USER GUIDE

ambion®
by life technologies™

LCM Staining Kit

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LCM Staining Kit

Contents

I. Introduction

A. Background

Laser Capture Microdissection (LCM) is a method for obtaining pure populations of cells from heterogeneous samples. LCM permits the selection and capture of cells, cell aggregates, and discrete morphological structures from thin tissue sections. Captured cells can then be used for nucleic acid studies, including SNP analysis, endpoint and real-time RT-PCR, and mRNA expression profiling. It is often necessary to stain tissue sections so that discrete structures within the tissue can be discerned. To isolate RNA from LCM samples, it is important to use a procedure that minimizes RNA degradation during staining.

Analysis of RNA recovered from tissue sections after each step of the conventional staining protocol for frozen samples revealed that significant RNA degradation occurred during exposure of the section to aqueous solutions, presumably due to reactivation of endogenous nucleases. To circumvent this problem, the LCM Staining Kit employs a novel staining procedure that avoids exposing the tissue sections to pure water at any step (Figure 1 on page 4). The procedure provides superior staining of frozen sections to facilitate identification of target cells for LCM, while preserving RNA quality.

B. Product Description

The LCM Staining Kit (patent pending) provides reagents and protocols for staining tissue sections by a method that minimizes RNA degradation during the staining process. Reagents are provided for processing 80 slides. Two different stains are provided with the kit: Cresyl Violet and Acridine Orange.

Cresyl Violet is a hydrophilic, basic stain that binds to negatively charged nucleic acids. The resulting nuclear staining, allows for visualization of variations in cell morphology, which can be useful for identifying malignant cells. Cresyl Violet is compatible with most mounting media. Cresyl violet-staining can be used to provide a preliminary assessment for identification of target cells before performing LCM with the dehydrated tissue.

In most cases, Cresyl Violet staining is adequate for identification of target cells for LCM. However, in cases where the tissue morphology has been damaged, Cresyl Violet might not clearly show cell distribution. In such cases, the Acridine Orange stain may provide better contrast between clusters of cells.

Acridine Orange is a fluorescent stain that intercalates with nucleic acids. To visualize this stain, the microscope must be equipped for fluorescent detection. Acridine Orange staining offers an advantage when microdissecting single cells, because cells isolated by LCM will fluoresce on the cap after the microdissection, making it easy to assess whether the tissue/cell was effectively microdissected and retrieved.

Using these optimized tissue processing and staining protocols in conjunction with the Ambion® RNAqueous®-Micro Kit, total RNA of excellent quality can be obtained from pure populations of cells selected by LCM.

C. Kit Components and Storage

Reagents are provided for the processing of 80 slides.

Amount	Component	Storage
25 mL	Acridine Orange Stain	4°C*
25 mL	Cresyl Violet Stain	4°C
10	Slide Chambers	room temp
15 g	Dehydration Beads	room temp
250 mL	RNase*Zap*® Solution	room temp
4	500 mL Bottles (empty)	room temp
1	Barrier Pen	room temp
1 L	Nuclease-free Water	any temp†

* Protect from light.
† Store the Nuclease-free Water at room temperature, 4°C, or –20°C.

D. Required Materials Not Included with the Kit

Histology supplies

We recommend the products below, although alternative products and sources are also available.

Materials	Suggested Source
Microscope slides	VWR® Superfrost® Plus Micro Slide VWR Cat #48311-703
Cryomold ®	VWR Cat #25608-916
Microslide Boxes	VWR Cat #48444-04
OCT Compound	VWR Cat #25608-930
Forceps	VWR Cat #25719-044 or 25601-008
Cytocool® II	VWR Cat #8323
Xylene, histological grade	Fisher Scientific Cat #1330-20-7
100% ACS grade ethanol	200-proof Alcohol VWR Cat #EM-EX0289-4
Desiccant	VWR Cat #22890-900
Lint-free paper towels	Kimberly-Clark code 34155 (Kimwipes® Wiper)

Equipment

- Cryostat with disposable microtome blades
- –80°C freezer and dry ice
- Fume hood
- LCM work station—This protocol was developed using the Arcturus® Pixcell® and Pixcell® Ile LCM Station.

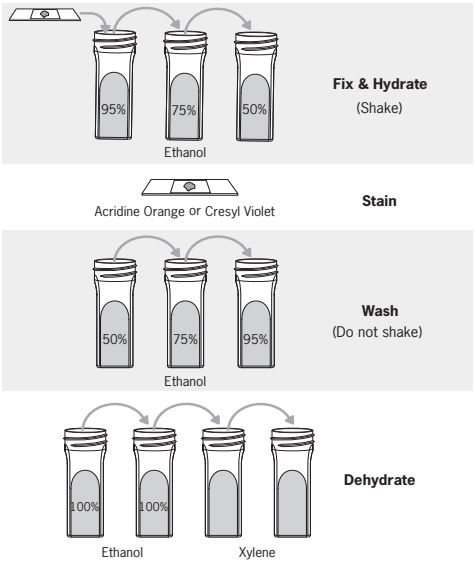
E. Related Products

RNAqueous®-Micro Kit P/N AM1931	RNAqueous-Micro Kit employs a simple and rapid procedure to purify total RNA without using organic solvents.The procedure is optimized for the purification of total RNA from micro sized samples, such as those obtained by laser capture microdissection, needle biopsies fine dissection, and low numbers of cultured cells. RNA suitable for most downstream applications can be purified in about 30 minutes.
MessageAmp™ II aRNA Amplification Kits See web or print catalog for P/Ns	Ambion® offers a full line of MessageAmp™ II Kits tailored for different array analysis applications. The MessageAmp™ II Kit offers maximum flexibility; samples can be amplified using either single- or double-round amplification, and the reagent cocktails are configured to accommodate modification. For arrays requiring biotin-labeled samples, Ambion® offers the MessageAmp™ II-Biotin *Enhanced* Single Round aRNA Amplification Kit. For preparation of fluorescently-labeled samples, we recommend the Amino Allyl MessageAmp™ II Kits which are available with and without Cy®3 and Cy®5. Bacterial RNA can be amplified using the MessageAmp™ II Bacteria RNA Amplification Kit. We also offer the MessageAmp™ II-96 and Amino Allyl MessageAmp™ II-96 aRNA Amplification Kits for high throughput applications.
RNase-free Tubes & Tips See web or print catalog for P/Ns	Ambion® RNase-free tubes and tips are available in most commonly used sizes and styles. They are guaranteed RNase- and DNase-free. See our latest catalog or our website (www.thermofisher.com) for specific information.
High quality water See web or print catalog for P/Ns	All water products are shown to be nuclease-free by stringent nuclease testing. DEPC-treated water is autoclaved both before and after packaging to assure sterility and inactivation of DEPC. Nuclease-free water that has not been treated with DEPC is also available.
RETROscript® Kit P/N AM1710	First strand cDNA synthesis kit for RT-PCR. When purchased with SuperTaq™ polymerase, this kit provides reagents, controls and protocols for reverse transcription and PCR. Both oligo(dT) and random primers for cDNA priming are included, as is RNase inhibitor.
SuperTaq™ P/N AM2050, AM2052	Thermostable DNA Polymerase (includes 10X buffers and dNTPs)
RNA 6000 Ladder P/N AM7152	The RNA 6000 Ladder is a set of 6 RNA transcripts designed for use as reference standards with the RNA 6000 Lab Chip Kits and the Agilent 2100 bioanalyzer.

II. LCM Staining Protocol

A. Procedure Overview

Figure 1. Procedure Overview of the LCM Staining Kit.



B. Before Beginning

More information on LCM

A CD with a video illustrating the protocols described in this Instruction Manual is included with this kit. If you are new to these techniques, we recommend watching the video before beginning the procedure.

RNase-free technique

RNA can be easily degraded if not handled properly. Below are suggestions for controlling exogenous RNases:

- Use the RNase*Zap*® Solution included with the kit to treat your work area and all the materials that will come in contact with the tissue (e.g., tweezers and brushes). See the package insert included with the RNase*Zap*® Solution for detailed instructions on its use. (Note: additional nuclease-free water is needed for cleaning.)
- Use RNase-free reagents.
- Wear clean disposable gloves while working with RNA.
- Do not reuse solutions or pour them back into the original containers.
- Clean the slide chambers with RNase*Zap*® Solution before each use.

Prepare ethanol solutions

100% Ethanol
Add the Dehydration Beads provided with the kit to the bottle labeled 100% Ethanol and add 500 mL of ACS grade 100% ethanol. The Dehydration Beads are used to remove trace amounts of moisture from the ethanol.


NOTE

Do not use the ethanol with dehydration beads for preparing the following solutions.

95% Ethanol
Add 475 mL of ACS grade 100% ethanol and 25 mL of Nuclease-free Water (provided in the kit) to the 500 mL bottle labeled 95% Ethanol. Mix well.

75% Ethanol
Add 375 mL of ACS grade 100% ethanol and 125 mL of Nuclease-free Water (provided) to the 500 mL bottle labeled 75% Ethanol. Mix well.

50% Ethanol
Add 250 mL of ACS grade 100% ethanol and 250 mL of Nuclease-free Water (provided) to the 500 mL bottle labeled 50% Ethanol. Mix well.

Store the ethanol solutions tightly closed at room temperature.

C. Specimen Preparation


NOTE

In general, it is important to carry out the procedures described in this protocol quickly, especially those steps that involve freezing the tissue. We recommend familiarizing yourself with the entire procedure before beginning.

1. Freeze tissue

There are two ways to freeze tissue that will protect RNA integrity and tissue morphology; ***with either method, the most important factor for success is to work quickly.***

Freeze in liquid nitrogen
Place freshly dissected tissue in a 50 mL polypropylene screw-cap tube. Close the tube and drop it into liquid nitrogen, where it will freeze immediately. Tissue frozen in liquid nitrogen usually yields higher quality RNA than tissue frozen in dry ice.

The tissue can be stored in the tube at –80°C until ready to embed.

Freeze in dry ice
Place a 50 mL polypropylene tube on dry ice and allow the tube to cool. Place the tissue into the tube and let it slowly freeze. It will freeze completely in a few minutes. The tissue morphology is better preserved by freezing in dry ice than by freezing in liquid nitrogen.

The tissue can be stored in the tube at –80°C until ready to embed.


NOTE

If the tissue is too big to fit into the 50 mL tube, cut it with a clean (RNaseZap® Solution-treated) sharp blade into approximately 1 cm² pieces.

2. Embed the tissue in OCT

a. Set up crushed dry ice and cryomolds.
Label the bottom of a disposable vinyl cryomold with the sample type and mark one edge for orientation. Cool the cryomolds in crushed dry ice.

b. Cool the embedding media in the cryomold until viscous.
Add OCT embedding media to the cryomold (about 2/3 full) and allow it to cool on dry ice until it is slushy and viscous (not solid). Remove the cryomold from dry ice if the OCT turns white, which indicates that it has frozen.

c. Set the tissue in the OCT. Allow OCT-embedded tissue to freeze by incubating ~5 min on dry ice.

i. Using forceps to orient the tissue in the OCT/cryomold, press the tissue down until it is flush with the bottom of the cryomold.

ii. Add more OCT media to the cryomold until the tissue is completely covered, then freeze the tissue completely by storing the cryomold in crushed dry ice for ~5 min.

d. Mark the frozen OCT block and store at –80°C or proceed to the next section.


IMPORTANT

This is a stopping point in the procedure. The samples can be stored at –80°C until ready to use.

When all the OCT media is completely frozen (it turns white when it freezes), mark the orientation of the tissue in the block to indicate which side to mount toward the blade. Proceed to tissue sectioning or store the frozen blocks at –80°C until ready to use. ***Do not allow the frozen blocks to warm up during transfer to or from the freezer.***

D. Tissue Sectioning and Slide Preparation

1. Prepare cryostat

a. Precool the cryostat to the temperature recommended by the manufacturer (usually –24°C to –30°C).

b. Clean the knife holder (not the knife blade itself) with 100% ethanol and treat the brushes that will be used to manipulate the tissue sections with RNase*Zap*® Solution.

c. Cool the specimen stage and brushes in the cryostat.

2. Mount embedded tissue on the specimen stage

a. Inside the cryostat, remove the frozen OCT-embedded tissue from its cryomold and quickly attach it to the metal specimen stage with OCT media. Mount the tissue securely to the metal grid. Orient the tissue according to regions of interest.

b. Inside the cryostat, let the OCT media harden (add more OCT media if needed to ensure the tissue is securely anchored to the metal specimen stage).

c. Equilibrate the tissue (at least 10 min) to the cryostat temperature (–24°C to –30°C).

3. Attach stage to cryostat and trim excess OCT

a. Attach and orient the specimen stage to the cryostat holder.

b. Using the coarse setting, carefully pulse forward to move the specimen toward the blade and cutting surface.

c. Install a fresh disposable blade into the blade holder.

d. Trim the block until the tissue becomes visible. Then set the cutting thickness to 8–10 µm. For most tissues, a section thickness of 8 µm will give the best staining results.

4. Section the tissue and transfer to a glass slide

a. Section the tissue. Use the small brushes to straighten out folded, curled, or wrinkled sections by pressing them against the cutting surface.

b. Manipulate the sections onto the central area of a precleaned glass microscope-slide.


NOTE

Keep a slide box on dry ice to store the finished slides during the sectioning step. Discard slides with folds or wrinkled sections. If you are cutting more than one tissue, use a new disposable blade for each tissue.

The microscope slide should be at room temperature before depositing the first tissue section on it; once the first section is mounted, the slide should be kept in the cryostat while the remaining sections are placed on it. Usually 2–4 sections can be positioned on each slide.

c. Put slides on dry ice.
It is very important to keep the slides on dry ice; allowing the tissue to thaw will result in deterioration of morphology and RNA.

5. Proceed to next section or store slides at –80°C

Proceed to the tissue staining protocol or store slides in a tightly closed box with desiccant at –80°C for up to 4 weeks depending on the tissue (tissues low in RNases such as brain may be stored longer).


STOPPING POINT

The slides can be stored at –80°C for up to 4 weeks at this point.


NOTE

Repeated cycling of the tissue block from –80°C to –20°C during cryosectioning will accelerate RNA degradation. It is recommended that the first time the tissue is sectioned, a sufficient number of slides be prepared for approximately 4 weeks of experiments.

E. Staining with Cresyl Violet

Cresyl Violet is a basic dye that stains negatively charged nucleic acids in the nucleus of a cell with a dark blue color. It also stains the rough endoplasmic reticulum in neurons (which may clump into Nissl bodies). In most cases Cresyl Violet staining will be sufficient for cell identification.

1. Prepare slide chambers

Label the slide chambers according to the table below. Add 25 mL of xylene in slide chambers "i" and "j" and place them in a fume hood. Add 25 mL of the ethanol solutions (prepared in section II.B) to the corresponding slide chambers.


NOTE

Treat the slide chambers with RNaseZap® Solution if they have been previously used.

Slide Chamber	Reagent
a.	95% Ethanol
b.	75% Ethanol
c.	50% Ethanol
d.	50% Ethanol
e.	75% Ethanol
f.	95% Ethanol
g.	100% Ethanol
h.	100% Ethanol
i.	Xylene (under fume hood)
j.	Xylene(under fume hood)

2. Fix tissue with decreasing concentrations of ethanol


NOTE

In the following steps, drain slides between transfers from one solution to another by gently blotting the edge of the slide on absorbent paper.

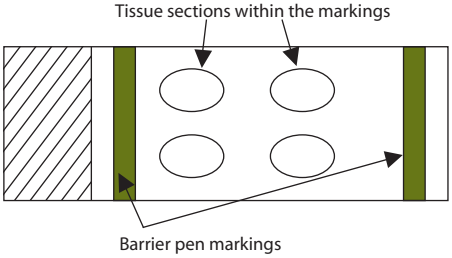
a. Place slide in 95% ethanol for 30–40 sec.
Place up to 4 slides in slide chamber "a" containing 95% ethanol for 30–40 sec. ***Do not allow slides to thaw before this step, transfer slides from the dry ice into the slide chamber as quickly as possible.***

b. Transfer slides to 75% ethanol for 30–40 sec.
Transfer slides to the chamber labeled "b" containing 75% ethanol; leave slides in the solution for 30–40 sec.

c. Transfer slides to 50% ethanol for 25–30 sec.
Transfer slides to the chamber labeled "c" containing 50% ethanol; leave slides in the solution for 25–30 sec. Slides may be gently agitated during this step to help dissolve OCT.

3. Stain for ~40 sec with 300 µL of dye

a. Mark the area to be stained with a Barrier Pen.
After draining off excess ethanol, lay slides on a flat surface and quickly mark the edges with the Barrier Pen as shown in the diagram below. The Barrier Pen marks will keep the dye in place, allowing it to penetrate and stain the tissue evenly.



b. Add 300 µL of Cresyl Violet to tissue section and stain for ~40 sec.
Add 300 µL of the Cresyl Violet stain directly onto the tissue section. Stain the tissue for 20 sec to 1 min (the ideal staining time may vary depending on the tissue, e.g., mouse brain stains well in 20 sec while small intestine requires at least 40 sec). Longer staining times will not compromise the RNA integrity. The tissue should be covered with the stain at all times during the incubation.

4. Wash tissue with increasing concentrations of ethanol

a. Drain off stain and place slides in 50% ethanol for 25–30 sec.
Tap off the stain on lint-free absorbent paper (e.g. Kimwipes® wiper) and place slides in the chamber labeled "d" containing fresh 50% ethanol solution for 25–30 seconds. ***Do not agitate the slides at this point or the tissue will begin to destain***.

NOTE
For best staining results change wash solutions d, e, f, and g every 4 slides or every time new tissue is stained to avoid cross contamination. Change xylenes every 6–8 slides.

b. Transfer the slides to 75% ethanol for 25–30 sec.
Transfer slides to the chamber labeled "e" containing 75% ethanol; leave slides in the solution for 25–30 sec.

c. Transfer the slides to 95% ethanol for 30–40 sec.
Transfer slides to the chamber labeled "f" containing 95% ethanol; leave slides in the solution for 30–40 sec.

d. Transfer the slides to the first 100% ethanol for 30–40 sec.
Transfer the slides to chamber "g" containing the first 100% ethanol for 30–40 sec.

e. Transfer the slides to the second 100% ethanol for 30–40 sec.
Transfer the slides to chamber "h" containing the second 100% ethanol wash and move them to the hood where the slide chambers containing the xylene are located. Do not let slides stay in this wash for more than 1 min.

5. Treat slides with xylene

Rinse the slides in the first xylene chamber, then transfer to the second xylene chamber for 5 min.
Transfer the slides to chamber "i" containing the first xylene and gently move each slide up and down a few times to rinse off the excess ethanol. After rinsing each slide transfer it to the second xylene container (slide chamber "j"). Allow the slides to stay in chamber "j" for 5 min without agitation.


CAUTION

Xylene is a hazardous chemical and must be handled with care under a fume hood. Use forceps to transfer the slides from xylene to xylene. Do not use fingers to do so since the xylene can penetrate common latex gloves. If your gloves come in contact with xylene, change gloves immediately and let contaminated gloves dry under the hood.

6. Remove slides from xylene and dry for 5 min

Remove the slides from the slide chamber and lean them against the inside wall of the hood on top of lint-free absorbent paper. This will allow the xylene to evaporate evenly and rapidly. Air-dry the slides under the hood for 5 min.

Place slides in a slide box with fresh desiccant and store at room temp until ready to perform LCM.


IMPORTANT

It is recommended that LCM be carried out immediately after staining for best RNA quality and yields.

F. Staining with Acridine Orange

Acridine Orange is a fluorescent intercalating dye with an excitation wavelength of 490 nm and an emission wavelength of 526 nm. If using the Arcturus Pixcell IIe instrument, this dye is visible under the Blue Filter Cube (Ex 455–495 nm/ Em >510 nm).

1. Prepare slide chambers

Label the slide chambers according to the table below. Add 25 mL of xylene in slide chambers "i" and "j" and place them in a fume hood. Add 25 mL of the ethanol solutions (prepared in section II.B) to the corresponding slide chambers.

NOTE

Treat the slide chambers with RNaseZap® Solution if they have been previously used.

Slide Chamber	Reagent
a.	95% Ethanol
b.	75% Ethanol
c.	50% Ethanol
d.	50% Ethanol
e.	75% Ethanol
f.	95% Ethanol
g.	100% Ethanol
h.	100% Ethanol
i.	Xylene (under fume hood)
j.	Xylene(under fume hood)

2. Fix tissue with decreasing concentrations of ethanol

NOTE

In the following steps, drain slides between transfers from one solution to another by gently blotting the edge of the slide on absorbent paper.

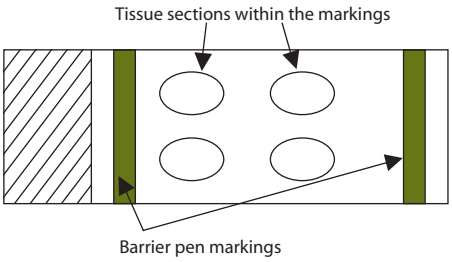
a. Place slide in 95% ethanol for 30–40 sec.
Place up to 4 slides in slide chamber "a" containing 95% ethanol for 30–40 sec. ***Do not allow slides to thaw before this step, transfer slides from the dry ice into the slide chamber as quickly as possible***.

b. Transfer slides to 75% ethanol for 30–40 sec.
Transfer slides to the chamber labeled "b" containing 75% ethanol; leave slides in the solution for 30–40 sec.

c. Transfer slides to 50% ethanol for 25–30 sec.
Transfer slides to the chamber labeled "c" containing 50% ethanol; leave slides in the solution for 25–30 sec. Slides may be gently agitated during this step to help dissolve OCT.

3. Stain for ~2 min in the dark with 300 µL of dye

a. Mark the area to be stained with a Barrier Pen.
After draining off excess ethanol, lay slides on a flat surface and quickly mark the edges with the Barrier Pen as shown in the diagram below. The Barrier Pen marks will keep the dye in place, allowing it to penetrate and stain the tissue evenly.



b. Add 300 µL of Acridine Orange to tissue section and stain for 2 min in the dark.

Before using, mix the Acridine Orange solution by vortexing for a few seconds. Add 300 µL of the Acridine Orange stain directly onto the tissue section and protect from it light (for example by covering the slide with a tent of aluminum foil; do not allow the foil to touch the stain). Stain for ~2 min (the ideal staining time may vary depending on the tissue). The tissue should be covered with the stain at all times during the 2 min incubation.

4. Wash tissue with increasing concentrations of ethanol

a. Drain off stain and place slides in 50% ethanol for 25–30 sec.

Tap off the stain on lint-free absorbent paper (e.g. Kimwipes® Wiper) and place slides in the chamber labeled "d" containing 50% ethanol solution for 25–30 seconds. ***Do not agitate the slides at this point or the tissue will begin to destain***.

NOTE

For best staining results, change wash solutions d, e, f, and g every 4 slides or every time new tissue is stained to avoid cross contamination. Change xylenes every 6–8 slides.

b. Transfer the slides to 75% ethanol for 25–30 sec.

Transfer slides to the chamber labeled "e" containing 75% ethanol; leave slides in the solution for 25–30 sec.

c. Transfer the slides to 95% ethanol for 30–40 sec.

Transfer slides to the chamber labeled "f" containing 95% ethanol; leave slides in the solution for 30–40 sec.

d. Transfer the slides to the first 100% ethanol for 30–40 sec.

Transfer the slides to chamber "g" containing the first 100% ethanol for 30–40 sec.

e. Transfer the slides to the second 100% ethanol for 30–40 sec.

Transfer the slides to chamber "h" containing the second 100% ethanol wash and move them to the hood where the slide chambers containing the xylene are located. Do not let slides stay in this wash for more than 1 min.

5. Treat slides with xylene

Rinse the slides in the first xylene chamber, then transfer to the second xylene chamber for 5 min.

Transfer the slides to chamber "i" containing the first xylene and gently move each slide up and down a few times to rinse off the excess ethanol. After rinsing each slide transfer it to the second xylene container (slide chamber "j"). Allow the slides to stay in chamber "j" for 5 min without agitation.

CAUTION

Xylene is a hazardous chemical and must be handled with care under a fume hood. Use forceps to transfer the slides from xylene to xylene. Do not use fingers to do so since the xylene can penetrate common latex gloves. If your gloves come in contact with xylene, change gloves immediately and let contaminated gloves dry under the hood.

6. Remove slides from xylene and dry for 5 min

Remove slides from the xylene slide chamber and lean them against the inside wall of the hood on top of lint-free absorbent paper. This will allow the xylene to evaporate evenly and rapidly. Protect the slides from light with aluminum foil during this drying step. Air-dry the slides under the hood for 5 min.

Place slides in a slide box with fresh desiccant and store at room temp until ready to perform LCM.

IMPORTANT

It is recommended that LCM be carried out immediately after staining for best RNA quality and yields.

III. Troubleshooting

A. Solving Problems at the LCM Step

The troubleshooting suggestions below are for LCM problems using the Arcturus Pixcell or Pixcell Ile LCM Station.

The tissue is not completely dehydrated

If the tissue was not completely dehydrated, the melted thermoplastic film may not bond to the tissue properly. The following suggestions may help correct this situation.

- Replace the ethanol washes and make sure that the 100% ethanol container is stored tightly closed to avoid introducing moisture from the air.
- Replace the washes with fresh xylene and make sure to keep the slides in xylene for the recommended 5 minutes.
- Make sure to wash the slides for the time recommended in the protocol; shorter washes will not effectively remove water from the tissue.
- Make sure that there is fresh desiccant in the slide box and always store stained slides inside the box.

The tissue is dehydrated but will not lift

- Increase/decrease the power and duration settings on the LCM station and test with a new tissue section.
- If power and duration cannot be increased, try more than one shot on the same target to lift single cells.
- It is important that the tissue does not dry between steps in the staining process. Work quickly through these steps to avoid drying the tissue.
- Try using a new LCM cap. Sometimes caps become scratched during handling, which can prevent them from fully contacting the tissue.
- If the tissue section has folds, try a different section or set the cap in a region away from the folds.
- We recommend using VWR® Superfrost® Plus Micro Slides (VWR Cat #48311-703). Use of other types of slides may result in the tissue sticking to the slide.
- If the humidity and temperature in the room are high, the tissue may become moist. Consider using a dehumidifier near the LCM station and lower the temperature in the room. Alternatively, try performing the LCM at a cooler/drier time.
- It is possible that the cap has reached its capacity. If you have been using the same cap for different tissues sections, try a new cap.
- Make sure the laser is properly focused to obtain optimal melting of the cap.

Cannot see tissue stained with Acridine Orange

- Make sure you have the upgraded Arcturus Pixcell IIe instrument with the fluorescence package that includes a high-sensitivity variable integration time, color CCD video camera and red, blue, and green filter cubes.
- Instrument check:
  - Warm up the lamp before using the fluorescent instrument for at least 15 min.
  - Use the blue filter to visualize Acridine Orange.
  - Make sure the illuminator control is completely down to block the white light.
  - Make sure the shutter is open.
- Increase the integration time until the dye is visible on the screen. Acridine Orange is a fluorescent dye; therefore, the slides need to be protected from light prior to use. If the slides were photobleached by excessive exposure to light, more integration time is needed to visualize the stain.
- Follow the protocol for staining with Acridine Orange, but increase the staining time in increments of 30 seconds. However, most tissues do not need longer than 2 minutes to stain.
- Perform LCM quickly; the fluorescent dye will slowly photobleach during LCM, requiring longer integration times. Mount only one or two sections per slide if cells are to be microdissected from dispersed areas, which requires more time.
- Try staining the tissue with a different dye.
- Test the instrument with another fluorophore; if this test fails contact Arcturus for Technical Support.
- If the troubleshooting suggestions above fail to resolve the problem, contact technical support at 1-800-888-8804.

The tissue is sticking to the plate or cutting surface

Simply spray the cutting holder, blade, and plate with Cytocool II aerosol to cool them. (See section *I.D. Required Materials Not Included with the Kit* on page 2.)

B. Poor Cell Definition

- If the staining procedure is not performed quickly, deterioration of the sample may occur. Be sure to familiarize yourself with the staining protocol before beginning.
- The tissue may have freeze-damage, which can disrupt cell structure. Repeat the procedure with new tissue or mount one section with standard mounting media to assess tissue quality. Try staining damaged tissue with Acridine Orange.

- Mount only as many sections on the slide as time permits before the slide becomes too cool for tissue for sections to attach. The structure of the tissue may be altered if the slide is warmed to promote tissue adherence.
- Do not store frozen slides more than 4 weeks.
- The tissue might have thawed before fixation. Be sure to transfer the tissue straight from the dry ice to the first ethanol solution.
- Do not shake the tissue or the slide chambers during the washing steps (after staining).
- Do not store slides longer than 1 minute in the last 100% ethanol wash.

C. Low Yields of RNA Isolated from LCM Samples

- The RNA isolation method you are using may not be optimized for LCM samples. We recommend using the Ambion® RNAqueous®-Micro Kit (P/N AM1931) for the best results.
- The starting material may have low RNA content. Microdissect more tissue for the assay or pool several LCM samples.
- The quality of the RNA in the tissue may have been compromised by surgical procedures and storage conditions prior to processing for LCM. See Appendix A for instructions on assessing RNA quality in the tissue prior to processing.

D. Troubleshooting Tissue Sectioning

The tissue is brittle

Sometimes the tissue temperature is not optimal for sectioning (too warm). Try spraying the tissue with Cytocool II rapid freezing aerosol (see section I.D).

The tissue is difficult to cut and cuts unevenly

Some cryostats will retract after each section. Do not mount every section you cut; try using every third section.

Tissue may also cut unevenly if it has not fully equilibrated to the temperature of the cryostat. Remedy this by waiting for the tissue temperature to match the cryostat temperature before attempting to cut sections.

The tissue sections increase or decrease in thickness

It is possible that the tissue was not properly embedded or that the block is too cold. Let the OCT freeze slowly during the embedding procedure on a bed of crushed dry ice. Allow tissue blocks to stabilize to the cryostat temperature for at least 10 min before sectioning.

Tissue distorts when sectioned

If the tissue is too difficult to section without creating folds or wrinkles, try cutting thicker sections (up to 10 µm).

IV. Additional Protocols

A. Assessment of RNA Quality

Checking the integrity of RNA in the tissue before sectioning

Isolate the RNA (we recommend using the Ambion® RNAqueous®-Micro Kit) from 1–2 (10 µm thick) tissue sections (depending on tissue) without mounting them onto the slide. After cutting, allow the tissue to roll into itself to facilitate handling. Add the sections to a precooled 1.5 mL microfuge tube and then add 100–200 µL of a guanadinium-based lysis solution. After RNA isolation, assess the yield and quality on a denaturing agarose gel or microfluidics bioanalyzer (such as Agilent 2100).

Checking the integrity of RNA in tissue already mounted on slides

Lyse the tissue by applying a guanadinium-based solution to the tissue on the slide. Scrape the lysate with a pipet tip, then pipet it into a microfuge tube. Extract the RNA and assess RNA quality as described above. It is a good idea to do this each time stored tissues are used for LCM to provide a reference point for RNA quality and yeild, since degradation may have occured during storage.

Determining RNA degradation during LCM

Process two extra slides when staining for LCM. Label them control "a" and control "b". After staining, immediately lyse control "a" using ~100 µL of a guanadinium-based lysis solution. Transfer the lysate to a microfuge tube and store at –20°C or –80°C. Store control "b" with the rest of the slides used for LCM. After completing the LCM, lyse control "b" in the same way as control "a" and freeze the lysate. Isolate RNA from both controls and assess RNA quality.

The RNA from control "a" will resemble the RNA quality from the first LCM sample you microdissected and the RNA from control "b" will resemble the RNA quality of the last sample you microdissected. If the RNA is degrading during the LCM step, prepare only one or two slides at a time and perform LCM as soon as possible.

The RNA isolated from the control slides can be quantitated by UV absorbance and used in downstream applications, for example to create standard curves for quantitation of RNA in LCM samples in qRT-PCR assays.

B. Analysis of RNA from LCM

RNA isolation

Use an RNA isolation method that is designed for RNA isolation from small amounts of tissue or LCM samples. The Ambion RNAqueous-Micro Kit (P/N AM1931) is recommended for RNA isolation from LCM samples.

One-step qRT-PCR

The following protocol can be used as a starting point for initial qRT-PCR experiments. The reagents can be substituted with reagents from other suppliers.

For a 25 µL reaction prepare the following master mix (prepare 10% overage):

Component	Amount
RNA from LCM sample	2– 5 µL
Nuclease-free Water	to 25 µL
10X RT buffer*	2.5 µL
dNTP mix (2.5 mM each) †	4 µL
Primer mix (forward+reverse 10 µm each)	1 µL
Probe (2 µM)	1 µL
50X ROX standard	0.5 µL
RNase Inhibitor (40U/µL)**	0.25 µL
DNA Polymerase (5U/µL) †	0.2 µL
MMLV-RT*	0.1 µL

† SuperTaq DNA Polymerase from Ambion P/N AM2052
*M-MLV Reverse Transcriptase 100U/µL from Ambion P/N AM2043
**RNase Inhibitor from Ambion P/N AM2682

qRT-PCR profile for most ABI PRISM® instruments:

reverse transcription	42°C – 15 min	
denaturation/enzyme activation	95°C – 10 min	
denaturation	95°C – 15 sec	40 cycles
extension	60°C – 40 sec–1 min	

Linear amplification of the RNA for microarrays

Two rounds of linear amplification using the Van Gelder and Eberwine technique are usually needed toproduce enough RNA for microarray studies. The Ambion MessageAmp™ II Kit (P/N AM1751) has been used to amplify LCM samples from two different mouse brain regions, and a microarray study was successfully completed using the amplified RNA.

C. 30% Sucrose Treatment

A 30% sucrose treatment may improve RNA quality and morphology of the tissue and it is especially appropriate for use with brain tissue.

1. Preparation of 30% sucrose in PBS

You will need high quality sucrose (VWR OmniPur® ultra pure RNase-free is recommended but other sources may be acceptable), nuclease-free water (P/N AM9932, not included with the kit), and nuclease-free 10X PBS (P/N AM9624).

To make 1 liter:

a. Dissolve 300 g sucrose in ~500 mL nuclease-free water plus 100 mL 10X PBS. Dissolve by stirring at room temperature.

b. Adjust the volume to 1 liter with nuclease-free water after all the sucrose has dissolved. Store in ~ 100 mL aliquots at 4°C, or –20°C for long-term storage.

2. Rinse tissue in 1X PBS

Rinse freshly dissected tissue briefly in cold, nuclease-free 1X PBS.

3. Soak tissue in 30% sucrose at 4°C for at least 4 hr

Place the tissue into a 50 mL conical tube containing ~40 mL of 30% sucrose and store at 4°C for at least 4 hours, preferably with intermittent gentle agitation. The tissue can be soaked in 30% sucrose for up to 24 hr.

4. Rinse tissue 1X PBS

Rinse tissue in cold, nuclease-free 1X PBS.

5. Proceed with sectioning

Continue from step *II.C. Specimen Preparation* starting on page 5.

V. Appendix

A. Quality Control

Functional Testing

Staining efficiency of both dyes is evaluated by comparing stained mouse kidney sections to pictures of previously stained tissue, which was set as the standard. The staining must be similar and the cell nuclei must be visible in both stains.

RNA integrity from stained tissue is evaluated by comparing RNA purified after staining with the LCM Staining Kit to RNA purified from the same tissue that was not stained. RNA is analyzed by bioanalyzer and must show no more than a 0.2 decrease of 28S/18S ratio from the control RNA (from unstained tissue) to the test RNA (from stained tissue).

B. Safety Information

Chemical Safety

To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.).
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.

- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

IMPORTANT Radioactive or biohazardous materials may require special handling, and disposal limitations may apply .

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Safety equipment also may include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, Biosafety in Microbiological and Biomedical Laboratories (BMBL), 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf
- World Health Organization, Laboratory Biosafety Manual, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

C. Documentation and Support

Obtaining SDS

Safety Data Sheets (SDSs) are available from **www.lifetechnologies.com/support**.

For the SDSs of chemicals not distributed by Life Technologies, contact the chemical manufacturer.

Obtaining Certificates of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to www.lifetechnologies.com/support and search for the Certificate of Analysis by product lot number, which is printed on the box.

Obtaining Support

For the latest services and support information for all locations, go to:

www.lifetechnologies.com/support

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**techsupport@lifetech.com**)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**

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