Morphological, Epigenomic and Mutational Analyses of PAXgene® Tissue Fixed, Paraffin-Embedded (PFPE) Colorectal Cancer (CRC) Specimens – Comparison to Formalin Fixed, Paraffin Embedded (FFPE) and Snap-Frozen Samples

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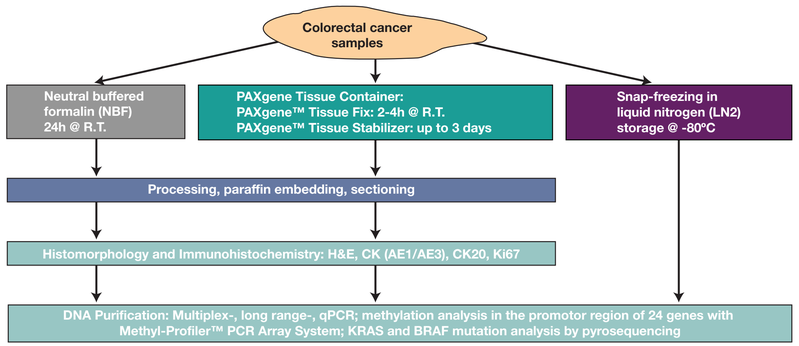
Introduction

The PAXgene Tissue System preserves tissue morphology, proteins and nucleic acids enabling multi-modal biomarker analyses from the same tissue sample.[1]

In this study, five cases of CRC were each divided for either snap-freezing in liquid nitrogen (LN2), or fixation in the PAXgene Tissue Container or formalin. Fixed tissue was embedded in paraffin and stained by H&E or immunohistochemically for cytokeratin (CK, clone AE1/AE3), cytokeratin 20 (CK20), and Ki-67.

DNA isolated from snap-frozen, PFPE and FFPE samples was analyzed by agarose gel electrophoresis, long-range, multiplex-, and q-PCR. Methylation status of the promoter regions of 24 genes were analyzed using the Methyl-Profiler™ PCR array system. Mutational status of KRAS and BRAF were determined by pyrosequencing.

Study Design

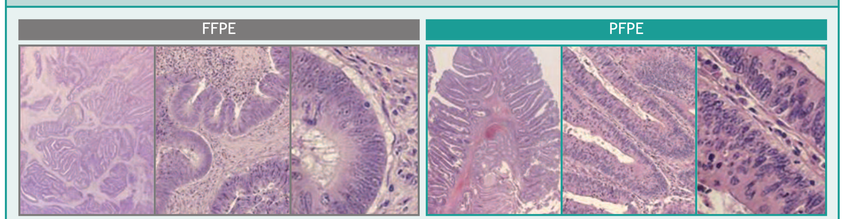


Materials and Methods

Tissue specimens	5 cases of colorectal cancer
Immunohistochemistry	Anti-Human Cytokeratins [clones AE1/AE3], Cytokeratin 20 [clone K_S 20.8] and Ki-67 [clone MIB-1] (Dako)
DNA purification	QIAamp® DNA Mini, QIAamp® DNA FFPE (QIAGEN), PAXgene® Tissue DNA Kit (PreAnalytiX)
PCR	QuantiTect® Probe PCR, QIAGEN® LongRange and QuantiTect Multiplex PCR Kits (QIAGEN)
DNA Methylation analysis	Methyl-Profiler™ DNA Methylation PCR Array System, Colon Cancer Signature Panels (SABiosciences)
Pyrosequencing	PyroMark® Q24 MDx; KRAS Pyro® Kit & BRAF Pyro Kit (QIAGEN)

Results

Figure 1: H&E staining



Hematoxylin and eosin (H&E) stained sections

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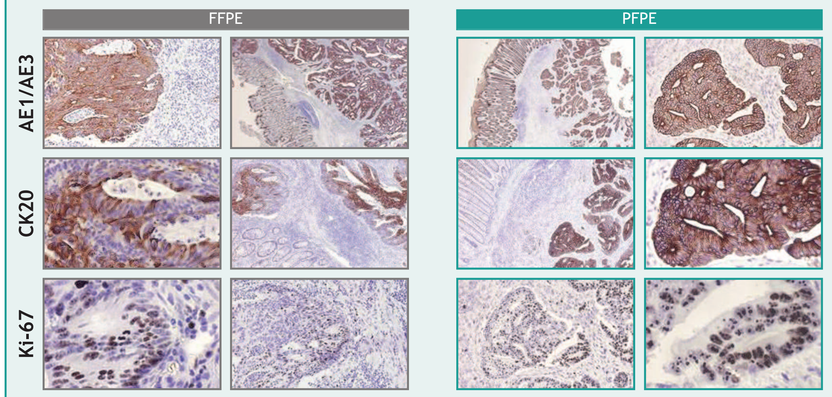
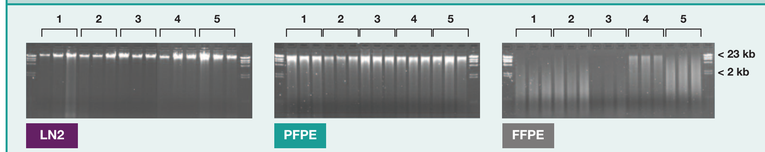
Figure 2: IHC staining of CKs (clones AE1/AE3), CK20 and Ki-67



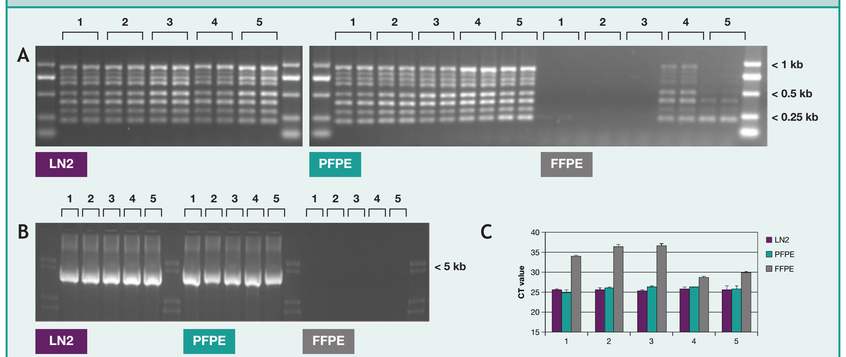
IHC staining of cytokeratins (AE1/AE3), cytokeratin 20 (CK20) and proliferation marker Ki-67 in streptavidin/biotin-labeled assays counterstained with hematoxylin. Staining was performed according to the recommendations from the manufacturer except for PFPE tissue to which were applied the following modifications: epitope retrieval was performed with all antibodies by incubation in Tris/EDTA buffer, pH9, for 10min at 70°C (AE1/AE3 and Ki-67) or 10min at room temperature (CK20).

Figure 3: Agarose gel electrophoresis with DNA from five CRC cases



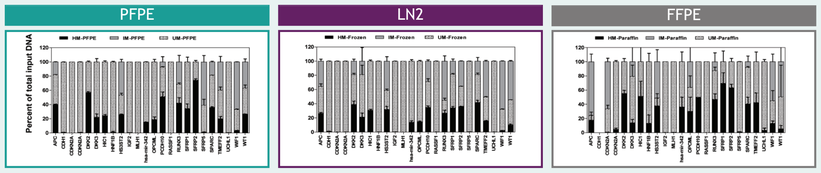
Agarose gel electrophoresis on 0.8% TBE buffered gels with 200ng genomic DNA isolated in triplicate from five cases (1-5) of human colorectal cancer, matched samples of frozen, PFPE and FFPE tissue.

Figure 4: Performance in long-range, multiplex, and real time TaqMan® PCR



Three different PCR applications with DNA from five cases (1-5) of human colorectal cancer, matched samples of frozen, PFPE and FFPE tissue. (A) Multiplex PCR of eight different genomic DNA fragments ranging from 222 to 955 bp, (B) long-range PCR of a 5 kb genomic DNA fragment and (C) quantitative β-actin real time PCR on TaqMan 7900.

Figure 5: DNA Methylation with the Methyl-Profiler™ System

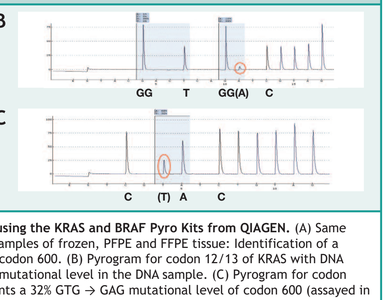


Methylation status of the promoter regions of 24 genes in DNA from Case 1 of human colorectal cancer in matched samples of frozen (LN2), PFPE and FFPE tissue analyzed with the Methyl-Profiler™ PCR Array System colon cancer signature panel (384-well format). Fractions of different DNA species are classified as hypermethylated (HM), intermediately methylated (IM) and unmethylated (UM).

Figure 6: KRAS and BRAF Mutational Analysis by Pyrosequencing

A

	KRAS		BRAF	
Case	Codon 12	Codon 13	Codon 61	Codon 600
1	GGT (WT)	GGC (WT)	GTT (WT)	GTG (WT)
2	GGT (WT)	GAC (mutant)	GTT (WT)	GTG (WT)
3	GGT (WT)	GGC (WT)	GTT (WT)	GAG (mutant)
4	GGT (WT)	GGC (WT)	GTT (WT)	GTG (WT)
5	GGT (WT)	GGC (WT)	GTT (WT)	GTG (WT)



Analysis of mutational status of KRAS and BRAF on a PyroMark Q24 MDx using the KRAS and BRAF Pyro Kits from QIAGEN. (A) Same results for DNA from five cases (1-5) of human colorectal cancer, matched samples of frozen, PFPE and FFPE tissue: Identification of a mutation in the DNA from case 2 in KRAS codon 13 and from case 3 in BRAF codon 600. (B) Pyrogram for codon 12/13 of KRAS with DNA from case 2, PFPE tissue. The small peak (○) represents a 12% GGC → GAC mutational level in the DNA sample. (C) Pyrogram for codon 600 of BRAF with DNA from case 3, PFPE tissue. The small peak (○) represents a 32% GTG → GAG mutational level of codon 600 (assayed in reverse orientation).

Discussion

H&E, IHC

H&E staining of PFPE tissue sections demonstrate intact morphology with slightly more contrast than that seen in FFPE mirrored samples (**Fig 1**). Immune reactivity for PFPE tissue was equivalent to or stronger than reactivity in FFPE tissue for all tested antibodies (**Fig 2**). In order to achieve strong and specific staining intensities in PFPE tissue, eptiope retrieval was performed in tris/EDTA buffer, pH 9.0. Incubation temperatures for epitope retrieval in PFPE tissue vary between room temperature and 98°C and must be determined for each antibody used.

DNA

Genomic DNA isolated from PFPE is of high molecular weight and appears on agarose gels as one distinct band with little smearing (**Fig 3**). Since the DNA is not burdened with crosslinks and chemical modifications, demanding downstream applications such as multiplex, long-range, and quantitative PCR give results comparable to DNA from snap-frozen samples (**Fig 4**).

DNA Methylation and Sequencing

Methylation patterns of DNA from PFPE can be analyzed with Methyl-Profiler™ PCR Array System, a technology based on methylation-dependent restriction and quantitative PCR. Fractions of different DNA species classified as hypermethylated, intermediately methylated and unmethylated are comparable between PFPE and frozen samples and resulted in small error bars. In contrast, DNA from FFPE samples showed extensive errors and larger differences compared to PFPE and frozen tissues (**Fig 5**). In addition, the DNA from PFPE is fully compatible with methods developed to work with highly fragmented DNA such as pryrosequencing (**Fig 6**).

Conclusion

- **Morphology in PFPE CRC tissue is equivalent to morphology in FFPE CRC samples.**
- **After optimization of the heat induced epitope retrieval step, immunohistochemical staining methods can be applied.**
- **High molecular weight DNA can be isolated from PFPE samples.**
- **In PCR assays, DNA from PFPE samples performs as well as DNA from snap frozen samples.**
- **The methylation pattern of DNA from PFPE can be analyzed with the Methyl-Profiler™ PCR Array System.**
- **DNA from PFPE is suitable for use in pyrosequencing.**

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 a commercial provider with prior written informed consent by the patient.