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Edvo-Kit #
274

Edvo-Kit #274

Identifying the Epstein Barr Virus Using ELISA

Experiment Objective:

In this experiment students will perform an enzyme-linked immunosorbent assay (ELISA) in order to screen simulated serum samples for antibodies to the Epstein Barr Virus (EBV) - a virus that causes most mononucleosis infections and that is associated with pediatric lymphomas.

See page 3 for storage instructions.

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Safety Data Sheets can be found on our website: **www.edvotek.com/safety-data-sheets**

Experiment Components

COMPONENTS **Check (✓)**

Store components A-G in the refrigerator.

A	10x ELISA Wash Buffer	❑
B	ELISA Dilution Buffer	❑
C	Antigen (Lyophilized)	❑
D	Primary Antibody (Lyophilized)	❑
E	Secondary Antibody (Lyophilized)	❑
F	ABTS Substrate (Lyophilized)	❑
G	ABTS Reaction Buffer	❑

Experiment #274 is designed for 10 lab groups.

All experiment components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

REAGENTS & SUPPLIES

Store all components below at room temperature.

- Small transfer pipets ❑
- Microtiter plate ❑
- 15 mL conical tubes ❑
- Snap-top microcentrifuge tubes ❑

Requirements *(not included with this kit)*

- Paper towels
- Distilled or deionized water
- Beakers or flasks
- Disposable lab gloves
- Safety goggles
- Recommended: Adjustable volume micropipettes (50 µL volume) and tips

NOTE: Make sure that glassware is clean, dry, and free of soap residue. For convenience, additional disposable transfer pipets can be purchased for liquid removal and washing steps.

This experiment does not contain Epstein-Barr virus (EBV) or its components. None of the components have been prepared from human sources.

Background Information

Nine out of ten individuals will be infected with the Epstein Barr Virus (EBV) by the time they are adults. During the initial stage of an infection, EBV goes into a lytic cycle and can cause a collection of symptoms often diagnosed as mononucleosis. Later, the virus enters a lysogenic life cycle where it "hides" and replicates within the genome of its human host. In some cases, latent viruses like EBV are classified as carcinogens because they have been linked to higher rates of certain cancers.

THE EPSTEIN BARR VIRUS AND MONONUCLEOSIS

You're running a fever, your throat and muscles are sore, there's a swollen bump on your neck the size of a tennis ball, and you're absolutely exhausted. If you're in your teens or twenties this may be glandular fever, also known mononucleosis ("mono"). Most symptoms of mono will disappear after a few weeks of rest and do not require medical treatment. Nevertheless, a doctor will carry out tests to confirm a diagnosis of mono because there are potential complications – such a spleen rupture – that are more serious.

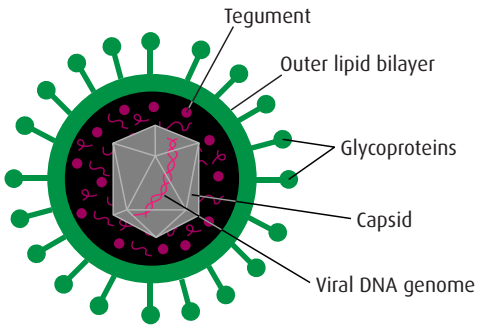


Figure 1: The Epstein Barr Virus.

Mono is mainly caused a member of *Herpesviridae* family know as the Epstein-Barr virus or EBV (Figure 1). A single EBV is compromised of four major parts: (1) a double-stranded DNA genome containing around 200 genes, (2) a 20-sided protein capsid that contains this genetic material, (3) an inner matrix – known as the tegument - which contains proteins and RNA, and (4) an outer envelope consisting of lipids and glycoproteins.

Like other members of the *Herpesviridae* family, EBV has two different replication strategies that result in either chronic or latent infections (Figure 2). During a chronic infection, the virus takes over the host cell's metabolic machinery and produces new virions. These new virions are released and, in turn, infect other cells. This is known as a lytic cycle(s). In contrast, during a latent infection, the virus' genome is largely unexpressed but remains in the nucleus of key host

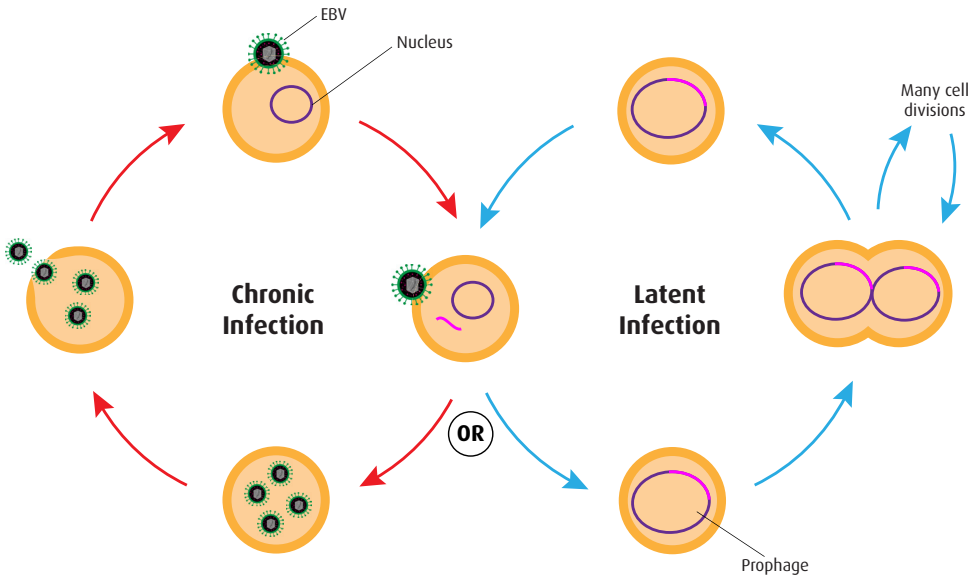


Figure 2: Lytic (left) and Lysogenic (right) Life Cycles.

cells. This is known as a lysogenic cycle(s). It is very hard for a host's immune system to detect and attack a virus when it is lysogenic. Because EBV can switch between lytic and lysogenic a person infected with mono will then carry the virus for the rest of their lives. This is also true of the virus' close relative the varicella zoster virus that causes chickenpox and shingles.

EBV is an extremely common human virus. It is found worldwide and is carried as a persistent infection by >90% of adults! Mono's nickname as "the kissing disease" derives from the fact that the EBV virus is often spread by direct contact with saliva, although it can also be transferred through tears, mucus, blood, and semen. Once inside the body, EBV seeks out epithelial cells in the pharynx or B cells of the immune system. When a virion finds one of these cells its viral envelope fuses with the host cell's membrane and its DNA is inserted. At this point, the virus begins one of its life cycles.

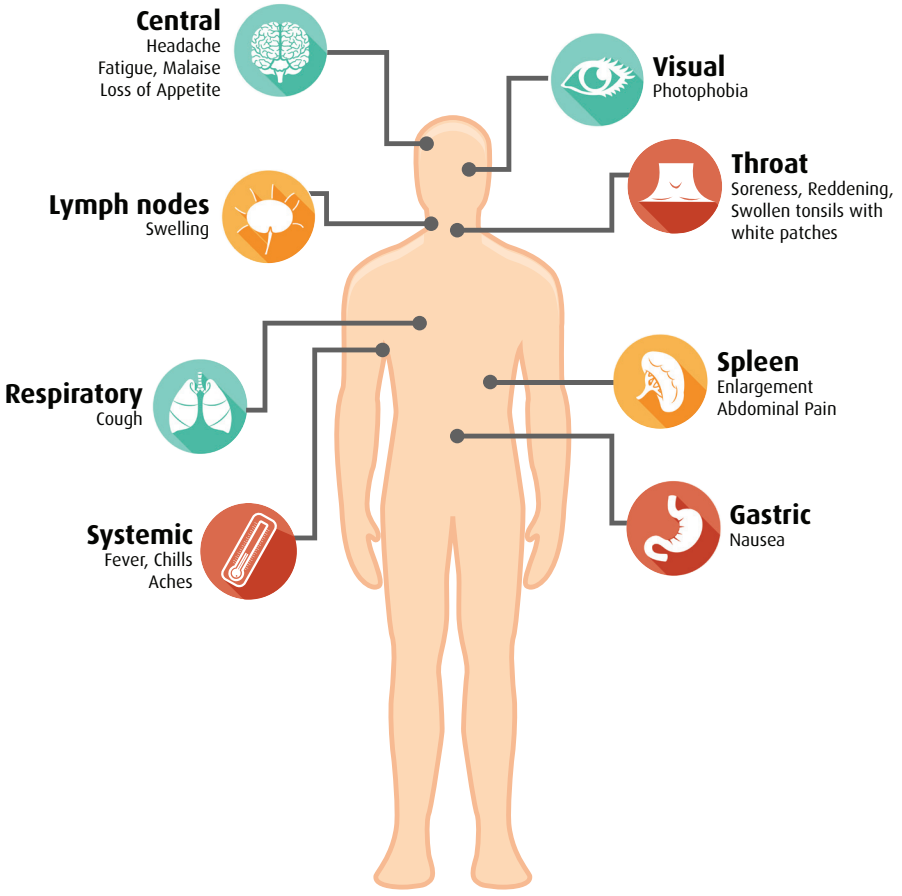


Figure 3: Symptoms of infectious mono.

Symptoms of mono appear 4-7 week after infection – if they appear at all. In fact, most people infected with EBV are asymptomatic. This is particularly true of children whose immune systems are unable to quickly recognize and strongly respond to the virus when it first enters the body. In adolescent and adults, the key diagnostic symptoms for mono are fever, sore throat, swollen glands, and fatigue. Headaches, abdominal pain, body aches, nausea, and a rash can also occur in some cases (Figure 3). Two to three weeks after these symptoms occur some patients also show splenomegaly (an enlarged spleen) and hepatomegaly (an enlarged liver). These symptoms are treated with rest, over the counter pain relievers, and in some cases steroids to reduce swollen tonsils.

EBV was first identified in 1964 by Drs. Anthony Epstein, Yvonne Barr, and Burt Achong. These three scientists were studying white blood cells taken from children with an aggressive cancer known as Burkitt's lymphoma with the hopes of discovering a link between this cancer and previous viral infections. However, while EBV was quickly confirmed as a new human virus, it was not until 1968 that the connection between mono and EBV was discovered and not until 1976 that the link between certain EBV infections and cancer was established.

LINKING VIRUSES AND CANCER

Cancer is a broad group of diseases involving abnormal cell growth caused by genetic mutations. These genetic mutations can occur as part of the natural life cycle of our cells or from environmental factors such as exposure to carcinogens, UV radiation, or - in some cases - previous infections. Viruses that can lead to cancer are called

oncoviruses and may be linked to ~18% of human cancers. One of the most well-studied oncoviruses is the Human Papilloma Virus (HPV). In 2002 HPV associated cancers made up ~5% of diagnosed cancer cases. This led to the development of a HPV vaccine and its widespread promotion.

How can a viral infection lead to cancer? One theory is that virus infected cells divide more frequently than normal cells, creating more opportunities for a DNA mutation to occur during a replication event. A similar theory is that as cells are damaged or lost during an infection the immune system instructs certain cells to divide more frequently, likewise increasing the chance of mitotic errors. Another possibility is that during an infection, the immune system releases chemicals that, over extended periods of time, may damage the bodies' own DNA.

However, identifying a specific oncovirus is extremely challenging. This is because so many other factors (life-style, environment, inherited genetics, and other infections) play a role in predisposing an individual to cancer and because cancer diagnosis often occurs many years after the viral infection. In addition, the vast majority of oncovirus infections do not lead to cancer which makes it hard to establish a clear pattern. EBV has been studied as an oncovirus since its discovery in 1965 mainly in association with Burkitt lymphoma – a cancer of the immune system and that causes fast-growing tumors. Globally this disease accounts for only 1% of adult lymphoma cases but up to 40% of pediatric lymphoma cases. In addition, EBV has also been linked to cancers of the nose, throat, and stomach. Researchers are also looking into possible links between EBV and arthritis, Parkinson's disease, and Chronic Fatigue Syndrome. However, it is important to remember that an EBV infection leads to these conditions only in a small minority of cases.

DETECTING EBV WITH AN ELISA

Clinical studies looking at the link between EBV and certain cancers or other medical conditions must first detect the EBV in patients and, if possible, determine what the viral concentration – or titration – is within certain tissues. Similarly, a doctor with a potential mono patient will want to confirm the presence and virulence of the virus before advising treatments like avoidance of contact sports. In both cases, an Enzyme Linked Immune Assay (ELISA) is used to test for the presence of three antibodies - VCA IgG, VCA IgM, and EBNA. Antibodies are specialized proteins that allow the immune system to distinguish between "self" and "non-self" proteins and polysaccharides. During a viral infection, an individual mounts a targeted immune response by creating several antibodies that bind to and flag viral molecules known as antigens.

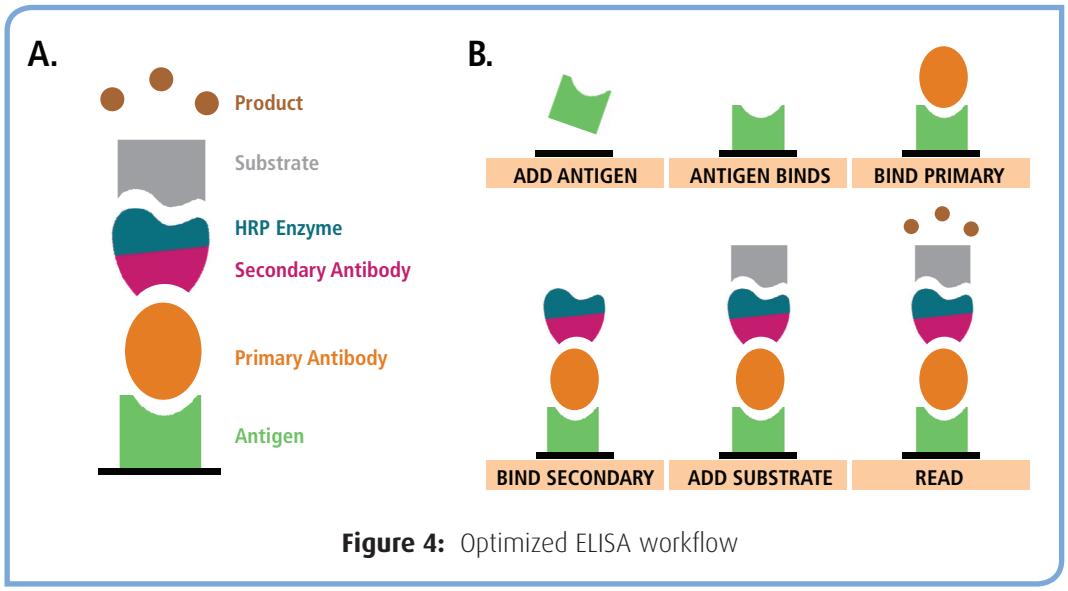


Figure 4: Optimized ELISA workflow

To perform an EBV ELISA (Figure 4) a solution containing EBV antigen is added to several small, clear wells. Non-specific hydrophobic associations cause this antigen to bind to the surface of the well and to remain there even after washings. Next, the patient samples and controls are added. If these samples contain EBV antibodies (primary antibodies), the antibodies will bind to the antigen and remain even after the well is washed again. A second antibody that can recognize the first antibody and that is covalently linked to an enzyme called Horseradish Peroxidase (HRP) is added next and the wells are again washed. HRP enables a fast change of color in certain substrates and has a high catalytic activity. Consequently it can create a strong and clear visual signal that indicates the presence of even trace amounts of antigen and EBV antibodies. Finally, a substrate is added to each well to enable this color change to occur.

Wells that tested a negative sample for the EBV antibody will contain only the antigen, not the primary or secondary antibody, and so will undergo a very slow color change. However, wells that tested a positive sample containing the EBV antibody will also contain the secondary antibody and HRP enzyme complex. These wells will undergo a strong colorimetric reaction when the substrate is finally added.

In this experiment, you will detect a patient's circulating viral capsid antigen immunoglobulin (VCA IgG). VCA IgGs are antibodies produced by the immune system in order to bind to and tag the EBV virus. While the concentration of these antibodies peaks at two to four weeks after an EBV infection, their presence in the body persists for the rest of an infected individual's life. Therefore, the presence of VCA IgG antibodies in a patient sample indicates that an infection has occurred. In a medical setting, this test would be followed by two additional ELISAs that would help determine whether the infection occurred relatively recently or long ago.

Experiment Overview

EXPERIMENT OBJECTIVE:

In this experiment students will perform an enzyme-linked immunosorbent assay (ELISA) in order to screen simulated serum samples for antibodies to the Epstein Barr Virus (EBV) - a virus that causes most mononucleosis infections and that is associated with pediatric lymphomas.

LABORATORY SAFETY

1. Gloves and goggles should be worn routinely as good laboratory practice.

2. Always wash hands thoroughly with soap and water after handling contaminated materials.

Wear gloves and safety goggles

LABORATORY NOTEBOOKS:

Address and record the following in your laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

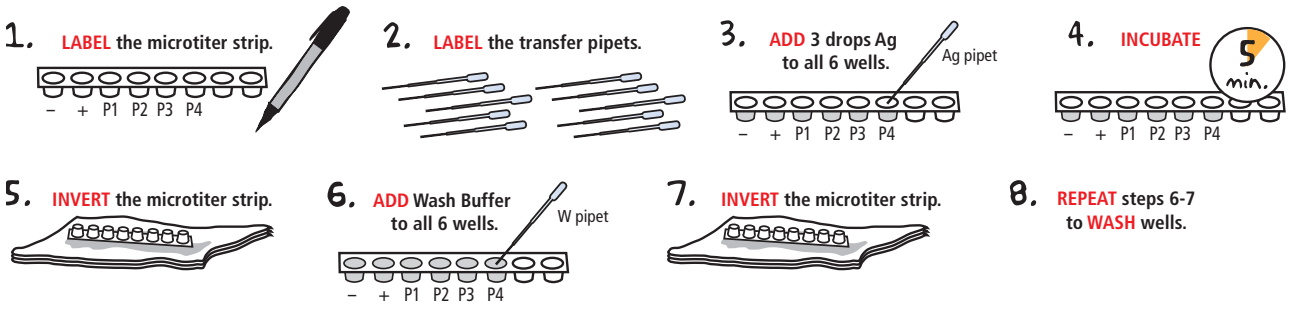
During the Experiment:

- Record (draw) your observations, or photograph the results.

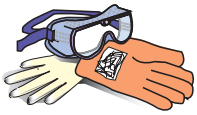
After the Experiment:

- Formulate an explanation from the results.
- Determine what could be changed in the experiment if the experiment were repeated.
- Write a hypothesis that would reflect this change.

Performing the ELISA



Loading of the EBV Antigen:



Wear gloves and safety goggles

1. **OBTAIN** an 8-well strip. Using a fine tipped marker, **LABEL** the first six wells with "-", "+", "P1", "P2", "P3", or "P4" as shown and add your initials or lab group number.
2. **LABEL** the transfer pipets as outlined in the box, below. These 10 pipets will be used to add liquid to the wells. If using an automatic micropipette, you will instead use a fresh tip for every step.

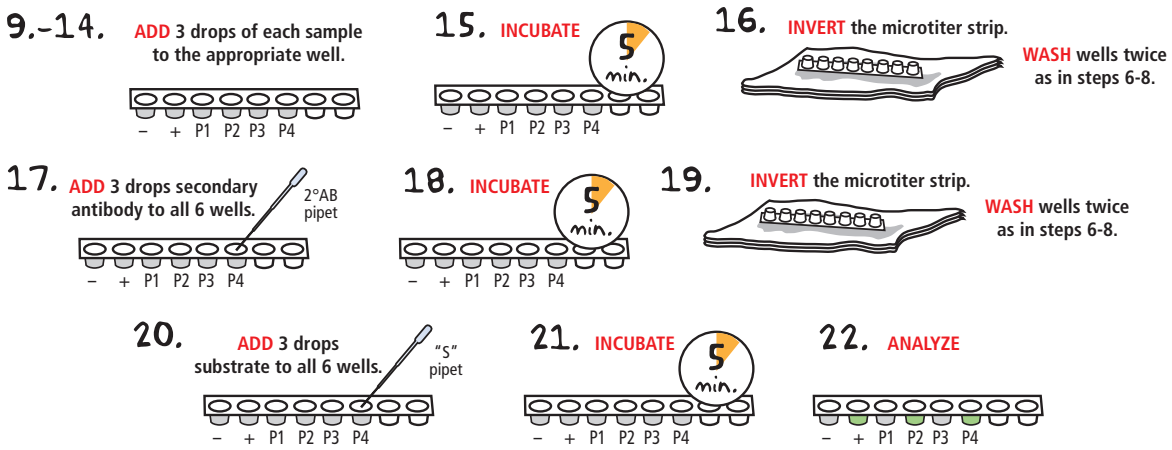
(W)	Wash Buffer (PBS)	(P1) Patient Serum 1
(Ag)	EBV Antigen	(P2) Patient Serum 2
(2°AB)	Secondary antibody	(P3) Patient Serum 3
(S)	Substrate	(P4) Patient Serum 4
(-)	Negative Control	
(+)	Positive Control	

3. Using the "Ag" transfer pipet or a micropipette, **ADD** 3 drops or 50 µL of Antigen to all six wells.
4. **INCUBATE** the strip at room temperature for 5 minutes.

Removal of Sample and Washing the Wells:

5. **INVERT** the strip over the sink or a stack of paper towels to remove the samples. Gently **TAP** the strip 4-5 times onto a fresh paper towel. **DISCARD** the wet paper towel.
6. Using the "W" transfer pipet, **ADD** Wash Buffer to each well until they are almost full (~200 µL). DO NOT allow the buffer to spill over into adjacent wells.
7. **INVERT** the strip over the sink or a stack of paper towels to remove the samples. Gently **TAP** the strip 4-5 times onto a fresh paper towel. **DISCARD** the wet paper towel.
8. **REPEAT** steps 6 and 7 to wash the wells once more.

Performing the ELISA, continued



Addition of Control and Patient Samples:

9. Using the "-" transfer pipet or a micropipette, **ADD** 3 drops or 50 µL of Negative Control to the appropriate well.
10. Using the "+" transfer pipet or a micropipette, **ADD** 3 drops or 50 µL of Positive Control to the appropriate well.
11. Using the "P1" transfer pipet or a micropipette, **ADD** 3 drops or 50 µL of Patient 1 to the appropriate well.
12. Using the "P2" transfer pipet or a micropipette, **ADD** 3 drops or 50 µL of Patient 2 to the appropriate well.
13. Using the "P3" transfer pipet or a micropipette, **ADD** 3 drops or 50 µL of Patient 3 to the appropriate well.
14. Using the "P4" transfer pipet or a micropipette, **ADD** 3 drops or 50 µL of Patient 4 to the appropriate well.
15. **INCUBATE** the strip at room temperature for 5 minutes.
16. **INVERT** onto paper towels and **TAP**. **WASH** the wells twice as in steps 6-8.

Addition of Secondary Antibody:

17. Using the "2°AB" transfer pipet or a micropipette, **ADD** 3 drops or 50 µL of Secondary Antibody (2°AB) to all six wells. **REPLACE** the pipette tip.
18. **INCUBATE** the strip at room temperature for 5 minutes.
19. **INVERT** onto paper towels and **TAP**. **WASH** the wells twice as in steps 6-8.

Addition of Substrate:

20. Using the "S" transfer pipet or a micropipette, **ADD** 3 drops or 50 µL of Substrate to all six wells.
21. **INCUBATE** the strip for 5 minutes at room temperature.
22. **ANALYZE** the plate. (If no color is develops after 5 minutes, incubate for a longer period of time. However, the negative control will eventually begin to show color if incubated for too long.)

Study Questions

1. What are the two life cycle strategies of EBV? How long can each last?
2. List at least three challenges that scientist face when classifying a virus as an oncovirus.
3. What is the ELISA? Briefly describe the purpose of the following solutions used in an ELISA: antigen, wash buffer, primary antibody, secondary antibody, and substrate.
4. Why do serum samples from EBV infected patients change colors while those from uninfected patients remain clear?

Instructor's Guide

OVERVIEW OF INSTRUCTOR'S PRELAB

The section outlines the recommended prelab preparations and approximate time requirement to complete each prelab activity.

What to do:	When:	Time Req.
Prepare strips	Anytime before the lab	5 min.
Prepare 1x wash buffer	Anytime before the lab. Cover and store in the refrigerator.	5 min.
Prepare Antigen	Up to one week before performing the experiment.	10 min.
Prepare patient and control samples	Up to one week before performing the experiment.	10 min.
Prepare Secondary Antibody	Day of the lab.	10 min.
Prepare ABTS Substrate	Up to one week before performing the experiment.	5 min.

Red = Prepare immediately before module.

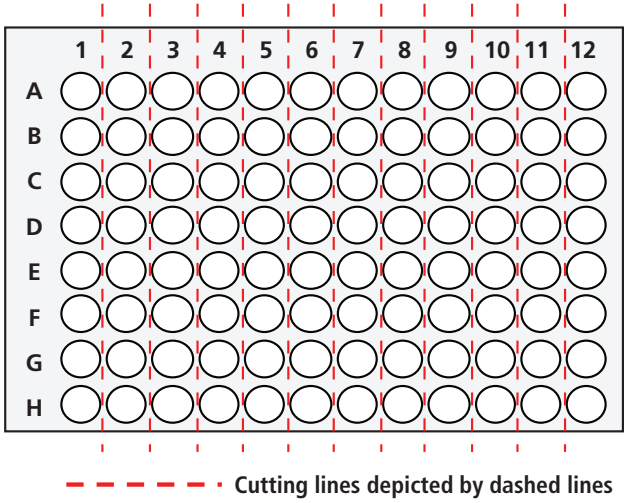
Yellow = Prepare shortly before module.

Green = Flexible

Pre-Lab Preparations

Preparation of Strips

1. Carefully divide the microtiter plate along the perforated line to create ten 8 well strips as shown in the figure.



Preparation of Wash Buffer

1. Add all of the 10x ELISA Wash Buffer (A) to 180 mL of distilled water and mix well.
2. Label as "Wash Buffer".
3. Dispense 12 mL into ten labeled small beaker or tubes for each lab group.

Preparation of Antigen

1. Transfer 7 mL of ELISA Dilution Buffer (B) to a 15 mL conical tube. Label as "Antigen".
2. Carefully remove the stopper from the vial of lyophilized Antigen (C) and transfer approximately 0.5 mL of the ELISA Dilution Buffer from the tube in step 1. Close the stopper and gently shake the vial to mix.
3. Transfer the entire volume of the Antigen back to the conical tube from step 1. Mix well.
4. Label ten microcentrifuge tubes as "Antigen"and dispense 500 µL of the prepared antigen into these tubes.

Preparation of Patient and Control Samples

1. Transfer 7 mL of ELISA Dilution Buffer (B) to a 15 mL conical tube. Label as "Primary Antibody".
2. Carefully remove the stopper from the vial of lyophilized Primary Antibody (D) and transfer approximately 0.5 mL of the ELISA Dilution Buffer from the tube in step 1. Close the stopper and gently shake the vial to mix.
3. Transfer the entire volume of the primary antibody back to the conical tube from step 1. Mix well.
4. Dispense 75 µL of the prepared primary antibody into thirty tubes. Label these tubes as "+" (10), "P2" (10), or "P3" (10).
5. Dispense 75 µL of ELISA Dilution Buffer (B) into thirty tubes. Label these tubes as "-" (10), "P1" (10), and "P4"(10).

Pre-Lab Preparations

Preparation of the Secondary Antibody

Prepare the same day as the experiment.

1. Transfer 7 mL of ELISA Dilution Buffer (B) to a 15 mL conical tube. Label as "Secondary Antibody".
2. Carefully remove the stopper from the vial of the Secondary Antibody (E) and transfer approximately 0.5 mL of the ELISA Dilution Buffer from the tube in step 1. Close the stopper and gently shake the vial to mix.
3. Transfer the entire volume of the secondary antibody back to the conical tube from step 1. Mix well.
4. Dispense 500 µL of the prepared secondary antibody into ten microcentrifuge tubes labeled "2°AB". Store on ice or refrigerate until needed. Do not freeze.

Preparation of Substrate

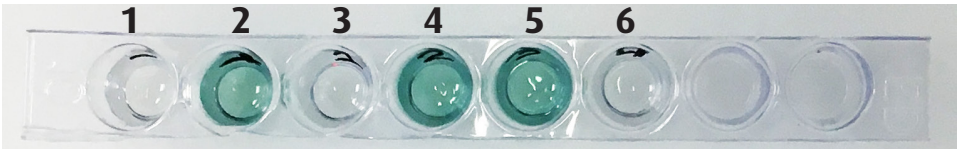
1. Transfer 7 mL of ABTS Reaction Buffer (G) to a 15 mL conical tube. Label as "Substrate".
2. Carefully remove the stopper from the vial of lyophilized ABTS (F) and transfer approximately 0.5 mL of the ABTS Reaction Buffer from the tube in step 1. Close the stopper and gently shake the vial to mix.
3. Transfer the entire volume of ABTS back to the conical tube from step 1. Mix well.
4. Dispense 650 µL of the prepared ABTS into ten microcentrifuge tubes labeled "Substrate".

STUDENT MATERIALS
Each Lab Group Should Receive:

- 1 Microtiter Strip (8 wells)
- 10 Transfer pipets
- 1 Small beaker with 12 mL Wash Buffer
- 1 Empty beaker for waste
- 1 tube with Antigen
- 1 tube with Positive Sample
- 1 tube with Negative Sample
- 4 tube containing Patient Samples
- 1 tube containing Secondary Antibody
- 1 tube containing Substrate

Experiment Results and Analysis

Patients 2 and 3 should be positive for EBV as represented in the figure below. The color of the positive patients should look similar to the positive control.



Experiment Results:

1. Negative Control
2. Positive Control
3. Patient 1 Serum Sample (Negative)
4. Patient 2 Serum Sample (Positive)
5. Patient 3 Serum Sample (Positive)
6. Patient 4 Serum Sample (Negative)

Please refer to the kit insert for the Answers to Study Questions