Processing Adherent Cultured Cells for Tissue Microarray Controls

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(Adapted from method of Anthony McCabe, PhD)

Objective and General Notes:

The Rimm lab uses cell lines as internal standard controls in TMA experiments. This protocol was developed to provide a standard size cell disk for coring. We grow all cell lines to 80% confluence and replace media 12-24 hours before fixation. We also grow two extra flasks, and collect cell lysate and freeze cells simultaneously. We use the lysate to QC antibodies by Western or measure protein by ELISA.

Cells: 60-80% (can be higher) subconfluent cells in 5-10 T75 or 2-4 T175 Flasks, depending on cell density at subconfluence. (Adjust volumes for other culture containers)

Tools:

- Suggested Cell Scraper: Falcon 3086
- Suggested probe for pellets: Heyman Type B microspoon, catalog #62411-B http://www.emsdiasum.com/microscopy/search/results.asp?Prod=heyman

Solutions:

- 1X PBS
- 80% Ethanol
- 10% Neutral-buffered Formalin (purchase ready-to-go)
- RIPA cell lysate buffer – add fresh inhibitors, 4uL per mL lysate buffer
  - CLAP, 4uL per mL lysate buffer

    CLAP

C- Chymostatin	*Inhibits chymotrypsin, papain, and most cysteine proteases*
L-Leupeptin	*Reversible inhibitor of trypsin-like and cysteine proteases*
A-Antipain	*Peptidyl arginine aldehyde protease inhibitor (like Leupeptin)*
P-Pepstatin A	*Reversible inhibitor of aspartyl-like (Cathepsin D, pepsin, rennin)*

    - *Make 1mg/mL stock solutions of each inhibitor. Mix together equally, store in 100uL aliquots in the -20 freezer. Use 4uL per mL lysate buffer.*
  - Pefabloc, 4uL per mL lysate buffer

    0.5M Pefabloc (FW=239.7)

    - *Irreversibly inhibits serine proteases (trypsin, chymotrypsin, plasmin, thrombin)* Dissolve 0.7 g in 5.84mL of ddH_2O, store in 100uL aliquots @ -20, use 4uL per mL lysate buffer
  - Activated Sodium Orthovanidate, 200mM pH 10.0 – add to lysate buffer, 20uL per mL

Cell Fixation (Recommended 4-6 T175 flasks @ 80% confluence)

1. Gently wash cells 2x in sterile 1XPBS, aspirate gently to remove trace PBS
2. Cover cell monolayers with 5mL (or enough to cover the cells) 10% neutral-buffered formalin (NBF) in each flask for 2-5min. *Some cells will need immediate scraping after fixation or they may adhere too tightly to the plastic*.
3. Scrape cells (with Falcon 35-3806 scraper or sterile rubber policeman) into NBF, and collect into labeled 50mL tubes – wash each flask 1-2X with 5mL 10% NBF if necessary to collect all cells.
4. Stored at 4C in NBF for at least 3-4 hours or until further processing is desired. (we believe cells can be stored under these conditions indefinitely, although pellets will become more difficult to process after ~3 weeks)

Pellet Processing

1. Prepare 10mL of 1% Low Melt agarose solution in 1XPBS (*microwave (cap off) for 10 seconds to get it into solution*) and incubate in hyb oven at 55C for at least one hour to get solution to consistent temp while you do subsequent steps.
2. Prepare Eppendorf tubes with ~200uL paraffin – this makes a "bed" for the cells to spin down on. Cut off tops of (another set of) eppendorf tubes, you'll use the space inside the cap as a mold to create a "disk" cell pellet
3. Centrifuge cells at 1200 rpm for 5min, gently remove supernatant and wash x2 in 1X PBS. (Resuspend in PBS and centrifuge to pellet each time)
4. Resuspend cell pellets in 0.5mL 80% ethanol and transfer to 1.5ml or 0.6mL eppendorf tubes (depending on volume of cells used) – use the same size cap in later steps.
5. Pack cells by centrifugation at 12,000 rpm for 5min. Aspirate off ethanol and gently repeat with another ethanol wash -- resuspend in ~0.5mL ethanol (or as much as will fit), centrifuge to pellet. This removes the last traces of PBS, and helps the pellet stick together.
6. Gently aspirate off 80% ethanol. Using a Heyman probe or similar tool, transfer cell pellet to eppendorf cap – mold into cap with probe. If necessary, using ~75uL of 1%Agarose, resuspend cells into caps of eppendorf tubes. Work quickly and put caps into freezer ASAP.
7. After a few hours, "Pop out " disks into embedding cassettes, (*hint: you may need to use a razor blade to cut the sides of the cap*) store ON in cassette submerged 80% ethanol
8. [Take to Research Histology for embedding] → Embed pellets in vertical position in the center of a paraffin block **without** eosin (eosin increases autofluorescence)

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Freezing cells

RULE OF THUMB: Freeze slow, Thaw fast.

- It is best to freeze cells at the beginning of experiments in case there is any drift or contamination of your line during the course of your experiment. Then you have a stock of your starting material. Freezing cells several times during the course of your experiments serves as insurance for a bad freeze.
- Cells freeze best when they are in the log phase of growth, when they are actively growing (50-80% confluent), rather than when they are static at confluence.

1. Make freeze media, usually FBS with 5-10% DMSO. Check your cell line info (ATCC) or a paper in the literature for freeze conditions.
2. Prepare your screw top cryovials. Label them with your initials, the date, the cell line name, and the passage number. Cells are not happy in DMSO and it kills them, so have your vials ready so you can aliquot your cells in freeze media to the tubes without letting them sit in freeze media while you label tubes.
3. Rinse cells with 1XPBS 2X and trypsinize as if you were splitting.
4. Quench cells with media, usually 5 mls.
5. Transfer cells to a sterile 15 ml conical; Spin 1200 rpm about 3 minutes room temp to pellet cells.
6. Aspirate media.
7. Resuspend cells in freeze media and aliquot to tubes – usually 1.0mL per tube.
8. Put cells Immediately into the –80 to freeze them – we have special containers that use for freezing.
9. The next day, or at least within one week, move the cells to the liquid nitrogen.

The number of freezes you make and the number of cells you freeze depends on the type of cells and their viability after thawing.
An alternative to this protocol is to add a counting step after trypsinizing and quenching the cells either on a coulter counter or hemocytometer. Then you can freeze a known number of cells.

Remember

- *If a pipette or tip or anything touches the outside of a flask, container or other object of suspicious sterility, throw it away. Plastic is cheap. Cells and lost experiments are not.*
- *Always use different plasticwear when going between cells. Cross-contamination would be disaster.*
- *Use ethanol liberally.*
- *Always record the passage number and date on the flasks.*
- *Cells don't like trypsin and it is possible to over-trypsinize them. Keep an eye on them.*
- *Cells don't like to be out in the cold world. Work with them swiftly to get them back to 37C ASAP.*
- *Yellow media is a BAD BAD thing. It means exhausted cells dying in their own waste, or worse, contaminated cells. Keep your cells well fed and they will love you.*

Preparing Cell Lysates

Need: Lysate buffer, Pefabloc, CLAP, phosphatase inhibitors if necessary.
Keep lysate buffer on ice:
RIPA buffer is "stronger", likely to disassociate weak complexes and denature proteins.
Triton buffer is a gentler approach.

RIPA Buffer for 250mL ***(mL)***

[Solution]	[Stock]	V2	C1	C2	(V2*C2)/C1
50mM TrisHCl pH 7.4	1M	250	1000	50	12.5
150 mM NaCl	1M	250	1000	150	37.5
5mM EDTA	500mM	250	500	5	2.5
(1%) NP40	100%	250	1	0.01	2.5
(0.5%) Na Deoxycholate	5%	250	0.05	0.005	25
(0.1%) SDS	10%	250	0.1	0.001	2.5
Water					167.5

1% Triton Buffer for 250mL ***(mL)***

[Solution]	[Stock]	V2	C1	C2	(V2*C2)/C1
20mM TrisHCl pH 8.0	1M	250	1000	20	5
150 mM NaCl	1M	250	1000	150	37.5
5mM EDTA	250mM	250	250	5	5
(1%) glycerol	100%	250	1	0.01	2.5
(1%) Triton	100%	250	1	0.01	2.5
Water					197.5

Everything @ 4ºC – keep on ice or perform protocol in cold room

1. Prepare lysate buffer (10mL) by adding inhibitors 1:250 (4uL for every 1 mL), chill and keep on ice. Use within 30 min – inhibitors have a short half-life.
2. Aspirate media, wash each dish with 1XPBS x2. Tip plates to side when aspirating to remove as much PBS as possible.
3. Add 1mL chilled lysate buffer to plate, immediately put on ice. Rock on ice or in cold room for 10-30min. (Check under microscope, first membranes will lyse, then eventually, cell nuclei. *If preparing lysates from multiple plates, re-use buffer to keep volume as low as possible.*
4. Transfer lysate to 1.5mL Eppendorf. Rinse dish with 500uL lysate buffer and add to tube. Shear DNA (slimy) with 26 guage needle 10-15X.
5. Spin @ 4ºC for 5min. Transfer supernatant to new, labeled 500uL tubes and freeze at -80C.
6. Save an aliquot for Bradford assay if moving straight to immunoblotting. Avoid freeze-thaw cycles with lysate, store in protein loading buffer after first thaw.