Neural Precursor Cell-Based Screening & Bioassay Kit

Catalog Number SC014

Reagents for screening toxins or other bioactive agents for their effects on neural precursor cell proliferation and neuronal differentiation.

This package insert must be read in its entirety before using this product.

FOR LABORATORY RESEARCH USE ONLY. NOT FOR DIAGNOSTIC USE.
THE SAFETY AND EFFICACY OF THIS PRODUCT IN DIAGNOSTIC OR OTHER CLINICAL USES HAS NOT BEEN ESTABLISHED.

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MANUFACTURED AND DISTRIBUTED BY:

R&D Systems, Inc.
614 McKinley Place NE
Minneapolis, MN 55413
United States of America

TELEPHONE: (800) 343-7475
(612) 379-2956
FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

R&D Systems Europe, Ltd.
19 Barton Lane
Abingdon Science Park
Abingdon, OX14 3NB
United Kingdom

TELEPHONE: +44 (0)1235 529449
FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

R&D Systems China Co. Ltd.
24A1 Hua Min Empire Plaza
726 West Yan An Road
Shanghai PRC 200050

TELEPHONE: +86 (21) 52380373
FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

INTRODUCTION

The ability to manipulate neural precursor cells *in vitro* has provided insight into central nervous system development as well as potential treatments for disease states. Identification of modulators of neural precursor cell proliferation and differentiation is essential to these studies. Bioactive agents including toxins, drugs, growth factors, and other substances that can affect the renewal and differentiation abilities of neural precursors impact the adult and developing nervous systems. The ability of neural precursors to self-renew and differentiate into neurons *in vitro* makes it possible to test how such bioactive agents influence neural proliferation and differentiation (1 - 8). The Neural Precursor Cell-Based Screening and Bioassay Kit is intended as an *in vitro* screening tool for determining the effect of such substances on neural precursor proliferation and differentiation.

PRINCIPLE OF THE ASSAY

This kit contains specially formulated media supplements for the maintenance and expansion of neural precursors and for the differentiation of neural precursors into neurons. Resazurin is included for the assessment of precursor proliferation. Mouse anti-neuron specific β-III tubulin antibody conjugated to horseradish peroxidase (HRP) is included for quantitation of differentiated neurons. The components included in this kit are sufficient for two 96-well plate format assays of neural precursor proliferation and two 96-well plate format assays of neuronal differentiation. The kit allows determination of both adverse effects on proliferation and differentiation, as with a toxin, as well as increased rates of proliferation and/or differentiation resulting from an agent that is stimulatory.

Neural Precursors

Assess Effects on Proliferation

Neural precursors are plated in an uncoated 96-well plate with Completed Media containing Maintenance Supplement and toxin or bioactive agent of interest.

48 hours

Neural precursor proliferation is assessed by Resazurin.

Assess Effects on Differentiation

Neural precursors are plated on a Poly-L-Ornithine/Fibronectin coated 96-well plate with Completed Media containing Maintenance Supplement.

48 hours

Toxin or bioactive agent of interest is presented in Completed Media containing Differentiation Supplement.

7 days

Neuronal differentiation is assessed by cell-based ELISA for neuron-specific β-III tubulin expression.

LIMITATIONS OF THE PROCEDURE

- FOR LABORATORY RESEARCH USE ONLY. NOT FOR DIAGNOSTIC USE.
- The safety and efficacy of this product in diagnostic or other clinical uses has not been established.
- Do not mix reagents with those from different lots or sources.
- Do not expose kit reagents to strong light during storage or incubation.
- Avoid contact of kit reagents with oxidizing agents and metals.
- Incubation times and temperatures other than those specified may yield invalid results.
- The quality of the neural precursors and any variation in the procedure can cause variation in the efficiency of cell proliferation and differentiation.

PRECAUTION

The acute and chronic effects of over-exposure to the reagents in this kit are unknown. Safe laboratory handling procedures should be followed and protective clothing should be worn when handling kit reagents.

MATERIALS PROVIDED

Maintenance Supplement (500X) (Part # 390497) - A lyophilized concentrated supplement containing recombinant human Fibroblast Growth Factor (FGF) basic and recombinant human Epidermal Growth Factor (EGF). Reconstitute with 600 µL of sterile deionized water.

Differentiation Supplement (100X) (Part # 390498) - A lyophilized concentrated supplement containing Insulin-like Growth Factor I (IGF-I) and fetal bovine serum (FBS). Reconstitute with 2 mL of sterile deionized water.

Fibronectin (100X) (Part # 965894) - Lyophilized fibronectin. Reconstitute with 250 µL of sterile 1X PBS.

Resazurin (Part # 965895) - 5 mL of a non-toxic, water soluble, redox-sensitive dye for measuring cell proliferation.

Wash Buffer (10X) (Part # 895600) - 50 mL of a buffered surfactant with preservatives.

Blocking Buffer (Part # 893185) - 50 mL of 10% FBS in Wash Buffer.

Anti-β-III Tubulin Antibody (50X) (Part 965893) - 1 mL of 50X concentrated mouse anti-neuron-specific β-III tubulin conjugated to HRP.

Substrate F1 Concentrate (Part 893239) - 100 µL of a 200X concentrated fluorogenic substrate for HRP.

F1 Diluent (Part 893238) - 20 mL of a solution for diluting the Substrate F1 Concentrate.

Plate Sealers (Part 640197) - 4 adhesive strips.

STORAGE

Unopened Kit	Store at 2 - 8° C. This kit is stable for up to 6 months from the date of receipt.	
Opened/ Reconstituted Reagents	Maintenance Supplement (500X)	Aliquot and store at ≤ -20° C in a manual defrost freezer for up to 3 months.* Avoid repeated freeze-thaw cycles. Store the aliquot in use at 2 - 8° C for up to 1 week.
	Differentiation Supplement (100X)	
	Fibronectin (100X)	
	Anti-β-III Tubulin Antibody (50X)	Store at 2 - 8° C.*
	Wash Buffer (10X)	
	Blocking Buffer	
	Unmixed Substrate F1 Concentrate	
	Unmixed F1 Diluent	
	Resazurin	

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

Materials

- Neural precursors (rat cortical stem cells; R&D Systems, Catalog # NSC001 or equivalent)
- 15 mL centrifuge tubes (Corning Costar, Catalog # 430052 or equivalent)
- Pipettes and pipette tips
- Serological pipettes
- Reagent reservoirs (Matrix Technologies Corp., Catalog # 8094 or equivalent)
- 96-well cell culture microplates for use as vessels in the proliferation assay (Corning Costar, Catalog # 3595 or equivalent)
- Black 96-well clear-bottom cell culture microplates and covers for use as vessels in the differentiation assay (Corning Costar, Catalog # 3603 or equivalent)
- 96-well round bottom plates (Corning Costar, Catalog # 3359 or equivalent)

Reagents

- StemXVivo™ Serum-Free NSC Base Media for neural stem cell expansion and differentiation (R&D Systems, Catalog # CCM002 or equivalent)
- Penicillin-Streptomycin 100X (Invitrogen, Catalog # 15140-122 or equivalent)
- Poly-L-Ornithine (Sigma, Catalog # P3655 or equivalent)
- 1X Phosphate Buffered Saline (PBS) (Irvine Scientific, Catalog # 9240 or equivalent)
- 4% Paraformaldehyde in PBS
- Sterile deionized water
- 30% H_2O_2 (Sigma, Catalog # H1009 or equivalent); refer to MSDS prior to use
- 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen, Catalog # D1306 or equivalent)

Equipment

- 37° C and 5% CO_2 incubator
- 37° C water bath
- Centrifuge
- Hemocytometer
- Inverted microscope
- Multi-channel pipette
- Horizontal orbital microplate shaker
- Fluorescent microplate reader

Assay for Effects of Bioactive Agent(s) on Neural Precursor Proliferation

REAGENT PREPARATION

Use serological pipettes to transfer and remove solutions.

Base Media - Thaw the StemXVivo Serum-Free NSC Base Media at 2 - 8° C or room temperature. Aliquot any remaining thawed media and store at ≤ -20° C or use within 10 days when stored **in the dark** at 2 - 8° C.

Completed Base Media - Add Penicillin-Streptomycin (100X) to the Base Media at a 1:100 dilution. Store **in the dark** at 2 - 8° C for up to 10 days.

Maintenance Supplement (500X) - Reconstitute the lyophilized Maintenance Supplement with 600 µL of sterile deionized water. Mix well. This volume is enough for four 96-well plates (two plates for proliferation and two plates for differentiation). If a smaller experiment is being run, aliquot and freeze any unneeded reconstituted Maintenance Supplement at ≤ -20° C in a manual defrost freezer. **Avoid repeated freeze-thaw cycles.**

Note: *The Base Media, Completed Base Media, and Maintenance Supplement are also needed for cell plating and maintenance in the Neuronal Differentiation assay.*

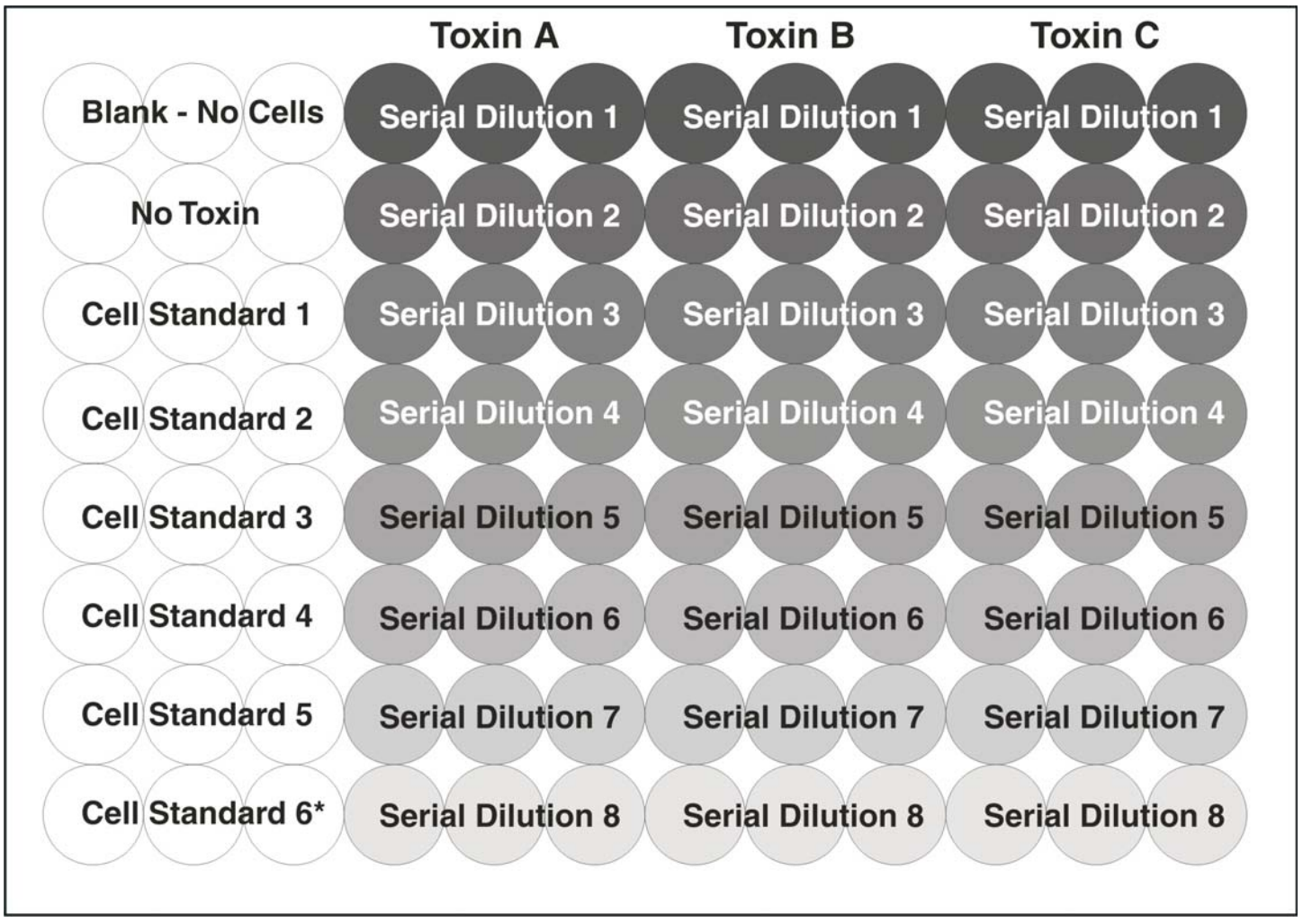
Maintenance Supplemented Media (MSM-1X) - Fresh supplemented media should be made for each usage. For initial cell plating and toxin/bioactive agent exposure in one 96-well plate, add 60 µL of reconstituted Maintenance Supplement to 30 mL of Completed Base Media to make MSM-1X.

Maintenance Supplemented Media (MSM-10X) - Fresh supplemented media should be made for each usage. For cell maintenance at 24 hours and 48 hours (one 96-well plate), add 50 µL of reconstituted Maintenance Supplement to 2.5 mL of Completed Base Media to make MSM-10X.

PROCEDURES

To plan an experiment, it is recommended to use serial dilutions of the bioactive agent(s) being tested. Running the dilution series in triplicate is recommended to account for variations between wells. It is also recommended to run a vehicle only control (if applicable), an untreated cell control (positive control for resazurin activity), and a no cell control (negative control for resazurin activity). If desired, a standard curve can be used as a relative measure of the magnitude of an effect a bioactive agent has on cell proliferation. A standard curve can be created by plating untreated cells over a range of densities (see diagram).

Below is a diagram of a sample plate that can be used as a guide in experimental design. This diagram assumes that 3 different bioactive agents are being tested at 8 concentrations. This can be modified to include a larger number of agents at fewer concentrations or fewer agents at a wider range of concentrations and also includes wells for a standard curve in the unshaded columns.



*Or vehicle only control if applicable.

These procedures describe a proliferation assay in one 96-well plate. If a smaller experiment is being run, refer to the table below for needed volumes.

Solution Preparation by Assay Size - Proliferation

Total Number of Wells for Assays	Volume of Reconstituted Maintenance Supplement Needed for MSM-1X Cell Plating and Bioactive Agent Exposure	Volume of MSM-1X Needed for Cell Plating and Bioactive Agent Exposure	Volume of Reconstituted Maintenance Supplement Needed for MSM-10X Cell Expansion and Maintenance	Volume of MSM-10X Needed for Cell Expansion and Maintenance	Volume of Resazurin Needed for Cell Proliferation Assay
96 wells	60 µL	30 mL	100 µL	5 mL	2.5 mL
48 wells	30 µL	15 mL	50 µL	2.5 mL	1.25 mL
24 wells	15 µL	7.5 mL	25 µL	1.25 mL	0.625 mL

I. Bioactive Agent Exposure and Cell Plating

Fresh MSM-1X should be made for cell plating and bioactive agent exposure. The recommended amount of MSM-1X for plating and exposure in one 96-well plate is 30 mL (200 µL/well).

1. Dilute bioactive agent in MSM-1X. These dilutions should be at 2X the desired final concentration of bioactive agent in a volume of 100 µL/well. Add 100 µL to each well being used. The addition of neural precursors in a volume of 100 µL/well will bring the final volume to 200 µL/well and bioactive agent concentration to 1X.
2. Suspend neural precursors in 12.5 mL of MSM-1X (1.3×10^5 - 2.5×10^5 cells/mL).
   Note: *Make sure to agitate the cell suspension by pipetting up and down immediately prior to plating the neural precursors, as cells will quickly begin to aggregate while in suspension. Cell aggregation will lead to significant variability in cell plating between wells.*
3. Seed the plate at 100 µL/well (1.3×10^4 - 2.5×10^4 cells/well).
   Note: *There should now be a final volume of 200 µL/well and a 1X bioactive agent concentration.*
4. Incubate the cells at 37° C and 5% CO_2.

II. Cell Expansion and Maintenance

Expansion and maintenance of neural precursor cells continues for 48 hours after initial cell plating. The cells in this assay grow in suspension culture. The plate containing MSM-1X and bioactive agent from the initial cell plating should be spiked with fresh MSM-10X. **Do not remove media.**

1. Twenty-four hours after initial plating, add 20 μL of MSM-10X to each well.
2. Incubate the plate at 37° C and 5% CO_2.
3. Cell proliferation is measured at 48 hours.
   Note: *Depending on the stability of the bioactive agent(s) of interest, the schedule for the addition of the bioactive agent can be modified.*

III. Cell Proliferation Assessed by Resazurin

Forty-eight hours after the initial cell plating, cell proliferation is assessed using Resazurin, which is light sensitive. The plate containing MSM-1X and bioactive agent from the initial cell plating should be spiked with fresh MSM-10X prior to the addition of Resazurin. **Do not remove media.** This is a suspension culture.

1. Add 20 μL of MSM-10X to each well.
2. Add 24 μL of Resazurin to each well.
3. Incubate the plate at 37° C and 5% CO_2 for 4 - 6 hours.
4. Read in a fluorescence microplate reader using 544 nm excitation and 590 nm emission wavelengths.

For sample data, refer to page 16.

Assay for Effects of Bioactive Agent(s) on Neuronal Differentiation

REAGENT PREPARATION

Use serological pipettes to transfer and remove solutions.

Base Media - Thaw the StemXVivo Serum-Free NSC Base Media at 2 - 8° C or room temperature. Aliquot any remaining thawed media and store at ≤ -20° C in a manual defrost freezer or use within 10 days when stored **in the dark** at 2 - 8° C.

Completed Base Media - Add Penicillin-Streptomycin (100X) to the Base Media at a 1:100 dilution. Store **in the dark** at 2 - 8° C for up to 10 days.

Maintenance Supplement (500X) - If not done previously, reconstitute the lyophilized Maintenance Supplement with 600 µL of sterile deionized water. Mix well. This volume is enough for four 96-well plates (two plates for proliferation and two plates for differentiation). If a smaller experiment is being run, aliquot and freeze any unneeded reconstituted Maintenance Supplement at ≤ -20° C in a manual defrost freezer. **Avoid repeated freeze-thaw cycles.**

Note: *The Maintenance Supplement is also needed in the Neural Precursor Proliferation assay.*

Maintenance Supplemented Media (MSM-1X) - Fresh supplemented media should be made for each usage. For cell plating in one 96-well plate, add 50 µL of reconstituted Maintenance Supplement to 25 mL of Completed Base Media to make MSM-1X.

Maintenance Supplemented Media (MSM-2X) - Fresh supplemented media should be made for each usage. For cell maintenance of one 96-well plate at 24 hours, add 50 µL of reconstituted Maintenance Supplement to 12.5 mL of Completed Base Media to make MSM-2X.

Differentiation Supplement (100X) - Reconstitute the lyophilized Differentiation Supplement with 2 mL of sterile deionized water. Mix well. This volume is enough for two 96-well plates. If a smaller experiment is being run, aliquot and freeze any unneeded reconstituted Differentiation Supplement at ≤ -20° C in a manual defrost freezer. **Avoid repeated freeze-thaw cycles.**

Differentiation Supplemented Media (DSM-1X) - Fresh supplemented media should be made for each usage. For days 0, 2, 4, and 6 of differentiation in one 96-well plate, add 250 µL of the Differentiation Supplement to 25 mL of Completed Base Media to make DSM-1X.

Poly-L-Ornithine Stock Solution (1000X) - Dissolve poly-L-ornithine in sterile 1X PBS to make a 15 mg/mL stock solution. Aliquot and store at ≤ -20° C in a manual defrost freezer for up to 6 months. **Avoid repeated freeze-thaw cycles.**

Poly-L-Ornithine Solution (1X) - Dilute the poly-L-ornithine stock solution 1:1000 in sterile 1X PBS to make a 1X solution (15 µg/mL). Prepare fresh as needed.

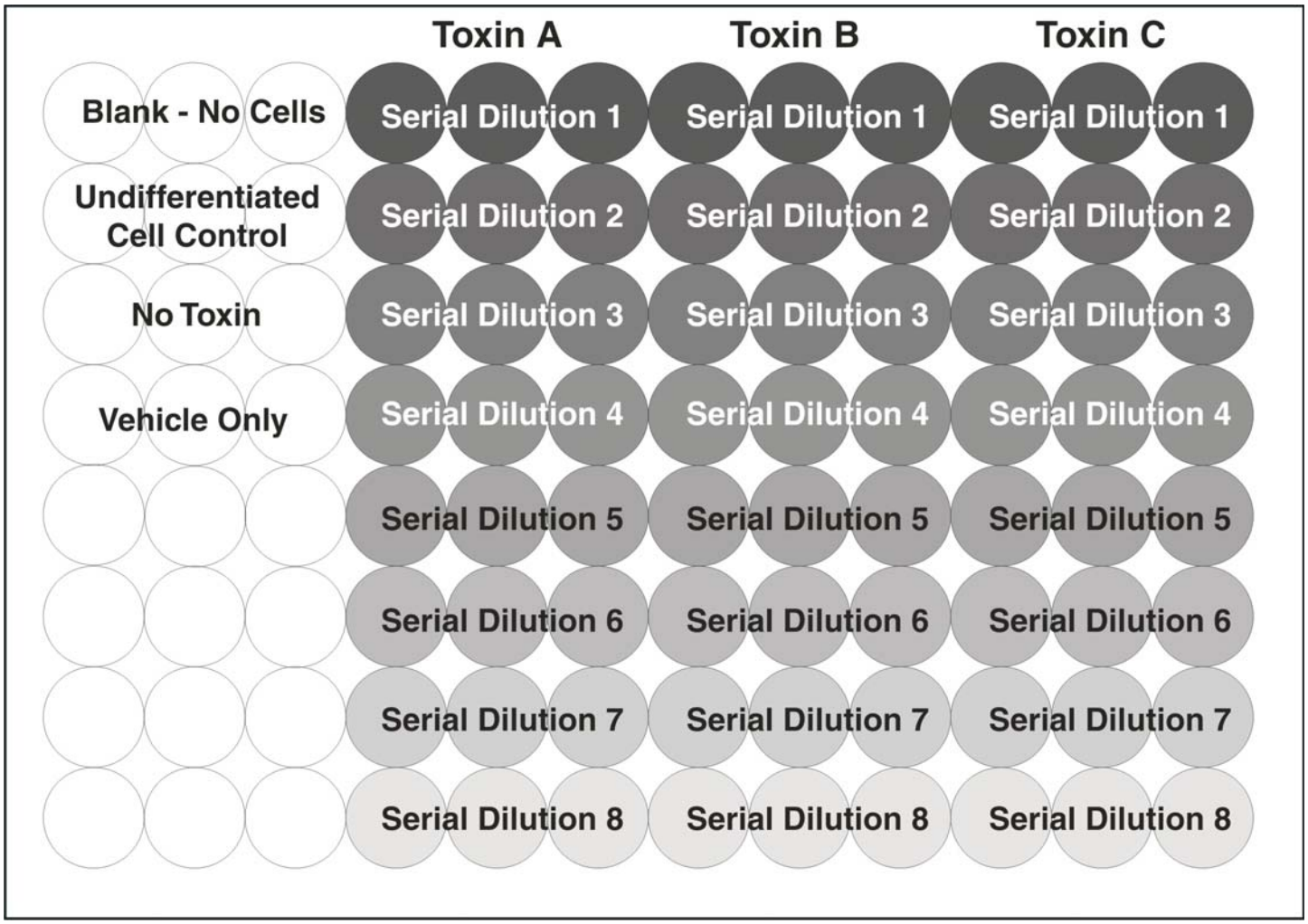
Fibronectin Stock Solution (100X) - Reconstitute the lyophilized fibronectin in 250 µL of sterile 1X PBS to make a 100X stock solution. This volume is enough for two 96-well plates. If a smaller experiment is being run, aliquot and freeze any unneeded Fibronectin Stock Solution at ≤ -20° C in a manual defrost freezer. **Avoid repeated freeze-thaw cycles.**

Fibronectin Solution (1X) - Dilute the fibronectin stock solution 1:100 in sterile 1X PBS to make a 1X solution. Prepare fresh as needed.

PROCEDURES

To plan an experiment, it is recommended to use serial dilutions of the bioactive agent(s). Running the dilution series in triplicate is recommended to account for variations between wells. It is also recommended to run a vehicle only control (if applicable), an untreated cell control (positive control for differentiation and β-III Tubulin-HRP staining), and a no cell control (negative control for β-III Tubulin-HRP staining). As an additional control for β-III Tubulin antibody specificity and/or quality of the cells being used, 3 wells can be reserved for plating of undifferentiated cells 48 hours prior to assessment with β-III Tubulin-HRP.

Below is a diagram of a sample plate that can be used as a guide in designing your experiment. This diagram assumes that 3 different agents at 8 concentrations are being tested. This can be modified to include a larger number of agents at fewer concentrations or fewer agents at a wider range of concentrations.



These procedures describe a differentiation assay in one 96-well plate. If a smaller experiment is being run, refer to the table below for needed volumes.

Solution Preparation by Assay Size - Differentiation

Total Number of Wells for Assays	Total Volume of Reconstituted Maintenance Supplement Needed for Precursor Maintenance	Total Volume of MSM-1X Needed for Precursor Maintenance	Total Volume of MSM-2X Needed for Precursor Maintenance	Total Volume of Reconstituted Differentiation Supplement Needed for Neuronal Differentiation	Total Volume of DSM-1X Needed for Neuronal Differentiation
96 wells	100 µL	25 mL	12.5 mL	1 mL	100 mL
48 wells	50 µL	12.5 mL	6.25 mL	500 µL	50 mL
24 wells	25 µL	6.25 mL	3.12 mL	250 µL	25 mL

I. Preparation of Poly-L-Ornithine and Fibronectin Coated Plate

The poly-L-ornithine and fibronectin coated plate will be used for neural precursor cell adherence.

1. Add 100 µL of 1X Poly-L-Ornithine Solution to each well of a black, clear bottom 96-well plate. Incubate overnight at 37° C.
2. Discard the Poly-L-Ornithine Solution. Wash each well 3 times with 100 µL of sterile 1X PBS.
3. Add 100 µL of sterile 1X PBS to each well. Incubate overnight at 37° C.
4. Discard the 1X PBS and add 100 µL of 1X Fibronectin Solution to each well.
5. Incubate overnight at 37° C.
6. Discard the Fibronectin Solution. Wash each well 3 times with 100 µL of sterile 1X PBS.
7. Use the plate immediately for cell plating.
   Note: *If an undifferentiated cell negative control will be included, 3 wells of the plate are left in the final 1X PBS wash until needed in step IV on page 13.*

II. Neuronal Differentiation Assay Plating Protocol

Fresh supplemented media should be made for cell plating. The amount of MSM-1X recommended for plating one 96-well plate is 25 mL (200 µL/well). The recommended cell plating density is 2.5×10^4 - 5.0×10^4 cells/well.

1. Suspend neural precursor cells in 25 mL (1.25×10^5 - 2.50×10^5 cells/mL) of MSM-1X.
2. Seed one poly-L-ornithine and fibronectin coated 96-well plate at 200 µL/well (2.5×10^4 - 5.0×10^4 cells/well).
   Note: *Pipette cell suspension up and down immediately prior to plating neural precursor cells as cells will quickly begin to aggregate while in suspension. Cell aggregates will lead to large amounts of variability in cell plating between wells. Additionally, do not seed the cells in undifferentiated cell negative control wells.*
3. Incubate the cells at 37° C and 5% CO_2.
4. Cells should become adherent to the poly-L-ornithine and fibronectin coated plate after 24 hours.

III. Differentiation Assay: Expansion and Maintenance

Expansion and maintenance of neural precursors continues for 48 hours after initial cell plating. Twenty-four hours after initial cell plating, replace 100 µL of the MSM-1X in each well with 100 µL of fresh MSM-2X.

1. Add 50 µL of the Maintenance Supplement to 12.5 mL of the Completed Base Media to make MSM-2X. Mix gently.
2. Carefully remove 100 µL of the 200 µL/well of MSM-1X from each well. Cells should be adherent.
3. Gently add 100 µL of fresh MSM-2X to each well to get a final 1X concentration of MSM.
4. Incubate the plate at 37° C and 5% CO_2.
5. DSM-1X containing bioactive agent will replace the MSM-1X at 48 hours.

IV. Differentiation Assay: Bioactive Agent Exposure

Day 0 of Differentiation
Forty-eight hours after initial cell plating replace the MSM-1X with fresh DSM-1X containing bioactive agent(s). This is considered Day 0 of differentiation.

1. In a round bottom 96-well microplate, make up agent serial dilutions in DSM-1X for a total volume of 200 μL/well.
2. Carefully remove the MSM-1X from the plated cells. Cells should be adherent.
3. Gently and immediately add the 200 μL of fresh DSM-1X and the bioactive agent to each well.
4. Incubate the plate at 37° C and 5% CO_2.

Days 2, 4, and 6 of Differentiation
Replace media plus bioactive agent every 48 hours (days 2, 4, and 6 of Differentiation).

1. In a round bottom 96-well microplate, make up serial dilutions of the agent of interest in fresh DSM-1X for a total volume of 200 μL/well.
2. Carefully remove the 200 μL of DSM-1X and toxin from each well of the plate containing the cells.
3. Gently and immediately add 200 μL of fresh DSM-1X containing the bioactive agent to each well.
4. Incubate the plate at 37° C and 5% CO_2.
   Note: *Depending on the bioactive agent being used, the addition of the agent with every media change may not be desired. In those cases, media changes on Days 2, 4, and 6 of differentiation can be in DSM-1X without bioactive agent. If an undifferentiated cell control is being used, using the wells left in 1X PBS from step I.7 on page 11, plate undifferentiated cells on day 5 of differentiation and maintain cells using maintenance supplemented media as described in sections II and III above. These cells are to remain undifferentiated and serve as a control for the specificity of the β-III tubulin antibody.*

Day 7 of Differentiation
On Day 7, cells are ready for assessment of neuronal differentiation by ELISA for neuron-specific β-III tubulin expression.

β-III Tubulin Expression ELISA

REAGENT PREPARATION

Use serological pipettes to transfer and remove solutions.

These procedures describe an assay in one 96-well plate. If a smaller experiment is being run, refer to the table below for needed volumes.

Wash Buffer - Add 25 mL of Wash Buffer (10X) to 225 mL of 1X PBS to prepare 1X Wash Buffer. Store at 2 - 8° C for up to 60 days.

Quenching Buffer - Add 200 µL of 30% H_2O_2 to 9.8 mL of 1X Wash Buffer to prepare 0.6% H_2O_2.

Anti-β-III Tubulin Antibody Solution (1X) - Dilute the needed amount of Anti-β-III Tubulin Antibody (50X) 1:50 in Blocking Buffer.

F1 Substrate - Add 50 µL of the Substrate F1 Concentrate to 10 mL of F1 Diluent. Store the F1 Substrate at 2 - 8° C for up to 60 days.

Solution Preparation by Assay Size - ELISA

Total Number of Wells for Assays	Volume of Wash Buffer	Volume of Blocking Buffer	Volume of Antibody Solution	Volume of Quenching Buffer	Volume of F1 Substrate
96 wells	250 mL	25 mL	12 mL	10 mL	10 mL
48 wells	125 mL	12.5 mL	6 mL	5 mL	5 mL
24 wells	62.5 mL	6.25 mL	3 mL	2.5 mL	2.5 mL

PROCEDURES

Neuron-specific β-III Tubulin-HRP is used as a marker for early differentiated neurons.

I. Neuron-Specific β-III Tubulin Expression ELISA

After seven days of culturing cells in DSM-1X, cells are fixed and analyzed with Neuron-specific β-III Tubulin-HRP antibody.

1. Carefully remove the DSM-1X from the 96-well monolayer plate.
2. Fix cells by replacing the medium with 100 µL of 4% paraformaldehyde in 1X PBS. Add the plate cover and incubate for 20 minutes at room temperature.
3. Remove the paraformaldehyde solution and wash the cells 3 times with 200 µL of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.
4. Remove the Wash Buffer and add 100 µL of Quenching Buffer. Add the plate cover and incubate for 20 minutes at room temperature.
5. Remove the Quenching Buffer and wash the cells 3 times with 200 µL of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.
6. Remove the Wash Buffer and add 100 µL of Blocking Buffer. Add the plate cover and incubate for 1 hour at room temperature.
7. Remove the Blocking Buffer and add 100 µL of 1X Anti-β-III Tubulin Antibody Solution to each well. Cover the plate with a plate sealer and incubate for 16 hours at 2 - 8° C. Protect the plate from direct light.
8. Remove the Antibody Solution and wash the cells 3 times with 200 µL of 1X Wash Buffer followed by 2 washes with 200 µL of 1X PBS. Each wash step should be performed for 5 minutes with gentle shaking. Protect the plate from direct light.
9. Remove the 1X PBS from the plate and add 75 µL of F1 Substrate to each well. Incubate for 20 - 60 minutes at room temperature. Protect the plate from direct light. A pale pink or rosy color should develop and indicates staining.
10. Read the plate using a fluorescence plate reader with excitation at 540 nm and emission at 600 nm.
11. Remove the F1 Substrate from each well and replace with 100 µL/well of 10 µM DAPI solution diluted in 1X PBS. Incubate at room temperature for 5 minutes.
12. Wash the cells 3 times with 200 µL of 1X PBS. Leave the final wash in the wells when reading the plate.
13. Read the plate using a fluorescence plate reader with excitation at 346 nm and emission at 460 nm.

SAMPLE DATA

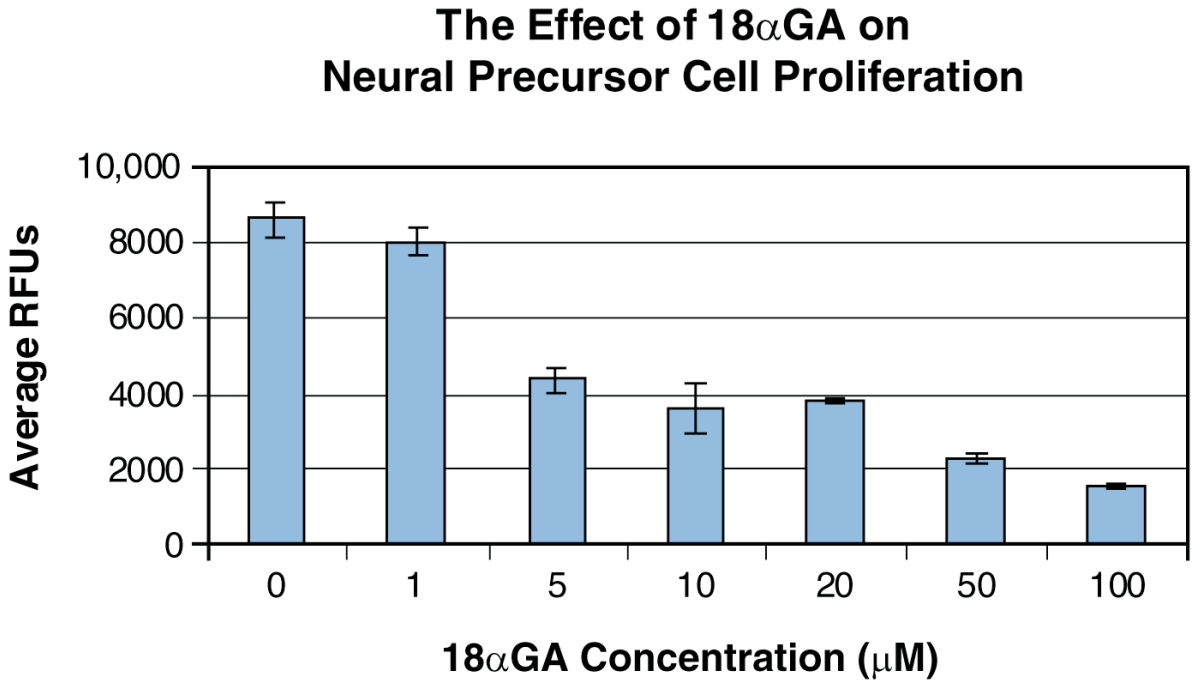


Figure 1: Effect of 18α-glycyrrhetinic acid (18αGA) on neural precursor proliferation. The gap junctional inhibitor, 18αGA, has been shown to decrease proliferation of neural precursors (9). Rat cortical stem cells (R&D Systems, Catalog # NSC001) were seeded at 2.5 x 10^4 cells/well and grown in the presence of the indicated concentrations of 18αGA. Cell numbers were determined using Resazurin. Error bars represent the standard deviation of triplicate samples.

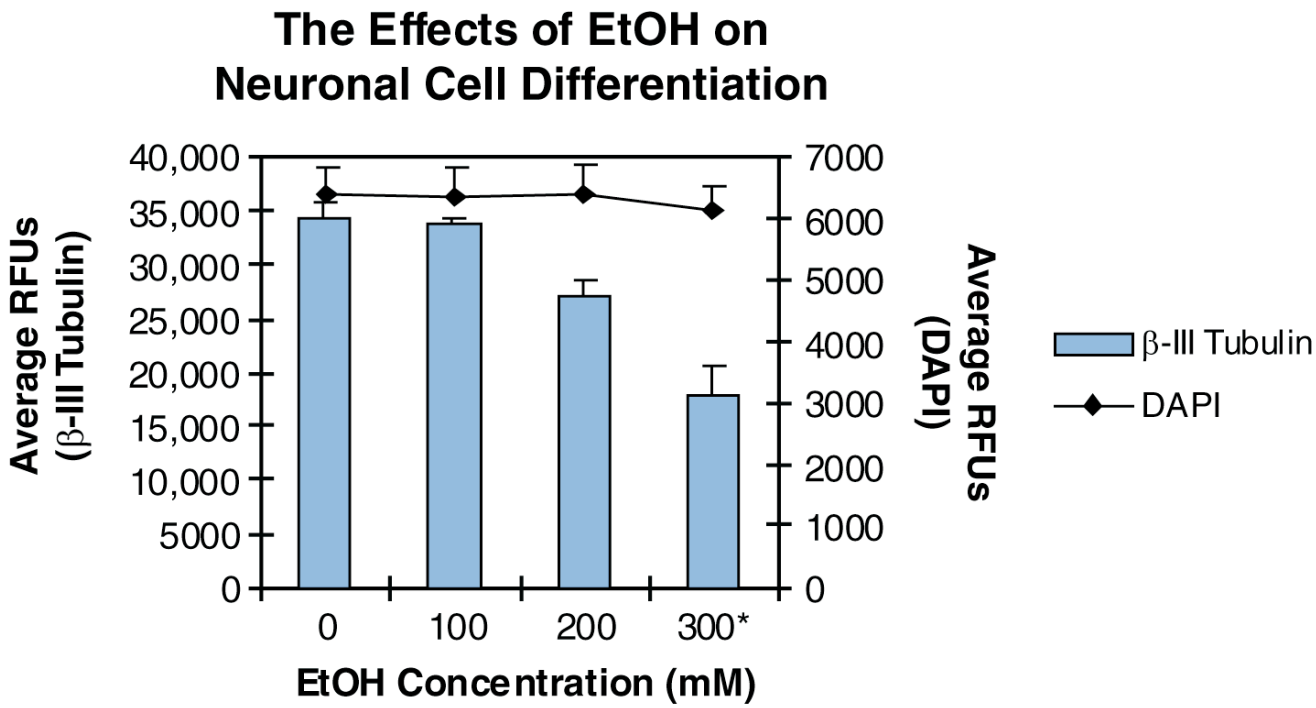


Figure 2: Effect of EtOH on neuronal differentiation of neural precursor cells. EtOH has been shown to reduce levels of neuronal differentiation of neural precursor cells (10). Rat cortical stem cells (R&D Systems, Catalog # NSC001) were seeded at 5.0 x 10^4 cells/well and differentiated in the presence of the above indicated amounts of EtOH. Levels of neuronal differentiation were determined by assessing β-III Tubulin levels and cells were stained with DAPI as a relative measure of total cell number. Error bars represent the standard deviation of triplicate or duplicate samples.

Note: *Because DAPI and β-III Tubulin are not measured at the same wavelength, it should not be assumed that similar RFU values indicate similar numbers of cells. Rather, the minimal variation in DAPI staining over the 0 - 200 mM EtOH concentrations indicates that the reduction in β-III Tubulin levels is not simply due to an overall decrease in cell numbers.*

*Only wells where the cells were alive (as assessed by DAPI) were used for analysis. The 300 mM EtOH concentration caused cell death in one of three wells. The results shown for this concentration are an average of two wells instead of three. EtOH concentrations higher than 300 mM caused most cells to die and were not used for this analysis.

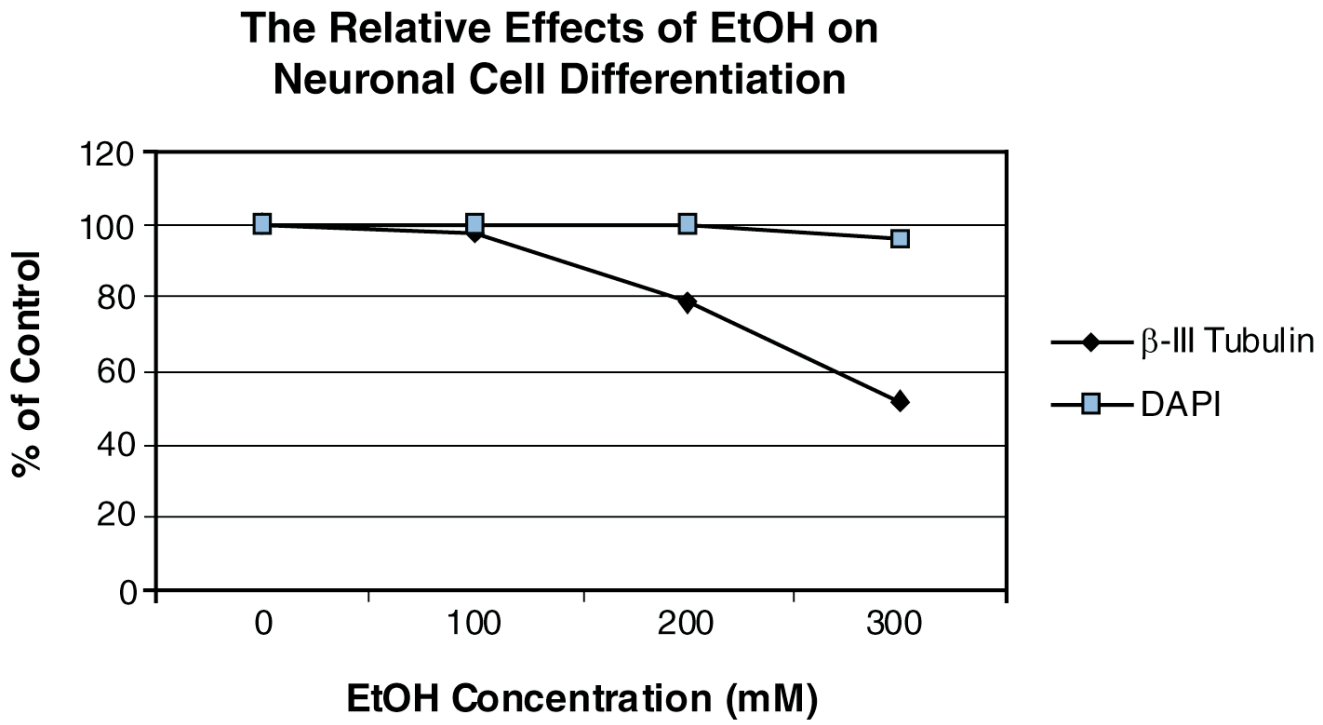


Figure 3: The relative effect of EtOH on neuronal differentiation of neural precursor cells. The data shown in Figure 2 was normalized to control levels (wells without EtOH) and graphed as a percentage of the control. While total cell numbers remained within 5% of untreated samples, at 300 mM EtOH, neuronal differentiation was reduced by approximately 50%.

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TROUBLESHOOTING GUIDE

Observation	Possible Cause	Possible Solution
High variability between wells/high amounts of cell death in wells	Variable cell numbers are being plated	**Proliferation/Differentiation Assay:** Agitate the cell suspension immediately prior to plating to prevent aggregation.
	The bioactive agent concentration is too high causing all cells to die	**Proliferation/Differentiation Assay:** Titrate down the concentration of bioactive agent used to determine a concentration that affects proliferation/differentiation without causing excessive cell death.
		Differentiation Assay: Use DAPI readings to ensure that decreases in β-III Tubulin are due to failure to differentiate and not due to cell death.
		Proliferation/Differentiation Assay: Change the schedule for addition of the bioactive agent. For the proliferation assay, bioactive agent can be added at a time point closer to assessing proliferation. For example, bioactive agent could be added on Day 2 with MSM-10X. For the differentiation assay, instead of adding bioactive agent with every media change, the agent can be added only on Day 0 of differentiation.
No/very low signal	Cell number too low	**Proliferation/Differentiation Assay:** Obtain an accurate viable cell count prior to plating to ensure that the proper number of viable cells is being plated.
		Proliferation/Differentiation Assay: If using cells other than RCSC from R&D Systems (Catalog # NSC001), optimal cell concentrations for this assay may need to be determined. A variety of cell concentrations can be used to determine optimal cell numbers for each assay.
	Poor quality cells	**Proliferation/Differentiation Assay:** Use quality undifferentiated cells that are able to proliferate.
	No cells present	**Proliferation Assay:** This is a suspension culture. Removing medium will also remove cells.
		Differentiation Assay: Add and remove all medium and solution carefully so as not to disturb/detach the cells. Use DAPI readings to ensure that cells are present.
Very high signal in all wells	Poor quality cells	**Differentiation Assay:** Include undifferentiated control wells to ensure that cells are not prematurely differentiated.