

Video Article

Scale-Up of Mammalian Cell Culture using a New Multilayered Flask

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Abstract

A growing number of cell-based applications require large numbers of cells. Usage of single layer T-flasks, that are adequate during small-scale expansion, may become cumbersome, laborious and time-consuming when large numbers of cells are required. To address this need, the performance of a new multi-layered cell culture vessel to facilitate easy scale up of cells from single layered T-flasks will be discussed. The flasks tested are available in 3- and 5-layer format and enable culture and complete recovery of three and five times the number of cells respectively, compared to T-175 flasks. A key feature of the BD Multi-Flask is a mix/equilibration port that allows rapid in-vessel mixing as well as uniform distribution of cells and reagents within and between layers of each vessel and consistently produce cells that can be cultured in an environment that is congruent to T-175 flasks.

The design of these Multi-Flasks also allows for convenient pipette access for adding reagents and cells directly into the flasks as well as efficient recovery of valuable cells and reagents and reduces risk of contamination due to pouring. For applications where pouring is preferred over pipetting, the design allows for minimal residual liquid retention so as to reduce wastage of valuable cells and reagents.

Video Link

The video component of this article can be found at <http://www.jove.com/video/3418/>

Protocol

1. Protocol to use Multi-Flasks for cell culture

1. *Preparation of vessels and adding cell suspension*
 1. Prepare cell growth medium as needed.
 2. Line up Multi-Flask vertically on its side with the cap facing up on the work surface (sterile laminar flow hood).
 1. *These flasks are available in 3 and 5-layer formats and use a similar work-flow pattern as a T-175 flask.*
 3. Loosen and remove caps. Add required amount of pre-warmed medium into flask using a 50 or 100 mL pipette or by pouring.
 1. *Pipettes that are ≤ 10 ml can reach the bottom of the vessel when Multi-Flasks are placed vertically with cap facing upwards. Pipettes ≥ 10 ml can reach into the vessel immediately past the logo on the Multi-Flask, thereby providing a convenient portal to add larger volumes of media to vessel.*
 4. To avoid bubbling of medium, allow liquid stream to flow along the inner wall of the Multi-Flask lid (logo-side).
 5. Add cell suspension from a concentrated cell stock into growth medium through the top layer using a 10 mL pipette and cap flasks.

Tips:

- Transport Multi-Flasks on a cart to incubator site and perform remaining steps.

- The cell seeding density will vary depending on the cell type, medium and culture duration need. Begin with the seeding density and media volume to that used in standard T-175 flasks and multiply by 3 or 5 depending on the Multi-Flask format used.

2. *Mixing of cells*
 1. **Mix position:** Hold the Multi-Flask upright with the logo facing you and turn counter clockwise to a 45° angle with mix port facing you.
 2. Holding at the same angle, gently tilt Multi-Flask from front to back (neck away from you) until liquid in the top layer drains fully downwards through the mix port. Pivot on mix port-side.
 3. Likewise, gently rock Multi-Flask from back to front (neck towards you) until medium drains fully from the bottom layer towards the top through the mix port. Repeat Steps 1.2.2 and 1.2.3 one more time to ensure proper mixing.
 4. Bring Multi-Flask back to mix position (Step 1.2.1) and proceed to Step 1.3
 5. Alternatively, cell suspension can be prepared externally from the Multi-Flask and cell suspension can be added to the vessel using a pipette or by gentle pouring.
 1. *Mix port allows in-vessel mixing of cells with media and eliminates the need to make large volumes of cell suspensions externally.*
 2. *It also allows media equalization across the layers of the Multi-Flask*

3. *Equilibration of fluid*
 1. After mixing/adding of cell suspension, place Multi-Flask vertically on a flat work surface to equalize liquid volume equally in all the layers.
4. *Partition liquid into each layer*
 1. Hold the Multi-Flask with the logo facing you and turn clockwise to a 45° angle to partition the liquid into each of the layers.

Tip:

- For best results, it is important to use a flat work surface for Steps 1.3-1.4

5. *Transportation*
 1. Once media containing cell suspension is partitioned, transport Multi-Flask at the same 45° angle (clockwise) as in Step 1.4.1 with media away from the mix port into incubator. This position is also suitable when removing flasks from incubator for viewing on a microscope.
6. *Placing Multi-Flask onto incubator shelf*
 1. Holding Multi-Flask at the 45° angle (clockwise, away from mix-port), gently rotate it down horizontally onto the incubator surface (using the corner away from the mix port as a pivot). Lay Multi-Flask with Logo facing up.
 1. Falcon Multi-Flask design allows vessels to be stacked and holds them in place by a sturdy stacking rib.
7. *Distribution of cells and reagents*
 1. After placing Multi-Flask flat on work surface, gently rock back and forth and side-to side to distribute cells evenly onto culture surfaces taking care not to spill liquid from each layer. Stack flasks.
 1. This Flask is made of optically clear material and cells on the last layer can be easily viewed on a microscope.
8. *Media exchange*
 1. Aspirate while tilting the Multi-Flask to the left (mix port-side) with the logo facing you.
 2. Then tilt, the Multi-Flask to the right, continuing to aspirate all residual media.
 3. Add appropriate amount of media and follow Steps 1.3-1.7

2. Harvesting cells from Multi-Flasks

1. To dissociate cells, line up Multi-Flasks vertically and bring each one to **Mix position** (Step 1.2.1). Gently invert flasks with neck facing you so as to allow media to drain to the top layer of Multi-Flask.
2. Insert a 5 or 10 mL aspirating tip through the neck until you reach liquid level near the mix port. Aspirate exhausted medium.
3. Add dissociation reagent/wash buffer and bring to **Mix position** as in Step 1.2.1, then proceed and follow steps 1.3-1.7. Neutralize with growth medium /neutralizing agent and pour cell suspension into a receiving tube OR invert flask such that cells drain to the top layer of the vessel and collect cells using a 10 mL pipette.

Tip: To enhance recovery of cells or reagents, bring Multi-Flask to mix position (Step 1.2.1), invert with neck towards operator to allow complete drainage of media from all layers to the top layer. Then, tilt Multi-Flask clockwise to 45° angle (away from the mix port) while the Multi-Flask remains inverted. Use a pipette (1-10 mL) to collect any remaining reagents.

3. Recommended working volume in Falcon Multi-Flasks

Growth media

3-Layer: 75-150 mL per Multi-Flask

5-Layer: 125-250 mL per Multi-Flask

Dissociation agent

3-Layer: ≥15 mL per Multi-Flask

5-Layer: ≥25 mL per Multi-Flask

Tip: Begin with medium volume used in standard T-175 flasks and multiply by 3 or 5 times depending on the Multi-Flask format evaluated so that mL per unit surface area remains the same.

4. Representative Results:

1. Design of Falcon Multi-Flask

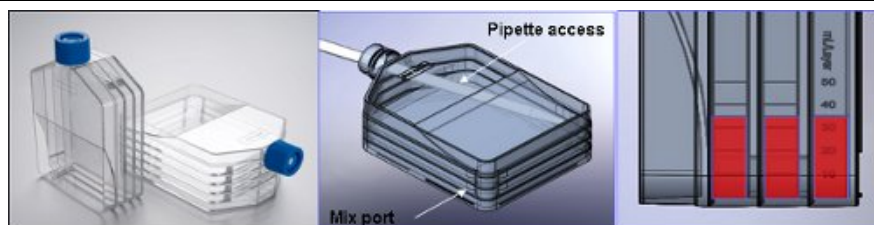


Figure 1. Multi-Flask Cell Culture vessels are available in a 3- and 5-layer stackable format for scale-up of cells providing 525 and 875 cm² growth surface area, respectively. Pipette access facilitates addition and removal of cells and reagents into-and out of the vessel. Presence of mix-port allows for rapid in-vessel mixing and equalization of media across all layers of the Multi-Flask.

2. Cell Yield using Multi-Flask:

These vessels are available in 3-layer and 5-layer formats which correspond to 3 and 5-times the surface area of T-175 flasks. Accordingly, ≥ 3 and 5 times ($130 \pm 6.8 \times 10^6$ and $218 \pm 23.6 \times 10^6$ cells, respectively) the number of BHK-21 (baby hamster kidney) cells were grown and recovered from Multi-Flask compared to T-175 flasks ($43.2 \pm 3.5 \times 10^6$ cells; Fig.2A). Cell yield per unit surface area was equivalent in 3- and 5-layer Multi-Flasks and T-175 flasks for BHK-21, LnCAP (human prostate adenocarcinoma cell line), Hep-G2 (human hepatocarcinoma cell line), EcoPack 2-293 (human kidney cell line) cultured for a period of 48-96h (Fig.2B) in growth media (35 ml per layer) as recommended by cell vendor (ATCC, Sigma and/or Clontech). Cells were enumerated on an automated Vi-CELL counter ⁽¹⁾.

Figure 2A : Three and five times the number of BHK-21 cells were grown and recovered from 3- and 5-layer Multi-Flasks compared to T-175 flasks. Expected yield was determined using mean cell yield from control T-175 flasks multiplied by three and five times for the 3- and 5-layer Multi-Flasks respectively (n=4 flasks/format).

Figure 2B : Cell yield per cm² was equivalent in 3- and 5-layer Multi-Flasks and T-175 flasks for BHK-21, LnCAP, HepG2 and EcoPack2-293 cells. Each bar represents mean of 4 to 6 flasks. BHK-21 cells (11,000 cells/cm²) were cultured for 72 hours, LnCAP cells (20,000 cells/cm²) and EcoPack2-293 cells (~35,000 cells/cm²) were cultured for 96 hours and HepG2 cells (25,000 cells/cm²) were cultured for 48 hours prior to harvest.

3. Media distribution among layers of Multi-Flask

Cell culture medium (DMEM; Invitrogen) was added to 5-layer Multi-Flasks (250 mL/5-layer vessel) and partitioned into layers according to protocol described above. Media distribution was measured by drilling holes in each layer and media pumped out from individual layers. Weight of fluid recovered from each layer was found to be relatively uniform from layer to layer as shown in Fig 3. They are as follows: 51.8 ± 0.73 , 50.3 ± 0.58 , 50.21 ± 0.13 , 49.88 ± 0.35 , 49.45 ± 0.37 (gm).

Figure 3. Uniform media distribution in each of the five layers of a 5-layer Multi-Flask. Cell culture medium was added to Multi-Flasks (250 ml/5-layer vessel), equilibrated & partitioned to individual layers. The media was pumped out through holes drilled into individual layers and fluid weight was recorded from each layer (n=6 flasks).

4. Cell distribution between layers of Multi-Flask

Cells can be added and mixed within the Multi-Flask. We simulated distribution of cells between layers of Multi-Flask using beads (10 μ m; PolySciences Inc.) similar in size to cells. Bead suspension was added into Multi-Flask vessel using a 10 mL pipette through the top layer and mixed with media in the vessel as described in the protocol above. Bead distribution was measured by drilling holes in each layer and fluid containing bead suspension was pumped out from individual layers. Bead concentration recovered from each layer was read on a Coulter Counter and recorded. Shown below are equivalent inter-layer bead distributions in 3-layer Multi-Flasks (Fig.4A). The mix-port enables homogenous distribution of cells and reagents between Multi-Flask layers.

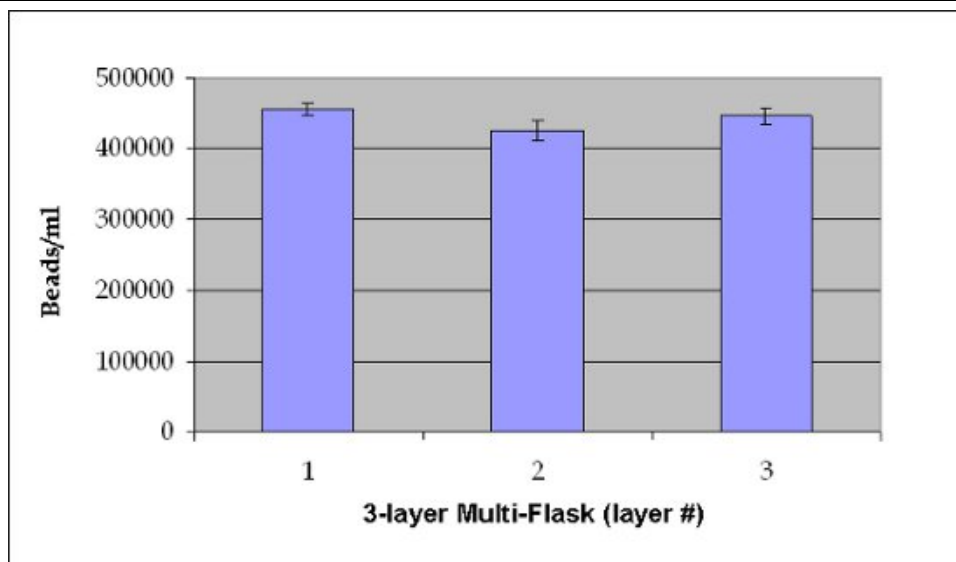


Figure 4A : Bead distribution in each of the three layers of a 3-layer Multi-Flask. A suspension of beads (3.6×10^6 /ml) was added to medium dispensed into Multi-Flasks (bead suspension:media volume is 1:10, vol:vol) and mixed, followed by equilibration and partition steps using the protocol described. Bead concentration recovered from each layer (medium pumped through holes drilled on each layer) was read on a Coulter Counter and recorded. Shown below are equivalent inter-layer bead distributions in 3- layer Multi-Flasks (n=5 flasks)

Shown below are representative images of EcoPack 2-293 staining patterns of cells grown to >80% confluence on 3-layer Multi-Flasks in supplemented growth media. Cell monolayers were fixed and stained with crystal violet and Multi-Flask layers were then cut and images scanned (2). Note, cell patterning was homogenous on all layers of Multi-Flask (Fig.4B). Similar results were obtained with multiple cell types evaluated (data not shown).

Figure 4B : This figure illustrates homogeneous cell growth between layers of Multi-Flasks. EcoPack-2-293 cells grown to >80% confluence in 3-layer Multi-Flasks and T-175 were fixed and stained with crystal violet. Multi-Flask vessels were cut and each stained layer was scanned.

5. Air supply in Multi-Flask:

Analysis of spent media using BioProfile FLEX analyzer (3,4) (Nova Biomedical) from EcoPack2-293 cells cultured for 96 hours revealed no difference in air saturation of cells grown in 5-layer Multi-Flasks vs. T-175 flasks (81.03 ± 1.9 vs. 83.4 ± 5.8 % ambient O_2).

Figure 5 : Air saturation (% ambient O_2) of spent media were similar in pre-mixed media from 5-layer Multi-Flasks vs. T-175 flasks. EcoPack2-293 cells were seeded at a density of 35,000 cells/cm² and cultured for 96 hours prior to media analysis (n=3 flasks).

Discussion

The current study demonstrates the increase in productivity that the Multi-Flask design offers researchers. Whilst it is important to follow the steps outlined above for optimal performance when using Multi-Flasks, there are few critical steps in this protocol that are deemed most essential. These include (i) mixing of cells and reagents using the mix port within the vessel (ii) transporting Multi-Flask at a 45° angle clockwise after partitioning fluid into each of the layers (iii) laying Multi-Flask flat post partitioning into the incubator.

Proper use of Multi-Flask leads to production of a homogeneous cell population within each vessel that is cultured in an environment that is congruent to T-175 flasks (5). These vessels provide 3 and 5 times more cells in a similar footprint as the T-175 flask. The 3 and 5-layer vessel provides 525 and 875cm² of growth area, respectively and offer both space and labor savings to users. Tissue Culture Surface treatment is comparable to standard flasks thus enabling scale-up without the need for re-optimizing existing culture conditions or compromising quality, homogeneity or performance of cells (6, 7). This also provides for comparability with previously collected data. These vessels can be also coated with reagents such as collagen, fibronectin, poly-D-lysine to provide a specialized substratum for attachment, growth and differentiation of certain cell types such as hepatocytes⁸, keratinocytes⁹, stem cells grown in serum-free media formulations. Coating solutions can be removed with minimal residual liquid retention so as to reduce wastage of valuable reagents as well as effective removal of coating solution prior to culturing cells. The recommended optimal media volume to culture cells in Multi-flasks range from 0.142-0.287 ml/cm² which translate to 25-50 ml per layer. Unlike another multilayered flask, this product offers a mix-port in the vessel that allows rapid in-vessel mixing as well as uniform distribution of cells and reagents within and between layers of each vessel. Diverse cell lines, primary cultures and stem cells have been scaled up efficiently using Multi-Flasks. These vessels are particularly advantageous in applications that demand a large number of cells such as in high throughput-screening, vaccine production, viral vector transfections and cell therapy.

Savings in time, space, labor and reduced waste generation are key winnings of the Multi-Flask versus conventional cultures in single-layered vessels. We can culture approximately three times the number of cells harvested from 5, T-175 flasks in the same space using 3, 5-layer Multi-Flasks. This advantage in space saving is not limited only to T-175 but can also be extended to other vessels: a standard roller bottle apparatus that fits into common laboratory incubators houses ~4 roller bottles (2200 ml) each of which provides 850cm² surface area. In the same area, ~20, 5-layer Multi-Flasks can be housed thus providing five-times the growth surface to culture cells. Furthermore, with an increase in the "Go-Green" awareness, options to reduce waste generation is highly desirable. In that respect, there is a 38% (5, T-175 flasks weigh ~640g whereas one, 5-layer Multi-Flask weighs ~ 400g) decrease in waste generation using Multi-Flasks compared to T-175 flasks and these advantages lead to decrease in waste storage and disposal costs and result in economic savings to the user.

Disclosures

All authors are employees of Becton Dickinson, Biosciences Segment, Discovery Labware Unit.

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