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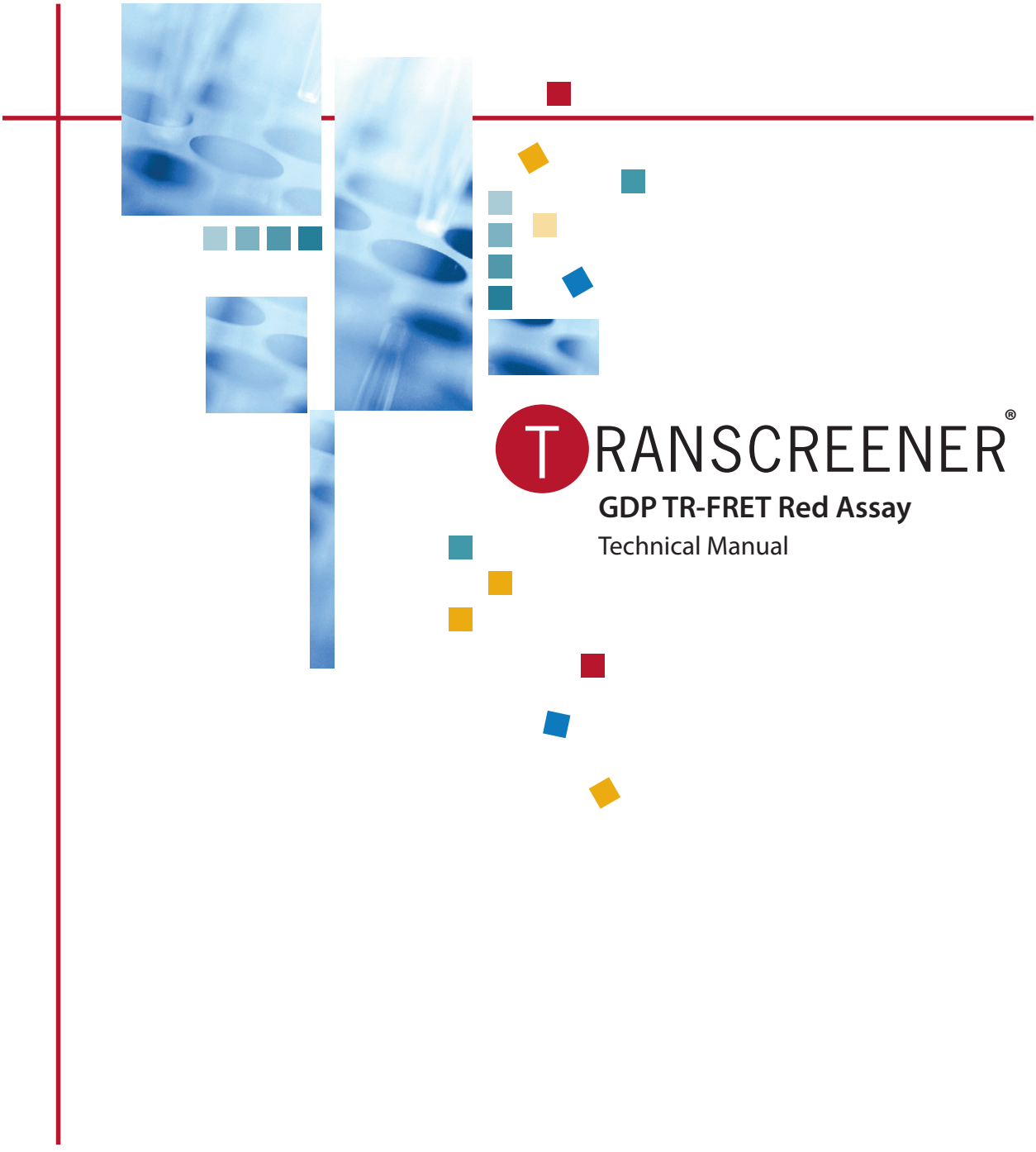
BellBrook Labs

FAR RED TR-FRET



TRANSCREENER®

GDP TR-FRET Red Assay

Technical Manual

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

TM#125 • Rev 2017-06

Transcreener® GDP TR-FRET Red Assay Technical Manual

Contents

1.0 Introduction

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. Because it is highly selective for GDP, the assay can be used with any enzyme that converts GTP to GDP, regardless of what other substrates are used. Examples of enzymes include GTPases and fucosyltransferases.

The Transcreener® assay is designed specifically for high-throughput screening (HTS), with a single-addition, mix-and-read format. It offers reagent stability and compatibility with commonly used multimode plate readers. 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 provides the following benefits:

- Accommodates GTP concentrations ranging from 0.1 µM to 1,000 µM.
- Excellent data quality (Z' ≥ 0.7) at low substrate conversion (typically 10–30%).
- Overcomes the need for time-consuming, one-off assay development for individual members within a group transfer enzyme family by using a single set of assay reagents that detect an invariant product.
- Time-gated detection method largely eliminates interference that can result from prompt fluorescence of test compounds.
- Far-red tracer further minimizes interference from fluorescent compounds and light scattering.

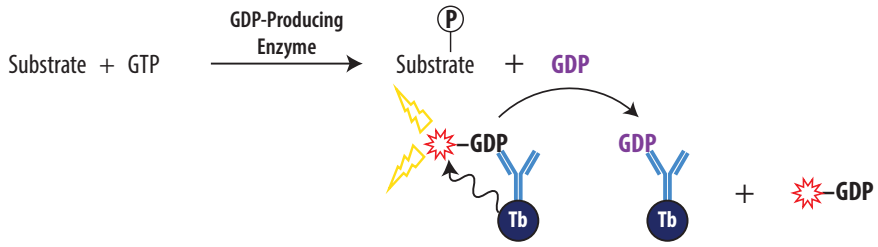


Figure 1. Schematic overview of the Transcreener® GDP TR-FRET Red Assay. The Transcreener® GDP Detection Mixture contains a GDP HiLyte647 tracer bound to a GDP antibody conjugated to terbium (Tb). Excitation of the Tb complex in the UV range (~330 nm) results in energy transfer to the tracer and emission 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.

2.0 Product Specifications

Product	Quantity	Part #
Transcreener® GDP TR-FRET Red Assay	1,000 assays*	3011-1K
	10,000 assays*	3011-10K

*The exact number of assays depends on enzyme reaction conditions. The kits are designed for use with 384-well plates, using 20 µL reaction volumes.

Storage

Store all reagents at –80°C upon receipt.

2.1 Materials Provided

Component	Composition	Notes
GDP HiLyte647 Tracer	10 µM solution in 2 mM HEPES (pH 7.5) containing 0.01% Brij-35	The concentration of GDP HiLyte647 Tracer needed for an enzyme target depends upon the GTP concentration and buffer conditions in the enzyme reaction (see Section 4.2). Sufficient tracer is included in the kit to complete 1,000 assays (Part # 3021-1K) or 10,000 assays (Part # 3021-10K) at a GTP concentration up to 100 µM GTP.
GDP Antibody-Terbium Conjugate	800 nM solution in HEPES-buffered saline	The final antibody concentration in the reaction is 4 nM in a 20 µL final reaction volume.
Stop & Detect Buffer C, 10X	500 mM HEPES (pH 7.5), 200 mM EDTA, and 0.2% Brij-35	The Stop & Detect Buffer C components will stop enzyme reactions that require Mg^{2+}. To ensure that the enzyme reaction is stopped completely, confirm that the EDTA concentration is at least equimolar to the magnesium ion concentration in the reaction. The final concentration of Stop & Detect Buffer C at the time of FRET measurement is 0.5X.
GTP	5 mM	The GTP supplied in this kit can be used for the enzyme reaction and to create a GDP/GTP standard curve, if desired.
GDP	5 mM	GDP is used to create the GDP/GTP standard curve.

Caution: GTP is a common reagent in many laboratories; however, it is imperative that a highly purified preparation be used for the Transcreener® assay. If the GTP stock contains impurities, such as GDP, the assay window will be compromised.

2.2 Materials Required but Not Provided

- ***Ultrapure Water***—Some deionized water systems are contaminated with nucleases that can degrade both nucleotide substrates and products, 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***—User-supplied enzyme buffer components include enzyme, buffer, acceptor substrate, $MgCl_2$ or $MnCl_2$, EGTA, Brij-35, and test compounds.
- ***Plate Reader***—A microplate reader configured to measure 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 with emission wavelengths at 615 nm and 665 nm. Validation was completed using PHERAstar Plus $Ex_{337}/Em_{620}/Em_{665}$ (BMG LABTECH) and Envision $Ex_{320}/ Em_{615}/Em_{665}$ (Perkin Elmer).
- ***Assay Plates***—It is important to use assay plates that are entirely white with a nonbinding surface. We recommend Corning® 384-well plates (Cat. # 4513).
- ***Liquid Handling Devices***—Use liquid handling devices that can accurately dispense a minimum volume of 2.5 µL into 384-well plates.

Note: Contact BellBrook Labs Technical Service for suppliers and catalog numbers for buffer components, and additional information regarding setup of TR-FRET instruments.

3.0 Before You Begin

1. Read the entire protocol and note any reagents or equipment needed (see **Section 2.2**).
2. Check the TR-FRET instrument and verify that it is compatible with the assay being performed (see **Section 4.1**).

4.0 Protocol

The Transcreener® GDP TR-FRET Red Assay protocol consists of 4 steps (**Figure 2**). The protocol was developed for a 384-well format, using a 10 µL enzyme reaction and 20 µL final volume at the time that the plates are read. The use of different densities or reaction volumes will require changes in reagent quantities.

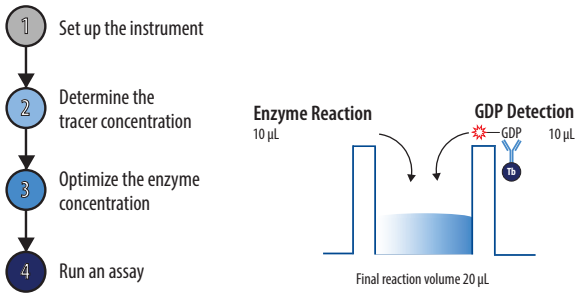


Figure 2. An outline of the procedure. The assay consists of 4 main steps with a mix-and-read format.

4.1 Set Up the Instrument

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

4.1.1 Verify That the Instrument Measures TR-FRET

Ensure that the instrument is capable of measuring TR-FRET (not simply fluorescence intensity) of the terbium:HiLyte647 TR-FRET pair ($Ex_{320}/Em_{615}/Em_{665}$).

Note: A complete list of instruments and instrument-specific application notes can be found online at: https://www.bellbrooklabs.com/technical-resources/instrument-compatibility Contact BellBrook Labs Technical Service if you have questions about settings and filter sets for a specific instrument.

4.1.2 Define the Maximum TR-FRET Window for the 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 in quantities sufficient to perform at least 6 replicates for each condition.

Use GTP and GDP HiLyte647 Tracer at 0.5X concentration in a 20 µL final reaction volume. This mimics the 2-fold dilution when adding an equal volume of detection mixture to an enzyme reaction. As an example, the 1X detection mixture may contain 10 µM GTP. After adding this to the enzyme reaction, the concentration in the final 20 µL reaction volume would be 5 µM.

High FRET Mixture

Prepare the following solution:

Component	Stock Concentration	Final Concentration	Example: 25 Assays	Your Numbers
GDP Antibody-Tb	800 nM	4 nM	2.5 µL	
10X Stop & Detect Buffer C	10X	0.5X	25.0 µL	
GDP HiLyte647 Tracer	10 µM	13.4 nM	0.7 µL	
GTP	5 mM	5 µM	0.5 µL	
Water			471.3 µL	
Total			**500.0 µL**	

The assay window will depend upon your initial GTP concentration. These volumes can be adjusted for fewer assays and different GTP concentrations.

Low FRET Mixture

Prepare the following solution:

Component	Stock Concentration	Final Concentration	Example: 25 Assays	Your Numbers
GDP Antibody-Tb	800 nM	4 nM	2.5 µL	
10X Stop & Detect Buffer C	10X	0.5X	25.0 µL	
GDP HiLyte647 Tracer	10 µM	15 nM	0.7 µL	
GDP	5 mM	5 µM	0.5 µL	
Water			471.3 µL	
Total			**500.0 µL**	

The assay window will depend upon your initial GDP concentration. These volumes can be adjusted for fewer assays and different GDP concentrations.

4.1.3 Measure the TR-FRET

Test the Z′ factor and assay window on your instrument by adding 20 µL of the Low FRET Mixture in 16 wells and 20 µL of High FRET Mixture in 16 wells. Calculate the Z′ factor using the equation below; values greater than 0.7 are acceptable.

Caution: Contact BellBrook Labs Technical Service for assistance if the calculated Z′ factor is less than 0.7.

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

4.2 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; the amount needed primarily depends upon the GTP concentration in the enzyme reaction.

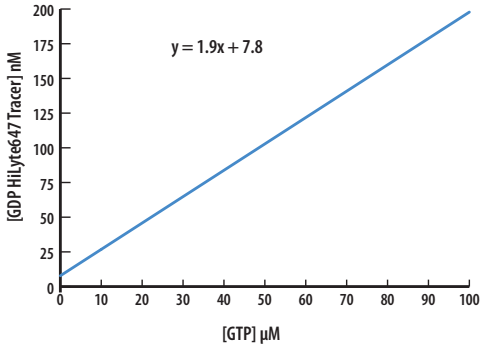


Figure 3. Linear relationship between [GTP] and [GDP Tracer]. The tracer concentration can be calculated using the equation: **y = 1.9x + 7.8**

4.2.1 Calculating the Tracer Amount

As shown in **Figure 3**, the relationship between GTP and GDP HiLyte647 Tracer concentrations is linear. (Though shown for 0.1–100 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** = [GTP] (µM) in the 10 µL enzyme reaction, **y** = [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 reaction volume of 20 µL.

For example, if you are using 3 µM GTP in a 10 µL enzyme reaction, the optimal GDP HiLyte647 Tracer concentration in the 1X GDP Detection Mixture (assuming 10 µL of GDP Detection Mixture was added to each 10 µL enzyme reaction) would be (1.9 × 3) + 7.8 = 13.6 nM.

4.2.2 Optimizing the Tracer Concentration

Using the GDP HiLyte647 Tracer concentration calculated using the equation in **Figure 3** will produce excellent results for most users. If it does not produce the results you require, simply optimize the tracer concentration in a stepwise fashion using the GDP HiLyte647 Tracer concentration (X) from the line as a starting point. Try performing a standard curve (see **Section 7.1**) at 0.5 × [Y], [Y], and 1.5 × [Y] tracer concentrations to find an assay window that suits your needs. See **Section 6** for troubleshooting suggestions.

4.3 Optimize the Enzyme Concentration

Perform an enzyme titration to identify the optimal enzyme concentration for the Transcreener® 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 **Section 4.2**. 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 $MgCl_2$, 2 mM EGTA, 1% DMSO (test compound solvent), 0.015% Brij-35, and GTP. Run your enzymatic reaction at its requisite temperature and time period. Refer to **Section 7.2** for the tolerance of different components for your buffer conditions.

4.3.1 Enzyme Titration Steps

To achieve the most robust assay and a high signal, the quantity of enzyme required to produce a 50–80% change in FRET signal is ideal (EC_{50} to EC_{80}) for screening of large compound libraries and generating inhibitor dose-response curves (see **Figure 4**). To determine the EC_{80} enzyme concentration, use the following equation:

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

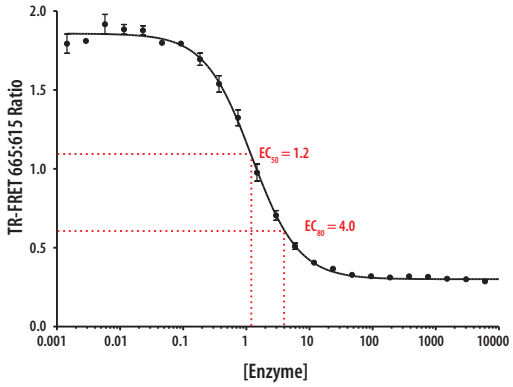


Figure 4. Enzyme titration curve. The ideal range of enzyme concentrations is shown in red.

4.3.2 Enzyme Assay Controls

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

Component	Notes
0% GTP Conversion Control	This control consists of the GDP Detection Mixture, the enzyme reaction components (without enzyme), and 100% GTP (0% GDP). It 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). It defines the lower limit of the assay window.
Minus-Nucleotide Control and Minus-Substrate 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, a GDP/GTP standard curve can be useful to ensure day-to-day reproducibility and that the assay conditions were performed using initial rates. It can also be used to calculate product formed and inhibitor IC_{50} values. See **Section 7.1** for a description of how to run the standard curve.
Background Control	This control contains 0.5X enzyme reaction conditions and Stop & Detect Buffer C.

4.4 Run an Assay

1. Add the enzyme reaction mixture to test compounds and mix on a plate shaker.
2. Start the reaction by adding GTP and acceptor substrate, then mix. The final volume of the enzyme reaction mixture should be 10 µL. Incubate at a temperature and time ideal for the enzyme target before adding the GDP Detection Mixture.
3. Prepare 1X GDP Detection Mixture as follows:

	GTP Concentration: Examples			
Component	**1 µM**	**10 µM**	**100 µM**	**Your Numbers**
GDP Antibody-Tb	100 µL	100 µL	100 µL	
GDP HiLyte647 Tracer	9.7 µL	26.8 µL	197.8 µL	
10X Stop & Detect Buffer C	1,000 µL	1,000 µL	1,000 µL	
Water	8,890.3 µL	8,873.2 µL	8,702.2 µL	
Total	**10,000 µL**	**10,000 µL**	**10,000 µL**	

Final concentrations in the detection mixture should be 8 nM GDP Antibody-Tb, 1X Stop & Detect Buffer C, and the tracer concentration calculated using the equation in **Figure 3**. An example is shown below:

	$y = 1.9x + 7.8$		
GTP	1 µM	10 µM	100 µM
GDP HiLyte647 Tracer	9.7 nM	26.8 nM	197.8 nM

4. Add 10 µL of 1X GDP Detection Mixture to 10 µL of the enzyme reaction. Mix using a plate shaker.
5. Incubate at room temperature (20–25°C) for 90 minutes and measure TR-FRET.

5.0 General Considerations

5.1 Assay Types

5.1.1. Endpoint Assay

The Transcreener® GDP TR-FRET Red Assay is designed for endpoint readout. The Stop & Detect Buffer C contains EDTA to stop Mg^{2+}-dependent enzyme reactions by chelating available Mg^{2+}. Contact BellBrook Labs regarding stop buffers for non-Mg^{2+}-dependent enzymes.

5.1.2 Real-Time Assay

You can perform real-time experiments by adding the GDP Detection Mixture, without the Stop & Detect Buffer C, directly to an enzyme reaction at initiation of the reaction. GDP detection equilibration time is not instantaneous, making it difficult to quantify GDP production; however, this method can provide insight into optimal enzyme concentration and incubation time. If Mn^{2+} or heavy metal ions, such as Cr^{3+}, Co^{2+}, $Fe^{2+/3+}$, or Cu^{2+} are present, they can negatively quench the terbium chelate at high enough concentrations, so this method may not be possible for all enzymes. As an alternative, the Transcreener® GDP FP Assay is recommended to perform real-time assays. Note that the optimal GDP HiLyte647 Tracer concentration may change when EDTA is omitted from the reaction.

5.2 Reagent and Signal Stability

The Transcreener® technology provides a robust and stable assay method to detect GDP.

5.2.1 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) for at least 24 hours at room temperature (20–25°C). If you plan to read TR-FRET on the following day, seal the plates to prevent evaporation.

5.2.2 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., when stored on the liquid handling deck).

6.0 Troubleshooting

Problem	Possible Causes and Solutions
Low selectivity	*Suboptimal tracer concentration* • Under the reaction conditions used in the Transcreener® GDP TR-FRET Red Assay, the GDP antibody is >140-fold selective for GDP over GTP. To achieve maximum sensitivity and assay window, the GDP tracer concentration must be optimized for each starting GTP concentration. *GTP concentration out of range* • Ensure that the starting GTP concentration is in the range of 1–1,000 µM.
No change in TR-FRET observed	*Low antibody/tracer activity* • The tracer and antibody are stable for up to 6 freeze-thaw cycles. For frequent use, aliquot the antibody and tracer and store the aliquots at –20°C. Use a minimum of 20 µL aliquots. *Interference from metal ions* • Mn^{2+} or heavy metals like Cu^{2+}, Fe^{2+}, Fe^{3+}, Cr^{3+}, or Co^{2+} can quench terbium at higher concentrations. This effect can be relieved by increasing EDTA concentration or adding additional quantities of EDTA-containing Stop & Detect Buffer C. Use a minimum molar ratio of at least 4X EDTA to metal ions.
High background signal	*Nonproductive GTP hydrolysis* • Certain kinases catalyze some level of nonproductive GTP hydrolysis, to the extent that water is able to get into the active site. However, the rates are generally low even in the absence of acceptor substrate and are even further reduced when acceptor substrate is present. If you are using the assay to screen for potential acceptor substrates, then background from GTP hydrolysis has to be taken into account on a case-by-case basis. We recommend a "no substrate" control to detect nonproductive GTP hydrolysis. *Interference from impurities* • Since the assay measures GDP production from any source, impurities that cause GDP production—such as a contaminating kinase, phosphatase, or GTPase—will interfere with accurate measurement of the desired GTPase activity. Care should be taken to minimize these potential contaminants in both GTPase and protein substrate preparations.

7.0 Appendix

7.1 GDP/GTP Standard Curve

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 and, therefore, the % GTP consumed (% GTP conversion). In this example, a 12-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, 20, 30, 60, and 100% GTP conversion (see **Table 1**). Commonly, 8- to 12-point standard curves are used.

% Conv.	GTP (µM)	GDP (µM)
100	0	100
60	40	60
30	70	30
20	80	20
10	90	10
5	95	5
2.5	97.5	2.5
1	99	1
0.75	99.25	0.75
0.5	99.5	0.5
0.25	99.75	0.25
0	100	0

Table 1. Concentrations of GTP/GDP to prepare a 12-point standard curve.

A

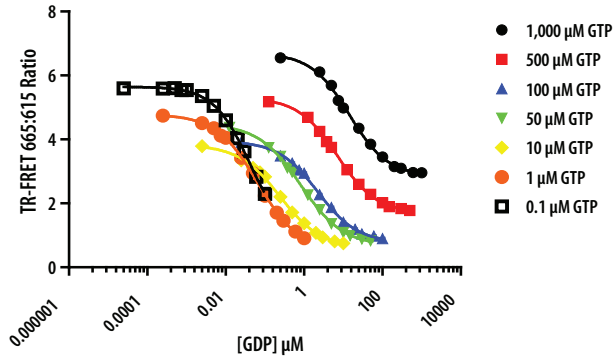


Figure 5. GTP/GDP standard curves. A) Sample data for 0.1 µM, 1 µM, 10 µM, 100 µM, and 1,000 µM GDP/GTP standard curves. The nucleotide concentration reflects the amount in the enzyme reaction, prior to the addition of the GDP Detection Mixture. Curves are obtained in a final 20 µL assay volume consisting of 25 mM Tris (pH 7.5), 2.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5% DMSO, 0.005% Brij-35, 4 nM GDP Antibody-Tb, GDP/GTP standards, and GDP HiLyte647 Tracer (concentration from equation in Figure 3) (n = 6–12). The data are plotted as FRET ratio and change in ratio vs. log [GDP] using 4-parameter nonlinear regression curve fitting. Alternatively, a 2-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). LLD = the concentration of GDP that generates Z′ > 0.

B

0.1 µM Std Curve		1.0 µM Std Curve		10 µM Std Curve		100 µM Std Curve		1,000 µM Std Curve	
Z′ at 30% Conversion	LLD (nM)	Z′ at 10% Conversion	LLD (nM)	Z′ at 10% Conversion	LLD (nM)	Z′ at 10% Conversion	LLD (nM)	Z′ at 10% Conversion	LLD (nM)
0.7	10	0.8	7.5	0.8	75	0.8	500	0.8	7,500

Use the following equations to calculate the Z′ factor:

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

$$Z' = 1 - \frac{[(3 \times SD_{\text{initial [GTP]}}) + (3 \times SD_{\text{sample}})]}{|(\text{ratio}_{\text{initial [GTP]}}) - (\text{ratio}_{\text{sample}})|}$$

7.2 Summary of Additive Effects on the Transcreener® GDP TR-FRET Assay

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

Component	5-Hour Tolerance (0–100% Conversion Signal)	5-Hour Tolerance (0–10% Conversion Signal)
Solvents		
Acetonitrile	>50%	12.5%
DMSO	3.13%	3.13%
Ethanol	>50%	12.50%
Methanol	>50%	25.00%
Glycerol	>50%	0.39%
Detergents		
Brij-35	0.94%	0.47%
CHAPS	1.25%	0.08%
NP40	0.16%	0.16%
SDS	0.04%	0.04%
Triton X-100	0.31%	0.31%
Sodium deoxycholate	0.00%	0.16%
N-lauroyl sarcosine	0.08%	0.04%
Metal chelates		
EDTA	125 mM	62.5 mM
EGTA	>250 mM	125 mM
Reductants		
Beta mercaptoethanol	6.25%	0.02%
Dithiothreitol	>500 mM	0.031 mM
Salts		
Ammonium acetate	125 mM	125 mM
Ammonium sulfate	3.91 mM	3.91 mM
Calcium chloride	15.62 mM	7.81 mM
Magnesium acetate	15.62 mM	3.91 mM
Magnesium chloride	15.62 mM	7.81 mM
Magnesium sulfate	1.95 mM	1.95 mM
Manganese chloride	15.62 mM	7.81 mM
Potassium chloride	500 mM	125 mM
Sodium azide	1.25%	1.25%
Sodium bromide	12.5 mM	3.12 mM
Sodium chloride	12.5 mM	6.25 mM

Component	5-Hour Tolerance (0–100% Conversion Signal)	5-Hour Tolerance (0–10% Conversion Signal)
Phosphatase Inhibitors		
Glycerol phosphate	15.62 mM	7.81 mM
Imidazole	62.5 mM	62.5 mM
Sodium fluoride	7.81 mM	3.91 mM
Sodium molybdate	31.25 mM	31.25 mM
Sodium tartrate	>400 mM	200 mM
Sodium orthovanadate	7.81 mM	7.81 mM
Sodium pyrophosphate	0.39 mM	0.78 mM
Carrier Proteins/Coactivators		
BSA	>1.0 mg/mL	>1.0 mg/mL
BGG	>2.5 mg/mL	>2.5 mg/mL

Not all combination of these components have been tested together. Results may vary depending on your assay conditions.

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