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Storage conditions and collection containers for various specimen types to preserve analyte stability: Review

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***Abstract*---**Background: Molecular tests for detecting nucleic acids in biological materials are crucial in clinical diagnosis and therapy decision-making. However, errors in molecular labs often stem from pre-analytical mistakes during specimen handling. The integrity of nucleic acids can be compromised by inappropriate collection, transit, and storage procedures. Despite the importance of pre-analytic factors, there is a lack of standardized recommendations for molecular assays. Aim of Work: This article aims to provide a comprehensive overview of key considerations in sample handling before molecular testing, covering various types of biological samples. Methods: We conducted a review of the literature to identify key pre-analytical factors that can impact molecular testing. We focused on specimen collection, transportation, and storage protocols for solid tissues, whole blood, serum, plasma, and other bodily fluids. We also discussed pre-analytical factors relevant to prenatal diagnostic samples. Additionally, we highlighted the importance of adhering to established procedures to minimize errors in molecular testing. Results: Our review identified several critical pre-analytical factors that can affect the quality of nucleic acids in biological samples. Factors such as inadequate sample collection, improper transportation conditions, and suboptimal storage can lead to degraded nucleic acids, affecting the accuracy of molecular tests. Following standardized protocols for sample handling is essential to ensure reliable results in molecular analysis. Conclusion: In conclusion, pre-analytical factors play a significant role in the success of molecular testing for nucleic acids. By understanding and

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addressing key considerations in sample handling, healthcare professionals can improve diagnostic accuracy and treatment decisions. Standardized protocols for specimen collection, transportation, and storage are crucial in minimizing errors and ensuring the reliability of molecular assays. Further research and consensus guidelines are needed to establish best practices for pre-analytic factors in molecular testing.

***Keywords*---**Biological fluids, Formaldehyde, Genetic material, Pre-analytical stage.

Introduction

Nucleic acid-based diagnostic assays are seeing fast expansion as they serve to supplement or even replace conventional diagnostic techniques in clinical labs. Additionally, they are used for prognostication and determining the optimal approach for treating several ailments. These tests need little quantities of DNA or RNA samples to be conducted and are very precise. The findings can be replicated, but like other diagnostic tests, they may have limitations due to analytic and pre-analytic variables. Several studies indicate that pre-analytical mistakes account for around 60-70% of all laboratory errors, both inside and outside of laboratory settings [1,2]. There are valuable suggestions for standardizing specimen processing in molecular labs, but there is a significant amount of variation in how specimens are handled before they are sent to these laboratories. This crucial stage of test performance has the potential to provide inaccurate outcomes, either in the form of false negatives or false positives. Implementing dependable protocols in sample processing will enhance the precision and consistency of these diagnostic tests. The primary concerns about sample handling are to the preservation of nucleic acid integrity and stability, as well as the potential impact of interfering agents during sample transit, extraction, and storage.

Recently, Compton et al. put out a set of guidelines specifically designed for tissue and blood specimens from cancer patients (Compton et al.) [3]. This article is a study from the Precision Medicine Project Team of the College of American Pathologists (CAP). The team has developed a set of evidence-based recommendations for pre-analysis procedures in tissue and blood specimens. Their review was restricted to primary data obtained from published recommendations by ASCO (American Society of Clinical Oncology), Biorepositories and Biospecimen Research Branch, CAP, European Committee for Standardization/Technical Committee, CLSI (Clinical & Laboratory Standards Institute), and other relevant guidelines. Their emphasis was only on pre-analytical parameters that affected the analysis data of nucleic acids utilized for cancer patients, namely in formalin-fixed, paraffin-embedded (FFPE) tissues or blood/plasma biospecimens.

Aim of Work

This article examines published papers and recommendations that discuss proper procedures for collecting, transporting, preserving, and storing various biological samples, such as tissue and blood. These procedures are commonly followed in pathology and translational research laboratories. Aside from the examination of cancer biospecimens, this study also included a comprehensive analysis of prevalent molecular assays used for the detection of various viral and bacterial pathogens. The majority of literature regarding pre-analytical research in human samples was gathered from the previous 20-year period. In order to choose papers for citation, we took into account both the citation rate of the article and the ranking of the journal in which it was published.

Form for Selecting and Requesting Tests

Multiple medical professionals often request molecular testing to accurately diagnose, choose an appropriate therapeutic approach, or determine the prognosis classification of patients. Clinicians who request a molecular test should be cognizant of the limits of the test's efficacy in guiding management and decision-making, as well as its cost-effectiveness. There are still several disorders for which some regular non-molecular diagnostics are more reliable than the currently available molecular testing. The doctors must ensure that the request has been determined adequately with a rational and cost-efficient step-by-step approach in patient care. Conducting essential but unsolicited molecular studies after to initial diagnosis by pathologists is a recognized approach in molecular labs [4]. Characterizing gene expression assays accurately and performing these tests by an in-house pathologist or laboratory medicine experts (reflex testing) is crucial for saving time in targeted treatment [5-12]. This guideline is particularly advisable when the proportion of samples with a specific diagnosis requiring further testing exceeds 10%. Some frequent examples of genetic abnormalities in different types of cancer are HER2 amplification in breast cancer, EGFR mutations and ALK rearrangements in lung cancer, BRAF mutations in malignant melanoma, RAS mutations in colorectal cancer, and BCR/ABL1 in chronic myelogenous leukemia [4].

Best Practices for Pre-analytical Phase in Various Biological Samples

Tissue specimens

Fixation is an essential process for preserving tissues in the long term, preventing self-destruction and the deterioration of molecular components. Fixed tissue specimens kept in paraffin blocks in pathology departments are often used as useful reservoirs of nucleic acids for molecular tests. Hence, it is crucial to examine pre-analytic factors in order to prevent any potential misunderstanding of the findings.

Categories of Fixatives

Commonly used fixatives include neutral buffered formalin (NBF), ethanol, methanol, Bouin, Carnoy, Zenker, and glutaraldehyde [13-15]. We provide a

concise overview of the impacts of various fixatives on nucleic acids. Formalin fixation causes the formation of cross-links between DNA and proteins, as well as between RNA and proteins. These cross-links might hinder the effective extraction of nucleic acids and can interfere with in situ hybridization. Nucleic acid fragmentation and loss might result from extended fixation of the material or changes in the fixative's pH. Fixation in un-buffered formalin causes a substantial reduction in the amount of extracted DNA compared to buffered formalin, resulting in less efficient DNA detection [16].

Formalin fixation may cause unpredictable damage to nucleic acids and result in erroneous mutations [17]. The postmortem interval, which is the time elapsed since death, and the cold ischemia time, which is the time between tissue removal from the body and preservation, should be restricted to 48 hours and 1 hour respectively when analyzing DNA using Fluorescence In Situ Hybridization (FISH). Similarly, for PCR analysis, these thresholds can be defined as less than 4 days and 24 hours. For maximum DNA integrity, it is recommended to fix the sample in formalin for fewer than 72 hours. However, PCR may still be performed successfully even if the fixation time is greater. This applies to both formalin fixation and the creation of formalin-fixed paraffin-embedded (FFPE) blocks [18]. Several authors have suggested that for optimal preservation of nucleic acids, it is advisable to begin formalin fixation within 2 hours of tissue removal, use cold fixation at 4°C with 10% neutral formalin, fix the tissue for a duration of 3 to 6 hours, add ethylene-diamine-tetra-acetic acid (EDTA) at a concentration of 20-50 mmol/L, and avoid exposure to low pH environments [12].

Formalin may chemically modify RNA, unlike fresh material. In an experimental investigation, the extracted RNA from tissues showed reverse transcription inhibition in quantitative RT-PCR experiments, particularly in samples with long length amplicons, while having satisfactory RNA integrity scores in FFPE samples. The PAXgene fixed samples did not exhibit this inhibition (19). For RNA analysis, the duration of fixation in NBF should not exceed 48 hours at 4°C or 8 hours at room temperature (uncontrolled RT). Additionally, the blocks should be examined within a maximum of 1 year. The research has generally neglected to consider the potential effects of many parameters, such as dehydration, clearing, paraffin reagents, and other circumstances, on DNA endpoints. To get more details, we suggest consulting a highly commendable study authored by Bass et al. [18]. Although there are several hurdles, genomic and gene expression data obtained from FFPE specimens often provide satisfactory outcomes when compared to the original fresh samples.

Ethanol and methanol are very effective fixatives for maintaining both high molecular weight DNA and RNA, while causing little chemical alterations to the nucleic acids [12]. Aside from its ability to preserve solid tissue, ethanol may also serve as a means of transporting and preserving tissue at room temperature (RT) [19,20]. Kilpatrick et al. proposed that tissues preserved in ethanol for a duration ranging from one day to two years may yield high molecular weight DNA with great efficiency. After a period of 6 months, significant DNA destruction was seen in tissues that were kept in 20% ethanol.

Carnoy's solution, consisting of 60% ethanol, 30% chloroform, and 10% glacial acetic acid, is considered one of the most effective fixatives for preserving nucleic acids, particularly high-molecular-weight RNA [12, 21]. Research has shown that RNA obtained from epithelial cells and preserved in Carnoy's solution retains the integrity of nucleic acids for the beta-actin gene, allowing amplification of up to 977 base pairs (bps) [22]. Carnoy's fixative demonstrates superior tissue preservation compared to methanol-acetone when evaluated after a post-fixation time of more than 3 months [22]. Li et al. performed DNA amplification on target DNA sequences extracted from cells that had been fixed in Carnoy's preservative and kept for varying periods of time, ranging from a few weeks to 6 years, at a temperature of -20°C. The researchers found no discernible variation in the amplification products' quality when comparing three different template sizes (400 bps for the retinoblastoma gene, 251 bps for the Duchenne muscular dystrophy gene, and 609 bps for the sex-determining region of the Y gene). This held true for both fresh tissue samples and Carnoy's-fixed cells [23].

The efficacy of extracting and retaining total RNA from tissue has been shown using a fixative solution called Methacarn. Methacarn is composed of 60% methanol, 30% chloroform, and 10% glacial acetic acid. RNA fragments ranging from 300 to 700 base pairs in length may be amplified from methacarn-fixed tissues [24].

Severe DNA degradation is found in Bouin's, B5, and Zenker's solutions due to the presence of heavy metals in these fixatives. Using a fixative containing mercury for nucleic acid separation is not recommended. Mercuric acid and chromium not only coagulate proteins, but also induce the development of large metal-nucleoprotein complexes, resulting in a decrease in the amount of DNA extracted [25-27]. Furthermore, Bouin's solution, which consists of acetic acid and formalin with a pH of 2, induces DNA de-purination and causes damage to both DNA and RNA [25, 28]. These ribonucleic acids develop resistance to ribonuclease enzymes, which may lead to their presence in DNA extraction products [29].

Glutaraldehyde is a commonly used fixative in the field of electron microscopy. Studies have shown that DNA with a high molecular weight (>50 kb) is better retained in tissues that have been fixed in a 0.2 M phosphate-buffered solution containing 1% glutaraldehyde (at pH 7.0) compared to tissues fixed in a 10% buffered formalin solution [13]. There is a lack of data on RNAs that have been preserved with glutaraldehyde.

Consequences of Decalcification

Decalcification agents often utilized, such as nitric acid and hydrochloric acid, include powerful acids that destroy nucleic acids and reduce their recovery rate from samples. The amount and purity of the extracted nucleic acids are satisfactory when decalcification is performed using either 14% EDTA or formic acid, as opposed to the previously described strong acids [30-32].

Paraffin

Paraffin waxes infiltrate the tissue in a liquid state, quickly solidify upon cooling, and maintain the structure of the tissue during sectioning [27]. There are many types of paraffin waxes with varying quality and melting temperatures, ranging from 47°C to 64°C. These variations may have a significant impact on the process of nucleic acid extraction, leading to potential confusing consequences. Paraffin, when exposed to a temperature of 60°C, has the potential to induce DNA damage and protein cross-linking [33-35]. Paraffin removal is essential for obtaining pure DNA since any remaining paraffin might hinder PCR, as shown in [36, 37]. Goelz et al. first reported the inhibitory impact of remaining paraffin on the amount of DNA obtained [38]. Chung et al. showed that thorough removal of paraffin is an essential need for extracting nucleic acids from specimens that are embedded in paraffin. The formalin fixation and paraffin embedding resulted in a 70% decrease in RNA production and a reduction in amplicon length to fewer than 300 bps, as shown in their demonstration [33]. However, a separate research demonstrated that reverse transcription-polymerase chain reaction (RT-PCR) successfully amplified all (100%) RNA fragments up to 151 base pairs in length for the housekeeping gene (glucose-6-phosphate dehydrogenase) in breast cancer samples that were preserved in paraffin. Nevertheless, it is important to acknowledge that advancements in nucleic acid extraction techniques have led to a reduction in the disruptive impact of paraffin [40].

While evaluating single nucleotide polymorphisms (SNPs) using real-time techniques, Huijsmans et al. suggested the use of extraction by silica membrane-based column and improved magnetic silica, despite the potential presence of amplification inhibitors in these approaches. The researchers discovered that the use of a silica membrane-based extraction technique, together with the addition of magnetic silica, could effectively amplify DNA fragments ranging from 400 to 600 base pairs [41]. RNA fragmentation may occur in paraffin-embedded tissue samples [42]. Páska et al. suggested that amplicons of up to 225 base pairs (bps) may provide satisfactory outcomes in gene expression investigations [42], but Godfrey et al. demonstrated that only RNA amplicons shorter than 130 bps could produce acceptable findings in reverse transcription polymerase chain reaction (RT-PCR) [43]. To enhance PCR efficiency, modifying some thermocycler settings, such as increasing the number of cycles and the length of each cycle, was beneficial [44, 45].

Microtomy

The ideal thickness of sections in FFPE tissue for nucleic acid assessment is contingent upon the size and cellularity of the tissues. Typically, it is advised to use 20-µm slices of big tissues or 40 to 80 µm thickness of smaller tissue for PCR analysis [46]. The recommended protocol suggests using three sections that are 10 micrometers thick for each response in kidney, prostate, stomach, colon, brain, bone marrow (BM), and lung samples. However, for breast tissue, it is necessary to use 10 sections with a similar thickness in order to carry out DNA extraction [47]. Based on our empirical observations, using five sections that are 10 micrometers thick may provide dependable outcomes in terms of both the quality and amount of nucleic acid (data that has not been published). Prior to

each block sectioning, it is advisable to clean the blade of microtomes by using 100% ethanol [46] and xylene or by employing a disposable blade [48]. To ensure the thorough cleaning of blades and prevent any contamination from previous blocks, it is recommended to cut at least two sections of a blank paraffin block without any tissue between each set of target blocks. The sections of these blanks should undergo DNA extraction procedures and be evaluated simultaneously with the samples of interest to guarantee the absence of any cross-contamination [46].

Tissue Conditions

While the composition of the tissue has a role, typically 1 to 2 grams of tissue would provide sufficient material for molecular analysis. A minimum of 2 grams of tissue is required for the analysis of low cellular specimens such as muscle, fibrous, and adipose tissue [46]. During surgical operations, extended use of anesthetic and ischemia techniques such artery ligation may result in tissue damage due to lack of oxygen and lowered pH levels. This can subsequently impact the expression of mRNA, leading to inaccurate quantification of different mRNAs [12, 49]. The age of a sample is a crucial component that must be taken into account when dealing with archival samples. According to Goelz et al., DNA fragment sizes obtained from samples maintained for four to six years were often lower compared to the DNA sizes of samples held for less than two years [38]. However, some labs successfully used 20-year-old FFPP blocks without encountering any issues [50, 51].

Watanabe et al. assessed the quantity, quality, and intactness of DNA in FFPE tissue samples. A research was done on twenty-five lung cancer FFPE samples that had been maintained at room temperature for 0.5, 3, 6, 9, and 12 years. The researchers used two DNA extraction techniques and observed that the duration of storage impacts the DNA's integrity, but not its overall yield and purity in FFPE samples, irrespective of the extraction method [52]. In a separate work, Wang et al. demonstrated effective amplification of DNA from 40-year-old FFPE tissues. However, the amount of DNA obtained was lower compared to fresh cells [53]. The target tissues for molecular analysis often consist of a heterogeneous combination of cells, not all of which are the primary focus of molecular tests. In order to prevent the weakening of nucleic acids from unwanted cells, it is essential to identify and designate the specific region that contains the desired cells on both the hematoxylin and eosin (H&E) slide and the associated paraffin block. One possible subsequent procedure may include performing shallow punching on the paraffin block or using laser micro-dissection on the slides. To summarize, while long-term storage of blocks has proven effective for DNA analysis, it is crucial to acknowledge that the length of amplifiable gene fragments may diminish with time. The literature has not thoroughly addressed the potential effects of many parameters, such as dehydration, clearing, paraffin reagents, and other circumstances, on DNA endpoints. These effects should be confirmed in future investigations.

Conclusion

It is recognized that putting into practice the suggestions provided here may need changes in processes, timetables, or supplies. However, it is vital to note that a

trustworthy documenting of the pre-analytical history of patients' specimens is a necessary need for its application. its documentation provides objective hints to identify false positive or negative results. Specimen pre-analytical variables in translational research facilities are neither regulated nor recorded. Furthermore, many labs do not regularly conduct essential quality control measures that are required for clinical purposes, which might potentially identify subpar specimen quality prior to testing. As a result, the results produced by these laboratories may be entirely incomprehensible or inconclusive. Hence, reaching a consensus on the precise factors related to patient specimen collection, handling, processing, storage, and transportation that lead to quality issues and molecular changes would guarantee that the research data is sufficiently reliable and reproducible. This, in turn, would facilitate the translation of findings from laboratory experiments to clinical applications.

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