

Protocol

Random Kinase Peptide Microarrays

Off-the-shelf peptide microarrays

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1 Introduction

The Random Kinase Peptide Microarrays offer a very efficient way to detect potential phosphorylation sites in peptide substrates using a randomized amino acid distribution surrounding a central phosphor-site (Ser, Thr, Tyr). Each of the displayed peptides represents one individual peptide sequence, NOT peptide mixtures. Following the incubation with the target kinase in the presence of radioactive ATP, incorporated phosphate can be detected by autoradiography or phosphor-imaging.

2 List of components

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 including relevant files for the specific peptide microarray (protocols as .pdf-file, sequence info as .gal-file and GalViewer software)

3 Storage and Handling

3.1 Storage of Kinase Peptide Microarray Slides

- Optimal storage conditions for JPT's peptide microarray slides are in a cool (approx. 4°C / 39°F) and dry environment. JPT's peptide microarrays are stable for at least 6 months 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 peptide microarray slides
- Please hold the microarray slides at the end, which carries the engraved data label. This label provides a unique identification of the array. 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.

**PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING YOUR EXPERIMENTS!
CAREFULLY NOTE THE HANDLING AND STORAGE CONDITIONS OF PEPTIDE
MICROARRAYS.**

**PLEASE CONTACT JPT PEPTIDE TECHNOLOGIES' TECHNICAL SERVICES FOR
ASSISTANCE IF NECESSARY.**

4 General considerations

4.1 Experimental basics

JPT's Kinase Peptide Microarrays are devices for detecting phosphorylation sites in peptides with randomly distributed amino acids surrounding a central acceptor residue 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 radioactively labelled phosphate moiety can be detected by phospho-imaging (see Figure 1)

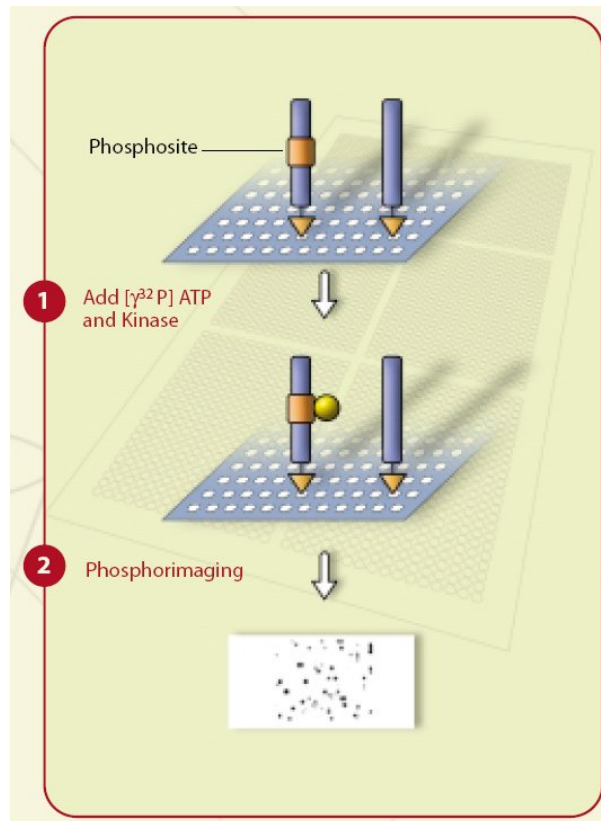


Figure 1: General principle of kinase peptide microarray based phosphosite detection

All peptides are displayed in three identical subarrays on each slide. Random kinase peptide microarray slide surfaces are delivered in a pre-treated form minimizing unspecific interaction of your target kinase. The data CD-ROM delivered with the Random Kinase peptide microarray contains 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 Kinase 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.

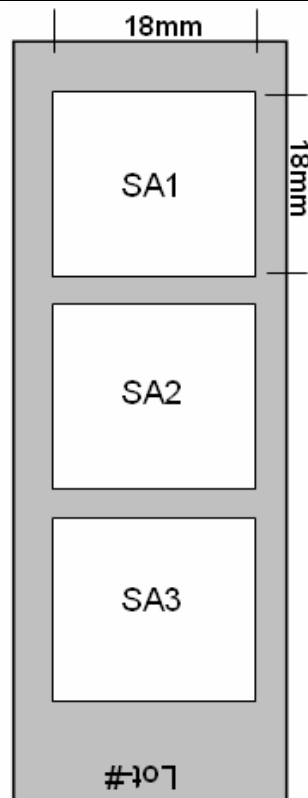


Figure 2: Schematic layout of peptide microarray (SA=subarray)

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 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.

The principal layout of the three subarrays is shown in Figure 3 and Figure 4.

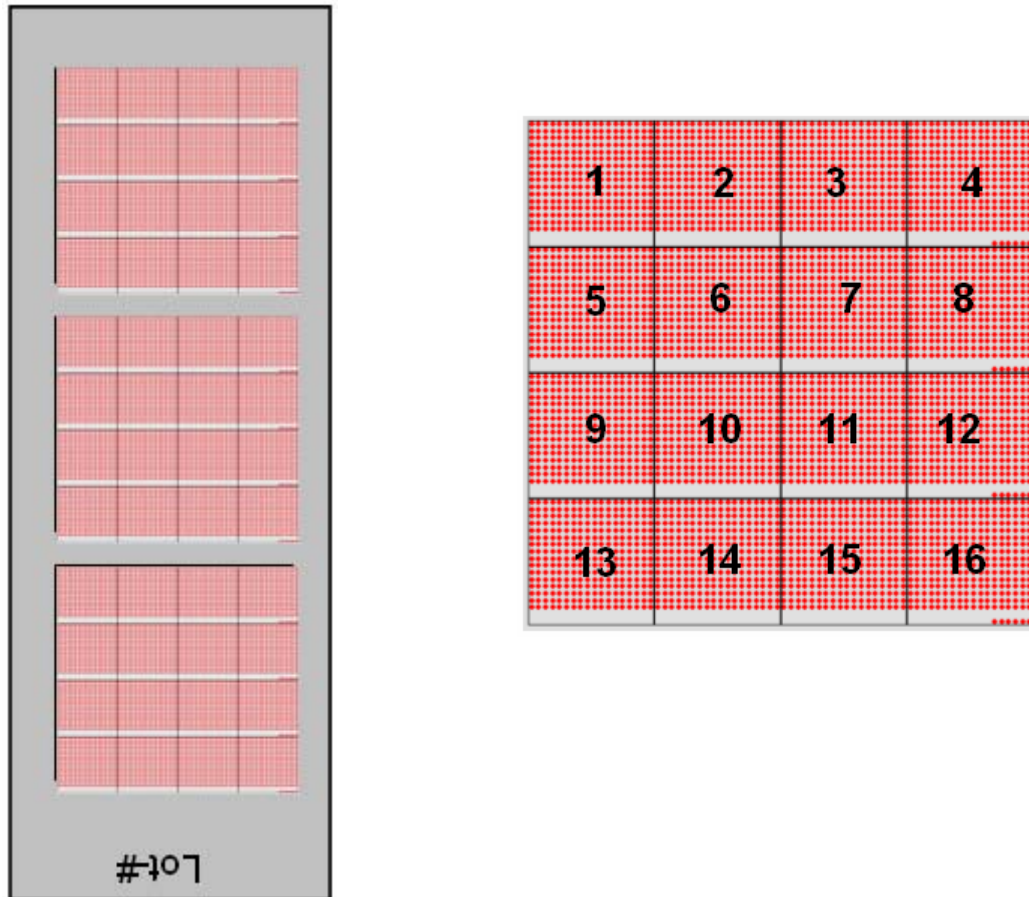


Figure 3: Exemplary view for a microarray slide with 3 subarrays (left) and a subarray consisting of 16 individual blocks (right).

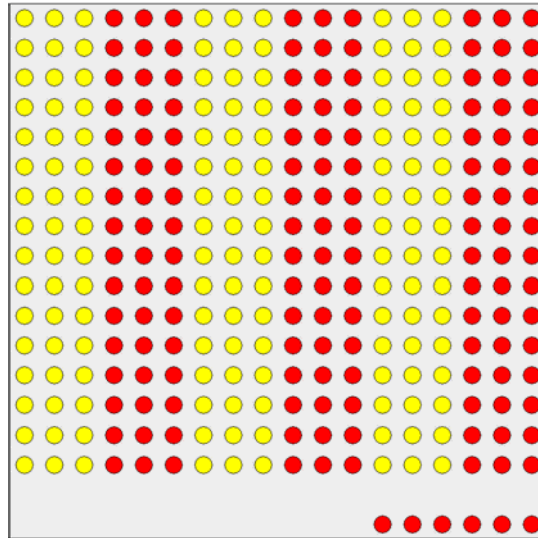


Figure 4: Exemplary block layout, each peptide is printed in triplicates (red/yellow spots), (for details please refer to .gal-file)

The three identical side by side peptides will result in a more intense signal which could be detected even with low-resolution scanning systems. Additionally, this arrangement will also be helpful to distinguish between real signals (looks like a short line or minus sign in phospho-image) and potential artefacts caused by dust particles (round spots in phospho-image).

4.3 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 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!

4.4 General principles for incubation

4.4.1 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 5).

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.

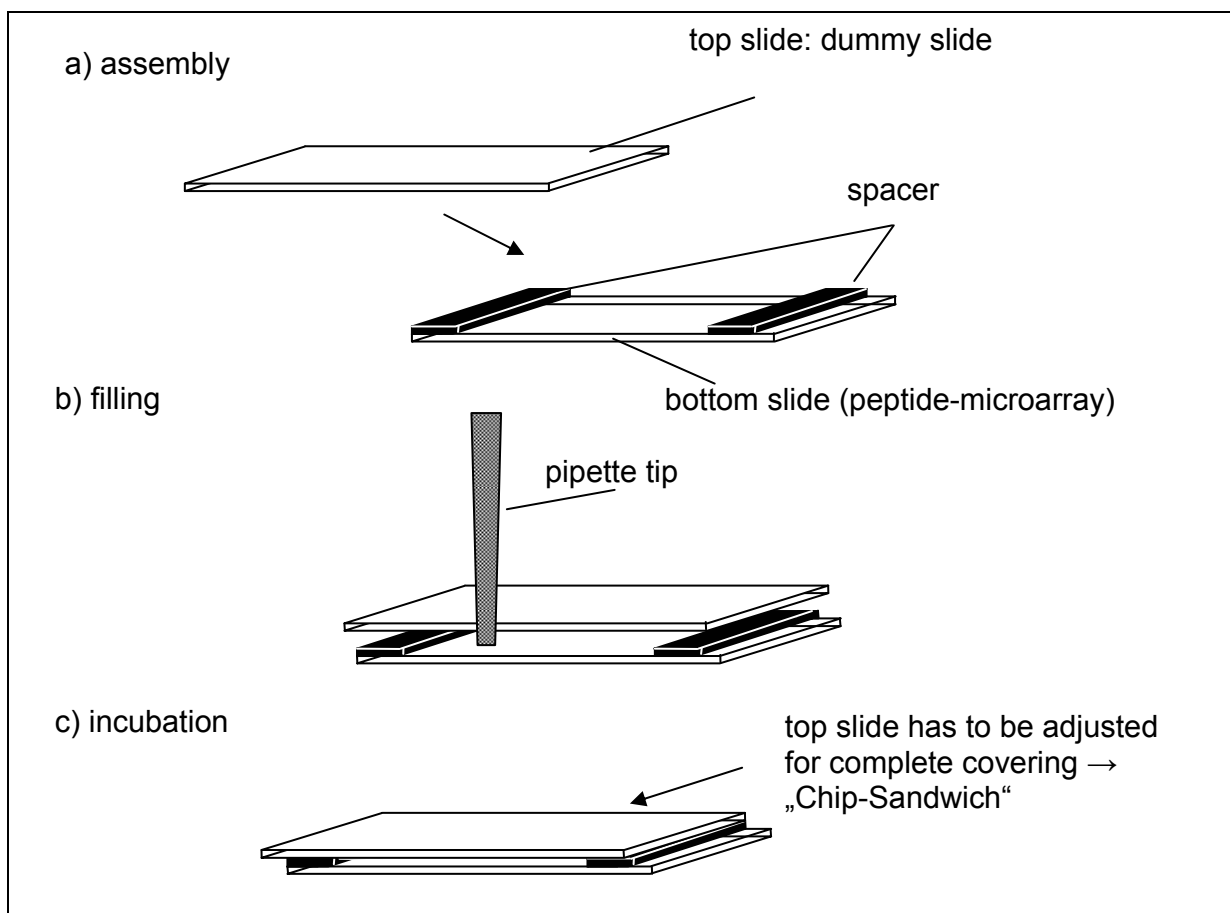


Figure 5: Assembly of "Chip-Sandwich"

- 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.
- 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.
- 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 can be replaced by another peptide-displaying chip. Please make sure that in such 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 200 μ m, which will result in a total volume of 300 to 350 μ L 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 Experimental protocols

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

5.1 Additional Materials and Solutions Required

- Catalytically active kinase
- Specific kinase-buffer
- cold ATP and [$\gamma^{32}\text{P}$] ATP or [$\gamma^{33}\text{P}$] ATP
- PBS-buffer
- Phosphoric acid (0.1M)
- De-ionized water



If no specific buffer is supplied with your kinase, JPT recommends to use a general kinase buffer as described in section 5.1.1

5.1.1 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:

- 50mM HEPES-NaOH, pH 7.5
- 5mM MgCl₂
- 5mM MnCl₂
- 3 μM sodium-orthovanadate
- 1mM DTT
- 0.1mg/mL BSA
- 10 μM ATP (non radioisotopic)
- 1 μM ATP (approx. 3×10^5 cpm [$\gamma^{32}\text{P}$] ATP) or [$\gamma^{33}\text{P}$] ATP

5.1.2 Additional Hardware

- Petri dish and sealing tape
- Phospho-imager or X-ray film exposure equipment

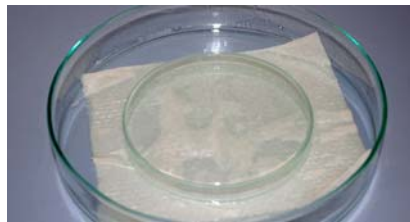
5.2 Radioactive Readout in microarray chip sandwich

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:

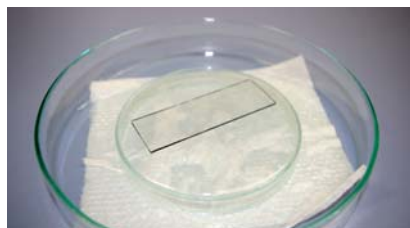
PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING THE INCUBATION!

5.2.1 Prepare the slide-environment for easy handling

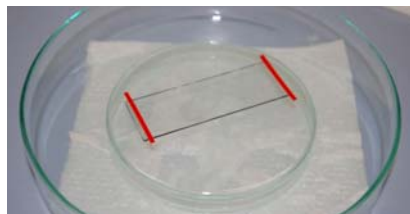
- I. Place a small Petri-dish upside down into larger Petri-dish to serve as support plate for the microarray. Place a piece of wet cloth underneath the small Petri-dish. This will keep the incubation solution from evaporating once the large Petri-dish is closed.



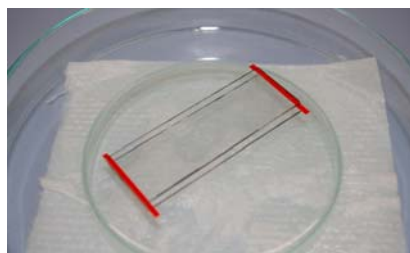
- II. Place the peptide microarray slide support plate facing upward (engraved label has to be readable from top).



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



- IV. Prepare the microarray sandwich according to description above (4.4.1). If two peptide microarrays are used make sure that peptide displaying sides are facing each other.



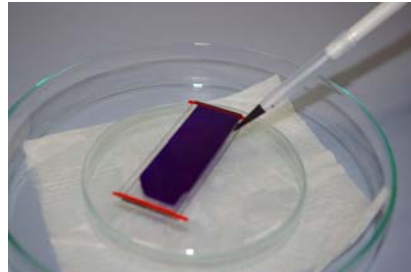
5.2.2 Pipette kinase solution and radioactively labelled ATP



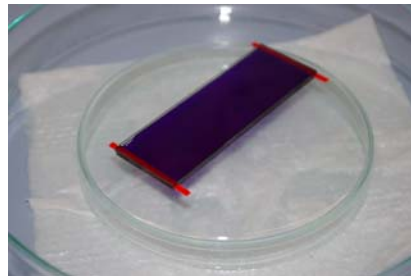
In case your kinase is performing an autophosphorylation, please make sure to add a small amount of non-radioactive ATP first. This would create a non-labelled autophosphorylated kinase. In case your kinase tends to stick to the surface during the incubation time the background signal will not be increased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

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

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).

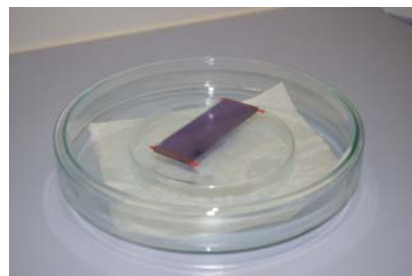


VII. Adjust the peptide microarray sandwich as described in point 4.4.1.



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 seal the chamber using sealing tape..



5.2.3 Incubation

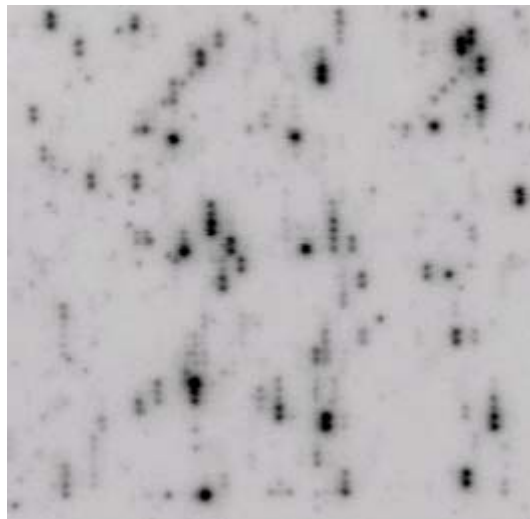
- IX. Incubate the peptide microarray for the appropriate time and temperature with your kinase/ATP solution. JPT recommends an incubation time of at least 2 hours at approximately 30°C (86°F).

5.2.4 Wash microarray

- 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.

5.2.5 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 [³²P] ATP. Phospho-imaging on a FLA 3000 Reader was used for readout.

6 Related products

- Avoid dust or other particles during each step of the experiment. Dust particles and resulting scratches will cause artefacts during the final signal readout.
- JPT recommends filtering all solutions prior performing incubation steps on peptide microarray. For filtering please use a 2µm or preferably a 0.4µm sterile filter.
- If your kinase tends to autophosphorylation, please preincubate your kinase with cold ATP only before adding hot ATP. In case your kinase shows hydrophobic interactions to the slide surface, the background will not be increased.

7 Related products

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

- Kinase Peptide Microarrays (annotated phosphosites)
- Phosphorylation Site Detector peptide microarrays
- 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)