iPSC culture and differentiation (updated 5/25/2020 by PC)
Add when to start/stop
Aliquots Preparation:

1. Matrigel (Growth factor reduced membrane matrix 354230) (Fisher catalog CB-40230)

The night before: Take a tall, circular ice bucket and fill it half-way with ice. Place the Matrigel in the center and then top the rest off with ice to completely cover it. Place at 4C and let sit overnight to thaw. Place 1000uL pipet tips, 0.5 mL tubes, and 1.5mL tubes at 4C.

To aliquot: **Do not filter.** Get a separate ice bucket. Pre-label all tubes and keep them on ice. Keep your pipet tips on ice (in a separate ice bucket, surround your tips in ice while keeping them in the holder). Aliquot 500uL/tube for matrigel plates, 100 uL/tube for devices, change tips whenever Matrigel starts sticking to the sides (this usually means they are getting warm – despite aliquoting close to 500uL in each tube, you will likely get ~450-475uL). Store immediately in Cardiac -20C.

2. Matrigel Plate preparation: Thaw Matrigel aliquots at 4C for 1 hour before use (or overnight). Aliquots can be kept for 3 weeks. To make plates you need 75uL of Matrigel to 6 mL of DMEM/F12 (makes one plate at 1ml mixture per well of 6 well, adjust other volumes to other plates). Use DMEM/Matrigel mixture immediately after making and coat plates (DMEM/F12 catalog 11330-057). Incubate at room temperature/in the incubator for one hour (or can be left for one week at 37C and used later as long as it hasn't dried up). If placed in 4C at least 24 hours, can leave out for 15 minutes before removing Matrigel, and plates can be stored at 4C for up to 3 weeks. Wash one time with 1xPBS before using

3. Y-27632 (Tocris 1254, 10mg), make 5mM stock solution**. Do not filter.**

In a sterile environment, add 6.24mL sterile 1xPBS to 10mg of Y-27632. Divide the solution into 100uL aliquots and store at -20C for up to 1 year.

4. mTeSR1 medium (Stem Cell, basal medium 05850, 5x supplement 05852)

NOTE: Thaw supplements or complete medium at room temperature (15 - 25°C) or overnight at 2 - 8°C. Do not thaw in a 37°C water bath (unless absolutely critical and you intend on using the entire supplement with a low density of cells. If this is done, try to change media the following day slightly earlier).

1. Thaw mTeSR™1 5X Supplement at 4C overnight and mix thoroughly. NOTE: Once thawed, use supplement immediately or aliquot and store at -20°C for up to 3 months. Do not exceed the shelf life of the supplement. After thawing the aliquoted supplement, use immediately. Do not re-freeze.
2. Add 100 mL of mTeSR™1 5X Supplement to 400 mL of mTeSR™1 Basal Medium. Mix thoroughly. Filter in two separate 250mL filters. NOTE: If not used immediately, store complete mTeSR™1 medium at 2 - 8°C for up to 2 weeks. Alternatively, aliquot and store at -20°C for up to 6 months. After thawing the aliquoted complete medium, use immediately or store at 2 - 8°C for up to 2 weeks. Do not re-freeze.

5. IWP4 (Tocris 5214), make 2.5mM stock. **Do not filter.**

Dissolve 10mg in 8.05mL DMSO. Do not filter. Takes time to solubilize (15mins). Aliquot in 25 uL aliquots and store at -20C. Use at 1:500 dilution. Be careful that IWP4 is in solution. Thaw each aliquot fresh – only use for one 24 hour period.

6. CHIR99021 (Tocris 4423), make 12mM stock. **Do not filter.**

Dissolve 10mg in 1.79mL DMSO (dissolves in 5 mins). Aliquot (200uL) and store at -20C. Use at 1:1000 dilution (if want 12uM final concentration).

7. FreSR™-S (Stem Cell, 05859). **Do not filter.**

Defined, serum-free cryopreservation medium for human ES and iPS cells. Keep 50mL bottle in fridge (stable at 4C).

8. Fibronectin (lab stock located in the 4C room, Fisher 356009)

Using a 20G needle, add 5 mL of sterile water to 5 mg of Fibronectin to make a 1 mg/mL stock. Allow the fibronectin to dissolve at room temperature. Make 500 uL aliquots and store at -20C. Keep at 4C for up to two weeks. Dilute 1:100 (10 ug/mL final concentration) in PBS and add 1 mL per 6 well plate. Incubate at room temperature for an hour. Plates can be stored for 2 weeks at 4C and ~5-7 days at 37C (check to make sure it is still covering the bottom).

Thawing iPSCs:

1. Thaw 150uL Matrigel aliquot on ice/in 4C. Add 150uL Matrigel to cold 12mL DMEM/F12, mix well and use quickly – keep cold and use ice if you will not use within 1-2 minutes of making. Add 1mL/well to two 6-well plates and shake to cover bottom. Incubate at 37C or at room temperature for >= 1hr. Prior to seeding cells, wash once with 1xPBS.--------Alternatively take a Matrigel-coated plate from 4C and place at RT for >15min to warm up.

2. Warm mTeSR1 medium to RT.

3. Thaw one aliquot (100uL) of Y-27632 stock (5mM). This is also commonly located in the TC fridge.

4. Remove a frozen vial of hiPSCs from liquid N2, immerse vial in 37C water bath without submerging the cap. Swirl the vial gently for 2-3min and remove immediately when content is completely thawed.

5. Spray vial with 70% ethanol and place into TC hood. Add 5mL PBS into a 15mL tube. Transfer cells into the tube.

6. Centrifuge at 1000 RPM for 4 min at RT. Aspirate supernatant. Check cell pellet to thaw at around 25% confluency of a six well plate.

7. Add 1.5 mL mTesr1 + 5uM Y-27632 per well of a 6 well plate.

8. Resuspend cell pellet in 1mL of mTeSR1 medium with 5uM Y-27632 (1:1000 dilution of 5mM stock Y-27632 into mTeSR1 medium). Pipet up and down with a P1000 slowly 3 times to break the pellet.

9. Add 1/3 – 1/2 of cells to one well, and 1/5 – 1/4 of cells to the other well (adjust based on pellet size). Put plate back into 37C incubator. Move the plate in three quick, short, back-and-forth and side-to-side motions to disperse the cells across the surface of the well.

10. On the next day, replace medium with fresh RT mTeSR1 medium 2mL/well. If cells are very low density and in single cell colonies, replace media with mTeSR1 + 5uM Y-27632 until colonies grow up.

11. Medium change daily until cells are ready (70%-90%, every 3-4 days) for passage. Check morphology and keep the better looking confluency going. Try not to under/over seed when thawing cells down – optimal time to spit is 2-4 days after thawing.

Accutase splitting of iPSCs:

1. Thaw 150uL Matrigel aliquot on ice/in 4C. Add 150uL Matrigel to 12mL DMEM/F12, mix well and use quickly – keep cold and use ice if you will not use within 1-2 minutes of making. Add 1mL/well to two 6-well plates and shake to cover bottom. Incubate at 37C or at room temperature for >= 1hr. Prior to seeding cells, wash once with 1xPBS.--------Alternatively take a Matrigel-coated plate from 4C and place at RT for >15min to warm up.

2. Prior to seeding cells, wash wells once with 1xPBS, remove and add 1.5mL of mTeSR1 medium with 5uM Y-27532 (dilution 1:1000 from 5mM stock). -------- should have 1mL extra mTeSR with Y-27632 to resuspend 1-well cell pellet. Warm Accutase in 37C bath.

3. Remove spent mTeSR medium from cells, wash once with 1xPBS.

4. Add 500uL/well accutase, incubate at 37C for 5-7min until cells detach.

5. Pipet gently 2-3x with P1000 pipet (waterfall down the well to collect as many cells as possible), add cell/accutase mixture to a 15mL tube with 5 mL of PBS to dilute accutase.

6. Spin at 1000rpm (**200**-250g) for 4min at 4C/RT.

7. Remove supernatant, add 1mL mTeSR with Y-27632, mix gently 3x with P1000.

8. Add 50-100uL cell suspension into a fresh well (with 1.5 mL mTeSR + Y-27632 added in step 2).

9. Put plate back into 37C incubator. Move the plate in three quick, short, back-and-forth and side-to-side motions to disperse the cells across the surface of the well.

10. On the next day, replace medium with fresh RT mTeSR1 medium (without Y-27632) 2mL/well.

11. Medium change daily until cells are ready (70%-90%, every 3-5 days) for passage.

Freezing iPSCs/CMs:

1. Freeze cells when the wells are 75-90% confluent – enough for 2 vials, or 50% if desperate into one vial.
2. Wash cells with 1xPBS.

3. Accutase detach cells, collect cells (dilute Accutase using 5 mL of 1xPBS per well), and spin down at 200g at 4C (as when passaging cells).

4. Remove supernatant.

5. On ice, suspend iPSCs in the freeze medium with 5uM Y27, mix gently. For iPSCs freeze a 70-90% confluent well in 1 mL of freezing media.

6. Transfer 500 uL into each cryovial.

7. Put the vials in RT Mr. Frosty (check isopropanol level). Put the Mr. Frosty into -80C.

8. The next day, transfer the vials to liquid N2.

iPSCs differentiation into CMs (Wnt pathway):

Preparation:
Day 0: Warm CHIR and IWP4 to 37C prior to use. Both are found in Rack 2 boxes 9-12 in the -20C.
Day 3: Be careful that IWP4 is in solution. If cloudy after several freeze thaws will require extended 37C to solubilize or need new aliquot. We now have small aliquots of IWP4 that's enough for two plates.
Note: Time sensitive steps include Day 0 – Day 1 (activating the WNT signaling pathway), and Day 3-Day 5 (inhibiting the WNT signaling pathway). Try to stay as consistent as possible with these timelines.

Warm RPMI/B27 w/ or w/o insulin.
RPMI/B27 w/o insulin = RPMI with Glutamax Supplement + 2% B27(w/o insulin).
RPMI/B27 w/ insulin = RPMI with Glutamax Supplement + 2% B27(w/ insulin).

Differentiation protocol:

Day 0: When cells are 85-100% confluent (usually Day 4 after seeding, minimum 3 days, up to 6 days. The starting density depends on specific iPSC cell lines or clones.). Wash cells once with 1xPBS, add 2mL/well RPMI/B27 w/o insulin + 12uM CHIR99021 (1:1000 dilution from 12mM stock).
Day 1 (24 hours later – time sensitive): Wash once with 1xPBS. Shake the plate gently during the wash. Replace with 2mL/well RPMI/B27 w/o insulin.
Day 2: Nothing.
Day 3: (around 48 hours following day 1): Add 2mL/well RPMI/B27 w/o insulin + 5uM IWP4 (1:500 dilution from 2.5mM stock).
Day 4: Nothing.
Day 5: (48 hours after day 3) Wash once with 1xPBS, add 2mL/well RPMI/B27 w/o insulin.
Day 6: Nothing.
Day 7: Replace with 2mL/well RPMI/B27 w/o insulin.
Day 8: Nothing.
Day 9: Replace with 2mL/well RPMI/B27 w/ insulin.
Day 11: Replace medium every other day with 2mL/well RPMI/B27 w/ insulin.
Day N: When cells are beating, PBS wash x1, RPMI no glucose +4mM sodium lactate (metabolic selection)
Day N+1: Replace with fresh RPMI no glucose +4mM sodium lactate.

- Selection for total 2~4 days.

Day N+4: Replace with RPMI/B27 w/ insulin.
Day N+6: Replate cells onto fibronectin coated plates by 0.25% Trypsin-EDTA . Replate in RPMI/B27 w/ insulin +2% FBS +5uM Y27.
Day N+7: Replace with fresh RPMI/B27 w/ insulin.
Use cells for assays 2 weeks after being replated onto FN.

Re-plating Cardiomyocytes:

1. Prepare a fibronectin coated plate and let sit at room temperature for 1 hour.
2. Remove media from IPSC-CMs and wash once with 1xPBS.
3. Add 500 uL of dissociation reagent (0.25% Trypsin-EDTA, Accutase, or TrypLE catalog A1285901 into a 6 well plate) with 10ug/mL DNAse and let at 37C for 5-30 minutes (5-10 minutes for 0.25% Trypsin, 15-30 for Accutase, and 15-30 for TrypLE). Check cells periodically and gently shake the plate to separate the cells and lift them from the bottom of the well.
4. Prepare a 15 mL conical with RPMI B-27 with insulin and 20% FBS (make 2mL of media for every well you are re-plating), or add 250 uL FBS to every 500 uL trypsin added to the cells to neutralize.
5. Gently pipet cells up and down with a P1000 to break up cell clumps.
6. Add cell mixture to the RPMI B-27 with insulin and 20% FBS conical (or 250 uL FBS per 500 uL trypsin).

7. Count cells (optional). For a confluent monolayer, 2-3 million cells/well works well in a 6 well plate.
8. Spin cells down for 4 minutes at 1000 rpm.
9. Remove the supernatant.
10. Add RPMI B-27 with 2% FBS and 5 uM Y27632 to the cell pellet (2 mL/well).
11. Remove fibronectin coating from the plate.
12. Wash once with 1x PBS.
13. Add cell mixture to fibronectin coated wells and gently shake the plate up and down, back and forth to evenly distribute the cells.
14. After 24 hours, replace media with fresh RPMI B-27 with insulin.