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

KSI Experienced Users Guide

This quick reference contains brief instructions for using the ProtoArray® Human Protein Microarray v5.0 for the kinase substrate identification (KSI) application, to identify potential substrates of a protein kinase of interest in the presence of radiolabeled ATP. It is intended for experienced users of ProtoArray® Microarrays.

If you are a first time user, refer to the **ProtoArray® Applications Guide** available at www.invitrogen.com, for detailed instructions for performing the KSI application, microarray specifications, ProtoArray® technology overview, troubleshooting, and license information.

Experimental Overview

1. Block the ProtoArray® Human Protein Microarray with KSI Blocking Buffer.
2. Probe the array with protein kinase probe and [γ^{33}P]ATP, or [γ^{33}P]ATP alone.
3. Dry the array for imaging.
4. Expose the array to a phosphorimager screen and scan the screen to obtain an array image.
5. Download the protein array lot specific information from ProtoArray® Central portal and acquire the image data using microarray data acquisition software.
6. Analyze results using ProtoArray® Prospector data analysis software available from www.invitrogen.com/protoarray.

Important Guidelines

To obtain the best results with the ProtoArray® Human Protein Microarray, follow these guidelines:

- The ProtoArray® Microarray can only be used once. **Do not re-use or re-probe** the array.
- We recommend probing one array with [γ^{33}P]ATP alone as a negative control for every assay.
- Always wear clean gloves while handling microarrays.
- **Do not** use [γ^{32}P]ATP in place of [γ^{33}P]ATP, as data quantitation with [γ^{32}P]ATP is not supported.
- **Do not** touch the surface of the array. Damage to the array surface can result in uneven or high background.
- Maintain the array and reagents at 2–8°C during the experiment.
- Avoid drying of the array during the experiment. Ensure the array is completely covered with the appropriate reagent during all steps of the protocol.
- Perform array experiments at a clean location to avoid dust or contamination. Filter solutions as needed (particles invisible to the eye can produce high background signals and cause irregular spot morphology).
- Dry the array by centrifugation prior to scanning. **Do not** dry the array using compressed air or commercial aerosol sprays. Scan the array immediately upon completion of the experiment.

Working with Radioactive Materials

Follow these general guidelines when working with radioactive material. Refer to the ProtoArray® Applications Guide for additional details.

- Do not work with radioactive materials until you have been properly trained.
- Follow all the radiation safety rules and guidelines mandated by your institution.
- Wear protective clothing (laboratory coat, disposable gloves, and eyewear), and use a radiation monitor.
- Work in areas designated for radiation use, and monitor continuously for radioactive contamination.
- Dispose of radioactive waste properly. This includes reagents discarded during the probing procedure (*e.g.* washes).

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Materials Needed

- Purified protein kinase of interest (see previous page for requirements), store on ice until use
- ProtoArray® Human Protein Microarray v5.0 and buffers (see recipes below)
- [γ^{33}P]ATP (3,000 Ci/mmol, 10 µCi/µl)
- 0.45 µm filters (Millipore, Cat. no. SLHVR25LS)
- Clean, covered 4-chamber incubation tray (Greiner, Cat. no. 96077307 or ISC Bioexpress, Cat. no. T-2896-1) , chilled on ice
- Forceps, and deionized water
- 50 ml conical tubes
- Shaker (capable of circular shaking at 50 rpm, place the shaker at 4ºC)
- Incubator set to 30°C
- 60 × 24 mm glass coverslips (VWR, Cat. no. 48404-454)
- X-ray film cassette and clear plastic wrap
- Microarray slide holder and centrifuge equipped with a plate holder (*Optional*)
- Cyclone Plus Phosphor Imager (Perkin-Elmer, Cat. no. C431200); Multisensitive Phosphor Screen (Perkin-Elmer, Cat. no. 7001723)
- Microarray data acquisition software (*e.g.* GenePix® Pro from Molecular Devices; refer to the ProtoArray® Applications Guide for details and optional software packages)
- Data analysis software (ProtoArray® Prospector available at www.invitrogen.com/protoarray, recommended)

Preparing Buffers

Prepare the following buffers **fresh**. Mix all buffers well, sterile filter, and store on ice until ready for use.

Buffer	Composition	Preparation
KSI Blocking Buffer 5 ml of buffer is needed for each microarray.	1X PBS, pH 7.4 1% BSA	1. Prepare 100 ml KSI Blocking Buffer **fresh** as follows: 10X PBS, pH 7.4 — 10 ml 30% ***protease free*** BSA — 3.3 ml Deionized water — to 100 ml 2. Mix well and store on ice until use.
Kinase Buffer 120 µl of buffer is needed for each microarray.	1% NP-40 100 mM MOPS, pH 7.2 100 mM NaCl 1% BSA 5 mM $MgCl_2$ 5 mM $MnCl_2$ 1 mM DTT	1. Prepare 1 ml Kinase Buffer **fresh** as follows: 10% NP-40 — 100 µl 1 M MOPS, pH 7.2 — 100 µl 5 M NaCl — 20 µl 30% ***protease free*** BSA — 33 µl 1 M $MgCl_2$ — 5 µl 1 M $MnCl_2$ — 5 µl 1 M DTT — 1 µl Deionized water — to 1 ml 2. Filter buffer with 0.45 µm filter. Store at -20°C until use.
0.5% SDS 80 ml of buffer is needed for each microarray.	0.5% SDS	1. Prepare 200 ml 0.5% SDS **fresh** as follows: 10% SDS — 10 ml Deionized water — to 200 ml 2. Mix well and store at room temperature until use.

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Protein Kinase Requirements

The recommended protein concentration range for probing an array is 50 nM. However, you may need to vary the concentration of the protein from 1–100 nM depending upon the activity of kinase and level of kinase autophosphorylation. Too much kinase may result in high background or a dark array. Too little kinase will result in no additional spots relative to a kinase-free control. Kinases are diluted in Kinase Buffer (see recipe on page 2) to a final volume of 120 µl. If purifying your own protein of interest, observe the following guidelines:

- **Purify the protein kinase under native conditions.**
- Proteins should be > 90% pure as determined by Coomassie® staining.
- Perform an activity assay of the protein kinase after purification using a method of choice.
- Make sure the protein kinase is soluble and active in buffers used for probing the microarray (see below).

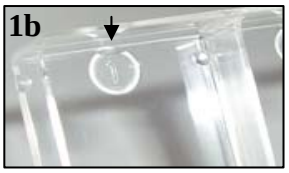
Preparing the Kinase

You need 120 µl Kinase Buffer containing your kinase of interest to probe **one** ProtoArray® Human Protein Microarray v5.0. At Step 2 of the **Probing the Microarray** 33 nM [γ^{33}P]ATP will be added to the diluted kinase.

1. Prepare a 120 µl dilution of your kinase of interest at a final concentration of 50 nM in Kinase Buffer.
2. Mix well (**do not** vortex) and store on ice until use. Immediately return the remaining kinase to –80°C.

Blocking the Microarray

1. Remove the mailer containing the ProtoArray® Human Protein Microarray v5.0 from storage and place immediately at 4°C. Equilibrate the mailer at 4°C for at least 15 minutes prior to blocking. Not doing so may result in condensation on the array which can reduce protein activity or alter spot morphology.
2. Place one microarray with the barcode facing up into each well of a chilled 4-chamber incubation tray (see previous page) such that the barcoded end of the microarray is near the end of the tray marked with an indented numeral (see figure 1a). The indentation in the tray bottom is used as the site for buffer removal (see figure 1b, arrow).
3. Using a sterile pipette, add 5 ml KSI Blocking Buffer equilibrated to 4°C into each chamber with an array. **Avoid pipetting buffer directly onto the array surface.**
4. Incubate the tray for 1 hour at 4°C on a shaker set at 50 rpm (circular shaking).
5. After incubation, remove array from 4-chamber incubation tray using forceps. Insert the tip of the forceps into the indentation at the numbered end of the tray and gently pry the array upward (see figure 2). Using a gloved hand, pick up the microarray by holding the array by its **edges** only. Tap to remove excess liquid from array surface.
6. Proceed immediately to the **Probing the Microarray** (next page).



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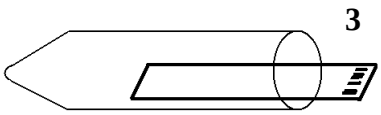
Probing the Microarray



1. Place the ProtoArray® Human Protein Microarray in a 50 ml conical tube with one-third of the slide extending outside of the tube (see figure 3). The barcode should be outside the tube, face up.
2. If probing two microarrays as recommended in the **Guidelines**:
   - Add 1 µl of [^{33}P]ATP (3000 Ci/mmol, 10 µCi/µl) to 120 µl of Kinase Buffer containing diluted kinase (see **Preparing the Kinase**) to obtain a final [γ^{33}P]ATP concentration of 33 nM for one microarray.
   - Add 1 µl of [γ^{33}P]ATP (3000 Ci/mmol, 10 µCi/µl) to 120 µl of Kinase Buffer (see recipe) **without kinase**

     Note: 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.
3. Pipet mixture gently onto the surface of the ProtoArray® Human Protein Microarray within the conical tube without touching the array surface as follows:
   - First microarray: apply 120 µl Kinase Buffer containing **your kinase of interest** and 33 nM [γ^{33}P]ATP.
   - Second microarray (negative control): apply 120 µl Kinase Buffer with 33 nM [γ-^{33}P]ATP and **no kinase.**
4. Using forceps, carefully lay a coverslip on the surface of the microarray without trapping any air bubbles. Align the coverslip to ensure the printed area of the array is completely covered.
5. Position the coverslipped array so that it is inside the conical tube with the printed side (barcode) facing up, and cap the tube.
6. Place the conical tube horizontally on a flat surface in an incubator set to 30°C with the printed side of the array facing up and the tube as level as possible. If needed, tape the tube to the flat surface to avoid any accidental disturbances.
7. Incubate the conical tube containing the array for 1 hour at 30ºC **without shaking**.
8. Remove the conical tube containing the array from incubator and add 40 ml of 0.5% SDS to the tube by dispensing the SDS down the sides of the tube. **Avoid pipetting SDS directly onto the array surface**. Remove glass coverslip from tube with forceps after it floats off, and discard as radioactive waste.
9. Cap tube and incubate at room temperature for 15 minutes **without shaking**. Discard the wash as radioactive waste.
10. Add 40 ml 0.5% SDS to the tube (dispense SDS as described in Step 8), cap tube, and incubate for 15 minutes **without shaking**. Discard the wash as radioactive waste.
11. Add 40 ml of water to the tube (dispense water as described in Step 8), and incubate for 15 minutes at room temperature **without shaking**. Discard the water wash as radioactive waste, and repeat the wash a second time.
12. Remove the array from the tube using forceps and place in a slide holder.
13. Proceed immediately to **Drying and Scanning the Microarray**, next page.

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Drying and Scanning the Microarray

1. Dry the array using a table top centrifuge. Spin the array at 200 × g for 1–2 minutes at room temperature in the slide holder (if using a centrifuge equipped with a plate rotor) or 50 ml conical tube (if using a swinging bucket rotor). Verify that the array is completely dry.
2. Place array in X-ray film cassette, cover with plastic wrap and overlay with phosphor screen or X-ray film.
3. Expose array to phosphor screen or X-ray film for 3 hours. You may need to vary the duration of the exposure from 1–12 hours depending upon experimental conditions.
4. Remove phosphor screen from cassette and scan with phosphorimager or develop film using film developer.
5. Obtain 16-bit TIFF image file by scanning X-ray film with scanner or retrieving file from phosphorimaging of phosphor screen.
6. Process image using imaging software (*i.e.* Prospector Imager or Adobe® Photoshop®). For Prospector Imager, refer to the ProtoArray® Prospector User Guide. For Adobe® Photoshop® process the image as follows:
   a) Crop 1″ × 3″ fixed rectangular areas from each TIFF file that correspond to each slide.
   b) Invert data.
   c) Change image file to 2,550 × 7,650 pixels (constrained proportions).
   d) Save cropped TIFF image with new name.

   Note: Do not adjust pixel levels of file in Adobe® Photoshop® as this will affect the dynamic range of the spots.
7. Proceed to **Data Acquisition and Analysis**, below.

Data Acquisition and Analysis

1. Connect to the portal at www.invitrogen.com/protoarray and then click on the **ProtoArray® Lot Specific Information** link that can be found under **BioMarker Discovery Resources**.
2. Enter the array barcode in the **Input Barcode Number** box and click on the **Search** button.
3. For each input barcode, various lot specific files are displayed. Download the .Gal file to your computer.
4. Start the GenePix® Pro microarray data acquisition software on the computer. Open the saved image (.tiff) from Step 6, (**Drying and Scanning the Microarray**) and open the .GAL files downloaded from ProtoArray® Central for protein arrays. The .GAL file defines the array grid required by the microarray data acquisition software.

   Important: Make sure you download the latest files for the specific barcode on your array. Lot specific information files are updated frequently based on recently available sequence or protein information.
5. Adjust the subarray grid to ensure the grid is in proper location for each subarray. After the grid is properly adjusted and all features are aligned, acquire the pixel intensity data for each feature by clicking the **Analyze** button in GenePix® Pro, and save/export the results as a .GPR (GenePix® Results) file.

   Option: We recommend increasing the spot diameter from 110 µm to 180 µm in each subarray for easier alignment and more accurate data quantitation.
6. Use the files from Step 5, above, for data analysis using ProtoArray® Prospector (available through the **Online Tools** link that can be found under **BioMarker Discovery Resources** at www.invitrogen.com/protoarray).
7. Install ProtoArray® Prospector.
8. Start ProtoArray® Prospector from the desktop icon. Set the **Application** to Kinase Substrate Identification.
9. Select the **Analyze** button from the Tool Bar.
10. Select the .GPR files from the "Files of type" pull-down list and navigate to your data file(s). Select the file(s) for analysis and click the **Open** button.
11. After analysis, ProtoArray® Prospector generates a list of potential kinase substrates. The proteins that score as positive in the experiment are proteins that satisfy the basic program options based on Z-Factor. We recommend validating the interactions as described in the ProtoArray® Applications Guide.

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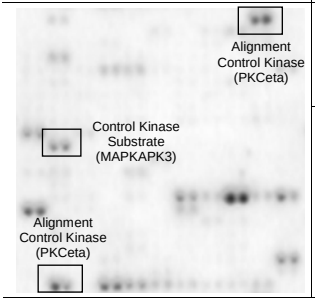
Expected Results

Use controls printed on the ProtoArray® Human Protein Microarray v5.0 in verifying the probing, detection, and scanning protocols. Refer to the ProtoArray® Applications Guide at www.invitrogen.com for details.

Results obtained after probing the ProtoArray® Human Protein Microarray v5.0 with 120 nM of a MAPK14 (p38 alpha) control kinase and radiolabeled ATP are shown below. Refer to the ProtoArray® Applications Guide for additional details on control features.

Note: To identify kinase substrates specific to your protein kinase, we recommend probing a second array simultaneously with buffer only that enables you to determine kinase-specific signals.

Image	Control	Description/Function
	Alignment Control Kinases	Autophosphorylating kinase used for orientation of the microarray image, and serving as control for proper radiolabel and assay conditions.
	Control Kinase Substrate	Substrate for control kinase serves as control for proper probing and scanning procedures.

Additional Products

The table below lists additional products available separately from Invitrogen. For more information about these products, visit www.invitrogen.com or contact Technical Support.

Product	Quantity	Catalog no.
ProtoArray® Products		
ProtoArray® Human Protein Microarray v5.0	1 array 20 arrays	PAH052501 PAH0525020
ProtoArray® Control Protein Microarray v5.0	1 array	PA10057
ProtoArray® Human Protein Microarray v5.0 KSI Kit *for kinase substrate identification*	1 kit	PAH0525065
Control Kinase (MAPK14, Active)	10 µg	PV3304
Phosphate Buffered Saline (PBS), 1X	500 ml	10010-023