Protocol Thawing GEMM-ESC clones in 2i medium

- adapted protocol from:
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Materials & Reagents

N2B27 (home-made) – see preparation and recipe

2i medium – see preparation and recipe

Complete Knock-Out DMEM (Washing medium) – see preparation and recipe

Knock-Out DMEM
Gibco-Invitrogen Cat.#: 10829018

Knock-Out Serum Replacement
Gibco-Invitrogen Cat.#: 10828028

Non-essential amino acids
Gibco-Invitrogen Cat.#: 11140035

L-Glutamine
Gibco-Invitrogen Cat.#: 25030024

LIF
Esgro-Chemicon ESG1107 (10^7 Units) – see preparation and recipe

Gelatin (from porcine skin) – see preparation and recipe

DMEM/F12
Gibco (Invitrogen) Cat.#: 31330-095

Neurobasal medium
Gibco (Invitrogen) Cat.#: 21103-049

N2 Supplement (100X)
Gibco (Invitrogen) Cat.#: 17502-048

B27 Supplement (50X)
Gibco (Invitrogen) Cat.#: 17504-044

ß-mercaptoethanol – see preparation and recipe
Sigma Cat.#: M7522

Bovine Albumin Fraction V Solution (7.5%) (BSA)
Invitrogen Cat.#: 15260-037

Accutase
Sigma Cat.#: A6964

CHIR99021 (GSK3 inhibitor)
Axon Medchem
Cat.#: Axon 1386

PD0325901 (MEK inhibitor)
Axon Medchem
Cat.#: Axon 1408

DMSO
Sigma Cat.#: D2650

Regenerated Cellulose filter
Corning (Sigma-Aldrich) Cat.#: CLS431222

Freezing Medium – see preparation and recipe

Methods & Comments

Day 0:

1. Gelatinize a 4-well plate (Multidish 4-well; Nunc, 1.8cm^2 surface); fill each well with 100µl and leave in the incubator for at least 30 minutes before use.

2. Slowly thaw the cryo-vial in a 37°C waterbath by gently shaking. Transfer the content to 9ml of pre-warmed Washing medium in a 15ml Falcon tube. Spin at 1200rpm for 5 minutes.

3. Aspirate supernatant and resuspend cells in 1ml N2B27 medium + 2i + LIF.

4. Aspirate gelatin from the 4-well plate and add 500µl N2B27 medium + 2i + LIF to the 2nd, 3rd and 4th well.

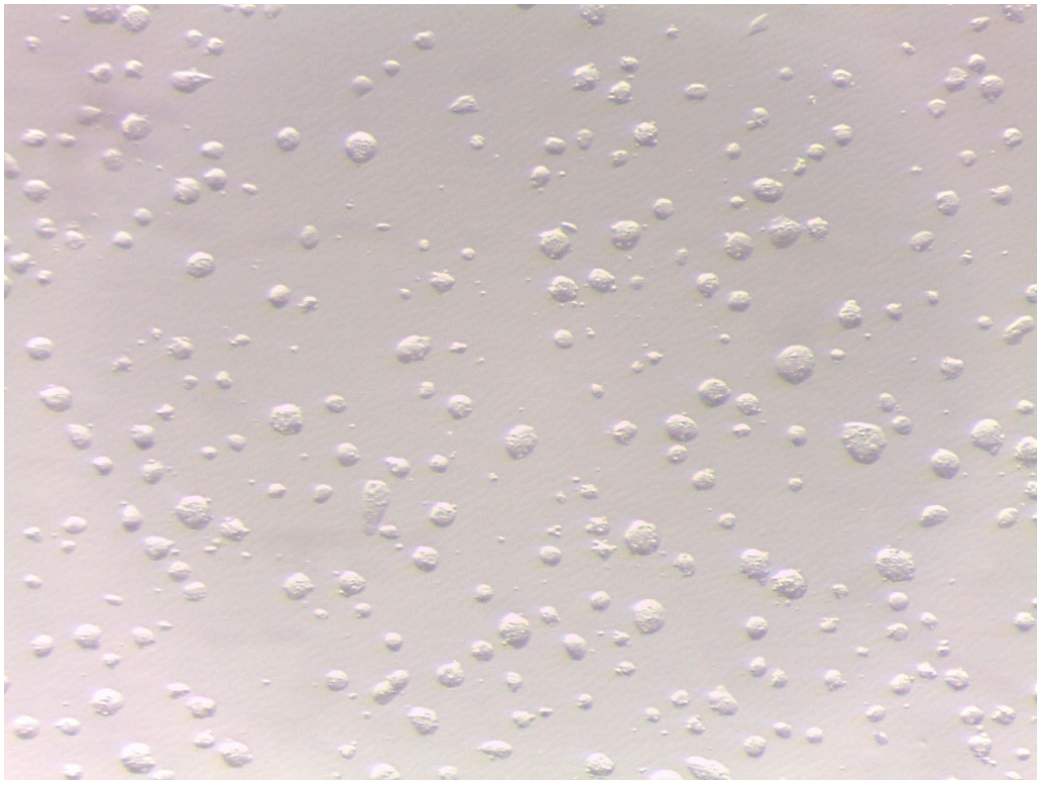
5. Add the cell suspension (1ml) to the 1st well. Transfer 500µl to the 2nd well and resuspend. Transfer 500µl from the 2nd well to the 3rd well and resuspend. Transfer 500µl from the 3rd well to the 4th and resuspend. Take 500µl from the 4th well and trash. This dilution series allows you to choose the cells in optimal condition. Incubate in 37°C/5% CO_2 incubator.

Day 1:

6. Gently remove medium with a P1000 pipet (Don't wash! Cells are very loosely attached) and gently add pre-warmed 500µl N2B27 medium + 2i + LIF per well.

Day 2:

7. The GEMM-ESC clones should look similar to the picture below. This might already be at Day 1, but can also be at Day 3, depending how cells come out of the thawing procedure. ES colonies at the density shown below can be split (see point 8.). We usually split 2 wells of the 4-well plate at the same time. We select the wells that look best; each well will be transferred to a separate well of a 6-well plate.



8. Gelatinize 2 wells of a 6-well plate (Costar, 10cm^2 surface); fill each well with 500µl and leave in the incubator for at least 30 minutes before use.

9. Gently remove medium with a P1000 pipet (Don't wash! Cells are very loosely attached) and add 100µl Accutase per well. Leave in the incubator for 5 min.

10. Add 900ul Washing medium per well and resuspend. Transfer to 15ml Falcon and spin at 1200rpm for 5 minutes.

11. Aspirate supernatant and resuspend cells in 3ml N2B27 medium + 2i + LIF.

12. Remove gelatin from 6-well plate and add 3ml cell suspension to each well. Incubate in 37°C/5% CO_2 incubator.

Day 3 - onwards:

13. Observe the cell growth for the next couple of days (refresh medium every other day). When they are confluent with ES colonies, passage them into bigger surface area dishes (we roughly split our cells at 1:3 or 1:6 ratio). Pas15 November 2013saging cells: Aspirate exhausted medium and add Accutase solution per well. Incubate in the incubator for approximately 5 minutes. Dislodge the cells, spin at 1200rpm for 5 minutes and resuspend cells in N2B27 medium + 2i + LIF. Seed into new gelatinized plate and place back in the incubator.

14. When the cells are at a 6-well plate, they can be frozen down at 2 vials per well. When cells are grown on a large tissue culture dish (100x20mm Style; BD Falcon; 57cm^2 surface), they can be frozen down at 10 vials per plate.

Freezing procedures:

Aspirate exhausted medium and add in Accutase.
Incubate at 37°C/5% CO² for approximately 5 minutes.
Neutralize with Washing medium and collect cells in a 15 ml tube.
Centrifuge at 1200rpm for 5 minutes.
Resuspend cell pellet in N2B27 medium + 2i + LIF and add in equal volume with the cold (4°C) freezing medium. Aliquot into cryovials and use an appropriate freezing program.
Long term storage is performed in liquid nitrogen.

Preparation and Recipe

0.1% Gelatin
Make a 0.1% gelatin in PBS and autoclave sterilized.

LIF (1000X)
Dilute 1mL LIF in 9mL N2B27 medium and divide into 50uL aliquots in an eppendorf. Keep aliquots frozen at -20°C.

1000X ß-mercaptoethanol
70µL ß-mercaptoethanol in 10mL distilled water.
Filter through 0.22µm. Store at 4°C and use within 1 month

1. Preparation of N2B27 medium

DMEM/F12-N2 medium Mix
To 500mL DMEM/F12 medium, add in 5mL N2 supplement (100X) and 335µL 7.5% stock BSA.

Neurobasal-B27 medium Mix
To 500mL Neurobasal medium, add in 10mL B27 supplement (50X) and 5mL L-Glutamine.

N2B27 medium
Mix DMEM/F12-N2 medium with Neurobasal-B27 medium in the ratio of 1:1. (Total of 1000mL)
Add ß-mercaptoethanol to a final concentration of 0.1mM from the 1000X (0.1M) stock solution. (Use 1mL per 1000mL of medium)
Filter sterilised through a 0.22µm filter, aliquot into 50mL tubes and store at 4°C and use within 1 month.

N2B27 medium + 2i + LIF (2i medium)
To 50mL N2B27 medium, add 50µL LIF and 50uL each of stock 1000X PD0325901 and CHIR99021.
Filter sterilised through a 0.22µm filter before use.
Store at 4°C and use within 2 weeks.

2i = home-made 2i which consist of 2 inhibitors (CHIR99021 & PD0325901)

Final concentration of CHIR99021 = 3µM
Final concentration of PD0325901 = 1µM

2. Preparation of 2i

CHIR99021

- dissolve powder (in the amber bottle) in appropriate amount of filtered DMSO to make a 10mM stock solution. Filter DMSO through a 0.22µm regenerated cellulose filter.
- make a 3mM stock solution (1000X) in filtered DMSO and aliquot 50µL per eppendorf. Keep aliquots frozen at -20°C.

PD0325901

- dissolve powder (in the amber bottle) in appropriate amount of filtered DMSO to make a 10mM stock solution. Filter DMSO through a 0.22µm regenerated cellulose filter.
- make a 1mM stock solution (1000X) in DMSO. Filter through a regenerated cellulose filter and aliquot at 50µL per eppendorf. Keep aliquots frozen at -20°C.

3. Preparation of Washing medium

To 500mL Knock-Out DMEM, add the following:
50mL Knock-Out Serum Replacement
5mL Non-essential amino acids
5mL L-Glutamine

- Filter the medium through a 0.22µm filter unit before use.
- Store medium at 4˚C.

4. Preparation of Freezing medium

Add 20% final concentration of DMSO to FBS.
Filter through a 0.22µm filter before use.
Store at 4˚C.