

There are four experiments to be performed, based on antibody-antigen interactions. Work in pairs throughout.

1. ENZYME LINKED IMMUNO SORBANT ASSAY (ELISA).

The solid-phase ELISA is a very widely used immunological assay which can be adapted to quantitate either antibodies or antigens. It is probably the most widely used diagnostic assay.

In this experiment you are going to use a two-site ELISA to determine the presence of human antibody. A population of rat hybridoma cells were transfected with an expression plasmid encoding a humanised monoclonal antibody. A number of clones were made from the transfected cell mixture and the tissue culture supernatants from two clones are provided. The ELISA plate wells have been coated with anti-human IgG antibody. You will titrate the supernatants and use Horseradish Peroxidase (HRP)-coupled anti-human kappa light chain to detect human IgG, κ . The HRP enzyme converts the substrate ABTS to a green product.

MATERIALS

1. ELISA plate rows coated with anti-human IgG.
2. Supernatants from two clones (X and Y), diluted 1:10 in saline.
3. HRP-anti-human kappa (at working dilution 1:2500; labelled HRP).
4. ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) substrate.

METHOD

1. Arrange your ELISA plate as two rows of 12 columns each, with the row letters [A-H] at the left hand side. Using a micropipette, add 100 μ l saline to all wells **EXCEPT** the left-hand side column (column 1) wells of both rows.
2. Add 100 μ l of clone X supernatant to left hand well and another 100 μ l to its immediate neighbour (columns 1 and 2) of the upper row and 100 μ l of clone Y supernatant to each of the left hand two wells of the lower row.
3. Using the micropipette, make a doubling dilution series from wells 2 to 11 [mix the contents of well 2 by pipetting up and down, transfer 100 μ l from well 2 to well 3, mix well 3 and transfer 100 μ l from well 3 to well 4. Repeat until you reach well 11 where you discard 100 μ l to waste to leave well 12 as a saline control and produce an even volume of 100 μ l in all wells].
4. Incubate for 20 minutes at room temperature (on the bench).
5. Wash the ELISA plate by discarding the contents of the wells into the sink and filling the wells from the wash bottles marked PBS. Repeat the wash step 3 times.
6. Add 100 μ l of the HRP anti-human kappa solution to all the wells. Incubate for 20 minutes on the bench.
7. Repeat the washing as at step 5.
8. Add 100 μ l ABTS substrate solution to all the wells. The reaction will develop with time. Read the result after ~10 minutes.

RESULTS

What is the antigen in this experiment?
What controls are included?
Can you suggest additional controls which might improve the experiment?
Draw a diagram to represent the molecular basis of the reaction.
Is either clone secreting human antibody?
If the sensitivity limit of detection is 0.1ng/well, make an estimate of the concentration of antibody in the positive supernatant.

2. REVERSE PASSIVE HAEMAGGLUTINATION (THE AGGLUTINATION OF ANTIBODY-COATED RED BLOOD CELLS WITH ANTIGEN)

As part of a murder investigation it is important to identify the source of a blood stain on a butcher's shirt as human or sheep. Distilled water has been added to the fragments of cloth to extract the serum proteins and lyse the red cells in the blood stain. You will determine if it is human blood by testing for the presence of human immunoglobulin using antibody specific for human immunoglobulin. The antibody has been chemically coupled to red blood cells for you - these will be agglutinated if the stain contains human immunoglobulin. Such agglutination is visible with the naked eye.

MATERIALS

Very small fragments of two stains (A & B), one of human and one of sheep's blood in water.

Saline

Red blood cells coupled to anti-human immunoglobulin antibody (C).

METHOD

1. Using a pastette, add 9 drops of saline to the bijoux containing the extract of each fragment

(A and B). This will give you a 1:10 dilution. Also add 9 drops of saline to each of two new bijoux labelled A/100 and B/100 respectively and add 1 drop of the appropriate diluted extract to give a 1:100 dilution.

2. Mark out your Microtitre tray as shown and add one drop of diluted extract to the appropriate wells.

(Caution: do not contaminate the reagent with your fingers. Why?)

STAIN EXTRACT

	1/10	1/100	Saline
A			
B			

- To each well add 1 drop of red cells coupled with IgG anti-human immunoglobulin (C) - take care to resuspend the cells well before use. Mix by tapping the wells very gently.
- Leave on the bench for 40-60 mins. Agglutination indicates the presence of human immunoglobulin, and thus human blood, in the extract.

Catalogue Number	Image
I_01.jpg	Haemagglutination

RESULTS

Which blood stain was human?

Suggest alternative ways of showing if this stain was human blood.

3. AGGLUTINATION INHIBITION-PREGNANCY TEST

This experiment shows that agglutination can be produced with antibodies against soluble antigens, by attaching the antigen to the surface of a particle (here a latex bead). This agglutination can be inhibited by first exposing the antibody to an excess of antigen. Free soluble antigen binds to the antibody and prevents the antibody from binding to the antigen attached to the latex beads. Thus excess free antigen inhibits agglutination.

In this experiment, human chorionic gonadotrophin (HCG) has been attached to the surface of latex beads, which will be agglutinated by antibody to HCG. If the antibody is first mixed with urine from a pregnant woman, agglutination is prevented by the HCG in the urine.

MATERIALS (ON ICE AT THE END OF THE BENCH).

- Latex suspension (latex coated with HCG)
- Antibody to HCG (anti HCG)
These reagents are precious-PLEASE do not contaminate them with used tips or distribute them about the bench
- Urine from woman A (labelled UA)
- Urine from woman B (labelled UB)

METHOD

- Using the pipettor and a yellow plastic tip (you will find these with the reagents), dispense 30 μ l of urine from woman A in the left circle on the ring tile provided.
- Now dispense 30 μ l of urine from woman B in the other circle.
- Add 20 μ l of antiserum to each urine drop and rock to mix.
- Resuspend the latex suspension by shaking with the cap screwed on. Add 30 μ l to each circle.
- Mix using a wooden stick and spread the mixture to fill the ring.
- As soon as the reagents are evenly mixed, note the time and rock the plate gently with a circular motion for 4-5 mins.

RESULTS

Which mixture agglutinates?
Which woman is pregnant?

4. PRECIPITATION IN GEL

Antigen and antibody molecules will diffuse in an agar gel; immune complexes form where an antibody meets an antigen for which it is specific; this can result in a visible precipitate where the proportions of antibody:antigen are optimal [think about why?]. Different antigens may form separate lines of precipitate with a single antibody preparation.

MATERIALS

These materials are in bijou bottles. Please do not let the contents of one bottle contaminate another.

Antiserum Anti-human serum labelled anti-HS

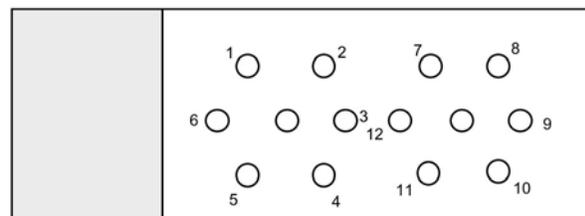
Antigens: Human serum labelled HS
 Human IgG labelled IgG
 Human albumin labelled Alb
 Fraction A labelled A
 Fraction B labelled B

METHOD

1. Lay the slide covered with agar over the diagram and cut holes in the agar using the metal punch.
2. Fill the central well of both clusters with anti-HS using a capillary tube.
3. Fill wells 1-12 as follows:

LHS

- 1. and 4. Alb
- 2. and 5. HS
- 3. and 6. IgG



RHS

- The IgG has been further purified into fractions A and B
- 7. and 10. A
 - 8. and 11. B
 - 9. Alb
 - 12. IgG

4. Keep a record of the position of each reagent. Label the slide with a pencil on the frosted end and place it in the container provided. Make a note in which container you have placed the slide.

RESULTS

After examining your results (which you will do in the next class), compare them with the demonstration photographs.

Think about why the lines form as they do.

Discuss this experiment with a demonstrator.

Draw a diagram to explain why you need optimal antigen/antibody proportions to see a visible line.

Please disinfect your bench area.

Push your stool under your bench.

Thank you.