Notes before starting:

- iPSCs are extremely sensitive to processing as single cells. It is critical to minimize the processing time and handling of iPSCs as much as possible. Ensure that all materials are prepared before hand and that everything is ready to go once the cells are lifted and ready for sort.
- mTeSR is prepared following manufacturer instructions (Stemcell Technologies #85850) and should be supplemented with antibiotics unless otherwise noted.
- The Sony MA900 is very gentle resulting in a very high viability for iPSCs. Only 1 cell will be plated per 96-well sized well. This should yield approximately 48 iPSC colonies per 96-well plate (ie approximately 1 out of 2 plated cells survive). Scale as needed, dependent on experimental requirements. Note that a single 6-well of ready-to-passage iPSCs contains 1-2x10^6 cells and is sufficient starting material to sort into many 96-well plates.
- If the Sony MA900 is not available for plating single cells, the following run parameters may help iPSC viability: 100um nozzle, sample pressure of 20psi, sample flow rate of 20-30ul/min, cell suspension and collection plate held at 4-5C.

Cloning Media = mTeSR adjusted to 10% CloneR (ie 9 mL complete mTeSR + 1 mL CloneR). Cloning medium can be stored at 4C for up to one week. (CloneR, Stemcell Technologies #05888)

1. Two hours prior to sorting, prewarm 5mL mTeSR supplemented with 10 μM ROCK inhibitor (Y-27632). Aspirate media of iPSCs and replace with 2mL mTeSR + ROCK inhibitor. Store remaining mTeSR + ROCK inhibitor at 37C. Warm cloning media to 37C (5mL + 10mL per plate). Warm 1mL accutase to room temperature.

2. During this time, coat a 96-well plate with hESC-matrigel (more than one plate if necessary). Coat each well with 50 μL of diluted hESC-matrigel (diluted following manufacturer instructions). Leave plate at RT for 1 hour. After 1 hour, aspirate excess hESC-matrigel and use a multi-channel pipet to add 100 μL cloning medium to each well. Incubate plate for 1 hr at 37C. Cells will be sorted directly into this plate.

3. After 2 hour incubation with Rock inhibitor, aspirate media from iPSCs and wash once with DPBS. Add 0.6 ml accutase for 8 -10 minutes at 37°C. At 4 minutes, gently rock the plate to mix and check progress. Do not let cells incubate for longer than 10 minutes.

4. After incubation, add 2ml mTeSR + 10 μM ROCK to each well to neutralize accutase. If cells are not fully lifted by 10 minutes, use a cell scraper to dislodge remaining cells. Collect the cells in a 15ml conical tube and spin down at 200 g for 2 minutes.

5. Aspirate media and gently resuspend pellet in 4 mL cloning medium [3-5x10^5 cells per mL].

6. Immediately take the resuspended cells and the 96-well plate(s) for sorting. Pass cells through a 40 μm cap and into a FACS sorting tube at the flow facility. Sort 1 cell into each well of the 96-well plate.
   Note: If this is the first time following the protocol, it is advisable to add DAPI as a viability stain. Viability is typically >95% and DAPI staining is not necessary.

7. Place all seeded 96-well plates in the incubator. Do not change media the following two days.

8. 72 hours after sort, replace media with fresh cloning medium (100 μL per well).

9. 96 hours after sort, perform a full media change using mTeSR **without CloneR**. After the media change, mark wells that have a detectable iPSC colony. Some wells may have more than one colony, mark these and avoid.

10. Continue to change every-other-day until colonies are ready to passage (about 12 days after sort).

11. When colonies grow large enough, passage using standard iPSC procedures. For routine passaging, we use 0.5mM EDTA (pH 8.0) diluted in DPBS. Expand into a 24-well. Do not plate cells with rock inhibitor. Continue expansion following standard iPSC culture procedures and then proceed with characterization.