Thawing and Culture Protocol for E14TG2a.4 feeder independent ES cell clones

Need:

- Feeder Medium (*see recipe*)
- GTES ES Cell Medium (*see recipe*)
- Injection Medium (*see recipe*)
- 0.25% Trypsin (*Invitrogen Cat# 25200-056*)
- DPBS (*Invitrogen Cat# 14190-250*)
- Corning CellBind plates (*E&K Cat# 680100, for 6-well*) or 0.1% gelatin-coated tissue culture dishes
- 15mL centrifuge tubes
- Nunc CryoTubes (*Fisher Cat# 12-565-167N*)
- 2x Freezing Medium (*DMSO, FBS, ES Cell Media, 1:1:3 ratio*)

**IMPORTANT SAFETY NOTE:** When you remove a vial from liquid nitrogen, please loosen the top immediately to release the pressure from rapidly evaporating nitrogen within the tube and thereby reduce the chance of an explosion. **Manufacturer's Alert:** To prevent cryogenic vials from exploding, never overfill liquid nitrogen storage units. Always examine vials before use to ensure no visible defects around the closure rims. Always use full face shields, heavy safety gloves and laboratory protective apparel when removing vials from cryogenic storage. Use care when thawing vials removed from nitrogen tanks. Never reuse cryogenic vials.

Thawing a Vial of Feeder-Independent ES Cells from Previously Expanded Stock

1. Remove cells from liquid/vapor nitrogen tank storage, loosen cap, and place immediately on dry ice. Vials should remain on dry ice until the moment they are thawed.
2. Warm Feeder and GTES ES Cell Media in a 37°C water bath for no less than 15 minutes.
3. After media is warm and transferred to hood, pre-aliquot 5 mL of feeder media to each 15 mL tube (one tube for each vial to be thawed).

***the DMSO used in the freezing process will begin to damage cells as soon as they are thawed, so steps 4-6 should be done quickly and carefully***

4. Remove a vial (or two at a time, if you prefer) from the dry ice. Place directly into 37°C water bath so that just the bottom half of the vial is submerged (a gentle swirling motion will ensure more uniform thawing)
5. Check the vial every few seconds until you see mostly liquid inside – ideally with just a small ice crystal left to ensure it has not warmed too long. After cleaning the vial with 70% EtOH, transfer the vial to the culture hood.
6. Open the cryovial containing the cells and remove media using a 1000 µL pipet. Transfer the entire volume to a pre-aliquoted 15mL spin tube. (For ease of identification, you may peel the label off of the cryovial and attach it to the tube).
7. Spin for 5 minutes at 1000 rpm
8. Aspirate medium from centrifuge tube, taking care not to disturb cell pellet.

9. Add 3 mL GTES ES Cell Medium to the 15 mL tube. Pipet up and down approximately eight times to resuspend pellet in media.
10. Plate media onto one well of a 6-well plate. Rock plate back and forth gently (both laterally and distally) to evenly distribute cells.
11. Place 6-well plate into 37°C incubator set at 6% $CO_2$.
12. Change media every day for optimum growth.

Passing Feeder-Independent ES Cells

1. Once cells become approximately 80% confluent (usually after two to three days), they must be passed to the next size dish to prevent differentiation. We recommend passing at a ratio of 1:5 or 1:6 for best results.
2. Aspirate old media from the dish and wash with 3 mL DPBS (for 6-well).
3. Aspirate DPBS and add 0.5 mL 0.25% trypsin. Incubate at 37°C for 4 minutes.
4. Add 3 mL feeder media (for 6-well) to neutralize trypsin. Pipet up and down approximately 8 times to break up cells into single-cell suspension.
5. Place cell suspension into 15 mL spin tube and spin for 5 minutes at 1000 rpm.
6. Aspirate medium from centrifuge tube, taking care not to disturb cell pellet.
7. Add 10 mL GTES ES Cell Medium to the 15 mL tube. Pipet up and down approximately eight times to resuspend pellet in media.
8. Plate onto a 10 cm dish. Rock plate back and forth gently (both laterally and distally) to evenly distribute cells.
9. Place 10 cm dish into 37°C incubator set at 6% $CO_2$.
10. Change media every day for optimum growth.

Freezing Feeder-Independent ES Cells (From 10 cm dish)

1. After 2-3 days on a 10 cm dish, the cells should be ~80% confluent. At this point, they are ready for freezing.
2. Aspirate old media from the dish and wash with 10 mL DPBS.
3. Aspirate DPBS and add 1.5 mL 0.25% trypsin. Incubate at 37°C for 4 minutes.
4. Add 10 mL feeder media to neutralize trypsin. Pipet up and down approximately 8 times to break up cells into single-cell suspension.
5. Place cell suspension into a 15mL centrifuge tube and spin for 5 minutes at 1000 rpm.
6. Aspirate medium from centrifuge tube, taking care not to disturb cell pellet.
7. Add 2 mL GTES ES Cell Medium to the 15 mL tube. Pipet up and down approximately eight times to resuspend pellet in media.
8. Add 2 mL 2x Freezing Medium to cell suspension. Mix by pipetting 2-3 times.
9. *Immediately* aliquot 0.5 mL of the mixture to each of 8 labeled cryotubes.
10. Place cryotubes in a slow-cooling apparatus (we use Sarstedt styrofoam boxes) in the -80. Leave overnight. For long-term storage, transfer to liquid/vapor phase nitrogen tanks.

Preparing Feeder-Independent ES Cells for Blastocyst Micro-injection

1. Thaw cells as described above onto a 6-well dish 48 hours prior to injection. Feed with ES cell media the next day.

2. About 20 minutes prior to injection time, remove the dish from the incubator. Aspirate off the old media.
3. Wash cells with 3 mL DPBS. Aspirate DPBS.
4. Add 0.5 mL 0.25% trypsin and incubate at 37°C for 4 minutes.
5. Add 3 mL feeder media (for 6-well) to neutralize trypsin. Pipet up and down approximately 8 times to break up cells into single-cell suspension.
6. Place cell suspension into 15 mL spin tube and spin for 5 minutes at 1000 rpm.
7. Aspirate medium from centrifuge tube, taking care not to disturb cell pellet.
8. Resuspend pellet in 100 µL of injection medium and transfer to a sterile, 1000 µL Eppendorf-style tube. Cells are now suitable to deliver for injection.

| **Feeder Medium** | | | **GTES Medium** | |
|---|---|---|---|---|
| DMEM | 435 ml | | GMEM | 470mL |
| FBS (10%) | 50 ml | | FBS (15%) | 87 mL |
| Sodium Pyr. | 5 ml | | L-Glutamine | 6 mL |
| P/S | 5 ml | | Na Pyr. | 6 mL |
| L-Glutamine | 5 ml | | NEAA | 6 mL |
| | | | Pen/Strep | 6 mL |
| | | | BME | 600 µL |
| | | | LIF | 58 µL |
| | | | | |
| **2x Freezing Medium** | | | **Injection Medium** | |
| GTES Media | 30mL | | DMEM w/ Hepes | 475mL |
| FBS | 10mL | | FBS | 25mL |
| DMSO | 10mL | | | |

GMEM *(Sigma # G5154)*
DMEM *(Invitrogen # 11965-092)*
DMEM w/Hepes *(Invitrogen #12430-054)*
Fetal Bovine Serum *(currently, Invitrogen #10437-028)*
Sodium Pyruvate *(Invitrogen # 11360-070)*
Non-Essential Amino Acids (NEAA) *(Invitrogen # 11140-050)*
L-Glutamine *(Invitrogen # 25030-081)*
Penicillin/Streptomycin *(Invitrogen # 15140-122)*
β-Mercaptoethanol (BME) *(Invitrogen # 21985-023)*
Dimethyl Sulfoxide (DMSO) *(Sigma # D2650)*
Leukemia Inhibitory Factor (LIF) *(Millipore # ESG1107)*
*Added to media for a final concentration of 1000 units/mL*