PTP1B Tyrosine Phosphatase Drug Discovery Kit
A colorimetric, non-radioactive assay designed to measure the phosphatase activity of purified PTP1B.

Instruction Manual
BML-AK822

For research use only
BACKGROUND

The *PTP1B Tyrosine Phosphatase Drug Discovery Kit* is a colorimetric, non-radioactive assay designed to measure the phosphatase activity of purified PTP1B. This 96-well assay is useful for screening inhibitors and modulators of PTP1B activity. The kit includes human, recombinant PTP1B (residues 1-322; MW=37.4 kDa), expressed in *E. coli*. The detection of free-phosphate released is based on the classic Malachite green assay and offers the advantages of convenient, 1-step detection and excellent sensitivity, without radioactivity.

PTP1B (protein tyrosine phosphatase-1B) is a ubiquitous, non-transmembrane protein tyrosine phosphatase, originally identified in human placenta. It is implicated in the negative regulation of insulin receptor signaling, and is a potential therapeutic target for treatment of type 2 diabetes and obesity.

The phosphopeptide substrate supplied with this kit ("IR5", Cat. BML-P315, pTyr-1158 (a.k.a. pTyr-1146, mature peptide numbering)) contains sequence from the insulin receptor ß 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 PTP1B inhibitor suramin (BML-KI285) 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. (See Fig. 4)

References:
3. B. Martin *et al.* *J. Biol. Chem.* 1985 **260** 14932
4. K.W. Harder *et al.* *Biochem. J.* 1994 **298** 395
6. C. Sun *et al.* *Cell Metabolism* 2007 **6** 307
7. B. Xue *et al.* *J. Biol. Chem.* 2007 **282** 23829
8. C. Ramachandran *et al.* *Biochemistry* 1992 **31** 4232
9. F.P. Ottensmeyer *et al.* *Biochemistry* 2000 **39** 12103
10. S.R. Hubbard *EMBO J.* 1997 **16** 5572
ALSO AVAILABLE SEPARATELY...

<table>
<thead>
<tr>
<th>PRODUCT</th>
<th>CATALOG #</th>
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<tbody>
<tr>
<td>PTP1B Enzyme (human, recombinant)</td>
<td>BML-SE332</td>
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<tr>
<td>“IR5”, PTP1B substrate</td>
<td>BML-P315</td>
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PLEASE READ ENTIRE BOOKLET BEFORE PROCEEDING WITH THE ASSAY. CAREFULLY NOTE THE HANDLING AND STORAGE CONDITIONS OF EACH KIT COMPONENT. PLEASE CONTACT ENZO LIFE SCIENCES TECHNICAL SERVICES FOR ASSISTANCE IF NECESSARY.

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. NO WARRANTY OR GUARANTEE OF PERFORMANCE USING THESE PROCEDURES IS MADE OR IMPLIED.
COMPONENTS

NOTE ON STORAGE: Store all components except the microtiter plate at -80°C for the highest stability. Components with storage temperatures other than -80°C can be stored at the temperature listed OR at -80°C. The PTP1B enzyme (component BML-SE332-9092) must be handled carefully in order to retain maximal enzymatic activity. Thaw quickly in a RT water bath or by rubbing between fingers, then immediately store on ice. The remaining unused enzyme should be ‘snap’ frozen, e.g. in liquid nitrogen or a dry ice/ethanol bath, and stored at -80°C. If only a few assays are to be performed each day, the PTP1B may be divided into several aliquots (best if ≥10 µL) to help minimize freeze/thaw cycles.

BML-SE332-9092 PTP1B ENZYME (human recombinant)
FORM: 0.1 µg/µL in 50 mM HEPES, pH 7.2, 1 mM EDTA, 1 mM DTT, 0.05% NP-40, 10% (v/v) glycerol
MW: 37.4 kDa
STORAGE: -80°C. AVOID FREEZE/THAW CYCLES!
QUANTITY: 5 µg

BML-P315-9090 SUBSTRATE ("IR5" Insulin Receptor β residues 1142-1153, pY-1146)
FORM: Lyophilized solid.
MW: 1703 Da
STORAGE: -20°C
QUANTITY: 1 mg net peptide/vial

BML-KI467-0020 2X ASSAY BUFFER
FORM: 100 mM MES, pH 6.0, 300 mM NaCl, 2 mM EDTA, 2 mM DTT, 0.1% NP-40
STORAGE: -80°C
QUANTITY: 20 mL

BML-KI468-0005 BIOMOL RED™ Concentrated Phosphate Detection Reagent
FORM: Liquid in screw-cap plastic bottle.
STORAGE: 4°C. Long-term at -20°C or lower.
QUANTITY: 5 mL

BML-KI285-0010 SURAMIN (PTP1B INHIBITOR)
FORM: Solid
MW: 1429.2
STORAGE: -20°C
QUANTITY: 10 mg
RECONSTITUTION: Water or Assay Buffer to 10 mM (10 mg in 0.70 mL).
Store solution at -70°C.

BML-KI470-0500 PHOSPHATE STANDARD
FORM: 100 µM in 1X Assay Buffer
STORAGE: -20°C
QUANTITY: 0.5 mL

80-2404 ½-VOLUME MICROPLATE
1 clear, 96-well
STORAGE: Room Temperature
OTHER MATERIALS REQUIRED

- Microplate reader capable of measuring OD_{620} to ≥3-decimal accuracy.
- Pipettors capable of accurately pipetting 2-100 µL
- Multi-channel pipettor capable of pipetting 25 and 50 µL (optional).
- Microcentrifuge.
- Ice bucket to keep reagents cold until use.
- Temperature controlled microplate warmer and/or water bath for performing assays at other than ambient temperature (optional).

EXPERIMENTAL METHODS

PRECAUTIONS: BIOMOL® Red 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 dH₂O or employ unused plasticware.

Some Things To Consider When Planning Assays.

1. 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, BIOMOL® Red. The 100 µL “reaction” may consist either of PTP1B phosphatase acting on the phosphopeptide substrate or simply a dilution of the free Phosphate Standard (BML-K1470). Bear these volumes in mind when, for example, planning how much 1X Assay Buffer to prepare for a given experiment.

2. Enough of the IR5 phosphopeptide substrate (0.59 µmol; BML-P315) 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 (See Fig. 3), 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.

3. 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 IR5 peptide substrate (BML-P315).

4. 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 solution and thereby ensure that the enzyme is exposed simultaneously to substrate and inhibitor.

To Prepare Reagents For The Assay:

1. Thaw all kit components and hold PTP1B, substrate, and assay buffer on an ice bath; Store BIOMOL RED™ reagent at room temperature (RT).

2. Reconstitute a vial of IR5 substrate (BML-P315) to 1.5 mM by adding 196 µL 2X Assay Buffer (BML-K1467) plus 196 µL dH₂O to 1 mg net peptide. Vortex. After use, store remaining substrate at -70°C.
To Prepare A Phosphate Standard Curve:
1. Prepare 1.2 mL 1X assay buffer by diluting 600 µL of the 2X assay buffer with 600 µL of dH2O.
2. Pipette into duplicate sets of six wells: 100, 97.5, 95, 90, 80 and 70 µL of 1X Assay Buffer.
3. Pipette, into those same wells, in the same order, 0, 2.5, 5, 10, 20, and 30 µL of the 100 µM Phosphate Standard (BML-KI470).
4. These wells will contain, respectively, 0, 0.25, 0.5, 1.0, 2.0 and 3.0 nmol of inorganic phosphate.

To Prepare A Time Course Assay:
1. Prepare 1 mL 1X Assay Buffer (dilute 500 µL of 2X assay buffer with 500 µL of dH2O) and keep on ice.
2. Designate reaction times for a desired number of wells (e.g.: 30, 20, 10, 5 and 0 min).
3. Equilibrate microtiter plate to reaction temperature (e.g.: 30 °C).
4. Add 45 µL 1X assay buffer (prepared in step 1) to each well and equilibrate to assay temperature (at least 5 min. at, for example, 30°C).
5. Prepare a dilution of PTP1B enzyme (BML-SE332-9092) 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.
6. Prepare a dilution of the 1.5 mM IR5 substrate stock at 2 times the desired assay concentration and warm to assay temperature. Example: Prepare 300 µL 150 µM IR5(2X Substrate)—30 µL 1.5 mM IR5 plus 270 µL Assay Buffer—and warm to 30°C.
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 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 BIOMOL® Red.
8. See Fig. 2. for plots of time courses performed at several concentrations of substrate.

To Prepare A Test Sample/Inhibitor Assay:
1. Prepare appropriate volumes of 1X Assay Buffer, a PTP1B dilution and 2X Substrate as described in “To prepare a time course assay:”
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 (BML-KI285) 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.
3. Add 35 µL of 1X Assay Buffer to each well and warm to assay temperature, e.g. 30°C.
4. Add 10 µL of test sample/inhibitor 10X stocks to appropriate wells. Add 10 µL of 1X Assay Buffer to control wells.
5. Add 5 µL of the PTP1B enzyme dilution to each well.
6. Initiate reactions by adding 50 µL of the warmed 2X Substrate.
7. Incubate samples at desired temperature, e.g. 30°C, for desired length of time, e.g. 30 min.
8. See Fig. 4 for examples of inhibition by various concentrations of suramin in 30 min. incubations with 75 µM IR5 substrate.
To Terminate Reactions And Read The Plate:
1. After incubating wells for desired duration, including the standard curve, terminate reactions by addition of 25 µL BIOMOL® Red Reagent (BML-KI468). Mix thoroughly by repeated pipetting, but take care to avoid producing bubbles.
2. Allow color to develop for 20-30 minutes. Be careful to assure samples spend approximately the same time with the reagent before reading on the microplate reader.
3. Read OD_{620nm} on a microplate reader.
4. Perform data analysis (see below).

**NOTE:** Retain microplate for future use of unused wells!

**FIGURE 1. Phosphate Standard Curve.** Dilutions of phosphate standard and a buffer blank were prepared as described (p. 2). The 100 µL samples were mixed with 25 µL BIOMOL® Red Reagent and incubated at 30 °C, 20 min to develop color. OD_{620nm} was read on a microplate-reading spectrophotometer (Bio-Tek). **A.** Least-squares fit to the entire set of phosphate amounts, from 0 to 3 nmol. **B.** A more accurate correlation of OD_{620} to phosphate is obtained by separate fits of the data from 0 to 1 nmol and 1 to 3 nmol.
FIGURE 2. Time Courses of PTP1B Phosphate Release from the IR5 Phosphopeptide. 2X Substrate solutions (150, 50 and 20 µM) and 2X Enzyme solutions (2 ng/well) were prepared and incubations at 30°C were performed as described on p. 2. Reactions were then terminated by addition of 25 µL of BIOMOL® Red and OD\textsubscript{620} read. OD\textsubscript{620} readings were converted to nmol of PO\textsubscript{4}\textsuperscript{2-} with a phosphate standard curve. Each point represents the mean of two determinations. See Fig. 1 and “Data Analysis”, p. 4.

FIGURE 3. Dependence of PTP1B Kinetics on IR5 Concentration. Initial rates of IR5 dephosphorylation by 2 ng of PTB1B were determined at 30°C and the indicated concentrations from 20 min. time course plots (See Fig. 2 and “Data Analysis”). The line is a non-linear least squares fit to the Michaelis-Menten equation. The K\textsubscript{m} for IR5 was 85 µM and the V\textsubscript{max} was 101 pmol/min.
FIGURE 4. Inhibition of PTP1B by Suramin. Phosphate release from 75 µM IR5 Substrate by 2.5 ng PTP1B (30°C) was measured at 10 min. intervals for 30 min at suramin concentrations of 0 to 100 µM. Data were converted from OD$_{620}$ to pmol of phosphate by means of standard curves (see Fig. 1B) and initial rates determined from best fits to the linear parts of plots of pmol phosphate vs. time (0-20 min. for 0 and 2 µM suramin, 0-30 min. for all other concentrations). The dose-response curve was derived from a least squares fit to the 4-parameter Hill-Slope model: 
\[ y = \text{bottom} + \left( \text{top} - \text{bottom} \right) / \left( 1 + \left( x / \text{IC}_{50} \right)^{\text{slope}} \right) \] ('Solver' tool, Microsoft, Excel). The fitted parameter values were: top = 101.9 pmol/min; bottom = -3.5 pmol/min; IC$_{50} = 9.5$ µM; slope = 1.8.

FIGURE 5. Z-Factor Analysis. PTP1B (2.5 ng) (red squares) or buffer (blue diamonds) was incubated for 20 minutes at 30°C with 75 µM IR5 Substrate. Reactions were then terminated by addition of 25 µL of BIOMOL® Red and OD$_{620}$ read. The Z’ factor for this assay was 0.84, (Z-factor = 1 - ((3SD$_{positive}$ + 3SD$_{negative}$))/ (mean$_{positive}$ – mean$_{negative}$)). Dashed lines indicate the 3*Standard deviation range.
**DATA ANALYSIS**

**Conversion of OD620 to nmol of Phosphate with a Standard Curve.**
1. Plot standard curve data as OD\textsubscript{620nm} versus nmol PO\textsubscript{4}\textsuperscript{2–} (see Fig. 1).
2. Obtain a line-fit or fits to the data using an appropriate routine. Note that, as in Fig. 1, the plot may not be linear over the entire span from 0 to 3 nmol of phosphate. As shown in Fig. 1B, 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 OD\textsubscript{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 of Fig. 1B):**

a) Measured OD\textsubscript{620} = 0.180. Since this OD falls below that of the 1 nmol point in Fig. 1B, choose the 0 to 1 nmol fit, i.e. y = 6.58x – 0.386.

b) Replacing x with OD\textsubscript{620} and y with nmol of PO\textsubscript{4}\textsuperscript{2–} produces:

\[
\text{nmol of PO}_4^{2-} = 6.58(\text{OD}_{620}) - 0.386
\]
\[
\text{nmol of PO}_4^{2-} = 6.58(0.180) - 0.386
\]
\[
\text{nmol of PO}_4^{2-} = 0.798
\]

**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 BIOMOL RED™ Reagent, and other experimental factors.

**Time Course Data and Rate Calculations.**
1. Using a standard curve, convert OD\textsubscript{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. (See Fig. 2.) 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:

\[
75 \times 10^{-6} \text{ mol/L} \times 100 \times 10^{-6} \text{ L} = 7500 \times 10^{-12} \text{ mol} = 7.5 \text{ nmol}
\]
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; see Fig. 2).
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 BIOMOL® Red Reagent (BML-KI468) into the 50 µL of 2X Enzyme and then mix in the 50 µL of 2X Substrate (see Time Course instructions, p. 2).

2. Calculate activity as a % of Control (See. Fig. 4):

\[
\text{% Activity} = \frac{\text{[Test sample (nmol PO}_4^{2-}) - \text{“time zero” (nmol PO}_4^{2-})]}{\text{[Control (nmol PO}_4^{2-}) - \text{“time zero” (nmol PO}_4^{2-})]} \]

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 BIOMOL® Red 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 IR5 substrate; see Phosphate Standard Curve instructions, p.2).
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