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| **Revision** | **06-July-2011 JSW** |
| **Synonyms** | **Protein Tyrosine Phosphatase 1B Assay Kit, Colorimetric** |
| **Form** | **96 Tests** |
| **Format** | **96-well plate** |
| **Detection method** | **Colorimetric** |
| **Storage** | **Upon arrival store the 1/2 Volume Plate at room temperature and the remaining components at -70°C to maintain the highest stability. The PTP1B must be handled very carefully in order to retain maximal enzymatic activity. Thaw it quickly in a RT water bath or by rubbing between fingers, then immediately store on an ice bath. The remaining unused enzyme should be quickly refrozen by placing at -70°C. To minimize the number of freeze/thaw cycles, aliquot the PTP1B into separate tubes and store at -70°C.** |
| **Intended use** | **PLEASE READ THE ENTIRE PROTOCOL BEFORE PROCEEDING WITH THE ASSAY. CAREFULLY NOTE THE HANDLING AND STORAGE CONDITIONS OF EACH KIT COMPONENT. PLEASE CONTACT CALBIOCHEM® TECHNICAL SERVICES FOR ASSISTANCE IF NECESSARY.**<br><br>**NOTE: THE FOLLOWING PROCEDURES ARE INTENDED ONLY AS A GUIDELINE. THE OPTIMAL EXPERIMENTAL CONDITIONS WILL VARY DEPENDING ON THE PARAMETERS BEING INVESTIGATED, AND MUST BE DETERMINED BY THE INDIVIDUAL USER.**<br><br>**The Calbiochem® PTP1B Assay Kit, Colorimetric is a nonradioactive assay designed for measuring PTP1B activity in purified prperations and for inhibitor or activator screening.** |
| **Principles of the assay** | **The Calbiochem® Protein Tyrosine Phosphatase 1B Assay Kit is a colorimetric, non-radioactive assay designed to measure PTP1B activity in purified preparations and for inhibitor or activator screening. The kit includes human, recombinant PTP1B (residues 1-322; M.W. 37,400), expressed in *E. coli*. The** |

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| | **phosphopeptide substrate supplied with this kit contains sequence from the insulin receptor β subunit domain that must be autophosphorylated to achieve full receptor kinase activation. This "activation loop" is the target of several protein phosphatase regulators of insulin signaling, including, notably, PTP1B. The detection of free phosphate released is based on the classic Malachite green assay and offers the following advantages: non-radioactive; convenient 1-step detection; excellent sensitivity. The PTP1B inhibitor Suramin is supplied as a control for inhibitor detection. Suramin is a reversible and competitive inhibitor of PTP1B, with a $K_i$ of 5.5 µM.** |
| **Materials provided** | **• PTP1B Enzyme (Kit Component No. KP8401-5UG): 1 vial, 5 µg, 100 ng/µl in 50 mM HEPES, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, 0.05% NP-40, pH 7.2 in a screw cap microcentrifuge tube**<br>**• PTP1B Substrate (IR5) (Kit Component No. KP31851-1MG): 1 vial, 1 mg, supplied as a lyophilized solid in a screw cap vial, composed of amino acids 1142-1153, $pY^{1146}$, M.W. 1703 kDa**<br>**• 2X Assay Buffer (Kit Component No. KP31852-20ML): 1 bottle, 20 ml, supplied as 2X buffer (300 mM NaCl, 100 mM MES, 2 mM DTT, 2 mM EDTA, 0.1% NP-40, pH 7.2) in a screw-cap plastic bottle**<br>**• Red Reagent (Kit Component No. KP31818-5ML): 1 bottle, 5 ml, supplied as concentrated phosphate detection reagent in a screw-cap plastic bottle**<br>**• Phosphate Standard, (Kit Component No. KP8405-500UL): 1 vial, 500 µl, supplied as a 100 µM solution in 1X Assay Buffer in a screw cap microcentrifuge tube**<br>**• Suramin Inhibitor (Kit Component No. KP31853-10MG): 1 vial, 10 mg**<br>**• 1/2 Volume Plate, (Kit Component No. KP8407-1EA): 1 plate** |
| **Materials Required but not provided** | **• Plate reader capable of measuring $A_{620}$ to >3-decimal accuracy**<br>**• Pipetman capable of pipetting 2-100 µl accurately**<br>**• Multi-channel pipetman capable of pipetting 25 and 50 µl (optional)**<br>**• Microcentrifuge**<br>**• Ice bucket to keep reagents cold until use**<br>**• Temperature controlled microplate warmer and/or water bath** |

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| | **for performing assays at other than ambient temperature (optional)** |
| **Precautions and recommendations** | **• The Red 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 assay. Detergents used to clean labware may contain high levels of phosphate. Use caution by either rinsing labware with $dH_2O$ or employ unused plasticware.**<br>**• This kit is designed to perform endpoint assays in which each well contains a 100 µl reaction in Assay Buffer and is terminated by the addition of 25 µl of the phosphate detection reagent, Red Reagent. The 100 µl "reaction" may consist either of PTP1B phosphatase acting on the phosphopeptide substrate or simply a dilution of the free Phosphate Standard. Bear these volumes in mind when, for example, planning how much 1X Assay Buffer to prepare for a given experiment.**<br>**• Enough of the IR5 phosphopeptide substrate (0.59 µmol) is provided to perform, for example, 96 assays at 60 µM, 84 assays at 70 µM, 78 assays at 75 µM or 72 assays at 80 µM. The $K_m$ of PTP1B for the IR5 substrate is ~85 µM, so any of the above concentrations would be reasonable choices for inhibitor screening. Note that the Phosphate Standard Curve experiment described below requires six wells, twelve when done in duplicate etc.**<br>**• Enough enzyme is provided (5 µg = 50 µl @ 100 ng/µl) to allow for a broad range of possible enzyme amounts per well. Typically, 2-3 ng/well will provide a useful signal (~1 nmol phosphate) with a 30 min incubation at 30°C, and a 75 µM final concentration of the PTP1B Substrate (IR5).**<br>**• The phosphatase reactions in the Time Course and Test Sample/Inhibitor experiments described below are initiated by mixing 50 µl of a prewarmed 2X Substrate solution into an assay well containing 50 µl of prewarmed 2X Enzyme solution. In the example given for the Test Sample/Inhibitor experiment, the addition of inhibitor is made to the 2X Enzyme solution. This would be a useful way to proceed if, for example, investigating an inhibitor that reacts covalently with the enzyme, but whose reaction would be blocked by the substrate. It may in some cases, however, be desirable to add inhibitor to the 2X Substrate** |

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| | **solution and thereby insure that the enzyme is exposed simultaneously to substrate and inhibitor.** |
| **Reagent preparation** | **1. Thaw all kit components and hold the PTP1B Enzyme, PTP1B Substrate (IR5), and Assay Buffer on an ice bath; store Red Reagent at room temperature (RT). 2. Reconstitute the PTP1B substrate to 1.5 mM by adding 88 µl 2X Assay Buffer and 88 µl of $dH_2O$ to 500 µg net peptide. Vortex. Following reconstitution, aliquot and freeze (-70°C). 3. Prepare a phosphate standard curve: a. Prepare 1.2 ml of 1X Assay Buffer by diluting 600 µl 2X Assay Buffer with 600 µl $dH_2O$. b. Pipette into duplicate sets of six wells: 100, 97.5, 95, 90, 80 and 70 µl 1X Assay Buffer. c. Pipette, into those same wells, in the same order, 0, 2.5, 5, 10, 20 and 30 µl of the 100 µM Phosphate Standard. d. These wells will contain, respectively, 0, 0.25, 0.5, 1.0, 2.0 and 3.0 nmol of inorganic phosphate. NOTE: PERFORM STANDARD CURVE IN DUPLICATE.** |
| **Detailed protocol** | **Time Course Assay:**<br><br>**1. Prepare 1 ml 1X Assay Buffer (dilute 500 µl of 2X assay buffer with 500 µl of $dH_2O$) and keep on ice.**<br>**2. Designate reaction times for a desired number of wells (e.g.: 30, 20, 10, 5 and 0 min).**<br>**3. Equilibrate plate to reaction temperature (e.g.: 30 °C).**<br>**4. Add 45 µl 1X Assay Buffer to each well and equilibrate to assay temperature (at least 5 min).**<br>**5. Prepare a dilution of PTP1B enzyme in cold 1X Assay Buffer, such that each 5 µl contains the desired amount of enzyme per well. Example: Dilute the PTP1B 200-fold, e.g. 2 µl plus 398 Assay Buffer, to prepare a 0.5 ng/µl stock for assay at 2.5 ng/well.**<br>**6. Prepare a dilution of the 1.5 mM IR5 PTP1B Substrate (IR5) stock at 2 times the desired assay concentration and warm to assay temperature. Example: Prepare 300 µl 150 µM IR5 (2X Substrate) by mixing with 30 µl 1.5 mM IR5 plus 270 µl Assay Buffer and warm to 30°C.**<br>**7. Just before adding substrate to start the reaction in each well, add 5 µl of the cold PTP1B dilution to the 45 µl of warmed Assay Buffer. Initiate reactions by then mixing in 50 µl of the warmed 2X Substrate. Make the additions in the reverse time order such that all incubations end at the same time (e.g., Add 30 min time** |

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| | **pt. at t=0; add 5 min at t=25 min, etc.). NOTE: A convenient way to prepare accurate 0 min reactions is to add the 2X Substrate to 'time zero, wells immediately after the termination of the reactions by the addition of Green Reagent.**<br><br>**Test Sample/Inhibitor Assay:**<br><br>**1. Prepare appropriate volumes of 1X Assay Buffer, a PTP1B dilution and 2X Substrate as described in Time Course Assay above.**<br>**2. Prepare test sample/inhibitor solutions in 1X Assay Buffer at 10 times the desired final concentration and warm to assay temperature, e.g. 30°C. Example: Prepare a 10 mM stock of Suramin by dissolving the 10 mg of solid in 0.7 ml of 1X Assay Buffer. For a final concentration of 10 µM, prepare a 10X stock (100 µM) by mixing 10 µl of the 10 mM stock with 990 µl Assay Buffer.**<br>**3. Add 35 µl of 1X Assay Buffer to each well and warm to assay temperature, e.g. 30°C.**<br>**4. Add 10 µl of test sample/inhibitor 10X stocks to appropriate wells. Add 10 µl of 1X Assay Buffer to control wells.**<br>**5. Add 5 µl of the PTP1B enzyme dilution to each well.**<br>**6. Initiate reactions by then in 50 µl of the warmed 2X Substrate.**<br>**7. Incubate samples at desired temperature, e.g. 30°C, for desired length of time, e.g. 30 min.**<br><br>**Reaction Termination:**<br><br>**1. After incubating wells for desired duration, including the standard curve, terminate reactions by addition of 25 µl Red Reagent. Agitate plate or triturate wells gently to mix.**<br>**NOTE: Avoid production of air bubbles in the wells.**<br>**2. Allow color to develop for 20-30 min. Be careful to assure samples spend approximately the same time with the reagent before reading on the plate reader.**<br>**3. Read the absorbance at 620 nm ($A_{620}$) on plate reader.**<br>**4. Perform data analysis (see below).**<br>**NOTE: Retain plate for future use of unused wells!** |
| **Calculations** | **Conversion of $A_{620}$ to nmol of Phosphate with a Standard Curve. 1. Plot standard curve data as $A_{620}$nm versus nmol $PO_4^{2-}$.** |

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| | **2. Obtain a line-fit or fits to the data using an appropriate routine. Note that the plot may not be linear over the entire span from 0 to 3 nmol of phosphate. As shown in figure below, two linear fits, one for 0 to 1 nmol phosphate and a second for 1 to 3 nmol, can produce a more accurate correlation of $A_{620}$ to amount of phosphate. 3. Use the slope and Y-intercept of the appropriate plot to calculate amount of phosphate released for a particular data point. 4. Example (sample calculation with the standard curve in the figure below): a) Measured $A_{620}$ = 0.180. Since this A falls below that of the 1 nmol point in the figure, choose the 0 to 1 nmol fit, i.e. y = 6.58x - 0.386. b) Replacing x with $A_{620}$ and y with nmol of $PO_4^{2-}$ produces: nmol of $PO_4^{2-}$ = 6.58($A_{620}$) - 0.386 nmol of $PO_4^{2-}$ = 6.58(0.180) - 0.386 nmol of $PO_4^{2-}$ = 0.798 NOTE: For highest accuracy, a standard curve should be performed for each new set of assay data. This will normalize for variations in free phosphate in samples, time of incubation with the Green Reagent, and other experimental factors. Time Course and Rate Calculations 1. Using a standard curve, convert $A_{620}$ measurements for a series of time points to nmol of phosphate, as described above. 2. Plot nmol of phosphate versus time and obtain the slope in nmol/min. If the slope is decreasing at later times, restrict the time points used in the slope determination to the earliest, linear part of the plot. 3. It may be useful to calculate the total amount of phosphopeptide per well contained at the chosen substrate concentration. For example, at 75 µM IR5 peptide in 100 µl reaction that is: 75 x $10^{-6}$ mol/L x 100 x $10^{-6}$ L = 7500 x $10^{-12}$ mol = 7.5 nmol 4. Although when possible it is best to obtain initial rate data from the first few percent of substrate converted to product, this may not generate a sufficient signal for an accurate rate estimate. In practice, with PTP1B at 75 µM IR5 substrate, time course plots remain linear for at least the first 15% of substrate hydrolyzed (~1 nmol of phosphate). Test Sample/Inhibitor Data 1. It is important to obtain a "time zero" measurement and to subtract this value, expressed as nmol of phosphate, from both the control and test sample/inhibitor values. Again, a convenient way to obtain an accurate t=0 measurement is to add and mix the 25 µl of Red Reagent into the 50 µl of 2X Enzyme and then mix in the 50 µl of 2X Substrate (see Time Course instructions). 2. Calculate activity as a % of Control** |

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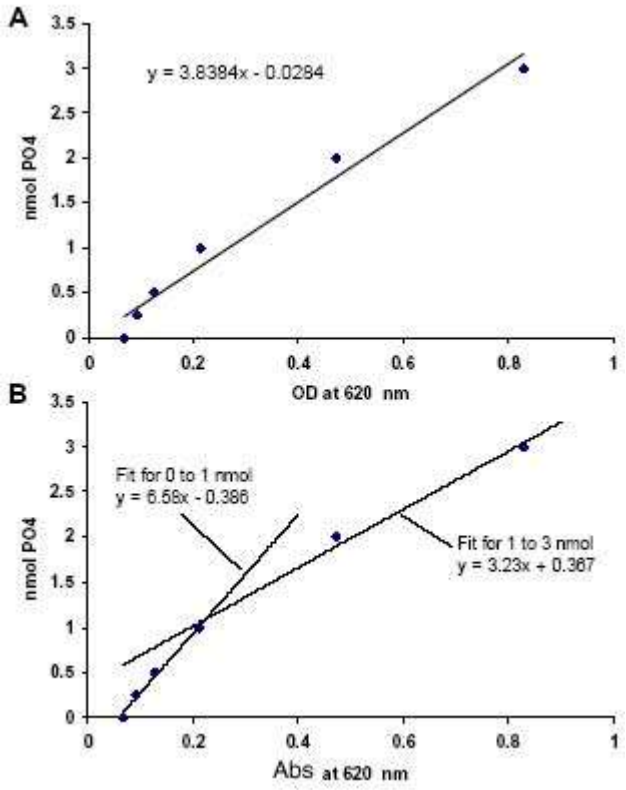
| | **Figure 1: Activity Calculation**<br><br>% Activity =<br><br>$\frac{[\text{Test sample (nmol } PO_4^{2-}) - \text{“time zero” (nmol } PO_4^{2-})]}{[\text{Control (nmol } PO_4^{2-}) - \text{“time zero” (nmol } PO_4^{2-})]} \times 100\%$<br><br>**3. Add additional controls as necessary. For example, while an extremely low reading will generally indicate that the test sample is a potent inhibitor, there is the possibility that the compound interfered with the color development. In such a case, an appropriate control would be to compare the color reaction obtained from 1 nmol of phosphate (10 µl of 100 µM Phosphate Standard) with and without added test compound [buffer alone with no enzyme or PTP1B Substrate (IR5)]; see Phosphate Standard Curve instructions).** |
| **Standard curve** | **Figure 2: Phosphate Standard Curve**<br><br>  <br><br><br>**Dilutions of phosphate standard and a buffer blank were prepared as described. The 100 µl samples were mixed with 25 µl Red Reagent and incubated at 30°C, 20 min to develop color. $A_{620nm}$ was read on a microplate spectrophotometer. A. Least-squares fit to the entire set of phosphate amounts, from 0 to 3 nmol. B. A more accurate correlation of $A_{620}$ to phosphate is** |

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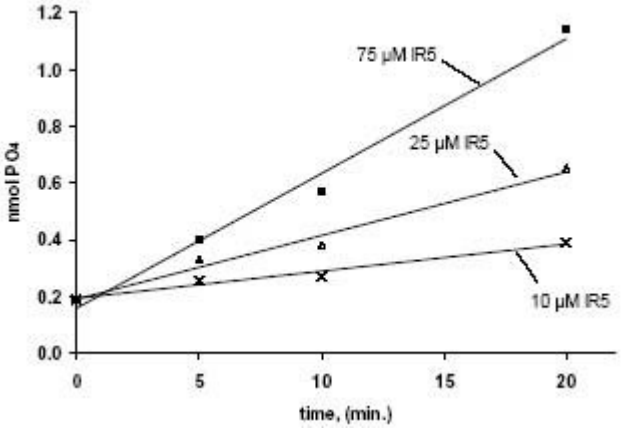
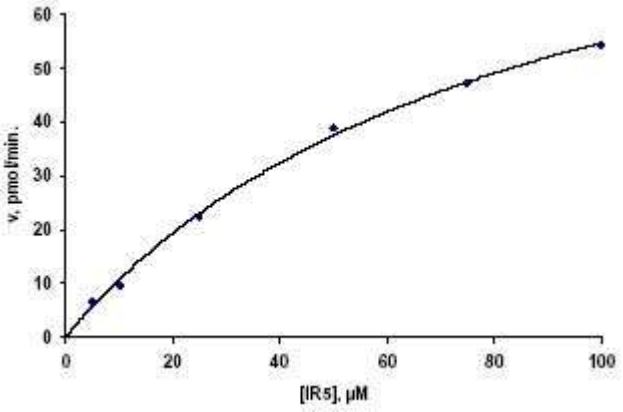
| | **obtained by separate fits of the data from 0 to 1 nmol and 1 to 3 nmol.** |
| **Example data** | **Figure 3: Time Courses of PTP1B Phosphate Release from the PTP1B Substrate (IR5)**<br>  <br><br>**2X PTP1B Substrate solutions (150, 50 and 20 µM) and 2X Enzyme solutions (2 ng/well) were prepared and incubations at 30°C were performed. Reactions were then terminated by addition of 25 µl of Red Reagent and $A_{620}$ read. $A_{620}$ readings were converted to nmol of $PO_4^{2-}$ with a phosphate standard curve. Each point represents the mean of two determinations.**<br>**Figure 4: Dependence of PTP1B Kinetics on the Concentration**<br>  <br><br>**Initial rates of PTP1B Substrate (IR5) dephosphorylation by 2 ng of PTB1B Enzyme were determined at 30°C and the indicated concentrations from 20 min time course plots. The line is a non-linear least squares fit to the Michaelis-Menten equation. The $K_m$ for IR5 was 85 µM and the $V_{max}$ was 101 pmol/min.**<br>**Figure 5: Inhibition of PTP1B by Suramin** |

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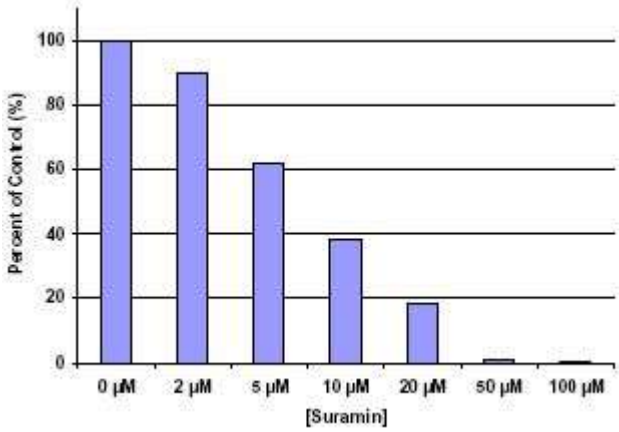
| |  <br>**Phosphate released in 30 min from 75 µM PTP1B Substrate (IR5) by 2.5 ng PTP1B Enzyme, at 30°C, was determined at the indicated concentrations of Suramin. Data was recalculated as percent of the '0 µM' Suramin control.** |
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