

Video Article

# Derivation of Stem Cell Lines from Mouse Preimplantation Embryos

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## Abstract

Mouse embryonic stem cell (mESC) derivation is the process by which pluripotent cell lines are established from preimplantation embryos. These lines retain the ability to either self-renew or differentiate under specific conditions. Due to these properties, mESC are a useful tool in regenerative medicine, disease modeling, and tissue engineering studies. This article describes a simple protocol to obtain mESC lines with high derivation efficiencies (60-80%) by culturing blastocysts from permissive mouse strains on feeder cells in defined medium supplemented with leukemia inhibitory factor. The protocol can also be applied to efficiently derive mESC lines from non-permissive mouse strains, by the simple addition of a cocktail of two small-molecule inhibitors to the derivation medium (2i medium). Detailed procedures on the preparation and culture of feeder cells, collection and culture of mouse embryos, and derivation and culture of mESC lines are provided. This protocol does not require specialized equipment and can be carried out in any laboratory with basic mammalian cell culture expertise.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/56171/>

## Introduction

Embryonic stem cells (ESC) are pluripotent cells derived from preimplantation embryos, which retain the ability to either self-renew or differentiate under specific conditions<sup>1</sup>. Based on these properties, ESC have become a useful tool for regenerative medicine, disease modeling, and tissue engineering studies<sup>2</sup>.

ESC were derived for the first time from preimplantation mouse embryos, originating mouse ESC (mESC) lines with low success<sup>3,4</sup>. For years, the derivation efficiency remained low due to the difficulty of maintaining pluripotency *in vitro*. Besides the presence of feeder cells<sup>5</sup>, which improve derivation efficiency, promote colony attachment, and enhance karyotypic stability<sup>6</sup>, several modifications of the protocol lead to an improvement in pluripotency maintenance. The most important were the addition of Leukemia Inhibitory Factor (LIF) to mESC culture medium<sup>7,8</sup>, the use of embryos from the 129S2 permissive background<sup>9</sup> and the culture of mESC with medium supplemented with defined serum, free of potential differentiation factors<sup>10</sup>. More recently, compelling evidence has shown that addition of a cocktail of two small-molecule modulators of signaling pathways to the mESC culture medium (known as 2i) is best to maintain pluripotency. The 2i cocktail consists of a combination of the MEK inhibitor PD0325901, which reduces the differentiation signals by inhibiting the mitogen-activated protein kinase (MAPK) pathway, and the GSK3 $\beta$  inhibitor CHIR99021, which enhances cell survival at low density by activating the Wnt pathway<sup>11</sup>.

In the present article, we describe a simple protocol to efficiently derive mESC lines from mouse blastocysts. We follow standard procedures, culturing embryos from permissive strains on feeder cells in derivation medium supplemented with LIF and a serum replacement<sup>12</sup>. Following this protocol, mESC lines can be derived with efficiencies equivalent to those obtained in 2i medium. In spite of this, 2i can be added to avoid possible problems with pluripotency maintenance or when working with non-permissive strains.

## Protocol

The use of the animals described in the sections below has been approved by the Ethics Committee on Animal and Human Research of the Universitat Autònoma de Barcelona and by the Departament d'Agricultura, Ramaderia, Pesca i Alimentació of the Generalitat de Catalunya (protocol #8741).

### 1. Feeder Cell Inactivation and Storage

NOTE: Human foreskin fibroblasts (HFF-1) were previously frozen in 1 mL of freezing solution consisting of 90% Fetal Bovine Serum (FBS) and 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen until use.

1. Thaw a cryogenic vial of HFF-1 (1 mL) by immersing the vial in a 37 °C water bath.

2. Add the thawed contents of the cryogenic vial to pre-warmed Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS to make a 1:10 dilution.
3. Seed the diluted cells (10 mL) in a T75 flask and culture them at 37 °C and 5% CO<sub>2</sub>.
4. The next day, change the medium to eliminate the DMSO remnants. Remove the medium and add 10 mL of pre-warmed DMEM supplemented with 10% FBS.
5. Culture the HFFs and change the medium every 48 h until the culture reaches the confluent stage. This may take approximately 7 to 10 days.
6. To prepare the inactivation solution, dilute mitomycin C in DMEM supplemented with 10% FBS at a final concentration of 10 µg/mL. Incubate the cells with the inactivation solution for 3 h at 37 °C and 5% CO<sub>2</sub>.
7. Remove the inactivation solution and rinse the cells thrice with 2 mL of Hanks Balanced Salt Solution (HBSS) to eliminate mitomycin C remnants.
8. To detach the inactivated cells, add 1 mL of pre-warmed Trypsin-EDTA solution and incubate for 5 min at 37 °C and 5% CO<sub>2</sub>.
9. Observe the cells under an inverted microscope to ensure they are detached (10x objective with 0.3 numerical aperture or NA). If they are not, incubate them for a couple more minutes at 37 °C and 5% CO<sub>2</sub>. Check the cells again under the inverted microscope.
10. Pipette the solution with a Pasteur pipette in order to dissociate the cell clumps.
11. Neutralize the enzymatic solution by adding 4 mL of DMEM supplemented with 10% FBS and resuspend.
12. **Take an aliquot of the cell suspension and make a 1:100 dilution in HBSS. To determine the number of cells, load 10 µL of the dilution into a Neubauer's chamber. Count the cells located in one of the big squares of the chamber under an inverted microscope (20x objective with a 0.45 NA).**
  1. Repeat the counting in a total of four big squares. Calculate the total number of cells (N) as  $N = \text{average number of cells counted} \times \text{total volume} \times \text{dilution} \times 10^4$ .
13. Centrifuge the rest of the sample for 5 min at 200 x g. Discard the supernatant and resuspend the pellet with freezing solution at a concentration of 10<sup>6</sup> cells per mL.
14. Aliquot the suspension in cryogenic vials with 10<sup>6</sup> cells (1 mL) each. Place the cryogenic vials in a freezing container and freeze at -80 °C.
15. The next day, store the cryogenic vials in liquid nitrogen.

## 2. Feeder Cell Culture

1. One to four days before starting the derivation process, thaw a cryogenic vial containing 1 x 10<sup>6</sup> inactivated HFF-1 cells in a water bath at 37 °C.
2. Make a 1:6 dilution of the thawed HFF-1 in pre-warmed DMEM supplemented with 10% FBS and keep the temperature at 37 °C.
3. Fill every well of a 4-well plate with 500 µL of pre-warmed 0.2% gelatin from porcine skin and keep the plate at 37 °C for 10 min.
4. Remove the 500 µL of gelatin from the wells and add 500 µL of the HFFs dilution. Gently rock the plate to ensure a uniform distribution of the HFFs on the bottom of the wells. Avoid circular movements, otherwise the cells will concentrate at the center of the well.
5. Incubate the plate at least 24 h at 37 °C and 5% CO<sub>2</sub> to obtain a monolayer of inactivated HFFs in each well.
6. The next day, observe the cells under an inverted microscope (10X objective with 0.3 NA) and check that they are attached and homogeneously distributed. Change the medium to eliminate the DMSO remnants. Remove the medium and add 500 µL of pre-warmed DMEM supplemented with 10% FBS.

## 3. Embryo Collection and Culture

1. Four days before embryo collection, administer 5 IU of pregnant mare's serum gonadotropin by intraperitoneal injection to 6-12 week old female mice.  
NOTE: We have used females from the 129S2 or B6CFAF1 strains, though females from other mouse strains can be used. Nevertheless, when working with females from non-permissive strains (e.g. CBA), medium should be supplemented with 2i as mentioned in step 4.2.
2. After 48 h, administer 5 IU of human chorionic gonadotropin by intraperitoneal injection, and mate the females with males, at least 8 weeks old, at a 1:1 ratio. Separate the females from the males the next day.
3. The day before embryo collection, prepare a 35-mm cell culture Petri dish with several 40 µL drops of KSOMaag medium supplemented with 4 mg/mL Bovine Serum Albumin (BSA) and fill the plate with mineral oil to fully cover the drops. Incubate the dish at 37 °C and 5% CO<sub>2</sub> to equilibrate the medium.
4. To prepare embryo manipulation pipettes, heat the thinner part of a long-tipped glass Pasteur pipette using a Bunsen burner. Once soft, withdraw it from the flame and pull the two ends apart. Break it to the desired length. The inner diameter at the tip should be 100-150 µm.
5. Euthanize the female mice by cervical dislocation 45-48 h *post-coitum* (p.c.).
6. Dissect the mice to expose the abdominal cavity by cutting the skin with curved smooth sharp scissors and the peritoneum with straight sharp scissors. Grab one uterus with forceps, cut off the oviduct using straight sharp scissors, and place it in Hepes-buffered CZB medium (HCZB)<sup>13</sup> at 37 °C. Repeat the process to remove the other oviduct.
7. Flush the oviducts with a 1 mL syringe loaded with HCZB held in place by watchmaker forceps to collect the 2-cell embryos.  
NOTE: To prepare the flushing needle take a 30 G hypodermic needle, cut the end, and ground to a blunt tip on an abrasive stone.
8. Using the mouth pipette apparatus, pick up the embryos, wash them in HCZB and culture them in the previously prepared embryo culture dish at 37 °C and 5% CO<sub>2</sub> until the blastocyst stage. Up to 50 embryos can be cultured in each 40 µL drop. Monitor embryo development every 24 h under a stereomicroscope.  
NOTE: Alternatively, embryos can be collected at the blastocyst stage to shorten *in vitro* embryo culture. In this case, euthanize the females at day 3.5 - 4.5 p.c., dissect the uterus. and flush it with HCZB.

## 4. Stem Cell Derivation

1. **Preparation of the derivation plates**

1. On the day of embryo seeding, prepare the derivation medium by supplementing DMEM medium with 100  $\mu$ M 2- $\beta$  mercaptoethanol, 1x Non-Essential Amino Acids, 20% of serum replacement, and  $10^{-3}$  U/mL LIF.
2. If desired, also add 1  $\mu$ M of the MEK inhibitor PD0325901 and 3  $\mu$ M of the GSK3 inhibitor CHIR99021 to obtain a 2i medium.
3. Take the 4-well plate with the monolayer of inactivated HFFs (step 2.6) from the incubator. Remove the medium and add 800  $\mu$ L of pre-warmed derivation medium to each well. Return the plate to the incubator.
2. **Zona pellucida removal and embryo seeding**
  1. Prepare a 35 mm cell culture Petri dish with three 30  $\mu$ L drops of acidic Tyrode's solution<sup>14</sup> and three 30  $\mu$ L drops of derivation medium. Cover the drops with mineral oil and keep the dish at 37 °C.
  2. Take the embryo culture dish from the incubator. One by one, transfer the blastocysts from the culture drops to an acidic Tyrode's solution drop using an embryo manipulation pipette. If possible, select only the expanded blastocysts to start the derivation process.
  3. Monitor the zona pellucida digestion under the stereomicroscope. As soon as the zona pellucida disappears, transfer the blastocyst to a derivation medium drop in order to wash out the acidic Tyrode's solution.
  4. Take the 4-well derivation plate from the incubator and seed one zona-free blastocyst on the feeder cells in each well.
  5. Return the 4-well derivation plate to the incubator and culture at 37 °C and 5% CO<sub>2</sub>.
3. **Monitoring stem cell growth and medium change**
  1. Monitor the cultures every 24 h under an inverted microscope (20X objective with 0.45 NA). It is important to note whether there are signs of stem cell differentiation, such as swollen refractive cells surrounding the outgrowth or even pushing the feeder cells aside.
  2. Every other day, change the medium of the culture. Take the 4-well plate from the incubator, remove the medium from each well, and add 800  $\mu$ L of pre-warmed derivation medium supplemented with LIF and 2i, if used. Maintain the culture until outgrowths are observed (6-8 days).

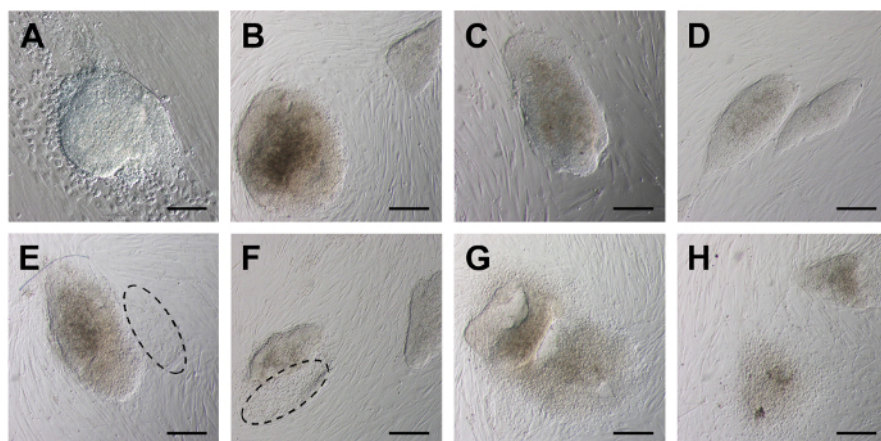
## 5. Stem Cell Culture and Maintenance

1. Prepare Kolle-like handles and stem cell manipulation pipettes. Follow step 3.4 to prepare stem cell manipulation pipettes with an inner diameter at the tip of 250-300  $\mu$ m. Use the other part of the pulled Pasteur pipette to prepare the Kolle-like handle. Heat and shape it until obtaining a hook.
2. Take the 4-well culture plate out of the incubator and locate the outgrowth under a stereomicroscope. Use the handle to push away the differentiated cells and the feeders that surround the outgrowth.  
NOTE: To avoid eventual differentiation of the outgrowth, it is important to remove all the differentiated cells from the edge of the outgrowth.
3. Aspirate the outgrowth with a stem cell manipulation pipette and place it in a new dish in a 50  $\mu$ L trypsin-EDTA drop.
4. Use a scalpel to cut the outgrowth in several parts in order to disaggregate it both mechanically and enzymatically.
5. Straight away, transfer the outgrowth fragments into a fresh drop of derivation medium to neutralize trypsin action.
6. Transfer the outgrowth fragments to a new 4-well plate with a monolayer of feeders. Transfer up to 10 fragments per well and culture them with derivation medium at 37 °C and 5% CO<sub>2</sub>.
7. Maintain the stem cell-like cultures for at least six weeks before considering the mESC line established. Change the medium every other day and subculture them once a week.

## Representative Results

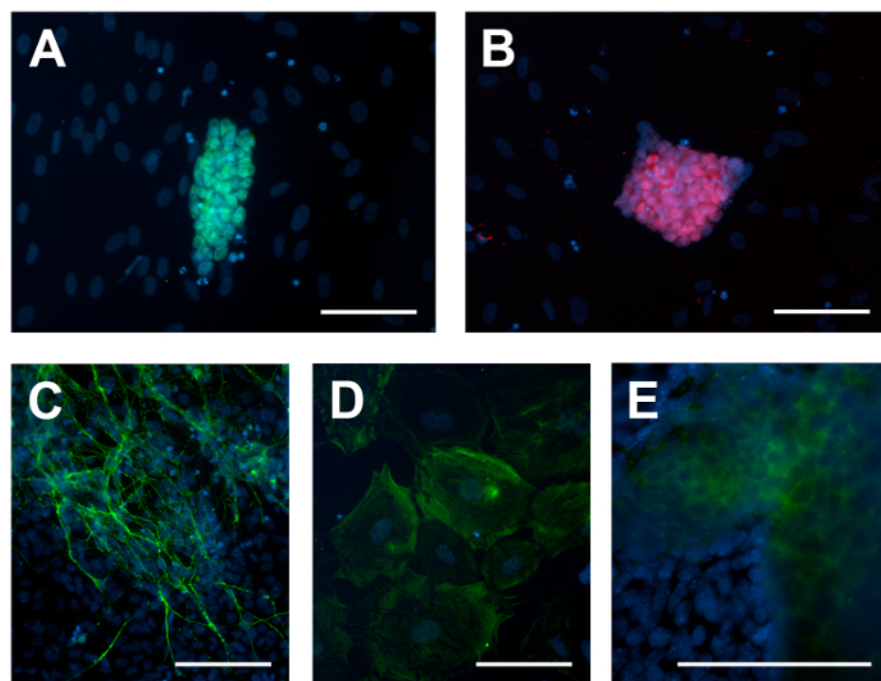
Following this protocol, over 95% of outgrowth formation should be achieved after the first week of culture (**Figure 1A**). Establishment of mESC lines after six passages is variable among experimental replicates, with an average success of 60-80%. The addition of 2i does not significantly improve the results when working with permissive mouse strains, such as those with a 129S2 background, but it is necessary when working with non-permissive strains.

Mouse ESC colonies should present a regular morphology with a flat shape and defined edges when cultured without treatment (**Figure 1B-C**) or cultured with 2i (**Figure 1D**). Colonies presenting peripheral differentiation signs should be discarded (**Figure 1E-F**). If the derivation efficiencies obtained are lower than the expected values, control the growth of mESC colonies and subculture them more often to avoid overgrowth, as this can cause cell death and would promote differentiation (**Figure 1G-H**).



**Figure 1: Representative images of mESC colonies in culture.** (A) Outgrowth derived from a blastocyst after the first week of culture (B, C) Several mESC colonies at passage 10 presenting defined edges and a flat shape. (D) Colonies from mESC lines treated with 2iL. (E, F) Examples of semi-differentiated mESC colonies presenting peripheral differentiation signs (highlighted with dashed ellipses). (G, H) Examples of overgrown mESC colonies presenting a bad morphology and differentiation signs. Scale bars = 100  $\mu$ m. [Please click here to view a larger version of this figure.](#)

To verify the stemness of the putative mESC lines obtained after six weeks of culture, the presence of pluripotency and differentiation markers should be assessed. The mESC lines should be positive for pluripotency markers such as Oct4 and Sox2 (**Figure 2 A-B**). Moreover, after induction of spontaneous differentiation by culturing the cells for 10 days without feeder cells in DMEM supplemented with 10% FBS, mESC lines are expected to be positive for the ectoderm marker  $\beta$ -tubulin class III (Tuj1) (**Figure 2C**), the mesoderm marker  $\alpha$ -Smooth Muscle Actin ( $\alpha$ SMA) (**Figure 2D**) and the endoderm marker Alpha-Fetoprotein (AFP) (**Figure 2E**). Only the lines positive for the five markers assessed should be considered true mESC lines and thus used for the calculation of the derivation efficiency. Usually, this corresponds to virtually all the mESC lines generated using the present protocol.



**Figure 2: Representative immunofluorescence against pluripotency and differentiation markers.** mESC colonies expressing (A) Oct4 (green) (B) and Sox2 (red). Differentiated mESC colonies expressing (C) Tuj1 (green), (D)  $\alpha$ SMA (green) and (E) AFP (green). In all images the nuclear material is counterstained with Hoeschst (blue). Scale bars = 100  $\mu$ m. [Please click here to view a larger version of this figure.](#)

## Discussion

Although mESC line derivation is a well-known procedure routinely used in many laboratories, its efficiency is not always as high as expected due to the multiple factors that can disturb pluripotency maintenance. In the present article we show, step by step, how to establish mESC lines with high efficiencies using a simple and reliable protocol. These efficiencies are similar to those reported in the literature.



To ensure maximum efficiency, it is important to control the cells daily or every other day, since the time points indicated are just illustrative. It is essential to avoid the overgrowth of colonies, since this promotes cell death and differentiation. It is also important to reduce as much as possible the exposure of the colonies to trypsin-EDTA during their subculture, because this can also reduce cell viability.

Another pivotal factor is the source of feeder cells and their culture. Several studies have demonstrated that HFF, Mouse Embryonic Fibroblasts (MEF) and mouse STO fibroblasts are equally able to support ESC derivation and culture<sup>15</sup>. However, HFF are up to two times more durable than MEF and STO in culture<sup>16</sup>, allowing more time for the mESC to grow. Thus, the use of HFF avoids extra mESC subcultures.

If the derivation efficiencies obtained are lower than the results reported, it is important to carefully check all the components of the derivation medium, especially serum replacement. Different batches of serum replacement can result in significant differences in the derivation efficiencies<sup>17,18</sup>. Therefore, each new batch must be tested prior to its use.

It is important to point out that the genetic background of the embryos used as a source for mESC derivation can also significantly affect the derivation efficiency. Indeed, mouse strains have been classified into permissive strains (such as 129S2 and C57BL6) and non-permissive strains (such as CBA, NOD and FVB) according to their ability to yield mESC lines under standard conditions<sup>9,19</sup>. The protocol reported here can be successfully applied to embryos from both types of strains as long as the 2i cocktail is included in the derivation medium when working with embryos from non-permissive strains. The 2i medium is able to modulate the signaling pattern of mESC lines to sustain pluripotency, irrespective of the genetic background<sup>6</sup>. The main limitation is that, due to the strong signaling pattern acquired during culture<sup>20</sup>, mESC lines cultured in 2i become resistant to differentiation. To overcome this limitation, mESC lines should be cultured without 2i for at least one passage before the induction of differentiation to weaken the signaling acquired.

Overall, this work can ease the practice of mESC derivation by showing the steps as simply as possible and pointing at the main difficulties and how to overcome them.

## Disclosures

The authors have nothing to disclose.

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