# ArunA Biomedical's hNP1™ Oris™ Cell Migration Assembly Kit - FLEX

This protocol describes the use of ArunA Biomedical's hNP1™ Neural Progenitor Cells in conjunction with an Oris™ Cell Migration Assembly Kit- FLEX to measure the effect of neuroactive compounds and biologics that modulate proliferation and migration of neural progenitor cells. Detailed instructions are given for plate coating procedures, thawing and expansion of the ArunA hNP1™ cells and use of the Oris™ Cell Migration Assembly Kit. A total of 96 wells may be used with this kit in up to 4 separate experiments.

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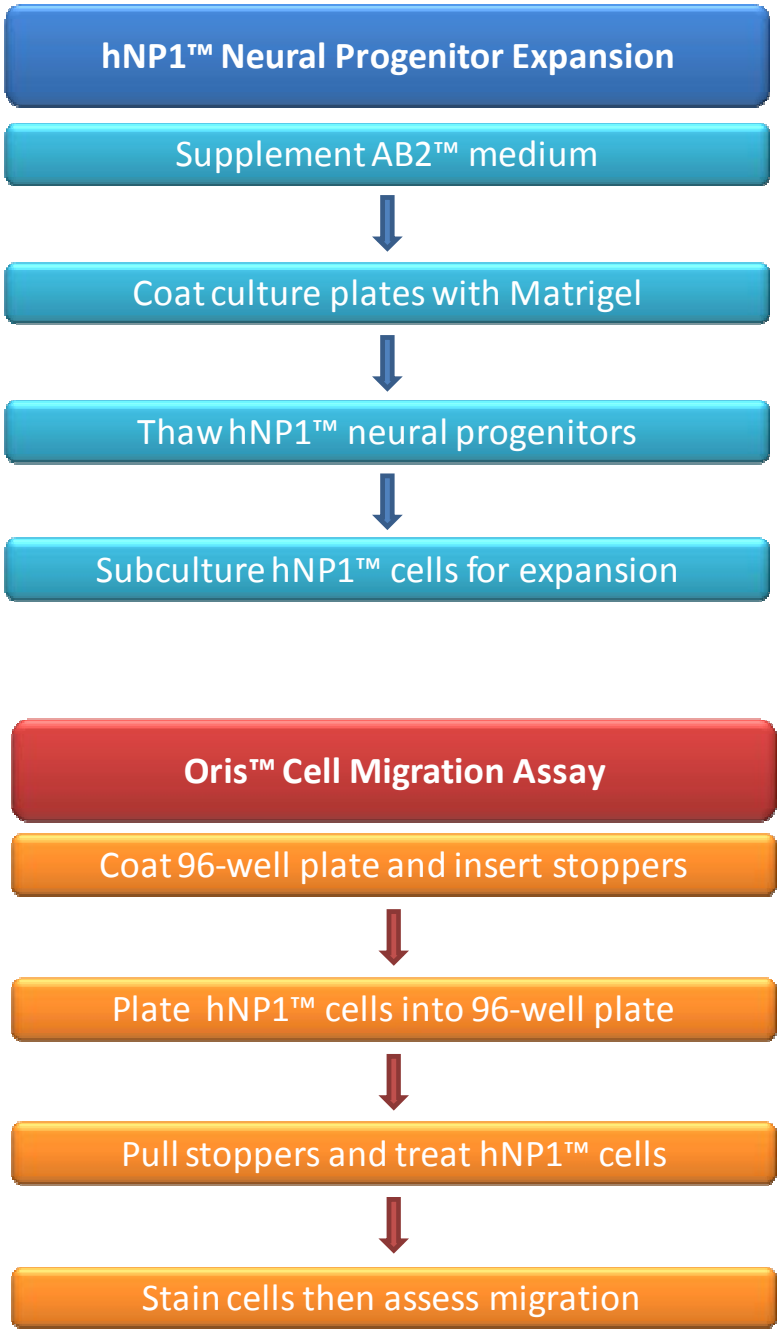
# 1. PROTOCOL OVERVIEW



# 2. Contents of Kits and Storage Instructions

**Kit Contents**

**Please note that the kits must be ordered separately from ArunA Biomedical and Platypus Technologies.**

***hNP1™Neural Progenitor Expansion Kit (Cat# hNP7013.1): ArunA***

- hNP1™ Human Neural Progenitor Cells, one vial (>$10^6$ viable cells)
- AB2™ Neural Culture Medium, one 500-mL bottle
- ANS™ Neural Supplement, five 2-mL vials

***Oris™ Cell Migration Assembly Kit – FLEX (Cat# CMAUFL4): Platypus Technologies***

- Oris™ compatible, 96-well plates, 4
- Oris™ Cell Seeding Stoppers, 4 packs of 24 *(sufficient for 96 tests)*
- Oris™ Detection Mask, 1
- Oris™ Stopper Tool, 1

**Required but not Supplied**

- bFGF (50 µg/mL)
- LIF (10 µg/mL)
- L-Glutamine (200 mM)
- BD Matrigel™ Basement Membrane Matrix
- Phosphate Buffered Saline (with calcium and magnesium)
- Neurobasal® Medium, phenol-red free
- Bovine Serum Albumin
- Calcein AM (1 mg/mL in DMSO)
- Hoechst 33342 (10 mg/mL in $H_2O$)

**Optional but not Supplied**

- Penicillin (5,000 U/mL) plus Streptomycin (5,000 µg/mL)

**Unpacking and Storage Instructions**

**hNP1™ Human Neural Progenitor Cells**

- Cells must be moved from dry ice to liquid nitrogen IMMEDIATELY. Temperature fluctuations will have adverse effects on cell health and viability.
- When stored in the recommended storage conditions (liquid nitrogen), hNP1™ Human Neural Progenitor Cells can remain stable in excess of 3 years.

**Medium and Supplements**

- Upon arrival, store the AB2™ Neural Medium at 2-8°C protected from light.
- Upon arrival, store ANS™ Supplement at -20°C.
- After supplements are thawed, use within one month.
- Do not refreeze

**Oris™ Cell Migration Assembly Kit – FLEX**

- Can be stored at 2-8°C for up to 1 year

## *Planning note:*

From an initial plating of ~$1x10^6$ hNP1™ cells post-thaw, approximately 3 doublings will yield ~$8x10^6$ cells. At 60,000 cells per well for the cell migration assay (see Section 4), this yield will be sufficient for an entire 96-well plate. With an approximate doubling time of 3-4 days, you should plan for a 2 week period for hNP1™ expansion. Since the Oris™ Cell Migration Assembly Kit – FLEX provides the flexibility for running 4 partial plate experiments of 24 wells each, you can adjust the cell number needed according to the number of wells you actually need for a given experiment.

# 3. hNP1™ Neural Progenitor Expansion

**3.1 Supplementing the AB2™ Basal Medium**

1. Decontaminate the external surfaces of all supplement vials and the medium bottle with ethanol or isopropanol.
2. Aseptically open each supplement vial and add the amount indicated below to the basal medium with a pipette.

| To make 100 ml of complete medium: | |
|---|---|
| AB2™ Neural Medium | 96 mL |
| ANS™ Supplement | 2 mL |
| bFGF, 50 µg/mL | 40 µL |
| LIF, 10 µg/mL | 100 µL |
| L-Glutamine, 200 mM | 1 mL |
| Penicillin (5,000 U/mL)/ Streptomycin (5,000 µg/mL) | 1 mL |

3. Supplemented medium should be stored at 2-8°C, protected from light. The complete medium should be given a 2 week expiration date. Dispense the complete medium into aliquots to avoid repeated heating prior to each use.

**3.2 Plate Coating Protocol for hNP1™ Neural Progenitor Expansion**

To coat dishes perform the following steps:

1. Thaw BD Matrigel™ at 2-8°C overnight. Matrix will gel rapidly at 22°C to 35°C. Keep Matrigel™ on ice and use pre-cooled pipettes, plates and tubes when preparing. Gelled Matrigel™ may be re-liquified if placed at 2-8°C on ice for 24 to 48 hours.
2. Handle using aseptic technique in a laminar flow hood.
3. Once BD Matrigel™ Matrix is thawed, swirl vial to be sure that material is evenly dispersed.
4. Place thawed vial of BD Matrigel™ Matrix in sterile area, decontaminate the external surfaces with ethanol or isopropanol and air dry. BD Matrigel™ Matrix may be gently pipetted using a pre-cooled pipette to ensure homogeneity.
5. Dilute Matrigel™ 1:200 with cooled Dulbecco's Modified Eagle's Medium. Keep on ice.
6. Add 2 mL diluted Matrigel™ to a 35-mm dish. Swirl to ensure the entire surface of the 35-mm dish is covered with the Matrigel solution.
7. Place dishes at 2-8°C for 1-3 hours.
8. Rinse thoroughly with PBS.
9. Remove PBS and use immediately.

**3.3 Cell Thawing Protocol for hNP1™ Neural Progenitor Expansion**

To plate the cells perform the following steps:

1. Do not thaw the cells until the recommended medium and appropriately coated plasticware and/or glassware are on hand.
2. Remove the vial from liquid nitrogen and incubate in a 37°C water bath. Closely monitor until the cells are completely thawed. Maximum cell viability is dependent on the rapid and complete thawing of frozen cells.
   *IMPORTANT: Do not vortex the cells. Breaking cells down to single cell suspensions will significantly increase cell death.*
3. As soon as the cells are completely thawed, disinfect the outside of the vial with 70% ethanol or isopropanol. Proceed immediately to the next step.
4. In a laminar flow hood, use a 1 or 2 mL pipette to transfer the cells to a sterile 15 mL conical tube. Be careful to not introduce any bubbles during the transfer process.

5. Using a 10 mL pipette, slowly add dropwise 9 mL of fully supplemented AB2™ Neural Medium (pre-warmed to 37°C) to the 15 mL conical tube.
   *IMPORTANT: Do not add the whole volume of medium at once to the cells. This may result in decreased cell viability due to osmotic shock.*
6. Gently mix the cell suspension by slow pipetting up and down twice. Be careful to not introduce any bubbles.
   *IMPORTANT: Do not vortex the cells. Breaking cells down to single cell suspensions will significantly increase cell death.*
7. Centrifuge the tube at room temperature at 200 x g for 4 minutes to pellet the cells.
8. Aspirate as much of the supernatant as possible. Steps 4-8 are necessary to remove residual cryopreservative (DMSO).
9. Resuspend the cells in a total volume of 2 mL of fully supplemented AB2™ Neural Medium (pre-warmed to 37°C).
10. Plate the 2 mL cell suspension of hNP1™ cells onto a Matrigel-coated 35 mm dish.
11. Incubate the cells at 37°C in a 5% $CO_2$ humidified incubator.
12. Exchange the medium with fresh fully supplemented AB2™ Neural Medium 24 hours post plating. Exchange with fresh medium every other day thereafter. Use caution not to dislodge the cells; do not pipette media directly onto the cells but rather onto the side of the culture dish.
13. Once the hNP1™ cells reach 100% confluence, they can be dissociated manually for passaging (e.g., by cell scraping or by gentle and slow pipeting up and down to detach the cells). The cells should be maintained at a high density at all times – the recommended passaging ratio is 1:2.

**3.4 Subculture of hNP1™ Cells**

1. Once the hNP1™ cells reach 100% confluence, carefully remove the medium from the 35 mm dish.
2. Apply 2 mL fully supplemented AB2™ Neural Medium (pre-warmed to 37°C) to the cells so that the cells can be harvested in fresh medium.
3. Using a pipette, manually detach the cells from the dish by slow pipeting up and down the dish. Be careful to avoid introducing any bubbles. We recommend using a 200 µL or 1000 µL manual pipette to dislodge the attached cells. Alternatively, cells can be dislodged with a sterile cell scraper.
   *IMPORTANT: We do NOT recommend enzymatic methods for passaging the hNP1™ cells. Doing so reduces the long term viability of the cells and can cause karyotypic abnormalities.*
4. Plates should be observed to ensure that all cells have been removed. This is most easily accomplished by working under a dissection microscope within a laminar flow hood, but can also be achieved by frequent observation under a bright field or phase contrast microscope.
5. Transfer the dissociated cells to a 50 mL conical tube. Inspect the plate to ensure that all the cells have been removed.
6. If necessary, count the cells and calculate the cell concentration. Cells can be centrifuged at 200 x g for 4 minutes in order to concentrate the cell suspension for higher plating densities.
7. Plate the cells at the desired density into the appropriately coated flasks, plates or wells (see section 4.1) in fully supplemented AB2™ Neural Medium. We recommend keeping the cells at a high cell density by passaging 1:2.
8. Incubate the cells at 37°C in a 5% $CO_2$ humidified incubator.
9. Exchange the medium with fresh fully supplemented AB2™ Neural Medium 24 hours post plating. Exchange with fresh medium every other day thereafter. Use caution not to dislodge the cells; do not pipette media directly onto the cells but rather onto the side of the culture dish.

# 4. Oris™ Cell Migration Assay – FLEX

## 4.1 Plate Coating Protocol for Cell Migration Assay

1. Thaw BD Matrigel™ at 2-8°C overnight. Since it will gel rapidly at 22°C to 35°C, keep Matrigel™ on ice and use pre-cooled pipettes, plates and tubes when preparing. Gelled Matrigel™ may re-liquefy if placed at 2-8°C on ice for 24 to 48 hours.
2. Handle using aseptic technique in a laminar flow hood.
3. Once the Matrigel™ is thawed, swirl vial to be sure that material is evenly dispersed.
4. Place thawed vial of Matrigel™ in sterile area, decontaminate the external surfaces with ethanol or isopropanol and air dry. Matrigel™ may be gently pipetted using a pre-cooled pipette to ensure homogeneity.
5. Dilute Matrigel™ 1:200 with cooled AB2™ Neural Culture Medium. Prepare ~1 mL diluted Matrigel for each column (8 wells) to be used. Keep on ice.
6. Add 100 µL of diluted Matrigel™ to each well intended for use in the 96 well plate.
7. Tap the plate gently to ensure the entire surface of the well is covered with diluted Matrigel.
8. Place dishes at 2-8°C for 1-3 hours.
9. Remove the residual coating solution and rinse each well twice with 200 µL of PBS per well.
10. Remove PBS and insert the Oris™ Cell Seeding Stoppers into the coated wells of the 96-well plate (page 7, section 4.3, Figure 1).
11. Visually inspect to ensure that the Oris™ Cell Seeding Stoppers are firmly sealed (page 7, section 4.3, Figure 2).

## 4.2 Cell Migration Assay Protocol

1. Harvest cells as described in steps 1-5 of section 3.4 – ***Subculture of hNP1™ Neural Progenitor cells*** (page 5)
2. Count cells and adjust cell suspension volume to the following concentration: 600,000 cells/mL
3. Plate 100 µL of suspended cells into each stoppered well for a cell density of 60,000 cells per well.
4. Incubate the cells at 37°C in a 5% $CO_2$ humidified incubator overnight (16-24 hours) to permit cell attachment.
5. Using the Oris™ Stopper Tool, remove all stoppers (page 7, Figure 3), except for those in "no migration controls" which will remain in place until time of staining.
6. Carefully remove the seeding media from the wells and add 200 µL medium containing the test compound per well.
7. Briefly examine the wells by phase contrast microscopy to ensure continued adherence of the cells.
8. Incubate the cells at 37°C/5% $CO_2$ for 72 hours to permit cell migration.
9. After 72 hours, mix 5 µL Calcein AM, 5 µL Hoechst 33342, and 10 mL phenol red-free Neurobasal medium with 0.1% BSA.
10. Carefully remove stoppers from the “no migration controls”.
11. Carefully remove the test medium from all wells and add 100 µL of diluted Calcein/Hoechst solution to each well.
12. Incubate plate at 37°C/5% $CO_2$ for 30 - 60 minutes with the lid on and in the dark (the darkness of a standard incubator will suffice).
13. For use with a fluorescence microplate reader, attach the Oris™ Detection Mask (page 7, section 4.3, Figure 4) and read promptly for Calcein fluorescence (ex 494 nm/ em 517 nm).
14. For image analysis, photomicrograph wells using epifluorescence illumination with or without the Oris™ Detection mask (see page 7). Images can then be analyzed using either area closure with the calcein stain or number of cells (nuclei) using the Hoechst stain. ImageJ freeware available from the NIH (http://rsbweb.nih.gov/ij/) can be used for migration data analysis as percent area closure or cellular enumeration.

For further information on the Oris™ Cell Migration Assembly Kit- FLEX visit http://www.platypustech.com/Oris_Protocol_AssemblyKit_FLEX.pdf

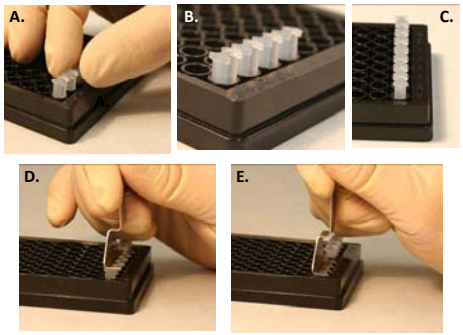



Figure 1. Stopper Insertion Process. A) Placement of Stoppers into Wells, B) Close-up of Stoppers Partially Inserted into Wells, C) Proper Placement of Stoppers, D) Pressing of Stoppers into Wells, and E) Fully Inserted Stoppers.

## 4.3 Instructions for using the Oris™ FLEX Kit stoppers and mask

**Figure 1**. Populate the plate with Oris™ Cell Seeding Stoppers (provided in 4-stopper strips) under sterile conditions:

- Vertically position the tip ends of two, 4-stopper strips into one full column of 8 wells at a time (Figure 1A).
- Gently press down on the strip backbone to partially insert the stoppers halfway into the well (Figure 1B).
- When both stopper strips have been partially inserted in 1 column, ensure that the position of the stoppers is vertical with respect to the well wall, making any necessary adjustments (Figure 1C).
- Using the Oris™ Stopper Tool, firmly press down on the strip backbone to fully insert the stoppers into each well (Figure 1D and 1E). Repeat for the remaining columns.
- Once the sterile pouch of Stoppers has been opened, handle the stoppers aseptically. Any unused stoppers can be kept in a sterile environment (i.e., laminar flow hood/UV light). Do not autoclave the stoppers.
- It is extremely important to ensure that the stoppers are fully inserted and perpendicular to the well bottom.

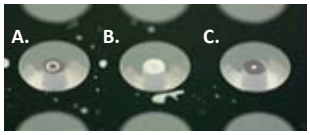



Figure 2. Stoppers that are: A) Partially Sealed, B) Unsealed and C) Completely Sealed

**Figure 2**. Visually inspect the populated plate to ensure that the Stoppers are firmly sealed against the bottom of the plate. To inspect the stoppers, turn the plate over and examine the stoppers for sealing, i.e., bullseye pattern. If incomplete sealing is observed, return the plate to the upright position and use a sterile instrument to gently push the stopper back into the well until sealing is observed.

**Figure 3**. Use the Oris™ Stopper Tool to remove stoppers

- The Stopper Tool can be made sterile by washing it with 70% alcohol.
- Secure the 96-well plate by holding it firmly against the deck of your work space. Slide the tines of the Stopper Tool under the backbone of the stopper strip, keeping the underside of the Stopper Tool flush with the top surface of the plate.
- Lift the Stopper Tool ***vertically*** to gently remove the stoppers.

**NOTE: DO NOT** use the Stopper Tool as a lever to pry the stoppers from the well (see Figure 3E), as this may displace seeded cells and distort the detection zone area.

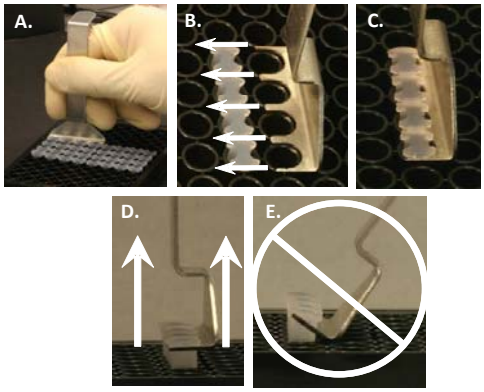



Figure 3. Removal of Stoppers. Panels A, B, and C) Position the Tines of the Stopper Tool between the Stopper Tips, D) Lift Vertically, and E) Do NOT Pry Stoppers

**Figure 4**. Apply the Oris™ Detection Mask to the bottom of the plate if microplate reader data is being collected. The Detection Mask is not necessary if collecting image-based data.

- Orient the chamfered corners of the mask with those of the 96-well plate, ensuring that the A1 corner of the mask is aligned with the A1 well of the plate (see Figure 4).
- Align the holes in the attachment lugs with the bosses on the bottom of the 96-well plate.
- Gently press the mask until it is flush with the bottom of the 96-well plate.

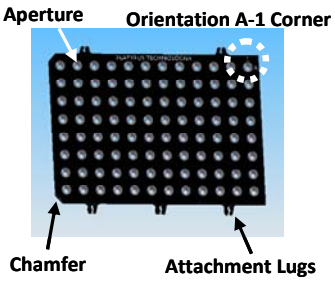



Figure 4. Mask Features.