Protocol Steps

Preparation of Complete Stem Cell Culture Medium

1. Thaw mTeSR1 5x Supplement at room temperature (15 - 25°C). Aliquot mTeSR1 5x supplement into 50-ml conical tubes, 20 ml/tube, and store at −20°C.
2. In a suitable vessel, mix 20 ml of mTeSR1 5x Supplement with 80 mL of mTeSR1 Basal Medium to generate complete medium. Mix thoroughly. This mTeSR1 medium is now ready for use. Store complete mTeSR1 medium at 4°C for up to 2 weeks if not used immediately.

Preparation of Matrigel-Coated Cell Culture Plate: It is imperative that the Matrigel remains on ice at all times, as it will quickly and irreversibly solidify at room temperature.

3. To aliquot the matrigel, place the matrigel bottle on ice in a cold room. The neck of the bottle should not be submerged in ice. Aliquot the matrigel into 1.5-ml pre-chilled microcentrifuge tubes, 0.5 mg/tube, and store at −80°C.
4. Pre-chill a clean 15-ml conical tube on ice, then add 6 ml of ice-cold DMEM/F12 medium.
5. Pipette 1 ml of the ice-cold DMEM/F12 medium into a 0.5 mg matrigel aliquot. Pipette up and down gently to thaw and dissolve the matrigel, avoiding bubbles.
6. Immediately transfer the dissolved matrigel to the 15-ml conical tube prepared in step 4 and mix by inverting the tube several times.
7. Pipette 1 ml of the mix into each well of a 6-well plate. Gently shake the plate to coat all surface with the matrigel. Place the plate in a 37°C incubator and wait for at least 1 hour before use.

Do not let the plated matrigel solution evaporate. If not used immediately, seal cultureware (e.g., with Parafilm) to prevent evaporation of the matrigel solution. Store up to 1 week at 2-8°C.

Before proceeding to the next step, let stored coated cultureware come to room temperature (15 - 25°C) for 30 minutes.

Sub-culture (Passaging) of Stem Cells

8. Check the confluency of the hPSCs daily.
9. Aliquot a sufficient amount (see below) of mTeSR1 and warm to room temperature (15-25°C).

Prepare approximately 1.5 ml per well of a 6-well plate of mTeSR1 for each well that will be used, plus 300 µl for pellet resuspension. Do not warm mTeSR1 in a 37°C water bath.

10. Collect spent media and transfer to a 15-ml conical tube.
11. Immediately wash cells (do not allow them to dry out) with 1 ml of D-PBS and aspirate.
12. Add 1 ml of ReLeSR per well of a 6-well plate and aspirate after 30-40 seconds
13. Incubate at 37°C for 3-5 minutes.

The incubation time may vary for different hPSC lines. However, do not incubate for

more than 5 minutes.
14. Add 1 ml of the spent media collected in step 10.
15. Use a 1-ml pipette to gently detach cells by pipetting the media up and down in a circular fashion around the well.
16. Transfer the detached cell aggregates to the 15-ml conical tube prepared in step 10.
17. Spin down the cell aggregate at 300 x g for 5 minutes at room temperature.
18. During the spin down, gently tilt the prepared matrigel-coated cultureware from step 7 onto one side so that any excess matrigel solution collects at the edge. Aspirate the excess solution collected at the bottom of the cultureware without touching the coated surface. The cultureware should now have a thin layer of Matrigel attached to the surface and no extra liquid.
19. Immediately add 1.5 ml of warm mTeSR1 medium per well to each well of the cultureware.
20. When the spin-down is completed (step 17), aspirate the supernatant and resuspend the cell pellet in 300µl of fresh pre-warmed mTeSR1 medium, gently pipetting up and down several times.
21. Plate the cell aggregate suspension at the desired density, usually 50 - 200 aggregates per well of a 6-well plate, onto the coated wells containing mTeSR1.
22. Move the plate back-and-forth and side-to-side to distribute the cell aggregates. Place the plate in a 37°C incubator.
23. Change the media daily with pre-warmed mTeSR1 and visually assess cultures to monitor growth until passaging again.
If the colonies are at an optimal density of 50 - 200 aggregates per well, perform 1-in-6 to 1-in-10 splits every 4-7 days.

Cryopreservation: Cryopreserve cultures when they would otherwise be ready for passaging. Each cryopreserving vial should have the cell aggregates from half of one well of a 6-well plate.
24. Prepare cryopreserving media (see Reagents and Solutions):Anticipate 1 ml of cryopreserving media for each cryovial.
25. Detach cells according to steps 10-17.
26. Aspirate supernatant and then carefully resuspend the pellet with 2 ml of cryopreserving media per well harvested. Try to avoid breaking up cell aggregates.
27. Transfer 1 ml of cell aggregate suspension into separate labeled cryovials.
28. Cryopreserve cell aggregates using a multi-step protocol: −20°C for 2 hours, −80°C overnight, and then long-term storage at −135°C (liquid nitrogen) or colder.
Cell Thawing: Stem cells should be thawed into matrigel-coated cultureware. In general, one vial of cryopreserved cells from step 28 can be thawed into two wells of a 6-well plate.
29. Prepare all tubes, room temperature mTeSR1 medium (15 - 25°C), and coated cultureware before starting the procedure to ensure that the thawing procedure is performed quickly.
Once mTeSR1 is at room temperature (15 - 25°C), follow steps 18-19 to replace the

excess matrigel solution in the cultureware plate with mTeSR1. If matrigel-coated cultureware was previously stored at 8°C, allow the plate to come to room temperature (15 - 25°C) for 30 minutes, as explained in step 7.
30. Wipe the outside of the cryovial with 70% ethanol.
31. Quickly thaw cells in a 37°C water bath by gently shaking the vial while it is submerged below the neck. Remove the vial from the water bath when a small frozen cell pellet still remains.
32. Clean the outside of the vial with 70% ethanol.
33. Use a 1-ml pipette tip to transfer cells from the cryovial to a 15-ml conical tube.
34. Add 1 ml of pre-warmed mTeSR1 dropwise to the 15-ml tube, gently swirling the tube as the medium is added.
35. Centrifuge cells at 300 x g for 5 minutes at room temperature.
36. Aspirate the supernatant, leaving the cell pellet intact. Gently resuspend the cell pellet in 1 ml of mTeSR1, maintaining the cells as aggregates but breaking up the pellet.
37. Transfer 0.5 ml of the cell mixture into one well of a matrigel-coated, mTeSR1-containing 6-well plate (step 29).
In general, more cell aggregates will need to be plated after thawing
than during routine passaging.
38. Move the plate back-and-forth and side-to-side to distribute the cell aggregates. Place the plate in a 37°C incubator.
39. Change medium (fresh mTeSR1) at 6 hours post plating and place the plate back in a 37°C incubator.
40. Change medium daily using mTeSR1 and visually assess cultures to monitor growth until passaging again.