Cell-Mate3D™ RNA Isolation Kit

RXT-1000

This protocol is optimized for isolation of RNA from cell embedded Cell-Mate3D™ cocoons. Each isolation is suitable for up to 100mg (100uL) of cell embedded Cell-Mate3D™.

REAGENTS AND EQUIPMENT SUPPLIED

- Reagent A: Dextran Sulfate, 2 ml vial
- Reagent B: Extraction Buffer, 10 ml brown vial
- $CHCl_3$: Isoamyl Alcohol (24:1), 60 ml reagent bottle
- Reagent C: 7M LiCl, 2 ml vial
- Reagent D: 4M GuHCl, 10 ml brown vial
- Reagent E: 0.4M LiCl, 10 ml brown vial
- Glassmilk Suspension: 440 mg/ml, 2 ml vial
- Precellys Ceramic Bead Tubes

EQUIPMENT NOT SUPPLIED

- Liquid N_2
- Block heater or water bath at 65^0 C
- Micro Centrifuge (14kxg capable)
- SpeedVac (optional)
- Mortar and Pestle

REAGENTS REQUIRED THAT ARE NOT SUPPLIED

- Chloroform
- Ethanol
- Isopropanol
- 2-mercaptoethanol
- Nuclease Free H_2O

REAGENT PREPARATION PRIOR TO START OF ISOLATION PROTOCOL

- Reagent A - rehydrate the dry dextran sulfate by adding 1.0 ml of nuclease free water
- Reagent B - add 2-mercaptoethanol (2% v/v), (300ul:6ul)
- Reagent E - dilute to proper molarity by adding 7.0ml of absolute ethanol to contents
- Prepare 70%, 75%, and 80% ethanol solutions using nuclease free water

Cell-Mate3D™ RNA Isolation Kit
Condensed Protocol

Step 1 – Cell-Mate3D™ Cocoon Disruption

1. Cool the mortar and pestle thoroughly in liquid N_2 bath and add liquid N_2 directly into Mortar
2. Add frozen cocoon (≤ 100mg) to the mortar containing N_2
3. Add 25 ul of Reagent A – Dextran Sulfate
4. Add 25 ul of Reagent B – Extraction Buffer already containing the 2-mercaptoethanol
5. Carefully fracture the contents, than commence grinding to a fine white powder
6. Carefully remove mortar from N_2 bath, tilt and evaporate remaining N_2
7. As N_2 evaporates, tease together the dry ground powder
8. With the cold spatula, transfer powder to the ceramic bead tube held in a liquid N_2 bath

Step 2 – RNA Extraction

1. Add 300ul of Reagent B – Extraction Buffer with the 2-mercaptoethanol to the tube
2. Warm slightly and vortex until thawed
3. Place in a 65°C block heater for 10 minutes, vortex about every 2 minutes
4. Cool to near room temperature (RT) and add 300 ul CHCl3:IsoAmyl Alcohol (24:1) reagent
5. Vortex and centrifuge at 12000 x g for 12 minutes
6. Carefully remove top layer and transfer to a 2 ml micro centrifuge tube
7. Wash x2 with 300 ul of $CHCl_3$ centrifuge at 5 x g for 3 min each time, always removing only the top layer

Step 3 – Isopropanol Precipitation

1. Estimate the volume of the top layer of the previous step and add equal volume of isopropanol
2. Invert gently several times
3. Place at 4°C for a minimum of 30 minutes
4. Centrifuge at 12000 x g for 12 min
5. Carefully remove the liquid and gently dislodge the pellet
6. Wash the pellet once with about 100 ul of 75% ethanol
7. Centrifuge at 5000 x g for 4 min
8. Remove the ethanol

Step 4 – LiCl Precipitation

1. Dissolve the pellet in 60 ul of nuclease free H_2O. This takes some time.
2. Add 30 ul of Reagent C. (Proportionally more depending on volume used in step 4.1)
3. Place the tube at 4^oC overnight
4. At 4^oC , centrifuge 14000 x g for 20 min
5. Carefully remove and discard the supernatant
6. Wash the pellet in about 600 ul of cold 80% ethanol
7. Centrifuge cold at 14000 x g for 15 min
8. Carefully remove as much ethanol as possible
9. Dry the pellet in a SpeedVac for about 40 s or air dry

Step 5 – Glass milk Binding and Elution

1. Dissolve the pellet in 100 ul of Reagent D
2. Add 15 ul of glass milk
3. Pulse vortex for 5 min at RT. Always be sure glass milk does not remain clumped
4. Centrifuge for 2 min at 3000 x g
5. Remove and discard the supernatant
6. Wash glass milk once with 75 ul of Reagent D.
7. Repeat step 4 (and after each following wash step)
8. Wash the glass milk once with 75 ul of Reagent E
9. Wash the glass milk twice with 75 ul of 70% ethanol
10. Remove as much ethanol as possible, pulse centrifuge if needed
11. Dry in a SpeedVac for about 40 s or air dry
12. Add 60 ul of nuclease free H_2O and vortex
13. Allow to sit idle for 5 min and then centrifuge at 12000 x g for 5 min
14. The RNA is in the supernatant

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Full Protocol

CELL-MATE3D™ COCOON DISRUPTION

Cool the mortar and pestle thoroughly in liquid nitrogen bath. Add liquid nitrogen to the mortar and proceed when a pool is sustained in the mortar. It is essential that the mortar, pestle, spatula, and ceramic bead tubes be kept at liquid nitrogen temperature during the disruption step (Hints: keep spatula and ceramic bead tube in a rack so that the bottom remains in liquid N_2. The nitrogen will stop boiling and thus evaporating once all equipment is cold). Cut the CM cocoon in about 0.5 cm sections (≤ 100mg) and drop into the pool of liquid N_2 in the mortar. Also dispense 25 ul of dextran sulfate solution (Reagent A) and 25 ul of Extraction Buffer (Reagent B containing the 2-mercaptoethanol) into the liquid N_2. The cocoon and the dextran sulfate will freeze almost immediately and sink to the bottom of the liquid N_2 pool. It is desirable to have a temporary cover over the pestle while grinding to prevent evaporation of the liquid nitrogen and for safety. Carefully commence grinding of the frozen CM and the dextran sulfate. It will take some amount of pressure to initially fracture the frozen material, but soon the grinding goes more smoothly and one will observe a fine white powder forming in the bottom of the mortar. Remove the cover if using one and the pestle, carefully lift the mortar from the box, hold the mortar in a towel or other suitable protection and tilt it to aid in the evaporation of the liquid N_2. During this evaporation time, use the cold spatula to tease the white powder together and when the N_2 is gone the white powder becomes a manageable clump that can be scooped into the cold tubes containing the ceramic beads. Note: The spatula must be at near liquid N_2 temperature to make this transfer go smoothly.

EXTRACTION

Add to the frozen powder in the ceramic bead tube, 300ul of extraction buffer (Reagent B) mixed with 6ul of 2-mercaptoethanol. Vortex at RT until the thawing is complete. Then place the tube in a block heater set at 65° C for 10 minutes, but with vigorous vortex mixing every 2 minutes. Cool to room temperature. Add 300ul of the $CHCl_3$: isoamyl alcohol reagent supplied. Vortex the tube thoroughly and centrifuge at 12000 x g for 12 minutes. Carefully remove the top layer which contains the RNA and transfer to an RNace free 2ml micro centrifuge tube. Wash twice with 300 ul $CHCl_3$. Centrifuge at 5000 x g for 3 minutes each time, always removing the top layer containing the RNA.

ISOPROPANOL PRECIPITATION

Get a good estimate of the volume remaining after the extraction step and add an equal volume of isopropanol. Invert the tube several times to mix but do not vortex which can inhibit maximum nucleic acid precipitation). The nucleic acids will begin to precipitate almost immediately. Store at 4°C for 30 minutes to further complete the precipitation. Centrifuge at 12000 x g for 12 minutes, remove liquid, and then gently dislodge the pellet from the bottom of the tube. This can usually be accomplished by tapping the tube several times. Wash the dislodged pellet with 75% ethanol; centrifuge at 5000xg for 4 minutes. Remove the ethanol thoroughly, but drying is not necessary.

LiCl PRECIPITATION

Dissolve the pellet from the isopropanol precipitation in 60 uL of nuclease-free water. This does not go rapidly, but give it time with frequent flicking of the tube. When the precipitate is completely dissolved, add 30ul of the 7M LiCl reagent (Reagent C). This results in a final concentration of 2.3 M LiCl for RNA precipitation. Place the tube at 4° C overnight (12h minimum). Centrifuge the tube at 4° C at 14000 x g for 20 minutes and carefully remove the supernatant from the LiCl precipitation by keeping the pipet tip 3mm from the bottom of the tube. Before drawing off the supernatant, slant the centrifuge tube at about the same angle as in the centrifuge and turn so the pellet containing the RNA is on the bottom. The pellet is very hard to discern at this step. Wash the pellet in about 600ul of 80% cold (4^{0}C) ethanol. Centrifuge cold at 14000 x g for 15 min. Remove as much ethanol as possible (pulse spin if necessary). Dry the pellet briefly (40 sec) in the SpeedVac, or air dry at RT with the tube lying on its side and tilted downward.

GLASS MILK BINDING AND ELUTION

Dissolve the pellet from the LiCl precipitation in 100 uL of 4 M guanidine hydrochloride (Reagent D). Add 15 ul of glass milk (440 mg/ml). Pulse vortex occasionally for 5 minutes at room temperature. Centrifuge at 3000 x g for 2 minutes to settle the glass milk. Remove and discard the supernatant. Wash the glass milk once with 75 ul of buffered guanidine hydrochloride (Reagent D) (Repeat the centrifugation at 3000 x g for 2 minutes and again after each of the following wash steps). Then, wash once with 75 ul of 0.4 M LiCl (Reagent E) in 70% ethanol and finally twice with 70% ethanol. Remove as much ethanol as possible (pulse on microfuge if needed). Dry the glassmilk pellet in the SpeedVac for about 30 to 45 seconds or air dry. Add 60 ul of nuclease-free water and vortex. Allow to sit idle at room temperature for 5 minutes and then centrifuge at 12000 x g for 5 minutes. The RNA is in the supernatant.

REFERNCES

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3. Kistner c and Matamoros M (2005) RNA Isolation Using Phase Extraction and LiCl precipitation. Lotus japonicus Handbood. pp. 123-124.

SAFETY DISCLAIMER

Only competent and trained personnel using appropriate personal protective equipment and working within a controlled environment should handle all chemicals and perform the protocol described herein. Prior to performing this protocol, users should review appropriate safety information, including the manufacturers MSDS, related to the components used in this protocol supplied and not supplied in this kit. Bioactive Regenerative Therapeutics, Inc. is not liable for any loss, injury or damage as a result from the use of this protocol.