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Notes & Tips

A robust RNA integrity-preserving staining protocol for laser capture microdissection of endometrial cancer tissue

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ABSTRACT

Laser capture microdissection of frozen tissue sections allows homogeneous cell populations to be isolated for expression profiling. However, this requires striking a balance between retaining adequate morphology for accurate microdissection and maintaining RNA integrity. Various staining protocols were applied to frozen endometrial carcinoma tissue sections. Although alcohol-based methods were superior to aqueous stains for maintaining RNA integrity, they suffered from irreproducible staining intensity. We developed a modified alcohol-based, buffered cresyl violet staining protocol that provides reproducible staining with minimal RNA degradation suitable for tissues with moderate to high levels of intrinsic RNase activity.

Laser capture microdissection (LCM)[1] permits direct visualization of tissue sections and the selection of specific cell populations for DNA, RNA, and protein profiling. However, this technology presents challenges, particularly with regard to isolating intact RNA for downstream applications such as expression arrays, where the recommended minimum RNA integrity number (RIN) is 7.0 [1,2]. Although frozen tissue yields more intact RNA than its formalin-fixed counterpart [3], issues of collection (time to freezing) and storage markedly affect RNA integrity [3–5]. A further challenge presented by frozen tissue LCM is retaining good tissue morphology while maintaining RNA integrity, which requires staining protocols that minimize endogenous tissue RNase reactivation. Because tissues vary widely in their intrinsic RNase levels [6], staining protocols optimized for tissues with very low RNase activity (e.g., brain) often prove to be unsuitable for other tissue types.

LCM has been used extensively on frozen endometrial tissue, although RIN values of extracted RNA are seldom reported. Indeed, to our knowledge, the highest quality RNA obtained through endometrial tissue LCM had a minimum cutoff RIN of 6 [7]. Therefore, we compared various staining protocols (including LCM application-specific commercial stains) with the analysis of human endometrioid endometrial carcinoma (EEC) frozen tissue sections. To ensure high starting RNA integrity levels, tissue collection and storage were streamlined; fresh EEC specimens (obtained with ethics approval) were transported to the laboratory on ice, where they were snap-frozen in liquid nitrogen and stored at −80 °C (uterus removal to freezing <1 h). Cryosectioning was performed on a CM305S cryostat (Leica Microsystems; Wetzlar, Germany) at a −16 °C chamber/chuck temperature after allowing 15 min for the tissue to equilibrate to temperature and using RNase-free water to mount the specimens onto the chuck. Sections (8 μm) were mounted onto room temperature RNase-free glass slides that were immediately placed on dry ice and fixed/stained on the same day. Following staining, whole sections were scraped into RLT buffer (Qiagen, Crawley, UK) containing 2-mercaptoethanol (1%, v/v) and extracted using RNeasy Plus microkits (Qiagen) after homogenization through QIAshredder columns (Qiagen), and RNA was stored at −80 °C. To simulate LCM, matched stained sections were kept at ambient temperature for 3 h prior to extraction.

For LCM, 12-μm sections were mounted onto polyethylene naphthalate (PEN)-coated slides (Carl Zeiss, Munich, Germany) and 2–4 × 10^6 μm^2 epithelial cells were microdissected into 500-μl adhesive cap tubes (Carl Zeiss) using the RoboLPC function of a PALM MicroBeam LCM microscope (Carl Zeiss). RNA was extracted as above but without the QIAshredder step and with additional Qiagen RPE buffer and 80% ethanol column washes (one each). Eluted RNA was concentrated using a Savant SC110 SpeedVac with a cold trap for 15–20 min at ambient temperature, and the RNA was redissolved in 5 μl of RNase-free water (to bring the RNA concentration within a measurable range).

For all experiments, starting RNA integrity was determined by extracting RNA from a whole unmounted section cut from the same tissue at the same time as the experimental sections. RNA

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[1] *Abbreviations used:* LCM, laser capture microdissection; RIN, RNA integrity number; EEC, endometrioid endometrial carcinoma; PEN, polyethylene naphthalate.

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yield and purity were assessed by Nanodrop 1000 spectrophotometry, and integrity was assessed using RNA-6000 nanochips on a Bioanalyzer (Agilent Technologies, Wokingham, UK). RNA integrity loss (ΔRIN) resulting from different staining procedures was calculated by subtracting RIN values from the starting RIN (above). ΔRIN values were compared using Mann–Whitney *U* tests.

We began by testing two LCM-specific commercial stains: Arcturus HistoGene aqueous stain (Applied Biosystems, Warrington, UK) and Ambion's alcohol-based cresyl violet stain (Applied Biosystems). The manufacturer's recommended protocols were followed except for the replacement of the final xylene dehydration step with anhydrous 100% ethanol because xylene makes tissue brittle and difficult to laser capture microdissect [8]. Ambion's LCM cresyl violet stain was superior to HistoGene in terms of RNA integrity given that the ΔRIN values were significantly lower from Ambion-stained sections both extracted immediately and extracted after a 3-h incubation at ambient temperature ($P < 0.05$) (Table 1). Although HistoGene is reported to yield high-quality RNA in frozen brain LCM [9], it has been found to cause significant RNA degradation in microdissected epithelia from other tissues (e.g., mammary, prostate, cervix) [10–12]. Although comparative studies of intrinsic RNase levels in endometrial tissue are lacking, data from the Cooperative Human Tissue Network indicate that obtaining high-quality RNA from this tissue type may be problematic [4], suggesting comparatively high RNase activity levels. We surmised that the superior RNA obtained using Ambion's cresyl violet stain was because it is alcohol based (as developed by Clément-Ziza and colleagues [13]), thereby minimizing endogenous tissue RNase reactivation.

Including a chemical RNA protection reagent (ProtectRNA, Sigma–Aldrich, Poole, UK) has been reported to improve RNA integrity obtained using aqueous stains [11,12]. Therefore, we evaluated the merit of this reagent in combination with aqueous stains (HistoGene and hematoxylin), whereby the reagent was added (1:500 dilution) to all solutions (including the stain). We found that although this approach improved RNA integrity, ProtectRNA caused significant precipitation of both stains, leading to very poor tissue visualization (Table 1).

Although initial results with Ambion's cresyl violet stain were excellent, a marked deterioration of staining intensity was noted in consecutive experiments over a period of approximately 3 weeks (despite correct stain storage) such that tissue became impossible to visualize. We reasoned that this was due to atmospheric CO_2 dissolving into the aqueous component of the stain, leading to a decreased pH and a loss of staining intensity over time. Cresyl violet belongs to a class of cationic dyes [14] that bind to negatively charged nucleic acid phosphate groups and, thus, are used to visualize nuclei and rough endoplasmic reticulum. Traditionally, aqueous solutions of these dyes are used, and differentiation (prevention of overstaining) is achieved using ethanolic acetic acid solutions [15]. Therefore, we hypothesized that alcoholic cresyl violet solutions may give intrinsically weaker staining, particularly at reduced pH. We tested this hypothesis by developing our own in-house alcohol-based cresyl violet stain using unbuffered RNase-free water (pH 6.5) to make the stain and adjusting the pH with 20 mM Tris–HCl to 7.0, 7.5, and 8.0. The protocol was as follows. Specimen slides were transferred from dry ice to 95% ethanol for 30 s and incubated in 75% ethanol for 30 s. Filtered cresyl violet stain (300 μl of 1% cresyl violet acetate in 75% ethanol) was added to the slides, which were incubated for 40 s. Slides were transferred to 75% ethanol for 30 s and then dehydrated through 95% ethanol (30 s), followed by 2 × 30-s and 1 × 5-min incubations in anhydrous 100% ethanol (prepared by adding 15 g of 3-Å molecular sieve beads to 500 ml of absolute ethanol). Slides were dried for 5 min at room temperature before LCM/RNA extraction.

Supplementary Fig. S1 (see Supplementary material) shows staining intensity obtained at different pH values. The best staining intensity (pH 8.0) was of equivalent quality to hematoxylin/eosin staining. Because pH was standardized, staining intensity was reproducible across more than 10 separate experiments over more than 4 months. We found it necessary to buffer the stain just before starting the experiments because it resulted in slight cresyl violet precipitation over the course of 1 h, although this had no discernible effects on staining quality. RNA integrity obtained from whole sections stained at pH 8.0 compared favorably with both unbuffered and Ambion stains (Table 1). Indeed, ΔRIN values of RNA from sections extracted immediately were slightly lower using our pH 8.0 in-house stain than with the Ambion stain ($P < 0.05$), although there was no significant difference for sections extracted after 3 h. Our staining protocol had no detectable impact on RNA integrity; ΔRIN values of RNA extracted immediately from stained sections were not significantly different from those of whole mounted sections that were simply fixed and dehydrated. There was also minimal loss of RNA integrity after storage of stained sections for 3 h at ambient temperature (20–22 °C and 27–44% relative humidity) to simulate LCM. Although it has been reported that laboratory relative humidities greater than 23% at 22 °C negatively affect RNA integrity of LCM specimens [9], this observation may reflect the authors' use of an aqueous stain (HistoGene). Indeed, our results with HistoGene show marked further RNA degradation following 3 h of incubation at ambient temperature.

We applied our optimized staining protocol to epithelial cell LCM from EEC sections (Fig. 1). Initially, we observed some reduction in RNA integrity in microdissected samples compared with whole sections extracted post-LCM, presumably due to the sensitivity of small amounts of RNA to residual tissue RNase

Table 1
Staining protocol comparisons of frozen endometrial cancer tissue sections.

Stain	0 h poststain		3 h poststain		Staining quality
	RIN (means ± SEM)	ΔRIN (means ± SEM)	RIN (means ± SEM)	ΔRIN (means ± SEM)	
Ambion's cresyl violet	8.0 ± 0.2	0.7 ± 0.2	7.6 ± 0.5	1.1 ± 0.5	Variable
HistoGene	6.3 ± 0.4	2.5 ± 0.4	3.6 ± 0.1	5.2 ± 0.1	+++
HistoGene + ProtectRNA	7.8 ± 0.1	1.4 ± 0.1	7.8 ± 0.2	1.4 ± 0.2	–
Hematoxylin + ProtectRNA	7.8 ± 0.2	1.4 ± 0.2	7.7 ± 0.1	1.5 ± 0.1	+
In-house cresyl violet (unbuffered)	8.3 ± 0.1	0.5 ± 0.1	7.8 ± 0.1	1.0 ± 0.1	–
In-house cresyl violet (pH 8.0)	8.7 ± 0.1	0.1 ± 0.1	8.3 ± 0.1	0.5 ± 0.1	+++
Fixed and dehydrated	8.5 ± 0.0	0.3 ± 0.0	ND	ND	NA

Note. SEM, standard errors; ND, not done; NA, not applicable. Matched sections were stained using Ambion's alcohol-based cresyl violet stain and two aqueous stains (Arcturus HistoGene and Mayer's hematoxylin). The inclusion of ProtectRNA was also tested with both aqueous stains. Sections were also stained using our in-house cresyl violet protocol with and without 20 mM Tris–HCl (pH 8.0). Control sections were fixed in 95% ethanol and dehydrated in 3 × 100% anhydrous ethanol washes without staining. RNA was harvested from whole sections immediately (0 h) and after 3 h of ambient temperature incubation (simulating LCM). RNA integrity from triplicate sections (mean RIN ± SEM) is indicated, as is the loss of integrity resulting from staining (mean ΔRIN ± SEM), calculated by subtracting RIN values from that of a matched whole unmounted section. Staining quality was evaluated in terms of visualizable tissue morphology and is rated "–" (very poor) to "+++" (excellent).

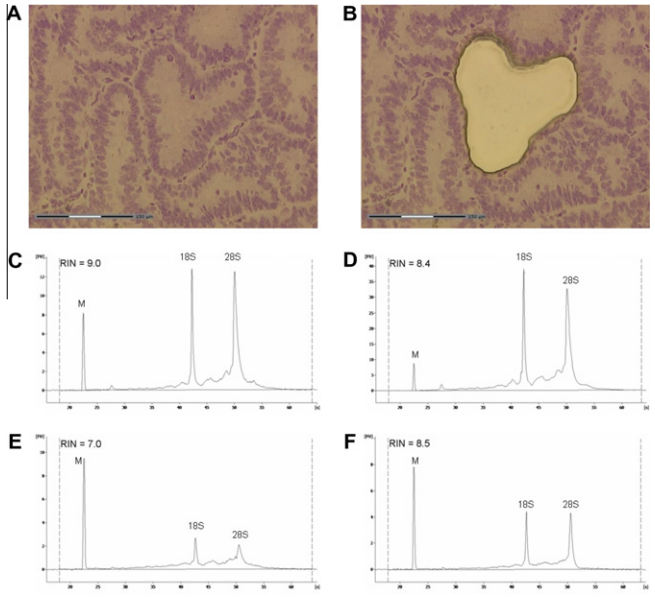

Fig.1. LCM and RNA extraction from endometrial carcinoma tissue. Matched frozen EEC tissue sections (same tumor) were stained using our optimized protocol. (A and B) Representative micrographs (20× magnification) are shown before (A) and after (B) epithelial cell LCM. (C–F) Representative Bioanalyzer electropherograms (fluorescence [ordinate] vs. time [abscissa]) with respective RIN values are shown for RNA extracted from a whole unmounted section (C), a whole section extracted post-LCM (D), and approximately 3×10^6 μm^2 laser-captured epithelial cells without (E) and with (F) SUPERase-In added directly after extraction. Positions of 18S and 28S ribosomal RNA and marker (M) peaks are indicated.

contamination. We excluded the SpeedVac apparatus as the source of contamination because concentration of highly pure cell line RNA (diluted to <10 ng/μl) did not affect RNA integrity (data not shown). However, adding 5 U of RNase inhibitor (SUPERase-In, Ambion) prior to sample concentration prevented this degradation. We tested our protocol on LCM material from EEC specimens from four patients. RIN values ranged from 7.8 to 8.5 (ΔRIN values $\leqslant$0.6, yield 15–36 ng/μm^2 [data not shown]), indicating that RNA extracted using our method comfortably exceeded the minimum RIN value required for expression array platforms.

To summarize, our protocol's key steps were as follows:

(i) using an alcohol-based cresyl violet stain that we demonstrated to be superior to aqueous stains in maintaining RNA integrity;
(ii) buffering the staining solution (pH 8.0) prior to use, which achieved reproducible staining intensities and excellent tissue visualization in contrast to other alcohol-based methods or aqueous stains that include a chemical RNase inhibitor; and
(iii) adding an RNase inhibitor (SUPERase-In) immediately after RNA extraction to preserve the integrity of RNA obtained from microdissected tissue.

Our protocol should also be applicable to other tissues with moderate to high intrinsic RNase activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2011.05.009.

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