Determination of Metabolic Stability Using Cryopreserved Hepatocytes from Rainbow Trout (*Oncorhynchus mykiss*)

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Trout provide a relatively easy source of hepatocytes that can be cryopreserved and used for a range of applications including toxicity testing and determination of intrinsic clearance. Standard protocols for isolating, cryopreserving, and thawing rainbow trout hepatocytes are described, along with procedures for using fresh or cryopreserved hepatocytes to assess metabolic stability of xenobiotics in fish by means of a substrate depletion approach. Variations on these methods, troubleshooting tips, and directions for use of extrapolation factors to express results in terms of in vivo intrinsic clearance are included. These protocols have been developed for rainbow trout, but can be adapted to other fish species with appropriate considerations. © 2015 by John Wiley & Sons, Inc.

Keywords: rainbow trout • metabolic stability • biotransformation • in vitro assay • fish • hepatocytes • substrate depletion

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INTRODUCTION

Isolated hepatocytes obtained from mammals have long been used in drug discovery and chemical hazard assessment (Houston, 1994; Obach, 1999; Hengstler et al., 2000; Gomez-Lechon et al., 2003; Hewitt et al., 2007; Hodgson et al., 2014). Although their preparation can be more cumbersome, hepatocytes offer several advantages over other in vitro systems derived from liver tissue, such as S9 fractions and microsomes. Because they possess intact cell membranes and membrane transport proteins (Nordell et al., 2013), hepatocytes more closely resemble intact tissue. For this reason, hepatic clearance estimates obtained using intact cells are often more predictive of measured in vivo clearance than estimates obtained using subcellular fractions (Ito and Houston, 2005; Brown et al., 2007). When used to study biotransformation, hepatocytes do not require inclusion of costly cofactors. Finally, because they maintain their biotransformational

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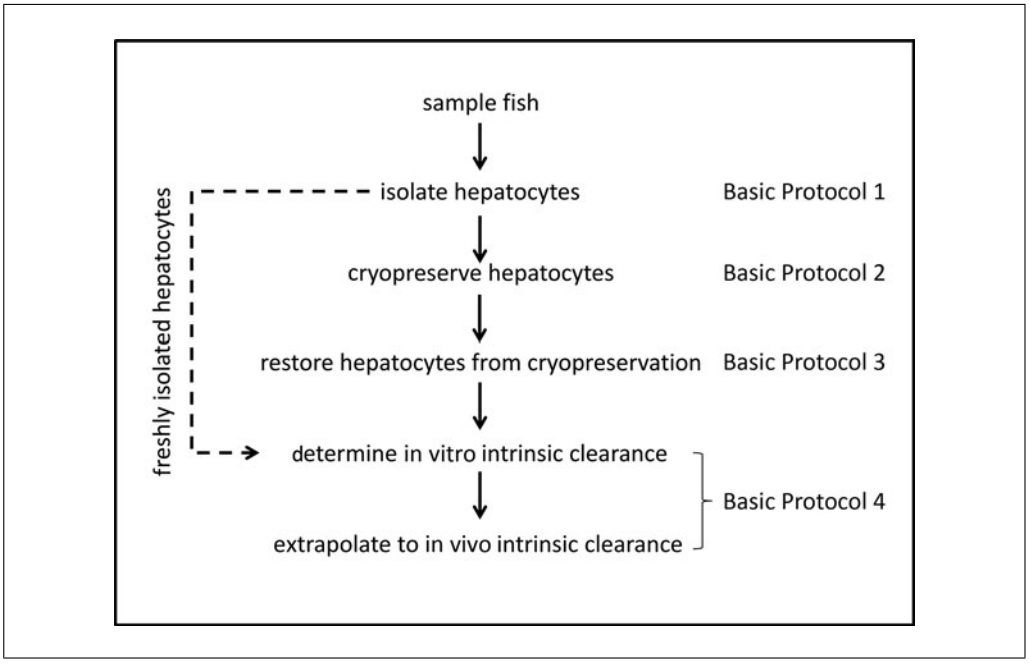

Figure 4.42.1 Flow chart of steps associated with individual protocols. The dotted line indicates an alternative route using freshly isolated hepatocytes for the determination of a metabolic depletion rate.

integrity longer, hepatocytes may be preferred over subcellular preparations for assessing slowly metabolized chemicals.

Fish hepatocytes have been used to perform toxicity assessments, screen for endocrine-active compounds, and predict biotransformation impacts on chemical accumulation (Baksi and Frazier, 1990; Pesonen and Andersson, 1997; Segner and Cravedi, 2001; Laville et al., 2004; Navas and Segner, 2006; Finne et al., 2007; Tollefsen et al., 2008; Markell et al., 2014). While most of this work has been conducted using hepatocytes isolated from rainbow trout, other species have been successfully employed (Baksi and Frazier, 1990; Segner, 1998; Braunbeck and Segner, 2000; Cowan-Ellsberry et al., 2008). However, trout provide a relatively easy source of hepatocytes, and the resulting cells have been shown to cryopreserve well, with minimal loss of xenobiotic metabolizing capability (Mingoia et al., 2010; Fay et al., 2014a). This feature makes it possible to freeze cells in one location and distribute them to other laboratories for later use. In a recent ring trial involving three different laboratories, Fay et al. (2014b) found that cryopreserved trout hepatocytes can be used to reproducibly measure in vitro intrinsic clearance rates for compounds covering a range of physico-chemical properties.

Detailed protocols describing the isolation of liver S9 fractions from rainbow trout and their use in conducting metabolic stability studies were recently published (Johanning et al., 2012). In this unit, a similar set of protocols for the isolation of primary trout hepatocytes, cryopreservation and thawing of cells, and their use in the assessment of metabolic stability via a substrate depletion approach is provided (Fig. 4.42.1). Additional information is provided to aid the reader in performing critical preliminary studies, selecting and using reference compounds, and interpreting test results.

CAUTION: All tissues should be handled with caution due to possible presence of infectious agents. Wear appropriate laboratory coat, gloves, and eye protection during all laboratory operations. Read the material safety data sheet for each organic solvent and test chemical, and employ recommended safety measures. Bulk solvents should be

handled in a fume hood. Liquid nitrogen and dry ice should be handled using cryogenic gloves in a well-ventilated area.

NOTE: Animal use should be reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) or equivalent at the institute where the procedure takes place.

RAINBOW TROUT HEPATOCYTE ISOLATION

BASIC PROTOCOL 1

The procedure for isolating hepatocytes from rainbow trout largely follows techniques used to obtain hepatocytes from mammals (Seglen, 1976; Mommsen et al., 1994; Segner, 1998; Mudra and Parkinson, 2001). The fish is anesthetized (but alive) during the isolation procedure to take advantage of the dilated hepatic vascular system, which allows for increased perfusion efficiency. The hepatic portal vein is cannulated, and the liver is perfused with a Ca^{2+}/Mg^{2+}-free balanced salt buffer to clear the liver of blood and loosen desmosomes. The liver is then perfused by a balanced salt solution (with Ca^{2+} and Mg^{2+}) containing the enzyme collagenase IV. After digestion, the collagenase reaction is terminated by perfusion with cell medium containing bovine serum albumin, and the cells are mechanically separated from the liver capsule. The primary hepatocytes are washed and purified using a density gradient. All buffers are adjusted to the blood plasma pH of the fish and chilled to its acclimation temperature. For rainbow trout, this is typically pH 7.8 at 12°C.

Generally, pooling hepatocytes from several fish (three to six) is recommended. This approach will diminish the influence of a single fish and better represent a population. Elapsed time from isolation to use, or cryopreservation, should be minimized. Quick isolation of cells from several fish may be accomplished by using several isolation stations simultaneously, or by splitting the perfusate line to accommodate more than one fish. This latter scenario is described in more detail below.

If possible, it is best to perform the isolation using sterile technique in a biosafety containment hood. Sterile technique is especially important if the hepatocytes will subsequently be cultured. If cells will be used in suspension for short-term assays, sterile technique may not be necessary, although sterile buffers and media should still be used.

Materials

Sexually immature rainbow trout (200 to 500 g)
Buffer I (see recipe)
Buffer II (see recipe)
Buffer III (see recipe)
70% (v/v) ethanol
Tricaine methanesulfonate (MS-222; Western Chemical, Inc) anesthetic (see recipe)
90% isotonic Percoll in Dulbecco's phosphate buffered saline (DPBS) (see recipe)
Leibovitz L-15 medium (L-15; Life Technologies, cat.no. 21083), pH 7.8 at 12°C

10-gallon buckets or tanks for transferring fish
Perfusion apparatus (Fig. 4.42.2) including:
- Recirculating water bath capable of chilling water to 12°C
- Peristaltic pump
- Pump tubing
- Water-jacketed glass coil condenser (45 × 260–mm or similar)
- Water-jacketed glass bubble trap with stopcock
- Surgical platform with catch basin for blood and perfusate (or tray lined with paper towels), optional

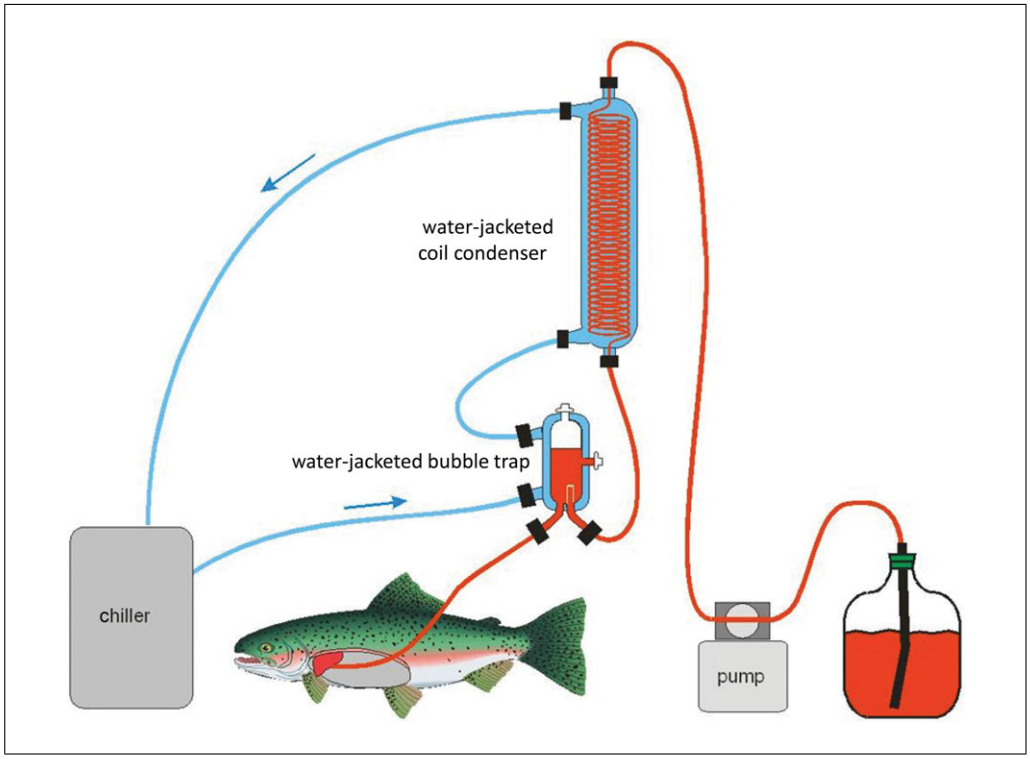

Figure 4.42.2 Set-up for perfusion of a fish liver to obtain primary hepatocytes. Perfusate is pumped first through a water-jacketed coil condenser followed by a water-jacketed bubble trap before perfusing the liver. Water is cooled by a chiller so that the perfusate exiting the bubble trap is maintained at the temperature in which the fish was acclimated. The perfusion line exiting the bubble trap may be split to perfuse two fish, simultaneously.

Surgical instruments:
- 3 × 3–in. weigh boats
- Forceps
- Large and small sharp surgical scissors
- 21-G × ¾ safety winged infusion set (butterfly catheter)
- Micro-bulldog clamps (Harvard Apparatus, cat. no. NP 52-3258) *or* sutures

100-μm nylon mesh
150-ml tall glass beaker
Fish net
Digital balance (1 to 2000 g)
50-ml conical centrifuge tubes
Refrigerated centrifuge
Serological pipets

Additional reagents and equipment for counting cells (see Support Protocol 1)

Perform preliminary preparation

1. Obtain sexually immature rainbow trout from fish supplier.

   Rainbow trout are typically raised at 10° to 15°C. The temperature of the holding tank in the laboratory should be similar and maintained at ± 2°C.

2. Acclimatize fish in laboratory for at least 2 weeks prior to use.

   Measure and record water chemistry characteristics weekly, including: pH, total alkalinity (as mg/liter $CaCO_3$), dissolved oxygen (mg/liter, converted to percent saturation), and total ammonia (mg/liter). Record fish maintenance details as well, including: photoperiod, feeding regime, feed type, water temperature, holding density (kg fish/liter tank

volume), and number of fish/tank. Guidelines for maintaining trout, including loading and feeding rates, are available in ASTM (1992) and Piper et al. (1982).

3. Fast fish 24 hr prior to sacrifice.
4. Set up the perfusion apparatus (Fig. 4.42.2).

   If perfusing two fish with one apparatus, the line exiting the bubble trap may be split. Use a clamp or valve to control the perfusate flow through the second line while starting perfusion on the first fish.

5. Turn chiller and circulator on, and adjust the temperature setting so that perfusate exiting the cannula is ~12°C (or the temperature at which the fish was acclimated).
6. Set perfusate flow to ~10 ml/min.

   If using a split perfusion line to isolate cells from two fish simultaneously, pre-determine the pump rate needed for 10 ml/min flow through the first line when the second line is clamped. Determine the necessary increase in pump rate required to maintain the flow rate when both perfusion lines are open. Note that the flow rate may need adjustment for fish of different sizes (e.g., 5 ml/min for 100-g fish).

7. Check pH of pre-prepared buffers within 2 hr prior to use and adjust to the target pH at the acclimation temperature of the fish (e.g., pH 7.8 at 12°C), if necessary.

   Particularly, buffers I and II will decrease in pH if prepared too far in advance due to the dissolution of CO_2 and formation of carbonic acid.

8. Flush tubing and bubble trap with 70% ethanol for ~10 min to clean out flow path. Then, flush for an additional 10 min with deionized water, and finally with buffer I for ~3 min just prior to start of liver perfusion.

   To flush the bubble trap, open the top and front valves to empty. Block the front valve and fill the bubble trap until fluid spills out of the top, and then discharge the majority of the fluid by releasing the front valve. Flush in this manner several times.

9. Set out all surgical instruments: forceps, large and small scissors, weigh boat, butterfly catheter set, and micro-bulldog clamp or sutures. Using a rubber band, secure a piece of 100-μm nylon mesh over rim of a 150-ml tall glass beaker. Place beaker on ice.

   The mesh should not be tight across the top of the beaker, but depressed in the center to filter the cell suspension. Poor quality nylon may not be sufficient for use in filtering cells. Stitching with ragged edges may damage cells.

10. Prepare tricaine methanesulfonate (MS-222) solution.

Prepare fish for surgery

11. Using a net, capture a fish and transfer it to a tank or bucket containing 8 liters of MS-222.

    The MS-222 solution can be used to anesthetize several fish without loss of anesthetic efficacy, but this number may depend on the size of the fish.

12. Once anesthetized, weigh fish and record weight (e.g., Fig. 4.42.3).

    The fish is properly anesthetized when opercular movement has ceased, there is a total loss of equilibrium and muscle tone, and no response to stimuli (a firm squeeze at the base of the tail may be used to determine response to stimuli).

13. Measure the total length of the fish and record (e.g., Fig. 4.42.3).

Hepatocyte isolation from rainbow trout

Initials of researchers: Date of isolation:

Hepatocyte batch ID	
Isolation start time	
Stop time	
Total number of fish pooled	
Volume of suspension frozen (ml)	
No. cryovials frozen (1.5 ml each)	
Location of cryovials	

Fish information	
Species	
Strain	
Source	
Date of arrival	
Last feeding	
Photoperiod	

Fish no.	Fish mass (g)	Fish length (cm)	Gender	Gonad mass (g)	GSI	Comments
1						
2						
3						
4, etc.						

Pre-count volume of cell suspension (ml): Post-count volume of cell suspension (ml):

Cell counts

Replicate 1

Dilution factor	Side A	Side B	Ave
Live count (4 corners)			
Total count (4 corners)			
% viable			
Concentration (10^6 cells/ml)			

Replicate 2

Dilution factor	Side A	Side B	Ave
Live count (4 corners)			
Total count (4 corners)			
% viable			
Concentration (10^6 cells/ml)			

Replicate 3

Dilution factor _____	Side A	Side B	Ave
Live count (4 corners)			
Total count (4 corners)			
% viable			
Concentration (10^6 cells/ml)			

Viable cell conc. (10^6 cells/ml):
Average concentrations from reps 1,2,3

Total yield from isolation (10^6 cells):
Viable cell conc. × pre-count volume

Total number of viable cells (post-count):
Viable cell conc. × post-count volume

Suspension volume required for 375×10^6 cells (ml):
(375×10^6 cells/viable cell conc.)

Figure 4.42.3 Example worksheet to record fish information, individual fish observations, and hepatocyte isolation details (see Basic Protocol 1). Tables for recording and summarizing cell count and cell viability measurements are provided, along with calculations for determining the isolation yield and preparing a suspension for cryopreservation (see Basic Protocol 2).

14. Place trout on the surgical platform with the ventral surface facing the technician.
15. Make a midline incision from the vent to the isthmus, taking care not to cut too deeply into the body cavity (Fig. 4.42.4).
16. Make a lateral incision at the caudal end of the midline incision extending about half way up to the dorsal surface (Fig. 4.42.4). Make a similar lateral incision just caudal to the operculum.
17. Fold back the resulting flap of the body wall and cut away, exposing the body cavity (Fig. 4.42.5). The exposed liver should be dark red and the heart should still be beating.

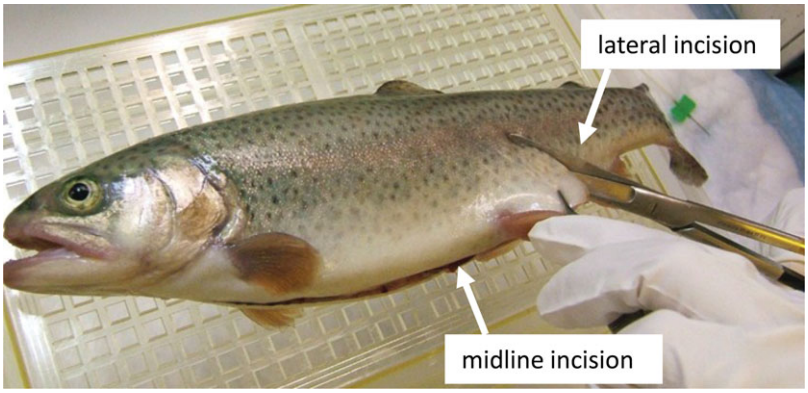

Figure 4.42.4 Photograph showing a midline incision from the vent to the isthmus, and a lateral incision extending dorsally from the vent.

Figure 4.42.5 Photograph showing the fish's liver, exposed by cutting away the body wall. The arrow indicates a ventral branch of the hepatic portal vein.

18. Locate a ventral branch of the hepatic portal vein, which runs from the intestine to the liver hilus, and carefully clear away any obscuring connective tissue (white arrow in Fig. 4.42.5).

19. Turn on the perfusate pump set to 10 ml/min.

    If perfusing two fish with one apparatus, the second perfusion line should be clamped initially. Both fish should be prepared to the point where they are ready for liver perfusion: anesthetized, weighed, body cavity exposed, and hepatic portal veins located.

20. With buffer I flowing, carefully insert a 21-G butterfly catheter into the portal vein in the direction of the liver, and secure in place with a micro-bulldog clamp (Fig. 4.42.6).

    Different gauge catheters may be preferred depending upon the size of the fish. If a micro-bulldog clamp is not available, sutures or pressure applied by fingers may be substituted (as described in Johanning et al., 2012).

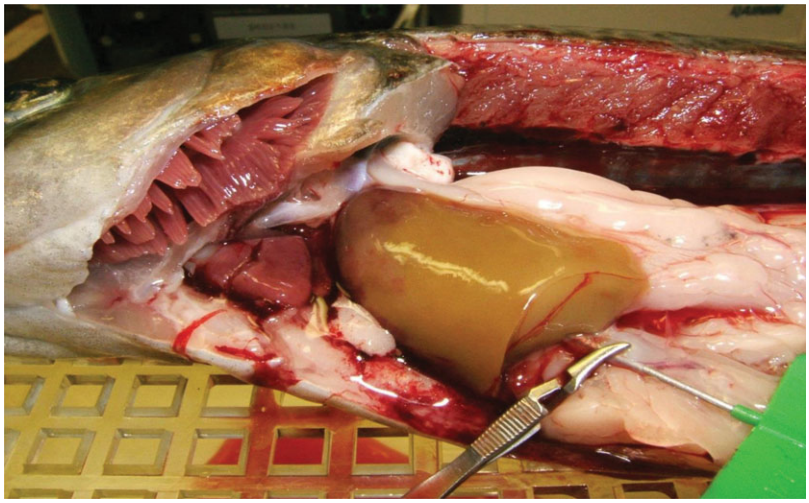

Figure 4.42.6 Photograph showing a cannulated liver. The liver will blanch immediately upon insertion of the catheter and perfusion with buffer I. Sever the vessels to the heart or the chambers of the heart to allow for perfusate efflux.

21. Sever the blood vessels leading from the anterior aspect of the liver to the heart. Alternatively, the heart may be severed or removed completely to allow efflux of perfusate (Fig. 4.42.6).

    If perfusing two fish from one apparatus, the second perfusion line should be unclamped just prior to cannulating the portal vein of the second fish, and the pump rate increased to provide the target perfusion flow rate (e.g., 10 ml/min) in both perfusion lines. Allow the liver of the first fish to perfuse with buffer I while the second fish is prepared (~1 min).

Perfuse liver

22. Perfuse the liver with buffer I for 8 to 12 min. Blanching of the liver should be evident within the first minute of perfusion (Fig. 4.42.6).

23. Switch to buffer II (collagenase-containing buffer) and perfuse for 12 to 15 min until the liver visibly softens.

    Switching perfusates may be accomplished by quickly transferring the draw tubing to the reservoir containing the next buffer, or by using a split line with a valve on the draw tubing to switch between buffers. The inclusion of a bubble trap in the perfusion apparatus will prevent any air introduced to the perfusion line during the buffer transition from reaching the liver. After ~5 min of perfusion with buffer II, the liver may be periodically gently prodded with blunt forceps to test for softening. Generally, perfusion with buffer II beyond 15 min will result in over-digestion and should be avoided.

24. Once the liver has sufficiently softened, switch to buffer III and continue perfusing for ~3 min. This buffer will terminate the collagenase digestion.

Isolate hepatocytes

25. Stop flow of buffer III, and remove the catheter. Using small, sharp scissors, excise the liver along with the intact gall bladder. Carefully cut away the gall bladder without rupturing and transfer liver to a weigh boat containing ~30 ml of ice-cold buffer III (Fig. 4.42.7).

    If the gall bladder ruptures during this process, rinse liver with buffer III to remove any bile prior to its transfer to the weigh boat containing buffer III.

Figure 4.42.7 Photograph showing removal of the liver.

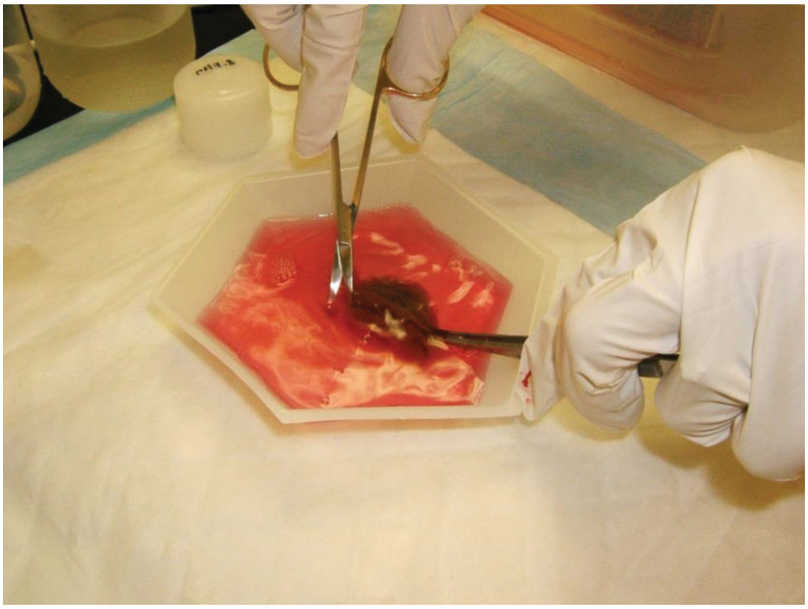

Figure 4.42.8 Photograph showing the mechanical collection of hepatocytes.

26. Using sharp forceps or the ends of small, sharp scissors, tear open the Glisson's capsule and gently shake the liver in buffer III to release the hepatocytes (Fig. 4.42.8).

    The liver may be gently raked with forceps or scissors to facilitate recovery of cells. The scraping and gentle shaking of the liver capsule may take several minutes to collect a sufficient number of cells.

27. Filter crude cell suspension through the 100-μm nylon mesh and collect hepatocytes into the beaker (Fig. 4.42.9). Gently push the remaining liver connective tissue against the mesh to increase cell recovery; however, excessive handling will produce poorer quality cells (e.g., blebbed).

    The mesh should be primed with a small amount of buffer III prior to pouring cells to minimize initial sheer stress. Alternatively, 100-μm nylon mesh tube inserts may be purchased for use with 50-ml conical tubes. These tube inserts should also be primed with a small amount of buffer III.

Figure 4.42.9 Photograph showing the crude cell suspension after filtration through a 100-μm nylon mesh.

28. Gently swirl the beaker to distribute the cells evenly, and transfer the filtered cells to 50-ml centrifuge tubes. Centrifuge crude suspension 3 min at 50 × *g*, 4°C, to sediment cells.

29. While the suspension is centrifuging, remove the gonads (ovaries or testes) in their entirety and weigh to the nearest 0.01 g. Determine the gonadosomatic index (GSI) of the donor animal as follows:

$$\text{GSI} = (100 \times \text{the gonad mass}) / \text{whole animal mass}$$

Record both the gonad mass and GSI (e.g., Fig. 4.42.3).

The gonads (testes or ovaries) appear as two strands of tissue that run along the length of the peritoneal cavity on the ventral side of the kidney. Sexual maturity in trout may be determined by the measured GSI. Generally, males with a GSI <0.05 and females with a GSI <0.5 may be considered sexually immature. Alternatively, the GSI may be determined histologically (Blazer, 2002). Detailed descriptions of gonadal development in trout may be found in the literature (Billard and Escaffre, 1975; Tyler et al., 1990; Gomez et al., 1999; Le Gac et al., 2001).

30. Aspirate the supernatant to the point where the centrifuge tube begins to taper (~4 ml mark), being careful not to disturb the cell pellet. Add 5 ml of buffer III and suspend cells by holding the centrifuge tube at an approximately 60° angle, and gently tapping the bottom of the centrifuge tube on the back of your opposite hand. After visually inspecting the tube for complete cell suspension (no visible clumps), bring the final volume up to 32 ml with buffer III.

The supernatant may be aspirated either manually by using a serological pipet, or by using a vacuum pump, but should not be poured.

31. From each 50-ml centrifuge tube, transfer 16 ml of cell suspension to a new 50-ml centrifuge tube (so that all tubes contain 16 ml of cell suspension). To each tube, add 14 ml of 90% Percoll solution and mix well by gentle inversion.

32. Centrifuge mixture 10 min at 96 × *g*, 4°C.

33. Immediately remove the supernatant by aspirating to just above the pellet, and suspend cells in ~20 ml of L-15 medium (pH 7.8 at 12°C). Combine two tubes of cells into one tube.
34. Centrifuge suspension 3 min at 50 × *g*, 4°C, to sediment cells and aspirate supernatant to just above the cell pellet.
35. Suspend cells in ~20 ml of L-15, and again combine two tubes of cells into one tube, if applicable.
36. Centrifuge cell suspension 3 min at 50 × *g*, 4°C, to sediment cells and aspirate the supernatant to just above the cell pellet.
37. Suspend cells in 20 to 40 ml of L-15 depending on how many cells are expected to be in the suspension.

    All suspensions should be combined into one tube at this point.
38. Determine cell yield and viability using a hemacytometer with trypan blue (see Support Protocol 1). Record cell counts and viability (e.g., Fig. 4.42.3)
39. Calculate the total yield from the isolation procedure as:

$$\text{Total yield of cells} = \text{viable cell concentration (cells/ml)} \times \text{suspension volume prior to cell counting (ml)}$$

    The total number of cells available (for assays or cryopreservation) is similarly calculated as viable cell concentration (cells/ml) × suspension volume post-cell counting (ml).

HEPATOCYTE CRYOPRESERVATION

BASIC PROTOCOL 2

This protocol is designed for 50 cryogenic vials containing 1.5 ml of 10×10^6 hepatocytes/ml each (15×10^6 hepatocytes per cryogenic vial). Fifty vials will require 750×10^6 hepatocytes, but the number of vials may be scaled up or down depending upon the number of cells available for cryopreservation.

Materials

Isolated primary trout hepatocytes (see Basic Protocol 1)
Cryopreservation buffer (see recipe)
Cryopreservation buffer with 12% DMSO (see recipe)
Cryopreservation buffer with 16% DMSO (see recipe)
Liquid nitrogen

50-ml centrifuge tubes
Refrigerated centrifuge
1.8-ml cryogenic vials
Cryogenic container

NOTE: Keep cells and media on ice throughout entire procedure unless specifically stated otherwise.

1. Adjust pH of all buffers at the fish maintenance temperature (e.g., 12°C) within 2 hr prior to use and maintain on ice or at 4°C.
2. Determine the suspension cell concentration and calculate the volume required for 375×10^6 cells (e.g., Fig. 4.42.3).

Cells should be concentrated such that the required volume is <50 ml (minimum cell concentration of 7.5 × 10^6 cells/ml).

Volume required (ml) = 375 × 10^6 cells/suspension concentration (cells/ml)

3. Transfer 375 × 10^6 cells into two clean 50-ml centrifuge tubes, and bring each tube to a final volume of 50 ml with cryopreservation buffer.
4. Centrifuge cells 5 min at 50 × *g*, 4°C, to sediment cells. For each tube, aspirate supernatant to just above pellet, and then add cryopreservation buffer up to 12.5 ml.

   Assuming no loss of cells in this step, the concentration will be 30 × 10^6 cells/ml.
5. Suspend cells by gentle inversion and tapping as described in Basic Protocol 1, step 30. Slowly add 6.25 ml of cryopreservation buffer containing 12% DMSO while gently swirling cells.
6. Maintain cells for 5 min on ice, then slowly add 18.75 ml of cryopreservation buffer containing 16% DMSO while gently swirling cells.

   The final volume is 37.5 ml at 10 × 10^6 cells/ml.
7. Maintain cells 5 min on ice, then suspend by gentle inversion. Transfer 1.5-ml aliquots of the bulk hepatocyte suspension into 1.8-ml cryogenic vials. To ensure proper cell concentration, gently mix or swirl the cells in the bulk suspension between each transfer.
8. Cryopreserve cells by placing vials into the vapor phase of liquid nitrogen (i.e., place vials into a cane or storage box within a cryogenic container).

   Cells may also be successfully cryopreserved using a controlled-rate freezer (see Alternate Protocol 1).
9. Store vials in the vapor phase of liquid nitrogen in a cryogenic container.

   Cryopreserved cells should be viable with minimal loss of enzymatic activity for up to 1 year and possibly longer.

BASIC PROTOCOL 3

THAWING CRYOPRESERVED TROUT HEPATOCYTES

Thawing cryopreserved hepatocytes results in a 25% to 45% yield (compared to the total number of cells frozen) with high viability (>85%) (Mingoia et al., 2010; Markell et al., 2014; Fay et al., 2014a,b).

Materials

Cryogenic vials containing 1.5 ml cryopreserved hepatocytes at 10 × 10^6 cells/ml (see Basic Protocol 2)
Recovery medium (see recipe)
Leibovitz-15 (L-15) with glutamine, without phenol red, pH 7.8, ice cold

50-ml centrifuge tubes
Room temperature water bath
Refrigerated centrifuge
Serological pipets

Set up for thawing

1. Determine total number of cryogenic vials needed to provide enough cells for the experiment.

   Users can expect 25% to 45% yield from trout hepatocyte suspensions cryopreserved using Basic Protocol 2. Thus, two vials containing 1.5 ml of 10 × 10^6 cells/ml suspension

each can be expected to provide ~7.5–13.5 × 10^6 hepatocytes. Substrate depletion experiments (see Basic Protocol 4) are typically conducted at 1–2 × 10^6 cells/ml. It is recommended that the user thaw two or three vials together for superior yield. Thawing one vial alone may result in <25% recovery from cryopreservation.

2. Prepare recovery medium, including any necessary pH adjustment and bring to room temperature.
3. Determine the number of 50-ml tubes required. One 50-ml tube is required for every two to three cryovials being thawed. Pipet 42 ml recovery medium into each 50-ml tube. Prepare one additional tube with recovery medium for cryogenic vial washes.
4. Set up an area specifically for thawing cryovials with a room temperature water bath, 50-ml centrifuge tubes containing room temperature recovery medium (caps loosened), and 1000-µl pipettor.

Perform thawing

5. Remove cryogenic vials containing frozen hepatocytes from the liquid nitrogen vapor. Immediately place vials in a room temperature water bath, holding them by their caps above the water level so that the frozen suspensions are below the water line. Gently move vials side to side until contents freely move and a small ice crystal remains (typically, the thawing process takes ~2 min, 15 sec).

   If the location of the cryopreserved cells and the laboratory used for thawing are different, it is recommended that the user transport the vials on dry ice.

6. Pour contents of two or three vials into one 50-ml centrifuge tube containing 42 ml of room temperature recovery medium.
7. Transfer 1 ml of the extra recovery medium into each emptied cryovial to suspend any remaining cells. Replace the caps and invert once to mix. Add contents from the rinse into the 50-ml tube containing the hepatocytes in recovery medium.

   The final volume expected is ~47 ml or 49.5 ml, depending on whether the contents of two or three cryogenic vials were combined into one 50-ml centrifuge tube.

8. Cap and gently invert the 50-ml centrifuge tube(s). Centrifuge 5 min at 50 × *g*, 4°C.
9. Aspirate supernatant to the point where the centrifuge tube begins to taper (~4 ml), being careful not to disturb the cell pellet.

   Aspirating close to the pellet may decrease yield. To obtain consistent results, aspirate the supernatant to the conical portion of the tube for all wash steps. The supernatant can be aspirated manually by using a pipet, or by using a vacuum pump. Do not discard the supernatant by pouring.

10. Add ~5 ml of L-15 medium (pH 7.8 ±0.1, 4°C or ice cold) to the centrifuge tube and suspend cell pellet (see Basic Protocol 1, step 30). Combine suspensions from two tubes into one tube, if applicable, and bring all tubes to a final volume of 45 ml with L-15. Invert tubes once and centrifuge 5 min at 50 × *g*, 4°C.
11. Repeat steps 9 and 10. Again, combine cells into one 50-ml centrifuge tube, if applicable, and add L-15 to a final volume of 45 ml.
12. Invert tube once and centrifuge 3 min at 50 × *g*, 4°C.
13. Aspirate supernatant to just below (~2 mm) the conical portion of centrifuge tube, add ~0.75 ml L-15 per cryogenic vial thawed, and suspend pellet (see Basic Protocol 1, step 30). Using a serological pipet, measure the volume of suspension.

14. Count cells using a hemacytometer and dilute to the desired cell concentration (see Support Protocol 1).

The volume of the cell suspension prior to cell counting (step 13), along with the cell count results, may be used to determine the % cell recovery from cryopreservation.

Number of recovered (live) hepatocytes = average live cell concentration × suspension volume prior to cell counting

% cell recovery = (100 × number of recovered hepatocytes)/ number of cells initially cryopreserved

15. Keep cell suspension on ice until use.

BASIC PROTOCOL 4

IN VITRO DETERMINATION OF METABOLIC STABILITY AND EXTRAPOLATION TO IN VIVO INTRINSIC CLEARANCE

Metabolic stability experiments are conducted using a substrate depletion approach. The biotransformation rate is determined by measuring the disappearance of parent chemical from the reaction vessel over time. The incubation system consists of hepatocytes suspended in L-15 medium, typically at a concentration of 1–2 $\times$ 10^6 cells/ml. Introduction of the test chemical to the system initiates the reaction. A minimum of six sample time points is recommended to provide a high-quality regression of log-transformed chemical concentration data. Reactions are terminated by transferring an aliquot of the suspension to a larger volume (four times or greater) of stopping solution (typically a solvent). Trout hepatocytes will generally maintain enzymatic activity for several hours, but metabolic stability experiments exceeding 4 hr are not recommended due to a decline in the performance of the system. The reaction temperature should be at the acclimation temperature of the source fish (e.g., 12°C). Biotransformation rates are temperature sensitive, and the assay temperature should be strictly controlled using a water bath, incubator, or thermomixer.

Preliminary incubations should be performed to determine the appropriate cell concentration, test chemical concentration, and sampling time points. Incubations using heat-denatured cells may serve as a negative control to distinguish between enzymatic metabolism and other potential loss processes, such as adsorption to the reaction vessel, volatilization, and abiotic degradation. It is recommended to evaluate a preparation of cells by using a reference chemical with well-documented in vitro intrinsic clearance rates. Details pertaining to appropriate reaction conditions, and the use of reference chemicals and negative controls are provided in the Commentary.

Materials

Test chemical
Reference chemical
Stopping solution (organic solvent or acid) containing internal standard, if appropriate
Leibovitz-15 (L-15) with glutamine, without phenol red, pH 7.8, ice cold

Sample incubation equipment:
- Shaking water bath with chiller
- Shaking incubator with heating and cooling functions
- Thermomixer block with shaking capabilities

7-ml glass vials with caps (e.g., scintillation)
Vial rack
1.5-ml microcentrifuge tubes
Timer

Refrigerated microcentrifuge
Multi-tube vortex mixer
HPLC/GC sample vials

Prepare reagents

1. Prepare stock solution(s) of the test chemical (and reference chemical).

   The solvent used to dissolve the test compound depends on its nature and aqueous solubility. If possible, water or buffer should be used. Other typical solvents include acetone, dimethyl sulfoxide, and acetonitrile. If an organic solvent is used, the total amount in the incubation should be ≤1% to avoid suppressing enzyme activity. The stability of the test compound in stock solution should be evaluated in advance of the experiment.

2. Prepare the stopping solution if an internal standard is used.

   In cases where the test chemical is detected by gas chromatograph/mass spectrometry, the addition of an internal standard (typically an isotope of the test chemical) can reduce variability in the final dataset due to volatilization of the stopping solvent, injection volume discrepancy, or changes in instrument sensitivity during a run. Researchers also may choose to include an internal standard for samples analyzed by other means (e.g., HPLC).

3. Adjust pH of L-15 medium (e.g., 7.8 at 12°C). Keep on ice or at 4°C until use.

Prepare vials and workstation

4. Turn on the shaking water bath or chilled incubator and adjust the temperature to the reaction conditions (e.g., 12°C).

5. Set up reaction vials (clear or amber 7-ml scintillation vials) in an appropriate rack and place on ice or at 4°C. The reactions will be performed by spiking a single suspension with test chemical and removing subsamples at each time point to determine the rate of loss. For a tractable chemical (i.e., non-volatile, does not bind to vessel walls, and distributes rapidly through the reaction system), this design generally produces the least variable results and is simplest to perform. For an alternative study design using independently spiked vials for each time point, see Alternate Protocol 2.

   Figure 4.42.10 shows a possible study design to test a tractable chemical at one test concentration. The use of a positive control or reference chemical is optional, but offers several benefits (see Commentary) and is included in this example.

6. Pre-fill 1.5-ml microcentrifuge tubes for each time point with stopping solution. Cap tubes until use.

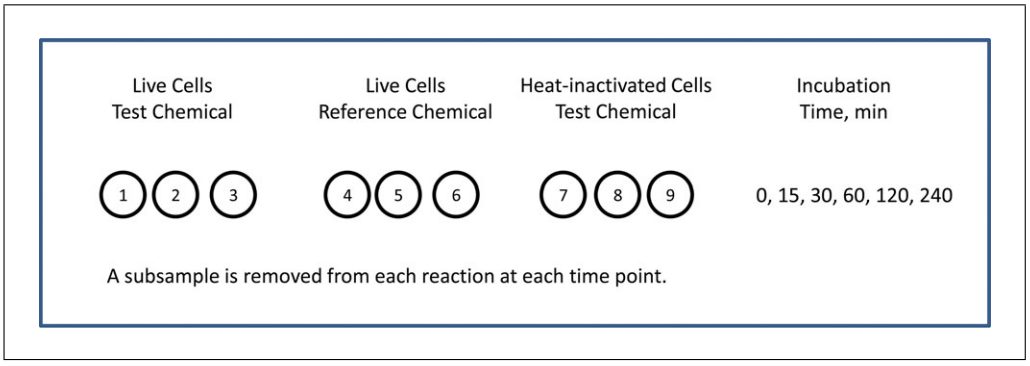

Figure 4.42.10 Example study design.

The samples removed at each time point are transferred to 1.5-ml microcentrifuge tubes containing the stopping solution (e.g., organic solvent, which may include an internal standard). Therefore, in the example study design given in Figure 4.42.10, 54 microcentrifuge tubes containing stopping solution are needed for the nine different reaction vials and six time points. The volume of stopping solution will depend on the volume of the aliquot removed and the detection capability of the analytical method. The ratio of sample to stopping solvent should be determined in preliminary experiments. Typical ratios of sample-to-organic stopping solvent range from 1:4 to 1:9 (e.g., 100 µl sample terminated in 400 to 900 µl solvent). Samples stopped using solvents such as acetonitrile and methanol should be at 4°C or kept on ice to facilitate precipitation. In some cases, depending on the test chemical and analytical method, acid may be used to stop the reaction in place of organic solvent.

7. Calculate the volume of cell suspension necessary for the number of active and heat-denatured samples that will comprise an experiment. An additional 25% is recommended to provide a modest excess of biological material.

   The volume of cell suspension required for each reaction vial depends on the number of desired time points as well as the volume to be removed for each time point. For example, the experiment shown in Figure 4.42.10 contains six live samples and three heat-inactivated controls for a total of nine reactions. If 1.0 ml per vial will be used in the experiment, a total of ~7.5 ml should be prepared for the live suspension and 3.8 ml for the heat-inactivated suspension (6.0 ml × 1.25 = 7.5 ml live suspension, 3.0 × 1.25 = 3.75 ml heat-inactivated suspension). Excess heat-inactivated biological material may be used to generate matrix spike samples. Excess live biological material may be used to prepare heat-inactivated control material for subsequent assays (see Support Protocol 2). Either may be used for matrix blanks.

8. Determine the cell concentration in the suspension and dilute cell suspension to the desired cell concentration in L-15 medium (see Support Protocol 1). To obtain highly accurate cell concentrations, additional cell counts may be performed on the cell suspension once it has been diluted to its reaction concentration.

   Metabolic stability assays are typically conducted using 1–2 × 10^6 cells/ml. Three additional cell counts (using separate dilutions with trypan blue) on the reaction suspension are recommended. This second set of counts may be performed during or after the incubations as time allows. If the three cell counts vary by >20% CV, an additional cell count may be needed to obtain an accurate estimation of the live cell concentration.

9. Dispense the desired volume of cell suspension to each reaction vial and loosely cap the vials.

   To ensure good mixing at the final time point, there should be at least enough cell suspension remaining after the final sample is removed to cover the bottom of the vial.

10. Pre-incubate the reaction vials at the assay temperature (e.g., 12°C) for 15 min with gentle shaking.

11. Spike the test chemical (or reference chemical) directly into the suspension of each reaction vial to initiate the reaction. Swirl cells to distribute the chemical. Loosely cap the reaction vials after dosing and between time points as time allows.

    The dosing of reaction vials and subsequent sampling at pre-selected time points may be time-staggered to facilitate precise sampling. An example is provided in Support Protocol 3. Use of a laboratory timer is important to facilitate this process as it is necessary to account for the time lapse that exists between samples during the dosing process. Accurate timing will decrease variability. Once familiar with the assay, researchers should be able to stagger the dosing/sampling every 20 sec. For novice users, 1-min intervals are recommended. In the time between dosing, the user must aspirate chemical stock solution, dispense into the reaction vial, swirl the vial to distribute the spike solution, recap, and discard pipet tip. For chemicals dissolved in organic solvents such as acetone, priming the pipet tip several times may also be required.

12. To sample the reaction vial at a specified time point, remove it from the water bath or incubator, gently swirl or shake to form a homogenous suspension, remove an aliquot with a pipet, and dispense directly into the organic solvent. To ensure quantitative transfer of the sample, pipet up and down in the solvent three times.

13. Place quenched samples on ice, if appropriate, until all incubation samples (all time points) have been collected.

    If the solvent used as the stopping solution is miscible with water (e.g., methanol or acetonitrile), samples should be kept cool (2° to 10°C) at all times. It may be useful to refrigerate such samples overnight to facilitate complete protein precipitation. Chemical extractions with immiscible solvents may not require this treatment. Preliminary experiments should be performed to confirm complete precipitation of proteins upon termination of the reaction.

14. After the experiment is complete, vortex microcentrifuge tubes containing the incubation samples (hepatocyte suspension plus stopping solution) on a vortex mixer.

15. Centrifuge samples 15 min at 20,000 × *g*, 4°C.

16. Transfer the supernatant or the organic phase, as appropriate, to analytical HPLC/GC sample vials.

17. Store below −10°C until analysis.

18. Analyze samples using a validated analytical method.

Determine in vitro intrinsic clearance

19. Plot $\log_{10}$-transformed substrate concentrations against time; a first-order elimination rate constant, k_e (1/hr), is determined as:

$$k_e\,(1/\text{hr}) = -2.3 \times \text{slope of the log-linear decline.}$$

20. Divide k_e by the measured live-cell concentration to obtain the in vitro intrinsic clearance ($CL_{\text{int, in vitro}}$; ml/hr/$10^6$ cells).

    If cell counts were performed pre- and post-dilution of cell suspension, use the post-dilution counts to determine the in vitro intrinsic clearance.

Extrapolate to in vivo intrinsic clearance

21. Multiply $CL_{\text{int, in vitro}}$ by a hepatocellularity value of 510 × 10^6 hepatocytes/g liver to obtain an in vivo intrinsic clearance value ($CL_{\text{int, in vivo}}$; ml/hr/g liver).

    Hepatocellularity is the number of hepatocytes present in a given mass of liver. The value referenced above has been determined as an appropriate hepatocellularity for sexually immature rainbow trout (Fay et al., 2014a). This value is likely to change as trout become sexually mature, and is also likely to vary by species. Extrapolation of in vitro intrinsic clearance obtained using hepatocytes from a source other than sexually immature trout requires confirmation of an appropriate scaling factor. Hepatocellularity of liver tissue is typically determined from ratios of liver/cell protein, DNA or CYP content (Carlile et al., 1997).

CONTROLLED RATE HEPATOCYTE CRYOPRESERVATION

ALTERNATE PROTOCOL 1

Trout primary hepatocytes have been successfully cryopreserved using the method described in Basic Protocol 2, as well as the following step-wise freezing protocol (Mingoia et al., 2010).

Materials

Cryogenic vials containing 1.5 ml of trout hepatocyte suspension (10×10^6 cells/ml; see Basic Protocol 2)
Liquid nitrogen
Cryogenic controlled-rate freezer, 4°C

1. Place cryogenic vials containing 1.5 ml of trout hepatocyte suspension (10×10^6 cells/ml; see Basic Protocol 2, step 7) into a cryogenic controlled-rate freezer set to 4°C. Place the temperature probe into one cryogenic vial.
2. Start the controlled rate freezing protocol as follows:
   a. Wait 30 min at 4°C
   b. Decrease 1°C/min until sample reaches −4°C
   c. Decrease 25°C/min until chamber reaches −40°C
   d. Increase 10°C/min until chamber reaches −12°C
   e. Decrease 1°C/min until chamber reaches −40°C
   f. Decrease 10°C/min until chamber reaches −140°C
3. Remove vials and place in the vapor phase of liquid nitrogen for long-term storage.

   Cryopreserved cells should be viable with minimal loss of enzymatic activity for up to 1 year and possibly longer.

ALTERNATE PROTOCOL 2

IN VITRO DETERMINATION OF METABOLIC STABILITY USING INDIVIDUAL VIALS FOR EACH TIME POINT

For chemicals that exhibit non-standard behavior when using the single-vial approach, an alternative approach using individual vials for each time point may improve results. These non-standard behaviors include apparent loss of chemical from both active and denatured samples due to volatilization and/or adsorption to the sides of the reaction vessel (see Commentary). Problems with chemical adsorption are minimized by adding the stopping solvent directly to each sample vial. Incubations with volatile test substances can be performed by sealing the vials with a septum-lined cap after the pre-incubation period. A syringe is then used to introduce both the test chemical and stopping solution.

Materials

Cell suspension
Test chemical
Stopping solution

Shaking water bath or chilled incubator
1.5-ml or larger glass reaction vials (e.g., gas chromatography amber glass DP T/S septa vials; National Scientific, cat. no. C400-2W)
Vial holder (e.g., VWR, cat. no. 89022-294)
Refrigerated centrifuge
HPLC/GC sample vials

1. Turn on the shaking water bath or chilled incubator and adjust the temperature to the reaction conditions (e.g., 12°C).
2. Set up one reaction vial for each time point, with appropriate replication, and place on ice or at 4°C.

   Note that the plastic microcentrifuge tubes containing stopping solution are eliminated in this protocol. Figure 4.42.11 shows an example study design with six time points, and three replicate assays each for live cells spiked with the test compound, live cells spiked with a reference chemical, and heat-inactivated cells spiked with the test chemical.

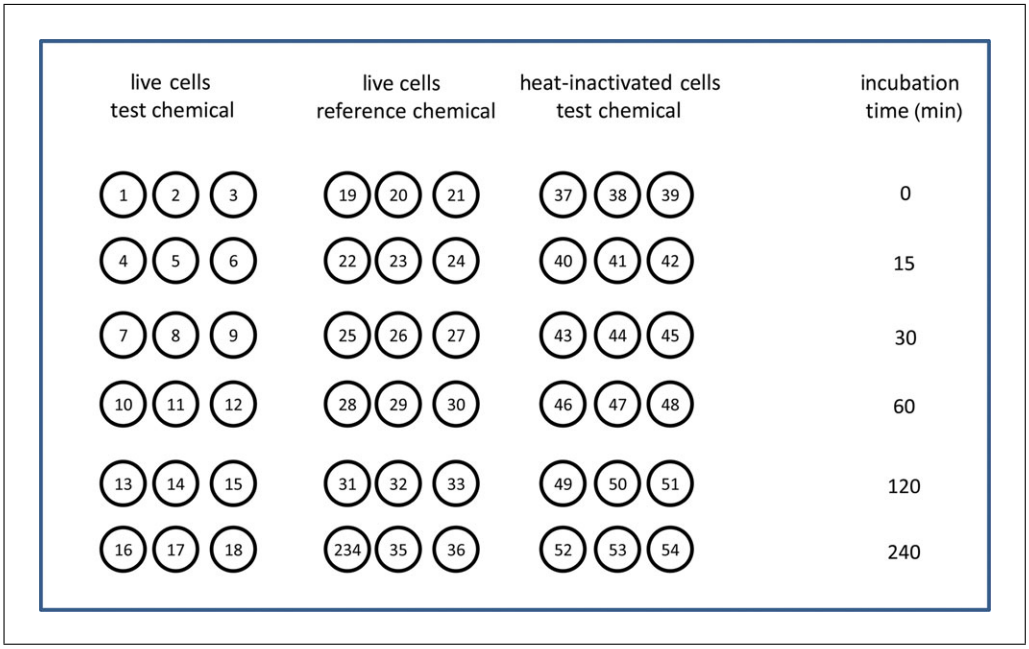

Figure 4.42.11 Example alternative study design using separate vials for each time point.

3. Dispense the desired volume of cell suspension to each reaction vial and cap.

   In this protocol, the volume of the cell suspension in each vial is reduced compared to the single-vial approach because no sub-samples are removed, and the volume of the suspension plus added organic solvent cannot exceed the total capacity of the vial. Generally, it is not recommended to add <250 µl of suspension per vial, in part, because the spike volume is likewise reduced. If an organic solvent is used to introduce the test chemical, the maximum spike volume is 1% of the suspension volume. Spiking small volumes of concentrated chemical requires careful pipetting, especially for organic solvents that may drip easily from the pipet tip.

4. Spike each vial independently, using time-staggering (e.g., spike each sample 30 sec apart).

5. To terminate the reaction, remove the vial from the water bath or incubator, and slowly add the entire volume of stopping solution. Swirl or vortex sample and place on ice, if appropriate, until the experiment is complete.

   Account for any time-staggering during the sample spiking when terminating reactions.

6. Centrifuge samples 15 min at 20,000 × *g*, 4°C.

   Samples may be transferred to a centrifuge tube if the reaction vials are not designed for centrifugation.

7. Transfer supernatant or organic phase, as appropriate, to analytical HPLC/GC sample vials.

8. Store below −10°C until analysis.

9. Analyze samples using a validated analytical method.

CELL STAINING AND COUNTING

One reliable and inexpensive method of determining the concentration of cells in a suspension is to use a hemacytometer. Incorporation of trypan blue allows for visualization of viable (unstained) and non-viable cells (blue). Several protocols are in use for staining

and counting cells using a hemacytometer, which are generally adapted from Freshney (1993).

Materials

Cell suspension
Leibovitz-15 (L-15)
0.04% trypan blue solution (available commercially in 0.81% NaCl and 0.06% K_2HPO_4)

1-ml microcentrifuge tubes
Hemacytometer (improved Neubauer) and coverslips
Microscope

1. Prepare a dilution of the cell suspension by transferring an aliquot to a 1-ml microcentrifuge tube containing L-15 and trypan blue. Gently invert the microcentrifuge tube to mix the cells with the dye.

   Uniformly suspend cells within the bulk suspension before removing an aliquot for dilution. The final concentration of trypan blue in the dilution should be 0.04%. For example, to make a five-fold dilution of a cell suspension, combine 100 μl of the cell suspension with 350 μl L-15 and 50 μl of 0.04% trypan blue solution. The dilution step should result in 50 to 150 total cells present in a given counting quadrant (200 to 600 total cells/side of a hemacytometer).

2. Carefully suspend cells in the microcentrifuge tube containing trypan blue, quickly transfer 10 μl of the suspension into one of the V-shaped wells of the hemacytometer with overlaying coverslip, and gently expel the sample. The area under the coverslip will fill by capillary action. Load each side of the hemacytometer.

3. Place the loaded hemacytometer onto the microscope stage and bring the counting grid on one side into focus at low power (20× magnification).

4. Count the unstained (live) cells in the four corners of the grid (Fig. 4.42.12, quadrants A, B, C, and D). When counting cells within a quadrant, start at the top left-hand corner and follow the direction shown by the arrows in the insert of Figure 4.42.12. Count all cells wholly contained within the limit lines of the quadrant (a triple line), and count cells that are touching a top or right limit line. On the top and right limit lines that are counted, the cell must be primarily contained within the quadrant or touching the center line. Do not count cells that are touching the bottom or left limit lines, unless they are wholly contained within the quadrant, but touching the inner-most limit line.

5. Record the total number of unstained (live) cells from all four corners (e.g., example worksheet in Fig. 4.42.3).

6. From the same four corners, count the stained (dead) cells and add this number to the live cell count to determine the total number of cells (stained + unstained). Record the total number of cells (e.g., example worksheet in Fig. 4.42.3).

7. Count both sides of the hemacytometer.

8. Determine percent (%) viability for each side and record.

$$\%\ \text{viable} = (\text{total live cells}/\text{total cell count}) \times 100$$

9. Determine the viable cell concentration for each side and record.

$$\text{Viable cell concentration (cells/ml)} = (\text{no. of live cells}/\text{number of fields counted}) \times \text{dilution} \times 10{,}000$$

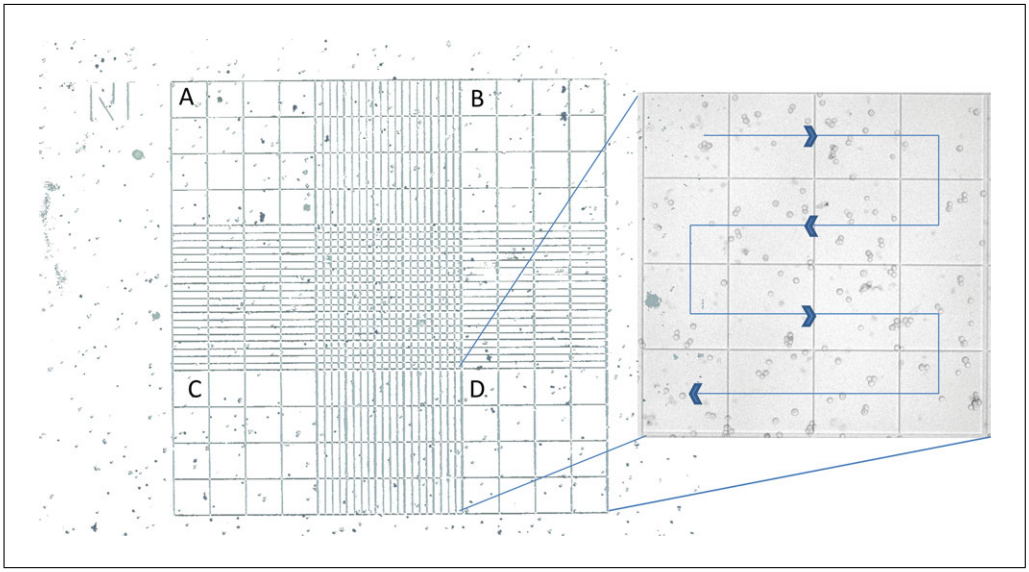

Figure 4.42.12 Image of cells loaded into a chamber of a hemacytometer (Neubauer improved). Cells contained within the four quadrants (A, B, C, and D) are counted. Within each quadrant (e.g., insert) the count should start at the top left-hand corner and follow the direction shown by the arrows. Count all cells wholly contained within the limit lines of the quadrant (a triple line), and count cells that are touching a top or right limit line. On the top and right limit lines that are counted, the cell must be touching the center line. Do not count cells that are touching the bottom or left limit lines, unless they are wholly contained within the quadrant, but touching the inner-most limit line.

The total number of live cells counted in the four corners from one side of the hemacytometer would be divided by four (the number of fields counted).

10. Perform three cell counts using both sides of the hemacytometer (six counts total). Prepare a new trypan blue dilution from the cell suspension just prior to each count so that the cells do not remain in tyrpan blue more than 2 min prior to counting. Average the percent viability and viable cell concentration from all counts and record (e.g., example worksheet in Fig. 4.42.3).

11. Once the viable cell concentration is determined, cells may be diluted to a desired cell concentration (e.g., 2×10^6 cells/ml). To determine the volume needed for a desired cell concentration, carefully measure the volume of the remaining suspension used for cell counting, multiply by the calculated average cell concentration and divide by the desired cell concentration. The amount of L-15 medium to add to the suspension is the difference between the current volume and the desired volume. For example:

    Volume of suspension = 3.3 ml
    Concentration determined from cell counting = 2.8×10^6 cells/ml
    (2.8×10^6 cells/ml × 3.3 ml)/ 2.0×10^6 cells/ml = 4.6 ml
    4.6 ml – 3.3 ml = 1.3 ml
    Add 1.3 ml of L-15 to the suspension.

HEAT-INACTIVATING CELLS FOR USE AS A NEGATIVE CONTROL

This process can take up to 1 hr; therefore, it is suggested that laboratories consider preparing a large volume of heat-inactivated cells at least 1 day prior to an experiment, and freeze them in 5-ml aliquots. Heating to a boil in a microwave is not recommended. Cells remaining unused at the end of an experiment may become material for subsequent heat-inactivation.

Materials

Cell suspension
Leibovitz-15 (L-15)

Heat-safe vessel (glass)
Hotplate
Beaker
Graduated cylinder

1. Dilute cell suspension in L-15 medium to the desired concentration for experimental use (e.g., 2×10^6 cells/ml).
2. Note the volume of the suspension and transfer to a heat-safe vessel (preferably glass).
3. Heat a beaker of water on a hotplate and bring water to boiling. Place vessel with cell suspension in the boiling water bath, and bring the suspension to a slow boil for 15 min.
4. After the suspension has cooled, transfer the suspension to a graduated cylinder and adjust the volume by adding L-15 medium to maintain the desired cell concentration.

SUPPORT PROTOCOL 3

TIME-STAGGERING

The time-staggering example in Table 4.42.1 applies to the first study design (see Basic Protocol 3; Fig. 4.42.10). Similar staggering may be used for alternative designs. In this example, each of the reaction vials is spiked with either the test chemical or reference chemical 30-sec apart. For example, the first live hepatocyte suspension (HEP 1) is spiked with test chemical at time 0′0″. The second and third live suspension (HEP 2, HEP 3) are spiked at times 0′30″ and 1′0″, respectively. Each subsequent live suspension (HEP 4, 5, and 6) is spiked with reference chemical every 30 sec, followed by the spiking of heat-inactivated hepatocyte suspensions with the test chemical. The reaction vials are then sampled at each time point 30 sec after HEP 1 to maintain a consistent elapsed time.

Table 4.42.1 Time-Staggering Example for Basic Protocol 3

Sample ID	Spiking = start clock time (min′ sec″)	Time point = 5 min Clock time (min′ sec″)	Time point = 20 min Clock time (min′ sec″)
HEP 1	0′	5′	20′
HEP 2	0′30″	5′30″	20′30″
HEP 3	1′	6′	21′
HEP 4	1′30″	6′30″	21′30″
HEP 5	2′	7′	22′
HEP 6	2′30″	7′30″	22′30″
HIHEP 1	3′	8″	23′
HIHEP 2	3′30″	8′30″	23′30″
HIHEP 3	4′	9′	24′

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Buffers I, II, and III

Combine reagents, as appropriate, listed in Table 4.42.2. The amounts provided are sufficient to perfuse three to four fish. These amounts may be scaled up or down, as needed. Buffers I and II should be made fresh the day of isolation. Buffer III may be made in advance and stored up to 1 week at 4°C. Adjust the pH of buffer III 1 to 2 hr prior to cell isolation to 7.8 at 12°C using 1.0 N NaOH or 1.0 N HCl.

Cryopreservation buffer

Cryopreservation buffer may be prepared 1 day before use (store at 4°C), but adjust pH 1 to 2 hr prior to cell isolation. Combine reagents provided in Table 4.42.3. The pH of the buffer may need to be adjusted to fully dissolve the albumin. Filter sterilize through a 0.2-μm PES filter. If needed, allow solution to sit overnight at 4°C to reduce foam. On the day of use, adjust buffer pH to 7.8 at 12°C using 1.0 N NaOH or 1.0 N HCl.

Cryopreservation buffer with 12% DMSO

Combine 1.8 ml of dimethyl sulfoxide (DMSO) for every 13.2 ml cryopreservation buffer (see recipe).

Cryopreservation buffer with 16% DMSO

Combine 7.2 ml of dimethyl sulfoxide (DMSO) for every 37.8 ml cryopreservation buffer (see recipe).

Table 4.42.2 Recipes for Perfusion Buffers I, II, and III

Buffer	Reagent	Per 600 ml preparation	Concentration
I	1× HBSS (no Ca^{2+}/Mg^{2+} salts)	600 ml	
pH 7.8	EDTA	510 mg	2.3 mM
	$NaHCO_3$	212 mg	4.2 mM
II	1× HBSS (with Ca^{2+}/Mg^{2+} salts)	600 ml	
pH 7.8	Collagenase, type IV	150 mg[a]	0.25 mg/ml[a]
	$NaHCO_3$	212 mg	4.2 mM
III	DMEM	600 ml	
pH 7.8	BSA	6.0 g	1% (w/v)

[a]Buffer II: Collagenase activity varies from lot to lot, and is not a pure preparation of enzyme, but contains other proteases, polysaccharidases, and lipases. It may be necessary to adjust the amount used depending on how well the liver digests.

Table 4.42.3 Cryopreservation Buffer (pH 7.8 at 12°C) Recipe

Reagent	Per 200 ml preparation	Concentration
DMEM	160 ml	
FBS	40 ml	20% (v/v)
BSA	0.5 g	0.25% (w/v)

Table 4.42.4 Recovery Medium Recipe

Reagent	Per 100 ml preparation	Concentration
DMEM	90 ml	
FBS	10 ml	10% (v/v)
BSA	0.25 g	0.25% (w/v)

Percoll solution, 90%

Using a biosafety hood and sterile technique, add 90 ml of chilled Percoll to a graduated cylinder. The temperature of the Percoll should match the experimental conditions (i.e., temperature at which the fish are acclimated). Adjust volume up to 100 ml with 10× DPBS solution. Mix well and titrate to pH 7.8 by slowly adding 1 N HCl. If the pH drops <7.8, do not add NaOH (NaOH forms a precipitate and turns the solution cloudy). Instead, add additional Percoll/DPBS to adjust the pH up. Store up to 14 days at 4°C.

Recovery medium

Combine reagents provided in Table 4.42.4. The pH of the medium may need adjustment to fully dissolve the albumin. Filter sterilize through a 0.2-μm PES filter. If needed, allow solution to sit overnight at 4°C to reduce foam. On the day of use, adjust the buffer pH to ~7.8 at 12°C using 1.0 N NaOH or 1.0 N HCl. Use within 1 week of preparation. Store at 4°C.

Tricaine methanesulfonate (MS-222), 150 mg/liter

Prepare the MS-222 anesthetic solution by filling a 10-liter plastic bucket or 10-liter glass fish tank with ~8 liters of water drawn from the same source used to maintain the fish. Add 1.2 g MS-222 (0.15 g/liter) to the water and mix until dissolved. Then add a predetermined amount of $NaHCO_3$ to maintain the source water pH. If the water is low-alkalinity, the required mass of sodium bicarbonate is approximately three times that of the MS-222. Make up fresh prior to use. Do not store.

COMMENTARY

Background Information

Primary hepatocytes obtained from trout, like those from mammals, largely maintain their epithelial phenotype, including functional glucose and lipid metabolism (Polakof et al., 2011), and the activities of phase I and II biotransformation enzymes (Segner and Cravedi, 2001). These cells possess functional membrane transporters, and have been studied using well-known transporter substrates and inhibitors (Sturm et al., 2001; Bains and Kennedy, 2005; Zaja et al., 2008; Hildebrand et al., 2009). Additional studies using primary trout hepatocytes have examined the effects of carcinogens (Bailey et al., 1987), hormones (Bailey et al., 1987; Sathiyaa et al., 2001), naturally derived toxins (Boaru et al., 2006), pharmaceuticals (Ellesat et al., 2010; Ings et al., 2012), nanoparticles (Massarsky et al., 2014), and a variety of other xenobiotic compounds or mixtures (Gagné et al., 2013; Lacaze et al., 2014; Sovadinová et al., 2014). Because they produce vitellogenin in response to estrogen receptor agonists, trout hepatocytes are being studied for use in an in vitro endocrine disruptor screening assay (Navas and Segner, 2000; Navas and Segner, 2006; Markell et al., 2014; Hultman et al., 2015). These cells retain their xenobiotic metabolizing capabilities and sensitivity to estrogen receptor agonists after they have been cryopreserved (Mingoia et al., 2010; Markell et al., 2014; Fay et al., 2014a), suggesting that they could be incorporated into testing protocols and promoted for widespread use.

One potential application of primary trout hepatocytes within a standardized testing protocol involves improving predictions of chemical bioaccumulation. The extent to which chemicals bioaccumulate in fish and other aquatic biota is one of three properties commonly used to perform hazard assessments for environmental contaminants (the other two are persistence and toxicity). Biotransformation

may substantially reduce the extent of chemical bioaccumulation in fish, particularly for lipophilic substances. However, the rate of this activity cannot be predicted from a simple physicochemical characteristic, such as the octanol-water partition coefficient (K_{OW}). As such, the influence of biotransformation represents the greatest source of uncertainty in many bioaccumulation assessments.

Nearly a decade ago, Nichols et al. (2006) suggested that in vitro intrinsic clearance rates, obtained using a substrate depletion approach, could be extrapolated to the whole animal and incorporated into existing mass-balance models for fish. Since then, several investigators have used in vitro systems derived from fish liver tissue to predict biotransformation impacts on the accumulation of selected test compounds (Han et al., 2007; Cowan-Ellsberry et al., 2008; Dyer et al., 2008; Mingoia et al., 2010; Laue et al., 2014). These studies have shown that incorporating measured rates of in vitro activity into the models substantially improves their performance; thus, predicted levels of accumulation are much closer to measured values than are model predictions obtained assuming no metabolism.

Standardized methods for assessing the metabolic stability of xenobiotic chemicals using trout liver S9 fractions were given previously by Johanning et al. (2012). Much of the commentary provided in Johanning et al. (2012) is applicable to the current protocol, including guidance for selecting test animals and ensuring first-order reaction conditions. Detailed information regarding sample extraction and processing are also provided by Johanning et al. (2012). In this commentary, focus is on hepatocyte-specific concerns, as well as considerations not previously covered.

Critical Parameters and Troubleshooting

Characterization of primary hepatocytes

Each cell lot should be evaluated for the ability to catalyze phase I and II biotransformation reactions. Because the hepatocytes retain their activity upon cryopreservation, these characterization assays may be performed on freshly isolated or thawed cells. Standardized assays for measuring CYP1A activity, glutathione S-transferase activity, and UDP-glucuronosyltransferase activity were given by Johanning et al. (2012). When applied to hepatocytes, these assays are generally performed on the lysate obtained from a sonicated suspension of cells. The results are then normalized to protein content, determined using the method described by Lowry et al. (1951), or similar. If the likely pathway for biotransformation of a particular test substance is known, it may be advisable to evaluate this pathway in advance, assuming that a standardized assay for this activity is available. In addition to enzymatic activity, freshly isolated hepatocytes should be characterized for percent (%) viability, as determined by trypan blue exclusion. Hepatocytes thawed from cryopreservation should be characterized for both % viability and % recovery (% viable cells obtained post-thaw compared to the number cryopreserved initially).

Incorporation of a reference chemical into substrate depletion assays

The user should consider incorporating a reference chemical to be run alongside each test compound when performing a substrate depletion assay. The reference chemical serves as an additional characterization assay, and may be used to identify inactive or sub-active lots of biological material. Because experiments with the reference chemical and the test chemical are conducted on aliquots of the same hepatocyte preparation, and are sampled under the same reaction conditions, the resulting data may alert the user to an experimental error, such as a change in assay temperature or inaccurate cell count. As experience with a given reference compound accumulates, it may be possible to use the data to improve comparisons within and among laboratories. For example, variability among cell lots could be accounted for by normalizing the depletion rate for a test substance to that of a well-understood reference. In principal, the same approach could be used to account for user-to-user differences in experimental technique. The full utility of this chemical "benchmarking" approach remains to be determined; nevertheless, it is clear that incorporating a reference compound into the experimental design substantially increases confidence in the experimental outcome.

The selection of a reference compound depends to some extent on the test compound that is being run. For example, it may be useful to incorporate a reference substance that is transformed by the same metabolic pathway(s). If the depletion rate for the reference chemical is similar to that of the test compound, the sampling schemes may be harmonized. Logistically, this synchronization may benefit the user. Thus, preliminary studies performed with the test substance may be useful for selecting

an appropriate reference substance (i.e., one metabolized at a similar rate). The reference compound should be well-documented with respect to its performance in the trout hepatocyte assay. Ideally, this performance data would be available from one's own laboratory. Depletion rates reported in the literature, while useful for guiding the selection of a reference compound, may differ among laboratories due to differences in biological material, starting substrate concentration, and experimental conditions. Additional considerations for selecting a reference compound include: commercial availability, cost, volatility, relative hydrophobicity, stability (especially in stock solution), and ease of analysis.

Substrate depletion negative controls

The inclusion of heat-denatured biological material as a negative control was addressed by Johanning et al. (2012). This material should be obtained by boiling cells in a water bath, as described in Support Protocol 2. Earlier efforts to denature cells by microwaving yielded unsatisfactory results.

Rate Determination Challenges

A decrease in biotransformation rate over time, as demonstrated by a reduction in the slope of the $\log_{10}$-transformed substrate depletion curve, may be caused by degradation of the biological material or depletion of cofactors. In this situation, it is appropriate to determine the intrinsic clearance rate from data collected during optimal system performance, since this is most likely to represent the in vivo level of activity. The authors' experience with the trout hepatocyte assay, as currently developed, suggests that its maximum working lifetime is ~4 hr.

An effect observed with some test substances assayed using the one-reaction vial method (see Basic Protocol 3) is an apparent increase in test chemical concentration at early time points (Fay et al., 2014b). This pattern may reflect slow dissolution and/or distribution of the test chemical. Alternate Protocol 2 may aid in correcting this behavior, or it may be possible to adjust the system composition to encourage more rapid distribution. Otherwise, the exclusion of early time points, or an adjustment of the sampling scheme so that the first time point occurs after the system has equilibrated, may resolve the issue. Further investigation is needed to identify chemicals likely to behave in this manner, and how best to approach their use in this type of assay.

Possible Improvement of the Cryopreservation Method

The protocols described in this unit derive from those used in drug metabolism studies with mammals (Berry et al., 1997; Mudra and Parkinson, 2001). While these procedures are amenable for use with trout, some methods might be further improved upon. One example is the protocol for cryopreservation, which employs a programmable cryofreezer (see Alternate Protocol 1). Currently, this protocol results in a cell recoveries of <50%. Similar values are obtained using Basic Protocol 3, which simply freezes the cells in the vapor phase of liquid nitrogen (Fay et al., 2014a). It is probable that modifications to the step-wise freezing protocol could result in improved cell recovery. Changes in the composition of the cryopreservation medium may also yield an increase in recovery, and would potentially improve both freezing methods. Additional research is needed to explore these questions.

Anticipated Results

Using Basic Protocol 1 for the isolation of hepatocytes from trout, researchers can expect to obtain $\sim 100 \pm 50 \times 10^6$ cells/g liver. The yield may vary substantially among individual fish perfused, ostensibly, for the same amount of time with equivalent buffers. This may, in part, be due to differences in hepatic vasculature. The viability of freshly isolated primary trout hepatocytes should be 90% or better after Percoll purification. If the viability is <80%, researchers may attempt to re-purify the cells. Hepatocytes thawed after cryopreservation should also exhibit relatively high viability (≥85%). Generally, using thawed cells in metabolic stability assays if their viability is below 80% is not recommended, as the unbound chemical fraction in the reaction system may be impacted by the presence of nonviable cells or cell debris. Furthermore, the release of lysosomes and autolytic enzymes from compromised cells can impact the performance of the viable cells. Researchers can expect hepatocyte recovery from cryopreservation to be >25%. However, in the authors' experience, cells obtained from preparations with very low yields (~10%) have comparable enzymatic performance to cells obtained from higher yield preparations.

Time Considerations

New users may require several months to become proficient in performing all of the protocols outlined in this unit. With experience, the liver perfusion and hepatocyte isolation

procedure (see Basic Protocol 1), including cell purification and counting, requires ~2 hr to complete. This estimate does not include time required for solution preparation, laboratory setup, or apparatus cleaning. Cryopreservation of the hepatocyte suspension (see Basic Protocol 2; not including buffer pH adjustment) will require an additional 30 to 45 min. Thawing and preparation of cells (see Basic Protocol 3) will require ~1 hr. Substrate depletion incubations (see Basic Protocol 4) may range from 5 min to 4 hr.

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Disclaimer

This document has been subjected to review by the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. ecommendation for use.

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