



## LabChip Assay: Off-Chip Incubation, Mobility Shift

# PTP1B and TCPTP Phosphatase Assays

## I. Introduction

The off-chip incubation, mobility shift phosphatase assay uses a microfluidic chip to measure the conversion of a fluorescent phosphorylated peptide substrate to a dephosphorylated product. The reaction mixture, from a microtiter plate well, is introduced through a capillary sipper onto the chip, where the phosphorylated substrate and the dephosphorylated product are separated by electrophoresis and detected via laser-induced fluorescence. The signature of the fluorescence signal over time reveals the extent of the reaction. This application note describes assay conditions for the phosphatases Protein Tyrosine Phosphatase 1B (PTP1B) and T-Cell Protein Tyrosine Phosphatase (TCPTP). Protein Tyrosine Phosphatases (PTPs) act as antagonists to Protein Tyrosine Kinases (PTKs) to regulate the phosphorylation status of specific substrate proteins. Aberrant PTP activity in human cells can lead to diseases including cancers and diabetes. PTP1B and TCPTP have emerged as important candidates for drug targets due to their roles in regulating insulin signaling and immune system responses. The off-chip mobility shift assay provides a novel means of screening compounds for effects on the activities of these PTPs.

## II. Methods

### Substrate



This peptide substrate has a molecular weight of 1324 and a net charge of -2.65 at pH 7.5. Upon dephosphorylation, the product ( $\text{FITC-AHA-DYRK-CONH}_2$ ) has a net charge of -0.85. PTP1B and TCPTP are closely related proteins with a highly conserved active site, and both enzymes effectively dephosphorylate the same substrate.

### PTP1B and TCPTP Assay Conditions (Final in reaction)

PTP1B: 0.025 mU/ $\mu\text{L}$  enzyme

TCPTP: 0.66 pg/ $\mu\text{L}$  enzyme

1  $\mu\text{L}$  Compound in 100% DMSO or water

1.5  $\mu\text{M}$  Substrate ( $\text{FITC-AHA-DY}(\text{PO}_3)\text{YRK-CONH}_2$ )

### 31 $\mu\text{L}$ Reaction

1  $\mu\text{L}$  Compound

15  $\mu\text{L}$  Enzyme

15  $\mu\text{L}$  Substrate

30 min reaction at 20  $^\circ\text{C}$

30  $\mu\text{L}$  Stop Solution

### Reaction Buffer

(Final Concentration in reaction)

100 mM Hepes, pH 7.5

5 mM DTT

0.015% Brij-35

### Chip/Trough Buffer

100 mM Hepes pH 7.5

5 mM  $\text{MgCl}_2$

0.015% Brij-35

0.1% Coating Reagent 3

20 mM EDTA

### Stop Solution

(Termination Buffer)

100mM Hepes, pH 7.5

0.015% Brij-35

0.1% Coating Reagent 3

50  $\mu\text{M}$  Sodium Orthovanadate

### Separation Conditions

	4-Sipper
Pressure (psi)	-1.8
Upstream Voltage (V)	-500
Downstream Voltage (V)	-1550
Sample Sip Time (sec)	0.2
Post-Sample Buffer Sip Time (sec)	30

Table 1: Assay Parameters for PTP1B and TCPTP Screening

## III. Results

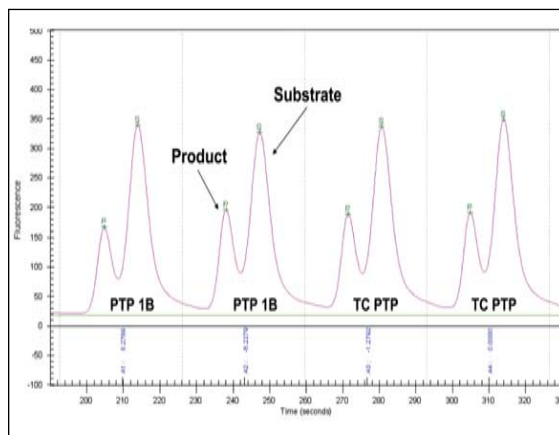


Figure 1: Caliper LabChip 3000 system Data Signature. Representative product and substrate peaks for PTP1B and TCPTP. The electropherogram illustrates the fluorescent signal detected from a single channel of a 4-sipper chip during 4 consecutive sips from different microtiter plate wells containing stopped phosphatase reactions.

### Substrate/Product Peak Separation

Figure 1 shows the separation of product and substrate on a 4-sipper chip using the parameters shown in Table 1. Phosphorylated substrate and dephosphorylated product are separated on the chip and appear as distinct peaks. The data analysis software (HTSWA) determines peak heights, from which the ratio of product to the peak sum  $P/(P+S)$  is calculated. The  $P/(P+S)$  value  $\times 100 = \% \text{ product formed}$ .

### Enzyme Titrations

The initial titrations for PTP1B and TCPTP are shown in Figures 2A and 2B. Reactions containing 60  $\mu\text{L}$  total volume with 1.5  $\mu\text{M}$  substrate and 4 different enzyme concentrations were assembled in duplicate on a 384-well microtiter plate. The plate was placed immediately onto the LabChip 3000 system and samples were introduced onto a 4-sipper chip every 4 minutes for 130 minutes. Temperature and humidity in the reaction chamber were maintained at 20  $^{\circ}\text{C}$  and 50%, respectively.

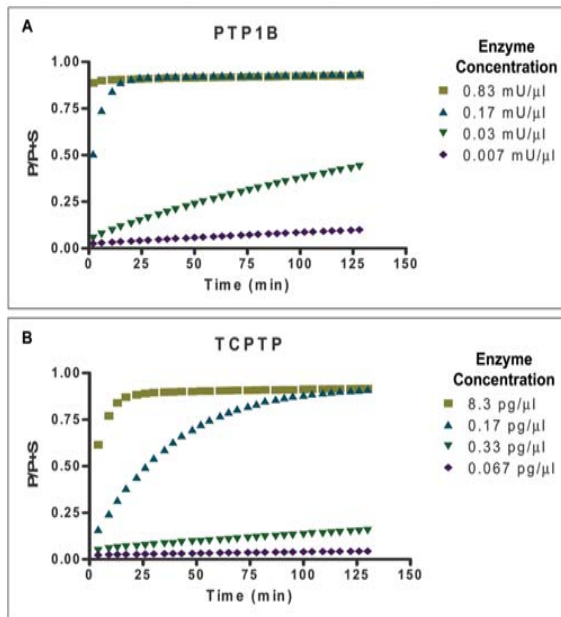


Figure 2: Enzyme Titrations. Real time kinetics of PTP1B (A) and TCPTP (B) dephosphorylation reactions containing varying enzyme concentrations. Data represents averages from duplicate reactions.

Substrate and dephosphorylated product were separated and detected on the chip. The enzyme concentration resulting in 30% product formed after 60 minutes incubation was extrapolated (PTP1B: 0.025 mU/ $\mu\text{L}$ , TCPTP: 0.66 pg/ $\mu\text{L}$ ) and chosen for further assay development studies.

### Reaction Linearity

Real-time kinetics were used to show that the phosphatase reactions remained linear for approximately 30 minutes (Figure 3). Reactions containing 60  $\mu\text{L}$  total volume with 1.5  $\mu\text{M}$  substrate, and 0.025 mU/ $\mu\text{L}$  PTP1B or 0.66 pg/ $\mu\text{L}$  TCPTP were assembled on a microtiter plate. The plate was immediately placed on the LabChip 3000 system and samples were introduced onto a 4-sipper chip every 3

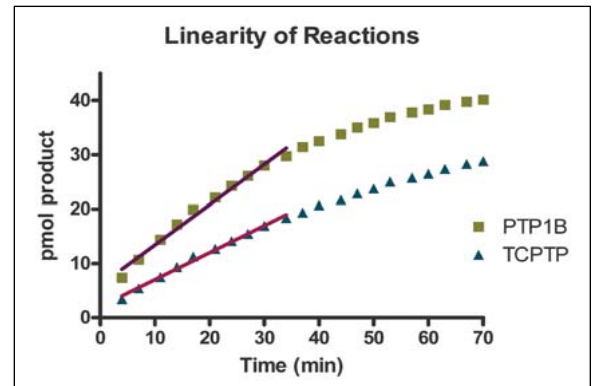


Figure 3: Linearity of the PTP1B and TCPTP phosphatase reaction rates.

minutes for 70 minutes. Temperature and humidity in the reaction chamber were maintained at 20  $^{\circ}\text{C}$  and 50%, respectively. Substrate and dephosphorylated product were separated and detected on the chip. The lines represent the linear regression from the initial 10 data points, collected during the first 35 minutes of reaction time. Reaction rates slowed significantly after 30 minutes. Varying the concentration of enzyme and/or substrate in the reaction did not increase the length of time for which linearity was observed (data not shown). Based on these observations, the recommended incubation time for screening assays is 30 minutes.

### K<sub>m</sub> Determinations

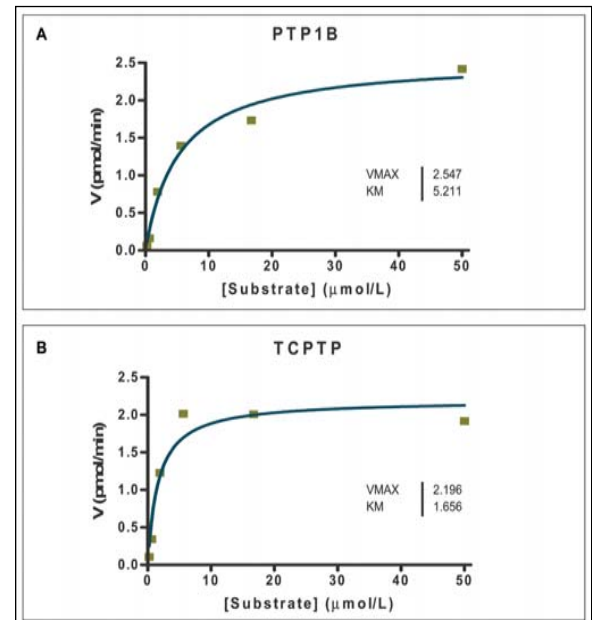


Figure 4: Substrate  $K_m$  determinations for PTP1B (A) and TCPTP (B). The plots show Michaelis-Menten non-linear regression analyses of initial reaction rates vs. substrate concentration.

Substrate  $K_m$  values were determined for each enzyme using real-time kinetics (Figures 4A and 4B). Initial reaction rates were determined by assembling 60  $\mu\text{L}$  reactions containing increasing concentrations of substrate (0.21,

0.62, 1.9, 5.6, 16.7, and 50  $\mu\text{mol/L}$ ) with either 0.025 mU/ $\mu\text{L}$  PTP1B or 0.66 pg/ $\mu\text{L}$  TCPTP. The plate was immediately placed on the LabChip 3000 system and samples were introduced onto a 4-sipper chip every 6 minutes for 60 minutes. Temperature and humidity in the reaction chamber were maintained at 20  $^{\circ}\text{C}$  and 50%, respectively. Substrate and dephosphorylated product were separated and detected on the chip. Initial rates  $V$  (pmol/min) were calculated for each substrate concentration by finding the slopes of product formed vs. time during the first 30 minutes of the reaction.  $K_m$  values were determined by plotting  $V$  (pmol/min) vs. substrate concentration  $[S]$  ( $\mu\text{mol/L}$ ) and applying non-linear regression analysis using the Michaelis-Menten equation. The substrate  $K_m$  values for PTP1B and TCPTP were found to be 5.2  $\mu\text{M}$  and 1.7  $\mu\text{M}$ , respectively.

#### DMSO Tolerance

The effect of DMSO on PTP1B and TCPTP activity was determined by running 30  $\mu\text{L}$  phosphatase reactions with increasing amounts of added DMSO (Figure 5). 100% DMSO (0.5  $\mu\text{L}$  to 5.5  $\mu\text{L}$ ) was added to wells of a microtiter plate, mimicking the addition of compounds dissolved in DMSO. Reactions were then assembled in the wells, incubated, and stopped as described in the Methods section.

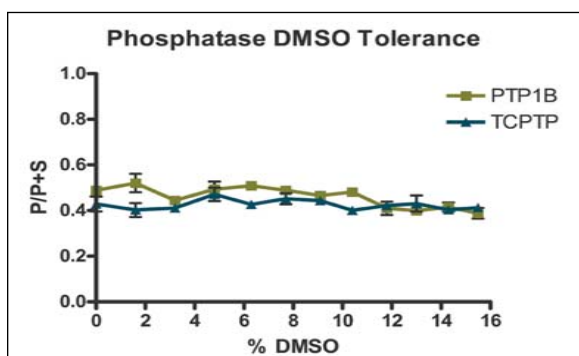


Figure 5: DMSO Tolerance. Increasing DMSO concentration has no significant effect on the activity of either PTP1B or TCPTP.

Substrate and dephosphorylated product in the stopped reaction were separated and detected on a 4-sipper chip. As shown in Figure 5, increasing DMSO concentration has no significant effect on the activity of either PTP1B or TCPTP. Both enzymes should tolerate the addition of up to 5.5  $\mu\text{L}$  of compound dissolved in DMSO to a 30  $\mu\text{L}$  reaction (final concentration of 15.5% DMSO).

#### Inhibitor $IC_{50}$ Determinations

Known PTP1B inhibitors, one general phosphatase inhibitor (sodium orthovanadate) and 5 PTP-specific inhibitors were selected for analysis. Reactions were assembled, incubated, and terminated as described in the Methods section. Substrate and dephosphorylated product were separated and detected on a 4-sipper chip.  $IC_{50}$  values (Table 2) were calculated using non-linear regression analysis of the fraction of product formed (P/P+S) vs. Log of inhibitor concentration. The inhibition curves are shown in Figure 6A (PTP1B) and 6B (TCPTP).

As expected from the literature, sodium orthovanadate and bpV(bipy) inhibited PTP1B activity in the nanomolar range, while the other four compounds inhibited in the micromolar range. TCPTP demonstrated reduced sensitivity to the less potent compounds, but higher sensitivity to orthovanadate and bpV(bipy).

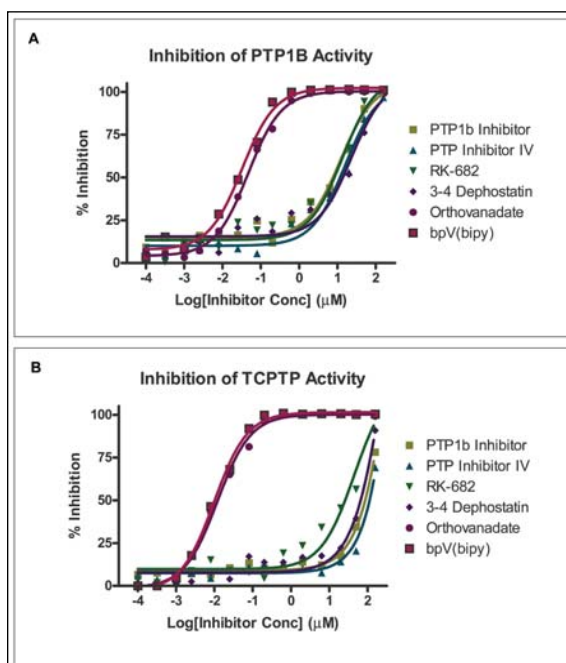


Figure 6: Inhibition curves showing the effects of 6 known PTP inhibitors on the activities of PTP1B(A) and TCPTP(B)

Compound	$IC_{50}$ for PTP1B ( $\mu\text{M}$ )	$IC_{50}$ for TCPTP ( $\mu\text{M}$ )
PTP1B Inhibitor	18	>100
PTP Inhibitor IV	33	>100
RK-682	19	45
3-4 Dephostatin	41	~100
Orthovanadate	0.046	0.014
bpV(bipy)	0.030	0.0094

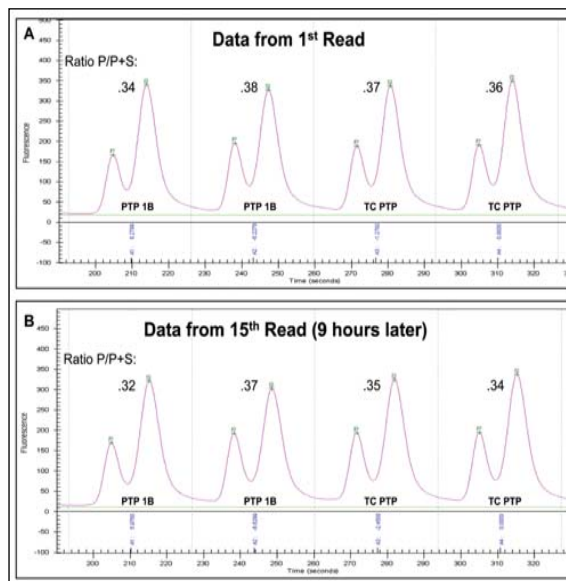
Table 2:  $IC_{50}$  values determined for inhibitors

#### Data Stability

For high throughput screening applications, it is necessary to assemble, incubate, and terminate enzymatic reactions in multiple microtiter plates during a single run of the assay. The LabChip 3000 system screening system may be programmed to read results from up to 60 plates in a single run. But, the quality of the results will depend on the stability of the substrate and product in the terminated reaction mixture. As shown in Figure 7, the PTP1B and TCPTP substrate and dephosphorylated product show a high degree of stability. Reactions were assembled, incubated, and terminated as described in the Methods section. The microtiter plate was immediately placed in the LabChip 3000 system and sampled with a 4-sipper chip.

The plate remained in the LabChip 3000 system chamber, with temperature and humidity maintained at 20 °C and 50%, and the reaction wells were sampled repeatedly over the course of 9 hours. The data from the first and last analyses are nearly identical in terms of substrate and product peak integrity, separation, and relative intensity.

*Figure 7: Stability of the substrate and de-phosphorylated product in the terminated reaction mixture. Data collected immediately after reaction termination (A) and collected from the same reaction wells after 9 hours in the LC3000 chamber (B). The electropherograms illustrate the fluorescent signal detected from a single channel of a 4-sipper chip during 4 consecutive sips from different microtiter plate wells.*



#### IV. Materials

ITEM	ITEM NAME	MANUFACTURER	CATALOG #	
Microfluidic System Components	LabChip 3000 Drug Discovery System	Caliper Life Sciences		
	Caliper Chip Module FS	Caliper Life Sciences		
	Caliper Chip Module TC	Caliper Life Sciences		
	Off-chip Mobility Shift Chip, 4-Sipper, with Coating Reagent 3	Caliper Life Sciences	761043-0266R	
	Off-chip Mobility Shift Chip, 12-sipper, with Coating Reagent-3	Caliper Life Sciences	761037-0372R	
Assay Components	PTP1B (full-length, human, recombinant) Lot# 27968 Specific Activity: 13U/ g	Upstate	14-358	
	TCPTP (AA 1-341, human, recombinant) Lot# 28205AU Specific Activity: 427U/mg	Upstate	14-646	
	Substrate: FITC-AHA-DY(P03)YRK-CONH2	Analytical Core Facility Tufts University	Custom	
	HEPES, Free Acid ULTROL	Calbiochem	391338	
	HEPES, Sodium Salt, ULTROL	Calbiochem	391333	
	Magnesium Chloride	Sigma	M2670	
	Brij-35 Solution (30%)	Sigma	B4184	
	Coating Reagent-3	Caliper Life Sciences	760050	
	EDTA, disodium salt, 0.5 mM	Sigma	E7889	
	DTT	Calbiochem	233153	
	18 M Water			
	Inhibitors	Sodium Orthovanadate	Sigma	S6508
		PTP1B Inhibitor	Calbiochem	539741
PTP Inhibitor IV		Calbiochem	540211	
RK-682		Calbiochem	557322	
3,4-Dephostatatin, Ethyl		Calbiochem	263203	
BpV(bipy)		Calbiochem	203694	

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