Chapter 12

Methods and Process Optimization for Large-Scale CAR T Expansion Using the G-Rex Cell Culture Platform

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Abstract

The G-Rex cell culture platform is based on a gas-permeable membrane technology that provides numerous advantages over other systems. Conventional bioreactor platform technologies developed for large scale mammalian cell expansion are typically constrained by the mechanics of delivering oxygen to an expanding cell population. These systems often utilize complex mechanisms to enhance oxygen delivery, such as stirring, rocking, or perfusion, which adds to expense and increases their overall risk of failure. On the other hand, G-Rex gas-permeable membrane-based bioreactors provide a more physiologic environment and avoid the risk and cost associated with more complex systems. The result is a more robust, interacting cell population established through unlimited oxygen and nutrients that are available on demand. By removing the need to actively deliver oxygen, these bioreactors can hold larger medium volumes (more nutrients) which allows the cells to reach a maximum density without complexity or need for media exchange. This platform approach is scaled to meet the needs of research through commercial production with a direct, linear correlation between small and large devices. In the G-Rex platform, examples of cell expansion (9–14 day duration) include; CAR-T cells, which have atypical harvest density of 20–30 $\times$ $10^6/cm^2$ (or 2–3 $\times$ 10^9 cells in a 100 cm^2 device); NK cells, which have a typical harvest density of 20–30 $\times$ $10^6/cm^2$ (or 2–3 $\times$ 10^9 cells in a 100 cm^2 device) and numerous other cell types that proliferate without the need for intervention or complex processes normally associated with large scale culture. Here we describe the methods and concepts used to optimize expansion of various cell types in the static G-Rex bioreactor platform.

Key words G-Rex, CAR T expansion, Process development, Large scale, T cell culture, Suspension culture, In vitro cell culture, Gas-permeable membrane, Bioreactor

1 Introduction

The enormous promise demonstrated by numerous cellular therapies has created demand for cell expansion platforms that can address the needs of autologous and allogenic applications (*see* **Note 1**). One established technology that addresses these needs is the G-Rex (gas-permeable rapid expansion) series of bioreactors. Currently, these devices are prominently used for cell therapy applications due to their effective ability to expand large numbers of T

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cells without the need for technician interaction or complicated supporting instrumentation. More specifically, G-Rex technology is based on a gas-permeable silicone membrane that allows for optimal gas exchange at the base of each device. This key element provides T cells with unlimited access to oxygen in a quiet, static environment without the need to perfuse, stir, rock, or shake the culture [1–4, 7]. T cells and peripheral blood mononuclear cells gravitate to this gas-permeable, liquid-impermeable membrane (*see* **Note 2**), where they distribute uniformly across the bottom. Further, these bioreactors are then filled with an unconventional (greater) media volume which results in access to a larger nutrient pool, when compared to other static systems [5, 8, 15]. For example, oxygen (ambient air in the incubator) freely moves across the gas-permeable membrane in response to cellular demand. Consequently, when G-Rex devices are filled with a large volume of media per surface area (compared to the 0.3 cm height limit of conventional static flasks), the need for technician intervention or addition of fresh nutrients is eliminated [6]. Nutrients move freely within the media so cells have access on demand without the need for mechanical assistance or complex mixing equipment. By uncoupling the need to oxygenate via media delivery, larger volumes lead to greater availability of nutrients and dilution of metabolic waste that, when combined with unlimited access to oxygen, creates a more physiologic or static environment for natural cell to cell interaction (Fig. 1). The result is high viability leading to greater cell numbers in a shorter time period [8], a more consistent phenotype, a greater central memory population [9] and significantly more cells in a smaller footprint. In addition, the static nature and consistent media to gas membrane surface area ratio, leads to linear scalability across the G-Rex product portfolio. These attributes combine to make the G-Rex platform well-suited for therapeutic and commercial cell therapy applications.

This demonstrated reliability and cost-effective approach, when compared to conventional bioreactor technologies, has led to numerous academic, biotechnology, and pharmaceutical cell therapy programs adopting G-Rex as their principal platform methodology [9–16].

2 Materials

G-Rex devices are designed to be used with all types of cell culture media and cytokines. Any laboratory cell culture procedure that is currently used to maintain or expand CAR T cells can be directly applied to any G-Rex device. This includes procedures that incorporate specific serum-based media (RPMI, MEM, etc.), serum free media (e.g., TexMACS, PRIME-XV T Cell Expansion XSFM, PRIME-XV T Cell CDM, & numerous other specialty media)

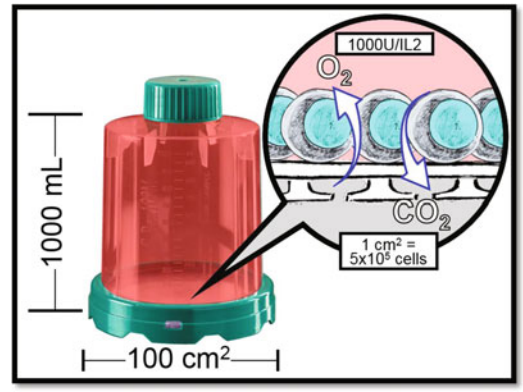

Fig. 1 The cells in G-Rex devices reside on a gas-permeable membrane (bottom of device) that allows for the exchange of O_2 and CO_2. As a result, oxygen and nutrients are available to the expanding cell population on demand, without intervention or need to add fresh media

and cytokines (R&D Systems) or other reagents (Such as magnetic beads for T cell activation or new reagents such as Quad Technologies' Cloudz) that are routinely used in T cell cultures. These can all be readily be applied to G-Rex multi-well plates, G-Rex10 series, G-Rex100 series, and G-Rex500 series devices, without the need for tedious adaptation or weaning procedures.

3 Methods

G-Rex technology is well-suited for suspension cell lines, such as CAR T, NK, TIL, TCR, and other T cell applications. When culturing a cell line in G-Rex for the first time, it is best to determine the maximum attainable cell density and total cell number. To do this, the following generalized protocol with a *G-Rex6 Well Plate or G-Rex24 Well Plate* should be followed to determine the maximum cell density. Once that has been determined, it is then possible to perform further process development studies to optimize the use of media and cytokines in a manner that does not affect the optimal final cell number:

3.1 G-Rex 6 Well Plate (10 cm² Surface Area per Well)

1. *Day 0*: Fill each well with 40 ml of culture medium and seed with 5×10^6 total cells per well (stated differently; 0.5×10^6 cells/cm^2). Additional seeding densities, such as 1×10^6 cells/cm^2, or 0.25×10^6 cells/cm^2 can also be evaluated at this same time within a single 6 well plate.
2. *Day 2*: Supplement with growth factor in a manner similar to conventional medium exchanges (typically after 2 days) performed in standard T flasks. Do not replace the medium, simply

add the IL-2 as a small bolus to each well. Do not mix or disturb the cells residing on the gas-permeable membrane.

3. *Day 4*: Remove 30 ml of spent media by pipetting from the top of the well downward, taking care not to remove or disturb cells residing on the membrane. When approximately 30 ml has been removed (10 ml remaining) from the well, gently swirl or pipet the remaining medium up and down to resuspend the cells. Remove a 500 μl sample to perform a cell count, then replenish with 30 ml of fresh media and place back into the incubator.
4. *Day 7*: Repeat day 4 and remove 30 ml of spent media by pipetting from the top of the well downward, taking care not to remove or disturb cells residing on the membrane. With 10 ml remaining in the well, gently swirl or pipette the remaining medium up and down to resuspend the cells. Remove a small sample for a cell count, then replenish with 30 ml of fresh media (remember to add cytokine) and place back into the incubator.
5. *Day 10*: Repeat day 7 and remove 30 ml of spent media by pipetting from the top of the well downward, taking care not to remove or disturb cells residing on the membrane. With 10 ml remaining in the well, gently swirl or pipette the remaining medium up and down to resuspend the cells. Remove a small sample for a cell count, then replenish with 30 ml of fresh media (remember to add cytokine) and place back into the incubator.
6. *Day 13–14*: At this point, each well should be at the maximum cell density. Gently remove 75% of the medium (pipetting from the top downward) and discard. Resuspend the cells in the remaining volume and perform a final cell count or other tests typically used to evaluate the final cell population.

3.2 G-Rex 24-Well Plate (2 cm^2 Surface Area per Well)

1. *Day 0*: Fill each well with 8 ml of complete culture medium and seed 1×10^6 total cells per well (stated differently; 0.5×10^6 cells/cm^2). Additional seeding densities, such as 1×10^6 cells/cm^2, or 0.25×10^6 cells/cm^2 can also be evaluated within a single 24 well plate.
2. *Day 2*: Supplement with growth factor in a manner similar to conventional medium exchanges (typically after 2 days) performed in standard T flasks. Do not replace the medium, simply add the IL-2 as a small bolus to each well. Do not mix or disturb the cells residing on the gas-permeable membrane.
3. *Day 4*: Remove 6 ml of spent media by pipetting from the top of the well downward, taking care not to remove or disturb cells residing on the membrane. When approximately 6 ml has been removed (2 ml remaining) from the well, gently swirl or

pipet the remaining medium up and down to resuspend the cells. Remove a 200 μl sample to perform a cell count, then replenish with 6 ml of fresh media and place back into the incubator.

4. *Day 7*: Repeat day 4 and remove 6 ml of spent media by pipetting from the top of the well downward, taking care not to remove or disturb cells residing on the membrane. With 2 ml remaining in the well, gently swirl or pipette the remaining medium up and down to resuspend the cells. Remove a small sample for a cell count, then replenish with 6 ml of fresh media (remember to add cytokine) and place back into the incubator.
5. *Day 10*: Repeat day 7 by removing 6 ml of spent media. Again, do so by pipetting from the top of the well downward, taking care not to remove or disturb cells residing on the membrane. With 6 ml remaining in the well, gently swirl or pipette the remaining medium up and down to resuspend the cells. Remove a small sample for a cell count, then replenish with 6 ml of fresh media (remember to add cytokine) and place back into the incubator.
6. *Day 13–14*: At this point, each well should be at the maximum cell density. Gently remove 75% of the medium (pipetting from the top downward) and discard. Resuspend the cells in the remaining volume and perform a final cell count, or other tests typically used to evaluate the final cell population.

3.3 Process Optimization

1. After the maximum achievable cell number has been determined, a next step is to establish the optimal seeding density [8]. This can be done as described above, using one or more wells within a standard G-Rex6 well or G-Rex24 well plate.
2. After the optimal seeding density has been determined, further process optimization should be done in the G-Rex6M well plate. These plates are part of the "M" series of devices, which incorporate the optimal media to gas-permeable membrane surface area ratio of 10 ml/cm^2 [8]. At this ratio, the nutrient supply is sufficient to achieve the maximum cell number without the need to replenish medium during the culture period. With exception of possibly adding cytokines, there is no need for technician intervention. Disturbing or interrupting the quiescent cell-to-cell interaction only serves to slow cell expansion and lengthen the time required to reach the maximum cell density (*see* **Note 3**).
3. Using the 6 M well plate, the next step in process optimization is to determine the quantity and frequency of cytokine addition. Many G-Rex users are finding cytokines to be more stable than originally believed (*see* **Note 4**). This is likely due to the optimal cell environment created by unlimited oxygen and

nutrient availability, combined with the physiologic cell-to-cell interaction established on the gas-permeable membrane. As such, supplementation of cytokines is either not necessary, or significantly less than what is typically required within conventional cultures. By varying the timing and quantity of cytokine addition, the impact on maximum cell number can quickly be determined in the 6 M plates. A similar approach can be done with other process variables to develop a no touch, cost-effective process for the rapid expansion of T cells.

4. To optimize the process, each comparison should be seeded in triplicate. For each condition, three wells should incorporate the same starting cell density, each filled with 100 ml of culture medium, and each well following the same cytokine supplementation schedule. To avoid resuspending the cell population, glucose and lactate levels can be monitored to provide a surrogate measurement for cell number [8]. Typically, glucose and lactate concentrations are the same throughout the culture volume, so samples (10–100 μl) can be removed anywhere above the cell bed. Carefully remove the samples and do not disturb or disrupt the cell population residing on the bottom silicone membrane. In the M series of devices, the maximum cell density will be obtained in the shortest duration if the cells are not resuspended and moved away from the gas-permeable membrane (the oxygen source).To correlate those measurements with an actual cell count, seeding in triplicate is important. Starting on Day 0, ensure there is an accurate glucose concentration for your media. On Day 3, remove a representative media sample from each of the three wells. Determine the glucose and lactate levels (*see* **Note 5**), then harvest the first well to manually perform a cell count. The glucose and lactate values can now be correlated with the manual count for Day 3. On Day 6, repeat the process by removing a small media sample for glucose/lactate determination, followed by harvest and cell count of the second well. Finally, remove a media sample for glucose/lactate determination in the third well and harvest on Day 9, or Day 10.
5. This glucose and lactate data can be used as follows to estimate cell counts for similar cultures. By using the formula (*see* **Note 6**), the glucose concentration can be used to predict the cell expansion rate and timing for the subsequent harvest. Glucose monitoring allows the culture to be tracked without disrupting the cell to cell interaction needed to reach a maximum cell number [8]. It often takes several hours for the CAR-T cells to gravitate back to the oxygen rich zone above the silicone membrane (approximately 300 μm above the membrane). After that, the cells must then recondition the microenvironment and reinitiate the paracrine signaling required for rapid

cell expansion. Since the best protocols are those where the cell bed is not disturbed, the ability to use glucose and lactate concentrations as a surrogate for cell number is paramount to attaining optimal expansion within the shortest period of time.

3.4 Large Scale Production with G-Rex Closed Systems (G-Rex100M-CS and G-Rex500M-CS)

1. After the process parameters have been established (optimal inoculation density, maximum achievable cell density, elimination of media changes by using the M series G-Rex devices, determining optimal cytokine administration schedule (*see* **Note 7**), and the ability to use glucose/lactate measurements as a surrogate to cell count), the next step is to transition the process into a closed system G-Rex device. As a result of the consistent ratio between media volume (ml) and gas membrane surface area (cm^2), there is a linear relationship between the different M series G-Rex devices. In other words, the optimal process developed in the G-Rex6M well plate will scale directly into the larger closed system G-Rex100M-CS (by a factor of 10) or G-Rex500M-CS (by a factor of 50) devices [8, 12, 14]. To achieve linear scalability, all of the M series devices should be inoculated with the same cells/cm^2, media volume ml/cm^2 and the same ratio of cytokines or other growth factor. The expansion rate, phenotype and other cell characteristics will remain the same throughout the various size G-Rex vessels (*see* **Note 7**).
2. The following example is based on the G-Rex100M-CS Device (Ref. #81100-CS) which is an FDA listed Class I Medical device. A typical optimized CAR T expansion in the G-Rex100M-CS involves seeding 50 million transduced T cells, filling the device with 1 l of media and frontloading with cytokines (*see* Subheading 3.3, **step 1** for more information). In most instances, this simple process will result in the expansion of 3 billion cells in 9 or 10 days. At maximum cell number (determined during process development studies), simply remove the device from the standard cell culture incubator and sterile weld the red-striped PVC tubing to a media collection bag (*see* **Note 8**). Connect the air line of the GatheRex Liquid Handling, Cell Harvest Pump (Wilson Wolf Corp., Ref. #80000E) to the G-Rex100M-CS (as shown in Fig. 2). The GatheRex creates positive air pressure to pressurize the head space of the vessel and push 90% of the culture media (now waste media) out of the device. The advantage to positive pressure is that cells remain undisturbed on the gas membrane and are not removed during the media reduction step [8, 12]. After the volume has been reduced, gently swirl the device to resuspend the cells in the remaining 100 ml. Sterile weld the clear PVC line to a collection vessel or bag and, again

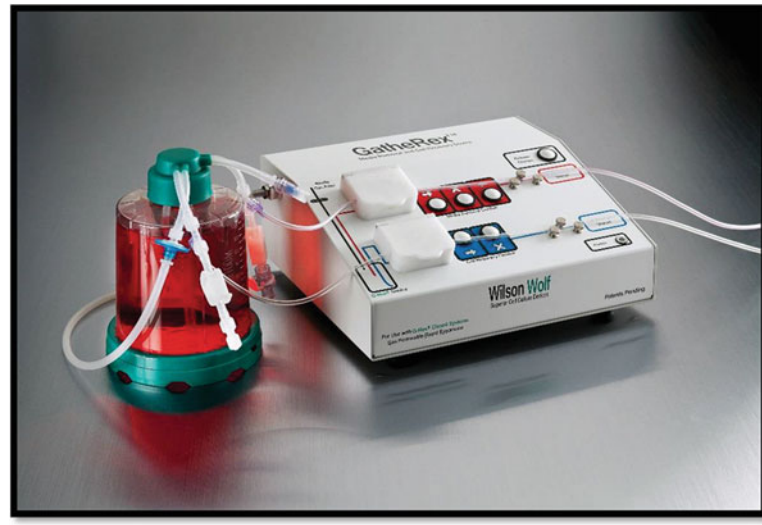

Fig. 2 The G-Rex100M-CS Cell Culture Device (REF 81100-CS) connected to the GatheRex Liquid Handling, Cell Harvest Pump (REF 80000E). The device is connected to the GatheRex using the air line attachment. The sterile weld compatible tubing (red-stripe and clear) are seated in the sensor housing that detects air bubbles which stops the fluid flow automatically once specific levels of fluid have been removed

using positive pressure from the GatheRex, harvest approximately 3 billion cells concentrated into a 100 ml volume.

3. As shown in Fig. 3, this section describes the various ports and options for moving media and cells into or out of the G-Rex100M-CS. Port A is outfitted with the sterile air filter. This filter will connect to the air line, which will connect to the GatheRex and allow air to be forced into the device. This increased pressure within the device serves to drive liquid (media & cell fraction) out of the G-Rex100M-CS and into the collection vessels. Port B is for sampling or cytokine addition. It is equipped with a needleless septum rated for multiple sterile connections. Internally, this sampling line is approximately level with the 500 ml demarcation. Port C is the media removal conduit. This port is connected to the internal tube that sits just above the gas-permeable membrane (about 1 cm above the membrane). The height of the tube above the membrane allows the user to remove 90% (100 ml remains) of media prior to harvesting the cells. The T-fitting at the top of this port has multiple attachments for various filling/removing options. They include an MPC (quick connect) fitting and the media removal tubing, which is marked with a red stripe. The end of this line is equipped with a male Luer cap and can be connected to a media bag for filling or waste bag for removal. The red-stripe tubing is compatible with sterile welders to

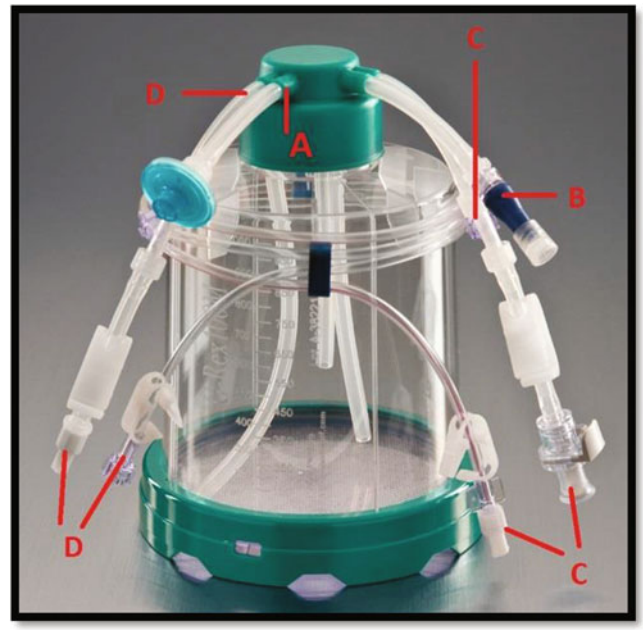

Fig. 3 The closed G-Rex100M-CS Cell Culture Device (REF 81100-CS). Port A is the vent filter port, outfitted with a 0.2 μm filter. Port B is the sampling port, outfitted with a Clave® Needleless Luer lock fitting for docking a syringe. Port C is the media addition/removal port, outfitted with an MPC (quick connect) fitting as well as 30 in. of sterile weld compatible PVC tubing (red-stripe) that terminates in a female Luer lock fitting. Port D is the cell recovery port, outfitted with female Luer lock fitting as well as 30 in. of sterile weld compatible PVC tubing (clear) that terminates in a male Luer lock fitting

perform as a functionally closed unit. This is the preferred method of operation. Port D is the cell recovery conduit. This port is fastened to the internal tube that resides at the base of the device near the outer edge of the gas-permeable membrane. The T-fitting at the top of this port is equipped on one side with a male Luer-capped end for media addition. A clamp is positioned to prevent the flow of liquid to this side of the T-fitting when collecting the cell fraction. The other side of this port connects to the cell recovery line (clear tubing). At the end of this line is a female Luer lock with a cap which can be connected to a media bag to initially fill the device just prior to inoculation. At the end of the culture, this same line is used to harvest cells when connected to a cell recovery vessel. The clear tubing is also compatible with sterile welders to perform as a functionally closed unit. This is the preferred method of operation.

4. As described above, there are several ways to fill the device with media: (a) Using a sterile tube welder, connect the media bag tubing to the red-striped tubing on the media removal conduit.

Station the media bag at a height above the G-Rex device and open any clamps to allow culture media to flow into the device. Fill to desired level; 1 l is suggested to expand the culture to maximum cell density without the need to replenish with fresh media; (b) If using a large media bag equipped with an MPC connecter, use the connection on Port C. Follow the same process using gravity to fill the G-Rex device to the desired level; (c) There are also male and female Luer lock ports which can be utilized for media addition.

5. Similar to adding media, the G-Rex100M-CS can be inoculated as follows: Using a sterile tube welder, the user can connect the cell recovery tubing to a culture bag that holds the cell inoculum or, using aseptic technique and Luer fittings, find the desired fitting to attach to the culture bag containing the inoculum. Port D has a male Luer fitting; Port B is outfitted with a needleless septum for inoculation if the cells are transferred via syringe.
6. Cytokines or other growth factors (if necessary) are typically added through the needless septum (Port B).

4 Notes

1. G-Rex technology was designed for expanding suspension cells. In addition to CAR T and other immune cells, the G-Rex platform can be used for cell expansion of many other cell types, such as hybridoma, K562, etc. The hydrophobicity of the gas-permeable membrane does NOT lend itself to cell adherence and will not support the expansion of anchorage-dependent cell lines.
2. The G-Rex membrane is liquid impermeable. Additionally, the membrane should not be thought of as a filter, but simply a highly gas-permeable surface on which cells reside. As an example, magnetic beads used for T cell activation will not "clog" the membrane because it does not consist of pores typically associated with membrane-based filters\.
3. Use care to avoid disrupting the cells residing on the gas-permeable membrane. The key to rapid CAR T expansion is the static culture environment that exists on G-Rex gas--permeable membrane. Cellular interaction, or cell to cell signaling between T cells is a normal process during in vivo expansion. Unlike other in vitro technologies, the G-Rex platform is a static culture environment. This allows for access to oxygen, nutrients, and importantly, cellular interaction to occur when needed. By eliminating any limitation associated with availability of these critical variables, cells maintain a high

viability which results in a more rapid population expansion rate in G-Rex when compared to other in vitro technologies [9, 15].

4. Interestingly, the widespread use of G-Rex for cell therapy has led to the understanding that many cytokines are more stable than originally believed. This is contrary to common understanding, meaning cytokine degradation is not as severe as previously assumed. Traditional culture vessels only hold small quantities of media so frequent changes or splitting of wells/flasks is conventional practice [15]. At the same time, researchers have typically supplemented or replenished the IL-2 and other cytokines with these media exchanges. It is likely that over time, this practice has led to a belief that many cytokines degrade readily and need to be supplemented every 1–3 days. Our "M" series G-Rex vessels, which eliminates the need to exchange media during the culture period, has challenged this assumption. Consequently, many investigators have discovered that several cytokines can be frontloaded at the onset and no further supplementation is required for the duration of the G-Rex culture. While it does seem to be common in G-Rex cultures, it is important to determine cytokine stability that is applicable to your unique media and cytokine combination. This is described in Subheading 3.3, **step 3** above, where we have outlined the way to evaluate cytokine stability using our G-Rex6M Well Plate.
5. As mentioned above, it is important to fill the device to the recommended volume. In the production devices (M series), this is 10 ml media/cm^2 of gas-membrane surface area. At this ratio, CAR T cells have an adequate supply of nutrients to reach a maximum number without the need to replenish with fresh media [12]. In the G-Rex devices, small molecules, such as glucose, amino acids, vitamins, etc., are constantly in motion during the culture period due to convection. As a result, small molecules are distributed evenly throughout the device and nutrients are readily available to the expanding cell population. Since nutrients are continually available and oxygen is available on demand via gas-permeable membrane at the base of the device, cells will expand at their maximum physiologic rate without the need to rock or stir the media. This static environment will result in a predictable harvest number and the shortest culture duration [8–16].
6. The glucose concentration can be used to estimate the viable cell number based on the following formula [8]:

$$\text{Estimated cell number} = (A - B)/C \times D/E \times F \times G$$

A = Initial glucose concentration
B = Current glucose concentration

C = Glucose consumed to achieve maximum cell number
D = Initial media volume
E = Total media volume required for maximum cell number
F = Maximum cell density
G = Surface area of the G-Rex gas-permeable membrane.

7. One of the principal advantages to the G-Rex platform is the linear scalability exhibited throughout the entire product line. When the process development studies are complete, cell expansion on a per cm^2 basis will be consistent across all M series devices. This is primarily the result of a uniform media (ml) to gas membrane surface area (cm^2). By using the same inoculation density (cells per cm^2) and filling the device to the recommended volume, the expansion rate and final number of cells per cm^2 will be identical, no matter which sized device is used for production [8].
8. The closed system G-Rex devices can easily be connected to upstream and downstream cell processing equipment in order to create functionally closed cell expansion protocols. One example is the work being done at the NIH's Cell Processing Section with TCR transduced T cells. These cells are expanded in the G-Rex500M-CS (averaged about 18 billion per device), harvested with the GatheRex Pump and finally docked onto the LOVO cell washing equipment from Fresenius Kabi for final processing [12].

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