Forrester Lab Human iPSC Protocols

A. Thawing human iPSC

1.Prepare a well of a 6-well plate with diluted CELLSTART

735ul PBS+Mg+Ca +15ul CELLSTART (1 in 50 dilution/well).

- Very important that PBS +Mg +Ca is used.
- Make sure well is completely coated.

2. Incubate at 37°C for 1 hour.

3. Warm Stempro + bFGF in water bath

5ml Stempro + 10ul of bFGF from stock (10ug/ml) to give final concentration of 20ng/ml.

4. Aliquot 2.5mls of the warmed Stempro + bFGF into a universal tube (30ml tube).

5. Thaw vial of frozen cells in the palm of hand. As last crystals disappear gently remove cells using a plastic pastette and add to the warmed Stempro + bFGF medium. Rinse the vial with some of the medium from the tube.

6. Centrifuge @1200rpm (200g) for 3 minutes.

7. Aspirate medium leaving pellet of cells intact.

8. Gently resuspend cell pellet with 1.5ml warmed Stempro + bFGF using pastette being careful to resuspend the pellet but not breaking up the clumps of cells.

● The cells were frozen down as little squares and it is important to leave these squares intact when resuspending the pellet.

9. Aspirate CELLSTART from well and replace with medium containing the resuspended cells

10.Add 5ul Rock Inhibitor to well from 1mg/ml stock (final concentration 10uM).

11. Check the cells by microscopy.

12. Place in the incubator (37°C : 5%CO_2) and gently rock the plate backwards and forwards a few times to evenly disperse the cells over the well.

13. On the following day remove medium and replace with fresh Stempro + bFGF medium.

● Cells only have Rock inhibitor when they are thawed then it is removed the following morning.

● When cells are thawed there is never a precise timescale as to when they can be passaged and often it takes time for them to recover. Very rarely they will be confluent at this stage. As the colonies they should be passaged into 2 wells when they look about 50-80% confluent. If after 7 days they have not reached this state of confluency, they should be replated. (1well-→1 well). Usually this encourages them recover and grow properly.

14. Media should be replaced everyday: remove media and replace with pre-warmed Stempro + bFGF (1.5ml/well of 6 well plate).

- Daily media change is very important
- Stempro is supplemented with bFGF (20ng/ml) **on the day of use** to prevent its degradation by repeating warming and chilling media.

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B. Passaging human iPSC.

iPSCs are ready for passaging when iPSC colonies are confluent (Figure 1), the medium is changed an hour before passaging.

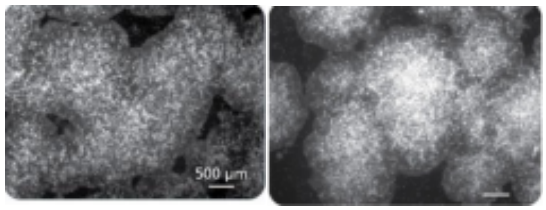

Figure 1. Examples of images of confluent iPSCs ready for passaging or freezing.

● When cells are maintained in Stempro under feeder-free conditions, there is sometimes a little differentiation round the edges of the colonies. This is nothing to worry about and quite normal.

1. Prepare an appropriate number of wells CELLSTART as described in step A1.

● 1 confluent well can be passaged to 4 new wells.

2. Cut the confluent well using EZ tool (Figure 2).

● Do not remove the medium from the well before cutting.

Hold the plate in one hand and pull (roll) the tool across the entire plate in one direction (left to right). Apply enough pressure so the entire roller blade touches the well and maintain uniform pressure during the rolling action. Keep rolling the EZ tool parallel to the first pass until the whole well has been covered, rotate the plate 90◦ and repeat.

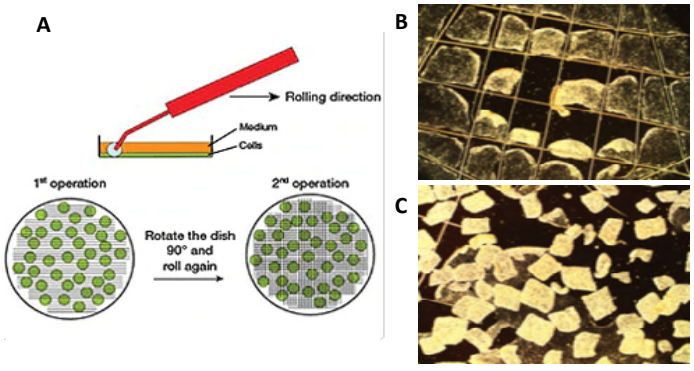

Figure 2. Use if EZ tool and images of iPSC colonies during (B) and after (C) the procedure.

3. Dislodge the cells from the base of the well by gently flushing using a pastette.

4. Remove CELLSTART from prepared wells and add 1.5ml Stempro + bFGF to each well.

5. Distribute the contents of the 'cut' well evenly to the prepared wells using a pastette.

6. Check the cells by microscopy.

7. Rock the plate to distribute the squares of cells evenly across the plate once they are in the incubator.

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C. Freezing human iPSC

Human iPSCs are ready for freezing when confluent (see Figure 1).

- 1 confluent well can be frozen into two cryovials.

1. Remove media and replace with fresh pre-warmed Stempro + bFGF.

2. Add 5ul Rock Inhibitor to well from 1mg/ml stock. (final concentration 10uM).

3. Incubate for at least 1 hour.

4. Cut the iPSC colonies with EZ tool as described in protocol B2, dislodge with pastette and then flush the squares of cells into a 30ml Universal tube.

5. Centrifuge @ 1200rpm (200g) for 3 minutes.

6. Remove supernatant and resuspend the cell pellet gently with 1ml CRYOSTOR using a pastette.

7. Using a pastette add 0.5ml cells + CRYOSTOR to labelled cryovials (from 1 confluent well of a 6well plate cells can be frozen in 2 cryovials)

- It is important to be very gentle when resuspending the pellet to leave the squares of cells intact.

8. Place cryovials in pre-chilled (4^{o}C) Mr. Frosty.

9. Place in -80C overnight.

10. Transfer vials to liquid nitrogen or -150C freezer for long term storage.