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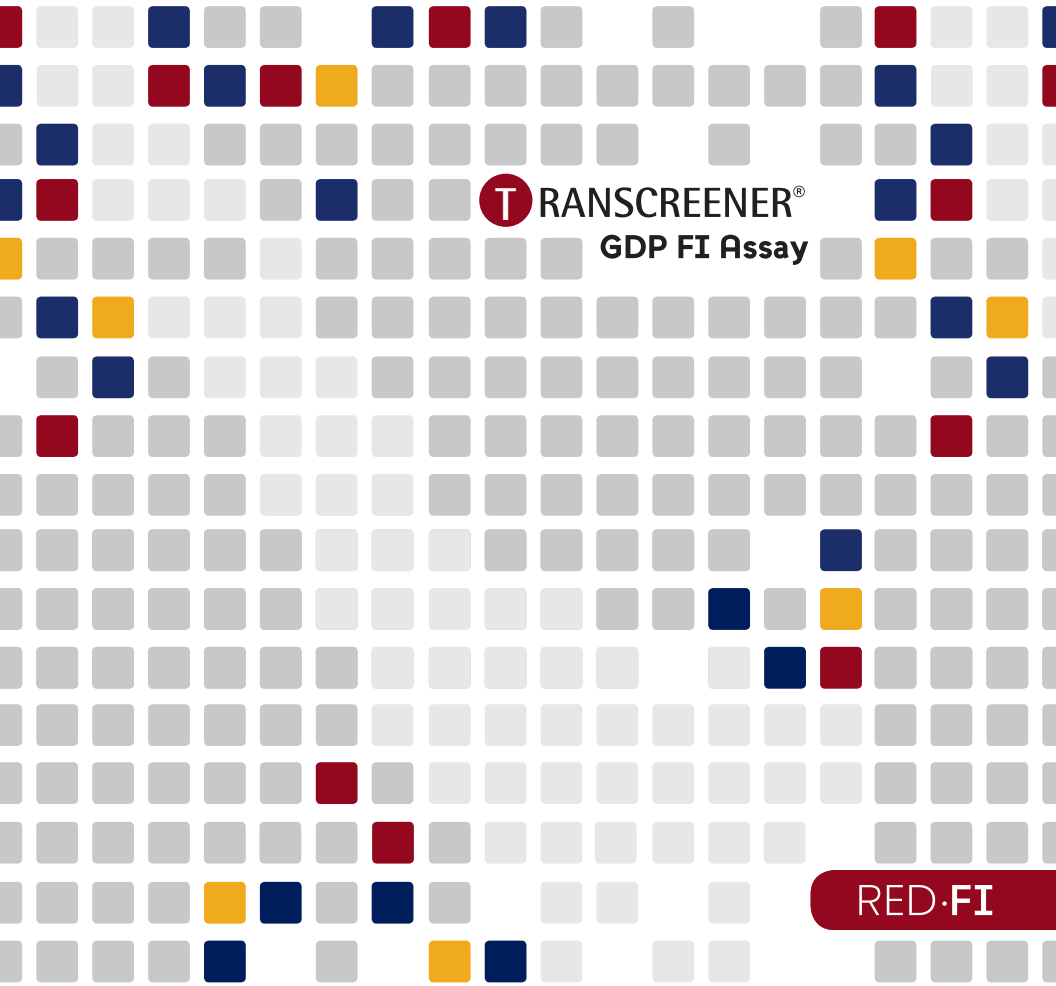


Transcreener® GDP FI Assay

Instructions for Part Numbers 3014-A, 3014-1K and 3014-10K

1.0 Introduction

The Transcreener GDP FI Assay is a competitive immunoassay for GDP with a red fluorescent intensity (FI) readout. Designed specifically for HTS and featuring a single addition mix-and-read format and room temperature reagent stability, the Transcreener GDP FI Assay is compatible with plate readers typically found in research labs as well as those used in HTS core facilities. The 3014-A kit is formatted for 96-well plates while the 3014-1K and 3014-10K kits are for 384-well and higher densities.

Able to accommodate GTP concentrations ranging from 0.1 to 1,000 µM, the Transcreener GDP FI Assay provides excellent data quality (Z′ ≥ 0.7) at low substrate conversion (typically 10% or less). Because the proprietary antibody used is highly selective for GDP, the assay can be used with any enzyme that converts GTP to GDP, including small G proteins. (Note that many commercially available GTPase preparations contain GDP as a stabilizer, which must be removed or diluted prior to use with this assay so as not to contribute to background signal.)

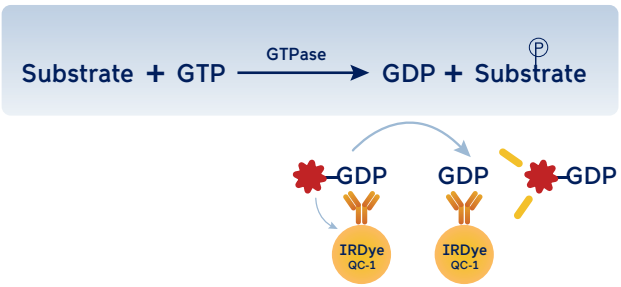


Figure 1. Transcreener GDP FI Assay Principle

The Transcreener GDP Detection Mixture comprises a GDP Alexa594 Tracer bound to the GDP Antibody- IRDye® QC-1 quencher conjugate (quencher licensed from LI-COR®). GDP produced by the target enzyme displaces the tracer from the Ab-quencher conjugate which causes an increase in fluorescence. The use of a red tracer minimizes interference from fluorescent compounds and light scattering.

2·0 Transcreener GDP FI Assay Components

Store reagents at -20°C. Individual reagents tolerate 10 freeze-thaw cycles. Sufficient reagents are provided to complete up to 200 assays (96-well format) with 3014-A, 1,000 assays (384-well format) with 3014-1K and 10,000 assays (384-well format) with 3014-10K at a GTP concentration up to 100 µM GTP. Please contact BellBrook Labs for custom packaging for enzyme reactions requiring >100 µM GTP.

GDP Antibody-IRDye® QC-1

A concentrated mouse monoclonal GDP Antibody-IRDye® QC-1 is provided in 100 mM KH_2PO_4 (pH 8.5). Spin the tube briefly after thawing if there is an evident precipitate. The concentration of GDP Antibody-IRDye® QC-1 needed for an enzyme target is dependent upon the GTP concentration and buffer conditions in the enzyme reaction, and can be calculated using a linear equation (Section 3.1). Sufficient antibody is included in the kit to complete 200 assays (96-well format) with 3014-A, 1,000 assays (384-well format) with 3014-1K and 10,000 assays (384-well format) with 3014-10K at an GTP concentration up to 100 µM GTP. Please contact BellBrook Labs for custom packaging for enzyme reactions using >100 µM GTP.

GDP Alexa594 Tracer, 800 nM

The GDP Alexa594 Tracer, 800 nM is provided in 2 mM HEPES (pH 7.5) containing 0.01% Brij-35. The final tracer concentration in the reaction is 4 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 Stop & Detect Buffer B components will stop Mg^{2+}- or Mn^{2+}- requiring enzyme reactions and aid in the detection and stabilization of the FI signal. Stop & Detect Buffer B is at a 0.5X concentration at the time of fluorescent 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 enzyme reaction.

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 FI Assay. If the GTP stock contains impurities, such as GDP, the assay window will be compromised. GTP supplied in this kit can be used for the enzyme reaction and to create the GDP/GTP standard curve. Contact BellBrook Labs for alternate suppliers and catalog numbers.

5 mM GDP

GDP is not common to all laboratories and therefore is a supplied reagent. GDP is used to create the GDP/GTP standard curve.

Materials Required but Not Provided

Enzyme

The Transcreener GDP FI assay is designed for use with purified enzyme preparations. Contaminating enzymes such as phosphatases or nucleotidases can produce background signal and reduce the assay window. Also, many commercial preparations of GTPase enzymes contain GDP as a stabilizer, which must be removed or diluted sufficiently to prevent background signal.

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 supplier recommendations.

Plate Reader

A multidetection microplate reader configured to measure fluorescence intensity of Alexa Fluor®594 is required. For a complete list of standard fluorescent plate readers that have been validated using the Transcreener assays, refer to Appendix 6.3. We will update this list as we expand our instrument validation testing. Contact BellBrook Labs Technical Service for additional information.

Assay Plates

It is important to use assay plates that are entirely black. We recommend Corning® 384-well plates (catalog #3676) and Greiner 96-well, half area plates (catalog# 675076).

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 FI Assay is a universal biochemical assay designed for enzymes that produce GDP. Optimal reagent concentrations are based on the initial GTP concentration used in the enzyme reaction and enzyme buffer conditions.

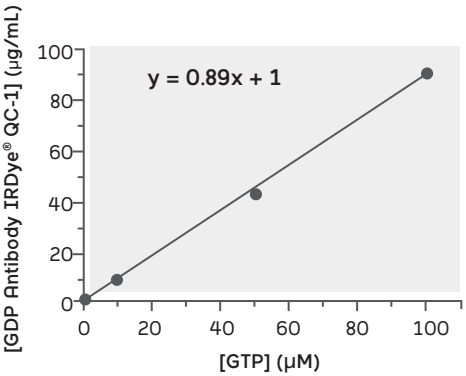
There are four steps to complete:

1. Determine antibody concentration
2. Set-up instrument
3. Titrate enzyme
4. Detect GDP

Completing these steps will provide optimal GDP detection results. The enzyme titration need not be repeated unless enzyme reaction conditions change.

3·1 Determine GDP Antibody-IRDye® QC-1 Concentration

Figure 2. Linear Relationship between [GTP] and [GDP Antibody-IRDye® QC-1]



The Transcreener GDP FI 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-IRDye® QC-1 determines the total assay window and the GDP detection range, and the amount needed is dependent upon the GTP concentration in the enzyme reaction.

As shown in Figure 2, the relationship between [GTP] and [GDP Antibody-IRDye® QC-1] 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 Antibody-IRDye® QC-1 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 enzyme reaction, y = [GDP Antibody-IRDye® QC-1] (µg/mL) in the 1X GDP Detection Mixture, m (slope) = 0.89, and b (y-intercept) = 1. We recommend a final volume of 20 µL (384-well plate) or 50 µL (96-well plate).

For example, if you are using 3 µM GTP in a 10 µL enzyme reaction, the optimal antibody concentration in the 1X GDP Detection Mixture (assuming 10 µL of GDP Detection Mixture added to each 10 µL enzyme reaction) would be [0.89 x 3] + 1 = 3.67 µg/mL.

Determining your GDP Antibody-IRDye® QC-1 concentration using this equation will provide excellent results for most assay conditions. If it does not provide the results you require, please refer to the Appendix 6.1 for instructions on preparing an GDP Antibody-IRDye® QC-1 titration in the buffer system ideal for your enzyme target.

3.2 Instrument Set-up

Becoming familiar with ideal instrument settings for fluorescence intensity is essential to the success of the Transcreener GDP FI Assay.

Verify Instrument Measures Fluorescence Intensity

Make sure your plate reader measures fluorescence intensity. The optimal excitation/emission settings for the GDP Alexa594 Tracer are excitation 590 nm (10 nm bandwidth) and emission 617 nm (10 nm bandwidth). The GDP Alexa594 Tracer has been successfully used at excitations of (580 - 590) and emissions of (610 - 620) with bandwidths of 10 nm. Please see Appendix 6.3 for filter recommendations on several standard fluorescent instruments. For any additional questions please view our series of application notes or contact BellBrook Labs Technical Services at 866-313-7881.

Define the Maximum Fluorescence Window for Your Instrument

Measuring low (tracer + antibody) and high (free tracer) relative fluorescence units (RFUs) will define the maximum assay window of your specific instrument.

Low RFU Mixture

Prepare 4 nM GDP Alexa594 Tracer/0.5X Stop & Detect Buffer B with one half of your GDP Antibody-IRDye® QC-1 concentration calculated using the equation in Figure 2.

High RFU Mixture

Prepare 4 nM GDP Alexa594 Tracer/0.5X Stop & Detect Buffer B without GDP Antibody-IRDye® QC-1.

Measure the Fluorescence Intensity

The difference between the low and high RFU values will give you your maximum assay window. The values will differ depending on the units from your plate reader but the ratio of the RFUs from the (High RFU mixture)/(Low RFU mixture) ratio should be >5. If not, please call BellBrook Labs Technical Service.

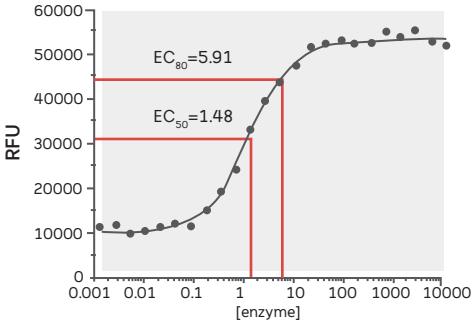
3.3 Enzyme Titration

An enzyme titration is performed to identify the optimal enzyme concentration for the assay. Use enzyme buffer conditions, substrate, and GTP concentrations that are optimal for your target enzyme and GDP Antibody-IRDye® QC-1 concentration calculated as described in Section 3.1. If a compound screen is planned, you should include the library solvent at its final assay concentration. We routinely use enzyme buffer containing 50 mM HEPES (pH 7.5), 4 mM MgC_2, 2 mM EGTA, 1% DMSO, 0.01% Brij-35 for many enzymes, and have tested the effect of a diverse panel of commonly used reagents including salts, detergents, metal ions and organic solvents. For more information please contact BellBrook Labs customer service at 866-313-7881. Run your enzymatic reaction at a temperature and time period that is convenient for your screening protocol.

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

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

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 (see p.8), the enzyme reaction components (without enzyme), and 100% ATP (0% GDP). This control defines the lower 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 upper 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.

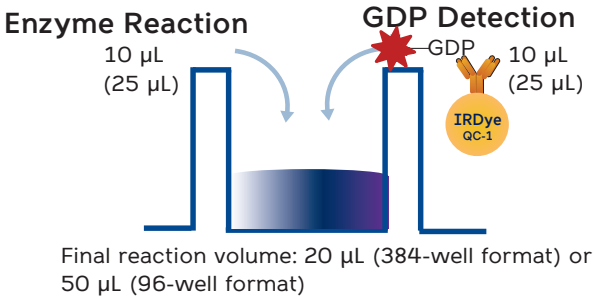
GDP/GTP Standard Curve

If desired, a standard curve mimicking the enzymatic conversion of GTP to GDP can be constructed and used to convert raw fluorescnce data to the amount of GDP produced in reactions. Because the relationship between fluorescence and GDP is non-linear, use of a standard curve is the most accurate way to obtain IC_{50} values. See Appendix 6.2 for a description of how to prepare a standard curve.

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 1 hour. Increase each volume to 25 µL if performing the assay in 96-well half-volume plates. The enzyme reaction components (including GTP) and the GDP Detection Mixture are 0.5X in the final 20 µL (384-well plate) or 50 µL (96-well plate) volume.

Figure 4. Protocol



Enzyme Reaction (10 µL or 25 µL)

Add the enzyme reaction mixture to test compounds and mix using a 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 or 25 µL)

The 1X GDP Detection Mixture is prepared by adding GDP Antibody-IRDye® QC-1 and GDP Alexa594 Tracer to Stop & Detect Buffer B. Final concentrations should be 8 nM GDP Alexa594 Tracer, 1X Stop & Detect Buffer B, and the antibody concentration calculated using the equation of line in Figure 2. Add the 1X GDP Detection Mixture to the enzyme reaction and mix using a plate shaker. Incubate at room temperature (20 - 25°C) for 1 hour, and measure fluorescence intensity.

GDP Detection Controls

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

Without Antibody (Free Tracer) Control

This sample contains the GDP Alexa594 Tracer without the GDP Antibody-IRDye® QC-1. This determines the maximum RFU achievable.

Without Tracer Control

This sample contains the GDP Antibody-IRDye® QC-1 without the GDP Alexa594 Tracer and is used as a sample blank for all wells. It is used as a 'buffer blank', but contains the same GDP Antibody-IRDye® QC-1 concentration used in all wells.

Endpoint Assay

The Transcreener GDP FI Assay is designed for endpoint readout. The Stop & Detect Buffer B 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 not be stopped with the addition of the buffer provided.

Real-Time 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-IRDye® QC-1 concentration may change when EDTA is omitted. Note: the equilibration time for the GDP tracer and GDP Antibody-IRDye® QC-1 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 RFU assay window at 10% substrate conversion was determined after the addition of the GDP Detection Mixture to the standard samples. The RFU assay window at 10% substrate conversion (10 µM) 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 RFU window at 10% substrate conversion (10 µM GTP) remains constant (<10% change) when up to 25% DMSO, acetonitrile, ethanol, or methanol are used in the enzyme reaction. Contact BellBrook Labs for further reagent compatibility information.

5·0 References

Lowery, R. G., & Kleman-Leyer, K (2006). Transcreener™: screening enzymes involved in covalent regulation. *Expert Opinon on Therapeutic Targets, 10*(1), 179-190.

6·0 Appendix

6·1 Optimize Antibody Concentration

The Transcreener GDP FI 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-IRDye® QC-1 determines the total assay window and the GDP detection range, and the amount needed is dependent upon the GTP concentration in the enzyme reaction.

If the EZ Protocol for selecting the appropriate concentration of GDP Antibody-IRDye® QC-1 does not yield a sufficient assay window, we recommend determining the EC_{10} antibody concentration in the specific conditions used for your enzyme reaction. This involves setting up an antibody binding curve in mock reactions containing all enzyme assay components (including GTP at the appropriate concentration) except enzyme.

Titrate GDP Antibody-IRDye® QC-1 in 1X Stop & Detect Buffer B

Prepare 8 nM GDP Alexa594 Tracer in 1X Stop & Detect Buffer B with and without GDP Antibody IRDye® QC-1 (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). If using a 96-well plate, adjust the volumes to 50 µL of mixture (with antibody) into wells in column 1 and 25 µL for the remaining dispensing steps.

Add Enzyme Reaction Buffer (Containing GTP)

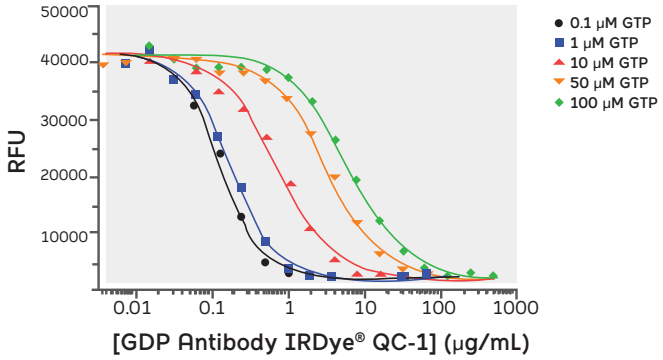
Prepare your enzyme reaction mixture (include substrate and GTP, but omit enzyme) and add 10 µL (25 µL for 96-well plates) to the titrated antibody. Mix the plate, equilibrate at room temperature (1 hour), and measure fluorescence intensity.

Plot RFU vs. log of GDP Antibody-IRDye® QC-1 Concentration and Calculate the EC_{10}

The antibody concentration at the EC_{10} is often used as a good compromise between sensitivity and maximal assay window. The EC_{10} is determined by inputting the EC_{50} and hillslope values from a sigmoidal dose response curve fit into the equation below.

$$EC_{10} = ((10/(100 - 10))^{1/hillslope})*EC_{50}$$

Figure 5. GDP Antibody-IRDye® QC-1 Titrations at Various GTP Concentrations



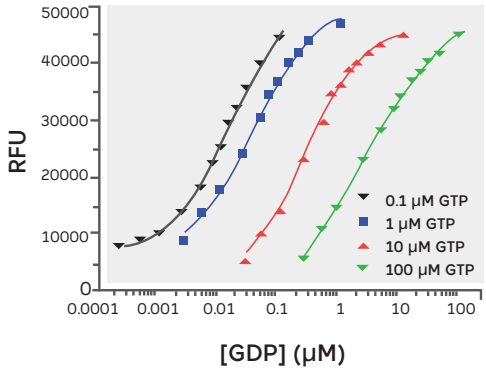
The final 20 µL assay volume consisted of 4 nM GDP Alexa594 Tracer, 0.5X Stop & Detect Buffer B, 0.5X enzyme reaction mixture (50 mM HEPES (pH 7.5), 2 mM $MgCl_2$, 1 mM EGTA, 0.5% DMSO, 0.01% Brij-35, and GTP) and GDP Antibody-IRDye® QC-1 (n=3).

6·2 GDP/GDP Standard Curve

The standard curve mimics an enzyme reaction (as GTP concentration decreases, GDP concentration increases); the guanosine 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%, 0.5%, 1%, 2.5%, 5%, 7.5%, 10%, 15%, 20%, 30%, 50% and 100% GTP conversion.

Figure 6. GDP/GTP Standard Curves

A



Sample data for 0.1 µM, 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. 10 µL of reaction mix (50 mM HEPES (pH 7.5), 4 mM $MgCl_2$, 2 mM EGTA, 1% DMSO, 0.01% Brij, GDP/GTP standards) was added to 10 µL detection mix (GDP Antibody IRDye QC-1, 8 nM GDP Alexa594 tracer, 1X Stop & Detect buffer B using GDP Antibody IRDye QC-1 concentration determined from line in Figure 2 (n=24)). The data are plotted as RFU vs log [GDP] using nonlinear regression curve fitting.

B

% GTP Conversion	0.1 µM GTP	1 µM GTP	10 µM GTP	100 µM GTP
0.5	nd	0.72	0.58	0.77
1	0.56	0.79	0.78	0.81
2.5	0.81	0.86	0.87	0.87
5	0.81	0.89	0.89	0.88
10	0.88	0.89	0.91	0.88
30	0.91	0.91	0.92	0.92
100	0.90	0.91	0.91	0.92

Excellent Z' values are obtained.

$$Z' = 1 - [(3*SD_{(x\% \text{ conversion})} + 3*SD_{(0\% \text{ conversion})}) / (RFU_{(x\% \text{ conversion})} - RFU_{(0\% \text{ conversion})})]$$

6·3 FI Assay Instrument Settings

BMG LABTECH PHERAstar *Plus*

Excitation Filter/Bandwidth	580/10nm
Emission Filter/Bandwidth	620/10nm

PerkinElmer EnVision®

Excitation Filter/Bandwidth	545/7nm	2100-5070
Emission Filter/Bandwidth	635/15nm	2100-5590
Mirror	Texas Red FP D595 single mirror	2100-4190

Tecan Safire²™ (monochromator based)

Excitation Wavelength/Bandwidth	580/10nm
Emission Wavelength/Bandwidth	620/10nm

Molecular Devices' SpectraMax M2

Excitation Wavelength	584nm
Emission Wavelength	612nm (with auto cut-off at 610nm)

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, and12/029,932; European Applications Nos. 04706975.2 and 05785285.7; Canadian Application No. 2,514,877; Japanese Application No. 2006-503179; and International Patent Application No. PCT/US07/088111.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. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) to not transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration 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 to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) 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 250, Madison, Wisconsin 53711. Phone (608)443-2400. Fax (608)441-2967.

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