| Humboldt-CIRM Shared Resource Lab | STANDARD OPERATING PROCEDURE |
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**Title:** Clump passaging of hPSCs using ReLeSR
**Purpose:** To describe standard procedure for thawing, maintaining, and freezing iPSCs
**Version: 1**
**Last Updated: 20260218**
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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 |
| **ReLeSR** | Clump-based, non-enzymatic dissociation reagent | Room Temperature | MSPP-100-0484 | VWR Funding Inc |
| **Matrigel (hESC-Qualified)** | Basement membrane matrix | -20°C | BD354277 | VWR Funding Inc |
| **DPBS (no $Ca^{2+}$, no $Mg^{2+}$)** | Ca2+/Mg2+ free wash buffer | Room Temperature | D8537-500ML | Sigma-Aldrich Inc |
| **ROCK Inhibitor (Ri)** | Optional: survival support if too many single cells are generated | -20°C | 101763-964 | VWR Funding Inc |
| **Advanced DMEM/F12 (ADF)** | Washing/quenching medium | 2–8°C | 12634010 | Fisher Scientific |
| **Penicillin-Streptomycin** | Optional antibiotic for contamination prevention | -20°C | 15-140-122 | Fisher Scientific |
| **70% Ethanol (EtOH)** | Sanitization of surfaces and materials | Room Temperature | — | — |
| **6-well TC Plates** | Multiwell culture plates (Advanced TC treated) | Room Temperature | 89131-688 | VWR Funding Inc |
| **Conical Tubes** | Centrifugation and media preparation | Sterile | 21008-936 | VWR Funding Inc |
| **Serological Pipettes** | Sterile liquid handling | Room Temperature | 76184-746 | VWR Funding Inc |
| **PPE (Gloves)** | Nitrile Exam Gloves | Room Temperature | 99452683 | Fastenal |
| **Permanent Marker** | Labeling culture vessels and tubes | Room Temperature | — | — |

## Clump Passaging using ReLeSR

Once wells reach 70–80% confluency, they can be passaged for expansion or other downstream applications e.g., generating embryoid bodies. Clump passaging is generally recommended over single-cell dissociation, as it lowers the vulnerability to apoptosis and subsequent genetic drift [1-3] while circumventing the necessity for ROCK inhibitor supplementation. While 0.5 mM EDTA is a common alternative for clump passaging, this protocol relies on ReLeSR for its ability to selectively detach undifferentiated colonies while leaving differentiated cells behind.

1. **Preparation:** Determine the optimal split ratio and coat the appropriate number of wells with Matrigel at 37°C for ≥ 1 hour (See: **Matrigel – Aliquoting, Thawing, and Coating**). Generally, a 1:6 split is recommended for standard maintenance. Calculate the total volume of mTeSR Plus required based on 2 ml per well (e.g., 12 ml for an entire 6-well plate), place this volume into a 50 ml falcon tube, and allow it to reach room temperature (do not warm it in a 37°C incubator). **Note:** the split ratio can be adjusted higher (e.g., 1:10) if the culture is very confluent or lower (e.g., 1:4) if growth is slow, depending on your desired timeline.

2. **ReLeSR Treatment:** Wash each well 2x with 1-2 ml DPBS (no Ca2+, no Mg2+). Add enough ReLeSR to entirely cover the bottom of the well (e.g., 1 ml per well of a 6-well plate) then immediately and completely aspirate the ReLeSR, leaving only residual liquid.

3. **Incubation:** Incubate at 37°C for roughly 5-7 minutes. Colonies should begin to ball up or detach entirely while differentiated cells remain. **Note:** the exact duration of incubation is less important than what is physically happening with the colonies themselves, and this will vary depending on numerous factors e.g., cell line, density, passage

number, DIV, etc. Therefore, it is **critical to observe the cells** every couple of minutes to identify the time window in which healthy colonies are lifting while any potential differentiated cells remain attached.

4. **Detachment:** Add exactly 1 ml of mTeSR Plus to each individual well. Firmly tap each side of the plate for a total of 30–60 seconds to detach the colonies. Once detached, gently transfer the 1 ml suspension from each well into its own fresh well; do not mix the contents of different wells.

   Gently and uniformly dissociate the suspension by pipetting the entire volume up and down to break the colonies into clumps of approximately 15–40 cells. Maintaining a uniform clump size is critical to reduce the potential for non-specific differentiation. Ensure you pipette the full volume of media and cells with a consistent, uniform force with each stroke to achieve maximum consistency. Monitor the aggregate size under the microscope after every 1 or 2 pipette strokes to ensure the desired clump size is obtained.

5. **Seed:** Dilute clumps in volume of mTeSR Plus, ***without Rock inhibito***r. Aspirate/remove Matrigel from fresh wells, and plate. **Note:** If there is a large proportion of single cells after detachment/dissociation, then it may be necessary to culture cells in the presence of Rock inhibitor (no longer than 24 hours). To prevent over dissociation in the future it may be necessary to incubate in ReLeSR for less time, or to break apart colonies with less force or frequency.

6. **Plating & Incubation:** Next, 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 (3-5 times) and then side-to-side (3-5 times), in order to disperse the clumps evenly throughout the well. Leave plate undisturbed overnight before moving.

## Troubleshooting

| Problem | Possible Cause | Solution |
|---|---|---|
| **Colonies remain attached / hard to dislodge** | Insufficient incubation time with ReLeSR. | Increase incubation time by 1–2 minutes. |
| **Aggregates are too large** | Insufficient pipetting | Pipette cells up and down a few more times and with more force. Check using the microscope. |
| **Aggregates are too small. Many single cells generated** | Over-incubation or excessive pipetting. | Decrease incubation time by 1–2 minutes. Minimize manipulation after dissociation. |
| **Differentiated cells detaching with colonies** | Incubation is too long or temperature is too high. | Decrease incubation time by 1–2 minutes. Decrease incubation temperature to room temperature (15–25°C). |
| **Non-uniform attachment** | Clumps were not evenly distributed during plating. | Move the plate in brisk side-to-side and forward-backward motions (avoid circular motions) before settling. |

## Further Reading:


1. Beers, J., Gulbranson, D.R., George, N., Siniscalchi, L.I., Jones, J., Thomson, J.A. and Chen, G., 2012. Passaging and colony expansion of human pluripotent stem cells by enzyme-free dissociation in chemically defined culture conditions. *Nature protocols*, *7*(11), pp.2029-2040.
2. Bai, Q., Ramirez, J.M., Becker, F., Pantesco, V., Lavabre-Bertrand, T., Hovatta, O., Lemaitre, J.M., Pellestor, F. and De Vos, J., 2015. Temporal analysis of genome alterations induced by single-cell passaging in human embryonic stem cells. *Stem cells and development*, *24*(5), pp.653-662.
3. Nguyen, H.T., Geens, M., Mertzanidou, A., Jacobs, K., Heirman, C., Breckpot, K. and Spits, C., 2014. Gain of 20q11. 21 in human embryonic stem cells improves cell survival by increased expression of Bcl-xL. *Molecular human reproduction*, *20*(2), pp.168-177.