

Laser-Assisted Microdissection of Membrane-Mounted Sections Following Immunohistochemistry and *In Situ* Hybridization

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Summary

Laser microbeam microdissection (LMM) is an increasingly important histological technique for obtaining homogeneous cell populations and tissue components in order to analyze target-specific changes in genes, gene expression, and proteins. The quality of data obtained with LMM is heavily dependent on the precision with which the target for microdissection can be identified. Since no cover slip is used during LMM, tissue morphology is poor compared with traditional light microscopy. This hampers morphological recognition of targets for microdissection in routinely stained sections and can be a limiting factor in the use of this technique. Immunohistochemistry (IHC) and *in situ* hybridization (ISH) can improve the identification of specific cell populations *in situ* in tissue sections, but there are a number of problems in applying these methods to slides prepared for LMM. In this chapter, we present optimized protocols that allow IHC to be performed for detecting a wide range of antigens in conjunction with LMM, both on formalin-fixed paraffin-embedded and on frozen sections. In addition, we present a quick, versatile protocol for performing ISH on archival material suitable for LMM.

Key Words: Laser-assisted microdissection; membrane-mounted tissue; MOMeNT; frozen tissue; formalin-fixed paraffin-embedded tissue; immunohistochemistry; *in situ* hybridization.

1. Introduction

This chapter focuses on methods for improved identification of cell populations in membrane-mounted sections intended for laser microbeam microdissection (LMM). Pathologically altered tissues are often markedly heterogeneous-malignant tumors, for example containing a variable (and often significant) admixture of non-neoplastic cells. Some tumors may be made up

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of neoplastic cells that are morphologically relatively similar to normal cell types (e.g., many lymphomas), making reliable identification of target cells in paraffin-embedded sections difficult and in frozen sections often impossible. Furthermore, since cover slips are not used during LMM, tissue morphology is poor compared with traditional light microscopy, hampering the morphological recognition of targets for microdissection in routinely stained sections. One approach to avoiding the problems associated with poor morphology is to immunostain target cells with antibodies against specific antigens not expressed in other cells in the tissue (**1–6**), thus increasing the specificity, precision, and speed of microdissection. LMM may be performed in combination with MOMeNT (microdissection of membrane-mounted native tissue), thus facilitating dissection and transfer of large intact specimens and reducing the risk of contamination. However, this technique is difficult to use in combination with traditional immunohistochemistry (IHC) and *in situ* hybridization (ISH) staining methods. Both the tissue section and the membranes are easily damaged using standard staining protocols. In particular, the use of microwave superheating for heat-induced epitope retrieval (HIER)—an often essential step if sufficiently sensitive staining of paraffin sections is to be achieved—may result in detachment of both the membranes and sections, and it is potentially damaging to target nucleic acids (**7**).

In response to these problems, we have optimized protocols for IHC and ISH using “low”-temperature HIER, which can be used reliably with membrane-mounted tissue sections. There are many different staining protocols for IHC and we have focused on the detection systems used routinely in our laboratory. The protocols presented here can be performed on formalin-fixed paraffin-embedded (FFPE) and frozen tissue, and are suitable for detection of a wide range of antibodies and different nucleic acids (**8**). The protocols are easy to perform, and have been tested in various different settings and organ-systems.

2. Materials

2.1. Immunohistochemistry for MOMeNT on Formalin-Fixed Paraffin-Embedded Slides

1. Humid chamber.
2. Disposable plastic pipets.
3. Membrane-mounted slides—two types as described in Chapter 11.
4. Xylene stored at room temperature (RT).
5. Graded alcohols (99%, 96%) at RT.
6. 0.5% (v/v) H₂O₂ in methanol: 100 mL methanol + 1.5 mL 35% w/w H₂O₂.
7. TEG buffer: Tris-EGTA, pH 9.0, 0.1% diethylpyrocarbonate (DEPC) at RT.

8. Primary antibody: Melan-A (M7196, DakoCytomation, Copenhagen, Denmark) or Ki-67 (M7240, DakoCytomation), both used 1:10 in TBS, stored at 4°C.
9. TBS buffer: 50 mM Tris-HCl, 150 mM NaCl₂, pH 7.6, 0.1% DEPC at RT.
10. EnVision™ + (peroxidase conjugated mouse; DakoCytomation K4001) at 4°C.
11. Chromogen: Diaminobenzidine (DAB) 4170 (Kem-En-Tec Diagnostics A/S, Copenhagen, Denmark).
12. Hydrogen peroxide (H₂O₂).
13. Histological counterstain: Mayer's hematoxylin, toluidine blue, or methyl green.

2.2. Immunohistochemistry for MOMeNT on Frozen Sections

1. Humid chamber.
2. Disposable plastic pipets
3. Membrane-mounted slides—two types as described in Chapter 11.
4. Absolute acetone at 4°C.
5. Coons buffer: 0.9% NaCl, 0.45 g Na₂HPO₄·2H₂O, 1.68 g NaH₂PO₄·H₂O at pH 7.1 per liter with 1% bovine serum albumin (BSA) at 4°C.
6. Primary antibody: Melan-A (M7196, DakoCytomation) 1:10 or Ki-67 (M7240, DakoCytomation) 1:5, both in Coons buffer at 4°C.
7. Secondary antibody (biotinylated swine anti-mouse immunoglobulins; DakoCytomation, E 0453) and avidin-biotinylated horseradish peroxidase complex (ABC, K0355, DakoCytomation) or EnVision™ + (peroxidase conjugated mouse; DakoCytomation K4001) at 4°C.
8. Tris buffer: 100 mM Tris-HCl, pH 7.6, 150 mM NaCl₂ at RT.
9. Chromogen: DAB (3,3'-diaminobenzidine tetrahydrochloride) 4170, Kem-En-Tec Diagnostics A/S).
10. H₂O₂.
11. Histological counterstain: Mayer's hematoxylin, toluidine blue, or methyl green.
12. Graded alcohols (99%, 96%) at RT.

2.3. In Situ Hybridization for MOMeNT on Formalin-Fixed Paraffin-Embedded Slides

1. Humid chamber.
2. Disposable plastic pipets.
3. Membrane-mounted slides—two types as described in Chapter 11.
4. Xylene at RT.
5. Graded alcohols (99%, 96%) at RT.
6. 0.1% DEPC/H₂O at RT.
7. 0.4% Paraformaldehyde/10x PBS (pH 7.4) at RT.
8. Proteinase K (S3004, 1:10 in TBS, DakoCytomation) at 4°C.
9. Cover slip (nonsiliconized).
10. EBER-specific probe (developed in our laboratory): hybridization mixture consists of 2 ng/μL probe, 50% deionized formamide, 2x standard saline citrate (SSC), 10% dextran sulfate, and 200 μg/mL tRNA. Store at 4°C until use.

11. Monoclonal anti-digoxin mouse primary antibody (D8156 [clone D1-22], 1:2500; Sigma-Aldrich Denmark A/S, Copenhagen, Denmark).
12. 0.5% (v/v) H₂O₂ in methanol: 100 mL methanol + 1.5 mL 35% w/w H₂O₂.
13. 2x SSC: 3 M NaCl₂, 0.3 M Na citrate, pH 7.0.
14. EnVision™+ (peroxidase conjugated mouse; DakoCytomation, K4001).
15. TBS buffer at RT.
16. Chromogen (DAB; 4170 Kem-En-Tec Diagnostics A/S).
17. Counterstain: Mayer's hematoxylin or toluidine blue.

3. Methods

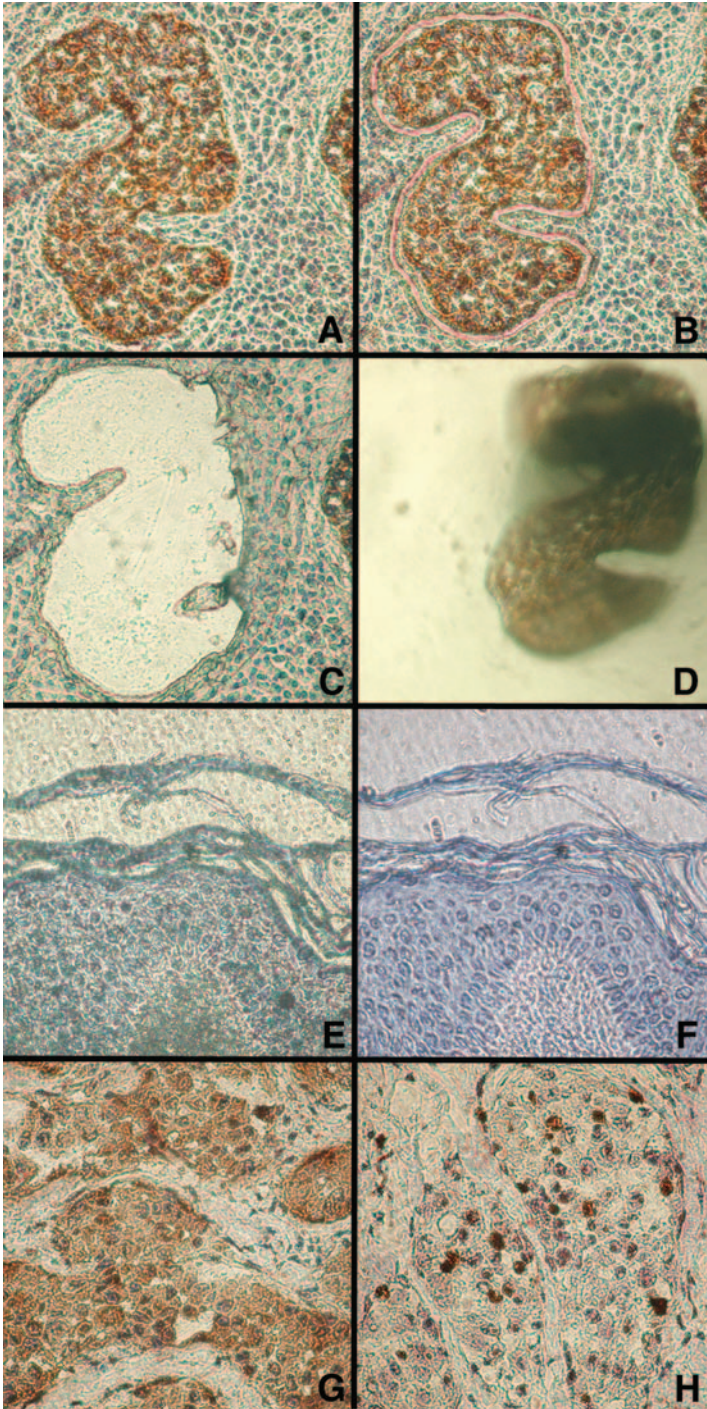
In the following section, we present brief staining protocols for IHC on FFPE and frozen tissues, and for ISH on FFPE. As a model for IHC protocols, we stained sections of malignant melanoma lymph node metastases for Melan-A and Ki-67 (*see Fig. 1G–H*), on both FFPE and frozen tissues. ISH was performed on paraffin sections from a post-transplantation B-cell lymphoma. This tumor was Epstein-Barr virus (EBV)-positive, as shown by nuclear positivity for EBERS (small EBV encoded early RNAs) detected using RNA-ISH with a digoxigenin-labeled polymerase chain reaction (PCR)-generated single-stranded DNA probe developed in our laboratory *see Fig. 2A–D*).

3.1. Immunohistochemical Stains for Melan-A and Ki-67 on FFPE Tissues Using EnVision + Staining Protocol

3.1.1. Removal of Paraffin and Blocking of Endogenous Peroxidase

1. Preparation of membrane-mounted slides and cutting of FFPE tissues has been described in detail in Chapter 11.
2. If the sections are handled carefully, no extra adhesion (e.g., with poly-L-lysine or APES) of the tissue is normally needed (*see Note 1*).
3. Removal of paraffin is carried out in xylene for 2 × 2 min, followed by 2 min in 99% and 96% alcohol each.
4. Endogenous peroxidase is blocked with 0.5% H₂O₂ in methanol (*see Note 2*) and sections are briefly washed in distilled water for 1 min.

Fig. 1. (*opposite page*) UV laser-assisted microdissection of membrane-mounted immunostained paraffin sections. (A–D) Membrane-mounted sections from Epstein-Barr-infected, undifferentiated nasopharyngeal carcinoma stained with pancytokeratin AE1/3. (A) Positive staining helps identify target carcinoma areas before microdissection. (B) Targeted area after cutting. (C) Same area after removal of target fragment. (D) Microdissected fragment on the needle tip. (E–F) Skin stained with H & E prior to microdissection. Before (E) and after (F) using optical medium (Cytotec) to improve morphology. (G–H) Membrane-mounted sections from lymph node metastasis from malignant melanoma stained for cytoplasmic Melan-A (G) and nuclear Ki-67 antigen (H).



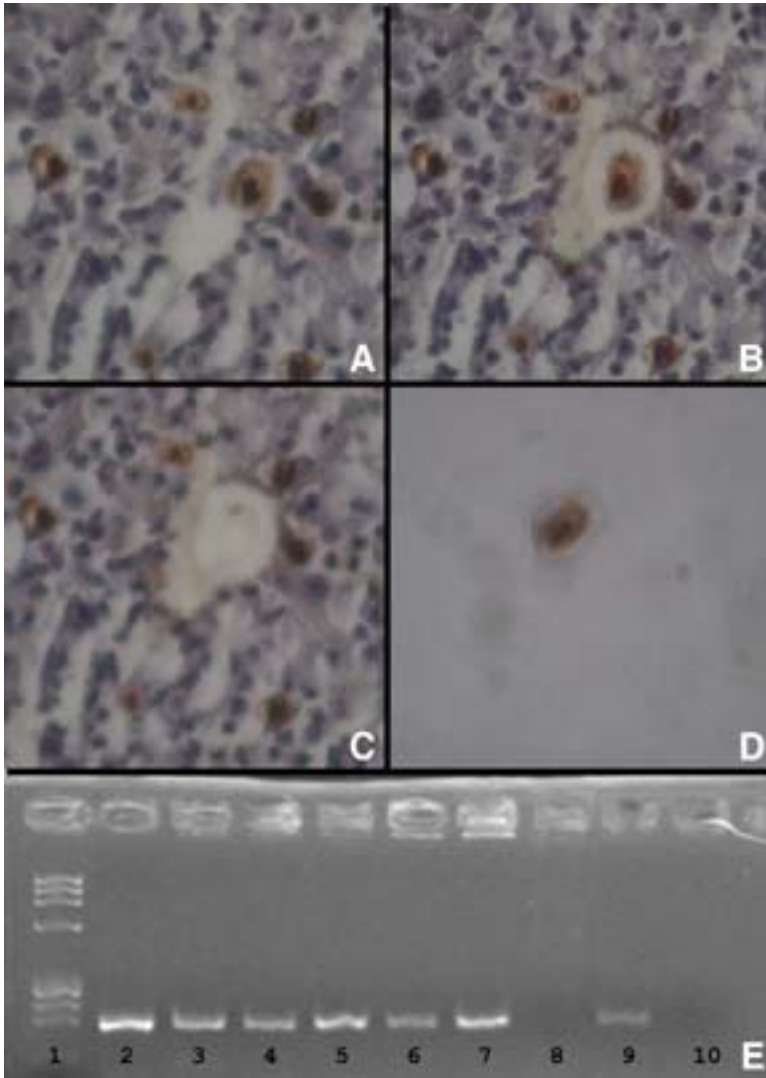


Fig. 2. UV laser-assisted single-cell microdissection of EBV-positive Hodgkin's lymphoma in paraffin sections (A–D). Hodgkin/Reed-Sternberg (HRS) cells show strong nuclear staining after *in situ* hybridization for EBERS. A single HRS cell is seen before microdissection (A) and after isolation by laser cutting (B). (C) The same field after the cell is removed. (D) High-power view of the adhesive cap in the PCR tube containing a single microdissected HRS cell. (E) Gel analysis of PCR products after amplification for the housekeeping gene NPM. Lane 1: size marker; lane 2: positive control consisting of 50 microdissected neoplastic and non-neoplastic cells; lane 3: 25 individually dissected HRS cells; lanes 4 and 5: 15 individually dissected HRS cells; lanes 6 and 7: 10 individually dissected HRS cells; lanes 8 and 10: negative controls; lane 9: positive whole section Hodgkin's lymphoma control.

3.1.2. "Low"-Temperature Heat-Induced Epitope Retrieval

1. Unmasking of epitopes is achieved by prolonged "low"-temperature incubation in TEG buffer (pH 9.0) at 60°C in a hot-air oven (**8**) (*see Note 3*).
2. The sections are placed in a glass Coplin jar, covered with ample buffer (to avoid drying out), and incubated overnight (approx 18 h).
3. The next day, the sections are allowed to cool down for 20 min in the same buffer.
4. The slides are then placed in a humid chamber and washed briefly in TBS buffer using disposable plastic pipets.

3.1.3. Immunostaining and Visualization

1. Sections are covered with primary antibody solution (200 μ L of Melan-A or Ki-67; both diluted 1:10 in TBS), incubated for 5 min, then washed in TBS using disposable plastic pipets for approx 1 min (*see Note 2*).
2. They are then incubated in 200 μ L undiluted EnVision + for 10 min, before again being washed in TBS.
3. One tablet of DAB is dissolved in 10 mL distilled water approx 30 min before use.
4. Immediately before application, 10 μ L H₂O₂ is added.
5. Slides are incubated in DAB for 10 min and washed in TBS.
6. The sections are counterstained with Mayer's hematoxylin for 2 min (*see Note 4*), washed in distilled water for 1 min, and dehydrated through graded alcohols (99%, 96%, 1 min each).
7. The sections are allowed to dry in a fume cupboard before microdissection (*see Note 5*).

3.2. Melan-A and Ki-67 Staining Protocols for Frozen Sections Using EnVision + and ABC Detection

3.2.1. Fixation

1. The cut frozen sections are placed on membrane-mounted sections as described in Chapter 11, before being fixed in cold absolute acetone for 3 min, and then dried for 15 s.
2. The slides are placed in a humid chamber and washed with Coons buffer for 1 min using a disposable plastic pipet.

3.2.2. Immunostaining and Visualization

1. Sections are covered with the primary antibody solution (Mela-A [1:10] or Ki-67 [1:5] in Coons buffer, 200 μ L each), incubated for 5 min, and then washed in Coons buffer.
2. For Melan-A staining, 200 μ L of undiluted EnVisionTM + is applied for 10 min and then washed in Tris-HCl.
3. Alternatively for Ki-67 staining, sections are first incubated for 5 min in 200 μ L of secondary biotinylated anti-mouse immunoglobulin antibody, washed in TRIS, incubated for 5 min in the tertiary layer avidin-biotinylated horseradish peroxidase complex (200 μ L), and finally washed in Tris-HCl.

4. Signal detection using DAB, counterstaining, dehydration and drying are carried out as described for paraffin section immunohistochemistry.

3.3. In Situ Hybridization for EBER on FFPE Tissues Using EnVision + Visualization System

3.3.1. Preparation of Slides and Sections

1. The preparation of the membrane-mounted glass slides and cutting of the tissue is described in detail in Chapter 11.
2. Adhesion of the tissue is achieved by heating for 15–30 min (*see Note 1*) in a hot-air oven (60°C).
3. Sections are deparaffined in xylene for 2 × 2 min, followed by 99% and 96% ethanol, for 2 min each.

3.3.2. Tissue Digestion and Fixation

1. The sections are immersed in 0.1% DEPC/H₂O for 2 × 2 min, and then digested with proteinase K (1:10 in TBS) for 4–10 min.
2. After washing in 0.1% DEPC/H₂O for 1 min (*see Note 2*), the sections are post-fixed for 15 min in 0.4% paraformaldehyde in PBS (*see Note 6*).
3. After washing in 0.1% DEPC/H₂O for 1 min the slides are dehydrated by dipping 5 times in 99% ethanol, and then air-dried for 2 min.

3.3.3. Hybridization and Visualization

1. Sections are hybridized with the probe for 60–90 min at 55°C (*see Note 7*).
2. Sections are covered with a standard nonsiliconized glass cover slip.
3. PAP pens (or similar hydrophobic slide markers) should not be used (*see Note 8*).
4. The cover slip is picked up carefully with forceps and excess probe is removed by sequential washing steps in graded SSC: 2x SSC at 55°C for 8 min; 0.2x SSC at 40°C for a further 8 min; and finally 0.1x SSC at RT for 4 min (*see Note 9*).
5. The slides are washed in TBS for 2 × 2 min, followed by labeling with anti-digoxin for 8 min.
6. After immunolabeling, the sections are blocked for endogeneous peroxidase using 0.5% H₂O₂ in methanol for 5 min, immersed in TBS for 2 × 3 min (*see Note 2*), and EnVision + is applied undiluted for 8 min.
7. Sections are rinsed in TBS for 2 × 3 min, followed by DAB for 5 min.
8. One tablet of DAB is dissolved in 10 mL of distilled water approx 30 min before use, and immediately before application, 10 μL H₂O₂ is added.
9. The slides are immersed in distilled water for 1 min, and then counterstained with 0.1% toluidine blue for 30 s (*see Note 4*).
10. The sections are dehydrated in graded ethanol (70%, 96%, and 99% ethanol for 2 min each) before being air-dried.
11. The slides are now ready for microdissection (*see Note 10, Fig. 1A–D*).

4. Notes

1. Adhesion of the cut sections to the membranes varies according to the tissue. Some tissues require firm adhesion, which can be achieved by coating the membrane with 8% APES (8). However, if the sections are handled gently during the washing steps, we find that most tissues stay on the membrane during the procedure. We normally place the MOMeNT slides in a hot-air oven at 60°C for 15 min to help ensure tissue adhesion. For sections to be used for ISH, we prefer to incubate the slides for 30 min as they undergo treatment in proteinase K.
2. In our standard protocols, we block endogenous peroxidase using methanol and H₂O₂ for 10 min, but in this short staining procedure, we find 5 min to be sufficient. All washing steps are reduced by several minutes, without any significant impact on the staining result. This is important, as it is deleterious to mRNA preservation for the slides to be immersed in aqueous media, in part because this activates tissue ribonucleases (RNAses). This is particularly important when dealing with cryostat sections (1,3). In FFPE sections, the internal RNAses are inactivated during formalin fixation. In these cases, the major threat to RNA integrity comes from external RNAses introduced from the outside environment (e.g., in wash buffers). We use DEPC-treated buffers and equipment to avoid this potential problem.
3. Formalin fixation results in extensive cross-linking of proteins that will often mask target epitopes from immunohistochemical detection. Optimal detection of many antigens in paraffin sections requires the use of short periods of superheating (e.g., to >100°C for 20 min) in buffers of variable pH to unmask these hidden epitopes. The membranes used in MOMeNT can rarely survive this treatment. As an alternative, the protocols described here make use of prolonged heating at a lower temperature (60°C) to achieve similar antigen retrieval without damaging the sections or membranes. “Low”-temperature HIER is performed in a hot-air oven at 60°C. The sections are placed in an ample volume of buffer (TEG, pH 9.0) for different retrieval times depending on the antibody used. For Melan-A and Ki-67, 18 h is sufficient to give clear positive staining with low background (see Fig. 1G–H), while 24 h is recommended for staining cytokeratins (e.g. pan-cytokeratins AE1/AE3 [DakoCytomation; see Fig. 1A–D], KL1, and high molecular weight cytokeratin) (8). For detection of antigens with other antibodies (e.g., CD30 antigen and EBV-encoded LMP-1, DakoCytomation) 48 h of retrieval is required to achieve strong positive staining with low background. During “low”-temperature HIER, it is important that the sections are completely immersed in TEG buffer, so that they do not dry out. The glue used for attaching the membranes to the slides is silicone-based and in some cases this can become detached. To overcome this problem, we apply a second layer of glue to the edge of the membrane, after UV light treatment. The metal slides for single step collection can undergo “low”-temperature HIER without any difficulties. Pronase digestion for antigen retrieval does not work well in our hands, resulting in either

weak stains with low background, or strong, false-positive stains with high background.

4. Mayer's hematoxylin binds to DNA; several papers have reported that this dye may interfere with PCR-based amplification of DNA (7,9,10). In contrast, other groups have not found this to be a problem (11) and single cell microdissection of hematoxylin-stained sections with subsequent PCR-based analysis of DNA is possible (12). It appears that RNA is not affected in the same way, and several papers have reported successful RT-PCR on single-cells microdissected from hematoxylin-stained FFPE (13,14). However, when working with small numbers of microdissected cells (<50) or single cells, it may be advisable to use methyl green or toluidine blue instead. In these circumstances we prefer to use toluidine blue as the counterstain.
5. It is difficult, and often impossible, to microdissect wet sections, so the slides must be allowed to dry. See **Note 9** in Chapter 11 for further details.
6. Paraformaldehyde (0.4%) is made by dissolving 8 g of paraformaldehyde (Sigma P-6148) in 100 mL of 20x PBS and 100 mL of 0.1% DEPC/H₂O. The solution is heated to a temperature of 70°C whilst mixing until it is clear (approx 60 min). Prolonged storage of this fixative is possible at -20°C. Thawed fixative should be replaced each week.
7. We use a digoxigenin-labeled PCR-generated single-stranded DNA probe developed within our laboratory for EBER-ISH. A number of alternative probes for EBER-ISH are available commercially. For example, fluoresceinated EBER-specific oligonucleotide probe (NCL-EBV, Novocastra, Newcastle, UK) can be used as recommended by the manufacturer. Hybridized probe can be detected in various ways, for example using monoclonal mouse anti-FITC antibody (M0878, DakoCytomation), followed by biotinylated second-stage antibody and ABC.
8. We do not normally use PAP pens (or similar hydrophobic slide markers) to encircle the tissue prior to immunostaining or hybridization, as these may cause either damage or wrinkling to the membranes.
9. This wash step is crucial. We have used different stringent washing solutions and some result in loss of both the hybrids and/or the tissue. For example, in our routine ISH protocol, we use stringent wash concentrate (DakoCytomation S3500, 1:50 for 25 min). However, this results in unacceptable section loss when used with membrane-mounted slides.
10. Extraction of DNA and RNA is described in detail in Chapter 11. No special kits or precautions are needed. These protocols all allow for extraction and amplification of standard quality template. Standard methods are used to visualize amplified DNA on ethidium bromide-stained agarose gels (8) (see **Fig. 2E**), and quantification of RNA is by real-time quantitative RT-PCR. Immunostaining in frozen tissues does decrease the overall yield of available RNA, compared to hematoxylin stain alone. The choice of antibody and staining system might also have an impact on the yield, but in our hands, quantification of RNA is still possible.

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