

Protocol

Phosphatase Peptide Microarrays *PhosphoSites-Tyrosine*

Ready-to-use microarrays for phosphatase profiling

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1 Introduction

Enzymatic dephosphorylation of proteins represents a key regulation event in biologically important processes like signal transduction and cell cycle regulation. To study these processes and the involved enzymes, the identification of detailed substrate information is critical. One of the most efficient ways to study phosphatase activities and substrate specificity is incubating a collection of potential substrate phosphopeptides displayed on peptide microarrays with the phosphatase of interest.

JPT Peptide Technologies' Phosphatase Peptide Microarrays (PhosphoSites-Tyrosine) represent a selection of more than 6000 phosphopeptides derived from human phosphorylation sites for rapid screening of protein tyrosine phosphatase activity. Upon incubation with your enzyme, the removal of the phosphate can be detected by reading the fluorescence intensity subsequent to treatment with fluorescently labelled anti-phosphotyrosine antibody. If the phosphatase is active against a displayed phosphopeptide the fluorescence intensity will be decreased.

2 List of components

Component	Quantity
Phosphatase Peptide Microarrays	2 x glass slides each displaying >6000 phosphopeptides
Data CD-ROM	one CD-ROM containing protocol as .pdf-file, .gal-viewer software and sequence info as .gal-file)

3 Storage and Handling

3.1 Storage of Phosphatase Peptide Microarray Slides

- Optimal storage conditions for JPT's Phosphatase Peptide Microarrays are in a cool (approx. 4°C/39°F) and dry environment. JPT's peptide microarrays are stable for at least 12 months when stored at 4°C (39°F).
- Do not freeze the peptide microarrays for prolonged storage.

3.2 Handling of Phosphatase Peptide Microarray Slides

- Always handle the delicate microarrays with care.
- Never touch the microarray slide surface.
- Always wear laboratory gloves when handling JPT's Phosphatase Peptide Microarrays.
- Please hold the microarrays at the end which carries the engraved data label. This label provides for unique identification of the microarray. It codes for the JPT batch and the position of the individual slide during production process.
- 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 2µm, preferably 0.4µm particle filters before use. Filtration of the final assay solution is recommended, too.

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING YOUR EXPERIMENTS! CAREFULLY NOTE THE HANDLING AND STORAGE CONDITIONS OF THE PHOSPHATASE PEPTIDE MICROARRAYS. PLEASE CONTACT JPT'S TECHNICAL SERVICES FOR ASSISTANCE IF NECESSARY.

3.3 Product Description

JPT Peptide Technologies' ready-to-screen Phosphatase Peptide Microarrays (PhosphoSites-Tyrosine) are comprised of more than 6000 phosphotyrosine-containing peptides derived from human phosphorylation sites. The 13meric peptides represent the amino acids around the phosphorylation site with the phosphotyrosine residue in the middle position. An optimized hydrophilic linker moiety is inserted between the glass surface and displayed phosphopeptide to avoid false negatives caused by steric hindrance. The peptides are displayed in three identical subarrays resulting in a total number of more than 18,000 peptides on one glass slide. Subsequent to incubation with the enzyme, dephosphorylation can be detected after treatment with anti-phosphotyrosine antibody followed by fluorescently labelled secondary antibody by imaging the microarray. Dephosphorylated peptides will result in a decrease of fluorescence signal intensity. The second slide is meant to perform a control incubation under identical conditions without addition of phosphatase. Thus, a base signal intensity value for each spot can be derived with which the signals from the phosphatase-treated slide can be subsequently be compared.

The data CD-ROM included with the microarrays contains all information needed for the detailed analysis of your data including peptide sequences as well as the Swiss-Prot accession numbers for the proteins containing these phosphorylation sites.

4 Additional Materials Required

- Phosphatase of adequate activity (we recommend a final activity of 0.1U per mL).
- Phosphatase assay buffer.
- Anti-phosphotyrosine antibody and fluorescently labelled secondary antibody (JPT recommends antibody anti-pTyr-100 (Cell Signalling #9411) in combination with anti-mouse-Dylight649 (Pierce, #35515))
- Double distilled water.
- Fluorescence scanner/imager capable of excitation of the fluorophore which is attached to the secondary antibody (pixel size should be at least 20µm). Pixel sizes smaller than 20µm will result in more accurate data points but may not be necessary.
- Software tool allowing the assignment of signal intensities to spots on the surface of the microarrays (JPT recommends GenepixPro 6.0; www.genepix.com)
- TBS buffer (50mmol Tris, 137mmol NaCl, 2.7mmol KCl; pH 8.0)

5 General considerations

5.1 Experimental basics

JPT's Phosphatase Peptide

Microarrays are devices for detecting potential dephosphorylation sites in annotated peptide sequences. Each spot in the microarray represents a single individual phosphorylated peptide.

During incubation of the peptide microarray with a phosphatase selective phosphate moieties are cleaved from the phosphorylated residue (see Figure 1, step 2)

The remaining phosphate moieties can be detected by incubation with a fluorescently labelled phosphospecific antibody or secondary antibody directed against a primary anti-pTyr-antibody (Figure 1, step 3)

By means of a fluorescence scanner, cleaved phosphopeptides can be detected by reduced signal intensity.

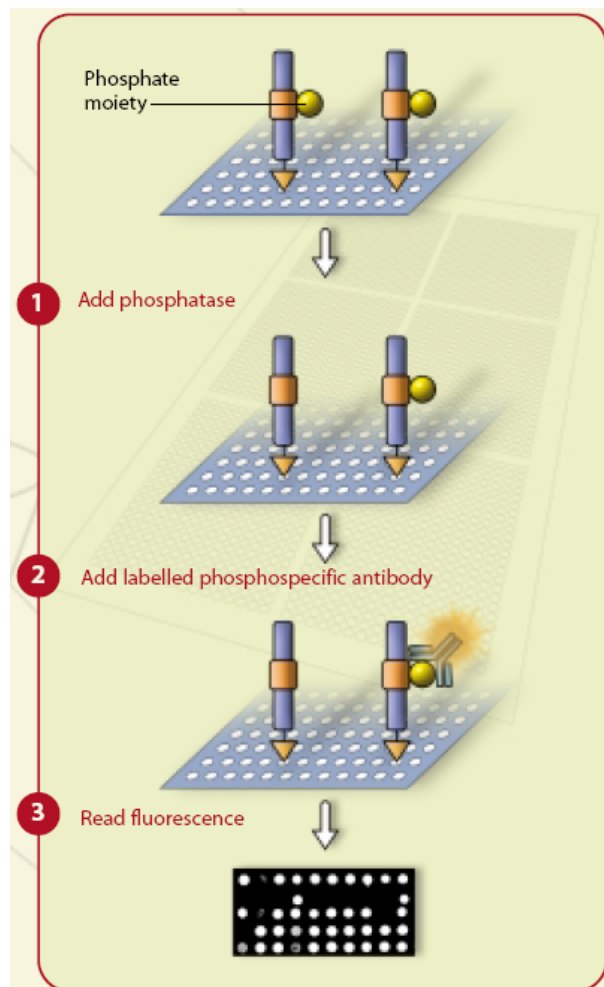


Figure 1: General principle of Phosphatase Peptide Microarray based phosphorylation site detection

5.2 Phosphatase Peptide Microarray Layout

Please refer to the .gal-file on the enclosed CD-ROM 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. The .gal-file can be opened with microarray evaluation software capable of evaluating high-density microarray slides. Since .gal-files are tab-separated text files, they can be processed with software modules such as Microsoft Editor (Notepad) or Microsoft Excel.

A schematic layout of the peptide microarray is shown in Figure 2.

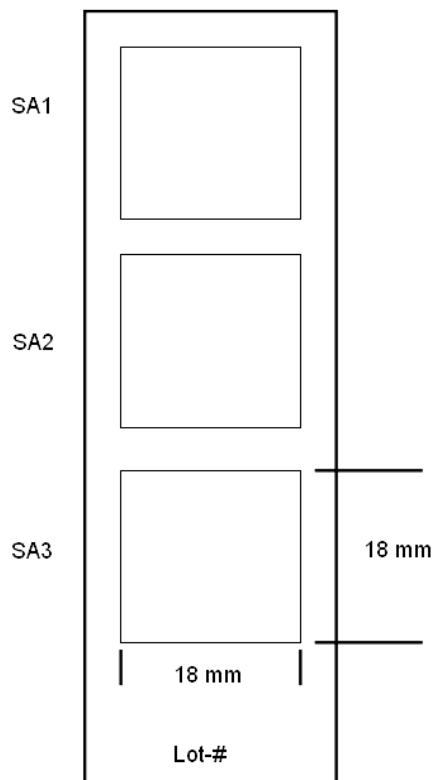


Figure 2: schematic layout of peptide microarray (SA=subarray)

As shown in Figure 2 the peptide array is printed in three identical subarrays (SA). This enables efficient intra-chip-reproducibility tests using scatter plots or correlation functions.

Each peptide subarray (SA) is printed in individual blocks (see Figure 3, details see Figure 4). Using the provided .gal-file, the evaluation can be performed using software modules like Genepix, ArrayPro or similar programmes which will align the .gal-file induced grid onto the resulting image.

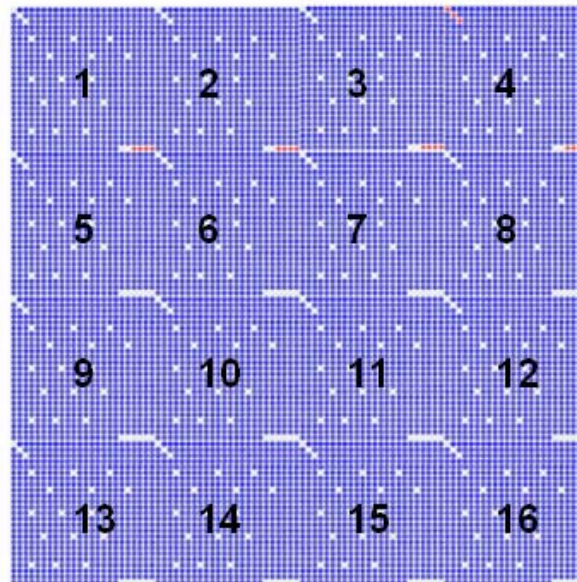


Figure 3: Exemplary view for a subarray consisting of 16 individual blocks.

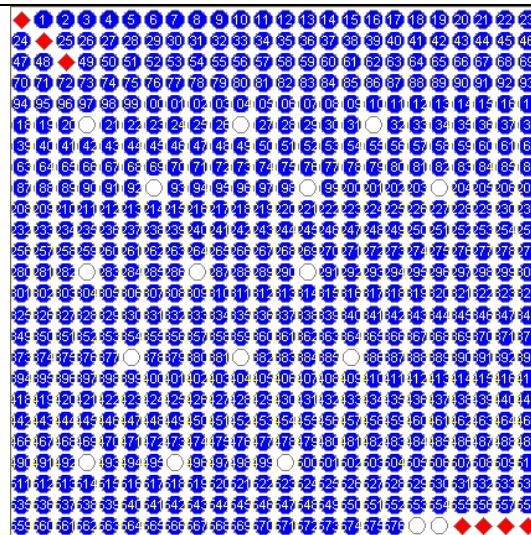


Figure 4: Exemplary block layout, red coloured spots represent fluorescent landmarks or protein/antibody positive controls respectively, white spots are background controls (for details please refer to .gal-file)

6 Experimental protocols

Note: The following procedure is given as a guideline only. The optimal experimental conditions will vary depending on the investigated parameters and cannot be predetermined - they must therefore be established by the user. No warranty or guarantee of performance using this procedure with your target enzyme can be made or is implied.

The Phosphatase Peptide Microarray (PhosphoSites-Tyrosine) is designed as a ready-to-use product to assay enzyme activity directly on the surface of the glass slides. There is no need to perform blocking steps on the surface prior to incubation with the target enzyme. However, if you would like to perform an additional blocking reaction, please ensure to only use protein-free solutions like PEGs (polyethyleneglycols, 0.5mg/mL) or PVPs (polyvinylpyrrolidones, 0.1mg/mL).

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.

6.1 General principles for incubation

6.1.1 Fully automated microarray processing station

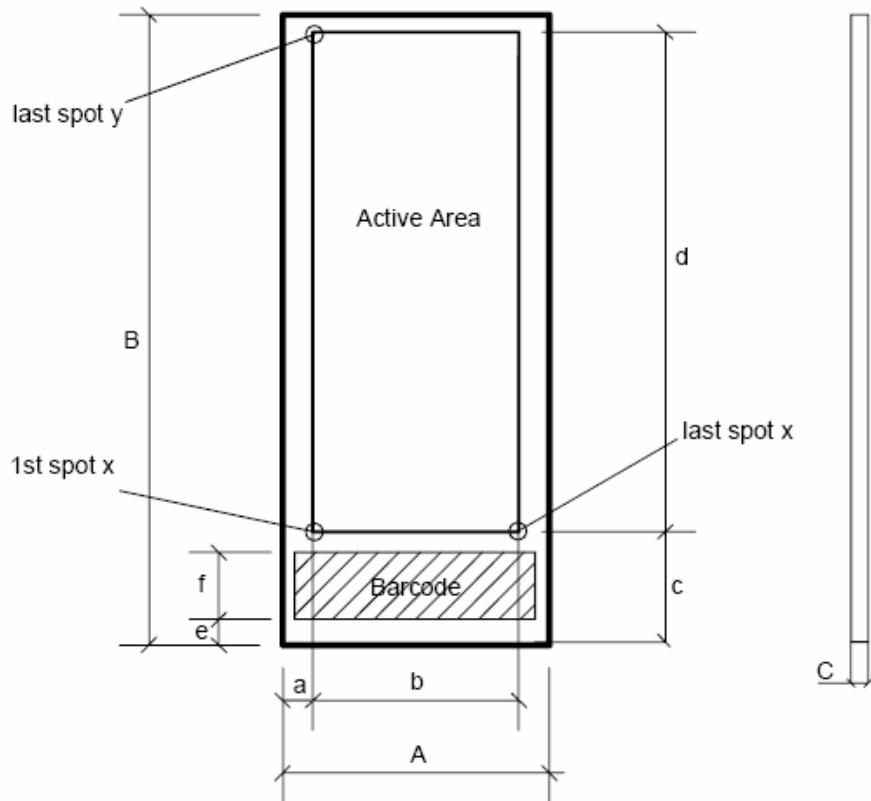
All high density peptide microarrays produced by JPT have an identical layout with regard to active area and spotted surface. Although the content of the microarrays varies the overall layout and dimensions are the same (see Figure 5).



Please check with the manufacturer of your microarray processing station for compatibility with the required liquids. Most microarray processing stations are sensible towards strong acids and organic solutions. Protocols have to be adapted to prevent permanent damage to your device.

All peptide microarrays produced by JPT are adjusted to fit in common fully automated microarray processing systems (see Figure 5). JPT recommends using Tecan HS4X00 Hybridisation systems.

Protocols and procedures for using Tecan HS4X00 systems can be provided by JPT if necessary.



	Distance (mm)
A	25
B	75
C	1
a	3.5
b	18
c	11
d	60
e+f	1+7

Figure 5: Maximum area dimension on JPT peptide microarrays

6.1.2 Microarray incubation using microarray-chip-sandwich

To create a simple incubation chamber, two slides, one displaying the peptides and another slide (Dummy-slide) without any peptides, have to be assembled according to Figure 6 in a sandwich-like format. If two peptide microarrays should be screened the top slide could be another peptide-displaying chip. Please make sure that in such a case the two peptide-displaying sides are facing each other. The two slides are separated by two spacers consisting of thin plastic strips (enclosed to delivery).

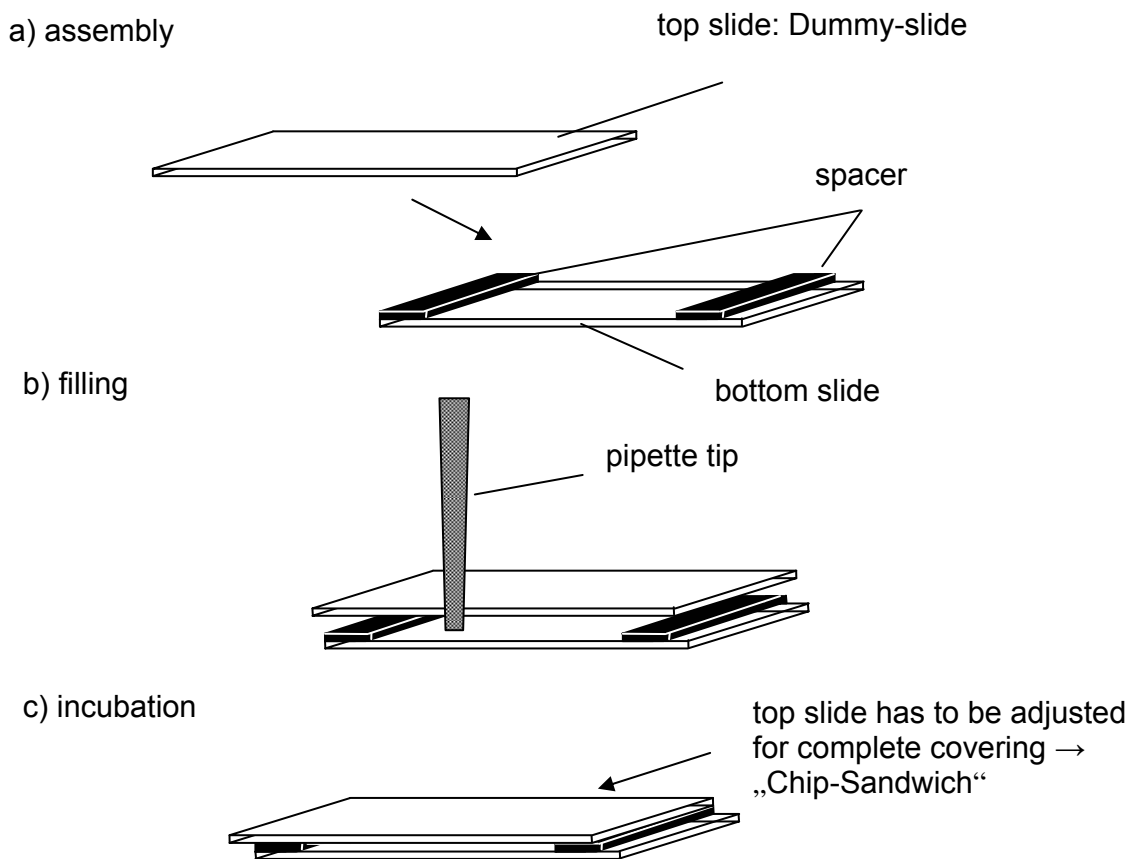


Figure 6: Assembly of "Chip-Sandwich" is shown

a) Two plastic spacers are placed between the peptide displaying microarray (bottom slide) and the Dummy-slide or second peptide displaying microarray (top slide) resulting in a defined reaction chamber.

b) Assay solution is applied via pipette tip into the reaction chamber formed by the two slides. Capillary forces will soak-in the solution without formation of bubbles.

c) Top microarray is shifted resulting in overlaying ends of the glass slides. This arrangement enables convenient disassembly after the incubation step.

The final assay volume will depend on the thickness of these plastic spacers (0.2mm thickness will result in 100µL assay volume, JPT recommends at least 0.5mm thickness resulting in about 500µL final assay solution. The sample has to be applied in between the two slides. Therefore, the top slide is shifted about 1mm to one side. If the pipette tip is adjusted on the position directly over the uncovered bottom slide the capillary forces allow proper distribution of the sample solution without formation of bubbles. Finally, readjust the top slide by sliding it back over the bottom slide so that all slide edges are aligned.

After the incubation is finished, open the microarray sandwich in TBS-buffer, remove the plastic spacers and rinse the peptide microarrays thoroughly with TBS-buffer before continuing with the assay protocol.

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



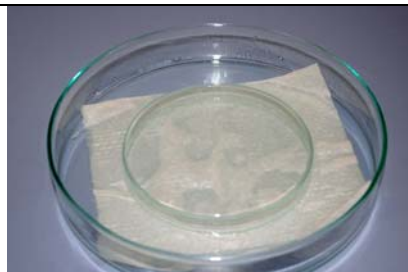
Since plastic surfaces might reduce enzyme-activity by adsorption, JPT recommends performing the enzyme reaction using a microarray chip sandwich. Antibody incubations for the final read-out may be performed using a fully automated microarray processing station.



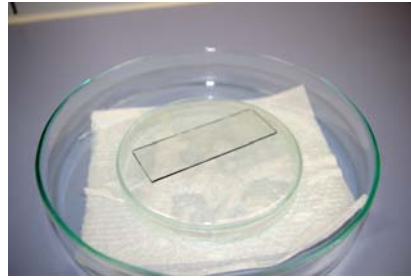
Perform a control incubation in parallel using an additional peptide microarray under identical conditions (without phosphatase solution)

6.1.3 Prepare the slide-environment for easy handling

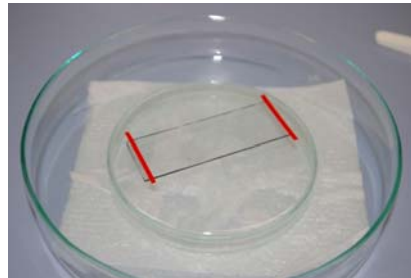
- I. Place a small Petri-dish upside down into a larger Petri-dish to serve as support plate for the microarray. Place a piece of wet cloth underneath the small Petri-dish. This will keep the incubation solution from evaporating once the large Petri-dish is closed.



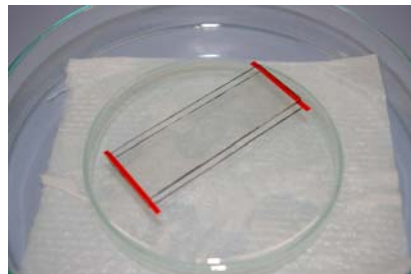
- II. Place the peptide microarray slide support plate facing upward (engraved label has to be readable from top).



- III. Place enclosed spacers on both ends of the microarray.



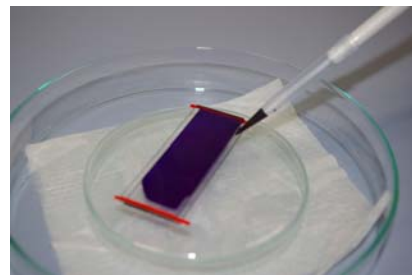
- IV. Prepare the microarray sandwich according to description above (6.1.2). If two peptide microarrays are used make sure that peptide displaying sides are facing each other.



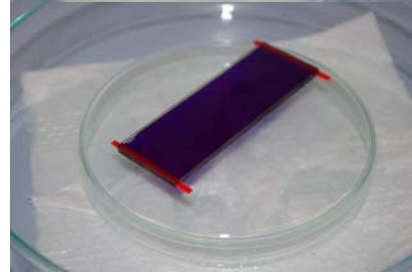
6.1.4 Pipette enzyme solution into microarray chip sandwich

- V. Prepare approx. 500 μ L of final assay solution (if enclosed spacer are used) containing your target enzyme.

- VI. Pipette the complete volume into microarray chip sandwich. Capillary forces will suck the solution in between the two slides. Make sure there are no air bubbles within the sandwich.

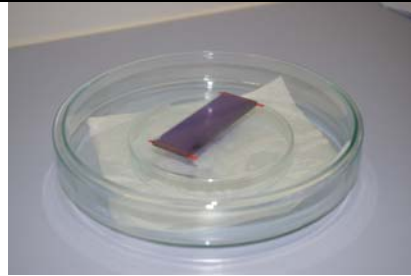


- VII. Adjust the peptide microarray sandwich as described in Point 6.1.2.



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

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



6.1.5 Incubate microarray slides

- IX. Incubate the slides with your enzyme for an appropriate time at appropriate temperature. JPT recommends an incubation time of at least 2 hours and an incubation temperature of 37°C

6.1.6 Wash microarray

- X. Wash the microarray slides 5 times for 5 min each with double distilled water. Take care that the slide is washed using sufficient volumes of solution.

In case a fully automated microarray processing station is to be used for the final incubation steps, please refer to the manual of your machine to program the remaining steps.

Protocols for using Tecan HS4X00 processing station could be obtained from JPT

6.1.7 Treat with anti-phosphotyrosine antibody

- XI. Incubate the slides with anti-phosphotyrosine antibody. We strongly recommend the use of anti-phosphotyrosine antibody anti-pTyr-100 from Cell Signalling (#9411). Dilute the antibody 1:1000 with blocking reagent (Pierce/Thermo, Superblock TBS-T20, #37536, final antibody concentration 1µg/mL). Incubate the Phosphatase Peptide Microarrays with the resulting antibody solution for 1 hour at room temperature (gentle shaking). Subsequent to antibody treatment wash the slides again according to point X (please see above).

6.1.8 Incubation with secondary antibody

XII. Incubate the peptide microarray with your fluorescently labelled secondary antibody. JPT recommends using anti-mouse Dylight 649 (Pierce/Thermo, #35515, final antibody concentration 1µg/mL) in connection with anti-phospho-Tyr-antibody from point XI and an incubation time of at least 45 minutes at room temperature (gentle shaking).

Alternatively a directly labelled anti-phospho-Tyrosine antibody can be used in which case this step is to be skipped.

6.1.9 Wash microarray

XIII. Wash the slide 5 times with TBS-buffer (3-4min each wash) to remove excess antibody. Ensure that microarray is properly washed with enough liquid rinsing over the slide.

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

XV. Dry the slides using a microarray centrifuge or by blowing a gentle stream of nitrogen on the microarray surface.

6.1.10 Imaging of Phosphatase peptide microarray

XVI. Perform fluorescence scans according to your scanner type and with laser settings corresponding to the fluorescence label of the secondary antibody. We recommend a resolution with a pixel size of at least 20µm.

6.1.11 Data analysis

XVII. Generate lists containing signal intensities of each peptide spot.

XVIII. Calculate the mean value for the signal intensities of spots with identical peptides (three identical subarrays) for the images from the control experiments (A) and from the images subsequent to phosphatase/antibody incubation (B).

XIX. Calculate the ratio between B and A (B/A). All values should be smaller or equal to 1!

XX. Arrange the results according to the B/A value. Start with smallest value. The smallest values indicate the spots displaying peptides dephosphorylated most effectively by the phosphatase.

XXI. Align identified top substrates to extract consensus amino acids or sequence motifs.

7 Notes

- Avoid dust or other particles during each step of the experiment. Dust particles and resulting scratches will cause artefacts during the final signal readout.
- Fluorescence scanning could be very sensitive depending on the scanner. Avoid any fluorescent impurities/contaminations inside your assay solution or wash solutions. You can easily check for such impurities by incubating and washing a Dummy-slide with the same solutions followed by imaging at the same filter settings.
- Phosphatases may have diverse optimal assay conditions (ionic strength, pH value, necessary additives like metal ions etc.). Therefore, a universal buffer system cannot be provided.
- Phosphatase buffers could contain components like metal ions which can bind to certain peptide sequences and influence the antibody/peptide recognition. Therefore, a control experiment should be performed on buffer-treated microarrays only.
- Anti-phosphotyrosine antibodies show some subsite specificity regarding the recognized phosphopeptides. Therefore, the signal intensities are not equal for each peptide spot in the control experiment. Data analyses adjust this variation by using the ratio (B/A, see chapter 6 point XIX) of intensities as opposed to differences.

8 Related products

For further information visit our homepage (www.jpt.com) or contact our customer support.

- Tyrosine Phosphatase Substrate Set
- Phosphatase Substrate Set
- Phosphatase Profiling Service