SAMPLE PREPARATION

1. QUANTITY OF LYSATE

The amount of protein requested for the Kinex™ KAM-880 Antibody Microarray service is 100 μg per sample at an approximate concentration of 2 mg/ml. If your samples have a higher concentration, we recommend sending it without further dilution and Kinexus will adjust the concentration as required during processing. In this case, we prefer a minimum volume of approximately 50 μl. If your samples have a lower concentration than 1.8 mg/ml, there are alternate steps that can be undertaken for ensuring optimum results. This includes concentrating your samples or providing additional dye-labeling reactions to your samples. Please contact a Kinexus Technical Service Representative for more information on how to proceed and the additional costs involved if your sample concentrations are low.

2. LYSIS BUFFER

The standard ingredients for our lysis buffer are listed below, however other lysis buffers commonly used for protein lysate preparation with non-ionic detergents should be compatible with the service. **However any lysis buffers containing Tris or reagents carrying reactive amine groups are not acceptable alternatives**. Your cell pellets or tissues should be homogenized in the following ice-cold lysis buffer and the final pH of the lysis buffer should be adjusted to 7.2.

1. 20 mM MOPS, pH 7.0 (any other buffer without Tris at this pH could be substituted);
2. 2 mM EGTA (to bind calcium);
3. 5 mM EDTA (to bind magnesium and manganese);
4. 30 mM sodium fluoride (to inhibit protein-serine phosphatases);
5. 60 mM β-glycerophosphate, pH 7.2 (to inhibit protein-serine phosphatases);
6. 20 mM sodium pyrophosphate (to inhibit protein-serine phosphatases);
7. 1 mM sodium orthovanadate (to inhibit protein-tyrosine phosphatases);
8. 1% Triton X-100 (can be substituted with 1% Nonidet P-40) *** for total cellular fractionation**

Protease inhibitors and DTT to be added just before use:

9. 1 mM phenylmethylsulfonylfluoride (to inhibit proteases);
10. 3 mM benzamidine (to inhibit proteases);
11. 5 μM pepstatin A (to inhibit proteases);
12. 10 μM leupeptin (to inhibit proteases);
13. 1 mM dithiothreitol (to disrupt disulfate bonds).

The protease inhibitors and dithiothreitol (DTT) must be added to lysis buffer immediately before use and samples should be processed as quickly as possible. Not all protease inhibitors are required, but it is optimal to use as many as possible. For convenience, the Roche Complete Mini Inhibitor Cocktail tablet can be used to replace the individual protease inhibitors. The samples must be frozen and shipped to Kinexus on dry ice after protein quantification without any SDS-PAGE sample buffer as the proteins are to remain in their native structure and nondenatured.

3. PREPARING YOUR SAMPLES

A large body of information and instruction is provided with this information package. A careful review of this package and in particular the sample preparation protocols, will ensure that we can offer the highest level of quality in providing our unique proteomics services to you.

Important points to remember include:

1. The cells or tissues should be processed quickly at 4°C or less;
2. Add the DTT and protease inhibitors to the lysis buffer just before processing;
3. Homogenization should not be performed in too large a volume of lysis buffer to obtain lysates at the concentration required;
4. Make sure the homogenization buffer does not include any free amines such as Tris buffer;
5. The detergent-soluble fraction should be obtained as quickly as possible after the cells or tissues are homogenized;
6. Sonication is required (do not over sonicate) and cannot be omitted;
7. The highest centrifugal forces available should be used to generate the detergent-soluble fraction; and
8. The supernatants should be frozen as quickly as possible if a protein assay cannot be performed immediately.

4. FRACTIONATIONS

There are different types of fractionations that can be performed, with the most common type being the total cellular or detergent soluble fraction. The type of lysis buffer used will vary depending on the type of fractionation you are considering to prepare.

Total Cellular Fractionation:

For quantitation of total cellular levels of cell signalling proteins, lysis and homogenization should be performed in the presence of a non-ionic detergent. We recommend the use of 1% Triton X-100 or 1% Nonidet P40, but comparable detergents are acceptable. This is the most common type of fractionation prepared by clients.

Subcellular Fractionation:

Detergents should be omitted from the homogenization buffer if the subcellular distribution of cell signalling proteins is to be examined. If a particulate-solubilized fraction is to be analyzed, a microsomal pellet should be obtained following the initial homogenization and ultracentrifugation in the absence of detergent and subsequent removal of the cytosolic supernatant. In this instance, the cytosolic extract should be removed and the microsomal pellet should then be resuspended in the homogenization buffer containing 1% Triton X-100 or 1% Nonidet P-40 and subjected to homogenization and ultracentrifugation once again. The resulting detergent-solubilized microsomal fraction should be removed and immediately assayed for its protein concentration.

Other Fractionations:

At this time, we do not recommend that you send samples from immunoprecipitation or antibody affinity pull-down experiments for the Kinex™ Antibody Microarray Services.

5. CELL LYSATE PREPARATION

A. Adherent Cell Lysates

1. Remove medium from culture dishes containing approximately 1×10^6 to 2×10^6 cells for each sample to be analyzed by our Kinex™ Antibody Microarray Service. For the validation immunoblotting service, you will need to prepare about 10 times more cells or 1×10^7 to 2×10^7;
2. Rinse the cells twice with ice-cold PBS to remove medium residue (serum must be completely removed from cells); remove as much PBS as possible after the last rinse;
3. Add 200 µl ice-cold lysis buffer to 150 mm culture dish per sample (more lysis buffer can be added if cells are concentrated); or add 100 µl ice-cold lysis buffer to 100 mm culture dish;
4. Scrape the cells in lysis buffer, collect the cell suspension from the dishes and transfer it into a 1.5-ml microcentrifuge tube;
5. Sonicate four times for 10 seconds each time with 10-15 second intervals on ice to rupture the cells and to shear nuclear DNA; **This step is crucial and cannot be omitted**;
6. Centrifuge the homogenate at 90,000 x g or higher for 30 minutes at 4°C in a Beckman Table Top TL-100 ultracentrifuge, Beckman Airfuge or equivalent. Alternatively clients can also centrifuge on maximum speed (15,000-17,000 x g) on a benchtop Eppendorf microcentrifuge for 30 minutes at 4°C;
7. Transfer the resulting supernatant fraction to a 1.5-ml microcentrifuge tube;
8. Assay sample for protein concentration using a commercial Bradford assay reagent (available from Bio- Rad, catalogue number 500-0201) or using the standard protocol of Bradford *(Bradford, M.M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254).* Bovine serum albumin should be used as the protein standard;
9. Aliquot at least 100 µg for each lysate to be analyzed with our Kinex™ Antibody Microarray Service;
10. Save any remaining lysate for future validation studies. If you wish to have Kinexus perform the custom Western blotting analysis, aliquot 350-500 µg for each 18 proteins to be tested, or if sample quantity is limited, aliquot 50 µg of sample for each 3 proteins to be tested; and
11. Label your microcentrifuge tubes and freeze immediately.

B. Suspended Cell Lysates

1. Place medium containing cells in appropriate sized tube and centrifuge at 500 x g for 2 minutes at 4°C in a swinging bucket benchtop centrifuge. Remove as much medium from the cell pellet as possible without disrupting cells;
2. Wash the pellet by gently resuspending the cells in ice-cold PBS, followed by centrifugation as above. Repeat once to ensure complete removal of serum;
3. Remove as much PBS as possible after the last wash;
4. Add an adequate amount of ice-cold lysis buffer to the sample (more lysis buffer can be added if the number of cells is high); and
5. Repeat steps # 5 through 11 as described in the Adherent Cell Lysate Section above.

6. PREPARATION OF CELL PELLETS

An additional charge will apply for submission of cell pellets to be processed at Kinexus. Please submit a sufficient number of cells (>2×10^6 cells) for processing. If you would like to have the Kinetworks™ validation performed, the number of cells required is ten-fold higher (>2×10^7 cells).

A. Adherent Cells

1. Remove the medium and rinse the cells in dish with ice-cold PBS once;
2. Detach cells with trypsin as one does in passaging cells or scrape the cells with a rubber policeman, followed by the addition of equal volume of medium;
3. Collect cells in a 15-ml conical tube and centrifuge at 500 x g for 2 minutes at 4°C in a swinging bucket benchtop centrifuge;
4. Wash the pellet twice with ice-cold PBS thoroughly, (the presence of serum from medium could skew the protein assay) and remove as much PBS as possible (the presence of liquid residue dilutes the sample and may also result in the damage of cells during freezing process); and
5. Freeze the pellets for shipping. Pellets must be shipped on dry ice.

B. Suspended Cells

Simply follow steps 3-5 above for "*for adherent cells"* and freeze the cell pellet immediately. Pellets must be shipped on dry ice.

7. TISSUE LYSATE PREPARATION

1. Use 1 ml of lysis buffer per 250 mg wet weight of the chopped tissue;
2. Rinse the tissue pieces in ice-cold PBS three times to remove blood contaminants;
3. Homogenize the tissue on ice with 15 strokes of a glass dounce (or 3 times for 15 seconds each time with a Brinkman Polytron Homogenizer or with a French Press as alternatives);
4. Sonicate the homogenate 4 times for 10 seconds on ice each time to shear nuclear DNA;
5. Centrifuge the homogenate at 90,000 x g or higher for 30 minutes at 4°C in a Beckman Table Top TL- 100 ultracentrifuge, Beckman Airfuge or equivalent. Alternatively clients can also centrifuge on maximum speed (15,000 – 17,000 x g) on a benchtop Eppendorf microcentrifuge for 30 minutes at 4°C;
6. Transfer the resulting supernatant fraction to a new tube and subject it to protein assay. Using a commercial Bradford assay or using the standard protocol of Bradford. Bovine serum albumin should be used as the protein standard;
7. Aliquot at least 100 μg for each lysate to be analyzed with our Kinex™ Antibody Microarray Service;
8. Save any remaining lysate for future validation studies. If you wish to have Kinexus perform the custom Western blotting analysis, aliquot 350-500 μg for each 18 proteins to be tested, or if sample quantity is limited, aliquot 50 μg of sample for each 3 proteins to be tested; and
9. Label your microcentrifuge tubes and freeze immediately.

8. PREPARATION OF TISSUES

An additional charge will apply for submission of tissue samples to be processed at Kinexus. Freshly harvested tissues are preferred if possible. When harvesting, the tissues should be cut into small pieces (no larger than 0.5 x 0.5 x 0.5 cm^3) and quickly rinsed in ice-cold PBS to remove any blood or other contamination on the surface. Wrap the tissues individually in tinfoil and snap freeze them in liquid nitrogen for 10 minutes before storing them at -80 °C. The tissues should be shipped on dry ice.

9. STORAGE OF SAMPLES

The final protein concentration of the cell/tissue samples should be approximately 2 mg/ml. Please record the actual concentration and volume of each sample on the Sample Description Form (KAM-NSDF or KAMCSDF). We request 100 µg of cell or tissue lysate for each sample submitted for analysis with the Kinex™ Antibody Microarray. If any of our custom validation immunoblotting studies are to be performed based on the analysis of your Kinex™ results, we recommend sending additional lysate at this time to save on future shipping costs. We request 350-500 µg of additional material for every 18 antibodies selected for validation Western blotting. Samples should be stored in screw cap vials. The vials should be clearly labeled with an indelible marker with a unique identification number,parafilmed to protect against leakage, and put into another support structure such as a small box or a 50-ml conical or centrifuge tube to provide extra protection during shipping. **All samples must be shipped on dry ice.**