RediPlate™ 96 EnzChek® Tyrosine Phosphatase Assay Kit (R-22067)

Quick Facts

Storage upon receipt:
- ≤ –20°C
- Desiccate
- Protect from light

Ex/Em of reaction product: 358/452 nm

Introduction

Molecular Probes' RediPlate™ 96 EnzChek® Tyrosine Phosphatase Assay Kit provides a fast, simple and direct fluorescence-based assay for detecting tyrosine phosphatases and their corresponding modulators and inhibitors. Protein tyrosine phosphatases (PTPases) represent a large family of enzymes that play a very important role in intra- and intercellular signaling. PTPases work antagonistically with protein tyrosine kinases to regulate signal transduction pathways in response to a variety of signals, including hormones and mitogens.[1–3] Our RediPlate 96 assay kit provides researchers with a sensitive and convenient means to monitor PTPase and screen PTPase inhibitors for a variety of research areas including insulin regulation,[4–6] cell proliferation and differentiation,[7] axonal outgrowth,[8,9] angiogenesis,[10] immune response and inflammation.[11] Unlike other microplate assays, this kit provides the necessary reagents predispensed into a 96-well microplate. Simply reconstitute the fluorogenic substrate in the assay wells with buffer, add the desired sample to the wells, incubate and then quantitate the fluorescence in any standard fluorescence-based microplate reader.

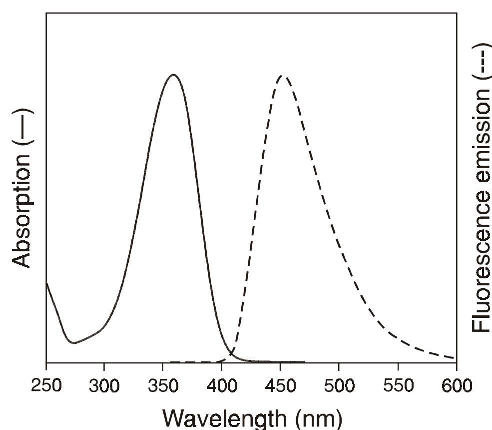
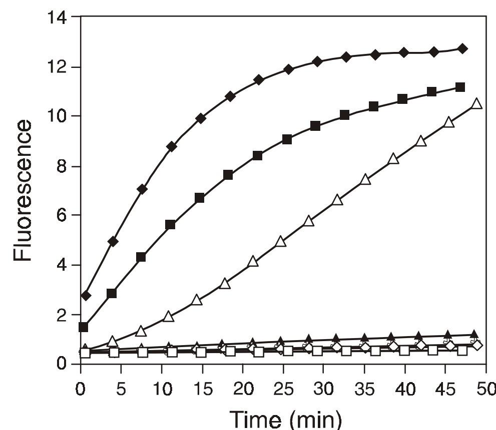


Figure 1. *Normalized absorption and fluorescence emission spectra of 6,8-difluoro-7-hydroxy-4-methylcoumarin (DiFMU).*



Symbol	Enzyme (class)	Enzyme Units*
◆	CD-45 (tyrosine phosphatase)	1 U/mL
■	PTP-1B (tyrosine phosphatase)	5 mU/mL
△	PTPase (tyrosine phosphatase)	1 U/mL
▲	Acid phosphatase	1 mU/mL
✩	Alkaline phosphatase	1 mU/mL
●	PP2A (ser/thr phosphatase)†	1 U/mL
□	PP1 (ser/thr phosphatase)†	1 U/mL
◇	PP-2B (ser/thr phosphatase)†	500 U/mL

* Enzyme unit (U) definitions are standard definitions for each enzyme.
† Serine/threonine phosphatase.

Figure 2. *Specificity of the RediPlate 96 EnzChek Tyrosine Phosphatase Assay Kit. The phosphatases listed in the tables were applied to a RediPlate 96 EnzChek tyrosine phosphatase assay microplate. At the indicated time points, the fluorescence was measured using excitation at 355 ± 20 nm and emission at 460 ± 12.5 nm.*

The microplate included with the RediPlate 96 EnzChek Tyrosine Phosphatase Assay Kit is pre-loaded with our patented 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP), a substrate whose reaction product, DiFMU, exhibits excitation/emission maxima of 358/452 nm (Figure 1) and possesses a low pK_a (~4.9) and a high quantum yield (~0.89). Inhibitors are included in each assay well to ensure that the assay is selective for tyrosine phosphatases — other phosphatases, including serine/threonine phosphatases do not significantly react with the substrate (Figure 2). Unlike phosphopeptide-based assays, DiFMUP can be used to assay a variety of PTPases, including PTP-1B, LAR and CD-45 (Figure 3). Additional advantages of our assay include compatibility with non-ionic detergents and insensitivity to free phosphate, resulting in minimal sample processing required before analysis. PTPase inhibitors can be evaluated quantitatively in the assay for their effect on PTPase activity (Figure 3).

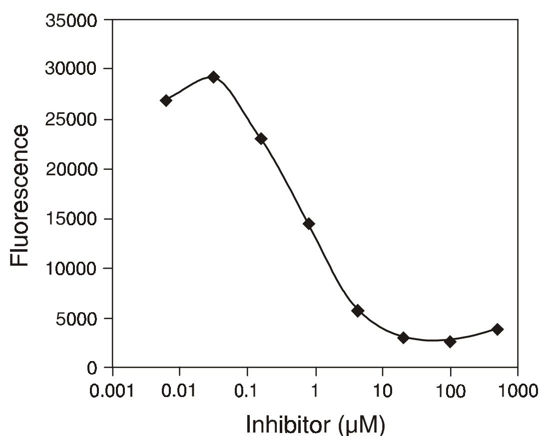


Figure 3. *Detection of LAR protein tyrosine phosphatase inhibition by sodium orthovanadate using the RediPlate 96 EnzChek Tyrosine Phosphatase Assay Kit. Each reaction contained 50 µM DiFMUP, 0.1 U/mL of LAR protein tyrosine phosphatase and the indicated concentration of sodium orthovanadate in the provided reaction buffer. Reactions were incubated at 37°C. After 30 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at 355 ± 20 nm and fluorescence detection at 460 ± 12.5 nm.*

The RediPlate 96 EnzChek Tyrosine Phosphatase Assay Kit includes one microplate and a bottle of reaction buffer. To ensure the integrity of the fluorogenic components, the microplate is contained in a resealable foil packet. The microplate consists of twelve removable strips, each with eight wells. Eleven of the strips (88 wells) are preloaded with the fluorescent reaction product, DiFMUP. The remaining strip, marked with blackened tabs, contains a dilution series of DiFMU as a fluorescence reference standard.

Materials

Kit Contents

- **RediPlate 96 EnzChek Tyrosine Phosphatase assay microplate** (Component A), one microplate
- **RediPlate tyrosine phosphatase reaction buffer** (Component B), 28 mL of 25 mM MOPS at pH 7.0 containing 50 mM NaCl, 1 mM DTT and 0.05% Tween® 20

Storage and Handling

Store the RediPlate 96 EnzChek Tyrosine Phosphatase Assay Kit at -20°C or below, desiccated and protected from light. When stored properly, the kit components should remain stable for at least six months.

Experimental Protocol

The following protocols describe the assay for PTPase or PTPase inhibitor activity in total volumes of 100 µL per microplate well. Each RediPlate 96 EnzChek Tyrosine Phosphatase Assay Kit contains one 96-well microplate with 88 wells (11 strips) intended for assays and 8 wells (1 strip, with blackened tabs) for a fluorescence reference standard curve. Because each strip is removable, researchers can perform as many or as few assays as needed.

RediPlate 96 Microplate Preparation

1.1 Allow the kit components to warm to room temperature. Remove the RediPlate Kit from the freezer and allow it to warm to room temperature. DO NOT OPEN THE FOIL PACKET UNTIL IT IS WARM. The plate (Component A) will typically take ~20 minutes to warm. Because the reaction buffer (Component B) may take longer than 20 minutes to thaw at room temperature, place the vial of buffer in a warm water bath to accelerate thawing.

1.2 Remove any extra strips. Determine the number of strips required, then carefully cut through the self-adhesive sealing film with a razor blade and remove any extra strips that are to be used at a later date. Return these to the protective foil bag. All of the strips, with the exception of the control strip with blackened tabs, contain equivalent amounts of the PTPase substrate. Empty strip holders from previously purchased RediPlate 96 kits are useful for storing extra assay strips.

Fluorescence Reference Standards

The standard curve can be used to convert fluorescence units obtained in an assay into nanomoles of phosphate. One mole of the included reference standard, DiFMU, is equivalent to one mole of phosphate cleaved from the substrate, DiFMUP. Furthermore, the fluorescence reference standards serve as controls for instrument-to-instrument variation, for day-to-day variation in single-instrument performance and for linearity of the fluorescence signal detection.

2.1 Prepare the fluorescence standards. Add 100 µL of the reaction buffer to the control strip of the RediPlate 96 microplate and mix by pipetting. Blackened tabs differentiate the control strip from strips containing DiFMUP. The control strip contains a series of the DiFMU reference standard (Table 1 and Figure 4). Well A has the highest amount of the reference standard (5000 picomoles); well H contains no reference dye.

2.2 Measure the fluorescence. The fluorescence standard samples are typically measured for fluorescence along with the samples from the PTPase or PTPase-inhibitor assays (see below).

PTPase Assay

3.1 Add 80 µL of the reaction buffer to the assay wells. Add the provided reaction buffer (Component B) or another appropriate buffer to as many assay wells as will be needed, and then mix

Table 1. *Reference standards for the RediPlate 96 Tyrosine Phosphatase Assay Kit.*

Well	Amount of DiFMU
A	5000 picomoles
B	3000 picomoles
C	1500 picomoles
D	500 picomoles
E	100 picomoles
F	50 picomoles
G	10 picomoles
H	0 picomoles

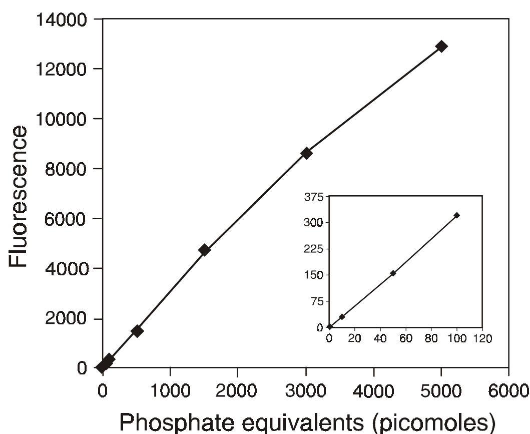


Figure 4. Dynamic range and sensitivity of the assay provided in the RediPlate 96 EnzChek Tyrosine Phosphatase Assay Kit. A 100 µL volume of the reaction buffer was added to each well containing the DiFMU reference standard. Fluorescence was measured in a fluorescence-based microplate reader using excitation at 355 ± 30 nm and emission at 440 ± 17.5 nm. Background fluorescence (59 arbitrary units) has been subtracted from each well. The inset shows an enlargement of the results obtained with 0 to 100 picomoles of DiFMU dye. In the assay, 1 picomole of DiFMU is equivalent to 1 picomole of phosphate.

by pipetting. It is important to fully solubilize the PTPase substrate in the microplate wells before adding the PTPase sample. To minimize background, rehydrate the substrate immediately before the assay.

3.2 Prepare a no-PTPase control. Include a negative control by adding an additional 20 µL of reaction buffer to any well containing the reconstituted substrate (prepared in step 3.1), and mix by pipetting.

3.3 Dilute the PTPase-containing samples. Dilute the samples in the provided reaction buffer or another appropriate buffer. A volume of 20 µL will be used for each reaction. The dilution factor required depends on the total amount of PTPase present in the sample. In the first trial, the samples should be serially diluted to determine the optimal amount of sample for the assay. Please note that these PTPase samples will be further diluted in the assay by a factor of five.

3.4 *(Optional)* Prepare a plus-PTPase positive control. Generate a positive control by diluting an appropriate enzyme standard of known activity in the reaction buffer. A 20 µL volume will be used. This plus-PTPase control will demonstrate hydrolytic activity of the enzyme on the fluorogenic substrate.

3.5 Begin the reactions. Pipet 20 µL of the PTPase-containing samples, including any positive control samples, into the wells of the microplate containing reconstituted substrate (prepared in step 3.1), and mix well.

3.6 Incubate the reactions. Incubate at the optimal temperature for the PTPase, protected from light. The exact interval will have to be determined experimentally. A suggested starting range is 20–30 minutes. Because the assay is continuous (not terminated), fluorescence can be measured at multiple time points to follow the kinetics of the reactions.

3.7 Measure the fluorescence. Use a fluorescence microplate reader equipped with appropriate filters. DiFMU has excitation/emission maxima of approximately 358/452 nm (see Figure 1). We have found that standard filters for blue-fluorescent dyes (e.g., excitation = 355 ± 20 nm, emission = 460 ± 12.5 nm) can be used to detect DiFMU.

3.8 Correct for background fluorescence. For each point, subtract the value derived from the no-PTPase control.

PTPase-Inhibitor Assay

The following provides one possible protocol for measuring PTPase inhibition with the RediPlate 96 EnzChek Tyrosine Phosphatase Assay; other protocols can also be devised. Please note that the spectral properties of the PTPase inhibitor alone may require evaluation prior to the experiment to see if the compound absorbs or fluoresces at wavelengths that might interfere with DiFMU's fluorescence. Intrinsic fluorescence or, to a lesser extent, absorbance of the inhibitor can complicate the interpretation of fluorescence observed in the reactions. Thus, it may be necessary to perform additional controls not specified in the following protocol.

4.1 Dilute the PTPase inhibitor in reaction buffer. A volume of 80 µL will be used for each reaction. A variable dilution will be required depending on the potency of the inhibitor and the total amount of PTPase present in the sample. Please note that these inhibitor samples will be further diluted in the assay. To account for this dilution, multiply the concentration of the sample by 0.8.

4.2 Add the PTPase inhibitor–containing samples to the assay. Pipet 80 µL of the PTPase inhibitor–containing samples into as many assay wells as will be needed, and then mix by pipetting. It is important to fully solubilize the PTPase substrate before adding the PTPase sample (see step 4.6, below).

4.3 Prepare no-inhibitor controls. For controls, add 80 µL of reaction buffer, alone, to two assay wells and mix by pipetting. The first well will serve as a no-inhibitor/no-PTPase control; the other will serve as a no-inhibitor/plus-PTPase control.

4.4 Dilute the PTPase in reaction buffer. A volume of 20 µL will be used for each reaction. An appropriate dilution will be required depending on the total amount of PTPase-inhibitor in the sample. The optimal PTPase concentration for the PTPase-inhibitor must be determined in advance. Please note that the PTPase sample concentration will be further diluted in the assay by a factor of five.

4.5 Prepare a no-inhibitor/no-PTPase control. Add an additional 20 µL of reaction buffer to the no-inhibitor/no-PTPase assay well (prepared in step 4.3), and mix by pipetting.

4.6 Begin the reactions. Pipet 20 µL volumes of the PTPase into each inhibitor-containing well. In addition, pipet 20 µL of the PTPase into the no-inhibitor/plus-PTPase control well (prepared in step 4.3).

4.7 Incubate the reactions. Incubate at the optimal temperature for the PTPase, protected from light. The optimal time will have to be determined experimentally. A suggested starting range is

20–30 minutes. Because the assay is continuous (not terminated), fluorescence can be measured at multiple time points to follow the kinetics of the reactions.

4.8 Measure the fluorescence. Use a fluorescence microplate reader equipped with appropriate filters. DiFMU has excitation/emission maxima of approximately 358/452 nm (see Figure 1). We have found that standard filters for blue-fluorescent dyes (e.g., excitation = 355 ± 20 nm, emission = 460 ± 12.5 nm) can be used to detect DiFMU.

4.9 Correct for background fluorescence. For each point, subtract the value derived from the no-inhibitor/no-PTPase control.

4.10 Report the change in fluorescence. Inhibition can be reported either directly as corrected fluorescence (from step 4.9, see Figure 3) or as percent inhibition. Percent inhibition is calculated by using the following formula:

$$\%\ \text{inhibition} = \left(1 - \frac{F_{\text{inhibitor}}}{F_{\text{control}}}\right) \times 100\%$$

where $F_{inhibitor}$ is the corrected fluorescence of the inhibitor-containing sample and $F_{control}$ is the corrected fuorescence of the no-inhibitor/plus-PTPase control.

References

1. Oncogene 19, 6607 (2000); **2.** Trends Cell Biol 11, 258 (2001); **3.** Curr Opin Cell Biol 13, 182 (2001); **4.** Curr Opin Chem Biol 5, 416 (2001); **5.** J Mol Med 78, 473 (2000); **6.** Biochem Pharmacol 60, 877 (2000); **7.** Cell Res 10, 279 (2000); **8.** Curr Opin Neurobiol 11, 95 (2001); **9.** Neuroreport 11, R5 (2000); **10.** Adv Exp Med Biol 476, 35 (2000); **11.** Biochem Biophys Res Commun 286, 721 (2001).

Product List

Current prices may be obtained from our Web site or from our Customer Service Department.

Cat #	Product Name	Unit Size
R-22067	RediPlate™ 96 EnzChek® Tyrosine Phosphatase Assay Kit *one 96-well microplate*	1 kit
R-22068	RediPlate™ 384 EnzChek® Tyrosine Phosphatase Assay Kit *one 384-well microplate*	1 kit

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