

Diagnostic Implication and Clinical Relevance of Ancillary Techniques in Clinical Pathology Practice



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ABSTRACT: Hematoxylin–eosin-stained slide preparation is one of the most durable techniques in medicine history, which has remained unchanged since implemented. It allows an accurate microscopic diagnosis of the vast majority of tissue samples. In many circumstances, this technique cannot answer all the questions posed at the initial diagnostic level. The pathologist has always been looking for additional ancillary techniques to answer pending questions. In our daily histopathology practice, we referred to those techniques as *special* stains, but nowadays, they are more than stains and are collectively called ancillary tests. They include a wide range of techniques starting from histochemical stains and ending in one or more advanced techniques, such as immunohistochemistry, immunofluorescence, molecular studies, cytogenetic studies, electron microscopy, flow cytometry, and polymerase chain reaction.

KEYWORDS: ancillary technique of pathology, special stain, histochemical stain, molecular studies, IHC, immunofluorescent staining, FISH, cytogenetic studies, flow cytometry, PCR, single-cell suspension

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Introduction

The daily histopathology practice mainly depends on simple and low-cost procedure of fixation and chemical processing of tissue samples to obtain the ultimate hematoxylin–eosin (H&E)-stained slides, which give rapid, comprehensive, and informative scope of the scene. In the H&E technique, hematoxylin stains the nuclei while eosin counterstains the cytoplasm and various extracellular materials. It is one of the most durable techniques in medicine history, which has remained unchanged since implemented. Moreover, it allows an accurate microscopic diagnosis of the vast majority of tissue samples sent for evaluation. Meanwhile, in many cases, this technique cannot answer all the questions posed at the initial diagnostic level, and it is clearly insufficient when the pathologist is seeking for etiologic factors or a histogenetic and pathogenetic relationship. Therefore, the pathologist has always been looking for additional ancillary techniques to answer those questions. In our daily histopathology practice, we referred to those techniques as special stains, but nowadays, they are more than stains and are collectively called ancillary tests. In this review, the current techniques that are most popular in diagnostic pathology practice are discussed.

Histochemical Stains

Almost all histochemical stains are suitable for formalin-fixed tissues, and they belong to different families of chemical stains designed for microscopic visualization of different types

of human tissues, cells, body secretions, pigments, minerals, parasites, and microorganisms. They remain an important diagnostic tool for many pathologists providing powerful complementary information, which may be followed by one of other ancillary techniques such as immunohistochemistry (IHC), flow cytometry, in situ hybridization, and other diagnostic technologies. Most of the histopathology laboratories run the protocol of chemical stains manually, but nowadays, the protocols of chemical stains have become increasingly automated, thus resulting in higher levels of productivity and flexibility. In general, a histochemical stain consists of the main chemical reaction that demonstrates cellular or tissue element of interest followed by chemical reactions to stain the background. The histochemical stains were widely used prior to the advent of immunohistochemistry; nowadays, the pathologist will find a relatively minority of them to be of real diagnostic utility, while the majority are rendered obsolete. Table 1 lists the most widely used chemical stains in the daily clinical practice of histopathology and their clinical uses.

Immunohistochemistry

IHC is a method for demonstrating the presence and location of proteins, such as *antigen*, in the tissue sections. In other words, it is the application of basic immunologic principles and techniques to demonstrate molecules in cells and tissues. However, it is quantitatively less sensitive than immunoassays, such as Western blotting or enzyme-linked immunosorbent

**Table 1.** Commonly used chemical stains.

| | CHEMICAL STAIN | APPLICATION | CLINICAL USES |
|-----|---|---|--|
| 1. | (Ziehl-Neelsen Stain) | Detects nocardioform-actinomycete, <i>Mycobacterium</i> Spp | To settle the diagnosis of Tuberculosis |
| 2. | Giemsa Stain | Stains <i>H. pylori</i> , <i>Plasmodium vivax</i> , <i>Rickettsia</i> , <i>Trypanosoma cruzi</i> , <i>Giardia lamblia</i> | Demonstration of various hematolymphoid elements (including mast cells) and microorganisms |
| 3. | Oil Red O | stains lipids in frozen sections and some lipoproteins in paraffin sections | <i>Detect neutral lipids</i> |
| 5. | sliver for reticulin | identification of reticulin fibers in tissue samples | Traditionally it used to differentiate epithelial tumors from different types of sarcoma. Now a day it is limited to hepatic disorders |
| 6. | Verhoeff Stain Van Gieson Stain | Identification of elastic fibers, Verhoeven is specific for elastic fibers, while Van Gieson Stain is specific for collagen | To identify extracellular collagen |
| 7. | Periodic Acid-Schiff (PAS) Stain | Mucin, collagen fibers, fungal bodies | Detect fungi, parasite, basement membrane, intracytoplasmic crystal of many disorders and neoplasm |
| 8. | Mayer Mucicarmine Stain | Mucin, encapsulated yeast-like fungus <i>Cryptococcus</i> | Demonstrate intestinal metaplasia of esophagus and stomach |
| 9. | Grocott methenamin Silver (GMS) Stain | Useful in identifying a variety of pathogenic fungi | Identification of fungi |
| 10. | Masson Trichrome Stain | stains mature connective tissue | To grade hepatitis and cirrhosis |
| 11. | Congo Red | Amyloid substance | Diagnosis of amyloidosis |
| 12. | Masson-Fontana Stain | stains melanin pigment | To differentiate between various body's brown pigments |
| 13. | Perls' Prussian Blue Stain | stains Haemosiderin | Detect iron deposition |
| 14. | Phosphotungstic Acid-Hematoxylin (PTAH) | stains intracytoplasmic filaments | Detect reactive astrocytosis |

assay, but it enables the observation of processes in the context of intact tissue, typically applicable for assessing the progression and treatment of diseases, such as breast cancer. In general, the information gained from IHC combined together with the initial morphological information provided by H&E staining reveals that data cannot be obtained by using any other available ancillary methods. Immunohistochemical staining is carried out for treating the tissue or the cell, which contains the specific antigen with antibodies that recognize the target antigen. As antibodies are highly specific, the antibody will bind only to the protein of interest in the tissue section. The antibody-antigen interaction is then visualized using chromogenic detection, in which an enzyme conjugated to the secondary antibody cleaves a substrate, containing chromogen, to produce a colored precipitate at the location of the antigen and can be visualized using light microscopy.¹

There is probably no other method that has so revolutionized the scene of clinical histopathology during the past 50 years as the immunohistochemical technique.² The obvious advantages are remarkable sensitivity and specificity; applicability to routinely processed tissue, which is the formalin-fixed, paraffin-embedded tissue section (FFPETS), even if stored for long periods; and ultimately the easiness and precise correlation with morphological features. The immunohistochemical technique is compatible with formalin, the most

widely used fixative in routine clinical pathology and earlier in H&E-stained microscopic sections. It is sometimes positive even in entirely necrotic material.³ It can also be used in cytology to detect antigen molecules in different types of cytologic preparations. It has replaced many of the conventional histochemical stains and rendered many of the ancillary diagnostic techniques, such as electron microscopy (EM), obsolete. In order to prevent this technique to be misleading rather than helpful, the pathologist should realize that it presents potential pitfalls, and hence all the stains must be interpreted in the context of morphological features.⁴ Many immunohistochemical detection techniques are available in the daily pathology practice, the most commonly used at present being the immunoperoxidase technique.

By using the traditional light microscopy, tissue and cellular antigen molecules cannot be visualized unless they are labeled by chromogen to permit their visualization. Detection systems attach certain labels to primary or secondary antibodies in order to visualize the target antibody-antigen localization in the tissue sections. There are two methods to visualize the antigen localization.

Direct method. The direct method is a one-step staining method and involves a labeled antibody reacting directly with the antigen in the tissue sections. This technique utilizes only one antibody, and therefore, it is simple and rapid,



the sensitivity is less than the indirect method due to no or little signal amplification, and it is less commonly used than the indirect methods. The direct conjugate procedure has the advantages of rapidity and ease of performance, while the practical disadvantage of the direct method is that it usually demands the primary antibody to be used at a relatively high concentration and it cannot be used to detect multiple antigens in the same section.⁵

Indirect method. This is a two-step method; primary antibodies are raised against an antigen of interest and are typically unconjugated (unlabeled). Secondary antibodies are raised against immunoglobulins (Igs) of the primary antibody. The secondary antibody is usually conjugated to a linker molecule, such as biotin, or enzyme–chromogen complex. It is the commonly used method in routine pathology practice. To amplify the outcome signal, various techniques have been developed to add more enzymes or fluorophores to the target of interest, such as avidin–biotin complex, labeled streptavidin biotin, HRP polymer, and Tyramide signal enhancing.¹

IHC Double Staining

IHC double-staining technique is used to visualize two or more antigens in the same section and analyze the relationship of their localization, within the same cell or tissue section. Currently, this technique is widely used in clinical pathology practice especially in lymphoma and prostatic carcinoma. It is usually done by immunoperoxidase staining through sequential staining technique (see Sequential staining section); however, new polymer-based methods and polyvalent detection systems have made concurrent staining possible.⁵ There are two methods to carry out the double-staining technique:

Sequential staining. This method is used when both the primary antibodies are produced in the same species of animals. It is an indirect technique using unlabeled primary antibody, the first sequence of staining for the first antigen is completed with the development of the peroxidase reaction using 3,3'-Di-amino-benzidine (DAB) as the substrate, and this procedure yields a brown color signal. Then, staining for the second antigen is carried out with a primary antibody of different specificity and a second different substrate and chromogen system for the peroxidase or phosphatase enzymes; this method yields a blue or red color signal.

Simultaneous staining. In this staining, the indirect method is used when both the primary antibodies are raised in different host species and it is less time-consuming than the sequential method, as we can mix both the primary antibodies in one incubation and the secondary labeled antibodies.⁶

Clinical uses of IHC. The widespread application of IHC in clinical pathology has entirely transformed diagnostic surgical pathology “from something resembling an art into something more closely resembling a science.”⁷ It is a notably a cost-effective powerful tool to settle the final diagnosis and should be used judiciously. As a matter of fact, the daily clinical practice can no longer be afforded without IHC.

Nowadays, it is rare to issue an informative and conclusive histopathological report without the usage of IHC, especially for tumor cases.

An ongoing major drawback of IHC is the lack of objective quantitative measurement for target antigens under investigation, especially for proper use of the different prognostic markers of many cancers. In general, the major clinical uses of IHC in daily pathology practice are as follows:

1. IHC is used to identify many intercellular substances, such as renal Igs of glomerulonephritis and amyloid.
2. It is used as tumor markers for diagnosis, typing, and classification of tumors.
3. It is used to discriminate in situ lesion from the real invasion of malignant lesion, as in breast and prostatic cancers.
4. It is a prognostic factor in the majority of tumors by using KI-67.
5. It is a predictive factor to guide specific therapy (response to therapy), particularly in breast cancers.
6. It is used to identify infectious (causative) viral agents, chlamydia, and other bacteria.
7. It is widely used in molecular pathology to detect many chromosomal abnormalities or to localize a specific protein or gene on the chromosomes.
8. It is also widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue.

Immunofluorescence (IF)

This ancillary study is used to support fixed tissue diagnosis and to provide additional diagnostic and prognostic information, particularly in autoimmune diseases, vesiculobullous skin diseases, tissue and organ transplantation, and renal glomerular diseases. IF is a technique allowing the visualization of a specific protein or antigen in cells or tissue sections usually fresh tissue, but FFPETS also can be used by binding a specific antibody chemically conjugated with a fluorescent dye (fluorochrome), such as fluorescein isothiocyanate, and the antigen–antibody complex is visualized using fluorescent microscopy. Fluorochromes are dyes that absorb ultraviolet rays and emit visible light. This process is called fluorescence. Commonly used fluorochromes are acridine orange, rhodamine, lissamine, and calcofluor-white.⁸ There are two major types of IF staining methods:

Direct method. It is a one-step histochemical staining in which the primary antibody is labeled with fluorescence dye. This technique is used to detect antigen in clinical specimens using a specific fluorochrome-labeled antibody. It can be used to detect viral, parasitic, and tumor antigens from specimens or monolayer of cells of patients. Another application is the identification of anatomic distribution of an antigen within a tissue or within compartments of a cell, particularly in renal diseases and immunobullous disorders of the skin.⁸



Indirect method. This is a two-step histological staining in which the secondary antibody is labeled with fluorochrome. This technique is mainly used to detect circulating antibodies in the body fluid; meanwhile, it is a highly sensitive technique currently used to recognize a primary antibody in FFPETS. Indirect IF uses two antibodies: the unlabeled first (primary) antibody specifically binds the target molecule and the secondary antibody, which carries the fluorophores, recognizes the primary antibody and binds to it. Multiple secondary antibodies can bind a single primary antibody. This provides signal amplification by increasing the number of fluorophore molecules per antigen. This protocol is more complex and time-consuming than the abovementioned primary (or direct) protocol, but it allows more flexibility because various different secondary antibodies and detection techniques can be used for a given primary antibody.⁹

Different cytological preparation and tissue sections (FFPETS) can be stained with IF staining.¹⁰ Then, the antibody–antigen interaction is visualized using fluorescein detection, in which a fluorescein, which is conjugated to the secondary antibody to produce a colored precipitate at the location of the antigen–antibody reaction, can be visualized using fluorescence microscopy.

IF applications can label antigens in cell suspensions, cultured cells, tissues, beads, and microarrays for the detection of specific proteins. IF techniques can label both fresh and fixed samples.¹¹

IF double staining. It is the demonstration of two independent antigens in identical cells or other compartments in the same tissue section, which has currently become an essential technique in cell biology. There are two methods to carry out the double-staining technique:

Simultaneous method. This technique simultaneously detects two antigens in single sections, when the colocalization protocols typically use primary antibodies raised in two different species.

Sequential method. This method is applicable when both of the antibodies used to localize the antigens are raised in the same species.

Molecular Studies

There were tremendous advancements during the past three decades made in molecular biology, reflecting a major impact on the clinical practice of pathology. In fact, the latest molecular techniques have the potential to alter the diagnostic and therapeutic profiles of cancers in a way that no other techniques ever had before.⁴ Molecular techniques can be performed in tissues handled as part of the routine pathology practice, such as FFPETS and frozen sections. Pathologists have been practicing molecular morphology (the microscopic localization of protein, DNA, and RNA) since the late 1970s. Most of the molecular studies depend on hybridization techniques based on the application of recombinant DNA technology,¹² by using a synthetic probes of synthetic oligo(deoxyribo) nucleotides,

single-stranded cDNA, and single-stranded antisense RNA. The probes are labeled by nonradioactive labeling – particularly with biotin and fluorescein.

Fluorescence in Situ Hybridization

Fluorescence in situ hybridization (FISH) uses tagged probes that bind to chromosome-specific DNA sequences of interest, thereby allowing for the identification of both structural and numeric aberrations that specify certain hematopoietic and nonhematopoietic malignancies. FISH is usually performed on dividing (metaphase) cells as well as on nondividing (interphase) cells. It is superior to the cytogenetic study in that it can be used to diagnose many congenital anomalies, as listed in Table 2. It can be performed on air-dried cytological preparation, fresh tissue, frozen sections, and FFPETS, it can detect molecular abnormalities of many tumors, and it can facilitate the detection of genetic and chromosomal numeric abnormalities.¹³ The principal use of FISH in daily practice of pathology is to detect somatic changes associated with cancers, which have a known diagnostic, prognostic, or therapeutic implication.

Signal fading is one of the major limitations of FISH in practical use. However, most of the laboratories overcome this disadvantage by capturing digital images as a permanent record using chromogenic in situ hybridization (CISH), yielding permanent signals. Still, multicolor CISH is not as simple as multicolor FISH. Other disadvantages, such as artifacts in FFPETS, are common.¹³ The most common clinical diagnostic, therapy predictive, and prognostic applications of FISH testing are listed in Table 3.

Cytogenetic Study

Cytogenetic technique is a conventional karyotypic analysis; it is the identification and study of each chromosome to detect specific chromosomal abnormalities associated with hereditary diseases and tumors. It has been only 50 years since the correct number of human chromosomes was established.

Table 2. Clinical and diagnostic uses of FISH (modified¹³).

| CLINICAL USES OF FISH | |
|-----------------------|--|
| 1. | Prenatal testing |
| 2. | Transplant pathology |
| 3. | XY FISH on sex-mismatched organ transplant |
| 4. | Disease relapse using known genetic alterations in primary tumor |
| 5. | Oncology (diagnostic, prognostic, and/or predictive markers) |
| 6. | Chromosomal aneusomies |
| 7. | Gene/Locus deletions |
| 8. | Gene amplifications |
| 9. | Translocation |
| 10. | Congenital abnormalities, such as Trisomy 13, 18, 21, XY aneusomies, Microdeletion syndromes, Cri-du-chat (5p), Prader-Willi/Angelman (15q). |

**Table 3.** Common clinical application of FISH.

| CLINICAL APPLICATION | | |
|----------------------|--------------------|---|
| 1. | Diagnostic | <ul style="list-style-type: none"> – Translocations associated with many hematologic, soft tissue, and pediatric malignancies – UroVysion testing in urine cytology specimens – 1p/19q deletion testing in gliomas |
| 2. | Therapy predictive | <ul style="list-style-type: none"> – Her2 amplification in Breast cancers – t(11;18) in mucosa-associated lymphoid tissue lymphoma |
| 3. | Prognostic | <ul style="list-style-type: none"> – Her2 amplification in Breast cancers – del(13q) and/or t(4;14) in multiple myeloma |

Cytogenetic study is usually performed to settle the diagnosis of congenital disorders at pre- and postnatal time. Meanwhile, it is a powerful technique to study the tumors and provides clues to the molecular mechanisms involved in their pathogenesis.¹⁴ The main applications of cytogenetic study in tumor pathology are as follows:

1. Defining the subsets within putatively histologically homogeneous tumor types,
2. Suggesting connections between histologically diverse tumors,
3. Suggesting the site of the primary tumor when a specific cytogenetic change is found in a metastasis, and
4. Providing clues for tumor classification, causation, and presence of cancer-related genes.¹⁵

These applications depend on the fact that some morphologically and clinically distinct subtypes of lymphoma or leukemia and soft tissue tumors have specific cytogenetic alteration closely and sometimes uniquely associated with those neoplasms.¹³

Traditional cytogenetic analysis is limited for the detection of numerical chromosomal abnormalities and gross structural rearrangements. It is not sensitive to detect mutations such as small deletions and amplifications and single-base pair substitutions or any other abnormalities at the gene level. Handling and delivery of specimen is critical to avoid sample growing *in vitro*. The specimen required for this technique must be fresh sample. The most commonly used samples in clinical practice are peripheral blood, bone marrow aspirate, and solid tissue, where each sampling has its unique preservative and delivery conditions. The most noticeable limitations of this technique are high cost, time-consuming, and fresh specimen requirement.

Flow Cytometry

Flow cytometry is a complex field that draws people from diverse scientific backgrounds. It is a biophysical technology based on laser light beam, widely and routinely used nowadays in clinical hematology and research purposes. It allows simultaneous multiparametric analysis of the physical and chemical characteristics

of up to thousands of particles per second; one or more laser beams interrogate each particle and, at a minimum, the system measures the degree and direction of the scattered light – indicators of the particles' size, shape, and structure. If a particle is stained with one or more fluorescent dyes, known as fluorochromes, the light source excites these dyes to provide additional biological information about each particle.¹⁶

The unique power of flow cytometers is that they can rapidly and quantitatively measure multiple simultaneous parameters on individual live cells and then isolate the cells of interest. General uses of flow cytometry include measurement of cell size, cytoplasmic granularity, cell viability, cell cycle time (S-phase fraction), DNA content (DNA ploidy), detection of surface marker phenotype, enzyme content, cell counting, and cell sorting.⁴

The test sample should be a single-cell suspension with a maximum cell dimension of 50 microns, which is regarded as the major limitation of this device; in other words, only body fluid and blood can be used as samples. A number of protocols are available for disaggregating tissue samples from solid tumors into suitable single-cell suspensions. These protocols typically involve enzymatic digestion and mechanical chopping and filtering. Hedley et al.¹⁷ prescribed the preparation of single-cell suspension from FFPE for flow cytometric analysis, and the original method has been subjected to a wide range of technical modifications, either to simplify the procedure or to improve single-cell harvesting. A modified method to prepare single-cell suspension from FFPE was described by Makki et al.¹⁸ as follows:

- i. Two or more 50 µm sections are cut using a microtome, depending on the number of tumor cells in the tumor section.
- ii. Dewaxing – the sections are placed in 10-mL glass centrifuge tubes and dewaxed using two changes of xylene, 3 mL, for 10 minutes at room temperature.
- iii. Rehydration – the sections are rehydrated in a descending ethanol range, with a sequence of 3 mL of 100%, 95%, 70%, and 50% ethanol for 10 minutes each at room temperature.
- iv. Washing twice in distilled water.
- v. Lysis of tissue section – the sections are minced by sharp seizer and incubated in 1 mL of 0.5% pepsin (0.5 mg pepsin/1 mL of 0.9% NaCl, adjusted to pH 1.5 with 2 N HCl). The tubes must be placed in a water bath at 37 °C for 60 minutes, with an intermittent vortex mixing, each 10 minutes, mincing with a pipette tip.
- vi. Filtration – the resulting digest is filtered through a 70-mm nylon mesh.
- vii. Washing – the pepsin is washed out by centrifugation at 400 g two times for two minutes using Phosphate Buffered saline (PBS), and the pellet is resuspended in Tris-buffered saline, pH 7.6, or PBS.
- viii. Cells counting – Cell count is then made using a hemocytometer, provided that more than 10⁵/mL cells were present.¹⁸



Meanwhile, the development of flow cytometric techniques for multiple variable analyses is rapidly extending its scope. Nowadays, the main clinical uses of flow cytometry in solid tumors are to:

- i. Confirm the diagnosis of malignancy when the microscopical features are equivocal,
- ii. Establish and classify borderline malignancy,
- iii. Provide prognostic information independent of stage and grade,
- iv. Evaluate the response of therapy,
- v. Confirm the relapse of malignant lesion, and
- vi. Establish the origin of synchronous or metachronous tumors.⁴

Electron Microscopy

It has been more than 50 years since the admission of EM to the anatomical pathology field for the diagnosis of body's different organ diseases and tumor pathologies.¹⁹ EM can visualize ultra cellular structures and any other cellular structural abnormalities that cannot be appreciated by light microscopy. The main diagnostic uses of EM in clinical pathology are in the fields of renal biopsy, peripheral nerve biopsies, and muscle biopsies. EM is useful in the evaluation of metabolic and inherited diseases as well as demonstration of infectious agents or any evidence of drug toxicity,¹³ in addition to its major role in tumor pathology^{19,20} where the ultrastructural features have proved very useful in the determination of tumor differentiation. However, reactive conditions of benign tumors share malignant tumors to those ultrastructural changes of the same cell type.⁴

Nowadays, the role of EM in clinical pathology has reduced remarkably since the advent of IHC and molecular techniques. EM has provided a great utility and remains a powerful tool to settle the diagnosis of controversial nature of some tumors, including granular cell tumor, schwannoma, Langerhans cell histiocytosis, spindle cell (sarcomatoid) carcinoma, mesothelioma, melanomas, spindle cell thymoma, neuroendocrine tumors, spermatocytic seminoma through the detection of tumor-specific cytoplasmic filaments, granules, melanosomes, mesaxons, membrane-bounded crystals, or other ultrastructural changes. It is of great benefit that an expert anatomical pathologist should handle EM examination who has carried out the light microscopy examination of the controversial case and should conclude that EM is indicated for that specific reason.⁴ This is because conclusive and informative EM report can be achieved only with close correlation with light microscopy findings. EM studies require fresh tissue to be fixed in a special fixative; however, formalin-fixed wet tissue can also be used, and even FFPE is useful. Moreover, from H&E-stained section, tissue for EM examination can be obtained when the diagnostic feature is present only focally.²¹ Different cytological preparations are suitable for EM studies.²²

The clinical diagnostic uses in daily practice of pathology are summarized as follows (modified⁴):

1. Poorly differentiated malignancy to differentiate between carcinoma, melanoma, and sarcoma,²³⁻²⁵
2. Differential diagnosis between adenocarcinoma and mesothelioma,
3. Origin of anterior mediastinal tumors, either thymic tumors, malignant lymphoma, or germ cell tumor,
4. Differential diagnosis of small round cell tumors, including neuroendocrine tumors,^{26,27} and
5. Differentiation between spindle cell tumors of soft tissues.²⁸

Polymerase Chain Reaction (PCR)

PCR is an advanced molecular diagnostic technique that was developed during the mid 80 s of the last century. It is quick, reliable, highly sensitive, and a specific central technology for much of clinical molecular genetic testing. It generates millions of copies of any specific DNA sequence within a few hours.⁴ PCR relies on the ability of DNA polymerases to copy a DNA strand using a short complementary DNA fragment as an initiating template.^{29,30} The PCR technique can also be used to amplify RNA, so that gene expression can be analyzed. Real-time (RT) PCR represents a new generation of PCR for quantitative measure, in which the incorporation of fluorescent markers in the reaction mixture permits RT monitoring of the amplification process.³¹ The major advantages of RT PCR are shorter procedural time, higher sensitivity and specificity, and low risk of laboratory contamination.⁴ Specimens for PCR could be fresh blood, bone marrow, fresh tissue biopsies, frozen sections, and FFPE, as all are sources of nucleic acids for molecular analysis. Specimens should be collected and transported to the molecular pathology laboratory using aseptic techniques, if possible. Transport on ice reduces cell lysis, minimizes nuclease activity, and reduces nucleic acid degradation.¹³ Specimen contamination is a major drawback of this technique. Fresh tissue sampling is superior to fixed samples because all fixatives, including formalin, induce chemical degradation of nucleic acid. Nevertheless, fixed specimens are more easily stored and transported. One of the major advantages of PCR technique in pathology practice resides in the fact that samples of FFPE can be used to analyze DNA and RNA^{32,33} and even from microdissected tissue sections.³⁴

The main applications of PCR technology in pathology practice especially in tumor pathology are as follows (modified⁴):

1. Diagnosis of leukemia or lymphoma through the detection of Igs to determine the clonality of B- or T-cell proliferations,³⁵
2. Detection of chromosomal translocations in hematologic and solid malignancies,
3. Detection of point mutations in oncogenes and tumor suppressor genes,



4. Detection of gene amplifications, such as *MYCN* in neuroblastoma and *HER2* in breast carcinoma,
5. Detection of microsatellite instability and demonstration of loss of heterozygosity of genes,
6. Detection of microorganisms, such as *Mycobacterium tuberculosis*,³⁶ HPV in squamous cell carcinomas, EBV in malignant lymphomas, HHV8 in Kaposi sarcoma,³⁷ protozoan, and parasites, and
7. Detection of circulating tumor cells in peripheral blood, such as thyroid carcinoma, melanoma, and prostatic carcinoma.^{38,39}

Conclusion and Recommendation

Clinical ancillary diagnostic methods have been integrated not only into histopathology but also into many laboratory disciplines. Guidelines and recommendations from both professional institutions and regulatory agencies have been developed to assist in the development and performance of ancillary testing. Most of the ancillary techniques are performed in surgical pathology for detecting markers specific for a particular disease or a tumor and for focusing on somatic or acquired DNA variations in the cells, providing essential information about the diagnosis, prognosis, prediction of effective treatment, with monitoring the treatment response and the patients at high risk of disease.¹³

Author Contributions

Conceived the concepts: JSM. Analyzed the data: JSM. Wrote the first draft of the manuscript: JSM. Developed the structure and arguments for the paper: JSM. Made critical revisions: JSM. The author reviewed and approved of the final manuscript.

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