Technical Manual

Product #: 3018-1K and 3018-10K

Transcreener® UDP^2 FP Assay Kit

Contents

1.0 Introduction

The Transcreener® UDP^2 FP 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 decrease in fluorescence polarization as the bound tracer is displaced from the Transcreener® UDP^2 Antibody. The assay is a simple single step mix-and-read format enabling the use of unmodified native substrate concentrations of 1 – 1000 µM. The assay provides excellent signal at low substrate conversion, with a Z' ≥0.7 and ≥100 millipolarization shift (mP) under normal reaction conditions.

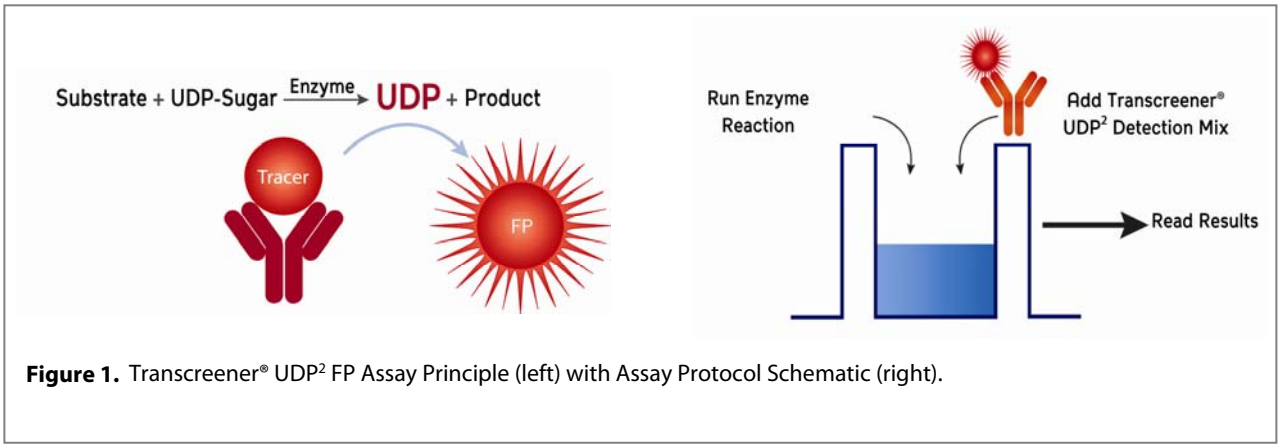


Figure 1. Transcreener® UDP^2 FP Assay Principle (left) with Assay Protocol Schematic (right).

2.0 Transcreener® UDP² Assay Components

Materials Provided

The kit contains sufficient reagents for 1,000 wells detecting concentrations up to 100 µM UDP. Store reagents at –20°C as indicated.

Component	Description
UDP² AlexaFluor® 633 Tracer, 800 nM	The UDP² AlexaFluor® 633 Tracer is provided at 800 nM in 2 mM HEPES, pH 7.5 containing 0.01% Brij-35. The UDP² AlexaFluor® 633 Tracer is 4 nM at the time of polarization measurement (20 µL).
UDP² Antibody	A concentrated monoclonal UDP² Antibody is provided in PBS with 10% glycerol. The concentration of UDP² Antibody needed for a particular target enzyme depends on enzyme conditions particularly the initial donor substrate concentration (i.e UDP-sugar).
Stop & Detect Buffer B, 5X	The Stop & Detect Buffer B 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+} -requiring enzyme reactions and aid in the detection and stabilization of the FP signal. Other reagents may be necessary to stop some enzymes.
5 mM UDP	UDP is used to create a UDP-sugar/UDP standard curve.

Materials Required

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 — Necessary enzyme reaction components include enzyme, enzyme cofactors, substrates, and test compounds.

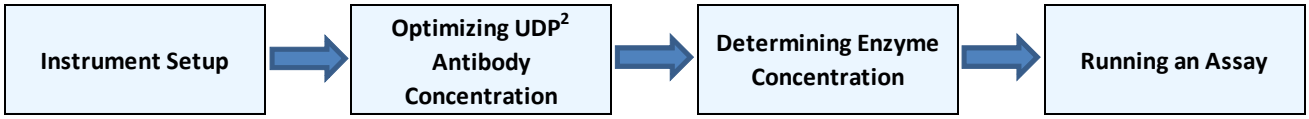
Plate Reader — A fluorescence polarization plate reader configured to measure fluorescence polarization of AlexaFluor® 633 is required. The Transcreener® UDP² FP Assay using has been successfully performed on the following instruments: BMG Labtech PHERAstar, Perkin Elmer EnVision, and Tecan Safire². NOTE: Contact BellBrook Labs for additional instruments, information regarding instrument set up, and fluorescence polarization measurements.

Assay Plate — It is important that assay plates be entirely black with a non-binding surface. We highly recommend Corning® 384 well plates (catalog #3676).

Liquid Handling Devices — Use liquid handling devices that can accurately dispense a minimum of 2.5 µL into **384 well plates**.

3.0 Protocol

The following protocol was developed for 384 well plate format with 15 µL enzyme reactions and 20 µL final volume at the time the plates are read. Use of the assay at different densities and well volumes will require proportional changes in reagent quantities.



3.1 Instrument Set Up

Determination of the optimal instrument settings for fluorescence polarization is essential to the success of the Transcreener® UDP² FP Assay. Refer to Table 1 for filter sets and settings for common multimode plate readers. If you are using a different instrument, verify that it can measure fluorescence polarization (not simply fluorescence intensity). Contact BellBrook Labs if you have questions about settings and filter sets for a specific instrument.

PerkinElmer EnVision®		
Mirror	D658fp/D688 dual mirror	Part# 2100-4260
Excitation Filter/Bandwidth	620/40 nm	Part# 2100-5760
Emission Filter(P-pol)/Bandwidth	688/45 nm	Part# 2100-5790
Emission Filter(S-pol)/bandwidth	688/45 nm	Part# 2100-5780
BMG LABTECH PHERAstar Plus (Transcreener FP Optic Module)		
Excitation Filter/Bandwidth	590/50 nm	
Emission A Filter/Bandwidth	675/50 nm	
Emission B Filter/Bandwidth	675 nm	
Tecan Safire2™ (monochromator based)		
Excitation Wavelength/Bandwidth	635/20 nm	
Emission Wavelength/Bandwidth	670/20 nm	
Read-FP Mode		

EnVision is a registered trademark of PerkinElmer. Safire² is a trademark of Tecan.

Table 1. Instrument Filters & Settings. Filter settings and filter part numbers for commonly placed multimode plate readers. A complete list of instruments and instrument-specific application notes can be found online: www.bellbrooklabs.com/transcreener_instrument_validation.html

i. Define the maximum polarization window for your instrument

Determine high (tracer + antibody) and low (free tracer) polarization values to define the maximum signal window for your specific instrument.

ii. Prepare High and Low polarization mixtures

High Polarization mixture (+ antibody): Add 5 µL of Detection Mixture (containing 20 µg/mL UDP² Antibody, 16 nM UDP² AlexaFluor® 633 Tracer, and 1X Stop & Detect Buffer B) to 15 µL enzyme reaction buffer devoid of enzyme and UDP.

Low Polarization mixture(- antibody): Add 5 µL of Detection Mixture (containing 16 nM UDP² AlexaFluor® 633 Tracer and 1X Stop & Detect Buffer B) to 15 µL enzyme reaction buffer devoid of enzyme and UDP.

iii. Measure the fluorescence polarization

The low and high polarization values should differ by >175 mP. Note that 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, and will depend on how far the UDP enzyme reaction proceeds. **If the signal window is <175 mP please contact BellBrook Labs for suggestions on optimizing the instrument filters or settings.**

3.2 Optimizing UDP^2 Antibody Concentration

The UDP^2 Antibody 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 UDP^2 Antibody concentrations up to 100 µM, perform an UDP^2 Antibody titration using the reaction conditions for your enzyme or donor sugar.

i. Titrate UDP^2 Antibody in Enzyme Reaction Buffer containing UDP-sugar

Prepare your enzyme reaction mixture with and without UDP^2 Antibody (1 mg/mL) (include substrate and UDP-sugar, but omit enzyme). Dispense 30 µL of mixture (with antibody) into wells in column 1. Dispense 15 µL of the mixture (without antibody) across a 384-well plate (columns 2 – 24). Remove 15 µL from column 1 and serially titrate the contents across the plate (to column 24).

ii. Add Stop & Detect Buffer containing UDP^2 AlexaFluor® 633 Tracer

Prepare a 1X Stop & Detect Buffer containing 16 nM UDP^2 AlexaFluor®633 Tracer. Add 5 µL to the titrated antibody. Mix the plate, equilibrate at room temperature (1 hour), and measure fluorescence polarization according to the Instrument Settings established in Section 3.1

iii. Plot mP vs. log of UDP^2 Antibody Concentration and Calculate the EC$_{85}$

The antibody concentration at the EC$_{85}$ is used as a good compromise between sensitivity and maximal assay window. The EC$_{85}$ is determined by inputting the EC$_{50}$ and hillslope values from a sigmoidal dose response curve fit into the equation below.

$$EC_{85} = ((85/(100 - 85))^{(1/hillslope)})*EC_{50}$$

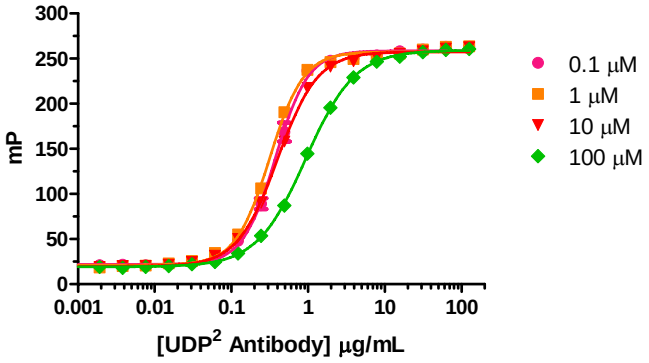


Figure 2. UDP^2 Antibody Titrations at Various UDP-glucuronic acid Concentrations. Sample data for 0.1 µM, 1 µM, 10 µM, and 100 µM UDP-glucuronic acid binding curves. The nucleotide concentration reflects the amount in the enzyme reaction prior to the addition of the UDP Detection Mixture. The UDP^2 Antibody (15 µL) was titrated in of 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 the Detection Mixture (16 nM UDP^2 AlexaFluor®633 Tracer and 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 4X EC$_{85}$.

3.3 Determining Enzyme Concentration

A polarization shift of 60-100 mP units and a Z' value of 0.5 indicates robust assay performance for HTS applications. For the initial UDP-glucuronic acid concentration of 1 µM this criteria was achieved when less than 7.5% conversion (75 nM) of the UDP was present (Figure 4, page 8). For 10 µM UDP-glucuronic acid concentration, this was achieved when 2% conversion (200 nM) was present (Figure 4, page 8). For 100 µM UDP-glucuronic acid concentration, this was achieved when 0.75% conversion (750 nM) was present (Figure 4, page 8).

***UDP enzyme reaction conditions*:** Run your enzymatic reaction with optimal buffer and additives at the requisite temperature. Initial donor substrate (i.e. UDP-sugar) concentration is critical, as it determines the UDP^2 Antibody concentration (Section 3.2). The acceptor substrate should be present at a concentration similar or higher than the donor substrate concentration to avoid non-linear kinetics resulting from substrate depletion.

UDP enzyme concentration: You will need to perform a pilot experiment to determine the optimal enzyme concentration to yield initial velocity conditions (substrate consumption ≤20%) and produce a sufficient assay window. The optimal enzyme concentration should be determined by serial titration using the same conditions that will be used for screening or profiling. We recommend an enzyme concentration that produces 50% to 80% of the maximal change in polarization (EC_{50} to EC_{80}) with a polarization change of at least 100 mP (Figure 3).

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

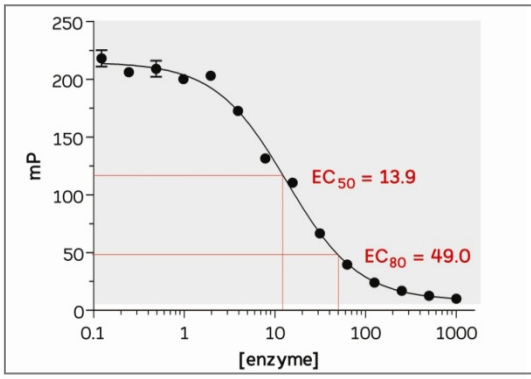


Figure 3. Typical UDP Enzyme Titration. Based on this curve, the optimal enzyme amount would be 20-40 units.

UDP^2 Detection Mixture: Prepare the UDP Detection Mixture (5 µL per well) containing 16 nM UDP^2 AlexaFluor®633 Tracer, 1X Stop & Detect Buffer B, and 4X EC_{85} UDP^2 Antibody as determined in Section 3.2. The UDP Detection Mixture is stable for at least 16 hours at room temperature (20-25°C); we recommend keeping reagents cold as much of the time as possible.

3.4 Running an Assay

The Transcreener® UDP^2 Assay is designed for endpoint readout. Following incubation of the enzyme reactions (15 µL) for the requisite time, 5 µL UDP Detection Mixture is added. Reactions are mixed and allowed to equilibrate at room temperature for 60 minutes before reading.

Assay Steps:

i. ***Run Enzyme Reaction (15 µL).*** Generally, a master mix containing all UDP enzyme reaction components, except donor substrate, is dispensed into wells and reactions are started by adding donor substrate. Mix the plate, and incubate at the desired temperature and time.

ii. ***Add UDP^2 Detection Mixture (5 µL).*** Add 5µL of UDP^2 Detection Mixture bringing the total volume to 20 µL. Note that the UDP enzyme reaction components (including donor substrate) will be diluted 1.3-fold and the UDP^2 Detection Mixture will be diluted 4-fold after this addition.

iii. ***Read Plates.*** Allow at least 60 minutes for the detection reaction to equilibrate before reading the plate. Our studies have indicated that the signal is stable (<10% change) for at least 24 hours at room temperature (20-25°C). If polarization measurement is to occur on the following day, seal the plates to prevent evaporation.

Control Reactions

When screening or profiling inhibitors, two types of controls are needed at a minimum: a) negative ("no enzyme") control wells to calculate the decrease in polarization caused by enzyme activity; this control defines the upper limit (highest polarization value) of the assay window b) positive ("no inhibitor") control wells lacking any test compounds to determine the full activity of the enzyme being screened. This control defines the lower limit of the assay window. Z' values are usually calculated from these two types of controls.

Standard Curves

A standard curve (Figure 4) is needed to convert polarization values to product formation (UDP) for quantitative data analysis. (Note that because the Transcreener® UDP^2 Assay relies on a competitive binding reaction, the response is non-linear, so the signal is not directly proportional to reaction progress). Whether to use a standard curve is a matter of choice. Many Transcreener® users do not use one for screening purposes, because the criteria for a hit are based on statistical analysis of the raw data. However, if quantitative enzyme turnover information is required for Michaelis-Menten kinetic analysis, a standard curve will be needed.

The wells for the standard curve should contain all UDP reaction components **except the enzyme** and receive UDP Detection Mixture. The curve is constructed to mimic an enzyme reaction: starting at the UDP-glucuronic acid concentration used for the screening reactions, UDP-glucuronic acid is decreased in increments and the UDP concentration is increased proportionately, keeping the sum of their concentrations [UDP-glucuronic acid + UDP] constant. We recommend using a twelve-point curve with concentrations of UDP-glucuronic acid and UDP corresponding to 0%, 0.5%, 1%, 2%, 3%, 5%, 7.5%, 10%, 15%, 30%, 50%, and 100% UDP-glucuronic acid conversion. Allow 60 minutes incubation prior to polarization measurement for the coupling enzyme reaction to reach completion.

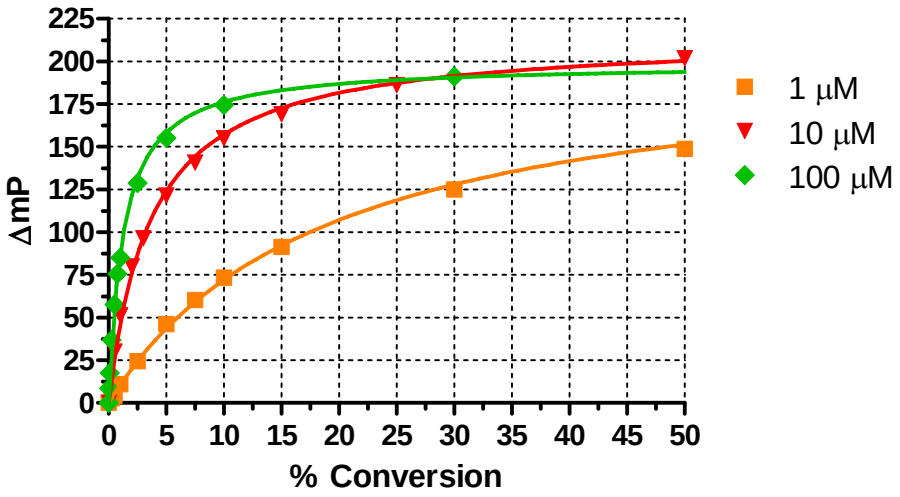


Figure 4. UDP-glucuronic acid/UDP Standard Curves. Sample data for standard curves starting at initial UDP-GA concentrations of 1, 10, and 100 μM are shown (UDP-glucuronic acid concentrations in the 15 μL mock enzyme reaction before the addition of Detection reagents). A polarization shift of 60-100 mP units and a Z' value of 0.5 indicates robust assay performance for HTS applications. For the initial UDP-glucuronic acid concentration of 1 μM this criteria was achieved when less than 7.5% conversion (75 nM) of the UDP was present. For 10 μM UDP-glucuronic acid concentration, this was achieved when 2% conversion (200 nM) was present. For 100 μM UDP-glucuronic acid concentration, this was achieved when 0.75% conversion (750 nM) was present.

4.0 FAQs

How sensitive is the assay?

The lower limit of UDP detection is dependent on the starting concentration of donor substrate in the enzymatic reaction. The assay becomes more sensitive with decreasing donor substrate concentrations. For example, the lower limit of detection (LLD) for UDP in enzyme reactions utilizing 100 µM UDP-glucuronic acid is 0.25 µM UDP, whereas reactions containing 1 µM UDP-glucuronic acid has an UDP LLD of 0.01 µM. LLD is defined as the minimum amount of UDP that generates a Z'>0.

Can this assay be run in continuous mode?

This assay can be performed in real time by eliminating stop reagents and including the UDP Detection Mixture components (antibody and tracer) in the enzyme reaction. However, this mode should only be used for relative activity comparisons because the extended signal equilibration time (1 hours) precludes accurate quantitation of UDP.

Why does my signal (mP units) change after addition of detection mix followed by the recommended incubation period?

- Enzyme reaction is not stopped by the Stop & Detect Buffer included in the kit. User will need to determine an alternative stop mix depending upon their target enzyme`s cofactors and modulator requirements.
- Nuclease contamination in the buffer can cause the window to collapse causing a change in mP units. We recommend using nuclease free water and buffer that has been prepared fresh.
- Non specific hydrolysis of UDP-sugars may also cause signal change. Aliquot your UDP-sugar or make it fresh each time to avoid frequent freeze thaw cycles.
- There is a rare possibility of compound interference with the detection mix may lead to change in signal.

5.0 References.

Wagner GK, Pesnot T. Glycosyltransferases and their assays. Chembiochem. 2010 Sep 24;11(14):1939-49. Review.

Terms & Conditions

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