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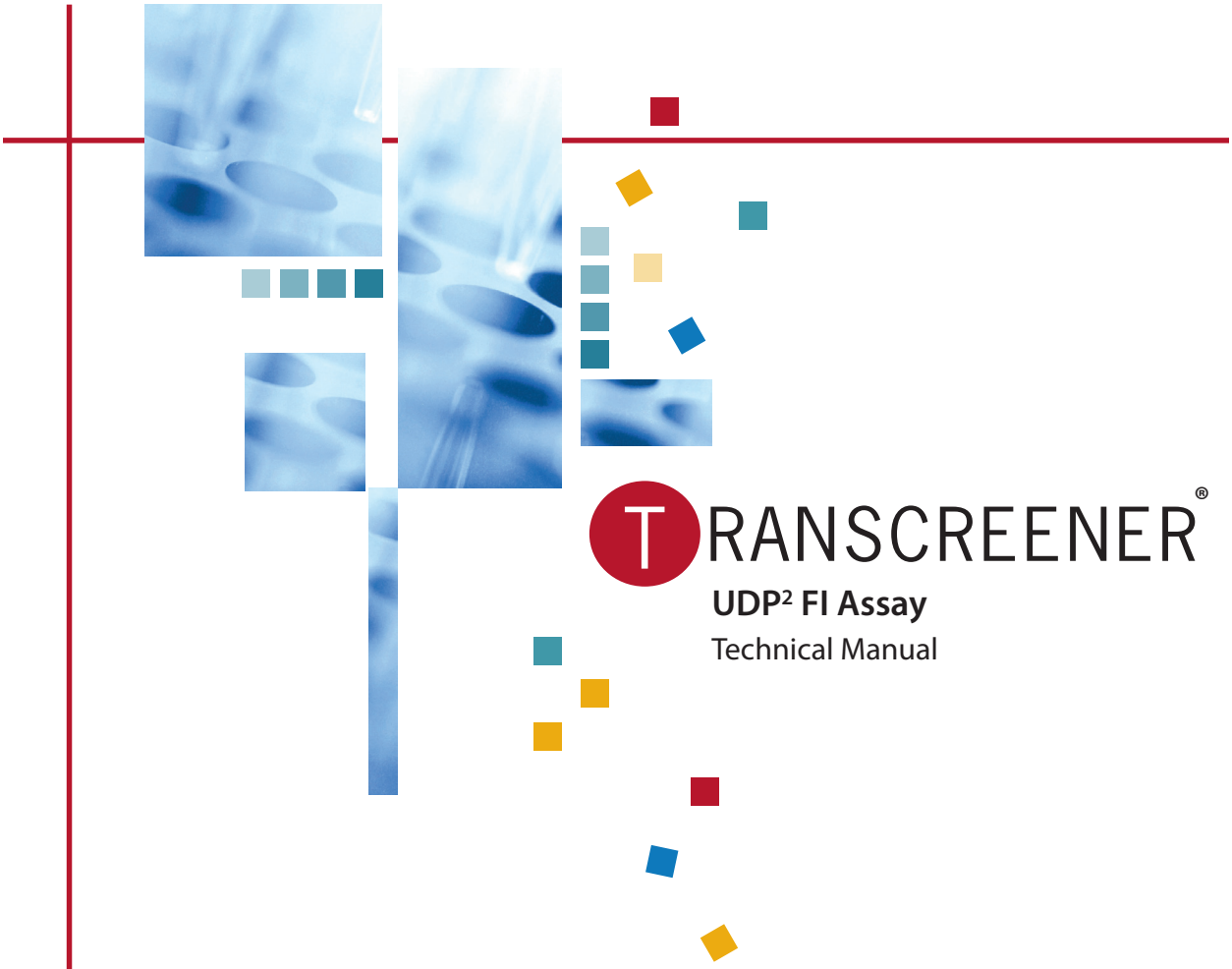
RED FI



TRANSCREENER®

UDP² FI Assay

Technical Manual

Instructions for Part Numbers 3019-A, 3019-1K, and 3019-10K

Rev 2023-05

Transcreener® ADP² FI Assay Technical Manual

Contents

1.0 Introduction

The Transcreener assay platform 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 FI Assay extends the Transcreener platform for UDP detection by utilizing a simple fluorescent intensity (FI) output. It can be used on fluorescence readers typically found in academic and therapeutic research laboratories, as well as more complex multimode plate readers more commonly used in core facilities and HTS facilities. Texas Red filter sets are compatible with the assay.

The assay is a red, competitive FI method (**Figure 1**).The Transcreener UDP² FI Assay is a universal biochemical HTS assay for enzymes that produce UDP, including glycosyltransferase, galactosyltransferase, glucuronyltransferase, N-acetylglucosamyltransferase, N-acetylgalactosyltransferase, xylosyltransferase, and glycogen, cellulose, lactose, and hyaluronan synthases. Enzyme activity is signaled by a increase in fluorescence intensity as the bound tracer is displaced from the Transcreener UDP² Antibody-IRDye QC-1. The assay is a simple single step mix-and-read format enabling the use of unmodified native substrate concentrations of 1 – 100 µM.

The Transcreener® UDP² FI Assay provides the following benefits:

- Accommodates UDP concentrations ranging from 1 µM to 100 µM.
- Excellent data quality (Z' ≥ 0.7) and signal at low substrate conversion using 1 µM UDP.
- 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.
- Red tracer further minimizes interference from fluorescent compounds and light scattering.

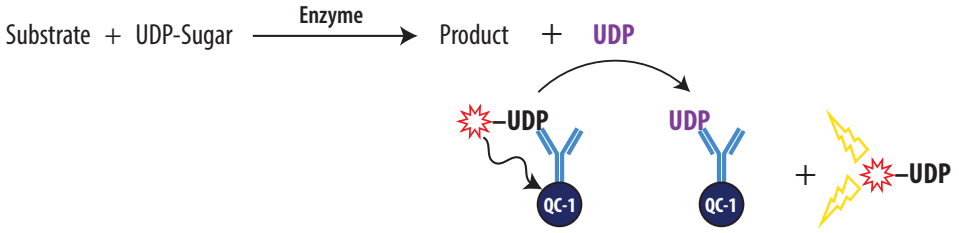


Figure 1. Schematic overview of the Transcreener UDP² FI Assay. The Transcreener UDP Detection Mixture contains a quenched UDP Alexa Fluor 594 tracer bound to an UDP² antibody conjugated to an IRDye QC-1 quencher. UDP produced by the target enzyme displaces the tracer, which is no longer quenched and causes a positive increase in FI.

2.0 Product Specifications

Product	Quantity	Part #
Transcreener® UDP² FI Assay	200 assays*	3019-A
	1,000 assays*	3019-1K
	10,000 assays*	3019-10K

*The exact number of assays depends on enzyme reaction conditions. The kits are designed for use with 96-well plates using 50 µL reaction volumes (3019-A), or 384-well plates (3019-1K and 3019-10K) using 20 µL reaction volumes.

Storage

Store all reagents at –20°C upon receipt. Although not included in the kit, please be sure to aliquot and store UDP-sugar as directed by the manufacturer. UDP-sugars commonly breakdown and can result in poor assay quality. Be sure to avoid multiple freeze-thaw cycles.

2.1 Materials Provided

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

Component	Composition	Notes
UDP² Antibody–IRDye® QC-1	1.4 mg/mL* solution in 100 mM KH_2PO_4 (pH 8.5)	The concentration of antibody needed for an enzyme target is dependent upon the UDP concentration and buffer conditions in the enzyme reaction (see **Section 4.2**). Sufficient antibody is included in the kit to complete 200 assays (Part # 3013-A), 1,000 assays (Part # 3013-1K), or 10,000 assays (Part # 3013-10K) at an UDP concentration up to 100 µM. **Note: The Antibody IR-Dye QC-1 can have precipitate upon thawing. If so, spin down the precipitate and continue using the supernatant in the assay. The antibody will perform as directed.**
UDP Alexa Fluor® 594 Tracer	800 nM solution 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	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 FI measurement is 0.5X.
UDP	5 mM	UDP is used to create a donor substrate/UDP standard curve.

Note: This kit contains sufficient reagents for up to 100 µM UDP. Please contact us if you plan on using greater than 100 µM UDP.

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, buffer, enzyme cofactors, substrates, and test compounds.
- ***Plate Reader***—A multidetection microplate reader configured to measure FI of the Alexa Fluor® 594 tracer is required. The Transcreener ADP² FI Assay has been successfully used on the following instruments: Perkin Elmer EnVision®; Molecular Devices Spectramax M2; and Tecan Infinite® M200 and Safire2™ (see **Table 1**).
- ***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) and Corning 96-well, half-area plates (Cat. # 3686).
- ***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 FI 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 FI instrument and verify that it is compatible with the assay being performed (see **Section 4.1**).

4.0 Protocol

The Transcreener® UDP² FI 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; increase each volume to 25 µL (final volume 50 µL) if performing the assay in 96 well half-volume plates. The use of different densities or reaction volumes will require changes in reagent quantities.

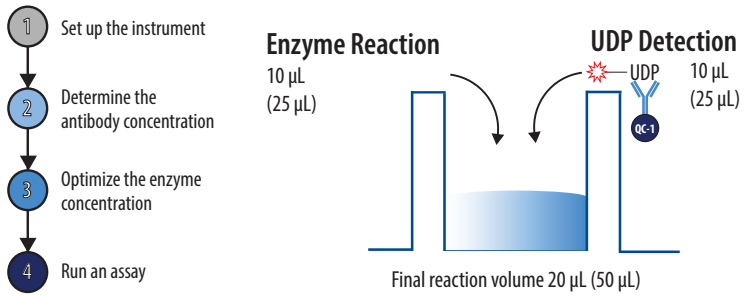


Figure 2. An outline of the procedure. The assay consists of 4 main steps with a mix-and-read format. Volumes shown are for 384-well plates (and 96-well plates).

4.1 Set Up the Instrument

Becoming familiar with ideal instrument settings for FI is essential to the success of the Transcreener UDP² FI Assay. **Table 1** shows common instrument parameters.

Note that use of narrow bandwidth filters is critical for assay performance because the Stoke's shift (separation between excitation and emission maxima) for the Alexa Fluor 594 is relatively narrow. It is possible to use wider bandwidth filters for some instruments, but it requires the use excitation and emission wavelengths different from those shown below in order to avoid spectral overlap.

Plate Reader	Excitation Filter/ Bandwidth	Emission Filter/ Bandwidth	Mirror Module	Other Parameters
Envision (Perkin Elmer)	545 nm/7 nm (Cat. # 2100-5070)	635 nm/15 nm (Cat. # 2100-5590)	D595	Mirror: Texas Red FP single mirror Cat. # 2100-4190
PHERAstar Plus (BMG Labtech)	580 nm/10 nm	620 nm/10 nm	NA	
Safire2 (Tecan)	580 nm/10 nm	620 nm/10 nm	NA	Monochromator-based
SpectraMax M2 (Molecular Devices)	584 nm	612 nm	NA	Emission filter auto-cutoff at 610 nm

Table 1. Instrument filters and settings for commonly used multimode plate readers. 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 FI

Ensure that the instrument is capable of measuring FI of Alexa Fluor 594. The optimal excitation/emission settings for the UDP Alexa Fluor 594 Tracer are excitation 590 nm (10 nm bandwidth) and emission 617 nm (10 nm bandwidth). The UDP Alexa Fluor 594 Tracer has been successfully used at excitations of 580–590 nm and emissions of 610–620 nm with bandwidths of 10 nm (see **Table 1**).

4.1.2 Define the Maximum FI Window for the Instrument

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

Use both tracer and antibody at 0.5X concentration in the final reaction volume. This mimics the 2-fold dilution when adding an equal volume of detection mixture to an enzyme reaction. As an example, if the calculated antibody concentration is 10 µg/mL, the concentration used here would be 5 µg/mL.

Low RFU Mixture

Prepare the following Low RFU Mixture as indicated in the table. Pipette 20 µL of the Total Low RFU 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–IRDye QC-1	1.4 mg/mL	5 µg/mL	1.8 µL	
10X Stop & Detect Buffer B	10X	0.5X	25.0 µL	
UDP Alexa Fluor® 594 Tracer	800 nM	4 nM	2.5 µL	
Water			470.7 µL	
Total			**500.0 µL**	

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

High RFU Mixture

Prepare the following High RFU Mixture as indicated in the table. Pipette 20 µL of the Total High RFU 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
10X Stop & Detect Buffer B	10X	0.5X	25.0 µL	
UDP Alexa Fluor 594 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 ratio is <5.0.

4.1.3 Measure the FI

Subtract the Low RFU Mixture readings from the corresponding High RFU Mixture readings. The difference between the low and high RFU values will give the maximum assay window. The values will differ, depending on the units from the plate reader, but the ratio (High RFU Mixture):(Low RFU Mixture) should be >5.0.

4.2 Determine the Optimal UDP² Antibody–IRDye QC-1 Concentration

The UDP² Antibody-IRDye QC-1 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 determined the optimal UDP² Antibody-IRDye QC-1 concentrations up to 100 µM, perform an UDP² Antibody-IRDye QC-1 titration using the reaction conditions for your enzyme or drug target.

4.2.1 Prepare a 500 µg/mL UDP² Antibody Stock-IRDye QC-1 Solution

Make a stock solution for UDP² antibody at 500 µg/mL in the enzyme reaction buffer.

4.2.2 Prepare the Enzyme Reaction Buffer

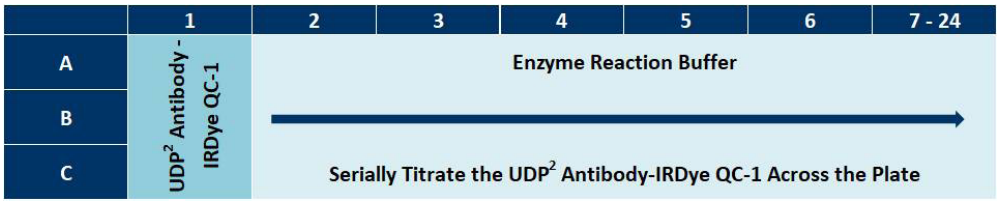
Prepare your enzyme reaction buffer **without enzyme**. The recipe for the enzyme reaction buffer depends upon the target enzyme.

4.2.3 Prepare Detection Mixture Containing UDP-Sugar Donor, Acceptor Substrate, UDP² AlexaFluor 594 Tracer in Stop & Detect Buffer B.

Prepare 8 nM UDP² AlexaFluor 594 Tracer in 1X Stop & Detect Buffer B with UDP-sugar and acceptor substrate (concentrations depend upon your target).

4.2.4 Titrate the UDP² Antibody-IRDye QC-1 Stock Solution.

Dispense 20 µL of UDP² Antibody-IRDye QC-1 Stock Solution into the column 1 wells. Dispense 10 µL of the enzyme reaction buffer 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).



4.2.5 Add Detection Mixture

Dispense 10 µL of the Detection Mixture (containing UDP-sugar, acceptor substrate, 8 nM tracer, and 1X Stop & Detect Buffer B) to all wells on the assay plate from column 1 to column 24. Do not titrate.

4.2.6 Incubate and Measure Fluorescence Intensity

Mix the plate, equilibrate at room temperature (1 hour), and measure fluorescence intensity according to the instrument settings established in **Section 4.1**.

4.2.7 Plot RFU vs. log of UDP² Antibody-IRDye QC-1 Concentration

The antibody concentration at the EC_{10} is 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.

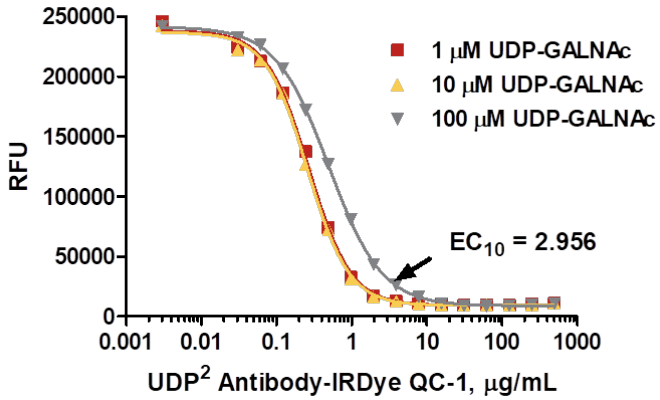


Figure 3. UDP² Antibody-IRDye QC-1 titration at various UDP-GALNAc concentrations. Sample data for 1 µM, 10 µM, and 100 µM UDP-GALNAc binding curves. The nucleotide concentration reflects the amount in the enzyme reaction prior to the addition of the UDP Detection Mixture. 10 µL of Detection Mix was added to 10 µL the enzyme reaction mix.

4.2.8 Calculate Antibody Concentration for Detection Mixture

Use the following equation or results from your plot to calculate the EC_{10}. The amount of antibody required in your 1X UDP Detection Mixture will be the EC_{10}. Please note that the graphical example starts at an antibody concentration of 500 µg/mL in the 10 µL enzyme buffer. If graphing from the final concentration of 250 µg/mL in the 20 µL volume you will need 2X the EC_{10} in your 1X UDP Detection Mixture.

$$EC_{10} = (10 / (100\text{-}10))^{(1/\text{hillslope})} \text{ X } EC_{50}$$

From the example above: The EC_{50} was found to be 0.499 µg/mL. Using the equation (or graphing software) the EC_{10} is 2.956 µg/mL. Once 10 µL of UDP Detection Mix is added to the enzyme reaction the final concentration will be your original 0.5X EC_{10}, or as in this example 1.478 µg/mL.

4.3 Optimize the Enzyme Concentration

Perform an enzyme titration to identify the optimal enzyme concentration for the Transcreener UDP² FI Assay. Use enzyme buffer conditions, substrate, and UDP-sugar concentrations that are optimal for your target enzyme. 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 TRIS (pH 7.5), 5 mM $MgCl_2$, 1% DMSO (test compound solvent), and 0.01% Brij-35. Run your enzymatic reaction at its requisite temperature and time period. Refer to **Section 5.2.3** 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 FI 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:

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

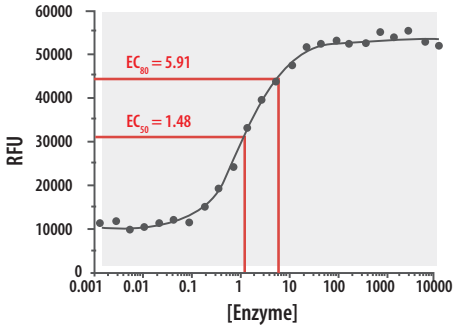


Figure 4. Enzyme titration curve. The ideal range of enzyme concentrations is shown in red.

4.3.2 Enzyme Assay Controls

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

Component	Notes
0% Conversion from UDP-Sugar Control	This control consists of the UDP Detection Mixture, the enzyme reaction components (without enzyme), and 100% UDP-Sugar (0% UDP). It defines the lower limit of the assay window.
100% Conversion to UDP Control	This control consists of the UDP Detection Mixture, the enzyme reaction components (without enzyme), and 100% UDP (0% UDP-Sugar). It defines the upper 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., UDP-Sugar) or acceptor substrate.
UDP/UDP-Sugar Standard Curve	Although optional, an UDP/UDP-Sugar 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	Use only 0.5X enzyme reaction conditions and Stop & Detect Buffer B.

4.4 Run an Assay

4.4.1 Experimental Samples

1. Add the enzyme reaction mixture to test compounds and mix on a plate shaker.
2. Start the reaction by adding UDP-Sugar and acceptor substrate, then mix. The final volume of the enzyme reaction mixture should be 10 µL (384-well plates) or 25 µL (96-well plates). Incubate at a temperature and time ideal for the enzyme target before adding the ADP Detection Mixture.
3. Prepare 1X UDP Detection Mixture as follows:

Component	Stock Concentration	Final Concentration	Example: 1000 Assays	Your Numbers
UDP² Antibody–IRDye QC-1	1.4 mg/mL	2.956 µg/mL*	21.1 µL	
UDP Alexa Fluor® 594 Tracer	800 nM	8 nM	100 µL	
10X Stop & Detect Buffer B	10X	1X	1000.0 µL	
Water			8,878.9 µL	
Total			**10,000.0 µL**	

*The EC_{10} obtained from **Section 4.2.** Your number will vary

Final concentrations in the detection mixture should be 8 nM tracer, 1X Stop & Detect Buffer B, and the antibody concentration calculated in **Figure 3 (Section 4.2)**.

5. Add 10 µL (384-well plates) or 25 µL (96-well plates) of 1X UDP Detection Mixture to the enzyme reaction. Mix using a plate shaker.
6. Incubate at room temperature (20–25°C) for 1 hour and measure FI.

4.4.2 UDP Detection Controls

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

Component	Notes
Minus Antibody (Free Tracer) Control	This control contains the UDP Alexa Fluor® 594 Tracer without the UDP² Antibody–IRDye® QC-1 and determines the maximum RFU value achievable.
Minus Tracer Control	This control contains the UDP² Antibody–IRDye® QC-1 without the UDP Alexa Fluor® 594 Tracer and is used as a sample blank. It contains the same antibody concentration in all wells.

5.0 General Considerations

5.1 Assay Types

5.1.1. Endpoint Assay

The Transcreener® UDP² FI Assay is designed for endpoint readout. The Stop & Detect Buffer B contains EDTA to stop Mg^{2+}-dependent enzyme reactions by chelating available Mg^{2+}.

5.1.2 Real-Time Assay

You can perform real-time experiments by substituting the Stop & Detect Buffer B, 10X (provided) with a detection buffer that does not contain EDTA. However, the equilibration time for the tracer and UDP² Antibody–IRDye® QC-1 is greater than 15 minutes, making it difficult to quantitate UDP produced during short-term enzyme reactions. Note that the optimal antibody concentration may change when EDTA is omitted.

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 FI assay window at 10% substrate conversion was determined after the addition of the UDP Detection Mixture to the standard samples. The RFU value at 10% substrate conversion (10 µM UDP) remained constant (<10% change) for at least 24 hours at room temperature (20–25°C). If you plan to read FI on the following day, seal the plates to prevent evaporation.

5.2.2 UDP Detection Mixture Stability

The ADP 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).

5.2.3. Solvent Compatibility

The RFU window at 10% substrate conversion (10 µM UDP) remains constant (<10% change) when up to 10% DMSO, DMF, ethanol, acetonitrile, ethanol, or methanol are used in the enzyme reaction. How solvents react to enzymes should be tested by the researcher. Contact BellBrook Labs for further reagent compatibility information.

6.0 Troubleshooting

Problem	Possible Causes and Solutions
Small Assay Window	*Suboptimal antibody concentration* • Under the reaction conditions used in the Transcreener® UDP² FI Assay, for the UDP² Antibody–IRDye® QC-1 to achieve maximum sensitivity and assay window, the antibody concentration must be optimized for each UDP/UDP-sugar concentration. *UDP concentration out of range* • Ensure that the starting UDP concentration is in the range of 1–100 µM.
No change in FI 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.
Does BSA Interfere with the assay?	Bovine Serum Albumin (BSA) interferes with the detection reagents and should be avoided. Detergent such as Brij-35 can be substituted in the enzyme reaction to prevent non-specific binding of enzymes and substrates to the plate,

7.0 Appendix

7.1 UDP/UDP-Sugar Standard Curve

The standard curve mimics an enzyme reaction (as UDP-sugar concentration decreases, UDP concentration increases). The UDP/UDP-sugar standard curve allows calculation of the concentration of UDP produced in the enzyme reaction and, therefore, the % UDP-sugar consumed (% conversion). In this example, a 12-point standard curve was prepared using the concentrations of UDP-sugar and UDP shown in **Table 2**. Commonly, 8- to 12-point standard curves are used.

Table 2. Concentrations of UDP-sugar/UDP to prepare a 12-point standard curve.

% Conv.	UDP-sugar(µM)	UDP (µM)
100	0	100
60	40	60
40	60	40
30	70	30
20	80	20
15	85	15
10	90	10
8.0	92	8.0
6.0	94.0	6.0
4.0	96.0	4.0
2.0	98.0	2.0
0	100	0

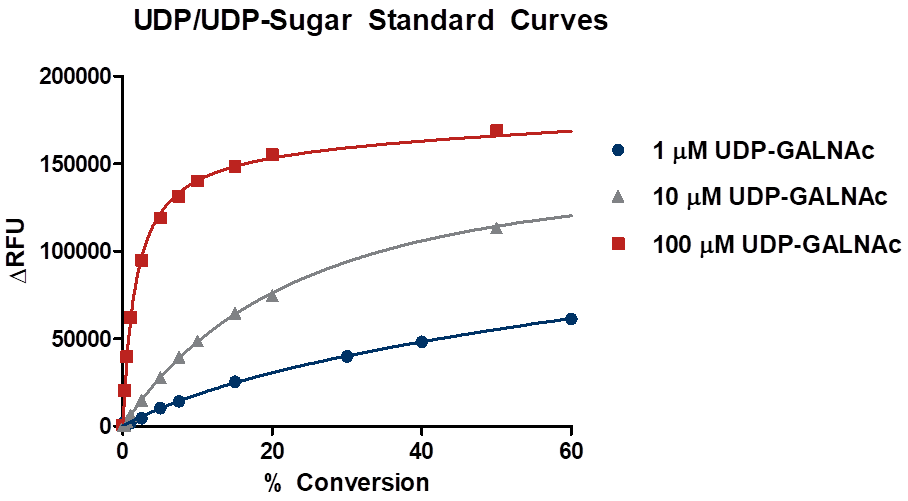


Figure 6. UDP/UDP-sugar standard curves. A) Sample data for, 1 µM, 10 µM, and 100 µM UDP/UDP-sugar standard curves. The nucleotide % conversion concentration reflects the amount in the enzyme reaction, prior to the addition of the UDP Detection Mixture. Curves were obtained in a final 20 µL assay volume after adding 10 µL of reaction mix (50 mM HEPES [pH 7.5], 4 mM $MgCl_2$, 1% DMSO, 0.01% Brij, and UDP-GALNAc/UDP standards) to 10 µL detection mix (UDP² Antibody–IRDye® QC-1, 8 nM UDP Alexa Fluor® 594 Tracer, 1X Stop & Detect Buffer. The data are plotted using 4-parameter nonlinear regression curve fitting.

Tabel 3. Z′ determination based on UDP-GALNAc Conversion using the antibody concentration determined from Figure 3 (n = 24). Excellent Z′ values are obtained for the range of UDP-GALNAc concentrations used.

% UDP Conversion	Z′ Values at Varying UDP-GALNAc Concentration		
	1 µM UDP-GALNAc	10 µM UDP-GALNAc	100 µM UDP-GALNAc
0.5	NA	0.5	0.8
1.0	0.0	0.6	0.8
2.5	0.5	0.8	0.9
5.0	0.7	0.9	0.9
10	0.7	0.9	0.9
100	0.8	0.9	0.9

Use the following equation to calculate the Z′ factor:

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

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