Genomic Medicine

A Practical Guide

Laura J. Tafe
Maria E. Arcila
Editors

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Preface

Molecular pathology is a rapidly evolving scientific discipline that encompasses the development of molecular and genetic approaches to the diagnosis, classification, monitoring, and risk assessment of human diseases. At the core of this discipline is the application of classical, novel, and cutting-edge technologies, developed in biochemistry, cell biology, molecular biology, proteomics, genetics, and bioinformatics, to the evaluation of pathologic processes. With the advent of high-throughput technology in recent years, the evolution of the field has gained such momentum, where it is difficult for the general pathologist, the clinician in practice, or young physicians in training to keep up with even the most basic concepts. While the information is readily available through various sources, it is extensive and is often difficult to find a central location with the "nuts and bolts" for quick reference.

As molecular fellows, we both looked for the ideal high-yield reference book that would compile critical information related to molecular biomarkers for various solid tumor and hematologic malignancy subspecialties. We hoped for a book to be succinct yet comprehensive enough to be suitable for fellows in training and medical professionals with an interest in molecular pathology and biomarkers. Several years passed and many comprehensive books have been published, yet there was still a gap for a quick reference resource. In this first edition of *Genomic Medicine: A Practical Guide*, we aimed to fill this gap and have brought together experts from various areas of molecular diagnostics with the same vision. The book covers many aspects of molecular diagnostics, from techniques to applications and comprehensive summaries of the molecular biomarkers of critical importance in solid and liquid tumors. Attention was also specifically devoted to bioinformatics and next-generation sequencing, as well as preanalytical issues related to molecular diagnostics which are commonly not extensively addressed in the literature.

In the first edition of this book, we concentrated on some of the key solid tumor and hematologic malignancies for which we felt consolidation of information would be most critical. A number of important organ systems such as central nervous system tumors and cutaneous and some head and neck malignancies were not captured in this edition but will be included, along with others in a future planned edition. We also welcome our readers' feedback for other important topics to be covered.

We'd like to thank our authors for generously contributing their efforts and expertise to our vision of this book and our families, friends, and colleagues in supporting us along this journey.

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Part I
General Concepts

Chapter 1
Pre-analytics, Current Testing Technologies, and Limitations of Testing

Alejandro Luiña Contreras, Jose Jasper L. Andal, Raymundo M. Lo, and Daphne C. Ang

Technical Terms and Abbreviations

aCGH	Array comparative genomic hybridization
CNV	Copy number variant
FISH	Fluorescence in situ hybridization
indel	Insertions and deletions
MLPA	Multiplex ligation-dependent probe amplification
NGS	Next-generation sequencing
RT-PCR	Reverse transcription polymerase chain reaction
SNV	Single-nucleotide variant
SV	Structural variants

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Key Points
- Molecular techniques are evolving rapidly.
- Pre-analytical variables can affect the results of an assay.
- Molecular methods can be divided in three broad categories: (1) methods used to detect chromosomal abnormalities, (2) methods used to detect changes in the DNA/RNA sequence, and (3) gene expression profiling.
- Clinicians should familiarize themselves in the different techniques in order to choose the best test to detect the genetic abnormality in question.

Key Online Resources
- My Cancer Genome [1]: https://www.mycancergenome.org/
- AMP Education Resources [2]: https://www.amp.org/education/education-resources/

Introduction

Molecular diagnostic tests have become routine in daily practice, and the landscape of analytical platforms is continuously evolving in the era of personalized medicine. An important aspect of molecular diagnostic tests is that the quality of biomarker information provided by these tests is dependent not solely on the analytical platform utilized but also on the quality of specimens used and the strict adherence to validated protocols. As such, all healthcare professionals need to familiarize themselves with pre-analytic variables that affect tissue for downstream testing, basic details of the platforms used to evaluate for these abnormalities, and limitations for each platform in order to effectively use and understand the provided results.

In this chapter, we will summarize the most common pre-analytic variables affecting samples and some of the most commonly used molecular testing technologies with their limitations.

Pre-analytic Variables in Surgical Pathology

Consideration of pre-analytic variables, including specimen handling, is a crucial requirement for ensuring accurate results of molecular diagnostic testing. The following pre-analytic variables impact subsequent molecular testing of tissue specimens: collection of the specimen, time to fixation (cold ischemia time), fixative type, duration of fixation, postfixation treatments, water quality, digestion, tissue processing time, macrodissection, section thickness, and the need for additional ancillary tests (e.g.,

immunohistochemistry). In general, nucleic acids from blood or bone marrow aspirate samples are of good quality; however, the appropriate anticoagulants must be used.

Prefixation

Time to fixation (cold ischemia time) is generally defined as the time from tissue removal to initiation of fixation. It should be recognized, however, that hypoxia, ischemia, metabolic stress, and tissue degradation start as soon as the first major vessel is ligated in surgery and not when the sample is removed. Embolization procedures prior to surgery, which often happened up to 2–3 days before surgery, can have major effects on tissue quality as well. High temperatures in the operating room can also speed up the autolysis process. Many of these factors are not within the control of the laboratory since the operating room staff are tasked with the immediate care of the sample. Proper communication and the establishment of interdisciplinary protocols for tissue handling are important, which take into consideration the type of testing that is to be performed downstream. While established guidelines do not exist for most uses at this time, tissue-handling requirements must be standardized at an institutional level and tracked on every specimen. It is generally recommended for the cold ischemia time to be less than an hour. Ideally, the surgical pathology request form should contain the information by allocating a "Time Specimen Out" and "Time Specimen Fixed" line on the forms for quality control purposes. For samples that are going to be delayed in the operating room, incubating in ice is recommended to preserve RNA.

Based on published literature, the effect of delay to tissue preservation will depend on the type of test to be performed and the target. For instance, DNA extracted from FFPE tissue that was subjected to a cold ischemia time of 1 hour displayed reduced fluorescence in situ hybridization (FISH) signals [3], while a cold ischemia time of 24 hours did not alter PCR amplification success rate [4].

It is believed that a prolonged time prior to fixation results in a time-dependent, autolysis-induced RNA degradation that starts early upon surgical removal of tissue [5]. RNA integrity numbers (RIN) were comparable between FFPE specimens subjected to a cold ischemia time of 0 and 2 hours, and a cold ischemia time of 0 hours versus 12 hours also did not lead to a difference in the relative expression of six transcripts [6, 7]. For immunohistochemical analysis, cold ischemia time of less than 12 hours is recommended to avoid negative effects [3].

The size of the biospecimen before fixation also plays a role in the PCR success rate. DNA extracted from tissue ranging from 3 to 10 mm in diameter has the highest success rate, as opposed to smaller specimens or larger specimens (encountered a higher background) [8].

In summary, the generally accepted cold ischemia time is less than 60 minutes. Unfixed specimens are best sent to the laboratory as soon as possible. The time points of collection and fixation should be established by the individual institution

depending on the projected downstream use of the sample and should be available for all personnel and clearly stated in laboratory records [9].

Fixation

Most diagnostic laboratories utilize 10% neutral buffered formalin for routine fixation and have validated tests from FFPE samples. The traditional formalin fixation process causes cross-linking of proteins, leading to shorter DNA fragments, and also random cytosine deamination. These limitations are mitigated by designing assays with shorter amplicon sizes and more robust assay protocols.

Different types of fixation used, temperature of fixation, and fixative delivery method affect downstream DNA analysis. DNA from neutral buffered formalin (NBF), as compared to unbuffered formalin, has greater DNA yields and better success rate in PCR, in situ hybridization (ISH), and genotype determination [10–15]. IHC staining is also optimal when fixed in a buffered 10–15% [16, 17] formalin solution with a neutral pH [18, 19]. Currently, 10% neutral buffered formalin (NBF) is the best and most broadly used and studied fixative. If an alternative fixative is considered, it must be validated against results for the same specimens fixed in 10% NBF. The laboratory director is responsible for the test performance using an alternative fixative. Other fixatives previously used before the molecular diagnostics era, including Zenker's, Bouin's, and B-5, have been shown to be harmful for downstream PCR testing [4].

Duration of fixation and its effects on ISH performance have been extensively studied, and the consensus is that excisional specimens should be fixed in 10% NBF for 6–72 hours, and this information should be documented in the pathology report. Fixation time of less than 72 hours in formalin is preferable for DNA integrity and yield, PCR, ISH, and single-nucleotide polymorphism detection assay performance [4, 10, 12, 20]. Amplification success after prolonged fixation was reportedly influenced by the target sequence, amplicon length, and tissue type [15]. Fixation at elevated temperatures (37 °C) has shown reductions in DNA yield and integrity and PCR success [21, 22], while fixation at 4 °C increased yield of high-molecular-weight DNA and PCR success [23].

Microwave-accelerated or ultrasound-accelerated fixation improves the yield of high-molecular-weight (HMW) DNA and PCR success from FFPE tissue [24–28]. Ultrasound-accelerated fixation also results in a more intense and uniform ISH staining and yielded longer amplicons and higher levels of amplifiable RNA as compared to conventional immersion fixation [27, 28].

For postfixation pretreatments in in situ hybridization (ISH) analysis, tissue processing time and digestion also impact the quality and interpretation. Different digestion enzymes may result in variable signal strengths as well. The ISH product might benefit from longer tissue processing times. The type of slides used (silanized versus positively charged) also affect adherence of tissues. Even water quality impacts the outcome. The use of highly purified water is recommended.

The generally accepted guidelines for fixation recommend 6–72 hours of fixation at 10% neutral buffered formalin. The laboratory records should document the date and time of fixation ideally within the pathology reports [9].

Decalcification

Decalcification methods may have marked adverse effects on nucleic acid analysis. Commonly used methods use acid-based decalcifiers, such as formic acid, hydrochloric acid, or nitric acid. Effects vary based on the concentration used and the length of tissue contact with the decalcifier. When different concentrations of formic acid are compared, decalcification with 5% formic acid for 12–18 hours produced FISH signals, while decalcification in a 10% formic acid solution for 7–10 days abolished the signal [29]. Decalcification with formic acid generally renders nucleic acid unsuitable for downstream testing by PCR methods in the majority of cases.

Recently, studies have shown that decalcification using ethylene diamine tetra acetic acid (EDTA) as opposed to acid methods is a better alternative. It allows amplification of longer PCR products [30], reduces background staining, provides stronger FISH signals [31–33], and provides superior determination of loss and gain of sequences for comparative genomic hybridization [31].

Processing and Storage

There are no studies showing the potential effects of dehydration, clearing, paraffin reagents, and embedding parameters in DNA and RNA yield.

Long-term storage of paraffin blocks has effects on DNA analysis, mostly in the length of amplifiable DNA. One study showed storage of FFPE sections for more than 10 years before DNA extraction and analysis was detrimental to PCR success rates; however, shorter durations have not been investigated [14].

Storage of 2–20 years leads to reduced RNA integrity as determined by RNA integrity number (RIN), as compared to those stored for 1 year or less [4, 20, 34, 35]. Humidity and temperature of the storage facility also plays a role in preserving (or degrading) the nucleic acid integrity.

Tissue Stewardship

A constant pre-analytic variable across all molecular testing technologies for solid tumor malignancies is tumor cellularity or the amount of viable tumor nuclei in the sample relative to the non-tumoral nucleated cells. A corresponding H&E slide

should be evaluated by a pathologist to identify the area(s) on the slides for testing and to quantify the amount of tumor in the selected areas. The choice of testing platform may be considered depending on the available sample and in cases wherein a repeat biopsy is not feasible. This limitation can be overcome by tumor enrichment or microdissection. For example, a cell block sample that contains less than 10% is not ideal for a Sanger sequencing assay that requires at least 30–50% tumor. Manual macrodissection or laser capture microdissection may enrich for tumor in these cases. More sensitive test methods may be utilized, such as droplet digital PCR or real-time PCR. These assays have greater analytic sensitivity (0.1% for ddPCR, 1% for rt-PCR), but are usually targeted mutational assays, frequently designed to cover only the most common mutations. It should be recognized, however, that the process of formalin fixation induces DNA damage that leads to low-level mutation detection. Highly sensitive assays may therefore lead to false positive results in such cases. The level of this DNA damage and risk for false positive results should be established at the time of validation of each high-sensitivity assay.

The amount of DNA yield from surgical pathology FFPE tissues to meet test requirements has been demonstrated to require at least nine square millimeters to produce 1ug of DNA in 99% of cases. This amount of DNA is deemed sufficient to meet most multigene assays [36]. It is up to each laboratory to validate the FFPE tissue requirements and DNA yields for each assay as part of the validation as these can be highly variable from assay to assay, laboratory to laboratory, and specimen type. Most laboratories will accept unstained slides for testing. The number of sections/slides and the tumor cellularity requirements will vary depending on the assay.

RNA Pre-analytic Considerations

RNA from formalin-fixed paraffin-embedded (FFPE) tissues is now commonly used in clinical practice. And, similar to DNA, pre-analytic variables that can influence RNA quality include the time to fixation, fixation time, tissue storage time, tumor cell content, and tumor percentage [37–39]. RNA analysis is commonly utilized for gene fusion detection and also gene expression profiling via reverse-transcriptase PCR (RT-PCR).

Pre-analytics of Circulating Tumor DNA or Cell-Free DNA

Liquid biopsy, especially plasma, obtained through a simple blood draw, has recently emerged as an alternative to surgical biopsy using blood samples of cancer patients for the detection of genetic alterations in plasma (cell-free) circulating tumor DNA (ctDNA) [40]. In cancer patients, plasma cfDNA is a combination of cell-free DNA

from normal cells and ctDNA. The levels of ctDNA can be highly variable and correlates with tumor size, degree of tumor invasion, disease stage, survival, and disease progression [41]. The cfDNA is usually fragmented to an average length of 140–170 bp and is present in limited quantities in the peripheral blood [42, 43].

Variability in blood collection affects the recovery of cfDNA. Peripheral blood collection tubes with cell stabilization agents that prevent cell lysis for several days are now commercially available, and several studies show the stability of cfDNA when the tubes remain at room temperature. Mehrotra et al., for example, shows that the yields of cfDNA in plasma from PB collected in Streck and K3-EDTA tubes were comparable when separated between 2 and 16 hours [40]. Plasma cfDNA integrity showed higher levels of larger fragments in the cfDNA from K3-EDTA tubes than in the cfDNA from Streck tubes [40]. In another study, blood sampled in Streck tubes, PAXgene® tubes (Qiagen), and cfDNA collection tubes from Roche GmbH (formerly from Ariosa) remained without noticeable contamination by high-molecular-weight DNA after 1 week at 22 °C [44]. Qualitative analysis of cfDNA from different blood collection tubes showed an average cfDNA fragment size of 100–120 bp at 2, 4, and 16 hours, with no HMW DNA [40].

Pre-analytical K2EDTA blood storage before cfDNA extraction in different conditions (room temperature for 4 hours, room temperature for 24 hours, and 4 °C for 24 hours) did not show significant difference in cfDNA concentrations [45]. However, a noticeable contamination by high-molecular-weight DNA, most likely originating from leukocyte lysis, is apparent by 24 hours [44]. Exposure to high temperatures, 39 °C for 5 hours and then 22 °C for another 19 hours, leads to high–molecular-weight DNA contamination in EDTA tubes, but not in the Streck tubes, PAXgene® tubes, and Roche tubes [45]. The mean concentration of cfDNA also increased proportionally in 3 ml compared to 1 ml plasma samples and in 5 ml compared to 3 ml samples by 2.9-fold and 3.44-fold [45].

Landscape of Current Testing Technologies

As a general rule, molecular methods can be divided in three broad categories: (1) methods used to detect large chromosomal or structural abnormalities; (2) methods used to detect small changes in the DNA sequence, including single-nucleotide changes, indels; and (3) gene expression profiling. Currently, several approaches and methodologies exist to detect each of the different types of alterations described above (Table 1.1). The choice of which approach to use is dependent on multiple factors including which type of alteration is being sought, medical necessity (diagnostic, prognostic, predictive), and tissue available for testing. The complexity of these tests can range from simple (interrogating a single variant) to very complex (multiplexed assays and NGS) depending on the need. In addition, each of these methods comes with strengths and limitations which are important to consider when choosing a test.

Table 1.1 Abnormalities detected by method

Method	SNV	Small duplications, insertions, or deletions	Large deletions	Copy number changes	Structural variants	Limits of detection	Turnaround time[a]
Karyotyping	−	−	+	+	+	Low, depends on cell growth	Weeks
FISH	−	−	+	+	+	Up to 0.5% depending on preparation	2–3 days
RT-PCR	+	+/−	−	−	−/+[b]	Below 1%	1–2 days
Fragment analysis and RFLP	+/−	+	−	−	−	~5%	1–2 days
dPCR	+	+/−	−	+/−	−/+[b]	Below 1%	1–2 days
MLPA	+	−	+	+	−/+[b]	~25%	2–3 days
aCGH	−	−	+	+	−	~25%	2–3 days
Allele-specific PCR	+	+/−	−	−	−/+[b]	1–5%	1–2 days
Sanger sequencing with capillary electrophoresis	+	+	−	−	+	~25%	3–5 days
Pyrosequencing	+	+	−	−	−	~5%	2–3 days
Melting curve analysis	+	+	−	−	−	10–20%	2–3 days
NGS-amplicon capture	+	+	+	−	+	5–10%	5–10 days
NGS-hybridization capture	+	+	+	+	+	2–5%	5–10 days
NGS-exon sequencing	+	+	+	+	−/+[c]	10%	5–10 days
NGS-whole genome sequencing	+	+	+	+	+	Depends on assay design	Weeks

RFLP restriction fragment length polymorphism

[a]In some laboratories, short turnaround times are not feasible due to specimen batching or fixed day runs

[b]Will detect specific rearrangements

[c]With difficulty, depending on the assay design

Tests to Detect Chromosomal Abnormalities

Chromosomal abnormalities include structural variants (SV) and changes in the number of chromosomes or copy number variations (CNV). SV are large structural changes and include rearrangements within or between different chromosomes and

inversions. The most common tests used to detect structural and numerical abnormalities include karyotyping (G-banding being the most common), fluorescence in situ hybridization (FISH), reverse transcription polymerase chain reaction (RT-PCR), array comparative genomic hybridization (aCGH), multiplex ligation-dependent probe amplification (MLPA), and next-generation sequencing (NGS). An overview of testing modalities used to detect chromosomal abnormalities is provided in Table 1.2. Even though all these tests are designed to detect SV or CNV,

Table 1.2 Tests to evaluate chromosomal abnormalities

Assay	Applications	Will not detect	Limitations	Sensitivity
Conventional karyotype (G-banding) [46]	Detect numerical and structural abnormalities	Small (cryptic) rearrangements; small insertions, deletions, or indels; SNV	Requires fresh tissue; long, turnaround time, low resolution; tumor cells might not grow in culture; necessitates high skills and experience in interpretation; difficult to detect complex rearrangements; might not detect cryptic translocations	Low, dependent on which cells grow
FISH [46, 47]	Detect rearrangements, large deletions and copy number variations of large chromosome segments	Small (cryptic) rearrangements; small insertions, deletions, or indels; SNV	Can only detect rearrangements, deletions, or copy number variations in the specific area of interest; insertions, small deletions, or inversions might not be detected	High, in touch imprints, and cytology specimens can be as high as 0.5%
Reverse transcriptase PCR [46]	Detect specific rearrangements; works well in formalin-fixed paraffin-embedded tissue	May not detect variants of the rearrangement	Can only detect rearrangements in a specific area. Not well suited for rearrangements with multiple fusion partners (e.g., the primer set might not cover the fusion present)	High, 1% to 5% when quantitative PCR is used
aCGH [48]	Can detect copy number at higher resolution	Copy neutral changes like balanced translocations or inversions	Requires normal DNA for comparison	Low, around 25%
MLPA [48, 49]	Can detect copy number, methylation, and point mutations	Copy neutral changes like balanced translocations or inversions	Single base pair changes can affect probe hybridization and be interpreted as a deletion; requires normal DNA for comparison	Low, around 25%

their design is significantly different, and they may not be used interchangeably and/or have different requirements for specimen submission.

Karyotyping (G-Banding)

Karyotyping can be done by several methods, the most common being Giemsa banding (G-banding). It is a cytogenetic technique used to visualize the complete karyotype by staining condensed chromosomes with the Giemsa stain. It is available in commercial laboratories and some hospitals. This technique allows visualization of all chromosomes [50] and is capable to detect copy number variants and large structural abnormalities [51, 52]. It is considered a low-resolution technique and requires prolonged turnaround time (weeks).

This technique suffers from several pre-analytical, technical, and interpretative limitations [53]. It requires rapid delivery of the sample to the laboratory because fresh tissue is needed to culture living cells. Timely delivery is important, but submission of the tissue in suitable culture media helps preserve the cells during delivery when shipping is delayed. During culture, insufficient cells may reach metaphase, yielding inconclusive results, or the culture may be overgrown by normal fibroblasts, which can lead to false negative results. Also, the cells may fail to grow or grow slowly.

The test is dependent on the availability of experienced interpreters and requires high skills. Also, a neoplasm may have numerous complex anomalies making interpretation difficult or mask recurrent rearrangements. Finally, a neoplasm can have cryptic rearrangements that cannot be detected by the test.

Fluorescence In Situ Hybridization

FISH is a molecular cytogenetic technique that uses fluorescence probes to detect specific areas in a chromosome. This technique can be performed in interphase (non-dividing) or metaphase (dividing) cells, in tissues procured fresh, frozen, or formalin-fixed, paraffin-embedded (FFPE). It can also be used in materials from blood smears, touch imprints, and cytology specimens like fine needle aspirations and cytospins, or other samples with minimal cellularity. It is considered a targeted approach, medium-resolution technique and, depending on the laboratory, can have a turnaround time of 1–3 days. It allows direct visualization of the abnormality in individual cells and when interphase cells are prepared from fresh tissue by touch prep or smear can have high sensitivity (up to 0.5% depending on how many cells can be visualized).

FISH is commonly used to detect copy numbers for specific chromosomes, amplifications, and rearrangements. Two methods to detect rearrangements exist. The first method uses break-apart probes, which are probes flanking the gene or region of interest. They detect the rearrangement when the fused signal separates into a split signal. This approach is frequently used for genes that have recurring breakpoints and also rearrange with multiple different partner genes (e.g., *EWSR1*, *ALK*). The second uses fusion probes. Fusion probes flank two genes or regions of

interest and detect the rearrangement when the separate signals fuse as a consequence of a rearrangement. With this approach, both partner genes are known.

The major limitation of FISH is its targeted approach, since it needs high suspicion for the specific abnormality being tested, and it is not well suited as a screening tool. In FFPE tissues, analysis may be limited by loss of nuclear material during sectioning (truncation artifact) that may lead to false positive or false negative results. For example, the nuclear material might be lost during sectioning, and it might falsely detect a deletion. This is mitigated by through validation and establishing cutoff values during the validation. These cutoff values may vary by laboratory.

Crushing, poor, or prolonged fixation time (usually more than 72 hours) and decalcification procedures may also affect interpretation and signal intensity. Because the probes are large, up to 500 kilobases (kb), it is not well suited to detect single-base mutations. Also, nomenclature and reporting may be inconsistent among laboratories although the vast majority adhere to standard nomenclature guidelines. The last major limitation is signal fading over time that does not allow for rereview of the slides after prolonged periods. Therefore, photo documentation is recommended.

Reverse Transcription Polymerase Chain Reaction

Reverse transcription PCR (RT-PCR) is well suited to detect chimeric transcripts as a consequence of chromosomal rearrangements [54, 55]. RT-PCR involves the conversion of RNA to complementary DNA (cDNA) sequences for subsequent testing. To detect rearrangements, both genes or one gene partner is needed to detect the fusion transcript [56]. This technique can be performed using formalin-fixed paraffin-embedded tissue, fresh or previously frozen tissues. It is considered a very specific technique, and the turnaround time is usually 1–3 days, similar to FISH.

RT-PCR is a highly sensitive technique that will allow detection of low-level disease down to 1% [57]. When performed in conjunction with real-time PCR, it can be used for quantitation of minimal residual disease to as low as 0.0001% depending on the amount of template used [58].

Because of its specificity, it is not a very good screening tool and requires high level of suspicion for a given rearrangement, like FISH. Another caveat for this test is that it will only detect one variant fusion, unless it is multiplexed [56]. For genes with multiple rearrangement partners or multiple fusion variants, like the *ALK* or *EWSR1* genes, it is difficult to design primer sets that will cover all possibilities. In these instances, it is easier to detect a rearrangement using a break-apart FISH probe or NGS technology, such as RNAseq or sequencing in conjunction with anchored PCR.

Multiplex Ligation-Dependent Probe Amplification

MLPA [59] is a molecular technique that utilizes ligation of specific probes to a target DNA sequence. If successful hybridization occurs, the probes are ligated and

then amplified. The assay can be used to detect single-nucleotide polymorphism or point mutations, copy number alterations, deletions, and small rearrangements. The main limitations of this methodology are that it cannot detect copy neutral loss heterozygosity (like uniparental disomy) or balanced rearrangements. It may also have problems detecting mosaicism, tumor heterogeneity, or alterations when there is excessive contamination with normal cells [60]. In addition, because this technique targets specific areas within an exon, point mutations or polymorphisms that hinder hybridization of the probe might be falsely interpreted as a deletion. Lastly, this technique requires a "normal" DNA for a control. The turnaround time is usually 2–3 days and the limit of detection is around 10%.

Array Comparative Genomic Hybridization (aCGH) and Single-Nucleotide Polymorphism (SNP) Arrays

Array comparative genomic hybridization (aCGH) is a molecular cytogenetic technique developed to detect whole genome copy number variations (CNV). It compares the target DNA to a reference sample using fluorescence probes. In order to achieve this, the target DNA and the reference DNA are hybridized in a solid support with immobilized capture probes (the array) [61, 62]. Its main advantages are that it does not need fresh tissue; it is a high-resolution technique; it can interrogate the whole genome and detect aneuploidies, deletions, duplications, and amplifications; and it has the added benefit of detecting submicroscopic chromosomal abnormalities. Its main disadvantage is the inability to detect chromosomal aberrations that do not result in copy number changes (like balanced translocations, inversions, or balanced insertions). It can be performed in fresh, frozen, or FFPE tissues. Lastly, aCGH has low detection limit (around 20%) and long turnaround time (weeks). Some newer arrays are based on single-nucleotide polymorphisms (SNPs) which have several added advantages including the ability to detect copy neutral loss of heterozygosity which is important in some cancers.

Methods Used to Detect Changes in the DNA Sequence

Changes in the DNA sequence that commonly cause disease include single-nucleotide variants (SNV), small duplications, insertions or deletions, and simultaneous insertions and deletions (indels). Numerous methods to detect mutations have been developed, and the most common ones used in routine clinical practice include polymerase chain reaction (PCR) and variants as the basic form of amplification followed by a detection method with or without subsequent sequencing (Table 1.3). Following PCR, detection may involve the measuring of the PCR products by size (fragment analysis, or restriction fragment length polymorphism analysis) or by specific melting curve characteristics (melting curve analysis). Alternatively, the PCR product may be sequenced by various methods including Sanger sequencing,

Table 1.3 Tests to evaluate changes in DNA sequence

Assay	Application	Will not detect	Limitations	Sensitivity
Allele-specific PCR	SNV	Deletions, duplications, insertions, deletions	Specific assay for the specific gene in question may not detect other mutations than the one the assay was designed for	1–5%
Sanger sequencing with capillary electrophoresis [63]	Considered the gold standard for sequencing, can be designed to detect changes in sequence in up to 1000 bp. Will detect insertions, deletions, and indels and rearrangements	Large chromosomal abnormalities, deletions	Low detection limit; labor intensive, costly	Approximately 25%
Pyrosequencing [63, 64]	Easy to implement; very good at detecting point mutations; high sensitivity (around 5%)	Very difficult to detect small insertions and deletions or rearrangements	Difficult to detect sequences with the same base (homopolymers when longer than 6–8 bp in length), requires a short template (around 40 bp, but can be designed up to 400 bp)	Around 5%
Melting curve analysis [63]	Fast and easy to implement; used to detect commonly known mutations		The specific mutation is not known; might require confirmation for unusual mutations	10–20%

pyrosequencing, mass spectrometry genotyping, or next-generation sequencing (NGS). NGS techniques, depending on the capture method, can be used to detect copy number variations, and they are also used to detect mutations, duplications, small insertions, deletions, and indels.

Next-generation sequencing methods are considered highly specific or of high resolution. One limitation that affects all sequencing methods is the size of sequence that can be evaluated at a given time. Depending on the assay, the sequences vary from around 40 bp (pyrosequencing) to up to 1000 bp (Sanger sequencing). However, most of the assays are optimized for DNA fragments ranging in size from 100 to 300 bp to allow use with FFPE tissue, since formalin fixation fragments the DNA.

The following is a description of some of these assays with their limitations.

Allele-Specific PCR

This is a targeted analysis for the detection of specific SNV. It is very specific for the mutation that the assay was designed for. It is also sensitive, and it can detect mutations when present at 1–5% concentrations. Its main limitation is its specificity. It cannot detect other mutations than the one the assay is designed for.

Sanger Sequencing with Capillary Electrophoresis

Sanger sequencing is a PCR technique and it is considered the gold standard for DNA sequencing. RNA can also be sequenced if converted to cDNA. Capillary electrophoresis is the method to separate the DNA strands by size and allow separation of up to 1 bp resolution. Sanger sequencing is well suited to detect SNVs, small deletions, insertions, and indels. It can also detect fusion transcripts, if appropriately designed. If good-quality DNA is used, it can sequence segments up to 1000 bp. The main benefits are that it will detect any mutation or variant in the DNA segment covered by the primer set. The biggest limitations are its costs and it is also labor intensive. It has low sensitivity, at around 20%, but sensitivity can be improved by using locked nucleic acids (LNA-PCR) [65]. Also, this method will not detect CNV. The turnaround time is around 3–5 days.

Pyrosequencing

Pyrosequencing is a sequencing-by-synthesis method that uses a series of reactions to measure the release of inorganic pyrophosphate as each nucleotide is incorporated into the DNA chain [66]. This technique is easy to implement and has fast turnaround time (can be performed in 1 or 2 days), and it is good at detecting SNV and has high sensitivity. It will detect a mutation in approximately 5% concentration. Also, it is well suited to detect mutations in less than optimal DNA, like decalcified bone specimens [67] because it requires short DNA templates (around 40 bp). When using this method, it is difficult to detect sequences with the same base (homopolymers when longer than 6–8 bp in length) and insertions and deletions. Also, it cannot sequence long templates well (more than 400 bp).

Melting Curve Analysis

Melting curve analysis is a technique developed to detect mutations by measuring the changes in fluorescence generated by the different dissociation curves between mutated and wild-type samples [68]. This technique is suitable to detect single base pair substitutions and small deletions and insertions. It can also be used to detect methylation status of a specific region [69]. Its main benefits are that it is a fast, closed-end system that is not labor intensive. The main limitations are that even

though it can detect a mutation in the area of interest, it cannot define which mutation is present. Also, in some instances, it might require confirmation by another method [63]. This assay can detect a mutation at approximately 10% concentration.

Next-Generation Sequencing

Next-generation sequencing (NGS) is a group of high-throughput methodologies that allow for low-cost sequencing of the genome or exome, transcriptome profiling (RNA-seq), protein-DNA interactions (ChIP-Seq), and epigenetic modifications. Depending on the manufacturer or platform, the assay may differ in the way the bases are incorporated and detected. The assays will also differ in the way the library is prepared and how the targets are enriched, when a targeted approach is used. The two most common methodologies for base incorporation and detection are sequence-by-synthesis using fluorescence (Illumina® dye sequencing) and hydrogen ions (Ion Torrent®) [70]. The most common target enrichment methods include hybridization or capture based and amplicon based (Table 1.4) [72]. All of these factors affect the performance characteristics of the assays [70–72].

Both methods of base incorporation can detect copy number variations and mutations, small insertions, and deletions. Between these base incorporation methods, dye sequencing appears to have a lower error rate (<0.4%) versus (~1.8%) [70]. The

Table 1.4 Next-generation sequencing approaches [70–72]

Method	Uses	Will not detect	Limitations
NGS-amplicon capture	SNV, small deletions, insertions, small duplications, structural rearrangements depending on design	Copy number alterations Limited capabilities for structural rearrangements Large structural change	Slow turnaround time, complex setup, need for bioinformatics support
NGS-hybridization capture	SNV, small deletions, insertions, small duplications, rearrangements (depending on design)	Large structural changes	Slow turnaround time, cumbersome to setup time, heavy bioinformatics support
NGS-whole exome sequencing	SNV, small deletions, insertions duplications	Structural rearrangements involving introns	Slow turnaround time, cumbersome to setup time, heavy bioinformatics support
NGS-whole genome sequencing	SNV, small deletions, insertions, duplications, structural changes		Expensive, slow, cumbersome to setup time, not practical for everyday use, few automated solutions Heavy bioinformatics support

base content of the template also influences the error rate as dye sequencing has difficulty reading areas rich in GC and ion semiconductor has difficulty detecting homopolymers (sequences with more than 6–8 base pairs) and is not well suited for AT-rich areas in the genome.

Additional limitations affecting next-generation sequencing are related to the general architecture of the DNA templates. The most important of these limitations is the presence of difficult-to-sequence areas in the genome. Areas of high-homology (e.g., pseudogenes), repetitive, and GC-rich regions [73–75] affect assay performance and sensitivity. The reason for poor performance in areas of high homology or repetitive regions of the genome is related to inaccuracies in the mapping to the reference sequence. In CG-rich regions, it has been proposed that the reason for the high error rate might be due to secondary structure formation, lower quality of reads, or high background noise [76, 77].

Additional limitations in next-generation sequencing are related to the analysis of the data which are discussed at length in both the bioinformatics section and dedicated NGS sections of this book. Genomic analysis and bioinformatics pipelines rely on the use of numerous public and private sources for analysis tools for which no specific guidelines and standardization have been applied [78]. This leads to significant variability among laboratories.

The next-generation sequencing limitations noted above are mainly due to the early stages of clinical implementation of these assays and will, in most likelihood, be resolved in the upcoming years. The bioinformatics pipeline limitations should be resolved as the "Standards for Clinical Grade Genomics Databases" is implemented [78].

Gene Expression Profiling

Gene expression profiles are most commonly used in the research setting, but have found clinical use in the prognostication of some malignancies [79]. In essence, these tests quantify the expression of multiple genes (signature panel) in an attempt to predict the likelihood of recurrence and/or selecting individual therapies. Depending on the assay, these may be performed in fresh, frozen, or FFPE tissues. Also, these tests require well-preserved tissue that has not seen prolonged cold ischemia time or is highly necrotic, since they utilize mRNA. The labile nature of RNA from FFPE has been overcome by specific extraction kits that can minimize RNA degradation and remove genomic DNA that can affect downstream applications. At least 30% of invasive tumor is needed to increase accuracy. Turnaround time is test dependent and could be 1–2 weeks. The limits of detection also vary by assay, but the norm is to submit tissue with at least 25% tumor.

Tissue stewardship and selecting and optimizing the use of tissues for ancillary testing are the responsibility of the pathologists in collaboration with other providers. It is essential to understand the pre-analytic variables at play as part of the tissue accrual, optimization, and management process. In addition, there is a spectrum of

molecular methods available in clinical laboratories, each designed for different applications and with different strengths and limitations. Keeping all of these factors in mind, an educated decision to select the test that will best serve the patient's clinical need can be made.

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