

Transfection Assays With Allele-Specific Constructs

Functional Analysis of UDP–Glucuronosyltransferase Variants

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Summary

Adverse drug reactions (ADRs) are a major clinical problem. A rapidly growing body of evidence suggests that genetic factors, at least in part, determine individual susceptibility to ADRs. A large number of pharmacogenetic studies have identified a number of polymorphisms as predictors of drug efficacy and/or adverse events. These candidate markers should be investigated further to ascertain the underlying mechanism of action, for example, changes in the kinetic parameters of an enzyme, or transcriptional activity of a promoter region. In this chapter, we describe a transient transfection assay for the functional characterization of naturally occurring variants of UDP–glucuronosyltransferase (UGT) 1A1. This phase II drug metabolizing enzyme is involved in the glucuronidation of SN-38, an active metabolite of the anti-cancer drug irinotecan. Single-nucleotide polymorphisms of the *UGT1A1* gene have been correlated to irinotecan-induced ADRs. Variant UGT1A1s are heterologously expressed in COS-1 cells and characterized in terms of the level of protein expression and enzyme kinetics.

Key Words: UDP–glucuronosyltransferase 1A1; single-nucleotide polymorphism; adverse drug reactions; SN-38 glucuronidation; kinetic analysis.

1. Introduction

UDP–glucuronosyltransferase (UGT) 1A1 catalyzes the glucuronidation of bilirubin, thereby rendering it soluble for excretion. A genetic defect in *UGT1A1*, therefore, can result in a phenotype of unconjugated hyperbilirubinemia, Crigler–Najjar syndrome, and Gilbert’s syndrome. UGT1A1, along with UGT1A7 and UGT1A9, also is known to play a dominant role in the glucuronidation of SN-38, an active metabolite of the anti-cancer drug irinotecan (**1,2**). Pharmacogenetic studies have revealed that several polymor-

phisms in *UGT1A1* affect the pharmacokinetics of irinotecan/SN-38 (3) and consequently the incidence of adverse side effects of SN-38, such as severe diarrhea and neutropenia (4,5). Large ethnic differences exist in *UGT1A1* polymorphisms, and non-synonymous variations in the coding region have been found in Japanese/Asian populations at relatively high frequencies; 211G>A (amino acid substitution of G71R), 247T>C (F83L), 686C>A (P229Q), and 1456T>G (Y486D) (6,7). We have characterized the functional alterations for some of these *UGT1A1* variants using the heterologously expressed recombinant proteins (8).

A simple way of evaluating the affect of a single nucleotide polymorphism on enzyme function is to perform transient transfection assays in COS-1 cells. Western blot analysis, with anti-UGT1A antibody, is used to determine protein expression levels of the variants, which often correlate with protein stability. Enzyme kinetic analysis is used to investigate the functional impact of amino acid substitutions.

2. Materials

2.1. Plasmid Construction

2.1.1. TA Cloning

1. Human adult normal liver complementary deoxyribonucleic acid (cDNA; BioChain Institute Inc., Hayward, CA).
2. TaKaRa LA Taq DNA polymerase (Takara, Kyoto, Japan).
3. TA cloning kit (Invitrogen, Carlsbad, CA).
4. Restriction enzymes: *NotI* and *BamHI* (Takara).
5. Calf intestinal alkaline phosphatase (Takara).
6. DNA ligation kit ver.2 (Takara).
7. pcDNA 3.1 (-) vector (Invitrogen).
8. Library efficiency *Escherichia coli* DH5 α -competent cells (Invitrogen).
9. *E. coli* culture media: luria broth (LB) medium and LB agar plate with 50 μ g/mL kanamycin or 100 μ g/mL ampicillin. Miller's LB powder (Invitrogen) or LB agar powder (Invitrogen) is dissolved in distilled water and autoclaved. The media is cooled to approx 50°C before adding the antibiotic from a 1000X stock solution (kanamycin, 50 mg/mL or ampicillin, 100 mg/mL).
10. ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kits version 2.0 (Applied Biosystems, Foster City, CA).

2.1.2. Site-Directed Mutagenesis

1. QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).
2. Mutagenic oligonucleotide primers (Table 1) were obtained from Proligo Japan, Kyoto, Japan.

Table 1
Primers Used for Plasmid Construction

Purpose	Primer name	Sequence ^a
TA cloning	UGT1A1_F	5'-CAAAGGCGCCATGGCTGT-3'
	UGT1A1_R	5'-CTTATTTCCCACCCACTTCTCA-3'
Site-directed mutagenesis	Mut_G71R_F	5'-CCTCGTTGTACATCAGAGACAGAGCATTTTACA CCTTGAAG-3'
	Mut_G71R_R	5'-CTTCAAGGTGTAAAATGCTCTGTCTCTGATGTA CAACGAGG-3'
	Mut_F83L_F	5'-CGTACCCTGTGCCACTCCAAAGGGAGGATGTG-3'
	Mut_F83L_R	5'-CACATCCTCCCTTTGGAGTGGCACAGGGTACG-3'
	Mut_P229Q_F	5'GCGACGTGGTTTATTCCCAAGTATGCAACCCT TGCCTC-3'
	Mut_P229Q_R	5'-GAGGCAAGGGTTGCATACTGGGAATAAACC ACGTCGC-3'
	Mut_Y486D_F	5'CCTCACCTGGTACCAGGACCATTCTTGGACG-3'
	Mut_Y486D_R	5'-CGTCCAAGGAATGGTCCTGGTACCAGGTGAGG-3'

^aBold letters show the nucleotides exchanged.

2.2. Transient Expression of UGT1A1s in COS-1 Cells

1. COS-1 cells from the Health Science Research Resources Bank (Osaka, Japan).
2. Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen).
3. Solution of trypsin (0.25%) and ethylenediamine tetraacetic acid (1 mM) from Invitrogen.
4. Opti-MEM (Invitrogen).
5. Lipofectamine 2000 reagent (Invitrogen).
6. Phosphate-buffered saline.
7. Buffered sucrose: 0.25 M sucrose, 5 mM *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonate, pH 7.4.

2.3. SDS-PAGE and Western Blotting

1. 10% Polyacrylamide gel (PAGE; READYGELS J) from Bio-Rad Laboratories, Inc. (Hercules, CA).
2. Running buffer (10X): 250 mM Tris, 1.92 M glycine, and 1.0% (w/v) sodium dodecyl sulfate (SDS). Store at room temperature.
3. Sample buffer (2X; Wako Pure Chemical Industries, Ltd. Osaka, Japan): 0.125 M Tris-HCl, 4% (w/v) SDS, 20% (w/v) glycerol, 0.002% (w/v) bromophenol blue, 10% (w/v) 2-mercaptoethanol. Store at 4°C.

4. Molecular weight markers: MagicMark XP Western protein standards (Invitrogen).
5. Polyvinylidene difluoride (PVDF) membrane (ATTO Corp., Tokyo, Japan).
6. Blotting buffer: 0.1 M Tris, 0.192 M glycine, 5% methanol.
7. Tris-buffered saline with Tween-20 (TBS-T): prepare 10X stock with 1.37 M NaCl, 0.2 M Tris-HCl, pH 7.6, 1% Tween-20.
8. Blocking buffer: 5% (w/v) non-fat dried milk (skim milk: Difco, BD Bioscience, Franklin Lakes, NJ) in TBS-T.
9. Primary antibody: rabbit anti-human UGT1A (BD Gentest, Woburn, MA), rabbit anti-calnexin polyclonal antibody (Stressgen Biotechnologies Inc., San Diego, CA).
10. Secondary antibody: donkey anti-rabbit Ig coupled to horseradish peroxidase (Amersham Biosciences, Piscataway, NJ).
11. Enhanced chemiluminescent (ECL) plus reagents from Amersham Biosciences.
12. Stripping buffer: 2% (w/v) SDS, 0.1 M 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.7.

2.4. Assay for SN-38 Glucuronidation

1. Reaction mixture for enzyme assay: 500 mM Tris-HCl buffer, pH 7.4, 100 mM MgCl₂. SN-38 (kindly provided from Yakult Honsha Co. Ltd. Tokyo, Japan) is dissolved in dimethyl sulfoxide/0.05 N NaOH (50:50) at 2.5–150 μ M. UDP-glucuronic acid (Wako) is dissolved in distilled water at 50 mM.
2. Standards: a stock solution (2.5 mM) of SN-38 glucuronide (kindly supplied by Yakult Honsha Co. Ltd.) is dissolved in 5 mL of methanol. A working solution (0.5–250 nM) for calibration curves is prepared by the serial dilution of the 2.5 mM stock solution with high-performance liquid chromatography (HPLC) elution buffer.
3. Termination solution for enzyme reaction: 10% (w/v) HClO₄ is prepared by dilution of the 60% (w/v) HClO₄ (Wako).
4. HPLC mobile phase: 50 mM KH₂PO₄ containing 3 mM sodium 1-octane-sulfonate, pH 2.5.
5. Acetonitrile (HPLC grade, Wako).
6. Methanol (HPLC grade, Wako).

3. Methods

3.1. Plasmid Construction

We describe here the cDNA cloning of UGT1A1 by the TA cloning method because it is one of the most well-known polymerase chain reaction (PCR)-based cDNA cloning methods. As an alternative strategy, we have also successfully applied the Gateway recombinational cloning method (Invitrogen) for the functional characterization of UGT1A9 and UGT1A10 variants (9,10).

3.1.1. TA Cloning

1. UGT1A1 cDNA is amplified by PCR from human liver cDNA. The 100- μ L amplification mixture contains 5 U of TaKaRa LA Taq DNA polymerase, 1X LA

PCR Buffer II, 1.5 mM MgCl₂, 50 μM dNTP, and 0.2 μM each of forward and reverse primers (**Table 1**; *see Note 1*). The cycling parameters are as follows: initial denaturation at 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 30 s and annealing/extension at 67°C for 2 min. Finally, the reaction is terminated by a 10-min extension at 72°C.

2. The PCR product is cloned into pCR 2.1 vector using a TA cloning kit. The 10-μL ligation reaction consists of 1 μL of 10X ligation buffer, 2 μL of the PCR product, 50 ng of pCR 2.1 vector, and 4 U of T4 DNA ligase. The reaction mixture is incubated overnight at 14°C. Chemically competent *E. coli* TOP10F' cells are then transformed with the ligation mixture and plated on LB medium containing 50 μg/mL kanamycin. Ten colonies are picked at random and plasmid DNA prepared from each. The insert DNA is then sequenced on both strands using an Applied Biosystems 3700 sequencer employing the BigDye Terminator Cycle Sequencing Ready Reaction kit, version 2.0.
3. The resulting plasmid containing the correct insert (designated as pCR-UGT1A1/WT) is double digested with *NotI* and *BamHI* for 4 h at 37°C in 0.5X Takara universal buffer K containing 0.01% BSA. Subsequently, the UGT1A1 cDNA fragment is ligated into a mammalian expression plasmid pcDNA3.1(-), which is previously digested with the same enzymes followed by the treatment with alkaline phosphatase, calf intestine for 30 min at 37°C (*see Note 2*). TE buffer (10 μL) containing 300 ng of the UGT1A1 fragment and 100 ng of linearized plasmid DNA is mixed with 10 μL of enzyme solution of DNA ligation kit, version 2. The reaction mixture is incubated for 30 min at 16°C and then chemically competent *E. coli* DH5α cells are transformed with the ligation mixture. The cells are then plated on LB medium containing 100 μg/mL ampicillin.

3.1.2. Site-Directed Mutagenesis

1. Mutagenic primers (**Table 1**) are designed using the following criteria: 1) the melting temperature (T_m) of the primers (25–45 bp) is $\geq 78^\circ\text{C}$, 2) GC content of the primer is $\geq 40\%$, 3) the primer terminates in one or more C or G bases, 4) the desired mutation is in the middle of the primer with 10 to 15 bases of correct sequence on both sides. T_m is calculated here as follows: $81.5 + 0.41 \times \text{GC content (\%)} - 675/\text{primer length (bp)} - \text{\% mismatch}$.
2. Mutations are introduced using a PCR-based site-directed mutagenesis kit (QuikChange site-directed mutagenesis kit). The reaction mixture (50 μL) consist of 5 μL of 10X reaction buffer, 1 μL of dNTP mix, 10 ng of pCR-UGT1A1/WT, 125 ng each of the forward and reverse mutagenic primers, and 2.5 U of *PfuTurbo* DNA polymerase (*see Note 3*). The cycling parameters are as follows: denaturation at 95°C for 30 s, followed by 12 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 6 min. The extension time corresponds to 1 min per kb of DNA template.
3. The methylated parental plasmid DNA is digested with *Dpn I* for 1 h at 37°C. XL1-Blue supercompetent cells are transformed with 1 μL of the reaction mixture and plated on LB medium containing 50 μg/mL kanamycin.
4. Five colonies for each UGT1A1 variant are picked at random and plasmid DNA prepared. The insert DNA is then sequenced on both strands. DNA verified as correct is then subcloned into pcDNA3.1(-) as described previously.

3.2. Transient Expression of UGT1A1s in COS-1 Cells

1. COS-1 cells are maintained in DMEM medium supplemented with 10% FBS and split at a ratio of 1:5 to 1:10 upon reaching 80 to 90% confluence. The day before transfection, the cells were plated in 100-mm culture dishes at a density of 5.5×10^4 cells/cm², or 3.0×10^6 cells/dish. For each UGT1A1 variant, three 100-mm dishes are required for the Western blot analysis and the enzyme assay.
2. Just before transfection, the culture medium is replaced with 8 mL of prewarmed Opti-MEM (see Note 4). The diluted plasmid DNA (14 μ g in 810 μ L of Opti-MEM) and the diluted Lipofectamine 2000 reagent (48 μ L in 810 μ L of Opti-MEM) are combined and incubated for 20 min at room temperature. The resulting plasmid DNA–Lipofectamine 2000 complex is then added directly to each dish. After 4 h, the medium is replaced with DMEM medium supplemented with 10% FBS.
3. Forty-eight hours after transfection, the cells are washed twice with 5 mL of ice-cold phosphate-buffered saline and harvested in 2 mL of buffered sucrose using a cell scraper. The cells are transferred into a 15-mL conical tube, precipitated by centrifugation at 1500g for 10 min at 4°C, and resuspended in 1 mL of buffered sucrose.
4. The chilled cell suspension is sonicated for 1 min at a pulse cycle of 1 s, using an ultrasonic processor VC130 equipped with a 3-mm probe (Sonics, Newtown, CT), followed by centrifugation at 105,000g for 60 min at 4°C.
5. The resulting membrane fractions are resuspended in appropriate volume of buffered sucrose; addition of 100 μ L of buffered sucrose per 100-mm dish will routinely produce approx 15 mg of protein per milliliter of suspension. The protein concentration of each membrane fraction is adjusted to 10 mg of protein per milliliter with buffered sucrose. Membrane fractions are stored at –80°C until used for Western blotting and SN-38 glucuronidation assay.

3.3. SDS-PAGE and Western Blotting

1. This protocol is intended to use the Bio-Rad Mini PROTEAN 3 cell and Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell. Any other apparatus would work well under similar conditions.
2. SDS-PAGE samples are prepared by adding equal amounts of 2X sample buffer to the membrane fractions (10 mg protein/mL), boiling for 5 min and cooling to room temperature.
3. A precast 10% gel (Ready Gels from Bio-Rad) is assembled in a Mini PROTEAN 3 electrophoresis module, and the 1X running buffer is added to the inner chamber and the mini tank. Each well is rinsed thoroughly with 1X running buffer before adding 4 μ L of sample containing 20 μ g of the membrane protein or 2 μ L of molecular weight marker. SDS-PAGE gels are run at a constant current of 10 mA.
4. During the run, PVDF membranes, cut just larger than the size of the gel, are immersed in 100% methanol for 15 s and then submerged and incubated in the blotting buffer with gentle agitation for at least 30 min. Complete wetting of the PVDF membrane is important to ensure proper blotting.

5. After the electrophoresis is finished, the gels are disassembled and equilibrated in blotting buffer for 10 min at room temperature.
6. Proteins on the gel are electrophoretically transferred to PVDF membranes. Extra thick blotting paper, pre-soaked in blotting buffer, is placed on to the platinum anode of a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell. Pre-wetted PVDF membrane is placed on the blot paper and then the equilibrated gel is placed on top of the PVDF membrane. Another pre-soaked blot paper is then placed on the gel. Air bubbles are carefully removed by rolling a glass pipette over the surface of the blot paper. The cathode is placed onto the stack (sandwich of blot paper-PVDF membrane-gel-blot paper) and the transfer is carried out at a constant current of 120 mA (2 mA/cm^2) for 30 min. Although the voltage limit is set to 15 V, to avoid excessive heating, transfer is usually completed below 10 V under these conditions.
7. After the transfer, the membrane is briefly rinsed with TBS-T and then incubated in 20 mL of blocking buffer for 1 h at room temperature on an orbital shaker to block non-specific binding sites of the PVDF membrane.
8. The blocking buffer is discarded and the membrane is incubated in a 1:5000 dilution of the anti-UGT1A antibody in blocking buffer for 1 h at room temperature on an orbital shaker (*see Note 5*).
9. After the membrane is washed three times for 5 min each with 50 mL of TBS-T, the membrane is incubated in 1:2000 dilution of the secondary antibody in blocking buffer for 1 h at room temperature on an orbital shaker.
10. The membrane is briefly rinsed with two changes of TBS-T, and then thoroughly washed four times for 10 min each with 50 mL of TBS-T.
11. During the final wash, ECL plus reagent (solution A and B), stored at 2 to 8°C, is equilibrated to room temperature. Solution A and B are mixed in a ratio of 40:1, or 4 mL of solution A and 0.1 mL of solution B (*see Note 6*).
12. The washed membrane is placed protein side up in the detection reagent and incubated for 5 min at room temperature, rotating by hand. The chemi-fluorescence signal is detected and quantified using the Typhoon 9400 variable mode imager (excitation; 457 nm, emission filter; 520BP40) and ImageQuant analysis software (Amersham Biosciences).
13. After the detection of chemifluorescence signal, the membrane is subsequently stripped and then reprobed with a polyclonal anticalnexin antibody. The membrane is incubated in 50 mL of stripping buffer for 30 min at 50°C with occasional agitation, extensively washed with distilled water until the lanes on the membrane become visible, and blocked again for 1 h in 20 mL of blocking buffer.
14. The membrane is then reprobed with anti-calnexin (1:100,000 in blocking buffer) by the same protocol as that for anti-UGT1A (*see Note 7*). A representative result of Western blotting is shown in **Fig. 1**.

3.4. Assay for SN-38 Glucuronidation

1. The assay mixture consists 40 μL of 500 mM Tris-HCl buffer (pH 7.4), 40 μL of 100 mM MgCl_2 , 266 μL of distilled water, 4 μL of SN-38 solution, 10 μL of membrane fraction of COS-1 cells (100 μg protein), and 40 μL of 50 mM UDP–

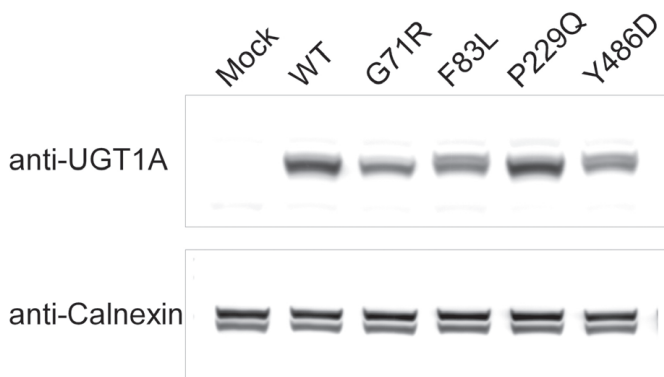


Fig. 1. Expression of wild-type (WT) and variant (G71R, F83L, P229Q, and Y486D) UGT1A1s in COS-1 cells. Aliquots (20 μ g) of the membrane fractions were subjected to SDS-PAGE, electrophoretically transferred to a PVDF membrane, and immunochemically detected with a rabbit anti-human UGT1A antibody (1:5000) and ECL plus reagents. The membrane was subsequently stripped and reprobed with a rabbit anti-calnexin antibody (1:100,000) to show that the samples were evenly loaded. The decreased expression of G71R, F83L and Y486D UGT1A1 proteins was reproducibly shown in several transfection assays without a significant reduction in their mRNA levels (data not shown), suggesting that the G71R, F83L and Y486D UGT1A1 proteins are less stable or more rapidly degraded than the wild-type protein.

glucuronic acid. Prepare the mixture in an ice-cold 1.5-mL microtube by adding each component other than 50 mM UDP-glucuronic acid (*see Note 8 [11]*).

2. After preincubation in a shaking water bath at 37°C for 1 min, the reaction is started by the addition of 40 μ L of 50 mM UDP-glucuronic acid.
3. The mixture is incubated at 37°C for 80 min, and the reaction is terminated with 100 μ L of 10% (w/v) HClO₄ and vortexing.
4. After centrifugation at 12,000g for 10 min at 4°C, the supernatant is filtered using a 0.45- μ m PTFE membrane filter (Millipore, Bedford, MA) and subjected to HPLC analysis.
5. HPLC analysis is performed using a Shimadzu LC-10AD_{VP} system (Kyoto, Japan) consisting of an SCL-10A_{VP} controller, three LC-10AD_{VP} pumps, a DGU-14A degasser, an SIL-10A_{VP} auto injector with sample cooler, a CTO-10A_{VP} column oven, an RF-10A_{XL} fluorescence detector, and a C-R7A plus chromatopac integrator. The samples are cooled at 4°C, and 20- μ L aliquots are injected into an Inertsil ODS-80A column (5 μ m, 150 \times 4.6 mm i.d., GL Sciences, Tokyo, Japan), which is kept at 40°C. The analyte is eluted isocratically with 50 mM KH₂PO₄ containing 3 mM sodium 1-octanesulfonate, pH 2.5/acetonitrile/methanol (72:22:6, v/v/v) at a flow rate of 1.0 mL/min. The excitation and emission wavelengths of the fluorescence detector are fixed at 370 and 425 nm, respectively (*see Note 9*).

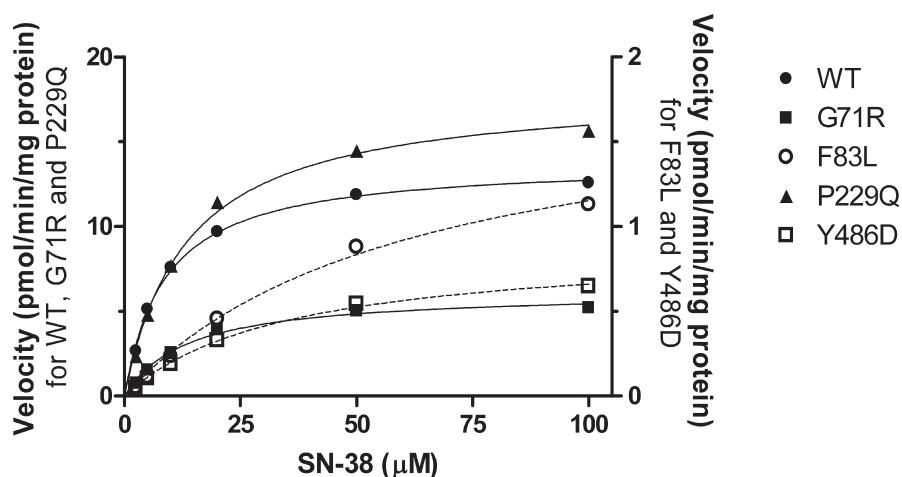


Fig. 2. Representative Michaelis–Menten kinetics of SN-38 glucuronidation by wild-type (WT) and variant (G71R, F83L, P229Q, and Y486D) UGT1A1s heterologously expressed in COS-1 cells. SN-38 glucuronidation by expressed UGT1A1s was assayed in the presence of the membrane fractions (100 μ g) at a substrate concentration range between 2.5 and 100 μ M. The solid and dashed lines indicate fitting of data to the Michaelis–Menten equation by nonlinear regression.

- Kinetic parameters are calculated with Prism 4.00 (Graph Pad Software, Inc., San Diego, CA), using nonlinear regression of the Michaelis-Menten equation. Representative Michaelis-Menten kinetics of SN-38 glucuronidation by UGT1A1s are shown in **Fig. 2**.

4. Notes

- To optimize the fidelity of PCR, concentrations of MgCl_2 and dNTP are lower than those used in the standard reaction conditions of 2.5 mM each.
- The strategy initially is intended to subclone the UGT1A1 cDNA into pcDNA3.1(+). All the correct clones of pCR-UGT1A1/WT, however, contain the insert in a reverse orientation. Therefore pcDNA3.1(-) is applied here, which has a multiple cloning site in the opposite orientation to pcDNA3.1(+).
- Mutation of the UGT1A1 cDNA is performed in the pCR 2.1 plasmid. The insert DNA is subsequently subcloned into the expression plasmid pcDNA3.1(-), thereby excluding possible mutations or PCR errors that may be introduced into the vector sequence.
- Although Lipofectamine 2000 is can be used in the presence of FBS, transfection is conducted under serum-free conditions to obtain the maximal transfection efficiency.
- Anti-UGT1A1 antibody is also commercially available from BD Gentest. However, we recommend anti-UGT1A antibody because of its higher affinity.

6. We preferably use ECL plus reagent for the detection of signals from Western blotting because this reagent is applicable for chemifluorescence detection as well as chemiluminescence detection. Fluorescence imaging by Typhoon 9400 offers a higher resolution and a wider linear dynamic range than chemiluminescence detection using a cooled-CCD camera.
7. It often is required that one ensure the SDS-PAGE samples are evenly loaded. Calnexin, an endoplasmic reticulum protein, is used for this purpose. Indeed, in some cases the expression level of a protein of interest (e.g., UGT1A1) is normalized by the amount of calnexin in each sample. In our experience, however, a Western blot of calnexin usually produce no detectable variation among the membrane fractions of COS-1 cells.
8. In the case of human microsome samples, pretreatment of the protein with alamethicin, a pore-forming peptide, is known to increase enzyme activity (**II**). This reagent is excluded from the SN-38 glucuronidation assay presented here because alamethicin has almost no effect on the glucuronidation activity of the COS-1 membrane fractions.
9. In the kinetic study, the amount of SN-38 glucuronide formed varies over a 300-fold range depending on the substrate concentrations and the membrane fractions of UGT1A1 variants. Therefore, it is important to adjust the dynamic range of the fluorescence detector appropriately. In this study, the dynamic range is achieved by setting the sensitivity to “medium” and the gain to “ $\times 1$ ” of the Shimadzu RF-10A_{XL} detector.

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