Who Has the Flu? Tracing Transmission with ELISA and PCR MiniLab

Student Guide

Cat# M6030

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Based on the original activity developed by

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Laboratory Safety

1. Wear lab coats, gloves, and eye protection whenever possible.
2. Use caution with all electrical equipment such as PCR machines and electrophoresis units.
3. The PCR machine has surfaces that can be extremely hot. Use caution when opening and closing the lid and when placing and removing tubes.
4. Heating and pouring molten agarose is a splash hazard. Use caution when handling hot liquids. Wear eye protection and gloves to prevent burns.
5. Wash your hands thoroughly after handling biological materials and chemicals.
6. Dispose of all materials in a biohazard bag or in a wash tub containing a 10% bleach solution.

Introduction and Lab Scenario

You wake up with a headache, fever, nausea and muscle aches. Is this the flu? Flu is short for influenza, a type of virus, but there are many causes of flu-like symptoms, including the common cold, pneumonia, strep throat, bronchitis, mononucleosis, and food poisoning (see "Did you know: viruses"). Some diseases with flu-like symptoms are caused by bacteria, some by viruses, and some have multiple causes. A visit to the doctor is needed to conclusively identify the **pathogen** and prescribe the appropriate treatment. Meanwhile, your body's immune system is mounting a sophisticated, multi-stage defense and generating an immunological memory that will protect you from future infections by the same pathogen.

The immune response can be compared to a symphony with many specialized cell types and proteins entering and exiting at their appropriate times, coordinating their responses to rid your body of the pathogen (see "Did you know: cells of the immune system"). The innate immune response is active within a few hours of infection, the adaptive immune response develops over the course of days, and immunological memory can last a lifetime.

Innate immune response

The innate immune system is your body's first line of defense against pathogens. Phagocytic cells called **macrophages** are present in all tissues and ready to act, engulfing pathogens and digesting infected cells. Macrophages secrete signals that activate other immune cells and stimulate other cells to produce antimicrobial proteins. **Granulocytes** in the bloodstream respond to signals from macrophages and migrate toward the infection site, where they help to engulf and destroy pathogens and contribute to inflammation. **Natural killer (NK)** cells of the lymphoid lineage are able to recognize and kill some infected cells.

Macrophages and **dendritic cells**, another phagocytic cell type found in most tissues, are messengers between the innate and adaptive immune systems. These phagocytic cells (or phagocytes) digest the pathogens they engulf and present fragments of the pathogens' proteins on the cell surface in a complex with **major histocompatibility complex class II (MHCII)** proteins. These foreign proteins derived from pathogens are called **antigens** and their display on the cell surface is called **antigen presentation**. Macrophages and dendritic cells migrate to the lymph nodes where they activate B and T cells of the adaptive immune system.

Adaptive immune response

The adaptive immune response is distinguished from the innate immune response by the specificity with which it is able to target individual pathogens. Although adaptive immunity takes longer to develop, it enables the body to "remember" a pathogen and be prepared for a faster response the next time it invades.

Adaptive immunity develops after the first exposure to an antigen, either through vaccination or infection by a pathogen (see "Did you know: vaccines"). B and T cells are responsible for the adaptive immune response. B and T cells are able to bind specific antigens through receptors on

their surface, but each individual cell expresses only one type of receptor and that receptor can only bind to a specific antigen. However, there are millions of possible receptors able to bind to millions of potential antigens (see "Did you know: antibody diversity").

B and T cells undergo gene rearrangement and begin expressing their particular antigen receptor during maturation. B cells that are able to bind to self-molecules in the bone marrow are either inactivated or undergo cell death, ensuring that mature B cells that are released into the periphery can distinguish between your own cells and a potential pathogen. Mature B cells leave the bone marrow and circulate through blood vessels and lymph vessels. There they may encounter pathogenic antigens.

Although B and T cells can both bind antigens, the way they interact with them is distinct. T cells can only interact with antigens that have been digested and presented bound to MHC molecules. B cells on the other hand can interact directly with intact antigens.

Adaptive Immunity - humoral response

To find cells to infect, viruses must travel through extracellular fluids like the blood and the lymph. The extracellular spaces are protected by the humoral immune response, so called because these bodily fluids were formerly called 'humors'. **Antibodies** play a major role in the humoral immune response.

Antibodies (Ab), the secreted form of immunoglobulin (Ig), are produced by B cells and can mediate the destruction of pathogenic microorganisms like bacteria and viruses. When naïve B cells encounter a pathogenic antigen, they migrate to lymphoid organs, such as the spleen or lymph nodes, where they are more likely to encounter a T cell that responds to the same antigen. Interaction with a T cell that responds to the same antigen induces an antigen-stimulated B cell to develop into an antibody-secreting plasma cell. B cells also process and display antigens in complex with MHCII proteins, and can activate T cells similar to macrophages and dendritic cells. Antigen-stimulated B cells that do not interact with a T cell that responds to the same antigen within 24 hours will die.

Antibodies help protect us from pathogens in two ways:

1. **Neutralization:** antibodies bind to proteins on the surface of the pathogen and keep them from binding to and entering host cells.
2. **Phagocytosis:** antibodies bound to the surface of a pathogen can be recognized by phagocytic cells, such as macrophages and dendritic cells, which then engulf and destroy the pathogen.

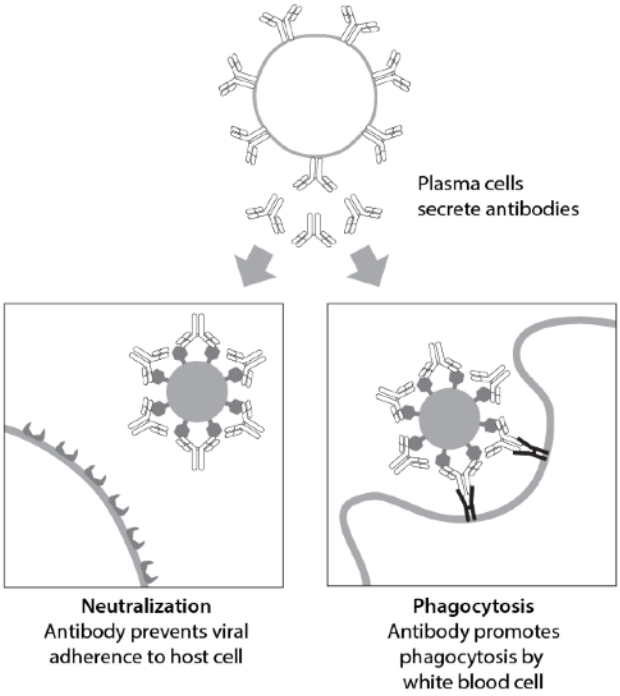


Figure 1. Antibodies protect us from viruses through neutralization and promoting phagocytosis. Image credit: Rick Simonson. Adapted from Janeway's Immunobiology, 9th Edition, Figure 1.28.

Adaptive immunity – cell-mediated response

If the infection is too strong for the innate and humoral immune responses, the cell-mediated immune response is the next line of defense. When antigen presenting cells (APCs) encounter T cells displaying a receptor capable of binding to the displayed antigen, that T cell is activated. These antigen-specific T cells can differentiate into cytotoxic T cells, which induce apoptosis of cells displaying the antigens that the T cell has been activated with. These cells may be infected with a virus or intracellular bacteria, so the apoptosis induced by cytotoxic T cells can stop the proliferation of the pathogen.

Immunological memory

B and T cells activated through exposure to an antigen that they can bind to can develop into **memory B and T cells**. Memory B cells remain in the body for a long time, but do not produce antibodies unless re-stimulated by the pathogenic antigen. Memory cells confer long-lived **protective immunity** against that pathogen because they are able to mount an immune response quickly if the pathogen they recognize enters the body again.

Tests for infection

Tests for infection begin with a culture. A doctor or nurse will insert a swab into your throat or nose and wipe it against your tissues extracting cells and mucus and possibly bacteria and viruses. Doctors can narrow down the type of bacteria by observing which types of media the bacteria will grow on. If the infection is bacterial, this will help the doctor choose the best antibiotic to treat the infection (See "Did you know: antibody or antibiotic?").

But what if your infection is not bacterial? The swab can also be used to diagnose a viral infection. The most common test for a viral infection is called a PCR (polymerase chain reaction) test, which detects viral DNA or RNA inside your own cells as a marker for infection (See "Did you know: Viruses").

The PCR test helps the doctor determine whether a patient is infected at the time of the test. However, when tracing the spread of an infectious disease like the flu, we also want to know who has been infected and recovered. When your body mounts an immune response, B cells make antibodies that circulate in the bloodstream. A doctor may order a blood draw to test for circulating antibodies, a marker for past infection. Based on the levels of influenza-specific antibodies in your serum, the doctor can determine whether you have been exposed, and if so, how recently.

Lab scenario

It was with great anxiety that high school senior Dani Rerio visited Dr. Mona Reinhardt. She had started experiencing flu-like symptoms and was nervous because she was getting ready to star as Titania in A Midsummer Night's Dream, opening in three days at Sandbeach Central High School.

A detail of Dani's personal life alerted Dr. Reinhardt to the potential source of her illness. Dani had another starring role: Sandy the Sea Slug, mascot for the undefeated Sandbeach Sea Slugs girls varsity basketball team. The Slugs had recently defeated the Westlake Water Bears, on the Water Bears' own court, to secure a place in the regional semifinals. Two days earlier, the fifteen-person basketball team had returned triumphantly to the tight knit community of Sandbeach along with their spirit squad, coach and assistant coach, two team managers, three parent chaperones, the bus driver, and Sandy Slug herself.

Dr. Reinhardt was aware that Westlake recently had an influenza outbreak that moved quickly through the town. Dr. Reinhardt suspected that Dani might have picked up influenza during the trip to Westlake. She called the school to recommend that everyone who had been on the bus get tested for influenza, so those infected could self-isolate to prevent an outbreak in Sandbeach.

In addition to the busload of students and chaperones, Dani told Dr. Reinhardt that there were also two carloads of spectators from Sandbeach who also attended the game and may have mingled with the infected Westlakers.

Your job is to test everyone who made the trip to Westlake for the presence of influenza virus

using a PCR assay and for antibodies against the influenza virus using an ELISA assay. Everyone being tested was also asked who they had been in contact with since returning. You will use the data to determine who has the flu and who may be immune due to vaccination or past exposure.

Did you know: Viruses

You may have heard that trillions of bacteria inhabit the human body, but did you know that viruses in the body might outnumber bacteria ten-to-one? Viruses are submicroscopic infectious agents varying widely in size, shape, and composition. Most are between 20 nm and 400 nm, but some, like the Ebola virus, can be one micron long (diagram showing diversity of viruses). For contrast, the diameter of a human skin cell is approximately 30 microns. All have a genome made of either DNA or RNA and a protective protein coat called a capsid.

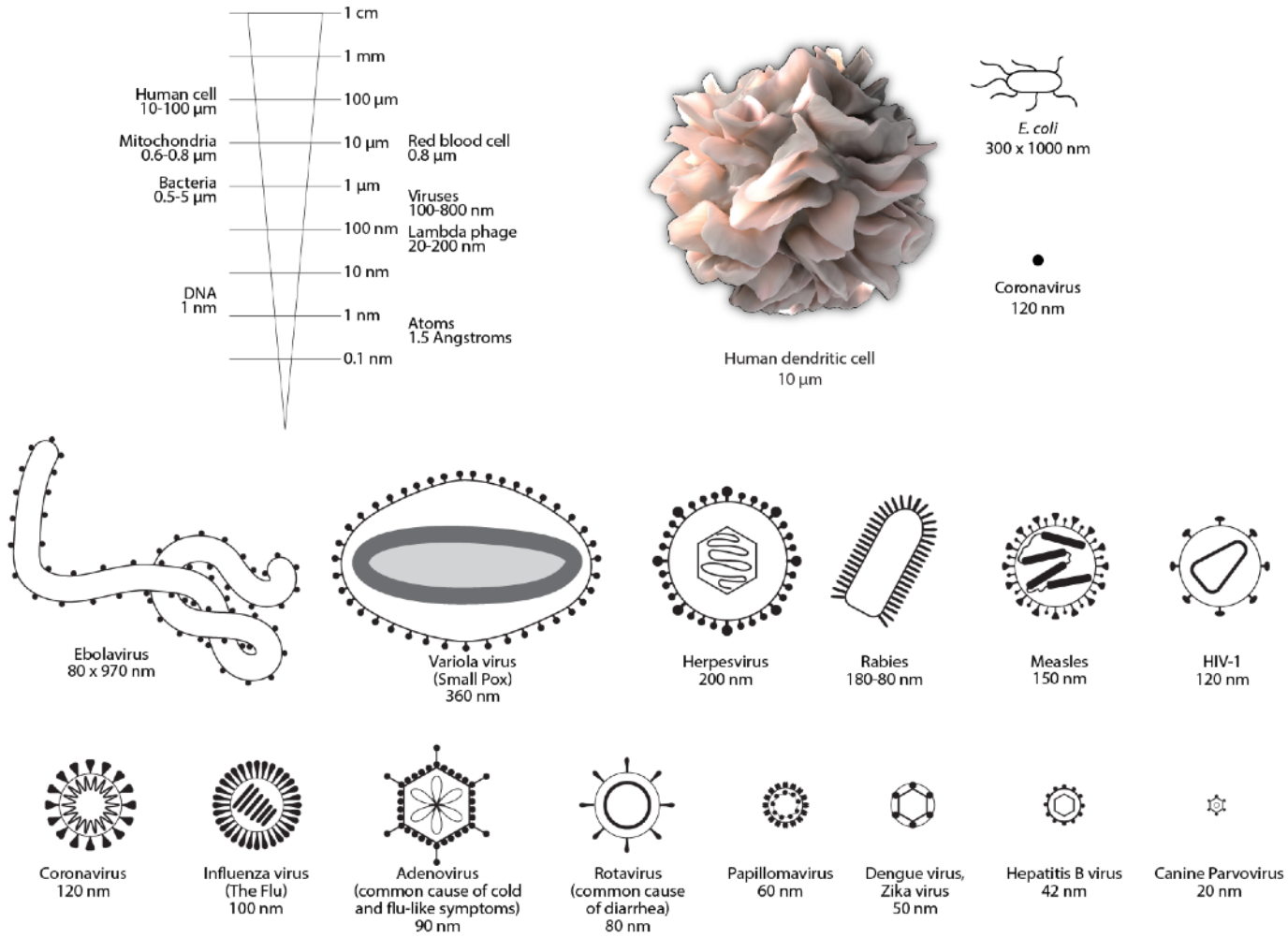


Figure 2. Viruses come in a wide range of sizes, shapes, and structures. Human dendritic cell and E. coli shown for scale. Image credit: Rick Simonson.

Viruses require a host cell to reproduce, making them obligate parasites. After infecting a host cell, the virus turns the host cell's cytoplasm into a factory for replicating its genome and producing the capsid proteins. The newly created genome and capsid proteins assemble into new virions, the complete infectious particles capable of infecting new cells. Some viruses, like the influenza virus and coronavirus, have a lipid layer covering the capsid called the viral envelope. Since soap disrupts the lipid envelope, these viruses are vulnerable to washing with soap and water.

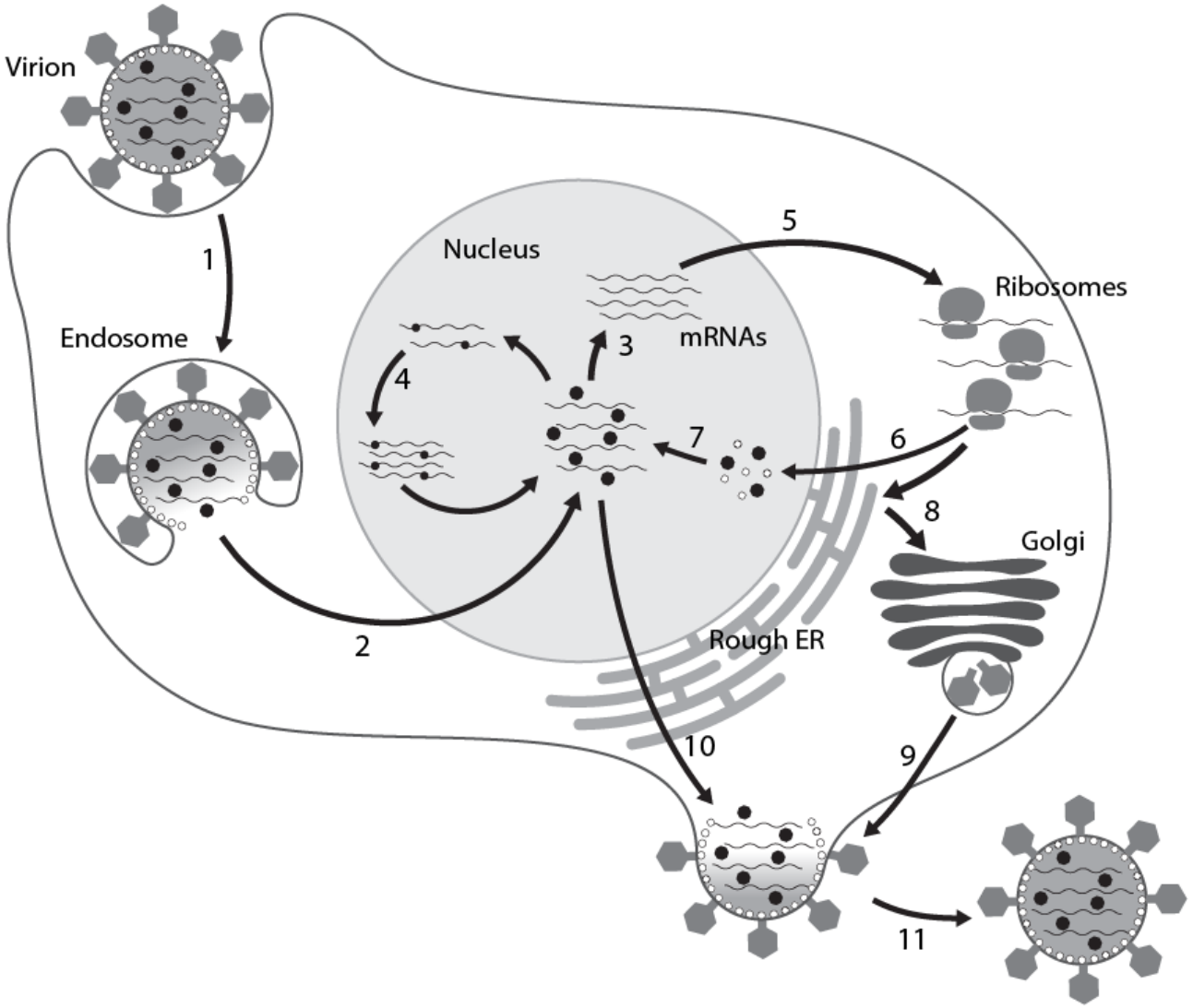


Figure 3. Life cycle of the influenza virus. 1) Virion binds to cell surface and is endocytosed by the cell, 2) viral membrane fuses with endosome membrane and genomic RNA enters the nucleus, 3) viral genome is transcribed into mRNAs, 4) genomic RNAs are replicated in the nucleus, 5) mRNAs exit the nucleus and are translated in proteins, 6) some viral proteins are imported into the nucleus, 7) where they form complexes with genomic RNAs, 8) other viral proteins are package for secretion in the rough endoplasmic reticulum and golgi, 9) secretory vesicles fuse with plasma membrane, 10) viral RNA and proteins are assembled in the core of the virion, 11) Mature virion buds off from the plasma membrane. Image credit: Rick Simonson. Adapted from: https://commons.wikimedia.org/wiki/File:Virus_Replication_large.svg

Viruses can infect bacteria, plants, and animals. Some viral strains are specific to one host species and some can move from one species to another. A virus that can be transmitted from animals to humans is called zoonotic. Most of the viruses in the human body do not infect human cells or cause disease. They are bacteriophages; viruses specialized for infecting our bacterial residents.

The influenza virus has an RNA genome. Some types of flu virus, like the 2009 swine flu, are zoonotic. Influenza virus has two major types of surface proteins: hemagglutinin (HA or H) and Neuraminidase (NA or N). Influenza strains are named for their versions of these two proteins (for example: H1N1, H1N5). (Figure showing basic structure of influenza virus and viral naming scheme)

When the influenza virus comes in contact with a cell, the HA protein on the virus binds to sialic acid sugar residues on the outside of the cell. Once bound, the virus enters the cell through endocytosis. Inside the cell, the virus releases its genome, which enters the host cell's nucleus. There, viral RNA polymerase and the host cell machinery transcribe the viral RNA (vRNA) into complementary RNA (cRNA) and then into mRNA. The viral mRNA travels out of the nucleus and into the cytoplasm where host cell ribosomes translate it into viral protein (Figure of influenza replication cycle, link to animation of viral replication). New virions formed from these proteins bud off of the cell's membrane, eventually killing the host cell.

The most common test for an active viral infection uses PCR (polymerase chain reaction). The PCR test examines the contents of your own cells taken from a swab. If viral RNA or DNA is found in your cells it indicates that your cells have been infected. Different PCR tests detect different types of viruses by specifically amplifying regions of the viral genome that make that virus unique. Based on the results of the PCR test a patient may be prescribed an antiviral medication, which interferes with the virus' ability to infect human cells. Different antiviral medicines work on different types of viruses making the PCR test essential for choosing the correct treatment.

Since the discovery of viruses, scientists have debated whether they are alive or inert. Based on what you have learned here, do you think that viruses are living organisms? How would you describe a virus to a family member who thinks they might have the flu?

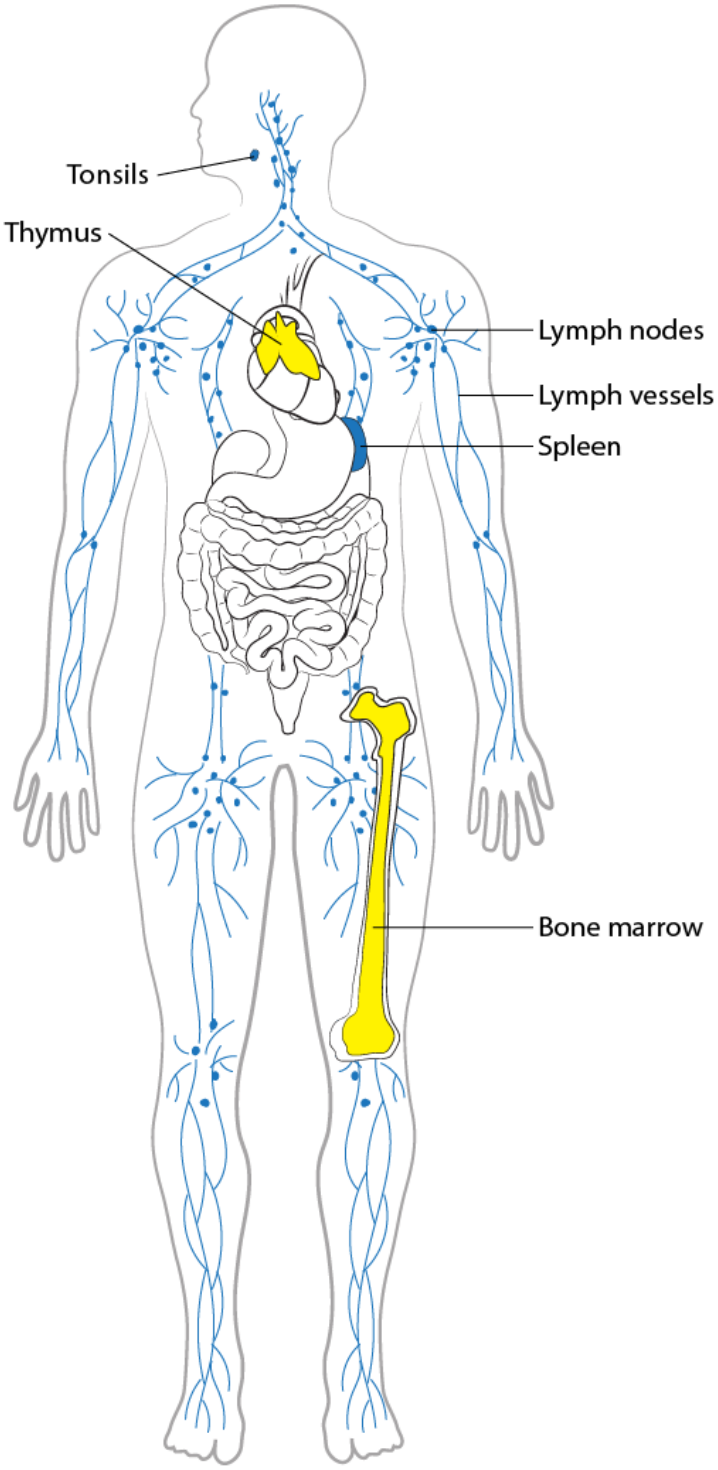


Figure 4. Major lymphatic organs of the human body. Image credit: Rick Simonson. Adapted from Janeway's Immunobiology, 9th Edition, Figure 1.18.

Did you know: Cells of the Immune System

Cells called **leukocytes**, meaning white cells, carry out the work of the immune system. Some leukocytes patrol the body by traveling through blood vessels and vessels of the lymphatic system looking for pathogens, while others reside in tissues.

Leukocytes develop in the bone marrow from pluripotent hematopoietic stem cells. These stem cells can develop into any type of blood cell depending on the stimuli they encounter. Some leukocytes leave the bone marrow and complete their maturation in other parts of the body. Other leukocytes complete their maturation in the bone marrow then migrate out into the body.

Leukocytes come from two lineages: **lymphoid** and **myeloid**. Cells from the myeloid lineage develop into **monocytes**. Cells from the lymphoid lineage develop into **lymphocytes**. There are multiple types of both monocytes and lymphocytes. Cell types involved in both innate and adaptive immune responses are described in more detail below.

Innate Immune Response

Myeloid Cells

Macrophages are mature monocytes. They are phagocytic, meaning they can engulf and kill pathogens. They reside in almost all tissues, making them a common first line of defense against pathogens. They can induce inflammation by secreting signaling proteins that recruit other immune cell types to the site of infection. Macrophages also present antigens to T cells, making them **Antigen Presenting Cells (APCs).** Antigen presentation is a critical link between the innate and adaptive immune systems.

Granulocytes are also called polymorphonuclear leukocytes because of their oddly shaped nuclei. They circulate in the bloodstream and migrate to the site of infection in response to signals secreted by macrophages. There are four types of granulocytes: **neutrophils, eosinophils, basophils, and mast cells.**

- **Neutrophils** are phagocytic, helping to ingest and destroy pathogens.
- **Eosinophils** and basophils are less well understood. They are also involved in allergic inflammatory reactions, and might be a first line of defense against parasites, which are too large to be ingested by macrophages and neutrophils.
- **Mast cells** contribute to inflammation and allergic reactions. They reside in connective tissues throughout the body.

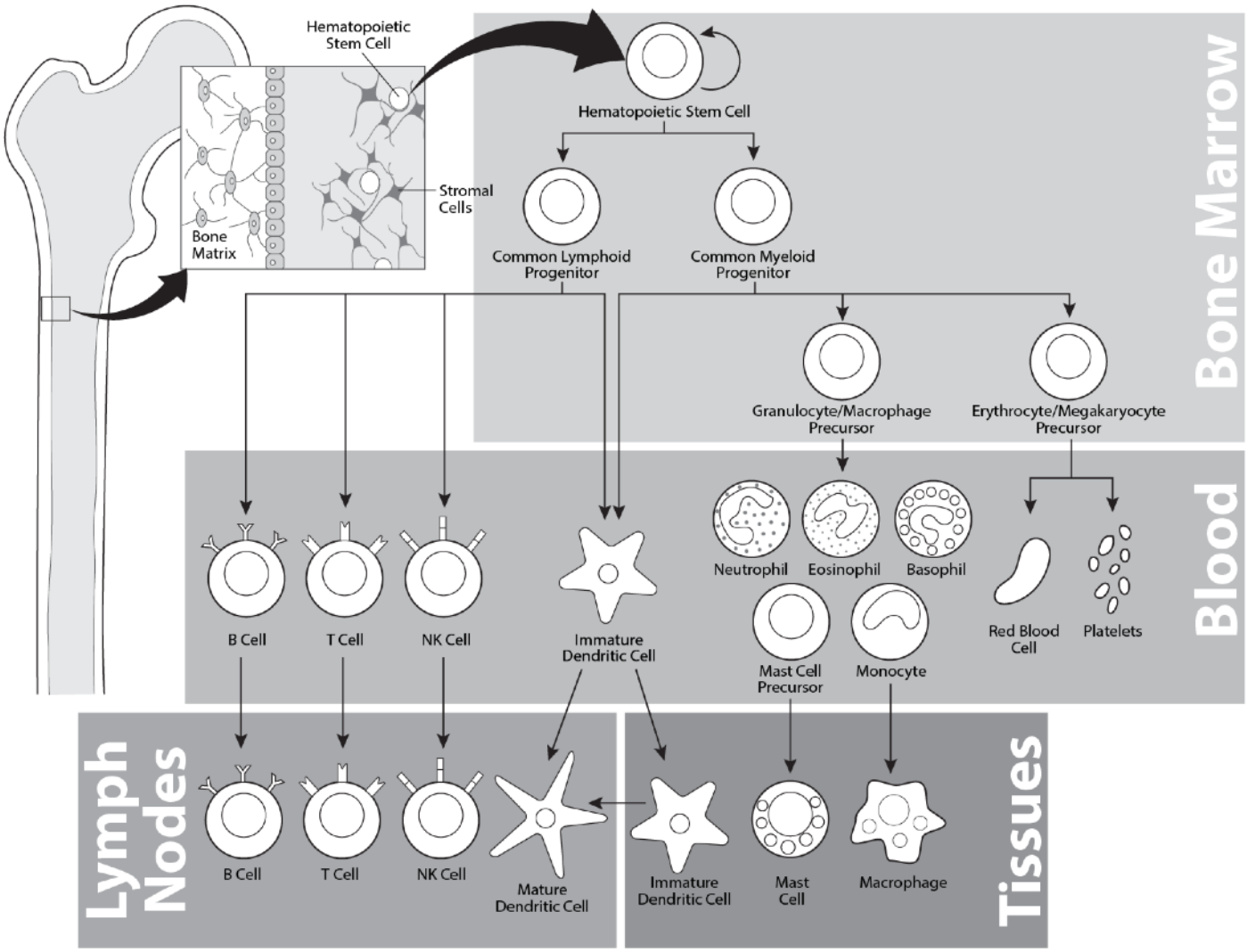


Figure 5. All blood cells arise from hematopoietic stem cells. In the bone marrow, hematopoietic stem cells give rise to common lymphoid precursors, which and common myeloid precursors. The lymphoid precursors give rise to B cells, T cells, NK cells, and immature dendritic cells, which mature in the lymph nodes. The myeloid precursors give rise to neutrophils, eosinophils, basophils, mast cells, macrophages (through the granulocyte/macrophage precursor), and to red blood cells and platelets (through the erythrocyte/megakaryocyte precursor). Image credit: Rick Simonson. Adapted from Janeway's Immunobiology, 9th Edition, Figure 1.3.

Dendritic cells are phagocytic cells that have long finger-like processes that like the dendrites of neurons. They are found in most tissues of the body. Dendritic cells also present antigens to T cells and are classified as **APCs.**

Lymphoid Cells

Natural Killer (NK) cells recognize and kill some abnormal cells including virally infected cells and some tumor cells.

Adaptive Immune Response (all lymphoid cells)

T lymphocytes (T cells) mature in the **thymus** and then enter the bloodstream. Before encountering antigen, they are called **naïve T cells.** All T cells express a **T cell receptor (TCR)** complex on their surface, which binds to antigens presented by APCs. Binding between antigen and TCR activates the T cell and it becomes an **effector T cell**. Effector T cells are responsible for the cell-mediated immune response (Janeway Fig. 8.1).

Types of effector T cell:

- **Helper T cells (Th)** produce cytokines after antigen presentation, which stimulate B cells to produce antibodies.
- **Cytotoxic T cells (CTL)** kill cells expressing the antigen they have been activated by, such as cells infected by bacterial or viruses.
- **Regulatory T cells (Treg)** control adaptive immune responses by producing inhibitory cytokines.

B lymphocytes (B cells) mature in the bone marrow and then travel to the bloodstream and the spleen (Janeway Fig. 7.5). B cells express immunoglobulin molecules on their surface, also called the B cell receptor (BCR). Each B cell expresses its own unique BCR (see "Did you know: antibody diversity"). Before encountering an antigen they are called **naïve B cells.** If the BCR binds to an antigen, the BCR sends an activating signal into the cell. The bound antigen is transported into the cell where it is degraded into peptides. These peptides are exposed on the B cell surface bound **Major Histocompatibility Complex (MHC)** proteins where they can activate helper T cells. Activated helper T cells then secrete cytokines that activate B cells to develop into immunoglobulin secreting plasma cells. Secreted immunoglobulins are known as **antibodies** and are responsible for the humoral immune response.

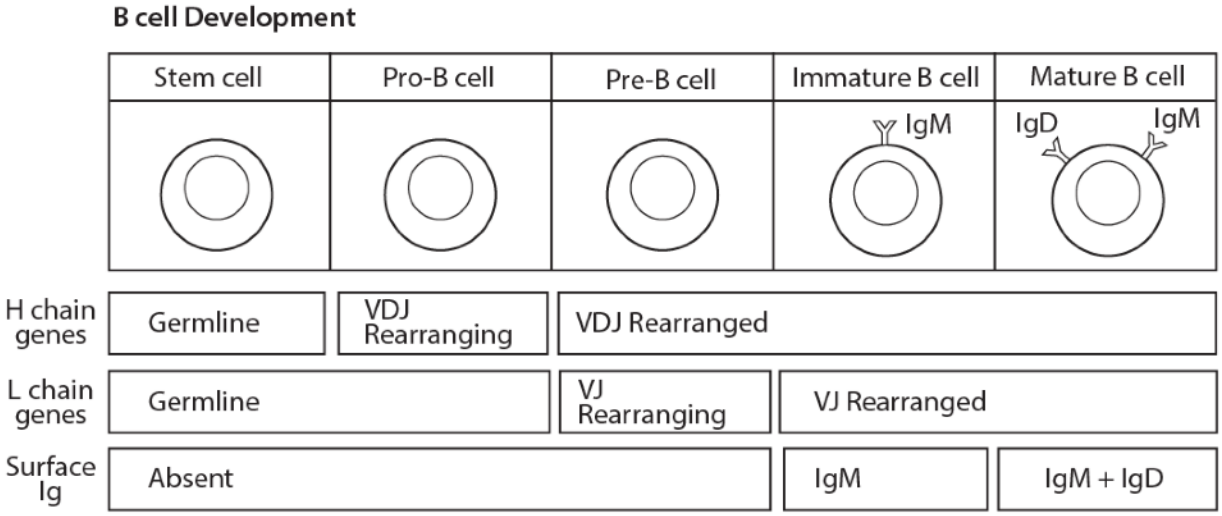


Figure 6. B cell development, antibody gene rearrangement, and surface Ig expression. Image credit: Rick Simonson. Adapted from Janeway's Immunobiology, 7th Edition, Figure 7.5

All effector B and T cells can become memory cells, which are responsible for maintaining long-lasting immunity that follows exposure to a pathogen or to vaccination.

Did you know: Immunization/Vaccination

In the late 18th century Dr. Edward Jenner observed that milkmaids who had cowpox were immune to the smallpox epidemics that happened regularly in Britain at that time. Cowpox made the milkmaids sick, resulting in lesions on their skin, but was not as lethal as smallpox.

In 1796, Jenner injected pus from a cowpox lesion into a cut made in the arm of an eight-year-old boy named James Phipps. Jenner then exposed Phipps to smallpox and Phipps did not become infected. Phipps was exposed to smallpox numerous times over the next 20 years as smallpox epidemics came and went in England in the early 1800s. He never became infected.

The immune response stimulated by the live cowpox protected against infection by the related virus that causes smallpox. The act of injecting cowpox to generate protection against smallpox became known as vaccination, from vaccinia, the Latin name of the cowpox virus. Today, vaccination refers to the act of injecting any material to generate immunity against an infectious agent. The term immunization can refer to the act of injecting a vaccine or to the injection itself.

Instead of injecting the live virus that could make us sick, most modern vaccines contain a killed virus, or only a piece of the virus, often purified viral capsid proteins. These proteins are usually the antigens that our body recognizes as foreign, triggering the immune response. The viral proteins are mixed with liquids for ease of injection.

Vaccines expose our bodies to viral antigens in a way that should not make us sick, but gives our immune system a head start on making antibodies before becoming infected by the live pathogen. As you've learned, each B cell makes a unique antibody. In most cases, some of these antibodies are able to bind to the viral proteins in the vaccine. The B cells that make antibodies that can bind the viral proteins are stimulated to divide and produce more antibodies.

Some of these B cells will become memory B cells, which circulate in our bodies for many years and make low amounts of the antibody to the antigen in the vaccine. If you are exposed to the live virus after you've been vaccinated, these memory B cells produce antiviral antibodies, neutralizing the virus before an infection can get underway.

How long does immunity conferred by a vaccine last? It depends on the vaccine and the virus. Immune responses to some vaccines are stronger than others. Often a second or third booster vaccination is needed to strengthen the immune response. For example the polio vaccine is given a total of 4 times – at 2 months of age, 4 months, 6-18 months and 4-6 years – to produce immunity that will last a lifetime. Vaccines against bacterial infection can also be designed. Familiar examples include vaccines against tetanus, typhus, and whooping cough.

Why is there a different flu vaccine EVERY year? Viral genomes mutate more rapidly than animal genomes. Unlike animal cells, viruses do not have enzymes that repair mistakes in their DNA sequence, so mutations accumulate rapidly. RNA-based viral genomes, like the flu virus genome, mutate even faster than DNA-based viral genomes. Mutations may change the structure of capsid proteins in such a way that antibodies stimulated by the previous year's flu vaccine can't bind. Without antibodies to neutralize the virus we may become sick.

How is each new flu vaccine created? Each flu vaccine is designed to protect against the 3 or 4 influenza strains that are most likely to cause illness during the coming flu season. National influenza centers in over 100 countries conduct influenza surveillance year round to determine which circulating strains are most likely to infect people in the coming flu season. These centers send virus samples to five World Health Organization (WHO) centers. One of these centers is in the US – in Georgia. Twice a year, the WHO reviews the data and makes recommendations for the next flu vaccine.

The antigen used in the influenza vaccine is the hemagglutinin (HA) coat protein. This is effective at preventing infection, since antibodies that bind to HA proteins can keep the virus from entering the patient's cells. However, the influenza HA protein mutates rapidly, so scientists are looking for other ways to stimulate long-lasting immunity against influenza.

Where do scientists get enough influenza virus to make a vaccine? The influenza virus has been traditionally made in live chicken eggs, which are easily infected with influenza virus. The virus replicates in the chicken egg and fluid containing the virus is harvested. The virus is then heat-killed and viral antigens are purified. Since 2013, HA antigens have been produced in cultured cells from armyworms using recombinant DNA technology. This method is much faster than purifying HA from whole viruses produced in chicken eggs.

Did you know: Antibody Diversity

We have seen that your body produces antibodies that bind to an antigen and help the immune system to destroy a pathogen. Antibodies bind to antigens like a lock and key, giving the immune response specificity and the ability to distinguish self from non-self proteins. Each antibody binds to only one antigen and there are millions of potential antigens. How does your body generate proteins that bind with high specificity to antigens it has never seen before?

To understand the remarkable adaptability of the immune system, we'll have to look at how antibodies are produced. Produced by B cells, Antibodies (Ab) are secreted forms of protein complexes called immunoglobulins (Ig). An 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 is linked to an L chain through one disulfide bond. The resulting complex is shaped like a letter "Y" (Janeway Figs 3.1 and 3.2).

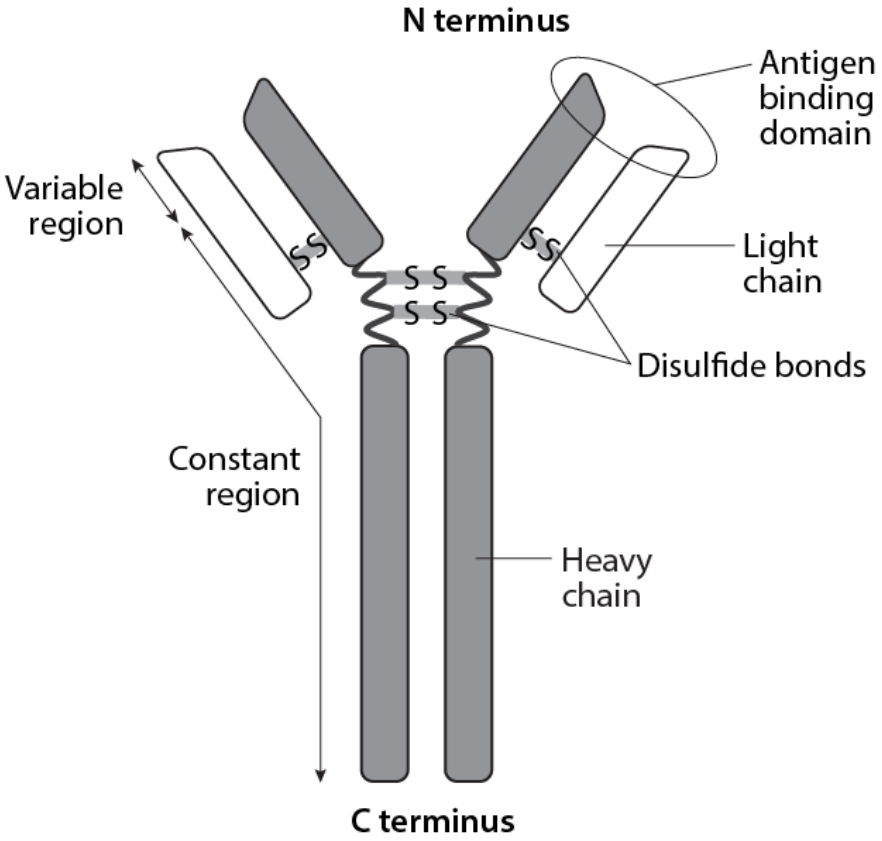


Figure 7. Simplified diagram of antibody structure showing heavy chains and light chains, disulfide bonds, and location of variable domains and antigen binding domains. Image Credit: Rick Simonson. Adapted from Janeway's Immunobiology, 9th Edition, Figure 4.1.

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" (Janeway Fig 3-1). The V region neutralizes the pathogen by binding to antigens on the pathogen surface, preventing the pathogen from binding to and entering host cells. The C regions of antibodies bound to a pathogen are recognized by phagocytic cells, which engulf and destroy the pathogen.

The human genome has a single heavy chain gene and two light chain genes, one encoding the kappa light chain and one encoding the lambda light chain. How is it possible for this small number of genes to generate proteins that can bind to such a wide range of antigens? The key is antibody gene rearrangement, discovered by Tsusumu Tonegawa's lab in the 1990s.

During gene rearrangement, an enzyme called V(D)J recombinase cuts stretches of DNA out of the genome guided by recombination signal sequences (RSS). Each H chain gene has multiple variable (V) regions, multiple diversity (D) regions and multiple joining (J) regions. The L chain genes contain multiple V regions and J regions, but no D regions. 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 (Janeway Fig 4-2).

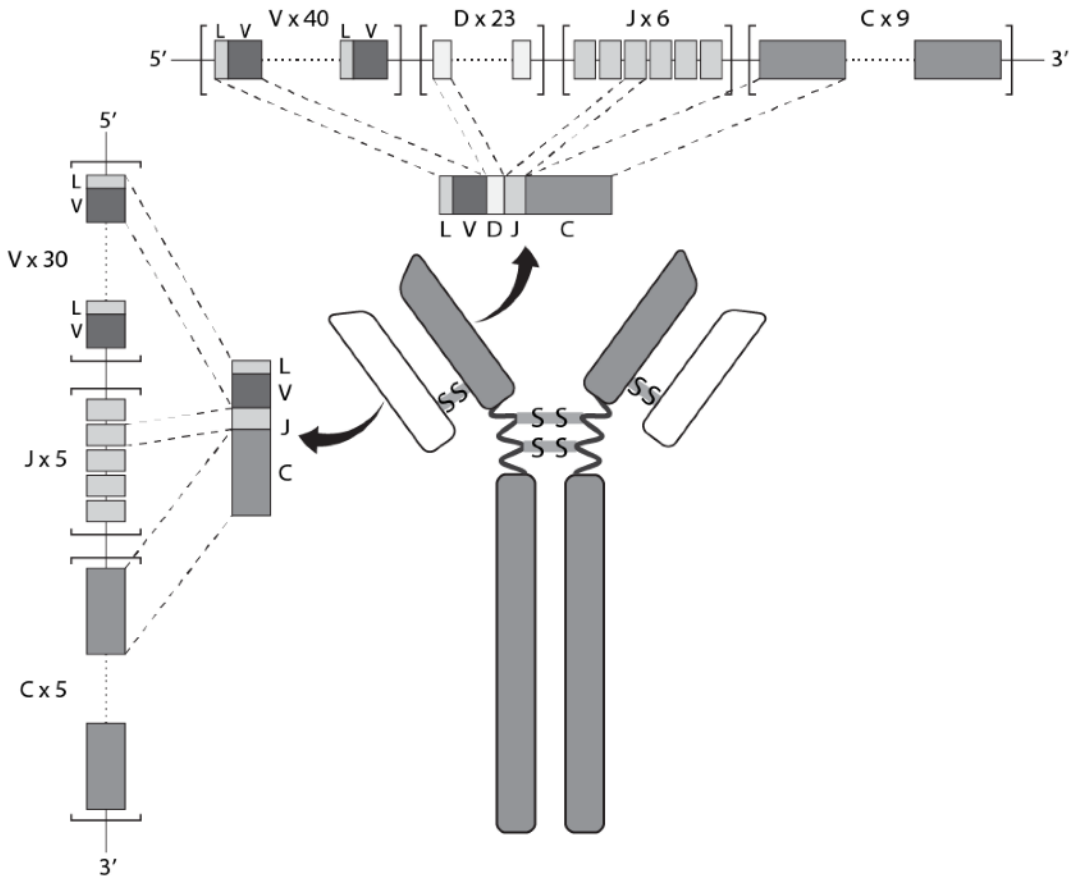


Figure 8. Generation of antibody diversity through gene rearrangement. Heavy chains are constructed by joining together LV, D, J, and C domains, while light chains are constructed by joining together LV, J, and C domains. Image Credit: Rick Simonson. Adapted from Janeway's Immunobiology, 9th Edition, Figure 5.3.

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. 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. 320 L chain gene possibilities x 6,000 possible H chain combinations gives 1.9 x 10^6 possible antibody V region specificities (Janeway Figs 4-3 and 4-4). 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.

As a hematopoietic stem cell begins to differentiate into an immature B cell, it begins to express Ig on its surface. At this point, each developing B cell will undergo its own rearrangement events to generate unique heavy chain and light chain genes. Each B cell will use either the kappa L chain gene or the lambda L chain gene to create light chain proteins.

The H chain gene also has multiple C regions that can be used and the C region determines the

class, or isotype, of the antibody. There are five classes of Ig: IgG, IgA, IgE, IgD and IgM. The protective mechanisms mediated by a particular Ig molecule depend on the class. Naïve B cells that have not yet encountered an antigen express IgM and IgD. After encountering an antigen, the B cell undergoes recombination of the C region and switches to producing IgA, IgG, or IgE.

Here we have just examined the mechanisms behind Ig diversity in B cells, but T cells also use genetic rearrangements to generate millions of unique antigen receptors.

Did you know: Antibody or Antibiotic?

What is the difference between an antibody and an antibiotic? You might associate both words with something that defends your body against illness, but antibody and antibiotic describe different types of molecules with different mechanisms of action.

Antibiotics are chemicals that kill bacteria. The word antibiotic is a combination of the Greek prefix anti (against) and the Greek word bios (life). Antibiotics interfere with bacterial growth through a variety of mechanisms. For example, ampicillin and penicillin interfere with bacterial cell wall synthesis, leading to cell rupture, while kanamycin blocks protein synthesis.

In nature, fungi and bacteria produce antibiotics as weapons for chemical warfare against other microorganisms. In the clinic, antibiotics are used to treat bacterial infections. Antibiotics do not work against viruses so are not an effective treatment for the flu. They also do not work against eukaryotic cells, so cannot be used to treat cancer.

Antibodies are proteins produced by your own body as a defense against foreign microorganisms that can make you sick. When your body mounts an immune response against influenza virus or bacterial infection, immune cells called B cells make antibodies that help your immune system destroy the pathogen (see "Did you know: cells of the immune system"). The "body" in "antibody" is the protein itself, so an antibody is a "body" that is against something. Antibodies circulate around the body via the bloodstream.

Pre-lab Questions

1. Describe, in sequence, the response of your immune system to a virus you have never been exposed to or vaccinated against, from the time the virus enters the body to the formation of immune memory for the virus's antigens.

2. How would an immune response to a virus differ between an individual who has been vaccinated against that virus and an individual who has not?

3. What results would you expect from a PCR test for influenza infection for each type of patient? Briefly state your reasoning.
    a. An individual who has not been infected with influenza
    b. An individual who was exposed to influenza but previously received a vaccination against this year's strain
    c. An individual who is infected with influenza but received a vaccination against a previous year's strain
    d. An individual who is infected with influenza but was not previously vaccinated against any influenza strain

4. What results would you expect from an ELISA test for influenza antibodies for each type of patient, assuming the ELISA test uses proteins from this year's influenza outbreak strain? Briefly state your reasoning.
    a. An individual who has never been vaccinated against influenza and has never been infected with the virus.
    b. An individual who has never been vaccinated, but has been infected with the influenza virus in a previous year.

c. An individual who received an influenza vaccine in a previous year.

d. An individual who has received the influenza vaccine every year including the current year.

5. Make a Venn diagram below showing the differences and similarities between PCR and ELISA in terms of their uses in detecting influenza exposure.

6. Optional activity: with your lab group, make a set of trading cards for the immune system cell types discussed in the introduction. Include a picture of the cell, information about the cell's special abilities or weaknesses, and its role in the immune system. Look at Pokemon cards, baseball cards, or Magic cards for inspiration.

Patient Data Table

As you go through the PCR and ELISA analysis modules, use the table below to keep track of the test results for each patient. Record the patient number in the first column. For PCR and ELISA results, record "++" for a high positive result, "+" for a medium positive result, or "-" for a negative result. Summarize your assessment of each patient in the "Diagnosis" column. Include whether you think they currently have the flu and whether you think they have immunity against the flu based on past exposure or vaccination.

Patient	PCR Results	ELISA Results	Diagnosis

Module 1: PCR Test for Influenza Virus Infection

PCR is used to test whether a patient currently has an influenza infection. The first step is to extract viral genomic RNA from the patient's nasal swab. A gene of interest is then copied into complementary DNA (cDNA) using a reverse transcriptase enzyme and gene-specific primers. The specific viral gene is then copied by Taq polymerase, creating millions of copies through exponential amplification. This amplification is necessary to generate detectable quantities of DNA.

The technique typically used is quantitative real time PCR (qRT-PCR) which can measure the concentration of RNA in the original sample, which is a proxy for the severity of the infection. Here we will only be able to observe the quantity of DNA amplified by the final PCR cycle. This amount is proportional to the starting template concentration, so you will be able to judge qualitatively from the brightness of the band whether the infection level is high, medium, or negative.

Materials at Your Stations

PCR Materials for each student group

- Influenza Primer Set
- FastTaq™ PCR Master Mix (2X)
- MiniOne® DNA Marker
- Sample Loading Dye (5X)
- 4 patient DNA samples
- 1 each positive and negative control DNA
- 6 X 0.2 mL PCR tubes
- 1 micropipette (2-20 µL) and 20 pipette tips
- PCR tube rack
- Fine point permanent marker

Electrophoresis Materials for each student group

- 1 MiniOne® Casting System
- 1 MiniOne® Electrophoresis System
- 1 micropipette (2-20 µL) and 10 pipette tips
- 1 agarose GreenGel™ cup (1.5%)
- TBE running buffer (135 mL)
- Sample Loading Dye (5X)
- MiniOne® DNA Marker

Common Workstation

- MiniOne® PCR Systems
- Tablets with MiniOne® PCR App
- Microwave
- Benchtop microcentrifuge

Experimental procedures - check the box when done

1. Check your workstation to make sure you have the required materials. ☐
2. Label the tops and sides of six thin-walled PCR tubes with your patient numbers and group number. ☐
3. Add reagents to each of the tubes according to the table below. Pipette the reagents directly into the bottom of the PCR tubes and try to avoid creating bubbles. Write your patient numbers into the table below. ☐

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6
Patient ID	Positive control	Negative control				
FastTaq™ PCR Master Mix (2X) (µL)	10	10	10	10	10	10
Patient DNA (µL)	5	5	5	5	5	5
Primer mix (µL)	5	5	5	5	5	5
Total volume (µL)	**20**	**20**	**20**	**20**	**20**	**20**

4. If there are some reagents stuck to the sides of the tubes, briefly spin down with a centrifuge to collect all liquid at the bottom of the tube. If a centrifuge is not available, tap the bottom of the tube on the bench. Gently flick the tube with your finger to make sure the reagents are well mixed and there are no bubbles trapped at the bottom of the tube. ☐
5. Turn on your MiniOne® PCR thermal cycler and place your tubes in the wells on the aluminum plate. Follow the instructions in your Getting Started Guide to program the following cycling parameters on the MiniOne® PCR mobile app and start the PCR protocol. ☐

PCR Step	Temperature (°C)	Time (s)	Cycles
Denaturation	94	5	20 cycles
Annealing	53	5	
Extension	72	5	

6. Monitor the progress of the PCR protocol with the real-time graph on the MiniOne® PCR mobile app. ☐

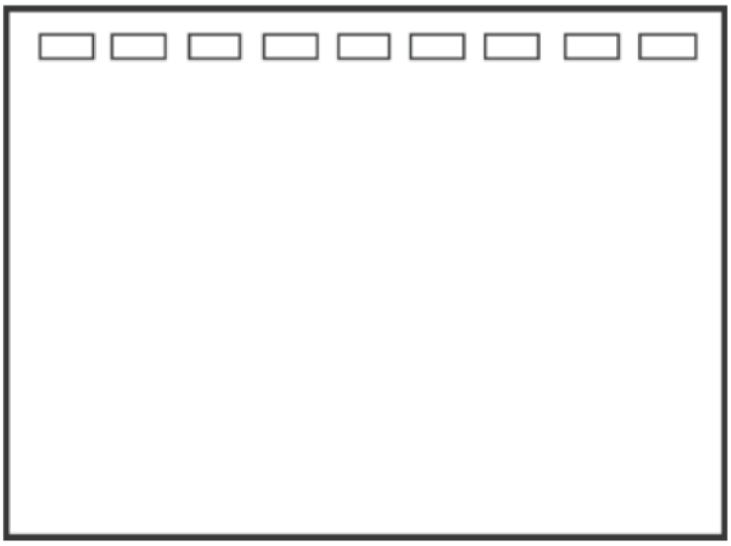
7. When the PCR protocol is complete, remove your tubes from the thermal cycler. Samples can be used immediately for gel electrophoresis or stored in the refrigerator overnight. **STOPPING POINT** ☐
8. Prepare your samples for gel electrophoresis by adding 5 µL of Sample Loading Dye (5X) to each sample. Flick with your finger to mix. If necessary, centrifuge or tap on the benchtop to bring all liquid to the bottom of the tube. ☐
9. Cast the 1.5% agarose GreenGel™ using the MiniOne® Casting Stand. Use the 9-well side of the comb. ☐
10. After the gel has solidified (~10 minutes) carefully remove the comb and follow the directions to set up the MiniOne® Electrophoresis chamber. ☐
11. Pour the buffer into one side of the tank to push air out from under the gel tray, creating a nice even background, without trapped air bubbles, for clear imaging of results. ☐
12. Load 10 µL of each sample and the MiniOne® DNA Marker (100, 300, 500, 1000 and 2000 bp) into the wells, keeping track of the placement using the Gel Analysis Worksheet. ☐
13. Run your gel for 20 minutes or until the bands have clearly separated. Document your results with your cell phone or camera and paste an image of your gel in your lab notebook. ☐
14. Complete the Gel Analysis Worksheet and the PCR analysis questions below. Record your patient results in the Patient Data Table. ☐

Gel Analysis Worksheet

Directions: Record an image of your gel and draw the results on the template below.

Note: Fragment sizes in the MiniOne® DNA Marker are 100, 300, 500, 1000 and 2000 bp.

Well	Sample
1	
2	
3	
4	
5	
6	
7	
8	
9	



PCR Analysis Questions

1. Based on the results of the PCR assay, which patients are infected with the influenza virus? Do these results match the predictions you made in the ELISA Analysis Questions?

2. Is there any correlation between the patients' vaccination status and the result of their PCR assay? What accounts for the differences?

3. Based on your results and the data on the patient information cards, are there any patients that might be infected with a different pathogen than influenza? Briefly justify your answer. Can you think of any alternative explanations for these results?

4. Based on combined results for your patients, which patients would you recommend to self-quarantine to stop the spread of influenza in Sandbeach?

5. Based on what you have learned in this lab, why is it important to get a flu vaccine every year?

6. In the scenario discussed here, the doctor in Sandbeach was able to determine that several of the patients showing flu-like symptoms were infected with a recent strain of influenza. However, we know that novel viruses emerge frequently and cause outbreaks in human populations. Some viruses may have very similar symptoms to the flu. How would the results of the PCR and ELISA assays have been different if these patients had been infected with a novel infectious agent, not influenza like the doctor suspected?

Optional activity: Using the cards that you made in the pre-lab activity, arrange the cells in order in which they respond to influenza virus infection in an individual who has not been vaccinated. Show your answer to your teacher. Next, rearrange your cards to show the order in which they respond to influenza virus infection in an individual who has been vaccinated.

Module 2: ELISA Test for Influenza Antibodies

In Module 1, we used PCR to test for the presence of viral RNA, an indication of current viral infection. In Module 2, we will use an enzyme-linked immunosorbent assay (ELISA) to test whether the patients have antibodies against flu antigens, an indication that they have either been infected with influenza or have been vaccinated against it.

ELISA begins with a blood sample from the patient followed by centrifugation to remove cells. This non-cellular fraction of blood, called plasma, contains the antibodies. Wells of a 96-well plate are coated with influenza virus proteins, then washed to remove unbound proteins. The patient's plasma is then applied to wells. Antibodies against influenza antigens, if present, will bind to the proteins in the wells. The quantity of antibody that binds will be proportional to the concentration in the patient's plasma. Alongside the patient samples, a dilution series starting with antibodies of known concentration is also applied to flu protein-coated wells. This dilution series serves as both a positive control and a set of standards. A sample with no antibodies is also applied as a negative control.

After unbound antibodies are washed out, a secondary antibody is applied. This secondary antibody binds to all human IgG antibodies. It's an antibody against another antibody! What makes this antibody different is that it is also attached to an enzyme that can catalyze a chemical reaction that produces a blue color. Since the amount of secondary antibody that will bind is proportional to the amount of patient antibody present, the intensity of the blue color produced by the enzyme is a proxy for the concentration of antibody in the patient's plasma. In the ELISA results you have been provided with, each positive control dilution is tested in duplicate and the patient samples are tested in triplicate.

Materials for each student group

Make sure your ELISA worksheet corresponds to the samples you amplified by PCR. **Make sure you identify and interpret just the patients you have been assigned**

- Samples A11 - A18, Worksheet A
- Samples B11 - B18, Worksheet B
- Samples C11 - C18, Worksheet C
- Samples D11 - D18, Worksheet D
- Samples E11 - E18, Worksheet E

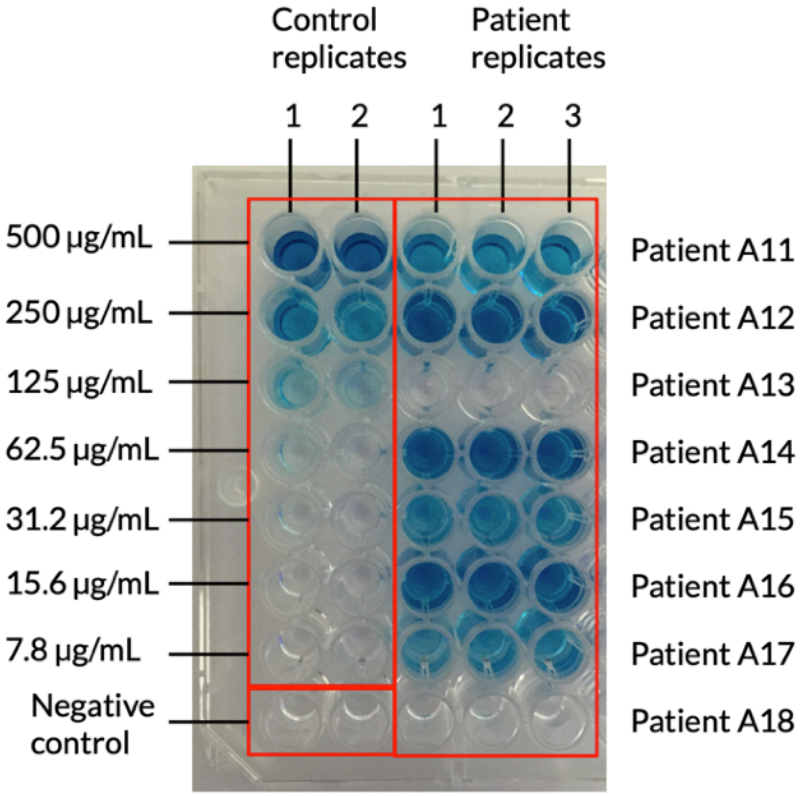
Experimental timing

This module can be completed in 20 minutes. Students can also work on interpreting the ELISA results while the PCR or electrophoresis is running.

Instructions

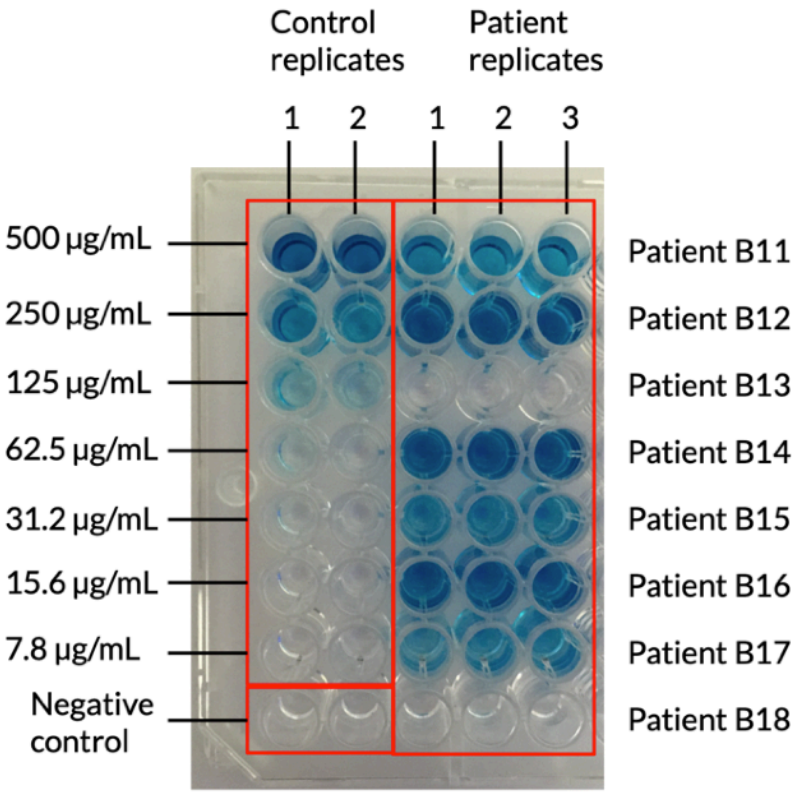
Examine the ELISA results on the influenza student worksheet. There are eight patient samples in each plate, so **make sure you identify and interpret just the patients you have been assigned**. Compare the color in the patient samples to the standard samples (positive controls) and the negative control. Determine an approximate concentration of influenza antibodies for each patient sample. Add this data to your student data table and complete the ELISA analysis questions below.

Influenza MiniLab ELISA worksheet - A



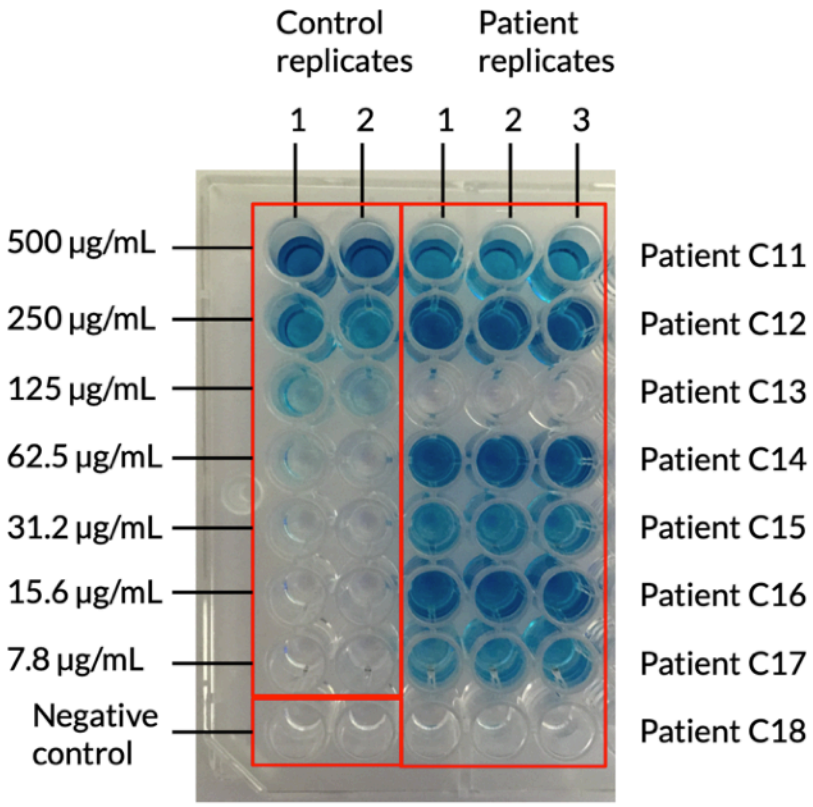
Patient ID	Role in scenario	Age	Symptoms	Vaccination
A11	bus driver	57	mild	old vaccine
A12	player (1)	16	none	new vaccine
A13	player (2)	18	severe	none
A14	spectator (1)	12	none	new vaccine
A15	player (3)	17	mild	none
A16	spirit squad dancer (1)	17	none	new vaccine
A17	spectator (2)	50	mild	old vaccine
A18	spirit squad dancer (2)	15	severe	none

Influenza MiniLab ELISA worksheet - B



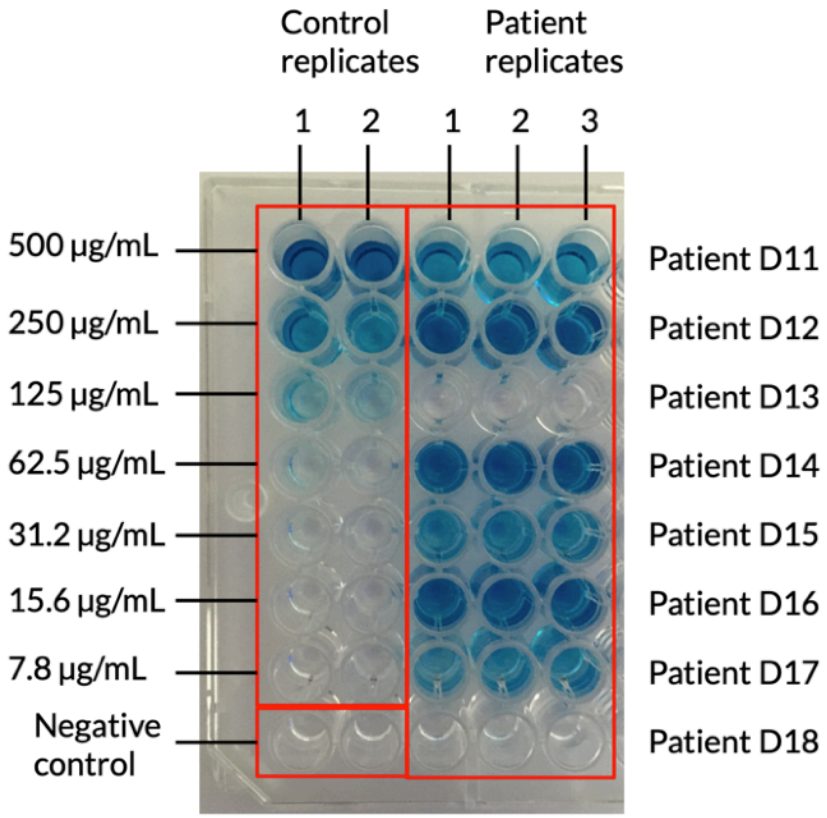
Patient ID	Role in scenario	Age	Symptoms	Vaccination
B11	player (4)	19	mild	old vaccine
B12	player (5)	16	none	new vaccine
B13	spirit squad dancer (3)	17	severe	none
B14	player (6)	17	none	new vaccine
B15	mascot	18	mild	none
B16	spirit squad dancer (4)	15	none	new vaccine
B17	coach	37	mild	old vaccine
B18	player (7)	15	severe	none

Influenza MiniLab ELISA worksheet - C



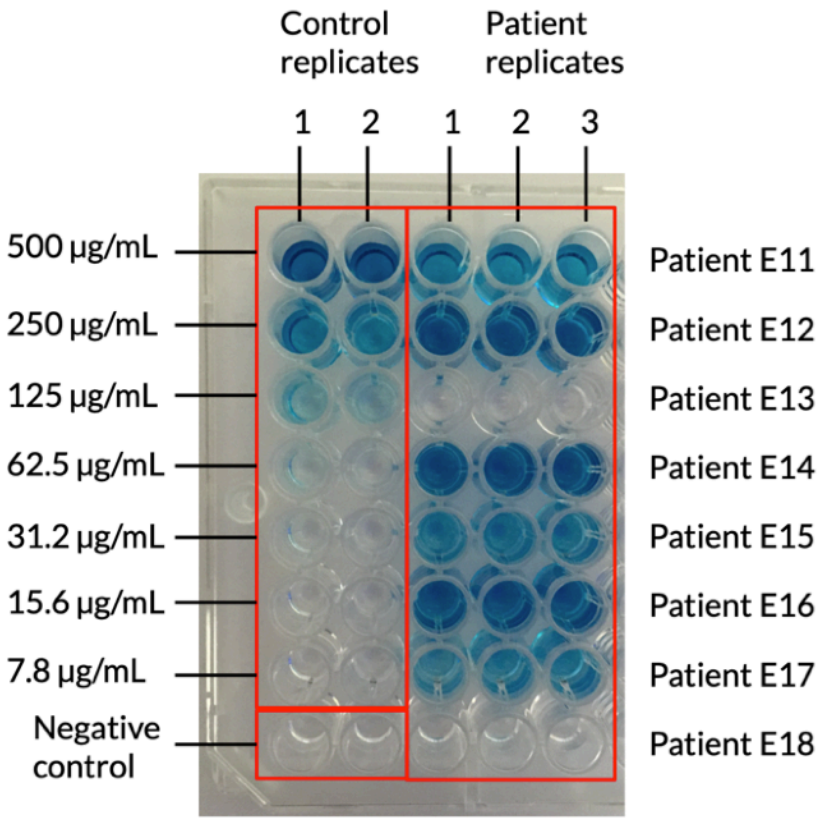
Patient ID	Role in scenario	Age	Symptoms	Vaccination
C11	team manager	17	mild	old vaccine
C12	spectator (3)	71	none	new vaccine
C13	parent chaperone (1)	40	severe	none
C14	spirit squad dancer (5)	14	none	new vaccine
C15	player (8)	16	mild	none
C16	spirit squad dancer (6)	15	none	new vaccine
C17	player (9)	16	mild	old vaccine
C18	spectator (4)	48	severe	none

Influenza MiniLab ELISA worksheet - D



Patient ID	Role in scenario	Age	Symptoms	Vaccination
D11	spirit squad dancer (7)	18	mild	old vaccine
D12	assistant coach	25	none	new vaccine
D13	player (10)	18	severe	none
D14	spectator (5)	13	none	new vaccine
D15	player (11)	17	mild	none
D16	player (12)	18	none	new vaccine
D17	spectator (6)	7	mild	old vaccine
D18	parent chaperone (2)	48	severe	none

Influenza MiniLab ELISA worksheet - E



Patient ID	Role in scenario	Age	Symptoms	Vaccination
E11	spectator (7)	73	mild	old vaccine
E12	spectator (8)	10	none	new vaccine
E13	assistant manager	15	severe	none
E14	player (13)	17	none	new vaccine
E15	parent chaperone (3)	45	mild	none
E16	spirit squad dancer (8)	17	none	new vaccine
E17	player (14)	17	mild	old vaccine
E18	player (15)	18	severe	none

ELISA Analysis Questions

1. Why are the standard curve samples and the patient samples analyzed in duplicate and triplicate? Did you see any variation between the two duplicates in either the patient samples or the standards?

2. Based on your understanding of the ELISA assay, why is there a more intense blue color for samples with higher concentrations of influenza antibodies?

3. Based on the results of the ELISA test, which patients have mounted an immune response against the influenza virus? Which patients had the highest levels of antibodies in their blood?

4. Do you see any connections between who has been vaccinated and who has the highest levels of antibodies in their blood?

5. Based on the results of your ELISA test and the data on the patient cards, which of your patients do you think might have an active viral infection?

Appendix A - Polymerase Chain Reaction

Polymerase chain reaction (PCR) addresses two major challenges in molecular biology producing usable quantities of DNA for analysis and isolating a specific region of the genome. First, the DNA that scientists want to analyze is often collected in extremely small quantities (for example, a single drop of blood from a crime scene). To have usable quantities of DNA we must make many copies using the original DNA as a template. Second, among the whole genome we must find only the part we want to analyze, whether it is a gene that causes a disease or a sequence that can help identify a species. This is no small matter since the human genome is over 3 billion base pairs (bp) long and we are often interested in regions less than 300 bp long.

The History of PCR

As with many ideas in biotechnology, nature provides us with most of the tools we need. Every time a cell divides it makes two replicates of its entire genome with the help of specialized enzymes. The phenomenon of complementary base pairing should give you a hint as to how a specific region can be targeted. In the late 1970s Frederick Sanger developed a method for copying DNA in vitro that used short pieces of DNA, called **primers** to initiate replication by a DNA polymerase, similar to the RNA primers in your model of cellular DNA replication. An artificial primer used in the Sanger method has a sequence that allows it to bind at only one location in the target DNA sequence.

Using only one DNA primer, the Sanger method can only produce one copy of the target at a time. In 1983 Kary Mullis proposed a modification to this method where a second primer is used to initiate replication using the first copy as a template. Recall that DNA has an antiparallel structure where one strand runs in the opposite direction relative to the second strand. To use the first copy as a target for replication, the second primer that Mullis added needed to initiate replication in the opposite direction, thus one primer is called the forward primer and the other is called the reverse. With the original sequence and its copy serving as templates, two copies are produced instead of one. Further, these two copies can each be used as templates in a second round of copying, which produces four copies. After each round, or cycle, the number of copies has doubled. Over multiple cycles, billions of copies of the DNA sequence between the two primers are generated. This method is called **Polymerase Chain Reaction (PCR)** – polymerase because of the enzyme that is used to copy DNA and chain reaction because the products of one cycle serve as templates for the next round of copying. This rapid and efficient technology for generating usable quantities of DNA won Mullis the Nobel Prize in Chemistry. The first application of PCR was a test for sickle cell anemia.

How PCR Works

Instead of trying to recreate the cell's intricate biochemical machinery in a test tube, scientists rely on heat to control the various steps of the PCR reaction. For DNA to be copied the nucleotide bases must be exposed, which means double stranded DNA must be separated into single strands. Just as heat applied to an ice cube weakens the hydrogen bonds between water molecules and causes a phase transition to liquid water, heat applied to double-stranded DNA weakens hydrogen bonds between bases causing separation of the strands into single-stranded

DNA. As with ice, this is sometimes called melting, but is commonly referred to as **denaturation**. In a PCR cycle, denaturation is performed at 90-98°C for 5-30 seconds. This temperature is just below the boiling point of water.

Once the template DNA has been separated into single strands, the temperature is lowered to encourage primers to bind to their target sequences. Just as in the water analogy, lower temperature favors the formation of hydrogen bonds between molecules, in this case the primer and the template DNA. This step, called **annealing**, is typically between 45 and 65°C for 5-30 seconds. The ideal temperature and duration of the annealing step depends on the sequences of the primers being used. It must be low enough that hydrogen bonds are able to form between the primer and specific complementary sequence, but not so low that nonspecific, or random, binding between primer and template occurs.

DNA polymerase binds to the primer-template complex and begins to add nucleotides (dNTPs) onto the 3' end of the primer. This step, called **extension**, results in a new copy attached to the template as double- stranded DNA. The duration of the extension step depends on the length of the DNA segment being copied and can be anywhere from 5 seconds to 5 minutes.

At this point you may have noticed a problem – DNA polymerase, which the entire PCR process relies on, is a protein enzyme that needs a very specific three-dimensional structure to copy DNA. Before getting to the extension step, the entire PCR mixture, including the polymerase, has already been through the denaturation step where the reaction was heated almost to boiling. Anyone who has cracked an egg into a frying pan will know what high heat does to proteins!

In the early days of PCR, fresh polymerase was added to the reaction tube every cycle to replace the polymerase that had been destroyed by the denaturation step. Having to open the tube and add new enzyme for up to 30 cycles was expensive, inefficient, and increased the chances of contaminating the reaction. The innovation that would remove these obstacles came from an unusual source – Yellowstone National Park.

In the 1970s, scientists had isolated a species of bacteria called *Thermus aquaticus* from a hot spring in Yellowstone. *T. aquaticus* thrives at 75-80°C and can survive much higher temperatures. Its enzymes are similarly heat-tolerant. *E. coli*, the original source for the polymerase used in PCR, thrives at 37.5°C, the same temperature as the gut of mammals.

Since the biochemistry of DNA is similar across the tree of life, polymerase from *T. aquaticus* (called Taq Polymerase) can replace *E. coli* polymerase in PCR, with the modification that the extension step is performed at 70-75°C.

This innovation led to PCR becoming the ubiquitous, inexpensive, and efficient tool that it is today. Reactions can be set up once, sealed inside a tube, and placed in an automated thermal cycler. The thermal cycler (or PCR machine) is a specialized instrument that accurately and rapidly changes the temperature of the tube between denaturation, annealing, and extension. After 20-40 cycles of these three temperatures, billions of copies of the desired DNA product can be produced. The amplified DNA can be analyzed with gel electrophoresis at the end of the experiment or detected as they are being formed using more advanced equipment.

Appendix B - What is Gel Electrophoresis?

Gel electrophoresis is a technique used in many areas of science to analyze the components of complex chemical mixtures. Mixtures of DNA, RNA, proteins, or dyes can be separated into their individual components based on molecular size and electrical charge using a separation matrix within an electric field.

The gel used in gel electrophoresis is a tangle of polymers forming a three-dimensional matrix with water-filled pores through which molecules migrate. A higher density of polymers creates smaller pores. Like the holes in a sieve or colander, the size of the pores has to be the appropriate size for the molecules being separated. Gels can be made from different substances depending on the application. One of the most commonly used and effective materials is agarose, a polymer extracted from seaweed. Agarose gels are formed (or cast) by pouring molten (melted) agarose into a tray where it solidifies into the desired shape as it cools. A comb is placed while the agarose is molten and then removed after it solidifies to create wells where the samples are loaded.

After the gel solidifies it is placed in an electrically conductive buffer between parallel positive ((+) anode) and negative ((-) cathode) electrodes

A voltage is applied between the electrodes, creating a uniform electric field within the gel. Molecules in the wells begin to move under the influence of the electric field: positively charged molecules migrate toward the (-) cathode and negatively charged molecules migrate toward the (+) anode.

The speed of a molecule's movement in an electric field is determined by the strength of its electric charge relative to its molecular weight. This is quantified as the charge to mass ratio. Speed of movement within a gel is also influenced by the size of the molecule relative to the pores in the gel. The polymers in the gel are like an obstacle course: smaller molecules maneuver easily through the pores, traveling faster and farther than large, bulky molecules. However, a large molecule can move faster through a gel than a smaller molecule when the strength of its charge relative to its mass is significantly higher. Shape can also affect how a molecule moves through the gel. Long spaghetti-like molecules will move slower than compact molecules, which slip easily through the pores. Molecules of the same size, shape, and charge will move together and form a distinct band. If multiple types or sizes of molecules are present in the sample, they will separate from each other and each will form a distinct band.

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