DEVELOPMENT, OPTIMIZATION AND EVALUATION OF HIV ELISA

Dissertation submitted in partial fulfilment of the requirement for the

degree of Masters of Science

In

Biotechnology

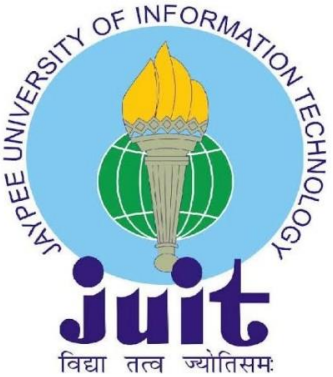
By

Varsha Sharma (225111021)

Under the supervision of

Dr. Shikha Mittal and Ms. Mamta Tewari

To



Department of Biotechnology & Bioinformatics

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MAY, 2024

DECLARATION

I hereby declare that the work presented in this report entitled **"Development, Optimization and Evaluation of HIV ELISA"** in partial fulfilment of the requirements for the award of the degree of **Master of Science in Biotechnology** submitted in the Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology, Waknaghat is an authentic record of my work carried out over a period from 5th February'2024 – 15th May'2024 under the supervision of **Ms. Mamta Tewari** J.Mitra & Co. Pvt. Ltd.A-180-181, Okhla Industrial Area, Phase-1, New Delhi-110020, INDIA and **Dr. Shikha Mittal** Department of Biotechnology and Bioinformatics, Jaypee University of information technology, Waknaghat, Solan Himachal Pradesh. I also authenticate that I have carried out the above-mentioned project work under the proficiency stream.

I further declare that the work reported in the major project has not been submitted and will not be submitted, either in part or in full, for the award of any other degree or diploma in this Institute or any other Institute or university by me.

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CERTIFICATE

This is to certify that the work reported in the M.Sc. Biotechnology thesis entitled "**Development, Optimization and Evaluation of HIV ELISA**", submitted by **Ms. Varsha Sharma (225111021) at Jaypee University of Information Technology, Waknaghat, India,** is a bonafide record of her original work carried out from 5th February 2024 – 15th May 2024 under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

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TO WHOM SO EVER IT MAY CONCERN

This is to certify that **Ms. Varsha Sharma,** student of M.Sc. Biotechnology (Final Year) of Jaypee University of Information Technology, Waknaghat, Distt. Solan (H.P.) India doing her internship from J. Mitra & Co. Pvt. Ltd., A-180-181, Okhla Industrial Area, Phase-1, New Delhi-110020, INDIA. Her Internship period is from 05-02-2024 to 04-07-2024. Ms. Varsha Sharma has completed a project for thesis entitled ***"Development, Optimization and Evaluation of HIV ELISA",*** is a bonafide record of her original work carried out from 05-02-2024 to 15-05-2024. This work has not been submitted elsewhere for any other degree or diploma.

This letter is issued on the request of Ms. Varsha Sharma for the submission of her M.Sc. Biotechnology thesis.

Ms. Mamta Tewari

R & D Head

J. Mitra & Co. Pvt. Ltd.

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My training in the J. MITRA and Co. Pvt. Ltd. has been a great experience which will help me a lot throughout my career. I would like to extend my love and thanks to my friends for their trust, support and love.

Thank you

Varsha Sharma

225111021

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LIST OF ABBREVIATIONS AND ACRONYMS

ELISA	Enzyme Linked Immunosorbent Assay
Ag	Antigen
Ab	Antibody
HRP	Horseradish Peroxidase
TMB	3,3',5,5'-Tetramethylbenzidine
PBS	Phosphate Buffer Saline
BSA	Bovine Serum Albumin
CBT	Checker Board Titration
CD	Conjugate Diluent
OD	Optical Density
H_2O_2	Hydrogen Peroxide
H_2SO_4	Sulfuric acid
Na_2CO_3	Sodium Carbonate
$NaHCO_3$	Sodium bicarbonate
DTT	Dithiothreitol
AP	Alkaline Phosphatase
HIV	Human Immunodeficiency Virus
AIDS	Acquired Immune Deficiency Syndrome
%	Percent
°C	Degree Celsius
µl	Microliter
ml	Milliliter
OD	Optical Density
nm	Nanometre

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ABSTRACT

Development of HIV ELISA is based on indirect ELISA, where envelope proteins like gp41, C terminus of gp120 and gp36 are coated on a microtiter plate with a coating buffer. If the serum contains the antibody specific for the antigen coated it will go and bind to that. Washing is done to remove unbound sites as they can produce background. Followed by the addition of secondary Ab which is HRP conjugated to antihuman IgG which can go bind Ab in serum. Finally, substrate is added having hydrogen peroxide which shows a colour change if the sample is positive. A stop solution is added and the plate is read at 450nm. The conditions like coating buffer, blocking buffer, time, temperature, pH, concentration of Ag coated, washing steps everything is optimized for developing an HIV ELISA plate. The sensitivity and specificity of the plate are then compared with the J. Mitra HIV ELISA market plate.

CHAPTER -1

INTRODUCTION

1. Introduction

In 1960 a new method was described by Rosalyn Yalow and Solomon Berson where they used radiolabelled antigen for detecting endogenous insulin [1]. Known as RadioImmunoAssay (RIA). Using the principle of RIA 2 scientists in 1970 in the Netherlands, Anton Schuurs and Bauke Van Weemen; in Sweden, Peter Perlman and Eva Engvall developed a method where instead of radioactive iodine 125 they conjugated enzyme with Ag /Ab [2, 3, 4]. Now known as ELISA.

ELISA is a technique used to detect and quantify antigen, antibody, vitamins and hormones. Where reaction components are adsorbed non-specifically or covalently on the surface of 96 well microtiter plate.

The reaction in ELISA is based solely on Ag - Ab interactions. Where Ag bounds on the solid surface and enzyme-linked Ab goes and binds to that Ag. The addition of substrate develops a blue colour which turns yellow as soon as it comes in contact with the stop solution.

1.1 Stages in ELISA

The general stages that are involved in ELISA are:-

1. Adsorption of Ab/ Ag on microtiter plate with coating buffer.

2. Blocking of the plate using blocking buffer.

3. Stabilization and drying of plate.

4. Addition of serum with sample diluent and incubation.

5. Washing to remove unbound and free reagents.

6. Addition of Ab linked with enzyme.

7. Addition of substrate and check for colour development.

8. Stopped the reaction using stop solution and took OD at 450nm.

Four types of ELISA are:

Direct, indirect, sandwich, and competitive [5, 6]. Direct ELISA Ag/Ab is coated on 96-well microtiter plate and then an enzyme linked Ag/Ab is used resulting in an Ag-Ab*Enzyme complex the only disadvantage was the labelling of Ag/Ab every time. To overcome this problem indirect ELISA was used that contains a second enzyme-linked antibody. In the sandwich ELISA Ag that needs to be detected is sandwiched between the coated Ab (capture Ab) and the same Ab but in enzyme-labelled form (detecting Ab). In indirect sandwich ELISA different species of Ab are used and the secondary enzyme-conjugated Ab is added followed by incubation, washing and addition of substrate. In competitive ELISA capture Ab specific to Ag is coated onto a plate and then incubated with a sample followed by the addition of labelled Ag to the wells they compete to bind the Ab sites [7].

1.2 HIV AIDS

Human immunodeficiency virus is a retrovirus that have the ability to infect and transmit among humans, which on further development causes AIDS. It can be transmitted through blood, sexual fluids or mother to child during the pre or post-natal period. It makes the immune system weak and the virus remains in inactive form before entering the host cell. This virus attacks our immune system and decreases the level of CD4+ which is the helper T cells that protect our body [8]. The antigen first appears in the body of HIV-positive people, but after a month or two, serological conversion causes the Ag to be eliminated and the level of antibodies against the virus to rise.

Fluids that have high concentrations of HIV are: Blood and its components, semen, vaginal fluids, and breast milk.

HIV entry into human cells

HIV makes copies inside the human cells when a person gets infected virus enters through the cell having CD4 on its surface. HIV infects T helper cells which makes our immune system weak. The reverse transcriptase enzyme helps in RNA-to-DNA conversion. The DNA goes and binds to the cell's DNA with the help of integrase which gets converted to mRNA. HIV protein and enzymes combine to create fresh virus

particles, which split off from the parent CD4 cell. The HIV protein's lengthy chains are broken up into smaller bits by the protease enzyme. These viruses can now infect additional cells [9].

1.3 Principle of ELISA for detection of HIV

Indirect ELISA forms the core of the ELISA HIV test. Microtiter wells are coated using the HIV envelope proteins gp41, gp36 for HIV-2, and the HIV-1's C terminus of gp 120 represents immunodominant epitopes. The microtiter wells are filled with specimens and controls, then they are incubated. If there are any antibodies to HIV-1 or HIV-2 in the samples, they will attach to the particular antigens that are absorbed onto the wall surfaces. Next, unbound material is removed from the plate by washing it. Each well receives an addition of antihuman IgG coupled with horseradish peroxidase (HRP). This combination will attach itself to the existing HIV antigen-antibody complex. Lastly, the wells are filled with a substrate solution that contains hydrogen peroxide and chromogen, and it is then incubated. The number of HIV antibodies in the serum will cause a blue colour to appear. A stop solution ends the colour reaction. The EIA reader measures the absorbance of the enzyme-substrate reaction at 450 nm. Enzyme conjugate will not bind in the absence of HIV antibodies in the sample, leaving the solution colourless or merely developing a light background.

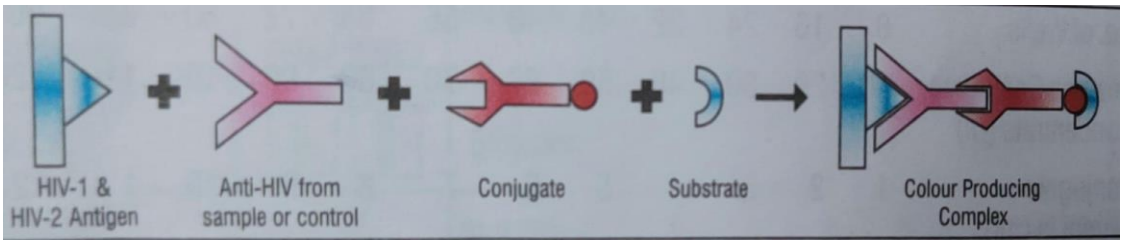


Figure 1 Indirect ELISA for detection of HIV where Ab in serum binds Ag of the coated plate and ant species enzyme-linked Ab goes and binds that Ab when the substrate is added it shows the colour change [Source J.Mitra Microlisa HIV manual]

1.4 Aims and Objectives

1. Developing an ELISA plate that is specific and sensitive for detecting HIV antibodies.
2. Optimizing various parameters like antigen concentration, antibody dilution, incubation times, and temperature conditions.
3. To evaluate the performance of the developed plate by performing the test and its comparison with the J. Mitra HIV ELISA plate.

CHAPTER 2

REVIEW OF LITERATURE

2. Review of literature

ELISA is a technique used for the clinical analysis of various immunological signals. In this technique, one component is passively adsorbed on the surface of the polystyrene plate while the other goes and binds to that component emitting colour when comes in contact with the substrate. In the most common approach Ab is adsorbed on the microtiter plate and Ag in the serum goes and binds to the bound antibody after washing secondary Ab that is enzyme-linked Ab is added forming a sandwich of Ab-Ag-Ab enzyme complex. On addition of substrate, colour develops indicating quantity of Ag in the body [10].

2.1 General principle of ELISA

ELISA is solely based on the interaction of Ag-Ab. Quantitative analysis of various proteins like peptides, hormones, and nucleic acid can be done with the help of ELISA. The detection is done by immobilizing the protein on the solid surface of polystyrene, polypropylene and polyvinyl chloride. The Ag reacts with the Ab and the enzyme labelled Ab generally horseradish peroxidase (HRP) [11], alkaline phosphatase (ALP) [12]and β-galactosidase [13, 14, 15] are commonly used. When substrates like TMB and o-phenylenediamine is added the enzyme labelled to the Ab converts it into a coloured product indicating the presence of Ag. Stopped reaction with the help of HCl, H_2SO_4, Na_2CO_3 and NaN_3 [16, 17].

2.2 General process of ELISA

Depending on the type of test different ELISA are used based on a different principle where the concentration of primary, secondary Ab, antigen, buffers and substrate can vary accordingly [18], where primary Ab binds to a specific Ag while secondary Ab is the detection Ab and it binds to primary Ab [19].

The steps followed in ELISA are:

1. Coating
2. Blocking
3. Testing

4. Reading at 450nm

For ELISA, a 96-well polystyrene microtiter plate (12 x 8) is frequently utilised. The material used to make the microtiter plate is extremely hydrophobic and is formed of a lengthy carbon chain with benzene rings bonded to each alternating carbon. This substance increases the ability of the microtiter plate to bind proteins by forming hydrophobic contacts between the solid matrix and the non-polar structure of the protein. The plate surface can be coated with antibodies either directly or indirectly [20].

In the testing stage detection is done by adding a substrate that gives a blue colour. After each step washing is done using wash buffer generally PBS and non-ionic detergent, to remove unbound materials.

2.3 Types of ELISA are:

1. Direct ELISA (Ag--Ab**Enz + S) – Detecting antibody.

2. Indirect ELISA (Ag--Ab--AntiAb**Enz + S) – Detecting antigen/antibody.

3. Sandwich ELISA (Ag—Ab—Ag** Enz +S) – Detecting antigen.

4. Competitive ELISA – Detecting antibody.

1. Direct ELISA:

In direct ELISA Ag is mainly coated onto a plate with a coating buffer of high pH (9.6) where carbonate and PBS are preferred. Mainly a concentration of 1 to 10µg/ml is preferred. Buffer is used as it contains no other protein that competes with the antigen. The plate is incubated at 37℃ for 1 hour or 4℃ overnight. Washing is a must after these steps to remove unbound Ag to the surface followed by blocking of the plate with suitable blockers like BSA, ovalbumin, aprotinin, or other animal proteins [21]. Conjugated Ab is diluted in a buffer that is proteins which can be added in high concentration for competing solid phase sites with Ab protein or detergents are used known as blocking agents. These blocking agents prevent non-specific binding.

Blocking is followed by rewashing of the plate and addition of detecting enzyme-linked Ab is added. Here conjugated Ab binds to antigen. The substrate is added and a colour change of the enzyme alkaline phosphatase (AP) or horseradish peroxidase (HRP) occurs. The colour of the sample changes as a result of either HRP oxidising substrates or AP hydrolyzing phosphate groups from the substrate [22]. This test shows low sensitivity but neglects cross-reactivity of secondary Ab and also it is rapid.

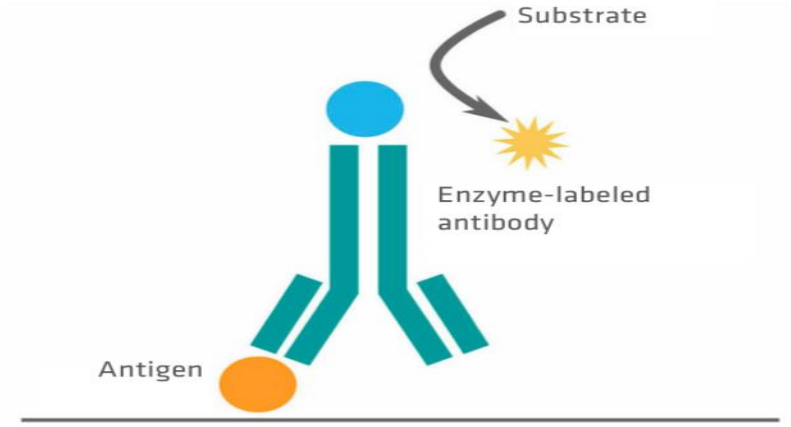


Figure 2 Direct ELISA (Source: The complete guide to ELISA Integra)

2. Indirect ELISA:

Indirect ELISA involves the addition of an enzyme labelled Ab which is usually diluted in conjugate diluent (CD) which is a buffer that helps prevent nonspecific binding. Followed by washing to remove unbound and nonspecific Ab. Substrate is then added to the bound conjugate [20]. The binding indicates colour change. Then stop solution is added and the reading is done in a spectrophotometer. Here Ab is targeted by another Ab that is enzyme-linked. Indirect ELISA uses antispecies antibodies, which are generated against Ig of the species from which primary antibodies are made [20].

The advantage of this ELISA is that several sera can be examined for binding Ag using single antispecies conjugate while the fact that each sera has a different level of nonspecific binding is a disadvantage [20].

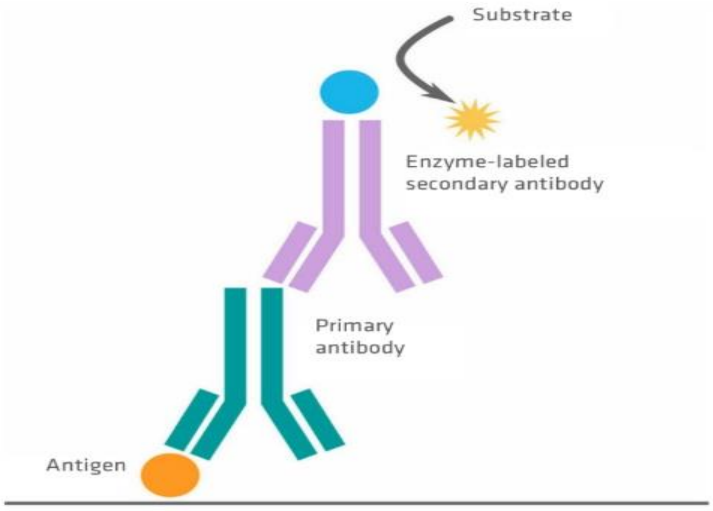


Figure 3 Indirect ELISA (Source: The complete guide to ELISA Integra)

3. Sandwich ELISA:

As the name suggests it is called a sandwich because Ag that needs to be detected is sandwiched between primary Ab (capture Ab) and secondary Ab (detection Ab) [23]. In this Elisa Ab are passively adsorbed to the solid surface of the plate these Ab are known as capture Ab. After that serum having the Ag is added if specific Ag is present it will it will go and bind to the Ab [20]. The Ag is diluted in sample diluent which is a buffer used to block the nonspecific attachment to solid surface of the plate. Ag and Ab form complex on attachment to solid surface after that secondary Ab conjugated with Enzyme are added which can be same as that of primary Ab. The sandwich is formed as Ab-Ag-Ab**Enz followed by the addition of substrate and stop solution after a particular time frame. Take OD at taken at 450nm [20].

4. Competitive ELISA

In this ELISA competition occurs between Ab in the sample and enzyme labelled Ab against the Ag coated onto the microtiter plate. These 2 Ab will compete against each other for binding Ag when added into wells. If the colour changes mean enzyme labelled Ab has bound to the Ag and the test will be declared negative while the absence in the development of colour means Ab are present in the sample and the test is declared positive [24].Thus higher the Ab in the sample, the lower the binding of the enzyme labelled Ab to Ag and vice versa. This is a simple method just like direct ELISA.

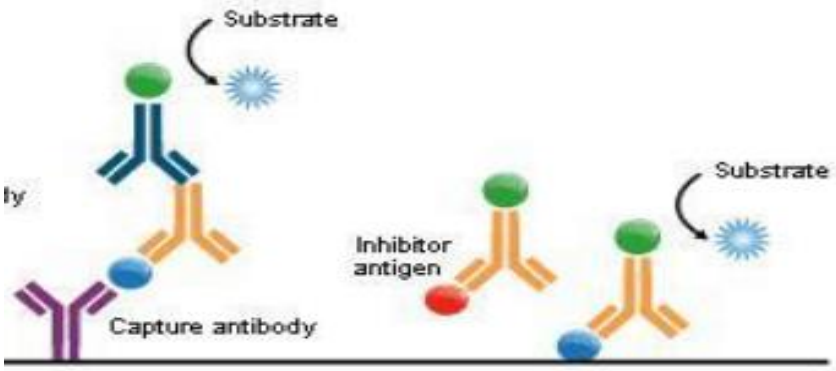


Figure 4 Sandwich ELISA and Competitive ELISA

2.4 Enzyme and substrate used in ELISA

Table 2.1 Enzymes and substrate used in ELISA [25,26]

Enzyme	**Substrate**
Alkaline phosphatase	p-nitrophenyl phosphate (PNPP)
Horseradish peroxidase	3,3',5,5'-Tetramethylbenzidine.
β-Galactosidase	o-nitrophenyl-β-D-galactopyranoside

2.4.1 HRP

Choosing the right concentration of HRP is a difficult task in the optimization process as low conc. May not provide an optimum signal for the detection and too much of enzyme may cause background resulting in the poor signal [27].

HRP, is a glycoprotein (40 kDa) that can be covalently coupled to various antigens and antibodies in a variety of ways, including free amino, carboxyl, and carbohydrate groups. It is also reasonably priced, well-purified, and very stable. It is extremely active even after chemical changes and has a very high turnover number. The removal of activating chemicals and activation of HRP are necessary for the conjugation of proteins (peptides, antigens, or antibodies). The protein and activated HRP then interact.

The concentration of HRP will become green as the reaction increases, shifting from a cool, brownish-gold colour if HRP is the enzyme being oxidised.

The polysaccharide chains in HRP are oxidised by sodium periodate to produce a reactive aldehyde group, which is then conjugated with an amine-containing molecule through reductive amination. The highly poisonous sodium cyanoborohydride is used in reductive amination to create cross-linking. For cross-linking with amine-containing antibodies, conjugation is always carried out at an alkaline pH [28].

2.4.2 TMB Substrate.

TMB undergoes oxidation when horseradish peroxidase breaks down H2O2 enzymatically.
A rich blue colour characterizes the TMB oxidised product. Once the reaction has been stopped with acid, measure the optical density (OD) of the yellow colour in a typical ELISA plate reader at 450 nm to identify oxidised TMB.

2.5 Titration of reagents

When a person is not sure about the quality and quantity of the reagents then performing titrations is the way to find out and use reagents with confidence.

Chessboard titrations: Working conc. of every component needs to be evaluated. Chessboard or checkerboard titration helps in this process. In this process, two-fold dilution is achieved by diluting 2 reagents against each other. Only 2 reagents are titrated at a time on the plate. The diluting and pipetting techniques need to be fundamental to the performance of ELISA [20].

2.5.1 CBT for Indirect ELISA

Indirect ELISA: HIV ELISA is based on indirect ELISA. Indirect ELISA have 3 components to titrate antigen, antibody and antispecies conjugate. CBT indirectly indicate optimum conditions and it tells us about the concentration range of all components. The objective is to identify reagents' usefulness [20].

It is used to find the amount of Ab that should be used against specific Ag, through full titration or Single dilution. That's why optimum amount of Ag must be coated to wells that will bind to Ab.

One time only 2 assays can be titrated 1) Enough Ag to bind Ab => it is done to find out the conc. at which Ag successfully binds to the plate as coating the plate with more conc. can waste the Ag. 2) Finding optimum conjugate as it avoids nonspecific binding to achieve required specificity.

Stage 1 Initial CBT

In the initial CBT Ag is diluted against positive and negative sera by using a recommended level of antihuman Ig conjugate with enzyme. A starting conc. of 1-10 ug/ml should be used.

(a) An example of 1/50 2-fold dilution was used where Ag was diluted from columns 1 to 11 and 12 remained as control. The plate was incubated and washed.

(b) Serum having Ab was diluted against Ag in one plate negative serum in another plate. Dilution occurs from A to G while H remains in control. Dilution occurs in blocking buffer. Followed by washing and the addition of antihuman Ig linked to conjugate. After checking OD if the plateau height decreases indicates there is a loss of Ag and Ab binding [20].

Stage 2 Titration of Sera & Conjugate

The Last dilution of Ag that gives good titration is usually preferred thus this titration is used to coat the plate with Ag. In this process Ag conc. is kept constant while serum is diluted from C-1 to C-11 against conjugate from R-A to R-G [20].

2.6 Structure of HIV

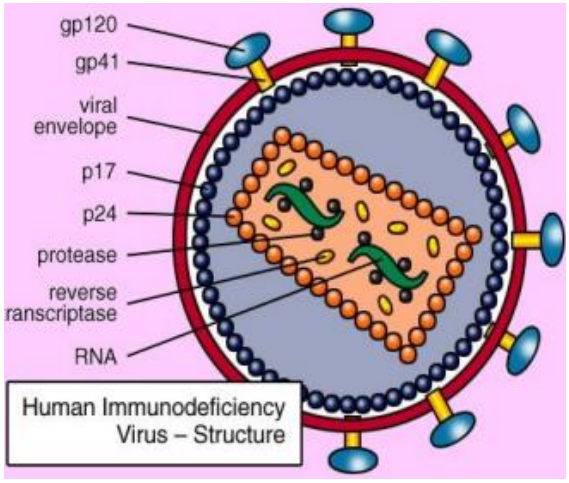


Figure 5 Structure of HIV [30].

Parts	Function
gp120	Its name, 120, is derived from its molecular weight. The enters the cells as it attaches itself to the cell surface receptors.
gp41	The human immunodeficiency virus is one of the retroviruses that contain this subunit in their envelope protein complex. Reverse transcriptase is a technique used by this family of enveloped viruses to replicate within their host cells. One host cell is the target
Viral envelope	The virus attaches itself to the envelope.
P17	Protein makes up the viral core. It mimics a bullet. Reverse transcriptase, integrase, and protease are the three enzymes needed for HIV replication.
P24	A part of the HIV capsid is P24.
Protease	It is a retroviral aspartyl protease that is necessary for HIV life cycle, which is the retrovirus responsible for AIDS. The natural protein components of an infectious HIV virion are created when this enzyme cleaves freshly synthesised polyproteins at the proper location.
Integrase	It is the enzyme that allows its genetic material to incorporated into the infected cell's DNA [29].
RNA	Long strands of DNA contain the genetic material of all living things, including the majority of viruses. Because their genes are made of RNA, retroviruses are an exception [2].

2.7 Detection of HIV using various screening tests

Existing antibodies for the envelope (gp120, gp41), as well as core (p24) proteins can be detected. Serological tests for the detection of HIV is:- screening tests E/R and confirmatory tests.[10]

E – ELISA

R – Rapid assay (dot blot, HIV spot and comb tests and latex and gelatine particle agglutination)

ELISA – In this test, Ag is coated onto a microtiter plate and serum is added if the serum contains Ab specific for Ag it will go and bound to it while the unbound serum is washed away. An enzyme linked to anti-human goat Ig is added and after that, a substrate is added that shows colour change. Development of colour is visualized using ELISA reader which shows if the test is positive or negative.

Rapid tests are the visual tests that comes in small packs. These assays employ distinct synthetic peptide antigens or HIV-1 or HIV-2 recombinant antigens. Control dots that signifies the right performance of the test. These are simple to perform and give results in 10 min. Yet expensive and give false results if sample are contaminated or stored.

Usually, a test tested positive should be confirmed by a confirmatory test based on another principle and different antigen preparation. The sample tested positive is then confirmed using the confirmatory test.[10]

Confirmatory tests: usually cross-reactivity occurs due to technical error or contaminated serum. So, these tests are done to confirm true positives as positive.

Western blot: The western blot (WB) usually detects antibodies to p24 (gag gene, core protein) and gp 120, gp 41, gp 160 (env gene, envelope protein [10]. SDS PAGE is run. The proteins are transblotted onto a nitrocellulose membrane. The membrane is sliced to react with the serum. Antibodies to HIV combine with various HIV protein fragments if they are found in the test serum. The enzyme-linked Ab is then supplied followed by the addition of substrate that shows colour change confirming the test to be positive.

Radioimmunoassay (RIA): The common detector, radioactive iodine 125 I, is covalently bonded to an antigen, an antibody, or reagents that attach to antibodies. The measurement of the gamma radiation generated by the bound 125 I is done using a gamma counter. This assay has a very high specificity and sensitivity. This test is expensive.

Immunofluorescence assay (IFA): In this assay infected cells are fixed onto a slide and they are allowed to react with test serum followed by the addition of fluorescein-conjugated anti-human gamma globulin. The positive results give apple-green colour. This test is expensive.

CHAPTER 3

OPTIZIMATION CONDITIONS FOR PLATE

Optimization is defined as choosing and improving the right proportion of known chemicals or reagents in a experiment, chemical reaction or a process. The main purpose of optimization is to achieve best efficiency and yield taking into consideration of providing stability and making the product cost-effective.

For the development of a ELISA plate there are different things starting from the buffer that is needed to coat the required Ag or Ab. On the basis of type of ELISA and medical condition that needs to be diagnosed there are different ELISA tests for detection and different conditions that are optimized and standardized for successful development of ELISA plate.

In this chapter different parameters like buffer, pH, temperature, time etc. are optimized for development of a 96 well microtiter plate for the detection of Ab against HIV by the test HIV ELISA.

The common procedure that was followed for the HIV ELISA testing are as follow:

1. Coating of Ag of a particular concentration by mixing the Ag in coating buffer.

2. Incubation of Plate followed by washing 4 times with wash buffer.

3. Blocking of plate using blocking buffer for some time at a particular temperature.

4.Stabilization and drying of Plate.

5. Testing of plate by adding 100µl of Sample diluent and 10µl of serum sample into each well. Incubating the plate for 30 min at 37℃.

6. Washing the plate with 1X PBS wash buffer for 4-5 times.

7. Added 100µl HRP by diluting it in CD (10µl in 1ml per strip).

8. Incubated plate as above followed by washing.

9. Added 100µl TMB substate in each well and incubating at room temperature for 30 min.

10. Took OD at 450 nm.

A. Optimization of buffers for coating.

Ag that needs to be coated requires a buffer of a particular pH.

Table 3.1 Different coating buffer and pH for optimization of buffer for coating.

S. No.	Name	Molarity	pH	Amount added / 100 ml
1.	Carbonate/ Bicarbonate buffer	0.1 M	8, 9, 9.8	$NaHCO_3$ = 0.84g Na_2CO_3= 1.04g
2.	Tris buffer	10mM	8	0.121g
3.	Borate buffer	0.1 M	9.5	3.81g

B. Optimization of pH of coating buffer.

A particular pH of buffer is required as it provides stability of Ag provides optimal binding and prevents non- specific binding of unwanted proteins to the plate surface.

Now the selected carbonate buffer was selected for the optimization of pH for this process following steps were followed:-

1. Weighed $NaHCO_3$ = 0.84g and Na_2CO_3 = 1.04g for 100 ml in different beakers.

2. Place them on magnetic stirrer to mix well.

3. Selected pH 9, 9.5, 9.8 for selecting best pH for coating.

4. Adjusted the pH of $NaHCO_3$ with the help of Na_2CO_3.

5. Filtered the buffers with 0.2 micron filter.

6. Coated the plate with 1 µg/ml of antigen on plate.

Followed above mentioned steps to find best pH

Result: After taking OD. pH 9 was selected for carbonate buffer as the panels were high at this pH and the background in the negative samples was comparatively low than the other pH of carbonate buffer.

C. Optimization of temperature and time for coating and blocking.

A particular temperature and time is required for the passive adsorption of Ag on the polystyrene plate otherwise the protein will denature or will not get sufficient time for the adsorption on plate.

The steps followed for coating are:-

Total 4 plates (6 strips for each temperature) at each temperature range were coated and placed for incubation. After the incubation time was over the plates were blocked and above-mentioned steps were followed for testing.

Table 3.2 Different temperature and time conditions for coating of plate.

S. No.	Temperature	Time
1	2-8°C	1 hr, 2 hr, overnight
2	25°C	1 hr, 2 hr, overnight
3	37°C	1 hr, 2 hr

Results: The plate coated for 1 hr at 37°C showed the panels having good OD. And the background for the -ve samples was also less.

After doing the coating experiment blocking experiment was also done. As plates that are coated needs to block for sufficient time and temperature so, there are no non-specific binding.

Blocking required to be done for more amount of time so to avoid non-specific binding. So, following time and temperature were selected. Same process was followed after blocking for testing.

Table 3.3 Different temperature and time conditions for blocking of plate.

S. No.	Temperature	Time
1	2-8°C	4 hr, overnight
2	25°C	4 hr, overnight
3	37°C	4 hr

Result: The best temperature was 2-8℃ and time was overnight incubation for the plate.

D. Optimization of blocking buffer

Three different blocking buffers were used having different composition buffer-A buffer- B and buffer- C. As plate was still showing background so more work needs to be done on blocking of plate. Above mentioned procedure was followed for the testing of plate.

Composition of the buffers were

Table 3.4 Three different buffers used for the blocking of plate to pick the best that can minimize nonspecific background.

Buffer - A	**Buffer - B**	**Buffer – C**
25X PBS	25X PBS	25X PBS
2.5 % BSA	2.5 % BSA	2.5 % BSA
5% Goat sera	5% Goat sera	2.5% Goat sera
0.01% Tween-20	0.05% Tween-20	0.01% triton-x

Out of these buffer buffer-B was selected as it helped in increasing sensitivity and removing background from the plate.

E. Optimization of different additives

As background was still their in the plate after testing different additives were tried during coating to remove non-specific binding.

The name, concentration and use of additives are as follows:

Table 3.5 Different additives and their concentration

S. No.	Name	Concentration
1	SDS	0.1%
2	Chaps	0.1%
3	DTT	25 mM
4	Triton-X	0.01%

4 plates were coated along with each additive added to the plate along with antigen and coating buffer.

Result: There was no such change in the OD. Of the plate. So, no addition of additive was selected for further experimentation.

F. Optimization of CD for plate

After the plate sensitivity was good the work needs to be done on plate specificity. For which experiments on different CD was done 4 different CD were provided (composition not disclosed by the company) and experiments were done to select the best CD for the plate.

10µl of HRP was added for each ml of CD. Same process was followed for checking the results.

Results: CD – 3 was selected as the background was minimum in this case and it was showing good OD for +ve panels.

G. Optimization of conc. Of ag to be coated on plate through CBT

Did many experiments to find out right conc. but there was background issues in the negative sample and panels were also not clear. So, both sensitivity and specificity was a issue. Followed a new method to find out right conc. Through checkerboard titration.

Steps Followed were:

1. 50 µl of CB was added in whole plate with the help of multichannel.

2. Dissolved 10, 8, 5, 2.5, 1 µg/ml of Ag in 1 ml CB now it is diluted in 1:1 ration with CB as conc. Of ag. was 2.29 mg/ml.

3. Added 50 µl of coating buffer in each well.

4. Added 50µl of these prepared ag concentrations in column-1 of plate with the help of multichannel and pipetted 4-5 times to mix well and transferred 50µl to the next column. Followed the same procedure of dilution from 1 to 11 after mixing in the 11th column discarded the 50µl. As the column 12 will act as control that contain no ag. In this way dilution were from 1:1 to 1:2048. Now allowed 1 hr incubation at 37℃.

5. Meantime prepared positive control (diluted human serum that is positive for HIV antibodies) and negative control dilution by adding 500µl of blocking buffer and 500 µl of positive control and negative control in 2 tubes and vortexed well.

6. After incubation plate was washed 4 times. Now plate is ready for vertical dilution from A – H for which 50µl of blocking was added in each well after that the prepared dilution of +ve control is added in row A from 1-12. This time dilution is from A-G vertically the last row will act as control containing no Ab.

7. The plates are incubated at 2-8℃ and same procedure was followed for testing starting from addition of enzyme conjugate.

8. Dilution of +ve and -ve serums to check the dilution that a plate can detect.

9. As the selected concentration of Ag was 2.5µg/ml so it is diluted in 1:1 ratio with coated buffer and 50µl of prepared dilution is added on plate. Coat the plate and incubate for 1 hr.

10. Dilution of 4 panel and 2 -ve serum is dono in 1:1 ratio with blocking buffer.Added panel 1 in A-1 and A-2 panel 2 in A-3 and A-4 and panel 3 in A-5, 6 and panel 4 in 7,8 and 2 different -ve serum in 9 to 12. Dilute the serum from A – H. Incubated at 2-8℃ and followed same procedure for testing from addition of enzyme conjugate. Result: 2.5µg/ml concentration of Ag was selected at 1:1 dilution. Same 1:1 dilution was selected for serum and panel.

CHAPTER 4

MATERIALS AND METHODS

4.1 Materials required

4.1.1 Chemicals

Table 4.1 List of Chemicals.

NaHCO3= 0.82g	Tween 20
Na2CO3= 1.04g,	10 mM phosphate buffer,
25 X PBS	Casein (0.1%)
2.5% BSA,	(0.1% triton-x),
5% Goat sera	1% BSA
Gp-41 P-11 (Antigen)	H_2SO_4
HRP	Conjugate diluent
TMB substrate	TMB diluent

4.2.2 Instruments required

Table 4.2 List of Instruments

pH meter	Magnetic stirrer
spectrophotometer	Incubator

4.2.3 Other requirements

Table 4.3 List of other material requirements.

96 well polystyrene microtiter plate	Reservoirs
Serum samples	Multichannel
Pipettes (50, 200, 1000µl)	Small test tubes
Beakers (250 ml)	0.2 micron filter
Magnetic stirrer and magnetic bead,	Syringe
Tips (10µl, 200µl, 1000µl)	Tissue

4.2 Methodology

4.2.1 Preparation of buffers

A. Preparation of coating buffer (100ml)

- Weighed 0.82g $NaHCO_3$ and Na2CO3= 1.04g and added in 2 different 100 ml distilled filter water.
- Added magnet in both beaker and mixed well on magnetic stirrer.
- With the help of pH meter set the pH of NaHCO3 by adding Na2CO3 to the beaker till pH reached 9.
- Filtered the prepared buffer to remove impurities.

B. Preparation of blocking buffer (100ml)

Measured 70 ml of filtered distilled water and added the reagents in the water.

Table 4.4 Reagents added in blocking buffer

Reagents	Amount added / 100 ml
25 X PBS	4ml
2.5% BSA	2.5 g
5% Goat sera	5 ml
0.05% Tween 20	0.125µl

C. Preparation of washing buffer

Took a beaker of 250 ml and added 100 ml of distilled water in it. Replaced 4 ml of distilled water with 25 X PBS. It remains stable at 2-8°C for 2 months.

D. Preparation of stabilizing.

- Added 100 ml of distilled water in beaker and replaced 4 ml of water with 25 X PBS.
- Weighed 2.5 g of sucrose in the prepared mixture and mixed well.
- 250µl of this prepared stabilizing is added to each well for 1 hr after blocking of plate.

4.2.2 Coating and blocking of plate

A. Coating of plate

1. Added 3 ml of coating buffer in a 15 ml falcon and added 3.27µl of gp41 P-11 and added another 3 ml of coating buffer to it to make 1:1 dilution of Ag.

2. Followed by addition of 50µl of prepared buffer in all wells of plate with the help of multichannel.

3. Covered the plate and incubated it for 1 hr at 37℃.

4. After incubation plates were washed with prepared wash buffer.

B. Blocking of plate.

1. In this procedure serum sample are blocked along with blocking in 1:1 ratio as blocking buffer is also acting as SD.

2. Took 46 small test tubes and added 500µl of random serum samples of human into these tubes and added 500µl of prepared blocking buffer into tubes to make 1:1 dilution.

3. Along with them added 6 panels same in 1:1 ratio and vortexed every sample.

4. In 2 wells added +ve and -ve control and added 50µl of panels and random samples in each well.

5. Covered the plate and left the plate at 2-8℃ for overnight incubation.

C. Stabilization of plate

1. It helps in maintain the integrity and stability of protein that is coated on plate.

2. The plate is tapped on tissue after overnight blocking and stabilized by adding 250µl of stabilizing for 1 hr.

D. Drying the plate

Tapped the plate on tissue paper and let it dry at room temperature for 5 hours. Now the plate is packed in aluminium pouch and it is ready to be used.

4.2.3 Plate Testing

1. The developed plate needs to be checked for the sensitivity and specificity of the plate. For which the developed plate tested as follows

2. The plate was taken out of the aluminium pouch. Working conjugate was prepared by adding Enzyme conjugate concentrate in CD in 1:100.

Table 4.5 Preparation of working conjugate.

No. of strips	1	2	3	4	5	6	7	8	9	10	11	12
No. of wells	8	16	24	32	40	48	56	64	72	80	88	96
Enzyme Conjugate concentrate(µl)	10	20	30	40	50	60	70	80	90	100	110	120
CD (ml)	1	2	3	4	5	6	7	8	9	10	11	12

3. Added 120µl of concentrated HRP in 12 ml of prepared CD.

4. Now load each well with 100µl of prepared HRP and covered the plate and incubated it at 37℃ for 30 min. Followed by washing with prepared wash buffer for 5 times. The wells need to fill completely and allow shaking of plate for 30 sec.

5. Tapped the plate and added substrate to the plate in 1:1 ratio. TMB diluent and TMB substrate are added in 1:1 ratio in plate.

Table 4.6 Preparation of working substrate.

No. of strips	1	2	3	4	5	6	7	8	9	10	11	12
No. of wells	8	16	24	32	40	48	56	64	72	80	88	96
TMB substrate (ml)	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0
TMB diluent (ml)	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0

6. Added 100µl of prepared substrate in each well and incubated the plate at room temperature for 30 min.

7. Added 100µl of stop solution(H_2SO_4).

8. Took OD at 450 nm.

4.2.4 Comparison of developed HIV ELISA plate with JMitra HIV Microlisa plate.

1. Above mentioned procedure was followed for the developed plate of HIV while for the market plate the procedure of the manual was followed.

2. 100µl of SD was added in all the wells except A-1 and B-1 as 100µl of -ve and +ve controls are added in these wells respectively.

3. 10µl of same serum samples used in developed plate are added to each well in the same order and incubated the plate at 37℃ ± 2℃ for 30 min.

4. Prepared working enzyme conjugate in the meantime. After the incubation time is over take out the plate and wash with prepared wash buffer 5 times as specified.

5. Added 100µl of prepared working enzyme conjugate in each well and incubate for 30 min at 37℃ ± 2℃. Followed by washing after incubation.

6. TMB substrate was added in 1:1 dilution with TMB diluent. And 30 min incubation is followed at room temperature in dark.

7. Added 100µl of stop solution in each well and took absorbance at 450nm.

CHAPTER 5

RESULTS

5.1 Results of Checker board titrations.

Below mentioned results show the optimum coating region of Ag by performing checker board titration of Ag against +ve and -ve sera.

The two-fold dilution of Ag starts from 1:1 to 1: 32 and Ab is diluted from 1:1 to 1:8. Where column 6 and row D are acting as control as it contains no Ag and Ab respectively. Ag colour development is seen till ratio 1/8 so, it can be diluted till column 4 as after that there is loss in colour. Reduction in the binding of Ag and Ab after column 5. Non-specific backgrounds have to be considered carefully when adapting indirect ELISA.

4 different titration were done for Ag against =ve and -ve Ab.

1. For 1.5µg/ml dilution of antigen were made from 1:1 to 1:32. Colour development decreased after column 3. The colour development was not that good indicating more Ag can be adsorbed passively to the plate.

	1	**2**	**3**	**4**	**5**	**6**
A	0.58	0.3	0.11	0.02	0.01	0.01
B	0.37	0.19	0.06	0.01	0.01	0.02
C	0.19	0.08	0.02	0.01	0.01	0.01
D	0.01	0.01	0.01	0.01	0.01	0.01

Positive serum dilution for 1.5 µg/ml coating.

1	**2**	**3**	**4**	**5**	**6**
0.01	0.02	0.03	0.02	0.02	0.02
0.01	0.01	0.01	0.02	0.01	0.01
0.01	0.01	0.01	00.01	0.01	0.01
0.01	0.01	0.01	0.01	0	0.01

Negative serum dilution for 1.5 µg/ml coating.

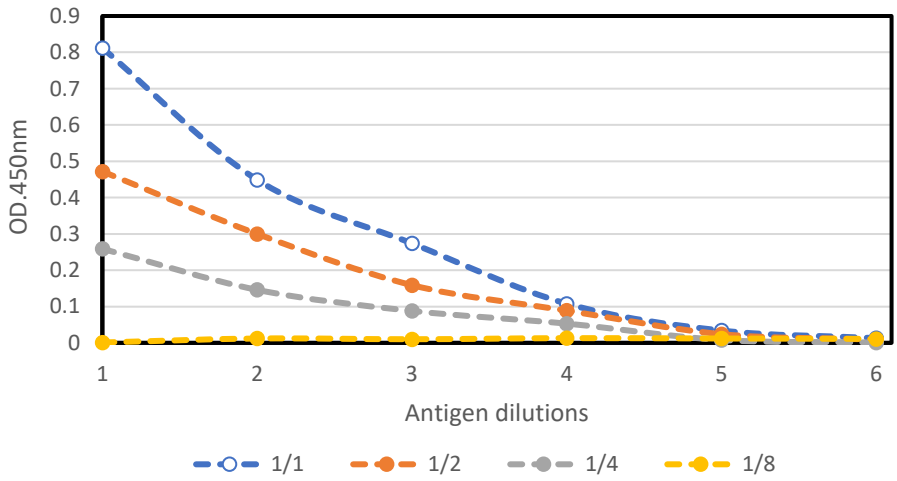
2. For 2.5µg/ml same dilution of Ag was made from 1:1 to 1:32. In this plate colour development decreased after row 4. Colour development was good and no background was seen for the same dilution and concentration of Ag for -ve serum. So, each non-specific background is considered carefully while adapting indirect ELISA.

	1	2	3	4	5	6
A	0.81	0.35	0.16	0.09	0.02	0.02
B	0.42	0.2	0.09	0.02	0.01	0.01
C	0.2	0.07	0.01	0.01	0.01	0.01
D	0.01	0.01	0.01	0.01	0.01	0.01

Positive serum dilution for 2.5 µg/ml coating.

1	2	3	4	5	6
0.02	0.01	0.01	0.02	0.01	0.02
0.01	0.01	0.01	0.01	0.01	0.01
0.01	0.01	0.01	0.01	0.01	0.01
0.01	0.01	0.01	0.01	0.01	0.01

Negative serum dilution for 2.5 µg/ml coating.



Graph 1: Showing that 1:1 dilution of 2.5µg/ml coating of antigen against positive serum.

5.2 Comparison of developed plate with J. Mitra HIV Microlisa plate

Table 5.1 Plate comparison between developed HIV ELISA plate and J. Mitra HIV Microlisa plate

	J.Mitra HIV Microlisa Plate						Developed HIV ELISA Plate					
A	0.014	1.615	0.022	0.079	0.073	0.028	0.018	1.67	0.067	0.042	0.026	0.192
B	0.015	2.583	0.014	0.099	0.018	0.012	0.022	2.368	0.023	0.012	0.014	0.118
C	2.455	0.579	0.011	0.068	0.011	0.231	2.193	0.526	0.073	0.019	0.023	0.352
D	2.31	0.594	0.202	0.013	0.076	0.027	2.18	0.393	0.086	0.04	0.037	0.175
E	1.109	0.071	0.055	0.014	0.032	0.021	1.759	0.02	0.032	0.414	0.065	0.044
F	2.312	0.034	0.016	0.008	0.014	0.012	2.343	0.032	0.071	0.282	0.012	0.299
G	0.421	0.01	0.048	0.099	0.053	0.015	1.045	0.059	0.021	0.224	0.019	0.218
H	0.551	0.014	0.01	0.009	0.015	0.286	2.137	0.021	0.217	0.014	0.009	0.098

= Negative Control

= Positive Control

= Positive Samples

= True Negative

= False Positive

$$\text{Cut-off Value} = \frac{\text{Mean Negative Control} + \text{Mean Positive Control}}{6}$$

J. Mitra HIV Microlisa plate cut-off value = 0.399

Developed HIV ELISA plate cut-off value= 0.364

Sensitivity and Specificity of plate

Sensitivity is defined as the ability to accurately show positive samples as positive.

Specificity is the ability to tell uninfected individuals as negative for the test.

Sensitivity = [True positives/ True positives + false negatives] x100

Specificity = [True negatives/ True negatives + false positives] x100

Sensitivity of J. Mitra HIV Microlisa plate = [8/8+0] x 100 = 100%

Specificity of J. Mitra HIV Microlisa plate = [34/34+2] x 100 = 94.44%

Sensitivity of developed HIV ELISA plate = [8/8+0] x 100 = 100%

Specificity of developed HIV ELISA plate = [34/34+2] x 100 = 94.44%

Table 5.2 Sensitivity and specificity of compared plates

	J. Mitra HIV Microlisa plate	**Developed HIV ELISA plate**
Sensitivity	100%	100%
Specificity	100%	94.44%

DISCUSSION

In this study we successfully developed a HIV ELISA plate by optimizing plate condition like time, temperature, pH, coating buffer, blocking buffer, concentration of antigen etc. and evaluated the performance of plate by comparing it with J. Mitra HIV Microlis plate and performing HIV ELISA test. The Developed plate was 100% sensitive as plate showed all 8 positive samples as positive which demonstrated that, these findings are quite encouraging since they show that our test is capable of accurately recognising every case of HIV positive case. While plate specificity was only 94% as plate picked 2 false positive samples out of 36 negative samples 2 samples showed to be false positive which is confirmed by checking the same sample on HIV Microlisa plate. The plate specificity was on a lower side but it can be refined by optimizing more conditions. Subsequent research effort have to concentrate on enhancing specificity while maintaining sensitivity and evaluating these results in various patient and hospitals.

CONCLUSION AND FUTURE ASPECTS

I conclude my training project **"DEVELOPMENT, OPTIMIZATION & EVALUATION OF HIV ELISA"** on the basis of my test result data. The plate was evaluated after optimization and development and it was100% sensitive and 94.73 % specific. In summary, this assay provides apparent advantages and shows great potential in the clinical diagnosis.

Enhancing sensitivity and specificity will probably be the main focus of future developments in HIV ELISA in order to lower false positives and negatives and increase diagnostic dependability. This technology's integration with automated platforms has the potential to expedite and improve testing procedures, especially in environments with limited resources. It is also possible to modify HIV ELISA for use in point-of-care testing, which would enable prompt treatment initiation and instant findings.

With a shift towards automation, newly emerging technique chemiluminescence immunoassay CLIA is approaching towards replacement of ELISA due to its easy handing & accuracy. Its applicability in terms of running multiple diagnostic tests at the same time is of great advantage of we look into its clinical implications, then it has reduced dependency over manpower & turn-around time. With ongoing research & development, furthur improvements, its usage is going to increase in upcoming years.

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