Humboldt-CIRM Shared Resource Lab	STANDARD OPERATING PROCEDURE

Title: Single-Cell Passaging using Accutase
Purpose: For passaging when precise cell counts are needed
Version: 1
Last Updated: 20260303
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Media and Supplies

Item	Purpose	Storage	Catalog Number	Supplier
mTeSR Plus	Primary maintenance culture media	2–8°C	MSPP-100-0276	VWR Funding Inc
Accutase	Gentle single-cell dissociation enzyme	-20°C	10761-312	VWR Funding Inc
Matrigel (hESC-Qual)	Basement membrane matrix	-20°C	BD354277	VWR Funding Inc
ROCK Inhibitor (Ri)	Y-27632; prevents apoptosis in single cells	-20°C	101763-964	VWR Funding Inc
DPBS (no Ca^{2+}, no Mg^{2+})	wash buffer	Room Temp	D8537-500ML	Sigma-Aldrich Inc
Advanced DMEM/F12	Washing/quenching medium	2–8°C	12634010	Fisher Scientific
Pen/Strep (Optional)	Antibiotic for contamination prevention	-20°C	15-140-122	Fisher Scientific
70% Ethanol (EtOH)	Sanitization of surfaces and materials	Room Temp	—	—
6-well TC Plates	Multiwell plates (Advanced TC treated)	Room Temp	89131-688	VWR Funding Inc
Conical Tubes				
Serological Pipettes				
Pipette Controller				
Inverted Microscope				

Single-Cell Passaging using Accutase

Once wells reach 70–80% confluency, they are ready for single-cell dissociation. This method is necessary for applications requiring precise cell counts, such as cryopreservation or seeding specific densities. We prefer to use Accutase as it is very gentle [(1)], other options e.g., recombinant trypsin alternatives are also used and are more cost effective [(2)].

Preparation: coat the appropriate number of wells with Matrigel at 37°C for ≥ 1 hour. Prepare the required volume of plating medium by adding ROCK inhibitor to mTeSR Plus to a final concentration of 10 µM and warm to room temperature. Accutase should be thawed in the 4°C and applied to the at this temp as well i.e., no need to warm the Accutase (**Note:** Accutase should be stored in single-use aliquots at -20°C to avoid repeated freeze-thaw cycles which degrade enzymatic activity.

1. **Wash:** Completely aspirate the media from the wells and wash 2x with 1 ml DPBS (no Ca2+, no Mg2+).

2. **Dissociate:** Add 1 ml of Accutase per well and incubate at 37°C for 5-10 minutes (Accutase is very gentle – do not rush this step, let the enzyme do its job). Firmly tap the side of the plate to detach cells.

3. **Quench:** *Gently* pipette the suspension 5 times to singularize the cell and then transfer the cell suspension to a conical tube containing 9 ml of Advanced DMEM/F12 to rinse the wells and dilute the Accutase.

4. **Centrifuge:** Pellet the cells by spinning for 5 minutes at 200 x g (RCF).

5. **Resuspend:** Aspirate the supernatant without disturbing the pellet. Tap the bottom of the tube firmly to flick-loosen the pellet then resuspend the cells in the required volume of mTeSR Plus + 10 µM ROCK inhibitor **Note:** ROCK inhibition is essential during single-cell dissociation to prevent apoptosis and ensure post-passaging viability and attachment [3,4].

6. **Plating:** Aspirate the Matrigel from fresh wells and **immediately** dispense **2 ml of the cell suspension** into each. Move quickly and do not allow the coated surface to dry out, as this compromises substrate integrity and cell attachment.

7. **Incubation:** Place the plate in the incubator on the shelf. While maintaining contact between the plate and the shelf, in a brisk manner, move the plate forward-backwards (3-5x) and then side-to-side (3-5x) to disperse the cells evenly. Leave the plate undisturbed overnight. **Note:** Some groups perform this entire sequence (forward-backwards and side-to-side) directly in the TC hood and then leave the plate undisturbed for roughly 20 minutes before carefully transferring to the incubator to prevent convection currents from causing uneven settling.

8. **Recovery:** The following day replace the medium with **2 ml of fresh mTeSR Plus** (without ROCK inhibitor). **Note:** Removal of ROCK inhibitor is critical because prolonged exposure can cause abnormal morphology, metabolic stress, and spontaneous differentiation, compromising iPSC pluripotency [5-7].

Troubleshooting

Problem	Possible Cause	Solution
Cells remain attached after 10 min	Insufficient incubation, insufficient rinsing, and/or degraded Accutase.	Increase incubation time up to 30 min. Remove Accutase from well, rinse 2x with DPBS (no Ca2+, no Mg2+) add a new ml of Accutase. Use new bottle or thaw a new aliquot.
High cell death / low viability	Mechanical stress due to over pipetting or pipetting before the cells have detached	Pipette gently and only after cells detach with gentle tapping.
Cell suspension is sticky or stringy	Lysed cells and leaked genomic DNA	Add 1-2 drops of a 1mg/ml stock solution of DNase I to the 10 ml cell suspension (Cells/Accutase/ADF). Incubate at 37°C for 5 – 10 min. Do not vortex.

Further Reading:

1. **Bajpai, R.** et al. (2008) 'Efficient propagation of single cells accutase-dissociated human embryonic stem cells', *Molecular Reproduction and Development*, 75(5), pp. 818–827. doi: 10.1002/mrd.20819.
2. **Ellerström, C.** et al. (2007) 'Facilitated expansion of human embryonic stem cells by single-cell enzymatic dissociation', *Stem Cells*, 25(7), pp. 1690–1696. doi: 10.1634/stemcells.2006-0590.
3. **Watanabe, K. et al.** (2007) 'A ROCK inhibitor permits survival of dissociated human embryonic stem cells', *Nature Biotechnology*, 25(6), pp. 681–686. doi: 10.1038/nbt1311.
4. **Ohgushi, M. et al.** (2010) 'Molecular Pathway and Cell State Responsible for Dissociation-Induced Apoptosis of Human Pluripotent Stem Cells', *Cell Stem Cell*, 7(2), pp. 225–239. doi: 10.1016/j.stem.2010.05.019.
5. **Vernardis, S. I. et al.** (2017) 'Mass spectrometry-based proteomics reveals that cell-cell adhesion is a key determinant of human pluripotent stem cell survival', *Scientific Reports*, 7, p. 42138. doi: 10.1038/srep42138.
6. **Alasmar, S. et al.** (2023) 'Human pluripotent stem cell-derived limbal epithelial stem cells: a review of current methods and future perspectives', *Stem Cells*, 41(11), pp. 1006–1021. doi: 10.1093/stmcls/sxad059.
7. **Matsumoto, T. et al.** (2022) 'Advanced Culture Systems for Mammalian Cells', *Bioengineering*, 9(11), p. 613. doi: 10.3390/bioengineering9110613.