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Guidelines

Part I - Pre-analytical phase

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Summary
Precision oncology relies on pathology to integrate morphological and genomic data for tailored treatment selection. The preanalytical phase, covering all steps from specimen collection to processing before analysis, is one of the major determinants of the quality of downstream molecular analyses, which are essential for selection personalized therapeutic strategies. Strict adherence to protocols and specific quality control programs are essential to ensure the reliability and standardization of molecular testing. This document intends to guide the multidisciplinary team (pathologists, technicians, molecular biologists) in pathology laboratories during the preparation of samples for molecular analyses.

Key words: Pathology, recommendations, preanalytics, precision medicine, standardization

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Introduction

Precision medicine in oncology aims at identifying the best therapeutic options in individual patients through the integrated analysis of tumor morphology and genomics traits [1]. Pathology laboratories are central to this process, as they are responsible for the handling and preparation of tissues (e.g. biopsy, surgical specimens) and/or fluids (e.g. plasma, ascitic fluid) for downstream molecular testing [2]. The pathology multidisciplinary team, comprising technicians, molecular biologists, and (molecular) pathologists, must receive specific training to ensure high-quality and consistent sample processing, preparation, and analysis [3].
The preanalytical phase in predictive molecular pathology includes all steps from specimen collection to processing that precede the actual molecular analysis [4]. This critical phase requires adherence to established guidelines, as errors can severely affect the accuracy and reliability of molecular diagnostics. This document outlines best practices for the pre-analytical phase to ensure high-quality molecular diagnostics in oncology and offers clear, evidence-based recommendations.

Tissue samples

Collection and transportation

Tissues and cell blocks are the primary source of samples processed and analyzed in pathology laboratories. Establishing a close and proactive collaboration among all healthcare professionals involved in tissue procurement, such as surgeons, radiologists and endoscopists, is essential to ensure a seamless workflow from sampling to specimen accessioning [5]. Small biopsies should be placed in formalin immediately after sampling, ensuring prompt initiation of fixation (ideally not less than 6 hours) without the strict need of immediate transfer to the laboratory [5,6]. In the case of surgical samples, different types of collection and transportation protocols can be adopted, and should be organized with respect to architecture, size, and specific needs of each Hospital or Institution [7]. Transportation of fresh specimens allows fresh sampling and snap freezing of tissue samples for intraoperative examination or tissue biobanking [8]. Depending to the type of sample (fresh or formalin-fixed), an appropriate sterile container, jar, or bottle should be used, or alternatively under-vacuum sealing can be employed [9-11]. In this context, it is essential to monitor the "cold ischemia time" (also referred to as "time to fixation"), i.e. the interval between tissue excision and initiation of fixation [12,13]. This is an important variable impacting on molecular analyses and shall not exceed 30 minutes [14]. The standardization of the time to fixation represents an important step to ensure the reliability of both molecular and immunohistochemical analyses [13]. The ASCO/CAP guidelines for HER2 and hormone receptor evaluation include specific sections on preanalytical factors, reflecting their profound impact on biomarker assessment, therapeutic decisions, and patient management [14,15]. These recommendations also state that it is the responsibility of the surgeon and operating room staff (or the radiologist and his/her staff) to document the collection time, ii) of the pathologist and laboratory staff to document the fixation initiation time. Of note, when surgical samples are preserved under vacuum, transfer to the to the pathology laboratory may be delayed for up to 72 hours, provided that they are stored at 4° C until transport [16]. However, earlier transfer is strongly recommended to preserve sample integrity. In such cases, the under-vacuum time is considered the time to fixation. Key aspects of this phase are reported in the green box of Figure 1.

RECOMMENDATION STATEMENTS

1. Monitoring sample collection, preservation, and transportation, and therefore cold ischemia time is a shared responsibility between pathology laboratories and tissue providers, including operating rooms, radiology suites, and other collection sites.

a. Tissue providers are responsible for recording the sample collection time (and eventually fixation initiation), which should be tracked and documented within the pathology laboratory information management system (LIMS).

b. Pathology laboratories are responsible for recording the fixation time, which should also be tracked within the LIMS.

2. Transporting specimens requires an institutional, documented workflow that is integrated into the quality management system and ensures timely fixation after sampling.

Fixation

Following accessioning and registration, samples are ready for processing [17]. Proper fixation is critical to preserve nucleic acids integrity and depends on both the tissue volume and the fixation conditions, including duration and specimen type [12]. The gold standard fixative is 10% neutral buffered formalin (NBF), prepared using a standardized and quality-controlled phosphate-buffered solution [18]. Given that most protocols and diagnostic assays have been standardized using NBF, and that clear evidence supporting the advantages of alternative fixation methods such as alcohol-based solutions is lacking, their use should be discouraged [19,20]. It is recommended that specimens be immersed in an adequate volume of formalin, with a tissue-to-formalin ratio of at least 1:10–though some sources suggest ratios up to 1:20 [4,21]. Fixation time should be adjusted based on tissue type and size: 6-12 hours for biopsies, 12-24 hours for larger specimens, and up to 48 hours for tissues with high fat content [19]. For tissues requiring decalcification, it is well established that the process can significantly reduce the quality and yield of both DNA and RNA [22]. EDTA-based decalcifying solutions better preserve quality and are therefore preferable [23-25]. Appendix 1 describes in detail the principles, parameters, and molecular mechanisms of formalin fixation.

RECOMMENDATION STATEMENTS

3. 10% neutral buffered formalin (NBF) is the gold standard fixative.

4. Fixation time should be adapted according to tissue type and size: 6–12 hours for biopsies, 12–24 hours for larger specimens, up to 48 hours for fatty tissues, and up to 72 hours for vacuum-sealed samples stored at 4 °C.

5. An adequate tissue-to-formalin volume ratio is defined as no less than 1:10.

6. Over- or under-fixation should be avoided.

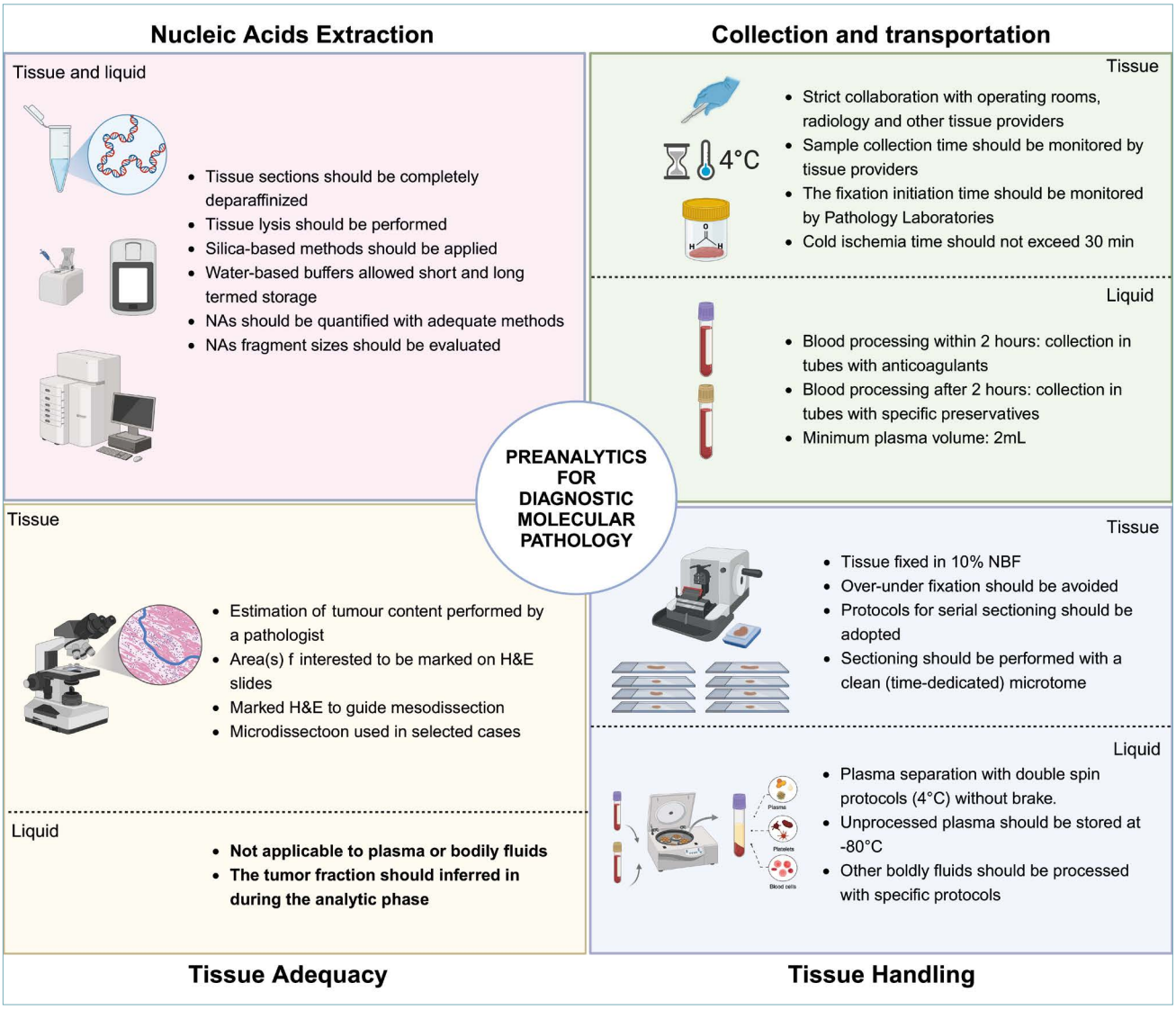

Figure 1. Key pre-analytical variables of molecular testing in solid tumors. Overview of critical steps and quality control parameters during the pre-analytical phase, including tissue procurement, fixation, sectioning, tumor cell content assessment, and nucleic acid extraction. Color-coded boxes summarize the main procedures: (green, tissue and liquid biopsy collection and transportation; violet, tumor cell content evaluation; yellow, area selection and dissection; pink, nucleic acid extraction). Standardization of these variables is essential to ensure high-quality molecular diagnostics.

Sectioning

After processing and paraffin embedding, samples are ready for sectioning. This step should be performed by pathology-trained laboratory technicians, specifically educated on the procedure rationale and on standard protocols designed to prevent cross-contamination between samples [26]. Molecular analyses are typically carried out after histopathological diagnosis; however, to optimize turnaround times, tissue sections for molecular testing can be prepared prior to the pathologist's review of the initial H&E slides [27]. Molecular pathology includes a broad variety of assays, from *in situ* methods, such as IHC and FISH, to *in vitro* assays requiring nucleic acid extraction, such as DNA/RNA sequencing. When designing tissue sectioning protocols, it is essential to consider the appropriate section thickness for each technique [28]. *In situ* methods have stricter requirements: sections should be 3-4 micron thick for IHC and exactly 4 micron-thick for FISH to prevent signal distortion. In contrast, *in vitro* assays are generally unaffected by section thickness. To balance workload and tumor cells yield, thicker but

fewer sections are often used for molecular analyses. However, in cases of limited material, such as small biopsies, using ~4 µm sections for both *in situ* and *in vitro* methods and preparing more slides is advisable to minimize tissue loss from suboptimal cuts.

RECOMMENDATION STATEMENTS

7. Tissue sectioning should be performed by pathology-trained laboratory technicians who are specifically trained in the rationale and standard procedures to ensure sample integrity and prevent cross-contamination.

8. Tissue sections for molecular analyses may be prepared prior to histopathological review of the initial H&E slides, when appropriate, to optimize turnaround times, provided that a subsequent diagnostic review is still performed.

9. Sections thickness should be adapted to the analytical method: 3–4 µm for immunohistochemistry (IHC), exactly 4 µm for fluorescence in situ hybridization (FISH); sequencing methods are generally not sensitive to section thickness.

10. In cases of limited tissue availability, such as small biopsies, it is recommended to use ~4 µm sections for both in situ and in vitro analyses and prepare multiple slides to reduce the risk of tissue loss due to suboptimal sectioning.

Tumor cell content evaluation

Estimating tumor cell content is essential to ensure sufficient material for molecular analysis and to accurately interpret results, including normalization of variant allele frequencies (VAFs) based on the testing method [29]. A recommended practice is to include an H&E slide at the end of the sectioning protocol to assess residual tissue Tumor cell content should be evaluated on both pre- and post-sectioning H&E slides by a trained pathologist [30]. When reviewing H&E slides to select the most representative tumor areas for molecular testing, necrotic, hemorrhagic, and stromal regions with abundant tumor infiltrating lymphocytes should be avoided [31-33]. Macro- or microdissection should be considered when necessary to prevent under-representation of tumor cells due to surrounding normal tissue components. The tumor cell content of the selected area must be sufficient for reliable molecular testing [31-33]. Although precise thresholds vary depending on the limit of detection (LOD) of the assay, a minimum of 100 tumor cells and a tumor-to-normal cell ratio of at least 50% is generally recommended for most molecular analyses [17,34-36]. For targeted NGS panels, a lower tumor fraction – typically around 20% – may still be acceptable, depending on assay sensitivity. When the tumor fraction falls below the LOD of the method (usually ~5% for NGS), the risk of false-negative results increases, particularly for low-frequency variants [37-40]. Key aspects of this phase are reported in the violet and yellow boxes of Figure 1.

RECOMMENDATION STATEMENTS

11. Tumor cell content should be evaluated by a trained pathologist on both pre- and post-sectioning H&E slides to confirm sample adequacy for molecular testing.

12. In selecting tumor areas for analysis, necrotic, hemorrhagic, and stromal regions with abundant tumor-infiltrating lymphocytes should be avoided.

13. Macro- or microdissection should be used when needed to enrich tumor cell content and avoid under-representation due to adjacent non-tumor tissue.

14. A minimum of 100 tumor cells and a tumor-to-normal cell ratio of at least 50% is recommended for most molecular analyses to ensure reliable variant detection.

15. For targeted NGS assays, a tumor fraction of approximately 20% is generally acceptable, depending on the sensitivity and limit of detection (LOD) of the platform.

16. When the tumor fraction is below the assay's LOD (typically ~5% for NGS), the likelihood of false-negative results increases, particularly for low-frequency variants; in such cases, molecular testing should be reconsidered or deferred.

Nucleic acids extraction

Nucleic acid (NA) extraction from FFPE samples begins with shared initial steps, followed by separate workflows for DNA and RNA. Extraction protocols lacking post-lysis purification (e.g., phenol-chloroform) should be avoided. Standard FFPE NA extraction includes three key steps: tissue deparaffinization, tissue lysis, and DNA and/or RNA purification. Effective removal of paraffin is essential to eliminate hydrophobic wax and enable interaction with aqueous buffers. Deparaffinization is typically performed on slides using xylene, followed by ethanol washes, as xylene can inhibit downstream enzymatic reactions. Safer alternatives, such as isoparaffinic solvents, may be used to reduce operator risk. In this workflow, mesodissection follows deparaffinization. In-tube deparaffinization is also a valid and efficient alternative [41-43]. Following deparaffinization, tissue is digested with a lysis buffer containing a protease, typically proteinase K, able to digest cellular and nuclear proteins. DNA extraction usually requires overnight incubation at 50–60 °C, while RNA extraction protocols typically involve shorter incubation times (2-6 hours). Extended incu-

bation may improve yield without significantly affecting nucleic acid quality. Most protocols also include a de-crosslinking step to reverse formalin-induced crosslinks. This is commonly achieved through heat during lysis, though chemical or enzymatic alternatives are also available [44]. The final step of extraction separates DNA or RNA from other components in the lysate. Silica-based methods, such as columns or beads, bind nucleic acids for purification, followed by optional RNase or DNase treatment to increase specificity. Automated extraction systems are also widely adopted in clinical laboratories and can standardize key steps of the extraction workflow, reduce hands-on time, and minimize cross-contamination. Their use should follow validation against manual methods to ensure comparable yield and nucleic acid quality. After buffer washes, nucleic acids are eluted with water or Tris-EDTA–based buffers, which help preserve stability. After extraction, DNA/RNA should be quantified and tested for integrity. Fluorometric methods offer high specificity, while the spectrophotometric absorbance ratios (260/280 and 260/230) provide additional information on purity. DNA and RNA fragmentation is a major limitation for molecular testing on FFPE tissue samples. Fragmentation can be assessed by qPCR-based assays (long/short amplicon ratio) or microfluidic electrophoresis (fragment size distribution). While no single method is preferred, using multiple QC approaches provides a more complete evaluation. Standardizing input based on consistent QC criteria is essential for downstream testing. Key aspects of this phase are reported in the pink box of Figure 1.

RECOMMENDATION STATEMENTS

17. Nucleic Acids extraction should begin with accurate transfer of the annotated area from H&E to unstained slides, followed by dissection of the region of interest.

18. Tissue sections must be completely deparaffinized to prevent hydrophobic-induced inhibition of subsequent water-based steps aqueous reactions caused by residual paraffin.

19. Complete tissue lysis should be ensured using lysis buffers and proteinase K; incubation time and temperature should be adjusted based on the NA type (DNA or RNA) and tissue characteristics.

20. DNA and RNA should be purified using validated protocols, typically involving silica-based binding and aqueous buffers; automated extraction systems may also be used, provided they are validated to ensure comparable yield, purity, and integrity.

21. Post-extraction quantification and quality assessment should be performed, tracked, and recorded.

Liquid biopsy samples (plasma cfDNA and cfRNA)

Plasma is currently the most commonly used biomaterial for liquid biopsy in predictive molecular pathology, due to its broad clinical applicability and ability to reflect tumor-specific alterations [45]. For the purpose of these recommendations (advanced yet aligned with routine clinical practice), only plasma-based liquid biopsy is considered. Other sources such as urine, saliva, cerebrospinal fluid, and pleural effusion are not included.

Blood collection and transportation

Plasma is obtained from blood, and proper venipuncture by qualified staff is essential to prevent hemolysis, clotting, and/or contamination [46,47]. Blood samples should never be frozen before plasma separation, regardless of the collection tube type. Samples must be collected in anticoagulant-containing tubes to prevent clotting. EDTA and citrate are both suitable for cfDNA preservation [48,49]. A critical factor is represented by the time between the blood collection and processing. This interval directly affects the integrity of circulating cell-free DNA (cfDNA), which has a short half-life of approximately two hours. Moreover, if processing is delayed beyond 3 hours, leukocyte lysis may occur, leading to the release of germline DNA into the plasma and causing dilution of the circulating tumor DNA (ctDNA) fraction. Importantly, storing blood at 4 °C does not prevent this leukocyte lysis, and therefore does not mitigate the risk of contamination from genomic DNA. If processing is delayed beyond 2 hours, tubes with preservatives that stabilize cfDNA and leukocytes should be used, ensuring sample preservation at room temperature (16–24 °C) during transport. The recommended maximum time from blood collection to plasma processing with preservative tubes is 3 days, despite manufacturers claims of cfDNA stability for up to 14 days [50]. A minimum of 2 mL of plasma is recommended for adequate cfDNA extraction, typically obtained from about 6 mL of peripheral blood. Once isolated from the blood, plasma must be frozen if not immediately processed for cfDNA purification and can be transported on dry ice to maintain the integrity of the analytes, stored in airtight containers to avoid contamination during transport. Key aspects of this phase are reported in the green box of Figure 1.

Plasma isolation

After collection, blood samples should be processed as quickly as possible to minimize cfDNA and cfRNA degradation. Ideally, plasma should be separated from whole blood within 1-2 hours by centrifugation to remove

cellular debris and eliminate leukocyte contamination. Among available protocols, the "double spin" method is highly recommended [51]. This involves two sequential centrifugations at 2300 g for 10 minutes each, performed without brake using a refrigerated centrifuge at 4 °C. If immediate analysis is not possible, plasma can be stored at –20 °C for short-term preservation. This is generally recommended for up to 1 month, although some protocols allow storage for up to 3 months, particularly for cfDNA. cfRNA is more sensitive and may degrade more rapidly under these conditions. For long-term preservation, plasma should be stored at –80 °C. Under these conditions, cfDNA remains stable for 6 to 12 months or longer. cfRNA, however, is less stable and should ideally be used within 3 to 6 months, even at –80 °C. In all cases, repeated freeze-thaw cycles should be avoided to maintain the integrity of the nucleic acids. Additionally, it is recommended to isolate and store the cellular fraction, specifically peripheral blood leukocytes (PBL), at the time of plasma separation. DNA extracted from PBL can be useful for identifying variants associated with clonal hematopoiesis [52].

Nucleic acids extraction

Several validated methods are available for cfDNA and cfRNA extraction from plasma and other body fluids, including both manual protocols using commercial kits and automated platforms. These systems typically rely on silica membrane or magnetic bead-based technologies, as previously discussed for tissue-derived nucleic acids. Automated methods are preferred in high-throughput settings due to their reduced hands-on time, lower contamination risk, and improved reproducibility. Unlike protocols developed for tissue samples, cfDNA extraction requires dedicated methods due to the low abundance and high fragmentation of circulating DNA (typically < 1,000 bp). Commercial kits allow for extraction from as little as 10 µL up to 10 mL of plasma, with optimal recovery achieved in low elution volumes (20–150 µL). A minimum of 2 mL of plasma is generally recommended to obtain sufficient cfDNA for downstream analysis [53]. However, cfDNA yield is highly variable and depends on clinical factors, including tumor burden and timing of blood draw [54]. Adapted extraction protocols also exist for other body fluids (e.g., ascites, pleural effusion), which may allow larger input volumes (10–50 mL). In these cases, at least 2–5 mL is recommended to detect cfDNA, while 5–10 mL is preferable for applications like NGS or qPCR. If cfDNA is low in concentration or heavily fragmented, higher input volumes or pre-concentration steps may be necessary. After extraction, cfDNA and cfRNA quantification and quality control are essential, and follow the same procedures to those used for tissue samples. Key aspects of this phase are reported in the pink box of Figure 1.

RECOMMENDATION STATEMENTS

22. Venipuncture should be performed by trained personnel using standardized procedures to prevent hemolysis, clotting, and contamination.

23. Blood must never be frozen prior to plasma separation, regardless of the type of collection tube used.

24. Samples should be collected in anticoagulant-containing tubes, with EDTA or citrate as the preferred options for cfDNA preservation.

25. Plasma should be separated within 1–2 hours to preserve cfDNA integrity; if processing is delayed beyond 2 hours, tubes with stabilizing preservatives can be used at room temperature (16–24 °C).

26. The maximum recommended time from blood collection to plasma separation is 3 days when using preservative tubes.

27. A minimum of 2 mL of plasma is recommended for sufficient cfDNA extraction, typically obtained from approximately 6 mL of whole blood.

28. Isolated plasma should be frozen immediately if not processed and transported on dry ice in sealed containers.

29. Plasma should be separated using a double-spin protocol, consisting of two centrifugation steps at 2300 g for 10 minutes each, without brake, using a refrigerated centrifuge at 4 °C.

30. Short-term plasma storage should be at –20 °C, preferably for no longer than 1 month. For cfDNA, some protocols allow storage up to 3 months; cfRNA is more sensitive and degrades faster.

31. Long-term plasma storage should be at –80 °C. cfDNA remains stable for 6–12 months or longer, while cfRNA should be used within 3–6 months.

32. Repeated freeze-thaw cycles should be avoided to preserve nucleic acid integrity.

33. The cellular fraction (PBL) should be isolated and preserved at the time of plasma separation, as it may be useful for identifying clonal hematopoiesis-associated variants.

Cytological samples

Cell blocks

Cell block (CB) preparation may be considered as a cytological surrogate of histological specimens. In fact, similarly to histological biopsies or surgical excision, cytological specimens are formalin-fixed and paraffin-embedded (FFPE). The main advantages of this kind of preparation compared to other cytological specimens are represented by the possibility to eval-

uate the architecture of the specific lesion (if the sample is highly cellular and small fragments of the lesion are represented in the sample) and the possibility to perform ancillary techniques, such as immunocytochemistry or molecular approaches, without the need of additional validation [55-57]. Remarkably, CB preparations suffer from the same limitation of histological specimens related to formalin fixation.

Neoplastic cells content within CBs is another crucial point for molecular testing. For this reason, it is fundamental to evaluate tumor cell content through H&E-stained slides prepared pre- and post-sectioning [58,59]. In addition, similarly to histological samples mesodissection of specific areas within CBs may be applied to enrich tumor cell content for DNA and RNA extraction. Similarly to histological specimens, the standard 4–5 µm CB sections represent part of the entire nuclei of tumor cells, resulting in a lower nucleic acid yields for molecular testing per cell than those obtained from other non-FFPE cytological preparations [58]. For this reason, cutting extra, unstained CB sections upfront ("strategic sectioning", as detailed above for histological specimens) would be crucial to save as much tumor tissue as possible for molecular testing [60].

In addition, a different number of CB preparations are commercially available. These include those based on agar or fibrin, which may require an additional validation.

Direct smears

Direct smears represent the main specimens for cytological diagnosis. However, despite the standardization in terms of morphology, an additional rigorous validation for each individual molecular assay should be performed on smear preparations [59]. Of note, International guidelines suggest the feasibility of direct smear for molecular tests [61]. Similar to CBs, mesodissection of specific areas can help increase tumor cell content before DNA and RNA extraction, however the smeared samples are typically more dispersed with a greater variation in the proportion of tumour/benign component in different areas of the slide [57]. Overall, cell isolation on smears can be obtained either via smear scraping or by cell lifting [62]. The first procedure is usually performed by a flat, single-edge scalpel blade. Cell lifting can be obtained by the Pinpoint solution of the Pinpoint Slide DNA Isolation System that is applied over the selected area [63]. Briefly, the solution is spread evenly over the area of interest and was air-dried for approximately 30–45 min. After the solution is completely air-dried a thin blue film appears. The embedded tissue together with the film is then loosened using a razor blade. The film is transferred to an Eppendorf tube and centrifuged briefly to collect the tissue at the bottom of the tube [62]. An alternative technique is represented by the adoption of a special commercially available medium, which is spread uniformly over the top of the cellular material on smears without coverslip. After slide heating, the media is hardened, cut and placed in an Eppendorf tube for DNA extraction and molecular testing [64]. Regarding smear preparation, both alcohol-fixed and air-dried smears are generally suitable for DNA and RNA extraction. Compared to CBs, these preparations do not suffer from formalin fixation and nuclei partial sectioning. Several studies demonstrated that molecular tests can be performed successfully using both Diff-Quik as well as Papanicolaou-stained slides. However, it has been suggested that Diff-Quik-stained smears should be preferred to Papanicolaou-stained slides in stored samples [65]. In fact, Pap-stained slides experienced DNA degradation as a function of age, instead the Diff-Quik stained smears provided high-quality DNA even if archived for a prolonged period.

The main limitation of smear adoption for molecular testing is represented by the fact that these samples are often unique and unrepeatable. For this reason, digitalization of smears may be useful to store the morphology of the smears sacrificed for molecular testing [66].

Liquid-based cytology

Liquid-based cytology (LBC) has become a widely adopted technique in cytopathology, offering several advantages over conventional smears, including improved specimen preservation, reduced artefacts, and suitability for ancillary molecular and immunocytochemical analyses [67]. After aspiration, the entire specimen is typically expelled into an alcohol-based fixative designed to preserve cellular morphology and allow for subsequent molecular and ancillary testing. These fixatives often contain methanol and may vary in their inclusion of formaldehyde, which can influence nucleic acid preservation [67,68]. Among the commonly used alcohol-based fixatives, those free of formaldehyde have been reported to provide superior preservation of nucleic acids, particularly RNA. In contrast, fixatives containing even low concentrations of formaldehyde – typically around 1% – may compromise DNA integrity due to cross-linking. Despite these biochemical differences, their impact on clinical performance appears limited. Comparative studies have shown that while conventional smears may yield higher DNA quantities and greater cellularity than liquid-based cytology slides, both approaches are generally equivalent in terms of diagnostic adequacy and mutation detection rates [69,70]. Cell-blocks derived from LBC samples allow for histological-level evaluation and a wide array of immunocytochemical and molecular studies. Both man-

ual and automated methods are currently employed to prepare cell-blocks from LBC material. In manual protocols, the sample is centrifuged to concentrate cells, and the supernatant is carefully decanted to isolate the pellet. This pellet is often resuspended in formalin for fixation and subsequently embedded in a supporting matrix such as plasma-thrombin clots, agar, or commercial gels [71]. After solidification, the cell block is processed and embedded in paraffin using standard histological techniques. Manual methods are simple and cost-effective but can be operator-dependent and variable in outcome [72]. Automated systems often use vacuum filtration or chamber-based centrifugation to compact cells into a uniform layer, followed by standardized fixation and processing steps. Automated approaches tend to yield more reproducible morphology and better antigen preservation, although they may require specific consumables and more upfront investment [73,74]. Liquid-based cytology systems that produce monolayered slides (e.g. ThinPrep) are widely used in cytopathology [75]. In addition to slide preparation, the residual material in the collection medium can also serve as a valuable source for cell-block preparation. Following centrifugation and removal of the supernatant, the resulting cellular pellet can be processed into a cell-block using either manual or automated techniques. This step is particularly important when ancillary studies such as immunocytochemistry or molecular testing are required. From a molecular standpoint, nucleic acids can be recovered not only from cell-blocks but also directly from LBC slides by means of cell scraping or from the residual liquid fixative. Several studies have demonstrated the feasibility of extracting high-quality DNA and RNA from these sources, expanding the diagnostic utility of cytology samples beyond morphology. However, attention to fixation protocols is essential, as prolonged formalin exposure can compromise nucleic acid integrity.

RECOMMENDATION STATEMENTS

34) Preparation of cell blocks stemming from cytological specimens follow the recommendations for tissue samples.

35) Formalin fixation, while essential for CB preparation, can impact nucleic acid integrity and should be managed carefully, particularly when planning molecular analyses.

36) Accurate evaluation of tumor cell content in CBs is critical for molecular testing and should be assessed on H&E-stained slides prepared before and after sectioning.

37) Due to partial nuclear sectioning and formalin-induced degradation, strategic upfront sectioning of CBs is recommended to preserve material for molecular testing.

38) Smears can be an alternative source of NAs when limited material is available; digitalization of slide before use for molecular downstream analyses is recommended.

39) LBC offers advantages over conventional smears, including improved cellular preservation, reduced artefacts, and enhanced compatibility with ancillary testing.

40) The type of fixative used in LBC (alcohol-based with or without formaldehyde) influences nucleic acid quality; formaldehyde-free fixatives are preferable for RNA preservation.

Conclusions

A strict adherence to protocols thus avoiding variability in the pre-analytical phase holds the promise to provide standardization and to guarantee quality of molecular pathology assays performed on a variety of tissue and liquid samples by using different methods. These recommendations aim at providing guidance to the multidisciplinary team (pathologists, technicians, molecular biologists) in Pathology Laboratories when preparing samples for molecular analyses in conjunction with the standard diagnostic process. Of note, several steps related to tissue procuration, handling, processing and preparation impact on the quality of digitalization of slides for those Pathology Laboratories that have already embraced a full digital transition for routine diagnostics. Although this at present impacts on the optimal visualization of digital slides, with the advent of artificial intelligence (AI) tools that can predict molecular alterations and/or surrogate molecular assays simple and basic preanalytics of slide preparation will have a profound impact on molecular analyses. Hence, a word of caution on the curation of this specific aspect should be voiced to foster a smooth preparation to AI-based molecular pathology diagnostics.

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Authors' contributions

Starting from an initial draft prepared by Enrico Berrino, all authors contributed substantially to the development of the manuscript and reviewed its final version. Caterina Marchiò coordinated the writing team and provided extensive input in both drafting and revising the text. Nicola Fusco served as the overall coordinator of the guidelines project, ensuring harmonization across all documents and conducting critical revision and editing of the manuscript. Umberto Malapelle and Fabio Pagni provided critical feedback and input throughout the writing process. Giancarlo Pruneri acted as the final reviewer.

Ethical consideration

Not applicable.

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Appendix 1: Mechanisms and implications of formalin fixation in tissue preservation and molecular analysis

The obvious advantage of formalin fixation is the prevention of ineluctable autolytic and putrefying processes that begin once tissue is removed from its physiological environment [1]. With tissue fixation we take snapshots of the surgically removed tissue, with the main goal to retain morphology and antigen immunoreaction [2]. However, since the gold standard for the fixation is a chemical process based on formaldehyde, we must consider that we create an iteration between a chemical substance with specific characteristics and the plethora of complex macromolecules included in a tissue [1]. While the NBF-induced crosslinking of proteins and nucleic acids stabilizes tissue architecture, it also accounts for the most detrimental effects on nucleic acid integrity [3]. From one hand, covalent protein-protein and protein-NAs links and methylene bridges between the amino groups of the nucleotides are associated with a poor yield in DNA-RNA purification [4]. In addition, NBF fixation impairs the backbone of the NAs, by breaking phosphodiester bonds and inducing a diffused fragmentation [5]. Moreover, NBF-fixation improves the spontaneous reaction of cytosine deamination, leading to C > T or G > A on the antisense strand, single base, sequence artifacts [6,7]. This complex scenario is complicated by numerous parameters that can strongly influence the quality of fixation: the chemical-physical laws, the time necessary for the process and the organ of origin of the tissue. Although neutrally buffered, the age-related NBF solution degradation could lead to the formation of formic acid, that can impact over the DNA structure and sequence [8]. Interestingly, the temperature for the tissue fixation represents an experimental paradox: from one hand, higher temperatures trigger NBF diffusion within the tissue and accelerate the rate of the chemical reaction between the fixative and tissue elements [9]. However, several studies have shown that cooling formalin to 4° C could preserve nucleic acid integrity [10]. Time of fixation, penetration rate, specimen dimensions and origins are strictly related. Each fixative shows a precise diffusion coefficient (K), calculated as the millimeters diffused per hour. For NBF, K is 0.78, and so formalin does not penetrate more than 1 mm/hour: a one-day fixation is approximately required to penetrate the center of a 10 mm thick sample [11]. This is related to the specimen size (bioptic withdrawal or surgical resection), but also to the organ of origin: a fat mammary gland requires a different type of protocol compared to a lung biopsy or a brain specimen. Altogether, these parameters could lead to over- or under-fixation. Over-fixation can result in an excessive cross-linking, potentially reversible with an appropriate antigen retrieval for immunohistochemical procedures, but with several sequencing artifacts over DNA and RNA [12,13]. On the other hand, insufficient tissue fixation allows cross-linking only on the external part of the sample, with the center potentially affected by tissue degradation [14], with strong issues for both antigen retrieval [15,16] and DNA/RNA quality [17].

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