

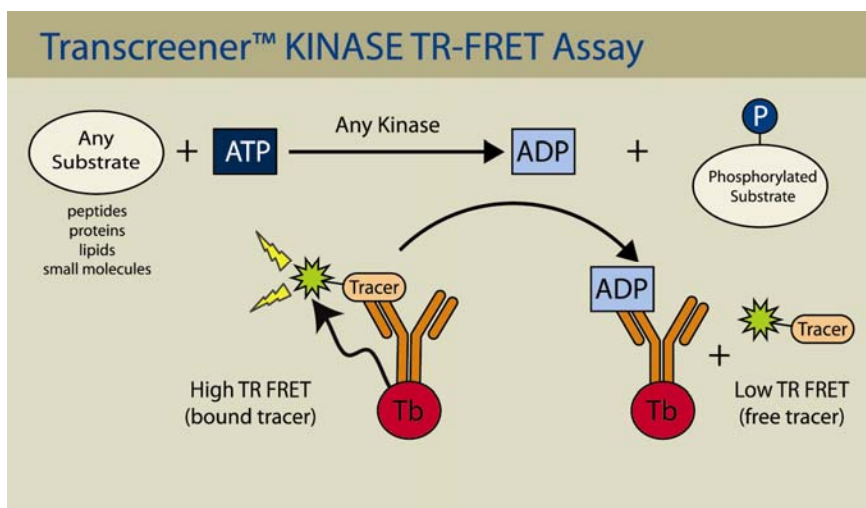
Expanding Transcreener™ Assay Technology: ADP Detection using TR-FRET

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Introduction

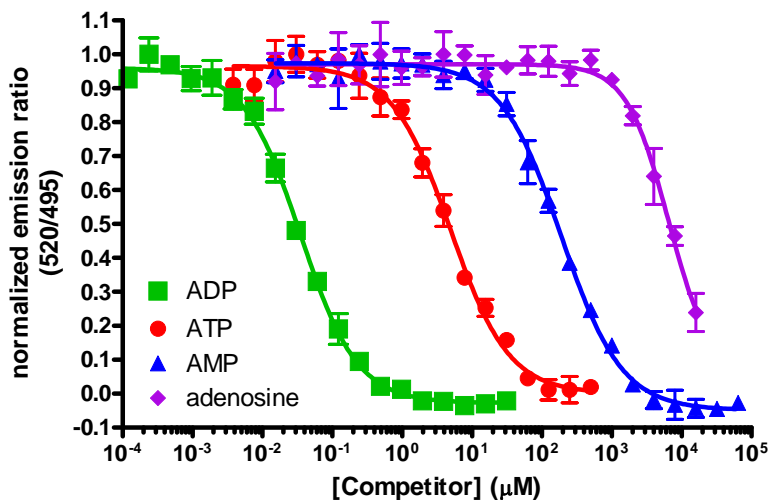
The Transcreener™ KINASE TR-FRET Assay is a competitive HTS assay that measures ADP, the invariant product of all kinases reactions. Previously we developed a monoclonal antibody and a fluorescent tracer that allow detection of ADP with >100-fold selectivity over ATP using a fluorescence polarization (FP) readout. To offer detection options to the HTS community, we formatted the Transcreener™ KINASE Assay to detect ADP production using time resolved fluorescent resonance energy transfer (TR-FRET). A lanthanide chelate was conjugated to the ADP antibody and coupled with an ADP tracer to enable TR-FRET based immunodetection of ADP. The assay was validated for robust detection of kinase activity using both protein and peptide substrates as well as kinase inhibitor screening.

Figure 1. Transcreener™ TR-FRET KINASE Assay Principle



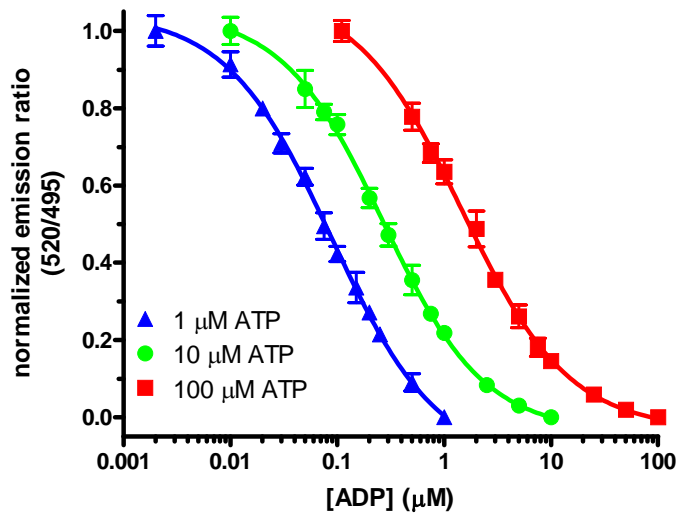
The Transcreener™ KINASE Assay is now adapted for time resolved fluorescent resonance energy transfer (TR-FRET). A terbium chelate was conjugated to the ADP monoclonal antibody. The Tb-labeled ADP antibody coupled with a fluorescein ADP tracer enables homogenous TR-FRET based immunodetection of ADP. TR-FRET is observed when the terbium (covalently bound to ADP antibody) is in close proximity to the fluorescein ADP tracer. As ADP is produced, TR-FRET decreases as the fluorescein ADP tracer is displaced from the Tb-labeled ADP antibody.

Figure 2. Transcreener™ Tb-labeled Antibody: High Selectivity for ADP.



The Transcreener™ ADP Antibody, which can distinguish between a single phosphate group, was labeled with a terbium chelate. The Tb-labeled ADP antibody and ADP fluorescein tracer complex was mixed with increasing concentrations of ATP, ADP, AMP, or adenosine to generate competition curves. The Tb-labeled mAb is 139-fold, 5,000-fold, and 18,000-fold more selective for ADP, relative to ATP, AMP, or adenosine, respectively.

Figure 3. The Transcreener™ TR-FRET KINASE Assay: Flexibility from 1 μ M to 100 μ M ATP.



ATP/ADP standard curves were generated for 1 μ M, 10 μ M, and 100 μ M ATP. To mimic ADP generated during a kinase reaction, the adenosine concentration remained constant for each standard curve (n = 10). The Transcreener™ TR-FRET KINASE Assay has excellent sensitivity for ADP. For the 1 μ M, 10 μ M, and 100 μ M ATP/ADP standard curves, the IC₅₀ values are 0.084, 0.27 μ M, and 1.47 μ M ADP, respectively.

Table 1. Excellent Z' Values at <10% ATP Conversion.

The Transcreener™ KINASE TR-FRET detection module is robust at less than 10% ATP conversion. The Z' value was calculated at different artificially produced % ATP conversions using 1 μ M, 10 μ M, and 100 μ M Standard Curve data points (n=10). Z values >0.52 for 5% ATP conversion were observed for each data set.

% ATP Conversion	1 μM ATP/ADP Z' Value	10 μM ATP/ADP Z' Value	100 μM ATP/ADP Z' Value
10%	0.69	0.81	0.88
7.5%	0.56	0.80	0.81
5%	0.52	0.66	0.77
3%	0.34	0.63	0.81
2%	0.16	0.58	0.57

Figure 4. The Generic Transcreeper™ KINASE TR-FRET Assay can Identify Optimal Kinase Substrates.

A single set of reagents was used for these PKA titrations using both protein and peptide substrates. The physiological substrate Histone H1 and the peptide are good PKA substrates, while MBP requires 10-fold more enzyme. The PKA titrations were performed at 10 μ M ATP using 50 μ M Kemptide, 5 μ M Histone H1, or 20 μ M MBP. The PKA reaction was successfully stopped and exhibited excellent signal stability over 24 hours (Kemptide data shown in inset).

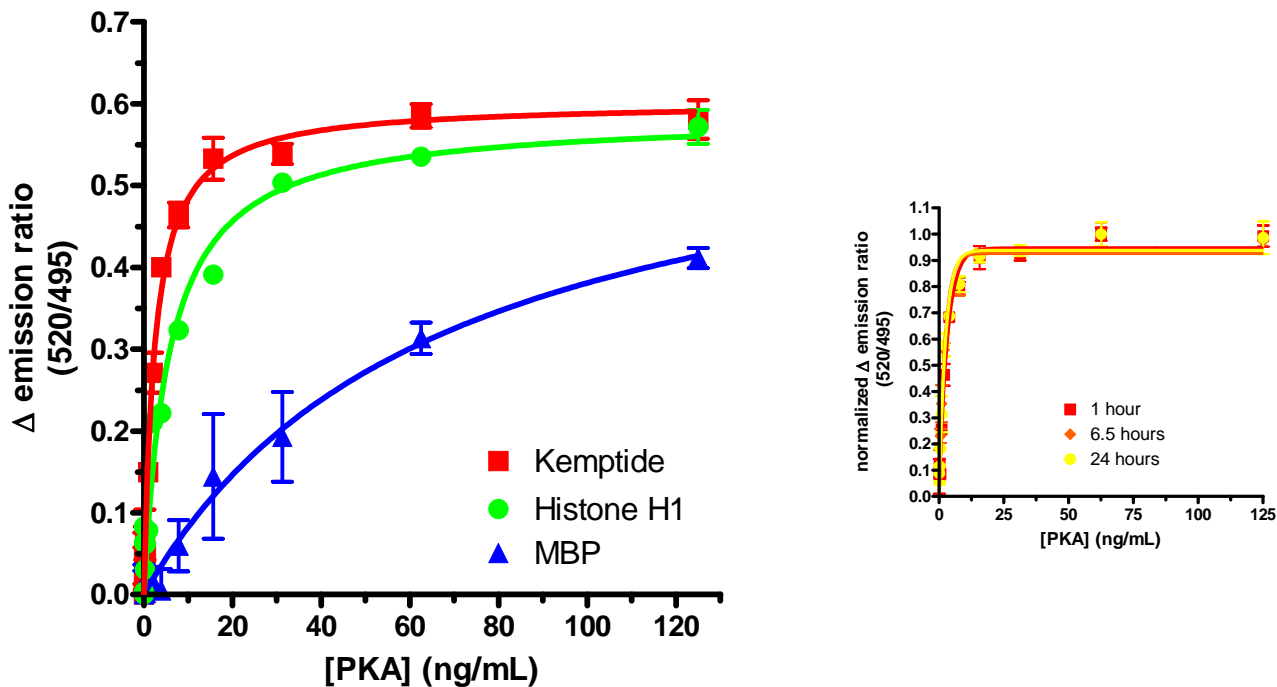
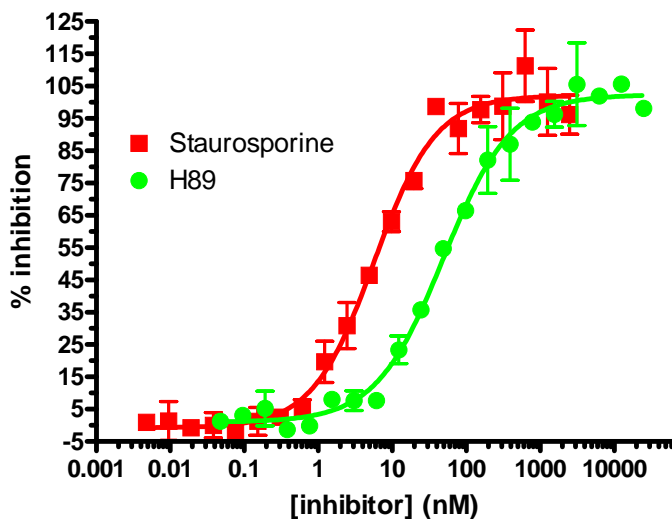


Figure 5. PKA Inhibitor Potency Confirmed Using Staurosporine and H89.



Dose dependent curves were generated using PKA kinase and two-fold serial dilutions of Staurosporine or H89. An excellent Z' value (= 0.76) at 4.2% ATP conversion was obtained. The IC_{50} values for PKA inhibition by Staurosporine and H89 were 5.7 nM and 49.0 nM, respectively. These values are similar to literature values of PKA inhibition by Staurosporine (2.6 nM to 15 nM) and H89 (40 nM to 135 nM). The PKA kinase assays were performed using 10 μ M ATP, 50 μ M Kemptide, and 7 ng/mL PKA.

Materials and Methods: PKA kinase and terbium chelate were purchased from Invitrogen. Kinase substrates were purchased from Chemicon or Sigma. Kinase inhibitors, and basic buffer components were purchased from Sigma or Fisher. The Transcreener™ KINASE TR-FRET Assay consisted of a one hour, 25°C kinase reaction (10 µL) initiated with the addition of ATP. The kinase reaction was then stopped and ADP detected by adding 10 µL of the Transcreener™ TR-FRET ADP Stop and Detect mixture, bringing the total volume to 20 µL. The PKA kinase reaction conditions were 50 mM HEPES (pH 7.5), 4 mM MgCl₂, 2 mM EGTA, 10 µM ATP, 5-50 µM substrate (50 µM Kemptide, 5 µM Histone H1, or 20 µM MBP), 1 % DMSO, and 0.02% Brij-35. The Transcreener™ TR-FRET ADP Stop and Detect mixture consisted of 50 mM HEPES (pH 7.5), 200 mM NaCl, 5 mM EDTA, 0.01% Brij-35, 2 nM Tb-labeled ADP Antibody, and various (4 nM to 75 nM) fluorescein ADP Tracer. Tecan Ultra filter sets were Ex_{340nm} (+/- 17.5 nm), Em_{495nm} (+/- 5 nm), Em_{520nm} (+/- 12.5 nm). Tecan Ultra and Tecan Safire²™ settings included a 100 µsec delay, 100-200 µsec integration time, 20 flashes at 30°C. Assays were performed in Corning® white (catalog # 3673) or black (catalog # 3676) 384-well, round bottom, low volume, polystyrene, non-binding surface plates. Curve fitting and IC₅₀ determinations were processed with Prizm software.

Conclusions:

- 1. BellBrook Labs has formatted the Transcreener™ KINASE Assay for time resolved fluorescent resonance energy transfer (TR-FRET).**
- 2. The Tb-labeled Transcreener™ ADP Antibody discriminates between one phosphate group and binds ADP selectively.**
- 3. Transcreener™ KINASE TR-FRET Assay can be used over a wide range of ATP concentrations (1 µM to 100 µM).**
- 4. The great sensitivity of the Transcreener™ KINASE TR-FRET Assay yields great Z' values at <10% ATP Conversion.**
- 5. Using the Transcreener™ KINASE TR-FRET Assay, a single set of reagents can be used with protein or peptide substrates.**
- 6. The Transcreener™ KINASE TR-FRET Assay was validated for robust detection of PKA kinase activity and inhibitor screening.**

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).

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