ETS-Embryo Medium

In Vitro Culture Media

Version 1.0

3D Culture Media

Product	Catalogue No	Storage
ETS-Embryo Medium, 25 ml (5 x 5 ml)	M13-25	-20°C
ETS-Embryo Medium, 100 ml	M13-100	-20°C

Additional Reagents

1. DMEM/F-12 (Gibco, Cat No 21331-020)
2. Fetal Bovine Serum (FBS)
3. L-glutamine
4. 2-mercaeptoethanol
5. MEM Non-essential Amino Acid Solution (NEAA)
6. Sodium pyruvate
7. Penicillin-streptomycin
8. PD0325901 (Cell Guidance Systems, Cat No SM26)
9. CHIR99021 (Cell Guidance Systems, Cat No SM13)
10. Leukaemia Inhibitory Factor (LIF) (Cell Guidance Systems, Cat No GFM200)
11. RPMI 1640 (Sigma, Cat No M3817)
12. Fibroblast Growth Factor 4 (FGF-4) (Cell Guidance Systems, Cat No GFH31)
13. Heparin (Sigma, Cat No H3149)
14. Phosphate Buffered Saline (PBS)
15. Trypsin-EDTA
16. Matrigel® (BD, Cat No 356234)
17. ibiTreat µ-plates, 8-well (Ibidi, Cat No IB-80826)

    Alternatives to ibiTreat µ-plates are given in table 1 below.

Storage conditions

Minimize freeze-thaw cycles. ETS-Embryo Medium can be purchased as individually aliquoted 5 ml volumes. If purchasing the 100 ml volumes, on first thawing of ETS-Embryo medium, aliquot the medium into individual volumes. When not in use the media should be kept at -20°C.

After thawing, the working aliquots can be kept at 4°C for up to 7 days.

The media should be kept in an incubator for a minimum of 30 minutes before use to allow pH equilibration.

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Protocol

1. Maintenance of Mouse Embryonic Stem Cells (ESCs) and Trophoblast Stem Cells (TSCs)

1.1 Maintenance of Mouse ESCs

Prior to production of ETS-embryo, mouse ESCs can be maintained in DMEM/F-12 (Gibco) with 15% Fetal Bovine Serum (FBS), 2 mM L-glutamine, 0.1 mM 2-mercaeptoethanol, 0.1 mM NEAA, 1 mM sodium pyruvate and 1% penicillin-streptomycin supplemented with 1 µM PD0325901, 3µM CHIR99021 and Leukaemia Inhibitory Factor (LIF) (0.1 mM) (a wide range of small molecules and LIF are available from Cell Guidance Systems).

In preparation for the ETS-embryo experiments, it is recommended that the ESCs are maintained in feeder-free conditions on gelatin-coated dishes at 37°C and 5% CO_2. ESCs should be passaged once they reach confluency. The medium should be prepared fresh every 7 to 10 days and changed in every 2 days and the day after passaging.

1.2 Maintenance of Mouse TSCs

Before the ETS-embryo experiment, TSCs can be cultured on MEFs using RPMI 1640 (Sigma) with 20% FBS, 2 mM L-glutamine, 0.1 mM 2-mercaeptoethanol, 1 mM sodium pyruvate and 1% penicillin-streptomycin, plus Fibroblast Growth Factor 4 (FGF-4) (Cell Guidance Systems) and heparin (Sigma). The addition of FGF-4 is essential to avoid differentiation.

In preparation for the ETS-embryo experiments, TSCs should be cultured on MEFs and passaged at 80% confluency. The medium should be prepared fresh every 7 to 10 days and exchanged every 2 days and on the day after passaging. If growing TSCs under different conditions, it is highly recommended to switch them to the described medium to increase their efficiency for ETS-embryo formation.

IMPORTANT POINTS:

If cells are freshly thawed from frozen vials, it is recommended to keep cells in adherent culture for at least one passage after thawing before attempting an ETS-embryo experiment.

Feeder cells need not be removed to begin the ETS-embryo experiment. Feeder-free conditions are preferable, but MEFs will not contaminate the culture if they are still present when you start the experiment.

2. ETS-embryo Generation

On day 0:

1.3 Step 1:

Wash ESC with Phosphate Buffered Saline (PBS) and treat with 0.05% trypsin-EDTA for 3 – 4 minutes at 37°C, then inactivate the trypsin using serum-based culture medium (e.g. DMEM/FBS) to obtain a single-cell suspension.

Treat TSCs with 0.05% trypsin-EDTA for 4 minutes at 37°C and inactivate the trypsin using RPMI/FBS to obtain small clumps of TSCs in suspension.

IMPORTANT POINT: At this stage, ESC colonies are dissociated to single cells and TSC colonies are dissociated into small clumps.

1.4 Step 2:

Centrifuge ESCs for 5 minutes at 1000 rpm. Resuspend in PBS and centrifuge a second time in order to 'wash' the 2i LIF from the ESC. Resuspend once again in PBS and centrifuge a third time. Agitate the ESC in PBS to obtain a single cell suspension. Cells can then be resuspended in PBS **without 2i LIF**.

Centrifuge the TSC suspension for 3 – 4 minutes at 1000 rpm, remove the supernatant, and resuspend the pellet gently in RPMI/FBS media. The handling of TSCs should be gentle to preserve small clumps of 2 to 8 cells.

IMPORTANT POINT: Over-washing and centrifugation can be problematic for TSCs, so the 'wash-centrifugation' step is not essential. However, washing is essential for ESCs, the media must be completely clear of 2i LIF so that the cells can exit naïve pluripotency and progress in the ETS-embryo system.

1.5 Step 3:

Count 5,000 - 10,000 cells for both ESC and TSC suspensions. Mix them in a 1:1 ratio in a small volume of PBS (e.g. 50 µl of ESCs in suspension with 50 µl of TSCs in suspension).

IMPORTANT POINT: Volumes and numbers of cells to seed depend on the culture plate being used and the rate at which the cells are growing. The goal is to get an approximately equal number of single ESC and TSC clumps, and to have them spread evenly through the Matrigel® (BD) drop at seeding.

1.6 Step 4:

Centrifuge this mixture, remove the supernatant, then re-suspend the pellet in ice cold liquid Matrigel®.

1.7 Step 5:

Plate the Matrigel®-cell suspension on µ-plates (Ibidi) and allow the matrix to solidify for 2 minutes at 37°C.

1.8 Step 6:

Once the Matrigel® is solid, add ETS-Embryo medium (see table 1) and culture at 5% CO_2 and 37°C for 120 hours / 5 days.

During the experiment:

1.9 Step 7:

On days 1 – 5 of culture, feed cultures with fresh ETS-Embryo Medium every other day.

IMPORTANT POINT: ESCs and TSCs secrete growth factors into the media which enhance ETS-embryo development. If the volume of the media is not depleted, do not change it, to avoid stripping the culture of these important secreted factors. ***At the same time, avoid cell death by ensuring the nutrients in the media are not depleted by regular changes of medium.***

Alternative Method:

For imaging a high number of structures, it is useful to have all the cells in the same plane.

For this purpose, a '3D on top' / sandwich approach (described in Lee *et al.*, 2007) may be effective. This alternative method involves seeding cells onto a layer of Matrigel®, and then a mixture of medium and 10% Matrigel® is put on top once the cells have attached.

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3. Complementary Information

Table 1. Approximate plating volumes for ETS in different culture plates- for the ‘3D on top’ method

	ibiTreat µ-plates, 8 well (Ibidi) IB-80826	Angiogenesis µ-plate (Ibidi) IB-81506	Glass-bottomed – culture dish (MatTek) P35G-1.5-14-C
Seeding Volume	25 µl	7 µl	100 µl
ETS-Embryo Medium Volume	200 µl	20 µl	2000 µl
Matrigel® Volume (3D on top)	35 µl	5 µl	60 µl

4. References

Lee, G. Y., et al. 2007. Three-dimensional culture models of normal and malignant breast epithelial cells. *Nature Methods* **4**(4): 359–365.

Assembly of embryonic and extra-embryonic stem cells to mimic embryogenesis in vitro. (2017) Sarah Ellys Harrison, Berna Sozen, Neophytos Christodoulou, Christos Kyprianou1 and Magdalena Zernicka-Goetz Science https://doi.org/10.1126/science.aal1810

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