Detection of rabbit IgGs using ELISA Immunoassay (Enzyme-Linked ImmunoSorbant Assay)

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1.0 SUMMARY

Enzyme-Linked ImmunoSorbent Assay (ELISA) is designed for detecting and quantifying substances such as peptides, proteins, antibodies, and many other analytes. An enzyme that reacts with a substrate to produce a colored product is covalently linked to a specific antibody that recognizes target antigen. If the antigen is present, the antibody-enzyme complex (conjugate) will bind to it, and the enzyme component of the conjugate will catalyze the reaction generating the colored product. Therefore, the presence of the colored product indicates the presence of the antigen.

The ELISA is a powerful immunological method. The method is easy to perform and yields graphic results making it well suited for the teaching laboratory.
In this experiment a goat-anti-rabbit IgG antibody (conjugate) is used to study the specificity of an antibody-antigen reaction. **Goat-anti-rabbit-IgG** antibodies, will recognize and bind to IgG molecules present in rabbit serum.

The objective of the experiment is the comparison of binding of goat-anti-rabbit IgG to IgGs in serum from rabbit, chicken and horse. The Goat-anti-rabbit IgG has been chemically linked to enzyme peroxidase (this type of complex is called conjugate), the enzyme catalyzes a color-producing reaction. This reaction enables to study the antibody-antigen interactions.

2.0 OBJECTIVE

Study the specificity and sensitivity of an antibody-antigen reaction using goat-anti-rabbit IgG antibody conjugate, rabbit serum containing rabbit IgGs, chicken serum and horse serum.

3.0 INTRODUCTION/BACKGROUND

Enzyme-Linked ImmunoSorbent Assay (ELISA) is designed for detecting and quantifying substances such as peptides, proteins, antibodies, hormones and many other analytes. An enzyme that reacts with a colorless substrate to produce a colored product is covalently linked to a specific antibody (conjugate) that recognizes a target antigen (analyte).
If the antigen is present, the antibody-enzyme complex will bind to it, and the enzyme component of the antibody-enzyme complex will catalyze the reaction generating the colored product. Therefore, the presence of the colored product indicates the presence of the antigen.

The most crucial element of the analyte detection and quantitation strategy is a highly specific antibody-antigen interaction.
An enzyme-linked immunosorbent assay, is rapid and convenient, and can very specifically detect less than a nanogram of a protein or other analyte of interest in the heterogeneous mixture.

Because the ELISA can be performed to evaluate the presence of antibodies in a sample, it is a useful tool for determining serum antibody concentrations in the individuals exposed to the infectious pathogens; such as antibodies against HIV or West Nile Virus. Thousands of medical diagnostic tests work by detecting the presence of many analytes of interest in blood serum and other biological specimen.
Immunoassays also found applications in the food industry in detecting potential food allergens such as milk, peanuts, walnuts, almonds, and eggs. ELISA can also be used in toxicology as a rapid presumptive screen for certain classes of drugs and as a powerful quality control tool in biomanufacturing.

For ELISA it is important that the antibody enzyme conjugate is of high specific activity. This is achieved when the antibody is affinity purified and the enzyme conjugation chemistry preserves antibody specificity as well as enzyme activity.

Most prevalent format of the ELISA is called sandwich format. The sandwich ELISA is called "sandwich" because the analyte (antigen) to be detected and/or quantified is bound between two antibodies (capture and detection antibody), that bind to different epitopes on the antigen.

There are two major types of ELISAs:

1. The indirect ELISA is called "indirect" because a secondary antibody linked to an enzyme is needed to detect an antibody that binds to a specific antigen.

The indirect, two-step method uses a labeled secondary antibody for detection. First, a primary antibody is incubated with the antigen.

This is followed by incubation with a labeled secondary antibody that recognizes the primary antibody.

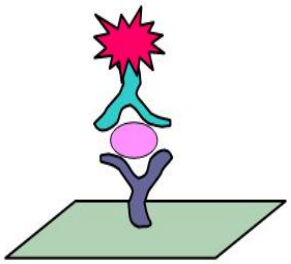
Indirect ELISA

2. The direct ELISA uses the method of directly labeling the primary antibody itself. Microtiter wells are coated with a sample containing the target antigen, and the binding of labeled antibody is quantified by a colorimetric, chemiluminescent, or fluorescent end-point.

Since the secondary antibody step is omitted, the direct ELISA is relatively quick, and avoids potential problems of cross-reactivity of the secondary antibody with components of the sample.

However, the direct ELISA requires the labeling of every antibody to be used, which can be a time-consuming and expensive proposition. In addition, certain antibodies may be unsuitable for direct labeling.

Direct methods also lack the additional signal amplification that can be achieved with the use of a secondary antibody.



Direct Sandwich ELISA

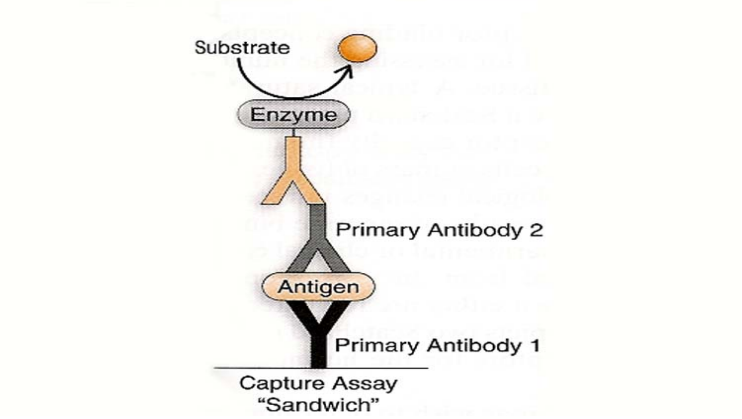
The sandwich ELISA measures the amount of antigen between two layers of antibodies.
The antigens to be quantified must contain at least two antigenic sites (epitopes), capable of binding to the antibody, since at least two antibodies act in the sandwich.
For this reason, sandwich assays are restricted to the quantitation of multivalent antigens such as proteins or polysaccharides.

To utilize this assay format, one primary antibody (the “capture” antibody) is bound to a solid phase typically attached to the bottom of a microtiter plate well.
Antigen is then added and allowed to complex with the first primary antibody attached to the well.

Unbound products are then removed with a wash, empty spaces on the well blocked with the blocking agent (usually gelatin) and a labeled second primary antibody (the “detection” antibody) is allowed to bind to the antigen, thus completing the “sandwich”.

The amount of analyte is determined by measuring the amount of colored product of enzymatic reaction performed by the second primary antibody conjugate, or secondary antibody conjugate bound to the matrix.

Major advantages of this technique are that the antigen does not need to be purified prior to use, and that these assays are very specific.
However, one disadvantage is that not all antibodies can be used.
Monoclonal antibody combinations must be qualified as "matched pairs", meaning that they can recognize separate epitopes on the antigen so they do not hinder each other's binding.



Indirect Sandwich ELISA

Dennis E Bidwell and Alister Voller created the ELISA tests to detect various infectious diseases, such as Malaria, Chagas' disease, and Johne disease.
The other examples of ELISAs used for vitro medical diagnostics include:

- detection of Mycobacterium antibodies in tuberculosis.
- detection of rotavirus in feces.
- detection of hepatitis B markers in the serum.
- detection of enterotoxin of *E. coli* in feces.
- detection of HIV in blood sample.

In mammals, there are 5 major types of antibodies, called **Immunoglobulins.**
The five types, or classes, are known as IgA, IgD, IgE, IgG, and IgM.

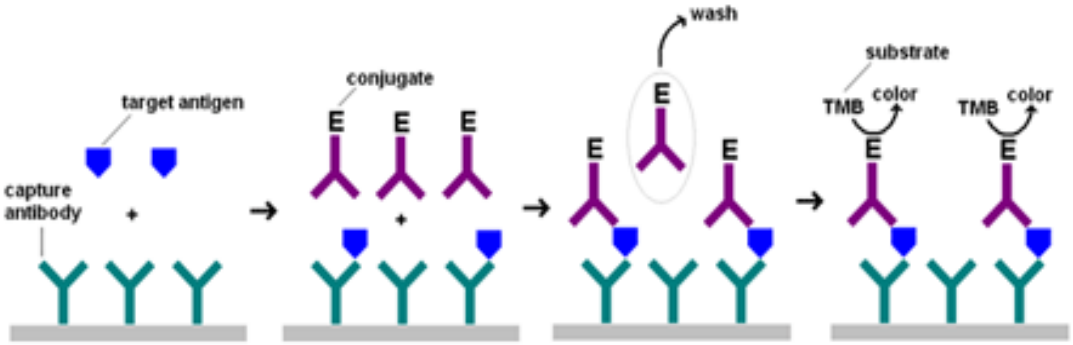
The major class of immunoglobulins in blood is IgG and this class constitutes about 10% of total proteins found in the serum (liquid) portion of blood. There is about 10 mg of IgG per ml of serum.

The amino acid sequence of IgG varies for each species of animal. Therefore, when IgG from rabbit is injected into a goat, the goat will produce antibodies in response to the rabbit IgG molecules. These antibodies produced by the goat are called **Goat-anti-rabbit-IgG** antibodies, and they will only recognize and bind to rabbit IgG molecules.

In this experiment a goat-anti-rabbit IgG antibody is used in order to study the specificity and sensitivity of an antibody-antigen reaction.

The analysis shows comparison of the binding of the goat-anti-rabbit IgG to IgGs in serum from rabbit chicken and horse. Conjugate is diluted (1:400 dilution), and (1:1000 dilution).

The anti-rabbit IgG has been chemically linked to peroxidase, which causes a color-producing reaction. TMB is a colorless substrate that changes into blue product in the presence of peroxidase. This color-producing reaction will enable to study the antibody-antigen interaction using an ELISA procedure.



Immunoassay performed in this experiment is **the direct two layers ELISA**.

Primary antibody (capture) is bound to the microtiter well, this antibody is also an antigen recognized by the second primary antibody. Second primary antibody (conjugate) binds to the first primary antibody resulting with two layers instead of three layers.

To minimize false negative results good pipetting skills are essential.
To minimize false positive results following blocking and washing procedure is essential.

4.0 MATERIALS
Per one team of 2 or 3 students
4.1 Pipetter: range 5-50 µl and tips
4.2 Pipetter: range 20-200 µl and tips
4.3 Approximately 100 Transfer pipettes
4.4 Pen and markers
4.5 Masking Tape

4.6 Stirring Bar
4.7 Stirring heating plate
4.8 Microtiter plate
4.9 Electronic balance

4.10 Beakers: one 200 ml, at least four 50 ml.
4.11 Di water
4.12 Graduated cylinders; 100 ml and 50 ml

4.13 Micro centrifuge tubes; 2ml for preparing conjugate dilutions
4.14 Small waste basket to toss used tips and transfer pipettes

4.15 Gelatin
4.16 TBS (Tris- Buffer Saline) working solution 0.1M, The buffer might be supplied as a 30-fold (3M) concentrate pH 8.0 and should be diluted.
4.17 TBS + Gelatin (TBS + 2% Gelatin)
4.18 TBS NP-40 (working solution TBS 0.1M, pH 8.0 + 0.05% Nonidet P-40)

4.19 Twenty microliters, 20µl of Goat anti-rabbit IgG - Peroxidase (Conjugate)

4.20 TMB -3,3',5,5'Tetramethylbenzidine. Liquid Substrate System for ELISA. Color development solution or substrate 1.0 ml-1.5 ml (Ready to use).

4.21 Fifty five microliters, 55µl Chicken Serum (1%)
4.22 Fifty five microliters, 55µl Horse Serum (1%)
4.23 Fifty five microliters, 55µl Rabbit Serum (1%)

4.24 0.1M NaOH

5.0 METHOD/PROCEDURE

5.1. Preparation of Solutions

5.1.1 TBS (Tris Buffer Saline) - The buffer can be supplied as a 30-fold (30X) stock solution. To prepare the working solution add 10ml of concentrate to 300 ml of r Di water. The buffer may be stored at room temperature for a few weeks.

5.1.2 TBS + NP-40 (TBS + Nonidet-P40) – Cane be prepared from the 30-fold concentrate as described above, also stable solution.

5.1.3 TBS + Gelatin- Add 5 ml of cold TBS to 3 grams of Gelatin, mix well, add the resulting slurry to boiling 150 ml of working solution TBS and mix on the heating/stirring plate until it dissolves cool down but not refrigerate. A solution is stable for up to 5 hours.

5.1.4 Color Development Solution or Substrate– This solution is ready to use, has to be refrigerated, take it from the refrigerator not earlier than 20min before use. One team needs about 1 ml of substrate; TMB ((Tetramethyl Benzidine).

5.1.5 1:100 dilution of Goat anti-rabbit IgG - Peroxidase - Add 20 µl of Goat anti-rabbit IgG - Peroxidase to 1980 µl (**Note! Microliters!)** of TBS buffer.

5.1.6 1:400 dilution of Goat anti-rabbit IgG – Peroxidase - Add 500 µl of the dilution 1:100 of Goat anti-rabbit IgG - Peroxidase to 1500 µl of TBS Buffer

5.1.7 1:1000 dilution of Goat anti-rabbit IgG – Peroxidase – Add 200 µl of the dilution 1:100 of Goat anti-rabbit IgG - Peroxidase to 1800 µl of TBS Buffer

5.2. Labeling the microtiter plate

A standard microtiter plate contains 96 wells. The plate can be labeled using a black marker pen, rows have to be labeled A, B, C, D, E, G, H (note! the rows can be already labeled by the manufacturer). Columns should be labeled (if not labeled by manufacturer) using numbers 1-12. Each well can now be identified by a single letter and number. For example, the first well in the top row is A-l and the last well in the bottom row is H-12.

5.3. Adsorption and serial dilutions of the Antigen

5.3.1. Using a Pipetter, place 50µl of TBS (working solution) into wells A2-6, B2-6, and C2-6, skip row D, and add 50ul of TBS to wells E2-6, F2-6, and H2-6.

5.3.2 Place 55µl of chicken serum into well A-I, 55µl of rabbit serum into well B-1, 55ul of horse serum into well C-1.

5.3.3 Repeat adding 55ul sera into wells E-1 chicken, F-1 rabbit, G-1 horse.

5.3.4 Dilution of the Sera:

5.3.4.1 Serial ten-fold dilutions are performed so that the concentrations of sera in the wells after dilution should be as follows:

Wells	A1, B1, C1, E1, F1, G1	1%	serum
Wells	A2, B2,C2, E2, F2, G2	0.1	serum
Wells	A3, B3, C3, E3, F3, G3	0.01	serum
Wells	A4, B4, C4, E4, F4, G4	0.01	serum
Wells	A5, B5, C5, E5, F5, G5	0.01	serum
Wells	A6, B6, C6, E6, F6, G6	no serum	

5.3.4.2 To perform the dilutions transfer 5µlof the serum from well A-1 to well A-2. Mix the contents in well A-2 thoroughly by drawing the sample into the pipette and expelling it back into the well a few times. Transfer 5µl of the sample from well A-2 to A-3, mix very well and transfer 5ul from A-3 to A-4 mix thoroughly, and transfer 5ul from A-4 to A-5. Mix between each transfer as described above. Do not transfer from well 5 into well 6.

5.3.5 Using the above procedure perform 1:10 serial dilutions of sera in wells B1-B5, C1-C5, E1-E5, F1-F5, and G1-G5.

5.3.6 The plate should be left undisturbed for about 20 minutes in order to provide time for the proteins to be adsorbed to the surface of the microtiter wells.

5.3.7 After 20 minutes, add two drops of TBS-gelatin to each well using a transfer pipette. The gelatin is added to block sites on the plastic that are not covered by serum proteins.

5.3.8 Using a transfer pipette, remove the liquid from **all wells** in the following order: start atA6 and end at A1, in the first row, repeat the procedure for all rows. The discarded liquid should be placed in your discard beaker. Discard pipettes after each row.

5.3.9 Using a transfer pipette, add 3-4 drops of TBS-gelatin to each well. After about 5 minutes, remove the solutions as described in the preceding step.

5.4. The Conjugate Binding and Plate Washing

5.4.1. Prepare 1:400 and 1:000 dilutions of goat anti-rabbit IgG peroxidase conjugate.

5.4.2. Add 50µI of the antibody conjugate, 1:400 dilution, to each well A1-A6 chicken and B1-B6 rabbit C1-C6 horse.

5.4.3. Add 50µI of the antibody conjugate, 1:1000 dilutions, to each well E1-E6 chicken and F1-F6 rabbit, G1-G6 horse

5.4.4. Rotate the plate to ensure that all surfaces at the bottoms of each well are in contact with the antibody solution.

5.4.5. Allow at least 20 minutes for the antibody conjugate to bind to the immobilized IgG.

5.4.6. Add two drops of TBS-gelatin to each well and then remove and discard the solutions in the wells as described in Steps 5.3.7- 5.3.8. **Use a new transfer pipette for each row.**

5.4.7. Add 3 drops of TBS-gelatin to each well, wait 3 min and remove and discard the solution as described in step 5.3.9

5.4.8. Add 3 drops of TBS-NP40 to each well and immediately remove and discard the solution as described in step 5.3.9, except remove TBS-NP40 right away (Note! No need to wait 3 min). Repeat the TBS-NP40 wash 2 more times (a total of 3 washes with TBS-NP40).

5.4.9. Add 3 drops of distilled water to each well and immediately remove and discard as described in step 5.4.8

5.5 Color Development

5.5.1. Add 50µI of TMB –Tetramethylbenzidine, ready to use, to each well. The color development solution should be taken out of the refrigerator immediately before use and has to be protected from light (keep in dark containers).

5.5.2. After about 15 minutes, place the plate over a white sheet of plain paper and examine the intensity of the blue color. The blue compound is the product of the peroxidase reaction.

6.0 RECORDING RESULTS AND STOPPING ENZYMATIC REACTION

6.1 Assign the following system to record relative color intensity

Legend: 0, for no color
+, for low color
++, for high color

6.2 Prepare the table to record the results

Well number	Conjugate Dilution 1:400			Conjugate Dilution 1:1000			Serum Concentration
	Chicken serum	Rabbit serum	Horse serum	Chicken serum	Rabbit serum	Horse serum	
							1%
							0.1%
							0.01%
							0.001%
							0.0001%
							No serum
							1%
							0.1%
							0.01%
							0.001%
							0.0001%
							No serum

6.3 Record the results as the relative color intensity or use a microplate reader to measure absorbance**. Do not wait with the recording longer than 30 min. TMB oxidizes spontaneously when exposed to light and oxygen at room temperature. All wells will eventually turn blue and you will get false positive results.**

6.4 Stopping Enzymatic Reaction. After 15- 20min of color development and when the recording of the results is completed add 1-2 drops of 0.1 M NaOH to each well. Blue product will change into yellow, enzymatic reaction and non-enzymatic TMB oxidation will be stopped. Sometimes this improves visibility of the color development.

ELISA Troubleshooting

Problem	Possible Causes	Action/solution
No signal	Assay set up incorrectly or used incorrect reagents	Review protocol. Repeat assay using a positive control
	Not enough antibody used	Increase concentration of the primary and/or secondary antibody

Problem	Possible Causes	Action/solution
No signal	Detection reagent too old or contaminated	Use fresh detection reagents
	Antigen not coated properly	Try longer coating times, different coating buffers, or avidin plates with biotinylated antigen
	Plate reader has the wrong settings	Check plate reader for wavelength, filters, gain etc.
	Antibody stored at 4°C for several weeks or subjected to repeated freeze/thaw cycles	Use a fresh aliquot of antibody that has been stored at -20°C or below
Weak signal	Insufficient amount of antigen was coated to microtiter plate	Use more antigen for coating or very coating buffer
	Not enough antibody used	Increase concentration of the primary and/or secondary antibody. Optimize antibody concentrations for your assay
	Detection reagent too old, contaminated, or used at the wrong pH	Use fresh detection reagents at the correct pH
	Detection reagent too dilute	Use a higher concentration of detection reagent
	Plate reader has the wrong settings	Check plate reader for wavelength, filters, gain etc
	Incubation temperature too low	Optimize the incubation temperature for your assay. Reagents should be at room temperature before beginning the assay
High background signal	Too much antibody used	Reduce the concentration of the primary and/or secondary antibody. Optimize antibody concentrations for your assay
	Non-specific antibody binding	Use a suitable blocking buffer or use an affinity-purified antibody
	Too much detection reagent used	Repeat assay with a higher dilution of detection reagent
	Too few washing cycles	Increase the number of washing cycles
	Contaminated blocking agent	Use fresh blocking agent
	Wrong concentration of blocking agent	Check the concentration of blocking agent in the recommended protocol
	Presence of blocking buffer interferes with antibody binding	Wash off blocking buffer before adding antibody
	Reaction not stopped	Use stop solution to prevent overdevelopment
	Plate left too long before reading	Start taking measurements shortly after the addition of detection reagent
	Wrong settings on the plate reader	Check settings and adjust as needed
	Insufficient amount of Tween® in the buffers	Use PBS containing 0.05% Tween®
	Incubation with substrate carried out in the light	Perform substrate incubation in the dark
	Incubation temperature too high	Optimize the incubation temperature for your assay
	Pipetting errors	Calibrate pipettes so that they dispense the correct volumes

Problem	Possible Causes	Action/solution
	Reagents were not mixed properly	Before pipetting solutions into wells, make sure all reagents and samples have been thoroughly mixed
	Inconsistent washing of wells	Take precautions to reduce variability of washes
	Uneven evaporation of solution from wells during incubation	Always incubate with a lid on the plate
Slow color development	Incubation temperature is wrong	Ensure plates and reagents are kept at room temperature
	Contaminated solutions	Make fresh solutions
	Detection reagent too old, contaminated or used at the wrong pH	Use fresh detection reagents at the correct pH

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