Accuracy of molecular data generated with FFPE biospecimens: lessons from the literature

Sarah R. Greytak[1], **Kelly B. Engel**[2], **B. Paige Bass**[1], and **Helen M. Moore**[3]

[1] Kelly Government Solutions, Rockville, MD USA

[2] Preferred Solutions Group, Arlington, VA USA

[3] Biorepositories and Biospecimen Research Branch, Cancer Diagnosis Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD USA

Abstract

Formalin-fixed and paraffin-embedded (FFPE) tissue biospecimens are a valuable resource for molecular cancer research. Although much can be gained from their use, it remains unclear whether the genomic and expression profiles obtained from FFPE biospecimens accurately reflect the physiological condition of the patient from which they were procured, or if such profiles are confounded by biological effects from formalin fixation and processing. To assess the physiological accuracy of genomic and expression data generated with FFPE specimens we surveyed the literature for papers investigating genomic and expression endpoints in case-matched FFPE and fresh or frozen human specimens using the National Cancer Institute's Biospecimen Research Database (http://biospecimens.cancer.gov/brd). Results of the survey revealed that the level of concordance between differentially preserved biospecimens varied among analytical parameters and platforms, but also among reports, genes/transcripts of interest, and tumor status. The identified analytical techniques and parameters that resulted in strong correlations between FFPE and frozen biospecimens may provide guidance when optimizing molecular protocols for FFPE use; however, discrepancies reported for similar assays also illustrate the importance of validating protocols optimized for use with FFPE specimens with a case-matched fresh or frozen cohort for each platform, gene or transcript, and FFPE processing regime. Based upon evidence published to date, validation of analytical parameters with a properly handled frozen cohort is necessary to ensure a high degree of concordance and confidence in the results obtained with FFPE biospecimens.

Keywords

Corresponding Author: Dr. Helen Moore, Biorepositories and Biospecimen Research Branch, National Cancer Institute, 9609 Medical Center Drive, Rm 3W422, MSC 9728, Bethesda, MD 20892, Phone: (240) 276-5713, Fax: (301) 480-4814, moorehe@mail.nih.gov.

The authors have no conflicting interests.

Introduction

Routine FFPE processing of surgical, biopsy and post-mortem tissue has resulted in a large collection of biospecimens uniquely representing a variety of cancer stages and subtypes. In many instances, case-matched slides of tumor and adjacent normal tissue have been previously screened by a pathologist for cancer-related biomarkers, making these biospecimens particularly attractive for the identification of molecular changes associated with cancer onset and progression. However, DNA and RNA isolated from FFPE biospecimens are of lower quality than those obtained from fresh or frozen biospecimens, displaying evidence of degradation and reduced assay efficacy (1-3). Such degradation may be due in part to sub-optimal fixation and processing conditions or extraction methods. Notably, all FFPE biospecimens are not "equal," as numerous effects on molecular analysis have been attributed to pre-analytical factors such as biospecimen size, the pH and composition of formalin, the temperature and duration of fixation, and FFPE block and slide storage (4). Such effects have been reported not only for DNA and RNA but for protein, phosphoprotein, and morphological endpoints as well (4,5). Further, DNA and RNA yield and quality can be adversely impacted by sub-optimal extraction methods, although optimal protocols are dependent upon the type of tissue and nucleic acid analyzed and the length of the nucleic acid fragment required (Greytak et.al., manuscript in preparation). Despite such challenges, genomic and gene expression data generated from FFPE biospecimens are often unquestionably accepted as an accurate reflection of the physiological condition of the patient. Literature evidence suggests, however, that analytical endpoints obtained with FFPE biospecimens reflect not only the physiology of the patient but a compilation of fixation, processing, and analytical influences. The aims of this review are (i) to assess the physiological accuracy of genomic and gene expression data generated with FFPE biospecimens across different platforms, assays, and diagnoses, and (ii) to identify analytical factors that can influence and maximize concordance between data generated with FFPE biospecimens and fresh or snap-frozen controls.

Methods

Published articles comparing genomic or gene expression analyses in case-matched FFPE and snap-frozen or fresh neoplastic tissue biospecimens were identified by searching the Biospecimen Research Database (BRD) (http://biospecimens.cancer.gov/brd), developed and maintained by the National Cancer Institute's Biorepositories and Biospecimen Research Branch. The BRD is a publicly available online database that summarizes and catalogs peer-reviewed journal articles investigating pre-analytical variability in human biospecimens. A preliminary list of articles obtained from the BRD was expanded through cross-referencing and targeted searches of the PubMed database (pubmed.gov; National Institutes of Health). The primary meta-analysis resulted in the identification of 68 relevant primary research articles; this list was further truncated to 34 representative articles due to reference limitations. Inclusion criteria favored reports that (i) were published within the last 10 years, (ii) analyzed biospecimens procured from 5 or more patients, and (iii) used current and relevant platforms for analysis. Exceptions to inclusion criteria were made when novel as opposed to supportive findings were reported (see Supplementary Table 1). Results discussed below were limited to those obtained by whole genome or transcriptome analysis

or targeted investigation of genes or transcripts relevant to cancer research using matched FFPE and frozen or fresh human biospecimens.

DNA analysis of FFPE biospecimens: success rates and challenges

Analysis of FFPE biospecimens by genotyping and copy number determination would permit genetic evaluation of surplus and archived pathology biospecimens, which could lead to a better understanding of the role that genetic variants play in cancer progression. However, success rates with FFPE biospecimens are often lower than those reported for fresh or frozen biospecimens, although the extent of the effect is dependent upon the analytical platform. For example, in FFPE biospecimens, genotyping success (measured by the number of sequencing reads) was strongly correlated with matched frozen controls when analyzed by next generation sequencing (NGS) (r=0.82)(6). In contrast, when utilizing microarray analysis, FFPE biospecimens exhibited lower success rates than frozen biospecimens with molecular inversion probe arrays (88% versus 100%) (7) and lower call rates with microarrays (31.86-86.30 versus 90.07-93.99) (8). Success rates for copy number analysis of FFPE biospecimens differ among platforms. Rates comparable to those obtained with frozen biospecimens were reported with traditional sequencing (6). However, a real-time PCR-based assay (9) and microarray (7,10) resulted in lower success rates for FFPE compared to frozen biospecimens. Despite robust detection rates (95-99%) for DNA methylation analysis of FFPE biospecimens by array (11-13), FFPE biospecimens yielded significantly fewer detectable loci by array (11) and a lower percentage of interpretable results by pyrosequencing (55-75% versus 87-97%) (14).

Genotyping: accuracy in FFPE tissue

The level of genotype concordance between FFPE and matched frozen biospecimens varies among analytical platforms and reports, ranging between >99% by high-throughput sequencing (15) and molecular inversion probe microarray (7), > 90% by microarray (8,16), and between 59% (17) and 82% (18) by traditional sequencing. Although the relatively high concordance values of some platforms provide support for the use of FFPE biospecimens for genotype determination, such values fail to capture potential differences in copy number or false discovery rates of cancer-relevant mutations. Large differences in false discovery rates have been reported among methods and reports, which cautions against the use of FFPE biospecimens without proper validation. For single nucleotide variants (SNVs) false discovery rates (defined as SNVs identified in FFPE but not frozen biospecimens) showed platform-specific differences ranging from <1-15% for NGS depending on coverage (5-80X) (15,19), 18% for SNP arrays (10), and 59% for traditional sequencing (17). Recognizing that platforms differ in scope as well as sensitivity, we examined discordance across reports for a single gene, the proto-oncogene KRAS. Variability in concordance between FFPE and frozen biospecimens for KRAS mirrored that reported for whole genome analysis; in FFPE biospecimens, 6% of KRAS variants were misclassified as wild type by high resolution melting analysis (18), and 18-20% were misclassified by traditional sequencing (18,20). Recognizing that any occurrence of misclassification could impose a risk of misdiagnosis, we questioned the sources of discordance and sought to identify analytical factors that could improve accuracy.

Discordance in genotyping results between FFPE and frozen biospecimens has been attributed, at least in part, to an increase in the number of mutations detected in FFPE biospecimens (6,21). More specifically, there were higher incidences of transversion mutations (19), transition mutations (21), and small insertions or deletions (21) in FFPE biospecimens compared to frozen controls. Also, while mutations at A:T base pairs were more commonly identified in FFPE biospecimens than mutations at G:C base pairs(21), it was the GC-content of the target sequence which ultimately influenced discordance between FFPE and frozen biospecimens (6,10), as the strongest correlation occurred when the GC-content of the sequenced fragments was 40% (r=0.97) (6). Similarly, single-nucleotide polymorphism (SNP) detection via microarray using FFPE biospecimens was most successful in regions with a GC-content of 35-55% where loss of detection and miscall counts were the lowest (10). Increasing the stringency of coverage during NGS from 20× to 40× coverage reduced discordant loci between FFPE and frozen biospecimens from 1% to 0.2% (15) and attenuated the effects of GC-content on correlation strength (6).

Copy Number determination: accuracy in FFPE tissue

The vast majority of published studies conflict as to whether copy number is similar between FFPE and frozen biospecimens (6,7,19), or whether small (7,19) or large differences (10,22) exist. Notably, the largest disparities between FFPE and frozen biospecimens occurred following whole genome amplification (WGA), which reduced concordance from 59 to 48% (10), or were attributable to platform choice. Copy number agreement between FFPE and frozen biospecimens was >98% using Agilent 4×44 K oligonucleotide arrays but was just 53.8-87.3% when determined by Affymetrix SNP 6.0 arrays using the same biospecimens (22). Tumor heterogeneity may also be a contributing factor in copy number disparity between FFPE and frozen biospecimens, as copy number data, when used in concert with allele ratio, can reportedly be used to extrapolate the extent of stromal contamination (7).

DNA Methylation: accuracy in FFPE biospecimens

The accuracy of promoter methylation data generated with FFPE biospecimens depends upon the context in which it will be used. Unsupervised cluster analysis of FFPE biospecimens using methylation chip arrays successfully separated tumor from normal adjacent biospecimens (11) and tumor biospecimens by CpG island methylator phenotype (CIMP) status, which included biospecimens with KRAS mutations (13); however, samples first clustered by preservation method then by diagnosis in all but one study (12). Methylation levels, on the other hand, were more variable, as correlations between FFPE and frozen biospecimens ranged from strong (r^2=0.97) for the entire biospecimen cohort (12) to only 10% of biospecimens displaying a strong correlation (r   0.6) (11). Commonality among tumor-specific differentially methylated loci (DML) was sub-optimal between case-matched FFPE and frozen biospecimens, as only 61% of DML identified in frozen biospecimens were also identified in FFPE biospecimens (13) and only 7 loci appeared on lists of the top 50 DML for both frozen and FFPE biospecimens (11). Two articles also reported lower efficiency for the required bisulfite conversion step with FFPE biospecimens compared to frozen biospecimens (13,14). While a number of variables differed between reports, the extraction method, DNA restoration method, and array type

were common among several of the conflicting reports; therefore it is unlikely that any of these factors alone are responsible for the reported effects. Conversely, the DNA region analyzed (14), batch effect (13), and FFPE block storage (13) have been demonstrated to be confounding variables to methlyation analysis within a study.

DNA analysis of FFPE biospecimens: Lessons from the literature

The accuracy of genotyping and copy number determination with FFPE biospecimens was variable across analytical platforms and publications. However, a high degree of concordance with frozen biospecimens has been achieved for genotyping when FFPE biospecimens were analyzed by microarray, NGS or high resolution melting analysis, and when targeted regions had a GC content of 30-55% (6,10), and when a NGS stringency of 40× coverage or greater was applied (6,15). For copy number analysis, discordance was minimized when WGA was omitted (10) and oligonucleotide arrays were used rather than SNP arrays (22). For DNA methylation analysis, detection rates were improved when DNA was extracted from FFPE biospecimens using a FFPE-specific kit (14). While favorable results were reported when a DNA restoration step was applied after bisulfite conversion, validation of the restoration step with proper controls has not yet been reported. Validation with a case-matched frozen cohort is required when optimizing analytical methods for use with FFPE biospecimens due to the elevated risk of misclassification, false discovery rate, and variable levels of concordance, all of which could confound the discovery of new mutations and the detection of known mutations in cancer diagnosis.

RNA expression analysis: success rates and challenges

RT-PCR and transcriptome analysis using FFPE biospecimens would facilitate rapid screening for and confirmation of important transcripts involved in disease causality or progression; however, success rates for such analytical platforms vary when used with FFPE biospecimens. Some studies report decreased success in the form of efficacy (1-3,23,24) or percent present calls (3,24-27) for RT-PCR, microarray, and cDNA-mediated Annealing, Selection, Extension, and Ligation (DASL) analysis of FFPE biospecimens in comparison to frozen counterparts. Other studies report equivalent performance among differentially preserved biospecimens (1,28,29). Of the studies that reported decreased success in FFPE biospecimens, differences were attributed to RNA degradation, leading to alterations in transcript abundance and length (2,3,23,24). Importantly, such alterations are not global, as transcript-specific effects have been noted (30). For example, the agreement between real-time qRT-PCR and microarrays using FFPE biospecimens was reported to be strong only when similar locations within the transcript are targeted (27). Analytical parameters such as amplicon size and priming method can be manipulated to improve assay success. While RT-PCR success with FFPE biospecimens is influenced by amplicon size (2,23,24,28), reliable qRT-PCR data has been obtained with both FFPE and frozen biospecimens for amplicons between 54 and 105 bp in length (2). One study reported that low percent present rates for FFPE biospecimens increased when samples were primed with both oligo dT and random hexamer primers and analyzed by Exon arrays (25), while another study reported an increase following transcript repair (24).

RT-PCR: accuracy in FFPE biospecimens

The accuracy of the RNA expression data generated with FFPE biospecimens, extrapolated by the degree of concordance with fresh or frozen counterparts, is dependent on a number of analytical factors including the transcript of interest, RNA amplification, and the number of genes used for normalization. Despite differences in raw cycle threshold (Ct) values of 3-10 cycles between FFPE and frozen biospecimens, a strong correlation (r=0.93) has been achieved when levels are normalized to one or more transcript(s) (2). Notably, transcript- and tumor-specific effects necessitate the evaluation of individual transcripts and tumor types. To illustrate, in FFPE carcinoma biospecimens, normalized levels of p21 were 104% higher than those of matched frozen biospecimens while those of VEGF were 498% higher than those of frozen controls (30), but this difference was smaller in non-tumor biospecimens (p21 was 56% and VEGF was 86% of frozen) (30). A pre-amplification step can also adversely affect concordance and assay efficacy, as amplification of RNA from FFPE biospecimens resulted in modestly weaker correlations to unamplified frozen biospecimens (0.88 versus 0.954) (31). However, when RNA amplification was preceded by a complementary-template reverse transcription repair step, in FFPE biospecimens, it resulted in longer transcripts than those obtained with untreated FFPE controls (maximum length: 750 bp versus <200 bp), although a number of tissue-specific transcripts were lost (24). Differences in relative expression between FFPE and frozen tissue were also influenced by the number of transcripts used for normalization of real time qRT-PCR (2), and while the strongest correlations were reported with 2 normalization transcripts, transcript choice and the number used are not universal, and verification by comparison with frozen biospecimens is still required (2).

Microarray and DASL

The accuracy of RNA expression data generated with FFPE biospecimens by microarray or DASL, extrapolated by the strength of correlation with a fresh or frozen biospecimen cohort, varied widely between reports and RNA subtypes. Correlations in mRNA microarray expression levels between FFPE and frozen biospecimens ranged from very weak (r=0.02-0.10) (24) to modest (r=0.45-0.62) (26,32) to strong (r=0.743-0.837) (24) to very strong (r=0.80-0.96) (28). When strong or very strong correlations were reported between mRNA levels from FFPE and frozen biospecimens, the level of variability observed among FFPE biospecimens was still greater than that observed among frozen controls (3,23,24). Conversely, correlations for miRNA microarray levels between FFPE and frozen biospecimens were stronger and less varied in the literature, ranging from modest (0.53-0.92) (33) to strong (r=0.71-0.94) (34) or very strong (r>0.94) (1). Differences in the stability of RNA subtypes in FFPE biospecimens were also reflected in cluster analysis where miRNA expression levels, detected by microarray, clustered by patient diagnosis rather than preservation method (33), while mRNA levels analyzed by DASL clustered first by preservation method then by diagnosis (29). An interaction between diagnosis and formalin fixation was also identified by a 3-way analysis of variance (ANOVA) for 772 genes (29). This interaction is particularly concerning as it resulted in the identification of differentially expressed transcripts between tumor and normal adjacent biospecimens for each preservation method, such that only 33% of the genes differentially expressed between tumor and normal tissue in FFPE biospecimens were also differentially expressed in frozen

biospecimens, and only 48% of those differentially expressed in frozen biospecimens were also differentially expressed in FFPE biospecimens (29). Affected transcripts included a number of breast cancer related genes such as APC, CDKN2A, IGF1R, TGFA, TSG101 and ESR1 (29). This interaction between diagnosis and preservation method was also reported with microarray, as only 57% of genes differentially expressed between neoplastic and normal adjacent FFPE biospecimens were also identified in frozen biospecimens (3). Further, transcript levels differed between neoplastic and normal adjacent tissue in a preservation method-specific manner; as such, manipulating the threshold of differential expression from > 2-fold to > 5-fold subsequently increased the concordance between the two preservation methods to 90% (3).

The discordance in RNA expression between FFPE and frozen biospecimens outlined above has been attributed, at least in part, to the location of the probe or primer set within the transcript, the GC-content of the targeted region, the priming method employed during reverse transcription, and assay choice. In FFPE biospecimens, probes that targeted the 5' end of the transcript produced a less intense signal than those located in the 3' untranslated region (UTR) (3). GC-content of the targeted region may also confound analysis, as the strongest correlations between FFPE and frozen biospecimens were generated with GC-rich probes (40-60%) (35). Similar to real-time qPCR, transcript repair increased both the percent present calls in FFPE biospecimens and the correlation coefficient with matched frozen specimens (24). Finally, use of Human exon 1.0 arrays rather than Affymetrix U133 Plus 2.0 arrays increased the gene level sensitivity from 75-80% to 93% and the specificity from 92% to 94-96% (25).

RNA analysis of FFPE biospecimens: Lessons from the literature

The literature shows that the use of FFPE tissue to conduct mRNA transcriptome analysis may result in artifactual findings unless validation studies are performed using fresh or frozen biospecimens. While reported correlations in miRNA expression were, at times, very strong between FFPE and frozen biospecimens (1,33), in other instances correlations were weaker than expected for replicates (33). Disparities in RNA expression between preservation methods have been attributed to location within transcript (3), target size (2,23,24,28), normalization methods (2), transcript-repair (24), analysis criteria (3), and platform choice (25). Further, in real-time qRT-PCR analysis, transcripts were not affected equally (30) and thus normalizers must be carefully validated (2). When performing RNA expression analysis by microarray, exon arrays performed better than the U133 arrays, especially in combination with NuGAN FFPE labeling (25). For microarray analysis, increasing the threshold of effect for differential expression between tumor and non-tumor biospecimens has increased concordance between FFPE and frozen biospecimens (3). In terms of concordance between analytical platforms, it is worthwhile to note that agreement between real-time qRT-PCR and microarrays is high only when similar locations within the transcript are targeted (27).

Discussion

The accuracy of genomic and gene expression data generated with FFPE biospecimens varied among analytical platforms, with the highest degree of concordance with frozen

biospecimens reported with oligonucletide microarrays, NGS or high resolution melting analysis for genotyping or copy number, and real-time PCR, microarray or DASL for gene expression analysis. However, discrepancies in concordance between FFPE and frozen biospecimens existed between reports, even for a common platform. Notably, relying solely on concordance is itself a hindrance, as correlations fail to capture such clinically relevant differences as false discovery rates, copy number differences, and transcript- or promoter-specific effects, which could translate to misclassification, misdiagnosis, or the false discovery of cancer-specific biomarkers. Such differences, coupled with the ability by some to obtain data from FFPE biospecimens that is strongly correlated with that of frozen biospecimens, indicate that factors beyond platform choice can influence data accuracy and ultimately affect the identification and study of cancer-specific nucleic acid markers. Based upon the evidence from the literature, GC content (6,10,35) and position of the probe within the transcript (3), the type of array (3,25), amplicon size (2,24,28), the selection and number of transcripts used for normalization (2), and the use and type of pre-amplification (10) and transcript repair (24) can be optimized for use with FFPE biospecimens using frozen biospecimens as a control, thereby increasing the accuracy of the data generated. Such validation has proven to be critical due to the specific nature of effects reported for different genes, transcripts, diagnoses, and tissue types. Further, certain FFPE regimes, such as formalin fixation for 72 h or more, have also been shown to alter nucleic acid endpoints (30), suggesting that validation may also be necessary for biospecimens processed under different fixation protocols. Whether optimization of biospecimen fixation and processing protocols for DNA and RNA analyses would translate to increased reliability of the data has yet to be determined. However, it is important to remember that, even after optimization, some disparities between frozen and FFPE biospecimens remain. While often these disparities have little impact on the study outcome, the identification of an interaction between fixation method and diagnosis has the potential to lead to false avenues for cancer research.

While analytical optimization and validation may allow researchers to extract valuable information from FFPE biospecimens, such procedures circumvent the underlying issue: the introduction of artifacts and variability by sub-optimal and non-standardized fixation and processing regimes. With regard to the collection of new biospecimens, many have reported successful and accurate molecular data with formalin-free fixatives, such as BHP (36), HOPE® (37), PAXgene Tissue™ (38), Streck® (39), and RCL-2® and Boonfix (40). Although formalin alternatives are promising, a single alternative fixative has yet to be universally adopted by researchers and biobanks and the impact of pre-analytical variability on biospecimens preserved by these new techniques must be addressed. Given that FFPE is so widely used today, it is important to note that realistic steps can be taken in order to improve FFPE protocols and thus improve the utility of FFPE for research and molecular diagnosis. Targeted improvements to FFPE protocols must be based on scientific evidence on specific pre-analytical factors that affect downstream analysis as well as acceptable thresholds. Such an evidence-based approach can permit both the reevaluation of banked FFPE biospecimens as well as the development of standardized protocols for prospective biospecimen collection. NCI is piloting such an approach through the concept of Biospecimen Evidence-Based Practices, procedural guidelines developed using literature

evidence. The first set of guidelines, related to snap-freezing of resected tissues, was published earlier this year (http://biospecimens.cancer.gov/resources/bebp.asp) (41), and additional documents for FFPE are in preparation. There is also a pressing need for biomarkers of biospecimen quality to enable fit-for-purpose use of archived FFPE blocks, which may be associated with a wealth of clinical information. Current measures of RNA and DNA integrity, such as RNA Integrity Number (RIN), can prove to be unsuitable for FFPE biospecimens. Development of platform-specific biomarkers of stability could qualify archived biospecimens for molecular analysis and improve both the reliability and confidence in the resultant data.

In conclusion, the accuracy of genotyping and mRNA expression data produced using FFPE biospecimens varies widely between reports and is affected by analytical parameters. The high levels of concordance produced by some studies, combined with advantages such as the ability to use biospecimens previously used for IHC or morphology, make these biospecimens particularly valuable for cancer research. However, discordance in nucleic acid analysis even when small can lead to the identification of biomarkers of biospecimen handling rather than those of disease state and could confound patient diagnosis. Based upon evidence published to date, validation of analytical parameters with a properly handled frozen cohort is necessary to ensure a high degree of concordance and confidence in the results obtained with FFPE biospecimens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Jang JS, Simon VA, Feddersen RM, Rakhshan F, Schultz DA, Zschunke MA, et al. Quantitative miRNA expression analysis using fluidigm microfluidics dynamic arrays. BMC Genomics. 2011; 12:144. [PubMed: 21388556]

2. Green TM, de Stricker K, Moller MB. Validation of putative reference genes for normalization of Q-RT-PCR data from paraffin-embedded lymphoid tissue. Diagn Mol Pathol. 2009; 18(4):243–9. [PubMed: 19861891]

3. Abdueva D, Wing M, Schaub B, Triche T, Davicioni E. Quantitative expression profiling in formalin-fixed paraffin-embedded samples by affymetrix microarrays. J Mol Diagn. 2010; 12(4): 409–17. [PubMed: 20522636]

4. Bass BP, Engel KB, Greytak SR, Moore HM. A review of pre-analytical factors affecting molecular, protein, and morphological analysis of formalin-fixed, paraffin-embedded tissue. Arch Pathol Lab Med. 2014; 138(11):1520–30. [PubMed: 25357115]

5. Engel KB, Moore HM. Effects of preanalytical variables on the detection of proteins by immunohistochemistry in formalin-fixed, paraffin-embedded tissue. Arch Pathol Lab Med. 2011; 135(5):537–43. [PubMed: 21526952]

6. Schweiger MR, Kerick M, Timmermann B, Albrecht MW, Borodina T, Parkhomchuk D, et al. Genome-wide massively parallel sequencing of formaldehyde fixed-paraffin embedded (FFPE) tumor tissues for copy-number- and mutation-analysis. PLoS One. 2009; 4(5):e5548. [PubMed: 19440246]

7. Wang Y, Carlton VE, Karlin-Neumann G, Sapolsky R, Zhang L, Moorhead M, et al. High quality copy number and genotype data from FFPE samples using Molecular Inversion Probe (MIP) microarrays. BMC Med Genomics. 2009; 2:8. [PubMed: 19228381]

8. Jacobs S, Thompson ER, Nannya Y, Yamamoto G, Pillai R, Ogawa S, et al. Genome-wide, high-resolution detection of copy number, loss of heterozygosity, and genotypes from formalin-fixed, paraffin-embedded tumor tissue using microarrays. Cancer Res. 2007; 67(6):2544–51. [PubMed: 17363572]

9. Ferguson AE, Cohn RJ, Ashton LJ. Use of formalin-fixed paraffin-embedded tumor tissue as a DNA source in molecular epidemiological studies of pediatric CNS tumors. Diagn Mol Pathol. 2012; 21(2):105–13. [PubMed: 22555093]

10. Liang CW, Lee YS, Marino-Enriquez A, Tsui K, Huang SH. The utility and limitation of single nucleotide polymorphism analysis on whole genome amplified mesenchymal tumour DNA in formalin fixed tumour samples. Pathology. 2012; 44(1):33–41. [PubMed: 22157688]

11. Jasmine F, Rahaman R, Roy S, Raza M, Paul R, Rakibuz-Zaman M, et al. Interpretation of genome-wide infinium methylation data from ligated DNA in formalin-fixed, paraffin-embedded paired tumor and normal tissue. BMC Res Notes. 2012; 5:117. [PubMed: 22357164]

12. Moran S, Vizoso M, Martinez-Cardús A, Gomez A, Matías-Guiu X, Chiavenna SM, et al. Validation of DNA methylation profiling in formalin-fixed paraffin-embedded samples using the Infinium HumanMethylation450 Microarray. Epigenetics. 2014; 9(6)

13. Dumenil TD, Wockner LF, Bettington M, McKeone DM, Klein K, Bowdler LM, et al. Genome-wide DNA methylation analysis of formalin-fixed paraffin embedded colorectal cancer tissue. Genes Chromosomes Cancer. 2014; 53(7):537–48. [PubMed: 24677610]

14. Tournier B, Chapusot C, Courcet E, Martin L, Lepage C, Faivre J, et al. Why do results conflict regarding the prognostic value of the methylation status in colon cancers? The role of the preservation method. BMC Cancer. 2012; 12:12. [PubMed: 22243995]

15. Kerick M, Isau M, Timmermann B, Sültmann H, Herwig R, Krobitsch S, et al. Targeted high throughput sequencing in clinical cancer settings: formaldehyde fixed-paraffin embedded (FFPE) tumor tissues, input amount and tumor heterogeneity. BMC Med Genomics. 2011; 4:68. [PubMed: 21958464]

16. Lips EH, Dierssen JW, van Eijk R, Oosting J, Eilers PH, Tollenaar RA, et al. Reliable high-throughput genotyping and loss-of-heterozygosity detection in formalin-fixed, paraffin-embedded tumors using single nucleotide polymorphism arrays. Cancer Res. 2005; 65(22):10188–91. [PubMed: 16288005]

17. Verhoest G, Patard JJ, Fergelot P, Jouan F, Zerrouki S, Dreano S, et al. Paraffin-embedded tissue is less accurate than frozen section analysis for determining VHL mutational status in sporadic renal cell carcinoma. Urol Oncol. 2012; 30(4):469–75. [PubMed: 20863722]

18. Solassol J, Ramos J, Crapez E, Saifi M, Mangé A, Vianès E, et al. KRAS Mutation Detection in Paired Frozen and Formalin-Fixed Paraffin-Embedded (FFPE) Colorectal Cancer Tissues. Int J Mol Sci. 2011; 12(5):3191–204. [PubMed: 21686179]

19. Menon R, Deng M, Boehm D, Braun M, Fend F, Biskup S, et al. Exome Enrichment and SOLiD Sequencing of Formalin Fixed Paraffin Embedded (FFPE) Prostate Cancer Tissue. Int J Mol Sci. 2012; 13(7):8933–42. [PubMed: 22942743]

20. Laosinchai-Wolf W, Ye F, Tran V, Yang Z, White R, Bloom K, et al. Sensitive multiplex detection of KRAS codons 12 and 13 mutations in paraffin-embedded tissue specimens. J Clin Pathol. 2011; 64(1):30–6. [PubMed: 21030527]

21. Quach N, Goodman MF, Shibata D. In vitro mutation artifacts after formalin fixation and error prone translesion synthesis during PCR. BMC Clin Pathol. 2004; 4(1):1. [PubMed: 15028125]

22. Nasri S, Anjomshoaa A, Song S, Guilford P, McNoe L, Black M, et al. Oligonucleotide array outperforms SNP array on formalin-fixed paraffin-embedded clinical samples. Cancer Genet Cytogenet. 2010; 198(1):1–6. [PubMed: 20303007]

23. Fedorowicz G, Guerrero S, Wu TD, Modrusan Z. Microarray analysis of RNA extracted from formalin-fixed, paraffin-embedded and matched fresh-frozen ovarian adenocarcinomas. BMC Med Genomics. 2009; 2:23. [PubMed: 19426511]

24. Loudig O, Milova E, Brandwein-Gensler M, Massimi A, Belbin TJ, Childs G, et al. Molecular restoration of archived transcriptional profiles by complementary-template reverse-transcription (CT-RT). Nucleic Acids Res. 2007; 35(15):e94. [PubMed: 17636051]

25. Linton K, Hey Y, Dibben S, Miller C, Freemont A, Radford J, et al. Methods comparison for high-resolution transcriptional analysis of archival material on Affymetrix Plus 2.0 and Exon 1.0 microarrays. Biotechniques. 2009; 47(1):587–96. [PubMed: 19594443]

26. Sadi AM, Wang DY, Youngson BJ, Miller N, Boerner S, Done SJ, et al. Clinical relevance of DNA microarray analyses using archival formalin-fixed paraffin-embedded breast cancer specimens. BMC Cancer. 2011; 11:253, 1–13. [PubMed: 21679412]

27. Linton KM, Hey Y, Saunders E, Jeziorska M, Denton J, Wilson CL, et al. Acquisition of biologically relevant gene expression data by Affymetrix microarray analysis of archival formalin-fixed paraffin-embedded tumours. Br J Cancer. 2008; 98(8):1403–14. [PubMed: 18382428]

28. Coudry RA, Meireles SI, Stoyanova R, Cooper HS, Carpino A, Wang X, et al. Successful application of microarray technology to microdissected formalin-fixed, paraffin-embedded tissue. J Mol Diagn. 2007; 9(1):70–9. [PubMed: 17251338]

29. Kibriya MG, Jasmine F, Roy S, Paul-Brutus RM, Argos M, Ahsan H. Analyses and interpretation of whole-genome gene expression from formalin-fixed paraffin-embedded tissue: an illustration with breast cancer tissues. BMC Genomics. 2010; 11:622. [PubMed: 21059268]

30. Macabeo-Ong M, Ginzinger DG, Dekker N, McMillan A, Regezi JA, Wong DT, et al. Effect of duration of fixation on quantitative reverse transcription polymerase chain reaction analyses. Mod Pathol. 2002; 15(9):979–87. [PubMed: 12218216]

31. Gloghini A, Canal B, Dal Maso L, Carbone A. Multiple gene expression analyses in human lymphoid tissues by taqman low-density array using amplified rna isolated from paraffin-embedded samples. Diagn Mol Pathol. 2009; 18(3):156–64. [PubMed: 19704261]

32. April C, Klotzle B, Royce T, Wickham-Garcia E, Boyaniwsky T, Izzo J, et al. Whole-genome gene expression profiling of formalin-fixed, paraffin-embedded tissue samples. PLoS One. 2009; 4(12):e8162. [PubMed: 19997620]

33. Løvendorf MB, Zibert JR, Hagedorn PH, Glue C, Ødum N, Røpke MA, et al. Comparison of microRNA expression using different preservation methods of matched psoriatic skin samples. Exp Dermatol. 2012; 21(4):299–301. [PubMed: 22417307]

34. Hasemeier B, Christgen M, Kreipe H, Lehmann U. Reliable microRNA profiling in routinely processed formalin-fixed paraffin-embedded breast cancer specimens using fluorescence labelled bead technology. BMC Biotechnol. 2008; 8:90. [PubMed: 19038028]

35. Mittempergher L, de Ronde JJ, Nieuwland M, Kerkhoven RM, Simon I, Rutgers EJ, et al. Gene expression profiles from formalin fixed paraffin embedded breast cancer tissue are largely comparable to fresh frozen matched tissue. PLoS One. 2011; 6(2):e17163. [PubMed: 21347257]

36. Mueller C, Edmiston KH, Carpenter C, Gaffney E, Ryan C, Ward R, et al. One-step preservation of phosphoproteins and tissue morphology at room temperature for diagnostic and research specimens. PLoS One. 2011; 6(8):e23780. [PubMed: 21858221]

37. Olert J, Wiedorn KH, Goldmann T, Kühl H, Mehraein Y, Scherthan H, et al. HOPE fixation: a novel fixing method and paraffin-embedding technique for human soft tissues. Pathol Res Pract. 2001; 197(12):823–6. [PubMed: 11795830]

38. Staff S, Kujala P, Karhu R, Rökman A, Ilvesaro J, Kares S, et al. Preservation of nucleic acids and tissue morphology in paraffin-embedded clinical samples: comparison of five molecular fixatives. J Clin Pathol. 2013; 66(9):807–10. [PubMed: 23750036]

39. Burns JA, Li Y, Cheney CA, Ou Y, Franlin-Pfeifer LL, Kuklin N, et al. Choice of fixative is crucial to successful immunohistochemical detection of phosphoproteins in paraffin-embedded tumor tissues. J Histochem Cytochem. 2009; 57(3):257–64. [PubMed: 19001637]

40. van Essen HF, Verdaasdonk MA, Elshof SM, de Weger RA, van Diest PJ. Alcohol based tissue fixation as an alternative for formaldehyde: influence on immunohistochemistry. J Clin Pathol. 2010; 63(12):1090–4. [PubMed: 20978021]

41. Engel KB, Vaught J, Moore HM. National Cancer Institute Biospecimen Evidence-Based Practices: a novel approach to pre-analytical standardization. Biopreserv Biobank. 2014; 12(2): 148–50. [PubMed: 24749882]

Table 1

Analytical parameters that influence concordance of human FFPE and frozen tissue biospecimens

Parameter	Optimal technique for FFPE biospecimens	Degree of concordance	Reference
DNA			
Platform/method	Agilent 4×44 K oligonucleotide arrays	98% agreement achieved with Agilent 4×44 K oligonucleotide array versus 53.8-87.3% by Affymetrix SNP 6.0 array	(22)
GC–content	40%	Strongest correlations (r=0.97) were observed when probes had a GC content of 40%	(6)
Stringency	NGS, 40× coverage	99.8% agreement was achievable when NGS had 40× coverage (vs. 99% at 20×).	(15)
RNA			
Platform	Human exon 1.0 array	Human exon 1.0 arrays (rather than Affymetrix U133 Plus 2.0 arrays) increased sensitivity from 75-80% to 93% and specificity from 92% to 94-96%	(25)
WTA	Unamplified	Amplification reduced the correlation coefficient from r=0.954 to r=0.88.	(31)
Amplicon size	100 bp	Amplification success was similar to frozen when the targeted region was 100 bp, unless prefaced by transcript repair.	(24,28)
Probe location	Close proximity to the 3′ end	Distance from the 3′ end had an exponential effect on probe intensity with FFPE biospecimens, compared to a linear effect with frozen biospecimens.	(3)
GC content	40-60% GC	Probes with a GC content of 40-60% were very strongly correlated between FFPE and frozen biospecimens (r>0.93), while probes with higher or lower GC content displayed weaker correlations (r<0.1).	(35)
Stringency	2 normalizer genes	Relative gene expression by normalization with 2 transcripts resulted in a stronger correlation (r=0.93) than when more than 2 transcripts were used (r=0.89).	(2)
	5 fold change	A differential expression threshold of 5-fold resulted in 90% agreement, compared to 55% at 2-fold.	(3)

WTA, whole transcriptome amplification