ADP Hunter™: a generic homogeneous assay for high throughput kinase inhibitor screening

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ABSTRACT

The DiscoveRx ADP Hunter™ assay measures the generation of ADP resulting from kinase phosphorylation of a substrate, in a format specifically designed for the high throughput screening laboratory. ADP is measured through a coupled enzyme system, which is linked to a positive, red-shifted fluorescent readout. The assay has a wide dynamic range (1.2 – 120 µM), and can be used with a wide range of ATP, from less than 5 µM up to 300 µM. No substrate labeling is required, and the assay can accommodate either peptide or whole protein substrates. The simple assay format, with a single sixty minute incubation, provides robust and reproducible results with signal to background ratios > 10 and typical Z' values > 0.7. The assay has been validated with several kinases, and against the LOPAC library with excellent results. Compound interference rates are low, and known kinase inhibitor IC_{50} values are consistent with literature references. In all, the ADP Hunter provides a useful, generic tool for screening novel inhibitors of kinase activity.

ADP Hunter Assay Principle

The ADP Hunter Assay is a **generic**, **non-antibody**, **non-radioactive** assay for monitoring phosphotransferase (i.e. kinases, ATPases) activity. It is designed to be compatible with either peptide or whole-protein substrates in an endpoint assay format for screening. Unlike other generic methods, such as ATP depletion, this positive signal read-out assay detects the generation of ADP produced as a result of enzyme activity.

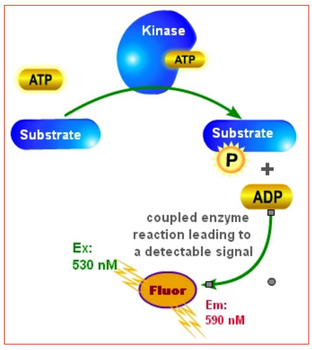

***Figure 1**: ADP Hunter Assay Principle*

ADP Hunter uses an enzyme-coupled reaction that produces a positive fluorescent signal. that is directly proportional to the amount of ADP in solution. The fluorescent signal is red-shifted, minimizing interference from fluorescent compounds.

ADP Hunter: Assay Protocol

Format: Homogeneous

Plate: 384-well low volume black plate

Signal: Fluorescent Intensity

Reader: Standard microplate reader

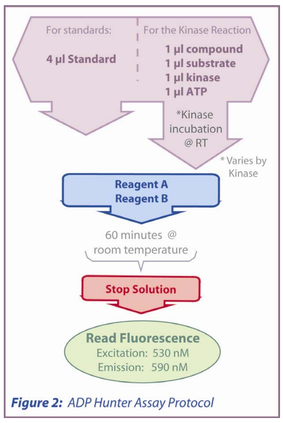

***Figure 2:** ADP Hunter Assay Protocol*

Materials and Methods

All kinase reactions and standard curves were performed in assay buffer containing 15 mM Hepes, 20 mM NaCl, 1 mM EGTA, 0.02 % Tween 20, 10 mM $MgCl_2$, 0.1 % bovine gamma globulins, pH 7.4. Protein kinases were purchased from Upstate. Staurosporine was obtained from Roche or AG Scientific. Lyn, CK1 and JNK2 peptide substrates were purchased from American Peptide Co., and all other reagents obtained from Sigma-Aldrich. All kinase reactions were incubated for 30-60 minutes at 30 °C unless otherwise noted.

The fluorescent intensity signal was measured using a Packard Fluorocount Plate Reader using excitation / emission wavelengths of 530 / 590 nm with 0.1 s integration and PMT voltage of 750 V.

ADP Hunter: Standard Curve

The ADP Hunter Standard curve is illustrated in the figure below. The dynamic range of the assay is 1.5 µM to 120 µM ADP and the data is shown using a third order polynomial curve fit. The ADP Hunter Assay format was designed to produce a positive signal in direct proportion to the amount of ADP produced.

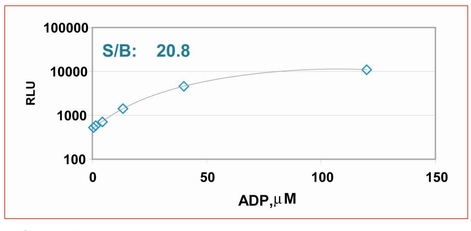

***Figure 3**: ADP Hunter standard curve*

Activity and Inhibition of Kinase Activity

ADP Hunter is designed as a generic assay for phosphotransferase enzyme activity, particularly in cases where the user does not have access to a modified substrate, a phosphorylation-state specific antibody, or the ability or desire to use radioactivity. It has broad applicability for both serine/threonine and tyrosine kinases. The figures below exhibit kinase activity and inhibition as measured using ADP Hunter.

Activity and Inhibition of Lyn Tyrosine Kinase

Kinase Reaction Conditions:

$p34^{cdc2}$
Substrate: 100 µM
ATP: 25 µM

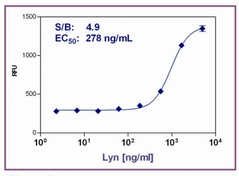

Figure 3a: *Lyn enzyme curve titration*

For inhibition only:
$p34^{cdc2}$
Substrate: 100 µM
ATP: 25 µM

Lyn: 400 ng/mL

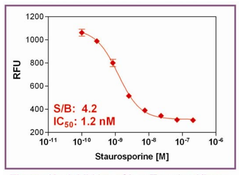

Figure 3b: *Inhibition of Lyn Tyrosine Kinase*

Activity and Inhibition of JNK2 Kinase

Kinase Reaction Conditions:

Substrate: 100 µM
ATP: 25 µM

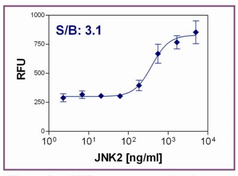

Figure 4a: *JNK2 enzyme curve titration*

For inhibition only:
Substrate: 100 µM
ATP: 25 µM

JNK2: 700 ng/mL

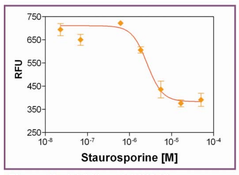

Figure 4b: *Inhibition of JNK2 Kinase*

Signal Stability for screening

For screening, ADP Hunter is designed for batch reading of microplates. A stop solution was incorporated to stop the enzyme reaction from further activity, allowing sufficient time for a series of plates to be read without compromising signal. The table lists the percentage of Time zero "X" hours after adding the stop solution.

% of Time 0	1 hour	2 hours	3 hours	4 hours
Background	114	121	129	134
Low standard	97	92	91	89
High standard	85	76	70	66

***Table 1**: ADP Hunter Signal Stability*

Compound Interference

The ADP Hunter assay is specifically designed for high throughput screening with its robust formulation that minimizes compound interference. Figure 4 summarizes the results obtained for the LOPAC library when screened with ADP Hunter reagents. Compound interference was <0.8%.

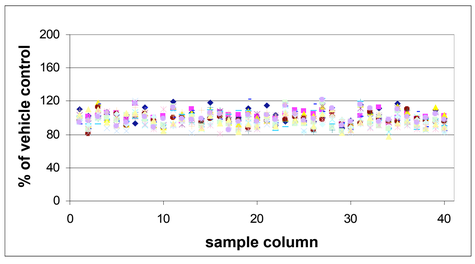

***Figure 4**: ADP Hunter LOPAC Library Screening*

ADP Hunter: Summary

- ADP Hunter is a generic, non-antibody, non-radioactive assay for monitoring phospho-transferase (i.e. kinases, ATPases) activity
  - A positive readout is generated in direct proportion to ADP accumulated as a result of enzyme activity
  - Broad ATP tolerance and robust signal to background ratios.
- The assay is simple and homogeneous with a single incubation
- ADP Hunter incorporates a kinase stop solution to provide signal stability for screening
- ADP Hunter has a robust formulation that minimizes compound interference (<0.8% false positives)
- Broad instrument applicability
  - Easy to automate
  - Compatible with a variety of fluorescent Intensity readers, PMT based or multimode CCD cameras