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Edvo-Kit #

274

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In Search of the "Kissing Disease"

Experiment Objective:

The objective of this experiment is to understand the experimental concepts and methodology involved with enzyme-linked immunosorbent (ELISA) assays in the context of the clinical screening of serum samples for antibodies to EBV. Primary infection with EBV results in infectious mononucleosis, which is popularly known as the "Kissing Disease".

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 **Store all components in the refrigerator.**		Check (✓)
A	EBV Antigens (simulated)	❑
B	Positive Control (primary antibody)	❑
C	Donor 1 Serum (simulated)	❑
D	Donor 2 Serum (simulated)	❑
E	Donor 3 Serum (simulated)	❑
F	Donor 4 Serum (simulated)	❑
G	Anti-IgG-peroxidase conjugate (secondary antibody)	❑
H	Substrate solution	❑
I	Phosphate buffered saline concentrate	❑

Experiment #274 is designed for 10 lab groups.

REAGENTS & SUPPLIES **Store all components below at room temperature.**	Check (✓)
• Microtiter strips	❑
• Transfer pipets	❑
• Microtest tubes with attached caps	❑
• 15 ml plastic tubes	❑

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.

Requirements *(not included with this kit)*

- Distilled or deionized water
- Beakers
- Disposable lab gloves
- Safety goggles
- Automatic micropipets (0 - 50 µl) and tips recommended

Make sure 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

IN SEARCH OF THE "KISSING DISEASE"

Infectious mononucleosis, popularly known as the "Kissing Disease", is a fairly common illness among students on college campuses around the world. It is called the "Kissing Disease" because it is believed that the virus which causes the infection is transmitted by saliva during kissing. The culprit responsible for this disease is the Epstein-Barr virus (EBV). It is a DNA virus and is a member of the herpes virus group.

EBV was first described by Epstein and coworkers. Infections due to EBV are usually of short duration, self-limiting and without long term effect, characterized by fever and sore throat. The virus is indigenous to all world populations and infects most humans at some stage of their life. Primary infections in third world countries usually occur in early childhood and are subclinical. In more developed countries primary infections usually occur at a later age and may present itself as acute infectious mononucleosis. Its presence can be easily demonstrated by immunological ELISA tests.

Primary infection with EBV results in infectious mononucleosis, but the virus is also associated with numerous other diverse diseases. These include Burkitt's lymphoma and nasopharyngeal carcinoma, acquired immune deficiency syndrome (AIDS), autoimmune diseases and chronic fatigue syndrome. There is increasing evidence for their association with cellular immune defects. These illnesses are usually chronic, and correlate with elevated levels of IgG antibodies to EBV. The common denominator for all these illnesses is the presence of elevated antibody titers to EBV, the recovery of virus or identifiable EBV DNA from patients.

Fatigue is a major symptom of EBV infectious mononucleosis. It is also the primary symptom for Chronic Fatigue Syndrome. Chronic Fatigue Syndrome is closely identified with EBV, and in addition to fatigue, its symptoms may include low-grade fever, headaches, sleep disorders, and depression which can last for a long time. IgG antibodies to EBV are elevated during the course of Chronic Fatigue Syndrome implicating EBV as a causative agent of the disease. However, current research indicates that patients who suffer from Chronic Fatigue Syndrome also suffer from immune deficiency problems.

Patients suffering from rheumatoid arthritis tend to have higher antibody titers to EBV antigens than do normal individuals although they do not show ill effects from EBV viral infections. The T-cell dysfunction observed in patients with rheumatoid arthritis appears to be specific for this illness and is not found in other autoimmune diseases.

Nasopharyngeal Carcinoma is restricted to certain geographical areas which include southern China, Tunisia and Greenland. Patients have increased antibody titers to EBV, and tumor cells from these patients contain EBV. It is known that soil in these geographical areas contains high levels of nickel, nitrosoamines and diterprene esters, all of which are suspected carcinogens. These chemicals induce EBV-infected B-cell proliferation, which in turn will stimulate T-suppressor cell production.

Burkitt's lymphoma is primarily associated with parts of Africa, New Guinea and Sweden. A large majority of patients with this cancer have recoverable EBV DNA from their tumors. It has been reported that individuals suffering from Burkitt's lymphoma have chromosomal translocation. Diagnosis for EBV in clinical laboratories is carried out by the patient's immune response to the virus using an enzyme-linked immunosorbent (ELISA) or immunofluorescence assay. As with many viral infections the immune response after the initial infection may be significantly delayed, especially in children and immunocompromised individuals. With Polymerase Chain Reaction (PCR), it is possible to detect viral DNA present in small quantities in blood and tissues.

Description of the Immunological Screening

Enzyme-linked immunosorbent assay (ELISA) tests were originally developed for antibody measurement. These immunoassays have also been adapted to successfully detect samples that contain antigens.

This ELISA simulation experiment has been designed to detect a hypothetical patient's circulating IgG directed towards the viral (EBV) antigen. ELISAs are done in microtiter plates which are generally made of polystyrene or polyvinyl chloride. The plates are somewhat transparent and contain many small wells, in which liquid samples are deposited. First, antigen(s) are added to

the wells where some are adsorbed by hydrophobic association to the walls of the well after washing away the excess. The antigens can be the whole EBV lysate, specific EBV proteins, or a mixture of the two. There is no specificity involved with the adsorption process although some substances may exhibit low binding to the walls. In certain cases the antigens can be covalently cross-linked to the plastic using UV light. After washing away unadsorbed material, the unoccupied sites on the walls of the plastic wells are blocked with proteins, typically bovine serum albumin.

Infection by EBV (positive for EBV) causes the individual to mount an antibody response which eventually results in plasma IgG molecules that bind to different EBV proteins. The EBV antibody present in the patient's plasma sample will bind to the viral proteins (antigen) in the well and remain there after washing. If the patient does not have EBV antibody in their serum, there will be no such binding. This will discontinue the ELISA steps that follow and not yield a color reaction which diagnoses the patient to be negative for the virus.

If EBV antibody remains bound, then the secondary antibody will bind to it and remain attached after washing. Secondary antibodies are raised in rabbits and goats immunized with human IgG fractions. Secondary antibodies are purified and covalently cross linked to an enzyme with a high turn number, such as horseradish peroxidase. The cross-linking does not significantly affect the binding specificity and affinity of the antibody or the enzymatic activity of the peroxidase (Figure 1).

After washing, a solution containing hydrogen peroxide and azino-diethylbenzthiazoline sulfonate (ABTS) is added to each well. Peroxidase possesses a high catalytic activity and can exceed turnover rates of 10^6 per second. Consequently, amplification of a positive sample can occur over several orders of magnitude. The substrate solution added is nearly colorless. Peroxidase converts the peroxide to $H_2O + O_2$ using the ABTS as the hydrogen donor. The oxidized ABTS is green and can be easily observed in wells containing anti-EBV IgG (positive plasma).

It should be noted that polyclonal antibody preparations to a given antigen can have variable binding affinities due to differences in the immunological responses between animals. Different immunizations with the same antigen in the same animal can also produce antibodies with variable binding affinities. The use of monoclonal antibodies directed against a single epitope eliminates this variability. Western blot analysis of positive ELISA samples are used to confirm infection by EBV.

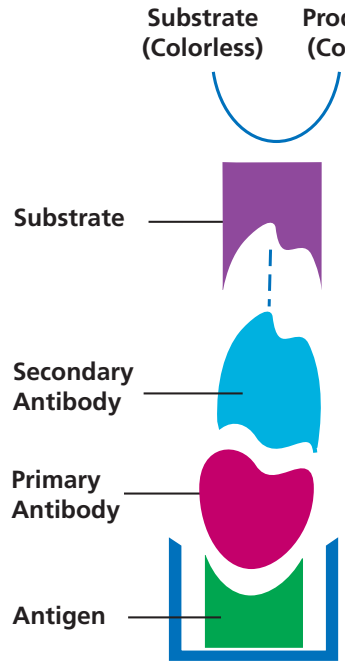


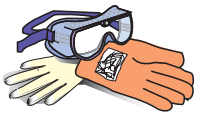
Figure 1: Schematic for ELISA

Experiment Overview

EXPERIMENT OBJECTIVE:

The objective of this experiment is to understand the experimental concepts and methodology involved with enzyme-linked immunosorbent (ELISA) assays in the context of the clinical screening of serum samples for antibodies to EBV. Primary infection with EBV results in infectious mononucleosis, which is popularly known as the "Kissing Disease".

LABORATORY SAFETY



Wear gloves and safety goggles

1. Gloves and goggles should be worn routinely as good laboratory practice.
2. Exercise extreme caution when working with equipment which is used in conjunction with the heating and/or melting of reagents.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS OR BULBS.
4. Always wash hands thoroughly with soap and water after handling contaminated materials.

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.

Student Experimental Procedures

GENERAL INSTRUCTIONS AND PROCEDURES

Labeling the Microtiter Plate:

1. Place the microtiter strip as shown in Figure 2.

2. Carefully mark the strip with your initials or lab group number and number the wells 1-6 down the side.

3. Do not separate the reaction wells.

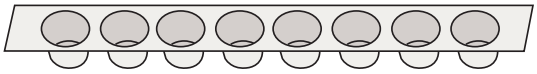


Figure 2: Microtiter plate.

Labeling the Plastic Transfer Pipets:

1. Use an automatic micropipet (50 µl) and disposable yellow tips or the transfer pipets.

2. Label 7 transfer pipets as follows:
   - (-) (negative)
   - (+) (positive)
   - DS 1 (Donor Serum 1)
   - DS 2 (Donor Serum 2)
   - DS 3 (Donor Serum 3)
   - DS 4 (Donor Serum 4)
   - PBS (Phosphate Buffered Saline)

   Use the appropriately labeled plastic transfer pipet for sample additions, removals, and washes as outlined in the experimental procedures.

3. Label 3 additional transfer pipets as follows:
   - EBV
   - 2°Ab
   - Substrate

Student Experimental Procedures, continued

INSTRUCTIONS FOR ADDING LIQUIDS AND WASHING WELLS

Adding Reagents to Wells:

- For adding reagents to the wells, use the labeled transfer pipets or use an automatic micropipet and disposable tips.

Liquid Removal and Washes:

- When instructed in the experimental procedures, remove liquids with the appropriately labeled transfer pipet, and then wash the wells as follows:

  A. Use the transfer pipet labeled "PBS", to add PBS buffer to each of the wells. Add PBS buffer until each well is almost full.

  The capacity of each well is approximately 0.30 ml. Do not allow the liquids to spill over into adjacent wells.

  B. With the appropriately labeled transfer pipet, remove all the liquid (PBS buffer) from each of the wells. Dispose the liquid in the beaker labeled "waste".

NOTE:
The general procedure for adding reagents to the wells will conserve the use of a large number of pipets in this simulated classroom ELISA.

If available, reagents should be dispensed with an automatic micropipet using disposable tips.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Wear gloves and safety goggles

1. To all 6 wells, add 50 µl or 3 drops of "EBV" (viral antigens). If using a transfer pipet, use the one labeled "EBV".
2. Incubate for 5 minutes at room temperature.
3. Remove all the liquid (viral antigens) with a transfer pipet.
4. Wash each well once with PBS buffer as described above ("Liquid Removal and Washes").

   In research labs, following this step, all sites on the microtiter plate are saturated with a blocking solution consisting of a protein mixture, such as BSA. We have designed this experiment to eliminate this step to save time.

5. Add reagents as outlined below:

   Remember to use the appropriately labeled transfer pipet for adding a new reagent. If you are using automatic micropipets, use a clean micropipet tip for each reagent.

   - Add 50 µl or 3 drops of PBS Buffer to the first well. (This is the negative control.)
   - Add 50 µl or 3 drops of "+" (positive) to the second well. (This is the positive control.)
   - Add 50 µl or 3 drops of Donor Serum "DS1" to the third well.
   - Add 50 µl or 3 drops of Donor Serum "DS2" to the fourth well.
   - Add 50 µl or 3 drops of Donor Serum "DS3" to the fifth well.
   - Add 50 µl or 3 drops of Donor Serum "DS4" to the sixth well.

Student Experimental Procedures, continued

6. Incubate at room temperature for 5 minutes.
7. Remove all the liquid from each well with the appropriately labeled transfer pipet.
8. Wash each well once with PBS buffer (as described under "Liquid Removal and Washes").

OPTIONAL STOPPING POINT:
The experiment can be stopped after step 8, but requires that PBS be added to all the wells for overnight storage at room temperature. The experiment can be resumed during the next lab period. Remove the PBS and continue with step 9.

9. Add 50 µl or 3 drops of the Anti-IgG peroxidase conjugate (2°Ab) to all 6 wells. If using a transfer pipet, use the one labeled "2°Ab".
10. Incubate at room temperature for 5 minutes.

    At this time you can obtain the substrate to be used in step 13.
11. Remove all the liquid from each well with the appropriately labeled transfer pipet.
12. Wash each well once with PBS buffer (as described under "Liquid Removal and Washes").
13. Add 50 µl or 3 drops of the substrate to all 6 wells. If using a transfer pipet, use the one labeled "substrate".
14. Incubate at room temperature for 5-10 minutes.
15. Remove the wells for analysis.

REMINDERS:

Adding Reagents:
Be sure to use a fresh tip for each reagent. (Steps 1, 5, 9, & 13). Alternatively, use the appropriately labeled transfer pipet for each reagent.

Liquid Removals:
Use the appropriately labeled transfer pipet to remove all liquid from each of the wells (steps 3, 7, & 11) and after washes (steps 4, 8 & 12).

Transfer pipet (-) Well 1
Transfer pipet (+) Well 2
Transfer pipet DS 1 Well 3
Transfer pipet DS 2 Well 4
Transfer pipet DS 3 Well 5
Transfer pipet DS 4 Well 6

Dispose of the liquid in a beaker labeled "waste".

Washes:
For all wells, use the transfer pipet labeled "PBS" to add PBS until each well is almost full. (Steps 4, 8, & 12).

QUICK REFERENCE:

The positive control, which contains IgG directed against EBV antigens, is the primary antibody. Positive serum samples will also contain anti-EBV IgG, while negative serum samples will not contain anti-EBV IgG.

Study Questions

1. Describe the reaction that generates the color in positive wells in the ELISA reaction.
2. How is the EBV infection transmitted through kissing?
3. Why was EBV suspected to be the cause of chronic fatigue?
4. (OPTIONAL) What is PCR and how can it be used to detect EBV virus?

Instructor's Guide

APPROXIMATE TIME REQUIREMENTS FOR PRE-LAB AND EXPERIMENTAL PROCEDURES

1. Pre-lab preparation of biologicals and reagents takes approximately one and one-half hours.

2. The student experiment requires approximately 45 minutes.

Class size, length of laboratory sessions, and availability of equipment are factors which must be considered in the planning and the implementation of this experiment with your students. These guidelines can be adapted to fit your specific set of circumstances.

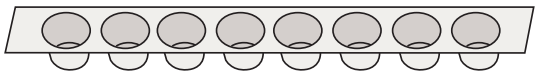
If you do not find the answers to your questions in this section, a variety of resources are continuously being added to the EDVOTEK® web site. In addition, Technical Service is available from 9:00 am to 6:00 pm, Eastern time zone. Call for help from our knowledgeable technical staff at 1-800-EDVOTEK (1-800-338-6835).

Pre-Lab Preparations

PREPARATIONS BEFORE THE LAB

Microtiter Wells

1. Carefully divide each microtiter plate into 8-well strips. Each student group requires one 8-well strip.



Dispensing Components A through F:

2. Use a clean pipet for dispensing each of the components A-F directly from the component tubes provided in this experiment kit. Label microtest tubes and dispense volumes as outlined in the chart "Preparation of Experiment Reagents" below.

PREPARATION OF EXPERIMENT REAGENTS

Component		Label	Dispense for each group
A*	EBV Antigens	EBV	0.5 ml
B*	Positive control	+	75 µl
C*	Donor Serum # 1	DS 1	75 µl
D*	Donor Serum # 2	DS 2	75 µl
E*	Donor Serum # 3	DS 3	75 µl
F*	Donor Serum # 4	DS 4	75 µl
G + PBS	Anti-IgG-peroxidase-conjugate	2°Ab	0.5 ml
H	Substrate Solution	Substrate	0.4 ml
I + water	Phosphate Buffered Saline	PBS	10 ml

* Components A - F can be dispensed before the actual day of the lab and stored in the refrigerator. If these components are dispensed on the day of the lab, leave at room temperature.

Pre-Lab Preparations

PREPARATIONS ON THE DAY OF THE LAB

Preparation of Phosphate Buffered Saline

1. Add all of the Phosphate Buffered Saline concentrate (I) to 135 ml of distilled water. Mix.
2. Label this diluted Phosphate Buffered saline as "PBS".
3. Dispense 10 ml into small beakers for each of the 10 lab groups.

Preparation of Anti-IgG Peroxidase Conjugate (Secondary Antibody)

Note: Prepare on same day as needed for the experiment.

4. Add 0.3 ml of diluted Phosphate Buffered Saline (PBS) to the concentrated Anti-IgG peroxidase conjugate (G). Mix thoroughly by tapping and inverting the tube.
5. Transfer 6 ml of diluted Phosphate Buffered Saline (PBS) to one of the 15 ml plastic tubes provided.
6. Transfer the entire contents of tube G prepared in step 4 to the 15 ml tube containing 6 ml of PBS prepared in step 5. Label the tube "2°Ab" (Secondary Antibody). Mix.
7. Dispense 0.5 ml of the diluted Anti-IgG peroxidase conjugate for each group.

NOTE:
The sample volume of the secondary antibody is very small - the tube can be centrifuged to collect the sample at the bottom.

Substrate Solution

8. Dispense 0.4 ml of the substrate solution for each of the 10 groups.

STUDENT MATERIALS
Each Lab Group Should Receive:

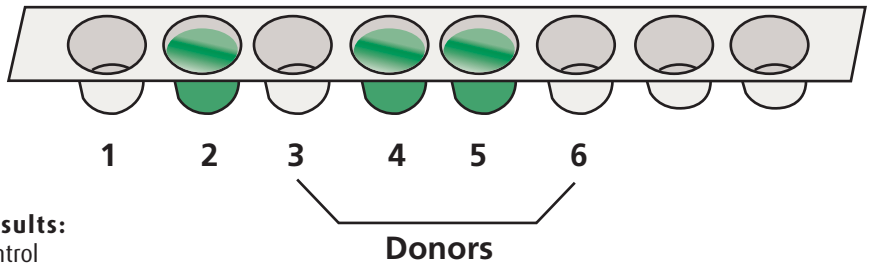
- 1 piece of microtiter strip
- 1 tube labeled "EBV"
- 1 tube labeled "+"
- 1 tube labeled "DS 1"
- 1 tube labeled "DS 2"
- 1 tube labeled "DS 3"
- 1 tube labeled "DS 4"
- 1 tube labeled "2°Ab"
- 1 automatic micropipet with tips (optional)
- 1 pipet pump
- 10 Transfer pipets
- 1 beaker or tube containing PBS
- 1 empty beaker labeled "waste"
- 1 tube labeled "Substrate"

Avoiding Common Pitfalls

1. Students should be advised to be very careful when transferring solutions into and out of the microtiter strips.
2. Use only clean or appropriately labeled pipets and avoid contaminating adjacent wells.
3. Do not attempt to empty the microtiter wells by shaking it out. This will not work - it will result in contaminating adjacent wells.
4. Wash the wells gently and slowly, without force.

Experiment Results and Analysis

Donors 2 and 3 should show positive for EBV as represented in the figure below. Actual liquid amounts in the wells are not drawn to scale, but the color of the positive donors should look similar to the positive control, which is in the second well.



Experiment Results:

1. Negative Control
2. Positive Control
3. Donor 1 (Negative)
4. Donor 2 (Positive)
5. Donor 3 (Positive)
6. Donor 4 (Negative)

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