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Tissue Scrape Protocol for Verifying RNA Quality Using the PicoPure® RNA Isolation Kits

Protocol #1

Introduction

Arcturus recommends verifying the integrity of RNA in the tissue sample before proceeding with staining and Laser Capture Microdissection (LCM) procedures. This enables you to understand the quality of the RNA in the experimental sample before proceeding with further downstream processing. This protocol is recommended for all new frozen tissue samples.

The protocol involves preparing and dehydrating a tissue section, then scraping the entire tissue section into a 0.5 ml tube. RNA is then extracted from the sample using a modified version of the PicoPure™ RNA Isolation Kit (Catalog # KIT0204) protocol for larger amounts of tissue. Finally, the Lab-on-a-Chip System (Agilent) or a gel can be used to assess 28S and 18S ribosomal RNA integrity. If ribosomal bands are detected, then the sample contains viable RNA and is therefore a good candidate for LCM. If the ribosomal RNA bands are faint or not present, then the sample may contain degraded RNA.

Equipment and Reagents

This protocol requires the following reagents:

- ❑ PicoPure® RNA Isolation Kit (Arcturus, Cat. # KIT0204)

The following laboratory materials are also required to complete the protocol:

- ❑ Disposable gloves
- ❑ Kimwipes® or similar lint-free towels
- ❑ RNase-free pipet tips
- ❑ Slides (Sigma, Cat. #S4651)
- ❑ Tissue-Tek® OCT compound (VWR, Cat. # 25608-903)
- ❑ Staining Jar (Evergreen Scientific, Cat. #222-5450-G8S)
- ❑ Tissue-Tek® Cryomold (VWR, Cat. # 25608-916)
- ❑ Xylene (VWR, Cat. #EM-XX0060-4)
- ❑ Dry ice
- ❑ Ethanol 100% (VWR, Cat. # 34172-020)
- ❑ Detergent (Fisher Scientific, Cat. # 0-355)
- ❑ Ethanol 95%. Dilute 100% EtOH with nuclease-free water
- ❑ RNase AWAY (Invitrogen, Cat. # 10328-011)
- ❑ Ethanol 75%. Dilute 100% EtOH with nuclease-free water
- ❑ Microcentrifuge tubes, nuclease-free
- ❑ Distilled water, nuclease-free (Life Technologies, Cat. # 10977-015)

The following laboratory equipment is required to complete the protocol:

- ❑ Cryostat with disposable blades
- ❑ Scalpels, #11 sterile
- ❑ Fume hood
- ❑ Tweezers
- ❑ -70°C freezer
- ❑ Cover glass forceps
- ❑ Pipettor: 1000 µL, 200 µL, 100 µL, 20 µL, 10 µL
- ❑ Microslide box – plastic (VWR Cat. # 48444-004)
- ❑ Centrifuge
- ❑ *Optional*: Agilent 2100 Bioanalyzer

RNase-free Technique

RNase contamination will cause experimental failure. Minimize RNase contamination by adhering to the following recommendations throughout your experiment:

- Wear disposable gloves and change them frequently.
- Use RNase-free solutions, glassware and plastic ware.
- Wash scalpels, tweezers and forceps with detergent and bake at 210° C for four hours before use.
- Use RNase AWAY® (Invitrogen) according to the manufacturer's instructions on the horizontal staining rack and any other surfaces that may come in contact with the sample.

⚠ **For best RNA preservation, freeze tissue specimens immediately after dissection. Wear clean disposable gloves throughout the Specimen Freezing procedure. Use clean, RNase-free instruments.**

⚠ **Wear clean disposable gloves throughout the Slide Preparation procedure.**

⚠ **Note that isopentane has a very low flash point and should be kept away from open flames. Perform procedure in a fume hood or a well-ventilated space.**

Specimen Freezing

Step	Procedure
1.	Place dry ice in an appropriate container.
2.	Slowly pour isopentane into container with dry ice, filling until the isopentane level is just above the layer of dry ice.
3.	Bubbling of the isopentane will occur upon its addition to the dry ice, once this has subsided the isopentane is ready for use.
4.	If necessary, identify specimen on cryomold using a sharpie pen.
5.	Take cryomold and place a thin layer of OCT on the bottom of it.
6.	Collect dissected tissue specimen and place tissue in desired orientation onto the layer of OCT in the cryomold.
7.	Carefully add more OCT until specimen is completely covered and the cryomold is filled.
8.	Carefully place prepared cryomold into the cooled isopentane.
9.	Wait for OCT to completely solidify. If freezing down additional specimens, the processed specimens can be held in a separate container with dry ice only.
10.	Store frozen specimen in the cryomold in a –70°C freezer or proceed to slide preparation.

Slide Preparation

Step	Procedure
1.	Pre-cool the cryostat to the temperature recommended by the manufacturer for the specimen you are preparing.
2.	Remove and discard old microtome blade. Wipe down the knife holder and antiroll plate in the cryostat with 100% ethanol to avoid sample cross-contamination. Do not use the 100% ethanol solution provided in the HistoGene Frozen Section Staining Kit for this step.
3.	Install a new disposable microtome blade in the cryostat.
4.	Set cutting thickness to 8 µm.
5.	Place a microslide box on dry ice near the cryostat.
6.	Transfer the cryomold containing the specimen from the –70°C freezer to the cryostat, transporting on dry ice if necessary.
7.	Wait a minimum of 10 minutes for the specimen to equilibrate with the temperature of the cryostat.
8.	Mount specimen to specimen holder with OCT. Cut 8 µm sections.
9.	Mount specimen towards the center of a room temperature LCM microslide. Place slide immediately into microslide box on dry ice. Do not allow slide to dry at room temperature.

10.	Discard slides with folded or wrinkled sections. If cutting more than one specimen, use a new area of the disposable microtome blade for each one. In addition, wipe down knife holder and anti-roll plate with 100% ethanol in between each specimen to avoid cross contamination.
11.	Proceed immediately to the "Staining and Dehydration" segment of the protocol or store at –70°C for up to two months.

Dehydration

⚠ **Perform this segment of the protocol in a hood. Wear clean disposable gloves. Change all solutions in the plastic slide jars between each batch of slides to avoid cross-contamination. Do not re-use solutions. Do not transfer solutions back into their original bottles.**

Step	Procedure
1.	Label six staining jars as follows: a. 75% ethanol b. water nuclease-free c. 75% ethanol d. 95% ethanol e. 100% ethanol f. xylene
2.	Fill the labeled staining jars with 25 mL of the appropriate solution.
3.	Remove slide from the slide box on dry ice containing freshly mounted sections and place on a clean Kimwipe towel (or similar lint-free towel) and allow to thaw for no more than 30 seconds.
4.	Place the slide in staining jar a containing 75% ethanol for 30 seconds.
5.	Transfer the slide to staining jar b containing nuclease-free distilled water for 30 seconds.
6.	Transfer the slide in plastic jar c containing 75% ethanol for 30 seconds.
7.	Place the slide in staining jar d containing 95% ethanol for 30 seconds.
8.	Transfer the slide to staining jar e containing 100% ethanol for 30 seconds.
9.	Transfer the slide into staining jar f containing xylene for five minutes.
10.	Place the slide on a Kimwipe towel to dry in the hood for five minutes.
11.	Proceed immediately to perform scrape of section.
12.	Discard all used dehydration solutions according to standard procedures.

Scrape and RNA Extraction

⚠ **Wear disposable gloves and chang ethem frequently. Use RNase AWAY, according to the manufacturer's instructions, on the horizontal staining rack and any other surfaces that may come in contact with the sample.**

Step	Procedure
1.	Pipette 100 µL of PicoPure Extraction Buffer into a 0.5ml microcentrifuge tube.
2.	Using a clean scalpel blade, scrape entire section off of slide and place it in the tube containing 100 µL of PicoPure Extraction Buffer. Use a new scalpel blade for each sample.
3.	Vortex slightly.
4.	Incubate at 42°C for 30 minutes.
5.	Spin briefly to collect extract in bottom of tube.
6.	Proceed with RNA isolation or freeze at –70°C.

Isolation

Step	Procedure
1.	Pre-condition the MiraCol™ Purification Column: a. Pipette 250 µL Conditioning Buffer (CB) onto the purification column filter membrane. b. Incubate the purification column with Conditioning Buffer for 5 minutes at room temperature. c. Centrifuge the purification column in the provided collection tube at 16,000 x g for one minute.
2.	Pipette 100 µL of 70% Ethanol (EtOH) into the cell extract from Part D (RNA Extraction). Mix well by pipetting up and down. DO NOT CENTRIFUGE.
3.	Pipette the cell extract and EtOH mixture into the pre-conditioned purification column. The cell extract and EtOH will have a combined volume of approximately 200 µL.
4.	Centrifuge for 2 minutes at 100 x g, immediately followed by a centrifugation at 8,000 x g for 30 seconds.
5.	Pipette 100 µL Wash Buffer 1 (W1) into the purification column and centrifuge for one minute at 8,000 x g.
6.	DNase treatment is performed directly within the purification column to reduce DNA interference. The following protocol utilizing the RNase-Free DNase Set (Qiagen, catalog#79254) may be used. Protocol is applied during RNA Isolation. DNA must be removed by DNase treatment as follows: a. Pipette 10 µL DNase I Stock Solution into 70 µL Buffer RDD (provided with RNase-Free DNase Set). Mix by gently inverting. b. Pipette the 80 µL DNase incubation mix directly into the purification column membrane. Incubate at room temperature for 15 minutes. c. Pipette 80 µL PicoPure RNA Kit Wash Buffer 1 (W1) into the purification column membrane. Centrifuge at 8000 x g for 15 seconds.
7.	Pipette 100 µL Wash Buffer 2 (W2) into the purification column and centrifuge for one minute at 8,000 x g.
8.	Pipette another 100 µL Wash Buffer 2 (W2) into the purification column and centrifuge for two minutes at 16,000 x g.
9.	Transfer the purification column to a new microcentrifuge tube provided in the kit.
10.	Pipette 22 µL Elution Buffer (EB) directly onto the membrane of the purification column, according to the Elution Volume.
11.	Incubate the purification column for one minute at room temperature.
12.	Centrifuge the column for one minute at 1000 x g then centrifuge at 16,000 x g for an additional minute to elute RNA. The entire sample may be used immediately or stored at –80°C until use.

⚠ Flowthrough waste following centrifugation is usually present at only a small volume; therefore, it is not necessary to discard the flowthrough waste after every centrifugation step. Make sure that the accumulated flowthrough waste does not make contact with the purification column. Flowthrough waste should be discarded when the waste fluid level approaches the surface of the purification column. Remove all traces of wash buffer prior to transferring purification column to the new microcentrifuge tube. To remove wash buffer, discard flow through waste and recentrifuge the column for one minute at 16,000 x g.

RNA Quality Gel

Use the Agilent Lab-on-a-Chip System with the Bioanalyzer 2100 (Agilent) or an RNA gel to check the quality of the ribosomal RNA in your tissue sample. You should see a 2 to 1 ratio between the 28S and 18S peaks using the Bioanalyzer 2100.

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