# SBP Recommendation

# Preanalytical conditions to prepare human DNA from FFPE Tissue for Next Generation Sequencing (NGS)

## Introduction

Pre-analytical factors include all factors that can influence the results of a sample analysis, particularly during the processes of sample collection, transport, processing, and storage. Their impact, often underestimated, can be critical. Identifying and controlling these factors at each step, and standardizing processes to obtain reliable, reproducible, and accurate results, will help ensure sample quality and suitability for advanced research applications, particularly for omics studies. Indeed, the integration of multi-omics data, facilitated by technological advancements, is crucial for accelerating both research progress and advancements in precision medicine. In this context, ensuring the reliability and integrity of biological samples is essential and biobanks and researchers play a key role in this.

The aim of this document is to support and guide researchers and biobanks to prepare quality DNA for genomic projects.

Before considering each process in detail and focusing on sample quality, you need to establish standardized procedures for tracking samples effectively (incl. naming, coding) and mitigate the risk of sample swapping.

For each pre-analytical process, from collection to storage, a set of recommendations including DNA quality criteria is given, highlighting which steps are important when handling samples to preserve the integrity of the DNA, minimize the possibility of any chemical modifications, and avoid any agents that could contaminate nucleic acid samples or inhibit downstream enzymatic manipulations (e.g., residual proteins, chemical reagents). These recommendations are aligned with the ISO 20166-3: Molecular in vitro diagnostic examinations - Specifications for pre-examination processes for formalin-fixed and paraffin-embedded (FFPE) tissue - Part 3: Isolated DNA[1].

To further support researchers and biobanks, a list of key pre-analytical variables to be documented is provided at the end of the document.

## Considerations about FFPE tissue samples

Formalin-fixed, paraffin-embedded (FFPE) tissue samples encompass a wide range of well-characterized pathological specimens, representing a vast resource that can be used to search for disease or diagnostic biomarkers, notably through the isolation of nucleic acids. However, extracting high-quality amplifiable DNA from FFPE tissues poses significant challenges due to the aggressive formalin-fixation process and resulting in cross-linking, often leading to low yields and DNA degradation. In addition, contamination with paraffin and other substances can also compromise the possible downstream analyses.

Nevertheless, extracting DNA from archived FFPE blocks can be useful, as they represent a valuable source of available, often unexploited, biological material. Indeed, samples stored in FFPE blocks are often associated with detailed clinical data, making possible to link genetic information with relevant health information. This can be particularly advantageous in biomarker research.

Furthermore, preparing FFPE blocks from tissue in a standardized way for cost-effective long-term storage, as no ultra-low temperatures are required, has the advantage to preserve clinical samples and ensure that they remain available for future analyses.

To maximize the success of this challenging processing, several factors need to be considered, especially during sample collection, preparation, tissue block storage and DNA isolation. By following appropriate protocols and ensuring favorable conditions in the pre-analytical phase, it is possible to preserve quality nucleic acids from these samples, enabling subsequent accurate molecular analyses.

The recommendations and requirements outlined in this document are intended to ensure quality DNA for next-generation sequencing (NGS), the most commonly used technology with DNA extracted from FFPE blocks.

This document is divided into three parts, so that it can be used according to the intended purpose:

- The first part is for those aiming to prepare FFPE blocks for cost-effective long-term storage while preserving DNA integrity. These blocks can be later used for DNA extraction.
- The second part is for those intending to isolate DNA from archived FFPE tissue while preserving to the best extent DNA integrity.
- A third part outlines the quality criteria that must be met for DNA to be suitable for NGS.

## PART 1 - FFPE BLOCK PREPARATION AND DNA QUALITY PRESERVATION[i]

It should be noted that making FFPE blocks with the sole aim of immediately extracting DNA is clearly not recommended. In this case, the use of fresh or frozen tissue is more appropriate and therefore encouraged.

### TISSUE COLLECTION

During the process of collecting a surgical specimen, a distinction is made between warm ischemia time, which corresponds to the *"condition before the tissue is removed from the body, but where it is deprived of its normal blood supply"*[1], and cold ischemia time, which corresponds to the *"condition after removal of the tissue from the body until stabilization or fixation"*[1]. Both phases can lead to molecular degradation, and their duration must therefore be minimized and recorded.

Tissue collection conditions, including duration and temperature can have a significant impact on DNA integrity and quality.

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[i] In all cases, and where applicable, the manufacturer's instructions must be followed precisely.

We recommend:

1) Use sterile instruments during the collection process.
2) Select the tissue to be sampled by appropriately trained pathologist with molecular pathology experience, in an appropriate location.
3) Immediately collect the tissue sample from the surgical specimen in an appropriate size.
   - Note that tissue specimens should not be bigger than 3cm × 2cm × 0.5cm [2].
4) Immediately place the tissue in a suitable sterile container.
   - Note that if the tissue sample collection cannot be collected immediately, keep the surgical specimen at 4°C in a refrigerator for a maximum of 3 hours[3].
5) For storage after collection, either immediately fix the tissue sample in a standard buffered formalin solution or store tissue sample at 4°C.
   - Note that tissue should be fixed as quickly as possible (in less than 1 hour).
6) Do not allow the tissue sample to dry out and never rinse it in water or physiological solution.

## TISSUE TRANSPORT / RECEPTION

During the transport and reception processes, duration and temperature are two important preanalytical factors to be considered in order to preserve DNA quality.

We recommend:

- Keep the tissue sample container on ice or at 4°C if not already placed in fixative. Make sure not to freeze the tissue during transport.

Or

- Transport the tissue sample at ambient temperature if it has already been placed in a fixative.

   - Note that vacuum-based systems for storing and transporting surgical specimens to the processing site could be considered[4].

## TISSUE PROCESSING

During processing, the tissue sample will first undergo a fixation step, followed by an embedding step. For each of these steps, the reagents used are harmful and must therefore be handled as per the applicable safety rules and according to the manufacturer's instructions. The following steps can be carried out using a tissue processor, and the tissue is generally placed in a cassette during processing (up to the embedding step).

### Formalin fixation

Three factors are important for the fixation step: tissue thickness, fixative volume and fixation time[5].

We recommend:

1) Use a Standard Buffered Formalin Solution "*10 % formalin solution in water with a mass fraction of 3.7 % formaldehyde (corresponding to a volume fraction of 4%), buffered to pH 6.8 to pH 7.2*"[1], also known as neutral buffered solution. Note that formalin lacking buffer has a limited shelf life and degrades rapidly.
2) Check the pH of the formalin solution, as despite the buffer, the solution may still degrade depending on storage conditions, especially light and temperature.
3) Use an adequate volume of fixative. The minimum ratio between standard buffered formalin solution and tissue is at least 10:1[5].
4) Carry out the fixing procedure at ambient temperature.

5) Avoid insufficient or excessive fixing[3,5,6]. Optimum fixation depends on tissue type and thickness. The infiltration rate is around 1 mm/h[3,5] and the optimum fixation time is generally between 12 and 24 hours.
6) Use DNase-free reagents and consumables during processing to avoid DNase contamination and thus DNA degradation.
7) Avoid adding elements and compounds (e.g. mercury compounds, zinc, EDTA, EGTA, acids) to formalin, which can alter the ionic balance[5].
8) Choose a suitable, well-closed container that allows complete immersion of the specimen in the formalin solution.
   - It Is possible to use a container pre-filled with a standard buffered formalin solution. In this case, follow the manufacturer's instructions.
9) If necessary, such as for bone biopsies, EDTA must be used instead of acidic decalcification[7]. Decalcification is not advised as it significantly reduces DNA yield and quality.

## Paraffin embedding

This step must be carried out according to a validated protocol. It is preferable to embed tissues in paraffin using automated systems specialized in tissue processing. Automated tissue processing involves immersing samples in a defined series of solutions comprising alcohols, xylene and paraffin.

The inclusion step thus consists of 3 steps:

- Dehydration (water is replaced by alcohol)
- Clearing (alcohol is replaced by xylene)
- Impregnation (xylene is replaced by paraffin)

<u>We recommend:</u>

1) Use high-quality reagents[5], especially for paraffin wax, which varies widely in terms of compositions, additives, heating temperature and characteristics. The choice of reagents is important in order to obtain quality blocks that are easy to process and cut.
2) Replace reagents regularly, according to the manufacturer's instructions.
3) Follow each step of the protocol precisely (e.g. number of reagent changes, concentration change, treatment temperature and processing time).
4) Do not allow samples to dry out during preparation. Samples must always be covered with reagent solutions.
5) Use synthetic paraffin wax with standardized composition and low melting point[5] to ensure correct impregnation of tissue.
6) Use pure paraffin and avoid the use of additives such as Beeswax[5] which can contain contaminants such as pollen and have a negative impact on the quality of the material to be extracted.
7) Check heating temperature, make sur not to overheat.
8) Choose a suitable mold, with an appropriate size for the tissue specimen and do not overfill the mold to avoid overflowing.
9) Correctly orientate the tissue according to the area of interest.

Particular care must be taken at the dehydration step to avoid any residual water later on. The tissue must be completely dehydrated, otherwise the water will not be properly replaced by the paraffin, which could influence the quality of the material to be extracted and thus lead to its degradation, as well as the shelf life of fixed tissue[8].

### FFPE BLOCKS STORAGE

The storage of FFPE blocks, although considered cost-effective as it is often carried out at room temperature, is a factor to be considered to preserve DNA integrity.

We recommend:

1) Store FFPE blocks at low temperature (4°C, -20°C) rather than at ambient temperature[5,9] to extend storage time and preserve DNA integrity.
2) Store them in an appropriate storage system and a controlled environment.
3) Protect from daylight, humidity, extreme temperatures, insects, and rodent[2,5].
4) Store the FFPE block without cut faces, if possible, to avoid exposure to air, humidity and other environmental factors that could lead to DNA degradation.
5) Do not cut sections in advance. For DNA extraction, sections must be freshly prepared and unstained[3,5].

## PART 2 - DNA ISOLATION FROM FFPE TISSUE[ii]

DNA isolation from archived FFPE samples is a multi-step process, several factors need to be taken into account to generate DNA of the highest possible quality.

It should be noted that, despite efforts to preserve nucleic acid integrity during the isolation step, quality can be poor or not as optimal as wished, as there is often no way to ensure a proper control of the FFPE block preparation nor the block storage.

Nevertheless, concerning the starting material and the method to be used,

We recommend:

1) Prefer to use blocks stored for up to 3 years[11,12] and avoid using blocks stored for more than 7 years[10,11].
2) Use diagnostic hematoxylin and eosin (H&E) slides, reviewed by appropriately trained pathologists with molecular pathology experience, for tumor/cellularity assessment[7].
3) Use freshly cut, unstained sections with a maximum thickness of 10 µm[13]. If the isolation procedure is not immediate, keep freshly cut sections temporarily at 4°C[3]. You can use H&E stained slide as a reference to select the area of interest for DNA isolation[13], using macrodissection to enrich the neoplastic/cellular content when needed[7,14].
4) Consider cellularity and % necrosis of the tissues by H&E examination. It is recommended to select a region with a little necrosis as possible[11,12].
5) Select sample containing a percentage of cells (tumor cells) that is at least three times the limit of detection of the method being used (e.g. if a method has a 5% limit of detection, the area of tumor/tissue sample selected for DNA extraction should contain at least 15% of cellularity/tumor content)[7].
6) Cut out the first section of the archived FFPE block before cutting for DNA isolation.
7) Use new disposable blades when sectioning different specimens to avoid contamination[7].
8) Do not perform the isolation step in the same area as the amplification step to avoid cross-contamination.
9) Use a commercially available isolation kit for DNA isolation[6].
    - Make sure you select a proper kit, as performance may vary, and follow the instructions carefully.
    - Avoid mixing products from different kits (they may not be compatible).
    - Choose a kit with proteinase K digestion, as digesting samples with proteinase K generally improves DNA yield and/or purity, and with an RNase step to obtain RNA-free genomic DNA.

[ii] In all cases, and where applicable, the manufacturer's instructions must be followed precisely.

# PART 3 - DNA SAMPLE REQUIREMENTS FOR NGS

Given that the quality of DNA extracted from FFPE tissue differs from that of DNA obtained from blood or fresh tissue, it seems imperative to reassess DNA sample requirements, taking into account the source material (FFPE block) and the challenges associated with obtaining quality DNA from it. DNA derived from FFPE tissues is generally highly fragmented, limiting the functional fraction available for genotyping and making the total amount of DNA an unreliable measure of usability. DNA fragmentation is therefore a crucial factor to assess[15]. Furthermore, it has been observed that the amount of amplifiable input DNA better predicts library complexity for NGS, compared to measurements in nanograms, because it takes into consideration the degree of fragmentation.

To obtain DNA suitable for NGS:

1) **Concentration**: Quantify DNA by a sensitive and precise analysis using specialized methods such as fluorescent dye quantification[2,7,16]. Alternatively, measurement by spectrophotometry is also possible. It should be noted that differences in concentration may be observed for the same sample, depending on the method used. A minimal amount of 50ng of double strand DNA (dsDNA, measured by fluorimetry) is generally required for NGS[11,17].
2) **Integrity:** Measure the DNA Integrity Number (DIN) or genomic quality number (GQN), which are metrics obtained by electrophoresis-based technologies. DIN values are distributed on a scale of 1 to 10. A high DIN indicates highly intact DNA, and a low DIN indicates highly degraded DNA sample. A value above 2.05 indicates that samples may be suitable for NGS[17,18].
3) **Amplifiability:** Assess amplifiability by measuring the ratio (Q-ratio) of longer amplicons to shorter ones, obtained through real-time PCR (qPCR). A Q129 bp / Q41 bp ratio > 0.1 is recommended[10,17].
4) **Purity:** Measure by spectrophotometry
   a. the A260/280 ratio, an indicator of contamination by proteins or RNA. A ratio of ~1.8[2,16] is generally accepted as 'pure' for DNA, and a range between 1.7 and 2.0 is accepted.
   b. the A260/230 ratio, an indicator of contaminants that absorb at 230 nm such as proteins, guanidine HCL (used for DNA isolations), EDTA, carbohydrates, lipids, salts, or phenol[2,16]. A ratio between 2.0 and 2.2 is accepted[16].

   Note that the 260/280 ratio may vary according to pH[19]. When comparing the 260/280 ratio for different DNA samples, it is important to ensure that the pH and ionic strength of the elution buffers used are the same. Absorbance at 260 nm and 260/280 values are reproducible when a low-salt buffer is used as elution buffer, but not water[16].

# PREANALYTICAL VARIABLES TO BE DOCUMENTED

Below is the list of the key variables to be documented as part of the standardization of data documentation to accompany your DNA sample and record key preanalytical data. Some of these variables are specific to FFPE tissues. For a complete list of variables, please refer to the document "SBP Dataset for Human Tissue Sample"[20].

| Process | Variables | Description |
|---|---|---|
| **Collection** | sample_ID | Unique identifier of primary sample |
| | collection_start_time | Date and time of sampling |
| | clamping time | Date and time when organ is deprived of its normal blood supply |
| | | Acquisition method of specimen |
| | collection_procedure* | Date and time when organ/tissue is removed from the body |
| | resection_time | Duration between clamping_time and resection_time |
| | warm ischemia_time* | Type of sample collected |
| | sample_type* | Additives or specific conditions applied |
| | collection_special_conditions | Temperature conditions before transport |
| | pre_transport_temp | |
| **Transport** | transport_sample_type | Precision if transported item is the unprocessed specimen or if specimen has already been processed (=sample) |
| | transport_start_time | Date & time when transport starts |
| | transport_temp | Temperature conditions during transport |
| **Reception** | reception_time | Date & time when sample arrives at reception |
| **Processing (fixation)** | processing_start_time | Date & time when processing starts |
| | processing_temp | Temperature conditions during processing |
| | sample_size | Size of the tissue or volume – often "predefine" sample size |
| | sample_size_unit | Unit of sample size |
| | storage_container | Storage container type for long term storage |
| | fixation_type* | Fixation or stabilization type (type of fixative) |
| | fixative_volume | Volume of fixative used |
| | fixation_start_time | Date and time when fixation/stabilization starts |
| | cold ischemia_time* | Duration between resection_time and fixation_start_time |
| | fixation_special_conditions | Additives or specific conditions applied |
| | fixation_stop_time | Date and time when fixation/stabilization stops |
| | fixation_time* | Duration between fixation_start_time and fixation_stop_time |
| **Processing (embedding)** | Processor_type | Type of processor used for embedding |
| | Paraffin_type | Type of paraffin used |
| | embedding_start_time | Date & time when embedding starts |
| | embedding_special_conditions | Additives or specific conditions applied |
| | embedding_stop_time | Date & time when embedding stops |
| **Block storage** | storage_start_time | Date & time when FFPE block is physically stored in the freezer or refrigerator |
| | storage_temperature | Storage temperature |
| | storage_place | Storage location of the sample |
| **DNA Isolation** | storage_stop_time | Date & time when FFPE block is physically removed from the freezer or refrigerator |
| | block_sectionning_time | Date & time the slide was prepared |
| | isolation_start_time | Date & time when isolation starts |
| | isolation_DNA_kit | Isolation kit used for DNA Isolation |
| **Quality measurements** | quantification_method | Type of quantification method used |
| | concentration | Concentration value obtained |
| | DIN | DIN value obtained |
| | Q-ratio | Q-ratio obtained |
| | A260/A280 ratio | A260/A280 ratio value obtained |
| | A260/A230 ratio | A260/A230 ratio value obtained |
| **DNA Storage** | storage_start_time | Date & time when DNA is physically stored in the freezer |
| | storage_temperature* | Storage temperature |
| | storage_place | Storage location of the sample |
| | freeze_thaw_cycle | Actual sample status - e.g. "2" = defrosted twice |

In addition to these variables, it is recommended to use the SPREC reporting system. This valuable tool documents pre-analytical conditions and is based on seven pre-analytical variables that are essential to identify potential non-conformities. These SPREC variables are highlighted with an asterisk (*) in the table above.

For more information, please visit the following website: SPREC.

# References

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