

## Protocol

# Kinase Peptide Microarrays

Off-the-shelf peptide microarrays

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## 1 Introduction

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Our Kinase Peptide Microarrays offer a very efficient way to detect potential phosphorylation sites in selected kinase substrate peptides derived from kinase substrates 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.

## 2 List of components

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Component	Quantity
Kinase peptide microarray	glass slide(s) displaying peptides in triplicates
Blank slide engraved with "Dummy"	one blank slide per Kinase peptide microarray
Plastic spacers	2 spacers per Chip-Sandwich
Data CD-ROM	one CD-ROM containing two files (protocol as .pdf-file and sequence info as .gal-file)

## 3 Storage and Handling

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### 3.1 Storage of Kinase Peptide Microarray Slides

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

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## 3.2 Handling of Kinase Peptide Microarray Slides

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- Always handle the delicate microarrays with care.
- Never touch the microarray surface.
- Always wear laboratory gloves when handling JPT's kinase 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 2 $\mu$ m, preferably 0.4 $\mu$ m particle filters before use. Filtration of the final assay solution is recommended, too.

## 4 Additional Materials Required

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### 4.1 Materials and Solutions for Radioactive Readout

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- Catalytically active kinase
- Specific kinase-buffer
- ATP and [ $^{32}\text{P}$ ] ATP or [ $^{33}\text{P}$ ] ATP
- Phosphoric acid (0.1M)
- De-ionized water
- Additional hardware (refer to point 4.1.2)



If no specific buffer is supplied with your kinase, JPT recommends to use a general kinase buffer as described in section 4.1.1

#### 4.1.1 General Kinase Buffer

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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:

- 50mM HEPES-NaOH, pH 7.5
- 5mM  $\text{MgCl}_2$
- 5mM  $\text{MnCl}_2$
- 3 $\mu\text{M}$  sodium-orthovanadate
- 1mM DTT
- 1 $\mu\text{M}$  ATP (approx.  $3 \times 10^5$ cpm [ $^{32}\text{P}$ ] ATP)

### 4.1.2 Additional Hardware

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- Hybridisation station capable of washing and incubating slides in a temperature controlled environment (JPT recommends Tecan Hyb Station HS4X00), alternatively a microarray incubation sandwich can be used (please refer to point 6 for further details)
- Phospho-imager or X-ray film exposure equipment

## 4.2 Materials and Solutions for Non-Radioactive Readout

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- Catalytically active kinase
- Specific kinase-buffer
- ATP
- BSA solution (1mg/mL)
- Blocking buffer (refer to point 4.2.2)
- TBS-Buffer 1x (refer to point 4.2.3)
- Phospho-specific antibody (refer to point 4.2.4)
- Pro-Q Diamond stain (refer to point 4.2.5)
- Additional hardware (refer to point 4.2.6)



If no specific buffer is supplied with your kinase, JPT recommends to use a general kinase buffer as described in section 4.2.1

***Prepare all solutions using de-ionized H<sub>2</sub>O.***

### 4.2.1 General Kinase Buffer

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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:

- 50mM HEPES-NaOH, pH 7.5
- 5mM MgCl<sub>2</sub>
- 5mM MnCl<sub>2</sub>
- 3μM sodium-orthovanadate
- 1mM DTT
- 1μM ATP

### 4.2.2 Blocking Buffer

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Following the incubation with the target kinase, peptide microarray has to be blocked to prevent unspecific binding of the antibody or the phospho-specific stain. JPT strongly recommends using the blocking reagent Pierce Biotechnology Inc. (#37536) which was validated not to interact with phospho-specific antibodies or phosphor-sensing stains like Pro-Q-Diamond.

### 4.2.3 TBS Buffer

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

Dilute all components in liter of de-ionized water and adjust pH Value to 8.0

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#### **4.2.4 Antibody solution**

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Anti-phospho-tyrosine antibodies may be used to detect tyrosine phosphorylation. JPT recommends to use the FITC-labelled monoclonal anti-phospho-tyrosine antibody clone PT 66 (Sigma Aldrich; [www.sigmaaldrich.com](http://www.sigmaaldrich.com); Cat.#: F3145). For longer wavelength settings JPT recommends to use a primary/secondary antibody readout system such as anti-phospho-Tyr-100 antibody (Cell-Signalling #9411) followed by anti-mouse-Dylight 649 antibody (Pierce Biotechnology Inc, #35515).

- Dilute the antibody-stock solutions with Blocking Buffer (described in 4.2.2) to a final concentration of 1µg/mL.

For more information about the listed phosphor-specific antibodies please refer to the data sheet delivered with the appropriate antibody.

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#### **4.2.5 Pro-Q-Diamond stain**

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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 microarrays are contained in the data sheet of the stain.

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#### **4.2.6 Additional Hardware**

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You may use any microarray scanner that is compatible with standard industry glass slides (75mm x 25mm x 1mm) and which enables excitation and readout of your chosen fluorescence label. The minimal resolution required to guarantee satisfying spot-recognition is 50µm pixel size.

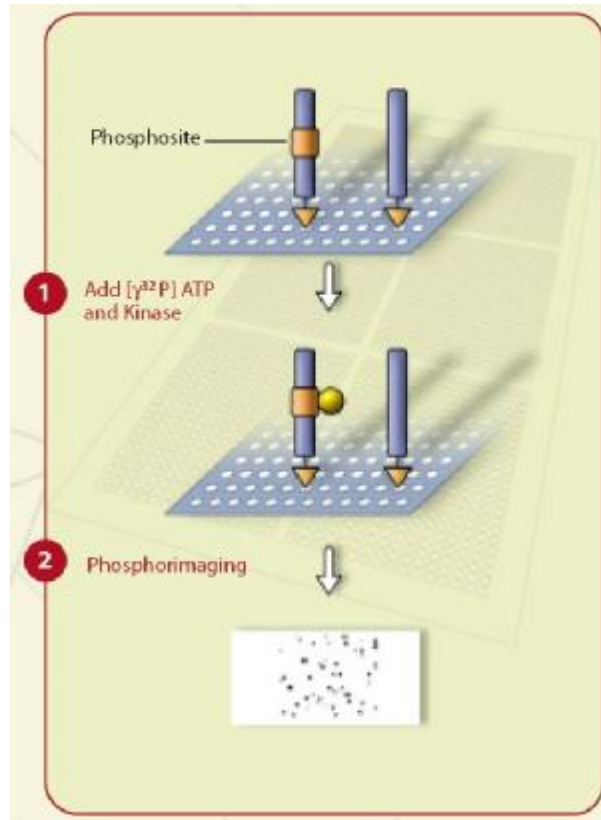
## 5 General considerations

### 5.1 Experimental basics

JPT's Kinase Peptide Microarrays are devices for detecting potential phosphorylation sites in peptides with randomly distributed amino acids surrounding a central phosphorylation site. Each spot in the microarray represents a single individual peptide.

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 phospho-imaging (if radio-actively labelled ATP was used) or by incubation with a fluorescence labelled phospho-specific antibody or dye (see Figure 1)

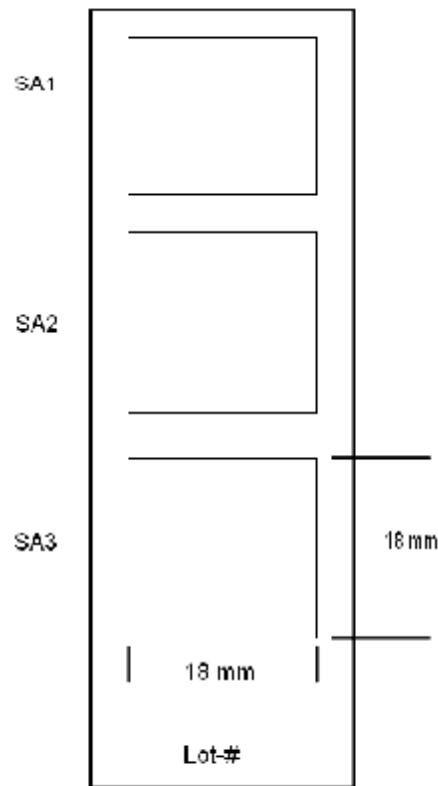


**Figure 1:** General principle of kinase peptide microarray based phosphosite detection

### 5.2 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.

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



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

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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.

### **5.3 Kinase Peptide Microarray Pretreatment**

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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 use protein-free solutions like PEG's (polyethyleneglycols, 1mg/mL) or PVP's (polyvinylpyrrolidones, 1mg/mL) only.

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

## 6 Experimental protocols

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### **6.1 General principles for incubation**

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#### **6.1.1 Fully automated microarray processing station**

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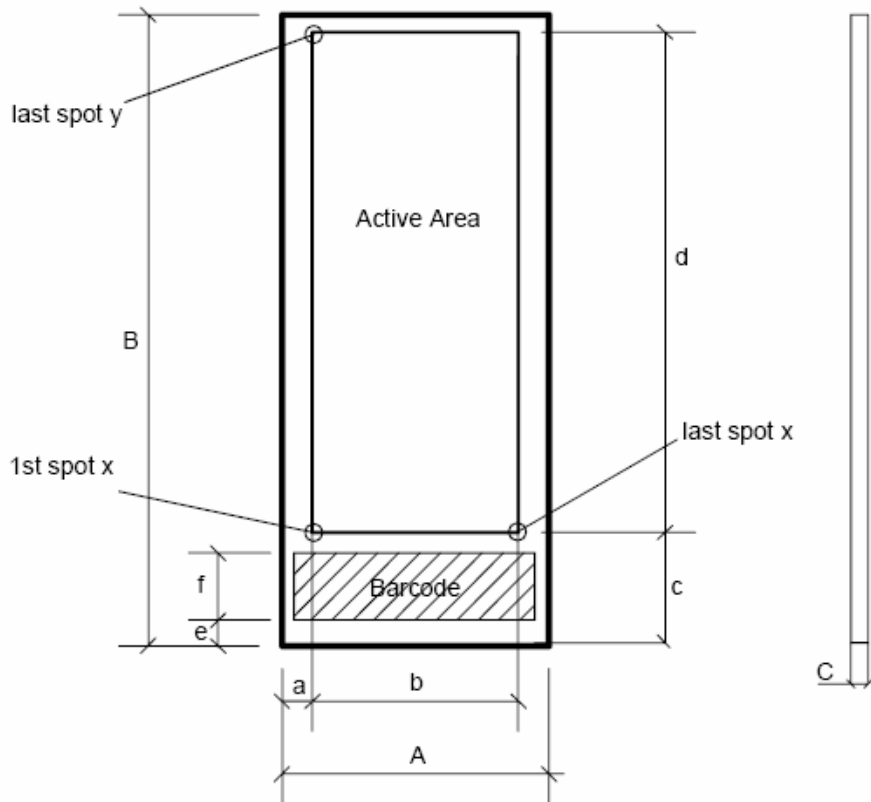
All high density peptide microarrays produced by JPT have an identical layout concerning active area and spotted surface. Although the content of the microarrays varies the overall layout and dimensions are the same (see Figure II).



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 3). JPT recommends using Tecan HS4X00 Hybridisation systems.

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

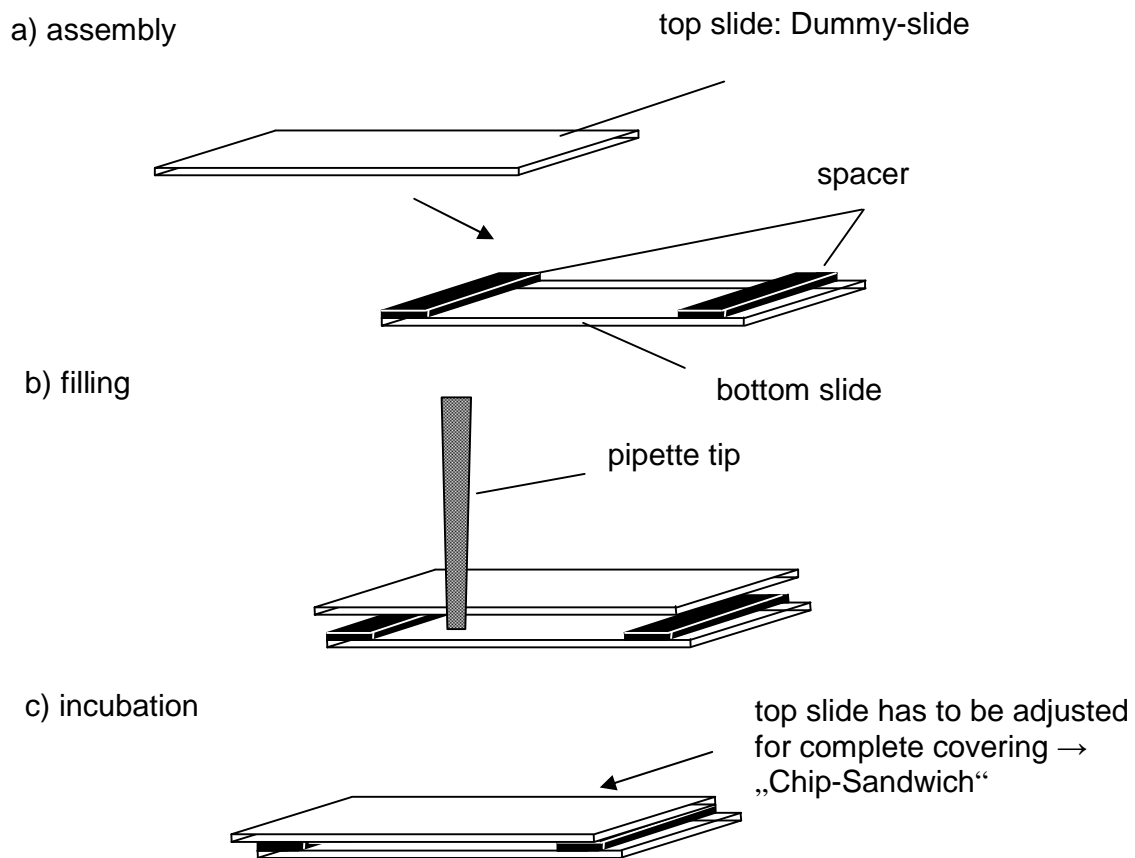


	Distance (mm)
A	25
B	75
C	1
a	3.5
b	18
c	9
d	57
e+f	1+8

**Figure 3:** 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 4 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 generated from a plastic sheet (see Figure 4).



**Figure 4:**

a) Assembly of “Chip-Sandwich” is shown. 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 1mm thickness resulting in about 500µL final assay solution (spacers enclosed). 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.

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.

## **6.2 Radioactive Readout in microarray processing station**

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**PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING THE INCUBATION!**

Please follow the instruction of the machine-manufacturer for processing microarray slides in your station.

In general the incubation and washing parameters are identical to the procedure described for the use of a microarray chip-sandwich.



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. **Especially the washing steps using phosphoric acid should be changed to an intensive washing step using buffers!**

For kinase incubation details please continue at point 6.3

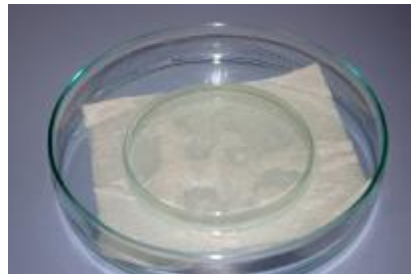
## 6.3 Radioactive Readout in microarray chip sandwich

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:

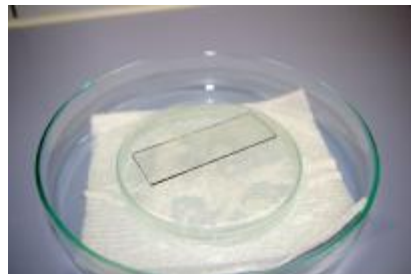
**PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING THE INCUBATION!**

### 6.3.1 Prepare the slide-environment for easy handling

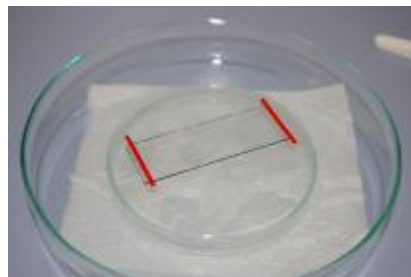
- I. Place a small Petri-dish upside down into larger Petri-dish to serve as support plate for the microarray. Place a piece of wet cloth underneath the small Petri-dish. This will keep the incubation solution from evaporating once the large Petri-dish is closed.



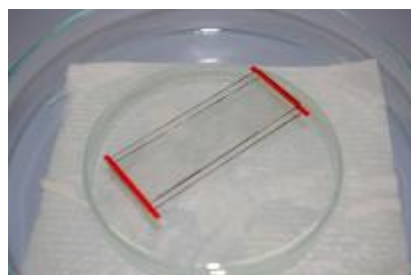
- II. Place the peptide microarray slide support plate facing upward (engraved label has to be readable from top).



- 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.



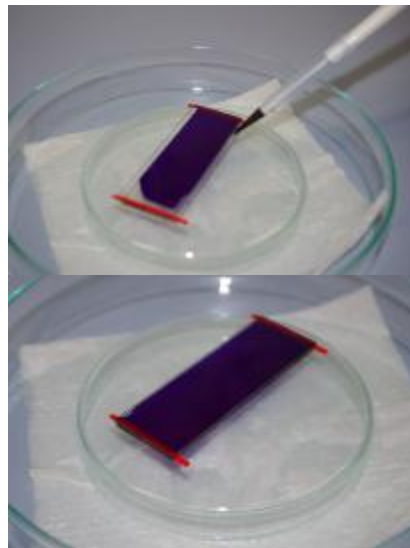
### 6.3.2 Pipette kinase solution and radioactively labelled ATP



In case your kinase is performing an autophosphorylation, please make sure to add a small amount of non-radioactive 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 autophosphorylation.

V. Prepare approx. 500 $\mu$ L of final assay solution (if enclosed spacer are used) containing your target antibody/sera.

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.

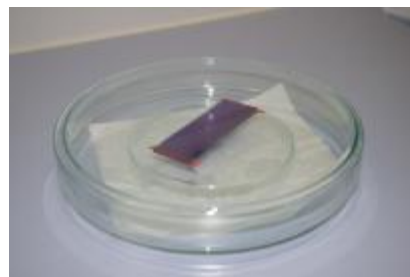


VII. Adjust the peptide microarray sandwich as described in Point 6.1.2.



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

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



### 6.3.3 Incubation

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- 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).

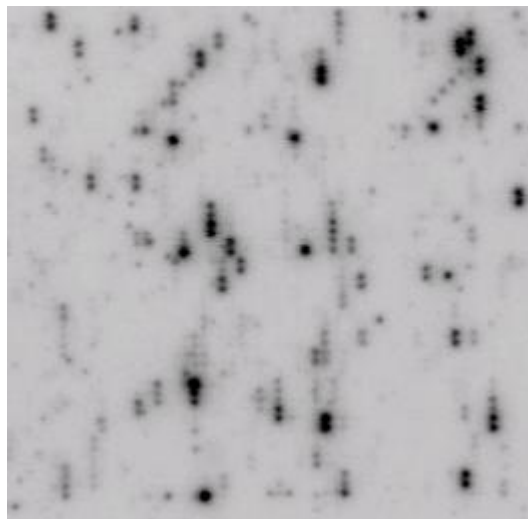
### 6.3.4 Wash microarray

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- X. Wash the slide 5 times 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.

### 6.3.5 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).



High density Peptide microarray was incubated with Abl kinase and [<sup>32</sup>P] ATP. Phospho-imaging on a FLA 3000 Reader was used for readout.

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## 6.4 Non-Radioactive Readout in microarray processing station

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**PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING THE INCUBATION!**

Please follow the instruction of the machine-manufacturer for processing microarray slides in your station.

In general the incubation and washing parameters are identical to the procedure described for the use of a microarray chip-sandwich and [<sup>32</sup>P] ATP.



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. **Especially the washing steps using phosphoric acid should be changed to an intensive washing step using buffers!**

For kinase incubation details, please continue at point 6.3

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## 6.5 Non-Radioactive Readout in microarray chip sandwich

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**PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING THE INCUBATION!**

For details of the peptide microarray incubation with kinase solution please refer to 6.3. There are no differences in handling the slides except for the radioactive ATP which has to be exchanged to non-radioactive ATP.

After kinase incubation is performed according to above protocol, please continue with Step 6.5.1

### 6.5.1 Prepare the slide for antibody or phosphor-specific-stain incubation

- I. Wash the peptide microarray for 1 hour in blocking-solution (refer to point 4.2.2). Make sure that the slide is properly washed using sufficient volumes of buffer solution.

### 6.5.2 Incubate with antibody

- II. Prepare antibody/stain solution (refer to point 4.2.4 and 4.2.5).
- III. 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.

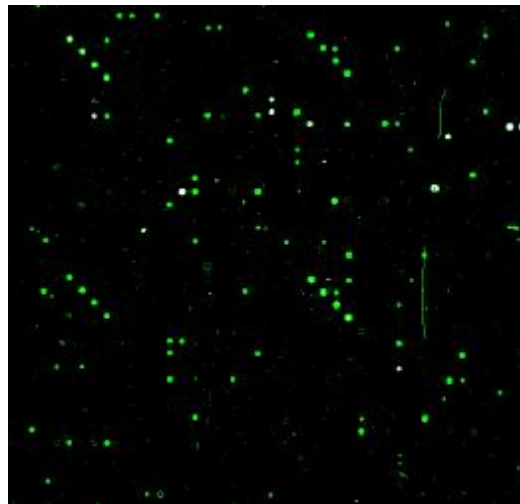
### 6.5.3 Final washing steps

- IV. Wash the peptide microarray 5 times with TBS-Buffer (refer to point 4.2.3). Make sure that the slide is properly washed using sufficient volumes of buffer solution.
- V. 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.

In case an incubation using a secondary antibody is required, please repeat steps II to VI.

### 6.5.4 Fluorescence image the peptide microarray

- VI. Perform fluorescence scans according to your microarray scanner type and antibody-label properties. Please refer to point 4.2.4 and 4.2.5 for antibody/stain recommendations



Example: JPT Peptide microarray was incubated with Abl-kinase and ATP. Fluorescent image was generated using a Genepix 4000B microarray scanner (Readout: Pro-Q phosphospecific stain)

## 7 Notes

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- 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 incubation and washing a Dummy-slide with the same solutions followed by imaging.
- Blocking with protein containing solutions like bovine serum albumin (BSA) can cause depending on the source and preparation high background signals impairing the final results. If you need such a blocking step (not recommended by JPT) please scan the Kinase peptide microarray subsequent to this blocking step and use that image as a starting point for your analysis.
- Avoid dust or other particles during each step of the experiment. Dust particles and resulting scratches will cause artefacts during the final signal readout.
- For incubation with the fluorescently labelled antibodies it is important to use metal trays with a cover or plastic trays completely covered with aluminium foil as these antibodies are sensitive towards light.
- Control incubations using antibodies alone (without prior treatment with kinase, but with addition of ATP) should be performed in parallel to the experiment to ensure that found signals are not caused by unspecific binding of the antibodies to the immobilized peptides or peptide/ATP/antibody interaction.
- Carefully adjust the final dilution of your fluorescently labelled antibodies. Microarray technology is very sensitive and therefore it could be possible to use the antibodies in a higher dilution as proposed by the manufacturer. Generally, 1:1000 dilutions of a 1mg/mL stock solution are working very well. Nevertheless, depending on the nature of the antibodies, such concentrations may yield high background signals caused by unspecific binding to the coated glass surface. If the signals within the peptide spots are high, you could test 1:5000 or 1:30000 dilutions of a 1mg/mL stock as well.

## 8 References

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Journal Biological Chemistry (2003) 277, 27839 - 27849

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

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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



## 9 Related products

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- Kinase Peptide Microarrays (Ser / Thr / Tyr based random libraries)
- Kinase Substrate Sets (Biotinylated peptides in microtiter plates)
- Full kinase profiling service using JPTs PepStar™ high density peptide microarrays
- Large collection of peptidic kinase substrates (biotinylated or fluorescently labeled)
- Phosphorylation Site Detector; collections of kinase substrate proteins displayed as peptide scans on PepStar™ microarrays
- Data packages resulting from incubation of PepStar™ high density peptide microarrays with commercially available kinases