DNA quality measurement and somatic mutation profiling in PAXgene tissue samples with qBiomarker Somatic Mutation PCR Arrays

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Abstract

Current formaldehyde-based tissue fixation methods are of limited use for molecular analysis due to degradation and chemical modification of nucleic acids that can cause inhibition in PCR or real-time PCR. The PAXgene Tissue Kit is a novel, powerful system that enables both molecular and traditional pathology analysis from the same specimen. The system consists of a tissue collection device (tissue container for collection, stabilization, storage, and transportation of tissue specimens) and kits for purification of nucleic acids. The fixation and stabilization method used in this system preserves tissue morphology and the integrity of nucleic acids for an extended period without the destructive cross-linking and degradation found in formalin-fixed tissues. In addition, samples stabilized using the PAXgene Tissue Kit can be embedded in paraffin for histological studies. Nucleic acids can be isolated from the stabilized samples either before or after embedding in paraffin.

In the present study, the specialized qBiomarker Somatic Mutation PCR Array for Human DNA QC assessment, which consists of a panel of 7 copy number PCR control assays, was employed to compare the quality of DNA extracted from fresh frozen, formaldehyde-fixed, and PAXgene tissues that were prepared at the same time, from the same set of mouse xenograft samples of human melanoma. The DNA quality of PAXgene tissue samples was found to be similar to that of fresh frozen samples (i.e. intact DNA), while more than 80% of the DNA fraction in formaldehyde-fixed samples exists in non-amplifiable fractions under typical real-time PCR conditions.

The Human EGFR Pathway qBiomarker Somatic Mutation PCR Array was used to profile the mutation status in these melanoma xenograft samples for the following genes: EGFR, BRAF, KRAS, NRAS, HRAS, c-MET, PIK3CA, AKT and PTEN. While the identical set of BRAF and NRAS mutations was identified in DNAs from samples of all three preparation methods, at all DNA input doses tested (200 ng per sample as the lowest dose for screening 87 mutations, and up to 1200 ng per sample), the mutation call was at least 4-fold more sensitive in DNA extracted from PAXgene samples and fresh frozen samples than that from the FFPE DNA. In addition, when duplicate samples were compared, among the three sample preparation methods, PAXgene sample DNA quality showed the least variability between duplicate samples. This consistency in sample quality also translated into the highest reproducibility in mutation calls among the three sample preparation methods.

The PAXgene Tissue System, with its excellent tissue morphology preservation and close-to-intact level of nucleic acid preservation, is an ideal system for molecular and histological testing of research samples. The combination of the PAXgene Tissue Kit with disease- or pathway-focused qBiomarker Somatic Mutation PCR Arrays allows specific, highly sensitive, and accurate somatic mutation detection in tumor samples (especially for mutations occurring at a low frequency), and has distinct advantages over other systems that are used for sample stratification.

The applications presented here are for research use only. Not for use in diagnostic procedures.

PAXgene Tissue System workflow and experimental design

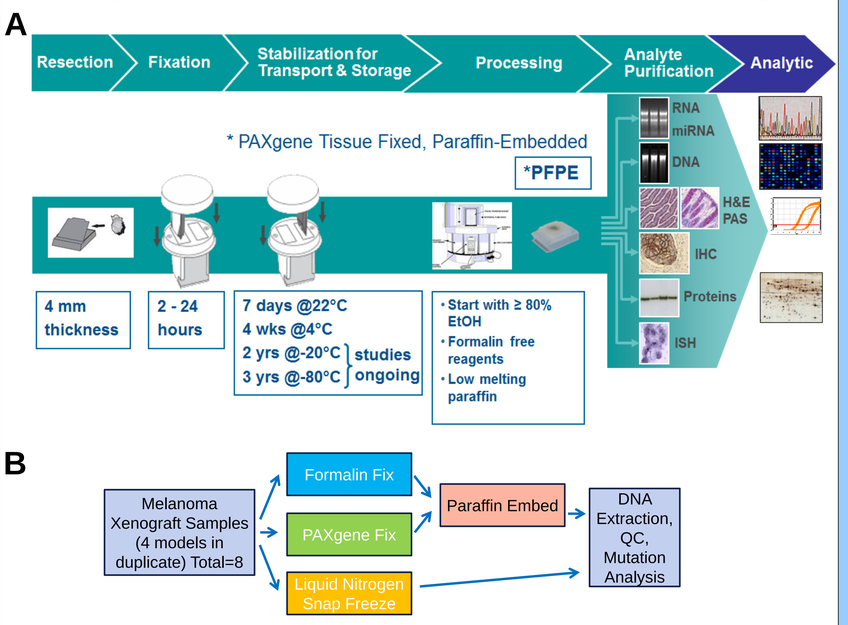


Figure 1. PAXgene Tissue System workflow and experimental design.
(A) The PAXgene Tissue System provides a fixation and stabilization method that preserves tissue morphology and the integrity of nucleic acids without destructive cross-linking and degradation found in formalin-fixed tissues. The system consists of a tissue collection device (the PAXgene Tissue Container for collection, stabilization, storage, and transportation of human tissue specimens) and kits for purification of total RNA, DNA, or miRNA. The Tissue Container is a dual-chamber container prefilled with two reagents. Chamber 1 contains PAXgene Tissue Fix. Tissue samples with a maximum size of 4 x 15 x 15 mm can be fixed in chamber 1 for 2 to 24 hours. After fixation, the tissue cassette is transferred to Chamber 2 containing PAXgene Tissue Stablizer for storage for a minimum of 7 days at room temperature or for a minimum of 4 weeks at 2–8°C, depending on tissue type. Tissues in PAXgene Tissue Stablizer can also be stored at –20 to –80°C for long-term storage without negative effects on tissue morphology or nucleic acid integrity. Stabilized samples can be processed and embedded in paraffin for histological studies. Nucleic acids and proteins can be isolated from the stabilized PFPE (PAXgene Tissue fixed, paraffin-embedded) which can be used for downstream analyses such as DNA analysis including mutation profiling (this study), gene expression quantitation, next generation sequencing and 2-D gel electrophoresis.
(B) Experimental design to compare the DNA quality (Figure 5C) and mutation detection performance (figure 6) in fresh frozen, PAXgene tissue and FFPE samples.

Performance of PAXgene Tissue Kit in preserving morphology of surgically resected tissues

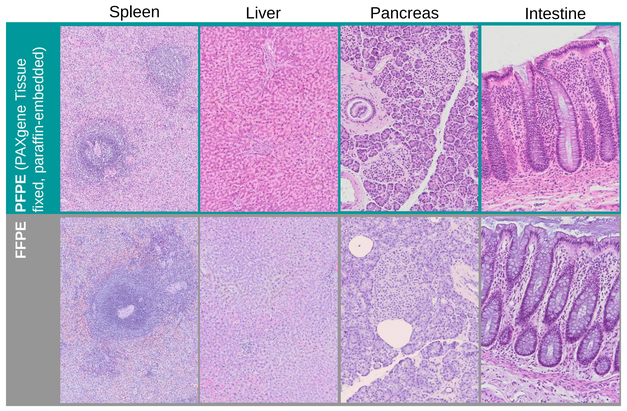


Figure 2. Tissue morphology preservation by the PAXgene Tissue Kit. Morphology of H&E (hemotoxylin and eosin) stained, mirrored human spleen, liver, pancreas and large intestine tissue sections (4 µm), preserved either by PFPE (PAXgene Tissue fixed, paraffin embedded) or FFPE (formalin-fixed). Surgically resected tissues were collected by a commercial tissue provider (Cureline) with prior written informed consent from respective patients. Spleen and liver: original magnifications x 10; pancreas and large intestine: original magnifications x 20.

qBiomarker Somatic Mutation PCR Assay principle and performance

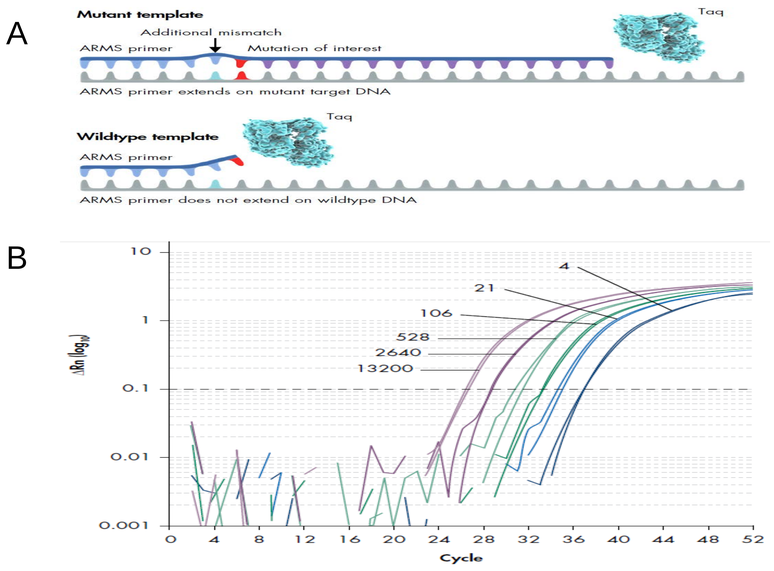


Figure 3. qBiomarker Somatic Mutation PCR Assay principle and performance. [A] qBiomarker Somatic Mutation PCR Assays employ ARMS primers to differentiate between mutant and wild-type alleles using template matches and mismatches at a primer's 3' end. An ARMS primer that has a match to the mutant template at the 3' end, which is mismatched with the wild-type, will extend on the mutant instead of the wild-type template. Additional mismatch(es) can be included in the ARMS primer to increase allele discrimination. Amplicons containing the mutation are subsequently detected by a probe. [B] Assay sensitivity test for p53 R280K qBiomarker Somatic Mutation PCR Assay. A series of 10 ng genomic DNA samples, which contain 4, 21, 106, 528, 2640 and 13200 copies of mutant DNA template respectively, from MDA-MB-231 cell line (mutant harboring the p53 R280K mutation) mixed with genomic DNA from the Coriell GM00131 cell line (wild-type), were tested with the p53 R280K mutation assay. Amplification plots for duplicate reactions are shown. Mutation detection limit for this assay is determined to be 0.03%.

qBiomarker Somatic Mutation PCR Array workflow

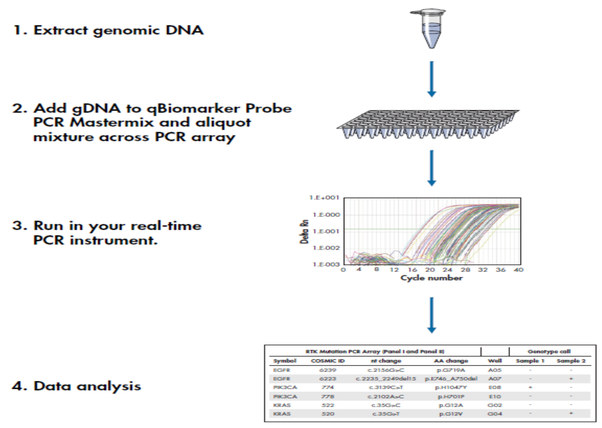
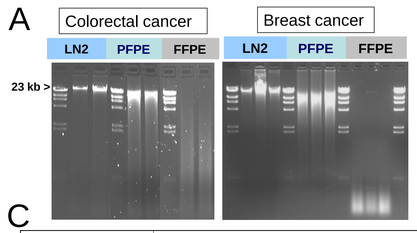


Figure 4. qBiomarker Somatic Mutation PCR Array workflow. The procedure involves DNA extraction, qPCR detection on qBiomarker Somatic Mutation PCR Arrays, and data analysis (using the qBiomarker Somatic Mutation Data Analysis Template or web portal). The arrays can be used on all real-time cyclers except the Cepheid® SmartCycler® and the Roche® LightCycler® 2.0.

DNA integrity of PAXgene-preserved tissues is far superior to that of FFPE samples



B

	1	2	3	4	5	6	7	8	9	10	11	12
A	BRAF	BRAF	BRAF	BRAF	BRAF	BRAF	BRAF	BRAF	BRAF	BRAF	BRAF	BRAF
B	KRAS	KRAS	KRAS	KRAS	KRAS	KRAS	KRAS	KRAS	KRAS	KRAS	KRAS	KRAS
C	HRAS	HRAS	HRAS	HRAS	HRAS	HRAS	HRAS	HRAS	HRAS	HRAS	HRAS	HRAS
D	NRAS	NRAS	NRAS	NRAS	NRAS	NRAS	NRAS	NRAS	NRAS	NRAS	NRAS	NRAS
E	MEK1	MEK1	MEK1	MEK1	MEK1	MEK1	MEK1	MEK1	MEK1	MEK1	MEK1	MEK1
F	PIK3CA	PIK3CA	PIK3CA	PIK3CA	PIK3CA	PIK3CA	PIK3CA	PIK3CA	PIK3CA	PIK3CA	PIK3CA	PIK3CA
G	PTEN	PTEN	PTEN	PTEN	PTEN	PTEN	PTEN	PTEN	PTEN	PTEN	PTEN	PTEN
H	SMPC	SMPC	SMPC	SMPC	SMPC	SMPC	SMPC	SMPC	SMPC	SMPC	SMPC	SMPC

C

	Fresh frozen								Set QC C_T average	Set QC C_T Std. dev
Sample ID	PHTX004683	PHTX004684	PHTX004685	PHTX004686	PHTX004687	PHTX004688	PHTX004689	PHTX004690		
Sample QC C_T average	29.14	29.06	29.23	29.24	28.02	28.73	28.92	27.87	28.78	0.54
	PAXgene									
Sample ID	PHTX004973	PHTX004974	PHTX004961	PHTX004963	PHTX004969	PHTX004971	PHTX004981	PHTX004983		
Sample QC C_T average	28.96	29.16	29.08	29.14	28.99	28.72	28.13	28.21	28.8	0.41
	FFPE									
Sample ID	PHTX004977	PHTX004978	PHTX004962	PHTX004964	PHTX004970	PHTX004972	PHTX004982	PHTX004984		
Sample QC C_T average	30.69	31.1	32.04	32.12	30.96	31.17	30.34	30.65	31.13	0.64

Figure 5. DNA integrity comparison among fresh frozen, PAXgene tissue, and FFPE samples. [A] Analysis of 300 ng DNA in agarose gel (0.8%) electrophoresis. DNA was isolated from mirrored human tumor tissues (left: colorectal cancer; right: breast cancer) that were fresh frozen (liquid nitrogen snap frozen; LN2), PAXgene Tissue fixed and embedded in paraffin (PFPE), or FFPE. [B] qBiomarker Somatic Mutation PCR Array DNA QC plate layout. 7 probe-based gene copy number reference assays are used to interrogate sample DNA quality, while the SMPC assay monitors the presence of PCR inhibitors in the sample. Each 96-well plate can be used to QC 12 DNA samples, and each 384-well plate can QC 48 DNA samples. [C] Real-time PCR DNA quality analysis of DNAs from 4 mouse xenografts of human melanoma (color-coded gray, light blue, pink and light green) that were preserved by liquid nitrogen snap frozen (fresh frozen), PAXgene tissue kit followed by paraffin embedding (PAXgene), or formalin fixation (FFPE) at the same time. For each preservation method, each sample was prepared in duplicates. About 8 weeks after preservation processing, DNA was isolated from the samples using QIAamp DNA Mini Kit (fresh frozen), PAXgene Tissue DNA Kit, and QIAamp DNA FFPE Tissue Kit, respectively. Purified DNA samples were then analyzed on the qBiomarker Somatic Mutation PCR Array DNA QC plate at an input of 2 ng DNA per reaction. Sample QC C_T average: the average of the C_T values of the 7 gene copy number reference assays for the sample. Set QC C_T average: the average of the "sample QC C_T average" for the 8 samples with the same preservation method. On average, more than 80% of the DNA fraction in FFPE samples exists in non-amplifiable fractions under typical real-time PCR conditions, as compared to fresh frozen and PAXgene sample.

Somatic mutation detection in PAXgene tissue samples using qBiomarker Somatic Mutation PCR Array

A

	1	2	3	4	5	6	7	8	9	10	11	12
A	AKT1 33765 c.49G>A p.E17K	BRAF 450 c.1391G>T p.G464V	BRAF 451 c.1393G>T p.G466V	BRAF 460 c.1406G>C p.G469A	BRAF 470 c.1796C>G p.L597V	BRAF 1130 c.1798G>A p.V600M	BRAF 476 c.1799T>A p.V600E	BRAF 18443 c.1799T>C p.V600A	BRAF 6137 c.1799T>G p.V600G	EGFR 6252 c.2155G>A p.G719S	EGFR 6253 c.2155G>T p.G719C	EGFR 6239 c.2156G>C p.G719A
B	EGFR 6223 c.2235_2249del15 p.E746_A750del	EGFR 6225 c.2236_2250del15 p.E746_A750del	EGFR 12728 c.2236_2253del18 p.E746_T751del	EGFR 12678 c.2237_2251del15 p.E746_T751>A	EGFR 12422 c.2238_2248>GC p.I747_A750>P	EGFR 6229 c.2238_2255del18 p.E746_S752>D	EGFR 6218 c.2239_2247del9 p.L747_E749del	EGFR 6255 c.2239_2256del18 p.L747_S752del	EGFR 12369 c.2240_2254del15 p.L747_T751del	EGFR 6241 c.2303G>T p.S768I	EGFR 12376 c.2307_2308insGCCAGCGTG p.V769_D770insASV	EGFR 12378 c.2310_2311insGGT p.D770_N771insG
C	EGFR 12377 c.2319_2320insCAC p.H773_V774insH	EGFR 6240 c.2369C>T p.T790M	EGFR 12366 c.2572C>A p.L858M	EGFR 6224 c.2573T>G p.L858R	EGFR 6213 c.2582T>A p.L861Q	KRAS 552 c.182A>G p.Q61R	KRAS 553 c.182A>T p.Q61H	KRAS 555 c.183A>T p.Q61H	KRAS 517 c.34G>A p.G12S	KRAS 518 c.34G>C p.G12R	KRAS 516 c.34G>T p.G12C	KRAS 521 c.35G>A p.G12D
D	KRAS 522 c.35G>C p.G12A	KRAS 520 c.35G>T p.G12V	KRAS 528 c.37G>A p.G13S	KRAS 529 c.37G>C p.G13R	KRAS 527 c.37G>T p.G13C	KRAS 532 c.38G>A p.G13D	KRAS 533 c.38G>C p.G13A	KRAS 534 c.38G>T p.G13V	KRAS 543 c.64C>A p.Q22K	HRAS 496 c.181C>A p.Q61K	HRAS 499 c.182A>G p.Q61R	HRAS 498 c.182A>T p.Q61L
E	HRAS 502 c.183G>T p.Q61H	HRAS 480 c.34G>A p.G12S	HRAS 482 c.34G>C p.G12R	HRAS 481 c.34G>T p.G12C	HRAS 484 c.35G>A p.G12D	HRAS 483 c.35G>T p.G12V	HRAS 486 c.37G>C p.G13R	HRAS 488 c.37G>T p.G13C	NRAS 580 c.181C>A p.Q61K	NRAS 582 c.182A>C p.Q61P	NRAS 584 c.182A>G p.Q61R	NRAS 583 c.182A>T p.Q61L
F	NRAS 563 c.34G>A p.G12S	NRAS 564 c.35G>A p.G12D	NRAS 565 c.35G>C p.G12A	NRAS 569 c.37G>C p.G13R	NRAS 573 c.38G>A p.G13D	NRAS 575 c.38G>C p.G13A	NRAS 574 c.38G>T p.G13V	NRAS 577 c.52G>A p.A18T	MEK1 167A>C Q56P	MEK1 171G>T K57N	MEK1 199G>A D67N	MEK1 371C>T P124L
G	PIK3CA 759 c.1616C>G p.P539R	PIK3CA 760 c.1624G>A p.E542K	PIK3CA 763 c.1633G>A p.E545K	PIK3CA 764 c.1634A>G p.E545G	PIK3CA 765 c.1635G>T p.E545D	PIK3CA 775 c.1740A>G p.H1047R	PIK3CA 776 c.3140A>T p.H1047L	PTEN 5033 c.389G>A p.R130Q	PTEN 5219 c.388C>G p.R130G	PTEN 5152 c.388C>T p.R130*	PTEN 5089 c.517C>T p.R173C	PTEN 5039 c.518G>A p.R173H
H	PTEN 5154 c.697C>T	AKT1 copy number assay	BRAF copy number assay	EGFR copy number assay	KRAS copy number assay	NRAS copy number assay	MEK1 copy number assay	PIK3CA copy number assay	PTEN copy number assay	SMPC positive PCR control	SMPC positive PCR control	

B

	Fresh frozen							
Sample ID	PHTX004683	PHTX004684	PHTX004685	PHTX004686	PHTX004687	PHTX004688	PHTX004689	PHTX004690
BRAF V600E Ct	40	40	28.58	29.14	40	40	27.7	26.82
NRAS Q61K Ct	40	40	40	40	27.07	27.72	37.19	40
	PAXgene							
Sample ID	PHTX004973	PHTX004974	PHTX004961	PHTX004963	PHTX004969	PHTX004971	PHTX004981	PHTX004983
BRAF V600E Ct	40	40	28.87	28.84	37.18	36.34	27.52	27.62
NRAS Q61K Ct	40	37.13	40	40	27.91	28.13	40	40
	FFPE							
Sample ID	PHTX004977	PHTX004978	PHTX004962	PHTX004964	PHTX004970	PHTX004972	PHTX004982	PHTX004984
BRAF V600E Ct	40	40	31.21	31.29	40	40	28.76	29
NRAS Q61K Ct	40	40	40	40	29.88	30	40	40

Figure 6. Somatic mutation profiling in mirrored fresh frozen, PAXgene and FFPE samples using the EGFR pathway qBiomarker Somatic Mutation PCR Array. [A] Assay layout of the EFGR pathway qBiomarker Somatic Mutation PCR Array. Three categories of probe-based assays are included on this array. Firstly, mutation detection assays are included for the most frequently occurring somatic mutations for genes in the EGFR pathway. Gene copy number reference assays are also incorporated for all genes covered on this array. These assays provide DNA quality and gene dosage information during data analysis. Finally, SMPC assays are included for monitoring PCR conditions, such as the presence of PCR inhibitors. [B] More robust performance of PAXgene samples compared to FFPE samples in real time PCR-based somatic mutation analysis. The samples in Figure 5(C) were profiled on the EGFR pathway qBiomarker Somatic Mutation PCR Array (4ng DNA per reaction) and C_T values for selected loci are shown. On average, mutation call was at least 4 fold more sensitive (i.e. Cts ≥2 Ct lower) in DNA extracted from PAXgene and fresh frozen samples than FFPE DNA. C_T values in black: wildtype calls; C_T values in green: mutant.

Conclusions

- Tissue morphology preservation in PAXgene tissue samples is comparable to or better than FFPE samples of the same age.
- DNA isolated from PAXgene tissues is mostly in 10 to 23 kb molecular weight range, which translates to significantly higher amplifiable fractions in real-time PCR.
- qBiomarker Somatic Mutation PCR Arrays consist of pathway- or disease-focused collections of mutation detection assays with sensitivity as high as 0.03%.
- Mutation detection in PAXgene samples is at least 4-fold more sensitive than in FFPE samples; the combination of the PAXgene Tissue Kit with qBiomarker Somatic Mutation PCR Arrays allows specific, highly sensitive, and accurate somatic mutation detection in tumor samples, and has distinct advantages over other systems that are used for sample stratification.