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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.

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Experiment Components

COMPONENTS **Check (√)**
Store all components in the refrigerator.

	Component	Check (√)
A	Antigens	❑
B	Positive Antibody	❑
C	Anti-IgG-peroxidase conjugate (secondary antibody)	❑
D	Hydrogen peroxide, stabilized (for S1)	❑
E	Peroxide co-substrate (for S1)	❑
F	ABTS substrate (S2)	❑
G	Phosphate buffered saline concentrate	❑

Experiment #269 is designed for 10 lab groups.

REAGENTS & SUPPLIES **Check (√)**
Store all components below at room temperature.

- Microtiter plates ❑
- 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.

None of the components have been prepared from human sources.

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Background Information

PRINCIPLES OF ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

During an infection, an individual mounts an antibody response which eventually results in production of plasma IgG molecules that bind to various parts of the infectious agent. If these antibodies are present in the sample, they will bind to the adsorbed antigens in the well and remain there after washing, and will be detected by the ELISA technique.

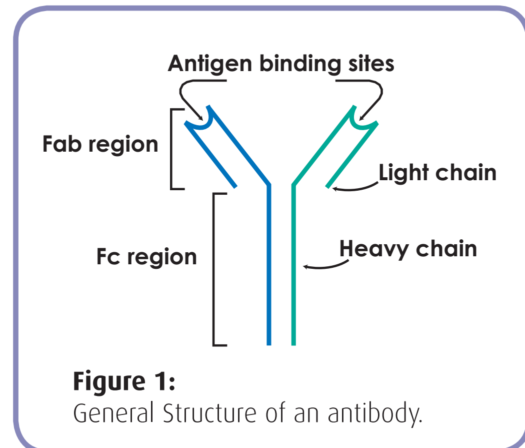

Figure 1: General Structure of an antibody.

All antibodies belong to a group of serum proteins known as globulins. Each antibody is made up of a heavy and light polypeptide chain (Figure 1). In general, antibodies are produced in response to the presence of a "non-self" antigenic response.

Antibodies obtained from animals, such as rabbits, in response to an antigen are known as polyclonal antibodies. Polyclonal antibodies are heterogeneous in structure and vary in their ability to bind to antigens. Antibodies that have a high affinity for non-specific antigens may give unwanted cross-reactions that can result in high backgrounds. Such antibodies can also give false negative results. By contrast, antibodies with weak binding constants may not be as sensitive.

Enzyme linked immunosorbent assay (ELISA) tests were originally developed for antibody measurement but have also been adapted to successfully detect samples that contain antigens. This ELISA experiment has been designed to detect an antibody directed against an antigen.

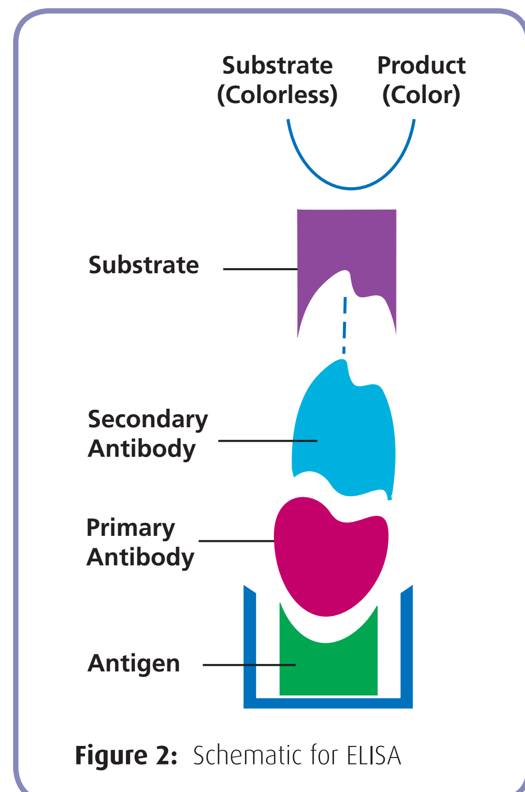

Figure 2: Schematic for ELISA

ELISAs (Figure 2) are done in microtiter plates usually made of polystyrene or polyvinyl chloride. The plates are somewhat transparent and contain many small wells, into which liquid samples are deposited. The following are the basic steps of the ELISA technique.

Step 1

The antigen is added to the wells where some remain adsorbed by hydrophobic association to the walls after washing away the excess. The antigens can be a lysate, a specific protein, 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.

Step 2

After washing away unadsorbed material, the unoccupied sites on the walls of the plastic wells are blocked with proteins, typically gelatin or bovine serum albumin.

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Step 3

A solution that may or may not contain the primary antibody is added to the wells. If present in the solution, the primary antibody will bind to the adsorbed antigen in the well and remain after washing.

Step 4

A solution containing the secondary antibody is then added to the wells. If the primary antibody has remained bound to the well, then the secondary antibody will bind to it and remain attached after washing. Secondary antibodies are purified and covalently cross linked to enzymes such as horseradish peroxidase. This coupling does not significantly affect the binding specificity and affinity of the antibody or the enzymatic activity of the peroxidase.

Step 5

The wells are washed with buffer to remove unbound secondary antibody.

Step 6

After washing the wells, substrate 1 (S1) will be added to all the wells in rows 1 and 2. Substrate 2 (S2) will be added to rows 3 and 4. The enzyme attached to the secondary antibody is a peroxidase. 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. Many hydrogen donor co-substrates can be used by peroxidase. These co-substrates include o-diansidine, aminoantipyrine, aminosalicylic acid and numerous phenolic compounds that develop color upon oxidation.

Substrate 1 (S1) contains hydrogen peroxide and amino salicylate. The substrate solution added is nearly colorless. Peroxidase converts the peroxide to $H_2O + O_2$ using the salicylate as the hydrogen donor. The oxidized salicylate is brown and can be easily observed in wells that have received each of the components required for completion of the reaction.

Substrate 2 (S2) contains hydrogen peroxide and azino-di ethylbenzthiazoline sulfonate (ABTS). 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 positive wells.

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 animals can also produce antibodies with variable binding affinities. The use of monoclonal antibodies directed against a single epitope eliminates this variability. Western blot analysis is usually used to confirm the ELISA results and to quantitate the size and amount of antigen. Western Blots and ELISA-based tests are used as diagnostic tools.

This experiment demonstrates two important concepts. The first is the effect of the absence of the antigen or the primary antibody which results in the disruption of the ELISA reaction. The second is the demonstration that the substrate utilized by the enzyme attached to the secondary antibody can result in different positive well colors.

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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

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.

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Student Experimental Procedures

GENERAL INSTRUCTIONS AND PROCEDURES

Labeling the Microtiter Plate:

1. Orient the microtiter plate as shown in Figure 3. Carefully mark the microtiter plate with your initials or lab group number.
2. If your microtiter plate is pre-labeled by the manufacturer, mark out the letters or numbers and relabel the plate as instructed as follows.
3. Label the microtiter plate A, B and C across the top.
4. Label the rows of wells consecutively 1, 2, 3 and 4 down the left side of the microtiter plate.

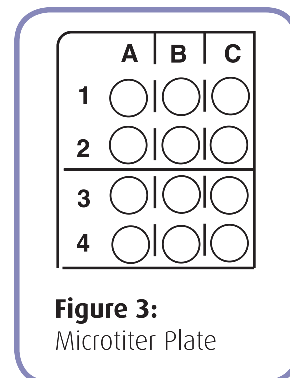

Figure 3: Microtiter Plate

Labeling the Plastic Transfer Pipets:

Label 10 transfer pipets as follows:

- PBS (Phosphate Buffered Saline)
- Ag (Antigen)
- 1°Ab (Primary Antibody)
- 2°Ab (Secondary Antibody)
- S1 (Substrate 1)
- S2 (Substrate 2)
- Row 1
- Row 2
- Row 3
- Row 4

Use the appropriately labeled plastic transfer pipet for sample additions, removals, and washes as outlined in the experimental procedures starting on page 8.

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Student Experimental Procedures, continued

INSTRUCTIONS FOR ADDING LIQUIDS AND WASHING WELLS

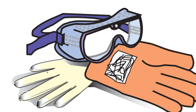
Adding Reagents to Wells:

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

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

Liquid Removal and Washes:

1. When instructed in the experimental procedures to remove liquid reagents (Antigen, Primary Antibody and Secondary Antibody), use the appropriately labeled transfer pipet designated for each row.



Wear gloves and safety goggles

2. To wash the wells, do the following:

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

   The capacity of each well is approximately 0.2 ml. Do not allow the liquids to spill over into adjacent wells.

   B. Remove all the PBS from each of the wells with the transfer pipet designated for each row.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Antigen:

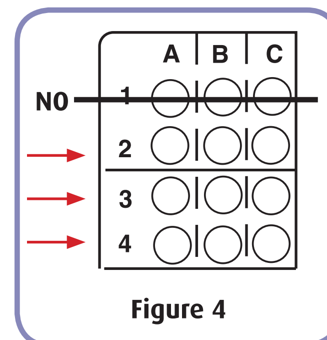


Figure 4

1. Add 50 µl or 3 drops of Antigen (Ag) to all the wells in Rows 2, 3, and 4. Do not add antigen to the wells in Row 1 (Figure 4).
2. Incubate for 5 minutes at room temperature.
3. Remove all the liquid with the transfer pipet labeled "Ag".
4. Wash all 12 wells once with PBS buffer as described in the previous section "Liquid Removal and Washes". If stopping at this point, leave PBS in wells - see OPTIONAL STOPPING POINT, below. If continuing with experiment, remove PBS from each well using the transfer pipet designated for each row.

OPTIONAL STOPPING POINT:
The experiment can be stopped after step 4, but requires that PBS be left in 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 5.

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

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Student Experimental Procedures, continued

Primary Antibody:

5. Add 50 µl or 3 drops of the primary antibody (1°Ab) to all the wells in Rows 1, 2, and 4. Do not add Primary Antibody to the wells in Row 3 (Figure 5).

6. Incubate for 5 minutes at room temperature.

7. Remove all the liquid using the transfer pipet designated for each row.

8. Wash each well once with PBS buffer as described in the section "Liquid Removal and Washes" on page 8. Remove PBS from each well using the transfer pipet designated for each row.

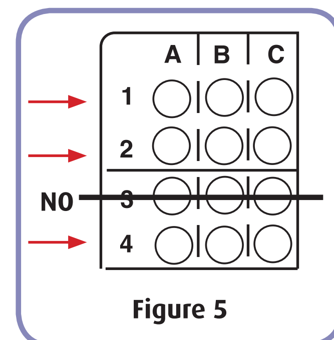

Figure 5

Secondary Antibody:

9. Add 50 µl or 3 drops of Secondary Antibody (2°Ab) to all the wells in Rows 1, 2, 3, and 4.

10. Incubate for 5 minutes at room temperature.

11. Remove all the liquid using the transfer pipet designated for each row.

12. Wash each well once with PBS buffer as described in the section "Liquid Removal and Washes" on page 8. Remove PBS from each well using the transfer pipet designated for each row.

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).

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).

Substrate:

13. Add 0.1 ml or 5 drops of the substrate S1 to each of the wells in rows 1 and 2 (Figure 6).

14. Add 0.1 ml or 5 drops of substrate S2 to each of the wells in rows 3 and 4 (Figure 6).

15. Incubate for 5 minutes at room temperature.

16. Remove the plate for analysis.

17. If color is not fully developed after 5 minutes, incubate for a longer period of time.

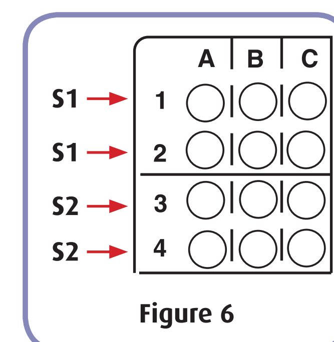

Figure 6

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Study Questions

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

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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 60 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.

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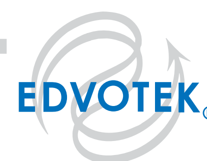


Pre-Lab Preparations

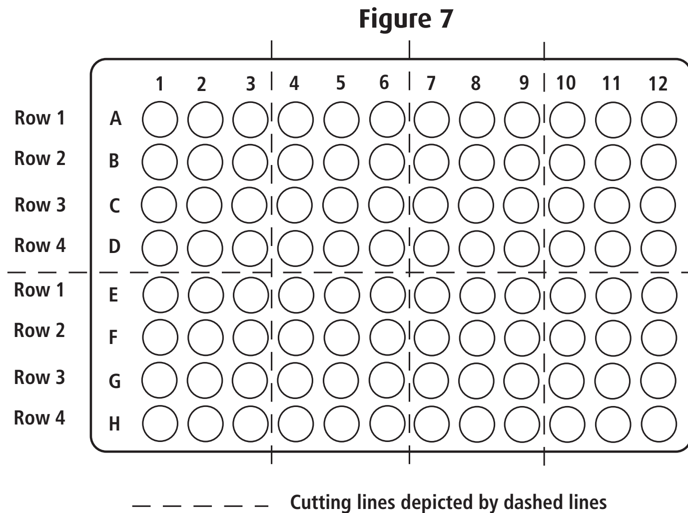
PREPARATIONS BEFORE THE LAB

Microtiter Plates

1. As shown in Figure 7, orient the microtiter plates so that numbers 1-12 are at the top and letters A-H are on the left.

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

Figure 7



PREPARATIONS ON THE DAY OF THE LAB

Dispensing Components A and B:

1. Use a FRESH 1 ml pipet for dispensing the Antigen (A) directly from the component tubes provided in this experiment kit. Label 10 microtest tubes "Ag" and dispense 0.6 ml per tube.

2. Use a FRESH 1 ml pipet for dispensing the Primary Antibody (B) directly from the component tubes provided. Label 10 microtest tubes "1°Ab" and dispense 0.6 ml to each tube.

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 12 ml into 10 small beakers for each lab group.

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

Preparation of Anti-IgG Peroxidase Conjugate (Secondary Antibody)
(Prepare on same day as needed for the experiment.)

1. Add 0.3 ml of diluted Phosphate Buffered Saline (PBS) to the concentrated Anti-IgG peroxidase conjugate (C). Mix thoroughly by tapping and inverting the tube.

2. Transfer 6 ml of diluted Phosphate Buffered Saline (PBS) to one of the 15 ml plastic tubes provided.

3. Transfer the entire contents of Tube C prepared in step 1 to the 15 ml tube containing 6 ml of PBS prepared in step 2. Label the tube "2°Ab" (Secondary Antibody). Mix.

4. Dispense 0.6 ml of the diluted Anti-IgG peroxidase conjugate for each group.

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Pre-Lab Preparations

PREPARATIONS ON THE DAY OF THE LAB

Preparation of Peroxidase Substrate
(Prepare DURING the lab experiment, 15 - 30 minutes before the last incubation.)

1. Dispense 9 ml of diluted Phosphate buffered saline (PBS) to the second 15 ml tube provided.
2. Add Peroxide co-substrate (E) to the 9 ml of PBS. Cap and mix thoroughly by shaking and/or vortexing. There is usually undissolved material remaining.
3. Add 1 ml of Hydrogen peroxide (D). Cap and mix.
4. Dispense 0.75 ml peroxidase substrate (S1) for each of the 10 groups.

Dispensing Component F

1. Dispense 0.75 ml ABTS substrate (S2) for each of the 10 groups.

QUICK REFERENCE:

Substrate 1 is prepared for the peroxidase enzyme, which is attached to the anti-IgG peroxidase conjugate (secondary antibody).

Prepare the substrate 15-30 minutes before students require it for plate development (last incubation).

PREPARATION OF EXPERIMENT REAGENTS

Component		*Label*	*Dispense for each group*
A*	Antigen	Ag	0.6 ml
B*	Positive control	1°Ab	0.6 ml
PBS + C	Anti-IgG-peroxidase-conjugate	2°Ab	0.6 ml
PBS + D + E	Peroxidase-enzyme substrate**	S1	0.75 ml
F	ABTS Substrate	S2	0.75 ml
G + water	Phosphate Buffered Saline	PBS	12 ml

* *Components A - B 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.*

** *Peroxidase-enzyme substrate should be prepared 15-20 minutes before the last incubation.*

STUDENT MATERIALS
Each Lab Group Should Receive:

1 Microtiter section
1 Tube labeled "Ag"
1 Tube labeled "1°Ab"
1 Tube labeled "2°Ab"
1 Automatic micropipet with tips (optional)
10 Transfer pipets
1 Beaker or tube containing PBS
1 Empty beaker labeled "waste"
1 Tube labeled "S1" (just before the last incubation)
1 Tube labeled "S2" (just before the last incubation)

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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 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

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.

		A	B	C
missing Ag	1			
	2			
missing 1Ab	3			
	4			

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