NCI Biospecimen Evidence-Based Practices	DNA AND RNA EXTRACTION FROM FORMALIN-FIXED, PARAFFIN-EMBEDDED TISSUE BIOSPECIMENS				
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1.0 PURPOSE

The purpose of this document is to provide evidence-based and expert-vetted guidance on nucleic acid extraction from formalin-fixed and paraffin-embedded (FFPE) human tissues. This guidance is intended to support the development and execution of evidence-based Standard Operating Procedures (SOPs) for DNA and RNA extraction from FFPE biospecimens.

2.0 SCOPE

This evidence-based, expert-vetted best practice document is applicable for nucleic acid extraction from standard-size FFPE blocks containing human tissue, although such guidance may also be applicable to tissue from mammalian animal models. Specifically, recommended procedures are suitable for standard cassette-sized, non-decalcified tissue biospecimens that were fixed in 10% neutral buffered formalin (NBF) for less than 48 h before paraffin-embedding. Biospecimens processed under these procedural guidelines are suitable for analysis by polymerase chain reaction (PCR), methylation analysis, Sanger sequencing and next-generation sequencing, and microarray hybridization. Much of the evidence presented in this document pertains to mRNA molecules and DNA, thus shorter RNA molecules such as microRNAs (miRNAs) may require additional consideration and optimization. This document is not applicable for analysis of biospecimens by *in situ* hybridization. Guidance relating to optimization of procedures for specific analytical platforms are located within Summaries of Literature Evidence (see 7.0) and Summaries of Expert Recommendations (see 8.3). Analytical platform specific guidelines for determining specimen suitability are listed in Tables 1 and 2. It is strongly advised that SOPs developed under this Biospecimen Evidence-Based Practice (BEBP) undergo experimental validation for each analytical platform anticipated (see 8.3.1). Details regarding the expert review of this document and resultant recommendations can be found within section 8.0 and the names and credentials of experts that participated on the review panel can be found within section 8.2.

3.0 DEFINITIONS

3.1 **Section** – the thickness of a slice of FFPE tissue cut from a paraffin block (usually expressed in µm).

3.2 **Block**– the paraffin block containing the FFPE tissue biospecimen.

3.3 **Section Storage**- the length of time elapsed between sectioning and deparaffinization.

3.4 **Demodification**- the method by which the effects of formalin on DNA and RNA structure are reversed.

3.5 **Digestion**- the process of breaking down proteins and tissue structures through the use of enzymes.

4.0 ENVIRONMENTAL HEALTH & SAFETY

Guidelines on precautions to prevent the transmission of infectious agents should be consulted and can be used for all phases of organ/tissue dissection and handling (Reference 9.1.1).

The National Institute for Occupational Safety and Health (NIOSH) Pocket Guide to Chemical Hazards should be consulted for all steps including formalin, xylene and other chemicals (Reference 9.1.2).

5.0 RECOMMENDED MATERIALS/EQUIPMENT

5.1 RNase removal spray or wipes.

5.2 Microtome.

5.3 Ice block.

5.4 Pre-labeled RNase-free microcentrifuge tubes.

5.5 Plastic-backed absorbent bench paper.

5.6 Xylene.

5.7 Ethanol.

5.8 Microcentrifuge.

5.9 An experimentally-validated extraction kit of the user's choice. Acceptable methods for DNA extraction include silica, magnetic-bead, heating, focused-ultrasonication, resin-filter or salting-out based methods, but organic extraction should be avoided

(see 7.8). Acceptable RNA extraction methods include silica, magnetic bead, organic focused-ultrasonication or salting-out based methods, but generally resin-filter and heat-based methods should be avoided (see 7.9).

5.10 Real-time PCR machine.

5.11 Spectrophotometer.

5.12 Fluorometric DNA quantification method of choice.

6.0 PROCEDURAL GUIDELINES

6.1 Recording of biospecimen pre-acquisition, acquisition and processing data

6.1.1 Whenever possible, extensive data should be recorded relating to pre-acquisition, acquisition and processing conditions that may affect the integrity of the biospecimen. Such data may consist of patient information (including age, gender, diagnosis, and treatment), details relating to surgery and biospecimen acquisition (including the use of anesthesia, warm ischemia time, and surgical procedure and duration), and FFPE processing (including fixation duration, temperature, embedding medium) and the duration of block storage (Reference 9.1.9).

6.2 Preparation of bench space

6.2.1 Pre-label microcentrifuge tubes.

6.2.2 Clean work area and metal equipment including microtome with a xylene-free deparaffinization reagent (see 8.3.2).

6.2.3 Wipe down all equipment, work area and all tools with RNase removal wipes or spray.

6.3 Sectioning paraffin blocks

6.3.1 Place paraffin blocks face-down on a cold plate for a minimum of 5 min or at room temperature if blocks were stored at -20°C. The 5 min incubation may

be reduced if the block has been stored under recommended conditions (see 7.1). Placement of paraffin blocks on wet ice is not recommended (see 8.3.2).

6.3.2 A new microtome blade should be used for each biospecimen.

6.3.3 Position the FFPE tissue block in the specimen clamp of the microtome.

6.3.4 Cut sections that are 5-10 µm thick (Reference 9.1.4) (see 7.2). Allow individual sections to roll up naturally. After discarding the first 3-4 sections, place the number of sections required to obtain the desired volume of tissue needed for extraction directly into a sterile RNase-free microfuge tube using RNase-free forceps. For reference, 1-8 sections will be required for a block with a tissue surface area of 250 mm^2 (Reference 9.1.10). However, the optimal number of sections for extraction is based on the tissue surface area and cellularity (determined by H&E staining) of a given block and the extraction kit used; thus, the number of sections or tissue area needed for extraction should be experimentally validated for each extraction kit and standardized within a specimen cohort (see 8.3.3).

6.3.5 Paraffin sections should be processed for extraction immediately, optimally within 24 h. While available literature is limited, evidence suggests that when necessary, sections may be stored for up to 90 days at room temperature (see 7.3) or 4, -20 or -80˚C in a sealed microcentrifuge tube (see 8.3.4) for some analyses.

6.4 Deparaffinization of sections

6.4.1 Add 1 ml of xylene to the microcentrifuge tube or slide and incubate at room temperature for 10 min to deparaffinze sections. Acceptable alternative deparaffinization methods are discussed in sections 7.4 and 8.3.5. For DNA extraction, acceptable alternatives include use of proprietary buffers, mineral oil, lysis buffer heated to 98-120°C, focused-ultrasonication, heptane or the xylene substitute HemoClear. For RNA extraction, acceptable alternatives include use of proprietary buffers, heptane, lysis buffer heated to 95-98°C, focused-ultrasonication or the xylene substitute HemoD. Microwave heating

should be avoided (see 7.4). The acceptable duration of deparaffinization will depend upon the method employed (see 7.5).

6.4.2 Centrifuge tubes for 2 min at maximum speed (approximately 16,000-20,000 x g) (References 9.1.4, 9.1.5, 9.1.6, and 9.1.7) and discard the superanatant.

6.4.3 Wash the pellet/slide in 100% ethanol, vortex, and centrifuge at maximum speed at room temperature. Discard the supernatant. A second ethanol wash may be necessary for some deparaffinization techniques and specimens of low quality (see 8.3.5).

6.4.4 Ensure the pellet is completely dry. Ethanol may be removed from the pellet by air-drying at room temperature or with the use of a speed vacuum.

6.5 Proteinase K digestion

6.5.1 Add lysis buffer containing proteinase K at the volume and concentration specified by the extraction kit and incubate at 55-56°C for 2-48 h for DNA or for 15 min-overnight for RNA. The optimal duration of digestion is dependent upon multiple factors, including buffer composition and enzyme concentration, and must be determined experimentally. Other digestion durations and lower temperatures may also be acceptable (see 7.6 and 8.3.6 for alternatives).

6.5.2 Heat tubes to 90°C for 10 min-2 h for DNA extraction or to 80°C for 0-15 min for RNA extraction. Optimal durations of demodification will be dependent upon the extraction kit used (see 7.7). This step may be unnecessary for some analyses, in which case it can be omitted. For alternate temperatures and durations see sections 7.7 and 8.3.7.

6.6 DNA and RNA extraction

6.6.1 Continue to DNA or RNA extraction using the method specified by the experimentally validated nucleic acid extraction kit (see 7.8 and 7.9). Acceptable methods for DNA extraction include silica, magnetic-bead, heating, resin-filter, ultrasonication or salting-out based methods, but organic extraction should be avoided (see 7.8 and 8.3.8). Acceptable RNA extraction

methods include silica, magnetic bead, organic, ultrasonication or salting-out based methods, but generally resin-filter and heat-based methods should be avoided (see 7.9 and 8.3.8).

6.7 Post-extraction treatment

6.7.1 Remove contaminating nucleic acids by RNase treatment of DNA or DNase treatment of RNA is advised but not required (see 7.10 and 8.3.9).

6.8 Nucleic acid quantification and quality assessment

6.8.1 Quantify DNA and RNA using a fluorometric or qPCR-based method. While spectrophotometry should be avoided for DNA quantification as it overestimates yield, it can provide valuable information on DNA purity and quality. Spectrophotometry may be acceptable for RNA quantification, although inconsistent and overestimated yields have been observed with incomplete DNAse treatment (see 7.11 and 8.3.10).

6.8.2 Verify DNA integrity using fit-for-purpose quality metrics. Suggested DNA quality guidelines are organized by analytical platform in Table 1 (see 7.12 and 8.3.11).

6.8.3 Verify RNA quality using the percentage of RNA fragments >200 nt (DV_{200}) and/or a combination of DV_{200} and real-time qRT-PCR amplification of products from the 5' and 3' end of the transcript. RIN is not an informative method of RNA quality assessment for FFPE specimens and should be avoided. Suggested RNA quality guidelines are listed by analytical platform in Table 2 (see 7.13 and 8.3.12).

7.0 SUMMARIES OF LITERATURE EVIDENCE

7.1 **Block Storage**. Nucleic acids are sensitive to paraffin block storage, although the magnitude of storage-attributable effects may also be influenced by fixation and processing parameters [1]. While some studies have reported relatively minor effects on DNA following storage for several years [2-10], others have shown that the length of amplifiable DNA [11, 12] and whole genome amplified fragments [13] decreased progressively with block storage, with effects observed between specimens stored

for <1 year and those stored for 4-5 years [4, 5]. Amplicon size is another confounding variable as the effect of block storage on PCR success was dependent on amplicon length, with an effect observed for 989 bp fragments but not 268 bp fragments [5] and 300 or 150 bp fragments relative to 75 bp fragments [12]. Notably, such effects on PCR yields have been shown to be influenced by tissue type [12]. The majority of studies report a decrease in mRNA amplification success and efficiency [2, 14-22] or RNA integrity [15, 16, 19] after block storage for 2-20 years in comparison to controls stored for less than a year. The prevalence and magnitude of observed storage-induced effects on mRNA amplification were determined by assay-specific variables including amplicon size [16, 17, 21] and primer/probe location within the transcript [18]. Conversely, several other reports investigating mRNA [23-25], microRNA (miRNA), and ribosomal RNA (rRNA) [26] observed no effect on amplification after 5 to 21 y of block storage compared to control blocks less than a year old. Although information is limited, FFPE block storage at 4°C or room temperature resulted in comparable mRNA levels, determined by real-time quantitative RT-PCR [22].

7.2 **Block Sectioning.** Use of 5-10 µm thick sections is preferable to a single thicker section (25- 50 µm) for DNA extraction, as greater yields of high molecular weight were reported when multiple 5µm thick sections were used rather than a comparable tissue volume of 50 µm sections [27]. Higher PCR success rates were also reported when one 5-10 µm section was used but it was unclear if tissue volume was standardized [28]. However, while another study found DNA yield increased with section thickness (5-50 µm), amplifiable DNA could be obtained from a single 5 µm section [29, 30]. For RNA, while section thickness was not experimentally compared, maximal yields were obtained from 16 sections (20 µm thick) with an tissue area of <75 mm^2 or four (20 µm thick) sections with a tissue area >150 mm^2 [31]. Importantly, extraction criteria such as lysis volume [29] and enzyme amounts [31, 32] must be adjusted to compensate for section number and cross-sectional area.

7.3 **Section Storage.** Although published evidence was limited, storage of FFPE sections for 10 y prior to DNA extraction was highly detrimental to PCR success rates [33]. Similarly, FFPE sections stored for 180 days at room temperature, 4°C or -80°C prior to extraction had reduced real-time qRT-PCR success rates than those stored for ≤ 90

days at room temperature, 4°C or -80°C and significantly lower β-actin levels than those analyzed immediately after sectioning [34]. Levels of β-actin were not significantly affected by 90 d of storage at room temperature, but significant declines were noted after 90 days at 4˚C or -80˚C (P=0.002) [34]. We were unable to identify information on the impact of waxing and dewaxing sections for storage on the quality of the extracted DNA and RNA.

7.4 **Deparaffinization Solution.** Compared to traditional deparaffinization with xylene, heating in lysis buffer led to higher PCR yields [35], increased genotyping success rates [36], and a higher percentage of probes with copy numbers in the normal range using multiplex ligation dependent probe amplification (MLPA) [37], but comparable RT-PCR success rates [38]. Use of an extraction kit that included focused-ultrasonication during deparaffinization resulted in larger DNA maximum amplicon size [39]. Other deparaffinization methods generated comparable or inferior results to those generated with xylene, but higher DNA yields were reported using hexadecane, pentadecane, tetradecane or mineral oil although these solvents were not removed prior to lysis [40]. Heating in mineral oil led to mean DNA fragments of comparable sizes with those obtained with xylene, but mineral oil resulted in superior homogeneity among high resolution melting peaks [41] and higher DNA yields [40]. Deparaffinization using HemoD should be avoided when PCR analysis is anticipated [41]. Reports conflict as to whether deparaffinization by microwave heating was detrimental to PCR success [8], or resulted in comparable [42] or increased PCR success [43], although isolation methods differed among reports. RNA yields were comparable when specimens were heated in a proprietary auto-dewaxer buffer at different temperatures and durations. The literature also supports deparaffinization with HemoClear for analysis by PCR [43], and HemoD for analysis by RT-PCR [32, 44] but not PCR. Other methods that have been optimized for paraffin removal prior to extraction of DNA or RNA from FFPE specimens include proprietary buffers (WaxFree Paraffin Sample RNA Preparation Kit, QuickExtract, VERSANT kPCR Molecular System)[35], d-limonene (Absolutely RNA FFPE), and heat (FormaPure RNA isolation kit), but experimental comparisons have not been published.

7.5 **Deparaffinization Duration/Condition.** Effects associated with the duration of deparaffinization are influenced by both the deparaffinization reagent and extraction

method used. Extending the duration of deparaffinization with xylene from 10 min to overnight did not affect PCR success rates when DNA was extracted using the QIAamp FFPE tissue kit [45], but extending deparaffinization from 2 h to 4 h increased PCR success following phenol chloroform extraction [3]. We were unable to locate published findings investigating the duration or temperature of xylene deparaffinization on RNA analyses, but when a proprietary dewaxer reagent was used RNA yield increased with the temperature of deparaffinization (80˚C versus 95˚C) and duration (1 day versus 3 days)[46]. However, FFPE-specific RNA and DNA extraction kits recommend deparaffinization for 10 seconds in room temperature xylene (QIAamp FFPE, ALLPrep FFPE, RNeasy), 3 min in 50°C xylene (RecoverAll FFPE), or use of proprietary buffers (AllPrep, WAXFree) or technologies (MaxWell 16).

7.6 **Proteinase K.** A single recent experimental study found no effect of extending the duration of proteinase K digestion from 1 h to 72 h on DNA yield or fragment size [41]. Although comparisons are lacking in the literature, DNA from FFPE specimens has been successfully analyzed by methylation bead chip array after digestion at 56°C overnight [47] or until completion [48]; pyrosequencing after digestion at 56°C for 1 h [49, 50] or 2 d [51]; NGS after digestion at 50°C until completion [52], 55°C overnight [53], 56°C for 1 h [54, 55], and 65°C for up to 24 h [56]; and real-time PCR after digestion at 56°C overnight [57] or for 24 h [58]. While the majority of studies report successful RNA analysis by microarray, nanostring, real-time RT-PCR, and NGS following proteinase K digestion for 15 min at 50°C in conjunction with the RecoverAll kit [59-61] or at 55-56°C with the RNeasy kit [55, 62-65], several studies report superior yields when digestion is extended. RNA yield increased when digestion was extended from 15 min to 5 h with the RecoverAll kit [66], or to 3 h [66] or overnight [67] with the RNeasy FFPE kit, although amplification was not affected [67]. Further, studies that altered the digestion conditions recommended by the manufacturer reported successful RNA analysis by NGS after digestion at 56°C overnight with the AllPrep extraction kit [68], and by Nanostring after digestion at 45°C overnight with the RecoverAll kit [63]. Successful analysis of miRNA has been reported after digestion for 1 h at 59˚C by NGS [69], and 15 min at 50˚C by NanoString [60] and TaqMan Low Density Arrays [59]. Lysis buffer composition may be a confounding factor [70], but recommendations are not possible due to the proprietary nature of many digestion buffers.

7.7 **Demodification.** A demodification step may be necessary during DNA and RNA extraction from FFPE specimens in order to reverse covalent modifications introduced during formalin fixation [71]. Experimental comparison of DNA demodification methods has been limited to studies that employ a demodification step instead of proteinase K digestion. Shi *et. al*. compared a variety of methods based on antigen retrieval procedures and found that the greatest amount of amplifiable DNA was obtained when specimens were heated to 100˚C or 120˚C in Britton and Robinson buffer (pH 11) for 20 min compared to 80°C or buffers at another pH [72]. DNA yields and amplificability were greater when specimens were heated to 120˚C in a buffer consisting of 1% SDS and 0.1M NaOH (pH 12.78) compared to other temperatures (80˚C or 100˚C) or other buffers containing NaOH, KOH, GITC, SDS or Tween-20 alone or in combination [73]. However, another study found superior PCR success rates when specimens were heated to 95˚C for 10 min following proteinase K digestion compared to those heated to 100˚C for 20 min in 0.1 M NaOH [74]. While not experimentally compared, demodification by heating specimens to 90-95°C for 10 min (WaxFree, reference 9.1.8), 1 h (QIAamp, reference 9.1.4), or 2 h (AllPrep, reference 9.1.7) in lysis buffer have been shown to be successful.

For RNA, demodification by incubation at 70°C for 45 min in 10 mM citrate buffer pH 4.0 prior to extraction with a silica-based kit was found to produce more reliable PCR results than incubation in TE (pH 7-8), sodium borate (pH 6.5-10), or 50 mM citrate buffer, or in 10 mM citrate buffer with a lower or higher pH or temperature [75]. When specimens were extracted using organic methods, incubation of RNA at 94°C for 20 min in ammonium chloride (pH 8.0) led to lower cycle threshold values than when no demodification was performed; intermediate cycle threshold values were obtained when incubation in ammonium chloride was conducted at room temperature or at pH 5.0 [76]. A study using mouse tissue reported incubation at 55˚C for 18 h in a pH 7.0 buffer resulted in increased RNA yield, RIN, and the number of amplifiable copies of RNA in specimens fixed for 3 months compared to when no demodification step was included; additionally, including an organocatalyst (2-amino-5-methylphenyl phosphonic acid) in the reaction buffer further increased the number of amplifiable RNA copies [77]. While not experimentally tested, the literature also reports successful analysis after demodification using kit-specific protocols, which

include heating in lysis buffer to 80°C for 15 min (RNEasy, RecoverALL, AllPrep) (References 9.1.5, 9.1.6, and 9.1.7) or 90°C for 10 min (WAx-Free RNA; Reference 9.1.8).

7.8 **DNA Extraction.** The majority of studies agree that, commercial/proprietary silica membrane-based extraction kits produce high quality DNA that resulted in equivalent [2, 11, 57, 78-80] or superior [8, 38, 55, 74, 81-86] results compared to those obtained using organic solvents, magnetic beads, heating, resin filters, and other methods in the majority of studies. However, several studies report superior DNA results in comparison to silica-based kits when FFPE specimens were extracted using Trimgen's resin-filter based WaxFree DNA kit or Promega's cellulose filter based ReliaPrep kit, which gave comparable or higher PCR success rates depending on amplicon length [11, 57], phenol chloroform which produced a longer maximum PCR amplicon size [87], the adaptive focused acoustics (AFA) based truXTRAC kit which produced a longer maximum PCR amplicon size [39], and the glass fiber-based Roche High Pure kit which led to increased success in methylation studies [88]. Reports conflict as to whether DNA extracted by magnetic bead-based kits performs superiorly [89], comparably [78, 81] or inferiorly [79, 82, 85] to DNA extracted by silica membrane-based kits. Similarly, DNA extracted by in-house boiling or heating methods has been shown to lead to higher [90], comparable [2] and lower [74] PCR success rates; a higher percentage of probes with copy numbers in the normal range with MLPA [37]; and increased DNA fragmentation [8] compared to when specimens were extracted using a silica membrane-based DNA extraction kit. The salting-out method should be avoided as it led to higher CT values [81] and less consistent PCR results [79] than obtained with silica-based kits.

7.9 **RNA Extraction.** RNA extraction with a silica membrane- or bead-based kit produced high quality RNA [61, 66, 67, 86, 87, 91-93] in the majority of published reports we identified, although extraction with phenol-chloroform yielded high quality RNA in several reports [11]. Reports conflict as to the performance of RNA extracted from magnetic bead-based kits [20, 94] [16, 32]. RNA extraction from FFPE specimens using resin-filters [11, 95] or heat [91] is not recommended.

7.10 **Contaminating nucleic acid digestion.** While DNA quantification by spectrophotometer is not recommended, should it prove necessary, purified DNA

should be treated with RNase and purified RNA should be treated with DNase in order to prevent interference by the respective contaminating nucleic acid, which has been reported in a rat model [96].

7.11 **DNA and RNA Quantification.** The literature suggests that quantification of DNA and RNA is most reliable using fluorometric- or qPCR-based assays. Available evidence indicates that spectrophotometric methods overestimate DNA yield compared to fluorometric methods for FFPE specimens [97-100], although this may be confounded by different levels of variability among quantification methods [89]. qPCR has also been found to be a reliable indicator of the amount of functional DNA [99, 101] and RNA [32, 102].

7.12 **DNA Quality Assessment.** The successful generation of amplicons longer than 100 bp by multiplex PCR and evaluation by denaturing high-performance liquid chromatography were reliable predictors of real-time qPCR success after whole genome amplification [103] and aCGH success [104]. In contrast, visual inspection of DNA by electrophoresis was independent of PCR success [103]. For mutation detection by NGS, the false negative rate was lowest when the input amount was adjusted based on real-time PCR amplification and the false positive rate was lowest when analysis was limited to specimens with at least 5-6% of amplifiable DNA [99, 105] . The ISBER Biospecimen Science Working Group recommends real-time PCR as the most reliable method of quantification [106], which also serves as a quality metric given quantification is limited to amplifiable DNA [100]. Further, ΔCT values obtained from the real-time PCR-based Illumina FFPE QC Kit were inversely correlated with NGS quality metrics, and application of a threshold of ΔCT<1.55 resulted in fewer sequencing artifacts [101].

7.13 **RNA Quality Assessment.** For FFPE specimens, RNA integrity number (RIN) was not a suitable predictor of performance for qRT-PCR [107], DASL assay [102, 108] or Affymetrix Exon microarray [109]. Suitability is best assessed by examination of real-time RT-PCR metrics [106], such as low raw CT values [102], small differences between 28S rRNA Ct values of the FFPE sample and a universal cell line control [32] and the absence of bias (slope of <0.15) in mean log ratio due to the probe's distance from the 3' end or its C-content [110], although firm cut-off values have not been identified [108]. Real-time RT-PCR metrics are most reliable when used in

combination with other approaches, as microarray success was determined with 100% accuracy when yield, A260/280 OD ratio, 3'/5' ratio of less than 100 determined with SYBR, and Cy-dye incorporations were examined [107], and acceptable DASL replicate reproducibility scores were obtained when FFPE samples were screened by yield, A260/280 OD ratio, and TaqMan Ct values for RPL13a [102].

8.0 EXPERT-VETTING

8.1 Details of Expert Review

Five experts were identified and invited to review the document based on their contributions to the literature regarding extraction of nucleic acids from FFPE specimens (see 8.2). Feedback from participants was collected and documented following initial review of the draft BEBP and in response to specific clarifying questions. Final thoughts and recommendations were captured from the expert panel during a scheduled teleconference after review of the BEBP document. Participating individuals did so voluntarily and without compensation.

8.2 Participating Experts

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8.3 Summaries of Expert Recommendations

8.3.1 **SOP optimization and validation.** Notably, the type of extraction kit used can influence the ideal specimen size, digestion duration and modification steps, as such these factors should be experimentally optimized for each extraction kit. Experts emphasized that any protocol generated using the BEBP should undergo experimental validation for each analytical platform of interest. To facilitate SOP validation, specific parameters predictive of NGS success were provided by major sequencing centers at the request of the expert panel.

8.3.2 **Work area preparation.** To reduce contamination risk, experts recommend the microtome be wiped down first with a solvent and then with RNase-removal wipes. The experts agreed that storage of FFPE blocks on wet ice should be avoided to prevent potential contamination.

8.3.3 **Input amount.** Given that the capacity of columns used for nucleic acid extraction can differ, the experts recommend that a fixed tissue volume be used for a given extraction kit but caution that the tissue volume required must be optimized.

8.3.4 **Section and curl storage.** Experiences of the expert panel yielded different recommendations on preferred conditions of FFPE section/curl storage. While two experts store slide-mounted sections at 4˚C or room temperature and

one expert noted that nucleic acid quality remains unaffected for up to 100 days, the third expert stores sections at -80˚C. Another practice noted by an expert was storage of paraffin-coated slides at 4°C, which have DNA and RNA yields that are comparable to fresh for up to 4 months of storage. For FFPE curls, one expert prefers storage at -20˚C.

8.3.5 **Deparaffinization.** Each expert employs a different method of deparaffinization within their respective laboratory based upon the extraction kit used: Covaris AFA, heptane followed by methanol, histoclear and Qiagen Blue. Notably, two of the three experts reported positive results with an adaptive focused ultrasonication method. If xylene is used for deparaffinization, a 10 min incubation is recommended; and although a second ethanol wash was noted by two experts to only be necessary for FFPE blocks of particularly low quality, it was preferred by the third to ensure removal of all xylene. An expert emphasized that deparaffinization protocols must take into account the amount of wax present.

8.3.6 **Digestion.** Research conducted by the expert panel suggests that the concentration and duration of proteinase K digestion affect DNA and RNA yield and quality. Although an expert reported the DNA yield was 2-fold higher when digested with twice as much enzyme for 24 h than when digested for 72 h, a slightly higher percentage of DNA extractions passed the Illumina FFPE QC assay when digested for 72 h rather than 24 h. However, two experts noted that prolonged proteinase K digestion may adversely affect the integrity of resultant DNA. One expert has found digestion for 48 h rather than 24 h leads to DNA degradation and thus recommends a 24 h incubation. Another expert recommends DNA extraction include an initial 45 min digestion at 55°C and a second 45 min digestion at 56˚C. For RNA, an expert noted that in his experience, excessive digestion results in decreased integrity as determined by DV_{200}. As a starting point for dual extraction, the sequential purification of RNA and DNA from a single lysate, he suggests an initial 45 min digestion at 55°C for RNA and a second 45 min digestion at 56˚C of the DNA pellet. The optimal duration of proteinase K digestion should be determined

experimentally using a freshly prepared high quality control FFPE block and should be governed by both yield and integrity.

8.3.7 **Demodification.** While experts agree that optimal demodification procedures are dependent on the extraction kit used, for DNA they recommend incubation at 90°C for 10 min-2 h. For RNA, a 15 min incubation at 80˚C was recommended. The expert panel noted that based on their collective expertise, incubations for longer than 15 min can lead to RNA degradation. One expert has observed a negative impact of demodification on the percentage of RNA fragments >200 nt (DV_{200}%).

8.3.8 **Extraction.** While the experts note that based on their experiences many kits are capable of producing high quality nucleic acids from FFPE specimens, they use kits based upon silica or adaptive focused ultrasonication methods. All experts stressed the need to experimentally validate any extraction kit chosen. An expert also emphasized that elution volume is a crucial consideration when optimizing an extraction protocol, as reducing the final elution volume can increase sample concentration, which in turn can improve the accuracy of quantification and downstream analyses, although ultimately reducing yield.

8.3.9 **Post-extraction nucleic acid treatments.** Experts advised DNA samples should be subjected to RNase digestion post-extraction to eliminate the possibility of contaminating RNA. Experts advised DNase digestion of RNA samples is required when RNA is to be quantified by spectrophotometry and noted that they include a DNase treatment step during RNA extraction as part of standard practice.

8.3.10 **DNA/RNA quantification.** Fluorometry was advised for DNA and RNA quantification as there was a consensus that spectrophotometry overestimates both DNA and RNA yield by as much as 30% and 15%, respectively. One expert also noted the degree of overestimation for RNA yields determined by spectrophotometry was inconsistent between specimens, making it unreliable for the standardization of input amounts.

Another expert found spectrophotometry to be reliable to RNA if DNAse digestion was completed.

8.3.11 **DNA quality assessment.** Expert recommendations on DNA quality metrics are analytical platform-specific. Double-stranded DNA yield was noted as an important metric for NGS, CGH microarrays, and whole genome amplification. An expert advised that the effective amplification of 100 bp, 200 bp, and 300 bp PCR amplicons can be used to characterize a specimen prior to NGS, CGH microarray and WGA analysis, respectively, while another expert noted that next-generation sequencing success is independent of DNA integrity metrics. One expert concludes that integrity assessment by electrophoresis can be beneficial for some analyses. Although spectrophotometric measurements are not suitable for DNA yields, the ratios may be used to investigate specimen purity.

8.3.12 **RNA quality assessment.** Expert recommendations on RNA quality metrics are also dependent upon the desired downstream analytical platform. Based on the collective experience of the experts surveyed, RNA from FFPE specimens with a DV_{200} greater than 30% are suitable for both real-time RT-PCR and NGS, although successful amplification of a 60 bp amplicon by semi-quantitative RT-PCR was also recommended as a predictor of real-time RT-PCR success.

8.3.13 **Downstream applications.** As noted by an expert, this document is unavoidably biased toward PCR and sequencing platforms given that little has been published on how DNA and RNA extraction methods for FFPE specimens affect methylation and copy number array platforms. However, one expert from the panel advises that although FFPE specimens are well suited for methylation studies and CNV analysis via exome or whole genome sequencing, analysis by SNP6.0 array should be avoided. In the experience of the expert panel, attempts at whole genome amplification using FFPE specimens have been unsuccessful due to low yield or a lack of reproducibility between technical replicates. Due to the complexity involved in next generation sequencing applications, the nature and scope of artifacts introduced through FFPE-derived specimens should be thoroughly evaluated

during the assay validation process. Bioinformatic pipelines optimized for use with fresh frozen specimens may require customization to appropriately handle FFPE derived nucleic acids.

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109. Hall, J.S., et al., *Exon-array profiling unlocks clinically and biologically relevant gene signatures from formalin-fixed paraffin-embedded tumour samples.* Br J Cancer, 2011. 104(6): p. 971-81.
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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 prior to sequencing. Successful libraries should have the majority of library fragments between 300-600 bp in size with a minimum yield of 15µl at 3nM.	BC Cancer Agency (http://www.bcgsc.ca/services/sequencing-libraries-faq and personal correspondence with Dr. Andy Mungall)
	Sample Intake QC- Minimum DNA integrity (>200 bp) and absence of	Personal Communication with Dr. Harsha Doddapaneni and Dr. David

	protein contamination evaluated by E-Gel. Library construction yields should be >300 ng with fragments between 200 bp and 800 bp when using 100ng input (manual preparation) or 250ng (robotic preparation). Post library capture should have >10 nM yield and devoid of primer dimers.	Wheeler (Baylor College of Medicine Human Genome Sequencing Center)
	150 ng dsDNA, Amplification of 100 bp product; ΔCt<2 using the FFPE QC kit	Expert (Dr. Betsou)
	>5% copies allow for amplification of a 254 bp product	[105]
	>10 ng of DNA quantified by a duplex SYBR green based assay	[111]
	>6% amplifiable copies; Input adjusted based on PCR amplification of tata-binding protein (TBP) or ferritin heavy polypeptide 1 (FTH1)	[99]
	ΔCT<1.55 real-time PCR based Illumina FFPE QC Kit	[101]
PCR	Comparative assessment of differentially sized GAPDH PCR amplicons: 100, 236, 299, 411, bp visualized by HPLC	[103]
aCGH	Amplification of a 200 bp fragment of GAPDH from 100 ng DNA	[104]
	Amplification of 200 bp product; > 2 µg DNA	Expert (Dr. Betsou)

Table 2: RNA quality guidelines by analytical platform.

Application	Criteria Employed	Reference
RT-PCR	Amplification of a 60 bp product; DV_{200} >30%	Expert (Dr. Betsou)
NGS	No RNA Integrity metric (RIN, DV200, etc.) useful in predicting if a library is successful. Predictors of success can come from library's that yield >4ng/µl concentration or miSeq test runs where goal is to see >10% of reads mapping to mRNA. RNA capture required between 100-200ng of total RNA derived from FFPE while Illumina Total RNAseq requires between 400-1000ng.	Personal Communication Dr. Hoadley (UNC)
	Following library construction and Agilent/Caliper QC, the majority of fragments should be between 200 bp and 500 bp in length. Final library concentration should be >1 nM in at least 10µl. Ribosomal RNA depletion for RNA-Seq requires a minimum of 400ng of total RNA input when quantified by Bioanalyzer/Caliper GX. Alternatively, 400ng of total nucleic acid quantified by Qubit or Quant-iT can be used.	Personal Communication Dr. Andy Mungall (BC Cancer Agency)
	Sample Intake QC- DV200 should be greater than 30%. RIN is not informative. Library construction yields should be >3 ng with cDNA fragments between 100 bp and 1,500 bp when using between 50 and 100 ng RNA input.	Personal Communication with Dr. Harsha Doddapaneni and Dr. David Wheeler (Baylor College of Medicine Human Genome Sequencing Center)

	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	[107]
	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 7 cycles of the same quantity of universal control RNA	[32]
	Mean log ratio slope <0.15 due to the probe's distance from the 3' end or its C-content in microarray hybridization	[110]
DASL	>100 ng RNA; A260/280 ratio >1.5; Rpll13a Ct values of <29	[102]