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Arcturus® HistoGene® Immunofluorescence Staining Kit

User Guide

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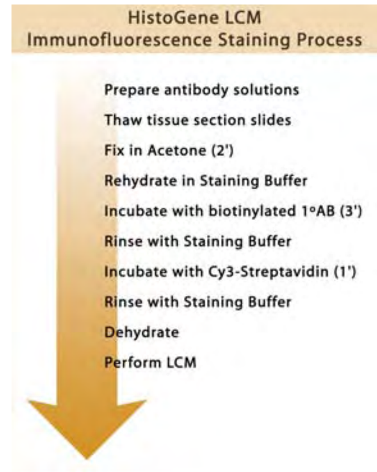
I. Introduction

A. Background

The HistoGene® LCM Immunofluorescence Staining Kit is the only kit designed to enable retrieval of high-quality RNA from immunofluorescently stained frozen tissue. It enables convenient and reliable staining, dehydration and Laser Capture Microdissection (LCM) of tissue sections with protocols streamlined and optimized both for optimal LCM captures and maintaining RNA quality for downstream applications that require intact RNA, like microarray analysis and RT-PCR.



The HistoGene Immunofluorescence Staining Kit uses a two-step immunostaining technique that works with any biotinylated monoclonal antibody. It is based on use of biotin-avidin system: a primary biotinylated antibody and Cy®3-conjugated Streptavidin. Avidin is an egg-white derived glycoprotein with an extraordinarily high affinity for biotin. Many biotin molecules can be coupled to a protein, enabling the biotinylated protein to bind more than one molecule of avidin. Cy3 dye is used because it offers the best staining intensity and works well with the biotin-avidin system.

Liver, kidney and some lymphoid tissues contain endogenous biotin and are therefore not recommended for staining with Streptavidin based IHC procedures.

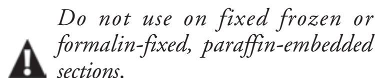
The advantages of using the biotin-avidin system are that it improves sensitivity and reduces background fluorescence. Furthermore, the high affinity between biotin and avidin assures the user of a rapidly formed and stable complex between primary antibody and the avidin-fluorophore conjugate. Thus, the biotin-avidin system provides highly specific staining with very little background and enables short staining times necessary to maintain RNA integrity.

This kit should not be used with antibodies requiring enzyme digestion or target retrieval.

The unique process is performed at 4°C using specially formulated Staining Buffer. This dramatically increases RNA yield from captured cells. The HistoGene Cold Block (HIS0101) makes the 4°C processing very convenient. It holds up to four slides and has places for reagent tubes. It is designed for use with the CoolSafe triple density polystyrene cooler (Cat. # CSF-BOX)

and –10°C cool brick (Cat. # BRIK-1520) from Diversified Biotech (www.divbio.com). The proprietary Staining Buffer perserves the quality of RNA in the tissue sections and preserves good, specific staining intensity.

The HistoGene Immunofluorescence Kit requires starting with unfixed, frozen tissues known to contain intact RNA. Thus, we recommend that you verify RNA quality before you begin. See Appendix A. All Kit components used are premium grade, LCM certified and nuclease free to ensure RNA quality is maintained during the process.


Do not use on fixed frozen or formalin-fixed, paraffin-embedded sections.

B. Storage and Stability

1. HistoGene LCM Immunofluorescence Staining Kit

Store the staining reagents as follows:

Buffer A (–70°C)
Buffer B (4°C)
Cy3 Streptavidin (4°C)

Store slides at room temperature and the Cold Block at 4°C. Do not freeze and thaw Buffer A repeatedly.

2. HistoGene Cold Block

Store at 4°C.

C. Safety Data Sheets

Safety Data Sheets (SDS) for kit chemical components are available from Technical Services.

Call: 1 800 831-6844 option 5.

You can also obtain these sheets from:
www.appliedbiosystems.com.

D. Related Products

PicoPure® RNA Isolation Kit
For extraction and isolation of total RNA from small samples particularly Laser Capture Microdissected (LCM) cells. The PicoPure RNA Kit comes with optimized buffers, MiraCol™ Purification Columns and an easy-to-use protocol to maximize recovery of high-quality total cellular RNA ready for amplification with the RiboAmp OA 1 Round RNA Amplification Kit.

RiboAmp® PLUS RNA Amplification Kit
The RiboAmp PLUS RNA Amplification Kit enables the production of microgram quantities of antisense RNA (aRNA) from nanogram quantities of total cellular RNA. Amplified RNA produced using the kit is suitable for labeling and use for probing expression microarrays. The kit achieves amplifications of up to 1000-fold in one round of amplification, and amplifications of up to 1,000,000-fold in two rounds. The RiboAmp PLUS Kit comes with all necessary enzymes, reagents, and MiraCol purification columns needed to complete the included amplification protocol.

RiboAmp® HS RNA Amplification Kit
The RiboAmp HS RNA Amplification Kit starts with picogram total cellular RNA input and enables the production of microgram quantities of antisense RNA (aRNA). The Kit provides the greatest level of sensitivity in starting RNA quantities to produce enough RNA suitable for labeling and hybridizing onto expression microarrays. The RiboAmp HS Kit comes with all necessary enzymes, reagents, and MiraCol Purification Columns needed to complete the included amplification protocol.

PicoPure® DNA Extraction Kit

The PicoPure DNA Extraction Kit is optimized to maximize the recovery of genomic DNA from 10 or more cells captured by LCM. The kit comes with reagents and protocol tested to ensure complete extraction of DNA from LCM samples prepared with any standard tissue preparation procedure. DNA prepared using the kit is *PCR-ready* and needs no additional purification to perform amplification.

HistoGene® LCM Frozen Section Staining Kit

The HistoGene LCM Frozen Section Staining Kit is used to process tissue sections for LCM that maximizes the quality and yield of RNA from LCM cells. The Kit comes with all dehydration and staining reagents, disposable staining jars, specially treated slides, and detailed protocol and troubleshooting guide.

Paradise® PLUS Reagent System

The Paradise PLUS Reagent System is the only reagent system designed to enable gene expression studies using formalin-fixed paraffin-embedded (FFPE) tissue samples. Components include sample preparation and staining reagents, RNA extraction and isolation reagents, RNA amplification reagents and a comprehensive user guide.

II. Kit Components

A. Reagents and Supplies in Kits

The HistoGene Immunofluorescence Staining Kit includes reagents to stain 32 tissue sections. **Fixation and dehydration reagents are not supplied with the Kit.** See additional materials required below.

- ❑ Buffer A (8 x 500 µl)
- ❑ Buffer B (60 ml)
- ❑ Cy3 Streptavidin (60 µl)
- ❑ Slides (72)
- ❑ User Guide

B. Additional Equipment and Materials Required

1. Materials

- ❑ PAP pen or ImmEdge pen (Vector Laboratories, Cat. # H-4000)
- ❑ RNase-free pipet tips
- ❑ 15 ml nuclease-free Falcon tubes
- ❑ Microcentrifuge tubes, nuclease-free
- ❑ RNaseAway (Life Technologies, Cat. # 10328-011)
- ❑ Kimwipes
- ❑ Tissue-Tek® OCT Compound (VWR Cat. # 25608-930)
- ❑ Staining jars (Evergreen Scientific # 222-5450-G8S)
- ❑ Dry ice
- ❑ Disposable gloves
- ❑ Tissue-Tek® Cryomold (VWR Cat. # 25608-916)

2. Equipment

- ❑ -70ºC freezer
- ❑ Fume hood
- ❑ Cryostat with disposable microtome blades
- ❑ Pipettors: 1000 µl, 200 µl, 100 µl, 10 µl
- ❑ Cover glass forceps
- ❑ HistoGene Cold Block (Applied Biosystems Cat. # HIS0101)
- ❑ Thermometer, celsius
- ❑ Microslide box, plastic (VWR Cat. # 48444-004)
- ❑ Refrigerator

2. Reagents

- ❑ Acetone
  (VWR Cat. # AX-125-4; Fisher Cat. # HC-300-GAL)
- ❑ Xylene
  (VWR Cat. # EM-XX0060-4; Fisher Cat. # HC-700-GAL)
- ❑ Ethanol 100%
  (VWR Cat. # 34172-020; Fisher Cat. # HC-800-GAL)
- ❑ Ethanol 95%
  (VWR Cat. # 34172-022; Fisher Cat. # HC-1100-GAL)
- ❑ Ethanol 70%
  (VWR Cat. # 34172-000; Fisher Cat. # HC-1000-GAL)
- ❑ Isopentane or 2-Methylbutane (VWR Cat. #JTQ223-7)

III. Preliminary Steps

A. Material and Protocol Review

This protocol is only for immunofluorescent staining of acetone fixed frozen tissue sections.

To get the most from your HistoGene LCM Immunofluorescence Staining Kit, take a few moments to examine the components of the kit and read through the information in this Section and Section IV.

B. Recommendations for RNase-free Technique

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

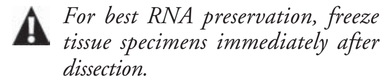
- Wear disposable gloves and change them frequently.
- Use RNase-free solutions, glassware and plasticware.
- Do not re-purify HistoGene LCM Immunofluorescence Staining Kit components. They are certified Nuclease Free.
- Wash scalpels, tweezers and forceps with detergent and bake at 210°C for four hours before use.
- Use RNase AWAY® solution (Life Technologies) according to the manufacturer's instructions on any surfaces that may come in contact with the sample.

C. Checking RNA Quality in Starting Material

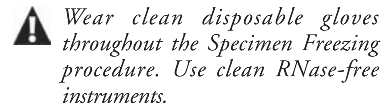
Before you begin preparing specimens, it is critical that you first check the quality of the RNA in the tissue to ensure that your samples contain intact, high-quality RNA. See Appendix A.

IV. Protocol

A. Specimen Freezing



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



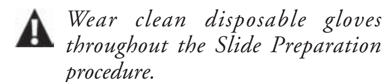
Wear clean disposable gloves throughout the Specimen Freezing procedure. Use clean RNase-free instruments.

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.

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.

It is okay to stop at this point in the protocol.

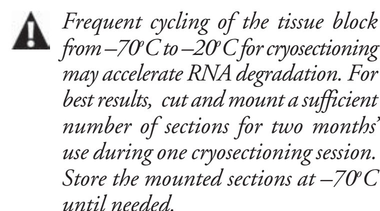
B. Slide Preparation



Wear clean disposable gloves throughout the Slide Preparation 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. Dry wet surfaces with another dry Kimwipe.
3. Install a new disposable microtome blade in the cryostat.
4. Set cutting thickness to 8-10 µm.
5. Place a microslide box on dry ice near the cryostat.
6. Transfer the cryomold containing your specimen from the –70°C freezer to the cryostat, transporting on dry ice if necessary.
7. Wait a minimum of 10 minutes for your specimen to equilibrate with the temperature of the cryostat.
8. Mount specimen to specimen holder with OCT. Cut 8-10µm sections.
9. Mount sections toward 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.
11. If you are cutting more than one specimen, use a new unused section of the disposable microtome blade or a new blade for each one. In addition, wipe down the knife holder and anti-roll plate with 100% ethanol in between each specimen to avoid cross-contamination.
12. Upon completion of sectioning, the surface of the specimen must be protected with a thin layer of OCT prior to storing at –70C. This can be accomplished by applying a small amount of OCT on the surface of the block and immediately applying a metal block that has been pre-cooled to the cryostat's temperature. To separate the two objects once the OCT has solidified, take a razor blade and place it between the metal block and the OCT. Once separated, carefully remove the specimen block from the specimen holder using the razor blade. Remove any excess OCT surrounding the block with the razor blade and replace the specimen into its cryomold.
13. Proceed immediately to the "Staining and Dehydration" segment of the protocol or store slides in a slide box at –70°C for up to two months.



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.

C. Primary Biotinylated Antibody Solution Preparation

When using the HistoGene LCM Immunofluorescence Staining Kit, it is important to use primary antibodies proven to be of high quality. They should be tested in a conventional immunostaining procedure and shown to produce good staining intensity with low background before proceeding with the HistoGene LCM Immunofluorescence Staining protocol.

The recommended concentration of the primary biotinylated antibody should be between 30–100 µg/ml. The final concentration of the primary antibody should be optimized by the user by performing a serial dilution staining experiment using the HistoGene Immunofluorescence Kit's Staining Buffer (Buffers A & B combined).

Biotinylated antibodies against many different antigens are now commercially available. If a certain antibody cannot be obtained from a commercial source the biotinylation can easily be performed using a biotinylation kit (Pierce cat # 21338, Sigma Cat. # B-TAG).

It should be noted, that some commercially available biotinylated antibodies have low IgG concentrations and need to be used undiluted.

To perform a negative control staining, use a biotinylated control antibody from the same animal species and of the same isotype as your primary antibody. Dilute to the same working concentration as the primary antibody. (Mouse control antibodies can be obtained from DAKO Cat. # X0928).

Working solution of the primary biotinylated antibody and the negative control antibody should be prepared in Staining Buffer (Buffers A & B) and held at 4°C. The recommended concentration of Buffer A in all antibody solutions is 10 mM. If preparing the antibody solution dilutes the Buffer A concentration below 10 mM, add an adequate volume of the Buffer A stock solution (100 mM) to the antibody solution.

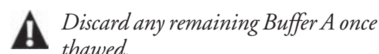
D. Cy3 Streptavidin Conjugate Preparation

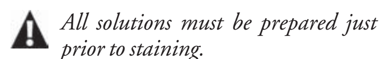
Working solution of Cy3 Streptavidin should be prepared in Staining Buffer (Buffer A & B) and held at 4°C, protected from light. The recommended dilution of the Cy3 Streptavidin is 1:100. User should, however, determine the optimal staining concentration of the Cy3 Streptavidin conjugate by performing a serial dilution staining experiment using the HistoGene Immunofluorescence Kit's Staining Buffer (Buffers A & B).

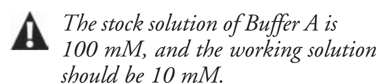
E. HistoGene LCM Immunofluorescence Staining Protocol

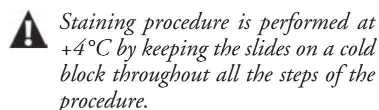
Minimizing the time required and keeping the samples cold during immunoflourescent staining is critical for obtaining high RNA quality and yield. For best results, the staining protocol should be performed within a maximum of 10 minutes, not including dehydration steps. This requires maintaining a steady workflow. To help minimize preparation time and keep the samples cold, we recommend that you do not process more than 4 slides at once. Also do not allow the sections to dry after Step 14 of the protocol. Drying may result in non-specific staining, loss of specific staining and it may compromise RNA quality.

1. Pre-cool the HistoGene Cold Block in a refrigerator to 4°C.
2. Take out one aliquot of frozen Buffer A and thaw.
3. Prepare fixative and dehydration reagents in the fume hood.
4. Label 5 plastic jars as follows:
   a Acetone
   b. 70% ethanol
   c. 95% ethanol
   d. 100% ethanol
   e. Xylene
5. Fill each labeled jar with 25 ml of solution.
6. Cool Acetone to 4°C.
7. Take the cold block out of the refrigerator and place it in the CoolSafe box or on wet ice. Just prior to staining, check the temperature of the cold block by placing a thermometer in one of the tube holes. The temperatures should be 4°C or


Discard any remaining Buffer A once thawed.

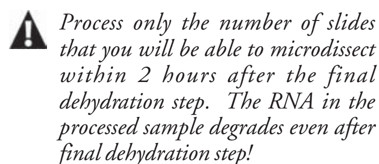

All solutions must be prepared just prior to staining.

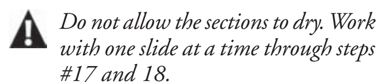

The stock solution of Buffer A is 100 mM, and the working solution should be 10 mM.

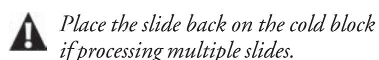

Staining procedure is performed at +4°C by keeping the slides on a cold block throughout all the steps of the procedure.

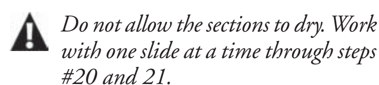
below.

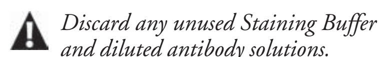
8. Prepare Staining Buffer by adding one aliquot (500 µl) of the Buffer A into 4500 µl of Buffer B. Store tube in the cold block. Discard any remaining Buffer A once thawed.
9. Prepare primary antibody solutions as described in Section IV.C and keep them in the cold block.
10. Prepare the Cy3 Streptavidin conjugate and keep it in the dark and at 4°C as described in Section IV.D.
11. Remove a maximum of four slides from the –70°C freezer, allow no more than 30 seconds for the condensation to disappear prior to fixing in acetone.
12. Place slides in cold acetone (4°C) for 2 minutes.
13. Remove from acetone and air dry the slides in the fume hood for 30 seconds.
14. Use hydrophobic barrier pen to circumscribe each section to keep the staining reagents localized on the tissue section.
15. Place all the slides on a cold block.
16. Apply 200 µl of Staining Buffer per section to each slide.
17. Drain off staining buffer and wipe the slide gently, without touching the section, with a clean Kimwipe.
18. Place the slides on the cold block and apply 100 µl per section of the diluted primary antibody and incubate for 3 minutes.
19. Drain off the antibody solution and rinse the sections by applying 200 µl of Staining Buffer per section, drain and repeat rinsing.
20. Drain off staining buffer and wipe the slide gently with a clean Kimwipe.
21. Place the slides on the cold block and apply 100 µl per section of the diluted Cy3 Streptavidin and incubate for 1 minute.
22. Drain off and rinse by applying 200 µl of Staining Buffer per section, drain and repeat rinsing.
23. Immediately proceed to the dehydration protocol.


Process only the number of slides that you will be able to microdissect within 2 hours after the final dehydration step. The RNA in the processed sample degrades even after final dehydration step!


Do not allow the sections to dry. Work with one slide at a time through steps #17 and 18.


Place the slide back on the cold block if processing multiple slides.


Do not allow the sections to dry. Work with one slide at a time through steps #20 and 21.


Discard any unused Staining Buffer and diluted antibody solutions.

Dehydration is performed at room temperature.

1. Place the slides in 70% ethanol for 30 seconds.
2. Transfer into 95% ethanol for 30 seconds.
3. Transfer into freshly dispensed 100% ethanol for 30 seconds.
4. Transfer into xylene for 5 minutes.
5. Dry the slides in a fume hood for 5 minutes.
6. Immediately perform Laser Capture Microdissection.

V. Troubleshooting

A. Targeted Cells Do Not Lift From the Slide During LCM

1. The sample may contain residual water. Ensure that the ethanol solutions are fresh. Ethanol is hygroscopic. Keep the 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.
2. The sample may have dried between protocol steps. Perform all steps of the protocol at a steady pace.

B. No Staining

1. Make sure that the primary antibody is used at right concentration and that it is active. Perform a titration experiment using both high and low concentration of the primary antibody.
2. You can test the activity of the antibody on a section taken from another known positive tissue.
3. Make sure that the Cy3 Streptavidin is not inappropriately diluted. Perform a titration experiment using both high and low dilutions of the Cy3 conjugate.

C. Background Staining

1. The primary antibody used is too concentrated. Perform a titration experiment using both high and low concentrations of the primary antibody to achieve clean specific staining without background.
2. The primary antibody may cross-react with other tissue epitopes and bind non-specifically. Change source or species of the primary antibody.
3. Make sure that sections are kept moist during all steps of the staining.
4. Endogenous biotin may be present in the tissue. To check if this is occurring perform a staining omitting the primary antibody application.
5. Tissue such as kidney, liver and adrenals as well as some lymphoid tissues contain different levels of endogenous biotin and should not be used with a biotin/avidin system.

D. RNA Cannot be Recovered from the Sample

1. The sample starting material may contain poor quality RNA. Freeze the sample immediately following dissection, and take care to use RNase-free technique.
2. RNA may become degraded during RNA isolation. Wear gloves; use RNase-free technique and RNase-free instruments and reagents. Wipe down the Laser Capture Microdissection System with RNase AWAY prior to use.
3. 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.
4. The starting material quantity may be insufficient. Use at least 500 captured cells.

VI. Appendix

A. Tissue Scrape Protocol for Verifying RNA Quality Using the PicoPure RNA Isolation Kit

Applied Biosystems recommends verifying the integrity of RNA in the tissue sample before proceeding with staining and Laser CaptureMicrodissection (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.

For more information, visit: www.appliedbiosystems.com, or call technical support at: 1 800 831-6844 option 5.

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