ELISA Technical Guide

biomat

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1. INTRODUCTION

The **enzyme-linked immunosorbent assay (ELISA),** also called *enzyme immunoassay* (EIA), is an effective method for detecting and quantifying the presence of specific substances, commonly proteins (or peptides, antibodies, and hormones) in a complex liquid mixture, using antibodies. ELISA is one of the most sensitive and reproducible plate-based technologies available. The assay you can set up is *rapid*, *simple to perform,* and *easy to automate*.

Biomat manufactures polystyrene microplates used in diagnostic and pharmaceutical fields since 1992. Biomat's products include:

- **Uncoated polystyrene Medium- and High-Binding Microplates**

- **Pre-coated ELISA Microplates** such as *Streptavidin, Neutravidin, Protein A and G, Lectins, Goat Anti-Mouse IgG and Goat Anti-Rabbit IgG*.

2. ELISA TECHNOLOGY

The **ELISA test** is a plate-based rapid test used for detecting and quantifying proteins, peptides, antibodies, and hormones in a liquid sample.

It may be used to diagnose:

- HIV
- Lyme disease
- Pernicious anemia
- Rocky Mountain spotted fever
- Rotavirus
- Squamous cell carcinoma
- Syphilis
- Toxoplasmosis
- Varicella-zoster virus
- Zika virus

ELISA test includes 4 different steps:

1. The **solid phase** is performed on a 96-well polystyrene plate. The function of the solid phase is to *immobilize a specific lipoprotein target*, which is passively adsorbed. This step is called **coating**.
2. The bound target is complexed with an excess of a specific anti-target linked to an enzyme, called **conjugate**.
3. After an **incubation step,** the plate is washed to eliminate any excess of unbound conjugate in the reaction medium.
4. Finally, the **enzyme activity of the bound enzyme is measured** using a substrate that changes color when modified by the enzyme. Light absorption of the product, formed after substrate addition, is measured and converted to numeric values.

Depending on the lipoprotein target-antibody combination, the assay is called:

- direct ELISA
- indirect ELISA
- competitive ELISA
- sandwich ELISA.

Direct ELISA

The target lipoprotein (antigen in the picture) is bound to the bottom of the microplate well and is recognized by a specific enzyme-conjugated antibody that allows detection through the Chromogen/Substrate reaction.

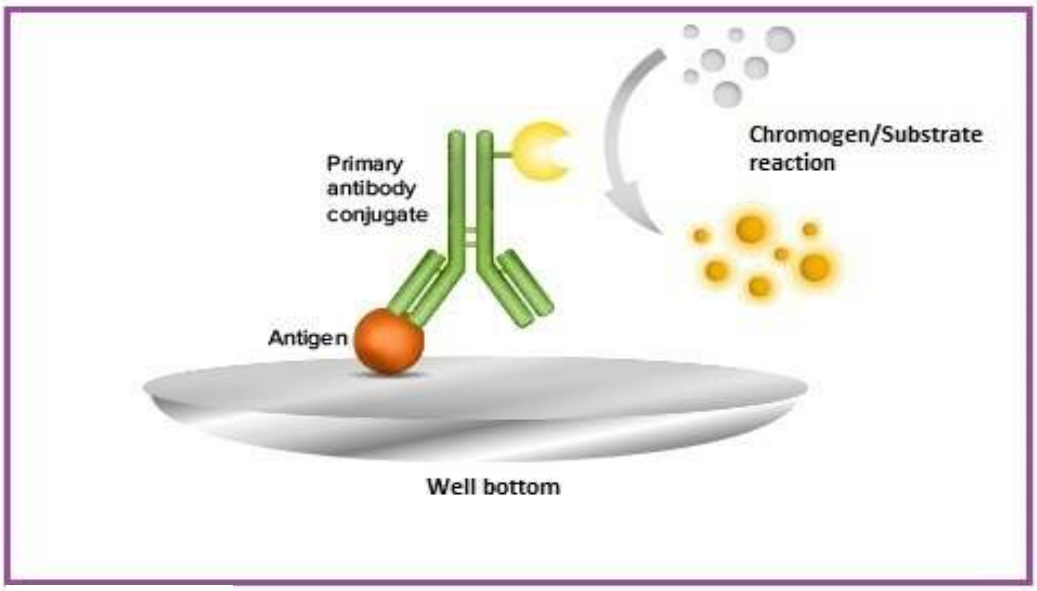


Image 1

Indirect ELISA

The target lipoprotein (antigen in the picture) is bound to the bottom of the microplate well, then an antigen-specific antibody (primary antibody in the picture) is added. A secondary enzyme-conjugated antibody that binds to the first antibody is added, allowing its detection through the Chromogen/Substrate reaction.

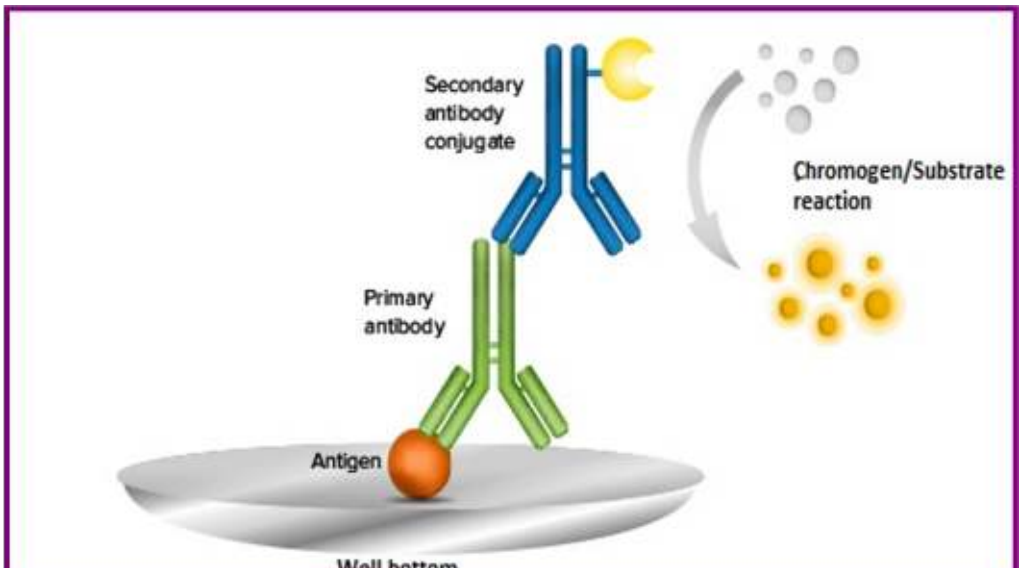


Image 2

Competitive ELISA

The target lipoprotein (antigen in the picture) is bound to the bottom of the microplate well.

The sample plus an antigen-specific enzyme-conjugated antibody (primary antibody conjugate in the picture) are added to the wells. If the sample includes an antigen (inhibitor antigen in the picture), it competes with the antigen bound to the well for binding to the specific antibody. Unbound material is washed away. The more antigen is in the sample, the less specific antibody ends up being bound to the coated antigen, and the lower is the final signal.

The Chromogen/Substrate reaction allows detecting the specific enzyme-conjugated antibody bound to the antigen.

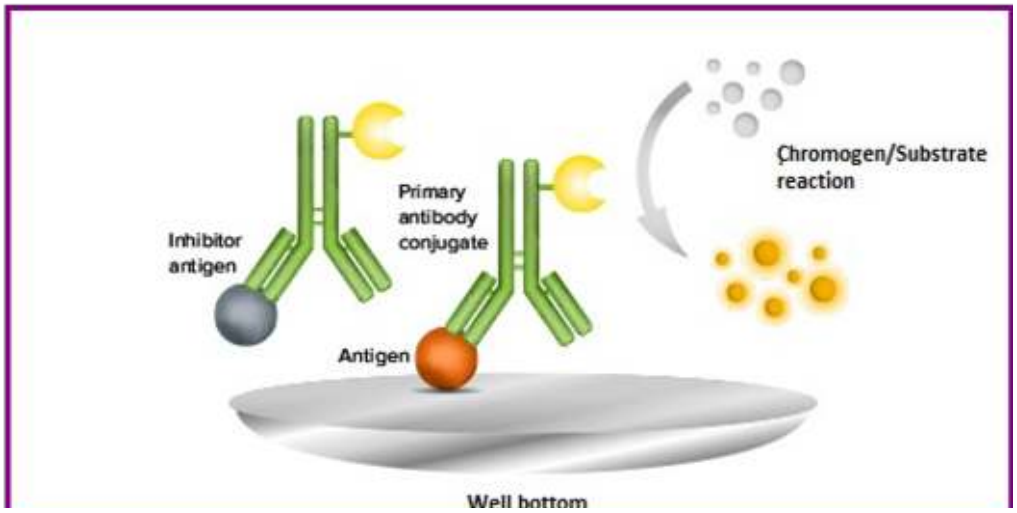


Image 3

Sandwich ELISA

For this type of ELISA, two antibodies specific to two different epitopes on the target lipoprotein (antigen in the picture) are used. The capture antibody (primary antibody in the picture) is bound to the bottom of the microplate well and binds to one antigen epitope. The detection antibody (secondary antibody in the picture) binds to a different antigen epitope and is conjugated to an enzyme, allowing detection through the Chromogen/Substrate reaction.

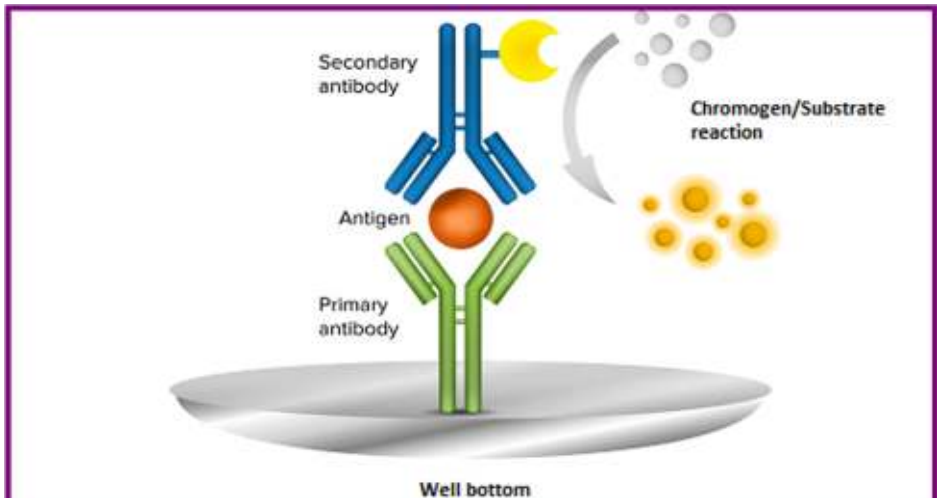


Image 4

ELISA: Example of an assay using a 96-well plate

The following picture shows a plate at the end of the assay. An HRP conjugate has been used with TMB + H_2O_2 as chromogen/substrate.

Image 5

The yellow color indicates that the target protein is present. A darker color means a higher concentration of the target protein.

3. SOLID SUPPORT FOR ELISA TESTS: MICROPLATES

Biomat offers both **Medium Binding Microplates** and a **High Binding Microplates** for passive adsorption. In addition, Biomat offers coated and treated surfaces, see **surfaces for microplates.**

Each surface type is tested to guarantee reproducibility of binding capacity, and coating uniformity. For assistance in your assay development or for assay development services, please contact us.

FORMAT & COLOR

Biomat 's full range of **96-well plates** for immunoassay includes:

- 96-Well Breakable Strip Plates (on single well-holding frame)
- 96-Well Strip Plates (assembled on 12 x 8 well-holding frame)
- 96-Well Solid Plates

They are made of **Clear, White,** and **Black Polystyrene** and can be used for *ELISA, Luminescence (White)*, and *Fluorescence (Black) assays*.

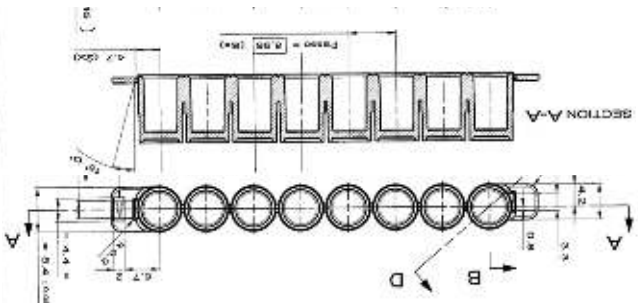
DESIGN

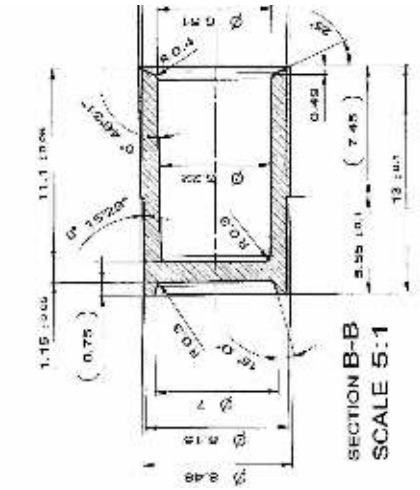
Biomat's microplates design offers the best performance for the following reasons:

- manufactured from pure polystyrene with low fluorescence
- optical quality, important to reduce the background signal, is pursued through the mold design
- inner bottom edged radius of the wells improves washing effectiveness
- external lid ensuring vertical alignment when using single wells
- rim protecting the bottom's external face from scratches
- plates comply with SBS standards, and the design ensures a good performance in automatic processing plants.

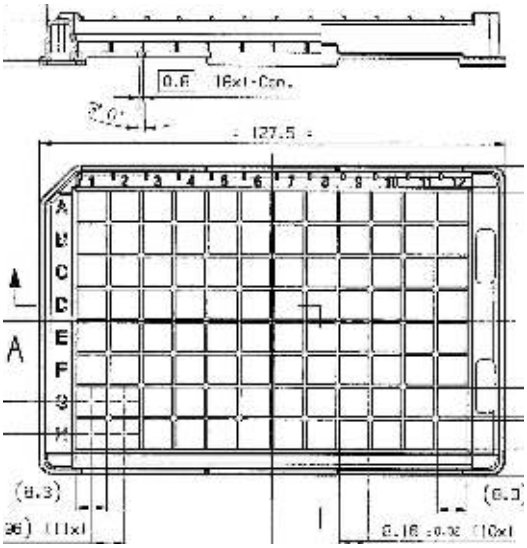
Biomat's 96-Well Strip Plates are offered with different well capacities:

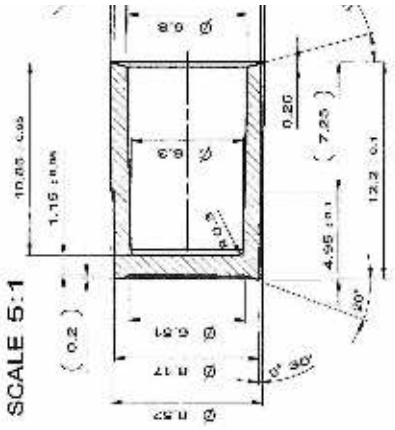
- 350 µl
- 360 µl
- 400 µl



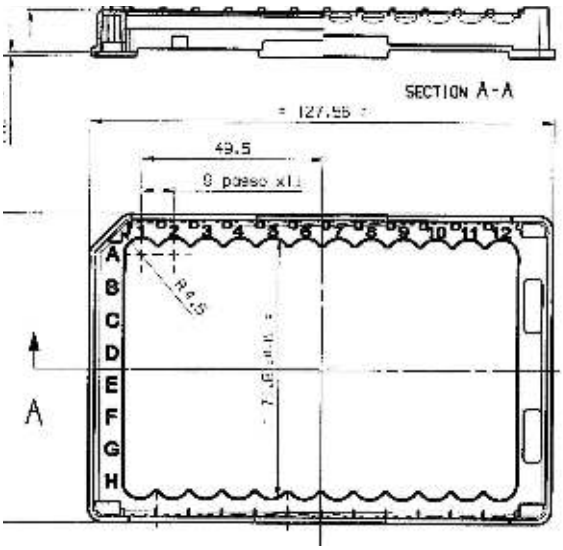


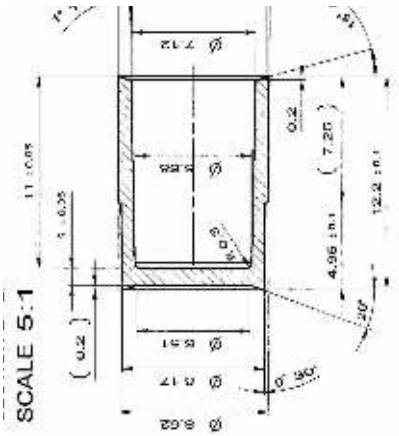
350µl





360µl





400µl

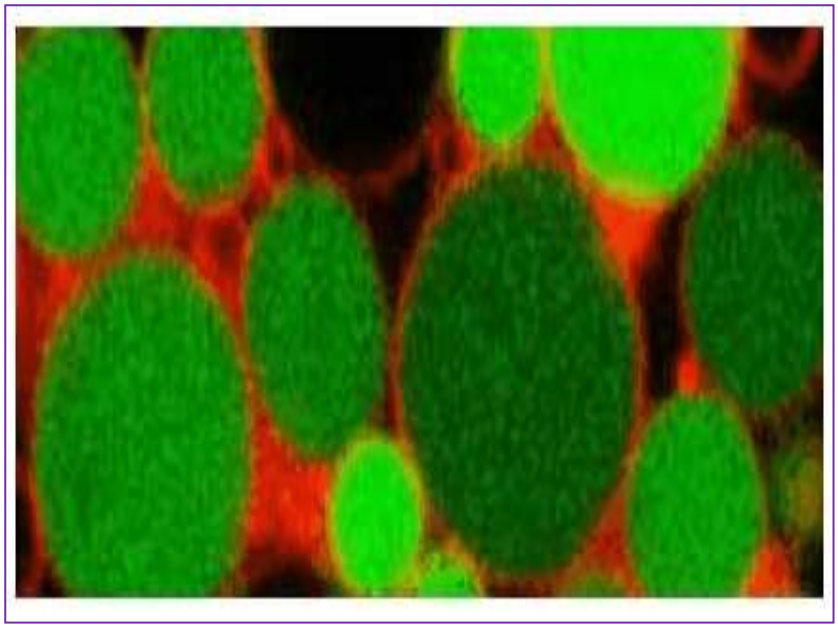
Image 6

Materials

Biomat's ELISA microplates are manufactured from polystyrene, the most widely used material for this application. Polystyrene is hydrophobic, however its properties can be modified by using surface treatments or coatings to accommodate different requirements for different applications. Thanks to these features, it is best suited for microscopic imaging and optical measurements.

Polystyrene is composed of an aliphatic carbon chain with pendant intermittent benzene rings. This provides a very **hydrophobic surface,** and this type of microplates is typically referred to as **Medium Binding microplates**.

The binding capacity can be enhanced through a surface treatment, such as irradiation, which breaks a certain number of benzene rings, yielding carboxyl (COOH) and hydroxyl (OH) groups. The presence of these groups provides an opportunity for hydrophilic interactions. Microplates modified in this way are typically referred to as **High Binding microplates**. The resulting surface is primarily hydrophobic with intermittent carboxylic and hydroxylic groups capable of ionic interactions with positively charged groups on biomolecules.



hydrophobic polystyrene
(Medium Binding)

hydrophobic/hydrophilic
polystyrene
(High Binding)

Image 7

The forces that passively adsorb biomolecules through the surface of *Medium Binding* and *High Binding* plates in increasing order of strength are:

- hydrophobic interactions
- ionic interactions
- van der Waals forces
- hydrogen bonding

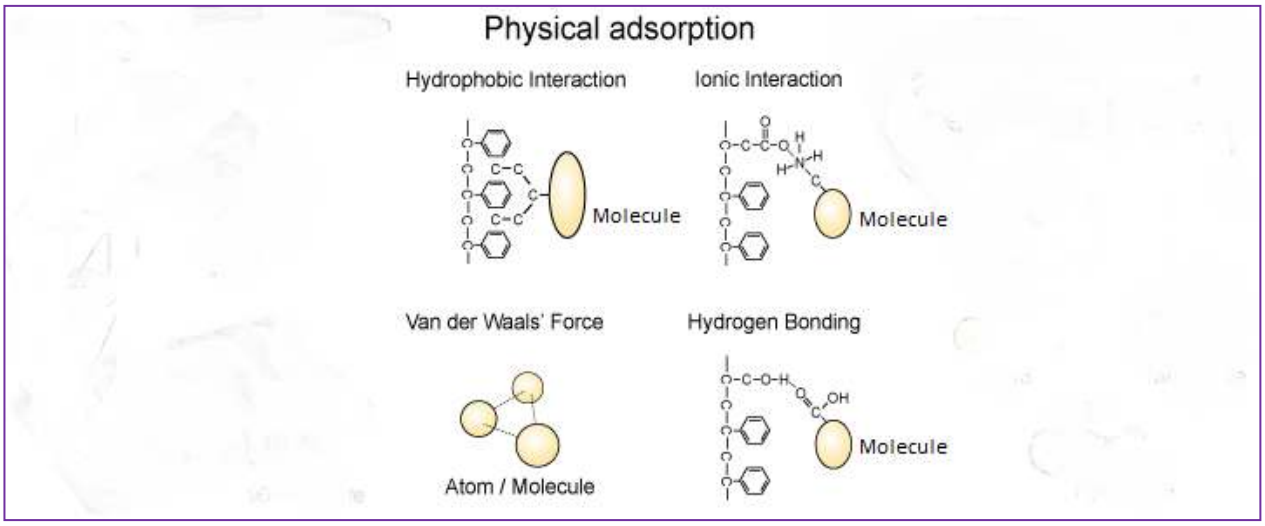


Image 8

The above picture describes the four physical adsorptions that occur when a bio-molecule comes into contact with the bottom of polystyrene microplates.

Quality/reliability

Biomat selected the best medical grade raw materials to produce its microplates. We mold all our products in clean conditions and following strict quality controls, in complete absence of contaminants.

Thanks to the ongoing clients' feedback, we were able to improve the stability of the wells in our frames, that come off easily but at the same time are stable in the washing phase.

All lots are tested to ensure they meet our quality standards.

HIGH BINDING vs. MEDIUM BINDING CAPACITY

High Binding Surfaces are ideal for **hydrophilic interactions**, whereas Medium Binding Surfaces are **hydrophobic**.

High Binding Capacity

Biomat High Binding Surface is a hydrophilic surface **suitable for passive adsorption of proteins with different degrees of hydrophilicity**. This surface is ideal for immunoassays with a binding capacity of 400 to 500 ng IgG/cm^2.

Furthermore, this surface is highly selective and shows **high affinity for molecules adsorption,** also when they are present in very small amounts (<50 ng/cm^2), thus allowing for maximum test sensitivity**.**

Available configurations

Biomat 's **High Binding surfaces** are available on **96-well plates:**

- High Binding 96-Well Breakable Strip Plates,
- High Binding 96-Well Strip Plates,
- High Binding Solid Plates

Competitors surface comparison

Test results showed that:
• a comparable binding capacity of proteins of Biomat HB8 and competitor strips
• the capacity of both types of samples to ensure specific binding between the coated protein and the protein to be revealed: our test results (on 232 sera, 94 positive and 128 negative) confirmed the sensitivity and specificity of both sample types with all tested sera
• the result of regression analysis, whose acceptable value had been established at R 0.95, was fully met
• the ranges of coefficients and values obtained in equation (1), which must not significantly differ from 0 and whose values range was $0.8 \leq b \leq 1.2$, proved the strict results correspondence.

Medium Binding Capacity

Biomat Medium Binding Surface is a hydrophobic surface **suitable for passive adsorption of proteins** with different degrees of hydrophobicity.

It is used in assays in which adsorbed molecules **present large hydrophobic regions**, such as antibodies.

Furthermore, this surface is highly selective and shows **high affinity for hydrophobic polypeptides with a molecular weight > 10kDa**.

Available configurations

Biomat's Medium Binding surfaces are available on **96-well plates:**

- Medium Binding 96-Well Breakable Strip Plates
- Medium Binding 96-Well Strip Plates
- Medium Binding 96-Well Solid Plates

Uniformity

Biomat High Binding and Medium Binding Microplates have a CV% of less than 5 using:

- HIgG as a coating molecule in an ELISA format, anti HIgG-HRP as detector and TMB as substrate

4. COATING BUFFERS, TIME OF ABSORPTION, TEMPERATURE, AND BIOMOLECULE CONCENTRATION

A key factor in the immobilization of molecules coating step is the pH of the coating buffer in which the biomolecules are dissolved.

Selecting a coating buffer with a pH between 6.0 and 9.6 can influence the total charge and the steric structure of the biomolecules' binding, thus affecting their immobilization.

Recommended buffers for immobilization are:
Coating Buffer - 0.1 M Phosphate buffer pH 6.0 (Biomat code 100-5)
Coating Buffer - 0.1 M Phosphate buffer pH 7.2 (Biomat code 100-1)
Coating Buffer - 0.1 M Carbonate/Bicarbonate buffer pH 9.6 (Biomat code 100-9)

Nevertheless, for some antibodies (IgG species), an acid pH lower than 6.0 can have a more favorable structure and charge for adsorptive binding to the microplate.

To this end, we suggest using citrate-phosphate and acetate buffers.

Moreover, other key factors need to be kept in mind to get a correct ELISA coating such as:

- temperature
- time of adsorption
- concentration of biomolecules being adsorbed

The most thorough adsorption and lowest well-to-well variation occurs overnight (16-18 hours) at + 4°C, with sealed wells to prevent evaporation. Adsorption time can be speeded up by incubation at room temperature for 4-8 hours or at 37°C for 1-4 hours.

A concentration range of 1-10 µg/ml of biomolecules, in a volume of 50-100 µl, is a good guide for the level of biomolecules needed to saturate available sites on a polystyrene microplate.

5. BLOCKING BUFFERS

Coating wells with a specific binding biomolecule leaves unoccupied hydrophobic/hydrophilic sites on the polystyrene bottom of the plate. These sites must be blocked in a specific step called *"post-coating"* to prevent non-specific binding of subsequent reactants.

Failure to perform this step will result in the assay suffering from high background signal and lowered specificity and sensitivity.

The following picture of a typical sandwich ELISA shows the problem.

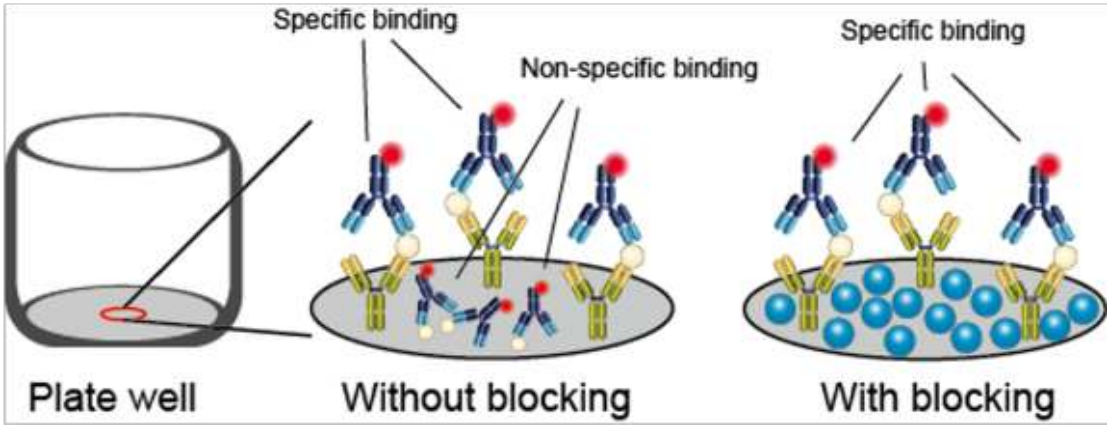


Image 9

As you can see, without the *blocking step* the enzyme labeled secondary antibody can be adsorbed onto the bottom of the plate in a non-specific manner. This will cause the background of the final reaction.

By performing the ***blocking step***, on the other hand, the enzyme labeled secondary antibody only binds to the antigen, creating a correct sandwich with the coated primary antibody.

The *blocking step* helps reducing non-specific binding and **increasing the signal-to-noise ratio**. To prevent non-specific binding, buffer solutions called "blocking buffers" are used after the *solid phase coating step* to block remaining open binding sites.

Blocking buffers are typically chosen in an empirical way. The best blocker for one assay may not perform well in other assays.

The two major classes of blocking agents that are currently used are proteins and non-proteins.

The most typical proteins used are:

- Bovine serum albumin (BSA) at 1-5% in PBS 0.1 M pH 7.2
- Non-fat-dry milk (NFDM) at 0.1-0.5% in PBS 0.1 M pH 7.2
- Normal serum 5-10% diluted in PBS 0.1 M pH 7.2
- Casein at 1-5% in PBS 0.1 M pH 7.2
- Fish gelatin at 1-5% in PBS 0.1 M pH 7.2

Recommended product: Biomat's Blocking Solution - ELISA Blocking - BSA based (Biomat code 300-1).

Example of non-proteins blockers include polymers such as:

- Polyethylene glycol (PEG)
- Polyvynil alcohol (PVA)
- Polyvynilpyrrolidone (PVP)

These blocking reagents are known for their ability to coat hydrophobic surfaces and make them both non-binding and hydrophilic. They should be used with a M.W. of 20 to 50 kDa.

Other most common *non-protein blocker agents* are non-ionic detergents such as Tween 20 and Triton X-100 at low concentrations, usually 0.01-0.1%.

An alternative would be to combine the use of a *non-ionic detergent* with a protein blocker. Recommended product: Biomat Blocking Solution - BlockerWell - non proteic (Biomat code 300-2).

6. ELISA ASSAY DILUENTS

Sample Diluent

A sample diluent is used to carry biomolecules during the first step of an ELISA assay. It is designed to reduce background noise and to help equalize matrix in problematic serum, plasma, or cell culture samples.

For all ELISA formats, it should contain mammalian serum proteins to reduce non-specific interactions between biomolecules and the microplate surface.

To avoid a potential target analyte under-recovery, it is mandatory to equalize the protein content of the diluted sample and diluted standard point curve proteins of the assay.

Standard curve points should also contain inhibitor complement, thrombin activity, and antimicrobial agent.

Recommended product: Biomat Diluent & Stabilizer - Sample Diluent (Biomat code 400-1)

Conjugate Diluent

The two commonly used enzymes in ELISA are *Horseradish Peroxidase (HRP)* and *Alkaline Phosphatase (AP)*. In order to be used in the assay, HRP and AP molecules must first be conjugated to the detection antibody or antigen.

After being conjugated to HRP or AP, these antibodies or antigens are stored at 2-8°C as concentrated stock solution or diluted in an appropriate buffer to the desired assay dilution factor.

Performing the assay starting from the concentrated stock solution requires diluting it every time at the desired assay dilution factor. You need a diluent that can ensure adequate reactivity and specificity during the test session. These diluents are known as disposable diluents.
Recommended product: Biomat Diluent & Stabilizer - Diluent for HRP conjugate (Biomat code 400-2)

To run the assay with a ready-to-use, long-term stored, diluted conjugate at the desired assay dilution factor, the diluent needs to have stabilizing properties to maintain its molecular conformation and prevent loss of activity over time.

Recommended product: Biomat Diluent & Stabilizer - HRP-AP Stabilizing buffer (Biomat code 400-3)

7. WASH BUFFER

ELISA washing steps remove unbound nonspecific materials and are very important within the ELISA protocol, as ELISA uses surface binding for separation.

The wash buffer is generally PBS (phosphate ions should be avoided with Alkaline Phosphatase), with a small concentration of a non-ionic detergent such as Tween 20.

Washing is typically repeated 3-5 times between each ELISA step, with a 30-second incubation for each washing step to thoroughly remove unbound non-specific material.

It was observed that a greater number of washes is more effective than a longer washing time. In the final washing step, excess of wash solution must be carefully removed to prevent the reagent added in the subsequent step to be diluted and possibly contaminated.

When the test is run manually, this can be achieved by simply tapping the washed plate upside down on absorbent paper.

When the test is performed on automated equipment, special care should be taken in positioning the needle height vacuum to suck up all the liquid from the bottom of the well.

Recommended product: Biomat Wash Buffer - Phosphate buffer saline 0.01 M pH 7.2 with Tween 20 , Wash Buffer (Biomat code 200-3) - Phosphate buffer saline 0.01 M pH 7.2 (Biomat code 200-1)

8. ELISA STEPS OVERVIEW

Below is a description of the main steps of the four assay types described at point 2.

Step	Direct	Indirect		Competitive	Sandwich
Coating (Adsorption to solid phase)	Antigen	Antigen		Antigen	Capture antibody
Wash		Separate bound / unbound biomolecules			
Blocking		Addition of blocking agent to prevent non-specific binding. Removal of blocking agent to start the assay			
Analyte (Addition of test sample)	Enzyme-conjugated antibody	Unconjugated antibody		Enzyme-conjugated antibody + unknow antigen being teste	Antigen sample
Wash		Separate bound / unbound analytes			
Secondary reagent	N/A	Enzyme-conjugated antibody		N/A	Enzyme-conjugated detection antibody
Wash		Separate bound / unbound analytes			
Signal development		Addition of chromogen/substrate for enzyme-conjugated antibodies			
Stop signal development		For end-point reading of enzyme-based detection systems			
Signal detection		Colorimetric detection			

N.B.: The same flow chart applies to fluorescent or chemiluminescent assays, using appropriate materials such as:

- black microplates for fluorescent assays or white microplates for chemiluminescent assays
- fluorophores for fluorescent antibody-labelled or acridinium ester for chemiluminescent assays
- antibody-labelled related chromogen/substrate for fluorescent or chemiluminescent assays.

9. Technical tips for ELISA

WELL SURFACE

The area covered by the liquid is the one effectively involved in the reactions.
At each level, the total area includes: the bottom surface, which is the same for every level, and the wall surface, which can vary.

OPTICAL PATH

The optical path is another important feature, since it can affect the results of the O.D. reading, sometimes giving a false impression of higher or lower sensitivity.
A smaller well diameter means that the same quantity of liquid will reach a higher level, so that the beam will have a longer path through the colored liquid. Some other factors as:
– the shape of the liquid meniscus
– the exact alignment of the light beam so that it passes through the very center of the well which may be lower or higher, depending upon the well wettability
– the wettability itself affects this value, therefore these figures are only indicative

Level ml	Optical path mm	Bottom surface mm^2	Wall surface mm^2	Total surface mm^2
100	2.98	30.68	61.27	91.95
200	5.98	30.68	117.37	148.05
300	8.97	30.68	176.05	206.73

*data refer to 96-Well Strip Plates.

10. COMMON ELISA TROUBLESHOOTING

ELISA involves multiple assay components and steps, which often require troubleshooting and optimization to correctly perform a test.

In this **ELISA troubleshooting guide** we listed solutions to some of the most common **problems** you may face during an **ELISA test**.

For specific help or support with an ELISA development, please contact our technical department at info@biomat.it.

Problem: Weak signal or no signal

Possible cause	Solution
Reagents are not at room temperature at start of assay	It is recommended that all reagents be at room temperature before starting the assay. Allow reagents to rest on the bench for 30-60 minutes to reach room temperature.
Expired reagents	Confirm expiration dates on all reagents. Do not use reagents that are past their expiration date.
Reagents added/prepared incorrectly	Check the protocol and ensure that reagents were added in the proper order and correctly diluted.
Plate read at incorrect wavelength	Ensure plate reader is set accurately for the substrate type being used.

Problem: High signal

Possible cause	Solution
Wells were insufficiently washed	Wash wells as per protocol recommendations.
Reagents prepared incorrectly	Check protocol, ensure reagents were prepared at the correct dilution.
Longer incubation times than recommended	Make sure to follow recommended incubation times.

Contaminants from laboratory glassware	Make sure reagents are prepared in clean glassware.
Waiting too long to read the plate after adding stop solution	Read plate immediately after adding stop solution.

Problem: High background

Possible cause	Solution
Insufficient washing	Increasing number of washing steps or adding soaking steps may help.
Substrate exposed to light prior to use	Ensure substrate is not exposed to light, store in a dark place. Limit exposure to light while running assay.
Pipetting errors	Calibrate pipettes so that they dispense correct volumes.
Longer incubation times than recommended	Make sure incubation times are correct.
Contamination of buffers	Always prepare fresh buffer.
Cross-reactivity	Ensure no cross-reactivity between detection antibodies and assay components occurs. Run appropriate controls.

Problem: High variation

Possible cause	Solution
Plate washing was not adequate or even	Make sure pipette tips are tightly secured. Confirm all reagents are completely removed in all wash steps.
Pipetting errors	Calibrate pipettes.
Cross well contamination	Care should be taken when using the same pipette tips for reagent addition. Ensure that pipette tips do not touch the reagent on the plate.

Inconsistent incubation temperature	Be aware of fluctuations in temperature due to environmental conditions.
Non-homogenous samples	Thoroughly mix samples before pipetting. Remove particulate matter if present.
Plates stacked during incubations	Stacking plates does not allow constant temperature Across plate wells. Avoid stacking.
Bubbles in wells	Ensure no bubbles are present prior to reading the plate.
Reagents are not well mixed	To ensure a consistent concentration across all wells, ensure all reagents and samples are mixed before pipetting onto the plates.
Evaporation	If there is a risk of evaporation during the assay steps, seal the plate completely with a plate sealer during incubation.

Sources

Sources: Images 1, 2, 3, 4 from Molecular Devices

Source: Image 9 from Creative Diagnostics

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Rev. 00