OVERVIEW OF KINEX™ KAM – 880 ANTIBODY MICROARRAY

1. INTRODUCTION

The Kinex™ signal transduction protein profiling services are convenient and very cost-effective solutions to assist scientists in the broad discovery of productive research leads such as biomarkers. These services utilize our unique antibody microarrays to track the differential binding of dye-labeled proteins in lysates prepared from cells and tissues including cell extracts, fresh or frozen tissues and serum samples. The results can provide productive insights into differences in protein expression, phosphorylation and protein-protein interactions, and define antibody reagents that can be used to follow up these findings. Our unique integrated platform of proteomics and bioinformatics services and proprietary technologies makes the Kinex™ Antibody Microarray superior to any other commercially available antibody microarrays. In this Customer Information Package, we explain how the KAM-880 Antibody Microarray performs and how it can be most effectively used to advance your research programs.

The Kinex™ KAM-880 Antibody Microarray is our most powerful and advance antibody microarray chip to date, it was released in January 2015. It is the culmination of continuous on-going efforts to steadily improve the power and accuracy of our antibody microarrays over the last 6 years. Advantages of our KAM-880-based services include: screening with 877 highly validated and well characterized pan- and phosphosite-specific antibody probes, with wide coverage of cell signalling proteins and pathways; proprietary methods and reagents to reduce the rate of false positives and improve sensitivity; extensive follow up immunoblotting services for validation; and complementary bioinformatics analyses for comparison purposes and better understanding of the results.

2. HIGHLY VALIDATED ANTIBODIES

Our current Kinex™ KAM-880 Antibody Microarray monitors changes in the expression levels and phosphorylation states of signalling proteins which includes 518 pan-specific antibodies (for protein expression) and 359 phospho- site-specific antibodies (for phosphorylation). These antibodies, which have been selected from more than 6000 different commercial antibodies from over 26 companies, have been independently tested by Kinexus to identify many of the best immunological reagents available today to track important signal transduction proteins. The top 20% of these antibodies that performed well in Western blotting applications have been incorporated into our Kinex™ Antibody Microarrays. In addition, Kinexus has started production of well characterized cell signalling antibodies, many of which are incorporated into this latest KAM-880 Antibody Microarray. Such cherry-picking is apparently not performed by other microarray companies, which rely only on one or two suppliers with dubious information about individual antibody performance. When our clients utlize the KAM-880 antibody microarray, upon request, we are pleased to disclose the commercial sources and in many cases, these antibodies are available directly from Kinexus. Immunoblots with the antibodies sold by Kinexus are available for easy viewing on our website. A complete listing of all the antibodies printed on the KAM-880 chip is provided or is downloadable from the Kinexus website. The classes of targeted proteins and phosphosites on the KAM-880 Antibody Microarray are listed in Table 1 below. The antibodies in our microarrays have been optimized to work in human, mouse and rat model systems, but have also been shown commonly to work in chicken, bovine, porcine, canine, rabbit, frog, sea star and many other diverse model systems.

Table 1. Families of protein targets for the KAM-880 Antibody Microarray chip. These statistics apply to Lot K00120 and may be slightly altered in future print runs of this microarray chip.

Content	Total %	Total Number
Total Number of pan-specific antibodies:	60%	518
Total Number of phospho-specific antibodies:	40%	359
Total Number of Antibodies	**100%**	**877**
Total Number of protein kinase pan-specific antibodies:	35%	307
Total Number of protein kinase phospho-specific antibodies:	23%	200
Total Number of protein phosphatase pan-specific antibodies:	7%	65
Total Number of protein phosphatase phospho-specific antibodies:	0.5%	4

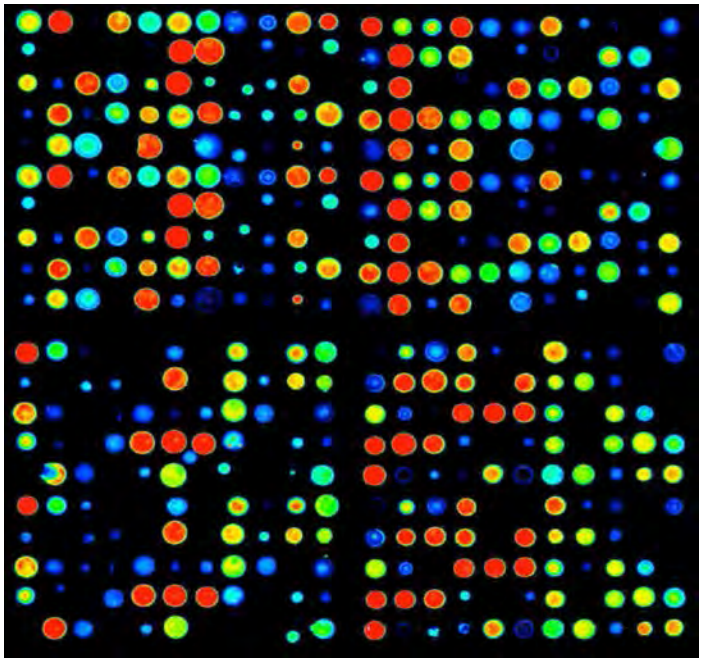
3. QUALITY CONTROL PROCEDURES

Our antibodies are covalently immobilized on a high quality glass surface coated with a proprietary 3-D polymer material to ensure high binding efficiency and specificity. Our microarrays are subjected to stringent quality control measures designed to ensure optimum antibody activity, printing consistency, and consistent intra-slide and inter- slide variability. Each microarray also has loading and antibody controls to ensure the amount of protein is consistent on all fields. The KAM-880 Antibody Microarray provides semi-quantitative analyses of the expression and phosphorylation states of cell signalling proteins in two samples. The quantitative analysis of the strength of the fluorescence signals for each target protein is based on duplicate measurements.

In internal studies with the latest KAM-880 Antibody Microarray and without chemical cleavage, we determined that the median spread between duplicate measurements with the same antibody in printed pairs was about 24% (i.e. the median range from the average of the duplicates was ±12% with a standard deviation of 2.0% from testing of 12 fields of 877 antibody pairs per field). With chemical cleavage, we determined that the median spread between duplicate measurements with the same antibody in printed pairs was about 30% (i.e. the median range from the average of the duplicates was ±15% with a standard deviation of 2.0% from testing of 54 fields of 877 antibody pairs per field). The frequency of flagged antibody spots dues to dust or mis-printing is less than 0.5%. When the average of duplicate measurements of antibody pairs on each chip was determined for the same sample applied to different KAM-880 Antibody Microarrays, we observed that the median value for the differences in the averages was ±8.1% with a standard deviation of 0.6% from testing of 4 pairs of fields. The dynamic range between the highest and lowest reproducible dye-bound protein signals from these Kinex™ chips was greater than 6,000-fold.

For learning more about the procedures that Kinexus follows in performing our KAM-880 Antibody Microarray analyses, a series of demonstration videos are available for viewing on our company's You-Tube Channel at https://www.youtube.com/channel/UC_GL-BCsGRrnKiQ_6qV1jeA

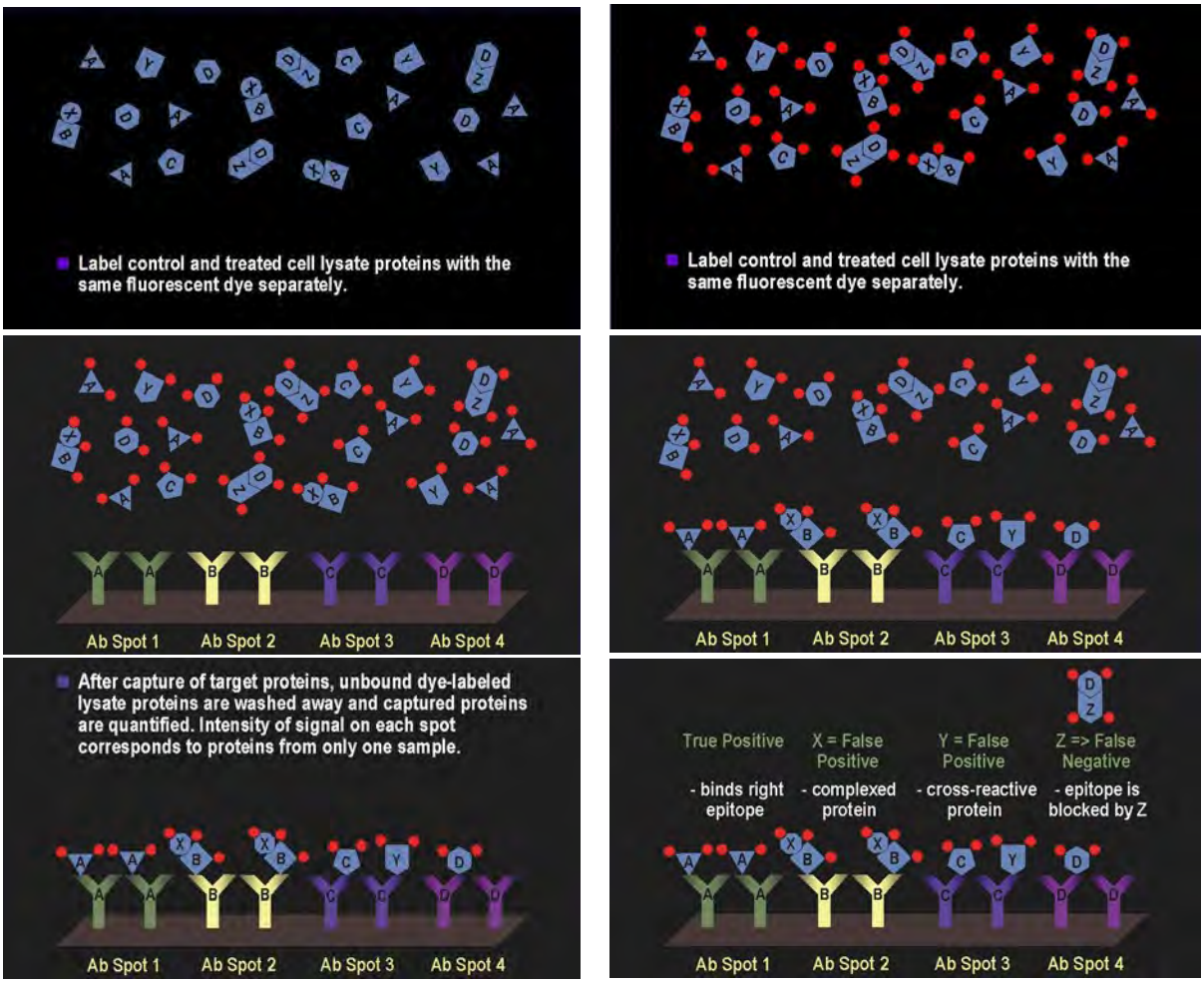
Figure 2. Close up scanned image of 4 of 32 grids that are divided into two fields on a Kinex™ KAM-880 Antibody Microarray chip. Decreasing signal intensity corresponds with a red to orange to yellow to green to blue transition.



4. PRINCIPLES OF BINDING AND DETECTION

The methodology behind the Kinex™ KAM-880 Antibody Microarray is illustrated in Figure 3. The issues of antibody cross-reactivity, protein complexes and epitope masking are highlighted in last panel.

Figure 3. Methodology used in Kinex™ KAM-880 Antibody Microarray.



5. PROPRIETARY DYE COMBINATION

One key advantage of our antibody microarrays is that lysate samples from control and treated cells are labeled with the same dyes and analyzed together on the same chip at the same time. These dyes are included with the Kinex™ KAM-880 Antibody Microarray Kit. In our experience, the use of a two dye, competitive binding system, in which a control sample is labeled with a different dye from the treatment sample and the two samples are mixed and co-incubated with the same regions of the same chips, generates a higher rate of false leads. Unlike oligonucleotides such as DNA and RNA, proteins display strong individual differences in their relative affinities for dyes. It should be appreciated that this problem also significantly impacts other proteomics approaches such as DIGE 2D gel analysis where two samples that are labeled with different dyes are mixed prior to electrophoresis

Colour changes seen with spots evident on a DIGE 2D gel may not be related to differences in protein expression but rather dye binding to individual protein species. Clients should also be aware that cell signalling proteins are typically present at concentrations that are 100- to 1,000-fold lower than structural proteins and metabolic pathway enzymes. Consequently, these low abundance proteins are usually not evident on 2D gels without some type of special pre-enrichment. This is why we feel that antibody-based detection of proteins with our Kinex™ KAM-880 Antibody Microarrays and our follow up Kinetworks™ Custom Screens are superior and complementary methods to undertake broad studies of proteins for signalling network analyses.

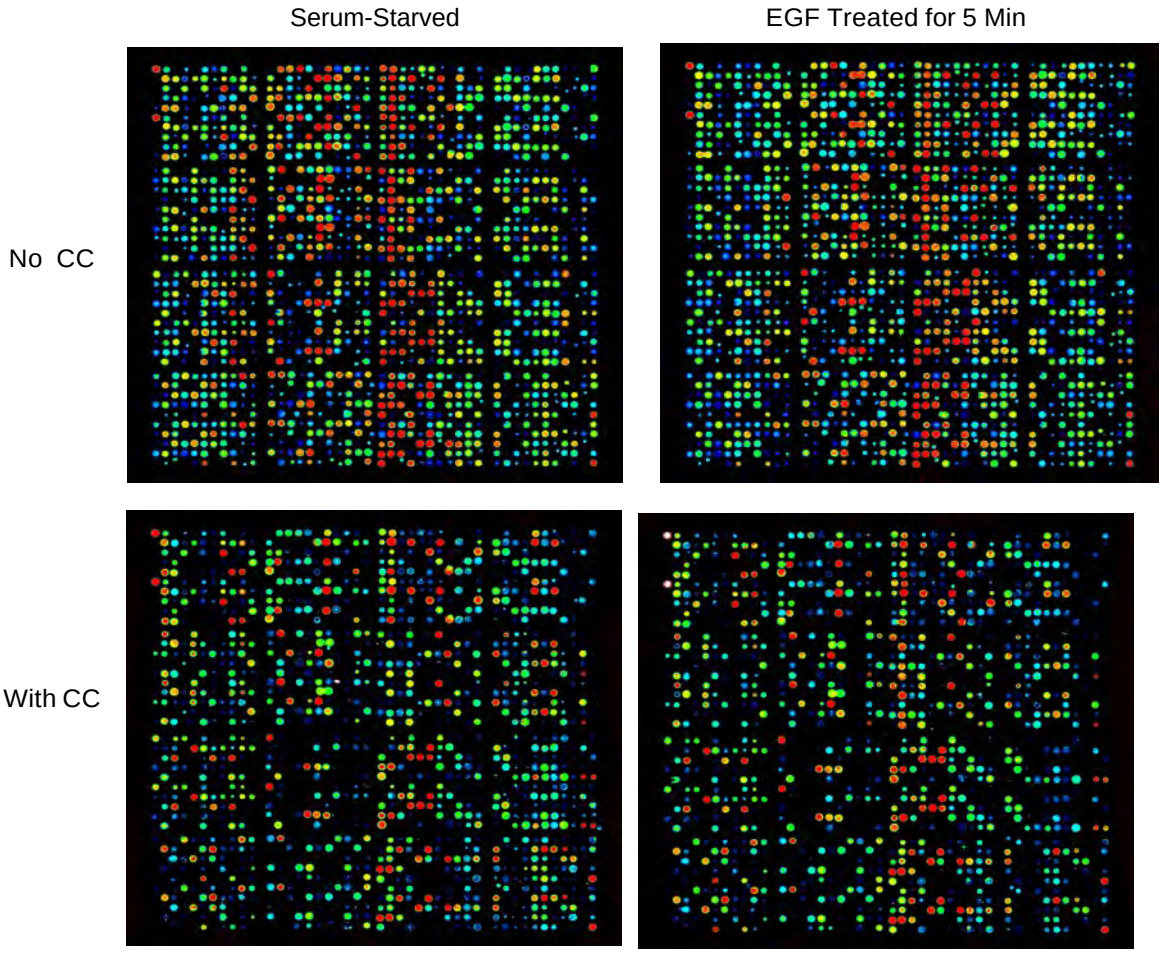
6. FALSE POSITIVES & FALSE NEGATIVES (CHEMICAL CLEAVAGE)

Since non-denatured proteins are commonly analyzed by this method, as illustrated in the last panel of Figure 3, there is increased opportunity for false positives and false negatives due to antibody cross-reactivity and blocked epitopes in protein complexes. Many proteins reside in complexes with other proteins and antibodies, and as it is normally necessary to use non-denaturing conditions with antibody microarrays, many apparent changes in protein expressions or phosphorylations may arise from alterations in protein-protein interactions. In our internal studies with cells from different cells, tissues and species, only between 30 to 45% of the protein changes detected on a protein microarray were reproduced by immunoblotting. In addition, about 20 to 30% of the protein changes could not be validated by immunoblotting, because no detectable immunoreactive proteins were evident in these studies as the antibody microarray appears to be about 10-fold or more sensitive than standard Western blotting. It should be appreciated that this high rate of false positives is an inherent problem with all commercial antibody microarrays due to the reliance on non-denaturing conditions for immune capture of target proteins. To help reduce the number of false positives that are typically generated on a protein microarray, we have developed a proprietary chemical digestion step in which native proteins are cleaved into larger fragments. This fragmentation leads to dissociation of complexes, but does not destroy most of the epitopes recognized by phosphosite-antibodies. Typical enzymatic cleavage of proteins with proteases such as trypsin causes the loss of most phosphosite epitopes as basic amino acids commonly surround phosphoserine and phosphothreonine sites. This chemical digestion step is an option to reduce the number of false positives for clients that are less interested in tracking protein-protein interactions changes in experimental model systems. The reagents for this chemical cleavage step are utilized with the Kinex Microarray Service, but not provided with the KAM-880 Kit unless ordered separately, as many users may wish to also observe changes in protein-protein interactions in lysate samples from specimens from humans and animals. Table 2 summarizes the differences in results in the analysis of lysates from growth factor treated cells analyzed with and without the chemical cleavage step.

Table 2. Effect of chemical cleavage on the detection of protein changes on the KAM-880 antibody microarray using lysates from epidermal growth factor-treated A431 cells. Overnight, serum-starved A431 cells were treated with and without 100 nM EGF for 5 minutes prior to preparation of cell lysates. The lysates were dye-labeled either without or with prior chemical cleavage (CC). In the Table 2, the data is restricted to those antibodies that yielded Intensity signals that were greater than 300. With chemical cleavage, the Median Intensity signal for the antibody spots was reduced by 80% to 1149. Without chemical cleavage, the Median Intensity signal was 5843. Based on the data below, which represent the averaged results from three separate experiments, we conclude that chemical cleavage step shows more marked changes with EGF treatment and even improves the detection signals with some antibodies. While chemical cleavage produced a marked decline in the Intensity signals (based on Median values), the signal strength is still very high.

Effect of EGF	# Ab with ≥ 100% increase	# Ab with ≥ 50% increase	# Ab with ≥ 50% decrease	# Ab with ≥ 75% decrease
Without CC	44	92	31	1
With CC	48	142	11	0

Figure 4. Scanned images of a Kinex™ Antibody Microarray following incubation with dye-labeled lysate proteins from serum-starved A431 human cervical carcinoma cells treated without (left fields) and with 100 nM epidermal growth factor for 5 minutes (right fields). Prior chemical cleavage of the lysate proteins before incubation with the chips was either not performed (upper fields) or carried out (lower fields).



To provide a sense of the typical performance of individual antibodies on the Kinex™ KAM-880 Antibody Microarray and enable comparison of the specific results obtained with a tested customer cell/tissue lysate, we have generated an MS-Excel spreadsheet entitled “KAM-880_Performance.xlsx”. This file, which is provided along with our client’s specific KAM-880 results, features the results obtained from the analyses of 12 different cell or tissue lysates samples without chemical cleavage and 54 lysate samples with chemical cleavage. It also indicates which antibodies printed on the KAM-880 chips can be ordered directly from Kinexus for follow up studies.

7. KAM-880 ANTIBODY MICROARRAY REPORTS

Following scanning and quantification of the intensities of dye-bound proteins captured on the KAM-880 chip, we use our proprietary software to average the intensities recorded for each pair of antibody spots to calculate the differences between the control and treated lysate samples. This includes data normalization to account for slight differences in lysate protein loading, calculations of Z scores, percent changes from control (%CFC) and application of our proprietary Kinections Pathway Mapping analyses. This permits the identification of the most promising biomarkers for further validation by immunoblotting. The Kinections Pathway Maps provides direct linkage of subsets of the KAM-880 results with local signalling network maps for many of the proteins and phosphosites tracked on the KAM-880 microarray. The Report is in PDF and MS-Excel formats.