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| epa_seal_verysmall.gifU.S Environmental Protection Agency  Office of Research and Development  **Center for Computational Toxicology & Exposure**  ***Chemical Characterization and Exposure Division***  ***Experimental Toxicokinetics and Toxicodynamics Branch*** | |
| **STANDARD OPERATING PROCEDURE** | |
| SOP Title: Preparation of Hepatocyte Metabolic Stability Assay Samples | |
| SOP ID: I-CCED-ETTB-SOP-4554 | Effective Date: December 01, 2021 |
| SOP was Developed:  In-house  Extramural: enter organization | |
| SOP Discipline: General Biology | |
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**Biennial Review**

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**Revision History**

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| **Revision No.** | **Name** | **Date of Revision** | **Description of Change(s)** |
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**SOP Title:** Preparation ofHepatocyte Metabolic Stability Assay Samples

# Scope and Applicability

This Center for Computational Toxicology and Exposure standard operating procedure (SOP) provides instruction for hepatocyte metabolic stability assay sample preparation. This SOP can be applied to sample preparation using both human and rodent hepatocyte suspensions. This assay can be used to assess hepatocyte metabolism of commercial chemicals (including drugs), environmental pollutants, and emerging contaminants. Resulting assay samples require subsequent quantitative analysis using ultra-high performance liquid chromatography or gas chromatography tandem mass spectrometry (UPLC-MS/MS or GC-MS/MS). Details about such analytical methodology are not specified in this SOP.

# Summary of Method

This SOP is applicable to both human and rodent hepatocyte suspensions. An experiment is to be defined as any/all samples generated from the same vial(s) of hepatocytes on the same day. To measure hepatic clearance, primary cryopreserved hepatocyte suspensions are resuspended in Williams’ Media E, evaluated for viability, and adjusted to the required assay cell density. To start the metabolic process, hepatocytes are added to wells in 96-well plates containing test agents (TAs) diluted in pre-warmed media. The plates are transferred to a humidified incubator (37°C, 5% CO2) and maintained on a shaker. No cell (ie, media only) and/or metabolically inactivated controls are included. At multiple time points, the metabolic reactions of a set of samples (triplicate for each sample type) and controls are stopped by the addition of ice-cold acetonitrile and centrifuged to precipitate protein and salt.., The clarified samples and controls are analyzed by UPLC-MS/MS or GC-MS/MS to quantitate loss of parent compound over time, in what is known as a “substrate depletion” approach. The targeted quantitative MS procedures used to report TA concentration are described in I-CCED-ETTB-SOP-3632-0, Assessing Per- and Polyfluoroalkyl Substances (PFAS) Solution Quality and Presence Using Ultra-High-Performance Liquid Chromatography (UPLC)-Tandem Mass Spectrometry (MS/MS), and (GC-MS/MS SOP).

# Definitions/Acronyms

**Williams’ Media E (WME); Complete Williams’ Media E (WME +/+)**

Williams’ Media E is a buffered solution enriched with various supplements to support hepatocyte viability in culture. Media and supplements are ordered and provided separately and are typically combined immediately prior to use in the assay. Media containing these supplements will be labeled as “WME +/+”.

**Test Agent (TA)**

The test agent (TA) is any chemical that is being evaluated in a certain assay.

**Cell Viability (CV)**

Hepatocyte cell viability (CV) will be monitored during the assay to ensure the cells remain functional during. CV measures will be taken throughout the assay time course.

**Assay Reference Compound (RC)**

An assay reference compound (RC) selected for inclusion in the assay ensures the assay is performing as expected. Compounds selected have documented hepatocyte clearance rates previously described either in peer-reviewed literature or Wetmore laboratory records. The specific compound selected will be determined on a case by case basis and may be selected based on anticipated clearance rates or physico-chemical properties of the test compounds. A RC may be considered a TA, but not all TAs are RCs.

# Health and Safety Warnings

Any individual performing this protocol must have completed the Safety Health and Environmental Management (SHEM) Initial Safety Health and Environmental Management (ISHEM) course and comply with SHEM annual safety training requirements. Personnel should complete the SHEM Bloodborne Pathogens training. Personnel must wear personal protective equipment (gloves, safety glasses, and lab coat) during the execution of this assay. Personnel must be thoroughly acquainted with the potential hazards of the reagents, products, solvents, equipment, and procedures described within this SOP. The Health and Safety Research Protocol for this laboratory activity (HSRP ID 856, “In Vitro Toxicokinetic Assessments”) and the relevant material safety data sheets are on file in Room E453A and in the Wetmore IVKM-Lab OneDrive folder and the O:PRIV\CCTE\_Wetmore-Lab\ folder. Care should be observed with the use of all compounds specified in this protocol as some may be hazardous if used incorrectly.

# Cautions/Interferences

**Use of Aseptic Technique** - Although this is a short-term assay, care should be taken to employ aseptic technique where possible. Prior to touching any items related to the assay, gloves should be sprayed with 95% ethanol. All surfaces (including the interior of the robot) should be wiped down with 95% ethanol prior to use. Any steps performed outside the incubator or the liquid handling robot should be executed in either a biological safety cabinet or a fume hood. After required supplies have been obtained for any assay, all bags/containers should be resealed and secured immediately to maintain sterility longer-term.

**Layouts** - It is recommended that individuals new to this assay do not deviate from the described layouts until they have demonstrated proficiency exemplified by maintaining good hepatocyte viability and/or achieving reference compound clearance rates within 20% of the reported

historical/literature values.

**Nonspecific Chemical Binding** - Given the varied range of TAs that may be screened in this assay, nonspecific chemical binding to glass or plastic may be a concern. As a rule, when no information is available following a literature search, polypropylene plastic is the preferred labware plastic for stock solution preparation and sample storage.

**Per- and Polyfluoroalkyl Substances (PFAS)** - Any work conducted on per- and polyfluoroalkyl substances (PFAS) should be conducted in polypropylene plastic to avoid sorption loss of PFCs on the container walls. Standard glassware may retain low levels of PFAS and may alter the precision and accuracy of the method

# Personal Qualifications/Responsibilities

This SOP assumes a thorough understanding of and proficiencies in the execution of basic laboratory skills, preparation of reagents, and usage of instrumentation. This document is designed to guide a competent laboratory worker in the execution of the hepatocyte stability assay. It is not intended to instruct individuals on the basic aspects of biochemistry techniques.

# Equipment and Supplies

## Equipment

* Cell culture CO2 incubator, humidified (Thermo Scientific Forma Series 3 or equivalent)
* Orbital shaker capable of reaching a speed of 200 rpm (Ohaus M/N SHLD0415DG or equivalent)
* Biological Safety Cabinet, Class II Type B2 (Nuaire M/N NU-430-400 or equivalent)
* Automated cell counter that can reliably quantitate hepatocytes (BioRad M/N TC20 or equivalent)
* Shaking water bath, heated (Julabo M/N SW23 or equivalent)
* Clinical centrifuge, refrigerated, that can hold 15 and 50mL centrifuge tubes and microplates (Thermo Scientific Heraeus Multifuge M/N X3R or equivalent)
* Microplate centrifuge, capable of achieving 4,200xg (Thermo Scientific Heraeus Multifuge M/N X3R or equivalent)
* Refrigerated microcentrifuge capable of achieving 13-15,000xg (Eppendorf M/N 5430R or equivalent)
* An electronic balance capable of weighing down to 10 mg (Sartorius Cubis M/N MSE124S-100-DU or equivalent)
* < -70°C freezer (Thermo Scientific M/N EXF-40086ARAK or equivalent)
* < -20°C freezer (Thermo Scientific M/N DXF32040A or equivalent)
* 4°C refrigerator (Thermo Scientific M/N TSX3005GA or equivalent)
* Single channel pipettors, variable volume, air displacement, capable of pipetting 2-20 μL (Gilson P/N F123600 or equivalent); 20-200 μL (Gilson P/N F123601 or equivalent); and 200-1000 μL (Gilson P/N F123602 or equivalent)
* Portable pipetting aid (Drummond Pipet Aid XP M/N 4-000-101 or equivalent)
* Multichannel pipettor, capable of pipetting 100-1200 μL (ThermoScientific Finnpipette Novus M/N 4630080 or equivalent)
* Heat block capable of reaching 90°C (Eppendorf ThermoMixer C M/N 5382-000-015 or equivalent)
* Note: a liquid handling robot (EpMotion M/N M5073 or equivalent) may be used during some of the liquid handling steps

## Supplies

* Polypropylene pipette tips to fit on variable volume air displacement single channel pipettes described above; capable of pipetting 2-20 μL (Thermo Scientific ART P/N 3521-05-HR or equivalent); 20-200 μL (Thermo Scientific ART P/N 3551-HR or equivalent; and/or 200-1000 μL) (Thermo Scientific ART P/N 3791-05-HR or equivalent)
* Sterile serological pipets (Capacity of: 5 mL (Corning P/N 357543 or equivalent); 10 mL (Corning P/N 356551 or equivalent); and/or 25 mL (Corning P/N 357535 or equivalent)
* Vacuum Filter/Storage System, 0.2 mM filtration, 150-500 mL capacity (Corning P/N 431153 (150mL, PES filter), 430767 (250 mL, CA filter) or equivalent)
* Centrifuge tube and cap, polypropylene, sterile, 15 mL and 50 mL (BD Falcon brand, BD, P/Ns 352096 and 352070, respectively, or equivalent)
* 96-well polypropylene plate with flat or U-bottom that can hold a working volume of 500 uL (Axygen Scientific P/N P-96-450R-C-S or equivalent)
* 96-well polystyrene plate lids (Costar P/N 3931 or equivalent)
* BioRad TC20 counting slides (Bio-Rad P/N 1450017)
* Matrix™ 1.4 mL polypropylene storage tubes (ThermoFisher P/N 4252-11 or equivalent)
* Matrix™ SepraSeal tube caps compatible with selected polypropylene storage tubes (ThermoFisher P/N 4464 or equivalent)
* Aluminum foil laminate sealing films (Brandtech Scientific P/N 701395ES) or equivalent

# Reagents and Standards

## Biological Products/Reagents

Primary cryopreserved hepatocyte suspensions, either human or rodent, typically pooled, 50% male and 50% female. (BioIVT P/N X008005 or equivalent). Cell post-thaw viability ≥ 70%, stored at ≤ - 130°C (gas phase, liquid nitrogen cryofreezer) prior to experiment. Metabolic enzyme characterization information from vendor should be obtained and retained with project binder and/or data. *More specifics (ie, age group targeted, sex, size of pool)* should be determined on a study-by-study basis.

***Note:*** *it is recommended that, prior to starting a major effort where large quantities of hepatocytes (e.g., >10-15 vials) are required, test vials are obtained from vendors under consideration to re-evaluate vendor-reported viability measures. Also, sufficient vials of cells from a single lot should be obtained to control for lot-to-lot variability.*

## Media, Supplements, and Other Reagents

* OptiThaw Hepatocyte Kit cell culture media (Sekisui/Xenotech P/N K8000)
* Williams’ Medium E, no phenol red (WME; Gibco/Life Technologies, P/N A1217601 or equivalent)
* Trypan Blue, 0.4%, 10 x 1.5 ml (Bio-Rad P/N 1450022 or equivalent)
* Primary Hepatocyte Maintenance Supplements (Gibco P/N CM4000). Two vials provided; one containing dexamethasone and a second containing a cocktail solution of penicillin-streptomycin, ITS+ (insulin, transferrin, selenium complex, BSA, and linoleic acid), GlutaMAX™, and HEPES

## Biological Products/Reagents

Primary cryopreserved hepatocyte suspensions, either human or rodent, typically pooled, 50% male and 50% female. (BioIVT P/N X008005 or equivalent). Cell post-thaw viability ≥ 70%, stored at ≤ - 130°C (gas phase, liquid nitrogen cryofreezer) prior to experiment. Metabolic enzyme characterization information from vendor should be obtained and retained with project binder and/or data. *More specifics (ie, age group targeted, sex, size of pool)* should be determined on a study-by-study basis.

***Note:*** *it is recommended that, prior to starting a major effort where large quantities of hepatocytes (e.g., >10-15 vials) are required, test vials are obtained from vendors under consideration to re-evaluate vendor-reported viability measures. Also, sufficient vials of cells from a single lot should be obtained to control for lot-to-lot variability.*

## Solvents and Additives

* Dimethyl sulfoxide (DMSO) (Sigma P/N 276855 or equivalent; 99.9% pure)
* Acetonitrile (ACN), LC-MS grade or equivalent (Honeywell Burdick & Jackson P/N LC-015)
* Water, LC-MS grade or equivalent (Honeywell Burdick & Jackson P/N LC-365)

*Note: Solvents and additives are stored at room temperature unless otherwise noted.*

## Test Agents

Test compounds typically at 20 mM, in DMSO or other solvent. Stored at ≤- 20°C; typically at <-70°C. Any stock solutions over 1 year of age must be evaluated by UPLC-MS/MS or GC-MS/MS for stability prior to use. Stock solutions will be discarded when deemed to be unstable or after 5 years, whichever comes first.

## Assay Reference Compounds and Associated Isotopically-Labeled Internal Standards

Below is a list of recommended assay reference compounds, of which a minimum of one should be included with each experiment (any/all samples run from the same vial of hepatocytes on the same day). Consideration of test agents under investigation (e.g., physicochemical properties, anticipated clearance rates) and to the required analytical technique determine which reference compound will be included in any experiment. Reference compounds may be substituted or added as needed. Any substitutions or additions should be noted in project records or laboratory notebooks along with the deviation from the SOP.

* Atenolol (Sigma Aldrich, P/N PHR1909; solid)
* *(Atenolol-D7 (CDN Isotopes, P/N D-6202; solid))*
* Diphenhydramine Hydrochloride (Sigma Aldrich, P/N D3630; solid)
* *(Diphenhydramine-D5 HCl (CDN Isotopes, P/N D6991; solid))*
* n-butyl paraben (Sigma Aldrich, P/N 54680; solid)
* *(n-butyl paraben-13C6 (Cambridge Isotopes, P/N CLM-8285-1.2; 1 mg/mL in MeOH))*
* Propranolol (Sigma Aldrich, P/N P0689; solid)
* *(Propranolol-D7 (CDN Isotopes, P/N D-2386; solid))*
* Verapamil Hydrochloride (Sigma Aldrich, P/N V4629; solid)
* *(Verapamil-D3 HCl (CDN Isotopes, P/N D-7546; solid))*
* Warfarin (Sigma Aldrich, P/N A2250; solid)
* *(Warfarin-D5 (CDN Isotopes, P/N D-7080; solid))*

# Procedures

## Reference Compound Selection

An assay reference compound (RC) will be selected for each experiment based on the anticipated clearance rates and/or analytic needs of the test agents. These compounds will be tested at 1 mM unless otherwise noted.

## Solution Preparation

### Williams’ Medium E, +/+ or Complete (ie, Hepatocyte cell media)

To 100 mL Williams’ Medium E, add 1 uL dexamethasone and 4 mL cell maintenance cocktail-B. Mix well. Store media at 4°C. Complete media is stable for 1 month at 4°C. Media should be warmed to 37°C prior to use in assay.

### Crash Solution

Crash solution will be comprised of 100% acetonitrile. The amount needed to process all samples generated during a given day’s run should be aliquoted to a separate tube and stored either at -20°C or on ice to ensure it is ice-cold throughout the assay. Depending on the analyte tested, an acceptable deviation to this protocol may incorporate formic acid (e.g., 1% of 0.1M solution) with the acetonitrile. Any such deviation should be noted in a project’s records or laboratory notebook and the accompanying report.

## Metabolic Inactivation of Hepatocytes

Metabolically inactivated hepatocytes are a negative control used to monitor for the abiotic loss of TA.

*Note: given the expense of the hepatocytes, any excess “active” cells remaining from earlier runs should be saved. When volumes/cell #s are sufficient, these cells should be pooled, counted, brought up to described density and volume and inactivated as described below. Also, any remaining inactive cells can be reserved for future use.*

### Remove one vial of hepatocytes from the cryofreezer and thaw in a water bath at 37°C for 90 seconds.

### Place the vial in a heat block set to 90°C and heat for 40 minutes.

### Transfer inactive cells to conical tube, rinse vial with 1 mL media, and transfer rinse to same conical tube. Dilute with media to 10 mL total volume.

### Transfer 10 µL suspended cells to a new 1.5 mL microcentrifuge tube; mix with 10 l trypan blue.

### Using the Bio-Rad TC20-cell counter (PN 1450102), take 2 measurements (See Section 9.6 for instructions). Record total cell count, live cell count, and % viability.

### Perform calculations to determine volume (mL) of media to add to achieve a cell density of 1,000,000 cells/ml:

### Add this volume to microcentrifuge tube with WME +/+.

*Note - These can be prepared in advance and stored at <-70°C until needed (up to 5 years).*

## Test Agent (TA) Stock and Working Stock Preparation

### TA stocks are typically received from Evotec contractor at 20-30 mM in DMSO and stored *at* 40ºC. If this is the case, please proceed to step 4 below.

### For TA stock preparation from neat compound, record TA name, CASRN, DTXSID (if available) and molecular weight in the project’s records or laboratory notebook. Use the following equation to guide stock preparation.

To calculate the amount (mg) of neat compound to dissolve 10 mL for a 30 mM solution:

Example: for a compound with a molecular weight of 150 g/mole:

### Weigh out compound and transfer to a 15 mL polypropylene centrifuge tube. Bring up to desired volume to achieve required mg/mL concentration in DMSO or acetonitrile. Be sure to adjust volume so that the desired concentration (e.g., 4.5 mg/mL) is achieved. (For example, if 45 mg weighed out, bring volume up to 10 mL in solvent). Vortex and visually inspect to ensure TA has gone into solution.

### Prepare smaller aliquots (e.g., 0.5-1.5 mL per 1.5 mL polypropylene microcentrifuge tubes); label each tube with the date, chemical name, stock concentration and solvent used. If prepared in advance, store at <-20ºC until use.

### Thaw TA stock solutions to RT. Dilute to 2 mM in DMSO, then dilute in Williams’ Medium E +/+ to a final working concentration of 2 µM as described in the table below. The final DMSO concentration ideally should be at 0.1% if TA solubility allows. If not strive, to target 0.2%, then 0.5% but flag this protocol deviation. When performing this 6-point time course (0, 15, 30, 60, 120, 240 min; controls at 0, 60, 240 min), 1800 mL of the 2 mM assay stock will be needed.

**TA Working Stock Preparation for 6-point Time Course**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Compound** | **TA Stock (in DMSO; mM)** | **Vol (mL) of X mM stock to make 2 mM** | **Vol (mL) DMSO** | **Vol (ml) of 2 mM stock make 2 mM** | **Vol (ml) WEM +/+ q.s. to 3 ml to prep 2 uM stock** | **Final % DMSO in assay incubations** |
| Test Agent A (30 mM) | 30 | 10 | 140 | 5 | 4995 | 0.1 |
| Test Agent B (20 mM) | 20 | 10 | 90 | 5 | 4995 | 0.1 |
| Test Agent C (10 mM) | 10 | 10 | 40 | 5 | 4995 | 0.1 |

### Following the plate map described in 9.5, add 50 µL of the test agent working stocks as indicated.

### To any wells in Columns 10-12 to be used for a cell viability check, add 50 µL unspiked WME +/+.

### Once prepared, place plates in humidified incubator (37°C, 5% CO2) on a shaker, set to shake at 200 rpm.

## Experimental Plate Map Layouts

*Note: Each hepatocyte vial contains approximately 5-7 million viable cells, which are sufficient to test 5-6 TAs in a 6-point time course performed in triplicate. A representative plate layout is provided below. Acceptable deviations to this protocol would be to expand the 6-point time course to assess 15-18 TAs, requiring 3 vials of hepatocytes and minor modifications to the plate layouts provided. Alternately, multiple time points could be consolidated to decrease the number of assay plates used from 6 to 3 (e.g., combining Times 15 and 60 min and Times 30 and 240 min). It is recommended that individuals new to this assay do not deviate from the described layouts until they have demonstrated proficiency exemplified by maintaining good cell viability and/or achieving reference compound clearance rates within 20% of the reported historical/literature values.*

**Plate Layout: 6-point Time Course** (Times 0, 15, 30, 60, 120, 240 min)

6 plates during assay; 4 sample storage plates stored at -70ºC

* 7 Test agents (TAs; e.g., TA1, TA2, etc.), plus 1 reference compound (RC) – 8 TAs total - as described below
* (More TAs may be added for increased throughput)
* Cell Viability (CV) in columns 10-12 (optional)

Time 0

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | ACTIVE | | | INACTIVE | | | MEDIA ONLY | | | Viability Assay | | |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | TA1 | TA1 | TA1 | TA1 | TA1 | TA1 | TA1 | TA1 | TA1 | CV-0 | CV-0 | CV-0 |
| B | TA2 | TA2 | TA2 | TA2 | TA2 | TA2 | TA2 | TA2 | TA2 |  |  |  |
| C | TA3 | TA3 | TA3 | TA3 | TA3 | TA3 | TA3 | TA3 | TA3 |  |  |  |
| D | TA4 | TA4 | TA4 | TA4 | TA4 | TA4 | TA4 | TA4 | TA4 |  |  |  |
| E | TA5 | TA5 | TA5 | TA5 | TA5 | TA5 | TA5 | TA5 | TA5 |  |  |  |
| F | TA6 | TA6 | TA6 | TA6 | TA6 | TA6 | TA6 | TA6 | TA6 |  |  |  |
| G | TA7 | TA7 | TA7 | TA7 | TA7 | TA7 | TA7 | TA7 | TA7 |  |  |  |
| H | RC | RC | RC | RC | RC | RC | RC | RC | RC |  |  |  |

Time 15

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | ACTIVE | | |  | | |  | | | Viability Assay | | |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | TA1 | TA1 | TA1 |  |  |  |  |  |  | CV-15 | CV-15 | CV-15 |
| B | TA2 | TA2 | TA2 |  |  |  |  |  |  |  |  |  |
| C | TA3 | TA3 | TA3 |  |  |  |  |  |  |  |  |  |
| D | TA4 | TA4 | TA4 |  |  |  |  |  |  |  |  |  |
| E | TA5 | TA5 | TA5 |  |  |  |  |  |  |  |  |  |
| F | TA6 | TA6 | TA6 |  |  |  |  |  |  |  |  |  |
| G | TA7 | TA7 | TA7 |  |  |  |  |  |  |  |  |  |
| H | RC | RC | RC |  |  |  |  |  |  |  |  |  |

Time 30

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | ACTIVE | | |  | | |  | | | Viability Assay | | |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | TA1 | TA1 | TA1 |  |  |  |  |  |  | CV-30 | CV-30 | CV-30 |
| B | TA2 | TA2 | TA2 |  |  |  |  |  |  |  |  |  |
| C | TA3 | TA3 | TA3 |  |  |  |  |  |  |  |  |  |
| D | TA4 | TA4 | TA4 |  |  |  |  |  |  |  |  |  |
| E | TA5 | TA5 | TA5 |  |  |  |  |  |  |  |  |  |
| F | TA6 | TA6 | TA6 |  |  |  |  |  |  |  |  |  |
| G | TA7 | TA7 | TA7 |  |  |  |  |  |  |  |  |  |
| H | RC | RC | RC |  |  |  |  |  |  |  |  |  |

Time 60

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | ACTIVE | | |  | | |  | | | Viability Assay | | |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | TA1 | TA1 | TA1 |  |  |  |  |  |  | CV-60 | CV-60 | CV-60 |
| B | TA2 | TA2 | TA2 |  |  |  |  |  |  |  |  |  |
| C | TA3 | TA3 | TA3 |  |  |  |  |  |  |  |  |  |
| D | TA4 | TA4 | TA4 |  |  |  |  |  |  |  |  |  |
| E | TA5 | TA5 | TA5 |  |  |  |  |  |  |  |  |  |
| F | TA6 | TA6 | TA6 |  |  |  |  |  |  |  |  |  |
| G | TA7 | TA7 | TA7 |  |  |  |  |  |  |  |  |  |
| H | RC | RC | RC |  |  |  |  |  |  |  |  |  |

Time 120

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | ACTIVE | | |  | | |  | | | Viability Assay | | |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | TA1 | TA1 | TA1 |  |  |  |  |  |  | CV-120 | CV-120 | CV-120 |
| B | TA2 | TA2 | TA2 |  |  |  |  |  |  |  |  |  |
| C | TA3 | TA3 | TA3 |  |  |  |  |  |  |  |  |  |
| D | TA4 | TA4 | TA4 |  |  |  |  |  |  |  |  |  |
| E | TA5 | TA5 | TA5 |  |  |  |  |  |  |  |  |  |
| F | TA6 | TA6 | TA6 |  |  |  |  |  |  |  |  |  |
| G | TA7 | TA7 | TA7 |  |  |  |  |  |  |  |  |  |
| H | RC | RC | RC |  |  |  |  |  |  |  |  |  |

Time 240

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | ACTIVE | | | INACTIVE | | | MEDIA ONLY | | | Viability Assay | | |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | TA1 | TA1 | TA1 | TA1 | TA1 | TA1 | TA1 | TA1 | TA1 | CV-240 | CV-240 | CV-240 |
| B | TA2 | TA2 | TA2 | TA2 | TA2 | TA2 | TA2 | TA2 | TA2 |  |  |  |
| C | TA3 | TA3 | TA3 | TA3 | TA3 | TA3 | TA3 | TA3 | TA3 |  |  |  |
| D | TA4 | TA4 | TA4 | TA4 | TA4 | TA4 | TA4 | TA4 | TA4 |  |  |  |
| E | TA5 | TA5 | TA5 | TA5 | TA5 | TA5 | TA5 | TA5 | TA5 |  |  |  |
| F | TA6 | TA6 | TA6 | TA6 | TA6 | TA6 | TA6 | TA6 | TA6 |  |  |  |
| G | TA7 | TA7 | TA7 | TA7 | TA7 | TA7 | TA7 | TA7 | TA7 |  |  |  |
| H | RC | RC | RC | RC | RC | RC | RC | RC | RC |  |  |  |

## Thawing, Resuspension, and Counting of Hepatocytes

### Prepare heat inactivated hepatocytes as described in 9.3 or remove vial of previously prepared heat-inactivated hepatocytes from <-70°C freezer and thaw in a 37°C water bath (latter is preferred).

### Pipet 2 mL of OptiThaw Media into a 15 mL centrifuge tube and warm to 37°C.

### Remove vial of hepatocytes from cryofreezer and immediately place in 37°C water bath. If immediate transfer to water bath not possible, place on dry ice for transport to water bath. Thaw vial for approximately 90 seconds, monitoring closely to note moment that ice crystals disappear.

### Immediately transfer suspension to 15 mL centrifuge tube containing pre-warmed OptiThaw media. Mix by inversion. Rinse original vial with an additional 1 mL OptiThaw media and transfer to same 15 mL tube.

### Spin vial with cells at 100xg for 5 min at room temperature, discard supernatant.

### Resuspend pellet in 37°C with 3 mL WME +/+.

### If the cell count will be performed using the Bio-Rad TC20 continue to Section 9.6.8., if the cell count will be performed using the hemacytometer, skip to Section 9.6.9.

### Cell counting using Bio-Rad TC20

#### Transfer 10 uL of suspension to a microcentrifuge tube (or a well of a 96-well plate) containing 10 uL 0.4% trypan blue; mix by pipetting up and down 3 times.

#### Carefully load 10 uL of solution from Step 6 onto BioRad TC20 cell counting chamber slides, filling entire cavity with no air bubbles.

#### Turn on BioRad TC20 counter; follow instructions on instrument to determine cell counts and viability.

### Cell counting using hemacytometer:

#### Use of hemacytometer step 1.

#### Use of hemacytometer step 2.

#### Use of hemacytometer step 3.

### Complete calculations in worksheet below to determine volume of WME +/+ needed to achieve a cell density of 1,000,000 cells/mL.

Sample Worksheet (note cell suspension in 3.0 mL WME +/+)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Total cell number | % viable cells | Number of viable cells | Final volume WME+/+ needed | Final viable cell density | Volume WME+/+ to add: |
| 6x10^6 | 85 % | 5.1x10^6 | 5.1 mL | 1x10^6 cells | 2.1 mL |

### Using numbers calculated in worksheet, bring up volume of WME +/+ to yield 1 million cells/mL.

### Cell viability check during time course (optional): Cell viability must be checked when cells are thawed as described above; this is assigned the Time 0 viability measure. To evaluate cell viability at other time points, empty wells (e.g., in columns 10-12 of experimental assay plate (or in an alternate plate)), add 50 ul cells and 50 ul warm media and place in incubator on shaker along with metabolic assay samples. At desired time points, follow the steps in Section 9.6.8. The chart below can be completed, with the Time 0 measure being set to 100%.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| ***Cell Viability Check\**** | | | |  |  |  |
|  | ***T0\****  ***% viable*** | ***T15***  ***% viable*** | ***T30***  ***% viable*** | ***T60***  ***% viable*** | ***T120***  ***% viable*** | ***T240***  ***% viable*** |
| ***Cell #*** |  |  |  |  |  |  |
| ***# live cells*** |  |  |  |  |  |  |
| ***%*** |  |  |  |  |  |  |
| ***Exptal % from start\*\**** | ***100*** |  |  |  |  |  |

\* T0 will reflect viability obtained from initial thawing and resuspension of hepatocytes.

\*\*“Experimental % viable from start” resets the number the T0 % viable to 100% and should be used to calculate % viable throughout time course.

## Hepatocyte Stability Assay

### Time 0 Plate: Remove the plate from incubator and add 200 µL cold crash solution to all wells containing test agent. Next, add 50ul of the appropriate solution - media, inactive cell suspension, or active cell suspension - to assigned wells. Swirl by hand to mix; Cover with plate lid and place in -20°C (or lower) freezer for a minimum of 10 minutes.

### *Note -* *Crashing the other plates exactly at their respective time points is more critical than processing the Time 0 plate exactly after 10 minutes has elapsed.*

### 

### Remaining Plates: Starting with Time 240 plate, remove from incubator and add 50 µl of the appropriate solution (media, inactive cell suspension, or active cell suspension) to wells as assigned in plate layout. Cover plate with lid, swirl to mix, and place in humidified incubator (37°C, 5% CO2) on a shaker, with slight shaking (150-200 rpm).

### Set the timer for 240 minutes and start timing.

### Working backwards through the time course, repeat steps described in Section 9.7.2 for the remaining plates.

### *Caution – keep plates at 37°C until right before hepatocyte addition to optimize hepatocyte viability.*

### At each time point remove the relevant plate from the incubator, add 200 µL cold crash solution to each well, and gently swirling to mix well. Cover with plate lid and move to -20°C freezer for at least 10 minutes.

### After 10 minutes has elapsed, remove from freezer and spin at 4000xG in the plate centrifuge for 30 minutes at 4°C. Delay processing until two or four plates are available to balance the centrifuge during operation. Store plates in -20°C freezer until they are processed.

### Carefully transfer 250 µL supernatant from each well to a matrix tube rack. Consolidate the 15, 30, 60, and 120 minute time-points into one rack as shown below.

### Cap, then transfer individual tubes to refrigerated microcentrifuge and spin at 12,000xg for 10 minutes. Transfer tubes back to matrix rack, maintaining alphanumeric order as printed on bottom of tubes.

### Transfer 200 µL of the resulting supernatant to a new tube in a matrix rack.

### Store plates at <-40°C until analysis.

### *Note - Calibration curve (CC) and quality control samples for quantitative analysis of TAs and RC should be prepared within two weeks of assay sample preparation. See Section 9.8 or 9.9 for CC preparation for ultra-high-performance liquid chromatography (UPLC) tandem mass spectrometry (MS/MS) and gas chromatography (GC) MS/MS, respectively.*

***Sample Storage Plate – 15, 30, 60, 120 min***

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | ACTIVE – 15 | | | ACTIVE-30 | | | ACTIVE-60 | | | ACTIVE – 120 | | |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | TA1 | TA1 | TA1 | TA1 | TA1 | TA1 | TA1 | TA1 | TA1 | TA1 | TA1 | TA1 |
| B | TA2 | TA2 | TA2 | TA2 | TA2 | TA2 | TA2 | TA2 | TA2 | TA2 | TA2 | TA2 |
| C | TA3 | TA3 | TA3 | TA3 | TA3 | TA3 | TA3 | TA3 | TA3 | TA3 | TA3 | TA3 |
| D | TA4 | TA4 | TA4 | TA4 | TA4 | TA4 | TA4 | TA4 | TA4 | TA4 | TA4 | TA4 |
| E | TA5 | TA5 | TA5 | TA5 | TA5 | TA5 | TA5 | TA5 | TA5 | TA5 | TA5 | TA5 |
| F | TA6 | TA6 | TA6 | TA6 | TA6 | TA6 | TA6 | TA6 | TA6 | TA6 | TA6 | TA6 |
| G | TA7 | TA7 | TA7 | TA7 | TA7 | TA7 | TA7 | TA7 | TA7 | TA7 | TA7 | TA7 |
| H | RC | RC | RC | RC | RC | RC | RC | RC | RC | RC | RC | RC |

## Preparation of Calibration Standards and Quality Control Samples for UPLC-MS/MS Analysis

### All Plates Except Time 0: Starting with Time 240 plate, remove from incubator and add 50ul of the appropriate solution - media, inactive cell suspension, and active cell suspension - to wells as assigned in plate layout. Cover plate with lid, swirl to mix, and place in humidified incubator (37°C, 5% CO2) on a shaker, with slight shaking (150-200 rpm). Set timer for 240 minutes.

### Working backwards through the time course, repeat Step 1 for the remaining plates, keeping at 37°C until right before hepatocyte addition to optimize hepatocyte viability.

### Time 0 Plate: Remove from incubator and add 200 uL cold crash solution to all wells containing test agent. Next, add 50ul of the appropriate solution - media, inactive cell suspension, or active cell suspension - to assigned wells. Swirl by hand to mix; Cover with plate lid and place in -20°C freezer for a minimum of 10 minutes. (Crashing the other plates exactly at their respective time points is more critical than processing the Time 0 plate.)

### At each time point, remove the relevant plate from the incubator and add 200 uL cold crash solution to each well, gently swirling to mix well. Cover with plate lid and move to freezer for at least 10 minutes.

### After 10 minutes has elapsed, remove from freezer and spin at 4000xG in plate centrifuge for 30 minutes at 4°C. Delay processing until two or four are ready so the plates are readily balanced in the centrifuge. Store plates awaiting processing in -20°C freezer.

### Carefully transfer 250 uL supernatant from each well to a matrix tube rack. Consolidate the 15, 30, 60, and 120 minute time-points into one rack as shown below.

### Cap, then transfer individual tubes to refrigerated microcentrifuge and spin at 12,000xg for 10 minutes. Transfer tubes back to matrix rack, maintaining alphanumeric order as printed on bottom of tubes.

### Transfer 200 uL of the resulting supernatant to a new matrix tube rack.

### Store plates at <-40°C until analytical analysis.

### Calibration curve for TAs and RC should be prepared within two weeks of assay sample preparation.

## Preparation of Calibration Standards and Quality Control Samples for GC-MS/MS Analysis

# Data and Records Management

The research notebook will be the record for any procedure conducted and will provide the objective, procedural details, results and comments relevant for project progress, execution and completion. These entries will provide sufficient information to describe the work completed, summarize the results, and provide location of any electronic instrument files or data summaries when not directly supplied in the notebook. The laboratory notebooks are the property of EPA and will be stored in accordance with EPA’s record management policy.

Within each notebook entry documenting execution of this SOP, TA and RC information, ranging from chemical identification information (including catalog numbers, lot numbers, sample ID numbers (if stocks provided by EPA contractor), certificates of analyses, safety data sheets, and other associated documentation will be described or the location of such documentation (in a networked folder, as described in the relevant QAPP) will be provided. Hepatocyte lot information and cell count and cell viability calculations as described in Section 9.6 will be documented.

The plate map that documents chemicals screened and location within the tube racks will be summarized in a chain of custody document either within or linked to the relevant electronic research notebook. This document will be shared with the analyst who will conduct targeted MS analysis upon sample transfer,and will contain a brief summary of the experimental details (assay date, assay technician, sample ID, sample map, assay concentration, sample contents.

Links or file directory information will be provided to pertinent electronic data, which will be housed on Agency network drives and subject to regular back-ups per Agency policy. Records generated will be maintained by the principal investigator until completion of the project. Upon completion, data should be stored in accordance with EPA’s record management policy.

# Quality Assurance/Quality Control

Initially, successful execution of this SOP will be determined upon review of hepatocyte cell viability data collected at the start of the assay. Ultimately the MS analytic data collected for the no cell and metabolically inactivated cell controls; and the *in vitro* clearance rates derived for the assay reference compound(s) will be used to evaluate performance and usability of the samples prepared by this assay.

These reviews will be conducted after test agent/compound quantitation of the samples has been performed by MS analysis. The table below provides a summary of assay-specific acceptance criteria that will be considered along with required corrective action should these samples fail QC.

|  |  |  |  |
| --- | --- | --- | --- |
| **QC Sample Type** | **Frequency** | **Acceptance Criteria** | **Corrective Action if QC Sample Fails** |
| Hepatocyte Cell Viability | Once per experiment | >70% upon resuspension | Flag in comments; reassess data; rerun assay if warranted |
| No Cell Control Check | One per experiment | Reported TA concentrations for T0 and T240 samples are ±25% of each other | If TA concentrations are >±25% but <±50%, flag but still proceed with Clint calculations. If >±50%, flag and reassess data, compare to inactivated cell controls; if >50%, assess compound stability in system |
| Inactivated Cell Control | One per experiment | Reported TA concentrations for T0 and T240 samples are ±25% of each other | See Corrective Action for No Cell Control Check |
| Reference Compound | One per experiment | ±20% of literature values or historical lab data | Flag in comments; assess reason; rerun assay if warranted |

# References

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Wetmore, B.A., Wambaugh, J.F., Ferguson, S.S., Li, L., Clewell, H.J., Judson, R.S., Freeman, K., Bao, W., Sochaski, M.A., Chu, T-M., Black. M.B., Healy, E., Allen, B., Andersen, M.E., Wolfinger, R.D., and Thomas R.S. (2013). The relative impact of incorporating pharmacokinetics in predicting i*n vivo* hazard and mode-of-action from high-throughput i*n vitro* toxicity assays. *Toxicol Sci* 132(2):327-46.

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