Human Hepatocyte Cell Culture Protocol

It is important to read and understand the following instructions prior to use. Improper handling may adversely affect cell quality and performance.

This protocol is specific to the use of normal healthy human hepatocytes. If you are using hepatocytes from donors with a high BMI (BMI ≥ 30) or NAFLD/NASH, please contact cells_tissues@lifenethealth.org for necessary protocol adjustments.

Medium: For optimal cell performance, LifeNet Health's Human Hepatocyte Thawing (HHTM), Plating (HHPM), and Culture (HHCM) Media are recommended. LifeNet Health's HHTM is formulated to maximize the yield and viability of hepatocytes following cryopreservation. HHPM is optimized for attachment of hepatocytes to cultureware. HHCM is serum-free and maintains healthy hepatocytes during cultivation.

I. Receiving Cryopreserved Human Hepatocytes

1. Transport the shipping container containing the cryopreserved hepatocyte vial(s) next to the cryogenic storage freezer.
2. Don appropriate Personal Protective Equipment (PPE) for liquid nitrogen (LN_2) and cryovial handling (Refer to OSHA Standard 29CFR.1910.132).
3. Fill insulated laboratory ice tray with sufficient amount of LN_2 to submerge only a few millimeters of a cryovial storage box.
4. Unseal the shipping container by opening the flaps and removing the cap/plug of the inner dewar. **NOTE:** You may notice a small amount of vapor rising from the dewar.
5. Open the cryogenic storage freezer and remove the appropriate rack and box for storing the received vials. Place the storage box in the LN_2-containing tray. If the vials were shipped in a cryovial storage bag, proceed to Step 6. If the vials were shipped in a cryovial storage box, proceed to Step 9.
6. Quickly lift the shipping bag from the shipping dewar using the handles provided.
7. Immediately open the bag and use forceps or tongs to transfer the vials to the cryovial storage box in LN_2.
8. Quickly replace the storage box into the rack of the cryogenic storage freezer.
9. When receiving larger numbers of vials, they may be shipped in cryovial storage boxes. Either place this box quickly into an empty slot in the cryogenic freezer rack, or place the shipped box into the LN_2 next to the cryovial storage box to be used and use forceps or tongs to transfer the vials from the shipping box to the storage box.
10. Repeat Steps 6-8 or Step 9, as applicable, until all vials have been transferred to the cryogenic storage freezer.
11. Follow the instructions provided with the shipping dewar for return shipment.

II. Preparation of LifeNet Health Cell Media

1. Remove HHTM bottle(s) from -20°C storage and thaw immediately prior to use in a 37°C water bath, protected from bright light, for approximately 20-30 minutes.

NOTES:

- Do NOT warm for an excessive period of time.
- One HHTM bottle can be used for up to three vials of cryopreserved human hepatocytes (maximum of 40-50 x 10^6 cells).

2. Remove HHPM and HHCM supplement vials from -20°C storage and thaw immediately prior to use in a 37°C water bath.
3. Remove HHPM and HHCM base media from 4°C storage.
4. Working in a biological safety cabinet (BSC), add 15 mL of thawed HHPM supplement to the HHPM base medium. Mix well by gently inverting the container several times. **NOTE:** If desired, an antibiotic can be added to the HHPM. Penicillin/Streptomycin, at a final concentration of 50 units/mL and 50 µg/mL, respectively, is recommended. Optional: Filter complete medium through a 0.2 µm filter.
5. Add 5 mL of thawed HHCM supplement to the HHCM base medium. Mix well by gently inverting the container several times. **NOTE:** If desired, an antibiotic can be added to the HHCM. Penicillin/Streptomycin, at a final concentration of 50 units/mL and 50 µg/mL, respectively, is recommended. Optional: Filter complete medium through a 0.2 µm filter.
6. Aliquot a minimum of 20 mL of fully supplemented HHPM into a sterile 50 mL conical centrifuge tube. Place in a 37°C water bath, protected from bright light, for approximately 20-30 minutes. **NOTE:** Do NOT warm for an excessive period of time.
7. Aliquot the needed amount of fully supplemented HHCM into a sterile conical centrifuge tube or bottle, depending on volume. Keep the aliquot at room temperature, protected from bright light, until ready to use. Place remaining HHCM in 4°C storage.

NOTE: Fully supplemented HHPM and HHCM should be prepared fresh daily for best results. If not prepared daily, use within 72 hours.

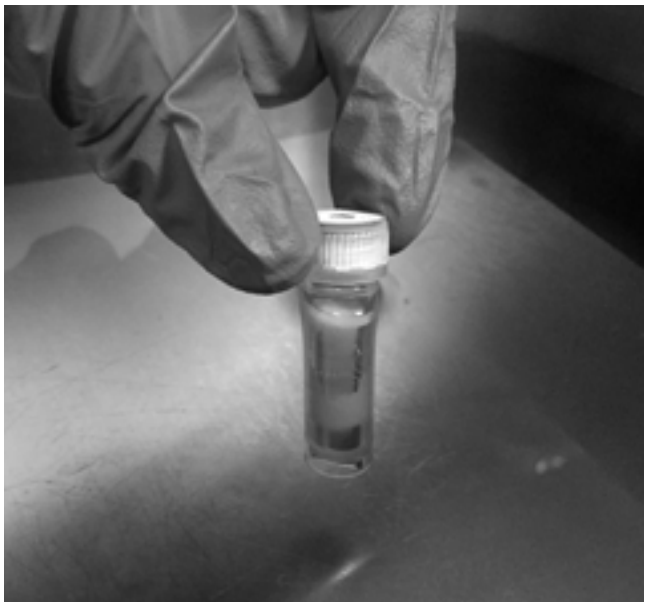
III. Thawing Cryopreserved Human Hepatocytes

1. Refer to the individual hepatocyte lot's CoA for average yield per vial and any special centrifugation instructions prior to initiating the following steps.
2. Don appropriate PPE for handling LN_2 and biohazardous materials (Refer to OSHA Standard 29CFR1910.132).
3. Fill portable LN_2 dewar or other suitable container with enough LN_2 to submerge a cryovial at least half of its height.
4. Remove HHTM bottle(s) from water bath, spray with 70% (v/v) alcohol, and place in a BSC.
5. Invert HHTM bottle to evenly mix, then filter HHTM through a 0.2 µm filter. Transfer filtered HHTM to a sterile 50 mL conical centrifuge tube. **NOTE:** If thawing a single vial of hepatocytes, transfer 49 mL HHTM to the tube. If thawing more than one vial, transfer 1 mL less of HHTM per vial being thawed, up to 3 mL (three vials) per tube.
6. Cap 50 mL conical centrifuge tube(s) and gently invert three times to mix HHTM thoroughly.
7. Place small insulated laboratory ice tray containing ice next to 37°C water bath.
8. QUICKLY remove vial(s) of cryopreserved human hepatocytes from the cryogenic storage freezer and place in the portable LN_2 dewar/container.
9. Carefully transport frozen vials in the LN_2 container to the laboratory area.

NOTE: Steps 11-12 should take no more than 10 seconds in order to maintain hepatocyte viability and functionality.

10. Using forceps or tongs, QUICKLY remove vial(s) of cryopreserved human hepatocytes from the LN_2, loosen the vial cap(s) slightly to release pressure and then re-tighten. **NOTE:** Do NOT completely remove vial cap(s).
11. Partially submerge vial(s) vertically in a 37°C water bath, to approximately 0.5 cm below the cap(s) (ensure the water level is at least even with the top of the frozen cell suspension; see Figure 1), to thaw for 90-100 seconds. **NOTE:** It is important to use a timer for these steps.

Figure 1: Vial in Water Bath



12. Remove vial(s) from 37°C water bath and invert. If the frozen cell suspension has begun to liquefy and slides freely without tapping or shaking the vial(s), return the vial(s) to upright and continue on to Step 14. If the frozen cell suspension does not slide freely, return the vial(s) to upright and place back in the water bath for an additional 5 seconds. Recheck the movement of the cell suspension before continuing on to Step 14. Repeat as necessary if frozen cell suspension continues to not move freely. **NOTE:** Place sufficiently thawed vials on ice while waiting for remaining vials to thaw.

III. Thawing Cryopreserved Human Hepatocytes continued

13. QUICKLY place vial(s) in ice tray for transport and wipe with 70% (v/v) alcohol prior to placement in the BSC.

14. Moving at a quick pace and working with 1 vial at a time, remove the vial cap and pour the hepatocytes into a 50 mL conical centrifuge tube containing HHTM. Using a 1000 µL pipette, rinse the vial once with approximately 1000 µL of HHTM from the 50 mL conical centrifuge tube, transferring the HHTM back to the tube. QUICKLY repeat this step for all remaining vials.

15. Tighten the tube cap(s) and gently invert the tube(s) 3 times to uniformly suspend the hepatocytes.

16. Centrifuge the hepatocytes at 100 x g for 8 minutes at room temperature. **NOTE:** Be sure to examine the individual hepatocyte lot's CoA for special instructions prior to centrifugation in order to maximize yield.

17. Return to the BSC and gently aspirate the supernatant without disturbing the cell pellet in the bottom of the tube(s).

18. Add 3-5 mL of warm supplemented HHPM or HHCM to the tube(s) (volume depends on expected cell yield; medium type depends on subsequent experiment). Gently rock the conical centrifuge tube(s) to resuspend the hepatocytes. Do NOT resuspend cells by swirling vigorously or using a pipettor or vortex mixer. **NOTE:** The typical cell concentration range is 1-2 x 10^6 cells/mL to allow for accurate cell counting. Refer to the individual hepatocyte lot's CoA for the average yield per vial.

19. Count the hepatocyte suspension by either the trypan blue exclusion method on a hemocytometer and/or AO/PI with an automated cell counter to determine cell yield and viability.

    NOTES:

    - Use a 10-fold dilution for the trypan blue exclusion method or 2-fold dilution for AO/PI staining.

    - An accurate concentration of your hepatocyte suspension is critical for proper plating. If a hepatocyte-specific program is available, use an automated cell counter. If not or your results do not match that of the CoA, perform manual counts.

20. Proceed to plating cells (Section IV) or using them for suspension assays.

IV. Plating Cryopreserved Human Hepatocytes

1. After thawing the cryopreserved human hepatocytes, refer to the individual hepatocyte lot's CoA for the recommended seeding density to use with standard collagen-coated 24-well plates. **NOTE:** If you are not using 24-well plates, see Table 1 for a suggested range of densities to use with other plate formats. Additionally, the correct cell density per lot for other plate formats can be determined by testing different densities or by contacting LifeNet Health for technical assistance.
2. Upon determining final cell yield, add warm supplemented HHPM to the cell suspension until the desired final cell density is reached. If the final volume is greater than 50 mL, transfer the cell suspension from the 50 mL conical centrifuge tube to an appropriate sterile bottle.
3. Gently rock or invert the tube or bottle to ensure the cell suspension is homogenously mixed, then pour the cell suspension into a sterile reagent reservoir until half full.
4. Using a multi-channel pipette, transfer the cell suspension from the reagent reservoir to the culture plate(s). **NOTE:** Hepatocytes settle out of suspension quickly. Gently rock the reservoir in all directions to ensure the cell suspension is homogenous prior to each refill of the pipette; likewise, gently invert the conical tube or bottle prior to refilling the reservoir.
5. Remove culture plate(s) from the BSC in stacks of 1-3 and place in a humidified incubator (37°C, 5% CO_2).
6. Shake culture plate(s) in a north-south, then east-west manner, followed by a figure 8, three times on the incubator shelf. The shaking should be gentle, yet vigorous enough to evenly distribute the cells across each well. Repeat the north-south and east-west motions at 15 minute intervals for the first 60 minutes of culture. Shake again at 90 minutes of culture. **NOTE:** 96-well plates do NOT need to be shaken prior to placement in the incubator nor at 15 minute intervals for the first 60 minutes of culture.

V. Culturing Cryopreserved Human Hepatocytes

1. At 2-4 hours post-plating (dependent upon cell attachment), remove culture plate(s) from the incubator and place in a BSC. Do not move more than 3 plates at a time. **NOTE:** Pre-warm only the necessary amount of supplemented HHCM to 37°C in a water bath, protected from bright light, 20-30 minutes prior to use. See Table 2 for recommended volumes.
2. Gently shake plates in a north-south, then east-west manner to dislodge dead cells.
3. Tilt plates and gently aspirate medium from the side of each well without touching the cell monolayer. **NOTE:** Avoid prolonged or excessive aspiration of wells which may cause dehydration of the cells.
4. Using a multi-channel pipette, add warm supplemented HHCM at the volume specified in Table 2.
5. Return plate(s) to the incubator for overnight or until the experimental protocol dictates. If warranted, overlay the hepatocytes with an extracellular matrix 8-10 hours post-plating.
6. Replace the medium daily with fresh, warm supplemented HHCM.

Table 1. Seeding Densities for Cryopreserved Human Hepatocytes

Culture Vessel	Seeding Density (cells x 10^6/mL)	Volume per Well
6-well plate	0.75 – 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	200 µL
96-well plate	0.4 – 0.6	150 µL

Table 2. Human Hepatocyte Culture Medium Well Volumes

Culture Vessel	Volume per Well
6-well plate	1.5 mL
12-well plate	1 mL
24-well plate	500 µL
48-well plate	200 µL
96-well plate	100 µL

Appendix I. Required Equipment and Consumables:

Large Equipment:

- Biological Safety Cabinet (BSC)
- Cryogenic storage freezer capable of temperatures ≤-150°C
- Portable liquid nitrogen (LN_2) dewar or other suitable container to transport frozen vials
- 37°C/5% CO_2 Incubator (humidified)
- 37°C water bath
- Centrifuge capable of achieving 100 x g (50-mL conical tube adaptors)
- -20°C freezer
- 4°C refrigerator
- Vacuum aspiration system with sterile aspiration tips (optional)

Small Equipment:

- Appropriate personal protective equipment (PPE)
- Laboratory ice tray capable of containing a small amount of liquid nitrogen and a cryovial box
- Hemocytometer or other cell-counting device
- Forceps or tongs
- Cryovial storage box
- Timer
- Pipettes (20, 200 and 1000 µL) with sterile tips
- Serological pipettor and sterile pipettes (1, 2, 5, 10, 25 mL)
- 1000 µL multichannel electronic pipette with sterile tips (optional)
- Collagen-coated culture plates (for plateable cells only)
- Sterile disposable or washable media bottles (100-250 mL)
- 0.2 µm Steriflip® filters (Sigma cat. # SCGP00525)

Consumables:

- 70% (v/v) alcohol (ethanol or isopropyl alcohol)
- 70% alcohol wipes (or lab wipes soaked in 70% (v/v) alcohol)
- Laboratory wipes
- Ice
- Liquid nitrogen (LN_2)
- Sterile 50 mL conical centrifuge tubes
- Sterile 50 or 100 mL reagent reservoir (optional)
- LifeNet Health Cryopreserved Human Hepatocytes (suspension or plateable)
- LifeNet Health Human Hepatocyte Thawing Medium (HHTM)
- LifeNet Health Human Hepatocyte Plating Medium (HHPM)
- LifeNet Health Human Hepatocyte Plating Medium Supplement
- LifeNet Health Human Hepatocyte Culture Medium (HHCM)
- LifeNet Health Human Hepatocyte Culture Medium Supplement
- Antibiotic (optional; product suggestion - Penicillin/Streptomycin)
- Trypan blue solution
- AO/PI (acridine orange/propidium iodide) stain

Appendix II. Quick Reference Guide:

Thawing Cryopreserved Human Hepatocytes

1. Carefully transport frozen vials in an LN_2 dewar or another suitable container to laboratory area. **NOTE:** Examine the individual hepatocyte lot's CoA for special instructions prior to centrifugation in order to maximize the yield for the lot.
2. Loosen vial cap(s) slightly to release pressure, re-tighten cap(s), and partially submerge vial(s) in a 37°C water bath (see Figure 1).
3. Thaw until frozen cell suspension liquefies and slides freely when vial is turned upside down (90-100 seconds; no tapping or shaking). **NOTE:** If thawing multiple vials, keep thawed vials on ice.
4. Transfer thawed vials on ice to a BSC, wipe vial(s) with 70% alcohol, and pour contents into sterile 50 mL conical centrifuge tube(s) containing warmed and filtered HHTM.
5. Using a 1000 µL pipette, rinse each vial with HHTM from the 50 mL tube(s) and transfer back to the corresponding tube. Repeat this step for all remaining vials.
6. Tighten cap(s) and gently invert tube(s) 3 times.
7. Centrifuge at 100 x g for 8 minutes at room temperature.
8. Aspirate medium without disturbing the pellet, add 3-5 mL of warm HHPM and gently resuspend the cells.
9. Count cells using a hemocytometer or automated cell counter to determine cell yield and viability.
10. Proceed to plating cells or using them in suspension assays.

Plating and Culturing Cryopreserved Human Hepatocytes

1. After thawing cryopreserved human hepatocytes, refer to the individual hepatocyte lot's CoA for recommended seeding density to use with standard collagen-coated 24-well plates (see Table 1 for additional plate formats).
2. Upon determining final cell number, add warm supplemented HHPM until desired final cell density is reached, and gently mix and pour the cell suspension into a sterile reagent reservoir until half full.
3. Using a multi-channel pipette, transfer the cell suspension from the reagent reservoir to the culture plates. **NOTE:** Gently rock the reservoir to ensure a homogenous cell suspension prior to each refill of the pipette; likewise, gently invert the conical tube or bottle prior to refilling the reservoir.
4. Place culture plates in a humidified incubator (37°C, 5% CO_2) and rock in a north-south, then east-west manner, followed by a figure 8, three times on the incubator shelf. Repeat the north-south, east-west motion at 15 minute intervals for the first 60 minutes of culture and again at 90 minutes of culture.
5. At 2-4 hours post-plating (dependent upon cell attachment), gently shake the plates and aspirate medium without disturbing the cell monolayer.
6. Using a multi-channel pipette, add fresh, warm supplemented HHCM at the volume listed in Table 2, and return plates to incubator for overnight or until experimental protocol dictates.
7. If warranted, overlay cells with extracellular matrix 8-10 hours post-plating. Replace medium daily with fresh, warm supplemented HHCM.

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