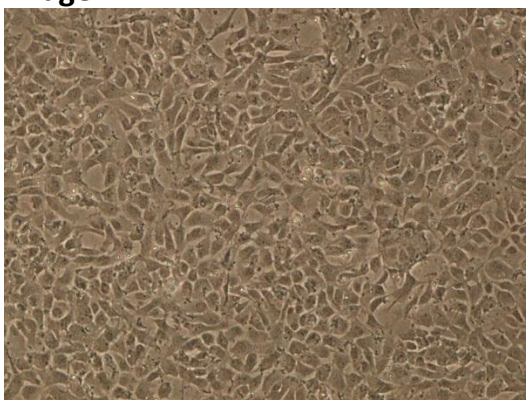
| Protocol: | NSC maintenance, freezing and thawing |
|---|---|

**Image:**



**Caption:**
**This is an image of NSC culture on geltrex surface.**

**Flow Chart:**

**Coating dishes with Geltrex**
**Enzymatic Passage of NSC**
**Maintenance of NSC**
**Cryopreservation of NSC**
**Thawing of NSC**

**Instructions:**

**Coating dishes with Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix**

1. Thaw Geltrex 5 ml in original bottle overnight at $4^0$C.
2. Aliquot to 1 ml/tube, store at -20°C (tips, pipets and tubes should be prechilled)
3. Thaw the tube 1-2 hours on ice or O/N at 4 degree
4. Dilute this 1:50 in cold Neurobasal media and coat dishes (2 ml/60mm, 1 ml/35 mm).
5. Incubate RT for 1 hr.

**Enzymatic Passage of NSCs**

1. Carefully aspirate medium from 60 mm dish containing confluent NSCs.
2. Add 1-2 mL of pre-warmed accutase onto the dish, place it in the cell culture incubator and for 3-5 min until cells detach.
3. Collect detached NSCs using P-1000 pipette and place cells in 15 mL conical tube. Rinse the dish once again with 3 mL of pre-warmed neurobasal medium and collect in the same tube.
4. Centrifuge cells at 580 x g for 4 min
5. Aspirate supernatant carefully and re-suspend cells in 3 mL of NSC medium.

**Note:** Cells from one confluent 60 mm dish shall be split onto three new 60 mm dishes (split 1:3). Approximately 2-2.5 million of cells per 60 mm dish will give a desirable density.

6. Aspirate geltrex solution from freshly coated dishes, add 4 mL of NSC medium into each plate and transfer 1 mL of NSCs (from step 5) into each plate.

7. Distribute NSCs evenly.
8. Incubate at 37°C/ 5% $CO_2$ and change medium every other day.

**Maintenance of NSC**

1. Observe and assess NSC culture every day. Generally media should be change every other day.
2. Condition aliquot of fresh NSC media in the water bath at $37^0$C.
3. Carefully aspirate media from the cell culture dish.
4. Slowly add fresh media on to the dish allowing fluid to slither along the wall, avoid pouring media directly onto the cells.

**Cryopreservation of NSCs**

This protocol can be applied for NSCs collected from several dishes.

1. Carefully aspirate medium from 60 mm dish containing confluent NSCs.
2. Add 1-2 mL of pre-warmed accutase onto the dish, place it in the cell culture incubator and for 3-5 min until cells detach.
3. Wash off NSCs from the dish using P-1000 pipette and place cells in 15 mL conical tube. Rinse the dish once again with 3 mL of pre-warmed neurobasal medium and collect in the same tube.
4. Centrifuge cells at 580 x g for 4 min
5. Re-suspend cells in a small volume of NSC-FREEZE-A medium (1 mL or more if multiple dishes are combined) and count them.
6. Dilute cells using the same medium to obtain desired cell density (4-5 millions cells/mL).
7. Carefully add an equal volume of NSC-FREEZE-B medium containing 20% DMSO under constant swirling (final cell density 2-2.5 millions cells/mL). Mix gently 2-3 times by pipetting.
8. Alternatively to steps 5-7: Resuspend cells in 500 µl of CryoStem Freezing medium to obtained cell density of 2-2.5 millions cells/mL.
9. Aliquot into cryogenic vials (1 mL/vial).
10. Immediately freeze at -80˚C using isopropanol contraption and transfer vials into liquid nitrogen tank the following day.

**Thawing NSC from Frozen Stocks.**

1. Thaw cells in 37°C water bath
2. Transfer cells to 15 mL conical tube containing 5 mL of pre-warmed Neurobasal medium under constant swirling. Wash the cryo-vial with additional 1 mL of medium and transfer to the same 15 mL tube.
3. Centrifuge cells at 580 x g for 4 min
4. Aspirate supernatant and re-suspend cells in 5 mL of NSC medium
5. Plate onto geltrex coated dishes (2 millions/35 mm dish)
6. Distribute evenly and incubate at 37°C/ 5% $CO_2$
7. Change medium every other day

**Materials:**

Neurobasal medium SFM, Life Technologies 21103049
B27 supplement, Life Technologies 17504044
MEM NEAA, Life Technologies 11140050
GlutaMax-I, Life Technologies 35050061
Rec Hu bFGF, Stemgent 03-002
DMSO, Sigma
Accutase, Life Technologies
Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix,
Life Technologies A1413202
D60, D35 CT Dishes Corning
Cryovial Corning
Conical tubes 15 ml, 50 ml Corning
CryoStem Freezing Medium, Stemgent 01-013-51

**Media :**

1. NSC Expansion Medium (NSC medium): Neurobasal medium supplemented with B27 (1x), 2 mM MEM NEAA MEM, 2 m*M* GlutaMAX-I, Rec Hu bFGF 10 ng/mL,
   For 100 mL: Neurobasal 96 mL, B27 supplemet 2 mL, MEM NEAA 1 mL, GlutaMAX-I 1 mL, Rec Hu bFGF 10 µL.
2. NSC freezing medium A (NSC-FREEZE-A): Neurobasal medium supplemented with B27 (1x), 2 m*M* MEM NEAA, 2 m*M* GlutaMAX-I. For 100 mL: Neurobasal 96 mL, B27 supplement 2 mL,MEM NEAA 1 mL, GlutaMAX-I 1 mL,
3. NSC freezing medium B (NSC-FREEZE-B): Neurobasal medium supplemented with 20% DMSO, 2 m*M* MEM NEAA, 2 m*M* GlutaMAX-I. For 100 mL: Neurobasal 78 mL, DMSO 20 mL, MEM NEAA 1 mL, GlutaMAX-I 1 mL

**FAQs:**

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**Last Updated: 02/03/2012**

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