Sample Preparation

1. QUANTITY OF LYSATE

The amount of protein required for the Kinex™ KAM-900P Antibody Microarray service is 100 μg per sample at an approximate concentration of 3 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 concentrations, 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**. These will interfere with lysate protein labelling. Please contact Kinexus for more information on the appropriate types of lysis buffers to use or email info@kinexus.ca to request an aliquot of our lysis buffer to be sent at no cost. We only require a courier account number to cover the shipping expenses. Your cell pellets or tissues should be homogenized in ice-cold lysis buffer.

The reagents in the Kinexus Lysis Buffer (pH 7.2) include:

1. 20 mM MOPS (pH 7.0)
2. 2 mM EGTA (to bind calcium);
3. 5 mM EDTA (to bind magnesium and manganese);
4. 50 mM sodium fluoride (to inhibit protein-serine phosphatases);
5. 60 mM β-glycerophosphate, pH 7.2 (to inhibit protein-serine phosphatases);
6. 25 mM sodium pyrophosphate (to inhibit protein-serine phosphatases);
7. 2.5 mM sodium orthovanadate (to inhibit protein-tyrosine phosphatases);
8. 50 nM phenylarsine oxide
9. 1% Triton X-100 * (can be substituted with 1% Nonidet P-40)

NOTE: *A detergent (Triton X-100) is required for preparing total detergent-solubilized lysates. The detergent should be omitted from the lysis buffer if a subcellular fractionation is to be performed.*

For chemical cleavage harvesting only:

10. 10 mM TCEP (Tris(2-carboxyethyl)phosphine hydrochloride)
11. 100 mM NTCB (2-nitro-5-thiocyanatobenzoic acid) (added after sonication)

Protease Inhibitors and Dithiothreitol

12. 0.5 μM aprotinin (to inhibit proteases);
13. 3 mM benzamidine (to inhibit proteases);
14. 1 mM Petabloc (to inhibit proteases);
15. 10 μM leupeptin (to inhibit proteases); and
16. 1 mM dithiothreitol (to disrupt disulphate 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. If the lysate proteins are to remain in their native structure and not denatured, the chemical cleavage step should not be used, and the samples must be frozen and shipped to Kinexus on dry ice. Samples that have been subjected to chemical cleavage or homogenized directly into 1X SDS-PAGE sample buffer can be sent to Kinexus without the need for refrigeration or freeze during shipping.

Important points to remember include:

1. The cells or tissues should be processed quickly at 4°C or less if the samples are not subjected to chemical cleavage at the time of homogenization;
2. Add the protease inhibitors and DTT to the lysis buffer just before processing samples;
3. Ensure the contents are completely dissolved and store on ice;
4. Homogenization should be performed in small volumes of lysis buffer to obtain protein lysates at high concentrations, ideally at 3-4 mg/ml or higher. The concentrations can be diluted later if required;
5. The detergent-soluble fraction should be obtained as quickly as possible after the cells or tissues are homogenized;
6. Sonication is required for optimal results (do not over sonicate);
7. The highest centrifugal forces available should be used to generate the detergent-soluble fraction;
8. The supernatants should be frozen as quickly as possible if a protein assay cannot be performed immediately. Lysates should be stored at -70°C, unless these have been subjected to chemical cleavage or processed in SDS-PAGE sample buffer.
9. We recommend harvesting cells and tissues with the chemical cleavage reagents (TCEP and NTCB) to help reduce the number of false positives that can arise from the use of non-denatured proteins on the antibody microarray. If you choose to prepare samples without the chemical cleavage method, then omit the sections below outlined in red. However, you should let us know and we can include the chemical cleavage step for you prior to probing your lysates on the microarray. Note that the best results are obtained if the chemical cleavage is performed during initial lysate preparation.

3. F RACTIONATIONS

There are many different types of fractionations that can be performed, and the choice of lysis buffer used will vary depending on the type of fractionation you are considering to prepare. The simplest type of lysate preparation is the total cellular extract obtained as a total detergent-solubilized fraction. To obtain just the soluble cytoplasmic proteins, detergent should not be included in the homogenizing buffer. The remaining microsomal pellet obtained following ultracentrifugation after removal of the cytoplasmic supernatant fraction can be re-sonicated in homogenizing buffer with detergent and re-ultracentrifuged to obtain the detergent-soluble membrane fraction.

Total Cellular Extract:

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 and is

optimal for monitoring changes in total protein expression. However, if proteins are re-distributed between cellular compartments as a consequence of a perturbation of an experimental model system, this will not be evident.

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 you send samples from immunoprecipitation or antibody affinity pull-down experiments for the Kinex™ KAM Antibody Microarray Services unless you consult with us first.

4. PROTEIN LYSATE PREPARATION WITH AND WITHOUT CHEMICAL CLEAVAGE

The optimum amount of protein recommended for the Kinex™ KAM-900P Antibody Microarray is 100 μg per sample at a concentration of 3.0 mg/ml or higher. We recommend preparing extra lysate, if possible, for follow-up studies. If the concentration of the lysate is below 2.0 mg/ml concentration, the sample can be concentrated using an Amicon Ultra-0.5 Ultracel-3 Membrane Centrifugal Filter with a M.W. cut-off of 3,000 (Catalog Number: UFC500308, Millipore, Billerica, MA). For more information about how to concentrate samples, please contact a Kinexus Technical Services representative at info@kinexus.ca or call 1-866-546-3987.

It is highly recommended to use the Kinexus Lysis Buffer included with this kit for protein lysate preparation, as it has been optimized for the use with KAM Antibody Microarray as well as any follow-up services. Other lysis buffers commonly used for protein lysate preparation containing non-ionic detergents may be compatible with the KAM-Antibody Microarray. **However, no lysis buffer containing Tris or reagents carrying reactive amine groups such as glycine and ammonia should be used to prepare lysates for the KAM Antibody Microarray.** The Kinexus Lysis Buffer contains phosphatase inhibitors and the Lysis Buffer Cocktail contains protease inhibitors and DTT. Immediately prior to use, transfer the content of the Kinexus Lysis Buffer into the Lysis Buffer Cocktail. Invert the tube several times to make sure the contents are completely dissolved and store on ice. Prepare the cell or tissue lysates according to protocols listed below. The resulting protein lysate samples prepared must be frozen at -70°C or below after protein quantification unless they are to be immediately subjected to protein labelling and purification.

It is also highly recommended to harvest cells and tissues at the time of homogenization with the chemical cleavage reagents (TCEP and NTCB) to help reduce the number of false positives that can arise from the use of non-denatured proteins on the antibody microarray. Samples prepared with the cysteine chemical cleavage (CCC)

method are stable at room temperature for at least 2 weeks. Use the appropriate set of instructions that follow depending on the type of cells or tissues to be analyzed and whether the CCC method is desired or not.

A) Preparation of Lysates from Cells with Chemical Cleavage

i) Adherent Cells:

1. Remove medium from culture dishes containing approximately 1×10^6 to 2×10^6 cells for each sample to be analyzed using the KAM-900P microarray.
2. Rinse the cells in the dishes twice with ice-cold Phosphate Buffered Saline (PBS) to remove medium residue (serum must be completely removed) and aspirate as much PBS as possible after the last rinse.
3. Mix the components in the **Kinexus Lysis Buffer** as listed in Section 11. Invert the tube several times to ensure the contents are completely dissolved and store on ice. Add 200 µl of the ice-cold Kinexus Lysis Buffer to a 150-mm culture dish, or add 100 µl ice-cold Kinexus Lysis Buffer to a 100-mm culture dish. Also, add 25 µl of 10 mM TCEP to 500 µl of lysis buffer for a final concentration of 0.5 mM TCEP. Adjust the pH of the lysis buffer containing 0.5 mM TCEP to pH 9 (approximately 2 µL of 10 N NaOH per 1 mL buffer).
4. Scrape the cells in Kinexus Lysis Buffer, collect the resulting cell suspension from dishes and transfer it into a 1.5-ml microcentrifuge tube. Check to make sure that the pH is 9.0.
5. Sonicate using a microprobe sonicator 4 times for 10 seconds each with 10-second intervals on ice to rupture the cells and to shear nuclear DNA. Alternatively, passing the cell suspension through a 26-gauge needle until the sample is no longer viscous is also acceptable if a sonicator is not accessible. This step is crucial and cannot be omitted. Add 6 µL of 100 mM NTCB per 100 µL cell homogenate for a final concentration of 6 mM NTCB, and make sure that the pH is 9.0 and adjust with 10 N NaOH if necessary). Incubate the homogenate at 45°C in a water bath for 30 minutes.
6. Centrifuge the resulting lysate homogenate at 90,000 x g or above for 30 minutes at room temperature in a Beckman Table Top TL-100 ultracentrifuge, Beckman Airfuge or equivalent. Alternatively, clearing homogenates at maximum speed (15,000-17,000 rpm) on a benchtop microcentrifuge for 30 minutes at room temperature is also acceptable.
7. Transfer the resulting supernatant to a new 1.5-ml microcentrifuge tube.
8. Remove a small aliquot and determine its protein concentration using a commercial Bradford assay reagent (available from Bio-Rad, catalogue number 500-0201) or following 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) is used as the protein standard. The protein concentration obtained should be approximately 3.0 mg/ml or higher. If the concentration obtained is less than 1.0 mg/ml, samples should be concentrated using an Amicon Ultra-0.5 Centrifugal Filter (Millipore).
9. Check the pH of the lysates and adjust to pH 7.0-7.4 with 1 M HCl if necessary. Aliquot and set aside 100 µg for each lysate to be analyzed with the KAM-900P chip.
10. If you wish to have Kinexus perform the custom immunoblotting follow-up analysis, aliquot 350-500 µg for each 18 antibodies to be tested, and boil in SDS-Sample Buffer following the protocols specified on our website. Chemically cleaved lysates are stable at ambient temperature for at least 2 weeks. Store any remaining lysates at -70°C for subsequent validation studies.

ii) Suspension Cells:

1. Transfer cells with medium from cell culture flasks into appropriate sized tubes 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 this step once to ensure complete removal of serum. Remove as much PBS as possible after the final wash.
3. Mix the components in the **Kinexus Lysis Buffer** as listed in Section 11**.** Invert the tube several times until dissolved and store on ice. Add 25 µl of 10 mM TCEP to 500 µl of lysis buffer for a final concentration of 0.5 mM TCEP, and adjust the pH to 9 (which is approximately 2 µL of 10 N NaOH per 1 mL buffer). Add an adequate amount of the ice-cold Kinexus Lysis Buffer to the sample based on the number and type of cells to achieve a final total protein concentration of approximately 3.0 mg/ml.
4. Follow Steps # 5 through 10 as described in the Adherent Cells Section above.

B) Preparation of Lysates from Cells without Chemical Cleavage

i) Adherent Cells:

1. Remove medium from culture dishes containing approximately 1×10^6 to 2×10^6 cells for each sample to be analyzed using the KAM-900P microarray.
2. Rinse the cells in the dishes twice with ice-cold Phosphate Buffered Saline (PBS) to remove medium residue (serum must be completely removed) and aspirate as much PBS as possible after the last rinse.
3. Mix the components in the **Kinexus Lysis Buffer** as listed in Section 11. Invert the tube several times to ensure the contents are completely dissolved and store on ice. Add 200 µl of the ice-cold Kinexus Lysis Buffer to a 150-mm culture dish, or add 100 µl ice-cold Kinexus Lysis Buffer to a 100-mm culture dish.
4. Scrape the cells in Kinexus Lysis Buffer, collect the resulting cell suspension from dishes and transfer it into a 1.5-ml microcentrifuge tube.
5. Sonicate using a microprobe sonicator 4 times for 10 seconds each with 10-second intervals on ice to rupture the cells and to shear nuclear DNA. Alternatively, passing the cell suspension through a 26-gauge needle until the sample is no longer viscous is also acceptable if a sonicator is not accessible. This step is crucial and cannot be omitted.
6. Centrifuge the resulting lysate homogenate at 90,000 x g or above for 30 minutes at 4°C in a Beckman Table Top TL-100 ultracentrifuge, Beckman Airfuge or equivalent. Alternatively, clearing homogenates at maximum speed (15,000-17,000 rpm) on a benchtop microcentrifuge for 30 minutes at 4°C is also acceptable.
7. Transfer the resulting supernatant to a new 1.5-ml microcentrifuge tube. The following steps should be performed as quickly as possible with the supernatant fraction kept in an ice bath.
8. Remove a small aliquot and determine its protein concentration using a commercial Bradford assay reagent (available from Bio-Rad, catalogue number 500-0201) or following 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) is used as the protein standard. The protein concentration obtained should be approximately 3.0 mg/ml or higher. If the

concentration obtained is less than 1.0 mg/ml, samples should be concentrated using an Amicon Ultra-0.5 Centrifugal Filter (Millipore).

9. Aliquot and set aside 100 μg for each lysate to be analyzed with the KAM-900P chip.
10. Store any remaining lysates at -70°C for subsequent validation studies. If you wish to have Kinexus perform the custom immunoblotting follow-up analysis, aliquot 350-500 μg for each 18 antibodies to be tested, and boil in SDS-Sample Buffer following the protocols specified on our website. Label and freeze remaining lysates.

ii) Suspension Cells:

1. Transfer cells with medium from cell culture flasks into appropriate sized tubes 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 this step once to ensure complete removal of serum. Remove as much PBS as possible after the final wash.
3. Mix the components in the **Kinexus Lysis Buffer** as listed in Section 11**.** Invert the tube several times until dissolved and store on ice. Add an adequate amount of the ice-cold Kinexus Lysis Buffer to the sample based on the number and type of cells to achieve a final total protein concentration of approximately 3.0 mg/ml.
4. Follow Steps # 5 through 10 as described in the Adherent Cells Section above.

C) Preparation of Lysates from Tissues with Chemical Cleavage

1. Mix the components in the **Kinexus Lysis Buffer** as listed in Section 11**.** Add 25 μl of 10 mM TCEP to 500 μl of lysis buffer for a final concentration of 0.5 mM TCEP. Invert the tube several times until dissolved and adjust the pH of the lysis buffer containing 0.5 mM to pH 9 (which is approximately 2 μL of 10 N NaOH per 1 mL buffer) and store on ice. Use approximately 1 ml of the Kinexus Lysis Buffer per 250 mg wet tissue.
2. Cut the tissue into smaller pieces and rinse them in ice-cold PBS three times to remove any blood contaminants.
3. Homogenize the tissue on ice with 15 strokes of a glass douncer (or 3 times for 15 seconds each time with a Brinkman Polytron Homogenizer or with a French Press as alternative).
4. Sonicate the homogenate 4 times for 10 seconds on ice each time to shear nuclear DNA.
5. Add 6 μL of 100 mM NTCB per 100 μL cell homogenate for a final concentration of 6 mM NTCB, and adjust the pH to 9.0 with 10 N NaOH if necessary. Incubate the homogenate at 45°C water bath for 30 minutes.
6. Centrifuge the homogenate at 90,000 x g or higher for 30 minutes at room temperature in a Beckman Table Top TL-100 ultracentrifuge, Beckman Airfuge or equivalent. Alternatively, clients can also centrifuge at maximum speed (15,000 – 17,000 rpm) on a benchtop microcentrifuge for 30 minutes at room temperature.
7. The following steps should be performed as quickly as possible once the supernatant fraction is obtained. Check that the pH of the lysates, which should be close to neutral (pH 7.0-7.4) and adjust with 1 M HCl if necessary.
8. Transfer the resulting supernatant fraction to a new tube and subject it to a protein assay using a commercial Bradford assay reagent or using the standard protocol of Bradford. BSA should be used as the protein standard. The protein concentration obtained should be approximately 15-20 mg/ml or higher, but a final

concentration of only 3 mg/ml for the antibody microarray is needed. If the concentration obtained is less than 1.0 mg/ml, samples should be concentrated using an Amicon Ultra-0.5 Centrifugal Filter (Millipore).

8. Aliquot 100 μg for each lysate to be analyzed with the KAM-900P antibody microarray.
9. Chemically cleaved lysates are stable at ambient temperature for at least 2 weeks. Store any remaining lysates at -70°C for subsequent validation studies.

D) Preparation of Lysates from Tissues without Chemical Cleavage

1. Mix the components in the **Kinexus Lysis Buffer** as listed in Section 11**.** Invert the tube several times until dissolved and store on ice. Use approximately 1 ml of the Kinexus Lysis Buffer per 250 mg wet tissue.
2. Cut the tissue into smaller pieces and rinse them in ice-cold PBS three times to remove any blood contaminants.
3. Homogenize the tissue on ice with 15 strokes of a glass douncer (or 3 times for 15 seconds each time with a Brinkman Polytron Homogenizer or with a French Press as alternative).
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 at maximum speed (15,000 – 17,000 rpm) on a benchtop microcentrifuge for 30 minutes at 4°C. The following steps should be performed as quickly as possible once the supernatant fraction is obtained.
6. Transfer the resulting supernatant fraction to a new tube, which is kept in an ice bath, and subject it to a protein assay using a commercial Bradford assay reagent or using the standard protocol of Bradford. BSA should be used as the protein standard. The protein concentration obtained should be approximately 15-20 mg/ml or higher. If the concentration obtained is less than 1.0 mg/ml, samples should be concentrated using an Amicon Ultra-0.5 Centrifugal Filter (Millipore).
7. Aliquot 100 μg for each lysate to be analyzed with KAM-900P and keep it on ice if it is to be used immediately.
8. Store any remaining lysates at -70°C for subsequent validation studies. Label the microcentrifuge tubes and freeze them immediately.

E) Additional Notes for KAM-900P Lysate Preparation

1. Note all cell lines are different so the suggested number of 1×10^6 to 2×10^6 cells for each sample is an estimate based on commonly used cell lines. For the validation immunoblotting service, you will need to prepare about 10 times more cells (1×10^7 to 2×10^7 cells).
2. Cells or tissues should be processed in a timely fashion at 4°C or below if the chemical cleavage step is not used.
3. The Kinexus Lysis Buffer with its phosphatase and protease inhibitors should be completely dissolved and kept over ice just prior to use.
4. Protein concentration of each sample should preferably be at or above 3.0 mg/ml.
5. 100 μg of lysate is recommended to be used, especially with the KAM-900P chip, since the phosphorylation of target proteins at specific sites is often found with very low stoichiometry. However, if sample material is

difficult to obtain, as little as 25 μg of lysate has been successfully used. (Note: The same amount of protein from each sample to be analyzed together must be applied to the microarray for optimal comparison purposes).

6. To minimize the volume and maximize the protein concentration of lysates, the lysis buffer used to recover the scraped cells from a culture dish can be transferred to the next dish if multiple dishes of cells for the same sample are to be used for lysate preparation. It is advised to use the *minimal amount* of lysis buffer for lysate preparation to achieve the protein concentration required for the KAM-900P antibody microarray analysis.
7. Nuclear DNA shearing by sonication or needle passing is necessary and cannot be omitted.
8. The highest centrifugal forces achievable on a microcentrifuge should be used to prepare the detergent-soluble fraction.
9. Detergents should be omitted from the lysis buffer if a particulate-solubilized fraction is to be prepared and analyzed.
10. Supernatants should be separated from pelleted precipitates and frozen as quickly as possible if the chemical cleavage is not performed. Removal of an aliquot for the protein assay is suggested so that the bulk of the lysate sample can be frozen quickly to preserve the phosphorylation state of the proteins in the extract.

Once we have received your lysate samples at Kinexus, they will undergo extensive processing according to your specifications. To get a sense of how they might be handled, demonstration videos are also available for viewing on our company's You-Tube Channel at https://www.youtube.com/channel/UC_GL-BCsGRrnKiQ_6qV1jeA .

5. PREPARATION OF CELL AND TISSUE PELLETS

An additional charge of $200 per sample will apply for submission of cell pellets to be processed at Kinexus. A sufficient number of cells (>2 x 10^6 cells) should be provided for each sample to be subjected to KAM-900P analysis. If Kinetworks™ multi-immunoblotting is desired for validation of the KAM-900P results, the number of cells required is ten-fold higher (>2 x 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 “A) Adherent Cells" and freeze the cell pellet immediately. Pellets must be shipped on dry ice.

C) Tissues

An additional charge of $200 per sample 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 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.