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Protocol to culture mESCs (LSCB, UPLUT)

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ABSTRACT

Standard protocol used for culturing mouse embryonic stem cells (mESCs) in the Lutolf Lab, EPFL.

GUIDELINES

This protocol assumes standard cell culture aseptic technique.

MATERIALS

NAME	CATALOG #	VENDOR
StemPro™ Accutase™ Cell Dissociation Reagent	A1110501	Thermo Fisher Scientific
PBS, pH 7.4	10010023	Thermo Fisher

MATERIALS TEXT

The following recipes were used to prepare culture and splitting media:

10% Serum Medium (home made fresh):

to make 500mL

- 434mL DMEM, high glucose, with GlutaMAX [CAT# 61965059, Gibco™/Life Technologies]
- 50mL ES-grade Foetal Bovine Serum [CAT#16141-079, Gibco™/Life Technologies]
- 5mL Penicillin-Streptomycin 10000U/mL [CAT#15140122, Gibco™/Life Technologies]
- 5mL Non Essential Amino Acids 100X [CAT#11140035, Gibco™/Life Technologies]
- 5mL Sodium Pyruvate 100mM [CAT#11360039, Gibco™/Life Technologies]
- 1mL beta-mercaptoethanol 50mM [CAT#31350010, Gibco™/Life Technologies]

for final concentrations of:

- Penicillin: 100U/mL
- Streptomycin: 100ug/mL
- Non Essential Amino Acids: 0.1mM
- Sodium Pyruvate: 1mM
- Glutamax (= L-Alanyl-Glutamine, included in DMEM): 3.97mM
- beta-mercaptoethanol: 0.1mM

to make **10% Serum 2i/LIF Medium (made fresh)**, add:

- 3uM CHIR99021 (1:1000 from our 3mM stock,) [CAT#361559, Merck/Millipore]
- 1uM PD0325901 (1:500 from our stock) [CAT#S1036, Selleck Chemicals]
- 100u/mL LIF (1:1000 from our stock), [sourced in house]

BEFORE STARTING

- Bring Accutase and small molecule inhibitor solutions to Room Temperature
- Bring medium and PBS to 37°C (preferably) or Room Temperature.

Cell detachment

1 Cell detachment:

1.1 Using a vacuum pump+glass pipette, aspirate all culture medium out of the well (but do not dry out the cells completely!) ^{10s}

1.2 Add 2mL PBS/- (against side of the well) to wash medium traces, and then aspirate it out again ^{10s}

1.3 Add 500uL Accutase, and let it digest ~  **00:03:00** min,  **Room temperature**, or until most cells are detached ^{3m}



You want to let Accutase digest until almost all cells have detached from the bottom of the well, even if they are still forming clumps in suspension. By the time you start collecting them up, they will have further dissociated into single cells.

Softly tapping with your hand on the side of the plate will aid detachment of loosely-adherent cells.



While the digestion is taking place, and if not done previously, you can start preparing the solutions and tubes required later in the protocol.

- ~6mL 10% Serum Medium to wash cells from the dish and, later, resuspend the pellet
- 1 empty 15mL Falcon tube where you will collect the cells
- 2mL "10%Serum 2i/LIF": 10% Serum Medium + 3uM CHIR99021 (1:1000 from our 3mM stock), 1uM PD0325901 (1:500 from our stock), 100u/mL LIF (1:1000 from our stock),

1.4 With a P1000, collect all cells while breaking down clumps. To do so, pipette repeatedly up and down (without introducing bubbles) and use the ejected liquid to wash the surface of the well. The goal is to collect as many cells as possible in suspension. At the end of the process, transfer to a 15mL Falcon tube. ^{1m}

1.5 Further collect leftover cells by washing the well one last time with with 4.5mL 10%Serum Medium; then adding such suspension to the tube with the accutase/cell suspension. The total volume in the tube should now be ~5mL. ^{30s}

1.6 Centrifuge  **200 x g** (1000rpm on our centrifuge),  **00:04:00 min**,  **4 °C** ^{4m}



While cells are centrifuging, add the 2mL of 10%Serum/2iLIF medium you prepared before to a new well of the 6well plate. Transfer this plate to the incubator to equilibrate it and have it ready to accept the new cells later on.

2 Cell counting:

2.1 Check that a nice pellet has formed at the bottom of the tube, and use a vacuum pump+glass pasteur^{30s} pipette to remove all medium.

2.2 Resuspend the pellet in 1mL 10%Serum Medium and pipette up and down for ~20 times. Change to a^{1m 30s} P200 and pipette up and down again for ~20 times to break down cells even finer.

2.3 Using a P20 or a P10 pipette, pipette up and down the cell suspension solution for ~5 times, and load^{7m} 10uL of the suspension into a chamber of the haemocytometer. Count cells and calculate their concentration in the original solution.



To count cells in the haemocytometer:

- tally the cells in each of the 4 big (4x4) squares at the four corners of the central cross
- divide the resulting total by 4, to find the average number of cells per square
- multiply *10'000
- this is your # cells/mL
- if you then need e.g. 70'000 cells, divide 70k by the number you just found, to know how many uL of cell suspension to use

2.4 Plate 70k cells in the new well of 2iLIF +10%Serum you had previously put to equilibrate in the incubator^{1m}

2.5 Slide the plate back and forth (cross pattern) across the surface of the laminar flow cabinet to^{30s} distribute them evenly, and then leave the cells undisturbed in the incubator to allow them to attach

2.6 Cells can be split every 2 or 3 days (choose one) without medium change in between. Daily checks are still recommended to monitor overcrowding and other possible issues.