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REVISED & UPDATED

Edvo-Kit #
269

Edvo-Kit #269

Introduction to ELISA Reactions

Experiment Objective:

This experiment introduces concepts and methodologies of enzyme-linked immunosorbent assays (ELISA).

See page 3 for storage instructions.

Table of Contents

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

Experiment Components

COMPONENTS **Store components A-H in the refrigerator.**		Check (✓)
A	Antigen reconstitution buffer	❑
B	Whey antigen (lyophilized)	❑
C	10x PBST (wash buffer)	❑
D	Primary Antibody (lyophilized)	❑
E	Secondary Antibody (lyophilized)	❑
F	ABTS substrate	❑
G	Aminosalicylic Acid (peroxide co-substrate)	❑
H	Hydrogen peroxide, stabilized	❑

Experiment #269 is designed for 10 lab groups.

REAGENTS & SUPPLIES **Store all components below at room temperature.**	Check (✓)
• Microtiter plates	❑
• Transfer pipets	❑
• Snap-top microcentrifuge tubes	❑
• 15 mL conical 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
- Recommended: Automatic micropipettes (50 µL) and tips

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.

None of the components have been prepared from human sources.

Background Information

PRINCIPLES OF ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Antibodies (also called immunoglobulins, or Igs) are specialized proteins that allow the immune system to distinguish between "self" and "non-self" proteins or polysaccharides. These Y-shaped molecules comprise four linked polypeptide chains: two identical "heavy chains" and two identical "light chains" (Figure 1). The antigen binding sites are located at the ends of the short arms of the Y. The amino acid sequence region is variable, allowing for each antibody to recognize a unique **epitope** (a particular location within an antigen).

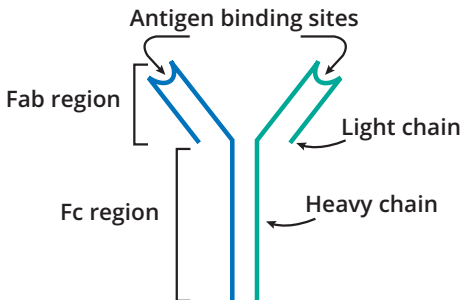


Figure 1:
General Structure of an antibody.

Antibodies used in scientific research are produced as an immune response when animals (i.e. rabbits, mice and guinea pigs) are injected with an antigen. The immune response will produce antibodies that are specific to the antigen, which are then purified from the serum. This solution will contain a mixture of antibodies because different immune cells will create antibodies that recognize different epitopes of the antigen. This heterogeneous mixture of antibodies is called a **polyclonal antibody**. If we isolate and culture individual immune cells from these animals, we can create **monoclonal antibodies**. These antibodies are directed against a single epitope, and thus are very specific.

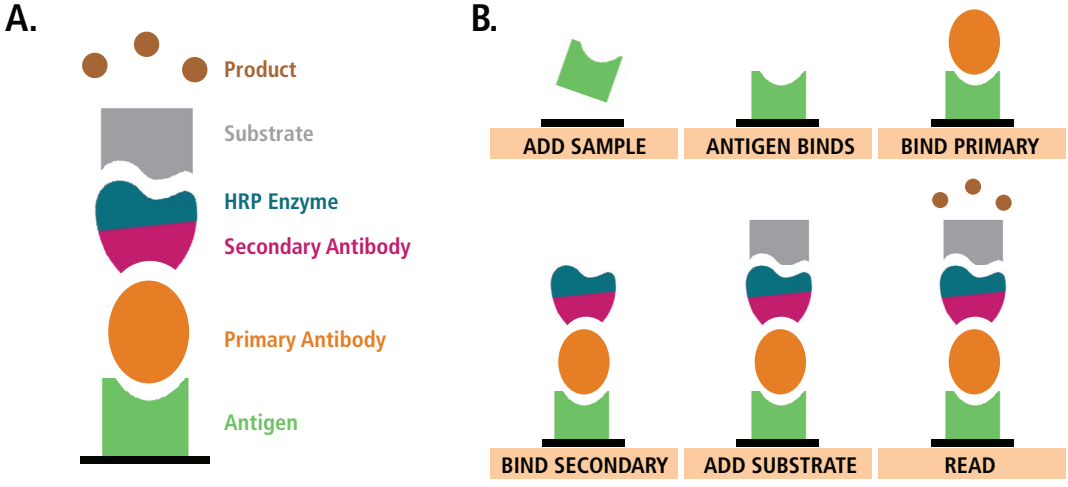


Figure 2: Optimized ELISA workflow.

Because of their specificity, antibodies can be used to detect the presence of specific biomolecules (i.e. peptides, proteins, antigens and hormones) in a sample. One common technique that uses antibodies in this way is the Enzyme Linked Immunosorbent Assay (ELISA). The ELISA is commonly used for medical diagnostics, as it can be used to identify antigens in blood, urine, spinal fluid, and other biological samples. An ELISA can be designed to provide qualitative or quantitative results. In a qualitative ELISA, the results will indicate if a sample is positive or negative for the antigen. This type of assay is simple to perform and is useful for situations where the exact concentration of molecules is not necessary, such as pregnancy or drug tests. Alternatively, quantitative ELISAs use a standard curve to determine the precise concentration of a substance in the sample.

The traditional ELISA requires two antibodies. One antibody, called the **primary**, recognizes the antigen of interest. For example, an ELISA that detects the HIV virus would use an antibody that recognizes one of the virion's coat proteins. The **secondary** antibody recognizes the primary antibody – if a rabbit produced our primary antibody, we would use a secondary antibody that recognizes rabbit antibodies. The secondary antibody is covalently linked to an enzyme called Horseradish Peroxidase (HRP) that lets us detect the presence of the antibody-antigen complex (Figure 2A). HRP has a high catalytic activity, allowing us to quickly detect even the smallest amount of antigen.

To perform an ELISA, the experimental samples are added to the wells and the antigens are allowed to adsorb to the wells during a brief incubation (Figure 2B). ELISAs are performed in transparent plastic microtiter plates, which allow scientists to easily visualize the results. After the incubation the wells are briefly washed, to remove any unbound sample, and the primary antibody is added to each well. If the antigen is present, the primary antibody will bind to it and remain attached after washing. Next, a secondary antibody is added and will only adhere where primary antibody has already bound. Finally, a substrate is added to each well – if the secondary antibody is present the HRP enzyme will catalyze a colorimetric reaction.

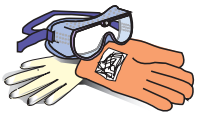
This experiment demonstrates the necessity of each component in the ELISA. For example, wells that are missing either the antigen or primary antibody will not be able to bind the secondary antibody and will therefore show a negative result. In addition, students will examine the colorimetric differences between two peroxidase substrates. Substrate 1 (S1) contains hydrogen peroxide and **aminosalicylic acid**, while Substrate 2 (S2) contains hydrogen peroxide and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), or **ABTS**. During the final step of the ELISA the peroxidase enzyme converts hydrogen peroxide into H_2O and O_2, using the substrate as a hydrogen donor. The oxidized salicylate is brown, while oxidized ABTS turns green. Both reacted substrates can be easily distinguished from the colorless unreacted substrate in negative wells.

Experiment Overview

EXPERIMENT OBJECTIVE:

The objective of this experiment is to understand the experimental concepts and methodology involved with enzyme-linked immunosorbent (ELISA) assays.

LABORATORY SAFETY



Wear gloves and safety goggles

1. Gloves and goggles should be worn routinely as good laboratory practice.
2. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS OR BULBS.
3. 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:

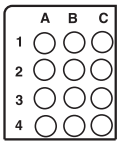
- Record (draw) your observations, or photograph the results.

After the Experiment:

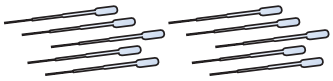
- Formulate an explanation from the results.
- Determine what could be changed in the experiment if the experiment were repeated.
- Write a hypothesis that would reflect this change.

Student Experimental Procedures

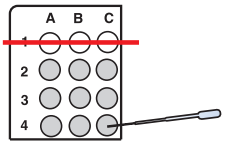
1. LABEL the microtiter plate.



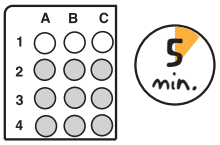
2. LABEL the transfer pipets.



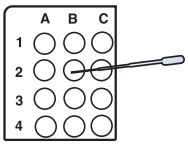
3. ADD 3 drops Ag to rows 2, 3, and 4.



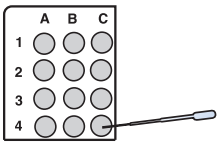
4. INCUBATE microtiter plate at room temp.



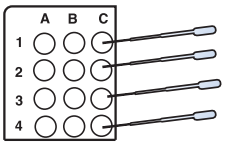
5. REMOVE liquid from wells.



6. WASH each well with 1X PBST buffer.



7. REMOVE all PBST using pipet for each row.



8. REPEAT Steps 6 and 7.

PERFORMING THE ELISA

Wear gloves and safety goggles

1. **LABEL** the wells of the microtiter plate as shown.

2. If not using an adjustable volume micropipette, **LABEL** the transfer pipets as outlined in the box below. These 10 pipets will be used to add and remove liquid from the wells.

(PBS)	Phosphate Buffered Saline	(S2)	Substrate 2
(Ag)	Antigen	(Row1)	Used to remove samples from row 1
(1°AB)	Primary antibody	(Row2)	Used to remove samples from row 2
(2°AB)	Secondary antibody	(Row3)	Used to remove samples from row 3
(S1)	Substrate 1	(Row4)	Used to remove samples from row 4

3. Using the "Ag" transfer pipet or a micropipette, **ADD** 3 drops or 50 µL of Antigen (Ag) to all of the wells in rows 2, 3, and 4. Do not add antigen to the wells in row 1.

4. **INCUBATE** the plate at room temperature for 5 minutes.

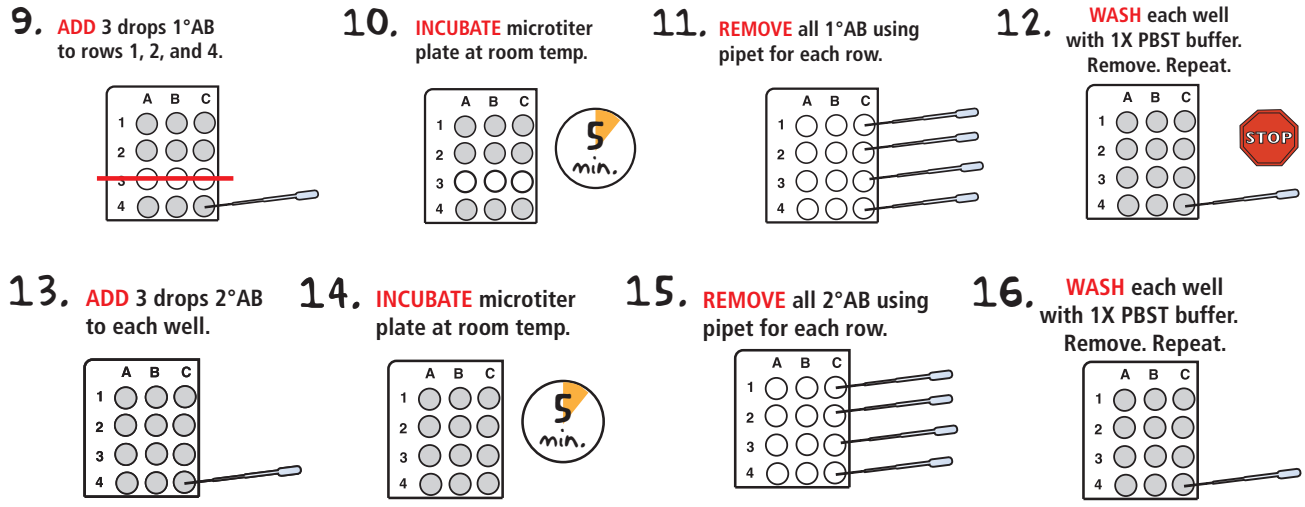
5. Using the "Ag" pipet **REMOVE** all of the liquid from the wells.

6. Using the "PBS" transfer pipet **WASH** each well by adding 1X PBST buffer until the wells are almost full (~200 µL). Do not allow the buffer to spill over into adjacent wells.

7. **REMOVE** all of the 1X PBST using the transfer pipet designated for each row.

8. **REPEAT** steps 6 and 7 to wash the wells once more.

Student Experimental Procedures, continued



9. Using the "1°AB" transfer pipet or a micropipette, **ADD** 3 drops or 50 µL of the primary antibody to the wells in rows 1, 2, and 4. Do not add primary antibody to the wells in row 3.

10. **INCUBATE** the plate at room temperature for 5 minutes.

11. Using the labeled transfer pipet for each row, **REMOVE** all of the primary antibody from each well.

12. **WASH** each well twice with fresh 1X PBST buffer. Between washes **REMOVE** all of the 1X PBST using the transfer piper designated for each row.

OPTIONAL STOPPING POINT: For overnight storage, **ADD** 200 µL of PBS to each well. Carefully cover the samples and place the plate in the refrigerator. The experiment should be resumed during the next lab period. Remove the 1X PBST and continue with Step 13.

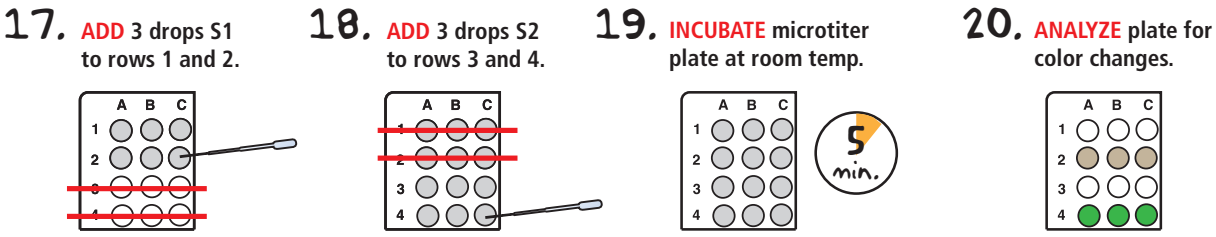
13. Using the "2°AB" labeled transfer pipet or a micropipette, **ADD** 3 drops or 50 µL of the secondary antibody to each well.

14. **INCUBATE** the plate at room temperature for 5 minutes.

15. Using the labeled transfer pipet for each row, **REMOVE** all of the secondary antibody from each well.

16. **WASH** each well twice with fresh 1X PBST buffer. Between washes **REMOVE** all of the 1X PBST using the transfer piper designated for each row.

Student Experimental Procedures, continued



17. Using the "S1" labeled transfer pipet or a micropipette, **ADD** 3 drops or 50 µL of Substrate 1 to rows 1 and 2.

18. Using the "S2" labeled transfer pipet or a micropipette, **ADD** 3 drops or 50 µL of Substrate 2 to rows 3 and 4.

19. **INCUBATE** the plate at room temperature for 5 minutes.

20. Immediately **ANALYZE** the plate for color changes in the substrates. If the color is not fully developed it can be left for a longer period of time.

Study Questions

1. What is the ELISA? Describe the purpose of each component used in an ELISA.
2. What is the effect of not including the antigen or the primary antibody in the ELISA reaction?
3. Why is it important to wash all the wells between the additions of the various components?
4. Can nucleic acids be detected by the ELISA format?

Instructor's Guide

OVERVIEW OF INSTRUCTOR'S PRELAB PREPARATION:

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

Preparation for:	What to do:	When:	Time Required:
Performing the ELISA	Divide microtiter plates	Before the class period	5 min.
	Prepare and Aliquot Reagents	Up to 1 week before the lab	30 min.
	Prepare substrate 1	During the lab (15-20 min. before last incubation)	10 min.
	Prepare secondary antibody	Same day as the lab	10 min.

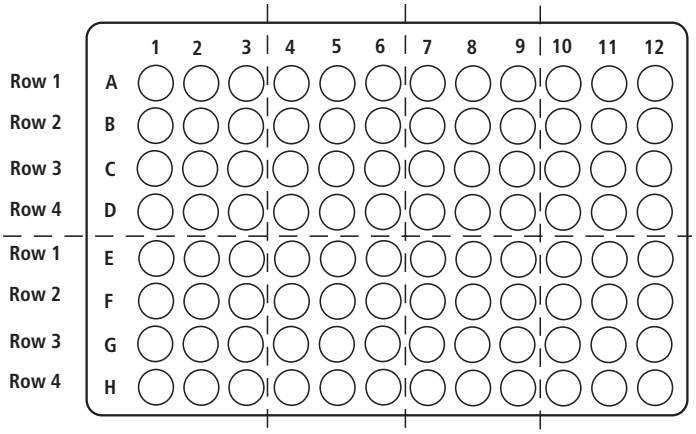
NOTE: If preparing and aliquoting reagents ahead of time, you must store all reagents at 4° C.

PRELAB PREPARATIONS

Preparing the Microtiter Plate:

1. As shown in the figure below, orient the microtiter plate so that the numbers 1-12 are at the top and the letters A-H are on the left.

2. Cut each plate on the dotted lines as shown in the figure. Each piece will contain 3 wells on one axis and 4 wells on the other axis. Each lab group will receive one piece.



— — — — — Cutting lines depicted by dashed lines

Pre-Lab Preparations - On The Day Of The Lab

Preparation of Whey Antigen

1. Transfer 7 mL of Antigen reconstitution buffer (A) to a 15 mL conical tube. Label the tube “Antigen”.
2. Carefully remove the stopper from the vial of lyophilized Whey antigen (B) and transfer approximately 0.5 mL of the antigen reconstitution buffer from the tube in step 1. Close the stopper and gently shake the glass vial to mix.
3. Transfer the entire contents of reconstituted Whey Antigen back to the 15 mL tube from step 1. Mix well.
4. Label 10 microcentrifuge tubes "Ag" and dispense 0.6 mL per tube.

Preparation of 1x PBST Wash Buffer

1. Add all of the 10x PBST (C) to 180 mL of distilled water and mix well. Label as “1xPBST”.
2. Dispense 16 mL into small beakers for each lab group.
3. Save the remaining 1xPBST for preparation of the primary and secondary antibodies and Substrate 1.

Preparation of Primary Antibody

1. Transfer 7 mL of 1xPBST to a 15 mL conical tube. Label the tube “1°AB”.
2. Carefully remove the stopper from the vial of lyophilized Primary Antibody (D) and transfer approximately 0.5 mL of the PBST from the tube in step 1. Close the stopper and gently shake the glass vial to mix.
3. Transfer the entire contents of reconstituted Primary Antibody back to the 15 mL tube from step 1. Mix well.
4. Label 10 microcentrifuge tubes "1°AB" and dispense 0.6 mL per tube.

Preparation of Secondary Antibody

(NOTE: Prepare on same day as needed for the experiment.)

1. Transfer 6.5 mL of 1xPBST to a 15 mL conical tube. Label the tube “2°AB”.
2. Carefully remove the stopper from the vial of lyophilized Secondary Antibody (E) and transfer approximately 0.5 mL of the PBST from the tube in step 1. Close the stopper and gently shake the glass vial to mix.
3. Transfer the entire contents of reconstituted Secondary Antibody back to the 15 mL tube from step 1. Mix well.
4. Label 10 microcentrifuge tubes "2°AB" and dispense 0.6 mL per tube.

Preparation of ABTS Substrate

1. Label 10 microcentrifuge tubes with “S2” for substrate 2.
2. Dispense 0.5 mL of ABTS substrate (F) into the labeled tubes.

Pre-Lab Preparations - During the Lab Experiment

Preparation of Aminosalicylic Acid Substrate

(NOTE: Prepare DURING the lab experiment, 15-20 minutes before needed.)

1. Dispense 9 mL of 1X PBST into a 15 mL conical tube.
2. Add the Aminosalicylic acid (G) to the PBS. Cap and mix thoroughly by shaking and/or vortexing. There may be undissolved material remaining in the tube.
3. Add 1 mL of Hydrogen peroxide (H). Cap and mix well.
4. Dispense 0.5 mL of prepared Aminosalicylic acid substrate into microcentrifuge tubes. Label tubes as "S1" for substrate 1.

Each Lab Student Group Should Receive:

1	Microtiter plate (3 x 4 well)
1	Microcentrifuge tube containing 0.6 mL Antigen
1	Microcentrifuge tube containing 0.6 mL Primary Antibody
1	Microcentrifuge tube containing 0.6 mL Secondary Antibody
1	Microcentrifuge tube containing 0.5 mL Substrate 1 (Aminosalicylic Acid)
1	Microcentrifuge tube containing 0.5 mL Substrate 2 (ABTS)
10	Transfer pipets
1	Beaker containing 16 mL 1X PBST
1	Empty beaker for waste

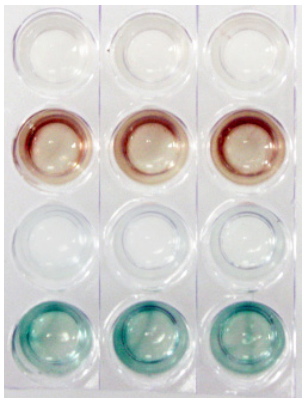
Avoiding Common Pitfalls

1. Students should be advised to be very careful when transferring solutions into and out of the microtiter plate wells.
2. Use only clean or appropriately labeled pipets.
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

Color should appear only in Rows 2 and 4. Rows 1 and 3 are each missing a critical component for the ELISA procedure. Row 2 will be a brown color and row 4 will be green in color.

Row	Antigen	1°AB	2°AB	S1	S2
1		√	√	√	
2	√	√	√	√	
3	√		√		√
4	√	√	√		√



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