

TRANSCREENER[®]
GDP TR-FRET Red
Assay
Technical Manual



Transcreener® GDP TR-FRET Red Assay

Instructions for Part Numbers 3021-1K and 3021-10K

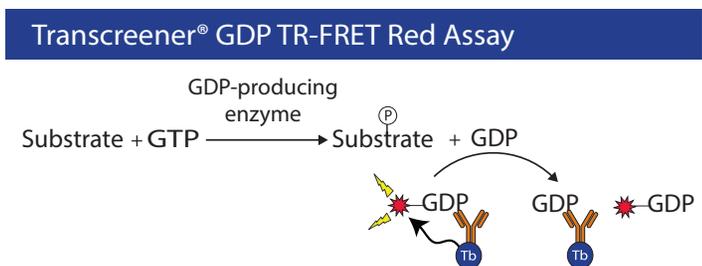
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1.0 Introduction

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 TR-FRET Red Assay is a competitive immunoassay for GDP with a far-red, time-resolved Förster-resonance-energy-transfer (TR-FRET) readout. It is designed specifically for HTS with a single addition mix-and-read format, reagent stability, and compatibility with commonly used multimode plate readers. Because it is highly selective for GDP, the assay can be used with any enzyme that converts GTP to GDP, Like GTPases, Fucosyltransferases, Etc. The assay can accommodate GTP concentrations ranging from 0.1 to 1,000 μ M and provides excellent data quality (Z' > 0.7) at low substrate conversion (typically 10-30%).

Figure 1. Transcreener® GDP TR-FRET Red Assay Principle



The Transcreener GDP Detection Mixture comprises an GDP HiLyte647 Tracer bound to an GDP Antibody-Tb conjugate. Excitation of the terbium complex in the UV range (ca. 330 nm) results in energy transfer to the tracer and emission at a higher wavelength (665nm) after a time delay. GDP produced by the target enzyme displaces the tracer which causes a decrease in TR-FRET. The time gated nature of the detection method largely eliminates interference that can result from prompt fluorescence

of test compounds. Use of a far red tracer further minimizes interference from fluorescent compounds and light scattering.

2.0 Transcreener® GDP TR-FRET Red Assay Components

Store reagents at -80°C. Sufficient reagents are provided to complete up to 1,000 assays with 3021-1K and 10,000 assays with 3021-10K; the exact number is dependent on your enzyme reaction conditions.

GDP HiLyte647 Tracer

A 10 μ M solution of GDP HiLyte647 Tracer is provided in 2 mM HEPES, pH 7.5 containing 0.01% Brij-35. The concentration of GDP HiLyte647 Tracer needed for an enzyme target is dependent upon the GTP concentration and buffer conditions in the enzyme reaction. In section 3.1, a linear relationship is illustrated between [GTP] in the enzyme reaction and the [GDP HiLyte647 Tracer] required for GDP detection. Sufficient tracer is included in the kit to complete 1,000 assays with 3021-1K and 10,000 assays with 3021-10K at a GTP concentration up to 100 μ M GTP. Please contact BellBrook Labs for custom packaging for enzyme reactions using > 100 μ M GTP.

GDP Antibody-Terbium Conjugate, 800 nM

The GDP Antibody-Tb, 800 nM is provided in HEPES buffered saline. The final antibody concentration in the reaction is 4 nM.

Stop & Detect Buffer C, 10X

The Stop & Detect Buffer C, 10X consists of 500 mM HEPES (pH 7.5), 200 mM EDTA, and 0.2% Brij-35. The Stop & Detect Buffer C components will stop Mg^{2+} -requiring enzyme reactions. To ensure the enzyme reaction is stopped completely, confirm that the EDTA concentration is at least equimolar to the magnesium ion concentration in the enzyme reaction. (Stop & Detect Buffer C is at a 0.5X concentration at the time of FRET measurement.)

5 mM GTP

GTP is a common reagent in many laboratories; however, it is imperative that a highly purified preparation be used for the Transcreener® GDP Assay. If the GTP stock contains impurities, such as GDP, the assay window will be compromised. The GTP supplied in this kit can be used for the enzyme reaction and to create an GDP/GTP standard curve, if desired. For additional quantities, pricing and alternate supplier information, please contact BellBrook Labs.

5 mM GDP

GDP is used to create the GDP/GTP standard curve.

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

Transcreener® GDP Assays are designed for use with purified enzyme preparations. Contaminating enzymes such as phosphatases or nucleotidases can produce background signal and reduce the assay window.

Enzyme Buffer Components

The enzyme buffer components supplied by the end-user include enzyme, buffer, acceptor substrate, MgCl₂ or MnCl₂, EGTA, Brij-35, and test compounds. Contact BellBrook Labs Technical Service for suppliers and catalog numbers.

Plate Reader

A microplate reader configured to measure time-resolved Förster-resonance-energy-transfer (TR-FRET) of the Tb:HiLyte647 donor:acceptor pair is required. This assay has been designed to provide high quality data on any HTS qualified instrument configured to measure TR-FRET using standard Europium or Terbium complexes measuring emissions at 615 nm and 665 nm. Validation was completed using BMG LABTECH's PHERAstar Plus (Ex337/Em620/Em665) and Perkin Elmer's Envision (Ex320/Em615/Em665). Contact BellBrook Labs Technical Service for additional information regarding instrument set-up and TR-FRET measurements.

Assay Plates

It is important to use assay plates that are entirely white with a non-binding surface. We recommend Corning® 384 plates (catalog #3673).

Liquid Handling Devices

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

3.0 EZ Protocol

The Transcreener GDP TR-FRET Red Assay is a universal biochemical assay designed for enzymes that produce GDP. It is designed around your initial GTP concentration and enzyme buffer conditions. There are four steps to complete:

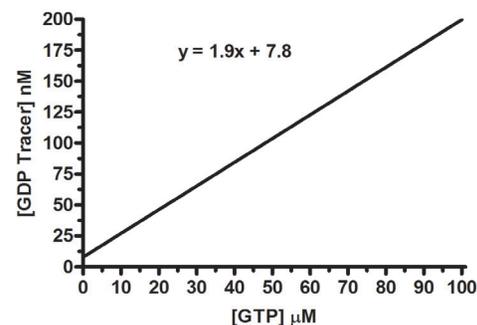
1. Determine antibody concentration
2. Instrument set-up
3. Enzyme titration
4. Detect GDP

Completing these steps will provide optimal GDP detection results.

3.1 Determine the GDP HiLyte647 Tracer Concentration

The Transcreener GDP TR-FRET Red 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 HiLyte647 tracer determines the total assay window and the GDP detection range, and the amount needed is primarily dependent upon the GTP concentration in the enzyme reaction.

Figure 2. Linear Relationship between [GTP] and [GDP Tracer]



- Determine the GDP HiLyte647 concentration.** As shown in Figure 2, the relationship between [GTP] and [GDP HiLyte647 Tracer] is linear. (Though shown for 0.1 µM to 100 µM GTP; the relationship is valid to 1,000 µM GTP.) Therefore the quantity of GDP HiLyte647 Tracer for enzyme reactions that use between 0.1 µM and 1,000 µM GTP can be determined using the equation $y = mx + b$, where $x = [\text{GTP}]$ (µM) in the 10 µL enzyme reaction, $y = [\text{GDP HiLyte647 Tracer}]$ (nM) in the 10 µL of 1X GDP Detection Mixture, m (slope) = 1.9, and b (y-intercept) = 7.8. We recommend a final volume of 20 µL.
- Sample GDP Detection Mixture.** For example, if you are using 3 µM GTP in a 10 µL enzyme reaction, the optimal GDP HiLyte Tracer concentration in the 1X GDP Detection Mixture (assuming 10 µL of GDP Detection Mixture added to each 10 µL enzyme reaction) would be $[1.9 \times 3] + 7.8 = 13.6$ nM.
- Further Tracer optimization (if necessary).** Determining your GDP HiLyte647 Tracer concentration using this equation will provide excellent results for most assay conditions. If it does not provide the results you require, simply optimize the tracer concentration in a step-wise fashion using the GDP HiLyte647 Tracer concentration (X) from the line as a starting point. Try performing a standard curve (described later) at 0.5x[Y], [Y], and 1.5x[Y] tracer concentrations to find an assay window that suits your needs. Please contact BellBrook Labs technical support for other suggestions.

3.2. Instrument Set-up

Becoming familiar with ideal instrument settings for fluorescence polarization is essential to the success of the Transcreener GDP TR-FRET Red Assay.

Verify Instrument Measures TR-FRET

Ensure the instrument is capable of measuring TR-FRET (not simply fluorescence intensity) of the terbium:HiLyte647 TR-FRET pair (Ex₃₂₀/Em₆₁₅/Em₆₆₅). Please call BellBrook Labs Technical Service if you have questions about settings and filter sets for a specific instrument.

Define the Maximum TR-FRET Window for Your Instrument

Measuring high (0% GTP conversion) and low (100% GTP conversion) FRET will define the maximum assay window of your specific instrument. Prepare High and Low FRET Mixtures below in quantities sufficient to perform at least 6 replicates for each condition.

High FRET Mixture

Prepare a solution containing: 4 nM GDP² Antibody-Tb, 0.5X Stop & Detect Buffer C, GDP HiLyte647 Tracer, and GTP. Use GTP at half the concentration present in your enzyme reaction and GDP HiLyte647 Tracer at half the concentration calculated using the equation in Figure 2. This mimics the 2-fold dilution when adding an equal volume of detection mixture to an enzyme reaction.

Low FRET Mixture

Prepare the same mix as used for High FRET Mixture substituting GDP for GTP.

Measure the Time-Resolved Förster-Resonance-Energy-Transfer

Calculate the Z'-Factor using the equation below; values greater than 0.7 are acceptable. Contact BellBrook Labs Technical Service for assistance if your calculated Z'-Factor is less than 0.7.

$$Z' = 1 - \frac{[(3 * SD_{\text{High FRET Mixture}}) + 3 * SD_{\text{Low FRET Mixture}}]}{[\text{mean of High FRET Mixture ratio } 665/615] - [\text{mean of Low FRET Mixture ratio } 665/615]}$$

3.3 Enzyme Titration

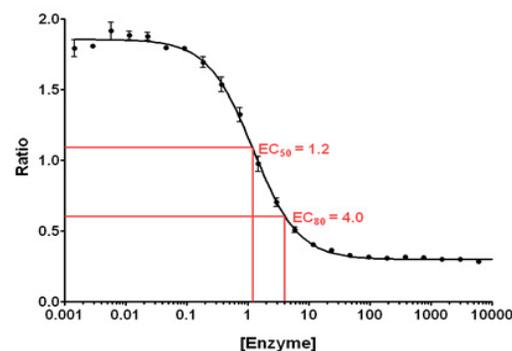
An enzyme titration is performed to identify the optimal enzyme concentration for the Transcreeper GDP TR-FRET Red Assay. Use enzyme buffer conditions, substrate, and GTP concentrations that are optimal for your target enzyme and GDP HiLyte647 Tracer concentration calculated as described in Figure 2. If a compound screen is planned, you should include the library solvent at its final assay concentration. We routinely use enzyme buffer containing 35 mM HEPES (pH 7.5), 4 mM MgCl₂, 2mM EGTA, 1% DMSO (test compound solvent), 0.015% Brij-35 and GTP. Run your enzymatic reaction at its requisite temperature and time period.

Enzyme Titration Steps

To achieve the most robust assay and a large signal, the quantity of enzyme required to produce a 50% - 80% change in FRET signal is ideal (EC₅₀ to EC₈₀) for screening of large compound libraries and generating inhibitor dose response curves. To determine the EC₈₀ [enzyme] use the equation below.

$$EC_{80} = (80 / (100 - 80))^{(1 / \text{hillslope})} * EC_{50}$$

Figure 3. Enzyme Titration Graph



Enzyme Assay Controls

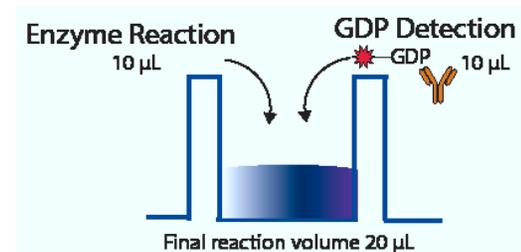
The enzyme reaction controls define the limits of the enzyme assay.

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) or acceptor substrate.

GDP/GTP Standard Curve

Although optional, an GDP/GTP standard curve can be useful to ensure day to day reproducibility and that the assay conditions were performed using initial rates, in addition to being used to calculate inhibitor IC₅₀ values. See Appendix for a description of how to run the standard curve.

Background Control

This control contains 0.5X enzyme reaction conditions and Stop and Detect Buffer C.

3.4 GDP Detection

The detection protocol is a single step as shown in Figure 4. 10 µL GDP Detection Mixture is added to the 10 µL enzyme reaction then mixed and incubated for 90 minutes. The enzyme reaction components (including GTP) and the GDP Detection Mixture are 0.5X in the final 20 µL.

Figure 4. Detection 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-Tb and GDP HiLyte647 Tracer to Stop & Detect Buffer C. Final concentrations should be 8nM GDP² Antibody-Tb Tracer, 1X Stop & Detect Buffer C, and the tracer concentration calculated using the equation in Figure 2. Add an equal volume of 1X GDP Detection Mixture to the enzyme reaction and mix using a plate shaker. Incubate at room temperature (20-25°C) for 90 minutes, and measure TR-FRET.

GDP Detection Controls

This control is used to verify background fluorescent levels are negligible and, if required, for subtraction of background signal.

Endpoint Assay

The Transcreeper GDP TR-FRET Red Assay is designed for endpoint readout. The Stop & Detect Buffer C contains EDTA to stop Mg⁺⁺ dependent enzyme reactions by chelating available Mg⁺⁺.

Real-time Assay

The end-user may perform real-time experiments by adding GDP Antibody-Tb and GDP HiLyte647 Tracer directly to an enzyme reaction. GDP detection equilibration time is greater than 30 minutes, making it difficult to quantify GDP produced during short term enzyme reactions, however this method can provide insight into optimal enzyme concentration and incubation time. Note that the optimal GDP HiLyte647 Tracer concentration may change when EDTA is omitted.

4.0 Reagent and Signal Stability

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

Signal Stability

The stability of the TR-FRET ratio assay window at 10% substrate conversion was determined after the addition of the GDP Detection Mixture to the standard samples. The ratio assay window at 10% substrate conversion (10 µM) remained constant (< 10% change) at least overnight at room temperature (20-25°C). If plates are to be read the following day, steps should be taken to prevent evaporation.

GDP Detection Mixture Stability

The GDP Detection Mixture is stable for at least 8 hours at room temperature (20-25°C) before addition to the enzyme reaction (i.e. stored on the liquid handling deck).

Solvent Compatibility

The assay window at 10% substrate conversion (10 µM GTP) remains constant (< 10% change) when up to 3% DMSO, 6.25% EtOH, 5% triton-X-100, 1% Brij 35, 300mM NaCl, 0.5mg/mL BSA are used in the enzyme reaction. Contact BellBrook Labs for further reagent compatibility information.

5.0 References

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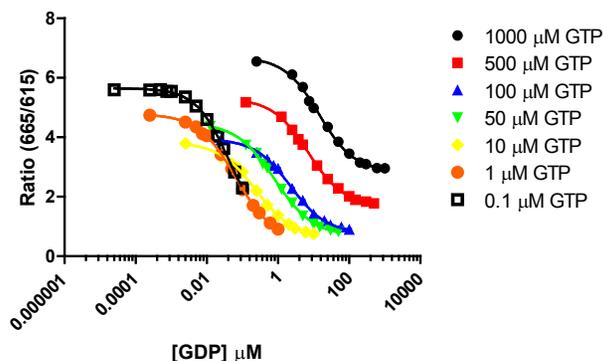
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6.0 Appendix

Standard Curve for Conversion of GTP to GDP

The standard curve mimics an enzyme reaction (as GTP concentration decreases, GDP concentration increases); the guanine nucleotide concentration remains constant. The GDP/GTP standard curve allows calculation of the concentration of GDP produced in the enzyme reaction (% GTP conversion). In this case a 16-point standard curve was prepared using concentrations of GDP and GTP corresponding to 0, 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, 10.0, 20, 30, 60, and 100% GTP conversion. Commonly 8 to 12 point standard curves are used.

Figure 6. GDP/GTP Standard Curves



	0.1 μM Std. Curve		1.0 μM Std. Curve		10 μM Std. Curve		100 μM Std. Curve		1000 μM Std. Curve	
	Z' at 30% Conversion	LLD (nM)	Z' at 10% Conversion	LLD (nM)						
GTP/GDP	0.7	10	0.8	7.5	0.8	75	0.8	500	0.8	7500

A) Sample data for 0.1 μM, 1 μM, 10 μM, 100 μM, and 1000 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 obtained in a final 20 μL assay volume consisting of 25mM Tris, 2.5 mM MgCl, 0.5 mM EDTA, 0.5% DMSO, 0.005% Brij-35, 4 nM GDP Antibody-Tb, GDP/GTP standards, and GDP HiLyte647 Tracer (Tracer concentration from equation Figure 2) (n= 6-12). The data are plotted as FRET ratio 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) Z' values for initial velocity detection (10% conversion for GTP/GDP standard curves and 30% for 0.1 μM and lower limits of detection. *LLD= Lower Limit of Detection, defined as the concentration of GDP that generates Z' > 0.

$$\Delta \text{ratio} = \text{ratio}_{\text{initial [ATP]}} - \text{ratio}_{\text{sample}}$$

and

$$Z' = 1 - \frac{[(3 * SD_{\text{initial [ATP]}} + 3 * SD_{\text{sample}}) / (\text{ratio}_{\text{initial [ATP]}} - \text{ratio}_{\text{sample}})]}{1}$$

U.S. Patent 7,332,278, 7,355,010 and 7,378,505 issued. U.S. Patent Application Nos. 11/353,500, 11/958,515 and 11/958,965, U.S. Divisional Application 12/029,932, and International Patent Application Nos. PCT/US07/088111, European Application Nos. 04706975.2 and 05785285.7, Canadian Application 2,514,877, and Japanese Application 2006-503179 applied. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes other than use of the product or its components to provide a service, information, or data. Commercial Purposes means any activity by a party for consideration other than use of the product or its components to provide a service, information, or data and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (3) resale of the product or its components, whether or not such product or its components are resold for use in research. BellBrook Labs LLC will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use, or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, BellBrook Labs LLC is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, BellBrook Labs LLC, 5500 Nobel Drive, Suite 230, Madison, Wisconsin 53711. Phone (608)443-2400. Fax (608)441-2967.

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