invitrogen™

ProtoArray® Kinase Substrate Identification (KSI) Kits

For identifying protein kinase substrates using a human or yeast protein microarray

Catalog nos. PA015, PA0121065, and PAH0524065

Version A
14 September 2006
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User Manual

Table of Contents

Kit Contents and Storage

Types of Kits

This manual is supplied with the following kits.

Product	Catalog no.
ProtoArray® Human Protein Microarray KSI Complete Kit v4.0 *for kinase substrate identification*	PAH0524065
ProtoArray® Yeast Proteome Microarray KSI Complete Kit v1.0 *for kinase substrate identification*	PA0121065
ProtoArray® Kinase Substrate Identification Application Kit	PA015

Kit Components

The ProtoArray® KSI Kits for kinase substrate identification (KSI) include the following components. For a detailed description on each kit component, see next page.

Note: Catalog nos. PAH0524065, and PA0121065 include **two** ProtoArray® Human Protein or Yeast Proteome Microarrays and **two** ProtoArray® Control Protein Microarrays.

Component	Catalog no.		
	PAH0524065	**PA0121065**	**PA015**
ProtoArray® Human Protein Microarray mg v4.0	√		
ProtoArray® Yeast Proteome Microarray mg v1.0		√	
ProtoArray® Control Protein Microarray mg v4.0	√	√	
ProtoArray® KSI Buffer Module A	√	√	√
ProtoArray® KSI Buffer Module B	√	√	√

Shipping and Storage

The components included in the ProtoArray® KSI Kits 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.

Components	Shipping	Storage
ProtoArray® Human Protein Microarray mg v4.0	Blue ice	-20°C
ProtoArray® Yeast Proteome Microarray mg v1.0	Blue ice	-20°C
ProtoArray® Control Protein Microarray mg v4.0	Blue ice	-20°C
ProtoArray® KSI Buffer Module A	Dry ice	see next page
ProtoArray® KSI Buffer Module B	Room temperature	Room temperature

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ProtoArray® Microarrays

Each ProtoArray® Microarray KSI Complete Kit contains mailers with the following ProtoArray® Microarrays:

- Human Kit (catalog no. PAH0524065): Contains two ProtoArray® Human Protein Microarrays mg v4.0 and two ProtoArray® Control Protein Microarrays mg v4.0
- Yeast Kit (catalog no. PA0121065): Contains two ProtoArray® Yeast Proteome Microarrays mg v1.0 and two ProtoArray® Control Protein Microarrays mg v4.0

Store the microarrays at -20°C.

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

For details on array specifications, see pages 6-11

ProtoArray® KSI Buffer Module A

The ProtoArray® KSI Buffer Module A includes the following reagents.

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 following reagents.

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

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 51).

Product	Quantity	Catalog no.
ProtoArray® Human Protein Microarray mg v4.0	1 array	PAH052406
ProtoArray® Yeast Proteome Microarray mg v1.0	1 array	PA012106
ProtoArray® Control Protein Microarray mg v4.0	1 array	PA1002
ProtoArray® Human Protein Microarray nc v4.0	1 array	PAH052401
ProtoArray® Yeast Protein Microarray nc v1.0	1 array	PA012101
ProtoArray® Control Protein Microarray nc v4.0	1 array	PA1007
ProtoArray® Kinase Substrate Identification Application Kit	1 kit	PA015
ProtoArray® Protein-Protein Interaction Buffer Modules	1 kit	PA014
ProtoArray® Human Protein Microarray PPI Complete Kit v4.0 *for biotinylated proteins*	1 kit	PAH0524011
ProtoArray® Yeast Proteome Microarray PPI Complete Kit *for biotinylated proteins*	1 kit	PA0121011
ProtoArray® Protein-Protein Interaction Application Kit *for biotinylated proteins*	1 kit	PA011
ProtoArray® Human Protein Microarray PPI Complete Kit v4.0 *for epitope-tagged proteins*	1 kit	PAH0524013
ProtoArray® Yeast Proteome Microarray PPI Complete Kit *for epitope-tagged proteins*	1 kit	PA0121013
ProtoArray® Mini-Biotinylation Kit	1 kit	AL-01
ProtoArray® Immune Response Biomarker Profiling Application Kit	1 kit	PA016
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® Transfer Buffer (20X)	125 ml	NP0006
NuPAGE® Antioxidant	15 ml	NP0005
NuPAGE® Sample Reducing Agent (10X)	250 µl	NP0004
NuPAGE® LDS Sample Buffer (4X)	10 ml	NP0007

Kinase

A variety of purified kinases is 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 51).

Introduction

Overview

Introduction

The ProtoArray® Human Protein and Yeast Proteome Microarray Complete Kits for Kinase Substrate Identification (KSI) allow rapid and efficient identification of potential human or yeast protein kinase substrates using a protein kinase of interest. The ProtoArray® Human Protein Microarray is printed with thousands of purified human proteins, while the ProtoArray® Yeast Proteome Microarray is a proteome microarray containing > 4,000 purified yeast proteins from *Saccharomyces cerevisiae*. In both cases, the proteins are printed in duplicate on a modified 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).

ProtoArray® Microarray KSI Applications

The ProtoArray® Human Protein and Yeast Proteome 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 or Yeast Proteome Microarray Complete Kits 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 or yeast 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

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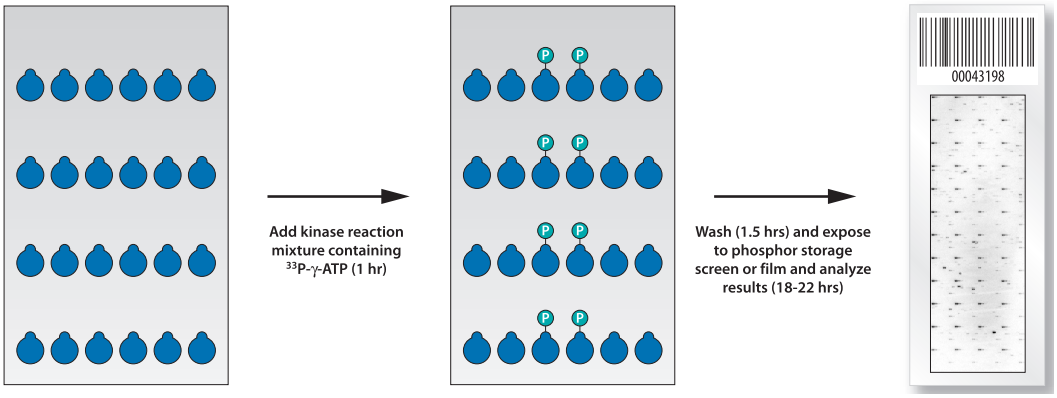
System Overview

To use the ProtoArray® Human Protein or Yeast Proteome Microarray KSI Complete Kits, you will:

- Purify your kinase of interest using a method of choice or purchase a purified protein kinase from Invitrogen (page vi).
- 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 or Yeast Proteome 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 phosphorscreen or X-ray film, acquire the array image to view results, and analyze data (see figure below). For a detailed experimental workflow, see page 28.



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Expected Results

ProtoArray® Human Protein Microarrays 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 48 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 kinase annotated substrates as 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 usually not 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 denaturing SDS as described in the assay protocol.

Purpose of the Manual

This manual provides the following information:

- An overview of the ProtoArray® Human Protein, Yeast Proteome, 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 Kits

The ProtoArray® Human Protein or Yeast Proteome Microarray KSI Complete Kits for kinase substrate identification include the following major components:

- The ProtoArray® Human Protein or Yeast Proteome Microarray on a modified glass surface, a high-density protein microarray that allows you to screen your kinase of interest (protein probe) against thousands of human proteins or the *Saccharomyces cerevisiae* proteome, respectively
- 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 and Yeast Proteome Microarrays

The ProtoArray® Human Protein and Yeast Proteome Microarrays mg are high-density protein microarrays containing human or *S. cerevisiae* proteins, respectively. Each human or *S. cerevisiae* open reading frame (ORF) is expressed as an N-terminal GST-(Glutathione-S-Transferase)-fusion protein, purified, and printed in duplicate on a modified glass (mg) slide.

The modified glass array is a thin nitrocellulose coated slide from GenTel® BioSciences, Inc., and is specifically selected for superior performance of the array in the KSI application. Thin-film nitrocellulose slides are manufactured by GenTel® BioSciences, Inc. using a proprietary surface chemistry owned by Decision Biomarkers, Inc.

Each ProtoArray® Microarray 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 or *S. cerevisiae* proteins in ~2 days to identify potential substrates for your kinase.

For array specifications and more details on how the human and yeast proteins are prepared, see pages 6-9.

ProtoArray® Control Protein Microarray

The ProtoArray® Control Protein Microarray mg contains protein kinase substrates and various controls printed on a modified glass (mg) slide, and is used to validate the assay **prior** to probing the ProtoArray® Human Protein or Yeast Proteome 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 11.

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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® KSI Application Kit

The ProtoArray® Kinase Substrate Identification Application Kit includes ProtoArray® KSI Buffer Modules A and B. You need to obtain a ProtoArray® Human Protein or Yeast Proteome Microarray separately from Invitrogen before performing a microarray screening experiment.

Note: The ProtoArray® KSI Application Kit is designed for use with the ProtoArray® Microarray mg v4.0 control and high content array only. The Control Kinase Substrate is printed only on the ProtoArray® Microarray mg v4.0 arrays and not on ProtoArray® Microarray v3.0 available previously from Invitrogen.

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 34), 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 4.0 (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 tab.

ProtoArray® Human Protein Microarray

Introduction

The ProtoArray® Human Protein Microarray mg 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 modified glass (mg) slide. This section provides details about the human protein microarray including array specifications and preparation of proteins.

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

Human Protein Microarray Specifications

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

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

The modified glass array is a thin nitrocellulose coated slide from GenTel® BioSciences, Inc., and is specifically selected for superior performance of the array in the KSI application. Thin-film nitrocellulose slides are manufactured by GenTel® BioSciences, Inc. using a proprietary surface chemistry owned by Decision Biomarkers, Inc.

Each microarray has a barcode for tracking samples. The barcode is also used to retrieve array specific information from the ProtoArray® Central Portal (page 35).

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 mg, go to the ProtoArray® Central portal at www.invitrogen.com/protoarray.

Total Subarrays:	48 (4 columns x 12 rows)
Subarray Size:	4400 µm x 4400 µm
Subarray Dimensions:	**20 rows x 20 columns**
Median Spot Diameter:	~150 µm
Spot Center to Center Spacing:	220 µm
Distance Between Subarrays:	100 µm
Replicates per Sample:	2
Total Human Proteins on v4.0 Array:	~8000*

*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 (next page) 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 human protein collection is derived from the human Ultimate™ ORF (open reading frame) Clone Collection available from Invitrogen (see http://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. Some of the kinases from the kinase collection have been cloned as catalytic domains rather than full-length proteins. About 250 proteins printed on the array are derived from the purified protein kinase collection available from Invitrogen. Approximately 25 purified proteins, peptides, and nucleic acids that have been demonstrated to be antigens in a variety of autoimmune diseases are also 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 Information File 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 a LR recombination reaction with a Gateway® destination vector, pDEST™20 to generate an expression clone. The expression clone is then used to express the protein as an N-terminus GST-fusion protein 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 that is used for preliminary expression experiments. After the baculoviral stock is amplified, the high-titer stock is used to infect Sf9 insect cells for large-scale expression of the recombinant protein of interest.

After verifying that each clone expresses a protein of the expected molecular weight, the proteins are expressed and purified using high-throughput procedures. The GST-tagged fusion proteins are purified under conditions optimized to obtain maximal protein integrity, function, and activity.

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ProtoArray® Human Protein Microarray, Continued

Note

Approximately 5000 human proteins printed on the ProtoArray® Human Protein Microarray mg v4.0 were also present on the previous version of the product, ProtoArray® Human Protein Microarray mg v3.0. However, not all proteins printed on ProtoArray® Human Protein Microarray mg v3.0 are also printed on ProtoArray® Human Protein Microarray mg v4.0. To obtain a list of ProtoArray® Microarray mg v3.0 proteins that are not printed on ProtoArray® Microarray mg v4.0, contact Technical Support (page 51).

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 12.

Printing the Human ProtoArray®

The purified human proteins are printed on modified glass 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® 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. 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. To control for the quality of the printing process, several microarrays from each lot are probed with an anti-GST antibody. Since the proteins contain a GST fusion tag, probing the microarrays with an anti-GST antibody allows identification of irregular spot morphology or missing spots. 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 Fiduciary Kinases.

For detailed product qualification, see page 52.

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ProtoArray® Yeast Proteome Microarray

Introduction

The ProtoArray® Yeast Proteome Microarray mg is a high-density protein microarray containing the majority of proteins from *S. cerevisiae*. Each *S. cerevisiae* open reading frame (ORF) is expressed as an N-terminal GST-6xHis fusion protein, purified, and printed in duplicate on a modified glass (mg) slide. This section provides details about the yeast proteome microarray including array specifications and preparation of proteins.

Note: The ProtoArray® Yeast Proteome Microarray KSI Complete Kit includes 2 ProtoArray® Yeast Proteome Microarrays mg.

Yeast Proteome Microarray Specifications

The specifications for the ProtoArray® Yeast Proteome Microarray mg are listed below.

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

The modified glass array is a thin nitrocellulose coated slide from GenTel® BioSciences, Inc., and is specifically selected for superior performance of the array in the KSI application. Thin-film nitrocellulose slides are manufactured by GenTel® BioSciences, Inc. using a proprietary surface chemistry owned by Decision Biomarkers, Inc.

Each microarray has a barcode for tracking samples. The barcode is also used to retrieve array specific information from the ProtoArray® Central Portal (see page 35).

Array Specifications

The array specifications for the yeast proteome 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 yeast and control protein spots on the ProtoArray® Yeast Proteome Microarray mg, go to the ProtoArray® Central portal at www.invitrogen.com/protoarray.

Total Subarrays:	48 (4 columns x 12 rows)
Subarray Size:	4400 µm x 4400 µm
Subarray Dimensions:	**16 rows x 16 columns**
Median Spot Diameter:	~150 µm
Spot Center to Center Spacing:	275 µm
Distance Between Subarrays:	100 µm
Replicates per Sample:	2

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ProtoArray® Yeast Proteome Microarray, Continued

Preparing Yeast Proteins

The yeast proteome collection is derived from the *S. cerevisiae* clone collection of 5,800 yeast ORFs (Zhu *et al.*, 2001). Each *S. cerevisiae* open reading frame (ORF) is expressed as an N-terminal GST-6xHis fusion protein in the modified version of the yeast expression vector pEG-KG (Mitchell *et al.*, 1993). The identity of each clone is verified using 5'-end DNA sequencing and the expression of the expected GST-tagged fusion protein is confirmed by western immunodetection using an anti-GST antibody. The yeast proteins are expressed and purified using high-throughput procedures and optimized conditions for maximal protein integrity, function, and activity.

Briefly, yeast stocks are initiated in growth media, protein expression is induced with galactose, and cell lysates prepared. The proteins are purified using glutathione affinity chromatography, eluted, and purified proteins are used to print the proteome microarray.

Printing the Yeast ProtoArray™

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

Controls

Various proteins and controls are printed on each ProtoArray® Yeast Proteome Microarray mg to allow you to verify background and detection conditions during probing. For details, see page 12.

Maintaining Stringent Quality Control

The ProtoArray® Yeast Proteome Microarrays are produced using the same rigorous production and pre-printing and post-printing quality control procedures used to produce the human protein microarrays (see page 8). For detailed product qualification, see page 52.

ProtoArray® Control Protein Microarray

Introduction

The ProtoArray® Control Protein Microarray mg contains kinase substrates and various controls printed on a modified glass (mg) slide. The Control Protein Microarray allows you to validate probing procedures prior to probing the ProtoArray® Human Protein or Yeast Proteome Microarray.

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

Note: The ProtoArray® Human and Yeast Microarray KSI Complete Kits include 2 ProtoArray® Control Protein Microarrays mg in each complete kit.

Control Microarray Specifications

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

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

The modified glass array is a thin nitrocellulose coated slide from GenTel® BioSciences, Inc., and is specifically selected for superior performance of the array in the KSI application. Thin-film nitrocellulose slides are manufactured by GenTel® BioSciences, Inc. using a proprietary surface chemistry owned by Decision Biomarkers, Inc.

Each microarray has a barcode for tracking samples. The barcode is also used to retrieve array specific information from the ProtoArray® Central portal (see page 35).

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 mg, go to the ProtoArray® Central portal at www.invitrogen.com/protoarray.

Total Subarrays:	48 (4 columns x 12 rows)
Subarray Size:	4400 µm x 4400 µm
Subarray Dimensions:	**16 rows x 16 columns**
Median Spot Diameter:	~150 µm
Spot Center to Center Spacing:	275 µm
Distance Between Subarrays:	100 µm
Replicates per Sample:	2

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ProtoArray® Control Protein Microarray, Continued

Controls Printed on Each ProtoArray® Microarray

Various proteins and other controls are printed on each ProtoArray® Human Protein, Yeast Proteome, 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	
Fiduciary Kinases	Kinases autophosphorylate and produce fiduciary (marker) 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	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.
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)	A positive control for protein-protein interaction application and interacts with the Array Control 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.

Continued on next page

ProtoArray® Control Protein Microarray, Continued

Maintaining Stringent Quality Control

The ProtoArray® Control Protein Microarrays are produced using the same rigorous production, and pre-printing and post-printing quality control procedures used to produce the ProtoArray® Human Protein and Yeast Proteome Microarrays (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 Fiduciary Kinases. For detailed product qualification, see page 52.

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.

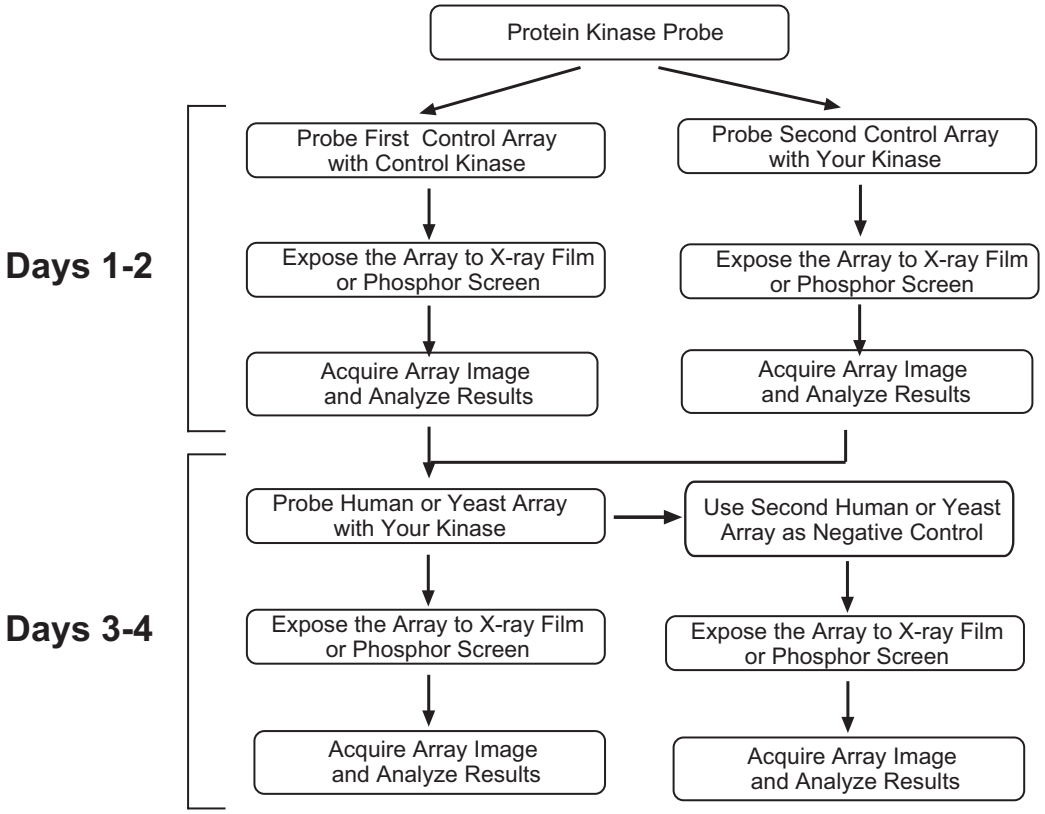
Important

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 17 and 28.



Methods

Preparing the Protein Kinase

Introduction

Before using the ProtoArray® Human Protein Microarray mg 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 51).

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 21).
- Make sure the protein kinase is soluble and active in buffers used for probing the microarray (see recipe on page 21).
- You need at least 120 µl of your purified protein kinase at a recommended initial protein concentration of **50 nM** to probe each ProtoArray® Microarray.

Probing the ProtoArray® Control Protein Microarray

Introduction

The ProtoArray® Control Protein Microarray mg allows you to verify probing conditions. Probe the Control Protein Array **prior to** probing the ProtoArray® Human Protein or Yeast Proteome Microarrays.

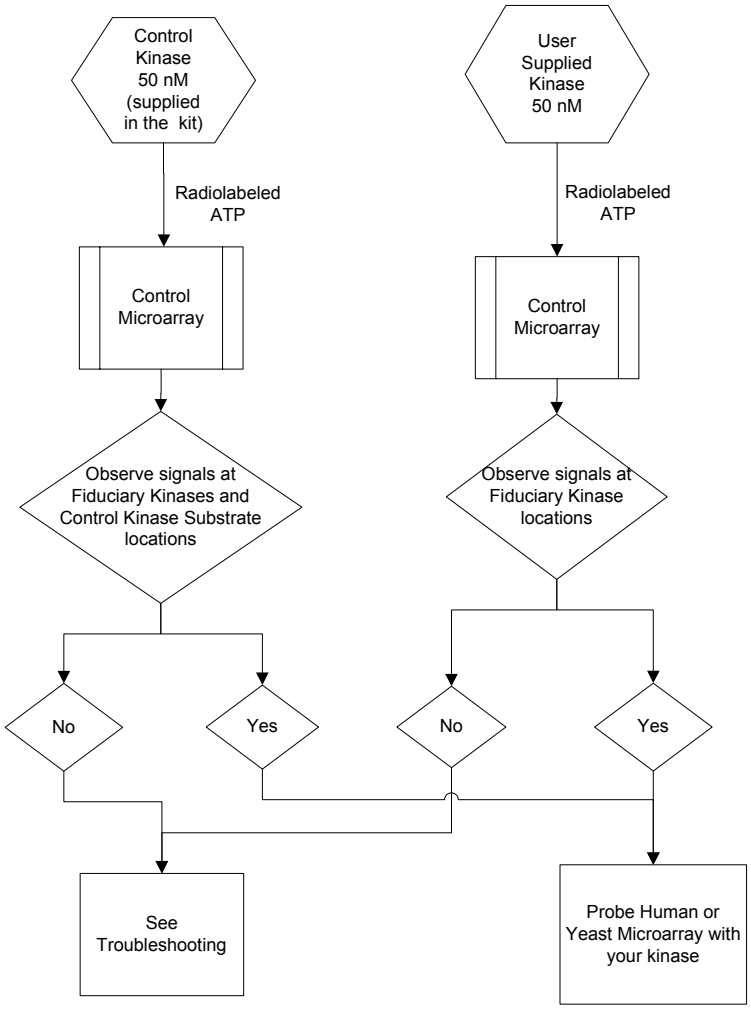
Instructions are provided in this section to probe the ProtoArray® Control Protein Microarray mg with the Control Kinase supplied with the kit.

ProtoArray® KSI Buffer Modules

The ProtoArray® KSI Buffer Modules A and B supplied with the complete kits 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.



Continued on next page

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 Kits 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 50 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 18-24 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 Fiduciary Kinases spotted on subarrays on each Control Microarray mg 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 Control Microarray mg 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 Fiduciary 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 performs as expected on the Control Microarrays, probe the Human or Yeast Protein Microarray with your kinase (page 27).

Continued on next page

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 to avoid any damage to the array surface resulting 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 and immerse the array immediately in blocking solution equilibrated at 4°C
- **Do not** use [γ-^{32}P]ATP for the assay, use [γ-^{33}P]ATP as the use of [γ-^{33}P]ATP supports increased signal resolution during data acquisition while [γ-^{32}P]ATP can be used for the assay but data quantitation with [γ-^{32}P]ATP is not supported
- Perform array experiments at a clean location to avoid dust or contamination and 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 and 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 or a sterile petri dish.
- The Incubation Chamber included with the ProtoArray® PPI Kits or other hybridization chambers may not be suitable for use, as you will need a container that seals tightly to prevent any leakage of radioactive material during the washing steps.
- You need cover slips that are able to completely cover the printed area (20 mm x 60 mm) of the glass slide and hold a small reagent volume to minimize the amount of valuable probe used and prevent evaporation of reagents. We recommend using glass cover slips (VWR catalog no. 48404-454).
- **Do not** use any 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 the 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 mg (included in complete kits only)
- ProtoArray® KSI Buffer Module A and B (included in the kit)
- Control Kinase in Kinase Buffer (included in the kits; page 22)
- Protein Kinase supplied by the user in Kinase Buffer (page 22)
- Incubator set to 30°C
- Sterile 50 ml conical tubes
- Ice bucket
- Shaker
- Deionized or ultra pure water
- Cover slips (VWR catalog no. 48404-454)
- X-ray film or phosphor screen (provide at least 50 µm resolution) and instrumentation to acquire the image (provide 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 mg. A significant signal for the Control Kinase substrate indicates that the assay was performed correctly.

Continued on next page

Preparing Buffers

Prepare the following buffers **fresh** using the reagents supplied in the kit. The recipe below provides sufficient buffer to probe two microarrays.

Blocking Buffer

1X PBS
1% BSA

1. 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 500 µl Kinase Buffer supplied with the kit, add 0.5 µl 1 M DTT supplied with the kit. 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 200 ml 0.5% SDS for washing two microarrays.

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

You need to calculate the molar concentration of your protein kinase as follows:

Use the protein concentration and molecular weight of your protein kinase for the calculation using the formula listed below.

Formula

Protein Concentration (μM) = [Protein concentration in mg/ml] x [1/(protein molecular weight in grams x 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] x [1/(50,000 x 10^{-6})]

μM = 10

Preparing the Kinase

You need 120 μl Kinase Buffer with 1 mM DTT containing the kinase to probe **one** Control 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	50 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 cover slips.
- Make sure the kinase in Kinase Buffer and Kinase Buffer are cold and stored on ice until use. Place 50-ml conical tubes on ice to chill the tube until 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 19 and **Working with Radioactive Material** on page 14, prior to starting the probing procedure.

Continued on next page

Probing the ProtoArray® Control Protein Microarray, Continued

Blocking Step

Instructions for blocking the Control Microarrays are described below:

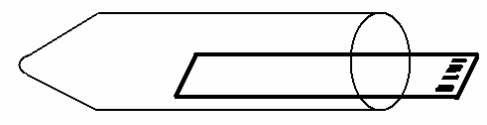
1. Remove two mailers containing ProtoArray® Control Protein Microarrays mg from storage at -20°C and immediately place the mailers at 4°C (be sure to use the microarray **before** the expiration date printed on the box).
2. Allow the mailer containing the array to equilibrate at 4°C for at least 15 minutes prior to performing the blocking step.
3. Perform blocking in the mailer. Ensure the microarray is placed in the mailer with the printed (barcode) side facing up. You can block 2 arrays simultaneously in the mailer using 30 ml Blocking Buffer. Add 30 ml Blocking Buffer (page 21) to the mailer containing the ProtoArray® Control Protein Microarray mg. Incubate for 2-3 hours at 4°C with gentle shaking (~50 rpm).
4. Decant the Blocking Buffer. Drain excess buffer by inverting the mailer on paper towels for a few seconds. Remove the array from the mailer. Tap one edge of the array gently on a laboratory wipe for a few seconds to drain any buffer **without allowing the array to dry**.
5. Proceed immediately to **Probing the Array**, next page.

Continued on next page

Probing the ProtoArray® Control Protein Microarray, Continued

Probing Control Arrays

1. Place each Control Microarray horizontally in a separate sterile 50-ml conical tube with the printed side (barcode) of the array facing up and about 1/3 of the array (barcode side) protruding to the outside of the conical tube as shown in the figure below. You may also use a petri dish.



2. To 120 µl kinase mixture prepared on page 22, add 1 µl [γ-^{33}P]ATP (3000Ci/mmol, 10 µCi/µl) to obtain a final radiolabel ATP concentration of 33 nM for each 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 more than 2 minutes prior to use on the array.

3. Pipette Kinase Buffer with the radiolabel and kinase on top of the array without touching the array surface.
   - First Control Microarray: add 120 µl Kinase Buffer containing 50 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. Carefully lift the cover slip from the package with forceps and lay the cover slip on the array to cover the array without trapping any air-bubbles. Align the cover slip flush with the top edge of the array to ensure the printed area of the array is completely covered. Gently adjust the cover slip to remove any air bubbles.
5. Gently slide each array with a cover slip into the conical tube with the printed side (barcode) of the array facing up. Cap the conical tube. If using the petri dish, place the array with cover slip on the dish with the printed side (barcode) of the array facing up and cover the dish.
6. Place each conical tube horizontally or the petri dish 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 or dish is as level as possible. If needed, you can tape the conical tube on the flat surface to avoid any accidental disturbances.
7. Incubate the array for 1 hour at 30°C **without shaking**. Remove tubes or dishes from the incubator. If you used a petri dish, transfer the arrays into a 50 ml conical tube.
8. Using a sterile pipette, add 40 ml 0.5% SDS (page 21) to 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 cover slip. Do not remove the cover slip with forceps if the cover slip is not dislodged from the array.
10. Using forceps, carefully remove the dislodged cover slip without touching the array surface. Discard the cover slip appropriately as radioactive waste.

Continued on next page

Probing the ProtoArray® Control Protein Microarray, Continued

Probing Control Arrays, Continued

Protocol continued from the previous page.

11. Cap the conical tubes and incubate arrays in 0.5% SDS for 15 minutes at room temperature.

    Note: Perform all washing steps with SDS and water without shaking to prevent any spill of radioactive waste.

12. Decant the 0.5% SDS. Be sure to dispose radioactive waste properly.
13. Slowly add 40 ml 0.5% SDS to the tubes, cap the tubes, and incubate for 15 minutes at room temperature.
14. Decant the 0.5% SDS. Be sure to dispose radioactive waste properly.
15. Add 40 ml ultrapure water to the tubes, cap the tubes, and incubate the arrays for 15 minutes at room temperature.
16. Decant the water. Be sure to dispose radioactive waste properly.
17. Add 40 ml ultrapure water to the tubes, cap the tubes, and incubate the arrays for 15 minutes at room temperature.
18. Decant the water. Be sure to dispose radioactive waste properly.
19. Proceed to **Drying Arrays**, below.

Drying Arrays

1. Remove the arrays from the tubes at the end of the probing procedure. Tap one edge of the array gently on a laboratory wipe for a few seconds to drain any buffer.
2. Place each array in a slide holder (or a sterile 50 ml conical tube, if you do not have a slide holder) in a vertical orientation. Ensure the array is properly placed and is secure in the holder to prevent any damage to the array during centrifugation.
3. Centrifuge the array in the slide holder or 50 ml conical tube at 800 x g for 3-5 minutes in a centrifuge (equipped with a plate rotor, if you are using the slide holder) at room temperature. Make sure that the array is completely dry; there should be no translucent areas.
4. Place the array in an X-ray film cassette. Cover the array with a clear plastic wrap. You can check the radioactivity on the array using a Geiger counter.
5. 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.
6. Expose the arrays for 18-24 hours.
7. Perform image analysis and data analysis as described 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 below. For details, see page 34.

1. Acquire an image (.tiff) from the X-ray film or phosphorscreen (page 31).
2. Use the barcode on the array to download the .GAL file from ProtoArray® Central as described on page 35.
3. Use the .GAL file and ProtoArray® Prospector (page 34) 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 Human Microarray are described on page 40. For troubleshooting, see page 44.

Probing the ProtoArray® Human or Yeast Microarrays

Introduction

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

Important

Each ProtoArray® Human Protein or Yeast Proteome 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 19 to obtain the best results with the arrays.

Materials Needed

You need the following items:

- ProtoArray® Human Protein Microarray mg **or** ProtoArray® Yeast Proteome Microarray mg, as appropriate (included in the complete kit)

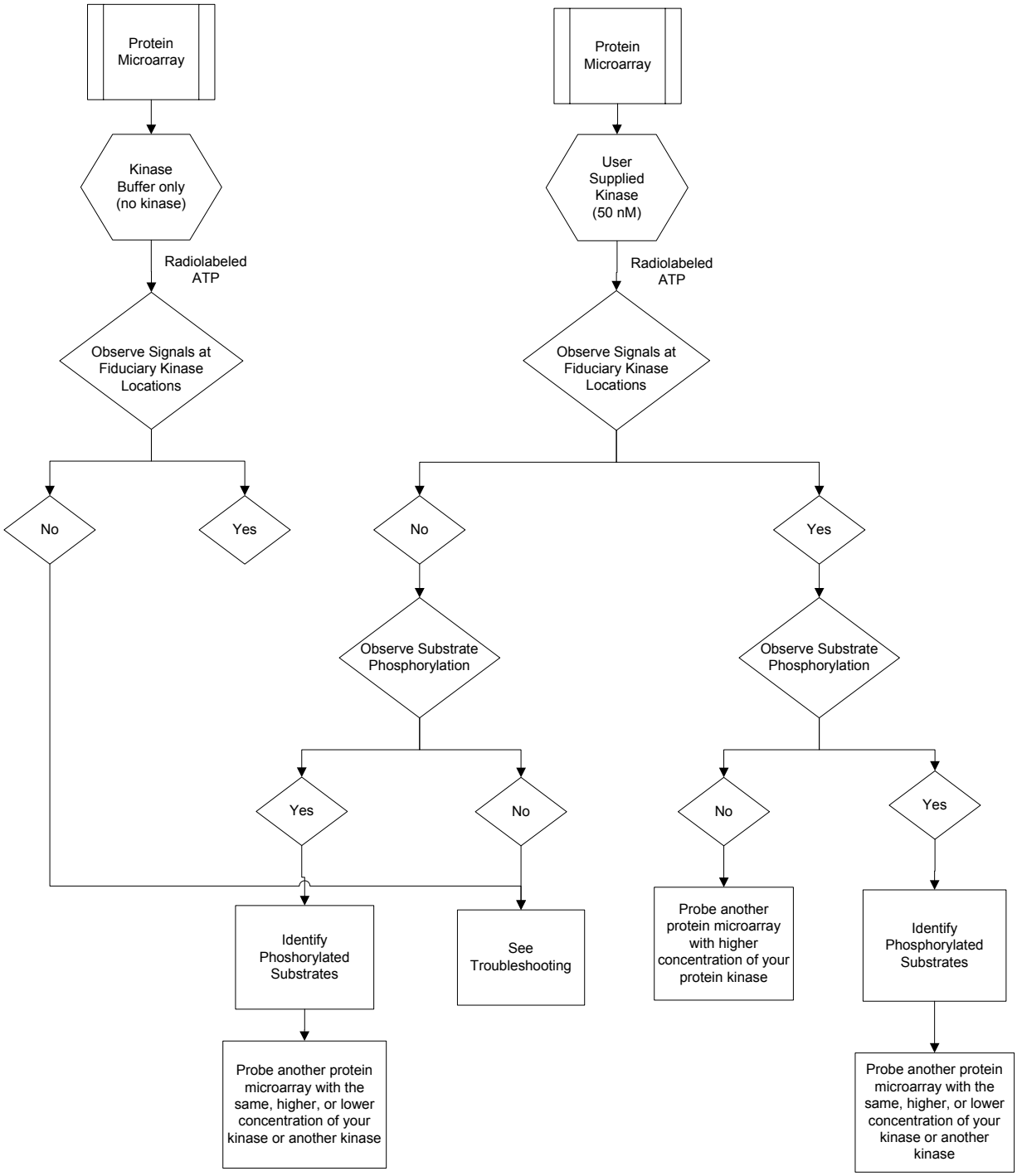
  Note: If you have purchased the ProtoArray® Kinase Substrate Identification Application Kit, you also need to purchase the ProtoArray® Human Protein or Yeast Proteome Microarray separately.
- [γ-^{33}P]ATP (3000 Ci/mmol, 10 μCi/μl)
- ProtoArray® KSI Buffer Module A and B (included in the kit)
- Protein Kinase supplied by the user in Kinase Buffer (see next page)
- Incubator set to 30°C
- Sterile 50 ml conical tubes
- Shaker
- Ice bucket
- Deionized or ultra pure water
- Cover slips (VWR catalog no. 48404-454)
- X-ray film or phosphor screen (provide at least 50 μm resolution) and instrumentation to acquire the image (provide 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

Probing the ProtoArray® Human or Yeast Microarrays,
Continued

Human or Yeast Array Experimental Workflow

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



Continued on next page

Probing the ProtoArray® Human or Yeast Microarrays,
Continued

Recommended Workflow

The ProtoArray® Human Protein or Yeast Proteome Microarray KSI Complete Kit contains 2 human or yeast arrays, respectively, and the arrays are probed using the workflow as described on the previous page and below.

The recommended protein kinase concentration for probing each array is 50 nM.

1. Simultaneously probe **two** ProtoArray® Human or Yeast Microarrays included in the complete kits as follows:
   - Probe the first array using your kinase (supplied by the user) at 50 nM in the presence of radiolabeled [γ-^{33}P]ATP to identify potential substrates
   - Probe the second array using only buffer and 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 18-24 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).

Preparing Buffers

Prepare Blocking Buffer, Kinase Buffer, and 0.5% SDS as described on page 21.

Preparing Your Kinase

You need 120 µl Kinase Buffer with 1 mM DTT containing your kinase of interest to probe **one** Human or Yeast Microarray mg.

To calculate the molar concentration of your kinase, use the protein concentration and molecular weight of your protein kinase for the calculation using the formula listed on page 22.

To the Kinase Buffer with 1 mM DTT, add your kinase to obtain a final concentration of 50 nM as follows:

Component	Amount
Kinase	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

Probing the ProtoArray® Human or Yeast Microarrays,
Continued

- To perform the washing and probing steps, we recommend using a sterile 50-ml conical tube or a sterile petri dish.
- The Incubation Chamber included with the ProtoArray® PPI Kits or other hybridization chambers may not be suitable for use, as you will need a container that seals tightly to prevent any leakage of radioactive material during the washing steps.
- **Do not** use any 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 protein kinase sample to 120 µl of Kinase Buffer. Addition of more than 10% of the kinase to the Kinase Buffer can decrease the 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 cover slips.
- Make sure the kinase in Kinase Buffer and Kinase Buffer are cold and stored on ice until use. Place a 50-ml conical tube on ice to chill the tube until 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 19 and **Working with Radioactive Material** on page 14, prior to starting the probing procedure.

Probing Arrays

See page 28 for the recommended workflow.

1. Simultaneously probe two ProtoArray® Human Protein or Yeast Proteome Microarrays using the procedure described on page 24 as follows:
   - Probe the first array using your kinase (supplied by the user) at **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
2. Dry the array as described on page 25.
3. Perform image analysis on the arrays as described on the next page and analyze results (page 34).

Examples of expected results obtained after probing the ProtoArray® Human Protein or Yeast Proteome Microarrays are shown on pages 41 and 42, respectively.

If you obtain weak signals or high background, see **Troubleshooting**, page 44.

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 desktop film scanner that provides at least 50 µm resolution (>600 dpi) to scan the X-ray film after developing the film 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 4.0 or higher (page 35). 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 standard 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 in the dark for use later if a longer exposure time is needed.
2. Develop the X-ray film.
3. Scan the X-ray film using a standard scanner or scan the phosphor screen using a phosphorimager to obtain a 16-bit TIFF file. Include the barcode in the area for maintaining a record and scan the array to provide a high-resolution image (at least 50 µm).
4. Save the image 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 (recommended) 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 4.0 (includes Imager and Analyzer) is available from Invitrogen at www.invitrogen.com/protoarray, and then click on the Online Tools tab. The ProtoArray® Prospector Imager allows image processing for data analysis. Install the **Complete** version of ProtoArray® Prospector installation package 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 x 1800 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 Fiduciary 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, which 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 300 dpi, set the fixed rectangular area to 300 x 900 pixels to cover the 1" x 3" array area.
4. Save the cropped and resized image (.tiff) file with a new name to a suitable location. Be sure the barcode is included in the name of the image.
5. Download lot-specific information from ProtoArray® Central, see next page.

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

Continued on next page

Scanning and Image Analysis, Continued

Image Processing Using Adobe® Photoshop®

1. Start Adobe® Photoshop® on the computer.
2. Open the microarray image (.tiff) acquired in Step 4, page 31.
3. Perform the following adjustments to the image:
   - Crop a fixed rectangular area (1″ x 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 2550 x 7650 pixels (constrained proportions).

     Important: Do not adjust the image quality (such as contrast or level) which 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 is included in the name of the image.
5. Download lot-specific information from ProtoArray® Central, see next page.

Data Acquisition and Analysis

Introduction

After scanning and saving an image of the array, download the protein array lot specific information (mainly the .GAL file) from ProtoArray® Central Portal. You use the lot specific information 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 Online Tools.

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, Yeast, 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 4.0 or higher (page 35). Microarray data acquisition software such as GenePix® Pro (Molecular Devices Corporation) or ScanArray® Software (PerkinElmer, Inc.) are also suitable for data acquisition.

Note

If you do not have access to any microarray data acquisition software, contact Technical Support (page 51).

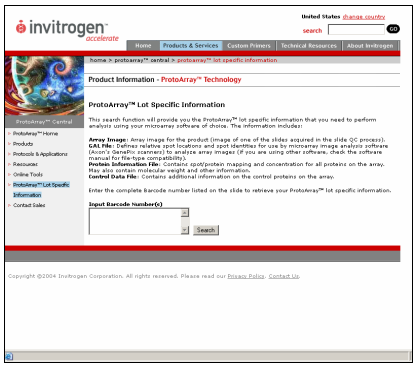
Continued on next page

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, then click on the link below to 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 Online Tools tab.
2. Click on the link to ProtoArray® Lot Specific Information.
3. Enter the array barcode in the Input Barcode Number(s) box. Click on the Search button.



Continued on next page

ProtoArray® Central, continued

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 the human proteins on the microarray.
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: the *(LotNumber_control.txt)*	This file contains a description of control spots on array.
Protein Application File: (LotNumber_application.PAI)	ProtoArray® Prospector uses the Protein Application Files for data analysis. Different PAI files are designed for different applications. For example, ProtoArray® Prospector uses the file HA10756 KSI.PAI to analyze data from KSI experiments performed on array from lot HA10756.
Slide Information File: (LotNumber_slide.txt)	This file contains a listing of all barcodes associated with a specific lot of arrays.

5. Download the files listed above for human or yeast array-specific information from a specific lot. Use these files to interpret your results with the ProtoArray® Human or Yeast Microarray.

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

6. Start the ProtoArray® Prospector Imager, GenePix® Pro Software, or equivalent microarray data acquisition software on the computer.

7. Open the saved image (16-bit TIFF file) from Step 4, page 32.

   Note: If the image is not saved as a 16-bit TIFF file, GenePix® Pro software is unable to open the file (image).

Continued on next page

ProtoArray® Central, continued

8. 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 Fiduciary Kinase 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 tab. Install the **Complete** version of ProtoArray® Prospector installation package 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 3, **Expected Results**, to help with data interpretation.

Based on the Z-score and available protein sequence information, we recommend validating the identified potential kinase substrates by *in vitro* solution assay or ProtoArray® Technology as described on the next page.

Continued on next page

The Next Step

After identifying potential kinase substrates on the Human or Yeast 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 or yeast array using a similar or a different kinase concentration to address reproducibility.
- **Specificity:** Probe a human or yeast 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 48.

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 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.

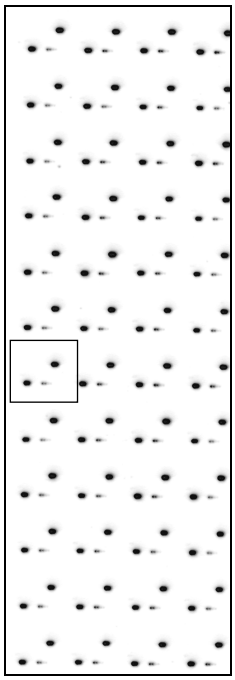
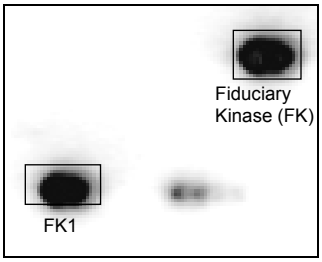
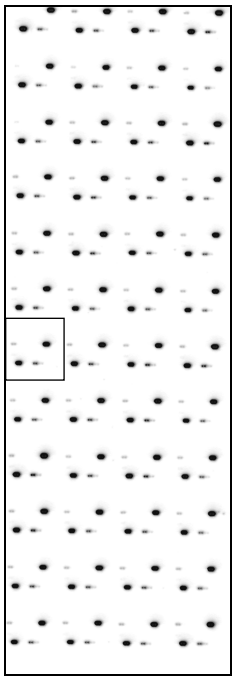
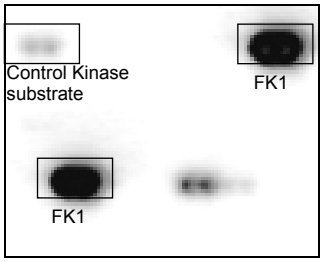
Visit www.invitrogen.com/clones to access our clone collections. Each Ultimate™ ORF Clone is full insert-sequenced and guaranteed to match the corresponding GenBank® amino acid sequence. Contact Technical Service (page 51) to order the purified protein kinases printed on the array.

Note: The yeast proteins printed on the array are derived from the Snyder collection (Zhu *et al.*, 2001). For information on obtaining the yeast clone corresponding to the potential protein substrate identified on the array, contact Technical Support (page 51).

Expected Results

Control Array Probing Results

Results obtained after probing the ProtoArray® Control Protein Microarray mg v4.0 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 50 nM Control Kinase	
Control Array Image	**Boxed area shown in detail**	**Control Array Image**	**Boxed area shown in detail**
			

- Fiduciary Kinase Signal

  Fiduciary Kinase on the arrays are autophosphorylated during the labeling reaction. The signals at Fiduciary 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 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.

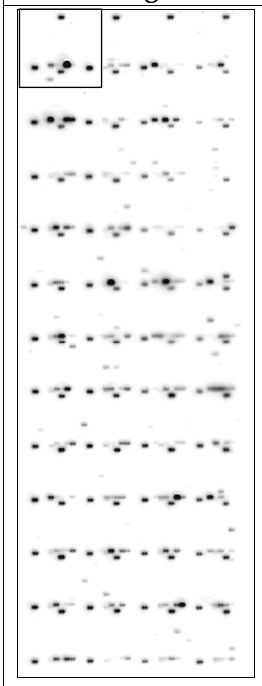
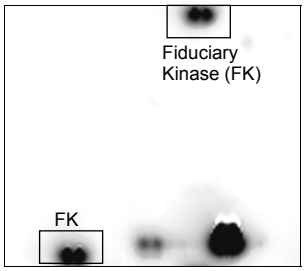
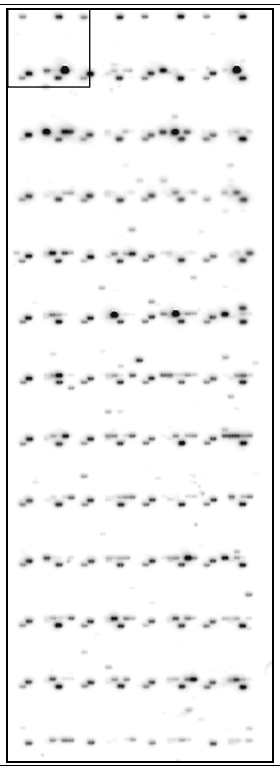
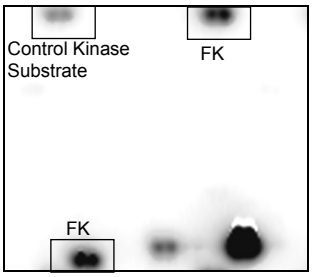
Continued on next page

Expected Results, Continued

Human ProtoArray® v4.0 Probing Results

The results obtained after probing the ProtoArray® Human Protein Microarray mg v4.0 with 50 nM Control Kinase is shown below. The Control Kinase phosphorylates the Control Kinase substrate printed on the array.

A negative control image of the Human Microarray mg v4.0 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**
			

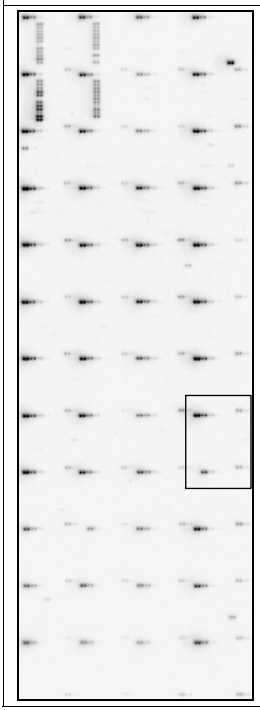
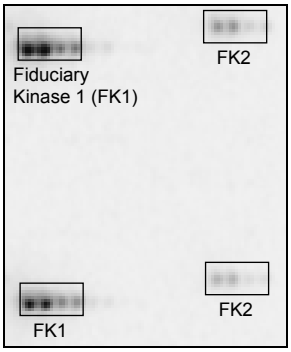
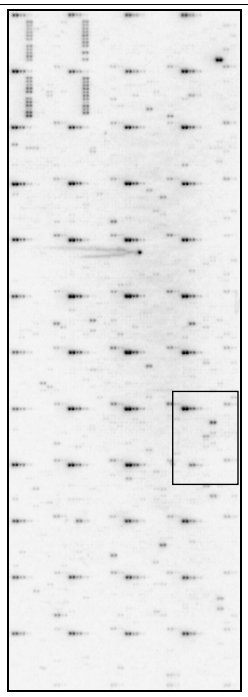
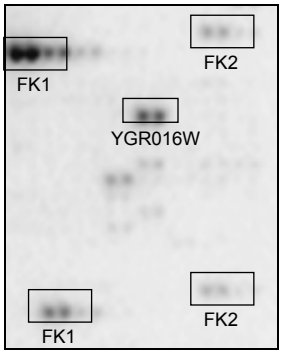
Continued on next page

Expected Results, Continued

Yeast ProtoArray® Probing Results

The results obtained after probing the ProtoArray® Yeast Proteome Microarray mg v1.0 with 10 nM Casein Kinase 2 alpha (CK2a) is shown below. The Casein Kinase 2 alpha phosphorylates a known casein kinase substrate, YGR016W.

A negative control image of the Yeast Proteome is also shown below.

Image of the Yeast Microarray when probed with labeled ATP only (negative Control)		Image of the Yeast Microarray when probed with 10 nM CK2a Kinase	
Yeast Array Image	**Boxed area shown in detail**	**Yeast Array Image**	**Boxed area shown in detail**
			

Note: The column of signal observed on the top left corner for the array is due to the autophosphorylation of yeast calmodulin kinase printed as a control spot on the ProtoArray® Yeast Proteome Microarray mg.

Continued on next page

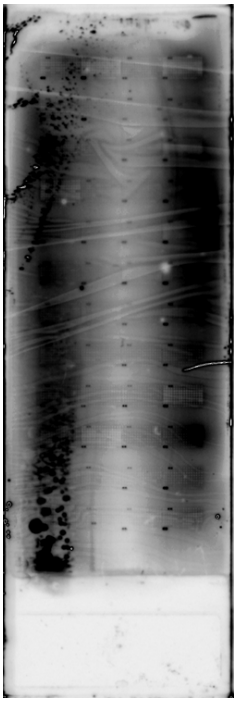
Example Showing High Background and Low Signal

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 Fiduciary Kinases on the array. The low signals may also be due to loss of activity of the Fiduciary Kinases when expired arrays are used.

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 or Yeast Proteome Microarray KSI Complete Kits.

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.
	Fiduciary 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 mg v4.0 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 21.
	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 cover slip 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 25 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 21.
	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 cover slip to the array as described in the manual. To avoid drying of the array, make sure the cover slip covers the printed area of the array and adjust the cover slip, 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

Troubleshooting, Continued

Problem	Cause	Solution
Human Protein or Yeast Proteome Array Results		
Weak or no signal with your protein kinase	Kinase of interest is not active or is inactivated by 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 or Yeast Proteome Array Results, continued		
High background	Improper blocking	Prepare the Blocking Buffer **fresh** as described on page 21.
	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 cover slip 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 25 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 using 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 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 after 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 a recipe
- [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 large variety of NuPAGE® Novex® Bis-Tris Pre-cast mini gels are available from Invitrogen for SDS-PAGE analysis of proteins. You may use any other 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 14 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 more 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 onto a well of the NuPAGE® Novex® Bis-Tris Gel (use an appropriate percentage of acrylamide gel 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 twice in 100 ml deionized water for 15 minutes each time.
6. Decant the water and blot of excess water from the gel using blotting paper.
7. Cover the gel with clear plastic wrap. You can check the radioactivity on the array 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:

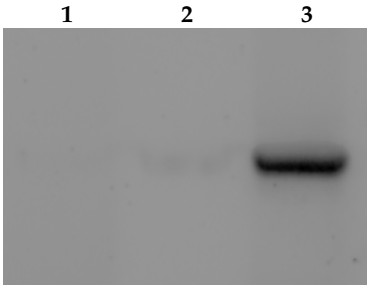
- Signal observed in the lane containing the kinase and kinase substrate at the position of the kinase substrate band
- None or very low signal observed in the kinase alone or substrate alone lanes at the position of the kinase substrate

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 Web site at www.invitrogen.com for:

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MSDS

MSDSs (Material Safety Data Sheets) are available on our website at www.invitrogen.com/msds.

Product Qualification

Introduction

The components supplied in the ProtoArray® Human Protein and Yeast Proteome Microarray KSI Complete Kits are qualified as described below.

ProtoArray® Human, Yeast, and Control Microarrays

The ProtoArray® Human Protein, Yeast Proteome, and Control Microarrays are visually examined for obvious defects.

The ProtoArray® Human Protein Microarray, Yeast Proteome Microarray, and Control Protein Microarray are probed with radiolabeled ATP only or the Control Kinase in the presence of radiolabeled ATP as described in this manual.

After image acquisition and data analysis, the following results must be observed:

- Fiduciary Kinase signals are observed at the expected locations
- Signal observed at the Control Kinase substrate location
- GST spots do not produce any significant signals

ProtoArray® KSI Buffers Module

Buffers

The Kinase Buffer, 10% SDS, 10X PBS, 1 M DTT are tested at 1X concentration for adherence to pH, refractive index, and/or conductivity specifications.

Control Kinase

The protein concentration and specific activity of the Control Kinase must be within the specified range.

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