Application Note

Improved Productivity of Mouse Embryonic Stem Cell Culture Using Millicell® HY Multilayer Culture Flasks

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Introduction

Traditionally, culture and expansion of mouse embryonic stem cells (mESC) is performed using several single-layer T-flasks or Petri dishes. Harvesting and analyzing stem cells from multiple culture vessels is a repetitive and time-intensive process that consumes valuable incubator space. Here, we report a method for expanding mESCs using a new multilayer cell culture flask. The Millicell HY multilayer culture flask makes expansion and harvesting of mESCs faster and easier than traditional culture methods. The method for using the new flasks is linearly scalable per unit surface area. Therefore, no reoptimization of procedure is required – culture conditions and media volume are identical, regardless of whether culture is performed in traditional single-layer devices or in a Millicell HY culture flask. Resulting morphology, cell recovery, viability, and pluripotency of mESCs were not significantly different between multilayer flask culture and single-layer flask culture methods.

Materials and Methods

mESC Culture Prior to Evaluation in Multilayer Flasks

T75 flasks were coated with 10 mL 0.1% gelatin in phosphate-buffered saline (PBS, Merck Millipore) and incubated for at least 30 minutes at room temperature (RT). Flasks were seeded with a suspension of mitomycin-treated mouse embryonic fibroblasts (MEFs, Merck Millipore Cat. No. PMEF-CF) at 5 x 10^6 cells in 15 mL MEF medium per T75 flask. After incubating 24 hours at 37 °C, 5% CO_2 and 95% relative humidity, the MEF medium was replaced with fresh mESC medium (Merck Millipore, Cat. No. ES-101-B). Flasks were seeded with mESCs (Merck Millipore, SCR012) at 5 x 10^6 cells in 30-35 mL mESC medium per T75 flask. Flasks were fed every day with fresh mESC medium.

mESC Culture for Evaluation of Feeder-Free Culture in Multilayer Flasks

Both T75 and Millicell HY T600 (3-layer) flasks were coated with gelatin, 10 mL of 0.1% gelatin for a T75 flask and 80 mL per T600 flask. Cultured mESCs were removed from the incubator and enzymatically dissociated at RT until they appeared rounded up by microscopy.* mESC medium was added to quench dissociation. Cells were mixed with a pipette to ensure a homogeneous cell suspension. Coated T75 and T600 multilayer flasks were seeded with 4 x 10^4 mESC per cm^2 without fibroblast feeder cells and incubated at 37 °C, 5% CO_2 and 95% relative humidity prior to next passage. mESCs were passaged five times prior to cell counting, viability, recovery and pluripotency analysis.

*Please refer to the Trypsinization Protocol on page 4 of this application note.

mESC Culture for Evaluation of Culture in Multilayer Flasks With Feeder Layer

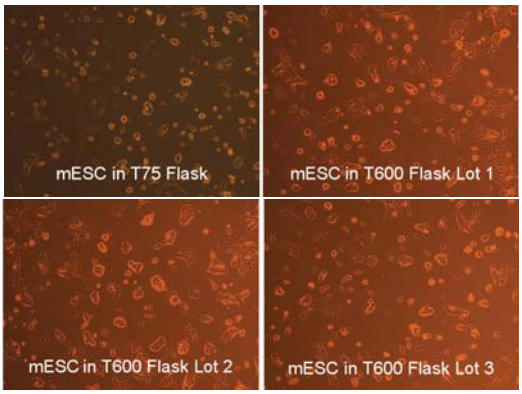
Both T75 and Millicell HY T600 (3-layer) flasks were coated with gelatin and seeded with MEF feeder layer. Flasks were incubated at 37 °C, 5% CO_2 and 95% relative humidity for 24 hours, then MEF medium was replaced with fresh mESC medium. Cultured mESCs were removed from the incubator and enzymatically dissociated at RT until they appeared rounded up by microscopy. mESC medium was added to quench dissociation. Cells were mixed with a pipette to ensure a homogeneous cell suspension. The T75 and T600 multilayer flasks containing MEF feeder layers were seeded with 4 x 10^4 mESC per cm^2 and incubated for two days before cell counting, viability, and recovery analysis.

Cell Count and Viability Determination

mESCs were harvested by enzymatic dissociation from both T75 and T600 flasks. Single cell suspensions were analyzed using the guava ViaCount® assay (Merck Millipore, Cat. No. 4500-0110) per kit instructions. Data were acquired and analyzed using a guava easyCyte™ benchtop flow cytometer (Merck Millipore).

Detection of SSEA-1 and Oct4 Expression

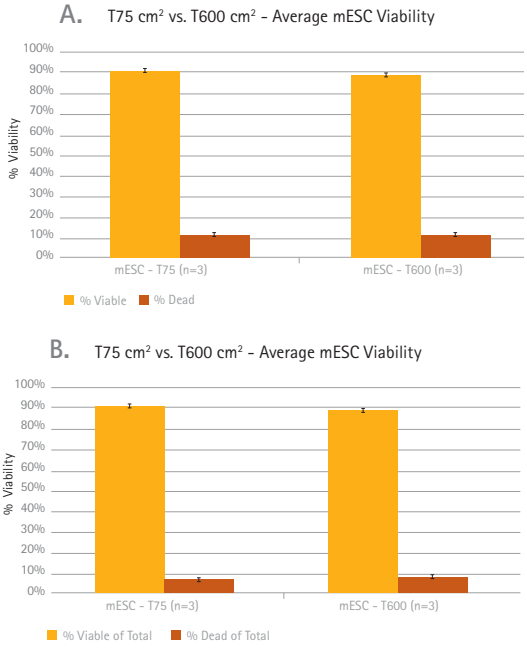
Single-cell suspensions of harvested mESCs were mixed with anti-SSEA1 (Merck Millipore, Cat. No. MAB4301, 20 µg/mL in 4% goat serum/96% PBS, 0.5 mL per 2 x 10^6 cells) or anti-Oct4 (Merck Millipore, Cat. No. MAB4419, 5 µg/mL in 4% goat serum/96% PBS, 0.5 mL per 6 x 10^5 cells). After primary antibodies were incubated for 1 hour, cells were washed and mixed with goat anti-mouse Alexa Fluor® 488 conjugated secondary antibody (1:1000 in 4% goat serum/96% PBS) and incubated for 30 minutes at RT. 1 mL of 4% goat serum/96% PBS was added to each sample. 300 µL of each sample was placed in each well of a 96-well plate and analyzed on the guava easyCyte flow cytometer.



Results

Visual inspection showed no significant difference in mESC morphology between T600 multilayer flasks and T75 flask cultures; furthermore, we observed no significant difference in mESC morphology between three separate lots of T600 flasks tested (Figure 1).

Figure 1. Visual assessment of mESC growth using brightfield microscopy. Only the bottom layer of the HY flasks are visualized.

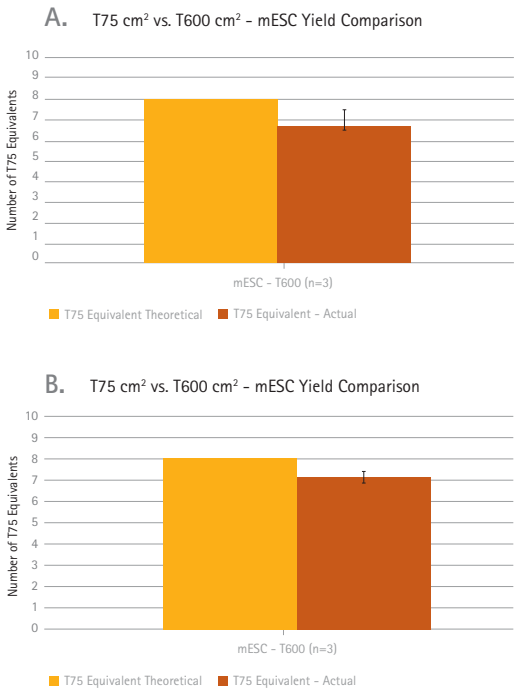


The average viability of mESCs passaged feeder-free in single-layer T75 flasks was 91% with no significant difference from average viability in T600 multilayer flasks, which was 90% (Figure 2A). When cultured on a feeder layer, mESC viability was 92% in T75 flasks and 91% in T600 multilayer flasks. We also observed no significant difference in viability between the 3 application lots of T600 multilayer flasks tested (data not shown).

Figure 2. Viability comparison between mESCs grown in three T75 single-layer flasks and mESCs grown in flasks from three lots of T600 multilayer flasks. mESCs were either passaged 5 times under feeder-free conditions (A) or cultured on a MEF feeder layer (B). Error bars indicate standard deviation.

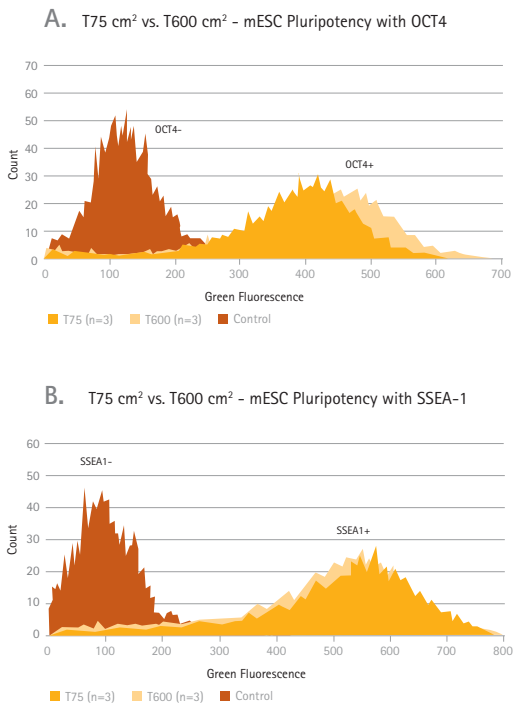
To calculate percent recovery, we first determined the number of cells recovered from single-layer T75 flasks. The actual surface area of a T600 prototype flask is equivalent to 8 T75 flasks, so we determined the theoretical cell yield to be 8 T75 flask-equivalents. The average cell recovery of mESCs passaged 5 times (feeder-free) in T600 multilayer flasks was equivalent to 6.72 (standard deviation = 1) T75 flasks (Figure 3A), which corresponded to 84% yield (standard deviation = 12%). The average cell recovery of mESCs cultured in T600 multilayer flasks with a feeder layer was equivalent to 7.1 (standard deviation = 0.3) T75 flasks (Figure 3B), which corresponded to 89% yield (standard deviation = 4%).

Figure 3. Recovery comparison between mESCs grown in three T75 single-layer flasks and mESCs grown in flasks from three lots of T600 multilayer flasks. mESCs were either passaged 5 times under feeder-free conditions (A) or cultured on a MEF feeder layer (B).



Using multiparametric flow cytometry analysis, we evaluated pluripotency of mESCs after 5 feeder-free passages in either T75 flasks or T600 multilayer flasks by staining for the pluripotency markers SSEA-1 (Figure 4A) and Oct4 (Figure 4B). On average, 92% (standard deviation = 1%) of mESCs from T75 flasks were SSEA-1 positive and 86% (standard deviation = 5%) were Oct-4 positive. mESCs passaged in T600 multilayer flasks were also 92% SSEA-1 positive (standard deviation = 1%) and 86% Oct4 positive (standard deviation = 5%). Clearly, passaging mESCs in multilayer flasks, while saving time and reagents, did not affect cell pluripotency at all.

Figure 4. Comparison of pluripotency of mESCs passaged 5 times (feeder-free) in T75 flasks or T600 multilayer flasks. Cells were stained with anti-SSEA-1 (A) and anti-Oct4 (B) to measure pluripotency. Unstained cells were used as negative control.



Cell Recovery

Table 1. A comparison of the number of T75 and HY T600 flasks needed to achieve a higher recovery of viable cells for mESCs cultured with and without MEF.

Experiment	Flask type	Average number mESCs recovered	T75 equivalents
mESC cultured with MEF	T75	2.6E+07	–
	T600	1.8E+08	7
mESC cultured without MEF	T75	1.7E+07	–
	T600	1.1E+08	6

Conclusion

The advantages of using Millicell HY multilayer culture flasks over using traditional single-layer cultureware for mouse embryonic stem cell culture include:

1. less time required when harvesting from a single flask rather than numerous culture vessels,
2. less incubator space required, and
3. uniform cell health and culture conditions, resulting in robust cell proliferation, yield, and pluripotency in comparison to culture in traditional single-layer flasks.

Millicell HY flasks have easy access for aspirating, dispensing, and pouring, which improves the overall handling and yield compared to other currently available multilayer cell culture devices.

Ordering Information

Description	Catalogue No.
Millicell HY 3-Layer Cell Culture Flask, T600, sterile	PFHYS0616
Millicell HY 5-Layer Cell Culture Flask, T1000, sterile	PFHYS1008
129/S6 Murine Embryonic Stem Cells	SCR012
Complete ES Cell Medium	ES-101-B
guava ViaCount Kit	4500-0110
Goat Serum	S26-100ML
Anti-Oct4	MAB4419
Anti-SSEA-1	MAB4301
guava easyCyte Flow Cytometer	0500-4008 and others
Sterile Phosphate-Buffered Saline	BSS-1006-A

Trypsinization Protocol

Harvest cells from T75 and HY T600 flasks

1. Wash each flask 2X with sterile PBS
   a. Add PBS to each flask
      i. 10 mL for a T75 flask
      ii. 80 mL for HY T600 flask
   b. Remove PBS from each flask
      i. Aspirate media from T75 flasks and HY T600 flasks
2. Lift cells by adding cell dissociation reagent (TrypLE) to each flask and incubating at 37°C
      i. T75 flask: 3 mL of cell dissociation reagent for 3-5 minutes
      ii. HY T600 flask: 24 mL of cell dissociation reagent for roughly 15-20 minutes
      iii. Visualize under microscope until cells are balled up
3. Add mESC media to each flask to inactivate cell dissociation reagent (TrypLE)
      i. 10 mL for a T75 flask
      ii. 76 mL for HY T600 flask
4. Mix cell suspension within flasks
      i. T75 flask: use a pipette
      ii. HY T600 flask: use a pipette or turn the flask on its side and back 4-5 times

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