

# Determination of FineFix fixation on histologic quality and recovery of RNA from human prostate tissue

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## Abstract

Preserving tissue specimens essentially entails either fixation and embedding or snap-freezing. Traditionally, fixation and embedding protocols have been used to preserve tissue for histologic analysis without regard to biomolecular preservation. Snap-freezing has been the gold standard for biomolecular preservation but poorly preserves histologic characteristics. It would be ideal to develop a fixation and embedding protocol that would not only maintain excellent histology, but would preserve biomolecules to the same extent as snap-freezing. Examining prostate tissue, this study compares FineFix-fixed/paraffin-embedded (FiPE) tissue with tissue that has been snap-frozen, formalin-fixed/paraffin-embedded (FFPE), and 70% ethanol-fixed/paraffin-embedded (EFPE) for histology and recovery of RNA. Tissue sections are H&E stained for histologic analysis and for RNA analysis, epithelial cells are microdissected, RNA purified, then reverse transcribed followed by polymerase chain reaction (PCR) using primers specific for 220 bp beta actin. The PCR products are run on an agarose gel. Histologic quality was comparable for FFPE, EFPE, and FiPE and were all superior to frozen. RNA recovery was comparable for frozen and FiPE and were superior to EFPE and FFPE. Tissue fixation using FineFix may be useful for performing gene expression analysis from microdissection of different cell populations showing subtle histologic differences.

## Introduction

There are essentially two methods of tissue preservation including fixation and embedding and snap freezing. Fixation and embedding has traditionally been used for the preservation of histology without regard to the preservation of biomolecules. Snap freezing has been the benchmark for preserving tissue for molecular analysis but gives poor histology. Crosslinking fixatives, such as 10% normal buffered formalin, is the routine fixative used in pathology departments and is the standard for histologic criteria. However, it has performed poorly for analyses of biomolecules. noncross-linking fixatives such as ethanol, acetone, methacarn, and Carnoy's are more promising (1-4). Besides fixative type, physical forces that facilitate fixation such as ultrasound(5) may influence biomolecular preservation.

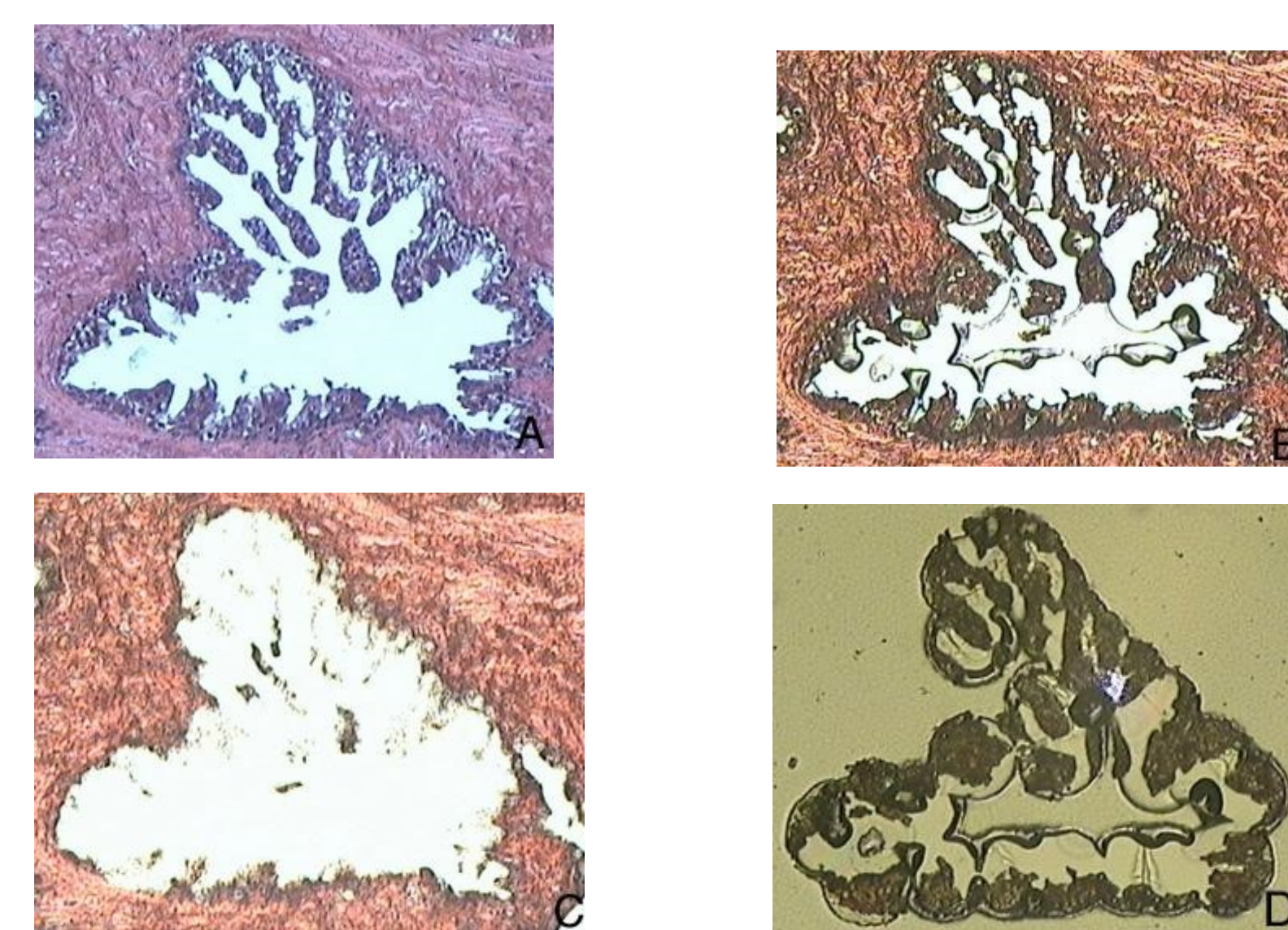
With the development of high-throughput molecular profiling techniques such as cDNA microarray and 2-D PAGE and tissue microdissection techniques, it would be ideal to develop a protocol for fixation and embedding that would not only preserve histology to the extent of formalin, but also preserve biomolecules to the extent of snap freezing. In this way, microdissection can be performed on tissue to dissect cells showing subtle histologic differences from other cells in the tissue.

This study examines the effect of FineFix fixation with microwave acceleration on histology and recovery of RNA from prostate tissue. Since RNA is unstable, it is an excellent measure of biomolecular preservation in tissue. Comparison of histology and recovery of RNA is performed for frozen, FiPE, EFPE, and FFPE prostate tissue. Equivalent numbers of epithelial cells from each of the preparations are laser capture microdissected for RNA recovery.

## Materials and Methods

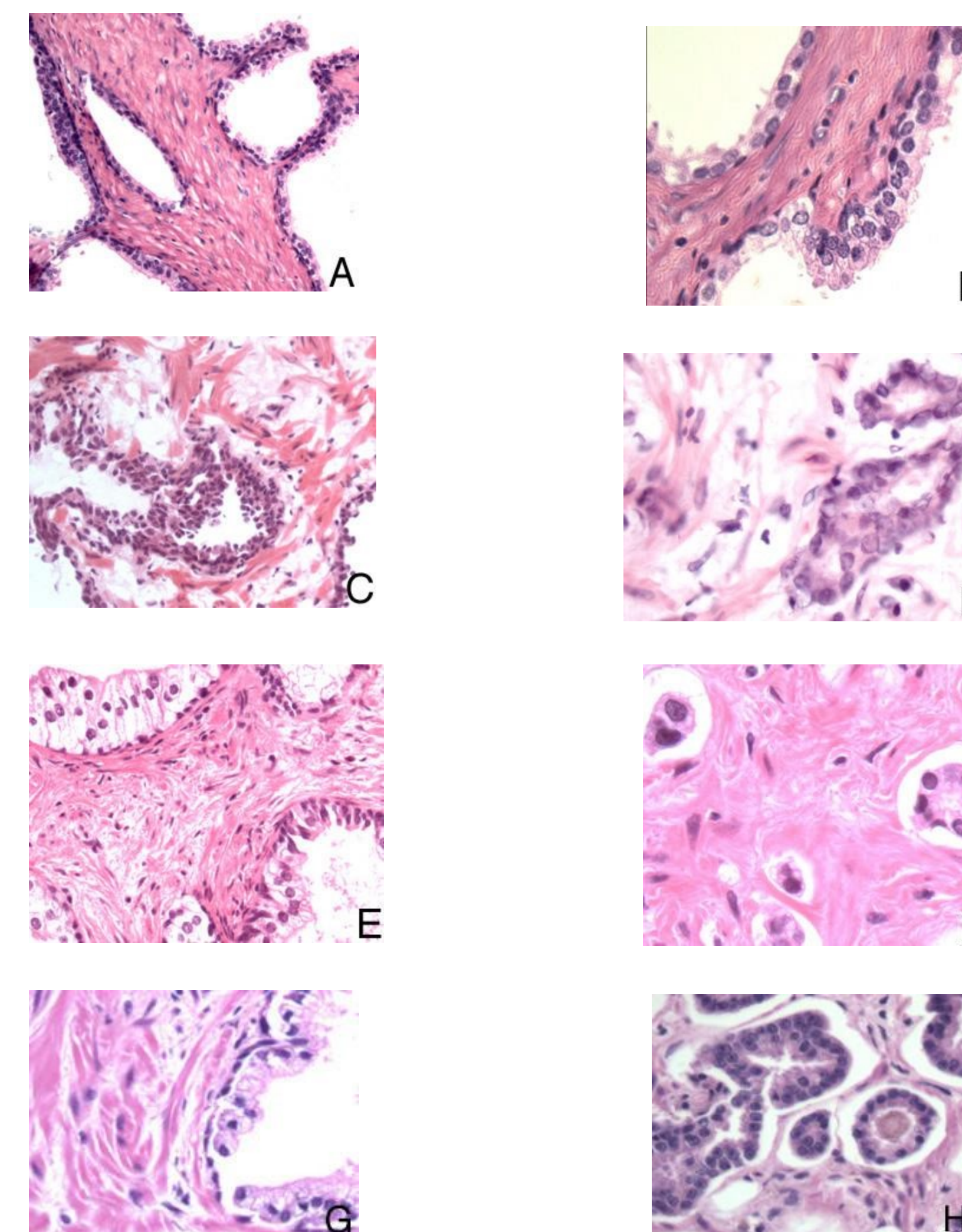
**Tissue preparation.** Snap-frozen, FFPE, and EFPE prostate tissue samples were received from the Urologic Oncology Branch through an IRB-approved protocol. FiPE prostate tissue was received from the Verona Hospital Department of Pathology (Italy). Frozen tissue was prepared by freezing in OCT on dry ice. Formalin-fixed tissue was routinely fixed overnight then processed in a tissue processor (Sakura FineTek, Torrance, CA), and embedded in paraffin. Ethanol-fixed tissue was fixed overnight in 70% ethanol, then processed in a tissue processor in increasing concentrations of ethanol followed by xylene, then paraffin embedding. Fixation of tissues with FineFix was accelerated by microwaves, followed by microwave paraffin processing (Milestone Medical).

**Histologic evaluation.** Three pathologists scored the histologic quality of the tissue preparations based on tissue architecture, staining, cellular and nuclear morphology with 1=poor, 2=fair, and 3=excellent. Score averages are shown in table 1.



**Figure 1. Laser capture microdissection of H&E-stained prostate tissue.** A. Roadmap showing high-quality histology of a hyperplastic gland to be microdissected. B. Before microdissection C. After microdissection D. Microdissected cells on cap.

**LCM.** Eight micron thick tissue sections were obtained and hematoxylin and eosin stained for LCM (Molecular Devices, Sunnyvale, CA). Following LCM (Fig. 1), cells were lysed from the cap and RNA purified using the PicoPure RNA Isolation kit (Arcturus/Molecular Devices, Sunnyvale, CA) including a DNase step.



**Figure 2. Representative histologic areas for different fixatives.**

A. Formalin fixed normal prostate (80X), B. Formalin fixed normal prostate (160X), C. Frozen normal prostate (80X), D. Frozen prostate adenocarcinoma (160X), E. FineFix fixed normal prostate (80X), F. FineFix fixed prostate adenocarcinoma (160X), G. Ethanol fixed normal prostate (80X), H. Ethanol fixed prostate adenocarcinoma (160X).

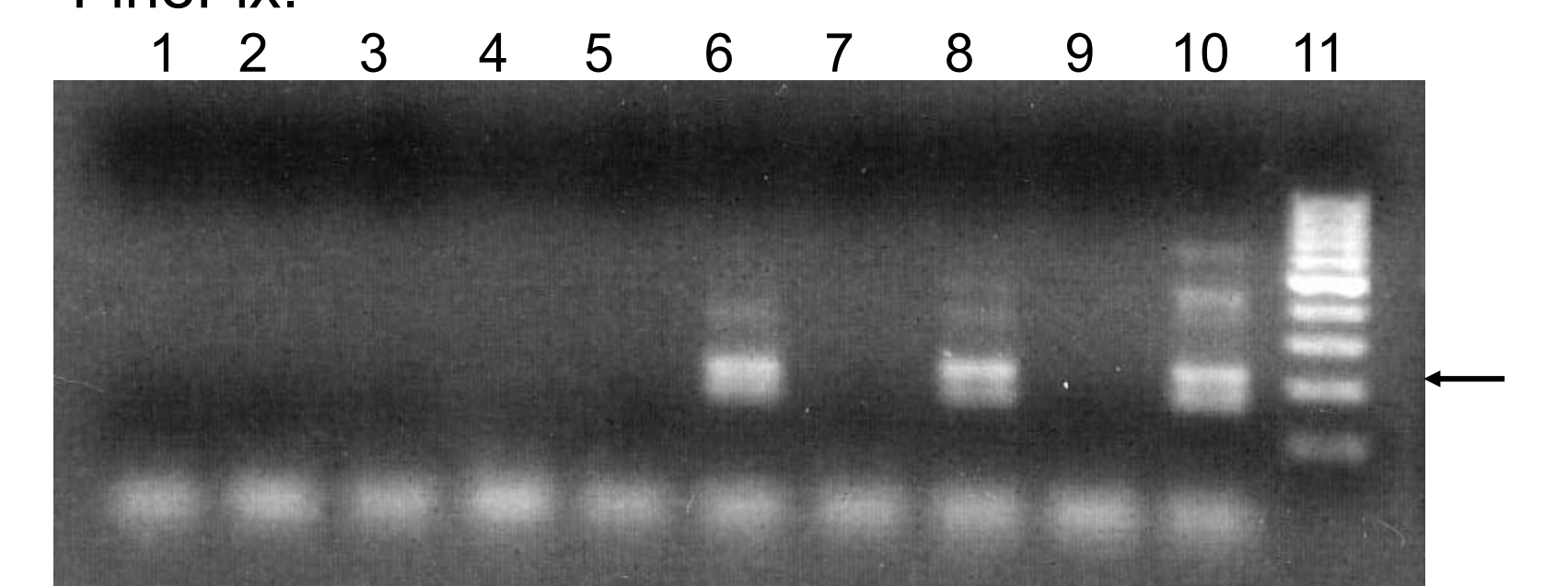
**RT-PCR.** Purified RNA then underwent reverse transcription and polymerase chain reaction with TaqMan and Applied Biosystem reagents, respectively. The amplification was carried out in a PTC-200 Thermal Cycler (Biorad) with the following conditions: 95C 10 min, 40 cycles with 95C 1 min, 60C 1 min, 72C 1 min, followed by 72C for 10 min, then 4C indefinitely. The PCR products were then run on a 1.5% agarose gel at 100V for 20 minutes. The gel was stained with ethidium bromide, visualized under a UV lamp, and photographed.

Sample	Architecture	Staining	Cellular Morphology	Nuclear Morphology
EFPE	3	2.33	3	2.33
FFPE	2.33	2.33	2.363	2
Frozen	1.67	2	1	1
FiPE	3	2.33	2.67	2.67

**Table 1. Histology scoring. Results**

After pathologists' scoring histologic quality (Table 1) for each of the tissue preparations, FiPE tissue shows histologic quality comparable to FFPE and EFPE tissue and is superior to frozen. Figure 2 shows representative histologic areas for the four prostate tissue preparations.

The gel corresponding to PCR showed an equal signal for the positive control, frozen, and Finefix-fixed samples (Figure 3) while the ethanol and formalin fixed samples gave no signal. This study underscores that biomolecular preservation may not only be influenced by the fixative, but also by physical conditions such as microwaves. Since PCR was performed with forty cycles, this may represent saturating conditions. However, this study does warrant that further studies be performed on FiPE tissue including quantitative RT-PCR, cDNA microarray, protein analytic platforms such as 2-D PAGE and western blot. Furthermore, it would be of great interest to determine histologic and biomolecular preservation months and years after fixation as well as to investigate other tissue types with FineFix.



**Figure 3. PCR of beta actin.** After random hexamer-primed RT, PCR products using primers specific for 220 bp actin, products run on an agarose gel visualized with ethidium bromide. Arrow denotes 220 bp product (1,2=FFPE, 3,4=EFPE, 5,6=FiPE, 7,8=Froz, 9,10=+control; 1,3,5,7,9=-RT, 2,4,6,8,10=+RT).

## References

- Paska, Csilla, et al. Effect of Formalin, Acetone, and RNAlater Fixatives on Tissue Preservation and Different Size Amplicons by Real-Time PCR from Paraffin-Embedded Tissue. *Diagn Mol Pathol.* 2004;13:234-240.
- Cox, M.L., et al. Assessment of fixatives, fixation, and tissue processing on morphology and RNA integrity. *Exp Mol Pathol.* 2006;80:183-191.
- Gillespie, John W., et al. Evaluation of Non-Formalin Tissue Fixation for Molecular Profiling Studies. *Am J Pathol.* 2002;160:449-457.
- Takagi, Hornori, et al. Microdissected Region-specific Gene Expression Analysis with Methacarn-fixed, Paraffin-embedded Tissues by Real-time RT-PCR. *J Histochem Cytochem.* 2004;52:903-913.
- Chu, Wei-Sing, et al. Ultrasound-accelerated formalin fixation of tissue improves morphology, antigen and mRNA preservation. *Mod Pathol.* 2005;18:850-863.