EXPERIMENT OBJECTIVE:

The objective of this experiment is to perform and master the experimental concepts and methodology involved with enzyme linked immunosorbent (ELISA) assays. This ELISA experiment is designed to detect circulating IgG directed towards two antigens. Observations in this experiment include specificity of antibodies, the effect of dilution on ELISA reactions, color development and quantitation.
# Quantitative ELISA

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Safety Data Sheets can be found on our website:  
www.edvotek.com/safety-data-sheets
This experiment is designed for 6 groups.

Upon receipt, store the perishable components (A-I) in the refrigerator.

None of the components have been prepared from human sources.

Requirements

- Distilled or deionized water
- Beakers or flasks
- 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 (Cat. #632) 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.

THIS EXPERIMENT DOES NOT CONTAIN HUMAN DNA. None of the experiment components are derived from human sources.
Antibodies are specific human and animal proteins that are produced by white blood cells in response to foreign material. Examples of such foreign material, known as antigens, include infectious agents and various environmental "non-self" materials. Biological antigens are high molecular weight biomolecules such as proteins, carbohydrates and nucleic acids which can be circulating freely or as part of a complex such as part of a virus coat or bacterial cell surface. Antibodies are made in response to antigens. They bind to antigens and play a significant role in the subsequent removal of such materials from circulation. For example, exposure to an infectious agent causes the individual to mount an antibody response which eventually results in plasma antibody molecules that bind to different viral proteins (and/or different areas of the same polypeptide).

When an antibody binds to a specific biological antigen, it can recognize specific chemical charges, sequences or structural conformational elements. These structural binding characteristics make up the specific fingerprint for an antigen. Each antibody molecule can bind two antigen molecules. This recognition and binding is highly specific and makes possible the differentiation between two circulating viruses that may be very closely related, as in the case of two strains of the same virus.

When an antigen and its antibodies form insoluble complexes, this highly specific binding reaction is known as immunoprecipitation. Precipitation of the complex is the result of various polyclonal antibodies binding to the antigens to form a network. In the traditional immunoprecipitation assay, antibodies are obtained from the serum of an animal exposed to the specific antigen. The serum, also known as plasma, is prepared by the removal of red blood cells. It contains the specific proteins for that particular animal and antibodies against a "non-self" antigen that is introduced in the animal by either design or an infection. Antibodies are purified from animal sera samples and can be used to detect particular antigens, such as human infectious agents.

Description of the Immunological Screening Test

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. 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, the antigens are added to the wells where some remain adsorbed by hydrophobic association to the walls after washing away the excess. The antigens can be the whole infecting agent, such as a virus or lysate, specific 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 gelatin, milk proteins or bovine serum albumin.

In this experiment, positive samples will have antibodies that will bind to the preadsorbed antigens in wells. If the primary antibody has remained in a well, then the secondary antibody will bind to it and also remain attached after washing. These secondary antibodies are usually raised in rabbits and goats immunized with "non-self" IgG fractions. The second IgG antibodies are purified and covalently cross-linked to horseradish peroxidase. This modification does not significantly affect the binding specificity and affinity of the antibody or the enzymatic activity of the peroxidase.
Background Information

After washing, a solution containing hydrogen peroxide and aminosalicylate 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. 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.

The substrate solution added is nearly colorless. Peroxidase converts the peroxide to $\text{H}_2\text{O} + \text{O}_2$ using the salicylate as the hydrogen donor. The oxidized salicylate is brown and can be easily observed in positive wells.

Figure 1 illustrated the ELISA assay. 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 variable binding affinities. The use of monoclonal antibodies directed against a single epitope eliminates this variability. Western blot analysis of positive samples can be used to confirm the presence and size of antibodies.

This ELISA experiment is designed to detect two separate circulating IgG molecules directed towards two distinct antigens. Observations in this experiment include specificity of antibodies, the effect of dilution on ELISA reactions, color development and quantitation.
Experiment Overview and General Instructions

EXPERIMENT OBJECTIVE:

The objective of this experiment is to perform and master the experimental concepts and methodology involved with enzyme linked immunosorbent (ELISA) assays. This ELISA experiment is designed to detect circulating IgG antibodies directed towards two antigens. Observations in this experiment include specificity of antibodies, the effect of dilution on ELISA reactions, color development and quantitation.

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 NOTEBOOK RECORDINGS:

Address and record the following in your laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Write a hypothesis that reflects the experiment.
- Predict experimental outcomes.

During the Experiment:

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

Following 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.
Experiment Overview and General Instructions

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

2. Place the microtiter plate as shown in the figure at right and carefully mark the plate with your initials or lab group number.

3. Using a permanent marker, label the columns 1-5 across the top and the rows A-H down the side.

4. As shown in the figure, draw lines across the plate between Rows B and C, Rows D and E, and Rows F and G. This will create four sections on the plate.

5. Label 4 large transfer pipets as follows (these are to be used for addition of reagents to the wells):
   - PBS for Phosphate Buffered Saline
   - Ag1 for Antigen 1
   - Ag2 for Antigen 2
   - block for blocking agent

6. Label 2 small transfer pipets Ag1 and Ag2. These are to be used for removal of liquid from the wells.

7. Proceed to dilution of Primary Antibodies 1 and 2 on pages 8 and 9.

CAUTION: To avoid cross-contamination and false results, use the appropriately labelled plastic transfer pipet for liquid removals and washes as outlined in the experimental procedures.
### Quantitative Enzyme Linked Immunosorbent Assay (ELISA)

**DILUTION OF PRIMARY ANTIBODIES 1 AND 2**

#### Primary Antibody 1

1. Label four tubes as follows: "Ab1 - 2". This is your 1:1600 dilution.
   "Ab1 - 3". This is your 1:6400 dilution.
   "Ab1 - 4". This is your 1:25,600 dilution.
   "Ab1 - 5". This is your 1:102,400 dilution.

2. Add 6 drops (330 µl) of PBS to each tube.

3. Obtain the “Ab1 - 1” tube from your instructor. This is your 1:400 dilution of Primary Antibody 1.

4. Use a fresh pipet tip or a fresh large transfer pipet to add 2 drops (110 µl) of “Ab1 - 1” Primary Antibody to the tube labeled “Ab1 - 2”. Pipet the solution up and down, cap the tube and mix well.

5. Use the same large transfer pipet to add 2 drops of “Ab1 - 2” to the tube labeled “Ab1 - 3”. Pipet the solution up and down, cap the tube and mix well.

6. Use the same large transfer pipet to add 2 drops of “Ab1 - 3” to the tube labeled “Ab1 - 4”. Pipet the solution up and down, cap the tube and mix well.

7. Use the same large transfer pipet to add 2 drops of “Ab1 - 4” to the tube labeled “Ab1 - 5”. Pipet the solution up and down, cap the tube and mix well.

See NOTE at bottom of page 9 for storage of Ab1 or just store diluted Ab1 in the refrigerator until needed.

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**NOTE:**

If using a micropipet for diluting the antibodies, dilute the 1:400 antibody solution 1:4 by combining 110 µl 1:400 antibody with 330 µl diluted PBS. Continue diluting the antibody 4-fold, up to 1:102,400.
Quantitative Enzyme Linked Immunosorbent Assay (ELISA)

Primary Antibody 2

1. Label four tubes as follows: “Ab2 - 2”. This is your 1:1600 dilution. “Ab2 - 3”. This is your 1:6400 dilution. “Ab2 - 4”. This is your 1:25,600 dilution. “Ab2 - 5”. This is your 1:102,400 dilution.

2. Add 6 drops (330 µl) of PBS to each tube.

3. Obtain the “Ab2 - 1” tube from your instructor. This is your 1:400 dilution of Primary Antibody 2.

4. Use a fresh pipet tip or a fresh large transfer pipet to add 2 drops (110 µl) of “Ab2 - 1” Primary Antibody to the tube labeled “Ab2 - 2”. Pipet the solution up and down, cap the tube and mix well.

9. Use the same large transfer pipet to add 2 drops of “Ab2 - 2” to the tube labeled “Ab2 - 3”. Pipet the solution up and down, cap the tube and mix well.

6. Use the same large transfer pipet to add 2 drops of “Ab2 - 3” to the tube labeled “Ab2 - 4”. Pipet the solution up and down, cap the tube and mix well.

7. Use the same large transfer pipet to add 2 drops of “Ab2 - 4” to the tube labeled “Ab2 - 5”. Pipet the solution up and down, cap the tube and mix well.

NOTE: Keep the Primary Antibodies 1 and 2 and their dilutions in the refrigerator until needed.
Experiment

The Enzyme Linked Immunosorbent Assay (ELISA)

1. Orient the microtiter plate so the wells labeled as columns 1-5 are on the horizontal axis and the wells labeled as rows A-H are on the vertical axis (as shown in the figure at left).

2. Read through the instructions and study the figure on the previous page before beginning to add antigens to the wells. Refer to the figure often during the experimental procedure to avoid making errors.

ADDITION OF ANTIGENS:

3. Using the appropriately labelled large transfer pipet or fresh pipet tip for each reagent, add reagents to the wells as described below.
   - Add 50 µl or 1 drop of Antigen 1 to the wells 1 - 5 in rows A, B, C and D.
   - Add 50 µl or 1 drop of Antigen 2 to the wells 1 - 5 in rows E, F, G and H.

4. Incubate the plate at room temperature for 5 minutes.

LIQUID REMOVAL OF ANTIGENS:

In the steps which follow, use the appropriately labelled small transfer pipets to remove liquid from the wells. It is important to follow directions carefully to avoid cross-contamination of the wells. Save the pipets for later steps.

5. Using the small pipet labelled "Ag1", remove the liquid from wells in rows A - D.

6. Using the small pipet labelled "Ag 2", remove the liquid from wells in rows E - H.

PBS WASH AND LIQUID REMOVAL OF PBS

7. Using the large transfer pipet labelled "PBS" add Phosphate Buffered Saline to wells 1 - 5 in all the rows. Fill until each well is almost full. If using an automatic micropipet, add 200 µl of PBS to each of the wells.

8. Remove the PBS from the wells using the same procedures outlined in steps 5 and 6.
Quantitative Enzyme Linked Immunosorbent Assay (ELISA)

**ADDITION OF BLOCKING AGENT**

9. Using a fresh pipet tip or the large transfer pipet labelled “block”, add 50 µl or 1 drop of “block” (blocking agent) to each of the wells in columns 1 - 5 in all rows (exclude wells in column 6).

10. Incubate the plate for 10 minutes at 37° C.

11. Using the small pipet labelled “Ag1”, remove the liquid from wells 1 - 5 in rows A - D.

12. Using the small pipet labelled “Ag 2”, remove the liquid from wells 1 -5 in rows E - H.

**PBS WASH AND LIQUID REMOVAL OF BLOCKING AGENT**

13. Using the large pipet labelled “PBS” add Phosphate Buffered Saline to all the wells. Fill until each well is almost full. If using a micropipet, add 200 µl of PBS to each of the wells.

14. Remove the PBS from the wells using the same procedures outlined in steps 11 and 12. Discard the small pipets when finished.

**OPTIONAL STOPPING POINT:**

The experiment can be stopped after addition of PBS (step 13) and resumed during next lab period. Cover microtiter plates with parafilm or plastic wrap and refrigerate overnight.
**Quantitative Enzyme Linked Immunosorbent Assay (ELISA)**

**ADDITION OF PRIMARY ANTIBODIES 1 & 2 (PREPARED ON PAGES 8 & 9)**

1. Label 3 large transfer pipets "PBS", "Ab1", and "Ab2". Use a fresh pipet tip or the appropriately labelled large transfer pipet for addition of PBS and each antibody.

2. Add 50 µl or 1 drop of PBS to the wells in rows C and E.

3. Add 50 µl or 1 drop of "Ab1 - 5" to wells A5, B5, and F5.

4. Add 50 µl or 1 drop of "Ab1 - 4" to wells A4, B4, and F4.

5. Add 50 µl or 1 drop of "Ab1 - 3" to wells A3, B3, and F3.

6. Add 50 µl or 1 drop of "Ab1 - 2" to wells A2, B2, and F2.

7. Add 50 µl or 1 drop of "Ab1 - 1" to wells A1, B1, and F1.

8. Add 50 µl or 1 drop of "Ab2 - 5" to wells D5, G5, and H5.

9. Add 50 µl or 1 drop of "Ab2 - 4" to wells D4, G4, and H4.

10. Add 50 µl or 1 drop of "Ab2 - 3" to wells D3, G3, and H3.

11. Add 50 µl or 1 drop of "Ab2 - 2" to wells D2, G2, and H2.

12. Add 50 µl or 1 drop of "Ab2 - 1" to wells D1, G1, and H1.

13. Incubate the plate at 37° C for 30 minutes.

**LIQUID REMOVAL OF ANTIBODIES:**

14. Label 4 small transfer pipets “AB”, “CD”, “EF”, and “GH”.

15. Starting with the most dilute antibody (column 5) in rows A and B, use the pipet labelled “AB” to remove the liquid from the wells moving from right to left (i.e. remove in order A5, B5, A4, B4, A3, B3, A2, B2, A1, B1).

16. Repeat the procedure with the other rows (C-H) using the appropriately labelled transfer pipets.
Quantitative Enzyme Linked Immunosorbent Assay (ELISA)

PBS WASH AND LIQUID REMOVAL OF PBS
17. Using the large pipet labelled “PBS” add Phosphate Buffered Saline to all the wells. Fill until each well is almost full. If using a micropipet, add 200 µl to each well.

18. Remove the PBS from the wells using the same procedures outlined in steps 2 and 3. Discard transfer pipets.

ADDITION OF SECONDARY ANTIBODY
19. Use a fresh pipet tip or a large transfer pipet to add 50 µl or 1 drop of Secondary Antibody to each of the wells in rows A-H (all 40 wells).

20. Incubate the plate at 37° C for 15 minutes.

LIQUID REMOVAL OF SECONDARY ANTIBODIES
21. Label 4 small transfer pipets “AB”, “CD”, “EF”, and “GH”.

22. Starting with the most dilute antibody (column 5) in rows A and B, use the pipet labelled “AB” to remove the liquid from the wells moving from right to left (i.e. remove, in order A5, B5, A4, B4, A3, B3, A2, B2, A1, B1).

23. Repeat the procedure with the other rows (C-H) using the appropriately labeled transfer pipets.

PBS WASH AND LIQUID REMOVAL OF PBS
24. Using the large pipet labelled “PBS” add Phosphate Buffered Saline to all the wells. Fill until each well is almost full. If using a micropipet, add 200 µl to each well.

25. Remove the PBS from the wells using the same procedures outlined in steps 22 and 23.

ADDITION OF SUBSTRATE
26. Start with column 5 and move to the left - use a fresh pipet tip or a fresh large transfer pipet to add 50 µl or 1 drop of Substrate to 40 wells (exclude wells in column 6).

27. Incubate at room temperature for 5 minutes.

28. Periodically observe the plate for color development.

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

Answer the following study questions in your laboratory notebook or on a separate worksheet.

1. To what do antibodies respond?
2. Can an antibody act as an antigen?
3. Describe the ELISA antigen antibody reactions.
4. Is a positive result always visualized as a brown color in the ELISA assay?
Instructor’s Guide

Notes to the Instructor & Pre-Lab Preparations

Class size, length of laboratory sessions, and availability of equipment are factors which must be considered in planning and implementing 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. 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).

APPROXIMATE TIME REQUIREMENTS FOR PRE-LAB AND EXPERIMENTAL PROCEDURES

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

• The student experimental activity requires approximately 2 hours with optional stopping points available.
Pre-Lab Preparations

PREPARATIONS BEFORE THE LAB

**Microtiter Plates**

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

2. Cut each plate along the solid lines as shown in the figure. Each piece will be 6 wells on one axis and 8 wells on the other axis. Each lab group will receive one piece containing 48 wells.

PREPARATIONS ON THE DAY OF THE LAB

**Preparation of Phosphate Buffered Saline**

1. Add all of the Phosphate Buffered Saline concentrate (comp. H) to 360 ml of distilled water. Mix.

2. Label this diluted Phosphate Buffered saline as “PBS”.

3. Dispense 50 ml into small beakers for each of the 6 lab groups.

**Antigens 1 and 2**

1. Label 6 tubes “Ag1” and “Ag2” and dispense 1.5 ml Antigen 1 (comp. A) into the tubes labeled “Ag1” and 1.5 ml Antigen 2 (comp. B) into the tubes labeled “Ag2”.

2. Distribute one tube of “Ag1” and “Ag2” each per group.

**Blocking Agent**

1. If the Blocking agent has gelled, place the bottle in a 37° C waterbath or oven to melt the gelatin.

2. Label 6 larger test tubes “block” and dispense 3.0 ml Gelatin Blocking Agent (comp. F) into the tubes.

3. Distribute one tube of Blocking agent per group.

**NOTES:**

The Phosphate Buffered saline may precipitate or crystalize. Warm the buffer in a 37° C water bath before using and check that there is no precipitate.

The blocking buffer will likely precipitate during storage. Warm @ 37° C for 5-10 minutes or until the precipitate has dissolved.
Pre-Lab Preparations

Primary Antibodies 1 and 2 (1:400)

1. Add 0.3 ml diluted PBS to tube C. Mix well and transfer the entire contents to a larger tube containing 5.7 ml diluted PBS. This is 1:400 Primary Antibody 1. Dispense 0.5 ml diluted Primary Antibody 1 (comp. C) into 6 tubes labeled “Ab1 - 1”.

2. Distribute one tube of “Ab1 - 1” per group.

3. Add 0.3 ml diluted PBS to tube D. Mix well and transfer the entire contents to a larger tube containing 5.7 ml diluted PBS. This is 1:400 Primary Antibody 2. Dispense 0.5 ml diluted Primary Antibody 2 (comp. D) into 6 tubes labeled “Ab2 - 1”.

4. Distribute one tube of “Ab2 - 1” per group.

Preparation of Secondary Antibody

1. Add 0.3 ml diluted PBS to the tube containing Secondary Antibody (comp. E). Add 18 ml diluted PBS into the 50 ml conical tube provided and transfer the entire contents of tube E into the PBS. Cap the tube and mix. Dispense 3.0 ml diluted secondary antibody into 6 tubes labeled “2°Ab”.

2. Distribute one tube of “2°Ab” per group.

Preparation of Peroxidase Substrate During the Lab Experiment

Prepare 15 - 30 minutes before the last incubation:

1. Dispense 16 ml of diluted Phosphate buffered saline (PBS) to the second 50 ml tube provided.

2. Add all of the Aminosalicylic acid (I) to the 16 ml of PBS. Cap and mix thoroughly by shaking and/or vortexing. There is usually undissolved material remaining.

3. Then add 1.8 ml of Hydrogen peroxide (G). Cap and mix.

4. Dispense 2.5 ml of the peroxidase substrate for each group.

5. Keep refrigerated in the dark until use.
## Pre-Lab Preparations

### PREPARATION OF EXPERIMENT REAGENTS

<table>
<thead>
<tr>
<th>Label</th>
<th>Dispense for each group</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Ag1 1.5 ml</td>
</tr>
<tr>
<td>B</td>
<td>Ag2 1.5 ml</td>
</tr>
<tr>
<td>C + PBS</td>
<td>Ab1 - 1 0.5 ml</td>
</tr>
<tr>
<td>D + PBS</td>
<td>Ab2 - 1 0.5 ml</td>
</tr>
<tr>
<td>E + PBS</td>
<td>2°Ab 3.0 ml</td>
</tr>
<tr>
<td>G + I + PBS</td>
<td>Substrate 2.5 ml</td>
</tr>
<tr>
<td>H + dH₂O</td>
<td>PBS 50 ml</td>
</tr>
<tr>
<td>F</td>
<td>block 3.0 ml</td>
</tr>
</tbody>
</table>

* Components A, B, C, D, E, F can be dispensed before the actual day of the lab and stored in the refrigerator.

### STUDENT MATERIALS

Each Lab Group Should Receive:

- 1 half piece of microtiter plate
- 1 tube labeled "Ag1"
- 1 tube labeled "Ag2"
- 1 tube labeled "block"
- 1 tube labeled "Ab1 - 1"
- 1 tube labeled "Ab2 - 1"
- 1 tube labeled "2°Ab"
- 1 automatic micropipet with tips (optional)
- 11 Transfer pipets (large)
- 11 Transfer pipet (small)
- 1 beaker containing PBS
- 1 empty beaker labeled "waste"
- 1 tube labeled "Substrate" (just before the last incubation)
- 8 microcentrifuge tubes

### 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.
Expected Results

The idealized schematic at right shows representative results. Actual results may vary.

<table>
<thead>
<tr>
<th></th>
<th>1:400</th>
<th>1:1600</th>
<th>1:6400</th>
<th>1:25,600</th>
<th>1:102,400</th>
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<tr>
<td>A</td>
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Please refer to the kit insert for the Answers to Study Questions