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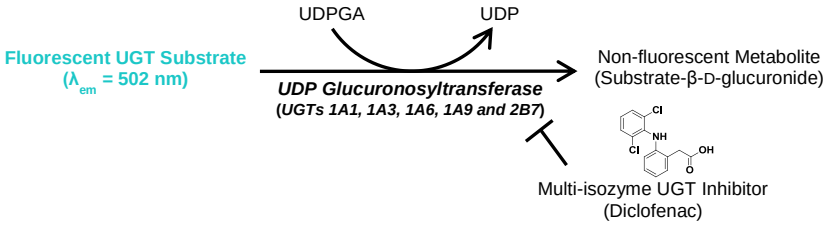
UGT Activity Assay / Ligand Screening Kit (Fluorometric)

rev 05/20

(Catalog # K692-100; 100 Reactions; Store at -20°C)

I. Introduction:

Uridine diphospho-glucuronosyltransferases (UGTs, EC 2.4.1.17) are a superfamily of microsomal membrane-bound glycosyltransferase enzymes. UGTs are responsible for the vast majority of Phase II biotransformation (conjugation) reactions, in which a hydrophilic moiety is attached to small molecule drugs and other xenobiotics to facilitate their rapid excretion from the body. UGTs are predominantly expressed in the liver, intestine and kidneys, where they catalyze the addition of a glucuronic acid moiety from the nucleotide sugar uridine-5'-diphospho-α-D-glucuronic acid (UDPGA) to nucleophilic hydroxyl, sulfhydryl or amino groups of pharmacologically active drugs, Phase I drug metabolites and certain endogenous substrates such as steroid hormones, bile acids and bilirubin. Isozymes of the UGT family have extremely broad, often overlapping substrate specificities and tend to exhibit modest affinity for substrates but high catalytic turnover rates. UGTs also contribute to 'first-pass' presystemic drug metabolism, which dramatically reduces the oral bioavailability of a number of drugs. **BioVision's UGT Activity Assay / Ligand Screening Kit** enables rapid measurement of native or recombinant UGT activity in biological samples such as liver microsomes and can also be used to assess the effect of drugs and other novel compounds on UGT activity. The assay utilizes a highly fluorescent UGT substrate with a large Stokes shift (Ex/Em = 415/502 nm) that allows determination of UGT activity by tracking the drop in fluorescence emission as the substrate is converted into a non-fluorescent glucuronide. The multi-isozyme substrate is glucuronidated by virtually all of the pharmacologically-relevant mammalian UGT1A and UGT2B enzymes. UGT specific activity is calculated by comparing the fluorescence loss versus a control reaction performed in the absence of the required cofactor UDPGA. The kit includes the pore-forming peptide antibiotic Alamethicin, which allows the UGT Substrate and UDPGA to rapidly diffuse across lipid membranes to access the UGT active site located in the lumen of microsomes. For verification of modulation of UGT activity by test ligands, diclofenac, a competitive inhibitor of most human and rodent UGT isozymes, is also included. The assay is highly sensitive, simple to perform and high-throughput adaptable. This assay can detect less than 0.1 mU UGT activity in biological samples. The kit contains a complete set of reagents sufficient for performing 100 reactions at a 100 µl reaction volume.



II. Applications:

- Rapid assessment of native/recombinant UGT activity in fractions prepared from tissues and cells.
- Screening of drugs and novel ligands for interaction with native/recombinant UGT enzymes.

III. Sample Type:

- Human or animal tissue microsomes and S9 fractions.
- Lysates of tissues and cultured cells (*e.g.* primary hepatocytes).
- Heterologously expressed recombinant UGT preparations.

IV. Kit Contents:

Components	K692-100	Cap Code	Part Number
UGT Assay Buffer	100 ml	NM	K692-100-1
UGT Inhibitor (Diclofenac)	1 vial	Clear	K692-100-2
UDPGA Stock (50X)	1 vial	Blue	K692-100-3
Alamethicin	50 µl	Orange	K692-100-4
UGT Substrate (250X)	1 vial	Yellow	K692-100-5
UGT Positive Control	1 vial	Violet	K692-100-6

V. User Supplied Reagents and Equipment:

- Multiwell fluorescence microplate top reader
- Precision multi-channel pipette and reagent reservoir
- Anhydrous (reagent grade) DMSO
- dH_2O
- Black 96-well plates with flat bottom

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C and protect from light. Briefly centrifuge all small vials prior to opening. Allow the UGT Assay Buffer to warm to room temperature (RT) prior to use. Read entire protocol before performing the assay procedure.

- **UGT Inhibitor (Diclofenac):** Reconstitute in 550 µl of dH_2O and vortex until fully dissolved to yield a 5 mM solution of diclofenac sodium. The solution should be stored at -20°C until use and is stable for at least 3 freeze/thaw cycles.
- **UDPGA Stock (50X):** Reconstitute with 220 µl dH_2O to yield a 50X stock solution. Aliquot the stock solution as desired and store the aliquots at -20°C. Avoid repeated freeze/thaw cycles and keep on ice while in use.

- **Alamethicin:** Alamethicin is provided as a solution in DMSO. Warm the solution to RT to melt the DMSO and vortex to ensure Alamethicin is completely dissolved. Aliquot and store at -20°C, stable for at least 3 freeze/thaw cycles.
- **UGT Substrate (250X):** Reconstitute with 110 µl reagent-grade DMSO and vortex until fully dissolved to obtain a 250X stock solution. The UGT Substrate should be stored at -20°C and is stable for at least 3 freeze/thaw cycles. Allow the vial to warm to RT before opening and promptly retighten cap after use to avoid absorption of airborne moisture.
- **UGT Positive Control:** Do not reconstitute until ready to use. Reconstitute with 22 µl UGT Assay Buffer and mix thoroughly to ensure a homogenous solution (the concentrated solution will be slightly viscous and have an opaque, milky appearance). The reconstituted UGT Positive Control may be aliquoted and stored at -80°C. Avoid repeated freeze/thaw cycles and use aliquots within one month. Thaw aliquots rapidly at 37°C and place on ice until use (thawed aliquots should be used within 4 hours).

VII. UDP Glucuronosyltransferase (UGT) Activity Assay Protocol:

1. Standard Curve Preparation:

a. Add 5 µl of the 250X UGT Substrate stock to 495 µl UGT Assay Buffer to generate a 0.1 nmole/µl UGT Substrate standard. Add 0, 2, 4, 6, 8, 12, 16 and 20 µl of the 0.1 nmole/µl solution into a series of wells in a black 96-well plate, yielding 0, 0.2, 0.4, 0.6, 0.8, 1.2, 1.6 and 2.0 nmole/well fluorescence standard. Adjust the volume of each well to 100 µl with UGT Assay Buffer.

b. Measure fluorescence at Ex/Em = 415/502 nm. Subtract the zero standard (0 nmole/well) reading from all of the standard readings, plot the background-subtracted values and calculate the slope of the standard curve.

2. Sample and Test Compound Preparation:

a. Standardized microsomal preparations may be purchased commercially (*e.g.* donor-pooled human liver microsomes) or prepared from tissue or cultured cells using the Microsome Isolation Kit (Cat. #K249). Alternatively, a crude enriched lysate can be prepared: start with ~50 mg tissue or ~5 x 10^6 pelleted, pre-washed cells and homogenize in 500 µl ice-cold UGT Assay Buffer with a Dounce homogenizer (Cat. #1998 or equivalent) on ice. Incubate the homogenate on ice for 5 min and then centrifuge at 15,000 x *g* for 15 min at 4°C. Collect the resultant clarified supernatant for the assay in a new pre-chilled microfuge tube and store on ice until use (cell and tissue lysates can also be stored at -80°C in aliquots for future experiments).

b. Prepare a 2X Sample Premix by diluting the sample with UGT Assay Buffer. For liver microsomes, we recommend that the 2X Sample Premix contain 0.05-0.2 mg protein/ml (resulting in a 0.025-0.1 mg/ml final concentration). For each well, 50 µl of 2X Sample Premix should be prepared (however, we recommend preparing enough of the premix for at least 10 wells to prevent pipetting inaccuracies). If using the UGT Positive Control, prepare a 2X Positive Control Premix solution by adding 10 µl of the reconstituted stock to 490 µl UGT Assay Buffer (a 1:50 dilution).

c. Place the 2X Sample Premix (and UGT Positive Control Premix, if applicable) on ice and add 5 µl of Alamethicin per ml to each of the 2X Sample Premix solutions (1 µl Alamethicin per 200 µl of premix solution) and incubate on ice for 15 min.

d. If desired, UGT activity in presence of test ligands may be measured. Test ligands should be dissolved into proper solvent to produce stock solutions. For each ligand, prepare a 5X solution by diluting in water or UGT Assay Buffer. We recommend running parallel solvent control well(s) to account for potential solvent effects. Final concentrations of organic solvents should be minimized to avoid impacting UGT activity; however DMSO has been shown to have little effect on UGT activity at a final concentration of ≤2% (v/v).

Notes:

- If assaying a crude tissue lysate or other uncharacterized sample, use the Bradford reagent (Cat. #K810) or an equivalent protein assay to determine sample protein concentration prior to preparing the 2X Sample Premix.
- Remember to account for any control reactions (such as the reaction blank, diclofenac inhibition and solvent control wells) when calculating the amount of 2X UGT Sample Premix to prepare.
- Dilute the UGT Positive Control directly before Alamethicin incubation. We do not recommend storing the diluted Positive Control.

3. Reaction Preparation:

a. Prepare a 10X working solution of UGT Substrate by diluting the 250X UGT Substrate stock in UGT Assay Buffer at a 1:25 ratio. Make a sufficient amount of 10X UGT Substrate solution to add 10 µl to each reaction to be performed (for 100 reactions, mix 40 µl of 250X UGT Substrate stock with 960 µl UGT Assay Buffer). The final concentration of UGT Substrate will be 40 µM, which in our experience is approximately equal to the K_m in donor-pooled human liver microsomes.

b. Set up the assay reaction wells according to the table below. In addition to the test samples, prepare a reaction blank (no-UDPGA control) well. If desired, you may also prepare an inhibitor control well using the UGT Inhibitor solution (5 mM diclofenac, for a final concentration of 1 mM). Adjust the volume of test sample, inhibitor control and positive control wells to 80 µl/well with UGT Assay Buffer. For measurement of UGT activity in the presence of test ligands, replace Assay Buffer with 5X concentrated test ligand solution:

	Test Sample	+Test Ligand	Blank (–UDPGA)	Inhibitor Control	Positive Control
Sample Premix (2X)	50 µl	50 µl	50 µl	50 µl	—
UGT Positive Control Premix (2X)	—	—	—	—	50 µl
UGT Substrate Working Solution (10X)	10 µl	10 µl	10 µl	10 µl	10 µl
Diclofenac 5 mM Solution (5X)	—	—	—	20 µl	—
UGT Assay Buffer	20 µl	—	40 µl	—	20 µl
Test Ligand (5X)	—	20 µl	—	—	—

c. Incubate the plate for 5 min at 37°C, protected from light. During the incubation, prepare a sufficient amount of 5X UDPGA solution to add 20 µl to each reaction well by diluting the 50X UDPGA Stock in UGT Assay Buffer at a 1:10 ratio.

d. Start the reaction by adding 20 µl of the 5X UDPGA solution to each well (except the no-UDPGA blank) using a multichannel pipette, yielding a final reaction volume of 100 µl per well.

4. Measurement:

Immediately measure the fluorescence at Ex/Em = 415/502 nm in kinetic mode for 30-40 min at 37°C. While the assay can be performed in either endpoint or kinetic mode, we strongly recommend reading in kinetic mode in order to ensure that the

measurements recorded are within the linear range of the reaction. Ideal measurement time for the linear range may vary depending upon the amount and variety of active UGT isozymes in the sample.

Notes:

- Since the reaction starts immediately after the addition of the UDPGA, it is essential to preconfigure the fluorescence microplate reader settings and use a multichannel pipette with a reagent reservoir to minimize lag time among wells.
- The fluorescent UGT Substrate may exhibit photobleaching if exposed to overly rapid excitation light pulses. To minimize the likelihood of photobleaching, we recommend setting the microplate reader scan interval to 20 seconds or greater.

5. **Calculation:** For each reaction well, choose two time points (T_1 and T_2) in the linear phase of the reaction progress curves, obtain the corresponding fluorescence values at those points (RFU_1 and RFU_2) and determine the absolute value of the change in fluorescence over the time interval: $\Delta F = \left| RFU_2 - RFU_1 \right|$. Calculate the specific fluorescence lost due to substrate glucuronidation (denoted by G) by subtracting the ΔF value of the blank (no UDPGA) reaction well from those of the test samples (S):

$$G_S = \Delta F_S - \Delta F_{blank}$$

Note: The UGT Substrate is glucuronidated by the majority of characterized human hepatic and non-hepatic UGT isozymes, aside from UGT1A4. Thus, the calculated UGT activity represents a composite of all of the isozymes expressed in a particular sample. In human liver microsomes, the major isozymes include UGT1A1, 1A3, 1A6, 1A9 and 2B7.

UGT activity is obtained by applying the G_S values to the substrate standard curve to get B nmole of substrate glucuronidated by sample UGT enzymes during the reaction time.

$$\textbf{UDP Glucuronosyltransferase Specific Activity} = \frac{B}{\Delta T \times P} = \textbf{nmole/min/mg} = \textbf{mU/mg}$$

Where: $\mathbf{B}$ is the amount of substrate consumed, calculated from the standard curve (in nmole)
$\Delta\mathbf{T}$ is the linear phase reaction time $T_2 - T_1$ (in minutes)
$\mathbf{P}$ is the amount of protein in the well (in mg)

UGT Unit Definition: One unit of UGT activity is the amount of enzyme that glucuronidates 1 µmole of fluorescent substrate per min (yielding a non-fluorescent conjugate) at 37°C and pH 7.5.

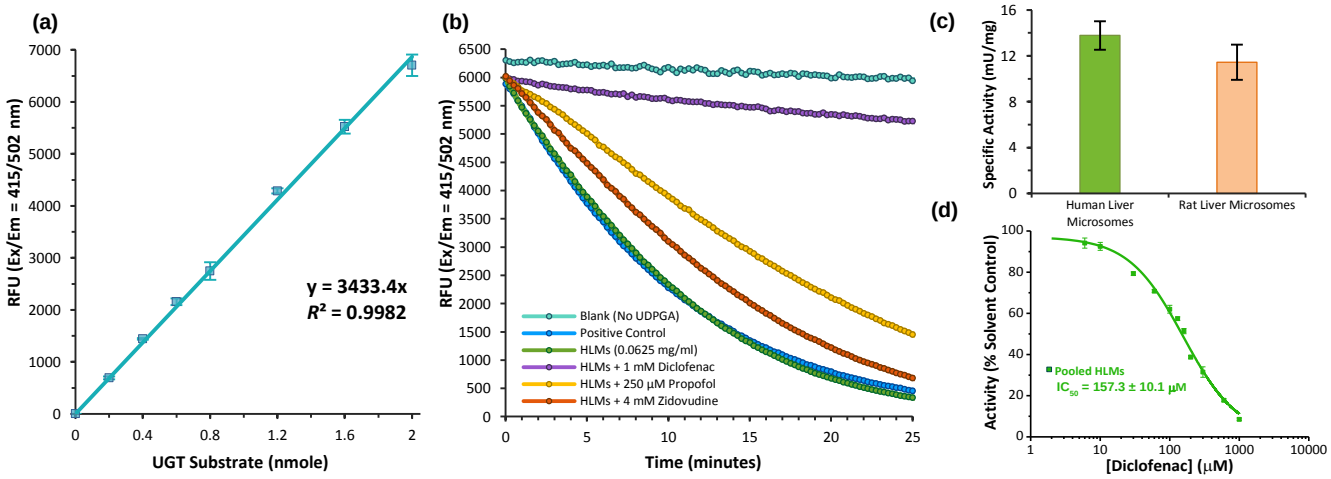


Figure: (a) Standard Curve of UGT Substrate fluorescence. Glucuronidation of one mole of UGT Substrate produces a decline in fluorescence equivalent to the fluorescence generated by one mole of the unconjugated substrate. **(b)** Reaction kinetics of fluorescent substrate glucuronidation in donor-pooled human liver microsomes (HLMs, 0.0625 mg/mL) at 37°C and inhibition of UGT activity in HLMs by the isozyme-selective ligands propofol (UGT1A9-selective) and zidovudine (UGT2B7-selective), as well as the non-selective UGT ligand diclofenac. For the blank reaction condition, vehicle (Assay Buffer) was substituted for the cofactor UDPGA. **(c)** Specific UGT activity in pooled human and rat liver microsomes (mean ± SEM of 3 replicates). **(d)** Dose-response curve for UGT inhibition by diclofenac in HLMs. Percent activity was calculated for each concentration by comparison to activity of reaction containing vehicle only. IC_{50} values were derived by 4-parameter logistic curve fitting with each point representing the mean ± SEM of at least 3 replicates. Assays were performed according to the kit protocol.

VIII. RELATED PRODUCTS:

Microsome Isolation Kit (K249)
Cytochrome P450 Reductase Activity Kit (K700)
Cytochrome P450 3A4 Activity Assay Kit (K701)
Cytochrome P450 3A4 Inhibitor Screening Kit (K702)
Cytochrome P450 2D6 Activity Assay Kit (K703)
Cytochrome P450 2D6 Inhibitor Screening Kit (K704)
Cytochrome P450 2C19 Activity Assay Kit (K848)
Cytochrome P450 2C19 Inhibitor Screening Kit (K849)
Cytochrome P450 1A2 Activity Assay Kit (K893)
Cytochrome P450 1A2 Inhibitor Screening Kit (K894)
Cytochrome P450 2C9 Activity Assay Kit (K895)
Cytochrome P450 2C9 Inhibitor Screening Kit (K896)

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