GUARDANT HEALTH®

OVERVIEW

of Molecular Diagnostics

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Introduction

Molecular diagnostics is a term used to describe a broad collection of techniques for detecting, diagnosing, and managing disease by studying molecules, such as DNA, RNA, and protein in a tissue or fluid.

Cancer biomarkers—also known as tumor markers—are substances produced by cancer or by other cells in the body in response to cancer. Many cancer biomarkers are produced by both normal cells and cancer cells; however, cancer cells may produce these markers at much higher levels. Biomarkers may be found in many locations, including the tumor tissue itself, but also in the blood, urine, stool, or other tissues or body fluids. Note that there is no single "cancer biomarker"—because cancer is a highly heterogenous disease, different cancers have different characteristic biomarkers.

Some cancer biomarkers are proteins that are overexpressed or aberrantly expressed on tumor tissues; patterns of gene expression and the direct study of mutations in DNA are also used as cancer biomarkers.

Cancer biomarkers are used in a number of different ways in the diagnosis, prognosis, and treatment of cancer (Table 1).

Table 1. Utility of Cancer Biomarkers

Use	Example
Estimating the risk for developing cancer	One well-known example of such a use are hereditary mutations in *BRCA1* and *BRCA2*, which can predict risk for breast cancer.
Screening for disease	Prostate-specific antigen (PSA) has been used as a rapid way to evaluate men for the presence of prostate cancer.
Determining the prognosis of disease	The presence/absence of certain genes may allow clinicians to predict the aggressiveness of disease. For example, *KRAS* mutations are associated with a poor prognosis.
Predict response to therapy	Many biomarkers can help clinicians select specific cancer treatments—both approved therapies and those that are in clinical trials. Activating *ALK* fusions are a good example of such a biomarker in lung cancer.
Monitor for response or progression	Some biomarkers may be used to follow patients routinely during or after treatment to determine whether disease has progressed or recurred. *BCR-ABL* transcript levels in chronic myelogenous leukemia are a classic example of this paradigm.

There have been rapid advances in the use of biomarkers to guide the management of cancer. Using lung cancer as an example, the National Comprehensive Cancer Institute guidelines for non-small cell lung cancer (NSCLC) have evolved over time—from not recommending the assessment of biomarkers at all, to recommending the assessment of biomarkers in selected patients, and finally, today, to strongly recommending broad molecular profiling with the goal of identifying driver mutations for which effective drugs may already be available, or to appropriately counsel patients regarding the availability of clinical trials.

> *Broad molecular profiling is a key component of the improvement in care of patients with NSCLC.*
>
> — National Comprehensive Cancer Institute, 2018

Similarly, the American College of Chest Physicians, in its evidence-based guidelines for the diagnosis of NSCLC, recognizes the central role of biomarker testing in the diagnosis and management of NSCLC.

> *Reporting a diagnosis of NSCLC is simply not enough. NSCLCs are clinically, pathologically, and molecularly heterogeneous tumors….*
> *In the past decade, paradigm shifts in the treatment of NSCLCs have emerged as the result of clinical trials that have shown us that NSCLCs respond to different therapeutic agents based on histologic phenotypes and molecular characteristics…*
>
> — American College of Chest Physicians, 2013

The number of biomarkers that are ideally assessed in every qualifying patient is increasing rapidly. In non-small cell lung cancer, as of 2018, there are 5 biomarkers that are commonly assessed for guiding treatment: *EGFR*, *ALK*, *ROS1*, *BRAF* V600E, and PD-L1, and there are many more markers that are relevant for prognosis and for agents that are currently under investigation. There are a number of different testing modalities that are used for the molecular evaluation of tumor samples. The following will be covered here:

- Fluorescence in-situ hybridization (FISH)
- Immunohistochemistry (IHC)
- Real-time polymerase chain reaction (PCR)
- Conventional sequencing
- Next-generation sequencing (NGS)

Learning Objectives

This module is intended to convey general information on molecular diagnostics and uses examples from the non-small cell lung cancer (NSCLC) treatment paradigm where applicable, although many of these techniques are routinely used or are under investigation for the evaluation of other types of cancers.

Upon completion of this module, you will be able to:

- Define anatomic and molecular pathology
- Describe the techniques and procedures used for anatomic and molecular pathology
- Discuss the methodology and interpretation of key molecular diagnostic tests used in cancer

Lesson 1: The Fundamentals of Anatomic and Molecular Pathology

1.0 Introduction

The evaluation of samples derived from tumor tissue is the responsibility of the pathologist—a healthcare provider who examines laboratory samples of body tissue for diagnostic purposes. In order for the pathologist to perform his or her function, a representative sample must be obtained from the patient.

Obtaining and processing tissue for analysis is thus the first step in the molecular diagnostic pathway. Until recently, all—or almost all—solid tumor samples were derived directly from the tumor using conventional biopsy techniques. As briefly introduced below, evidence now shows that "liquid biopsies," which involve analysis of blood or other fluid samples from the patient that contain tumor cells or **nucleic acids** shed from the tumor, can provide similar information with the need only for a blood draw, minimizing the risks associated with conventional invasive biopsy techniques and reducing the potential for sampling bias.

1.1 Clinicians, Pathologists, and the Pathology Team

1.1.1 Pathology Specialties

Anatomic pathology is the branch of medicine that studies the effect of disease on the structure of body organs, both as a whole and microscopically. Anatomic pathology is directed at identifying abnormalities to diagnose disease and manage treatment, and is commonly used to identify and help manage various types of tumors.

There are two main subdivisions within anatomic pathology:

- Histopathology involves examination of intact tissue under the microscope. This is often aided by staining and using antibodies to identify different components of tissue.
- Cytopathology (cytology), which is the examination of single cells or small groups of cells. Specimens are examined microscopically by a pathologist, who provides a definitive diagnosis to the treating physician.

Clinical pathology involves the measurement of the chemical constituents of the blood and other body fluids, analysis of blood cells (hematology), and identification of microorganisms, among other duties. Clinical pathology is, itself, divided into a number of subspecialties, the key ones being clinical chemistry, clinical **hematology**/blood banking, hematopathology, and clinical microbiology. In addition, emerging subspecialties include molecular diagnostics and proteomics.

Often, physicians will practice both anatomic and clinical pathology, a combination sometimes known as general pathology. Regardless of title, there is significant overlap between the 2 disciplines, especially with regard to molecular diagnostics. Some pathologists may be referred to specifically as molecular pathologists; these physicians focus on using molecular biology tools to identify genetic alterations associated with diseases and to gauge response to specific treatments.

1.1.2 Other Members of the Pathology Laboratory Team

Pathologist Assistants

Pathologist assistants (PAs) provide services under the direction and supervision of a pathologist. They are trained to process a variety of laboratory specimens, including most pathological specimens. Note that the pathologist is always responsible for the final diagnosis. Pathologist assistants may be responsible for gross examination and dissection of anatomic pathology specimens and the performance of postmortem examinations. They prepare tissue for pathological tests, including frozen sections, **flow cytometry**, and immunohistochemical staining.

Cytotechnologists

Cytotechnologists assist in screening cellular samples, rather than tissue sections. Cytotechnologists may screen cells and refer abnormal cases for pathologist review. Samples examined by cytotechnologists include fine-needle aspirates, washings, scrapings, and other body fluids.

Histotechnologists

Histotechnologists manage tissue processing and prepare slides from tissue for evaluation by the pathologist. Duties include fixing tissue, embedding it in paraffin, sectioning tissue onto slides, and staining tissue on slides.

1.1.3 Clinicians Involved in the Care of Lung Cancer

All cancer treatment—including treatment of lung cancer—is multidisciplinary. Members of the multidisciplinary clinical team involved in the care of lung cancer are summarized in Table 2.

Table 2. Clinicians Involved in Lung Cancer Care

Medical Oncologists	Medical oncologists are often the main healthcare provider for patients with cancer. They have specialized training in diagnosing cancer and in treating it using chemotherapy, hormonal therapy, biological therapy, and targeted therapy. They also: • Read and interpret biomarker tests and make decisions on targeted therapies to use • Provide supportive care • Coordinate treatment given by other specialists
Pulmonologists	Pulmonologists manage a broad range of respiratory diseases, such as asthma, chronic obstructive pulmonary disease. They also play a key role in the prompt diagnosis, staging, and treatment of patients with lung cancer. They may also: • Administer and interpret pulmonary function tests throughout treatment • Manage comorbidities, such as shortness of breath and respiratory failure, and may manage pulmonary adverse events associated with both the cancer and its treatment
Surgical Oncologists	Surgical oncologists perform surgical procedures related to cancer. They can perform open surgeries, minimally invasive surgeries, and biopsies.
Radiation Oncologists	Radiation oncologists specialize in the management of cancer using radiation.
Pathologists	(Discussed in 1.1.1 and 1.1.2 above)
Primary Care Physicians	PCPs play role in cancer therapy because: • They are often the first point of contact for patients at diagnosis • They counsel patients about treatment options • They help monitor treatment progress • They assist in managing routine side effects PCPs may also manage comorbid conditions, chronic pain, and depression.

1.2 Obtaining and Preparing Tissue Samples

1.2.1 Conventional Biopsy Techniques

The first step in the pathologic evaluation of tissue is obtaining tissue. There are 2 primary goals when selecting the best method for obtaining tissue samples from solid tumors:

- Maximizing the tissue yield of the selected procedure for both diagnosis and staging, while ensuring patient safety
- Avoiding unnecessary invasive tests for the patient, with special attention to the projected treatment plan

As an example, the American College of Chest Physicians (ACCP) guidelines for establishing the diagnosis of lung cancer summarize the primary techniques for obtaining sufficient biopsy tissue. In this example, the modality employed depends on the type of lung cancer (small cell lung cancer or non-small cell lung cancer), the size and location of the primary tumor, the presence of metastases, and the overall clinical status of the patient (Table 3).

Table 3. Conventional Clinical Biopsy Techniques for Lung Cancer

Noninvasive	Sputum cytology	Examination under a microscope of cells found in sputum (mucus and other matter brought up from the lungs by coughing). The test checks for abnormal cells, such as lung cancer cells. Used for assist in initial diagnosis, *but must be confirmed with further testing.*
Minimally Invasive	Percutaneous needle biopsy	A fine needle is inserted into the site of the tumor, removing a core of tumor tissue. Accuracy is enhanced by using imaging techniques to guide the needle. May be used for diagnosis or rebiopsy at progression. Biopsies with simpler imaging modalities, such as ultrasound, may be performed in a nonspecialist clinic; however, more complicated procedures may require a radiologist or trained surgeon in the hospital.
	Thoracentesis	Removal of **pleural fluid** for cytologic examination. May be used at diagnosis or at progression.
	Medical thoracoscopy/ video-assisted thoracoscopic surgery (VATS)	A minimally invasive procedure involving insertion of an endoscope (a narrow-diameter tube with a viewing mirror or camera) through a small incision in the chest wall. May be used at diagnosis or at progression; may be performed with curative intent or just to gather biopsy samples.
	Flexible bronchoscopy	A procedure that uses a flexible bronchoscope to examine the inside of the trachea, bronchi (air passages that lead to the lungs), and lungs. A bronchoscope is a thin, tube-like instrument with a light and a lens for viewing. It may also have a tool to remove tissue to be checked under a microscope for signs of disease. The bronchoscope is inserted through the nose or mouth. Bronchoscopy may be used to detect cancer or to perform some treatment procedures.
	Electromagnetic navigation (EMN) bronchoscopy	Consists of a steerable probe combined with an electromagnetic field generator that allows the exact position of the probe to be determined.
	Radial endobronchial ultrasound (R-EBUS)	Uses a flexible catheter housing a rotating ultrasound probe that produces a 360-degree “radar” image to visualize the tumor.
Highly Invasive	Open surgical biopsy	Usually performed during surgery for the primary tumor; involves extracting large amounts of tissue. There are two types of surgical biopsy: incisional and excisional. • Incisional surgical biopsies remove parts of abnormal tissue, similar to fine-needle and core biopsies but in a larger amount. • Excisional surgical biopsies remove the entire abnormal area or tumor, and may additionally excise normal tissue around it. Excisional biopsies may have a curative intent to remove affected parts of an organ or the entire organ itself, with tissue analysis following the excision. Lymph nodes may also be removed from the area surrounding the tumor. Surgical biopsies are typically performed in the hospital with local or general anesthesia depending on the extent of analysis.

1.2.2 Processing Conventional Biopsy Samples

Conventional biopsy samples are processed using a rigorous, step-by-step procedure that ensures that the final products are suitable for the molecular diagnostic techniques that are planned for assessing the tissue.

1	**Intraoperative frozen sections and touch preps** At times during a surgical procedure, a rapid diagnosis of a pathological process is required. Freezing makes the tissue solid enough to section with a microtome, a device that consists of a knife and a mechanism for advancing the tissue block in small increments, creating hair-thin sections that can undergo further processing. During a surgical procedure, the pathologist examines frozen sections, and may provide guidance to the surgical team. Samples are also frozen to deliver to the pathology laboratory. Instead of freezing, "touch preps" (imprint smears) may be used. In this case, the tissue is cut and pressed or smeared onto a glass slide, then stained and examined under the microscope.
2	**Specimen Accessioning** Tissue specimens arriving in the pathology lab are accompanied by a request form that lists: • Patient information • Patient history • Site of origin Samples are given a number that identifies each specimen, allowing tracking throughout the process.
3	**Gross Examination** Tissues that are removed from the body are examined by a pathologist. Gross examination describes the specimen, including its histology (for example, **squamous** vs non-squamous in lung cancer). When a malignancy is suspected, the tumor may be inked to mark the margins of the specimen.
4	**Fixation** Tissues that are removed from the body are placed in fixatives—chemicals that are intended to preserve tissues permanently in as life-like a state as possible.
5	**Tissue Processing and Sectioning** After fixation, the tissue must be processed into a form that can be easily sectioned. This is most often done by embedding tissue in paraffin, which is similar in density to a tissue. Sectioning is carried out with a microtome—a bladed device that can make sections as thin as 3 microns (although usually 6 to 8 microns). The tissue block itself may be stored for future analysis or for extraction of nucleic acids for testing.
6	**Staining** Tissues may be subjected to various staining processes to visualize certain structures or for the evaluation of biomarkers (discussed below). In order to stain the tissue, the paraffin embedding process must be reversed to get paraffin out of the tissue and allow water-soluble reagents to penetrate the sections.
7	**Coverslipping** The stained slide is covered with a thin piece of glass or plastic to protect the tissue and provide better optical quality.
8	**Biobanking** Samples are stored for future analyses or for research purposes. For example, biobanked tissue may be re-evaluated among patients who fail first-line therapy to determine qualification for a clinical trial.

Tissue Requirements for Molecular Diagnostics

The amount of tissue needed for molecular diagnostics varies widely and depends on the number of tests that are conducted. For example:

- Fluorescence in-situ hybridization (FISH) requires at least 2 slides of adequate tumor material—one stained with hematoxylin and eosin (H&E), and the other is used for hybridization of ALK probes
- Immunohistochemistry (IHC) requires at least 2 slides of adequate tumor material—one stained with H&E, the other stained with the assay antibody
- Real-time polymerase chain reaction (PCR) requires a single slide for extraction of DNA, amplification, and analysis
- Next-generation sequencing (NGS) requires more tissue but assesses multiple genes simultaneously. For example, the FoundationOne CDx provides comprehensive results with an FFPE tissue block or 10 slides (and 1 H&E slide)

1.2.3 Limitations of Conventional Biopsies

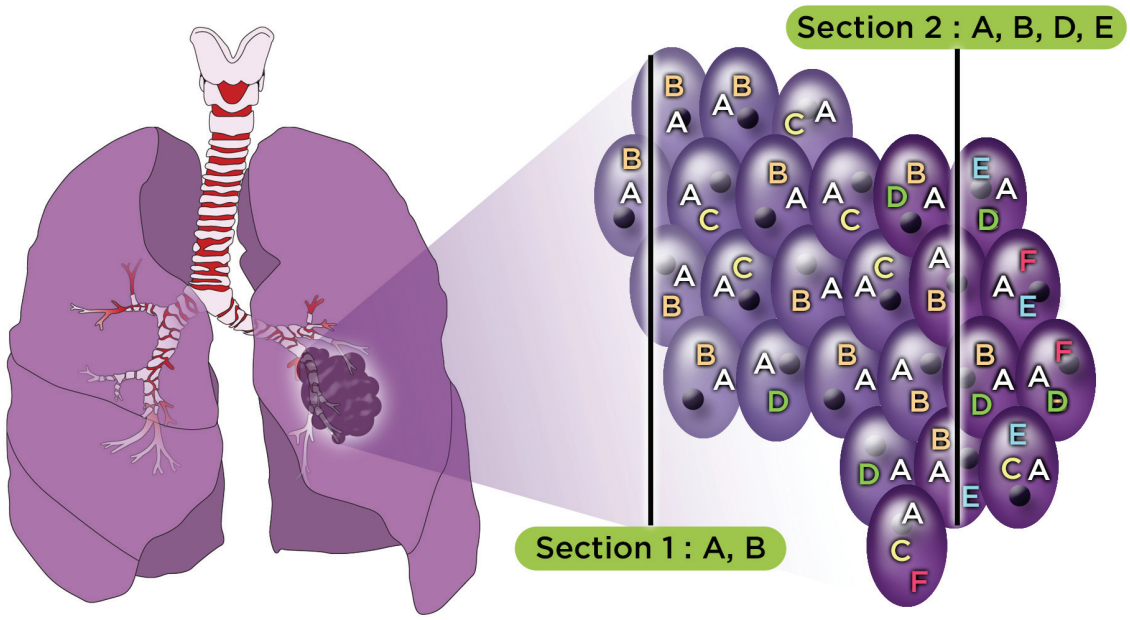
While use of tissue derived directly from the tumor has long been considered the gold standard for biomarker testing, this approach is associated with a number of important limitations.

Tissue availability: Among patients who are diagnosed with metastatic disease, tumor tissue may not be available or easily accessible. In these cases, surgical removal of the tumor is not indicated. Alternative sources of material, such as biopsies or cytology specimens, are associated with variable quantity of tumor cells and quantity of material. Thus, the amount and quality of DNA extracted from these tissues is highly variable. Tissue may also have already been used for previous testing.

Sampling bias: Tumors are genetically unstable, and tumor tissue can be highly heterogeneous at the molecular level (Figure 1). This leads to the potential for sampling bias. Simply put, any single conventional tissue section that is evaluated by a molecular diagnostic may not be representative of the breadth of mutations in a tumor. Further, the genetic profile of the primary tumor and metastatic sites may differ. For this reason, specific actionable mutations may be missed even by the best pathology lab.

Figure 1. Sampling Bias

Tumors are highly heterogeneous; the evaluation of a single section of fixed tumor tissue has the potential to miss targetable mutations, resulting in *intratumor* sampling bias. In this figure, the letters A through F represent different genetic alterations. A potential for *intertumor* sample bias also exists because the genetic profiles of the primary tumor and metastatic site(s) may differ (not shown in this figure).



Tumor evolution: As just noted, tumors are genetically unstable. As disease progresses, there are often changes in the genetic composition of the tumor that have the potential to affect both prognosis and treatment choice. For example, in NSCLC, many patients taking the EGFR inhibitor Tarceva® (erlotinib) or other EGFR TKIs may develop a specific mutation in EGFR, known as T790M, that renders them unresponsive to the initial targeted therapy. These patients can be subsequently treated with Tagrisso® (osimertinib), a second EGFR inhibitor that has demonstrated efficacy in EGFR-positive patients with T790M mutations.

Unfortunately, because of the potential risks associated with conventional biopsy, following the evolution of tumors over time can be quite difficult, particularly in frail patients who may not be able to tolerate repeated biopsies.

Need for tissue fixation: Methods of tumor preservation can actually alter DNA at the sequence level, potentially leading to inaccurate sequencing results.

Risks of conventional biopsy*:* Most biopsy procedures are associated with risks to the patient, which vary based on the method chosen. Many patients with metastatic disease may be too frail to undergo biopsy. In one study, adverse events during biopsy were observed in 19.3% of patients; most commonly in 35.2% of thoracoscopies, 33.3% of surgical excisions, and 29.7% of core-needle biopsies. These adverse events most commonly included **pneumothorax** (13.5%), respiratory failure requiring ventilation (5.7%), and hemorrhage (1.5%).

Costs of conventional biopsy: Conventional biopsies are associated with variable and sometimes high costs, depending on the modality chosen. Data suggest that the costs of conventional biopsy are approximately $14,587 per procedure in lung cancer; any adverse event increases the mean cost per biopsy to $37,745.

1.2.4 A Brief Introduction to Liquid Biopsy

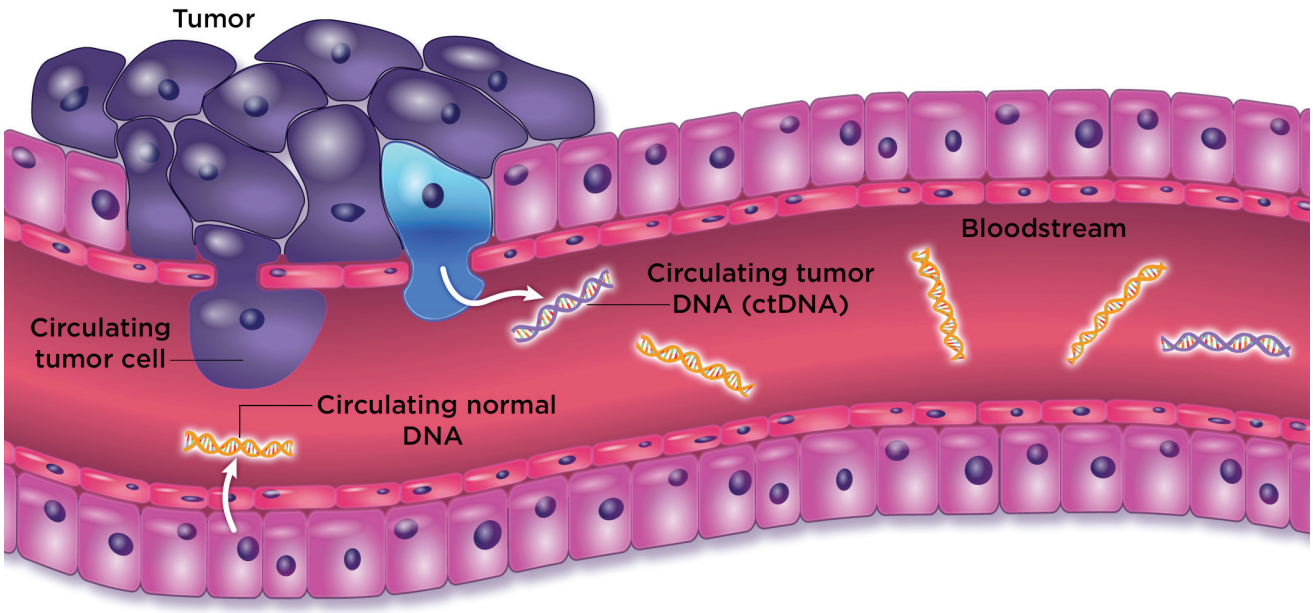
At present, tumor tissue remains the routine source for clinical molecular analyses; however, cancer-derived material circulating in the bloodstream has become an appealing alternative, showing promise for overcoming many of the challenges described above. Liquid biopsy is an increasingly accepted alternative to standard biopsies.

> You will receive detailed training on liquid biopsy, cell-free and circulating tumor DNA, and other concepts in this section elsewhere in this learning system.

Several sources of tumor material can be assessed by liquid biopsy (Figure 2):

- Cell-free or complexed nucleic acids including circulating cell-free DNA (cfDNA), of which a subset represents circulating *tumor* DNA (ctDNA). Cell-free DNA is composed of small fragments of DNA that are not associated with cells or cell fragments; instead, this DNA is derived from dead or dying tumor and normal cells that, as they die, release their contents—including their nucleic acids—into the bloodstream
- Cell-free RNA (cfRNA) as with cfDNA, cfRNA is derived from dead or dying cells that release RNA into the bloodstream (not shown in figure)
- Circulating tumor cells (CTCs) represent intact, viable non-blood cells with malignant features that can be isolated from blood (5). CTCs are released into the bloodstream during the spread of cancer through the bloodstream and present as single cells or clusters
- Exosomes (not shown in figure) are cell-derived, lipid-membrane bound packages that are released into the bloodstream. Exosomes may contain nucleic acids

Figure 2. Tumors Can Shed Cells or DNA Into the Bloodstream, Where They Can Be Collected in a Standard Plasma Sample



It is noteworthy that small tumors containing ~50 million malignant cells can release sufficient DNA for the detection of ctDNA in the blood. In contrast, the most advanced imaging technologies require the tumor to be of 7 to 10 mm in size, at which they contain approximately 1 billion cells or more.

Liquid biopsy provides the same information as tissue biopsies; however, it has a number of advantages over conventional biopsies:

- It is a source of fresh tumor-derived material that has not been subjected to preservatives like formalin
- It is minimally invasive and avoids the complications of biopsies and can be used in cases where tumor sampling is unavailable, inappropriate, or difficult to obtain. The minimal invasiveness also allows for the tumor to be followed over time for changes in driver mutations. In principle, this may allow clinicians to detect resistance mutations early—before clinical relapse—and institute appropriate therapy. It should be noted, however, that the clinical utility of ongoing monitoring with repeat liquid biopsies has not been established.
- The processing of whole blood and preparation of the sample is straightforward and is becoming more and more automated. Plasma-based testing may also have a substantially shorter turnaround time than tissue-based methods
- Liquid biopsies can better capture tumor heterogeneity when compared with a sectioned tissue sample, as the tumor nucleic acids derived from the blood are more likely to reflect the global molecular status of the patient

Lesson 2: Molecular Diagnostic Tests

2.0 Introduction

A broad range of technologies are available for the assessment of cancer biomarkers. This section will review some of the more common techniques, including:

- Fluorescence in-situ hybridization (FISH)
- Immunohistochemistry (IHC)
- Real-time polymerase chain reaction (PCR)
- Conventional sequencing
- Next-generation sequencing (NGS)

2.1 Overview and Key Terminology

There has been an explosion in the number of cancer treatments that address the specific needs of individual patients on the basis of genetic, biomarker, and/or tumor behavior that distinguish a particular patient's disease from that of other patients with similar clinical presentations. Biomarker-driven therapies address a specific molecular abnormality in the cancer cell, and—as the name suggests—are ideally used only in patients whose tumors have been quantitatively shown to have the abnormalities that are targeted by a specific therapy.

This approach has the potential to optimize the risk/benefit profile of treatment, not only by proactively identifying patients who have a higher likelihood of responding to the therapy, but also by ensuring that patients are not inappropriately exposed to treatments that will not address the pathophysiology of their tumor. As biomarker-driven treatments have become more common, biomarker assessment has become the standard of care in many types of cancer.

Molecular diagnostic testing has a language all its own. This section summarizes some of the key terms you should be familiar with in order to understand and discuss molecular diagnostics.

Biomarker-driven therapy may also be referred to as "targeted therapy," "precision medicine," "personalized medicine," or "individualized medicine." All terms emphasize the fact that treatment is tailored to the individual's disease characteristics.

2.1.1 *In Vitro* Diagnostics

The FDA defines *in vitro* diagnostics as "those reagents, instruments, and systems intended for use in the diagnosis of disease or other conditions, including a determination of the state of health, in order to cure, mitigate, treat, or prevent disease or its sequelae. Such products are intended for use in the collection, preparation, and examination of specimens taken from the human body."

In vitro diagnostics may also be used in precision medicine to identify patients who are likely to benefit from specific treatments or therapies. These *in vitro* diagnostics can include next-generation sequencing tests, which scan a person's DNA to detect genomic variations.

In vitro diagnostics for cancer fall into 2 broad categories: companion diagnostics (sometimes referred to by the acronym CDx) and laboratory-developed tests (LDTs).

Companion Diagnostics

According to the FDA, *companion diagnostics* are medical devices that provide information that is essential for the safe and effective use of a corresponding drug or biological product. Companion diagnostics help healthcare professionals determine whether a particular treatment's benefits to patients outweigh potential side effects or risks. If the diagnostic test is inaccurate, then the treatment decision based on that test may not be optimal.

Companion diagnostics can:

- Identify patients who are most likely to benefit from a particular therapeutic product
- Identify patients likely to be at increased risk for serious side effects as a result of treatment with a particular therapeutic product
- Monitor response to treatment with a particular therapeutic product for the purpose of adjusting treatment to improve safety or effectiveness

The US Food and Drug Administration (FDA) suggests that companion diagnostics be co-developed with drugs if there is an identified need for such a test.

Laboratory-developed Tests

A *laboratory-developed test* (LDT) is a type of *in vitro* diagnostic test that is designed, manufactured, and used within a single laboratory. While the uses of an LDT are often the same as the uses of FDA-cleared or -approved *in vitro* diagnostic tests, some labs may choose to offer their own test. For example, a hospital lab may run its own vitamin D assay, even though there is an FDA-cleared test for vitamin D currently on the market. The FDA does not consider diagnostic devices to be LDTs if they are designed or manufactured completely, or partly, outside of the laboratory that offers and uses them.

LDTs are important to the continued development of personalized medicine, so it is important that *in vitro* diagnostics are accurate so that patients and health care providers do not seek unnecessary treatments, delay needed treatments, or become exposed to inappropriate therapies.

2.1.2 Single-analyte vs Multiplexed Tests

Many molecular diagnostics use a one-gene, one-test approach (Table 4). These tests are often referred to as “single-analyte” tests. This approach is reasonable when there are only a few targets that require assessment; however, as the number of actionable targets has grown, it has quickly become clear that the ability to evaluate multiple targets in a single test is highly desirable. Tests that are able to simultaneously detect 2 or more targets in a single reaction are known as “multiplexed tests.”

Table 4. Single-analyte vs Multiplexed Tests

	Single Analyte	Multiplexed
FISH	✓	
IHC	✓	
Real-time PCR	✓	
Conventional sequencing	✓	
Next-generation sequencing		✓

2.1.3 Sequential vs Comprehensive Testing

Biomarkers may be tested simultaneously or sequentially. As you might imagine, as increasing numbers of genomic alterations are found to be clinically relevant and actionable, sequential or parallel testing of individual driver mutations using independent tests is becoming a significant resource and time challenge for clinical laboratories. Further, regardless of the test used, each requires tissue. As the number of tests grows, limited tissue from biopsies may constrain the number of tests that can be done. If sequential testing is chosen, the most commonly occurring biomarker is generally tested first; among patients who are negative for this biomarker, further tests may be conducted.

2.1.4 The Language of Molecular Abnormalities

At present, several genes have been identified in NSCLC that impact therapy selection. A number of different mutations can be seen at the level of the DNA sequence:

Point mutations are small, single-base changes in the DNA sequence. Although they affect only 1 base, they can change the amino acid sequence of a protein or even result in an abnormal shortening of the protein product.

Amplifications occur when regions of DNA are reproduced. This can lead to abnormally high amounts of the protein whose DNA coding region has been duplicated.

Indels or insertions and deletions, can result in changes in the function of the resulting protein or premature termination of that protein.

Fusions occur when parts of chromosomes invert or there is a swap between chromosomes. This can lead to an abnormal fusion protein that consists of parts of 2 different proteins or abnormal expression of a protein.

Note that PD-L1 is a protein marker that is also assessed in cancer, however, the assessment involves protein rather than genomic analysis:

You will learn more about specific molecular abnormalities in Module 2.

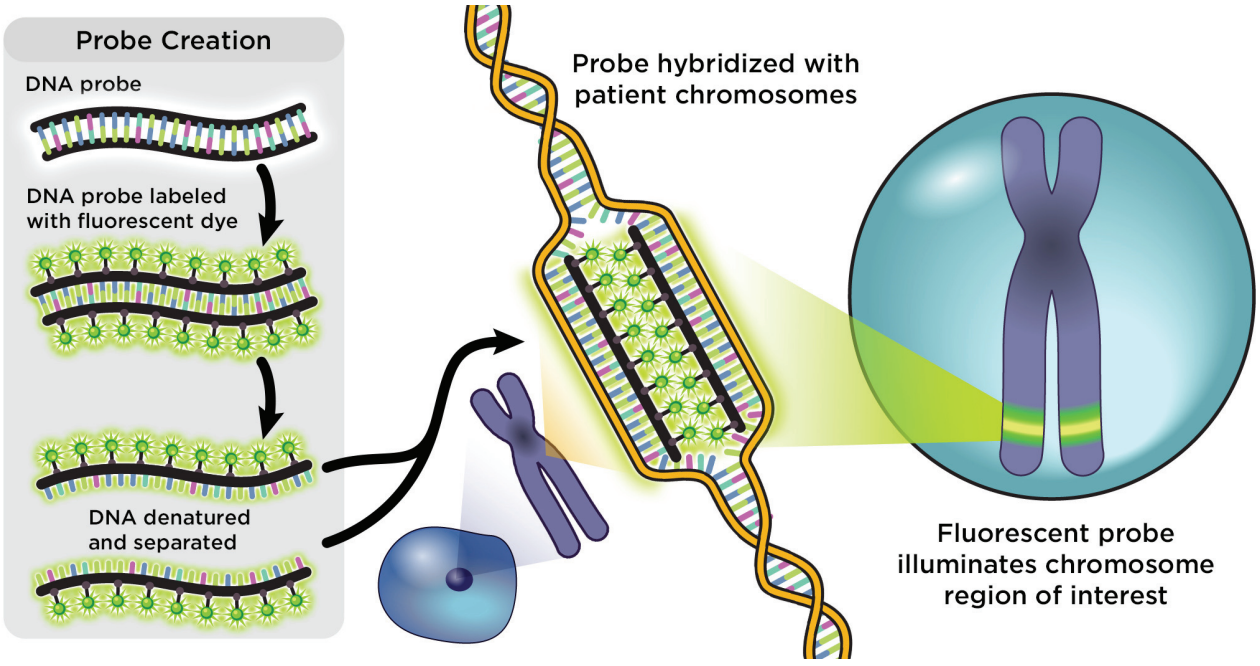
2.2 Molecular Diagnostic Modalities

2.2.1 Fluorescence In-situ Hybridization (FISH)

Fluorescence in-situ hybridization (FISH) is a single-analyte test that has multiple uses in cancer. First, short, single-stranded DNA that matches portions of a gene of interest is synthesized. These "probes" are labeled with a fluorescent dye.

DNA is composed of 2 strands of complementary molecules that bind to each other like chemical magnets. The fluorescently labeled DNA probes are able to bind to, or "hybridize," the complementary strand of DNA wherever it is located in the patient's chromosomes. The labeled probe allows for visualization of the location of that sequence. FISH is performed on cells fixed on a glass slide, which—after hybridization of probes—are viewed using a fluorescence microscope (Figure 3).

Figure 3. Steps in Fluorescence In-situ Hybridization



As an example, FISH is an approved companion diagnostic for the detection of *ALK* rearrangements in NSCLC, and may also be used for *ROS1*. In normal cells, the *ALK* and *EML4* genes are located in distinct areas of the short arm of chromosome 2. A small inversion in chromosome 2 results in the formation of a fusion gene that comprises portions of both the *EML4* gene and the *ALK* gene. This can be detected by FISH, which involves synthesizing 2 probes that are complementary to different parts of the *ALK* gene—one labeled with a red fluorescent dye, and the other with green fluorescent dye.

In normal cells without an *EML4-ALK* fusion (Figure 4a), the red and green signals are closely juxtaposed, and the cell—when viewed under a fluorescence microscope—shows either the red and green signals with little space between them, or yellow signals that are the result of complete juxtaposition of the 2 signals (Figure 4b). In cells with the *EML4-ALK* fusion, the ALK signals are "broken apart" – and thus the red and green signals are widely separated (Figures 4c and 4d). Positive cells are manually counted; a sample is considered positive if >25 cells out of 50 (>25/50 or >50%) are positive.

Figure 4a. Normal positions of *ALK* and *EML4* on the short arm of chromosome 2. The red and green probes are closely juxtaposed.

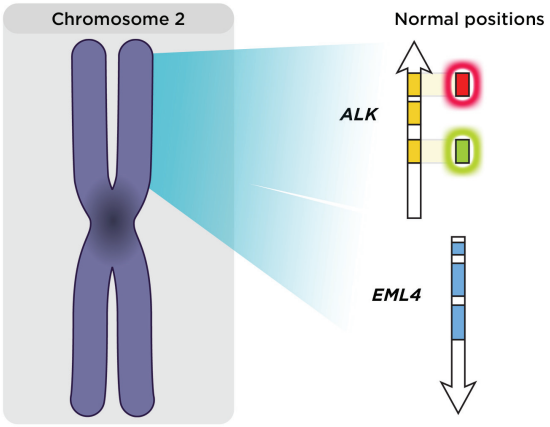


Figure 4b. Normal FISH result

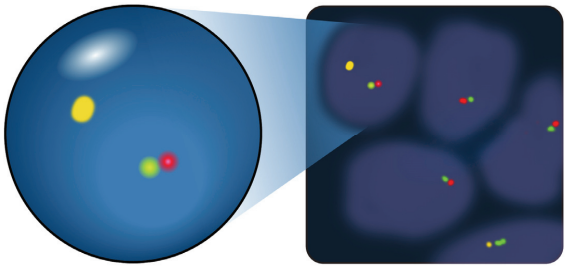


Figure 4c. A small inversion of chromosome 2 "breaks apart" the ALK signal.

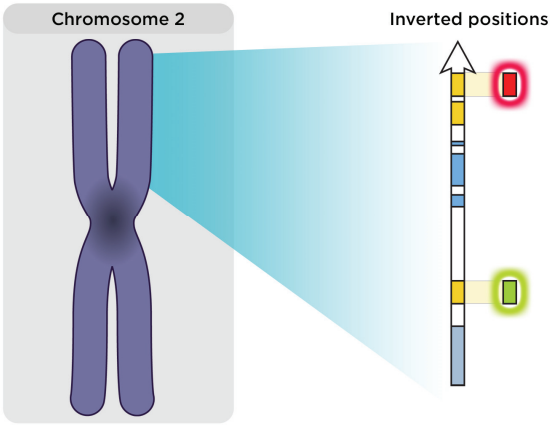
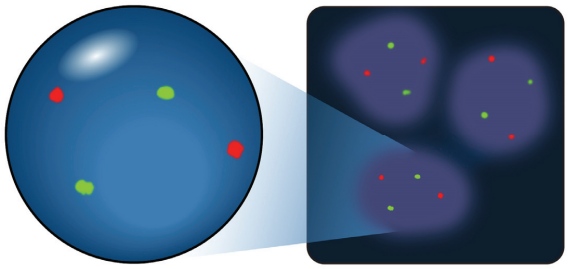


Figure 4d. Break-apart FISH signal, indicating the presence of an *EML4-ALK* fusion



2.2.2 Immunohistochemistry (IHC)

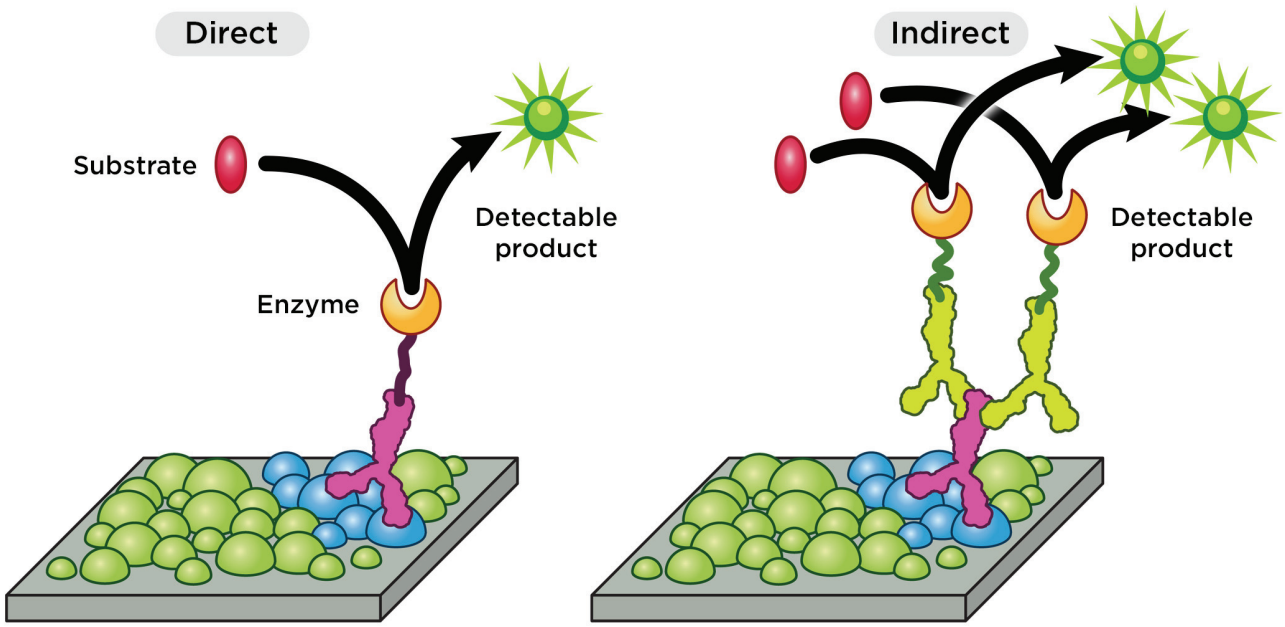
Immunohistochemistry is a single-analyte test that is used to detect proteins expressed in or on cells. In lung cancer, it is used for the detection of ALK and for PD-L1.

IHC utilizes highly specific **antibodies** that bind to specific proteins (antigens) inside the cell or on the cell surface of tissue fixed to a slide. The antibody can be labeled with a fluorescent molecule or with an enzyme for subsequent reaction with a substrate to create a colored, visible molecule. The enzymatic method may be direct or indirect:

- In the direct method, a labeled antibody specific for the protein of interest is applied to the tissue (Figure 5).
- In the indirect method, an unlabeled primary antibody is applied to the tissue and attaches to the antigen of interest. Then, in a second stage, a second, labeled antibody will bind with the primary antibody. Although more technically complex, indirect IHC allows for the amplification of signals, allowing small amounts of target protein to be detected.

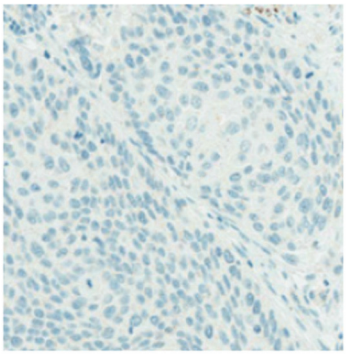
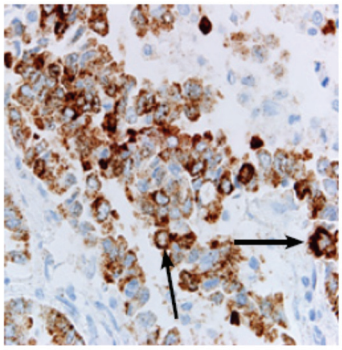
The choice of the method used depends on the level of expression of the target protein.

Figure 5. Direct and Indirect IHC



An example of an IHC assay for ALK is shown in Figure 6. As with FISH, IHC involves manual review and quantitation.

Figure 6. Interpretation of IHC, Using Example of an ALK Assay

Clinical Diagnosis Negative	Clinical Diagnosis Positive (Example)
 No staining	 Strong cytoplasmic staining tumor cells

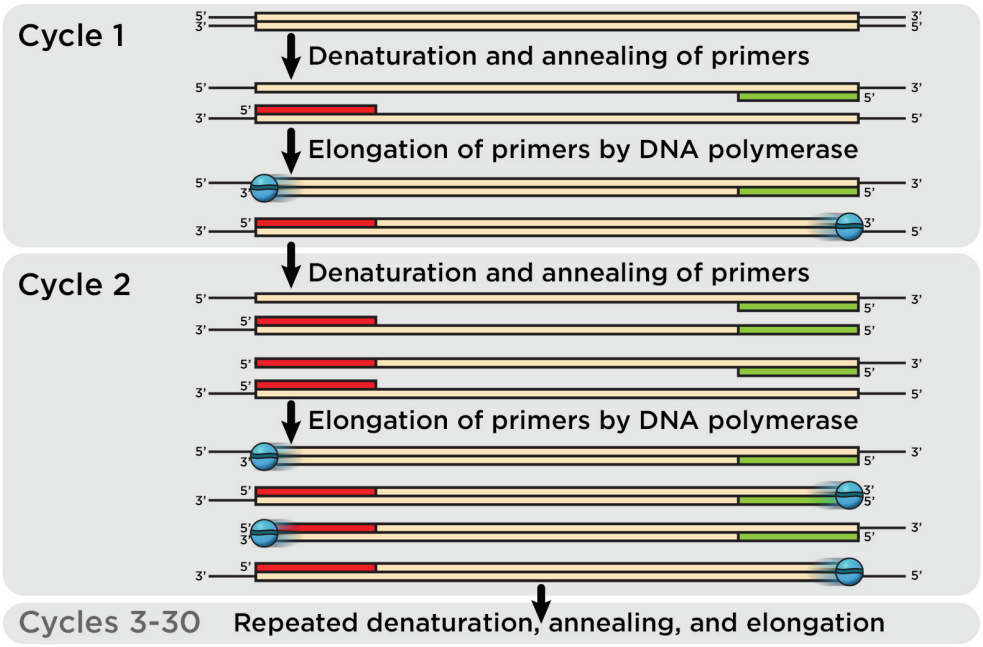
2.2.3 Real-time PCR

In NSCLC, real-time PCR is a single-analyte test used for the detection of certain mutations in EGFR. The cobas® EGFR Mutation Test is an approved companion diagnostic for Tarceva® (erlotinib) and Tagrisso® (osimertinib), treatments that are directed against EGFR. Use of real-time PCR requires knowledge of the most commonly occurring or clinically important mutations in a specific gene, and each real-time PCR test will detect only the selected mutations—mutations that are not specifically addressed by a particular test will not be detected.

First, let's examine the principles of basic PCR—a method for massively amplifying regions of interest in the DNA (Figure 7).

DNA is double-stranded. To amplify DNA, the double-stranded DNA and short, complimentary strands of DNA known as primers are mixed together in a tube. Heating the tube results in the 2 strands of DNA "denaturing," or separating into 2 single strands. Cooling the denatured DNA with the primers in the mix results in the primers binding, or "annealing," to their complimentary segments of DNA. This provides a starting point for **DNA polymerase**, the enzyme responsible for synthesizing DNA, to extend the primers, resulting in the production of a segment of DNA that is bounded on both sides by the primers. Repeating this process over and over—denaturing, annealing, and extension—results in massive amplification of the segment of DNA to be assessed.

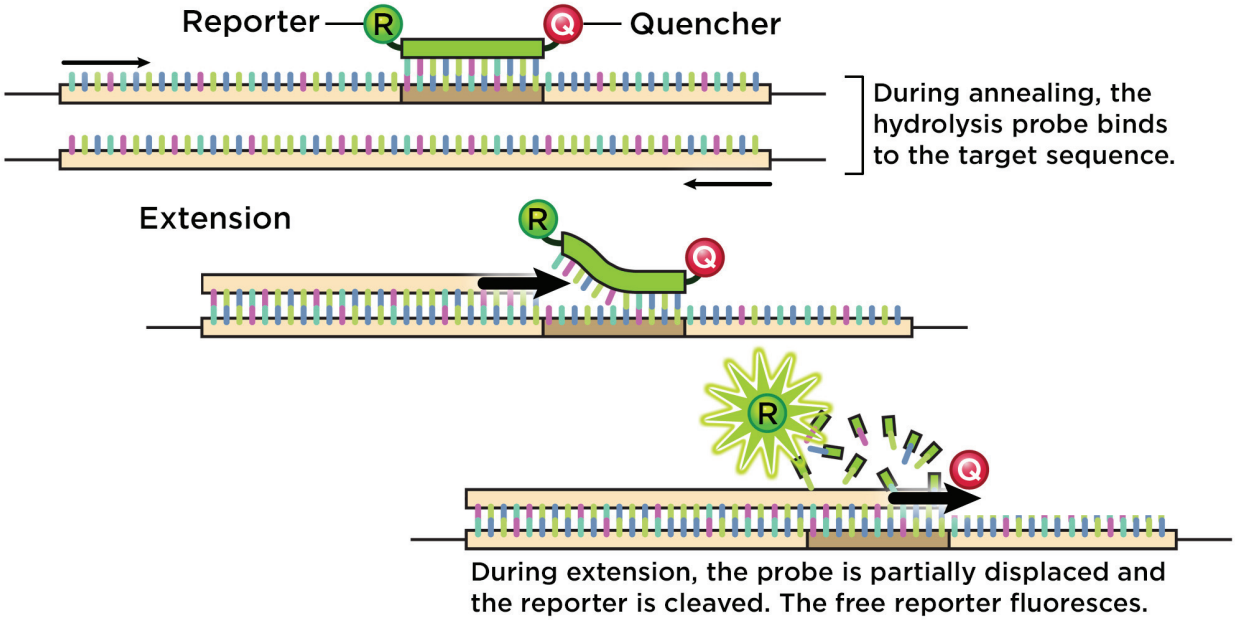
Figure 7. Basic PCR



The cobas EGFR test uses probes that amplify specific sequences, including each of the mutations detected by the test, using DNA derived from formalin-fixed, paraffin-embedded tumor tissue or plasma. Mutation-specific probes are included in the PCR mixture. During each cycle of amplification, the mutation-specific probe anneals to the site of the mutation, if present. Each probe includes a fluorescent dye and a quencher molecule.

When both the fluorescent dye and quencher molecule are attached to the probe, there is no fluorescence. During amplification, DNA polymerase cleaves the probe, separating the fluorescent dye and quencher and resulting in fluorescence. In this manner, the fluorescence of the sample increases only if the mutation is present (Figure 8). Multiple different mutations can be detected with the use of different-color fluorescent reporters.

Figure 8. Real-time PCR



2.2.4 Reverse-transcriptase PCR

Reverse-transcriptase PCR is a method in which RNA, isolated from a tumor sample, is first reverse-transcribed into complementary DNA (cDNA) by an enzyme known as reverse transcriptase. The cDNA generated by this reaction is then used as a template for PCR reaction. It can be performed as a single-step assay, in which both the reverse transcriptase and DNA polymerase are added to the same tube, or a 2-step assay, in which the reverse transcription and PCR steps are performed in different tubes. reverse-transcriptase PCR is not an approved companion diagnostic for any agent in NSCLC and is currently a laboratory-developed test only in this setting. Although it is highly sensitive when the right primer pairs are used, it has a high false-negative rate and failure rate. A technique known as quantitative reverse-transcriptase PCR (QPCR) is used in such conditions as chronic myeloid leukemia (CML) as a diagnostic tool and to follow the effects of treatment.

2.2.5 Conventional Sequencing

Conventional sequencing is a method of obtaining a base-by-base read of a specific gene. Direct DNA sequencing after PCR-based amplification was one of the earliest methods used to detect mutations in lung cancers, such as those involving *KRAS* and *EGFR*. This method was developed in the late 1970s by Frederick Sanger and is sometimes called Sanger sequencing. Sanger sequencing was more widely used in the past and has largely been supplanted by the multiplex or high-throughput assays that are described later in this module. Although the method is less frequently used for diagnostic purposes today, it is important to understand the general principles of how conventional sequencing works as a foundation for your understanding of next-generation sequencing technologies.

In Sanger sequencing:

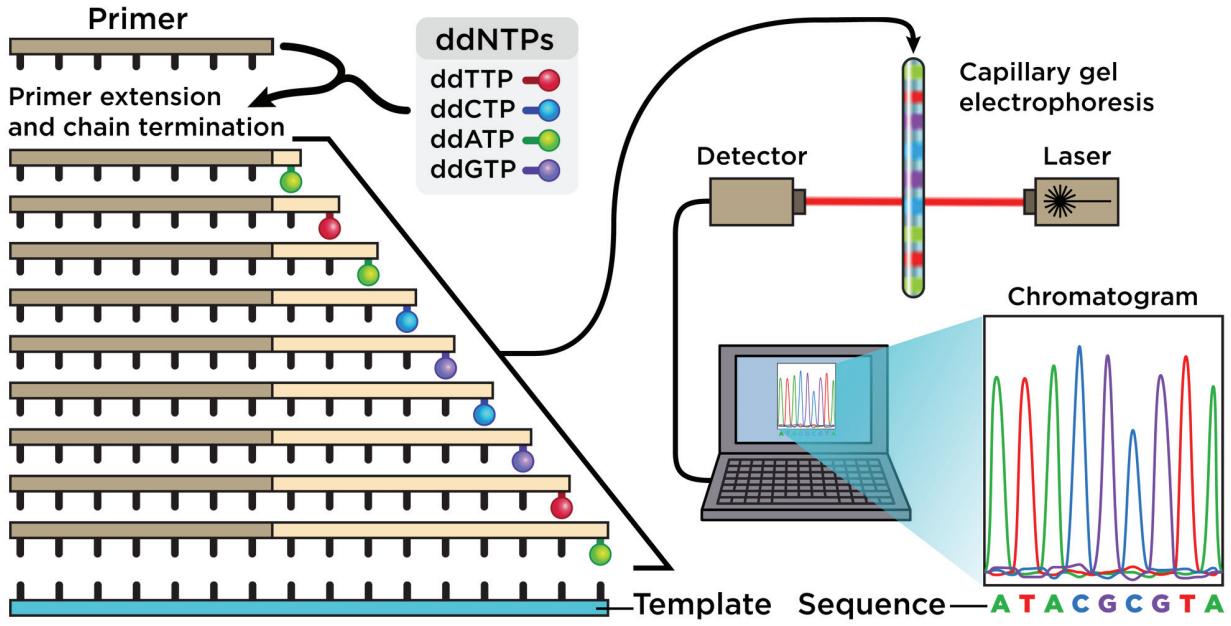
- The DNA sample is combined in 4 tubes with primer, DNA polymerase, and DNA deoxynucleotides (either dATP, dTTP, dGTP, or dCTP) (Figure 9). In addition, 4 dye-labeled, chain-terminating nucleotides, known as dideoxynucleotides, are added in much smaller amounts than the ordinary nucleotides.
- The mixture is first heated to denature the template DNA (separate the strands).
- The mixture is cooled so that the primer can anneal to the single-stranded template.
- Once the primer has bound, the temperature is raised again, allowing DNA polymerase to synthesize new DNA starting from the primer.
- DNA polymerase will continue adding nucleotides to the chain until it adds a terminator dideoxynucleotide instead of a normal one. At that point, no further nucleotides can be added, so the strand will end with the fluorescently labeled dideoxynucleotide.

This process is repeated in a number of cycles. By the time the cycling is complete, it is virtually guaranteed that a fluorescent terminator dideoxynucleotide will have been incorporated at every single position of the target DNA in at least one reaction. This will result in a sample containing fragments of different lengths, each of which ends at a nucleotide position in the original DNA.

After the reaction is complete, the fragments are run through a long, thin tube containing a gel matrix—in a process called capillary gel electrophoresis. Short fragments move quickly through the pores of the gel, while long fragments move more slowly. As each fragment crosses the “finish line” at the end of the tube, it is illuminated by a laser, allowing the attached dye to be detected.

The smallest fragment (ending 1 nucleotide after the primer) crosses the finish line first, followed by the next-smallest fragment (ending 2 nucleotides after the primer), and so forth. The colors of dyes are registered one after another on the detector; thus, the sequence of the original piece of DNA can be read one nucleotide at a time. The data recorded by the detector consist of a series of peaks in fluorescence intensity. The DNA sequence is read from these peaks.

Figure 9. Conventional (Sanger) Sequencing



Lesson 3: Next-generation Sequencing

3.0 Introduction

Like Sanger sequencing, next-generation sequencing (NGS) technologies produce a base-by-base read of target genes. While the Sanger method requires separate steps for sequencing, separation, and detection, NGS technologies sequence in a massively parallel, high-throughput manner that allows for multiple genes of interest to be rapidly evaluated at comparatively low cost. In fact, the human genome sequencing projects that took years with Sanger sequencing can now be completed in hours with NGS.

NGS, in general, is conducted in 4 steps:

1. Library preparation, in which random fragments of DNA from the subject are ligated to custom linkers
2. Amplification, in which the random fragments of DNA are amplified using various techniques
3. Sequencing, in which the amplified DNA is read base-by-base
4. Analysis and reporting of results

The first 3 of these steps occurs in a “wet lab”— a laboratory in which chemicals, drugs, or biological matter are tested and analyzed. The last step occurs in a “dry lab”—a laboratory where computers are used for analysis.

At present, 2 NGS tests are FDA-approved companion diagnostics in NSCLC:

- The ThermoFisher Oncomine™ Dx Target Test is an FDA-approved companion diagnostic for *EGFR*, *ROS1*, and *BRAF V600E*
- The Foundation Medicine FoundationOne test is an FDA-approved companion diagnostic for *EGFR*, *ALK*, and *BRAF V600E*

Both NGS tests also evaluate broad panels of other genes of interest in NSCLC.

Note that the Oncomine Dx Target Test and FoundationOne test are approved for different subsets of the 4 key targets in NSCLC that can be evaluated by sequencing (EGFR, ALK, ROS1, BRAFV600E; the fourth, PD-L1, involves protein expression and cannot be evaluated by sequencing).

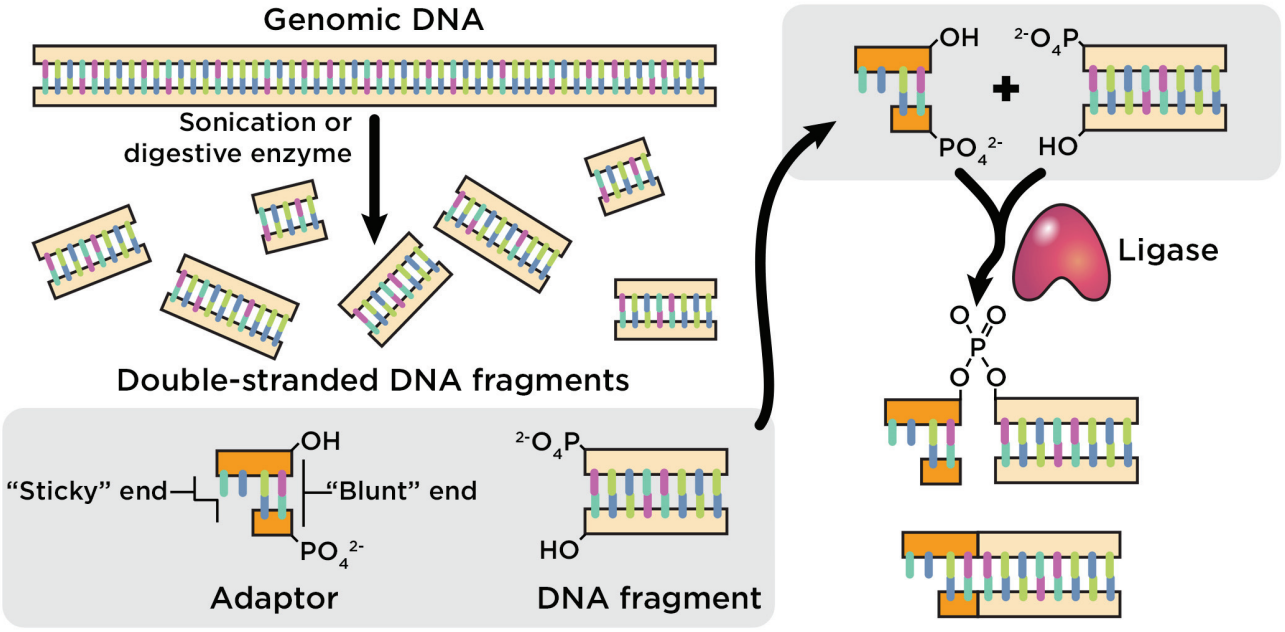
NGS technologies will be covered in greater detail in Phase 2 training.

3.1 Library Preparation

DNA derived from the sample is fragmented, either using enzymes or by sonication. Adapters—which are short, double-stranded pieces of synthetic DNA—are enzymatically joined to these fragments in a process known as ligation. These adapters serve as the “landing pad” for complementary primers, allowing the random sequences that are obtained through fragmentation to be amplified (Figure 10).

Figure 10. Library Preparation

In the first steps of NGS, the DNA is fragmented, and adapters are ligated to the ends of the resulting fragments, generating a library of DNA fragments, each of which is bounded by DNA adapters on each end.



3.2 Amplification

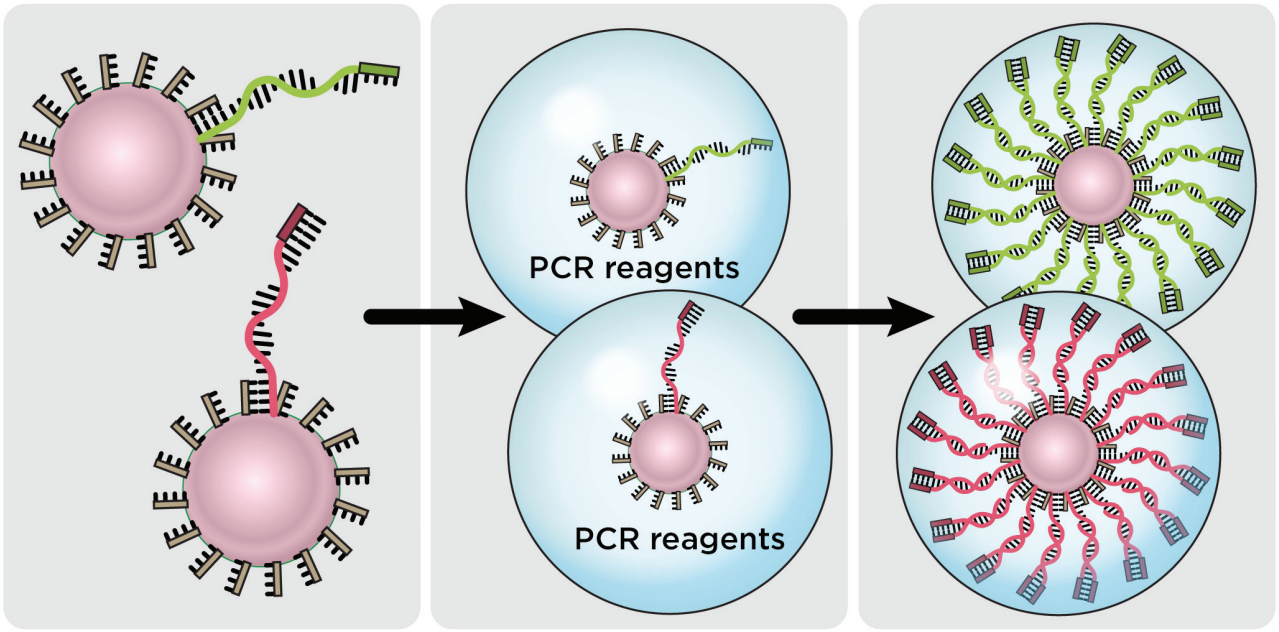
The next step is amplification of each fragment. This is required so that the signal from the sequencer is strong enough to be detected accurately.

3.2.1 Emulsion PCR

Emulsion PCR, with minor differences, is the amplification technique used by Roche 454, ABI SOLiD, and ThermoFisher Ion Torrent protocols. The process is summarized in Figure 11.

Figure 11. Emulsion PCR

In (a), adapters are used to capture single molecules of template onto microbeads by primer hybridization. In (b), beads are incorporated into a carefully controlled emulsion in which each bubble constitutes a microreactor containing DNA template, primer and reagents for PCR. (c) Following amplification, each bead is coated with clonally amplified molecules.

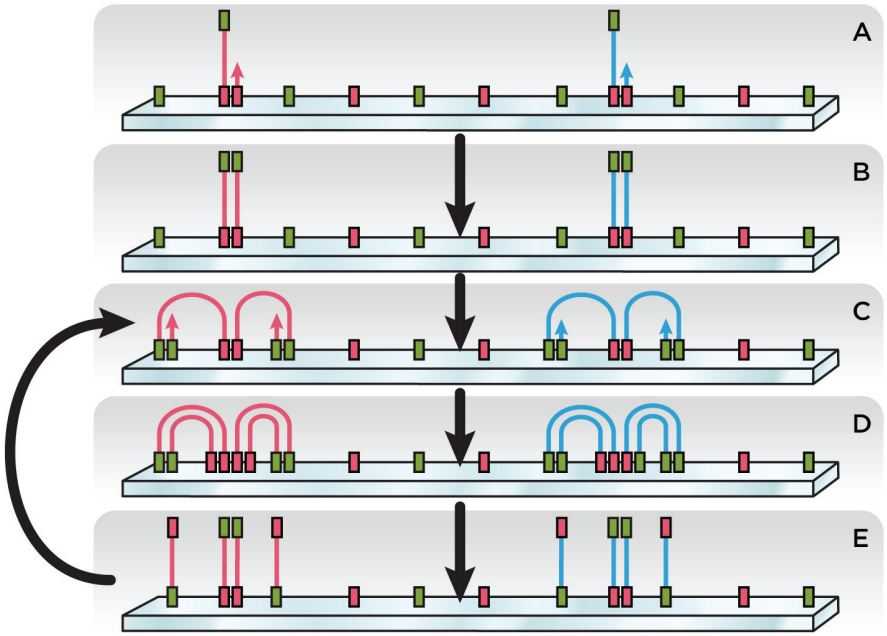


3.2.2 Bridge Amplification

Bridge amplification is the technique used by Illumina. The process is summarized in Figure 12.

Figure 12. Bridge Amplification

In (a), a single-stranded template is annealed to a glass plate by hybridization to a complementary primer; (b) The primer forms the basis for extension; (c) The free end of each single-stranded molecule can anneal to a second anchored primer in close spatial proximity, forming a “bridge” that acts as a template for (d) a second round of amplification; This results in (e) 4 single-stranded linear molecules; Stages (c)–(e) are repeated to generate clonally amplified islands or clusters for subsequent sequencing.

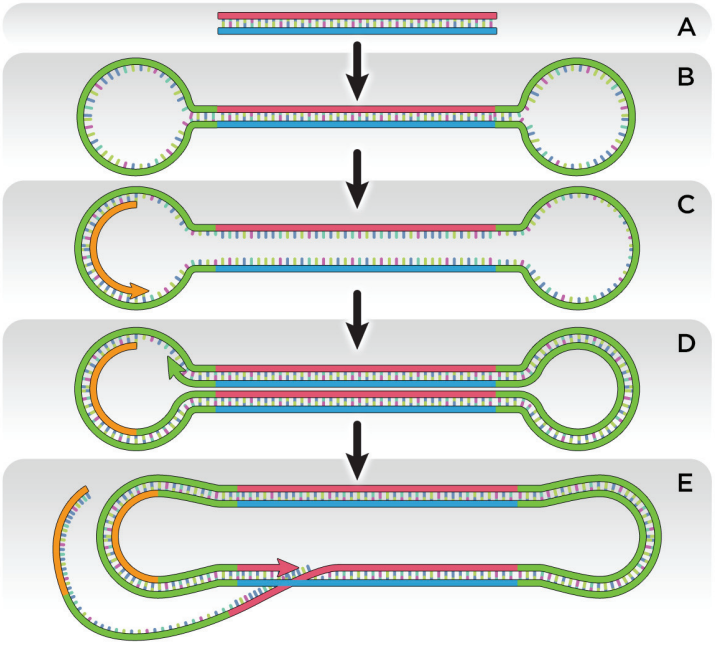


3.2.3 Linear Amplification

Linear amplification is the process used by the Pacific Biosciences protocol. The process is summarized in Figure 13.

Figure 13. Linear Amplification

(a) Double-stranded DNA is used as the template; (b) “Hairpin” adapters are bound to the ends of the double-stranded DNA, creating a single-stranded circular template; (c) A primer that is complementary to the hairpin sequence is bound as a starting point for replication; (d–e) Linear amplification and strand.



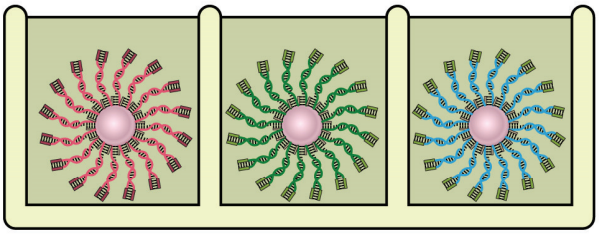
3.3 Sequencing

After the library has been amplified, the next step is to get a base-by-base read of each DNA fragment. As with amplification, there are a number of different technologies used in this process.

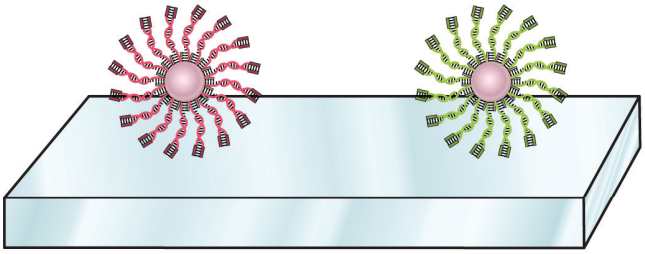
3.3.1 Separating Sequencing Reactions

In order to sequence the fragments, each fragment must be somehow separated from the others. There are a number of ways that this can be done (Figure 14).

Figure 14. Separating Sequencing Reactions

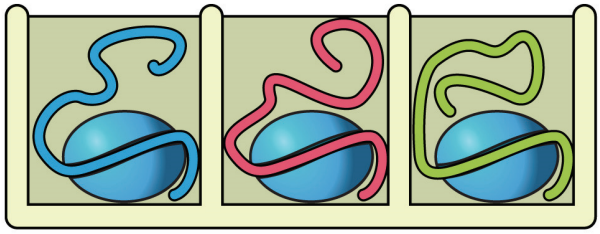


In Roche 454 or ThermoFisher protocols, microbeads are separated into a high-density array, such that each well contains a single bead.



In SOLiD sequencing, beads are attached to the surface of a glass slide.

The Illumina process generates separate "islands" of amplified product.



In the PacBio protocol, the amplified molecule is captured by a single DNA polymerase molecule at the bottom of a microcell.

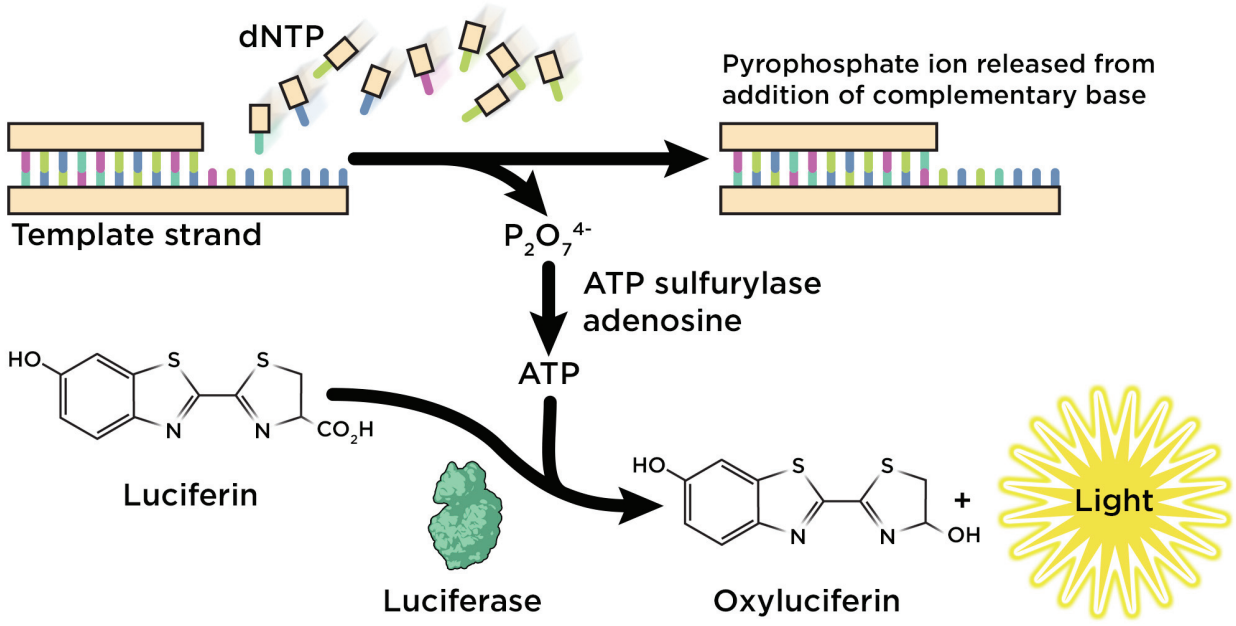
3.3.2 Sequencing

Pyrosequencing

Pyrosequencing is based on the "sequencing by synthesis" principle, where a complementary strand is synthesized in the presence of polymerase enzyme (Figure 15). Pyrosequencing detects the release of a pyrophosphate, which occurs when a nucleotide is added to the growing DNA chain.

The process begins when a single-stranded primer hybridizes to the end of the strand. The 4 different dNTPs sequentially flow into and out of the wells. When the correct dNTP is incorporated into the growing DNA strand, it results in the release of pyrophosphate. The pyrophosphate is converted into ATP, which catalyzes the conversion of luciferin to oxyluciferin, which produces light that can be detected with a camera.

Figure 15. Pyrosequencing Detects Release of Pyrophosphate When Nucleotides Are Added to DNA Chain

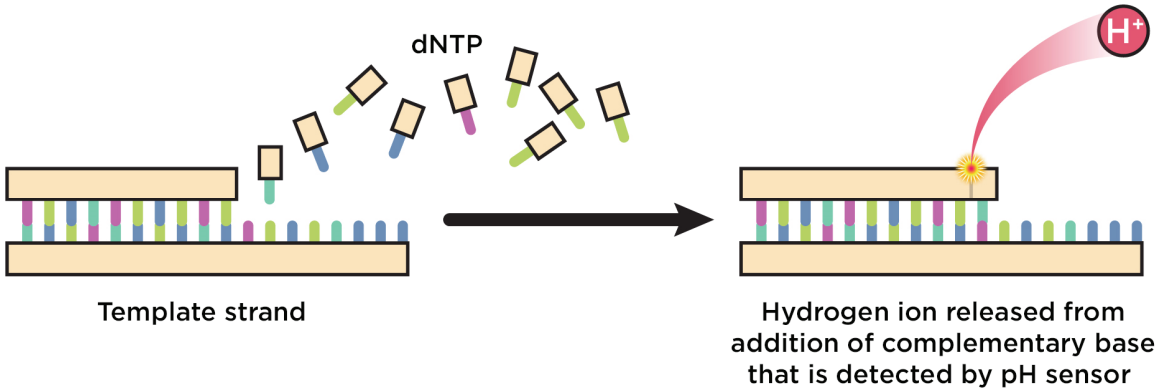


ThermoFisher Ion Torrent Sequencing

Ion torrent sequencing is based on the "sequencing by synthesis" principle, where a complementary strand is synthesized in the presence of polymerase enzyme (Figure 16). Ion Torrent™ Sequencing detects the release of hydrogen ions, which changes the pH inside of the microwell.

Figure 16. Ion Torrent Sequencing

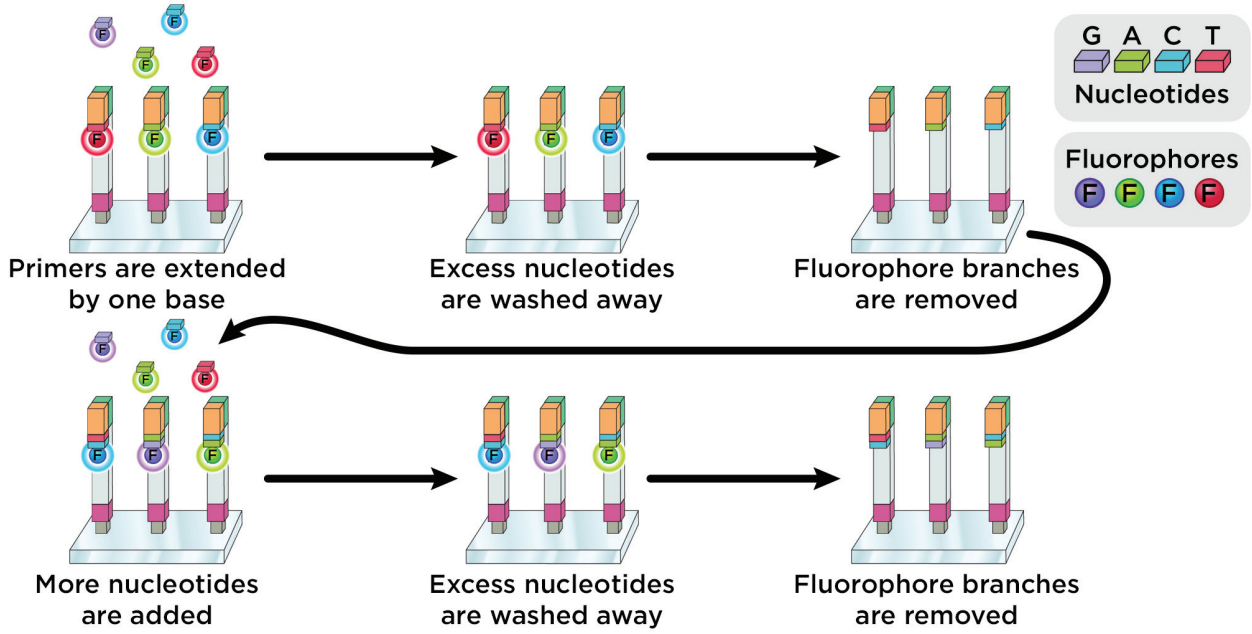
This method detects the release of hydrogen ions, which occurs when the correct nucleotide is incorporated at a specific position.



Illumina Reversible Terminator Sequencing

Reversible terminator sequencing uses fluorescently labeled reversible terminators, such that each single base incorporation on each molecule temporarily terminates the reaction (Figure 17). A high-resolution digital image is used to determine which nucleotide is incorporated in each DNA clonal cluster. After imaging, the terminator is reversed chemically, allowing the template molecule to be extended again in the next round of sequencing.

Figure 17. Reversible Terminator Sequencing



Third-generation Sequencing

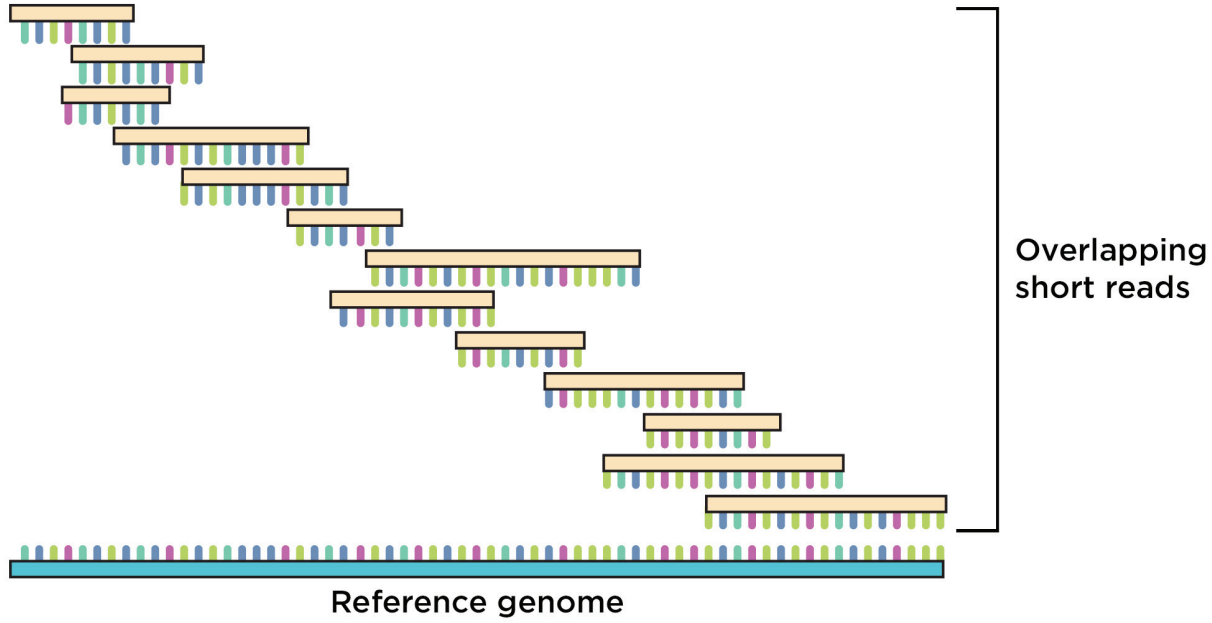
New techniques have been developed that allow for single-molecule sequencing. These techniques remove the need for PCR amplification, potentially reducing errors caused by PCR, reducing the complexity of library preparation, and providing greater read length. Two platforms—Pacific Biosciences (PacBio) SMRT Sequencing and Helicos BioSciences—utilize these technologies.

3.4 Analysis and Reporting of Results

Current NGS technologies generate short, overlapping, and duplicative reads that redundantly span the sequence, thus NGS generates much more raw data than would be expected based on a single, end-to-end read of the entire sequence (Figure 18). This sequence data must be aligned to a reference genome; moreover, since sequencing is not perfect, every base has a score attached to it to evaluate the quality of sequencing.

Figure 18. NGS Short-read Sequences

NGS generates short-read sequences that must be aligned, either internally, if no reference genome is available, or to a reference genome.



Because of the volume of data generated by NGS, it has been necessary to develop file formats that code the maximum amount of information in the minimum amount of space.

Raw Data Output

The raw output of Illumina-based NGS machines, as an example, is in the .bcl output—a binary file that contains both the base call and the quality of that base call. The .bcl format, however, is not useful as is for further analysis. In order to conduct further analyses, it is converted to the universally used FASTQ format. FASTQ, a text-based format that stores the nucleotide sequence and its corresponding quality scores, is one of the more commonly used file formats (Figure 19).

Figure 19. Example of NGS File Format—FASTQ

The FASTQ format normally includes 4 lines: Line 1 begins with a "@" and is followed by a sequence identifier. Line 2 is the raw sequence. Line 3 begins with a "+".Line 4 encodes the quality value for each base in Line 2 and contains the same number of characters as in the sequence.

NGS file format—FASTQ

```
@SEQ_ID
GATTTGGGGTTCAAAGCAGTATCGATCAAATAGTAAATCCATTTGTTCAACTCACAGTTT
+
!''*((((***+))%%%++)(%%%%).1***-+*''))**55CCF>>>>>>CCCCCCC65
```

Sequence Alignment and Analysis

Alignment of sequenced FASTQ data will result in the generation of a SAM file, which is the universal file format for mapped sequence reads. This format contains the sequence and quality scores of each read, but is more detailed as it also specified information about the location in the genome and the confidence of the aligner in mapping that location, as well as other information. A BAM file is the compressed binary version of SAM, and is otherwise identical. Other file formats include the Variant Call Format (VCF), which is a standardized text file that provides information on single-nucleotide polymorphisms, insertions and deletions, and structural variations.

Module Summary

- Molecular diagnostics is a term used to describe a broad collection of techniques for detecting, diagnosing, and manage disease by studying molecules, such as DNA, RNA, and protein in a tissue or fluid.
- Cancer biomarkers may be used to:
  - Estimate risk for developing cancer
  - Screen for cancer
  - Determine prognosis
  - Predict response to therapy
  - Monitor for response or progression
- Pathologists and the pathology team perform and interpret molecular diagnostics.
- A variety of techniques are used for obtaining tissue for analysis, ranging from entirely noninvasive modalities to direct excision of tumor tissue during surgery.
- Conventional biopsies have been the gold standard for biomarker testing; however, they are associated with a number of important limitations, including tissue availability, sampling bias, difficulties associated with repeated biopsies to follow tumor evolution, the need for tissue fixation, and the risks and costs of conventional biopsy.
- Molecular diagnostics provide information on cancer biomarkers that can be used to select appropriate treatments:
  - FISH utilizes labeled probes to assess chromosomal rearrangements
  - IHC uses specific antibodies to evaluate for protein expression
  - Real-time PCR selectively identifies mutations at the sequence level
  - Conventional (Sanger) sequencing provides a base-by-base read of the sequence of a single gene
- Like Sanger sequencing, next-generation sequencing technologies produce a base-by-base read of target genes.
  - While the Sanger method requires separate steps for sequencing, separation, and detection, NGS technologies sequence in a massively parallel, high-throughput manner that allows for multiple genes of interest to be rapidly evaluated at comparatively low cost
- All current NGS technologies follow the following steps; the exact technologies that are used in each of these steps vary
  - Library preparation, in which the DNA is fragmented and linkers attached
  - Amplification, during which DNA fragments are amplified
  - Sequencing, during which the amplified DNA fragments are read base-by-base
  - Analysis and reporting of results

Glossary

Antibody: a blood protein, often Y-shaped, that is produced in response to and counteracting a specific antigen

DNA polymerase: The enzyme responsible for synthesizing DNA

Flow cytometry: A technology used for cell counting, sorting, and biomarker detection. Involves suspending cells in a stream of fluid and passing them through an electronic detection apparatus

Hematology: The study of the blood

Nucleic acids: DNA and RNA

Pleural fluid: A fluid surrounding the lungs; may build up excessively in patients with lung disease

Pneumothorax: Collapsed lung

Squamous: A type of epithelial cell that appears very thin and flattened

Study Questions

1. In NSCLC, all of the following biomarkers are associated with approved therapies, except:
   A. PD-L1
   B. *ROS1*
   C. BRAF V600E
   E. KRAS
   F. *EGFR*
   G. ALK

2 Which of the following techniques is/are routinely used to *directly* identify gene fusions? (Select all that apply.)
   A. IHC
   B. FISH
   C. Real-time PCR
   D. NGS

3. Which of the following members of the pathology laboratory team is responsible for managing tissue processing and preparing slides for evaluation by the pathologist?
   A. Pathologist assistant
   B. Cytotechnologist
   C. Histotechnologists

4. Adverse events are observed in approximately ____% of conventional biopsies.
   A. 10
   B. 19
   C. 23
   D. 41

5. All of the following are single-analyte tests *except*:
   A. FISH
   B. NGS
   C. Real-time-PCR
   D. Conventional sequencing
   E. IHC

6. Which of the following molecular diagnostics detects *protein*, as opposed to relying on nucleic acids?
   A. IHC
   B. FISH
   C. NGS
   D. Real-time PCR

7. Which of the following tests involves the use of a reporter probe consisting of a reporter and quencher?
   A. IHC
   B. FISH
   C. NGS
   D. Real-time PCR

8. Which of the following correctly describes the general steps of NGS?
   A. Amplification → sequencing → library preparation → analysis
   B. Library preparation → sequencing → amplification → analysis
   C. Sequencing → amplification → library preparation → analysis
   D. Library preparation → amplification → sequencing → analysis

9. Which of the following sequencing technologies relies on the liberation of a hydrogen ion, changing local pH?
   A. Ion Torrent sequencing
   B. Pyrosequencing
   C. Reversible terminator sequencing
   D. Third-generation sequencing

10. True or false: sequencing data output in .bcl form is ready to use for analysis
    A. True
    B. False

11. Cancer biomarkers are used in a number of ways, including which of the following? Select all that apply.
    A. Estimating the risk of developing cancer
    B. Determining the prognosis of disease
    C. Predicting a response to therapy
    D. Screening for disease
    E. All of the above

12. Cancer biomarkers can be used to monitor for therapeutic response or disease progression. True or false?
    A. True
    B. False

13. What definition most closely matches cytopathology?
    A. The examination of single cells or small groups of cells
    B. The examination of intact tissues under the microscope
    C. None of the above

14. Which of the following is a main subdivision of anatomic pathology? Select all that apply.
    A. Hematopathology
    B. Cytopathology
    C. All of the above
    D. None of the above

15. What most closely defines thoracentesis?
    A. Examination of microscopic cells found in sputum
    B. Insertion of a fine needle into the tumor site
    C. Removal of pleural fluid for cytological examination
    D. Insertion of an endoscope through a small incision in the chest wall

16. Fill in the blank: A _____ involves the insertion of a fine needle into the site of the tumor, removing a core of tumor issue.
    A. Thoracentesis
    B. Percutaneous needle biopsy
    C. Stereotactic biopsy
    D. Open surgical biopsy

17. How much tissue is required for immunohistochemistry?
    A. One slide of adequate tumor material
    B. Two slides of adequate tumor material
    C. Three slides of adequate tumor material
    D. Up to 10 slides of adequate tumor material

18. Fill in the blank: Intratumor sampling bias is when the evaluation of a single section of fixed tumor _____.
    A. Limits access to targetable mutations within that tumor
    B. Limits access to the genetic profile of metastatic sites
    C. Limits access to future targetable mutations due to tumor evolution
    D. None of the above

19. Fill in the blank: A study of traditional tissue-biopsy patients found that adverse events were observed in _____ patients.
    A. 35.2%
    B. 25.4%
    C. 19.3%
    D. 10.2%

20. Approximately, how much does a conventional lung cancer tissue biopsy cost, if the patient does not experience an adverse event?
    A. Less than $2,000
    B. Between $2,000- $8,000
    C. Between $14,000- $16,000
    D. Above $40,000

21. FISH is a companion diagnostic for the detection of which genomic mutations?
    A. BRAF
    B. EBBB2
    C. ALK
    D. EGFR
    E. ROS1

22. Fill in the blank: Immunohistochemistry, a single-analyte test that is used to detect proteins expressed in or on cells, is used to detect _____ in lung cancer.
    A. RET and p53
    B. BRAF and ROS1
    C. ALK and PD-L1
    D. MET and RET

23. What is real-time PCR?
    A. A single-analyte test used to detect certain mutations in EGFR
    B. A multiplexed test used to detect certain mutations in EGFR
    C. A single-analyte test used to detect certain mutations in ERBB2 (HER2)
    D. A multiplexed test used to detect certain mutations in ERBB2 (HER2)

24. Fill in the blank: Real-time PCR _____.
    A. Utilizes labeled probes to assess chromosomal rearrangements
    B. Uses specific antibodies to evaluate for protein expression
    C. Provides a base-by-base read of the sequence of a single gene
    D. Selectively identifies mutations at the sequence level

STUDY ANSWERS

1. D. KRAS
2. B. FISH; D. NGS
3. C. Histotechnologists
4. B. 19
5. B. NGS
6. A. IHC
7. D, Real-time PCR
8. D. Library preparation → amplification → sequencing → analysis
9. A. Ion Torrent sequencing
10. B. False
11. E. All of the above - Estimating the risk of developing cancer; Determining the prognosis of disease; Predicting a response to therapy; Screening for disease
12. A. True
13. A. The examination of single cells or small groups of cells
14. C. All of the above. Cytopathology; Histopathology
15. C. Removal of pleural fluid for cytological examination
16. B. Percutaneous needle biopsy
17. B. Two slides of adequate tumor material
18. A. Limits access to targetable mutations within that tumor
19. C. 19.3%
20. C. Between $14,000- $16,000
21. C, D. ALK; ROS1
22. C. ALK and PD-L1
23. A. A single-analyte test used to detect certain mutations in EGFR
24. D. Selectively identifies mutations at the sequence level

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