Technical note: A rapid method for 5-bromo-2'-deoxyuridine (BrdU) immunostaining in bovine mammary cryosections that retains RNA quality¹

R. K. Choudhary,* K. M. Daniels,†² C. M. Evock-Clover,† W. Garrett,‡ and A. V. Capuco†³

*Department of Animal and Avian Sciences, University of Maryland, College Park 20742

†Bovine Functional Genomics Laboratory, and

‡Animal Biosciences and Biotechnology Laboratory, USDA-ARS, Beltsville, MD 20705

ABSTRACT

A rapid method of 5-bromo-2'-deoxyuridine (BrdU) immunostaining was developed in cryosections of bovine mammary tissue while preserving RNA quality of the stained section. A thymidine analog that is incorporated into DNA of proliferating cells, BrdU serves as a proliferation marker. Immunostaining of BrdU-labeled cells within a histological section requires heat, enzymatic or chemical-mediated antigen retrieval to open double-stranded DNA, and exposure to the BrdU antigen. Although these established treatments permit staining, they preclude use of cells within the tissue section for further gene expression experiments. Additionally, long antibody incubations and washing steps lead to extensive RNA degradation and elution. A protocol was developed for immunolocalization of BrdU-labeled cells in cryosections of bovine mammary tissue, which does not require harsh DNA denaturation and preserved RNA integrity and quantity. This protocol used an initial acetone:polyethylene glycol 300 [9:1 (vol/vol)] fixation (2 min) followed by staining with methyl green (0.5% aqueous; 2 min) to stabilize macromolecules, antigen retrieval with deionized formamide (70% in nuclease-free phosphate buffered saline; 4 min incubation), antibody incubation in the presence of RNase inhibitors (5 min), and minimal washing to facilitate recovery of RNA from cells from the stained sections. Applicability of this protocol to other nuclear antigens was evaluated by testing its suitability for staining estrogen receptor α and Ki-67 antigen. In both cases, use of the protocol provided good immunostaining and tissue morphology. The RNA quality of estrogen receptor α - and Ki-67-stained sections was not evaluated. Quality of the isolated RNA from BrdU-stained sections was evaluated

by micro-fluidic electrophoresis and its utility was confirmed using quantitative reverse transcription-PCR. Staining intensity obtained with this labeling protocol was similar to that obtained using conventional immunohistochemistry protocols. When coupled with laser microdissection and RNA or cDNA amplification, this immunostaining protocol provided a means for future transcriptome analysis of BrdU-labeled cells within a complex tissue.

Key words: 5-bromo-2'-deoxyuridine, cryosection, gene expression, laser microdissection

5-Bromo-2'-deoxyuridine (BrdU) has been used extensively to identify proliferating cells, study cell lineages, and identify putative stem cells (Plickert and Kroihner, 1988; Capuco, 2007; Conboy et al., 2007). A thymidine analog, BrdU is incorporated into DNA during the S-phase of the cell cycle and can be detected by standard immunohistochemical procedures. But these procedures have limitations for subsequent transcriptome analysis of BrdU-labeled cells. Antibodies against BrdU can access only the antigen in single-stranded DNA (Gratzner, 1982), so staining procedures require DNA denaturation before antibody incubation. These procedures involve high heat (~100°C) in sodium citrate buffer or treatment with harsh chemicals (e.g., HCl; Tang et al., 2007). Such treatments affect RNA integrity and can affect cell morphology. Establishing a procedure that could identify BrdU-labeled cells without damaging cellular RNA would facilitate subsequent *in situ* hybridization or isolation of cells by laser microdissection for subsequent analysis of gene expression in these cells. In this study, a novel strategy was developed whereby good-quality RNA may be extracted from BrdU-immunostained cryosections of bovine mammary gland. Our future application will be to use this protocol to identify putative bovine mammary stem cells for laser excision and subsequent downstream analyses. Putative bovine mammary stem cells can be identified on the basis of their ability to retain BrdU for extended periods of time after initial labeling, because of selective segregation of parental DNA strands during asymmetric cell division (Capuco, 2007).

Received October 20, 2009.

Accepted February 1, 2010.

¹Mention of a trade name or proprietary product does not constitute a guarantee or warranty by the USDA and does not imply approval to the exclusion of others not mentioned.

²Current address: Department of Animal Sciences, The Ohio State University, Wooster 44691

³Corresponding author: tony.capuco@ars.usda.gov

Table 1. Fixatives and antigen retrieval agents, their mode of action, and effects on morphology, 5-bromo-2'-deoxyuridine (BrdU) staining, and RNA quality in bovine mammary cryosections^{1,2}

Item	Mode of action	Morphology	Staining	RNA quality
Fixative				
Methacarn ³	Nonprecipitating + precipitating	+++	+	-
75% ethanol	Precipitating	++	+	+
Acetone	Precipitating	++	++	++
UMFIX ⁴	Precipitating	+++	++	++
ACEPEG ⁵	Precipitating	+++	+++	+++
Antigen retrieval agent				
2 N HCl	Denatures DNA	++	+++	-
DNaseI	Cleaves 5' end of DNA	+++	-	-
0.01 N NaOH	Denaturing and hydrolysis	Destroyed	-	-
UV irradiation	Breaks double-stranded DNA	Destroyed	-	-
Formamide 100%	Lowers DNA melting temperature	+	++	-
Formamide 10%	Lowers DNA melting temperature	Destroyed	-	-
Formamide 70%	Lowers DNA melting temperature	++	++	++

¹RNA quality was tested on slides subjected to the protocol under evaluation by microcapillary electrophoresis (Agilent Bioanalyzer, Agilent Technologies, Wilmington, DE). Companion slides were used to determine whether the protocol was suitable for BrdU staining.

²Quality graded as bad (-), poor (+), moderate (++), or good (+++). RNA integrity numbers corresponded as follows: - = RIN 1 to 3; + = RIN 3 to 4; ++ = RIN 4 to 6; +++ = RIN 6 to 8.

³Methanol + chloroform + glacial acetic acid [6:3:1 (vol/vol)].

⁴Methanol + polyethylene glycol 300 [9:1 (vol/vol)].

⁵Acetone + polyethylene glycol 300 [9:1 (vol/vol)].

Tissues used were obtained from an experiment that was performed in compliance with the Beltsville Agricultural Research Center's Animal Care and Use Committee. Five Holstein heifers (3 mo of age) were used. Heifers were injected with BrdU (Sigma-Aldrich Co., St. Louis, MO) intravenously once daily for 5 d as described previously (Capuco, 2007). Forty-five days after the last BrdU injection, heifers were humanely killed at the Beltsville Agricultural Research Center abattoir. Udders were removed and mammary tissue was collected from parenchymal regions within rear mammary glands (i.e., quarters) of the udder. Individual tissue samples (5 × 5 × 5 mm³) were immediately embedded in optimal cutting temperature (OCT) compound (Sakura, Torrance, CA), frozen in liquid nitrogen vapor, transported on dry ice, and finally stored at -80°C until cryosectioning. Serial sections (8 µm thick) were thaw-mounted on UV-irradiated (to destroy potential nuclease activity) glass slides covered with a membrane (2 µm thick) of polyethylene naphthalate (PEN slides, Leica AS, Wetzlar, Germany) and stored at -80°C until the time of microdissection (~2 wk maximum storage).

Tissue cryosections were fixed with a variety of non-crosslinking fixatives including alcohols and acetone. Crosslinking fixatives, such as formalin, severely impair the ability to extract quality RNA from tissue (Lewis et al., 2001) and were not used. A list of fixatives evaluated is provided in Table 1. Sections were treated with these fixatives to optimize morphology and BrdU staining while maintaining good RNA quality. Sections fixed

in acetone:polyethylene glycol (**ACEPEG**) 300 [9:1 (vol/vol); Sigma-Aldrich], our newly developed fixative made on the day of staining, at -20°C for 2 min possessed good morphology and consistent immunostaining and yielded RNA of highest quality. Preliminary experiments with acetone alone as a fixative yielded acceptable results. These results were much improved with the addition of polyethylene glycol (**PEG**) 300 to acetone. This fixative was derived from the composition of universal molecular fixative, which was a combination of methanol and PEG [9:1 (vol/vol)]. Further, PEG was a component of OCT compound, so adding it to the fixative would aid morphology by providing a more gradual transition from OCT compound to acetone fixative.

Different methods were evaluated for their ability to enhance the quality of RNA extracted from the tissue sections. These methods included 1) use of methyl green to precipitate RNA in aqueous solution without affecting immunostaining and RNA isolation procedures; 2) use of RNase inhibitors in antibody incubation buffer (RNasin Plus RNase inhibitor, Promega, Madison, WI); and 3) use of small volumes of wash solutions (200 µL instead of submersion in wash jar) and abbreviated wash time.

Effects of different antigen retrieval agents on immunostaining, tissue morphology, and RNA quality were evaluated. These treatments were time consuming and yielded unsuitable morphology and unacceptable RNA quality (Table 1). Yet, denaturation of DNA with 70% deionized formamide (Amresco, Solon, OH) in nuclease-

free PBS (**nfPBS**) for 4 min at 60°C maintained tissue morphology and facilitated immunostaining. This approach was an application of results obtained by Mayer et al. (2006), who successfully used deionized formamide for BrdU antigen retrieval in an in situ hybridization experiment. Formamide lowers the melting temperature of DNA by destabilizing its helical structure (Blake and Delcourt, 1996).

In all cases, slides were processed singly in RNase-free LockMailer microscope slide jars (Ted Pella Inc., Redding, CA). The optimized short method for immunostaining started with ACEPEG fixation at -20°C for 2 min followed by air drying for 1 min. Air drying was essential for best fixation and adherence of the tissue to the slide during subsequent steps. Drying of the slide was followed by incubating the section with 200 μ L of 0.5% aqueous methyl green (Vector Laboratories Inc., Burlingame, CA) for 2 min at room temperature (**RT**). After a brief (10 s) wash with nfPBS, the slide was incubated with 400 μ L of prewarmed 70% deionized formamide at 60°C on a metal block placed in a dry bath for 4 min. Immediately after formamide treatment (antigen retrieval), the section was washed twice with ice-chilled antibody dilution buffer at 4°C using 200 μ L of buffer for 30 s/wash. Antibody dilution buffer consisted of nfPBS with 1% normal goat serum and 0.1% triton X-100 (Sigma-Aldrich) and was formulated to reduce background staining and to enhance antibody penetration in the tissue. Washing the section with ice-chilled buffer immediately after formamide treatment was essential to prevent reannealing of denatured DNA. One-step immunostaining was performed using mouse monoclonal anti-BrdU antibody conjugated to Alexa 488 fluorophore (Clone PRB-1, Invitrogen, Carlsbad, CA) at 1:10 dilution in antibody dilution buffer for 5 min at RT in the dark. The section was then briefly washed with 200 μ L of nfPBS (twice for 10 s each), counterstained with 100 μ L of propidium iodide (2.5 μ g/ μ L in nfPBS) for 20 s, and rinsed in 200 μ L of nuclease-free water twice for 10 s each. The slide was dehydrated in freshly prepared ascending concentrations of ethanol: 70% for 10 s, 95% for 10 s, 100% for 10 s and 100% for 1 min. The slide was air-dried at RT in the dark for 5 min before sample acquisition for total RNA isolation. Whole sections of slides were lysed with the manufacturer's lysis buffer and total RNA was isolated using an RNeasy micro kit according to the purification of total RNA from microdissected cryosections protocol (Qiagen, Valencia, CA). The optional on-column DNase digestion was performed (Qiagen). Quality and quantity of RNA were evaluated using a Bioanalyzer (Agilent Technologies, Wilmington, DE) and a comparison was made between the RNA isolated from immunostained versus unstained cryosections.

The RNA integrity number (**RIN**) generated by analysis (Agilent Bioanalyzer) of the RNA electropherogram provided an objective measure of RNA quality (Fleige and Pfaffl, 2006; Schroeder et al., 2006).

Applicability of the protocol to other nuclear antigens (not specifically DNA) was tested using indirect immunolabeling and bright field microscopy in the presence and absence of 70% formamide, our antigen retrieval agent. Test antibodies were estrogen receptor α (**ER α**) and Ki-67. Because of the preliminary nature of this test, an indirect (2-step) immunostaining protocol was used and RNA quality was not evaluated after staining. For ER α labeling, slides were incubated with mouse ER α antibody (Clone c-311, cat. no. SC-787, Santa Cruz Biotechnology, Santa Cruz, CA) used at a concentration of 0.01 μ g/ μ L in antibody dilution buffer for 10 min at RT. Similarly, for Ki-67 labeling, slides were incubated with prediluted mouse Ki-67 antibody (Clone MIB-1, cat. no. 08-0156, Invitrogen) for 10 min at RT. Slides were then incubated with horseradish peroxidase conjugated broad spectrum secondary antibody (SuperPicture HRP Polymer Conjugate Broad Spectrum, cat. no. 87-9663, Invitrogen) after brief washings with PBS. Positively labeled cells were visualized with the 3,3'-diaminobenzidine (DAB) reaction (2-3 min incubation) and then rinsed in water. Slides were counterstained with hematoxylin for 30 s and washed briefly with water and then PBS. Finally, slides were dehydrated in ascending grades of ethanol and viewed through bright field microscopy.

All steps of the immunostaining method were optimized to ensure acceptable tissue morphology, consistent BrdU staining, and good RNA quality. Because the immediate application of this derived protocol was for isolation of BrdU-labeled cells using laser microdissection and subsequent transcriptome analysis, the slides were viewed without coverslips and digital micrographs were captured using a Leica AS-LMD laser microdissection system (Leica Microsystems, Bannockburn, IL). The initial fixatives tested resulted in tissue morphology of variable quality and low RNA quality and quantity (Figure 1A and B; only representative micrographs are presented among all fixatives tried). The newly developed ACEPEG fixative yielded the most desired morphology and RNA quality. Similarly, several antigen retrieval agents were evaluated and resulted in morphology and RNA quality that varied according to the retrieval agent tested (Figure 1C; only 1 micrograph from all retrieval agents initially tested is depicted). The results led to adaptation of 70% formamide, which ensured good morphology, antigen retrieval, and preservation of tissue RNA quality. The BrdU-labeled cells were identified using an Alexa-conjugated mouse monoclonal anti-BrdU antibody (Figure

Figure 1. Effects of selected fixatives and antigen retrieval agents on tissue morphology and bromodeoxyuridine (BrdU) staining. A) Acetone-fixed cryosection permitted good BrdU staining (arrows) when coupled with 70% deionized formamide as antigen retrieval agent. B) Methanol-polyethylene glycol (UMFIX)-fixed section resulted in good BrdU staining when coupled with 70% deionized formamide as antigen retrieval agent; UMFIX provided for good morphology and better preservation of stromal tissue compared with acetone-fixed sections. C) Deoxyribonuclease 1 failed to expose BrdU in the current protocol contrary to expectation. D) Acetone-polyethylene glycol (ACEPEG) fixation and formamide antigen retrieval provided an effective fixative and antigen retrieval agent, yielding good morphology, immunostaining, and best RNA quality (see Figure 3F). Section was labeled with Alexa 488-conjugated anti-BrdU (green; arrow indicates labeled nucleus of epithelial cell) and propidium iodide (red; arrow indicates labeled nucleus of stromal cell). Inset shows the highlighted labeled epithelial cell at greater magnification. Scale bar = 100 μ m.

1D). Adoption of fluorescently conjugated primary antibody minimized protocol length and potential for RNA degradation during multiple incubations. Sections were counterstained with propidium iodide. (4',6-Diamidino-2-phenylindole or Hoechst stains were not used as nuclear stains because the polyethylene naphthalate membrane fluoresced with similar excitation–emission characteristics.) To maintain RNA integrity, RNase inhibitors were added to the antibody dilution buffer and exposure to aqueous buffers was limited to a total of 15 min.

The ER α staining was restricted to mammary epithelium, consistent with previous results (Capuco et al., 2002), and Ki-67-labeled cells were primarily seen in mammary epithelium (Figure 2). Convincingly, more ER α -labeled cells were seen in formamide-treated slides (Figure 2A) than in PBS-treated slides (Figure 2B). Also, formamide enhanced overall tissue morphology of cryosections with clear nuclear staining. Similar findings were observed with Ki-67 staining, with formamide increasing detection of Ki-67-stained cells (Figure 2C) in comparison with a control slide having no forma-

Figure 2. Serial cryosections of bovine mammary gland stained with estrogen receptor α (ER α) and Ki-67 antibody. A) Immunostaining with ER α antibody in formamide-treated cryosection. B) Immunostaining with ER α antibody in PBS-treated cryosection. Good tissue morphology and improved ER α labeling (more intensely labeled nuclei in terminal ductal units) were evident in formamide-treated sections. C) Immunostaining with Ki-67 antibody in formamide-treated cryosections. D) Immunostaining with Ki-67 antibody in untreated cryosections. Good tissue morphology and more Ki-67-labeled cells were noted in formamide-treated sections. Arrows indicate examples of labeled nuclei in each panel. Scale bar = 100 μ m. Color version available in online PDF.

mide treatment (Figure 2D). This provided suggestive evidence for the applicability of our protocol to other non-DNA-specific nuclear antigens.

An increase in RNA quality (assessed by increase in RIN value) was achieved by addition of RNase inhibitors to the antibody incubation buffer (Figures 3A, B, C, and D). Antibody incubation time was limited to 5 min to minimize RNA degradation, but less than 5 min of incubation resulted in inconsistent immunostaining. The adverse effect of longer incubation times on RNA quality was consistent with the findings of Fend et al. (1999). To successfully utilize a short incubation time, a high concentration of antibody was employed as suggested for staining tissues for laser microdissection (Murakami et al., 2000). The

dilution used for BrdU (1:10) was titrated for our system. Finally, RNA quality of tissue sections that were immunostained using the optimized protocol were analyzed and compared with the RNA quality of fixed but unstained sections. The results showed that tissue RNA quality was not compromised using the current immunostaining method (Figures 3E and F; Figure 3F corresponds to micrograph in Figure 1D). The RIN obtained for stained sections from 5 heifers did not differ ($P > 0.05$) from that for unstained sections. Furthermore, the utility of the isolated RNA for gene expression analysis was tested using quantitative reverse transcription-PCR. Successful amplification of genes using specific primers confirmed good-quality RNA (data not shown).

Figure 3. Electropherograms of total RNA obtained from stained serial tissue sections. Effect of incorporating RNase inhibitor in incubation buffer on RNA quality was tested and is depicted in comparison with fixed, unstained sections and sections stained with the final optimized protocol. A) No RNase inhibitor; RNA integrity number (RIN) = 2.1. B) One unit of inhibitor (RNasin plus)/ μ L; RIN = 4.7. C) Two units of inhibitor/ μ L; RIN = 5.0. D) Three units of inhibitor/ μ L; RIN = 5.7. E) RNA isolated from acetone-polyethylene glycol (ACEPEG)-fixed, unstained slide from different serial sections yielded a maximum RIN = 7.2. F) Optimized protocol with ACEPEG-fixed, stained sections yielded RIN = 7.1 (micrograph provided in Figure 1D). Both panels E and F had similar RIN, indicative of good-quality RNA.

In conclusion, a rapid nuclear immunostaining method was developed with BrdU as the main antigen of study for bovine mammary cryosections. The method resulted in clear localization of BrdU-labeled cells and retention of good-quality RNA in the stained sections. This was achieved by fixation in ACEPEG, antigen retrieval with 70% formamide, and brief antibody incubation in the presence of RNase inhibitors. This protocol also resulted in clear localization of 2 other nuclear antigens, ER α and Ki-67, in cryosections.

REFERENCES

- Blake, R. D., and S. G. Delcourt. 1996. Thermodynamic effects of formamide on DNA stability. *Nucleic Acids Res.* 24:2095–2103.
- Capuco, A. V. 2007. Identification of putative bovine mammary epithelial stem cells by their retention of labeled DNA strands. *Exp. Biol. Med.* 232:1381–1390.
- Capuco, A. V., S. Ellis, D. L. Wood, R. M. Akers, and W. Garrett. 2002. Postnatal mammary ductal growth: Three-dimensional imaging of cell proliferation, effects of estrogen treatment and expression of steroid receptors in prepubertal calves. *Tissue Cell* 34:143–154.
- Conboy, M. J., A. O. Karasov, and T. A. Rando. 2007. High incidence of non-random template strand segregation and asymmetric fate determination in dividing stem cells and their progeny. *PLoS Biol.* 5:e102.
- Fend, F., M. R. Emmert-Buck, R. Chuaqui, K. Cole, J. Lee, L. A. Liotta, and M. Raffeld. 1999. Immuno-LCM: Laser capture microdissection of immunostained frozen sections for mRNA analysis. *Am. J. Pathol.* 154:61–66.
- Fleige, S., and M. W. Pfaffl. 2006. RNA integrity and the effect on the real time pRT-PCR performance. *Mol. Aspects Med.* 27:126–139.
- Gratzner, H. G. 1982. Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science* 218:474–475.
- Lewis, F., N. J. Maughan, V. Smith, K. Hillan, and P. Quirke. 2001. Unlocking the archive—Gene expression in paraffin-embedded tissue. *J. Pathol.* 195:66–71.
- Mayer, W., A. Smith, R. Fundele, and T. Haaf. 2006. Spatial separation of parental genomes in preimplantation mouse embryos. *J. Cell Biol.* 148:629–634.
- Murakami, H., L. Liotta, and R. A. Star. 2000. IF-LCM: Laser capture microdissection of immunofluorescently defined cells for mRNA analysis. *Kidney Int.* 58:1346–1353.
- Plickert, G., and M. Kroiher. 1988. Proliferation kinetics and cell lineages can be studied in whole mounts and macerates by means of BrdU/anti-BrdU technique. *Development* 103:791–794.
- Schroeder, A., O. Mueller, S. Stocker, R. Salowsky, M. Leiber, M. Gassmann, S. Lightfoot, W. Menzel, M. Granzow, and T. Ragg. 2006. The RIN: An RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol. Biol.* 7:3.
- Tang, X., D. L. Falls, X. Li, T. Lane, and M. B. Luskin. 2007. Antigen-retrieval procedure for bromodeoxyuridine immunolabeling with concurrent labeling of nuclear DNA and antigens damaged by HCl pretreatment. *J. Neurosci.* 27:5837–5844.