COSMO BIO CO., LTD.
Inspiration for Life Science

Sample prep 情報 for Multi-immunoblotting_200803

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. Clients must add their own protease inhibitors to the lysis buffer immediately before use. For convenience, they may choose to use the Roche Complete Mini inhibitor cocktail tablet with the addition of pepstatin A as opposed to individual protease inhibitors.

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℃ 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.

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℃ 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℃ 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℃ 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.

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℃ 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.

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℃ in the SDS-PAGE Sample Buffer. (See Appendix A for detailed instructions on preparing the Sample Buffer).

PREPARATION FOR STORAGE AND SHIPPING 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.