Kelly Lab
6/29/07

Rat Neonatal Cardiocyte Prep Using Kit

Reagents:
NCIS kit: Worthington, catalog number

Cat#	Pack Size	Price	
LK003300	1 kit	175.00	Purchase Online
LK003303	3 kit	475.00	Purchase Online

- BrdU: (SIGMA B5002) 10 mM stock solution (100X) 307.1 mg BrdU/100 mL tissue culture grade water, store at -20°, ok for 6 months at -20, make single use aliquots
  OR 1gram/326ml
- Penicillin/streptomycin (1000X), store at -20°
- Insulin (Sigma I-1882), 10 mg/mL stock in water (1,000,000X), store at 4°
- Transferrin (Sigma T-8158), 10 mg/mL stock in water (1000X), store at 4°
- Fatty Acid Free BSA (Sigma B-6003), 200 mg in 3 mL water, add to 400 mL of differentiation media before filtering (final concentration is 0.5 mg/mL)
- Gelatin, 0.1% (Specialty Media 1-800-543-6029 cat #ES-006-B)

Other Supplies:
Straight scissors for sternotomy (Fine Science Tools 1-800-521-2109 cat #14101-14)
Fine straight scissors for mincing (Fine Science Tools 1-800-521-2109 cat #14090-09)
Curved forceps (Fine Science Tools 1-800-521-2109 cat #11003-12)
Gauze sponges (4"X4"), PSS #2237

Hypertrophy Stimulants:
Phenylepherine: make fresh just before use, protect from light. Historically, the lab has used a range of 40 to 100 µM.
Endothelin 1: 10^{-8} M

Ligands:
9-cis retinoic acid (Sigma R-4643): 1 mg (Prepare 10^{-2} M solution in 334 µL DMSO; dilute 1:10,000 for final concentration of 10^{-6} M)
Oleate, albumin-conjugated: Sigma O-3008, molar ratio of albumin:oleate varies by lot
Call SIGMA when receiving a new lot number and ask for Oleate concentration and BSA concentration
See "Day after Harvest" for the addition of ligands

Rats

From Sasco, order "# litters of 1-day old rat pups. Ship with mother, separate litters.
Do NOT HOUSE. Call 2-8912 upon arrival
Order by Wed at 2pm to receive them the following week. Can either arrive on Tuesday (late am) or Thursday (early am).
Each litter usually contains 10-12 pups.

NOTE:
If litter has 15 pups, they are usually smaller than normal so only count them as 12

Calculation of Hearts Needed for an Experiment:
This may differ slightly with different hands harvesting the hearts

Roughly, figure that you will need 2 litters for 3 12-well plates.

				Lower density used	Lower density used
	24-well	12-well	6-well	100 mm	T-75
Surface area (cm^2)	2	4	10	60	75
Ratio to 24-well	1	2	5	30	37.5
Hearts to plate	0.25	0.5	1.25	6	7.5
Media volume	500ul	1ml	2ml	10ml	15ml

***The density of hearts used for a 100 mm plate or T-75 flask is slightly lower, but this density seems to work better on the larger plates. Keep this in mind when calculating amount of DNA or virus to use.

NOTE:
Each kit contains 5 sets of vials. Each set of vial can be used for up to 2 litters. (We have gotten away with using 3 litters as well, but this is pushing the limits.)

If you have 2 litters, perform the trypsin digest in 2 100mm plates and then combine for Day 2.

Day -1: **Preparation the Day Before Harvest**

Initial Media:

	Final Conc.	Amount for 200 mL
DMEM (4.5 g/L glucoseL)		q.s. to 200 mL
Horse Serum	10%	20 mL
Fetal Calf Serum	5%	10 mL
BrdU (100X)	100 µM	2 mL
L-glutamine (100X)	2 mM	2 mL
Pen/Strep (1000X)		0.2 mL

Sterile filter and store at 4°.
You will need 1ml of initial media per well of a 12-well.

Differentiation Media:

	Final Conc.	Amount for 400 mL
DMEM (1.0 g/L glucose)		q.s. to 400 mL
BrdU (100X)	100 µM	4 mL
L-glutamine (100X)	2 mM	4 mL
Pen/Strep (1000X)		0.4 mL
Transferrin (1000X)	10 µg/mL	0.4 mL
Insulin (1,000,000X)	10 ng/mL	0.4 µL
BSA (fatty acid free)	0.5 mg/mL	Dissolve 200 mg in 3 mL H2O; add to 400 mL media

Sterile filter and store at 4°.
You will need to prepare at least 2ml of diff media per well of a 12-well.

***For long time courses or hypoxia experiments, using a higher glucose concentration (4.5 g/L) and higher insulin (10 µg/mL) may be desirable.

Insulin calculations:
Final insulin concentration in diff media = 10ng/ml (or 0.00001g/L)
1mol/5734g * 0.00001g/L * 1×10^9 nM/M = 1.74nM

Supplies to Prepare:
Dissecting Instruments (**autoclaved**): large scissors, small scissors, curved forceps
Bag for Carcasses
250 mL Erlenmeyer flask for waste
2 150 mL glass beakers with 70% ethanol for washing dissecting instruments
Sterile gloves
100 mm dishes: 1 per litter
70% EtOH
CO_2 killing chamber

Day 1: Prepare Solutions for Digestions

Fill 1 or 2 ice buckets.
Make sure the HBSS (Reagent #1) is ice cold. Place 30-40ml in a 50ml conical on ice labeled "HEARTS". (1 for each litter)
Reconstitute Vial#2 (Trypsin) with 2ml of ice cold Reagent 1

PREPARE THE TISSUE CULTURE HOOD:
Right side of hood is "dirty"; left side is "clean." [left side: killing chamber, pups, gauze, EtOH bath; middle: instruments; right side: ice bucket, pipets]
Unwrap 4X4 gauze and stack them (*use sterile technique)* in back left corner of hood.
Add 70% ethanol to the bottom of one box of 4X4 gauze—this will be the EtOH bath for the pups. Place on left side of the hood.
Add 70% EtOH to the two 150 mL beakers. With sterile technique, unwrap the large scissors and forceps and place each into a beaker. (Keep small scissors wrapped for now.) Place in center of hood.
Place pipets and ice bucket on right side of the hood.
Hang a plastic bag off the left side of the hood (to hold carcasses).
Put the killing chamber and tank on the left side of the hood.
Hang a bag on the right side of the hood to collect pipets
**Place 100mm dish on ice

EUTHANIZE THE MOTHERS one at a time (you can save this for later if you want). Put live pups in the bottom of empty pipet tip boxes and place on the left side of the hood.

HARVESTING THE HEARTS

Open the tube labeled "HEARTS." Keep open and on ice during the harvest.

Put on sterile gloves. From this point on, the right hand should be kept sterile, and the left hand will be the "dirty" hand.

Using left hand, place 4-6 pups in killing chamber. When they are not moving, use left hand to transfer them to the ethanol bath, and immediately put the next 4-6 pups into the chamber.

With left hand, take one 4X4 gauze from the stack and hold it in left hand. With right hand, pick up a pup from the ethanol bath and place it on the gauze with its head toward you, feet away from you, and ventral surface up.

With right hand, pick up large scissors from EtOH bath. Using the point of the scissors (*not the blunt side),* create a median sternotomy. Scissors enter at the superior aspect of the sternum and cut through the bone to just above the diaphragm in a single cut.

Grip the back of the pup from underneath with your left hand; twist and squeeze. The heart pops up in the midline. With right hand, take the curved forceps, blot it on the sterile gauze to remove the EtOH, and remove the heart. (To take ventricles only, pinch the heart about 2/3 of the way up from the ventricles).

After the heart is removed, blot it on the gauze to remove as much blood as possible. Place the heart in the tube marked "HEARTS."

After all hearts have been harvested, cap the "HEARTS" tube and gently swirl to remove blood. DO NOT SHAKE!!

Let hearts settle, then draw off liquid with a 5 mL pipet to avoid pulling up the hearts. Replace the media with 10 mL fresh cold HBSS (vial #1). Swirl again, and use a 5 mL pipet to remove as much of the media as possible.

Pour the hearts into the cold 100 mm dish (1 litter per dish). Mince finely (about 5-10 minutes) with the small scissors to increase surface area of tissue before digestion.

Add HBSS to the Petri dish to a final volume of 9ml

Transfer <u>1ml</u> of the trypsin vial (vial #2) into the Petri dish and mix by swirling.

Place the lid on the Petri dish and immediately place in refrigerator overnight in the tissue culture room. Place a note on the door so that people are careful when opening the refrigerator. Incubate 16-20 hours.

Day 2:
Begin the following the next morning or early afternoon:

**Take INITIAL MEDIA out of refrigerator to warm to Room temp
**If transient transfection is planned, prepare DNA dilutions before beginning.

Prepare:
- Reagent #1, HBSS: 10ml ice cold
- Reagent #3. Trypsin inhibitor: Reconstitute one of Vial #3 with 1ml Reagent #1, Room temperature
- Reagent #4 Collagenase: Reconstitute one of Vial #4 with 5ml prepared Leibovitz L-15. Room temperature

Remove petri dish from refrigerator after overnight incubation and bring to sterile hood on ice. Pool and transfer tissue and buffer from 1-2 plates (1-2 litters) to the same 50ml conical tube on ice using 10ml pipet.

Transfer contents of Vial #3 (trypsin inhibitor) into tube and mix.

Warm tissue and buffer to 30-37°C in water bath, maintaining sterility (approx 20 min)

Slowly transfer the contents of Vial #4 (collagenase) into tube and mix. Cap tube tightly.

Place tube on a slowly rotating shaker at 37°C and incubate for 30-45 minutes. (Room 826, large glass front incubator has a shaker that you can use)

NOTE: ALL SUBSEQUENT STEPS AT ROOM TEMP!!

Remove tube from incubator and return to sterile hood. With 10ml pipet, triturate 10 times to release the cells, approximately 3ml/sec. Do not be too vigourous. Avoid bubbling the cell suspension.

Rinse a cell strainer with 1ml of the L15- culture medium. Allow tissue residue to settle 3-4 minutes, then filter the supernatant through the cell strainer into a fresh 50ml conical tube.

Add 5ml additional L-15 to tissue residue. Repeat tituration step. Allow tissue to settle as before then filter the cells through the same strainer.

Rinse mesh gently with 2ml culture medium. Allow to sit undisturbed for about 20 minutes at Room Temp. This allows complete digestion of the partially degraded collagen. Do not exceed 1 hour at this step.

During the 20 minute incubation:
- Prepare plates: **Coat plates** to be used for cardiocyte culture at this time with 0.1% gelatin by placing 1 ml to cover the bottom of the well (12-well plate).

Incubate at 37° until about 10 minutes before use. Aspirate gelatin off culture plates. Let dry in hood for 10 minutes.

- If transiently transfecting, prepare DNA for transfection at this time by adding A and B reagent to the DNA (see Transfection Protocol). Can use calcium/phosphate method for these cells.

Swirl cells gently; if no clumps have formed and appearance is uniform, sediment cells at 50-100xg for 5 minutes (900 rpm in IEC). If clumping is observed continue to incubate the cells at RT for an additional 5-10 minutes or until a uniform suspension is obtained. (spinning at 4C seems to work fine)

Resuspend this cell pellet in 10mls of Initial Media for every 10 hearts.

PLATING THE CELLS

Counting cells (Optional):
If you would like to count the cells to determine your yield proceed as follows:

Put 10ul of cell suspension into one end of the hemocytometer.

Count cells three different ways. For instance, count one row across and multiply x5. Then a different row etc. Average the three numbers and multiply by 10,000. This answer will equal the number of cells that you have per ml of suspension. Multiply by the number of mls in order to get total cells and then divide by the number of hearts to get cell per heart. A good yield is 2-3 million cardiocytes per heart. Plate approx 1,200,000 cells per well of a 12-well plate.

If you are experienced and continue to get the same yields each time, you may proceed without counting using this chart.

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If transfecting, add DNA to cells immediately after plating.

THE DAY AFTER HARVEST

Warm differentiation media and HBSS (no Ca++, phosphate, or phenol red).
Wash plates vigorously with warm HBSS to remove debris, including RBC's.
Wash 4 times with HBSS.
Refeed with differentiation media.
Check under the microscope to see that most of the debris is gone and that individual cardiocytes are visible. **A few may be beating at this stage**.

Several hours later, wash the plates 2X with HBSS, and feed with differentiation media.
If using a ligand (e.g., retinoic acid, oleate), add ligand at this time. For standard reporter assays, harvest about 60 hours after adding ligand.
If infecting with adenovirus, add adenovirus at this time.
Wash virus off the next day and add ligand at this time. Harvest 60 hours later.

For hypertrophy stimulation, add at the time of removal of serum. Change media daily with fresh phenylepherine, and allow 48 hours before harvesting for reporter activity or RNA.