

Protocol

Tyrosine Phosphatase Substrate Set

Ready-to-use peptide set for phosphatase profiling

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Table of content

1	INTRODUCTION	3
2	LIST OF COMPONENTS	3
3	STORAGE AND HANDLING	4
3.1	Storage of Phosphatase Substrate Set	4
3.2	Product description	4
4	ADDITIONAL MATERIALS REQUIRED	5
5	GENERAL CONSIDERATIONS	6
5.3	Experimental basics	6
5.4	Phosphatase Substrate Set microtiter plate Layout	7
6	EXPERIMENTAL PROTOCOLS	8
6.1	Assay Procedure	9
7	NOTES	11
8	RELATED PRODUCTS	13

1 Introduction

Enzymatic protein phosphorylation and de-phosphorylation are key regulation events 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 peptides with the phosphatase of interest.

Tyrosine Phosphatase Substrate Set is a selection of 360 phosphopeptides derived from human phosphorylation sites for rapid screening of protein phosphatase activity. These phosphopeptides contain one phosphorylation site, which is chemically phosphorylated. Upon incubation with your phosphatase, the released phosphate ions can be detected in a non-radioactive assay using complex formation to phosphate-binding dyes like molybdate/malachite green.

2 List of components

Component	Quantity	Format
Tyrosine Phosphatase Substrate Set	1	384 well format
Control microtiter plate	1	384 well format
Data CD-ROM	1	Microsoft Excel

3 Storage and Handling

3.1 Storage of Phosphatase Substrate Set

- Phosphatase 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 HANDLING AND STORAGE CONDITIONS OF THE PHOSPHATASE SUBSTRATE PLATE.

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

3.2 Product description

JPT Peptide Technologies' Ready-to-screen Tyrosine Phosphatase Substrate Set is comprised of a total of 360 phosphopeptides derived from human phosphorylation sites. The Tyrosine Phosphatase Substrate Set comes in a 384-well microtiter plate (250 pmol per well) with several empty wells available for positive and negative controls (wells P1-P24). Subsequent to incubation with the target phosphatase, released phosphate ions can be detected by complexation to phosphate-binding dyes, i.e. molybdate/malachite green. The sensitivity of the assay is sufficient to detect low nanogram levels of phosphatase using the molybdate/malachite green dye (Van Veldhoven, P.P & Mannaerts, G.P. (1987) *Anal. Biochem.* **161**, 45-48; Ekman, P. & Jager, O. (1993) *Anal. Biochem.* **241**, 138-41; Harder, K.W. et al. (1994) *Biochem. J.* **298**, 395-405).

Please use the provided control plate to determine the phosphate content of every single component used for the assay buffer! Obviously, any exogenous phosphate ions introduced with the reagents would corrupt the results of your experiment.

The data CD-ROM included with the set 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 phosphosites. In case the individual human peptide sequence is also found in other organisms, all respective Swiss-Prot accession numbers are given.

4 Additional Materials Required

- Phosphatase of adequate activity (we recommend a final activity of 0.1 U per well)
- Phosphatase assay buffer (please refer to chapter VII. Notes)
- Agent to capture released phosphate ions subsequent to incubation with target phosphatase. We recommend the malachite green additive (AK-111 *BIOMOL GREEN*[™] Reagent) from BIOMOL (www.biomol.com, AK111-0250).
- Microtiter plate reader capable of measuring absorbance at 620 nm to at least a 3-decimal accuracy. Wavelengths in the range of 620-660 nm may be used with very similar sensitivities.

5 General considerations

5.3 Experimental basics

JPT Peptide Technologies' Tyrosine Phosphatase Substrate Set includes one 384-well MTP containing 360 phosphopeptides derived from human phosphorylation sites (0.25nmol per well). The MTPs can be used in all standard phosphatase assays, which use phosphate-binding dyes to capture phosphopeptides (e.g. streptavidin-coated Flashplates, streptavidin-coated SPA-Beads or streptavidin-coated membranes). Since the Phosphatase Substrate Set is provided in a standard 384 MTP format, it can be easily adapted to your automated phosphatase assay or allows for hand-made assays within less than one day (see Figure 1 for assay demonstration).

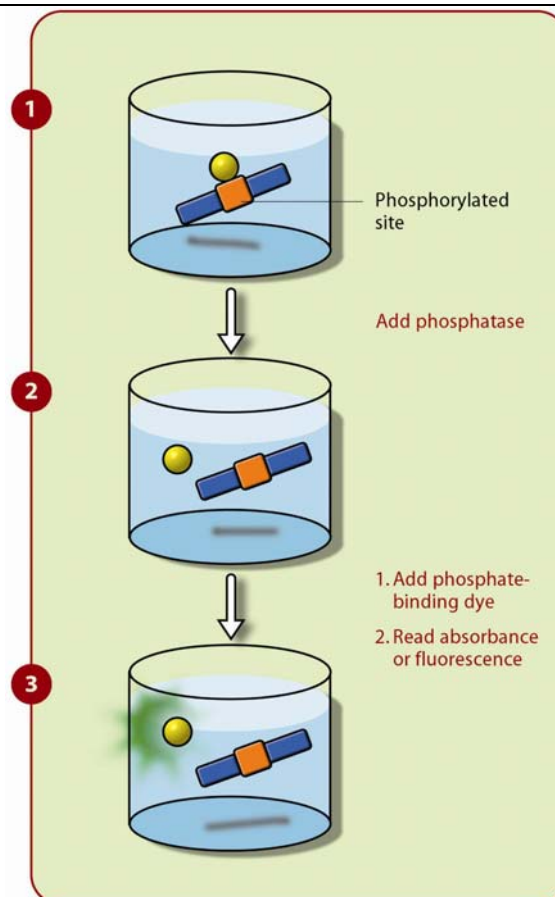


Figure 1: demonstration of Tyrosine Phosphatase Substrate Set

5.4 Phosphatase Substrate Set microtiter plate Layout

The data CD-ROM 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.

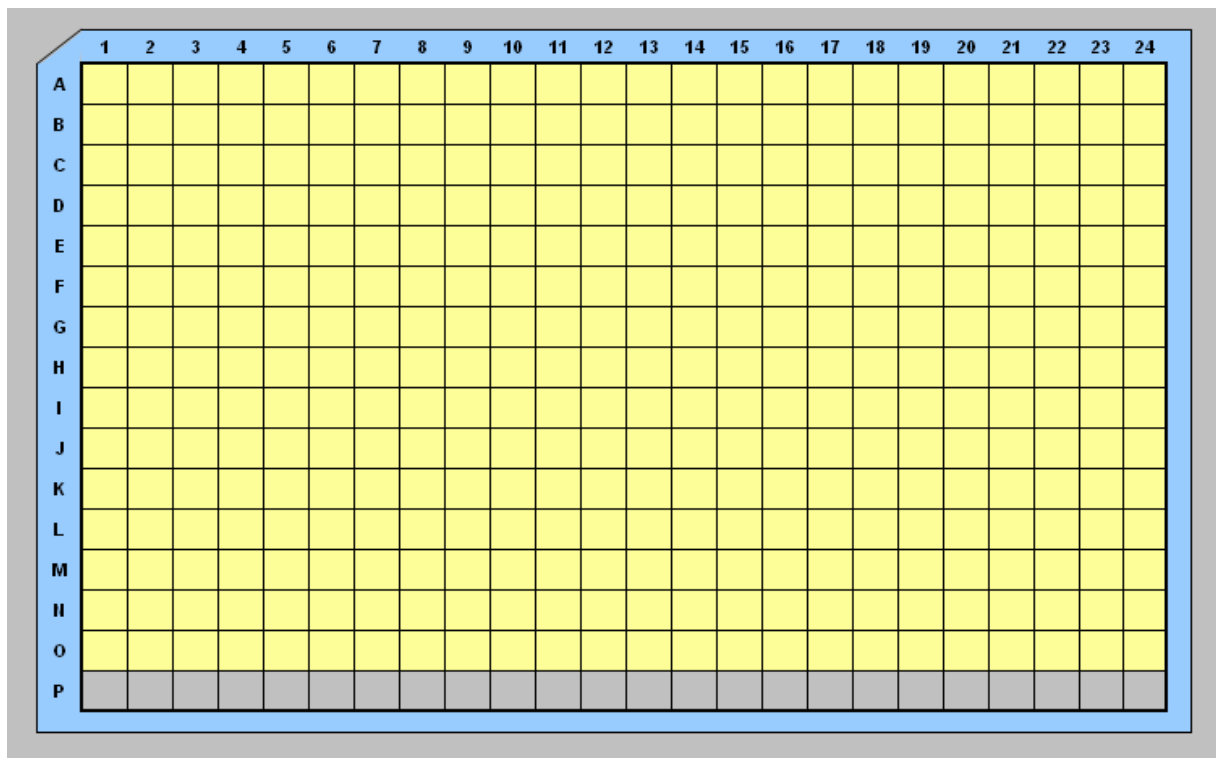


Figure 2: schematic layout of microtiter plate

6 Experimental protocols

The following procedure is recommended for the Tyrosine Phosphatase Substrate Set assay using the malachite green additive from BIOMOL. Please note that you will need to optimize buffer and reaction conditions for your specific target phosphatase.

Note: The following procedure is given as 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 Tyrosine Phosphatase Substrate Set is designed to assay enzyme activity directly in the 384-well microtiter plate. Phosphatase reactions are performed in a final volume of 25-50 μL resulting in a phosphopeptide concentration of 10 to 5 μM , respectively.

Before starting the experiment confirm that your enzyme preparation, water, and buffer components used do not contain free phosphate.

We are providing an additional empty microtiter plate (control plate) for this purpose. To determine possible phosphate contamination, add at least 25 μL of the malachite green additive solution to 25 μL of each single buffer component, to 25 μL of the phosphatase preparation, or to 25 μL of the experimental sample. Any sample containing free phosphate will turn green within 15-30 min of incubation at room temperature. Read absorbance at 620-660 nm.

6.1 Assay Procedure

1. Let the Tyrosine Phosphatase Substrate Set adjust to room temperature.
2. Carefully remove the foil covering the microtiter plate.
3. Prepare your final assay buffer containing adequate activity of phosphatase. We recommend a final phosphatase activity of about 0.1 U per well.
4. Transfer an adequate volume of assay buffer including the phosphatase (we recommend 25 μ L/well resulting in a 10 μ M phosphopeptide solution) into each phosphopeptide-containing well (A1-O24) to adjust to your desired final substrate concentration. Normally, the peptides are dissolved within 5 min at room temperature. Gentle agitation of the microtiter plate will help to dissolve the peptides. Add background controls to the empty wells P1-P24. Be careful in selecting the controls.

We strongly recommend using every assay buffer component as single controls, as well as the final phosphatase assay solution without peptide.

Make sure that the final volume in all wells is similar!

5. Incubate the microtiter plate with the phosphatase solution for 20 min to 2 hours (45 min recommended) at 37°C. Please note that reaction conditions may need optimization for your target phosphatase regarding incubation temperatures and reaction time.
6. Terminate phosphatase reaction by adding malachite green additive solutions. JPT recommends a volume similar to or higher as the assay volume (if 25 μ L assay volume was used, please add at least 25 μ L of the malachite green additive solution to the wells). This mixture is a strong acid and will stop the enzymatic reaction allowing accurate time points for absorbance readout. Other termination solutions such as SDS should be used with caution as these reagents may react with the molybdate/malachite green mixture and generate high backgrounds reducing the sensitivity of the assay.

Avoid bubbles during the addition of the malachite green solution! Bubbles in the wells or water drops on the bottom of the plate will adversely affect the absorbance readings.

7. Allow 15 min at room temperature for completion of color development.
8. Measure absorbance at a wavelength between 620 and 640 nm. Blank against the absorbance of control wells. Generally, absorbance is higher at 620 nm as compared to 650-660 nm. We recommend to blank against the control well containing the phosphatase in assay buffer without peptide.
9. Some assay results from this lot are shown in the table below. Backgrounds (water and dye solution) range between 0.04 and 0.11 after 15 min time for color development depending on the wavelength used.

Malachite Green Assay		
PTP-1B	well (@10 μ M)	absorbance (corrected*)
0.25 U per well	D1	0.0099
	J18	0.2625
	N6	0.2026
	N10	0.2409

* = corrected means that the absorbance for the control well containing the phosphatase in assay buffer without peptide was subtracted from the measured value.

7 Notes

- The malachite green reagent is a highly sensitive phosphate detection solution. Free phosphate present on labware and in reagent solutions will greatly increase the background absorbance of the final assay. This contamination can easily be detected visually as a change from yellow to green in a control experiment. Detergents used to clean labware may contain high levels of phosphate. Use caution by either thoroughly rinsing all labware with double distilled water or use disposable plasticware.
- Phosphatases possess diverse optimal assay conditions. Therefore, a universal buffer system cannot be provided. The pH optima of phosphatases are also substrate dependent. For example, phosphatases appear to have higher V_{max} values with phosphopeptide substrates (which are components of the Tyrosine Phosphatase Substrate Set) at lower pH values as compared to phosphoproteins. Additionally, lower pH may result in higher K_m values. It is recommended that an assay buffer pH between 5.0 and 7.5 is used when performing phosphatase assays in the supplied microtiter plate.
- Incubate the Tyrosine Phosphatase Substrate Set for 30 min for complete color development, if more than 5 μg of protein is present per well. This is because high protein concentrations will delay color development.
- Once color development is complete, the color will remain stable for at least two hours.
- For obvious reasons, phosphate buffers are not compatible with this system. Additionally, buffers containing sulfonic acid functions, like HEPES (1-Piperazineethane sulfonic acid), cannot be used with the malachite green assay. Reaction components that contain phosphate (i.e. glycerol phosphate or nucleotides) may interfere with the analysis in a concentration dependent manner. High concentrations of reductants like 2-mercaptoethanol may bleach the molybdate/malachite green dye resulting in lower sensitivity. A final concentration of

0.02% 2-mercaptoethanol is compatible with the assay, 0.05% will influence the final readout slightly, and 0.1% will yield a reduction of approximately 20% in sensitivity. Detergents may increase the background. If such reagents are necessary for the assay, the background should be determined using the provided control plate. To test the suitability of various components, add the individual or combined components at the highest concentration to be used in a total of 25 μ L to the wells of the control plate. Add 25 μ L of pre-mixed molybdate/malachite green dye and incubate at room temperature for 15 min (30 min, if more than 5 μ g protein will be present in the final assay). Prepare a control reaction with distilled, phosphate free water. Components that remain to be yellow and do not cause precipitation are compatible with the assay system.

- In case a protein precipitate forms during the addition of the pre-mixed molybdate/malachite green dye, either use a lower concentration of the protein, or (upon completion of the reaction) treat the reaction solution with 5 μ g of Proteinase K for 5 min at 30°C in a buffer containing 5 μ M CaCl₂ (final concentration) before adding the pre-mixed molybdate/malachite green dye.

8 Related products

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

- Phosphatase Substrate Set
- Phosphatase Peptide Microarrays
- Phosphatase Profiling Service