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Certificate

This is to certify that this dissertation entitled **Development, Optimisation and Standardization of a method for the analysis of free polysaccharide content in the final multivalent pneumococcal vaccine** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Asmi Gaikwad** at **Serum Institute of India, Pune** under the supervision of **Dr. Asha Mallya, Deputy Director, Monoclonal Antibody Research and Development Laboratory**, during the academic year **2024-2025**.

Dr. Asha Mallya

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Date: 21/03/2025

Development, Optimisation and Standardization of a method for the analysis of free polysaccharide content in the final multivalent pneumococcal vaccine

A Thesis

submitted to

Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

by

Asmi Gaikwad

Indian Institute of Science Education and Research Pune

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Date: **21st March, 2025**

Under the guidance of

Supervisor: **Dr. Asha Mallya**

Deputy Director

From **20th May 2024** to **21st Mar 2025**

Serum Institute of India Pvt. Ltd. (SIIPL), Pune

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Dr. Asha Mallya

Committee:

Dr. Asha Mallya 

Dr. Vineeta Bal 

This thesis is dedicated to my parents for their unwavering support and encouragement in nurturing my passion for science

Declaration

I hereby declare that the matter embodied in the report entitled **Development, Optimisation and Standardization of a method for the analysis of free polysaccharide content in the final multivalent pneumococcal vaccine** are the results of the work carried out by me at the Monoclonal Antibody Research and Development Laboratory (MARDL), **Serum Institute of India, Pune**, under the supervision of **Dr. Asha Mallya** and the same has not been submitted elsewhere for any other degree or publication.

Asmi Gaikwad

Date: 21.03.25

Table of Contents

List of Abbreviations

Abbreviation	Full form
Ab	Antibody
Ag	Antigen
BSA	Bovine serum albumin
DMEM/F12	Dulbecco's Modified Eagle Medium/Factor-12
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
HAT	Hypoxanthine Aminopterin and Thymidine
HRP	Horseradish Peroxidase
HPSeC	High Performance Size Exclusion Chromatography
MAb	Monoclonal Antibody
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
IgG	Immunoglobulin G
kDa	kilo Dalton
LAB	Luminex Assay Buffer
MFI	Median Fluorescence Intensity
SDS	Sodium Dodecyl Sulfate
PAGE	Polyacrylamide Gel Electrophoresis
PE	Phycoerythrin
RPM	Revolutions per minute
RT	Room temperature
TMB	3,3',5,5'-Tetramethyl Benzidine
WFI	Water for Injection
RBC	Red Blood Cell
DC	Detergent Compatibility
BBA	Bead Based Assay
TEMED	Tetramethylenediamine
APS	Ammonium Persulfate
LD	Limiting Dilution
BBCIA	Bead Based Competitive Inhibition Assay
µg	Microgram
µl	Microlitre

mg	Microgram
ml	Milliliter
min	Minute
ng	Nanogram
M	Molarity
N	Normality
mA	milliampere
mAU	Milli Absorbance Unit

Abstract

Streptococcus pneumoniae, a gram-positive bacterium is the causative agent for community acquired pneumonia. The infection results in pus filled alveolar sacs in the lungs leading to chest pain and respiratory distress. To date, about 106 strains have been discovered. With the evolution of each new strain, an increasing resistance to antibiotics is seen. A glance at the morphology reveals the capsular polysaccharide layer as the main virulence factor aiding the survival of the organism. Infants (< 2 years), elderly (> 65 years) and immunocompromised individuals are most susceptible to the disease. The first vaccine developed against *S. pneumoniae* was a purified capsular polysaccharide-based vaccine. This vaccine generated T-cell independent immune responses- which provided short-term immunity and no memory B-cell formation. However, this vaccine failed to prove its immunogenicity in infants due to presence of immature B-cells. Thus, the capsular polysaccharides were conjugated in-vitro using carrier proteins- CRM197, Diphtheria Toxoid and Tetanus Toxoid, resulting in a polysaccharide-conjugate vaccine. This type of vaccine was able to generate T-cell dependent immune responses leading to formation of memory B-cells. Estimation of free polysaccharide in conjugate vaccine is necessary to evaluate the stability of vaccine that has direct impact on immunogenicity. Traditional methods like ELISA, are laborious and time consuming. Additionally, with the development of higher valent vaccines, it is necessary to look for alternate high throughput methods for estimation of free polysaccharide levels. Therefore, through this project, I aim to develop a high throughput method for detection and quantification of free polysaccharide content from the multivalent pneumococcal vaccine formulations using multiplexed sandwich bead-based assay.

Acknowledgement

I would like to express my sincere gratitude to **Dr. Asha Mallya**, HOD, Monoclonal Antibody Research and Development Laboratory (MARDL), for giving me the opportunity to work in her laboratory at the Serum Institute of India, Pune.

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Contributions

Contributor name	Contributor role
Mr. Dipen Soni, Mr. Sandip Naikwade, Mr. Somnath Sargar, Asmi Gaikwad	Conceptualization Ideas
Mr. Dipen Soni, Mr. Sandip Naikwade, Mr. Somnath Sargar, Asmi Gaikwad	Methodology
Mr. Dipen Soni, Mr. Sandip Naikwade, Mr. Somnath Sargar	Software
Mr. Dipen Soni, Mr. Sandip Naikwade, Asmi Gaikwad	Validation
Mr. Dipen Soni, Mr. Sandip Naikwade, Asmi Gaikwad	Formal analysis
Mr. Dipen Soni, Mr. Sandip Naikwade, Asmi Gaikwad	Investigation
Serum Institute of India	Resources
Mr. Dipen Soni, Mr. Sandip Naikwade, Asmi Gaikwad	Data Curation
Asmi Gaikwad	Writing - original draft preparation
Asmi Gaikwad	Writing - review and editing
Mr. Dipen Soni, Mr. Sandip Naikwade, Mr. Somnath Sargar	Visualization
Dr. Asha Mallya, Mr. Dipen Soni, Mr. Sandip Naikwade	Supervision
-	Project administration
-	Funding acquisition

Chapter 1
Introduction

Introduction

1.1 Pneumonia:

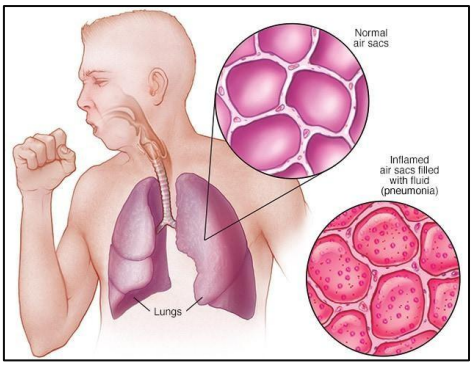
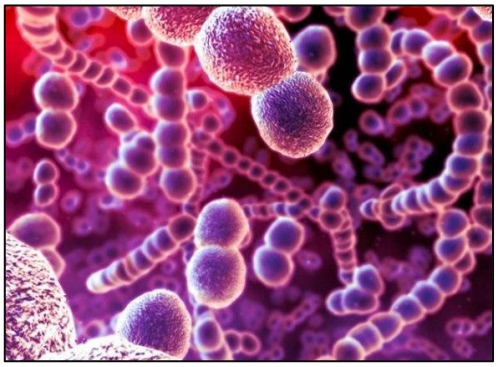


Fig 1.1: Schematic of a person suffering from pneumonia (source: https://tinyurl.com/zxwvvanb)

Pneumonia is a lower respiratory tract disease which results in inflamed or pus-filled air sacs[1]. It can occur in one or both lungs[1]. Common symptoms include cough, fever, chills, difficulty in breathing and chest pain [2]. Some of the known causative agents include bacteria (*Streptococcus pneumoniae*), viruses, and fungi[3]. This disease is highly contagious as it may easily spread through aerosols/ droplets – close contact with the patient or with contaminated surfaces can further ease transmission[3]. Infants (less than 2 years old) [4], elderly (greater than 65 years) and immunocompromised individuals are highly susceptible to being infected due to the presence of immature and weak immune system. [2]

1.2 Streptococcus pneumoniae:



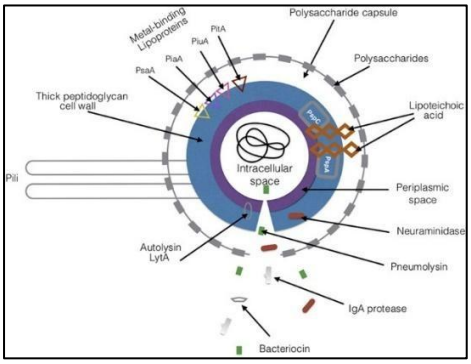


Fig 1.2 (a) *Fig 1.2 (b)*

Fig 1.2 a): 3D representation of S. pneumoniae strains (source: https://bit.ly/4j7VE67)

Fig 1.2b) Detailed morphology of Streptococcus pneumoniae ([5])

Streptococcus pneumoniae is the causative agent for community acquired pneumonia. This bacterium occurs as a pair of short chains, with a lancet shaped structure[6]. It is gram positive and has a rigid capsular polysaccharide layer made of peptidoglycan that enables its survival within the host cell[6]. It contains pili for attachment to the host cell. Some other key features include the presence of various surface proteins like Pneumococcal surface protein A (PsaA): Choline-binding protein A (CbpA), Pneumococcal surface protein C (PspC), PhtA, PhtB, PhtD, and PhtE (Polyhistidine triad (Pht) proteins)[6]. The key virulent factors include capsular polysaccharide layer, cytotoxic products, and surface proteins[6]. Moreover, this bacterium is mainly known to infect humans and colonises the mucosal surfaces of respiratory tract, leading to Pneumonia. it can also cause meningitis, otitis media and sinusitis [7].

1.3 Global disease burden:

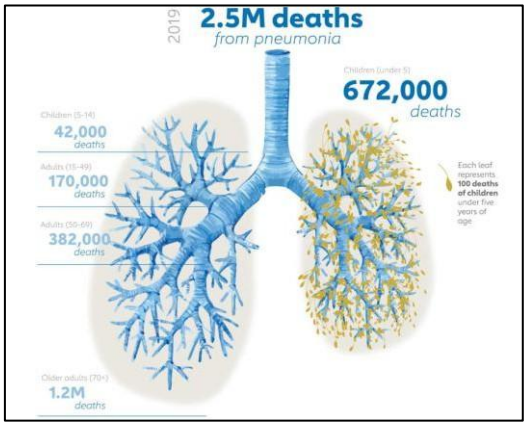


Fig 1.3: Global burden of pneumococcal disease (Source: Global Burden of Disease, 2019. https://bit.ly/4bUkkN2)

Infection caused by Streptococcus pneumoniae accounts for nearly 30-50% cases in children and adults [8]. In children, under five, according to World Health Organisation, over 3,00,000 deaths occur annually[8]. The disease burden is higher in low- and middle-income countries where access to quality health care is limited. More than a 100 strains of S. pneumoniae have been discovered so far. With every evolving strain, there is a stronger antibiotic resistance. Therefore, effective vaccines are being developed and tested to control the spread of this disease.[9]

1.4 Vaccine Development and its antigenicity:

Fig 1.4: Pneumosil, India's first polysaccharide-conjugate vaccine (10 valent) made by Serum Institute of India (source: https://bit.ly/4iqmS83)

As stated earlier, more than 90 strains of the bacterium have been discovered so far [6]. Thus, identification of the potential disease-causing strains was crucial. Considering this factor, the first vaccine against Streptococcus pneumoniae was a polysaccharide-based vaccine (14 valent) developed in 1977[10]. Later in 1983, 23 valent pneumococcal polysaccharide vaccine was developed. This vaccine being purely polysaccharide-based cause T-cell independent immune responses[10]. Therefore, when administered to adults, this vaccine led to the generation of short-term immune responses i.e production of only IgM based antibody. However, this Polysaccharide based vaccine failed to prove its antigenicity in infants (less than 2 years old), mainly due to the immature immune system present in these individuals. [11]

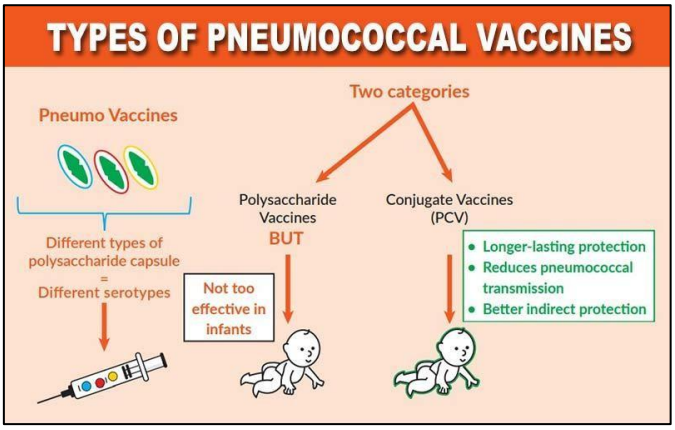


Fig 1.5: Difference between polysaccharide based and polysaccharide-conjugate based vaccines (source: https://bit.ly/4hOXyYh)

To overcome the barriers laid by the polysaccharide vaccines, the capsular polysaccharides were then chemically conjugated with a carrier protein in vitro. The carrier proteins included Cross Reactive Material 197 (CRM197), Tetanus Toxoid (TT) and Diphtheria Toxoid (DT)[12]. This process was performed to enhance the immunogenicity of the antigen to generate T-cell dependent immune cell responses eventually leading to the production of memory B-cells. Eventually, this led to the discovery of the first Pneumococcal Conjugate

Vaccine (PCV) in 2000 which covered 7 serotypes. Over time, PCV10, PCV 13, PCV15 and PCV 21 vaccines were developed.[13]

Now that these Polysaccharide conjugate vaccines were made, it was necessary to check their stability when introduced within the host. Literature states that Polysaccharide conjugate vaccines having free polysaccharide content above 10% show low immunogenicity as compared to those having considerably lesser percent of free polysaccharides[14]. Conventional methods like the Enzyme Linked Immunosorbent Assay (ELISA) have been used to check for the antigen- antibody reactivity. However, this method is highly time consuming and laborious too![15] Therefore, to overcome this drawback, the Multiplex bead-based assay was introduced to perform the task in lesser time and with more efficiency [16].

1.5 Antibodies:

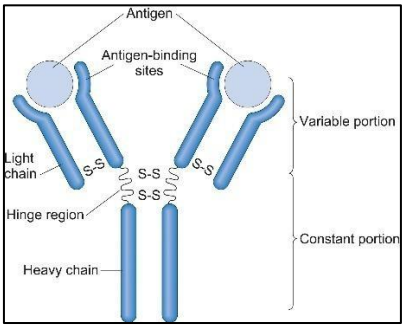


Fig 1.6: Structure of an antibody (source: https://bit.ly/4kN5POW)

Antibodies are Y-shaped Immunoglobulins consisting of two light and two heavy chains joined by disulphide linkages[17]. An antibody molecule is can be subdivided into two parts based on its regions namely- Fc region and Fab region[17]. It comprises of heavy and light chains. Light chains are a part of the Fab region and form the antigen binding sites. These proteins are secreted by the B-cells of the adaptive immune system in response to antigen invasion. There are five major classes of Immunoglobulins namely IgG, IgM, IgE, IgD and IgA [18]. IgM has a pentameric structure and is the first to be secreted post entry of the antigen. Out of the other immunoglobulins, IgA has a monomeric structure and all others occur as dimers[18]. They are secreted after affinity maturation of the B-cells as per requirement [19].

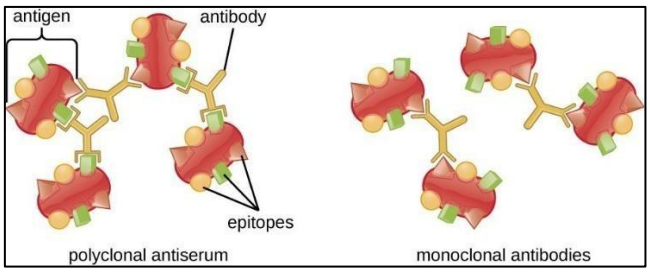


Fig 1.7: Difference between polyclonal and monoclonal antibodies (source: https://bit.ly/41OxKp4)

Further, they can be broadly classified as monoclonal and polyclonal based on the affinity towards various epitopes. Polyclonal antibodies (pAbs) target multiple epitopes of one or many antigens. Monoclonal antibodies (mAbs) on the other hand target a specific epitope for a given antigen. [20]

Antibodies serve as useful diagnostic tools for accurate detection and identification of antigens. [20]

1.6 Antibody Production:

Mammalian blood is comprised of four major components- plasma, red blood cells, white blood cells and platelets. On centrifugation of the blood, the red blood cells, white blood cells and platelets settle down, what remains in the supernatant is the plasma comprising of polyclonal antibodies. [21]

However, to produce monoclonal/ higher affinity antibodies, the B-cell clones must be screened and isolated such that every clone in that population targets the same antigen epitope[22]. For obtaining single B-cell clones, multiple methods are used – hybridoma technology, phage display and recombinant DNA technology [23]. For this project, the mouse monoclonal antibodies used are produced through hybridoma technology [24].

1.7 Monoclonal antibody production using Hybridoma Technology:

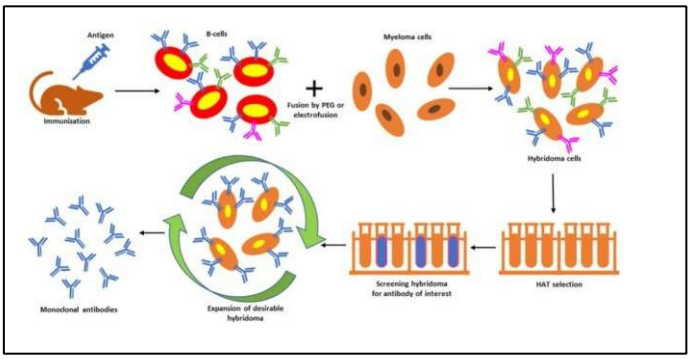


Fig 1.8: Production of hybridoma cells (source: https://bit.ly/4iQomrW)

Murine B-cells/ splenocytes are collected from the spleen of mature Balb/c mice. These B-cells are then fused with the myeloma cells using a fusating agent like Polyethylene glycol/ electrofusion, thus resulting in the formation of hybridoma cells. The selection media comprises of Hypoxanthine Aminopterin and Thymidine mixture (HAT). These cells acquire the antibody secreting property of the B-cells as well as the infinitely dividing property of the myeloma cells. Once the Hybridoma cells are ready they are expanded through 25, 75, 175 and 300 cm^2 tissue culture flasks. Once the 300 cm^2 flask is about 80-90% confluent, the polyclones are then subjected to limiting dilution where the cells are transferred into a 96-well plate such that there exists one cell/ well. Wells containing stable single cell clones are selected and later the cells from such wells are further expanded into a 24 well plate, followed by 6-well. At every

stage of transfer, the clones are screened for their reactivity. Finally, the stable single cell clones that have reached the 6-well stage, are expanded through 25, 75, 175 and 300 cm^2 tissue culture flasks. After this, the master cell bank is prepared and the cells are cryopreserved. Of the various clones of the master cell bank, the highest antibody secreting clones are selected and labelled as the Working cell bank. Now, from the Working cell bank, the clone is revived and passage through multiple tissue culture flasks and finally transferred into the roller bottle [24]. From the roller bottle, the supernatant is collected and filtered to remove any cell debris. This filtered supernatant is then purified using Affinity and Cation exchange Chromatography [25].

1.8 Antibody Purification Methods- Affinity and Cation Exchange chromatography:

1.8.1 Affinity Chromatography [25]:

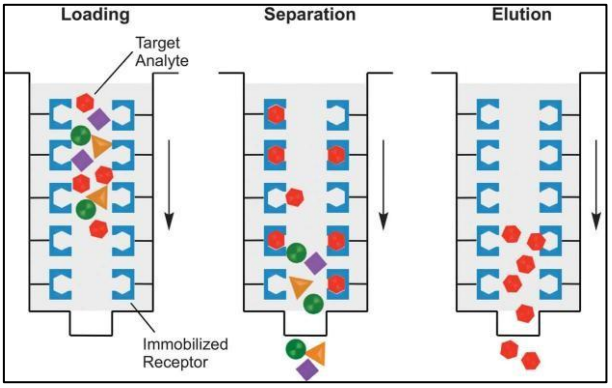


Fig 1.9: Schematic for affinity chromatography (source: https://bit.ly/4j7V3RV)

Affinity chromatography, as the name suggests, is a method that relies on the binding of a target molecule to an immobilized ligand on the stationary phase. This molecule could be a protein, nucleic acid or a hormone. Based on its affinity towards the ligand the molecule shall bind to it. The column designed for affinity chromatography consists of the following steps:

1) Stationary phase: consisting of Resin, agarose or sepharose beads that are covalently attached to a ligand.
2) Mobile Phase: Various buffers used for the purpose of sanitation and equilibration are passed through the column. The buffers at different pH ensure that the impurities are cleared out and pH is the same as the loaded sample, before sample is loaded onto the column.
3) Sample addition: While the sample is being loaded onto the column, the desired component in the sample binds to the Immobilized ligand on the stationary phase and the rest just moves out as a flow-through.
4) Washing: The various buffers are used to get rid of the loosely bound material on the ligand while ensuring that the desired component doesn't leave the ligand during impurity clearance.

5) Elution: This is the final stage of affinity-based purification where the desired component (antibody) is detached from the ligand and eluted out of the column at a lower pH.
6) Storage: The column is then stored in ethanol

1.8.2 Ion Exchange Chromatography [26]:

This chromatography is based on the principle of electrostatic interactions between charged particles. The stationary phase consists of Fractogel that contains negatively charged functional groups (like sulfonic acid groups for example) which attract and bind to positively charged molecules (cations). The various steps involved include the following:

1) Stationary phase: Consists of Fractogel containing negatively charged groups like sulfonate ($-SO_3^-$) or carboxyl groups ($-COO^-$)
2) Mobile Phase: Various buffers used for the purpose of sanitation and equilibration are passed through the column. The buffers at different pH ensure that the impurities are cleared out and pH is the same as the loaded sample, before sample is loaded onto the column.
3) Sample addition: While the sample is being loaded onto the column, the desired component in the sample binds to the negatively charged functional groups via electrostatic attraction on the stationary phase and the rest just moves out as a flow-through.
4) Washing: The various buffers are used to get rid of the loosely bound material on the ligand while ensuring that the desired component doesn't leave the ligand during impurity clearance.
5) Elution: The purified component (antibody) is finally eluted from the column using increasing amount of salt concentration which competes with the antibody for binding to the negatively charged functional groups.

1.9 Characterisation of the Antibody:

To check the integrity of the Immunoglobulin post purification, characterisation needs to be performed. The action of an antibody is at its best when it is in its monomeric form. The various methods of characterisation include performing High Performance Size Exclusion Chromatography (HPSeC) [27], Protein estimation[28] and Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)[29].

1.9.1 High Performance Size Exclusion Chromatography (HPSeC):

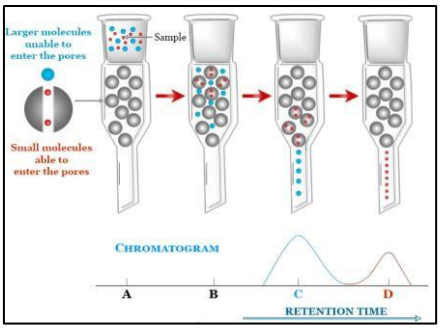


*Fig 1.10: Schematic for HPSeC (source: **bit.ly/4bRUYPG**)*

This technique separates molecules based on their sizes. Antibodies separated via Affinity and Cation exchange need to be ensured that they are monomeric. Therefore, this technique helps in estimating the same. In a chromatographic column, the smaller molecules enter the pores of the stationary phase whereas the larger molecules elute out without entering the pores Therefore, elution order starts with larger molecules and then proceeding towards the smaller ones. Graphical analysis confirms the presence of multiple sized molecules within system. [27]

1.9.2 Protein estimation using Detergent compatible (DC) Protein Assay:

The concentration of the total purified protein is determined using the DC protein Assay kt. This kit contains two Reagents- Reagent A and Reagent B. Reagent A is the Copper tartrate solution which formed a complex with the desired protein. This complex further reacts with Folin Reagent (Reagent B) to reduce it, thus resulting in a blue colour solution. The absorbance of this solution is measured at 450nm and the concentration is determined from the standard curve. [28]

1.9.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE):

To check for integrity and intactness of the antibody molecule, SDS PAGE is performed. There are two types of SDS PAGE performed- reducing and non-reducing. While the non-reducing PAGE confirms the intactness of the protein at 150kDa, the reducing PAGE confirms the presence of heavy and light chains of the antibody molecule obtained at 50kDa and 25kDa. [29]

1.10 Multiplex Bead based Assay System (Protein Suspension Array System):

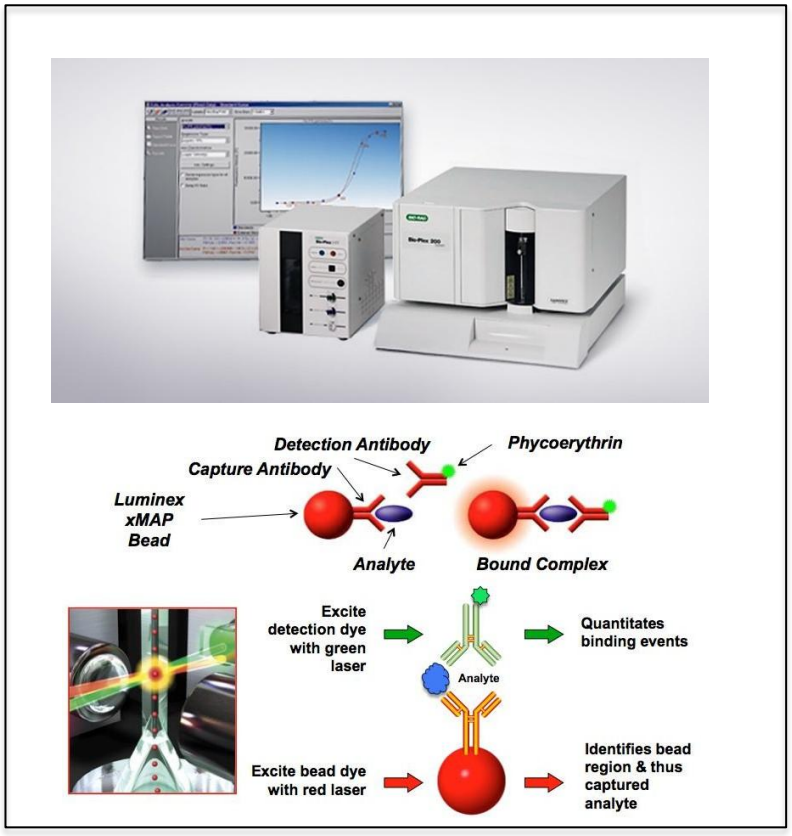


Fig 1.11: Schematic representation of Protein Suspension Array System (source: http://bit.ly/4bSVo8u)

This system is immunoassay based and makes use of antigen/ antibody coated Polystyrene beads to simultaneously detect and quantify multiple analytes in a sample (like polysaccharides, cytokines, or proteins). The Polystyrene beads are fluorescently colour coded, with each bead assigned a unique signature. Once these beads are incubated with the sample, they are conjugated using a biotinylated detection antibody like Phycoerythrin, which gives the readings proportional to the given analyte. Further these bead - analyte complexes are detected and quantitated using the Luminex Based detection system. The different types of assays that can be performed using this system include – Bead based assay (BBA), Sandwich Bead Based Assay (SBBA) and Bead Based Competitive Inhibition Assay (BBCIA). Bead Based assay is used for antibody quantitation, whereas SBBA and BBCIA are used to quantitate the antigen. In my project, I will be using BBA and SBBA. [30]

1.10.1 Indirect Bead Based Assay:

This method involves indirect way of determining antigen-antibody interaction. Here, the antigen coated beads are incubated with the desired purified antibody, then then detected using a secondary antibody against the primary antibody tagged with a fluorophore. The readings are then obtained using a Luminex based detection system [30]

1.10.2 Sandwich Bead Based Assay:

This method involves quantitating the presence of antigen. The antigen is sandwiched between two antibodies, and detected with a secondary antibody tagged with a fluorophore.[30]

Aim of the project: *To develop, standardize and optimize a high throughput method for the analysis of free polysaccharide content in the final multivalent pneumococcal vaccine*

Objectives of this project:

1) *To develop a model/ method for efficiently detecting/quantitating the pneumococcal polysaccharide content*
    a) *Generation of Rabbit polyclonal antibodies*
    b) *Production of mouse monoclonal antibodies*
2) *To isolate free polysaccharides from the given multivalent vaccine formulation*
3) *To quantitate free polysaccharides using Sandwich Bead Based Assay*
4) *To confirm the presence of polysaccharide-conjugates using Enzyme Linked Immunosorbent Assay System (ELISA)*

Chapter 2
Materials and Methods

2. Materials and Methods

2.1 List of materials:

2.1.1 List of Chemicals

Sr. No	Name of Chemical	Manufacturer
1	Dulbecco's modified eagles medium: Nutrient mixture f-12 (HAM) 1:1	gibco
2	HEPES (N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)) (C8H18N2O4S)	Sigma
3	Sodium Bicarbonate ($NaHCO_3$)	Sigma
4	Fetal Bovine Serum (FBS), sterile, filtered	Morgate
5	Dimethyl sulfoxide for molecular Biology (C_2H_6OS)	Sigma
6	Trypan blue ($C_{34}H_{28}N_6O_{14}S_4$) solution	Sigma
7	Antibiotic, antimycotic (anti-anti)	gibco
8	Sodium di-hydrogen Phosphate dihydrate (NaH_2PO_4)	Sigma
9	Di-Sodium Hydrogen phosphate (anhydrous) (Na_2HPO_4)	Sigma
10	Sodium Chloride (granular) (NaCl)	Sigma
11	Sodium Citrate (tribasic) dihydrate ($Na_3C_6H_5O_7$)	Sigma
12	Citric acid (anhydrous) ($C_6H_8O_7$)	Sigma
13	Ethanol Absolute (C_2H_5OH)	Spectrochem
14	DC Protein Assay- Reagent A and Reagent B	BioRad
15	Acrylamide (C_3H_5NO)	Sigma
16	Glycerol ($C_3H_8O_3$)	Sigma
17	Sodium Dodecyl Sulphate (SDS) ($NaC_{12}H_{25}SO_4$)	Sigma
18	Bromophenol blue ($C_{19}H_{10}Br_4O_5S$)	Sigma
19	B- Mercaptoethanol (C_2H_6OS)	Sigma

20	Trizma Base ($C_4H_{11}NO_3$)	Sigma
21	Potassium chloride (KCl)	Sigma
22	Sodium Hydroxide Pellets (NaOH)	Sigma
23	Glycine ($C_2H_5NO_2$)	Sigma
24	Methanol (CH_3OH)	Sigma
25	Acetic acid (CH_3COOH)	Sigma
26	Coomassie Brilliant Blue G ($C_{47}H_{48}N_3NaO_7S_2$)	Sigma
27	Ammonium Persulphate (APS) ($(NH_4)_2S_2O_8$)	Sigma
28	N, N, N, N -Tetramethyl ethylenediamine (TEMED) ($C_6H_{16}N_2$)	Sigma
29	D-Glucose ($C_6H_{12}O_6$)	Sigma
30	Bovine Serum Albumin (BSA)	MyBiosource
31	Sodium Azide (NaN_3)	Sigma
32	TWEEN 20	Sigma
33	TWEEN 80	Sigma
34	Histidine Buffer	In-house
35	N-N' Methylene-Bis-Acrylamide ($C_7H_{10}N_2O_2$)	Sigma
36	Pre-stained Protein Ladder	BioRad
37	Potassium di-hydrogen Phosphate (KH_2PO_4)	Sigma
38	3, 3' 5, 5' Tetramethylbenzidine (TMB) Peroxidase Substrate for ELISA	Sigma
39	Anti-mouse IgG R-Phycoerythrin	Jackson Immunoresearch

2.1.2List of Equipments

Sr. No	Instrument	Manufacturer
1	Analytical balance	Sartorius
2	pH meter	Thermo Fisher
3	Centrifuge 5804 R	Eppendorf
4	Water Bath	Thermo Fisher
5	Inverted microscope	Olympus
6	Biosafety cabinet (Class II)	ESCO
7	CO_2 incubator	Thermo Scientific
8	Liquid Nitrogen (LN_2) System	Azenta Life Science
9	Vacuum manifold assembly	Pall
10	Microplate Spectrophotometer	BioTek
11	Roll-in incubator	Bellco
12	Syringe driven filter unit (0.22 micron)	Pall
13	Shaker/ Incubator	New Brunswick Innova
14	Refrigerator (4℃)	Cryo Scientific
15	Deep Freezer (-20℃)	Angelantoni
16	Deep Freezer (-70℃)	Thermo Fisher Scientific
17	Protein Suspension Array System (BioPlex 200)	Bio-Rad
18	Sterile serological Pipettes (1ml, 10ml, 25ml and 50ml)	Costar
19	Sterile 96- well plate, 24, 6 well plate	Tarson/ Cyto One
20	Glassware Bottles (50ml, 100ml, 1L, 2L and 5L)	Scott/ Borosil
21	Water Purification System	Thermo Scientific
22	Affinity and Cation Exchange Chromatography System	Cytiva-AKTA PURE
23	$25cm^2$, $75cm^2$, $175cm^2$, $300cm^2$ tissue culture flasks	Corning, TPR
24	Cell scraper	Corning

2.2 Methodology:

2.2.1 Production of the Rabbit polyclonal sera (This was performed by the scientists at Serum Institute):

- Rabbits were immunized with the respective pneumococcal capsular polysaccharides-conjugates.
- After 4-8 weeks, blood was drawn out and centrifuged to separate the serum from the blood.
- The reactivity and titre of the antibodies in the serum was then checked using Bead based assay.

2.2.1.1 Bead Based assay for assessing the specificity and reactivity of the rabbit polyclonal sera:

- 1:1000. 1:2000, 1:4000 and 1:8000 dilutions of the rabbit polyclonal sera were prepared in LAB.
- A mixture of serotype specific antigen coated beads was prepared in LAB as 5µl/ml. The details were as follows:

Polyclonal sera to be tested	Antigen coated on to the beads	Regions
Anti-Serotype A polyclonal antibodies	Serotype A	12
Anti-Serotype B polyclonal antibodies	Serotype B	23
Anti-Serotype C polyclonal antibodies	Serotype C	8
Anti-Serotype D polyclonal antibodies	Serotype D	28
Anti-Serotype E polyclonal antibodies	Serotype E	52

Table 2.2.1: Region details *(PS: Bead regions are unique identification numbers – indicative of the ratio of fluorescent dye coated on to them)* of the antigen coated polystyrene beads for checking reactivity of polyclonal sera

- A 96- well filter plate was pre-wet with 100 µl/ well LAB, and excess LAB was aspirated using vacuum manifold assembly
- Antigen coated beads were added to the pre-wet filter plate as 50 µl/ well. Excess diluent was aspirated using vacuum manifold assembly
- Diluted polyclonal sera was then added to filter plate as 50 µl/ well
- The plate was then incubated at 37 ^{o}C, 150 rpm in the dark for 1 hour.
- After 1 hour, the plate was washed three times with 100µl/ well LAB
- 1:250 rabbit IgG R- Phycoerythrin solution was prepared in LAB and added to the filter plate as 50 µl/ well.
- This plate was incubated at 37 ^{o}C, 150 rpm in the dark for next 30 minutes.

- After 30 minutes, the plate was washed three times with 100µl/ well LAB
- The plate was read using the protein suspension array system (BioPlex 200)
- The readings for sera reactivity were quantified using Bio-Plex manager software
- The following parameters were considered – Mean fluorescent intensity, %CV, Observed/ theoretical values (back fit) and cross reactivity of the Polyclonal sera. The calculations for the same are as follows:

  i) Mean Fluorescent Intensity (MFI)

$$MFI = \frac{\sum(I_i \times N_i)}{\sum N_i}$$

- I_i = Fluorescence intensity of individual events (e.g., cells, beads)
- N_i = Number of events with intensity I_i
- $\sum N_i$ = Total number of events

  ii) Percent coefficient of variance:

$$CV\% = \left(\frac{\sigma}{\mu}\right) \times 100$$

- σ = **Standard deviation** of the dataset
- μ = **Mean** of the dataset

  iii) Cross reactivity calculation: $\frac{MFI\ value\ of\ heterogenous\ reactivity\ of\ Polyclonal\ sera}{MFI\ value\ of\ homogenous\ reactivity\ of\ Polyclonal\ sera} * 100$

2.2.2 Production of murine monoclonal antibodies:

2.2.2.1 Revival and expansion of cells from the Working Cell Bank (WCB):

- Hybridoma clones secreting specific antibodies (A, B, C, D, E) were removed from the LN_2 system. The vial was 70% thawed at 37°C in the water bath.
- Vial was then opened into the Biosafety Cabinet (Class II) and the cells from the cryo-vial were resuspended in a centrifuge tube containing 10ml of complete DMEM media (composed of 20% FBS and 1X antibiotic-antimycotic).
- The cells were then gently mixed using a 10ml serological pipette by aspirating and dispensing. The tube was then centrifuged at 1600 rpm for 5 mins. The cell pellet was retained at the bottom of the tube. Supernatant was discarded completely.
- The bottom of tube was then gently tapped to loosen the pellet (cells) followed by resuspension of the pellet in 6ml fresh DMEM complete media.
- A sterile 25cm^2 tissue culture flask was taken and labelled with name of hybridoma clone, date of cryopreservation and passage number. Resuspended cells were transferred into 25cm^2 tissue culture flask. The flask was observed daily under the inverted microscope to check the confluency and cell viability and mortality.

- Flask was incubated in humidified incubator at 37°C and supplied with 5% CO_2. On successive days, the cells were passaged to 75, 175 and 300cm^2 tissue culture flasks. Finally, when cell confluency reached 80 to 90%, cells were transferred into roller bottles, allowed to grow and secrete abundant number of monoclonal antibodies. The harvest collection was begun when cell viability started dropping below 80%. Collected harvest was stored in a 5L glass bottle overnight at 2-8 ^oC to allow cells to settle down.
- The harvest was filtered using a 0.22-micron filter to remove cell debris. The filtered harvest was sent for purification using Affinity and Ion-Exchange Chromatography.

2.2.2.2 Harvest Purification – Affinity and Ion-exchange Chromatography:

Purification was carried out using Cytiva Akta Pure system. Unicorn software was used to monitor different steps occurring during purification. The purification was performed in two steps – i) Affinity Chromatography followed by ii) Ion-exchange chromatography. Beginning with Affinity based purification, the column designated for the same was loaded with Protein A Resin beads as the stationary phase.

(*Note: Protein A was isolated from a bacteria-streptococcus aureus. This protein A has a strong affinity to the Fc region of the antibody. Hence, this protein has been immobilized on the Resin beads via covalent interaction and will serve as a ligand for binding to the Fc region of the monoclonal antibody in the harvest*).

The following steps were followed after harvest filtration:

2.2.2.2.1 Affinity Chromatography:

a) Sanitation: Column was sanitized using 0.1N NaOH to remove impurities and traces of ethanol (*when not in use the column is stored in Phosphate buffer containing 20% ethanol*)
b) Column Equilibration: Column was then washed with Wash I buffer at a pH of 7.2 (prepared using 0.1M Sodium phosphate buffer and 1M Sodium Chloride solution)
c) Loading of the desired harvest: Filtered harvest containing antibodies against the desired pneumococcal serotypes (A, B, C, D, E) was then loaded onto the column. The monoclonal antibodies were reversibly bound to the Protein A ligand in the column and the remaining components came out as a flow-through. The rate of solvent flow was maintained at 15ml/minute. Graph for UV absorbance was monitored during this time.
d) After loading completion, column was washed using wash buffer II (containing 0.1M Sodium Phosphate buffer & 1M Sodium Chloride solution at pH 7.2) and wash buffer III (containing 0.2M Sodium Citrate & 0.2M Citric acid solution at pH 6) to remove loosely bound material.
e) Elution of the purified monoclonal antibodies: The antibodies bound to protein A were eluted in a buffer containing 0.2M Sodium Citrate and 0.2M Citric acid solution at pH of 3.5 and immediately diluted to pH of 6 to avoid aggregation. The elution was monitored using a UV absorbance curve.

f) Sanitation, followed by column storage: Post elution of the monoclonal antibodies, the column was sanitized using 0.1N NaOH. Finally, the column was stored in Phosphate Buffer containing 20% ethanol

2.2.2.2.2 Ion-exchange chromatography

Antibodies eluted from affinity chromatography column were loaded on to column packed with Fractogel resin. The following steps were followed:

a) Sanitation: The column was sanitized using 0.1N NaOH to remove any impurities and traces of ethanol (*when not in use the column is stored in Phosphate buffer containing 20% ethanol*)
b) Column equilibration: Colum was washed with Wash I buffer (containing 0.1M Sodium phosphate buffer at pH of 7.2) to remove traces of NaOH
c) Loading elutes collected post affinity chromatography: Antibodies (against serotypes A, B, C, D and E) eluted from affinity chromatography column were loaded in Fractogel-based column.
d) Post loading completion, column was washed using Wash I buffer
e) An increasing gradient of buffer 'B' containing 0.1M Sodium Phosphate solution at pH 6 & 1M NaCl was passed through the column to elute the bound monoclonal antibodies.
f) Sanitation, followed by column storage: Post elution of the monoclonal antibodies, the column was sanitized using 0.1N NaOH and stored in Phosphate Buffer containing 20% ethanol

2.2.2.3 Assessment of specificity of purified monoclonal antibodies using bead-based assay:

- 1:1000, 1:2000, 1:4000 and 1:8000 dilutions of purified mouse monoclonal antibodies were prepared in LAB.
- A mixture of serotype specific antigen coated beads was prepared in LAB by adding 5µl/ml of serotype specific coupled beads.

  The details were as follows:

Monoclonal Antibodies to be tested	Antigen coated on to the beads	Regions
Anti-Serotype A monoclonal antibodies	Serotype A	12
Anti-Serotype B monoclonal antibodies	Serotype B	23
Anti-Serotype C monoclonal antibodies	Serotype C	8
Anti-Serotype D monoclonal antibodies	Serotype D	28
Anti-Serotype E monoclonal antibodies	Serotype E	52

Table 2.2.2: mAb and Region details for antigen coated polystyrene beads for checking specificity of monoclonal antibodies

- A 96- well filter plate was pre-wet with 100 µl/ well LAB. Excess LAB was aspirated using vacuum manifold assembly
- Polyclonal sera coated beads were added to the pre-wet filter plate as 50 µl/ well. Excess diluent was aspirated using vacuum manifold assembly
- Diluted monoclonal antibodies were then added to filter plate as 50 µl/ well.
- The plate was then incubated at 37 ^{o}C, 150 rpm in the dark for 1 hour.
- After 1 hour, the plate was washed three times with 100µl/ well LAB
- 1:250 mouse IgG R- Phycoerythrin solution was prepared in LAB and added to the filter plate as 50 µl/ well.
- This plate was incubated at 37 ^{o}C, 150 rpm in the dark for next 30 minutes.
- After 30 minutes, the plate was washed three times with 100µl/ well LAB
- The plate was read using the protein suspension array system (BioPlex 200)
- MFI values were generated against each serotype specific antibodies using Bio-Plex manager software and were used for calculating % homologous (Specificity) and heterologous reactivity (Cross reactivity).

2.2.2.4 Characterisation of purified antibodies:

2.2.2.4.1 Estimation of Protein concentration:

- Concentration of purified monoclonal antibodies (against serotype A, B, C, D & E) was estimated using BioRad Detergent Compatible (DC) protein assay kit.
- Standard and samples weres diluted in 96-well flat bottom plate as mentioned below.
- Protein assay standard was diluted two-fold five times from original concentration of 1.29mg/ml (Neat) in DM water.
- Given monoclonal antibody samples (against Serotype A, B, C, D and E) were diluted two-fold six times in DM water as Neat, 1:2, 1:4, 1:8, 1:16, 1:32.
- 5µl of standard and samples were transferred from the dilution plate to another 96-well plate (labelled as assay plate) in duplicates.
- 5µl of DM water was added in duplicates in wells designed as blank.
- 25 µl of Reagent A followed by 200 µl of Reagent B was added to the wells containing standard and samples in the assay plate.
- Assay plate was then kept in the dark at 150 rpm, 37 ^{o}C for 15 minutes.
- Meanwhile, the protocol for plate reading was prepared on Biotek Gen5 software. Post 15 minutes, the plate was read using the microplate spectrophotometer system and optical density values were obtained at 750 nm.
- Calculations for OD values (system generated) are given below:

$$A = \text{OD} = -\log_{10}\left(\frac{I}{I_0}\right)$$

- A or **OD** = Absorbance/Optical Density
- I_0 = Incident light intensity (before passing through the sample)
- I = Transmitted light intensity (after passing through the sample)

According to Beer-Lambert Law, absorbance of a given sample is directly proportional to its concentration.

$$A = \epsilon c l$$

- ϵ = Molar absorptivity (L/mol·cm)
- c = Concentration (mol/L)
- l = Path length of the cuvette (cm)

2.2.2.4.2 SDS PAGE:

- SDS-PAGE was performed to verify the molecular sizes of purified monoclonal antibodies by comparing their migration to a molecular weight marker.
- Two types of electrophoresis were performed – non-reducing SDS PAGE (10% resolving gel) and reducing SDS PAGE (12% resolving gel). 5% stacking gel was prepared for both.
- For non-reducing PAGE, the resolving gel (10%) was prepared using 4.07ml of DM water, 3.33ml of 30% acrylamide/ bis-acrylamide solution, 2.5ml of 1x Tris-HCl (pH 8.8), 100 µl of 10% SDS, 50 µl of 10% APS and 10 µl of TEMED
- For reducing PAGE, the resolving gel (12%) was prepared using 3.4ml of DM water, 4ml of 30% acrylamide/ bis-acrylamide solution, 2.5ml of 1x Tris-HCl (pH 8.8), 100 µl of 10% SDS, 50 µl of 10% APS and 10 µl of TEMED
- The two resolving gels were then poured in two separate gel cast containing glass slides clamped on to a stand and overlayed with butanol to prevent bubbles during polymerization.
- Meanwhile, 5% stacking gel was prepared by mixing 5.7ml of DM water, 1.7ml of 30% acrylamide/ bis-acrylamide solution, 2.5ml of 1x Tris-HCl (pH 6.8), 100µl of 10% SDS, 50 µl of 10% APS and 5µl of TEMED
- After polymerization of the resolving gel, the layer of isopropanol was removed.
- Stacking gel was then poured on top of the resolving gel in the cast and a comb was inserted to form wells. The gel was then allowed to polymerize at room temperature for another 30-60 minutes.
- For reducing PAGE sample preparation: 15 µl of reducing buffer (containing SDS, glycerol, bromophenol blue, and β-mercaptoethanol) was mixed with 10 µl of DM water and 5 µl of monoclonal antibody samples. The samples were then heated at 95°C for 5 mins
- For reducing PAGE sample preparation: 15 µl of reducing buffer (containing SDS, glycerol and bromophenol blue) was mixed with 10 µl of DM water and 5 µl of monoclonal antibody samples.
- Once samples were ready, they were loaded onto the gel. In addition to the monoclonal antibody samples, a protein molecular weight marker (170kDa - 15kDa) was loaded into one of the wells to provide a reference for the protein sizes of sample.

- Glass plates with the casted gel was placed in the electrophoresis chamber, and the chamber was filled with 1x electrode buffer (containing Trizma base, Glycine, SDS and DM water at a pH of 8.3 ± 0.2)
- The gel was run at a constant current of 25mA until the dye front reached the bottom of the gel. Smaller proteins migrated towards the positive end faster than the larger ones.
- After electrophoresis, the gel was stained for ~2 hours with Coomassie Brilliant Blue G to visualise the bands.
- Gel was de-stained using an aqueous solution (containing 400ml Methanol, 100ml of Acetic acid and 500ml of DM water) until background was cleared and protein bands were clearly visible.
- Finally, the monoclonal antibody protein bands were imaged using Gel Doc system.

2.2.3 Sandwich Bead Based Assay:

- *Experimental design: The method was designed such that the purified pneumococcal polysaccharide (PnPs)/ polysaccharide-conjugate as antigen was sandwiched between the rabbit polyclonal sera coupled onto the polystyrene beads and mouse monoclonal antibody against the respective serotype. Antigen detection was performed using anti-mouse IgG R-Phycoerythrin. As standard, for estimating the total Ps content, polysaccharide-conjugate mix can be used and for free Ps content, purified polysaccharides.*

2.2.3.1 Total Polysaccharide estimation from given vaccine formulation:

Vaccine formulation include the antigens (polysaccharide-conjugates) and the adjuvant i.e., alum onto which the polysaccharide-conjugates are adsorbed. For estimating the total polysaccharide content, the alum in the formulation needs to be dissolved and then the detection and quantification of the total polysaccharides is performed using sandwich bead-based assay.

- 1ml of vaccine formulation was taken in a 5ml tube. 10% of Sodium Citrate (tribasic) dihydrate crystals were added to the formulation. Then the tube was vortexed thoroughly until no crystals were visible. The pH of this formulation was then adjusted to 9.5 using 3M NaOH. The tube was kept on plate shaker for dissolution for ~2 hours
- Meanwhile, 1ml of polysaccharide-conjugate standard was prepared in histidine buffer.

Serotype	Stock Conc (µg/ml)	Required Conc (ng/ml)	Total Volume needed (µl)	Required Volume needed (µl)	Pre-dilution (1:100) (µl)
A	1150	50	1000	0.043	4.3
B	1160	250	1000	0.2155	21.6
C	900	200	1000	0.222	22.2
D	940	1000	1000	1.06	-
E	1270	100	1000	0.078	7.8

Table 2.2.3: Details for polysaccharide conjugate standard preparation

- Standard was diluted as 1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 in LAB.
- Sample (dissolved formulation) was diluted 2-fold in LAB eight times starting from 1:150.
- In a 5ml tube, rabbit polyclonal sera coupled polystyrene beads mixture was prepared in LAB as 5µl/ml. The details for the same were as follows:

Rabbit polyclonal sera coupled polystyrene beads	Regions
Serotype A	21
Serotype B	32
Serotype C	9
Serotype D	82
Serotype E	25

Table 2.2.4: Region details for rabbit polyclonal sera coupled polystyrene beads

- A 96- well filter plate was pre-wet with 100 µl/ well LAB. Excess LAB was aspirated using vacuum manifold assembly
- Rabbit polyclonal sera coupled beads were added to the pre-wet filter plate as 50 µl/ well. Excess diluent was aspirated using vacuum manifold assembly
- Diluted standard and samples were added to the filter plate as 50 µl/ well
- Plate was then incubated at 37 ^{o}C, 150 rpm in the dark for 30 minutes.
- After 30 minutes, the plate was washed three times with 100µl/ well LAB
- Diluted monoclonal antibodies were then added to filter plate as 50 µl/ well. Dilutions for mAb were as follows:

Mab against Serotype	Dilutions (in µl)
A	1:500
B	1:2000
C	1:1000
D	1:5000
E	1:5000

Table 2.2.5: Details for monoclonal antibody dilutions

- The plate was then incubated at 37 ºC, 150 rpm in the dark for next 30 minutes.
- After 30 minutes, plate was washed three times with 100µl/ well LAB
- 1:250 rabbit IgG R- Phycoerythrin solution was prepared in LAB and added to the filter plate as 50 µl/ well.
- This plate was incubated at 37 ºC, 150 rpm in the dark for next 30 minutes.
- After 30 minutes, the plate was washed three times with 100µl/ well LAB
- Plate was read using the protein suspension array system (BioPlex 200)
- The readings were quantified using Bio-Plex manager software.

2.2.3.2 Estimation of free Polysaccharide content from the vaccine formulation:

- Vaccine formulation was centrifuged at 4000 rpm for 10 minutes.
- Supernatant was collected and divided into three parts - first part was kept aside for sandwich bead-based assay, the second part was processed using an undisclosed method and the third part was spiked using 100ng/ml of purified polysaccharides and then subjected to processing using the same method as that for second part.
- Two sets of purified polysaccharide mixture were prepared in histidine buffer for standard and spiking.

Serotype	Stock Conc (µg/ml)	Required Conc (ng/ml)	Total Volume (µl)	Required Volume needed (µl)	Pre- dilution (1:100) (µl)
A	1480	25	1000	0.02	2
B	250	50	1000	0.20	20
C	500	25	1000	0.05	5
D	250	200	1000	0.80	80
E	250	25	1000	0.10	10

Table 2.2.6: Details for standard preparation containing purified polysaccharides

Serotype	Stock Conc (µg/ml)	Required Conc (ng/ml)	Total Volume needed (µl)	Required Volume needed (µl)	Pre- dilution (1:100) (µl)
A	1480	100	1000	0.07	7
B	250	100	1000	0.40	40
C	500	100	1000	0.20	20
D	250	100	1000	0.40	40
E	250	100	1000	0.40	40

Table 2.2.7: Details for standard preparation containing purified polysaccharides to be used for spiking

- Next mixture of rabbit polyclonal sera coupled polystyrene beads were prepared in LAB:

Polyclonal sera coupled polystyrene beads	Regions
Serotype A	21
Serotype B	32
Serotype C	9
Serotype D	82
Serotype E	25

Table 2.2.8: Region details for rabbit polyclonal sera coupled polystyrene beads

- 1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 dilutions of the polysaccharide standard, processed and unprocessed formulation were prepared LAB in separate columns in a 96-well sterile plate.
- A 96- well filter plate was pre-wet with 100 µl/ well LAB. Excess LAB was aspirated using vacuum manifold assembly
- Rabbit polyclonal sera coupled beads were added to the pre-wet filter plate as 50 µl/ well. Excess diluent was aspirated using vacuum manifold assembly
- Diluted standard and samples were added to the filter plate as 50 µl/ well
- Plate was then incubated at 37 ^{o}C, 150 rpm in the dark for 30 minutes.
- After 30 minutes, the plate was washed three times with 100µl/ well LAB
- Diluted monoclonal antibodies were then added to filter plate as 50 µl/ well. Dilutions for mAb were as follows:

Mab against Serotype	Dilutions (in µl)
A	1:500
B	1:2000
C	1:1000
D	1:5000
E	1:5000

Table 2.2.9: Details for monoclonal antibody dilutions

- The plate was then incubated at 37 ^{o}C, 150 rpm in the dark for next 30 minutes.
- After 30 minutes, plate was washed three times with 100µl/ well LAB
- 1:250 rabbit IgG R- Phycoerythrin solution was prepared in LAB and added to the filter plate as 50 µl/ well.
- This plate was incubated at 37 ^{o}C, 150 rpm in the dark for next 30 minutes.
- After 30 minutes, the plate was washed three times with 100µl/ well LAB

- Plate was read using the protein suspension array system (BioPlex 200)
- The readings were quantified using Bio-Plex manager software.

2.2.4 Enzyme Linked Immunosorbent Assay (ELISA):

ELISA was performed to check the presence of conjugates in the processed vaccine formulation samples.

- Four ELISA strips per serotype were coated overnight with murine monoclonal antibodies against the respective S. pneumoniae strains.
- Next day, a blocking solution of 2% Bovine Serum Albumin was prepared in 1X Phosphate Buffer.
- The excess buffer used in coating was decanted and then the blocking buffer was added as 250 µl/ well for 1 hour at 37°C.
- After 1 hour, the ELISA strips were washed 3 times with 1X PBST using ELISA microplate washer system.
- 1, 1:2, 1:4 dilutions of the standard and sample prepared in LAB. Fourth well was kept blank. Samples were incubated on the blocked ELISA strips for 1 hour at 37°C.
- After 1 hour, the ELISA strips were washed 3 times with 1X PBST using ELISA microplate washer system.
- 1:500 dilution of anti-CRM, DT and TT HRP conjugates mixture was prepared in the in LAB and added on to the ELISA strips for 1 hour at 37°C.
- After 1 hour, the ELISA strips were washed 3 times with 1X PBST using the ELISA microplate washer system.
- Later, dissolvable TMB solution was added on to the ELISA strips for 20 minutes in the dark at room temperature.
- After 20 minutes, reaction with TMB was quenched using 1N HCl solution.
- Then the ELISA plate was read using microplate spectrophotometer system after protocol preparation and absorbance measured at 450nm.

Chapter 3
Results

3. Results

3.1 Specificity of the Rabbit Polyclonal sera

The following table highlights the reactivity and specificity of the polyclonal sera against homologous and heterologous serotypes. The accepted cross reactivity of the polyclonal sera was less than 30%.

	Type A (12)	Type B (23)	Type C (8)	Type D (23)	Type E (52)	Dilutions
	Mean Fluorescent Intensity (MFI) values					
Blank	2	1	4	3	2	
Type A sera	29632	1991	2486	1974	4468	1000
	29754	1974	2292	1750	2490	2000
	29478	1772	1920	1338	1970	4000
	27666	1558	1798	1132	1772	8000
Type B sera	1120	31330	4020	4660	3900	1000
	914	29273	3680	3920	3640	2000
	888	25435	3380	3460	3240	4000
	460	13202	2560	2680	2960	8000
Type C sera	1560	314	29116	4620	4960	1000
	1358	235	29762	3720	3980	2000
	1140	124	29123	3280	3640	4000
	914	110	26308	2700	3000	8000
Type D sera	818	422	4960	29009	4360	1000
	690	364	3900	29159	3360	2000
	478	306	3380	27716	2700	4000
	294	250	2780	21341	2280	8000
Type E sera	1960	372	3640	3900	30407	1000
	1556	326	3360	3120	28390	2000
	1360	276	2380	2520	19492	4000
	1160	202	2180	2240	12376	8000

Table: Mean Fluorescent Intensity (MFI) values for reactivity of Rabbit polyclonal sera against various antigens

The percent cross reactivity was calculated:

	Type A (12)	Type B (23)	Type C (8)	Type D (23)	Type E (52)	Dilutions
	% Cross reactivity					
Blank	2	1	4	3	2	
Type A sera	100	7	8	7	15	1000
	100	7	8	6	8	2000
	100	6	7	5	7	4000
	100	6	6	4	6	8000
Type B sera	4	100	13	15	12	1000
	3	100	13	13	12	2000
	3	100	13	14	13	4000
	3	100	19	20	22	8000
Type C sera	5	1	100	16	17	1000
	5	1	100	12	13	2000
	4	0	100	11	12	4000
	3	0	100	10	11	8000
Type D sera	3	1	17	100	15	1000
	2	1	13	100	12	2000
	2	1	12	100	10	4000
	1	1	13	100	11	8000
Type E sera	6	1	12	13	100	1000
	5	1	12	11	100	2000
	7	1	12	13	100	4000
	9	2	18	18	100	8000

Table 3.1: Percent cross reactivity of Rabbit polyclonal sera against various other antigens

Inference: Qualitatively, it is evident that the homogenuous reactivity of the rabbit polyclonal sera was higher than the heterogenuous reactivity. However, at the four dilutions considered, one can see saturation of the mean fluorescent intensity (MFI) values, thus to observe a concentration gradient in the reactivity of antibodies in the sera, more dilutions of the sera need to be taken. Additonally, the heterogenous reactivity was observed to be less than 30 percent for all serotypes considered. Thus, this sera can now be used in our sandwich bead based assay method.

3.2 Chromatograms obtained post Affinity based purification:

The following chromatograms represent the yield of antibodies obtained from the purified harvest. The x-axis represents volume (in ml) and the y-axis represents UV absorbance (in milli Absorbance Units). In the give graphs, blue shows UV absorbance, orange for conductance, green is for concentration for Buffer B (no role here)

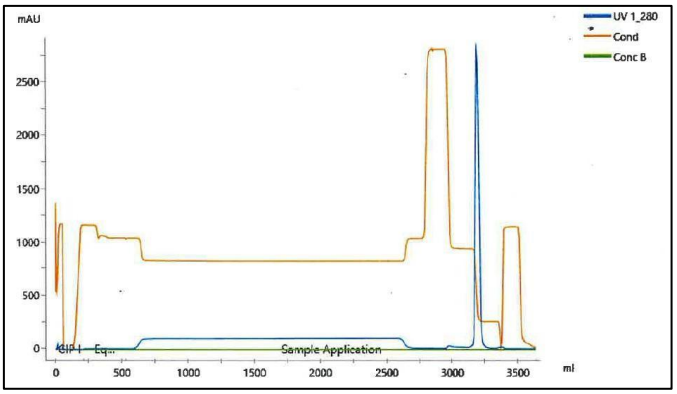


Fig 3.1: Chromatogram for monoclonal antibody against Serotype A. The UV absorbance peak was obtained at ~3000 mAU (milli Absorbance units)

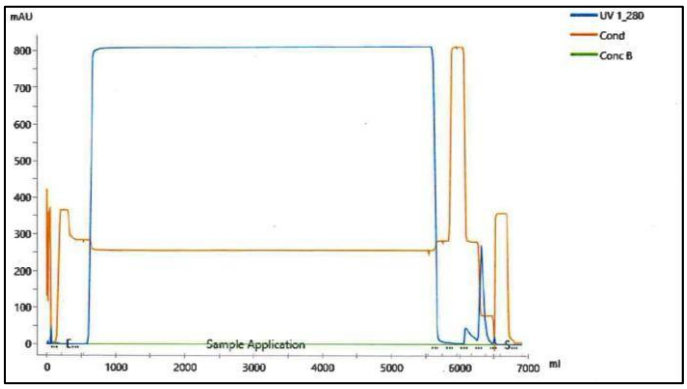


Fig 3.2: Chromatogram for monoclonal antibody against Serotype B. The UV absorbance peak was obtained at ~280 mAU (milli Absorbance units).

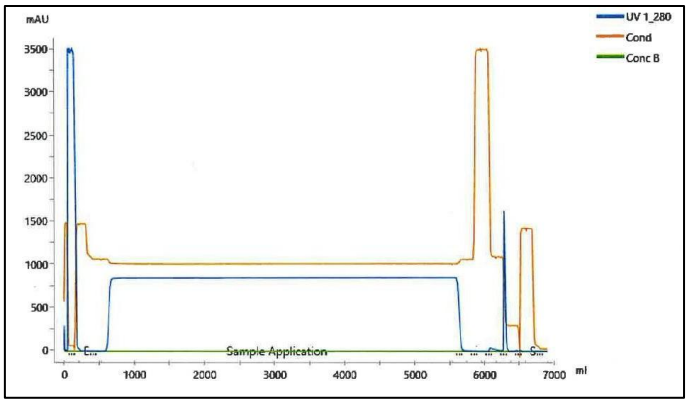


Fig 3.3: Chromatogram for monoclonal antibody against Serotype C. The UV absorbance peak was obtained at ~1600 mAU (milli Absorbance units).

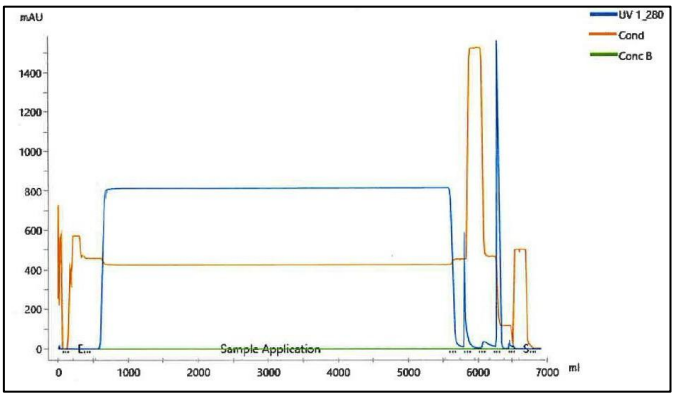


Fig 3.4: Chromatogram for monoclonal antibody against Serotype D. The UV absorbance peak was obtained at ~1500 mAU (milli Absorbance units).

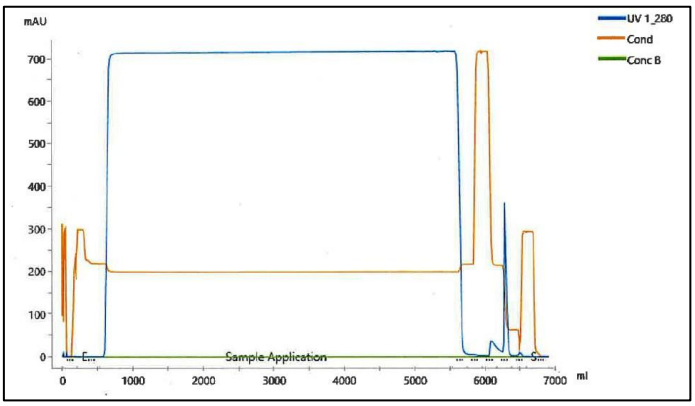


Fig 3.5: Chromatogram for monoclonal antibody against Serotype E. The UV absorbance peak was obtained at ~350 mAU (milli Absorbance units).

The yield of the monoclonal antibody per serotype can be tabulated as follows:

Serotype	UV absorbance
A	~3000 mAU
B	~280 mAU
C	~1600 mAU
D	~1500 mAU
E	~350 mAU

Table 3.2: Compiled yield for monoclonal antibodies after affinity-based purification

Inference: The height of the peaks indicates the yield of monoclonal antibodies per serotype. This peak in the chromatogram was measured in milli Absorbance units (the peaks are a result of change in the absorbance of Ultraviolet light as the antibody is eluted from the column at a lower pH). The yield was highest for monoclonal antibody against Serotype A and lowest for Serotype B.

3.3 Chromatograms obtained post Ion-exchange chromatography:

The following chromatograms were obtained after the affinity based purified antibody was loaded onto the Fractogel column for further purification.

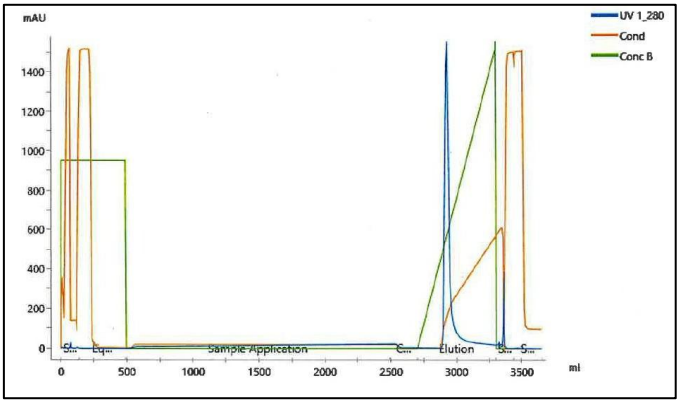


Fig 3.6: UV absorbance peak for monoclonal antibody against Serotype A was obtained at ~1500 milli Absorbance Units

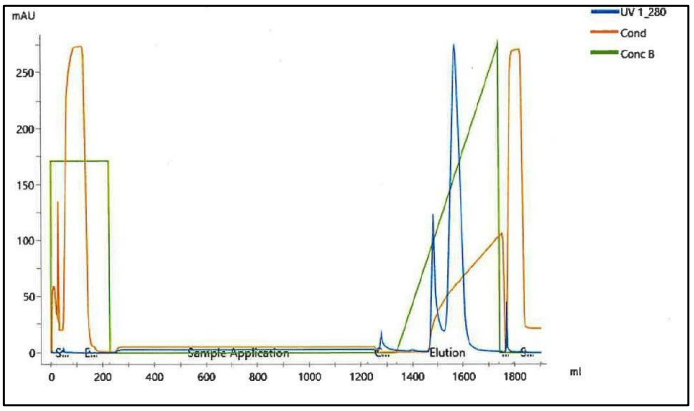


Fig 3.7: UV absorbance peak for monoclonal antibody against Serotype B was obtained at ~300 milli Absorbance Units. Two peaks were observed, the first was for non-specific bound impurities and the second one was for pure antibody

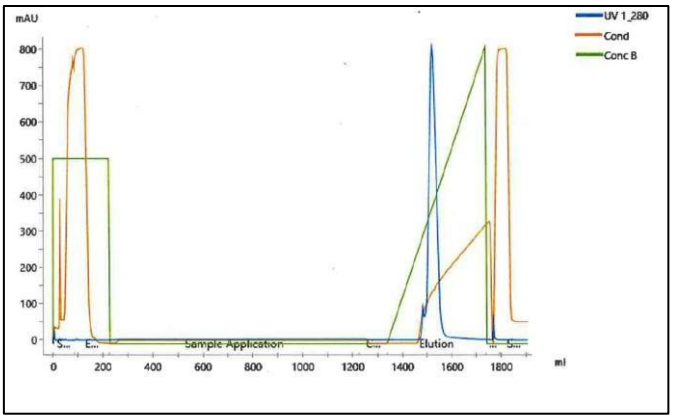


Fig 3.8: UV absorbance peak for monoclonal antibody against Serotype C was obtained at ~300 milli Absorbance Units. Single peak with a slight kink was observed.

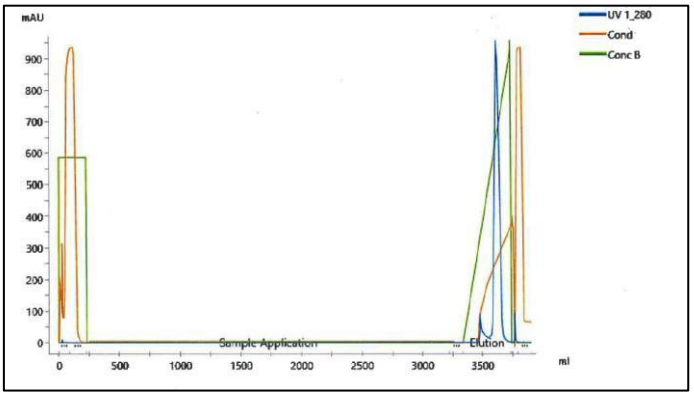


Fig 3.9: UV absorbance peak for monoclonal antibody against Serotype D was obtained at ~1000 milli Absorbance Units. Single peak with a slight kink was observed.

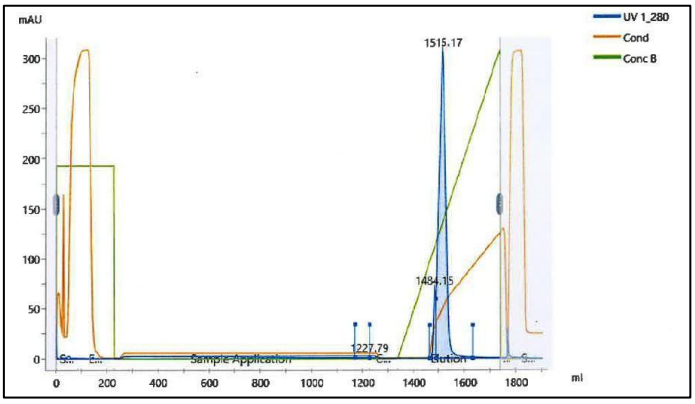


Fig 3.10: UV absorbance peak for monoclonal antibody against Serotype D was obtained at ~320 milli Absorbance Units. Single peak was observed.

Inference: The height of the UV absorbance peaks measured in milli absorbance units indicates the levels of the eluted antibodies. The antibodies passed through the Fractogel column were eluted against the increasing NaCl concentration at a lower pH. The separation was charge based. Thus, the separation between impurities and purified antibodies was clearly visible in the chromatogram.

3.4 Specificity of monoclonal antibodies:

The following table highlights the specificity of the purified monoclonal antibodies. Blank indicates the non-specific readings obtained when buffer with antigen coated beads without mAb was used. The readings obtained are the mean fluorescent intensity (MFI) values based on reactivity of the monoclonal antibodies with the antigen at dilutions 1:1000, 1:2000, 1:4000 and 1:8000.

Beads details	Type A (12)	Type B (23)	Type C (8)	Type D(28)	Type E(52)	Dilutions
Description	FI-Bkgd	FI-Bkgd	FI-Bkgd	FI-Bkgd	FI-Bkgd	
Blank	2	1	4	3	2	
Monoclonal antibody against Serotype A	24838.5	6	2	3	2	1000
	23634.5	6	2	3	2	2000
	24361	6	2	3	2	4000
	20226	5	2	3	2	8000
Monoclonal antibody against Serotype B	2	22122.5	2.5	2	2.5	1000
	2	20628	2.5	2	2.5	2000
	2	18042.5	2	2	2	4000
	2	13512	2	2	2	8000
Monoclonal antibody against Serotype C	3	2.5	24136.5	2.5	2	1000
	3	2	24677.5	2	2	2000
	3	1	24389	2	2	4000
	3	1	24098	2	2	8000
Monoclonal antibody against Serotype D	12	6	2.5	23987	2	1000
	10	4	2.5	22876	2	2000
	8.5	3	2.5	21777	2	4000
	4	3	2.5	21341	2	8000
Monoclonal antibody against Serotype E	4	2.5	2	4	29198	1000
	2	2	2	2	29100	2000
	1	1	2	1	28345	4000
	1	1	2	1	28123	8000

Table 3.3: Specificity and Cross-reactivity of the monoclonal antibody against respective strains

Beads details	Type A (12)	Type B (23)	Type C (8)	Type D(28)	Type E(52)	Dilutions
Description	FI-Bkgd	FI-Bkgd	FI-Bkgd	FI-Bkgd	FI-Bkgd	
Blank	1	1	2	2	2	
Monoclonal antibody against Serotype A	100.00	0.02	0.01	0.01	0.01	1000
	100.00	0.03	0.01	0.01	0.01	2000
	100.00	0.02	0.01	0.01	0.01	4000
	100.00	0.02	0.01	0.01	0.01	8000
Monoclonal antibody against Serotype B	0.01	100.00	0.01	0.01	0.01	1000
	0.01	100.00	0.01	0.01	0.01	2000
	0.01	100.00	0.01	0.01	0.01	4000
	0.01	100.00	0.01	0.01	0.01	8000
Monoclonal antibody against Serotype C	0.01	0.01	100.00	0.01	0.01	1000
	0.01	0.01	100.00	0.01	0.01	2000
	0.01	0.00	100.00	0.01	0.01	4000
	0.01	0.00	100.00	0.01	0.01	8000
Monoclonal antibody against Serotype D	0.05	0.03	0.01	100.00	0.01	1000
	0.04	0.02	0.01	100.00	0.01	2000
	0.04	0.01	0.01	100.00	0.01	4000
	0.02	0.01	0.01	100.00	0.01	8000
Monoclonal antibody against Serotype E	0.01	0.01	0.01	0.01	100.00	1000
	0.01	0.01	0.01	0.01	100.00	2000
	0.00	0.00	0.01	0.00	100.00	4000
	0.00	0.00	0.01	0.00	100.00	8000

Table 3.4: Percent homologous and heterologous reactivity of the monoclonal antibody against respective strains

Inference: The homologous reactivity of the monoclonal antibodies was higher than heterologous reactivity indicating the highly specific nature of the monoclonal

antibodies towards their respective serotypes. The heterologous reactivity was found to be less than 10%.

The acceptance criteria are as follows.

i) To say antibody is specific, Heterologous reactivity should be less than 20% of homologues reactivity.

Thus, given mAbs are highly specific and could be used in assay.

3.5 Characterization of the eluted murine monoclonal antibody:

3.5.1 Protein Estimation using Detergent Compatible Protein Assay:

	1	2	3	4	5	6	7	8
A	STD1 1.29 Standard	STD1 1.29 Standard	SPL1:1 1 MAb against Serotype A	SPL1:1 1 MAb against Serotype A	SPL2:1 1 MAb against Serotype B	SPL2:1 1 MAb against Serotype B	SPL3:1 1 MAb against Serotype C	SPL3:1 1 MAb against Serotype C
B	STD2 0.645 Standard	STD2 0.645 Standard	SPL1:2 2 MAb against Serotype A	SPL1:2 2 MAb against Serotype A	SPL2:2 2 MAb against Serotype B	SPL2:2 2 MAb against Serotype B	SPL3:2 2 MAb against Serotype C	SPL3:2 2 MAb against Serotype C
C	STD3 0.3225 Standard	STD3 0.3225 Standard	SPL1:3 4 MAb against Serotype A	SPL1:3 4 MAb against Serotype A	SPL2:3 4 MAb against Serotype B	SPL2:3 4 MAb against Serotype B	SPL3:3 4 MAb against Serotype C	SPL3:3 4 MAb against Serotype C
D	STD4 0.16125 Standard	STD4 0.16125 Standard	SPL1:4 8 MAb against Serotype A	SPL1:4 8 MAb against Serotype A	SPL2:4 8 MAb against Serotype B	SPL2:4 8 MAb against Serotype B	SPL3:4 8 MAb against Serotype C	SPL3:4 8 MAb against Serotype C
E	STD5 0.080625 Standard	STD5 0.080625 Standard	SPL1:5 16 MAb against Serotype A	SPL1:5 16 MAb against Serotype A	SPL2:5 16 MAb against Serotype B	SPL2:5 16 MAb against Serotype B	SPL3:5 16 MAb against Serotype C	SPL3:5 16 MAb against Serotype C
F	Blank Blank	Blank Blank	SPL1:6 32 MAb against Serotype A	SPL1:6 32 MAb against Serotype A	SPL2:6 32 MAb against Serotype B	SPL2:6 32 MAb against Serotype B	SPL3:6 32 MAb against Serotype C	SPL3:6 32 MAb against Serotype C

	1	2	3	4	5	6
A	STD1 1.29 Standard	STD1 1.29 Standard	SPL1:1 1 MAb against Serotype D	SPL1:1 1 MAb against Serotype D	SPL2:1 1 MAb against Serotype E	SPL2:1 1 MAb against Serotype E
B	STD2 0.645 Standard	STD2 0.645 Standard	SPL1:2 2 MAb against Serotype D	SPL1:2 2 MAb against Serotype D	SPL2:2 2 MAb against Serotype E	SPL2:2 2 MAb against Serotype E
C	STD3 0.3225 Standard	STD3 0.3225 Standard	SPL1:3 4 MAb against Serotype D	SPL1:3 4 MAb against Serotype D	SPL2:3 4 MAb against Serotype E	SPL2:3 4 MAb against Serotype E
D	STD4 0.16125 Standard	STD4 0.16125 Standard	SPL1:4 8 MAb against Serotype D	SPL1:4 8 MAb against Serotype D	SPL2:4 8 MAb against Serotype E	SPL2:4 8 MAb against Serotype E
E	STD5 0.080625 Standard	STD5 0.080625 Standard	SPL1:5 16 MAb against Serotype D	SPL1:5 16 MAb against Serotype D	SPL2:5 16 MAb against Serotype E	SPL2:5 16 MAb against Serotype E
F	Blank Blank	Blank Blank	SPL1:6 32 MAb against Serotype D	SPL1:6 32 MAb against Serotype D	SPL2:6 32 MAb against Serotype E	SPL2:6 32 MAb against Serotype E

Table 3.5: Plate protocol for estimating protein concentration of monoclonal antibodies generated against Serotype A, B, C D and E

Well ID	Conc/Dil	Blank 750	[Concentration] x Dil	Count	Mean	Std Dev	CV (%)	Average Conc (mg/ml)
MAb against Serotype A	1	0.561	>1.350	0	?????	?????	?????	3.74
	1	0.559	>1.350					
	2	0.344	>2.701	0	?????	?????	?????	
	2	0.333	>2.701					
	4	0.2	3.525	2	3.508	0.024	0.689	
	4	0.198	3.491					
	8	0.111	3.788	2	3.791	0.005	0.127	
	8	0.111	3.795					
	16	0.053	3.722	2	3.735	0.019	0.5	
	16	0.054	3.749					
	32	0.027	3.971	2	3.916	0.078	1.991	
	32	0.027	3.861					
MAb against Serotype B	1	0.454	>1.350	0	?????	?????	?????	2.63
	1	0.438	>1.350					
	2	0.261	2.434	2	2.398	0.052	2.156	
	2	0.255	2.361					
	4	0.151	2.599	2	2.565	0.047	1.842	
	4	0.148	2.532					
	8	0.083	2.838	2	2.807	0.045	1.587	
	8	0.081	2.775					
	16	0.039	2.745	2	2.795	0.071	2.541	
	16	0.04	2.846					
	32	0.019	2.767	2	2.608	0.224	8.588	
	32	0.017	2.45					
MAb against Serotype C	1	0.582	>1.350	0	?????	?????	?????	3.72
	1	0.578	>1.350					
	2	0.342	>2.701	0	?????	?????	?????	
	2	0.343	>2.701					
	4	0.194	3.413	2	3.473	0.085	2.446	
	4	0.2	3.534					
	8	0.109	3.703	2	3.694	0.012	0.325	
	8	0.108	3.686					
	16	0.056	3.874	2	3.95	0.107	2.717	
	16	0.058	4.026					
	32	0.025	3.584	2	3.743	0.225	6.015	
	32	0.027	3.902					

Name	Conc/Dil	Blank 750	[Concentration] x Dil	Count	Mean	Std Dev	CV (%)	Averge (mg/ml)
Mab against Serotype D	1	0.168	0.766	2	0.772	0.008	1.085	0.76
	1	0.17	0.778					
	2	0.099	0.852	2	0.859	0.009	1.055	
	2	0.1	0.865					
	4	0.051	0.859	2	0.852	0.011	1.286	
	4	0.05	0.844					
	8	0.023	0.764	2	0.743	0.03	3.991	
	8	0.022	0.722					
	16	0.009	0.509	2	0.565	0.079	14.036	
	16	0.01	0.621					
	32	0.003	<0.785	0	?????	?????	?????	
	32	0.004	<0.785					
Mab against Serotype E	1	0.187	0.873	2	0.863	0.014	1.622	1.00
	1	0.183	0.853					
	2	0.103	0.889	2	0.858	0.044	5.127	
	2	0.096	0.827					
	4	0.013	0.21	2	0.59	0.537	91.048	
	4	0.057	0.97					
	8	0.028	0.934	2	0.894	0.056	6.303	
	8	0.026	0.855					
	16	0.013	0.79	2	0.698	0.13	18.616	
	16	0.01	0.606					
	32	0.016	2.069	1	2.069	?????	?????	

Table 3.6: Protein concentration of monoclonal antibodies against Serotype A, B, C, D and E

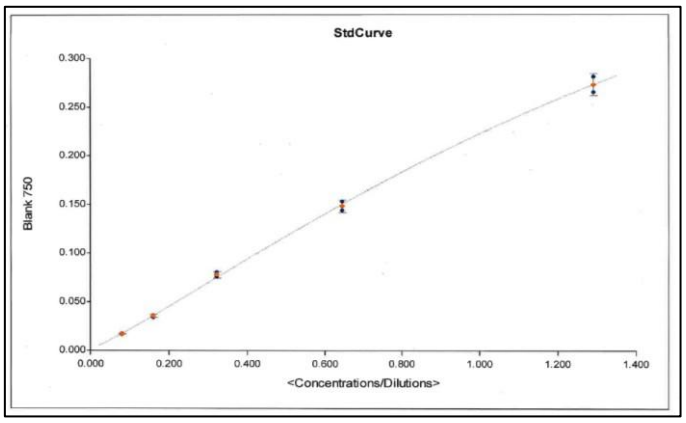


Fig 3.11: Protein standard curve

Curve Name	Curve Formula	A	B	C	D	E	R2	Fit F Prob
StdCurve	Y = (A-D)/(1+(X/C)^B)^E + D	0.00177	1.16	200	0.493	274	1	?????

Table 3.7: Values of the 5 - parameter curve obtained above

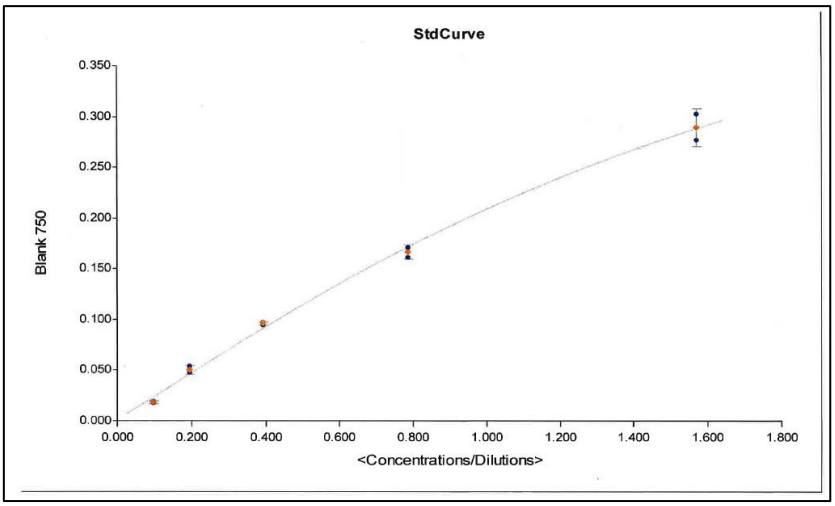


Fig 3.12: Protein standard curve

Curve Name	Curve Formula	A	B	C	D	E	R2	Fit F Prob
StdCurve	Y = (A-D)/(1+(X/C)^B)^E + D	0.00267	1.1	2.62E+03	0.449	3.55E+03	0.999	?????

Table 3.8: Values of the 5 – parameter curve obtained above

Inference: The protein concentrations of monoclonal antibodies against various serotypes were accurately estimated using the Detergent Compatible Protein Assay. Protein concentration was the highest for monoclonal antibody against Serotype A and lowest for that of against Serotype D. The following table shows the combined protein concentrations of monoclonal antibodies generated against Serotypes A, B, C, D and E.

Serotype	Protein Concentration (mg/ml)
A	3.74
B	2.63
C	3.72
D	0.76
E	1.00

Table 3.9: Compiled protein concentration values for monoclonal antibodies

3.5.2 SDS PAGE

The murine monoclonal antibodies were run on two separate gels of 10 and 12%. The protein ladder is shown on the left. The monoclonal antibodies were loaded in successive wells. The ladder and the samples have been labelled.

3.5.2.1 12% Reducing PAGE:

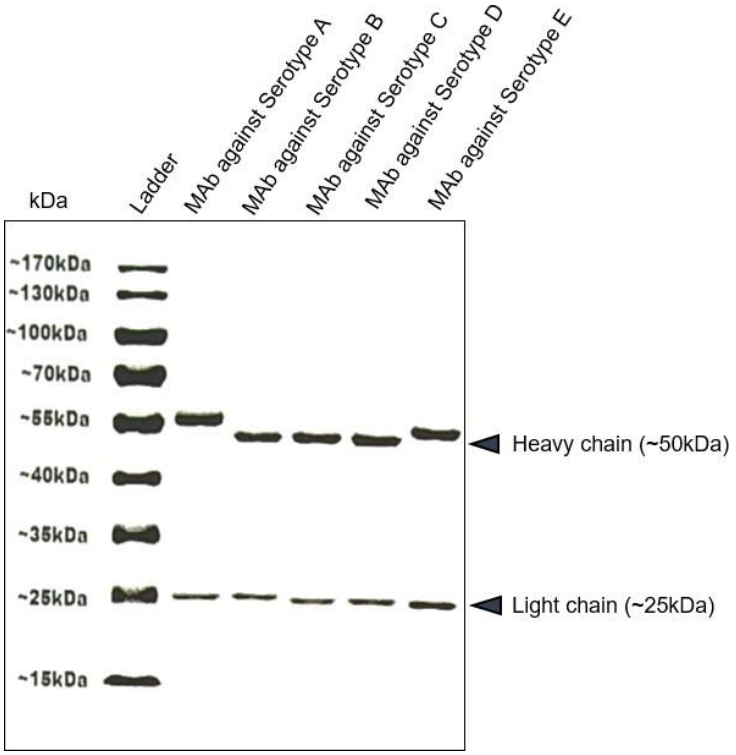


Fig 3.13: SDS PAGE reducing gel

Inference: Two bands of each monoclonal antibody were obtained indicating the presence of heavy chain (obtained ~ 50kDa) and light chain obtained ~25kDa.
Due to post translational modifications like glycosylation pattern of these wild type monoclonal antibodies, some of the bands are seen at a higher molecular weight.

3.5.2.2 10% non-reducing SDS PAGE:

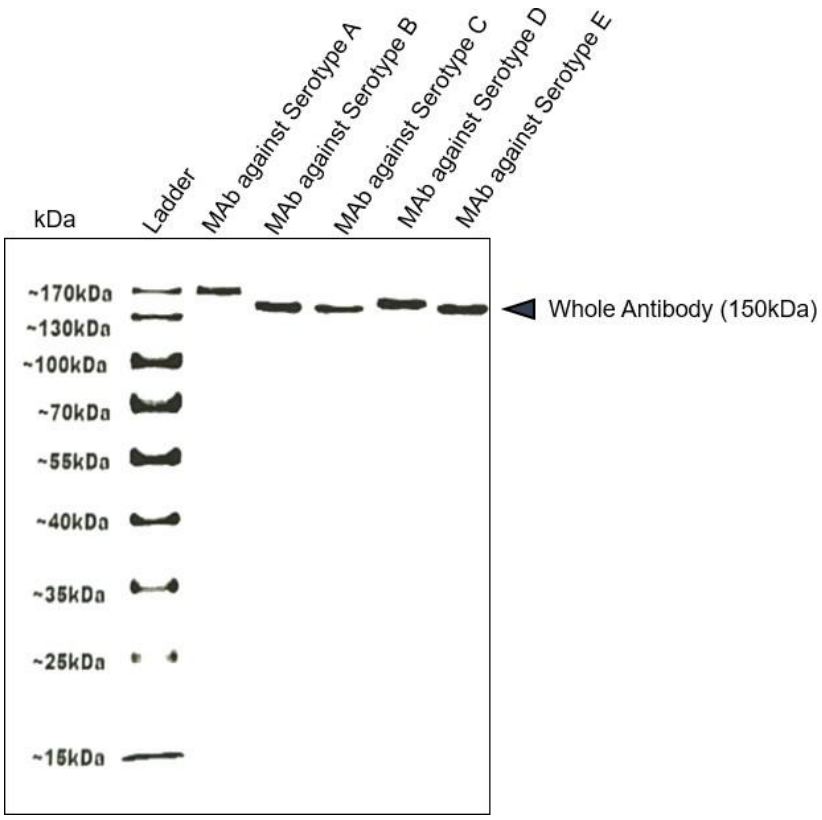


Fig 3.14: SDS PAGE non-reducing gel

Inference: Single band for each monoclonal antibody was observed at ~150kDa. Total molecular weight of the antibody molecule was obtained at ~150kDa.

3.6 Total Polysaccharide estimation from vaccine formulation using Sandwich Bead Based Assay (SBBA):

The total polysaccharide content in the given vaccine formulation was estimated using the sandwich bead- based assay model. In the graphs for the standard curves, the y-axis represents the fluorescence intensity and the x-axis represents concentration in ng/ml. The expected concentration of each polysaccharide-conjugate shall be 4400ng/ml.

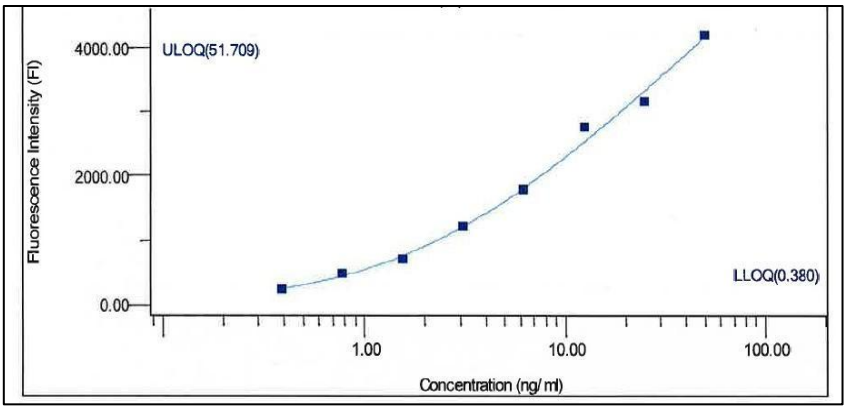


Fig 3.15: Standard curve for Polysaccharide Conjugate against Serotype A

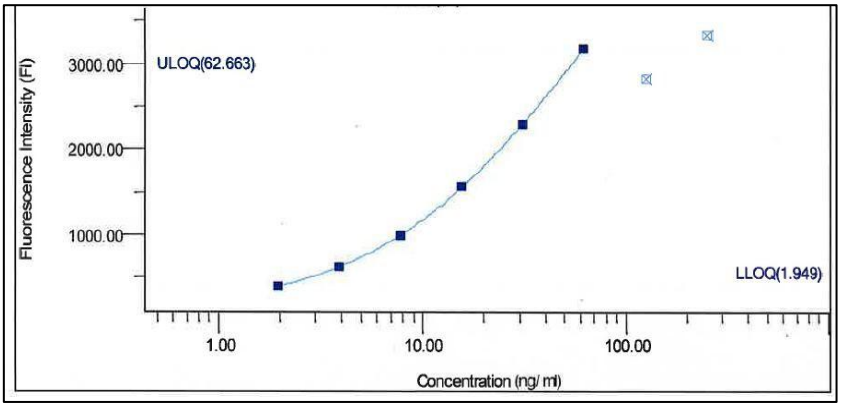


Fig 3.16: Standard curve for Polysaccharide Conjugate against Serotype B

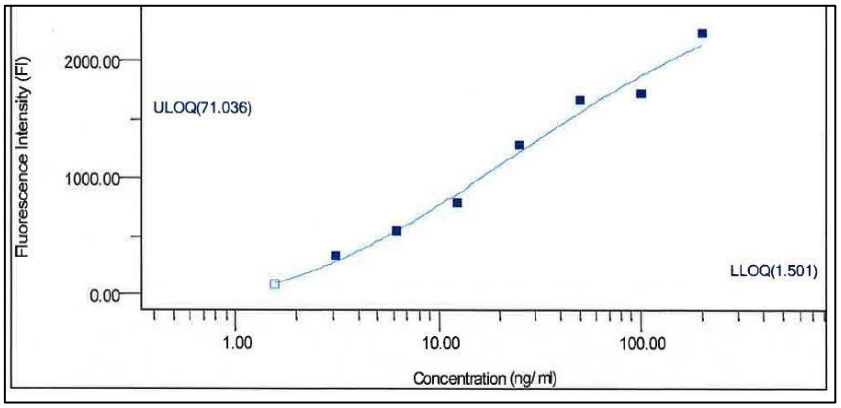


Fig 3.17: Standard curve for Polysaccharide Conjugate against Serotype C

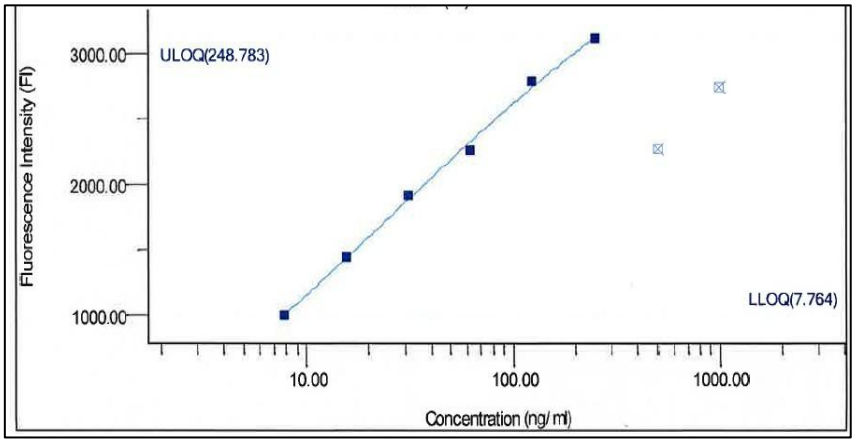


Fig 3.18: Standard curve for Polysaccharide Conjugate against Serotype D

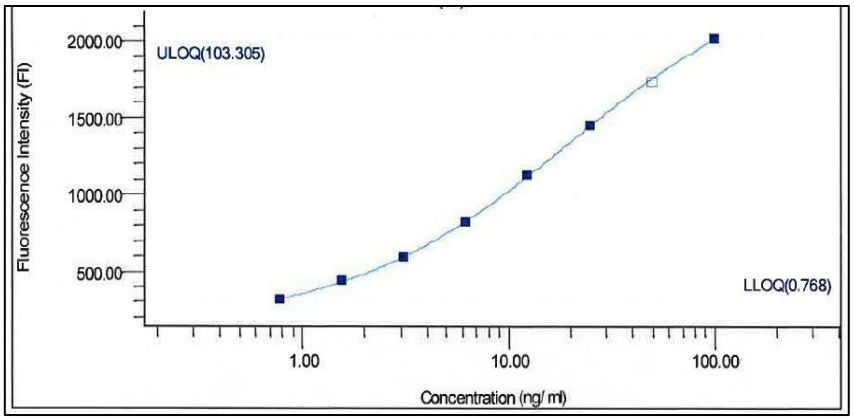


Fig 3.19: Standard curve for Polysaccharide Conjugate against Serotype E

Serotype	Average Concentration (ng/ml)	Percent Recovery
A	4582	104
B	3995	91
C	4630	105
D	4189	95
E	4730	108

Table 3.10: Compiled data for average concentration and percent recovery of total Ps

Inference: The concentration of polysaccharide-conjugates in a given vaccine formulation is 4400ng/ml. The acceptance criteria for recovery were 70-130%. Hence, in the serotypes considered for this experiment, all of them show percent recovery within range. The assay is accurately estimated content of total polysaccharide from the sample.

3.7 Free Ps estimation using sandwich bead-based assay:

Free polysaccharide content in the processed and unprocessed vaccine formulation (Sample) were estimated using the sandwich bead-based assay. In the standard curves obtained, the x-axis represents concentration in ng/ml and y-axis represents fluorescence intensity.

Accuracy of assay was checked by spiking known concentration of PnPs (100 ng/ml) in the sample.

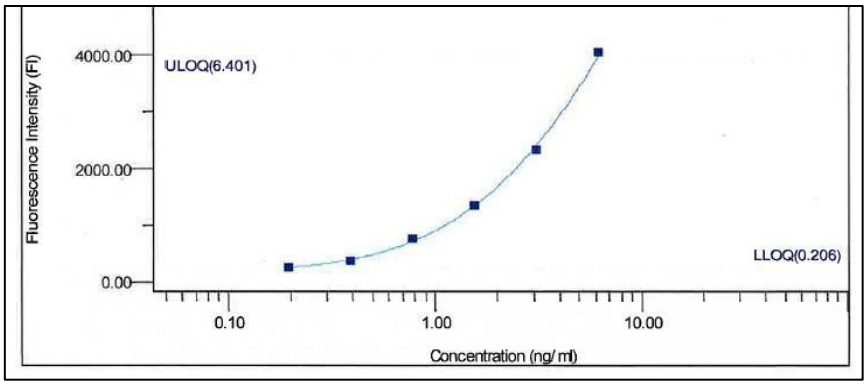


Fig 3.20: Standard curve for Purified Polysaccharide against Serotype A

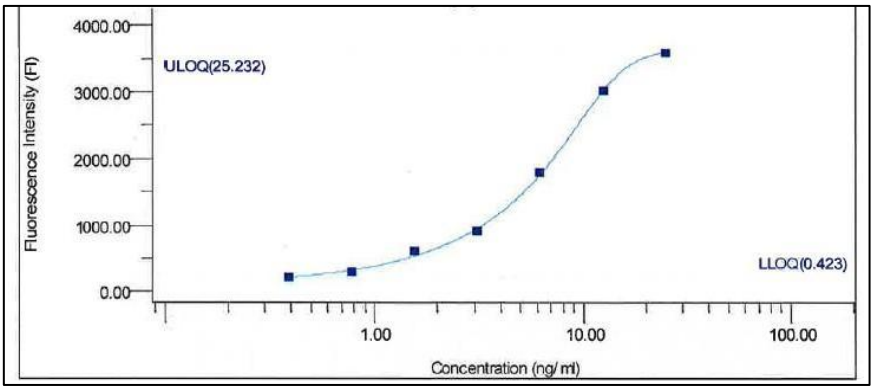


Fig 3.21: Standard curve for Purified Polysaccharide against Serotype B

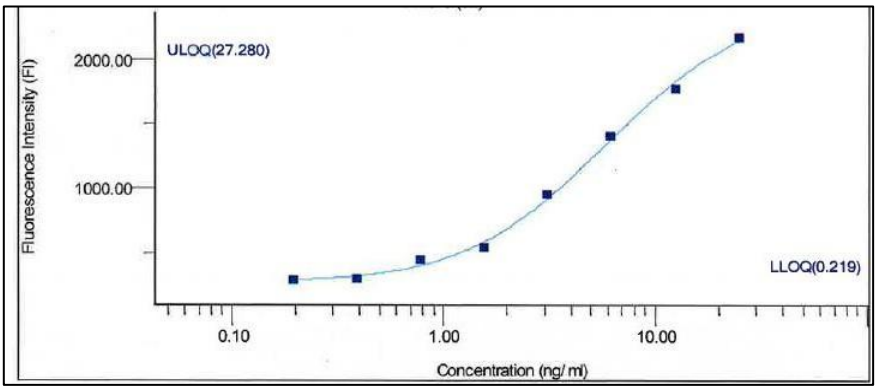


Fig 3.22: Standard curve for Purified Polysaccharide against Serotype C

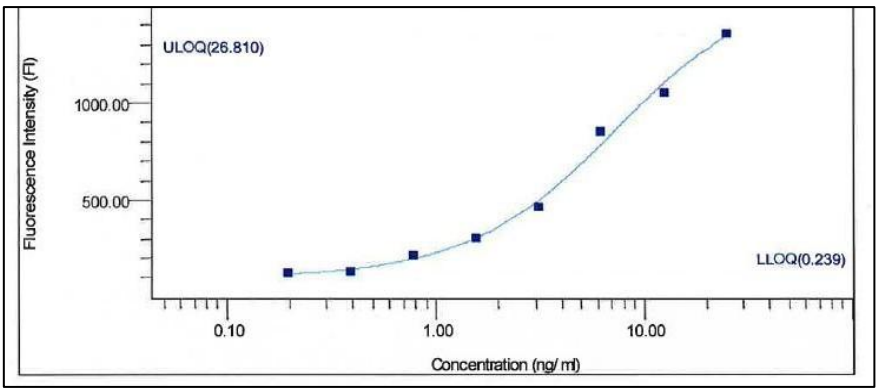


Fig 3.23: Standard curve for Purified Polysaccharide against Serotype D

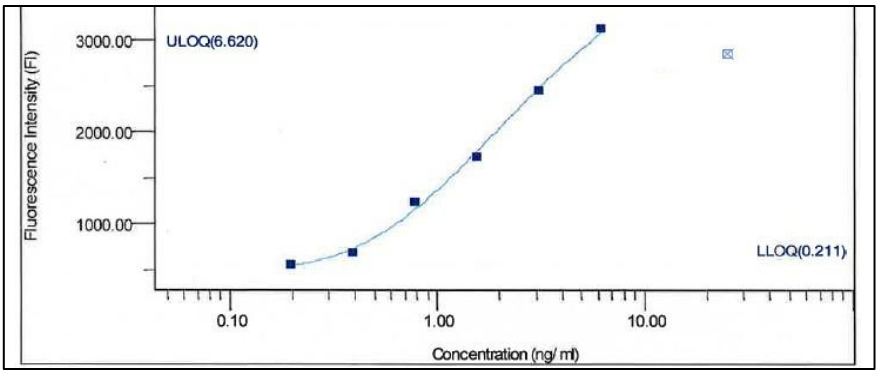


Fig 3.24: Standard curve for Purified Polysaccharide against Serotype E

Serotype	Vaccine Formulation (unprocessed supernatant) Conc. (ng/ml)	Processed formulation supernatant (free Ps), Conc (ng/ml)	Percent free Ps
A	60.66	58.73	1.33
B	266.88	141.89	3.22
C	415.95	71.95	1.64
D	49.96	33.22	0.76
E	256.03	83.67	1.90

Table 3.11: Compiled data for free Polysaccharide content

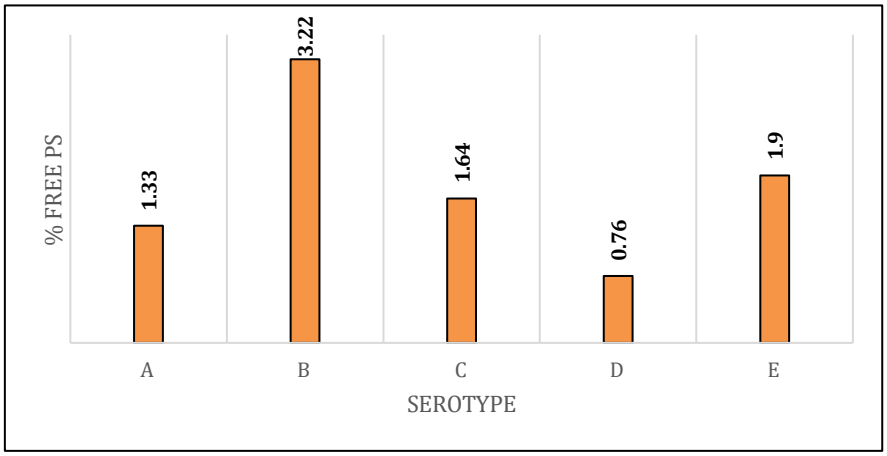


Fig 3.25: Bar graph for percent free Ps

Serotype	Vaccine Formulation (unprocessed supernatant) Conc. (ng/ml)	Spiked Processed sample (to check for Ps recovery), Conc (ng/ml)	Percent recovery after spiking (acceptance criteria: 70-130%)
A	60.66	79.92	99
B	266.88	139.04	76
C	415.95	237.1	92
D	49.96	65	87
E	256.03	170.1	96

Table 3.12: Compiled data for spike recovery of purified polysaccharide

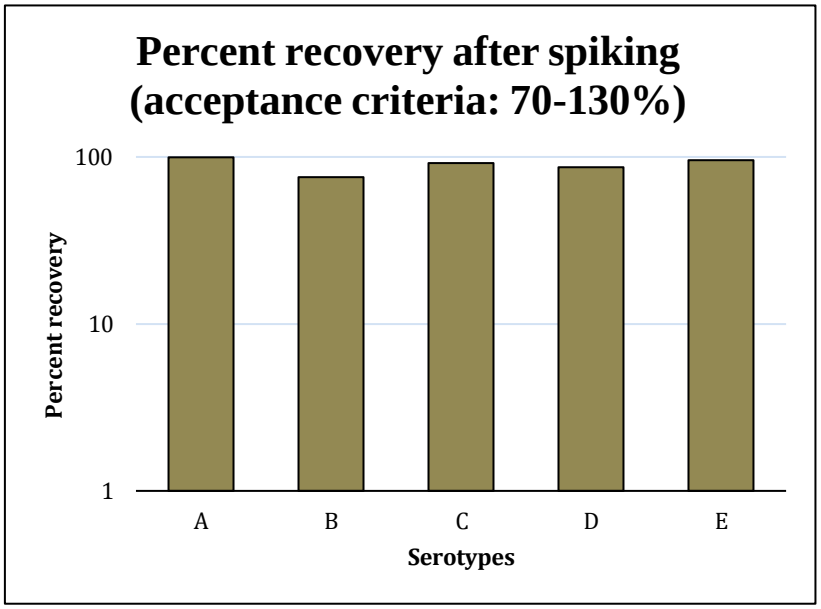


Fig 3.26: Bar graph for percent recovery of Ps after spiking

Inference: The percentage of free polysaccharides in the given vaccine formulation was less than 10 percent. Polysaccharide recovery after spiking was obtained between the acceptable range of 70 and 130%.

3.8 To confirm the presence of polysaccharide-conjugates in processed and unprocessed vaccine formulation supernatant using ELISA

Two controls and four samples were used to check the presence of polysaccharide-conjugates in the processed and unprocessed vaccine formulation supernatant. Purified polysaccharide mix used for spiking was used as the negative control and polysaccharide-conjugate mix was used as the positive control. 1:2, 1:4 and 1:8 dilutions of the standard and samples were taken. The fourth well in each column was blank.

Sample used:

i) PS standard mix as Negative control (PS-Cj should be absent)

ii) Unprocessed vaccine formulation sup

iii) Processed

(Monoclonal antibody against serotype A, B, C, D & E coated)					
SPL1:1 2 PS standard mix for spiking(100ng/ml) (negative control)	SPL2:1 2 Unprocessed vaccine formulation supernatant	SPL3:1 2 Processed vaccine formulation supernatant (to estimate free Polysaccharides)	SPL4:1 2 Spiked processed sample (to check for Ps recovery)	SPL5:1 2 Spiked unprocessed formulation supernatant	SPL6:1 2 Polysaccharide Conjugate mix (positive control)
SPL1:2 4 PS standard mix for spiking(100ng/ml) (negative control)	SPL2:2 4 Unprocessed vaccine formulation supernatant	SPL3:2 4 Processed vaccine formulation supernatant (to estimate free Polysaccharides)	SPL4:2 4 Spiked processed sample (to check for Ps recovery)	SPL5:2 4 Spiked unprocessed formulation supernatant	SPL6:2 4 Polysaccharide Conjugate mix (positive control)
SPL1:3 8 PS standard mix for spiking(100ng/ml) (negative control)	SPL2:3 8 Unprocessed vaccine formulation supernatant	SPL3:3 8 Processed vaccine formulation supernatant (to estimate free Polysaccharides)	SPL4:3 8 Spiked processed sample (to check for Ps recovery)	SPL5:3 8 Spiked unprocessed formulation supernatant	SPL6:3 8 Polysaccharide Conjugate mix (positive control)
Blank	Blank	Blank	Blank	Blank	Blank

Table 3.13: ELISA Plate format

	PS standard mix for spiking(100ng/ml) (negative control)	Unprocessed vaccine formulation supernatant	Processed vaccine formulation supernatant (to estimate free Polysaccharides)	Spiked processed sample (to check for Ps recovery)	Spiked unprocessed formulation supernatant	Polysaccharide Conjugate mix (positive control)	
MAb against Serotype A coated	0.08	0.323	0.063	0.061	0.193	0.163	450
	0.085	0.462	0.067	0.066	0.221	0.118	
	0.096	0.194	0.084	0.084	0.176	0.102	
	0.126	0.097	0.087	0.09	0.086	0.093	
MAb against Serotype B coated	0.081	0.66	0.084	0.08	0.45	0.395	450
	0.087	0.472	0.105	0.089	0.32	0.233	
	0.076	0.303	0.086	0.095	0.212	0.147	
	0.087	0.147	0.098	0.106	0.195	0.123	
MAb against Serotype C coated	0.058	1.846	0.129	0.09	1.675	1.448	450
	0.068	1.806	0.14	0.11	1.276	1.113	
	0.073	1.124	0.147	0.1	0.729	0.578	
	0.081	0.098	0.113	0.066	0.088	0.07	
MAb against Serotype D coated	0.066	0.667	0.085	0.068	0.654	3.119	450
	0.098	0.714	0.111	0.088	0.466	2.532	
	0.1	0.51	0.085	0.096	0.474	1.477	
	0.101	0.101	0.078	0.135	0.166	0.094	
MAb against Serotype E coated	0.094	1.576	0.269	0.092	0.829	0.266	450
	0.083	1.009	0.116	0.086	0.65	0.167	
	0.095	0.735	0.12	0.097	0.481	0.147	
	0.096	0.096	0.087	0.099	0.107	0.072	

Table 3.14: Optical density values obtained at 450nm

.	PS standard mix for spiking(100ng/ml) (negative control)	Unprocessed vaccine formulation supernatant	Processed vaccine formulation supernatant (to estimate free Polysaccharides)	Spiked processed sample (to check for Ps recovery)	Spiked unprocessed formulation supernatant	Polysaccharide Conjugate mix (positive control)	
MAb against Serotype A coated	-0.026	0.218	-0.042	-0.044	0.088	0.057	Blank 450
	-0.02	0.356	-0.038	-0.039	0.115	0.013	
	-0.009	0.089	-0.021	-0.021	0.071	-0.003	
	0.021	-0.008	-0.018	-0.016	-0.02	-0.012	
MAb against Serotype B coated	-0.024	0.555	-0.021	-0.025	0.344	0.29	Blank 450
	-0.018	0.366	0	-0.016	0.214	0.127	
	-0.029	0.198	-0.019	-0.01	0.107	0.042	
	-0.018	0.042	-0.007	0	0.089	0.018	
MAb against Serotype C coated	-0.048	1.741	0.024	-0.016	1.57	1.343	Blank 450
	-0.038	1.701	0.035	0.005	1.171	1.008	
	-0.033	1.019	0.042	-0.005	0.624	0.472	
	-0.024	-0.007	0.008	-0.04	-0.017	-0.035	
MAb against Serotype D coated	-0.039	0.562	-0.02	-0.037	0.548	3.014	Blank 450
	-0.008	0.608	0.006	-0.017	0.36	2.427	
	-0.006	0.405	-0.02	-0.01	0.369	1.372	
	-0.004	-0.004	-0.027	0.03	0.061	-0.011	
MAb against Serotype E coated	-0.007	1.476	0.168	-0.008	0.728	0.165	Blank 450
	-0.018	0.908	0.015	-0.015	0.549	0.066	
	-0.006	0.634	0.019	-0.004	0.38	0.046	
	-0.005	-0.005	-0.014	-0.002	0.006	-0.028	

Table 3.15: Optical density values obtained at Blank 450nm

Inference: The absorbance at 450nm in the column displaying processed samples are close to blank, indicating the absence of polysaccharide-conjugates. On the other hand,the unprocessed formulation supernatant shows the presence of polysaccharide- conjugates.

Chapter 4
Discussion

4. Discussion

Children less than two years of age and adults above 65 years are individuals most prone to being infected by Streptococcus pneumoniae. Therapeutic treatments like antibiotics are no longer useful against the evolving strains of this pathogen which are antibiotic resistant. Therefore, discovery of pneumococcal vaccines has been important for controlling the spread of the disease. The first vaccine developed was a polysaccharide-based vaccine which was able to generate T-independent immune cell responses. This vaccine was preferred for adults due to mature immune system, however, in children it failed due to amateur B-cells. Thus, to make the polysaccharide-based vaccine immunogenic to infants, capsular polysaccharides were conjugated to a carrier protein like CRM197, DT and TT. This led to the formation of memory B-cells in children thus providing long lasting immunity.

The quality control methods employed to test the antigen content in vaccine include ELISAs which are laborious and highly time consuming. Moreover, with the development of higher valent vaccines, it would be difficult to estimate antigen content of each strain in the formulation. Thus, development of multiplex bead-based assay was a boon to simultaneously quantitate and compare the antigenic content in a vaccine formulation.

In this study, we developed a method to estimate total and free polysaccharide content in vaccine formulations. We opted for a sandwich-based assay model to enhance the sensitivity of polysaccharide detection. Rabbit polyclonal sera were directly coupled onto polystyrene beads, with the polysaccharide antigen bound to these sera and monoclonal antibodies subsequently attached to the antigen.

We began by assessing the cross-reactivity of the polyclonal sera. The acceptance criterion for cross-reactivity was set at <30%, and all polyclonal sera selected for proof of concept met this requirement. Next, we produced murine monoclonal antibodies that demonstrated high specificity and less than 10% cross-reactivity.

With the rabbit polyclonal sera and murine monoclonal antibodies in place, the polyclonal sera were coupled to the beads using a method developed by the scientists at Serum Institute. The initial experiment focused on standard optimization, ensuring the sample curve aligned with the standard curve. Following optimization, the total polysaccharide content was estimated using the sandwich bead-based assay model.

This experiment enabled us to quantify both conjugated and non-conjugated polysaccharides in the vaccine formulation.

Subsequently, we proceeded to estimate the free polysaccharide content. In this step, we spiked the unprocessed vaccine formulation supernatant with 100 ng/ml of free polysaccharide to assess recovery. Our results demonstrated that the model accurately estimated the free polysaccharide levels across all serotypes. The spiked sample recovery fell within the acceptable range of 70-130%. These findings confirmed that the developed model could reliably estimate free polysaccharide content, even in higher-valent vaccine formulations.

Finally, the presence of conjugates in both the unprocessed and processed vaccine formulations was confirmed using an Enzyme-Linked Immunosorbent Assay (ELISA).

In conclusion, we successfully developed, standardized, and optimized a model for accurately detecting and quantifying free polysaccharide content in multivalent pneumococcal vaccines. The future prospects of this project lie in performing animal studies to evaluate the impact of free polysaccharide in final vaccine to observe IgG response in animal post immunization. This will help to assess formulation stability without performing in-vivo studies in animals.

Appendix

(Preparation of buffers and media)

1. **Freezing Medium:** Prepared by mixing 90% FBS and 10% DMSO. It is then incubated at -20°C till ice crystals form
2. **DMEM/F12 Incomplete culture medium (5L):** 60.0gm of DMEM/F12, 16gm of HEPES and 12gm of Sodium Bicarbonate were weighed and dissolved in 5000ml of WFI (pH 7.2 ± 0.2). The media was filtered in the biosafety cabinet using 0.2-micron filter
3. **20% DMEM/F12 Complete culture medium (2L):** 1600ml of DMEM Incomplete medium, 400 ml of heat inactivated FBS and 1% anti-anti.
4. **10X Phosphate Buffer Saline (PBS) (1L):** 80g of NaCl, 1.6gm of KCl, 27.53 gm of Na_2HPO_4 and 3.14 gm of KH_2PO_4 were weighed and dissolved in 700ml of WFI and then the volume was made up to 1000ml with the same. This was filtered through 0.2-micron filter
5. **1X Phosphate Buffer Saline (PBS) (1L):** Mix 100 ml of 10X PBS in 900 ml of WFI (pH 7.2 ± 0.2). Filter it through 0.2-micron filter
6. **20% Sodium azide:** 10 gm of Sodium Azide in 20 ml WFI. Volume up to 50 ml using WFI.
7. **Luminex assay buffer (5 Lt):** 12.5 ml of sodium azide solution, 10gm BSA, 500 ml 10X PBS dissolved in 3000 ml WFI. Make up the volume to 5 Lt using WFI. Add 5 ml tween-20. Measure pH 7.2 +- 0.5. Filter it through 0.2 m filter
8. **2% BSA (Blocking solution):** 200 mg BSA was weighed and dissolved in 10 ml of 1X PB.
9. **10% APS:** 50 mg APS is dissolved in 500 WFI.
10. **30% Acrylamide:** 29.2 gm acrylamide, 0.8 gm methylene bisacrylamide was dissolved in 100 ml WFI and stored at 4 degree Celsius.
11. **0.5 M Tris HCL buffer:** 6 gm trizma base was dissolved in 100 ml WFI. Adjust the pH to 6.8 using 6N HCL. Store at room temperature.
12. **1.5 M Tris HCL buffer:** 27.23 gm trizma base was dissolved in 150 ml WFI. Adjust the pH to 8.8 using 6N HCL. Store at room temperature.
13. **10% SDS:** 1 gm SDS dissolved in 10 ml WFI.
14. **10X Electrode buffer:** 30.3 gm trizma base, 144 gm glycine, and 10 gm SDS dissolved in 500 ml WFI. Volume up to 1000 ml using WFI. Adjust the pH to 8.32 +- 0.2 using 6N HCL.
15. **Staining Solution:** Mix 500 mg brilliant blue powder, 500 ml methanol, 100 ml acetic acid, in 400 ml WFI. Store at room temperature.

16. **Destaining Solution:** Mix 400 ml methanol, 100 ml acetic acid, in 500 ml WFI. Store at room temperature.
17. **0.5% bromophenol blue solution:** 50 mg bromophenol dye in 10 ml WFI.
18. **Reducing buffer:** 3.55 ml WFI, 1.25 ml 0.5M tris HCL, 2.5 ml glycerol, 2 ml SDS, 0.5% bromophenol blue solution, and 50µl beta mercaptoethanol. Store at 4 degree Celsius.
19. **Non- reducing buffer:** 3.55 ml WFI, 1.25 ml 0.5M tris HCL, 2.5 ml glycerol, 2 ml SDS, and 0.5% bromophenol blue solution. Store at 4℃

References:

1. Jain V, Vashisht R, Yilmaz G, Bhardwaj A. Pneumonia Pathology. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2025.

2. Regunath H, Oba Y. Community-Acquired Pneumonia. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2025.

3. Musher DM, Abers MS, Bartlett JG. Evolving Understanding of the Causes of Pneumonia in Adults, With Special Attention to the Role of Pneumococcus. *Clin Infect Dis*. 2017;65(10):1736-1744.

4. Ebeledike C, Ahmad T. Pediatric Pneumonia. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2025.

5. Brooks LRK, Mias GI. Streptococcus pneumoniae's Virulence and Host Immunity: Aging, Diagnostics, and Prevention. *Front Immunol*. 2018;9:1366.

6. Dion, C.F., and J.V. Ashurst. "Streptococcus pneumoniae." *StatPearls*. Treasure Island (FL): StatPearls Publishing, 2025.

7. Yahiaoui, R.Y., et al. "Prevalence and Antibiotic Resistance of Commensal *Streptococcus pneumoniae* in Nine European Countries." *Future Microbiology* 11 (2016): 737–744.

8. Moore, M.R., and C.G. Whitney. "Use of Pneumococcal Disease Epidemiology to Set Policy and Prevent Disease during 20 Years of the Emerging Infections Program." *Emerging Infectious Diseases* 21, no. 9 (2015): 1551–1556.

9. Zarabi, N., et al. "Clinical and Economic Burden of Pneumococcal Disease Among Adults in Sweden: A Population-Based Register Study." *PLoS ONE* 18, no. 7 (2023): e0287581.

10. Masomian, M., et al. "Development of Next Generation *Streptococcus pneumoniae* Vaccines Conferring Broad Protection." *Vaccines (Basel)* 8, no. 1 (2020).

11. Amanna, I.J., N.E. Carlson, and M.K. Slifka. "Duration of Humoral Immunity to Common Viral and Vaccine Antigens." *New England Journal of Medicine* 357, no. 19 (2007): 1903–1915.

12. Sood, R.K., and A. Fattom. "Capsular Polysaccharide-Protein Conjugate Vaccines and Intravenous Immunoglobulins." *Expert Opinion on Investigational Drugs* 7, no. 3 (1998): 333–347.

13. Fitzwater, S.P., et al. "The Worldwide Impact of the Seven-Valent Pneumococcal Conjugate Vaccine." *Pediatric Infectious Disease Journal* 31, no. 5 (2012): 501–508.

14. Dransfield, M.T., et al. "Long-Term Comparative Immunogenicity of Protein Conjugate and Free Polysaccharide Pneumococcal Vaccines in Chronic Obstructive Pulmonary Disease." *Clinical Infectious Diseases* 55, no. 5 (2012): e35–e44.
15. Concepcion, N.F., and C.E. Frasch. "Pneumococcal Type 22f Polysaccharide Absorption Improves the Specificity of a Pneumococcal-Polysaccharide Enzyme-Linked Immunosorbent Assay." *Clinical Diagnostic Laboratory Immunology* 8, no. 2 (2001): 266–272.16.
16. Kellar, K.L., and J.P. Douglass. "Multiplexed Microsphere-Based Flow Cytometric Immunoassays for Human Cytokines." *Journal of Immunological Methods* 279, no. 1–2 (2003): 277–285.
17. Aziz, M., F. Iheanacho, and M.F. Hashmi. "Physiology, Antibody." *StatPearls*. Treasure Island (FL): StatPearls Publishing, 2025.
18. Justiz Vaillant, A.A., et al. "Immunoglobulin." *StatPearls*. Treasure Island (FL): StatPearls Publishing, 2025.
19. Malik, B., and A. Ghatol. "Understanding How Monoclonal Antibodies Work." *StatPearls*.
Treasure Island (FL): StatPearls Publishing, 2025.
20. Lipman, N.S., et al. "Monoclonal Versus Polyclonal Antibodies: Distinguishing Characteristics, Applications, and Information Resources." *ILAR Journal* 46, no. 3 (2005): 258–268.
21. Mathew, J., P. Sankar, and M.A. Varacallo. "Physiology, Blood Plasma." *StatPearls*. Treasure Island (FL): StatPearls Publishing, 2025.
22. Tiller, T. "Single B Cell Antibody Technologies." *New Biotechnology* 28, no. 5 (2011): 453–457.
23. Kohler, G., and C. Milstein. "Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity." *Nature* 256, no. 5517 (1975): 495–497.
24. Zhang, C., *Hybridoma technology for the generation of monoclonal antibodies.* Methods Mol Biol, 2012. **901**: p. 117-35.
25. Cassedy, A. and R. O'Kennedy, *Antibody Purification Using Affinity Chromatography.* Methods Mol Biol, 2022. **2466**: p. 3-22.
26. Duong-Ly, K.C. and S.B. Gabelli, *Using ion exchange chromatography to purify a recombinantly expressed protein.* Methods Enzymol, 2014. **541**: p. 95-103.
27. Schrag, D., M. Corbier, and S. Raimondi, *Size exclusion-high-performance liquid chromatography (SEC-HPLC).* Methods Mol Biol, 2014. **1131**: p. 507-12.
28. Kielkopf, C.L., W. Bauer, and I.L. Urbatsch, *Methods for Measuring the Concentrations of Proteins.* Cold Spring Harb Protoc, 2020. **2020**(4): p. 102277.

29. Kielkopf, C.L., W. Bauer, and I.L. Urbatsch, *Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis of Proteins.* Cold Spring Harb Protoc, 2021. **2021**(12).
30. Rathod, V., et al., *Multiplexed bead-based assay for the simultaneous quantification of human serum IgG antibodies to tetanus, diphtheria, pertussis toxin, filamentous hemagglutinin, and pertactin.* Front Immunol, 2023. **14**: p. 1190404.