every jurisdiction where there is a potential commercial market for our product candidates. For each of our programs, our decision to seek patent protection in specific foreign markets, in addition to the U.S., is based on many factors, including:

- our available resources;
- the number and types of patents already filed or pending;

- the likelihood of success of the product candidate;
- the size of the commercial market;
- the presence of a potential competitor in the market; and
- whether the legal authorities in the market effectively enforce patent rights.

We continually evaluate our patent portfolio and patent strategy and believe our owned and licensed patents and patent applications provide us with a competitive advantage; however, if markets where we do not have patents or patent applications become commercially important, our business may be adversely affected. A discussion of certain risks and uncertainties that may affect our patent position, regulatory exclusivities and other proprietary rights is set forth in Item 1A. Risk Factors included in this report, and a discussion of legal proceedings related to the key patents protecting our product and product candidates is set forth below in the footnotes that immediately below the tables in this section.

Certain of our product candidates are in therapeutic areas that have been the subject of many years of extensive research and development by academic organizations and third parties who may control patents or other intellectual property that they might assert against us, should one or more of our product candidates in these therapeutic areas succeed in obtaining regulatory approval and thereafter be commercialized. We continually evaluate the intellectual property rights of others in these areas in order to determine whether a claim of infringement may be made by others against us. Should we determine that a third party has intellectual property rights that could impact our ability to freely market a compound, we consider a number of factors in determining how best to prepare for the commercialization of any such product candidate. In making this determination we consider, among other things, the stage of development of our product candidate, the anticipated date of first regulatory approval, whether we believe the intellectual property rights of others are valid, whether we believe we infringe the intellectual property rights of others, whether a license is available upon commercially reasonable terms, whether we will seek to challenge the intellectual property rights of others, the term of the rights, and the likelihood of and liability resulting from an adverse outcome should we be found to infringe the intellectual property rights of others.

Currently, U.S. patents, as well as most foreign patents, are generally effective for 20 years from the date the earliest regular application was filed. In some countries, the patent term may be extended to recapture a portion of the term lost during regulatory review of the claimed therapeutic. For example, in the U.S., under the Drug Price Competition and Patent Term Restoration Act of 1984, commonly known as the Hatch-Waxman Act, a patent that covers an FDA-approved drug may be eligible for patent term extension (for up to 5 years, but not beyond a total of 14 years from the date of product approval) as compensation for patent term lost during the FDA regulatory review process. In the U.S., only one patent may be extended for any product based on FDA delay. In addition to patent term extension, patents in the U.S. may be granted additional term due to delays at the U.S. Patent and Trademark Office ("USPTO") during prosecution of a patent application. We actively strive to maximize the potential for patent protection for our product and product candidates in accordance with the law.

Key Patents & Regulatory Exclusivities

Our product candidates and our technologies are primarily protected by composition of matter and use patents and patent applications. A summary of granted composition of matter and/or use patents that we own or control in the U.S. and Europe, which cover our product and late-stage clinical product candidates, is provided below. To the extent the product or product candidate indicated above the tables that immediately follow the name of such product is covered by a patent that is licensed to Sarepta, we may owe milestones and/or royalties to the indicated licensor in connection with the development and/or commercial sale of the product or product candidate.

Exhibit 2

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

WILSON WOLF MANUFACTURING CORPORATION, Plaintiff, vs. SAREPTA THERAPEUTICS, INC., Defendant.	Case No.: ~~_____~~19-2316-RGA **JURY TRIAL DEMANDED**

FIRST AMENDED COMPLAINT FOR PATENT INFRINGEMENT

Plaintiff Wilson Wolf Manufacturing Corporation ("Wilson Wolf"), for its First Amended Complaint against Defendant Sarepta Therapeutics, Inc., ("Sarepta"), states and alleges as follows:

THE PARTIES

1. Plaintiff Wilson Wolf is a corporation organized under the laws of the State of Minnesota, with its principal place of business in New Brighton, Minnesota.

2. Upon information and belief, Defendant Sarepta is a corporation organized under the laws of the state of Delaware with its designated registered agent located at 251 Little Falls Drive, Wilmington, Delaware.

JURISDICTION AND VENUE

3. This Court has jurisdiction over the subject matter of this action pursuant to 28 U.S.C. §§ 1331 and 1338(a), as this is an action for patent infringement arising under the patent laws of the United States, 35 U.S.C. § 1 *et seq.*

4. This Court has personal jurisdiction over Sarepta because it is a Delaware corporation and essentially "at home" in this District. Further, Sarepta has caused tortious injury

to Wilson Wolf through its acts of patent infringement, and on information and belief, regularly does or solicits business, or engages in a persistent course of conduct in this District or derives substantial revenue from things used or consumed in this District.

5. Venue is proper in this District under 28 U.S.C. §§ 1391(b)(1) and 1400(b), because Sarepta is incorporated under the laws of Delaware and has its designated registered agent located in this District, and therefore "resides" in this District within the meaning of those statutes.

FACTUAL ALLEGATIONS

I. Wilson Wolf Develops Innovative Devices and Methods to Grow Cells

6. Wilson Wolf is a leader in the design of innovative devices and methods to grow cells in a laboratory environment.

7. The process of growing cells in a laboratory environment is called "culturing" cells. Innovative cell culture technology allows a lab to grow cells in greater volume, to grow cells faster, and to grow cells with lower risks of contamination.

8. Cell culture technology is critical to many fields, including biology and medicine. Cell culture technology is important, for example, when cells are grown for purposes of scientific investigation and research. Scientists grow cells to study how cancer develops and evolves. In contrast, doctors grow cells to diagnose cancer in a particular patient, and to select and calibrate treatment options for that patient. Cell culture technology is also used when cells are grown for commercial production of medications. For example, drug companies grow cells that produce monoclonal antibodies and other proteins that are used to treat diseases. These medications produced by cells are sometimes referred to as "biopharmaceuticals."

9. Cells in culture can also be used to replicate specially engineered "viral vectors" in large quantities. These viral vectors can be introduced into a patient to treat genetic disorders. This is known as "gene therapy."

10. Another rapidly-expanding field of cell culture technology involves the production of cells which can *themselves* be used to treat diseases. Some cells naturally occurring as part of the body's immune system are very good at fighting illnesses. For example, certain lymphocytes naturally infiltrate tumors and attack cancerous cells, while "natural killer" cells help the body fight viral infections. Unfortunately, the patient's body typically does not have enough of these cells to mount an effective immune system response to overcome the illness. Using cell culture techniques, a small quantity of these cells from the patient can be expanded into an "army" of cells that can be reintroduced to the patient to support recovery.

11. Wilson Wolf has developed devices and methods that have revolutionized the process of culturing cells.

12. In order to grow, cells need food and oxygen. To provide food, cells are typically grown in a liquid medium that contains nutrients for the cells. To provide oxygen, many devices rely on the oxygen in the gas residing above the liquid medium. Oxygen enters the liquid medium through the gas-liquid interface and is available to the cells.

13. Prior to Wilson Wolf's innovations, the conventional wisdom was that nutrients do not move very far in the liquid medium. As a result, cells only benefit from liquid medium very close to them; excess medium is wasted, and medium is very expensive. Based on that conventional wisdom, cells were typically being grown in flasks with a very thin (2-3 mm) layer of liquid medium; the vast majority of the flask contained no medium and no cells, wasting a significant amount of space. Also according to conventional wisdom, oxygen could only travel a short way into the liquid medium. If a flask contained more than a very thin layer of liquid medium, the medium would suffocate the cells.

14. The traditionally shallow depth of liquid medium led to inefficient use of space. For example, one manufacturer recommends a working volume of 0.2 mL to 0.3 mL per square centimeter of cell growth surface area in the cell culture flask. For a standard 225 cm^2 flask with 850 mL of total volume, the recommended working volume is 45 mL to 67.5 mL. With a recommended working volume of 45 mL to 67.5 mL, only a small fraction of the space that the flask occupies is being used to grow cells. The remaining space is just gas. This wasted space above the thin layer of liquid medium is often referred to as "head space."

15. The image below illustrates the traditional shallow depth of medium in a cell culture flask. The liquid medium is the thin red/orange layer in the bottom of the flask. The flask is mostly empty. The empty space above the thin layer of liquid medium is the headspace.

16. The traditional limits on the amount of liquid medium per flask meant that one had to culture cells in multiple flasks in order to obtain a given volume of culture. For example, to obtain a 1000 mL volume of culture, one would need to culture cells in 15 to 22 T-225 cm^2 flasks with a working volume of 45 mL to 67.5 mL each. The requirement that 15 to 22 devices be fed and monitored increases labor costs and contamination risks.

17. The inefficient use of space in a cell culture flask is compounded by the fact that cells are typically cultured in an incubator. The incubator provides a controlled temperature and gas environment. Incubator space is limited. And only so many flasks can fit within a given volume of incubator space. Inefficient use of flask space therefore leads to inefficient use of incubator space. Based on conventional wisdom about medium thickness, decades of cell culture devices and methods made inefficient used of flask and incubator space. As a result, the process of culturing cells was slower, more cumbersome, and more prone to contamination than necessary.

18. Wilson Wolf challenged the conventional wisdom and developed devices and methods that grew more cells, in less space, with less labor and lower risk of contamination. Wilson Wolf challenged the conventional wisdom in at least two related ways. First, instead of

having cells “breath” through a thin layer of liquid medium, Wilson Wolf had cells “breath” through a gas permeable membrane. With gas permeable material, instead of relying on the headspace within the device as a source of oxygen, cells can get oxygen from outside the device. This eliminated the need for headspace within the device. Second, Wilson Wolf found that nutrients and oxygen could move further in the medium than the conventional wisdom taught. This eliminated the design constraint imposed by the conventional wisdom that the liquid medium should be confined to a thin layer above the cells.

19. By using these insights, Wilson Wolf pioneered several new device designs and cell culture methods. In one design, a device with a single gas-permeable growth surface could support far more medium than taught by the conventional wisdom, allowing cell growth to proceed for a longer time before replenishing the medium. In another design, multiple growth surfaces could be stacked in a single device filled with medium, increasing the number of cells grown in a given volume of space. Other designs combined multiple growth surfaces with more medium than taught by the conventional wisdom. Wilson Wolf has been awarded several U.S. patents for its innovative cell culture devices and methods, including the patents in suit.

II. Wilson Wolf's Asserted Patents

20. Wilson Wolf owns U.S. Patent No. 9,441,192 ("the '192 Patent"), entitled "Cell culture methods and devices utilizing gas permeable materials," which issued on September 13, 2016. A copy of the '192 Patent is attached as Exhibit A.

21. Independent claim 1 of the '192 Patent is set forth below:

> 1. A method of culturing cells comprising:
>
> adding medium and animal cells into a static cell culture device that is not compartmentalized by a semi-permeable membrane, at least a portion of said cell culture device is comprised at least in part of a non porous gas permeable material, ambient gas is in contact with at least a portion of said gas permeable material, and
>
> placing said cell culture device in a cell culture location that includes ambient gas at a composition suitable for animal cell culture, wherein said cell culture device is oriented in a position such that at least a portion of said cells reside upon at least a portion of said gas permeable material, the uppermost location of said medium is elevated beyond 2.0 cm from the lowermost location of said medium, and said device is in a state of static cell culture.

22. Wilson Wolf owns U.S. Patent No. 8,697,443 ("the '443 Patent"), entitled "Cell culture methods and devices utilizing gas permeable materials," which issued April 15, 2014. A copy of the '443 Patent is attached hereto as Exhibit B.

23. Independent claim 26 of the '443 Patent is set forth below.

> 26. A method of culturing cells in a cell culture device comprised at least in part of a gas permeable material and including at least one access port and including at least two scaffolds, the method comprising:
>
> a) adding cells and a volume of liquid medium into said cell culture device;
>
> b) orienting said cell culture device into an inoculation position such that said scaffolds reside at different elevations within said cell culture device;
>
> c) allowing cells to settle upon said scaffolds;
>
> d) adding enough liquid medium to prevent a unique gas-liquid interface from forming directly above at least one scaffold when the device is oriented in the

inoculation position and to have at least a portion of the liquid medium in contact with at least a portion of said gas permeable material;

e) placing the cell culture device in a cell culture location that includes ambient gas at a composition suitable for cell culture, said ambient gas making contact with said gas permeable material; and

f) not perfusing said liquid medium when said device is in said cell culture location.

III. Sarepta Infringes Wilson Wolf's Patents

24. Sarepta has infringed the patents in suit through its use of cells and/or cell-derived products including viral vectors manufactured using the Corning HYPERStack cell culture device. Such cells and cell-derived products are products made by a process patented in the United States, within the meaning of 35 U.S.C. § 271(g). The HYPERStack is a multiple-shelf device that uses gas-permeable material to oxygenate cells. In use, the device is filled with liquid medium.

25. The processes and methods patented by the '192 and '443 Patents, as well as products, such as the HYPERStack, that enable the use of these patented processes and methods, are research tools that are used in laboratories and manufacturing facilities in the development of cells and cell-derived products.

26. The processes and methods patented by the '192 and '443 Patents are not subject to any regulatory approval process that applies to the cells and cell-derived products that are developed using them.

27. As research tools, the processes and methods patented by the '192 and '443 Patents and the products that enable the use of these patented processes and methods do not constitute "patented inventions" within the meaning of 35 U.S.C. § 271(e)(1).

~~25.~~28. A 2019 Sarepta presentation entitled "A New Era of Medicine is Upon Us," reflects that one or more Sarepta products have been manufactured using the HYPERStack. See Exhibit C. In an earnings call for the third quarter of 2019, Doug Ingraham, Sarepta's president and CEO

stated that Sarepta's SRP-9001 product was made using Corning HYPERStacks. See Exhibit D, at 14 (excerpts from call transcript).

29. On information and belief, although some batches of SRP-9001 were manufactured for use by Sarepta in connection with submissions to the FDA, other batches of SRP-9001 were not manufactured for use by Sarepta for FDA purposes, and were instead used for other business purposes.

30. While some of Sarepta's infringement was strictly to generate infringement for the FDA, some of its infringement was for both FDA filings and other non-FDA purposes, and some of their infringement was solely for non-FDA purposes. For example, Sarepta had some batches of such products manufactured using Wilson Wolf's patented processes and methods for use to develop, improve, and optimize its manufacturing process for commercialization purposes. Sarepta also had some batches of such products manufactured using Wilson Wolf's patented process and methods for use in manufacturing capacity development and yield optimization for purposes of commercialization of the SRP-9001 product.

31. Even while conducting its clinical trials of SRP-9001, Sarepta moved forward in anticipation of commercialization of that product. For example, in 2018 Sarepta entered into a "manufacturing partnership" with Brammer Bio to build manufacturing capacity for the SRP-9001 product. *See* Exhibit F (Sarepta Press Release). The arrangement with Brammer Bio was designed to "integrate process development, clinical production and testing, and commercial manufacturing with the goal of bringing micro-dystrophin gene therapies to the patient community urgently and in sufficient supply." *Id.*

32. In 2019, Sarepta entered into a license agreement with Roche for commercialization of the SRP-9001 product outside of the U.S. that has been described as the single biggest such

license in biopharma history. Roche agreed to pay more than $1.1 billion up front for the commercial rights to SRP-9001 outside of the United States. The manufacture of Sarepta's SRP-9001 product using Wilson Wolf's patented processes and methods supported that commercialization agreement.

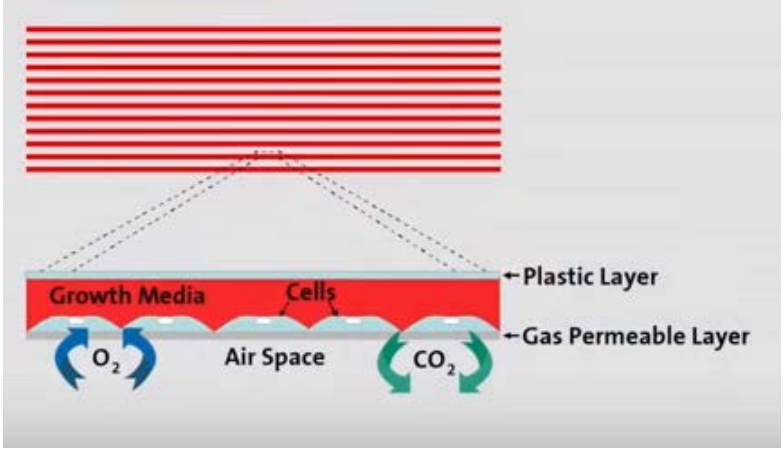
33. The manufacture of some of Sarepta's SRP-9001 product using Wilson Wolf's patented processes and methods was done to assist in commercialization of the product, and was not done to create information for FDA submissions. Sarepta itself stated that it developed its program to "expedite development and commercialization" of its gene therapy products, including SRP-9001. *See* Exhibit G at 9.

34. Because the manufacture of some of Sarepta's SRP-9001 product using Wilson Wolf's patented processes and methods was done to advance and support commercialization of the product, and was not done to create information for FDA submissions, Sarepta's use of that product falls outside of the Safe Harbor of 35 U.S.C. § 271(e)(1). Moreover, even if all of Sarepta's usage were strictly to provide information to the FDA, Wilson Wolf's intellectual property relates to research tools, and research tools are not included in the Safe Harbor.

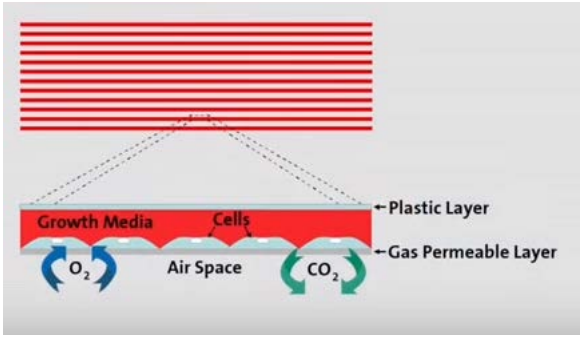
~~26.~~35. Sarepta has infringed at least claim 1 of the '192 Patent through its use of cells and/or cell-derived products including viral vectors manufactured using the HYPERStack, as set forth in the table below. The left side of the table contains the language of claim 1 of the '192 Patent. The right side of the table contains information on the HYPERStack and its use, including quoted text from an article entitled "Closed System Cell Culture Protocol Using HYPERStack Vessels with Gas Permeable Material Technology," authored by six Corning staff members, attached as Exhibit E, and images from Corning video entitled "Filling and Emptying the Corning®

HYPERStack® Cell Culture Vessel," posted on YouTube at https://www.youtube.com/watch?v=6CPcW_qWu_w.

A method of culturing cells comprising:	The HYPERStack is used to culture cells. "The HYPERStack Vessel is a multilayered vessel for . . . culturing of cells. . . ." Exhibit E (Protocol ¶ 1(1)) .
adding medium and animal cells into a static cell culture device	In use, medium and animal cells are added to the HYPERStack. The HYPERStack is a static cell culture device. "Inoculating Media": "Inject the Cell Suspension into the Media Bag and Mix well." "Using the bag stand, raise the media bag to help the cell suspension flow into the vessel." Exhibit E (Protocol ¶¶ 5(2) 6(5)).
that is not compartmentalized by a semi-permeable membrane,	The HYPERStack does not have a semi-permeable membrane.
at least a portion of said cell culture device is comprised at least in part of a nonporous gas permeable material,	"The HYPERStack vessels function via gas permeable material which allows gas exchange to occur. . . ." Exhibit E (Abstract ¶ 1).
ambient gas is in contact with at least a portion of said gas permeable material, and	"Rather than containing this 'headspace' for gas exchange within the vessel, the gas permeable products have air spaces . . . beneath each culture chamber which is open to the atmosphere." Exhibit E (Protocol ¶ 1(2)).
placing said cell culture device in a cell culture location that includes ambient gas at a composition suitable for animal cell culture,	"Move the HYPERStack vessel to the incubator." Exhibit E (Protocol ¶ 7(6)). Incubators used in cell culture contain ambient gas at a composition suitable for cell culture.

wherein said cell culture device is oriented in a position such that at least a portion of said cells reside upon at least a portion of said gas permeable material,	The HYPERStack is placed in the incubator such that at least some of the cells reside on the gas permeable material.  
the uppermost location of said medium is elevated beyond 2.0 cm from the lowermost location of said medium,	The uppermost location of medium is elevated more than 2.0 cm from the lowermost location of said medium, as can be seen in the picture above, from which the dimensions of the device filled with medium can be appreciated.
and said device is in a state of static cell culture.	The HYPERStack is cultured in a static state.

~~27.~~36. Sarepta has infringed at least claim 1 of the ‘443 Patent through its use of cells and/or cell-derived products including viral vectors manufactured using the HYPERStack, as set forth in the table below. The left side of the table contains the language of claim 1 of the ‘443 Patent. The right side of the table contains information on the HYPERStack and its use, including information authored by Corning staff, attached as Exhibit E.

A method of culturing cells in a cell culture device comprised at least in part of a gas permeable material	The HYPERStack is a cell culture device comprised at least in part of gas permeable material. See Exhibit E (Protocol ¶ 2(1)) ("The Stackette is the individual cell culture compartment that is made up of the top plate and gas permeable film.").
and including at least one access port and including at least two scaffolds, the method comprising:	The HYPERStack has at least one access port. See Exhibit E (Protocol ¶ 2(5)) ("The Liquid handling tube is connected to the liquid manifold and is used to make all closed system fluid manipulations."). The HYPERStack has at least two scaffolds. See Exhibit E (Protocol ¶¶ 2(1), 2(2)) ("The Stackette is the individual cell culture compartment that is made up of the top plate and gas permeable film. The cells are cultured within this compartment.") ("The Liquid Manifold connects each of the 12 stackette layers together within a HYPERStack module.").
a) adding cells and a volume of liquid medium into said cell culture device;	Cells and media are added into the HYPERStack. See Exhibit E (Protocol ¶ 6(6)) ("Using the bag stand, raise the media bag to help the cell suspension flow into the vessel.").
b) orienting said cell culture device into an inoculation position such that said scaffolds reside at different elevations within said cell culture device;	The device is oriented into a position such that the scaffolds reside one above the other at different elevations in the device as shown below. 
c) allowing cells to settle upon said scaffolds;	Cells settle upon the scaffolds, as shown in the diagram above.

d) adding enough liquid medium to prevent a unique gas-liquid interface from forming directly above at least one scaffold when the device is oriented in the inoculation position and to have at least a portion of the liquid medium in contact with at least a portion of said gas permeable material;	The user adds enough liquid medium to the HYPERStack to prevent a unique gas-liquid interface from forming above at least one scaffold when the device is in the inoculation position. See Exhibit E (Abstract ¶ 1) ("The HYPERStack vessels function via gas permeable material which allows gas exchange to occur, therefore eliminating the need for internal headspace within a vessel. The elimination of headspace allows the compartment where cell growth occurs to be minimized to reduce space, allowing more layers of cell growth surface area with the same volumetric footprint.") This can also be seen in the image below. 
e) placing the cell culture device in a cell culture location that includes ambient gas at a composition suitable for cell culture, said ambient gas making contact with said gas permeable material; and	The HYPERStack is placed in an incubator as shown in the image above. Incubators contain ambient gas at a composition suitable for cell culture. The HYPERStack has "air spaces . . . beneath each culture chamber which is open to the atmosphere." See Exhibit E (Protocol ¶ 1(2)).
f) not perfusing said liquid medium when said device is in said cell culture location.	The liquid medium in the HYPERStack is not perfused when the device is in the incubator.

COUNT I

INFRINGEMENT OF THE '192 PATENT AND THE '443 PATENT

~~28.~~37. Wilson Wolf incorporates by reference the above paragraphs as if stated herein.

~~29.~~38. The '192 Patent and the '443 Patent (collectively "the Patents-in-Suit") are valid and enforceable.

~~30.~~39. Sarepta has directly infringed at least one claim of the '192 Patent, including, without limitation, Claim 1 of the '192 Patent to the harm and detriment of Wilson Wolf, and to the benefit and profit of Sarepta.

~~31.~~40. Sarepta has directly infringed at least one claim of the '443 Patent, including, without limitation, Claim 1 of the '443 Patent to the harm and detriment of Wilson Wolf, and to the benefit and profit of Sarepta.

~~32.~~41. Sarepta's acts of direct infringement include, but are not limited to, its use in the United States of cells and/or cell-derived products including viral vectors manufactured according to Wilson Wolf's patented methods using the HYPERStack cell culture vessel.

42. Sarepta's use of cells and/or cell-derived products including viral vectors manufactured according to Wilson Wolf's patented methods using the HYPERStack cell culture vessel falls outside of the Safe Harbor of 35 U.S.C. § 271(e)(1).

~~33.~~43. Sarepta's infringement is irreparably harming Wilson Wolf.

~~34.~~44. Wilson Wolf is entitled to money damages in an amount to be determined at trial, and to preliminary and permanent injunctive relief.

PRAYER FOR RELIEF

WHEREFORE, Wilson Wolf prays for relief as follows:

1. A judgment that Sarepta has infringed the ‘192 Patent and the ‘443 Patent;

2. A judgment awarding Wilson Wolf damages in an amount to be determined at trial, but not less than a reasonable royalty;

3. An order enjoining Sarepta preliminarily, and permanently thereafter, from infringing, inducing infringement, and from contributing to the infringement of the ‘192 Patent and the ‘443 Patent;

4. A judgment awarding Wilson Wolf its costs incurred herein, including attorneys’ fees for an exceptional case pursuant to 35 U.S.C. § 285; and

5. A judgment awarding Wilson Wolf such other and further relief as the Court may deem just and equitable.

JURY DEMAND

Pursuant to Rule 38 of the Federal Rules of Civil Procedure, Wilson Wolf hereby demands a jury trial as to all issues so triable.

Dated: ~~December 20, 2019~~April 22, 2020

Respectfully submitted,

/s/ Kenneth L. Dorsney

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