**ENprotTC001  
Culture of feeder-free mouse Embryonic Stem Cells (mESCs)**

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Summary of update from previous version: included conditions for 2i+Serum+LIF, updated various cat#

**FYI:** each cell line will have distinct requirements. Below are procedures you can use with E14 (or E14tg2A mESCs).

**Critical:** if you are unsure about the mycoplasma status of the vial you want to use (e.g. you just got it from a collaborator) you MUST isolate the culture in quarantine and test it as soon as possible. Do not share any media, pipets or even hoods with non-quarantine cultures.

**MATERIALS AND REAGENTS**

* 1X PBS, sterile pH~7 (Corning MT21030CV)
* TryplE express 1X, sterile (ThermoFisher cat#12605010)
* ES culture medium, sterile
* Tissue culture flasks (see cat# at the end of this protocol)
* Frozen mESCs tested negative for mycoplasma
* 0.1% Gelatin Solution Millipore ES-006-B EmbryoMax® ES Cell Qualified sterile - or make your own (below)

**Critical:** not all lots of serum are compatible with mESCS. Either use Serum characterized for mESC culture or test aliquots of lots before any critical experiment. Note when changes in lot number and avoid comparing experiments performed with cells grown in different batches (even if it is the same catalog number).

**“Serum +LIF” mESC Medium**

DMEM + Glutamax ThermoFisher 10566-016 **500 mL**

Fetal Bovine Serum (mESC-grade) ThermoFisher SH30071.03 **90 mL**

b-Mercaptoethanol (55mM) ThermoFisher 21985-023 **0.6 mL**

MEM Non-essential amino-acids (100X) ThermoFisher 11140-050 **6 mL**

Sodium pyruvate (100mM) ThermoFisher 11360-070 **6 mL**

LIF (ESGRO 10^7 U/mL) Millipore ESG1107 **60µL  
Filter sterilize, keep at 4°C**

**Note:** filtering optional but helps removing debris from the serum which occur in variable amounts depending in each bottle and will give better consistency in the healthiness of the cultures.

**“2i+Serum+LIF” mESC Medium**“Serum +LIF” mESC Medium above +1mM PD0325901 **600µL** +3mM CHIR99021 **600µL**

**2i inhibitors stocks:**

1mM PD0325901: 5mg Sigma PZ0162-5MG in **10.4ml** sterile **DMSO**, 630μl aliquots at -20°C

3mM CHIR99021: 25mg Sigma SML1046-25MG in **17.9ml** sterile **DMSO**, 630 μl aliquots at -20°C

**1% Gelatin solution (10X stock), sterile** [if not buyingMillipore ES-006-B]

Weight 5g of Gelatin powder Sigma G1890 (cat# is critical)

Add 500mL sterile tissue-culture grade H20 (e.g. Sigma W3500)

Autoclave and store at room temp or 4deg (will gelify until brought back to room temp)

**0.1% Gelatin working solution, sterile** [if not buyingMillipore ES-006-B]

55mL 1% Gelatin, autoclaved sterile

500mL sterile 1X PBS, sterile pH~7 (Corning MT21030CV)

store at room temp

**PROCEDURE**

**mESC thawing**

1| Place a vial of frozen E14 mESCs in the 37°C water bath until the cells begin to thaw.

2| Add 1 mL of warmed ES medium to the vial, resuspend gently the cells and transfer the cell suspension to a 15-mL tube containing 4 mL of warmed ES medium

3| Centrifuge for 5 min at 950rpm, RT.

4| Aspirate supernatant

5| Resuspend the cells gently with ES medium and transfer the cells to a gelatin-coated dish.

**mESC expansion**

1| Aspirate medium and replace with fresh pre-warmed ES medium until cells become 60% confluent. This should occur 1-3 d after thawing.

**Critical:** Change the medium every day. Cells are typically split every day or every other day. Avoid having to wait more than 48h.

**mESC passage**

1| Remove medium and rinse the cells with warmed 1X PBS. Aspirate the PBS and add TrypLE.

2| Incubate for 7-10 min at 37°C in the incubator.

3| Add ES medium, disaggregate the cells by pipetting and transfer 1/3 (around 1 million in a t25) or 1/6 (around 0.5 million in a t25) to a 15-mL tube, depending on desired dilution factor.

4| Centrifuge for 5 min at 950rpm, RT.

5| Aspirate the supernatant, add ES medium to the pellet, resuspend gently and transfer to a gelatin-coated dish.

**FYI:** E14 mESC colonies will look much better if flasks are gelatinized at least overnight. You can leave flasks with gelatin for up to a week in the 37C incubator if needed.

**mESC freezing**

1| proceed as for passaging until centrifugation.

2| Resuspend the cell pellet in freezing medium: 90% ES-grade Fetal Bovine Serum – 10% sterile DMSO (Sigma D2650-100ML)

3| Aliquot in externally threaded screw-cap cryotubes, with 0.5-1.5mL per tube. For reference, freezing aliquot of around 1.5 million cells (around ½ T25) can be thawed into one T25.

4| Label each tube with

1. Cell line ID#
2. Passage number
3. Number of cells or flask equivalent (e.g. ½ T25)
4. Date

5| Place the labeled cryotubes in a freezing container, transfer at -80C at least overnight. Transfer into liquid nitrogen for long-term storage as soon as possible. Significant cell death will occur after a few weeks at -80C.

FYI: You will thaw cells by plating the content of a vial on a surface equal to two or three times the area from which you initially detached them prior to freezing (e.g. if you froze 1/2 or 1/3 of a T25 you will thaw it in a T25).

**Recommended flasks**

**Critical:** Not all flasks will work with mESCs. We have had consistent success with these:

|  |  |  |
| --- | --- | --- |
| Ibidi Slides for microscopy (8-well) | Ibidi | 50305795 |
| 6 well plates | Costar | 657160 |
| 12 well plates | Costar | 665180 |
| 24 well plates | Costar | 662160 |
| 48 well plates | Costar | 677180 |
| 96 well plates | Costar | 655180 |
| 10cm plates | Costar | 664160 |
| T25 | Corning | 430639 |
| T75 | Corning | 430641U |

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Gelatin mL (coating)** | 0.5 | 0.1 | 0.2 | 0.5 | 1 | 2 | 5 | 5 | 10 |
| **TryplE mL** | 0.5 | 0.1 | 0.2 | 0.3 | 0.4 | 0.6 | 1.5 | 1.5 | 3 |
| **1X PBS wash mL** | 0.1-0.2 | 0.2-.04 | 0.5-1 | 1-2 | 2 | 4 | 10 | 8 | 20 |
| **Culture medium mL** | 0.15 | 0.3 | 0.5 | 1 | 2 | 4 | 10 | 8 | 20 |
|  |  |  |  |  |  |  |  |  |  |
| **mESCs when ready to split** | 0.05 x 106 | 0.1 x 106 | 0.3 x 106 | 0.7 x 106 | 1.5 x 106 | 3 x 106 | 10 x 106 | 8 x 106 | 20 x 106 |
| **mESCs to seed (ready next day)** | 0.02 x 106 | 0.05 x 106 | 0.1 x 106 | 0.3 x 106 | 0.5 x 106 | 1 x 106 | 3 x 106 | 2.5 x 106 | 6 x 106 |
| **Surface cm2** | 0.32 | 1.1 | 1.9 | 3.5 | 9.6 | 25 | 75 | 56.7 | 145 |
|  | **96-well** | **48-well** | **24-well** | **12-well** | **6-well** | **T25** | **T75** | **10cm dish** | **15cm dish** |