ADP Hunter™ Plus: A Rapid, Quantitative HTS Assay for GDP and UDP

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Introduction

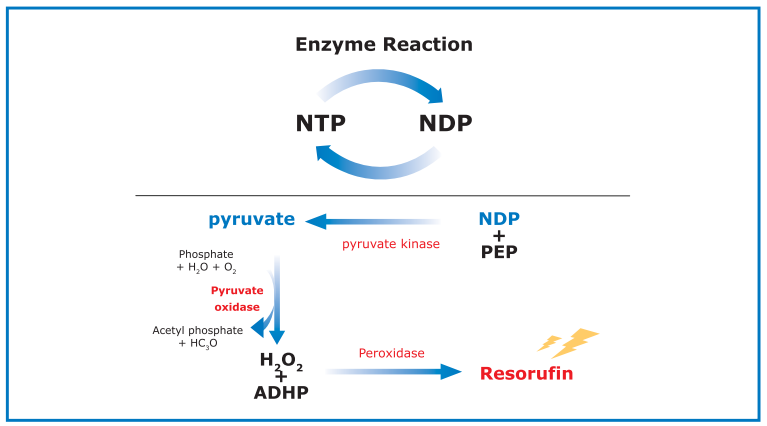
There are numerous assays available to measure the activity of enzymes involved in the transfer of a phosphate from ATP to a substrate macromolecule. However, there are only a few assays for enzymes that utilize other nucleotide triphosphates, opening opportunities to address pharmacologically relevant targets.

GTPases are a broad family of enzymes with a wide variety of activities such as signal transduction, Ras-mediated cell signaling and endocytosis. Hydrolysis of GTP to GDP is a critical step for GTPase activity and hence can be used to characterize these enzymes.

UDP-glycotransferases are involved in polysaccharide biosynthesis, post-translational modification and glucuronidation of small molecules. The latter is particularly relevant for drug discovery since UDP-transferases are important in drug metabolism and removal. There are over 200 glycotransferases in humans which are increasingly being targeted for therapeutic intervention.

DiscoveRx has developed ADP Hunter Plus and ADP Quest, an homogenous fluorescent assay for detection of nucleotide diphosphates, including ADP, GDP and UDP. ADP Quest was specifically developed for real time kinetics of enzymes. This is a non-antibody, general purpose biochemical assay that does not require a labeled substrate and generates a positive red-shifted fluorescent read-out that is compatible with any fluorescent plate reader. There are currently limited available approaches for GTPase such as HPLC, ELISA, IP or Pi assays. The DiscoveRx assay offers much greater ease of use, with an HTS-friendly procedure, excellent sensitivity and reproducibility. We will present data here highlighting the key features of the assay, the versatility of the technology, as well as results using our assay with an enzyme that produces UDP.

Figure 1: ADP Hunter™ Plus Assay Principle for NDP's



Methods

- All enzyme reactions and standard curves were performed in assay buffer containing 15 mM Hepes, 20 mM NaCl, 1 mM EGTA, 0.02 % detergent, 10 mM $MgCl_2$, 0.1 % bovine gamma globulins, pH 7.4.
- Enzymes and substrates were from Sigma.
- For the β-(1-4) Galactosyltransferase assay the reaction was performed in the presence of 2 mM $MnCl_2$.
- Fluorescent intensity signal was measured using a Packard Fluorcount Reader using excitation/emission wavelengths of 530/590 nm with 0.1 s integration and PMT voltage of 750 V.

ADP Hunter™ Plus NDP Assay

Assay modification

- Assay based on ADP Hunter Plus
  - For kinetic assay use ADP Quest
- Only the standard has been modified
  - UDP for the UDP assay
  - GDP for the GDP assay
- Reagents and assay protocol identical to ADP Hunter Plus

Assay Protocol

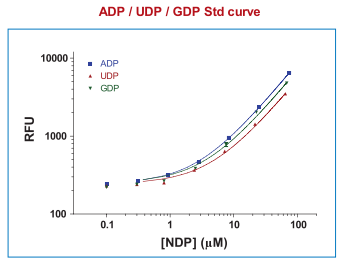
In a 384 well plate,

- 20 μL Enzyme Reaction
  - Incubation time and temperature - optimize for enzyme
- Add 10 μL ADP Hunter Plus Reagent A
- Add 20 μL ADP Hunter Plus Reagent B
- Incubate 15-60 minutes at room temperature, then read fluorescent signal (ex 530/em 590)
  - 60 minutes required for UDP detection
- Add 5 μL Stop solution (optional)

Figure 2. UDP and GDP Standard Curves Using ADP Hunter Plus

Standard Curve

- All NDPs generate similar dynamic range and sensitivity in the assay
  - Sensitivity <1 μM NDP
  - Linear range 0-25 μM NDP



- S/B for the assays
  - ADP > 25
  - GDP > 25
  - UDP > 15

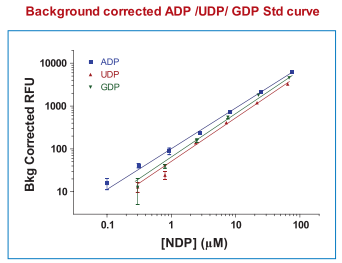


Figure 3. Detection of UDP Galactosyltransferase activity using ADP Hunter Plus

β-(1-4) Galactosyltransferase catalyzes formation of galactoside from UDP-galactose and an acceptor sugar such as N-acetyl glucosamine (GlcNAc). Millimolar substrate is required for this enzyme which can provide a challenge for other assays. The assay tolerated high concentrations of substrate and produced a S/B of 3.5-fold.

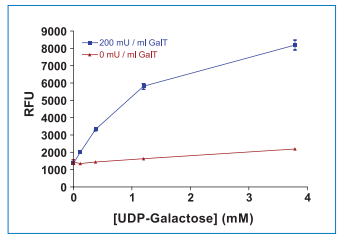


Figure 4. K_m determination of enzyme kinetics for UDP-Galactosyltransferase

K_m determination:

Linear relationship between signal and activity makes it ideal for examining substrate dependencies for these enzymes. The substrate was titrated in the assay in the presence of 2 mM $MnCl_2$ to measure the effect of substrate on enzyme activity. A K_m of 1 mM was obtained by plotting activity against substrate concentration.

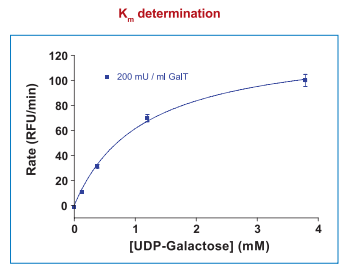


Table 1: Performance comparison with different NTP analytes using ADP Hunter™ Plus

Characteristic	ADP	UDP	GDP
Sensitivity	0.6 μM	1 μM	0.6 μM
Linear range	0-25 μM	0-25 μM	0-25 μM
DTT tolerance	2 mM	2 mM	2 mM
Compound Interference	Low	Low	Low
S/B	>25	>15	>25

Summary & Features of ADP Hunter™ Plus

- **DiscoveRx's ADPHunter and ADPQuest assay technologies provides a generic, non-radioactive detection of enzymes producing ADP, UDP, and GDP.**
  - The assay technology can be modified to a generic NTP assay
  - High sensitivity and S/B with a positive linear signal readout
  - Suitable for UDP-Galactotransferases and GTPases with a wide range of substrate requirements
  - High tolerance of substrate concentrations
  - Ideal for examining substrate dependencies for such UTPases

Benefits and Versatility of ADP Hunter™ and ADP Quest™ Assay Technology

- **Ideal for NTP enzyme & inhibitor characterization**
  - Fast and accurate tool for determining K_m, K_i and mode of action
  - Linear activity to signal relationship
- **Universal and homogenous assay, applicable to most NTPases including UTPases, GTPases, kinases and ATPases by measuring direct NDP accumulation**
- **Flexibility in NTP concentrations**
  - Can be used below, at, or above K_m
- **Endpoint and/or kinetic readout**
  - ADP Quest is the ONLY commercial assay where you can detect activity in a kinetic mode at constant NTP levels!
- **Non-antibody method**
- **Non-radioactive**
- **Fluorescence intensity read-out (red-shifted)**
- **Designed for HTS**
  - Optimized for high throughput screening with minimal compound interference
  - Compatible with reducing agents such as DTT
- **Broad instrument applicability**
- **Easily miniaturized**
  - Validated for use in 1536 well format

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