Khurana Lab BRIGHAM HEALTH BRIGHAM AND WOMEN'S HOSPITAL HARVARD MEDICAL SCHOOL	SOP-VK	**HCNT—Cortical Neuron Transdifferentiation**	Author: Zoe Approved:	Revision:	Issued 10-31-2020 Revised

1. **Purpose**
   To differentiate human iPS cells into cortical neurons by doxycycline induction of NGN2 transgene expression. Use iPSCs transfected with an NGN2-puro-SNAP PiggyBac construct, with a blasticidin-resistance backbone. Based on protocol from Yumanity/Xin Jiang/05-15-18.

2. **Scope**
   Begins with expanded iPSCs in culture and finishes with aged cortical neurons ready for use in imaging, biochemistry, sequencing, etc.

3. **Materials**
   Filtration system PES 0.2 µm, 250 mL
   6-well plates (Corning, #3506), or
   100 mm TC-treated Cell Culture Dishes (Westnet, #353003)
   Cell culture hood
   Centrifuge for 15mL conical tubes
   Pipets (10, 20, 1000p)
   15mL and 50mL conical tubes
   Autopipette
   10mL pipets
   Microcentrifuge tubes
   Counting slides
   Countess
   Filtration system PES 0.2 µm, 500 mL (Corning, #974102)
   96-well plates (Brooks, #MGB096-1-2-LG-L)
   Poly-L-Ornithine/Laminin 24-well TC treated plates (Corning, #354659)
   Steriflip 50mL 0.22 µm PES sterilized filter (Millipore, #SCGP00525)
   Multichannel pipet
   Reagent Reservoir (Fisher Scientific, #21385104)

4. **Reagents**
   Knock-out serum replacement (KO-SR) (Gibco, #10828-028)
   Matrigel growth factor reduced (Corning, #354230)
   DPBS without Ca and Mg, 500mL (Corning, #21-031-CV)
   Accutase (Life Technologies, #00-4555-56)
   Blasticidin (Invivogen, #ANT-BL-1)
   StemFlex medium (Gibco, A3349401)
   StemFlex Supplement
   ROCK inhibitor (Peprotech, #1293823)
   Doxycycline hyclate (Sigma, #D9891-5G)
   Trypan blue (Life Technologies, #15250-061)
   DMEM/F12 (Gibco, #11330-032)
   Non-essential amino acids (NEAA), 100X (Gibco, #11140-050)

GlutaMAX, 100X (Gibco, #35050-061)
Beta-mercaptoethanol 1000X (Gibco, #21985-023)
Neurobasal medium (Life Technologies, #21103-049)
B27 supplement (Life Technologies, #17504-044)
N2 supplement (Life Technologies, #17502-048)
Penicillin-Streptomycin (PenStrep; Life Technologies, #15070-063)
Puromycin 10mg/mL (Fisher Scientific, #ANT-PR-1)
Laminin (Sigma, #L2020)
DPBS with calcium and magnesium (Life Technologies, #14040-133)
Brain-derived Neurotrophic Factor (**BDNF**; Peprotech, #450-02)—see aliquoting protocol
Glia-derived Neurotrophic Factors (**GDNF**; Peprotech, #450-10)—see aliquoting protocol
Dibutyryl cyclic AMP (**cAMP**; Sigma, #D0627)
AraC
20x borate buffer (Thermo, #28341)
Distilled water ()
5% PEI (poly-ethylenimine, stored at 4C)
Neurobasal PLUS (Life Technologies, #A35829-01)
B27 PLUS supplement (Life Technologies, #A35828-01)

5. **Solution preparation:**

StemFlex complete medium

Simply pour entire bottle of StemFlex Supplement into bottle of StemFlex Basal Medium. Use 5mL serological pipet and some of the medium to wash out Supplement bottle. Label StemFlex bottle with date, initials, and ‘COMPLETE’.

Human embryonic stem cell (hES) medium

Pour 195-200 mL DMEM/F12 in the filtration system. No need to measure exact volume with serological pipet.

Add 50 mL KO-SR (final concentration of 20%).
Add 2.5 mL GlutaMax.
Add 2.5 mL NEAA.
Add 250 µL Beta-mercaptoethanol.
Total volume: 250 mL
Filter the medium.

Matrigel coating solution

Aliquot 25mL cold DMEM/F12 to 50mL falcon. Reserve serological pipet (cooled from contact with medium).
With 1000p, add ~1mL of the DMEM to an aliquot of Matrigel (~150µL, stored at -20°C)--
Pipet up and down to thaw Matrigel aliquot; transfer to 50mL falcon.
Mix 50mL falcon with same cooled serological pipet.

If dark pink particles of Matrigel are present, incubate tube in 4°C fridge.
Transfer 1mL solution per well of 6-well plate to be coated.
Let Matrigel solidify at 37°C for at least 1hr.
Store extra coating solution at 4°C.

Neurobasal N2/B27 Complete medium

Combine in a 500mL filter/storage bottle:
- 500mL Neurobasal medium
- 10 mL B27 supplement
- 5mL N2 supplement
- 5mL NEAA
- 5mL Glutamax
- 5mL PenStrep

Neurobasal PLUS N2/B27 PLUS Complete medium

Combine in a 500mL filter/storage bottle:
- 500mL Neurobasal PLUS medium
- 10 mL B27 PLUS supplement
- 5mL N2 supplement
- 5mL NEAA
- 5mL Glutamax
- 5mL PenStrep

6. Procedure

Note: Use early-passage iPSC-NGN2 stocks; avoid culturing for >4 passages.

Day -2 When iPSCs are expected to become ~70-80% confluent in two days, feed with 2mL StemFlex plus 5ug/mL **blasticidin** (if flat cells arise, use 10ug/mL)

Day -1 Feed iPSCs with 2mL StemFlex plus 5ug/mL **blasticidin** (if flat cells arise, use 10ug/mL)

Day 0 1) Use Accutase to dissociate stable Dox-NGN2 inducible iPSCs and count single cells

2) Plate at a density of 1.0-1.25x10^6 cells per well of a Matrigel-coated 6-well plate (in 2mL StemFlex plus 10ug/ml **Rock inhibitor** and 0.5ug/mL **doxycycline** (or plate 10x10^6 cells per 10cm dish, in 10mL medium)

3) Record in calendar date started (D0) and day cells will need to be dissociated

Note: Please use HCNT—Cortical Neuron Transdifferentiation; Day 0 Dissociation as a detailed protocol for Day 0

Khurana Lab BRIGHAM HEALTH BRIGHAM AND WOMEN'S HOSPITAL HARVARD MEDICAL SCHOOL	SOP-VK	HCNT—Cortical Neuron Transdifferentiation; **Day 0 Dissociation**	Author: Zoe Approved:	Revision:	Issued 10-31-2020 Revised

1. **Purpose**
   To initiate differentiation of human iPS cells into cortical neurons by doxycycline induction of NGN2 transgene expression. Use iPSCs transfected with an NGN2-puro-SNAP PiggyBac construct, with a blasticidin-resistance backbone.

2. **Scope**
   Covers only Day 0 of HCNT differentiation; use in conjunction with HCNT—Overview protocol.

3. **Materials**
   Filtration system PES 0.2 µm, 250 mL
   6-well plates (Corning, #3506), or
   100 mm TC-treated Cell Culture Dishes (Westnet, #353003)
   Cell culture hood
   Centrifuge for 15mL conical tubes
   Pipets (10, 20, 1000p)
   15mL and 50mL conical tubes
   Autopipette
   10mL pipets
   Microcentrifuge tubes
   Counting slides
   Countess

4. **Reagents**
   Knock-out serum replacement (KO-SR) (Gibco, #10828-028)
   DMEM/F12 (Gibco, #11330-032)
   Non-essential amino acids (NEAA), 100X (Gibco, #11140-050)
   GlutaMAX, 100X (Gibco, #35050-061)
   Beta-mercaptoethanol 1000X (Gibco, #21985-023)
   Matrigel growth factor reduced (Corning, #354230)
   DPBS without Ca and Mg, 500mL (Corning, #21-031-CV)
   Accutase (Life Technologies, #00-4555-56)
   StemFlex medium (Gibco, A3349401)
   StemFlex Supplement
   ROCK inhibitor (RI; Peprotech, #1293823)
   Doxycycline hyclate (DOX; Sigma, #D9891-5G)
   Trypan blue (Life Technologies, #15250-061)

5. **Solution preparation:**

   StemFlex complete medium

Simply pour entire bottle of StemFlex Supplement into bottle of StemFlex Basal Medium. Use 5mL serological pipet and some of the medium to wash out Supplement bottle. Label StemFlex bottle with date, initials, and 'COMPLETE'.

Human embryonic stem cell (hES) medium

Pour 195-200 mL DMEM/F12 in the filtration system. No need to measure exact volume with serological pipet.

Add 50 mL KO-SR (final concentration of 20%).
Add 2.5 mL GlutaMax.
Add 2.5 mL NEAA.
Add 250 µL Beta-mercaptoethanol.
Total volume: 250 mL
Filter the medium.

Matrigel coating solution

Aliquot 25mL cold DMEM/F12 to 50mL falcon. Reserve serological pipet (cooled from contact with medium).
With 1000p, add ~1mL of the DMEM to an aliquot of Matrigel (~150µL, stored at -20°C)--
Pipet up and down to thaw Matrigel aliquot; transfer to 50mL falcon.
Mix 50mL falcon with same cooled serological pipet.
If dark pink particles of Matrigel are present, incubate tube in 4°C fridge.
Transfer 1mL solution per well of 6-well plate to be coated.
Let Matrigel solidify at 37°C for at least 1hr.
Store extra coating solution at 4°C.

6. Procedure

6.1. Begin with a culture of Dox-NGN2 transfected iPS cells that have been exposed to 5µg/mL blasticidin for two days (to kill any cells which no longer express NGN2) and have become ~70-80% confluent (aim for maximum surface coverage, but passage before colonies touch and cells begin to differentiate under stress at the junctions).

6.2. Feed 1mL StemFlex per well of a 6-well plate early the day of passaging.

6.3. Allow Accutase aliquot to come to room temperature (about 1 hour). Do not warm Accutase at 37°C, or repeatedly cool and warm an entire bottle— potency of the enzyme may decline.

6.4. Prepare Matrigel plates and incubate at 37°C for at least 30-60 minutes.

6.5. Prepare enough StemFlex+10µg/mL rock inhibitor+0.5µg/mL doxycycline for plating and resuspension and warm at 37°C.

6.6. Aspirate media and wash wells thoroughly with 1X PBS, at least 1mL/well.

6.7. Aspirate PBS, mix Accutase well, and add 1mL Accutase to each well.

6.8. Move plate to incubator at 37°C for 4 minutes.

6.9. Prepare a 15mL (or 50mL) Falcon tube for each cell line; add 1.5mL hES medium per well of each line to be passaged.

6.10. Tap side of plate and check that cells are lifting. If not, return to incubator for another 1-2 minutes. If cells are on Matrigel, 4-5 minutes should be enough; laminin or cultrex may require 5-10 minutes at 37°C.

6.11. With a 1000p pipet, quickly pipet Accutase in a circular motion to gently wash cells into solution; gently but deliberately pipet up and down in plate to dissociate (keep number of pumps relatively consistent across lines).

6.12 Collect cells in prepared Falcon tubes (hES medium dilutes Accutase). Use another 1mL hES to wash wells again.

6.13. Pipet up and down in tube with 10mL autopipette.

6.14. Centrifuge at 900 rpm for 4 minutes to sediment single cells.

6.15. After spin, remove supernatant; resuspend pellet in 2mL StemFlex+RI+DOX and mix well with a 1000µL pipet.

6.16. Add 3-8mL more StemFlex+RI+DOX and pipet up and down with 10mL autopipette to ensure single-cell dissociation. Avoid bubbles.

6.17. Optional: Pass through 40µm filter into new 50mL falcon tube.

6.18. Confirm single cell suspension under microscope.

6.19. Immediately after pipetting, add 15µL cell solution from middle of tube to 15µL trypan blue in a microcentrifuge tube. With a new tip, mix and aliquot 10µL to each side of a cell-counting slide; count live cells with Countess and take average. If measurements are inconsistent, dilute in more SF+RI+DOX (Countess is accurate up to ~3x10^6 cells/mL).

6.20. Distribute 1-1.25 million live cells to each new well of a 6-well plate, or 10 million cells to each 10 cm dish; total volume: 2mL (for 6-well) or 10mL (for 10cm).

6.21. Move to incubator at 37°C; shake horizontally and vertically (pausing in between) to evenly distribute cells; leave undisturbed overnight.

Day 1 Change media to **Neurobasal N2/B27 Complete** media; add 0.5ug/mL **doxycycline** and 5ug/mL **blasticidin**

Day 2 Change medium to **Neurobasal N2/B27 Complete** media with 0.5ug/mL **doxycycline** and 1ug/mL **puromycin**

Days 3-6 Same as Day 2 (one weekend day can be skipped if cells are fed with extra 50% media)

Note: Do not allow neurons to grow long processes, or dissociation will break the fragile neurites and cells will fail to attach.

Day 6 (or one day before re-plating) Prepare PEI and coat plates; keep at 4°C overnight

Day 7 [Day 10 for A53T/CORR lines]

1) Remove PEI from plates, wash twice with distilled water and once with PBS, and add laminin (in PBS with Mg and Ca, see plating protocol); leave at 37°C ~2 hours. May also use poly-ornithine-laminin pre-coated plates—let these come to room temperature prior to plating

2) Use Accutase to dissociate neurons and re-plate in plates and densities necessary for experiments— recommended: 50,000 cells per well of 96-well plate; 1-1.5 million cells per well of 24-well plate

3) Plate in **Neurobasal N2/B27 Complete** media (100µL per well of 96-well plate, 1mL per 24-well) plus 0.5µg/mL **doxycycline** and 10µg/mL **Rock inhibitor**

Note: Please use HCNT—Cortical Neuron Transdifferentiation; Day 7 Dissociation as a detailed protocol for Day 7

Khurana Lab BRIGHAM HEALTH BRIGHAM AND WOMEN'S HOSPITAL HARVARD MEDICAL SCHOOL	SOP-VK	**HCNT—Cortical Neuron Transdifferentiation; Day 7 Dissociation**	Author: Zoe Approved:	Revision:	Issued 10-31-2020 Revised

1. **Purpose**
   To replate neurons after seven days of cortical transdifferentiation and prepare cells for longer-term culture.

2. **Scope**
   Covers only replating, usually performed on Day 7 of differentiation. Use in conjunction with HCNT—Overview protocol.

3. **Materials**
   Cell culture hood
   Centrifuge for 15mL conical tubes
   Pipets (10, 20, 1000p)
   15mL and 50mL conical tubes
   Autopipette
   10mL pipets
   Microcentrifuge tubes
   Counting slides
   Countess
   Filtration system PES 0.2 µm, 500 mL (Corning, #974102)
   96-well plates (Brooks, #MGB096-1-2-LG-L)
   Poly-L-Ornithine/Laminin 24-well TC treated plates (Corning, #354659)
   Steriflip 50mL 0.22 µm PES sterilized filter (Millipore, #SCGP00525)
   Multichannel pipet
   Reagent Reservoir (Fisher Scientific, #21385104)

4. **Reagents**
   20x borate buffer (Thermo, #28341)
   Distilled water ()
   5% PEI (poly-ethylenimine, stored at 4C)
   DPBS without Ca and Mg, 500mL (Corning, #21-031-CV)
   Accutase (Life Technologies, #00-4555-56)
   ROCK inhibitor (RI; Peprotech, #1293823)
   Doxycycline hyclate (DOX; Sigma, #D9891-5G)
   Trypan blue (Life Technologies, #15250-061)
   Non-essential amino acids (NEAA), 100X (Gibco, #11140-050)
   GlutaMAX, 100X (Gibco, #35050-061)
   Neurobasal medium (Life Technologies, #21103-049)
   B27 supplement (Life Technologies, #17504-044)
   N2 supplement (Life Technologies, #17502-048)
   Penicillin-Streptomycin (PenStrep; Life Technologies, #15070-063)

5. **Solution preparation:**

Neurobasal N2/B27 Complete medium (NB)

Combine in a 500mL filter/storage bottle:

500mL Neurobasal medium
10 mL B27 supplement
5mL N2 supplement
5mL NEAA
5mL Glutamax
5mL PenStrep

6. Procedure

Preparation Steps:

6.1. Follow PEI/laminin coating protocol, and/or bring pre-coated plates to room temperature.

6.2. Prepare medium: **NeuroBasal Complete** medium (NB) with **0.5µg/mL doxycycline** and **10µg/mL rock inhibitor**. Make enough for 1mL per well of 24-well plate, 100µL per well of 96-well plate, etc (half of eventual full medium volume), plus 1mL per well to be lifted (to dilute accutase), plus ~10mL extra per line to be dissociated (this isn't necessary for small differentiations— only if you have ≥20 million cells).

6.3. Let accutase come to room temperature (do not warm at 37°C)— 1mL per well of a 6-well plate, 4mL per 10cm dish, etc.

6.4. Label tubes— 15mL for each line for small differentiations, 50mL for >10 million cells. Aliquot about as much NB+DOX+RI medium per tube as accutase to be used for that line (eg. for 4x 10cm plates, 4x5mL accutase=20mL—> dilute with about 20mL medium).

Dissociation:

6.5. Aspirate medium and wash cells once with 1mL PBS. Add 1mL accutase. (For 10cm plates, use 4mL.)

6.6. Leave in incubator at 37°C for 4 minutes. Check for lifting and tap sides of plate. Return to 37°C for 1 minute. If cells remain adhered or sheet of cells doesn't easily break apart with tapping, incubate additional 2 minutes.

6.7. Use 1000p pipet to pipet accutase up and down gently but deliberately about the same number of times in each well to dissociate to single cells, then collect cells into labeled tubes. Wash wells with medium.

6.8. Pipet up and down in tube with 10mL autopipette to ensure single-cell dissociation.

6.9. Centrifuge for 4 minutes at 900 rpm.

6.10. Decant supernatant into a waste container (50mL falcon). Use a vacuum pasteur pipet to clean inner walls of tubes, but do not aspirate near pellets.

6.11. Resuspend pellet in 1-5mL prepared NB+Dox+RI medium. Pipet up and down with 1000p and then with 10mL auto-pipet to ensure single cell dissociation (again, keep number of times relatively consistent).

6.12. Optional: Pass through 40µm filter into new 50mL falcon tube (especially important for very distribution-sensitive applications such as Seahorse assays).

6.13. Examine tubes under microscope and confirm single-cell dissociation.

6.14. Add enough NB+Dox+RI to dilute to about 3 million cells per 1mL and mix.

6.15. Aliquot 15µL trypan blue to a microcentrifuge tube for each line. Immediately after mixing cell suspension, take 15µL cell suspension from the middle of the tube and mix with dye. Add 10µL mixture to each side of a slide and count cells with Countess; take average.

Troubleshoot: if counts for the same line are very different and >3 million cells per mL, dilute suspension and recount.

6.16. Dilute cell suspensions as needed to plate 1-1.5 million cells per well of 24-well plate in 1mL, 30,000-100,000 per well of 96-well plate in 100µL.

6.18. Aspirate laminin from plates.

6.19. Immediately before distributing cells, mix again with a 1000p or multi-channel pipet. When distributing, do not eject the last volume in the pipet, as this contains any cell clumps.

6.20. Add PBS to outer wells of 96-well plates to avoid drying.

6.21. With plate flat on microscope stage, shake horizontally a few times and stop, then vertically a few times and stop. Verify that cells are evenly distributed.

6.22. Transfer plates to incubator. Repeat shaking, including pauses. Leave undisturbed overnight.

Day 8 Don't remove media from plated neurons; GENTLY (drop-wise!) add additional 100µL (or enough to double volume) of **Neurobasal N2/B27 Complete** medium with:

	Aliquot	Example	Final
Neurobasal N2/B27		7mL	7+7 mL already in culture=14 mL
BNDF	20 ug/mL	7 uL	10 ng/mL
GNDF	20 ug/mL	7 uL	10 ng/mL
cAMP	1000x (0.5M)	14 uL	0.5mM
Laminin	1mg/mL	14 uL	1 µg/ml
AraC	500 uM	14 uL	0.5 µM

Day 11 Remove as much medium as possible but leave a layer of liquid over cells; feed (GENTLY) with a mixture of **1:1 Neurobasal N2/B27 Complete** and **Neurobasal PLUS N2/B27 PLUS Complete** media with growth factors but NOT AraC.

Prepare 1:1 mixture with factors added:

	Aliquot	Example	Final
Neurobasal		7 mL	
Neurobasal PLUS		7 mL	
BNDF	2000x (20 ug/mL)	7 uL	10 ng/mL
GNDF	2000x (20 ug/mL)	7 uL	10 ng/mL
cAMP	1000x (0.5M)	14 uL	0.5mM
Laminin	1000x (1mg/mL)	14 uL	1 µg/ml

Day 14 Remove ½ (100µL per well of 96-well) of existing medium; replace (GENTLY) with 100µL **Neurobasal PLUS N2/B27 PLUS Complete** medium with growth factors:

	Aliquot	Example	Final
Neurobasal PLUS		7 mL	
BNDF	2000x (20 ug/mL)	3.5 uL	10 ng/mL
GNDF	2000x (20 ug/mL)	3.5 uL	10 ng/mL
cAMP	1000x (0.5M)	7 uL	0.5mM
Laminin	1000x (1mg/mL)	7 uL	1 µg/ml

Day 17 etc. Continue to change half of existing medium every 3 days, or twice a week.

Note: If transfecting neurons, remove PenStrep from medium 24 hours prior to transfection.

Note: Add medium very gently, drop by drop, from a multichannel or serological pipette. Any forceful pipetting can cause cell layer to lift.

Khurana Lab BRIGHAM HEALTH BRIGHAM AND WOMEN'S HOSPITAL HARVARD MEDICAL SCHOOL	SOP-VK	**PEI/Laminin Coating**	Author: Zoe Approved:	Revision:	Issued 08.27.2020 Revised

1. **Purpose**
   To prepare plates for iPSC-derived neuron maturation.

2. **Scope**
   Part of the NGN2 cortical neuron differentiation protocol: coat plates in preparation for Day 7 dissociation and replating.

3. **Materials**
   50mL Falcon tube
   Steriflip 50mL 0.22 µm PES sterilized filter (Millipore, #SCGP00525)
   96 square-well plates (Brooks, #MGB096-1-2-LG-L)
   Cell culture hood
   Multichannel pipet
   Reagent Reservoir (Fisher Scientific, #21385104)

4. **Reagents**
   20x borate buffer (Thermo, #28341)
   Distilled water ()
   5% PEI (poly-ethylenimine, stored at 4C)
   Laminin (Sigma, #L2020, 1mg/mL)
   DPBS with calcium and magnesium (Life Technologies, #14040-133)
   DPBS without Ca and Mg, 500mL (Corning, #21-031-CV)

5. **Procedure**
   PEI Coating

5.0. At least one day before plating, make 2x borate buffer with 4mL 20x borate buffer (Thermo, cat. no. 28341) in 36mL water in a 50mL Falcon tube.

5.1. Dilute 800µL 5% PEI (poly-ethylenimine; stored at 4°C) in 40mL 2x borate buffer to make 0.1% PEI.

5.2. Filter-sterilize with SteriFlip: attach filter and tube to 50mL tube with PEI solution; flip and attach to vacuum; when medium drains through, remove filter and original tube.

5.3. Add 150µL to each of the inner 60 wells of a 96 square-well plate (Brooks, cat. no. MGB096-1-2-LG-L); 500µL for wells of 24-well plate (Corning, #3527).

Note: Poly-ornithine-laminin pre-coated plates (6-well, 24-well) can be substituted for this plating protocol—only 96-well must be PEI/laminin coated.

5.4. On 96-well plates, leave a border of empty wells to avoid edge effects; fill these with PBS to maintain humidity.

5.5. Label plate(s), wrap sides with parafilm and cover in plastic wrap; store at 4°C overnight.

Laminin Coating

5.6. On the day of passaging, make laminin 5µg/mL in 4°C PBS (with calcium and magnesium ions), ex. 1mL PBS with 5µL laminin (Sigma, cat. no. L2020, 1mg/mL).

5.7. Wash PEI-coated plates twice with distilled water (300µL for 96-well plates).

5.8. Wash once with PBS.

5.9. Add 150µL laminin to wells of 96-well plate, and 0.5mL to wells of 12-well.

5.10. Incubate plates at least 2 hours at 37°C before re-plating neurons.