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Mouse embryonic stem (ES) cell culture - basic procedures

This protocol presents something like a brief "best of" collection from various sources plus some or the autor's own experiences. A very good and more elaborate protocol can for example be found in:

M.P. Matise, W. Auerbach and A.L. Joyner
Production of targeted embryonic stem cell clones
in: A.L. Joyner (ed.) - Gene Targeting, a practical approach, 2nd edition)
The Practical Approach Series, Oxford University Press 2000
(book is available in our library)

Important rules for ES cell culture

(It is assumed that differentiation of ES cells is to be prevented...)

- ES cells do not like to be alone, chose appropriate size of dish at thawing, do not routinely split by more than 1/10
- do not overgrow, split when semiconfluent, otherwise cells will differentiate
- routinely cells should be passaged every 2-3 days
- to prevent differentiation always dissociate cultures into single cells after trypsinization
- do not grow cells for too long before starting an experiment, always try to reduce time the cells are in culture
- water and serum quality are very important (for the latter test different batches from different manufacturers)
- it is best to feed cells, i.e. change medium, every day; also refeed cells about 3 hours before passaging or freezing them

Signs of differentiation

(spontaneously differentiated ES cells do not always look the same depending on the cause of differentiation: low feeder quality, inappropriate feeder density and/or low LIF concentration - see below)

- colonies are surrounded by flattened cells that differ morphologically from the undifferentiated ES cells in the centre, often these cells at the colony's edge appear dark and spiky
- large colonies with necrotic centres, these appear as cells with defined boundaries: happens to cells which were not passaged for several days
- colonies appear as individual cells rather than as syncial mass, often cell nucleus is clearly visible in differentiated ES cells
- flattened colonies that appear as single-cell layers surrounded by a circle-like boundary

General tips

- keep a record of how often cells are passaged
- for passaging and freezing it is best if cells are at subconfluence.
- growing conditions should be optimal regarding growth rate and plating efficiency - suboptimal conditions can e.g. be due to a bad batch of FCS (see ingredients of medium below)

ES medium

ES cells are grown at 37°C / 5% CO^2 / 95% humidity in dishes coated with a feeder layer of mitotically inactivated primary mouse embryonic fibroblasts (see accompanying protocol "Isolation and handling of primary mouse embryonic fibroblasts (MEFs)"). In contrast to the feeder cells, ES cells form colonies rather than a single-cell layer

DMEM (high glucose, Gibco 41966-052, store in fridge) minimal medium supplemented before use with

- 15% (v/v) FCS (serum from newborn calf, liquid, tested for ES culture, store aliquots at -20°C)
- 1/100 (v/v) L-glutamine (200mM: Gibco 25030-024, store aliquots at -20°C), stable in solution for 10d only
- 1/100 (v/v) non-essential amino acids (Gibco 11140-035, store aliquots in fridge)
- 1/100 (v/v) pen/strep (Gibco 15140-122, store aliquots at -20°C)
- 1/500 (v/v) 2-mercaptoethanol (Gibco 31350-010, 0.1mM final conc., store aliquots in fridge)
- 1/10,000 (v/v) LIF ("ESGRO" from Chemicon, No. ESG1107, 1000 U/ml final conc. or less (depending on properties of respective cell line), make up 1/100 dilution in DMEM with 10% (v/v) or so serum to be further diluted by 1/100 to achieve the working conc., very expensive, store at -20°C)

Remarks

- feeder layers and exogeneously added LIF both serve to prevent differentiation
- medium should be changed every or must be changed when it turns orange (timepoint depends on state of culture and on when medium has been made up), discard cells if medium has turned yellow (acid pH).
- store medium in fridge, warm up in waterbath before use
- better make up small amounts of fresh medium every few days rather than living on a larger amount for weeks

Thawing ES cells (quickly)

When thawing ES cells, always have feeder plates prepared (mitomycin C treatment at leastone day before use, see accompanying protocol "Isolation and handling of primary mouse embryonic fibroblasts (MEFs)")

- remove ES cells from freezer/liquid nitrogen and quickly thaw in a 37°C waterbath
- transfer cell suspension (cell concentration is not very important) to a sterile tube containing several ml of warm medium
- gently mix and pellet the cells by centrifugation @ low speed for 5min
- aspirate off supernatant (removal of DMSO in freezing medium) and resuspend cells into 12ml (5ml) of warm ES medium and plate out in a 10cm (5cm) feeder plate
- ideally refeed cells daily with fresh ES medium
- upon subconfluence, cells need to be passaged or frozen or used for doing experiments

Passaging ES cells

routinely passage ES cells every 2-3 days (except having colonies under selection or so), otherwise cells will spontaneously differentiate

- check cells under the microscope for subconfluence
- refeed cells 3h before passing them (very important), warm up reagents briefly before use
- aspirate medium off, wash once or twice with PBS (Gibco 14190-169, without bivalent cations), add about 1ml (2ml) of trypsin/EDTA (Gibco 25300-096) to each 5cm (10cm) dish and incubate at 37°C until colonies float off when flicking the plate
- carefully transfer trypsin/cell suspension to a sterile falcon - this is to enrich the ES/feeder mix for ES cells - and trypsinize for a few more minutes at 37°C
- dissociate colonies into single cells by "Gilson pipetting", then add several ml of medium (no need to use expensive ES medium at this point) to inactivate the trypsin, pellet cells by low-speed centrifugation
- remove supernatant, resuspend cells in appropriate volume of ES medium depending on

plate format and splitting ratio. Splitting ratios for ES cells can vary from 1:1 to 1:10. Do not routinely exceed 1:10. The area relationships for several dish formats are given below:

dish	diameter	area(cm^2)	rel. area	final vol.	trypsin
96well	0.6cm	0.3	1/200	0,2ml	ca. 50µl
24well	1.5cm	1.8	1/ 30	1 ml	ca. 300µl
5cm	5.1cm	20	1/ 3	5 ml	ca. 1ml
9cm	8.5cm	57	1	10 ml	ca. 2ml
10cm	10.0cm	79	1.4	12 ml	ca. 3ml

Some typical passaging ratios:
1:6 = 1 x 5cm to 2 x 9cm
1:6 = 1 x 96well to 1 x 24well

- plate out cell suspension and rock plates gently to achieve a uniform CFU distribution. Believe it or not, the optimal movement depends on the plate format - I found a "rotating" movement to be optimal for dishes/wells below a diameter of 5cm. For 9 and 10cm dishes better move plate back and forth a few times, then to the left and right. For 5cm dishes do something in between, i.e. describing an "eight". Return plates to the 37°C incubator.
- always check feeder quality before using them. Feeder cell density is very important to achieve optimal growth conditions: They should be almost confluent, but each feeder cell should have enough space around itself to "spread". Use feedes that are at least one day old (because then they will have settled nicely and flattened)

Freezing ES cells (slowly)

reduce the time the cells are in culture before freezing and freeze at a density that allows recovery of the culture even if 90% of the cells die during the freezing and thawing process

- check cells under the microscope for subconfluence
- refeed cells about 3h before freezing them. Have a precooled styrofoam box as well as cryo-vials and freezing medium (10% DMSO, e.g. Sigma D2650, in ES medium) ready on ice
- aspirate medium off, wash once or twice with PBS (Gibco 14190-169, without bivalent cations), add about 1ml (2ml) of trypsin/EDTA (Gibco 25300-096) to each 5cm (10cm) dish and incubate at 37°C until colonies float off when flicking the plate
- carefully transfer trypsin/cell suspension to a sterile falcon - this is to enrich the ES/feeder mix for ES cells - and trypsinize for a few more minutes at 37°C
- dissociate colonies into single cells by "Gilson pipetting", then add several ml of medium (no need to use expensive ES medium at this point) to inactivate the trypsin, pellet cells by low-speed centrifugation
- remove supernatant, resuspend cells in appropriate volume of pre-cooled freezing medium and immediately transfer into freezing vials on ice (1ml per vial). Transfer vials into pre-cooled styrofoam box (inside ideally about 0°C) and then to a -80°C freezer. It is important to work quickly.
- next day or later, transfer cells to a liquid nitrogen freezer