

LIQUID BIOPSY TUTORIAL

FOR RESEARCH ADVOCATES



Research Advocacy Network

Advancing Patient-Focused Research

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Chapter 1: Introduction to Liquid Biopsies

Several months ago, Glenda learned that she had a suspicious mass of tissue in her left lung. Her doctor surgically removed a small piece of the tissue for analysis and, after looking at it under the microscope, confirmed that it was cancerous. The question that Glenda and her doctor then asked was which of the available treatments was best for her particular cancer? Not enough tissue was left for more specific tests that might help answer this question. Glenda was faced with having to undergo another surgery to get more of the cancerous tissue, but she was not happy at the prospect of another surgery and her doctor was worried that the tumor was in a sensitive location. Fortunately, medical advances have led to an alternative type of test that may be useful for Glenda and her doctor: the liquid biopsy.

As it pertains to cancer, a liquid biopsy is the sampling and analysis of biological liquids to potentially help identify cancer, develop treatment plans, and detect recurrences. In the story above, the liquid biopsy is being used to help determine which type of treatment is best matched to Glenda's tumor abnormalities and therefore may be the most effective. However, liquid biopsies have many other potential uses that we will explore in subsequent chapters.

A LIQUID BIOPSY is the sampling and analysis of biological liquids.

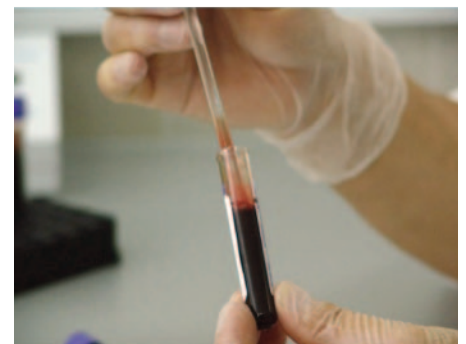
At the writing of this tutorial, only two liquid biopsy tests are commercially available in the United States: 1) cobas® EGFR Mutation Test v2 and 2) CellSearch® Circulating Tumor Cell Test. The former is used to determine whether certain lung cancers contain a specific type of mutation (change in DNA) that can be targeted with a medication. The latter is used to determine the circulating tumor cell status of patients with metastatic breast, prostate, or colorectal cancers. This information can indicate whether patients have a favorable or unfavorable prognosis. Many more liquid biopsies are in development and these are poised to gain widespread use. This will take time and effort on the part of scientists, physicians, advocates, collaborative consortia and patients. Although we all want more liquid biopsies available as soon as possible, it is important that these tests are supported by strong data before we can rely on them to help us make life-altering decisions. This means waiting for the results of rigorous scientific and medical tests, many of which are ongoing.

This tutorial gives an overview of liquid biopsies, along with an explanation of what they detect and how they can be used. The tutorial then considers the methods and techniques behind liquid biopsies, followed by a discussion of test validation and clinical utility. The final chapter describes ways advocates can use this information. We begin here with a definition of liquid biopsies.

What Is a Liquid Biopsy?

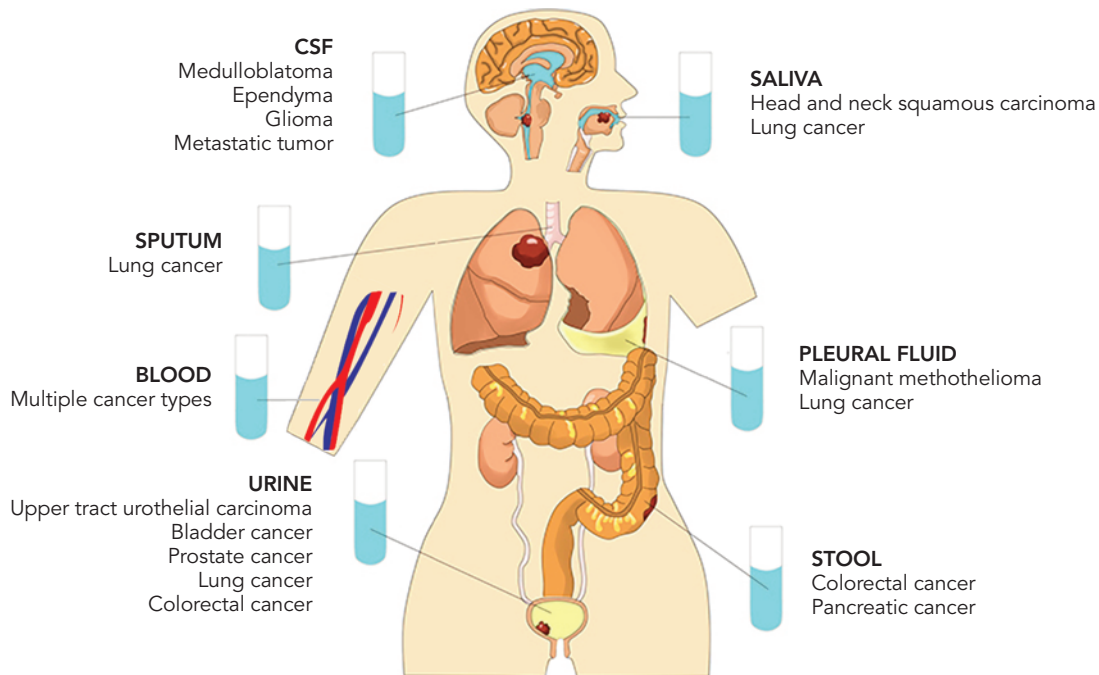
We've already noted that a liquid biopsy is the sampling and analysis of biological fluids. All of us have undergone a form of liquid biopsy whether we realize it or not—it's just been called a blood test. Blood tests have been used for decades to sample levels of glucose, hormones, cholesterol, calcium, red blood cells, white blood cells, and other blood components.

Most of us have also had urinalyses, or tests performed on our urine. Urine tests are commonly used in medical clinics to examine levels of hormones and drugs, to determine pregnancy, and to help diagnose diabetes, urinary tract infections, and other diseases. Indeed, urine tests have been used for many years to help diagnose bladder cancer, which can be associated with blood and cancer cells in the urine. Like blood tests, urinalyses are also liquid biopsies.



Other biological fluids that may be the target of liquid biopsies are saliva, sputum (liquid coughed up from the respiratory tract), pleural fluid (liquid in the lungs), and cerebrospinal fluid (the liquid surrounding brain and spinal cord). Given that all of these biological liquids are fluids, liquid biopsies are also sometimes called fluid biopsies.

So, why call these tests liquid biopsies? Why not call them blood tests or urinalyses? Even though the former terms are technically correct, the phrase liquid biopsies emphasizes their similarity to solid tissue biopsies in terms of the information obtained and potential clinical uses.



Biological Liquids Used in Liquid Biopsies

Graphic adapted from Peng M, Chen C, Hulbert A, Brock MV, Yu V. Non-blood circulating tumor DNA detection in cancer. *Oncotarget*. 2017; 8:69162-69173. <https://creativecommons.org/licenses/by/3.0/>

Why Have Liquid Biopsies Recently Become So Popular in Cancer?

If liquid biopsies have been around for years, why have they recently become such a popular topic in cancer? Several reasons account for this interest. First and foremost, our ability to detect exceedingly small amounts of substances and cells in biological fluids has improved dramatically. Researchers and physicians have known for many years that cancer cells from solid tumors—tumors that grow in solid tissues like the breast, lung, bone, skin, pancreas, etc.—can be found in the blood. However, until recently, it hasn't been possible to detect them because they were greatly outnumbered by normal blood cells, at least in people whose cancer has not spread.

The second reason for the popularity of liquid biopsies is our enhanced understanding of cancer. As discussed in subsequent chapters, cancers can have numerous mutations that may not be the same throughout the tumor. It isn't feasible to take samples from multiple areas of a solid tumor in an attempt to get all the cells with different mutations. Moreover, the mutations driving the cancer can change over time, especially in response to treatment. Solid tumor tissue can't be sampled each week to determine whether the mutations driving their growth have changed—most sampling procedures are simply too invasive. Preliminary evidence suggests that liquid biopsies, which are performed on blood or other fluid samples, may provide information about how the mutations change, which would make them incredibly useful for making treatment decisions over time. Such information may also help researchers understand how cancers change or evolve in the body, leading to the development of better medications.

How Do Liquid Biopsies Differ from Solid Tumor Biopsies?

As noted earlier, one of the most attractive features of liquid biopsies is that they are much less invasive than solid tissue biopsies. At a minimum, solid tissue biopsies require physicians to insert a needle or endoscope into the tumor area to obtain a tiny piece of tissue, which may or may not be sufficient for all the tests that are needed. In other cases, patients must undergo a full surgical procedure to obtain a larger amount of tissue. In contrast, biological liquids are much more accessible. None of us looks forward to a blood test, but it is usually painless and doesn't require surgery. Urine and saliva are even more accessible. Obtaining cerebrospinal fluid requires local anesthesia because a needle must be inserted into the area surrounding the spinal cord. However, even this procedure is preferable to brain or spinal cord surgery.

Given the relative ease with which liquid biopsies can be obtained, they can be performed repeatedly over the course of treatment. This allows the treatment team to have a "real time" picture of the cancer, including the mutations it shows at time of biopsy and how the mutations driving the cancer may change over time. This type of monitoring is not as timely with solid tissue biopsies.

Additionally, as alluded to in Glenda's story at the beginning of this chapter, tumors are sometimes found in sensitive locations. They may be close to important blood vessels, in the brain, or near other structures that are best left undisturbed. In such cases, solid tissue biopsies run the risk of harming patients. Other times the tumor may be so tiny that it is difficult for surgeons to obtain the cancerous tissue. In these cases, liquid biopsies are also an attractive alternative.

The last chapter of this tutorial includes a table comparing liquid biopsies to solid tissue biopsies. We left this until last because it is helpful to have a thorough background on these tests in order to fully appreciate their advantages and disadvantages.

Finally, we've discussed the features of liquid biopsies as they apply to solid tumors. However, people with blood cancers (also called hematologic cancers) such as leukemias, lymphomas, or myelomas can also undergo liquid biopsies. This is intuitive because blood tests are, by definition, liquid biopsies. Blood tests have long been used to help diagnose and monitor blood cancers. But, blood cells begin their lives in a solid tissue known as the bone marrow. People with blood cancers often must undergo bone marrow biopsies, which can be painful. Liquid biopsies may offer an alternative to bone marrow biopsies for patients with blood cancers.

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Chapter 2: What Do Liquid Biopsies Detect?

The year was 1869. The Blue Danube Waltz had premiered in Vienna a few years before, the Suez Canal had just opened, and the US was emerging from the aftermath of the Civil War. In Melbourne, Australia, a young resident physician named Thomas Ashworth was studying a blood sample from a cancer patient who had recently died. The unfortunate patient had had 30 tumors underneath the skin and physicians could do little to help him at the time. But in that single blood sample, Dr. Ashworth made an astounding discovery that would have ramifications far into the next two centuries. Using a light microscope, he saw cells whose shape looked identical to those obtained from the patient's tumors. This observation led him to suggest that the tumor cells in the blood originated from one of the solid tumors.

Over time, Dr. Ashworth's observation was proven correct: cells from solid tumors can escape into the blood (circulatory) system. Dr. Ashworth also correctly predicted that these circulating tumor cells may be the initiators of distant tumors, or metastases. In fact, this is the reason that Dr. Ashworth was able to spot the cells using a regular microscope—there were so many of them in the patient's blood because the tumor had metastasized (spread to other areas of the body). We now know that levels of circulating tumor cells high enough to be seen with a microscope in 1869 only occur when the cancer is extremely advanced and the patient is close to death.

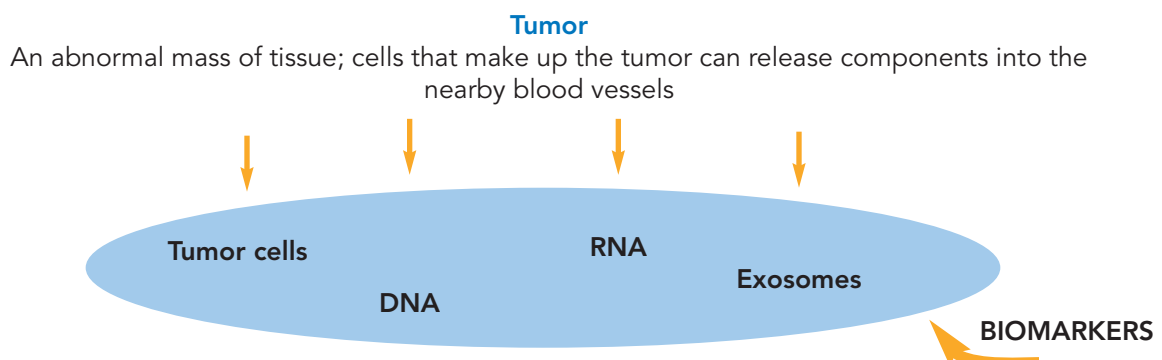
As technology progressed over the ensuing century, scientists were able to detect lower levels of circulating tumor cells in the blood in cancer patients with metastatic disease. It became clear these cells would be quite useful if only we could detect them at earlier stages of cancer when treatments are more effective and possible cures more likely. But at the early stages of cancer, circulating tumor cells in the blood are vanishingly rare. Just how rare? In every milliliter of blood, there are 1 billion red blood cells, several million white blood cells, and 1 to 10 circulating tumor cells.

METASTASIS is the development of a secondary malignant tumor distant from the site of the primary tumor.

Liquid Biopsies Detect Biomarkers

Liquid biopsies don't actually detect the solid tumor itself, but instead detect some component of bodily fluids that provides information about the tumor. In other words, liquid biopsies detect biomarkers for solid tumors. Biomarkers are biological substances, characteristics, or images that provide an indication of the biological state of an organism (for more about biomarkers see the Biomarkers tutorial at www.researchadvocacy.org). As you can see, the definition of biomarkers is quite broad. With liquid biopsies, we are interested in biomarkers that the tumor releases into the blood or other bodily fluid. The biomarkers that are currently garnering the most interest in liquid biopsies are circulating tumor cells, circulating tumor DNA, RNA, and exosomes.

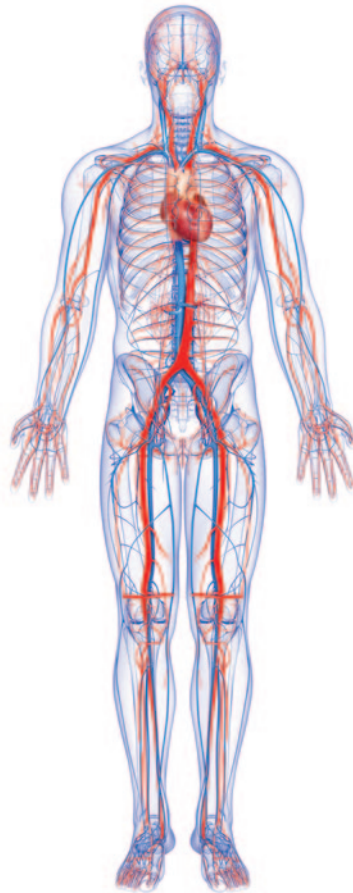
BIOMARKERS are biological substances, characteristics, or images that provide an indication of the biological state of an organism.



Circulating Tumor Cells

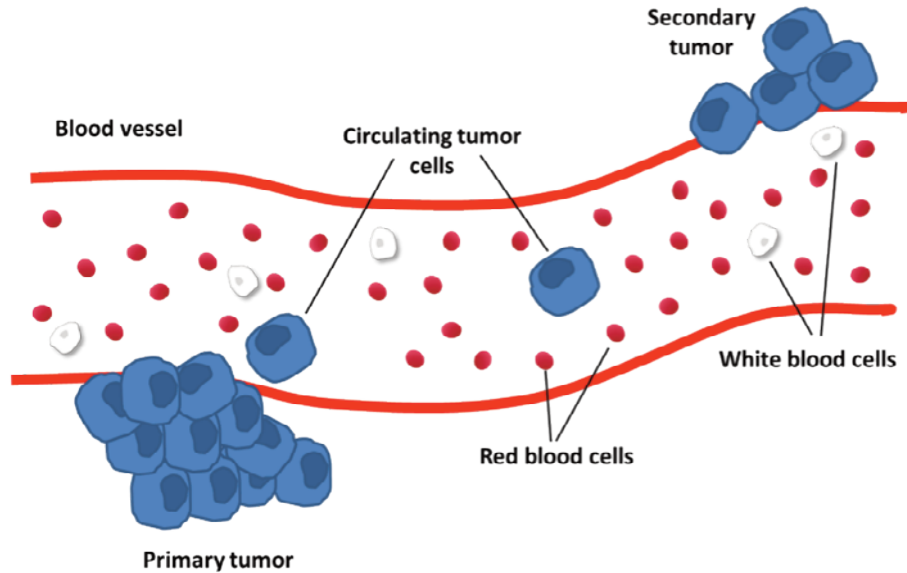
As noted earlier, circulating tumor cells are under intense study as biomarkers because we can now detect them at low levels. Circulating tumor cells, or CTCs, are cancer cells shed from tumors into the blood, where they may then travel to distant locations in the body and form metastases. Circulating tumor cells in the blood are usually pushed there by tumor growth, but other mechanisms are possible, including mechanical disruption of the tumor during surgery.

CIRCULATING TUMOR CELLS are cancer cells that are shed from tumors into the blood, where they travel to distant locations in the body.



Circulatory System

The graphic above shows the cardiovascular, or circulatory system. When cells from solid tumors enter the blood, they can be transported throughout the body. Some of these cells can act as seeds for tumors to grow in distant organs, resulting in metastatic cancer.



In the blood, nearly all tumor cells are eliminated by the body. The immune system attacks them, blood flow breaks them apart, or they die because they are no longer attached to tissue. Few of the circulating tumor cells—about 1 in 10,000—actually survive in the blood and are capable of generating a new tumor in a distant location. However, as metastatic disease progresses, the cancer diminishes the body's defenses, reducing its ability to rid the blood of tumor cells. Thus, levels of circulating tumor cells increase as cancer progresses.

Circulating DNA

Instead of looking for circulating tumor cells, some liquid biopsies examine the circulating tumor DNA in the blood. Tumor cells can release circulating tumor DNA, or ctDNA, whether the cells are alive or dead. Even normal cells that die can release DNA fragments, which are known as circulating free DNA, or cfDNA. The terminology gets a bit jumbled here, as circulating free DNA is sometimes used to refer to DNA from both normal cells and tumor cells, and is sometimes also called cell free DNA. The percentage of cell free DNA in the blood that comes from tumors can be extremely low (1 in 10,000) and may vary with tumor type, making it easier to detect for some types of tumors.

Like circulating tumor cells, circulating tumor DNA may not be just an innocent bystander floating in the blood. Under laboratory conditions, these DNA fragments can promote distant metastases. The body can normally rid itself of circulating DNA, but as tumors metastasize, chronic inflammation and cell death lead to a build-up of cell parts, including DNA. As with circulating tumor cells, the body can no longer efficiently eliminate the circulating DNA and thus high levels are associated with advanced cancer.

CIRCULATING TUMOR DNA, or ctDNA, are fragments of DNA released from tumor cells that are alive or dead.

CIRCULATING FREE DNA or CELL FREE DNA are fragments of DNA derived from tumor cells or normal cells.

Circulating RNA

Circulating RNA is another blood component that can be examined in a liquid biopsy. Several different types of RNA exist in the blood, including those involved in the gene expression (for more information, see the *Molecular Diagnostics* tutorial at <http://www.researchadvocacy.org/general-resources>) and those that perform other functions, such as turning genes off or on. One type of RNA that has been examined in liquid biopsies as a cancer biomarker is messenger RNA, or mRNA. mRNA has shown some promise as a biomarker, but it degrades rapidly in blood and is therefore difficult to study. Another type of RNA that has been investigated is microRNA (miRNA). miRNAs are abnormal in cancer and are relatively stable, making them good, potential candidates for liquid biopsies.

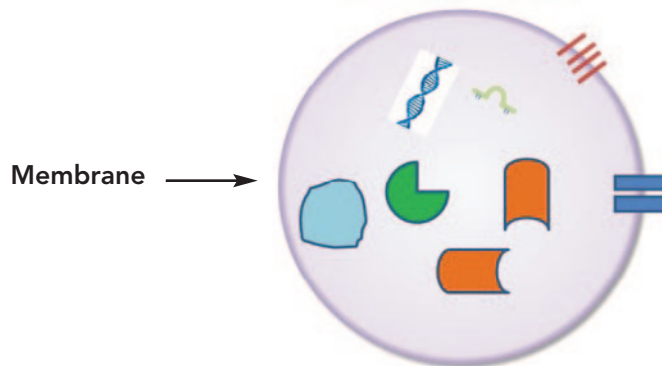
Exosomes

Exosomes are small sacs or vesicles that nearly all cells release into blood and other biological liquids. Exosomes contain DNA, RNA and proteins from inside the cell that are protected from degradation by a membrane surrounding the vesicle. The vesicle acts like a preservation container, keeping the molecules intact, which is clearly an advantage when trying to analyze them. This is particularly advantageous for RNA because, while it contains important information about the proteins that tumor cells are actively producing and the genes that are being regulated, it is notoriously unstable in blood. Like circulating tumor DNA and DNA from circulating tumor cells, the DNA found in tumor exosomes may provide genomic information about tumor cells mutations.

CIRCULATING RNA are fragments of RNA in the blood.

EXOSOMES are small, membrane-enclosed sacs (vesicles) that are released into the blood by nearly all cells. They contain fragments of DNA, RNA, and proteins from their cells of origin that can influence the extracellular environment and other cells.

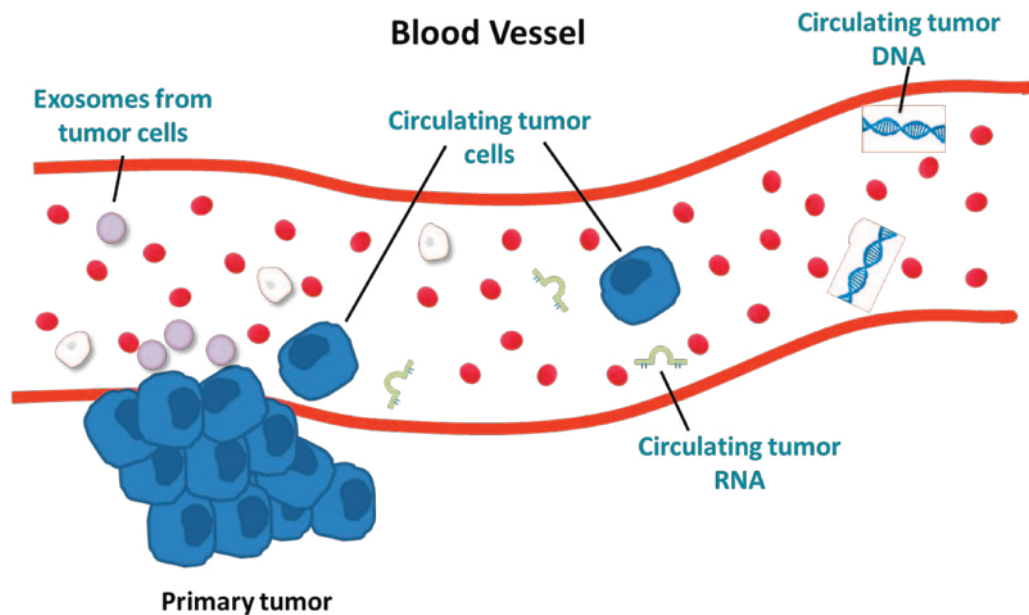
Exosomes contain DNA, RNA, proteins, and lipids (fats)



Tumor exosomes are not merely packets of information about tumor cells. They can affect the extracellular environment near tumors and transfer their contents to other cells, which can change gene expression. In this way, exosomes can cause tumor growth and metastasis, suppress immune responses, and induce the growth of new blood vessels (such as those needed by tumors).

Tumor cells produce and release more than 10,000 vesicles each day, where they join the hundreds of billions of vesicles produced by normal cells found in each milliliter of blood. Researchers have not yet reported a reliable method for separating tumor exosomes from healthy cell exosomes, but this is a popular area of research and it will likely be a matter of time before such methods are identified.

Circulating tumor cells, circulating tumor DNA, and exosomes can all stimulate cancer metastasis, making them potential targets of therapy in addition to their roles as indicators of cancer status.



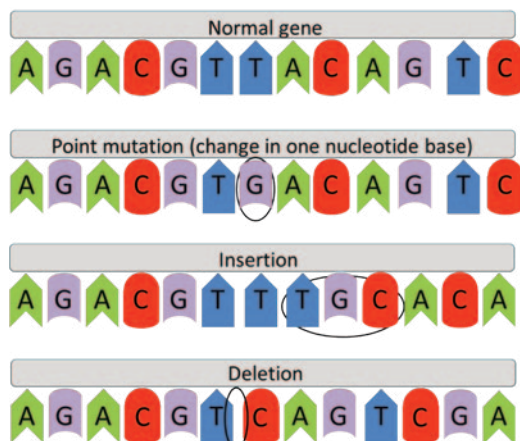
Review of Mutations

Gene mutations can cause cells to grow uncontrollably, which can lead to cancer. Mutations are one of the main types of biomarkers detected in liquid biopsies. Mutations are changes in DNA sequence. These can be inherited or acquired over the course of a person's life. For example, mutations can be acquired when DNA is being copied (e.g., if a copying mistake is made) or in response to smoking or exposure to ultraviolet light.

Several different types of mutations can occur, as shown in the following graphic. Single nucleotide bases can be changed to different bases (called point mutations), new nucleotide bases can be inserted into the sequence (called insertions), and nucleotide bases can be deleted (called deletions).

MUTATIONS are changes in DNA sequence. The word mutation usually but not always refers to DNA sequence changes that can have negative health consequences.

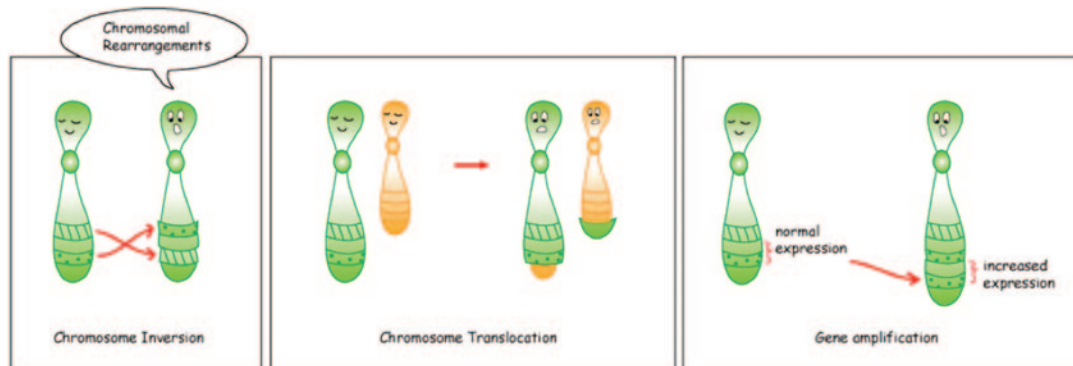
More information about mutations and other changes in DNA can be found at Scitable (<https://www.nature.com/scitable/definition/mutation-8>) and in the Genomics in Cancer tutorial on the Research Advocacy Network website (<http://www.researchadvocacy.org/general-resources>).



Examples of Mutations That Can Occur in Genes

In the normal gene in the top row, the sequence beginning at the blue T reads TTA. In the second row, the second T has been changed to a G. In the third row, TGC has been inserted between the second T and the A, and in the final row, the T and A have been deleted.

In our cells, DNA is coiled into chromosomes. Changes in chromosomes, such as the deletion, insertion, or rearrangement of chromosome parts, can result in overactive or underactive genes, dysfunctional genes, or too many copies of a gene. These changes can result in defective cells. Most of these defective cells will be detected and destroyed by the body's defenses, but if this doesn't happen, the cells may replicate uncontrollably, causing cancer.



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Examples of Some Chromosomal Abnormalities Associated with Cancers

For more information on chromosomes, DNA, nucleotide bases, genes, and mutations, you may want to refer to the following tutorials and training manuals at www.researchadvocacy.org: Molecular Diagnostics tutorial, Biomarkers in Cancer tutorial, and Genomics in Cancer training manual.

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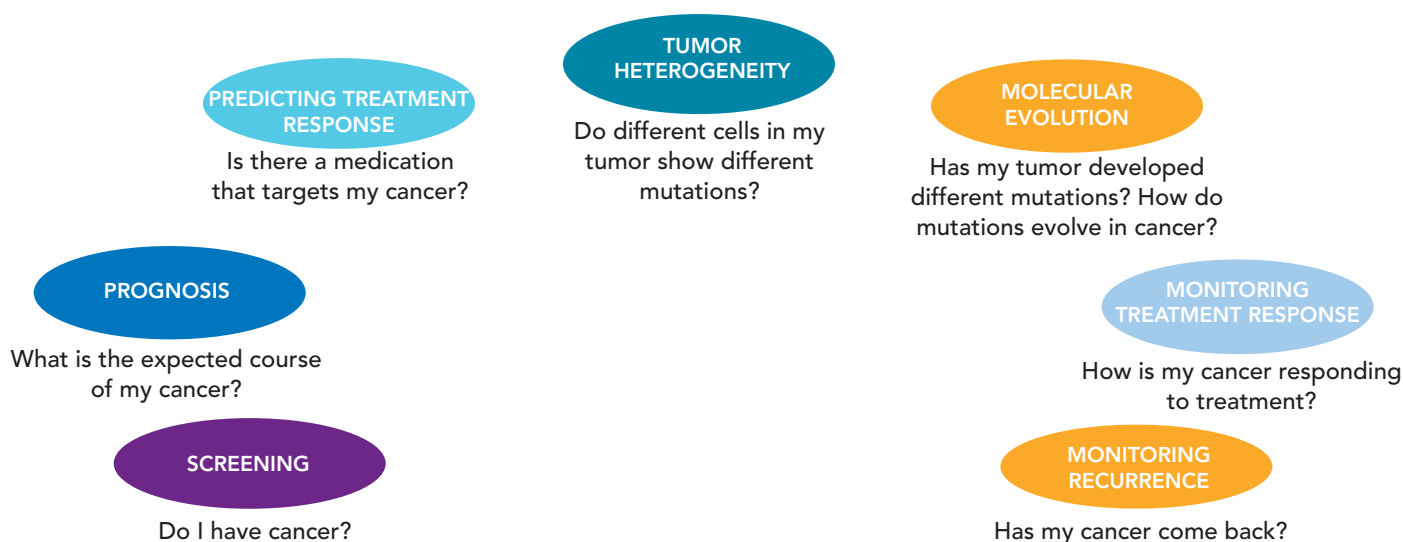
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Chapter 3: How Are Liquid Biopsies Used?

Imagine that you visit your physician's office for a routine annual check-up and have a blood test to analyze glucose and cholesterol levels. It is the year 2030, so the routine tests also examine your blood for cancer. The test comes back positive for colon cancer and, moreover, shows that your cancer has a mutation that can be targeted with a specific medication. You begin the medication, and have blood tests every few months that show the medication is working and the cancer is in remission. After a few years, your blood test once again comes back positive for colon cancer, but this time the mutation driving the cancer is different. You begin a combination of medications—one to target the driving mutation and one to target another common mutation that cancers often develop over time. The medication regimen is again successful and you live out the rest of your life cancer free.

This anecdote illustrates several potential uses of liquid biopsies: screening, diagnosis, predicting treatment response, detecting novel mutations, monitoring treatment response, and monitoring recurrence. Some of these potential uses are likely still several decades away as we wait for the techniques to be refined and the proper research to be conducted. Moreover, not all biomarkers in liquid biopsies provide all of this information. Some liquid biopsy tests are now combining different biomarkers to enhance the predictive ability (e.g., circulating tumor cells plus circulating DNA; circulating DNA plus circulating proteins). In the following sections, we consider the potential uses of liquid biopsies and give a few examples of research findings. The optimal biomarkers in liquid biopsies for different uses and different cancers have not yet been identified, but research is progressing rapidly.



Potential Uses of Liquid Biopsies in Cancer

At the writing of this tutorial, only two liquid biopsy tests are cleared for clinical use commercially available in the United States, the cobas® EGFR Mutation Test v2 and the CellSearch® Circulating Tumor Cell Test. The former is used to determine whether certain lung cancers contain a specific type of mutation (change in DNA sequence) that can be targeted with a medication. In this case, the test is being used to predict treatment response. The latter test is used to inform the prognosis of patients with metastatic breast, prostate, or colorectal cancers.

Screening

By definition, circulating tumor cells come from tumors, so they should only be present in the blood when people have cancer. As such, the presence of circulating tumor cells may be useful for cancer screening. As techniques improve, the ability of these tests to identify people with cancer is getting better. For example, one small study of patients with stomach cancer found that

97% of 102 people who had two or more circulating tumor cells per 7.5 mL were correctly identified as having cancer (<https://www.ncbi.nlm.nih.gov/pubmed/28662130>). However, 17 of 45 people known to have stomach cancer had levels of circulating tumor cells that were lower than this cut-off value (and would have been erroneously classified as cancer free), indicating that there is still a need to optimize the test.

A group of researchers at Johns Hopkins recently reported the results of a liquid biopsy that examines circulating DNA along with selected proteins in the blood to detect the presence of cancer (<http://science.sciencemag.org/content/early/2018/01/17/science.aar3247>). This blood test, called CancerSEEK (still in the research stage at the writing of this tutorial) examines 8 protein biomarkers and 16 different DNA sequences that contain frequent mutations in cancer. In a group of 1005 patients known to have cancer, the test correctly identified the presence of cancer in 69% to 98% of patients with cancer types for which there are virtually no screening tests: ovarian, liver, stomach, pancreatic, and esophageal cancers. The highest rate of correct identification was for ovarian cancer at 98%, followed closely by liver cancer, whereas the lowest was for breast cancer at 33%. Another important aspect of this test was that it gave positive results in only 7 of 812 (0.8%) people without cancer. Ideally, tests would never give these so-called false positive results, but in reality they always do and it's important that the number is as low as possible. For comparison, at least one study found false positive results for lung cancer in nearly 60% of smokers who underwent an existing screening test.

Prognosis

Liquid biopsies may also be useful for cancer prognosis or determining the expected course of cancer in the absence of treatment. Some cancers are more aggressive than others and knowing this can help guide treatment. If a biomarker in a liquid biopsy can help distinguish a cancer that is likely to grow rapidly from one that is likely to grow slowly, then patients with these two types of cancers might receive different treatments. Additionally, patients with slowly-growing tumors may be spared aggressive treatment.

Most of the research examining circulating tumor cells used a cutoff value for the number of tumor cells as opposed to the actual number of tumor cells detected in a person's blood. Consequently, patients or participants in research studies are either deemed to be above or below the cutoff value—sometimes referred to as CTC status (positive or negative). Cutoff values vary in different studies and between cancer types. As in all biological tests and definitions that include cutoff values, there is always the possibility of mis-categorizing someone with values close to the cutoff point. However, the cutoff values are designed to give the best possible predictions based on the data gleaned from validation studies.

As noted in Chapter 1, CellSearch® Circulating Tumor Cell Test is cleared by the United States Food and Drug Administration (FDA) for prognosis of metastatic breast, prostate, and colorectal cancers. The CellSearch® System has undergone clinical validation studies. This test uses the circulating tumor cell cutoff values shown in the table below.

Cutoff Values* for Circulating Tumor Cells in the CellSearch® Circulating Tumor Test			
Prognosis	Type of Metastatic Cancer		
	Breast	Prostate	Colorectal
Favorable	0-4 CTCs	0-4 CTCs	0-2 CTCs
Unfavorable	≥ 5 CTCs	≥ 5 CTCs	≥ 3 CTCs

CTCs=circulating tumor cells. *Number of CTCs per 7.5mL blood



In addition to circulating tumor cells, circulating tumor DNA has been studied as a biomarker for the prognosis of metastatic triple negative breast cancer. Triple negative breast cancer means the three most common types of receptors (proteins on the cell's exterior) stimulating breast cancer growth are not present on tumor cells. Consequently, medications that target these three receptors (estrogen, progesterone, and the receptor encoded by HER-2/neu gene) will not be effective. In one study, patients with triple negative breast cancer were categorized into two groups based on their level of circulating tumor DNA (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5815405/>). The group with higher levels of circulating tumor DNA fared significantly worse than the group with lower levels, as demonstrated by shorter survival. This information is being used to help researchers determine why one group fares better than another.

What Does It Mean to Be Cleared Versus Approved by FDA?

Before medical devices like the CellSearch® System or test kits can be marketed for clinical use, they must be either approved or cleared by the FDA. The regulatory route that a medical device is allowed to follow depends on whether or not it is similar to a medical device already legally marketed for the same use. If a medical device is "substantially equivalent" to an existing product, it may follow the so-called "510(k)" pathway. This pathway is named for a section of the Food, Drug, and Cosmetic Act. This pathway does not require the manufacturer to provide the FDA with data showing the device's safety and effectiveness, but rather that it is sufficiently similar to an already-approved medical device. Medical devices that follow the 510(k) pathway and are given the green light for marketing by the FDA are said to be "cleared."

In contrast, medical devices that are not substantially equivalent to an already-approved product must follow the pre-marketing approval (PMA) pathway. This pathway requires that the manufacturer show evidence of the product's safety and effectiveness, which often involves clinical studies. Even if a product qualifies for the 510(k) pathway, the manufacturer can choose to follow the more rigorous PMA pathway or can perform additional clinical studies, such as validation. Medical devices that follow the PMA pathway and are given the green light for marketing by the FDA are said to be "approved."

Predicting Treatment Response

Another useful piece of information that can be gleaned from liquid biopsies is the presence of mutations in the cancer that may render it responsive to drugs targeting those mutations (i.e., targeted therapies). For more detailed information on targeted therapies, please see the Targeted Therapies tutorial at www.researchadvocacy.org.

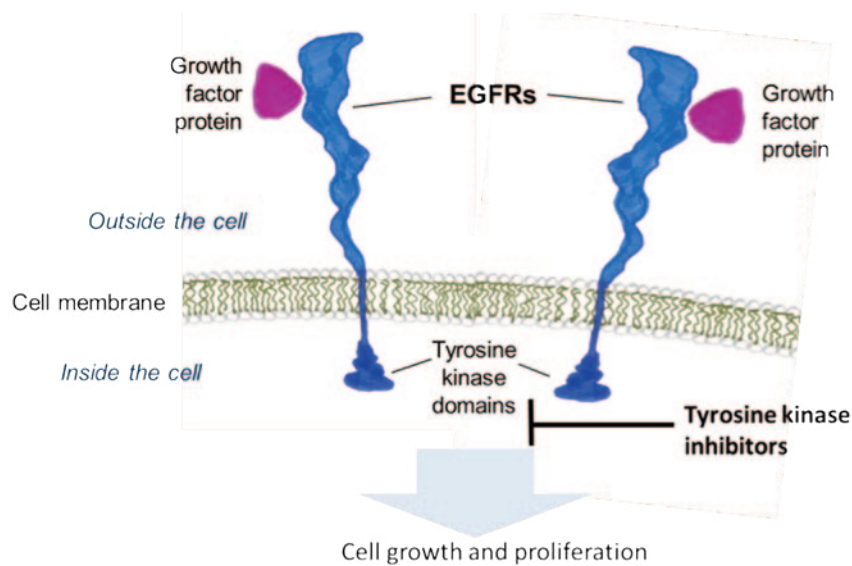
One of the biomarkers in lung cancer that also serves as a "target" for targeted therapy is EGFR. EGFR stands for epidermal growth factor receptor, a protein in the cell membrane that induces cell growth and proliferation when activated by growth factor proteins. Healthy cells have EGFRs that lead to controlled growth and cell proliferation when needed by the body. However, in about 15% of non-small cell lung cancers, a portion of the EGFR gene known as the tyrosine kinase domain contains one or more mutations that cause the EGFR to be overactive. This overactivity in turn causes cells to grow out of control, leading to tumor growth.

Tests are available to determine whether people with non-small cell lung cancer have EGFR mutations in the tyrosine kinase region, so they can then be treated with medications that inhibit overactivity. These medications are referred to as tyrosine kinase inhibitors because they target the tyrosine kinase domain. As noted in Chapter 1, a liquid biopsy test is currently available to test for EGFR mutations

TARGETED THERAPIES IN CANCER are drugs or other substances that block the growth and spread of cancer by interfering with specific molecules involved in cell growth and cancer progression.

EPIDERMAL GROWTH FACTOR RECEPTOR or EGFR is a protein in the membrane of normal and cancer cells that induces cell growth and proliferation.

(cobas® EGFR Mutation Test v2). This test can be performed on solid or liquid biopsies, and for the latter, the test uses circulating free DNA.



Epidermal Growth Factor Receptors

Epidermal growth factor receptors are proteins embedded in cell membranes. In some people with non-small cell lung cancer, the EGFRs contain mutations in the tyrosine kinase domains that make them overactive. The overactive tyrosine kinase domains can be inhibited with medications known as tyrosine kinase inhibitors. Liquid biopsies on circulating free DNA can now determine whether cells from people with non-small cell lung cancer have tyrosine kinase mutations in their EGFRs and thus are likely to respond to treatment with tyrosine kinase inhibitors.

Tumor Heterogeneity and Molecular Evolution of Cancer

Cancer cells divide to produce new, genetically identical cancer cells (i.e., clones). However, cancer cells are notorious for developing additional mutations. Not all cells in the tumor develop the same mutations, though, meaning that not all cells within a tumor are genetically identical. This leads to intra-tumor heterogeneity.

Tumor biopsies are obtained from a small site in the tumor and therefore may only include cancer cells with the same type of mutation. These biopsies may miss cells on the other side of the tumor that have different mutations. If the first mutation is used to make a treatment decision, such as selecting a targeted therapy, the treatment may work for a while by preventing cells with the first mutation to grow and proliferate. Eventually, though, tumor cells with other mutations divide and grow. This is an example of molecular evolution of cancer and it is a major reason for the development of drug resistance.

Even in the absence of targeted therapy, cells with some types of mutations will be more sensitive to chemotherapy and radiation therapy. These cells will die, whereas others that are better able to withstand these treatments will live, grow, and proliferate. Eventually, cancer cells shed from tumors will make their way into the circulatory system. Here they are transported to distant sites of the body, where they can form new tumors that may not be genetically identical to the primary (original) tumor. This is known as inter-tumor heterogeneity.

INTRA-TUMOR HETEROGENEITY refers to the differences in DNA among cells within a tumor. This can result in differences in cell **PHENOTYPE** (observed characteristics).

INTER-TUMOR HETEROGENEITY refers to DNA differences between cells in the primary tumor and secondary tumors that establish and grow at distant sites.

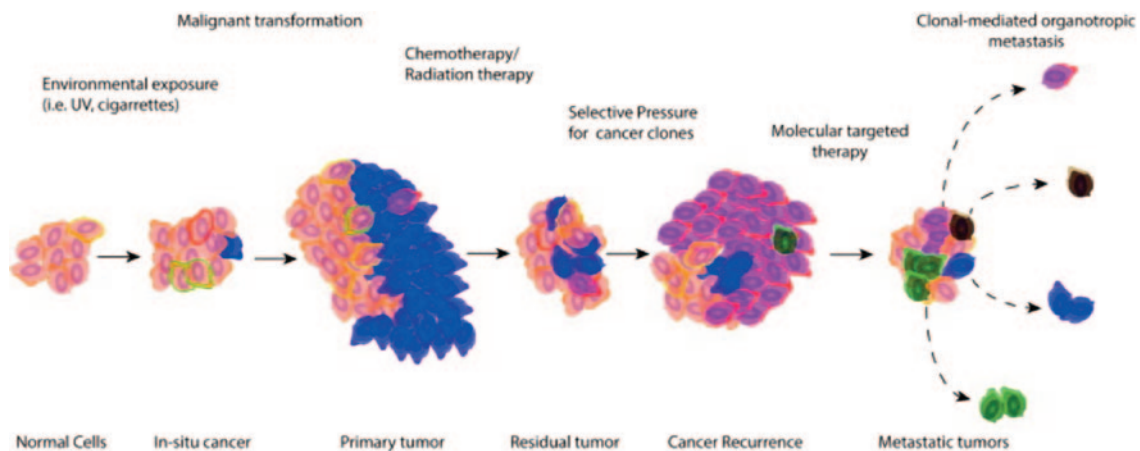


Image Credit: Rapisuwon S, Vietsch EE, Wellstein A. Circulating biomarkers to monitor cancer progression and treatment. *Comput Struct Biotechnol J.* 2016 Jun 1;14:211-22. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4913179/>
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Tumor Heterogeneity and Molecular Evolution of Cancer

This graphic shows tumor heterogeneity and molecular evolution of the cancer. The leftmost clump of orange cells are normal, but develop a mutation shown in blue following exposure to a carcinogen in the environment. Under some circumstances, such as the accumulation of other mutations, the blue, mutated cells multiply generating a primary tumor. A few of the cells in the primary tumor develop different mutations, shown in pink and green. Treatment with chemotherapy or radiation therapy can dramatically reduce the tumor's size, leaving a small residual tumor. Cells with the pink mutation are better able to divide and grow in the post-treatment environment than are the blue cells. Thus, they become an important mutation driving the development of a new tumor. A targeted therapy for the pink cells (called molecular targeted therapy in the graphic) dramatically reduces the size of the tumor by inhibiting its division and growth. After a time, cells with multiple different mutations divide and grow, eventually leading to metastatic disease that may be dominated by different mutations.

As we have learned, the tumor cells entering blood circulation are now fair game for liquid biopsies. Given that liquid biopsies do not sample a single small area of the tumor, but rather the body's blood system, they may be more likely than solid tissue biopsies to find cancer cells with a variety of different mutations. This may allow patients to receive medications targeting several of the cancer-driving mutations, perhaps resulting in longer remissions.

There are a couple of caveats to this prediction, however. First, we don't yet have medications that target many of the mutations driving cancers, known as driver mutations. Second, we don't know the identity of all the mutations driving cancers. This leads to another potential use of liquid biopsies: the ability to study the molecular evolution of cancer in the body—the different mutations and other molecular changes that occur over time and in response to treatment. This may lead to better treatments that can thwart cancer's ability to survive and proliferate.

Some evidence now suggests that circulating tumor cells change a certain way when they invade specific tissues or organs, such that there may be specific "lung circulating tumor cells" or "breast circulating tumor cells." These tissue- or organ-specific cells would be highly valuable for diagnosis of tumor metastases.

A final note to this section is that the different characteristics of circulating tumor cells may eventually be used to identify the organ or tissue of origin—to diagnose the primary or original tumor. It is useful to know in which tissue or organ (e.g., liver, brain, lung, etc.) the tumor originated because treatments and prognoses can differ.

DRIVER MUTATIONS are mutations that push cells toward cancer. **PASSENGER MUTATIONS** occur during the growth of the cancer, but do not contribute to the development of cancer.

Monitoring Treatment Response

A major advantage of liquid biopsies over solid tissue biopsies is that doctors can monitor a patient's treatment response over time. A recent analysis of 50 studies that included 6712 patients with breast cancer evaluated circulating tumor cell status following various treatments (<https://www.nature.com/articles/srep43464.pdf>). Results showed a significant reduction in the number of patients deemed positive for circulating tumor cells following treatment. When individual treatments were examined, all types of treatment except surgery alone reduced the number of positive patients. Another important finding was that the reduction was observed in patients with HER2+ or HER2- tumors, but not in patients with triple negative tumors. (For an explanation of HER2 status, you may want to read this article from the American Cancer Society: <https://www.cancer.org/cancer/breast-cancer/understanding-a-breast-cancer-diagnosis/breast-cancer-her2-status.html>). Negative circulating tumor cell status was significantly linked to reduced disease progression, longer overall survival, and lower progression-free survival. These results show that the status of circulating tumor cells in breast cancer can indicate whether treatments are working and may help guide additional treatment decisions.

Monitoring Cancer Recurrence

Various biomarkers in liquid biopsies, including circulating tumor cell status, are under investigation as indicators of cancer recurrence. In non-small cell lung cancer, chemical changes to circulating DNA have been linked to disease recurrence, and thus monitoring the blood for these changes may eventually be used to determine disease status.

One study of patients with bladder cancer evaluated a liquid biopsy that examined a combination of one protein and three DNA biomarkers in urine to identify those with and without recurrence (<https://www.ncbi.nlm.nih.gov/pubmed/24199181>). The test correctly identified nearly everyone who had no cancer recurrence. However, the test was less successful in identifying people who did have a recurrence (based on solid tissue biopsies). Thus, this test may be useful for identifying patients with bladder cancer who are unlikely to have recurrence, and the remainder may need to undergo additional diagnostic procedures.

The multitude of potential uses for liquid biopsies has stimulated an enormous amount of research. Some groups are looking at circulating tumor cells, whereas others are examining combinations of biomarkers in blood or other fluids. In most cases, it is not yet clear which strategy will provide the best information, and the optimal tests may vary based on the cancer type and location. The field is moving rapidly, and many experts are hopeful that additional liquid biopsies will soon be available for patients.

Making Sure the Tests Are Done Right: Clinical Laboratory Improvement Amendments (CLIA) and College of American Pathology (CAP) Laboratory Accreditation Program

Liquid biopsy tests performed as services (as opposed to physical products) are regulated under the Clinical Laboratory Improvement Amendments (CLIA) of 1988. According to these regulations, all laboratories performing these tests must be certified to conduct testing on human tissue samples, including blood. The essential ingredients needed to conduct the tests are regulated by the FDA. These ingredients can only be sold to authorized manufacturers, qualified clinical laboratories, or organizations that use the ingredients to make tests for non-medical purposes (such as academic laboratories). More information on CLIA regulations may be found at the following website: <http://wwwn.cdc.gov/clia/>.

The College of American Pathologists (CAP's) Laboratory Accreditation Program can help clinical laboratories meet CLIA regulations, ensure compliance, and maintain accuracy of test results. CAP provides regulatory and educational coaching through a team of practicing professionals in a peer-based approach. CAP accreditation is generally recognized as a high quality standard, and laboratories must pass inspection every 2 years to maintain accreditation.

Given the rapid changes in this field, one of the best ways to stay updated on the status of liquid biopsies is to search the internet. To find new liquid biopsy tests that are cleared or approved for marketing in the United States, simply type “liquid biopsy FDA” into your search engine. You may then need to search down the list to determine whether any new tests have been approved or cleared. Another internet search that gives interesting updates related to liquid biopsies is to type in “liquid biopsies cancer” and limit to news. This search is also likely to show new approvals or clearances for liquid biopsy tests, but also shows items undergoing research, such as sound waves being tested as a method for improving liquid biopsies.

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Chapter 4: How Are Liquid Biopsy Tests Performed?

Consider the number of seconds in 32 years. This large number is nearly impossible for us to imagine. If you multiply 60 seconds in 1 minute, times 60 minutes in 1 hour, times 24 hours in 1 day, times 365 days in 1 year, times 32 years, you have 1 billion seconds—the number of seconds in 32 years. Of that number, 1 second is a circulating tumor cell and the rest are red and white blood cells. It's no wonder scientists have trouble detecting that 1 tumor cell!

Adding to the problem is the fact that not all circulating tumor cells are the same shape or express the same proteins. The latter is especially relevant because it is difficult to use existing procedures that can “grab” proteins on the outside of the tumor cells to separate them from blood cells. Separating tumor cells from other cells in the blood is called enrichment, and it's typically the first step in detecting circulating tumor cells.

Enriching and Enumerating Circulating Tumor Cells

Methods of Enrichment

Enrichment based on biophysical differences. Several methods of enrichment rely on the differences in cells' biophysical properties. Circulating tumor cells are generally larger and stiffer than blood cells and can be filtered out using 2-dimensional or 3-dimensional filtration devices. This method has the advantages of being easy to use and allowing a relatively large amount of blood to be run through the filters. However, depending on the rate at which blood is filtered and several other factors, some circulating tumor cells may get through and some blood cells may get stuck in the filter.

Another method of enrichment relies on the differences in density between the blood cells and circulating tumor cells. In density-gradient centrifugation, the blood sample is spun around very quickly, which concentrates the blood cells at the top and the tumor cells at the bottom. Although this method is also easy to perform and can process a relatively large number of blood samples, up to 30% of circulating tumor cells are lost using this procedure.

A third method that has been used is separation of cells based on their electrical properties and their behavior in flowing liquid. This method, referred to as di-electrophoresis, is good at preserving the circulating tumor cells so that they can be further studied, but also loses about 30% of them in the process.

Enrichment based on biological differences. Other methods of enrichment rely on differences in the cells' biological properties. Antibodies against proteins on the surface of circulating tumor cells can be used to bind circulating tumor cells but not blood cells. Conversely, different antibodies can be used to bind blood cells but not tumor cells. Although both of these methods are useful, neither is perfect, with the antibodies against tumor cells inevitably missing some and the antibodies against blood cells possibly binding some tumor cells. At least one method of enrichment combines a physical method, density-gradient centrifugation, with the antibody method.

ENRICHMENT (as it pertains to circulating tumor cells) is the process of separating circulating tumor cells from other cells in the blood, thereby concentrating them to facilitate detection and identification.

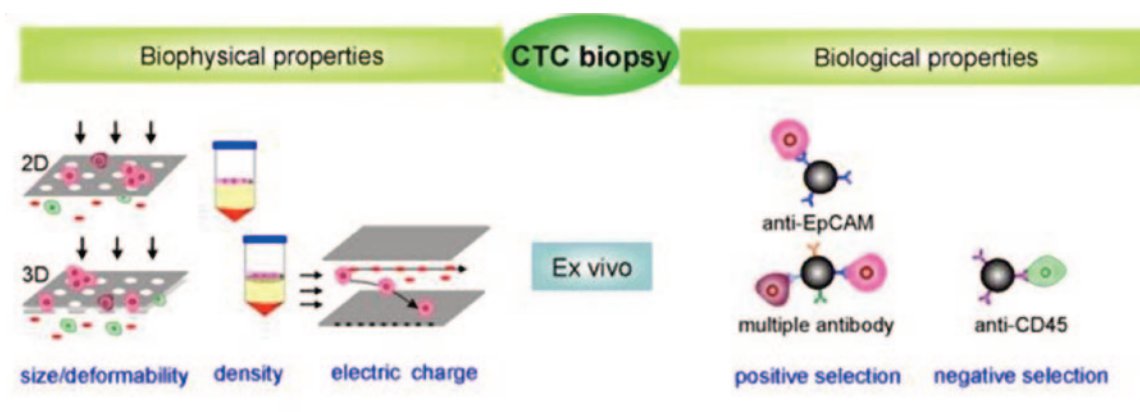


Image credit: Chen L, Bode AM, Dong Z. Circulating Tumor Cells: Moving Biological Insights into Detection. *Theranostics* 2017; 7(10):2606-2619. doi:10.7150/thno.18588. <http://www.thno.org/v07p2606.htm>
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Physical and Biological Methods of Circulating Tumor Cell Enrichment

The left side of the graphic demonstrates biophysical methods of separating circulating tumor cells from blood cells. The methods shown are all *ex vivo*, meaning that they are done outside the body. The cells can be separated based on size and stiffness or deformability, whereby the blood cells proceed through the filter and the tumor cells stick on the filter where they can be removed. In density centrifugation, the blood cells are left floating on top of the liquid where they can be removed. Differences in electrical charge properties allow blood cells and tumor cells to move along different paths in electrophoresis. Biological methods of separation are shown on the right. Single or multiple antibodies can be used to bind proteins only on the outside of tumor cells in "positive selection" procedures. Alternatively, antibodies can be used to bind only proteins on the outside of blood cells in "negative selection" procedures.

Devices for Enrichment

Various devices have been developed that use one or more methods of enrichment described above. These include the CellSearch[®] system and various nanochips that employ the antibody method along with strategies designed to optimize cell separation without damaging the cells or disturbing their properties or interactions. Another system under development uses a microchip to generate vortices (whirling fluids) that separate the tumor cells based on their physical differences from blood cells. The tumor cells can then be purified and analyzed in another test, such as one designed to examine mutations (see the following section on Genomic Characterization).

Enumeration of Circulating Tumor Cells

Once the circulating tumor cells are separated from the blood cells, they need to be counted (ie, enumerated) and/or characterized. The methods employed depend on the purpose for which the cells are being used. If the tumor cells are being used to determine prognosis, the number of cells is often counted, and many studies have used the CellSearch[®] system for this purpose. This device uses a combination of centrifugation, magnetic separation, and multiple antibody binding steps. The system then shows results of the probable circulating tumor cells to the person operating the system for review and verification. For more information about this system, you may wish to visit Cell Search website at www.cellsearchctc.com.

Genomic Characterization

Genomic characterization, or the assessment of DNA, can be performed for circulating tumor cells or circulating free DNA using a variety of different techniques. These methods are used to determine the mutations present in the tumor, which can help predict treatment response to targeted therapies, determine tumor heterogeneity, follow molecular evolution of the tumor(s), and monitor treatment response. Techniques for assessing DNA include real-time polymerase chain reaction (RT-PCR), next-generation DNA sequencing, microarray analysis, and comparative genomic hybridization (CGH).

Polymerase Chain Reaction (PCR)

PCR is a method that amplifies small amounts of DNA so that they can be more easily detected and analyzed. In fact, it has been referred to as “xeroxing DNA.” The following figure shows the basic steps in PCR. First, double-stranded DNA is heated to separate the strands. The temperature is then lowered and primers bind to the single strands of DNA. The DNA copying enzyme (DNA polymerase—the P in PCR) then copies each single strand. The sample is again heated to separate the newly created double strands, and the process is repeated. Each time the process is repeated, the number of DNA strands doubles. Within a few hours, PCR can produce a billion copies of the DNA sequence.

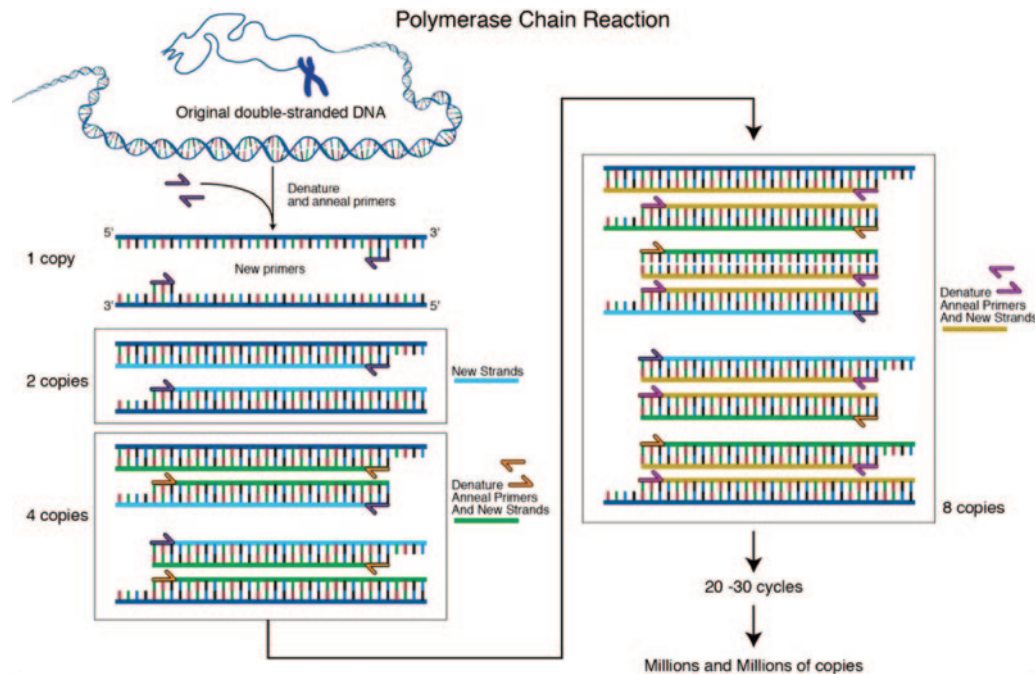


Image credit: Darryl Leja, National Human Genome Research Institute

Polymerase Chain Reaction (PCR)

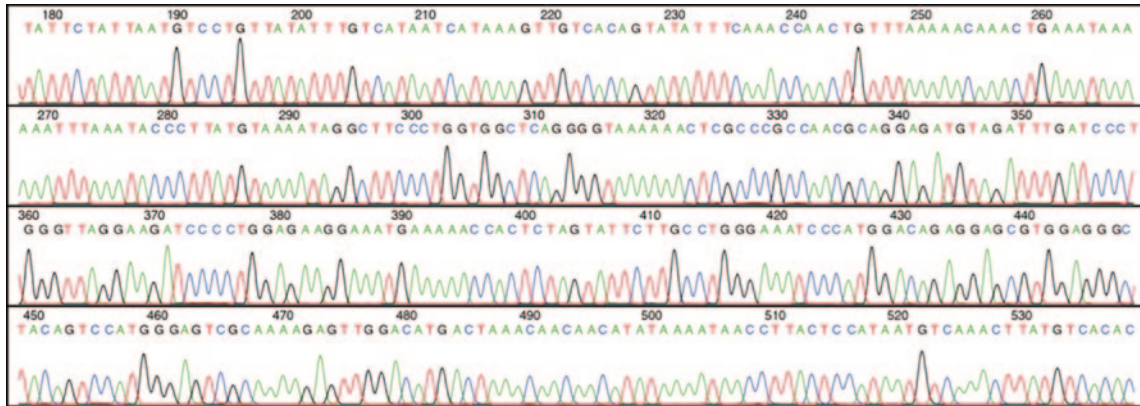
In PCR, the original double-stranded DNA is heated to separate it into single strands. The strands are cooled and exposed to primers, which signal the enzyme DNA polymerase to begin copying. The enzyme makes one copy of each single strand, resulting in two new strands, or 2 full copies of double-stranded DNA. The DNA is again heated to separate the strands and the process is repeated.

Today, most PCR performed is referred to as real-time PCR. In real-time PCR, the progress of the reaction is monitored as it occurs using fluorescence detection. In regular PCR the end product is analyzed after the reaction is complete, which requires an extra step that makes the procedure more laborious.

DNA Sequencing

DNA sequencing is a method in which the nucleotide bases in the DNA are directly determined. Nucleotide bases make up the backbone of DNA and are the components that allow it to form its unique paired helix structure and unzip for copying and generating new DNA. In one of the original methods of DNA sequencing, known as the Sanger method, the DNA is first separated into two strands. One strand is copied multiple times using chemicals that stop the copying process at different places along the DNA strand. This process results in numerous smaller DNA strands of different lengths. The researchers know which nucleotide is on the end of each fragment because

of the chemicals they used to stop the copying process. This allows them to assemble the pieces of DNA like a jigsaw puzzle to reveal the sequence of the original DNA strand. A video of this process is available at the DNA Learning Center website: <https://www.dnalc.org/view/15479-Sanger-method-of-DNA-sequencing-3D-animation-with-narration.html>.



DNA sequence data from an automated sequencing machine

Image credit: Darryl Leja, National Human Genome Research Institute

DNA Sequence Data From an Automated Sequencing Machine

Methods of direct DNA sequencing used today, called next generation sequencing, use a similar process, but are automated. In this method, up to 500 million separate sequencing reactions are run at the same time on a slide the size of a Band-Aid. This slide is put into a machine that analyzes each reaction separately and stores the DNA sequences in a computer.

DNA Microarrays

DNA microarrays were developed to detect thousands of genes at once. In DNA microarrays, small sections of DNA are “arrayed” or spotted in a grid pattern on a very small glass surface. The DNA microarray actually looks like thousands of tiny dots arranged in precise rows and columns. Each dot contains a single DNA sequence designed to pair with a complementary DNA sequence in the tissue sample (this could be, for example, a selected mutation). Because there are thousands of spots for probes, many different DNA sequences can be detected at the same time.

After the DNA probes are placed in the microarray, a sample containing the person’s DNA is prepared for analysis. The double-stranded DNA in the sample is denatured or separated into two complementary single strands. The strands are then cut into smaller fragments and attached to fluorescent dye. The labeled DNA in the sample is placed into the chip and allowed to bind with the DNA probes. The microarray is then washed; DNA that has bound will not wash off, but DNA that has not bound will wash off. Bound and unbound DNA is then detected as fluorescence. If the DNA in the sample has hybridized with the DNA probe, that spot on the array will light up. Computers contain information about which spot corresponds to which DNA sequence and can identify the presence or absence of that sequence in the sample. Depending on the type of technology, the array may use between one and four colors in the detection scheme.



Image courtesy of U.S. Department of Energy Genomics:GTL Program, <http://doegenomestolive.org>.

Image of a DNA microarray (AKA: An Array “Heat Map”)

This graphic shows a DNA microarray. Each part of the grid contains a DNA probe. After exposure to a sample containing a person’s DNA, some of the probes bind and some do not. Those that do bind light up, indicating that the specific DNA sequence is present in the sample. The different colors of the dots correspond to different levels of expression. Sometimes different colored dots such as red, black, and green are used in microarrays.

Comparative Genomic Hybridization

DNA microarrays can be combined with a procedure known as comparative genomic hybridization to detect copy number variations and regions of DNA that are gained or lost. In this test, DNA samples from someone with cancer and someone without cancer are placed in a microarray and labeled with different colored fluorescent dyes. The samples are allowed to bind to DNA sequences of interest and then the amount of fluorescence is compared using a specialized scanner.

Methods Used to Detect RNA

Several of the methods just described for the detection of DNA have been adapted to detect RNA and can be used to assess circulating RNA and microRNA.

Reverse Transcription-PCR

Reverse transcription-PCR is similar to PCR except that it detects RNA instead of DNA. Reverse transcription-PCR uses the same steps as PCR.

MicroRNAs

MicroRNAs are not a method but rather a type of RNA that was discovered in 1993. MicroRNA or miRNAs are small, single-stranded RNA molecules approximately 19 to 25 nucleotide bases in length that bind to specific parts of larger RNA molecules, preventing them from making proteins. In this way, microRNAs inhibit gene expression. Some of the tests for microRNA use quantitative real-time, reverse-transcription PCR.

MICRORNAs are or miRNAs are small, single-stranded RNA molecules approximately 19 to 25 nucleotide bases in length that bind to specific parts of larger RNA molecules, preventing them from making proteins.

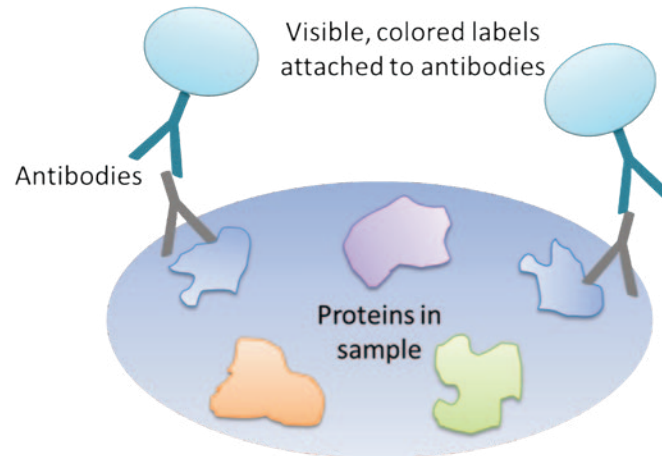
Methods Used to Detect Proteins

Molecular diagnostics may also detect proteins directly instead of the DNA that encodes them. The proteins of interest in liquid biopsies may be freely circulating in the blood, embedded in the membranes of circulating tumor cells, or associated with exosomes.

Immunohistochemistry

One of the most common methods used to detect proteins in biological liquids or embedded in biological membranes is called immunohistochemistry. This technique takes advantage of the method our immune systems use to rid the body of foreign proteins – namely, antibodies. In immunohistochemistry, a sample in which we are trying to identify a specific protein is placed

together with antibodies that bind to that protein. The antibodies are labeled beforehand with some sort of marker, often a fluorescent one that can be seen under a fluorescence microscope. The antibodies are mixed with the sample in a test tube and given time to bind. The sample is then washed off. If the protein of interest is present in the sample, the antibodies will bind and a visible colored label will be seen under the microscope. Unbound antibodies will wash off. Immunohistochemistry can be qualitative (is the protein present?) or quantitative (how much of the protein is present?).



Cartoon Showing Method of Immunohistochemistry

The sample contains many different types of proteins. Specific antibodies, shown as inverted gray Ys, bind to the protein. Secondary antibodies shown as inverted teal Ys are attached to a visible label. The secondary antibodies bind to the first antibodies and amplify the signal. If the colored marker is visible after washing, the antibodies have bound to the protein in the sample, and the presence of the protein is confirmed.

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Chapter 5: Testing the Tests: Validity, Reliability, and Clinical Utility of Liquid Biopsies

Seth is a university researcher who is developing a screening test for multiple cancers. He has genomic information from a group of 100 people with skin cancer and a group of 100 people without skin cancer, and he's trying to identify a set of biomarkers in the blood that would reliably distinguish the two. He first evaluates a test that includes a set of genes known to be mutated in skin cancer. This test correctly identifies 65 of the 100 people with skin cancer, but also gives a positive result in 25 of the 100 people without skin cancer. Seth tries to refine the test by including a protein that has been linked to skin cancer. This test correctly identifies 85 of the people with skin cancer, but also gives a positive result in 40 of the people without skin cancer. That is, the new test with genes + protein is better at identifying people who actually do have cancer, but also picks up more people who don't.

This example illustrates a common difficulty that researchers face when developing tests for cancer: the trade-off between correctly identifying all people in a certain group (e.g., those with skin cancer, those with a certain mutation, those whose disease is like to recur) while excluding all people not in that group. These features of a test are known as its sensitivity and specificity and they are key components of a test's validity.

Analytical Validity

Liquid biopsies must exhibit two types of validity in order to be useful: analytical and clinical. This section focuses on analytical validity and the next section addresses clinical validity. Analytical validity refers to how well a test measures what it is supposed to measure. For example, a test designed to detect a mutation associated with melanoma should not give a positive result for an unrelated mutation that does not cause melanoma.

ANALYTICAL VALIDITY is a measure of how well the test measures what it purports to measure.

Two different aspects of validity that good tests must show are specificity and sensitivity, which are really two sides of the same coin, as we saw in the paragraph at the beginning of this chapter. These concepts can apply to analytical validity of the liquid biopsy test as well as to the clinical validity of the biomarker being detected. Let's consider sensitivity first.

SENSITIVITY is the ability of the test to correctly identify those patients with the biomarker or condition.

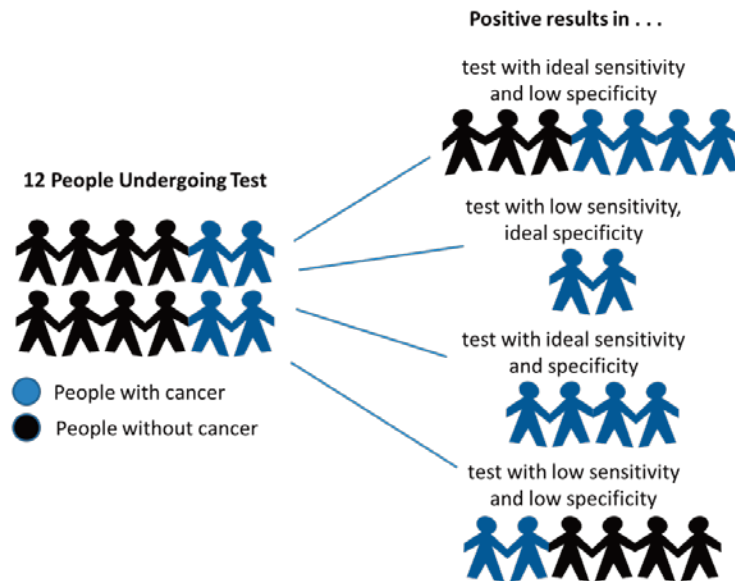
Sensitivity

Sensitivity is the ability of the test to correctly identify those patients with the biomarker or condition; in other words, it should correctly identify everyone who has cancer or who has a certain mutation. With a sensitive test, you can be relatively certain that, if you have the biomarker or condition, you will get a positive result on the test. In our example of Seth and his tests above, the first test had a sensitivity of 65% because it correctly identified 65 of the 100 people with cancer. Low sensitivity of a test is clearly problematic because it will miss some people with the biomarker or condition.

SPECIFICITY is the ability of the test to correctly identify those patients without the condition. Stated another way, a test is specific if it gives a positive result only when the condition is present. For instance, a specific test for a cancer mutation would give a negative result for everyone who does not have the mutation and only give a positive result if someone does have the mutation.

Specificity

Specificity is the ability of the test to correctly identify those patients without the biomarker or condition. Said another way, a specific test is one that gives a positive result only when the biomarker or condition is present. Returning to the example at the beginning of this chapter, the first test gave a positive result in 25 of the 100 people (25%) without cancer. Low specificity is problematic because it can cause mental anguish and can lead people to undergo unnecessary follow-up procedures and treatments that are associated with risks.



In this imaginary population of 12 people, 4 have cancer (blue figures) and 8 do not (black figures). A biomarker with ideal sensitivity but low specificity would be evident in 4 people with cancer but might also be evident in 3 people without. In other words, it would correctly identify all people who have cancer, but would falsely identify some people as having cancer when they actually do not. A biomarker with low sensitivity but ideal specificity might be evident in 2 people with cancer but 0 without. In other words, it would miss some of the people with cancer, but wouldn't falsely identify someone without cancer. A biomarker with ideal sensitivity and specificity would be evident in all 4 with cancer but none without. A biomarker with low sensitivity and low specificity might be evident in 2 people with cancer and 4 without (not a useful test at all!).

True and False Positives and Negatives

Results of liquid diagnostics (or any tests, for that matter) can be classified as correct or incorrect. When a test correctly identifies a person with a given biomarker or condition, the result is said to be a true positive. When a test correctly determines that a person does not have a given biomarker or condition, the result is said to be a true negative. This is the ideal situation of perfect specificity and sensitivity—we always want the results of tests to be correct or true. However, when a test is incorrect, the results are said to be false. If the test incorrectly identifies a person as having a biomarker or condition, the result is a false positive. If the test incorrectly identifies a person as not having the biomarker or condition, the result is a false negative.

Does the person actually have the biomarker or condition being tested for?	Test Result	
	Positive (yes)	Negative (no)
Yes	True positive	False negative
No	False positive	True negative

Accurate and useful tests have high true positive and true negative rates and low false positive and false negative rates. In the ideal case, the test would identify only those people with the biomarker or condition, but would also identify everyone with the biomarker or condition.

In biological testing, these terms are often called the positive and negative predictive values (PPV and NPV). A highly specific test will have a high positive predictive value; that is, if it is positive in a patient, it is very likely that patient has the condition. A highly sensitive test will have a high negative predictive value; that is, if the test is negative in a patient, it is very unlikely that the patient has the condition. However, no test is perfect. Depending on the condition, it may be better to have a higher positive predictive value or negative predictive value.

Positive predictive value is calculated as the number of true positives divided by the sum of the number of true positives and false positives (ie, all the positives).

Negative predictive value is calculated as the number of true negatives divided by the sum of true negatives and false negatives (ie, all the negatives).

For example, let's presume that 100,000 people undergo cancer screening and 200 of them actually have cancer. Let's apply a test with high sensitivity, say 90%. In this case, the test would correctly identify 180 of the 200 patients who have cancer. Let's also give this test what appears to be a high specificity, say 90%. In this case, 10%, or 10,000, of the screened patients will have a false positive test, leading them to worry needlessly and undergo additional screenings and procedures even though they are not necessary. So, in this case, the positive predictive value is the number of true positives (180) divided by all the positives (180 + 10,000). This gives 180 divided by 10,180, which is approximately 2%. Thus, of the people who tested positive, the percentage with cancer is only 2%!

The positive and negative predictive values depend on three issues:

- The prevalence of the condition in the population
- The sensitivity of the test
- The specificity of the test

In the example above, if the prevalence were much higher, let's say 50%, occurring in half of the 100,000 people, then we would have detected 45,000 true positives, but would still have had 10,000 false positives. In this case, the positive predictive value would be calculated as the number of true positives (45,000) divided by all of the positives (45,000 + 10,000). This gives 45,000 divided by 55,000, which is approximately 82%—a much higher positive predictive value. This shows how much the positive predictive value depends on the prevalence of the biomarker or condition in the population being tested.

Test Reliability

Another aspect of analytical validity is test reliability. Test reliability means that the results of the test are repeatable. If a liquid biopsy performed on Monday indicates that a person's blood contains circulating tumor cells, it should also give the same result when conducted on Tuesday. Clearly, unreliable tests are not useful in making diagnoses or treatment decisions.

Standardization

Another important consideration in liquid biopsy tests is standardization. Ideally, liquid biopsies would be standardized, meaning that they would be performed exactly the same way on the same equipment with the same chemicals each time. Because these tests often require precise measurements, complicated equipment, and/or different mixtures of chemicals, standardization can be difficult to achieve.

One feature that can contribute to the variability of results from a molecular diagnostic is the way samples are collected, processed, and stored—so-called pre-analytic factors. For example, one study of circulating tumor cells found that the type of test tubes in which the blood was stored affected cell shape. Cell shape is an important feature of some enrichment techniques and thus could substantially influence the test results. Consequently, the blood sample evaluated in the same liquid biopsy test may give different results depending on how it is collected, processed, and stored.

POSITIVE PREDICTIVE VALUE is the probability that people with a positive test result truly have the biomarker or condition.

NEGATIVE PREDICTIVE VALUE is the probability that people with a negative test result truly don't have the biomarker or condition.

PRE-ANALYTIC FACTORS are factors that occur prior to the test, including sample collection, processing, and storage, that can affect test outcome.

Ideally, the pre-analytic factors would be standardized, but at the very least, it is important that they are consistently reported. In 2011, experts published recommendations on Biospecimen Reporting for Improved Study Quality (BRISQ). These guidelines describe the pre-analytic details that must be reported anytime human biospecimens are used; this information is designed to help evaluate, interpret, compare, and reproduce the experimental results.

Sometimes tests that are sold by the manufacturer as kits include an internal standard—a test sample that contains a given, known amount of the biomarker being detected. This standard can then be used to calibrate the test. For example, a test kit might contain an internal standard that consists of 100 micrograms of a protein. When that standard sample is run in different laboratories, they should also find that it contains 100 micrograms. In this way, laboratories can make sure that their test is giving the correct results and that they are comparable to those of other laboratories.

Standardization in laboratory tests may be improved by requiring laboratories to undergo proficiency testing. For example, blood samples may be sent to participating laboratories for the determination of the substance of interest. The results from all participating laboratories are sent to a central facility where they are evaluated and the laboratory is either certified or not based on its ability to obtain accurate results.

To overcome the problem of reliability, some companies have designed liquid biopsies that require samples for testing to be sent to the company's own laboratory. In this case, the test can be performed the same way each time and the company has control over the reliability of the results. This is the case for Guardant360®, a circulating tumor DNA test that examines 70 different genes. For this test, healthcare professionals obtain two test tubes of the patient's blood and then send them to the company's laboratory for analysis. (See later in this chapter for a more thorough description of this test.) In other cases, companies sell testing kits that provide materials that help standardize the pre-testing procedures, as well as the actual test. This is the case with the cobas® EGFR Mutation Test v2, which tests for the EGFR mutation in non-small cell lung cancer, and can be performed on blood or solid tissue (using different test kits).

Clinical Validity

Clinical validity refers to the ability of the test to provide clinically relevant information. Clinical validity depends on close association of the biomarker with a clinically important outcome such as response to a medication or aggressiveness of the cancer.

CLINICAL VALIDITY is the measure of a test's ability to provide clinically relevant information.

Clinical validity is related to the concept of clinical utility, which is described more fully in a subsequent section. Some experts define clinical utility as a test's ability to provide clinically relevant information—which is the exact definition used for clinical validity. In contrast, other experts believe that clinical utility is a broader concept that involves a practical aspect of usefulness in the clinic.

Many experts, including a committee of the Institute of Medicine, consider clinical validity a measure of whether the test reliably divides one population into two or more with different biological or clinical characteristics or outcomes. However, if this difference is insufficiently large to justify treating those two groups differently (even if it is statistically significant) OR if knowing the difference is not associated with a therapeutic option that improves clinical outcomes, then it does not have clinical utility. Notably, there may be many different tests or assays for a single biomarker. They may have different analytical and clinical validities and clinical utilities. Each must be tested separately before it is recommended to guide therapy in a specific use context for patient care.

How Are Liquid Biopsies Clinically Validated?

Liquid biopsies are clinically validated by conducting studies that document the relationship of the test's outcome with an important medical or clinical outcome. For instance, if the liquid biopsy purports to detect response to therapy, then test results would need to show a relationship with reduced tumor growth, patient survival, or another important variable in a clinical study. Such studies provide scientific proof of the liquid biopsy's accuracy. Without validation in a clinical study, the test's accuracy must be considered unproven.

Clinical Utility

Clinical utility refers to the overall usefulness of a test in clinical practice that is determined by weighing its benefits and drawbacks. As discussed earlier, liquid biopsies must provide some clinically useful information in order to be clinically valid. This information should aid in diagnosis or clinical decision making. A test that could reliably detect 20 common genes associated with a tumor may not be clinically useful if those genes don't predict anything of value for the patient or physician.

CLINICAL UTILITY is the overall usefulness of a test in clinical practice, determined by weighing its benefits and drawbacks. This definition is still evolving, with some experts equating clinical validity and clinical utility.

In addition to being valid and reliable, liquid biopsies should also be practical. For example, a test that is extremely difficult to perform or requires rare technical equipment may not have clinical utility in routine clinical practice, even if it provides clinically useful information. Practical concerns are an important part of the equation, although not all experts consider them an aspect of "clinical utility." Instead, some experts limit the definition of clinical utility to mean providing clinically useful information. Irrespective of whether you consider practical concerns and drawbacks as part of clinical utility, they are important determinants of how useful liquid biopsies are.

Potential Reasons for Lack of Clinical Utility with Liquid Biopsies

- The test doesn't really work.
- The test works but doesn't distinguish one group of patients from another with sufficient magnitude that they would receive different treatments or procedures.
- The test works and distinguishes patient groups adequately, but no better treatments are available for either group.
- Insufficient evidence that the test reliably distinguishes one group of patients from another.

As you can see, some of the concerns in this chapter relate to the tests, and others to the biomarkers themselves. Even perfect tests will not lead to perfect outcomes given our imperfect understanding of cancer, its causes, and individual differences related to cancer and its treatment.

Ease of Use and Interpretation

Another aspect of clinical utility is the ease with which the tests can be used or conducted, and the ease with which results can be interpreted. Tests that are easy to perform and interpret are likely to be more useful than those that aren't. Moreover, tests that are difficult to perform may introduce a greater potential for error. The highly technical nature of some liquid biopsy tests can make them challenging to perform and interpret, and ease of use is definitely a concern as we will discuss when talking about test regulation in the next chapter.

Weighing Benefits and Drawbacks

Weighing the benefits and drawbacks of liquid biopsies is an important undertaking for patients as well as physicians. Low test validity can lead people to undergo unnecessary procedures and treatments or can miss some people for whom a given treatment might be effective. However, unlike solid tissue biopsies, the procedure for obtaining a liquid biopsy is associated with minimal risk and invasiveness. As discussed throughout this tutorial, this is a major advantage of liquid biopsies.

Example of a Liquid Biopsy Test Validation Study: Guardant360®

An example of a liquid biopsy test that has undergone a large validation study is Guardant360®, which received Expedited Access Pathway designation from the FDA in early 2018. The test uses next generation sequencing to analyze circulating tumor DNA for the presence of mutations and chromosomal rearrangements in 70 cancer-related genes.

The validation study was conducted using blood samples from >10,000 patients with more than 50 cancer types (<https://www.ncbi.nlm.nih.gov/pubmed/29691297>; only abstract is freely available). The researchers determined whether the frequency and distribution of alterations in the 70 cancer genes detected using the liquid biopsy were comparable to those in previously published studies in solid tissue biopsy samples. Results showed that the frequencies of critical mutations in known cancer-related genes (eg, EGFR, BRAF, KRAS, and PIK3CA) identified in the liquid biopsy were similar to those in the tumor biopsy samples, with correlations of 94% to 99%. Indeed, the positive predictive value for treatment-relevant alterations in lung cancer approached 100%. More than 60% of patients in this study were identified as having tumor biomarkers that could help physicians make treatment decisions, including targeted therapy with FDA-approved medications or medications being tested in clinical trials.

In another part of the study that included 400 patients for whom solid tumor biopsy tissue was available, results were compared with those of the liquid biopsy. The liquid biopsy was 87% accurate compared with the solid tissue biopsy, but 98% accurate when the blood and tumor samples were collected within 6 months of one another. This raises the possibility that the liquid biopsies were detecting more recent genetic alterations in the tumor. Indeed, the liquid biopsy identified genetic alterations known to make tumors resistant to treatment that were not found in solid tissue biopsy samples. The liquid biopsy identified resistance mutations in 27% of patients, which may not have been present at the time of the solid tissue biopsy. Instead, these mutations probably evolved as the patients were treated.

These results are exciting and extremely promising. To help stimulate the development and validation of even more liquid biopsies, the National Cancer Institute is supporting an initiative for tests that detect early-stage cancers, aid in diagnosis, and distinguish slow from fast growing tumors. The American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) have developed a joint review that assessed the evidence for circulating tumor DNA tests (<https://www.ncbi.nlm.nih.gov/pubmed/29504847>). This review analyzed the literature documenting results of published studies with circulating tumor DNA. The authors concluded that, although some circulating tumor DNA tests have demonstrated clinical validity and utility with certain types of advanced cancer, most have insufficient evidence. They further note a mismatch between the results of liquid biopsies and solid tissue biopsies. They found “no evidence of clinical utility and little evidence of clinical validity of ctDNA assays in early-stage cancer, treatment monitoring, or residual disease detection. There is no evidence of clinical validity and clinical utility to suggest that ctDNA assays are useful for cancer screening, outside of a clinical trial.” However, the authors do note that research is proceeding rapidly and these conclusions may be modified based on those results.

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Chapter 6: How Can Advocates Use This Information?

Liquid biopsies are a hot topic in cancer because of their immense potential to provide previously unavailable information about cancer that may help save people's lives. Moreover, liquid biopsies are extremely convenient for patients and potentially cost effective. As advocates, it is important for us to understand the basic features of these tests, their current and potential clinical effectiveness, and their current status in the research and development pipeline. The information will also help us communicate with physicians and researchers, give us added credibility for our viewpoints, and permit us to monitor developments in the field.

Issues in Liquid Biopsies of Interest to Advocates

For advocates, among the important issues with liquid biopsies are the following:

- The use of liquid biopsies for cancer varies with cancer type. For example, some cancers are more likely than others to release tumor cells into circulation. Similarly, different liquid biopsies are more useful for some cancer types than others. The CellSearch® Circulating Tumor Cell Test is currently cleared for use with breast cancer, but a test in development known as CancerSEEK found that breast cancer was associated with the lowest rate of correct identification among the cancer types studied (see earlier chapters for full description and references). Consequently, each specific liquid biopsy test is more useful for some types of cancers than others.
- Not every known “driver” mutation has a targeted therapy. In the coming years, liquid biopsies may become extremely good at detecting specific mutations that stimulate cancer growth. However, such information may not directly benefit patients unless there is a targeted therapy available to specifically attack the driver mutation(s). Fortunately, the numbers of targets and targeted therapies are growing rapidly.
- Liquid biopsies are important tools for cancer research. In the coming years, liquid biopsies will likely help scientists understand how cancers evolve because they can be performed repeatedly over the course of cancer treatment and into survival. This information is essential for understanding how driver mutations change over time, particularly in response to drugs. Eventually, this may reduce drug resistance or lead to the use of multiple medications that make drug resistance less probable.
- Like all tests, liquid biopsies must be validated to ensure that they are reliable and provide useful information. These tests may be used to inform or guide treatment decisions that could have life or death consequences. For this reason, we want to be sure that they are as accurate as possible—information that comes from validation studies.
- Standardization is important for liquid biopsies, as it is for all tests. We want to be sure that the tests are conducted under optimal conditions so that we can be confident in the results. Some companies are not leaving this to chance: they are requiring that the liquid biopsies be sent to their company laboratories for testing. Even in these cases, it is critical that the blood samples are obtained, stored, and shipped under strict conditions. One group that is interested in promoting standardization, as well as other important features of liquid biopsies, is the Blood Profiling Atlas in Cancer Consortium, described in the following paragraph.

Blood Profiling Atlas in Cancer Consortium (Blood PAC)

The Blood Profiling Atlas in Cancer Consortium is an organization that was founded to accelerate the development and validation of liquid biopsies to improve the outcomes of patients with cancer. Its specific goals are to aggregate, make freely available, and harmonize scientific and clinical data related to circulating tumor cells, circulating tumor DNA, exosomes, and proteins, as well as sample collection, preparation and handling protocols. This non-profit organization is open to new members, and more information can be found on their website: <https://www.bloodpac.org/> or in a recent article, available here: <https://www.ncbi.nlm.nih.gov/pubmed/28187516>. This organization is advocate friendly and includes advocates as members of their group (but may not provide travel compensation for advocates to attend their meetings).

Comparison of Liquid Biopsies and Solid Tissue Biopsies

Liquid biopsies are extremely promising, but not yet perfect. The following table compares the key advantages and disadvantages of liquid biopsies and solid tissue biopsies, but it is important to note that patients can undergo both types of tests—they are not mutually exclusive.

Point of Comparison	Liquid Biopsies	Solid Tissue Biopsies
Experimental support and validation	Some clinical uses established for some tests, in progress for others, not established for others	Established, gold standard
Invasiveness	Noninvasive (eg, urine) to minimally invasive (eg, blood) to less invasive than alternatives (eg, cerebrospinal fluid vs. brain surgery)	More invasive than liquid biopsies, but range from thin, fine needles to surgical removal of tissue (much more invasive)
Good for sensitive or hard-to-access tumors?	Yes	No
Good for people who are unable to undergo surgery, tolerate general anesthesia?	Yes	Mostly no, but some procedures may be acceptable (eg, fine needle biopsies)
Good for detecting early stage cancer?	Potentially, but require validation and may lead to over-treatment in some cases	No
Identification of intra-tumor heterogeneity in a single sample?	Potentially, but require validation	Not typically (tissue usually from a small portion of the tumor)
Identification of inter-tumor heterogeneity in a single sample?	Potentially, but require validation	No
Real time tumor monitoring for mutations or tumor status (ie, tracking tumor evolution)?	Yes	No
Easy to obtain adequate analytes of interest?	No, requires highly sensitive detection technologies	Usually

Additional Information on Liquid Biopsies

Additional information on liquid biopsies is provided by the following organizations:

- American Cancer Society: <https://www.cancer.org/latest-news/liquid-biopsies-past-present-future.html>
- National Cancer Institute: <https://www.cancer.gov/news-events/cancer-currents-blog/2017/liquid-biopsy-detects-treats-cancer>
- International Association for the Study of Lung Cancer: <https://www.iaslc.org/news/iaslc-issues-statement-paper-liquid-biopsy-lung-cancer>; <https://www.iaslc.org/webinars/liquid-biopsy-management-non-small-cell-lung-cancer>
- Peer-reviewed, published article by British pathologist Dr. Cree: <https://www.sciencedirect.com/science/article/pii/S2214663615000024>

The Research Advocacy Network (RAN) was formed in 2003 to bring together participants in the research process with the focus on educating, supporting, and connecting patient advocates with the medical research community. Research Advocacy Network is committed to improving patient care through research. Our goals are to get results of research studies (new treatments) to patients more quickly, to give those touched by the disease an opportunity to give back and to help the medical community improve the design of its research to be more attractive to potential participants.

To provide those touched by the disease an opportunity to give back, RAN created the Advocate Institute™. This virtual learning center provides advocates with multiple methods of learning to improve their effectiveness in interactions with the research “world.” The Institute uses an innovative curriculum, on-site presentations and online learning opportunities. RAN has used the latest technology to reach a larger audience of advocates through Focus on Research™. This is a system of preparatory conference calls, virtual classrooms (webinars), learning materials and mentoring to prepare advocates to attend research-oriented meetings. RAN applies best practices from the world of market research to inform research design. Using the models of focus groups and structured interviews, RAN was able to inform the design of the PACCT-1 (now renamed as TAILORx) clinical trial. Patient advocacy in research has many opportunities to make a contribution. RAN has training and educational programs, publications and tools for advocates on our Web site, and experience in effectively working with researchers in cancer centers. RAN works with advocates and organizations to effectively integrate advocates into research activities. Please learn more about us at our Web site at www.researchadvocacy.org or contact us about our work by e-mailing us at info@researchadvocacy.org or by FAX at 888-466-8803. We look forward to hearing from you!

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