Molecular Probes

Product Information

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RediPlate™ 96 EnzChek® Serine/Threonine Phosphatase Assay Kit (R-33700)

Quick Facts

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

Ex/Em: 358/452 nm

Introduction

Molecular Probes' RediPlate™ 96 EnzChek® Serine/Threonine Phosphatase Assay Kit provides a fast, simple and direct fluorescence-based assay for detecting serine/threonine phosphatases and their corresponding modulators and inhibitors. The majority of protein phosphorylation occurs on serine and threonine residues with <0.01–0.05% on tyrosine residues.[1] Serine/threonine phosphatases (Ser/Thr PPases) represent a large family of enzymes that have been implicated in regulation of metabolism,[2] transcription,[3] translation,[4] differentiation,[5] cell cycle,[1] cytoskeletal dynamics,[6] oncogenesis[7,8] and signal transduction.[9,10] Our RediPlate 96 Assay Kit provides researchers with a sensitive and convenient means to monitor Ser/Thr PPase activity and to screen Ser/Thr PPase inhibitors. 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. The substrate incorporated in the RediPlate 96 EnzChek Serine/Threonine Phosphatase Assay Kit is our patented 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP). This substrate generates DiFMU, which 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 Ser/Thr PPases — other phosphatases, including tyrosine phosphatases do not significantly react with the substrate (Figure 2). Unlike phosphopeptide-based assays, DiFMUP can be used to assay a variety of Ser/Thr PPases including PP-1, PP-2A and PP-2B (Figure 2). Additional advantages of our assay include compatibility with non-ionic detergents and insensitivity to free phosphate, resulting

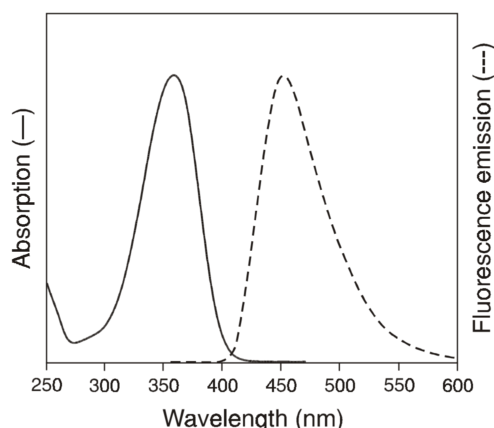
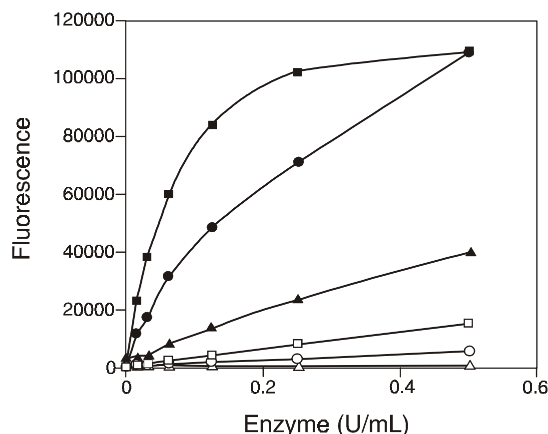


Figure 1. *Normalized absorption and fluorescence emission spectra of 6,8-difluoro-7-hydroxy-4-methylcoumarin (DiFMU).*



Symbol	Enzyme (Class)
■	PP-2A (ser/thr phosphatase *)
●	PP-1 (ser/thr phosphatase *)
▲	PP-2B (ser/thr phosphatase *)
□	Alkaline phosphatase
○	Acid phosphatase
△	LAR (PTPase †)

* Serine/threonine phosphatase. † Tyrosine phosphatase.

Figure 2. *Specificity of the RediPlate 96 EnzChek Serine/Threonine Phosphatase Assay Kit for serine/threonine phosphatases. The phosphatases listed in the table were applied at the indicated concentrations to a RediPlate 96 EnzChek serine/threonine phosphatase assay microplate. Reactions were incubated at 37°C. After 1 hour, the fluorescence was measured in a fluorescence-based microplate reader using excitation at 355 ± 20 nm and emission at 460 ± 12.5 nm.*

in minimal sample processing required before analysis. Ser/Thr PPase inhibitors can be evaluated quantitatively in the assay for their effect on Ser/Thr PPase activity (Figure 3).

The RediPlate 96 EnzChek Serine/Threonine Phosphatase Assay Kit includes one 96-well microplate, a bottle of reaction buffer and several buffer additives. 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 fluorogenic substrate, 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 serine/threonine phosphatase assay microplate** (Component A), one 96-well microplate
- **RediPlate serine/threonine phosphatase 2X reaction buffer** (Component B), 12 mL of 100 mM Tris-HCl at pH 7.0 containing 0.2 mM $CaCl_2$, 250 µg/mL BSA and 0.1% Tween® 20
- **$NiCl_2$** (Component C), 1.0 mL of a 40 mM solution in water
- **$MnCl_2$** (Component D), 1.0 mL of a 20 mM solution in water
- **Dithiothreitol (DTT)** (MW = 154.2, Component E), 50 mg

Storage and Handling

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

Materials required but not provided

Due to the diverse nature of Ser/Thr PPases, the reaction buffer may require additional components not included with this kit. Specifically, assays for PP-2C will require EGTA and $MgCl_2$ and assays for PP-2B will require calmodulin (see Table 1).

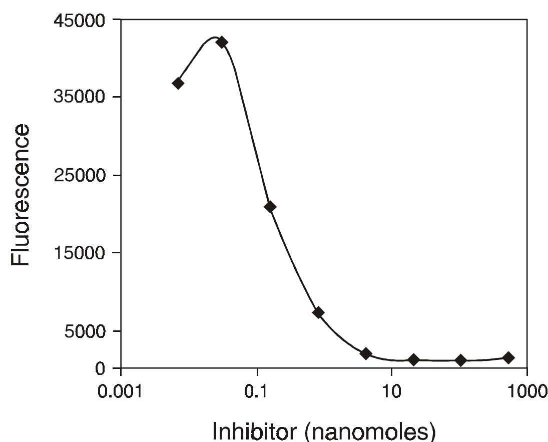


Figure 3. *Detection of PP-2A inhibition by okadaic acid using the RediPlate 96 EnzChek Serine/Threonine Phosphatase Assay Kit. Each reaction contained 50 µM DiFMUP, 10 mU/mL of PP-2A and the indicated concentration (log scale) of okadaic acid in reaction buffer containing 50 mM Tris-HCl, 0.1 mM $CaCl_2$, 1 mM $NiCl_2$, 125 µg/mL BSA and 0.05% Tween 20. 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.*

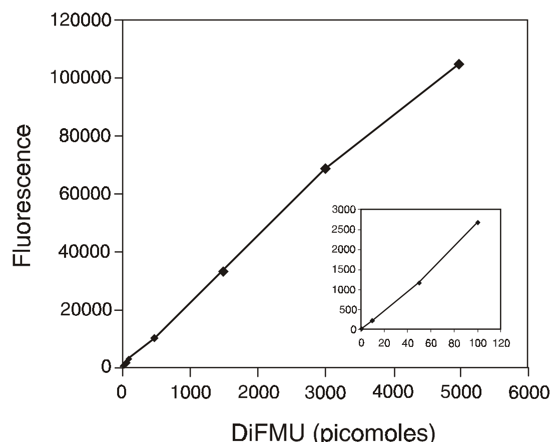


Figure 4. *Dynamic range and sensitivity of the serine/threonine phosphatase assay. A 100 µL volume of the reaction buffer optimized for PP-2A was added to each well containing the DiFMU reference standard. Fluorescence was measured in a fluorescence-based microplate reader using excitation at 355 ± 30 nm and emission at 440 ± 17.5 nm. Background fluorescence (121 arbitrary units), determined for well H, 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.*

Experimental Protocol

The following protocols describe the assay for Ser/Thr PPase or Ser/Thr PPase inhibitor activity in total volumes of 100 µL per microplate well. Each RediPlate 96 EnzChek Serine/Threonine Phosphatase Assay Kit contains one 96-well microplate with 88 wells (11 strips) intended for assays and 8 wells (1 strip, with blackened tabs) containing DiFMU for a fluorescence reference standard curve. Because each strip is removable, one 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 warm it 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. After thawing, the buffer may be stored at 2–6°C, for convenience.

1.2 Remove any extra strips. Determine the number of strips required and 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 Ser/Thr PPase substrate. Empty strip holders from previously purchased RediPlate 96 kits are useful for storing extra assay strips.

Reaction Buffer Preparation

Due to the diverse nature of Ser/Thr PPases, the provided reaction buffer may require the addition of salts, proteins or chelators depending upon the specific enzyme to be assayed. Consult Table 1 for a listing of recommended components and their final concentrations in the reaction buffer. Please note that the buffer composition is only a recommendation and may require additional optimization for a specific Ser/Thr PPase.

2.1 (*For PP-1 and PP-2C*) Prepare a 1 M stock solution of dithiothreitol (DTT). Dissolve the contents of the vial of DTT (Component E) in 325 µL of deionized water (dH_2O).

2.2 Optimize the reaction buffer. Using Table 1 as a guide, add directly to the 2X reaction buffer (Component B) any additional components required by the Ser/Thr PPase for optimal activity.

2.3 Prepare 1X reaction buffer. Dilute the concentrated reaction buffer containing any additional components to a final volume of 24 mL with dH_2O.

Fluorescence Reference Standards

A standard curve derived from the provided reference standards 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 released by cleavage of 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.

3.1 Prepare the fluorescence standards. Add 100 µL of the 1X reaction buffer (prepared in step 2.3) to each well of the reference standard strip of the RediPlate 96 microplate, and mix by pipetting. Blackened tabs differentiate this strip from strips containing DiFMUP. The control strip contains a series of DiFMU reference standards (Table 2 and Figure 4). Well A has the highest amount of the reference standard (5000 picomoles); well H contains no reference dye.

Table 2. *Reference standards for the RediPlate 96 Serine/Threonine 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

3.2 Measure the fluorescence. The fluorescence standard samples are typically measured for fluorescence along with the samples from the Ser/Thr PPase or Ser/Thr PPase-inhibitor assays (see below).

Ser/Thr PPase Assay

4.1 Add 50 µL of the reaction buffer to the assay wells. Add the 1X reaction buffer (prepared in step 2.3) or another appropriate buffer to as many assay wells as will be needed, and then mix by pipetting. It is important to fully solubilize the Ser/Thr phosphtase substrate (DiFMUP) in the microplate wells before adding the Ser/Thr PPase sample. To minimize background, rehydrate the substrate immediately before the assay. The concentration of the reconstituted DiMUP is now 100 µM but will be 50 µM in the final assay volume.

4.2 Prepare a no-PPase control. Include a negative control by adding an additional 50 µL of reaction buffer to any well containing the reconstituted substrate (prepared in step 4.1), and mix by pipetting.

4.3 Dilute the Ser/Thr PPase-containing samples. Dilute the samples in the 1X reaction buffer (prepared in step 2.3) or an-

Table 1. *Additional reaction buffer components suggested for specific Ser/Thr phosphatases.*

Ser/Thr Phosphatase	Additive (Kit Component)	Stock Concentration	Volume *	Final Concentration †
PP-1	DTT (E)	1 M **	48 µL	2 mM
	$MnCl_2$ (D)	20 mM	240 µL	200 µM
PP-2A	$NiCl_2$ (C)	40 mM	600 µL	1 mM
PP-2B	Calmodulin ‡	NA ‡	NA ‡	10 ug/mL
	$NiCl_2$ (C)	40 mM	600 µL	1 mM
PP-2C	DTT (E)	1 M **	48 µL	2 mM
	EGTA ‡	NA ‡	NA ‡	1 mM
	$MgCl_2$ ‡	NA ‡	NA ‡	20 mM

* Volume added to 12 mL of 2X Reaction Buffer. † Final concentration in 1X Reaction Buffer. ‡ Not included as a component of the RediPlate 96 EnzChek Serine/Threonine Phosphatase Assay Kit. ** Prepared in step 2.1. ‡ Will need to be supplied or determined by the end user. NA = not applicable.

other appropriate buffer. A volume of 50 µL will be used for each reaction. The dilution factor required depends on the total amount of Ser/Thr PPase 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 Ser/Thr PPase samples will be further diluted in the assay by a factor of two.

4.4 ***(Optional)*** **Prepare a plus-PPase positive control.** Generate a positive control by diluting an appropriate enzyme standard of known activity in the reaction buffer. A 50 µL volume will be used. This plus-PPase control will validate the performance of the fluorogenic substrate.

4.5 Begin the reactions. Pipet 50 µL of the Ser/Thr PPase-containing samples, including any positive control samples, into the wells of the microplate containing reconstituted substrate (prepared in step 4.1), and mix well.

4.6 Incubate the reactions. Incubate at the optimal temperature for the Ser/Thr PPase, 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.

4.7 Measure the fluorescence. Use a fluorescence microplate reader equipped with appropriate filters. DiFMU has excitation/emission maxima of approximately 358/452 nm. 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.

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

Ser/Thr PPase-Inhibitor Assay

The following provides one possible protocol for measuring Ser/Thr PPase inhibition with the RediPlate 96 EnzChek Serine/Threonine Phosphatase Assay; other protocols can also be devised. Please note that the spectral properties of the Ser/Thr PPase 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 absorbance or fluorescence 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.

5.1 Dilute the Ser/Thr PPase 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 Ser/Thr PPase present in the sample. Please note that these inhibitor samples will be further diluted in the assay. The final inhibitor concentrations in the assay will be 0.8-fold lower than these initial concentrations.

5.2 Add the Ser/Thr PPase inhibitor–containing samples to the assay wells. Pipet 80 µL of the Ser/Thr PPase inhibitor–containing samples into as many assay wells as will be needed, and then mix by pipetting. It is important to fully solubilize the Ser/Thr PPase substrate (DiFMUP) before adding the PTPase sample (see step 5.6, below). The concentration of the reconstituted DiFMUP is now 62.5 µM but will be 50 µM in the final assay volume.

5.3 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-PPase control; the other will serve as a no-inhibitor/plus-PPase control.

5.4 Dilute the Ser/Thr PPase 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 Ser/Thr PPase-inhibitor in the sample. The optimal Ser/Thr PPase concentration for the Ser/Thr PPase-inhibitor must be determined in advance. Please note that the Ser/Thr PPase sample concentration will be further diluted in the assay by a factor of five.

5.5 Prepare a no-inhibitor/no-PPase control. Add an additional 20 µL of reaction buffer to the no-inhibitor/no-PPase assay well (prepared in step 5.3) and mix by pipetting.

5.6 Begin the reactions. Pipet 20 µL volumes of the Ser/Thr PPase into each inhibitor-containing well. In addition, pipet 20 µL of the Ser/Thr PPase into the no-inhibitor/plus-PPase control well (prepared in step 5.3).

5.7 Incubate the reactions. Incubate at the optimal temperature for the Ser/Thr PPase, 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.

5.8 Measure the fluorescence. Use a fluorescence microplate reader equipped with appropriate filters. DiFMU has excitation/emission maxima of approximately 358/452 nm. 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.

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

5.10 Report the change in fluorescence. Inhibition can be reported either directly as corrected fluorescence (from step 5.9, 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 fluorescence of the no-inhibitor/plus-PPase control.

References

1. Oncogene 19, 6607 (2000); **2.** Proc Natl Acad Sci USA 98, 13710 (2001); **3.** Biochem Biophys Res Commun 285, 1192 (2001); **4.** J Biol Chem 276, 14829 (2001); **5.** J Neurosci Methods 105, 87 (2001); **6.** Eur J Immunol 30, 3422 (2000); **7.** Proc Natl Acad Sci USA 97, 3207 (2000); **8.** Eur J Biochem 263, 605 (1999); **9.** Eur J Biochem 269, 1060 (2002); **10.** Proc Natl Acad Sci USA 98, 13613 (2001).

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

Cat #	Product Name	Unit Size
R-33700	RediPlate™ 96 EnzChek® Serine/Threonine Phosphatase Assay Kit *one 96-well microplate*	1 kit

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