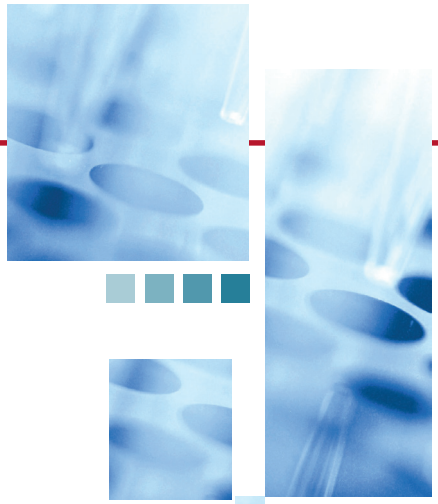
BellBrook Labs

FAR RED TR-FRET



TRANSCREENER®

UDP² TR-FRET Red Assay

Technical Manual

Instructions for Part Numbers 3022-1K and 3022-10K

TM#126 • Rev 2017-06

Transcreener® UDP² TR-FRET Red Assay Technical Manual

Contents

1.0 Introduction

The Transcreener® UDP² TR-FRET Red Assay is a competitive immunoassay for UDP with a far-red, time-resolved Förster-resonance-energy-transfer (TR-FRET) readout. Because it is highly selective for UDP, the assay can be used with any enzyme that generates UDP from a UDP-sugar donor. Examples include glycosyltransferase, galactosyltransferase, glucuronyltransferase, *N*-acetylglucosamyltransferase, *N*-acetylgalactosyltransferase, xylosyltransferase, and glycogen, cellulose, lactose, and hyaluronan synthases.

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® UDP² TR-FRET Red Assay provides the following benefits:

- Accommodates UDP-sugar donor concentrations ranging from 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 an 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.

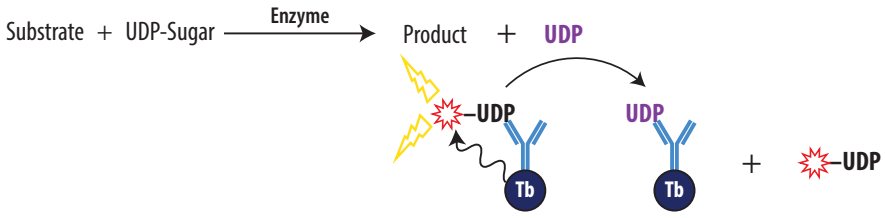


Figure 1. Schematic overview of the Transcreener® UDP² TR-FRET Red Assay. The Transcreener® UDP Detection Mixture contains a UDP HiLyte647 tracer bound to a UDP² antibody conjugated to terbium (Tb). Excitation of the Tb complex in the UV range (~330 nm) results in energy transfer to the tracer and emission at a higher wavelength (665nm) after a time delay. UDP produced by the target enzyme displaces the tracer, which causes a decrease in TR-FRET.

2.0 Product Specifications

Product	Quantity	Part #
Transcreener® UDP² TR-FRET Red Assay	1,000 assays*	3022-1K
	10,000 assays*	3022-10K

*The exact number of assays depends on enzyme reaction conditions.

Storage

Store all reagents at –20°C upon receipt.

2.1 Materials Provided

Component	Composition	Notes
UDP HiLyte647 Tracer	10 µM solution in 2 mM HEPES (pH 7.5) containing 0.01% Brij-35	The concentration of UDP HiLyte647 Tracer needed for an enzyme target depends upon the UDP-sugar 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 # 3022-1K) or 10,000 assays (Part # 3022-10K) for most UDP-sugar concentrations up to 100 µM.
UDP² Antibody-Terbium Conjugate	800 nM solution in HEPES-buffered saline	The final antibody concentration in the reaction is 2 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 most, but not all, enzyme reactions that require Mg^{2+}. The final concentration of Stop & Detect Buffer C at the time of FRET measurement is 0.5X.
UDP	5 mM	The UDP supplied in this kit can be used to create a UDP-sugar/UDP standard curve, if desired.

Caution: If Mn^{2+} or heavy metal ions such as $Cr^{3+/6+}$, Co^{2+}, $Fe^{2+/3+}$ or Cu^{2+} are present, they can negatively quench the terbium chelate. To overcome this problem, adjust the EDTA concentration so that it is at least equimolar or greater than the metal ion concentration in the reaction, by adding EDTA or additional Stop & Detect Buffer C.

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® UDP² 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, enzyme cofactors, substrates, 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® UDP² TR-FRET Red Assay protocol consists of 4 steps (**Figure 2**). The protocol was developed for a 384-well format, using a 15 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.

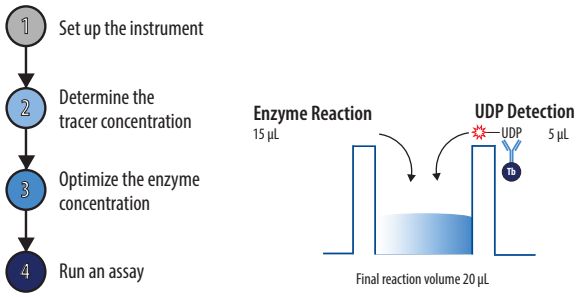


Figure 2. An outline of the procedure. The assay consists of 4 main steps with a mix-and-read format.

4.1 Set Up the Instrument

Becoming familiar with ideal instrument settings for TR-FRET is essential to the success of the Transcreener® UDP² TR-FRET Red Assay.

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.

4.1.1 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 ($Ex_{320}/Em_{615}/Em_{665}$).

4.1.2 Define the Maximum TR-FRET Window for the Instrument

Measuring high (No UDP) and low (100 µM UDP) 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. Note that, after optimizing conditions for a particular UDP-sugar, the assay window may be different.

High FRET Mixture

Prepare the following solution:

Component	Stock Concentration	Final Concentration	Example: 25 Assays	Your Numbers
UDP² Antibody-Tb	800 nM	2 nM	1.3 µL	
10X Stop & Detect Buffer C	10X	0.25X	12.5 µL	
UDP HiLyte647 Tracer	10 µM	15 nM	0.8 µL	
Water			485.5 µL	
Total			**500 µL**	

Low FRET Mixture

Prepare the following solution:

Component	Stock Concentration	Final Concentration	Example: 25 Assays	Your Numbers
UDP² Antibody-Tb	800 nM	2 nM	1.3 µL	
10X Stop & Detect Buffer C	10X	0.25X	12.5 µL	
UDP HiLyte647 Tracer	10 µM	15 nM	0.8 µL	
UDP	5 mM	100 µM	10 µL	
Water			µL	
Total			**µL**	

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 Optimal UDP HiLyte647 Tracer Concentration

The UDP HiLyte647 Tracer is the only assay component that requires adjustment for different reaction conditions. Its concentration will define the dynamic range of the assay, and it should be adjusted based on the initial donor substrate concentration used in the enzyme reactions. To determine the optimal UDP HiLyte647 Tracer concentration, perform a UDP HiLyte647 Tracer titration using the reaction conditions for your enzyme and UDP-sugar donor.

4.2.1 Titrate the UDP HiLyte647 Tracer

Caution: If Mn^{2+} or heavy metal ions such as $Cr^{3+/6+}$, Co^{2+}, $Fe^{2+/3+}$ or Cu^{2+} are present, they can negatively quench the terbium chelate. To overcome this problem, adjust the EDTA concentration so that it is at least equimolar or greater than the metal ion concentration in the reaction, by adding EDTA or additional Stop & Detect Buffer C.

1. Prepare two batches of enzyme reaction mixture, with and without UDP HiLyte647 Tracer at 1,000 nM. Include the substrate and UDP-sugar, but omit the enzyme.
2. Dispense 30 µL of the mixture from step 1 containing UDP HiLyte647 Tracer into the wells in column 1 of a 384-well plate.
3. Dispense 15 µL of the mixture from step 1 without UDP HiLyte647 Tracer across columns 2–24.
4. Remove 15 µL from column 1 and serially titrate the contents across the plate to column 24.

4.2.2 Add Stop & Detect Buffer Containing UDP² Antibody-Tb

1. Prepare a 1X Stop & Detect Buffer containing 8 nM UDP² Antibody-Tb.
2. Add 5 µL to each well of the plate containing the titrated UDP HiLyte647 Tracer from **Section 4.2.1**. Mix the plate and equilibrate at room temperature for 1 hour.
3. Measure TR-FRET according to the instrument settings established in **Section 4.1**.

4.2.3 Calculate the Optimal Concentration of UDP HiLyte647 Tracer

To calculate the optimal tracer concentration, plot the TR-FRET 665:615 ratio vs. log [UDP Hilyte647 Tracer] and calculate the EC_{85} value by inputting the EC_{50} and hillslope values from a sigmoidal dose-response curve fit into the following equation.

$$EC_{85} = (85 \div (100 - 85))^{(1 \div \text{hillslope})} \times EC_{50}$$

An example is shown in **Figure 3**.

The EC_{85} value is the UDP HiLyte647 Tracer concentration in the final 20 µL reaction (use 4 × [EC_{85}] in the 5 µL UDP Detection Mixture). It is a good compromise between sensitivity and the maximal assay window for UDP-sugar concentrations up to 100 µM.

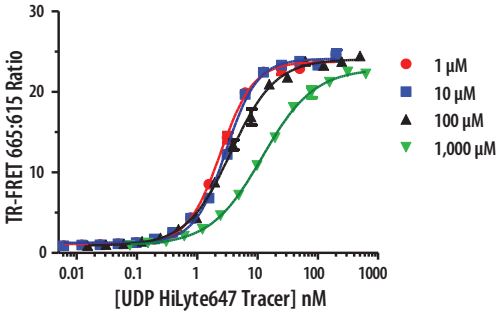


Figure 3. Optimizing the UDP HiLyte647 Tracer concentration for UDP-glucose. A sample tracer titration was performed with 1, 10, 100, and 1,000 µM UDP-glucose. The nucleotide concentration reflects the amount in the enzyme reaction prior to the addition of the UDP Detection Mixture. The UDP HiLyte647 Tracer (15 µL) was titrated in the enzyme reaction mix as described in Section 4.2. In this example, the calculated EC_{85} values were 7.3, 8.5, 15.7, and 67 nM UDP HiLyte647 tracer for 1, 10, 100, and 1,000 µM UDP-glucose respectively.

4.2.4 Further Tracer Optimization (If Necessary)

Determining the optimal UDP HiLyte647 Tracer concentration using the EC_{85} value 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 EC_{85} UDP HiLyte647 Tracer concentration as a starting point. Try performing a standard curve (see **Section 7.1**) at 0.75 × [EC_{85}], [EC_{85}], and 1.25 × [EC_{85}] tracer concentrations to find an assay window that suits your needs. For UDP-sugar concentrations greater than 100 µM, using the [EC_{50}–EC_{60}] is typical.

4.3 Optimize the Enzyme Concentration

Perform an enzyme titration to identify the optimal enzyme concentration for the Transcreener® UDP² TR-FRET Red Assay. Use enzyme buffer conditions, substrate, and UDP-sugar concentrations that are optimal for your target enzyme and UDP 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. 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 (**Figure 4**). To determine the EC_{80} enzyme concentration, use the following equation:

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

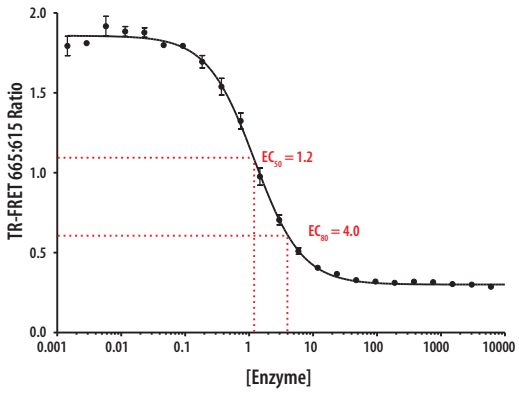


Figure 4. Enzyme titration curve. The ideal range of enzyme concentrations is shown in red.

4.3.2 Enzyme Assay Controls

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

Component	Notes
Negative (No Enzyme) Control	This control is used to to calculate the decrease in FRET caused by enzyme activity. It consists of UDP Detection Mixture, the enzyme reaction components (without enzyme) and 100% UDP-Sugar. It defines the upper limit of the assay window.
Positive (No Inhibitor) Control	This control is used to determine the full activity of the enzyme being screened. It consists of UDP Detection Mixture, and the enzyme reaction components (including enzyme) but without any potential test compounds that might interfere with the full activity of the enzyme being screened. It defines the lower limit of the assay window.
Minus-Nucleotide Control	To verify enzyme purity, perform an enzyme reaction in the absence of UDP-sugar.
UDP-Sugar/UDP Standard Curve	Although optional, a UDP-sugar/UDP 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 inhibitor IC_{50} values. See **Section 7.1** for a description of how to run the standard curve.
Background Control	Use only 0.5X enzyme reaction conditions and Stop & Detect Buffer C.

Note: Because the Transcreener® UDP² Assay relies on a competitive binding reaction, the response is nonlinear, so the signal is not directly proportional to reaction progress.

4.4 Run an Assay

1. Prepare a master mix containing all UDP enzyme reaction components except the donor substrate and mix on a plate shaker.
2. Start the reaction by adding the donor substrate, then mix. The final volume of the enzyme reaction mixture should be 15 µL. Incubate at a temperature and time ideal for the enzyme target before adding the UDP Detection Mixture.
3. Prepare 1X UDP Detection Mixture as follows:

Component	Stock Concentration	Final Concentration	Example*	Your Numbers
UDP² Antibody-Tb	800 nM	8 nM	100 µL	
10X Stop & Detect Buffer C	10X	1X	1,000 µL	
UDP HiLyte647 Tracer	10 µM	4 × [EC_{85}]*	34 µL*	
Water			8,866 µL	
Total			**10,000 µL**	

*The EC_{85} value is calculated as described in Section 4.2. The example shown here is for 10 µM UDP-glucose.

Note: The UDP enzyme reaction components (including donor substrate) will be diluted 1.3-fold and the UDP Detection Mixture will be diluted 4-fold after this addition.

4. Add 5 µL of 1X UDP Detection Mixture to 15 µL of the enzyme reaction. Mix using a plate shaker.
5. Incubate at room temperature (20–25°C) for at least 60 minutes and measure TR-FRET.

5.0 General Considerations

5.1 Assay Types

5.1.1. Endpoint Assay

The Transcreener® UDP² 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 UDP Detection Mixture, without the Stop & Detect Buffer C, directly to an enzyme reaction at initiation of the reaction. UDP detection equilibration time is not instantaneous, making it difficult to quantify UDP 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® UDP² FP Assay is recommended to perform real-time assays. Note that the optimal UDP 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 UDP.

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 UDP 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 UDP Detection Mixture Stability

The UDP 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* • To achieve maximum sensitivity and assay window, the UDP HiLyte647 Tracer concentration must be optimized for each starting UDP-sugar concentration. *UDP-sugar concentration out of range* • Ensure that the starting UDP-sugar 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	*Interference from impurities* • Since the assay measures UDP-sugar conversion from any source, impurities that cause UDP production—such as a contaminating enzyme—will interfere with accurate measurement of the desired enzyme activity. Care should be taken to minimize these potential contaminants in both UDP-sugar and substrate preparations.

7.0 Appendix

7.1 UDP-Sugar/UDP Standard Curve

The standard curve mimics an enzyme reaction (as UDP-sugar concentration decreases, UDP concentration increases). The UDP-sugar/UDP standard curve allows calculation of the concentration of UDP produced in the enzyme reaction and, therefore, the % UDP-sugar consumed (% UDP-sugar conversion). In this example, a 12-point standard curve was prepared using concentrations of UDP-glucose and UDP ranging from 1 µM to 1,000 µM (see **Table 1**). Commonly, 8- to 12-point standard curves are used.

Table 1. Concentrations of UDP-glucose/UDP to prepare a 12-point standard curve.

% Conv.	UDP-Glucose (µM)	UDP (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

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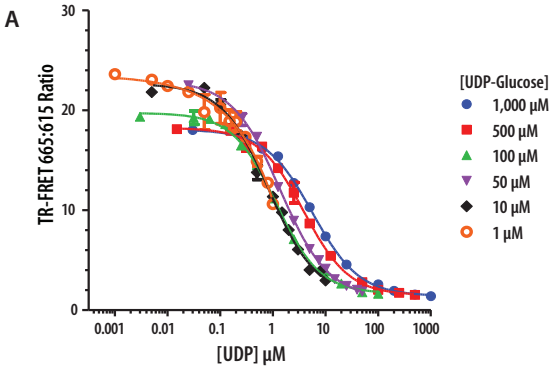

Figure 5. UDP-glucose/UDP standard curves. A) Sample data was plotted for for 1 µM, 10 µM, 50 µM, 100 µM, 500 µM, and 1,000 µM UDP-glucose/UDP standard curves. The nucleotide concentration reflects the amount in the enzyme reaction, prior to the addition of the UDP Detection Mixture. Curves were obtained in a final 20 µL assay volume consisting of 25 mM Tris (pH 7.5), 2.5 mM $MgCl_2$, 0.5% DMSO, 2 nM UDP Antibody-Tb, UDP-glucose/UDP standards, and UDP HiLyte647 Tracer (concentration from equation in Figure 3) (n = 6–12). The data are plotted as FRET ratio vs. log [UDP] 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 10–1,000 µM and 30% for 1 µM) and lower limits of detection (LLD). LLD = the concentration of UDP that generates Z′ > 0.

B

1 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)
0.7	150	0.7	250	0.7	500	0.7	630

Use the following equation to calculate the Z′ factor:

$$Z' = 1 - \frac{[(3 \times SD_{0\%\ conversion}) + (3 \times SD_{x\%\ conversion})]}{|(\text{mean 0\% conv. ratio}_{665:615}) - (\text{mean x\% conv. ratio}_{665:615})|}$$

7.2 Summary of Additive Effects on the Transcreener® UDP² TR-FRET Assay

The assay window at 10% substrate conversion remains constant (<10% change) when up to 3% DMSO, 6.25% ethanol, 5% Triton X-100, 1% Brij-35, 300 mM NaCl, and 0.5 mg/mL BSA 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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