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# Optimized Protocol for Preparing and Staining LCM Samples from Frozen Tissue and Extraction of High-Quality RNA

## PROTOCOL #2

### Introduction

Laser Capture Microdissection (LCM) enables gene expression pattern analysis in cells captured from frozen specimens. Obtaining accurate results from mRNA analysis experiments, including microarray hybridization and quantitative PCR, depends on careful preservation of intact RNA molecules in captured cells.

The Optimized Protocol for Preparing and Staining LCM Samples from Frozen Tissue for Extraction of High-Quality RNA described in this Application Note produces high contrast staining while preserving intact nucleic acid and protein species in captured cell populations. The protocol describes RNase-free laboratory technique, specifies RNase-free reagents and materials, and makes optimal use of Arcturus' HistoGene™ Stain, a new stain developed by Arcturus with staining results equivalent to hematoxylin and eosin, and protects nucleic acids from degradation.

Research in Arcturus' laboratories shows that this protocol yields high quality RNA from a variety of tissues, including human foreskin, ileum and jejunum, and mouse kidney, liver, brain, salivary gland, thymus and small intestine. Researchers may call Arcturus Technical Support at (888) 446-7911, send an e-mail inquiry to techsupport@arctur.com, or visit www.arctur.com for an updated list of tested tissues.

The specialized materials needed to complete the protocol described in this Application Note are available from Arcturus under the HistoGene label as individual items or as a complete kit (HistoGene LCM Frozen Section Staining Kit, Arcturus, Cat. # KIT0401).

### Equipment and Reagents

This protocol requires the following reagents:

- ❑ Xylene (VWR, Cat. # EM-XX0060-4)
- ❑ 100% ethanol, (VWR, Cat. # 34172-020)
- ❑ Distilled water, nuclease-free (Life Technologies, Cat. # 10977-015)
- ❑ 95% and 75% ethanol. Dilute 100% ethanol with nuclease-free water to obtain 95% and 75% ethanol solutions.
- ❑ HistoGene Staining Solution (Arcturus, Cat. # KIT0415)
- ❑ Isopentane ro 2-methylbutane (VWR, Cat. # JTQ223-7)

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

- ❑ Staining jars (Evergreen Scientific, Cat. # 222-5450-G8S )
- ❑ Slides (VWR Cat. # 48312-705, or VWR Plus Cat. # 48311-703, or Sigma Silane prep Cat. # S4651 )
- ❑ Tissue-Tek® OCT Compound (VWR, Cat. # 25608-903)
- ❑ Tissue-Tek® Cryomold (VWR Cat. # 25608-916)
- ❑ Detergent (Fisher Scientific, Cat. # 04-355)
- ❑ RNase AWAY (Life Technologies, Cat. # 10328-011)
- ❑ Dry Ice
- ❑ Pipettor: 1000µl, 200µl, 100µl, 20µl, 10µl
- ❑ RNase-free pipet tips
- ❑ Disposable gloves
- ❑ Kimwipes or similar lint-free towels

The following laboratory equipment is required to complete the protocol:

- ❑ Cryostat with disposable blades
- ❑ Fume hood
- ❑ -70°C freezer
- ❑ Tweezers
- ❑ Cover glass forceps
- ❑ Microslide box – plastic (VWR Cat. # 48444-004)
- ❑ Horizontal staining rack (optional)
- ❑ Optional: Humid chamber (Sigma Cat. # H6644)

### 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.

## Specimen Freezing

⚠ **For best RNA preservation, freeze tissue specimens immediately after dissection.**

⚠ **Wear clean disposable gloves throughout the Specimen Freezing procedure. Use clean, RNase-free instruments.**

⚠ **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.**

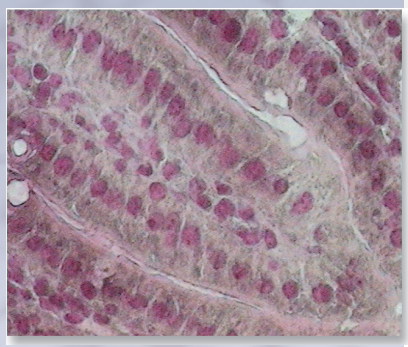
| Step | Procedure |
|---|---|
| 1. | Place dry ice in an appropriate container. |
| 2. | Slowly pour isopentane into container with dry ice, filling until the isopentane level is just above the layer of dry ice. |
| 3. | Bubbling of the isopentane will occur upon its addition to the dry ice, once this 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. |

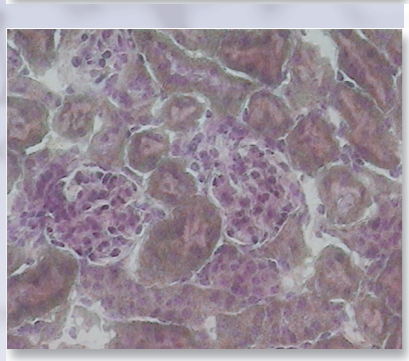
OK **It is okay to stop at this point.**

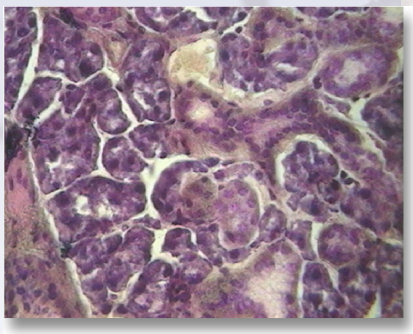
## Slide Preparation

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

| 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. |







**Mouse tissues stained for LCM using the Optimized Protocol for Preparing and Staining LCM Samples.** Arcturus HistoGene Stain for LCM samples provides contrast and specificity equivalent to hematoxylin and eosin.

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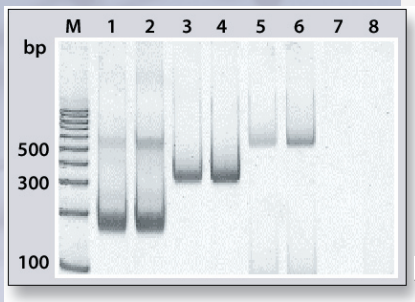
|---|---|
| 7. | Wait a minimum of 10 minutes for the specimen to equilibrate with the temperature of the cryostat. |
| 8. | Mount specimen to specimen holder with OCT. Cut 8 µm sections. |
| 9. | Mount sections towards the center of a room temperature LCM microslide. Place slide immediately into microslide 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 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. |

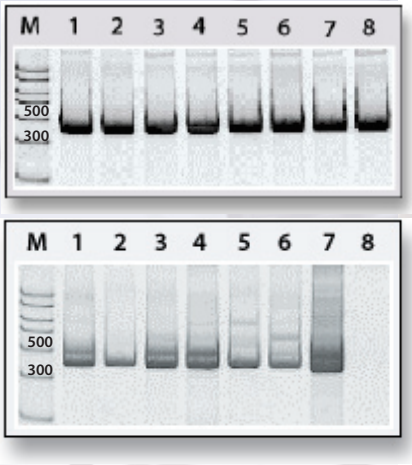
## Staining and Dehydration

⚠ **Carry out the "Staining and Dehydration" segment of the protocol in a hood. Wear clean disposable gloves. Divide the work batchwise, with a maximum of four slides per batch. Change all solutions in the staining jars between each batch of slides to avoid cross contamination. Do not reuse solutions. Do not transfer solutions back into their original bottles.**

| Step | Procedure |
|---|---|
| 1. | Label seven staining jars as follows:<br>a. 75% ethanol<br>b. distilled water<br>c. distilled water<br>d. 75% ethanol<br>e. 95% ethanol<br>f. 100% ethanol<br>g. xylene |
| 2. | Fill the labeled staining jars with 25 ml of the appropriate solution. |
| 3. | Remove up to four slides from the slide box and place them on a clean Kimwipe towel (or similar lint-free towel) and allow to thaw for no more than 30 seconds. |
| 4. | Place the slides in staining jar "a" containing 75% ethanol for 30 seconds. |
| 5. | Transfer the slides to staining jar "b" containing distilled water for 30 seconds. |
| 6. | Place the slides on a Kimwipe towel or a horizontal staining rack. |
| 7. | Using an RNase-free pipet tip, apply 100µl of the HistoGene Staining Solution to each section for 20 seconds. |
| 8. | Place the slides in staining jar "c" containing distilled water for 30 seconds. |
| 9. | Transfer the slides to staining jar "d" containing 75% ethanol for 30 seconds. |
| 10. | Transfer the slides to staining jar "e" containing 95% ethanol for 30 seconds. |
| 11. | Transfer the slides to staining jar "f" containing 100% ethanol for 30 seconds. |
| 12. | Transfer the slides into staining jar "g" containing xylene for five minutes. |




**RT-PCR products of low, medium and high abundance genes in 500 cells captured from mouse tissues.** Lane 1: kidney, elongation factor-1a (high abundance gene, 187 bp). Lane 2: liver, elongation factor -1a. Lane 3: kidney, GADPH (glyceraldehyde-3-phosphate dehydrogenase, medium abundance gene, 357 bp). Lane 4: liver, GADPH. Lane 5: kidney, protein phosphatase 1 (low abundance gene, 498 bp). Lane 6: liver, protein phosphatase 1. Lane 7: no RT control. Lane 8: no cDNA control. The mRNA profiles of all samples appear free of degradation. Mouse kidney protein phosphatase 1, a 498 bp mRNA, expresses under 300 copies per cell.




**RT-PCR analysis of RNA from mouse and human LCM Samples following Application Note protocol.** Amplification products of RT-PCR reaction using primers for 450 bp mouse GADPH on RNA recovered from 500 cells captured from mouse tissue following Application Note protocol. (A): Lanes 1-3: brain. Lanes 4-5: salivary gland. Lanes 6-7: small intestine. Lane 8: thymus.
Amplification products of RT-PCR reaction using primers for 449 bp cytoskeletal γ actin on RNA recovered from 500 cells captured from human tissues following the Application Note protocol . (B): Lanes 1-2: ileum. Lanes 3-4: foreskin. Lanes 5-6: jejunum. Lane 7: total human placental RNA. Lane 8: no RT (jejunum). RT-PCR analysis of RNA recovered from mouse and human cells captured following Application Note protocol indicates excellent RNA preservation.

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| 13. | Remove slides. Place the slides on a Kimwipe towel to dry in the hood for five minutes. Slides can be held in xylene. |
| 14. | Proceed immediately to perform LCM. |
| 15. | Discard all used staining and dehydration solutions according to standard procedures. |

⚠ **Frequent cycling of the tissue block from -70°C to -20°C for cryosectioning may accelerate RNA degradation. For best results, cut and mount a sufficient number of sections for two months use during one cryosectioning session. Store the mounted sections at -70°C until needed.**

## Cleaning Staining jars

**The staining jars provided with the HistoGene Frozen Section Staining Kit can be reused, but must be cleaned between each batch of slides. Rinse jars with 100% ethanol, followed by distilled water, then treat with RNase AWAY according to the manufacturer's protocol. Rinse jars thoroughly with nuclease-free water and allow to dry completely in the hood.**

## RNA Extraction and Isolation

**A complete extraction and purification method for preparing RNA from cells captured following treatment with the HistoGene LCM Frozen Section Staining Kit is available in the PicoPure™ RNA Isolation Kit (Arcturus, Cat. # KIT0204). The kit comes with a detailed, validated protocol and RNase-free materials. The kit materials and protocol produce total cellular RNA in a 10µl volume ready for analysis. To order the PicoPure RNA Isolation Kit, please contact Arcturus at (888) 446-7911 or (650) 962-3020, or send an e-mail to orders@arctur.com.**

## Checking RNA Integrity with Gel Analysis of RT-PCR Products

**The following RT-PCR protocol can detect the presence of specific mRNA in samples prepared from small numbers of cells. Quantitative fluorescence imaging confirms the size and relative abundance of mRNA transcripts from tissues prepared using the HistoGene Frozen Section Staining Kit and the PicoPure RNA Isolation Kit.**

| Step | Procedure |
|---|---|
| 1. | Mix 10µl of RNA isolated from 500 cells captured by LCM with 10µl Reverse Transcriptase master mix for cDNA synthesis as described in the Sensiscript Reverse Transcriptase Handbook (Qiagen) using oligo-dT primers and RNase inhibitor (Life Technologies, Cat. # 18418-012 and Cat. # 10777-019). Carry out the reverse transcriptase reaction for one hour at 37°C followed by a five minutes denaturation step at 95°C as described by the manufacturer. |
| 2. | Perform PCR using primer sets for three different abundance level genes as provided in the Stratagene Control RT-PCR Primers Kit (Cat. # 720170). Using a 0.5 ml thin-walled tube, add 2µl of the synthesized cDNA solution to 23µl of master mix containing forward and reverse primers at a final concentration of 200nM along with one Ready-to-Go bead (Amersham Pharmacia Biotech, Cat. # 27-9555-01). Denature samples at 95°C for five minutes, then amplify using the following thermal cycle parameters for 35 cycles, with a final extension of 72°C:<br>95°C — 15 seconds<br>primer anneal temp. — 30 seconds<br>72°C — 1 minute |

| | |
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| 3. | Add gel loading buffer to the PCR sample and separate 20µl of the mixture on a 6% Novex acrylamide gel (Invitrogen, Cat. # EC6265). Stain the gel with SYBR Gold Nucleic Acid Gel Stain (Molecular Probes, Cat. # S11494), then visualize on a FluorImager system (Molecular Dynamics). |

## Troubleshooting

I. Targeted cells do not lift from the slide during LCM

A. Sample may contain residual water. Ensure that ethanol solutions are fresh. Ethanol is hygroscopic. Keep ethanol bottles tightly capped, and do not pour ethanol solutions until you are ready to use them. If you suspect that the 100% ethanol solution has absorbed water, purchase a new bottle.

B. Sample may have dried between protocol steps. Carry out the "Staining and Dehydration" segment of the protocol at a steady pace.

II. RNA cannot be recovered from the sample

A. Sample starting material may contain poor-quality RNA. Freeze sample immediately following dissection, and take care to use RNase-free technique.

B. RNA may become degraded during RNA isolation. Wear gloves; use RNase-free technique and RNase- free instruments and reagents. Wipe down the PixCell® Laser Capture Microdissection System with RNase AWAY prior to use.

C. RNA may not be fully extracted and isolated from cells on the LCM cap. Use the Arcturus PicoPure RNA Isolation Kit or another guanidinium extraction method. Perform RNA extraction immediately after LCM to ensure complete extraction and optimum recovery of RNA.

D. Starting material quantity may be insufficient. Use at least 10 capture cells when performing RT-PCR. The isolation procedure used in the PicoPure RNA Isolation Kit provides reproducible recovery of RNA from the equivalent of 10 cells (200 pg).



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