Receiving Cryopreserved Human Hepatocytes

TECHNICAL BULLETIN

Purpose

The purpose of this bulletin is to provide instructions and recommendations for the proper receiving and transfer of cryopreserved human hepatocytes to minimize risk of product degradation.

Precautions

Observe universal precautions when handling human-derived tissues and cells as they are potentially biohazardous. Wear appropriate personal protective gear and follow institutional guidelines for the collection and disposal of all solid and liquid waste that has been in contact with these products.

Materials

- Appropriate personal protective equipment
- Foam or other laboratory ice tray capable of containing a small amount of liquid nitrogen (and a cryovial box)
  if liquid nitrogen is not available, dry ice can be used if you proceed with the process very quickly
- Tongs or forceps
- Cryogenic storage freezer (capable of temperatures ≤-150°C); if using liquid nitrogen storage device, store vials without submerging in liquid nitrogen)
- Cryovial storage box

Receiving Cryopreserved Human Hepatocytes

1. Transport the shipping container containing the liquid nitrogen shipping dewar next to the cryogenic storage freezer.
2. Unseal the shipping box.
3. Don appropriate PPE for liquid nitrogen and cryovial handling (i.e. face shield, insulated gloves, labcoat).
4. Fill the foam/ice tray with a small amount of liquid nitrogen (enough so that the cryovial storage box will be submerged by only a few millimeters).
   - If liquid nitrogen is not available, fill the container with a layer of dry ice instead, but proceed with the remaining steps more quickly.
5. Open the flaps of the shipping box and remove the cap/plug of the inner dewar. Note: you may notice a small amount of vapor rising from the dewar.
6. Open your cryogenic storage freezer and remove the appropriate rack and box for storing your vials. Place this box in the liquid nitrogen in the foam/ice tray.
   If you are receiving 30+ vials per lot, proceed to step 12; if you are receiving less than 30 vials per lot, proceed to step 7.
7. Quickly, lift the paper shipping bag using the handles provided.
8. Unseal the paper bag and remove the small labeled cloth bag.
9. Immediately open the bag and use the tongs to transfer the vials to your cryo box in the liquid nitrogen.
10. Quickly replace the box into the appropriate rack in your cryogenic storage freezer.
11. Repeat steps 6-10 for any additional lots.
12. When receiving larger numbers of vials they may be shipped in boxes. Either place this box quickly into an empty slot in your cryogenic freezer rack, or place the shipped box into the liquid nitrogen next to your current box and use the tongs to transfer the vials from the shipping box to your storage box.
13. Repeat steps 6-12 for any additional lots.
14. When all vials have been placed into your cryogenic storage freezer, follow the instructions provided with the liquid nitrogen vapor shipping dewar for return shipment.

Thawing Cryopreserved Human Hepatocytes

TECHNICAL BULLETIN

Purpose

The purpose of this bulletin is to provide instructions and recommendations for the proper handling of cryopreserved human hepatocytes to maintain optimal integrity and performance of the cells for standard cell culture applications.

Precautions

Observe universal precautions when handling human-derived tissues and cells as they are potentially biohazardous. Wear appropriate personal protective gear and follow institutional guidelines for the collection and disposal of all solid and liquid waste that has been in contact with these products.

Materials

- Appropriate personal protective equipment
- 70% alcohol (ethanol or isopropyl alcohol)
- 70% alcohol wipes (or lab wipes soaked in 70% alcohol)
- Small laboratory ice tray
- Portable dewar or other container to transport frozen vials in liquid nitrogen
- Tongs or forceps
- Ice
- Liquid nitrogen
- 37°C water bath (operating range 35-38°C)
- Centrifuge with 50 mL tube carriers
- Timer
- 50 mL bottle
- 1000 µL pipette
- 1000 µL pipette tips
- Serological pipettor (i.e. Pipet-Aid)
- 1, 2, 5, 10, 25 mL serological pipettes
- Plastic or glass aspiration tips with vacuum aspiration system (optional)
- 0.4% Trypan blue solution and hemocytometer (or equivalent cell staining and counting device)
- LifeNet Health Human Hepatocyte Plating Medium (HHPM)
- LifeNet Health Human Hepatocyte Thawing Medium (HHTM) ***At least 1 50 mL conical tube of Human Hepatocyte Thawing Medium (HHTM) is needed per lot of cryopreserved human hepatocytes. Up to five (5) vials of cryopreserved human hepatocytes can be placed in one 50 mL conical tube with HHTM (Do NOT overload HHTM tube with excess cells).*

All applicable steps should be performed using sterile technique

Table 1. Supplementing Human Hepatocyte Plating Medium

	Component A	Component B	Component C	Component D	Component E
250 mL	12.5 mL	2.5 mL	2.5 mL	25 µL	250 µL
100 mL	5 mL	1.0 mL	1.0 mL	10 µL	100 µL
50 mL	2.5 mL	0.5 mL	0.5 mL	5 µL	50 µL

Preparation of LifeNet Health Human Hepatocyte Thawing and Plating Media (HHTM and HHPM)

1. Remove HHTM bottle(s) from -20°C storage and thaw away from light (this can be done overnight at 4°C or immediately prior to use). *Note: One bottle can be used for up to 4-5 vials of cryopreserved human hepatocytes.*
2. Remove HHPM supplement pack from -20°C and thaw components A-D (this can be done overnight at 4°C or immediately prior to preparing the medium).
3. Remove HHPM base medium from 4°C and add components A-E (see Table 1 for amounts). *Optional: Filter through 0.2 m filter.*
4. Aliquot fully supplemented HHPM (approximately 20 mL per vial) into a sterile bottle.
5. Warm both HHTM and HHPM to 37°C in a water bath (typically 20-30 minutes).
    - Keep away from light and do NOT warm for excessive periods of time.

Thawing Cryopreserved Human Hepatocytes

1. Refer to the COA for average yield per vial as well as any special centrifugation instructions and note these prior to moving on to the following steps.
2. Don appropriate PPE for liquid nitrogen and human hepatocyte handling.
3. Fill a portable dewar or other liquid nitrogen container with a small amount of liquid nitrogen (enough to submerge at least half of the vial).
   Note: A precharged portable dewar drained of liquid nitrogen (or similar container) is the easiest and best method for transferring the vial(s)
4. Set up biological safety cabinet (BSC)
    - 50 mL conical tube
    - 1000 µL pipette with 1000 µL tips
    - Small laboratory ice tray containing ice
    - Aspiration tips
    - Serological pipettor with various serological pipettes
5. Remove 50 mL conical tube of HHTM from water bath, spray with 70% ethanol, and place in biological safety cabinet.
    - Invert tube to mix thoroughly.
    - Pour approximately 30 mL of HHTM into a 50 mL conical tube.

*****The following steps should be completed as quickly as possible to maintain hepatocyte viability and functionality*****

6. QUICKLY remove vial(s) with cryopreserved human hepatocytes from cryogenic storage freezer and place in the portable dewar or other liquid nitrogen container.
7. Carefully transfer cryopreserved cells in liquid nitrogen container to culture area.
8. QUICKLY remove vial(s) from liquid nitrogen (using tongs or forceps) and slightly loosen cap(s) to release pressure. Re-tighten cap(s).
   Do NOT remove cap(s) or expose the contents to air

9. QUICKLY submerge vial(s) vertically into 37°C water bath, until approximately 0.5 cm below cap, in water bath to thaw (see Figure 1).
    - Transferring vials from the liquid nitrogen, loosening caps, and submerging into water should take less than 10 seconds.
    - Thaw for 60-120 seconds – until a thin spindle of frozen cells remains in the center of the vial(s) (see Figure 2).
10. QUICKLY wipe vial(s) with a 70% ethanol wipe and place in ice tray and move tray to biological safety cabinet.
    If thawing multiple vials, remove individual vials from the water bath and place on ice when properly thawed regardless of the readiness of the remaining vial(s)
11. Moving at a quick pace and working with one vial at a time, remove vial cap and pour hepatocytes into 50 mL conical tube of HHTM.
    - Using 1000 µL pipette, rinse vial with 1000 µL of the HHTM and then transfer back into the 50 mL tube of HHTM. Repeat this step for all other vials (working quickly).
12. Once contents from all vials have been placed in the 50 mL conical tube of HHTM, QC to 50 mL with remaining fresh HHTM. Tighten cap on tube and gently invert tube three times to uniformly suspend hepatocytes.
13. Centrifuge hepatocytes at 100 x g for 8 minutes at room temperature.
    Note: Cells from individuals with a high BMI may have slightly different centrifugation conditions. Make sure you have examined the COA for the lot for any special instructions prior to centrifugation in order to maximize the yield for your lot.
14. Return to BSC and gently vacuum aspirate the supernatant without disturbing the pellet in the bottom of the tube.
    Note: you can also gently and very carefully pour the supernatant into a waste container without disturbing or shaking the pellet.
15. Add 3-5 mL of HHPM (volume depends on size of pellet/average yield) and gently rock the conical tube to resuspend hepatocytes (do NOT resuspend cells using a pipette or by vortexing).
    Note: Typical concentration for cell counting should be 1-2 x 106 cells/mL to allow for accurate trypan or automated counter analysis. Average yield per vial is stated on the COA for each lot.
16. Count hepatocyte suspension to determine yield and viability.
    - Use trypan blue exclusion test and/or AO/PI staining on a hemocytometer or automated cell counter (i.e. HepatoMeter, Cellometer, etc.).
17. Proceed to plating cells or using them in suspension (see Technical Bulletins X and X).
    Note: Automated cell counters are not ideal for hepatocyte counting unless a hepatocyte-specific program is used. Accurate concentration of your suspension is critical to proper plating. Use manual counting if a hepatocyte-specific program is not available.

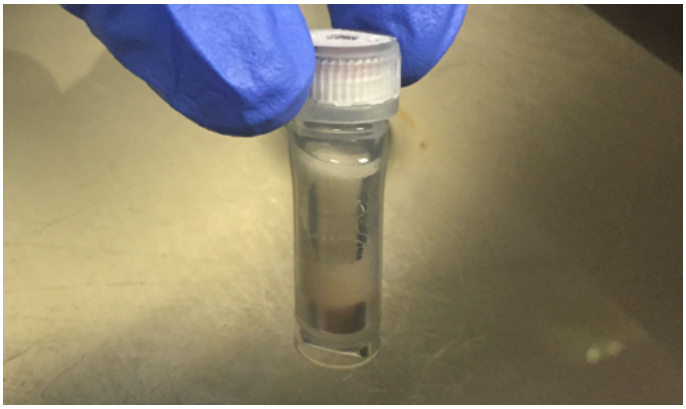

Figure 1. Correct placement of vial in water bath for thawing.
Note that the vial is submerged to the top of the frozen cell suspension, however the cap is above the water line (arrow).

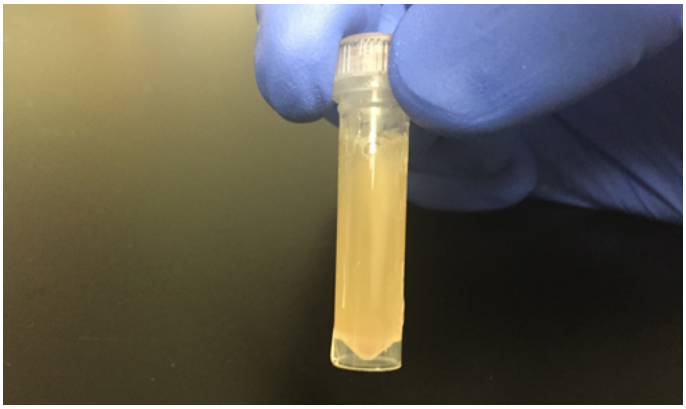

Figure 2. Remove vial from water bath when all ice is thawed except a thin spindle of frozen cells in the center of the vial (arrow). Clean cap and neck of vial with 70% ethanol wipe, and place on ice.

Plating Cryopreserved Human Hepatocytes

TECHNICAL BULLETIN

Purpose

The purpose of this bulletin is to provide instructions and recommendations for the proper handling and plating of cryopreserved human hepatocytes to obtain optimal monolayer formation and performance for standard cell culture applications.

Precautions

Observe universal precautions when handling human-derived tissues and cells as they are potentially biohazardous. Wear appropriate personal protective gear and follow institutional guidelines for the collection and disposal of all solid and liquid waste that has been in contact with these products.

Materials

- Appropriate personal protective equipment
- 37°C water bath (operating range 35-38°C)
- Timer
- 1000 µL multichannel electronic pipette
- 1000 µL multichannel electronic pipette tips
- Serological pipettor (i.e. Pipet-Aid)
- 1, 2, 5, 10, 25 mL serological pipettes
- Plastic or glass aspiration tips and vacuum aspiration system (optional)
- 50 or 100 mL reagent reservoir
- Culture vessels (multiwell plates, dishes)
- Disposable plastic or washable glass media bottles (100-250 mL)
- LifeNet Health Human Hepatocyte Plating Medium (HHPM)
- LifeNet Health Human Hepatocyte Culture Medium (HHCM)

All applicable steps should be performed using sterile technique

Table 2. Supplementing Human Hepatocyte Culture Medium (HHCM)

	Component A	Component B	Component C	Component D
500 mL	5 mL	5 mL	5 mL	5 µL
250 mL	2.5 mL	2.5 mL	2.5 mL	2.5 µL
150 mL	1.5 mL	1.5 mL	1.5 mL	1.5 µL
100 mL	1 mL	1 mL	1 mL	1 µL
50 mL	0.5 mL	0.5 mL	0.5 mL	0.5 µL
25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 µL

Preparation of Human Hepatocyte Plating and Culture Media

Ensure non-expired complete Human Hepatocyte Plating Medium (HHPM) is available (approximately 15 mL per plate). This may be performed immediately prior to thawing hepatocytes (see Technical Bulletin Thawing Cryopreserved Human Hepatocytes).

1. Remove HHCM supplement pack from -20°C freezer and thaw components A-D (this can be done overnight at 4°C or at 37°C immediately prior to preparing the medium).
2. Remove HHCM base medium from 4°C refrigerator and add components A-D (see Table 2 for amounts). Mix well after adding components by gently inverting container several times. *Optional: Filter through 0.2 µm filter.*
   Note: Fully supplemented HHCM should be prepared fresh daily for best results. If not prepared daily, use within 72 hours.
3. Aliquot sufficient volume of fully supplemented HHCM for a single day's use (approximately 15 mL per standard multiwell plate) into a sterile bottle or conical tube.
4. Warm both HHPM and HHCM to 37°C in a water bath (typically 20-30 minutes).
   - Keep away from light and do NOT warm for excessive periods of time.

Table 3. Seeding Densities for Cryopreserved Human Hepatocytes

Culture Vessel	Seeding Density (cells x106/mL)	Volume per Well
6 well plate	0.9-1.1	2 mL
12 well plate	0.8-1.0	1 mL
24 well plate	0.7-1.0	500 µL
48 well plate	0.6-0.8	250 µL
96 well plate	0.9-1.1	65 µL medium cell suspension and 65 µL *Note: add medium to wells prior to adding cell suspension*

Plating of Thawed Cryopreserved Human Hepatocytes

1. Place HHPM in 37°C water bath prior to thawing cryopreserved human hepatocyte vials until temperature is 37°C (typically 20-30 minutes).
2. After thawing cryopreserved human hepatocytes, refer to the COA for each lot of LifeNet Health cryopreserved human hepatocytes for recommended seeding density for standard collagen-coated 24-well plates.
   ***This density will be unique to each lot of cryopreserved human hepatocytes. A range of densities is provided in Table 3, but the final density for a specific lot is provided in the CoA. If you are not using 24-well plates, you may determine correct cell density per lot by testing different densities (refer to Table 3 for ranges) or contact LifeNet Health for technical assistance.*
3. After determining final cell yield, add HHPM to cell stock until desired final cell density is reached (first transferring cell stock from the 50 mL conical tube to an appropriate sterile bottle if final volume is over 50 mL).
4. After ensuring the suspension is homogenously mixed (by gentle rocking or inverting), pour cell suspension into the sterile reagent reservoir until half full.
5. Using an electronic multichannel pipette transfer cell suspension from reagent reservoir to culture vessels.
   ***Hepatocytes settle out of suspension fairly quickly. Gently rock the reservoir in all directions to ensure cell suspension is homogenous when refilling pipette; likewise mix cell suspension in the conical tube or bottle prior when re-filling the reservoir.*
6. Remove culture vessels from the biological safety cabinet in stacks of 1-5 and place in an incubator (37°C, 5% CO2).
   - Shake/rock culture plates in a N-S ↕ , E-W ↔ manner three times on the incubator shelf. Repeat this motion at 15 minute intervals for the first 60 minutes of culture.
     ***Note: this shaking should be gentle yet vigorous enough to displace cells grouped in the center of each well.*

Table 4. Human Hepatocyte Culture Medium Well Volumes

Culture Vessel	Volume per Well
6 well plate	1.5 mL
12 well plate	800 µL
24 well plate	300 µL
48 well plate	200 µL
96 well plate	75 µL

7. Warm Human Hepatocyte Culture Medium (HHCM) to 37°C in an appropriate water bath at approximately 3.5 - 5.5 hours post plating. [Note: aliquot and warm only the amount of medium you will need to add to the number of plates seeded, see Table 4, and do not warm or leave in water bath for longer than necessary.]
8. At 4-6 hours post plating, gently shake plates and remove HHPM by gentle vacuum aspiration and replace with fully supplemented HHCM.
    - Remove plates from the incubator and place in the biological safety cabinet (no more than 5 culture plates at one time).
    - Gently shake plates in N-S ↕ , E-W ↔ manner to dislodge dead cells.
    - Tilt plates and gently vacuum aspirate the medium from the side of each well without touching the cell monolayer.
    - Using a serological, micro-, or electronic multichannel pipette add HHCM in volumes shown in Table 4.
9. Return plates to incubator for overnight or until experimental protocol dictates.
    - If warranted, overlay cells with extracellular matrix the next morning.
    - Replace medium with fresh warm HHCM daily.

In Situ Metabolism for Cryopreserved Human Hepatocytes in Suspension

BIO-IMPLANT BRIEF

Purpose

The purpose of this bulletin is to provide instructions and recommendations for the proper handling and use of cryopreserved human hepatocytes in suspension culture for in vitro CYP activity profiling. Note: Portions of this protocol must be carried out in advance of thawing cells.

Precautions

Observe universal precautions when handling human-derived tissues and cells as they are potentially biohazardous. Wear appropriate personal protective gear and follow institutional guidelines for the collection and disposal of all solid and liquid waste that has been in contact with these products.

Materials

- Appropriate personal protective equipment
- 37°C water bath (operating range 35-38°C)
- CO_2 incubator set for 36.5-37.5°C and 5% CO_2
- Orbital shaker capable of speeds of 90-200 rpm and able to be placed in a CO_2 incubator
- Vortex shaker (for tubes or deepwell sample blocks)
- Centrifuge capable of 1000 x g
- Timer
- Insulated rectangular ice tray
- Ice
- Multiple pipettes (i.e. 200, 1000 µL)
- Multiple pipette tips
- Multichannel pipette with tips (optional)
- Serological pipettor (i.e. Pipet-Aid)
- 1, 2, 5, 10, 25 mL serological pipettes
- Plastic or glass aspiration tips and vacuum aspiration system (optional)
- 50 or 100 mL reagent reservoir
- 24-well non-collagen coated plates
- Sample tubes and/or deepwell sample blocks
- Disposable plastic or washable glass media bottles (100-250 mL)
- 0.2 µm PES filter unit (optional)

LifeNet Health Human Hepatocyte Culture Medium (HHCM)

All applicable steps should be performed using sterile technique

Preparation of Human Hepatocyte Culture Medium (HHCM)

Note: Resuspend cells post thaw in culture medium (HHCM) instead of plating medium (HHPM) if using cells for suspension assay.

1. Remove HHCM supplement pack from -20°C freezer and thaw components A-D (this can be done overnight at 4°C or at 37°C immediately prior to preparing the medium).
2. Remove HHCM base medium from 4°C refrigerator and add components A-D (see Table 1 for amounts). Mix well after adding components by gently inverting container several times. Optional: Filter using a 0.2 µm PES filter unit.

Note: Fully supplemented HHCM should be prepared fresh daily for best results. If not prepared daily, store medium at 4°C protected from light and use within 72 hours.

3. Aliquot sufficient volume of fully supplemented HHCM for a single day's use (approximately 12-36 mL per standard multiwell plate) into a sterile bottle or conical tube.
4. Warm HHCM to 37°C in a water bath (typically 20-30 minutes).
   - Keep away from light and do NOT warm for excessive periods of time.

Table 1. Supplementing Human Hepatocyte Culture Medium (HHCM)

	Component A	Component B	Component C	Component D
500 mL	5 mL	5 mL	5 mL	5 µL
250 mL	2.5 mL	2.5 mL	2.5 mL	2.5 µL
150 mL	1.5 mL	1.5 mL	1.5 mL	1.5 µL
100 mL	1 mL	1 mL	1 mL	1 µL
50 mL	0.5 mL	0.5 mL	0.5 mL	0.5 µL
25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 µL

Preparation of Suspension Culture Substrates

1. Prepare all substrate stock solutions to be 1000× of the required working substrate solution (see Table 2 for standard CYP probe substrates).
2. Calculate the minimum volume of substrate solution required by multiplying the number of wells by the volume of substrate solution added per well. Prepare working solutions using the following equation:
   - Working solution (µM) x Volume substrate solution needed (mL) = Volume stock needed
   - Add 10% for additional volume for pipette error.
3. Prepare 2× substrate solutions and add time-matched substrate solutions into adjacent wells of appropriate-sized multiwell plate(s) according to your assay plate map (this will be termed your "substrate plate" in subsequent steps).
4. Set up CO_2 incubator at 36.5-37.5°C. Place the orbital shaker and substrate plate in the incubator and set the shaker to 150 rpm.

Suspension Culture Substrate Incubations

1. Calculate the number of cells required for each study using the following equation as a guide:
   - [Number of substrates (not including 7-HC or 7-EC) x (cells/mL x mL/well) x number replicates] + [number of 7-HC and/or 7-EC substrates x (cells/mL x mL/well) x number replicates] = number of cells needed
   - i.e. for a test of phenacetin, testosterone, and 7-HC:
     * [2 x (2 x 106 cells/mL x 0.25 mL) x 3] + [1 x (2 x 106 cells/mL x 0.5 mL) x 3] = 6 x 106 cells required

Note: A total of ~12 x 106 cells are required for each multiwell plate; therefore the number of vials of cryopreserved hepatocytes required will vary from batch to batch. Refer to the CoA for the average yield per vial for your particular batch prior to starting experiments.

2. Thaw cryopreserved hepatocytes as indicated in Thawing Protocol section of Technical Bulletin (making sure to resuspend cells post centrifugation in culture medium NOT plating medium).
3. Dilute cells to 2 x 106 cells/mL using culture medium.
4. Add 250 µL (most substrates) or 500 µL (7-Hydroxycoumarin and 7-Ethoxycoumarin) cell suspension into respective wells of 24-well plate and place plate onto orbital shaker in incubator for 10 minutes.
5. Remove cell and substrate plates from the incubator and add the 2× substrate solutions into the appropriate wells of the cell plate to start reactions.

Note: Time-matched substrate solutions can be added using a multichannel pipettor.

6. Replace cell plate with substrates onto the orbital shaker (150 rpm) inside the incubator at 37°C and 5% CO_2 and allow plate to incubate for the appropriate times required per substrate (see Table 2).
7. At the end of the incubation period, terminate reactions by adding equal volume of ice-cold acetonitrile (ACN) containing the internal standard.
8. Transfer sample:ACN samples from cell culture plate to tubes or deepwell sample block and vortex for 30 seconds at high speed
9. Centrifuge samples at 1000 x g for 10 minutes at 4°C.
10. Transfer supernatants (typically ≥150 µL) to new tubes or deepwell sample block and process immediately or freeze at -80°C until analysis by LC/MS/MS.

Table 2. Prototypical Suspension Culture Substrate Probes and Incubation Times

CYP Activity	Substrate	Final Concentration (1X, in µM)	Incubation Time (minutes)	Marker Metabolite
CYP1A2	Phenacetin	100	15	APAP
CYP2A6	Coumarin	5	15	7-Hydroxycoumarin
CYP2B6	Bupropion	500	15	Hydroxybupropion
CYP3A4 #1	Testosterone	200	15	6β-Hydroxytestosterone
CYP3A4 #2	Midazolam	10	10	1-Hydroxymidazolam
CYP2C8	Paclitaxel	20	30	6α-Hydroxypaclitaxel
CYP2C9	Tolbutamide	25	15	4-Hydroxytolbutamide
CYP2C19	(S)-Mephenytoin	250	30	4-Hydroxymephenytoin
CYP2D6	Dextromethorphan	15	15	Dextrorphan
CYP2E1	Chlorzoxazone	250	15	6-Hydroxychlorzoxazone
Phase 1 (ECOD)	7-Ethoxycoumarin	100	30	7-Hydroxycoumarin (7-HC)
Phase II (7-HCG)	7-Hydroxycoumarin	100	30	7-Hydroxycoumarin Glucuronide
Phase II (7-HCS)	7-Hydroxycoumarin	100	30	7-Hydrozycoumarin Sulfate

For questions relating to these instructions, email **cells_tissues@lifenethealth.org**.

LifeNet Health helps to save lives, restore health and give hope to thousands of patients each year. We are the world's most trusted provider of transplant solutions, from organ procurement to new innovations in bio-implant technologies and cellular therapies—a leader in the field of regenerative medicine, while always honoring the donors and healthcare professionals that allow the healing process.

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