TECHNICAL MANUAL

Identification and Enumeration of Rat Neural Stem and Progenitor Cells with the NeuroCult™ Neural Colony-Forming Cell (NCFC) Assay Kit

STEMCELL™ TECHNOLOGIES

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1.0 Overview of the NeuroCult™ NCFC Assay

Neural stem cells (NSCs) are defined as cells with the ability to: 1) proliferate, 2) self-renew and 3) produce a large number of functional progeny that can differentiate into mature neurons, astrocytes and oligodendrocytes. The understanding of neural stem cells and their therapeutic potential relies heavily on robust functional assays that can identify and measure NSC activity. Recent publications have highlighted the limitations of the Neurosphere Assay as an accurate assay for measuring NSC frequency in relation to NSC regulation.[1,2] One of the major limitations of the Neurosphere Assay is that not all neurospheres are derived from an NSC.[2] Because the Neurosphere Assay does not discriminate between neurospheres derived from a neural stem or progenitor cell, enumerating neurosphere numbers and equating this read-out to NSC numbers leads to an overestimation of NSC frequency. In addition, neurosphere fusions occur significantly, even at limiting dilution plating[3] and the lack of standardization in the neurosphere culture system makes it difficult to compare experimental results between laboratories.[4,5]

The **NeuroCult™ Neural Colony-Forming Cell (NCFC) Assay** has been developed to allow for the identification and discrimination of mouse or rat (for mouse, refer to Document #28709) neural stem and progenitor cells (see Figure 1 for a procedure overview).[2] Using the NeuroCult™ NCFC Assay, clonally-derived colonies of different sizes are generated and colony size is used to enumerate stem cells, as their proliferative potential is greater than that of progenitors and more mature cells. Cells with high proliferative potential form the largest-sized colonies (≥ 2 mm diameter) and fulfill all of the functional criteria for a neural stem cell (Figures 2 - 4). Cells with low and limited proliferative potential form colonies that are < 2 mm in diameter. They are designated as progenitor cells and do not meet all of the functional criteria of a neural stem cell.

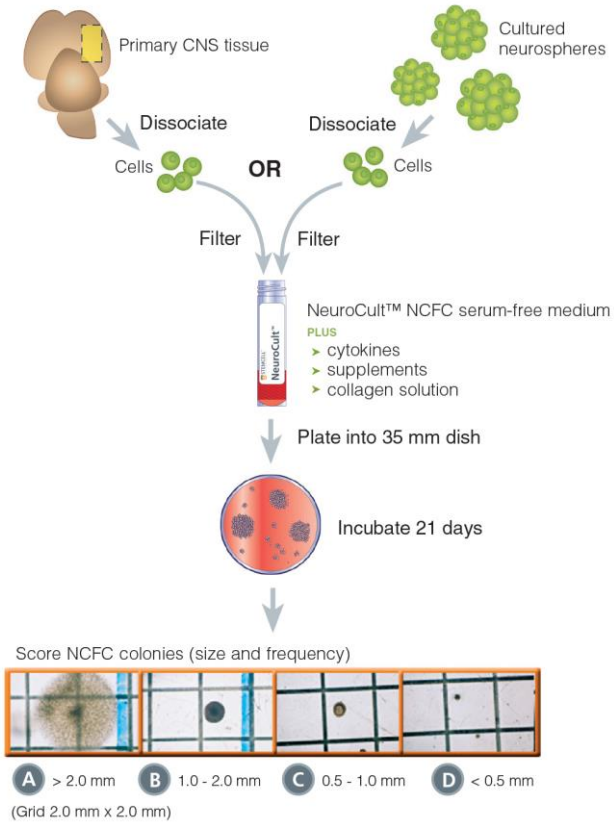

Figure 1. Overview of NeuroCult™ NCFC Assay Procedure for Rat

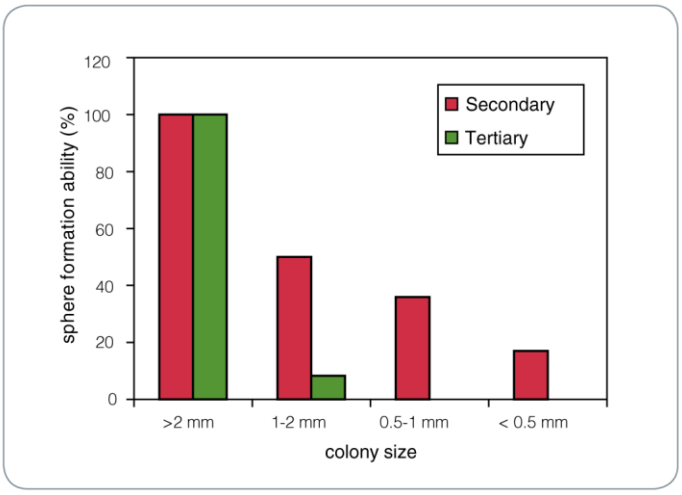

Figure 2. Secondary and Tertiary Neurosphere Formation Ability of Cells Isolated From NeuroCult™ NCFC Assay Colonies of Different Sizes (Data from Mouse Cells)

Cells isolated from colonies ≥ 2 mm diameter always formed tertiary neurospheres. No tertiary neurosphere formation was observed from cells isolated from colonies < 1 mm diameter.

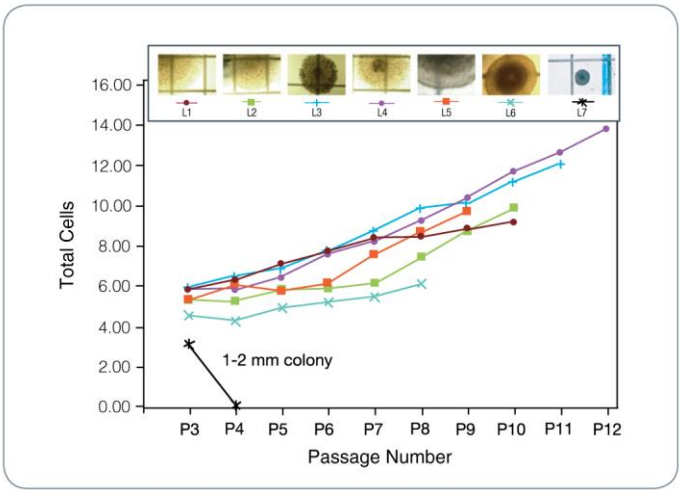

Figure 3. Proliferative Potential of Cells Isolated From NeuroCult™ NCFC Assay Colonies (Data from Mouse Cells)

Cells isolated from 6 representative colonies (L1 - L6) ≥ 2 mm diameter were passaged in liquid suspension neurosphere cultures and showed high fold expansion and self-renewal ability. Cells isolated from a colony (L7) 1 - 2 mm diameter failed to form neurospheres past passage 3.

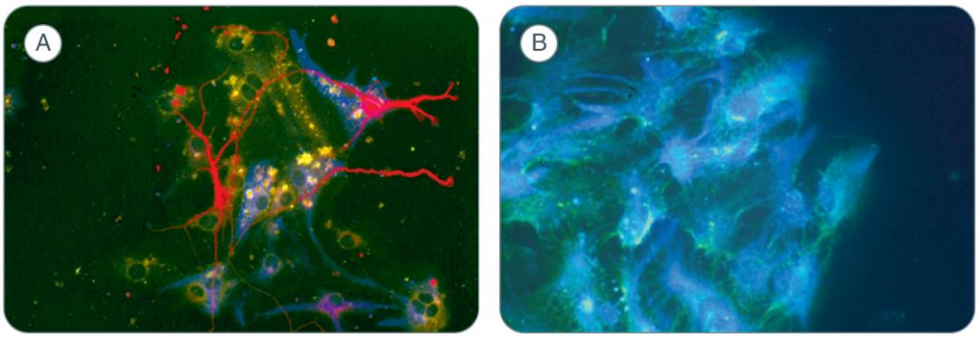

Figure 4. Differentiation Potential of Cells Isolated From NeuroCult™ NCFC Assay Colonies of Different Sizes (Data from Mouse Cells)

A. Cells isolated from colonies ≥ 2 mm diameter and cultured until passage 10 retain the ability to differentiate into neurons, astrocytes and oligodendrocytes.

B. Cells isolated from colonies 1 - 2 mm diameter and cultured until passage 2 do not retain multi-lineage differentiation potential. No neurons were observed in (B).

Neurospheres derived from cells from colonies ≥ 2mm were dissociated into single cells, and cells were differentiated by culturing in Complete NeuroCult™ Differentiation Medium (Mouse) for 10 days. Immunofluorescent staining was performed to identify the differentiated cell types. Neurons (red) were identified with Anti-Beta-Tubulin III Antibody, Clone TUJ1 (Catalog #60052), astrocytes (blue) were identified with Anti-GFAP Antibody, Polyclonal (Catalog #60128) and oligodendrocytes (green) were identified with Anti-Oligodendrocyte Marker O4 Antibody, Clone 81 (Catalog #60053).

2.0 Materials

2.1 NeuroCult™ NCFC Assay Kit (Rat) Components

Table 1. The NeuroCult™ Neural Colony-Forming Cell (NCFC) Assay Kit (Rat; Catalog #05742) contains the following components:

COMPONENT #	DESCRIPTION	UNIT SIZE
05720	NeuroCult™ NCFC Serum-Free Medium without Cytokines‡	50 mL
05773*	NeuroCult™ Proliferation Supplement (Rat)	50 mL
05770*	NeuroCult™ Basal Medium (Rat)	450 mL
04902	Collagen Solution	35 mL
07914	NeuroCult™ Collagenase Solution	5 mL
27100	35 mm Culture Dishes	70 dishes
27500	60 mm Gridded Scoring Dishes	5 dishes

‡ Use of NeuroCult™ NCFC Serum-Free Medium without cytokines allows the researcher to add cytokines of choice.

* Sold under license from StemCells California, Inc. US Patent Nos. 5,750,376; 5,851,832; 5,980,885; 5,968,829; 5,981,165; 6,071,889; 6,093,531; 6,103,530; 6,165,783; 6,238,922

For component storage and stability information, refer to the Product Information Sheet for the NeuroCult™ NCFC Assay Kit (Rat; Document #29578) available on our website at www.stemcell.com or contact us to request a copy.

2.2 Additional Required Reagents

- Recombinant Human Epidermal Growth Factor (rh EGF; Catalog #02633)
- Recombinant Human Basic Fibroblast Growth Factor (rh bFGF; Catalog #02634)
- 0.2% Heparin Sodium Salt in PBS (Catalog #07980)
- 10 mM acetic acid
- Bovine Serum Albumin (BSA)
- Trypan Blue (Catalog #07050)
- 70% Ethanol

2.3 Required Equipment

- Biohazard Safety Cabinet certified for Level II handling of biological materials
- 37°C incubator with humidity and gas control to maintain > 95% humidity and an atmosphere of 5% CO_2 in air
- Low speed centrifuge
- Pipette-aid and sterile 1 mL, 2 mL, 5 mL pipettes
- Micropipette with sterile 200 µl and 1 mL pipette tips

- Hemacytometer
- Forceps
- Routine light microscope for hemacytometer cell counts
- 100 mm Treated Tissue Culture Dish (Catalog #27125) or 245 mm x 245 mm Square Treated Tissue Culture Dish (Catalog #27120)
- Tubes: 15 mL polypropylene (Corning Catalog #352096) and 50 mL conical (Corning Catalog #352070)
- 40 µm Cell Strainer (Catalog #27305)

3.0 Preparation of Reagents

3.1 Basal Medium + Proliferation Supplement

1. Thaw NeuroCult™ Proliferation Supplement overnight at 2 - 8°C or for 1 - 2 hours at 37°C.
2. Prepare a 1 in 10 dilution of NeuroCult™ Proliferation Supplement in NeuroCult™ Basal Medium (i.e. 1 mL NeuroCult™ Proliferation Supplement for every 9 mL NeuroCult™ Basal Medium).

   Note: If not used immediately, store at 2 – 8°C for up to 1 month.

3.2 rh EGF Stock Solution (10 µg/mL)

1. Dissolve rh EGF (200 µg/vial) in 0.1 mL sterile 10 mM acetic acid containing at least 0.1% BSA.
2. Add 19.9 mL NeuroCult™ Basal Medium containing NeuroCult™ Proliferation Supplement (section 3.1).
3. Store 0.5 mL aliquots of the stock solution at -20°C. Do not freeze/thaw each vial more than 3 times.

3.3 rh bFGF Stock Solution (10 µg/mL)

1. Dilute rh bFGF (25 µg/vial) in sterile PBS containing 0.1% BSA to a final concentration of 10 µg/mL rh bFGF.
2. Store 0.5 mL aliquots of the stock solution at -20°C. Do not freeze/thaw each vial more than 3 times.

3.4 Complete NeuroCult™ Proliferation Medium (Rat)

1. To each 10 mL NeuroCult™ Basal Medium containing NeuroCult™ Proliferation Supplement (section 3.1), add the following:
   - 20 µL of 10 µg/mL rh EGF (to give a final concentration of 20 ng/mL rh EGF)
   - 10 µL of 10 µg/mL rh bFGF (to give a final concentration of 10 ng/mL rh bFGF)
   - 10 µL of 0.2% Heparin (to give a final concentration of 0.0002% Heparin (w/v) equals 2 µg/mL)
2. Mix well. Complete NeuroCult™ Proliferation Medium (Rat) is now ready for use.

   Note: If not used immediately, store Complete NeuroCult™ Proliferation Medium (Rat) at 2 – 8°C for up to 1 week.

3.5 Complete NeuroCult™ Replenishment Medium (Rat)

1. To 10 mL NeuroCult™ Basal Medium containing NeuroCult™ Proliferation Supplement (section 3.1), add the following:
   - 500 µL of 10 µg/mL rh EGF (to give a final concentration of 0.5 µg/mL)
   - 250 µL of 10 µg/mL rh bFGF (to give a final concentration of 0.25 µg/mL)
   - 250 µL of 0.2% Heparin (to give a final concentration of 0.005%; w/v equals 0.1 mg/mL)
2. Mix well. Complete NeuroCult™ Replenishment Medium (Rat) is now ready for use.

   Note: If not used immediately, store Complete NeuroCult™ Replenishment Medium (Rat) at 2 – 8°C for up to 1 week.

   Note: The concentrations of rh EGF, rh bFGF and Heparin in Complete NeuroCult™ Replenishment Medium (Rat) are higher than the concentrations in Complete NeuroCult™ Proliferation Medium (Rat). This is necessary because only a small amount of concentrated medium is used to replenish the growth factors.

4.0 NeuroCult™ NCFC Assay Procedure

Note: The number of dissections that will need to be performed depends on a number of variables, including the region of interest, the skill of the individual performing the dissection and the number of cells needed for the experiment.

4.1 Culture Set-Up

4.1.1 Preparation of Embryonic Rat Brain-Derived Cells

1. Dissect central nervous system (CNS) tissue from embryonic rats according to the procedure described in the Technical Manual: In Vitro Proliferation and Differentiation of Rat Neural Stem and Progenitor Cells (Neurospheres) Using NeuroCult™ (Document #28725) available on our website at www.stemcell.com or contact us to request a copy.
2. Filter the single-cell suspension of neural cells through a 40 µm Cell Strainer to remove undissociated cells or clumps of cells.
3. Count viable cells using Trypan Blue on a hemacytometer.
4. Dilute primary embryonic brain-derived cells to a concentration of 6.5 x 10^5 cells/mL in Complete NeuroCult™ Proliferation Medium (Rat).

   Note: This will result in the plating of ~7500 cells per 35 mm culture dish and should obtain an appropriate number of colonies for counting. It may be necessary to do a cell titration to determine the optimal plating density for your specific cell type.

4.1.2 Preparation of Neurosphere-Derived Cells

For information on culturing rat neurospheres, refer to the Technical Manual: In Vitro Proliferation and Differentiation of Rat Neural Stem and Progenitor Cells (Neurospheres) Using NeuroCult™ (Document #28725) available on our website at www.stemcell.com or contact us to request a copy.

1. Collect cultured neurospheres and dissociate into a single-cell suspension.
2. Filter the single-cell suspension of neural cells through a 40 µm Cell Strainer to remove undissociated cells or clumps of cells.
3. Count viable cells using Trypan Blue on a hemacytometer.
4. Dilute neurosphere-derived cells to a concentration of 2.2 x10^5 cells/mL in Complete NeuroCult™ Proliferation Medium (Rat).

   Note: This will result in the plating of ~2500 cells per 35 mm culture dish and should obtain an appropriate number of colonies for counting. It may be necessary to do a cell titration to determine the optimal plating density for your specific cell type.

4.2 Assay Set-Up

1. Thaw NeuroCult™ NCFC Serum-Free Medium without Cytokines and NeuroCult™ Proliferation Supplement overnight at 2 - 8°C or for 1 - 2 hours at 37°C.
2. Place thawed medium and supplement at 37°C and place the Collagen Solution on ice.
3. To prepare the semi-solid collagen NCFC Cloning Medium, add the following components **in the given order**. Choose the appropriate volumes to add based on the desired number of replicates. **Do not add Collagen Solution until Step 4**.

Note: If multiple tubes are being set up, add cells to a single tube, then add the Collagen Solution and plate the cells immediately (before adding the cells to subsequent tubes). Do not let the cells sit in the medium for an extended period of time before plating.

CATALOG #	DESCRIPTION	2 REPLICATES	3 REPLICATES	6 REPLICATES
05720	NeuroCult™ NCFC Serum-Free Medium without Cytokines	1.7 mL	2.8 mL	5.7 mL
05773	NeuroCult™ Proliferation Supplement (Rat)	330 µL	0.550 mL	1.1 mL
02633	rh EGF (10 µg/mL)	6.6 µL	11 µL	22 µL
02634	rh bFGF (10 µg/mL)	3.3 µL	5.5 µL	11 µL
07980	0.2% Heparin Sodium Salt in PBS	3.3 µL	5.5 µL	11 µL
	Rat cells at: 6.5×10^5 primary cells/mL OR 2.2×10^5 cultured cells/mL	25 µL	42 µL	83 µL
04902	Collagen Solution **(ADD LAST – refer to step 4)**	1.3 mL	2.2 mL	4.3 mL

4. Mix the medium containing cells by pipetting. Using a pipette, transfer the appropriate amount of cold Collagen Solution to the tube and mix again by pipetting. Remove 1.5 mL of this mixture and dispense into a 35 mm Culture Dish, repeating as necessary until the desired number of replicates are plated. Remove any air bubbles by gently touching bubble with the end of the pipette.
5. Gently tip each culture dish using a circular motion to allow the mixture to spread evenly over the surface of the dish.
6. Place 35 mm Culture Dishes into a 100 mm Treated Tissue Culture Dish. This dish must also contain an open 35 mm Culture Dish filled with 3 mL sterile water to maintain optimal humidity during the incubation period. Replace the lid of the 100 mm dish. Dishes can also be placed in a covered 245 mm x 245 mm Square Treated Tissue Culture Dish with 2 or 3 open 35 mm Culture Dishes containing sterile water.
7. Transfer the plates to a 37°C incubator at 5% CO_2 and > 95% humidity.

   Note: Gel formation will occur within approximately 1 hour. It is important not to disturb the cultures during this time.
8. Incubate cultures for 21 days (differences in colony size can be clearly discerned after 21 days).
9. As cultures are incubated for an extended period of time, cultures should be replenished with Complete NeuroCult™ Replenishment Medium (Rat). Gently (so as not to disrupt the gel) add 60 µL of Complete NeuroCult™ Replenishment Medium (Rat) into the center of each NCFC Assay dish once every 7 days during the entire NCFC Assay culture incubation (21 days).
10. Cultures should be visually assessed regularly for overall colony growth and morphology using an inverted microscope.

    Note: Do not leave cultures at room temperature for extended periods of time as the collagen gel will begin to liquefy and the colonies will not stay intact.

5.0 Counting NeuroCult™ NCFC Assay Colonies

Within 4 - 7 days of plating, neural stem and progenitor cells begin to proliferate and form small colonies. By day 14, these small colonies will have grown enough such that small differences in size between colonies can be discerned. A number of the colonies appear to stop growing after 10 - 14 days, while other colonies continue to expand. By day 21, colonies can be classified into one of the four categories:
1) less than 0.5 mm diameter, 2) 0.5 - 1 mm diameter, 3) 1 - 2 mm diameter and 4) ≥ 2mm in diameter.

1. Place an individual 35 mm Culture Dish on a 60 mm Gridded Scoring Dish. Place both the culture dish and gridded dish on the microscope stage.
2. Scan the entire dish using a low power (2.5X - 5X) objective lens, noting the relative proximity of the colonies to each other. Counting can then be performed with the same lens. Use a higher power (10X) objective to examine colonies in greater detail.
3. Classify colonies into one of the four categories:
    - Less than 0.5 mm diameter
    - 0.5 - 1 mm diameter
    - 1 - 2 mm diameter
    - ≥ 2 mm diameter
4. Quantification of neural stem and progenitor cells:
    - The original cell that forms a colony ≥ 2 mm diameter is referred to as a Neural Colony Forming Cell - Neural Stem Cell (NCFC-NSC) as this cell has high proliferative potential and multi-lineage potential.
    - Colonies < 2 mm contain cells which lack self-renewal ability and multi-potency and are likely produced by a progenitor.

6.0 Appendix 1 - Harvesting Colonies from Collagen Gel for Functional Characterization (Optional)

6.1 Procedure Overview

If desired, cells can be isolated from colonies of interest for further study (refer to Figure 5 for a procedure overview). This step is not necessary if the assay is only being used to quantify neural stem and progenitor cells.

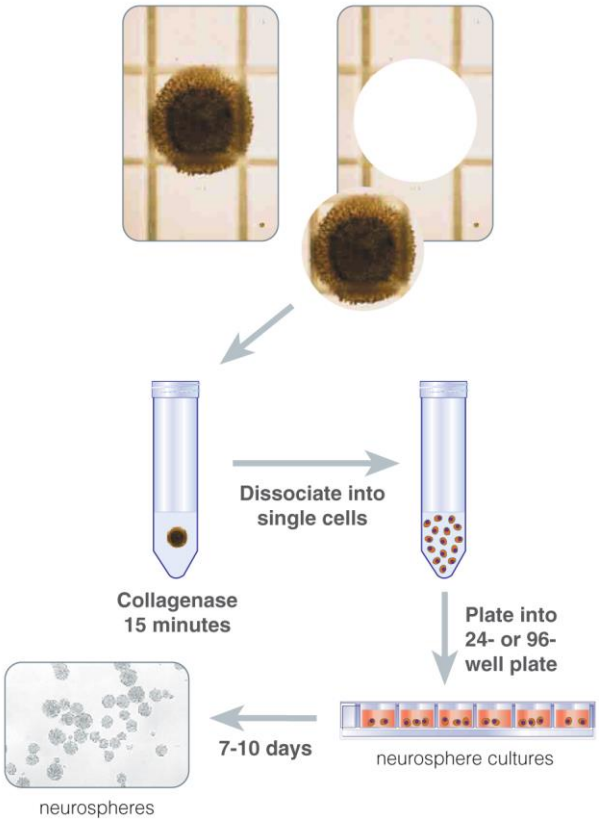

Figure 5. Overview of the Procedure for Harvesting Neural Colonies from the NeuroCult™ NCFC Assay Collagen Gel

6.2 Method

1. Sterilize a pair of extra fine spring microscissors (e.g. Fine Science Tools, Catalog #15396-01) in a bead sterilizer or using 70% ethanol.
2. Mark the colony that is to be isolated with a felt tip pen on the bottom of the dish.
3. Aliquot 100 µL of NeuroCult™ Collagenase Solution into individual sterile 1.5 mL Eppendorf tubes (one tube for each colony being isolated).

4. Looking through the dissecting microscope, cut individual colonies from the collagen matrix with the sterilized microscissors.

   Note: This can be difficult. We use the microdissection scissors to cut all around the colony until the colony is separated from the collagen (there will be a cut ridge). Sometimes it may be easier to stick the pointed tip of the scissors in the centre of the colony and scoop it up.

5. Transfer the individual colonies into the Eppendorf tubes containing the NeuroCult™ Collagenase Solution using the microscissors.
6. Incubate for 10 minutes at 37°C (do not exceed 15 minutes).
7. Mechanically disrupt the excised colony using a 200 µL micropipette to break up the matrix and produce a single-cell suspension.
8. Centrifuge the cells in the Eppendorf tubes at 150 x *g* for 5 minutes in a microcentrifuge.
9. Remove the supernatant and add 1 mL Complete NeuroCult™ Proliferation Medium (Rat; section 3.4).
10. Resuspend the cells by gently triturating with a 200 µL micropipette.
11. Centrifuge the cells at 150 x *g* for 5 minutes in a microcentrifuge. Remove the supernatant and resuspend in 100 µL Complete NeuroCult™ Proliferation Medium (Rat).
12. Aliquot an appropriate volume of Complete NeuroCult™ Proliferation Medium (Rat) into each well of a 24- or 96-well culture dish (e.g. 24-well, Corning Catalog #3527; 96-well, Corning Catalog #3596).

    Note: Generally, cells isolated from colonies ≥ 2 mm in diameter should be plated into a 24-well plate, cells isolated from colonies < 2 mm in diameter should be plated into a 96-well plate.

13. Plate all of the cells isolated from a single colony into an individual well of a 24- or 96-well culture dish containing Complete NeuroCult™ Proliferation Medium (Rat).
14. Incubate at 37°C in 5% CO_2 with > 95% humidity.
15. Collect neurospheres from wells 7 - 14 days later and passage as per standard neurosphere passaging procedures. For complete instructions, refer to the Technical Manual: In Vitro Proliferation and Differentiation of Rat Neural Stem and Progenitor Cells (Neurospheres) Using NeuroCult™ (Document #28725) available on our website at www.stemcell.com or contact us to request a copy. This subculture procedure should be repeated every 7 - 14 days, passaging cells in appropriate-sized culture vessels depending on numbers of cells generated (e.g. 6-well tissue culture dishes, Corning Catalog #3506; T-25 cm^2 flasks, Nunc Catalog #156367).

7.0 Appendix 2 - Differentiation of Cells Isolated from NeuroCult™ NCFC Assay Colonies (Optional)

7.1 Method

Cells can be isolated from NeuroCult™ NCFC Assay colonies to determine if they have the ability to form neurons, astrocytes and oligodendrocytes. This procedure is optional and may be performed at the researchers' discretion.

Cells should be harvested from the NeuroCult™ NCFC Assay colonies as described in section 7.0 and cultured as neurospheres until a sufficient number of cells have been obtained (it may be necessary to passage the cells isolated from the colonies 1 - 3 times as neurospheres). It is important to have sufficient cells from the colonies prior to differentiation. If cell numbers are limiting, the differentiation may not work well. These clonally-derived cells can then be differentiated using a serum-containing medium such as Complete NeuroCult™ Differentiation Medium (NeuroCult™ Basal Medium [Rat; Catalog #05770] plus NeuroCult™ Differentiation Supplement [Rat; Catalog #05774]).

Neural stem and progenitor cells may be differentiated as whole intact neurospheres or as dissociated cells. For complete instructions on the differentiation of neural cells into neurons, astrocytes and oligodendrocytes, refer to the Technical Manual: In Vitro Proliferation and Differentiation of Rat Neural Stem and Progenitor Cells (Neurospheres) Using NeuroCult™ (Document #28725) available on our website at www.stemcell.com or contact us to request a copy.

8.0 References

1. Reynolds BA & Rietze RL. (2005) Neural stem cells and neurospheres--re-evaluating the relationship. Nat Methods 2(5): 333–6.
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TOLL FREE PHONE 1 800 667 0322 • PHONE +1 604 877 0713
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