# Large-Scale Expansion of Mouse Embryonic Stem Cells on Microcarriers

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**Running Head:** Large-scale expansion of mouse ES cells

**Abstract**

A large-scale stirred culture system for the expansion of mouse embryonic stem (mES) cells on spinner flasks under serum-free conditions was established using macroporous microcarriers for cell attachment and growth. This type of microcarriers was chosen since they potentially offer more protection to cells against shear stress in the absence of serum compared to microporous ones. In addition, methods to characterize ES cells after large-scale expansion were established. The pluripotency of expanded mES cells was evaluated based on both flow cytometry and alkaline phosphatase staining. Envisaging the application of ES cells as a potential source of neural progenitors, the neural commitment potential of cells after expansion in the spinner flask was also determined by culturing cells on serum-free adherent monolayer conditions.

**Key Words:** Embryonic stem cells; expansion; large-scale; microcarriers; serum-free medium; neural commitment; spinner flask; pluripotency.

## 1. Introduction

Embryonic stem (ES) cells are pluripotent cells which have the capacity for self-renewal and that can give rise to differentiated cells of the three embryonic germ layers: ectoderm, endoderm and mesoderm ***(1)***. ES cells derivatives are potentially very attractive for many applications in cellular therapies ***(2)***, tissue engineering ***(3)*** and drug screening ***(4, 5)*** and can be potentially used as a reliable alternative to animal models. In particular, the generation of pure populations of neural progenitors from ES cells and their further differentiation into neurons, astrocytes and oligodendrocytes ***(6)*** allows the potential use of these cells for the cure of neurodegenerative diseases and for neural drugs testing. However, this type of applications requires a wide available source of

both undifferentiated ES cells and their neural differentiated derivatives, which constitutes an enormous challenge in terms of the large-scale *in vitro* expansion and controlled differentiation of ES cells.

Standard procedures for the expansion of ES cells rely on the use static culture systems such as T-flasks and tissue-culture Petri dishes. However, these systems have serious limitations concerning their non-homogeneous nature, resulting in concentration gradients in the culture medium ***(7)***, and are also limited in their productivity by the number of cells that can be supported by a given surface area ***(8)***. In order to circumvent these limitations, the scale-up of mouse ES cell expansion has been performed using simple laboratory scale stirred bioreactors, the so-called spinner flasks.

Mouse ES cells have been expanded in this type of bioreactors both in the form of aggregates ***(9)*** or using microporous microcarriers ***(10, 11)*** for cell attachment. In general, these procedures rely on the use of serum-containing medium supplemented with leukemia inhibitory factor (LIF). However, this medium can cause cells to acquire karyotypic changes due to the presence of fetal bovine serum (FBS) which is poorly defined and potentially exposes ES cells to animal pathogens ***(12)***. Several attempts have been made in order to develop serum-free formulations that are capable of maintaining ES cell properties during expansion. A specific complete serum-free medium was developed that maintains the undifferentiated state of mES cell during prolonged expansion, even from single cells ***(12)***. In addition to LIF, this medium is supplemented with bone morphogenetic protein 4 (BMP4), whose molecular signals are necessary for suppression of neural differentiation in the absence of serum ***(12)***. mES cells can also be expanded under serum-free conditions using a proprietary serum replacement designed to directly replace FBS. Nevertheless, serum-free conditions potentially exacerbate the harmful effects of shear stress on cultured cells ***(13)***. To

circumvent these harmful effects, the use of macroporous microcarriers can be advantageous in offering a more protective environment to cells ***(14)***, favouring cell expansion. This strategy was recently followed with success for the scale-up of mES cell expansion under serum-free conditions ***(15)*** and will be described in this chapter. As a model cell line, 46C mES cells were used. In this cell line, the open reading frame of the *Sox1* gene, an early marker of the neuroectoderm in the mouse embryo, is replaced with the coding sequence for the Green Fluorescent Protein (GFP) which allows the monitoring of neural commitment (Sox1-GFP expression) by fluorescence microscopy and flow cytometry ***(16, 17)***.

## 2. Materials

### *2.1 mES cell thawing and expansion under static conditions prior to spinner flask inoculation*

1. Phosphate buffered saline (PBS) solution. This solution is made by dissolving PBS powder (Gibco) in 1 L of water. The solution is filtered and stored at room temperature.
2. Knockout Dulbecco's modified Eagle's medium (KD-MEM) (Gibco) supplemented with 15% knockout serum replacement (KSR) (Gibco), 1% Glutamine 200 mM (Gibco), 1% Penicillin (50U/ml)/Streptomycin (50 μg/ml) (Gibco), 1% non-essential amino acids 100 X (Sigma), 0.1% 2-mercaptoethanol 0.1 mM (Sigma) (store this solution for up to 15 days at 4 ºC) and supplemented with human LIF produced by 293-HEK cells (*see* **Note 1**). This medium should be stored at 4ºC during only one week and make fresh when necessary. Exposition of KSR or serum-free complete media to light and to 37ºC water bath should be minimized.
3. Serum-free ESGRO complete clonal-grade medium (Millipore). This medium is stored in aliquots at -20ºC and protected from light.

4. Solution of trypsin 0.025%. Prepare 50 mL with 500 μL of trypsin 2.5% (Gibco), 650 μL of ethilendiamine tetraacetic acid (EDTA) (Sigma) 0.1 M and 50 μL of heat-inactivated chicken serum (Sigma) in PBS. This solution is sterilized by filtration and stored in aliquots at -20 ºC and can be maintained at 4 ºC after thawing.

5. Accutase® solution (Sigma) (*see* **Note 2**). Store at 4 ºC after thawing.

6. 0.1% Gelatine solution. Dilute 12.5 mL of gelatine 2% (Sigma) in 237.5 mL of PBS and store at 4ºC.

7. 0.1% Trypan blue dye solution. Prepared from a 0.4% trypan blue dye solution (Gibco) by diluting in PBS (*i.e.* 1mL trypan blue : 3mL PBS). Store at room temperature.

**2.2 *mES cell expansion under stirred conditions***

1. Spinner Flask *Stem Span* (StemCell Technologies) of 50 mL working volume equipped with an impeller with 90º paddles (normal paddles) and a magnetic stir bar.

2. Microcarriers *Cultispher®* S (Sigma). Store at room temperature.

***2.3 Cell culture monitoring in the spinner flask***

***2.3.1 Cell Counts and viability***

1. PBS solution.

2. Solution of trypsin 1% in PBS. Prepare 37.5 mL with 15 ml of 2.5% trypsin solution (Gibco), 1.5 mL chicken serum (heat-inactivated) (Gibco) and 1.5 mL 0.1 M EDTA (Sigma) in PBS. The solution is filter-sterilized and stored in 10 mL aliquots at -20ºC. After thawing, the trypsin aliquots can be maintained at 4 ºC.

3. 0.1% Trypan blue dye solution.

**2.3.2 Determination of the expression of pluripotency markers**

1. PBS solution.

2. PBS 10X solution. This solution is made by dissolving PBS powder (Gibco) in 100 mL of water. The solution is filtered and stored at room temperature.

3. 2% Paraformaldehyde (PFA) solution. Dissolve 2 g of PFA in 100 mL of PBS (*see* **Note 3**). Filter before use and maintain at 4 ºC.

4. 3% Normal goat serum (NGS) solution in PBS. Dilute 3 mL of NGS in 97 mL of PBS. Filter before use and maintain at 4 ºC.

5. 1% NGS solution in PBS. This solution can be made by diluting 10 mL of 3% NGS in 20 mL of PBS. Store at 4 ºC.

6. 5% Bovine serum albumin (BSA) in PBS. Dilute 1 mL of BSA solution 30% (Sigma) in 5 mL of PBS. Filter before use and store at 4 ºC.

7. 1% saponin solution in PBS. Dissolve 1 g of saponin (Sigma) in 100 mL of PBS. Filter before use and store at 4 ºC.

**2.3.3 Alkaline phosphatase staining**

1. Solution of 10% cold neutral-buffered formalin (Sigma; store at RT).

2. Aliquots of Fast Violet Solution: one capsule of Fast Violet B Salt (Sigma; store at 4ºC) in 48 mL Mili-Q water. Make aliquots and store at -20ºC.

3. Naphthol AS-MX Phosphate Alkaline Solution 0.25% (Sigma; store at 4ºC)

4. Reagent X: Add 4% (v/v) Naphthol AS-MX Phosphate Alkaline Solution 0.25% to a pre-thawed aliquot of Fast Violet Solution. Protect from light and use immediately.

**2.3.4 Neural Commitment of mES cells**

1. Neural differentiation medium RHB-A (Stem Cell Sciences). Store at 4 ºC or in aliquots at -20 ºC. Avoid freeze-thaw cycles and protect from light.

**2.3.5 Flow Cytometric quantification of neural conversion**

1. Solution of 4% FBS in PBS. Dilute 4 mL of FBS (Gibco) in 96 mL of PBS. This solution should be filtered before use and stored at 4 ºC.

2. FACSCalibur flow cytometer (Becton Dickinson Biosciences).

3. FACSFlow™ sheath fluid (BD Biosciences).

## 3. Methods

### *3.1 mES cell thawing and expansion under static conditions*

1. A cryogenic vial of frozen 46C mES cells (approximately 1 mL) is removed from liquid nitrogen and promptly thawed in a 37 ºC water bath.

2. The content of the cryogenic vial is resuspended in 4 mL of pre-warmed culture medium (ESGRO complete or KD-MEM supplemented with KSR).

3. Cell suspension is centrifuged at 1000 rpm for 2 minutes, the supernatant is discarded and the pellet is resuspended in 5 mL of pre-warmed (37 ºC) culture medium (ESGRO complete or KD-MEM supplemented with KSR).

4. Cell suspension is seeded on a 60 mm tissue culture plate previously coated with gelatine 0.1% during at least 15 minutes.

5. Cells are expanded during 48 hours at 37 ºC under a 5% $CO_2$ fully humidified atmosphere.

6. After expansion during 48 hours, cells are washed twice with PBS and incubated with Accutase® or with trypsin 0.025% at 37ºC during 2 minutes (*see* **Note 2**).

7. After detachment, the suspension of cells in trypsin 0.025% or Accutase® is diluted in culture medium (*see* **Note 4**) and centrifuged during 2 minutes at 1000 rpm.

8. Cells are resuspended in serum-free expansion medium and re-plated at a density of approximately $2x10^4$ cells/$cm^2$.

9. After 48 hours of expansion, the passaging procedure is repeated. In each passage, viable and dead cells are determined by counting in a hemocytometer under an optical microscope using the trypan blue dye exclusion test.

### 3.2 *mES cell expansion under stirred conditions*

1. Before cell expansion, *Cultispher®* S microcarriers (*see* **Note 5**) are hydrated overnight, sterilized by autoclaving (20 minutes at 120 ºC) and equilibrated in pre-warmed (37 ºC) serum-containing medium (D-MEM with 10% FBS) during at least 12 hours (*see* **Note 6**).

2. 46C mES cells ($5x10^4$ cells/mL) previously expanded for at least 2 passages (*see* **Note 7**) under static conditions in 60 mm culture plates are mixed with 1 mg/mL of *Cultispher®* S microcarriers.

3. Cells and microcarriers are incubated at 37 ºC in 1/6 of the final medium volume (5 mL) during 30 minutes, with gentle agitation every 10 minutes.

4. Fresh pre-warmed (37 ºC) medium is gently added until half of the final volume (15 mL) and cell suspension is transferred to the spinner flask.

5. After a 24 hour seeding period with intermittent stirring (15 minutes of stirring at 30-40 rpm followed by 60 minutes statically), medium is added up to the final volume (30 mL) and the speed adjusted to 40 rpm.

6. Feeding is performed every day by replacing 50% of the medium with fresh prewarmed medium (*see* **Note 8**).

**Fig. 2.1** shows a scanning electron microscopy ***(18)*** of 46C mouse ES cells adherent to *Cultispher®* S microcarriers after expansion on the spinner flask.

### *3.3 Monitoring of cell culture in the spinner flask*

#### *3.3.1 Cell Counts and viability*

1. Everyday, duplicate samples of an evenly mixed culture are collected from the spinner flask (0.5 mL).

2. After the microcarriers settle down, 0.3 mL of the supernatant is removed. The microcarriers are washed with pre-warmed (37ºC) 2 mL of PBS and incubated until complete microcarrier dissolution in a 37 ºC water-bath after adding 0.8 mL of trypsin 1% (*see* **Note 9**).

3. After dissolution of the microcarriers, 1 mL of 0.1% trypan blue solution is added and the viable and dead cells are determined on a hemacytometer.

**Fig. 2.2** shows the expansion of 46C mouse ES cells on the *Cultispher® S* microcarriers in the spinner flask system during 8 days both in terms of viable cells per millilitre and total cell fold increase. Two different serum-free media were compared in terms of cell expansion, the KD-MEM supplemented with KSR and the ESGRO complete clonal-grade medium.

***3.3.2 Determination of the expression of pluripotency markers***

After expansion in the spinner flask during 8 days under serum-free conditions, cell suspension is analysed for the presence of antibody markers specific of mouse ES cells by flow cytometry after intracellular (Oct4 and Nanog) or surface (SSEA1) staining.

1. Detach cells from the microcarriers using trypsin 1% for 15-20 minutes, centrifuge cell suspension for 7 minutes at 1250 rpm, perform a cell count and centrifuge again.

2. Wash cells with 10 mL of PBS, centrifuge for 7 minutes at 1250 rpm, resuspend the pellet in 10 mL of 2% PFA solution and incubate at room temperature for 20 minutes (*see* **Note 10**).

3. Centrifuge PFA-fixed cells for 7 minutes at 1250 rpm, wash with 5 mL of 1% NGS and centrifuge again for 7 minutes at 1250 rpm. This procedure should be performed twice.

4. Resuspend cells in 3% of NGS (0.5 mL for each antibody and negative control tested).

5. Transfer cell suspension to 1.5 mL tubes previously coated with 1 mL of 5% BSA solution during at least 15 minutes, centrifuge the tubes at 1000 rpm for 3 minutes and aspirate the supernatant from each tube carefully.

6. Resuspend cell pellet with blocking solution and/or detergent: 150 µL of 3% NGS and 150 µL of 1% saponin for intracellular staining and 300 µL of 3% NGS for surface staining.

7. Incubate at room temperature for 15 minutes.

8. Centrifuge all 1.5 mL tubes at 1000 rpm for 3 minutes at room temperature, remove the supernatant, resuspend in 300 µL of 3% NGS and incubate at room temperature for 15 minutes.

9. Centrifuge all tubes at 1000 rpm for 3 minutes at room temperature, carefully aspirate the supernatant and add the primary antibody diluted in 3% NGS in a final volume of 300 µL (in the case of the negative controls, cells are maintained in 3% NGS).

10. Incubate the tubes for 1 hour and 30 minutes at room temperature protected from light.

11. Centrifuge all tubes at 1000 rpm for 3 minutes at room temperature and resuspend the pellet with 500 µL of 1% NGS. This procedure should be performed twice. This step does not apply to the negative control tube.

12. Add the secondary antibody diluted in 3% NGS to all tubes in a final volume of 300 µL and incubate the tubes for 45 minutes at room temperature in the dark.

13. Centrifuge all tubes at 1000 rpm for 3 minutes at room temperature and resuspend the pellet with 500 µL of 1% NGS. This procedure should be performed two more times.

14. Add 0.5 mL of PBS, transfer the resuspended pellets to flow cytometry tubes and perform flow cytometry analysis (*see* **Note 11**).

**Fig. 2.3A** shows an example of the expression profiles obtained by flow cytometry for 46C ES cells expanded during 8 days in the spinner flask obtained after intracellular staining with the anti-Oct4 and Nanog antibodies and after surface staining with the anti-SSEA1 antibody. In this example, more than 95% of the 46C ES cells analysed expressed the three different pluripotency markers.

***3.3.3 Alkaline phosphatase staining***

In addition to flow cytometry analysis, the pluripotency of mES cells after expansion in the spinner flask can also be determined based on the activity of alkaline phosphatase.

1. Samples of microcarriers containing mES cells were washed with PBS and fixed in 10% cold neutral-buffered formalin for 15 minutes.

2. After fixing, cells were washed and kept in distilled water for another 15 minutes.

3. Following the washing step, the cells were incubated with the Reagent X for 1 hour in the dark and washed 3 times in distilled water.

4. Cells were kept in distilled water and observed under an optical microscope.

**Fig 2.3B** shows 46C ES cells adherent to the *Cultispher®* S microcarriers after alkaline phosphatase staining after 8 days in culture.

***3.3.4 Neural commitment of 46C mES cells***

The neural commitment potential of 46C ES cells is determined by plating the cells obtained after expansion in the spinner flask under serum-free adherent monolayer conditions ***(16, 17)***.

1. Samples of microcarrier-containing cells are taken from the spinner flask and dissolved with 1% trypsin.

2. After the complete dissolution of the microcarriers, cells are expanded at a relatively high density ($10^5$ cells/cm$^2$) during 24 hours in expansion medium ESGRO complete ***(17)***.

3. After expansion at high density, cells are detached from the plate with trypsin 0.025% or Accutase® (*see* **Note 2**), resuspended in serum-free RHB-A medium and re-plated in 2 wells of a 12-wells plate (1 mL/well) pre-coated with gelatine 0.1% (*see* **Note 12**) at a density of $10^4$ cells/cm$^2$.

4. The medium is changed every two days.

***3.3.5 Flow cytometric quantification of neural conversion***

The use of 46C mES cells allows the quantification of neural conversion by flow cytometry based on GFP expression ***(16)***.

1. After 6 days in culture with neural differentiation medium, cells are detached from the plate and resuspended in PBS containing 4% of FBS.

2. Cells are analysed by flow cytometry using appropriate analysis software (e.g. CellQuest, BD Biosciences). Gates are set at 10 units of fluorescence, which excludes more than 99% of undifferentiated cells. Cell debris and dead cells are excluded from the analysis based on electronic gates using the forward scatter (size) and side scatter (cell complexity) criteria. Undifferentiated 46C ES cells are used as negative control. **Fig 2.4** shows a typical flow cytometry profile of 46C ES cells after expansion in the spinner flask using serum-free ESGRO complete medium followed by neural commitment during 6 days under adherent monolayer conditions in RHB-A medium. In this example, more than 90% of the 46C ES cells expanded in the spinner flask underwent neural commitment, assessed by GFP expression along FL1 axis.

## 4. Notes

1. Human LIF was produced in HEK-293 cells. The optimal dilution of LIF for ES cells expansion was previously determined by testing the effect of different dilutions of LIF on standard ES cell cultures. The optimal dilution was determined based either on ES cell fold increase, expression of pluripotency markers (Oct4, SSEA1 and Nanog) and direct microscope observation of ES cell morphology. LIF formulations (ESGRO, Millipore) can also be used to supplement ES cell expansion medium (1000 U/mL).

2. When mouse ES cells are cultured in the absence of serum, Accutase® or a low concentrated trypsin solution (0.025%) should be used for passaging in order to preserve cell attachment properties. This step is especially critical when cells are plated for neural differentiation.

3. PFA powder should be firstly dissolved in a low volume of water at a high temperature (lower than 70 ºC) in order to facilitate dissolution. Then pH should be set to 7.3 and the final volume completed with PBS 10X.

4. When using trypsin for cell detachment, its action must be stopped using serum-containing medium which typically contains trypsin inhibitors. When using Accutase® for cell detachment, cell suspension can be diluted in serum-free medium and centrifuged immediately.

5. *Cultispher®* S microcarriers are spherical macroporous microcarriers made of gelatine. Other commercially available microcarriers, namely microporous, can also be successfully used to support the expansion of mouse ES cells under stirred conditions ***(10)***.

6. The adhesion efficiency of mES cells to microcarriers under dynamic conditions is greatly improved if the microcarriers are previously incubated in serum-containing medium. Indeed, cells are dependent on adhesion factors that are absent from serum-free media.

7. Before initiating ES cell expansion on microcarriers under stirred conditions, cells are expanded under static conditions at least during 2 passages in order to obtain enough cells to inoculate the spinner flask. Moreover, if cells are previously expanded under serum-containing medium, it will be necessary to adapt cells to serum-free conditions during several passages before beginning expansion under stirred conditions.
8. The removing/replenishment of the culture medium is performed immediately after the sedimentation of the microcarriers containing cells during 10-15 minutes.
9**.** Cells can be released from the *Cultispher®* S microcarriers by completely digesting the gelatine matrix. To perform this digestion, a more concentrated trypsin solution should be used (1%). Occasional flicking must be performed in order to facilitate gelatine matrix digestion. When cells attain large densities on the microcarriers, it becomes difficult to dissolve the gelatine matrix even when using this concentrated trypsin solution. In this case, it may be necessary to use longer incubation times. In alternative, a microcarrier dissolution protocol *in situ* (*i.e.* inside the spinner flask) was successfully tested. After the microcarrier-containing cells settle-down, the supernatant is removed and 30 mL of PBS is used to wash the cells/microcarriers. After the washing step, 15 mL of trypsin 1% is added and the spinner flask put into the incubator under agitation until the microcarriers are dissolved. After the dissolution of the microcarriers, 45 mL of medium-containing serum are added to stop trypsin action, and the cell suspension is removed from the spinner flask. It might be needed to use a cell strainer (*e.g.* 100 μm pore size) to separate the cells from the viscous gelatine matrix that did not dissolved completely.
10. If necessary, cells can be maintained during several days on 2% PFA at 4ºC until performing flow cytometry acquisition.

11. After addition of the secondary antibody, cell samples should be protected from light.

12. In order to improve cell attachment under serum-free conditions, prior to the neural commitment protocol, gelatine coating should be performed during at least 1 hour.

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