Edvo-Kit # 270

Edvo-Kit #270

Antigen-Antibody Interaction: The Ouchterlony Procedure

Experiment Objective:

The objective of this experiment is to introduce the principles of antigen-antibody interactions using the Ouchterlony procedure.

See page 3 for storage instructions.

Version 270.050418

SAMPLE LITERATURE
Please refer to included weblink for correct version

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Experiment Components

Components		Storage	Check (✓)
A	Antiserum (antibody)	Refrigerator	❑
B	Whole serum (antigen)	Refrigerator	❑
C	Albumin (antigen)	Refrigerator	❑
D	IgG (antigen)	Refrigerator	❑
E	Powdered buffer	Room Temp.	❑

Experiment #270 contains enough reagents for 10 lab groups.

REAGENTS & SUPPLIES

Store all components below at room temperature.

- UltraSpec-Agarose™ ❑
- Practice loading solution ❑
- Transfer pipets ❑
- Petri plates ❑
- Well cutters ❑
- Template for cutting wells ❑
- Microtest tubess ❑

Experiment Requirements *(NOT included in this experiment)*

- Micropipette and tips
- Plastic container or Pyrex baking dish
- Plastic wrap
- Distilled Water
- Pipets - 5 mL or 10 mL
- Marking pen
- Measuring spatula or toothpicks
- Heat plate, Bunsen burner, or microwave
- Paper towels
- Water bath

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. None of the experiment components are derived from human sources..

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Background Information

The interactions of an antibody (Ab) with an antigen (Ag) is the fundamental reaction of immunology.

$$Ab + Ag \rightarrow Ab - Ag$$

Most antigens are proteins. The exact identity of the groups that react with the antibody are usually not known. Macromolecular antigens and antibodies form complexes that become insoluble and precipitate from solution. This property makes it possible to perform qualitative and quantitative assays on the antibody-antigen system.

Precipitation occurs with most antigens because the antigen is multivalent, i.e., has several antigenic determinants per molecule to which antibodies can bind. Antibodies have at least two antigen binding sites, thus large aggregates or lattices of antigen and antibody are formed. Experimentally, an increasing amount of antigen is added to a constant amount of antibody in solution. Initially at low antigen concentration, all of the antigen is contained in the precipitate. This is called the antibody-excess zone. As more antigen is added, the amount of protein precipitated increases until the antigen and antibody molecules are at an optimal ratio. This is called the equivalence zone, or equivalence point, where maximum precipitation occurs. When the amount of antigen in solution exceeds the amount of antibody, the amount of precipitation will decrease. This is known as the antigen-excess zone (Figures 1 and 2).

When antibodies and antigens are inserted into different areas of an agarose gel, they diffuse toward each other and form opaque bands of precipitate at the interface of their diffusion fronts. Precipitation reactions of antibodies and antigens in agarose gels provide a method of analyzing various antibody-antigen reactions.

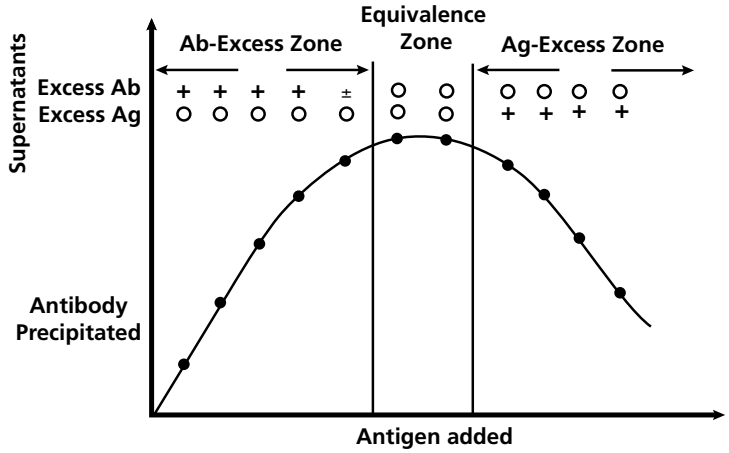
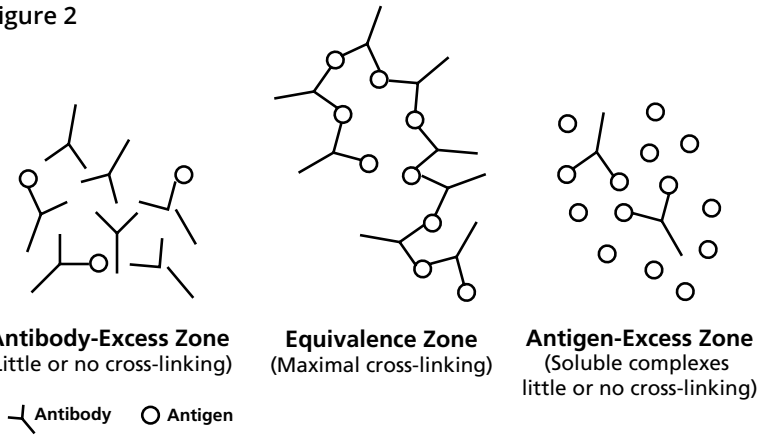


Figure 1

Figure 2



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THE OUCHTERLONY PROCEDURE

Double diffusion in two dimensions is a simple procedure invented by and named after the Swedish scientist, Örjan Ouchterlony. Antigen and antibody solutions are placed in separate wells cut in an agarose plate. The reactants diffuse from the wells toward each other and precipitate where they meet at equivalent proportions. A single antigen will combine with its homologous antibody to form a single precipitation line. When two antigens are present, each behaves independently of each other. Thus, the number of precipitin bands indicates there are at least that many antibody-antigen pairs present (see Figure 3). Arrows indicate diffusion patterns of antigens and antibodies.

Double diffusion in two dimensions is a useful technique for comparing antigens for the number of identical or cross-reacting determinants. If a solution of antigen is placed in two adjacent wells and the homologous antibody is placed in the center well, the two precipitin bands that form will join at their closest ends and fuse. This is known as a reaction of identity (Figure 4).

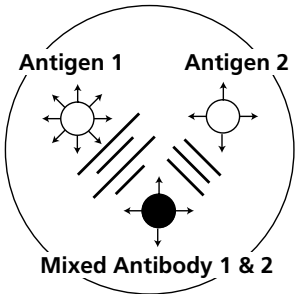

Figure 3

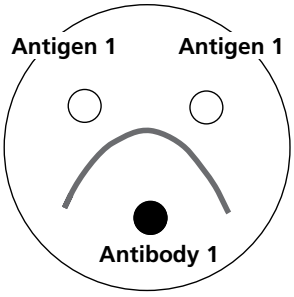

Figure 4:
Reaction of Identity

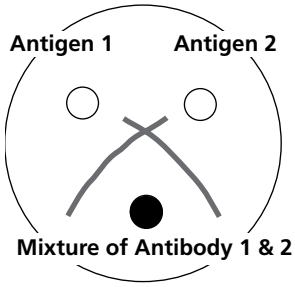

Figure 5
Reaction of Non-identity

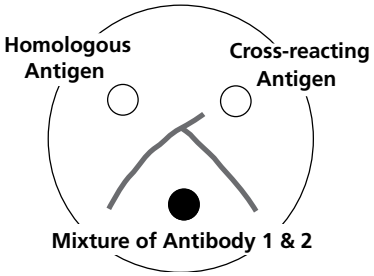

Figure 6
Reaction of Partial Identity

When unrelated antigens are placed in adjacent wells and the center well is filled with antibodies for each antigen, the precipitin bands will form independently of each other and will cross. This is known as a reaction of non-identity (Figure 5).

If two purified antigens cross-react, then placing them in adjacent peripheral wells with antibody to one in the central well will give a single band with the homologous and cross-reacting antigen. Since the cross-reacting antigen lacks some of the antigenic determinants present in the homologous antigen, it is not able to precipitate all of the antibody. The remaining antibody will diffuse beyond the line of cross-reacting precipitate to react with the homologous antigen to produce a spur. The spur that forms projects toward the antigen with the fewer determinants, i.e., the cross-reacting antigen. This is called a reaction of partial identity. Since these non-cross-reacting antibodies often are only a fraction of the total antibody involved in the homologous precipitin reaction, the spur is usually less dense (often difficult to visualize) than the precipitin band from which it projects (See Figure 7).

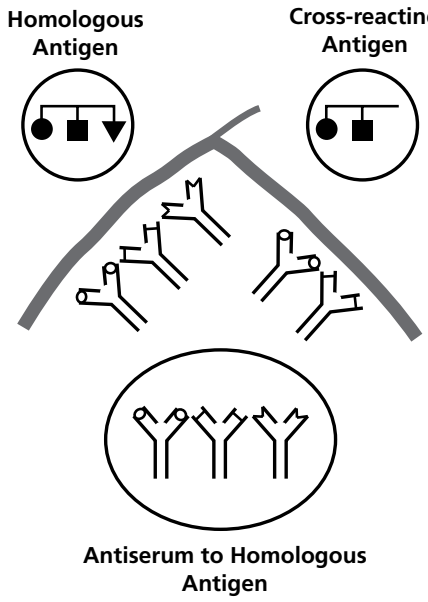

Figure 7

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Experiment Overview

EXPERIMENT OBJECTIVE:

The objective of this experiment is to introduce the principles of antigen-antibody interactions using the Ouchterlony procedure.

LABORATORY SAFETY

1. Gloves and goggles should be worn routinely as good laboratory practice.
2. Exercise extreme caution when working with equipment that is used in conjunction with the heating and/or melting of reagents.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
4. Exercise caution when using any electrical equipment in the laboratory.
5. Always wash hands thoroughly with soap and water after handling reagents or biological materials in the laboratory.

Wear gloves and safety goggles

LABORATORY NOTEBOOKS

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.

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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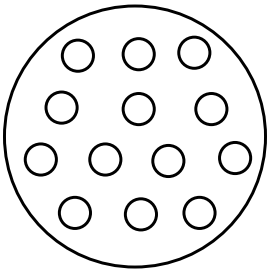
Student Experimental Procedures

A. PREPARATION OF AGAROSE AND POURING OF OUCHTERLONY PLATES

1. Each group requires 4 plates: 1 practice loading plate and 3 experimental plates. Using a 5 mL or a 10 mL pipet, carefully pipet 5 mL of the cooled agarose (55°C) into each plate, rotating the plate to cover the bottom with agarose. Repeat with the remaining plates.
2. If the molten agarose contains bubbles, gently swirl to remove the bubbles.
3. Allow the agarose to solidify. This will take approximately 10-15 minutes, at which time the gel will appear slightly opaque.
4. If the plates are not to be used that day, the plates can be wrapped with plastic wrap and stored inverted in the refrigerator for two weeks.

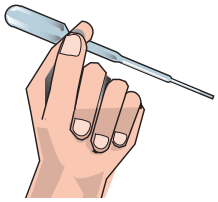
B. PRACTICE WELL LOADING (OPTIONAL)



This experiment contains practice loading solution. This solution is included to allow instructors and students to practice loading the sample wells before performing the actual experiment . Use a micropipetting device, or one of the plastic transfer pipets included in your experiment kit to practice loading the sample wells with the practice loading solution.

1. One practice plate should be prepared for each group. Enough reagents have been provided for this purpose.
2. Using the well cutters provided, cut several rows of wells as shown in the diagram at left.
3. Practice loading the sample wells with the plastic, disposable transfer pipets. (See "Sample Loading of Wells with Transfer Pipets").
4. If you are using an automatic micropipetting device, the amount of sample that should be loaded is 30 microliters.

Sample Loading of Wells With Transfer Pipets



1. Squeeze the pipet stem, not the bulb, to slowly draw a portion of the sample up into the pipet. The sample should remain in the lower portion of the pipet.

   If the sample is overdrawn and becomes lodged in the bulb or on the walls, tap until the sample moves down into the lower stem of the pipet. Eject it back into the tube. Try step 1 again.
2. While holding the pipet tip above the tube, **slowly** squeeze the pipet stem until the sample is nearly at the opening of the pipet tip.
3. Place the pipet tip just over, **not** inside, the sample well. Maintain steady pressure on the pipet stem to prevent sample from being drawn back up into the pipet.
4. **Slowly** squeeze the pipet bulb to eject two (2) drops of sample. The well should appear full, but be careful not to overfill the wells and cause spillage on the agarose surface. Put any remaining sample in the pipet back into the tube.

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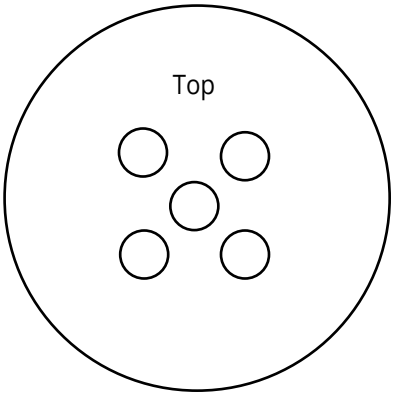
Student Experimental Procedures, continued

C. PREPARATION OF SAMPLE WELLS



Template

1. Make several copies of the template (at left) for your lab group.
2. Place the template under one of the plates so that the pattern is in the center of the plate. The distances between the wells is important. Try to follow the template as accurately as possible.
3. Cut the five wells using the well cutter (provided in the kit) in a gentle punching motion. Remove the agarose plugs with a flat-edged toothpick or spatula.
4. If well placement is not accurate, there should be enough room on the plate to re-cut the wells using the template.
5. Repeat steps 2 and 3 with the remaining two plates.

D. LOADING THE SAMPLES

1. Orient your lab number or group designation at the top before loading samples.
2. Using the same pipet, fill the center wells of all three plates with 30 microliters (2 drops with a transfer pipet) of antiserum (antibody) from Tube A. Wells should appear full, but be careful not to overfill the wells and cause spillage on the agarose surface. This may affect your results.
3. Fill the outer wells with 30 microliters of antigen using a clean pipet tip for each antigen as follows:

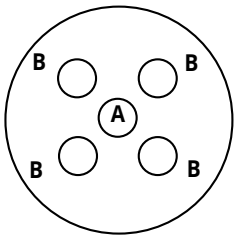


Plate 1 Center well: antiserum to the fluid containing antibodies (Tube A)
Left upper well: Whole serum (Tube B)
Right upper well: Whole serum (Tube B)
Left lower well: Whole serum (Tube B)
Right lower well: Whole serum (Tube B)

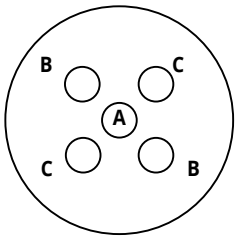


Plate 2 Center well: antiserum to the fluid containing antibodies (Tube A)
Left upper well: Whole serum (Tube B)
Right upper well: albumin (Tube C)
Left lower well: albumin (Tube C)
Right lower well: Whole serum (Tube B)

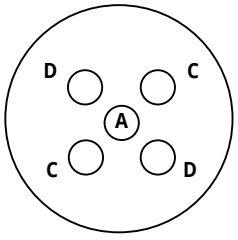


Plate 3 Center well: antiserum to the fluid containing antibodies (Tube A)
Left upper well: IgG (Tube D)
Right upper well: albumin (Tube C)
Left lower well: albumin (Tube C)
Right lower well: IgG (Tube D)

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Student Experimental Procedures, continued

E. INCUBATION

Replace lids onto plates. Carefully place the covered plates in the incubation chamber on top of the wet paper towel layer. Do not invert the plates. Cover the chamber with plastic wrap and let incubate at room temperature 24-48 hours to allow precipitin lines to form or the chamber can be placed in a 37°C incubation oven.

F. READING THE RESULTS

The precipitin lines will be visible in 24-48 hours. Carefully hold a plate up so that the overhead room lights shine through it. You should be able to see opaque white arcs in each side of the plate where the antibody and antigen precipitated. A drawing of the results should be made.

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Study Questions

1. Explain how qualitative observations can be performed on the antigen-antibody system.

2. What is the equivalence zone or equivalence point?

3. When would you observe the antigen-excess zone? What effect does this have on the amount of precipitation?

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Instructor's Guide

NOTES TO THE INSTRUCTOR

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. In addition, Technical Service is available from 8:00 am to 5:30 pm, Eastern time zone. Call for help from our knowledgeable technical staff at 1-800-EDVOTEK (1-800-338-6835).

APPROXIMATE TIME REQUIREMENTS

Your individual schedule and time requirements will determine when the Ouchterlony plates should be prepared. It takes approximately 20 to 30 minutes to prepare the plates (generally 10 minutes of this time is required for solidification). Students can prepare the plates, if time allows.

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Pre-Lab Preparations

A. PREPARATION OF AGAROSE AND POURING OF OUCHTERLONY PLATES

1. In a 500 mL Erlenmeyer flask or beaker, add the entire contents of powdered buffer package (Component E) to 225 mL of distilled water. Swirl the flask or beaker to dissolve the powder.

2. Add the entire contents of the agarose package to the flask or beaker. Swirl to disperse large clumps. With a marking pen, indicate the level of solution volume on the outside of the flask or beaker.

3. You must boil the solution to dissolve the agarose. This can be accomplished with a hot plate or Bunsen burner. Cover the beaker with foil. Heat the mixture to boiling over a burner with occasional swirling. Wear safety goggles and a hot glove. Boil the mixture until all the gelatinous agarose is dissolved. During heating, occasionally remove the beaker from the heat and check to see that there are no small, clear particles of agarose. Continue heating with occasional swirling. The final solution should be clear.

   A microwave can also be used to melt the agarose (no foil cover) on high in 30 sec. pulses for 2 minutes if required. Swirl the flask in between pulses and microwave for an additional 1-2 minutes. Check to see that there are no small clear particles of agarose. The final solution should be clear.

4. If detectable evaporation has occurred, add distilled water to bring the solution up to the original volume as marked on the flask or beaker in step 2. Total volume should be a minimum of 230 mL.

5. Cool the agarose solution to 55°C in a water bath. Swirl to promote even dissipation of heat.

6. Aliquot 25 mL of the agarose solution (55°C) for each of the ten groups.

B. PREPARATION OF INCUBATION CHAMBER
(Prepare day of laboratory)

Line the bottom of a plastic container or glass container (such as a pyrex dish) with several paper towels. Soak the paper towels with distilled water. There should not be any layer of liquid above the paper towels. All liquid should be absorbed into the paper toweling. Cover the entire chamber with plastic wrap.

C. PREPARATION OF ANTIBODY AND ANTIGENS

Antibody (Ab) and the three antigens (Ag) have been supplied in bulk. It is recommended that the Ab and Ag are aliquoted for each group.

1. Label 10 microtest tubes "A".
2. Label 10 microtest tubes "B".
3. Label 10 microtest tubes "C".
4. Label 10 microtest tubes "D".
5. Aliquot 100 µL of Sample A into each tube "A".
6. Aliquot 200 µL of Sample B into each tube "B".
7. Aliquot 150 µL of Sample C into each tube "C".
8. Aliquot 80 µL of Sample D into each tube "D".
9. Each group requires one each of tube A, B, C, and D.
10. Aliquot 10 microtest tubes 100 µL of practice solution per group.

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Avoiding Common Pitfalls

1. Follow instructions carefully when preparing the gel for the plates. Make sure the agarose is completely dissolved.
2. Make neat, clean wells with the well cutters. Take measures to ensure that the wells are properly spaced according to the template on page 8.
3. Add samples to the wells carefully and precisely. Avoid overfilling the wells.
4. Do not tip or invert plates when transferring to the humidity chamber.
5. Placing the humidity chamber in a 37°C incubation oven will expedite the formation of precipitin arcs.
6. Absence of precipitin lines is usually due to disproportionate pipetting between Ag and Ab wells, or distance between Ag and Ab wells.
7. Spurs may be faint. IgG and albumin are immunologically non-identical components of whole serum. They are both recognized by different antibodies in the antiserum made against whole rabbit serum.

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Experiment Results and Analysis

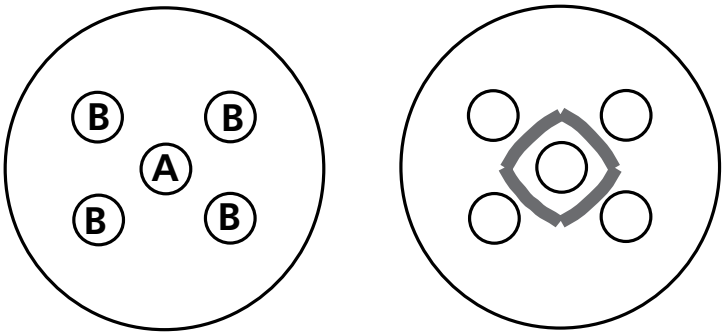

Plate 1: Reaction of Identity

Note the series of precipitation lines indicating many Ab-Ag systems may be visible.

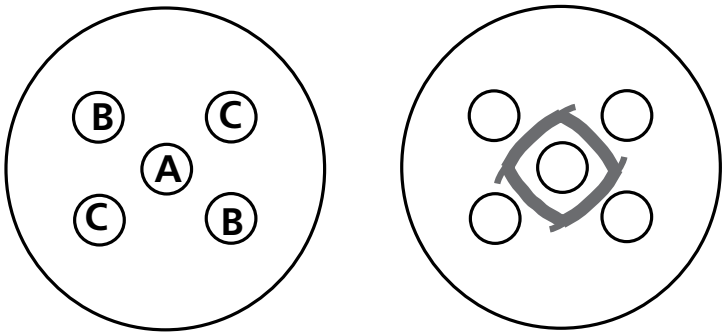

Plate 2: Reaction of Partial Identity

Spurs may be faint. Albumin is recognized by the antiserum in purified form or as a serum component. The antiserum also recognizes many other serum components.

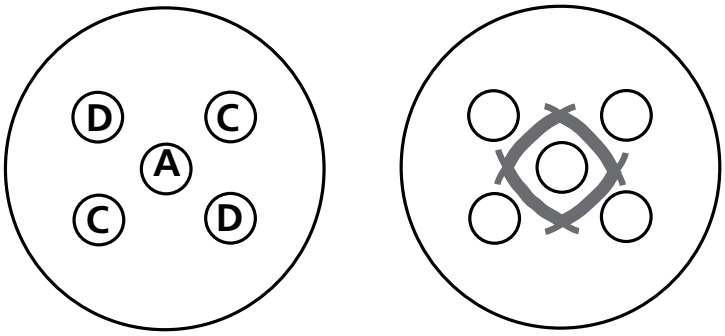

Plate 3: Reaction of Non-Identity

Spurs may be faint. IgG and albumin are immunologically non-identical components of whole serum. They are both recognized by different antibodies in the antiserum made against whole rabbit serum.

Technically, this is not a reaction of partial identity. It is a reaction of non-identity since albumin is in the serum too. The spur is all the other proteins reacting with the antiserum that antigenically have nothing in common with albumin.

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