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Detection of Total UDP-Glucuronosyltransferase (UGT) Activity in Melanoma Cells

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Abstract

The UDP-glucuronosyltransferases (UGTs) are integrally involved in the clearance of a wide range of drugs used to combat human diseases. UGT expression levels and activity can be induced by drug addition to cells and has been proposed as a potential intratumoral drug resistance mechanism. Traditional methods of assaying UGT activity are drug-centric and require HPLCs with multiple detectors (dependent on individual drug). Here, we describe a generalized method to detect total UGT activity (intrinsic or induced) via the UGT-Glo assay which utilizes a general UGT substrate with luminescence as the readout eliminating the need for multiple HPLC detectors to detect total UGT activity in a given sample. The method detailed here can be applied for any UGT containing sample, allowing for the efficient detection of total UGT activity to be the functional endpoint using a plate reader. In this manner, global changes in UGT activity can be monitored in response to a wide variety of stimuli.

**Keywords:** UDP-glucuronosyltransferase, Glucuronidase activity, UGT-Glo, Melanoma, Drug resistance

1 Introduction

The UGT family of enzymes is integrally involved in the detoxification of many carcinogens, the clearance of drugs and the metabolism of a variety of endogenous substrates such as bilirubin, steroid hormones, and bioactive lipids [1, 2]. Although UGTs are primarily expressed in liver, they also play vital roles in other tissues in the body. For example, UGT2B15 and UGT2B17 are expressed in the prostate where they regulate local androgen levels through glucuronidation [3] and UGT1A10 and UGT2B7 are expressed in the breast where they regulate estrogens [4]. There is also ample evidence that the UGTs play important roles in the aerodigestive tract, gastrointestinal tract, lung, colon, bladder, kidneys, and brain [5–9]. Recently, UGT2B7, UGT2B10, and UGT2B15 were identified as being normally expressed in human melanocytes [10]. However, UGT expression was observed to be lost during progression of melanocytes to melanoma [10]. Treatment of melanoma cell lines which lack UGT expression with anticancer agents induced expression of UGT2B7, UGT2B10, and UGT2B15

demonstrating that melanoma cells retain the ability to re-express these same three UGTs [10]. The corresponding increase in glucuronidation activity in melanoma cells following anticancer treatment was also observed using the UGT-Glo assay. Since the UGTs are drug metabolism enzymes, this re-expression of the UGTs is proposed to constitute a previously unsuspected mechanism for intratumoral drug resistance in melanoma [10].

Traditional methods of detecting UGT activity involve thin layer chromatography (with a radiolabeled co-substrate) or HPLC [11, 12]. In either method, a limiting factor in detecting total UGT activity is in the selection of a general UGT substrate which is a decent substrate for all UGT family members present in the sample. Further limitations can be access to sample, cost, or detection of the substrate. Some substrates have decent UV absorption; others require fluorescence or mass spectrometry detection. Here, we describe a general method to detect total UGT activity in melanoma cells using the UGT-Glo assay. This method has the distinct advantage of using a general UGT substrate that can react with a luciferin detection reagent to produce light that can be detected on a luminometer, unless that substrate is glucuronidated first (Fig. 1).
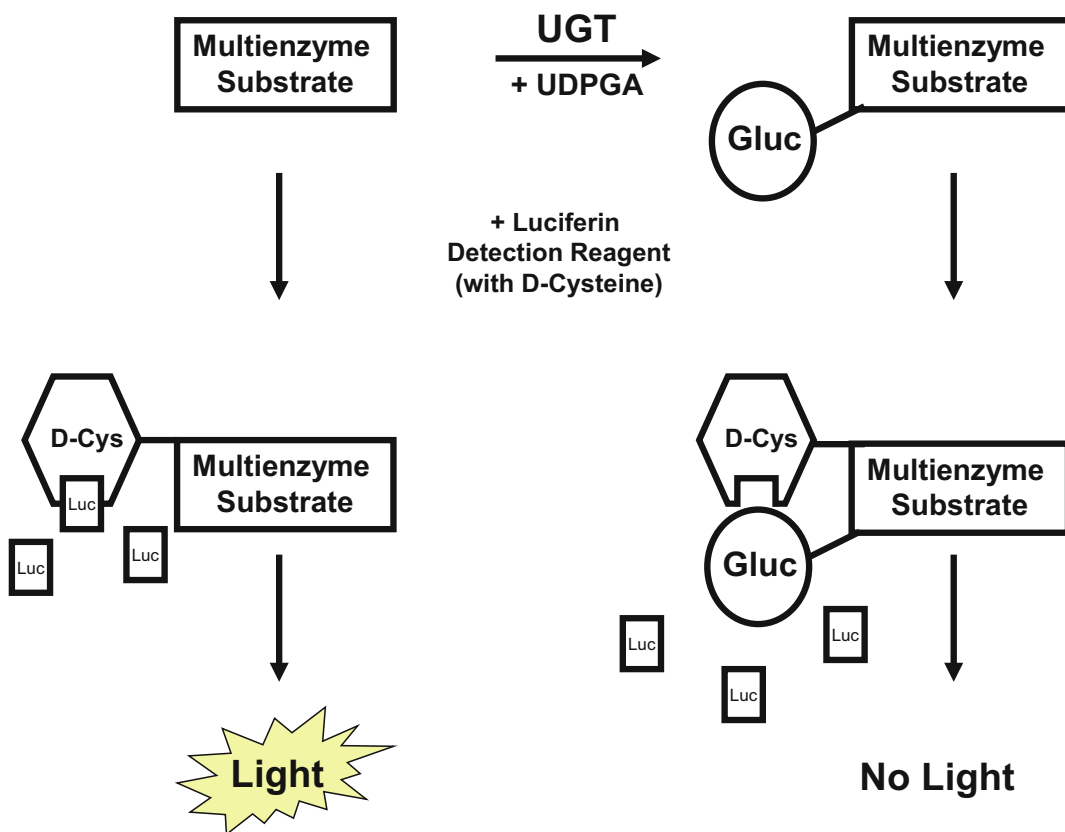
Fig. 1 Schematic of UGT-Glo activity assay. Stylized representation of glucuronidation detection using the UGT-Glo assay. *Gluc* glucuronide, *Luc* luciferin detection reagent, *D-Cys* D-cysteine. For actual chemical structures and processes please see the manufacturer's protocol

If the substrate is glucuronidated, it will no longer react with the detection reagent and no light will be emitted. Thus, reactions are run in parallel for each reaction, one with co-substrate (UDPGA) and one without co-substrate. In each case the (−) UDPGA reaction is the negative control that will emit light (Fig. 1). The (+) UDPGA reaction is the experimental reaction and any reduction detected in emitted light, as compared to negative control, indicates UGT activity (Fig. 1). These reactions are carried out in a 96-well plate (or 384 if desired) and as such several experimental conditions can be assayed at once (even in triplicate) as opposed to injecting one sample at a time onto an HPLC with run times of 15–30 min (or longer) each. However, it is important to note that this technique is not applicable to UGT kinetics assays, studies identifying UGT/substrate specificity or any other substrate-specific investigations. In those instances the traditional UGT activity assays are required.

2   Materials

Prepare all solutions with ultrapure water. Always keep any enzymatically active preparation on ice and minimize the time spent on ice prior to experiment to retain optimum activity.

1. Tris Buffered Saline (TBS) homogenate buffer: 25 mM Tris base, 138 mM NaCl and 2.7 mM KCl, pH 7.4. Add 8 g of NaCl, 3 g of Tris base, and 0.2 g of KCl to 800 mL water. Adjust pH to 7.4 and then add water to a final volume of 1 L. Can store at room temperature. To make working solution of TBS homogenate buffer add 10 mL to a clean 15 mL conical and dissolve one pellet of Complete mini protease inhibitor cocktail EDTA-free (Roche). Must be EDTA free as no chelating agents can be present in UGT assay since Mg^{+2} is required for optimal activity.

2. DMEM complete media: To 450 mL sterile DMEM, add 50 mL fetal bovine serum (FBS) and 5 mL penicillin/streptavidin solution.

3. Phosphate buffered saline (PBS): purchase sterile PBS (Fisher).

4. 96-well plates (one clear bottomed and one white opaque).

5. Alamethicin (sigma): 10 mg powder resuspended in 500 μL ethanol (20 μg/μL stock).

6. UGT-Glo assay (Promega).

7. UDP-Glucuronic acid (UDPGA): provided in the UGT-Glo assay as a 50 mM stock. Dilute to 16 mM in water before use.

8. Dounce homogenizer 2 mL.

9. Human Liver Microsomes (Xenotech, Lenexa, KS).

3 Methods

All cell culture should be carried out using sterile technique in a laminar flow hood (class II).

3.1 Cell Culture

1. Culture SKMel28 metastatic melanoma cells in 10 cm dishes with 10 mL DMEM complete media. Incubate cells in humidified 37 °C incubator with 5 % CO_2 (*see* **Note 1**).

2. Allow cells to grow to ~60 % confluency. One plate per experimental condition is required. Treat cells with desired drug. An example from our recent publication [10] is SKMel28 cells were treated with epirubicin at 0.1 or 1.0 μM or treated with vehicle alone (DMSO) for 24 h (*see* **Note 2**).

3.2 Preparation of Cell Homogenates

1. Aspirate media from cells and wash cells with 5 ml PBS. Aspirate PBS and add 1 mL trypsin to the cells. Incubate cells at 37 °C for 5 min (*see* **Note 3**).

2. Once cells have detached, add 1 mL of trypsin neutralizing solution (TNS) and transfer cells to a 15 mL conical tube. Pellet cells by centrifugation at $1000 \times g$ at 4 °C for 1 min. Discard supernatant.

3. Place pellets on ice. Resuspend the pellet in 200 μL TBS homogenate buffer.

4. Lyse cells via freeze thaw. Place pellets in liquid nitrogen (or dry ice–ethanol bath) to freeze them. Thaw cells in 37 °C water bath. Repeat three times.

5. Add homogenate to a 2 mL glass Dounce Homogenizer. Insert plunger and physically disrupt cells. Pull plunger out and reinsert. Repeat ten times (*see* **Note 4**).

6. Transfer homogenate to clean 1.5 mL eppendorf and keep on ice.

3.3 BCA Protein Quantification Assay

1. Place 2 μL of each homogenate into a clean eppendorf. Add 8 μL of water. We recommend triplicate reactions to ensure accurate protein concentration numbers.

2. Set up standard curve of known protein standard. We use Bovine Serum Albumin (BSA) at a stock solution of 2 μg/μL. Set up seven reactions for standard curve, 0, 2, 4, 6, 8, 10, and 20 μg/μL. From BSA stock solution add 0, 1, 2, 3, 4, 5, 10 μL to each tube, respectively. Then add the appropriate amount of water so the total volume equals 10 μL per tube (10, 9, 8, 7, 6, 5, 0 μL, respectively) (*see* **Note 5**).

3. In a clean 15 mL conical tube, mix the BCA working reagent (WR). Add 5.0 mL of Solution A and 100 μL of solution B for a

ratio of 50:1. Total volume can be adjusted depending on number of samples to be examined.

4. Add 200 μL of WR to each tube. Mix with pipette and transfer to a 96-well plate.

5. Cover plate and incubate at 37 °C for 30 min.

6. Cool plate to room temperature and then read on plate reader at 562 nm. Compare each unknown with the standard curve to get protein concentration. Divide by 2 to get your final unknown concentration since we analyzed 2 μL of unknown.

3.4 Total Glucuronidation Assay Using UGT-Glo

1. In a white, opaque 96-well plate, add 1 μL of alamethicin (20 μg/μL stock in ethanol) to all wells that will be assayed. Place 96-well plate at 37 °C to allow ethanol to evaporate (ethanol can interfere with UGT activity). Remove plate and add 50 μg of homogenate to each well and incubate on ice for 10 min (*see* **Note 6**). As depicted in Fig. 2, there will be six wells for each homogenate (triplicate reactions for both the (−)

(-) UDPGA						(+) UDPGA					
No UGT	No UGT	No UGT				No UGT	No UGT	No UGT			
CON 1	CON 1	CON 1				CON 1	CON 1	CON 1			
CON 2	CON 2	CON 2				CON 2	CON 2	CON 2			
CON 3	CON 3	CON 3				CON 3	CON 3	CON 3			
CON 4	CON 4	CON 4				CON 4	CON 4	CON 4			
CON 5	CON 5	CON 5				CON 5	CON 5	CON 5			
CON 6	CON 6	CON 6				CON 6	CON 6	CON 6			
HLM	HLM	HLM				HLM	HLM	HLM			

Fig. 2 Schematic of sample 96-well plate set up for the UGT-Glo assay. Every experimental condition (CON) is run in triplicate pairs. Three reactions minus UDPGA and three reactions with UDPGA. Only the (+) UDPGA reaction will have UGT activity. *HLM* human liver microsome (positive control)

UDPGA and the (+) UDPGA conditions). To keep total volume equivalent, add water to each sample up to 10 μL total. For a negative control, include a no UGT condition (six wells) in which only 10 μL of water is present. For a positive control, add 1 μL of human liver microsomes (HLM) + 9 μL of water (six wells). Since most UGT family members are highly expressed in HLM, 1 μL is plenty to serve as a positive control.

2. Prepare master mix for UGT activity reaction. For each well, 20 μL is required consisting of 8.0 μL UGT-Glo buffer (5×), 5 μL UGT multienzyme substrate (0.4 mM stock) (*see* **Note 7**), and 7 μL of water. Determine the total number of reactions to be set up, then add two reactions to compensate for pipetting errors. For example, if 50 total reactions (8 conditions × 6 wells each + 2 additional reactions) then add 400 μL of UGT-Glo buffer, 250 μL of the multienzyme substrate and 350 μL of water to a clean 1.5 mL eppendorf and mix thoroughly with pipette.

3. Once 10 min incubation is over remove plate from ice and place at room temperature. Add 20 μL of master mix to each experimental well.

4. Add 10 μL water to all (−) UDPGA wells. Total volume is now 40 μL.

5. Add 10 μL of UDPGA (16 mM) to all (+) UDPGA wells.

6. Incubate plate at 37 °C for 90 min (*see* **Note 8**).

7. Prepare Luciferin Detection Solution. Add entire contents of reconstitution buffer to the amber vial containing powered Luciferin Detection agent. Mix by inverting (do not vortex). Remove the appropriate amount of Detection solution needed for the experiment (40 μL per well). For example, for our 50 total reactions above place 2 mL (50 × 40 μL) of Detection solution into a clean 15 mL conical. Add 20 μl of D-Cysteine (100× stock solution). Mix by inverting (do not vortex) and place at room temperature until ready for use (*see* **Note 9**).

8. Remove plate from 37 °C incubator. Add 40 μL of Luciferin Detection reagent (plus D-Cysteine) to all experimental wells. Mix briefly on plate shaker or by gentle tapping of plate.

9. Incubate at room temperature for 20 min to stabilize the luminescent signal.

10. Read luminescence on a plate-reading luminometer or CCD camera. Values will be reported in relative light units (RLU).

3.5 Data Analysis

1. Only the (+) UDPGA wells will have UGT activity and that activity will reduce the amount of RLUs detected. Remember this is due to the fact that if the multienzyme substrate is glucuronidated then it will NOT bind to the D-Cysteine and

subsequently react with the Luciferin Detection Reagent (yielding light). Thus, to calculate total UGT activity the RLU value in the (+) UDPGA wells have to be subtracted from the corresponding (−) UDPGA wells for that same experimental condition.

2. Repeat this analysis for each well pair. Since all conditions were performed in triplicate an average and standard deviation can easily be calculated.

3. Final RLUs can then be compared across experimental condition by normalizing to total protein content which was obtained in Section 3.3 above.

4 Notes

1. SKMel28 metastatic melanoma cells were used in this experiment since they lack UGT expression. Using these cells it is easy to detect which drugs induce UGT activity since any detection of UGT activity would indicate the UGTs had been reactivated. Normal passage conditions for SKMel28 are 1:4 twice a week. Although SKMel28 is used in this example, the procedure is applicable to any cell line to detect intrinsic (or inducible) total UGT activity.

2. Any desired drug, hormone, growth factor, cytokine, carcinogen, etc. can be used here to test the effect on global UGT activity in any desired cell line. We find it is important to treat cells with various concentrations of drug with the lowest concentration being below the IC_{50} concentration for the given drug.

3. The time needed to trypsinize cells varies. For SKMel28 cells, 5 min at 37 °C is required. That time can be anywhere from 5 s to 10 min depending on how difficult the cells are to remove from the plate. Just keep checking on the cells until the majority has lifted off the plate.

4. We find that by not adding detergent (which are present in most lysis buffers), the UGTs stay active. Also, we find that homogenates maintain all UGT activity levels, where if you spin the homogenate down, or isolate just the microsomal fraction, the activity of some UGT family members may be lost. For example, UGT1A10 is known to be present in low levels in the microsomal fraction [13] and UGT2B7 and UGT1A6 have been shown to be nuclear [13, 14].

5. We find that protein concentrations are routinely between 5 and 10 mg/mL so we use a standard curve that covers this range. The standard curve can be adjusted if desired.

6. Alamethicin is a pore-forming peptide that has been demonstrated to increase UGT activity in vitro [12, 13, 15, 16]. Since UGTs are membrane bound enzymes normally found in the endoplasmic reticulum, their catalytic domains can be masked in the inside of membrane bound organelles or vesicles. Thus, by adding a pore-forming peptide we allow the substrate increased access to the catalytic domain of the UGT.

7. The UGT multienzyme substrate is used here and is the best option for measuring total UGT activity in a sample. It is important to note however that UGT1A4 has very little activity against this substrate. There is a UGT1A4 specific substrate also included in the UGT-Glo kit and this substrate should be used additionally to the multienzyme substrate if UGT1A4 is known to be present in the homogenate. In our SKMel28 cells UGT1A4 is not present and so only the multienzyme substrate is used.

8. Incubation time for glucuronidation assay can vary. Ninety minutes was plenty for our example, but this time can be extended as desired (even overnight). The caveat is that once the substrate or co-substrate is used up (or UGT enzyme degrades) then the activity will no longer be in the linear range for effective comparisons. Still, if detection of activity is the endpoint then an overnight reaction may be more fitting.

9. The resuspended Luciferin Detection Agent (without D-Cysteine) can be stored at 4 °C for 1 week or −20 °C for 3 months without loss of activity (per manufacturer instructions).

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