Culturing cast x 129 hybrid mESC clone F121-9

Cell identification

This is a female line derived by crossing male castaneous and female 129. Originally F121 was made in Jaenisch lab and F121-9 which has a relatively stable karyotype was subcloned in Gribnau lab. (PMID: 10471737 is the most appropriate reference to cite.)

Culture Media

Serum free ES medium (SFES) - 1000 ml

Solution (stock)	Cat. No.	Final conc.	Volume
NEUROBASAL MEDIA	Gibco 21103-049	50%	500ml
DMEM/F12	Gibco 11320-033	50%	500ml
N2-SUPPLEMENT	Gibco 17502-048	0.5x	5ml
B27(+RA)	Gibco 17504-044	0.5x	10ml
7.5% BSA	Gibco 15260-037	0.05%	6.66ml

Aliquot if needed and keep at 4°C out of light. Expiration: 1 month
N2 and B27 come frozen. Aliquot them to avoid repeated freeze/thaw cycle if you need to prepare SFES in a smaller scale.

Complete "2i" medium - 100 ml

Solution (stock)	Cat. No.	Final conc.	Volume
SFES	N/A	1x	100ml
PD03259010 (10mM)	Reprocell 04-0006-02C	1 µM	10 µl
CHIR99021 (10mM)	Reprocell 04-0004-02C	3 µM	30 µl
GLUTAMINE (200mM)	Gibco 25030-081	2mM	1ml
Monothioglycerol (11.9M)	Sigma M6145-25ML	1.5x10-4M	1.26 µl
LIF (1x10^6 U/ml)*	Cell Guidance Systems GFM200, see "stock solutions" section	1,000U/ml	100 µl*

*Larger volume of further dilution (10^5U/ml) can be used if you prefer. Since SFES contains 0.05% (0.5mg/mL) BSA, diluting with SFES is not a problem.

Keep at 4˚C out of light. Warm up only as much as you use for the day in 37˚C water bath. Expiration: 2 weeks

Stock solutions

NEUROBASAL MEDIA, DMEM/F12, N2-SUPPLEMENT, B27(+RA), 7.5% BSA: The consortium use the same lot of these reagents with **Reserve Reference Number:** 35116575. ALthough the lot reservation was made for the entire consortium, each PI needs to contact one's local ThermoFisher/Life Technologies rep to set up one's quote (using **Reserve Reference Number:** 35116575) reflecting each institute's contract status. Also, each PI needs

to purchase the reserved amount within the reserve period. The expiration of these reagents is much longer than reserve period.

PD03259010, CHIR99021: Reprocell Cat# 04-0006-02C and 04-0004-02C are special batches set aside for 4DN. These come as 10mM solution. Aliquot in single dose (10 µl of PD03259010, 30 µl of CHIR99021 if you always make 100 ml complete 2i medium) and store at -20˚C, light protected. Avoid freeze/thaw cycle. Expiration: 6 months at -20˚C after shipping

LIF: Cell Guidance Systems GFM200-5, 20, 100, 1000 (Please use the quotation found here: https://drive.google.com/file/d/0B548E2qA0rVAVHlOZUR2SldLeG8/view?usp=sharing) Comes lyophilized and is very stable at -20˚C. The specific activity of this product is approximately 10^8U/mg. To make 1x10^6 U/ml stock solution, centrifuge the vial before opening, open the vial in the tissue culture hood and add sterile 0.1% BSA/water to make 0.1mg/ml solution (50 µl for 5 µg vial, 200 µl for 20 µg vial ,,,). This becomes 1x 10^7U/ml. Gently pipette and wash down the side of the vial to ensure full recovery of protein into solution. This can be further diluted in SFEF 1:10 to make 10^6U/ml stock solution. Aliquot in single doze and store in -20˚C if the reconstituted solution is not used all at once. Expiration after reconstitution: 3 months

Other reagents
ESGRO COMPLETE GELATIN (Ultrapure Water with 0.1% Gelatin): EMD Millipore SF008
DPBS, calcium, magnesium free: Gibco 14190144 or equivalent
ESGRO Complete Accutase: EMD Millipore SF006
HyCryo-Stem: GE Healthcare SR30002

Procedures

Gelatin coating of the plate/flask: Apply 0.1% gelatin for 25-30 min (e.g. 2.5 ml in a T25 flask) at room temperature. This can continue ~ a week if evaporation is prevented.
Aspirate gelatin, rinse with PBS and aspirate again. The plate is now ready to use. (Use plates of this stage promptly as expiration is unknown.)

Thawing: The initial stock comes as 2 x 10^6 cells/vial. Thaw quickly in a 37˚C water bath, then wash once with basal media (transfer the cell suspension to a 15 mL conical tube, add 10mL media, centrifuge at 1,000 rpm for 5 minutes at room temperature, remove supernatant and resuspend the pellet in 5mL complete 2i) and plate into gelatin-coated T25 flask. Label the vessel with cell line, passage number, date at least. Change the media next day.

Passage: (This is for T25 flask. If different size of vessel is used, change the volume of the solutions accordingly.)

1. Remove carefully “2i” media.
2. Add 0.5 ml of accutase and agitate the vessel to spread accutase evenly. Incubate the cells with the accutase for several minutes at room temperature until cell colonies dislodge from the plate and single cells begin to separate out.
3. Add 5 mL of SFES and pipet up and down using a 5mL pipette to make single cell suspension (~10 times).

4. Transfer the cell suspension to a 15mL conical tube and centrifuge at 1,000 rpm for 5 minutes at room temperature, remove supernatant.
5. Resuspend the pellet in complete 2i media and plate into new gelatin-coated vessel at 1:6-1:10. Label the vessel with cell line, passage number/PDL, date at least. Place the culture into a tissue culture incubator (37˚C, 5% CO_2-95% air, 99% humidity).
6. Observe the cells change the media daily. If cells are splitted at 1:6, they will be ready for the next passage in 2 days. Cells do not become “confluent” (cover the entire surface of the vessel) but grow as colonies. If the medium becomes yellow the day after medium change, it’s time to split.

Freezing:

1. Detach cells from the vessel and make single-cell suspension as described in steps 1-3 of the “Passage”. Transfer the cell suspension to a 15mL conical tube.
2. Count the cells while cell suspension is centrifuged at 1,000 rpm for 5 minutes at room temperature and label necessary number of cryovials with cell line, passage/PDL, date, operator initial.
3. Remove the supernatant and resuspend the pellet in SFES to make 4 million cells/mL. Add the same volume of Hycryo-Stem (this is 2x) and mix. Aliquot 1 mL each into labeled cryovials.
4. Freeze in a styrofoam rack in -80˚C overnight (or any temperature control system to ensure slow freezing) then transfer the vials to liquid nitrogen storage or -150˚C freezer.

Quick reference (recommended liquid volume for each culture vessel)

vessel	**0.1% gelatin***	**accutase****	**medium (for inactivating accutase and plating/feeding)**
6-well/35mm dish	0.5mL	0.1mL	2mL
T25 flask/60mm dish	2.5mL	0.5mL	5mL
90mm dish	5mL	1mL	10mL
T75 flask	7.5mL	1.5mL	15mL

*Larger or smaller volume of gelatin is ok as far as the vessel surface to which cells should attach is covered.

** Accutase should also cover the cell surface completely, but here smaller volume than gelatin is recommended, because accutase is applied to already wet surface while gelatin is applied to dry surface where spreading liquid needs to compete with surface tension, plus application of gelatin is longer than that of accutase. If you want to increase the volume of accutase, you also need to increase the volume of SFES to inactivate accutase and/or repeat washing with SFES before plating detached cells.