Protocol for growing and maintaining Human Cortical Spheroids –
adapted from Yoon et al, 2019 and Pasca, 2015

DMEM-F12	Thermo Fisher	11320082
DMEM With L-Glutamine and 4.5g/L Glucose; Without Sodium Pyruvate	Thermo Fisher	MT10017CV
Knockout Serum Replacement	Gibco Life Technologies	10828028
Newborn Calf Serum,USA origin, Heat Inactivated, sterile-filtered, suitable for cell culture	Sigma-Aldrich	N4762-500ML
Penicillin-Streptomycin (10,000 U/mL)	Gibco Life Technologies	15140163
MEM-NEAA (100x)	Gibco Life Technologies	11140050
Gibco™ GlutaMAX™ Supplement	Gibco Life Technologies	35050061
HEPEs buffer	Sigma-Aldrich	H0887-100ML
Neurobasal™ Medium	Gibco Life Technologies	21103049
B-27™ Supplement (50X), minus vitamin A	Gibco Life Technologies	12587010
Dorsomorphin, ≥98% (HPLC)	Sigma-Aldrich	P5499-5MG
SB431542	Selleck Chemicals	S1067
Y-27632 – ROCK Inhibitor	Chemdea	CD0141
Recombinant Human BDNF Protein, CF	R&D Systems	11166-BD-050
NT-3	Sigma	SRP3128
Heat Stable Recombinant Human bFGF	Thermo Fisher Scientific	PHG0367
Recombinant Human EGF Protein, CF	R&D Systems	236-EG-01M
Dimethyl sulfoxide (DMSO)	Thermo Fisher Scientific	BP231-100
Accutase	Thermo Fisher Scientific	SCR005
DPBS w/o calcium and magnesium	Corning	MT21031CV
CountessTM Cell Counting Chamber Slides	Thermo Fischer Scientific	C10228
AggreWell™800 (6-well plate)	StemCell Technologies	34821

Costar® 6-well Clear Flat Bottom Ultra-Low Attachment 6-well plate	Corning	3471

Media Formulations:

hESC KSR Media (500 ml)

400 ml DMEM/F12
100 ml KSR
5 ml Pen-strep
5 ml NEAA
5 ml Glutamax
5 mL HEPES buffer

hESC Wash Media (500 ml)

500 ml DMEM
25mL ml Calf Serum
5 ml Pen-strep

hCS media (500 ml)

500 ml Neurobasal media
10 ml B27 (minus vitamin A)
5 ml pen-strep
5 ml Glutamax
5 mL HEPES buffer

Neural precursor expansion media

50 ml hCS media
40 µl 25 ug/ml FGF (final concentration, 20 ng/ml)
25 µl 40 µg/ml EGF (final concentration, 20 ng/ml)

Neural induction media

50 ml hESC KSR Media
50 µl 10 mM SB431542 (final concentration, 10 μM)
25 µl 10 mM dorsomorphin (final concentration, 5 µM)

Cortical differentiation media

50 ml hCS media
50 µl 20 µg/ml BDNF (final concentration, 20 ng/ml)
50 µl 20 µg/ml NT-3 (final concentration, 20 ng/ml)

Resuspend Dorsomorphin in DMSO at a concentration of 10 mM (1:2000)
Resuspend SB431542 in DMSO at a concentration of 10 mM (1:1000)
Resuspend Rock Inhibitor in DMSO at a concentration of 10 mM (1:1000)
Resuspend BDNF in water at a concentration of 20 µg/ml (1:1000)

Resuspend NT3 in water a concentration of 20 µg/ml (1:1000)
Resuspend FGF at a concentration of 25 µg/ml (1:1250)
Resuspend EGF at a concentration of 40 µg/ml (1:2000)

1. Grow stem cells feeder-free on 6-well plates as described in (dx.doi.org/10.17504/protocols.io.b4mcqu2w)
2. On day -1 of differentiation remove hESC Media and feed with hESC KSR Media + 10uM Rock Inhibitor (1:1000)
3. Pre-coat an aggrewell plate according to manufacturer instructions.
4. On day 0 of differentiation, aspirate media and add 1 ml of PBS- onto each well
5. Immediately Remove PBS- and add 1 ml of Accutase (1:3 Diluted with PBS-) onto each well of the 6 well plate.
6. Return plate to incubator for ~30 mins or until cells are dissociated into single cell suspension.
7. Remove plate from incubator. Pool the cells into a 15mL conical tube per plate (6mL total volume).
8. Using 6 mL hESC Wash media, rinse the wells and dilute the cell-Accutase suspension.
9. Spin down cells for 5 mins at 200-300 g.
10. Remove the supernatant from the falcon tube using a sterile glass pipette. Be careful not to aspirate any of the cells.
11. Resuspend the cells in 5 ml of hESC KSR Media + 10uM Rock Inhibitor (1:1000)
12. Using a 5mL serological pipette, filter cells through a 40 µm cell strainer into a new 50 ml conical tube.
13. Take two sets of 10 µl of cell suspension. Mix each set with 10 µl trypan blue dye, which comes with the Countess Cell Counting Chamber Slides.
14. Count cells with Countess automated cell counter or hemocytometer, averaging the counts from the two sets.
15. Resuspend the cells to a concentration of 18 million cells per 5 mL of media (3.6 million cells per mL) in hESC KSR Media + 10µM Rock Inhibitor (1:1000).
    1. If needed, combine tubes from multiple plates and re-concentrate by spinning down at 200-300 g and resuspending in an appropriate volume of hESC Media + Rock Inhibitor 1:1000
16. Transfer 5 ml of suspension to one well of a pre-coated 6-well Aggrewell 800 plate. Return plate to the incubator.
17. The next day (Day 1), prepare neural induction media.
18. Remove the aggrewell plate from the incubator and use a serological pipette to transfer the newly formed Embryoid Bodies (EBs) to a 15 ml conical tube.
19. Allow EBs to settle (1-2 mins, but check before aspirating to ensure EB retention) and aspirate off media, taking special care to not suck up the EBs.
20. Resuspend in 5 ml of neural induction media and deposit into one well of a 6-well ultra-low attachment plate.
21. Repeat steps 17-20 to change media each day until Day 6.

22. On Day 6, remove cell suspension from the plate and deposit into a 15 ml falcon tube
23. Allow spheroids to settle to the bottom of the tube (~1-2 mins).
24. While settling, make neural precursor expansion media.
25. Remove the supernatant from the settled spheroid suspension.
26. Resuspend spheroids in neural precursor expansion media.
27. Return to the Ultra-Low Attachment plate and return the plate to the incubator.
28. Repeat steps 22-27 to feed cells every day until Day 16 and then every other day until Day 25
29. On day 25, prepare Cortical differentiation media.
30. Remove cell suspension from the plate and deposit into a 15 ml falcon tube.
31. Allow spheroids to settle to the bottom of the tube (~1-2 mins).
32. Remove the supernatant from the settled cell suspension.
33. Resuspend cells in Cortical differentiation media.
34. Return to the Ultra-Low Attachment plate and return the plate to the incubator.
35. Repeat steps 30-34 every four days (d29, 33, 37, 41) day until Day 43
36. On day 43, change the media as above, but replace with only hCS media with no additives.
37. Change media by sedimenting the spheroids, removing the supernatant, resuspending in hCS Media, and returning to the Ultra-Low Attachment plate every four days until the termination of the experiment.