



Protocols Using Plateable Human Hepatocytes in ADME Assays

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Presentation Overview

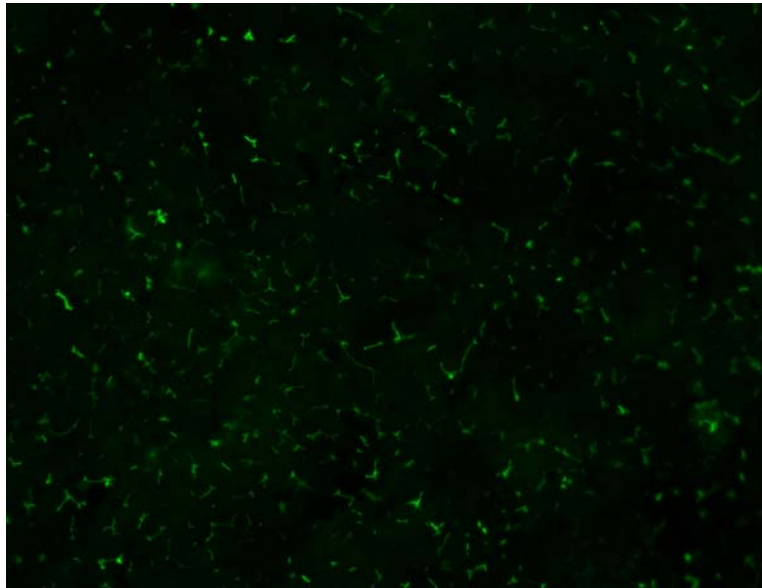
- Human Hepatocyte Products and Applications
 - The gold Standard in drug metabolism studies
- Protocols Using Plateable Human Hepatocytes in ADME Studies
 - Thawing and Plating
 - BD Matrigel™ Overlay
 - Metabolic Stability
 - CYP Induction
 - Drug Efflux/Uptake Transport Study



Human Hepatocytes

- Gold Standard for Drug Metabolism Studies

- **Primary human hepatocytes are considered the Gold Standard model for metabolism studies**
 - Contain all the hepatic enzymes, including transporters and co-factors needed for drug metabolism/hepatic transport studies: NADPH, UDPGA, GSH, PAPS etc.
 - Eliminate species difference for IV-IVE compared to animal hepatocytes.
 - Contain full machinery for enzyme regulation, important for CYP induction studies.
 - Can form *in vivo* like hepatobiliary uptake/efflux network for drug transport studies.



Hepatocyte Applications in ADME

Fresh Hepatocytes

- Fresh cells in suspension
 - Metabolic stability
 - Drug uptake studies
 - Metabolite ID
- Fresh cells on tissue culture plates
 - Primarily used for Induction studies
 - Drug efflux and uptake studies (with sandwich culture)



Cryopreserved hepatocytes (more convenient vs fresh cells)

- Cryo hepatocytes in suspension
 - Metabolic stability (drug half-life); characterized for all the major CYPs and UGTs
 - Drug uptake studies; characterized for important SLC uptake transporters
- Plateable cryopreserved hepatocytes
 - CYP induction studies (CYP3A4, 1A2 and 2B6)
 - Drug efflux studies using sandwich culture (P-gp, MRP2, BSEP)
 - Metabolic stability studies (slowly metabolized drugs requiring long incubation period in order to observe metabolites)
 - Toxicity



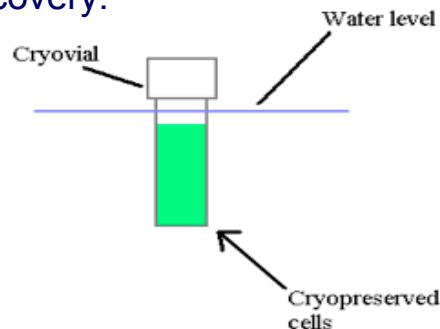
Hepatocyte Protocols in ADME Studies



Thawing CryoHepatocytes – Protocol

Note: BD Gentest™ High Viability Recovery Kit (454534) include Recovery Media and Plating Media

1. Add 5 mL FBS to the Plating Media tube, warm the Recovery Media and Plating Media to 37°C.
2. Place the cryo vial into water bath, but do not completely submerge the vial, be careful to keep the cap above the water (see illustration below).
3. Gently shake the vial back and forth to achieve even thawing while continuously monitoring the contents.
4. When only a few small ice crystals present in the vial, remove the vial from the water-bath, spray the vial with 70% alcohol and wipe dry, proceed to the hood.
5. Transfer the contents to the Recovery Media tube, rinse the vial with Recovery Media and combine the wash.
6. Centrifuge at 100 g for 10 minutes (middle acc/low brake).
7. Carefully aspirate and discard the supernatant containing dead cells and cell debris without disturbing the pellet.
8. Add 2 mL/vial of pre-warmed Plating Media with 10% FBS by pouring along the side of the tube, avoid adding the media directly onto the cell pellet.
9. Resuspend the pellet using a gentle rocking motion. When necessary, use a 2 mL pipette to titrate two to three times very gently to obtain a homogeneous cell suspension.
10. Measure cell volume, viability, and recovery.



Plating CryoHepatocytes – Protocol

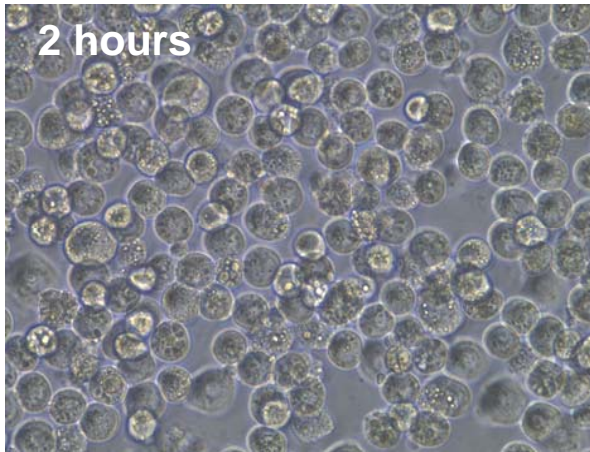
11. Dilute resuspended hepatocytes to 10^6 cells/mL.
12. Use repeat pipet or multichannel pipet to dispense cells into BD BioCoat™ Collagen I coated plate (eg, 400 μ L/well of 24-well plate).
13. After cell plating is completed, gently move the plate in a star pattern on a level surface to distribute the cells evenly over the bottom of the plate. See illustration below.
14. Place plates in a 37°C, 5% CO₂ incubator.
15. Every 20 to 30 minutes during the first 2 hours plating, remove plates from incubator and gently rock the plates to redistribute the cells evenly in the wells. Gently tapping the edge of the plate may also be helpful to redistribute the cells. Excessive accumulation of cells in the center of the wells can cause cell death.
16. After 2 to 4 hours, gently aspirate the Plating Media and gently refeed cells with complete Hepatocyte Culture Media (or customer preferred hepatocyte culture media).
17. Keep plates in the incubator overnight for further experiments as required.



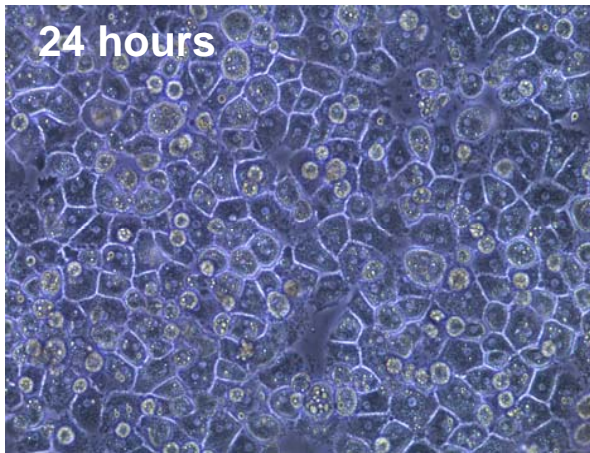
Note: swirling will cause the cells to accumulate excessively in the center of the well and can cause cell death due to anoxia.



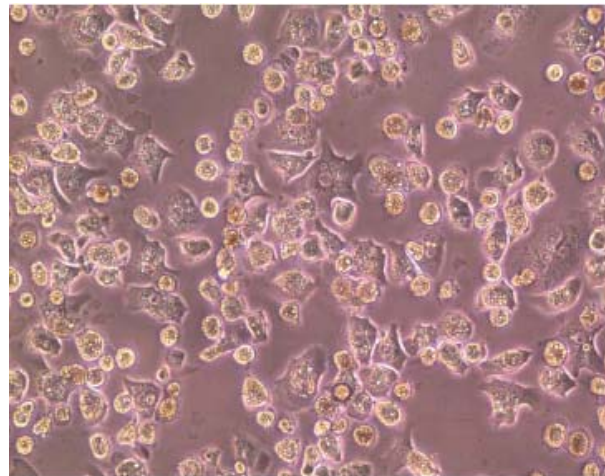
Cell Attachment Post Thaw



Cells are not completely adherent after 2 hours



After 24 hours, cells show typical cuboidal morphology (confluence 95-100%)



Cells do not attach well (confluence <50%)

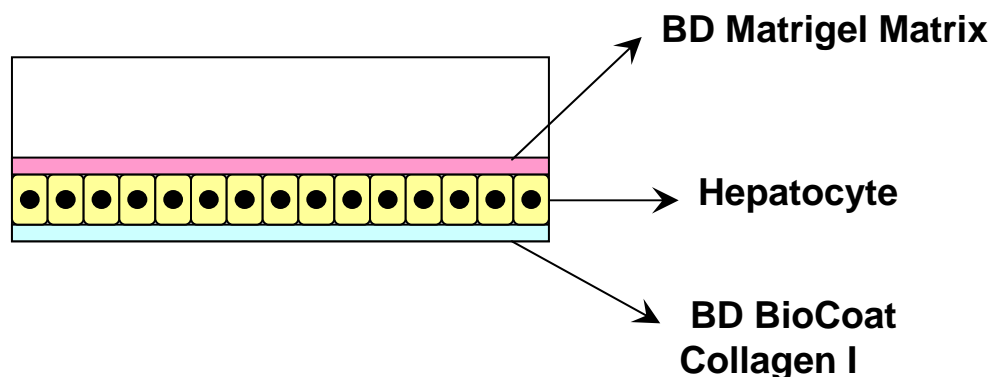
Keys to Success

- Thawing cells should be quick (<2 mins) to minimize viability loss.
- Care should be taken when aspirating dead cells after centrifugation to avoid contaminating live cells. While aspirating, move pipette tips in a circle just touching the surface of the supernatant (which contains mostly cell debris and dead cells) can help the purification.
- Gently shaking plates every 20-30 min during 1st 1-2 hours post plating to distribute cells evenly.
 - Do not allow cells to accumulate in the center of the well
- Care should be taken when aspirating and replacing old medium during media change so as not to disturb hepatocyte monolayer. Tilting plate while aspirating medium helps to minimize the opportunity of disturbing the monolayer.
- Work with 1 plate at a time to avoid over-drying the plates during medium change.



Human Hepatocyte Collagen I/BD Matrigel™ Overlay Sandwich Culture

Collagen I/Matrigel Sandwich Culture



- Promotes an *in vivo* like hepatobiliary network which is critical for studying hepatic uptake and biliary efflux transporter activity.
- BD Matrigel overlay creates a more physiological-like environment, maintains hepatocyte specific morphology, and improves CYP450 activities.

Overlaying Human Hepatocytes with BD Matrigel Matrix - Protocol

1. Prepare medium for diluting BD Matrigel matrix according to the following ratios.

Note: Medium can be prepared up to 1 month in advance and stored at 4°C.

Component	Volume
WME	500 mL
L-Glutamine	10 mL
Gentamycin Sulfate	0.5 mL
Dexamthasone	0.005 mL
ITS+Supplement	10 mL

2. Thaw BD Matrigel matrix on wet ice. This may take up to 8 hours depending on the size of the aliquot.

Note: Thawing overnight may be convenient for your work flow.

3. Prepare a BD Matrigel matrix working solution by diluting chilled BD Matrigel matrix to a final concentration of 0.25 mg/mL with ice cold medium prepared in Step 1.

Note: Prechilled, positive displacement pipettes are recommended due to the viscous nature of BD Matrigel matrix. Mix by inverting the tube three times. Keep this Working Solution on wet ice.

4. Aspirate the culture medium from the plated hepatocytes.



Overlaying Human Hepatocytes with BD Matrigel Matrix - Protocol

5. Add the following volumes of Working Solution to each well with a prechilled positive displacement pipette tip.

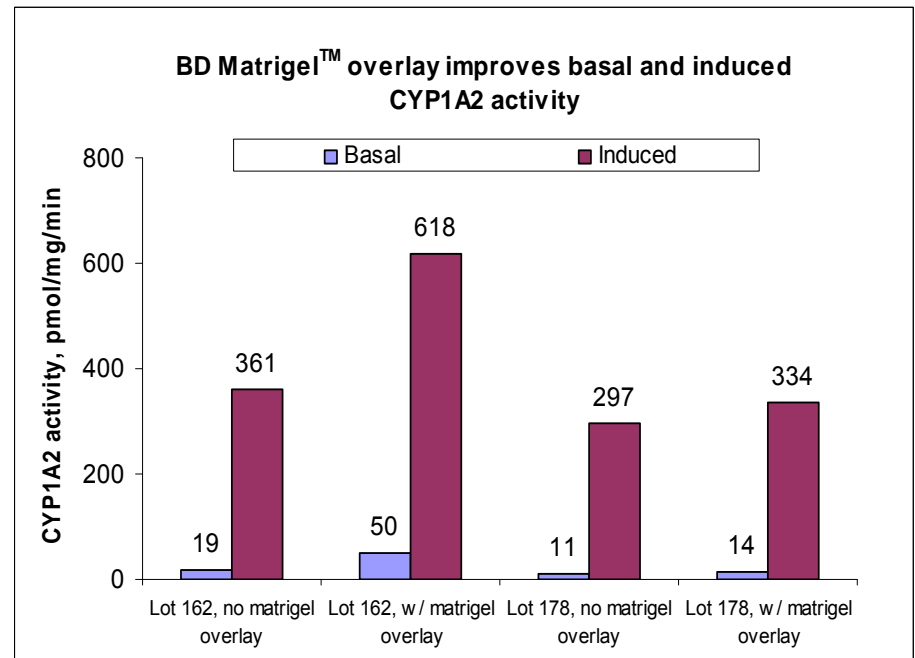
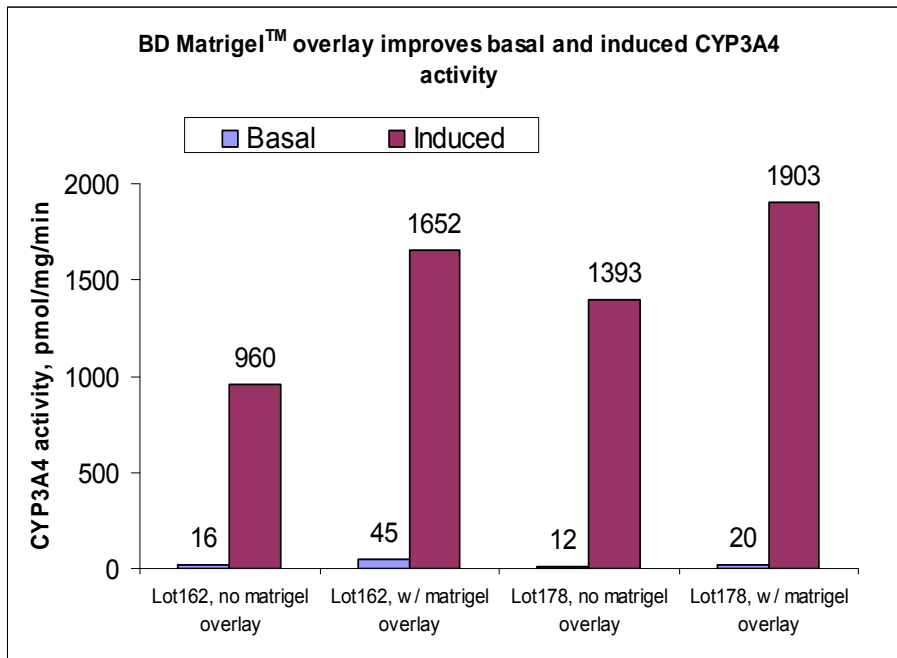
Plate Size	Volume per well
6 well	2 mL
12 well	1 mL
24 well	0.5 mL
48 well	0.2 mL
96 well	0.1 mL

6. Incubate the overlaid culture at 37°C, in a humidified, 5% CO₂ atmosphere overnight.
7. Tilt the plate and carefully aspirate the Working Solution taking care not to disturb the BD Matrigel matrix overlay. Replace with the same volume of prewarmed (37°C) medium.

Note: With the media aspirated, the hepatocyte monolayer overlaid with BD Matrigel matrix will have a “glossy” appearance relative to a medium only control.



BD Matrigel Overlay Improves CYP450 Activities



BD Matrigel overlay improves both basal and induced activity of CYP1A2 and CYP3A4 in cryopreserved human hepatocyte culture



***In Vitro* Evaluation of
Metabolic Stability Using
Plated Human Hepatocytes**

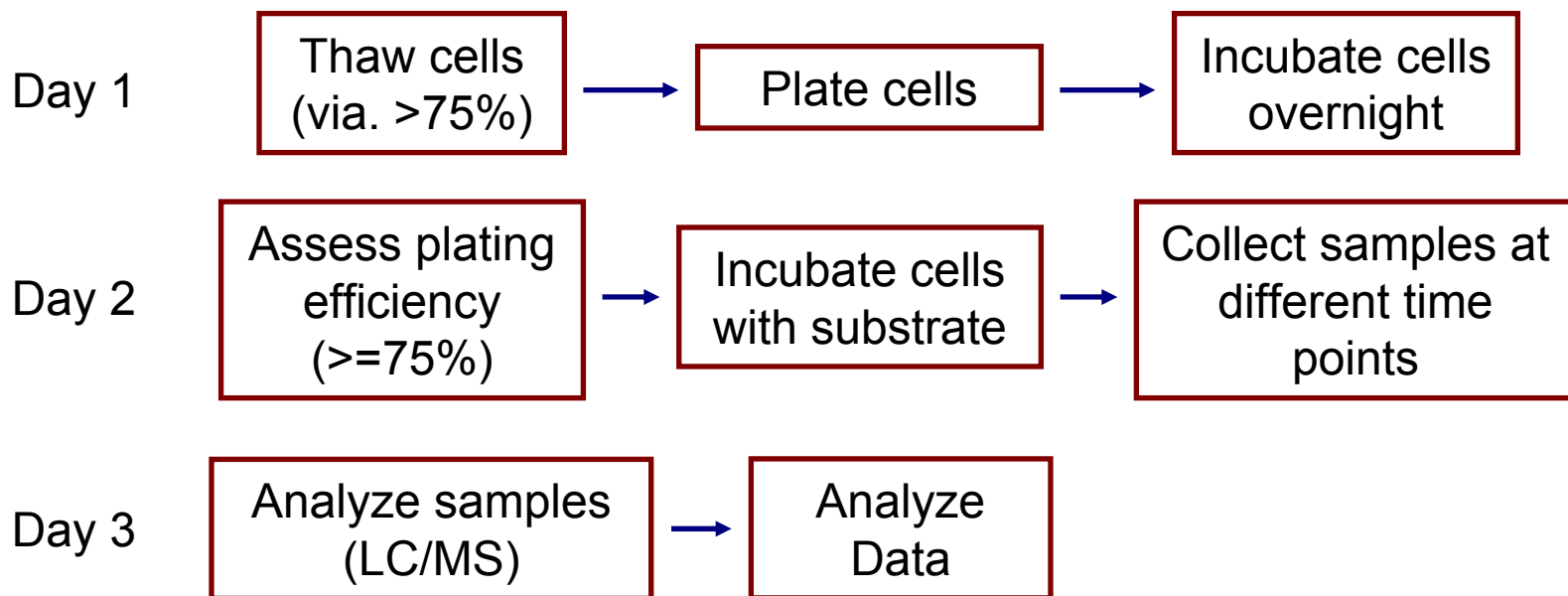


Choice of Enzyme Source

- **Hepatocytes**
 - Fresh or cryopreserved cells; Plated or in suspension
 - Contain the full complement of drug metabolizing enzymes and need no supplementation to function properly.
 - Experiment is slower; harder to automate, more technical expertise required
 - Enhancement in *in vitro/in vivo* predictivity
- **Liver microsomes**
 - Thaw and go; concentrated source of P450 isoforms; amenable to HTS
 - Containing membrane bound drug metabolism enzymes; but not cytosolic metabolic enzymes
 - Need to complement with NADPH or NADPH-regenerating system for function
- **Selection of test systems**
 - Microsome: screen compounds in a high-throughput manner in early discovery phase
 - Hepatocytes: more definitive work



Metabolic Stability Assay Using Plated Human Cryopreserved Hepatocytes



HLM time course: 0, 10, 30, 60, 90, 120 min

Suspension hepatocyte time course: 0, 30, 60, 90, 120, 180, 240 min

Plateable hepatocyte time course: 0, 1, 2, 3, 4, 6, 8, 24 hr

Plateable hepatocytes are useful for determining the half life of stable compounds that may require longer incubation times not possible in suspension culture.



Metabolic Stability Assay using Plated Human Cryopreserved Hepatocytes - Protocol

1. **Day 1.** Thaw human cryopreserved hepatocytes using BD Gentest High Viability CryoHepatocyte Recovery kit (Cat. No. 454534). Resuspend cells in plating medium containing 10% FBS and seed on 48-well BD BioCoat Collagen I coated cultureware at a seeding density of 168,000 cells/well (200 μ L cell suspension at a concentration of 0.84×10^6 cells/mL). Incubate plate at 37°C with 5% CO₂.
2. During the first 2 hours of seeding, re-distribute the cells in the plate every 20 - 30 minutes by gently rocking the plates.
3. Two to four hours after seeding, remove the plate, gently tap the side of the plate to loosen dead cells. Aspirate the plating medium, and replace with 200 μ L of fresh supplemented Williams' Medium E.
4. Continue incubating cells at 37°C with 5% CO₂.
5. **Day 2.** Approximately 18 - 24 hours after plating, change medium with 100 μ L of fresh supplemented Williams' Medium E containing probe substrates or test articles.
6. Continue to incubate cells for 8 hours or overnight at 37°C with 5% CO₂.
7. At different time points during incubation, remove an aliquot, e.g. 80 μ L from the 100 μ L incubation media and dispense into an appropriate 96-well plate or Eppendorf tubes with preloaded stop solution.
8. Centrifuge the sample/stop solution mixture at 14,000 rpm for 3 minutes if using Eppendorf tubes or 4000 rpm for 20 min if using a 96-well plate. Collect supernatant for LC-MS/MS analysis.



Determination of *in vitro* Intrinsic Clearance $CL_{int. \text{ In vitro}}$ - Data Analysis

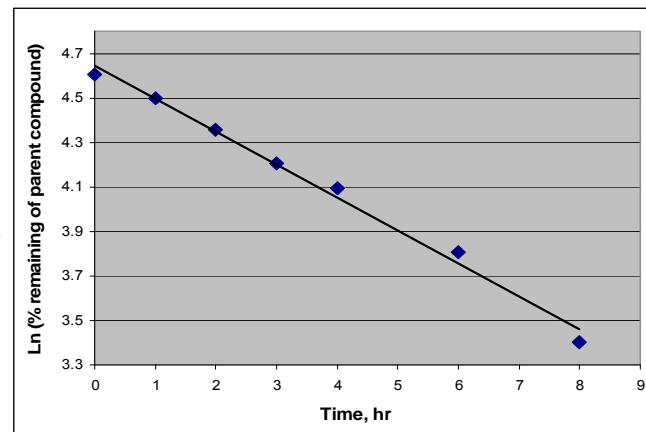
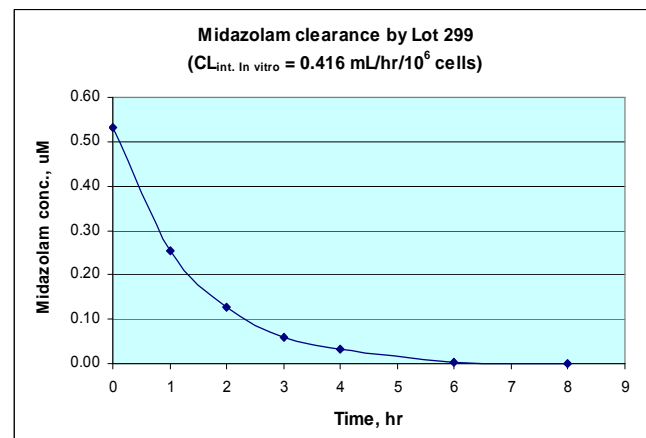
1. Plot natural log percent remaining of parent compound vs. incubation time.

2. In vitro half life may be determined using equation: $t_{1/2}$ (hour) = $\ln 2 / -k$, here k is terminal elimination rate constant which is calculated as the slope of the line defined by the linear regression of the natural log percent remaining of parent compound vs. incubation time. The slope may be calculated using the "SLOPE" function in Microsoft® Excel software.

3. Determine in vitro intrinsic clearance ($CL_{int. \text{ in vitro}}$) according to the following calculation.

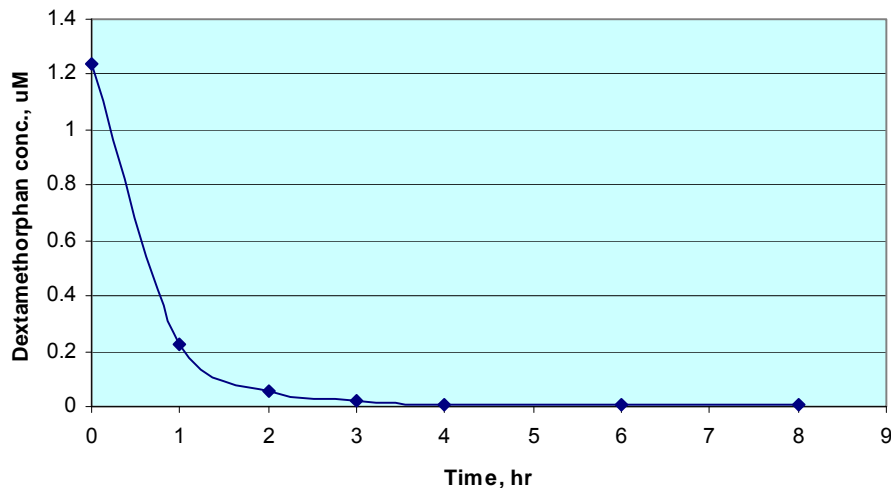
$$CL_{int. \text{ in vitro}} (\text{mL/hour}/10^6 \text{ cells}) = \frac{0.693}{t_{1/2} (\text{hour})} \times \frac{\text{Incubation volume (mL)}}{\text{Hepatocyte cell number } (10^6)}$$

4. Use scaling factors (99×10^6 hepatocytes/g liver and 20 g liver/kg body weight) to extrapolate in vivo intrinsic clearance $CL_{int. \text{ in vivo}}$ (mL/hour/kg body weight) from $CL_{int. \text{ in vitro}}$.

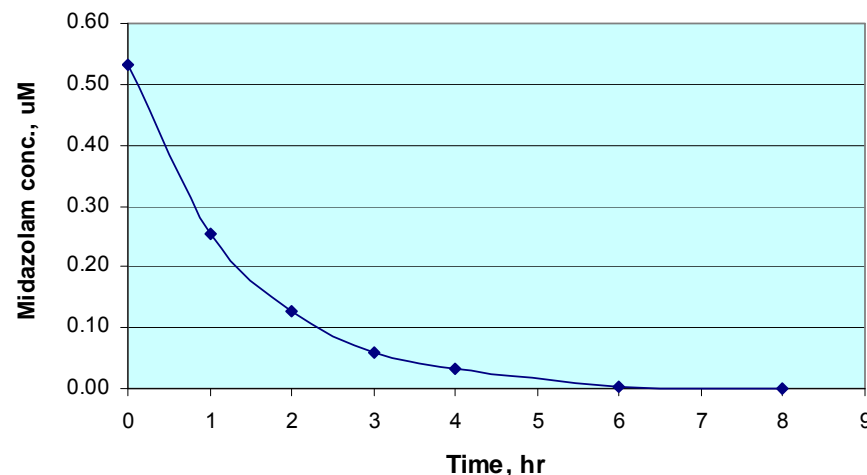


Cryo Lots Characterized for Plated Metabolism – Sample Data

Dexamethorphan clearance by Lot 296
($CL_{int. \text{ In vitro }} = 0.733 \text{ mL/hr/10}^6 \text{ cells}$)



Midazolam clearance by Lot 299
($CL_{int. \text{ In vitro }} = 0.416 \text{ mL/hr/10}^6 \text{ cells}$)



BD Lot No.	Intrinsic clearance (mL/hr/10 ⁶ cells)		Metabolite formation velocity (nmol/hr/10 ⁶ cells)		
	(Midazolam)	(Dextromethorphan)	(Phenacetin)	(Diclofenac)	(Estradiol)
Metabolism-Qualified Cat. No. 454543 ≥ 5 Million cells/vial					
261	0.302	0.258	2.3	3.6	0.78
281	0.221	0.124	1.37	2.38	1.11
303	0.25	0.457	0.26	2.8	0.83
304	0.262	0.69	0.4	10.1	0.95



Keys to Success

- **Important general tips for working with plateable cryopreserved hepatocytes**
 - Good post-thaw viability: low viability may affect cell attachment.
 - Good cell attachment: poor attachment may skew metabolism activity.
- **Test substrate concentration**
 - When measuring loss of parent for estimating intrinsic clearance, it is customary to use a low substrate concentration that is $< K_m$, for example 1 μM (at $< K_m$ concentrations the observed rate approximates Intrinsic Clearance).
 - When measuring metabolite formation velocity, higher substrate concentration (V_{max}) can be used to assess the activity of individual drug metabolizing enzymes.
- **Metabolite analysis**
 - Metabolites can be analyzed using either HPLC or LC-MS/MS. The analytical method should be adapted based on the metabolite(s) to be detected.



***In Vitro* Evaluation of
CYP450 Induction Using
Plateable Human CryoHepatocytes**



CYP Induction Assay

- CYP induction
 - Represents one scenario for metabolic Drug-Drug Interaction (opposite of what is seen with CYP inhibition).
 - CYP induction is an increase in the amount of protein and enzyme activity (cause increase in the metabolism of other drugs or itself).
 - More enzyme means more clearance and less drug in circulation.
 - Causes a reduction in the efficacy of co-administered drug.
 - Can lead to drug “tolerance” when drug can stimulate its own metabolism.
 - Can lead to generation of toxic metabolites.
 - **This is a complex assay and is typically done later in lead optimization or in development.**
 - **FDA expects data for enzyme induction potential.**



In Vitro CYP450 Induction Assay – Experimental Design

- **Choice of models**

- Primary cultures of human hepatocytes (fresh/cryopreserved) - gold standard.

- **Choice of enzymes**

- FDA requires evaluation of induction potential in CYP1A2, CYP2B6, and CYP3A4.

- **Selection of positive control inducers and concentrations**

- CYP1A2 (Omeprazole 25-100 μ M, β -Naphthoflavone 33-50 μ M), CYP3A4 (Rifampin 10-50 μ M), CYP2B6 (Phenobarbital 500-1000 μ M)

- **Concentrations of test articles**

- At least 3 concentrations spanning the therapeutic range including 1 concentration that is an order of magnitude > the average expected plasma drug concentration.

- **Exposure time**

- A 2-3 day treatment is recommended in the FDA draft guidance.

- **Choice of endpoints**

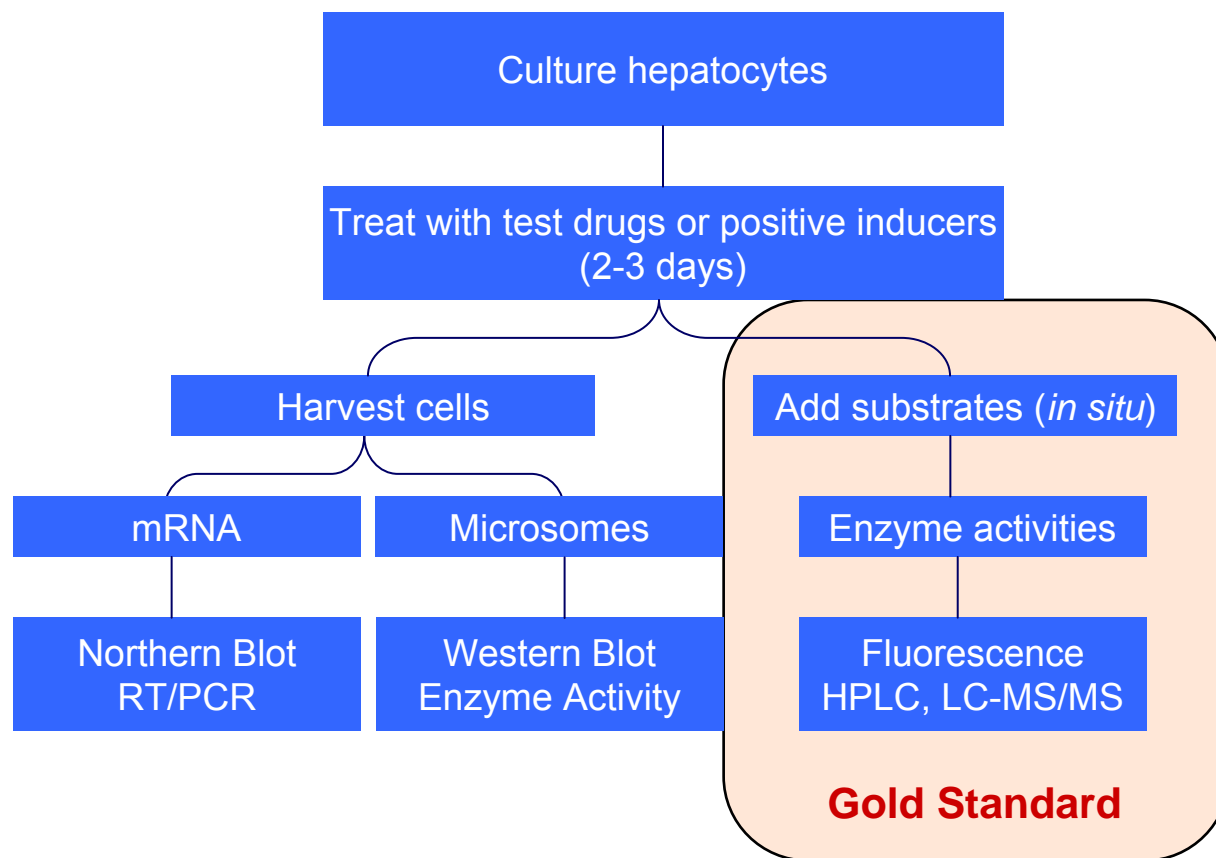
- Enzyme activity (most reliable), mRNA or protein expression (help to identify inhibition).

- **Choice of probe substrates**

- Phenacetin (CYP1A2), Bupropion (CYP2B6), Testosterone (CYP3A4)



In Vitro CYP450 Induction Assay – Flow Chart



In Vitro CYP450 Induction Assay - Protocol

1. **Day 1.** Thaw human cryohepatocytes using BD Gentest High Viability CryoHepatocyte Recovery Kit. Resuspend cells in plating medium containing 10% FBS and seed on 24-well collagen I coated cultureware at a seeding density of 400,000 cells/well. Incubate the plate at 37°C with 5% CO₂.
2. During the first 2 hours of seeding, re-distribute the cells in the plate every 20 - 30 minutes by gently rocking the plates.
3. Two to four hours after seeding, remove the plate, gently tap the side of the plate to loosen dead cells. Aspirate the plating medium, and replace with fresh BD Hepatocyte Culture Media (Cat. No. 355056).
4. Continue incubating cells at 37°C with 5% CO₂.
5. **Day 2.** Approximately 18 - 24 hours after plating, change medium with fresh hepatocyte culture medium containing the positive control inducer, or negative control (the solvent vehicle control), or test articles.
6. Repeat step 5 for day 3 and day 4.
7. **Day 5.** Remove induction incubation medium. Perform a wash step for all wells with pre-warmed hepatocytes culture medium, and start enzyme reaction by adding 200 µL of fresh hepatocyte culture medium containing the probe substrate and return to incubator.
8. At the end of the enzyme reaction period, remove an aliquot, e.g. 100 µL from the 200 µL assay media and dispense into a 96-well plate or Eppendorf tubes with preloaded stop solutions. Store the plate or tubes on ice.
9. Centrifuge the mixture of enzyme assay sample and stop solution from step 8 at 14,000 rpm for 3 minutes if using Eppendorf tubes or 4000 rpm for 20 min if using a 96-well plate at room temperature. Collect supernatant for LC-MS/MS analysis.



In Vitro CYP450 Induction Assay – Protocol (Cont.)

10. Protein sample preparation

- After the enzyme assay is completed, aspirate media and wash cells once with 400 μ L/well of 1X PBS buffer.
- Aspirate the buffer and add 1 mL/well of detergent (1% SDS freshly prepared from 10% SDS).
- Incubate the plates with detergent for at least 10 minutes at 37°C in an incubator.
- Transfer cell lysate into Eppendorf tubes by pipetting and store sample tubes at -20°C until protein analysis is performed.

11. RNA sample preparation

- If mRNA expression needs to be measured, at the end of step 8, aspirate media and wash cells once with 400 μ L/well of 1x PBS buffer.
- Immediately freeze and store plate at -80°C until RNA isolation



CYP Induction Calculation

Calculation of enzyme activity, fold of induction, % of positive control response

$$\text{Activity (pmole / mg protein / min)} = \frac{(\text{Calculated Conc } \mu\text{M}) \times (\text{Incubation volume } \mu\text{L})}{(\text{mg Protein / well}) \times \text{incubation time (min)}}$$

$$\text{Fold of Induction} = \frac{\text{Activity of treated sample}}{\text{Average activity of solvent vehicle control}}$$

$$\% \text{ of Positive control response} = 100 \times \frac{\text{Activity of treated samples} - \text{Average activity of SVC}}{\text{Average activity of positive control} - \text{Average activity of SVC for positive control}}$$

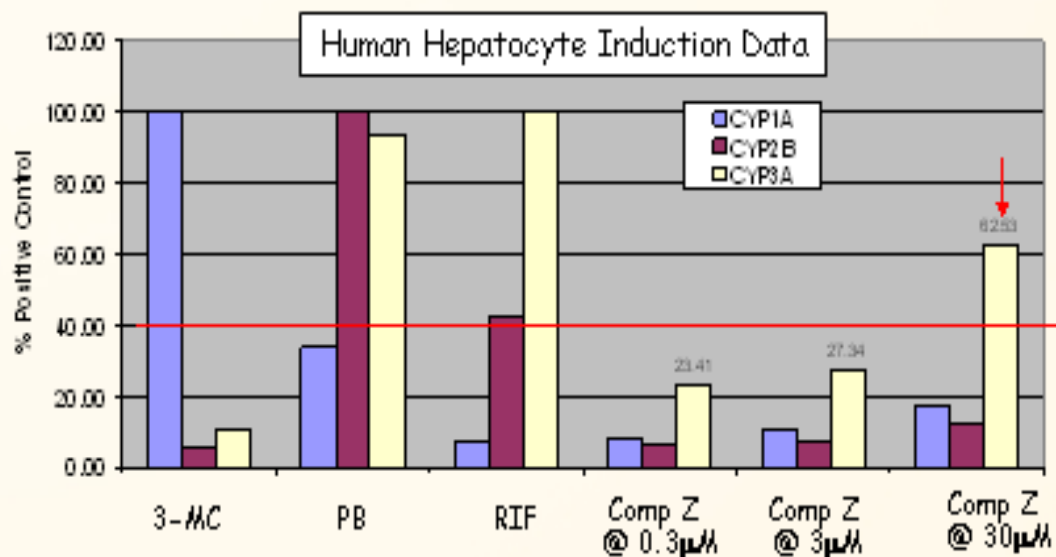
Definition of Inducer: “A drug that produces a change that is equal to or greater than 40% of the positive control can be considered as an enzyme inducer *in vitro* and *in vivo* evaluation is warranted.”



Induction Sample Data

Cryo Lots characterized for Induction

ID Lot No.	Basal activity (pmol/min/mg) CYP3A4	Induced activity (pmol/min/mg) CYP3A4	Fold induction CYP3A4	Basal activity (pmol/min/mg) CYP1A2	Induced activity (pmol/min/mg) CYP1A2	Fold induction CYP1A2
Inducible-Qualified Cat. No. 454551 ≥ 5 Million cells/vial						
303	36.0	1600.0	44	6.8	210	31
304	55.0	860.0	16	4.3	180	42
311	16.0	1200.0	75	4.8	160	33
314	31.0	1600.0	52	4.7	71	15
318	31.0	1300.0	42	1.7	160	94
319	11.0	360.0	33	2.1	120	57
320	11.0	220.0	20	2.4	57	24
321	21.0	1800.0	86	8.9	250	28



Induction by Test Compound Z



Keys to Success

- **Important general tips for working with plateable cryopreserved hepatocytes**
 - Good post-thaw viability: low viability may affect cell attachment
 - Good cell attachment: poor attachment may skew metabolism activity
- **Choice of endpoints**
 - **Most reliable:** Enzyme activity of human primary hepatocyte cultures treated with test articles, compared with enzyme activity of hepatocyte culture treated with positive control inducer, and the induction potential is expressed as “% of positive control response”.
 - **Alternative endpoints:** mRNA analysis by RT-PCR (helps to identify inhibition/”down regulation”)
- **BD Matrigel Basement Membrane Matrix as an overlay (Optional)**
 - **Note:** Overlay hepatocyte monolayer with BD Matrigel at 4 - 6 hours after cell plating for induction assay.
- **Inter-individual variability**
 - FDA requires using fresh or cryopreserved hepatocytes from at least 3 different donor livers due to the presence of inter-individual differences in induction potential.



***In Vitro* Evaluation of Drug Transport Using Plateable Human Hepatocytes**



Application of Transporters in ADME/Tox

- Understand the function of transporters in the process of drug absorption, distribution and elimination
 - **Pharmacokinetics:** Change AUC (area under the curve), C_{\max} , etc. (Metformin by hOCT1)
 - **Pharmacodynamics:** Drug cannot reach target
- Evaluate and improve drug bioavailability
 - Quantitative Structure-Activity Relationship (QSAR) strategy to improve permeability through a transporter prodrug approach
- Enhance tissue and target specific drug transport, and minimize side effects
 - MDR1 prevented the penetration of toxic compounds across BBB (blood brain barrier)
 - hENT1 caused the mitochondrial toxicity of nucleoside drug (Fialuridine)
- Predict and avoid transporter involved DDI
 - P-gp involved
 - OATP involved



Hepatic Transport Models

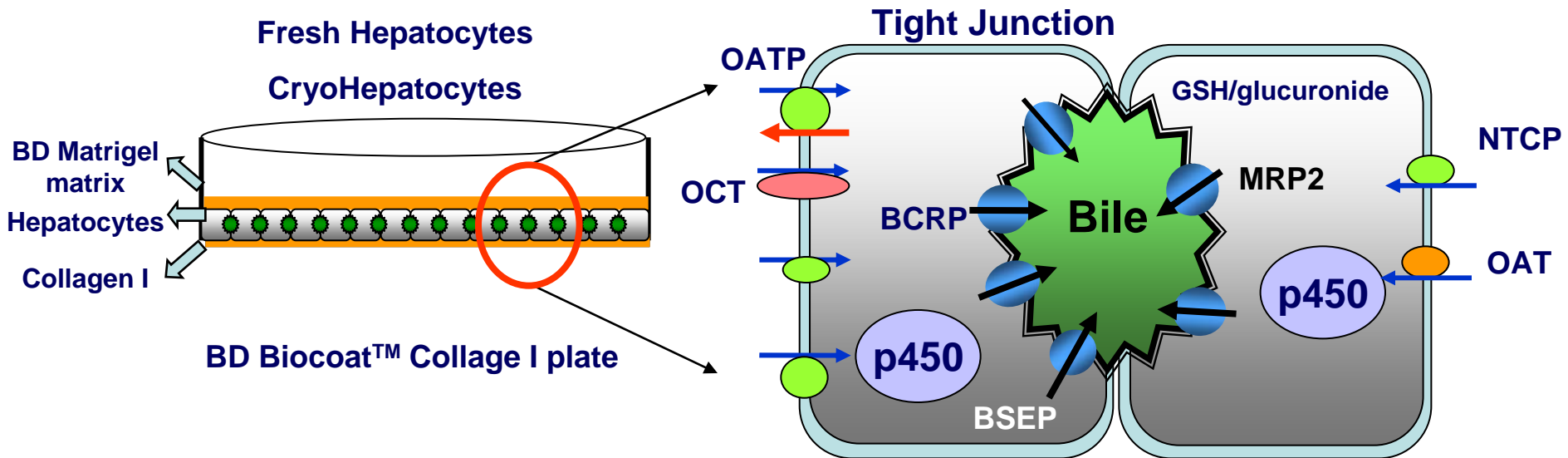
In vivo to in vitro, complex to simple

- *In vivo* studies (animal models)
- Hepatocytes
 - Sandwich culture: Multiple SLC and ABC transporters
 - Suspension: Multiple SLC transporters
- Canalicular membrane vesicle (CMV)
 - Multiple ABC transporters
- Recombinant models
 - Cell lines
 - Inside out vesicles

Simple to complex, in vitro to in vivo

Sandwich Cultured Hepatocytes

- Hepatic Uptake and Biliary Excretion



Features of SCHH:

- Repolarization of hepatocytes
- Form intact bile canaliculi, mimic *in vivo* hepatobiliary network
- Quantitative estimation of hepatic uptake clearance & biliary excretion

Efflux Transporter Study in Sandwich Cultured Human Hepatocytes - Protocol

Thawing/Plating/Matrigel Overlay

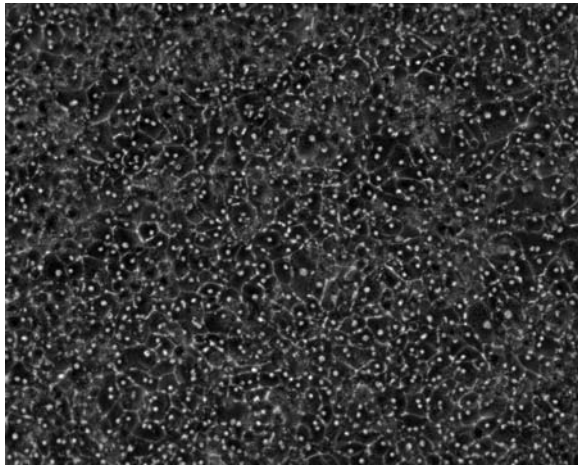
1. **Day 1.** Thaw human cryohepatocytes using BD Gentest High Viability CryoHepatocyte Recovery kit and plate cells on on BD Collagen I coated plate at a seeding density of 400,000 cells/well.
2. **Day 2.** Overlay hepatocyte culture with BD Matrigel matrix.
3. **Day 3 and Day 4.** Change medium daily with 0.5 mL pre-warmed supplemented Williams' Medium E.
4. **Day 5.** Assess drug uptake and efflux transporter activities as described below.

Fluorescence efflux assay

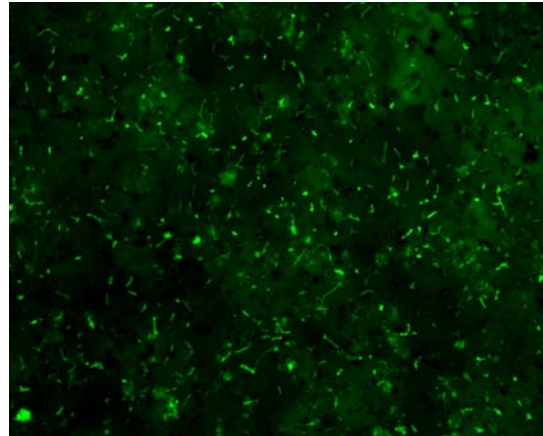
5. Wash cells twice with 0.5 mL/well pre-warmed HBSS (with $\text{Ca}^{2+}/\text{Mg}^{2+}$). Add 0.5 mL/well pre-warmed HBSS (with $\text{Ca}^{2+}/\text{Mg}^{2+}$), then incubate the plate at 37°C with 5% CO_2 for 10 -15 mins.
6. Carefully aspirate all the media from the cell plate. Add 0.5 mL/well of warm HBSS (with $\text{Ca}^{2+}/\text{Mg}^{2+}$) containing the efflux transporter substrate (e.g., 5 μM CDFDA) with or without inhibitor (e.g., 50 μM MK-571) and incubate cells for 20 minutes in a 37°C incubator with 5% CO_2 .
7. At the end of the incubation, aspirate the substrate and inhibitor solutions, wash twice with 0.5 mL/well of pre-warmed HBSS (with $\text{Ca}^{2+}/\text{Mg}^{2+}$) buffer. Add 0.5 mL/well of pre-warmed HBSS (with $\text{Ca}^{2+}/\text{Mg}^{2+}$).
8. Assess cell morphology and bile canaliculi formation (CDF accumulation in bile canaliculi) with phase contrast and fluorescence microscopy analysis respectively.



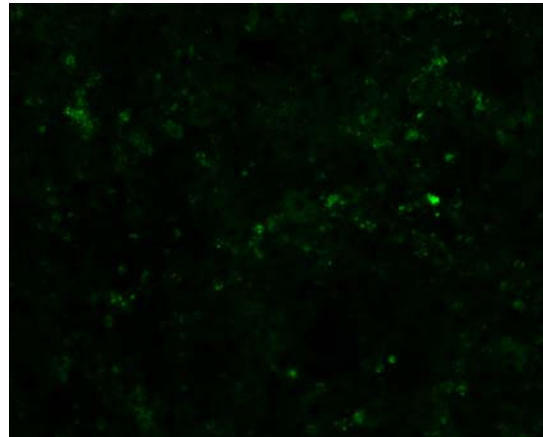
Bile Canaliculi Formation in Collagen I/Matrigel Sandwich Cultured Human Hepatocytes



Phase contrast image of monolayer in Day 5 sandwich culture



Bile canaliculi formation in Day 5 sandwich culture



Bile canaliculi formation in the presence of MRP2 Inhibitor MK571 in Day 5 sandwich culture

CDFDA in Cells

Esterase

CDF in Cells

MRP2

CDF in Bile

CDF Accumulation in the Bile

CDF: Carboxydichlorofluorescein

CDFDA: Carboxydichlorofluorescein diacetate



Uptake Transporter Assay in Sandwich Cultured Human Hepatocytes - Protocol

1. On assay day, wash cells twice with 0.5 mL/well of pre-warmed HBSS (with $\text{Ca}^{2+}/\text{Mg}^{2+}$). Add 0.5 mL/well of pre-warmed HBSS (with $\text{Ca}^{2+}/\text{Mg}^{2+}$), incubate the plate at 37°C with 5% CO_2 for 10 - 15 mins.
2. Carefully aspirate all the media out of cell plate. Add 0.5 mL/well of pre-warmed HBSS containing substrates into the appropriate wells (refer to Table below for the example of substrate preparation, 1 μM TCA). Incubate at 37°C with 5% CO_2 for 2 mins and 10 mins (one plate per time point).

Substrate 1: 1 μM TCA	Volume	Concentration in Solution
1 mM Taurocholate in DMSO ("cold" compound)	9 μL	0.9 μM
[3H]-Taurocholate ("hot" compound) - 200 μM	5 μL	0.1 μM
1X HBSS (Ca^{2+} , Mg^{2+}) buffer	10 mL	1X

3. At the desired time point, quickly take plate out of incubator, aspirate the media quickly, and wash twice with 0.5 mL/well of pre-chilled HBSS buffer twice.
4. Add 0.5 mL/well of 0.5% Triton-X-100. Incubate the plates for 20 - 30 mins at room temperature. The cell monolayer should clearly detach during this time. Gently shake the plate every 5 - 10 mins. If cells have not completely detached after 30 mins, gently pipet the solution up and down to aid in detachment.
5. Transfer 0.4 mL of the assay sample from each well into scintillation vials containing 5 mL of scintillation fluid.



Uptake Transporter Assay in Sandwich Cultured Human Hepatocytes - Protocol

6. For normalization, add 0.4 mL of each substrate solution into separate blank scintillation vials containing 5 mL of scintillation liquid. This will provide the Reference DPM (Disintegrations Per Minute) value for each substrate (DPM/pmol substrate) which can then be used to normalize the scintillation signal of substrate treated samples and to determine the corresponding uptake accumulation in pmol.
7. Analyze samples using a scintillation counter.
8. Determine cellular protein content using wells similarly treated except without being exposed to substrates.
9. Uptake activity is calculated using the formula shown below:

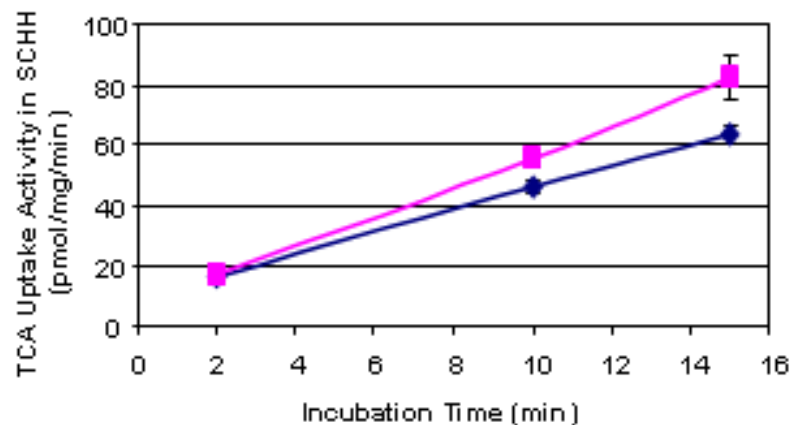
Uptake activity (pmol/mg protein/min) =

$$\frac{\text{Substrate accumulation}_{10\text{min}} (\text{pmol}) - \text{Substrate accumulation}_{2\text{min}} (\text{pmol})}{\text{Protein (mg)} \times \text{Time}_{10\text{min} - 2\text{min}} (\text{min})}$$

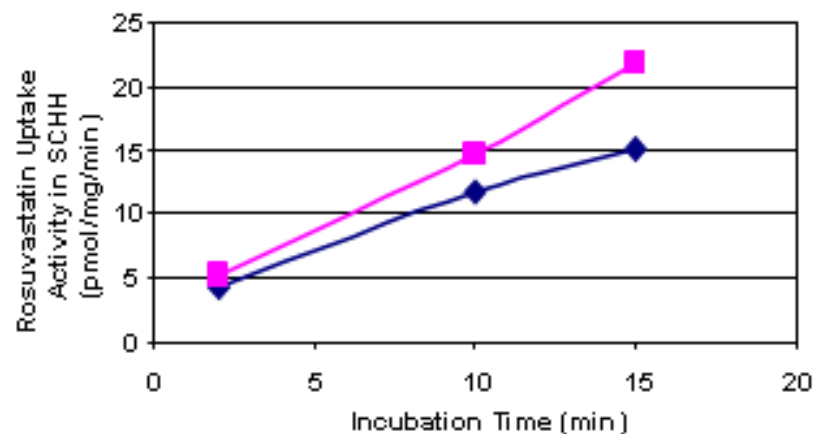


Uptake Activity Sample Data

NTCP Activity in SC BD Gentest Human CryoHepatocytes



OATP Activity in SC BD Gentest Human CryoHepatocytes



Lot 262 Lot 295

	Uptake Activity (pmol/mg/min)	
	TCA (NTCP)	Rosuvastatin (OATP)
Lot 262	16.4	4.2
Lot 295	17.9	4.5



Keys to Success

- **Important general tips for working with plateable cryopreserved hepatocytes**
 - Good post thaw viability: low viability may affect cell attachment
 - Good cell attachment: poor attachment will compromise formation of hepatobiliary network
- **BD Matrigel Basement Membrane Matrix as an overlay**
 - **Note:** overlay hepatocyte monolayer with BD Matrigel 18-24 hours after cell plating provides optimal formation of hepatobiliary network
- **For efflux assay**
 - Minimize exposure of assay plates to fluorescent light during substrate incubation (suggested to wrap assay plates with aluminum foil) to prevent photobleach.
 - Fluorescence efflux assay can be used to characterize efflux transporter inhibitor. Prepare substrate solution as described in the procedure with or without inhibitors (test articles) at defined or a series of concentrations. Fluorescence image can be analyzed quantitatively using imaging software.
 - Other fluorescent efflux transporter substrates such as BD Gentest CLF (Cholyl-lysyl-fluorescein, Cat. No. 451041) can be used in the fluorescence efflux assay to characterize hepatobiliary excretion and cholestasis.
- **For uptake assay**
 - Transporter uptake assays can be conducted with non-radiolabeled compounds or radiolabeled compounds. When non-radiolabeled compounds are used, acetonitrile or methanol is recommended for lysing the cells for HPLC or LC-MS/MS analysis.





Questions?

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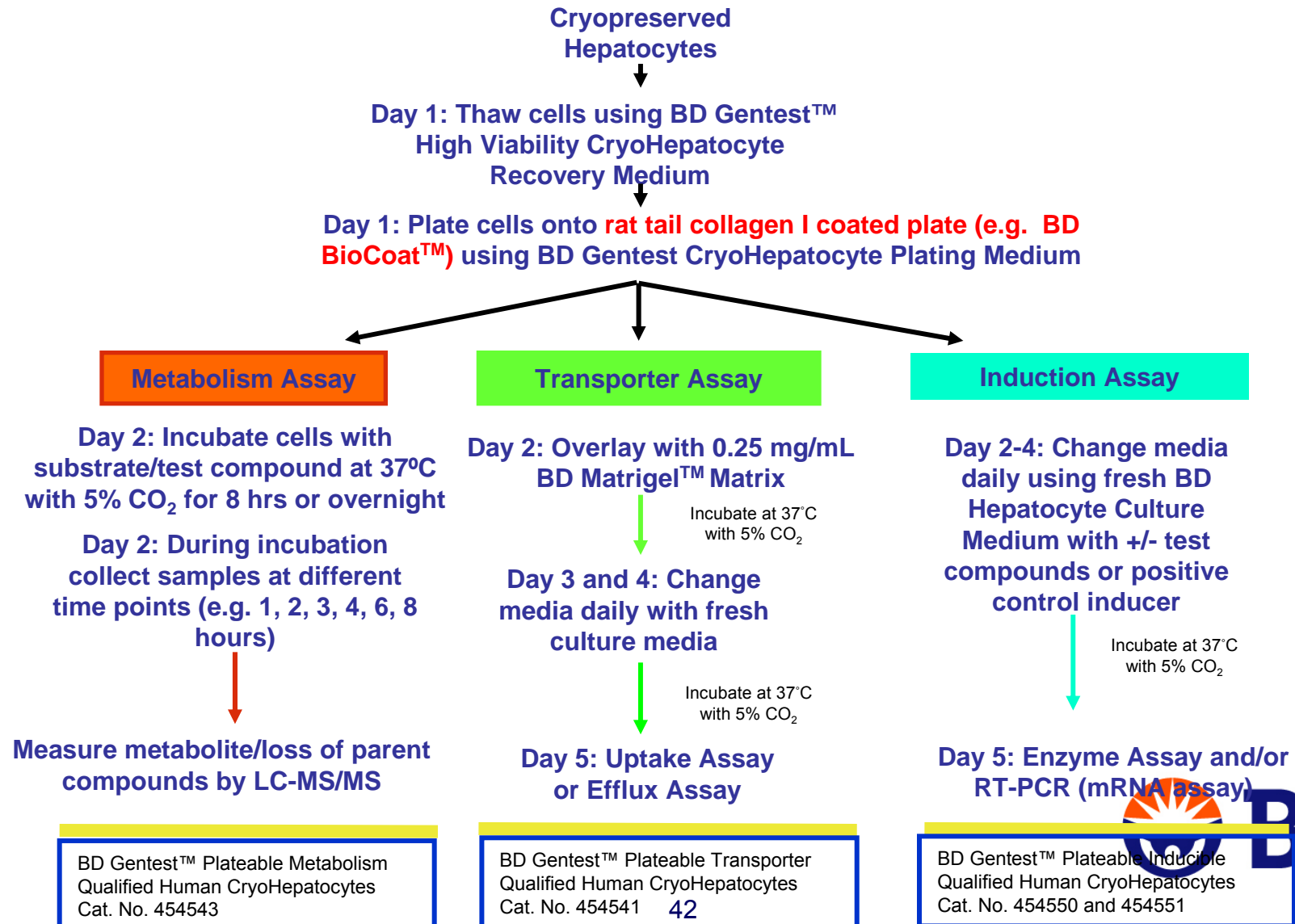


REFERENCE

Assay Conditions



Quick Guide for Assays Using Plateable Cryopreserved Human Hepatocytes



***In Vitro* CYP450 Induction Assay - Materials**

- **BD Gentest High Viability Human CryoHepatocyte Recovery Kit (Cat. No. 454534)**
- **Inducible Qualified Human Cryopreserved hepatocyte (Cat. No. 454550, 454551)**
- BD BioCoat Collagen I coated 24-well plate (Cat. No. 354408)
- BD Matrigel Basement Membrane Matrix (optional)
- Hepatocyte Culture Media Kit (Cat. No. 355056)
- Positive Control Inducers: Rifampin, Omeprazole, Phenobarbitol
- Negative Control (solvent vehicle control): e.g. DMSO
- Probe substrates
- Metabolite Standards
- Stop solutions



Plated Metabolism Assay - Materials

- **BD Gentest High Viability Human CryoHepatocyte Recovery Kit (Cat. No. 454534)**
- **Metabolism Qualified Plateable Human Cryopreserved hepatocyte (Cat. No. 454543)**
- BD BioCoat Collagen I coated 48-well plate (Cat. No. 354505)
- Williams' Medium E (Sigma Cat. No. 1878) supplemented with BD™ ITS Universal Culture Supplements (BD Cat. No. 354352 or equivalent, 1:100 dilution in final medium), L-Glutamine (Invitrogen Cat. No. 21051 or equivalent, 4 mM final concentration), Dexamethasone (Sigma Cat. No. D4902 or equivalent, 0.1 μ M final concentration), and Penicillin-Streptomycin (Sigma Cat. No. P4333 or equivalent, 1:100 dilution in final medium)
- Probe Substrates
- Metabolite standards
- Stop solutions



Drug Transport Assay - Materials

- **BD Gentest High Viability Human CryoHepatocyte Recovery Kit (Cat. No. 454534)**
- **Transporter Qualified Human Cryopreserved hepatocyte (Cat. No. 454541)**
- BD BioCoat Collagen I coated 24-well plate (Cat. No. 354408)
- BD Matrigel Basement Membrane Matrix
- Williams' Medium E (Sigma Cat. No. 1878) supplemented with BD™ ITS Universal Culture Supplements (BD Cat. No. 354352 or equivalent, 1:100 dilution in final medium), L-Glutamine (Invitrogen Cat. No. 21051 or equivalent, 4 mM final concentration), Dexamethasone (Sigma Cat. No. D4902 or equivalent, 0.1 μ M final concentration), and Penicillin-Streptomycin (Sigma Cat. No. P4333 or equivalent, 1:100 dilution in final medium)
- Probe substrates
- Radiolabeled compounds: used to normalize the uptake accumulation of corresponding substrates.
- Assay buffer: Hanks' Balanced Salt Solution (HBSS, 1X with Ca^{2+} / Mg^{2+} , Invitrogen, Cat. No. 14025-092). It is important to have Ca^{2+} and Mg^{2+} ions present in the assay buffer to maintain intact tight junctions between cells which define the bile canaliculi network on the apical surface of the hepatocytes.



Assay Conditions - Positive Control Inducers and Probe Substrates for Induction Assay

Enzyme	Induction Incubation		Enzyme Assay		Metabolite Standard
	Inducer	Final Inducer Conc. (μM)	Substrate	Final Substrate Conc. (μM)	
CYP3A4	Rifampicin	10 – 20	Testosterone	200	6 β -Hydroxytestosterone
CYP1A2	β -Naphthoflavone	20	Phenacetin	100	4-Acetamidophenol
	Omeprazole	50			
CYP2C8	Rifampicin	10 – 20	Amodiaquine	100	Desethylamodiaquine
CYP2C9	Rifampicin	10 – 20	Diclofenac	100	4'-Hydroxydiclofenac
CYP2C19	Rifampicin	10 – 20	S-Mephenytoin	200	4'-Hydroxymephenytoin
CYP2B6	Phenobarbital	1000	Bupropion	250	Hydroxybupropion
			S-Mephenytoin	200	(S)-N-DesmethyImephenytoin
UGT1A1	β -Naphthoflavone	20	Estradiol	100	Estradiol 3-Glucuronide



Assay Conditions - Probe Substrates and Metabolites for Plated Metabolism

Enzyme	Assay	Probe Substrate	Probe Substrate Conc., μM	Metabolite Standard
CYP3A4	Intrinsic Clearance	Midazolam	0.5	1'-Hydroxymidazolam
CYP2D6		Dextromethorphan	1	Dextrophan
CYP1A2	Metabolite Formation Velocity	Phenacetin	100	4-Acetamidophenol
CYP2C9		Diclofenac	100	4'-Hydroxydiclofenac
UGT1A1		Estradiol	100	Estradiol 3-Glucuronide



Assay Conditions - Probe Substrates for Drug Transporter Assay

Transporter	Transporter Assay	
	Substrate	Final Conc. (μ M)
MRP2 (Efflux)	CDFDA (hydrolysis product CDF, 5-(and-6)-carboxy-2',7'-dichlorofluorescein)	5
OATP1B1/OATP1B3 (Uptake)	Rosuvastatin	2
NTCP (Uptake)	Taurocholic Acid	1

In Vitro CYP450 Induction Assay – Conditions Overview (LC-MS Analysis Method)

	CYP3A4	CYP1A2	CYP2B6
Seeding density in 24-well plate	400,000cells/well in 24-well plate	400,000cells/well in 24-well plate	400,000cells/well in 24-well plate
Plating medium	ISOM's medium with 10% FBS	ISOM's medium with 10% FBS	ISOM's medium with 10% FBS
Solvent vehicle control	0.08% DMSO in Hepatocyte Culture Medium	0.08% DMSO in Hepatocyte Culture Medium	0.5% PBS in Hepatocyte Culture Medium
Positive control inducer	400 μ L of 20 μ M Rifampicin in Hepatocyte Culture Medium	400 μ L of 20 μ M β -Naphthoflavone in Hepatocyte Culture Medium	400 mL of 1000 μ M Phenobarbitol in Hepatocyte Culture Medium
Induction time	72 hrs	72 hrs	72 hrs
Enzyme substrate	200 μ L of 200 μ M Testosterone in Hepatocyte Culture Medium	200 μ L of 100 μ M phenacetin in Haptocyte Culture Medium	200 mL of 250 μ M bupropion in Haptocyte Culture Medium
Enzyme assay incubation time	30 min	60 min	30 min
Stop solution	aliquote 100 μ L enzyme assay sample and mix with 25 μ L stop solution		
	5 μ M 6 β -Hydroxytestostertone-D ₇ in 100% Acetonitrile with 0.1% Formic Acid	10 μ M Acetamidophenol- ¹³ C ₂ ¹⁵ N in 100% Acetonitrile with 0.1% formic acid	0.1 μ M Hydroxybupropion-D ₆ in 100% Acetonitrile with 0.1% Formic Acid
Standard / Metabolite	6 β -Testosterone	Acetamidophenol	Hydroxybupropion

In Vitro CYP450 Induction Assay – Plate Format

Solvent vehicle control (0.08% DMSO), 0 min substrate incubation	Solvent vehicle control (0.08% DMSO), 0 min substrate incubation	Solvent vehicle control (0.08% DMSO), 0 min substrate incubation	Solvent vehicle control (0.08% DMSO), 30 min substrate (200 uM Testosterone) incubation	Solvent vehicle control (0.08% DMSO), 30 min substrate (200 uM Testosterone) incubation	Solvent vehicle control (0.08% DMSO), 30 min substrate (200 uM Testosterone) incubation
Test comp concentration 1x, 30 min substrate (200 uM Testosterone) incubation	Test comp concentration 1x, 30 min substrate (200 uM Testosterone) incubation	Test comp concentration 1x, 30 min substrate (200 uM Testosterone) incubation	Test comp concentration 10x, 30 min substrate (200 uM Testosterone) incubation	Test comp concentration 10x, 30 min substrate (200 uM Testosterone) incubation	Test comp concentration 10x, 30 min substrate (200 uM Testosterone) incubation
Test comp concentration 100x, 30 min substrate (200 uM Testosterone) incubation	Test comp concentration 100x, 30 min substrate (200 uM Testosterone) incubation	Test comp concentration 100x, 30 min substrate (200 uM Testosterone) incubation	Test comp concentration 1000x, 30 min substrate (200 uM Testosterone) incubation	Test comp concentration 1000x, 30 min substrate (200 uM Testosterone) incubation	Test comp concentration 1000x, 30 min substrate (200 uM Testosterone) incubation
Positive control inducer (20 uM Rifampin) incubation, 30 min substrate (200 uM Testosterone) incubation	Positive control inducer (20 uM Rifampin) incubation, 30 min substrate (200 uM Testosterone) incubation	Positive control inducer (20 uM Rifampin) incubation, 30 min substrate (200 uM Testosterone) incubation	Fill with culture medium	Fill with culture medium	Fill with culture medium



REFERENCE

Ordering Information



BD Gentest™ Human Plateable Cryopreserved Hepatocytes

Product Name	Quantity	Cat. No.
BD Gentest™ Plateable Transporter-Qualified Human CryoHepatocytes	≥ 5 million cells	454541
BD Gentest™ Plateable Metabolism-Qualified Human CryoHepatocytes	≥ 5 million cells	454543
BD Gentest™ Plateable Inducible-Qualified Human CryoHepatocytes	≥ 5 million cells	454551
BD Gentest™ Plateable Inducible-Qualified Human CryoHepatocytes	2-5 million cells	454550



Reagents

Product Name	Format	Cat. No.
BD Gentest™ High Viability CryoHepatocyte Recovery Kit	Kit	454534
BD Gentest™ High Viability CryoHepatocyte Recovery Medium	50 mL Tube	454560
BD Gentest™ CryoHepatocyte Plating Medium	50 mL Tube	454561
BD™ Hepatocytes Culture Media Kit	Kit	355056
BD Gentest™ Midazolam	5 mg	451028
BD Matrigel™ Basement Membrane Matrix, Phenol Red-free	10 mL	356237
BD BioCoat™ collagen-coated plates, dishes and flasks	6, 12, 24, 48, 96 – well plates; 60 mm, 100mm, 150mm dishes; 25 cm², 75 cm² flasks	See BD Catalog
BD™ Collagen I, rat tail, 100 mg	100 mg	354236
BD Matrigel™ Matrix thin layer plates	6, 24 & 96 - well plates	See BD Catalog
BD Falcon™ cell cultureware	6, 12, 24, 48 & 96 - well plates	
BD Falcon™ pipets	1, 2, 5, 10, 25, 50 & 100 mL	
BD Gentest™ ADME/Tox chemicals including substrates, inhibitors and metabolites		
BD Gentest™ Contract Research Services including induction and metabolic stability assays using hepatocytes		



Reagents

Enzyme	Probe Substrate/Catalog No.	Metabolite/ Catalog No.	Stable labeled Internal Standard for LC/MS Analysis/ Catalog No.	Positive Control Inducer/Catalog No.
CYP1A2	Phenacetin / Sigma (A-2375)	4-Acetamidophenol/ Sigma (A-5000)	Acetamidophenol-13C2, 15N / BD (451001)	β -Naphthoflavone / Sigma (N-3633), Omeprazole / Sigma (O-104)
CYP2B6	Bupropion / BD (451710)	Hydroxybupropion / BD (451711)	Hydroxybupropion-d6 / BD (451003)	Phenobarbital / Sigma (P-5178)
CYP2C9	Diclofenac / Sigma (D6899)	4'-Hydroxydiclofenac / BD (451743)	4'-Hydroxydiclofenac-13C6 / BD (451006)	Rifampicin / Sigma (R-3501)
CYP2D6	Dextromethorphan / Sigma (D2531)	Dextrorphan / BD (451030)	Dextrorphan-d3 / BD (451008)	Not inducible
CYP3A4	Midazolam / BD (451028)	1'-Hydroxymidazolam / BD (451038)	1'-Hydroxymidazolam-13C3 / BD (451010)	Rifampicin / Sigma (R-3501)
UGT1A1	Estradiol / Sigma (E-8875)	Estradiol 3-Glucuronide / Sigma (E-2127)	N/A	β -Naphthoflavone / Sigma (N-3633)



Reagents

Transporter	Transporter Assay		
	Substrate (Conc. μ M) / Catalog No.	Inhibitor (Conc. μ M) / Catalog No.	Radiolabeled Substrate / Catalog No.
MRP2 (Efflux)	CDFDA (5) / Invitrogen (C369)	MK-571 (50) / Sigma (M7571)	n.a.
OATP1B1/OATP1B3 (Uptake)	Rosuvastatin (2) / American Radiolabeled Chemicals (ARCD 0409)	n.a.	[3H]-Rosuvastatin / American Radiolabeled Chemicals (ART 1628)
NTCP (Uptake)	1 mM Taurocholic Acid (1) / Sigma (T4009)	n.a.	[3H]-Taurocholate / Perkin Elmer (NET322)