Chapter 5

In Vitro Culture and Analysis of Adult Hippocampal Neural Progenitors

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Abstract

Adult hippocampal neural progenitor cell (AHNPC) culture is a useful technique for gaining insight into adult neurogenesis, studying disease, and high throughput drug screening. The ability of AHNPCs to proliferate and differentiate into the three cell lineages of the adult brain in cell culture provides the researcher a powerful platform to study the extracellular and intracellular regulatory mechanisms in a well-controlled environment. In this chapter, we describe some of the *in vitro* techniques necessary to study hippocampal progenitors in the adult rat. This chapter details routine culture techniques for passaging and differentiating hippocampal progenitors. We also describe techniques for analyzing the culture state, such as proliferation and expression of cell fate markers by quantitative RT-PCR and immunofluorescence.

Key words: Neural, Hippocampus, Progenitors, Rat, *In vitro*, Adult, Quantitative RT-PCR, Immunostaining, Differentiation, Proliferation, Neurons, Astrocytes

1. Introduction

The ability to culture and analyze hippocampal neural progenitor cells *in vitro* is crucial to researching their function and behavior in the adult brain. *In vitro*, adult hippocampal neural progenitor cells (AHNPCs) can undergo self-renewal as well as generate nearly all major cell types in the mammalian brain, including neurons, astrocytes, and oligodendrocytes (1). Therefore, culturing these cells and analyzing them in a culture dish provides a facile, initial means of testing hypotheses before conducting *in vivo* experiments that are often resource-intensive and time-consuming.

Here, we describe the protocols necessary for culture, differentiation, and analysis of AHNPCs *in vitro* (see Chapter 4

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for isolation of primary culture from the adult rat). Once derived, these cells have stable karyotype for up to 35 population doublings (2) and can readily be cryo-preserved for long-term storage. We also describe typical techniques for the *in vitro* analysis of cell behavior. These include proliferation and expression of cell fate markers as analyzed by quantitative RT-PCR and immunofluorescence.

We have used these techniques to investigate the inter- and intra-cellular signals responsible for AHNPC proliferation, self-renewal, and differentiation (3, 4). When designing an experimental plan involving these assays, it is especially important to be familiar with the strengths and weaknesses of each technique. Often, more than one of these techniques is required to convincingly demonstrate the existence of a phenomenon. A proliferation assay is not a measure of multipotency. Rather, it merely provides a measure of cell number and therefore growth rate. Other assays, such as quantitative RT-PCR and/or immunofluorescence for a multipotent maker, must accompany proliferation results to demonstrate multipotency. Additionally, quantitative RT-PCR measures a population-averaged concentration of mRNA in an entire culture. If cell-to-cell variability within a culture is important, then we recommend analyzing the culture via immunostaining to gain single cell data. Another key difference between quantitative RT-PCR and immunofluorescence is that the former measures mRNA concentration while the latter measures protein concentration. The two concentrations may not necessarily follow the same trend.

Many of the culture techniques outlined here are necessary for the completion of protocols detailed in other chapters describing the isolation (Chapter 4), *in vivo* engraftment and analysis (Chapter 6), and viral transduction (Chapter 7) of AHNPCs. Therefore, we recommend that the reader become comfortable with the routine culture described here before proceeding to the other chapters.

2. Materials

2.1. Culture, Cryo-Preservation, and Differentiation of Neural Progenitors

2.1.1. Laminin-Coated Culture Plates

2.1.1.1. Reagents

1. Sterile tissue culture water.
2. Sterile phosphate buffered saline (PBS), pH 7.4 (Invitrogen, Bethesda, MD).
3. Poly-L-ornithine hydrobromide (Sigma, St. Louis, MO) dissolved in tissue culture water at 10 mg/ml. Sterilize by 0.22 µm syringe filtration, aliquot, and store at –20°C. Avoid repeated freeze/thaw.

4. Natural mouse laminin (Invitrogen). Store at –80°C. Thaw slowly at 4°C before use. Avoid repeated freeze/thaw and vortexing.
5. Plastic wrap.

2.1.1.2. Preparation of Plates

6. Dilute poly-L-ornithine in tissue culture water to a final concentration of 10 µg/ml and add to tissue culture plates such that plates are well covered (~7 ml for 10 cm plate). Incubate overnight at room temperature. To maintain sterility, leave the plates in a biological safety cabinet with blower off and sash closed. Leave UV light OFF.
7. Rinse plates twice with approximately the same amount of sterile water as used for poly-L-ornithine incubation. Do not allow plates to dry out.
8. Dilute laminin in sterile PBS to a final concentration of 5 µg/ml and add to plates at the same volume as was used for poly-L-ornithine.
9. Incubate at 37°C overnight.
10. Wrap in plastic wrap and store at –20°C for up to 6 months. Freeze plates on a level surface to avoid pooling and dry spots.

2.1.2. Cell Culture, Proliferation, and Differentiation Reagents

1. Dulbecco's Modified Eagle Medium/Nutrient Mix F-12 (DMEM/F-12) with HEPES and L-Glutamine (Invitrogen) supplemented with 1% (v/v) N-2 Supplement (Invitrogen). Store supplemented medium at 4°C for up to 1 month.
2. Accutase (Innovative Cell Technologies, San Diego, CA).
3. Basic fibroblast growth factor (FGF-2, Peprotech, Rocky Hill, NJ). FGF-2 stock solution: dissolve in DMEM/F-12 + N-2 at 0.1 mg/ml, aliquot, and store at –20°C. Once thawed, aliquot can be stored at 4°C for up to 1 week.
4. Poly-L-ornithine/laminin coated culture plates (see Subheading 2.1.1).
5. Sterile cotton-plugged glass Pasteur pipettes.
6. 2× freezing medium: 20% (v/v) DMSO dissolved in DMEM/F-12 + N-2. Use immediately.
7. Fetal bovine serum (FBS).
8. Retinoic acid (RA), all-trans (Biomol, Plymouth Meeting, PA). Reconstitute to 1 mM in DMSO. Aliquot and store at –80°C protected from light. Avoid freeze/thaw. Reconstituted RA is stable for up to 3 months at –80°C. Use care when handling and disposing, as RA is a potent toxin and teratogen.
9. Forskolin (Biomol). Reconstitute to 5 mM in DMSO. Aliquot and store at –20°C protected from light. Avoid freeze/thaw. Reconstituted forskolin is stable for up to 2 months at –20°C.

10. Mixed population differentiation medium: 1 µM RA (1 µl RA stock per ml) + 1% (v/v) FBS (10 µl/ml) in DMEM/F-12 + N-2. Use immediately.
11. Neuronal differentiation medium: 1 µM RA (1 µl RA stock per ml) + 5 µM forskolin (1 µl forskolin stock per ml) in DMEM/F-12 + N-2. Use immediately.
12. Optional: Hemacytometer.

2.2. Analyzing Culture State

2.2.1. Proliferation Assay

1. All reagents in Subheading 2.1.2 items 1–5; however, DMEM/F-12 + N-2 should be without phenol red to avoid interference with the colorimetric WST-1 Reagent.
2. Tissue culture plate (96-well) coated with poly-L-ornithine/laminin (see Subheading 2.1.1).
3. Hemacytometer.
4. Cell Proliferation Reagent WST-1 (Roche, Indianapolis, IN). Upon receipt, thoroughly thaw at 37°C and swirl/pipette to ensure all aggregates dissolve. Then aliquot and store at –20°C. Protect from light. Aliquots can be freeze/thawed if necessary. Before use, warm again to 37°C to dissolve aggregates.
5. Multichannel pipettes appropriate for 10–100 µl volumes.
6. Microplate reader (96-well).

2.2.2. Immunostaining

1. Poly-L-ornithine/laminin-coated (see Subheading 2.1.1) CultureSlides (BD Falcon, Bedford, MA). We recommend 8-well CultureSlides, and the volumes used here are for the 8-well format; however, other formats may be used with properly adjusted volumes.
2. PBS, pH 7.4 (Invitrogen).
3. 10× PBS, pH 7.4 (Invitrogen).
4. 4% (w/v) paraformaldehyde. While stirring, carefully heat 400 ml water on a hot plate in a fume hood to 60°C. Do not heat above 60°C, because aldehyde will form and cause autofluorescence. While maintaining temperature, add 20 g paraformaldehyde and stir. As necessary, add NaOH pellets one at a time until paraformaldehyde dissolves. Generally, 1–2 NaOH pellets over the course of 10–15 min will fully dissolve the paraformaldehyde. There will still be small particulates in suspension. Cool to room temperature. Add 50 ml 10× PBS and pH to 7.4 with HCl. Bring total volume to 500 ml with water and sterile filter to remove particulates. Paraformaldehyde must be disposed as chemical waste.
5. Triton X-100 (EMD Biosciences, San Diego, CA).
6. Donkey serum (Sigma), or other appropriate normal serum for blocking (see Note 1). Upon receipt, thaw at

Table 1
Recommended primary antibodies, dilutions, and storage conditions for immunostaining

Name	Species	Supplier	Dilution	Storage
β-Tubulin III	Mouse	Sigma	1:250	Aliquot –20°C
MAP2a + 2b	Mouse	Sigma	1:250	Aliquot –20°C
GFAP	Rabbit	Abcam (Cambridge, MA)	1:500	4°C
O4 (see Note 16)	Mouse	Millipore (Billerica, MA)	1:250	4°C
Nestin	Mouse	BD Pharmingen (San Jose, CA)	1:1,000	4°C

room temperature, aliquot, and store at –20°C. Avoid freeze/thaw.

7. Primary antibodies. See Table 1 for recommended antibodies, dilutions, and storage conditions.
8. Cy3 and Cy5 conjugated donkey anti-rabbit, and antibodies (Jackson ImmunoResearch, West Grove, PA). Upon receipt, reconstitute in 1:1 glycerol:water according to package insert (volume varies by lot), aliquot, and store at –20°C protected from light. Avoid freeze/thaw.
9. TO-PRO®-3 nuclear stain (Invitrogen). Upon receipt, aliquot and store at –20°C. Protect from light and avoid freeze/thaw.
10. Alexa Fluor® 488 conjugated donkey anti-mouse and anti-rabbit antibodies (Invitrogen). Upon receipt, aliquot and store at –20°C protected from light. Avoid freeze/thaw.
11. Microscope cover glasses (24 × 50 mm, Fisher, Pittsburgh, PA).
12. Clear nail varnish.
13. ProLong® Antifade Kit (Invitrogen).

2.2.3. Quantitative RT-PCR

2.2.3.1. RNA Isolation

1. RNase Away (Molecular BioProducts, San Diego, CA).
2. RNase-free, presterilized pipette tips (e.g., ART® sterile, self-sealing, barrier pipette tips, Sigma).
3. RNase-free water (Invitrogen).
4. RNase-free micro-centrifuge tubes (Fisher).
5. TRIzol® Reagent (Invitrogen). Causes burns. Always use gloves when handling, and dispose of residual with chemical waste.
6. Cell Lifter (Corning, Corning, NY).
7. Chloroform (ACS Grade).
8. DNase I, RNase-free, 10 U/μl (Roche, Indianapolis, IN).

9. Glycogen for molecular biology, 20 mg/ml (Roche).
10. Isopropanol (ACS Grade).
11. 75% ethanol, prepared with RNase-free water.

2.2.3.2. Reverse Transcription

12. ThermoScript™ RT-PCR System (Invitrogen) containing:
    ThermoScript™ reverse transcriptase
    5× cDNA synthesis buffer
    0.1 M dithiothreitol (DTT)
    10 mM dNTP mix
    RNase OUT™
    Random hexamers primer
    DEPC-treated water
    RNase H
13. RNase Away (Molecular Biology Products).
14. RNase-free, presterilized pipette tips (e.g., ART® sterile, self-sealing, barrier pipette tips, Sigma).

2.2.3.3. QPCR

15. Distilled/de-ionized water (Invitrogen).
16. Antibody/Taq polymerase. Mix Taq DNA polymerase (Invitrogen) and Anti-Taq JumpStart™ Antibody (Sigma) in a 1:1 ratio. Incubate at room temperature for 10 min, aliquot, and store at –20°C.
17. Taq DNA polymerase 10× PCR Buffer (Invitrogen). Aliquot and store at –20°C.
18. 25 mM $MgCl_2$ (Thermo Scientific, Waltham, MA). Aliquot and store at –20°C.
19. 10 mM dNTP mix. Formulated from 100 mM dNTP Set (Invitrogen). Mix all four dNTPs and dilute with distilled/de-ionized water such that the final concentration of each dNTP is 10 mM. Store at –20°C.
20. 2× PCR supermix. For 1 mL, mix 200 µl 10× PCR Buffer, 240 µl 25 mM $MgCl_2$, 40 µl 10 mM dNTP mix, and 520 µl distilled/de-ionized water. Can be kept at –20°C for long-term storage.
21. Real-time PCR detection system (e.g., iQ5, Bio-Rad Laboratories, Hercules, CA).
22. Thermowell® 96-well PCR Plate (Corning).
23. Microseal B Adhesive Seals (Bio-Rad).
24. Primer and probe sets specific for the gene(s) of interest (Biosearch Technologies, Novato, CA). All primer and probe stocks should be diluted to 10 µM in distilled/de-ionized water. Table 2 lists primer and probe sets for common differentiation markers.

Table 2
Primers, probes, and amplicons for quantitative PCR

Name	Sequence	T_M (°C)
β-Tubulin III		
Sense primer	5′-GCATGGATGAGATGGAGTTCACC-3′	65.2
Antisense primer	5′-CGACTCCTCGTCGTCATCTTCATAC-3′	65.4
Probe	5′-FAM490-TGAACGACCTGGTGTCTGAG-BHQ-3′	59.9
Amplicon	GCATGGATGAGATGGAGTTCACCGAGGCCGAGAGCAACATG AACGACCTGGTGTCTGAGTACCAGCAGTACCAGGACGCC ACTGCCGAGGAGGAGGGGGAGATGTATGAAGATGACGAC GAGGAGTCG	
GFAP		
Sense primer	5′-GACCTGCGACCTTGAGTCCT-3′	61.8
Antisense primer	5′-TCTCCTCCTTGAGGCTTTGG-3′	61.8
Probe	5′-FAM490-TCCTTGGAGAGGCAAATGCGC-BHQ-3′	68.9
Amplicon	GACCTGCGACCTTGAGTCCTTGCGCGGCACGAACGAGTCC TTGGAGAGGCAAATGCGCGAACAGGAGGAGCGCCACGCT CGGGAGTCGGCCAGTTACCAGGAGGCACTCGCTCGGCTG GAGGAGGAGGGCCAAAGCCTCAAGGAGGAGA	
MBP		
Sense primer	5′-ACTTGGCCACAGCAAGTACC-3′	61.8
Antisense primer	5′-GTGTGAGTCCTTGCCAGAGC-3′	62.0
Probe	5′-FAM490-TTGACTCCATCGGGCGCTTCT-BHQ-3′	68.3
Amplicon	ACTTGGCCACAGCAAGTACCATGGACCATGCCCGGCATGGC TTCCTCCCAAGGCACAGAGACACGGGCATCCTTGACTCC ATCGGGCGCTTCTTTAGCGGTGACAGGGGTGCGCCCAAG CGGGGCTCTGGCAAGGACTCACAC	
Nestin		
Sense primer	5′-GAGCTCTCTGGGCAAGTGGA-3′	63.0
Antisense primer	5′-CTCCCACCGCTGTTGATTTC-3′	62.9
Probe	5′-FAM490-AGGACAGTCAGCAGTGCCTGCA-BHQ-3′	67.8
Amplicon	GAGCTCTCTGGGCAAGTGGAACGTAGAGACCAGGGTAGA GGACAGTCAGCAGTGCCTGCAAGTAGAAGAGGGTCT GCAGGAGGAACAGCACCAAGAGTCTCTGAGAGAGGTG AAGCAGGAGCTGCCTAGCTCTGGAAATCAACAGCGGT GGGAG	
18S		
Sense primer	5′-GTAACCCGTTGAACCCCATTC-3′	62.6
Antisense primer	5′-CCATCCAATCGGTAGTAGCGA-3′	62.2

(continued)

Table 2 (continued)

Name	Sequence	T_M (°C)
Probe	5′-CAL610-AAGTGCGGGTCATAAGCTTGCG-BHQ-3′	67.6
Amplicon	GTAACCCGTTGAACCCCATTCGTGATGGGGATCGGGGATT GCAATTATTCCCCATGAACGAGGAATTCCCAGTAAGTGC GGGTCATAAGCTTGCGTTGATTAAGTCCCTGCCCTTTG TACACACCGCCCGTCGCTACTACCGATTGGATGG	

β-Tubulin III: neuronal marker; GFAP (glial fibrillary acidic protein): astrocytic marker; MBP (myelin basic protein): oligodendrocytic marker; Nestin: multipotent marker; 18S (18S ribosomal subunit): internal control; FAM490: FAM490 fluorophore; CAL610: CAL610 fluorophore; BHQ: Black Hole Quencher®; T_M: Melt temperature

25. PCR standard. Amplify the fragment of interest from recently prepared cDNA using the primers shown in Table 2. PCR clone this fragment into a plasmid. To do this, we recommend using either the TOPO-TA Cloning® Kit (Invitrogen) or the StrataClone™ PCR Cloning Kit (Stratagene, La Jolla, CA). Miniprep using a kit such as the Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI), perform sequence analysis to confirm that the plasmid contains the correct amplicon (see Table 2), and measure A_{260} to quantify DNA concentration. Dilute to 1 ng/μl working stock and aliquot. Store at –20°C.

3. Methods

3.1. Culture, Cryo-Preservation, and Differentiation of Neural Progenitors

Adult hippocampal neural progenitors can be cultured *in vitro* for multiple passages and later cryopreserved for long-term storage. Morphologically, multipotent progenitors are very spherical, approximately 10–15 μm in diameter, phase-bright with a high nucleus to cytoplasmic volume ratio, and have only one or two short projections when viewed under a phase-contrast microscope at 40–100× (Fig. 1a). A culture is ready for passage when it is approximately 80% confluent (Fig. 1b), and cultures should be seeded at approximately 25,000 cell/cm². Therefore, the ideal passage protocol is a 1:4 subculture followed by 2 days of growth. Do not allow a culture to overgrow, as the cells will begin to differentiate and detach from the plate (unpublished observation).

Nearly any size culture dish is suitable for cell growth, provided that the surface is coated with poly-L-ornithine/laminin to allow cell attachment. We have successfully cultured cells in all formats ranging from 96-well plate to 10 cm culture dish,

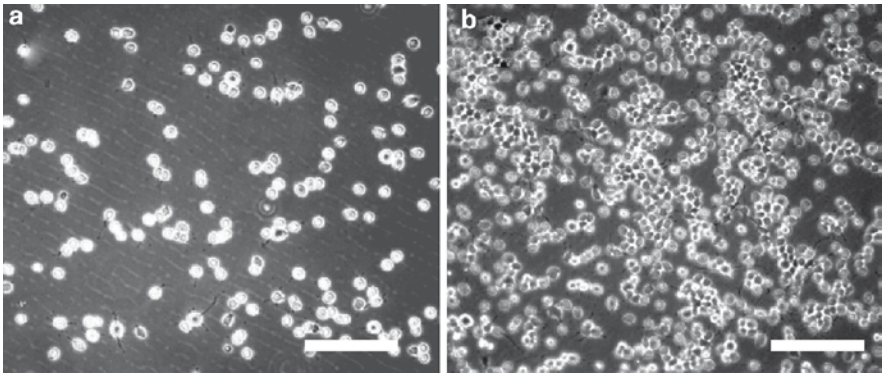

Fig. 1. Bright field images of adult neural hippocampal progenitors cultured in DMEM/F-12 + N-2 supplemented with 20 ng/ml FGF-2. (**a**) Approximately 1 h after plating on poly-L-ornithine/laminin coated dish. (**b**) Cells are ~80% confluent after 48 h in culture. Scale bars: 100 µm

including CultureSlides. However, we recommend using 3.5, 6, or 10 cm culture dishes for serial propagation of cells.

Once progenitors are isolated, avoid serum or antibiotics in culture to prevent differentiation and slow growth, respectively. Good aseptic technique is critical for the growth of AHNPCs in the absence of antibiotics. As with all cell culture, all open steps should be performed in a HEPA filtered biological safety cabinet by a person trained in good aseptic technique.

Progenitors are easily cryopreserved and generally recover quickly when thawed. The cells are best preserved in DMEM/F-12 + N-2 with 10% DMSO. As with most cell types, it is important to manipulate the cells gently when they are exposed to DMSO. Also, it is optimal to minimize the time that cells are exposed to DMSO in the liquid state, as DMSO will cause cells to differentiate (unpublished observation). Do not rush when freezing/thawing the cells, but do prepare all reagents and supplies (media warmed, vials labeled, etc.) before beginning those procedures.

3.1.1. Cell Culture

1. Examine the culture under a microscope to determine whether it is ready for passaging. The plate should be approximately 80% confluent (see Fig. 1b).
2. Warm DMEM/F-12 + N-2 to 37°C and thaw poly-L-ornithine/laminin coated plates. Plates can be thawed at either room temperature or 37°C, but avoid the formation of dry spots on the plate, as these will subsequently not effectively support cell attachment.
3. Aspirate all but ~0.5 ml of medium from the culture plate to prevent the plate from completely drying.
4. Add approximately 1 ml of cold Accutase per 75 cm^2 culture surface area. Never warm the Accutase stock and return it to 4°C immediately after use.

5. Incubate at 37°C for approximately 2 min to detach cells. Gently rock and shake the plate to fully dislodge progenitors. Do not bang the plate on a hard surface. Confirm under the microscope that all cells have detached (see Note 2).
6. Dilute the Accutase cell suspension in 5 ml of warm DMEM/F-12 + N-2. Gently wash the plate with this suspension 2–3 times to break up large cell clumps, then pipette into a 15 ml centrifuge tube.
7. Pellet cells at 200 g for 2 min (see Note 3).
8. Flame-polish a cotton-plugged Pasteur pipette (preferably inside a biological safety cabinet to maintain sterility of the full pipette). Gently rotate the tip of the pipette in the edge of a Bunsen burner flame until the flame turns yellow. Continue rotating the pipette and hold the tip in the flame for approximately 3 s. Rotating the pipette is crucial for achieving an even polish. After polishing, examine the tip. It should be smooth and evenly circular, and the diameter of the bore should be approximately half of its original size. Keep the polished pipette sterile by storing upright (in a tube rack, for example) in the biological safety cabinet.
9. Aspirate supernatant from the cell pellet, and add 2 ml DMEM/F12 + N-2. Other volumes may be used, but we recommend 2 ml for most applications.
10. Using the flame polished pipette, triturate the cell pellet by drawing nearly the whole 2 ml into the pipette and expelling it against the bottom of the centrifuge tube. Repeat 3–4 times. The pellet should be completely dissociated, and the solution should be turbid. Use care to prevent blowing bubbles when expelling the solution. Likewise, be careful to avoid drawing air into the pipette when aspirating (air–liquid interfaces promote cell lysis).
11. Optional: The number of cells in this suspension can now be quantified by hemacytometer counting. This is not necessary for routine cell maintenance; however, precise knowledge of cell concentration is required for experiments where inoculation density is critical.
12. Aspirate the PBS from the freshly thawed poly-L-ornithine/laminin coated dish, and immediately add warm DMEM/F-12 + N-2. Do not allow the plate to dry (see Note 4). Medium volume will depend on plate size and inoculum volume. Recommended culture volumes: 10 cm dish, 8 ml; 6 cm dish, 4 ml; 3.5 cm dish, 2 ml; 6-well plate, 2 ml; 12-well plate, 1 ml; 24-well plate, 0.5 ml; 96-well plate, 100 µl; 8-well CultureSlide, 500 µl.
13. Pipette cell suspension up/down several times to mix, because the cells settle very quickly. Then, add the appropriate volume

to the culture dish. Immediately after adding the inoculum, rock the plate back and forth several times to mix. To ensure a homogeneous inoculation, it is critical to thoroughly mix the plate immediately after adding the inoculum because the cells will settle and attach very quickly. Do not swirl the plate, as this will force the cells to edge of the plate.

14. Thaw an FGF-2 aliquot at room temperature and supplement the culture at 20 ng/ml (0.2 µl of FGF-2 stock per ml of culture). Gently rock the plate back and forth to mix.
15. Incubate at 37°C in 5% CO_2 atmosphere. Most cells should be attached within 30 min.

3.1.2. Cryopreservation

1. Follow Subheading 3.1.1 steps 1–10.
2. Determine the number of vials to be frozen. We recommend freezing approximately 2×10^6 cells per 1 ml vial. Therefore, each 80% confluent 10 cm dish will generate 4 vials. However, lower or higher cell concentrations can be used if desired.
3. Appropriately label all cryovials before continuing.
4. Prepare 20% DMSO freezing solution – 0.5 ml per vial plus 1 ml contingency.
5. Slowly and with constant and gentle mixing/swirling, add an equal volume of freezing solution to the cell suspension. Pipette up/down gently to mix.
6. Aliquot into cryovials at 1 ml per vial. If aliquotting a large number of vials, be sure to keep the cell suspension well-mixed.
7. Immediately transfer to –80°C. It is important to minimize the time that cells are exposed to liquid DMSO.
8. Store at –80°C at least overnight before transferring to liquid nitrogen storage. Cells can be stored at –80°C for up to 3 months without appreciable loss of viability, but longer-term storage requires liquid nitrogen.

3.1.3. Cell Thawing

1. Warm DMEM/F-12 + N-2 to 37°C and thaw a poly-L-ornithine/laminin coated plate. A standard vial frozen with 2×10^6 cells will require a 6 cm culture dish, but the dish size can be adjusted based on the application and number of cells. Plates can be thawed at either room temperature or 37°C.
2. Remove the vial from cryopreservation and immediately thaw in 37°C water bath with gentle swirling. Thawing will take 2–3 min (see Note 5).
3. Gently pipette the entire contents of the vial to a 15 ml centrifuge tube, taking care to avoid bubbles.
4. Slowly and with gentle mixing, add 5 ml warm DMEM/F-12 + N-2 to the cell suspension. Gently pipette up/down several times to mix.

5. Pellet cells at 200 g for 2 min.
6. Flame-polish a cotton plugged Pasteur pipette as in Subheading 3.1.1 step 8.
7. Aspirate supernatant and add 2 ml DMEM/F-12 + N-2 to the cell pellet.
8. Using the flame polished pipette, gently triturate the cell pellet as in Subheading 3.1.1, step 10.
9. Aspirate PBS from the freshly thawed poly-L-ornithine/laminin coated plate and immediately add warm DMEM/F-12 + N-2. Medium volume will depend on plate size (see Subheading 3.1.1 step 12).
10. Add the entire 2 ml of cell suspension to the plate. Immediately after adding the inoculum, gently rock the plate back and forth several times to mix.
11. Supplement the culture with 20 ng/ml FGF-2 (0.2 µl FGF-2 stock per ml of culture) and again rock the plate to mix.
12. Incubate overnight at 37°C in 5% CO_2 atmosphere.
13. To remove residual DMSO, aspirate and replace the medium with fresh, warm DMEM/F-12 + N-2 the day after inoculation. Resupplement with 20 ng/ml FGF-2.
14. Incubate at 37°C in 5% CO_2 atmosphere and monitor daily to determine when to pass the culture, which is generally the next day. Cells are ready for experimentation after one passage.

3.1.4. Differentiation

1. Follow the protocol in Subheading 3.1.1 to dissociate, count, and seed cells at 20,000 cells/cm^2. If, after differentiation, the culture will be analyzed by immunostaining (Subheading 3.2.2), we recommend using 8-well CultureSlides. If, after differentiation, the culture will be analyzed by quantitative RT-PCR (Subheading 3.2.3), we recommend using 3.5 cm dishes or 6-well culture plates (see Note 6).
2. Supplement the culture with 20 ng/ml FGF-2 and incubate overnight at 37°C in 5% CO_2 atmosphere.
3. Day 1: Remove FGF-2 medium and replace with differentiation medium of your choice (see Note 7):

Mixed population:	1 µM RA + 1% (v / v) FBS in DMEM / F - 12 + N - 2
Neuronal:	1 µM RA + 5 µM forskolin in DMEM / F - 12 + N - 2

Incubate at 37°C in 5% CO_2 atmosphere for 2 days.

4. Day 3: Replace media.

5. Day 5: Cultures are now ready for analysis. Proceed immediately to immunostaining protocol (Subheading 3.2.2) or quantitative RT-PCR protocol (Subheading 3.2.3).

3.2. Analyzing Culture State

3.2.1. Proliferation Assay

Quantifying the ability of neural progenitors to proliferate when exposed to variety of culture conditions and genetic manipulations is crucial for developing an understanding of the mechanisms governing the expansion of these cells. It is important to note, however, that this assay is not a measure of multipotency and simply measures cell number. Further experimentation is required to assess the lineage commitment capabilities of the cells (see Subheadings 3.2.2 and 3.2.3).

Proliferation can readily be measured with Cell Proliferation Reagent WST-1. This is a tetrazolium salt cleaved by mitochondrial dehydrogenase activity, producing formazan dye whose absorbance is quantified by spectrophotometry. After culturing cells in a 96-well plate format for 5 days, WST-1 is directly added to the culture, incubated for 2–3 h, and quantified on a microplate reader. Cell number is determined by comparison to a standard curve, where the linear range of absorbance vs. cell number is 1,000–50,000 cells per well.

Before beginning any experimentation, it is extremely important to plan the layout of the 96-well plate. We discourage using the edge wells for culturing the cells because evaporation over the 5-day culture period will affect the culture volume, which in turn will affect the spectrophotometric reading at the end of the experiment. The edge wells should be completely filled (300 µl) with sterile PBS until the final day of the experiment when they can be used for the blank and standard curve. We also recommend culturing each experimental condition in quintuplicate (5 wells) to ensure sound statistics. However, quadruplicate is acceptable. To help organize the 96-well plate, create a spreadsheet with a map of the plate, print it out, and refer to it often.

3.2.1.1. Day 0: Inoculation

1. Thaw 96-well poly-L-ornithine/laminin coated plate. This can be done at room temperature or 37°C.
2. Prepare all media solutions at 2× working concentration. For instance, prepare DMEM/F-12 + N-2 with 40 ng/ml FGF-2 if the experiment calls for 20 ng/ml FGF-2.
3. If not using the edge wells, which we do not recommend using, fill them completely (~300 µl) with sterile PBS.
4. Aspirate PBS from the other wells and immediately add 50 µl of 2× media. Do not allow the wells to dry out (see Note 4); therefore, this is best done by aspirating the PBS and adding media one row at a time. Place plate in 37°C incubator to warm the media.

5. Prepare cells. Follow Subheading 3.1.1 steps 1–11. Determine the cell concentration of the cell suspension by hemacytometer. This solution is generally quite concentrated, so a 1:10 dilution in DMEM/F-12 + N-2 prior to counting is usually necessary. This 1:10 dilution is generally sufficient for use in step 6. Keep cell suspensions well-mixed throughout the counting and diluting process.
6. Dilute the 1:10 solution used to quantify cell concentration to 20,000 cells/ml in DMEM/F-12 + N-2.
7. Using a multichannel pipette, add 50 µl of 20,000 cell/ml suspension to each well. To ensure that cells are evenly plated from one row to the next, the cell suspension in the pipette basin should be well mixed between each row. This can be achieved by pipetting up/down with the multichannel pipette multiple times.
8. Follow Subheading 3.1.1 steps 12–15 to continue culturing the leftover cells. They will be used on day 5 to generate a standard curve.
9. Incubate at 37°C in 5% CO_2 atmosphere. After 30 min, visually confirm under the microscope that cells are evenly plated across all wells.

3.2.1.2. Day 1–4: Media Changes

10. Perform daily 50% media changes on all wells. Using a multichannel pipette as appropriate, remove 50 µl of media and replace it with 50 µl fresh 1× media.
11. Continue passaging the leftover cells in parallel to give an ~80% confluent 10 cm dish on day 5.

3.2.1.3. Day 5: Plate Standard Curve and Quantify Cell Number by WST-1

12. Dissociate, dilute, and count the leftover cells maintained in parallel by following Subheading 3.1.1 steps 1–11.
13. Prepare standard curve and plate 100 µl per well of each cell concentration. Quadruplicate is best, but triplicate is sufficient. The cell concentrations can be varied based on the application, but we recommend 40,000, 20,000, 10,000, 5,000, 2,500, 1,250, and 0 (DMEM/F-12 + N-2 only) cells per well. There is no need to wait for cells to attach.
14. Thaw WST-1 at 37°C for at least 5 min. Briefly vortex to ensure all aggregates dissolve.
15. Using a multichannel pipette, add 10 µl WST-1 to each well. Gently rock plate to mix.
16. Incubate at 37°C for 2 h.
17. Rock on shaking platform for approximately 1 min.
18. Measure samples against blank (media only) on microplate reader. Measure absorbance at 440 and 700 nm. Subtract A_{700} from A_{440}. This is the experimental readout.

19. Plates can then be returned to the incubator and reanalyzed up to 4 h after WST-1 addition. Use the standard curve to determine the best read. This will depend on the cell concentrations of the standard curve, but we have generally found that 2–3 h post-WST-1 addition provides the best results.
20. Use the standard curve to interpolate cell numbers and perform statistical analyses as necessary.

3.2.2. Immunostaining

Immunostaining allows the researcher to directly visualize proteins expressed in a cell by probing with primary antibodies directed against the proteins with the appropriate secondary antibodies conjugated to fluorophores. This technique is typically used to determine a cell's lineage by staining for markers of neuronal (e.g., β-tubulin III or microtubule associated protein (MAP) 2a+2b), astrocytic (e.g., glial fibrillary acidic protein (GFAP)), oligodendrocytic (e.g., O4), and multipotent (nestin) cells. The resultant staining can be imaged and quantified to determine the percentage of cells positive for lineage markers of interest.

We recommend using a confocal microscope for generating high-quality images; however, a conventional fluorescent microscope is adequate to qualitatively assess the monolayer culture. Be sure to use the proper secondary antibodies and nuclear stain for your microscope. Because of the high associated costs, many confocal microscopes do not have a UV laser line, and therefore are unsuitable for imaging fluorophores that excite in the UV. This includes the common nuclear stain DAPI. Similarly, without the proper light-gathering hardware, it is impossible to visualize fluorophores that emit in the infrared with most fluorescent microscopes.

We recommend using the following fluorophores for secondary antibody staining: Alexa Fluor® 488 (green), Cy3 (red), and Cy5 (infrared). If nuclear staining is necessary, we recommend TO-PRO®3, which emits in the infrared and therefore should not be used simultaneously with Cy5. Before beginning staining, design the staining protocol and ensure that all of the proper primary and secondary antibodies are in stock. If costaining, do not use multiple primary antibodies raised in the same species and do not use fluorophores with similar emission spectra.

1. Culture the experiment on CultureSlides according to Subheading 3.1.1 and/or Subheading 3.1.4, depending on the experiment. The 8-well CultureSlides allow for multiple experimental conditions without sacrificing culture and staining convenience. However, other size CultureSlides may be used. Regardless of size, the CultureSlides must be coated with poly-L-ornithine/laminin (Subheading 2.1.1).
2. Fix cells with 0.5 ml of 4% paraformaldehyde per well for no longer than 10 min at room temperature (see Note 8).

This and all subsequent incubations should be done with gentle shaking, such as with an orbital shaker. If not immunostaining immediately, the cells can be stored in PBS at 4°C. If staining for O4, see Note 9 before proceeding.

3. Rinse three times with 0.3 ml PBS, 5 min per rinse at room temperature.
4. Block/permeablize for 30 min at room temperature with 0.3 ml PBS containing 5% donkey serum (or the appropriate normal serum) and 0.3% Triton X-100 (PBS-DT). (see Note 1).
5. Incubate with primary antibody diluted in PBS-DT (0.25 ml) for 24–48 h at 4°C. See Table 5.1 for recommended antibody dilutions.
6. Rinse twice with 0.5 ml PBS for 10 min at room temperature.
7. Rinse once with 0.5 ml PBS for 30 min at room temperature.
8. Incubate with 1:250 secondary antibody + 1:1000 TO-PRO®3 (1 μM) in PBS-DT (0.25 ml) for 2 h at room temperature. While incubating, cover with aluminum foil to protect from light. Care should be taken in all subsequent steps to minimize light exposure and avoid photobleaching.
9. Repeat steps 6–7.
10. Slides are now ready to be mounted. Remove gasket from the CultureSlide and allow sample to dry for 15 min.
11. Follow instructions in ProLong® Antifade Kit to prepare antifade/mounting medium. Pipette a line of ~200 μl antifade/mounting medium lengthwise along the edge of the slide, and slowly lay a coverslip over the slide, starting at the edge with the antifade/mounting medium. Proceed slowly and with care to avoid bubbles. Antifade/mounting medium should evenly coat the slide.
12. Dry the slide at room temperature in the dark for at least 2 h (overnight drying is acceptable). Seal the slide with clear nail varnish. Apply the varnish carefully to avoid covering the samples. Dry at room temperature in the dark for approximately 30 min.
13. Slides are now ready to be imaged and can be stored in the dark at 4°C. Alexa Fluor® 488 excites at 495 nm and emits at 519 nm (green). Cy3 excites at 550 nm and emits at 570 nm (red). Cy5 excites at 650 nm and emits at 670 nm (infrared). TO-PRO®-3 nuclear stain excites at 642 nm and emits at 661 nm (infrared). See Fig. 2 for typical β-tubulin III, MAP 2a + 2b, GFAP, O4, and nestin staining images.

3.2.3. Quantitative RT-PCR

Quantitative reverse transcription (RT)-PCR is a useful tool for determining the state of a cell population by measuring the concentration of a specific mRNA transcript. It is important to

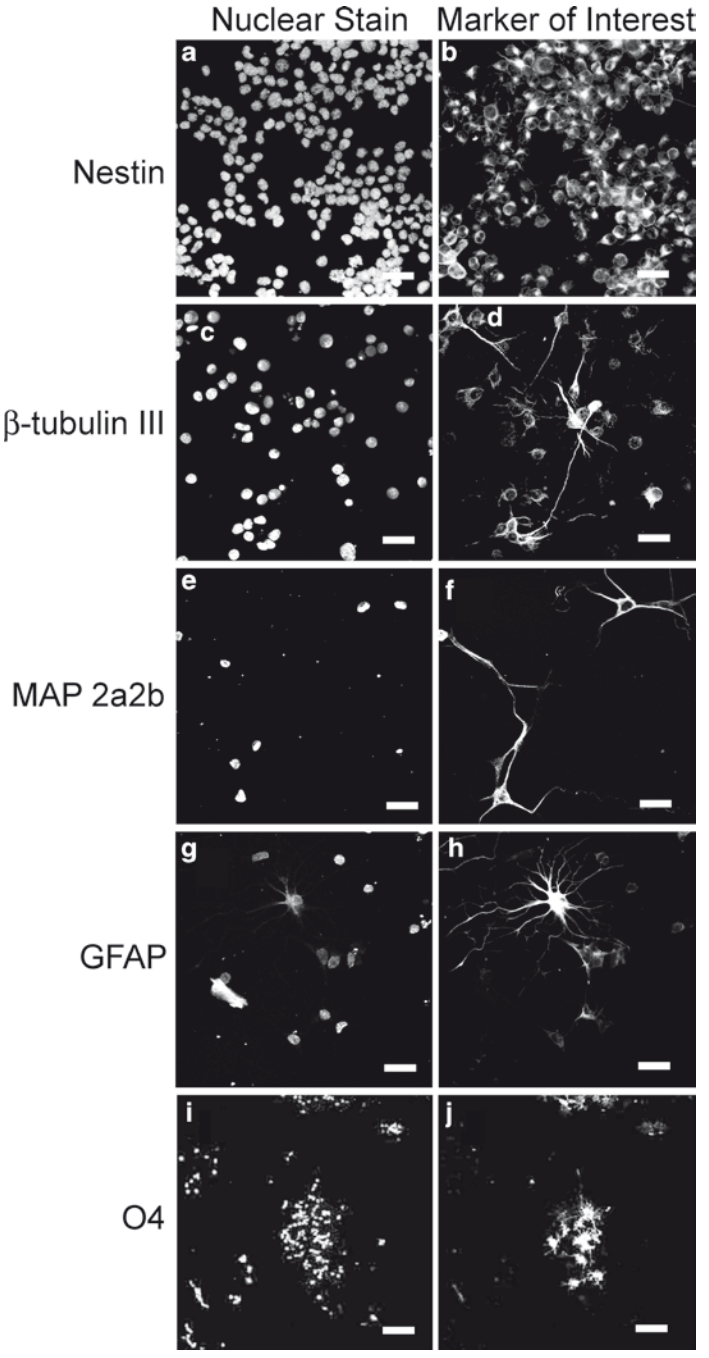

Fig. 2. Representative immunostaining of (**b**) the multipotent marker nestin, (**d**) neuronal marker β-tubulin III, (**f**) neuronal marker MAP 2a + 2b, (**h**) astrocytic marker GFAP, and (**j**) oligodendrocytic marker O4. (**a, b**) Cultures were grown in DMEM/F-12 + N-2 supplemented with 20 ng/ml FGF-2 for 2 days and stained for nestin. (**c, d**) Cultures were grown in neuronal differentiation medium (DMEM/F-12 + N-2 supplemented with 1 μM RA + 5 μM forskolin) for 5 days and stained for β-tubulin III. (**e**–**j**) Cultures were grown in mixed population differentiation medium (DMEM/F-12 + N-2 supplemented with 1 μM RA + 1% FBS) for 4 days and stained for MAP 2a + 2b, GFAP, or O4, respectively. (**a**, **c**, **e**, **g**, **i**) TO-PRO®-3 nuclear stain. (**b**, **d**, **f**, **h**, **j**) marker of interest. Scale bars: (**a**–**h**) 16 μm, (**i**, **j**) 50 μm

note that quantitative RT-PCR measures the population-averaged concentration of mRNA for an entire culture. If cell-to-cell variability within a culture is important, then we recommend analyzing the culture via immunostaining to gain single cell data (Subheading 3.2.2). We have demonstrated the utility of quantitative RT-PCR to quantify the cell differentiation state through the measurement of neuronal (β-tubulin III), astrocytic (GFAP), oligodendrocytic (myelin basic protein (MBP)), and multipotent (nestin) markers (5). Here, we present a method for quantifying expression of these marker genes in neural progenitors; however, we do not cover the design and testing of new primer and probe sets. We refer the reader to standard references for a detailed explanation of this procedure (6).

Quantitative RT-PCR is accomplished in four steps: cell culture, RNA isolation, reverse transcription, and quantitative PCR. The cell culture conditions will depend on the experimental design and have been discussed previously (Subheading 3.1). To ensure sound statistics, we recommend culturing each experimental condition in triplicate. RNA isolation is accomplished with TRIzol® reagent, and this is followed immediately by reverse transcription. Both of these methods draw heavily from the manufacturer's (Invitrogen) instructions. The resulting cDNAs are quantified by Taqman probe QPCR using the 18S ribosomal subunit as an internal control. Again for statistical purposes, we recommend running technical triplicates for each cDNA sample. Combined with the biological triplicate, this results in nine QPCR reactions per condition.

3.2.3.1. RNA Isolation

1. The RNases that exist on most surfaces easily degrade RNA. Therefore, it is extremely important to use only RNase-free reagents and supplies and to wash all equipment, surfaces, and gloved hands with RNase Away before beginning work.
2. Aspirate media from cells, wash once with PBS, and add TRIzol®. The ideal TRIzol® volume for sample processing in micro-centrifuge tubes is 1 ml. This volume is adequate to cover 3.5 cm, 6 cm, and 6-well plates; however, this and all subsequent steps must be scaled appropriately for larger plates.
3. Scrape the surface of the plate with a cell lifter to detach all cells. Then, triturate with 1 ml Pipetman to dissociate clumps.
4. Pipette TRIzol® solution into micro-centrifuge tubes (see Note 10) and incubate at room temperature for 5 min. Samples can now be stored long-term at −80°C and slowly thawed on ice for further processing. Beyond this point, samples should be kept on ice at all times unless otherwise noted.
5. Add 200 µl chloroform and vortex vigorously for 15 s.
6. Let samples phase separate on ice for 2–3 min. Spin at 12,000 g for 15 min at 4°C.

7. Pipette the upper, aqueous phase to a fresh, RNAse-free, micro-centrifuge tube. The interface between the aqueous and organic phases contains cellular proteins. Do not disturb this interface when pipetting, or protein contamination may result.
8. Add 1 µl DNase and incubate at room temperature for 15 min.
9. Repeat steps 5–7 to remove DNase and residual TRIzol®.
10. Add 5 µl of glycogen. This will improve visibility of the RNA pellet and increase yield.
11. Add 500 µl of isopropanol and incubate at room temperature for 10 min (see Note 11).
12. Centrifuge at 12,000 *g* for 10 min at 4°C.
13. Pour off the supernatant and wash the pellet with 1 ml 75% ethanol. Spin at 7,500 g for 5 min at 4°C.
14. Pipette off the supernatant (this pellet is looser) and briefly air-dry the pellet for 5–10 min at room temperature. Do not overdry.
15. Resuspend the pellet in 20 µl RNase-free water.
16. Proceed immediately to reverse transcription. Alternatively, RNA can be stored at –80°C and thawed on ice for later processing.

3.2.3.2. Reverse Transcription

17. Continue to use care to prevent RNase contamination (see step 1) until after the reverse transcription reaction is complete.
18. Quantify RNA concentration by measuring A_{260} on a spectrophotometer. Predilute samples in RNase-free water if necessary, depending on the spectrophotometer. A 3.5 cm plate of cells should yield roughly 20–40 µg of RNA, depending on experimental conditions (see Note 12).
19. For each sample, mix the following in a PCR (0.2 ml) tube:

Random hexamer primer:	1 µL
10 mM dNTP mix:	2 µL
RNA (10 pg – 5 µg):	*x*µL
DEPC – water:	to 12 µL

The volume of RNA will depend on the concentration of the sample. We recommend normalizing all samples within an experiment such that each reverse transcription reaction has the same amount of RNA. Each reaction should have 10 pg to 5 µg RNA; however, it is best to normalize all reactions to the highest concentration possible.

20. Denature this mixture by incubating in a thermal cycler at 65°C for 5 min. Immediately place on ice (see Note 13).
21. Prepare the following master reaction mix on ice. Volumes shown below are per reaction. Make approximately 10% more master reaction mix than needed as a contingency. Vortex to mix.

5× cDNA synthesis buffer:	4 µl (vortex before use)
0.1 M DTT:	1 µl
RNase OUT™:	1 µl
DEPC water:	1 µl
ThermoScript™ RT:	1 µl (keep on ice at all times)

22. Add 8 µl master reaction mix to each PCR tube. Keep on ice.
23. Perform the reverse transcription reaction by incubating in thermal cycler.

    25°C 10 min

    55°C 50 min

    85°C 5 min
24. To digest the remaining RNA templates, add 1 µl RNase H to each reaction and incubate at 37°C for 20 min (see Note 14). DO NOT use RNase-free supplies for this step, as they will become contaminated with RNase.
25. The resulting cDNAs can be used immediately for quantitative PCR or stored at –20°C for up to 2 weeks. Quantitative data are unreliable from cDNAs older than 2 weeks; however, these samples are adequate for semi-quantitative experiments or cDNA amplification for cloning.

3.2.3.3. QPCR

26. Prepare standard curve in micro-centrifuge tubes. Mix 10 µl of 1 ng/µl gene-of-interest standard with 10 µl of 1 ng/µl 18S ribosomal subunit standard. Using distilled/de-ionized water, serially dilute in tenfold increments to generate a standard curve spanning 7 orders of magnitude.
27. Add 6 µl of each cDNA sample and each standard curve sample to individual micro-centrifuge tubes.
28. Prepare PCR reaction mix. See Table 2 for primer and probe sequences. Mix the following components in the order shown. Volumes shown are per 20 µl PCR reaction. As a contingency, make approximately 10% more reaction mix than needed. Vortex to mix.

2× Supermix	10 µl
Distilled/de-ionized water	7 µl
Gene-of-interest sense primer(10 µM stock)	0.5 µl
Gene-of-interest antisense primer (10 µM stock)	0.5 µl
18S sense primer (10 µM stock)	0.5 µl
18S antisense primer (10 µM stock)	0.5 µl
Gene-of-interest probe (10 µM stock)	0.4 µl
18S probe (10 µM stock)	0.4 µl
Antibody/Taq polymerase	0.25 µl

Table 3
Quantitative PCR protocol

Cycle	Repeats	Step	Time (mm:ss)	Setpoint (°C)
1	1	1	05:00	95
2	50	1	00:30	95
		2[a]	00:30	58
		3	00:15	72

[a]Indicates the real-time detection step

29. Add 60 µl PCR reaction mix to each sample. Pipette up/down to mix.
30. Transfer 20 µl to each well on a 96-well PCR Plate. Plate each sample and standard in triplicate. The resulting standard curve now has concentrations from 1 ng/well to 10^{-7} ng/well of both the 18S and gene-of-interest standards.
31. Seal the plate with the Microseal B Adhesive Seal. Run a hard, clean, straight edge across the top of the plate to create a strong seal around each well.
32. Transfer the plate to real-time PCR detection system, and run the protocol shown in Table 3. Be sure to select the correct fluorophores (FAM-490 for gene-of-interest and CAL-610 for 18S ribosomal subunit – the Texas Red setting may be substituted for CAL-610).
33. PCR efficiencies should be near 100% (±10%). Construct standard curves for the gene-of-interest and 18S ribosomal subunit and interpolate unknown sample values. Normalize gene-of-interest values to 18S ribosomal subunit and perform appropriate statistical analyses (see Note 15).

4. Notes

1. Normal serum is serum derived from the species in which the secondary antibody was raised. Use of nonmatched secondary antibodies and blocking serum can lead to nonspecific, background staining.
2. Differentiated cells are much more adherent than multipotent progenitors. If, after several minutes of Accutase incubation and gentle rocking, there are large areas of attached cells, do not attempt to forcibly remove them. Most likely, they are lineage committed and not desirable for further passaging.
3. The medium does not contain serum; therefore, it will not inactivate the Accutase. The Accutase must be removed by centrifugation for cell pelleting followed by aspiration of the Accutase-containing medium.
4. Always add media to the plate before adding cells to prevent drying, as the dry regions will not support cell attachment.
5. If the vial was stored in liquid nitrogen, proceed immediately to thawing. Do not transfer to −80°C or dry ice for any longer than a few minutes. This will result in extremely poor viability after thaw.
6. If a quantitative RT-PCR experiment involves a time-course study, then do not use 6-well plates. RNA extraction requires lysing cells directly from the plate using the highly toxic TRIzol® reagent. Residual TRIzol® in a well, which contains volatile components, is sufficient to kill cells in neighboring wells.
7. If a long-term (10–14 days) differentiation experiment is required, use DMEM/F-12 + N-2 supplemented with 1 ng/ml FGF-2 + 10 ng/ml neurotrophic factor 3 (NT-3, Peprotech) + 10 ng/ml brain derived neurotrophic factor (BDNF, Peprotech). Change media every other day.
8. All volumes in the immunostaining protocol are for 8-well CultureSlides. Other sizes may be used, but volumes must be adjusted accordingly.
9. If immunostaining for O4, do not permeablize with Triton X-100. O4 is a surface marker which can be disrupted by Triton X-100. For *in vitro* staining, follow Subheading 3.2.2 steps 1–9 without Triton X-100, postfix with 4% paraformaldehyde, and then continue with permeablization and costaining at step 3.
10. TRIzol® already contains RNase inhibitors, so there is no need to use RNase-free tubes for this step.
11. As a convenient stopping point, samples may be incubated overnight on ice at 4°C.

12. To verify the integrity of the isolated RNA, the ratio A_{260}/A_{280} should be 1.9–2.1. Additionally, a small aliquot of RNA can be analyzed on a 1% agarose gel and visualized with ethidium bromide. The predominant bands are 18S and 28S rRNA at approximately 1 and 2 kilobases, respectively, when compared to a DNA ladder.
13. Set the thermal cycler to automatically drop to 4°C after denaturation. This avoids accidentally overheating the samples at the end of the run.
14. RNase H cleaves RNA phosphodiester bonds in DNA:RNA duplexes. Also note that RNase H is not inhibited by RNase OUT™.
15. The purpose of the standard curve is only to provide a reference on which to base experimental values. The experimental "concentrations" obtained from interpolating the standard curve do not correspond to the absolute quantities of mRNA in the culture. These concentrations provide relative values for comparing data from multiple experiments.
16. The mouse anti-O4 antibody is an IgM. Confirm that the intended secondary antibody will recognize IgM before use.

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