# CELL CULTURE PROTOCOLS

NAN QIN
Jan., 13, 2014
Division of Clinical Neurochemistry
Institute of Clinical Chemistry and Laboratory Medicine
and the Department of Medicine
University of Dresden
Fetscherstr 74
01307 Dresden
Germany

# Contents

# 1 MPC Cluture

## 1.1 Making collagen A flask

### 1.1.1 Items required

- Gibco, DPBS # 14190
- Biochrom, Collagen A # L7220

### 1.1.2 Procedure

- make 4% Collagen A solution in DPBS (fresh)
- put corresponding amount of collagen A solution into incubation flask.
- distribute collagen A solution over bottom of flask
- after 2 hours, remove collagen A solution and wash flask 2 times with DPBS

## 1.2 Making up MPC media

### 1.2.1 Items required

- Gibco, Horse Serum # 26050
- Gibco, FBS # 10108165
- Gibco, DPBS # 14190
- Gibco, RPMI 1640 # 52400
- Promo Cell, TNS= Trypsin inhibitor # C-41120
- Promo Cell, Trypsin/EDTA # C-41020
- Antibiotics Aliquot

### 1.2.2 Procedure

- Clean hood with ethanol and bleach.
- making Madium
  - RPMI 1640 contant: 10% Horse Serum, 5% FBS and 0.1% antibiotics
- Remove 75ml of RPMI 1640 from the 500mL bottle (this number represents the total volume of constituents to be added to the bottle), and discard into a waste container
- To the RPMI 1640 add the following:
  - 50 ml donor horse serum
  - 25ml of fetal calf serum
  - 0.5mL antibiotics
- Mix the contents by rocking the bottle up and down gently
- Store media in 4° C fridge, and monitor for color changes and growths.

## 1.3 Feeding MPC cells

### 1.3.1 Procedure

- Clean hood with ethanol and bleach.
- Remove cells from incubator, and monitor media for variables such as color and turbidity
- Remove media from flask and discard
- Add new media to flask and return flask to incubator
- Store media in 4° C fridge, and monitor for color changes and growths.

## 1.4 Splitting MPC cells

### 1.4.1 Items required

- 70% ethanol & bleach
- Clean 50ml falcon tubes
- Serological pipettes
- Trypsin-EDTA
- DPBS

### 1.4.2 Procedure

- Clean hood with ethanol and bleach.
- Remove cells from incubator, and monitor media for variables such as color and turbidity
- Remove media from flask and discard
- Wash cell monolayer with 10ml of DPBS (DPBS should be specifically for cell culture use only). Rotate PBS over monolayer gently (dont want to remove any of the cells).
- Put 4ml of Trypsin-EDTA into flask. Place in incubator for 5 minutes. After this time, the cells should be sufficiently removed from the flask. if not, repeat pipetting by using 10 ml pipette.
- Add 4ml TNS
- Replace the contents of flask into the 15ml falcon tube.
- Spin tube for 5 minutes 480g at room temperature.
- Remove tube from centrifuge, and return to hood. Discard supernatant (into waste container).
- Resuspend cells and split into new flasks. Usually split about 1:4

# 2 Cell Release Assay

## 2.1 Making up Krebs Buffer

**Krebs Buffer:**

- 0.126 M NaCl; 7.36 g/L NaCl
- 2.5 mM KCl; 0.186 g/L KCl
- 25 mM NaHCO3; 2.1 g/L NaHCO3
- 1.2 mM NaH2PO4; 0.166 g/L NaH2PO4.H2O
- 1.2 mM MgCl2; 0.244 g/L MgCl2.6H2O
- 25 mM CaCl2; 0.368 g/L CaCl2.2H2O

## 2.2 Making up Krebs Buffer+K

**Krebs Buffer+K:**

- 25 mM NaCl; 1.461 g/L NaCl
- 100 mM KCl; 7.455 g/L KCl
- 25 mM NaHCO3; 2.1 g/L NaHCO3
- 1.2 mM NaH2PO4; 0.166 g/L NaH2PO4.H2O
- 1.2 mM MgCl2; 0.244 g/L MgCl2.6H2O
- 25 mM CaCl2; 0.368 g/L CaCl2.2H2O

## 2.3 Making up Krebs Buffer+K

**Krebs Buffer+100$\mu$M Nicotine:** 1.59 $\mu$l Micotine +100 ml Krebs Buffer

## 2.4 Release

- Cells cultured in 24 well plate
- After 48 hours culture calculation the cell numbers
- Remove media from wells and discard
- Add release buffer 500$\mu$L/well
- Return flask to incubator, 10 min at 37°C.
- Remove release buffer to label tubes
- Add 500$\mu$L Perchloride solution into control well
- Remove cell perchloride solution into label tubes
- Release samples are ready for HPLC assay

### 2.5 HPLC analyst

- Buffer 1: 20 dilution
- 300 $\mu$l extraction
- Perchloride Solution 1: 40 dilution
- 90 $\mu$l injection

**PHEO cell**

- Buffer (without dilution) 50 $\mu$l extraction
- Perchloride Solution 1: 10 dilution
- 90 $\mu$l injection

# 3 Human Pheochromocytoma Dissociation

## 3.1 General Considerations

- Individual tumors have distinct characteristics. Try to cover all bases
- Different lots of collagenase can dramatically affect yield. It may be necessary to test several lots and stock up on one that works well.
- A modest amount of starting tissue (no more than 2 cm cube) will often give a better yield than a huge amount.
- Sedimentation at unit gravity is an easy and efficient method for separating dissociated tumor cells from blood and debris (see #1 under Tumor Dissociation below).
- Plan to freeze dissociated cells right away. Don't expect to "grow them up" first because they probably won't grow. Replicate vials of frozen cells can be used to repeat experiments! Similarly, if you plan to inject tumor into mice do it right away. Small minced tissue fragments can be injected sc or ip with a 16 gauge or larger needle.
- To determine whether pheo cells are truly growing, perform double staining for TH and incorporation of BrdU at 2 wks. This is usually the point at which you can stop wasting your time.

## 3.2 Tumor Dissociation

- Mince tumor tissue into 2-3mm chunks in a 100 mm dish containing 10 ml calcium- and magnesium-free Hank's (CMF HBSS; Gibco cat # 12444-014) in a laminar flow hood. Use 2 scalpels, one in each hand, and be sure to slice, not crush the tissue. OCCASIONALLY, this by itself will dislodge a sizable number of tumor cells. Therefore, collect the CMF HBSS (if the chunks they are very bloody rinse in CMF HBSS and collect the rinse to pool with the CMF HBSS that was used for mincing), spin ( 250g $\times$ 5 min), aspirate sup and re-suspend pellet in culture medium with 15% FBS. Fill a 15 ml conical tube with the suspension, close it and leave it in a vertical position at room temperature for at least 1/2 hr. A gray-tan sediment containing the tumor cells will accumulate at the bottom of the tube while RBCs and debris remain suspended. Aspirate everything above the cloud of sediment after it looks like the sediment is all down. Examine a drop of the sediment microscopically to decide whether anything from this step is worth keeping.
- Rock the minced tissue pieces in a 15 ml conical tube containing collagenase B solution (1.5 mg/ml, Boehringer Manheim, cat # 1088 823, 0.22 $\mu$m filter-sterilized) in HBSS (not CMF) for 45 min at 37°C. Volume of enzyme soln should be at least 10$\times$ volume of tissue pieces. DNAase is often not necessary during collagenase digestion. If a DNA gel begins to form add DNAse 1 (to final concentration 50 ug/ml, from a 20$\times$ frozen stock soln).
- Stand the tube vertically, let the pieces settle out, aspirate enzyme soln. Fill tube gently with CMF HBSS, invert, let pieces settle out again and aspirate again. Add 35 ml of CMF HBSS to the tube and this time triturate the tissue pieces in CMF HBSS. Trituration can be performed first in a 1ml plastic pipette from which the tip has been broken off under sterile conditions (snap it off while the pipette is still in its disposable sleeve), then in an intact plastic pipette and then in 9" Pasteur pipettes. Don't brutalize the cells or tissue. After a couple of minutes of trituration, all cells that are going to dislodge will have done so (Chunks that have not completely dissociated can either be discarded or subjected to

an additional round of digestion in fresh collagenase). Collect the cell suspension, add a few ml of culture medium with 15% FBS (even a small amount of serum will protect cells during centrifugation). Spin, (discard sup), re-suspend pellet in complete medium and sediment the tumor cells as in #1 above. At this point, the cells will be mostly in small spherical clusters.

### 3.3 Tumor Cell Purification (optional)

Pheo cells can often be highly enriched by differential attachment or detachment but it is not predictable whether or not this will work with any particular tumor. Pheo cells dissociated in collagenase or mechanically dislodged during initial mincing often attach very poorly to collagen. However, they attach well after subsequent trypsinization.

- Plate cells in complete medium (RPMI 1640 with L-glutamine, Gibco cat# 11875-093; 15% fetal bovine serum, Gibco cat# 10437-028; 100 units/ml penicillin, 100 $\mu$g/ml streptomycin, Gibco cat# 15140-122 ) in a collagen coated dish (we recommend collagen prepared according to Tischler lab collagen protocol- All collagen preps are not equivalent!). Also try dish with no collagen. Place dishes in the tissue cultur incubator at 37°C and examine periodically. It may become apparent in as little as 1 hour that fibroblast-like cells have selectively attached. If so, gently pipette off and save the tumor cells. If this rapid effect is not apparent, culture the cells for 2-3 days, then gently spritz the culture medium with a 9" Pasteur pipette to selectively dislodge and collect loosely attached tumor cells.

### 3.4 Lysis Buffer

add 8.26 g NH4Cl, 1g KHCO3 (or NaHCO3). 0.037g EDTA in 1L water (adjust pH 7,35). after standing 5 minutes at RT. The cell suspension was centrifuged with 200 g for 5 minutes at RT.

### 3.5 Trypsinization (optional but often desirable) and Long-term Culture

Mechanically dislodged cells, cells freshly dissociated in collagenase or cells purified and collected as above may eventually attach in the absence or presence of collagen. However, trypsinization serves to break up cell clusters and improve attachment.

- Centrifuge the cells and rinse/spin $\times 2$ in CMF HBSS. Re-suspend pellet in a 15 ml conical tube in 5 ml trypsin (0.25%, in CMF HBSS without EDTA). Add DNAse 1 (to final concentration 50 $\mu$g/ml from a 20x frozen stock soln).
- Rock tube for 45 minutes at 37°C.
- Add 2 ml of complete culture medium (RPMI 1640 with L-glutamine, Gibco cat# 11875-093, 15% fetal bovine serum, Gibco cat# 10437-028, 100 units/ml penicillin, 100 $\mu$g/ml streptomycin, Gibco cat# 15140-122). Centrifuge (500 $\times$ g, 5 minutes at room temperature). Aspirate sup, re-suspend pelleted cells in 2ml complete medium. Triturate the cells using a flame constricted (about 50% of original diameter) glass 9 inch pasteur pipette (pre-wetted with complete medium to prevent sticking), dilute as required and plate. Most pheos will attach to collagen after trypsinization. Also, use of McCoy's 5A medium instead of RPMI promotes attachment, flattening and neurite outgrowth.

### 3.6 Freezing

Until recently we have frozen cells in complete culture medium with 15% serum and 10% DMSO. This year our laboratory started using pre-prepared freezing medium (Gibco cat.# 11101-011), which so far seems to be giving better results. Freezing should be done in some sort of controlled-rate freezing device and the vials should be kept in liquid N2 for long-term storage.

# 4 Human Pheochromocytoma Primary Cell Phenotype Control

- Day 1: Freeze a small piece fresh tissue for analyzing catechols content.
- Day 2: analyze the primary cell catechols content.
  - Take small amount of cell suspension (500 $\mu$L).
  - Account the cell number from the cell suspension.
  - Centrifuge the cell suspension with 200 g for 5 minutes at RT.
  - Remove the supernatant. Resuspense the cell pellet with 300 $\mu$L perchloride solution.
  - Storage the cell pellet perchloride solutions in -80°C fro HPLC analyze.
- This step will be repeated every 6 days.

# 5 Bovine Chromaffin Cell Isolation

## 5.1 Primary culture

- Briefly immerse adrenal glands with 70% ethanol, and transfer to PBS.
- Remove the fat tissue on the glands. Perfuse the gland with PBS through vein until most of blood is flushed out.
- Inject digestion solution (Collagenase 0.3%, 0.01% DNase in PBS) in to gland. Incubate the tissue under 37°C for 15 minutes. Repeat this step for 2 more times (45 minutes in tatol).
- cut the tissue, remove the medulla. If the tissue is not digested well, roughly cut with scalpel. collect all medulla tissue in PBS. Repeat pipteeing by using 25 ml pipette, and 10 ml pipette until the tissue is homogenized.
- Filter the homogenized tissue with sieve that the undissociated tissue will be removed. Further filter it through 100 $\mu$m sieve in tunnel and cell strainer.
- Put the filtrate into 50 ml tubes, add PBS until around 45-50 ml. Centrifuge with 200 g for 8 minutes. Remove the supernatant. Resuspense the pellet with PBS wash again.
- Pool all the pellet together and resuspense in 20-50 ml medium. Filter again with 100 $\mu$m cell strainer if aggregate forms.
- Culture the cells in 10% steroid-free FBS in DMEM/F12 (Gibco 31330) and 1% Pen Strep (Gibco 15140).

## 5.2 Differential Plating

- After overnight culturing, the cells suspense in the medium are removed and transferred to a new flask. Further culture the cells for 1.5 hr. Repeat this step for 2 more times.
- Collect the cells by centrifuging under 200 g for 8 minutes. Count the cell number. Seed the cells in 7000 cells/ml with 10% steroid-free FBS in DMEM/F12 in low-attachment plate. Culture the cells for 2 weeks until chromospheres are ready for further experiment.

# 6 Tissue and Cells for Measurments of Catecholamines

## 6.1 Materials

- 0.4 M Perchloric acid containing 0.5 mM EDTA (250 mL to 500 mL stock). Use deionized milli-Q-filtered H2O.
- Polytron homogenizer with suitably sized small 5-7 mm generator. Required for most tissue specimens (exceptions brain). Note: A small (5-7 mm) sized generator is suitable for most tissue specimens of less than 400 mg homogenized in 12 x 75 mm polypropylene tubes.
- Ultrasonic cell or tissue disrupter with suitably sized probe Required for cell culture specimens - can also be used for small brain tissue samples. Note: Small probes suitable for volumes of up to 1 mL (at the most 2 mL) in eppendorf tubes (up to 1 mL) or 12 $\times$ 75 mm polypropylene tubes (up to 2 mL).
- Beaker (100-250 mL).
- Deionized milli-Q water (for washing generator or probe).
- Precision weighing balance (scales) accurate to 0.0001 gm (tissues).
- Cell counter (cell culture).
- Centrifuge (refrigerated for samples spun for more than 2 minutes).
- Ice.
- Dry ice.

## 6.2 Procedures

### 6.2.1 Tissue samples

- Tissue samples (5-400 mg) are weighed frozen (requires appropriate setting of precision balance)transfer from dry ice with clean forcepssamples may be weighed on disposable foil/plastic weighing material or in processing tubes (e.g., on a tared balance) and should be immediately transferred back to dry ice in processing tubes once weighing is complete (should take at the most 6 seconds). Weights must be recorded down to at least 0.0001 gm (0.1 mg).
- An appropriate (at least 5 volumes - v/w) accurately measured volume of 0.4 M perchloric acid is added to each processing tube. Volumes must be accurately recorded (easiest to keep to a set volume - e.g., a constant 500 l for tissue samples weighing between 5 to 100 mg or 1 mL for samples up to 200 mg).
- Tissue samples are then homogenized using a polytron (use 12 $\times$ 75 mm processing tubes with a polytron). This should take 30 seconds, at the most 1 minute. Samples should be kept cold during homogenization using ice in a beaker placed around the processing tube.
- Tubes are then spun for 15 minutes at 3000 rpm in a refrigerated centrifuge to separate the precipitated proteins and cell debris (refrigerated centrifuge generally required for 12 $\times$ 75 mm tubes, but with a fast 1 minute spin on a microfuge refrigeration is not necessary).
- The perchloric acid extract is separated from the pellet (use disposable Pasteur pipettes), frozen on dry ice and stored at -80oC until assayed for catecholamine contents.

### 6.2.2 Cell culture samples

- Cells are counted (this may be done using a separate representative sample) and incubating media is removed.
- A suitable and accurately measured volume (generally less than 1 mL per 20,000 cells) of perchloric acid is added to the cells (this may be added directly to cell culture wells). Added volumes of perchloric acid must be recorded.
- Use of an ultrasonic cell disrupter is recommended for liberation of catecholamines from stores. For this it may be most appropriate and convenient to place the tip of the probe directly into each well before transferring the contents into labeled microfuge tubes. If this is inconvenient then plates may be frozen at -80oC after which the contents of each well can be transferred to labeled microfuge tubes before use of the ultrasonic disrupter.
- Microfuge tubes are then spun at high speed for up to 2 minutes to separate the precipitated proteins and cell debris.
- The perchloric acid extract is separated from the pellet (use disposable pasteur pipettes), frozen on dry ice and stored at -80oC until assayed for catecholamine contents.
- For chromaffin cells (e.g., primary cultures of bovine chromaffin cells; PC12 cells) a total of 10,000 cells should be sufficient for reliable measurement of cellular catecholamine contents. Higher cell numbers may be required for other cell types or chromaffin cells with decreased capacity for catecholamine synthesis and storage.

# 7 Western Blotting

## 7.1 Selection Guide: Secondary Antibodies

Most primary antibodies are produced in mouse or rabbit host species; therefore, anti-mouse IgG and anti-rabbit IgG are the most popular classes of secondary antibodies. Goat is the host species most easily and frequently used by manufacturers to produce polyclonal anti-mouse and anti-rabbit secondary antibodies. Consequently, goat secondary antibodies against mouse IgG and rabbit IgG are commercially available in the widest variety of forms. Several kinds of anti-mouse and anti-rabbit secondary antibodies from other host species are also available. See the table below for help locating secondary antibodies with specificity for mouse or rabbit antibodies and their conjugation options.

## 7.2 Protocol

- Sample preparation: 25% LDA Sample Buffer + 10% DTT 10× (6.25$\mu$l LDA Sample Buffer + 2.5$\mu$l DTT 10× + 16.25$\mu$l protein samples) 3 min 100∘C
- 4 ml low Gel + 2 ml upper Gel
- The amount of protein that should be loaded to the gel varies with the experiment; it can be 25 to 100 $\mu$g per sample.
- After putting the sample in the gel, run until the blue marker goes to the end of the gel.

| Power Suppply: | | | |
|---|---|---|---|
| 0:30 | 60 V | Max. mA | Max. W |
| 2:30 | 100 V | Max. mA | Max. W |

| Power Suppply from ClearPage: | | | | |
|---|---|---|---|---|
| fresch lower runn buffer | 60-90 min | 175 Vcon | 80 mA | 14 W |
| used lower runn buffer | 60-90 min | 150 Vcon | 60 mA | 9 W |

- Transfer
  - Wet the membrane in methanol for 15 seconds. Membrane should uniformly change from opaque to semi-transparent.
  - Carefully place the membrane in Milli-Q water and soak for 2 minutes.
  - Carefully place the membrane in transfer buffer and let equilibrate for at least 5 minutes.

| Power Suppply: | | | |
|---|---|---|---|
| 2:30 | 200 V | Max. mA | Max. W |

| Power Suppply (ClearPAGE): | | | |
|---|---|---|---|
| 1.5-2 hours | 220 Vcon | 160 mA | 36 W |

| Power Suppply (G Bio.): | | | |
|---|---|---|---|
| 2 hours | 100 V | 200 mAcon | 30 W |

- Blocking: block with 5 % milk in PBS. 2% milk for antibody. Block for 30 min shaking (Slowly) at room temperature.
- Primary antibody: After blocking, without washing, put the membrane in 1×PBS with the antibody in a 1:1000 dilution and incubate it "overnight" at 4°C in the shaker. Note that the optimal dilution for a specific antibody will vary and should be determined by the enduser.
- First wash: After the primary antibody incubation wash the blot three times with 1×TBS and then wash 3 times (10 min with 1×PBS + 0.1% Tween).
- Secondary antibody: Incubate for 1 hours in 1×PBS + 0.1% Tween with the secondary antibody concentration 1: 6000.
- Secondary wash: After the secondary incubation wash rapidly 3 times with 1×PBS + 0.1% Tween.
- Cemiluminescence Reaction: Incubate the membrane in the 1ml working Detection Solution(RT) for 3-5 minute.

# 8 RNA Extraction

- 300$\mu$L **ice-cold** Cell Disruption Buffer for $10^6$ cells
- 100$\mu$L **ice-cold** Cell Disruption Buffer for 10$\mu$g Tissue
- Homogenize on ice
- Transfer the lysate that will be used for RNA isolation to a tube containing an equal amount of 2× Denaturing solution **at room temp**.
- Immediately mix thoroughly.
- Incubate the mixture on ice for 5 min.
- Add a volume of Acid-Phenol:chloroform**Be sure to withdraw the bottom phase** equal to the total volume of the sample lysate plus the 2× Denaturing solution.
- Vortex for 30-60 sec to mix.
- Centrifuge for 5 min at maximum speed (10,000× g) **at room temp** to separate the mixture into aqueous and organic phases.
- Remove the aqueous (upper) phase, and transfer it to a fresh tube. **Note the volume recovered**.
- Add 1.25 volumes of **room temp** 100% ethanol to the aqueous phase and mix thoroughly.
- Pipet the lysate/ethanol mixture onto the Filter Cartridge (700$\mu$L/ Cartridge).
- Centrifuge for 30 sec.
- Discard the flow-through, and repeat until all of the lysate/ethanol mixture is through the filter.
- Apply 700$\mu$L miRNA Wash Solution 1 to the Filter Cartridge and cenrifuge for 15 sec. Discard the flow-through.
- Apply 500$\mu$L Wash Solution 2/3 and draw it though the Filter Cartridge.
- Repeat with a second 500$\mu$L of Wash Solution 2/3.
- After discarding the flow-through, spin the assembly for 1 min.
- Transfer the Filter Cartridge into a fresh Collection Tube. Apply 100$\mu$L of **95°C** Elution Solution or nuclease-free water.
- Centrifuge for 30 sec to recover the RNA.

| Name | Weight | Total Cell Disruption Buffer | Cell Disruption Buffer for RNA | 2×Denaturing solution **at room temp** | Acid-Phenol:chloroform (Bottom) | Aqueous | Ethanol | H2O |
|---|---|---|---|---|---|---|---|---|
| | mg | $\mu$L | = $\mu$L | Total $\mu$L | $\mu$L | $\mu$L | 1.25 × $\mu$L | $\mu$L |

# 9 PNMT Enzyme Activity

## 9.1 Stock solution

- **Incubation Buffer** 0.4 M Tris pH 8.5, 4.85 g Tris in 80 ml H2O; adjust pH to 8.5 with 6 M HCL
- **Incubation Time** 20 min 37 C
- **100 mM NE** Use NE-HCl 20.564 mg/mL
- **2.5 mM D3-SAM** 5 mg D3-SAM in 1.836 mL (0.2 M HAC pH 2.6)
- **Calibration curve**
  - **IS1 for calibration curve** 1000 ng/mL D6-EPI
  - **IS2 for sample preparation** 50 ng/mL D6-EPI (20$\mu$L)
  - **Calibration curve using D3-EPI**

| Name | D3-EPI concentration | Volume of D3-EPI ($\mu$) | final concentration of calibrator | volume of MP ($\mu$L) | Volume of IS1($\mu$L) |
|---|---|---|---|---|---|
| STD1 | 1 ng/mL | 100 $\mu$L | 0.1 ng/mL | 880 | 20 |
| STD2 | 100 ng/mL | 10 $\mu$L | 1 ng/mL | 970 | 20 |
| STD3 | 10 $\mu$g/mL | 10 $\mu$L | 100 ng/mL | 970 | 20 |
| STD1 | 10 $\mu$g/mL | 50 $\mu$L | 500 ng/mL | 930 | 20 |

## 9.2 Procedure

The reaction mixture, consisting of 920 $\mu$L of 0.4 M Tris/HCl buffer (pH 8.5), 20 $\mu$L of 2.5 mM d3-SAM, 20 $\mu$L of 2.5 mM d3-EPI and 40 $\mu$L of tissue preparation in a total volume of 1 mL in 1.6 mL polypropylene eppendorf tubes, was incubated for 20 min at 37°C in a water bath. The reaction was stopped by addition of 20 $\mu$L of glacial acetic acid and then mixed for 20 min on a Vortex-Genie 2T (VWR, Darmstadt, Germany) with 0.25mM d6-EPI (in 20 $\mu$L) as internal standard, 400 $\mu$L of 1M Tris/HCl (pH 8.6 with EDTA) and 5 mg alumina (stored at 100°C before use). After brief centrifugation the supernatant was aspirated and the alumina washed once with Milli-Q water. Following aspiration of the water wash, 50 $\mu$L of a solution consisting of water/acetonitrile (98:2, V/V) and 2% formic acid was added to the alumina and vortexed for 45 sec in order to elute the catecholamines. After centrifugation, the supernatant was transferred into sample vials ready for injection onto the autosampler.

# 10 3D-Nucleofecotor Optimization Protocol

## 10.1 Prepare SiRNA for transfection

| SiRNA | Company | RNase free buffer | final concentration | Volume require for 20 $\mu$L transfection mix | Volume require for 100 $\mu$L transfection mix |
|---|---|---|---|---|---|
| MAX (m) | Santa Cruz | 66 $\mu$L | 50 $\mu$M | 4 $\mu$L | 20 $\mu$L |
| H2A (r) | Santa Cruz | 66 $\mu$L | 50 $\mu$M | 2.5 $\mu$L | 12.5 $\mu$L |
| MAX (m) | Origene | 20 $\mu$L | 100 $\mu$M | 0.85 $\mu$L | 4.25 $\mu$L |
| H2A (m) | Gen Skript | | 1 $\mu$g/$\mu$L | | 2 $\mu$L |
| c-Myc/MAX/Control shRNA | Santa Cruz | 100 $\mu$L | 200 ng/$\mu$L | 2 $\mu$L | 10 $\mu$L |

## 10.2 Plasmid information

| Plasmid Vector | Company | final concentration | Volume require for 20 $\mu$L transfection mix | Volume require for 100 $\mu$L transfection mix |
|---|---|---|---|---|
| HIF2alpha | Genscript | 1$\mu$g/ $\mu$L | 0.8 $\mu$L | 4 $\mu$L |
| MAX | Genscript | 2.2$\mu$g/ $\mu$L | 0.4 $\mu$L | 2 $\mu$L |
| Empty Vector | Genscript | 4.7$\mu$g/ $\mu$L | 0.2 $\mu$L | 1$\mu$L |

## 10.3 Master mixes for 16 samples (20 $\mu$L transfection)

| | |
|---|---|
| Suspension cells | $3.2\times10^6$ to $1.6\times10^7$ |
| Adherent cells | $1.6\times10^6$ to $0.8\times10^7$ |
| SE/SF/SG X solution | 279 $\mu$L |
| Supplement | 62 $\mu$L |

## 10.4 Master mixes for 100 $\mu$L transfection

| | |
|---|---|
| Suspension cells | $1\times10^6$ to $0.5\times10^7$ |
| Adherent cells | $0.5\times10^6$ to $0.25\times10^7$ |
| SE/SF/SG X solution | 85.5 $\mu$L |
| Supplement | 19 $\mu$L |

## 10.5 Procedure

### 10.5.1 Cell culture recommendations for adherent cells

- Subculture 1 - 2 days before Nucleofection
- Optimal confluency for Nucleofection: 70 - 80%. Higher cell densities may cause lower Nucleofection Efficiencies

### 10.5.2 Nucleofection

- Select for the appropriate Nucleofector Program (DG-134 for MPC, CM-167 for PC12, CM-150 for MTT). Note for 3T3 (Ruben): SE solution and program CM-137.
- Prepare cell culture plates by filling appropriate number of wells with desired volume of recommended culture media and pre-incubate plates in 37°C and 5% CO2 incubator.
- Prepare Mixes
- Harvest the cells by trypsinization
- Count an aliquot of the cells
- Centrifuge the required number of cells at 90×g for 10 minutes at room temperature. Remove supernatant completely
- Resuspend each cell pellet carefully in room temperature Nucleofector Mixes (320 $\mu$L/16 well)
- Transfer 20 $\mu$L of the aliquots into the wells.
- OR Resuspend each cell pellet carefully in room temperature Nucleofector Mixes (100 $\mu$L/well) and transfer 100 $\mu$L into the well.

## 11 Medium Summary for Transfection Cell line

| Cell | Fetal Bovine Serum | Horse Serum | Base Medium | Antibiotics for Selective Culture |
|---|---|---|---|---|
| MPCwt & MPC+H2A | 5% | 10% | RPMI 1640 | G418 500 $\mu$g/mL |
| PC12wt & PC12+MAX | 2.5% | 15% | F-12K | G418 500 $\mu$g/mL |
| PC12wt/wt, PC12wt/-EPAS1 | 2.5% | 15% | F-12K | G418 500 $\mu$g/mL & Puromycin 12.5 $\mu$g/mL |
| PC12+MAX/wt, PC12+MAX/-EPAS1 | 2.5% | 15% | F-12K | G418 500 $\mu$g/mL & Puromycin 12.5 $\mu$g/mL |
| MPCwt & MPC-c-Myc | 5% | 10% | RPMI 1640 | G418 500 $\mu$g/mL & Puromycin 0.5 $\mu$g/mL |

# 12 Immunostaining for Cells and Tissue

## 12.1 Products

- Triton-x100 (cat. No. 934418, Fluka): 10% Trition in PBS
- Bovine Serum Albumin BSA (cat. No. A1470, Sigma)
- Donkey Serum (cat. No. S30, Millipore)
- Permibilisation Solution (donkey Serum/Triton/BSA (5%/0.3%/1%) in PBS
  - For 50 mL staining solution: 47.5 mL PBS + 2500 $\mu$L donkey serum + 1500 $\mu$L Trition (10%) + 100 mg BSA
  - Aliquote in 5 mL and freeze at -20°C
- Staining Solution (donkey Serum//BSA (5%//1%) in PBS
  - For 50 mL staining solution: 47.5 mL PBS + 2500 $\mu$L donkey serum + 500 mg BSA
  - Aliquote in 5 mL and freeze at -20°C
- DAPI working stock (1:10000 in PBS) lift protected and stored in 4 °C
- Primary antibody (diluted with staining solution)
- Secondary antibody (diluted with staining solution)

## 12.2 Cell Staining

| Step | Time |
|---|---|
| Wash culture well with PBS | 2 × 5 min, RT |
| Fix cells in 4% PFA | 30 min, RT |
| Wash culture well with PBS | 1 × quickly wash, 2 × 5 min, RT |
| Block with permibilisation solution | 30 min, RT |
| Staining with primary antibody | 2 hours , RT or overnight 4°C |
| Wash culture well with PBS | 1× quickly wash, 2 × 5 min, RT |
| Staining with secondary antibody | 1 hours , RT |
| Wash with PBS | 1 × quickly wash, 1 × 5 min, RT |
| Staining with DAPI | 1:10,000 in PBS, 5 min, RT |
| Wash with PBS | 2 × 5 min, RT |
| Mount with Aqua Poy-Mount on glass slides | |
| Dry slides at RT and keep slides at 4°C in dark | |

### 12.3 PFA Fixed Tissue Staining

| Step | Time |
|---|---|
| take slide from -80° | |
| Dry slides at RT | |
| Scratch the tissue tek with pipette tip | |
| Put around the hydrophobic marker | |
| Wash culture well with PBS | 2 × 5 min, RT |
| Block with permibilisation solution | 30 min, RT |
| Staining with primary antibody | 2 hours , RT or overnight 4°C |
| Wash culture well with PBS | 1× quickly wash, 2 × 10 min, RT |
| Staining with secondary antibody | 1 hours , RT |
| Wash with PBS | 1 × quickly wash, 1 × 5 min, RT |
| Staining with DAPI | 1:10,000 in PBS, 5 min, RT |
| Wash with PBS | 2 × 5 min, RT |
| Mount with Aqua Poy-Mount on glass slides | |
| Dry slides at RT and keep slides at 4°C in dark | |

# 13 Cell Cycle

## 13.1 Materials

- PBS
- Culture Medium
- 1.0 % bovine serum albumin (BSA) (w/v) in PBS
- 0.5 % Tween 20 (v/v) plus 1.0 % BSA (w/v) in PBS
- 1 mM BrdU. Reconstitute in PBS to make a working solution. Aliquots may be frozen for later use.
- Direct Immunofluorescence Staining: FITC-conjugated Anti-BrdU
- Indirect Immunofluroescence Staining: Anti-BrdU
- Propidium iodide stock, 1 mg/mL in PBS. Dilute to 5 ug/mL in PBS for flow cytometric analysis.
- 2N HCl with 0.5 % Triton X-100 (v/v)
- 0.07N NaOH
- 0.1 M sodium tetraborate (Na2B4O7.10H2O), pH 8,5

## 13.2 Reagent make

- 10 $\mu$M Brdu stocksolution
- 2 N HCl/0.5% Triton = 83.33 mL conc. HCl 2.5 ml of Triton X-100 bring up to 500 ml in dH2O
- 0.1 M Na2B4O7 = 19.07 g sodium borate bring up to 500 mL in dH2O, pH to 8.5 with HCl

## 13.3 Defination

Cell cycle analysis is a method in cell biology that employs flow cytometry to distinguish cells in different phases of the cell cycle. Before analysis, ,the cells are permeablised and treated with a fluorescent dye that stains DNA quantitatively, usually propidium iodide (PI). The fluorescence intensity of the stained cells at certain wavelengths will therefore correlate with the amount of DNA they contain. As the DNA content of cells duplicates during the S phase of the cell cycle, the relative amount of cells in the G0 phase and G1 phase (before S phase), in the S phase, and in the G2 phase and M phase (after S phase) can be determined, as the fluorescence of cells in the G2 /M phase will be twice as high as that of cells in the G0 /G1 phase. Cell cycle anomalies can be symptoms for various kinds of cell damage, for example DNA damage, which cause the cell to interrupt the cell cycle at certain checkpoints to prevent transformation into a cancer cell (carcinogenesis). Other possible reasons for anomalies include lack of nutrients, for example after serum deprivation.

### 13.4 Method

- Plate 1-100 million cells on 100 mm dish
- Add BrdU directly to the culture medium to achieve a final concentration of 10 $\mu$M. Incubate the cells for 60 minutes, 120 minutes, 240 minutes in the CO2 incubator at 37° C.
- Prepare 70% EtOH, and place 5 mL of 70% EtOH into glass tubes and store at -20°C until ready for use.
- Remove labeling medium. Wash 2× with PBS. Trypsinize, neutralize (using 12 mL plastic tube), spin down (500× g, 15 min).
- Wash 1× with cold PBS-spin down
- Resuspend cell pellet in 0.2 mL PBS.
- Slowly add cells, a few drops at a time, into the ethanol (5 mL ethanol, keep in -20°C) while maintaining a vortex. Incubate on ice for 30 minutes. The cells are now fixed.
- Centrifuge cells at 500 ×g for 5 minutes at 10C. Aspirate the supernatant carefully. Loosen peller by vortexing.
- Slowly add 1mL of 2N HCl/triton X-100 to the cells, a few drops at a time, while maintaining a vortex. Incubate at RT for an additional 30 minutes. This denatures the DNA to produce single-stranded molecules.
- Centrifuge the cells at 500 × g for 10 minutes. Aspirate the supernatant. (Sedimentation of fixed cells may require higher speed. Remove Super.)
- Resuspend in 1ml of 0.1 M Na2B4O7, pH 8.5 to neutralize the acid. Look at cells to see if too clumpy, and count cells. (Brdu labeled cells may be stored at this stage by spining down, and resuspending in 1 ml 70%ETOH with storage at -20°C). Aliquot to 1× $10^6$/tube, and spin down.
- Resuspend in 50 $\mu$L of staining buffer (0.5% Tween 20/1%BSA/PBS) containing 5 $\mu$L Anti-Brdu-Fitc (Boehringer) for 45 minutes at 37°C.
- Add 1 mL staining buffer, spin down at 500 ×g for 6-10 minutes; resuspend in 0.5 mL PBS containing 5 $\mu$g/ml PI and 100 $\mu$g/ml Rnase(5 $\mu$L of 10 mg/ml stock)
- Filter for FACS, and store in refrigerator overnight. Or incubate at RT 30 minutes and do FACs directly.

# 14 Migration and Invasion, Corning Incorporated 3421, migration kit

## 14.1 Definition

Cell migration, the movement of cells from one area to another generally in response to a chemical signal, is central achieving function such as wound repair, cell differentiation, embryonic development and the metastasis of tumors.
Cell invasion is similar to cell migration; however, is requires a cell to migrate through an extracellular matrix (ECM) or basement membrane extract (BME) barrier by first enzymatically degrading that barrier and to then become established in a new location.
Cell invasion is exhibited by both normal cells in responses such as inflammation and by tumor cells in the process of metastasis.
Transwell inserts from Corning Life Sciences, provides a relatively simple in vitro approach to performing chemotaxis and cell invasion assays.
Common barriers employed for invasion assays include collagen, fibronection and laminin coating as well as more complex extracellular or basement membrane extracts.
**Recommended Cell Seeding Concentrations and Volumes**

| Assay plate format | Cells/well | Seeding volume/Insert (mL/Insert) | Reservoir volume (mL/well) |
|---|---|---|---|
| 96 Well | 0.5 | 0.05 | 0.15 |
| 24 Well | 1.0 to 2.0 | 0.1 | 0.65 |
| 12 Well | 1.0 to 2.0 | 0.385 | 1.0 |
| 6 Well | 3.0 to 4.0 | 1.5 | 2.0 |

## 14.2 Methods

- Lower well containing (RPMI 1640 5% FBS, 10%HS, and 50 nM rhEGF-I as a chemoattractant. (using 10 ug/mL IGF-I. diluted with full serum medium (1:25 for migration))
- using typsine stripping cells
- count cell number
- 100 $\times$ 1000 cells were added to the collagen coated insert with 100 $\mu$L full serum medium
- after 18-24 h of incubation at 37°C in 5% CO2, the cells remaining on the upper membrane surface we're removed with a cotton swab
- cells on the lower surface of the filter were fixed and stained
  - wash with PBS 1 min
  - Fix with PFA 4% 10 min
  - wash with water 2-3 min
  - Incubate with crystal violet 60 min RT: Crystal violet (0.2% w/v in 2% v/v 100% ethanol) = 200 $\mu$L EtOH 100% + 9800 $\mu$L water + 20 mg crystal violet
  - wash with water 4 times, 5 min each
  - cut membranes carefully. do it fast to avoid dryness
  - count cell number in lower medium and membrane

# 15 CytoSelect 24-Well Wound Healing Assay Cat. CBA-120

## 15.1 Definition

Wounded tissue initiates a complex and structured series of events in order to repair the damaged region. These events may include increased vascularization by angiogenic factors, an increase in cell proliferation and extracellular matrix deposition, and infiltration by inflammatory immune cells as part of the process to destroy necrotic tissue. The wound healing process begins as cells polarize toward the wound, initiate protrusion, migrate, and close the wound area. These pricesses reflect the behavior of individual cells as well as the entire tissue complex.

## 15.2 Assay Protocol

### 15.2.1 Cell Migration

- Create a cel suspension containing 300,000 cells/ml in media.
- Add 50 $\mu$L of cell suspension to each well by carefully inserting the pipet tip through the open end at the top of the insert. For optimal cell dispersion, add 250 $\mu$L of extra medium to either side of the open ends at the top of the insert.
- Incubate cells in a cell culture incubator overnight or until a monolayer forms.
- Carefully remove the insert from the well to begin the wound healing assay.
- Slowly aspirate and discard the media from the wells. Wash well with media to remove dead cells. Finally, add media to wells to keep cell hydrated.
- Visualize wells under a light microscope. Repeat wash if wells still have debris or unattached cells.
- When washing is complete, add media to continue cell culture and wound healing process.
- For best results, use a reticle with micrometer measurements to create a defined surface area in order to monitor the closing, or healing of the wound.
- Monitor the wound closure with a light microscope or imaging software.

### 15.2.2 DAPI Florescence Labeling

- Cells can be fixed by removing media and adding 0.5 mL of Fixing Solution to each well (4% PFA).
- Allow the cells to fix for 10 minutes a RT. Aspirate and discard the solution.
- Carefully wash each well 3 $\times$ with PBS.
- Dilute DAPI 1:1000 in PBS
- Add 0.5 mL of DAPI solution to each well to be stained. Incubate 15 minutes at RT.
- Carefully wash each well well 3 $\times$ with PBS. Add 1mL PBS to each well to keep cells hydrated.

### 15.2.3 Cell Staining

- Remove the media and add 400 $\mu$L of Cell Stain Solution to each well.
- Aloow the stain to incubate with the cells for 15 minutes at RT. Aspirate and discard the solution.
- Carefully wash each stained well 3 $\times$ with deionized water. Discard washes and allow cells to dry at RT.

## 15.3 Calculation of Results

### 15.3.1 Percent Closure

- Determine the surface are of the defined wound area. Total Surface Area = 0.9mm $\times$ length
- Determine the surface area of the migrated cells in to the wound area. Migrated Cell Surface Area = length of cell migration (mm) $\times$ 2 $\times$ length
- Percent Closure (%) = Migrated Cell Surface Area / Total Surface Area $\times$ 100

### 15.3.2 Migration Rate

- Dtermine the migration rate of cells into the defined wound area: Migration Rate = length of cell migration (mm) / migration time (hr).

## 16 List of Stock Solution

| Name | Company | Ser. No. | Concentration |
|---|---|---|---|
| G418 | Life Technologies | 10131-027 | 50 mg/mL |
| Puromycin | Life Technologies | A1113803 | 10 mg/mL |
| rhIGF-I | Sigma Aldrich | I3769 | 1 mg/mL |

# 17 For Western Blotting

## 17.1 4°C

| Product Name | P/N | Firma | Size (kDa) | Dilution | Species reactivity |
|---|---|---|---|---|---|
| Anti-H2A (rabbit) | NB100-122 | Novus | 118 | 1:100 - 1:1000 | Fish, Rat, Ms, Hu |
| Anti-Rabbit IgG (HRP) | NB730-H | Novus | | 1:1000- 1:30,000 | |
| PHD3 | NB100-303 | Novus | | | |
| Max (C-17) (rabbit) | sc-197 | Santa Cruz | 26 | 1:100-1:1000 | Ms, Rat, Hu |
| Anti-H1A (rabbit) | NB100-479 | Novus | 93 | 1:500-1:2000 | Hu, Ms, Rimate, Rat |
| Anti-H2A (rabbit) | ab199 | Abcam | 100 | 1:200-1:1000 | Hu, Ms, Rat |
| Anti-Goat IgG (HRP) | NB710-H | Novus | | 1:1000-1:30,000 | |
| Anti-Actin (mouse) MAB 1510R | Millipore | 43 | 1:1000 | | |
| goat anti-rabbit IgG (HRP) | SC-2004 | Santa Cruz | | 1:500-1:10,000 | |

## 17.2 -20°C

| Product Name | P/N | Firma | Size (kDa) | Dilution | Species reactivity |
|---|---|---|---|---|---|
| Protein Marker A5418 | | AppliChem | | | |
| Precision Plus Marker | | Biorad | | | |
| Chemiluminescent Marker | | Magic | | | |
| Anti-Rest | HPA006079 | Sigma | | | |
| Overexpression Lysat REST | | Novus | | | |
| Transfected Lysate REST | | Novus | | | |
| Anti-PNMT (rabbit) | AB110 | Millipore | 31 | 1:1000-1:5000 | Hu, Rat |
| Anti-MAX (goat) | NB100-793 | Novus | 23 | 1:1000-1:5000 | Hu, Ms, Rat |

### 17.3 -80°C

| Product Name | P/N | Firma | Size (kDa) | Dilution | Species reactivity |
|---|---|---|---|---|---|
| Anti-beta-Actin (rabbit) | ab8227 | Abcam | b38 | 1:1000-1:5000 | Hu, Ms, Rat, Chk, Cow, Dog, Fish |
| H2A control (-) | NBL1-10286 | Novus | | | |
| H2A control (+) | NBL1-10286 | Novus | 96.3 | | |
| Anti-PNMT (rabbit) | ab90862 | Abcam | 31 | 1:600 | Rat, Ms, Hu |
| COS-7 Hypoxic control | NB800-PC26 | Novus | 96 | 20 $\mu$L + 4 $\mu$L sample buffer | |
| Human MAX protein | H00004149 | | 42 | 3 $\mu$L for loading | |
| PC12-Nuclear extraction | NB810-55229 | Novus | 97 | | |