Derivation of Multipotent Neural Progenitors from Human Embryonic Stem Cells for cell therapy and biomedical applications

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Summary/Abstract

Long-term neuroepithelial-like stem cells (lt-NES) derived from human embryonic stem cells are a stable self-renewing progenitor population with high neurogenic potential and phenotypic plasticity. lt-NES are amenable to regional patterning towards neurons and glia subtypes and thus represent a valuable source of cells for many biomedical applications. For use in regenerative medicine and cell therapy, lt-NES and their progeny require derivation with high quality culture conditions suitable for clinical use. In this chapter, we describe a robust method to derive multipotent and expandable lt-NES based on Good manufacturing Practice and cell therapy-grade reagents. We further describe fully defined protocols to terminally differentiate lt-NES towards GABA-ergic, dopaminergic and motor neurons.

Key Words

Human embryonic stem cells, Neural progenitors, lt-NES, stem cells, GMP, differentiation, cell therapy, regenerative medicine.

1. Introduction

In the decades since their first derivation *[1]*, human embryonic stem cells (hESCs), have gone from the theoretical promise of a new unlimited source of human cell types [*2*] to the reality of the first in human trials based on their derivatives *[3,4]*. HESCs have been successfully differentiated into a large collection of cells that are otherwise unavailable or difficult to isolate without ethical challenges, such as human neural progenitors [5-7]. Indeed, hESC-based cell therapy represents a game-changing strategy for neurological diseases involving tissues that are permanently damaged and unable to regenerate, as in the case of multiple sclerosis, Parkinson's disease or spinal cord injury [*6,8*]. Furthermore, therapeutic advancements crucially depend on a reliable source of high-quality neurons and glia to model the pathophysiology of neurological disorders and drug responses.

Long-term neuroepithelial-like stem cells (lt-NES) have been generated from pluripotent stem cells as a viable alternative to foetal tissue. Their extensive self-renewal capacity, stable karyotype and phenotypic plasticity provide an advantageous platform to model a multitude of disease and for biomedical applications [*9-14*]. Lt-NES retain multipotency as a rosette-type population when in presence of EGF and FGF growth factors while differentiate with a bias for GABAergic neurons upon growth factors withdrawal [*9,10*]. Furthermore, upon addition of patterning cues they can be directed towards midbrain dopaminergic, motoneuron, or astroglia phenotypes [*10,9,11*].

Despite the tremendous applications offered by lt-NES in vitro, fully defined set of highly qualified Good Manufacturing Practice (GMP)-grade protocols based on cell therapy-grade reagents are required for regulatory compliance and in order to deploy them for clinical use [*15-17*]. In this chapter, we describe a step-by-step method (Fig. 1) for the derivation, maintenance and differentiation of lt-NES based on GMP-grade and cell therapy-grade reagents including substrates and growth factors [*18*]. In particular, we describe methods to terminally differentiate lt-NES into GABAergic neuron and into midbrain dopaminergic neurons

or motoneurons phenotypes (Figure 1). In essence, this protocol involves as a first step the formation of same-sized embryoid bodies from pluripotent hESCs (step 3.1), followed by neural induction resulting in the formation of neural rosettes (step 3.2). lt-NES are then sorted from these neural rosettes to be passaged at high density in presence of EGF and FGF to promote self-renewal (step 3.3). Once established, lt-NES lines are maintained in continuous presence of EGF and FGF (step 3.4) while spontaneous and terminal neural differentiation is induced by the withdrawal of EGF and FGF (step 3.5). Patterning cues involving addition of FGF8b and SHH direct lt-NES to a midbrain dopaminergic phenotype after 21 days (Figure 1, step 3.6). Patterning cues involving retinoid acid and SHH direct lt-NES towards motoneuron phenotype after 21 days of treatment (step 3.7). Overall, this method provides a GMP and cell therapy-grade platform to generate multipotent and bankable lt-NES amenable for downstream differentiation into clinically useful cell types.

2. Materials

All the cell culture reagents are stored, reconstituted and used as specified in the manufacturer instruction unless stated. Procedures are performed at Room Temperature (RT) unless otherwise specified.

2.1 Reagents grade and quality specifications

1.   The reagents used in this protocol are of cGMP quality, unless stated, regardless of this being written in their product name. Refer to each manufacturer for specific details on documentation and quality specifications provided.

2.   Cell Therapy Systems (CTS™) reagents are GMP-grade, serum-free, xeno-free and animal origin-free formulations. Moreover, they have cell and gene therapy specific intended use statements.

https://www.thermofisher.com/uk/en/home/clinical/cell-gene-therapy/cell-therapy/cell-therapy-systems.html

3.   GMP growth factors from R&D are produced following cGMP guidelines "that allow for their use as ancillary materials in cell therapy or for further manufacturing processes. GMP proteins also come with extensive documentation and traceability, as well as additional quality control testing."

https://www.rndsystems.com/products/good-manufacturing-practices-gmp-grade-proteins

4.   Laminin 521 substrate is available as cell therapy grade (CTG) from the Biolamina manufacturer. This product is a seamless transition from the Laminin 521 of the same company used in this protocol. Notably, the CTG grade was in the pipeline at the time of this protocol development and was chosen in view of the availability of this grade as well as its high performance.

5.   StemPro Accutase and STEMdiff™ Neural Rosette Selection Reagent would need custom due diligence qualification for therapy usage. STEMdiff™ Neural Rosette Selection Reagent is enzyme and animal derivatives-free. The manufacturer is committed to working with cell therapy developers to support the use of this product in cell therapy manufacture and can assist with performing due diligence for specific regulators. Overall, these reagents provide the best solution to date for effectiveness and clinical compliance.

2.2 Equipment and consumables

1.   Class II biological safety cabinet.

2.   Inverted microscope.

3.   Incubator set at 5% CO_2, 5% O_2 and 37 °C.

4.   Fridge (4°C) / Freezers (-20°C/ -80°C).

5.   Centrifuge fitted with 15 ml tubes holders.

6.   Centrifuge with swinging rotor fitted with plate holder (capacity up to 2000 g).

7.   Thermostat filled with beads (i.e. LabArmor Beads, Thermo Scientific) at 37°C.

8. Pipettor.

9. Haemocytometer.

10. Sterile and individually plastic-wrapped serological stripettes.

11. Pipettes and sterile tips (suitable for p1000, p200, p10).

12. Corning® CoolCell™ LX Cell Freezing Container (CLS432001, SDS)

13. Sterile 6-, 12-, 24-, and 48-well cell culture plates of polystyrene.

14. Sterile 50 and 15ml tubes.

15. Sterile polypropylene 0.5 and 1.5ml tubes.

16. Sterile 2ml cryovials.

17. AggreWell™800 (27865, StemCell Technologies).

18. 1000μl Filter Tip, Wide Orifice, Sterile, Racked (E1011-9618, Starlab UK).

19. 37 μm Reversible Strainer (27215, StemCell Technologies)

2.3 Cell culture

1. CTS™ VTN-N Vitronectin (A27940, Life Technolgies).

2. CTS™ Essential 8™ (A2656101, Life Technologies).

3. UltraPure™ 0.5M EDTA (15575020, Invitrogen).

4. CTS™ DPBS$^{-/-}$, (A1285601, Life Technologies).

5. StemPro Accutase (A1110501, Life Technologies).

6. Essential 6 (A1516401, Life Technologies).

7. CTS™ DPBS$^{+/+}$ (A1285801, Life Technologies).

8. CTS™ DMEM-F12 (A1370801, Life Technologies).

9. CTS™ N2 (A1370701, Life Technologies).

10. CTS™ B27 (A1486701, Life Technologies).

11. CTS™ Glutamax (A12860-01, Life Technologies).

12. Laminin 521 (LN521, Biolamina).

13. STEMdiff™ Neural Rosette Selection Reagent (5832, StemCell Technologies).

14. GMP FGF (233-GMP, Bio-Techne).

15. GMP EGF (236-GMP, Bio-Techne).

16. CryoStem (K1-0640, Geneflow).

17. Revitacell supplement (A2644501, Life Technologies).

18. Sterile water (10114292, Gibco™)

2.4 Additional reagents for spontaneous lt-NES differentiation

1. Neurobasal CTS™ (A1371001, Life Technologies).

2. cAMP (A9501, Sigma Aldrich).

2.5 Additional reagents for lt-NES differentiation to dopaminergic neurons

1. Neurobasal CTS™ (A1371001, Life Technologies).

2. GMP Sonic Hedgehog (SHH, 130-095-727, Miltenyi Biotec).

3. GMP FGF8b (130-095-740, Miltenyi Biotec).

4. GMP GDNF (212-GMP-010, Bio-Techne).

5. GMP BDNF (248-GMP-025, Bio-Techne).

6. Dibutyryl-cAMP (D0260-25mg, Sigma Aldrich).

7. Ascorbic acid (95210-250G, Sigma Aldrich).

2.6 Additional reagents for lt-NES differentiation to motoneurons

1. Neurobasal CTS™ (A1371001, Life Technologies).

2. GMP Sonic Hedgehog (SHH, 130-095-727, Miltenyi Biotec).

3. GMP GDNF (212-GMP-010, Bio-Techne).

4. GMP BDNF (248-GMP-025, Bio-Techne).

5. cAMP (A9501, Sigma Aldrich).

6.   Retinoid Acid (R2625, Sigma Aldrich).

2.7 Coating cell culture plates with VTN-N

1.   Thaw one VTN-N vial at room temperature and aliquot in $60\mu l$ volume in sterile polypropylene tubes. Store aliquots at -80°C.

2.   Dilute the appropriate volume of VTN-N stock in CTS™ DPBS^{-} based on the number of plates required to a concentration of 0.5 μg/cm^2.

3.   Add 6 ml of CTS™ DPBS^{-} to a 15 ml tube followed by $60\mu l$ of VTN-N.

4.   Gently resuspend the VTN-N solution with a 5 ml stripette.

5.   Add 1 ml of VTN-N solution to each well of a 6-well plate.

6.   Swirl plate to assure even coverage.

7.   Incubate at room temperature for 1 hour.

1.   Aspirate the diluted VTN-N solution from the wells prior to use (*see* **Note 1**).

2.8 Coating cell culture plates with Laminin 521

1.   Thaw the laminin 521 stock at 2-8°C. Once thawed is stable at 2-8°C for up to 3 months.

2.   Dilute the appropriate volume of laminin 521 stock in CTS™ DPBS^{++} based on the number of plates required to a concentration of 10 μg/ml (see **Note 2**).

3.   Incubate for 3 hours at 37°C or overnight at 4°C with plate sealed with parafilm to avoid evaporation. Plates can be kept at 4°C up to 4 weeks.

4.   Visualize if the coating solution still covers the entire surface, particularly the centre of the well, since after prolonger storage some coating may have evaporated.

5.   The plates are ready to use, prior removal of the laminin 521 coating.

2.9 Preparation of N2 base differentiation medium (N2 medium)

1.   Thaw CTS™ N2 and CTS™ B27 at room temperature.

2. Remove 10.5 ml of media from the CTS™ DMEM/F12 500 ml bottle.

3. Add 5 ml of CTS™ N2.

4. Add 500 μl of CTS™ B27.

5. Add 5 ml of Glutamax.

6. Mix well the components by inverting the bottle upside down.

7. Store media at 4°C up to 3 weeks. It is recommended to make 50ml aliquots for the weekly requirement.

8. Always warm the media at room temperature 20 minutes before use.

2.10 Growth Factors/Molecules stock concentration

All growth factors and molecules must be stored according to the manufacturer's instructions. In this protocol, we recommend reconstituting with the following solvents and final stock concentrations:

1. EGF: Reconstitute 200 μg in CTS™ DPBS⁺ to 10 μg/ml final stock concentration.

2. FGF: Reconstitute 25 μg in CTS™ DPBS⁺ to 4 μg/ml final stock concentration

3. FGF8b: Reconstitute 25 μg in CTS™ DPBS/ to 100 μg/ml final stock concentration

4. BDNF: Reconstitute 25 μg in CTS™ DPBS⁺ to 100 μg/ml final stock concentration

5. GDNF: Reconstitute 10 μg in CTS™ DPBS⁺ to 100 μg/ml final stock concentration

6. SHH: Reconstitute 25 μg in sterile water to 250 μg/ml final stock concentration

7. Ascorbic Acid: Reconstitute 50 mg in sterile water to 200 mM final stock concentration

8. cAMP: Reconstitute 1 g in sterile water to 300 μg/ml final stock concentration

9. dy-cAMP: Reconstitute 25 mg in sterile water to 50 mM final stock concentration

10. Retinoid Acid: Reconstitute in DMSO to 3mM final stock concentration

3. Methods

Perform all work at room temperature unless specified

3.1 HESCs maintenance, passaging, freezing

1. HESCs are maintained in Essential 8™ (E8) medium on VTN-N coated plates with an EDTA passaging method, performed without centrifugation (see **Note 3**).

2. Passage the cells when they are 70-80% confluent.

3. Wash the cells once with DPBS⁻.

4. Aspirate the DPBS⁻ and replace with 0.5 mM EDTA solution (prepared in DPBS⁻).

5. Incubate the cells at room temperature for 1-2 minutes.

6. Aspirate the EDTA solution entirely when the cells start to round up, and the colonies appear to have holes in them.

7. Add 1 ml/well of E8 with a p1000 tip to gently detach cells as small clumps

8. Add cell suspension in a 15 ml tube.

9. Repeat step 7 and 8 until the majority of the cells are collected.

10. Gently mix the cell suspension with a 5 ml or 10 ml stripette.

11. Resuspend the cells in half of the final volume of media needed for a 1:6 passaging ratio.

12. Remove DPBS⁻ from the VTN-N coated plates and replace with 1ml/well (for a 6-well plate format) of E8 medium.

13. Add 1ml/well of cell suspension drop-by-drop across each well.

14. Move the plate in the incubator and assure an even distribution of the cells by rocking side to side and top to bottom. Do not move for 24 hours.

15. Replace E8 medium after 24 hours and feed daily afterwards.

16. Freezing: cells are frozen in CryoStem and thawed in E8 plus GMP ROCK inhibitor Revitacell added to the media for the first 24 hours.

3.2 Embryoid body formation (Day 0)

1. HESCs should be checked for suitability for differentiation by ensuring a level of spontaneous differentiation that is less than 2-5% of the entire culture.

2. The protocol requires two wells of 80% confluent hESCs in a 6-well plate format.

3. Prepare 5 ml of E6 medium plus 50 μl of Revitacell.

4. Prepare Aggrewell for cell seeding:

a. Wash a single well of Aggrewell with 2 ml of E6 media.

b. Aspirate the media and add 500 μl of E6 plus Revitacell to the well.

c. Seal the plate with parafilm and centrifuge at 2000 g for 5 minutes in a centrifuge fitted with plate holder.

d. Check under an inverted microscope that the microwells inside the Aggrewell does not contain bubbles.

5. Aspirate the E8 medium from two wells of hESCs in a 6-well plate and proceed for each well with the following steps (6-11)

6. Add 1 ml of DPBS$^{-/-}$ and swirl gently to ensure even coverage.

7. Aspirate the DPBS$^{-/-}$.

8. Add 1 ml of Accutase and swirl gently to ensure even coverage.

9. Incubate the wells at 37°C for 1-2 minutes until the cells start to detach but are not all floating in suspension.

10. Neutralize the Accutase by adding 1 ml of E6 media to each well.

11. Pipette with a p1000 tip to collect all cells and possibly attached colonies.

12. Pull together the cell suspension from the two 6-wells into a 15 ml tube containing 8 ml of E6.

13. Pipette up and down 5-7 times with a 5 ml stripette in order to produce single cell suspension (*See* **Note 4**).

14. Remove 10 μl of cell suspension and place it in a hemotocytometer for cell counting.

15. Centrifuge the 10 ml cell suspension at 300 g for 5 minutes.

16. Count cells by averaging the number of cells in the 16 squares of 4 quadrants of a hemotocytometer and multiply by 10^4 and then 10 ml, to obtain the total cell number. It is expected to obtain at least 4 x 10^6 cells.

17. Aspirate the supernatant and re-suspend the cell pellet into 1 ml of E6 plus Revitacell with a p1000 pipette and mix well.

18. Based on the total cell count previously calculated, remove the appropriate volume of cell suspension corresponding to 3 x 10^6 cells and place it into a separate 15 ml tube.

19. Top up to 1.5 ml with E6 plus Revitacell.

20. Add 1.5 ml of cell suspension to the Aggrewell containing 500 μl of E6 plus Revitacell with a p1000 tip by gently pipetting few times to disperse the cells.

21. Inspect the Aggrewell at the microscope to assure cells are evenly distributed.

22. Seal the plate carefully with parafilm and centrifuge at 100 g for 3 minutes with a corresponding plate balance.

23. Remove the parafilm and inspect under the microscope to confirm that the cells are concentrated at the bottom of the microwell.

24. Incubate at 37°C for 24 hours.

25. Day 1. Remove all the media carefully with a p1000 tip and replace with 2 ml of fresh E6 for a full media change (*see* **Note 5)**

26. Incubate at 37°C for 24 hours.

27. Day 2 till Day 4. Feed the EBs with half-media change by gently replacing only 1 ml of E6 per well. Incubate at 37°C for 24 hours.

3.3 Neural induction (Day 5)

For each EB preparation (1 well of Aggrewell plate), 1 well of a 6-well plate coated with laminin 521 needs to be ready at day 5.

1. On day 5 of EB formation, prepare a 15 ml tube fitted with a 37 μm reversible strainer with arrow pointing up.

2. Prepare N2 base medium.

3. With a standard p1000 tip remove 1 ml of medium from the Aggrewell containing EBs and expel it back into the Aggrewell with vigor in order to detach EBs (see **Note 6**).

4. Use a large bore p1000 tip to collect all the media containing floating, and visible, EBs, and slowly add this suspension to the top of the cell strainer.

5. Repeat stem 3 and 4 to collect all EBs (see **Note 7**).

6. Remove the Laminin 521 coating solution from a precoated 6-well plate and place the cell strainer upside-down (arrow pointing down) on top of the corner of the well by holding it with one hand.

7. Add 1 ml of N2 base media with a standard p1000 tip slowly around the top of the cell strainer to release the EBs into the well.

8. Repeat step 7 in order to release all EBs in the well.

9. Incubate at 37°C to start neural induction and do not disturb for 24 hours. (*see* **Note 8)**

10. Day 1 of neural induction. Using a stripette, replace media with 2 ml of fresh N2 base medium.

11. Day 2 of neural induction. Using a stripette, replace media with 2 ml of fresh N2 base medium.

3.4 lt-NES derivation (Day 8/10)

1. Neural rosettes should be derived between day 3 to day 5 of neural induction when the rosettes appear, at microscopic inspection, to be flat, round-shaped and with lumens, while the confluence of the surrounding differentiated cells has not yet reached 80% of the entire plate.

2. Prepare, or have already, at least 4 wells of a 48-well plate coated with Laminin 521.

3. Remove the N2 base medium from the rosette well.

4. Add 1 ml of rosette selection solution and incubate for 45 minutes at 37°C. (see **Note 9**).

5. Remove the rosette selection solution entirely.

6. Using a p1000 tip firmly release 1 ml of N2 media on top of the rosette clusters (visible cell clumps by naked eye).

7. With the same tip, aspirate the media containing the rosettes and transfer it to a 15 ml tube. (see **Note 10**)

8. Repeat step 5 to 6 until 70 % of rosettes have been removed (see **Note 11**).

9. Spin the rosette suspension at 300 g for 5 minutes.

10. Aspirate supernatant.

11. Suspend the rosettes pellet in 400 μl of N2 media plus 10 ng/ml of EGF and FGF (named N2 EF) supplemented also with Revitacell (see **Note 12**).

12. Distribute the rosette suspension equally in 4 wells of a 48-well plate coated with Laminin 521.

13. Top up each well to a final volume of 500 μl with N2 EF media plus Revitacell.

14. Monitor cell attachment after 10 minutes and around 5-6 hours (*see* **Note 13**).

15. After 24 hours perform a media change with fresh N2 EF media (*see* **Note 14**).

16. Prepare, or have already, at least 4 wells of a 24-well plate coated with Laminin 521.

17. On day 2, if lt-NES are 100% confluent proceed with a p0 to p1 expansion from a 48- to a 24-well format, for each of the wells, as following:

    a. Remove media from one well of the 48-well plate.

    b. Add 300 μl of Accutase and incubate at 37°C for 1 minute.

    c. Once the cells have started to look round but are not floating, use a p1000 to aspirate accutase and pipette back on the cells to physically detach them.

    d. Transfer the lt-NES suspension to a 15ml tube containing 10 ml N2 media.

    e. Centrifuge at 300 g for 3 minutes.

    f. Aspirate supernatant and resuspend the lt-NES into 600 μl of N2 EF plus Revitacell.

    g. Plate lt-NES into a single well of a 24-well format coated with Laminin 521.

18. Daily feed p1 lt-NES with 0.6 ml of N2 EF until reaching 100% confluency (see **Note 15**).

19. Keep one well of p1 lt-NES cell as security or for early stock (*see* **Note 16**).

20. Prepare, or have already, at least 3 wells of a 12-well plate coated with Laminin 521.

21. Once p1 lt-NES are 100% confluent, proceed with a p1 to p2 expansion from a 24- to a 12-well format, for each of the wells, as following:

    a. Remove media from one 24-well vessel.

    b. Add 500 μl of Accutase and incubate at 37°C for 1 minute.

    c. Once the cells have started to look round but are not floating, use a p1000 to aspirate accutase and pipette back on the cells to physically detach them.

    d. Transfer the lt-NES suspension to a 15ml tube containing 10 ml N2 media.

    e. Centrifuge at 300 g for 3 minutes.

    f. Aspirate supernatant and resuspend the lt-NES into 1 ml of N2 EF plus Revitacell.

    g. Plate lt-NES into a single well of a 12-well plate coated with Laminin 521.

22. Daily feed p2 lt-NES with 1 ml of N2 EF until reaching 100% confluency.

23. Prepare, or have already, at least 6 wells of a 12-well plate coated with Laminin 521.

24. Once p2 lt-NES are 100% confluent, proceed with a p2 to p3 expansion from a 12- to a 12-well format, for each of the wells, as following:

    a. Remove media from one 12-well vessel.

    b. Add 500 μl of Accutase and incubate at 37°C for 1 minute.

    c. Once the cells have started to look round but are not floating, use a p1000 to aspirate accutase and pipette back on the cells to physically detach them.

    d. Transfer the lt-NES suspension to a 15ml tube containing 10 ml N2 media.

    e. Centrifuge at 300 g for 3 minutes.

    f. Aspirate supernatant and resuspend the lt-NES into 2 ml of N2 EF plus Revitacell.

    g. Plate lt-NES into 2 wells of a 12-well format coated with Laminin 521.

25. Daily feed p3 lt-NES with 1 ml of N2 EF until reaching 100% confluency.

26. Prepare, or have already, at least 3 wells of a 6-well plate coated with Laminin 521.

27. Once p3 lt-NES are 100% confluent, proceed with a p3 to p4 expansion from a 12- to a 6-well format, for two of the wells, as following:

    a. Remove media from two 12-well vessels.

    b. Add 500 μl of Accutase and incubate at 37°C for 1 minute.

    c. Once the cells have started to look round but are not floating, use a p1000 to aspirate accutase and pipette back on the cells to physically detach them.

    d. Transfer the lt-NES suspension to a 15ml tube containing 10 ml N2 media.

    e. Centrifuge at 300 g for 3 minutes

    f. Aspirate supernatant and resuspend the lt-NES into 2 ml of N2 EF plus Revitacell

    g. Plate lt-NES into 1 well of a 6-well format coated with Laminin 521.

28. Daily feed p4 lt-NES with 2 ml of N2 EF until reaching 100% confluency.

29. Once p4 lt-NES are 100% confluent, proceed with a 1:2 passage in a 6-well format (*see* **Note 17**).

    a. Remove media from one 6-well vessel.

    b. Add 1 ml of Accutase and incubate at 37°C for 1 minute.

    c. Once the cells have started to look round but are not floating, use a p1000 to aspirate accutase and pipette back on the cells to physically detach them.

    d. Transfer the lt-NES suspension to a 15ml tube containing 10 ml N2 media.

    e. Centrifuge at 300 g for 3 minutes.

    f. Aspirate supernatant and resuspend the lt-NES into 4 ml of N2 EF plus Revitacell.

    g. Plate lt-NES into 2 wells of a 6-well format coated with Laminin 521.

30. Daily feed p5 lt-NES in N2 EF until reach 100% confluency.

31. Once lt-NES reach a comfortable expansion in a 6-well plate format, they are ready for characterisation (*see* **Note 18**).

3.5 lt-NES maintenance

Passaging:

1. Lt-NES are split at a ratio of 1:2 or 1:3 every 3 days when culture is 95-100% confluent.

2. Remove media from one 6-well vessel.

3. Add 1 ml of Accutase and incubate at 37°C for 1 minute.

4. Once the cells have started to look round but are not floating, use a p1000 to aspirate accutase and pipette back on the cells to physically detach them.

5. Transfer the lt-NES suspension to a 15ml tube containing 10 ml N2 media.

6. Centrifuge at 300 g for 3 minutes.

7. Aspirate supernatant and resuspend the lt-NES into 4 ml (or 6 ml) of N2 EF

8. Plate NES into 2 (or 3 wells) of a 6-well format coated with Laminin 521.

9. Perform a media change with N2 EF (10ng/ml) every day from passage 0 to passage 10 and every other day from passage 10 onwards.

Freezing:

1. Remove media from one 6-well vessel.

2. Add 1 ml of Accutase and incubate at 37°C for 1 minute.

3. Once the cells have started to look round but are not floating, use a p1000 to aspirate accutase and pipette back on the cells to physically detach them.

4. Transfer the lt-NES suspension to a 15ml tube containing 10 ml N2 media.

5. Centrifuge at 300 g for 3 minutes.

6. Aspirate supernatant and resuspend the lt-NES pellet into 1 ml Cryostem freezing medium.

7. Immediately transfer cell suspension into a labelled Cryovial and place into a CoolCell freezing device at -80 °C for 24 hours.

8. After 24 hours cells can be removed from the CoolCell device and transferred to liquid or vapour phase Nitrogen tanks for long-term storage.

Thawing:

1. Remove one cryovial from the liquid nitrogen tank and place immediately into a dry ice box.

2. Place the cryovial to thaw in a beads-filled thermostat. Keep the vial at 37°C until visibly there is only a 1/10 of the solution in the solid state, in the form of a small piece of ice.

3. Remove the cell solution from the cryovial with a 5ml stripette and transfer it into a 15 ml tube containing 10 ml of pre-warmed N2 base media.

4. Centrifuge at 300 g for 3 minutes.

5. Aspirate supernatant and resuspend the lt-NES pellet into 2 ml of N2 EF plus Revitacell.

6. Plate lt-NES into 1 well of a 6-well format coated with Laminin 521.

7. After 24 hours remove N2 media containing Revitacell and add fresh N2 EF medium.

8. Place the cell culture 37°C in the incubator.

3.6 lt-NES spontaneous differentiation

1. Remove media from one 6-well vessel containing lt-NES.

2. Add 1 ml of Accutase and incubate at 37°C for 1 minute.

3. Once the cells have started to look round but are not floating, use a p1000 to aspirate accutase and pipette back on the cells to physically detach them.

4. Transfer the lt-NES suspension to a 15ml tube containing 10 ml N2 media.

5. Remove 10 μl of suspension and count the cells by using a haemocytometer.

6. Centrifuge at 300 g for 3 minutes to pellet the cells.

7. Calculate the volume of cell suspension required to seed lt-NES at 40000 cells/cm^2

8. See cell on wells (48-well format or higher, depending on the experimental design) coated with Laminin 521 in N2 media without EF.

9. After 24 hours remove N2 media.

10. Prepare terminal differentiation media by mixing 50:50 parts of DMEM-F12 (with N2 1:100): Neurobasal (with B27 1:50).

11. Add terminal differentiation media plus freshly added 300 ng/ml cAMP. This constitutes Day 1 of the terminal differentiation.

12. Daily feed the cells with differentiation medium plus cAMP till day 21.

13. Cells are ready for characterization from day 21 (see **Note 19**).

3.7 lt-NES directed differentiation towards dopaminergic neurons

1. Remove media from one 6-well vessel containing lt-NES.

2. Add 1 ml of Accutase and incubate at 37°C for 1 minute.

3. Once the cells have started to look round but are not floating, use a p1000 to aspirate accutase and pipette back on the cells to physically detach them.

4. Transfer the lt-NES suspension to a 15ml tube containing 10 ml N2 media.

5. Remove 10 μl of suspension and count the cells by using a haemocytometer.

6. Centrifuge at 300 g for 3 minutes to pellet the cells.

7. Calculate the volume of cell suspension required to seed lt-NES at 40000 cells/cm^2

8. See cell on wells (48-well format or higher, depending on the experimental design) coated with Laminin 521 in N2 media without EF.

9. After 24 hours remove N2 media.

10. Day 1. Change the medium to patterning medium composed of DMEM-F12 (with N2 1:100) medium plus freshly added 200ng/ml SHH, 100ng/ml FGF8 and 160 μM Ascorbic Acid.

11. Daily feed the cells with patterning medium till day 13.

12. Day 14. Change medium to dopaminergic terminal differentiation medium composed of DMEM-F12 (N2 1:100): Neurobasal (B27 1:50) plus freshly added 20ng/ml BDNF, 10ng/ml GDNF, 160 μM Ascorbic Acid, 500 μM dy-cAMP.

13. Daily feed neurons with dopaminergic terminal differentiation medium till day 21.

14. From day 21 the neurons are ready for immunofluorescence characterization, although differentiation can continue longer in the same medium for long-term functionality and maturity studies (see **Note 20**).

3.8 lt-NES directed differentiation towards motoneurons

1. Remove media from one 6-well vessel.

2. Add 1 ml of Accutase and incubate at 37°C for 1 minute.

3. Once the cells have started to look round but are not floating, use a p1000 to aspirate accutase and pipette back on the cells to physically detach them.

4. Transfer the lt-NES suspension to a 15ml tube containing 10 ml N2 media.

5. Remove 10 μl of suspension and count the cells by using a haemocytometer.

6. Centrifuge at 300 g for 3 minutes to pellet the cells.

7. Calculate the volume of cell suspension required to seed lt-NES at 40000 cells/cm^2

8. See cell on wells (48-well format or higher, depending on the experimental design) coated with Laminin 521 in N2 media without EF.

9. After 24 hours remove N2 media.

10. Day 1. Change the medium to patterning medium composed of DMEM F12 (With N2 1:100, B27 1:50) plus 10ng/ml EGF, 10ng/ml FGF and 1 μM Retinoid Acid.

11. Daily feed cells with patterning medium till day 5.

12. Day 5. Supplement the patterning medium with 1μg/ml GMP SHH.

13. Day 6. Feed cells with patterning medium plus 1μg/ml GMP SHH.

14. Day 7. Feed cells with patterning medium modified by removing EGF and FGF and reducing the concentration of Retinoid Acid to 0,01 μM.

15. Day 8 till Day 11. Feed cells with patterning medium as per step 9.

16. Day 12. Change feeding media to motoneuron terminal differentiation media composed of equal parts of DMEM-F12 (N2 1:100) : Neurobasal (B27 1:50) plus 20 ng/ml GMP BDNF, 20 ng/ml GMP GDNF, 50ng/ml SHH and 300 ng/ml cAMP.

17. Daily feed neurons with motoneuron terminal differentiation medium till day 21.

18. From day 21 the neurons are ready for immunofluorescence characterization, although differentiation can continue longer in the same medium. (see **Note 21**).

4. Notes

1. It is important that wells do not dry therefore it is recommended to add Essential 8™ Medium to the wells as soon as you have aspirated the Vitronectin solution. Culture vessels coated with Vitronectin can be stored at 2—8°C wrapped in parafilm for up to two days. However, it is recommended that the plates are coated on the day of use.

2. Instruction for one well of several formats. 6-well: 100 μl L521 to 900 μl of CTS™ DPBS+/+ . 12-well: 50 μl L521 to 450 μl of CTS™ DPBS+/+. 24-well: 30 μl L521 to 270 μl of CTS™ DPBS+/+. 48-well: 17.5 μl L521 to 157.5 μl of CTS™ DPBS+/+.96-well: 6 μl L521 to 54 μl of CTS™ DPBS+/+.

3. Media should be changed every day; it is acceptable once a week to add double the quantity of media and leave the cells without media change for 48 hours.

4. Do not overly stress cells as this will affect their survival, over pipetting will result in cell death and hamper the quality of EBs in the subsequent steps.

5. After 24 hours the cells should have aggregated in EB-like shape and the first media change is performed. Remove media very gently, without touching the microwells. Likewise, addition of the media needs to be performed very slowly by touching the border of the well plastic with the top of

the tip. Failure to be gentle at this step will dislodge EBs form the microwells with the result of having more than 1 EB per microwell or loss of EBs.

6. Do not aspirate back the suspension with the standard p1000.

7. Do not pipette EBs up and down in the Aggrewell, simply do many more rounds if not all the EBs appear to be detached once inspected under microscope.

8. Neural rosettes, characteristic morphologies indicating a successful neural induction, will start to emerge around day 2-3 in the centre of the EBs. Sometimes rosettes are already visible after 24 hours, but these are at the earliest stages and maturation generally takes up to 3 days. Neural rosette should be visible with a 2x to 10x magnification in an inverted microscope

9. The solution will promote detachment of neural rosettes. Time of incubation may require optimization based on the cell line used, from 45 minutes up to 1.15 hours.

10. Do not do reuse the media containing rosettes to target new areas.

11. It is important to check under the microscope that only the rosettes have been removed while the surrounding cells remain largely attached as these non-progenitors cells can affect the derivation of pure lt-NES.

12. Assess if there are large clusters of fully formed round rosettes, if so, pipette with a p200 firmly until producing clusters of around 5-20 cells and some single cells. Do not pipette to produce a 100% single cell suspension, the ideal preparation will have small aggregates and also single cells.

13. One of the main challenges in the derivation of lt-NES is the proper attachment of the cells to the matrix. Improper coating can lead to poor attachment, visible already after a couple of hours from plating. Lt-NES cells generally attach to the laminin after 5-10 minutes from plating and within 6 hours start to spread to the surface. If clumps or round cells are visible, remove the cells (with gentle pipetting or short addition of Accutase) and place in a new laminin plate. Monitor attachment of lt-NES and prepare extra reserve plates with Laminin made on different days. Batch testing of Laminin lots is highly recommended.

14. Cells should be between 90% to 100% confluent. Inspect under the microscope in phase contrast for the presence of typical neural rosette progenitors morphology. Due to high cell density the right rosette morphology may also not be visible as cells are too compacted and rounded. If cells are on top of each other they need passaging 1:2 into a 48-well format.

15. This can vary from 24 hours to 2 days. Typical Lt-NES morphology should be visible at this stage.

16. Plan ahead a freeze of passage 1 and following early passages of lt-NES, even from a 48-well format. This will save work if lt-NES do not attach to the laminin for various reasons and also guarantee an early stock.

17. It is highly recommended to freeze 1:1 one 6-well of confluent NES at this passage, and split 1:2 the other well.

18. Immunofluorescence for rosette and lt-ES markers can be performed with the following antibodies: SOX2 (1: 100, Bio-techne); Nestin (1: 100, 10C2, Abcam); PAX6 (1: 100, Cambridge Bioscience); DACH1 (1: 100, Proteintech); PLZF (1: 100, Life Technologies); ZO-1 (1: 50, Bio-techne). Gene expression assessment of lt-ES markers can be performed with following genes (Taq man assays from Life Technologies). SOX2(Hs01053049_s1); PLZF(Hs00957433_m1);PLAGL1(Hs00414677_m1);MMNR1(Hs00201182_m1);DACH1(Hs003 62088_m1);NOTCH1(Hs01062014_m1);PAX6(Hs00240871_m1);SOX1(Hs01057642_s1);NANO G (negative) (Hs04260366_g1); PBGD (housekeeping gene) (Hs00609296_g1).

19. Spontaneously differentiated Lt-NES are positive for the following markers by immunofluorescence: GABA (1:500, Sigma), MAP2 (1:500, Sigma), Tubulin TuJ1 clone (1:1000, Biolegend).

20. After 21 days Lt-NES are positive for the following dopaminergic markers by immunofluorescence: Tyrosine Hydroxylase (1:200, Millipore);Tubulin β3 [Clone: TUJ1] (1:1000,

Biolegend);FOXA2 (1:100, Santa Cruz);LMX1alfa (1:500, Millipore);GIRK2 (1:500, Millipore); Nurr1 (1:500, Santa Cruz).

21. Motoneurons immunofluorescence markers: Tubulin β3 [Clone: TUJ1] (1:1000, Biolegend), HB9.

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Figure Legend

Figure 1. Schematic overview of the step-by-step protocol. HESCs are dissociated to generate embryoid bodies of the same size in a microwell format (Step 1). After five days the formed EBs are dislodged from the microwell and plated for neural induction (Step 2). Neural induction will follow for up to five days when characteristic neural rosettes morphologies appear by microscopic inspection, at which point they are ready for purification (Step 3). Scalable expansions of dissociated rosettes in presence of EGF and FGF leads to the establishment of a pure population of lt-NES, which is self-renewing, bankable and multipotent (Step 4). The downstream differentiation of lt-NES depends on the chosen patterning conditions, with a high neurogenic potential towards GABAergic neurons upon EGF and FGF withdrawal (Step 5). Specific patterning conditions including FGF8b and SHH lead lt-NES towards dopaminergic phenotypes (Step 6). On the contrary, with patterning media containing retinoid acid and SHH, lt-NES are redirected towards motoneurons phenotypes (Step 7). (Figure Created with BioRender.com).

hESCs → Step 1 (D0-D4) → EBs → Step 2 (D5-D8/10) → Neural Rosettes → Step 3 (p1+) → lt-NES → Step 4

lt-NES differentiation

Step 7 → Motoneurons

Step 6 → Dopaminergic neurons

Step 5 → GABAergic neurons

Step 1   Step 2
Step 3   Step 4