The Biotechnology Education Company®

EDVOTEK®

269

EDVO-Kit #

Introduction to ELISA Reactions

Storage:
Some components require refrigerator storage. See page 3 for details.

EXPERIMENT OBJECTIVES:

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

Table of Contents

Experiment Components

A Antigens
B Primary 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

- 2 Microtiter plates
- Transfer pipets
- Microtest tubes with attached caps
- 15 ml plastic tubes

This experiment is designed for 10 groups.

Upon receipt, refrigerate Components A-G.

None of the components have been prepared from human sources.

Requirements

- Distilled or deionized water
- Beakers
- 37°C Incubation oven
- 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.

All 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.

Introduction to ELISA Reactions

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.

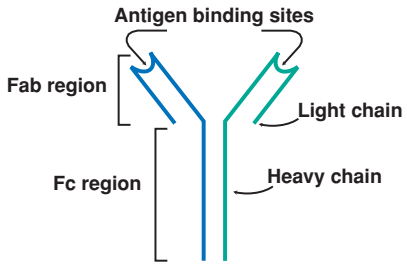


Figure 1:
General Structure of an antibody

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.

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.

Introduction to ELISA Reactions

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.

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.

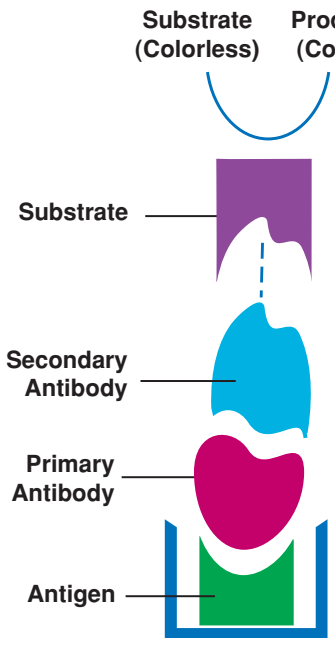


Figure 2:
Schematic of the ELISA Reaction

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.

Introduction to ELISA Reactions

Background Information

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.

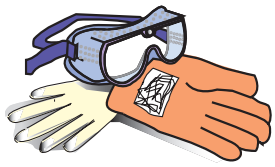
It should be noted that polyclonal antibody preparations to a given antigen can have variable binding affinities due to differences in the immunological responses between animals. Different immunizations with the same antigen in animals can also produce antibodies with variable binding affinities. The 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.

Experiment Overview

EXPERIMENT OBJECTIVE:

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



Wear gloves and safety goggles

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.

General Instructions and Procedures

Experiment Procedures

Equilibrate a 37°C incubation oven before starting the experiment.

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 re-label 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.

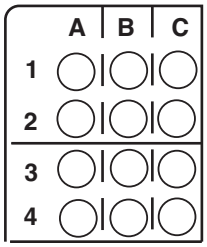


Figure 3

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)
- Sub 1 (Substrate 1)
- Sub 2 (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 9.

General Instructions and Procedures

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

INSTRUCTIONS FOR ADDING LIQUIDS AND WASHING WELLS

Adding Reagents to wells:

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

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.

WEAR SAFETY GOGGLES AND GLOVES

EXPERIMENTAL STEPS FOR THE ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Antigen:

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" on page 9. If continuing with experiment, remove PBS from each well using the transfer pipet designated for each row.

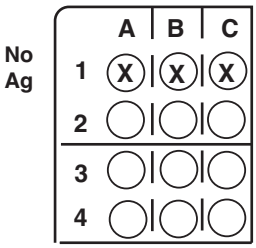


Figure 4

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.

Experiment Procedures

General Instructions and Procedures

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.

REMINDERS:

ADDING REAGENTS:

Be sure to use a fresh tip for the addition of 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 the liquid into 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).

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 37°C.
7. Remove all the liquid using the transfer pipet designated for each row.

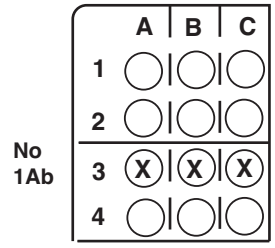


Figure 5

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.

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 37°C.
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.

General Instructions and Procedures

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

15. Incubate for 5 minutes at 37°C.

16. Remove the plate for analysis.

17. If color is not fully developed after 5 minutes, incubate at 37°C for a longer period of time.

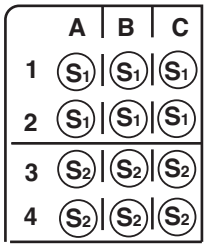


Figure 6

Edvotek 269 "Introduction to ELISA" – study questions

Name:

1. What does the acronym ELISA mean?

2. Of the different types of biological macromolecules, which ones most commonly serve as antigens to which antibodies will bind?

3. Describe the roles of the 1^{o} Antibody and the 2^{o} Antibody during an ELISA test.

4. Diagram a well containing all components necessary for a positive ELISA result.

5. After the addition of the 2^{o} Antibody, how did you determine which wells contain the Antigen bound to 1^{o} Antibody bound to 2^{o} Antibody?

6. Describe how you could use ELISA to determine if the human and cow versions of a specific protein (e.g. the blood protein albumin) have a similar epitope.