Successful biomarker profiling from FFPE samples

Sample to Insight

The nature of FFPE samples

FFPE tissue sections are a valuable and extensive source of material for biomedical research. However, formalin fixation makes molecular analysis from FFPE samples challenging. Formalin fixation causes a chemical reaction between formaldehyde and nucleic acids resulting in crosslinking with proteins and other biomolecules (Figure 1)

Factors that influence molecular analysis from FFPE samples

FFPE preparation

- Degree of nucleic acid fragmentation
- Use of unbuffered, diluted formalin
- Overfixation or underfixation (Figure 2)
- Inappropriate fixative to sample ratio
- Inappropriate storage conditions
- Inappropriate paraffin embedding

FFPE sample processing for analysis

- Inefficient deparaffinization
- Inefficient nucleic acid purification
- Inefficient or excessive crosslink removal
- Cytosine deamination
- Overestimation of nucleic acid yield

Critical factors for successful molecular analysis of FFPE samples

Formalin fixation and paraffin embedding

Fixation of tissues involves placing specimens in a formalin solution which can vary in composition (a typical 10% formalin solution contains 3.7% formaldehyde and 1–1.5% methanol). For optimal results, use neutral-buffered formalin solution instead of unbuffered or acidic formalin solutions. Also, pay equal attention to other factors that influence the extent of tissue fixation such as the thickness of the tissue specimen, the volume of formalin solution and the duration of fixation.

Did you know?
Formalin starts penetrating tissues at a rate of about 1 mm per hour.
However, the rate decreases after the first millimeter.

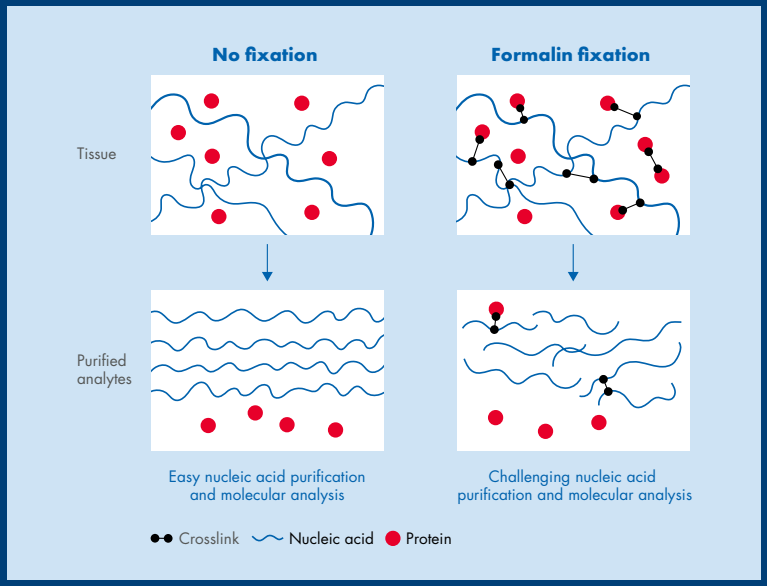


Figure 1. Fixation causes crosslinking. Formalin crosslinking makes nucleic acid purification and molecular analysis challenging.

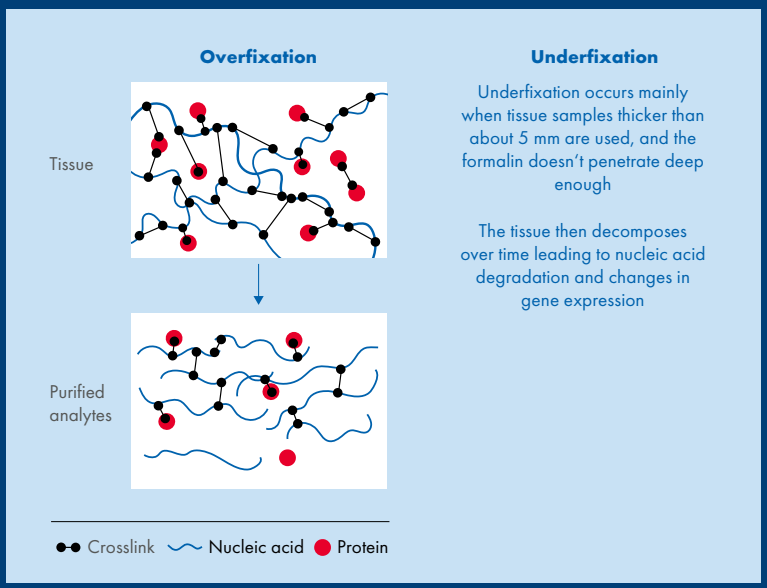


Figure 2. Avoid the consequences of overfixation or underfixation.

Nucleic acid fragmentation in FFPE samples is a result of various parameters:

- Cellular processes and tissue autolysis during the fixation process
- Elevated temperatures during the embedding process
- Prolonged storage of the embedded samples (aging)
- Oxidation of nucleic acids on the exposed surface during slicing
- Staining procedures

Document the following:

Sample collection

- ID of the specimen donor/patient, relevant health status, medical treatment, appropriate consent
- Information about the specimen:
  - Start of ischemia within the body
  - Time, date and method of removal from the body
  - Description of tissue type, tissue conditions and organ of origin
- ID of the person responsible for specimen collection
- Time (cold ischemia) and storage conditions until fixation
- Any additions or modifications to the specimen after removal from the body (e.g., further grossing, ink-marking, stitches, incisions)

FFPE

- Formalin used for fixation, ratio of formalin to tissue, temperature and duration
- Dehydration and paraffin infiltration steps (temperature, duration, vacuum), quality and concentration of alcohol and paraffin
- Storage of FFPE samples (time, temperature, humidity)

Processing for analysis

- Sectioning for DNA/RNA extraction:
  - Number of sections and their thickness
  - Information about grossing into the block before removal of sections for DNA/RNA extraction
  - Extraction of DNA/RNA directly from sections of FFPE or from areas excised from sections mounted on slides

Do's and don'ts during fixation and embedding:

DO

- Document all steps from sample collection to isolation of the analyte
- Use standard-buffered formalin solution as fixative – check pH and concentration before use
- Use thin tissue pieces, preferably ≤5 mm
- Ensure at least 10:1 (V/V) formalin to tissue ratio for optimal fixation
- Reduce time to fixation to the minimum
- Keep fixation duration between 12 and 24 hours for 5 mm tissue pieces
- Use low-melting-temperature paraffin
- Store your FFPE samples at 4°C or below, if possible

DON'T

- Try not to exceed a temperature of 60°C during embedding
- Avoid sample staining – if absolutely required, avoid procedures that involve high pH or heavy metal ions, instead, use a nucleic acid-compatible stain such as cresyl violet

For standardization of the pre-examination process refer to:

ISO 20166-1 Molecular in vitro diagnostic examinations – Specifications for pre-examination processes for formalin-fixed and paraffin-embedded tissue – **Part 1: Isolated RNA**

ISO 20166-3 Molecular in vitro diagnostic examinations – Specifications for pre-examination processes for formalin-fixed and paraffin-embedded tissue – **Part 3: Isolated DNA**

Cytosine deamination and implications on sequencing

In FFPE samples, deamination, which occurs randomly during formalin fixation and with aging, turns cytosine into a uracil base (Fgure 3). Deaminated cytosine pairs with adenine as uracil. In DNA sequencing reactions, this will manifest as a C>T|G>A transition. This can be mitigated by UNG treatment (Figure 4).

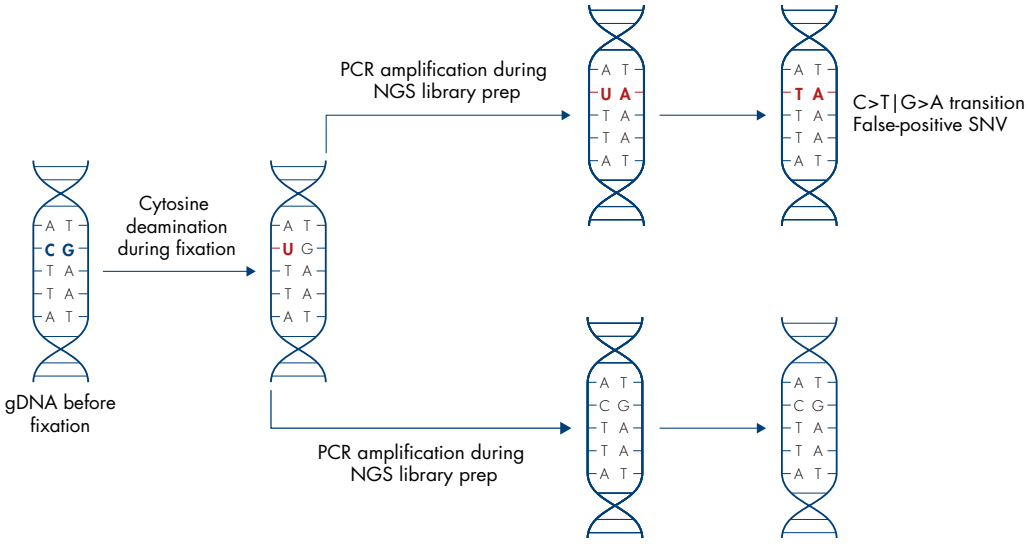


Figure 3. Potential for false positives
Cytosine deamination can lead to false positives during DNA sequencing.

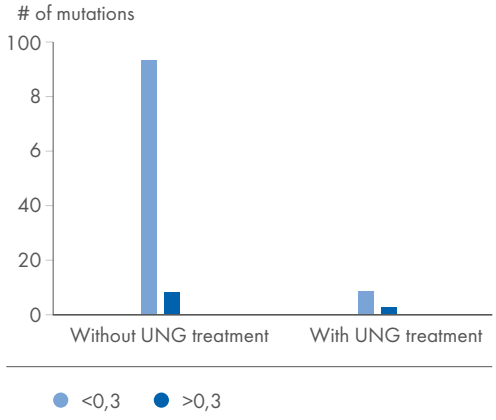


Figure 4. Dramatic reduction in artifactual C→T | G→A transitions
DNA was extracted from liver cancer samples, either with the incorporated UNG treatment step – a feature of the QIAamp DNA FFPE Advanced UNG Kit – or without UNG treatment. The results show that UNG treatment removed over 90% of low-frequency novel mutations that are most likely to be artifactual.

Did you know?
To improve the reliability of low-frequency variant detection bywhole genome or targeted DNA sequencing, uracil- N-glycosylase (UNG)treatment is recommended during DNA sample preparation. UNG removes uracil to prevent mismatch amplification.

Retrieval of usable nucleic acids from FFPE samples

The ideal starting material for nucleic acid purification from FFPE tissues are freshly-cut sections. Omit the first 1–2 sections because they are prone to oxidation of DNA caused by exposure of the block to the atmosphere. Due to this potential oxidation, freshly-cut sections should be processed immediately and not stored. If storage cannot be avoided, make sure to store the paraffin sections at lower temperatures, preferably at –20°C or –80°C. Additionally, use 5–10 µm sections for best results. Thicker sections may result in lower nucleic acid yields, even after prolonged incubation with proteinase K. Three steps (Figure 5) result in efficient deparaffinization and successful DNA extraction (Figure 6).

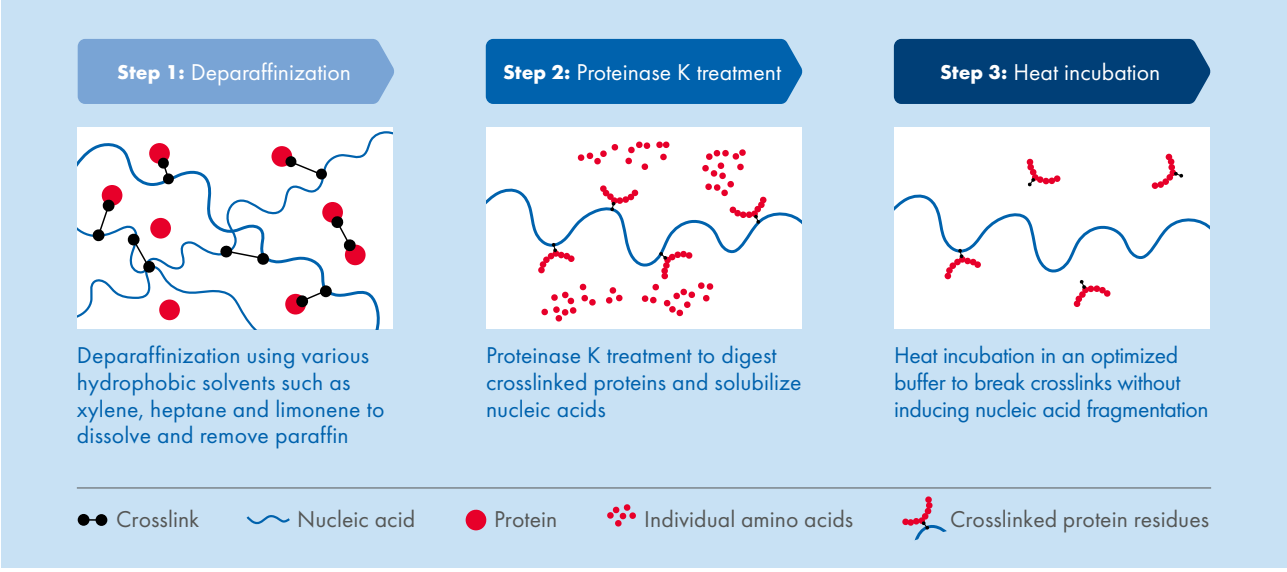


Figure 5. Key steps
Nucleic acid sample preparation from FFPE samples.

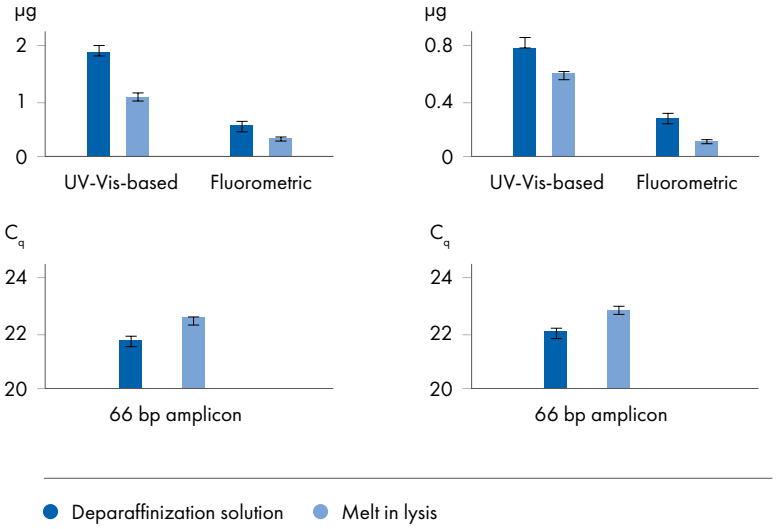


Figure 6. Efficient deparaffinization
Deparaffinization of 2 x 10 µm FFPE sections from human lung and human liver was carried out either by treatment with the QIAGEN Deparaffinization Solution or by melting of paraffin before lysis. Genomic DNA was purified using the QIAamp® DNA FFPE Tissue Kit. DNA yields were measured using UV-Vis-based and fluorometric technologies (Qubit®). The same volume of the diluted eluate was used as template in a PCR assay amplifying a 66 bp amplicon of the 18S rDNA. Samples treated with Deparaffinization Solution show significantly higher yields and lower C_q values than samples where paraffin was melted. This indicates that inefficient deparaffinization leads to insufficient release and poor recovery of DNA.

Purification of genomic DNA

FFPE samples contain DNA molecules that are crosslinked mainly to protein molecules. Such cross-linked DNA is less efficiently recovered during the purification procedure and is a poor substrate for PCR and other enzymatic assays. Therefore, breaking these crosslinks is a necessary step during gDNA purification.

Crosslink remnants like nucleic acids attached to proteinase K-digested protein fragments should also be reversed if possible, as they can lead to sequence artifacts during DNA analysis. However, decrosslinking and reversing chemical modifications should be carried out with care, because harsh reaction conditions may result in further DNA fragmentation.

For efficient decrosslinking, incubate at elevated temperatures such as 90°C for 1 hour. Alternatively, incubate longer at slightly lower temperatures, for example, 4 hours at 80°C, for probably gentler yet equally efficient decrosslinking.

However, overnight incubation at 56°C is not recommended because, without crosslink removal at higher temperatures, performance in downstream qPCR remains poor (Figure 7B). Also, UV-Vis-based quantification was unreliable for such samples (Figure 7A).

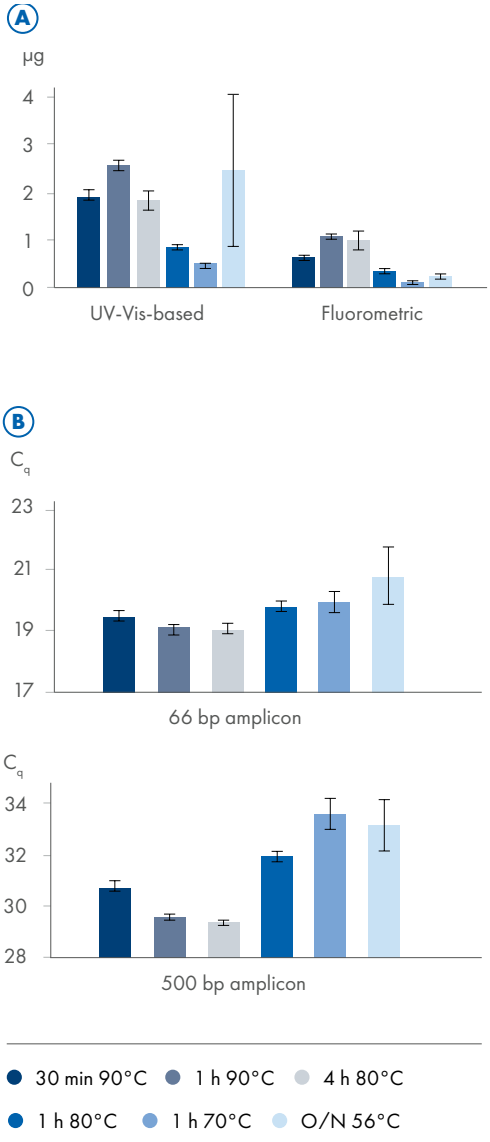


Figure 7. Efficient decrosslinking
2 x 10 µm FFPE sections from human lung were treated with proteinase K at 56°C for 1 h and then incubated as indicated. Genomic DNA was purified using the QIAamp DNA FFPE Tissue Kit. DNA yields were measured using UV-Vis-based and fluorometric technologies (Qubit). The same amount of DNA was used as template in a duplex PCR assay amplifying a short (66 bp) and a long amplicon (500 bp) of the 18S rDNA. **A:** Incubation for 1 h at 90°C and 4 h at 80°C achieved similarly high DNA yields. **B:** Incubation for 1 h at 90°C and 4 h at 80°C achieved similarly low C_q values, in contrast to the shorter incubations at 80°C or incubations at lower temperatures. Hence, shorter incubations at high temperatures or longer incubations at slightly lower temperatures work best for efficient decrosslinking.

Purification of total RNA and miRNA

Crosslinking and chemical modifications also affect RNA. Since RNA is more easily fragmented by heat treatment, optimized buffer conditions and lower incubation temperatures are critical. While incubation above 80°C may slightly improve RNA performance in RT-PCR further, it will also result in greater RNA fragmentation. On the other hand, incubation at lower temperatures will result in lower yields and significantly poorer performance in RT-PCR and other applications. Isolation of short RNA in a separate fraction to enrich miRNA (and other short RNA) is usually not feasible from FFPE specimens, because of the presence of short fragments derived from long RNA species.

Simultaneous purification of DNA and RNA

Purification of DNA and RNA from FFPE sections allow reliable comparison of genomic and transcriptomic data. This is particularly important when working with tumor tissues as they often contain a heterogeneous distribution of healthy and malignant cells, which means different sections from the same sample may differ in their cellular composition.

Simply dividing a sample in half for separate DNA and RNA purification procedures results in the purification of DNA and RNA from different populations of cells, which may differ in their properties Simultaneous purification of DNA and RNA from the same sample prevents wastage of precious FFPE samples.

Did you know?
Recent advances in NGS chemistries, platforms and bioinformatics pipelines have empowered users to efficiently interrogate DNA and RNA modifications in biological samples. Current approaches, however, require the use of two separate library prep workflows – one each for DNA and RNA. QIAseq® Multimodal Panels overcome these limitations by consolidating targeted DNA and RNA enrichment and analyses.

How to calculate sample input volume for optimal nucleic acid yield

Determining the exact amount of input material can be tricky with FFPE materials. However, accurate input calculations can avoid overloading the lysis and extraction system and ensure efficient purification of the target molecule. First, distinguish between tissue material and paraffin in the FFPE block, and consider only the actual tissue material for calculation (i.e., excluding the paraffin area). Then, calculate the starting material (amount of tissue in volume) from the surface area of the tissue (Figure 8), the number of sections and the thickness of sections. This method can be considered as the most accurate way of calculating the exact amount of input material.

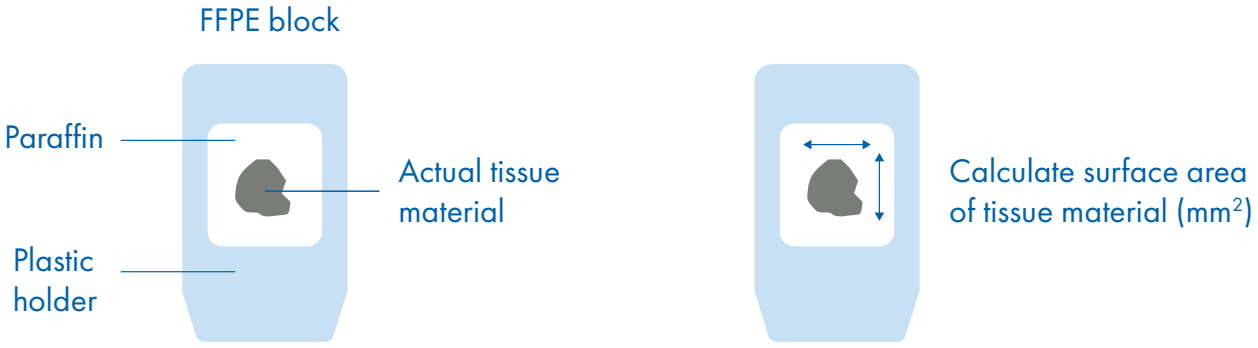


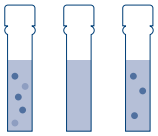
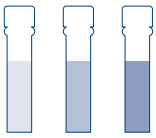
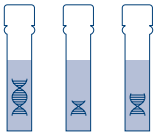
Figure 8. Sample volume
Calculating the sample input volume for FFPE tissues.

Table 1. Examples of sample input volume calculations for FFPE tissues

Surface area	No. of sections (10 µm thickness)	Total volume
50 mm^2	1	0,5 mm^3
	2	1 mm^3
	4	2 mm^3
	8	4mm^3
100 mm^2	1	1 mm^3
	2	2 mm^3
	4	4 mm^3
200 mm^2	1	2 mm^3
	2	4 mm^3
400 mm^2	1	4 mm^3

Nucleic acid quality control (QC)

Proper quality control of nucleic acids obtained from FFPE material is important regardless of the downstream assay. QC plays an important role in developing routine procedures and troubleshooting experiments.

QC criteria		Impact on analysis
Purity		• Impurities such as phenol, ethanol and salts can jeopardize sensitivity and efficiency of downstream enzymatic reactions • Large amounts of unwanted nucleic acids can lead to overestimation of the molecule of interest and interfere with downstream applications
Quantity		• Wrong estimation of DNA/RNA amounts can lead to incorrect sample-enzyme ratios resulting in inhibitory effects and suboptimal yields during NGS library preparation • Overestimation of nucleic acid amounts can also result in low-complexity libraries
Integrity		• Poor nucleic acid integrity can lead to PCR errors and/or lower yields (e.g., irrelevant C_T values) • Degraded samples can produce false negatives and lead to overestimation in concentration measurement (OD) • Highly-fragmented RNA could result in lower uniformity in target coverage and a higher probability of gaps in NGS • High fractions of single-stranded DNA (ssDNA) may impact adapter ligation during NGS library preparation

Elevated temperatures during decrosslinking denature double-stranded DNA (dsDNA), impacting DNA quantification.

In UV-VIS-based systems (e.g., NanoDrop®), dsDNA and ssDNA have different conversion factors. Conformation changes due to elevated temperatures, such as increase of ssDNA, may lead to the overestimation of gDNA quantity. Fluorometric systems (e.g., Qubit), which use dyes that intercalate into dsDNA but not to single-stranded molecules such as ssDNA or RNA, usually underestimate the gDNA quantity (see Figure 6A).

For subsequent DNA library preparation for NGS, check the quantity and quality of the DNA amenable to amplification using a qPCR system. The level of fragmentation should be checked using a gel electrophoresis method.

RNA quantity (yield) and RNA integrity (quality) are often poor due to various factors such as fixation procedures, storage time and temperature. Analysis of isolated RNA by electrophoretic methods (e.gs., QIAxcel®, Bioanalyzer®) shows only the physical integrity of RNA, ignoring chemical modifications by formaldehyde. The same is true for quantitation of RNA by OD measurement (e.g., NanoDrop) or fluorometric assays (e.g., Qubit). Therefore, routine QC methods provide information about RNA purity and integrity. They do not provide information about proper RNA decrosslinking and thus, cannot predict a successful downstream experiment such as qRT-PCR or NGS.

Importantly, fragment length distribution, often a mark of integrity or quality of isolated RNA, is affected by the isolation method, specifically the extent to which short RNA transcripts or fragments are recovered. For applications such as qRT-PCR, shorter fragments (down to 60 nucleotides or less) can provide meaningful results, whereas, for transcriptome analysis by most RNA-seq workflows, the minimum length is 200 nucleotides (exemplified by the DV200 quality score). In many cases, RNA isolation workflows can be adapted to recover or not recover shorter RNA fragments.

Recommendations for RNA quality control:

- Do not rely too much on routine RNA yield or RNA integrity measurements like RIN numbers – information is limited
- Use peak fragment length (which can be deduced from the electropherogram) as a measure of RNA integrity for strongly-degraded RNA
- Use a set of RT-PCR assays to determine the upper limit with regard to amplicon size
- Perform a qRT-PCR QC assay using different amplicon lengths to assess FFPE RNA performance in downstream applications

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