ATCC®

EXTRACTION PROTOCOL

Extracting RNA from ATCC® HepatoXcell™ Primary Human Hepatocytes

HepatoXcell™ by ATCC

Equipment

- Biological safety cabinet (BSC)
- Chemical fume hood
- Micropipettes (Various sizes)
- Bio-Rad C1000 Touch Thermal Cycler (or equivalent)
- Incubator (37°C, 5% CO_2)
- NanoDrop (or equivalent)
- Centrifuge (high-speed, 1.5 mL tubes)
- Centrifuge (high-speed, 50 mL tubes)
- Minifuge
- Ice bucket
- Water bath (37°C)
- Microscope and hemocytometer or automated cell counter (or equivalent)
- Ultra-low temperature freezer (-80°C) (if long-term storage needed)
- Qubit fluorometer (optional)

Materials

- HepatoXcell™ Eco: Normal Human Hepatocytes (ATCC® PCS-450-012™), HepatoXcell™ Pro: Normal Human Hepatocytes (ATCC® PCS-450-011™), or HepatoXcell™ Plus: Normal Human Hepatocytes (ATCC® PCS-450-010™)
- HepatoXcell™ Primary Hepatocyte Thawing Medium (HTM) 1X (ATCC® PCS-450-032™)
- HepatoXcell™ Primary Hepatocyte Maintenance Medium (HMM) 1X (ATCC® PCS-450-034™)
- 50 mL conical tubes (Corning, catalog number 352070 or equivalent)
- Filter-barrier, sterile micropipette tips for 10, 20, 200, and 1000 µL pipettes
- Sterile 96-well plates (VWR, catalog number 89049-178 or equivalent)
- 96-well plate seals (Bio-Rad, catalog number MSB1001 or equivalent, as needed)
- 1.5 mL RNase-free Eppendorf tubes (Thermo Fisher, catalog number AM12400 or equivalent)
- Ice
- RNase AWAY (Thermo Fisher, catalog number AM9780 or equivalent)
- DNA AWAY (Thermo Fisher, catalog number 7010 or equivalent)
- 200 proof ethanol (Fisher, catalog number BP2818100 or equivalent)
- TRIzol reagent (Fisher, catalog number 15-596-026 or equivalent)
- Chloroform (Thermo Fisher, catalog number J67241.AP or equivalent)
- Nuclease-free water (Thermo Fisher, catalog number R0582 or equivalent)
- Thermo Fisher Scientific PureLink RNA Kit (Thermo Fisher, catalog number 12183018A or equivalent)
- Verso cDNA Synthesis Kit (Thermo Fisher, catalog number AB-1453/A or equivalent)
- TaqMan Gene Expression Master Mix for qPCR (Thermo Fisher, catalog 4369016; or equivalent)
- Primers, probes
- GAPDH probe or other housekeeping probe (Thermo Fisher, catalog number 4448489 or equivalent)

Safety Precautions

- Use appropriate personal protective equipment (lab coat, safety glasses, sleeves, gloves, etc.).
- Dispose of all biological materials into appropriate biological waste containers throughout the process.
- Before beginning and after completing work with cultures, decontaminate all surfaces with 70% ethanol.
- When working with chloroform it is recommended to work inside a chemical fume hood.

Note: The working area should be treated with RNase AWAY or equivalent prior to starting work

Procedure

Hepatocyte Collection

For plated hepatocytes:

1. Sanitize and prepare the BSC. Remove cells from the incubator and place them into the BSC.
2. Using an aspirator or a micropipette, remove HepatoXcell™ Primary HMM.
3. Using a micropipette, add 500 µL of TRIzol reagent into each well. Gently triturate the solution by pipetting up and down several times.
4. Transfer the contents of each well into separate 1.5 mL RNase-free Eppendorf tubes. Ensure tubes are properly labeled for downstream processing.
5. If storing after collection, store samples in a -80°C freezer for up to one month. Otherwise, proceed to **RNA Purification**.

   Note: Lysis buffer from the Purelink RNA kit can be used in place of TRIzol, but it is recommended to perform RNA purification the same day as collection to avoid degradation. If using lysis buffer in place of TRIzol, proceed to **Binding, Washing, and Elution**.

For suspended hepatocytes:

A. If using vials directly from liquid nitrogen (LN_2):

1. Prewarm HepatoXcell™ Primary HMM to approximately 37°C in a water bath.
2. Thaw the vial of HepatoXcell™ Primary Human Hepatocytes in a water bath set to 37°C for approximately two minutes.
3. Transfer the contents of the vial to a 50 mL conical tube containing 19 mL of room temperature HepatoXcell™ Primary HTM.
4. Centrifuge cells at 100 x g for 10 minutes at room temperature (RT). Once centrifugation is complete, move the 50 mL conical tube containing the cell pellet to the BSC then aspirate or remove the supernatant.
5. Resuspend cell pellet in 3 mL of warmed HepatoXcell™ Primary HMM. Count the hepatocytes using a hemocytometer or automated cell counter.
6. Aliquot cells into an appropriate number of 1.5 mL RNase-free Eppendorf tubes.

   Note: It is recommended to have at least $5x10^5$ - $1x10^6$ cells per aliquot
7. Centrifuge tubes using a minifuge for about 5 seconds at maximum speed. Once centrifugation is complete, transfer tubes back into the BSC.
8. Aspirate the supernatant carefully to avoid agitating the cell pellet.
9. Use a micropipette to add 500 – 1000 µL of TRIzol reagent to each sample. Gently triturate the mixture by pipetting up and down.
10. If storing after collection, store samples in a -80°C freezer for up to a month. Otherwise, proceed to **RNA Purification**.

    Note: Lysis buffer from the Purelink RNA kit can be used in place of TRIzol, but it is recommended to perform RNA purification the same day as collection to avoid degradation. If using lysis buffer in place of TRIzol, proceed to **Binding, Washing, and Elution**.

B. If the cells are already in suspension or you are using leftover cells from primary human hepatocyte plating:

1. Aliquot cells into an appropriate number of 1.5 mL RNase-free Eppendorf tubes.

   Note: It is recommended to have at least $5x10^5$ - $1x10^6$ cells per aliquot.
2. Centrifuge tubes using a minifuge for about 5 seconds at maximum speed. Once centrifugation is complete, transfer tubes back into the BSC.
3. Aspirate the supernatant.
4. Use a micropipette to add 500 - 1000 µL of TRIzol reagent to each sample. Gently triturate the mixture by pipetting up and down.
5. If storing after collection, store samples in a -80°C freezer for up to a month. Otherwise, proceed to **RNA Purification**.

   Note: Lysis buffer from the Purelink RNA kit can be used in place of TRIzol, but it is recommended to perform RNA purification the same day as collection to avoid degradation. If using Lysis buffer in place of TRIzol, proceed to **Binding, Washing, and Elution**.

RNA Purification (For samples collected in TRIzol only)

1. In a chemical fume hood use a micropipette, add 100 µL of chloroform per 500 µL of TRIzol reagent and sample. Shake the tube vigorously by hand for 15 seconds.

   Note: Avoid vortexing if the downstream application is sensitive to the presence of DNA as it increases DNA contamination. If vortexing is used, perform a DNase digestion step during RNA purification or after purification. Refer to the PureLink RNA mini kit manual.

2. Incubate the sample at room temperature for two to three minutes.
3. Centrifuge the sample at 12,000 x g for 15 minutes at 4°C.

   Note: After centrifugation, the mixture separates into three phases: a lower phenol-red chloroform phase, an interphase, and a colorless upper aqueous phase that contains the RNA. The volume of the upper aqueous phase should be approximately 300 µL.
4. Prepare a solution of 70% ethanol using 200 proof ethanol and nuclease-free water in an appropriately sized tube. The solution should not be the same as the ethanol used in the sterilization and cleaning procedures.
5. Transfer approximately 200 µL of the aqueous phase containing the RNA to a fresh RNase-free tube.

   Note: Avoid collecting any of the lower phases (i.e., interphase or phenol-red chloroform phase). These phases contain materials that negatively affect the final amount of RNA.
6. Add an equal volume of 70% ethanol to the amount removed in step 5 of **RNA Purification** to obtain a final ethanol concentration of 35%. Mix well by inversion.
7. Invert the tube to disperse any precipitate that may form.
8. Continue to **Binding, Washing, and Elution**.

Binding, Washing, and Elution

1. Follow the instructions provided by the Thermo Fisher Scientific PureLink RNA Kit (or equivalent).

   Note: If the application requires sequential elution of the same sample due to large volume, collect all elutes into the same tube.

   Note: Store the purified RNA on ice if it will be used within a couple hours. For long-term storage, store the purified RNA at -80°C.

cDNA Synthesis

1. Using a NanoDrop, calculate the purity and concentration of the RNA sample.
2. **Optional:** Calculate concentration using a Qubit Fluorometer.
3. Dilute the RNA sample in a second tube to 1 ng/µL using RNase-free water. This is now the working stock RNA sample. For a single reaction of cDNA synthesis, use 1 µL of the 1 ng/µL RNA sample.
4. Prepare tube(s) containing the sample and all reagents on ice (Refer to the Verso cDNA Synthesis Kit protocol).
5. Prepare the reagents using a micropipette; combine 4 µL of 5X cDNA synthesis buffer, 2 µL of dNTP mix, 1 µL of Verso Enzyme mix, 1 µL of RT enhancer, 1 µL of RNA primer, and 10 µL of nuclease-free water, totaling 19 µL of reagent/reaction.

   Note: Ensure that the final volume of reagent/reaction without RNA is 19 µL. Multiply the number of reactions needed by 19 µL to obtain the final required volume. To include overage, multiply the final required volume by ~1.1 to account for pipetting error. Multiply as needed for larger reactions.
6. Transfer 19 µL of reagent from step 5 of **cDNA Synthesis** to a tube or plate suitable for a thermal cycler or PCR machine. Using a micropipette, add 1 µL of the RNA sample to the tube. The sample is now prepared for the cDNA synthesis reaction. The total volume should be 20 µL.
7. Place the cDNA synthesis tube into a compatible thermal cycler or PCR machine using the following protocol settings.

Component	Temperature	Time	Number of Cycles
cDNA synthesis	42°C	30 minutes	1
Inactivation	95°C	2 minutes	1

Note: If using cDNA within the next several hours, store the cDNA on ice. For long-term storage, store at -80°C.

PCR Amplification

1. Using a NanoDrop, calculate the purity and concentration of the cDNA sample.
2. Optional: Calculate concentration using a Qubit Fluorometer.
3. Dilute the cDNA prepared in **cDNA Synthesis** to 1 µg/µL.

   Note: cDNA concentration is dependent on the RNA concentration prior to reverse transcription. For ease of use, dilute the cDNA to 1–1.7 µg/µL. If the cDNA is too concentrated, the PCR can become overloaded and yield a false positive result. If the cDNA is not concentrated or not enough time was given for cDNA to amplify, a false negative result may be given.
4. Calculate the volume of components needed to prepare the wanted number of reactions. Make sure to include the negative controls for each condition, and one to two reactions worth of overage.
5. 20 µL is required for each reaction. Include the following reagents in the working master mix: 1 µL of the selected housekeeping probe, 1 µL of the desired probe (multiple probes can be used if desired, but different types of probes or separate master mixes should be prepared), and 10 µL of TaqMan Gene Expression master mix. The remaining volume should be compensated for with nuclease-free water for a final volume of 19 µL (See the Thermo Fisher Scientific user guide TaqMan Gene Expression Master Mix, Publication Number:

4371135, for more detailed information). Swirl or mix the TaqMan gene expression master mix tube to thoroughly mix the solution. This is now the working stock master mix.

a. Different tests require different primers. Add 1 µL of the test probe to the GAPDH/master mix solution, ensuring that the test primer is a different type than the housekeeping probe (e.g., if using VIC type GAPDH as the housekeeping probe, use FAM type primers or other primers).

Note: Run each sample in duplicate or triplicate.

6. Organize the plate map as preferred, taking care to have empty wells in between each set of tests to avoid light bleed. It is recommended to place duplicate or triplicate wells horizontally adjacent to the sample.
7. Open a sterile plate and add 19 µL of the working master mix to both the control and test wells, including duplicates and triplicates.
   a. Add 1 µL of cDNA (the final volume in the well should be 20 µL), excluding negative control wells.
   b. If running more than one test set, repeat this step as needed for each condition or test.
8. Seal the plate with an adhesive PCR plate seal or equivalent and ensure that all edges are firmly sealed.
9. On a thermal cycler, enter the following information as the protocol:

   Step 1: 50°C for 2 minutes
   Step 2: 95°C for 10 minutes
   Step 3: 95°C for 15 seconds
   Step 4: 60°C for 60 seconds

   Note: Steps 3 and 4 should repeat 50 times, or 50 cycles.
10. Analyze the results after all thermal cycler steps are completed.

Acceptance Criteria

1. Amplification from qPCR should be within the 20 to 30 range for the cycle threshold (Ct). A singular instance of <20 or >30 Ct is not enough for disqualification; however, more than three instances of such occurring should be addressed.
2. Amplification in the >35 Ct range is considered extremely poor and should be considered non-standard. The <20 Ct range is extremely unusual and likely a sign of contamination or too much cDNA.

References

1. Thermo Fisher Scientific. PureLink™ RNA Mini Kit User Guide. Publication Number MAN 0000406. Thermo Fisher Scientific, Waltham, MA, USA.
2. Thermo Fisher Scientific. PureLink™ RNA Mini Kit Quick Start Reference Guide. Publication Number MAN0019347. Thermo Fisher Scientific, Waltham, MA, USA.
3. Thermo Fisher Scientific. Verso™ cDNA Synthesis Kit User Guide. Publication Number MAN0012822. Therm Fisher Scientific, Waltham, MA, USA.
4. Thermo Fisher Scientific. TaqMan™ Gene Expression Master Mix User Guide. Publication Number 4371135. Thermo Fisher Scientific, Waltham, MA, USA.

Troubleshooting

RNA Purification:

- Minimize the risk of contamination by using RNase-free tools and maintaining a RNase-free working environment. Though RNA can be processed on the benchtop, it can be beneficial to reduce the risk of RNases by working inside a BSC.
- When not working with sample(s), store at -80°C for long-term storage and -20°C if planning to use within a few months. This prevents degradation of the sample as RNA is very unstable.
- If the yield is lower than expected or desired, it is recommended to start with more starting material.
- Avoid overloading columns to prevent degradation of sample(s).

cDNA Purification:

- Ensure starting material is sufficient. If sample is too small, it may not yield enough cDNA for further downstream applications.
- Store properly to avoid degradation of sample(s).

PCR:

- Verify that material has not degraded before starting the PCR.
- Ensure the starting material is not too concentrated as this can overload the PCR and give a false positive result.
- Ensure that starting material is not too diluted or the protocol not given enough time for cDNA to amplify as both can give a false negative result.
- Verify that probes are different types. For example, If using GAPDH FAM for the housekeeping probe, the testing probes should be a different type like VIC or another equivalent probe type.

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