

# **ab139465**

## **PTP1B Inhibitor**

### **Screening Assay Kit**

#### **Instructions for Use**

For the screening of PTP1B inhibitors:

This product is for research use only and is not intended for diagnostic use.



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# 1. Background

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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.

## 2. Principle of the Assay

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Abcam PTP1B Inhibitor Screening Assay Kit (ab139465) 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.

The phosphopeptide substrate supplied with this kit (PTP1B Substrate, IR5 Insulin Receptor  $\beta$  residues 1142-1153, pTyr-1158 (a.k.a. pTyr-1146, mature peptide numbering)) contains sequence from the insulin receptor  $\beta$  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 is supplied as a control for inhibitor detection. Suramin is a reversible and competitive inhibitor of PTP1B, with a  $K_i$  of 5.5  $\mu\text{M}$ .

### 3. Protocol Summary

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#### **Prepare Phosphate Standard curve**

Dilute Phosphate Standard in 1X Assay Buffer

#### **Prepare time course assay**

Dilute PTP1B Substrate



Add assay buffer to each well



Dilute PTP1B Enzyme



Add PTP1B Substrate to standard curve wells

### **Prepare test sample/inhibitor assay**

Prepare samples containing PTP1B Inhibitor and test compound and add to appropriate wells.



Add PTP1B Enzyme dilution to each well.



Initiate reaction adding PTP1B Substrate



Incubate samples at appropriate temperature and time



Terminate reaction by adding Red Assay Reagent



Read OD<sub>620nm</sub> on a microplate reader.



Perform data analysis

## 4. Materials Supplied

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| Item                                                                       | Quantity | Storage |
|----------------------------------------------------------------------------|----------|---------|
| PTP1B Enzyme (Human recombinant)<br>(0.1 µg/µL)                            | 5 µg     | -80°C   |
| PTP1B Substrate<br>(IR5 Insulin Receptor β residues 1142-1153,<br>pY-1146) | 1 mg     | -80°C   |
| 2X PTP1B Assay Buffer                                                      | 20 mL    | -80°C   |
| Red Assay Reagent                                                          | 5 mL     | -80°C   |
| PTP1B Inhibitor (Suramin)                                                  | 10 mg    | -80°C   |
| Phosphate Standard (100 µM in 1X PTP1B<br>Assay Buffer)                    | 0.5 mL   | -80°C   |
| 96-well Clear Microplate (½ Volume)                                        | 1        | RT      |

## 5. Storage and Stability

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- Please note that all components at or below the temperatures listed for highest stability.
- The PTP1B Enzyme must be handled particularly 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 '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  $\geq 10 \mu\text{L}$ ) to help minimize freeze/thaw cycles.
- Red Assay 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  $\text{dH}_2\text{O}$  or employ unused plasticware.



## 6. Materials Required, Not Supplied

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- Microplate reader capable of reading OD<sub>620nm</sub> to  $\geq 3$ -decimal accuracy.
- Pipettes or multi-channel pipettes capable of pipetting 2-100  $\mu\text{L}$  accurately.
- 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).

## 7. Assay Protocol

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### A. General Considerations

1. This kit is designed to perform endpoint assays in which each well contains a 100  $\mu\text{L}$  reaction in PTP1B Assay Buffer and is terminated by the addition of 25  $\mu\text{L}$  of the phosphate detection reagent, Red Assay Reagent. The 100  $\mu\text{L}$  “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.
2. Enough of the PTP1B Substrate (0.59  $\mu\text{mol}$ ) is provided to perform, for example, 96 assays at 60  $\mu\text{M}$ , 84 assays at 70  $\mu\text{M}$ , 78 assays at 75  $\mu\text{M}$  or 72 assays at 80  $\mu\text{M}$ . The  $K_m$  of PTP1B for the PTP1B Substrate is  $\sim 85 \mu\text{M}$  (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  $\mu\text{g}$  = 50  $\mu\text{L}$  at 100 ng/ $\mu\text{L}$ ) to allow for a broad range of possible enzyme amounts per well. Typically, 2-3 ng/well will provide a useful signal ( $\sim 1$  nmol phosphate) with a 30 minute incubation at 30°C, and a 75  $\mu\text{M}$  final concentration of the PTP1B Substrate.

4. The phosphatase reactions in the Time Course and Test Sample/Inhibitor experiments described below are initiated by mixing 50  $\mu$ L of a prewarmed 2X PTP1B Substrate solution into an assay well containing 50  $\mu$ L of prewarmed 2X PTP1B Enzyme solutions. In the example given for the Test Sample/Inhibitor experiment, the addition of inhibitor is made to the 2X PTP1B 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 PTP1B Substrate solution and thereby ensure that the enzyme is exposed simultaneously to substrate and inhibitor.

## **B. Reagent Preparation**

1. Thaw all kit components and hold PTP1B, Substrate, and Assay Buffer on an ice bath; Store Red Assay Reagent at room temperature (RT).
2. Reconstitute the vial of PTP1B substrate to 1.5 mM by adding 196  $\mu$ L 2X PTP1B Assay Buffer plus 196  $\mu$ L dH<sub>2</sub>O to 1 mg net peptide. Vortex. After use, store remaining substrate at -80°C.
3. Reconstitute PTP1B Inhibitor to a 10mM solution: 10 mg in 0.7 mL 1X PTP1B Assay Buffer.

### **C. Preparing a Phosphate Standard Curve**

1. Prepare 1.2 mL 1X Assay Buffer by diluting 600  $\mu\text{L}$  of the 2X PTP1B Assay Buffer with 600  $\mu\text{L}$  of  $\text{dH}_2\text{O}$ .
2. Pipette into duplicate sets of six wells: 100, 97.5, 95, 90, 80 and 70  $\mu\text{L}$  of 1X Assay Buffer.
3. Pipette, into those same wells, in the same order, 0, 2.5, 5, 10, 20 and 30  $\mu\text{L}$  of the 100  $\mu\text{M}$  Phosphate Standard
4. These wells will contain, respectively, 0, 0.25, 0.5, 1.0, 2.0 and 3.0 nmol of inorganic phosphate.

#### **D. Preparing a Time Course Assay**

1. Prepare 1 mL 1X Assay Buffer (dilute 500  $\mu$ L of 2X PTP1B Assay Buffer with 500  $\mu$ L of dH<sub>2</sub>O) 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  $\mu$ L 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 in cold 1X Assay Buffer, such that each 5  $\mu$ L contains the desired amount of enzyme per well. Example: Dilute the PTP1B Enzyme 200-fold, e.g. 2  $\mu$ L plus 398  $\mu$ L Assay Buffer, to prepare a 0.5 ng/ $\mu$ L stock for assay at 2.5 ng/well.
6. Prepare a dilution of the 1.5 mM PTP1B Substrate stock at 2 times the desired assay concentration and warm to assay temperature. Example: Prepare 300  $\mu$ L 150  $\mu$ M PTP1B Substrate (2X) – 30  $\mu$ L 1.5 mM PTP1B Substrate plus 270  $\mu$ L Assay Buffer – and warm to 30°C.
7. Just before adding substrate to start the reaction in each well, add 5  $\mu$ L of the cold PTP1B Enzyme dilution to the 45  $\mu$ L of warmed 1X Assay Buffer. Initiate reactions by then mixing in 50  $\mu$ L of the warmed 2X PTP1B 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 Red Assay Reagent.

8. See Fig. 2. For plots of time courses performed at several concentrations of substrate.

## **E. Preparing a Test Sample/Inhibitor Assay**

1. Prepare appropriate volumes of 1X Assay Buffer, a PTP1B dilution and 2X Substrate as described in "Preparing 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 PTP1B Inhibitor by dissolving the 10 mg of solid in 0.7 mL of 1X Assay Buffer. For a final concentration of 10  $\mu$ M, prepare a 10X stock (100  $\mu$ M) by mixing 10  $\mu$ L of the 10 mM stock with 990  $\mu$ L Assay Buffer.
3. Add 35  $\mu$ L of 1X Assay Buffer to each well and warm to assay temperature, e.g. 30°C.
4. Add 10  $\mu$ L of test sample/inhibitor 10X stocks to appropriate wells. Add 10  $\mu$ L of 1X Assay Buffer to control wells.
5. Add 5 $\mu$ L of the PTP1B Enzyme dilution to each well.
6. Initiate reactions by adding 50  $\mu$ L of the warmed 2X PTP1B 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 PTP1B Inhibitor in 30 min. incubations with 75  $\mu$ M PTP1B Substrate.

## **F. Terminating Reactions and Reading the Plate**

1. After incubating wells for desired duration, terminate reactions by addition of 25  $\mu$ L Red Assay Reagent. 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<sub>620nm</sub> on microtiter-plate reader.  
Note: Retain microtiter plate for future use of unused wells.
4. Perform data analysis (see below).



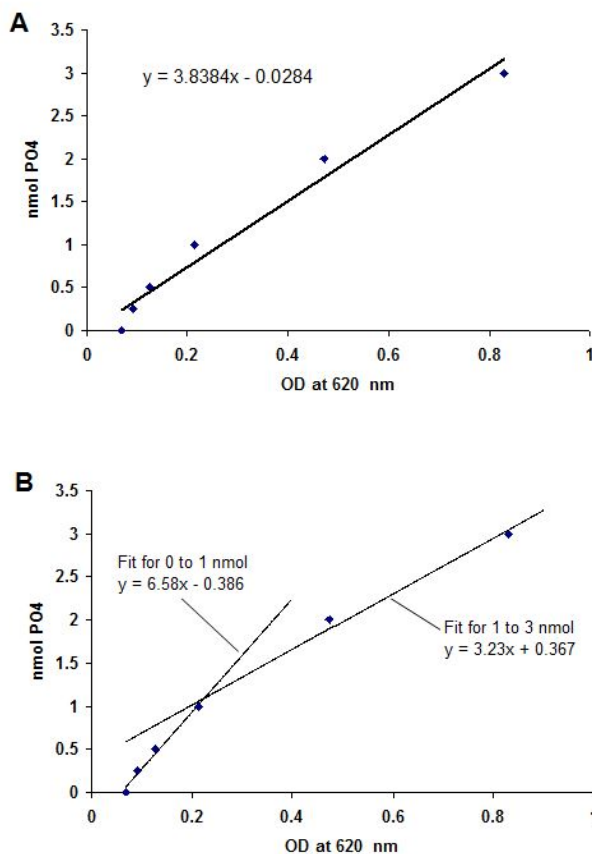
## 8. Data Analysis

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### A. Conversion of OD<sub>620</sub> to nmol of Phosphate with a Standard Curve.

1. Plot standard curve data as OD<sub>620nm</sub> versus nmol PO<sub>4</sub><sup>2-</sup> (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 1-3 nmol, can produce a more accurate correlation of OD<sub>620</sub> 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<sub>620</sub> = 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<sub>620</sub> and  $y$  with nmol of PO<sub>4</sub><sup>2-</sup> produces:  
$$\text{nmol of PO}_4^{2-} = 6.58(\text{OD}_{620}) - 0.386$$
$$\text{nmol of PO}_4^{2-} = 6.58(0.180) - 0.386$$
$$\text{nmol of PO}_4^{2-} = 0.798$$

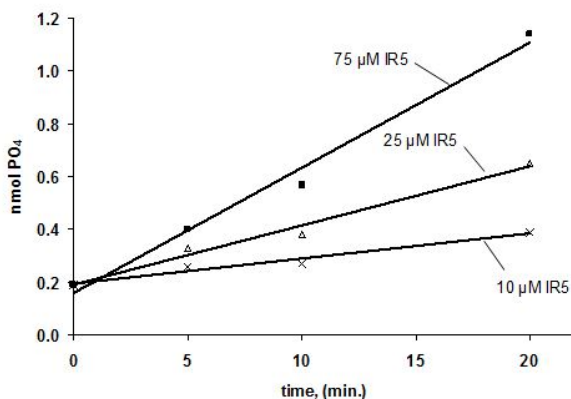
NOTE: For highest accuracy, a standard curve should be performed for each new set of assay data. This will normalize for variations in free phosphate in samples, time of incubation with the Red Assay Reagent, and other experimental factors.



**Figure 1. Phosphate Standard Curve.** Dilutions of phosphate standard and a buffer blank were prepared as described. The 100  $\mu$ L samples were mixed with 25  $\mu$ L Red Assay Reagent and incubated at 30°C, 20 min to develop color. OD<sub>620nm</sub> was read on a microplate-reading spectrophotometer. **A.** Least-squares fit to the entire set of phosphate amounts, from 0 to 3 nmol. **B.** A more accurate correlation of OD<sub>620</sub> to phosphate is obtained by separate fits of the data from 0 to 1 nmol and 1 to 3 nmol.

## **B. Time Course Data and Rate Calculations.**

1. Using a standard curve, convert OD<sub>620</sub> 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 (PTP1B Substrate) per well contained at the chosen substrate concentration. For example, at 75  $\mu$ M PTP1B Substrate in 100  $\mu$ L 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 Enzyme at 75  $\mu$ M PTP1B Substrate, time course plots remain linear for at least the first 15% of substrate hydrolyzed (~1 nmol of phosphate; see Fig. 2).



**Figure 2. Time Courses of PTP1B Phosphate Release from the IR5 Phosphopeptide.** 2X PTP1B Substrate solutions (150, 50 and 20  $\mu\text{M}$ ) and 2X PTP1B Enzyme solutions (2 ng/well) were prepared and incubations at 30°C were performed as described. Reactions were then terminated by addition of 25  $\mu\text{L}$  of Red Assay Reagent and  $\text{OD}_{620}$  read.  $\text{OD}_{620}$  readings were converted to nmol of  $\text{PO}_4^{2-}$  with a phosphate standard curve. Each point represents the mean of two determinations.

### C. Test Sample/Inhibitor Data

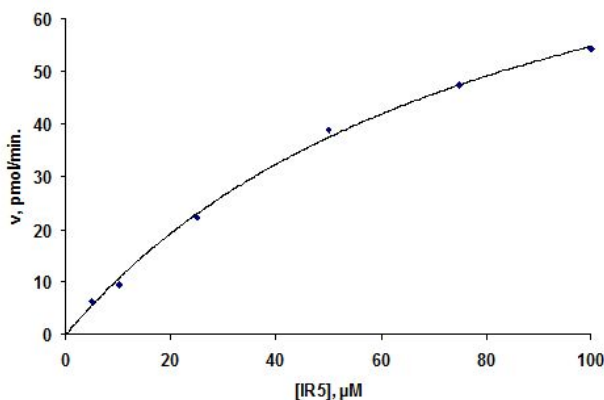
1. It is important to obtain a “time zero” measurement and to subtract this value, expressed as nmol of phosphate, from both the control and test sample/inhibitor values. Again, a convenient way to obtain an accurate t=0 measurement is to add and mix the 25 µL of Red Assay Reagent into the 50 µL of 2X PTP1B Enzyme and then mix in the 50 µL of 2X PTP1B Substrate (see Preparing a Time Course Assay instructions).

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

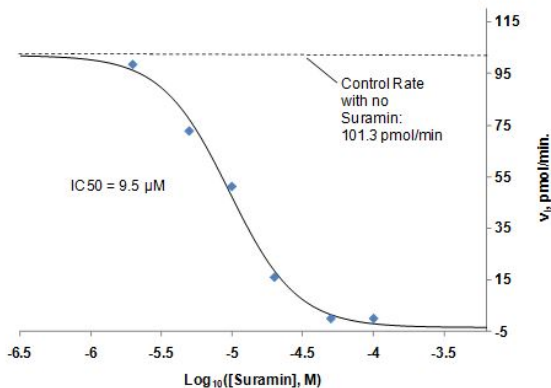
% Activity =

$$\frac{[\text{Test sample (nmol PO}_4^{2-}) - \text{“time zero” (nmol PO}_4^{2-})]}{[\text{Control (nmol PO}_4^{2-}) - \text{“time zero” (nmol PO}_4^{2-})]} \times 100$$

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 Red Assay Reagent 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; see Preparing a Phosphate Standard Curve instructions).



**Figure 3. Dependence of PTP1B Kinetics on IR5 (PTP1B Substrate) Concentration.** Initial rates of IR5 (PTP1B Substrate) dephosphorylation by 2 ng of PTP1B 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  $K_m$  for IR5 (PTP1B Substrate) was 85  $\mu\text{M}$  and the  $V_{\max}$  was 101 pmol/min.



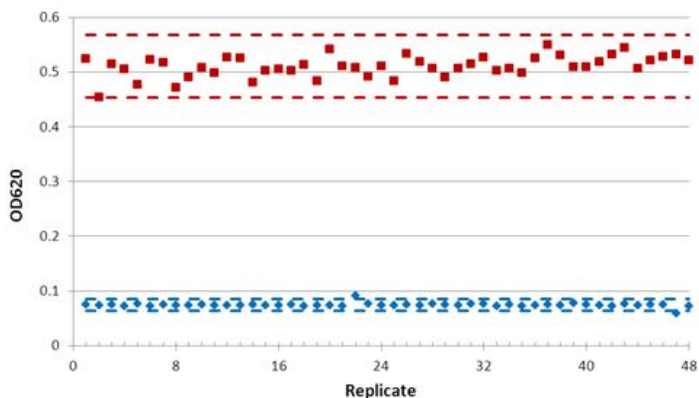
**Figure 4. Inhibition of PTP1B by Suramin (PTP1B Inhibitor).**

Phosphate release from 75  $\mu\text{M}$  PTP1B Substrate by 2.5 ng PTP1B (30°C) was measured at 10 minute intervals for 30 min at suramin concentrations of 0 to 100  $\mu\text{M}$ . Data were converted from  $\text{OD}_{620}$  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  $\mu\text{M}$  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:

$y = \text{'bottom'} + (\text{'top'} - \text{'bottom'}) / (1 + (x/\text{IC}_{50})^{\text{slope}})$  ('Solver' tool, Microsoft, Excel).

The fitted parameter values were:  $\text{top} = 101.9$  pmol/min;  $\text{bottom} = -3.5$  pmol/min;  $\text{IC}_{50} = 9.5$   $\mu\text{M}$ ; 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  $\mu$ M PTP1B Substrate. Reactions were then terminated by addition of 25  $\mu$ L of Red Assay Reagent and OD<sub>620</sub> read. The Z' factor for this assay was 0.84, (Z-factor =  $1 - ((3SD^{\text{positive}} + 3SD^{\text{negative}}) / (\text{mean}^{\text{positive}} - \text{mean}^{\text{negative}}))$ ). Dashed lines indicate the 3\*Standard deviation range.





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