MILLIPORE

Protocol

Kinase Peptide Microarray – Random Serine Library

Off-the-shelf and customized peptide microarrays

Catalog # 30-051

upstate
now part of Millipore

USA & Canada
Phone: +1(800) 437-7500 • Fax: +1 (951) 676-9209 • Europe +44 (0) 23 8026 2233
Australia +61 3 9839 2000 • Germany +49-6192-207300 • ISO Registered worldwide
www.upstate.com • custserv@upstate.com • techserv@upstate.com

Table of contents

1 Introduction

Millipore's Kinase Peptide Microarrays, using JPT technology, offer a very efficient way to detect potential phosphorylation sites in selected kinase substrate peptides derived from kinase substrate proteins. Following the incubation with the target kinase in the presence of radioactive ATP, incorporated phosphate can be detected by autoradiography or phosphorimaging. Alternatively, phospho-specific antibody-based fluorescent readout may be used for phospho-peptide detection. Moreover, phospho-specific stains like the Pro-Q-Diamond reagent from Molecular Probes could be used to identify peptidic kinase substrates. This product consists of human phosphorylation sites (13-mers) containing one serine residue in the center position, displayed on 4 peptide microarrays in three identical subarrays (3 x 384 = 1156 peptide spots per slide).

2 List of Components

- Microarray glass slide(s)
- CD-ROM containing array layout and sequence information
- Data-sheets

3 Storage and Handling

3.1 Storage of Kinase Peptide Microarray Slides

- Optimal storage conditions for JPT's kinase peptide microarray slides are in a cool (approx. 4°C / 39°F) and dry environment. JPT´s peptide microarrays are stable for at least 18 month when stored at 4°C (39°F).
- Do not freeze the microarray slides for storage.

3.2 Handling of Kinase Peptide Microarray Slides

- Always handle the delicate microarray slides with care.
- Never touch the microarray slide surface.
- Always wear laboratory gloves when handling JPT's kinase peptide microarrays.
- Please hold the microarrays slides at the end, which carries the engraved data label (frosted area). This label provides for unique identification of the array.

- Please take care when dispensing solutions onto the microarray surface. Make sure not to touch the surface with pipette-tips or dispensers.
- Never whisk the surface of the slide with a cloth.
- Never use other chemicals as described. Inappropriate chemicals may destroy the chemical bonding of the peptides to the glass surface.
- Avoid dust or other particles during each step of the experiment.
  Dust, particles and resulting scratches will cause artefacts during the final signal readout.
- Please filter all solutions for the washing steps through 2 µm, preferably 0.4 µm particle filters before use.

4 Additional Materials Required

4.1 Materials and Solutions for Radioactive Readout

- Catalytically active kinase
- Specific kinase-buffer
- ATP and [γ^{32}P] ATP or [γ^{33}P] ATP
- Phosphoric acid (0.1 M)
- De-ionized water
- Additional hardware (refer to point 4.1.A)

If no specific buffer is supplied with your kinase, JPT recommends to use a general kinase buffer as described in section 0

4.1.A General Kinase Buffer

The general kinase buffer is only needed if no specific buffer is supplied with or known for your kinase. JPT recommends the following final buffer conditions for kinase mediated phosphate transfer from ATP to microarray bound substrate peptides:

50 mM HEPES-NaOH, pH 7.5

5 mM $MgCl_2$

5 mM $MnCl_2$

3 µM sodium-orthovanadate

1 mM DTT

1 µM ATP (approx. $3x10^5$ cpm [γ ^{32}P] ATP)

4.1.A Additional Hardware

- Liquid blocker pen
  (Pelco International; www.pelcoint.com; order# 22309)
- Phosphorimager or X-ray film exposure equipment

4.2 Materials and Solutions for Non-Radioactive Readout

- Catalytically active kinase
- Specific kinase-buffer
- ATP
- BSA solution (1mg/mL)
- Blocking buffer (refer to point 4.2.B)
- TBS-Buffer 1x (refer to point 4.2.C)
- Phospho-specific antibody (refer to point 4.2.D)
- Pro-Q Diamond stain (refer to point 4.2.E)
- Additional hardware (refer to point 4.2.F)

If no specific buffer is supplied with your kinase, JPT recommends to use a general kinase buffer as described in section 4.2.A

Prepare all solutions using de-ionized H_2O.

4.2.A General Kinase-Buffer

The general kinase buffer is only needed if no specific buffer is supplied with or known for your kinase. JPT recommends the following final buffer conditions for kinase mediated phosphate transfer from ATP to microarray bound substrate peptides:

50 mM HEPES-NaOH, pH 7.5

5 mM $MgCl_2$

5 mM $MnCl_2$

3 µM sodium-orthovanadate

1 mM DTT

1 µM ATP

4.2.B Blocking Buffer

Following the incubation with the target kinase, the peptide microarray has to be blocked to prevent unspecific binding of the antibody or the phospho-specific stain. JPT strongly recommends to use the blocking reagent from Roche Diagnostics GmbH (please find details in the next section).

Protocol for preparation of blocking buffer:

- Dilute the blocking reagent (Roche Diagnostics GmbH; Cat.# 1 096 176; www.roche-applied-sience.com) prepared according to the manufactures protocol 1:10 with buffer prepared as follows:
    - 100 mM maleic acid
    - 150 mM NaCl
    - adjust to pH 7,5 with NaOH
    - stock solution should be stored at 4°C (39°F)

Always prepare fresh blocking buffer before use!

For further information about the blocking buffer, please refer to the data sheet provided by the manufacturer.

4.2.C TBS-Puffer

- 50 mM Tris/Cl pH 8.0
- 137 mM NaCl
- 2.7 mM KCl

4.2.D Antibody solution

Anti-phospho-tyrosine antibodies may be used to detect tyrosine phosphorylation. JPT recommends to use the FITC-labeled monoclonal anti-phospho-tyrosine antibody clone PT 66 (Sigma Aldrich; www.sigmaaldrich.com; Cat.#: F3145).

- Dilute the antibody-stock solution with Blocking Buffer described in 4.2.B 1:1000.

For more information about the anti-phospho-tyrosine antibody please refer to the data sheet delivered with the antibody.

4.2.E Pro-Q-Diamond stain

For the detection of serine/threonine phosphorylation, JPT recommends to use the Pro-Q-Diamond stain from Molecular Probes. Details for incubation of the peptide microarray slides are contained in the data sheet of the stain.

4.2.F Additional Hardware

- Disposable incubation chamber (Abgene; Microarray Gene-Frame™, 300 µL (19 x 60 mm ID); Cat.# AB-0630)
- Slide Scanner; you may use any scanner that is compatible with 75 x 25 x 1 mm slides and with your chosen fluorescence label.

5 General considerations

5.1.A Experimental basics

Millipore's Kinase Peptide Microarrays are devices for detecting potential phosphorylation sites within selected peptides. Each spot in the microarray represents a single peptide, derived from the primary structure of a kinase substrate protein

During incubation of the peptide microarray with a kinase in the presence of ATP a phosphate moiety is added to the substrate peptides

The incorporated phosphate moiety can be detected by phosphorimaging (if radio-actively labelled ATP was used) or by incubation with a fluorescence labeled phospho-specific antibody or dye

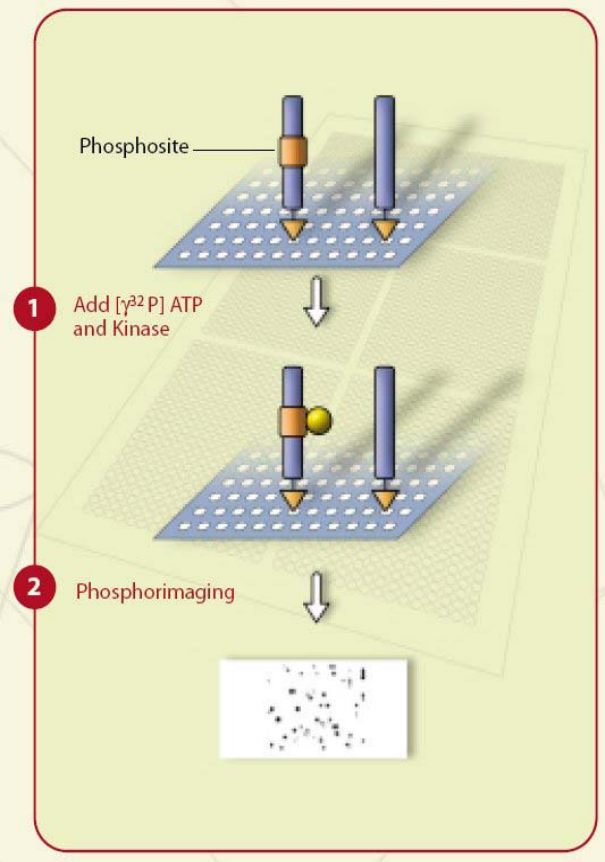


Figure I: General principle of kinase peptide microarray based phosphosite detection

5.1.B Kinase Peptide Microarray Layout

Please refer to the files on the enclosed CD-ROM and to the corresponding data sheets for the identity and location of the spots on the microarray surface. The side of the slide with the engraved label represents the surface displaying the attached peptides.

5.1.C Kinase Peptide Microarray Pretreatment

The kinase peptide microarray is designed as a ready-to-use product. There is no need to perform blocking steps on the surface prior to incubation with the target kinase. However, if you would like to perform an additional blocking reaction, please ensure to only use protein-free solutions like PEGs (polyethyleneglycols, 0.5 mg/mL) or PVPs (polyvinylpyrrolidones, 1 mg/mL).

This keeps your kinase of interest from phosphorylating the protein used for blocking!

6 Experimental protocols

6.1 Radioactive Readout

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING THE INCUBATION!

From experience, we recommend the following hybridization technique, which keeps handling of the slide to a minimum once radioactive material is dispensed on the microarray:

Simply use a liquid blocker pen to draw a defined incubation area on the slide.

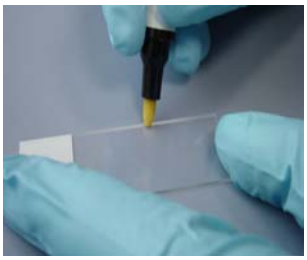
This virtual incubation chamber does not require sophisticated handling steps and also reduces the volume of kinase solution needed.

However, if the volume of your kinase solution is very limited a commercially available incubation chamber may also be used.

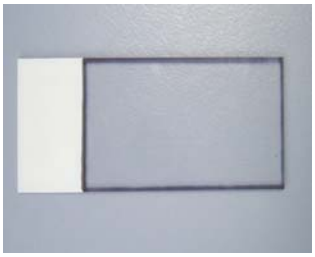
When carrying out enzyme reactions in an incubation chamber, we recommend to treat the surface of the chamber prior to the enzyme reaction with a solution of 1mg/mL BSA for at least 6 hours. If you are using the Gene-Frame™ incubation chamber, it is sufficient to pre-treat the needed coverslip with BSA.

6.1.A Define the incubation area using a liquid blocker pen

I. Use a liquid blocker pen to define an incubation area surrounding the microarray.



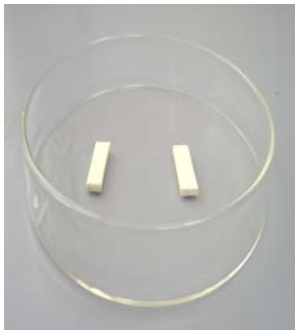
II. Make sure that this barrier blocking the liquid does not touch the area of deposited peptides. The best way to achieve this, is to place the barrier as close to the edge of the chip as possible (For visualization, the liquid-blocking barrier is in black.)



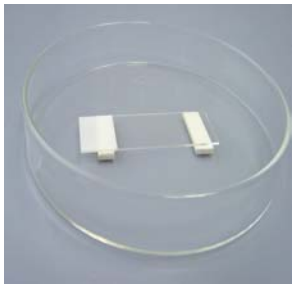
The liquid blocker pen should not be used, if your kinase is sensitive against lipids. In this case, please use an incubation chamber or cover the peptide microarray with a dummy slide treated with BSA.

6.1.B Prepare the slide-environment for easy handling

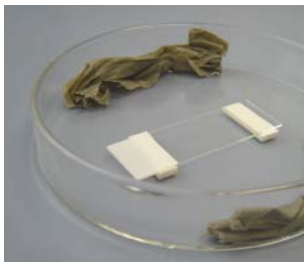
III. Place two spacers into a petri dish spaced apart to fit the size of the peptide microarray slide. The spacers will allow you to conveniently handle the slide with tweezers, once the radioactive solution was placed onto the surface of the microarray. Alternatively, use a smaller Petri dish turned upside down as support plate.



IV. Place the peptide microarray slide with the defined incubation area on the spacers facing upward.



V. Place pieces of wet cloth next to the slide. This will keep the incubation solution from evaporating in a closed incubation chamber.
(See step 6.1.C VIII)



6.1.C Pipette kinase solution and radioactively labeled ATP

VI. Prepare approx 550 µL of final assay solution containing kinase, kinase buffer, ATP and radioactively labeled ATP.

VII. Pipette the complete volume into the defined incubation area. The barrier (refer to point 6.1.A) will contain the solution.
(For visualization, the solution is in blue).

Make sure not to touch the microarray slide with the pipette tip. Scratches and marks on the surface may destroy the deposited microarray and will cause artefacts!

VIII. Close the petri dish with a matching cover to create an incubation chamber.

6.1.D Incubation

IX. Incubate the peptide microarray for the appropriate time and temperature with the kinase. Millipore recommends an incubation time of at least 1 hour at approximately 30-35°C (86-95°F).

6.1.E Wash microarray

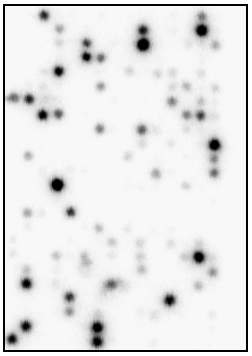
X. Wash the slide 5 times with 0.1 M phosphoric acid (3-4 min each wash) to stop the reaction and to remove excess radioactively labeled ATP. Ensure that the slide is properly washed with enough liquid rinsing over the slide.

XI. Wash the slide 5 times with de-ionized water (3-4 min each wash). Ensure that the slide is properly washed with enough liquid rinsing over the slide.

XII. Wash the slide with methanol and dry it preferably by gently using a stream of nitrogen.

6.1.F Image the radioactive phosphorous

XIII. Use the dry slide for phosphorimaging. JPT strongly recommends the detection of incorporated phosphate by phosphorimaging using a FLA 3000 Reader. For best results, use the highest resolution possible (at least 50 µm pixel size).



Peptide microarray was incubated with casein kinase II an [□^{32}P] ATP. Phosphoimaging o a FLA 3000 Reade was used for readout.

6.2 Non-Radioactive Readout

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING THE INCUBATION!

We recommend to use an incubation chamber for best results. In our hands, the disposable Gene-Frame™ from Abgene House (# AB-0630, http://www.abgene.com) with a volume of 300 µL worked best with our slides under a wide range of volumes and conditions.

The incubation may be performed with other incubation chambers as well without loss of quality. In this case, please refer to the instructions of the manufacturer. Alternatively, the incubation technique for radioactive experiments (refer to point 0) may also be used for non-radioactive experiments.

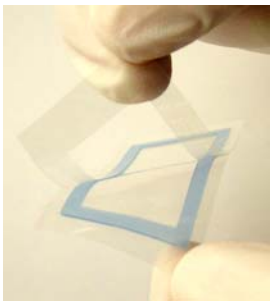
The following protocol describes the incubation of our kinase peptide microarray with the disposable incubation chamber Gene-Frame™. It consists of two parts: a blue adhesive frame, sandwiched between a thick and a thin polyester sheet, and a matching transparent coverslip.

When carrying out enzyme reactions in an incubation chamber, we recommend to treat the surface of the chamber prior to the enzyme reaction with a solution of 1mg/mL BSA for at least 6 hours. If you are using the Gene-Frame™ incubation chamber, it is sufficient to pre-treat the needed coverslip with BSA.

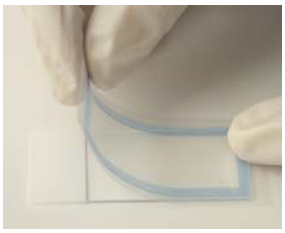
6.2.A Attach chamber to peptide microarray

I. Ensure that the surface area of the microarray slide is dry and clean.

II. Each adhesive frame is sandwiched between a thin and a thick polyester sheet (while the thin sheet covers the adhesive frame completely, the thick sheet has the central part removed). Please remove the thick polyester sheet carefully ensuring that the blue frame stays on the thin polyester sheet.

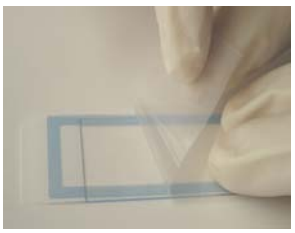


III. Carefully place the adhesive blue frame on the thin polyester sheet onto the microarray avoiding contact with the peptide displaying microarray surface.



IV. Press down the blue frame firmly without trapping air under the adhesive.

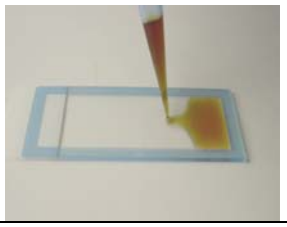
V. Remove the second, thin polyester sheet from the top of the adhesive blue frame.



6.2.A Pipette kinase solution and ATP into chamber

VI. Prepare approximately 330 µL of final assay solution containing kinase, kinase buffer and ATP.

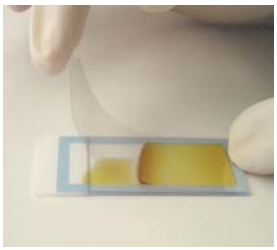
VII. Pipette the complete volume starting at one end of the adhesive frame (for visualization, the solution is in yellow).



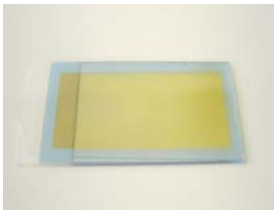
Make sure that the microarray surface is not touched by the pipette tip. Scratches and marks on the surface may destroy the deposited microarray and will cause artefacts during the fluorescence scanning.

6.2.B Seal incubation chamber

VIII. Carefully place the polyester coverslip over the adhesive frame. Start at the end where the assay solution was first pipetted on and slowly press down the coverslip over the blue frame.



IX. The deposited solution will then spread across the whole microarray surface without trapping any air bubbles.



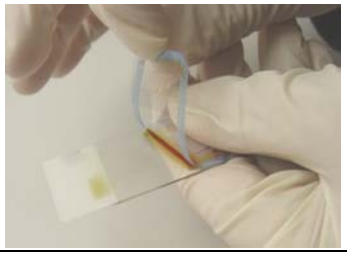
X. Press the cover onto the adhesive frame with a blunt instrument for approximately 10 seconds.

6.2.C Incubate

XI. Incubate the peptide microarray for appropriate time and temperature with the kinase. Millipore recommends an incubation time of at least 1 hour at approximately 30-35°C (86-95°F).

6.2.D Remove incubation chamber

XII. To remove the incubation chamber after incubation hold the slide with one hand and gently pull back the tab of the coverslip along the length of the slide. Use the disposable incubation chamber for one experiment only.



Take care to remove the incubation chamber completely. Any remaining adhesive might disturb the following washing steps.

6.2.E Wash

XIII. Wash the microarray slide 5 times for 5 min each wash with TBS-Buffer (refer to point 4.2.C). Take care that the slide is properly washed using sufficient volumes of buffer solution.

6.2.F Prepare the slide for the antibody incubation

XIV. Wash the peptide microarray for 1 hour in blocking-solution (refer to point 4.2.B). Make sure that the slide is properly washed using sufficient volumes of buffer solution.

6.2.G Incubate with antibody

XV. Prepare antibody solution (refer to point 4.2.D).

XVI. Incubate the peptide microarray for 3 hours with the antibody solution at room temperature. Make sure that the slide is properly washed using sufficient volumes of buffer solution.

6.2.H Final washing steps

XVII. Wash the peptide microarray 5 times with TBS-Buffer (refer to point 4.2.C). Make sure that the slide is properly washed using sufficient volumes of buffer solution.

6.2.I Scan the peptide microarray

XVIII. Perform fluorescence scans according to your scanner type and antibody-label properties. For the detection of phosphotyrosine JPT recommends to use the PT-66 anti-phosphotyrosine antibody (refer to point 4.2.D). See figure to the side.

Example: Peptide microarray was incubated with Abl-kinase and ATP. Fluorescent image was generated using a FLA 3000 Reader at 473 nm for emission and an extinction wavelength of 515 nm.

7 References

Jose M. Lizcano, Maria Deak, Nick Morrice, Agnieszka Kieloch, C. James Hastie, Liying Dong, Mike Schutkowski, Ulf Reimer, and Dario R. Alessi:

Molecular Basis for the Substrate Specificity of NIMA-related Kinase-6 (NEK6)

Journal Biological Chemistry (2003) 277, 27839 - 27849

Leszek Rychlewski, Maik Kschischo, Liying Dong, Mike Schutkowski, and Ulf Reimer:

Target Specificity Analysis of the Abl Kinase using Peptide Microarray Data

Journal Molecular Biology (2004) 336, 307 - 311

Sören Panse, Liying Dong, Antje Burian, Robert Carus, Mike Schutkowski., Ulf Reimer, and Jens Schneider-Mergener:

Profiling of generic anti-phosphopeptide antibodies and kinases with peptide microarrays using radioactive and fluorescence-based assays

Molecular Diversity (2004), in press

Mike Schutkowski, Ulf Reimer, Sören Panse, Liying Dong, Jose M. Lizcano, Dario R. Alessi, and Jens Schneider-Mergener:

High content peptide microarrays for deciphering kinase specificity and biology

Angewandte Chemie (2004) 116, 2725 - 2728

8 Related products

- Kinase Substrate Sets (Biotinylated peptides in microtiter plates)
- Full kinase profiling service using PepStar™ high density peptide microarrays
- Large collection of peptidic kinase substrates (biotinylated, fluorescent labeled...)
- PhosphoSite Detector; collections of kinase substrate proteins as peptide scans on PepStar™ microarrays
- Data packages resulting from incubation of PepStar™ high density peptide microarrays with commercially available kinases

Warranty

These products are warranted to perform as described in their labeling and in MILLIPORE® literature when used in accordance with their instructions. THERE ARE NO WARRANTIES, WHICH EXTEND BEYOND THIS EXPRESSED WARRANTY AND MILLIPORE® DISCLAIMS ANY IMPLIED WARRANTY OF MERCHANTABILITY OR WARRANTY OF FITNESS FOR PARTICULAR PURPOSE. MILLIPORE®*'s* sole obligation and purchaser's exclusive remedy for breach of this warranty shall be, at the option of MILLIPORE®, to repair or replace the products. In no event shall MILLIPORE® be liable for any proximate, incidental or consequential damages in connection with the products.

Cat No.30-051

January 2007
Revision A, 4003713