Icahn School of Medicine at Mount Sinai LINCS Center for Drug Toxicity Signatures

Standard Operating Procedure: Preparation of Adherent Cell Lysates for Microwestern Array

DToxS SOP Index: A-10.0
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Quality Assurance/Control (QA/QC) steps are indicated with green highlight.

Metadata recording is highlighted with yellow highlight and superscript indices.

NOTE: This protocol is based on a protocol written by Mark Ciaccio (Ciaccio MF, Wagner JP, Chuu CP, Lauffenburger DA, Jones RB (2010) Systems analysis of EGF receptor signaling dynamics with microwestern arrays. Nat Methods 7:148–155; Youtube videos on MWA, www.youtube.com/watch?v=0iUhoWL1IC0).

Preperation of Reagents

1) Prepare inhibitor stock solutions and reagents. Aliquots are single use for a 10 mL batch of lysis buffer and can be scaled accordingly. For optimal function do not freeze-thaw aliquots but rather safely discard any unused portion.
   a) 200 mM activated sodium orthovanadate (Na_3VO_4)(FIVEphoton Biochemicals #ActVO-[1]):
      i) Aliquot the activated sodium orthovanadate (60 μL per aliquot) and store the aliquots at -20°C. Good for ~1 year (per manufacturer).
      ii) QA/QC 1. Check color of final solution. It must be colorless. If not, return to the company.
   b) 1mg/mL Aprotinin
      i) Weigh 1mg Aprotinin (Fisher/MP Biomedicals #194559[2]) into a 1.7mL microcentrifuge tube (VWR, #20172-698).
      ii) Add MilliQ water (Millipore Advantage A10 system 18.2 MΩ) to bring final volume to 1mL.
      iii) Aliquot for single use (12 μL per aliquot into a 0.6mL microcentrifuge tube (Fisher #05-408-120). Store at -20°C for up to one year.

c) 1mg/mL Leupeptin
   i) Weigh 1mg leupeptin (MP Biochemicals, #195624[3]) into a 1.7mL microcentrifuge tube.
   ii) Add MilliQ water to bring final volume to 1mL.
   iii) Aliquot for single use (12 μL per aliquot into a 0.6mL microcentrifuge tube). Store at -20°C for up to one year.
d) 1mg/mL Pepstatin A
   i) Weigh 1mg pepstatin A (MP Biochemicals, #195368[4]) into a 1.7mL microcentrifuge tube
   ii) Add MilliQ water to bring final volume to 1mL.
   iii) Aliquot for single use (12 μL per aliquot into a 0.6mL microcentrifuge tube). Store at -20°C for up to one year.
e) 1M ß-glycerophosphate (ß-GP)
   i) Weigh 1.53g ß-GP (Santa Cruz Biotechnology #sc203323[5]) into a 15 mL conical tube (Fisher, #50-754-1410)
   ii) Add MilliQ water to bring final volume to 5mL.
   iii) Aliquot for single use (110 μL per aliquot into a 0.6mL microcentrifuge tube). Store at -20°C for up to one year.
f) 100mM Ethylenediaminetetraacetic acid (EDTA)
   i) Weigh 146 mg EDTA (Sigma # E6758[6]) into a 15 mL conical tube
   ii) Add MilliQ water to about 3 mL.
   iii) Adjust pH to 8.0 with 10 M NaOH (Fisher #SS255-1[7])
   iv) Bring final volume to 5 mL with MilliQ water.
   v) Aliquot for single use (110 μL per aliquot into a 0.6 mL microfuge tube). Store at -20°C for up to one year.

2) Prepare 10 mL of lysis buffer.
   a) Note: Lysis buffer should be prepared fresh the day of the cell lysis. Recipe can be scaled accordingly.
   b) Combine 2 mL of stock 5x Gel Buffer (SOP A-9.0, step 2), 1 mL of 10% Sodium Dodecyl Sulfate (SDS) (Fisher #BP2436-1[8]), 50 μL glycerol (Sigma, #G5516[9]), 100 μL of stock 100 mM EDTA (step 1f) and 7 mL of MilliQ water in a 15 mL conical centrifuge tube.
   c) Put tube on ice and allow it to cool.
   d) Add the following immediately before cell lysis: 10μL stock aprotinin (step 1b); 10μL stock leupeptin (step 1c); 10μL stock pepstatin A (step 1d); 100μL stock ß- GP (step 1e); 50μL stock Na_3VO_4 (step 1a); 500μL 1M DL-1, 4-Dithiothreitol (DTT) (Fisher, # AC426380100[10]).
   e) Keep lysis buffer on ice.
3) Cell lysis
   a) NOTE: For a 24 well microwestern experiment, we find that ~ 150 μL at 1 mg/mL total protein concentration is needed after lysis (or equivalently e.g. 300 μL at 0.5 mg/mL). One should empirically determine the number of cells to plate prior to preparation for microwestern by measuring protein concentration as in Step 4g below. We recommend 500k cells as a conservative starting point but routinely see adequate results with as low

as 150k cells. Optimization to reduce these amounts is ongoing, and of course everything depends on the abundance of the epitopes and antibody quality.

b) Prepare cells for adding the lysis buffer. This typically consists of washing cells 2x in ice-cold phosphate-buffered saline (PBS) (Corning #21-040-CV) [11].
   i) Place plate / dishes on ice.
   ii) Add 5 mL ice-cold PBS per 10 cm dish (scale volume according to culture area for different size dishes). Aspirate fully. Repeat. It is important at this stage that aspiration is complete.

c) Add 1.0 mL ice-cold lysis buffer per 10 cm dish (scale volume by culture area for different size dishes).

d) Scrape cells with a cell scraper (Denville Scientific, #T0139), comprehensively going over the entire culture area multiple times. Tilt the dish / plate by leaning it on the side of the ice tray at an approximately 30 degree angle and allow lysate to gravity pool for at least 20 to 30 seconds (can leave up to ~10 minutes if lysing batches of dishes).

e) Transfer lysate into a pre-cooled 1.7 mL microcentrifuge tube using a pipette. Keep on ice.
   i) NOTE: At this point, the lysate at the end of step d) may be applied to a separate dish which has gone through step b), to increase the protein concentration.
   ii) Pipette carefully to avoid introducing bubbles into the lysate, which increases difficulty of maximum collection.

f) Place lysate-containing tube into a 95^{o}C heat block (Fisher, #11-718-20) for 5 minutes. Vortex for ~10 sec. At this point, one can freeze sample at -80^{o}C if necessary.

4) Process and concentrate lysate

a) If lysates are frozen, thaw on ice for at least 10 min. Vortex briefly (~two seconds)..

b) Sonicate. There are two options for sonication, depending on the lysate volume.
   i) Large volumes (>~ 1 mL). Sonicate at Power 9 on a probe sonicator (Misonix; model XL2000) for one second while keeping lysate on ice. Turn off sonicator for 1 sec. Repeat on / off cycle 9 more times (20 seconds total).
   ii) Small volumes use the VialTweeter from Hielscher Ultrasonics placed in a 4^{0}C cold room. Note the sonotrode is machined by the company for specific microfuge tubes. The tubes we use are listed below.
      (1) Place lysates up to 200 μL in 0.25 mL polypropylene tubes (Fisher #02-681-230).
      (2) Place tubes into sonotrode and sonicate for 10 seconds followed by a 30 second rest period. Repeat this cycle 10x. Amplitude is set at 100%.
      (3) For lysates with a volume of 200 μL to 500 μL we recommend a Protein LoBind 1.5mL tube (Eppendorf #022431081); however, note we have not yet optimized the VialTweeter for these tubes and volumes.
   iii) NOTE: The microwestern array facility at Univ. of Chicago uses a Covaris bath sonication apparatus.

c) Large volumes (step 4.b.i above) are needle sheered with a 25 gauge needle (Fisher, #NC0779671), by drawing the sample into and out of the needle and a 1.0 mL plastic syringe five times (Fisher #22-253-260).

d) Place tubes into a 95^{o}C heat block for 2 minutes and immediately place in 500 μL Centricon spin columns (Amicon Ultra – 0.5mL centrifugal filter; Millipore #UFC501096).

e) Spin at 14,000g, room temperature for 15 min.
f) Each sample should be concentrated ~5X to 10X by volume. If not, spin longer.
g) As per the protocol for the Amicon centrifugal filters, recover concentrated samples by placing the filter device upside down in one clean microcentrifuge tube (tube included in the Amicon kit) and spin at 1,000g for 2 minutes.
h) Freeze at -80°C or proceed directly to measuring protein concentration using the Pierce 660nm Protein Assay kit, (Fisher #22660[12] and Fisher #22663[13]). Follow the manufacturer's protocol. We perform triplicates.
    i) QA/QC 2. Check the turbidity of the lysate after prepared for Pierce assay. If it is turbid or "clumpy" this suggests inadequate sonication and/or not enough lysis buffer volume was used during cell lysis.
    ii) QA/QC 3. Check total protein concentration. We aim for 5mg/mL of total protein, but have had suitable blotting results with as little as 1 mg/mL. If protein concentration is too low, repeat steps d) – f).
i) Equalize protein concentration of all lysates with ice-cold lysis buffer. Aliquot lysates into 1.7 mL microfuge tubes, if necessary, to avoid repeated freeze-thaw cycles (21 μL aliquots work well). Freeze at -80°C until use.

Metadata

Record lot # for the following:

The specific lot #'s used in our experiments (as of July 2015) are:

1. Sodium Orthovanadate (FIVEphoton Biochemicals) Lot #26711-1

2. Aprotinin (Fisher/MP Biomedicals #194559) Lot #MR30363

3. Leupeptin (Fisher/MP Biochemicals, #195624) Lot #M8546

4. Pepstatin A (Fisher/MP Biochemicals, #195368) Lot #8372K

5. ß- GP (Santa Cruz Biotechnology #sc203323) Lot #H1115

6. EDTA (Sigma # E6758) Lot # 011M0123V

7. NaOH 10N (Fisher #SS255-1) Lot # 932721-247.

8. 10% SDS (Fisher #BP2436-1) Lot #121099

9. Glycerol (Sigma, #G5516) Lot #SHBC7796V

10. 1M DTT (Fisher, #AC426380100). Lot #A0345819

11. PBS (Corning #21-040-CV) Lot # 12815001

12. Pierce 660nm Protein Assay Reagent (Thermo Scientific # 22660) Lot # QE216003

13. Ionic Detergent Compatibility reagent (Fisher #22663). Lot #QG220132

Quality Assurance/ Quality Control

QA/QC 1. Yellow tinge indicates Na Orthovanadate is not activated.

QA/QC 2. We have found that if one can see “clumps” or if the solution is turbid immediately before absorbance measurements in the Pierce 660 assay, then protein concentration measurements will be unreliable as also might be the microwestern results.

QA/QC 3. Be mindful that a minimum volume for adequate nanoplotter dispensing performance is ~18 uL in the microtiter plate. We pipette 18 uL into the plate from 21 uL in the tube. Higher protein concentration gives a better signal but sample must have low viscosity for the arraying process to be functional. All of this also depends on the cell type and antibodies, so there is often some optimization and iteration.