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Applied StemCell

Datasheet

Human ESC/iPSC - Neural Stem Cell Differentiation and Characterization Kit

Catalog Number ASK-4002

Size 1 Kit (5 Reactions)

Description

Applied StemCell's Neural Stem Cell (NSC) Differentiation/Characterization Kit contains all the reagents necessary to differentiate up to five human iPSC/ESC lines starting with a 35 mm plate. The kit also includes primary and fluorescent-labeled secondary antibodies for NSC characterization.

Kit Contents

All of the reagents supplied in the kit are sufficient for five reactions according to the protocol below. Reagents are also available separately from Applied StemCell Inc.

Component	Size	Concentration	Catalog #	Storage & Stability
NI-1	5 mg		ASC-4101	Short term (up to 1 week): 4ºC Long term (2-3 months): -20ºC to -80ºC
NI-2	1 mg		ASC-4102	
ESC-Sure™ Conditioned Medium (CM)	100 ml	1×	ASM-5008	Short term (up to 2 week): 4ºC Long term (6 months): -20ºC to -80ºC
B27	2.5	50×	ASA-0435	Short term (up to 1 week): 4ºC Long term (2-3 months): -20ºC to -80ºC
Rabbit anti-Sox2	800 µl	1×	ASA-0120	4ºC
Mouse anti-Nestin	800 µl	1×	ASA-2010	
Goat-anti rabbit-Alexa 488	2 ml	1×	ASA-0006	4ºC, in dark
Goat-anti mouse-Alexa 590	2 ml	1×	ASA-0001	
Cell Fixation Solution	15 ml	1×	ASB-1010	Short term (up to 1 week): 4ºC Long term: -20ºC to -80ºC
Permeabilization Solution	15 ml	1×	ASB-0102	4˚C, in dark
Blocking Solution	15 ml	1×	ASB-0103	
DNA Staining Solution	1.5 ml	1×	ASB-0104	
Mounting Solution	5 ml	1×	ASB-0105	

Storage and Handling

Storage:
NI-1, NH-2, B27, Cell Fixation Solution: Aliquot, store at -20ºC to -80ºC. Avoid repeat freeze/thaw cycles
ESC-Sure™ Conditioned Medium, Antibodies, staining solutions: 4°C

Shipping: Dry Ice

Usage Statement

The **hESC/iPSC - Neural Stem Cell Differentiation and Characterization Kit** is for research use only and not intended for human or animal diagnostic or therapeutic uses.

Background

Recent advances in methods to differentiate either human embryonic stem (hES) cells or induced pluripotent stem (iPS) cells offers great promise for cell replacement therapies and disease modeling. Currently, methods used to differentiate hES or iPS cells into neural cells can be placed into three categories: Initials methods relied on co-culture with neural inducing stroma cells (1-3). Another method bases on generating embryoid bodies which are then induced to differentiate into specific neuronal cell types (4). Lastly, dual inhibition of key SMAD signaling pathways has been used used to induce ectodermal/ neuroectodermal pathways (5).

Although each method has been successful in generating neurons from either hES or iPS cells, they all have disadvantages. Co-culture based methods induce hES/iPS cells to neuronal fates, but the use of a poorly defined neural inducing stroma makes it difficult to utilize this method for future studies. Likewise, the heterogeneous nature of embryoid body formation and the protracted length of differentiation make this method inconsistent. Lastly, the dual SMAD inhibition method can produce only a finite number of neurons that cannot be expanded or frozen down for future use (6-7).

In order to realize the full potential of hEC or iPS cells for the study of neural cells, our **Human ESC/iPSC Neural Stem Cell (NSC) Differentiation and Characterization Kit** is designed to produce NSCs without the requirement of either stroma cells or embryoid body formation. Included in the kit are our optimized, neural inducing reagents, reagents for identification and characterization of NSC and a detailed protocol. The kit provides a fast, user-friendly method for the generation of NSCs which then can be further differentiated to the particular neuronal cell type of the investigator's choice. Antibodies for NSC identification (Sox2 and Nestin), are provided (8).

Materials and Instruments Required but Not Provided

- Geltrex™ Matrix (Invitrogen Cat. #A10480-01)
- N-2 Supplement, 100x (Invitrogen Cat. #17502-048)
- Neural Basal Medium (Invitrogen Cat. # 21103049)
- Accutase™(Innoative Cell Technologies Inc., Cat.#AT-104)
- Tissue Culture Grade DMSO (Sigma Aldrich Cat. #C6295)
- 18 gauge needle (VWR BD305766)
- StemPro^{R} EZPassage^{TM} Disposable Stem Cell Passaging Tool (Invitrogen Cat. #23181-010)

Reagent Formulas

Component	Concentration	Notes
NI-1 Stock Solution		
DMSO	3.13 ml	Dissolve NI-1 in tissue culture grade DMSO
NI-1	5 mg	Prepare 20 ul aliquots and store at -20ºC for up to 3 months
NI-2 Stock Solution		
DMSO	237 µl	Dissolve NI-2 in tissue culture grade DMSO
NI-2	1 mg	Prepare 10 ul aliquots and store at -20ºC for up to 1 month
Complete Conditioned Medium (Complete CM)		
ESC-Sure™ CM		
Pen/Strep (optional)	1x	
FGF-2	10 ng/ml	
RIM (Rosette Induction Medium)		
ESC-Sure™ CM	10 ml	
NI-1	5 µl	Store at 4ºC for up to four days. Protect from direct light.
NI-2	10 µl	
NIM (Neural Induction Medium)		
ESC-Sure™ CM		
Non-essential amino acid	1x	
L-glutamine (2 mM)	2x	Add FGF-2 after filtering the medium
N2 Supplement	1x	
FGF-2	20 ng/ml	
NPM (Neural Proliferation Medium)		
ESC-Sure™ CM		
Non-essential amino acid	1x	
L-glutamine (2 mM)	2x	Add B27 and FGF-2 after filtering the medium
B27 Supplement	1x	
FGF-2	20 ng/ml	
NSC-FRA		
Neurobasal medium		
Non-essential amino acid	1x	Add B27 after filtering the medium
L-glutamine (2 mM)	2x	
B27 Supplement	1x	
NSC-FRB		
Neurobasal medium		
Non-essential amino acid	1x	
L-glutamine (2 mM)	2x	Add B27 after filtering the medium
B27 Supplement	1x	
DMSO	20%	

Protocol

Starting Criteria:

- At least 1 well of a 6-well plate of human ES or iPS cell colonies
  - On a feeder free matrix in **Complete Conditioned Medium** (**Complete CM**)
  - About 80% confluent, ready for passaging (~4-7 days old)
  - Minimal (less than 5%) or no differentiation

PART 1: ECs/iPSCs to Rosettes

1. Clean the pluripotent hES or hiPS cell culture by removing colonies that have the appearance of differentiated cells, uneven boarders or transparent centers.
2. Aspirate the medium and wash the plate 2-3 times with PBS. Be sure that the plate is completely free of differentiated cells. Add fresh **Complete CM** to the plate.
3. Using a StemPro^{R} EZPassage^{TM} Disposable Stem Cell Passaging Tool, divide the colonies into optimally sized pieces.
4. Gently transfer the colony pieces to a Geltrex-coated plate containing **Complete CM** such that the plate is at least 80% confluent

NOTE: Use a freshly prepared Geltrex-coated plate. Do not transfer differentiated cells.

5. Allow the colony pieces to attach for at least 24 hours before you proceed.
6. Prepare 10 ml of **Rosette Induction Medium** (**RIM**). After addition of NI-1 and NI-2 store **RIM** at 4ºC for up to four days. Wrap the medium container in aluminum foil to protect from direct light.
7. To initiate differentiation, remove the Compete CM and add an appropriate amount of **RIM** to each well/dish. Change half of the medium every day while monitoring for signs of rosette formation. Rosettes normally appear around day 10.
8. Once rosettes appear, transfer to a new plate containing 100% **Neural Induction Medium (NIM)**.

PART 2: Rosettes to Neural Stem Cells (NSCs)

9. Once rosettes appear: manually isolate the rosettes (Fig. 1a) from any non-rosette cell types.
10. Transfer the rosettes to a Geltrex-coated well of a 12-well plate. Repeat this process to obtain a homogeneous population of rosettes.
11. Allow the rosettes to attach for 12-24 hours in **NIM**.
12. Change the **NIM** every day while the NSCs grow out from the rosette border.
13. Once the NSCs have started to expand from the rosette border for 2-3 days, dissociate the rosette/NSC cells into single cells using Accutase® (~1 ml per well). Transfer all cells into a 15 ml conical tube by washing the plate with an equal amount of **NIM**.
14. Centrifuge the cells at 3,000 RPM for 3 minutes. Aspirate the medium and resuspend the cells in **NSC Proliferation Medium** (**NPM**). Plate the cells on a 35 mm dish coated with Geltrex.

NOTE: The density of the replated NSCs is critical. Plate the NSCs at a minimal density of 30-40%.

PART 3: Maintaining NSC Cultures

15. Change the **NPM** every day by tilting the Geltrex-plate and carefully aspirating the medium from the side.

NOTE: NSCs weakly attach to the plate surface. Do not tilt the plate at too steep an angle and slowly remove the NPM.

16. Passaging the NSC Cultures
    a) Add the appropriate amount of Accutase® to the plate (~0.5 – 1 ml for a 35 mm plate).
    b) Incubate at RT for 1-3 minutes.
    c) Wash the NSCs from the dish by gently pipetting the medium onto the plate surface.
    d) Transfer the medium to a 15 ml conical tube and centrifuge for 3-4 minutes at 1300-1600 rpm.
    e) Gently resuspend the NSCs in **NPM** and plate onto recently prepared Geltrex coated dishes.
    f) Equally distribute the NSCs on the plate surface by gentle tilting the plate in a cross pattern.

PART 4: Freezing NSC Cultures

Starting Materials:

- **NSC-FRA** and **NSC-FRB** media
- Cryotubes
- Mr. Frosty freezing container

17. Repeat steps 14 a-c.
18. Count the NSCs after resuspending in a small volume of **NSC-FRA** medium (<1 ml).
19. Adjust the final volume such that the concentration is 4 x 10^6 cells/ml **NSC-FRA** medium.
20. Add an equal volume of **NSC-FRB** medium by slowly pipetting the medium while gently swirling. Mix the entire solution **once** by gently pipetting up and down and aliquot 1 ml per cryovial.
21. Place the cryovials into a freezing container and immediately place at -80ºC
22. Transfer vials to liquid N_2 after 24 hours.

PART 4: Thawing NSC Cultures

Starting Materials:

- 15 ml conical tube containing 5 ml of pre-warmed **Neurobasal medium**
- **NPM**
- 35 mm Geltrex-coated plate
- All volumes should result in 50% confluency on a 35mm plate

23. Immediately place the retrieved cryovial of NSCs in a 37°C water bath.
24. Once thawed transfer the NSCs to a 15 ml conical tube containing 5 ml of pre-warmed **Neurobasal medium**. Wash the cryovial with 1 ml of **Neurobasal medium** and add to the cell suspension.
25. Centrifuge the cells for 3-4 minutes as 1300-1600 rpm.
26. Gently resuspend the NSCs in **NPM** and plate onto a freshly prepared Geltrex-coated dish.
27. Immediately distribute the NSCs on the plate surface by gently tilting the plate in a cross pattern.

PART 6: Immunocytochemical Staining of Neural Stem Cells (NSCs)

Starting Materials

- Expanded NSCs on glass cover slips coated with Poly-D-Lysine (Sigma, Cat. #P3655) and Laminin (BD Bioscienes, Cat. #354087 or equivalent).
- Anti-human Sox2 and Nestin monoclonal antibodies (provided)
- Appropriate secondary antibodies (provided)
- Fluorescent microscope

Note: See protocol for coating glass cover slips with Poly-L-ornithine and Laminin on page

28. Transfer isolated NSCs in **NPM** to a 24-well plate containing 13 mm glass cover slips coated with poly-L-ornithine and laminin and allow for attachment for at least 12 hours.
29. Rinse the wells twice with PBS and fix the cells by immersing the cover slips in Fix Solution for 1-2 hours at room temperature.
30. Wash the cells three times with PBS for 5 min at room temperature.
31. Permeabilize the cells the by immersing the cover slips in Perm Solution for 30 minutes at room temperature.
32. Aspirate the solution and wash the cells twice with PBS at room temperature.
33. Block the nonspecific antigen sites by adding Block Solution for 1 hour at room temperature.
34. Aspirate Block Solution and wash the cells three times with PBS at room temperature.
35. Place a strip of Parafilm® in a humidified chamber. Pipette 30 ul of the Sox2 primary antibody onto the strip. Using forceps, place the cover slip (cells facing **down**) on top of antibody mix. Place the humidified chamber in a secure place and incubate at room temperature for two hours. Alternatively, incubate at 4ºC overnight.
36. Using forceps invert the glass slides such that the cell surfaces are facing upward. Aspirate the primary antibody solution and wash the cells three times with PBS for 5 minutes at room temperature.
37. Repeat steps 36 and 37 using 30 ul of the anti-Nestin primary antibody.
38. Aspirate the PBS and then add one drop (~45 µl) of goat anti-rabbit Alexa 488 secondary antibody to the cover slip. Incubate for one hour at room temperature.

NOTE: Keep the cover slips in the dark from here on through the remaining of the procedure!

39. Aspirate the antibody solution and wash twice with PBS for 5 minutes at room temperature.
40. Add one drop (~45 ul) of goat anti-mouse Alexa 590 secondary antibody to the cover slip. Incubate for one hour at room temperature in the dark.
41. Aspirate the antibody solution and wash twice with PBS for 5 minutes at room temperature.
42. Aspirate the PBS and add one drop (~45 ul) of DNA staining solution. Incubate for eight minutes at room temperature.
43. Aspirate the DNA staining solution and wash the cover slips with PBS at room temperature.
44. Place one drop (~20 ul) of Mounting Solution on a labeled glass slide. Using forceps, place the cover slip (cells facing **down**) on top of the drop. Place a larger glass cover slip on top.
45. Wrap the slide in aluminum foil and store at 4°C. The slides can be stored under these conditions for up to seven days.
46. Examine the slide under a fluorescence microscope. Nestin and Sox2 staining are located within the NSCs (Fig. 1c).

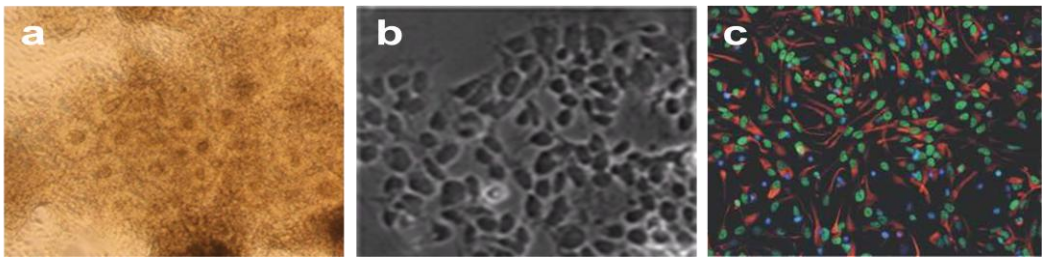

Figure 1. Rosettes and Neural Stem Cells (NSC). Representative images of (a) rosette clusters and (b) NSCs under light microscope. (c) NSCs stained with antibodies against Nestin (red) and Sox2 (green), counterstained with DAPI (blue). Adapted from Elkabetz *et al* (8).

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