INSTRUCTOR GUIDE

Experiment 801

NOTE: The procedure for antibody reaction described on page has recently been changed and the revised procedure follows.

I. Chemicals and Materials Provided

	Amount
Goat Serum	150µl
Sheep Serum	150µl
Horse Serum	150µl
Chicken Serum	150µl
Cow Serum	150µl
Cow Transferrin	150µl
Cow Albumin	150µl
Anti-cow Albumin	150µl
Anti-cow Gamma Globulins	150µl
Cow Gamma Globulins	150µl
Goat Anti-Rabbit IgG Peroxidase	150µl
Nitrocellulose	4 sheets
Blotting Paper	16 sheets
10 x Ponceau S (Protein Blot Stain)	20ml
30xTBS	30ml
30 x TBS + NP40	30ml
20 x Color Development Buffer	20ml
Chloronapthol	5ml
Hydrogen Peroxide	1ml
Plastic Dishes for Blot Incubation	4
Gelatin	6g

The serum proteins, albumin, transferrin, and gamma globulins are dissolved in electrophoresis sample buffer which contains glycerol and bromophenol blue. The compositions of the other solutions are described below.

II. Chemicals and Materials that are Needed but Not Provided

PROCELL Electrophoresis Unit and MB-170 Power Supply or equivalent Accessory Kit or equivalent

Water Bath

Agarose and Electrophoresis Buffer (Tris-Glycine, pH 8.6) from Electrophoresis Package 1/8. - To prepare the buffer, add the contents of one package to 3 liters of water.

III. Preparation of Solutions

TBS (Tris Buffer Saline) -The buffer is supplied as a 30-fold concentrate. To prepare the working buffer (0.15M NaCl, 10mM Tris, pH 8.0), add the 30ml of concentrate to 870ml of distilled or deionized water. The buffer may be stored at room temperature for a few weeks.

TBS + NP-40 (TBS + Nonidet-P40) -To prepare the working buffer (0.15MNaCl, 10mM Tris, pH 8.0, 0.05% NP40), add the 30ml of concentrate to 870ml of distilled or deionized water. The buffer may be stored at room temperature for a few weeks.

TBS + Gelatin - To prepare the solution, add the 6g of gelatin to 300ml of boiling TBS and stir until the gelatin is dissolved. Cool to room temperature before use. The solution may be stored for 1-2 days at room temperature or up to 3-4 days in the refrigerator.

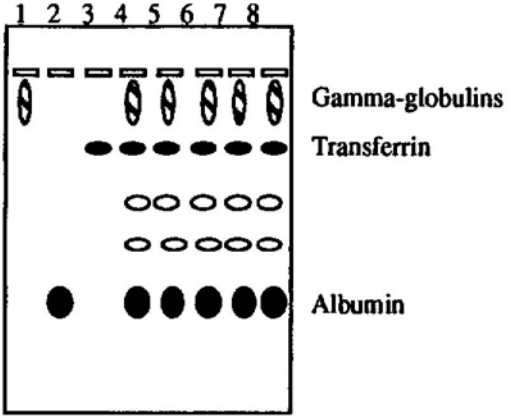
Color Development Solution - This solution is prepared immediately before use by adding the following to 130ml of distilled water:

1. 7ml of 20 x Color Development Buffer (This buffer contains Tris-HCl, pH8.0)
2. 5ml Chloronapthol (The chloronapthol is dissolved in methanol)
3. 0.5ml of Hydrogen Peroxide

Ponceau S - Protein Blot Stain- The stain is provided as a 10-fold concentrate in 5% acetic acid. To prepare the working stain, add 20ml of concentrate to 180ml of water. Do not mouth pipet this solution.

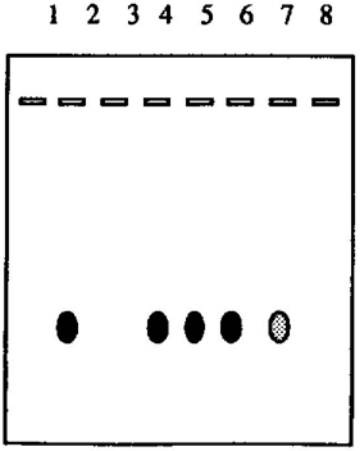
IV. Experimental Results *(in diagram form)*

Proteins Detected by
Protein Blot Stain
(page 25)

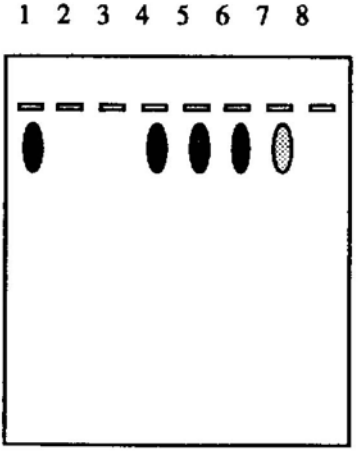


Proteins Detected by
Antibody Reactions
(pages 26 and 27)

BLOT INCUBATED WITH
ANTIBODY TO
ALBUMIN



BLOT INCUBATED WITH
ANTIBODY TO
GAMMA GLOBULINS



V. Data Analysis and Answers to Study Questions

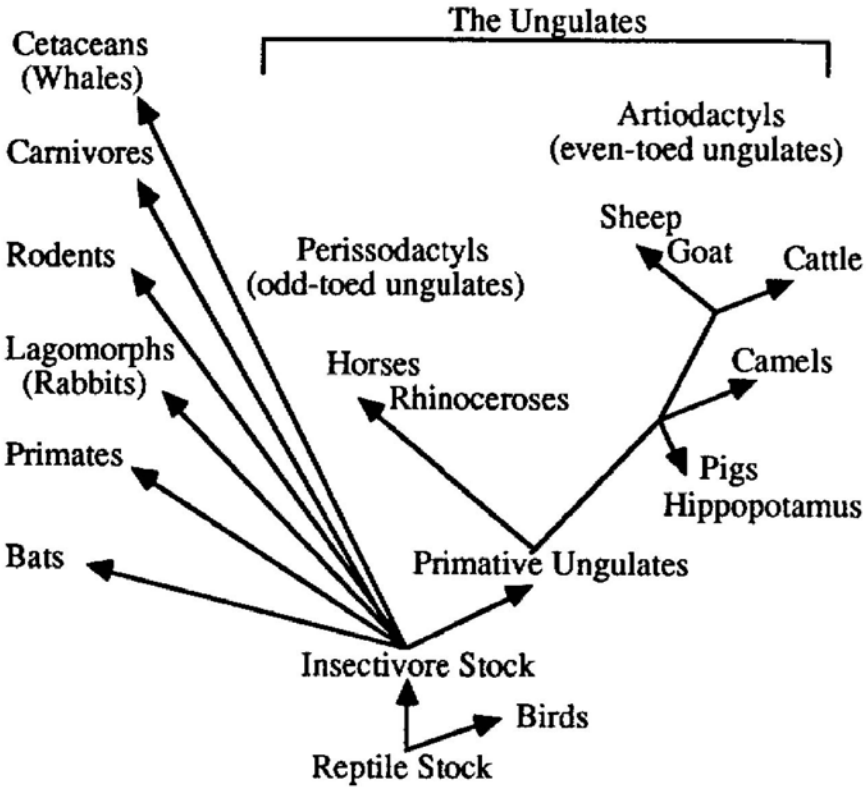
1. Diagrams are given on the previous page.

2. The relative reactions of both antibodies should follow the order: cow > sheep = goat > horse > chicken.

3. A diagram of a family tree of the mammals studied in this exercise is shown below. The results of the antibody analysis are in agreement with traditional taxonomic relationships as shown by this tree.

4. The antibody would probably react strongly with serum from chicken but not with sera from cow, goat, sheep, or horse.

A Family Tree of the Mammals



EXPERIMENT 1 (801)

Identification and Evolutionary Relationships of Serum Proteins by the Western Press-Blot Procedure

Background Information

The theory of organic evolution is based upon the belief that present-day organisms have descended with modifications from forms of life that lived in the past. Phylogeny is the evolutionary history of a species, genus or larger group and taxonomy is the science of classification of organisms according to the degree of kinship and evolutionary relationships. The doctrine of organic evolution is one of the most important generalizations in science. It is supported by evidence drawn from genetics, paleontology and geographical distribution and from comparative anatomy and embryology. Results from the modem biochemistry laboratory have also provided strong evidence for the doctrine of organic evolution and bave suggested possible mechanisms by which evolutionary changes occur.

Each protein carries in its amino acid sequence information pertaining to its own evolutionary history and origin, and clues to the evolutionary history of the organism in which it is found. Indeed, the millions of proteins existing today are, in effect, living fossils. Two of the methods that have been used to study the evolution of proteins at the molecular level are described below.

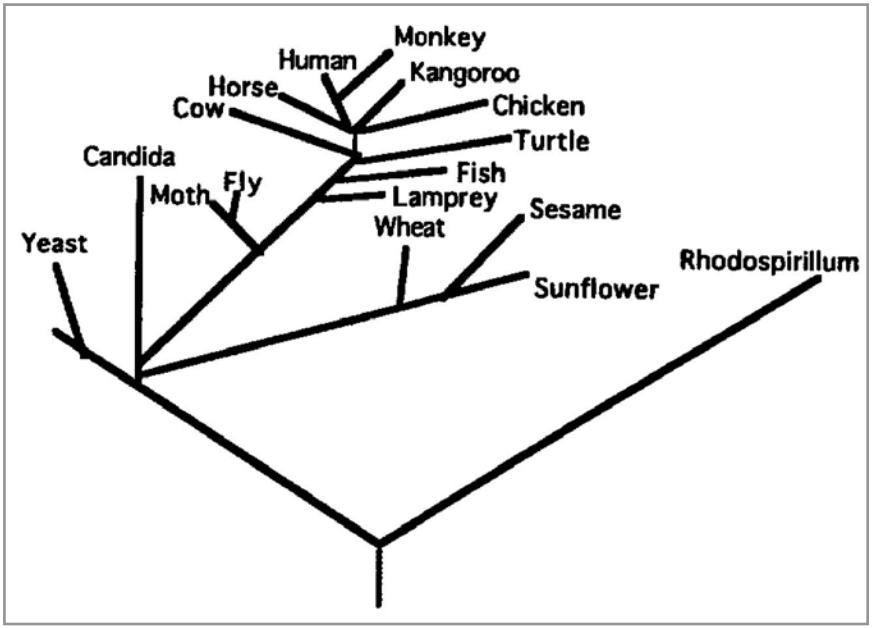
I. Protein Sequence

A comparison of the amino acid sequence of the same protein in different organisms has provided a direct way to study molecular evolution. A comparison of the amino acid sequence of cytochrome C from over 80 species has revealed that the amino acid sequence of this protein from different species varies and the degree of variation corresponds to the distance that separates two species on the evolutionary tree. That is, the greater the taxonomic difference, the more the cytochromes are likely to differ in their order of amino acid residues. For example, the cytochrome C molecules in men and chimpanzees contain 104 amino acid residues and the order of their amino acid residues is exactly the same. In contrast, the cytochrome C in man differs from the cytochrome C found in yeast in 44 out of the 104 amino acid residues. The numbers of amino acid replacements in cytochrome C of 12 species are compared in the table below. This type of information has led to the construction of family trees of organisms that agree remarkably well with those obtained from the classical anatomical record (See Figure 1-1). In fact, on a number of occasions, comparative protein studies have been used to clarify and expand on phylogenetic relationships that were derived from classical analysis.

Variations in the Amino Acid Sequence of Cytochrome C

Cytochromes compared to human cytochrome	Number of variant amino acid residues
Chimpanzee	0
Rhesus monkey	1
Kangaroo	10
Dog	11
Horse	12
Chicken	13
Rattlesnake	14
Tuna fish	21
Dogfish	23
Moth	31
Wheal	35
Neurospora	43
Yeast	44

Figure 1-1. A Family Tree of Organisms Based on the Amino Acid Sequence of Cytochrome C



II. Immunological Procedures

The immune system consists of a diverse set of cells, tissues and organs and about 1012 antibody molecules. The major function of the immune system is to protect the organism from viruses, bacteria, protozoans and larger parasites. When these organisms enter the vertebrate body, macromolecules on their surfaces induce the production of specific antibodies that appear in the serum of the infected animal. The antibodies, in turn, combine with these foreign macromolecules thereby rendering the invading organisms inactive and noninfective. Central to this protective mechanism is the ability of the immune system to distinguish between foreign macromolecules and those that are a natural part of the individual's body.

The macromolecules that elicit antibody production are called antigens and are most often proteinaceous in nature. Although antigens are frequently components of foreign organisms, purified foreign proteins will serve as antigens in that they will stimulate the formation of antibodies when injected into a suitable test animal such as rabbit. Each antigen possesses features that are recognized by the antibody and these features constitute the antigenic determinants or epitopes. An antigenic determinant recognized by an antibody molecule may be a unique shape or a sequence of about 5 to 10 amino acid residues on the protein molecule. It follows that each protein possess a large number of potential antigenic determinants and when a foreign protein is injected into an animal, antibodies to different determinants on the protein are produced and appear in the serum. This antibody containing serum is called an antiserum. Thus, an antiserum generated by immunization with one purified protein usually contains a large number of different antibodies which recognize different determinants along the protein.

Antibodies are frequently used to study evolutionary relationships because they recognize unique antigenic determinants along a protein molecule. For example, human serum albumin injected into a rabbit makes the animal produce anti-human albumin antibodies. These antibodies are directed against determinants of human albumin (shapes and/or amino acid sequence) that are not found on the rabbit albumin molecule. When the anti-albumin serum is mixed with human albumin, the antibodies in the serum react strongly with the albumin and an antibody-antigen complex is formed. Similarly, the antiserum will react strongly with albumin isolated from gorilla, orangutan and baboon. In contrast, the anti-human antiserum will react only weakly with albumin from deer, horse and ox and not at all with chicken or frog albumin.

In this experiment, you will be comparing the evolutionary relatedness of albumin and gamma globulins from various vertebrates. First, you will observe the patterns of electrophoretically separated proteins present in sera from cow, goat, sheep, horse, and chicken. You will then perform an immunological procedure known as Western Blotting. Through a series of steps (described below), this procedure enables the investigator to visualize proteins that react with a specific antiserum after the proteins have been separated by electrophoresis. Thus, the relatedness of the proteins in sera samples can be compared both in terms of the number of cross-reactive proteins and in terms of their electrophoretic mobilities.

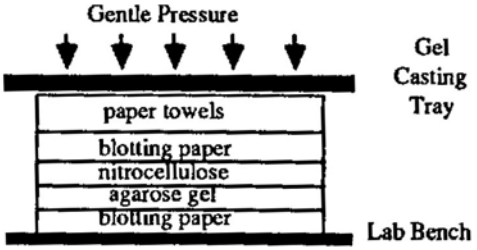
The first step in Western Blotting is to electrophoretically separate the serum protein samples in agarose gels. Next, the gel is placed against a specialized membrane made of nitrocellulose. The proteins are then forced out of the gel by applying gentle pressure and are trapped on the nitrocellulose membrane. As a result, a replica (blot) of the electrophoretically separated proteins is produced on the nitrocellulose membrane (see Figure 1-2).

Figure 1-2. Transfer Procedure for the Western Press-Blot

1. Run agarose gel.

2. Build "sandwich" consisting of gel casting tray, paper towel, blotting paper, gel, and nitrocellulose membrane.

3. Transfer separated proteins to nitrocellulose membrane by applying gentle pressure on the gel casting tray.



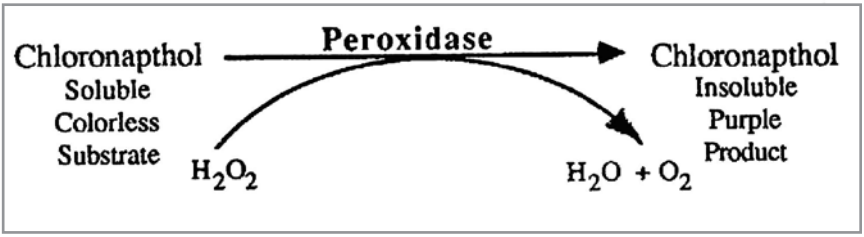
4. Detect all proteins on the membrane with protein blot stain.

5. Detect specific proteins on the nitrocellulose membrane with immunological techniques.

The next step is to incubate the membrane with antibodies which react with the proteins trapped on the membrane. In the present experiment, you will use antibodies that were generated in rabbits against cow serum albumin and cow gamma globulins. These antibodies will bind to the membrane-trapped cow albumin and cow gamma globulins and to the proteins from the other species that are structurally related to the cow proteins. Gelatin is added to the membrane before and during antibody incubation in order to minimize non-specific protein• antibody interaction.

Since the antigen-antibody complexes are not colored, they must be treated in some way in order to visualize them. A commonly used method involves the coupling of a color producing enzyme to the antibody. Enzymes which catalyze the reaction of soluble, colorless substances to insoluble, colored products are often coupled to antibodies to permit visualization. In this experiment, the antibodies you will use have been coupled to Horseradish Peroxidase (HRP) which catalyzes the reaction diagrammed in Figure 1-3. Following incubation of the membrane with the antibody, the final step in the Western Blotting procedure is to incubate the blot in a color development solution containing hydrogen peroxide and 4-chloro-1-naphthol. The immobilized HRP then converts the 4-chloro-1-naphthol to an insoluble purple product which is deposited at the site of the antigen bands and allows antigen visualization.

Figure 1-3. The Reaction Catalyzed by Horseradish Peroxidase



III. Serum Proteins

Blood is a remarkable tissue containing cellular elements (erythrocytes, leukocytes and platelets) suspended in a liquid medium called plasma. Whole blood or plasma clots upon standing and if the clot is removed, the remaining straw colored fluid is called serum. Serum has basically the same composition as plasma except that it lacks certain proteins (fibrinogen and some clotting factors) that are involved in the clotting process.

Serum contains a variety of small molecular weight components as well as hundreds of different serum proteins. Serum proteins, such as the antibodies, are important in fighting disease. Other proteins in serum, such as albumin and transferrin, function as carrier molecules for the transport of small molecular weight compounds such as metals, fatty acids, amino acids, hormones and drugs.

In this laboratory, you will identify specific proteins in serum and then determine the evolutionary relationships of these proteins by using the western blot procedure. A brief description of these proteins is given on the following page.

Serum Albumin - Serum Albumin is the major protein found in serum and this protein transports a number of small molecules in blood. Although albumin is not naturally colored, the protein binds to bromophenol blue. This feature will enable you to identify albumin after electrophoresis since bromophenol blue has been added to the samples and some of this dye will remain bound to the albumin during the electrophoretic run.

Transferrin- Transferrin binds iron and this protein transports iron in the circulation.

Gamma Globulins - The numerous antibodies in blood are found in the gamma globulin fraction of serum.

Objective

To identify albumin, transferrin, and gamma globulins in serum and to study the evolutionary relationships of albumin and gamma globulins in vertebrates.

Laboratory Schedule

This experiment was designed such that two students will work together, sharing one gel and one blot. If students work in pairs, the samples of four students should be analyzed on one gel and one blot The entire procedure can be performed in one 3-hour laboratory period. Alternatively, steps I - IV can be done in one two-hour lab and steps V and VI can be performed during the next lab session.

Materials Provided

Goat Serum
Sheep Serum
Cow Serum
Horse Serum
Chicken Serum
Cow Transferrin
Cow Gamma Globulins
Cow Albumin
Antibodies to Cow Albumin
Goat anti Rabbit IgG Peroxidase
Antibodies to Cow Gamma Globulins - This antibody was prepared by injecting rabbits with cow gamma globulins. It is coupled to the enzyme HRP.
Nitrocellulose (4 sheets)
Blotting paper (16 sheets)
*Gelatin solution
*TBS (Tris-Buffer Saline)
*TBS + NP40 (Tris-Buffer Saline + Nonidet (P40)
*Color Development Solution (contains chloronapthol and hydrogen peroxide dissolved in development buffer - this solution should be prepared immediately before use.)
*Ponceau S - Protein "Blot" Stain
4 plastic petri dishes for blot incubation - These dishes should be used for the antibody reaction described under Part V.

*Prepared as described in the Instructor Guide.

The sera samples, albumin, transferrin, and gamma globulins are dissolved in electrophoresis sample buffer which contains glycerol and bromophenol blue.

Materials not provided

Gloves
A water bath maintained at 37"C
4 containers for blot washing - These containers should hold about 100 ml and be used for all steps described below except the antibody reactions.
Paper towels
4 razor blades
4 scissors

Procedure

I. Preparation of the Agarose Gels

1. To prepare one 0.8% agarose gel,dispense 15 ml of electrophoresis buffer (Tris-Glycine, pH 8.6) into a 25 ml glass test tube and add 0.12 grams of agarose. Four gels will be used in the experiment

2. Boil the agarose and then pour the gel as described on page 14.

II. Electrophoresis

1. Load 15µl of the following samples into the sample wells.

Sample Well	Sample	
1	Cow Gamma Globulins	
2	Cow Serum	Group 1
3	Albumin Cow Transferrin	
4	Cow Serum	
5	Goat Serum	
6	Sheep Serum	Group 2
7	Horse Serum	
8	Chicken Serum	

2. During the electrophoretic run, perform Step III-I (below).

3. Electrophorese until the albumin (blue bands) in the sera samples and in lane 2 has migrated to within 1.5cm of the positive end of the gel. At 170 volts, this should take about 50 minutes.

III. Preparation Of The "Western Press - Blot"

1. . During the electrophoretic run, perform the following tasks:

A. Wet one sheet of nitrocellulose by floating it in a container with about 20ml of distilled water. After wetting, pour off the water and replace with 5-10ml of TBS. In a separate container, wet two sheets of blotting paper with 5ml of TBS.

Note: Gloves should be worn when handling nitrocellulose to prevent transfer of proteins from your hands to the membrane. If gloves are not available, use forceps. Touch only the edges of the membranes with gloves or forceps.

B. Cut 6 paper towels (to about 5cm x 7cm) to form a stack that is l-2cm high.

C. Clean a small section of your laboratory bench top.

2. After electrophoresis, build a "sandwich" on your clean bench top by sequentially overlying each of the following materials. Smooth with your gloved index finger after each addition **except for step C** to eliminate air bubbles between layers.

A. One sheet of wet blotting paper

B. Agarose gel (place the gel upside down onto the blotting paper so that the nitrocellulose will be in contact with the bottom of the gel).

C. One nitrocellulose membrane- After the addition of the nitrocellulose membrane, trim a **small** comer of the membrane at the bottom of lane 1. This marking will enable you to identify the positions of the gel lanes on your blot.

D. One sheet of wet blotting paper.

E. One sheet of dry blotting paper.

F. One sheet of dry blotting paper.

G. One stack (1-2cm) of dry paper towels.

3. Place a gel casting tray, right side up, on top of the stack and press down firmly on the tray for three minutes. (Note: you should apply moderate steady pressure during this time. **Do not** press so hard that the gel collapses completely). During this time, proteins that exit the gel will he trapped on the nitrocellulose.

IV. Staining the Nitrocellulose Blot

1. Disassemble the blotting "sandwich",remove the nitrocellulose membrane and place it in 30ml of protein - blot stain.

2. After 5 minutes, pour off and discard the stain, rinse the blot 3 times with distilled water and note the red protein bands.

3. In the space provided below, draw a diagram of the protein bands on your blot. By examining the positions of the standard proteins on your blots (lanes 1-3), you should he able to identify these proteins in the sera samples. Label, as best you can, the gamma globulins, albumins and transferrins on your diagram for each sera sample.

4. After completion of your diagram, place the blot in 25ml of gelatin solution and incubate at 37°C for 10 minutes. Replace the solution with 25ml of fresh gelatin and incubate for an additional 5 minutes. The gelatin serves to block sites on the nitrocellulose that are not occupied by protein. The dyes will dissociate from the proteins during this step and the blots should appear colorless at the end of the incubation.

5. The blots can be processed immediately as described below or they can be stored in gelatin for a few days in the refrigerator. If stored in the refrigerator, allow them to warm to 37°C before continuing.

V. Antibody Reaction

Two of the blots will be incubated with antibodies to cow albumin while the remaining two blots will be incubated with the antibodies to gamma globulins. The four blots will be compared at the end of the experiment

1. Place 50ul of either the cow albumin antibody or 50ul of the cow gamma globulin antibody into a peal dish. Add 5m1 of gelatin to each dish. Transfer the blot to the dish and gently swirl the dish to ensure that all surfaces of the blot are exposed to the antibody solution.

2. Place the lid on the dish,and float the dish in a water bath at 37°C for 25 minutes.

   Note: Great care should be taken not to bump the dishes during the incubation.

3. Transfer the blot to a suitable small container (e. g.a clean gel staining tray) and wash for 2 minutes each in 40 ml of the following solutions. Manual rocking or shaking of the container should be performed during these washes.

1.	TBS+NP40
2.	TBS+NP40
3.	TBS

4. While the blot is washing, wash the petri dish with water. Dilute the HRP-goat anti-rabbit antibody 1:200 by adding 25ul of antiserum to 5 ml of gelatin solution in the clean petri dish.
5. After the last wash, transfer the blot to the dish containing the HRP - antibody and gently swirl the dish to ensure that the blot is exposed to the antibody solution. Place the lid on the dish and float the dish in a water bath at 37°C for 20 minutes.
6. Transfer the blot to a small container and wash with rocking or shaking for 2 minutes each in 30m1 of the following solutions.

1.	TBS+NP40
2.	TBS+NP40

7. While the blots are washing, the instructor should prepare the color development solution by adding 7m1 of development buffer, 0.5m1 of hydrogen peroxide and 5m1 chloronapthol to 130m1 of water.

VI. Color Development Reaction

1. Place 30ml of the Color Development Solution into a small container and transfer the blot to the solution. Gently rock blot in color development solution until purple bands appear. This should take about 5-15 minutes. Rinse the blot in water and carefully examine both sides for purple bands. Blots may be stored protected from heat and light (between 2 sheets of black construction paper, for example).

Data Analysis and Study Questions

1. Examine your blot carefully and compare your results to the results of your classmates. In the space provided below, prepare diagrams of the indicated blots.

BLOT INCUBATED WITH ANTIBODY TO ALBUMIN	BLOT INCUBATED WITH ANTIBODY TO GAMMA GLOBULINS
LANES	LANES
1 2 3 4 5 6 7 8	1 2 3 4 5 6 7 8

2. Compare the reaction of the antibodies to cow albumin and gamma globulins from the various vertebrates. Which species show the greatest similarity to cow with respect to the reaction? Which show the least?

3. How does this analysis compare with the traditional taxonomic relationships reported for these animals? You may need to consult a zoology or comparative anatomy text book to determine what is known about the relationships between cow, sheep, goat, and horse.

4. Describe how your results would have been affected if the antibody had been made in rabbits against duck albumin.