Detecting ATP-utilizing Enzyme Activity Using a Simple Fluorescence Intensity Quenching ADP Assay

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Introduction

The Transcreener® ADP fluorescence polarization and TR-FRET assays have been validated for evaluation of PI3K pharmacology (*J Biomol Screen 2008; 13(6):476-485*). BellBrook Labs extends the robust Transcreener technology to include a fluorescence intensity (FI) readout. The Transcreener® ADP² FI Assay allows the facile detection and screening of established drug targets including protein and lipid kinases, as wells as other ATPases by directly measuring ADP formation. This homogenous, single-step competitive fluorescence intensity quenching immunoassay employs a selective ADP monoclonal antibody conjugated to an IRDye® QC-1 quencher licensed from LI-COR® and a red Alexa Fluor®594 tracer with >100-fold selectivity for ADP vs. ATP. The tracer emission is quenched upon antibody binding. As ADP is produced in the enzyme reaction, the tracer is displaced from the antibody resulting in an increase in the fluorescence signal. Validated in 96 and 384 well formats, the ADP² FI assay is compatible with most plate readers commonly found both in academic labs and high throughput screening settings. This generic ADP detection system enables robust detection for 0.1 µM to 100 µM ATP for any lipid kinase or other ADP-producing enzymes under initial velocity conditions (<10% ATP conversion). Furthermore, lipid kinase and PKA dose-dependency curves were generated using known inhibitors.

The Transcreener® ADP² FI Assay Principle

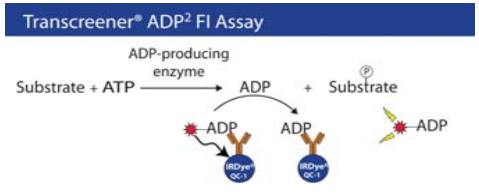


Figure 1. The Transcreener ADP² FI Assay. The Transcreener ADP² FI Assay was developed to follow the progress of any enzyme that produces ADP. The Transcreener ADP Detection Mixture comprises a quenched ADP Alexa594 Tracer bound to a selective ADP monoclonal antibody conjugated to an IRDye® QC-1 quencher licensed from LI-COR®. The tracer is displaced by ADP, the invariant product generated during an enzyme reaction. The displaced tracer becomes un-quenched in solution leading to a positive increase in fluorescence intensity. Therefore, ADP production is proportional to an increase in fluorescence. The red tracer minimizes interference from fluorescent compounds and light scattering.

EZ Assay Format Facilitates Assay Development

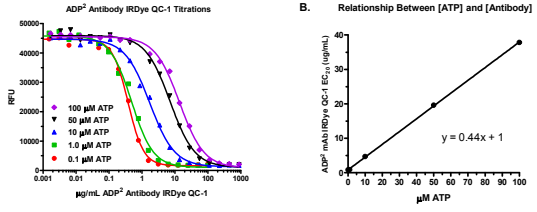
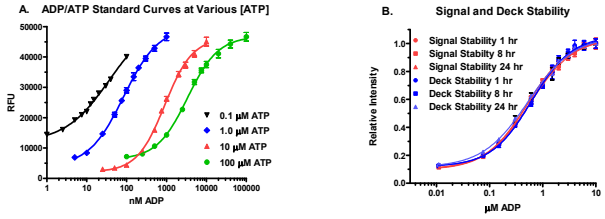


Figure 2. An EZ format provides a simple way to get started with any [ATP]. In **2A** the optimal amount of ADP² Antibody IRDye QC-1 was determined by performing antibody titrations at various [ATP] in a simple buffer system. 10 µL of reaction mix (50 mM HEPES pH7.5, 4 mM $MgCl_2$, 2 mM EGTA, 1% DMSO, 0.01% Brij, XuM ATP) was added to 10 µL antibody titration mix (ADP² Antibody IRDye QC-1, 8 nM ADP Alexa594 tracer, 1X Stop and Detect buffer B). Optimal sensitivity was determined for each [ATP] to be the EC_{50}. The appropriate EC values were plotted vs [ATP] to determine their linear relationship in **2B**. By using the equation y = 0.44x + 1, the amount of antibody needed can be determined for any [ATP], where x = [ATP] and y = the amount of ADP² Antibody IRDye QC-1 needed.

Excellent Z' Signal and Stability at Initial Velocity Conditions

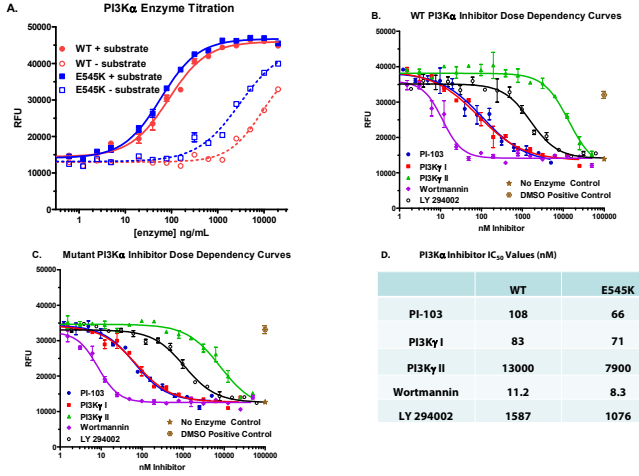


C. Excellent Z' Values at Low ATP Conversions

% ATP Conversion	0.1 µM ATP	1 µM ATP	10 µM ATP	100 µM ATP
0.5	nd	0.4	0.7	0.6
1	nd	0.6	0.8	0.8
2.5	0.3	0.8	0.9	0.9
5	0.7	0.8	0.9	0.9
10	0.7	0.9	0.9	0.9
100	0.9	0.8	0.9	0.9

Figure 3. ATP/ADP standard curves were performed from 0.1 µM to 100 µM ATP. 3A shows ADP/ATP standard curves that mimic the conversion of ATP to ADP in an enzyme reaction using the amount of ADP² Antibody IRDye QC-1 determined from the equation in Figure 2B. **3B** Excellent signal and deck stability for up to 24 hours. **3C** Excellent Z' values of >0.7 were achieved at 1% ATP conversion for 10 µM, and 100 µM ATP and 5% conversion for 0.1 µM and 1 µM ATP.

Wild Type PI3Kα and E545K Mutant Assay Development



D. PI3Kα Inhibitor IC_{50} Values (nM)

	WT	E545K
PI-103	108	66
PI3Kγ I	83	71
PI3Kγ II	13000	7900
Wortmannin	11.2	8.3
LY 294002	1587	1076

Figure 4. Assay development is quick and easy. In **4A** PI3Kα wild type and E545K were titrated (1:2). Reactions were performed in a 10 µL assay volume with 30 µM ATP with or without 200 µM PI(4,5)P_2 C8 substrate, and incubated for 1.5 hours. Detection Mixture (10 µL) was added and equilibrated for one hour before reading. The final 20 µL (384-well) assay volume including the Detection Mixture consisted of 4 nM ADP Alexa594 tracer, 0.5X Stop & Detect Buffer B, 9 µg/mL ADP² Antibody IRDye-QC1, and 0.5X enzyme reaction mixture (25 mM HEPES pH 7.5, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 50 mM NaCl, 0.5% DMSO, 0.005% Brij-35, 15 µM ATP and 100 µM PI(4,5)P_2 C8). **4B** and **4C** show dose dependency curves with known inhibitors using 300 & 225 ng/mL WT and E545K PI3Kα respectively. Assays went to ~7.5% completion (Z' = 0.6) for DMSO control (N = 16). Table **4D** shows similar IC_{50} inhibitor values of for wild type and mutant PI3Kα.

SpectraMax M2 and Tecan Safire² Compatible

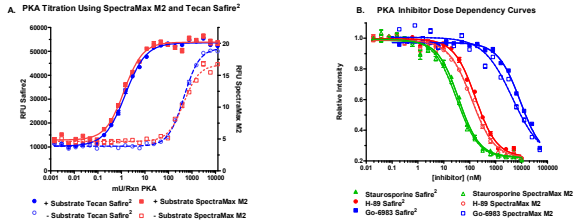


Figure 5. The Transcreener ADP² FI Assay is compatible with both academic and HTS readers. 5A Shows the data as PKA was titrated (1:2). Reactions were performed in a 10 µL assay volume with 10 µM ATP and 50 µM kemptide substrate for one hour. Detection Mixture (10 µL) was added and reactions equilibrated for one hour before reading. The final 20 µL assay volume with the Detection Mixture consisted of 4 nM ADP Alexa594 tracer, 0.5X Stop & Detect Buffer B, 4.5 µg/mL ADP² mAb QC-1 antibody, and 0.5X enzyme reaction mixture (25 mM HEPES pH 7.5, 2 mM $MgCl_2$, 1 mM EGTA, 0.5% DMSO, 0.005% Brij-35, 5 µM ATP and 25 µM kemptide). EC_{50} was 1.0 mU/rxn (Safire²), and 1.1 mU/rxn (SpectraMaxM2). **5B**. Dose dependency curves were generated. Staurosporine, H-89, and GO-6983 (known PKA inhibitors) were serially titrated into the PKA reaction following the assay conditions noted previously. Similar IC_{50} values are shown using both instruments (Safire²/SpectraMax M2) for staurosporine, H-89 and GO-6983 were 37/32 nM, 165/124 nM and 9.0/4.7 µM respectively.

Use the ADP² FI Assay for GDP Detection

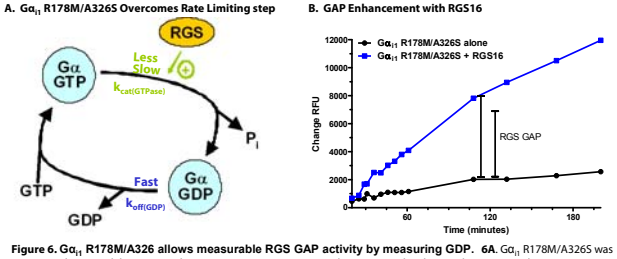


Figure 6. $G\alpha_{i1}$ R178M/A326 allows measurable RGS GAP activity by measuring GDP. 6A. $G\alpha_{i1}$ R178M/A326S was engineered as a model protein to detect RGS GAP activity in a steady state assay by altering the $G\alpha_{i1}$ GDP dissociation rate, which is the rate limiting step, and the intrinsic GTPase rate. **6B**. 50 nM $G\alpha_{i1}$ R178M/A326S was added to a reaction mix (20 mM Tris 7.5 pH, 1 mM EDTA, 10 mM $MgCl_2$, 10 µM GTP, 20 µg/ml ADP2 Antibody IRDye QC-1 , 4 nM ADP Alexa594 Tracer) with 250 nM RGS16, or without in a 384 well low volume assay plate and incubated at 30°C. The plate was read at various time points. N = 16. Change RFU = RFUGα(+/- RGS) - RFU no Gα(+/-RGS). This indicates that RGS16 is a GAP for $G\alpha_{i1}$ R178M/A326S

Conclusions

- The Transcreener® ADP² FI Assay allows the facile detection and screening of established drug targets including protein and lipid kinases, as wells as other ATPases by directly measuring ADP formation.
- The ADP² monoclonal antibody has greater than 100-fold selectivity for ADP relative to ATP.
- This generic ADP detection system enables robust signal for 0.1 µM to 100 µM ATP for any ADP-producing enzyme using initial velocity conditions (<10% ATP conversion).
- The ADP² FI assay has excellent compatibility with plate readers commonly found both in academic labs and HTS settings.
- The Transcreener® ADP² FI Assay is a valuable tool for GDP detection.

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