KINETWORKS™ MULTI-IMMUNOBLOTTING

1. QUANTITY OF LYSATE REQUIRED

The Protein Kinase (KPKS 1.2) Screening Service for the expression level of 76 kinases requires 750 µg of crude cell/tissue lysate, while all other standard screens require 500 µg respectively. For the Custom Screens, the KCPS-1.0 Multi-Antibody Screen (1 sample/18 antibodies) also requires 500 µg of lysate, while the KCSS-1.0 Multi-Sample Screen (8 samples/3 antibodies) requires 50 µg for each sample submitted. The final protein concentration in SDS-PAGE sample buffer should be 1 mg/ml, although a range of **0.6 - 2.0 mg/ml** is acceptable. If your concentration is higher or lower, please speak to our customer service representatives.

The cell pellet or tissue should be homogenized in the following ice-cold lysis buffer:

1. 20 mM MOPS, pH 7.0 (any other buffer 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)
   Important Note: *Do not add if you intend to first prepare a cytosolic fraction.*
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 reduce disulphide linkages)

The final pH of the homogenizing buffer should be adjusted to 7.2.

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.

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. Important things to remember are that the cells or tissues should be processed quickly at 4°C or less. Homogenization should not be performed in too large a volume to obtain lysates at the concentration required. The detergent-soluble fraction should be obtained as quickly as possible after the cells or tissues are homogenized. **Sonication is required and cannot be omitted.** The highest centrifugal forces available should be used to generate the detergent-soluble fraction. The supernatants should be frozen as quickly as possible if a protein assay cannot be performed immediately.

2. PREPARATION OF CELL LYSATES

A. Adherent Cells

1. Remove medium from culture dishes containing about 1×10^7 to 2×10^7 cells;
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 is a crucial step and cannot be omitted;
6. Centrifuge the homogenate at 90,000 x g or more for 30 min at 4°C in a Beckman Table Top TL-100 ultracentrifuge or Beckman Airfuge;
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) 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 (BSA) should be used as the protein standard. **Make sure that the protein concentration is determined before the addition of SDS-PAGE Sample Buffer**.

B. Suspension Cells

1. Place medium containing cells in appropriate sized tube and spin 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 200 µl ice-cold lysis buffer per sample (more lysis buffer can be added if the number of cells is high);
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 more for 30 min at 4°C in a Beckman Table Top TL-100 ultracentrifuge or Beckman Airfuge;
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) 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 (BSA) should be used as the protein standard. **Make sure that the protein concentration is determined before the addition of SDS-PAGE Sample Buffer**.

3. PREPARATION OF CELL PELLETS

For an additional fee per sample, Kinexus will process your cell pellets into a lysate for processing with any of our Kinetworks™ screens. To prepare your cell pellets for shipping to Kinexus, please follow steps 1-4 below and label the tubes containing your pellets accordingly. Cell pellets must be shipped on dry ice. Clients may need to prepare as much as 2×10^7 cells to ensure sufficient quantity.

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, 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); **Remove as much PBS as possible** (The presence of liquid residue dilutes the sample and may also result in the damage of cells during the freezing process);
5. Freeze the pellet for shipping. **Pellet must be shipped on dry ice.**

B. Suspension cells:

Simply follow steps 1-3 in the Section 3B and freeze the cell pellet immediately. **Pellets must be shipped on dry ice at the expense of the client.**

4. TISSUE 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. Sonciate the homogenate 4 times for 10 seconds on ice each time to shear nuclear DNA. **This step is crucial and cannot be omitted;**
5. Centrifuge the homogenate at 90,000 x g or more for 30 min at 4°C in a Beckman Table Top TL-100 ultracentrifuge or Beckman Airfuge;
6. Transfer the resulting supernatant fraction to a new tube and subject it to a protein assay using a commercial Bradford assay (available from Bio-Rad) 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 (BSA) should be used as the protein standard. **Make sure that the protein concentration is determined before the addition of SDS-PAGE Sample Buffer.**

5. SAMPLE BUFFER PREPARATION

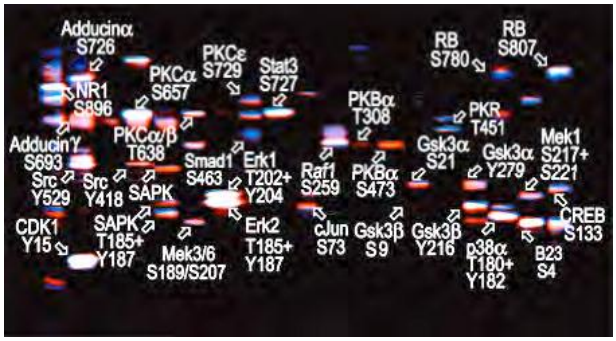
We recommend the final composition of SDS-PAGE Sample Buffer in the sample be: 31.25 mM Tris-HCl (pH 6.8), 1% SDS (w/v), 12.5% glycerol (v/v), 0.02% bromophenol blue (w/v), and 1.25 % β-mercaptoethanol. The cell/tissue samples should be boiled for four (4) min at 100°C in the SDS-PAGE Sample Buffer. (See Appendix A for detailed instructions on preparing the Sample Buffer).

6. STORAGE OF SAMPLES

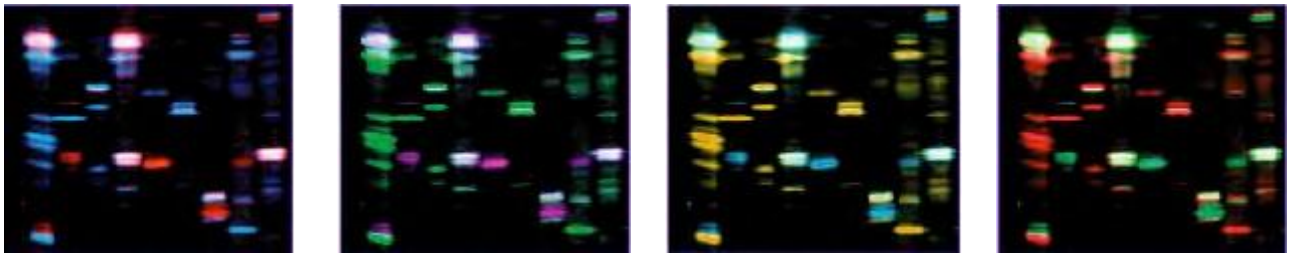
The final protein concentration of the cell/tissue samples should be **1 mg/ml** in SDS-PAGE Sample Buffer as specified by Laemmli *(Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-684).* For all screens, the minimum acceptable protein concentration of the cell/tissue samples in the SDS-PAGE Sample Buffer is **0.6 mg/ml** and the maximum concentration is **2.0 mg/ml**. Please record the actual concentration and volume of each sample on the Sample Description Form (Box B of KW-NSDF-01 or KW-CSDF-01).

For the KPKS–1.2 Screen, exactly 750 µg (i.e. 750 µl of 1 mg/ml protein) of boiled cell/tissue extract protein in the SDS-PAGE Sample Buffer should be aliquoted into a 1.5 ml Eppendorf *screw cap* vial. For all other standard screens, exactly 500 µg (i.e. 500 µl of 1 mg/ml protein) should be aliquoted into a 1.5-ml Eppendorf *screw cap* vial. For the Custom Screens, the KCPS-1.0 Multi-Antibody Screen also requires at least 500 µg of cell/tissue lysate, while the KCSS-1.0 Multi-Sample Screen requires at least 50 µg per sample. There should be one vial per sample for each screen requested, except for the KCSS-1.0 Multi-Sample Custom Screen

which can have up to 8 different samples. The vials should be clearly labeled with an indelible marker with an unique identification number (recorded on the Sample Description Form), parafilmed, and then put into a secondary support container such as a 50-ml conical centrifuge tube to provide extra protection to prevent accidental leakage during shipping. It is not necessary to refrigerate or freeze the samples during shipping once they are in SDS-PAGE Sample Buffer.



Example of a labeled colour overlay of two Kinetworks™ multiblots



Examples of different colour overlay possibilities of two Kinetworks™ multiblots.

Appendix A

KINETWORKS™ SAMPLE BUFFER PROTOCOL

SPECIFICATIONS

Reagent	Volume of Stock	[4X Sample Buffer]
100 % Glycerol	5.00 ml	50 %
1 M Tris-HCl, pH 6.8	1.25 ml	125 mM
20 % SDS	2.00 ml	4 %
1% Bromophenol blue	0.80 ml	0.08 %
Distilled Water	0.45 ml	-
*β -*mercaptoethanol*	0.50 ml	5 %
Total Volume	**10.00 ml**	

INSTRUCTIONS FOR USE

1) Prepare 4X SDS-PAGE Sample Buffer

Prepare the 4X SDS-PAGE Sample Buffer according to the specifications described above (the volume can be adjusted as required). The SDS-PAGE Sample Buffer can be stored at ambient temperature for up to 1 year but for best results, do not store 4X SDS-PAGE Sample Buffer with the β-mercaptoethanol.

2) Adding Reducing Agent

Add 50 µl of β-mercaptoethanol per 950 µl of 4X SDS-PAGE Sample Buffer for a final concentration of 5% β- mercaptoethanol in the 4X stock. Add the * β-mercaptoethanol to the 4X SDS-PAGE Sample Buffer just before mixing with the protein Sample.

3) Sample Dilution Ratio: 1 part 4X SDS-PAGE Sample Buffer to 3 parts Sample

The volume of 4X SDS-PAGE Sample Buffer to add is 25% of the total final volume.

The KPSS-1.3, 10.1, 11.0 and 12.1 and KPPS-1.2 Screens require at least 500 µg of protein. More protein is desirable if possible in case of unforeseen problems. Based on the formula ($C_1V_1 = C_2V_2$), at a protein concentration of 1.85 mg/ml, 270 µl of protein is required to obtain 500 µg with the addition of 125 µl of 4X SDS-PAGE Sample Buffer and 105 µl of phosphate-buffered saline (PBS) or Lysis Buffer, for a total volume of 500 µl (see Example 1).

The KPKS-1.2 Screen requires exactly 750 µg of protein. Based on the formula ($C_1V_1 = C_2V_2$), at a protein concentration of 2.80 mg/ml, 268 µl of protein is required to obtain 750 µg with the addition of 188 µl of 4X Sample Buffer and 294 µl of phosphate-buffered saline (PBS) or Lysis Buffer for a total volume of 750 µl (see Example 2).

Screen	***Example 1***	***Example 2***
Protein required (µg)	500 µg @ 1 mg/ml	750 µg @ 1 mg/ml
Sample concentration	1.85 mg/ml	2.80 mg/ml
Volume required	270 µl	268 µl
4X Sample Buffer	125 µl	188 µl
PBS or Lysis Buffer	105 µl	294 µl
Total Volume	**500 µl**	**750 µl**

For all screens, the lowest protein concentration acceptable of the cell/tissue samples in SDS-PAGE Sample Buffer is 0.6 mg/ml and the maximum protein concentration is 2.0 mg/ml

Prepare samples by heating in a boiling water bath for 4 minutes at 100 °C. The sample should be shipped in a 1.5- ml Eppendorf *screw cap* vial, clearly labeled with an indelible marker for its identification, and parafilmed to prevent accidental opening or leaking.