PAXgene® Vs. Formalin Fixed Tissue: A Comparison of Tissue Morphology and RNA Quality

[1]Daniel Grölz, [1]Ralf Wyrich, [1]Lynne Rainen, [2]Carolyn C. Compton, [2]Philip A. Branton, [3]Leslie Sobin

[1]PreAnalytiX GmbH, Hombrechtikon, CH; [2]Office of Biorepositories and Biospecimen Research (OBBR), National Cancer Institute, Rockville, MD 20852; [3]Applied Biomedical Science and Informatics Program, SAIC-Frederick, Inc., NCI-Frederick, Frederick, MD 21702

Background

Preanalytical treatment of tissue samples can influence bioanalyte quality and, ultimately, outcome of analytical results. PAXgene Tissue (PAXgene) is a two-step, formalin-free fixation and stabilization system, intended to standardize tissue fixation for simultaneous preservation of morphology, nucleic acids and proteins.[1,2]

In this study, we compared RNA quality and tissue morphology in mirrored samples of formalin fixed paraffin-embedded (FFPE) rat liver to that of PAXgene Tissue fixed, paraffin-embedded (PFPE) tissue samples in an animal model under highly controlled and standardized conditions.

Tissue fresh frozen in liquid nitrogen served as control for RNA quality. In addition effects of reversal of the PAXgene fixation/stabilization protocol were examined.

Materials and Methods

Tissue
- *Rattus norvegicus* liver
- Size: < 10 x 5 x 4 mm
- Ischemia: < 10 min
- Fixed with:

Fixation

FF	PFPE	FFPE
• Fresh frozen in liquid nitrogen (FF) • Storage @ -80°C	• PAXgene Tissue Fix: 4h @ R.T. • PAXgene Tissue Stabilizer: 72h @ R.T.	• Neutral buffered formalin (NBF) • 24h @ R.T.

Processing
- EtOH 70%, 80%, 90%, 99% (x2) EtOH
- Isopropanol (x2), Xylene (x2)
- Low melting paraffin at 56°C (x3)
- 1 hour each position

Analyte Purification

FF	PFPE	FFPE
miRNeasy® Mini (QIAGEN)	PAXgene Tissue miRNA (PreAnalytiX)	miRNeasy FFPE (QIAGEN)

Analytic
- Staining with hematoxylin and eosin (H&E)
- Endpoint RT-PCR
- Real time RT-PCR

Results

Morphology preservation: PAXgene fixed tissues were histologically comparable to formalin-fixed tissues (Fig. 1). The cytoplasm of PAXgene-fixed tissues tended to be somewhat more eosinophilic compared to formalin-fixed tissues. Red blood cells were lysed in PAXgene-fixed tissues.

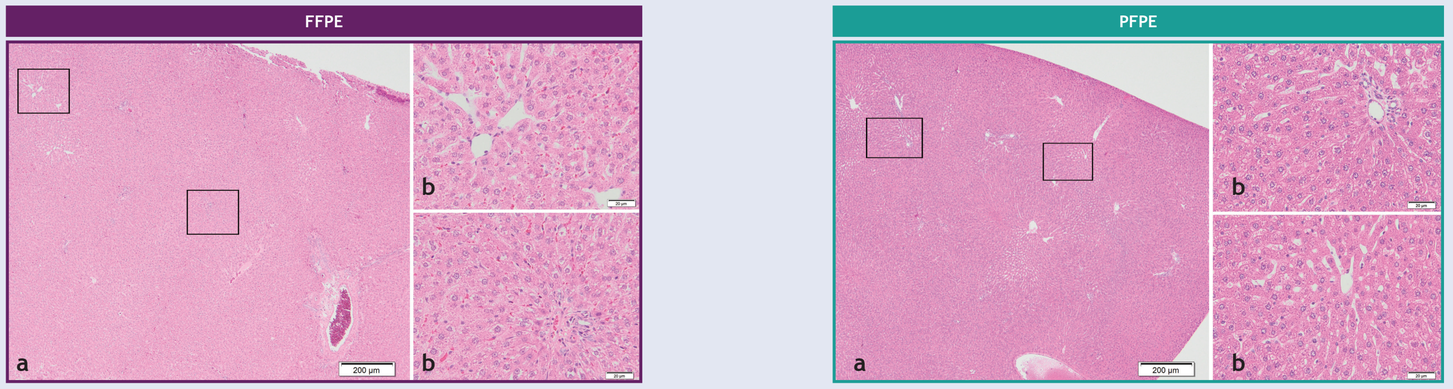

Figure 1: Hematoxylin & Eosin stained sections of formalin (FFPE) or PAXgene (PFPE) fixed rat liver, (a) 4x, (b) 40x objective.

RNA preservation: Under highly standardized conditions comparable results could be achieved for RNA from both FFPE and PFPE tissue with regard to yield, purity and RNA integrity numbers (RIN) using dedicated extraction methods (Fig. 2).

RNA from FFPE tissue was burdened by chemical modifications. These modifications were not reflected by the RIN value but could be identified in the electropherogram and gel analysis after microcapillary electrophoresis by a slower migration as well as washed out, less focused bands for 18s and 28s rRNA (Fig. 3).

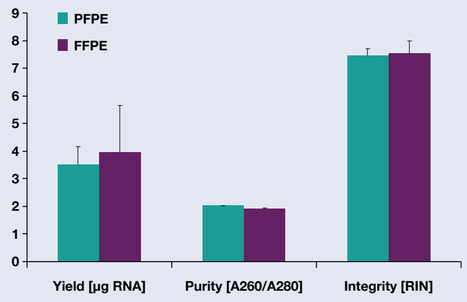

Figure 2: RNA extraction in triplicates from 3 x 10 µm sections of rat liver formalin (FFPE) or PAXgene (PFPE) fixed tissue; RNA yield and purity determined by spectrophotometry (Nanodrop ND-1000 spectrophotometer, Nanodrop Technologies, Wilmington, USA), RNA integrity by microcapillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, Waldbronn, Germany).

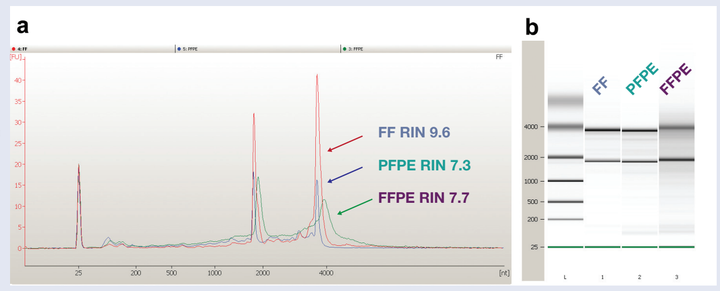

Figure 3: Analysis of total RNA isolated from rat liver fresh frozen (FF), PAXgene (PFPE) or formalin (FFPE) fixed tissue by microcapillary electrophoresis; (a) electropherogram (b) gel analysis.

Performance in RT-PCR: High RIN values for RNA from formalin (FFPE) fixed tissues could not be used as a predictor for performance in endpoint RT-PCR. Assays for amplification of sequences longer than 404 bp were completely inhibited. In contrast RNA from fresh frozen (FF) and PAXgene (PFPE) fixed tissues performed equally and did not inhibit endpoint RT-PCR (Fig. 4).

In real time RT-PCR, RNA from FFPE liver tissue inhibited amplification depending on amplicon length. In contrast RNA from PFPE liver performed equally compared to RNA from fresh frozen tissue independent of amplicon length (Fig. 5 and 6).

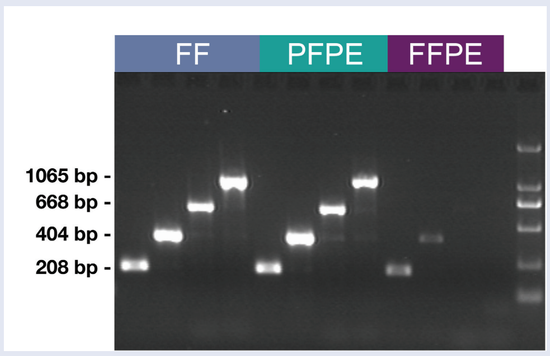

Figure 4: Analysis of total RNA isolated from rat liver fresh frozen (FF), PAXgene (PFPE) or formalin (FFPE) fixed tissue by one-step RT-PCR (QIAGEN OneStep RT-PCR kit, QIAGEN GmbH, Hilden, Germany). Amplicons of increasing lengths from the rat hypoxanthine phosphoyribosyl transferase (HPRT) mRNA were amplified with 10 ng RNA each in 35 cycles.

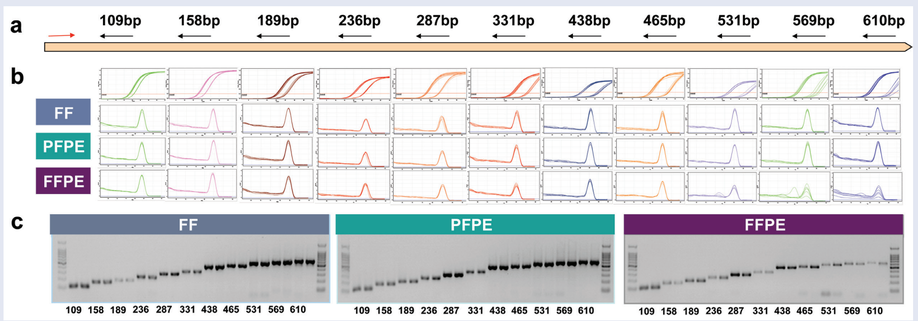

Figure 5: SYBR® Green real time RT-PCR assay design for amplification of rat beta-actin gene; (a) one common forward primer and different reverse primer to generate amplicon lengths from 109 to 610 base pair; (b) amplification plot, (c) melting curve and (d) agarose gel electrophoresis of 10 ng RNA from rat liver fresh frozen (FF), PAXgene (PFPE) or formalin (FFPE) fixed tissue amplified on RotorgeneQ (QIAGEN GmbH, Hilden, Germany) in a one-step RT-PCR reaction using the Quantitect SYBR Green RT-PCR kit (QIAGEN). RT-PCR was performed in duplicates from triplicate extractions of FF, PFPE and FFPE respectively, for each assay.

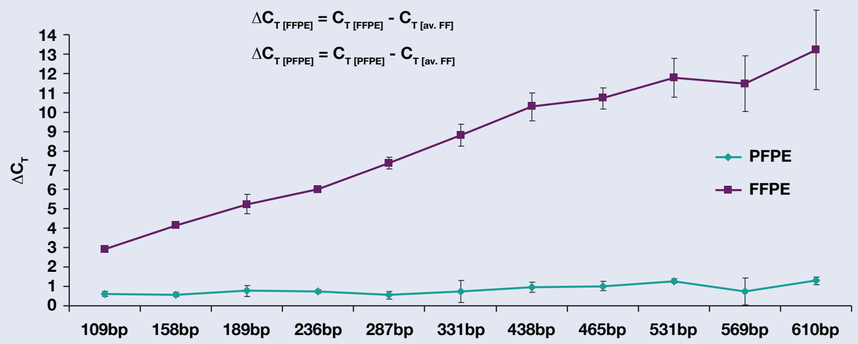

Figure 6: ΔC_T value calculation from beta-actin SYBR Green real time RT-PCR assays from Figure 5. ΔC_T were calculated by subtraction of the average C_T values obtained from fresh frozen (FF) samples (duplicate PCR from triplicate extraction) from each single C_T value obtained with RNA from FFPE or PFPE rat liver tissue.

Detection of improper use: Accidental immersion of tissue into PAXgene Tissue Stabilizer before fixation could be easily detected by histological examination. Placing tissues in PAXgene stabilizer prior to fixation left a characteristic eosinophilic coagulated tissue border. This border increased with the time the tissue was placed in the stabilizer before fixation.

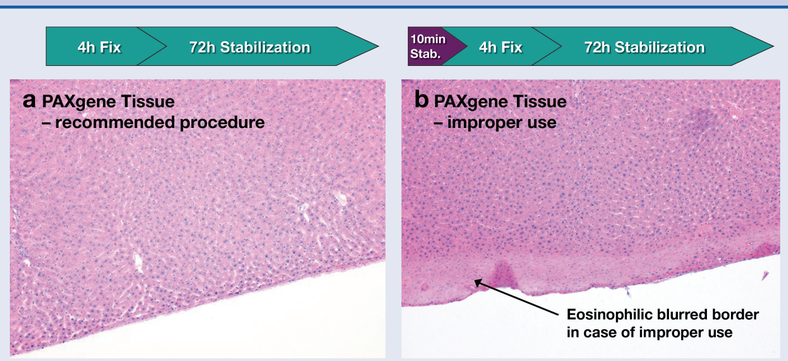

Figure 7: Hemotoxylin & Eosin stained sections of PAXgene (PFPE) fixed rat liver, 10x objective, (a) recommended procedure with 4 hours fixation followed by 72 hours stabilization and (b) improper use with 10 min incubation in the stabilizer before fixation.

Conclusions

Histology of PAXgene Tissue fixed tissue was comparable to formalin-fixed tissue. While in a highly controlled workflow, under ideal conditions RNA with acceptable RIN values could be isolated from both FFPE and PFPE tissue, only RNA from PFPE tissue performed in real time RT-PCR assays equally to RNA from fresh frozen tissue.

Acknowledgment
Funded by NCI Contract No. HHSN261200800001E

References
1. Ergin B, Meding S, Langer R, *et al*. Proteomic analysis of PAXgene-fixed tissues. *J Proteome Res* 2010;9:5188-5196
2. Kap M, Smedts F, Oosterhuis W, *et al*. Histological Assessment of PAXgene Tissue Fixation and Stabilization Reagents. *PLoS ONE* 2011;6:e27704