Screening Diverse Glycosyltransferases Using Homogenous, Fluorescent Transcreener HTS Assays For Direct Detection Of Nucleotide Products

Robert G. Lowery, Tom Zielinski and Meera Kumar
BellBrook Labs, Madison, WI, USA

Abstract

Glycosyltransferase enzymes participate in extremely diverse metabolic and regulatory roles by catalyzing the transfer of sugar molecules to protein, lipid and carbohydrate acceptors as well as endogenous and xenobiotic small molecules. From a drug discovery point of view, they are gaining increasing interest as targets for "substrate reduction therapy" in lysosomal storage diseases such as Gaucher and Fabry diseases, and as anti-microbial targets for disrupting bacterial cell wall biosynthesis. From an HTS assay perspective, they are a challenging target class because of the diversity of both donor and acceptor substrates. Most glycosyltransferase screening efforts have relied on either enzyme-coupled assay methods, which are prone to interference, or mass-spectrometry, which is not available to many labs. We noted however that most of the more than 200 glycosyltransferases in humans use one of nine sugar-nucleotides as donors, all of which have either GDP, UDP or CMP as the nucleotide moiety. Based on this observation, we explored the use of various Transcreener HTS assays for detection of glycosyltransferases that produce these three nucleotides, including GDP detection for a fucosyltransferase, UDP detection for glucosylceramide synthase and CMP detection for a sialyltransferase. All of the Transcreener assays rely on direct, highly selective immunodetection of the nucleotide products in a homogenous (mix-and-read) format with far-red, ratiometric readouts; e.g. fluorescence polarization or TR-FRET, and have been extensively validated in pharma HTS campaigns. The results indicate that a suite of Transcreener assays may be a powerful approach for screening and profiling diverse glycosyltransferases in humans, and it is expected that the same approach may be extended to many of the microbial enzymes that use similar sugar-nucleotides.

A Suite of Transcreener Assays for Glycosyltransferases

TRANSCREENER® UDP² Assay

UDP-producing Enzymes

Glucosyltransferase
Galactosyltransferase
Glucuronyltransferase
N- acetylglucosamyltransferase
N-acetylgalactosyltransferase
Xylosyltransferases

TRANSCREENER® AMP²/GMP² Assay

CMP-producing Enzymes

Sialyltransferases

TRANSCREENER® GDP Assay

GDP-producing Enzymes

Fucosyltransferase
Mannosyltransferase

- There are more than 200 glycosyltransferases (GTs) encoded in the human genome.
- Acceptor substrates are very diverse, including proteins, proteoglycans, lipids and small molecules (e.g., xenobiotics). Some GT enzymes use more than one acceptor substrate.
- Donor molecules include just nine sugar-nucleotides: UDP-glucose, UDP-galactose, UDP-GlcNAc, UDP-GalNAc, UDP-xylose, UDP-glucuronic acid, GDP-mannose, GDP-fucose, and CMP-sialic acid.
- These Three Transcreener Assays allow detection of all human Glycosyltransferases that use a sugar-nucleotide donor.

Transcreener HTS Assays: Homogenous . Direct Immunodetection of Nucleotides

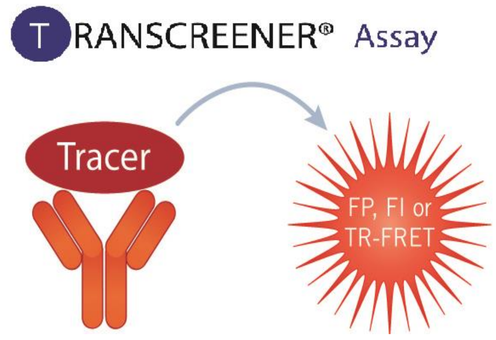


Figure 1. Transcreener platform for glycosyltransferases: Transcreener assays rely on the homogenous fluorescent detection of the invariant reaction product of a group transfer reaction. The assay antibodies differentiate between nucleotides on the basis of small structural differences, such as a single phosphate group, with 100-fold or greater selectivity. There is only one "donor product" for each type of group transfer reaction, so a single set of Transcreener detection reagents can be used for all family members, regardless of the acceptor substrate. For the glycosyltransferase family the nucleotide product is either UDP, GDP, or CMP. Transcreener assays are available for ADP, AMP and GMP as well.

Selective Detection of UDP vs UDP Sugars

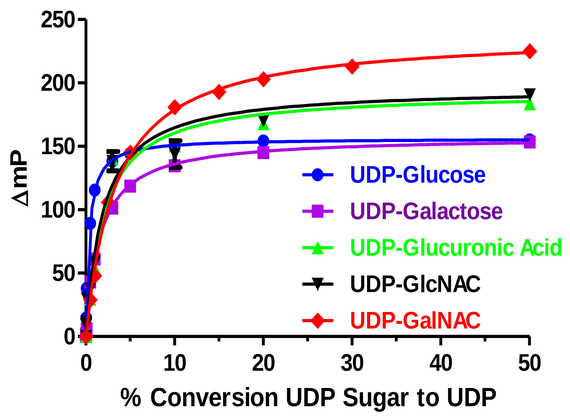


Figure 2. Standard curves for conversion of UDP sugars to UDP : 10 µM UDP-sugar/UDP standards were prepared in enzyme reaction buffer; total uridine concentration was held constant. 10 µL of each standard was added to wells in a 384-well plate followed by addition of 10 µL UDP Detection Mixture. The plate was read after 1 hr incubation at RT. ΔmP was calculated by subtracting each percent conversion from 0% conversion.

Detection of Glucosylceramide Synthase (GCS) Activity and Inhibition by PDMP

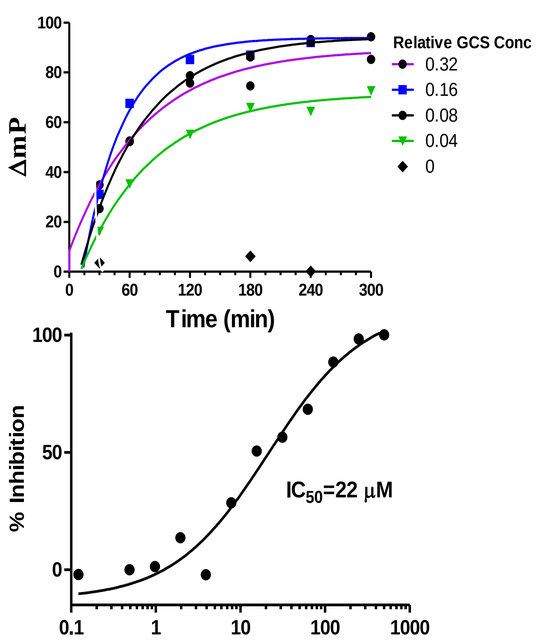


Figure 3. GCS Time Course Showing initial progress curves: 20 µL reactions were performed in GCS assay buffer containing 20 µM UDP-Glucose , +/- 5 µM C-8 Ceramide, 25 µg/mL UDP antibody, and 2 nM UDP DyLight™ 632 Tracer. Polarization (mP) was measured with a PheraStar Plus. Control reactions lacked the C-8 ceramide acceptor.

Figure 4. Dose dependent inhibition by PDMP: PDMP was titrated in the presence of optimal concentration of GCS in the assay buffer. The reactions were initiated with the addition of UDP-Glucose +/- 5µM C-8 Ceramide. The plate was incubated for 2 hours at 37°C. After the addition of detection mix, the plates were read. The mP data was converted to UDP formation to show the percent inhibition.

Detection of Polypeptide GalNAC Transferases

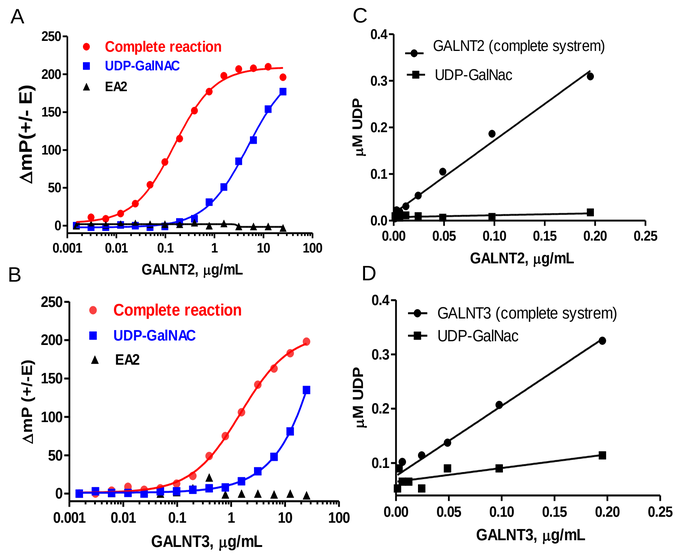


Figure 5. Titration of Polypeptide GalNAC Transferases. Titration of GALNT2 (A) and GALNT3 (B) in the presence of 10 µM EA2 peptide substrate and 10 µM UDP-GalNAC. C, D. Conversion of polarization data to UDP formation shows a linear correlation of the enzyme conc with product formed.

Selective Detection of GDP vs GDP Fucose

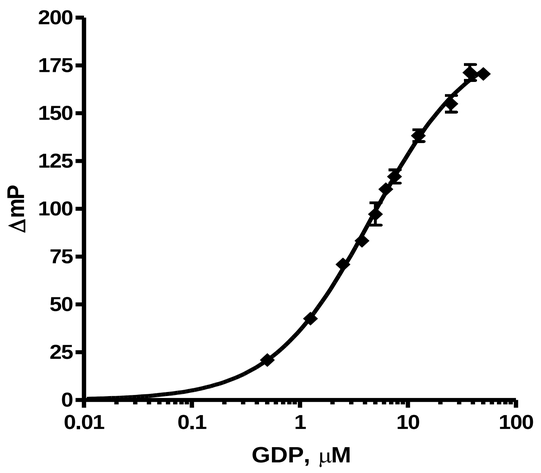
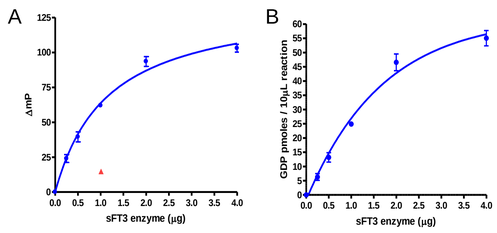


Figure 6. Standard curve for the conversion of GDP Fucose to GDP: 50 µM GDP-fucose/GDP standards were prepared in enzyme reaction buffer; total guanidine concentration was held constant. 10 µL of each standard was added to wells in a 384-well plate followed by addition of 10 µL GDP Detection Mixture. The plate was read after 1 hr incubation at RT.

Detection of Fucosyltransferase, FT3 Activity



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sFT3 (µg)	GDP (pmol)	Specific Activity
0.25	6.28	1.26
0.5	13.19	1.32
1	24.89	1.24
4	55.02	0.69

Figure 7. Titration of Fucosyltransferase, FT3. A. FT3 was added to enzyme buffer containing Lec and the reaction was initiated with GDP-fucose (10 µL total volume). The reaction was incubated at 30°C for 20 minutes.10 µL GDP Detection Mixture was added to the enzyme reaction and equilibrated for 1 hour at room temperature prior to reading the plate. B. The amount of GDP produced was calculated from a standard curve that was prepared on the same plate. C. Specific activity of FT3 calculated as pmol GDP produced/min/µg of FT3.

Selective Determination of CMP vs CMP-NAN for Detection of Sialyltransferases

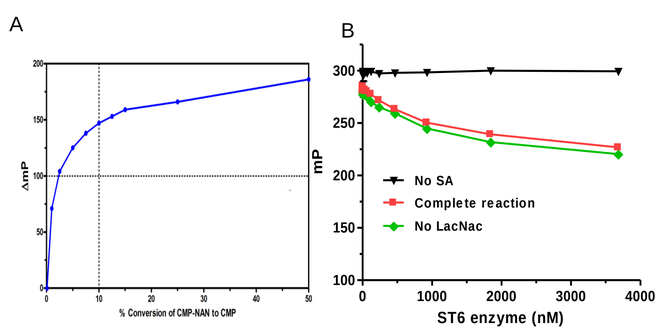

Figure 8. Titration of Sialyltransferase, ST6 and a standard curve mimicking the conversion of CMP from CMP-NAN. A. Starting with 10 µM CMP-NAN, CMP was titrated into reactions and CMP-NAN was decreased proportionately to mimic an enzyme reaction. B. Titration of Sialyltransferase, ST6 in the presence of 10 µM CMP-NAN and 25 nM LacNac . Control reactions lacked enzyme; CMP production in the absence of acceptor LacNac represents sialylation of the AMP antibody.

Conclusions

- **A suite of Transcreener assays are available to study diverse glycosyltransferases.**
- **The Transcreener UDP assay was used to detect GCS and GALNT enzyme activties.**
- **The Transcreener GDP assay was used to detect the activity of fucosyltransferase FT3.**
- **The Trancreener AMP assay was used to measure CMP production by sialyltransferase, ST6.**

BellBrook Labs, 5500 Nobel Drive, Suite 230, Madison, WI 53711
866.313.7881 or 608.443.2400