Protocol for Tissue Lysis for Reverse Phase Protein Array

Reagents provided by Proteomics Core:

3 ml RPPA Lysis Buffer (store at 4°C)

1 ml 5X Protease Inhibitors (store at -20°C)

1 ml 5X Phosphatase Inhibitors (store at -20°C)

Choose one of the following sample buffers based on your estimated protein concentration:

1 ml 2X SDS Sample Buffer (store at 4°C)

0.5 ml 4X SDS Sample Buffer (store at 4°C)

Please note this is the buffer validated specifically for our RPPA platform. Please don't use RIPA or any other buffer with NP40 or Triton X detergent. If you wish to make your own buffer for a large number of samples, please reach out to us for further information.

Reagents/supplies required, but not supplied by Proteomics Core

2-mercaptoethanol

Sample Tubes RB (Qiagen, Cat# 990381) for tissue aliquot

1.5 ml microcentrifuge tube with tight cap for lysate aliquots.

We recommend using the microcentrifuge tube from Fisherbrand, catalog number 02-681-320.

Equipment (please contact Proteomics Core for training, assisted or unassisted usage)

TissueLyser II (Qiagen, Cat# 85300)

RPPA Working Solution (5 mL) Preparation

- Obtain Lysis Buffer components from Proteomics Core and store in appropriate location. Prepare working solution fresh on the day samples will be lysed.
- Add the 1 ml of Protease Inhibitors (5X) and 1 ml Phosphatase inhibitor (5X) to the tube containing 3 ml RPPA Lysis buffer to create a 5 ml solution. RPPA Working solution is now ready.
- Discard remaining buffer. Always use fresh RPPA Working Solution.

Tissue Lysis Procedure (must be done at 4°C as quickly as possible without stopping):

1) Store samples in -80°C.
2) Transfer samples to labeled *Sample Tubes RB* (work in 4°C cold room for entire transfer).
    a. While keeping the tissue sample tubes on dry ice, transfer tissue piece from original sample tube to labeled *Sample Tubes RB*.
    b. Work quickly but steadily using a pipette tip to transfer the tissue. For small tissue samples, be careful not to lose the tissue.
    c. Be sure to keep all tubes on dry ice so that tissue samples don't begin to thaw prematurely. Premature thawing of tissue samples may cause degradation of proteins and affect results.
    d. Transfer 10 samples at a time. Once tissue samples have been transferred to *Samples Tubes RB*, place samples in -80°C until it is time for lysis procedure.
3) Add appropriate volume of RPPA Working Solution into *Sample Tubes RB*.
    a. Test tissue to volume ratio before doing large scale lysate preparation, since different tissues lyse differently.
    b. Small samples (10-15 mg tissues) can use as little as 150 µl of RPPA Lysis Buffer. For larger tissue piece (20-30 mg), add 300 µl of RPPA Lysis Buffer.
4) Lyse tissues using TissueLyzer (Qiagen).
    a. Keep TissueLyzer and adapter at cold room all the time or precool at least 1 hour.
    b. Add 1 pc of 5 mm stainless steel bead to each tube.
    c. Check to make sure tubes are properly closed.
    d. Homogenize tissues on the TissueLyzer using the following settings: 2 minutes at 23Hz.
    e. Take apart the Adapter Set and reverse the order of the tubes to ensure that all the tubes are evenly homogenized [Samples on the inside of the adapter rack move more slowly than samples on the outside, merely rotating the adapter set is not sufficient]
    f. Repeat TissueLyzer homogenization using the same settings as above.

g. Examine each tube after homogenization. Repeat homogenization if tissue pieces can still be seen.

5) Spin at 14,000 x g for 15 min at 4°C then transfer supernatant to fresh tube. Repeat centrifugation THREE to FIVE more times at 14,000 x g for 15 min at 4°C then transfer supernatant to a new tube. Supernatant should be clear (i.e. no fats or particulates). If supernatant is still cloudy, repeat spin and transfer step. Most tissue samples require FIVE spins and transfers; FAT TISSUES generally require SIX or SEVEN centrifugations to avoid debris and floating fat. Exceptionally fatty tissues may require even more spins and transfers. For optimal homogenization quality of tissue samples, it is crucial to ensure that the protein concentration does not surpass 5 mg, with the ideal range showing below.

6) Determine protein concentration using BCA Assay or Bradford. **Use <u>RPPA Working Solution</u> as a blank and diluent for standards and samples when measuring protein concentration.**
The desired protein concentration is between 1.1 mg/ml – 3 mg/ml.

7) Prepare the lysates for RPPA analysis by adding SDS Sample Buffer, β-mercaptoethanol, and RPPA Working Solution to obtain a final concentration of **<u>0.5 mg/ml</u>**. Depending on your protein concentration, choose one of the tables below to prepare the final RPPA ready samples.
NOTE: Prepare all your samples the same way using the same type of SDS Sample buffer.

For lysates ≥1.1 mg/ml	
__ μl	Lysate
__ μl	RPPA Working Solution
30 μl	2X SDS Sample Buffer
1.5 μl	β-mercaptoethanol (2.5% final concentration)
60 μl	Total Volume

For lysates <1.1 mg/ml	
__ μl	Lysate
__ μl	RPPA Working Solution
15 μl	4X SDS Sample Buffer
1.5 μl	β-mercaptoethanol
60 μl	Total Volume

8) Heat samples for 5 min at 95°C.
9) Bring to room temperature & spin down to collect volumes.
10) Aliquot into <u>two</u> tubes (<u>30 μl</u> for each tube). Please use 1.5 ml microcentrifuge tubes with tight cap to avoid sample loss during heating.
   If your sample volume is low, please contact us for advice and DON'T just distribute it evenly between #1 & #2 tubes!
11) Clearly label sample tubes and put them into a freezer/cardboard box labeled with your name/PI name, contact number/date etc.
   Label **tubes** as follows:
   PI initials_Auto# (ex: SH_1)

   SH_1 RPPA-30ul   SH_2 RPPA-30ul
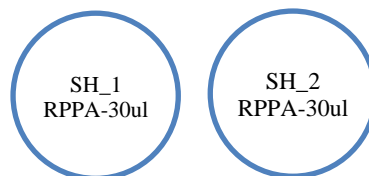
   <u>Label **freezer/cardboard box** as follows:</u>

   Investigator Name/PI name
   Contact number
   Date

12) Store tubes at -80°C.
13) Send your *RPPA Sample Submission Form* by email **<u>before</u>** you deliver your samples to the Core.
14) Deliver samples on dry ice to the Core.

*DO NOT use homemade or any other concentration of SDS Sample Buffer as it may generate higher background based on previous observations.

Use the same SDS Sample Buffer (either 2X or 4X) for all your samples. **DO NOT prepare some samples in 4X and some samples in 2X Sample Buffer.

Please contact us if you have any questions.