

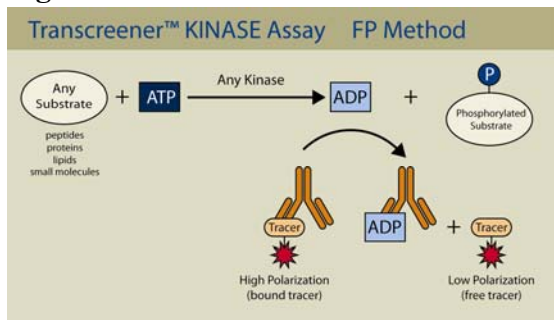
## Application of Transcreeper KINASE HTS Assays in Evaluating PI3 Lipid Kinases

Tony Klink, Andrew Kopp, Thane Westermeyer, and Karen Kleman-Leyer  
BellBrook Labs, Madison, Wisconsin, USA, 53711

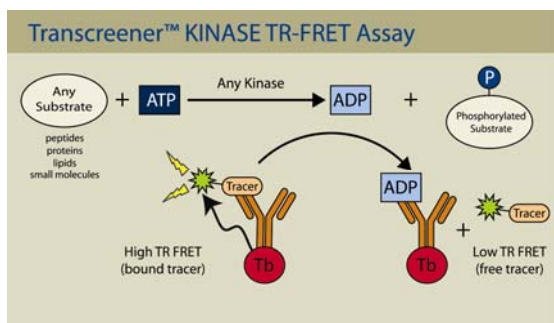
### Introduction

Development of drug candidates for Phosphoinositide 3-kinase (PI3K) isoforms has been delayed by the lack of robust screening assays. In addition, lipid substrate preparation can be laborious; choosing an optimal technique to prepare the lipid substrate can be daunting. The Transcreeper KINASE Assays alleviate these bottlenecks to drug discovery. Here, we describe generic HTS competitive immunoassays for direct detection of the invariant kinase reaction product, ADP. Each Transcreeper KINASE Assay is designed to eliminate fluorescent compound interference with readouts available in far-red fluorescence polarization (FP) and time-resolved fluorescence resonance energy transfer (TR-FRET). The Transcreeper platform can characterize lipid preparation techniques, determine ideal lipid concentration, identify lipid substrates with a single set of reagents, and ascertain pharmacology of inhibitors of PI3K isoforms. Excellent  $Z'$  values were obtained under initial velocity conditions (<10% ATP conversion) using the phosphatidylinositol 4,5 bisphosphate substrate with each of the PI3K $\alpha$ , PI3K $\beta$ , and PI3K $\gamma$  isoforms. Dose-dependency curves were generated with six known inhibitors (wortmannin, PI103, PI3K $\gamma$ , PI3K $\gamma$ II, LY 294002, and quercetin). In addition, PI3K $\alpha$  is shown to have intrinsic ATPase activity and generates similar pharmacology, relative to reactions with substrate.

**Figure 1 Transcreeper KINASE FP and TR-FRET Assays**

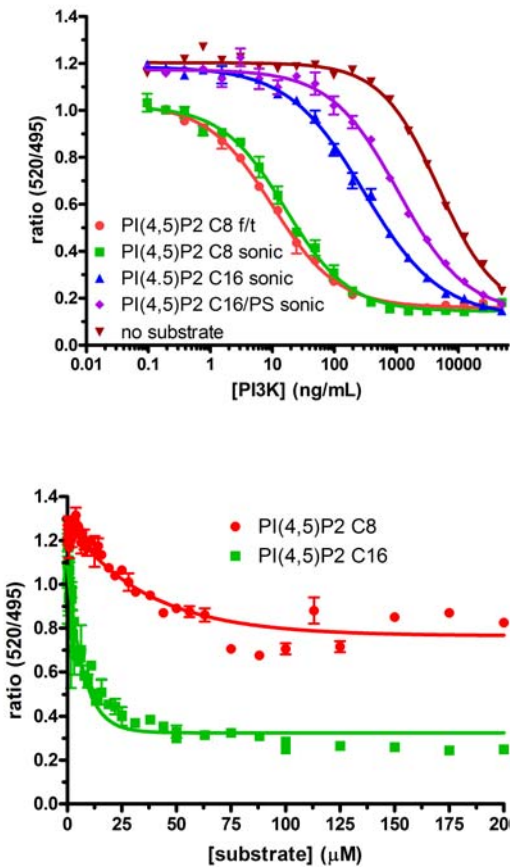


With FP detection, high polarization is observed when the monoclonal ADP Antibody is bound to the ADP Alexa Fluor®633 Tracer. ADP generated during the enzyme reaction displaces the ADP Tracer from the ADP Antibody causing a decrease in polarization.



TR-FRET is observed when terbium (covalently bound to the ADP Antibody) is in close proximity to the ADP-fluorescein (FAM) Tracer. ADP displaces the ADP-FAM Tracer from the ADP Antibody-Tb, thereby decreasing TR-FRET.

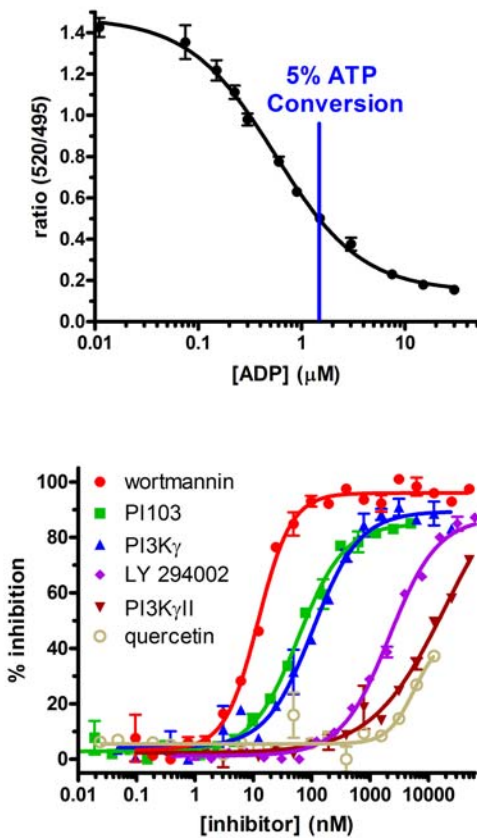
## Figure 2 Identify Optimal Lipid Substrate Preparation and Concentration



**A.** The Transcreener platform can characterize lipid preparation techniques. PI3 $\alpha$  Kinase activity was not only observed with two lipid substrates, but also without lipid substrate (intrinsic ATPase activity). PI3 $\alpha$  Kinase was serially diluted two-fold in reaction buffer using phosphatidylinositol 4,5 biphosphate (C8 and C16) substrates (as well as without substrate) using 30  $\mu$ M ATP.

**B.** Optimal substrate concentration was identified by serially titrating substrate with constant enzyme. The phosphatidylinositol 4,5 biphosphate substrate  $K_m$  values are 5  $\mu$ M for C16 and 25  $\mu$ M for C8 which are similar to literature values. Kinase reactions (10  $\mu$ L) were incubated for 90 minutes at 30°C followed by addition of an equal volume (10  $\mu$ L) of ADP Detection Mixture.

## Figure 3 Inhibitor Dose-Dependency with PI3 $\alpha$ Kinase (TR-FRET)



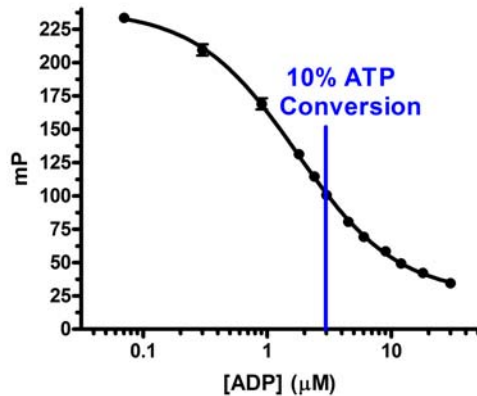
**A.** ADP/ATP Standard Curves for 30  $\mu$ M ATP using Transcreener Kinase TR-FRET Assay. The ADP/ATP standard curve mimics a typical enzyme reaction (as ADP is produced, ATP is depleted). Optimal signal at initial velocity conditions (5% ATP conversion) is indicated.

**B.** Inhibitor potency confirmed for PI3 $\alpha$  in a two-fold serial dilution experiment using six known lipid kinase inhibitors. The PI3 $\alpha$  (0.36 nM) reaction used phosphatidylinositol 4,5 biphosphate (C16) and was performed at 30°C for 90 minutes and progressed to 4.7% ATP conversion with a  $Z'$  = 0.82. The  $IC_{50}$  values for wortmannin, PI103, PI3K $\gamma$ , LY 294002, PI3K $\gamma$ II, and quercetin were 12 nM, 55 nM, 105 nM, 2.8  $\mu$ M, 13.7  $\mu$ M, and >20  $\mu$ M, respectively.

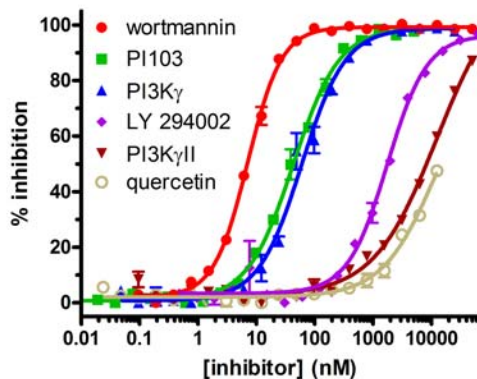
**Table 1 Pharmacology of PI3K isoforms using Transcreener™ TR-FRET Assay**

|                                |                 | wortmannin<br>IC <sub>50</sub> (nM) | PI103<br>IC <sub>50</sub> (nM) | PI3K $\gamma$<br>IC <sub>50</sub> (nM) | LY<br>294002<br>IC <sub>50</sub> ( $\mu$ M) | PI3K $\gamma$ II<br>IC <sub>50</sub> ( $\mu$ M) | quercetin<br>IC <sub>50</sub> ( $\mu$ M) |
|--------------------------------|-----------------|-------------------------------------|--------------------------------|--|---|---|--|
| <b>PI3K<math>\alpha</math></b> | Literature      | 1 - 5                               | 4.4 - 8                        | 60                                     | 0.5 - 9.3                                   | 4.5   | 3.8                                      |
|                                | PI(4,5)P2 (C16) | 12                                  | 55                             | 105                                    | 2.8   | 13.7  | >20                                      |
|                                | PI(4,5)P2 (C8)  | 55                                  | 44                             | 39                                     | 2.9   | 11.8  | >20                                      |
| <b>PI3K<math>\beta</math></b>  | Literature      | N/F                                 | 13 - 88                        | 270                                    | 0.3 - 2.9                                   | >20   | N/F                                      |
|                                | PI(4,5)P2 (C16) | 50                                  | 111                            | 717                                    | 2.2   | >20   | >20                                      |
|                                | PI(4,5)P2 (C8)  | 19                                  | 56                             | 166                                    | 1.0   | >20   | >20                                      |
| <b>PI3K<math>\gamma</math></b> | Literature      | 1.5 - 2                             | 76 - 150                       | 8                                      | 2.8 - 3.5                                   | 0.25  | 1.4                                      |
|                                | PI(4,5)P2 (C16) | 13                                  | 434                            | 36                                     | 5.6   | 2.6   | 11.3                                     |
|                                | PI(4,5)P2 (C8)  | 7                                   | 537                            | 64                                     | 5.5   | 5.7   | >20                                      |

Transcreener TR-FRET results show similar pharmacology regardless of lipid substrate. Inhibitor potency (IC<sub>50</sub>) confirmed for PI3K $\alpha$ , PI3K $\beta$ , and PI3K $\gamma$  isoforms. The PI3 kinase reactions (0.4 nM to 5.8 nM) used phosphatidylinositol 4,5 biphosphate (C16 or C8) substrate and were performed at 30°C for 60-180 minutes. Each reaction progressed between 1.1% to 8.0% ATP conversion with a Z' values > 0.45 with the exception of the PI3K $\gamma$  reactions, which converted 1.6% ATP. Literature values were determined using various assay conditions (N/F=not found).

**Figure 4 Inhibitor Dose-Dependency with with PI3 $\alpha$  Kinase (FP)**

**A.** ADP/ATP Standard Curves for 30  $\mu$ M ATP using Transcreener KINASE FP Assay. The ADP/ATP standard curve mimics a typical enzyme reaction (as ADP is produced, ATP is depleted). Optimal signal at initial velocity conditions (10% ATP conversion) is indicated.



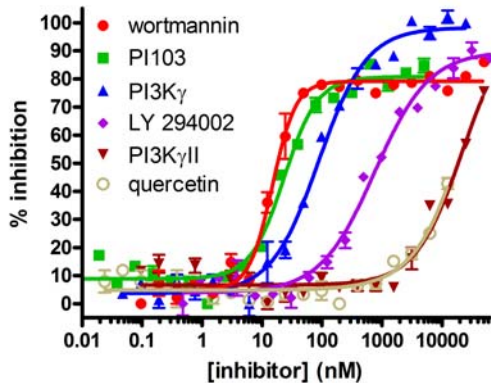
**B.** Inhibitor potency confirmed for PI3K $\alpha$  in a two-fold serial dilution experiment using six known lipid kinase inhibitors. The PI3K $\alpha$  kinase (1.0 nM) reaction used phosphatidylinositol 4,5 biphosphate (C16) and was performed at 30°C for 90 minutes and progressed to 10.3% ATP conversion with a Z' = 0.89. The IC<sub>50</sub> values for wortmannin, PI103, PI3K $\gamma$ , LY 294002, PI3K $\gamma$ II, and quercetin were 7 nM, 43 nM, 59 nM, 1.4  $\mu$ M, 8.0  $\mu$ M, and 14.3  $\mu$ M, respectively.

**Table 2 Pharmacology of PI3K isoforms using Transreener™ FP Assay**

|                                |                 | wortmannin<br>IC <sub>50</sub> (nM) | PI103<br>IC <sub>50</sub> (nM) | PI3K $\gamma$<br>IC <sub>50</sub> (nM) | LY 294002<br>IC <sub>50</sub> ( $\mu$ M) | PI3K $\gamma$ II<br>IC <sub>50</sub> ( $\mu$ M) | quercetin<br>IC <sub>50</sub> ( $\mu$ M) |
|--------------------------------|-----------------|-------------------------------------|--------------------------------|--|--|---|--|
| <b>PI3K<math>\alpha</math></b> | Literature      | 1-5                                 | 4.4-8                          | 60                                     | 0.5-9.3                                  | 4.5   | 3.8                                      |
|                                | PI(4,5)P2 (C16) | 7                                   | 43                             | 59                                     | 1.4                                      | 8.0   | 14.3                                     |
|                                | PI(4,5)P2 (C8)  | 13                                  | 49                             | 39                                     | 1.0                                      | 11.0  | >20                                      |
| <b>PI3K<math>\beta</math></b>  | Literature      | N/F                                 | 13-88                          | 270                                    | 0.3 - 2.9                                | >20   | N/F                                      |
|                                | PI(4,5)P2 (C16) | 35                                  | 93                             | 990                                    | 2.4                                      | 11.8  | 2.0                                      |
|                                | PI(4,5)P2 (C8)  | 11                                  | 62                             | 203                                    | 0.76                                     | 13.3  | >20                                      |
| <b>PI3K<math>\gamma</math></b> | Literature      | 1.5-2                               | 76 -150                        | 8                                      | 2.8 - 3.5                                | 0.25  | 1.4                                      |
|                                | PI(4,5)P2 (C16) | 4                                   | 182                            | 36                                     | 3.3                                      | 1.3   | 6.9                                      |
|                                | PI(4,5)P2 (C8)  | 4                                   | 289                            | 24                                     | 4.0                                      | 1.9   | 6.6                                      |

Transreener FP results show similar pharmacology regardless of lipid substrate. Inhibitor potency (IC<sub>50</sub>) confirmed for PI3K $\alpha$ , PI3K $\beta$ , and PI3K $\gamma$  isoforms. The PI3 kinase reactions (0.4 nM to 5.8 nM) used phosphatidylinositol 4,5 biphosphate (C16 or C8) substrate and were performed at 30°C for 60-180 minutes. Each reaction progressed between 2% to 11% ATP conversion with a Z' values >0.64 with the exception of the PI3K $\gamma$  reactions (C16), which were at 2.0% conversion (Z'=0.44). Literature values were determined using various assay conditions (N/F=not found).

**Figure 5 and Table 3 PI3 $\alpha$  Kinase Intrinsic ATPase Pharmacology**



Inhibitor potency confirmed for the PI3K $\alpha$  intrinsic ATPase activity (without substrate) in a two-fold serial dilution experiment with six known inhibitors using both TR-FRET and FP formats. The PI3K $\alpha$  kinase (29 nM) reaction was performed at 30°C for 4 hours and progressed to 2.7% ATP conversion with a Z' = 0.64. The IC<sub>50</sub> values have good correlation to those with substrate. FP data is shown on graph, TR-FRET detection results are below (curves not shown).

|                | wortmannin<br>IC <sub>50</sub> (nM) | PI103<br>IC <sub>50</sub> (nM) | PI3K $\gamma$<br>IC <sub>50</sub> (nM) | LY 294002<br>IC <sub>50</sub> ( $\mu$ M) | PI3K $\gamma$ II<br>IC <sub>50</sub> ( $\mu$ M) | quercetin<br>IC <sub>50</sub> ( $\mu$ M) |
|----------------|-------------------------------------|--------------------------------|--|--|---|--|
| <b>FP</b>      | 14                                  | 23                             | 89                                     | 0.76                                     | >20   | >20                                      |
| <b>TR-FRET</b> | 21                                  | 21                             | 107                                    | 0.62                                     | 19.1  | >20                                      |

## Materials and Methods:

Terbium chelate and Alexa Fluor®633 were from Invitrogen. PI3 Kinase isoforms were from Chemicon/Upstate. PI3 Kinase inhibitors were from EMD Biosciences. Lipid substrates were purchased from either Avanti Polar Lipids or Cellsignals. Lipid substrates were prepared using either 5 cycles of freeze/thaw (snap freeze in isopropanol/dry ice bath followed by rapid thawing in 40°C water bath) or by sonication (50/60 Hz/80 watts/117 volts for 1 hour at 30 °C). In general, PI3K reactions (10 µL) were performed in 50 mM HEPES (pH 7.1), containing 100 mM NaCl, 4 mM MgCl<sub>2</sub>, 2 mM EGTA, 2 mM DTT, 30 µM ATP, and either PI(4,5)P<sub>2</sub> C8 (100 µM) or C16 (30 µM) for 60 to 90 minutes at 30°C. Intrinsic ATPase reactions were performed between 2 to 6 hours. ADP produced in the kinase reaction was measured after the addition of an equal volume (10 µL) of TR-FRET or FP ADP Detection Mixture. TR-FRET ADP detection consists of 50 mM HEPES (pH 7.5), 200 mM NaCl, 5 mM EDTA, 0.01% Brij-35, 2 nM ADP-Antibody-Tb, and 30 nM ADP FAM Tracer. FP ADP detection conditions were 50 mM HEPES (pH 7.5), 400 mM NaCl, 10 mM EDTA, 0.01% Brij-35, 2 nM ADP Alexa Fluor®633, and ADP antibody (31 µg/mL). Assays were performed in Corning white (#3673) or black (#3676) 384-well, round bottom, low volume, polystyrene, non-binding surface plates. TR-FRET measurements were performed on the Tecan Ultra and Tecan Safire2 plate readers (100 µsec delay, 100-200 µsec integration time, 10 flashes at 30°C). Tecan Ultra filter sets were Ex<sub>340</sub> (+/- 17.5nm), Em<sub>495</sub> (+/- 5nm), Em<sub>520</sub> (+/- 12.5nm). FP measurements were performed on the Tecan Ultra (Ex<sub>612</sub> (+/- 5nm), Em<sub>670</sub> (+/- 12.5nm)) and Tecan Safire<sup>2</sup> (Ex<sub>635</sub> (LED), Em<sub>670</sub> (+/- 5nm)) plate readers. Curve fitting and IC<sub>50</sub> determinations were processed with Prizm software.

## Conclusions:

1. The Transcreener™ KINASE Assay is available in both far-red FP and TR-FRET formats. Each assay utilizes two-component detection methodologies for the direct measurement ADP, the invariant product of all kinase and ATPase reactions.
2. The Transcreener™ KINASE Assays are optimized for excellent signal at initial rate conditions (<10% ATP conversion). Sensitive detection at initial rate conditions decreases reagent costs for the end-user.
3. Transcreener™ can easily identify optimal lipid substrate concentrations and preparation techniques.
4. PI3K isoform pharmacology is similar to literature values using both FP and TR-FRET Transcreener™ methods.
5. PI3α Kinase has quantifiable intrinsic ATPase activity which shows similar pharmacology, relative to its kinase activity.

This work was supported by NIH SBIR grant CA110535-01A1. Transcreener™ HTS Assay Platform is patent pending. Transcreener™ is a trademark of BellBrook Labs. Alexa Fluor® is a registered trademark of Molecular Probes, Inc (Invitrogen).

©2007 BellBrook Labs. All rights reserved.

BellBrook Labs 525 Science Drive, Suite 110, Madison, WI 53711 866-313-7881  
www.bellbrooklabs.com