

1) ISOLATION and PRIMARY CULTURE of BROWN FAT PREADIPOCYTES

Principal Investigator: C.R. Kahn

Rationale: To prepare primary brown preadipocytes for immortalization: useful for metabolic studies from knockout mice. This consists of the following five protocols.

References: Fasshauer, M., J. Klein, K M. Kriauciunas, K. Ueki, M. Benito, and C.R. Kahn. 2001. Essential role of insulin substrate 1 in differentiation of brown adipocytes. *Mol Cell Biol* **21**: 319-329.

Fasshauer, M., J. Klein, K. Ueki, K.M. Kriauciunas, M. Benito, M.F. White, and C.R. Kahn. 2000. Essential role of insulin substrate-2 in insulin stimulation of glut4 translocation and glucose uptake in brown adipocytes. *J. Biol Chem* **275**:25494-25501.

Reagents:

Primary cell culture medium (500ml)

DMEM-high (450 mg/dl glucose)	345 mls
FBS (Fetal Bovine Serum)	100 mls (final conc. 20%)
1M HEPES buffer solution (Life Technologies, catalog #15630-080)	10 mls (final concentration = 20mM)
100X penicillin/streptomycin (Life Technologies, catalog #15140-122)	5 mls (=1X)

Isolation buffer (100ml)

NaCl (3M)	4.1 mls (123.0mM final)
KCl (0.154M)	3.25 mls (5.0mM final)
CaCl ₂ (100mM)	1.3 mls (1.3mM final)
Glucose (50 mM)	10 mls (5.0mM final)
HEPES (1M)	10 mls (100mM final)
ddH ₂ O	70.35 mls
pen/strep (100X)	1.0 ml
BSA (Bovine Serum Albumin) (Fisher, FRAC V, catalog # BP 1605-100)	4.0 g (4% final)

Filter sterile (0.2 micron), freeze 20ml aliquots

Protocol:

Prepare:

- shaking waterbath 37°C
- Thaw isolation buffer and add collagenase (1.5mg/ml) (Collagenase A, B&M Roche, catalog #103 578) vortex
- Sterile PBS (Phosphate Buffered Saline)
- Reserve tissue culture hood
- Warm up primary cell culture medium (which includes 20mM HEPES and 20% FBS)

1. Remove interscapular brown fat pad from mice (age late fetal to post natal day 2)
2. Dissect and mince tissue on parafilm with 500 ul PBS (sterile), use sterile pipette tips, indent parafilm in center so PBS will stay in the center.
3. Pipette tissue into 500 ul collagenase solution in Eppendorf vials (total volume now about 1000 ul).
4. Vortex for 10 seconds.
5. Put Eppendorf vials into shaking waterbath at 90 cycles/minutes (use plastic box and red weights) for 30 seconds and vortex for 10 seconds every 5 minutes- digestion should not exceed 40 minutes.
6. Filter digested tissue through 100 um filters (LABCOR PRODUCTS, INC. P.O. Box 7277, Gaithersburg, Maryland 20898-7277, catalog # 30-1, Sterile Nytex Membrane Filter, 38 x 38 mm, 100 Micron, 50/pkg, Individually Wrapped, order from: PGC Scientific, 7311 Governors Way, Frederick, Maryland 21704, 800-424-3300, catalog # 358-201) into fresh, autoclaved Eppendorf vials.
7. Centrifuge Eppendorf vials at RT at 1500 rpm (Eppendorf centrifuge) for 5 minutes.
8. During centrifugation add 2 ml pre-warmed culture medium to 12 well plates.
9. Remove supernatant (use 1 ml automatic pipette).
10. Re-suspend pellet in 1 ml culture medium per sample (use 1 ml automatic pipette) and transfer into a 12 well plate.
11. Leave cells overnight to get plated (day 1).
12. Next day: wash cells with 3mls culture medium and add 3mls fresh culture medium (day 2). (N.B. Cells are sensitive to cold and aspirating therefore hand aspirate.)

2) IMMORTALIZING BROWN FAT CELLS-ESTABLISHING CELL LINES

Principle Investigator: C. R. Kahn

After cells are plated overnight, (day 1), next day wash cells (day 2), on day 3 need to add virus (pBabe SV40 Large T antigen puromycin). (*preparation of viral stock see protocol 3*)

To each well add: VIRUS SOLUTION which consists of: 1) 50% primary culture media, 2) 50% viral stock, 3) 1X polybrene- 8 mg/ml stock solution dissolved in ddH₂O-final conc.4 ug/ml(1:2000 dilution) (hexadimethrine bromide, Sigma, catalog # H-9268)

1), 2), 3), should add up to approximately 0.9 ml/well. For example: if there are 6 wells with cells to be immortalized you need $6(X)(0.9 \text{ ml})= 5.4 \text{ ml}$ total volume containing primary culture media, viral stock and polybrene. Thus, take 2.7 mls of primary culture media, 2.7 mls of viral stock, and 2.7 ul of polybrene stock, mix by pipetting, filter sterile using a 0.45 micron filter. Then, for each well, pipette off media, DO NOT USE VACUUM (vacuum will disrupt cells too much), rinse well with 2 mls pre-warmed primary culture media, pipette off rinsing media and add VIRUS SOLUTION *VERY SLOWLY!!!!!!*

NEXT DAY: split cells into 15 cm dishes

- pipette off VIRUS SOLUTION
- rinse well with 2 mls DMEM-high (without FBS)-pipette off, put in $\cong 0.2$ ml trypsin for $\cong 3$ minutes (until cells just come off plate)
- add 2 mls primary culture media, transfer to 15 cm dish containing 25 mls primary culture media.

TWO DAYS LATER: start puromycin (Sigma, catalog # P 8833) selection (2ug/ml puromycin into DMEM high (+) 20% FBS). Leave in puromycin media for one week. (At this point some cells can be split off to differentiate or do some experiments with.)

After puromycin selection cells need to be put into media directed against *mycoplasma infection* (BM Cyclin, Boehringer Mannheim, catalog # 799 050). This will take approximately 2-3 weeks. Once cells have been in puromycin and mycoplasma selection they can be frozen down.

3) SV40 LARGE T ANTIGEN VIRUS PRODUCTION

Principle Investigator: C.R. Kahn

Pharmacia's Cell Plect protocol with slight modifications (Cell Plect Transfection Kit, Pharmacia Biotech, Cat. # 27-9268-01)

-Grow ϕ NX Cells (see below) in 15 cm plates, 50-70% confluent

4 pm evening

1. Dissolve 15 ug of SV40T-pBabe-puro in 480 ul distilled water.
2. Add 480 ul buffer A
3. Vortex briefly and incubate for 10 minutes at room temperature.
4. Add 960 ul buffer B with blue pipette tip while at the same time you are "bubbling" the solution with an automatic pipette.
5. Incubate for 15 minutes at room temperature.
6. Add the precipitate to the ϕ cell culture (in 15 cm plates, 50-70% confluent) as evenly as possible and support distribution by rotating the dish in a figure eight configuration a few times.

Next morning (i.e. 10 a.m.):

1. Aspirate the medium and rinse the cells *CAREFULLY* with 25 mls ROOM TEMPERATURE DMEM-H (no FBS) twice.
2. Add 25 mls ROOM TEMPERATURE DMEM-H + 10% FBS to the cells.

Same day (i.e. 4 pm.):

1. Change the medium to 25 mls fresh ROOM TEMPERATURE DMEM-H + 10% FBS.

Next evening (24 hours later, i.e. 4 pm.):

First viral harvest:

1. Aspirate supernatant with a 50cc sterile syringe + 18G sterile needle.
2. Remove needle and put 0.45 micron filter in front of the syringe.

3. Press the supernatant through the filter into a 50 ml blue top tube.
4. Add polybrene (4ug per ml) to the supernatant and mix.
5. Freeze 1 ml aliquots in sterile freezing vials and store in (-) 80°C freezer.
6. Add 25 mls fresh ROOM TEMPERATURE DMEM-H + 10% FBS to the ϕ cells.

Next evening (24 hours later, i.e. 4 pm.):

Second harvest exactly like the first one.

NOTES ON HANDLING fNX CELLS

1. The cells are grown in DMEM-High + 10% FBS at 5% CO₂.
2. The cells grow rapidly.
3. The cells come off of the dish very easily so be careful when washing/feeding them.
4. The cells are frozen in 90% FBS + 10% DMSO (dimethyl sulfoxide)
5. It is recommended that the cells are not allowed to reach confluence and that they are periodically grown in selection media to maintain their retroviral packaging “goodies”.

fNX Cell Selection Media

DMEM-High +10% FBS + 300ug/ml Hygromycin B + 1 ug/ml Diptheria Toxin A

The cells will round up some, they don't look very good but it's OK.

The cells need to be in selection only about 4-7 days, then place them back into regular media and allow them to “recover” to their normal morphology before using and/or freezing them.

Additional information and more details regarding the ϕ NX cells can be found on the Nolan lab homepage at: www.Stanford.edu/group/Nolan/.

4) CULTURING BROWN FAT CELLS

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Media:

- 1) DMEM high (+) 10% FBS
- 2) DMEM high no FBS (for rinsing cells)-always use new pipette
- 3) DMEM high no FBS (for starving cells)
- 4) DMEM high (+) 10% FBS (+) insulin and T3- Differentiation Media
- 5) Induction Media (see below)

Typical Differentiation Time Course:

Day 1- split into Differentiation Media

2

3

Day 4 - induce confluent cells

5

Day 6- put back into Differentiation media

7

8- aspirate off old media and add fresh Differentiation media

9

Day 10- fully differentiated cells ready to be used for: Oil Red O staining, RNA/DNA extraction, Western analysis etc.

MASTER PLATE-one large dish 15cm

Always in DMEM high (+) 10% FBS

To split: if confluent a 1:20 split (ie.- 1 ml cells plus 25 mls media will reach confluence in 3 days)

Aspirate off media

Rinse cells using DMEM high (no FBS)- fill 25 ml pipette to top- rinse each Master plate with 18 ml

Add 2.5mls trypsin/plate (always use new pipette)

Put back into CO₂ for at least 20 mins. (while trypsinizing label other plates/dishes)

(leave cells alone while trypsinizing- this seems to help avoid cells from clumping)

Preparation for "Splitting" Plates

-decide what kind and how many plates are needed:

ie: 6 well plate- gets 4mls differentiation media plus 12 drops of cells

12 well plate: gets 2mls differentiation media

10cm dish- gets 8 mls Differentiation media (+) 3 ml cells-usually can get 2-4mg protein

15cm dish-Master plate –ALWAYS gets 25 mls DMEM high (+) 10% FBS

After labelling plates:

Take out trypsinized Master Plate

Add 25 mls DMEM high (+) 10% FBS, pipette up and down three times to disperse cells and break up clumps- add- 1 ml to Master plate,
12 drops into 4 mls differentiation media-6 well plate,
3 mls cells into 8 mls differentiation media- 10cm. Dish

- while plating out one cell type- others can remain in trypsin in the hood

- when done “splitting” cells take Master plate and 10 cm dish and “distribute” cells evenly by moving dishes in a “figure eight” configuration ten times (for 6 well plates use a “faster” figure eight)

FREEZING CELLS- freeze cells in DMEM high (+) 20% FBS (+) 10% DMSO

DIFFERENTIATION MEDIA—DMEM high (+) 10% FBS

1) 500 ml filter bottle- 450 mls DMEM high

2) 50 mls FBS

add:

10ul of 1mM insulin (10^{-3} M)- 1:50,000

50ul of 10 uM T3 (Sigma, cat# T-2877)

FILTER STERILE (0.2micron filter)

INDUCTION MEDIA- when cells are confluent- prepare fresh –use only on the day you need it

Aspirate off differentiation media and add induction media-no rinsing- 11mls per plate

For 200 mls Media: add 200 mls Differentiation media to top of 500 ml filter bottle add:

1) 0.125 M indomethacin (1000X)-200 ul (Sigma, catalog # I-7378)

2) 2 mg/ml dexamethasone- vortex (1000X)-200 ul (Sigma, catalog # D-1756)

3) 0.25 M IBMX (500X)- 400 ul- (Sigma, catalog # I-5879) must add immediately after thawing- thaw- vortex-add back to heating block

-all three are dissolved in 100% ethanol and stored frozen in (-) 20 freezer

-1) and 3) must be heated to 75°C (use heating block)

5) OIL RED O STAINING PROTOCOL

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This protocol is used to test for lipid accumulation in fully differentiated cells.

Stock Oil Red O: 0.5g Oil Red O (Sigma, catalog # 0-0625) in 100 ml isopropanol

1. Rinse plates with PBS
2. Fix cells by covering with PBS + 10% formaldehyde or with commercially available buffered formalin (Sigma, catalog # HT50-1-640)
3. Let plates sit at least 15 min. or as long as overnight, at room temperature, alternatively plates can be left @ 4°C until multiple plates are collected.
4. Prepare Oil Red O working solution (make fresh each time, as working solution is unstable) by adding 6 ml stock to 4 ml ddH₂O. Mix and filter through Whatman #1 filter paper. (The filtration step takes awhile; it is not advisable to hasten the process by applying a vacuum because there is too much stuff to filter out.) Make about 5 ml per 10 cm plate to be stained.
5. Remove fixation agent, add Oil Red O stain.
6. Sit 1 hour (or longer) at room temperature.
7. Rinse several times, carefully, with distilled water to remove excess stain and any precipitate that forms.
8. Maintain dishes in water if intact cells are desired.
9. Pour off distilled water and allow dishes to air dry.
10. Scan plates.