SNAP AND ASTROCYTE INDUCTION PROTOCOL
WELLS LAB—AUGUST 2023

mTeSR Plus (500 mL)

Reagent	Volume	Dilution
mTeSR+ Base	400 mL	
5X Supplement	100 mL	1:5
Normocin	1 mL	1:500

NGN2 Induction Base (250 mL)

Reagent	Volume	Dilution
DMEM:F12	244 mL	
Glutamax	2.5 mL	1:100
20% Glucose	3.75 mL	1:66.7

NGN2 Day 1 Induction (25 mL)

Reagent	Volume	Dilution	Conc.
Induction Base	25 mL		
N2 Supplement	250 µL	1:100	
Doxycycline	2.5 µL	1:10,000	2µg/mL
LDN-193189	5 µL	1:5,000	200nM
SB431542	25 µL	1:1,000	10µM
XAV939	5 µL	1:5,000	2µM

NGN2 Day 2 Induction & Selection (25 mL)

Reagent	Volume	Dilution	Conc.
Induction Base	25 mL		
N2 Supplement	250 µL	1:100	
Doxycycline	2.5 µL	1:10,000	2µg/mL
LDN-193189	2.5 µL	1:10,000	100nM
SB431542	12.5 µL	1:2,000	5µM
XAV939	2.5 µL	1:10,000	1µM
Puromycin	12.5 µL	1:2,000	5µg/mL

NES Base (500 mL; Sterile Filter)

Reagent	Volume	Dilution
DMEM:F12	480 mL	
Glutamax	10 mL	1:50
Pen/Strep	5 mL	1:100
MEM NEAA	5 mL	1:100

NES Complete & Selection (50 mL)

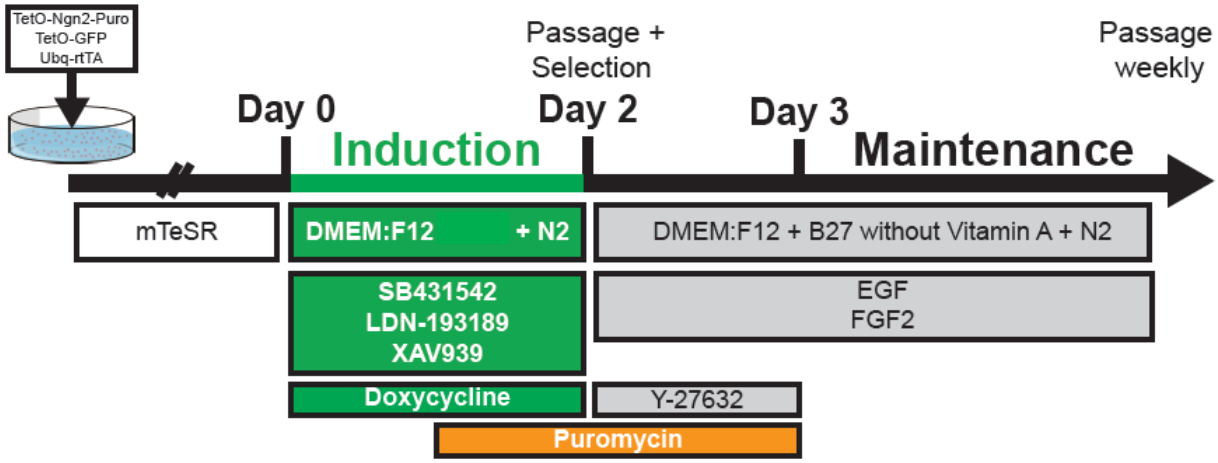
Reagent	Volume	Dilution	Conc.
NES base	48.5 mL		
B27 w/o Vitamin A	1 mL	1:50	
N2 Supplement	500 µL	1:100	
EGF	5 µL	1:10,000	10ng/mL
bFGF	5 µL	1:10,000	10ng/mL
Puromycin	25 µL	1:2,000	5µg/mL
ROCK inhibitor	50 µL	1:1,000	10µM

NES Complete (50 mL)

Reagent	Volume	Dilution	Conc.
NES base	48.5 mL		
B27 w/o Vitamin A	1 mL	1:50	
N2 Supplement	500 µL	1:100	
EGF	5 µL	1:10,000	10ng/mL
bFGF	5 µL	1:10,000	10ng/mL

Astrocyte Media (500 mL)

Reagent	Volume	Dilution
AM Base	500 mL	
FBS	10 mL	1:50
Pen/Strep	5 mL	1:100
Growth Supplement	5 ml	1:100



Day -X: Lentiviral transduction of pluripotent stem cells (PSCs)

1. Accutase and count PSCs

2. Re-suspend 1 million cells in 1.5 mL of mTeSR-Plus and ROCK inhibitor (Y-27632; 10 µM; 1:1000)

3. Add TetO-Ngn2(mouse)-Puromycin (from Wernig lab; Addgene #52047), Ubq-rtTA, and TetO-GFP (Addgene #30130; optional) lentiviruses at a multiplicity of infection (MOI) of 1-3. Mix well by gently pipetting up and down. I typically use MOI = 2, though this may need to be optimized depending on the cell line.

NOTE: This protocol has only been adapted for overexpression of mouse Ngn2 delivered through lentiviral transduction. No other configurations (e.g. human NGN2, electroporation, etc) have been optimized.

4. Add 750 µL of the virus/cell suspension per well of a 12-well plate pre-coated with Geltrex

5. The next day (16-24 hours later), remove the media and replace with fresh mTesR-Plus media. Feed cells daily until 30-50% confluent. (**Note:** It is normal to see cell death beginning 48-72 hours post-plating.)

6. When the cells have reached the desired confluency, passage for expansion or freeze for future use. (**Note:** We typically do not use transduced PSCs after 10 passages.)

Day -1: Plating of pluripotent stem cells for SNaP induction

1. Accutase and count transduced PSCs. (**Note:** If taking from frozen stock of transduced PSCs, it is best to passage 1-2 times before using as starting material for any induction or differentiation protocol.)

2. Plate PSCs at 75,000 cells/cm^2 in mTesR-Plus media with ROCK inhibitor in a 12-well or 6-well plate pre-coated with Geltrex. (**Note:** This protocol has not yet been optimized for the 10 cm^2 plate.)

3. Make sure cells are evenly distributed and incubate overnight at 37°C/5% CO_2.

Day 0: Start induction

1. Aspirate media.

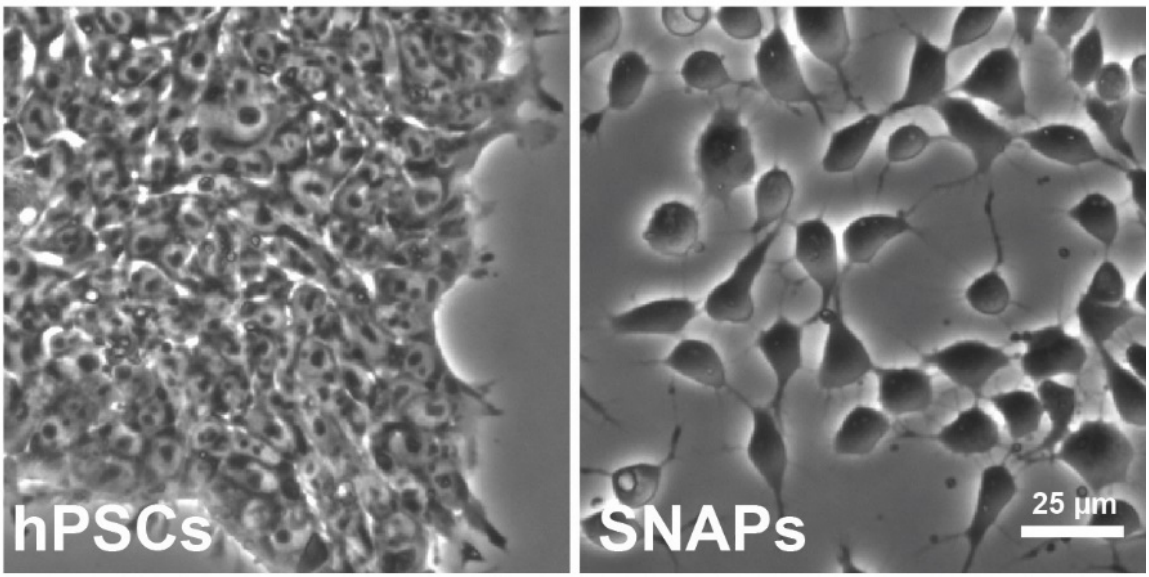
2. Add 2 mL (for 12-well) or 3 mL (for 6-well) of *NGN2 Day 1 Induction Media*:

Day 1: Feed and selection (exactly 24 hours post-induction)

1. Check the cells under a light microscope—small morphological changes should be visible. If the TetO-GFP virus was included, bright green cells should be present. (**Note:** The proportion of cells that are green is a good estimate of the transduction efficiency and therefore the SNaP production efficiency. If less than 50% of the cells are green, the SNaP output may be low and re-transduction of the PSCs may be necessary.)

2. Exactly 24 hours after starting induction, aspirate media and add 2 mL (for 12-well) or 3 mL (for 6-well) of *Day 2 Induction and Selection Media*:

Day 2: Passage and selection (48 hours post-induction)



SNaP morphology (right) at 48 hours post-induction. SW7388-1 iPS cell starting material (left) for reference.

1. Exactly 24 hours later (48 hours post-induction), accutase and count the cells.

2. Prepare *NES Base Media* and sterile filter; make *NES Complete and Selection media.*

3. Plate 120,000—150,000 cells/cm^2 in 12-well or 6-well plates pre-coated with Geltrex in *NES Complete and Selection media*.

Day 3: Feed and selection

1. Aspirate the media and feed with *NES complete.*

(**Note:** Do NOT leave the cells in the NES complete and selection media for more than 24 hours, as the Puromycin resistance will diminish and the cells will die. I typically change media 12-18 hours post-plating)

Day 4-onwards: Daily feeds and passaging for maintenance

1. Feed the cells daily with 1 mL (24-well), 2 mL (12-well), or 3 mL (6-well) of *NES complete* media.

2. Passage cells weekly at 120,000—150,000 cells/cm^2. (Note: We have passaged the SW7388-1 iPSC line 15 times without any noticeable changes in morphology or proliferation rate.)

Glial differentiation using ScienCell Astrocyte Media

1. Passage and plate SNaPs at 10,000—15,000 cells/cm^2 in ScienCell *Astrocyte Media* (ScienCell, #1801) plus ROCK inhibitor.

2. The next day, aspirate media to remove ROCK inhibitor and replace with fresh ScienCell Astrocyte Media.

3. Feed cells 2-3 times per week for the first 2 weeks (feed when media is yellow). Cells may need to be passaged multiple times during this high growth rate phase (cell confluency will be high). There is no need to use ROCK inhibitor during these passages.

4. Starting in the third week (Day 15), cell proliferation will slow, and cells can be fed 1-2 times per week for the remainder of the culture. These cells can be passaged every 10-14 days. Note: Immunostaining of these cells should reveal mostly CD44+/S100B+ cells.

5. By Day 30-40, most cells should be CD44+/S100B+/GFAP+.

6. Cells can be frozen and banked anytime after Day 7 and thawed for continued differentiation.

Protocol Notes:

- This protocol has been used successfully to generate SNaPs in 111 of 115 stem cell lines, including hESCs and hiPSCs, as determined through immunocytochemistry of the NPC markers PAX6, NESTIN, and SOX1, as well as the negative marker OCT4.

- Validation of this technique at the functional level (i.e. spontaneous differentiation, neurosphere formation, Zika infection, etc) has been conducted using the SW7388-1 iPSC line and the H09 hESC line. Some of these validation assays have also been performed on a pool of 48 hESC lines.
- The largest source of line-to-line and batch-to-batch variation in this protocol is the initial NGN2 viral transduction efficiency of the pluripotent stem cells. We typically achieve 70-95% efficiency and were able to establish SNaP cultures with transduction efficiencies as low at 30-40%. We have found that transduction of hPSCs in StemFlex dramatically reduces transduction efficiencies, so we suggest mTeSR media for this step.
- With high transduction efficiencies (>80%), Day 2 SNaPs can be used for any type of neural progenitor cell-based experimentation (i.e. differentiation or proliferation assays, Zika virus infections). Low transduction efficiencies may require a week of expansion in NES complete media.
- **IMPORTANT**: If expansion of Day 2 SNaPs is desired, the most sensitive stage of this protocol is the initial passaging step at that begins on Day 2. After this passage, some SNaPs will appear neuronal in morphology for a few days, partially due to the presence of ROCK inhibitor and partially due to confluency effects. In fact, we typically observe a 1-2% contamination of post-mitotic neurons during this time. We have found that in most cases, the neuronal contamination and morphological changes resolve themselves over the course of 3-5 days with daily feeds, at which point the proliferating progenitors take over the culture. At this point, the culture should be more pure and ready for another passage.

Culture Feed Log

Date	Media	Completed? (Checkmark)	Notes