VNIVERSITAT DE VALÈNCIA Facultat de Ciències Biològiques

Laboratory Sessions in Immunology

Bachelor's Degree in Biology

<u>Experiment 1</u>. Quantification of microbicidal and phagocytic activity (measured by flow cytometry) of human blood.

<u>Experiment 2</u>. Double Immunodiffusion Technique or Ouchterlony: a qualitative or semi-quantitative method used for the population-level analysis of the presence of specific antibodies and antigens in serum.

<u>Experiment 3</u>. Indirect ELISA (enzyme-linked immunosorbent assay) for quantifying specific antibodies in serum, and competitive ELISA for antigen quantification.

EXPERIMENT 1

Quantification of phagocytic and microbicidal activity of human blood

Introduction and objectives

Phagocytosis and microbial killing by polymorphonuclear leukocytes (PMNs) and macrophages (professional phagocytes) represent the most important defense mechanism across all phyla of the animal kingdom. Despite the array of defensive components that vertebrates have developed and that have culminated in the complex immune systems of mammals, phagocytosis still remains as the primary effector mechanism for the elimination of invasive microorganisms, particles and unnecessary cells. Phagocytic functions have clearly co-evolved with other components of the innate and adaptive immune systems, such as antibodies and the complement system, forming a tightly interdependent network.

Methods to assess phagocytosis typically involve the use of bacteria (*Staphylococcus aureus*, *Escherichia coli*, etc.) or fungi (*Candida albicans*), but results can vary significantly between laboratories. However, those using *C. albicans* as the phagocytosed microorganism tend to yield more consistent results. Furthermore, evaluating the ability to phagocytose and kill *C. albicans* is particularly relevant, as the incidence of invasive fungal infections has increased considerably over the last decades due to the concurrence of several factors, such as the use of intensive immunosuppressive regimens in cancer and transplant patients. Of note, *C. albicans* is an opportunistic pathogen that causes a variety of infections, with invasive candidiasis (in which the fungus enters the bloodstream and invades internal organs) being one of the leading causes of mortality in immunocompromised patients.

The experimental protocol we will follow is designed to assess, on the one hand, the ability of blood cells to ingest yeast cells, and on the other, their ability to destroy them. For this purpose, we will use whole peripheral blood from two healthy donors, which will be co-cultured with live yeast cells for a defined period of time.

To measure phagocytosis, we will use yeasts inactivated by fixation from a *C. albicans* strain that expresses GFP (Green Fluorescent Protein). Therefore, by analyzing the co-cultures by flow cytometry, after red blood cell lysis and sample fixation, we will be able to detect the yeasts by measuring fluorescence emission.

To measure the fungicidal activity of blood, we will use live *Saccharomyces cerevisiae* yeasts as a model organism (since working with live *C. albicans* requires a biosafety level 2 laboratory). After co-culture, we will determine the number of viable yeast cells by quantifying colony-forming units (CFUs) through plate counting. As a control, yeasts will also be cultured in the presence of the corresponding blood plasma.

IMPORTANT NOTE*: The use of human blood samples requires handling them as if they were infectious material. Students must follow the general safety guidelines they already know. Additionally, lab coats and gloves must be worn at all times. All blood-contaminated material must be disposed of in the appropriate biohazard containers.

EXPERIMENT 1-A. Assessment of the fungicidal activity of human blood cells.

Materials and reagents

- Liquid YPD culture of *S. cerevisiae*
- Peripheral blood samples collected by venipuncture with lithium heparin as anticoagulant (n = 2)
- Sabouraud dextrose agar plates
- RPMI 1640 medium (Gibco)
- Sterile phosphate-buffered saline (PBS)
- Sterile water
- Micropipettes and tips from 2 to 1000 µl
- Test tubes and racks for preparing dilutions
- Digradlsky loops and alcohol jars
- Microcentrifuge
- Microscopes
- Neubauer counting chambers
- Mechanical shakers

Experimental protocol

1. Plasma preparation:
- Centrifuge 1.5 ml of whole blood at 3000 × g for 5 min.
- Collect the supernatant (plasma) and transfer it to a clean eppendorf tube.
- Centrifuge the plasma at 3000 x g for 5 min.
- Collect the supernatant (cell-free plasma) and transfer it to a clean tube.

2. Yeast preparation and counting:
- Centrifuge 1.5 ml of the microbial culture at 3000 × g for 5 min to pellet the yeast cells.
- Wash the yeast cells with PBS.
- Resuspend the cells in PBS and perform a microscopic count using a Neubauer chamber (see appendix).
- Prepare a suspension containing 50×10^6 yeast cells in 1 ml of PBS.

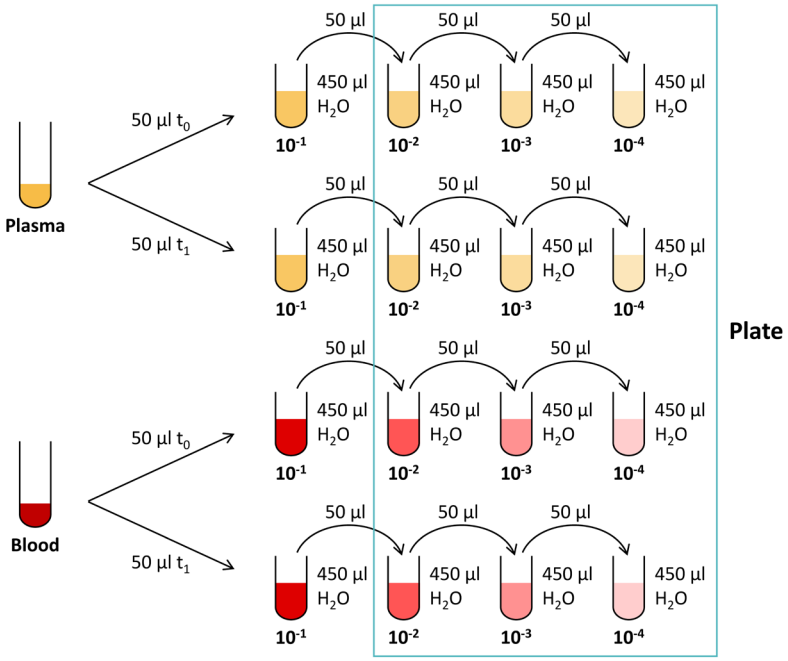
3. Prepare the following mixtures (final volume per tube 200 µl):

Tube #	RPMI (µl)	Blood (µl)	Plasma (µl)	Yeasts (µl)
1 - Plasma	90	-	100	10
2- Blood	90	100	-	10

The number of yeast cells added (0.5×10^6 yeasts per 100 µl of blood) corresponds, in individuals with a normal leukocyte count, to a 1:1 ratio (yeast:leukocyte).

- Mix well and take an aliquot from each tube at t = 0 h. Perform serial dilutions in sterile water for each sample: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} (mix 50 µl of the sample with 450 µl of sterile water to make the first 10^{-1} dilution).
- Incubate the mixtures for 1 h at 37 °C.

- Mix again thoroughly and, at t = 1 h, perform the same serial dilutions in sterile water for each sample.
- Plate 100 µl of the 10^{-2}, 10^{-3}, and 10^{-4} dilutions on Sabouraud dextrose agar, spreading the inoculum with a Digradlsky loop.



- Incubate the plates overnight at 37 °C until colonies become visible.

Interpretation of results

- Count the number of CFUs in the dilution plates that contain between 30 and 300 colonies, and determine the number of CFUs in the co-cultures with plasma and with blood at t_0 and t_1.

$$\frac{CFUs}{ml} = counted\ CFUs\ x\ \frac{1}{dilution}\ x\ \boxed{5}\ x\ \boxed{0,4}$$

initial Vi = 200 µl

100 µl were plated

- Calculate the percentage of fungal survival in the presence of blood cells and the percentage of yeast killing by blood cells as follows:

$$\%\ CFUs\ blood = \frac{CFUs\ blood\ t_1}{CFUs\ blood\ t_0}\ x\ 100 \qquad \%\ CFUs\ plasma = \frac{CFUs\ plasma\ t_1}{CFUs\ plasma\ t_0}\ x\ 100$$

$$\%\ survival = \frac{\%\ CFUs\ blood}{\%\ CFUs\ plasma}\ x\ 100 \qquad \%\ killing = 100\ -\ \%\ survival$$

- Complete the following table with the obtained results.

Survival of *S. cerevisiae* in plasma and blood from both donoes.

S. cerevisiae	**CFUs at 0h plasma**	**CFUs at 1h plasma**	**CFUs at 0h blood**	**CFUs at 1h blood**	**% CFUs plasma**	**% CFUs blood**	**% survival**	**% killing**
Donor 1								
Donor 2								

Tasks

- Analyze and discuss the results obtained by all students in the group as a whole.
- Compare the results between the two blood and plasma samples.
- Discuss what types of studies this technique could be applied to.
- Explain why it is important to perform the counts at time 0 h.

EXPERIMENT 1-B. Quantification of phagocytic activity of human blood cells.

Materials and reagents

- Fixed yeasts from a GFP- expressing *C. albicans* strain
- Peripheral blood samples collected by venipuncture with lithium heparin as anticoagulant (n = 2)
- Sterile phosphate-buffered saline (PBS)
- Sterile water
- RPMI 1640 medium (Gibco)
- Micropipettes and tips from 2 to 1000 µl
- Test tubes and racks for preparing dilutions
- BD FACS Lysing solution 1X
- Microcentrifuge
- Microscopes
- Neubauer counting chambers
- Flow cytometer

Experimental protocol

1. Prepare the yeast suspension:

- We start with a tube containing 100 x 10^6 fixed *C. albicans*-GFP yeasts, previously frozen at -80 ºC as a dry pellet.
- Resuspend the yeasts in 1 ml of RPMI

2. Prepare the following mixtures (final volume per tube 100 µl) in 2 ml eppendorf tubes:

Tube #	RPMI (µl)	Blood (µl)	Yeast (µl)
1	10	90	-
2	90	-	10
3	5	90	5
4	-	90	10

The number of yeast cells added (0.5 × 10^6 yeasts in tube 3 and 1 x 10^6 in tube 4) corresponds, in individuals with a normal leukocyte count, to approximately a 1:1 and 2:1 ratio, respectively (yeast:leukocyte).

- Incubate at 37 ºC for 20 min so that yeast phagocytosis by blood phagocytes takes place.
- Add 1.9 ml of 1X lysing solution (to lyse red blood cells and fix the remaining cells)
- Mix well and let stand for 10 min at room temperature.
- Centrifuge at 3000 x g for 5 min.
- Resuspend the cell pellet in 0.5 ml of PBS.
- Keep the tubes at 4 ºC until flow cytometry analysis, which will be performed on the following day.

<u>Interpretation of results:</u> quantification of phagocytosis by Flow cytometry.

We will use the **BD FACSVERSE 3L 8C** flow cytometer (Beckton Dickinson). This instrument is located at the Cell Culture and Flow Cytometry Facility of the "Servicio Central de Soporte a la Investigación Experimental" (SCSIE) at the University of Valencia. The facility is located on the 5th floor of the "Jerónimo Muñoz" Research Building, on the Burjassot campus.

This cytometer is equipped with a blue laser (488 nm) and a red laser (633 nm). It allows for the detection of six fluorescence parameters and two light scatter parameters: Forward Scatter and Side Scatter (FS and SC). It also includes a digital signal processing system through the FACSDiva software.

Before acquisition, samples should be gently mixed to remove any possible cell aggregates.

For each sample, we will analyze: forward-scattered light (FS), which is proportional to the relative size of the particle; side-scattered light (SS), which is proportional to the granularity and internal complexity of the particle; and green fluorescence emission (emitted by GFP), resulting from the excitation of the sample by the blue laser.

Sample 1. Blood only. We should be able to identify the different leukocyte populations (lymphocytes, monocytes and granulocytes), based on their FSC and SSC properties, in FSC vs. SSC dot plots (two-parameter histograms).

Sample 2. Yeasts only. We will identify yeast cells, based on their FSC and SSC properties. In addition, we will detect green fluorescence emission using single-parameter histograms, plotting green fluorescence intensity versus the number of cells. GFP is excited by the blue laser and has a peak emission at 509 nm; therefore, in the cytometer, fluorescence will be detected using the 510/520 nm filter.

Samples 3 and 4. Blood co-incubated with two different yeast amounts (0.5 and 1 million yeasts). We will detect green fluorescence associated with granulocytes using FSC or SSC vs. fluorescence intensity dot plots.

This will allow us to determine the percentage of granulocytes that have phagocytosed yeasts, for each of the two ratios.

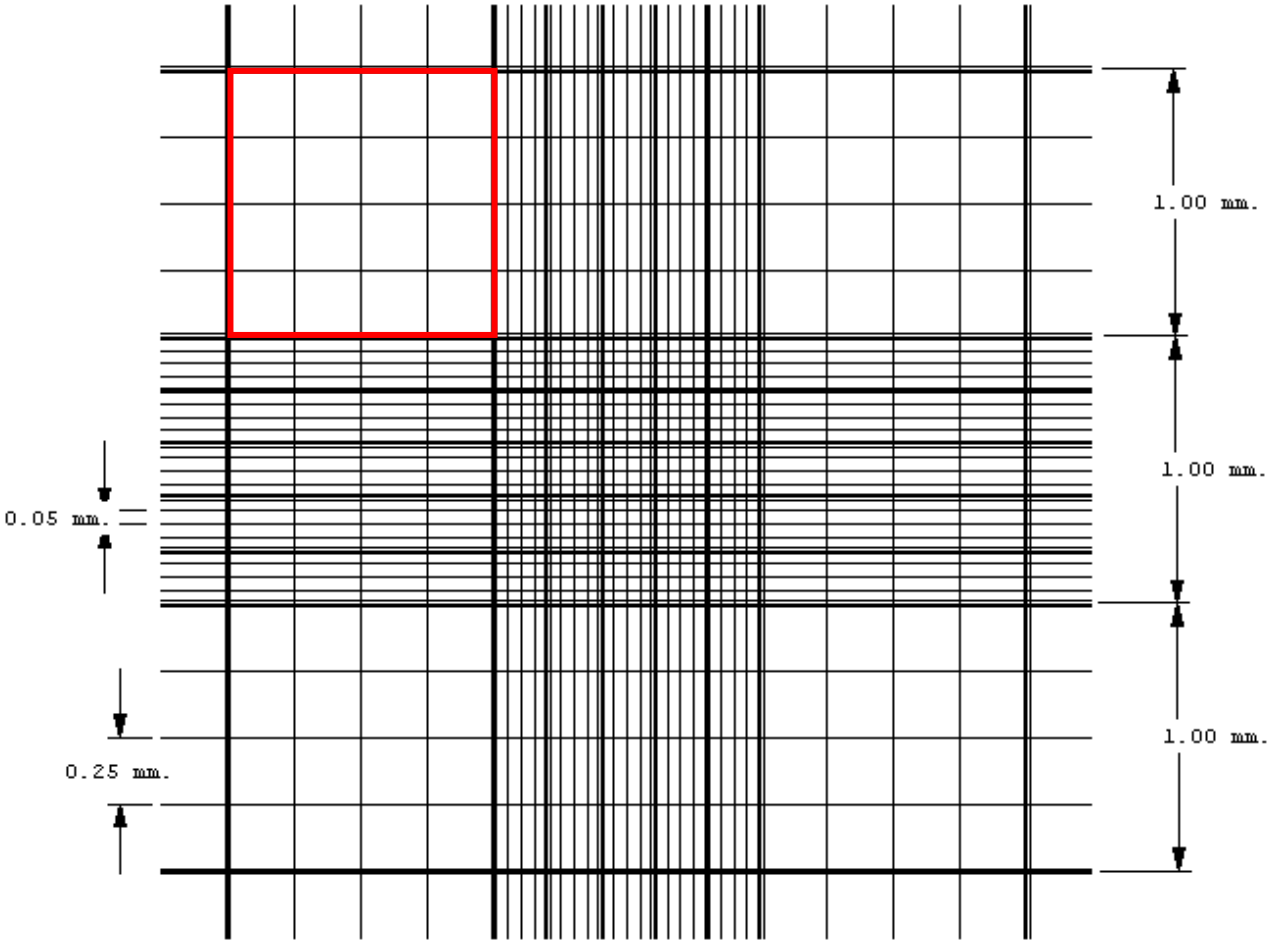
<u>Tasks</u>

- Observe the histograms obtained from the Flow cytometry data, as well as the statistics.
- Discuss the biological significance of your observations.
- Suggest improvements to the experimental protocol used.

APPENDIX

Cell counting using a Neubauer chamber.

- Prepare and clean the Neubauer counting chamber
- Place a coverslip over the Neubauer chamber
- Using a pipette, load 10 µl of the cells suspension between the chamber and the coverslip
- Let the cells settle for a couple of minutes
- Observe under the microscope using a 40x objective
- Count the number of cells (yeasts, in this case) in the four big squares (16 small squares x 4). Calculate the average of the four counts. (One big square corresponds to the square outlined in red).
- Multiply the resulting number by 10^4 (since the volume between one big square an the coverslip is 1/10000 ml).
- Multiply by the dilution factor to obtain the cell concentration per ml.



INTRODUCTION TO THE LAB SESSIONS

Immunoassays: Detection techniques based on the antigen-antibody reaction

All techniques that use antibodies and rely on the specificity of the antigen-antibody reaction are referred to as immunoassays.

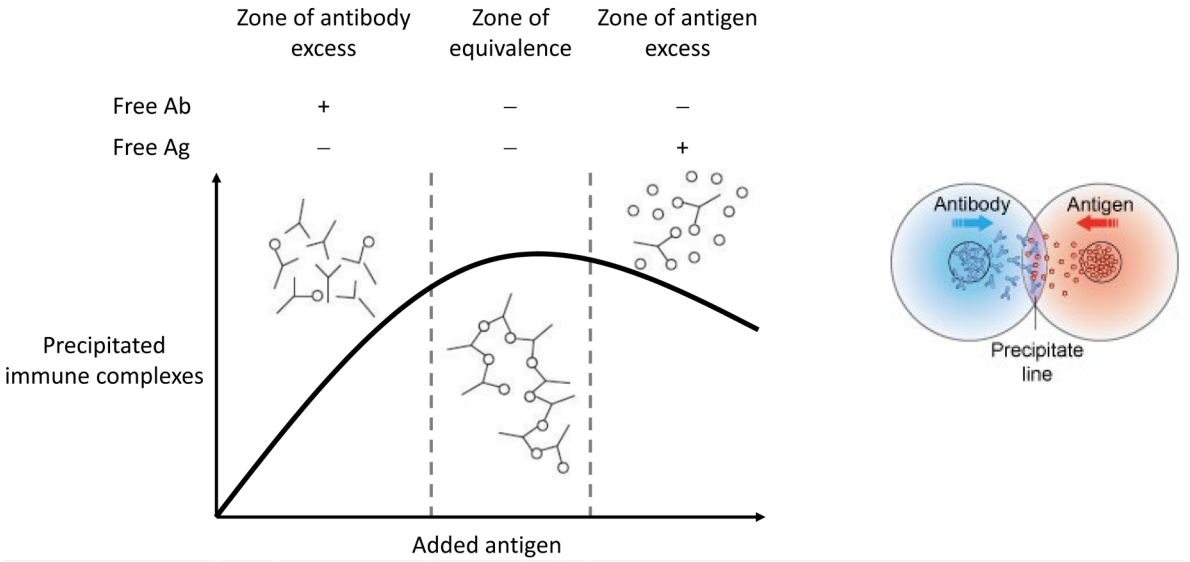
The ability of immunoglobulins to bind antigens, the specificity of this binding, and the fact that this interaction can be made visible through precipitation, agglutination or other indirect mechanisms (labeling with fluorochromes, radioisotopes, or enzymes) has led to these methods being widely used.

Immunoassays are highly important for disease diagnosis, assessing the degree of humoral immune response, and identifying and purifying molecules and cells of interest.

EXPERIMENT 2
Double immunodiffusion technique or Ouchterlony

1.-Introduction and objectives

Immunoprecipitation Reactions: when a soluble antigen interacts with its specific antibody, antigen-antibody complexes (or immune complexes) are formed, which precipitate when the antigen and antibody are present at a certain molecular ratio. When the amount of antigen is increased while keeping the amount of antibody constant, the amount of precipitated proteins initially increases, reaches a maximum, and then decreases.

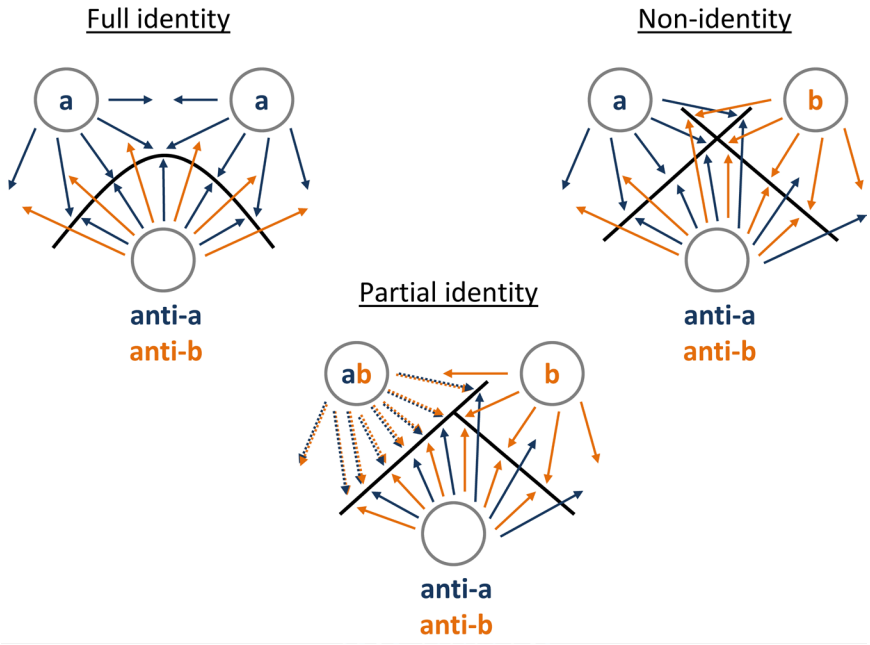


The **Double Immunodiffusion Technique**, or **Ouchterlony, is an immunoprecipitation technique performed in a solid medium.**

It is based on the diffusion of antigens and antibodies through a solid medium (agarose gel) from opposing wells. Upon diffusion, antigen and antibody molecules encounter each other, and if recognition occurs, immune complexes will precipitate in the zone of equivalence. That is, the area where the concentrations of both solutions are optimal for antigenic sites to be saturated by antibodies. The precipitates appear as whitish arcs visible to the naked eye. Each arc corresponds to a single antigen-antibody system. Therefore, if one antibody recognizes two different antigens, two distinct precipitation arcs will appear.

Three different precipitation patterns can be observed:

- **Full identity**: antigens in the samples share the same epitope recognized by the antibody.
- **Partial identity**: antigens in the samples share some common epitopes, but not all of them.
- **Non-identity**: antigens in the samples do not share any common epitopes.



Clinical relevance: this technique can be used to detect the presence of specific antibodies in a serum sample, to reveal the presence of an antigen in a biological fluid, to determine the zone of equivalence, or to assess the degree of identity (complete, partial, or none) between different antigens. Moreover, since the position of the precipitate depends on the relative concentrations of antigens and antibodies, this is considered a semiquantitative method.

This technique can be performed either at the bottom of small Petri dishes (60 mm in diameter) or on glass slides, where an agarose gel is prepared. Equidistant wells are cut using a template, into which the antiserum and antigen solutions are placed. After 12–24 hours of diffusion, the resulting precipitation arcs are analyzed.

In our case, the antibody solutions we will be using are rabbit hyperimmune sera against two antigens (bovine serum albumin, or BSA, and chicken egg albumin, or OVA), and the antigenic solutions consist of either albumin solutions or sera from different animals containing their corresponding serum albumins.

2.- Materials, reagents and solutions

- PBS buffer (phosphate-buffered saline)
- 6 mm diameter Petri dishes containing agarose gel
- Micropipettes and tips from 2 to 1000 µl
- 10 ml pipettes
- Templates and well punchers
- Antigen solutions (BSA and OVA at 1 mg/ml in PBS)
- Animal sera (horse serum, rabbit serum, goat serum, calf serum) and primary antibodies (anti-BSA serum and anti-OVA serum)
- Test tubes and racks for preparing dilutions

3.- Experimental protocol

*Preparation of agarose plates (0,5%) (this step will be performed by the lab technicians)

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- Mix 3 g of agarose with 200 ml of PBS buffer in a flask (144 ml are needed for 4 student pairs). Heat in a microwave, stirring occasionally, until the solution becomes transparent.
- Once the agar is completely melted and the flask is no longer hot to the touch (approximately 60°C), pipette about 6 ml and pour them immediately into the bottom of small Petri dishes (60 mm in diameter), forming a gel approximately 1-1.5 mm thick. The plates must be placed on a perfectly level surface.
- Allow the agar to cool and solidify (this takes a few minutes).

* You will be provided with pre-prepared agarose plates. Students begin the experimental protocol form this point onward.

- Using a puncher and a template, create the wells in the agar at the appropriate distances.
- Load the antigen and antibody solutions into the wells, being careful not to overfill or spill.
- Allow the antigens and antibodies to diffuse at room temperature for 24 h to enable the formation of immune complexes.
- After this time, observe the plates against the light to detect opalescent white precipitation arcs.

Each student pair will prepare 4 agar plates and will test the combinations of antigen and antibody solutions indicated for plates 1, 2, 3, and 4, or alternatively 1, 2, 5, and 6. (The instructor will indicate which plates each pair should prepare).

Load 4 microliters per well in all cases. Fill the peripheral wells starting with the one directly above the central well, then proceed clockwise.

<u>Plate 1:</u>
Central well: anti-BSA antiserum
Peripheral wells: horse serum, rabbit serum, goat serum, calf serum, BSA, PBS

<u>Plate 2:</u>
Central well: anti-BSA and anti-OVA antisera (4µl of each).
Peripheral wells: OVA, PBS, PBS, BSA, BSA, OVA

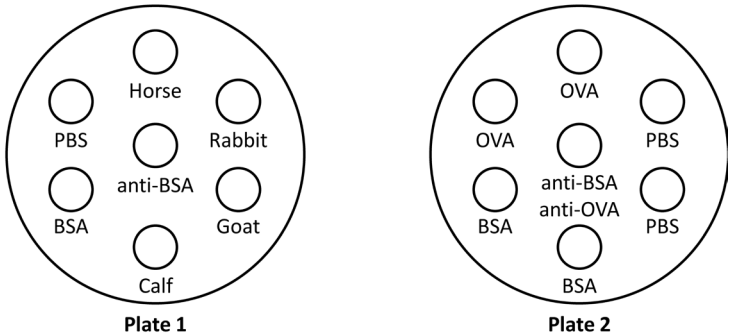


Plate 3:
Central well: BSA
Peripheral wells: anti-BSA antiserum 1/1, 1/2, 1/4, 1/8, 1/16, 1/32
(note: prepare dilutions in 10 µl)

Plate 4:
Central well: OVA
Peripheral wells: anti-OVA antiserum 1/1, 1/2, 1/4, 1/8, 1/16, 1/32

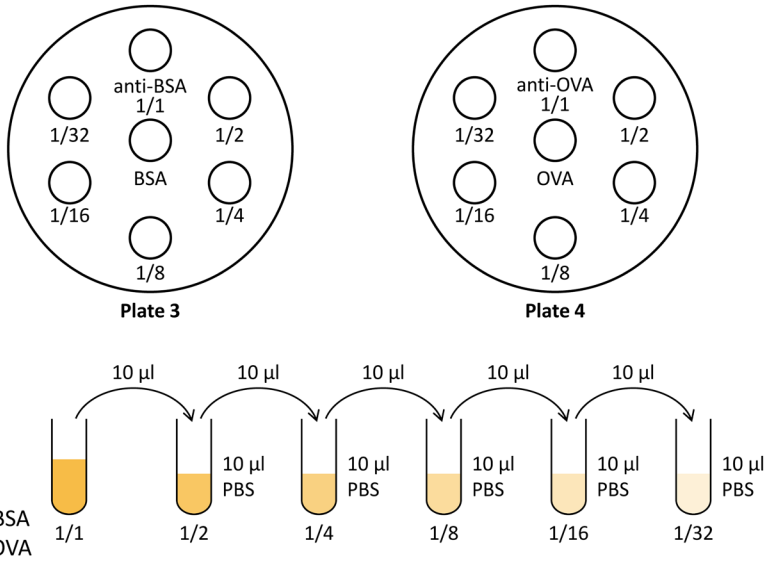
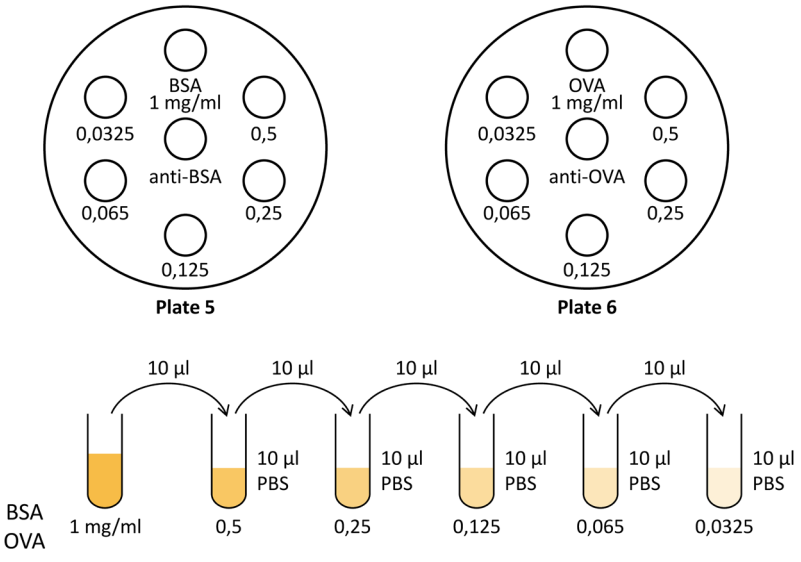


Plate 5:
Central well: anti-BSA antiserum
Peripheral wells: BSA at 1mg/ml, 0,5 mg/ml, 0,25 mg/ml, 0,125 mg/ml, 0,065 mg/ml, 0,0325 mg/ml

Plate 6:
Central well: anti-OVA antiserum
Peripheral wells: OVA at 1mg/ml, 0,5 mg/ml, 0,25 mg/ml, 0,125 mg/ml, 0,065 mg/ml, 0,0325 mg/ml



Guide for creating the wells.
Appearance of the wells.
Example of a precipitation arc.

Interpretation of results:

- Deduce the specificity of the antisera based on the results from plates 1 and 2. Determine whether BSA and OVA share any common antigenic determinants.
- Determine the titer of both antisera based on the results from plates 3 and 4.
- Identify the zone of equivalence based on the results from plates 5 and 6. Observe the distance of the precipitation arcs from the central well in relation to antigen concentration in plates 5 and 6.

Reminder:

"Antibody titer" refers to how much the serum can be diluted before the test becomes negative (it is, therefore, the last dilution that still produces a precipitation arc). The titer depends both on the concentration of antibodies in the serum and their affinity, and it may vary depending on the sensitivity of the assay.

The zone of equivalence corresponds to the thinnest and clearest precipitation line.

EXPERIMENT 3
ELISA (enzyme-linked immunosorbent assay)

1.- Introduction and objectives

The Enzyme-Linked Immunosorbent Assay (ELISA) is currently the most widely used technique for quantifying nearly any soluble protein in most body fluids or culture media in the laboratory. It is a liquid-phase immunoassay in which one of the components (Ag or Ab) is adsorbed onto a plastic surface. The technique is based on the use of enzyme-labeled antibodies, so that immune complexes can be detected by adding a substrate specific to the enzyme, which yields a colored product that is easily quantifiable with a spectrophotometer. This method combines the specificity of antibody binding with the signal amplification provided by enzymatic reactions, making it the most commonly used immunological technique in clinical practice due to its simplicity and sensitivity.

There are multiple variations of the technique, although the most common are indirect ELISA, competitive ELISA, and sandwich ELISA.

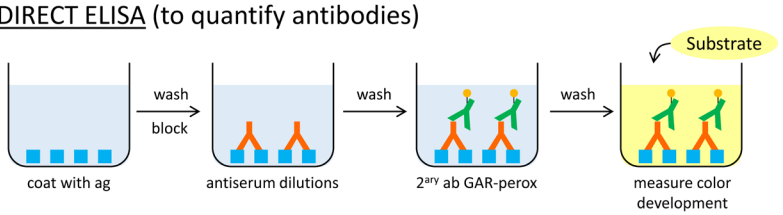
1.- **Indirect ELISA** is the variation used when we want to quantify specific antibodies in a serum sample (for example, to diagnose an infectious or autoimmune disease). The Ag is adsorbed onto a solid surface (typically the wells of a microtiter plate), and a dilution of the patient's serum is added. If antibodies are present, they will bind to the antigens. To detect this binding, an enzyme-labeled antibody against human immunoglobulins (secondary antibody) is added. Finally, the enzyme's substrate is added, and the appearance of a colored product is measured. A quantitative estimation of antibody levels can be made by determining the “Antibody titer”, that is, how much the serum can be diluted before the test becomes negative. Note that the titer of a given serum will vary depending on the sensitivity of the assay.

2.- When the goal is to quantify a serum protein (for example, a cytokine, a hormone or a bacterial toxin), either competitive ELISA or sandwich ELISA is used.

Sandwich ELISA. In this case, an antibody is first adsorbed onto the plastic surface. The test sample is then added and if it contains the target protein, it will bind to the antibody. A second antibody, specific for the same protein and labeled with an enzyme, is then added. In this way, the antigen is “sandwiched” between the two antibodies. Finally, the enzyme substrate is added, and the appearance of a colored product is measured. By using two monoclonal antibodies that recognize different epitopes of the same antigen, the system achieves greater specificity and can better discriminate between different antigens. Comparison with a standard curve allows for quantification of the antigen.

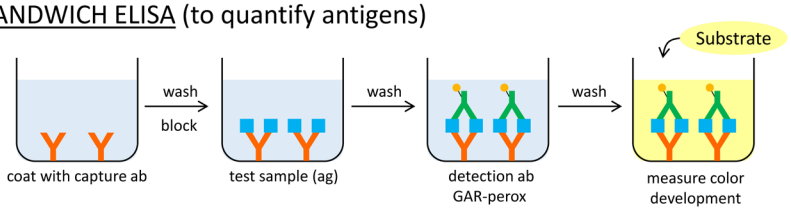
Competitive ELISA. The specific antibody for the antigen is incubated in a solution containing the antigen. This mixture is then added to wells previously coated with an excess of antigen. After washing, an enzyme-conjugated secondary antibody is added, which binds to the primary antibody that did not originally bind antigen. Finally, the enzyme substrate is added. The antigen concentration, in the biological fluid, is inversely proportional to the color intensity produced. Comparison with a standard curve allows for quantification of the antigen.

➢ INDIRECT ELISA (to quantify antibodies)

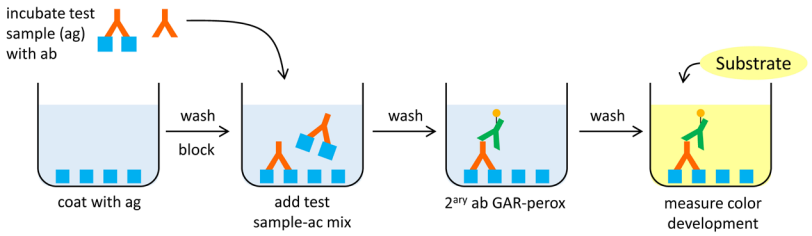


Titrate anti-OVA and anti-BSA antisera by testing them against OVA and BSA antigen solutions, respectively.

➢ SANDWICH ELISA (to quantify antigens)



➢ COMPETITIVE ELISA (to quantify antigens)



Determine the concentration of BSA in a test sample by interpolating its absorbance value on a standard curve.

In this experiment, we will perform an **indirect ELISA** and a **competitive ELISA**. The antibody solutions used will be hyperimmunized rabbit sera (polyclonal antisera) against two antigens (bovine serum albumin: BSA and chicken egg albumin: OVA), that is, anti-BSA and anti-OVA.

Indirect ELISA

The anti-BSA antiserum will be tested against the BSA solution, and the anti-OVA antiserum will be tested against the OVA solution. This will allow us to determine the titer of both antisera.

Competitive ELISA

We will determine the concentration of an antigen (BSA) in an unknown test sample by interpolating the absorbance value on a standard curve.

2.- Materials, reagents and solutions

- Antigen solutions (BSA and OVA at 1 mg/ml in PBS)
- Test sample: BSA solution of unknown concentration
- Primary antibodies (anti-BSA and anti-OVA antisera)
- Secondary antibody (GAR-perox, goat anti-rabbit antisera conjugated to peroxidase)
- 96-well ELISA microtiter plates
- PBS buffer
- PBS buffer with 0.1% Tween 20 for washing

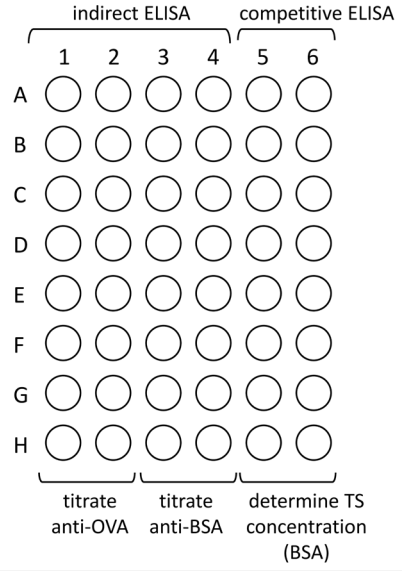
- 60 mM carbonate/bicarbonate buffer, pH 9.6
- Blocking solution (1% gelatin in PBS)
- Enzyme substrate solution (3,3',5,5'-Tetramethylbenzidine (TMB) with hydrogen peroxide)
- Stop solution (0.5 M sulfuric acid)
- Micropipettes and tips from 2 to 1000 µl
- Automated ELISA plate reader
- Flasks, test tubes, and racks for preparing dilutions

3.- Experimental protocol

Day 1

- Coat the plate with 50 µl per well of the antigen solution, dissolved in carbonate buffer. OVA (columns 1 and 2) and BSA (columns 3-6) at 25 µg/ml, and incubate the plates overnight at 4 °C.

Layout of the two ELISA assays on the microtiter plate →



Day 2

- For the competitive ELISA, prepare a series of **tubes** for the pre-incubation of the antigen (standard and test sample) with the antibody, using the amounts indicated below. Incubate at room temperature for two hours with occasional mixing.

Tube	BSA at 1mg/ml (µl)	PBS (µl)	Test sample (µl)	Anti-BSA antiserum (1:2500) (µl)	Position in the plate
1	-	100	-	100	A
2	2,5	97,5	-	100	B
3	5	95	-	100	C
4	10	90	-	100	D
5	20	80	-	100	E
6	30	70	-	100	F
7	40	60	-	100	G
8	-	-	100	100	H

- Wash the wells of the plate three times with PBS containing 0.1% Tween 20 (100 µl/well).

- Block the wells with 100 µl/well of a 1% gelatin solution in PBS for 45 minutes at 37 ºC.

- Wash the wells three times with PBS containing 0.1% Tween 20 (100 µl/well).

- For the indirect ELISA, add 50 µl of the different dilutions of primary antibodies in PBS to the wells. The primary antibodies will be tested at various dilutions (prepared directly on the plate), starting at 1/5000.

- For the competitive ELISA, load 50 µl from each tube into the corresponding wells (in duplicate).

- Incubate overnight at 4 ºC.

Day 3

- Wash the wells three times with PBS containing 0.1% Tween 20 (100 µl/well).

- Add 50 µl of the secondary antibody (GAR-peroxidase) diluted 1/10,000 in PBS to each well and incubate for 45 min at 37 ºC.

- Wash the wells four times with PBS containing 0.1% Tween 20 (100 µl/well).

- Add 50 µl of the peroxidase substrate to each well and incubate until color develops. Stop the reaction by adding 25 µl of stop solution.

- Measure the color development at 450 nm using an automated ELISA plate reader.

Interpretation of results:

- Indirect ELISA. After reading the absorbance for each dilution of the antiserum (for each antigen-antibody system), determine the titer of both antisera and compare it with the result obtained using the Ouchterlony method.
- Competitive ELISA. Plot the absorbance values at 450 nm against the BSA concentration (mg/ml) to generate the standard curve. Determine the BSA concentration of the test sample.