Lateral Flow - Breast Cancer Metastasis MiniLab

Student Guide

Cat# M3024

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Students will learn about antibodies, how a rapid test or lateral flow assay works by using a model, then run patient samples by lateral flow to test for metastasis biomarker of breast cancer patients who had previously been diagnosed with a form of breast cancer. This lab is ideal for high school biology students, especially honors and advanced placement, and college level biology. This MiniLab contains enough supplies for **10 groups** to perform experiments.

Laboratory Safety

1. Wear lab coats, gloves, and eye protection when handling samples.
2. Wash your hands thoroughly after handling biological materials and chemicals.
3. Dispose of all materials in a biohazard bag.

Introduction

The Lateral Flow Assay (LFA) is an immunological assay that utilizes the binding capacity and specificity of antibodies to detect proteins of interest called biomarkers. In our recent past, citizens of the world became acquainted with using a rapid test to test for COVID. Rapid test is another name for a Lateral Flow Assay.



Figure 1. Example lateral flow test that shows positive for COVID antibodies.

Lateral flow assays have several advantages. They are low cost, easy to run, deliver results quickly, and are stable at room temperature and over relatively long storage times. They can be used in doctors offices or even at home to provide a quick assessment of health conditions. These assays are used in a broad range of applications including determining the presence of biomarkers indicative of disease (viral infection, cancer, etc) or of a biological state (pregnancy), as well as the detection of microorganisms that compromise food safety, environmentally hazardous chemicals, or illegal drugs.

The substances detected using the LFA kit could include:

- Biomarkers of disease: cancer biomarkers, infectious disease biomarkers etc
- Biomarkers of human health: hormones, metabolites
- Biomarkers of veterinary health: detection of biomarkers of infectious diseases such as bovine spongiform encephalitis (cattle), Giardia (dogs), feline leukemia virus (cats)
- Biomarkers of plant health: pathogen (viral or bacterial) detection in the plant or in the soil
- Environmental toxins: lead (in water), pesticides (in soil or water)

In order to understand how these assays work, we need to understand how antibodies recognize foreign objects and how the immune system creates the wide variety of them, giving us a chance to use the technology to test for a multitude of substances.

Antibodies

Before discussing antibodies, you will first need to learn a few vocabulary terms. A pathogen is any disease-causing microorganism, such as bacteria or viruses. An antigen is a protein molecule on the surface of the pathogen that triggers an immune response, and an epitope is a specific part of the antigen that antibodies bind to. Below is an image of an epitope on an antigen on a pathogen.



Figure 2. https://www.creative-biolabs.com/vaccine/epitope-predicting-algorithm-for-vaccine-design.htm

When a pathogen first enters your body, immune cells in your body recognize the antigens present on the pathogen as foreign or 'non-self'. Upon detection, your innate immune response launches a quick and general defense, often resulting in inflammation or a fever. Meanwhile, the B cells and T cells in your adaptive immune system are working to make antibodies that recognize and attack specific epitopes on the antigens. In fact, the term antigen comes from the combination of the terms **anti**body and **gen**erator.

Antibodies are Y-shaped proteins that specifically recognize and neutralize or attack the antigen-presenting pathogen. Antibodies bind to the epitope they are specific for like a lock and key, giving the immune response specificity and the ability to distinguish self from non-self proteins.

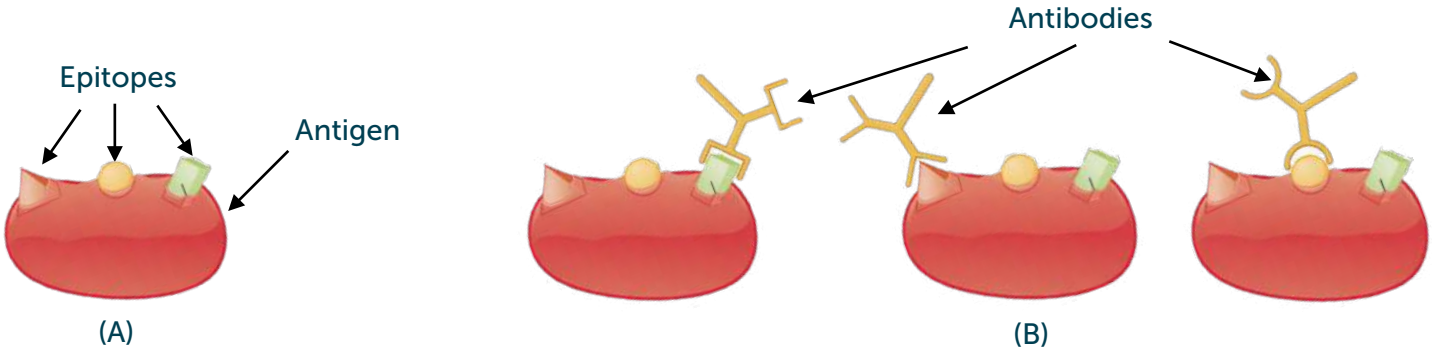


Figure 3. (A) Antigens can have multiple epitopes. (B) Each epitope is recognize by a specific antibody.

Each antibody binds to only one antigen and there are millions of potential antigens. This is possible through a process called antibody rearrangement.

To understand how antibodies work, let's look at their structure. **Antibodies (Ab)** are produced by B cells. They are secreted forms of protein complexes called **immunoglobulins (Ig)**. Every Ig has two subunits, the **heavy (H) chain** and the light **(L) chain**, encoded by different genes. Each Ig is made up of two H chain proteins and two L chain proteins. The H chains are linked to each other by two disulfide bonds and each H chain is linked to an L chain through one disulfide bond. The resulting protein complex is shaped like a letter 'Y'.

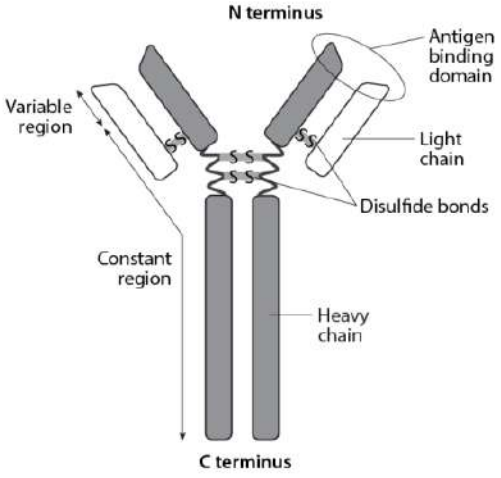


Figure 4. Simplified diagram of antibody structure showing heavy chains and light chains, disulfide bonds, and location of variable domains and antigen binding domains. Image Credit: Rick Simonson. Adapted from Janeway's Immunobiology, 9th Edition, Figure 4.1.

Every Ig has variable **(V)** regions, the parts of the H and L chains at the tips of the arms of the Y, and a constant **(C)** region, which is the rest of the "Y". The V region is the part of the Ig that binds to an antigen. The binding of the V region to an antigen on the surface of a pathogen neutralizes the pathogen because this prevents the pathogen from binding to and entering host cells.

Each immunoglobulin H chain gene is a complex locus made up of multiple variable (V) regions, multiple diversity (D) regions and multiple joining (J) regions, and each L chain has multiple (V) and (J) regions. Antibodies undergo a process called gene arrangement in which V-D-J recombinase cuts and rejoins the DNA such that one V region, one D region and one J region are joined together and all other V, D and J regions are spliced out on the H chain. Similarly V-J regions are spliced out on the L chain leaving one V and one J region on the L chain.

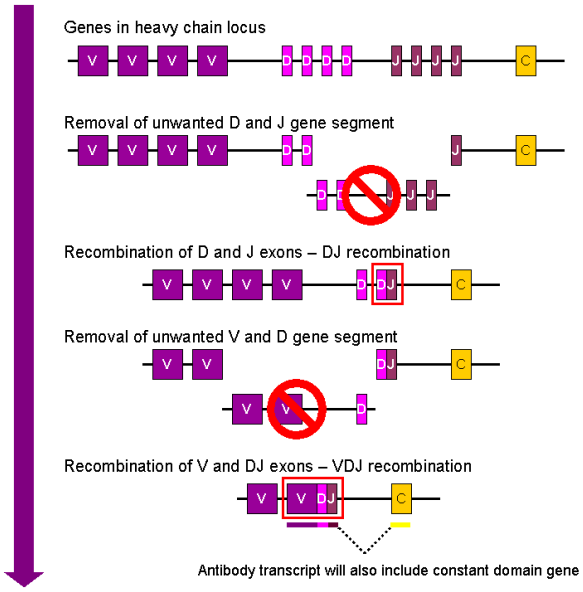


Figure 5. By gustavocarra after en:Ciar - en:Image:VDJ_recombination.png, Public Domain, https://commons.wikimedia.org/w/index.php?curid=4329676

The number of different antibody isoforms that can be generated is staggering. The Ig H chain gene contains at least 40 V regions, 25 D regions and 6 J regions, for a total of 40 x 25 x 6 = 6,000 possible combinations. Meanwhile, there are two types of light (L) chains in antibodies, kappa and lambda. The kappa L chain gene contains at least 30 V regions and 5 J regions, for a total of 200 possible combinations. The lambda L chain gene contains at least 30 V regions and 4 J regions, for a total of 120 possible combinations. 200 kappa L chain possibilities and 120 lambda L chain possibilities gives us a total of 320 L chain possibilities. 320 L chain gene possibilities x 6,000 possible H chain combinations gives 1.9×10^6 possible antibody V region specificities. The variety of possible genetic rearrangements is called combinatorial diversity. Additional diversity can be introduced as nucleotides are added and subtracted at the joints between regions.

Because antibodies bind so specifically to the antigen that triggered the B cell that made them, they can be used as research tools to detect specific proteins. Antibodies are the basis of immunoassays such as an ELISA and lateral flow assay. These assays can be used to detect the presence of protein biomarkers in the bodily fluids of patients.

Pre Activity Questions

1. What do you remember about antibodies?

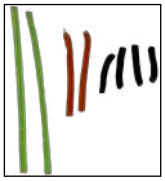
2. What is the function of antibodies in the human body?

3. What is an antigen? What is an epitope? Draw and label a diagram showing an antigen and epitope.

Activity I: Making Antibodies

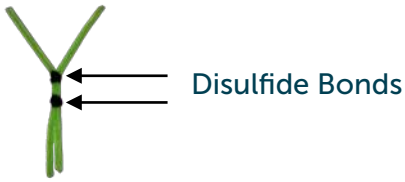
In this activity you will make an antibody based on some of the basic rules of how antibodies are made in your body. This activity is courtesy of the Fred Hutch Cancer Center.

1. Gather the following parts to make your antibody:

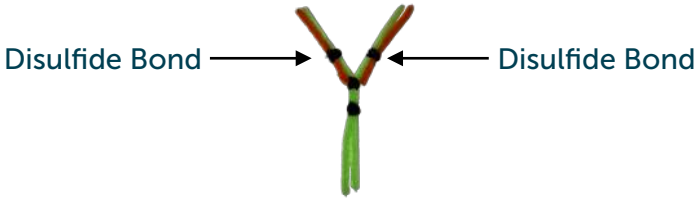


- 2 long stems of the same color of your own choice = **2 heavy chains**
- 2 medium stems of the same color of your own choice = **2 light chains**
- 4 short length black stems = **disulfide bonds**

2. Wrap two black 'disulfide bonds' about halfway up around the two heavy chains to attach the two heavy chains to each other, then bend the two 'arms' of the antibody model apart



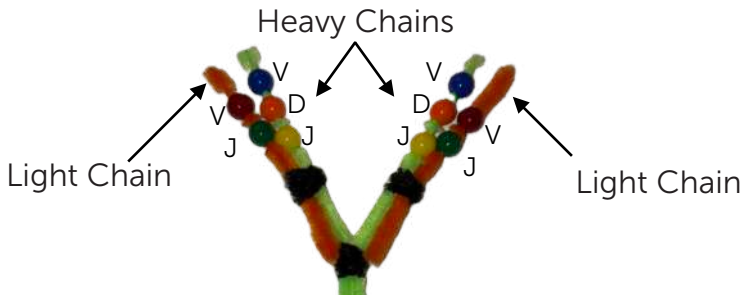
3. Attach each light chain to each heavy chain using 1 disulfide bond each



4. To make the variable regions of the antibodies, gather the following:

Heavy Chains	Light Chains
Choose 2 "V" beads	Choose 2 "V" beads
Choose 2 "D" beads	Choose 2 "J" beads
Choose 2 "J" beads	Light chains do not have D regions

5. Place one each of the 3 V, D and J beads on each heavy chain
6. Place one each of the 2 V and J beads on each light chain



Post-Activity Questions

1. How does the structure of the antibody contribute to its function in the immune response?

2. How does the model help illustrate the concept of antibody diversity? Compare your antibody to your classmates, did anyone make the same one as yours?

3. Why is antibody diversity important?

Lateral Flow Components

Biomarkers

The word 'biomarker' is a combination of the words '**bio**logical **marker'**. A biomarker is a measurable substance or characteristic of the body that indicates a normal or abnormal process, condition, or disease. The most commonly measured biomarkers are proteins or other substances that can be found in bodily fluids such as blood, urine, and saliva. Biomarkers can also be specific types of cells, DNA or RNA.

Biomarkers are important in medicine and can be used in a number of ways to:

- **Indicate the need to test for specific medical issues.** High levels of lead in the blood would suggest the need for cognitive or neurological tests.
- **Diagnose a specific disease or type of disease.** Hemoglobin A1c (HbA1c) levels can be used to diagnose pre-diabetes and diabetes.
- **Monitor disease treatment.** Biomarkers can be used to assess how well a treatment for a disease is working or whether the treatment causes toxic side-effects. Biomarkers can be used to monitor the effects of investigational drugs during clinical trials. The presence of cardiac troponin can predict cardiac damage and cardiac toxicity caused by some types of chemotherapy.
- **Predict disease prognosis.** Biomarkers can be used to predict how likely it is that a particular patient's disease will progress. Elevated levels of CA 27.29 may indicate a higher probability that a patient's breast cancer has metastasized (spread to other sites in the body).

Although biomarkers are most often thought of as ways to measure problems in the body, biomarkers can also be used to measure bodily health. Many substances in our blood, such as cholesterol or blood sugar, can be found at various amounts or concentrations. These levels may be within a healthy range or within an unhealthy range.

Protein biomarkers can often be detected using immunoassays, or tests, that use antibodies in order to detect proteins, such as a lateral flow assay.

Lateral Flow Assay Description

There are several common formats for these assays, namely the sandwich and competitive assays. The format you will be using is the '**sandwich**' format.

The LFA test strip consists of:

- a sample application pad - where the sample to be tested is loaded.
- a conjugate release pad - contains the labeled antibody that will bind to the biomarker.
- an analytical membrane - immobilizes the Test Line and Control Line of antibodies.
- a wicking pad - provides the capillary action to draw the solution through the membrane.

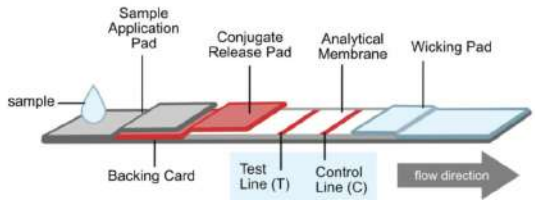


Figure 6. Image source:https://www.milenia-biotec.com/en/universal-lateral-flow-development-tool/

The capture (test line) and control antibodies are immobilized in two separate places on the nitrocellulose membrane. The test line contains an antibody that binds to the specific biomarker being tested for. The control line contains a different antibody that will bind to any other antibody.

During detection, the sample solution moves through the membrane towards the wicking pad by capillary action. If the sample contains the specific target biomarker, the biomarker will bind to the labeled antibody in the conjugate pad creating an Ab-conjugate/biomarker complex. Gold nanoparticles are a common conjugate, and are covered in antibodies with 2 recognition sites, one for the biomarker, and a more generic one that will bind to another antibody. As this Ab-conjugate/biomarker complex passes the test line, it will bind to the antibody to the biomarker on the test line, causing a color change and the formation of a line. This forms the 'sandwich' where the biomarker is sandwiched between the two detection antibodies.

Any excess Ab-conjugate that does not bind to the biomarker will continue to pass along the strip until it encounters the antibodies in the control line. These antibodies will bind to other non-specific antibodies, immobilizing the unbound Ab-conjugate, causing a color change and the formation of a second line. The figure below shows a diagram of a positive test for the pregnancy biomarker, the hormone, human chorionic gonadotropin (hCG).

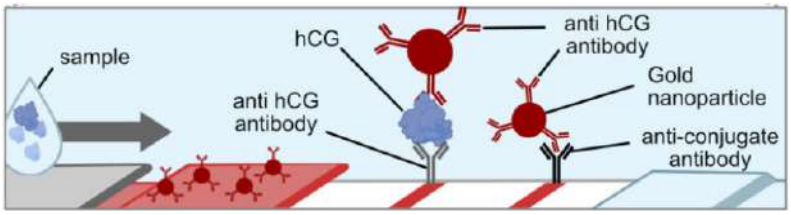
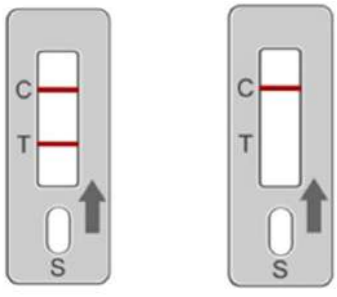


Figure 7. Image source:https://www.milenia-biotec.com/en/universal-lateral-flow-development-tool/



A. **B.**

Figure 8. (A) The conjugate pad contains Ab-conjugate in excess, so there is more Ab-conjugate than biomarker. Therefore, a positive result will show two lines. (B) If there is no biomarker present in the sample, the Ab conjugate will flow past the test line and will not bind to it, continuing down the strip to bind to the Ab in the control line. Therefore, a negative result will show only one line at the control line.

Image source:https://www.milenia-biotec.com/en/universal-lateral-flow-development-tool/

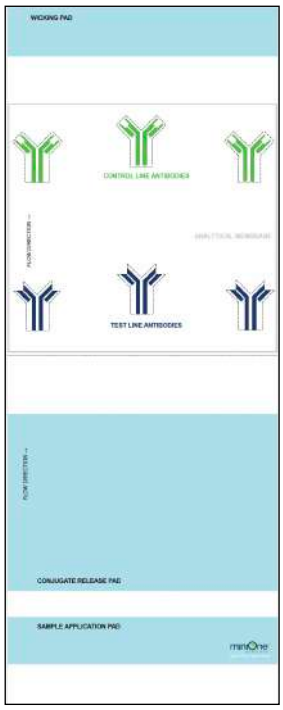
Activity II: Lateral Flow Paper Model

Check your station for the following:

- 1 Lateral Flow Backing
- 1 page of Gold-Ab Conjugates
- 1 strip of 6 Biomarker stickers
- 1 strip of 3 clear stickers

In this activity, you will use a model to see how the various parts of a lateral flow test work together to produce a positive or negative test result.

1. Unfold the backing card and make note of what and where the parts of the test strip are.



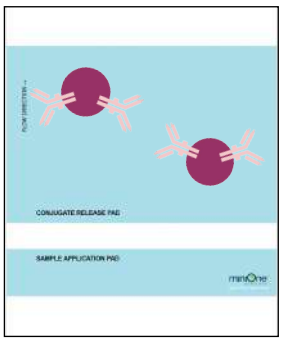
2. Find the three **Control Line Antibodies (green)** and carefully poke the antibodies out of the flat card from behind, then fold at the base of each antibody to make the antibody stand upright.



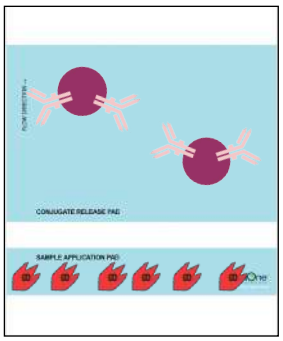
3. Find the three **Test Line Antibodies (blue)** and carefully poke the antibodies out of the flat card, then fold at the base of each antibody to make the antibody stand upright.



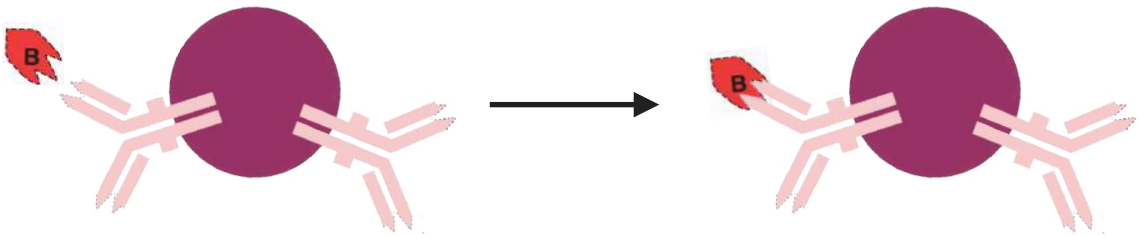
4. Carefully pop out the **Gold-Ab Conjugates** and place on the conjugate release pad.



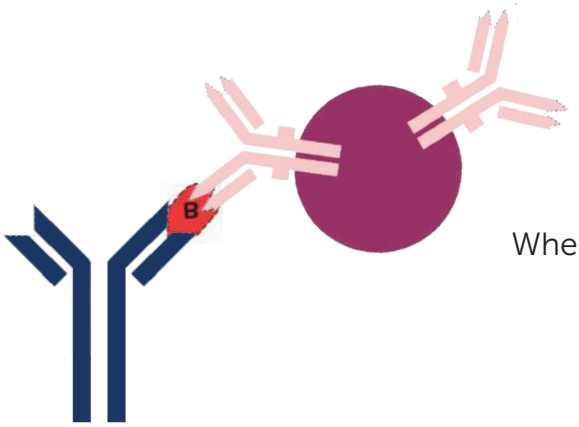
5. Place your Biomarkers on the **Sample Application Pad.**



6. As your biomarkers flow through the assay they will first bind to a Gold-Ab conjugate that has antibodies specific for the biomarker on its surface. Bind (stick) your biomarkers to **some** of the Gold-Ab conjugates at the binding region. You should also have some Gold-Ab conjugates with NO biomarker.

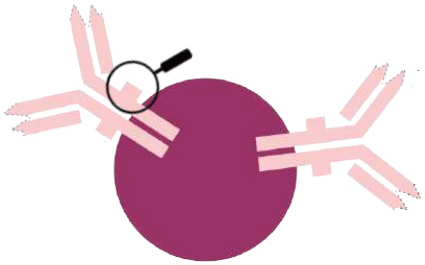


7. Your Biomarker-Gold-Ab Conjugates molecules will now travel to the test line. Find where the exposed side of the biomarker matches the test line antibodies and stick together. Repeat until all of your Biomarker-Gold-Ab Conjugates molecules have been attached to the test line antibodies.

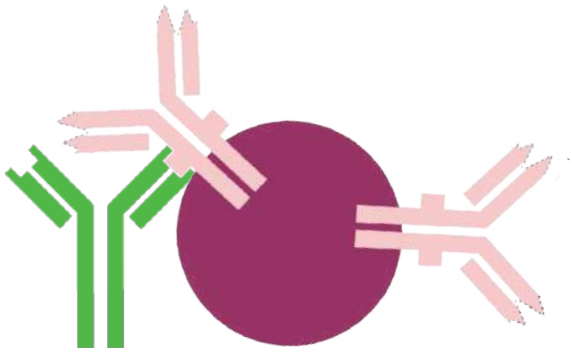


Where is the “sandwich”?

8. Gold-Ab Conjugates that **do not** have a Biomarker attached to it will not attach to the test line. They will instead attach to the antibodies on the control line but notice, the antibody on the Gold-Ab Conjugate has another point of attachment.



9. Attach the Gold-Ab Conjugates to the control line antibodies.



Post Lab Questions

1. Which lines show up with a positive test and why?

2. Which lines show up with a negative test?

3. What does it mean if you get a line at the Test but not the Control lines?

Cancer Metastasis

CA 27.29 Biomarker

In this MiniLab, you will be using a lateral flow assay to detect the presence or absence of the biomarker CA 27.29 in the serum of a cohort (group) of fictional breast cancer patients. CA 27.29 is a biomarker for metastasis of breast cancer. High levels of CA 27.29 in patient serum is an indicator of a higher probability of metastasis. This means there is a greater chance that the cancer of these patients has metastasized.

But what is CA 27.29? CA 27.29 is an '**epitope**', or part of an altered form of a protein called MUC-1. This 'epitope' can be used as an antigen to make antibodies against this specific altered form of MUC-1. MUC-1 is a mucin. It is a glycoprotein, a protein that has sugar residues attached to it. It is found on the surface of many types of epithelial cells. In MUC-1 diagram below, the yellowish line is the MUC-1 protein core. The reddish branches are the sugar residues attached to the MUC-1 protein core. Mucins have a lot of sugar residues attached to them. These sugars stick to pathogens that enter our bodies, preventing them from contacting the epithelial cell surface. If a pathogen is not able to contact the cell surface, it cannot enter and infect the cell.

For reasons that are not understood, breast cancer patients express a version of MUC-1 that has fewer sugars. Because there are fewer sugars, the protein core of this version of MUC-1 is more exposed. Antibodies made to this protein core can bind the altered version of MUC-1 called CA 27.29. The antibodies cannot bind the unaltered version of MUC-1 because the protein core is completely coated with sugar residues.

This altered MUC-1 breaks off from the patient's epithelial cells during metastasis and enters the blood stream. The CA 27.29 version of MUC-1 can be detected in the serum using specific antibodies. The higher the amount of CA 27.29 in the patient serum, the more likely it is that the patient's cancer has metastasized.

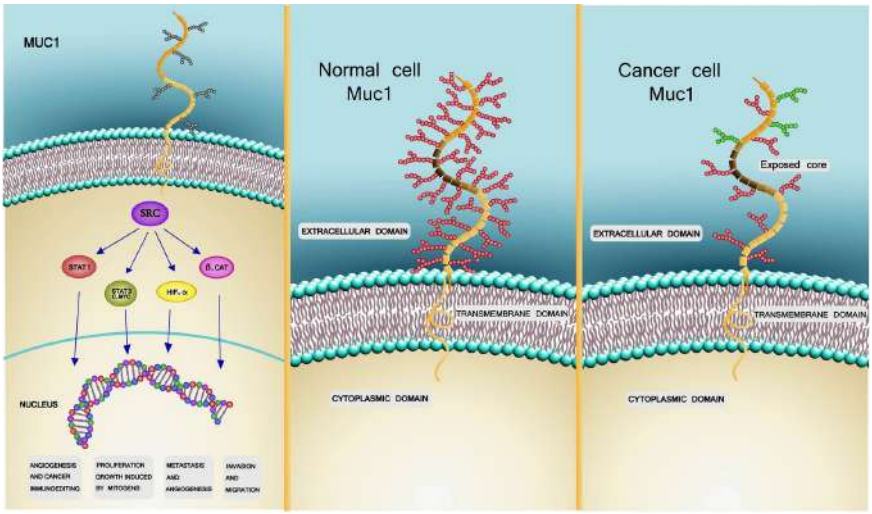


Figure 9. Image source: https://cancerci.biomedcentral.com/articles/10.1186/s12935-021-02398-6

What is Metastasis?

Metastasis is a process in which cancer cells break away from the primary tumor, travel through the blood or lymph, and form tumors in other parts of the body.

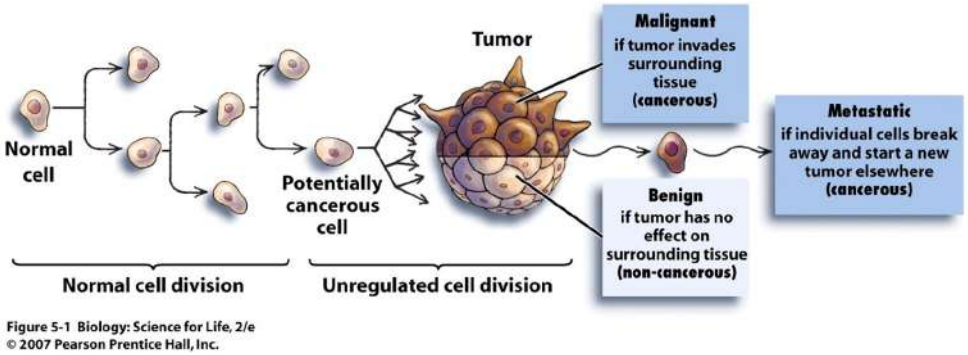


Figure 10. Progression of a normal cell to a cancerous cell that has metastasized.

There are several stages in the process of metastasis:

- Dissociation: In this stage cells dissociate (detach) from the primary tumor mass.
- Invasion: In this stage, the cells invade the nearby tissues including blood vessels or lymph vessels.
- Intravasation: In this stage the cells move through vessel walls and enter the blood vessels or lymph vessels. Many cells do not survive the harsh conditions in the vessels, but some do.
- Extravasation: In this stage the cells move out of the vessels and into distant tissues.
- Colonization: In this stage, cells form tumors in distant tissues

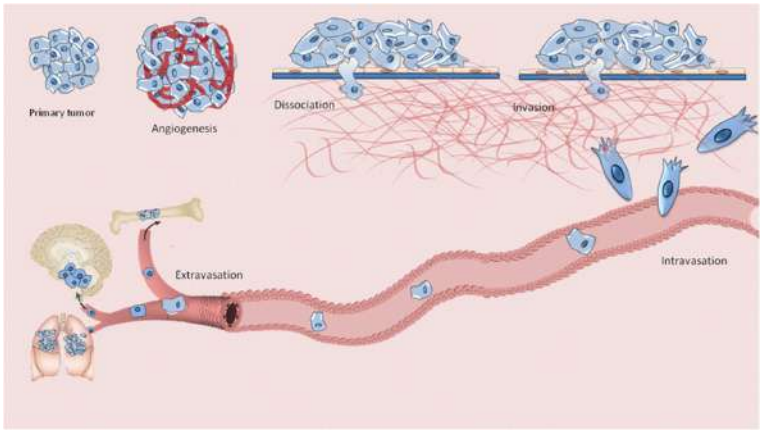


Figure 11. Image source: https://link.springer.com/article/10.1007/s13277-014-2421-z

Each of these stages requires changes in gene expression, leading to the expression of different types of proteins with different functions. The process of metastasis is not well understood and is an active area of research in cancer biology.

For further information on current research in this area please read this article from the Fred Hutchinson Cancer Research Center in Seattle, WA: https://www.fredhutch.org/en/news/center-news/2016/04/science-of-cancer-metastasis.html

Activity III: Breast Cancer Scenario – Lateral Flow Assay

As a medical laboratory technologist, you have been assigned to a cohort (group) of 12 people who are diagnosed with breast cancer and are in need of treatment.

But what is the best treatment for each patient? It is important for oncologists to determine whether or not a patient's cancer has metastasized (spread to other organs). The treatment regimen that is best for a cancer that has not spread is different than it is for a cancer that has spread to other parts of the body.

Your goal is to identify which patients are more likely to have cancers which have metastasized. Your task is to test the serum from the 12 patients with a lateral flow assay that detects the presence of a biomarker of breast cancer metastasis called CA 27.29. The higher the amount of CA 27.29 in the patient sample, the more likely it is that the cancer has metastasized. By examining the results, you will identify which patients are more likely to have cancers that have metastasized.

Finally, you will present your findings, determining the relative level of the CA 27.29 biomarker in each patient sample. Based on this information, you will recommend appropriate therapies and/or further testing. Be sure to justify your treatment suggestions based on the molecular characteristics of each patient's cancer.

These 12 patients are labeled A through L to protect their private health information according to the Health Insurance Portability and Accountability Act of 1996 (HIPAA).

Pre-Lab Questions

1. What kinds of biomedical tests can you think of?

2. Why might it be important for a biomedical test to be accurate?

3. Label the parts of a 'sandwich' lateral flow assay strip in the diagram below:

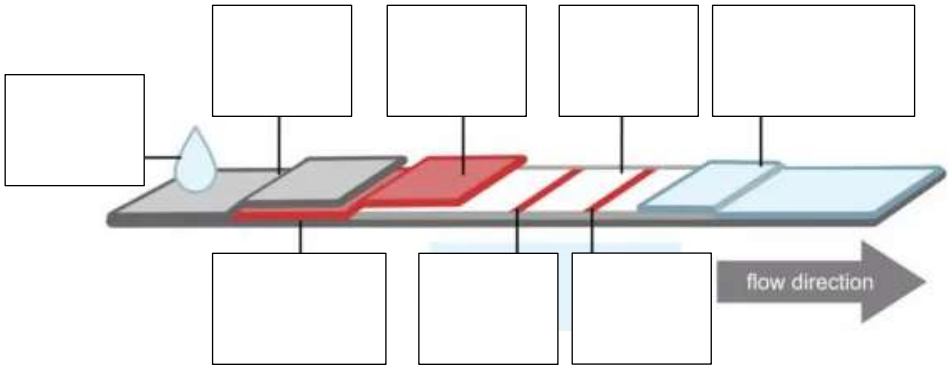


Figure 12. Image source: https://www.milenia-biotec.com/en/universal-lateral-flow-development-tool/

4. What is MUC-1?

5. How is MUC-1 different between cancer patients and non-cancer patients?

Activity III: Lateral Flow Assay Lab

Objective: Perform a lateral flow assay to detect the CA 27.29 biomarker related to breast cancer metastasis.

You will test:

- A negative control
- A positive control
- Patient samples A-F **OR** G-L

Materials:

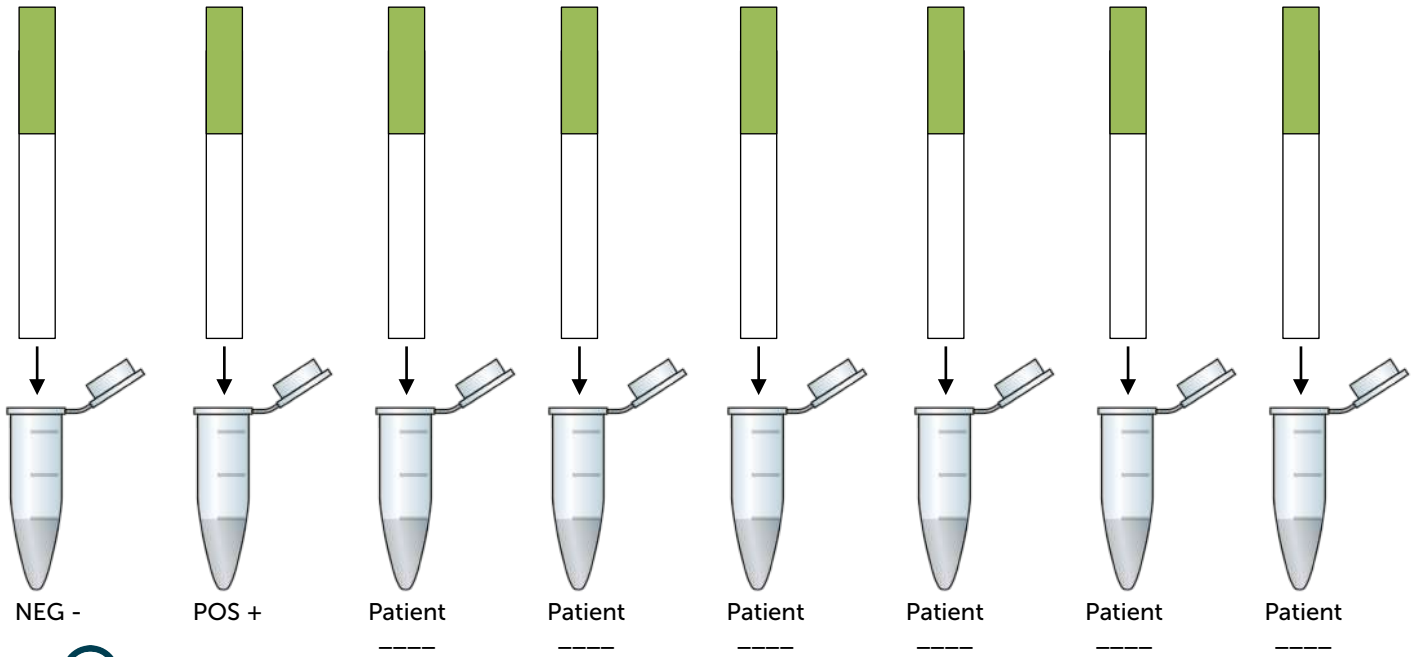
- Sample tubes (Neg ctrl, Pos ctrl, patient samples A-F **OR** patient samples G-L)
- Microcentrifuge tube racks for holding 1.5/2.0 mL tubes
- Lateral flow test strips
- Gloves (for safety)
- Timer or stopwatch
- Fine point marker

Instructions:

1. Line up the sample tubes in a rack in the following order:
   - Neg ctrl, Pos ctrl, A-F **OR**
   - Neg ctrl, Pos ctrl, G-L
2. Label Test Strips: Use a thin tipped sharpie pen to write the sample name on the colored portion of each test strip.



3. Insert Test Strips: Open each tube while keeping it in the rack and place each test strip into the tube labeled with the same sample name, ensuring the ensuring the white end of the strip is partially submerged in the liquid.



4. Wait for Results: Start the timer and wait for 2-5 minutes for the samples to wick up the strips and observe the results.
5. Record Results: After the waiting period, check for the appearance of lines at the test and control positions on each strip, and record the results in the data table below.

Sample	Label the strip and stick to this column	Results
Neg. Control		
Pos. Control		
Patient A		
Patient B		
Patient C		
Patient D		
Patient E		
Patient F		

Sample	Label the strip and stick to this column	Results
Neg. Control		
Pos. Control		
Patient G		
Patient H		
Patient I		
Patient J		
Patient K		
Patient L		

Post-Lab Questions

1. Is your experiment valid? Explain how you know based on your positive & negative controls.

2. Are your patients positive for CA 27.29? Explain your reasoning based on the Lateral Flow Assay Lab.

3. How might the results of this assay influence further clinical decisions for patients?

4. What potential sources of error could affect the accuracy of your assay results?

5. What are some other applications of lateral flow assays in the medical field?

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Patents issued: US 10,641,731 B2, US 20110253541 A1, US 11879118-B2, US 12,384,994,
US 11,879,117, US 11,879,118