Protocol

PepStar™ Microarrays

Ready-to-use peptide microarrays for kinase profiling

Revision 1.2

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1 Introduction

The Kinase Peptide Microarrays offer a very efficient way to detect phosphorylation sites in selected kinase substrate peptides derived from proteins. Following the incubation with the target kinase in the presence of radioisotopiclly labeled ATP, incorporated phosphate can be detected by autoradiography or phospho-imaging.

2 List of Components

1. **PepStar™ peptide microarray**
   Glass slide displaying peptides in three identical subarrays (see: 4.2)
2. **Blank slides engraved with *"Dummy"* or *"Blank"***
   One blank slide per PepStar™ Peptide Microarray
3. **JPT Peptide Microarray Spacers**
   Vials containing 20 spacers each
   Two spacers per PepStar™ peptide microarray are needed
4. **Data Files**
   Relevant files for the specific peptide microarray (protocols as pdf-files, sequence info as gal-file and JPT's GalViewer software) are provided by customer support

3 Storage and Handling

3.1 Storage of PepStar™ Peptide Microarray Slides

- Optimal storage conditions for peptide microarray slides are in a cool (approx. 4°C / 39°F) and dry environment.
- Peptide microarrays are stable for at least 6 months when stored at 4°C (39°F).
- Do **not** freeze the peptide microarrays!!!

3.2 Handling of PepStar™ Peptide Microarray Slides

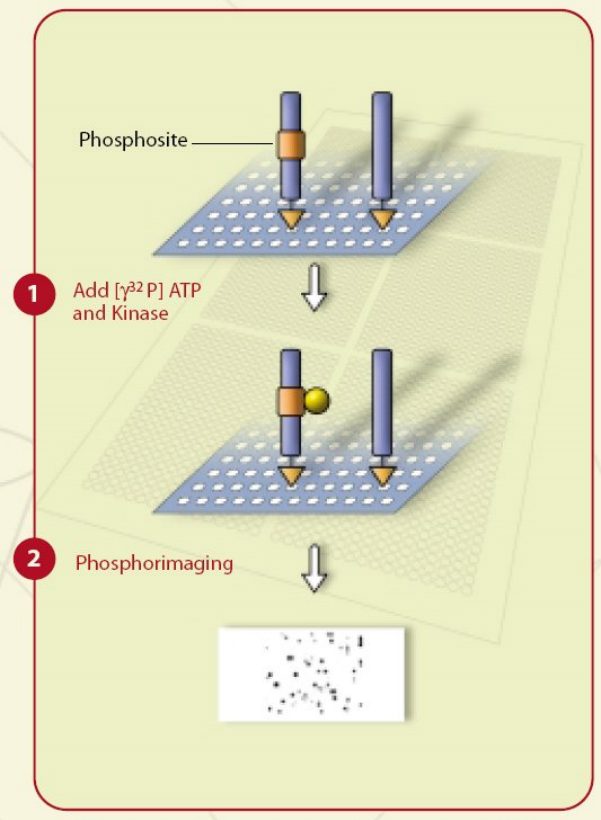
- Always handle the peptide microarrays with care.
- Never touch the peptide microarray slide surface.
- Always wear powder-free laboratory gloves when handling peptide microarray slides.
- Hold peptide microarray slides at the end, which carries the engraved data label. This label provides a unique identification of the specific microarray.
- Take care when dispensing solutions onto the slide surface. Make sure not to touch the surface with pipette-tips or dispensers.
- Inappropriate chemicals may destroy the chemical bonding of the peptides to the glass surface. Never use chemicals with corrosive activity.
- Usage of strong alkaline or acidic solutions should be avoided.
- Avoid dust or other particles during each step of the experiment. Dust, particles and resulting scratches will cause artifacts during the final signal readout.
- Filter all solutions for the washing steps through 0.2 µm particle filters before use.

READ THE ENTIRE PROTOCOL BEFORE STARTING YOUR EXPERIMENTS! CAREFULLY NOTE THE HANDLING AND STORAGE CONDITIONS OF JPT's PEPTIDE MICROARRAYS.
PLEASE CONTACT JPT PEPTIDE TECHNOLOGIES´ CUSTOMER SUPPORT FOR ASSISTANCE IF NECESSARY.

4 General Considerations

4.1 Experimental Basics

JPT Peptide Technologies' PepStar peptide microarrays comprise purified synthetic peptides derived from proteins or other sources (principle of Phosphorylation Site detection see Figure 1) that are chemoselectively and covalently immobilized on the glass surface. An optimized hydrophilic linker moiety is inserted between the glass surface and the peptide sequence to avoid false negatives caused by sterical hindrance. For technical reasons all peptides contain a C-terminal glycine.



The PepStar peptide microarray is a device for detecting potential phosphorylation sites within selected and annotated phosphosites. Each spot in the microarray represents a single peptide, derived from the primary structure of the target protein

During incubation of the peptide microarray with a kinase in the presence of ATP, a phosphate moiety is added to the substrate peptides (see point 1 in Figure 1).
The incorporated phosphate moiety can be detected by phospho-imaging (see point 2 in Figure 1).

Figure 1: General principle of peptide microarray based phosphorylation site detection

All peptides are displayed in three identical subarrays on each slide. PepStar peptide microarray slide surfaces are delivered in a pre-treated form minimizing unspecific interaction of your target kinase. The data files provided by customer support contain all information needed for detailed analysis of your data, including peptide sequences and positions on the glass surface by means of a .gal-file.

4.2 PepStar™ Peptide Microarray Layout

Please refer to the .gal-file provided together with data files for identity and location of the spots on the microarray surface. The microarray side carrying the engraved label represents the surface displaying the attached peptides. The .gal-file can be opened using microarray evaluation software-modules capable of evaluating high-density microarray slides or JPT's GalViewer-software (part of data files sent by customer support). Since .gal-files are tab-separated text files, they can also 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.

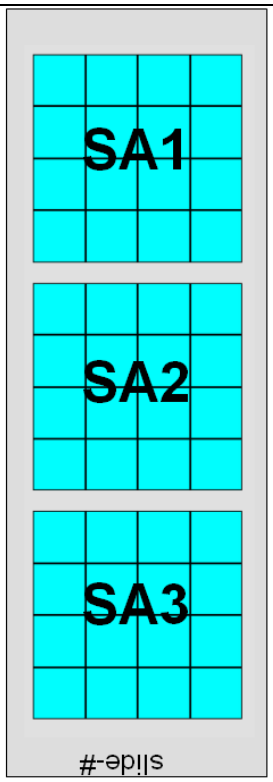


Figure 2: Schematic layout of a peptide microarray (SA=subarray).

The peptide microarray is printed in three identical subarrays (SA, see Figure 2). This enables efficient intra-chip-reproducibility tests using scatter plots or correlation functions.

For high-density peptide microarrays, each peptide subarray (SA) is printed in individual blocks (see Figure 3, details in 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.

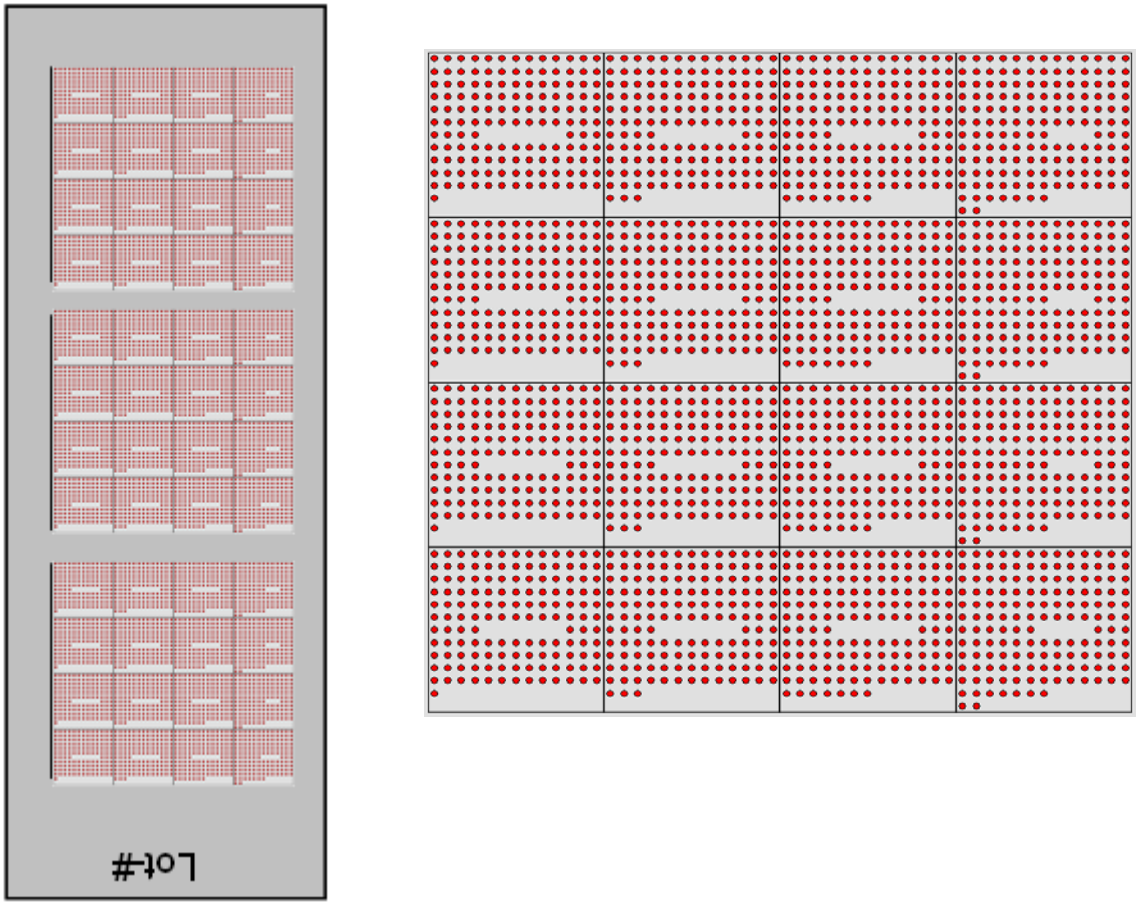


Figure 3: Exemplaric view for a subarray (left) consisting of 16 individual blocks. Each block (right) is calculated by number of rows vs. number of columns

Each peptide is printed three times in a row per subarray. With the .gal-file provided, evaluation can be performed using software modules like GenePix, ArrayPro or similar programs, which align the .gal-file induced grid onto the resulting image. JPT´s GalViewer software can be applied for qualitative analysis and spot identification.

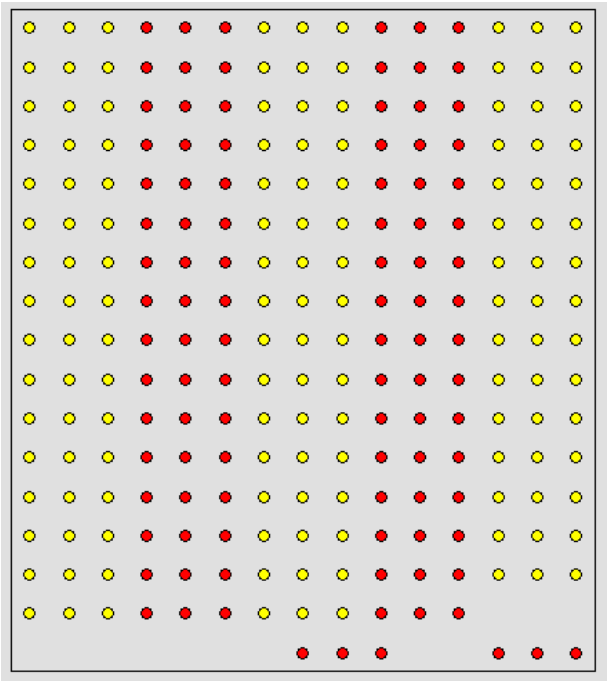


Figure 4: Exemplary block layout

Each spot represents an individual peptide, triple spots are visualized yellow/red (for details please refer to .gal-file). Blank spots between individual peptide groups are empty

5 Experimental Protocols

Note: The following procedure is provided as a guideline only. The optimal experimental conditions will vary depending on the investigated sample and instruments used and can, therefore, not be predetermined. The optimal experimental conditions must be established by the user. No warranty or guarantee of performance using this procedure with a target antibody or serum can be made or is implied.

The PepStar 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 use protein-free solutions like PEGs (polyethyleneglycol, 1 mg/mL) or PVPs (polyvinylpyrrolidones, 1mg/mL).
This keeps your kinase of interest from phosphorylating the protein used for blocking!

5.1 Additional Materials Required

1. **Catalytically active kinase:**

   JPT recommends a final concentration of about 1-10 µg/ml

2. **Specific kinase-buffer**

   For sample dilution (e.g. 3% BSA in 1x TBS-Buffer + 0.1% Tween20 (TBS-T))

3. **Detecting reagent**

   cold ATP and [γ-^{32}P]-ATP or [γ-^{33}P]-ATP

4. **PBS-buffer**

5. **Phosphoric acid (0.1M)**

   1x TBS-Buffer + 0.1% Tween20 (TBS-T)

6. **De-ionized water**

   For final washing steps of the microarrays

5.2 Additional Hardware and Software

1. **Tweezers**

   For handling of PepStar™ Peptide Microarrays

2. **Petri dish and sealing tape**

3. **Microarray Centrifuge**

   Or access to a stream of nitrogen to dry the microarray slides

4. **Phospho-imager or X-ray film exposure equipment**

5. **Analysis Software**

   Allowing quantification of the image and the assignment of signal intensities to individual peptides using the provided gal-file

5.3 General principles for incubation

5.3.1 Microarray incubation using microarray-chip-sandwich

All peptide microarrays produced by JPT have an identical layout concerning active area and spotted surface. Although the content of the microarrays varies the overall layout and dimensions are the same (see Figure 3).

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 5 in a sandwich like format.

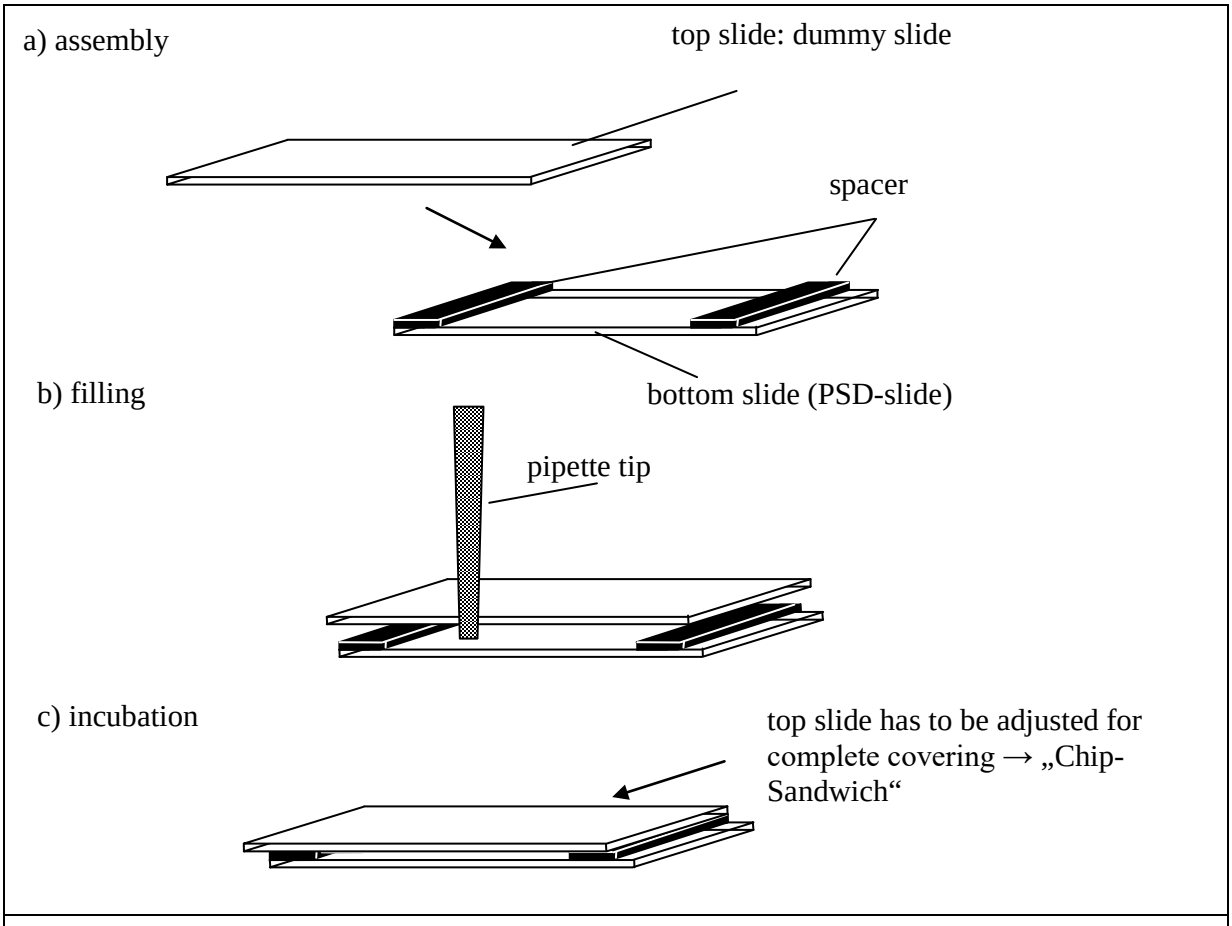


Figure 5: Assembly of “Chip-Sandwich”

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.

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 which are enclosed to the delivery (see Figure 5).

The final assay volume will depend on the thickness of the plastic spacers (enclosed spacers show a thickness of approx 200µm, which will result in a total volume of 300 to 350µL in between the two slides). The sample has to be applied between the two slides. For that end, the top slide is shifted about 1mm to one side. If the pipette tip is adjusted to a position directly over the uncovered bottom slide the capillary forces allow proper distribution of the sample solution without formation of bubbles.

After the incubation is finished, open the microarray sandwich in PBS-buffer, remove the plastic spacers and rinse the peptide microarrays thoroughly with PBS-buffer and phosphoric acid in order to remove excess ATP before continuing with the assay protocol.

5.4 Radioactive Readout in microarray chip sandwich

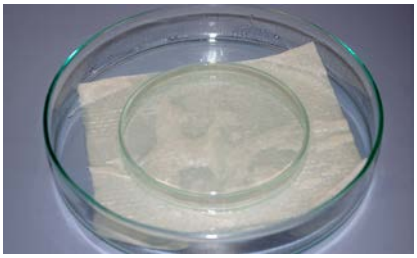
5.4.1 Workflow for Incubation using a Microarray-Chip-Sandwich*

I.	**INCUBATION**	***WITH ACTIVE KINASE*** @30°C (86°F) 1-2 hrs *Note: Final assay volume of the chip-sandwich ~ 300 µl !*
II.	**DISASSEMBLING**	in PBS-T
III.	**WASHING**	with phosphoric acid 5x 3-4 min
V.	**WASHING**	De-Ionized water
VI.	**SLIDE DRYING**	Using microarray centrifuge / by blowing a gentle stream of nitrogen on the microarray surface
VII.	**IMAGING**	Phospho-imaging or X-Ray - scanning *Note: Scan the image with highest possible resolution, at least 50 µm pixel size !*
		JPT recommends to perform several imaging runs using different exposure times. Standard exposure times used at JPT are 1-2h, 8h and 24h and 48h. If saturated signals are observed, no additional exposure time is applied.
VIII.	**DATA ANALYSIS**	Determination of signal intensities of each peptide spot. Bioinformatic evaluation of data.

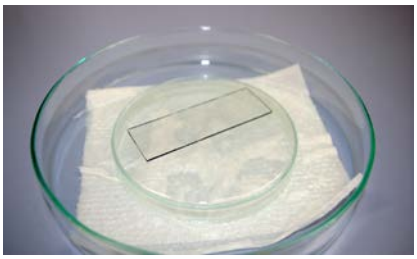
* For the design of a PepStar experiment, the attached PepStarTM Microarray Incubation Protocol can be used.

5.4.2 Prepare the slide-environment for easy handling

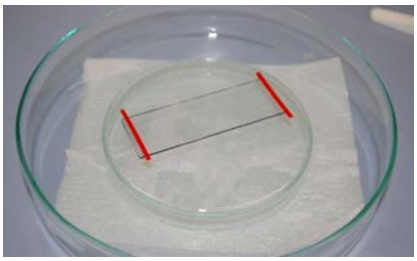
II. Place a small Petri-dish upside down into larger Petri-dish to serve as support plate for the microarray. Place a piece of wet cloth underneath the small Petri-dish. This will keep the incubation solution from evaporating once the large Petri-dish is closed.



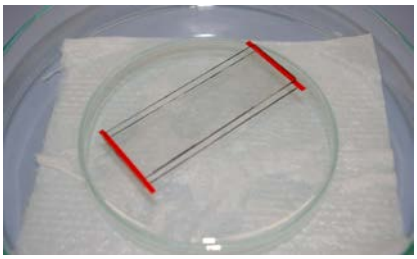
III. Place the peptide microarray slide support plate facing upward (engraved label has to be readable from top).



IV. Place enclosed spacers on both ends of the microarray.



V. Prepare the microarray sandwich according to description above. If two peptide microarrays are used make sure that peptide displaying sides are facing each other.

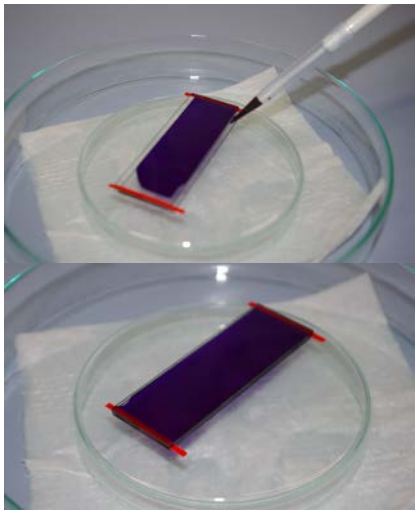


5.4.3 Pipette kinase solution and radioactively labelled ATP

	In case your kinase is known for performing autophosphorylation, please make sure to add a small amount of non-radioisotopic ATP first. This would create a non-labelled autophosphorylated kinase. In case your kinase tends to stick to the surface during the incubation time the background signal will not be increased due to autophosphorylated kinase sticking on the slide surface.

Prepare approx. 400µL of final assay solution (if enclosed spacer are used) containing your target kinase and ATP (JPT recommends a ratio of hot : cold ATP of 1:200

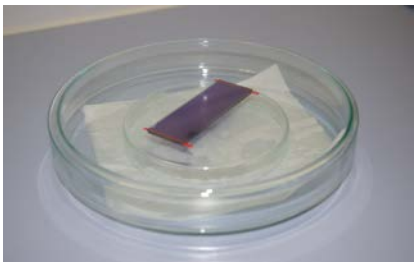
VI. Pipette the complete volume into microarray chip sandwich. Capillary forces will suck the solution in between the two slides. Make sure there are no air bubbles within the sandwich (for visualisation, the solution is coloured blue in photo)



VII. Adjust the peptide microarray sandwich.

	Make sure not to touch the microarray 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 and seal the chamber using sealing tape.



5.4.4 Incubation

IX. Incubate the peptide microarray for the appropriate time and temperature with your kinase/ATP solution. JPT recommends an incubation time of at least 2 hours at approximately 30°C (86°F).

5.4.5 Wash microarray

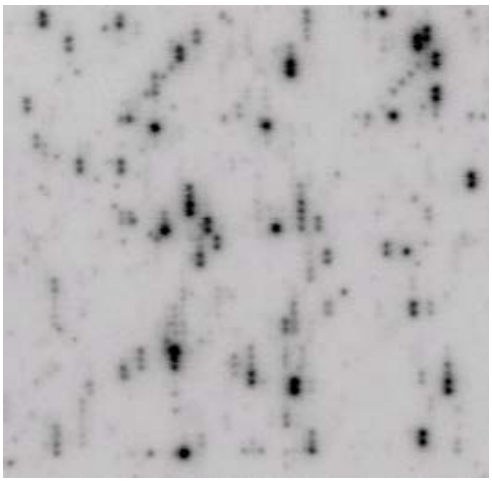
X. Open the peptide microarray sandwich in a beaker with PBS-buffer and wash the slide 5 times in a petri dish with 0.1M phosphoric acid (3-4min each wash) to stop the reaction and to remove excess radioactively labelled ATP. Ensure that the slide is properly washed with enough liquid rinsing over the slide.

XI. Wash the slide 5 times with de-ionized water (3-4min 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 using a gentle stream of nitrogen. Alternatively, a microarray centrifuge could be used for drying the microarray.

5.4.6 Image the radioisotopically labelled phosphorous

XIII. Use the dry microarray for phospho-imaging. JPT strongly recommends the detection of incorporated phosphate by phospho-imaging. For best results, use the highest resolution possible (at least 50 µm pixel size).



Peptide microarray was incubated with Abl kinase and [^{32}P] ATP. Phospho-imaging on a FLA 3000 Reader was used for readout.

5.4.7 Data Analysis

For details about application and modification of .gal files, refer to the protocol: "reading a _gal-file" enclosed with the Galviewer software.

1. Generation of a list containing signal intensities of each peptide spot by means of microarray evaluation software.
2. Calculation of the mean value for the signal intensities of spots with identical peptides (three identical spots per subarray).
3. The highest values indicate the spots showing the highest phosphorylation in your experiment.
4. Create heatmap or bar-plot diagram for visualization and identification of major binding sites (examples see Figure 6). Generate amino acid specific plots to identify key residues responsible for kinase interaction.

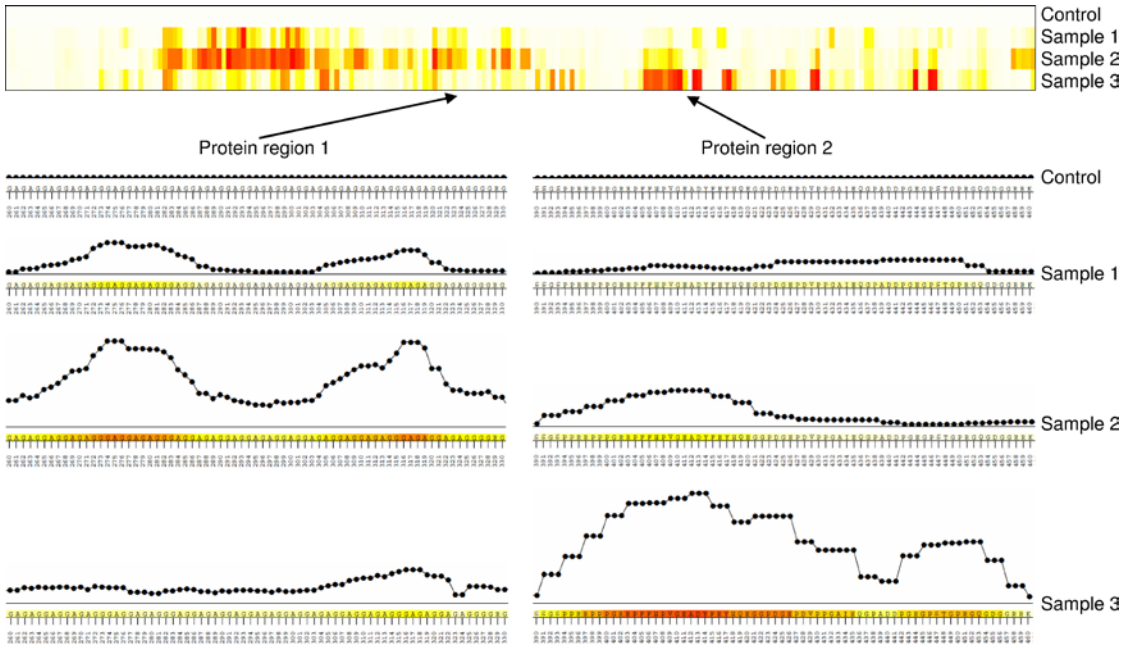


Figure 6: Visualization of microarray incubation experiment.

The numerical data were processed using JPT's proprietary evaluation and visualization bioinformatics tools. Upper panel: visualization of results by heatmap diagram. The peptides are sorted on the x-axis according to their position in the scanned protein. Lower panel: for two regions of protein, the contribution of each individual residue to the epitope recognized was calculated using information from overlapping peptides.

6 PepStar™ Microarray Incubation Protocol

General Information	**Experiment-#:**	
	Experiment Title:	
	PepStar™ Name:	
	Date:	
	Operator:	
	Comments:	

	PepStar Slide-#:				
I. Incubation	**Kinase**				
	Lot-#				
	Stock concentration				
	Assay concentration				
	Assay buffer				
	Volume Sample [µl]				
	Volume buffer [µl]				
	Incubation Temperature				
	Incubation Time				

II.	**Disassembling:**	

III.	**Washing:**	
	phosphoric acid	
	de-ionized water	

IV.	**Slide Drying:**	

VII.	**Imaging Parameters:**	
	Exposure time	
	Position on film	
	Resolution:	
	Data management:	
	Comments:	

7 Notes / Troubleshooting

Problem	Cause	Solution
Artifacts	• Dust particles and resulting scratches	• Avoid dust or other particles during each step of the experiment • Use filtered buffers and solutions only
High background	• Nature of the sample	• Make sure to perform an autophosphorylation step prior application of kinase sample to microarray sandwhich with cold ATP only
	• Type of ATP	• Make sure the hot ATP you are using doesn't contain any stabilizing agent. This could interfere with the microarray surface and create inhomogeneous background
	• Insufficient washing	• Adjustment of washing conditions • All buffers and solutions should be prepared freshly every day
	• Contaminated wash buffer	
Little or no signals	• Incubation time	• Warranty of sufficient incubation time
	• Kinase activity	• Make sure to adjust the incubation conditions to optimize the activity of your kinase sample. Parameters to take into account are incubation time, kinase buffer ingredients and incubation temperature
	• Scanning / Imaging conditions	• Make sure to use a phospoimaging film with a resolution sufficient for readout • Make sure to expose the phosphoimaging film long enough to collect enough signal

8 Related Products

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

- Phosphorylation Site Detector peptide microarrays
- Kinase Peptide Microarrays (Ser / Thr / Tyr based random libraries)
- Full kinase profiling service using JPT's PepStar™ high density peptide microarrays
- Kinase Substrate Set (biotinylated peptides in microtiter plates)
- Large collection of peptidic kinase substrates (biotinylated or fluorescently labeled)