Chapter 4

Peptide Microarrays for Real-Time Kinetic Profiling of Tyrosine Phosphatase Activity of Recombinant Phosphatases and Phosphatases in Lysates of Cells or Tissue Samples

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Abstract

A high-throughput method for the determination of the kinetics of protein tyrosine phosphatase (PTP) activity in a microarray format is presented, allowing real-time monitoring of the dephosphorylation of a 3-nitro-phosphotyrosine residue. The 3-nitro-phosphotyrosine residue is incorporated in potential PTP substrates. The peptide substrates are immobilized onto a porous surface in discrete spots. After dephosphorylation by a PTP, a 3-nitrotyrosine residue is formed that can be detected by a specific, sequence-independent antibody. The rate of dephosphorylation can be measured simultaneously on 12 microarrays, each comprising three concentrations of 48 clinically relevant peptides, using 1.0–5.0 μg of protein from a cell or tissue lysate or 0.1–2.0 μg of purified phosphatase. The data obtained compare well with solution phase assays involving the corresponding unmodified phosphotyrosine substrates. This technology, characterized by high-throughput (12 assays in less than 2 h), multiplexing and low sample requirements, facilitates convenient and unbiased investigation of the enzymatic activity of the PTP enzyme family, for instance by profiling of PTP substrate specificities, evaluation of PTP inhibitors and pinpointing changes in PTP activity in biological samples related to diseases.

Key words Tyrosine phosphatase, Phosphatase activity, Peptide microarray, Multiplex assay, Phosphatase substrate identification, Phosphatase activity profiling, Phosphatase kinetics, Phosphatase inhibition, Phospho-nitrotyrosine, Nitrotyrosine

1 Introduction

Reversible tyrosine phosphorylation is a fundamental signaling mechanism controlling a diversity of cellular processes. Whereas protein tyrosine kinases have long been implicated in many diseases, aberrant protein tyrosine phosphatase (PTP) activity is increasingly being associated with a wide spectrum of disorders. PTPs are now regarded as key regulators of biochemical processes, e.g., cell differentiation, proliferation, and oncogenic

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transformation, instead of simple "off" switches operating in tyrosine kinase signaling pathways [1].

The interplay between tyrosine phosphatase and tyrosine kinase assays can be illustrated by the phosphatase SHP-2. The levels of tyrosine phosphorylated SHP-2 in CD4+ T-cells of human melanoma specimens decrease with tumor progression, leading to a modulated downstream activity of SHP-2. CTLA4 or PD1 is able to recruit and activate SHP-2 which contributes to the negative regulation of T cell activation. This suggests an important role for SHP-2 in preventing melanoma progression and metastasis [2].

Measuring both phosphorylation as well as dephosphorylation of different kinase and tyrosine phosphatase substrates in a multiplex mode might provide a better understanding of cascades.

Modification of the flow-through microarray technology used for measuring (rates of) kinase substrate phosphorylation [3–6] resulted in the development of a kinetic PTP assay [7, 8].

The principle of the assay is shown in Fig. 1. Three concentrations of 48 tyrosine phosphatase peptide substrates containing a 3-nitro-phosphotyrosine residue are covalently coupled onto Anopore aluminum oxide membranes that are activated with a functionalized spacer. The peptide substrates have been selected for their therapeutic relevance, e.g., SIGLEC2 for its role in autoimmune diseases [9] and ZAP70 for its importance in leukemia [10]. As a positive control for detection, a nitrotyrosine peptide substrate (nSTAT3) is present among the substrates spotted on the array.

The dephosphorylated 3-nitro-tyrosine is detected by a monoclonal mouse anti-nitrotyrosine antibody and a FITC-labeled goat

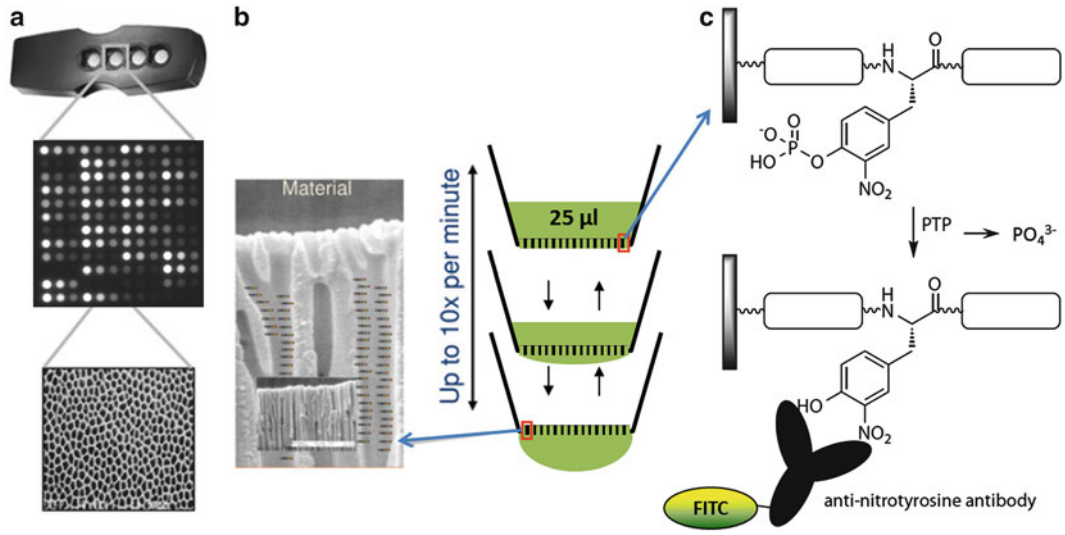

Fig. 1 Schematic view of the PTP assay. (**a**) PamChip® disposable with four arrays on which peptide substrates are spotted, 12 × 12 per array. (**b**) PTP containing sample is pumped through the array. (**c**) 3-nitro-phosphotyrosine detection strategy. Fluorescent images are recorded when the solution is underneath the membrane

anti-mouse secondary antibody that is simultaneously present in this assay. The reaction kinetics of dephosphorylation of the substrate is monitored in time by imaging the membranes every 5 min for 1 h. The fluorescence intensity of every peptide spot on every image is quantitated to yield reaction rates. The method is rapid, because apart from preparation of the lysates, no preprocessing is required. The activity of the enzymes in the lysate can be determined and modulated by addition of phosphatase inhibitors, allowing the investigation of their effect ex vivo on phosphatases with relevant post-translational modifications in naturally occurring protein complexes.

Note that this dynamic, sensitive, high-throughput PTP substrate microarray assay is inventive because it allows detection of product formation rather than disappearance of the substrate. Dephosphorylation by a PTP leaves a 3-nitrotyrosine residue that can be detected by a selective, sequence-independent antibody, which uniquely allows a real-time product formation assay (Fig. 1c) [8]. Several phosphatase assays have been described [11–16] but they suffer from several drawbacks, like the necessity for the PTP to compete with phosphotyrosine antibody prior to reaction and/or problems associated with detector-signal saturation, as discussed extensively in [8].

In summary, in combination with the existing protein tyrosine kinase (PTK) peptide microarray-based assay, the novel PTP assay creates a tool to measure phosphorylation as well as dephosphorylation in a multiplex way that will contribute to a better understanding of (the regulation of) biological processes.

2 Materials

Prepare all dilutions with ultrapure water. Use analytical grade chemicals to prepare solutions that are not provided in the reagent kit.

2.1 Preparation of Lysates for Analysis of Tyrosine Phosphatase Activity

1. Mammalian extraction buffer (M-PER) (Thermo Scientific).
2. Halt Protease Inhibitor Cocktail, EDTA free (Thermo Scientific).
3. Phosphate buffered saline (PBS, 10× stock).
4. Bradford protein concentration assay reagents.

2.2 Analysis of the Tyrosine Phosphatase Activity of a Recombinant or Purified Phosphatase or of a Cell Lysate

1. PamChip® PTP peptide microarrays (PamGene International BV, 's-Hertogenbosch, The Netherlands).
2. PamStation®12 (PamGene International BV, 's-Hertogenbosch, The Netherlands).
3. PTP reagent kit (PamGene International BV, 's-Hertogenbosch, The Netherlands). The kit contains:

- Blocking buffer: 20 mg/ml Bovine Serum Albumin (BSA).
- 10× PTP buffer (*see* **Note 1**).
- DTT (1 M).
- 100× BSA (10 mg/ml).
- Mouse anti-nitrotyrosine antibody (StressMarq).
- Goat anti-mouse FITC antibody (Southern Biotech).

All materials should be stored as indicated in the information provided with the kit.

3 Methods

Phosphatase activity can be measured with phosphatases expressed as recombinant and purified proteins or with crude lysates prepared from cell lines or tissues. When phosphatase inhibitors are added before thawing, the same lysates can be used for kinase activity determination. The preparation of these lysates is described in Subheading 3.1. The phosphatase activity assay itself is described in Subheading 3.2.

3.1 Preparation of Lysates for Analysis of Tyrosine Phosphatase Activity

1. Lyse at most four to six samples simultaneously. Aliquot final lysates in multiple tubes. Biological replicates are samples prepared separately.
2. Cool on ice Mammalian Extraction Buffer (M-PER) supplemented with protease inhibitor cocktail as indicated by the supplier (1:100) (*see* **Note 2**).
3. Label tubes for lysate aliquots of every sample and precool tubes on dry ice. This will cause the lysate to freeze immediately. Alternatively, lysates can be snap-frozen in liquid nitrogen.
4. *Cells*. Remove culture medium from cells and wash twice with ice cold PBS. Add 100 µl of cold lysis buffer per 1×10^6 cells (*see* **Note 3**). Proceed with **step 9**.
5. *Tissue*: Fine needle biopsies and endoscope biopsies can be lysed without cutting of sections. For core biopsies, cut a number of sections that gives about 1 mm^3 of tissue. For cutting sections from larger tissue blocks several options are possible (*see* **Notes 4** and **5**). Either cut one section of 60 µm, or several thinner sections (total of 60 µm of material) of a 5 × 5 mm tissue block of a fresh frozen specimen (*see* **Note 6**). Make sure that the sections remain frozen during the cutting process. The integrity of the sections is less important than in histology, since protein will be extracted from the sections. Use first and last or middle sections for histological investigations if possible.
6. Place sections at the bottom of a precooled vial.

7. The material can be lysed immediately or stored at −80 °C for later use.
8. For lysis of tissues, add 100 µl cold lysis buffer to the first vial with frozen tissue to start the lysis procedure. Keep on ice.
9. Promote the lysis by slowly pipetting the mixture up and down (approximately ten times) in the vial until the solution is clear (no lumps should be visible). Expel the fluid gently from the pipette tip in order to prevent foaming and denaturation of the proteins.
10. Keep the lysate on ice for 15 min. Check the lysis process visually. Promote lysis by pipetting the liquid up and down a few times at intervals of about 5 min. Prolong the incubation time to 30 min when solution is not clear.
11. Centrifuge the lysate for 15 min at 16,000 × *g* at 4 °C in a precooled centrifuge.
12. Transfer the supernatant of each lysed sample to new precooled vials, mix and aliquot (for instance four times 5 µl/vial and four times 20 µl/vial). Lysates can be placed at the bottom of vials precooled on dry ice. Alternatively, vials can be snap-frozen in liquid nitrogen before storage. Store vials immediately at −80 °C. Keep a 5 µl aliquot for protein quantification purposes.
13. Repeat the procedure described above for the remainder of the samples.
14. Determine protein concentration with a standard Bradford protein quantitation assay.

3.2 Analysis of the Tyrosine Phosphatase Activity of a Recombinant or Purified Phosphatase or of a Cell Lysate

In a PamStation®12 instrument three PamChips® with four arrays each are placed, which allows analysis of 12 samples simultaneously (Fig. 2).

PamChip® 90121 comprises 48 peptide substrates spotted at three concentrations (125, 250, and 500 µM) on the same microarray. The rate of dephosphorylation depends on peptide concentration (Fig. 2) and on sample input as shown in Fig. 3.

Before the start of the experiment, an experimental set up and a pipetting scheme must be prepared. The experimental setup describes the distribution of the samples over the chips and arrays, based on the research question to be answered by the experiment and the desired statistical analysis. The pipetting scheme aims at reducing experimental variation between the arrays by making master mixes with as many common ingredients for the assays as possible. The scheme is based on the volumes per array and is shown in Table 1. Multiply the volumes by the number of arrays and add at least 10% additional volume to account for pipetting loss during transfer of solutions (e.g., for four arrays, multiply volumes with at least 4.4).

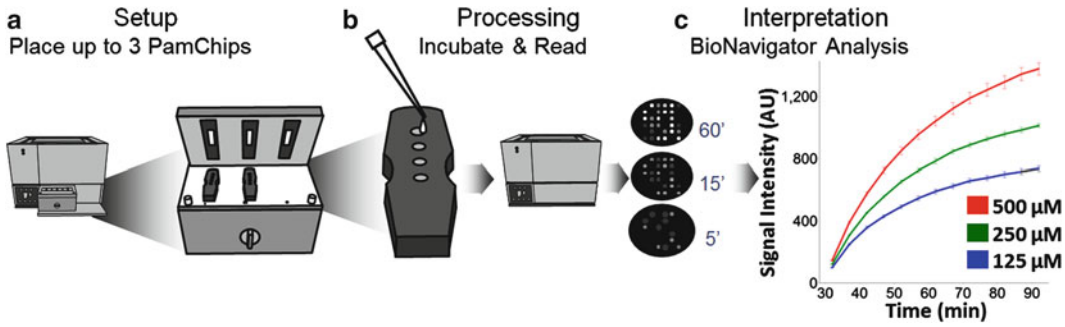

Fig. 2 Workflow for testing three PamChips® (12 microarrays) involving the setup (**a**) and processing (**b**). Dephosphorylation activity (**c**) depends on peptide substrate concentration and increases in time. Data are shown for a selected peptide substrate at three spot concentrations

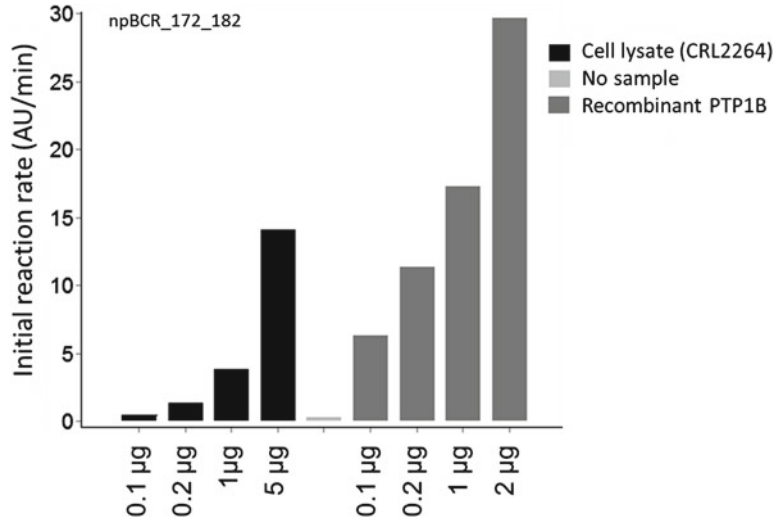

Fig. 3 The dephosphorylation rate depends on sample input for both recombinant phosphatase and lysate. Kinetic curves are fitted to signal intensities as a function of time and initial reaction rates are calculated [17]. In the concentration range tested, a sample input of 0.1–5 µg/array for the CRL2264 lysate and 0.1–2 µg/array for recombinant PTP1B enzyme results in good signals

Keep all solutions on ice, unless indicated otherwise.

1. Allow the PamChip® disposable(s) to come to room temperature.
2. Cool some ultrapure water on ice.
3. To prepare 5 ml 1× PTP wash buffer, dilute 10× PTP buffer tenfold in ultrapure water and add DTT from a freshly prepared 100 mM stock to a final concentration of 1 mM.
4. When performing experiments with inhibitors in the assay mix, pre-dilute the inhibitors to 50× the final concentration in DMSO. From these dilutions, add inhibitors just before the assay (*see* **Notes 7–9**).

Table 1
Composition of assay master mix per array

Solution	Volume (µl)
Water	To a final volume of 25 µl
10× PTP buffer	2.5
100 mM DTT	0.25
100× BSA stock solution	0.25
Mouse anti-nitrotyrosine	0.25
Goat anti-mouse FITC	0.05
(Inhibitor in 100% DMSO)	(0.5)
Sample (recombinant PTP or lysate)	max 10

5. Turn on the PamStation®12 following the instructions of the manufacturer and load the desired assay protocol into the Evolve software program. A more detailed protocol is given in the manual provided with the PamStation®12.
6. Place 1, 2, or 3 PamChip® PTP disposables in the incubator.
7. Place a syringe with 1× PTP wash buffer in the instrument at the position indicated in the assay protocol.
8. Apply 30 µl of blocking buffer to each array (*see* **Note 10**).
9. Start the blocking step in the assay protocol (*see* **Note 11**).
10. Prepare the assay master mix while the arrays are being blocked and washed. The assay master mix is prepared just before application onto the array and should not be stored. Mix gently, *do not vortex* and keep the assay mix on ice. Add components in the order indicated in Table 1. To reduce variation between arrays, prepare an assay master mix containing all components common to all arrays.
11. Divide the desired amount of the assay master mix over pre-cooled tubes representing the different conditions to be tested. Complement with other ingredients, according to experimental design.
12. Add the sample to the assay mix just before application to the arrays. The amount of input material required depends on the sample type used.
13. For recombinant phosphatases, the amount can vary from 1 to 100 nmol, depending on the activity of the recombinant phosphatase (*see* **Notes 12–14**).
14. For cell or tissue lysates, in general 0.2–5.0 µg protein per array will result in a robust phosphatase activity signal (*see* **Notes 15–18** and Figs. 3 and 4).

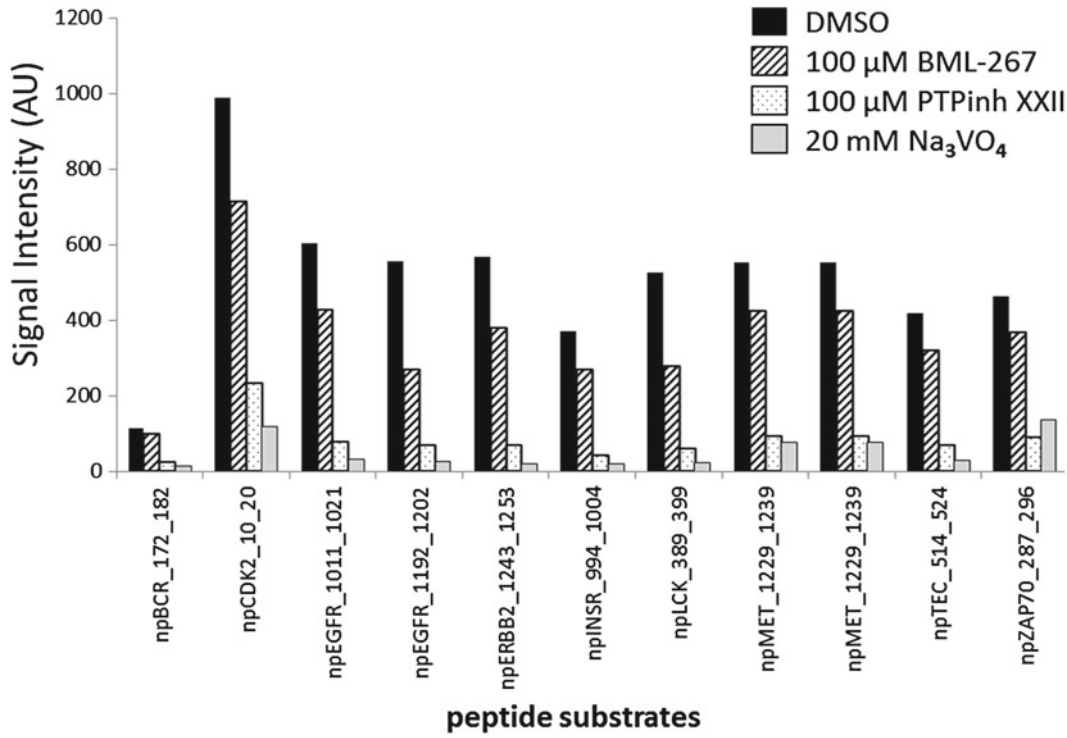

Fig. 4 Inhibition of dephosphorylation activity of recombinant full length PTP1B by three phosphatase inhibitors

15. Apply 25 µl assay mix per array (*see* **Note 19**).
16. Run the assay protocol.
17. During the run, (a run takes 60 min in the standard assay protocol), images are taken (Fig. 2), showing the signal increase on all peptide spots. At the end of the run, an automated washing step is performed, and images are taken at different exposure times.
18. When the running of the assay protocol is completed, remove the PamChip® disposable(s) and shut down the instrument according to the information in the PamStation®12 manual.
19. Use BioNavigator software (PamGene International BV, 's-Hertogenbosch, the Netherlands) to quantitate the signal intensities of all peptide spots on all images (*see* **Notes 20** and **21**). The software has an algorithm that recognizes the spots and their identity and places a grid on the array of spots on each image.
20. In BioNavigator, inspect the correct placement of each grid and identification of spots by the software as well as the occurrence of irregularities, fluorescent particles and other phenomena affecting data quality (*see* **Note 22**). The software calculates the intensity in each spot, the local background around each

spot and detects saturation in the pixels in the images. Subtraction of local background yields the value SigmBg (signal minus background) for each spot. These values are used for further analysis.

21. The data points can be inspected as a time series of signal intensity per peptide (Fig. 2). The initial rate of reaction can be calculated and used as basis for data analysis. Alternatively, the SigmBg end levels either before or after the washing step can be used for further analysis. In general, these values are very similar. In case a high phosphatase activity leads to saturation of many spots, the values at an earlier time point can be used as input for data analysis.
22. The dynamic range of the assay can be increased by making use of a combination of different exposure times. Therefore the slope of the signal as function of exposure time is used. Saturated signals can be excluded from this analysis.
23. The BioNavigator software provides several predefined statistical analysis and visualization methods for quick data analysis and interpretation. Furthermore the software provides building blocks for construction of alternative analysis methods and can easily be linked to R-modules.
24. A database function allows easy combination of the results of multiple experimental runs and analysis of such combined data sets.

4 Notes

1. Composition of the 10× PTP buffer: 250 mM potassium phosphate adjusted to pH 7.4, 500 mM NaCl, 50 mM EDTA, 0.5% Brij-35.
2. Some tissues are known to have a high protease content. Use 1:50 diluted inhibitors for such tissues.
3. Preferably scrape adhering cells because trypsin is known to induce kinase activity. The influence of trypsin treatment on phosphatase activity has not been investigated.
4. Grinding frozen tissue with a mixer-mill or pestle is a good alternative (Detailed instructions are available from PamGene International BV).
5. Cutting of sections makes it possible to use sections before and after for HE staining to determine for example the percentage of tumor cells.
6. Avoid the use Tissue-Tek® or OCT™, a wax-like substance used for embedding tissues before cryo-section. If it is used, remove as much as possible. Embedded material is compatible

with PamChip® array analysis when tissue sections contain less than 10% of such material.

7. When working with inhibitors, usually a stock solution of 10 mM inhibitor in DMSO is made that is then diluted in DSMO till 50× the desired final concentration. Just before the assay, this solution is diluted in the final assay mixture. Per 25 μl assay mix, 0.5 μl of this solution is added, resulting in 2% DSMO in the final assay mix. When no replicates are tested, prepare higher volumes to prevent variation due to small volumes.
8. When dissolving and diluting inhibitors, the experimenter should check that all material dissolves. Depending on the inhibitor concentration, dilution in water may result in precipitation. When this happens, a less concentrated solution can be made. Up to 2% DMSO in the assay mixture is tolerated by phosphatases, but may result in lower activity.
9. Some inhibitors might bind slowly to the phosphatase. To assure proper binding, pre-incubation of the inhibitor to the phosphatase can be considered.
10. The material the arrays are made of is very brittle. Do not touch the arrays with the pipette tip to prevent breaking. Place the pipette tip in the dedicated area in the plastic housing adjacent to the array or let the fluid wet the array by touching the surface with the drop hanging from the pipette tip.
11. Complete drying of arrays after blocking affects assay performance negatively. When a long time passes between washing and application of the assay mixture, the arrays may dry completely (white appearance). When it is expected that the time between the end of the washing step and application of assay mix will be more than 15 min, the PamChip® disposable(s) may be removed from the instrument and stored in the original pouch to prevent further drying.
12. For a recombinant phosphatase the optimal input must be determined by testing a concentration series of the phosphatase. The input suggested by the supplier is a good starting point. When such information is not available, a range between 1 and 100 nmol or 1 and 1000 ng per array can be tested to determine the optimal concentration.
13. When preparing a concentration series of a phosphatase, one assay master mix containing the highest concentration phosphatase is made and one without. The latter is used to dilute the phosphatase containing assay master mixture. This method avoids errors due to variation in pipetting small volumes.
14. Since recombinant phosphatases may be sensitive to freeze–thaw cycles, they must be treated according to the instruction of the manufacturer. In many cases, preparing aliquots of the recombinant phosphatase is suggested to avoid freeze–thaw cycles.

15. Lysates should be centrifuged shortly in a precooled centrifuge before addition to the assay mix to remove precipitates that could be present.
16. The optimal amount of lysate per array must be determined. In general, 0.2–5 μg of protein per array results in a robust signal (Fig. 3). For an optimization experiment, it is suggested to test three input concentrations, with the middle concentration repeated with a generic phosphatase inhibitor like 1.0 mM sodium vanadate spiked-in to confirm that the activity determined can also be inhibited. Signals increase with input concentration till an optimum is reached.
17. It is advised to dilute the samples in M-PER lysis buffer with freshly added protease inhibitors since the inhibitors are not resistant to freeze–thaw cycles.
18. Although the phosphatase activity in some samples can withstand several freeze–thaw cycles, other lysates are more susceptible to freezing–thawing. Therefore, it is advised to aliquot samples after lysis so as to avoid freeze–thaw cycles.
19. To establish that *bona fide* phosphatase activity is measured controls should be performed to assure that activity depends on the phosphatase input concentration and that it can be inhibited by a generic phosphatase inhibitor like sodium vanadate.
20. Other programs can be used for signal quantitation, but are not designed to deal with a large number of arrays nor with the kinetic readouts.
21. The grid is placed on the last image of a series. When the camera position has slightly shifted during the run, grid placement may be inaccurate. In such cases separate analysis of images (see manual for more instructions) is helpful.
22. When fluorescent speckles are visible on many arrays, precipitates in the antibody solution may be the cause. Remove those by centrifugation of the antibody solutions and transfer to clean tubes.

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