A New Tissue Fixative for Biomarker Discovery: Gene expression and miRNA in PAXgene® Tissue Fixed Paraffin-Embedded (PFPE) Colorectal Cancer (CRC) and Breast Cancer Tissue vs. Formalin Fixed Paraffin-Embedded (FFPE) Tissue

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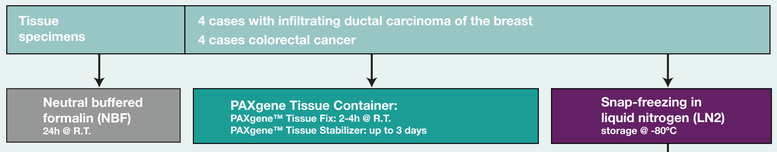
Introduction

A major impediment to accurate and consistent analysis of RNA from tissue is the poor recovery and quality of RNA from FFPE specimens. We have developed a new tissue fixative and stabilizing system, the PAXgene Tissue System for preservation of histomorphology, proteins, and nucleic acids in paraffin embedded tissue samples.[1] The system is comprised of a collection container for formalin-free fixation and stabilization of tissue specimens and purification kits for isolation of DNA, RNA or microRNA (miRNA) from tissues stored in PAXgene Tissue Stabilizer or PFPE tissue.

In this study, mirrored samples of human infiltrating ductal carcinoma (HIDC) of the breast or colorectal cancer (CRC) specimens were each divided for snap-freezing in liquid nitrogen (LN2), or fixation in either the PAXgene Tissue Container or formalin. Fixed tissue was embedded in paraffin. RNA and miRNA was extracted from snap frozen, FFPE and PFPE tissue. RNA expression was analyzed in different RT-qPCR arrays and miRNA expression in up to 16 different single assays. After normalization expression results of FFPE versus LN2 and PFPE versus LN2 were compared.

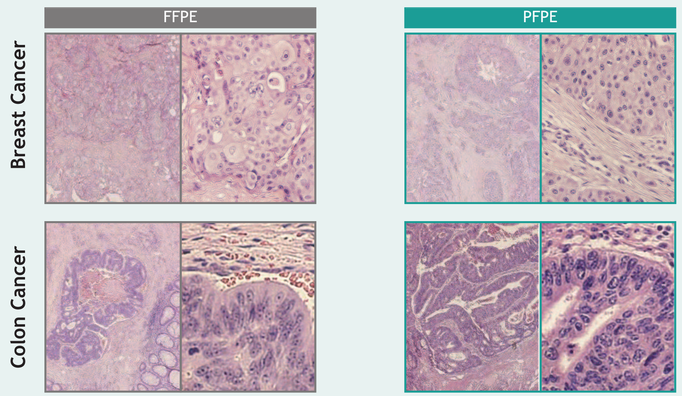
Materials and Methods



RNA and miRNA purification	PFPE: PAXgene® Tissue RNA and PAXgene® Tissue miRNA Kit (PreAnalytiX) FFPE: RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion) or RNeasy® FFPE (QIAGEN) Snap frozen (LN2): RNeasy Mini and miRNeasy Mini (QIAGEN)
RT-qPCR arrays	RT² Profiler™ PCR Arrays (SABiosciences) and TaqMan Array Gene Signature 96-Well Plates (Applied Biosystems)
miRNA specific RT-qPCR assays	TaqMan® MicroRNA assays (Applied Biosystems), miScript Primer Assays and miScript PCR Control Set (QIAGEN)

Results

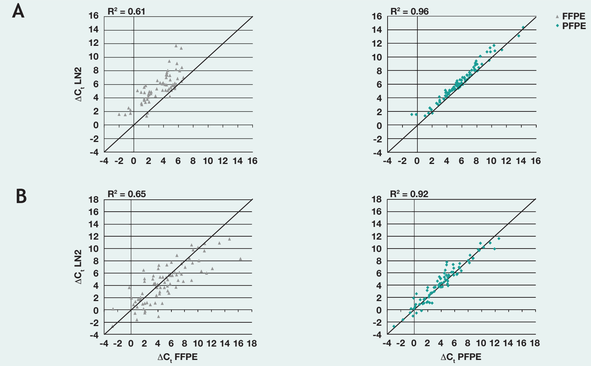
Figure 1: H&E staining



Hematoxylin and eosin (H&E) stained sections.

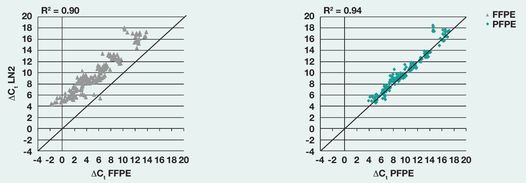
The applications presented here are for research use only. Not for use in diagnostic procedures. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of disease.

Figure 2: Gene Expression Analysis With RNA from HIDC Breast Cancer on RT-qPCR Arrays



Scatterplots with ΔC_t values from PCR arrays: ΔC_t FFPE or ΔC_t PFPE *versus* ΔC_t from snap-frozen tissue
RT-qPCR with RNA from mirrored samples of FFPE, PFPE and LN2 snap-frozen human breast cancer specimens. C_t values were normalized with average C_t values from housekeeping genes (HKG): $\Delta C_t = C_t$(target gene) − Ct(av. HKG); R^2: coefficient of determination
A: 500ng RNA from case 1 in TaqMan Array 'human molecular mechanisms of cancer' with primer/probe assays of 92 genes associated with cancer and 4 HKG.
B: 200ng RNA from case 2 amplified in RT² Profiler PCR Array 'human epithelial to mesenchymal transition' with Sybr-Green assays of 84 genes involved in this process and 5 HKG. In case of RNA from FFPE samples a 8 cycle pre-amplification of cDNA was performed prior to RT² Profiler Array analysis. RNA from LN2 or PFPE samples without preamplification.

Figure 3: miRNA Expression Analysis With Total RNA from HIDC Breast Cancer on miRNA Specific RT-qPCR Assays



Scatterplots with ΔC_t values from 15 different single miRNA specific RT-qPCR assays: ΔC_t FFPE or ΔC_t PFPE *versus* ΔC_t from snap-frozen tissue; six primer/probe TaqMan microRNA assays (miR10a, -16, -29a, -30b, -103, -192) and nine sybr green miScript assays (miR9, -10a, -10b, -29a, -103, -125b, -143, -145, -192).
C_t values were normalized with average C_t values from six miScript PCR controls (RNUA1, RNU5A, RNU6B, SNORD25, SCARNA17, SNORA73A HKG): $\Delta C_t = C_t$(target gene) − C_t(av. controls); R^2: coefficient of determination.
RNA from case 3 and 4 was purified in triplicates from mirrored samples FFPE, PFPE and LN2 snap-frozen human breast cancer specimens from each of two different patients.

Results

H&E stained sections of PFPE were similar to, or indistinguishable from, FFPE tissue (**Fig 1**).

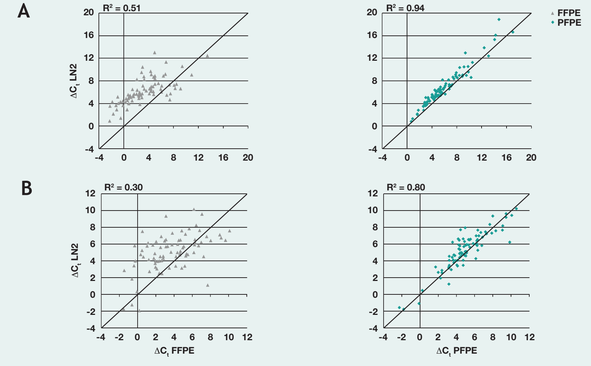
RNA quality from fresh frozen tissue was excellent with RIN values ranging from 5.8 − 9.9 (average 8.4). In contrast RNA from paraffin embedded tissue varied with RIN values of 2.2 − 6.8 (average 4.1) in the case of PFPE and RIN values of 2.1 − 2.7 (average 2.4) in case of FFPE.

Despite the differences in RIN values, a high correlation of gene expression results (normalized C_t values) was observed between RNA from PFPE and RNA from snap frozen tissue samples as determined by the coefficient of determination (R^2) value. R^2 values of 0.96 and 0.92 in the case of breast cancer samples (**Fig 2**) and 0.94 and 0.80 in the case of CRC samples (**Fig 4**) were achieved in profiling experiments with primer/probe based TaqMan Gene Signature and the Sybr-green based RT² Profiler arrays. A preamplification step in case of the RT² Profiler PCR array, mandatory for RNA from FFPE samples, wasn't necessary. The gene expression profile from PFPE tissue was comparable to fresh frozen tissue with (data not shown) or without pre-amplification (**Fig 2** and **4**).

The correlation of the same measurements of RNA from FFPE tissue to snap frozen tissue samples was much lower. R^2 values were significantly lower with 0.61 and 0.65 for breast cancer (**Fig 2**) and 0.51 and 0.30 for CRC (**Fig 4**) in TaqMan Gene Signature and RT² Profiler arrays (with preamplification) respectively.

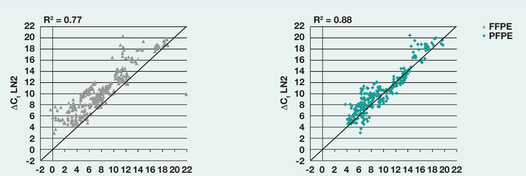
Correlation of miRNA expression between FFPE and fresh frozen was higher with R^2 values of 0.90 and 0.77 (breast cancer and CRC samples respectively), but still not as high as the correlation between PFPE and fresh frozen with R^2 values of 0.94 and 0.89 (breast cancer and CRC samples respectively) (**Fig 3** and **5**).

Figure 4: Gene Expression Analysis With RNA from Colon Cancer on RT-qPCR Arrays



Scatterplots with ΔC_t values from PCR arrays: ΔC_t FFPE or ΔC_t PFPE *versus* ΔC_t from snap-frozen tissue
RT-qPCR with RNA from mirrored samples of FFPE, PFPE and LN2 snap-frozen human colon cancer specimens. C_t values were normalized with average C_t values from housekeeping genes (HKG): $\Delta C_t = C_t$(target gene) − C_t(av. HKG); R^2: coefficient of determination
A: 500ng RNA from case 1 in TaqMan Array 'human colorectal cancer metastasis' with primer/probe assays of 92 genes associated with cancer and 4 HKG.
B: 500/ 250ng RNA from case 1 amplified in RT² Profiler PCR Array 'human cell cycle' with Sybr-Green assays of 84 genes involved in this process and 5 HKG. In case of RNA from FFPE samples a 8 cycle pre-amplification of cDNA was performed prior to RT² Profiler Array analysis. RNA from LN2 or PFPE samples without preamplification.

Figure 5: miRNA Expression Analysis With Total RNA from Colon Cancer on miRNA Specific RT-qPCR Assays



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C_t values were normalized with average C_t values from six miScript PCR controls (RNUA1, RNU5A, RNU6B, SNORD25, SCARNA17, SNORA73A HKG): $\Delta C_t = C_t$(target gene) − C_t(av. controls); R^2: coefficient of determination.
RNA from cases 2-4 was purified in triplicates from mirrored samples FFPE, PFPE and LN2 snap-frozen human breast cancer specimens from each of three different patients.

Conclusions

- **Morphology: The PAXgene Tissue System preserves morphology similarly to that seen in FFPE tissue.**
- **Gene expression profile: In PFPE tissue comparable to fresh frozen tissue, in FFPE tissue, poor correlation to fresh frozen tissue even with pre-amplification steps.**
- **miRNA expression profile: FFPE tissue profile correlates to fresh frozen tissue profile, but PFPE tissue profile correlates to frozen tissue profile better than FFPE tissue.**

Summary

The PAXgene Tissue System provides comparable preservation of tissue morphology to formalin, but the PAXgene Tissue System is superior to formalin in preserving RNA oncology biomarkers.

References

1. Bilge, E.; Meding, S.; Langer, R.; Kap, M.; Viertler, C.; Schott, C.; Ferch, U.; Riegman, P., Zatloukal, K.; Walch, A.; Becker K-F. Proteomic Analysis of PAXgene-Fixed Tissues. *J Proteome Res.* **2010** Oct 1; 9(10):5188-5196

Acknowledgment

Surgically resected tissue was collected by commerical providers with prior written informed consent by the patient.