

Transcreener™ UGT Assay: Optimization for Commercial Enzyme Preparations and Poor Substrates

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Introduction

The Transcreener™ UGT Assay relies on detection of the invariant UGT reaction product UDP using a competitive fluorescence polarization immunoassay (Figure 1). This assay can be used to detect UGT activity with any enzyme source and any acceptor substrate, enabling direct screening of compounds for glucuronidation. This study focuses on optimization of the UGT Transcreener™ Assay for two parameters. 1) Because UGTs are only available as crude microsomal preparations, an important consideration in the development of the assay was optimization of conditions to maximize UGT activity while eliminating the deleterious effects of contaminating enzymes such as phosphatases that degrade UDP. 2) Some compounds, such as ketoprofen and valproic acid, that have been reported as UGT2B7 substrates, were not detected using the standard Transcreener™ UGT Assay conditions. We used a radioassay to show that these compounds are very poor substrates, and to identify conditions that allow their detection in the Transcreener™ UGT assay. This poster summarizes the optimization studies for recombinant expressed UGT2B7 and human liver microsomes. Also, we report on recent studies incorporating longer wavelength fluorors to minimize the optical interference that can result from using high concentrations (1mM) of poor substrates.

Figure 1.

Transcreener™ UGT Assay Principle

UDP produced during transfer of glucuronic acid to acceptor substrate is detected using a competitive fluorescence polarization immunoassay. Because it relies on detection of the invariant reaction product, the same detection reagents can be used for any UGT and any acceptor substrate.

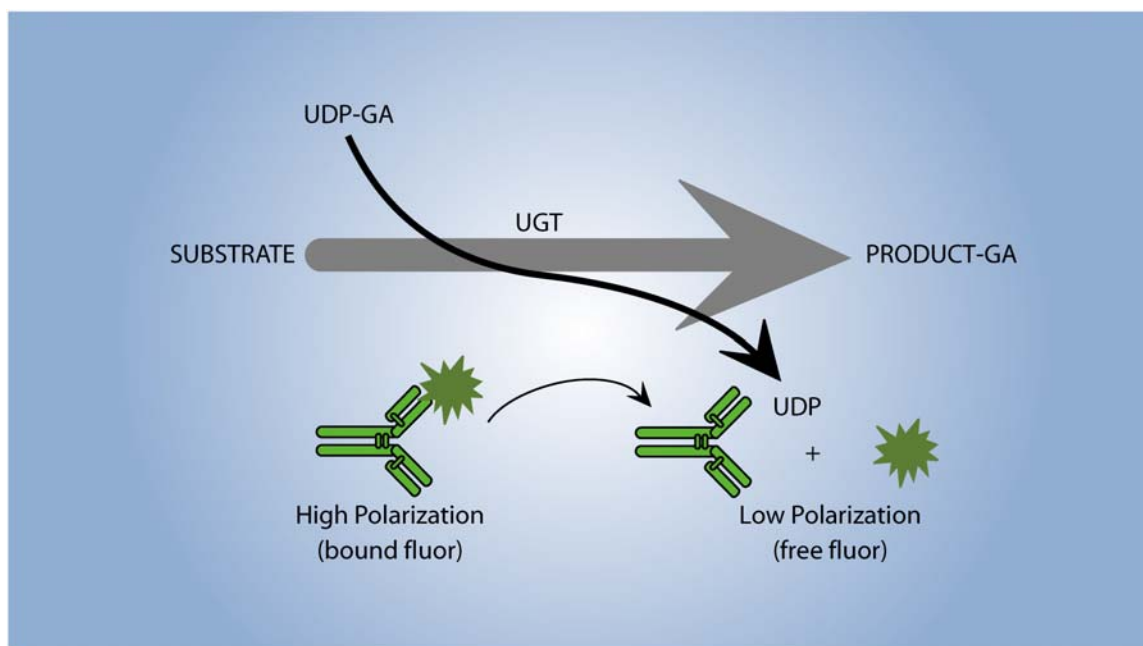


Table 1
Effect of Agents Tested to Enhance UGT Activity and UDP Stability

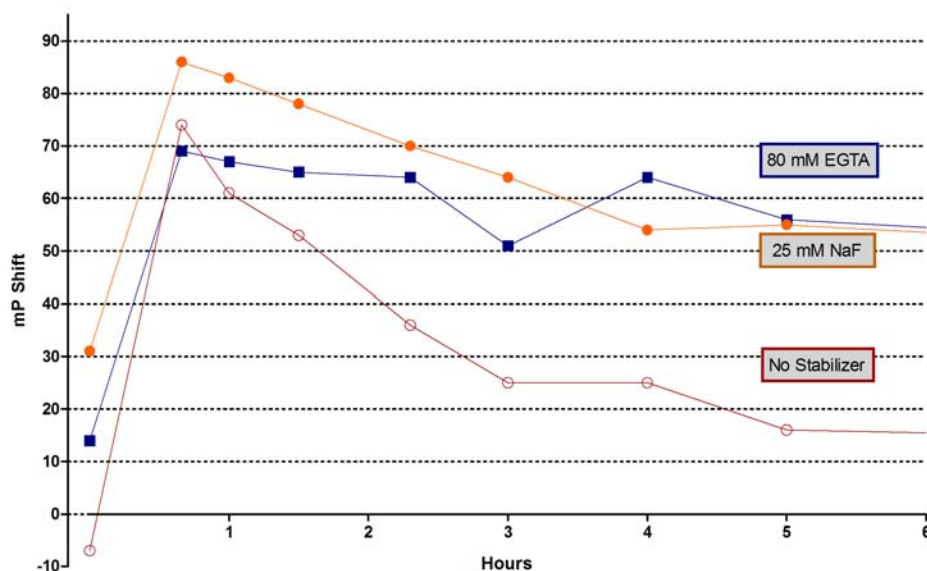
		EGTA	NaF	Na ₃ VO ₄	Saccharo-lactone	AMP	Alamethicin	KPO ₄
Purpose		Stabilize UDP	Stabilize UDP	Stabilize UDP, quench UGT rxn	Stabilize glucuronides	Stabilize UDPGA	Substrate access	Comm on UGT buffer
Effects in ¹⁴C-UDPGA Radioassay	Recombinant UGT2B7	Inhibits ~50% @ 80mM	Inhibits ~50% @ 50mM	Inhibits 70-80% @ 25mM	No effect	No effect	No effect	Inhibits ~50% @ 50mM
	HLMs	Inhibits ~50% @ 30mM	Inhibits ~50% @ 40mM	Inhibits 70-80% @ 25mM	No significant effect	No significant effect	2x stimulation @ 50-150 µg/mg	No effect

EGTA is included in the Transcreener™ UGT Assay buffer and Na₃VO₄ is used to quench the reactions.

Testing of effectors in UGT radioassay. A radioassay that quantifies incorporation of ¹⁴C-UDPGA into acceptor substrates was used to independently determine the effect of various agents on UGT activity using recombinant UGT2B7 Baculosomes™ (Invitrogen) and pooled human liver microsomes (Xenotech). Typical reactions contained 100mM Tris, pH 7.5, 5mM MgCl₂, 100 µM UDPGA, 50nCi ¹⁴C-UDPGA, and hyodeoxycholic acid, ketoprofen, or both as acceptor. ¹⁴C-labelled glucuronides were isolated using organic extraction with ethyl acetate and counted in a scintillation counter as described (H. Matern, et al (1994) Analytical Biochem. 219:182-188). Blanks lacked acceptor or enzyme.

Figure 2.
The Stability of UDP in Commercial UGT Preparations can be Enhanced by Phosphatase Inhibitors

Effect of phosphatase inhibitors on UDP stability. 5mM UDP was incubated at 37 °C in 30 µl reactions (384 well plates) containing 50 µg/ml UGT2B7 Supersomes™ (Becton-Dickinson) in 50mM KPO₄, 5mM MgCl₂, 70 µM UDPGA, 0.7% Anti-UDP Ab, 2nM UTP-488 tracer in the presence and absence of predetermined concentrations of EGTA and NaF. Polarization



values were read in a Tecan Ultra plate reader; values are shown as the shift in polarization relative to control reactions lacking UDP. Similar results were observed for other UGT sources including UGT2B7 Baculosomes™ (Invitrogen) and pooled human liver microsomes (Xenotech).

Figure 3.
Transcreener™ UGT Assay Signal is Significantly Enhanced by Phosphatase Inhibitors

Effect of phosphatase inhibitors on Transcreener™ assay signal. Various acceptors at 100 μM were incubated in 384 well plates with 50 μg/ml UGT2B7 Supersomes™ (Becton-Dickinson) for 0.5 hours either under standard Transcreener™ UGT Assay conditions (50mM KPO₄, 5mM MgCl₂, 70 μM UDPGA, 0.7% Anti-UDP Ab, 2nM UTP-488 tracer, 80mM EGTA) or under the same conditions lacking EGTA. Reactions were quenched with 10 μl of 100 mM Na₃VO₄, and polarization values were read in a Tecan Ultra plate reader (Ex_{485nm}/Em_{535nm}); values are shown as the shift in polarization relative to control reactions lacking acceptor.

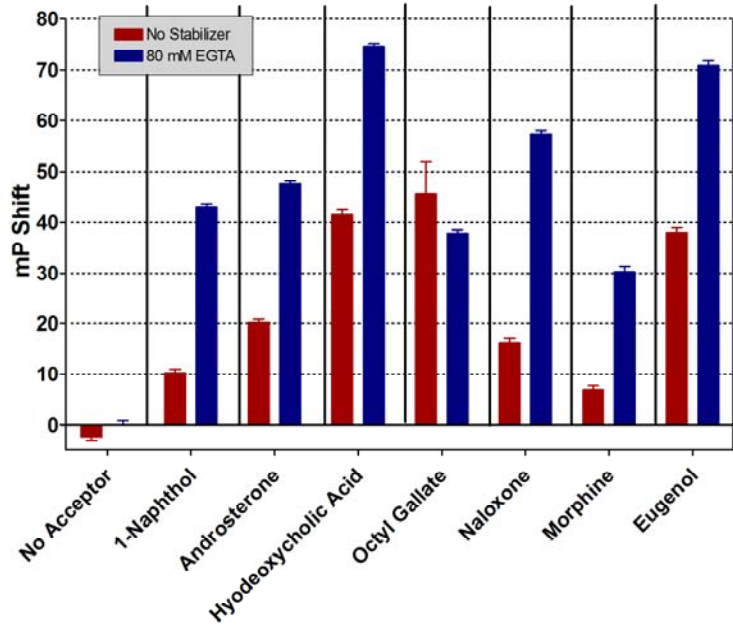


Figure 4.
Stabilization of UDP with EGTA Increases the Transcreener™ Assay Window

Effect of phosphatase inhibitors on Transcreener™ assay Z' values. The effect of phosphatase inhibitors on Transcreener™ assay Z' values was studied. Replicates of Transcreener™ UGT reactions with recombinant UGT2B7 and hyodeoxycholic acid in the presence and absence of 80mM EGTA (conditions and methods described in legend to Figure 3).

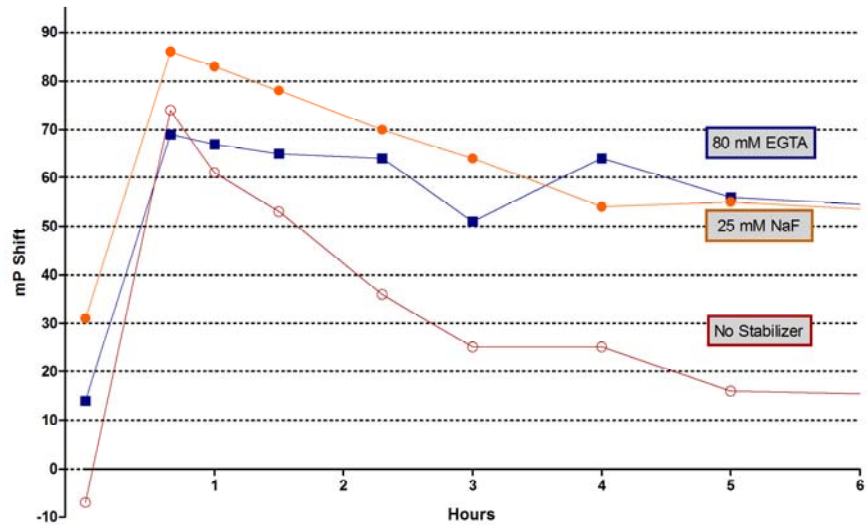
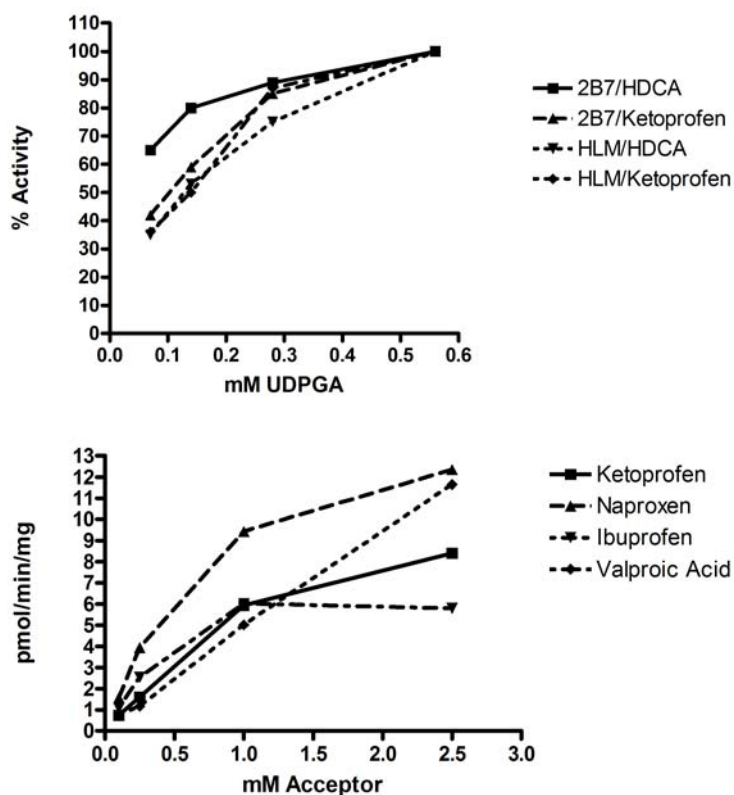


Table 2.
Radioassay Data for Some Poor Acceptor Substrates that are not Detected Using Standard Transcreener™ UGT Assay Conditions

Acceptor	Specific Activity (pmol/min/mg)	
	Recombinant UGT2B7	Human Liver Microsomes
Hyodeoxycholic Acid (HDCA)	160-230	3,100-5,800
Ketoprofen	5.9	307
Naproxen	9.4	nd
Ibuprofen	6.0	nd
Valproic Acid	5.0	nd

Hyodeoxycholic acid, which yields an excellent signal in the Transcreener™ Assay, is shown for comparative purposes. Poor acceptors were tested at 1mM using the standard radioassay conditions described above. Hyodeoxycholic acid was tested at 250 μM.

Figure 5.
Dependence of Enzyme Activity on Donor (UDPGA) and Acceptor Concentrations with UGT2B7 Baculosomes™ and HLMs



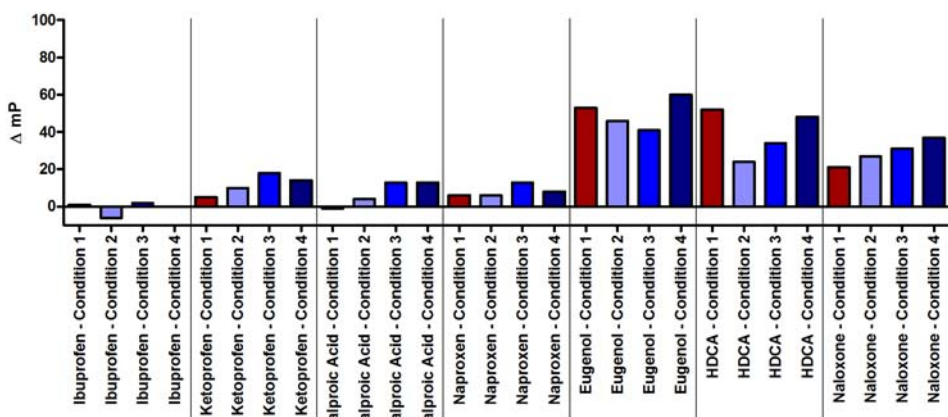
UDPGA and acceptors were titrated in the radioassay. The standard Transcreener™ UGT Assay conditions include 70 μM UDPGA, and we recommend testing compounds as potential acceptors at 100 μM. These data show that increasing the concentrations of both UDPGA and acceptors will increase product formation, which should enhance the Transcreener™ signal for poor acceptors.

Figure 6A. Changes in Transcreener™ UGT Assay Conditions Enables Detection of Some Poor Substrates

Effect of higher acceptor and UDPGA concentrations and different buffer/phosphatase cocktail on Transcreener™ signal with various acceptors using 488nm tracer. Transcreener™ UGT Assays were run for 1.5 hours in 384 well plates essentially as described for Figure 3, using 24 replicates for each acceptor.

Condition 1 was

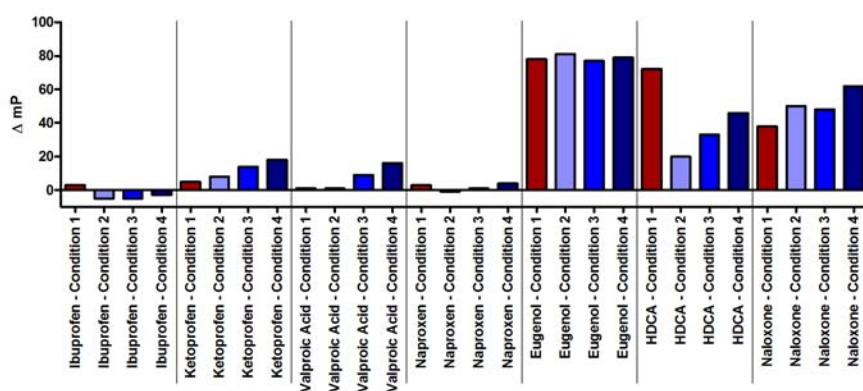
standard Transcreener™ UGT Assay conditions: 50 mM KPO₄, 5 mM MgCl₂, 70 μM UDPGA, 0.7% Anti-UDP Ab, 2 nM UTP-488 tracer, 80 mM EGTA, 300 μg/ml UGT2B7 Baculosomes™. conditions 2-4 were the same basal conditions, but included changes that yielded higher UGT activity in the radioassay: increased donor and acceptor concentrations and use of a different buffer/phosphatase inhibitor cocktail. (Conditions 3 and 4 include compensatory increases in Ab to accommodate higher UDPGA.)



Condition 1: Standard Transcreener UGT Assay conditions (incl. 100 μM acceptor and 70 μM UDPGA)
 Condition 2: Acceptor increased to 1mM
 Condition 3: Acceptor increased to 1mM, UDPGA increased to 250 μM
 Condition 4: Acceptor increased to 1mM, UDPGA increased to 250 μM, 100mM Tris, pH 7.5 buffer instead of KPO₄, 30mM NaF/40mM EGTA as phosphatase inhibitors

Figure 6B. Use of Red-Shifted Tracer Increases the Transcreener™ Signal for Some Acceptors

Effect of higher acceptor and UDPGA concentrations and different buffer/phosphatase inhibitor cocktail on Transcreener™ signal with various acceptors using 568nm tracer. Transcreener™ UGT Assays were run as described above using a red-shifted tracer (568-UTP tracer) and purified antibody (IgG fraction).



Condition 1: Standard Transcreener UGT Assay conditions (incl. 100 μM acceptor and 70 μM UDPGA)
 Condition 2: Acceptor increased to 1mM
 Condition 3: Acceptor increased to 1mM, UDPGA increased to 250 μM
 Condition 4: Acceptor increased to 1mM, UDPGA increased to 250 μM, 100mM Tris, pH 7.5 buffer instead of KPO₄, 30mM NaF/40mM EGTA as phosphatase inhibitors

Conclusions:

1. UDP is unstable in commercial UGT preparations, and can be stabilized by phosphatase inhibitors including EGTA, NaF and Na_3VO_4 . All of these agents inhibit UGT activity as well, but EGTA and NaF can be used at concentrations that stabilize the UDP signal while not inhibiting UGT activity severely. The standard Transcreener™ UGT Assay conditions include 80mM EGTA, and Na_3VO_4 is used to quench the reactions.
2. AMP and saccharolactone do not significantly affect UGT activity for recombinant UGT2B7 or HLMs; alamethicin (@100-15- $\mu\text{g}/\text{mg}$ protein) stimulates UGT activity in HLMs approximately 2-fold, but has no significant effect on recombinant UGT2B7 activity.
3. Some poor 2B7 acceptors such as Ketoprofen , Naproxen and Valproic Acid are not detected at 100 μM using the standard Transcreener™ UGT assay conditions, but can be detected by increasing UDPGA to 250 μM and using 1mM acceptor. Use of the higher UDPGA concentration requires a two-fold increase in the amount of anti-UDP antibody used.
4. Use of a red-shifter Tracer (568-UTP tracer vs. 488-UTP tracer) and purified antibody in the Transcreener™ UGT Assay increases the signal for the good acceptors , but does not improve the detection of poor substrates.

This work was supported by NIH SBIR grant GM59542-02A1. Transcreener™ Assay Platform is patent pending. Transcreener™ is a trademark of BellBrook Labs. Supersomes™ is a trademark of Becton-Dickenson. Baculosomes™ is a trademark of Invvitrogen.

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