BellBrook Labs

FAR RED FP

TRANSCREENER
GDP FP Assay

Technical Manual

v042809

Transcreener® GDP FP Assay

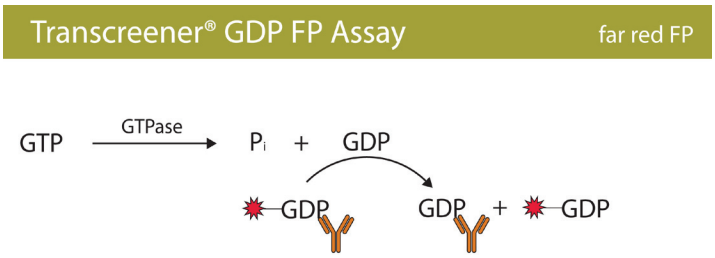
Instructions for Part Numbers 3009-1K and 3009-10K

1.0 Introduction

The Transcreener® HTS Assay platform overcomes the need for time-consuming, one-off assay development for individual members within a group transfer enzyme family by utilizing a single set of assay reagents that detect an invariant product. The generic nature of the Transcreener HTS Assay platform eliminates delays involved in assay development for new HTS targets, and greatly simplifies compound and inhibitor profiling across multiple target families.

The Transcreener® GDP FP Assay is a far-red, competitive fluorescence polarization (FP) assay based on the detection of GDP and therefore is compatible with any enzyme that produces GDP, including GTPases such as CDC42. The Transcreener GDP FP Assay is a simple one step homogenous detection assay, and is extremely flexible with regard to GTP concentration (1 to 1,000 µM GTP). The assay provides excellent signal at low substrate conversion, with a Z′ ≥ 0.7 and ≥85 polarization shift (mP) at 10% GTP conversion using 10 µM GTP.

Figure 1. Transcreener® GDP FP Assay Principle



The Transcreener GDP FP Assay was developed to follow the progress of any enzyme that produces GDP. The Transcreener GDP Detection Mixture comprises a GDP Alexa633 Tracer bound to a GDP Antibody. The tracer is displaced by GDP, the invariant product generated during the enzyme reaction (Figure 1). The displaced tracer freely rotates leading to a decrease in fluorescence polarization. Therefore, GDP production is proportional to a decrease in polarization. The assay uses a far red tracer to minimize interference from fluorescent compounds and light scattering.

2.0 Transcreener® GDP FP Assay Components

Store reagents at -20°C. Individual reagents tolerate 10 freeze-thaw cycles. Sufficient reagents are provided to complete the protocol when using GTP within a range of 1 to 100 µM. Please contact BellBrook Labs for custom packaging for enzyme reactions requiring >100 µM GTP.

GDP Antibody
A concentrated mouse monoclonal GDP Antibody is provided in PBS. The concentration of GDP Antibody needed for an enzyme target is dependent upon the GTP concentration and buffer conditions in the enzyme reaction. To produce the most sensitive and robust assay signal, it is necessary to perform an GDP Antibody titration in the buffer system ideal for your enzyme target.

GDP Alexa633 Tracer, 400 nM
The GDP Alexa633 Tracer, 400 nM is provided in 2 mM HEPES, pH 7.5 containing 0.01% Brij-35. The final tracer concentration in the reaction is 2 nM.

Stop & Detect Buffer B, 10X
The Stop & Detect Buffer B, 10X consists of 200 mM HEPES (pH 7.5), 400 mM EDTA, and 0.2% Brij-35. The 1X Stop & Detect Buffer B components will stop Mg^{2+} or Mn^{2+}-requiring enzyme reactions and aid in the detection and stabilization of the FP signal. Stop & Detect Buffer B is at a 0.5X concentration at the time of polarization measurement. To ensure the enzyme reaction is stopped completely, confirm that the EDTA concentration is at least equimolar to the magnesium ion concentration in the reaction.

5 mM GTP and 5 mM GDP
GTP and GDP are not common to all laboratories and therefore are supplied reagents. These reagents are used to create the GTP/GDP standard curve. It is important to use pure reagents as certain contaminants can affect the assay window.

Materials Required but Not Provided

Ultrapure Water
Some deionized water systems are contaminated with nucleases that can degrade both nucleotide substrates and products, therefore reducing assay performance. Careful handling and use of ultrapure water eliminates this potential problem.

Enzyme Buffer Components
The enzyme buffer components supplied by the end-user include enzyme, enzyme buffer, acceptor substrate, $MgCl_2$ or $MnCl_2$, EGTA, Brij-35, and test compounds. Contact BellBrook Labs Technical Service for suppliers and catalog numbers.

Plate Reader
A multidetection microplate reader configured to measure fluorescence polarization of Alexa Fluor®633 is required. The Transcreener GDP FP Assay has been successfully used on the following instruments: BioTek Synergy™2 and Synergy™4, BMG Labtech PHERAstar and POLARstar, Molecular Devices Analyst (AD, HT, and GT), Perkin Elmer EnVision, ViewLux, Victor²™ V, and Victor ³™ V, and Tecan Ultra, Infinite® F500, Safire²™,

and GENios Pro. Contact BellBrook Labs Technical Service for additional information regarding instrument set-up and fluorescence polarization measurements.

Assay Plates
It is important to use assay plates that are entirely black with a non-binding surface. We recommend Corning® 384 plates (catalog #3676).

Liquid Handling Devices
Use liquid handling devices that can accurately dispense a minimum of 2.5 µL into 384-well plates.

3.0 Protocol

The Transcreener GDP FP Assay is designed around the customer's initial GTP concentration and enzyme buffer conditions. The GDP Detection system must be established before running the enzyme assay. These steps include instrument set-up and antibody optimization. Completing these initial steps will provide optimal GDP detection results and need not be repeated unless assay reaction conditions change.

- Instrument Set Up
- Antibody Titration
- Enzyme Reaction + GTP/GDP Standard Curve
- GDP Detection

Set up GDP Detection System

Instrument Settings
Becoming familiar with ideal instrument settings for fluorescence polarization is essential to the success of the Transcreener GDP FP Assay.

Verify instrument measures fluorescence polarization
Ensure the instrument is capable of measuring fluorescence polarization (not simply fluorescence intensity) of Alexa Fluor®633. Please call BellBrook Labs Technical Service if you have questions about settings and filter sets for a specific instrument.

Define the maximum mP window for your instrument
Measuring high (tracer + antibody) and low (free tracer) polarization will define the maximum assay window of your specific instrument.

High polarization mixture
Prepare 20 µg/mL GDP Antibody in 2 nM GDP Alexa633 Tracer/0.5X Stop & Detect Buffer B.

Low polarization mixture
Prepare 2 nM GDP Alexa633 Tracer/0.5X Stop & Detect Buffer B without GDP Antibody.

Measure the fluorescence polarization
The difference between the low and high polarization values should be >175 mP. If the assay window is <175 mP, please call BellBrook Labs Technical Service.

Optimize GDP Antibody Concentration

The Transcreener GDP FP Assay requires detection of GDP in the presence of excess GTP (assuming initial velocity enzyme reaction conditions) using an antibody with a finite selectivity for the diphosphate vs. the triphosphate. The concentration of GDP Antibody determines the total assay window and the GDP detection range, and the amount needed is dependent upon the GTP concentration in the enzyme reaction. To produce the most sensitive and robust assay signal, it is necessary to perform a GDP Antibody titration in the buffer system ideal for your enzyme target.

To determine the optimal antibody concentration, titrate the GDP Antibody using the reaction conditions for your enzyme or drug target. The nucleotide substrate concentration in the enzyme reaction generally determines the appropriate concentration of GDP Antibody. We recommend using the EC_{85} concentration of antibody.

Titrate GDP Antibody in 1X Stop & Detect Buffer B

Prepare 4 nM GDP Alexa633 Tracer in 1X Stop & Detect Buffer B with and without GDP Antibody (1 mg/mL). Dispense 20 µL of mixture (with antibody) into wells in column 1. Dispense 10 µL of the mixture (without antibody) across a 384-well plate (columns 2-24). Remove 10 µL from column 1 and serially titrate the contents across the plate (to column 24).

Add Enzyme Reaction Buffer (containing GTP)

Prepare your enzyme reaction mixture (include substrate and GTP, but omit enzyme) and add 10 µL to the titrated antibody. Mix the plate, equilibrate at room temperature (1 hour), and measure fluorescence polarization.

GDP Detection Controls

These controls are used to calibrate the fluorescence polarization plate reader controls and are added to wells that do not contain enzyme.

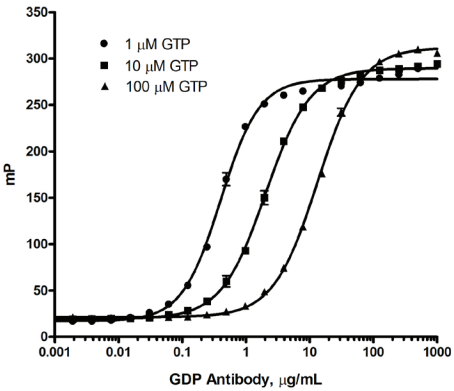
> ***Without Antibody (free tracer) Control***
> This sample contains the GDP Alexa633 Tracer without the GDP Antibody and is set to 20 mP.
>
> ***Without Tracer Control***
> This sample contains the GDP Antibody without the GDP Alexa633 Tracer and is used as a sample blank for all wells. It is used as a 'buffer blank', but contains the same GDP Antibody concentration used in all wells.

Plot mP vs. log of GDP Antibody concentration and calculate the EC_{85}

The antibody concentration at the EC_{85} is often used as a good compromise between sensitivity and maximal polarization value. The EC_{85} is determined by inputting the EC_{50} and hillslope values from a sigmoidal dose response curve fit into the equation below.

$$EC_{85} = ((85/(100-85))^{1/hillslope}) * EC_{50}$$

Figure 2. GDP Antibody Titrations at Various GTP Concentrations

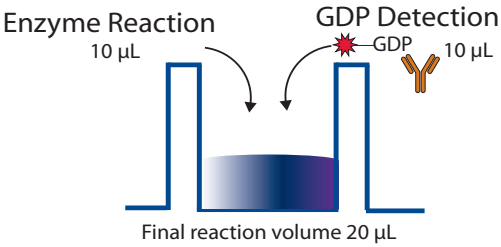


The final 20 µL assay volume consists of 2 nM GDP Alexa633 Tracer , 0.5X Stop & Detect Buffer B, and 0.5X enzyme reaction mixture, which was 25 mM HEPES (pH 7.5), 2 mM $MgCl_2$, 1 mM EGTA, 0.5% DMSO, 0.01% Brij-35, 20 mM EDTA, GTP, and GDP Antibody (n=3).

Detect GDP in an Enzyme Reaction

The Transcreener GDP FP Assay is a universal biochemical assay designed for enzymes that produce GDP. In the one-step detection protocol, 10 µL GDP Detection Mixture is added to the 10 µL enzyme reaction then mixed and incubated for 1 hour. The enzyme reaction components (including GTP) and the GDP Detection Mixture are 0.5X in the final 20 µL.

Figure 3. Protocol



Enzyme Reaction (10 µL)

Add the enzyme reaction mixture to test compounds and mix on plate shaker. Start the reaction by adding GTP and mix. Incubate at temperature and time ideal for enzyme target before addition of the GDP Detection Mixture.

GDP Detection Mixture (10 µL)

The 1X GDP Detection Mixture is prepared by adding GDP Antibody (2*EC_{85}) and 4nM GDP Alexa633 Tracer in 1X Stop & Detect Buffer B. Add the 1X GDP Detection Mixture and mix on plate shaker. Incubate at room temperature (20-25°C) for 1 hour, and measure fluorescence polarization.

Enzyme Reaction Conditions

Choose enzyme buffer conditions and GDP Detection Mixture that are ideal for your enzyme target. Perform your enzymatic reaction at its requisite temperature and optimal duration. An enzyme titration can be performed to identify the optimal enzyme concentration for the Transcreener GDP FP Assay. Quality control of the Transcreener GDP FP Assay components is performed in 50 mM HEPES (pH 7.5), 4 mM $MgCl_2$, 2 mM EGTA, 1% DMSO (test compound solvent), 0.01% Brij-35 and varying GTP concentrations (1 to 1000 µM).

Enzyme Assay Controls

The enzyme reaction controls define the limits of the enzyme assay.

0% GTP Conversion Control

This control consists of the GDP Detection Mixture, the enzyme reaction components (without enzyme), and 100% GTP (0% GDP). This control defines the upper limit of the assay window.

100% GTP Conversion Control

This control consists of the GDP Detection Mixture, the enzyme reaction components (without enzyme) and 100% GDP (0% GTP). This control defines the lower limit of the assay window.

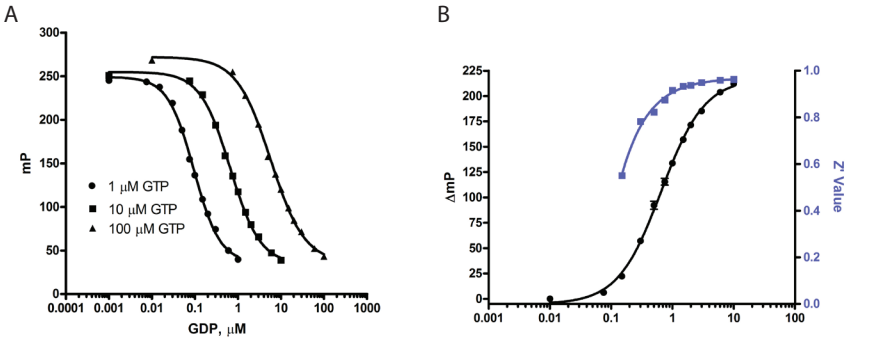
Without Nucleotide Control

To verify that the enzyme does not interfere with the detection module, perform an enzyme titration in the absence of nucleotide (i.e. GTP) and acceptor substrate.

GDP/GTP Standard Curve

The standard curve mimics an enzyme reaction (as GTP concentration decreases, GDP concentration increases); the guanine concentration remains constant. The GDP/GTP standard curve allows calculation of the concentration of GDP produced in the enzyme reaction and therefore the % GTP consumed (% GTP conversion). Prepare a twelve-point curve using concentrations of GDP and GTP corresponding to 0%, 2%, 4%, 6%, 8%, 10%, 15%, 20%, 30%, 40%, 60%, and 100% GTP conversion.

Figure 4. GDP/GTP Standard Curves



A) Sample data for 1 µM, 10 µM, and 100 µM GDP/GTP standard curves. The nucleotide concentration reflects the amount in the enzyme reaction, prior to the addition of the GDP Detection Mixture. Curves are shown in a final 20 µL assay volume consisting of 50 mM HEPES (pH 7.5), 2 mM $MgCl_2$, 1 mM EGTA, 0.5% DMSO, 0.01% Brij-35, 20 mM EDTA, 2 nM GDP Alexa633 Tracer, GDP/GTP standards, and GDP Antibody (EC_{85} concentration) (n=24). The data are plotted as mP vs log [GDP] using four-parameter nonlinear regression curve fitting. Alternatively a two-phase exponential decay and nonlinear regression can be used to present the data (GraphPad Prism). B) Excellent Z' values are obtained at <10% GTP conversion for the 10 µM GTP standard curve.

$$\Delta mP = mP_{\text{initial [GTP]}} - mP_{\text{sample}}$$

and

$$Z' = 1 - [(3*SD_{\text{initial [GTP]}} + 3*SD_{\text{sample}})/|(mP_{\text{initial [GTP]}} - mP_{\text{sample}})|]$$

Endpoint Assay

The Transcreener GDP FP Assay is designed for endpoint readout. The Stop & Detect Buffer B, 10X contains EDTA to stabilize the signal. EDTA stops the enzyme reaction by chelating available Mg^{2+} or Mn^{2+}, which is required for enzyme turnover. The activity of some GTPase enzymes that produce GDP but do not require metal ions will note be stopped with the addition of the buffer provided.

Realtime Assay

The end-user may perform real-time experiments by substituting the Stop & Detect Buffer B, 10X (provided) with a detection buffer that does not contain EDTA. Note that the optimal GDP Antibody concentration may change when EDTA is omitted. Note: the equilibrium rate for the GDP detection reagents is about 1 hour, therefore GDP formation is not recommended for exact quantitation of realtime assays.

4.0 Reagent and Signal Stability

The Transcreener technology provides the end-user with a robust and stable assay method to detect GDP.

Signal Stability

The stability of the mP assay window at 10% substrate conversion was determined after the addition of the GDP Detection Mixture to the GDP/GTP standard samples. The mP assay window at 10% substrate conversion (10 µM GTP) remained constant (< 10% change) for at least 24 hours at room temperature (20-25°C). If plates are to be read the following day, they should first be sealed to prevent evaporation.

GDP Detection Mixture Stability

The GDP Detection Mixture is stable for at least 24 hours at room temperature (20-25°C) before adding to the enzyme reaction (i.e. stored on the liquid handling deck).

Solvent Compatibility

The mP assay window at 10% substrate conversion (10 µM GTP) remains constant (< 10% change) when up to 10% DMSO, ethanol, or acetonitrile are used in the enzyme reaction. Contact BellBrook Labs Technical Service for further reagent compatibility information.