Protocol

Kinase Substrate Sets

Ready-to-use peptide sets for kinase profiling

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1 Introduction

Enzymatic protein phosphorylation by protein kinases is one of the key regulation events in biologically important processes. To study protein kinases the identification of detailed substrate information for protein kinases is critical.

JPT Peptide Technologies' Kinase Substrate Set is a selection of biotinylated peptides derived from human phosphorylation sites for rapid screening of protein kinases. These biotinylated peptides bind strongly to streptavidin making them compatible with standard protein kinase assays, e.g. scintillation proximity assays (SPA), Flashplate assays, or the direct transfer to streptavidin-coated membranes.

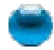
2 List of Components

Component	Quantity	Format
Kinase Substrate Set (red/blue plate)	1x2 plates	384 well format
Replicator	2	384 well format
Product Documantation	1	Microsoft Excel

3 Storage

- Kinase Substrate Set microtiter plates should be stored at -20°C.
- All other components may be stored at room temperature.

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING YOUR EXPERIMENTS! CAREFULLY NOTE THE STORAGE CONDITIONS OF THE KINASE SUBSTRATE SET.
PLEASE CONTACT JPT PEPTIDE TECHNOLOGIES´ TECHNICAL SERVICES FOR ASSISTANCE IF NECESSARY.

 4 Additional Materials required

- Kinase of adequate activity (we recommend a final activity of 1 U per mL)
- Kinase buffer with ATP
- [γ-^{32}P]ATP or [γ-^{33}P]ATP (we recommend [γ-^{32}P]ATP)
- Agent to capture biotinylated peptides subsequent to incubation with target kinase. We recommend streptavidin-coated SPA-Beads, Flashplates or membranes (e.g. Sam^{2}® Biotin Capture Membrane, Promega Corporation, USA) depending on your favored assay.
- Instrumentation to detect radioactivity such as Phosphorimager or X-ray film exposure equipment

5 General considerations

5.1 Experimental basics

JPT Peptide Technologies' Kinase Substrate Set includes two 384-well MTPs each containing 360 biotinylated peptides derived from human phosphorylation sites (>95% purity, 0.25 nmol per well). The MTPs can be used in all standard kinase assays, which use streptavidin to capture biotinylated peptides (e.g. streptavidin-coated Flashplates, streptavidin-coated SPA-Beads or streptavidin-coated membranes). Since the Kinase Substrate Set is provided in a standard 384 MTP format, it can be easily adapted to your automated kinase assay or allows for hand-made assays within less then one day (see Figure 1 for potential assay variations)

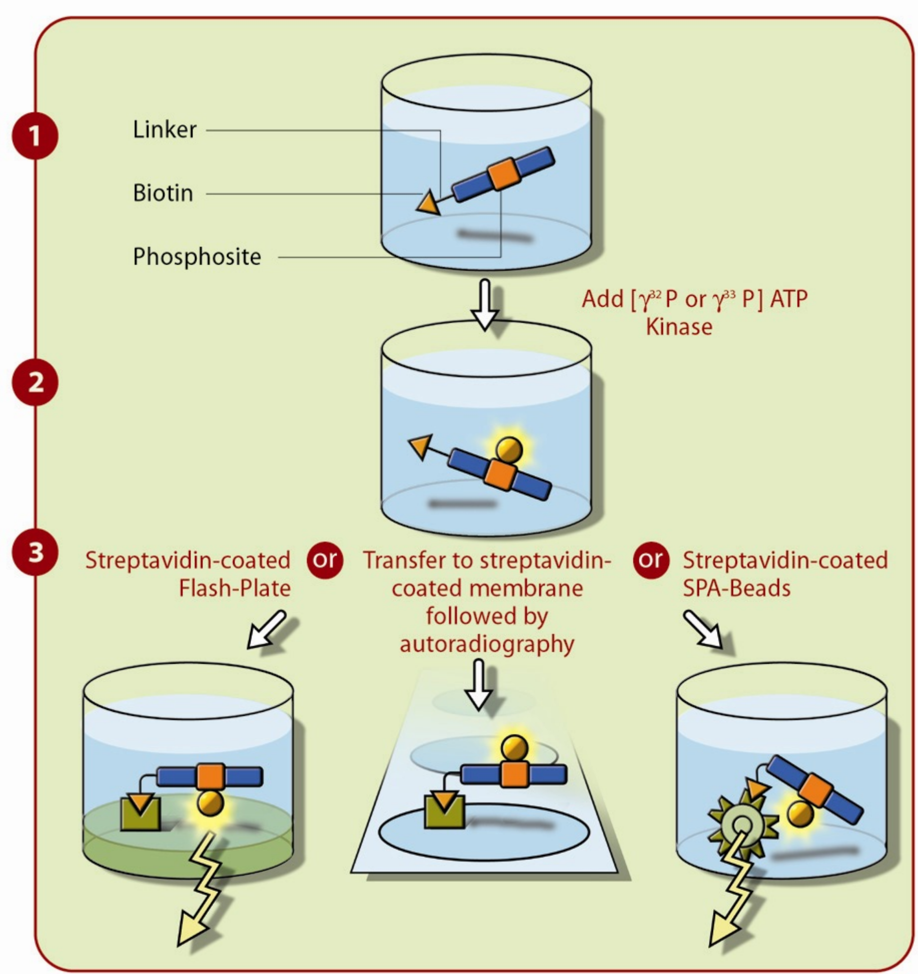


Figure 1: demonstration of potential assay variations

5.2 Microtiterplate Layout

The product documentation provided with the set contains all information needed for the detailed analysis of your data including peptide sequences, Swiss-Prot accession number, and names of the appropriate human proteins. The schematic layout of the microtiterplate is shown in Figure 2.

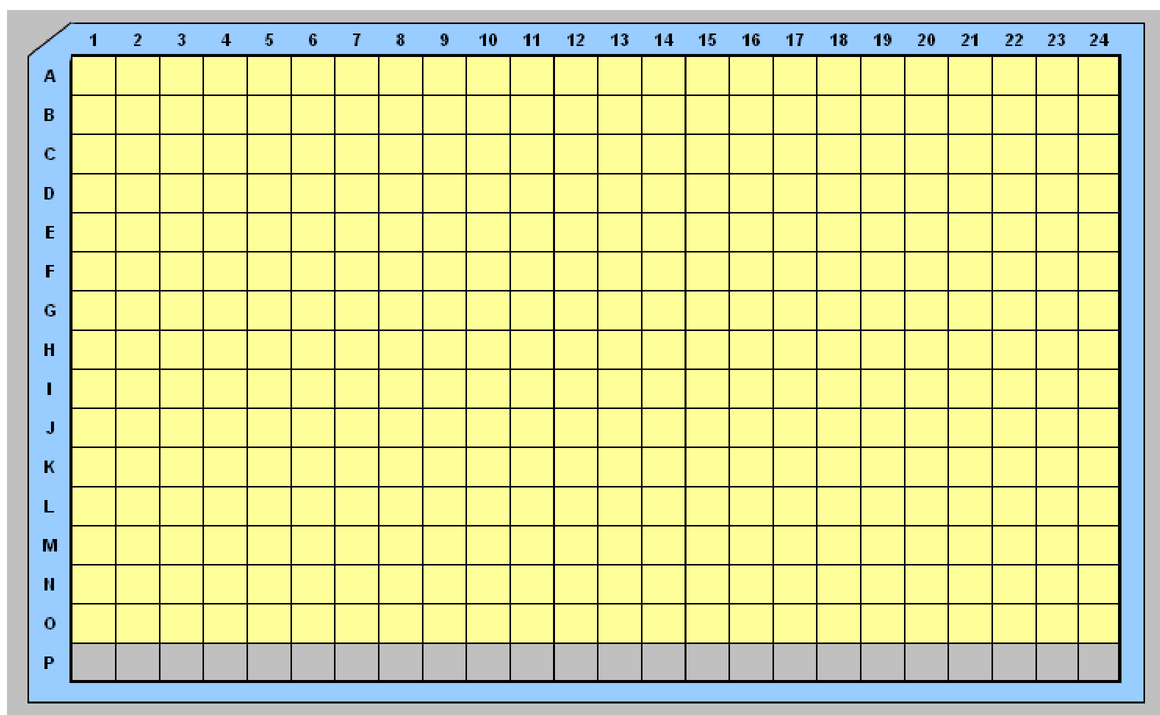


Figure 2: schematic layout of microtiterplate. Cavities P1 to P24 are empty, so that buffer, ATP and kinase controls can be included in assay procedure

6 Experimental protocols

The following procedure is recommended for the Kinase Substrate Set Assay using Sam$^{2®}$ Biotin Capture Membranes. Please note that you will need to optimize buffer and reaction conditions for your specific target kinase.

> Alternatively, you may also use streptavidin-coated SPA-Beads or Flashplates to capture the biotinylated peptides subsequent to incubation. Please note that you might have to optimize washing procedures, transfer of samples, etc. depending on your protocol used for SPA-Beads or Flashplates.

6.1 Preparation of Kinase Substrate Set

- Let the Kinase Substrate Set adjust to room temperature.
- Using tweezers, carefully remove the foil covering the MTPs.
- Prepare your assay buffer containing ATP, [γ-^{32}P]ATP and adequate activity of kinase. We recommend a final ATP concentration of 10 µM supplemented with about 50 µCi/ml [γ-^{32}P]ATP. Please note that some kinases may need higher ATP concentrations for effective catalysis of phosphoryl transfer. In these cases, we recommend to keep the ratio of ATP and [γ-^{32}P]ATP constant.

6.2 Pipette kinase solution and radioactively labelled ATP

- Transfer an adequate volume of assay buffer (we recommend 20 µl/well resulting in a 12.5 µM peptide solution) into each well to adjust to your desired final substrate concentration. Normally, the peptides are dissolved within 5 min at room temperature. Gently mixing of the MTPs will help to dissolve the peptides. (see Figure 3, 1)

6.3 Incubation and Termination of reaction

- Incubate the MTPs at 30°C for 2 hours. Please note that reaction conditions might need to be optimized for your kinase regarding incubation temperatures and reaction time.
- Terminate the assay by adding 0.5 volumes of 7.5 M guanidine hydrochloride solution in water (resulting in a final concentration of 2.5 M).

6.4 Transfer of solution – Replicate Step

- Use the provided replicator to transfer a sample from each well to the streptavidin-coated flat surface (we recommend Sam$^{2®}$ Biotin Capture Membranes from Promega), please refer to Figure 3, 2a and 2b.
  - Unpack the replicator carefully avoiding any rough handling, which might lead to twisted pins. Place the streptavidin-coated surface and the microtiter plate on a flat horizontal place in front of you close to each other. Please use both your hands to gently hold the replicator at the long sides. Slowly move the replicator over the microtiter plate so that each pin is exactly above its respective well on the microtiter plate. This is most conveniently done with your eyes just above the level of the microtiter plate.
  - Slowly move down the replicator so that each pin fits into its respective well and delicately touches the bottom of the well. Move up the replicator **very slowly** until all pins have left their well. Move the replicator slowly over the streptavidin-coated flat surface in a way that all pins will touch the membrane. Move the replicator down slowly onto the streptavidin-coated flat surface so that all pins touch the surface of the membrane simultaneously. Let the replicator rest for 5 seconds on the membrane and then slowly move it up off the membrane.
  - Mark the corner of the streptavidin-coated flat surface that corresponds to the sample taken from position A1 in the microtiter plate

6.5 Washing of the SAM² Biotin Capture-Membrane

- Put the membrane in a dish that can be filled with at least 200 ml of liquid. Wash the membrane according to the following protocol using 100 ml each time using an orbital shaking system (Make sure that shaking speed is not too high to avoid spillage!):
  - 1 time for 30 seconds with 2 M NaCl.
  - 3 times for 2 minutes each with 2 M NaCl.
  - 4 times for 2 minutes each with 2 M NaCl in 1% H3PO4.
  - 2 times for 30 seconds each with dionized water.
  - Optional: Final wash with 95% ethanol for 15 seconds.
- Dry the membrane on a piece of aluminium foil under a heat lamp for 5-10 minutes or air-dry at room temperature (30-60 minutes). Drying can be shortened by the optional final 95% ethanol wash.

6.6 Image the radioactive phosphorous

- Expose Sam$^{2®}$ Biotin Capture Membranes to a Phosphor-Imager Screen or X-Ray film. For easier handling we recommend to wrap the membranes still slightly wet in plastic foil (e.g. Saran Wrap®) before exposure (see Figure 3, 3)

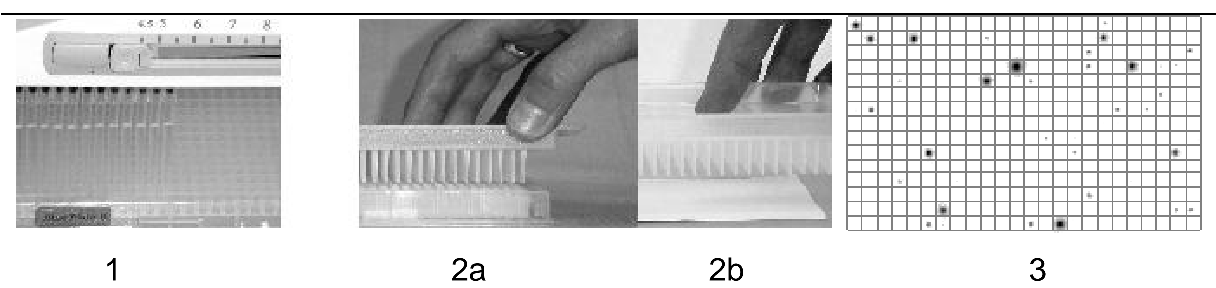


Figure 3

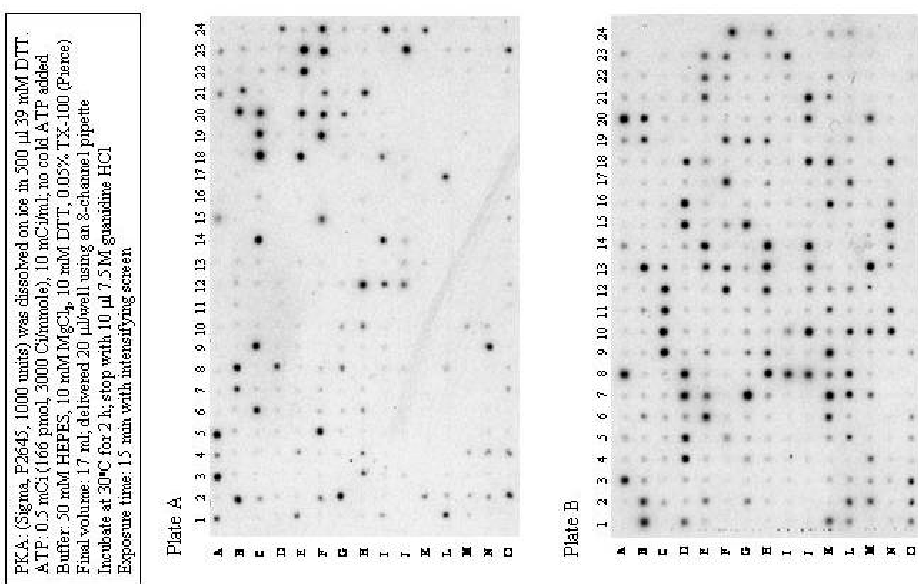
Pipette and Incubate	Replicate (Visualization, always wear gloves!)	Image and Evaluate (CKII in Blue Plate)

7 Notes

- Use radioisotopes with due caution in accordance with the regulations of your institution.
- Kinases vary in activity and the concentration of ATP they require.
- Due to the nature of these enzymes, the optimal concentration of ATP, [γ-^{32}P]ATP, and buffer as well as the specific assay conditions need to be determined empirically.
- Please contact us, if you require further information and assistance!

We would very much appreciate your comments and suggestions and would be absolutely delighted to hear of your successful experiments like in the following:

... Chana Rabiner, a doctoral student, carried out the experiment shown below. We were very pleased with how easy it was to do the experiment with the equipment we had on hand - for example, we just used our 8-channel micropipette to add the enzyme/ATP mix.
Betty A. Eipper, Ph.D., Dep. of Neuroscience, U of CT, Health Center, Jan. 2004, with kind permission



8 Related products

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

- Kinase Peptide Microarrays (annotated phosphosites)
- Kinase Peptide Microarrays (Ser / Thr / Tyr based random libraries)
- 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
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