Advances in Biology Laboratory Education
Publication of the Association for Biology Laboratory Education
Volume 43, Article 9, 2023

Who has the flu? Tracing transmission using ELISA and PCR in the classroom

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With outbreaks in the news and misinformation spreading faster than a virus, immunology and vaccination are urgent topics for high school and introductory college biology. In this engaging, hands-on lab, students will learn how the immune system protects us against disease while tracing an outbreak of influenza in a small community. Students will use real-world molecular techniques to screen a group of fictitious individuals with varying vaccination status for influenza infection: enzyme-linked immunosorbent assay (ELISA) to detect antibodies and polymerase chain reaction (PCR) to detect viral RNA in patient samples. At the conclusion of the lab, students will pool their data and discuss the patterns of infection among vaccinated and unvaccinated individuals. The data highlights the need to update the influenza vaccine each year on account of the constant evolution of the influenza virus. This lab activity is suitable for advanced high school and early college, but can be adapted for students with different levels of preparation. Both the ELISA protocol and the PCR protocol are carried out as done in research labs, however lower-cost reagents have been substituted in certain steps, making this lab affordable and accessible to a larger number of teachers and students.

Keywords: influenza, infectious diseases, diagnostics, vaccines, immune system, PCR, ELISA

Introduction

The ongoing COVID-19 pandemic has made infectious disease and immunology critical topics for science education and public outreach. PCR, antibodies, and viral variants have become common topics of household conversation, as the spread of SARS-CoV-2 impacts every aspect of our lives. At the same time, the spread of misinformation, driven in part by lack of public understanding of the immune system, complicates our efforts to stop the spread of the virus through vaccination and public health precautions.

Broadening public understanding of immunology is clearly an urgent need. Creating more opportunities for exploring immunological concepts in high school and undergraduate biology courses is one path to accomplishing this. Besides the clear relevance for students in pre-health career tracks, as an interdisciplinary subject, immunology has the potential to create connections between multiple scientific fields and engage students with diverse interests (Bruns et al. 2021). Training in this subject, however, has typically been reserved for the graduate level (Bruns et al. 2019).

One barrier to bringing immunology into high school and undergraduate biology courses is the specialized vocabulary and volume of content that must be mastered (Stranford et al. 2020). Educators in the field advocate an active learning approach in which the student engages with solving problems and becomes the “architect of their own knowledge” (Stranford et al. 2020). Active learning is especially valuable for helping students integrate new knowledge and draw connections to what they’ve learned in other parts of the course.

To promote active learning in immunology for high school students and undergraduates we have developed a hands-on laboratory activity that reinforces key concepts in a context that the students will likely find familiar. The lab is based around a scenario where forty people of varying age, vaccination status, and infection history were exposed to influenza. Following the exposure, students are tasked with testing this group for presence and quantity of antibodies against the current strain of influenza using an enzyme-linked immunosorbent assay (ELISA) and for presence and quantity of viral RNA using polymerase chain reaction (PCR). Pooling their data for this group of patients, students can analyze patterns of infection severity related to vaccination status and spot ambiguous cases that may require further investigation. Overall the data highlights the importance of yearly vaccination against influenza to combat the constantly evolving strains of this virus.

A separate challenge to teaching immunology at the high school and undergraduate level is the expense and complexity of reagents. An ELISA requires horseradish peroxidase (HRP)-coupled secondary antibodies and flu protein antigens which are sold in kits that cost hundreds of dollars. For this reason, we have taken a simulation approach where the students perform the same steps as they would in doing an ELISA and get comparable results, but with less costly reagents. Phosphate buffered saline (PBS) is substituted for most reagents, and HRP-coupled protein A is used in place of patient antibodies and positive control. The HRP-protein A is diluted to give two concentrations for patient samples, simulating two levels of antibody titer. A serial dilution of HRP-protein A serves as a positive control and concentration standard.

For the PCR test, students amplify a short fragment (200-500 bp) from a plasmid, lambda phage genomic DNA, or other available source. Two concentrations of template DNA are used for patient samples with high and low viral load. Quantitative PCR (qPCR) is typically used to test for viral infection, but even with conventional PCR, students will be able to see the difference in band intensity between the high and low template concentration samples.

This lab activity is most appropriate for advanced high school (e.g. honors or AP biology) or introductory college biology, but could be adapted for students with different levels of preparation. It could also be used as part of a specialized undergraduate course in biotechnology, microbiology, or anatomy and physiology.

Much of the background information presented here is adapted from Janeway's Immunology, 9th Edition (Murphy and Weaver 2016), which is an excellent resource for more detailed information. We assume that laboratory instructors will cover the mechanisms of PCR and ELISA tests and do not provide details here.

Student Outline

Introduction

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. A visit to the doctor is needed to 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. Please see Appendix A for background on the immune system and the molecular tests we will be running in this lab.

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 whom 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.

Laboratory Safety

- Wear lab coats, gloves, and eye protection whenever possible.
- Use caution with all electrical equipment such as PCR machines and electrophoresis units.
- Do not touch the heated lid or the tube block in the PCR thermal cycler, as these can be extremely hot. Use caution when opening and closing the lid and when placing and removing tubes.
- Heating and pouring molten agarose is a splash hazard. Use caution when handling hot liquids. Wear eye protection and gloves to prevent burns.
- Wash your hands thoroughly after handling biological materials and chemicals.
- Dispose of all materials in a biohazard bag or in a washtub containing a 10% bleach solution.

Experimental Procedures

Part A: ELISA Test for Influenza Antibodies

In Part A of this lab you will use an enzyme-linked immunosorbent assay (ELISA) to test four patient samples for the presence of antibodies against the current strain of influenza. Alongside the four patients, you will also run a negative control and a set of eight standards, prepared through serial dilution of a positive control of known concentration. This will allow you to estimate the relative antibody titer of the patient samples.

Materials for each group

- One aliquot each:
    - Protein Coating Buffer
    - Flu Proteins
    - ELISA Blocking Buffer
    - Positive Control Antibodies
    - Negative Control Antibodies
    - Antibody Diluent
    - ELISA Wash Buffer
    - Anti-Human HRP Secondary Antibody
    - ELISA TMB Substrate (3,3',5,5'-tetramethylbenzidine)
- Patient samples (prepare according to table and keep track of which patient samples are assigned to each group)
- One ELISA plate
- 20-200 µL adjustable volume micropipette and tips
- Twelve 0.65 mL microcentrifuge tubes
- Stack of paper towels
- Fine point permanent marker
- Waste container
- Microcentrifuge tube rack (optional)

Common workstation

- 37°C incubator (optional)
- Sink or large plastic bin

Coating your ELISA plate

1. Check your workstation to make sure you have all the required materials.

2. Label the side of your ELISA plate with the date and your group number. There is a flat area on the side that is designed for labeling.

3. Dilute the **Flu Proteins** to 1 µg/mL using **Protein Coating Buffer**. The concentration of the Flu Proteins stock solution is 150 µg/mL.

    a. Use the equation C1 * V1 = C2 * V2. C1 is the final concentration, 1 µg/mL. V1 is the final volume, 5 ml (5,000 µL). Make sure to use the same units on both sides of the equation. C2 is our starting or "stock" concentration, 150 µg/mL and V2 is the unknown amount of our stock solution that we must add to the Protein Coating Buffer.

    b. Rearrange the equation to calculate V2 from the other quantities. Plug in the other quantities and calculate V2. V2 = _____.

    c. Add the quantity of stock you calculated to the 5 ml tube of **Protein Coating Buffer**, put the lid on the tube, and mix by inverting 3-5 times.

4. Add 100 µL of the 1 µg/mL **Flu Proteins** solution to each of the wells, marked either Positive Control, Negative Control, or Sample in the **ELISA Plate Plan** (Figure 1).

5. Cover your ELISA Plate with the ELISA plate cover or slide it back into the plastic bag that it came in and close it with tape. Make sure it is well sealed. Follow your instructor's directions to either store it at 4°C overnight or at room temperature for 5 minutes (possible stopping point).

6. Remove the cover from your ELISA plate. Take your ELISA plate to the sink and discard the liquid by flinging the contents of the ELISA plate into the sink.

7. Return to your bench and add 200 µL of **ELISA Blocking Buffer** to each of your sample wells that you coated with influenza proteins.

8. Follow your instructor's directions to incubate your ELISA plate in **ELISA Blocking Buffer** for at least 5 minutes at room temperature. You can also incubate it overnight at 4°C (possible stopping point).

9. While your plate is incubating in the blocking buffer, label seven microcentrifuge tubes as follows: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128. If using a microcentrifuge tube rack, arrange the labeled tubes in the order listed.

10. Add 200 µL of **Antibody Diluent** to each tube.

11. Add 200 µL of the **Positive Control Antibodies** to the 1:2 tube and mix by pipetting up and down three times, then discard your pipette tip. You should now have 400 µL of your first 1:2 dilution.

12. Prepare serial dilutions of the positive control to make all of your **Positive Control Samples**. You can use the same pipette tip for each step.

    a. Take 200 µL from the 1:2 tube, add it to the 1:4 tube, and mix by pipetting up and down three times.

    b. Take 200 µL from the 1:4 tube, add it to the 1:8 tube, and mix by pipetting up and down three times.

    c. Continue to take 200 µL from one tube in the serial dilution series, add it to the next one, mix, and then move on to the next dilution until you reach the last tube, the 1:128 tube.

Testing patient samples

13. Record the sample IDs of the patients you will be testing in the space below:

14. Take a moment to familiarize yourself with the ELISA Plate Plan in Figure 1. This is the plan that you will use for loading samples in step #16.

Figure 1. ELISA plate plan. Image credit: Rick Simonson.

15. Remove the cover from your ELISA plate. Take your ELISA plate to the sink and fling the liquid contents into the sink. Pound the plate on a stack of paper towels to help remove the last of the blocking buffer.

16. Use the ELISA Plate Plan above to add 100 µL of the correct sample to each well.

17. Cover your plate and follow your instructor's directions to either store it at 4°C overnight or at 37°C for 15 minutes (possible stopping point).

18. After the incubation of your patient samples is complete, take your ELISA plate to the sink and fling the liquid contents into the sink. Pound the plate on a stack of paper towels to help remove the last of the samples.

19. Wash your ELISA plate by adding 200 µL of **ELISA Wash Buffer** to each well that has control or patient samples. These are the same wells labeled on the ELISA plate plan.

20. Remove the **ELISA Wash Buffer** by flinging the liquid contents of the plate in a sink and then pound out the excess on a stack of paper towels.

21. Repeat the wash in steps 19-20 two additional times, for a total of three washes.

22. Add 100 µL **Anti-human-HRP Secondary Antibody (2° Ab)** to each of the control and patient sample wells, referring to the ELISA plate plan.

23. Let the antibody incubate in your ELISA plate for at least 5 minutes at room temperature.

24. When the incubation is complete, take your ELISA plate to the sink and fling the liquid contents into the sink. Pound the plate on a stack of paper towels to help remove the last of the secondary antibody.

25. Add 200 µL of **ELISA Wash Buffer** to each well that has control samples and patient samples.

26. Remove the **ELISA Wash Buffer** by flinging the liquid contents of the plate in a sink and then pound out the excess on a stack of paper towels. Repeat the wash in steps 25-26 two additional times for a total of three washes.

27. After your third wash, add 100 µL of **ELISA TMB Substrate** to each well that has control samples and patient samples.

28. Let the **ELISA TMB Substrate** incubate in your ELISA plate **until your positive control samples are blue**. The most concentrated positive controls, the 1:2 dilutions in wells A1 and A2, should be very dark blue (See Appendix D for examples). The other positive control samples will get progressively lighter blue as the positive control becomes more dilute. The longer you allow the plate to develop, the darker the blue colors will become.

29. Use the table below to record your sample IDs and note which samples were high positive, low positive, and which were negative for influenza antibodies by comparing them to the positive and negative controls. Remember that the Negative Control has no anti-influenza antibodies and the 1:2 dilution of the Positive Control has a high level of anti-influenza antibodies. A low positive result will appear as an intermediate shade of blue. Each patient may have different levels of antibodies (i.e., different shades of blue).

Table 1. Fill in results of ELISA test of patient samples.

Sample ID	**ELISA test result**

Part B: PCR Test for Influenza Virus Infection

In Part B you will use polymerase chain reaction (PCR) to test four patient samples for the presence of influenza viral RNA. The influenza virus genome is single stranded RNA, so in a previous step RNA was reverse transcribed into DNA. This DNA will serve as the template for PCR amplification. You will then analyze the results of your PCR using gel electrophoresis. By comparing the intensity of bands on the gel, you will be able to determine whether the patients have high or low viral load, or are negative for influenza infection.

PCR Materials for each student group

- Aliquots of:
    - Influenza primer set
    - PCR Taq MasterMix 2X
    - Four patient samples
    - Positive control sample
    - Negative control sample
- Six 0.2 mL PCR tubes
- One micropipette (2-20 µL) and 20 pipette tips
- PCR tube rack
- Fine point permanent marker

Electrophoresis Materials for each student group

- One electrophoresis gel casting system
- One electrophoresis system with power supply
- Blue light illuminator
- One micropipette (2-20 µL)
- 20 pipette tips
- 2% agarose gel with TBE buffer and GelGreen™ dye
- TBE running buffer
- 5x loading dye
- DNA molecular weight marker

Common Workstation

- PCR thermal cycler
- Microwave
- Benchtop microcentrifuge

Instructions: PCR Amplification of Influenza DNA

1. Check your workstation to make sure you have all 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 Table 2. Pipette the reagents directly into the bottom of the PCR tubes and try to avoid creating bubbles. Record the patient you are testing for each tube.

Table 2. PCR Setup.

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6
Patient number	Negative control	Positive control				
2x Taq PCR MasterMix (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 PCR thermal cycler and place your tubes in the plate. Program your thermal cycler according to the cycling parameters in Table 3.

Table 3. PCR Cycling Protocol.

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

6. 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 (possible stopping point). We suggest freezing the samples if storing them for longer than overnight.

Instructions: Electrophoresis analysis of PCR Products

7. Prepare your samples for gel electrophoresis by adding 5 µL of 5x loading dye to each sample. Flick with your finger to mix. If necessary, centrifuge or tap the tube on the benchtop to bring all liquid to the bottom of the tube.

8. Cast the 2% agarose gel with GelGreen™ using your casting system. Use a 1:10,000 dilution of GelGreen™ in the molten agarose. Make sure you have enough wells for the number of samples you are testing and the molecular weight marker.

9. After the gel has solidified, carefully remove the comb and follow your instructor's directions to set up the electrophoresis chamber and add the TBE running buffer to cover the gel.

10. Load 10 µL of each sample and the molecular weight marker into the wells, keeping track of the placement using the gel template.

11. Run your gel until the bands in the DNA molecular size marker have clearly separated or the leading dye front has moved three quarters of the distance toward the end of the gel.

12. View your gel in a blue light illuminator and document your results with your cell phone or camera and paste an image of your gel in your lab notebook.

Discussion

Answer the following questions in your lab notebook, supporting each answer by referencing your data from the ELISA and PCR tests. Your instructor may ask you to pool your results with the other groups and discuss the entire dataset.

1. 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?

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

3. Based on the results of the PCR assay, which patients are infected with the influenza virus?

4. Is there a connection between the patients' vaccination status and the result of their PCR assay?

5. Are there any patients that might be infected with a pathogen other than influenza?

6. Which patients would you recommend to self-quarantine to stop the spread of influenza in Sandbeach?

Materials

These products are provided as examples. Any comparable product can be substituted.

Reagents

- 10X phosphate buffered saline (PBS) (ThermoFisher Scientific, product code: AM9624).
- Carbonate/Bicarbonate (Carb/Bicarb) coating buffer, pH 9.6 (Fisher Scientific, product code: 50-100-8800).
- Tween-20 (Sigma-Aldrich, product code: 1379).
- Protein A/HRP conjugate (Millipore Sigma, product code: 18-160).
- Tris Borate EDTA (TBE) buffer concentrate for making electrophoresis running buffer (Millipore Sigma, product code: T4415).
- Agarose powder (Millipore Sigma, product code: A9539).
- GelGreen™ nucleic acid stain (Biotium, product code: 41005).
- Colorimetric TMB substrate (Millipore Sigma, product code: RABTMB3).
- 2X PCR master mix (Fisher Scientific, product code: FERK0171).
- Forward and reverse PCR primers (see Notes for the Instructor).
- Patient DNA samples (high, low, and negative; see Notes for the Instructor).
- Electrophoresis sample loading dye, 5X (BioLine, product code: BIO-37045). We recommend using a loading buffer with Orange G as the leading dye.
- 100 bp DNA molecular size marker for electrophoresis (Qiagen, product code: 239901).

See Appendix B for instructions for preparing reagents before class.

Consumables

- ELISA plates, one per student group (Millipore Sigma, product code: M8CHNFX00).
- 15 mL conical tubes, one per student group and one for teacher prep (Falcon, product code: 352095).
- 5 mL plastic tubes, five per student group (Corning, product code: SCT-5ML-S).
- 0.65 mL microcentrifuge tubes, 20 tubes per group (Millipore Sigma, product code: T9661).
- 0.2 mL thin-wall PCR tubes, six per student group (Millipore Sigma, product code: CLS6571).
- Tips for 2-20 µL, 20-200 µL, and 100-1000 µL adjustable volume micropipettes (Millipore Sigma, product codes: CLS4860 and CLS4867).

Molecular biology lab equipment

- PCR System, 1-3 per class depending on capacity (see Notes for the Instructor).
- Horizontal gel electrophoresis system with casting system, power supply, and blue light illuminator, one set per group (see Notes for the Instructor).
- 2-20 µL adjustable volume micropipette, one per student group (Thermo Scientific, product code: 4641060N).
- 20-200 µL adjustable volume micropipette, one per student group (Thermo Scientific, product code: 4641080N).
- 100-1000 µL adjustable volume micropipette, one per student group (Thermo Scientific, product code: 4641100N).
- Bench-top micro-centrifuge, 2-3 per class, optional (ThermoFisher Scientific, product code: 75004061).

Other equipment and supplies

- Deionized water (dH_2O) for diluting buffers.
- Stack of paper towels for each student group.
- Timer or stopwatch.
- Biological waste disposal container.
- Fine point permanent markers.
- Microwave oven (Magic Chef, product code: HMM770W).
- Microcentrifuge tube racks (Bel-Art, product code: F18845-0003).

- 37°C incubator, optional (Corning, product code: 6800).
- PCR tube racks, optional (Millipore Sigma, product code: R6901).

Notes for the Instructor

The ELISA portion of the lab can be completed in 120 minutes. It can be completed in a single period or split into multiple periods by stopping at steps labeled “possible stopping point.” PCR and electrophoresis analysis will take 90 minutes and can be completed in one or two periods.

This lab procedure is optimized for use with the MiniOne PCR System controlled by mobile app (Embi Tec, product code: M4000) and the MiniOne Electrophoresis System with power supply and casting stand (Embi Tec, product code: M1000), but can be adapted to any PCR and electrophoresis system. The reagents can also be purchased as a kit from Embi Tec (product code: M6030).

Details of reagent preparation and aliquoting reagents for student groups are described in Appendix B. For the PCR test you can amplify any short fragment of DNA. Plasmids and lambda phage genomic DNA are convenient templates. Appendix B provides recommended concentrations of template DNA and primers. Adjust PCR parameters for the polymerase and target fragment you are amplifying. Parameters in the student outline are provided as an example.

Please see Appendix C for an answer key showing expected rsults for each patient and expected responses for the students discussion questions. Appendix D shows example electrophoresis results of the PCR analysis and example ELISA results.

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Acknowledgments

The authors thank Dina Kovarik, PhD, of Shoreline Community College for initial development of this lab and Rita Wong, Erika Fong, and Richard Chan of Embi Tec for feedback on the protocol. We would also like to thank the Washington area high school teachers who contributed to the development and classroom testing of the lab.

This material is based upon work supported by the National Science Foundation under Grant No. 1700441. START Immuno Biotech: Strategies for Accelerating Recruitment and Training in Immuno-Biotechnology. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

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Appendix A: Student Background Information on the Immune System

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. 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 foriegn 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. 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.

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.

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.

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. 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. The influenza virus genome is single stranded RNA, so the RNA must be reverse transcribed into DNA before amplification with PCR.

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. The test for antibodies in patient serum is called an enzyme linked immunosorbent assay (ELISA). ELISA detects antibodies against a specific antigen through a chemical reaction that generates a visible color, the intensity of which is proportional to the antibody concentration.

Appendix B: Teacher Prep Details

Student groups and patient samples

We recommend that each student group analyze four patient samples, in addition to negative and positive control. To cover all combinations of PCR and ELISA results, have Group 1 analyze samples 1-4, Group 2 analyze samples 5-8, etc. All instructions assume that you are preparing 40 samples for 10 student groups. Adjust the volumes of solutions and number of aliquots if you are preparing for a different number of groups. For ELISA, patients can have either high, low, or negative antibody titer, and for PCR patients can have high, low, or negative DNA concentration. Use Appendix C to determine how many aliquots of each sample type you will need to prepare and label the tubes with the patient ID number.

Prepare buffers for ELISA

1. **1X PBS**: PBS can be purchased or prepared as a 10X stock solution and will be used in the lab activity as the Protein Coating buffer (CB), Flu proteins (FP), Blocking Solution (Block), Negative control (Neg Ctrl), Anti-Human HRP (secondary antibody) (2° Ab), and for making the wash buffer in step 2. To dilute, mix 1 part 10X PBS with 9 parts deionized water (dH_2O). For ten student groups, prepare 500 mL of 1X PBS by mixing 50 mL of 10X PBS with 450 mL of dH_2O. Store in a bottle with a lid until use. 1X PBS can be stored for 2 years at room temperature.

2. **Wash Buffer**: Prepare the Wash Buffer (1X PBS / 0.05% Tween-20) using the 1X PBS made above. For each class, mix 190 ml of 1X PBS with 10 ml of 1% Tween-20. Store in a bottle with a lid until use. Wash buffer can be stored for 2 years at room temperature.

3. **Antibody high positive patient samples:** Prepare 7 mL of a 1:5,000 dilution of Protein A-HRP by adding 1.4 µL of Protein A-HRP to 7 mL of Carb/Bicarb Buffer. This solution should be prepared on the same day that the students add the patient samples to the ELISA plate.

4. **Antibody low positive patient samples:** Prepare 5 mL of a 1:10,000 dilution of Protein A-HRP by adding 0.5 µL of Protein A-HRP to 5 mL of 1X Carb/Bicarb Buffer. This solution should be prepared on the same day that the students add the patient samples to the ELISA plate.

Prepare sample aliquots

Prepare the solutions listed in Table 4 and aliquot for each student group.
Use a 15 mL plastic conical tube for the blocking solution and a 5 mL plastic tube for the protein coating buffer, antibody diluent, wash buffer, Anti-human HRP secondary antibody, and the TMB.

Table 4. ELISA reagent preparation.

Referred to in protocol as:	Label for tube	What it really is	Total volume for 10 groups	Volume for each student group
Protein Coating Buffer	CB	PBS	50 mL	5 mL
Flu Proteins	FP	PBS	500 µL	50 µL
ELISA Blocking Buffer	Block	PBS	150 mL	15 mL
Patient samples	Patient ID - see table	High positive: 1:5,000 ProA-HRP in carb/bicarb buffer Low positive: 1:10,000 ProA-HRP in carb/bicarb buffer	Varies, see table	350 µL each patient

		Negative: Carb/bicarb buffer		
Positive Control Antibodies	Pos Ctrl	1:5,000 ProA-HRP	2.5 mL	250 µL
Negative Control Antibodies	Neg Ctrl	PBS	2.5 mL	250 µL
Antibody Diluent	Ab Dil	Carb/bicarb buffer	50 mL	5 mL
ELISA Wash Buffer	Wash	PBS-Tween	200 mL	20 mL
Anti-Human HRP (Secondary Antibody)	2° Ab	PBS	50 mL	5 mL
ELISA TMB Substrate	TMB	TMB	50 mL	5 mL

Prepare electrophoresis running buffer

Prepare 1X electrophoresis running buffer by diluting the concentrated Tris Borate EDTA (TBE) stock solution with dH_2O. For example, add 100 mL 10X TBE stock to 900 mL of dH_2O to make 1 L of 1X running buffer.

Prepare agarose gels

Prepare 2% agarose gels in 1X TBE running buffer according to the volume required for your gel casting system. For example, to make 100 mL gel, add 2 g of agarose powder to 100 mL of 1X TBE. Microwave until the powder is completely dissolved. Add GelGreen™ nucleic acid stain at a final dilution of 1:10,000 in the melted agarose. Agarose gels can be cast by the teacher ahead of time or done by the students as part of the lab while the PCR is running. See Step 8 under Part B of the student laboratory protocol.

Prepare sample aliquots for each student group

Aliquot PCR and electrophoresis reagents into 0.65 mL microcentrifuge tubes.

- 65 µL 2X Taq Master Mix
- 35 µL Influenza primer set (1 µM forward and reverse primers)
- 7 µL DNA for each of four patients, refer to Appendix C for which samples to provide to each group. High = 0.2 ng/µL, med = 0.02 ng/µL, negative = dH_2O.
- 7 µL deionized water (dH_2O) for negative control
- 7 µL 0.2 ng/µL DNA for positive control
- 12 µL DNA molecular size marker
- 35 µL 5x sample loading dye

Appendix C: Answer Key

Patient information					Sample prep		Expected results	
Patient ID	Role in scenario	Age	Symptoms	Vaccination	Protein A-HRP Dilution	PCR template conc.	ELISA-Ab	PCR
1	bus driver	57	mild	old vaccine	1:10,000	0.02 ng/µL	+	+
2	player (1)	16	none	new vaccine	1:5,000	0 ng/µL	++	-
3	player (2)	18	severe	none	0	0.2 ng/µL	-	++
4	spectator (1)	12	none	new vaccine	1:5,000	0 ng/µL	++	-
5	player (3)	17	mild	none	1:10,000	0.02 ng/µL	+	+
6	spirit squad dancer (1)	17	none	new vaccine	1:5,000	0 ng/µL	++	-
7	spectator (2)	50	mild	old vaccine	1:10,000	0.02 ng/µL	+	+
8	spirit squad dancer (2)	15	severe	none	0	0 ng/µL	-	-
9	player (4)	19	mild	old vaccine	1:10,000	0.02 ng/µL	+	+
10	player (5)	16	none	new vaccine	1:5,000	0 ng/µL	++	-
11	spirit squad dancer (3)	17	severe	none	0	0.2 ng/µL	-	++
12	player (6)	17	none	new vaccine	1:5,000	0 ng/µL	++	-
13	mascot	18	mild	none	1:10,000	0.02 ng/µL	+	+
14	spirit squad dancer (4)	15	none	new vaccine	1:5,000	0 ng/µL	++	-
15	coach	37	mild	old vaccine	1:10,000	0.02 ng/µL	+	+
16	player (7)	15	severe	none	0	0 ng/µL	-	-
17	team manager	17	mild	old vaccine	1:10,000	0.02 ng/µL	+	+
18	spectator (3)	71	none	new vaccine	1:5,000	0 ng/µL	++	-
19	parent chaperone (1)	40	severe	none	0	0.2 ng/µL	-	++
20	spirit squad dancer (5)	14	none	new vaccine	1:5,000	0 ng/µL	++	-
21	player (8)	16	mild	none	1:10,000	0.02 ng/µL	+	+
22	spirit squad dancer (6)	15	none	new vaccine	1:5,000	0 ng/µL	++	-
23	player (9)	16	mild	old vaccine	1:10,000	0.02 ng/µL	+	+

24	spectator (4)	48	severe	none	0	0 ng/µL	-	-
25	spirit squad dancer (7)	18	mild	old vaccine	1:10,000	0.02 ng/µL	+	+
26	assistant coach	25	none	new vaccine	1:5,000	0 ng/µL	++	-
27	player (10)	18	severe	none	0	0.2 ng/µL	-	++
28	spectator (5)	13	none	new vaccine	1:5,000	0 ng/µL	++	-
29	player (11)	17	mild	none	1:10,000	0.02 ng/µL	+	+
30	player (12)	18	none	new vaccine	1:5,000	0 ng/µL	++	-
31	spectator (6)	7	mild	old vaccine	1:10,000	0.02 ng/µL	+	+
32	parent chaperone (2)	48	severe	none	0	0 ng/µL	-	-
33	spectator (7)	73	mild	old vaccine	1:10,000	0.02 ng/µL	+	+
34	spectator (8)	10	none	new vaccine	1:5,000	0 ng/µL	++	-
35	assistant manager	15	severe	none	0	0.2 ng/µL	-	++
36	player (13)	17	none	new vaccine	1:5,000	0 ng/µL	++	-
37	parent chaperone (3)	45	mild	none	1:10,000	0.02 ng/µL	+	+
38	spirit squad dancer (8)	17	none	new vaccine	1:5,000	0 ng/µL	++	-
39	player (14)	17	mild	old vaccine	1:10,000	0.02 ng/µL	+	+
40	player (15)	18	severe	none	0	0 ng/µL	-	-

Patient color codes:	
	Patient with old vaccine and mild infection due to partial immunity against new strain
	Patient with new vaccine and no infection due to full immunity to new strain
	Patient who has never been vaccinated and has severe flu symptoms and influenza infection since they have no immunity
	Patient who has never been vaccinated, but has antibodies against influenza, possibly due to past infection, and shows mild symptoms after new infection
	Patient with severe flu-like symptoms but no flu infection, possibly they are infected with a different pathogen, possibly false negative PCR test

PCR results:	
-	No amplification (negative)
+	Faint band (low)
++	Strong band (high)
ELISA results:	
-	No reaction (negative)
+	Light blue color (low)
++	Dark blue color (high)

Answers for Discussion Questions:

1. 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?
   Please see the Patient Information Table above. All patients with a "+" or "++" in the "ELISA-Ab" column have mounted an immune response against the influenza virus. The students should identify the patients with a "++" in the ELISA-Ab column as having the highest levels of antibodies in their blood.

2. Do you see any connections between who has been vaccinated and who has the highest levels of antibodies in their blood?
   Students should observe that patients with the new vaccine have the highest levels of antibodies in their blood ("++"). Patients who received the old vaccine have moderate levels of antibodies in their blood ("+"). Students may also notice that some patients have never receive a vaccine but still have anitbodies in their blood. They may suggest that this is because these patients have been infected with influenza in a past year so have partial immunity to the current year's strain.

3. Based on the results of the PCR assay, which patients are infected with the influenza virus?
   All patients with "+" or "++" in the "PCR" column of the table above are infected with the influenza virus.

4. Is there a connection between the patients' vaccination status and the result of their PCR assay?
   Patients who have received the new vaccine will have negative PCR results ("-" in the "PCR" column of the table above). Patients who have received the old vaccine have PCR test results that indicate a low level of infection ("+"). Students will also recognize that patients with PCR test results that indicate a low level of infection ("+") but who have never been vaccinated may have parial immunity because they were infected by a previous year's strain of the virus.

5. Are there any patients that might be infected with a pathogen other than influenza?
   Students should recognize that a patient with severe flu-like symptoms but with no antibodies against the influenza virus and negative PCR results probably has a different viral or bacterial infection.

6. Which patients would you recommend to self-quarantine to stop the spread of influenza in Sandbeach?
   Anyone who is infected with the influenza virus currently should self-quarantine to stop the spread. This would include anyone with a positive PCR test, both "+" and "++".

Appendix D: Example Results

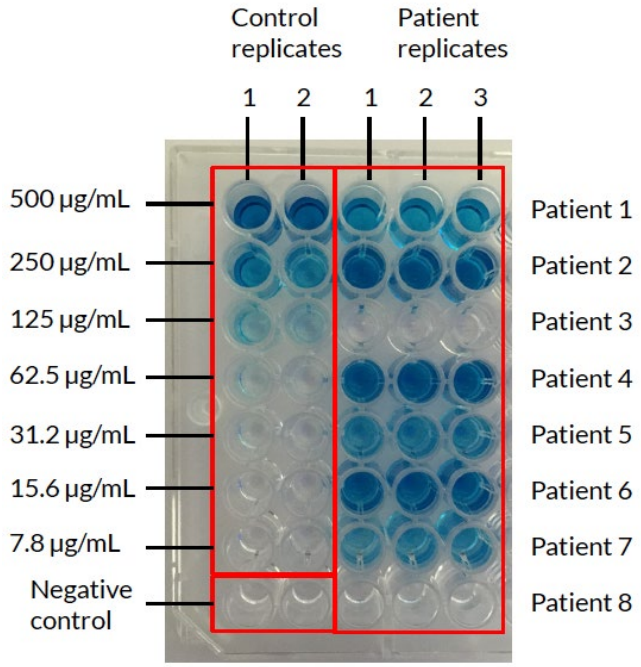

Figure 2. Example ELISA results for eight patient samples, one negative control and a positive control dilution series of protein A-HRP. Patients 1, 5 and 7 demonstrate low (+) results, while Patients 2, 4 and 6 demonstrate high (++) results, and Patients 3 and 8 show negative (-) results.

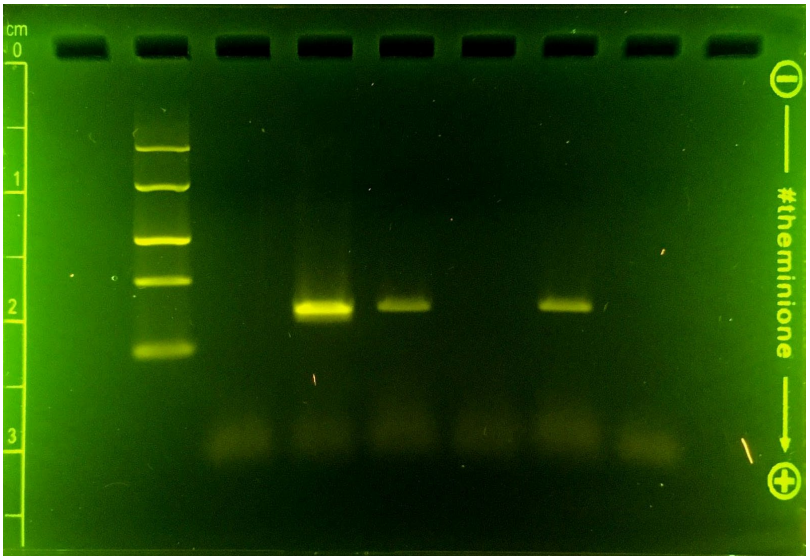

Figure 3. Example student electrophoresis gel showing PCR amplification results. Lane 2: Molecular weight marker (bands are 2000, 1000, 500, 200, 100 bp), Lane 3: negative control, Lane 4: positive control (identical results for high (++) results, Lanes 5 + 7: low positive (+) results, Lanes 6 + 8: negative results (-).

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Citing This Article

Hyland C. and Chalupny NJ. 2023. Who has the flu? Tracing transmission with ELISA and PCR. Article 9 In: Boone E and Thuecks S, eds. *Advances in biology laboratory education*. Volume 43. Publication of the 43rd Conference of the Association for Biology Laboratory Education (ABLE).
https://doi.org/10.37590/able.v43.art9