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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. Among its offering Biomat proposes:

- 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
- *Activated microplates* such as Aminated, Carboxylated, Maleimide coated

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, as an example, the following disease states:

- Hepatitis
- Retrovirus
- ToRCH (Toxoplasmosis, Rubella, Cytomegalovirus, Herpex Simples)
- EBV
- MMVZ (Measles, Mumps, Varicella, Zooster)
- Syphilis
- Tubercolosis
- Autoimmunity

ELISA test consists of 4 different main phases:

1. In ELISA technology the **solid phase** is performed on a 96-well polystyrene plates. The function of the solid phase is to *immobilize a specific lipo-proteic target*, which is passively adsorbed. This phase is called **coating**.
2. Then the bound target is complexed with an excess of a specific anti target that is linked to an enzyme, called **conjugate**.
3. After an **incubation step** the plate is washed to eliminate the unbound conjugate that remains free in the reaction medium.
4. Finally, the **enzyme activity of 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 lipo-proteic target-antibody combination, the assay is called:

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

Direct ELISA

The target lipo-protein (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, by the Chromogen/Substrate reaction.

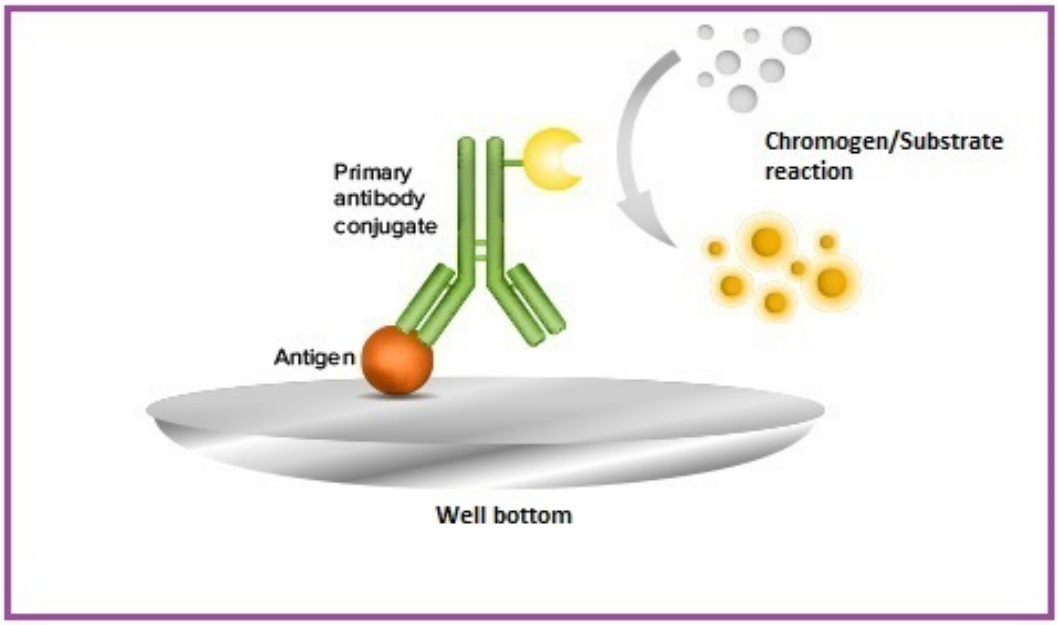


Image 1

Indirect ELISA

The target lipo-protein (antigen in the picture) is bound to the bottom of the microplate well, then a specific antibody to the antigen (primary antibody in the picture) is added. Secondary enzyme conjugated antibody that binds to the first antibody is added, allowing its detection, by Chromogen/Substrate reaction.

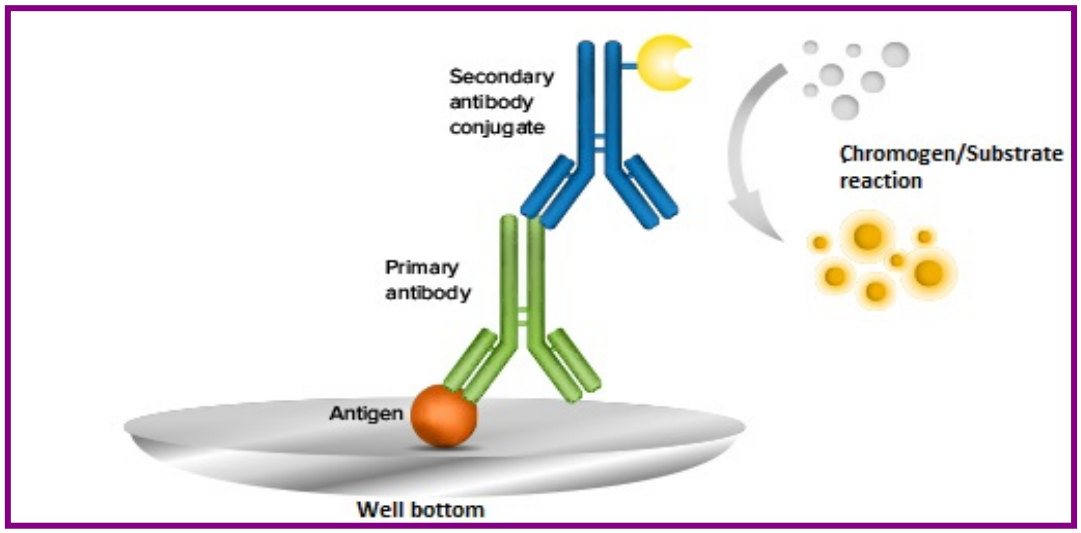


Image 2

Competitive ELISA

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

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

The Chromogen/Substrate reaction allows the final detection of the specific enzyme conjugated antibody bound to the antigen.

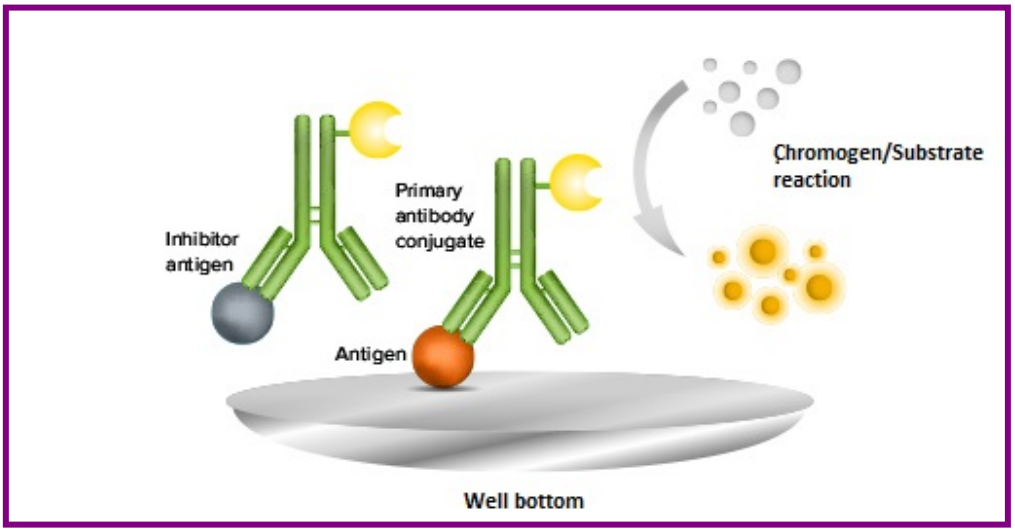


Image 3

Sandwich ELISA

For this type of ELISA two antibodies specific to two different epitopes on the lipo-protein target (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 one epitope of the antigen. The detection antibody (secondary antibody in the picture) binds the antigen at a different epitope and is conjugated to an enzyme, allowing detection, by the Chromogen/Substrate reaction.

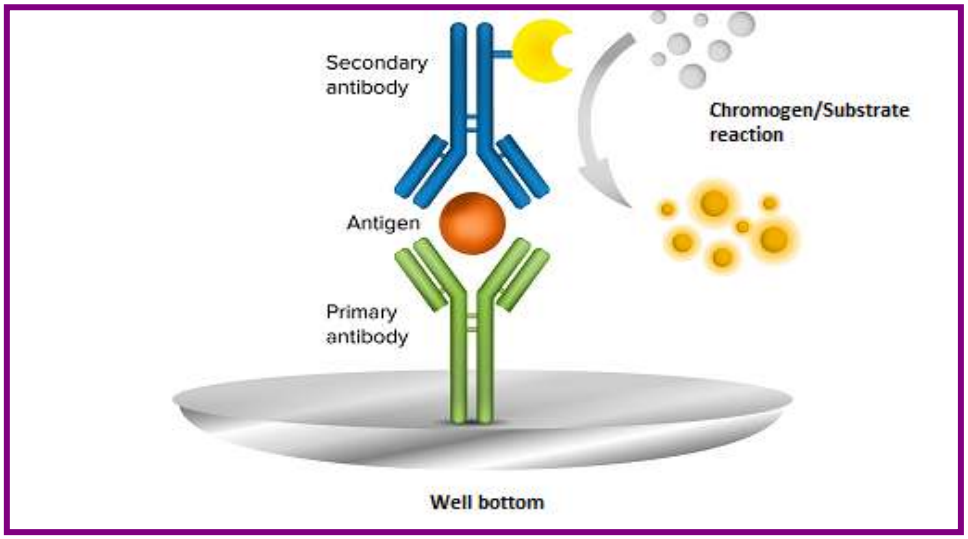


Image 4

ELISA: An example of an assay using a 96-well plate

The following picture shows, as example, a plate at the end of the assay. In 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. The higher degree of the color, the higher concentration of the target protein.

3. SOLID SUPPORT FOR ELISA TESTS: MICROPLATES

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

Each type of surface is tested in order to guarantee reproducibility of binding capacity and uniformity of the coating. If you need assistance in your assay development or are looking for assay development services, please contact us.

FORMAT & COLOR

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

- solid,
- 8 well strip format (assembled on 12 x 8 well-holding frame) and in
- 8-well breakable strip format (on single well-holding frame)

allowing the maximum flexibility for the user.

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

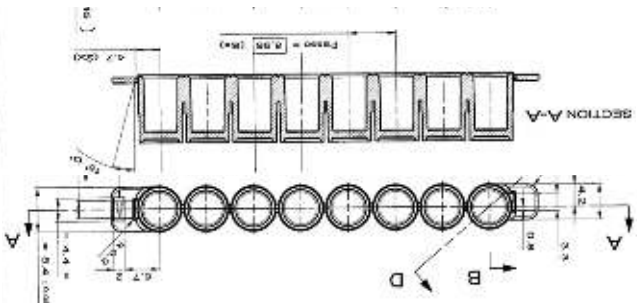
DESIGN

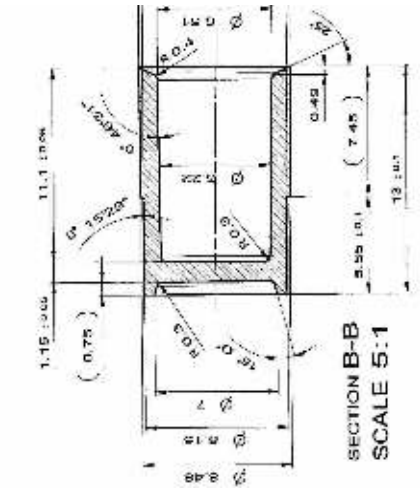
Biomat's microplates design offers the best performances for the following reasons:

- manufactured in pure polystyrene with low fluorescence
- optical quality, important to reduce the background signal, is pursued through the mould design
- inner bottom edged radius of the wells improves the efficiency of washings
- the external lid warrants vertical alignment when using single wells
- a rim protects the external face of the bottom from scratches
- the plates comply with SBS standards and the design assures a good performance in automatic processing plant

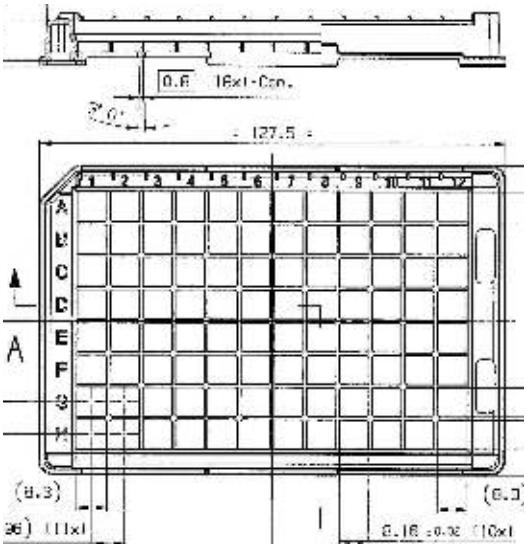
Biomat's 96 Wells Strip Plates are offered with different Well capacities:

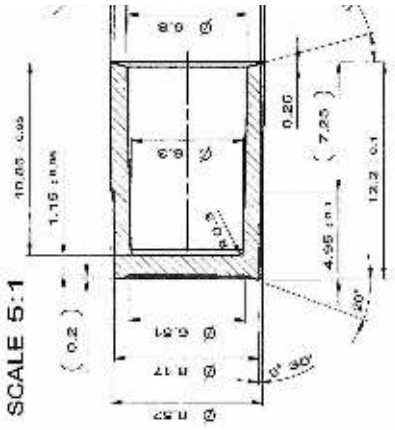
- 350 µl
- 360 µl
- 400 µl



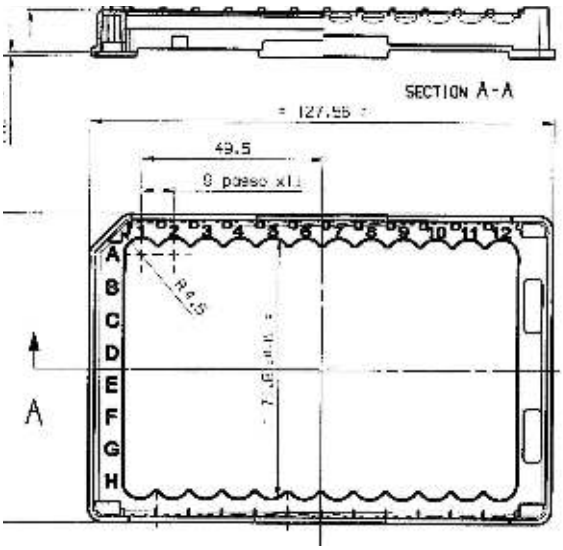


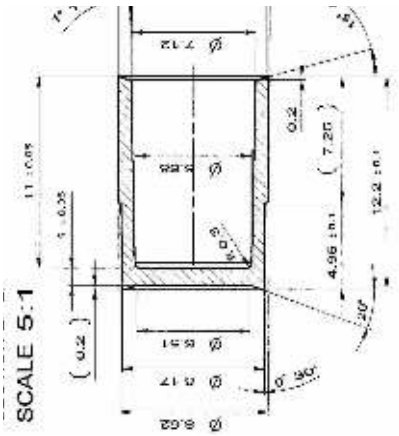
350µl





360µl





400µl

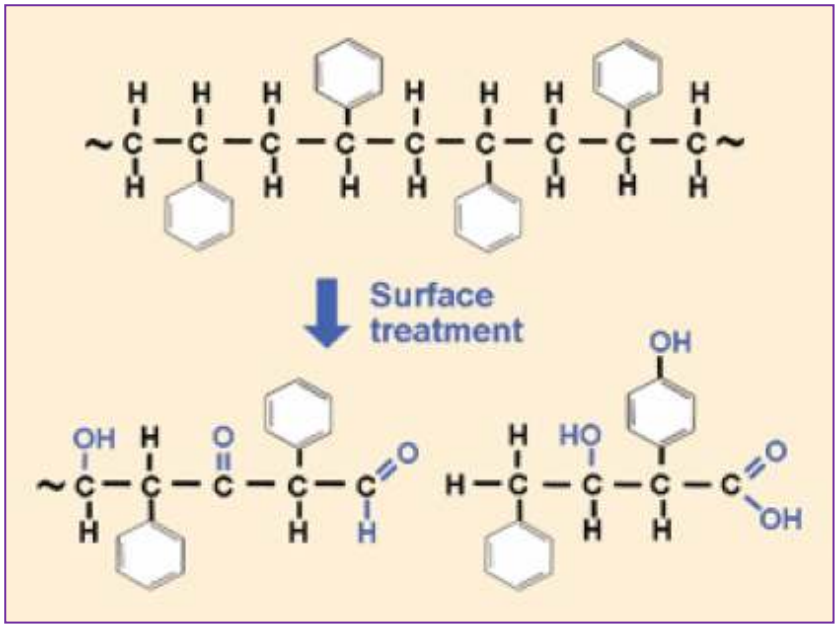
Image 6

Materials

Biomat's ELISA microplates are produced in polystyrene, the most widely used material for this application. Polystyrene is hydrophobic however its properties can be modified by performing surface treatments or coatings to accommodate different requirements for diverse applications and its characteristics allow it to be the 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 microplates of this type are typically referred to as **Medium Binding**.

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**. 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* in order of increasing strength are:

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

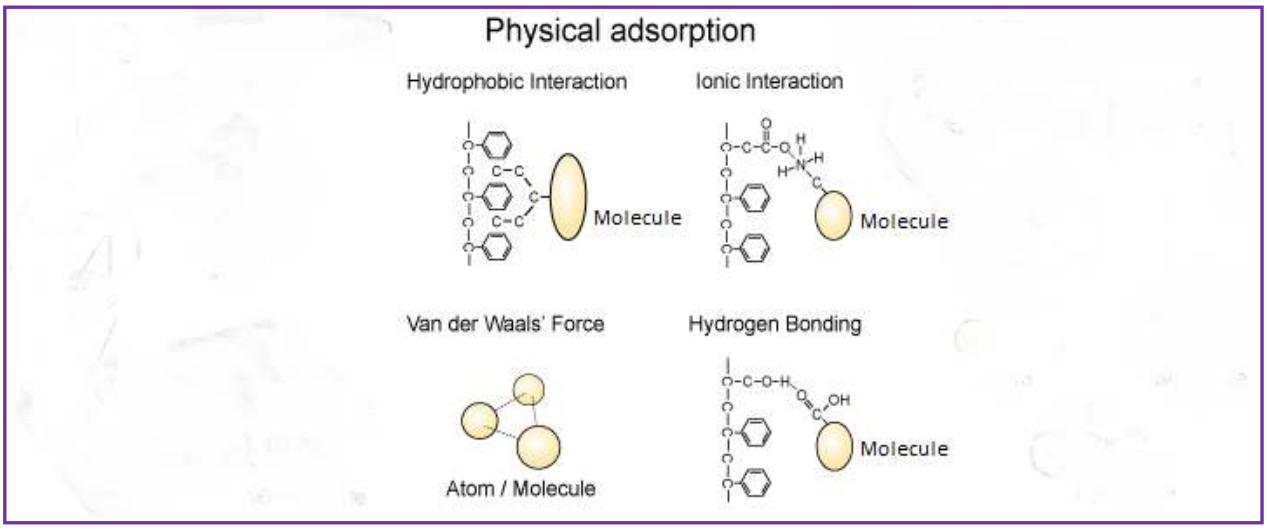


Image 8

The above pictures describe the four physical adsorptions that occur when the bio- molecule comes in contact with the bottom of polystyrene microplate.

Quality/reliability

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

Thanks to the continue feedback of the clients we have improved the stability of our wells in our frames that come off easily but are also stable in the washing phase.

All lots are tested to guarantee their quality.

HIGH BINDING vs. MEDIUM BINDING CAPACITY

The High Binding Surface provides an opportunity for **hydrophilic interactions**, whereas Medium Binding Surface provides a **hydrophobic surface**.

High Binding Capacity

Biomat High binding surface is a hydrophilic surface **suitable for passive adsorption of proteins with different grades 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 towards the adsorption of molecules** also when they are present in very small amounts (<50 ng/cm^2) allowing to obtain the maximum sensitivity of the test**.**

Available configurations

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

- High Binding Solid Plates,
- High Binding 8 Well Strip Plates
- High Binding 8-Well Breakable Strip Plates

Competitors surface comparison

Analysis results show:

• a comparable binding capacity of proteins of both Biomat HB8 and competitor's strips

• the capacity of both types of samples to assure the specific binding between the coated protein and the protein to be revealed: 100% of results of our tests (on 232 sera, 94 positive and 128 negative) were confirmed stating the sensitivity and specificity of both types of samples with all the tested sera

• the result of regression analysis, whose acceptable value had been fixed at R 0.95 has been fully respected

• the ranges of coefficients and values obtained in equation $y = a+bx$ where the *a* values must not significantly differ from 0 and the *b* values must fall in the range 0.8 – 1.2 proved the strict correspondence of results

Medium Binding Capacity

Biomat Medium binding surface is a hydrophobic surface **suitable for passive adsorption of proteins** with different grades of hydrophobicity.

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

Furthermore this surface is highly selective and shows **high affinity towards hydrophobic polypeptides that present a molecular weight > 10kDa**.

Available configurations

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

- solid plates
- 8 well strip plates
- 8-well breakable strip plates

Uniformity

Biomat High Binding and Medium Binding Microplates show a CV% 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 immobilization of molecules coating step, can be the pH of the coating buffer in which the biomolecules are dissolved in.

Selecting a coating buffer between pH 6.0 and pH 9.6 can have an effect on the total charge and the steric structure of the biomolecules' binding and this affects biomolecules 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.

For this scope citrate-phosphate and acetate buffers are suggested.

Moreover other key factors such as:

- temperature
- time of adsorption
- concentration of the biomolecule being adsorbed have to be kept in mind to get a correct ELISA coating

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

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

5. BLOCKING BUFFERS

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

If this step is not performed, the assay will suffer from high background signal and lowered specificity and sensitivity.

The following picture, representing a typical sandwich ELISA assay, highlights the problem.

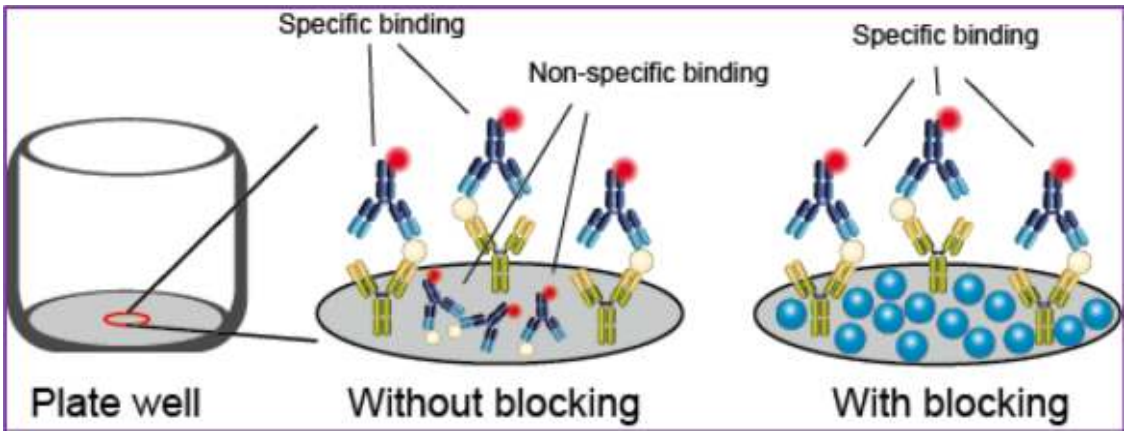


Image 9

It is possible to see, that without the *blocking step*, the enzyme labeled secondary antibody can be adsorbed to the bottom of the plate in a non-specific manner. This will cause the background of the final reaction.

On the contrary, by performing the ***blocking step*** the enzyme labeled secondary antibody binds only the antigen, creating a correct sandwich with the coated primary antibody.

The *blocking step* works to reduce non-specific binding and allowing to **increase 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 manner. The optimum 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 render them both non-binding as well as hydrophilic. They should be used with a M.W. comprise between 20 and 50 kDa.

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

Moreover, 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

The sample diluent in an ELISA assay is used to carry the biomolecule during the first step of the assay. It is thought and designed to reduce background noise and to assist in matrix equalization in problematic serum, plasma or cell culture sample.

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

In order to avoid the possible cause of target analyte under-recovery it is mandatory to equalize the protein content of 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 assays 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.

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

If you want to work the assay starting from the concentrated stock solution you have to dilute it every time at the desired assay dilution factor. You need a diluent that is able to guarantee a correct reactivity and specificity during the test session. This kind of diluents are the so-called disposable diluents.
Recommended product: Biomat Diluent & Stabilizer - Diluent for HRP conjugate (Biomat code 400-2)

If you want to work the assay with a diluted conjugate, ready-to-use, stored for long time at the desired assay dilution factor, the diluent needs stabilizing properties to maintain the 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 wash steps remove unbound nonspecific materials and are very important component of ELISA protocol as ELISA assay uses surface binding for separation.

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

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

It has been observed that a greater number of washes are more effective than a longer time of washing. In the final wash step excess of wash solution must be carefully removed to prevent the dilution and possible contamination of the reagent added in the subsequent stage.

This aim can be achieved when the test is run manually simply tapping the washed plate upside down on an absorbent paper.

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

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

8. ELISA ASSAY STEPS OVERVIEWS

Below is the description of the main steps in the four type assays, discussed 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; removing the blocking agent to start the assay			
Analyte (Addition of testing sample)	Enzyme-conjugated antibody	Unconjugated antibody	Enzyme-conjugated antibody + unknown antigen under evaluation	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			

NOTE: the same flow chart is valid for fluorescent or chemiluminescent assays, using the appropriate materials such as:

- black microplate for fluorescent assay or white microplate for chemiluminescent assay
- fluorophores for fluorescent antibody-labelled or acridinium ester for chemiluminescent 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 is variable.

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 diameter of the well means that the same quantity of liquid will reach a higher level, so that the beam will have a longer path through the coloured liquid. Obviously some other factors as:
– the shape of the meniscus of the liquid
– the exact alignment of the light beam so that it passes at the very center of the well which is lower or higher, depending upon the wettability of the well
– the wettability itself affects this value, so these figures are intended only to be indicative

Level µl	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

Performing ELISA requires multiple assay components and steps, and therefore, there is often a need for troubleshooting and optimization to carry out a correct test.

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

If you need specific help or support with an ELISA development please contact our technical department at info@biomat.it

Problem: Weak 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 stay on the bench or 30-60 minutes to reach room temperature.
Expired reagents	Confirm expiration dates on all reagents. Do not use reagents that are past the expiration date.
Reagents added/prepared incorrectly	Check the protocol and ensure that regents were added in the proper order and prepared to correct dilution.
Plate read at incorrect wavelength	Ensure plate reader is set accurately for type of substrate being used.

Problem: High signal

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

Contaminants from laboratory glassware	Ensure 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 dispense the correct volumes.
Longer incubation times than recommended	Make sure your incubation times are correct.
Contamination of buffers	Always make fresh buffer.
Cross-reactivity	Ensure no cross reactivity among detection antibody with constituents of assay. Run appropriate controls.

Problem: High variation

Possible cause	Solution
Plate washing was not adequate or uniform	Make sure pipette tips are tightly secured. Confirm all reagents are removed completely 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. Pay attention at removing particulate matter if present.
Plates stacked during the incubations	Stacking of plates does not allow constant temperature across the wells of plates. 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 evaporation can occur during the assay steps, seal the t plate completely with a plate sealer during incubations.

Sources

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

Source: Image 9 from Creative Diagnostics

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