

## Fluorescent HTS assay for Glycosyltransferases

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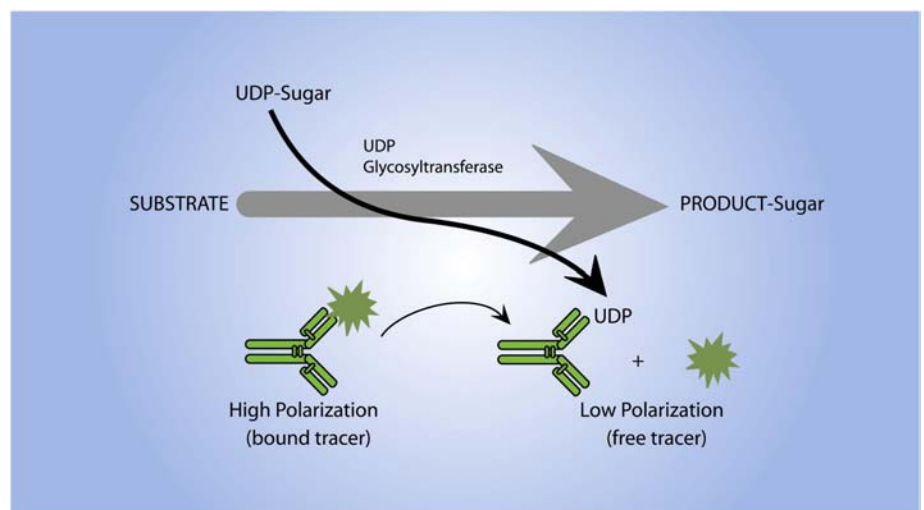
### Introduction

Glycosyltransferases (GTs) are increasingly being targeted for therapeutic intervention, both in humans and microbes. There are over two hundred glycosyltransferases in humans, and the diversity of donors and acceptor substrates complicates development of assays that can be used across family members. Current conventional assay methods rely on costly radioactive assays and/or on cumbersome methods that separate the conjugated product from the reactants. To overcome these difficulties, we have developed the Transcreener™ group transfer assay platform, a universal, homogenous, fluorescence - based technology that relies on detection of a common donor by-product. The Transcreener™ assay platform is universal in that, for a given type of group transfer reaction, the principle assay reagents, protocol and readout are the same regardless of the acceptor substrate or isozyme.

Many GT's use UDP- activated sugars, and in this study, we show that a competitive fluorescence polarization immunoassay (FPIA) for UDP (Transcreener™ UGT Assay) can be used with high sensitivity to detect the activities of a mammalian galactosyltransferase, alpha-1,3-galactosyltransferase (alpha-1,3-GalT), using lactose as an acceptor substrate. We have shown previously that the Transcreener™ UGT Assay can be used for detection and screening of hepatic UDP-glucuronosyltransferases. Alpha-1,3-GalT transfers galactose from uridine-5'-diphosphogalactose (UDP-Gal) to terminal N-acetylglucosamine (GlcNAc) residues. There is considerable medicinal interest in this enzyme as its products are the major cause of hyperacute xenotransplant rejections, and selective inhibitors against this enzyme may find use in xenotransplantation. This validated drug target was used to establish proof of concept for a broadly applicable HTS assay to detect and profile modulators across diverse members of the GT superfamily.

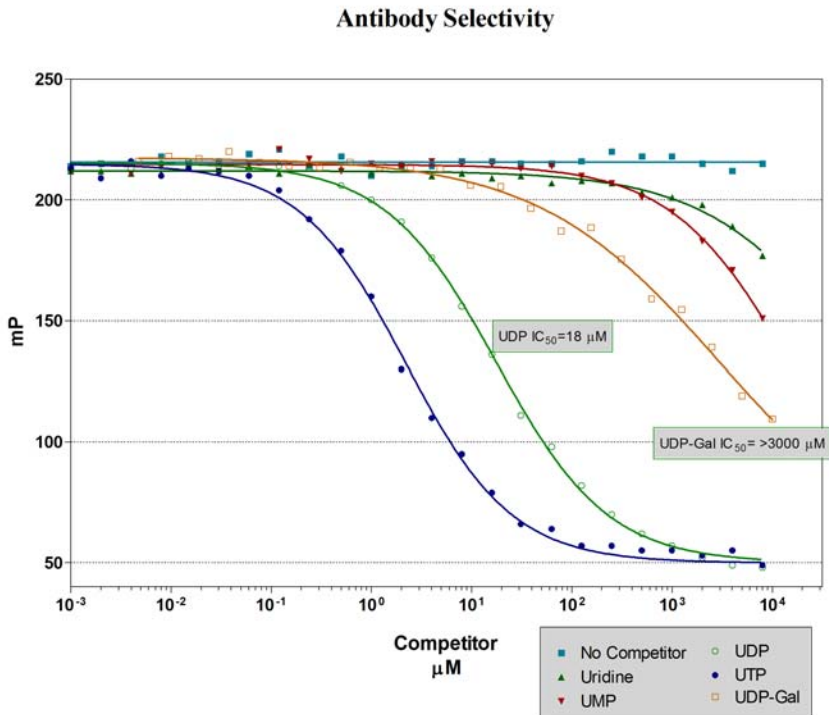
### Figure 1. Transcreener™ Assay Principle for Glycosyltransferases

The assay principle is based on a fluorescence polarization immunoassay (FPIA) for the reaction product UDP, which is formed during the transfer of galactose from the donor molecule (UDP-Gal) to an acceptor substrate and displaces AlexaFluor® 488 labeled UTP (tracer) from an anti-UDP Antibody. The unbound, freely rotating tracer no longer polarizes light, resulting in a decrease in the overall mP signal (Figure 1). Thus, a decrease in mP signal is proportional to the amount of UGT activity. The Transcreener™ assay is universal in that any substrate or any UGT isoform can be used.



## Figure 2. Transcreener™ UGT Antibody is Highly Sensitive for UDP over UDP-Gal

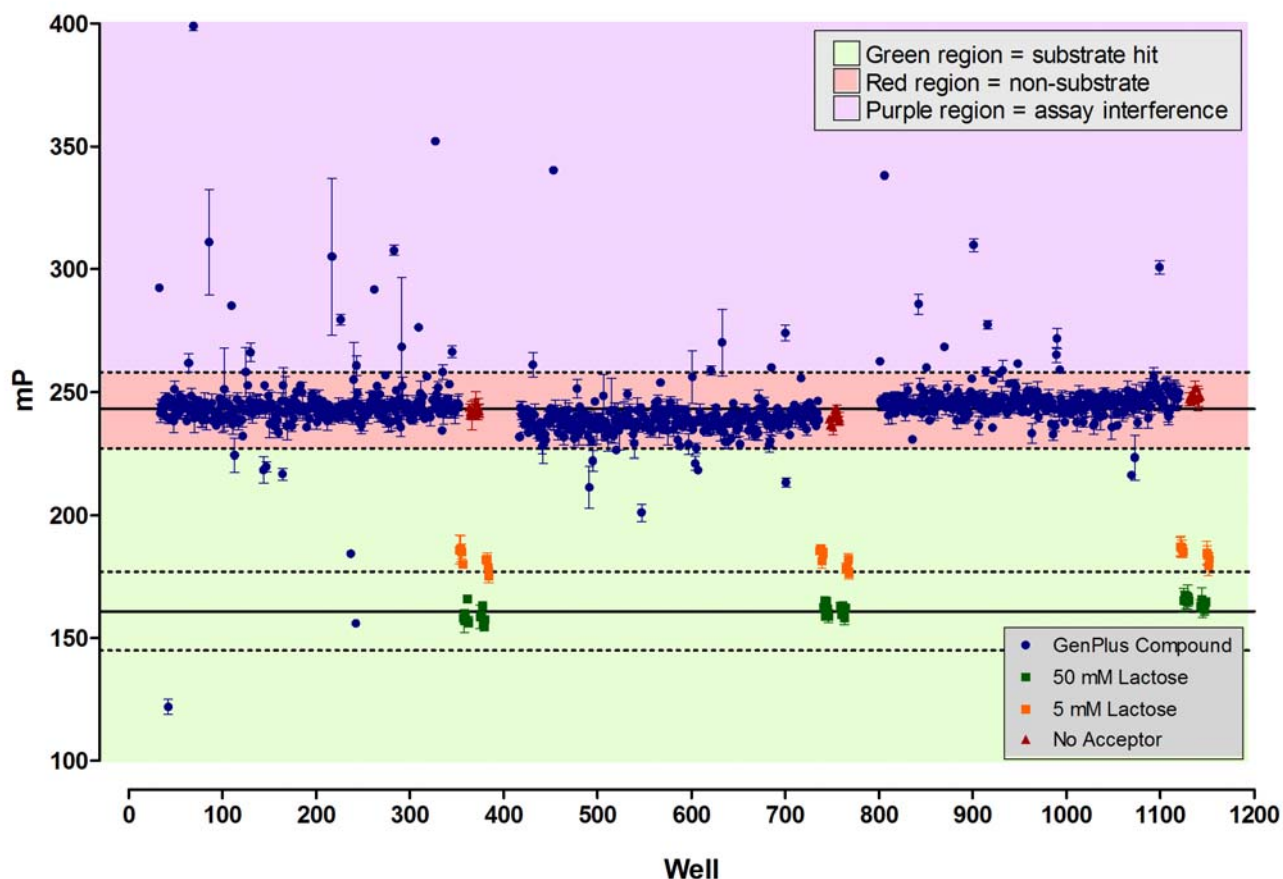
These data demonstrate a greater than 100 fold selectivity of the product (UDP) over the donor substrate (UDP-Gal). An anti-UDP Antibody and UTP-Alexa Fluor® 488 (tracer) were added to wells containing indicated amounts of uridine nucleotides in 50 mM KPO4 (pH 7.5), 150 mM NaCl, and 0.1 mg/ml BGG (Invitrogen.). Polarization was read in a TECAN ULTRA plate reader after 1 hour of equilibration at room temperature using a Ex485/Em535 filter set.



## Figure 3A and B. Transcreener™ UGT Assay Enables Direct Screening for Substrates and Inhibitor Screening Using Physiological Substrates

A small pharmacologically active compound library was screened for both substrates and inhibitors of galactose conjugation catalyzed by alpha-1,3-Galactosyltransferase (alpha-1,3-GalT) using the Transcreener™ UDP Assay. The Transcreener™ assay platform is unique in that it provides the means to screen for both substrates and inhibitors. Because the assay is not dependent on a substrate specific for the detection method, the physiological substrate can be preferentially used. Both substrates and inhibitors were identified by these screens as detailed in the legends for Figure 3A and 3B. A secondary screen of the resulting hits is underway to confirm that these compounds do not interfere with detection of the signal molecule, UDP. Assay conditions are described in the legend of figure 4. The compound library screened was the GenPlus compound library supplied by MicroSource Discovery Systems containing 960 compounds of known pharmacological activity. Compounds were screened at 100 µM.

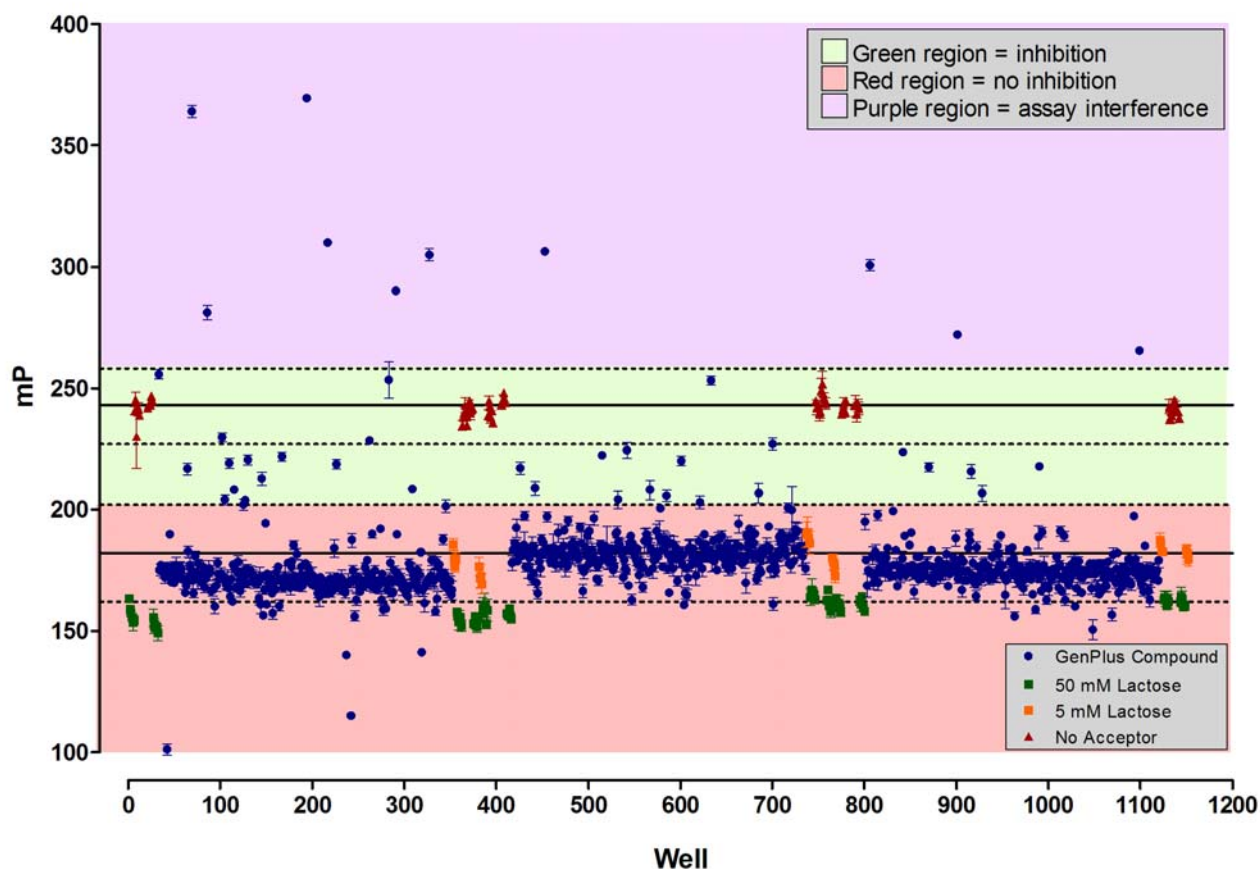
**Figure 3A.**  
**UDP Transcreeper™ Substrate Screen of alpha-1,3-Galactosyltransferase**



Because of alpha-1,3-Galactosyltransferase (alpha-1,3-GalT) involvement in xenotransplant rejection, identification of alpha-1,3-GalT interacting compounds is of significant pharmacological interest. Compounds may affect alpha-1,3-GalT activity by interaction as a substrate or as a non-substrate inhibitor. Here Figure 3A shows results of a screen of 960, structurally diverse, pharmacologically active compounds for substrates of alpha-1,3-GalT.

A decrease in the mP signal denotes product (UDP) formation. Lactose, a common substrate for alpha-1,3-GalT was used as a control substrate at both 5 mM and 50 mM. A substrate hit (green region) was defined as a compound with a negative mP shift greater than 3 standard deviations from the negative control (no acceptor). Compounds with a positive mP shift greater than 3 standard deviations from the negative control were classified as compounds interfering with assay output (purple region of graph). Compounds within 3 standard deviations of the negative control were classified as having no effect (red region). Of the compounds in this library: 1.7% were classified as substrate hits, 94.4% demonstrated no activity, and 2.6% interfered with assay signal.

**Figure 3B.**  
**UDP Transcreeper™ Inhibitor Screen of alpha-1,3-Galactosyltransferase**



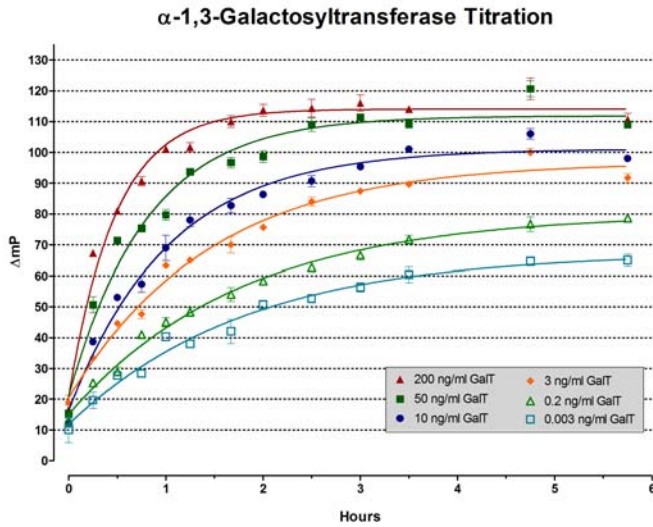
This figure is the results of an inhibitor screen of the same compound set used in the substrate screen in Figure 3A. Compounds were tested for their ability to inhibit galactose conjugation of the acceptor substrate lactose. Product formation (UDP) results in a negative shift in the mP signal and inhibition results in a positive shift from the control wells containing 5 mM lactose, the substrate concentration at which 90% of the maximum assay signal is observed.

The No Acceptor control was used to define the mP value where no product is formed (complete inhibition). Compounds having mP values statistically above the No Acceptor control mP value were defined as compounds causing assay interference (purple region). The 5 mM Lactose wells (substrate only) were used to define the mP value where there is no inhibition. Compounds with an mP less than 3 standard deviations from the 5mM Lactose control wells were defined as compounds with no activity (red region). Inhibition (green region) was defined as a statistically significant positive mP shift (greater than 3 standard deviations) observed when using 5 mM Lactose as substrate

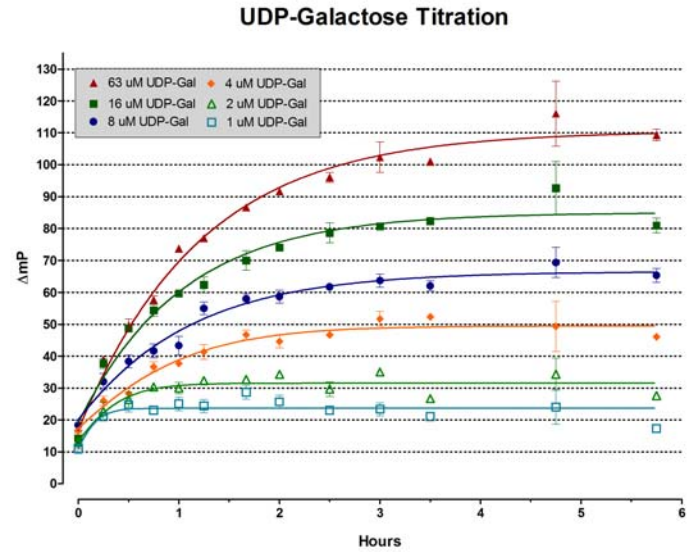
Of the compounds in the library: 3.2% were inhibitor hits, 92.2% demonstrated no activity, and 1.0% interfered with assay signal.

**Figure 4.**  
**UDP Production is Enzyme and Substrate Dependent**

**Figure 4A.**  
**Alpha-1,3-Galactosyltransferase Dependence**

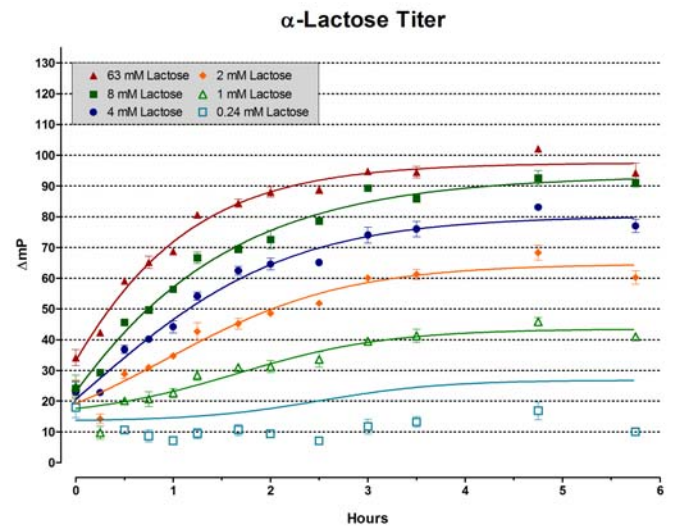


**Figure 4B.**  
**UDP-Galactose Dependence**



Collectively, these data demonstrate assay signal dependence on enzyme, donor substrate, and acceptor substrate. Similar experiments were used to develop and optimize the screening protocol. Transcreer™ assays were performed in 30  $\mu l$  volumes with 50 ng/ml alpha-1,3-GalT (or titration), 50  $\mu M$  UDP-Gal (or titration) and 50 mM lactose (or titration). The enzyme used in this study was a His-tagged, recombinant alpha-1,3-GalT expressed in and purified from *E. coli*. The standard assay conditions were: 10 mM Tris-HCl pH 7.0, 10 mM  $MnCl_2$ , 1.25% v/v UDP Antibody, 2nM UTP-488 AlexaFluor® tracer was synthesized in house. Reactions were incubated at 37 °C for 1 hour followed by 0.5 hour incubation at room temperature. Polarization measurements were taken with a Tecan Ultra plate reader using an Ex<sub>485nm</sub>/Em<sub>535nm</sub> filter set at 30 °C.

**Figure 4C.**  
**Alpha-Lactose Dependence**



## **Conclusions:**

1. The Transcreener™ UGT Assay is a universal, HTS-amenable assay has been developed for the UDP-glycosyltransferase family using and FPIA-based assay for UDP production, the common product of all UDP-glycosyltransferases.
2. The Transcreener™ UGT Assay was used to screen a small panel of drug compounds for both substrates and inhibitors of alpha-1,3-galactosyltransferase, an enzyme involved xenotransplant rejection, against a small panel of drug compounds.
3. Screening results show proof-of-concept for the use of the Transcreener™ platform for screening UDP-glycosyltransferases. The robust, fluorescent, homogenous format make this assay highly amenable for high-throughput and secondary screening.

## **Acknowledgements:**

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