

Automation of a Luminescent Assay to Measure UDP-glucuronosyltransferase (UGT) Enzyme Activity

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Abstract

The UDP-glucuronosyltransferase (UGT) enzyme is responsible for the catalyzation of the glucuronidation reaction. This reaction involves the transfer of a hydrophilic glucuronosyl group from uridine 5'-diphosphoglucuronic acid (UDPGA) to a substrate. Through this function, the UGT enzyme family plays an important role in the elimination of drugs and other chemicals from the body. This has caused an increased interest in the study of how altered UGT function can contribute to potential drug-drug interactions in the body.

Here we demonstrate the automation of a 384-well luminescent assay which incorporates pro-luminogenic substrates to assess UGT activity. Automation consists of an inexpensive pipetting station to perform serial dilution of compounds to be tested as well as low volume reagent addition. Z'-Factor values, as well as pharmacology confirm that this combination of assay and instrumentation provides an easy to use, robust solution for automated UGT enzyme profiling.

Introduction

The UDP glucuronosyltransferase (UGT) family of enzymes is involved in the metabolism of various compounds in the body. These enzymes transfer a hydrophilic glucuronic acid moiety to their substrates, rendering them more water soluble and suitable for excretion. The UGTs act on various endogenous substrates, such as bilirubin, β -estradiol, and testosterone, as well as xenobiotics and drugs, such as diclofenac, morphine, and valproic acid. The function of these enzymes is essential for the clearance of xenobiotics and other toxins from the body. Therefore, drug induced alteration of UGT activity could potentially cause adverse health effects for patients with lowered UGT function, or drug-drug interactions *in vivo*. The current methods for this study involve protein precipitation or chromatographic separation. These are laborious and are not amenable to higher throughput compound profiling applications using multiple UGT isoforms.

The UGT-Glo™ assay from Promega Corporation, combined with instrumentation from BioTek Instruments, provides an ideal solution to overcome these obstacles. The UGT-Glo™ assay is a bioluminescent method for measuring UGT enzyme activity and inhibition *in vitro*. BioTek's instrumentation provides sensitive, easy to use ways to automate compound titration and microplate formatting steps, as well as read the luminescent output from assay plates. This combination creates an ideal solution for high-throughput UGT profiling of lead compounds in drug discovery campaigns.

UGT-Glo™ Luminescent UGT Assay

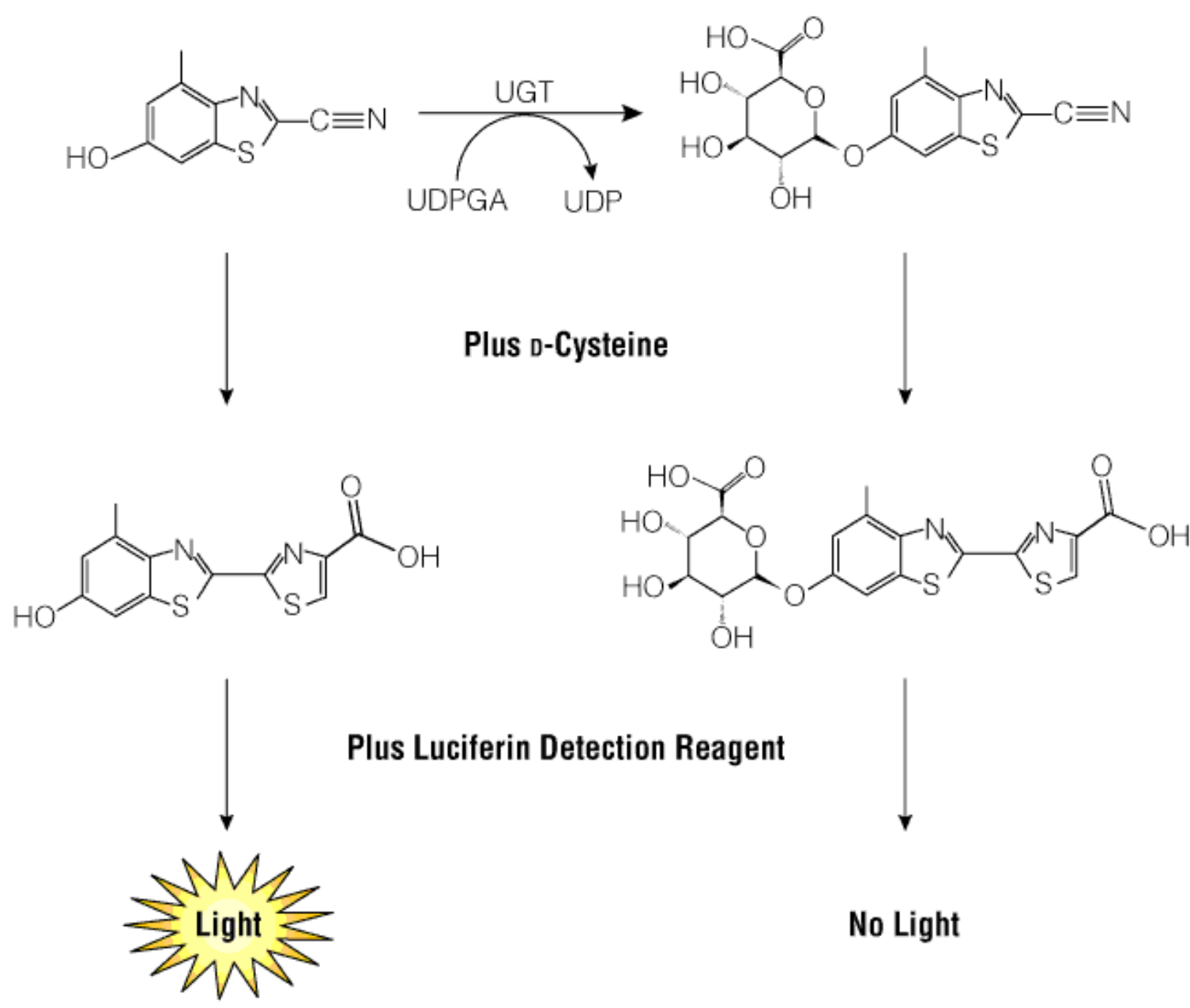


Figure 1 – The UGT-Glo™ assay is performed by incubating UGT with a proluciferin substrate; a portion of the substrate gets conjugated with UDP, while the remainder is unmodified. Upon the addition of D-Cysteine, the unconjugated proluciferin is converted into luciferin and, in a coupled reaction with luciferase/luciferin, is converted into light. Conjugated proluciferin remains intact and does not contribute to the luminescence. Thus, the signal generated is inversely correlated with UGT activity present in the sample.

BioTek Instrumentation



Figure 2 – Precision™ XS Microplate Sample Processor

The Precision™ XS combines a single-channel sample processing head, an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. The instrument was used to serially titrate compounds across a 96-well plate, as well as transfer compound and assay components to the 384-well assay plates.



Figure 3 – Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader

The Synergy™ Mx uses a dedicated optical system, separate from the fluorescence optics, for high-performance luminescence detection. An ultra low noise digital photon integration system and high-quality optics ensure the best sensitivity available today. The instrument was used to read the luminescent signal from all assay plates. The Luminescence Detection Method was chosen, and a 1.0 second integration time was used. Automatic Sensitivity Adjustment was used to detect the well on the plate containing the highest luminescent signal. All wells were appropriately adjusted to that well.

Automated Profiling Method Validation

Z'-Factor assays were performed in order to validate the automated profiling method for the UGT1A1, 2B7, and 1A4 assays in 384-well format prior to inhibitor testing. Component conditions and reaction incubation times used are listed in Table 1. 20 μ M represents the substrate K_m value for UGT1A1 and 2B7 with the UGT Multienzyme Substrate. 50 μ M represents a concentration slightly below the K_m for UGT1A4 with this substrate.

Selective inhibitors, at concentrations of 0 and 100 μ M, were used as the negative and positive controls, respectively. Raloxifene was used as the UGT1A1 and 2B7 inhibitor, while Ritonavir was used as the UGT1A4 inhibitor. Microplate shaking was performed using an orbital shaker, and incubations were performed using a 37°C incubator to simulate typical robotic profiling conditions.

UGT Isoform	UGT per Reaction	Substrate Concentration [1X]	1X UDPGA Conc. (mM)	37°C Incubation Time (Min.)
UGT1A1	0.1 mg/ml	20 μ M UGT Multienzyme Substrate	4 mM	150
UGT2B7	0.1 mg/ml	20 μ M UGT Multienzyme Substrate	4 mM	120
UGT1A4	0.4 mg/ml	50 μ M UGT1A4 Substrate	4 mM	240

Table 1 – Component concentrations and incubation times for UGT-Glo™ assays

- Dispense forty-eight 5 μ L replicates of 0 or 400 μ M inhibitor in 0.8% DMSO to the 384-well assay plate.
- Dispense 5 μ L of 16 mM UDPGA to 24 replicates of each condition, and 5 μ L of water to the remaining 24 replicates.
- Dispense 10 μ L of enzyme/substrate mix to all 96 replicates. Plate shaken for 30 seconds using an orbital shaker, and incubated at 37°C for appropriate time listed in Table 1.
- 20 μ L of Luciferin Detection Reagent plus D-Cysteine dispensed to microplate, followed by 30 second shake using an orbital shaker, and 20 minute RT incubation.
- Luminescence read on Synergy™ Mx.

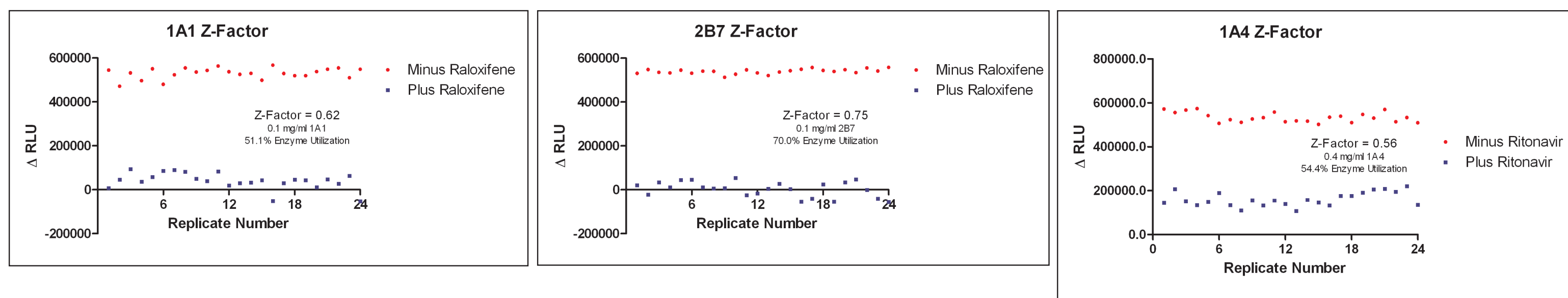


Figure 2 – Z'-Factor validation results for UGT1A1, 2B7, and 1A4 Automated UGT-Glo™ Assays

Precision™ XS UGT Profiling Methods

Compound Titration Method

The compound titration method to be used for UGT profiling involved creating serial 1:3 dilutions of compounds. Prior to the compound plate being placed onto the automated platform, 130 μ L of the appropriate diluent was placed into two rows of column 1 of the plate. 130 and 180 μ L of the top inhibitor concentration were then placed into columns 2 and 3 of the same two rows of the plate, respectively.

Automated Protocol

- Transfer 120 μ L of the appropriate diluent from a 96-well deep well microplate to columns 4-12 of two rows of the 96-well microplate.
- Aspirate 60 μ L from column 3, containing the highest concentration of test compound, dispense to column 4, and perform a 12X mix.
- Repeat the procedure for columns 4-11, leaving column 12 as a no compound control.
- Transfer 5 μ L aliquots, in quadruplicate, from each well of the 96-well compound plate to the 384-well assay plate.

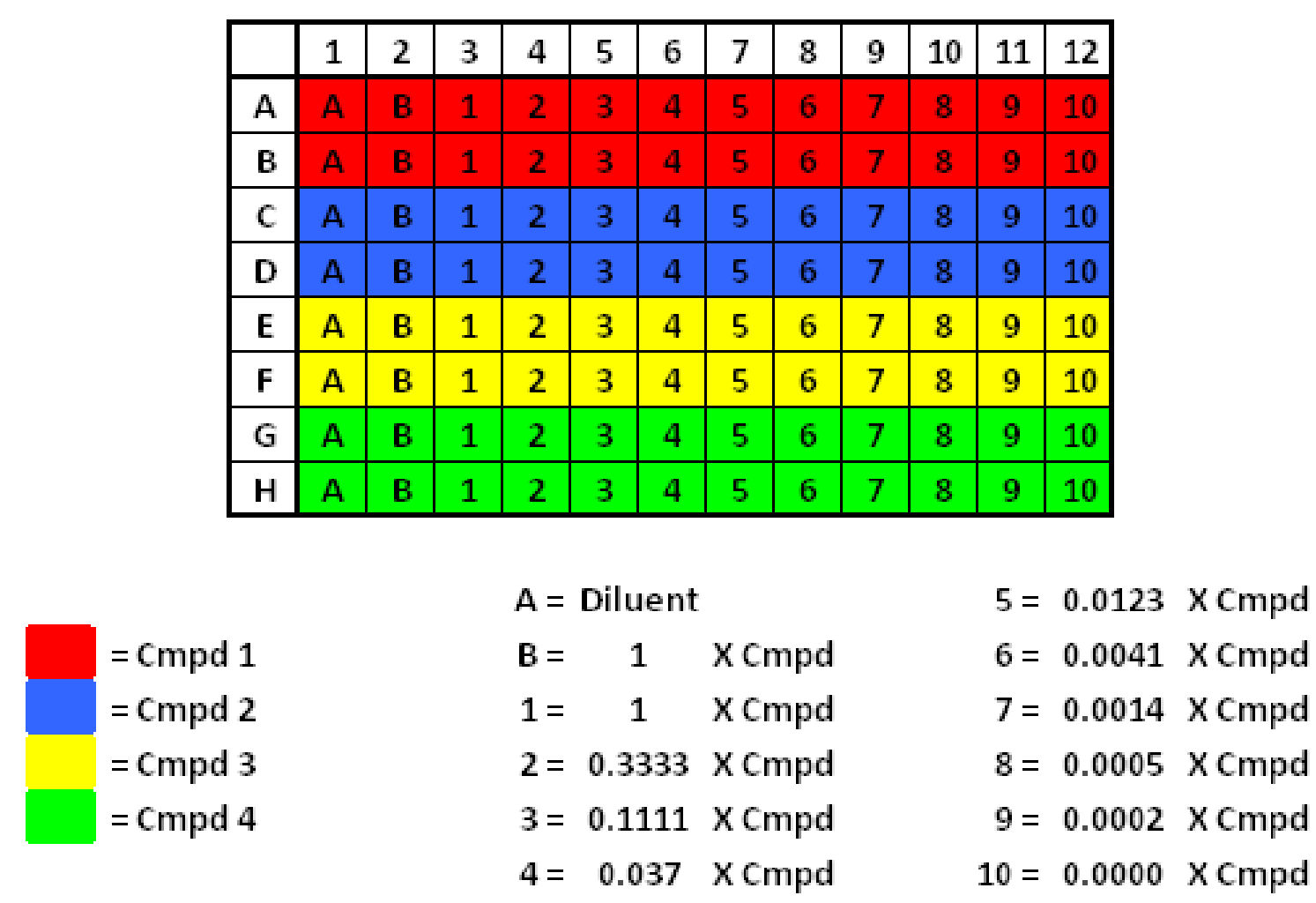


Figure 4 – Plate layout for Precision XS compound titration method

Reagent Addition Method

In addition to the compound titration being tested with enzyme/substrate, the top concentration of compound, as well as diluent, was also tested with control membrane/substrate mixes containing no enzyme. This allowed for the determination of the effect of the compound on the detection reagent reaction.

Automated Protocol

- Transfer 5 μ L of 16 mM UDPGA to the top two rows of each set of controls plus compound titration, and 5 μ L of water to the bottom two rows.
- Transfer 10 μ L of control membrane/substrate mix to the first four columns of the assay plate.
- Transfer 10 μ L of enzyme/substrate mix to the compound titration wells.
- Shake the plate for 30 seconds using an orbital shaker, and incubate at 37°C for the appropriate time listed in Table 1.
- Transfer 20 μ L of Luciferin Detection Reagent plus D-Cysteine to the assay plate. Repeat shaking procedure and incubate for 20 minutes at RT.
- Read luminescence on Synergy™ Mx.

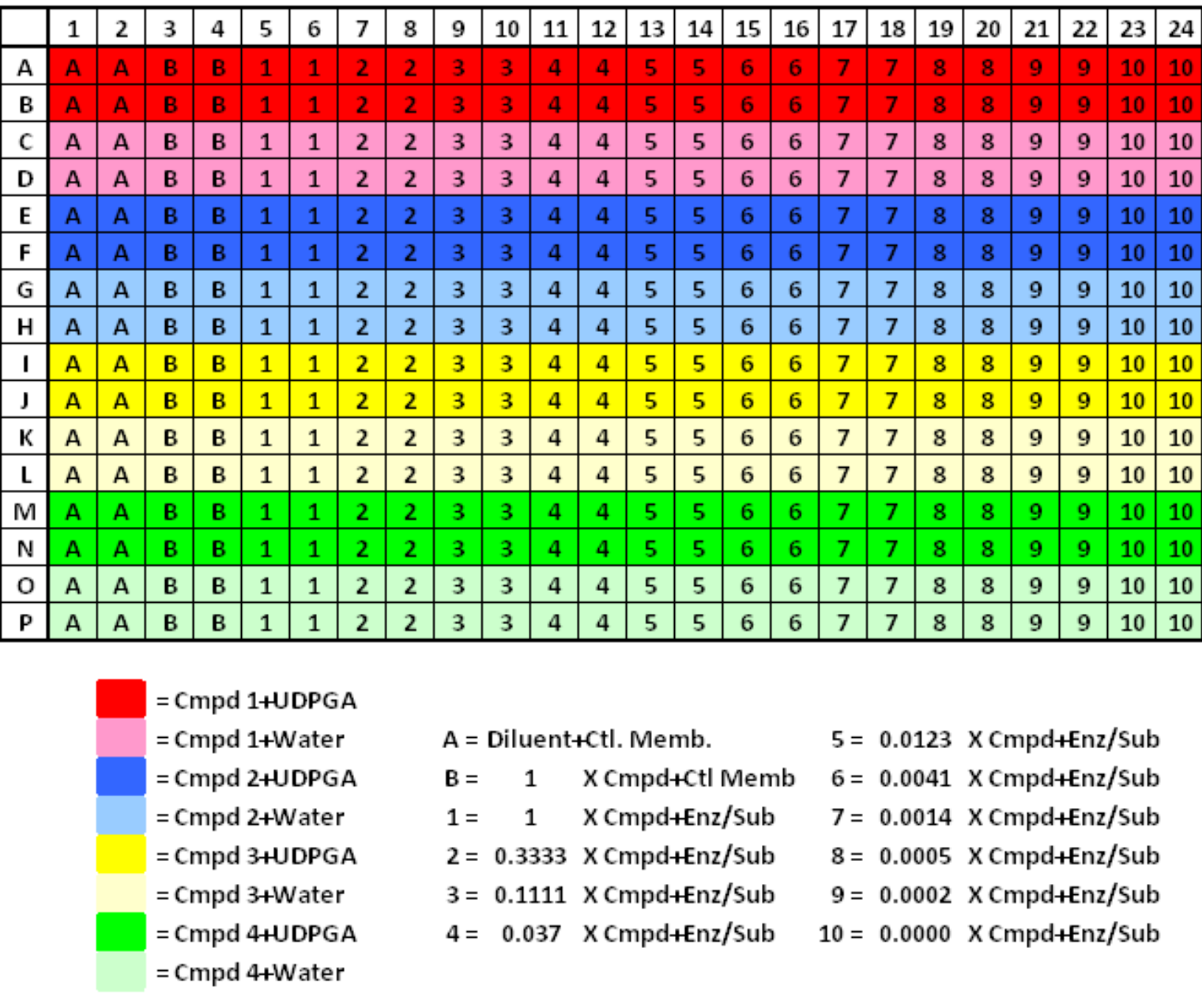


Figure 5 – Plate layout for Precision XS reagent addition method

UGT Inhibitor Profiling Results

A panel of compounds known or suspected to have activity as UGT substrates or inhibitors was profiled using the three validated assays. IC₅₀ values were obtained by fitting background corrected substrate utilization data to a sigmoidal dose-response curve using GraphPad Prism. K_i values were then estimated from the IC₅₀ values by using the concentration of the substrate from the reaction, and the substrate K_m.

Results

Compound	UGT Isoform	Measured IC ₅₀ (μ M)	Estimated K _i (μ M)	Literature K _i or K _m (μ M)	Literature Substrate
Bilirubin	UGT1A1	11.7	5.9	5	Bilirubin
	UGT2B7	>250	>125	nd	
	UGT1A4	>250	>125	nd	
Diclofenac	UGT1A1	73	36.5	52	4-methylumbelliferone
	UGT2B7	33	16.5	13-25	Diclofenac
	UGT1A4	318	187.1		
Ritonavir	UGT1A1	16	8	9.5	Bilirubin
	UGT2B7	>500	>250	>50	7-hydroxy trifluoromethyl coumarin
	UGT1A4	12	7.1	1.0	Trifluoperazine
Indinavir	UGT1A1	14	7	43.5	Bilirubin
	UGT2B7	>500	>250	>50	7-hydroxy trifluoromethyl coumarin
	UGT1A4	~325	191.2	>50	Trifluoperazine
Raloxifene	UGT1A1	11.7	5.85	nd	
	UGT2B7	11.4	5.7	nd	
	UGT1A4	15.2	8.9	nd	
Tamoxifen	UGT1A1	111	55.5		
	UGT2B7	46	23		
	UGT1A4	110	64.7	2	Tamoxifen
Fluconazole	UGT1A1	>500	>250	none reported	
	UGT2B7	>500	>250	627	zidovudine
	UGT1A4	>500	>250	none reported	
Benazepril	UGT1A1	69	34.5		
	UGT2B7	>500	>250		
	UGT1A4	>500	>250		

Table 2 – Automated UGT Profiling Results

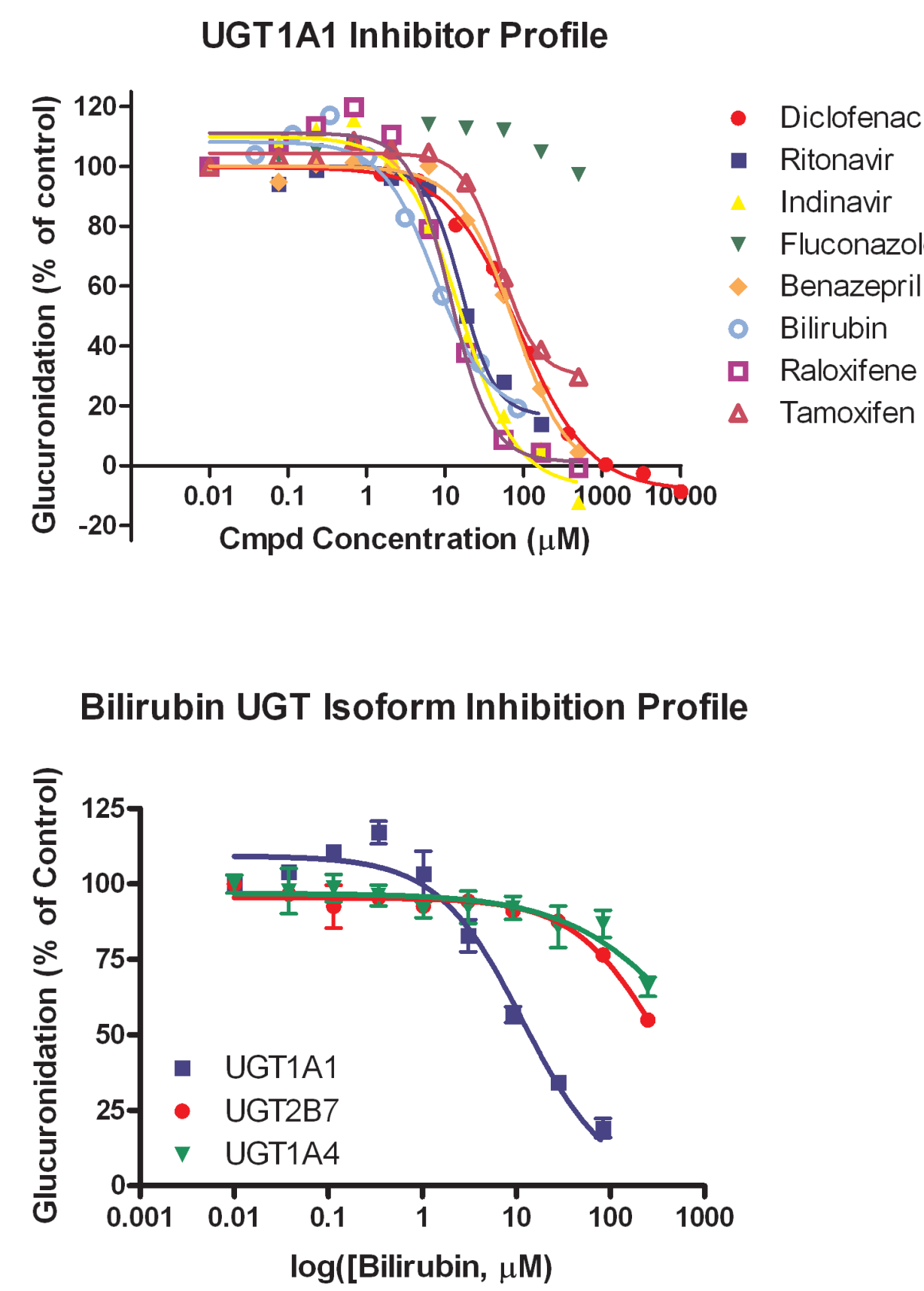


Figure 6 – Representative UGT profiling results. Data shown here for the UGT1A1 isoform and bilirubin

Conclusions

- Promega's UGT-Glo™ Assay provides straight-forward, rapid UGT inhibition data that agrees with established literature values.
- The Precision™ XS provides an easy-to-use solution for accurate and reproducible compound titration, and assay component transfer for high-density assay plates.
- The Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader is suitable for luminescence measurements.
- The combination of BioTek's instrumentation, and Promega's UGT-Glo™ Assay creates a practical solution for high-density, automated UGT profiling.