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BRIEF REPORT

National Cancer Institute Biospecimen Evidence-Based Practices: Harmonizing Procedures for Nucleic Acid Extraction from Formalin-Fixed, Paraffin-Embedded Tissue

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Introduction

Variable and suboptimal biospecimen handling practices have been identified as impediments to biomarker discovery,[1,2] including predictive biomarkers for oncology[3] indicating a clear and present need for evidence-based, standardized practices. The United States and international efforts have been launched to better understand and mitigate variability during the preanalytical phase and decrease associated effects by promoting harmonization of procedures both within and across institutions. The Biorepositories and Biospecimen Research Branch (BBRB) of the United States National Cancer Institute (NCI) publishes best practice documents aimed at improving the quality of data generated from human biospecimens (https://biospecimens.cancer.gov/bestpractices/overview.asp) and sponsors research initiatives (https://biospecimens.cancer.gov/programs/default.asp) and the Biospecimen Research Database (BRD; http://biospecimens.cancer.gov/brd) to better understand thresholds and effects of individual preanalytical factors in biospecimen handling. The BRD allows users to query both a curated literature repository and standard operating procedure (SOP) library for a specific preservative, diagnosis, analyte, or preanalytical factor. The BRD incorporates information from international efforts, including those by the International Society for Biological and Environmental Repositories (ISBER) and the European Union-sponsored SPIDIA program (standardization and improvement of generic preanalytical tools and procedures for *in vitro* diagnostics; www.spidia.eu), among others.

To facilitate the implementation of evidence-based practices in biospecimen handling, BBRB has developed a document series termed Biospecimen Evidence-Based Practices (BEBP), which contains step-by-step procedural guidelines derived from peer-reviewed primary research articles and expert experience.[4] The aim of the BEBP series is to promote a practical level of standardization and improve overall biospecimen quality and data reproducibility by specifying both optimal methods and suitable alternatives, while merging published evidence with practical knowledge of experts in the field. The intent of the BEBP is not to serve as a SOP, but to facilitate the development of evidence-based SOPs by individual laboratories.

The present BEBP focuses on nucleic acid extraction from formalin-fixed, paraffin-embedded (FFPE) tissue biospecimens (see Supplementary Data; Supplementary Data are available online at www.liebertpub.com/bio). Such biospecimens are being increasingly utilized in genomic research, and it has become clear over the past decade that variable and suboptimal FFPE biospecimen collection and processing practices can alter the quantity and/or quality of extracted DNA and RNA.[5] Concordance between the molecular data generated with FFPE and snap-frozen biospecimens varies widely among reports, with correlations ranging from weak to very strong for the same analytical method.[6] While lack of concordance may be partially attributable to differences in biospecimen handling during FFPE processing, available evidence suggests that the extraction method can compound or mitigate effects introduced during biospecimen collection, processing, and storage, thus affecting the suitability of samples for downstream analysis.[7–11] When deciding on a nucleic acid extraction procedure, it is paramount to consider artifacts that may have been introduced during formalin fixation and processing, such as nucleic acid fragmentation,[12] nucleic acid–protein crosslinking,[13,14] denaturation,[15,16] and

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additions of methylol groups to nucleic acids.[15,17] Importantly, many such formalin-induced modifications to DNA and RNA are reversible[13,18–21]; for example, the addition of a demodification step as well as optimization of multiple steps during the extraction procedure can attenuate effects introduced during fixation and processing. However, FFPE-specific optimization steps are interdependent and must be considered collectively when developing a strategy for extraction. While several commercial extraction kits are marketed as being tailored for FFPE biospecimens, experts contributing to the BEBP advised validating any kit for the intended tissue type, processing regime, and analytical platform before implementation in experimental studies. Validation includes experiments to confirm the feasibility and accuracy of the intended analytical use, the reproducible performance of the kit, and the robustness to processing regimes.

Materials and Methods

The BEBP for nucleic acid extraction from FFPE biospecimens was built upon a framework of key procedural steps (or preanalytical factors) during DNA and RNA extraction from FFPE tissue biospecimens that was generated through an initial survey of the literature and available SOPs. These preanalytical factors were used to guide targeted searches of the National Library of Medicine (NLM)'s PubMed database (pubmed.gov) and NCI's BRD. Published evidence was compiled regarding how each step or extraction-specific preanalytical factor affects nucleic acid yield, quality, integrity, or downstream analysis. The list of potential preanalytical factors was expanded through crossreferencing and pertinent articles were curated for inclusion in the BRD. Once a draft document was available for review, experts were invited to participate on a review panel based on their expertise and publication history in the field. Feedback based on the knowledge, experiences, and opinions of participating experts was collected primarily through electronic correspondence both upon review of the original document and in response to detailed questions. Additional input was captured during a teleconference after experts had reviewed a complete draft.

Results

The final expert-vetted BEBP document contains step-by-step procedural guidelines for the extraction of nucleic acids from FFPE tissue (Supplementary Data). Recommendations outlined within the procedural guideline section are a culmination of the summarized literature, protocols, and details from commercial extraction kits, published guidelines by the Centers for Disease Control and Prevention and the Clinical & Laboratory Standards Institute Laboratory, and guidance from the expert panel. As more than one method was often

Table 1. DNA Quality Guidelines by Analytical Platform

Application	*Criteria employed*	*Reference*
NGS	Monitor yield after library preparation and mean insert size as predictors for characterization success. Age of sample is not a predictor of success, as fixation technique plays a greater role.	BROAD Institute (http://genomics.broadinstitute.org/data-sheets/DTS_FFPE_4-2017.pdf)
	A minimum of 100 ng DNA is used for library construction. DNA integrity is not assessed by gel electrophoresis. Library metrics are used to determine pass/fail status before sequencing. Successful libraries should have the majority of library fragments between 300 and 600 bp in size with a minimum yield of 15 μL at 3 nM.	Dr. Andrew Mungall BC Cancer (www.bcgsc.ca/services/sequencing-libraries-faq)
	Sample intake QC-minimum DNA integrity (>200 bp) and absence of protein contamination evaluated by E-Gel.	Dr. Harsha Doddapaneni and Dr. David Wheeler (Baylor College of Medicine Human Genome Sequencing Center)
	Library construction yields should be >300 ng with fragments between 200 and 800 bp when using 100 ng input (manual preparation) or 250 ng (robotic preparation).	
	Post-library capture should have >10 nM yield and devoid of primer dimers.	
	150 ng double-stranded DNA, amplification of 100 bp product; ΔCt <2 using the FFPE QC Kit	Personal communication Dr. Betsou (IBBL, Luxembourg)
	>6% amplifiable copies; Input adjusted based on PCR amplification of TBP or FTH1	Sah et al.[22]
	ΔCt <1.55 real-time PCR-based Illumina FFPE QC Kit	Serizawa et al.[23]
PCR	Comparative assessment of differentially sized GAPDH PCR amplicons: 100, 236, 299, 411, bp visualized by HPLC	Wang et al.[24]
aCGH	Amplification of a 200 bp fragment of GAPDH from 100 ng DNA	van Beers et al.[25]
	Amplification of 200 bp product; >2 μg DNA	Personal Communication Dr. Betsou (IBBL, Luxembourg)

aCGH, array comparative genomic hybridization; FFPE, formalin-fixed paraffin-embedded; FTH1, ferritin heavy polypeptide 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HPLC, high-performance liquid chromatography; NGS, next-generation sequencing; PCR, polymerase chain reaction; TBP, tata-binding protein.

Table 2. RNA Quality Guidelines by Analytical Platform

Application	*Criteria employed*	*Reference*
RT-PCR	Amplification of a 60 bp product; $DV_{200} > 30\%$	Personal Communication Dr. Betsou (IBBL, Luxembourg)
NGS	No RNA Integrity metric (RIN, DV200, etc.) useful in predicting if a library is successful.	Dr. Hoadley (UNC)
	Predictors of success can come from libraries that yield >4 ng/μL concentration or MiSeq test runs, where goal is to see >10% of reads mapping to messenger RNA.	
	RNA capture required between 100 and 200 ng of total RNA derived from FFPE while Illumina Total RNA-Seq requires between 400 and 1000 ng.	
	Following library construction and Agilent/Caliper QC, the majority of fragments should be between 200 and 500 bp in length. Final library concentration should be >1 nM in at least 10 μL.	Dr. Andrew Mungall (BC Cancer)
	Ribosomal RNA depletion for RNA-Seq requires a minimum of 400 ng of total RNA input when quantified by Agilent Bioanalyzer/Caliper GX. Alternatively, 400 ng of total nucleic acid quantified by Qubit or Quant-iT can be used.	
	Sample Intake QC-DV200 should be >30%. RIN is not informative.	Dr. Harsha Doddapaneni and Dr. David Wheeler (Baylor College of Medicine Human Genome Sequencing Center)
	Library construction yields should be >3 ng with complementary DNA fragments between 100 and 1,500 bp when using between 50 and 100 ng RNA input. Greater input is required for samples with lower DV200 values.	
	Post-library capture should have >10 nM yield and devoid of primer dimers.	
Microarray	>600 ng total RNA (by spectrophotometer); OD 260/280 ratio >1.5; 3′/5′ ratio <100 (as determined by TaqMan-based real time qRT-PCR of beta-actin using primers located 300 bp apart); Cy-dye incorporation >4.5 pmol/ng	Penland et al.[26]
	Ratio of real-time PCR amplicons of the 3′ to the 5′ end of beta-actin <20; Cycle threshold of the amplicon of the 5′ end of ACTB within seven cycles of the same quantity of universal control RNA	Roberts et al.[27]
	Mean log ratio slope <0.15 due to the probe's distance from the 3′ end or its C-content in microarray hybridization	Duenwald et al.[28]
DASL	>100 ng RNA; A260/280 ratio >1.5; Rpll13a Ct values of <29	Abramovitz et al.[29]

ACTB, beta-actin; DASL, cDNA-mediated Annealing, Selection, Extension and Ligation; RT-PCR, reverse transcription polymerase chain reaction.

supported in the literature and/or by the expert panel, optimal procedures as well as acceptable alternatives are specified within the procedural guidelines. In cases where a method or reagent has detrimental effects, the procedural guidelines state that it is inadvisable for use with FFPE biospecimens. Literature summaries within the BEBP are organized by preanalytical factor and reflect the data presented in the peer-reviewed primary research articles identified. Literature summaries are internally cited with pertinent published literature. In addition to standard bibliographic information, literature references include hyperlinks to both the original article through PubMed and the BRD curation entry. Expert vetting of the BEBP document resulted in: (1) guidance on specimen suitability, (2) recommendations for procedural steps based on the panel's collective experience, (3) recommendations for platform-specific quality metrics (Tables 1 and 2), and (4) identification of extraction steps for which there was no consensus from the panel.

SOPs generated based on the guidance provided in the expert-vetted BEBP will require experimental validation for the tissue type examined, extraction method employed, and the downstream analysis that is anticipated. Furthermore, it is crucial that such optimization and validation are performed on similarly handled and stored FFPE biospecimens of the same tissue type, as preanalytical factors associated with the FFPE processing regime and FFPE block storage may also affect downstream molecular analysis.

Discussion

The BEBP series represents a more granular approach than the *NCI Best Practices for Biospecimen Resources* (https://biospecimens.cancer.gov/bestpractices). The BEBP document presented in this study reflects an extensive but pragmatic vetting process that reflects discussions of topics with limited research evidence as well as assurance that the recommendations are practical for implementation. Application-specific guidance for DNA and RNA quality is provided (Tables 1 and 2), enabling researchers to make an informed decision as to whether nucleic acids extracted using a SOP developed under the BEBP are in fact fit for purpose. Development and implementation of evidence-based SOPs

using the BEBP should prove a valuable tool in improving the quality and reproducibility of molecular data generated with FFPE biospecimens. This document can also be found on BBRB's website (http://biospecimens.cancer.gov).

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Author Disclosure Statement

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