# SignalScout Phosphatase Profiling System

## Instruction Manual

**Catalog #257001**

Revision B

**Research Use Only. Not for Use in Diagnostic Procedures.**

257001-12

Agilent Technologies

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# SignalScout Phosphatase Profiling System

## CONTENTS

# SignalScout Phosphatase Profiling System

## MATERIALS PROVIDED

### SignalScout Phosphatase Profiling System I

| Materials Provided | Concentration | Quantity |
|---|---|---|
| Human Phosphatase Vector | 1 µg/µl | 20 µg |
| Substrate Trapping Mutant Human Phosphatase Vector | 1 µg/µl | 20 µg |
| Anti-c-Myc Monoclonal Antibody, Clone 9E10 (Catalog #257260) | 250 µg/ml | 25 µg |

### SignalScout Phosphatase Profiling System II

| Materials Provided | Concentration | Quantity |
|---|---|---|
| Human Phosphatase Vector | 1 µg/µl | 20 µg |
| Substrate Trapping Mutant Human Phosphatase Vector | 1 µg/µl | 20 µg |
| Active Phosphatase | varies[a] | varies[a] |
| Anti-c-Myc Monoclonal Antibody, Clone 9E10 (Catalog #257260) | 250 µg/ml | 25 µg |

### Individual SignalScout Phosphatase Products

| Materials Provided, sold separately | Concentration | Quantity |
|---|---|---|
| Human Phosphatase Vector | 1 µg/µl | 20 µg |
| Substrate Trapping Mutant Human Phosphatase Vector | 1 µg/µl | 20 µg |
| Active Phosphatase | varies[a] | varies[a] |

[a] Refer to the lot-specific Certificate of Analysis for enzyme concentration and quantity.

**For up-to-date, comprehensive phosphatase product offerings, please visit:**
***www.genomics.agilent.com***

## STORAGE CONDITIONS

**All vectors:** –20°C
**All active enzymes:** –80°C
**Anti-c-Myc Antibody:** Store at –20°C for long term storage. The antibody may be stored at 4°C for up to one month. Avoid repeated freeze-thaws.

Revision B

## ADDITIONAL MATERIALS REQUIRED

Mammalian expression cells
6-well plates
96-well plates
RNase free DNase I (Stratagene Catalog # 600031)
Fluorescence plate reader
Optional: Anti-c-Myc monoclonal antibody (Clone 9E10, Stratagene Catalog # 257260 and 257261)
DiFMU (Molecular Probes Catalog # D6566)
DiFMUP (Molecular Probes Catalog # D6567)
DiFMUP reagent and assay buffer[§]
Lysis buffer[§]
Malachite Green, Solution A (Upstate Catalog #20-105)
Malachite Green detection solution[§]
pNPP reagent and assay buffer [§]
Protein G beads
PTEN Malachite Green Assay Kit (Upstate Catalog #17-351)
Transfection reagents

## NOTICE TO PURCHASER

### CMV Promoter

The use of the CMV Promoter is covered under U.S. Patent Nos. 5,168,062 and 5,385,839 owned by the University of Iowa Research Foundation and licensed FOR RESEARCH USE ONLY.

[§] See *Preparation of Media and Reagents.*

# INTRODUCTION

The Stratagene SignalScout phosphatase profiling system enables researchers to specifically study dephosphorylation by protein tyrosine phosphatases (PTPs). PTPs are widely studied because of their critical role in regulating normal cell growth through their role in cellular signaling pathways. PTPs work antagonistically with protein tyrosine kinases (PTKs) to regulate signal transduction in a cell: PTKs phosphorylate tyrosine residues on a substrate protein and PTPs remove these phosphates from substrate tyrosines (see Figure 1). Key residues contributing to the catalytic activity of a PTP are shown below in Figure 1. While the numbering of these active site amino acids differs among PTPs, the overall structure of the active site and the identities of the key amino acid residues is conserved.

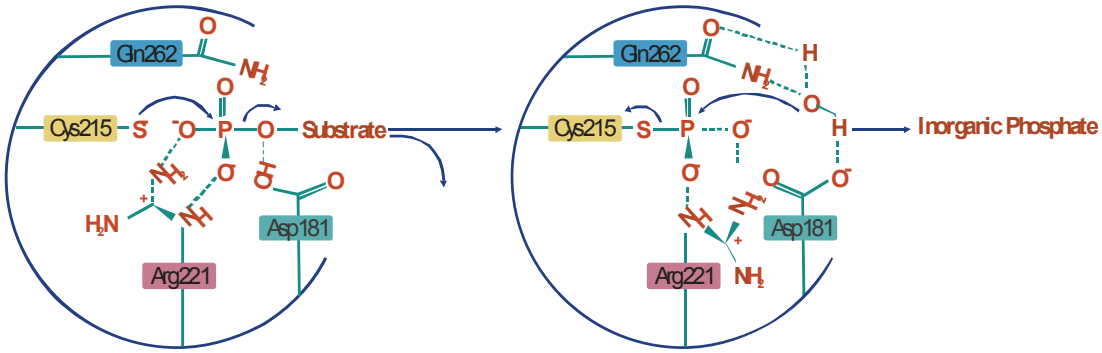



**FIGURE 1** The activity of the human phosphatase PTP1B as an example of the specific mechanism of phosphatase catalysis.

Since the phosphorylation status of a protein can modulate its function, PTKs and PTPs work together to regulate protein function in response to a variety of signals, including hormones, mitogens, and oncogenes through a process called protein phosphorylation. This process, whereby a phosphate group is either attached to or removed from a protein, can have profound effects on a given protein's activities and properties, and nearly all aspects of cell life are regulated by this reversible mechanism. Protein phosphorylation provides an equilibrium of protein function by allowing proteins to be reversibly activated and deactivated; a process dependent on the antagonistic interplay between protein kinases and phosphatases (see Figure 2). Because it is important that the effects of an activated protein are reversible, it is generally observed that a substrate-specific kinase is matched by a corresponding phosphatase, allowing the necessary equilibrium of protein phosphorylation.

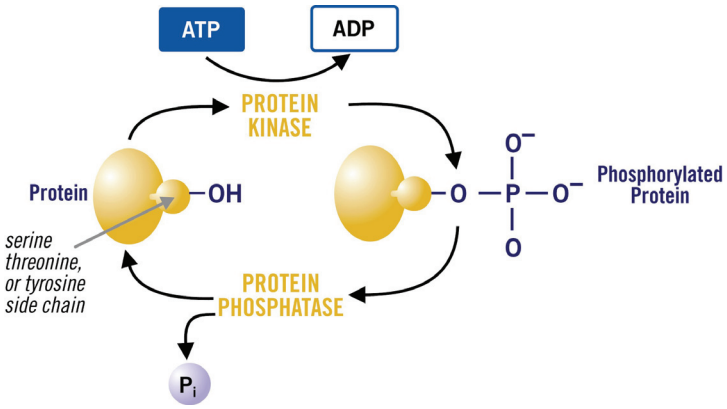



**FIGURE 2** Kinase/phosphatase regulation of phosphorylation.

The SignalScout phosphatase profiling system enables users to study dephosphorylation by protein tyrosine phosphatases. This system includes tagged human phosphatase expression vectors, matching tagged substrate trapping mutant expression vectors, and purified GST-tagged phosphatases, available as separate components. Complete kits are available for select phosphatases, and include the anti-c-myc antibody for detection of the c-myc-tagged phosphatase expression product.

# SIGNALSCOUT HUMAN PHOSPHATASE VECTORS

SignalScout human phosphatase vectors can be transfected into mammalian cells and the fusion protein can be easily detected using a commercially available c-Myc antibody (Anti-c-Myc clone 9E10 antibody, Stratagene catalog # 257260 and 257261). These protein tyrosine phosphatase clones are expressed from either the pCMV-Tag 3, pCMV-Tag 5, pCMV-3Tag 2, or pCMV-3Tag 4 vector and contain sequences for the c-Myc epitope at either the N or C-terminus (see Figure 3). These specific epitope tags are small, highly immunoreactive, and are not likely to interfere with the function of the target protein. The c-Myc epitope is derived from the human c-Myc gene and contains ten amino acid residues (EQKLISEEDL).[1] All vectors contain either one copy or three contiguous copies of the c-Myc tag fused to the phosphatase ORF (see individual Certificates of Analysis for c-Myc copy number). In addition to the epitope tag sequences, the pCMV-Tag vectors contain features for expression of fusion proteins in eukaryotic cells. The cytomegalovirus (CMV) promoter allows constitutive expression of the cloned DNA in a wide variety of mammalian cell lines. The neomycin-resistance gene is under control of both the prokaryotic β-lactamase promoter to provide kanamycin resistance in bacteria and the SV40 early promoter to provide G418 resistance in mammalian cells.

Successful transfection can be detected using either Western blot analysis or immunoprecipitation (Anti-c-Myc clone 9E10 antibody, Stratagene catalog # 257260 and 257261). Proteins collected using immunoprecipitation can be further analyzed by measuring the activity of the immunoprecipitated phosphatase (see Figure 4). Two phosphatase assays are highlighted in the protocol section of this manual: the para-nitrophenyl phosphate (pNPP) assay, and the 6, 8-difluoro-4-methyllumbelliferyl phosphate (DiFMUP) assay. Both the pNPP and DiFMUP assays can be used to detect alkaline phosphatases, acid phosphatases, protein tyrosine phosphatases and serine/threonine phosphatases. The pNPP assay yields para-nitrophenolate which can be measured spectrophotometrically at 405 nm. The DiFMUP assay yields 4-methylumbelliferone which can be measured at an emission wavelength of 455 nm when excited at a wavelength of 358 nm. The malachite green assay relies on interaction between molybdate and malachite green to detect hydrolyzed phosphate which can be measured spectrophotometrically at 620–660 nm. The assay can be used to measure the activity of any type of phosphatase depending upon the substrate provided (e.g., lipid phosphatases use phospholipid vesicle (PLV) as substrate).

## SIGNALSCOUT SUBSTRATE-TRAPPING MUTANT HUMAN PHOSPHATASE VECTORS

It has been found that by mutating certain key amino acids in the phosphatase active site, either singly or in paired combinations, substrate trapping mutants (STMs) can be generated. STMs are phosphatases mutated to retain specific binding activity but lacking catalytic activity. In other words, STMs bind to phosphorylated substrates, but cannot cleave off the phosphate, and the substrate remains bound to the STM. We have created candidate STMs in which both the key glutamine and aspartic acid shown in Figure 1 have been mutated to alanine.[2] These residues are conserved across all classical PTPs and the mutations to alanine have been shown to convert PTPN1 into an effective STM. Similar mutations in the other classical PTPs are predicted to result in STMs as well (though not all have been tested for specific substrate binding). These mutant protein tyrosine phosphatase clones are expressed from either the pCMV-Tag 3, pCMV-Tag 5, pCMV-3Tag 2, or pCMV-3Tag 4 vector and contain sequences for the c-Myc epitope at either the N or C-terminus (see Figure 3). All vectors contain either one copy or three contiguous copies of the c-Myc tag fused to the mutant phosphatase ORF (see individual Certificates of Analysis for c-Myc copy number).

Mutant human phosphatases with D/A and Q/A STM motifs can be detected using Western blot or immunoprecipitation with the anti-c-Myc antibody. However, unlike the SignalScout human phosphatase clones, immunoprecipitated mutant proteins are inactive in a phosphatase assay (see Figure 4). The immunoprecipitated protein may also co-immunoprecipitate with specific phosphorylated protein substrates from the cell lysate.

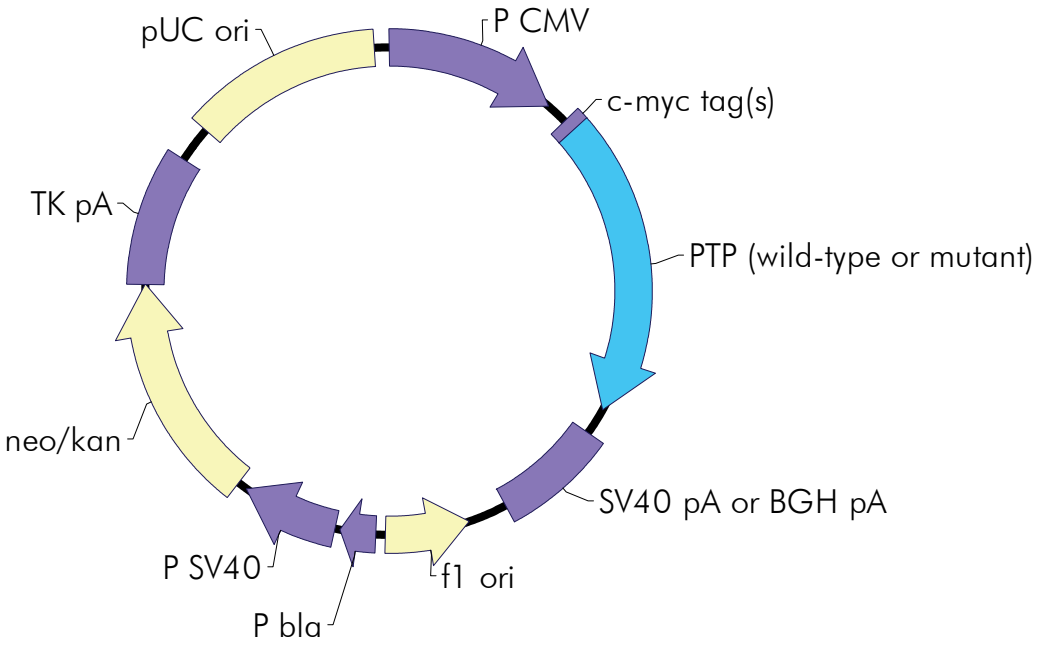
When used in conjunction with the corresponding wild-type PTP vector, the STMs might be used to:

- activate or inhibit known signaling pathways.
- identify new signaling relationships through monitoring their effect on known signaling molecules or cell phenotype.
- determine, in screening assays, which small molecules or transgenes affect the phosphatase in mammalian cells.
- obtain, through c-Myc immunoprecipitation, active mammalian phosphatase enzyme for use in in vitro studies.
- pull out native phosphatase substrates from cell lines and determine how the substrate profile changes upon cell treatments.

## SIGNALSCOUT ACTIVE GST-TAGGED HUMAN PHOSPHATASE ENZYMES

In addition to the in vivo expression vectors, matched purified affinity-tagged phosphatases are available for in vitro assay studies. These phosphatases are enzymatically active either as a full-length construct, a truncated protein, or a catalytic domain. An affinity tag is fused to each purified phosphatase (see individual Certificates of Analysis for affinity tag type and location).

**A**




**B**

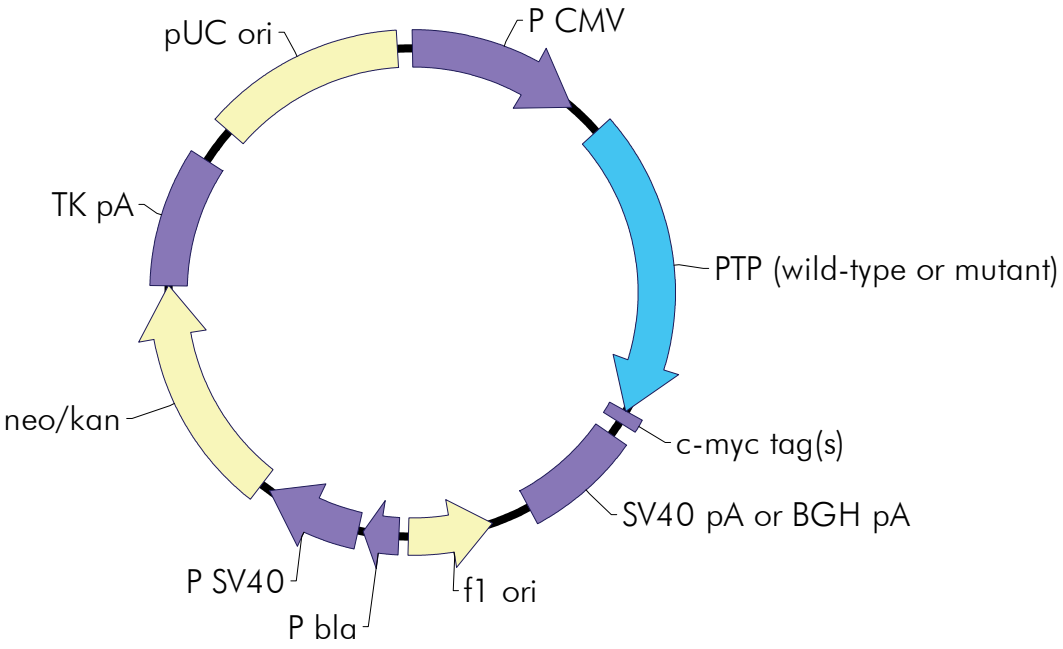



**FIGURE 3** Generalized vector elements for the SignalScout human phosphatase (wild-type) and substrate trapping mutant human phosphatase (mutant) vectors with a c-Myc tag positioned at the N-terminus (A) or C-terminus (B) shown to illustrate the location of specific vector elements. All vectors contain either one copy or three contiguous copies of the c-Myc tag fused to the phosphatase ORF. For specific vector maps, please visit *www.genomics.agilent.com*.

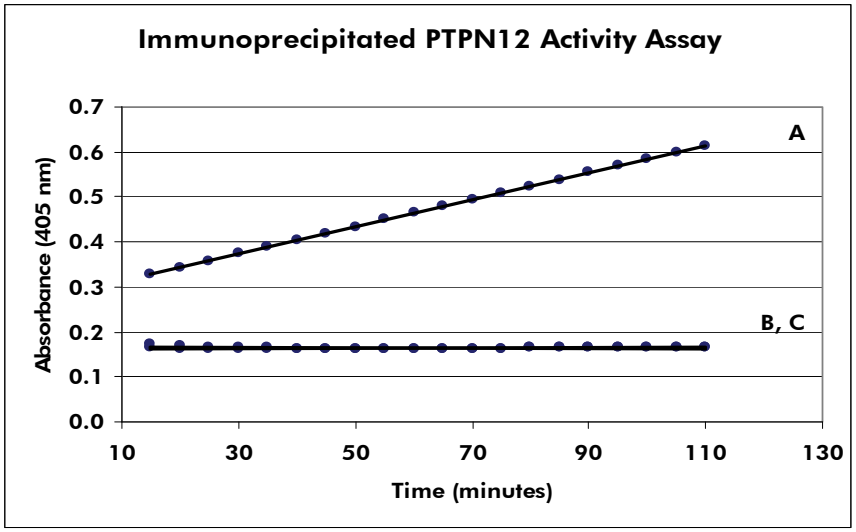



**FIGURE 4** Immunoprecipitated PTPN12 wild-type and catalytically inactive mutant PTPN12 were tested in a pNPP phosphatase assay. Trendlines and data points plotted for a phosphatase assay compare the activity of SignalScout PTPN12 human phosphatase clone vector (A, $r^2$=1.0) to a mutant strain (B, $r^2$=.88) and to untransfected cells (C, $r^2$=0.95). The anti-c-Myc tag antibody was used for the immunoprecipitation.

# PROTOCOL

**Note** *The SignalScout phosphatase profiling system is designed to work for a variety of different research applications. Each application may or may not use each of the steps detailed in the protocol section of this manual.*

## Transfecting the SignalScout Human Phosphatase Vectors

The efficiency of transfection will vary depending on the host cell line used. Choose a transfection method that will give the highest transfection efficiency for the chosen cell line. The amount of phosphatase vector required for expression of the gene of interest may vary depending upon the expression level of the gene, the transfection efficiency, and the host cell line used. Varying the amount of vector may be required to achieve optimal gene expression. In most cases, expression of the genes should be detectable 24–72 hours after transfection. The length of time is dependent upon mRNA and protein stability. A time course is recommended to identify the optimal time to detect gene expression. Perform the appropriate assays to determine protein expression levels (e.g., Western blotting).

When transfecting 293T cells, grow the cells in DMEM + 10% FBS and antibiotics in a 6-well dish overnight to a confluency of approximately 60%. Perform a lipid-based transfection following the manufacturer's instructions and incubate the cells at 37°C for 24 hours. Add 1 ml of PBS to each well containing approximately $1 \times 10^6$ cells each and transfer the cell suspension(s) to a 1.5 ml Eppendorf tube.

## Cell Lysis

1. Pellet the cells at 1,000 × *g* for 2 minutes in a microfuge.
2. Remove the supernatant.
3. Add 200 µl Lysis Buffer[§] to each tube.
4. Incubate for 15 minutes at room temperature.
5. Add 2.0 µl of 1M $MgCl_2$ and 1.0 µl of RNase-free DNase I [Stratagene catalog # 600031] to each tube.
6. Incubate for 15 minutes at room temperature.
7. Centrifuge at 9,000 × *g* for 20 minutes at 4°C.
8. Collect the supernatant. The supernatant may be stored at –80°C for use in phosphatase assays, Western blot analysis or immunoprecipitation.

[§] See *Preparation of Media and Reagents*

## Immunoprecipitation

### Cell Lysate Preclearing

1. Wash 10 µl of a Protein G bead slurry with 100 µl of cold Lysis Buffer twice.
2. Resuspend the Protein G beads in 10 µl of cold Lysis Buffer.
3. Add 200 µl of the cell lysate to the Protein G beads.
4. Incubate the mixture on ice for 60 minutes.
5. Pellet the beads at 10,000 × *g* for 30 seconds and transfer the supernatant to new tubes.

### Antibody Binding

1. Add 1 µg of anti c-Myc antibody (Clone 9E10) to the cold precleared lysates and incubate for 1 hour at 4°C.
2. Add 10 µl of Protein G slurry (pre-washed in cold Lysis Buffer).
3. Incubate the mixture for 1 hour at 4°C on a rotator.
4. Pellet the beads at 10,000 × *g* for 30 seconds at 4°C and remove the supernatant completely.
5. Wash the beads 5 times with 100 µl of cold Lysis Buffer.
6. Remove and discard the supernatant.

   **Note** *Steps 7–8 should be performed if an analysis of phosphatase activity is desired.*

7. Resuspend the beads in 25 µl of a buffer that is appropriate to the assay chosen (e.g. a pNPP or DiFMUP assay).
8. Conduct phosphatase assay if desired.

   **Note** *Steps 9–10 should be performed if Western blot analysis is desired.*

9. For Western analysis, resuspend the beads in an appropriate volume of 1× sample buffer (sufficient to fill a well of the SDS-PAGE gel).
10. Boil the suspension for 1 min, then spin to pellet the beads, and run the supernatant on a gel. See *Western Blot using Anti-c-Myc Antibody* for more information.

## pNPP Phosphatase Assay

1. Add 1–10 µl of each phosphatase (from the crude lysate or immunoprecipitation, or using one of the purified GST-tagged phosphatases available from Stratagene Products Division) to separate wells of a 96-well plate. Bring the volume up to 80 µl with Complete Assay Buffer[§].

2. Incubate the plate at 37°C for 15 minutes.

3. Add 120 µl of 1× pNPP Substrate[§] to each well.

   **Note** *Highly active phosphatases can be standardized to a room temperature reaction. Enzymes with low activity should be kept at 37°C to maximize reaction velocity.*

4. A single timepoint assay or dynamic time course assay may be performed.

   **Note** *Filters that read 400–410 nm are adequate for this procedure.*

   **Endpoint assay**
   For the single timepoint assay, incubate the plate at 37°C for 30 minutes. Read the absorbance at 405 nm. Calculate phosphatase activity using the equations given below.

$$\text{Specific Activity (SA) (nmoles/min/}\mu\text{g)} = \frac{V\ (\mu l) \times OD_{405nm}}{\text{E.C.} \times \text{incubation time (min)} \times \text{pathlength of light (cm)} \times \text{enzyme } (\mu g)}$$

Where E.C. = extinction coefficient for pNPP = 17.8 $\mu l\ nmol^{-1} cm^{-1}$

$$\text{Enzyme turn-over number } (min^{-1}) = 10^{-3} \times \text{SA (nmoles/min/}\mu\text{g)} \times \text{enzyme MW (daltons)}$$

[§] See *Preparation of Media and Reagents*

**Time course assay**

To obtain enzyme activity over time, incubate the plate at 37°C, reading the absorbance at 405 nm at 2–5 minute intervals up to at least 60 minutes. Graph the absorbance vs. time (minutes) and compute the slope. Calculate phosphatase activity using the equation given below.

$$\text{Specific Activity (SA) (nmoles/min/}\mu\text{g)} = \frac{\text{Sample Volume (}\mu\text{l)} \times \text{Slope (abs/min)}}{\text{E.C.} \times \text{Pathlength of light (cm)} \times \left[\begin{array}{c}\mu\text{g of enzyme} \\ \text{OR} \\ \mu\text{l of lysate} \\ \text{OR} \\ \text{cell number}\end{array}\right]}$$

Where E.C.= extinction coefficient for pNPP = 17.8 $\mu$l nmol$^{-1}$cm$^{-1}$

***Note*** *One Unit is defined as 1 nmol of pNPP hydrolyzed per minute and is computed on a per microgram-, lysate-, or cell number- basis using the above formula.*

## DiFMUP Phosphatase Assay

### Sample Preparation

1. Prepare a 10 mM stock solution of **DiFMUP** by dissolving 10 mg in 3.42 ml of DMSO.

2. Prepare a 10 mM stock solution of **DiFMU** by dissolving 10 mg in 4.72 ml of DMSO.

3. Prepare a 200 µM **DiFMUP** stock solution using the 10 mM stock prepared in step 1.

   **Note** *The remainder of the protocol can be done using phosphatase, crude lysate or immunoprecipitated protein. Take care to read the protocol carefully and to be consistent in your use of one of the three.*

4. Add 10 ng–1 µg phosphatase OR 1–20 µl crude lysate OR 1–20 µl immunoprecipitate to a single well of a 96-well plate. Bring to a total volume of 50 µl with 1× DiFMUP Reaction Buffer[§].

   **Note** *Because phosphatase activity varies, the amount to be added to each assay is different for individual phosphatases and should be titrated.*

5. Add 50 µl of the 200 µM stock DiFMUP solution (from step 3) to each well.

6. Immediately place the plate in a fluorescence plate reader and take reading(s) using detection settings of: excitation at 358 nm and emission at 455 nm.

7. Read the fluorescence every 5 minutes as the reaction progresses at room temperature until a clear trend has been established.

8. Calculate pmol DiFMU generated per minute per microgram of phosphatase OR per microliter crude lysate OR per microliter immunoprecipitate by comparison to a standard curve.

[§] See *Preparation of Media and Reagents*

## DiFMU Standard Calibration Curve Preparation

1. Using the table below, prepare a series of standards that will allow you to convert fluorescence readings (Relative Fluorescence Units or RFU) to pmoles of DiFMU. Each standard well should have a total volume of 100 µl.

| Well Number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| 100 µM DiFMU[a] | 0 µl | 1 µl | 2 µl | 3 µl | 4 µl | 5 µl | 8 µl | 12 µl |
| 100 µM DiFMUP[b] | 100 µl | 99 µl | 98 µl | 97 µl | 96 µl | 95 µl | 92 µl | 88 µl |

[a] Diluted from the 10 mM DiFMU solution from Step 2 of the sample preparation.

[b] Diluted from the 10 mM DiFMUP solution from Step 1 of the sample preparation.

2. Plot the RFU obtained from the data above versus the pmoles of DiFMU in the table below:

| Well Number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| pmoles DiFMU | 0 | 100 | 200 | 300 | 400 | 500 | 800 | 1200 |
| Substrate Conversion | 0% | 1% | 2% | 3% | 4% | 5% | 8% | 12% |

***Note*** *Calculated specific activity will be less than actual specific activity at substrate conversion values above 15%. The value at which the calculated specific activity will vary from the actual specific activity is unique to each enzyme tested.*

## DiFMU Concentration Standard Curve Example

Once the RFU have been plotted against the concentration of DiFMU (pmoles) in a standard curve, the standard curve can be used to determine the concentration of DiFMU in the experimental samples. Figure 5 is an example of such a standard curve. For every DiFMUP Assay performed, a new set of standards must be made and a new standard curve plotted.

Another important consideration when deriving the concentration of DiFMU from your standard curve is the RFU value at which the curve plateaus. Any RFU which fall in the plateau region is subject to large inaccuracy and should not be used to determine the concentration of DiFMU.

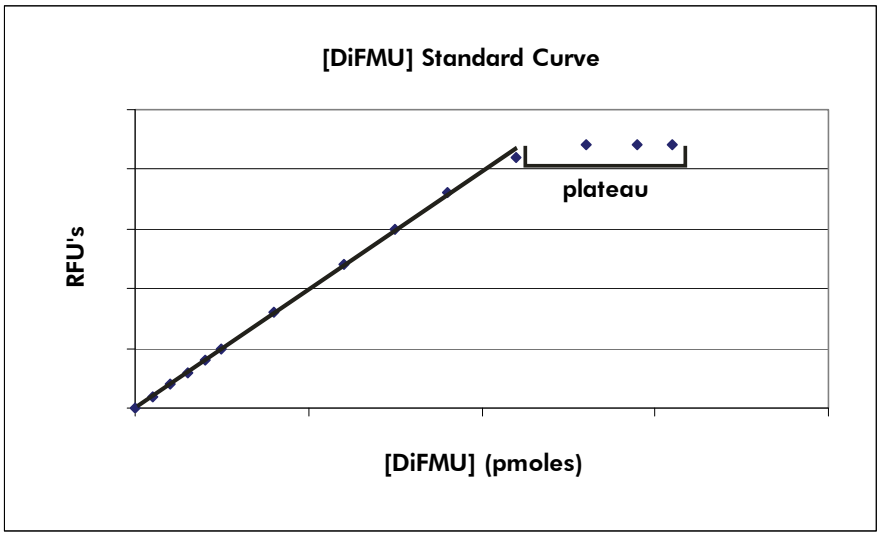



**FIGURE 5** In order to determine the value for each enzyme, plot the enzyme concentration verses product formed per unit time. As the enzyme concentration increases, the proportional relationship between concentration and time will plateau. At the point of the plateau, the standard curve is no longer useful.

## Malachite Green Assay

**Note** *Before proceeding, verify that the enzyme preparations and reaction solutions are free of contaminating phosphate by adding Malachite Green to a sample of each solution and measuring the absorbance at 620–660 nm.*

### Sample Preparation

1. Add 10 µl of enzyme assay buffer and 10 µl of phospholipid vesicle (PLV) preparation to a single well of a 96-well plate. Bring to a total volume of 25 µl by adding 10 ng–1 µg phosphatase OR lysate OR immunoprecipitate. The optimal sample amount must be determined by performing a titration. Incubate the reaction(s) for ~10–15 minutes at 37°C.

   **Note** *The enzyme assay buffer and PLV preparation are specific to the phosphatase being assayed.*

   *Because phosphatase activity varies, the amount to be added to each assay is different for individual phosphatases and should be titrated.*

2. Add 100 µl of Malachite Green detection solution[§] to each well. Allow 15 minutes for color development.

3. Place the plate in a plate reader and measure absorbance of each well at 620–660 nm.

4. Calculate pmol of phosphate generated per microgram of phosphatase OR per microliter crude lysate OR per microliter immunoprecipitate by comparison to a standard curve.

[§] See *Preparation of Media and Reagents*

### Phosphate Standard Curve Preparation

1. Prepare a 0.1 mM stock solution of potassium phosphate monobasic. Using the table below, prepare a series of known phosphate standards that will allow you to convert absorbance readings to pmoles of phosphate.

| Tube Number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **0.1 mM $KH_2PO_4$** | 200 µl | 180 µl | 160 µl | 140 µl | 120 µl | 100 µl | 80 µl | 60 µl | 40 µl | 20 µl | 0 µl |
| **Water or Diluent** | 50 µl | 70 µl | 90 µl | 110 µl | 130 µl | 150 µl | 170 µl | 190 µl | 210 µl | 230 µl | 250 µl |
| **Phosphate Concentration per 25 µl** | 2000 pmol | 1800 pmol | 1600 pmol | 1400 pmol | 1200 pmol | 1000 pmol | 800 pmol | 600 pmol | 400 pmol | 200 pmol | 0 pmol |

2. Add 25 µl from each standard tube into separate wells of a 96-well plate. Add 100 µl of Malachite Green detection solution to each well and mix carefully to avoid creating bubbles. Place the plate in a plate reader and measure absorbance of each well at 620–660 nm.

### Phosphate Standard Curve Example

Once the absorbance values have been plotted against the concentration of phosphate (pmoles) in a standard curve, the standard curve can be used to determine the concentration of phosphate in the experimental samples. For every assay performed, a new set of standards must be made and a new standard curve plotted.

## Western Blot using Anti-c-Myc Antibody

1. Load 20 µl of the supernatant containing your phosphatase of interest (from step 9 of *Antibody Binding* protocol) in 1× sample buffer onto an SDS-PAGE gel.

2. Resolve the protein preparation by SDS-PAGE.

3. Transfer the resolved proteins to a membrane.

4. Detect the phosphatase of interest using 1µg/ml anti-c-Myc antibody.

# TROUBLESHOOTING

| Observations | Suggestions |
|---|---|
| No expression of transfected phosphatase by Western blot | Western-blot with anti-c-Myc should show appropriate sized band. If not then there could be protein degradation in the lysates prior to Western blotting. Include protease inhibitors in the lysis procedure and keep all reactions on ice. |
| | The phosphatase may be expressed at very low levels in a particular cell type. Try expressing the protein in 293T cells which should give high expression. |
| | Primary or secondary antibody concentration is too low. Titrate antibody conjugates for optimal concentrations. |
| | Transfer of proteins is poor. Repeat transfer and optimize time of transfer, current and gel concentration. Verify transfer using molecular weight markers that cover the range to be transferred. |
| | Membrane preparation is inadequate. Ensure proper membrane hydration. |
| | Confirm that all Western blot reagents are working properly. |
| | Protein has degraded during storage of the membrane. Use fresh blots. |
| The Western produces excessive background | Insufficient blocking solution may have been used or the membrane was not thoroughly washed. Check the concentration of the blocking solution and/or wash thoroughly. |
| | Too much protein was loaded on gel. Load less protein on gel. |
| | Contamination by fingerprints and/or keratin has occurred. Use fresh membranes. Avoid touching the membrane. Use gloves and blunt forceps. |
| | The concentration of the anti-c-Myc, or secondary anti-mouse antibody, is too high. Check the concentration of the antibodies and dilute if necessary. |
| No apparent phosphatase activity | Check for protein expression by Western blot. |
| | Confirm phosphatase assay reagents are working by using another phosphatase source. Commercial sources are available. |
| | Test plasmid in 293T cells which should make a high level of protein. |
| | Poor isolation of tagged protein. Use a different cell lysis procedure. |
| | Proteolytic cleavage. Include protease inhibitors in lysis buffer. |

## PREPARATION OF MEDIA AND REAGENTS

| | |
|---|---|
| **Lysis Buffer**<br>100 mM NaCl<br>10 mM Tris-HCl, pH 8.0<br>1 mM EDTA<br>1% Triton X<br>5 mM DTT | **PBS (Phosphate Buffered Saline)**<br>150 mM NaCl<br>20 mM $Na_2HPO_4$<br>adjust to pH 7.4 with HCl |
| **5× pNPP Assay Buffer**<br>125 mM HEPES, pH 7.2<br>250 mM NaCl<br>12.5 mM EDTA | **1× Complete Assay Buffer**<br>1.276 ml 5× pNPP Assay Buffer<br>0.424 ml 0.1 M DTT<br>6.8 ml $H_2O$ |
| **1× DiFMUP Reaction Buffer**<br>50 mM HEPES, pH 7.2<br>1 mM DTT<br>0.1% BSA | **TE Buffer**<br>10 mM Tris-HCl, pH 7.5<br>1 mM EDTA |
| **100× pNPP Substrate (0.4 M)**<br>150 mg pNPP<br>1 ml Complete Assay Buffer | **4× SDS-PAGE Sample Buffer (with reducing agent)**<br>10% (w/v) electrophoresis-grade SDS<br>20% (v/v) glycerol<br>0.1% (w/v) bromophenol blue<br>25% 0.5M Tris-HCl, pH 6.8<br>2–5% (v/v) β-Mercaptoethanol dissolved in $dH_2O$ |
| **Malachite Green, Solution A (Upstate Catalog #20-105)**<br>0.034% malachite green<br>10 mM ammonium molybdate<br>1 N HCl<br>3.4% ethanol | **Malachite Green Detection Solution**<br>1 ml Malachite Green, Solution A (Upstate Catalog #20-105)<br>10 µl 1% Tween®-20 |

## ENDNOTES

Tween® is a registered trademark of ICA Americas, Inc.

## REFERENCES


1. Evan, G. I., Lewis, G. K., Ramsay, G. and Bishop, J. M. (1985) *Mol Cell Biol* 5(12):3610-6.
2. Xie, L., Zhang, Y. L. and Zhang, Z. Y. (2002) *Biochemistry* 41(12):4032–9.


## MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.