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RECENTLY UPDATED!

Edvo-Kit # 267

Edvo-Kit #267

Single Antibody Diagnostics

Experiment Objective:

This experiment introduces a rapid and sensitive one antibody procedure for Enzyme Linked Immunosorbent Assays (ELISA).

See page 3 for storage instructions.

Version 267.190513

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

Experiment Components

COMPONENTS **Upon receipt, store components A-E in the refrigerator.**		Check (√)
A	10X ELISA Wash Buffer	❑
B	ELISA Dilution Buffer	❑
C	Antibody (lyophilized)	❑
D	ABTS (lyophilized)	❑
E	ABTS Reaction Buffer	❑

Experiment #267 is designed for 10 lab groups.

REAGENTS & SUPPLIES **Store all components below at room temperature.**	Check (√)
• Microtiter plates	❑
• Transfer pipets	❑
• Microtest tubes with attached caps	❑
• Plastic tubes	❑

Requirements *(not included with this kit)*

- Distilled or deionized water
- 15 mL Conical tubes
- Beakers
- Paper towels
- Disposable lab gloves
- Safety goggles
- Automatic micropipettes (0-50 µL) and tips (OPTIONAL)

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.

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.

Background Information

Organisms are incredibly complex, consisting of an elaborate mixture of proteins, carbohydrates, and nucleic acids. In addition, different organisms, and even different cells within an organism, can contain an entirely distinct mixture of these compounds. Because of this, examining the function of one specific protein can become extremely challenging.

Fortunately, scientists have developed tools to measure the presence of a desired protein in a complicated mixture. One of the most common of these is the Enzyme-Linked ImmunoSorbent Assay, or ELISA. The ELISA uses antibodies to determine if a specific protein is present in a sample. ELISAs are commonly performed to test patient samples, monitor environmental specimens, and to ensure food and drug safety.

ANTIBODIES – THE FOUNDATION OF THE ELISA

The most important components in any ELISA are the specific antibodies that will be used to detect the target protein. Antibodies are specialized proteins consisting of four linked polypeptide chains: two identical "heavy chains" and two identical "light chains" that form a "Y" shaped molecule (Figure 1). The primary function of antibodies is to allow the immune system to distinguish between "self" and "non-self" proteins or polysaccharides. Every antibody will selectively bind to a unique target, known as an antigen, using sites at the ends of the short arms of the "Y". The amino acid sequence in this region is variable, allowing for each antibody to recognize a unique epitope (a particular location within an antigen).

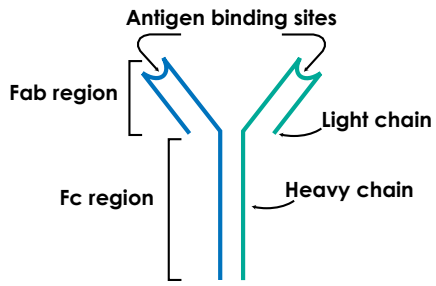


Figure 1:
General Structure of an antibody.

To create the antibodies used in scientific research, scientists inject animals (i.e. rabbits, goats, and guinea pigs) with the antigen of interest. In response to the injection, the animal's immune cells create antibodies that recognize different epitopes of the antigen. Blood is recovered from the immunized animal and the antibody-containing serum is separated from the blood cells.

Because of their specificity, researchers can use antibodies to detect the presence of unique biomolecules (i.e. peptides, proteins, antigens and hormones) in a complex sample. Most ELISAs use a combination of two antibodies. First, a primary antibody is selected to detect the target protein. These antibodies are very specific and are designed to only bind to a single protein. Next, a secondary antibody is chosen to target the primary antibody. For example, if the primary antibody is made from rabbit immune cells, a secondary antibody will be selected to identify rabbit proteins.

In addition to targeting the primary antibody, the secondary antibody is also covalently linked to a Horseradish Peroxidase (HRP) enzyme. This enzyme can oxidize certain substrates to produce a visible color change, which is used to indicate the presence of our target protein. For example, HRP can react with the substrate ABTS ((2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)). In the presence of hydrogen peroxide, the HRP enzyme will oxidize ABTS, turning the clear solution a vivid blue-green. HRP has a high catalytic activity – the substrate turnover rates exceed 10^6 per second – allowing us to quickly detect even the smallest amount of antigen present within the sample.

THE ELISA PROCEDURE

In the laboratory, ELISAs are performed in transparent plastic microtiter plates. Scientists add the test samples and controls to the wells of the plastic plate, where they non-specifically stick to the wells through hydrophobic and electrostatic interactions (Figure 2). This means that any protein in the sample, and not just the desired target, can stick to the plastic. Next, the primary antibody is added to the wells, and the mixture is allowed to incubate for a short time. This antibody specifically recognizes and binds to the target molecule (Figure 2). Therefore, if any target protein is present in the microtiter wells it will be recognized by the primary antibody.

Following a brief incubation period, the wells are washed to remove any primary antibody that did not bind with the antigen. After the wash, an HRP-linked secondary antibody is added to the wells where it recognizes and binds to the primary antibody (Figure 2). The excess secondary antibody is removed from the wells by washing several times with buffer. However, if the secondary antibody has bound to the primary antibody it will stay in the well.

Finally, a substrate solution of ABTS and hydrogen peroxide is added to each well. The HRP enzyme linked to the secondary antibody can oxidize ABTS in wells where the antigen-antibody complex is present, turning the clear substrate solution blue-green.

Importantly, the enzyme-substrate reaction can only occur if every step of the ELISA was successful. If the target protein is not present in the sample solution, or if the concentration is too low, the primary and secondary antibodies will not have anything to bind to and will be washed out of the well. Similarly, even if the antigen is present it will not be detected if the wrong primary antibody is used. In each of these scenarios, there will be no HRP enzyme available and the ABTS substrate will remain colorless.

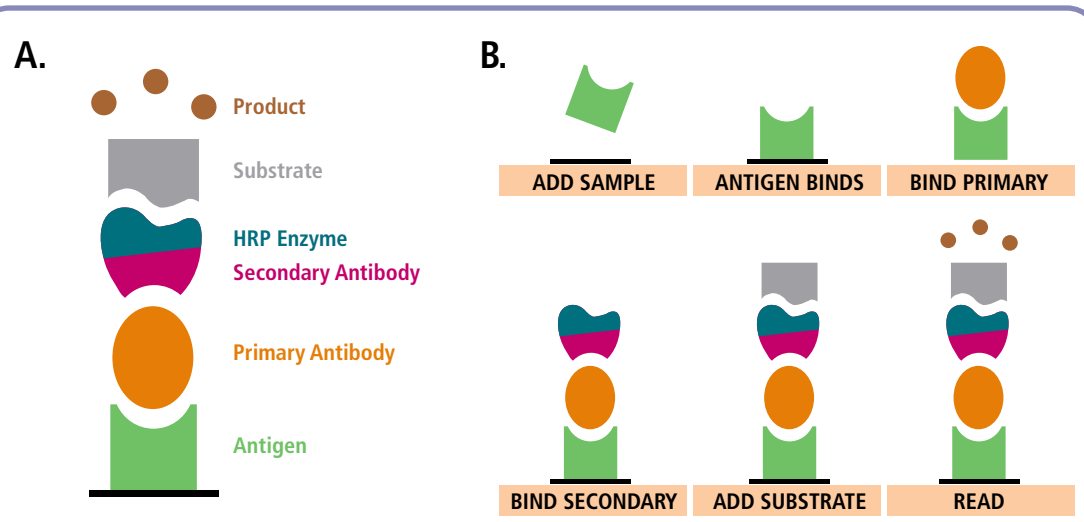


Figure 2: Optimized ELISA workflow

SINGLE ANTIBODY ELISA DETECTION

While most ELISA reactions use both primary and secondary antibodies, it is much faster and economical to use a single-antibody ELISA. In this type of reaction, the primary antibody is already coupled to HRP, eliminating the need for a second antibody. Instead, the substrate is added right after the primary antibody washes. Single-antibody ELISA detection reactions can be less specific and lead to higher background than the traditional double-antibody ELISA. However, they are less prone to error because there are fewer steps, and it takes significantly less time. Single-antibody ELISA detection is commonly used to identify markers of infection such as antibody levels in the blood or viral levels.

This experiment demonstrates the use of a rapid and sensitive one antibody ELISA to detect a unique protein in patient samples. This is a direct ELISA where one antibody is used to which the enzyme is bound. The microtiter wells are pre-treated with the antigen. After the simulated patient serum sample is added to the wells, washed, and substrate is added, the conversion of the substrate to product results in the color formation for positive samples.

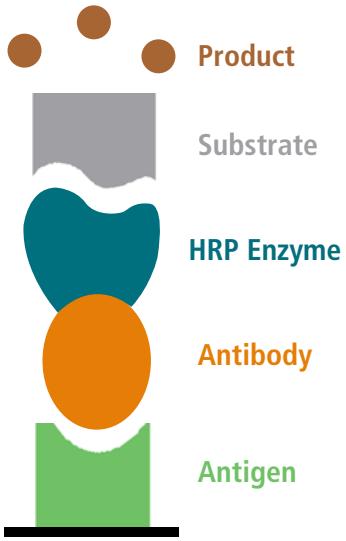


Figure 3:
Single antibody ELISA

Experiment Overview

EXPERIMENT OBJECTIVE

This experiment introduces a rapid and sensitive one antibody procedure for Enzyme Linked Immunosorbent Assays (ELISA).

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.

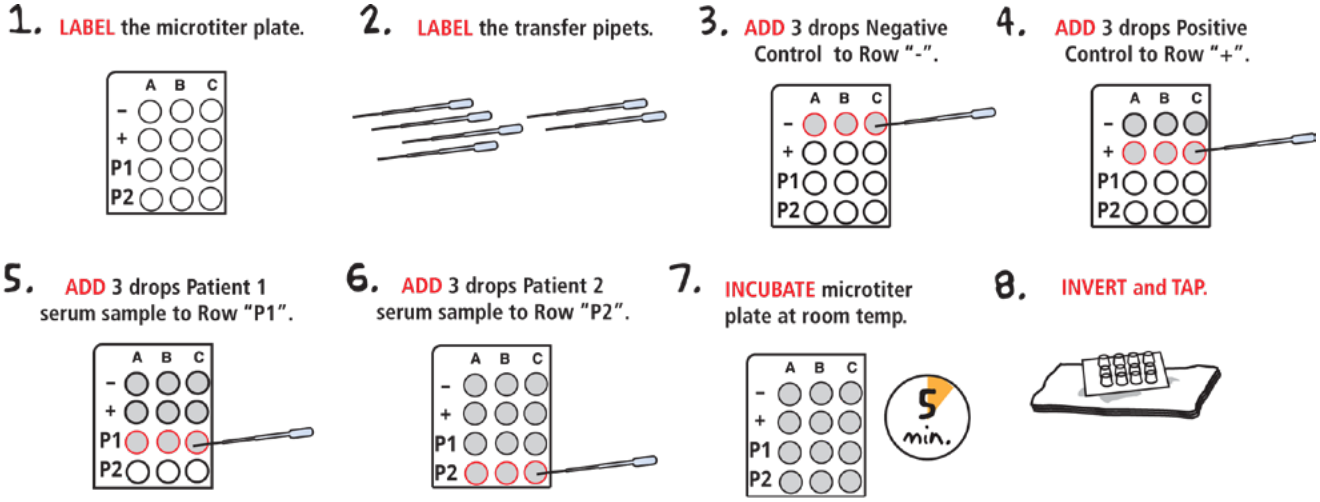
After the Experiment:

- 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.

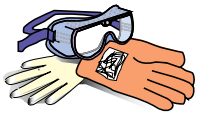
AVOIDING COMMON PITFALLS

1. Be very careful when transferring solutions into the microtiter plate wells.
2. Use only appropriately labeled transfer pipets to avoid contamination. Alternatively, if using automatic micropipets, use fresh tips for each step.
3. Wash the wells gently and slowly, without force. DO NOT allow the buffer to spill over into adjacent wells.
4. Empty the microtiter wells by carefully inverting onto stacks of paper towels and gently tapping.

Student Experimental Procedures



PERFORMING THE ELISA



Wear gloves and safety goggles

This is a direct ELISA where one antibody is used to which the enzyme is bound. The microtiter wells are pre-treated with the antigen. After the simulated patient serum sample is added to the wells, washed, and substrate is added, the conversion of the substrate to product results in the color formation for positive samples.

1. **LABEL** the wells of the microtiter plate as shown and add your initials or lab group number.

2. **LABEL** the transfer pipets as outlined in the box, below. These 7 pipets will be used to add liquid to the wells. ***If using an automatic micropipette, you must use a fresh tip for every step.***

(–)	Negative Control		
(+)	Positive Control	(W)	Wash Buffer
(P1)	Patient 1 serum sample	(Sub)	Substrate
(P2)	Patient 2 serum sample		

3. Using the "–" transfer pipet, **ADD** 3 drops or 50 µL of Negative Control to all of the wells in Row "–".

4. Using the "+" transfer pipet, **ADD** 3 drops or 50 µL of Positive Control to all of the wells in Row "+".

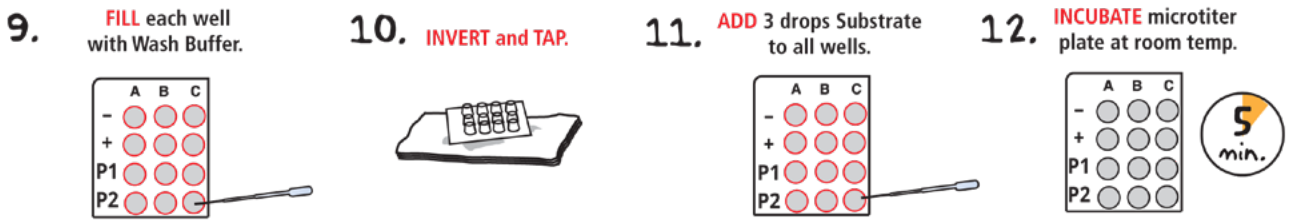
5. Using the "P1" transfer pipet, **ADD** 3 drops or 50 µL of Patient 1 serum sample to all of the wells in Row "P1".

6. Using the "P2" transfer pipet, **ADD** 3 drops or 50 µL of Patient 2 serum sample to all of the wells in Row "P2".

7. **INCUBATE** the microtiter plate for 5 minutes at room temperature.

8. **INVERT** the plate over a stack of paper towels to remove the samples. Gently **TAP** the plate 4-5 times onto a fresh paper towel. **DISCARD** the wet paper towels.

Student Experimental Procedures, continued



9. 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.***

10. **INVERT** the plate over a stack of paper towels to remove the wash buffer. Gently **TAP** the plate 4-5 times onto a fresh paper towel. **DISCARD** the wet paper towels.

11. Using the "Sub" transfer pipet, **ADD** 3 drops or 50 µL of the Substrate to all the wells.

12. **INCUBATE** the microtiter plate for 5 minutes at room temperature.

13. **ANALYZE** the plate. If no color is developed after 5 minutes, it can be incubated 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 advantages/disadvantages to doing a single-antibody ELISA as opposed to a traditional two-antibody ELISA?
2. What would be the effect of not including the antigen or the 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

What to do:	When:	Time Required:
Prepare 1X wash buffer	Anytime before the lab.	5 min.
Prepare antibody	Can be prepared the day before the lab, but best to do it the day of the lab.	5 min.
Prepare ABTS	Up to 1 week before the lab.	30 min.
Aliquot reagents	Up to 1 week before the lab.	30 min.

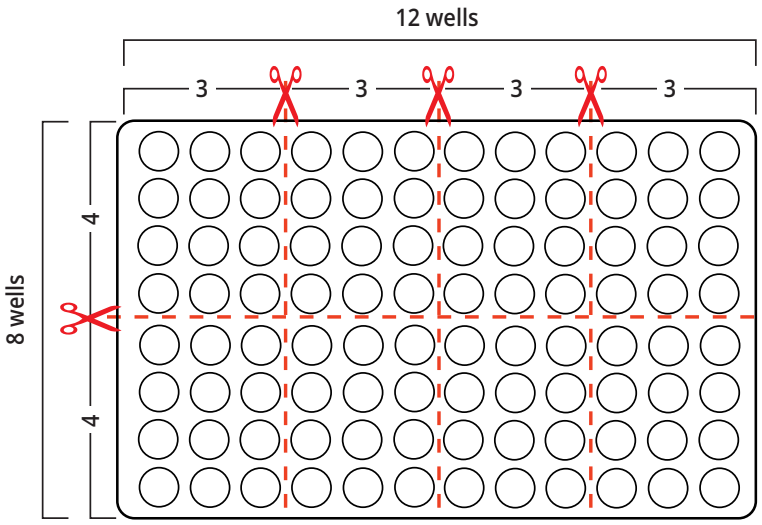
Yellow = Prepare shortly before module. Green = Flexible / prepare up to a week before the module.

Red = Prepare immediately before module.

PRELAB PREPARATIONS

Preparing the Microtiter Plate

Carefully cut and divide the 8 x 12 well microtiter plate to create EIGHT 4 x 3 well pieces (as shown in the figure to the right). Each piece will contain 3 wells on one axis and 4 wells on the other axis. Each group will receive one piece.



Preparing the Wash Buffer

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

Pre-Lab Preparations

Preparing the Patient and Control Samples

1. Transfer 7 mL of ELISA Dilution Buffer (Component B) to a 15 mL conical tube labeled "Positive".
2. Remove 0.5 mL of ELISA Dilution Buffer from the conical tube above and add to the vial of Antibody (Component C).
3. Carefully mix to dissolve.
4. Transfer the entire volume of the Antibody vial back to the conical tube from step 1 and mix.
5. Aliquot 200 µL of "Positive" per group and label "Positive Control".
6. Aliquot another 200 µL of "Positive" per group and label "Patient 2".
7. Aliquot 200 µL of ELISA Dilution Buffer (Component B) per group and label "Patient 1".
8. Aliquot 200 µL of ELISA Dilution Buffer (Component B) per group and label "Negative Control".

Preparing the Substrate

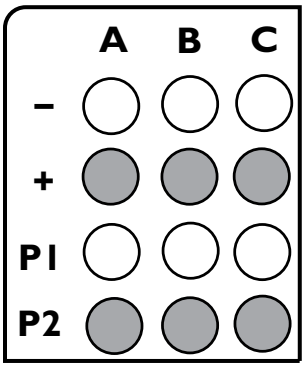
1. Transfer 7 mL of ABTS Reaction Buffer (Component E) into a 15 mL conical tube labeled "Substrate"
2. Remove 0.5 mL of ABTS Reaction Buffer from the conical tube above and add to vial of ABTS (Component D).
3. Carefully mix to dissolve.
4. Transfer the entire volume of the ABTS vial back to the conical tube from step 1 and mix.
5. Aliquot 650 µL per group and label "Substrate".

Each Lab Student Group Should Receive:

- 1 Microtiter plate (3 x 4 well)
- 7 Transfer pipets or an Automatic micropipette with tips
- 1 Tube labeled "-" for negative control
- 1 Tube labeled "+" for positive control
- 1 Tube labeled "Patient 1"
- 1 Tube labeled "Patient 2"
- 1 Tube labeled "Substrate"
- 1 Beaker or tube containing Wash Buffer
- 1 Empty beaker labeled "waste"
- • Paper towels

Experiment Results and Analysis

- Color should appear only in Rows 2 and 4.
- Row 1 is the negative control "-".
- Row 2 is the positive control "+".
- Row 3 is the negative patient sample "P1".
- Row 4 is the positive patient sample "P2".



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