Development of a Ligand binding assay for detection of Neutralizing antibodies against Monoclonal Antibody X in Human Serum

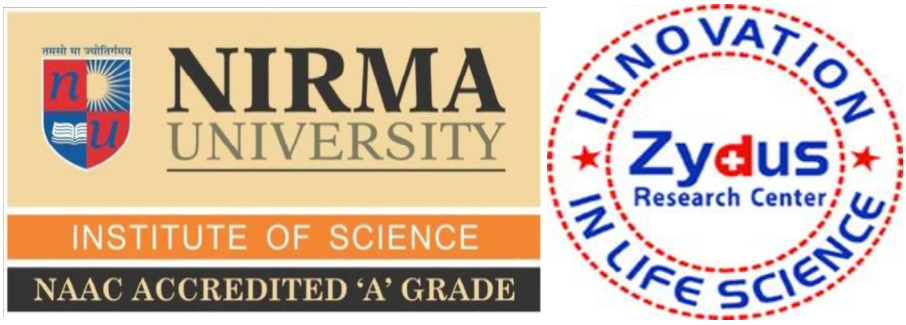
Dissertation thesis submitted to Nirma University in partial fulfillment for the Degree

Of

MASTER OF SCIENCE

IN

BIOCHEMISTRY



Submitted

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Date: Dhruti Dave

Place: Ahmedabad

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LIST OF SYMBOLS, ABBREVIATIONS & NOMENCLATURE

ABBREVIATION	SYMBOL NAME
%	Percentage
°C	Degree centigrade
µg	Microgram
ADA	Anti-drug antibody
BP	Biopharmaceutics
ELISA	Enzyme Linked Immunosorbent Assay
DNA	Deoxy Ribose Nuclicacid
HQC	High quality control
IgG	Immunoglobulin G
LQC	Low quality control
mL	Mili liter
IgG	Immuno globulin G
ng	Nano gram
O.D	Optical density
PBS	Phosphate buffered saline
WSA	Working solution A
WSB	Working solution B
MOA	Method of Analysis
NA	Not applicable
RT	Room temperature
TMB	3,3',5,5' - tetramethylbenzidine
IgM	Immuno globulin M
NAb	Neutralizing Ab
EPO	Erythropoietin
CLBA	Competitive Ligand Binding Assay
OD	Optical Density
FDA	Food and drug Administration
NHS	N-hydroxysuccinimide
BSA	Bovine serum Albumin
VEGF	Vascular endothelial growth factor
RPM	Rotation per minute
PRCA	Antibody (Ab)-mediated pure red cell aplasia

ABSTRACT

- **INTRODUCTION:** Biotherapeutics or large molecules such as Monoclonal Abs when administered in the patient can generate unwanted immune response by the production of neutralizing Abs.The Assessment of the Neutralizing Ab becomes important to understand the immunogenic response of a Drug/ Biopharmaceutic for which the Ligand Binding Neutralizing Assay can be developed. With the help of Nab assay,the presence of Neutralizing Ab production against the Therapeutics can be detected.

- **HYPOTHESIS:**

  1. Neutralizing Abs maybe present complexed with Therapeutic MAb in the serum. The acid dissociation of the samples will dissociate the Therapeutic Monoclonal Ab X from the Neutralizing Abs (Immune complex) in the serum. Further by Affinity purification, the Neutralizing Ab specifically will be obtained in the free form from the matrix.

  2. The Developed Ligand Binding Neutralizing Ab Assay will show detect Neutralizing Abs in the patient serum by preventing the binding of Therapeutic Monoclonal Ab X with VEGF, thereby confirming the presence or Absence of Neutralizing Ab in patient with the help of this assay.

- **OBJECTIVE:**
  1. To Develop a Ligand binding assay for detection of neutralizing antibodies against Monoclonal Antibody X in Human Serum.
  2. To check the stability and precision of the assay by running different Pre-Validation Parameters.

- **DESIGN:** To obtain the Neutralizing Ab in the serum free medium monoclonal Ab X coated sepharose beads were prepared and the double time affinity purification method was employed to prepare the samples which further can be analyzed with the help of ELISA. And once the Assay was developed the different Pre-Validation Parameters were checked.

- **RESULT:** A ligand binding assay was successfully developed to detect the presence of neutralizing Abs, produced against the therapeutic Monoclonal Ab X in normal human serum. The stability of the assay was proven with the help of different Pre-Validation parameters such as Accuracy and Precision, sensitivity, Selectivity and Drug-tolerance.

- **CONCLUSION:** The Developed Ligand Binding Assay for the detection of Neutralizing Ab which is produced against the therapeutic Monoclonal Ab X was found to be having the sensitivity of 300 ng/ml and the successful Matrix free Isolation of Neutralizing Ab was obtained by the double time affinity purification and Acid dissociation method.

1.INTRODUCTION AND REVIEW OF LITERATURE

1.1.Immunogenecity Of Large Molecules:

When the Drugs are derived from any living organism which can also be derived from any live Bio-Organism.This can be achieved with the help of Recombinant DNA technology or other gene expression relatedmethods,the derived products can be polypeptide or Nucleic acid in nature,their Biochemical characteristics are very much complex then the conventional chemical Drugs.
The Bio-Product thus produced can be affected by various variables such as the type of expression system,the growth conditions or be it the post transcription modification changes that take place during its production.It may affect the resulting activity and it turns out to be having some intrinsic molecular changes, the changes can be quantified.The structural variabilities which takes place in the production process are variable in nature,that brings down the reacativity of the BP in contrast to the conventional drugs.with small molecular weight which are obtained from a living or non-living organism via some specific chemical reactions.The conventional Bio drugs are molecules with very small,defined and composed chemnical structure,it is easy to characterize by analytical measurements.

The Biotherapeutic variability is further increased by the means of their sensitivity ,they can turn out to be extremely sensitive towards the external products,the modifications are not very likely to occur,the major modification can be protein denaturation ,agglutination, oxidation or the protein degradation due to their respective environmental conditions.This modifications are not very likely to take place in the conventional low molecular weight drugs because they are smaller in size and are very well predictable and could be controlled in the system.(Swanson, 2008).

The BP in contast to the conventional chemical drugs can turn out to be potentially high in immunogenicity response, in this aspect it is important to understand that some basic structural variations such as the little differences in the type and number of end product takes place in case of BP,it can interfere highly with the final Bio therapeutic drug.Product and production related impurities can further stimulate the immunogenic response of the bio pharmacheutic.Because of their large size the therapeutic monoclonal Abs have many possible epitopes which can be detected by the immune mechanism of the body.This epitopes are responsible for the increasing chances of immunogenicity development against the Bio-therapeutics(Swanson, 2008).

Table 1: Overview of the main differences between chemical and biological drugs	
Chemical	**Biological**
Produced by chemical synthesis	Produced by living cell cultures
Low molecular weight	High molecular weight
Well-defined structure	Complex, heterogeneous structure
Mostly process-independent	Strongly process-dependent
Completely characterised	Impossible to fully characterise the molecular composition and heterogeneity
Stable	Unstable, sensitive to external conditions
Mostly non-immunogenic	Immunogenic

Figure 1: The major differences between conventional low molecular weight chemical drugs and biological drugs/BP .

As described in the table above the comparison between the small and large sized molecules shows the biological variability of the both. Hence larger the molecule becomes; more are the chances for it to generate an immune response.

This could be further understood by the Example of Aspirin which is a compound of relatively smaller molecular weight (180 Daltons), Because of its small size there are pretty rare chances for it to develop unwanted immune response. As the molecular weight shifts to higher molecular weight complexes such as calcitonin (3,455 Daltons) And MAbs,(1,50,000 Daltons), the chances for developing adverse immune response increases greatly. Thus the therapeutic Monoclonal Abs have a very increased risk of immune response Development.

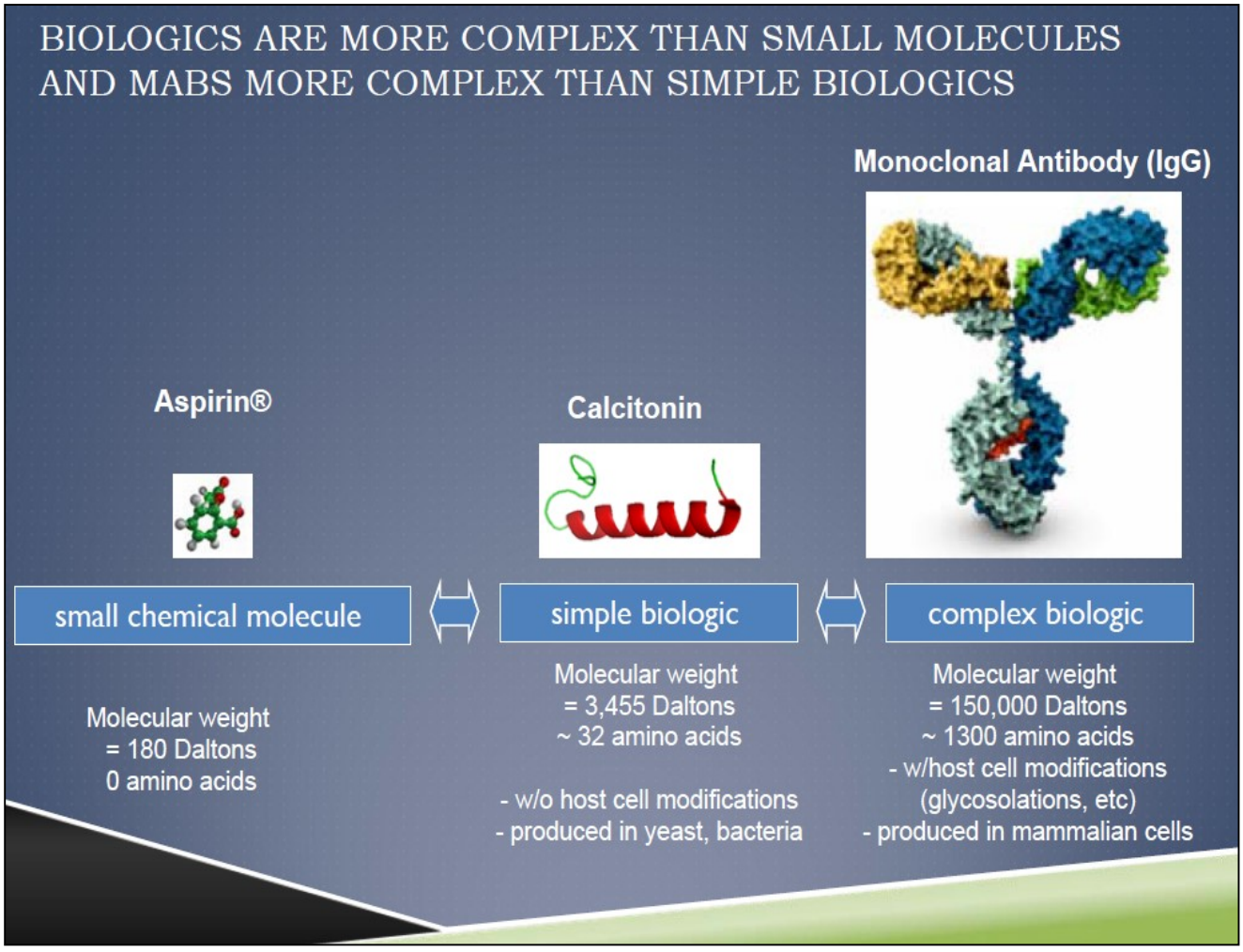


Figure 2: The illustrated relationship between the increasing molecular weight and increased risk of developing immunogenic response.

1.2.Ada Responseand Bio-Pharmaceutics:

Biotechnology derived proteins or Abs are potentially immunogenic in nature. The chances of this immunogenicity alters from no evidence or resulting effect to severe, life-threatening responses.Anti-drug antibodies (ADA) are also found to be involved in infusion reactions and anaphylaxis (Scheid et al., 2009) ,immune complex-mediated diseases. ADA can also cause secondary treatment failures such as efficacy-loss (Bartelds et al., 2007) and, in some instances, more severe adverse events like deficiency syndromes like thrombocytopenia and pure red cell aplasia.(Casadevall et al., 2002)may take place so, ADA have become a main concern in terms of safety after drug administration and long-time efficacy of the drug and it is very crucial to closely understand their Production in all patients during clinical-Trial studies, not just in a

symptom-driven method(Agency, 2015). To finely guide the medical practice, the knowledge of ADA responses and their characteristics in regards to clinical consequences is very essential.

Anti-drug antibodies (ADA) are termed as the drug-reactive antibody that can react with the administered biologic drug/Protein . ADA types mainly have two subparts which are neutralizing and non-neutralizing ADA. Other terms which has been used for ADA are such as anti-therapeutic antibody (ATA), anti-product antibody (APA), and anti-biologic antibody (ABA)

Binding ADA: All ADA are immanently "binding" antibodies because of their binding with the biologic drug molecule, irrespective of their in vivo pertinence (depends on their ability to produce clinical effect). A common misconception of this term is to apply it solely in terms of non-neutralizing antibodies.Neutralizing antibodies, itself are a subset of binding antibodies.

Neutralizing antibodies: Antibodies which are produced as the end Product of an unwanted immune response, after the administration of a drug product are termed as Neutralizing antibodies. These antibodies can hinder the biological effectiveness of the drug product either by binding to specific active-sites on the drug product and reducing their efficacy or because of steric hindrance at their site of action (Jolicoeur & Tacey, 2012).

Non-neutralizing ADA :ADA which bind to the biologic drug molecule but can not inhibit its pharmacological potential in an in vitro analysis or animal-based bioassay method, irrespective of its in vivo clinical pertinence.

Drug-sustaining ADA response: An ADA immune response related to a decresed clearance rate of the drug; the situation where the drug's half-life is longer in its ADAbound state than in its unbound state. (determined via statistic analysis). The pharmacological activity of the drug may or may not be affected by binding with a NAb or non-Nab.

Clearing ADA response: The ADA type which renders high clearance of the drug in terms such as it can be considered to be "clearing" in nature The effect of antibodies on drug clearance is a multifaceted mechanism which involves ADA,other circulating immune complex lattice, complement binding, Fc receptor binding, etc.

Pre-existing ADA: The type of antibodies reactive with the biologic drug that is present in subjects before to the treatment (or even before initiation of the clinical trial). This term is comparable with "baseline ADA," it can be used stringiently on the basis of obtaining antibodies which are reactive to drugs prior to treatment induction, irrespective of the statement of cause of this reaction (Shankar et al., 2008).

Treatment-induced ADA: The newly developed ADA response following the biologic drug induction (i.e., Production of ADA after the treatment initiation in a subject without any evidence of pre-existing ADA) (Shankar et al., 2008).

Treatment-boosted ADA: ADA which are boosted to a further enhanced level after a booster biologic drug dose given to the subject(Shankar et al., 2014).

1.3.The Importance Of Neutralizing Antibody Detection:

The importance of Neutralizing Ab detection can be understood by the illaustration of PRCA .Antibody (Ab)-mediated pure red cell aplasia (PRCA) is an immunological pathology which is related to the production of neutralizing Abs in this case the produced Abs inhibit the erythropoietic efficiency of endogenous erythropoietin (EPO) .It can occur due to recombinant erythropoiesis-stimulating agents (ESAs). Although this disorderis a rarely occurring one, Patients with Ab-mediated PRCA develops refusal to EPO and severe anemic conditions take place it a in this condition successful period of erythropoietic response is observed, and unveil a characteristic reduction in blood hemoglobin (Hb) level and in the number of circulating reticulocytes. it was found that found that the increased concentration of polysorbate 80 in the formulation of EPO-α is leading to micelle formation and that EPO molecules gets integrated into the surface of these formulated micelles. As aresult, EPO molecules are presented to the patient immune system in a regular spatial configuration, which in turn can trigger the immune system.And this Becomes a very life-threatening condition for the patients (Having Developed the Ab against one very essential internal protein)(Casadevall, 2005).

Thus the detection of Neutralizing Ab Presence is very important and there are several different ways for developing an assay for the detection of neutralizing Abs (Casadevall, 2005).

1.4.Development Of Nab Assays:

The FDA, the European Medicines Agency and other regulatory departments have issued some guidance documents about the development of sensitive immunoassays and also the cell-based assays as part of the drug-development,pharmacokinetic study and immunogenicity assessment process (FDA/CDER/CBER, 2016). The commonly used immunoassays, such as **enzyme-linked immunosorbent assays** (ELISA) and RIA,SPR assays, can check antidrug antibodies,yet these tests fails to deduce how these antibodies interact with the drug product. Detecting Nabs demands the use of more specialized, *in vitro* mammalian-cell-based assays or non-cell-based competitive Ligand-binding assays.(Jolicoeur & Tacey, 2012).

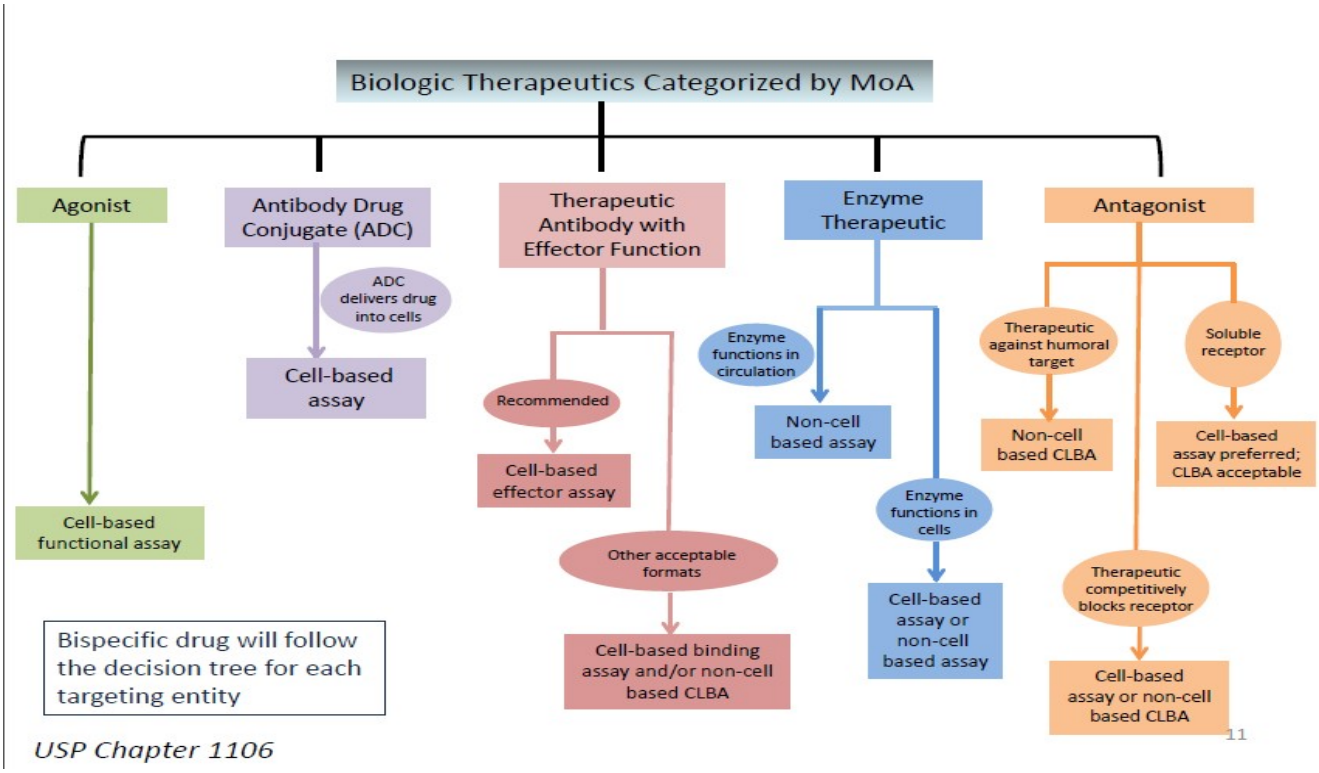


Figure 3: Strategies for selecting Nab Assay format – European Bioanalysis Forum 2016

Every NAb assay is unidentical and depends on the drug product or BP (e.g., monoclonal antibody, protein, or soluble receptor), the population of study (normal or diseased) and the study (preclinical or clinical). The type of NAb assay to be chose,for assay development(direct or indirect) relies on the drug's mechanism of action. Majority NAb assays are performed in a 96-well plate, but this can be changed for high-throughput and efficiency if required. Major steps in designing and testing a cell-based NAb assay are:

- Choosing the proper cellular response mechanism (end point method);
- proper control selection;
- Optimization of assay parameters;
- Validation.

NAb development in non-clinical studies is also fruitful since NAbs stops the drug product from being active.Hence the presence or absence of any toxicological effect becomes very difficult to interpret.for understanding the patient safety the immunogenic assessment and the Nab or non-Nab development is very important. Therefore, it is required to develop dependable NAb assays of suitable sensitivity, specificity, and robustness they can help in detection and differentiate NAbs from non-neutralizing antibodies in non-clinical or clinical samples which are collected for antibody testing. In certain cases, detected NAbs may require mathematical calculation;but, due

to the lack of proper reference standards for immunogenicity assays, any effort to calculate anti-drug product antibodies yields a putative positive result(Shankar et al., 2008).

For developing a NAb assay mostly a Multi-tired algorithm is employed. testing samples for the presence of neutralizing antibodies is promoted.

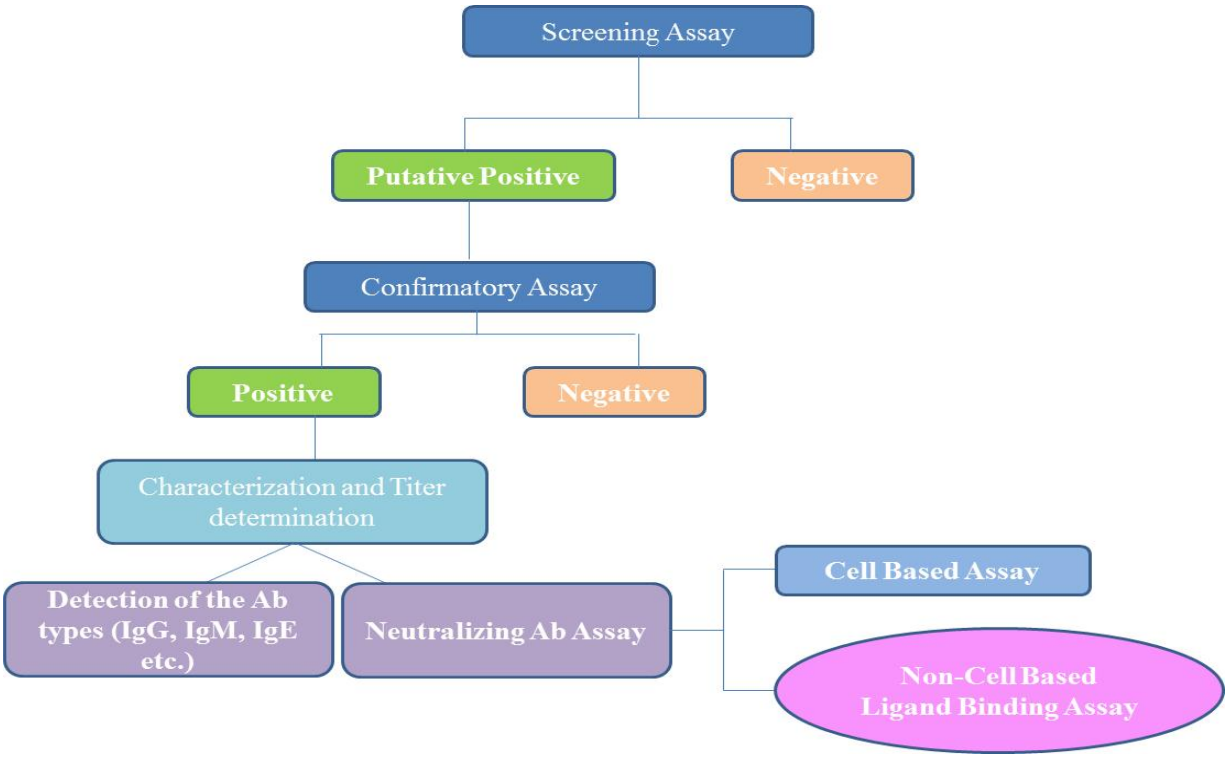


Figure 4: : Multi-Tiered algorithm for testing samples for the presence of neutralizing antibodies

1.4.1 Screening Assay: In this type of analysis the samples are fist screened prior to any kind of Drug response to randomly check for the presence of neutralizing Abs. First in a screening assay rapid identification of positive samples is performed.Screening assay In amulti phased testing strategy, the assay is commonly used to distinguish between potentially positive samples (based on cut-point) and negative samples(Gupta et al., 2007).

1.4.2 Confirmatory Assay:

This assay is used to confirm the putative positive results of the screening assay. The putative positive samples that have been obtained in the Screening assay are confirmed for the existence of ADA in the confirmatory assay. This is done by spiking a known volume or concentration of Drug/Therapeutic in the sample to obtain an inhibition of signal due to its binding with the ADA present in the sample (Gupta et al., 2007).

Samples with a positive result in the confirmatory assay can then be tested for anti-drug neutralizing activity with the help of an in-vitro cell-based assay or competitive non-cell-based ligand binding assay(Gupta et al., 2007).

1.4.3 ADA characterization assay: The characterization assay is designed to obtain some additional information on the type of the Ab , for example based on the immunoglobulin type the onset and prevalence of Nab in patient can be analysed

1.4.4 Qualitative assay: This assay enables us to clearly state the Positive or negative results.

1.4.5 Quasi-quantitative Assay: with the help of a quasi-quantitative assay, relative response for the ADA titer can be understood .

1.4.6 Titre assay: Titer determination gives the measurable or detactable amount of drug or antibody present in the sample.it is reciprocal to the mininmal dilution that can generate a positive response towards the assay.

1.4.7 Assay cut point/cut off value:

Cut-point are the statistical values obtained from a quasi quantitative parameters, because it can determine the positive samples by the means of threshold limit detection, for non specific blank.Hence it is termed s cut-point or cut-value.

Three types of cut points are used in the industry:

- **Fixed cut point**: Is the type of cut point obtained in pre-study or Pre-validation analysis and it stands true for any amount of the samples which are to be detected in the future.The assay is known as accuarate if the type of cut point used is a fixed one.

- **Floating cut point**: In floating cut point method the specific cut factor value is obtained during the pre-validation check and the cut factor is multiplied in the study phase.

- **Dynamic cut point**: This is the most robust type of the cutpoint it allows the variation between the plates, different runs and respective study sample analysis. This type of cut-points are highly variable and allows a wide and flexible range,but one of the limiting factor for this type of cut-point is to establish a new value of cut-points a lot of samples should be screened in each run and that demands a lot of well space in the plate and higher quantity of serum is also required so this is generally avoided.

Floating cut point is most commonly used one in the industry, the same can be used for the ADA assays in practice.

1.5.Cell Based And Non Cell-Nbased Ligand Binding Nab Assay:

According to the FDA guideline, cell based assays are more preferable for the detection of NAb because they closely reflect the in vivo situation. However, the Non cell based Ligand Binding assay can be used:

- If the target is soluble in nature
- It does not act upon the cellular cascade mechanism for its mode of action
- When the secondary messenger system is not involved in the response mechanism,

Also another difficulty with the cell-based assay is selection and availability of suitable cell lines(Finco et al., 2011). The behavior of the cell line can turn out different than expected outcome. In such instances the Non cell based ligand binding assay turns out to be more suitable approach for assay development(Lemaillet et al; 2015).

1.6.Pre-Validation Parameters

Assay Development is followed by a Method Validation which enables the developed method to be accurate, specific, selective, robust etc for studying the samplesprior to the Validation experiments, the major regulatory parameters are checked during the Method Development stage. These parameters are termed as Pre Validation Parameters.

Pre-validation parameter is helpful to understand the effect of pre established controls on developed assay and assay performance. These data and the scientific conclusion,sets the acceptance criteria for the assay validation and further studies. The acceptance criteria are generally produced before the actual final assay validation.

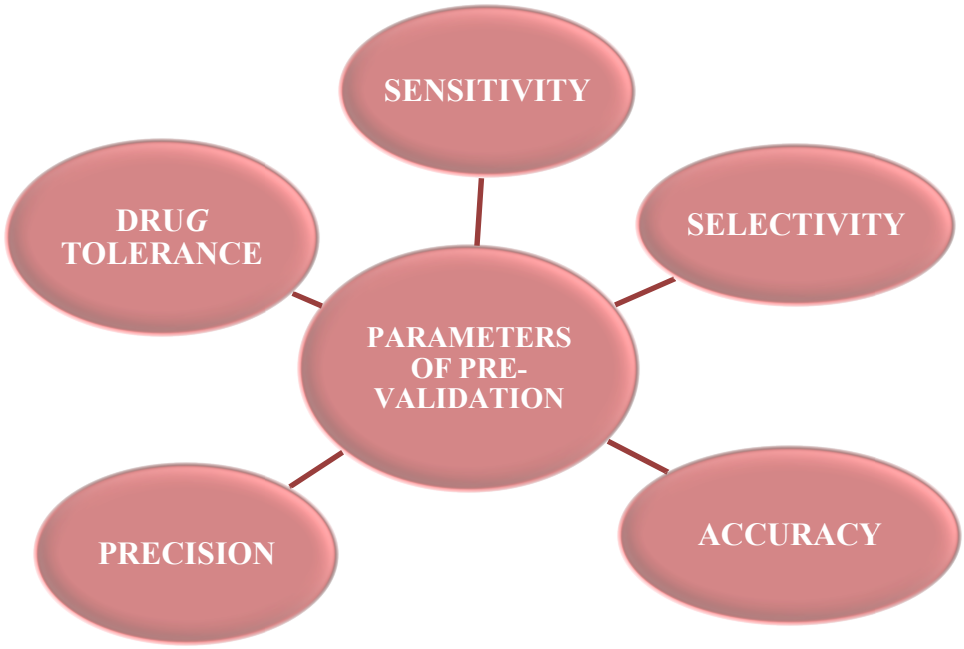


Figure 5: The Different Types of Pre-Validation Parameters.

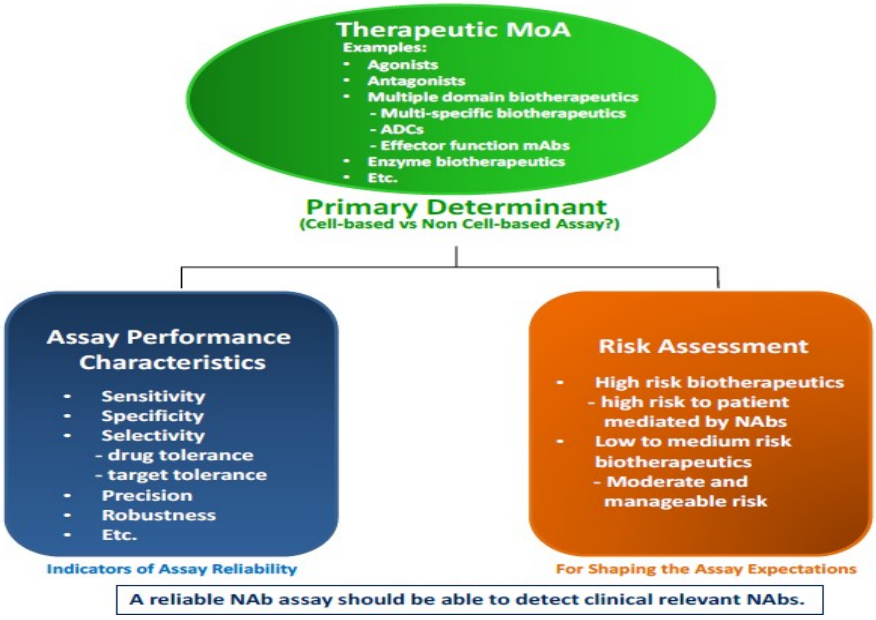


Figure 6: Ref. Strategies to determine Nab Assay mrthod for the assessment of Neutralizing Antibody Responses to Bio-Medicines(Wu et al., 2016)

1.6.1 Accuracy and Precision:

- **Accuracy** can be termed as the closeness to a certain value that can be obtained in the developed assay. The value can either be conventional true value or the accepted reference value.
- **Precision i**s the fluctuations in the data from reproducible determinations of the same sample under the normal assay conditions. The precision of any assay shows the higher chances of obtaining the same value in multiple assay runs .

1.6.2 Drug Tolerance:

Specificity of neutralizing Ab is often confirmed by a decreased binding of the Nab in the presence of excess soluble drug .The excess presence of soluble drug in the patient's serum sample for Nab analysis can obscure the assay from detecting anti-drug antibodies .Tolerance is obtained by checking assay sensitivity in the presence of increased amounts of added soluble drug . Drug tolerance explains the sensitivity of the ADA assay towards the Presence of excess amount of soluble drug. NAb assaysare generally more reluctant to drug presence than other immunoassays which are used for detecting binding antibodies.

The amount of interference that the soluble drug can create depend on many aspects, such as the drug concentration, the nature of positive contol Ab, and the final structure of the assay, thus a single "drug tolerance level" is not feasible to establish. The obtained level of drug interference in the NAb is included in the design of mehod development of the assay and analysis strategies. While the assay is being developed or standardizaed, drug interference can be examined by using

increasing drug spiking into a positive control neutralizing antibody, it is behaving as the mock sample for development. The level of soluble drug concentration that will interfere Nab detection in the assay will highly rely on the concentration and other characteristics such as affinity of the antibodies used as the positivecontrol or mock sample and those Nabs which are expected to be present in the test sample.

1.6.3 Selectivity:

The selectivity parameter is the ability of an assay to measure the molecule or protein of interest independently from any possible matrix effect.

The detection of drug in samples to be analysed is obtained by various drug-treated subjects which interfere with the ability of developed assays to detect binding antibodies or neutralizing antibodies in different serum samples .The selectivity mainly proves the stability of the assay in various unidentical serum samples.

1.7.Therapeutic Monoclonal Ab X And Its Role In Angiogenesis Inhibition:

New growth in the vasculatures of cancer tumor is one very essential step for the cancer proliferation .The metastasis and the spread, of cancer cells depends on meeting the needs of required suppliment of oxygen and nutrients and the proper excretion of waste products. The blood and lymph vessels are produced in increasing amount and this is termed as angiogenesis. Respectively,. Angiogenesis is achieved by altering the activity of both activator and inhibitor molecules. Many different proteins have been identified to be angiogenic activators and inhibitors. Level of angiogenic factor expression showcases the wide-spread and aggression of tumor cells. (Nishida et al; 2006)

Angiogenesis in cancer

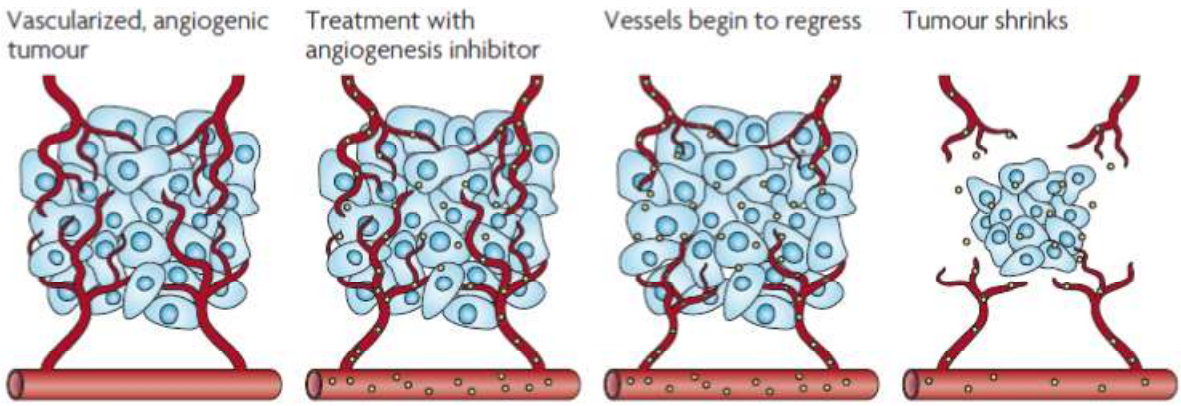


Figure 7: The effect of Angiogenesis inhibitor factor on the vascularizing tumor growth (R.zetter, 2008).

The tumor angiogenesis is a very necessary component for tumor growth, and based on this was the conclusion that antagonism of the angiogenic process can continue to give a new form of cancer therapy

Monoclonal Ab X is the type of Ab that is used to treat cancer predominantly in lung cancer and also in other types such as colorectal cancer or renal cell carcinoma the Ab X is used to block the process of angiogenesis by inhibiting the VEGF receptor signaling and there by stopping the angiogenesis promoting characteristics in the cancer growth.

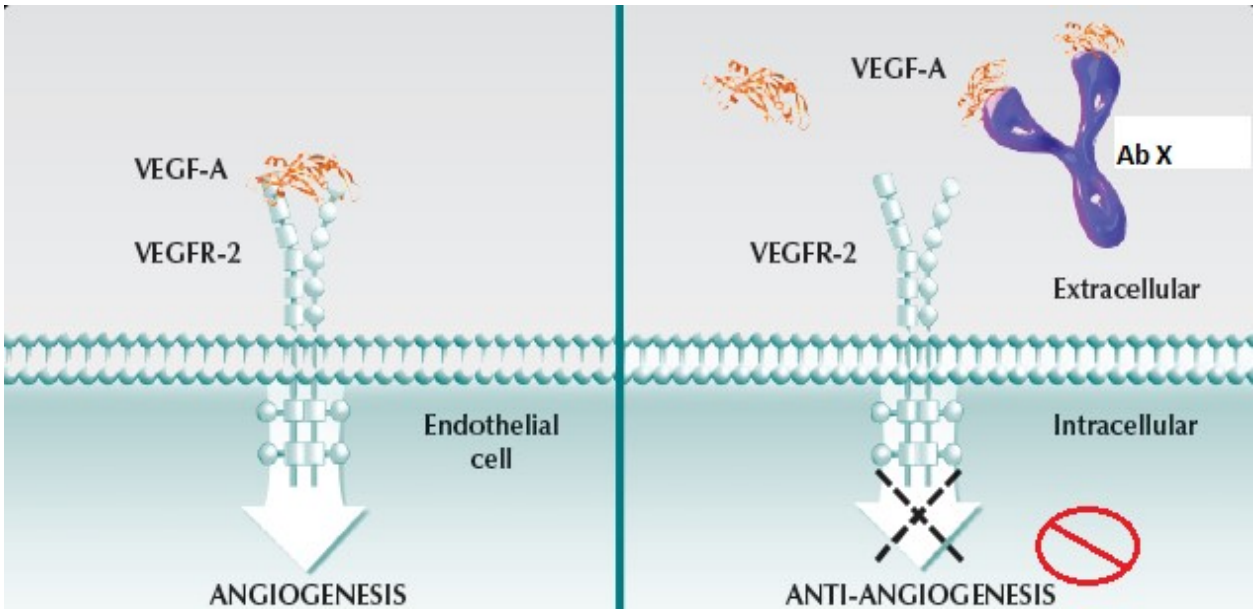


Figure 8: Role of Monoclonal Ab X in inhibition of angiogenic promoting factor VEGF during cancer growth (Hicklin, 2005)

When the patients are treated with this Monoclonal Ab X, they tend to develop immune response in the form of Neutralizing Ab production to assess the production of these Nabs the assay can be developed.(Folkman, 2010)

2.MATERIALS AND METHODS

2.1.Method for the Preparation of protein coated sepharose Beads:

The preparation of protein coated (Monoclonal Ab X) sepharose beads was done by NHS- (N-hydroxysuccinimide)- Activated Sepharose chemistry. The coupling of the Monoclonal Ab X to the sepharose was done in coupling buffer and then it was sequentially washed to get the bound Monoclonal Ab X-Sepharose bead, which could be further used in the assay as an important reagent.

2.1.1 NHS- (N-hydroxysuccinimide)- Activated Sepharose chemistry:

NHS- (N-hydroxysuccinimide)- Activated Sepharose is a bead having highly cross-linked reactivated matrix which is prepared by the coupling of sepharose with 6-aminohexanoic acid, with the help of a spacer arm, Here the terminal Carboxyl group is activated by etherification with N-hydroxysuccinimide. This active ester provides a very strong binding to the Ligands containing primary amino group by a stable amide linkage.

2.1.2 Dialysis of the protein and Coupling Reaction:

In the Dialysis step, buffer exchange of the protein is performed with the help of dialysis membrane, to bring the protein into the coupling buffer. The protein which is loaded in the dialysis membrane was incubated overnight with the help of magnetic stirrer at 4˚C after the incubation of the dialyzed protein is collected in the eppendorf tube.

This is followed by a coupling Reaction: In this step, the NHS activated beads are washed with 10-15volumes of cold 1mM HCl, so that the beads become free of alcohol or the storing medium. Then a fixed concentration of Monoclonal Ab X was added in the tube along with the coupling solution. This step will enable the binding of the protein onto the surface of the bead. Now to ensure that the sites other than those on which the molecule of our interest is positioned, are blocked, the column is kept in 0.5M ethanolamine for three hours. By this reaction step , all those remaining sites on which Monoclonal Ab X is not there are blocked, thus they cannot participate in the reaction anymore. After this step the beads are given multiple washes of Tris and acetate buffer alternatively and ultimately stored in the Tris buffer.

2.2.Procedure for Beads Preparation:

2.2.1 Dialysis:

1) The protein /Ab was Dialyzed to bring it in the coupling buffer
2) Dialysis bag was prepared from the Dialysis membrane by taking a sufficient amount of membrane and tying one of its ends with the help of string
3) protein/Ab was loaded and other remaining end was tied tightly with the string and was closed
4) It was then Incubated overnight in coupling buffer in stirring condition on magnetic stirrer at 2-8 ´C
5) Dialysis bag was removed after incubation and the dialyzed protein Ab was collected in an eppendorf tube.

2.2.2 Coupling:

1) 1 ml pipette tip was taken and cut to make it blunt ended then the required quantity of bead slurry was taken into a column
2) NHS activated sepharose beads were washed with 10-15 volumes of cold 1mM HCL immediately before use
3) The HCl was allowed to pass through completely and then beads were given a wash with the coupling solution
4) The protein/Ab was taken at the fixed concentration, in the tube and the coupling solution was added into it at the ratio of 0.5:1
5) The column was sealed with the parafilm on the top and rubber cap at the bottom by mixing and tapping it.
6) It was incubated overnight at 2-8´C
7) Next Day medium was kept in 0.5 M ethanolamine ,0.5M Nacl (pH 8.3) for three hours washed with 3 bed volumes of 0.1M Tris-HCl buffer (pH8.5-9.0)and 0.1M acetate buffer (pH 4 to 5) respectively alternately thrice.
8) The Beads were stored in 0.1 M Tris buffer

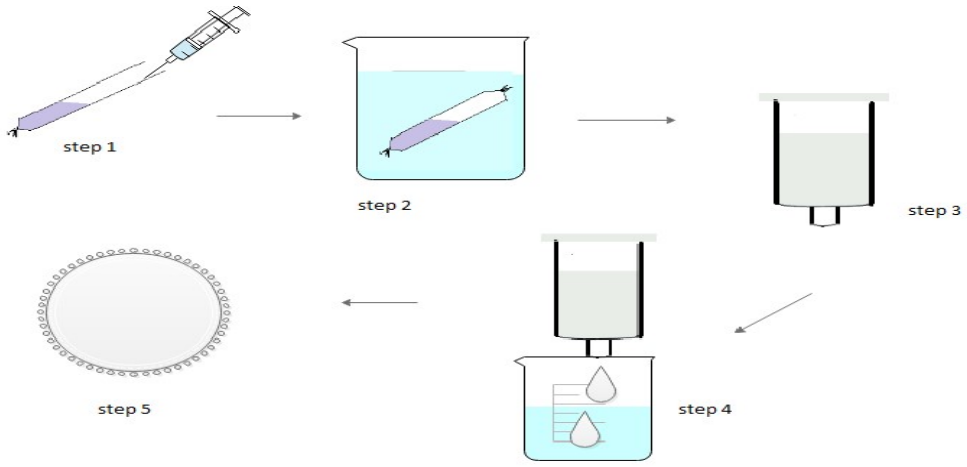


Figure 9: Schematic Diagram of Preparation of Monoclonal Ab X coated Sepharose Beads

Step 1: The therapeutic Monoclonal Ab was loaded in the dialysis bag and the bag was sealed on both the ends with the help of thread

Step 2: The Monoclonal Ab X coated dialysis bag was kept in the coupling buffer overnight for its complete purification

Step 3: After the Washed NHS- Activated sepharose Beads were loaded in the column, the therapeutic monoclonal Ab X was added at the fixed concentration, in the tube and coupling solution was added into it at the ratio of 0.5:1

Step 4: Next Day medium kept in 0.5 M ethanolamine,0.5M NaCl (pH 8.3) for three hours. It was then washed with 3 bed volumes of 0.1M Tris-HCl buffer (pH8.5-9.0)and 0.1M acetate buffer (pH 4 to 5) respectively alternately thrice

Step 5: The Beads were stored in 0.1 M Tris buffer.

2.3.Sample Preparation method and principle :

Sample preparation with the help of Double time affinity purification is a very crucial step for the proper liberation of the Neutralizing Ab. These Nabs are present in the bound form with the therapeutic monoclonal AbX in the patient serum. The presence of even a smallest amount of monoclonal Ab X in the sample can give background by generating the matrix effect. To avoid any chances of the presence of Pre-existing Drugin the serum and to ensure the proper liberation of Neutralizing Ab, affinity purification is performed twice. Hence there are no chances of cross-reactivity and the therapeutic Ab (Drug) is completely eliminated from the system

(Dai et al;2014).

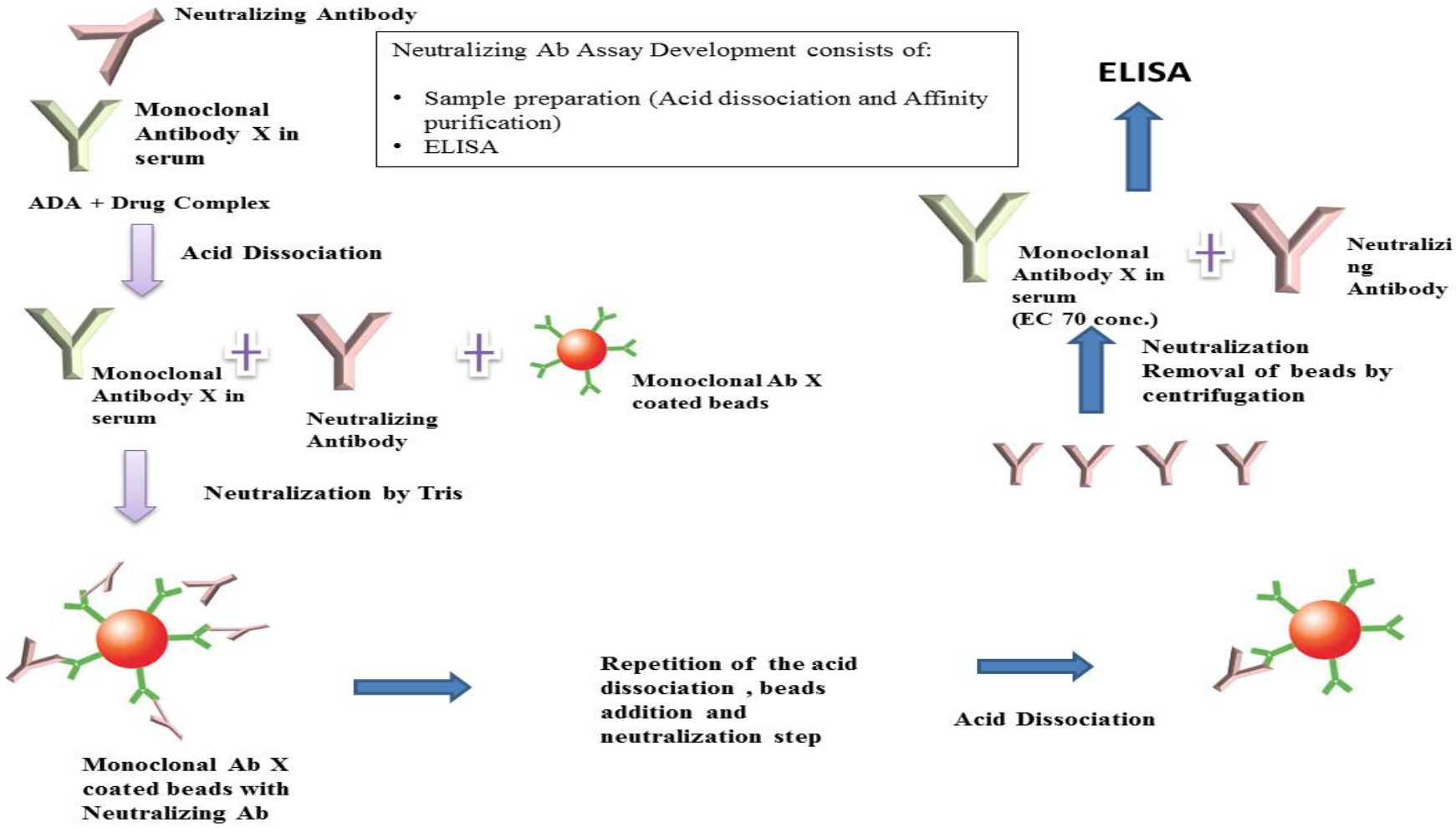


Figure 10: The schematic diagram of the Pre-treatment that is being given to the samples for the ligand binding Nab assay.

2.3.1 Pre-Treatment of the samples

All serum samples were centrifuged at 10,000 rpm for 5 min prior to use

1 50 µl of positive control/negative control/study sample was taken in a 1.5 mL tube and 400 µL of glacial acetic acid was added in it. Itwas then incubated on shaker at normal speed at 25*C for 30 min.

2 After incubation, 20 µL of Monoclonal Ab X coated sepharose beads were added using 200 µL pipette tips which are cut blunt followed by immediate addition of 160µL of 1M Tris.

3 Note: the proper mixing of beads was ensured before addition by vortexing it multiple times.

4 The tube caps were sealed with parafilm and kept on shaker at 700 RPM, in inverted position for 60 min at RT 450 RPM.

5 After incubation, the parafilm was removed from the caps and thetubes were centrifuged at 10000 rpm for 5 min at RT. The supernatant was removed then.

6 420µl of 300 mM Glacial acetic was added to the sample and was incubated on shaker at 700 rpm at 25°C for 30 mins

7 It was then centrifuged at 10000 rpm for 5 mins. Supernatant was removed then.

8 400µl of centrifuged sample was taken 20 µl of Monoclonal Ab X coated beads (second time) were added +160µl 1M Tris immediately. It was incubated on shaker at 700 rpm at 25°C for 1 hr.

9 Centrifuged at 10000 rpm for 5 mins. The supernatant was removed then

10 220µl of 300 mM Glacial acetic acid was added and it was incubated on shaker at 700 rpm at 25°C for 30 mins.

11 Centrifuged at 10000 rpm for 5 mins.

12 200µl of centrifuged sample was taken + 80µl 1M Tris buffer was added and stored at -70°C.

13 Next day Monoclonal Ab X calibration curve was prepared and loaded on the blocked VEGF Coated Plate.

The prepared samples were then run in the ELISA plate for the following day in the plate coated with VEGF.

2.4.Principle of ELISA:

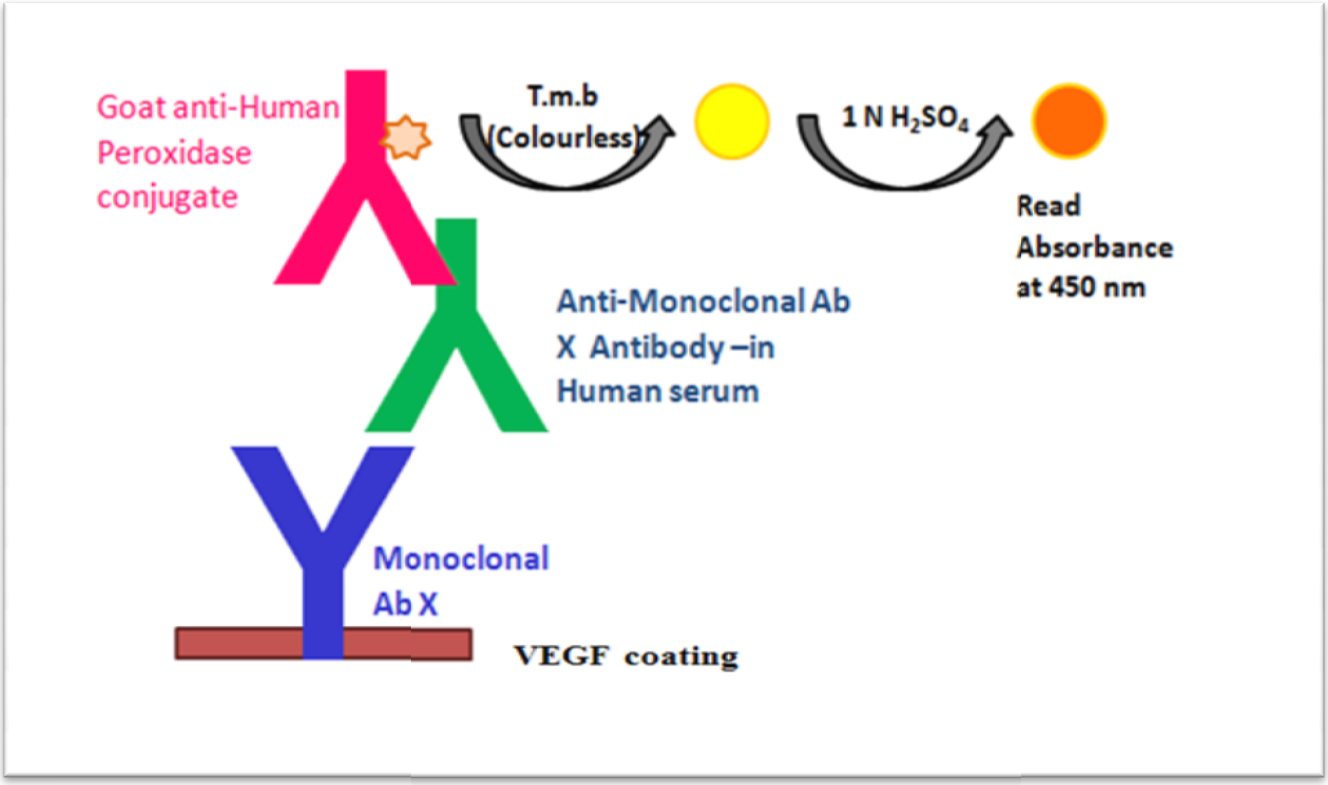


Figure 11: The schematic diagram of the ELISA principle used for the Assay Development

For the Detection of Nabs with the help of ELISA, the VEGF coated Plate is loaded with Monoclonal Ab X. The Presence of Neutralizing Ab/Human Anti-Monoclonal Ab X can be detected by the Detection Ab/Goat Anti-Human Peroxidase conjugated Ab .The Detection Ab can further be Analyzed upon the addition of the substrate TMB and the generated signal is read at the 450 in the ELISA Reader.

2.4.1 The standardized assay protocol for the ELISA:

Sr No.	Steps	Reagents	Volume	Incubation Time	Condition
1	Coating	Coating of 0.125µg/ml of VEGF in 1XPBS-Stock conc. 0.15 mg/ml	100ul/well	overnight incubation	4°C Humid Chamber
2	Blocking	2% Skim milk in PBST	300ul/well	60 mins	450 rpm on Shaker at 25°C in incubator
3	Washing	PBST	300ul/well	NA	3 times
4	Sample	Monoclonal Ab X standardization curve	100 ul/well	60 mins	450 rpm on Shaker at 25°C in incubator
5	Washing	PBST	300ul/well	NA	5 times
6	Peroxidase conjugated antibody	Peroxidase conjugated Goat anti human1/1 Lac in diluent	100ul/well	45 mins	450 rpm on Shaker at 25°C in incubator
7	Washing	PBST	300ul/well	NA	5 times
8	Substrate	TMB	150ul/well	15mins	Static at RT in dark
9	Stop	1N H_2SO_4	100ul/well	NA	Read at 450nm
Diluent: 0.1% Skim milk in PBST					

Table 1:The standardized assay protocol for the ELISA

2.5.Reagents:

2.5.1 Coating solution

Composition: 0.125 µg/mL of VEGF Proteinin 1 X PBS

Preparation: 9.16 µl of VEGF (Stock: 0.15 mg/ml) was added in 10.990 mL of 1 X PBS.

2.5.2 Blocking solution

Composition: 1X PBS, 2% skim milk and 0.05 % Tween 20

For 35 mL:

28 mL of 1X PBS was taken, 7.0 mL of 10% skim milk was added along with 17.5 µL of Tween 20 and it was mixed well.

2.5.3 Sample diluent

Composition: 1X PBS, 0.1% skim milk and 0.05 % Tween-20.

For 50 mL:

49.500 mL of 1X PBS was taken,1 mL of 10%skim milk was added along with 25 µl of Tween 20, and it was mixed well.

2.5.4 Control matrix for preparation of negative and positive control

This assay has been established using neat serum without any dilution. Pooled normal human serum was used. Pooled normal human serum was centrifuged at 10,000 rpm for 5 min prior to the use for preparation of controls sample for the assay.

2.5.5 Positive control samples for assay

System suitability positive controls were prepared at low and high concentration as described below and used for each analytical run.

Stock solution of positive control was diluted(1 µg/µL) 20-fold with sample diluent to get the working solution concentration 50 ng/ µL (WSA).

Solution concentration 50 ng/ µLwas diluted (WSA) 200-fold with sample diluent to get the working solution concentration 2.5 ng/ µL (WSB).

Sr No.	ID	Concentration	Preparation
1	HQC*	7500 ng/mL	7.5 µL of Pooled Normal Human serum+ 42.5 µL of WSA
2	LQC*	300 ng/mL	6 µL of Pooled Normal Human serum + 44.0µL of WSB

"*"concentration of HQC and LQC will changed based on method sensitivity data generated during Development test and it were to be prepare freshly for each assay.

Note: Pooled Normal Human serum was centrifuged at 10,000 rpm for 5 min prior to the use for preparation of positive controls sample for the assay.

2.5.6 Peroxidase conjugated antibody

Peroxidase conjugated Goat anti Human IgG Fc specific (1:1,00,000)

5.0 mL diluents were taken and 5.0 µL of Peroxidase conjugated Goat anti Human IgG Fc specific was added and mixed properly by vortexing to obtain 1:1000 dilutions.

For 10 mL : 100 µL from 1:1000 dilution was added to 10 mL of diluent to obtain a final dilution of 1: 1,00,000, Mixed well by vortexing.

2.5.7 Substrate solution

TMB (3, 3',5, 5 Tetra methyl benzidine) is a ready to use substrate solution; stored at 4°C.

2.5.8 Stop solution

To prepare 36 mL of stop solution, carefully 1 mL of 36 N H_2SO_4 was added to 35 mL of purified water.

❖ System suitability for assay

The criteria for acceptance of validation runs are summarized in the table below.

Blank	Blank sample prepared with pooled Normal human serum spiked with EC_{70} of Monoclonal Ab X and O.D. will be measured
HQC	% inhibition should be more than 50%
LQC	OD value should be below the assay cut point

Precision of HQC and LQC should be within ± 20%. At least 67% of the QC samples and at least 50% at each concentration level should comply with this criterion. The %CV of at least 50% of the Blank/NC should be within ± 20%.

2.6.Data Processing

Measurement parameters

Instrument name: Molecular Devices Spectra Max i3x

Plate Description: [GRE96ft] – Greiner 96 Flat transparent

Measurement Format: Absorbance

Measurement wavelength: 450 nm

Measurement Bandwidth: 9 nm

2.7.Pre-Validation Parameters:

2.7.1 EC_{70} determination:

Multiple assay runs were prepared at 9 different concentrations (200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 ng/mL) of Monoclonal Ab X and OD response was determined. The EC_{70} concentration was derived using validated Graph Pad Prism® software version 6.01at Department of clone Development, Zydus Research Centre, Ahmedabad, India.

2.7.2 Selectivity

The ability of the assay to differentiate a quantifiable response of positive control in the presence of serum proteins, serum factors, therapeutic protein present as endogenous human protein, co-administered drug during the treatment period present in serum was evaluated.

HQC and LQC samples were prepared in 10 individual lots of serum of normal human serum. The OD response of the samples was measured. The % inhibition and precision were calculated for HQC, and precision was calculated for LQC.

2.7.3 Drug tolerance

In ADA assay, the main interference is drug itself and it is expected that the drug would bind with drug-specific antibody. The detection reagent may then fail to detect circulating drug-specific antibody and generate false-negative results. The concentration of drug where signal of positive control is nearest and below the assay cut point, characterized the "drug tolerance limit" of the assay.

The positive control (1000 ng/mL) was prepared in neat serum in the presence of different concentrations of drug: 10, 7.5, 5, 2.5, 1, 0.5 and 0.25 µg/mL, and OD were analyzed.

2.7.4 Accuracy &Precision

HQC and LQC samples in replicates of 6, in normal human serum were prepared in neat serum and OD value was measured. Separate run was analyzed to assess precision for inter-plate variation

3.RESULTS

3.1. To screen different coating concentrations of VEGF to finalize the optimum coating concentration for the Development of ELISA to detect the Neutralizing Ab.

Plate Layout for Screening of coating conc.				
	1	2	3	
A	100ng/ml	100ng/ml	100ng/ml	Monoclonal Ab X conc. in Human Serum
B				
C	10ng/ml	10ng/ml	10ng/ml	
D				
E	2ng/ml	2ng/ml	2ng/ml	
F				
G	Blank	Blank	Blank	
H				
VEGF Coating Conc.	0.125µg/ml	0.25µg/ml	0.5µg/ml	

Table 2:The details of the plate layout for the different VEGF coating concentration taken.

Mean OD values			
	1	2	3
A	3.216	4.000	4.000
B			
C	0.689	1.042	1.436
D			
E	0.200	0.324	0.467
F			
G	0.095	0.136	0.206
H			

Table 3: the mean OD values observed in the experiment

Monoclonal Ab X	S/N Ratio		
100 ng/mL	33.919	29.455	19.389
10 ng/mL	7.269	7.672	6.962
1ng/mL	2.108	2.386	2.264
	0.125µg/ml	0.25µg/ml	0.5µg/ml
	VEGF coating conc.		

Table 4: The calculated signal to noise ratio for the three different concentrations used (0.125µg/ml, 0.25µg/ml and 0.5µg/ml respectively)

Conclusion 3.1: with the coating concentration of 0.125 µg/ml good signal to noise ratio was observed, hence this coating concentration of VEGF can be used further for development of ELISA assay

3.2. To optimize the final Blocker, diluent and Peroxidase conjugated Goat Anti-Human IgG Ab dilution by screening different blockers, diluents and different dilutions in the same

(Note: All the blockers were screened at the concentration of 2% and All the Diluents were screened at the concentration of 0.5%)

Plate Layout for Screening Blockers and Peroxidase conjuagated Ab dilution									
Blocker	1% Gelatin in PBST			2% Skim milk in PBST			2% BSA in PBST		
	1	2	3	4	5	6	7	8	9
A	100ng/ml			100ng/ml			100ng/ml		
B									
C	10ng/ml			10ng/ml			10ng/ml		
D									
E	2 ng/ml			2ng/ml			2ng/ml		
F									
G	Blank			Blank			Blank		
H									
Peroxidase conjugated Ab	1:1 lac	1:1.5 lac	1:2 lac	1:1 lac	1:1.5 lac	1:2 lac	1:1 lac	1:1.5 lac	1:2 lac

Table 5:The plate layout for the screening of different blockers Along with their respective Diluents accordingly and also the different dilutions of the peroxidase conjugated Ab goat Anti-Human FcϒY fragment specific are as mentioned in the grid

Mean OD value								
	1	2	3	4	5	6	7	8
A	4.000	3.458	2.338	2.537	1.912	1.400	3.696	3.054
B								
C	0.797	0.584	0.349	0.257	0.205	0.157	0.535	0.420
D								
E	0.262	0.206	0.134	0.100	0.084	0.075	0.195	0.151
F								
G	0.209	0.154	0.091	0.083	0.069	0.062	0.144	0.119
H								

Table 6:The Mean of Respective OD values obtained in the above mentioned experiment.

Blockers	Gelatin Blocker			Skimmed milk Blocker			BSA Blocker		
S/N ratio	19.175	22.515	25.802	30.437	27.713	22.556	25.692	25.622	25.414
	3.819	3.805	3.857	3.086	2.970	2.534	3.718	3.522	3.463
	1.256	1.340	1.474	1.198	1.223	1.203	1.355	1.267	1.257
Peroxidase conjugated Goat anti Human dilutions	1:1 lac	1:1.5 lac	1:2 lac	1:1 lac	1:1.5 lac	1:2 lac	1:1 lac	1:1.5 lac	1:2 lac

Table 7:The calculated signal to noise ratio for the three different Blocker, their Respective Diluent and the Different dilutions of the Peroxidase conjugated Ab goat Anti-Human FcϒY fragment specific accordingly

Conclusion 3.2: From this experiment it was concluded that the 2% skim milk blocker and 0.5% skim milk Diluent along with the 1/1 lac dilution of Peroxidase conjugated Goat Anti-Human FcϒY fragment specific were observed to be giving the good signal to noise ratio hence their combination can be used for successive Assay Development.

3.3. To run the Calibration curve of Monoclonal Ab X multiple times with the standardized blocker (skim milk), coating concentration (0.125 µg/ml) and Peroxidase conjugated Ab Goat anti human FcϒY fragment specific(1/1 lac dilution) the assay was performed in Normal human serum.

	1	2	3
A	200 ng/ml Monoclonal Ab X		0.781 ng/ml Monoclonal Ab X
B	100 ng/ml Monoclonal Ab X		
C	50 ng/ml Monoclonal Ab X		Blank unspiked
D	25 ng/ml Monoclonal Ab X		
E	12.5 ng/ml Monoclonal Ab X		
F	6.25 ng/ml Monoclonal Ab X		
G	3.125 ng/ml Monoclonal Ab X		
H	1.562 ng/ml Monoclonal Ab X		
Peroxidase conjugated Goat anti human Ig G 1/1 lac			

Table 8:The plate layout for the standard curve of Monoclonal Ab X

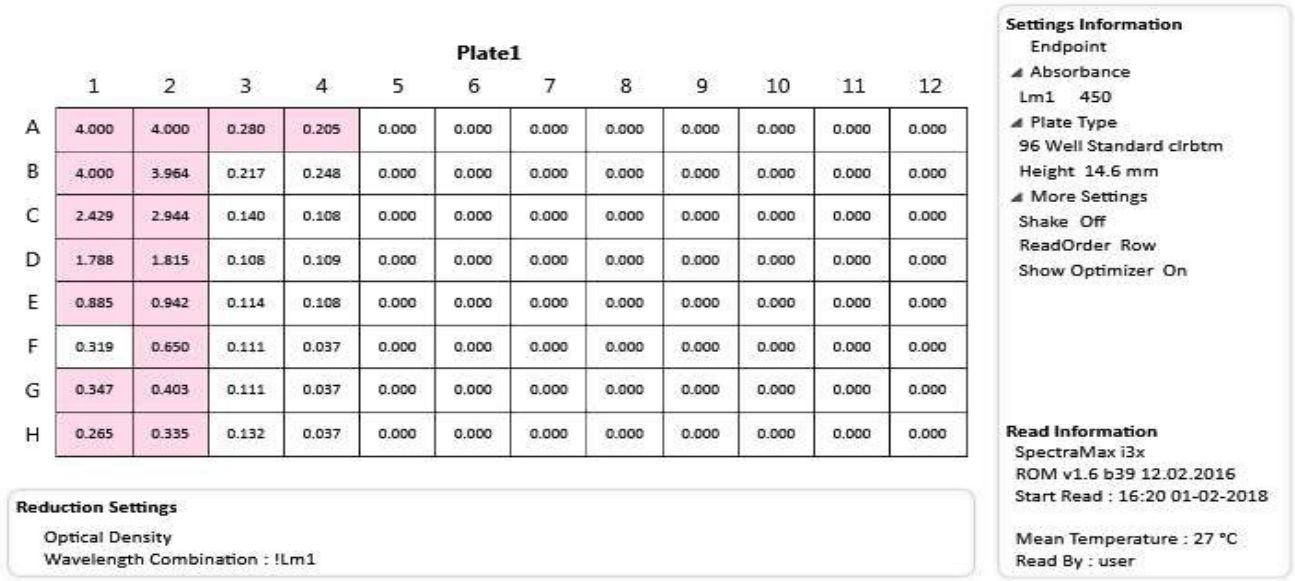


Plate1

	1	2	3	4	5	6	7	8	9	10	11	12
A	4.000	4.000	0.280	0.205	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
B	4.000	3.964	0.217	0.248	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
C	2.429	2.944	0.140	0.108	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
D	1.788	1.815	0.108	0.109	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
E	0.885	0.942	0.114	0.108	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F	0.319	0.650	0.111	0.037	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
G	0.347	0.403	0.111	0.037	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
H	0.265	0.335	0.132	0.037	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Settings Information
Endpoint
Absorbance
Lm1 450
Plate Type
96 Well Standard clrbtm
Height 14.6 mm
More Settings
Shake Off
ReadOrder Row
Show Optimizer On

Read Information
SpectraMax i3x
ROM v1.6 b39 12.02.2016
Start Read : 16:20 01-02-2018

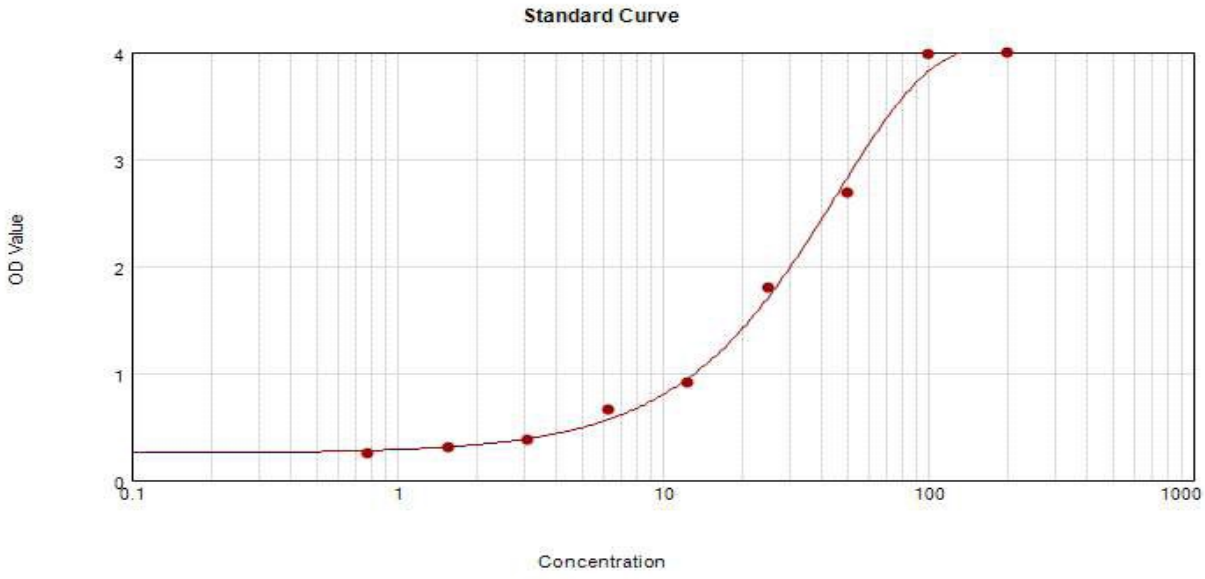
Mean Temperature : 27 °C
Read By : user

Reduction Settings
Optical Density
Wavelength Combination : !Lm1

Figure 12: The OD values of the standard curve of Monoclonal Ab X as observed in the ELISA reader Spectra Max i3X

Sample	Concentration ng/mL	BackCalcConc	Wells	Value	MeanValue	SD	CV	Avg-BackCalc	Cv-Bac
01	200.000	132.654	A1	4.000	4.000	0.000	0.0	132.654	0.000
		132.654	A2	4.000					
02	100.000	132.654	B1	4.000	3.982	0.025	0.6	127.861	5.302
		123.068	B2	3.964					
03	50.000	39.446	C1	2.429	2.687	0.364	13.6	46.264	20.841
		53.082	C2	2.944					
04	25.000	26.461	D1	1.788	1.802	0.019	1.0	26.703	1.282
		26.945	D2	1.815					
05	12.500	11.375	E1	0.885	0.913	0.040	4.4	11.829	5.422
		12.283	E2	0.942					
06	6.250	7.593	F2	0.650	0.650	0.000	0.0	7.593	0.000
07	3.125	2.305	G1	0.347	0.375	0.039	10.4	2.833	26.377
		3.362	G2	0.403					
08	1.563	0.455	H1	0.265	0.300	0.049	16.4	1.257	90.216
		2.059	H2	0.335					
09	0.781	0.838	A3	0.280	0.243	0.053	21.7	0.838	0.000
		Range?	A4	0.205					

Figure 13: The Details of the observed concentrations and the Mean OD values along with the CV and Bac-clalculated CV values in the standard curve of Monoclonal Ab X



● Std (Standards: MeanValue vs Concentration) Weighting: Fixed

Curve Fit Results ▲

Curve Fit : 5-Parameter Logistic $y = D + \frac{A - D}{\left\{1 + \left(\frac{x}{C}\right)^{B}\right\}^{G}}$

	Parameter	Estimated Value	Std. Error	Confidence Interval
Std R^2 = 0.996 EC50 = 33.86	A	0.252	0.115	[-0.067, 0.572]
	B	1.236	0.336	[0.303, 2.169]
	C	4.30e+6	2.81e+12	[-7.80e+12, 7.80e+12]
	D	4.090	0.170	[3.619, 4.562]
	G	1.41e+6	1.14e+12	[-3.15e+12, 3.15e+12]

Figure 14: The observed standard curve of Monoclonal Ab X in the ELISA Reader Spectra Max i3X

Conclusion: The experiment should be repeated to check the repeatability of the curve and to establish the range of EC-70 for the therapeutic Monoclonal Ab X.

3.4. To run the Calibration curve of Monoclonal Ab X to establish the EC-70 concentration and find the Range of the therapeutic Drug Monoclonal Ab X

	1	2	3
A	200 ng/ml Monoclonal Ab X		0.781 ng/ml Monoclonal Ab X
B	100 ng/ml Monoclonal Ab X		
C	50 ng/ml Monoclonal Ab X		Blank unspiked
D	25 ng/ml Monoclonal Ab X		
E	12.5 ng/ml Monoclonal Ab X		
F	6.25 ng/ml Monoclonal Ab X		
G	3.125 ng/ml Monoclonal Ab X		
H	1.562 ng/ml Monoclonal Ab X		
Peroxidase conjugated Goat anti human Ig G 1/1 lac			

Table 9:The plate layout for the standard curve of Monoclonal Ab X

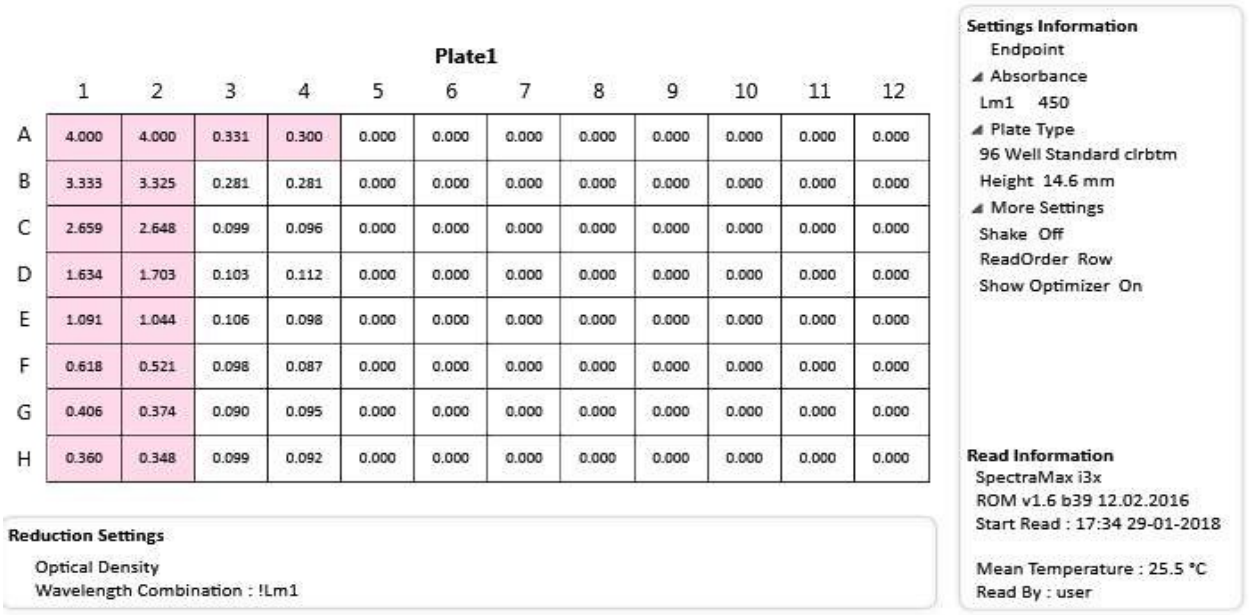


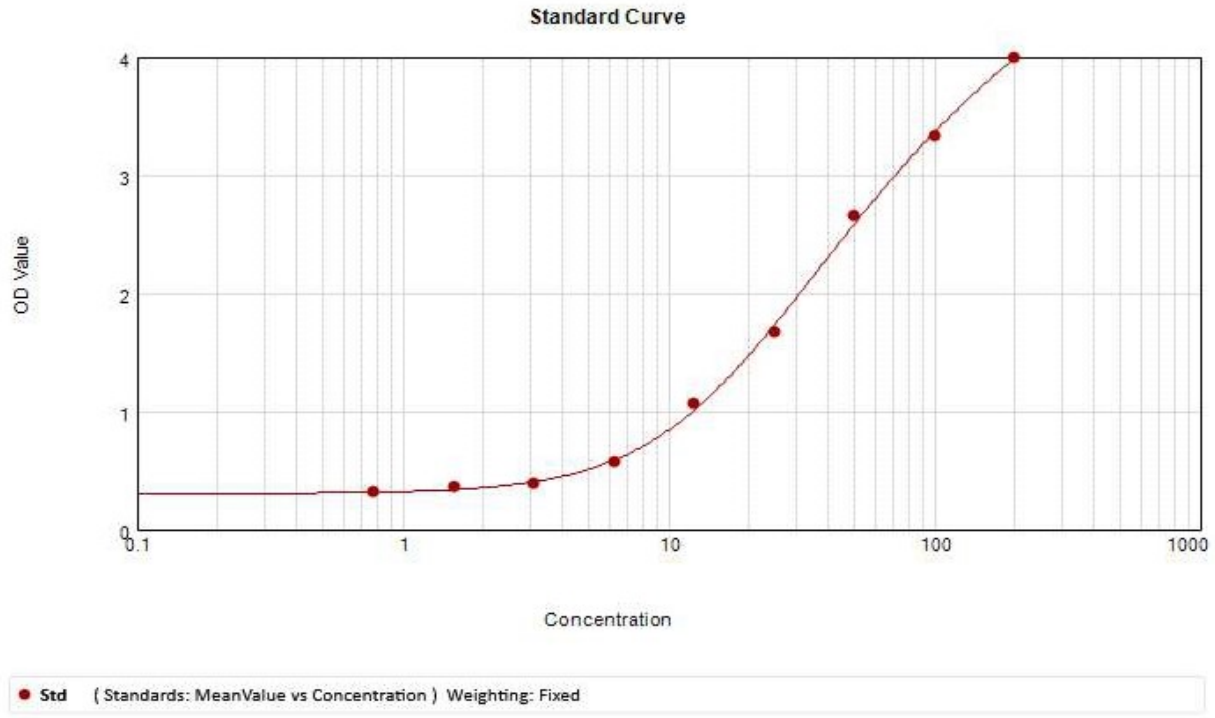
Plate1

	1	2	3	4	5	6	7	8	9	10	11	12
A	4.000	4.000	0.331	0.300	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
B	3.333	3.325	0.281	0.281	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
C	2.659	2.648	0.099	0.096	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
D	1.634	1.703	0.103	0.112	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
E	1.091	1.044	0.106	0.098	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F	0.618	0.521	0.098	0.087	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
G	0.406	0.374	0.090	0.095	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
H	0.360	0.348	0.099	0.092	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Figure 15: The OD values of the standard curve of Monoclonal Ab X as observed in the ELISA reader Spectra Max i3X

Sample	Concentration ng/mL	BackCalcConc	Wells	Value	MeanValue	SD	CV	Avg-BackCalc	Cv-Bac
01	200.000	202.694	A1	4.000	4.000	0.000	0.0	202.694	0.000
		202.694	A2	4.000					
02	100.000	96.669	B1	3.333	3.329	0.006	0.2	96.299	0.544
		95.929	B2	3.325					
03	50.000	52.925	C1	2.659	2.653	0.007	0.3	52.698	0.612
		52.470	C2	2.648					
04	25.000	22.944	D1	1.634	1.669	0.049	2.9	23.635	4.138
		24.327	D2	1.703					
05	12.500	13.643	E1	1.091	1.067	0.033	3.1	13.287	3.795
		12.930	E2	1.044					
06	6.250	6.679	F1	0.618	0.569	0.069	12.1	5.923	18.051
		5.167	F2	0.521					
07	3.125	3.126	G1	0.406	0.390	0.022	5.7	2.795	16.783
		2.463	G2	0.374					
08	1.563	2.135	H1	0.360	0.354	0.008	2.3	1.986	10.577
		1.838	H2	0.348					
09	0.781	1.316	A3	0.331	0.315	0.021	6.8	1.316	0.000
		Range?	A4	0.300					

Figure 16: The Details of the observed concentrations and the Mean OD values along with the CV and Bac-clalculated CV values for the standard curve of Monoclonal Ab X



Curve Fit Results ▲

Curve Fit : 5-Parameter Logistic $y = D + \frac{A - D}{\left\{1 + \left(\frac{x}{C}\right)^{B}\right\}^{G}}$

	Parameter	Estimated Value	Std. Error	Confidence Interval
Std R^2 = 0.999 EC50 = 75.12	A	0.305	0.052	[0.161, 0.449]
	B	1.621	0.415	[0.469, 2.773]
	C	15.97	8.937	[-8.841, 40.79]
	D	5.823	1.716	[1.058, 10.59]
	G	0.268	0.266	[-0.470, 1.006]

Figure 17: The observed standard curve of Monoclonal Ab X in the ELISA Reader Spectra Max i3X

Conclusion: standard curve observed in the experiment 3.3 was comparable with that of the experiment no 3.4 so the concentrations and their respective mean OD values can be further used to establish the range of EC-70 for the Development of Nab assay for the therapeutic Monoclonal Ab X.

3.5.Determination Of Ec-70:

From the Mean OD values obtained for the standard curve of therapeutic Monoclonal Ab X obtained in the experiments above a **statistical analysis** was done in the software Graph-pad Prism and after the calculation the EC 70 concentration was obtained to be 53.7 ng/ml which can be further analyzed for the development of Nab assay for the Drug , Therapeutic Monoclonal Ab X

3.6. To run the calibration curve of Human anti Monoclonal Ab X (positive control curve) with spiking of EC-70. (53.725 ng/ml) in the Normal human serum Matrix.

plate layout				
	1	2	3	4
A	1000 ng/ml human anti Monoclonal Ab X+Ec 70 spiked (53.725 ng/ml)		39.05 ng/ml human anti Monoclonal Ab X+Ec 70 spiked (53.725 ng/ml)	
B	5000 ng/ml human anti Monoclonal Ab X+Ec 70 spiked (53.725 ng/ml)		Blank + EC-70 spiked (53.725 ng/ml)	
C	2500 ng/ml human anti Monoclonal Ab X+Ec 70 spiked (53.725 ng/ml)		Blank + EC-70 spiked (53.725 ng/ml)	
D	1250 ng/ml human anti Monoclonal Ab X+Ec 70 spiked (53.725 ng/ml)		Blank + EC-70 spiked (53.725 ng/ml)	
E	625 ng/ml human anti Monoclonal Ab X+Ec 70 spiked (53.725 ng/ml)		Blank unspiked	
F	312.25 ng/ml human anti Monoclonal Ab X+Ec 70 spiked (53.725 ng/ml)			
G	156.25 ng/ml human anti Monoclonal Ab X+Ec 70 spiked (53.725 ng/ml)			
H	78.125 ng/ml human anti Monoclonal Ab X+Ec 70 spiked (53.725 ng/ml)			
Peroxidase conjugated Goat anti- Human Fc ϒ fragment specific	1/1 lac dilution			

Table 10:plate layout for the calibration curve of Human anti Monoclonal Ab X (positive control curve) with spiking of EC-70.(53.725 ng/ml) in the Normal human serum Matrix

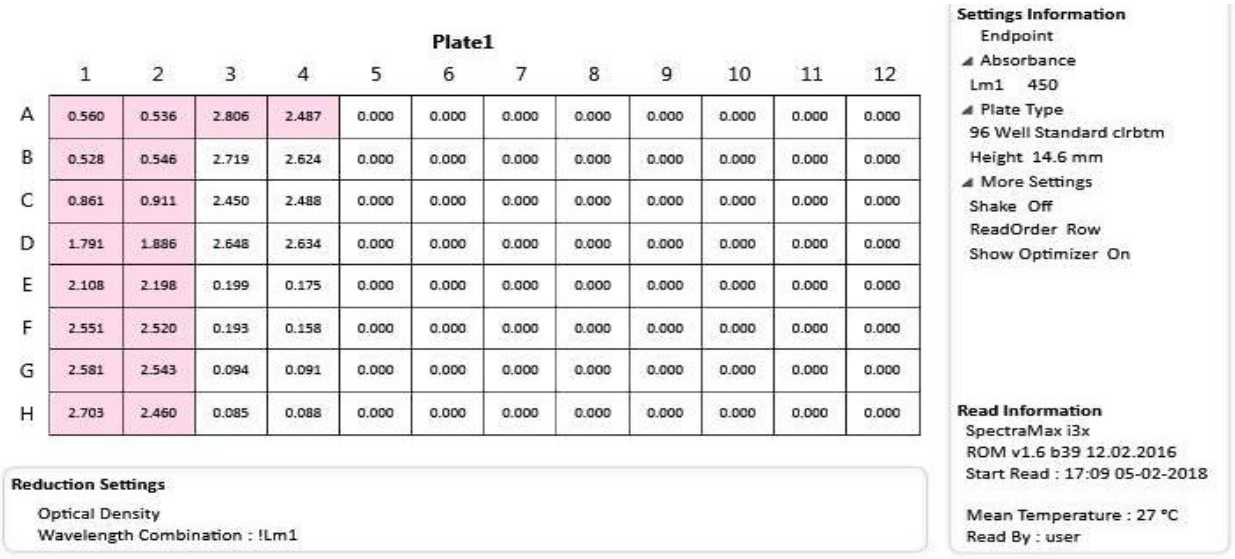


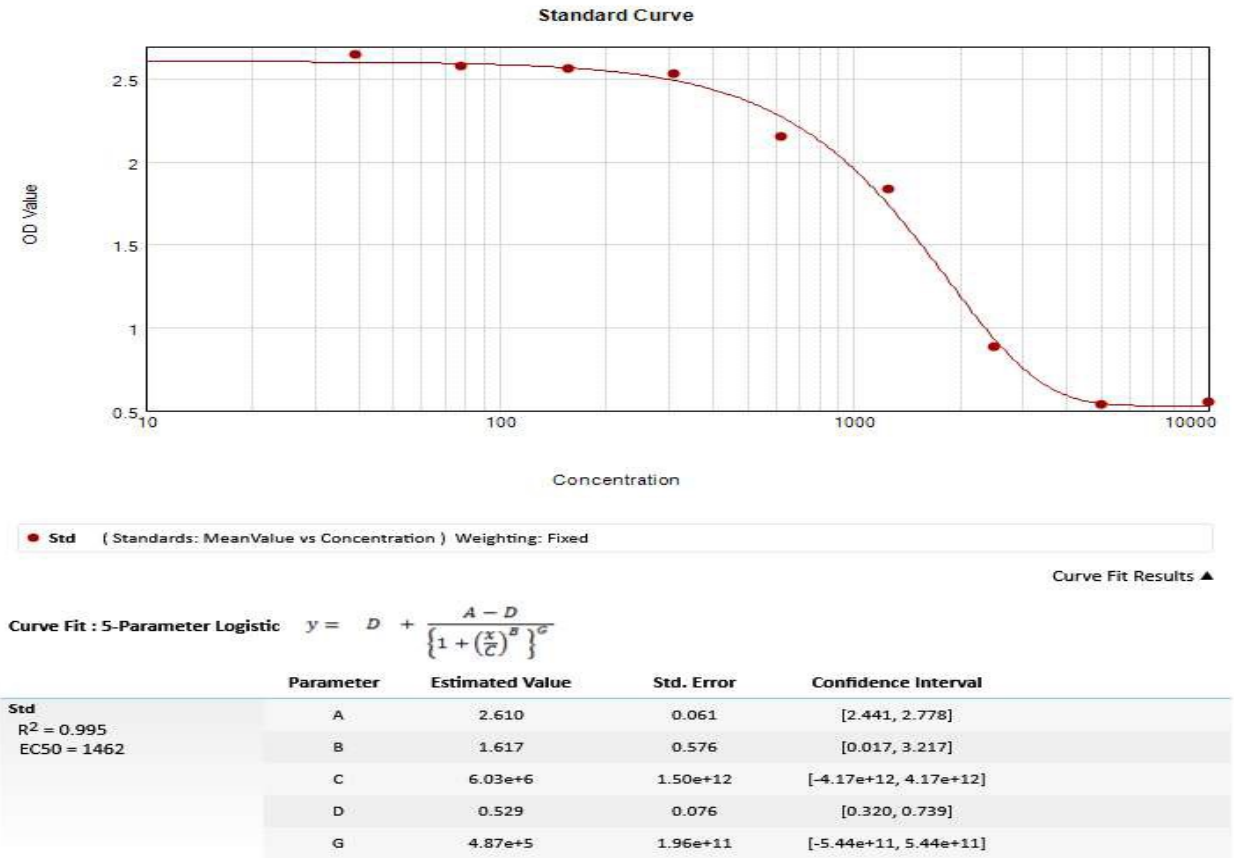
Plate1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.560	0.536	2.806	2.487	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
B	0.528	0.546	2.719	2.624	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
C	0.861	0.911	2.450	2.488	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
D	1.791	1.886	2.648	2.634	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
E	2.108	2.198	0.199	0.175	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F	2.551	2.520	0.193	0.158	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
G	2.581	2.543	0.094	0.091	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
H	2.703	2.460	0.085	0.088	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Figure 18: The OD values observed in the calibration curve of Human anti Monoclonal Ab X (positive control curve) with spiking of EC-70.(53.725 ng/ml) in the Normal human serum

Sample	Concentration ng/mL	BackCalcConc	Wells	Value	MeanValue	SD	CV	Avg-BackCalc	Cv-Bac
01	10000.000	4473.519	A1	0.560	0.548	0.017	3.1	4946.441	13.521
		5419.363	A2	0.536					
02	5000.000	Range?	B1	0.528	0.537	0.013	2.4	4849.471	0.000
		4849.471	B2	0.546					
03	2500.000	2671.521	C1	0.861	0.886	0.036	4.0	2606.639	3.520
		2541.757	C2	0.911					
04	1250.000	1194.974	D1	1.791	1.838	0.067	3.7	1139.807	6.845
		1084.640	D2	1.886					
05	625.000	826.549	E1	2.108	2.153	0.063	2.9	773.214	9.755
		719.879	E2	2.198					
06	312.500	204.416	F1	2.551	2.535	0.022	0.9	235.067	18.440
		265.718	F2	2.520					
07	156.250	128.927	G1	2.581	2.562	0.027	1.1	175.165	37.331
		221.402	G2	2.543					
08	78.125	Range?	H1	2.703	2.581	0.172	6.7	368.977	0.000
		368.977	H2	2.460					
09	39.062	Range?	A3	2.806	2.646	0.226	8.5	324.789	0.000
		324.789	A4	2.487					

Figure 19: The Details of the observed concentrations and the Mean OD values along with the CV and Bac-clalculated CV values for the calibration curve of Human anti Monoclonal Ab X (positive control curve) with spiking of EC-70.(53.725 ng/ml) in the Normal human serum.



	Parameter	Estimated Value	Std. Error	Confidence Interval
Std R^2 = 0.995 EC50 = 1462	A	2.610	0.061	[2.441, 2.778]
	B	1.617	0.576	[0.017, 3.217]
	C	6.03e+6	1.50e+12	[-4.17e+12, 4.17e+12]
	D	0.529	0.076	[0.320, 0.739]
	G	4.87e+5	1.96e+11	[-5.44e+11, 5.44e+11]

Figure 9: The observed calibration curve of Human anti Monoclonal Ab X (positive control curve)in the ELISA reader spectra Max i3X

Conclusion 3.6: The observed curve of positive control (Human anti Monoclonal Ab X) (exper is found to be completely inverted in nature from the standard curve of the Monoclonal Ab X, thus the activity of the Human anti Monoclonal Ab X (positive control) can be understood by the developed Nab assay which could be further used for the detection of Presence of Neutralizing Ab in patient serum.

Note: From the above experiments the value of controls HQC (7500 ng/ml) and LQC (300 ng/ml) were established and further used in the following assays:

3.7. To check the Selectivity parameter in developed Nab assay for the drug therapeutic Monoclonal Ab X the assay by screening the 10 different lots of normal human serum matrices with the pre-established quality control ranges.

plate layout for selectivity									
	1	2	3	4	5	6	7	8	9
A	HQC 7500 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml for **serum 1**		Blank+ EC 70 spiked 53.725 ng/ml for **serum 3**		LQC 300 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml for **serum 6**		HQC 7500 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml for **serum 9**		Blank+ EC 70 spiked 53.725 ng/ml
B	LQC 300 ng/ml Monoclonal Ab X + EC 70 spiked 53.725 ng/ml for **serum 1**		HQC 7500 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml for **serum 4**		Blank+ EC 70 spiked 53.725 ng/ml for **serum 6**		LQC 300 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml for **serum 9**		
C	Blank+ EC 70 spiked 53.725 ng/ml for **serum 1**		LQC 300 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml for **serum 4**		HQC 7500 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml for **serum 7**		Blank+ EC 70 spiked 53.725 ng/ml for **serum 9**		
D	HQC 7500 ng/mlMonoclonal Ab X+ EC 70 spiked 53.725 ng/ml for **serum 2**		Blank+ EC 70 spiked 53.725 ng/ml for **serum 4**		LQC 300 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml for **serum 7**		HQC 7500 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml for **serum 10**		
E	LQC 300 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml for **serum 2**		HQC 7500 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml for **serum 5**		Blank+ EC 70 spiked 53.725 ng/ml for **serum 7**		LQC 300 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml for **serum 10**		
F	Blank+ EC 70 spiked 53.725 ng/ml for **serum 2**		LQC 300 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml for **serum 5**		HQC 7500 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml for **serum 8**		Blank+ EC 70 spiked 53.725 ng/ml for **serum 10**		
G	HQC 7500 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml for **serum 3**		Blank+ EC 70 spiked 53.725 ng/ml for **serum 5**		LQC 300 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml for **serum 8**		Blank+ EC 70 spiked 53.725 ng/ml		
H	LQC 300 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml for **serum 3**		HQC 7500 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml for **serum6**		Blank+ EC 70 spiked 53.725 ng/ml for **serum 8**		Blank+ EC 70 spiked 53.725 ng/ml		
Matrix	Normal Human serum								
Peroxidase conjugated Antibody	1/1 Lac Goat anti human								

Table 11:The plate Layout for the Selectivity parameter in developed Nab assay for the Drug, Therapeutic Monoclonal Ab X screening of the 10 different lots of normal human serum matrices

Table 9: The plate Layout for the Selectivity parameter in developed Nab assay for the Drug, Therapeutic Monoclonal Ab X screening of the 10 different lots of normal human serum matrices

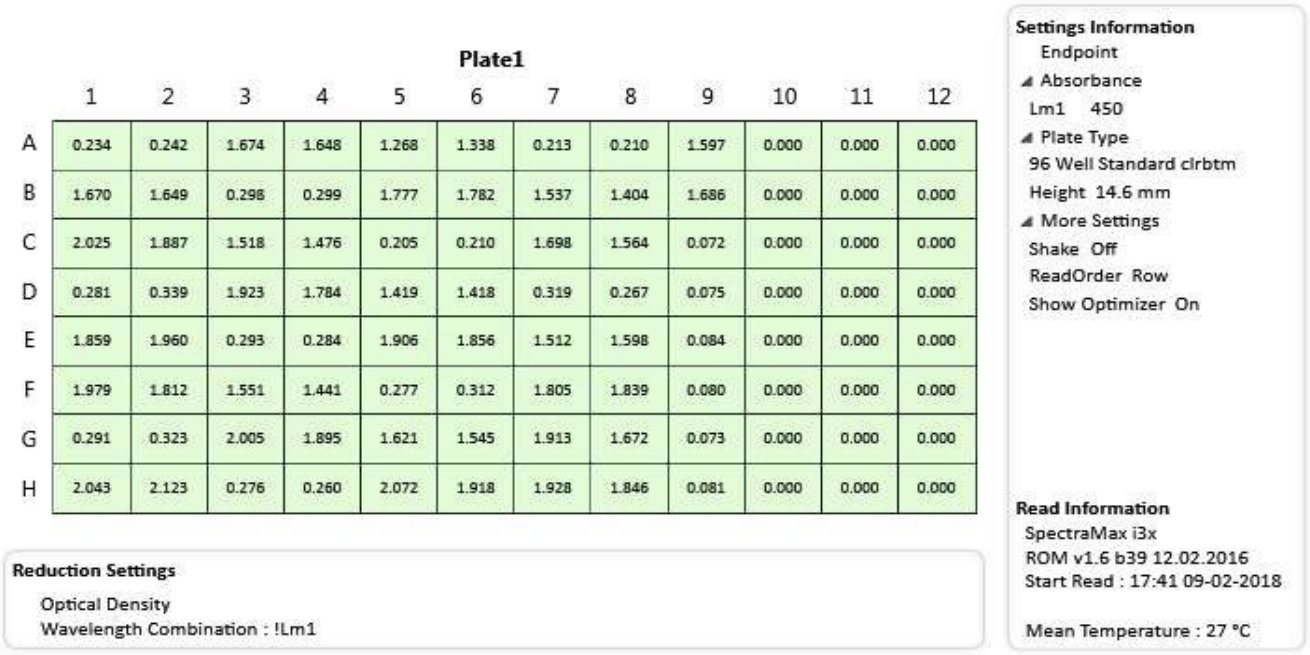


Plate1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.234	0.242	1.674	1.648	1.268	1.338	0.213	0.210	1.597	0.000	0.000	0.000
B	1.670	1.649	0.298	0.299	1.777	1.782	1.537	1.404	1.686	0.000	0.000	0.000
C	2.025	1.887	1.518	1.476	0.205	0.210	1.698	1.564	0.072	0.000	0.000	0.000
D	0.281	0.339	1.923	1.784	1.419	1.418	0.319	0.267	0.075	0.000	0.000	0.000
E	1.859	1.960	0.293	0.284	1.906	1.856	1.512	1.598	0.084	0.000	0.000	0.000
F	1.979	1.812	1.551	1.441	0.277	0.312	1.805	1.839	0.080	0.000	0.000	0.000
G	0.291	0.323	2.005	1.895	1.621	1.545	1.913	1.672	0.073	0.000	0.000	0.000
H	2.043	2.123	0.276	0.260	2.072	1.918	1.928	1.846	0.081	0.000	0.000	0.000

Figure 20: The OD values observed for the Selectivity parameter in developed Nab assay for the Drug, Therapeutic Monoclonal Ab X in ELISA Reader Spectra MAX i3X

	HQC	LQC	BLANK
1	0.238	1.659	1.956
2	0.31	1.909	1.895
3	0.307	2.083	1.661
4	0.298	1.497	1.853
5	0.288	1.496	1.95
6	0.268	1.303	1.78
7	0.208	1.418	1.881
8	0.294	1.583	1.995
9	0.211	1.47	1.631
10	0.293	1.555	1.822
AVERAGE	0.272	1.597	1.842
SD	0.039	0.234	0.116
%CV	14.272	14.670	6.284

Table 12:The observed Mean OD values of pre-established quality controls in 10 individual lots of normal human serum for the selectivity parameter for the Drug, Therapeutic Monoclonal Ab X

Conclusion: The Developed Nab assay for the therapeutic Monoclonal Ab X can successfully detect the presence of neutralizing Ab in different lots of Normal Human Serum.

3.8. To check the Drug tolerance in developed Nab assay for the drug therapeutic Monoclonal Ab X the assay by spiking the higher concentrations of the therapeutic Monoclonal Ab X to check the response of the Developed Nab assay at the presence of pre-existing higher levels of the Drug Therapeutic Monoclonal Ab X

Plate Layout for Drug tolerance				
	1	2	3	4
A	10 µg/ml of Drug concentration + 53.725 ng/ml Monoclonal Ab X (EC 70)		1000 ng/ml Human anti Monoclonal Ab X + 53.725 ng/ml Monoclonal Ab X (EC 70)	
B	7.5 µg/ml of Drug concentration + 53.725 ng/ml Monoclonal Ab X (EC 70)		Blank + EC 70 (53.725 ng/ml)	
C	5 µg/ml of Drug concentration + 53.725 ng/ml Monoclonal Ab X (EC 70)		Blank + EC 70 (53.725 ng/ml)	
D	2.5 µg/ml of Drug concentration + 53.725 ng/ml Monoclonal Ab X (EC 70)		Blank unspiked	
E	1 µg/ml of Drug concentration + 53.725 ng/ml Monoclonal Ab X (EC 70)			
F	0.5 µg/ml of Drug concentration + 53.725 ng/ml Monoclonal Ab X (EC 70)			
G	0.25 µg/ml of Drug concentration + 53.725 ng/ml Monoclonal Ab X (EC 70)			
H	1000 ng/ml Human anti Monoclonal Ab X + 53.725 ng/ml Monoclonal Ab X (EC 70)			
Peroxidase conjugated Goat Anti- Human FCγ fragment specific	1/1 lac dilutioin			
Normal Human serum				

Table 13:plate layout to check the Drug tolerance parameter in the developed Nab Assay

(The spiked higher concentration of the Drug, Therapeutic Monoclonal Ab X was compared with one point of positive control (1000 ng/ml Human Anti- Monoclonal Ab X)

Sample	Wells	Value	MeanResult	SD	CV
01	A1	2.583	2.716	0.188	6.9
	A2	2.849			
02	B1	2.539	2.723	0.260	9.5
	B2	2.907			
03	C1	2.362	2.461	0.141	5.7
	C2	2.561			
04	D1	2.407	2.530	0.175	6.9
	D2	2.654			
05	E1	2.196	2.302	0.149	6.5
	E2	2.407			
06	F1	2.352	2.482	0.184	7.4
	F2	2.612			
07	G1	2.269	2.427	0.223	9.2
	G2	2.585			
08	H1	1.775	1.820	0.063	3.5
	H2	1.865			
09	A3	1.728	1.963	0.332	16.9
	A4	2.197			
10	B3	2.818	2.693	0.177	6.6
	B4	2.568			
11	C3	2.583	2.627	0.062	2.4
	C4	2.671			
12	D3	0.181	0.189	0.011	5.8
	D4	0.197			
13	E3	0.102	0.106	0.005	5.1
	E4	0.110			
14	F3	0.108	0.118	0.014	12.1
	F4	0.128			
15	G3	0.102	0.102	0.000	0.4
	G4	0.102			
16	H3	0.110	0.110	0.000	0.2
	H4	0.110			

Figure 21: The mean OD values observed in the ELISA reader Spectra Max i3X, for the Drug tolerance check in the developed Nab assay

	Drug Concentration	Mean OD values
1.	10 µg/ml	2.716
2.	7.5 µg/ml	2.723
3.	5 µg/ml	2.461
4.	2.5 µg/ml	2.530
5.	1 µg/ml	**2.302**
6.	0.5 µg/ml	**2.482**
7.	0.25 µg/ml	**2.427**

Table 14: The observed mean OD values at the different spiked concentrations of the therapeutic Drug monoclonal Ab X

Conclusion: The Developed ligand binding neutralizing Ab assay is sensitive enough until the pre-existing range of 1 µg/ml of the therapeutic monoclonal Ab X present in the patient serum saturation was observed in the ranges above 1 µg/ml.

3.9. To Check the Accuracy and precision Parameter in the developed Ligand binding Neutralizing assay for the Detection of Neutralizing Ab produced against the therapeutic Monoclonal Ab X

Plate layout for Accuracy and precision parameter								
	1	2	3	4	5	6	7	8
A	HQC 7500 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml		Blank+ EC 70 spiked 53.725 ng/ml		LQC 300 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml		Blank+ EC 70 spiked 53.725 ng/ml	
B	LQC 300 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml		HQC 7500 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml		Blank+ EC 70 spiked 53.725 ng/ml		Blank+ EC 70 spiked 53.725 ng/ml	
C	Blank+ EC 70 spiked 53.725 ng/ml		LQC 300 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml		HQC 7500 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml		Blank unspiked	
D	HQC 7500 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml		Blank+ EC 70 spiked 53.725 ng/ml		LQC 300 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml			
E	LQC 300 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml		HQC 7500 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml		Blank+ EC 70 spiked 53.725 ng/ml			
F	Blank+ EC 70 spiked 53.725 ng/ml		LQC 300 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml		HQC 7500 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml			
G	HQC 7500 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml		Blank+ EC 70 spiked 53.725 ng/ml		LQC 300 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml			
H	LQC 300 ng/ml Monoclonal Ab X+ EC 70 spiked 53.725 ng/ml		HQC 7500 ng/ml Monoclonal Ab X+ EC 70 spiked		HQC 7500 ng/ml Monoclonal Ab X+ EC 70 spiked			
Matrix	Normal Human serum							
Peroxidase conjugated Antibody	1/1 Lac Goat anti human							

Table 15: The plate layout for the Accuracy and precision parameter for the developed Ligand binding Neutralizing assay for the Detection of Neutralizing Ab produced against the therapeutic Monoclonal Ab X; here the pre-established quality controls were checked 6 times along with two system suitability controls.

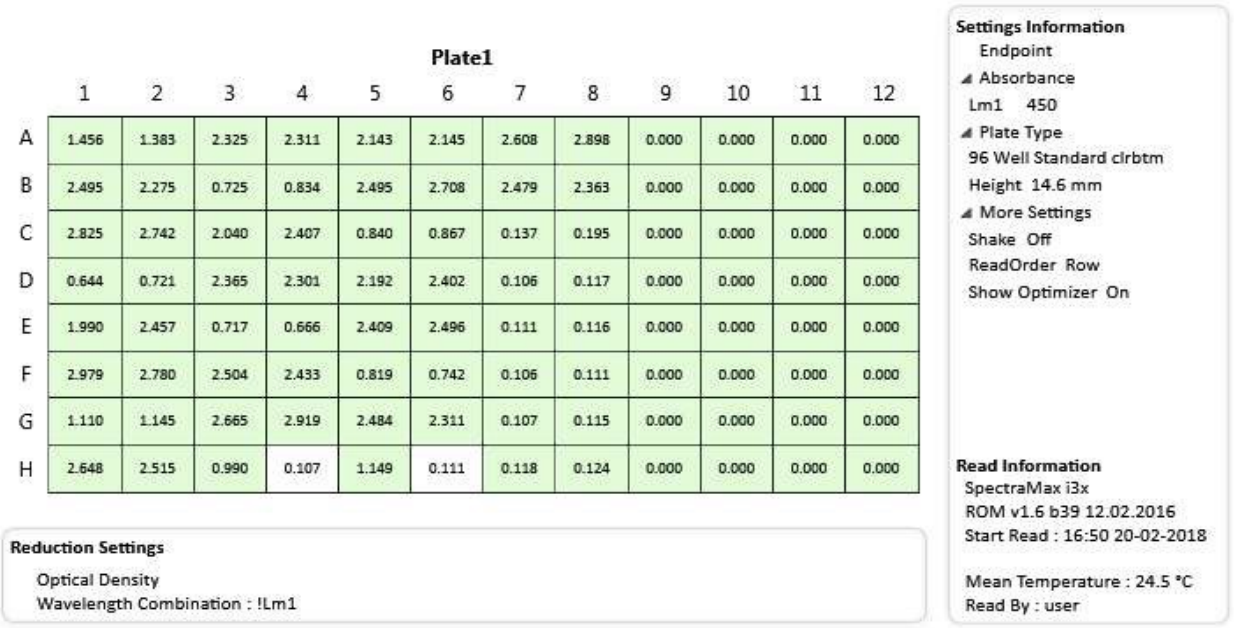


Plate1

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.456	1.383	2.325	2.311	2.143	2.145	2.608	2.898	0.000	0.000	0.000	0.000
B	2.495	2.275	0.725	0.834	2.495	2.708	2.479	2.363	0.000	0.000	0.000	0.000
C	2.825	2.742	2.040	2.407	0.840	0.867	0.137	0.195	0.000	0.000	0.000	0.000
D	0.644	0.721	2.365	2.301	2.192	2.402	0.106	0.117	0.000	0.000	0.000	0.000
E	1.990	2.457	0.717	0.666	2.409	2.496	0.111	0.116	0.000	0.000	0.000	0.000
F	2.979	2.780	2.504	2.433	0.819	0.742	0.106	0.111	0.000	0.000	0.000	0.000
G	1.110	1.145	2.665	2.919	2.484	2.311	0.107	0.115	0.000	0.000	0.000	0.000
H	2.648	2.515	0.990	0.107	1.149	0.111	0.118	0.124	0.000	0.000	0.000	0.000

Figure 22: The OD values observed in the ELISA reader spectra Max i3x for the Accuracy and precision Parameter in the developed Ligand binding Neutralizing assay for the Detection of Neutralizing Ab produced against the therapeutic Monoclonal Ab X

	HQC	LQC	Blank+EC 70 spiked
1	1.420	2.385	2.784
2	0.683	2.224	2.880
3	1.128	2.224	2.333
4	0.780	2.369	2.792
5	0.692	2.244	2.602
6	0.854	2.297	2.453
7	0.781	2.398	2.753
8	0.854	2.297	2.421
Average	**0.905**	**2.306**	**2.656**
SD	**0.236**	**0.067**	**0.191**
cv%	**26.079**	**2.914**	**7.193**

Table 16: The observed Mean OD values of the 6 different quality controls along with the two system suitability controls for the Accuracy and Precision parameter of the Developed Nab Assay for the detection of therapeutic Monoclonal Ab X.

Conclusion: The Developed Nab assay passes in the criteria of Accuracy and precision check.

4.CONCLUSION

Determination of Neutralizing Ab is important to understand the immunogenicity oftherapeutics.The Neutralizing Abs are present in the bound form with the Therapeutic drug(Monoclonal Ab X) and with the help of acid dissociation step they can be rendered free in the matrix.with the help of affinity purification step the Neutralizing Ab are liberated into an almost matrix free medium. The Developed Assay can detect the presence of Neutralizing Abs against the therapeutic Monoclonal Ab X in the Normal Human serum .Sensitivity of 300 ng/ml was achieved with the developed assay.The Assay also passes in various pre-validation Parameters such as selectivity ,Drug tolerance (1µg/ml drug concentration) and it was Proven to be Accurate and Precise.

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