invitrogen™

ProtoArray® Human Protein Microarray v5.0 Kinase Substrate Identification (KSI) Complete Kit

for kinase substrate identification

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User Manual

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Kit Contents and Storage

Shipping and Storage

The components included in the ProtoArray® Human Protein Microarray v5.0 KSI Complete Kit *for kinase substrate identification* are shipped as detailed below. Upon receipt, store as indicated.

The components of the buffer modules are stable for 6 months when stored properly. The **expiration date** is printed on the package for each array. Use the array before the expiration date for best results.

Component	Shipping	Storage
ProtoArray® Human Protein Microarray v5.0	Blue ice	–20°C
ProtoArray® Control Protein Microarray v5.0	Blue ice	–20°C
ProtoArray® KSI Buffer Module A	Dry ice	see next page
ProtoArray® KSI Buffer Module B	Room temperature	Room temperature

ProtoArray® Microarrays

Each ProtoArray® Human Protein Microarray KSI Complete Kit contains **two** ProtoArray® Human Protein Microarrays v5.0 and **two** ProtoArray® Control Protein Microarrays v5.0

Store the microarrays at –20°C.

For best results, use microarrays before the specified expiration date.

For details on array specifications, see pages 6-9.

Continued on next page

ProtoArray® KSI Buffer Module A

The ProtoArray® KSI Buffer Module A includes the reagents listed in the table below.

Upon receipt, store components as follows:

- Store Control Kinase at –80°C
- Store the remaining components at –20°C

Note: Sufficient reagents are supplied to perform 4 microarray screening experiments.

Item	Composition	Amount
Bovine Serum Albumin (BSA)	30% BSA in 0.85% NaCl	5 mL
DTT	1 M DTT in deionized water	400 µL
Kinase Buffer	100 mM MOPS, pH 7.2, 1% Nonidet P40 (NP40), 100 mM NaCl, 10 mg/mL BSA, 5 mM $MgCl_2$, 5 mM $MnCl_2$	10 mL
Control Kinase (concentration of the kinase is listed on the tube label)	Control Kinase in 30 mM potassium phosphate, pH 7.4, 50% glycerol, 150 mM KCl, 1 mM EDTA and 1 mM DTT	10 µL

ProtoArray® KSI Buffer Module B

The ProtoArray® KSI Buffer Module B includes the reagents listed in the table below.

Store at room temperature.

Note: Sufficient reagents are supplied to perform 4 microarray screening experiments.

Item	Composition	Amount
10X Phosphate Buffered Saline (PBS)	10X PBS, pH 7.4	14 mL
SDS	10% SDS in deionized water	20 mL

Intended Use

For research use only. Not intended for human or animal diagnostic or therapeutic uses.

Accessory Products

Additional Products

The table below lists additional products available separately from Invitrogen. For more information about these products, refer to our website (www.invitrogen.com) or call Technical Support (page 48).

Product	Quantity	Catalog no.
ProtoArray® Human Protein Microarray v5.0	1 array	PAH052501
ProtoArray® Human Protein Microarray v5.0	20 arrays	PAH0525020
ProtoArray® Control Protein Microarray v5.0	1 array	PA10057
ProtoArray® Human Protein Microarray v5.0 PPI Kit *for V5-tagged proteins*	1 kit	PAH0525013
ProtoArray® Human Protein Microarray v5.0 PPI Kit *for biotinylated proteins*	1 kit	PAH0525011
Biotin-XX Microscale Protein Labeling Kit and FluoReporter® Biotin Quantitation Assay Kit	1 kit	B30756
NuPAGE® Novex® 4-12% Bis-Tris Gel (1.0 mm, 10-well)	1 box	NP0321BOX
NuPAGE® MOPS SDS Running Buffer (20X)	500 mL	NP0001
NuPAGE® MES SDS Running Buffer (20X)	500 mL	NP0002
NuPAGE® Sample Reducing Agent (10X)	250 µL	NP0004
NuPAGE® Antioxidant	15 mL	NP0005
NuPAGE® Transfer Buffer (20X)	125 mL	NP0006
NuPAGE® LDS Sample Buffer (4X)	10 mL	NP0007

Kinase

A variety of purified kinases are available from Invitrogen for use with ProtoArray® Microarrays. For more information about these products, refer to our website at www.invitrogen.com or contact Technical Support (page 48).

Introduction

Overview

Introduction

The ProtoArray® Human Protein Microarray v5.0 Kinase Substrate Identification (KSI) Complete Kit (for kinase substrate identification) allows rapid and efficient identification of potential human protein kinase substrates using a protein kinase of interest. The ProtoArray® Human Protein Microarray is printed with thousands of purified human proteins printed in duplicate on a nitrocellulose-coated glass slide. See the next page for an overview of the system.

The ProtoArray® technology is based on the protein microarray technology developed by Zhu *et al*., 2001 to detect molecular interactions with proteins. The ProtoArray® Technology has recently been shown to be a powerful method to rapidly identify substrates for protein kinases (Mah *et al.*, 2005; Ptacek *et al.*, 2005; Boyle *et al.*, 2007).

ProtoArray® Microarray KSI Applications

The ProtoArray® Microarrays for KSI allow you to:

- Identify potentially biologically relevant protein kinase substrates
- Validate previously observed signals for KSI application
- Test various experimental conditions for the kinase of interest

Advantages

Using the ProtoArray® Human Protein Microarray v5.0 KSI Complete Kit to identify kinase substrates offers the following advantages:

- Provides a simple, rapid, and sensitive method to identify kinase substrates
- Includes qualified buffers and reagents for blocking, probing, and washing steps, eliminating the need to prepare reagents
- Allows screening of your kinase of interest against thousands of human proteins in an easy-to-use format
- Built-in controls are printed on each array to control for background, detection, and analysis
- Simple signal detection using autoradiography or phosphorimaging

Continued on next page

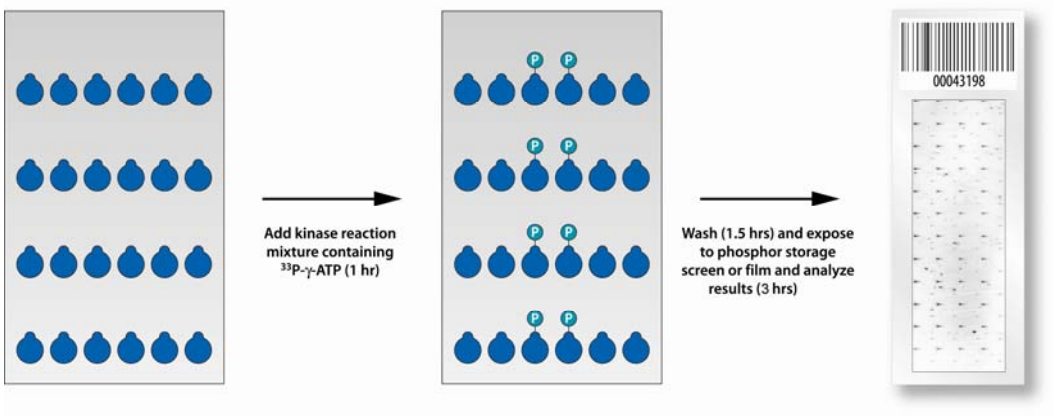
Overview, continued

System Overview

To use the ProtoArray® Human Protein Microarray v5.0 KSI Complete Kit, you will:

- Purify your kinase of interest using a method of choice or purchase a purified protein kinase from Invitrogen.
- Probe the ProtoArray® Control Protein Microarrays supplied in the kit in the presence of labeled [γ-^{33}P]ATP with the Control Kinase and your kinase of interest. Probing the Control Microarray with the Control Kinase allows you to demonstrate specific Control Kinase substrate phosphorylation while probing with your kinase allows you to assess the performance of your kinase under the specified assay conditions.
- Probe the ProtoArray® Human Protein Microarray with your kinase of interest in the presence of labeled [γ-^{33}P]ATP to identify potential substrates for your kinase.

The ProtoArray® KSI protocol includes instructions to block the array, probe the array with your kinase in the presence of radiolabeled [γ-^{33}P]ATP, wash to minimize non-specific binding, dry, expose the array to phosphor screen or X-ray film, acquire the array image to view results, and analyze data (see figure below). For a detailed experimental workflow, see page 27.



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Expected Results

ProtoArray® Microarrays for KSI are designed for kinase substrate identification. After performing the KSI assay and identifying potential kinase substrates, we recommend that you validate the observed substrate phosphorylation using another method such as *in vitro* solution assay.

Using ProtoArray® Human Protein Microarrays, we have typically observed a true positive rate of ~80% for serine-threonine protein kinases.

A true positive signal is defined as a phosphorylation signal observed on a protein microarray that is validated as a substrate using an *in vitro* solution assay (page 45 for details).

The kinase substrate identification assay depends on various factors such as the buffer composition, kinase activity/concentration, assay conditions, ATP concentration, protein sequence, and the amount of protein on the array.

It is possible that some proteins reported in literature as substrates for the kinase may not be identified as kinase substrates on the array. When comparing the kinase substrate data obtained from ProtoArray® experiments to annotated kinase substrates reported in the literature, it is important to review the experimental conditions used for identifying a protein as a substrate for the kinase. In many cases, several proteins annotated in the literature as kinase substrates have been identified using *in vivo* based approaches, which are not always conclusive. Sometimes the identified signals on the array may be due to the interaction of an array protein with radiolabeled ATP or autophosphorylated protein kinase, thereby causing false positive results. To minimize the number of false positive signals arising due to non-specific interaction and to decrease the number of signals not arising from protein kinase phosphorylation of array proteins, wash the kinase-treated microarray with the denaturing detergent SDS as described in the assay protocol.

Purpose of the Manual

This manual provides the following information:

- An overview of the ProtoArray® Human Protein and Control Protein Microarrays
- Instructions to probe the ProtoArray® Microarray
- Guidelines to perform data analysis
- Expected Results and Troubleshooting

Description of Kit Components

Components of the ProtoArray® KSI Complete Kit

The ProtoArray® Human Protein Microarray v5.0 KSI Complete Kit for kinase substrate identification include the following major components:

- The ProtoArray® Human Protein Microarray printed on a nitrocellulose coated slide; a high-density protein microarray that allows you to screen your kinase of interest (protein probe) against thousands of human proteins
- The ProtoArray® Control Protein Microarray and Control Kinase for verification of the probing conditions
- The ProtoArray® KSI Buffer Modules A and B containing pre-made, qualified reagents for performing the blocking and washing steps during probing

ProtoArray® Human Protein Microarray

The ProtoArray® Human Protein Microarray is a high-density protein microarray containing human proteins. Each human open reading frame (ORF) is expressed as an N-terminal GST-(Glutathione-S-Transferase)-fusion protein, purified, and printed in duplicate on a nitrocellulose-coated slide.

The thin nitrocellulose-coated slide from GenTel® BioSciences, Inc. Thin-film nitrocellulose slides are manufactured by Gentel® Biosciences, Inc. using a proprietary surface chemistry owned by Decision Biomarkers, Inc. Thin-film nitrocellulose slides are covered by US Patent 6,861,251, 7,297,497, and 7,384,742.

Each ProtoArray® KSI Complete Kit includes two protein microarrays to allow you to identify potential kinase substrates. Using a protein kinase of interest in the presence of radiolabeled ATP, you can screen against human proteins to identify potential substrates for your kinase in ~2 days.

For array specifications and more details on how the human proteins are prepared, see page 7.

ProtoArray® Control Protein Microarray

The ProtoArray® Control Protein Microarray contains protein kinase substrates and various controls printed on a nitrocellulose-coated slide, and is used to validate the assay **prior** to probing the ProtoArray® Human Protein Microarray.

Two Control Microarrays are included in each kit. Probe one Control Microarray with the Control Kinase supplied in the kit to validate assay conditions and obtain the expected phosphorylation results of the Control Kinase substrate printed on the array. Probe the second Control Microarray with your kinase of interest to determine the compatibility of the kinase with the array surface and assay conditions.

For specifications and more details on the ProtoArray® Control Protein Microarray, see page 9.

Continued on next page

Description of Kit Components, continued

ProtoArray® KSI Buffers Module

The ProtoArray® KSI Buffers Module A and B include qualified reagents used in the blocking and washing steps during probing of the ProtoArray® Microarrays. The pre-made buffers provide consistent results and eliminate the time required to prepare reagents.

ProtoArray® Central Portal

The ProtoArray® Central Portal at www.invitrogen.com/protoarray provides a web-based user interface to access ProtoArray® specific information including online tools, applications, and other resources. You also use the portal to retrieve ProtoArray® Lot Specific Information (see page 33), which is required for analysis of the array data and identification of statistically significant hits (potential substrates).

ProtoArray® Prospector Software

The ProtoArray® Prospector software version 5.0 (includes Imager and Analyzer) quickly analyzes the microarray data and easily identifies significant hits, saving you time and effort. In addition, the software has features that allow you to modify the analysis method and compare data obtained from different microarrays.

The ProtoArray® Prospector software and manual are available free-of-charge to ProtoArray® users, and are accessible online at the ProtoArray® Central Portal. To download the ProtoArray® Prospector software or manual, go to www.invitrogen.com/protoarray, and click on the **Online Tools** link under **BioMarker Discovery Resources**.

ProtoArray® Human Protein Microarray

Introduction

The ProtoArray® Human Protein Microarray v5.0 is a high-density protein microarray containing thousands of human proteins. Each human open reading frame (ORF) is expressed as an N-terminal GST-fusion protein, purified, and printed in duplicate on a nitrocellulose-coated slide. This section provides details about the human protein microarray including array specifications and preparation of proteins.

Note: The ProtoArray® Human Protein Microarray v5.0 KSI Complete Kit includes 2 ProtoArray® Human Protein Microarrays.

Human Protein Microarray Specifications

The specifications for the ProtoArray® Human Protein Microarray v5.0 are listed below.

Dimensions:	1 inch × 3 inch (25 mm × 75 mm)
Material:	Glass slide coated with a thin layer of nitrocellulose

The nitrocellulose-coated slide is from GenTel® BioSciences, Inc. Thin-film nitrocellulose slides are manufactured by Gentel® Biosciences, Inc. using a proprietary surface chemistry owned by Decision Biomarkers, Inc. Thin-film nitrocellulose slides are covered by US Patent 6,861,251, 7,297,497, and 7,384,742.

Each microarray has a barcode for tracking samples. The barcode number is also used to retrieve array specific information from the ProtoArray® Central Portal (page 34).

Array Specifications

The array specifications for the human protein microarray are listed below.

The proteins on the microarray are printed in 48 subarrays and are equally spaced in vertical and horizontal directions.

For details on the subarray layout, and human and control protein spots on the ProtoArray® Human Protein Microarray v5.0, go to the ProtoArray® Central portal at www.invitrogen.com/protoarray.

Total Subarrays:	48 (4 columns × 12 rows)
Subarray Size:	4400 µm × 4400 µm
Subarray Dimensions:	**22 rows × 22 columns**
Median Spot Diameter:	~110 µm
Spot Center to Center Spacing:	200 µm
Distance Between Subarrays:	100 µm
Replicates per Sample:	2
Total Human Proteins on v5.0 Array:	>9,000*

*Refer to ProtoArray® Central Portal for exact number of human proteins printed on the microarray.

Continued on next page

ProtoArray® Human Protein Microarray, continued

Human Proteins

The human proteins printed on the microarray are expressed in insect cells using a baculovirus expression system (below) and an optimized process to maximize the production of soluble recombinant proteins in a high-throughput format (Schweitzer *et al*., 2003). Proteins are expressed in insect cells at high expression levels and are similar to those expressed in mammalian cells with respect to protein folding and post-translational modifications such as phosphorylation and glycosylation (Bouvier *et al*., 1998; Hollister *et al*., 2002; Predki, 2003). In contrast to proteins expressed in *E. coli*, insect expressed proteins are more likely to be functional.

Array Content

The majority of the human protein collection is derived from the human Ultimate™ ORF (open reading frame) Clone Collection available from Invitrogen (see orf.invitrogen.com for more information). Each Ultimate™ ORF Clone is full insert sequenced and is guaranteed to match the corresponding GenBank® amino acid sequence.

Some of the human proteins printed on the array represent the human protein kinase collection derived from full insert sequenced clones but are not Ultimate™ ORF Clones. Other kinases from the kinase collection have been cloned as catalytic domains rather than full-length proteins. About 313 proteins printed on the array are derived from the purified protein kinase collection available from Invitrogen. Approximately 40 additional proteins printed on the array are purified cytokines available from Invitrogen. Approximately 28 proteins, peptides, and nucleic acids that have been demonstrated to be antigens in a variety of autoimmune diseases are also printed on the array. New content for ProtoArray v5.0 arrays was enriched for proteins relevant to disease processes, for a total of >6,100 potential drug targets printed on the array.

For accession number and amino acid sequence for each protein, as well as information on peptides and nucleic acids printed on the array, download the Protein Content List from www.invitrogen.com/protoarray.

Expression and Purification of Human Proteins

Almost all clones used to generate the human protein collection are entry clones consisting of a human ORF cloned into a Gateway® entry vector. Each entry clone is subjected to an LR recombination reaction with a Gateway® destination vector to generate an expression clone. The expression clone is then used to express the protein (as an N-terminus GST-fusion protein in some clones) using the Bac-to-Bac® Baculovirus Expression System available from Invitrogen. For more information on the Bac-to-Bac® Baculovirus Expression System, visit www.invitrogen.com.

The LR reaction mix obtained after performing the LR reaction is transformed into competent DH10Bac™ *E. coli* to generate a recombinant bacmid. The high molecular weight recombinant bacmid DNA is isolated and transfected into Sf9 insect cells to generate a recombinant baculovirus for preliminary expression experiments. After the baculoviral stock is amplified, the high-titer stock is used to transduce Sf9 insect cells for expression of recombinant proteins of interest.

The expressed proteins are purified by affinity chromatography under high-throughput conditions optimized to obtain maximal protein integrity, function, and activity. Following purification, each protein is assayed for purity and expected molecular weight.

Continued on next page

ProtoArray® Human Protein Microarray, continued

Controls

Various proteins and controls are printed on each ProtoArray® Human Protein Microarray to allow you to verify background and detection conditions during probing. For details, see page 10.

Printing the Human Protein ProtoArray®

The purified human proteins are printed on nitrocellulose-coated slides in a dust-free, temperature, and humidity controlled environment to maintain consistent quality of the microarrays. The arrays are printed using an automated process on an arrayer that is extensively calibrated and tested for printing ProtoArray® Human Protein Microarrays.

Maintaining Stringent Quality Control

The ProtoArray® Human Protein Microarrays are produced using rigorous production and quality control procedures with an integrated data management system to ensure consistent results with every array and maximize inter- and intra-lot reproducibility.

Pre-Printing Quality Control

Prior to production, the arrayer and supporting components are tested and adjusted to production specifications. The quality and performance of pins is critical and all pins are extensively tested and calibrated. To maintain protein stability and function, arrays are printed at 6ºC under controlled environmental conditions.

Post-Printing Quality Control

After production each microarray is visually inspected for obvious defects that could interfere with the experimental results. The presence of each control and human protein spot is assessed by fluorescent scan of a representative number of arrays and acquisition of signals due to fluorescence of the printing buffer. Signal-to-background ratios (SBR) are determined for each spot, and spots with a SBR less than 3 are labeled "missing." The probability that the control or human protein spot is missing from the entire lot is then calculated. The percentage of missing spots is estimated as the average missing probability of all the spots. That estimation must indicate that at least 95% of spots are present.

Consistent print quality is determined for all sub-arrays prior to starting the printing of each array lot. Proteins of a particular type or class are distributed randomly across all sub-arrays, and therefore several spots missing from a single sub-array is essentially no different from random spots missing across several sub-arrays. The arrays are functionally qualified by probing with radiolabeled ATP in the absence and presence of the Control Kinase to confirm phosphorylation of Control Kinase Substrate and Alignment Control Kinases.

Continued on next page

ProtoArray® Control Protein Microarray

Introduction

The ProtoArray® Control Protein Microarray v5.0 contains kinase substrates and various controls printed on a nitrocellulose-coated slide. The Control Protein Microarray allows you to validate probing procedures prior to probing the ProtoArray® Human Protein Microarray v5.0.

Details about the ProtoArray® Control Protein Microarray are described in this section.

Note: The ProtoArray® Human Microarray v5.0 KSI Complete Kit include 2 ProtoArray® Control Protein Microarrays.

Control Microarray Specifications

The specifications for the ProtoArray® Control Protein Microarray v5.0 are listed below.

Dimensions:	1 inch × 3 inch (25 mm × 75 mm)
Material:	Glass slide coated with a thin layer of nitrocellulose

The nitrocellulose-coated slide is from GenTel® BioSciences, Inc. Thin-film nitrocellulose slides are manufactured by Gentel® Biosciences, Inc. using a proprietary surface chemistry owned by Decision Biomarkers, Inc. Thin-film nitrocellulose slides are covered by US Patent 6,861,251, 7,297,497, and 7,384,742.

Each microarray has a barcode for tracking samples. The barcode number is also used to retrieve array specific information from the ProtoArray® Central portal (see page 34).

Control Array Specifications

The control array specifications are listed below.

The proteins on the microarray are printed in 48 subarrays and are equally spaced in vertical and horizontal directions.

For details on the subarray layout and control protein spots on the ProtoArray® Control Protein Microarray v5.0, go to the ProtoArray® Central portal at www.invitrogen.com/protoarray.

Total Subarrays:	48 (4 columns × 12 rows)
Subarray Size:	4400 µm × 4400 µm
Subarray Dimensions:	**22 rows × 22 columns**
Median Spot Diameter:	~110 µm
Spot Center to Center Spacing:	200 µm
Distance Between Subarrays:	100 µm
Replicates per Sample:	2

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ProtoArray® Control Protein Microarray, Continued

Controls Proteins

Various proteins and controls are printed on each ProtoArray® Human and Control Protein Microarray to allow you to verify reagents, background, and detection conditions used during probing.

The table below lists the controls printed on each ProtoArray® Microarray.

Note: Some of the controls printed on the arrays are not required for analysis using the KSI protocol.

Protein	Function
Control Spots required for KSI Data Analysis	
Alignment Control Kinases (PKCeta)	Kinases autophosphorylate and produce signals which are used for orientation of the microarray image; also serves as a positive control for the radiolabel and assay conditions.
Control Kinase Substrate (MAPKAP)	A substrate for the Control Kinase used to verify assay conditions. The Control Kinase phosphorylates the Control Kinase Substrate.
GST Protein Gradient	Serves as a negative control and signals are used by ProtoArray® Prospector software for background and statistical significance calculations.
Control Spots NOT required for KSI Data Analysis	
Alexa Fluor® Antibody (Rabbit anti-mouse IgG Antibody labeled with Alexa Fluor® 647, Alexa Fluor® 555, and Alexa Fluor® 488)	Serves as a positive control for fluorescence scanning and for orientation of the microarray image.
Bovine Serum Albumin (BSA)	A negative control for non-specific protein interactions.
Biotinylated Anti-mouse Antibody Gradient	A positive control for interaction with streptavidin-labeled detection reagent.
V5 Control Protein (biotinylated, V5-tagged control protein)	A positive control for detection with the Anti-V5-Alexa Fluor® 647 Antibody and streptavidin-labeled detection reagent. Also used as an optional normalization control for immune response serum profiling when anti-V5 antibody is added to the detection reagent.
Human IgG Protein Gradient	A positive control for the immune response serum profiling application. Interacts with Alexa Fluor® 647 goat anti-human IgG.
Anti-Human IgG Antibody Gradient (goat anti-human IgG)	A positive control for the immune response serum profiling application. Interacts with serum IgG antibodies which are then bound by Alexa Fluor® 647 goat anti-human IgG.
Yeast calmodulin (Cmd1p) or human calmodulin (CALM2)	A positive control for the protein-protein interaction application through activity between calmodulin printed on the array and the Array Control Protein. Refer to the lot specific .GAL file for the specific identity of the protein.
CAMK2A (Calcium/calmodulin-dependent protein kinase II alpha)	A human protein kinase that is used as a positive control for the small molecule profiling application.

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ProtoArray® Control Protein Microarray, Continued

Protein	Function
Control Spots NOT required for KSI Data Analysis, continued	
Anti-biotin Antibody (mouse anti-biotin antibody)	Detects biotin labeled protein probes and serves as a control for anti-mouse antibody detection reagent.
Anti-Human IgA Antibody Gradient (goat anti-human IgA)	A positive control for the immune response serum profiling application. Interacts with serum IgA antibodies which are then bound by Alexa Fluor® 647 anti-human IgA.
Estrogen Receptor Alpha	Binds to tritiated estradiol to produce marker signals which are used for orientation of the microarray image for the radiometric small molecule profiling application.
Human IgA Protein Gradient	A positive control for immune response serum profiling of IgA antibodies. Interacts with Alexa Fluor® 647 anti-human IgA
Mdm2	Serves as a control substrate for ubiquitin ligase profiling.
RanBP2ΔFG	Serves as a control substrate for sumo ligase profiling.

Maintaining Stringent Quality Control

The ProtoArray® Control Protein Microarrays are produced using the same rigorous production and quality control procedures used to produce the ProtoArray® Human Protein Microarray (see page 8). In addition, the control arrays are functionally qualified by probing the arrays with the Control Kinase to verify the phosphorylation of Control Kinase Substrate and validate autophosphorylation of the Alignment Control Kinases.

Pre-Printing Quality Control

Prior to production, the arrayer and supporting components are tested and adjusted to production specifications. The quality and performance of pins is critical and all pins are extensively tested and calibrated. To maintain protein stability and function, arrays are printed at 6ºC under controlled environmental conditions.

Post-Printing Quality Control

After production each microarray is visually inspected for obvious defects that could interfere with the experimental results. The arrays are functionally qualified by probing with radiolabeled ATP in the absence and presence of the Control Kinase to confirm phosphorylation of Control Kinase Substrate and Alignment Control Kinases

Working with Radioactive Material

Introduction

This section provides general guidelines and safety tips for working with radioactive material. For more information and specific requirements, contact the safety department of your institution.

Use extreme caution when working with radioactive material. Follow all federal and state regulations regarding radiation safety. For general guidelines when working with radioactive material, see below.

General Guidelines

Follow these general guidelines when working with radioactive material.

- Do not work with radioactive materials until you have been properly trained.
- Wear protective clothing, vinyl or latex gloves, and eyewear, and use a radiation monitor.
- Work in areas with equipment and instruments that are designated for radioactive use.
- Plan ahead to ensure that all the necessary equipment and reagents are available and to minimize exposure to radioactive materials.
- Monitor work area continuously for radiation contamination.
- Dispose of radioactive waste properly.
- After you have completed your experiments, monitor all work areas, equipment, and yourself for radiation contamination.
- Follow all the radiation safety rules and guidelines mandated by your institution.

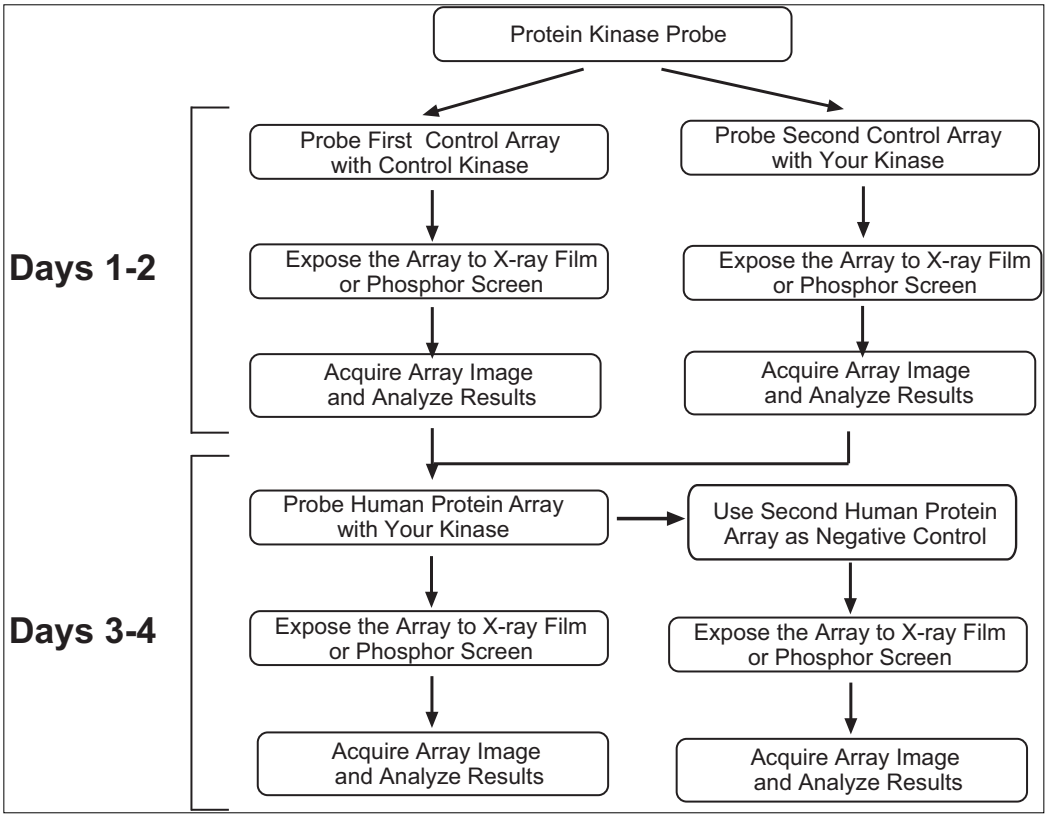
Important

Any material in contact with a radioactive isotope must be disposed of properly. This includes any reagents that are discarded during the probing procedure (*e.g.* washes). Contact your safety department for regulations regarding radioactive waste disposal.

Experimental Overview

Experimental Timeline

The recommended experimental timeline is outlined below. Detailed experimental workflows are shown on pages 15 and 27.



Methods

Preparing the Protein Kinase

Introduction

Before using the ProtoArray® Human Protein Microarray for KSI, you need to purchase or purify the protein kinase of interest to probe the microarray.

You may purify the protein kinase using any method of choice. You can use proteins purified from *E. coli*, yeast cells, or higher eukaryotes to probe the ProtoArray® Microarray.

A large variety of highly purified protein kinases are available from Invitrogen. For details, visit www.invitrogen.com or contact Technical Support (page 48).

The amount of protein and quality of protein required for probing are described below.

Protein Amount and Quality

- **Purify the protein kinase under native conditions.**
- Proteins should be > 90% pure as determined by Coomassie® staining.
- Check the activity of the protein kinase after purification using a method of choice.
- Dilute the kinase for use during probing in the Kinase Buffer (see recipe on page 19).
- Make sure the protein kinase is soluble and active in buffers used for probing the microarray (see recipe on page 19).
- You need at least 120 µL of your purified protein kinase at a recommended final protein concentration of **50 nM** to probe each ProtoArray® Microarray.

Probing the ProtoArray® Control Protein Microarray

Introduction

The ProtoArray® Control Protein Microarray allows you to verify probing conditions. Probe the Control Protein Array **prior to** probing the ProtoArray® Human Protein Microarray.

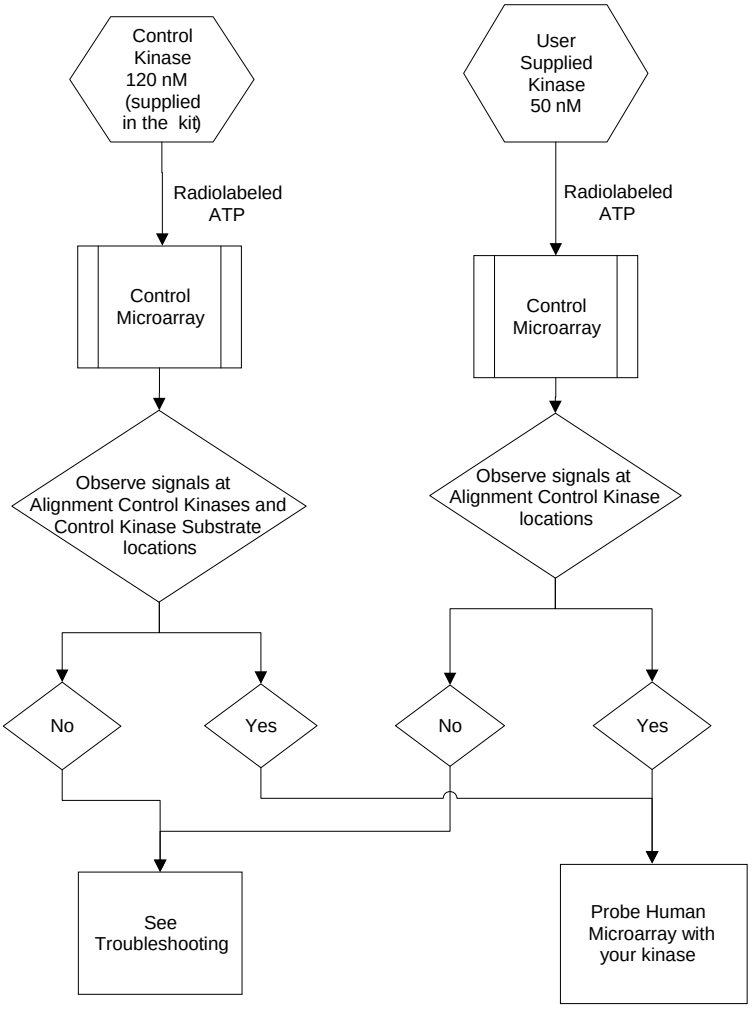
Instructions are provided in this section to probe the ProtoArray® Control Protein Microarray with the Control Kinase supplied with the kit.

ProtoArray® KSI Buffer Modules

The ProtoArray® KSI Buffer Modules A and B supplied with the complete kit include qualified reagents for blocking, washing, and probing during the microarray probing procedure. The pre-made buffers provide consistent results and eliminate the time required to prepare reagents.

Control Array Workflow

The recommended experimental workflow for probing the ProtoArray® Control Protein Microarray with the Control Kinase and your kinase is shown below.



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Probing the ProtoArray® Control Protein Microarray, Continued

Recommended Workflow

The recommended experimental workflow for probing the ProtoArray® Control Protein Microarray with the Control Kinase and your kinase of interest is shown on the previous page.

To obtain the best results and verify the probing procedure, use the ProtoArray® KSI Kit according to the workflow shown on the previous page and described below.

1. Simultaneously probe **two** ProtoArray® Control Protein Microarrays included in the kit using the following probes:
   - Probe the first array using the Control Kinase at 120 nM supplied in the kit in the presence of radiolabeled [γ-^{33}P]ATP to verify the probing procedure
   - Probe the second array using your protein kinase at 50 nM in the presence of radiolabeled [γ-^{33}P]ATP to assess the performance of your kinase with the array surface and array proteins
2. After the probing procedure, expose arrays to X-ray film or a phosphor screen for 3 hours. Acquire the array image to produce a 16-bit TIFF file. The array image can be acquired by scanning the phosphor screen using a phosphorimager or develop the X-ray film and scan the X-ray film using a scanner.
3. Process the microarray images, and acquire and analyze data using ProtoArray® Prospector (recommended).

If the assay is performed properly, you should observe the following results:

Results with Control Kinase

- The Alignment Control Kinases spotted on subarrays on each ProtoArray® Control Microarray autophosphorylate and form a pattern on the array that is necessary for data acquisition by the microarray data acquisition software.
- The Control Kinase phosphorylates a Control Kinase substrate spotted on each ProtoArray® Control Microarray and produces a signal indicating that the assay was performed correctly.

Results with Your Kinase

If your kinase concentration and activity is suitable for the assay, the signals for the autophosphorylating Alignment Control Kinases on the array are statistically significant above background and signals for the negative control proteins on the array are not significantly above background.

Once you have verified that the Control Kinase and your kinase perform as expected on the ProtoArray® Control Microarrays, probe the ProtoArray® Human Protein Microarray with your kinase (page 26).

Continued on next page

Probing the ProtoArray® Control Protein Microarray, continued

Important Guidelines

Since proteins are sensitive to various environmental factors, each array is produced in an environmentally controlled facility to ensure protein integrity and maintain consistency. To obtain the best results and avoid any damage to the array or array proteins, always handle the ProtoArray® Microarrays using the following guidelines:

- Always wear clean gloves while handling microarrays.
- **Do not** touch the surface of the array. Damage to the array surface can result in uneven or high background.
- To prevent condensation on the array that may reduce protein activity or alter spot morphology, allow the mailer containing the array to equilibrate at 4°C for at least 15 minutes prior to removing the array from the mailer. Use Blocking Buffer equilibrated at 4°C to immerse the array immediately.
- **Do not** use [γ-^{32}P]ATP in place of [γ-^{33}P]ATP for the assay. The use of [γ-^{33}P]ATP supports increased signal resolution during data acquisition, and while [γ-^{32}P]ATP can technically be used for the assay, data quantitation with [γ-^{32}P]ATP is not supported.
- Perform array experiments at a clean location to avoid dust or contamination. Filter solutions if needed (particles invisible to eyes can produce high background signals and cause irregular spot morphology).
- Avoid drying of the array during the experiment. Ensure the array is completely covered with the appropriate reagent during all steps of the protocol.
- Always dry the array prior to exposing to X-ray film or phosphor screen.
- **Do not** dry the array using compressed air or commercial aerosol sprays.

- To perform the washing and probing steps, we recommend using a sterile 50 mL conical tube.
- Incubation chambers are not suitable for use in the probing portion of the KSI application. A container that seals tightly is required to prevent any leakage of radioactive material during the washing steps.
- Use coverslips that are able to completely cover the printed area (20 mm × 60 mm) of the microarray. The coverslip should hold a small reagent volume to minimize the amount of valuable probe used and prevent evaporation of reagents. We recommend using glass coverslips (VWR catalog no. 48404-454).
- **Do not** use cold ATP for the kinase probing steps. If your kinase is stored in a buffer containing ATP, make sure the final concentration of cold ATP is less than 1 nM during the kinase probing step.
- Avoid adding more than 10% (v/v) of the kinase sample to 120 µL of Kinase Buffer. Addition of more than 10% of the kinase to the Kinase Buffer can decrease assay performance.

Continued on next page

Probing the ProtoArray® Control Protein Microarray, continued

Materials Needed

You need the following items:

- [γ-^{33}P]ATP (3,000 Ci/mmol, 10 µCi/µL), if the specific activity/concentration of the ATP is different, dilute the ATP to the recommended specific activity/concentration using the Kinase Buffer included in the kit
- ProtoArray® Control Protein Microarray v5.0 (included in the kit)
- ProtoArray® KSI Buffer Module A and B (included in the kit)
- Control Kinase in Kinase Buffer (included in the kit; page 20)
- Protein Kinase supplied by the user in Kinase Buffer (page 20)
- Incubator set to 30°C
- Clean, covered 4-chamber incubation tray (Greiner, Cat. no. 96077307 or ISC Bioexpress, Cat. no. T-2896-1) , chilled on ice
- Sterile 50 mL conical tubes
- Ice bucket
- Shaker
- Deionized or ultra pure water
- Coverslips (VWR, Cat. no. 48404-454)
- X-ray film or phosphor screen (with at least 50 µm resolution) and instrumentation to acquire the image (with at least 50 µm resolution)
- X-ray film cassette
- Clear plastic wrap
- *Optional*: Microarray slide holder and centrifuge equipped with a plate holder

Important

The ProtoArray® Control Protein Microarray can only be used once. Do not re-use the microarray or reprobe the same microarray with another kinase.

Control Kinase

The Control Kinase included with the kit is a protein kinase that phosphorylates a broad spectrum of substrates. The Control Kinase is >90% pure as assessed by SDS-PAGE.

The Control Kinase phosphorylates a Control Kinase substrate printed on the ProtoArray® Control Protein Microarray. A significant signal for the Control Kinase substrate indicates that the assay was performed correctly.

Continued on next page

Probing the ProtoArray® Control Protein Microarray, continued

Preparing Buffers

Prepare the following buffers **fresh** using the reagents supplied in the kit. Adjust volumes in recipe to provide sufficient buffer to probe the number of microarrays you will be assaying.

Blocking Buffer

1X PBS
1% BSA

1. For blocking in incubation trays, 5 mL of Blocking Buffer is needed for each microarray. Use reagents provided in the kit to prepare 30 mL Blocking Buffer as follows:

PBS (10X)	3 mL
30% BSA	1 mL
Deionized water	to 30 mL

2. Mix well (do not vortex) and store on ice until use.

Kinase Buffer with 1 mM DTT

You need 120 µL Kinase Buffer with 1 mM DTT for probing one microarray. To prepare Kinase Buffer with 1 mM DTT, add 0.5 µL 1 M DTT (supplied with the kit) to 500 µL Kinase Buffer. Mix well (do not vortex) and store on ice until use.

After preparing Blocking Buffer and Kinase Buffer with DTT, immediately return the remaining 30% BSA, Kinase Buffer, and 1 M DTT to –20°C.

0.5% SDS

You need ~80 mL 0.5% SDS for washing each microarray. Prepare 0.5% SDS from 10% SDS (included with the kit) as follows:

10% SDS	10 mL
Ultrapure water	190 mL
Total Volume	200 mL

Mix well and store at room temperature until use.

Continued on next page

Probing the ProtoArray® Control Protein Microarray, Continued

Calculating the Protein Molar Concentration

Calculate the molar concentration of your protein kinase using the protein concentration and molecular weight of your protein kinase of interest as shown in the formula listed below:

Formula

Protein Concentration (µM) = [Protein concentration in mg/mL] × [1/(protein molecular weight in grams × 10^{-6})]

Example:

For a kinase (50,000 Da) at a protein concentration of 0.5 mg/mL, the µM protein concentration is:

µM = [0.5 mg/mL] × [1/(50,000 × 10^{-6})]

µM = 10

Preparing the Kinase

You need 120 µL kinase diluted in Kinase Buffer with 1 mM DTT to probe **one** ProtoArray® Control Protein Microarray.

Note: The molar concentration of the Control Kinase is marked on the tube label. Prepare dilutions of the kinase in the Kinase Buffer included with the kit.

Prepare 2 tubes, each containing Kinase Buffer with 1 mM DTT and kinase as follows:

Component	Control Kinase	User Kinase
Kinase	120 nM	50 nM
Kinase Buffer with 1 mM DTT	to 120 µL	to 120 µL

Mix well (do not vortex) and store on ice until use.

Immediately return the remaining Control Kinase and your kinase to –80°C.

Before Starting

- Before starting the probing procedure, make sure you have all items on hand especially buffers (previous page), kinase in Kinase Buffer (above), and coverslips.
- Make sure the kinase in Kinase Buffer with 1 mM DTT and Kinase Buffer are cold and stored on ice until use. Place 50 mL conical tubes on ice to chill the tube prior to use.
- Do not store the 0.5% SDS solution on ice. Store the 0.5% SDS solution at room temperature.
- Review **Important Guidelines** on page 17 and **Working with Radioactive Material** on page 12, prior to starting the probing procedure.

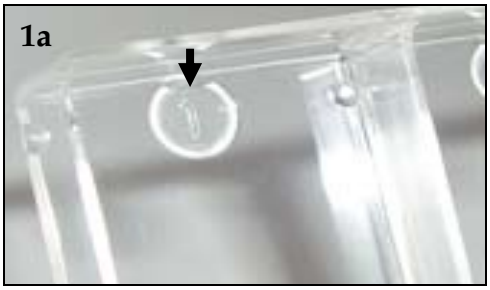
Continued on next page

Probing the ProtoArray® Control Protein Microarray, continued

Blocking Step

Instructions for blocking the ProtoArray® Control Protein Microarray are described below:

1. Remove the mailer containing the ProtoArray® Control Protein Microarray from storage at –20°C and place immediately at 4°C (be sure to use the microarray **before** the expiration date printed on the box).
2. Allow the array to equilibrate in the mailer at 4°C for at least 15 minutes prior to blocking. Failure to do so may result in condensation on the array which can reduce protein activity or alter spot morphology.
3. Place one microarray with the barcode facing up into each well of a chilled 4-chamber incubation tray such that the barcoded end of the microarray is near the end of the tray marked with an indented numeral (see figures 1a and 1b).



4. Using a sterile pipette, immediately add 5 mL Blocking Buffer into each chamber containing an array. **Avoid pipetting buffer directly onto the array surface**.
5. Incubate the tray for 1 hour at 4°C on a shaker set at 50 rpm (circular shaking).

Continued on next page

Probing the ProtoArray® Control Protein Microarray, continued

Blocking Step, continued

Protocol continued from the previous page.

6. After incubation, remove array from 4-chamber incubation tray using forceps. Insert the tip of the forceps into the indentation at the numbered end of the tray and gently pry the array upward (see figure 2). Using a gloved hand, pick up the microarray by holding the array by its **edges** only. Tap to remove excess liquid from array surface.

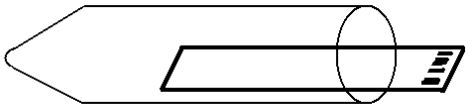
7. Proceed immediately to **Probing Control Arrays**.

Continued on next page

Probing the ProtoArray® Control Protein Microarray, continued

Probing Control Arrays

1. Place the ProtoArray® Control Protein Microarray in a 50 mL conical tube with one-third of the slide extended outside of the tube (see figure below). The barcode should be outside the tube, face up.



2. Add 1 µL [γ-^{33}P]ATP (3000Ci/mmol, 10 µCi/µL) to 120 µL Kinase Buffer containing diluted kinase (see **Preparing the Kinase**, page 20) to obtain a final radiolabeled ATP concentration of 33 nM for a single array.

   Important: Once the ATP is added to the kinase, use the kinase-ATP mixture **immediately** for probing the array. **Do not store** the prepared kinase-ATP mixture on ice for longer than 2 minutes prior to use on the array.

3. Pipette Kinase Buffer with the radiolabeled ATP and kinase on top of the array without touching the array surface.
   - First Control Microarray: add 120 µL Kinase Buffer containing 120 nM **Control Kinase** and 33 nM [γ-^{33}P]ATP (Step 2)
   - Second Control Microarray: add 120 µL Kinase Buffer containing 50 nM of **your kinase** and 33 nM [γ-^{33}P]ATP (Step 2)
4. Using forceps, carefully lay a glass coverslip on the surface of the array without trapping any air bubbles. Align the coverslip flush with the top edge of the array to ensure the printed area of the array is completely covered. If necessary, gently adjust the coverslip to remove any air bubbles.
5. Position the coverslipped array so that it is inside the conical tube with the printed side (barcode) facing up. Cap the conical tube.
6. Place the conical tube horizontally on a flat surface in an incubator set to 30°C such that the printed side of the array is facing up and the tube is as level as possible. If needed, tape the conical tube to the flat surface to avoid any accidental disturbances.
7. Incubate the array for 1 hour at 30°C **without shaking**. Remove the tube from the incubator.
8. Using a sterile pipette, add 40 mL 0.5% SDS (page 19) to the tube by dispensing the SDS down the sides of the tube. **Avoid pipetting SDS directly onto the array surface.**
9. Incubate the array in SDS for 1 minute at room temperature **without shaking**. Gently move the array in the tube to dislodge the coverslip. **Do not remove the coverslip with forceps if the coverslip does not float away from the array**.

Continued on next page

Probing the ProtoArray® Control Protein Microarray, continued

Probing Control Arrays, continued

Protocol continued from the previous page.

10. Using forceps, carefully remove the dislodged coverslip without touching the array surface. Discard the coverslip properly as radioactive waste.
11. Cap the conical tube and incubate the array in 0.5% SDS for 15 minutes at room temperature.

    Note: Perform all washing steps with SDS and water **without shaking** to prevent any spillage of radioactive waste.
12. Decant the 0.5% SDS. Discard the wash properly as radioactive waste.
13. Slowly add 40 mL 0.5% SDS to the tube (dispense SDS as described in Step 8), cap the tube, and incubate for 15 minutes at room temperature.
14. Decant the 0.5% SDS. Discard the wash properly as radioactive waste.
15. Add 40 mL ultrapure water to the tube (dispense water as described in Step 8), cap the tube, and incubate the array for 15 minutes at room temperature.
16. Decant the water. Discard the wash properly as radioactive waste.
17. Add 40 mL ultrapure water to the tube, cap the tube, and incubate the array for 15 minutes at room temperature.
18. Decant the water. Discard the wash properly as radioactive waste.
19. Proceed to **Drying Arrays**, below.

Drying Arrays

1. Remove the array from the tube using forceps at the end of the probing procedure. Touch one edge of the array gently against a laboratory wipe for a few seconds to drain any excess buffer.
2. Dry the array using a table top centrifuge. Centrifuge the array at 200 × g for 1–2 minutes at room temperature in the slide holder (if using a centrifuge equipped with a plate rotor) or 50 mL conical tube (if using a swinging bucket rotor). Verify that the array is completely dry. Ensure the array is properly placed and is secure in the holder to prevent any damage to the array during centrifugation.
3. Place the array in an X-ray film cassette. Cover the array with clear plastic wrap. You can check for radioactivity on the array using a Geiger counter.
4. Overlay the array with an X-ray film or a phosphor screen (at least 50 µm resolution). Be sure the phosphor screen was erased prior to exposure.
5. Expose the array to the phosphor screen or X-ray film for 3 hours.
6. Perform imaging and data analysis as outlined on the next page.

Continued on next page

Probing the ProtoArray® Control Protein Microarray, continued

Imaging and Data Analysis

Analyze the image and data to identify potential substrates as outlined below. For details, see page 33.

1. Acquire an image (.tiff) from the X-ray film or phosphor screen (page 30).
2. Use the barcode information on the array to download the .GAL file from ProtoArray® Central as described on page 34.
3. Use the .GAL file and ProtoArray® Prospector (page 33) to acquire pixel intensity values for all features on the array and analyze data to determine significant signals.

   Note: The expected results obtained after probing a Control Protein Microarray are described on page 38. For troubleshooting, see page 41.

Probing the ProtoArray® Human Protein Microarray

Introduction

After using the ProtoArray® Control Protein Microarray to verify the probing conditions, you may proceed to probe the ProtoArray® Human Protein Microarray using your protein kinase of interest. Follow the guidelines provided in this section.

Important

Each ProtoArray® Human Protein Microarray can only be used once. Do not re-use the array or reprobe the same array with another kinase.

Important Guidelines

Follow the **Important Guidelines** on page 17 to obtain the best results with the arrays.

Materials Needed

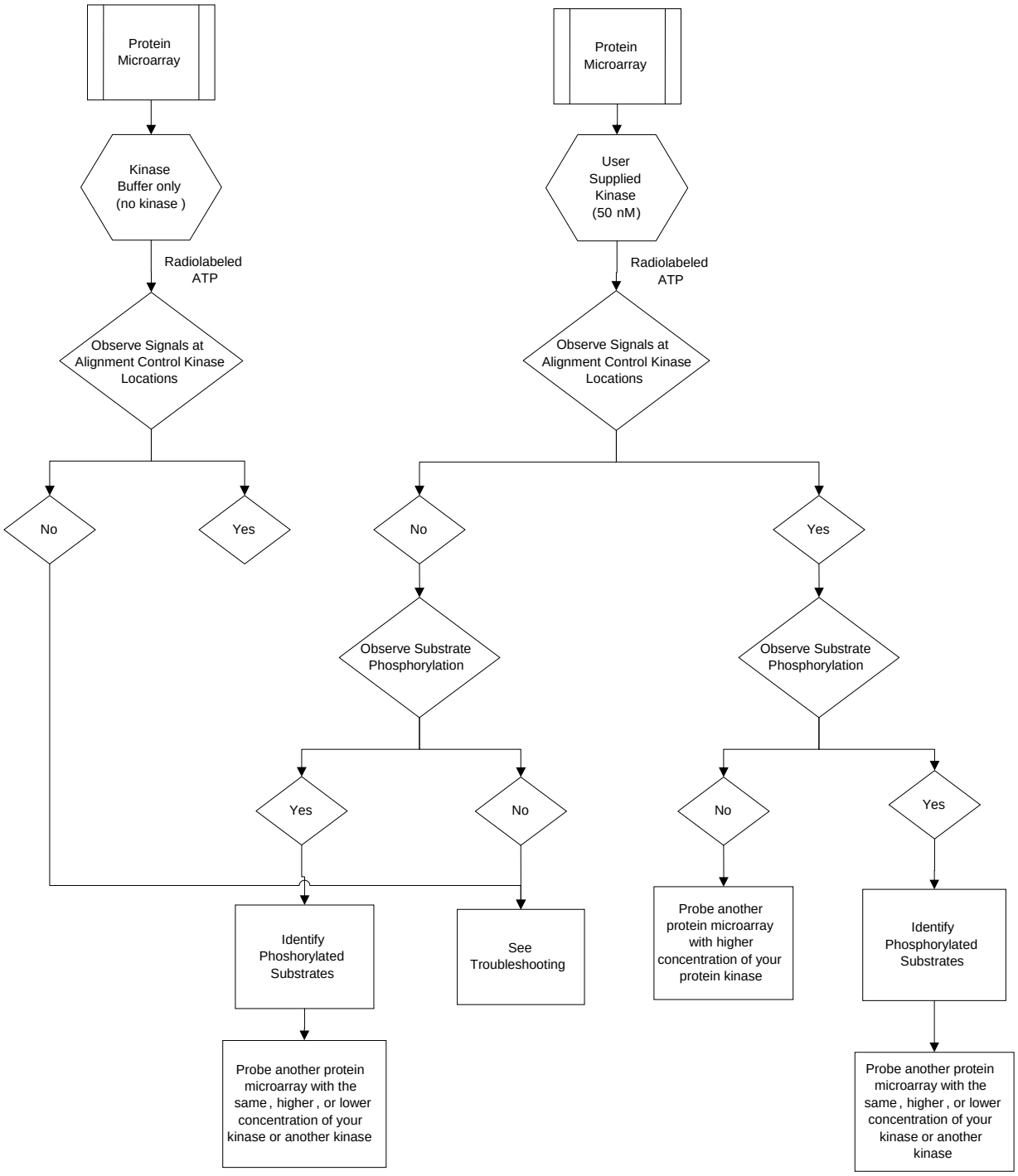
You need the following items:

- ProtoArray® Human Protein Microarray v5.0 (included in the kit)
- [γ-^{33}P]ATP (3,000 Ci/mmol, 10 µCi/µL)
- ProtoArray® KSI Buffer Module A and B (included in the kit)
- Protein Kinase supplied by the user in Kinase Buffer (see page 20)
- Incubator set to 30°C
- Clean, covered 4-chamber incubation tray (Greiner, Cat. no. 96077307 or ISC Bioexpress, Cat. no. T-2896-1) , chilled on ice
- Sterile 50 mL conical tubes
- Shaker
- Ice bucket
- Deionized or ultra pure water
- Coverslips (VWR, Cat. no. 48404-454)
- X-ray film or phosphor screen (with at least 50 µm resolution) and instrumentation to acquire the image (with at least 50 µm resolution)
- X-ray film cassette
- Clear plastic wrap
- *Optional*: Microarray slide holder and centrifuge equipped with a plate holder

Continued on next page

Human Array Experimental Workflow

The recommended experimental workflow for probing the ProtoArray® Human Protein Microarray with your kinase of interest is shown below.



Continued on next page

Probing the ProtoArray® Human Protein Microarray, continued

Recommended Workflow

The ProtoArray® Human Protein Microarray v5.0 KSI Complete Kit contains two human arrays. Probe arrays using the workflow as described on the previous page and below.

The recommended protein kinase concentration for probing each array ranges from 1–50 nM.

1. Simultaneously probe **two** ProtoArray® Human Protein Microarrays as follows:
   - Probe the first array using your kinase (supplied by the user) at 1–50 nM in the presence of radiolabeled [γ-^{33}P]ATP to identify potential substrates
   - Probe the second array **using Kinase Buffer with no kinase** (negative control) in the presence of radiolabeled [γ-^{33}P]ATP to determine which signals are specific to your kinase
2. After the probing procedure, expose arrays to X-ray film or a phosphor screen for 3 hours. Acquire the array image to produce a 16-bit TIFF file. The array image can be acquired by scanning the phosphor screen using a phosphorimager or developing the X-ray film and scanning the X-ray film with an image scanner.
3. Process the microarray images, and acquire and analyze data using ProtoArray® Prospector (recommended).

Preparing Buffers

Prepare Blocking Buffer, Kinase Buffer, and 0.5% SDS as described on page 19.

Preparing Your Kinase

The recommended protein concentration for probing an array can range anywhere from 1–50 nM. Activity of the kinase and level of kinase autophosphorylation will influence the optimal concentration. Too much kinase may result in high background or a dark array. Too little kinase will result in no additional spots relative to a kinase-free control.

You need 120 µL Kinase Buffer with 1 mM DTT containing your kinase of interest to probe **one** ProtoArray® Human Protein Microarray.

To calculate the molar concentration of your kinase of interest, use the protein concentration and molecular weight of your protein kinase for the calculation using the formula listed on page 20.

Add your kinase of interest to Kinase Buffer with 1 mM DTT, to obtain a final concentration of 1–50 nM as follows:

Component	Amount
Kinase	1–50 nM
Kinase Buffer with 1 mM DTT	to 120 µL

Mix well (do not vortex) and store on ice until use. Immediately return the remaining kinase to –80°C.

Continued on next page

- To perform the washing and probing steps, we recommend using a sterile 50 mL conical tube.
- The Incubation Chamber included with the ProtoArray® PPI Kits or other hybridization chambers are not suitable for use in the probing portion of the KSI application, as you will need a container that seals tightly to prevent any leakage of radioactive material during the washing steps.
- Use coverslips that are able to completely cover the printed area (20 mm × 60 mm) of the microarray. We recommend using glass coverslips (VWR, Cat. no. 48404-454).
- **Do not** use cold ATP for the kinase probing steps. If your kinase is stored in a buffer containing ATP, make sure the final concentration of cold ATP is less than 1 nM during the kinase probing step.
- Avoid adding more than 10% (v/v) of the kinase sample to 120 µL of Kinase Buffer. Addition of more than 10% of the kinase to the Kinase Buffer can decrease assay performance.

Before Starting

- Before starting the probing procedure, make sure you have all items on hand especially buffers, kinase in Kinase Buffer (above), and coverslips.
- Make sure the kinase in Kinase Buffer with 1 mM DTT and Kinase Buffer are cold and stored on ice until use. Place 50 mL conical tubes on ice to chill the tube prior to use.
- Do not store the 0.5% SDS solution on ice. Store the 0.5% SDS solution at room temperature.
- Review **Important Guidelines** on page 17 and **Working with Radioactive Material** on page 12, prior to starting the probing procedure.

Probing Arrays

See page 27 for the recommended workflow.

1. Block microarrays using the procedure described on page 21.
2. Simultaneously probe two ProtoArray® Human Protein Microarrays using the procedure described on page 23 as follows:
   - Probe the first array using your kinase (supplied by the user) at **1–50 nM** in the presence of radiolabeled [γ-^{33}P]ATP to identify potential substrates
   - Probe the second array (negative control) using only buffer and no kinase in the presence of radiolabeled [γ-^{33}P]ATP to determine which signals are specific to your kinase
3. Dry the array as described on page 24.
4. Perform image analysis on the arrays as described on the next page and analyze results (page 33).

Examples of expected results obtained after probing the ProtoArray® Human Protein Microarray are shown on pages 39.

If you obtain weak signals or high background, see **Troubleshooting**, page 41.

Scanning and Image Analysis

Introduction

Once you have exposed the ProtoArray® to X-ray film or phosphor screen, scan the film or phosphor screen, to acquire a TIFF image that is required for microarray data analysis.

Materials Needed

Scanning the X-ray film

You need a standard flatbed image scanner that provides at least 50 µm resolution (>600 dpi) to scan the X-ray film after development to produce a 16-bit TIFF file.

Scanning the Phosphor Screen

You need a phosphorimager that provides at least 50 µm resolution to acquire the microarray image from the phosphor screen to produce a 16-bit TIFF file.

The following phosphorimagers have been tested with the ProtoArray® Microarrays:

- Cyclone® Storage Phosphor System (PerkinElmer, Inc.)
- Typhoon™ Imager (Amersham Biosciences)

Data acquisition software

To acquire ProtoArray® data from the image, you need ProtoArray® Prospector 5.2 or higher (page 34). Microarray data acquisition software such as GenePix® Pro (Molecular Devices Corporation) or ScanArray® Software (PerkinElmer, Inc.) are also suitable for data acquisition.

Experimental Outline

1. Develop the X-ray film or process the phosphor screen according to the manufacturer's recommendations.
2. Scan the X-ray film on a image scanner or scan the phosphor screen on a phosphorimager to generate a 16-bit TIFF image file.
3. Process the image using ProtoArray® Prospector.
4. Save the adjusted microarray image.

Scanning Guidelines

After exposing the X-ray film or phosphor screen to the ProtoArray® Microarray, scan the film or phosphor screen to obtain a 16-bit TIFF image file that is required for microarray data analysis.

Brief scanning guidelines are described below. For details, refer to the manufacturer's recommendations on using the scanner or phosphorimager.

1. Remove the X-ray film or phosphor screen from the cassette. Keep the array covered in clear plastic wrap for later use if a longer exposure time is required.
2. Develop the X-ray film.
3. Scan the X-ray film using a standard image scanner or scan the phosphor screen using a phosphorimager to obtain a high-resolution image (at least 600 dpi or 50 µm). Include the barcode in the scan area for maintaining experimental records.
4. Save the image as a 16-bit TIFF file to a suitable location.

Continued on next page

Scanning and Image Analysis, continued

Processing the Image

To make the image compatible with the microarray data acquisition software, process the image using ProtoArray® Prospector or Adobe® Photoshop® image analysis software as described below.

Instructions are provided below and on the next page using ProtoArray® Prospector Imager or Adobe® Photoshop® image analysis software. You may use any equivalent image analysis software. For details on using any specific image analysis software, refer to the manual supplied with the software.

Image Processing Using ProtoArray® Prospector Imager

ProtoArray® Prospector software version 5.2 (includes Imager and Analyzer) is available from Invitrogen at www.invitrogen.com/protoarray, by clicking on the **Online Tools** link that can be found under **BioMarker Discovery Resources**. The ProtoArray® Prospector Imager is used to process images for data analysis. Install ProtoArray® Prospector to install ProtoArray® Prospector Imager.

1. Start ProtoArray® Prospector Imager on the computer.
2. Open the microarray image (.tiff) acquired in Step 4, previous page.
3. Perform the following adjustments to the image (refer to ProtoArray® Prospector Imager manual for detailed instructions)
   - Invert the data (convert the image from white background with black spots to black background with white spots which is required for analysis).
   - Rotate the image such that the array image is vertical and the barcode is located at the bottom
   - Crop a fixed rectangular area (600 × 1,800 pixels, if scanned at 600 dpi) from each image (.tiff) file corresponding to the array. If the spots are not aligned vertically, rotate the crop rectangle by holding the Ctrl key and rotating the selection angle with the mouse.

     First rotate and align the rectangle against the Alignment Control Kinase spots, release the Ctrl key and move the rectangle to cover the whole array area. Crop the image using the Crop button. If needed, adjust the image contrast/brightness in Imager for better visualization; this will not affect the final saved image.

     Note: If the image is scanned at a different dpi, set the fixed rectangular area accordingly. For example, if the image is scanned at 900 dpi, set the fixed rectangular area to 900 × 2,700 pixels to cover the 1" × 3" array area.
4. Save the cropped and resized image (.tiff) file with a new name to a suitable location. Be sure the barcode number is included in the name of the image.
5. Download lot specific information from ProtoArray® Central, see page 34.

Note: Follow instructions in the Prospector manual to download lot specific information and perform data analysis.

Continued on next page

Image Processing Using Adobe® Photoshop®

1. Start Adobe® Photoshop® on the computer.
2. Open the microarray image (.tiff) acquired in Step 4, page 30.
3. Perform the following adjustments to the image:
   - Crop a fixed rectangular area (1″ × 3″) from each image (.tiff) file corresponding to the array. If the spots are not aligned vertically, rotate the image to correctly align the spots.
   - Invert the data (convert the image from white background with black spots to black background with white spots).
   - Resize the image file to 2,550 × 7,650 pixels (constrained proportions).

     Important: Do not adjust the image quality (such as contrast or level) as this can compress the dynamic range of the data set and affect data analysis.
4. Save the cropped and resized image (.tiff) file with a new name to a suitable location. Be sure the barcode number is included in the name of the image.
5. Download lot specific information from ProtoArray® Central, see page 34.

Data Acquisition and Analysis

Introduction

After scanning and saving an image of the array, download the protein array lot specific information (*i.e.* the .GAL file) from ProtoArray® Central Portal. The lot specific information is used to acquire and analyze the data to identify potential kinase substrates.

Note: To familiarize yourself with the array and subarray layout, you may also download a file showing the subarray layout from ProtoArray® Central. To access the file, go to www.invitrogen.com/protoarray and click on the **ProtoArray® Lot Specific Information** link under **BioMarker Discovery Resources**.

Important

While downloading the lot specific information files, ensure that you are downloading files that are associated with your specific barcode on the array. Since lot specific information files are updated frequently based on recently available sequence or protein information, make sure that you download the latest version of the lot specific information files.

GAL File

The .GAL (GenePix® Array List) files describe the location and identity of all spots on the Human and Control microarrays and are used with the microarray data acquisition software to generate files that contain pixel intensity information for feature/spot and non-features of the slide.

The .GAL files are available for downloading from the ProtoArray® Lot Specific Information available on ProtoArray® Central, see below.

Note: The .GAL files are text files that contain the data in a format specified by GenePix® Pro Microarray data acquisition software. If you are using any other microarray data acquisition software, you can use data from the .GAL files to generate files that are compatible with your microarray data acquisition software.

Materials Needed

To acquire ProtoArray® data from the image, you need ProtoArray® Prospector 5.2 or higher (page 34). Microarray data acquisition software such as GenePix® Pro (Molecular Devices Corporation) or ScanArray® Software (PerkinElmer, Inc.) are suitable for data acquisition.

Note

If you do not have access to any microarray data acquisition software, contact Technical Support (page 48).

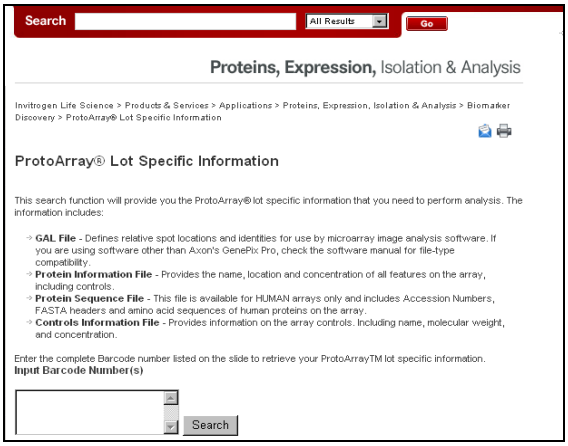
Continued on next page

ProtoArray® Central

The ProtoArray® Central Portal provides a web-based user interface to retrieve ProtoArray® Lot Specific Information. This information (.GAL file) is required for acquiring the array data.

If the scanner computer is connected to the Internet, connect to the portal. If the scanner computer is not connected to the internet, download the array-specific information to portable media as described below and then download the information onto the scanner computer.

1. Connect to the portal at www.invitrogen.com/protoarray and then click on the **ProtoArray® Lot Specific Information** link under **BioMarker Discovery Resources**.
2. The ProtoArray® Lot Specific Information page is displayed.
3. Enter the array barcode in the Input Barcode Number(s) box. Click on the Search button.



4. For each input barcode, the following files are displayed:

   .GAL file (LotNumber.gal):

   This file is essential for data acquisition by the software and defines spot locations and identities of all protein spots on the array. The file also includes the "equivalent solution protein concentration" in nM for use during data analysis.

   Protein Information File (LotNumber_info.txt):

   This file contains a listing and description of human proteins on the array.

   Protein Sequence File (LotNumber_seq.txt):

   This tab-delimited text file lists the GenBank® accession number, Ultimate™ ORF Clone ID number (if available), FASTA header, and amino acid sequence of the ORF for each array protein.

   Control Data File (LotNumber_control.txt):

   This file contains a description of control spots on the array.

   Slide Information File (LotNumber_slide.txt):

   This file contains a listing of all barcodes associated with a specific lot of arrays.

   Note: The file size for some files such as the Protein Sequence File may be larger than 1 MB.

Continued on next page

Data Acquisition and Analysis, continued

Data Acquisition

1. Start the ProtoArray® Prospector Imager, GenePix® Pro Software, or equivalent microarray data acquisition software on the computer.
2. Open the saved image (16-bit TIFF file) from Step 4, page 31.
3. **Note:** If the image is not saved as a 16-bit TIFF file, GenePix® Pro software is unable to open the file (image).Acquire data from ProtoArray® experiments as follows:
   - For ProtoArray® Prospector Imager, download the .GAL files from ProtoArray® Central, which defines the array grid required by the microarray data acquisition software. Load the .GAL file into Imager using the Array List button. Make adjustments to the blocks as described in the Imager manual. Use spots corresponding to the Alignment Control Kinase (PKCeta) as reference spots to orient the microarray image. Scroll through the image to ensure that the grid is in the proper location for each subarray. Adjust the subarray grid manually, if needed. After the grid is adjusted properly and all features are aligned, save the Project and analyze the results. Imager automatically opens the Analyzer component of ProtoArray® Prospector for data analysis, and allows you to select the KSI application and specify the experimental conditions. Analyzer then performs the data analysis and shows a summary of results (see ProtoArray® Prospector manual for details).
   - For GenePix® Pro Software, download the .GAL files from ProtoArray® Central, which defines the array grid required by the microarray data acquisition software. Analyze the data and save/export the results as a .GPR (GenePix® Results) file for data analysis using ProtoArray® Prospector (see next page). The results contain the pixel intensity information for each spot/feature on the array and information on additional parameters depending on the type of software used for data acquisition.
   - For other microarray data acquisition software, use data from the .GAL files from ProtoArray® Central to generate files that are compatible with your microarray data acquisition software to define the microarray grid.

Alternatively, save/export the results with an .xls extension or rename the .tab or .gpr file using the .xls extension for data analysis using Microsoft® Excel.

Analyzing Data

After data acquisition, analyze the data to identify potential kinase substrates. Once significant signals are identified, we recommend confirming these signals using visual identification.

We recommend using the ProtoArray® Prospector software available from Invitrogen for data analysis. This software allows rapid data analysis without the need to perform any manual calculations. For more information, see next page.

Performing the data analysis by importing the data file into Microsoft® Excel or an equivalent spreadsheet program to identify potential substrates is not recommended. This approach requires a certain degree of expertise with statistics and Excel, or another spreadsheet program.

Continued on next page

Data Acquisition and Analysis, continued

Data Analysis Using ProtoArray® Prospector

The ProtoArray® Prospector Analyzer software quickly analyzes the data acquired from the ProtoArray® Prospector Imager or image acquisition software and easily identifies statistically significant hits (potential substrates), saving you time and effort. The Analyzer software is designed to analyze data and identify potential substrates with a low false positive rate as compared to performing manual calculations using a spreadsheet program. In addition, the software has features that allow you to modify the analysis method and compare data obtained from different microarrays.

The ProtoArray® Prospector software and manual are available for FREE to ProtoArray® users. To download the ProtoArray® Prospector software and manual, go to www.invitrogen.com/protoarray, and click on the **Online Tools** link under **BioMarker Discovery Resources**. Install ProtoArray® Prospector to install ProtoArray® Prospector Imager and Analyzer.

The ProtoArray® Prospector software also accepts the output files (.GPR) generated by the GenePix® Pro microarray data acquisition software, and analyzes the data using specified algorithms to generate a list of human proteins as potential substrates with the protein kinase.

If .GPR files are not available, consult the ProtoArray® Prospector manual for guidelines to format a results file that is compatible for import into ProtoArray® Prospector.

Analyzing ProtoArray® Prospector Results

After data analysis, ProtoArray® Prospector presents a summary of the analyzed data in a table format (see ProtoArray® Prospector manual for details).

The proteins that score as positive in the experiment are proteins that satisfy the basic program options. Review the information on page 38, **Expected Results**, to help with data interpretation.

We recommend validating the identified potential kinase substrates by *in vitro* solution assay or ProtoArray® Technology as described in the Appendix.

Continued on next page

The Next Step

After identifying potential kinase substrates on the Human ProtoArray®, you may reproduce the observed results using the ProtoArray® Technology or validate the result using *in vitro* solution assays.

Using the ProtoArray® Technology, validate the potential protein kinase substrate by performing experiments with additional arrays to ensure:

- **Reproducibility:** Probe the human array using a similar or a different kinase concentration to address reproducibility.
- **Specificity:** Probe a human array with different kinases to identify substrates specific to your protein kinase of interest.

You can use an *in vitro* solution assay to validate the protein kinase substrates as described briefly below. For detailed protocol, see page 45.

To verify substrate phosphorylation in solution, perform solution assays in the presence of radiolabeled ATP using the purified protein kinase and potential kinase substrate (available as a DNA clone or purified protein from Invitrogen, see below) using the probing conditions described in this manual. Be sure to include appropriate positive and negative control reactions. Analyze the results using SDS-PAGE and autoradiography.

Accessing Clones

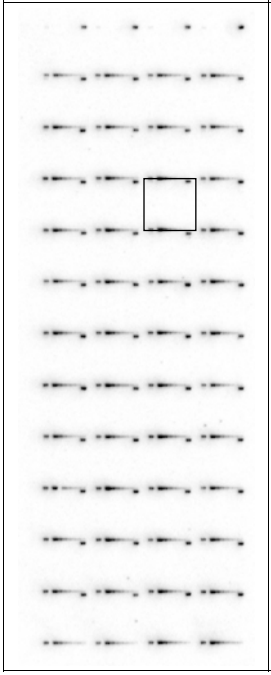
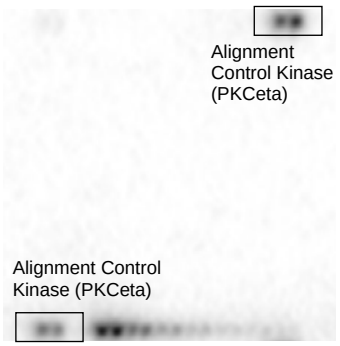
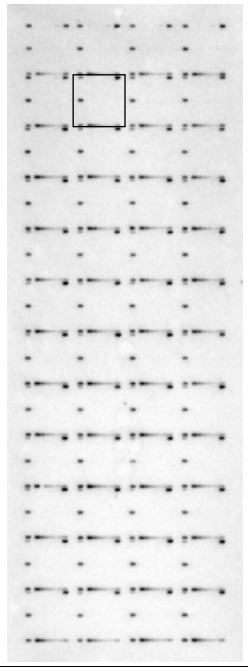
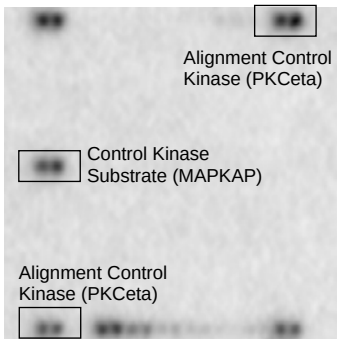
Since the majority of human proteins printed on the array are derived from the Ultimate™ ORF Clone Collection or purified proteins (protein kinases) available from Invitrogen, it is very easy to order the clone or purified protein corresponding to the protein identified on the array and validate the interaction.

Visit www.invitrogen.com/clones to access our clone collections. Each Ultimate™ ORF Clone is full insert-sequenced and guaranteed to match the corresponding GenBank® amino acid sequence. Contact Technical Support (page 48) to order the purified protein kinases printed on the array or to request information about custom production of additional proteins present on the array.

Expected Results

ProtoArray® Control Protein Array Probing Results

Results obtained after probing the ProtoArray® Control Protein Microarray v5.0 with the Control Kinase and radiolabeled ATP are shown below.

Image showing the Control Array when probed with labeled ATP only (negative control)		Image showing the Control Array when probed with 120 nM Control Kinase	
Control Array Image	**Boxed area shown in detail**	**Control Array Image**	**Boxed area shown in detail**
			

- Alignment Control Kinase Signal

  Alignment Control Kinase (PKCeta) on the arrays are autophosphorylated during the labeling reaction. The signals at Alignment Control Kinase locations indicate that the probing procedure and scanning is performed properly, and are used as reference spots to orient the microarray image and help assign spot identities.
- Control Kinase Substrate Signal

  The Control Kinase substrate (MAPKAP) is printed on the microarray. The Control Kinase phosphorylates the Control Kinase substrate producing a signal. These signals indicate proper probing and scanning procedures.

Note

To orient the results obtained from the .GAL file and ProtoArray® Prospector with the array image, position the microarray image such that the barcode is at the bottom of the image. In this orientation, the top left corner of the microarray image is Block 1.

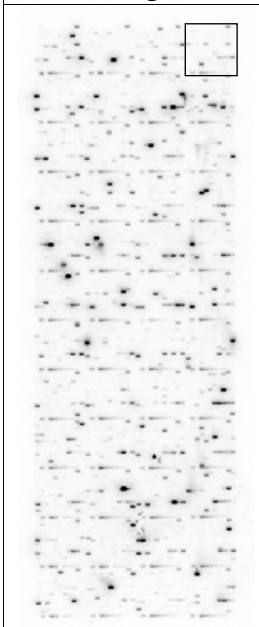
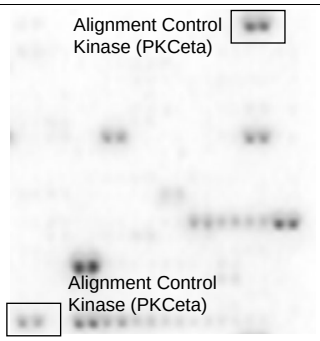
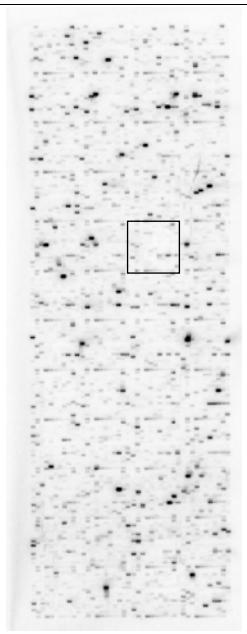
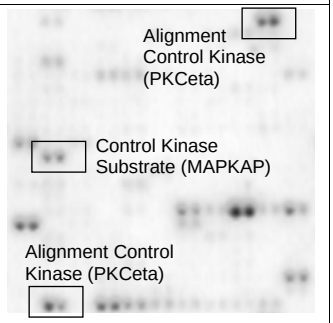
Continued on next page

Expected Results, continued

ProtoArray® Human Protein Microarray v5.0 Probing Results

The results obtained after probing the ProtoArray® Human Protein Microarray v5.0 with 120 nM Control Kinase is shown below. The Control Kinase phosphorylates the Control Kinase substrate printed on the array.

A negative control image of the ProtoArray® Human Protein Microarray v5.0 is also shown below.

Image of the Human Microarray when probed with labeled ATP only (negative control)		Image of the Human Microarray when probed with 50 nM Control Kinase	
Human Array Image	**Boxed area shown in detail**	**Human Array Image**	**Boxed area shown in detail**
			

Continued on next page

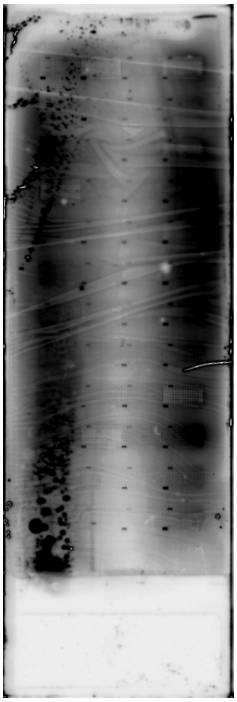
Example Showing High Background and Low Signal

High Background

In this example, the ProtoArray® Microarray displays high background due to poor washing of the array.

Low or No Signal

In this example, the ProtoArray® Microarray displays low signals due to too much activity of the exogenous kinase. The high activity of the exogenous kinase depletes the radiolabeled ATP which is not available for autophosphorylation of the Alignment Control Kinases on the array. The low signals may also be due to loss of activity of the Alignment Control Kinases when expired arrays are used, the use of old radiolabeled ATP, or an exposure time that is too short.

Array Image Showing High Background	Subarray Image Showing Low or No Signal
	

Troubleshooting

Introduction

The table below provides some solutions to possible problems you may encounter when using the ProtoArray® Human Protein Microarray KSI Complete Kit.

Problem	Cause	Solution
Control Array Results		
No signal with Control Kinase	Poor incorporation of radiolabel	Use fresh [γ-^{33}P]ATP. Be sure to check the array using a Geiger counter to verify that the radioactive signal is obtained after the probing procedure.
	Incorrect scanning or imaging	For X-ray film, develop the film and acquire the image using a standard film scanner. For phosphor screen, acquire the image using a phosphorimager. Follow the manufacturer's recommendations on using the scanner or phosphorimager to scan the array correctly. Be sure to use a scanner or phosphorimager that provides at least 50 µm resolution and generates 16-bit TIFF image files.
	Improper handling of Control Kinase	Store the Control Kinase at –80°C upon receipt. Use the recommended concentration (50 nM) of the Control Kinase for probing. Avoid repeated freezing-thawing of the Control Kinase.
	Alignment Control Kinases printed on the array not active	Do not use the ProtoArray® beyond the expiration date printed on the mailer.
	Used incorrect array	Be sure to use ProtoArray® Microarray v5.0 with the ProtoArray® KSI Application Kit.
Weak or no signal of the Control Kinase substrate printed on the array with Control Kinase	Poor incorporation of radiolabel	Use fresh [γ-^{33}P]ATP. Be sure to check the array using a Geiger counter to verify that the radioactive signal is obtained after the probing procedure.
	Improper handling of Control Kinase	Store the Control Kinase at –80°C upon receipt. Use the recommended concentration (50 nM) of the Control Kinase for probing.
	Kinase-ATP mixture not added immediately to the array	After preparing the kinase-ATP mixture, immediately add the mixture to the array. Do not store the prepared kinase-ATP mixture on ice for more than 2 minutes prior to use on the array.
	Incorrect assay conditions	Perform incubation of the arrays at 30°C during the probing procedure. Use the Kinase Buffer included with the kit for best results.
Weak signal with your kinase	Radiolabeled ATP not available for substrate phosphorylation	High concentration of the kinase used that depletes the radiolabeled ATP. Decrease the kinase concentration.
		Kinase phosphorylates BSA in the blocking buffer depleting radiolabeled ATP. Use alternate blocking buffer without BSA.

Continued on next page

Troubleshooting, continued

Problem	Cause	Solution
Control Array Results, continued		
High signal with your kinase	Kinase interacts non-specifically with array proteins	Increase the time for blocking step.
High Background	Improper blocking	Prepare the Blocking Buffer **fresh** as described on page 19.
	Improper washing	For the best results, perform the recommended washing steps using 0.5% SDS and water as outlined in the protocol.
	Incorrect amount of radiolabel used	Use the recommended concentration of the [γ-^{33}P]ATP (33 nM). Use **fresh** [γ-^{33}P]ATP.
	Array dried during probing or washing	Do not allow the array to dry during probing or washing procedure. Ensure the coverslip completely covers the printed area of the array. During the incubation step at 30°C, make sure the 50 mL conical tube is capped to minimize drying. During all wash steps, ensure the array is completely covered in buffers.
	Array not dried properly before scanning	Dry the array as described on page 24 before scanning.
Uneven background	Uneven blocking or washing	During the blocking or washing steps, ensure the array is completely immersed in buffers and use at least 40 mL buffer in the 50 mL conical tube to cover the array completely with buffer.
	Improper washing	To obtain the best results, perform the recommended washing steps. Prepare the 0.5% SDS **fresh** as described on page 19.
	Portions of array have dried	Do not allow the array to dry during probing.
	Improper array handling	Always wear gloves and avoid touching the surface of the array with gloved hands or forceps. Take care while inserting the array into the tube to avoid scratching the array surface.
	Control Kinase probe not applied properly	Apply the Control Kinase solution and coverslip to the array as described in the manual. To avoid drying of the array, make sure the coverslip covers the printed area of the array and adjust the coverslip, if needed.
	Buffer or radiolabeled ATP contains precipitates	Centrifuge the buffer or [γ-^{33}P]ATP to remove precipitates prior to probing the array.

Continued on next page

Problem	Cause	Solution
Human Protein Array Results		
Weak or no signal with your protein kinase	Kinase of interest is not active or is inactive in the assay buffer	Check the activity of the kinase after purification using a method of choice. Ensure the kinase is active under the conditions used for probing. Avoid repeated freezing-thawing of your kinase.
	Low specific activity of the kinase	Perform probing with higher kinase concentration, higher kinase specific activity, or increase the incubation time. Avoid repeated freezing-thawing of your kinase.
	Incorrect scanning or imaging	For X-ray film, develop the film and acquire the image using a standard scanner. For phosphor screen, acquire the image using a phosphorimager. Follow the manufacturer's recommendations on using the scanner or phosphorimager to scan the array correctly. Be sure to use a scanner or phosphorimager that provides at least 50 µm resolution and generates 16-bit TIFF image files.
	Incorrect assay conditions	Perform incubation of the array at 30°C during the probing procedure. Use the Kinase Buffer included with the kit for best results.
	Poor incorporation of radiolabel	Use fresh [γ-^{33}P]ATP. Be sure to check the array using a Geiger counter to verify that the radioactive signal is obtained after the probing procedure.
	Kinase-ATP mixture not added immediately to the array	After preparing the kinase-ATP mixture, immediately add the mixture to the array. Do not store the prepared kinase-ATP mixture on ice for more than 2 minutes prior to use on the array.
	Kinase specific substrates are not present on the array	Use another kinase.

Continued on next page

Troubleshooting, continued

Problem	Cause	Solution
Human Protein Array Results, continued		
High background	Improper blocking	Prepare the Blocking Buffer **fresh** as described on page 19.
	Improper washing	For the best results, perform the recommended washing steps using 0.5% SDS and water as outlined in the protocol.
	Array dried during probing or washing	Do not allow the array to dry during probing or washing procedure. Ensure the coverslip completely covers the printed area of the array. During the incubation step at 30°C, make sure the 50 mL conical tube is capped to minimize drying. During all wash steps, ensure the array is completely covered in buffers.
	Array not dried properly before scanning	Dry the array as described on page 24 before scanning.
	High kinase concentration	Decrease the kinase concentration/specific activity or decrease the incubation time.
Uneven background	See page 42 for details	See page 42 for details.
Poor spot resolution	Incorrect scanner or phosphorimager used	Be sure the scanner or phosphorimager is capable of providing at least 50 µm resolution.
	Improper array handling	Be sure to equilibrate the mailers with the array at 4°C for at least 15 minutes prior to use.
	Improper covering of arrays	Properly cover the arrays with a clear plastic wrap without any creases.
Signals from duplicate spots are merged	--	It is normal for signals from duplicate spots to merge sometimes. The merging of spots does not affect data analysis.

Appendix

Solution Kinase Assay Protocol

Introduction

Instructions for performing an *in vitro* solution assay to validate the protein kinase substrates identified using the ProtoArray® Technology are described in this section.

Briefly, perform solution assays in the presence of radiolabeled ATP following the protein kinase and potential kinase substrate using the assay conditions described below. Analyze the results using SDS-PAGE and autoradiography. A true positive signal identified on the array should also produce positive results using the solution assay while a false positive signal identified on the array should not produce any positive results using the solution assay.

Note

To perform the solution assay you will need your purified kinase of interest (20–50 nM) and the purified potential kinase substrate (20–500 ng). The proteins should be >90% pure as determined by Coomassie® staining.

Experimental Outline

1. Perform *in vitro* solution assay of your kinase of interest and the potential kinase substrate (identified on the array) in the presence of radiolabeled ATP.
2. At the end of the assay, analyze results by electrophoresis and autoradiography.
3. Acquire the image by autoradiography.

Materials Needed

- Purified kinase of interest (20–50 nM)
- Purified potential kinase substrate (20–500 ng)
- Kinase Buffer (supplied in the kit) or see page iv for composition
- [γ-^{33}P]ATP (1 µCi/µL)
- Water bath or heat block
- NuPAGE® Novex® Bis-Tris Gels (page vi)
- NuPAGE® Sample Buffer (page vi)
- NuPAGE® SDS Running Buffer (page vi)
- NuPAGE® Reducing Agent (page vi)
- Gel Fixing Solution (45% methanol, 10% acetic acid in ultrapure water)
- X-ray film or phosphor screen
- X-ray film cassette
- Clear plastic wrap
- Protein molecular weigh markers

NuPAGE® System

A wide variety of NuPAGE® Novex® Bis-Tris Pre-cast mini gels are available from Invitrogen for SDS-PAGE analysis of proteins. You may use any type pre-cast gels for analysis.

Continued on next page

Solution Kinase Assay Protocol, Continued

Amount of Kinase

The amount of kinase used for the solution assay will depend on the specific activity of the kinase of interest. If the specific activity of the kinase is 2 µmoles of phosphate transferred per minute per milligram of protein, use 20 nM kinase for the solution assay. Based on these guidelines, adjust the amount of kinase accordingly for the solution assay.

Solution Kinase Assay

The solution assay is performed using radiolabeled ATP. Be sure to handle radioactive samples and waste as mandated by your safety department. See page 12 for general guidelines on working with radioactive material.

Note: Do not add more than the recommended volumes listed in the table below. If needed dilute the kinase, kinase substrate, and radiolabeled ATP in the Kinase Buffer appropriately such that you can add the recommended volume for the assay.

1. Set a water bath or heat block to 30°C and 70°C each.
2. To sterile microcentrifuge tubes, add the following.

Reagents	Test	Kinase Only	Substrate Only
Kinase Substrate (20–500 ng)	≤2.5 µL	—	≤2.5 µL
Kinase (20–50 nM)	≤2 µL	≤2 µL	—
Kinase Buffer	to 10 µL	to 10 µL	to 10 µL
Add ATP to the reaction, just **prior to** starting the assay.			
[γ-^{33}P]ATP (1 µCi/µL)	1 µL	1 µL	1 µL

Note: Add the radiolabeled ATP and kinase, **immediately** before starting the assay. **Do not** prepare the kinase-ATP mixture and store on ice for longer than 2 minutes prior to use on the array.

3. Mix well and incubate at 30°C for 1 hour.
4. Add NuPAGE® Sample Buffer (4X) and heat the samples at 70°C for 10 minutes to stop the reaction. Briefly centrifuge the samples at high speed.
5. Proceed to **SDS-PAGE and Autoradiography**, next page.

Continued on next page

Solution Kinase Assay Protocol, Continued

SDS-PAGE and Autoradiography

1. Load the entire sample into a well of the NuPAGE® Novex® Bis-Tris Gel (use a gel with an acrylamide percentage that best resolves your proteins of interest).
2. Load appropriate protein markers on the gel.
3. Assemble the electrophoresis apparatus and perform electrophoresis at 200 V for 50 minutes for NuPAGE® Gels.
4. After electrophoresis is complete, remove the gel from the cassette and fix the gel in 100 mL Gel Fixing Solution for 45 minutes at room temperature.
5. Wash the gel in 100 mL deionized water for 15 minutes. Decant water and repeat the wash step.
6. Decant the water and blot excess water from the gel using blotting paper.
7. Cover the gel with clear plastic wrap. You can check for radioactivity on the gel using a Geiger counter.
8. Place the gel in an X-ray film cassette. Overlay the gel with an X-ray film.
9. Expose the gel overnight at room temperature.
10. Remove the X-ray film from the cassette. Develop the X-ray film.

Expected Results

To validate the protein kinase substrates identified using ProtoArray® Technology, you should observe the following results:

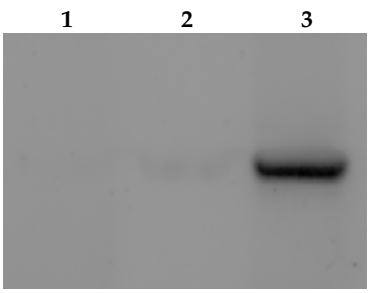
- Positive signal (band) in the lane containing the kinase and kinase substrate at the position corresponding to the molecular weight of the kinase substrate
- No or very low signal in the kinase alone or substrate alone lanes at the position corresponding to the molecular weight of the kinase substrate

An example of results obtained after performing an *in vitro* solution assay is shown below. The solution assay was performed with purified casein kinase 2 alpha (CK2a) and purified potential kinase substrate BC001600 (identified on the array) in the presence of radiolabeled ATP as described in this manual. The results were analyzed using SDS-PAGE and autoradiography. The signal of the phosphorylated kinase substrate is observed in lane 3 only and not in lanes 1 or 2.

Lane 1: Sample contains radiolabeled ATP and CK2a only

Lane 2: Sample contains radiolabeled ATP and kinase substrate, BC001600 only

Lane 3: Sample contains radiolabeled ATP, CK2a, and kinase substrate, BC001600



Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
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- Additional product information and special offers

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For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our Web page (www.invitrogen.com).

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SDS

Safety Data Sheets (SDSs) are available at www.invitrogen.com/sds.

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References

Bouvier, M., Menard, L., Dennis, M., and Marullo, S. (1998) Expression and Recovery of Functional G-protein-coupled Receptors Using Baculovirus Expression Systems. Curr. Opin. Biotechnol. *9*, 522-527

Boyle, S. N., Michaud, G. A., Schweitzer, B., Predki, P. F., and Koleske, A. J. (2007) A Critical Role for Cortactin Phosphorylation by Abl-Family Kinases in PDGF-Induced Dorsal-Wave Formation. Current Biology *17*, 445-51.

Hollister, J., Grabenhorst, E., Nimtz, M., Conradt, H., and Jarvis, D.L. (2002) Engineering the Protein N-glycosylation Pathway in Insect Cells for Production of Biantennary, Complex N-glycans. J. Biochemistry *41*, 15093-15104

Mah, A. S., Elia, A. E., Devgan, G., Ptacek, J., Schutkowski, M., Snyder, M., Yaffe, M. B., and Deshaies, R. J. (2005) Substrate Specificity Analysis of Protein Kinase Complex Dbf2-Mob1 by Peptide Library and Proteome Array Screening. BMC Biochem *6*, 22-33

Mitchell, D., Marshall, T., and Deschenes, R. (1993) Vectors for the Inducible Overexpression of Glutathione S-Transferase Fusion Proteins in Yeast. Yeast *9*, 715-722

Predki, P. (2003) Functional Protein Microarrays - Ripe for Discovery. Curr. Opin. Chem. Biol. *8*, 8-13

Ptacek, J., Devgan, G., Michaud, G., Zhu, H., Zhu, X., Fasolo, J., Guo, H., Jona, G., Breitkreutz, A., Sopko, R., McCartney, R., Schmidt, M., Rachidi, N., Lee, S. J., Mah, A., Meng, L., Stark, M., Stern, D., De Virgilio, C., Tyers, M., Andrews, B., Gerstein, M., Schweitzer, B., Predki, P., and Snyder, M. (2005) Global Analysis of Protein Phosphorylation in Yeast. Nature *438*, 679-684

Schweitzer, B., Predki, P., and Snyder, M. (2003) Microarrays to Characterize Protein Interactions on a Whole-Proteome Scale. Proteomics *3*, 2190-2199

Zhu, H., Bilgin, M., Bangham, R., Hall, D., Casamayor, A., Bertone, P., Lan, N., Jansen, R., Bidlingmaier, S., Houfek, T., Mitchell, T., Miller, P., Dean, R. A., Gerstein, M., and Snyder, M. (2001) Global Analysis of Protein Activities Using Proteome Chips. Science *293*, 2101-2105

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