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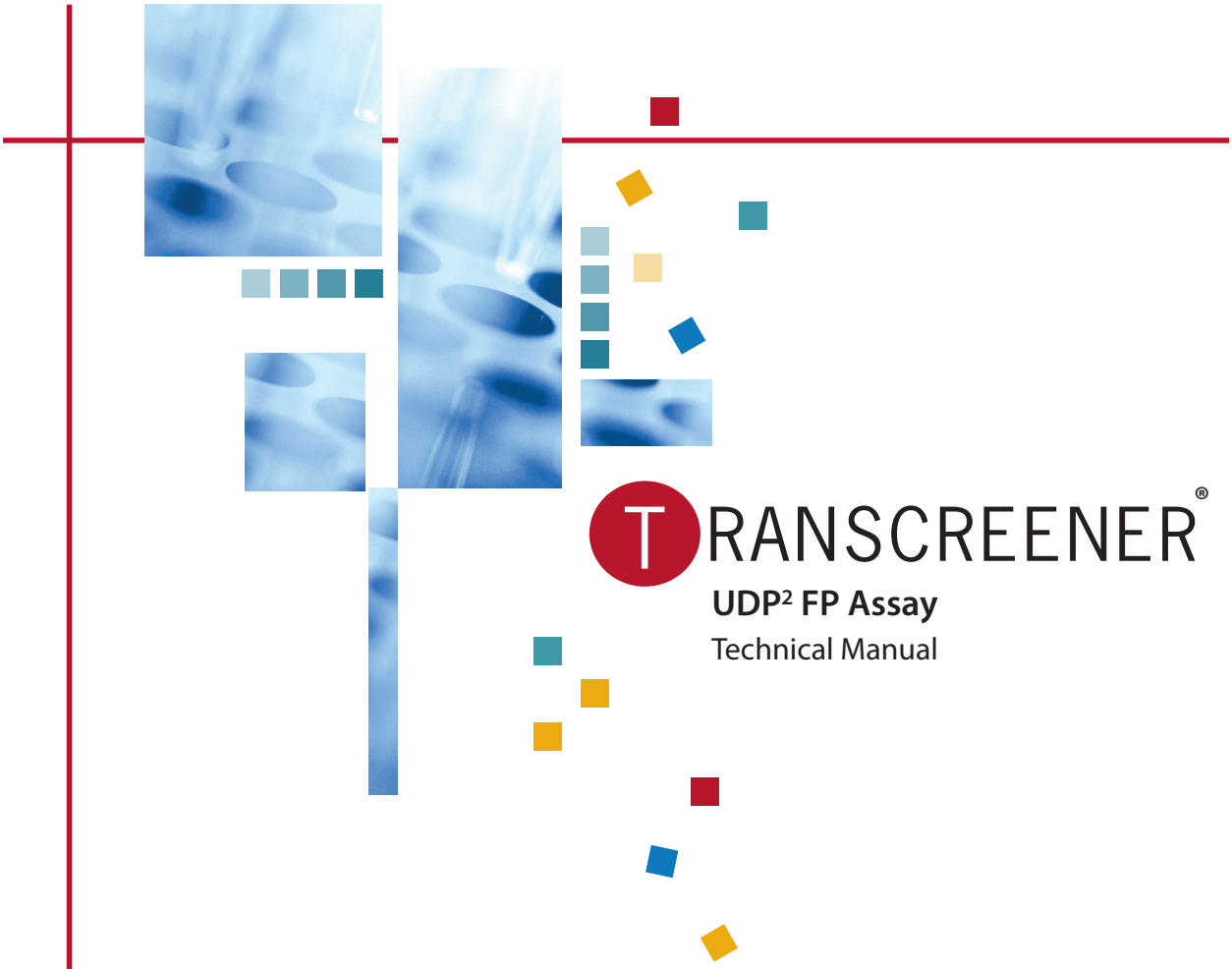
FAR RED FP



TRANSCREENER®

UDP² FP Assay

Technical Manual

Instructions for Part Numbers 3018-1K and 3018-10K

TM#130 • Rev 2020-07

Transcreener® UDP² FP Assay Technical Manual

Contents

1.0 Introduction

The Transcreener® UDP² FP Assay is a far-red, competitive fluorescence polarization (FP) assay (**Figure 1**). Because it is highly selective for UDP, the assay can be used with any enzyme that produces UDP, regardless of what other substrates are used. Examples of enzymes include glycosyltransferases, galactosyltransferases, glucuronyltransferases, N-acetylglucosamyltransferases, *N*-acetylgalactosyltransferases, xylosyltransferases, 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 FP Assay provides the following benefits:

- Accommodates UDP 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 a group transfer enzyme family by using a single set of assay reagents that detect an invariant product.
- Far-red tracer further minimizes interference from fluorescent compounds and light scattering.

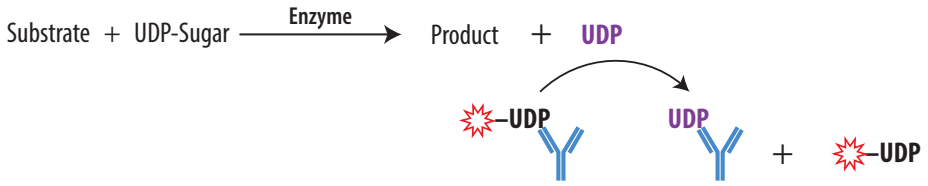


Figure 1. Schematic overview of the Transcreener® UDP² FP Assay. The Transcreener® UDP Detection Mixture contains a UDP Alexa Fluor® 633 tracer bound to a UDP² Antibody. UDP produced by the target enzyme displaces the tracer, which rotates freely, causing a decrease in FP.

2.0 Product Specifications

Product	Quantity	Part #
Transcreener® UDP² FP Assay	1,000 assays*	3018-1K
	10,000 assays*	3018-10K

*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 –20°C upon receipt.

2.1 Materials Provided

Component	Composition	Notes
UDP² Antibody	3.1 mg/mL solution in PBS with 10% glycerol*	The concentration of UDP² Antibody needed for an enzyme target is dependent upon the UDP-sugar concentration and buffer conditions in the enzyme reaction (see **Section 4.2**). Sufficient antibody is included in the kit to complete 1,000 assays (Part # 3018-1K) or 10,000 assays (Part # 3018-10K) to detect UDP at a concentration up to 100 µM.
UDP² Alexa Fluor® 633 Tracer	800 nM solution in 2 mM HEPES (pH 7.5) containing 0.01% Brij-35	The final tracer concentration in the 20 µL reaction is 4 nM.
Stop & Detect Buffer B, 5X	200 mM HEPES (pH 7.5), 400 mM EDTA, and 0.2% Brij-35	The Stop & Detect Buffer B 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 B at the time of FP measurement is 0.25X.
UDP	5 mM	UDP is used to create the UDP-sugar/UDP standard curve.

*The exact concentration may vary from batch to batch. Please refer to the Certificate of Analysis for an accurate concentration.

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® UDP 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 $MnCl_2$, Brij-35, and test compounds.
- ***Plate Reader***—A multidetection microplate reader configured to measure FP of the Alexa Fluor® 633 tracer is required. The Transcreener GDP FP Assay has been successfully used on the following instruments: BioTek Synergy™2 and Synergy™4; BMG Labtech PHERAstar; Perkin Elmer EnVision; and Tecan Safire²™.
- ***Assay Plates***—It is important to use assay plates that are entirely black with a nonbinding surface. We recommend Corning® 384-well plates (Cat. # 4514). The suggested plate has a square well top that enables easier robotic pipetting and a round bottom that allows good Z′ factors. It has a recommended working volume of 2–20 µL.
- ***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 FP 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 FP instrument and verify that it is compatible with the assay being performed (see **Section 4.1**).

4.0 Protocol

The Transcreener® UDP² FP 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.

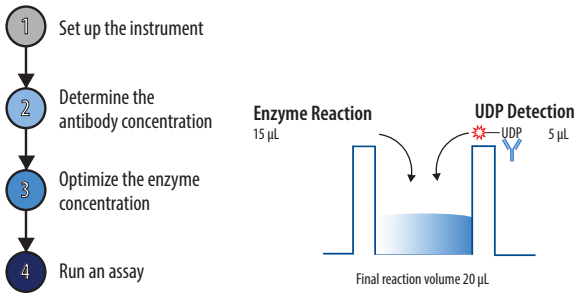


Figure 2. An outline of the procedure. The assay consists of 4 main steps with a mix-and-read format.

4.1 Set Up the Instrument

Becoming familiar with ideal instrument settings for FP is essential to the success of the Transcreener® UDP² FP Assay.

4.1.1 Verify That the Instrument Measures FP

Ensure that the instrument is capable of measuring FP (not simply fluorescence intensity) of Alexa Fluor® 633.

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 mP Window for the Instrument

Measuring high (tracer + antibody) and low (free tracer) FP will define the maximum assay window of your specific instrument. Prepare High and Low FP Mixtures in quantities sufficient to perform at least 6 replicates for each condition.

Use UDP² Alexa Fluor® 633 Tracer and Stop & Detect Buffer B at 0.25X concentration in a 20 µL final reaction volume. This mimics the 4-fold dilution when adding 5 µL of detection mixture to 15 µL of an enzyme reaction. As an example, the 1X detection mixture may contain 16 nM tracer. After adding this to the enzyme reaction, the concentration in the final 20 µL reaction volume would be 4 nM.

High FP Mixture

Prepare the following High FP Mixture as indicated in the table. Pipette 20 µL of the Total High FP Mixture to each well (from the example: 20 µL from 500 µL). Do not further dilute.

Component	Stock Concentration	Final Concentration	Example: 25 Assays	Your Numbers
UDP² Antibody	3.1 mg/mL	5 µg/mL	0.8 µL	
5X Stop & Detect Buffer B	5X	0.25X	25 µL	
UDP² Alexa Fluor® 633 Tracer	800 nM	4 nM	2.5 µL	
Water			471.7 µL	
Total			**500.0 µL**	

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

Low FP Mixture

Prepare the following Low FP Mixture as indicated in the table. Pipette 20 µL of the Total Low FP Mixture to each well (from the example: 20 µL from 500 µL). Do not further dilute.

Component	Stock Concentration	Final Concentration	Example: 25 Assays	Your Numbers
5X Stop & Detect Buffer B	5X	0.25X	25 µL	
UDP² Alexa Fluor® 633 Tracer	800 nM	4 nM	2.5 µL	
Water			472.5 µL	
Total			**500.0 µL**	

Caution: Contact BellBrook Labs Technical Service for assistance if the assay window is <150 mP.

4.1.3 Measure the FP

Subtract the Low FP Mixture readings from the corresponding High FP Mixture readings. The difference between the low and high FP values should be >150 mP. This measurement gives the *maximal signal window* that the instrument is capable of generating with these reagents. The *assay window* will be less than this value, and will depend on how far the UDP enzyme reaction proceeds.

4.2 Determine the Optimal UDP² Antibody Concentration

The Transcreener® UDP² FP Assay requires detection of UDP in the presence of excess UDP-sugar (assuming initial velocity enzyme reaction conditions) using an antibody with a finite selectivity for free UDP. The concentration of UDP² Antibody determines the total assay window and the UDP detection range; the amount needed primarily depends upon the UDP-sugar concentration in the enzyme reaction. To produce the most sensitive and robust assay signal, it is necessary to perform a UDP² Antibody titration in the buffer system ideal for your enzyme or donor sugar.

4.2.1 Titrate the UDP² Antibody

1. Prepare the reaction buffer: 35 mM HEPES (pH 7.5), 5 mM $MgCl_2$, and 0.01% Brij-35. Include the UDP-sugar and substrate but omit the enzyme.
2. Dispense 15 µL of the reaction buffer into each well of columns 2–24.
3. Dispense 30 µL of UDP² Antibody (at 2 mg/mL in the same reaction buffer) into each well of column 1.
4. Remove 15 µL from each well of column 1 and add it to the corresponding well of column 2.
5. Repeat step 4 for the remaining columns, thereby performing a 2-fold serial dilution across the plate to column 24.
6. Add 5 µL of UDP² Alexa Fluor® 633 Tracer (to a final concentration of 4 nM) in 1X Stop & Detect Buffer B to each well.
7. Mix the plate, equilibrate at room temperature for 1 hour, and measure FP.

4.2.2 Calculate the Optimal UDP² Antibody Concentration

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

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

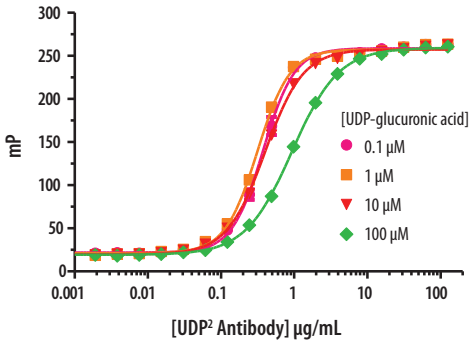


Figure 3. UDP² Antibody titration at various UDP-glucuronic acid concentrations. The nucleotide concentration reflects the amount in the enzyme reaction prior to the addition of the UDP Detection Mixture. The UDP² Antibody (15 µL) in enzyme reaction mix (50 mM Tris [pH 7.5], 5 mM $MgCl_2$, 8 mM EGTA, 1% DMSO, and UDP-glucuronic acid) was added to 5 µL of the Detection Mixture (16 nM UDP² Alexa Fluor® 633 Tracer in 1X Stop & Detect Buffer B) (n = 2). The data are plotted as mP vs. log [UDP] using nonlinear regression curve fitting. The amount of antibody required in your UDP Detection Mixture for future experiments is 4 × EC_{85}.

4.3 Optimize the Enzyme Concentration

Perform an enzyme titration to identify the optimal enzyme concentration for the Transcreener® UDP² FP Assay. Use enzyme buffer conditions, substrate, and UDP-sugar concentrations that are optimal for your target enzyme and UDP² Antibody 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 FP 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:

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

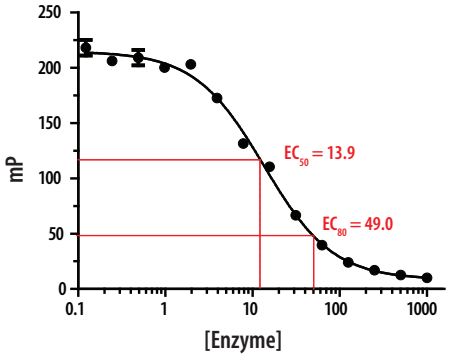


Figure 4. Enzyme titration curve. The ideal range of enzyme concentrations is shown in red.

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 FP 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 B.

4.4 Run an Assay

4.4.1 Experimental Samples

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:

	Stock Concentration	Detection Mixture Concentration*	Example	Your Numbers
UDP² Antibody	3.1 mg/mL	20 µg/µL*	32.3 µL	
UDP² Alexa Fluor® 633 Tracer	800 nM	16 nM	100 µL	
5X Stop & Detect Buffer B	5X	1X	1,000 µL	
Water			3,867.7 µL	
Total			**5,000 µL**	

*Final concentrations in the detection mixture should be 16 nM tracer, 1X Stop & Detect Buffer B, and 4 × [EC_{85}] UDP² Antibody concentration as determined in **Section 4.2**.

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 FP.

4.4.2 UDP Detection Controls

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

Component	Notes
Minus Antibody (Free Tracer) Control	This control contains the UDP² Alexa Fluor® 633 Tracer without the UDP² Antibody and is set to 20 mP.
Minus Tracer Control	This control contains the UDP² Antibody without the ADP Alexa Fluor® 633 Tracer and is used as a sample blank for all wells. It contains the same UDP² Antibody concentration in all wells.

5.0 General Considerations

5.1 Assay Types

5.1.1. Endpoint Assay

The Transcreener® UDP² FP 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 B, 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. Note that the optimal UDP² Antibody 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 FP assay window at 10% substrate conversion was determined after the addition of the UDP Detection Mixture to the standard samples. The mP value at 10% substrate conversion (10 µM UDP-glucuronic acid) remained constant (<10% change) for at least 24 hours at room temperature (20–25°C). If you plan to read FP 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 24 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 antibody concentration* • To achieve maximum sensitivity and assay window, the UDP² Antibody 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 1–100 µM.
No change in FP observed	*Low antibody/tracer activity* • The tracer and antibody are stable for up to 10 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.
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 the concentrations of UDP-glucuronic acid and UDP shown in **Table 1**. Commonly, 8- to 12-point standard curves are used.

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

% Conv.	UDP-Glucuronic Acid (µM)	UDP (µM)
100	0	100
50	50	50
30	70	30
15	85	15
10	90	10
7.5	92.5	7.5
5	95	5
3	97	3
2	98	2
1	99	1
0.5	99.5	0.5
0	100	0

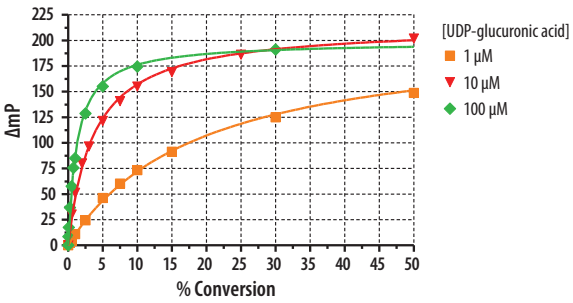


Figure 5. UDP-glucuronic acid/UDP standard curves. A) Sample data was plotted for for 1 µM, 10 µM, and 100 µM UDP-glucuronic acid/UDP standard curves. The nucleotide concentration reflects the amount in the enzyme reaction, prior to the addition of the UDP Detection Mixture. A polarization shift of 60–100 mP and a Z' value of 0.5 indicates robust assay performance for HTS applications. For the initial UDP-glucuronic acid concentration of 1 µM, these criteria were achieved at <7.5% conversion; for 10 µM UDP-glucuronic acid, at 2% conversion ; and for 100 µM UDP-glucuronic acid concentration, at 0.75% conversion.

Use the following equations to calculate the Z' factor:

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

$$Z' = 1 - \frac{[(3 \times SD_{\text{initial [UDP-sugar]}}) + (3 \times SD_{\text{sample}})]}{|(mP_{\text{initial [UDP-sugar]}}) - (mP_{\text{sample}})|}$$

7.2 Summary of Additive Effects on the Transcreener® UDP² FP Assay

The assay window at 10% substrate conversion (10 µM UDP-sugar) 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)[a]	5-Hour Tolerance (0–10% Conversion Signal)[b]
Solvents		
Acetonitrile	>50%	>50%
DMSO	25.00%	1.56%
DMF	12.50%	6.25%
Ethanol	>50%	25.00%
Methanol	>50%	>50%
Glycerol	>50%	6.30%
Detergents		
Brij-35	0.47%	0.06%
CHAPS	0.16%	0.04%
NP40	0.08%	0.08%
SDS	0.08%	0.02%
Triton X-100	0.08%	0.08%
Sodium deoxycholate	0.16%	0.01%
N-lauroyl sarcosine	0.08%	0.01%
Metal chelates		
EDTA	>250 mM	15.6 mM
EGTA	>250 mM	16.6 mM
Reductants		
Beta mercaptoethanol	3.13%	1.60%
Dithiothreitol	250 mM	125 mM
Salts		
Ammonium acetate	>500 mM	16.3 mM
Ammonium sulfate	31.3 mM	7.8 mM
Calcium chloride	15.6 mM	7.8 mM
Magnesium acetate	125 mM	7.8 mM
Magnesium chloride	62.5 mM	31.3 mM
Magnesium sulfate	15.6 mM	3.9 mM
Manganese chloride	15.6 mM	15.6 mM
Potassium chloride	>1000 mM	62.5 mM
Sodium azide	5.00%	0.63%
Sodium bromide	>1000 mM	125 mM
Sodium chloride	1250 mM	78.1 mM

Component	5-Hour Tolerance (0–100% Conversion Signal)[a]	5-Hour Tolerance (0–10% Conversion Signal)[b]
Phosphatase Inhibitors		
Glycerol phosphate	62.5 mM	15.6 mM
Imidazole	>1000 mM	125 mM
Sodium fluoride	31.3 mM	15.6 mM
Sodium molybdate	250 mM	125 mM
Sodium tartrate	>400 mM	50 mM
Sodium orthovanadate	31.3 mM	0.39 mM
Sodium pyrophosphate	1.6 mM	7.8 mM
Carrier Proteins/Coactivators		
BSA	0.625 mg/mL	0.16 mg/mL
BGG	0.01 mg/mL	0.001 mg/mL
Calmodulin	0.16 mg/mL	0.08 mg/mL

a. <10% drop in ΔmP observed at the listed concentration and below.

b. mP at 0% or 10% increased or decreased <3 standard deviations of the plate controls at the listed concentration and below.

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

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BellBrook Labs

5500 Nobel Drive, Suite 230
Madison, Wisconsin 53711 USA
Email: info@bellbrooklabs.com
Phone: 608.443.2400
Toll-Free: 866.313.7881
FAX: 608.441.2967