GDP Detection Using the Transcreener™ HTS Assay Technology

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Abstract

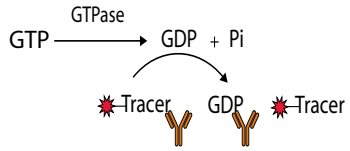
Proteins that utilize purine nucleotides as substrates or cofactors, collectively referred to as the purinome, account for approximately 13% of the coding capacity of the human genome. The Transcreener HTS Assay Platform relies on homogenous, fluorescent immunodetection of nucleotides, and thus provides generic detection for many enzymes in the purinome. We previously developed Transcreener Assays for ADP, AMP, and GMP detection, enabling facile screening of established target families such as protein kinases and phosphodiesterases as well as emerging targets such as lipid kinases and ATPases.

In this poster, we describe the development of a homogeneous, fluorescence polarization-based Transcreener™ Assay for GDP. The binding and selectivity properties of three monoclonal antibodies (mAb) raised against a purine nucleotide immunogen were characterized using far-red NDP tracers. Differential effects of reaction components, including salts and metals, on the antibody/tracer complex were observed with the different antibodies. A standard curve (10 µM GTP) prepared with a mouse monoclonal antibody and an ADP-AlexaFluor®633 tracer demonstrated a polarization shift of 90 mP units at 10 % GTP conversion. This robust assay is ideal for performing HTS GTPase assays under initial rate conditions. The Transcreener technology will facilitate the search for new inhibitors of GTPases, which control a variety of cellular events including actin cytoskeletal organization, cell growth, proliferation, differentiation, and survival.

Figure 1.

Assay Principle

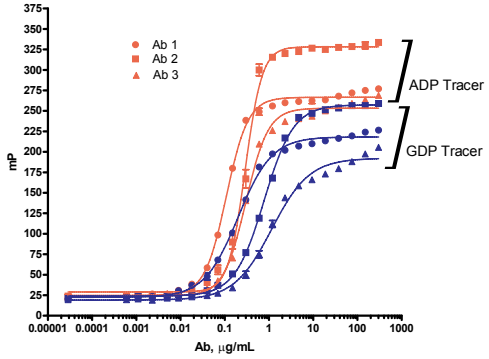
GDP detection using Transcreener™ Technology



The Transcreener™ GDP Assay is a competitive fluorescent polarization immunoassay employing a far red tracer bound by a selective antibody. GDP produced in a GTPase reaction displaces the tracer from the bound antibody resulting in an overall decrease in the polarization signal. An equal volume of the GDP detection mixture consisting of the antibody and tracer is added to the enzyme reaction mixture in this two-step, homogeneous endpoint assay.

Figure 2.

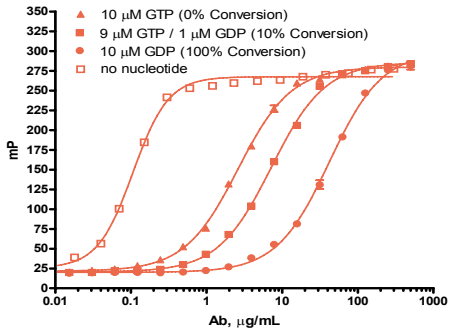
Tracer Structure Affects Antibody Binding



To identify suitable antibody/tracer pairs for FP assay development, antibodies were titrated two-fold in conditions typical of a quenched GTPase reaction (Complex Buffer). Maximal polarization shifts of >200 mP and Kd values ranging 0.7 nM-2 nM were observed with the ADP tracer with all three antibodies.

Figure 3.

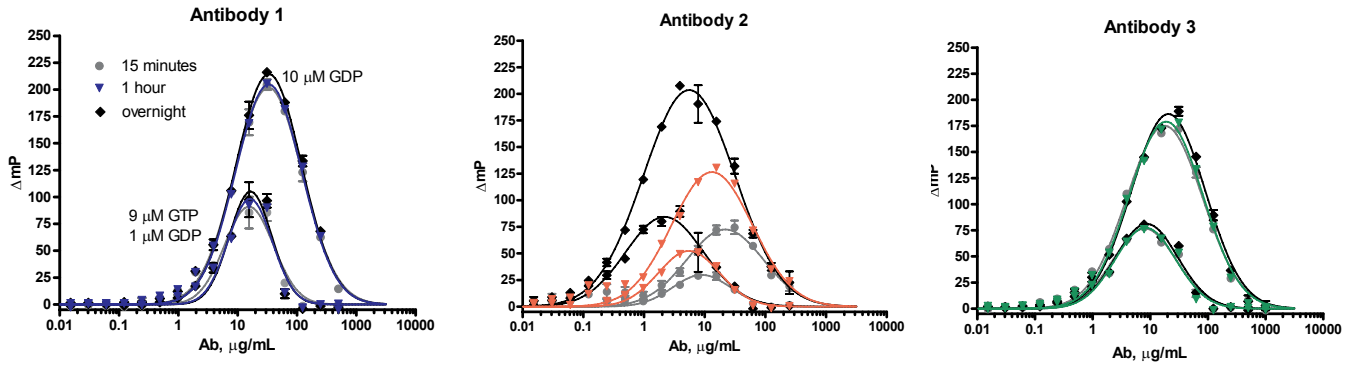
Cross-reactivity with GTP dictates the antibody concentration in the assay



Cross-reacting GTP causes the binding isotherm to shift to the right (no nucleotide vs. 10 µM GTP). Graphing the difference in polarization values between mock GTPase reaction antibody binding curves and the 0% conversion (10 µM GTP) curve enables determination of the antibody concentration that yielded the maximal polarization shift (ΔmP_{max}), or assay window for each level of GTP conversion (See Figure 4).

Figure 4.

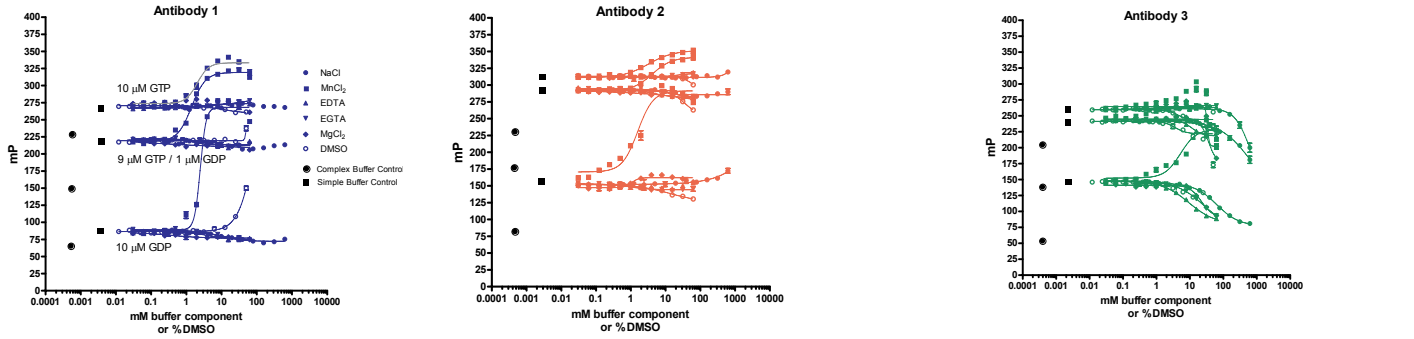
Assay window and equilibration times differ among mAbs raised against the same immunogen



Three distinct antibodies were produced from the same conjugated nucleotide as evidenced by differences in antibody requirement, assay window, and overall equilibration rates. Although Antibody 1 required the highest concentration per well (13µg/mL), it provided the greatest assay window at 10% GTP conversion (ΔmP= 100) and had a reasonable equilibration time of <1hr. The equilibration rate for Antibody 2 was much slower requiring 10x more time than the other antibodies.

Figure 5.

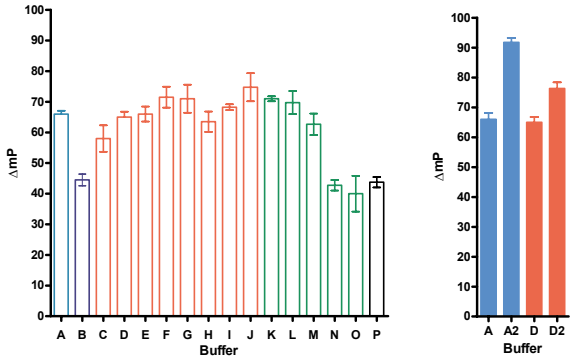
Ab/Tracer complexes are variably affected by individual components in the reaction buffer



An increase in the control mP values was observed with all three antibodies when simple buffer (■) was used in place of the complex buffer (●) at all mock GTP utilization reactions. To explore the sources of these changes common enzyme buffer components were individually introduced into the mixture. $MnCl_2$ had the most profound effect on all complexes causing a general increase in polarization signal at concentrations as low as 0.5 mM. Signal generated with Antibody 1 was the most stable with the remaining test reagents (except >10% DMSO). In addition to DMSO, increasing concentrations of EDTA, $MgCl_2$, and NaCl each altered the raw mP values of reactions prepared with Antibody 2 and to a greater extent with mixtures containing Antibody 3.

Figure 6.

Assay window is enhanced by adding EDTA to the GDP detection mixture



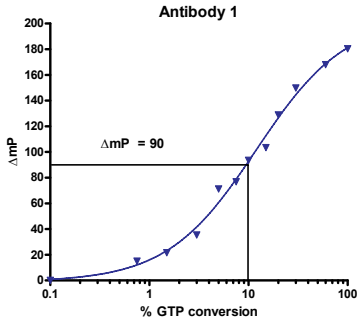
Key reagents in the complex buffer were tested alone and in combination to evaluate their effects on the 10% GTP conversion assay window (Fig. 6A). Assays containing either $MgCl_2$ and/or EDTA displayed higher maximum polarization shifts than those without (Buffer B=simple buffer). Adding the metal-chelating EDTA in the antibody/tracer mixture and including DMSO in the enzyme reaction mix enhanced the polarization shift to > 90 mP units (Fig. 6B). Buffer A2=Buffer A reagents, except EDTA was added later with the Ab/tracer ; D2= Buffer D except EDTA was added later with the Ab/tracer.

A	Hepes, Brij, EDTA, $MgCl_2$, EGTA, DMSO

B	Hepes, Brij	G	EDTA, EGTA	L	$MgCl_2$, DMSO
C	EDTA, $MgCl_2$	H	EDTA, DMSO	M	$MgCl_2$
D	EDTA, EGTA, $MgCl_2$	I	EDTA	N	EGTA, DMSO
E	EDTA, $MgCl_2$, DMSO	J	$MgCl_2$, EGTA, DMSO	O	EGTA
F	EDTA, EGTA, DMSO	K	$MgCl_2$, EGTA	P	DMSO

Figure 7.

10 μM GTP/GDP Standard Curve



Materials and Methods

Reagents, plates, and plate reader settings. Nucleotide conjugates were synthesized at BellBrook Labs and used as immunogens for mouse monoclonal antibody production and tracer synthesis. Antibodies were produced and protein-G purified at Harlan Biosciences (Madison, WI). All assays were performed in black Corning® 384 Well Microplates (Corning, NY). Fluorescence intensity and polarization measurements utilizing the ADP/GDP-Alexa Fluor®633 tracers were performed on a Tecan Safire²™ plate reader using the following filters and settings: 635 nm excitation (LED), 670 nm emission (10 nm bandwidth), 10 flashes per well, 30°C. Data from binding, competition and standard curves were fit to a variable slope sigmoidal dose-response algorithm. EC_{50} and EC_{85} values (the antibody concentration that led to 85% of the signal response) were calculated using GraphPad Prism® (GraphPad Software, San Diego, CA).

Equilibrium binding assays. Antibodies were titrated two-fold in conditions typical of a quenched GTPase reaction (50 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 0.01% v/v Brij-35, 1% v/v DMSO, 20 mM EDTA, 2 nM ADP or GDP-Alexa633 tracer). The maximum assay window (polarization shift) was determined by optimizing the antibody concentration in the presence of a GTP/GDP mixture in ratios representing different points in a GTPase reaction progress curve; (e.g. 0% conversion, 10% conversion = 9 μM GTP/1 μM GDP, 100% conversion). Antibody titrations were performed in 10 μL GDP Detection Buffer (50 mM HEPES (pH 7.5), 40 mM EDTA, and 0.02% BRIJ-35®) containing 4 nM ADP-Alexa633 tracer, followed by the addition of an equal volume of common GTPase buffer (Complex Buffer = 50 mM HEPES (pH 7.5), 4 mM $MgCl_2$, 2 mM EGTA, and 1% DMSO) containing nucleotide. Graphing the difference in polarization values between these mock enzyme reaction antibody binding curves and the 0% conversion (10 μM GTP) curve enables determination of the antibody concentration that yielded the maximal polarization shift ($\Delta\ mP_{max}$), or assay window for each level of GTP conversion. The optimal antibody concentration for a given initial GTP concentration was also determined by calculating the EC_{85} from the 0% GTP conversion binding curve.

Reagent effects on mP signal. Reagents commonly found in antibody binding and GTPase reaction buffers were titrated alone and in combination in simple buffer (50 mM Hepes, pH 7.5 and 0.01% BRIJ-35®) containing different GTP/GDP concentrations. An equal volume of the GDP antibody #1 (30 μg/mL) and 4 nM ADP-Alexa633 tracer in simple buffer was then added (Figure 5). Various combinations of 1 mM EGTA, 20 mM EDTA, 5 mM $MgCl_2$, and 1% DMSO were evaluated for their effects on the assay window representing 10% GTP conversion (Figure 6).

Standard curves. Equal volumes of pre-equilibrated GDP Detection Mixture(4 nM ADP-Alexa633 tracer and 30μg/mL Antibody #1 (2x EC_{85}) in GDP Detection Buffer) was added to GTP/GDP standards prepared in the complex buffer. % GTP Conversion = (μM GDP/ μM GDP+ μM GTP)* 100.

Conclusions

- Co-development of the antibody and tracer was required to identify an optimum antibody/tracer pair for fluorescence polarization immunodetection of GDP.
- Multiple monoclonal antibodies differing in their GDP detection properties (e.g. GDP affinity, selectivity, and equilibration rate) were derived from the same purine immunogen.
- Assay buffer components (namely $MnCl_2$, $MgCl_2$, EDTA, and DMSO) and the order they were added required optimization because they impacted the maximal mP shift at 10% GTP conversion.
- The proof of concept for GDP detection at low % GTP conversion was established with the Transcreener technology providing the framework for future development of an HTS assay.

Funded by SBIR RGS Phase I grant R43NS059082 from the NIH.

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