



# Optimization of Tissue Handling and Processing in the Era of Precision Medicine: A Practical Recommendation from a Multidisciplinary Panel of Indian Experts

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## Abstract

Molecular analysis of biospecimens is the key to diagnostic and therapeutic decisions in clinical practice. However, there is a lack of consolidated guidelines for biospecimen collection, tissue handling, and storage in India. Therefore, this study aims to generate expert recommendations for the optimization of tissue handling and processing practices in India in the era of precision medicine. This study aimed to evaluate the clinical gaps related to tissue handling for molecular analysis and develop expert recommendations to mitigate preanalytical issues associated with biospecimen processing. These expert recommendations will help in increasing the diagnostic yield and accuracy of biomarker testing in clinical practice. A virtual advisory board meeting was convened with 19 experts, including pathologists, molecular biologists, medical oncologists, surgical oncologists, interventional radiologists, and a senior histology technician from 10 hospitals in India, along with an accreditation officer for testing and calibration of laboratory procedures. The scientific coordinators developed specific questions to address the salient issues associated with the preanalytic phase of tissue specimen preparation. The experts discussed each question until a complete set of

## Keywords

- ▶ biospecimens
- ▶ cold ischemia time
- ▶ fixation
- ▶ tissue processing
- ▶ paraffin blocks

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recommendations was obtained. The expert panel provided recommendations for tissue collection, processing, fixation, and block preparation to ensure high-quality biospecimens. As per the expert panel recommendations, tissue sampling can be performed from any easily accessible site, regardless of the primary or metastatic locations. In addition, the cold ischemia time should be <1 hour, 10% neutral-buffered formalin should be used as the fixative, isopropyl alcohol should be used as the dehydrating agent, the volume of tissue to fixative ratio should be 1:10, and all the paraffin blocks should be archived in dry, pest-free conditions at room temperature. The experts suggested that the formalin used for fixation should be freshly prepared and its pH should be checked daily; moreover, the pH and date of formalin preparation should be mentioned on the containers. The experts highlighted the need to educate multidisciplinary teams on the optimization of tissue handling practices and emphasized that a pathologist should always check the tissue for adequate quality and quantity for biomarker testing. The existing routine clinical procedures for collecting and handling biospecimens adversely affect their quality. The expert recommendations for preanalytical quality control would ensure high-quality biospecimens for molecular analysis and precision medicine.

## Introduction

Biospecimens play a crucial role in the current era of precision medicine and translational research as they are a primary source of molecular information on a patient's disease in clinical practice.<sup>1</sup> These biospecimens are processed in multiple steps that begin with morphological diagnosis by immunohistochemistry to evaluate the histological type, followed by suitable molecular profiling of the tumor.<sup>2,3</sup> The importance of biospecimens for molecular diagnosis has been highlighted in several reports from different countries.<sup>4</sup> However, the success rate of molecular diagnoses based on biospecimens differs across institutions and depends on the institutes' acquisition and processing practices, in addition to the testing methodologies used.<sup>4</sup> Although single-gene assays are more commonly used in clinical practice, they provide limited information.<sup>5</sup> With the advent of multiple clinical biomarkers and the availability of potential targeted therapies, massive parallel sequencing platforms using next-generation sequencing (NGS) technology have been adopted by several laboratories in recent times. NGS assays are conducted with a more extensive deoxyribonucleic acid (DNA)/ribonucleic acid (RNA) input, where the optimal quality and quantity of the nucleic acid is extremely important, which is usually challenging to obtain when small tissues are not appropriately handled.<sup>4</sup> Amplicon-based NGS assays require minimal input material compared with hybrid capture technology-based assays.<sup>4,6</sup>

High-quality biospecimens are essential for personalized medicine.<sup>7</sup> To ensure the same, tissue samples should be collected and handled according to standard protocols that minimize chemical, mechanical, and thermal degradation and protect the molecular composition and consistency of the samples.<sup>8</sup> Several variables can affect the molecular integrity of the biospecimens, and result in potential errors

during the determination of molecular and physical characteristics of biospecimens.<sup>9,10</sup> The variables affecting the quality of the biospecimens can be divided into three general categories: "preanalytical factors," "analytical factors," and "postanalytical factors."<sup>11</sup> The preanalytical phase involves the handling of specimens by a multidisciplinary team and is based on several processes that might introduce errors at different levels and hamper the biomarker testing results.<sup>11</sup> As per a recent study, approximately 60 to 70% of laboratory-associated errors in tissue specimen handling occur in the preanalytical phase.<sup>1,12</sup> Therefore, the preanalytical phase plays a vital role in the evaluation of cellular pathology.<sup>11</sup> Some preanalytical variables that affect the quality of biospecimens are the acquisition of adequate samples and processing of samples, including specimen fixation, temperature, fixation time, pH of the fixative, postfixation processing, and methodology of testing.<sup>2,13</sup>

There are many challenges associated with tissue handling and processing in India, which include a lack of awareness among the surgical fraternity during the collection of samples, poor tissue fixation practices, and climatic conditions/changes that adversely affect the quality of specimens.<sup>11</sup> There are several published international and national guidelines for the preanalytical steps that ensure valid and reliable molecular testing.<sup>1,14-17</sup> However, the existing guidelines for biospecimen collection and handling are not applied consistently.<sup>1,18</sup> Adherence to guidelines is further impacted because many guidelines are patented, which limits their accessibility to all members of the biomedical fraternity, particularly in India.<sup>1</sup> Hence, due to diverse existing clinical practices for biospecimen handling and preparation, differences in results can occur between different laboratories, making molecular diagnosis challenging.<sup>19</sup> Moreover, as multiple stakeholders are involved in tissue handling, including interventional radiologists, surgeons,

pathologists, molecular scientists, technologists, hospital administration staff, and researchers, increasing awareness regarding biospecimen handling and associated guidelines are crucial.<sup>20</sup> Thus, there is an unmet need to frame expert recommendations that serve as a consistent and up-to-date reference for multidisciplinary teams for the optimization of tissue handling and processing for precision medicine. This white paper aims to highlight the challenges in the field of biospecimen handling and provide recommendations on the standardization of variables in the preanalytical phase of tissue processing for optimal biomarker testing.

## Methodology

A virtual advisory board meeting was conducted in August 2021 to develop recommendations for the optimization of tissue handling and processing for precision medicine. The panel comprised 19 experts including pathologists ( $n=10$ ), molecular biologists ( $n=2$ ), medical oncologists ( $n=4$ ), a surgical oncologist ( $n=1$ ), an interventional radiologist ( $n=1$ ), and a senior histology technician ( $n=1$ ) from 10 hospitals in India. The expert panel also included an accreditation officer for testing and calibration of laboratory procedures in India. The meeting was conducted in two sessions. In the first session, the experts acknowledged and recognized the challenges in the tissue journey from acquisition to storage (►Table 1). The experts discussed and provided recommendations to overcome those challenges in the second session.

To better align the discussion, the lead scientific coordinators independently developed relevant questions address-

**Table 1** Key challenges according to the expert panel for biomarker testing in India

1. Challenges in tissue acquisition and handling <ul style="list-style-type: none"> <li>• Adequate quantity of tissue to permit morphologic diagnosis and ancillary biomarker profiling</li> <li>• Lack of uniform and standardized protocols for tissue management and processing across laboratories (no benchmark in histopathology)</li> <li>• Assays and platforms to assimilate data from the same pathology specimen</li> </ul>
2. Excessive reliability on cytology specimens in the diagnostic workup
3. Lack of multidisciplinary approach: Local practices and ancillary analyses affect the tissue journey
4. Quality- and quantity-related issues of the tissue for biomarker testing
5. Lack of facilities for multiplex/next-generation sequencing testing
6. High turnaround time
7. Testing in the referral laboratories
8. Cost
9. Challenges with rebiopsy
10. Lack of training facilities for molecular diagnostics
11. Lack of appropriate archival facilities

ing important issues on the subject, such as practical issues with biomarker testing and different steps involved in the preanalytic phase of tissue specimen preparation, for the panel to address. The questionnaires are summarized in ►Table 2. During the advisory board meeting, each question was discussed and edited by the entire group through rounds of discussion and drafts until a complete set of recommendations was obtained. Based on the available literature and experts' clinical experience, recommendations for optimizing tissue handling were proposed. The workflow of the expert recommendation development is presented as an illustration in ►Fig. 1.

## Results

The preanalytical handling of the biospecimen from acquisition to storage is based on several successive procedures, such as tissue collection, fixation, processing, embedding, and storage. Additional factors affecting the integrity and molecular structure of the tumor include the quantity of the tumor within the collected tissues, tissue quality, and the characteristics of the genomic material depending on the test and diagnostic modality used.

The following are the practice recommendations based on evidence from current literature and the experts' clinical experience to control preanalytical determinants and variables.

### Clinical Requirements of Biomarker Testing/NGS Testing

Present diagnostic and prognostic classifications, based on clinical and pathologic factors, are insufficient to accurately characterize tumors due to their clinical heterogeneity.<sup>21</sup> This is more relevant in cases of metastatic cancers wherein multiple lines of standard chemotherapy have been utilized and/or have failed. Testing such metastatic tissues for targets has become imperative, given the availability of various approved biomolecules.<sup>2,22,23</sup> Therefore, NGS has emerged as a technique that can be used to screen and diagnose both germline (inherited) and somatic (acquired) genomic mutations and is mainly used for genomic and transcriptomic analyses.<sup>2</sup> The novel and rare somatic mutations can accurately be detected by NGS technology (►Box 1).<sup>24</sup>

### Tissue Acquisition

#### Issues Impacting the Tissue Journey

A key barrier to implementing biomarker testing is ensuring adequate tissue acquisition to provide sufficient material not only to permit morphologic diagnosis but also downstream ancillary biomarker profiling.<sup>2</sup> Obtaining an adequate tumor sample can be clinically challenging due to an inaccessible tumor location or late presentation with metastatic disease at diagnosis.<sup>2</sup>

The quality of one-third of core biopsy specimens was not found to be appropriate for novel immune biomarker discovery, NGS, and histopathologic testing.<sup>25</sup> It is now imperative that, whenever possible, the aim of any diagnostic

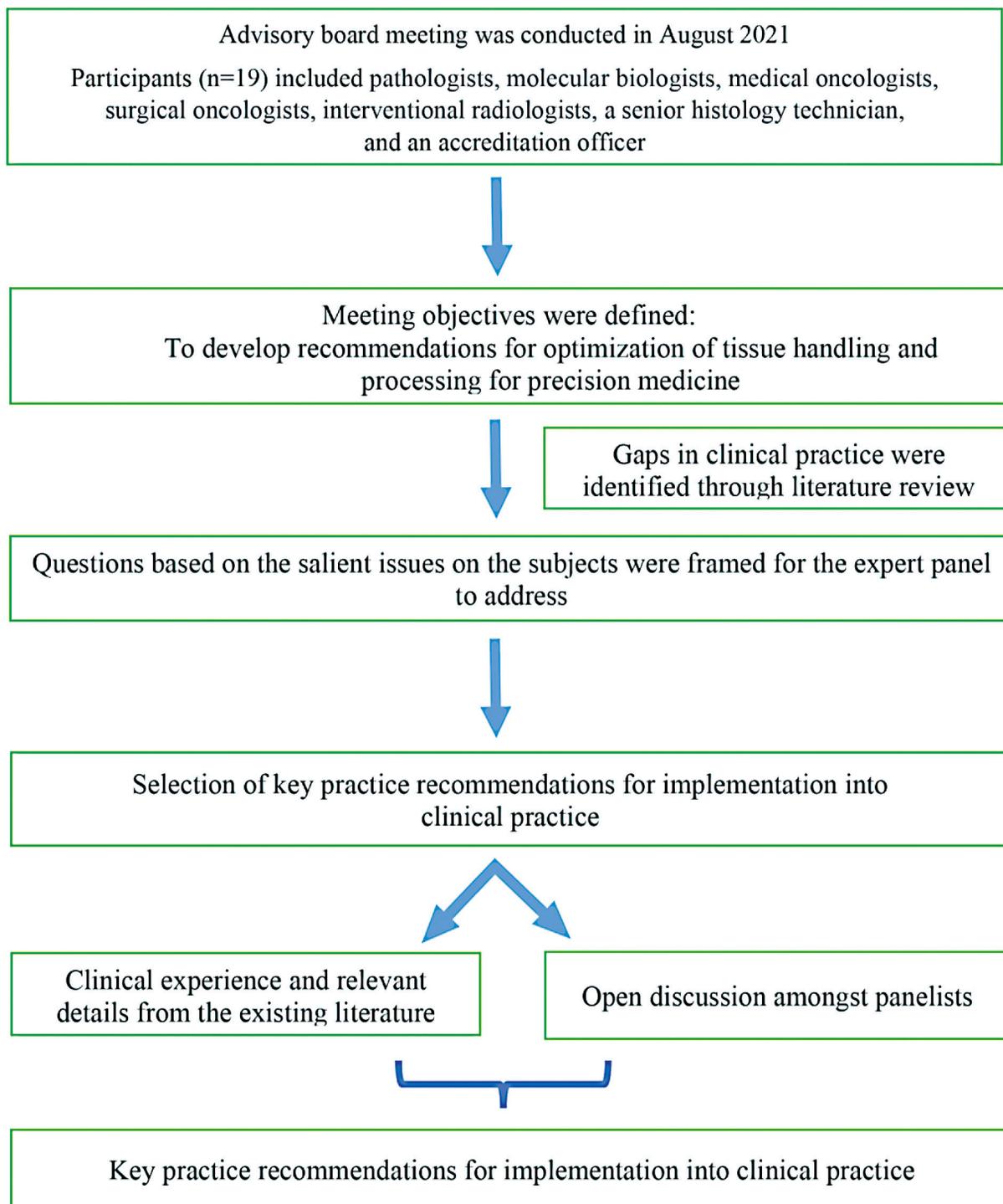
**Table 2** Questions provided to the expert panel

<b>A: Clinical requirements (questions specific to a medical oncologist)</b>
1. Who are the candidates for biomarker testing?
2. How often do they need biomarker testing in routine oncology practice?
3. What are the common site-specific molecular tests performed?
4. What type of tissue sample should be sent for molecular testing? Fresh tissue/RNALater/snap-freeze on or only paraffin blocks
5. In what type of patients do you suggest high-end genomic tests such as NGS in the course of their disease? Are the medical oncology community and colleagues aware of these tests? Do you counsel patients at the beginning of treatment regarding these issues?
6. What is the preferred testing modality while referring your patients for oncology biomarker testing? Germline or somatic?
7. What are the key challenges from the clinician's perspective while requesting these tests?
8. What is the reliability of small gene NGS panels?
<b>B: Tissue acquisition (questions specific to an intervention radiologist/surgeon)</b>
1. How easy/difficult is it to approach deep-seated tissues?
2. How do you assess the quality of the obtained tissue? How often do you take multiple passes? Do you take help from any surgeon? Any advice to be given at the outset to the clinician with regard to obtaining tissues?
3. Should touch imprint (ROSE) be practiced in evaluating tissue yield? How feasible is it to practice it commonly?
4. What is the ideal cold ischemia time to be followed with regard to small and big specimens? Is there any constant interaction on this among pathologists and surgeons? What is the experience of its impact on breast cancer specimens and predictive biomarker expression?
<b>C: Tissue processing and handling (questions specific to a pathologist and laboratory personnel)</b>
1. What are the critical factors for consideration for fixation—a type of fixative or time to fixation? a. Which is better—immediate fixing in the operating room or the frozen section room/pathology department? b. What is the size of the tissue bit to be processed? c. Shelf life: How frequently should the fixative be replaced? d. What should be the ratio of the fixative volume to tissue volume? e. Do you check these with your technical colleagues?
2. What is the optimum tissue processing protocol to process FFPE tissues?
3. How can the step of paraffin infiltration be standardized? What is the recommended melting temperature of paraffin used? What is the quality of paraffin, or any other parameters related to it?
4. How do you segregate a tissue block for molecular testing, especially in the case of lung cancer or GI malignancies if you have multiple bits?
5. Is there a dilemma on how much IHC testing is to be done for fear of losing out on tissue for molecular testing?
6. Tissue block selection: Which block is to be selected? What about tissue tumor content? When do you want to do the test? Do you want this test to be done on treatment naïve vs. post-NACT biopsy samples and whether to use the primary vs. metastatic site tissue? Are some guidelines available, or is it based on your practice?
7. How to ensure adequate storage conditions so that the RNA and DNA retain their integrity in FFPE tissue?
8. What is the best procedure to get maximum yield and accurate results in NGS?
9. Comment on various preanalytical and analytical aspects of NGS: The platform to be looked at, the reagent that is specific for the platform or usage of in-house reagents, the quality control EQA, troubleshooting, repeats, when to call inadequate/equivocal/when to give up on this test
10. How often do you face issues with the yield of DNA/RNA during the genomic test?

Abbreviations: DNA, deoxyribonucleic acid; EQA, external quality assessment; FFPE, formalin-fixed paraffin-embedded; GI, gastrointestinal; IHC, immunohistochemistry; NGS, next-generation sequencing; NACT, neoadjuvant chemotherapy; RNA, ribonucleic acid; ROSE, rapid on-site evaluation.

biopsy/tissue sampling procedure performed should be to maximize the amount of tissue acquired without compromising patient safety.<sup>26</sup> The quality of the biospecimens depends on the expertise of the surgeons and interventional radiologists in tissue collection. The best approach for individual patients can be determined by involving a

multidisciplinary team comprising pathologists, radiologists, surgeons, and oncologists.<sup>26</sup> Usually, patient safety, accessibility of the site, and probable tissue yield determine the choice of the site to be sampled.<sup>2,20,26</sup> This will reduce the number of repeat biopsies and prevent unnecessary delays in treatment.<sup>2</sup> Surgeons and interventional radiologists need to



**Fig. 1** Flowchart representation of expert recommendation development.

be trained to obtain adequate samples, especially from deep-seated tumors. A minimum of three tumor cores of different viable areas, avoiding necrotic regions of the tumor, should be obtained.<sup>27</sup> Rebiopsy for molecular workup may be required if diagnostic samples are inadequate for mutation analysis or the patient has disease progression (**– Box 2**).<sup>28</sup>

#### **Tissue Handling: Cold Ischemia Time**

Ischemia time is defined as the duration between specimen removal and proper fixation. Ischemia time is important as this allows activation of tissue enzymes, protein, and nucleic acid degradation, especially RNA, and autolysis. Ischemia time includes warm ischemia time and cold ischemia time.<sup>29</sup>

**Box 1** Expert opinion on clinical requirements of biomarker testing/NGS testing

- Genetic testing can be performed to identify any actionable mutation for solid tumors whenever all the lines of standard therapy have been exhausted.
- A minimal panel of biomarkers should be performed depending on the tumor type and site (e.g., *EGFR*, *ALK*, *ROS1*, *BRAF*, and *PD-L1* for lung cancer).
- Multiplex testing should be preferred over single-gene assays whenever feasible, especially in cases showing progression on initial therapy (through repeat biopsies).
- NGS should be utilized for tumors when multiple genes need to be tested in cancers such as ovarian cancer, lung cancer, uncommon tumors such as tumors of the salivary gland, thyroid, colon, and rare tumor neurotrophic tyrosine receptor kinase fusion cancer; thus, the judicious use of tissues as well as reduced turnaround time is essential.
- Liquid biopsy can be considered for molecular testing when tissue diagnosis is not feasible.
- Germline testing should be done depending on the clinical requirement, considering their utility pertaining to the specific tumor type.
- Appropriate consent and genetic counseling should be done before and after germline testing as they have implications for the patient and family members.
- Small gene hotspots are of practical utility, cost-effective, and reliable. At the same time, the issues of interlaboratory quality assurance need to be addressed.

Abbreviations: ALK, anaplastic lymphoma kinase; BRAF, B-Raf proto-oncogene serine/threonine kinase; EGFR, epidermal growth factor receptor; NGS, next-generation sequencing; PD-L1, programmed death ligand 1; ROS1, receptor tyrosine kinase 1.

**Box 2** Expert opinion on tissue acquisition

- Tissue sampling can be done from easily accessible sites irrespective of primary and metastatic sites.
- Choose less invasive technology or modality for the patient's diagnostic procedures. The collected tissue should be sufficient for both diagnosis and molecular testing.
- Image-guided biopsy is recommended for sampling small or extremely small tumors and deep-seated tumors, preferably by expert interventional radiologists trained in such procedures.
- A minimum of three, 1–2 cm biopsy cores per tumor, and sampling from different areas of the same tumor are recommended. A single-core biopsy is not recommended for deep-seated tumors.
- Rapid on-site evaluation may be used for adequacy assessment, wherever feasible.
- Cell blocks should be prepared for cytology specimens whenever possible, which aid in molecular testing.

Warm ischemia time is the interval between vessel ligation and specimen removal, whereas cold ischemia time starts from specimen removal to fixation.<sup>1,29,30</sup> Warm ischemia time can vary significantly, from a few minutes to several hours, and depends on the complexity of the surgical procedure, the expertise of the surgeon, the organ in question, and the modality of intervention. Cold ischemia time, which can take a few minutes or several hours, depends on the type of tissue, tumor size, collection method, surgeon, nursing staff, and procedures followed in various institutions.<sup>19,29</sup> Previous reports suggest that the changes occur in the RNA and protein of the tissue during this interval.<sup>29,31</sup>

Recent literature suggests that a cold ischemia time of 1 hour can minimize the rate of biomolecular degradation (– **Box 3**).<sup>1</sup>

**Specimen Type for Biomarker Testing**

Both tissue biopsy (fresh and formalin-fixed, paraffin-embedded [FFPE]) and cytology specimens can be used for diagnosis and molecular testing.<sup>2</sup> Biospecimens should be preserved in a way that they will remain reliable for both expected and unforeseen uses.<sup>9</sup> The removed tissue specimen can be used in three ways for molecular evaluation: by freezing the tissue, keeping it fresh, or stabilizing the tissue in a fixative.<sup>32</sup> Fresh frozen specimens (FFSs) (–80 to –190°C) and FFPE specimens

**Box 3** Expert opinion on cold ischemia time

- Tissues must be fixed immediately to avoid the rapid deterioration of DNA, RNA, and proteins.
- Cold ischemia time should be as minimal as possible and not exceed 1 hour.
- Ensure the training of nurses, trainees, and technologists on proper and prompt fixation of tissues and maintaining cold ischemia time below 1 hour.
- Maintaining a record of the time of administration of anesthesia and removal of the specimen may be submitted to the pathology department as a part of the details of the specimen.
- Timing of the beginning of fixation should be recorded.

**Box 4** Expert opinion on the type of biospecimens

- Formalin-fixed paraffin-embedded specimens are the most preferred specimens for biomarker testing and tissue storage as it increases the shelf life of the tissues to several years.
- Separate standardization and validation need to be done for cytology specimens.
- Cell blocks should be prepared whenever feasible.

may be used as sample archival methods after surgical removal of biopsy for the preservation of nucleic acids and proteins.<sup>2,33</sup> FFS is considered the gold standard for molecular analysis as it provides high-quality nucleic acid yield and facilitates superior preservation.<sup>34</sup> However, FFS disrupts the features of the tissues morphologically and hinders the assessment of tumor fraction; it requires highly controlled conditions and expensive infrastructure for proper handling and storing of the frozen samples.<sup>33,35</sup> FFPE tissues are easy to handle logistically as many biomarker assays are now standardized using these samples; however, it can cause adulteration in molecular tests, resulting in false-positive or false-negative results.<sup>27,36</sup>

Cytology samples (fine-needle aspiration cytology, fluid samples, direct stained smears, or liquid-based preparations) can be used reliably for molecular testing,<sup>26,37</sup> provided upfront planning is done at the time of sample acquisition. The protocol for the testing needs to be separately validated for the cytology specimens. Cell blocks should be prepared for cytology specimens whenever feasible, as the same protocols can be used for histology (► **Box 4**).<sup>26</sup>

### Tissue Processing and Handling

#### Tissue Fixation: Tissue Thickness, Time, and Volume of Fixative

Fixation is the preservation of cells, tissue structures, and their chemical constituents through chemicals.<sup>19,29</sup> This process involves submerging the tissue into a fluid called the fixative.<sup>29</sup> Fixation prevents decay or autolysis of cells and preserves tissue morphology. Poor fixation can lead to the generation of inappropriate and false-positive results that might preclude optimal diagnosis and further treatment.<sup>27,38</sup> Most biopsy specimens are small and are fixed quickly when placed in 10% neutral-buffered formalin (NBF) of pH 6.8 to 7.4 at 25°C; however, larger surgical specimens necessitate controlled fixation for a longer duration.<sup>39,40</sup>

The fixation process is predominantly dependent on three components: tissue thickness, time, and volume of fixative.<sup>19</sup> Inadequate compliance to any of these components will result in either under-fixation or over-fixation of the tissue.<sup>19</sup> Both over-fixation and under-fixation can adversely affect the molecular profile of the tumor. The rate of penetration of formalin in the tissue is variable depending on the type of tissue but is approximately 1 mm/h<sup>19</sup>; hence, a minimum dedicated time for fixation should be given. The infiltration of the fixative solution in the tissues occurs at varying speeds as it depends on the thickness of the sample. If the thickness is increased twice, the time allowed for the penetration of the fixative solution should be increased by four times.<sup>41</sup>

The duration for which the tissue is immersed in the fixative plays a crucial role in tissue processing in surgical pathology. The fixation time may vary depending on the size of surgical specimens.<sup>35</sup> The standard time duration required for the complete fixation process varies from a minimum of 6 hours for needle core and endoscopic biopsy specimens to more than 12 hours for sections derived from larger specimens.<sup>19</sup> The ratio between the volume of the tissue to the volume of fixative can vary from 1:10 to 1:20; generally, 1:10 is accepted as an optimum ratio for good fixation.<sup>41</sup>

The temperature and pH of the fixative can also impact the DNA yield. Fixation at room temperature triggers higher DNA degradation than at lower temperatures.<sup>2</sup> Therefore, it is recommended to initiate an immediate fixation at a low temperature of 4°C.<sup>42</sup> This may, however, take more time than fixation at room temperature and might decrease the staining intensity of the specimen.<sup>2</sup> A tissue fixed in formalin at low pH can cause extensive damage to DNA compared with tissue fixed at a neutral pH (► **Box 5**).<sup>2</sup>

#### Tissue Processing from Fixative to Paraffin

The tissue processing protocols must be standardized, which largely depends on the thickness of the tissue.<sup>43</sup> Suboptimal processing of tissues can adversely affect the recovery of biomolecules from the tissue specimen. The tissue processing after fixation is crucial for maintaining the quality of the biospecimen.<sup>19</sup> For fixed tissues, the biospecimens are processed across several steps, including sequential dehydration with ethanol, successive replacement by xylene in a process called clearing, and the process of replacement of xylene with paraffin, which is known as impregnation.<sup>19</sup> The quality of reagents, time, and temperature affect tissue quality. The time taken for this whole process may vary from 4 to 12 hours. Freshly prepared and high-quality reagents should be used in the process.<sup>19</sup> A delay in tissue processing could lead to incomplete dehydration of the tissue because of diluted alcohols and xylenes that are carried over from previous steps. Complete dehydration during processing is crucial, as the remaining water will not be replaced by paraffin and may cause tissue degradation. Inadequate dehydration of tissue is generally associated with improper fixation. Poor fixation leads to incomplete coagulation of proteins, which leads to the trapping of water within the tissue (► **Box 6**).<sup>19</sup>

#### The Impact of Paraffin on Fixed Tissues

Tissue fixation is followed by infiltrating the tissue through an embedding medium, usually wax. After infiltration, the tissue is embedded into a mold with the same medium to

**Box 5** Expert opinion on tissue fixation

- Fixative: 10% neutral-buffered formalin at a pH of 6.8–7.4 is the recommended fixative of choice.
- The date of preparation of formalin should be recorded.
- Freshly prepared formalin is always preferred, and it is recommended to mention the expiry date on the containers used for biopsy sample collection (within 5 days of preparation) (► **Supplementary Fig. S1**, available in the online version).
- Fixation time should be recorded and controlled.
- Recommended time in formalin: At least 6 hours (for small biopsies) and 12–18 hours (for large specimens). Fixation beyond 24–36 hours should be discouraged. Tissue with high-fat content may require 48 hours.
- Particular care should be taken regarding fixation timing for procedures conducted before a weekend or public holiday, as over-fixation can impact the molecular testing results.
- Special care should be taken for transportation time if the sample is to be transported to the referral laboratory (especially for samples collected on the weekend) for further testing as the likelihood of over-fixation increases.
- The pH of formalin should be mandatorily checked daily, and if it is less than 6.8, it should be discarded.
- Formalin fixation of tissue specimens at 4°C leads to better nucleic acid preservation (but this may be difficult to implement in clinical practice). If fixation is done at 4°C, in-house standardization and validation are recommended not only for routine histopathology and immunohistochemistry evaluation but also for molecular diagnostic assays.
- Acidic or heavy metal fixatives or decalcified specimens should be avoided for biomarker testing.
- Specimen dimensions: Tissue section thickness should not be more than 4 mm (cassette size 3.5 × 2.5 × 0.5 cm).
- Volume to mass ratio: Volume to mass ratio of 4:1 at a minimum, preferably 10:1, with tissue completely submerged.

**Box 6** Expert opinion on tissue processing

- Optimum processing time for core biopsies should be not less than 3 hours for smaller tissues and 8–16 hours for large tissues.
- Preferred dehydrating agent: Isopropyl alcohol.
- Standardized, automated processing protocols are preferred over manual tissue processing.
- Details of tissue processing conditions should be recorded.
- The solutions in the automated tissue processors must be changed periodically as per the manufacturer's recommendation and the record of the same must be kept in the laboratory.
- The tissue processing quality should be evaluated by checking the histomorphological features, in addition to optimal DNA/RNA yield.

form a block that is stored at room temperature.<sup>39,44</sup> Ideal characteristics for the embedding material are inertness, ability to repel moisture and readily penetrate the tissue, and reliability at room temperature.<sup>39</sup> Several types of paraffin wax can be used as the embedding material.<sup>39</sup> Different kinds of paraffin have different melting points and textures and are affected by the characteristics of the sections of the final blocks.<sup>19</sup> The use of high-melting point paraffin leads to inadequate deparaffinization, reducing the recovery of biomolecules from the tissue, and in the intensity and extent of immunostaining.<sup>1</sup>

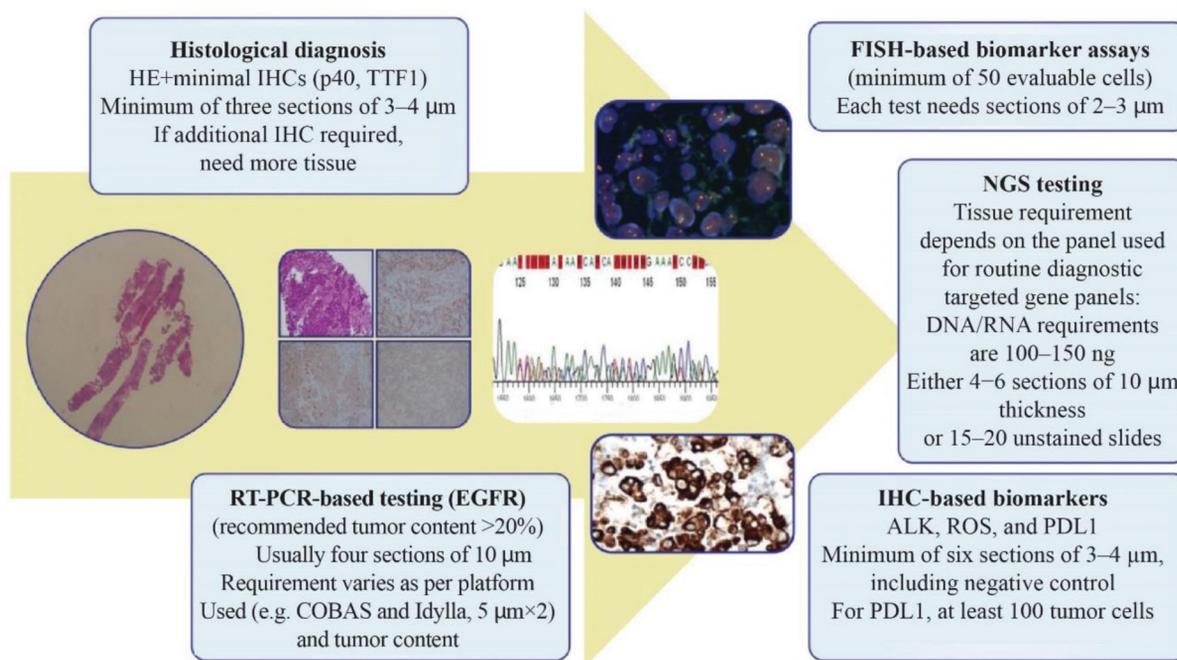
#### Tissue Quality and Quantity: How to Improve the Diagnostic Yield of Molecular Testing

It is pertinent to manage tissue specimens not only for diagnosis but also to maximize the availability of tissues for molecular studies. Hence, it is recommended that the histology technician follows the protocol for tissue preservation in the laboratory as well as that of reporting by the pathologist. The tumor fraction or purity of the specimen plays an important role in determining the success of diagnostic testing.<sup>2</sup> Areas of necrosis, hemorrhage, extracellular

mucin, and marked fibrosis may result in low tumor content despite the presence of adequate tissue volume in the biopsy sample. A viable tumor fraction is a vital factor to consider as a low tumor fraction can result in unknown reliability of molecular diagnostics, leading to false-negative results.<sup>2</sup> Different types of diagnostic testing need different quantities of the specimen. A prototype example of a lung is illustrated in ► **Fig. 2**. For instance, 50 evaluable tumor nuclei per section are required for fluorescence in situ hybridization analysis,<sup>45</sup> whereas genomic sequencing and mutational analysis need at least 10 to 20% of tumor content.<sup>2,20</sup> The ideal tumor fraction for NGS assays varies from >10 to 20% (► **Box 7**).<sup>2</sup>

#### Postanalytical Variables—Tissue Storage Conditions

There is a requirement for the proper storage of the block after processing the specimen. Research has revealed a reduced recovery of nucleic acids from older tissue blocks (FFPE) compared with recent FFPE tissues.<sup>19</sup> The reduction rate is 5 to 50% for each decade of age.<sup>19</sup> However, the cause of this reduction is ambiguous. It is assumed that the decreased recovery can be due to embedding media, quality



**Fig. 2** Illustration of the minimum tissue requirements for various molecular testing procedures using lung cancer as an example.

**Box 7** Expert opinion on biospecimen requirements for molecular testing

- Only a trained technician should handle the small biopsies.
- It is recommended not to club all the tissue cores into one paraffin block; at least two separate blocks should be made (one can be used for routine histopathological diagnosis, including IHC, and the other one for molecular workup (→ Fig. 3).
- It is recommended that highly purified paraffin wax (which melts at 58–60°C) is used for tissue embedding.
- Review all available pathology material together so that unnecessary repetition of the same test is not done.
- IHC should be performed only when deemed necessary; it should always be performed judiciously.
- Reflex testing is preferred over sequential testing, and blocks should be cut a minimal number of times.
- Tumor fraction should be maintained at more than 10% for DNA extraction.
- Microdissection and macrodissection can be performed to enrich the tumor content.
- Low cellularity tumors require more unstained sections for DNA extraction.

Abbreviation: IHC, immunohistochemistry.

of tissue processing, or characteristics of reagents used.<sup>19</sup> The long-term storage conditions determine the long-term stability of tissue-based biomarkers.<sup>46</sup> The biospecimens are stored for a longer duration in two ways: FFPE tissues at ambient temperatures and frozen tissue at ultra-low temperatures (from –80 to –190°C).<sup>33,47</sup> At ultra-low temperatures, nucleic acids with higher molecular weights and enzymatically active proteins in tissues can be preserved for several years. Still, RNA may be degraded.<sup>33</sup> The embedding of the tissue specimen with paraffin increases its shelf life. Freezing the tissues at ultra-low temperatures is more expensive than FFPE blocks due to high maintenance, more space requirements, and increased laboratory costs.<sup>33</sup> Usually, the FFPE blocks are widely stored by the pathology departments compared with frozen tissues. This storage of tissues for a longer duration facilitates the formation of an

extensive repository of tissue material and clinical details, thus, helping in translational clinical research (→ Box 8).<sup>33</sup>

## Discussion

Molecular analysis of the biospecimen is the keystone for diagnostic and therapeutic decisions in clinical practice. However, high-quality specimens are a prerequisite for accurate diagnosis. The procedures followed in the preanalytical phase of the biospecimen processing journey directly determine tissue quality.<sup>1</sup> The preanalytical phase includes collecting, processing, and storing the biospecimen until further molecular analysis.<sup>9</sup> The relevance of these preanalytical tissue factors is largely ignored in India.<sup>48</sup> This results in the wastage of resources,<sup>49</sup> particularly the expensive analytic components of molecular testing, yielding



**Fig. 3** Illustration of preparation of blocks for molecular testing (routinely employed in lung biopsies).

**Box 8** Expert opinion on postanalytical variables—long-term tissue storage conditions

- Storage conditions: Dry, pest-free conditions at room temperature (defined as 18–25°C).
- Paraffin blocks should be stored in a temperature-controlled environment, protected from excessive humidity, dryness, and light.
- Establishment of a tissue repository for the fresh tissues that can be stored for several years for research purposes.
- Follow the College of American Pathologists guidelines for preanalytical precision medicine and tissue repository establishment.
- The date of preparation of tissue block and slide, conditions of block storage, temperature, and humidity of storage area should be recorded.
- Block retrieval and back filing should have some defined protocol so that this block becomes accessible whenever required.

suboptimal quality of nucleic acids, and thus, affecting the downstream processing and results. The expert panel provided recommendations for the collection, processing, fixation, and block preparation of the tissue to ensure the high quality of biospecimens. However, an audit of practices and improvements is suggested.

According to the American Society of Clinical Oncology and College of American Pathologists, for cold ischemia time, the interval between the collection and fixation of the biospecimen should be 1 hour or less.<sup>1,50</sup> As per the latest research, cold ischemia time of less than 1 hour for breast cancer specimens facilitates the assessment of structural and functional parameters for prognosis and treatment.<sup>1,41,51</sup> The expert panel proposed that cold ischemia time should be 1 hour or less based on literature evidence. As per the experts, the date, time, and place of collection of the specimen should be mentioned in the requisition form and submitted to the pathology department along with the specimen. This record would also serve the purpose of auditing these processes and enabling improvement in case of inadequacies.

Clinicians, researchers, and pathologists should carefully plan and efficiently coordinate if a patient/specimen is considered for molecular profiling. This is done to ensure that tissues are handled optimally and carefully to preserve the integrity of

DNA, RNA, and proteins.<sup>2</sup> Initial handling includes transporting the specimen from the operating room to the pathology department and cutting the tissue into sufficiently small sections. Degradation of biomolecules can occur during this period, specifically proteins and RNA.<sup>2,29</sup> The method of transportation of surgical specimens may differ in hospitals according to the physical location of surgical rooms and pathology laboratories. Ideally, the biospecimen should be immediately fixed in the procedure room or transported as early as possible to the pathology department. The experts highlighted the importance of educating and training nurses, new trainees, and technologists on the proper fixation of tissues and keeping the cold ischemia time below 1 hour.

Fixation is the most critical step in tissue handling because this process is irreversible, and if fixation is poorly performed, it is impossible to recover the tissue. Generally, immunohistochemical staining is optimal when tissue specimens are fixed in 10 to 15% NBF.<sup>52</sup> Moreover, tissues should be fixed for 6 to 24 hours at ambient temperature to ensure optimal immunostaining, as per reports. Studies also suggest that fixation for more than 72 hours is counterproductive.<sup>53,54</sup> Under-fixation can cause nucleic acids and protein degradation or might change gene expression within tissue regions that have not been permeated by the fixative solution. Over-fixation can lead

to fragmentation of the DNA and extensive cross-linking, which challenges the extraction of usable nucleic acids and proteins.<sup>2</sup>

As per the experts, many laboratories in India presently use Bouin's fixative. The choice of fixative is critical. The experts approved the use of 10% NBF phosphate-buffered formalin at a pH of 7.0 as a fixative. They emphasized that the fixation time should not be less than 6 hours and not more than 24 to 36 hours.

The experts suggested that the formalin used for fixation should be freshly prepared and should be used within 5 to 7 days. The experts also recommended that while preparing formalin in the laboratory, standard procedures should be followed to ascertain the accurate concentration and pH of the solution. The pH of the commercially prepared formalin should be checked regularly, and it should be mentioned on the containers. Freshly prepared formalin should be available in operating room and endoscopy and radiology units. The stock solutions of formalin should be stored in sealed containers to prevent the conversion of formalin to formic acid. The volume of tissue to fixative ratio is recommended to be 1:10.

Regarding tissue storage and processing, experts agreed that FFPE is the most preferred method for tissue storage as it increases the shelf life of the tissues to several years. During tissue processing, the biospecimen is dehydrated and cleared. The experts recommended optimum dehydration time for core biopsies to be 3 hours and 8 to 10 hours for large tissues. The experts approved isopropyl alcohol as the dehydrating agent. The details such as time, temperature, presence of vacuum, equipment, and reagent used should be documented. The quality of the reagent should be monitored regularly. Studies comparing the conditions of processing the specimens and alternative reagents used in the tissue processing techniques are warranted.

The experts also highlighted the significance of the post-analytical phase of tissue processing and preservation. For tissue block preparation, the experts recommended the maintenance of paraffin blocks in dry, pest-free conditions at room temperature (25°C). Temperature and humidity should be monitored daily. The experts also suggested that housekeeping measures should be followed, and the storage area should be dust and pest free. Based on previous literature,<sup>55</sup> the acceptable thresholds for preanalytical factors for specific analytes were recommended by the experts (**►Supplementary Table S1**, available in the online version). In some instances, no relevant studies were available to provide evidence-based conclusions ("evidence not available"). However, for a few other factors, limited studies were available that were insufficient to draw definitive conclusions. The available evidence for those factors was constrained in terms of quality or relevance, which hindered a comprehensive examination of the investigated aspect ("evidence was insufficient") (**►Supplementary Table S1**, available in the online version).

The experts highlighted the significance of biorepositories in pathology. It serves as a reserve of high-quality tissues for biomarkers and provides information to the investigators about the content and characterization of the biospecimens

along with the patient information.<sup>56</sup> The experts suggested that a tissue repository must be established where tissues can be stored for several years and used for research purposes later. They emphasized that these repositories facilitate a better understanding of the disease and patients because they collectively provide genetic, clinical, and lifestyle-related information. Moreover, they concluded that the appropriate identification and validation of biomarkers could help in the advancement of personalized medicine through novel drug development and pharmacogenomic studies.

## Conclusion

The preanalytical handling of biospecimens is conducted in several steps, from surgical removal to paraffin embedding and storage of tissues. Each step is crucial and facilitates the preservation of morphological characteristics, antigens, and nucleic acids for molecular analysis. There are differences in specimen preparation practices that impact molecular quality and composition. The experts' recommendations for preanalytical quality control would ensure high-quality biospecimens for molecular analysis and precision medicine. These recommendations can enhance investigators' knowledge in clinics and pathology laboratories on optimal handling, collection, and storage of tissues for appropriate patient management and clinical trials.

### Patient Consent

Patient consent is not required.

### Authors' Contributions

All authors have contributed equally toward the conception, design, manuscript preparation, manuscript editing, review, and finalization of the manuscript.

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### Conflict of Interest

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