WiCell Neural Stem Cell Protocols

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Preface

This booklet of protocols is intended to serve as a primer for culturing neural stem cells in NSC culture medium. These protocols are representative of how the cells were cultured and banked. WiCell recommends that neural stem cells (NSCs) should be first thawed and established in the conditions in which they were initially frozen prior to transfer to alternate culture platforms. We recommend that you read through these protocols prior to thawing your cells, and follow them until you have established your own cell bank of frozen vials. As you thaw and expand your initial cell vials, we strongly encourage you to establish your own bank of frozen vials as soon as possible. Once the culture is established, freeze back a portion of the material, and freeze again when you have enough cells for 20 vials. This will ensure you have an adequate stock of material to expand from as you conduct experiments.

Because this bank will be the basis of all future work with this cell line, WiCell recommends that it be screened for karyotype and STR post thaw to ensure that the stability and identity of the banked material is as expected. These and other tests are available from WiCell's full-service Cytogenetics Laboratory; to learn more about WiCell Cytogenetic's offerings and how to submit samples visit www.wicell.org/cytogenetics.

If you have any additional questions, please contact us through technical support on the WiCell website at www.wicell.org.

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- 1.2.2. 10ml sterile serological pipettes (Fisher, 13-678-27F)
- 1.2.3. 15ml centrifuge tubes (Corning, 430052 or Fisher, 05-527-90)
- 1.2.4. 50ml centrifuge tubes (Corning, 43029 or Fisher, 05-539-10)
- 1.2.5. 6-well plates (Falcon, 08-772-1B or Nunc, 140675)
- 1.2.6. 250ml Nalgene Filter (Thermo Scientific, 568-0020)

1.3. Required Reagents

- 1.3.1. MEM NEAA (Invitrogen, 11140050)
- 1.3.2. Neurobasal medium (Invitrogen, 21103049).
- 1.3.3. B27 Supplement (Invitrogen, 17504-044).
- 1.3.4. Basic fibroblast growth factor, human Rec. (Life Technologies, PHG0314 or WCBL, rec.hum.FGF2)
- 1.3.5. GlutaMAX-1 (Invitrogen, 35050061)
- 1.3.6. PBS without $CaCl_2$ and without $MgCl_2$ (Invitrogen, 14190-250)
- 1.3.7. PBS with $CaCl_2$ and $MgCl_2$ (Invitrogen, 14040-141)
- 1.3.8. Bovine Serum Albumin (Sigma, A2153)
- 1.3.9. Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Invitrogen, A1413202) or equivalent such as Growth Factor Reduced Matrigel™ (Corning, 354230)
- 1.3.10. DMEM/F-12 medium (Invitrogen, 11330-032)

1.4. Matrix, Reagent, and Medium Preparation

1.4.1. PBS+0.1% BSA Solution

- 1.4.1.1. Add 250mg of Bovine Serum Albumin (BSA) to 250ml PBS+/+ (with calcium and with magnesium) and filter-sterilize. Store at 2-8°C, and freeze the other aliquots for up to 12 months at -20°C.

1.4.2. 2ng/ul β-FGF solution

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Note: This β-FGF solution preparation may not be needed if working with an equivalent that is already in solution.

1.4.2.1. Combine 100µg Basic Fibroblast Growth Factor (β-FGF) and 50ml of PBS+0.1% BSA Solution (from above). Solution is now 2ng/ul.

1.4.2.2. Aliquot into working volumes and store aliquots according to manufacturer's direction.

1.4.3. **Neural Stem Cell Culture Medium (NSC Medium)**

1.4.3.1. To make 100ml, combine the following in a 100ml filter.

Component	Amount	Final Conc.
Neurobasal medium	96ml	96%
B27 Supplement	2.0ml	2%
GlutaMAX	1.0ml	1%
MEM NEAA	1.0ml	1%
β-FGF solution at 2ng/ul	200ul	10ng/ml

1.4.3.2. Filter sterilize the medium, store at 2-8ºC, use for up to 14 days.

1.4.4. **Thawing and Aliquoting Geltrex™** LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Matrigel™ may also be used. See section below for preparation).

1.4.4.1. Thaw Geltrex™ overnight at 4°C.

1.4.4.2. Place 1.5ml microtubes, microtube holders and micropipette tips in a -20°C freezer overnight.

1.4.4.3. NSCs require 1:50 dilution of Geltrex™ for the culture matrix, 1ml total per well.

1.4.4.4. Different amounts may be aliquoted depending on the usage. NSCs require a 1:50 dilution of Geltrex™ for the culture matrix for a total of ~1ml per well of a 6-well plate (i.e. 1ml ÷ 50 = 0.020ml→ 20ul Geltrex™ + 1ml neuralbasal medium for 1 well)

2wells: 20ul Geltrex™ + 2ml neuralbasal medium; plate 1ml/well

6wells: 120ul Geltrex™ + 6ml neuralbasal medium; plate 1ml/well

12wells: 240ul Geltrex™ + 12ml neuralbasal medium; plate 1ml/well

1.4.5. **Coating 6 wells of a 6-well plate with Geltrex**

1.4.5.1. Thaw aliquot 1-2 hours on ice at 4°C or overnight at 4°C.

1.4.5.2. Add 5ml of cold sterile neuralbasal medium to a 15ml conical tube.

1.4.5.3. Use 1ml of the neuralbasal medium to dilute and transfer the contents of one tube.

1.4.5.4. Mix well, add 1ml/well. Ensure the entire surface of the well is coated with liquid. Label plate with "Geltrex" and the date.

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1.4.5.5. Incubate 1 hour at room temperature before using.

1.4.5.6. Extra plates may be wrapped in parafilm and stored at 4°C for up to one week. Warm the extra plates for 1 hour at room temperature before using.

1.4.6. **Preparation of Growth Factor Reduced Matrigel™ as an alternative to Geltrex**

1.4.6.1. **Preparing to aliquot Matrigel™**

1.4.6.1.1. Each lot of Matrigel™ is a different concentration. See the Matrigel™ Certificate of Analysis to learn the concentration for your lot. Calculate the volume of Matrigel™ needed for 1.5mg, the amount needed to coat 6 wells of a 6-well plate. If smaller or larger volume aliquots are more appropriate for your use, adjust calculations appropriately.

1.4.6.1.2. The day before aliquoting, place 3-5 centrifuge tube racks and sterile 1.5ml tubes in the in the -20ºC or -80°C freezer. Place an unopened box of the appropriate sized pipette tips (based on calculation in previous step) in the -20ºC freezer.

1.4.6.2. Thaw Matrigel™ overnight on ice in a covered ice bath in a 4°C refrigerator. Ensure the neck of the bottle is not submerged in the ice to reduce risk of contamination.

1.4.6.3. **Aliquoting Matrigel™**

1.4.6.3.1. Fill two small containers with ice and place both in the sterile biosafety cabinet (BSC). One of the containers will hold the Matrigel™ bottle; the other one will hold the box of pipette tips.

1.4.6.3.2. In the sterile biosafety cabinet, open the metal seal on the Matrigel™ bottle and carefully remove the rubber cover. Place bottle on ice. Retrieve one tube rack from the freezer, place in the BSC and carefully add sterile tubes to it.

1.4.6.3.3. Add Matrigel™ to each tube according to the calculations above. Switch pipette tips every 5-7 tubes to ensure sterility and prevent clogging of the tip.

1.4.6.3.4. Transfer tubes in batches to the -20ºC or -80°C freezer as soon as the rack is filled. Work quickly - if the Matrigel™ is allowed to warm at all, it will congeal and will not be appropriate for plating. Retrieve a new rack from the freezer at the same time you place the aliquot in the freezer. Repeat process until all Matrigel™ is aliquoted.

1.4.6.4. **Thawing Aliquots and Coating Plates with Matrigel™**

1.4.6.4.1. Place a sterile 15ml conical tube and a bottle of cold, sterile DMEM/F-12 medium it the BSC. Add 11ml cold DMEM/F-12 medium to the conical tube.

1.4.6.4.2. Remove one 1.5mg Matrigel™ aliquot from the freezer; use a micropipette to add 1ml of cold DMEM/F-12 medium.

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1.4.6.4.3. Gently pipette up and down to thaw and dissolve the Matrigel™. Immediately transfer it to the 15ml conical tube with 11ml DMEM/F-12.

1.4.6.4.4. Using a 5ml or 10ml pipet, mix gently (avoid vigorous mixing which will create undesirable tiny bubbles which will compromise the matrix when plated) and plate 2ml/well of a 6-well plate. This will be enough for 6 wells.

1.4.6.4.5. Allow to set one-two hours in a 37°C incubator (overnight in 37ºC incubator yields best attachment) before use.

1.4.6.4.6. Extra plates maybe stored in a 37°C incubator and should be used within 10 days after preparation. Prior to use, ensure there are no dry areas in the wells, as cells will not attach to it.

Section 2: Thawing Neural Stem Cells Protocol

Note: Before thawing, check the certificate of analysis included in the shipping packet insert to acquire the recommended number of wells one vial should be thawed into (this can vary among different lots).

2.1. Required Equipment

2.1.1. Biosafety cabinet

2.1.2. 37ºC / 5% CO2 incubator

2.1.3. 37ºC water bath

2.1.4. Centrifuge

2.2. Required Supplies

2.2.1. Forceps

2.2.2. Cryogenic handling gloves and eye protection

2.2.3. 5ml sterile serological pipettes (Fisher,13-678-27E) or equivalent

2.2.4. 95% Ethanol

2.2.5. Sterilized Pasteur pipettes (Fisher, 13-678-20D)

2.3. Required Reagents

2.3.1. Culture plates prepared with Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Invitrogen, A1413202) or equivalent

2.3.2. NSC Medium

2.4. Thawing Neural Stem Cells

2.4.1. **Neural Cells**

2.4.1.1. Prior to removing vial from storage, acquire the thaw recommendation (number of wells one vial should be thawed into) found in the certificate of analysis included in the shipping packet insert.

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2.4.1.2. Wear eye protection as vials stored in liquid nitrogen may accidentally explode when warmed.

2.4.1.3. Wear ultra-low temperature cryo gloves. Remove the pluripotent stem cell vial from the liquid nitrogen storage tank using forceps.

2.4.1.4. Using some metal forceps, immerse the vial in a 37˚C water bath without submerging the cap. Swirl the vial gently.

2.4.1.5. When only an ice crystal remains, remove the vial from the water bath.

2.4.1.6. Ensure the vial cap is tightly closed and immerse the vial into a 95% ethanol solution to sterilize the outside of the tube, and remove with forceps.

2.4.2. Removal of Cryoprotectant and Re-suspension of Neural Cells

2.4.2.1. Transfer the vial to a sterile biosafety cabinet.

2.4.2.2. Using a sterile 1, 2 or 5ml glass pipette, remove cells from cryovial and gently transfer into a sterile 15ml conical tube.

2.4.2.3. Slowly add 10ml of pre-warmed sterile neural basal medium drop-wise to the cells in the 15ml conical tube. While adding medium, gently move the tube back and forth to mix the cells. It is critical to add the medium slowly to reduce osmotic shock to the cells.

2.4.2.4. Centrifuge the cells at 580 x g for 4 minutes.

2.4.2.5. During centrifugation:

2.4.2.5.1. Prepare the plate onto which you are going to transfer the cells. For every ~2 million cells being thawed, prepare 1 well of a 6-well plate by aspirating Geltrex™ plating liquid and add 1ml/well of NSC Medium.

2.4.2.6. After centrifugation, aspirate the supernatant; take care not to disturb the cell pellet. Vacuum aspiration should be used with extreme caution, as there is significant risk of aspirating the cell pellet.

2.4.3. Plate Neural Stem Cells

2.4.3.1. If thawing in to one well, suspend the cell pellet in 1ml of NSC medium and add to well. If thawing into 2 wells, re-suspend the pellet in 2 ml and add 1ml/well.

2.4.3.2. Place plate gently into the incubator and gently shake the plate back and forth and side to side to evenly distribute the cells—avoid circular motions to prevent cells from pooling in the center of the well.

Note: While cells are attaching, try to limit opening and closing the incubator doors, and if you need to access the incubator, open and close the doors carefully. This will prevent disturbing the even distribution of cells across the well.

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Section 3: Feeding of Neural Stem Cells Protocol

3.1. Required Equipment

3.1.1. Biosafety cabinet

3.1.2. 37°C / 5% CO_2 incubator

3.1.3. Light microscope (a camera is recommended)

3.2. Required Supplies

3.2.1. 5ml sterile serological pipettes (Fisher,13-678-27E) or equivalent

3.2.2. Sterilized Pasteur pipettes (Fisher, 13-678-20D)

3.3. Required Reagents

3.3.1. NSC Medium

3.4. Feeding NSCs

3.4.1. Observe and assess NSC culture every day. Generally, medium should be changed every other day.

3.4.2. Aliquot what is needed for the day and warm in 37ºC water bath.

3.4.3. Carefully aspirate medium from the cell culture dish.

3.4.4. Slowly add 2-3ml/well of fresh medium to the dish allowing fluid to slither along the wall, avoid pouring medium directly onto the cells.

Section 4: Passaging of Neural Stem Cells Protocol

4.1. Required Equipment

4.1.1. Biosafety cabinet

4.1.2. 37°C / 5% CO_2 incubator

4.1.3. 37°C water bath

4.1.4. P1000 pipette

4.1.5. Centrifuge

4.2. Required Supplies

4.2.1. 5ml sterile serological pipettes (Fisher, 13-678-27E) or equivalent

4.2.2. 10ml sterile serological pipettes (Fisher, 13-678-27F)

4.2.3. Sterilized Pasteur pipettes (Fisher, 13-678-20D)

4.2.4. 15ml centrifuge tubes (Corning, 430052 or Fisher, 05-527-90)

4.3. Required Reagents

4.3.1. Culture plates prepared with Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Invitrogen, A1413202)

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4.3.2. NSC Medium

4.3.3. Neurobasal medium (Invitrogen, 21103049)

4.3.4. Accutase (MP Biomedicals, 091000449)

4.4. Passage Neural Stem Cells

Note: Neural cells are mortal, but lifespan should exceed 20 passages. Banking should always be completed in as few passages as possible to allow maximum usefulness.

4.4.1. Cells are ready to be passaged once they reach 100% confluence, about 3-5 days post thaw/split. Some cell lines can go up to 7 days between passaging.

4.4.2. Aspirate medium from wells to be split.

4.4.3. Add 1ml/well of warmed, sterile Accutase. Treat for 4 minutes at 37ºC until cells detach.

4.4.4. Wash off the NSCs from the plate using a P1000 pipette and transfer cells to a 15ml conical tube. Rinse the plate again with 3-4ml of sterile, pre-warmed neurobasal medium and collect in the same tube.

4.4.5. Centrifuge cells at 580 x g for 4 min.

4.4.5.1. While spinning, prepare wells of the new plate. Confluent cells can typically be split 1:3 to 1:6. Some cell lines may be split as much as 1:15.

4.4.5.2. Aspirate Geltrex™ plating liquid; add 1ml/well of NSC Medium.

4.4.6. Once spun down, aspirate the supernatant and re-suspend cells with NSC medium so that there is 1ml for every prepared well. Add 1ml of cells to each prepared well.

4.4.7. Place plate gently into the incubator and gently shake the plate back and forth and side to side to evenly distribute the cells—avoid circular motions to prevent cells from pooling in the center of the well.

Note: While cells are attaching, try to limit opening and closing the incubator doors, and if you need to access the incubator, open and close the doors carefully. This will prevent disturbing the even distribution of cells across the well.

Section 5: Freezing Neural Stem Cell Protocol

5.1. Required Equipment

5.1.1. Biosafety cabinet

5.1.2. 37°C / 5% CO_2 Incubator

5.1.3. 37°C water bath

5.1.4. Centrifuge

5.1.5. Light microscope

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5.1.6. Liquid Nitrogen storage tank, and liquid nitrogen

5.2. **Required Supplies**

5.2.1. Metal forceps

5.2.2. Cryogenic handling gloves and eye protection

5.2.3. Isopropanol freezing containers, and isopropanol

5.2.4. 1.5ml Cryovials, (Nunc, 5000-1020)

5.2.5. Plastic cryovial holders

5.2.6. 5ml and 10ml sterile serological pipettes (Fisher,13-678-27E, 13-67827F) or equivalent

5.2.7. 95% Ethanol

5.3. **Required Reagents**

5.3.1. **Cryopreservation Medium A (make day of freeze)**

5.3.1.1. Combine following components and filter sterilize, keep on ice. Volume of medium needed depends on how many cells will be frozen.

5.3.1.2. Discard any medium remaining after freeze.

Component	Amount
Neurobasal medium	96ml
B27 Supplement	2.0ml
MEM NEAA	1.0ml
GlutaMAX	1.0ml

5.3.2. **Cryopreservation Medium B (make day of freeze)**

5.3.2.1. Sterilely combine following components, keep on ice. Volume of medium needed depends on how many cells will be frozen.

Component	Amount
Sterile Neurobasal medium	78.0ml
Sterile MEM NEAA	1.0ml
Sterile GlutaMAX	1.0ml
Sterile DMSO	20.0ml

5.3.2.2. Do not filter DMSO, it will degrade the filter membrane.

5.3.2.3. Discard any medium remaining after freeze.

5.4. Prepare for Freeze

5.4.1. Prepare Cryopreservation Media A and B. Adjust volumes according to how many vials will be frozen.

5.4.2. Label cryovials with the cell line, passage number (add 1 to the passage number on the plate), the freeze date, and your initials. Use an alcohol proof pen or labels that resist liquid nitrogen and ethanol.

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5.4.3. Sterilize the biosafety cabinet (with the labeled vials in it) for 20 minutes with UV light. Turn on the blower and open the sash. Spray down the whole surface with ethanol and allow it to evaporate for 20 minutes prior to initiating cryopreservation.

5.4.4. Obtain a recharged isopropanol freezing container. Each container can hold 18 vials. The isopropanol must be replaced every 5 uses.

5.4.5. View all cells under the microscope. Cells are ready to be cryopreserved once they have reached confluence. Discard any contaminated plates.

5.5. Harvest and Spin Cells

5.5.1. Aspirate medium from wells to be split.

5.5.2. Add 1ml/well of warmed, sterile Accutase. Treat for 4 minutes at 37ºC until cells detach.

5.5.3. Wash off the NSCs from the plate using a P1000 (or other) pipette and transfer cells to a 15ml conical tube.

5.5.4. Rinse the plate again with 4ml of sterile pre-warmed plain neural basal medium, move from well to well to collect any remaining cells, and add to the same conical tube.

5.5.5. Centrifuge cells at 580 x g for 4 min.

5.5.6. Once spun down, aspirate the supernatant and re-suspend cells with 2ml of Cryo A medium for every plate collected.

5.5.7. Count cells and dilute cells to 4-5x10^6cells/ml using Cryo A medium.

5.5.8. Slowly add and equal volume of Cryo B medium. Once all has been added, mix one time by gentle pipetting up and down.

5.5.9. With the same pipet, distribute 1ml of cell suspension to each of the prepared cryovials. Mix the cell pool every 6-10 vials for even distribution.

5.5.10. Quickly, tighten caps and place cryovials into an isopropanol containing freezing container. Place the freezing container in a -80°C freeze overnight.

5.5.11. Transfer cell vials to liquid nitrogen storage the following day.

Section 6: Transitioning Between Culture Platforms

Transfer between platforms should be done only after an initial bank of material has been frozen back in the original conditions. We recommend maintaining the initial cultures in the original conditions in parallel until the user can be confident that the transition is successful.

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Section 7: Revision History and Protocol Approvals

7.1. Revision History

Version	Change Description	Effective Date
A	Document Initiation.	05/02/17
B	CC00722, Added details on preparing Matrigel™ as a Geltrex alternative.	See SPDC

7.2. Approvals

10/31/2017 X JKG JKG Quality Assurance Manager Signed by: Gay, Jenna	10/31/2017 X JLB JLB Media Optimization and Core Lab Manager Signed by: Brehm, Jenny

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