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Dedication

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Rahma



Dedication

To the One Who Bestows Blessings and Makes the Difficult Easy; Alhamdolilah, praise befitting His majesty, for His completeness and success.

To my dear father…

my most loyal friend, the companion of my journey, and my greatest support that never bends.

To the one who was my strength in hardship, my reason in confusion, and the heart that overflows with limitless giving… to you, who devoted your life so I could build mine, I offer the fruit of my efforts as a gesture of loyalty and gratitude.

To the paradise that walks on earth, and the one whose prayers have surrounded me with purity… my beloved mother.

To my steady support and the pride I cherish… my brothers and sisters.

To all of you, I dedicate this success.

Bilal

Résumé

Cette revue bibliographique porte sur la production des anticorps et leur importance dans les applications thérapeutiques et diagnostiques. Les anticorps sont des molécules immunologiques hautement spécifiques largement utilisées dans la recherche biomédicale, le diagnostic des maladies et les traitements ciblés. Les progrès de la biotechnologie et de l'ingénierie des anticorps ont considérablement amélioré les méthodes de production et élargi leurs applications biomédicales.

Ce travail présente la structure, la classification et les mécanismes immunologiques des anticorps, ainsi que les principales technologies utilisées pour la production des anticorps polyclonaux, monoclonaux et recombinants. Les différentes stratégies de production, les systèmes d'expression, les techniques de purification et les procédures de contrôle qualité sont également abordés afin d'évaluer la spécificité, la stabilité et les performances analytiques des anticorps.

Par ailleurs, cette étude met en évidence les applications thérapeutiques des anticorps dans l'immunothérapie, l'oncologie, les maladies auto-immunes et les maladies infectieuses. Elle examine également leur rôle dans les techniques diagnostiques et analytiques telles que l'ELISA, le Western blot, l'immunofluorescence, l'immunohistochimie et les tests diagnostiques rapides.

Une attention particulière est accordée aux défis actuels liés à la production des anticorps, notamment la standardisation, la reproductibilité, les coûts de production, la fiabilité analytique et la fabrication à grande échelle. Enfin, les perspectives futures concernant l'ingénierie des anticorps, la production locale et le développement des systèmes diagnostiques biotechnologiques sont discutées.

Mots-clés : Production, Anticorps, polyclonaux, monoclonaux, recombinants Immunothérapie, Tests sérologiques, diagnostiques, système de production.

الملخص

تتناول هذه المراجعة الببليوغرافية إنتاج الأجسام المضادة وأهميتها في التطبيقات العلاجية والتشخيصية. تُعد الأجسام المضادة جزيئات مناعية جد متخصصة تُستخدم على نطاق واسع في البحث البيوطبي، وتشخيص الأمراض، والعلاجات الموجهة. وقد ساهم التقدم في التكنولوجيا الحيوية وهندسة الأجسام المضادة في تحسين طرق إنتاجها وتوسيع مجالات استخدامها الطبي والحيوي.

يعرض هذا البحث بنية الأجسام المضادة وتصنيفها وآلياتها المناعية، بالإضافة إلى أهم التقنيات المستخدمة في إنتاج الأجسام المضادة متعددة النسائل، وحيدة النسيلة، والمؤتلفة. كما يستعرض مختلف استراتيجيات الإنتاج، وأنظمة التعبير، وتقنيات التنقية، وإجراءات مراقبة الجودة بهدف تقييم النوعية، والثبات، والأداء التحليلي للأجسام المضادة.

كما تسلط هذه الدراسة الضوء على التطبيقات العلاجية للأجسام المضادة في العلاج المناعي، وعلاج السرطان، والأمراض المناعية الذاتية، والأمراض المعدية. بالإضافة إلى ذلك، تناقش دور الأجسام المضادة في التقنيات التشخيصية والتحليلية مثل اختبار**ELISA** ، وتقنية**Western blot** ، والتألق المناعي، والكيمياء النسيجية المناعية، والاختبارات التشخيصية السريعة.

وقد تم التركيز كذلك على التحديات الحالية المرتبطة بإنتاج الأجسام المضادة، خاصة ما يتعلق بالتقييس، وقابلية إعادة الإنتاج، وتكاليف الإنتاج، والموثوقية التحليلية، والتصنيع على نطاق واسع. وفي الختام، تمت مناقشة الآفاق المستقبلية المتعلقة بهندسة الأجسام المضادة، والإنتاج المحلي، وتطوير الأنظمة التشخيصية البيوتكنولوجية.

الكلمات المفتاحية: الإنتاج، الأجسام المضادة متعددة النسائل، الأجسام المضادة وحيدة النسيلة، الأجسام المضادة المؤتلفة، العلاج المناعي، الاختبارات المصلية، الاختبارات التشخيصية، نظام الإنتاج.

Abstract

This bibliographic review focuses on antibody production and its importance in therapeutic and diagnostic applications. Antibodies are highly specific immunological molecules widely used in biomedical research, disease diagnosis, and targeted treatments. Advances in biotechnology and antibody engineering have considerably improved antibody production methods and expanded their biomedical applications.

This S presents the structure, classification, and immunological mechanisms of antibodies, as well as the principal technologies used for the production of polyclonal, monoclonal, and recombinant antibodies. Different production strategies, expression systems, purification techniques, and quality control procedures are also reviewed in order to evaluate antibody specificity, stability, and analytical performance.

In addition, this review highlights the therapeutic applications of antibodies in immunotherapy, oncology, autoimmune diseases, and infectious diseases. It also examines their role in diagnostic and analytical techniques such as ELISA, Western blotting, immunofluorescence assays, immunohistochemistry, and rapid diagnostic tests.

Special attention is given to the current challenges associated with antibody production, including standardization, reproducibility, production costs, analytical reliability, and large-scale manufacturing. Finally, future perspectives related to antibody engineering, local production, and the development of biotechnological diagnostic systems are discussed.

Keywords: Production, polyclonal antibodies, monoclonal antibodies, recombinant antibodies, immunotherapy, serological tests, diagnostic tests, production system.

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List of abbreviations

3Rs: Replacement, Reduction, and Refinement

ABC: Avidin–Biotin Complex

AC: Affinity Chromatography

ACE2: angiotensin-converting enzyme 2

ADC / ADCs: Antibody–Drug Conjugate(s)

ADCC: antibody-dependent cellular cytotoxicity

ADCP: Antibody-Dependent Cellular Phagocytosis

ADE: antibody-dependent enhancement

ADIPL: antibody-dependent increase in pathogen load

AI/ML: Artificial Intelligence / Machine Learning

AIT: allergen immunotherapy

AMS: ammonium sulfate

AOX1: Alcohol Oxidase 1

APC / APCs: Antigen-Presenting Cell(s)

ASSURED: Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, Deliverable to end-users

BAFF: B-cell Activating Factor

BCR / BCRs: B-cell Receptor(s)

BSA: Bovine Serum Albumin

bsAbs: bispecific antibodies

CAP: College of American Pathologists

CAR-T: Chimeric Antigen Receptor T-cell

CD40–CD40: signal between T cells and B cells that activates antibody production and immune response

CDC: Complement-Dependent Cytotoxicity

cDNA: complementary Deoxyribonucleic Acid

CDR / CDRs: Complementarity-Determining Region(s)

CH: Constant Heavy (domain)

CHO: Chinese Hamster Ovary

CL: Constant Light (domain)

COVID-19: Coronavirus Disease 2019

CpG: Cytosine-phosphate-Guanine

CrAg: Cryptococcal Antigen

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

CTLA-4: cytotoxic T-lymphocyte-associated antigen 4

CXCL12: C-X-C Motif Chemokine Ligand 12

DAB: Diaminobenzidine

DAR: Drug-to-Antibody Ratio

DIF: Direct Immunofluorescence

DMARDs: Disease-Modifying Anti-Rheumatic Drugs

DNA: Deoxyribonucleic Acid

DsFv: Disulfide-stabilized Fragment variable

DSP: Downstream Processing

E. coli: Escherichia coli

EBF1: Early B-cell Factor 1

EBV: Epstein–Barr Virus

EGFR: Epidermal Growth Factor Receptor

F(ab')2: Fragment Antigen Binding 2

Fab: Fragment Antigen Binding

FBC: Full Blood Count

Fc gamma Rs: Fc gamma receptors

Fc: Fragment Crystallizable

FcRn: Neonatal Fc Receptor

FcγR / FcγRs: Fc Gamma Receptor(s)

FcεR: Fc Epsilon Receptor

GAD: Glutamic Acid Decarboxylase

H1N1: Hemagglutinin type 1, Neuraminidase type 1

H2O2: hydrogen peroxide

HAMA: Human Anti-Mouse Antibodies

HAT: Hypoxanthine-Aminopterin-Thymidine

HbA1c: Hemoglobin A1c (Glycated Hemoglobin A1c)

HCPs: Host Cell Proteins

HEK293: Human Embryonic Kidney 293 Cells

HER2: Human Epidermal Growth Factor Receptor 2

HIC: Hydrophobic Interaction Chromatography

HIER: Heat-Induced Epitope Retrieval

HIV: Human Immunodeficiency Virus

HPLC: High-Performance Liquid Chromatography

HRP: horseradish peroxidase

HRP2: Histidine-Rich Protein 2

HSC / HSCs: Hematopoietic Stem Cell(s)

ICC: Immunocytochemistry

IEX: Ion-Exchange Chromatography

IF: Immunofluorescence

Ig: Immunoglobulin

IgA: Immunoglobulin A

IgD: Immunoglobulin D

IgE: Immunoglobulin E

IgG: Immunoglobulin G

IgG1 / IgG2 / IgG3 / IgG4: Immunoglobulin G subclasses 1, 2, 3, and 4

IgM: Immunoglobulin M

IgY: Immunoglobulin Y

IHC: Immunohistochemistry

IL: Interleukin

IMAC: Immobilized Metal Affinity Chromatography

IPTG: Isopropyl β-D-1-thiogalactopyranoside

J chain: Joining Chain

kDa: Kilodalton

KiH: knobs-into-holes

KLH: Keyhole Limpet Hemocyanin

koff: kinetic off-rate constant

LAG-3: Lymphocyte Activation Gene 3

LAMP: loop-mediated isothermal amplification

LB: lymphocyte B

LC-MS: Liquid Chromatography–Mass Spectrometry

LFAs: lateral flow immunoassays

LILRB1: leukocyte immunoglobulin-like receptor B1

LMPP: Lymphoid-Primed Multipotent Progenitor

LoD: Limit of Detection

LSAB: Labeled Streptavidin–Biotin

M13K07: M13 Helper Phage K07

mAbs: Monoclonal Antibodies

MAM: Multi-Attribute Method

MHC II: Major Histocompatibility Complex Class II

mIHC: Multiplex Immunohistochemistry

MPP: multipotent progenitors

mRNA: messenger Ribonucleic Acid

NAATs: Nucleic Acid Amplification Tests

Nb / Nbs: Nanobody / Nanobodies

NPV: Negative Predictive Value

NS0: NS0 Myeloma Cell Line

omab: Murine monoclonal antibodies

pAbs: Polyclonal antibodies

PAT: Process Analytical Technologies

PAX5: Paired Box gene 5

PCR: polymerase chain reaction

PD-1: Programmed Cell Death Protein 1

PD-L1: Programmed Death-Ligand 1

PEG: polyethylene glycol

PFA: Paraformaldehyde

PIER: Proteolytic-Induced Epitope Retrieval

POC: Point-of-Care

PPV: Positive Predictive Value

PTMs: Post-Translational Modifications

PVDF: polyvinylidene difluoride

QA: Quality Assurance

QC: Quality Control

RA: rheumatoid arthritis

rAbs: Recombinant antibodies

RAG1 / RAG2: Recombination Activating Genes 1 and 2

RBD: receptor-binding domain

RDT / RDTs: Rapid Diagnostic Test(s)

RNA: Ribonucleic Acid

RPLC: Reverse-Phase Liquid Chromatography

RSV: Respiratory Syncytial Virus

SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2

scFv: Single-Chain Variable Fragment

SCIT: Subcutaneous Immunotherapy

sdAb: Single-domain Ab (Antibody)

SDS-PAGE: Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

SEC: Size-Exclusion Chromatography

SERS: surface-enhanced Raman scattering

SLE: Systemic Lupus Erythematosus

SLIT: Sublingual Immunotherapy

SnNout: Sensitive test when Negative rules out disease

SpPIn: Specific test when Positive rules In disease

SPR: surface plasmon resonance

SPRI: Surface Plasmon Resonance Imaging

Syk: spleen tyrosine kinase

TAT: turnaround times

TB: Tuberculosis

TDM: therapeutic drug monitoring

TGF-β: Transforming Growth Factor Beta

TIGIT: T cell immunoglobulin and ITIM domain

TIM-3: T-cell Immunoglobulin and Mucin-domain containing-3

TN: True Negatives

TNF: Tumor Necrosis Factor

TP: True Positives

Treg: Regulatory T Cell

TSA: Tyramide Signal Amplification

umab: Fully human monoclonal antibodies

V(D)J: Variable, Diversity, and Joining Recombination

VEGF: Vascular Endothelial Growth Factor

VEGFR: Vascular Endothelial Growth Factor Receptor

VH: Variable Heavy Chain

VHH: Variable Domain of Heavy-Chain Antibody

VISTA: V-domain Ig Suppressor of T-cell Activation

VL: Variable Light Chain

WB: Western Blot

WHO: World Health Organization

WSI: Whole Slide Images

ximab: Chimeric monoclonal antibodies

zumab: Humanized monoclonal antibodies

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Introduction

Introduction

Antibodies, also known as immunoglobulins, are specialized glycoproteins produced by B lymphocytes as part of the adaptive immune response. They are characterized by their exceptional ability to specifically recognize and bind antigens with high affinity, which has made them essential tools in biotechnology, biomedical research, clinical diagnostics, and therapeutic applications. Since the early discoveries of humoral immunity in the nineteenth century, antibody-based technologies have continuously evolved, leading to major advances in both medical and life sciences.

In recent decades, antibodies have become key components of diagnostic and therapeutic systems. In clinical diagnostics, they are widely used in immunoassays for the detection and monitoring of infectious, autoimmune, and neoplastic diseases. In parallel, therapeutic antibodies have transformed modern medicine by enabling highly targeted and effective treatments for cancer, autoimmune disorders, inflammatory conditions, and infectious diseases. Advances in monoclonal antibody technology, recombinant antibody production, and antibody engineering have significantly expanded their clinical and biotechnological potential.

However, despite these considerable advances, antibody development and production still face several scientific, technical, and economic limitations, including issues related to production efficiency, cost, scalability, reproducibility, stability, and specificity. These constraints continue to limit the large-scale application and accessibility of antibody-based technologies in both research and healthcare systems. Therefore, a key scientific question arises: how can current advances in antibody production and engineering overcome these limitations to ensure more efficient, scalable, cost-effective, and widely accessible diagnostic and therapeutic applications

Within this context, this literature review aims to provide a comprehensive overview of antibodies, focusing on their biological foundations, production technologies, diagnostic and therapeutic applications, as well as current limitations and future perspectives.

The general objective of this work is to analyze the principles governing antibody structure, production, and applications while highlighting both advances and remaining challenges in the field. More specifically, this study aims to:

- Present the historical development, structure, classification, and immunological mechanisms of antibodies.

- Describe the main technologies used for the production of polyclonal, monoclonal, and recombinant antibodies.
- Review strategies for antibody engineering, expression, purification, and quality control.
- Analyze therapeutic applications in oncology, autoimmune diseases, and infectious diseases.
- Examine diagnostic applications including ELISA, Western blotting , immunohistochemistry, immunofluorescence, and rapid diagnostic tests.
- Discuss the main biotechnological, clinical, and economic challenges associated with antibody-based systems.
- Explore future perspectives, particularly local antibody production platforms and biotechnological capacity building.

The methodology adopted in this work is based on a literature review of peer-reviewed scientific articles, academic books, review papers, and international reports in immunology, biotechnology, antibody engineering, diagnostics, and therapeutic applications. Relevant data were collected, analyzed, and critically synthesized to provide a coherent and comprehensive understanding of the subject.

This thesis is organized into five chapters. The first chapter presents the general aspects of antibodies, including their history, structure, classification, biological origin, and immunological mechanisms. The second chapter focuses on antibody engineering, production technologies, purification strategies, and quality control. The third chapter discusses therapeutic applications in modern medicine. The fourth chapter examines diagnostic applications and antibody-based analytical techniques. The fifth chapter provides a critical discussion of current challenges and future perspectives in antibody research and biotechnological development.

Chapter I.

General Aspects of Antibodies

I.1. History of antibodies

The historical evolution of antibody production represents a fundamental pillar of modern biomedicine and molecular biotechnology, reflecting a transition from empirical serum-based therapies to highly advanced molecular engineering platforms (Lu et al., 2020). The field originated in 1890 with the pioneering work of Emil von Behring and Shibasaburo Kitasato, who demonstrated that serum from immunized animals contained protective components capable of neutralizing diphtheria and tetanus toxins. This discovery established the foundation of humoral immunity and introduced serotherapy as the earliest form of antibody-based treatment strategies (Lu et al., 2020). Early biochemical studies subsequently revealed that these protective factors were associated with the globulin fraction of serum proteins, marking the first step toward the biochemical characterization of antibodies, which initially relied on these heterogeneous polyclonal mixtures (Strohl, 2018). By the mid-twentieth century, advances in cellular immunology identified plasma cells, derived from B lymphocytes, as the specialized cells responsible for immunoglobulin production, thereby defining the cellular basis of antibody synthesis (Strohl, 2018).

A major structural milestone was achieved in 1959 when Edelman and Porter elucidated the Y-shaped architecture of immunoglobulin G (IgG), composed of two identical heavy chains and two light chains linked by disulfide bonds. This structural model established the functional division of antibodies into antigen-binding fragments (Fab) and the crystallizable fragment (Fc), providing a framework for understanding antibody effector functions (Strohl, 2018). Subsequently, Susumu Tonegawa demonstrated that antibody diversity arises from somatic V(D)J recombination, explaining how a limited number of genes can generate a vast repertoire of antigen receptors (Strohl, 2018). This understanding paved the way for a transformative breakthrough in 1975, when Köhler and Milstein developed hybridoma technology, enabling the production of immortal cell lines that secrete monoclonal antibodies with high specificity against a single epitope. This innovation marked the beginning of industrial antibody production and led to the first therapeutic monoclonal antibody, muromonab-CD3, approved for clinical use in 1986 (Lu et al., 2020; Strohl, 2018).

However, early murine monoclonal antibodies exhibited significant immunogenicity in humans, leading to human anti-mouse antibody (HAMA) responses that limited their clinical effectiveness (Strohl, 2018). To overcome this limitation, antibody engineering introduced chimeric antibodies combining murine variable regions with human constant regions, followed by

humanized antibodies in which only complementarity-determining regions (CDRs) were grafted onto human immunoglobulin frameworks (Strohl, 2018).

The development of recombinant antibody technologies further advanced the field, particularly through phage display systems. First introduced by George P. Smith, phage display enables the presentation of antibody fragments on the surface of bacteriophages, allowing *in vitro* selection of high-affinity binders from large antibody libraries without the need for animal immunization (Smith, 1985; Alfaleh et al., 2020; Roth et al., 2021). In parallel, large non-immunized human antibody libraries have enabled the isolation of antibodies with extremely high affinities, including sub-nanomolar binding capabilities, demonstrating the power of *in vitro* selection systems in antibody engineering (Vaughan et al., 1996). Continuous improvements in library design and selection strategies have significantly enhanced the efficiency and versatility of these platforms, making them a cornerstone of modern antibody discovery against infectious agents, toxins, and self-antigens (Zhang, 2023).

Modern antibody engineering has expanded beyond conventional immunoglobulin formats to include highly specialized therapeutic constructs. These include bispecific antibodies capable of simultaneously targeting two different antigens, antibody–drug conjugates (ADCs) for selective drug delivery, and engineered antibody scaffolds designed to enhance stability, affinity, and effector function (Strohl, 2018; Lu et al., 2020). Collectively, these innovations illustrate the convergence of immunology, genetic engineering, and biotechnology in the development of next-generation targeted therapies.

I.2. Definition, Structure, and Properties of Antibodies (Immunoglobulins)

I.2.1. Definition and Functional Dynamics of Antibodies

Antibodies, also known as immunoglobulins (Ig), are glycoproteins produced by differentiated B lymphocytes and play a central role in adaptive immunity. These molecules exist in two main forms: membrane-bound immunoglobulins, known as B-cell receptors (BCRs), which determine antigen specificity and initiate cellular activation, and soluble antibodies secreted by plasma cells, which circulate in biological fluids to neutralize pathogens and promote their elimination (Sadeghalvad & Rezaei, 2021).

The diversity of antibodies is not random but results from somatic gene rearrangement within the variable (V) regions of immunoglobulin genes. This process generates a vast repertoire of antigen-binding specificities. Each B lymphocyte expresses immunoglobulin receptors with

identical specificity, a property referred to as clonal specificity, ensuring that each clone is directed against a single epitope (Zheng, 2024).

Upon exposure to complex multivalent antigens, the immune response typically involves the activation of multiple B-cell clones simultaneously. This reflects the presence of multiple epitopes on a single antigen and results in a polyclonal antibody response. Such a response enhances antigen recognition and improves the efficiency of pathogen elimination through complementary binding activities (Goldsby et al., 2003; Sadeghalvad & Rezaei, 2021).

Following antigen clearance, most effector plasma cells undergo apoptosis, leading to a gradual decline in circulating antibody levels. However, immunological memory is preserved through long-lived memory B cells, which retain antigen-specific information. Upon re-exposure to the same antigen, these cells mediate a faster and more robust secondary immune response (Zheng, 2024).

I.2.2. Structural Architecture of Immunoglobulins

The fundamental unit of an immunoglobulin is a Y-shaped heterotetramer composed of four polypeptide chains: two identical Heavy (H) chains (~50 kDa each) and two identical Light (L) chains (~25 kDa each), stabilized by both non-covalent interactions and interchain disulfide bonds.

According to the structural framework provided by Schroeder and Cavacini (2010) and North et al. (2011), each chain is divided into functional domains:

- Variable Regions (VH and VL): Located at the amino-terminal ends, these regions exhibit high sequence variability. Together, they constitute the Antigen-Binding Site (paratope), which determines the antibody's exquisite specificity.
- Constant Regions (CH and CL): These regions remain relatively conserved and define the antibody’s Isotype (IgG, IgM, IgA, IgD, or IgE). The heavy chain constant regions (specifically CH2 and CH3) are critical for mediating effector functions, such as complement activation and binding to Fc receptors on immune cells.

For a standard monomeric IgG, this configuration results in a total molecular weight of approximately 150 kDa, providing a stable scaffold for molecular recognition

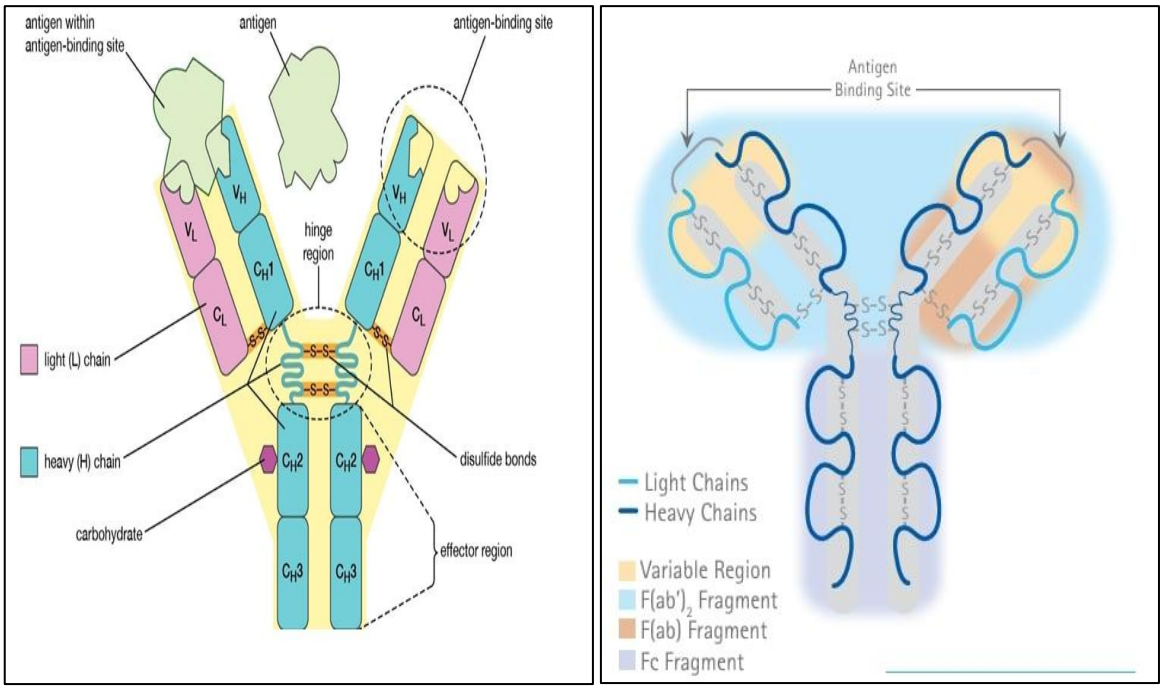


Figure 1 : Antibody structure "Integrated structural representation of a monomeric IgG and its functional domains". (Ahmed, 2024, and Mutneja et al., n.d.)

I.3. Classes of antibodies (IgG, IgM, IgA, IgE, IgD)

Characterization of Immunoglobulin Isotypes

The classification of immunoglobulins into distinct isotypes is determined by the amino acid sequence of the heavy chain constant regions (gamma γ \mu μ \alpha α \delta δ \and \epsilon ε). These structural variations dictate the unique biological effector functions and anatomical distribution of each class.

I.3.1. Immunoglobulin G (IgG):

The Dominant Systemic Isotype

As the most prevalent antibody in human serum (approximately 75–80% of total Ig), **IgG** serves as the cornerstone of long-term humoral immunity.

- **Structural Configuration:** IgG exists primarily as a monomer with a molecular weight of approximately **150 kDa**. It is further divided into four subclasses (**IgG1, IgG2, IgG3, and IgG4**), which differ in their hinge region flexibility and disulfide bond arrangement (Schroeder & Cavacini, 2010).
- **Effector Functions:** Due to its ability to bind with high affinity to **Fc gamma receptors (Fc gamma Rs)**, IgG is highly effective in opsonization and antibody-dependent cellular

cytotoxicity (ADCC). Furthermore, it is the only isotype capable of crossing the **placental barrier** via the neonatal Fc receptor (FcRn), providing critical passive immunity to the fetus.(Schroeder & Cavacini, 2010).

- **IgG Subclasses:** Subclass of an antibody play a decisive role in determining its biological activity and therapeutic performance. Structural variations within the constant regions of immunoglobulins directly influence effector functions, pharmacokinetics, and immunogenicity. Among antibody-based biologics, IgG subclasses—particularly IgG1—are the most widely utilized due to their favorable stability, long serum half-life, and well-characterized functional properties.

I.3.1.1. IgG1

IgG1 is the most abundant IgG subclass in human serum, accounting for approximately two-thirds of total IgG. Structurally, IgG1 antibodies are monomeric molecules with a molecular weight of approximately 146 kDa and a hinge region of intermediate length, which provides sufficient flexibility for effective bivalent antigen binding and immune complex formation. This subclass efficiently interacts with all Fcγ receptors, enabling key effector mechanisms such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP). Additionally, IgG1 is capable of forming hexameric complexes on target cell surfaces, thereby activating the classical complement pathway and mediating complement-dependent cytotoxicity (CDC).

From a therapeutic perspective, IgG1 exhibits a favorable balance between structural stability and functional potency. Although it is less prone to aggregation than IgG2 and IgG4, it may be more susceptible to chemical degradation, potentially due to its relatively longer hinge region. IgG1 antibodies also display genetic polymorphisms in their constant regions, known as allotypes. While these allotypic variations are theoretically immunogenic, clinical evidence suggests that therapeutic antibodies do not necessarily elicit increased anti-drug antibody responses in individuals lacking the corresponding allotype. (Goulet & Atkins, 2020).

I.3.1.2. IgG2

IgG2 is the second most prevalent IgG subclass in circulation and is predominantly associated with immune responses against carbohydrate antigens, such as those present on encapsulated bacteria. It possesses a shorter and more rigid hinge region than IgG1, stabilized by multiple disulfide bonds, which results in limited flexibility of the Fab arms. IgG2 displays weaker binding to Fcγ receptors and reduced complement activation, rendering it a relatively

immunologically silent isotype. Consequently, IgG2 is considered suitable for therapeutic applications requiring antigen neutralization or receptor blockade without strong immune activation. (Goulet & Atkins, 2020)

I.3.1.3. IgG3

IgG3 is distinguished by its extended hinge region, which confers exceptional flexibility and results in the strongest effector functions among all IgG subclasses. It exhibits high affinity for Fcγ receptors and is the most effective subclass in activating the complement system. However, these advantages are offset by significant limitations, including proteolytic susceptibility and a markedly reduced serum half-life. Furthermore, IgG3 demonstrates extensive polymorphism and hinge length variability, contributing to structural heterogeneity that complicates its development as a stable therapeutic antibody. (Goulet & Atkins, 2020)

I.3.1.4. IgG4

IgG4 is the least abundant IgG subclass and is typically associated with prolonged antigen exposure and anti-inflammatory immune responses. A defining characteristic of IgG4 is its ability to undergo Fab-arm exchange, resulting in bispecific antibodies that are functionally monovalent. While this phenomenon may reduce immune complex formation in vivo, it poses challenges for therapeutic development. To overcome this limitation, specific mutations are commonly introduced to stabilize the hinge region and prevent Fab-arm exchange. IgG4 exhibits weak effector functions, limited complement activation, and preferential binding to inhibitory Fcγ receptors, making it suitable for therapies where immune activation is undesirable. (Goulet & Atkins, 2020)

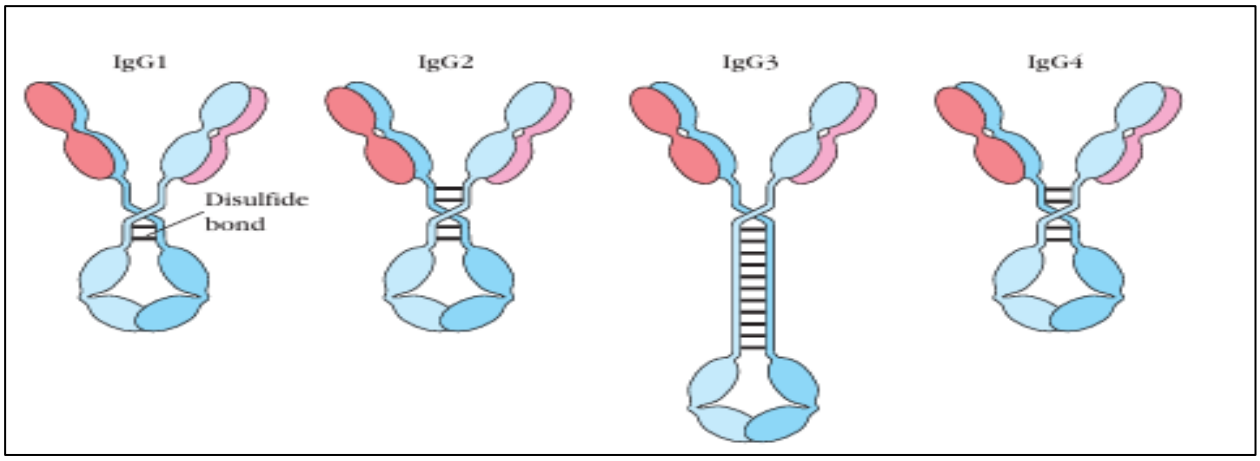


Figure 2 : General structure of the four subclasses of human IgG, which differ in the number and arrangement of the interchain disulfide bonds (thick black lines) linking the heavy chains. (Goldsby et al. (2013)

I.3.2. Immunoglobulin M (IgM):

The Primary Response Isotype

Immunoglobulin M (IgM) is the earliest antibody isotype produced during the primary immune response and plays a critical role in initial host defense. Although individual IgM antigen-binding sites exhibit relatively low affinity, the antibody compensates for this limitation through its polymeric organization, which confers very high avidity. Secreted IgM is predominantly found as a pentameric structure stabilized by a joining (J) chain, resulting in a large molecular complex with multiple antigen-binding sites. This multimeric arrangement enables IgM to efficiently neutralize pathogens, promote agglutination, and, most importantly, activate the classical complement pathway through effective C1q binding. Consequently, IgM is considered one of the most potent mediators of complement-dependent pathogen clearance. While early therapeutic applications of IgM antibodies were constrained by issues related to toxicity and manufacturing complexity, recent advances suggest renewed potential for IgM-based therapeutics, particularly in the treatment of cancer and infectious diseases (Goulet & Atkins, 2020; Schroeder & Cavacini, 2010).

I.3.3. IgA

IgA antibodies play a central role in mucosal immunity and are the predominant immunoglobulin class found in secretions such as saliva, breast milk, and mucosal surfaces. Although IgA has a shorter serum half-life than IgG due to the lack of FcRn binding, it mediates potent effector functions through interaction with Fcα receptors on myeloid cells. IgA exists in

both monomeric and polymeric forms, with secretory IgA providing a critical first line of defense against pathogens at mucosal interfaces. Structural and allotypic differences between IgA1 and IgA2 influence their tissue distribution and susceptibility to proteolysis. (Goulet & Atkins, 2020)

I.3.4. IgD

IgD is co-expressed with IgM on the surface of naïve B cells, yet its biological function remains incompletely understood. Evidence suggests that IgD may contribute to immune regulation by selectively responding to highly multivalent antigens, thereby influencing B-cell activation and tolerance. Although soluble IgD has been shown to participate in pro-inflammatory signaling, its functions are not sufficiently characterized to support therapeutic application. (Goulet & Atkins, 2020)

I.3.5. IgE

IgE is primarily associated with allergic responses and defense against parasitic infections. Despite its low concentration and short serum half-life, IgE exhibits prolonged tissue persistence through high-affinity binding to Fcε receptors on mast cells and basophils. Upon antigen-mediated crosslinking, IgE triggers rapid cellular degranulation and the release of inflammatory mediators. Recent preclinical studies suggest that IgE may offer unique advantages in cancer immunotherapy; however, safety concerns related to hypersensitivity reactions remain a significant challenge. (Goulet & Atkins, 2020)

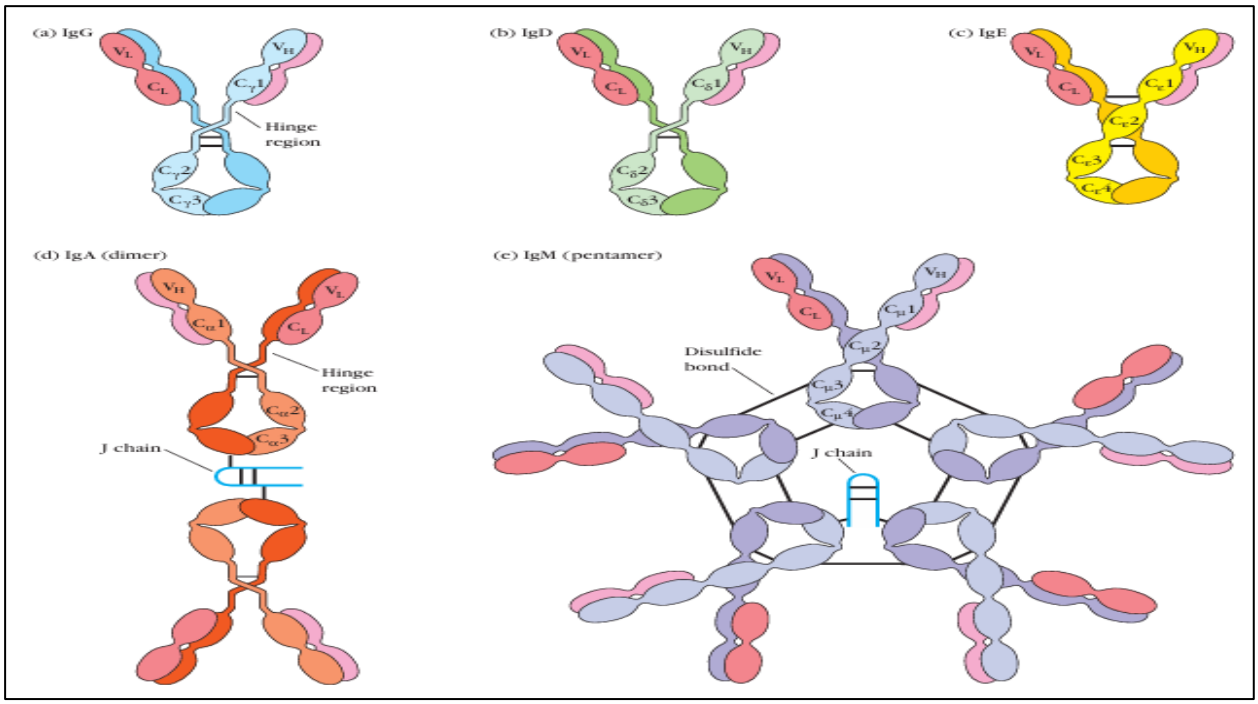


Figure 3 : Structural organization of the main classes of secreted antibodies. (Goldsby et al,2013)

Property/Activity	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	IgM‡	IgE	IgD
Molecular weight†	150,000	150,000	150,000	150,000	150,000–600,000	150,000–600,000	900,000	190,000	150,000
Heavy-chain component	γ1	γ2	γ3	γ4	α1	α2	μ	ε	δ
Normal serum level (mg/ml)	9	3	1	0.5	3.0	0.5	1.5	0.0003	0.03
In vivo serum half life (days)	23	23	8	23	6	6	5	2.5	3
Activates classical complement pathway	+	+/−	++	−	−	−	+++	−	−
Crosses placenta	+	+/−	+	+	−	−	−	−	−
Present on membrane of mature B cells	−	−	−	−	−	−	+	−	+
Binds to Fc receptors of phagocytes	++	+/−	++	+	−	−	?	−	−
Mucosal transport	−	−	−	−	++	++	+	−	−
Induces mast-cell degranulation	−	−	−	−	−	−	−	+	−

*Activity levels indicated as follows: ++ = high; + = moderate; +/− = minimal; − = none; ? = questionable.

†IgG, IgE, and IgD always exist as monomers; IgA can exist as a monomer, dimer, trimer, or tetramer. Membrane-bound IgM is a monomer, but secreted IgM in serum is a pentamer.

‡IgM is the first isotype produced by the neonate and during a primary immune response.

Figure 4 : Properties and biological activities*of classes and subclasses of human serum immunoglobulins (Goldsby et al. (2013)

I.4. Biological Origin and Immunological Mechanisms

I.4.1. B-Cell Ontogenesis and the Bone Marrow Microenvironment

B-cell ontogenesis is a tightly regulated and progressive process that begins during fetal development in the liver and subsequently shifts to the bone marrow, which remains the principal site of B-lymphocyte production throughout adult life. This developmental pathway originates from hematopoietic stem cells (HSCs) residing in specialized niches within the bone marrow. (Zheng, 2024).

The bone marrow microenvironment plays a central role in orchestrating early B-cell development. Stromal cells, in association with cytokines such as IL-7 and chemokines like CXCL12, provide essential survival and differentiation signals that drive lineage commitment. These interactions ensure not only the proliferation of precursor cells but also the maintenance of a controlled developmental environment. (Zheng, 2024).

At each stage, genetic and epigenetic regulatory mechanisms act in a coordinated manner to generate a highly diverse and self-tolerant B-cell repertoire capable of recognizing a wide array of antigens (Aribi, 2020). As HSCs differentiate, they progressively lose their self-renewal capacity and give rise to multipotent progenitors (MPP), followed by lymphoid-primed multipotent progenitors (LMPP). This transition is accompanied by the activation of recombination-activating genes (RAG1 and RAG2), which initiate immunoglobulin gene rearrangement, a fundamental step in B-cell receptor formation (Zheng, 2024).

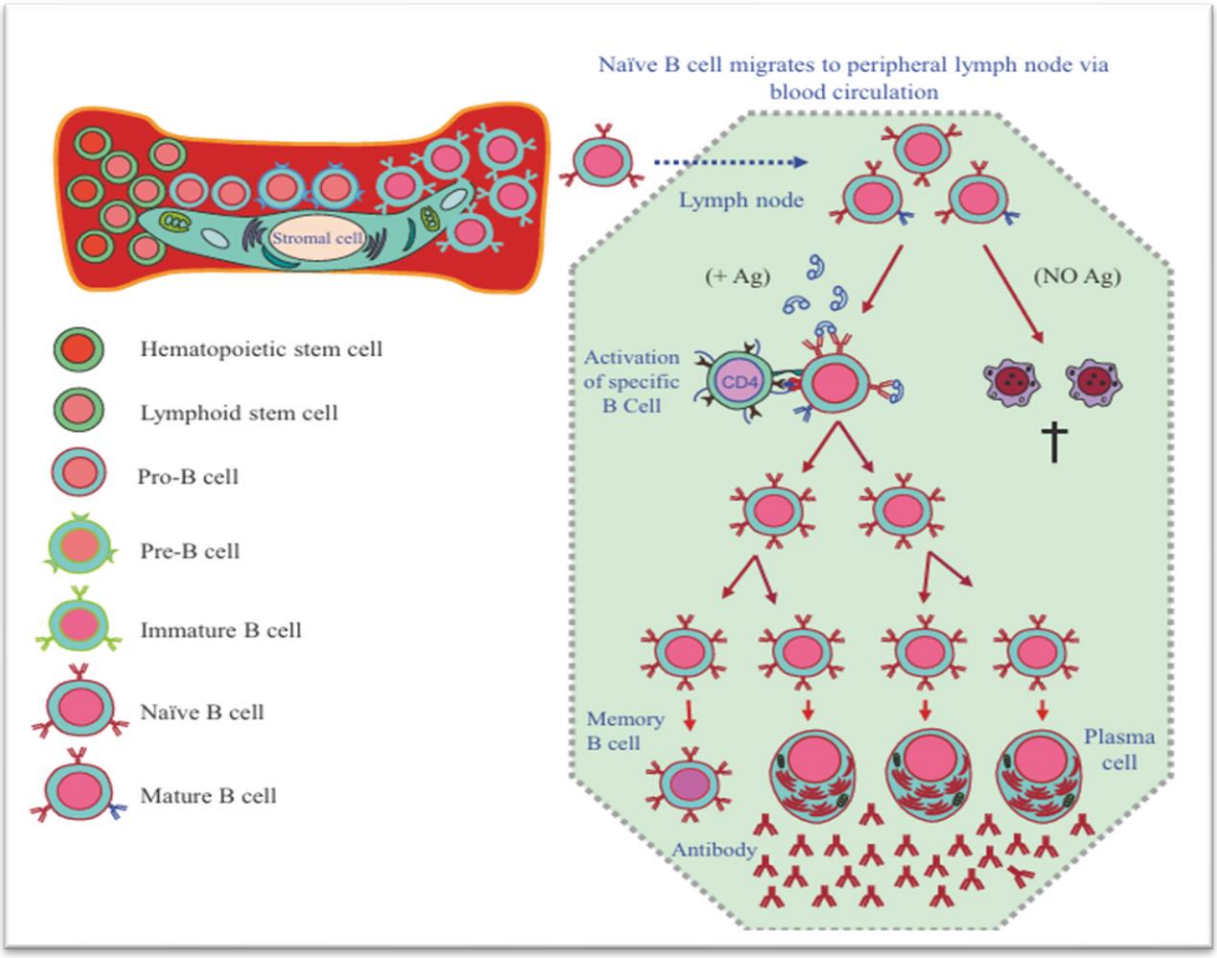


Figure 5 : Schematic representation of B-cell development, maturation, proliferation, and differentiation from hematopoietic stem cells to plasma and memory B cells. (Zheng, S. J, 2024).

I.4.2. Stages of B-Cell Development and V(D)J Recombination

The development of B lymphocytes into fully functional immune cells follows a sequence of well-defined and tightly regulated stages, each characterized by specific molecular and genetic events.

The earliest identifiable stage, the pro-B cell stage, is marked by the initiation of immunoglobulin heavy-chain gene rearrangement through V(D)J recombination. This process is under the control of key transcription factors such as EBF1, PAX5, and Aiolos, which ensure stable and irreversible commitment to the B-cell lineage (Plonquet, 2013). Successful rearrangement leads to the expression of the μ heavy chain, marking the transition to the pre-B cell stage.

At this stage, the μ chain associates with surrogate light chains (VpreB and λ5) to form the pre-B cell receptor (pre-BCR). Signaling through this receptor induces cellular proliferation and

enforces allelic exclusion, thereby ensuring monospecificity of the B-cell receptor. Subsequently, during the immature B cell stage, light-chain gene rearrangement occurs, leading to the expression of a complete IgM molecule on the cell surface. At this point, each B cell acquires a unique antigen specificity, representing a critical checkpoint in B-cell development (Zheng, 2024; Plonquet, 2013).

I.4.3. Central Tolerance and Receptor Editing

Before exiting the bone marrow, immature B cells undergo central tolerance, a crucial mechanism that prevents the survival of autoreactive clones.

When B-cell receptors (BCRs) strongly recognize self-antigens, several outcomes are possible. Cells may undergo clonal deletion through apoptosis, enter a state of anergy characterized by functional inactivation, or initiate receptor editing. This latter mechanism allows secondary rearrangement of light-chain genes, thereby altering antigen specificity and reducing autoreactivity.

Receptor editing is particularly important as it provides a means of salvaging potentially useful B cells while maintaining self-tolerance. Failure of these mechanisms results in elimination of the autoreactive cells, thus preserving immune homeostasis (Mak & Saunders, 2014; Zheng, 2024) .

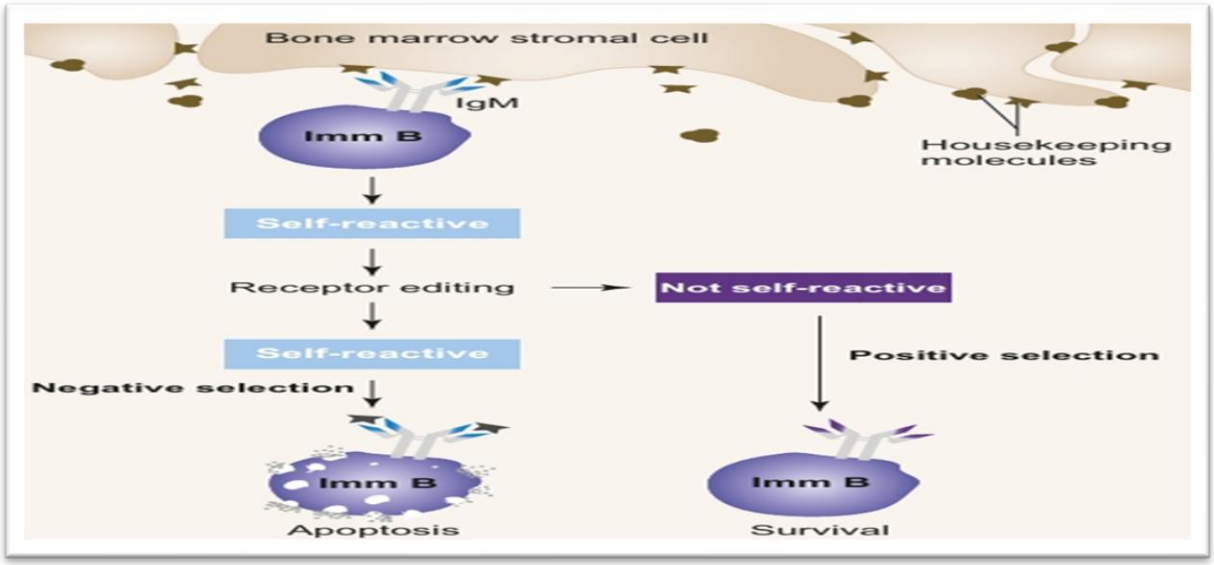


Figure 6 : Negative selection and receptor editing during B-cell central tolerance.

(Mak & Saunders (2014).

I.4.4. Peripheral Maturation and Selection

Following successful central tolerance, immature B cells migrate to peripheral lymphoid organs, primarily the spleen, where they complete their maturation.

These cells initially appear as transitional B cells (T1) and subsequently progress to the T2 stage. Their survival and maturation depend largely on signals mediated by the B-cell activating factor (BAFF). During this phase, selection processes ensure that only functionally competent and non-autoreactive cells persist.

Mature naïve B cells are distinguished by the co-expression of IgM and IgD on their surface, resulting from alternative RNA splicing. This dual expression allows the cells to remain in a quiescent state while maintaining readiness to respond to antigenic stimulation (Plonquet, 2014; Zheng, 2024).

I.4.5. B-Cell Activation and the Clonal Selection Theory

B-cell activation is governed by the clonal selection theory, which states that each B cell expresses a unique receptor specificity, and only those cells that recognize their cognate antigen are selected for proliferation and differentiation (Murphy et al., 2017).

Two major pathways of activation can be distinguished. T-independent responses involve antigens such as polysaccharides that directly cross-link B-cell receptors, leading to a rapid but generally low-affinity IgM response. In contrast, T-dependent responses require antigen processing and presentation via MHC class II molecules, followed by interaction with helper T cells.

Co-stimulatory signals, including CD40–CD40L interactions and cytokines such as IL-21, are essential for full activation. These interactions lead to the formation of germinal centers, specialized microenvironments in which B-cell responses are further amplified and refined (Zheng, 2024; Murphy et al., 2017).

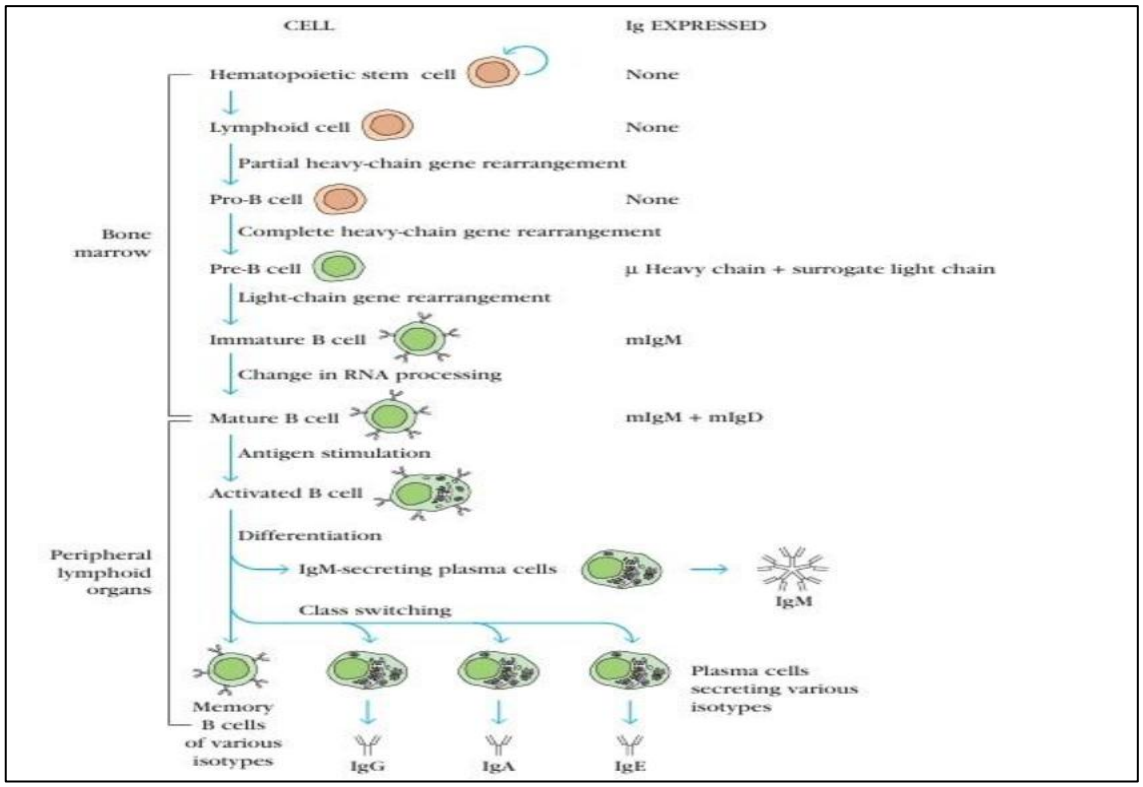


Figure 7 : Overview of B-cell development and immunoglobulin expression. **(**Goldsby et al. (2003)

I.4.6. The Germinal Center Reaction

Within germinal centers, activated B cells undergo critical processes that enhance both the specificity and effectiveness of the humoral immune response.

Somatic hypermutation introduces point mutations into the variable regions of immunoglobulin genes, generating a diverse pool of variants. These variants are then subjected to selection, favoring clones with increased affinity for the antigen, a process known as affinity maturation.

In parallel, class-switch recombination enables B cells to produce different antibody isotypes (such as IgG, IgA, and IgE) without altering antigen specificity. This functional diversification allows antibodies to adapt to different immunological contexts.

Ultimately, B cells differentiate into long-lived plasma cells, which continuously secrete antibodies, or memory B cells, which ensure rapid and efficient responses upon subsequent exposure to the same antigen (Hoffman et al., 2016; Zheng, 2024).

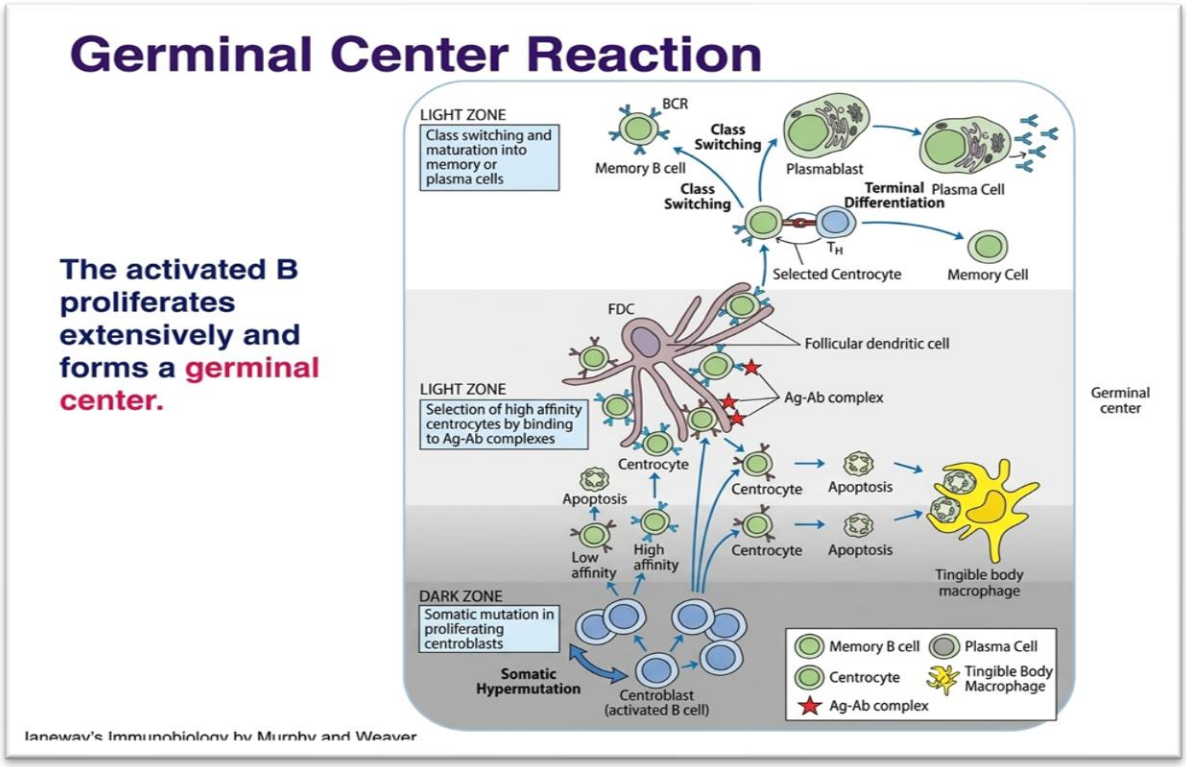


Figure 8 : The dynamic processes within the Germinal Center**.** The diagram illustrates the functional compartmentalization into the Dark Zone (B-cell proliferation and somatic hypermutation) and the Light Zone (affinity-based selection and class-switching). (Murphy & Weaver, 2017)

I.5. Dynamics of Antigen–Antibody Interactions

I.5.1. Fundamental Mechanisms of Binding

Antigen–antibody interactions are reversible and rely on non-covalent forces, including hydrogen bonds, electrostatic interactions, and van der Waals forces. These interactions are fundamental to immune defense mechanisms such as neutralization of toxins, opsonization of pathogens for phagocytosis, and activation of the complement system, ultimately leading to pathogen elimination (Zheng, 2024).

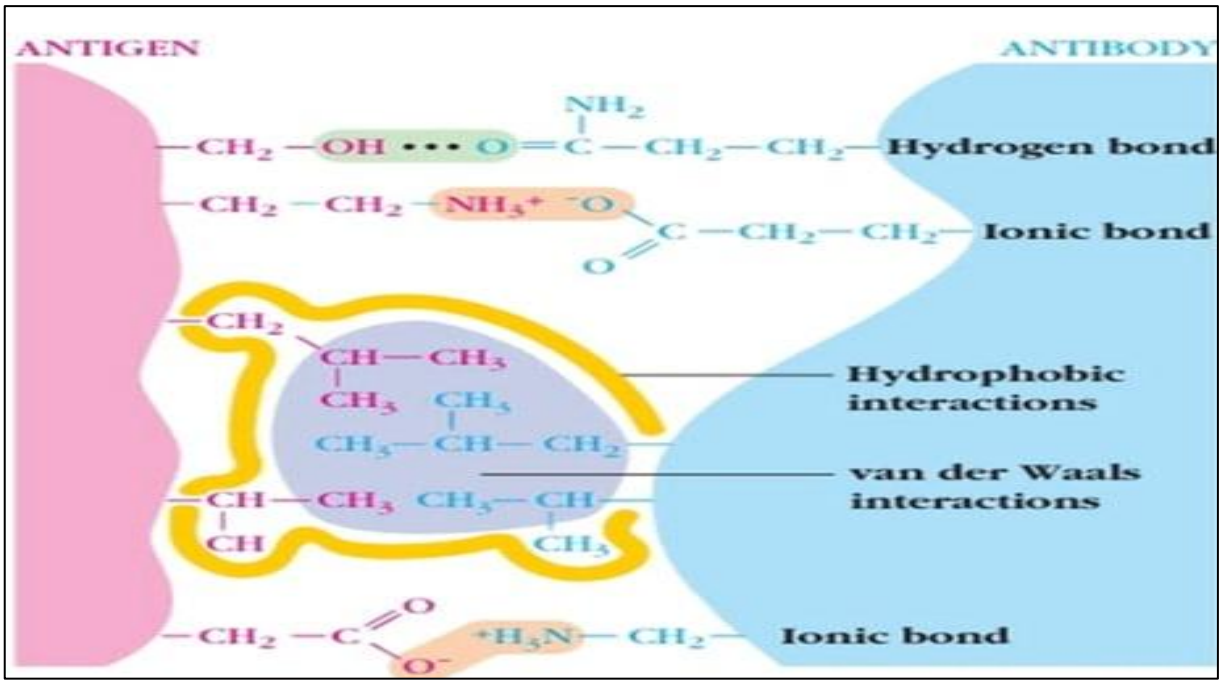


Figure 9 : Noncovalent forces involved in antibody–antigen interactions. (Goldsby et al. 2003, p. 138)

I.5.2. Specificity, Selectivity, and Affinity Maturation

Specificity refers to the precise structural complementarity between an antibody and its corresponding antigen, whereas selectivity describes the ability to discriminate between structurally related molecules (Karush, 1970). Interestingly, lower-affinity antibodies may exhibit higher selectivity due to the requirement for near-perfect structural matching.

Affinity maturation represents a hallmark of adaptive immunity, leading to the production of high-affinity antibodies that are more effective in pathogen clearance. In clinical settings, antibody avidity measurements are widely used to distinguish between primary and secondary infections, particularly in prenatal diagnostics (Goldblatt, n.d.; Zheng, 2024) .

I.5.3. Structural Basis of Maturation

At the molecular level, affinity maturation is driven by mutations within the complementarity-determining regions (CDRs), particularly in the VHCDR3 region. These mutations enhance antigen binding by improving structural complementarity and reducing the dissociation rate (koff).

As a result, mature antibodies exhibit increased binding stability and specificity, allowing for more efficient immune responses. These structural optimizations are essential for the long-term effectiveness of humoral immunity (Sundberg, 2009; Zheng, 2024)

I.6. Types of Antibodies Used in Biotechnology

I.6.1. Polyclonal antibodies (pAbs)

Polyclonal antibodies (pAbs) represent a diverse population of immunoglobulin molecules secreted by different B-cell lineages. They are characterized by their unique capacity to recognize and bind to multiple epitopes on a single antigen, a feature that provides comprehensive immune coverage and makes them indispensable in both diagnostic biotechnology and clinical therapeutics (Garcia-Calvo et al., 2021).

The biotechnical production of pAbs relies on rigorous immunization protocols designed to elicit a robust humoral immune response in various animal models. According to Garcia-Calvo et al. (2021), the selection of the host animal is a critical factor; common sources include lagomorphs (rabbits), rodents, and equines (horses). Furthermore, there is an increasing technological shift toward avian systems, specifically chicken embryos, for the production of Immunoglobulin Y (IgY) (Garcia-Calvo et al., 2021).

This process is typically illustrated by the immunization of different species with a target antigen to yield diverse antibody populations. As demonstrated in the schematic representation of animal immunization pathways, the methodology involves the following key stages (Garcia-Calvo et al., 2021):

- **Initial Immunization:** Injection of emulsified antigen (e.g., gliadin and glutelin fragments) combined with Freund's complete adjuvant.
- **Booster Administration:** Subsequent injections with Freund's incomplete adjuvant at two and four-week intervals to enhance the secondary immune response.
- **Harvesting and Purification:** Once high serum titers are confirmed, the antibodies are harvested—either from the blood serum of mammals or the egg yolks of avian hosts—and purified using techniques such as ammonium sulfate precipitation.

Beyond their role as high-sensitivity diagnostic reagents, these antibodies—particularly anti-gluten IgY—have demonstrated prospective therapeutic utility in mitigating intestinal damage associated with Celiac disease (Garcia-Calvo et al., 2021).

The clinical application of pAbs has a historical trajectory spanning over a century, beginning with the use of immunized animal sera to neutralize *Clostridium tetani* toxins (Lee et al., 2010). In the context of modern biotechnology, these antibodies are strategically sourced from convalescent plasma or highly purified immunoglobulin preparations.

As highlighted by Lee et al. (2010), high-titer immune plasma has been a critical tool during major respiratory crises, such as the 1918 influenza pandemic and the H1N1 outbreaks. The primary biotechnical advantage of polyclonal therapy is the provision of immediate passive immunity through broad-spectrum epitope recognition. Clinical evidence underscores that early intervention with these polyclonal preparations can significantly reduce mortality rates in infected populations (Lee et al., 2010).

I.6.1.1. Technical Challenges and Clinical Constraints

Despite their robust binding capabilities, the integration of pAbs into standardized biotechnological applications faces several constraints. According to **L**ee et al. (2010), these challenges include:

1. **Inter-donor Variability:** Inconsistencies in antibody affinity and concentration between different host animals or donors.
2. **Adverse Reactions:** Potential risks of hypersensitivity or transfusion-related complications in clinical settings.
3. **Temporal Efficacy:** A noted decline in therapeutic effectiveness during the advanced stages of disease progression.

Consequently, the successful application of pAbs necessitates precise timing in administration and rigorous patient stratification to ensure optimal therapeutic outcomes (**Lee et al., 2010**).

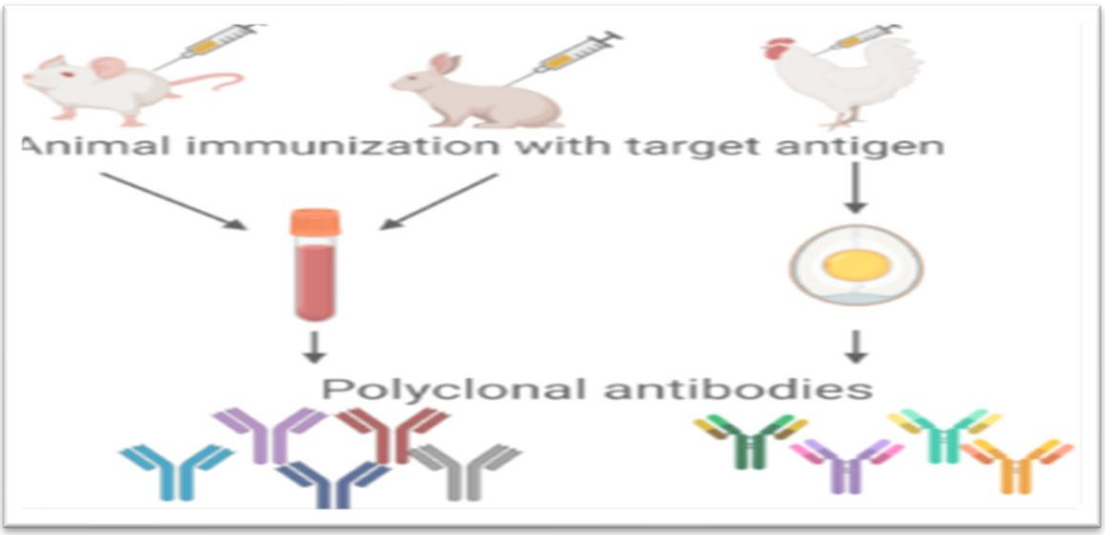


Figure 10 : Production and Characterization of Polyclonal Antibodies. (Garcia-Calvo et al ,2021)

I.6.2. Monoclonal Antibodies:

Monoclonal antibodies (mAbs) are glycoproteins produced by identical clones of B lymphocytes that are directed against a specific antigen. They are widely used in research, diagnostics, and therapy due to their high specificity and affinity for target molecules. Monoclonal antibodies against human targets can be generated through traditional immunization of animals, such as mice, or via advanced recombinant DNA technologies. To minimize potential immunogenicity in humans, genetic engineering approaches are applied to “humanize” these molecules (Lee et al., 2010; Sadeghalvad & Rezaei, 2021).

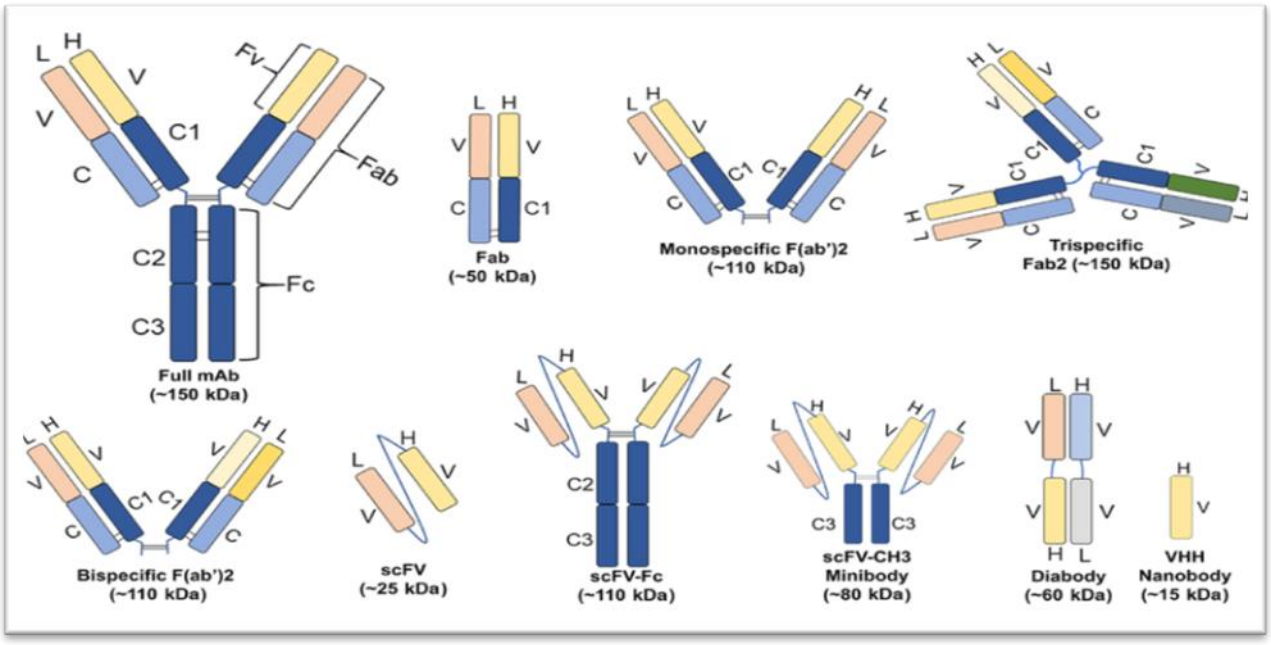


Figure 11 : Schematic representation of the structural diversity of monoclonal antibodies (mAbs) and their engineered fragments. (Saha et al., 2025)

The architecture of a full-length IgG antibody (extreme left) serves as the foundational template, comprising two antigen-binding fragments (Fabs) and a crystallizable region (Fc) maintained by interchain disulfide bonds. Recent advancements in biochemical engineering have enabled the development of truncated and multivalent derivatives to optimize therapeutic efficacy. These include Fab fragments and F(ab')2 constructs (monospecific, bispecific, or trispecific) designed for enhanced epitope targeting. Furthermore, smaller recombinant formats such as single-chain variable fragments (scFv), diabodies, and minibodies (scFv-CH3) offer improved tissue penetration. Notably, the nanobody (VHH) represents the minimal functional antigen-binding unit, derived solely from the variable heavy-chain domain, providing a scaffold for high-affinity recognition in a compact molecular size."

Based on their structural similarity to human immunoglobulins, mAbs are classified into four main generations, each progressively reducing immunogenicity while maintaining antigen specificity.

I.6.2.1. Murine monoclonal antibodies (-omab)

The first generation of mAbs is derived entirely from murine immunoglobulins. These antibodies are produced using hybridoma technology and are highly specific to the target antigen. However, because of their fully murine origin, they can elicit a strong immune response in humans, including the production of human anti-mouse antibodies (HAMA), which may reduce therapeutic efficacy and cause adverse effects (Sadeghalvad & Rezaei, 2021).

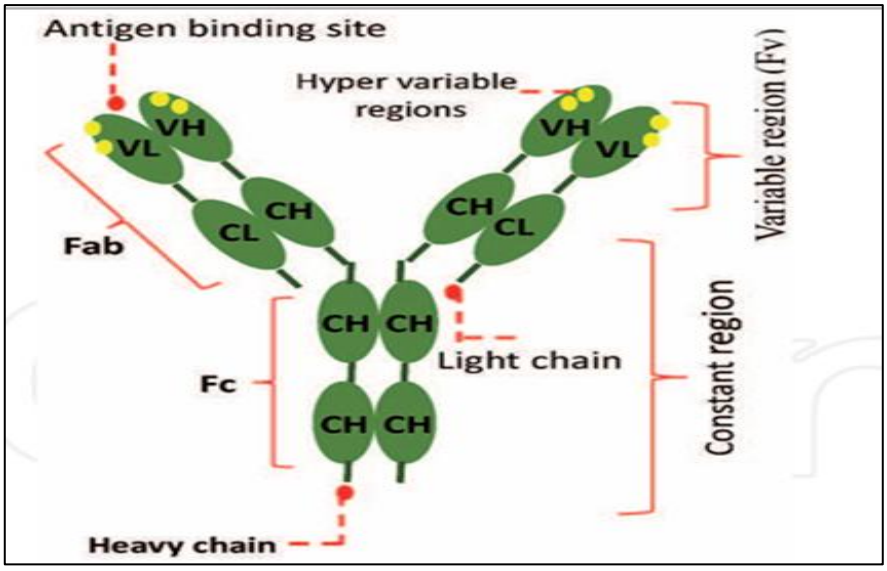


Figure 12 : Structure of murine monoclonal antibody showing entirely murine variable and constant regions (Sadeghalvad & Rezaei, 2021)

I.6.2.2. Chimeric monoclonal antibodies (-ximab)

Chimeric antibodies were developed to overcome the immunogenicity of murine mAbs. In these molecules, the murine variable regions responsible for antigen binding are fused with human constant (Fc) regions. This design results in antibodies that are approximately 25% murine, significantly reducing immune reactions while preserving antigen specificity (Lee et al., 2010; Sadeghalva &Rezaei, 2021).

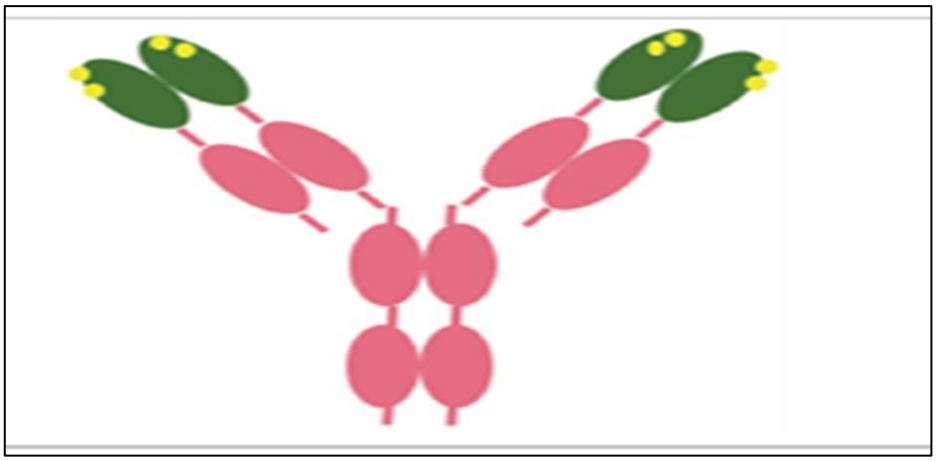


Figure 13 : Structure of chimeric monoclonal antibody illustrating human constant regions fused with murine variable regions in both heavy and light chains.

*(*Sadeghalvad & Rezaei, 2021)

I.6.2.3. Humanized monoclonal antibodies (-zumab)

Humanized antibodies represent further refinement. Only the murine complementarity-determining regions (CDRs) are retained and grafted into an otherwise fully human antibody framework. This approach produces antibodies that are about 95% human, thereby minimizing immunogenicity while maintaining high specificity for the target antigen (Lee et al., 2010; Sadeghalvad & Rezaei, 2021).

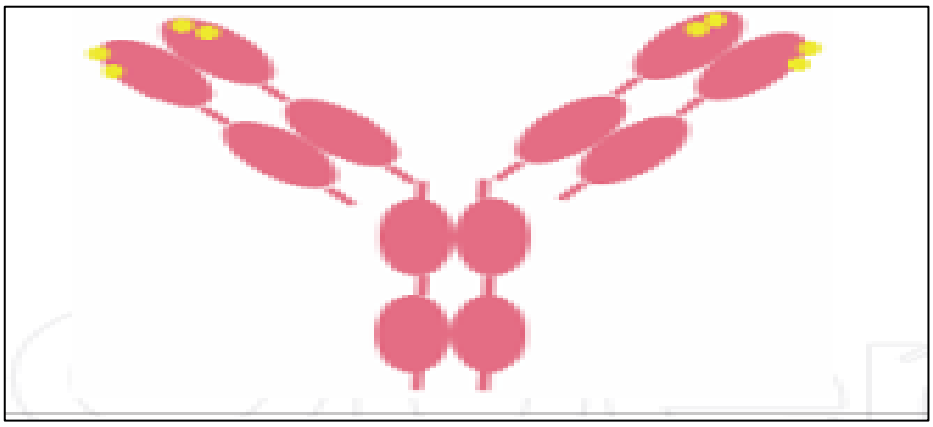


Figure 14 : Structure of humanized monoclonal antibody where only the hypervariable complementarity-determining regions are murine, grafted onto a human antibody framework.

(Sadeghalvad & Rezaei, 2021)

I.6.2.4. Fully human monoclonal antibodies (-umab)

Fully human mAbs are entirely composed of human immunoglobulin sequences and exhibit the lowest immunogenic potential. They are generated using advanced biotechnology platforms,

including transgenic mice engineered to produce human antibodies, Epstein–Barr virus (EBV)-transformed human B cells, or repertoire cloning techniques such as phage display or large cDNA libraries to isolate specific human CDRs. Fully human antibodies provide optimal safety and efficacy for therapeutic applications (Lee et al., 2010; Sadeghalvad & Rezaei, 2021).

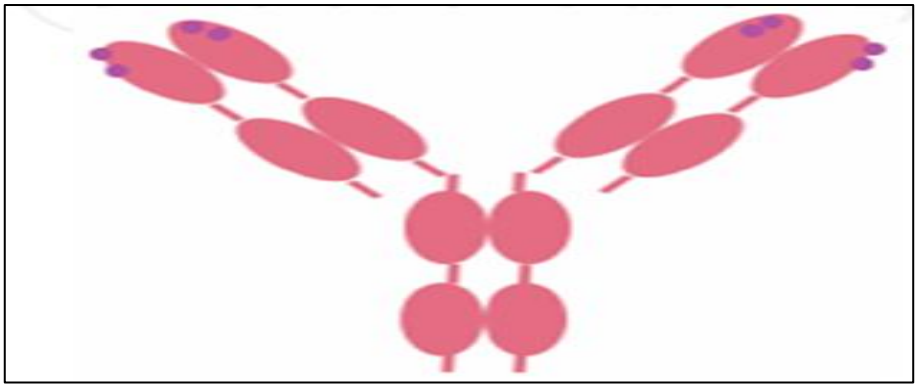


Figure 15 : Structure of fully human monoclonal antibody composed entirely of human immunoglobulin sequences (Sadeghalvad & Rezaei, 2021)

This classification highlights the progressive strategies used to reduce immunogenicity while enhancing therapeutic effectiveness, from fully murine to fully human constructs, illustrating the sophistication and versatility of modern antibody engineering.

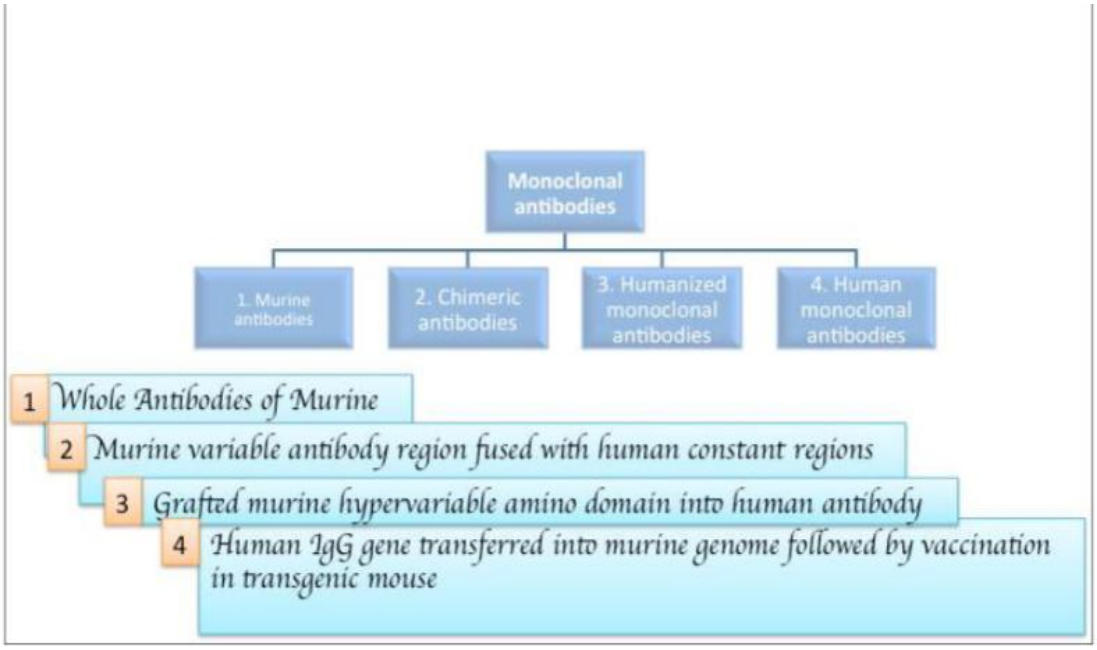


Figure 16 : Molecular Evolution of Monoclonal Antibodies: From Murine Scaffolds to Transgenic Human Platforms (MAHMUDA A. et al,2017)

Table 1: Simplified International Classification of Monoclonal Antibodies Based on Degree of Humanization (Scheen, 2009).

Antibody Category	Suffix	Approximate Human Content (%)	Immunogenicity	Representative Examples
Murine	-momab	0%	High (+++)	Muromonab (Orthoclone®), Ibritumomab (Zevalin®)
Chimeric	-ximab	60–70%	Moderate (+)	Infliximab (Remicade®), Rituximab (MabThera®)
Humanized	-zumab	>90%	Low (±)	Trastuzumab (Herceptin®), Bevacizumab (Avastin®)
Fully Human	-umab	100%	Minimal (±)	Adalimumab (Humira®)

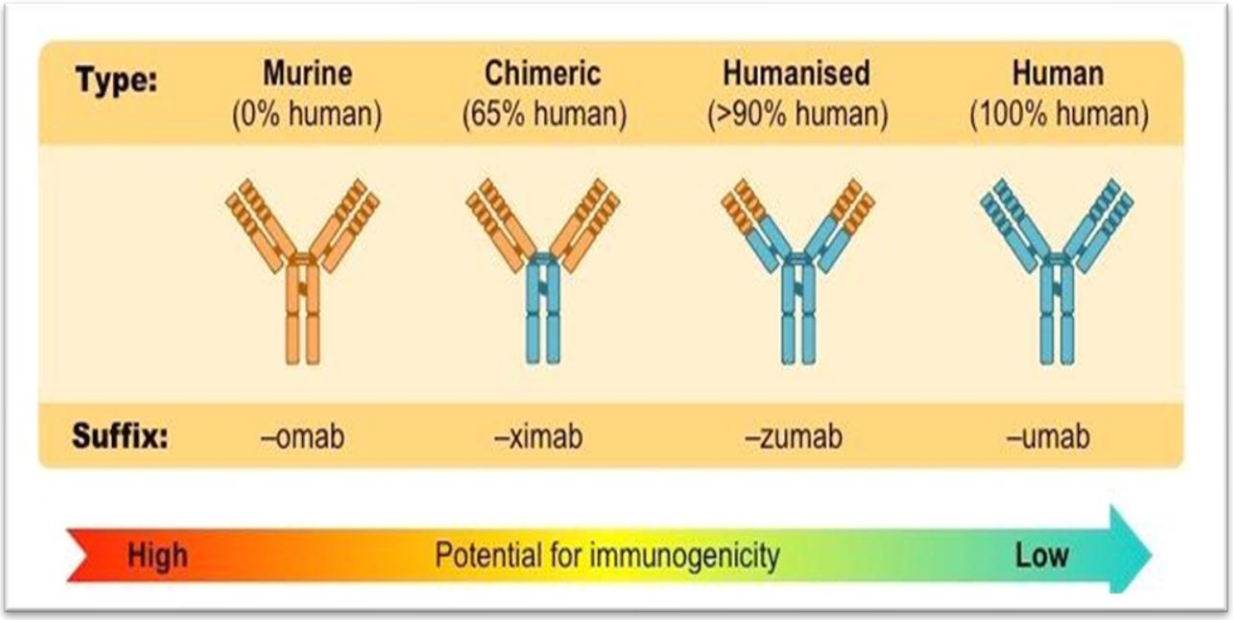


Figure 17 : Overview of Monoclonal Antibody Classes (Sherine et al. (2023).

I.6.3. Recombinant Antibody Fragments: Fab vs. scFv

The transition from full-length monoclonal antibodies (mAbs) to recombinant antibody fragments represents a significant development in modern biotechnology. This shift has been largely driven by the need for molecules with improved tissue penetration and more accessible, cost-effective production systems.

I.6.3.1. Antigen-Binding Fragment (Fab)

The Fab fragment corresponds to a monovalent portion of the antibody responsible for antigen recognition. Although smaller than the intact IgG molecule, it preserves a well-defined and stable structural organization.

- Structure: It consists of one constant and one variable domain of each of the heavy (VH, CH1) and light (VL, CL) chains. These two chains are linked by a natural disulfide bond.
- Stability: Due to the presence of the constant domains (CH1 and CL), Fab fragments exhibit high thermodynamic stability and a lower tendency to aggregate compared to smaller fragments.
- Binding Affinity: In most cases, Fab fragments retain the affinity and specificity of the parental antibody. This is mainly due to the preservation of the spatial arrangement of the antigen-binding site (paratope), ensuring functional equivalence to the native IgG (Benjouad et al., 2009).

I.6.3.2. Single-Chain Variable Fragment (scFv)

The single-chain variable fragment (scFv) represents the minimal antibody format capable of maintaining full antigen-binding specificity.

- Structure: It is a fusion protein composed of the variable regions of the heavy (VH) and light (VL) chains. These domains are connected by a short, flexible peptide linker (usually rich in Glycine and Serine) which allows the two domains to associate correctly.
- Functional Advantages: Due to its reduced size, the scFv demonstrates enhanced penetration into dense tissues, such as solid tumors. However, this small size also results in rapid clearance from the bloodstream.
- Production: scFvs are particularly well-suited for phage display technologies and can be efficiently produced in microbial expression systems such as *E. coli*. This greatly simplifies

production and reduces associated costs compared to full-length antibodies (Morrison et al., 1984, as cited in Benjouad et al., 2009).

Table 2: Comparison of Structural and Functional Characteristics of Fab and scFv Fragments (Benjouad et al. 2009).

Feature	Fab Fragment	scFv Fragment
Molecular Weight	~50 kDa	~25–30 kDa
Composition	Two chains (Heavy + Light)	Single polypeptide chain
Linkage	Disulfide bond (Natural)	Peptide linker (Synthetic)
Stability	High	Moderate (May require engineering)
Tissue Penetration	Good	Excellent
Applications	Diagnostics, Imaging, Therapeutics	Phage Display, CAR-T cells, Radio-imaging

Chapter II.

Antibody Engineering and Production

II.1. Production of Polyclonal Antibodies

II.1.1. Production Principles

The generation of polyclonal antibodies (pAbs) is fundamentally governed by the dynamics of the humoral immune response and the biochemical properties of the immunogen. This process is built upon several core scientific pillars:

• **Multi-epitope Recognition and Clonal Diversity:** When a complex antigen (such as a protein or glycolipid) is introduced, the host's immune system targets diverse antigenic determinants (epitopes). This triggers the activation of multiple B-cell clones, each secreting antibodies specific to different structural or conformational sites. This collective response ensures a broad-spectrum recognition profile (Dunbar & Schwoebel, 1990; Ascoli & Aggeler, 2018).

• **Phylogenetic Distance and Host Selection**: Selecting the appropriate animal species (e.g., rabbits, goats, or sheep) is a strategic decision based on the required volume of antiserum and the taxonomic relationship between the antigen source and the host. Increasing the phylogenetic distance typically enhances "immunogenicity" as the host's immune system more readily recognizes the antigen as foreign (Leenaars & Hendriksen, 2005).

• **Functional Role of Adjuvants:** Adjuvants are indispensable for augmenting the immune response to weak antigens. They function via the "depot effect," which ensures the slow, sustained release of the antigen, and by stimulating Antigen-Presenting Cells (APCs) through localized inflammatory signals (Dunbar & Schwoebel, 1990).

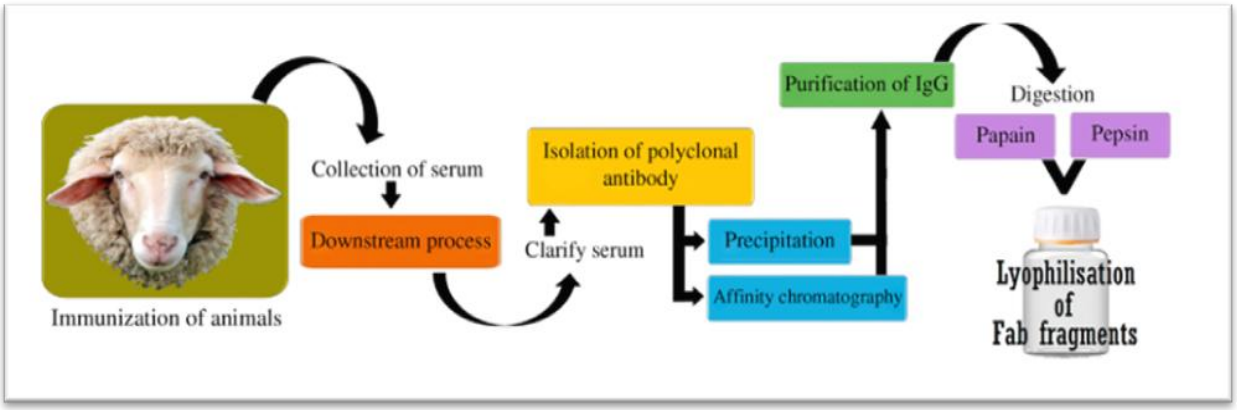


Figure 18 : Schematic Representation of the Production Process for Hyperimmune Polyclonal Therapeutics (Büyükköroğlu & Şenel, 2018)

II.1.2. Experimental Protocol and Immunological Mechanisms of Polyclonal Antibody Production:

The generation of polyclonal antibodies (pAbs) relies on a structured temporal framework designed to maximize antibody titers while maintaining rigorous ethical standards for animal welfare. This process integrates strategic laboratory interventions with the host's natural humoral immune response through the following phases:

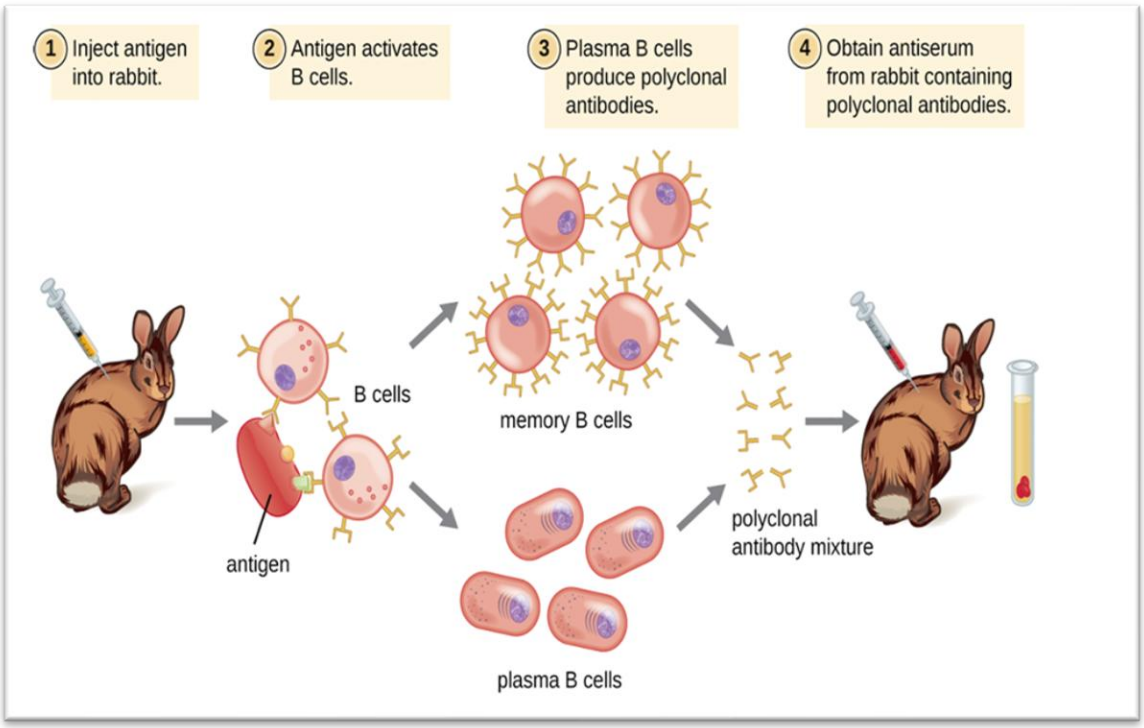


Figure 19 : Systematic Workflow for Polyclonal Antibody Production (Sophia Learning, 2025)

Phase I: Antigen Preparation and Primary Immunization

The protocol initiates with the meticulous purification of the target antigen to mitigate off-target immunological interference. For low-molecular-weight molecules (**haptens**), chemical conjugation to large carrier proteins, such as Keyhole Limpet Hemocyanin (KLH) or Bovine Serum Albumin (BSA), is essential to render them immunogenic (Dunbar & Schwoebel, 1990). Following preparation, an antigen-adjuvant emulsion is systemically administered to the host (e.g., a rabbit) to elicit the **primary immune response**. This "priming" stage is critical for the activation of naive B-cell populations (Leenaars & Hendriksen, 2005; Sophia Learning, 2025).

Phase II: Booster Strategy and Lymphocyte Differentiation

To ensure a robust and sustainable immune response, a **prime-boost strategy** is employed. Subsequent booster injections are administered at specific intervals (typically 2–4 weeks) to achieve two primary biological objectives:

1. **Affinity Maturation:** Stimulating B-cells to produce antibodies with increased binding strength.
2. **Class Switching:** Facilitating the transition from IgM secretion to high-affinity IgG isotypes (Leenaars & Hendriksen, 2005; Sophia Learning, 2025). During this phase, antigen-specific B-lymphocytes undergo rapid **clonal expansion**, differentiating into effector **plasma cells** for immediate secretion and **memory B cells** to ensure long-term humoral surveillance (Sophia Learning, 2025).

Phase III: Titer Monitoring and Polyclonal Secretion

Antibody concentration is monitored through periodic test bleeds, evaluated via Enzyme-Linked Immunosorbent Assay (ELISA) (Ascoli & Aggeler, 2018). At this stage, the heterogeneous lineages of activated plasma cells synthesize a diverse pool of **polyclonal antibodies**. Unlike monoclonal counterparts, this mixture recognizes multiple distinct **epitopes** across the complex surface of the antigen, providing a broad spectrum of detection (Sophia Learning, 2025).

Phase IV: Antiserum Harvesting and Affinity Purification

Upon reaching peak titers, the host's blood is collected, and the **antiserum** is isolated from cellular components through centrifugation (Ascoli & Aggeler, 2018). To achieve the necessary analytical specificity, the crude serum undergoes rigorous **affinity chromatography**. In this process, the target antibodies are isolated based on their specific binding to immobilized antigens, effectively separating the immunoglobulins of interest from other serum proteins and impurities (Ascoli & Aggeler, 2018; Sophia Learning, 2025).

II.1.3. Advantages

- **Superior Sensitivity:** Due to multi-epitope binding, pAbs can significantly amplify signals in assays such as Western Blotting and Immunohistochemistry (IHC), making them ideal for detecting low-abundance targets (Ascoli & Aggeler, 2018).
- **Robustness to Conformational Changes:** pAbs remain functional even if some epitopes are masked or altered by sample fixation or denaturation, as other antibodies in the mixture recognize alternative sites (Dunbar & Schwoebel, 1990).

- **Economic and Temporal Efficiency:** pAbs are relatively inexpensive and faster to produce compared to the complex hybridoma technology required for monoclonal antibodies (Leenaars & Hendriksen, 2005).

II.1.4. Limitations

- **Cross-reactivity:** Clonal diversity increases the probability of antibodies binding to structurally similar non-target proteins, which may lead to false-positive results (Ascoli & Aggeler, 2018).
- **Batch-to-Batch Variability:** Since production relies on the biological response of different animals, the composition and performance of the antiserum can vary significantly between production lots (Leenaars & Hendriksen, 2005).
- **Ethical Constraints:** The process involves live animal use and invasive procedures, necessitating strict adherence to the "3Rs" principles: Replacement, Reduction, and Refinement (Leenaars & Hendriksen, 2005).

II.2. Production of Monoclonal Antibodies

Monoclonal antibodies (mAbs) are essential tools in modern diagnostics and therapeutics. They are produced using several biotechnological approaches, most notably hybridoma technology and phage display technology. In this section, two principal techniques for the production of monoclonal antibodies (mAbs), namely hybridoma technology and phage display (Sadeghalvad & Rezaei, 2021), are discussed

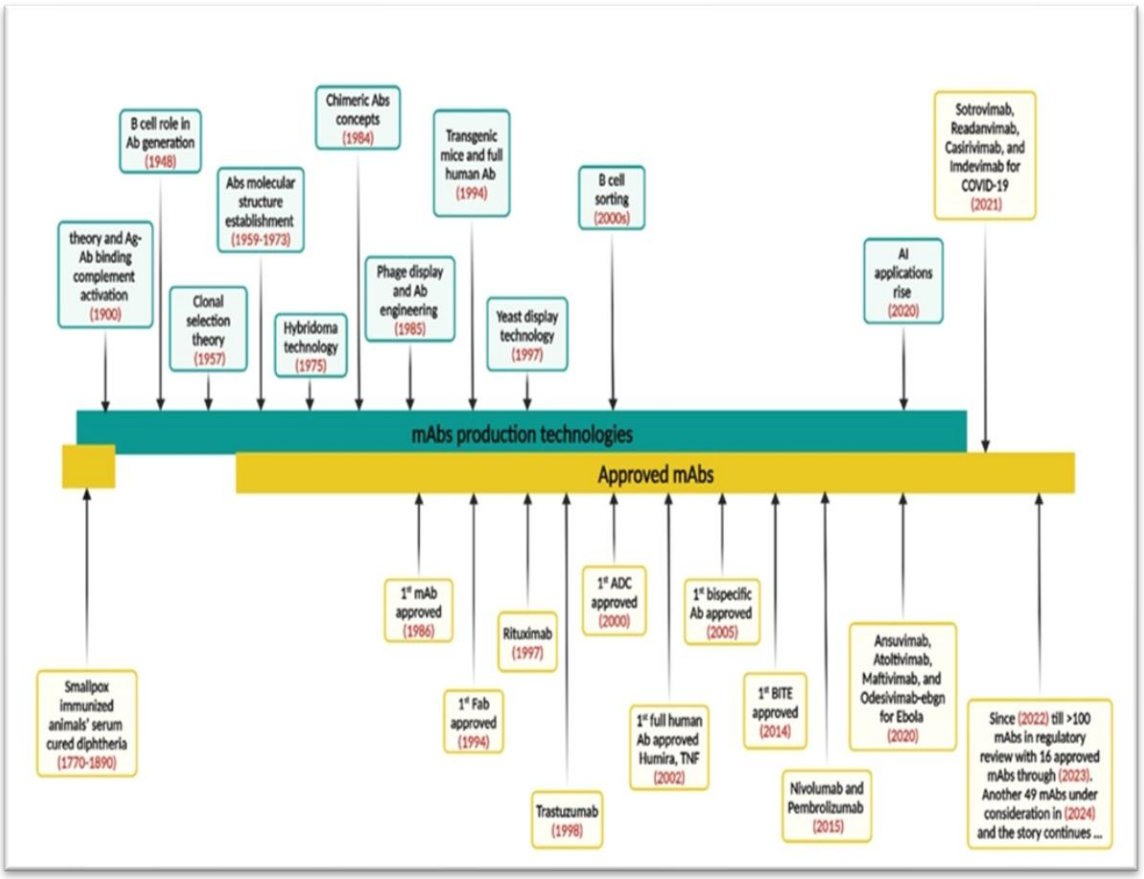


Figure 20 : Evolutionary Timeline of mAb Production Technologies and Key Regulatory Approvals (Aboul-Ella et al., 2024)

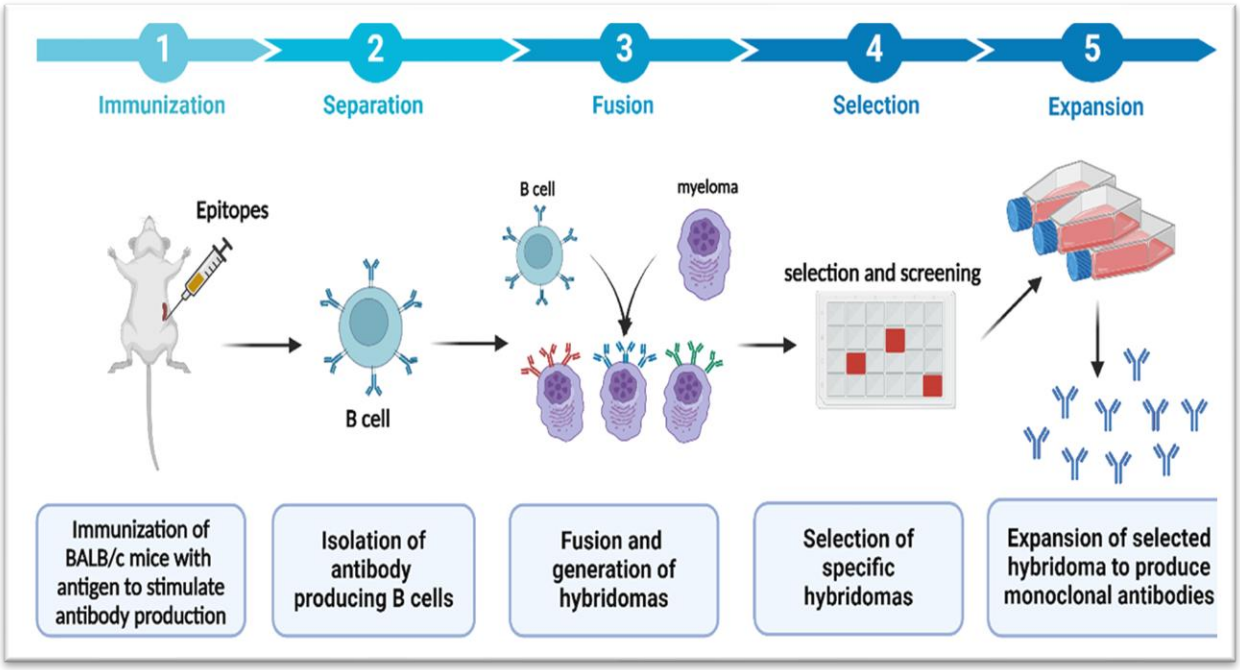


Figure 21 : Schematic Representation of the Sequential Workflow of Hybridoma Technology for Monoclonal Antibody Production (Aboul-Ella et al., 2024)

First:

II.2.1. Hybridoma Technology (Overview):

Hybridoma technology is based on the fusion of antibody-producing B cells with immortal myeloma cells to form hybridomas capable of continuous monoclonal antibody production. After immunization of a mouse with a specific antigen, spleen-derived plasma cells are fused with myeloma cells using polyethylene glycol (PEG). The resulting cells are cultured in HAT medium, where only fused hybridomas survive.

The selected hybridomas proliferate and form clones producing identical antibodies. These clones are screened, commonly using ELISA, to identify those with high specificity toward the target antigen. Positive clones are expanded in suitable culture media to produce large quantities of monoclonal antibodies.

Finally, antibodies are purified using techniques such as affinity chromatography or ion-exchange chromatography. Improvements in hybridoma technology, including optimized fusion methods and the use of non-secreting myeloma cells, have enhanced antibody yield and efficiency (Saleem et al., 2025).

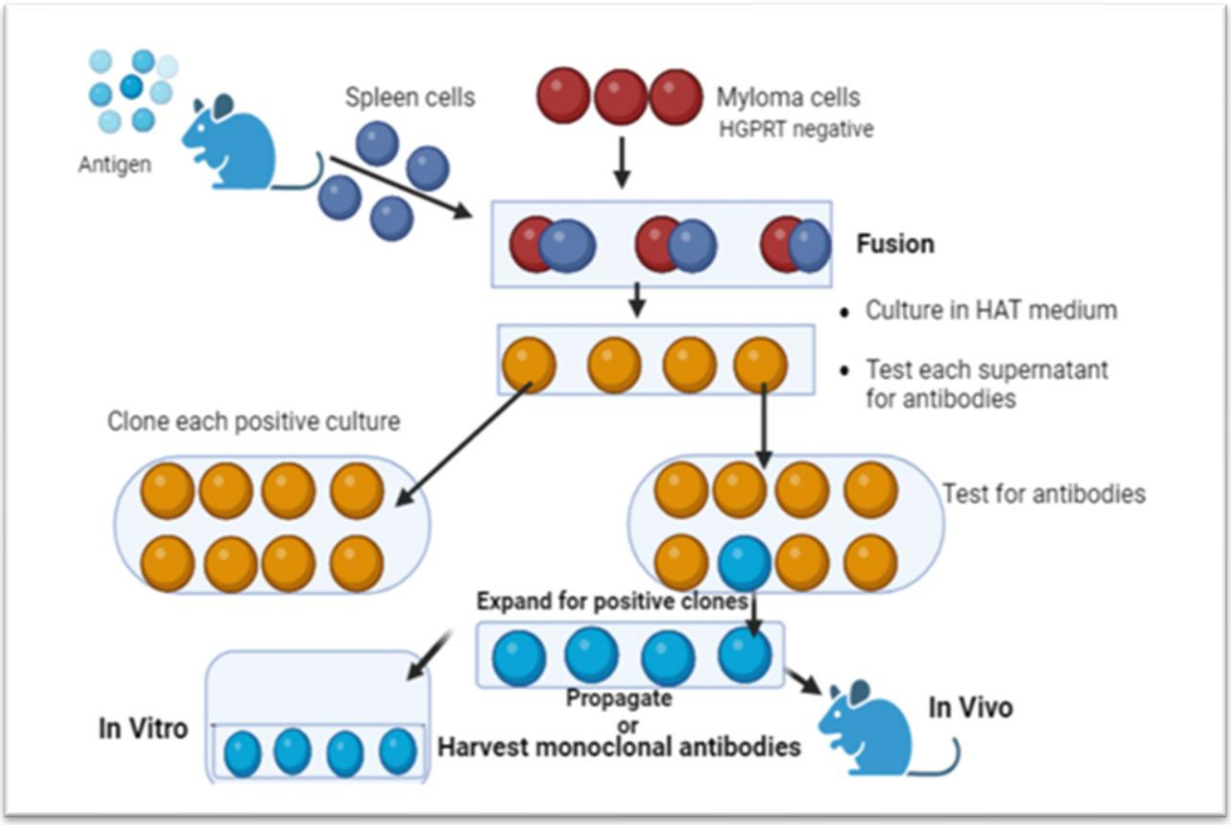


Figure 22 : Schematic diagram showing the sequential steps involved in hybridoma technology for the production of monoclonal antibodies (Saleem et al., 2025).

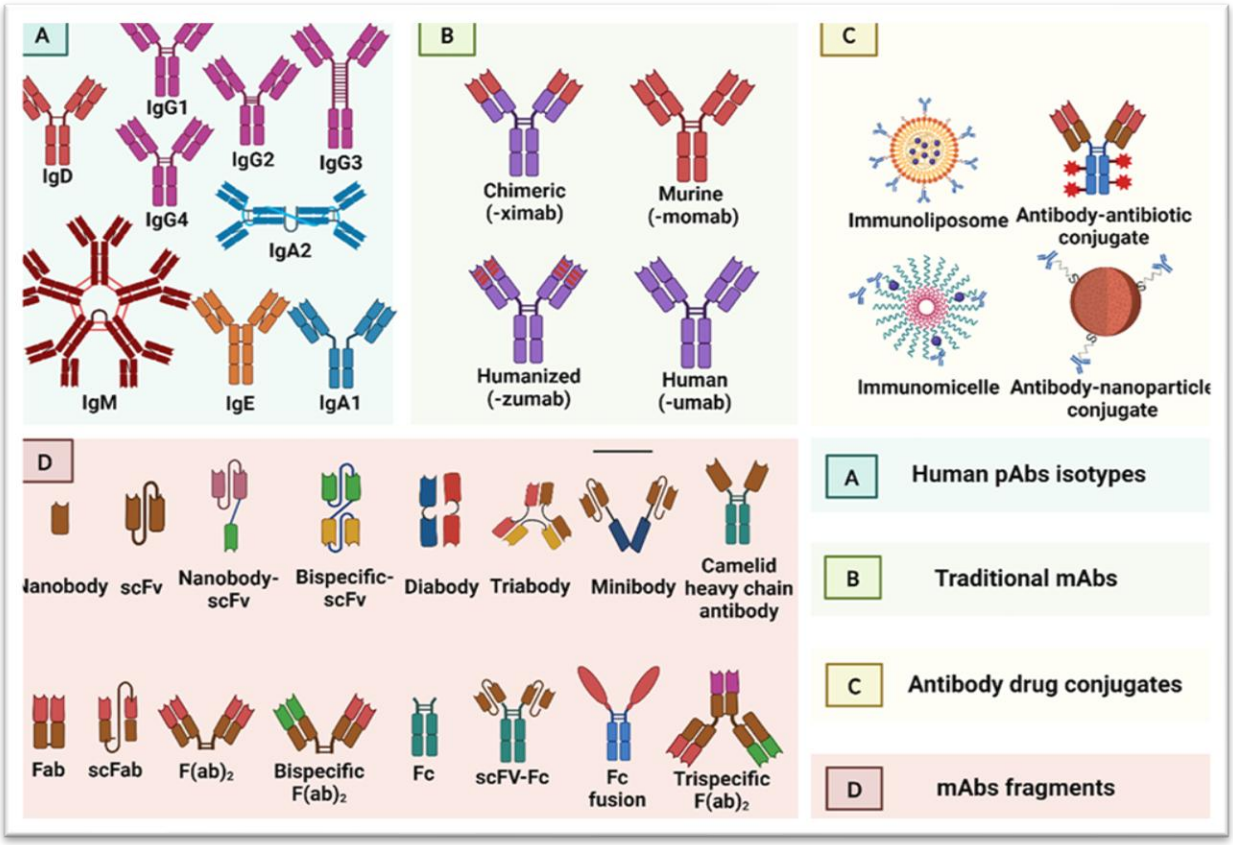


Figure 23 : Comparative Schematic Representation of the Structural Similarities and Differences Among Antibody Classes and Subtypes (Aboul-Ella et al., 2024)

II.2.1.1. Mouse Hybridomas:

Mouse hybridomas are widely used due to robust antibody production, ease of handling, and structural similarity to human antibodies. They support reproducible, long-term mAb production and have enabled therapeutics like OKT3 for organ transplantation (Parray et al., 2020).

II.2.1.2. Rabbit Hybridomas:

Rabbit hybridomas generate high-affinity antibodies with broad epitope recognition, including targets poorly immunogenic in rodents. Stable production was achieved using hetero-hybridoma approaches and specialized fusion partners such as 240E-W, making them valuable for research, diagnostics, and therapeutic applications (Parray et al., 2020).

II.2.1.3. Human Hybridomas:

Human hybridomas allow direct production of fully human antibodies, ideal for therapeutics. Challenges include low B cell fusion efficiency and scarcity of antigen-specific cells, mitigated by

EBV transformation or CpG stimulation to enhance hybridoma yield. These approaches have enabled antibodies against HIV, SARS-CoV, and other pathogens (Parray et al., 2020)

II.2.1.4. Chicken (Avian) Hybridomas:

Chicken hybridomas exploit phylogenetic distance to generate antibodies against conserved mammalian targets. IgY antibodies are abundant in serum and egg yolk, and transgenic chickens expressing human immunoglobulin loci allow direct production of human-compatible mAbs, though phage display is often preferred due to limited fusion partner availability (Parray etal.,2020)

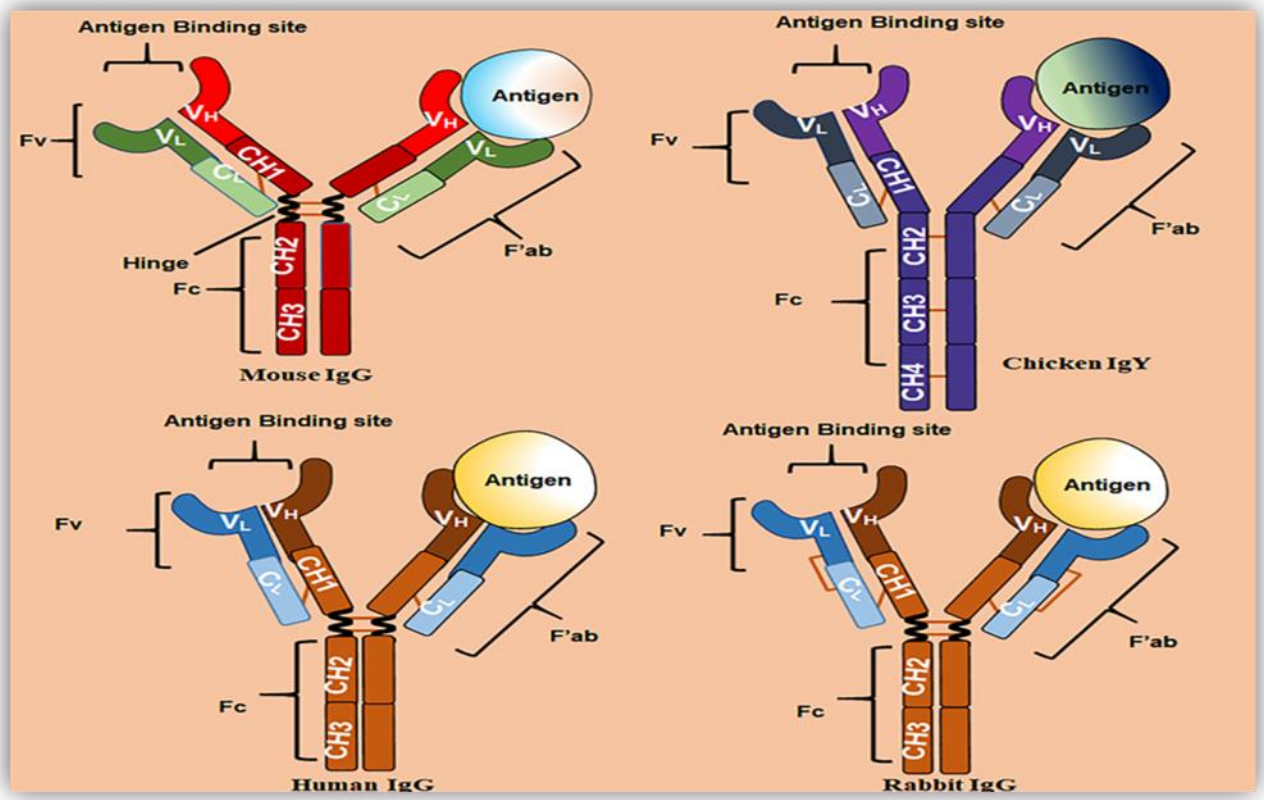


Figure 24 : Comparative Schematic Representation of Natural IgG Antibodies in Rabit, Mouse, Chicken, and Human (Parray et al,2020)

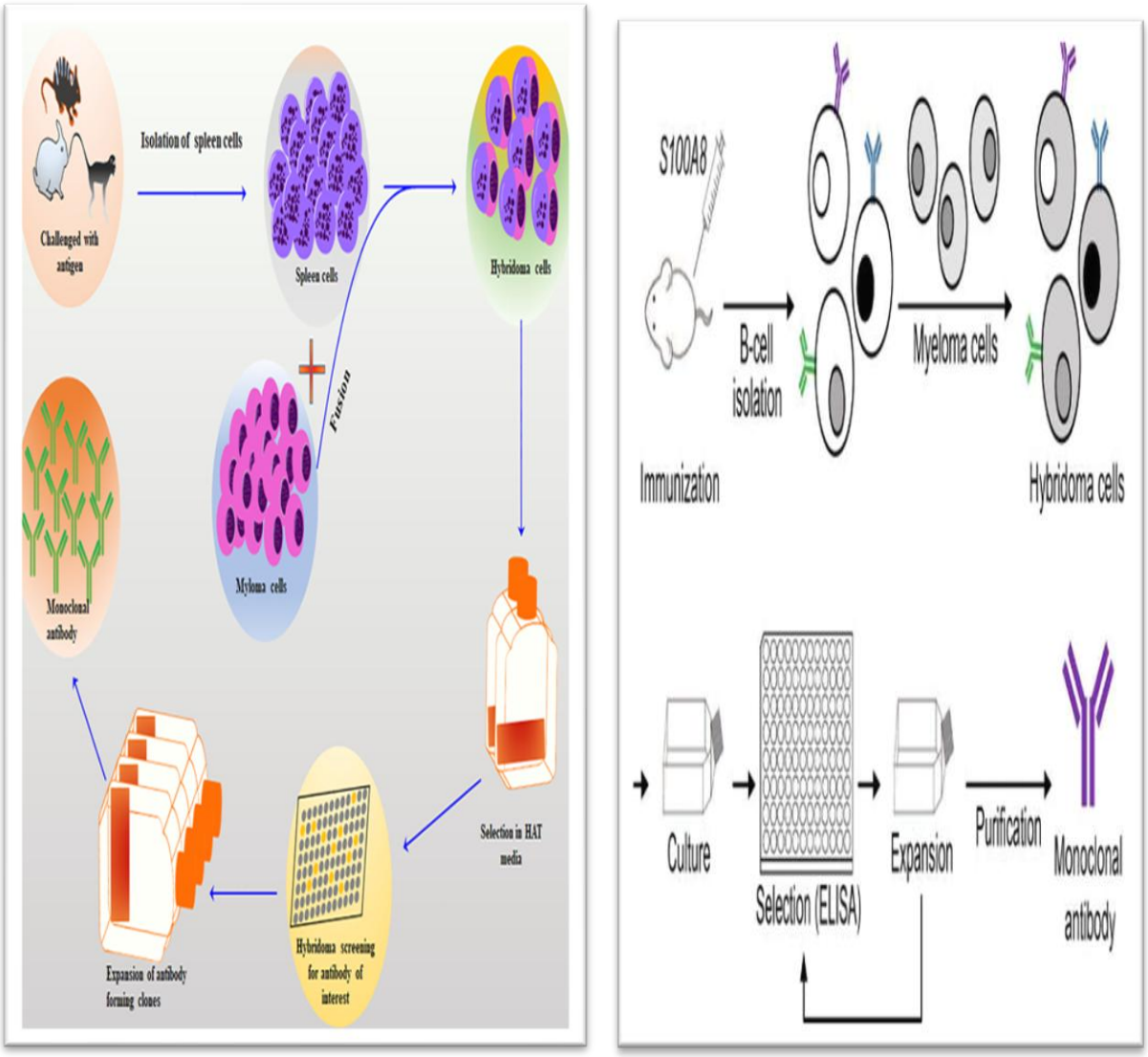


Figure 25 : Schematic of Monoclonal Antibody Production Using Hybridoma Technology. Laboratory animals are immunized with a target antigen, followed by fusion of antigen-specific B lymphocytes with immortal myeloma cells. Hybridoma cells are selected in HAT medium, and clones secreting the desired monoclonal antibodies are subsequently screened and expanded. (Parray et al,2020) and (Khalaf, Abdel Razaaq, & Hamad, 2025)

II.2.1.5. Steps of Hybridoma Technology

The process illustrated in the figure involves several key steps.

First, the experimental animal is immunized with the target antigen to stimulate the production of antigen-specific B lymphocytes. Once a sufficient immune response is achieved, spleen cells are collected and fused with myeloma cells using polyethylene glycol (PEG), resulting in the formation of hybridoma cells.

The fused cells are then cultured in HAT medium, where only hybridomas survive, while unfused cells die. These hybrid cells are subsequently screened, commonly using ELISA, to identify those producing the desired antibodies.

Positive hybridomas are then cloned using techniques such as limiting dilution to ensure monoclonality. Finally, selected clones are expanded for large-scale antibody production and may be further characterized and preserved for long-term use (Sherine et al., 2023).

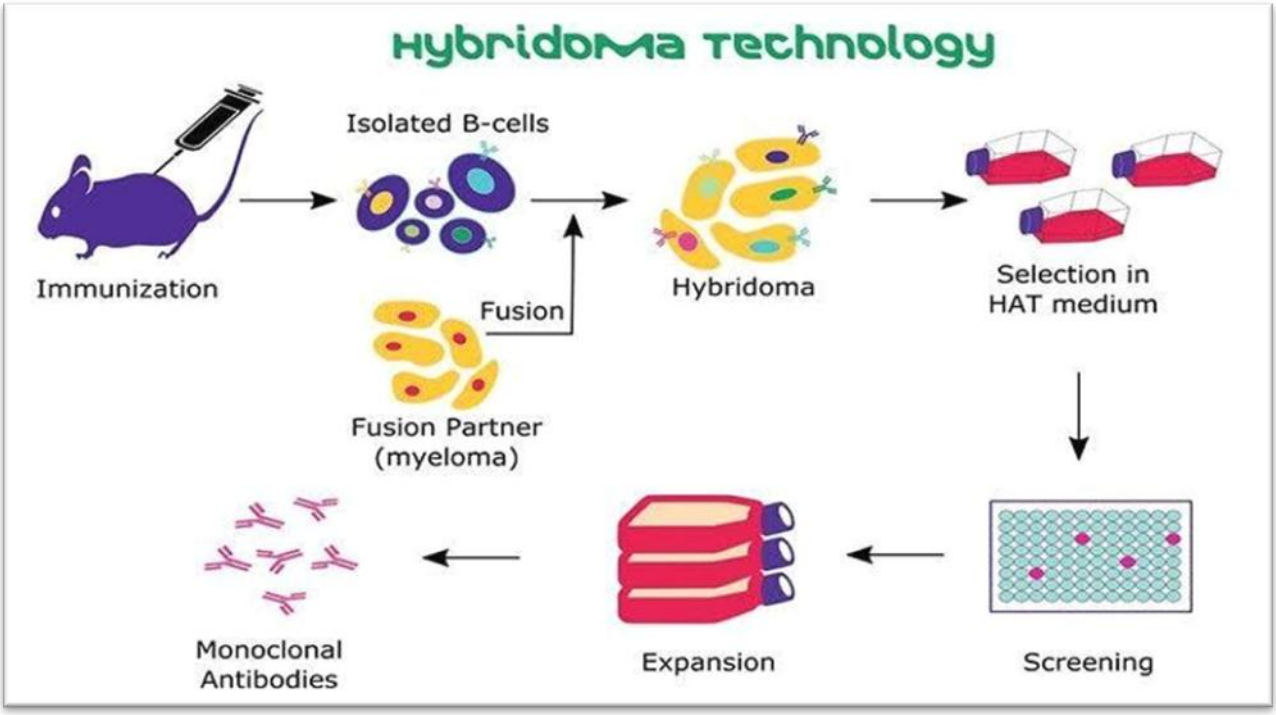


Figure 26 : Schematic representation of the main steps involved in hybridoma technology for monoclonal antibody production. (Sherine et al, 2023).

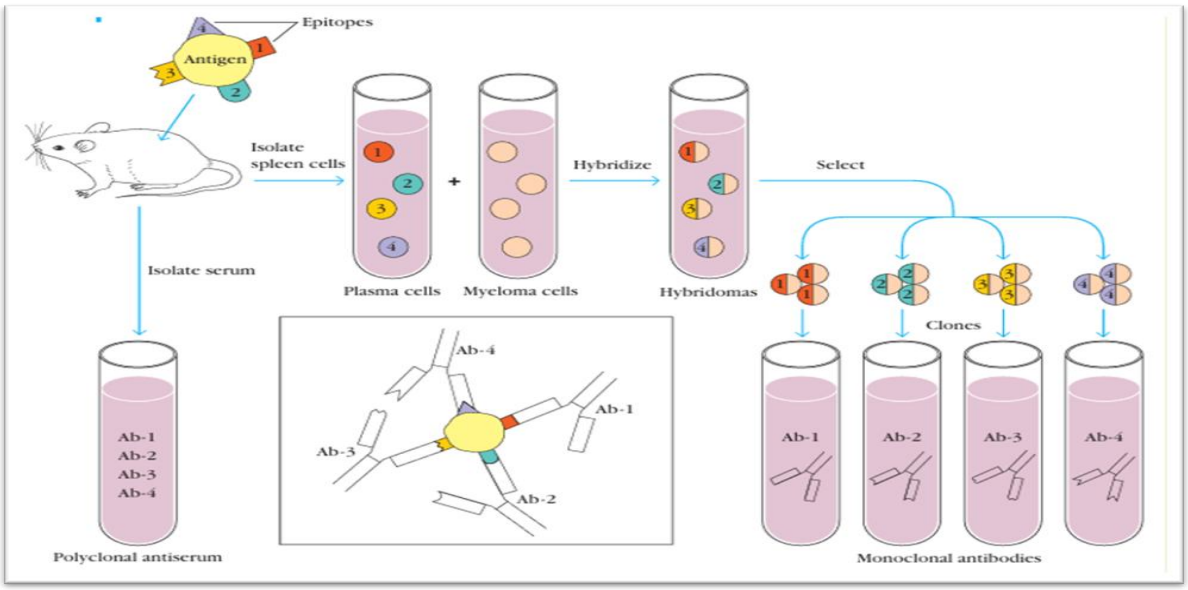


Figure 27 : Differentiation between multi-clonally derived antisera and single-clone monoclonal antibody production (Goldsby et al., 2003)

Second

II.2.2. Phage Display

Phage display is a technique first developed by George P. Smith in 1985. It is used to produce fully human antibodies in the laboratory without the need for animal immunization. For this reason, it is considered an important alternative to hybridoma technology (Sadeghalvad & Rezaei, 2021; Sherine et al., 2023).

This method works by displaying small parts of antibodies on the surface of viruses called bacteriophages, especially M13. These antibody fragments are genetically linked to proteins on the phage surface, which allows them to interact directly with specific antigens. One of the main advantages of this technique is the connection between the displayed antibody (protein) and its genetic information (DNA). This makes it easy to identify and select the antibodies that bind best to the target. In addition, very large libraries containing billions of variants can be tested efficiently.

The selection of specific antibodies is done through several repeated steps known as bio-panning. These steps include binding to the target antigen, washing away non-specific phages, recovering the bound ones, and then amplifying them in *Escherichia coli* cells (Chan et al., 2017).

Unlike antibodies produced by hybridoma technology, which may cause immune reactions such as HAMA, phage display allows the direct production of fully human antibodies, reducing

this risk. In this technique, antibody fragments such as scFv and Fab are commonly used because they are easy to produce in bacteria. However, when these fragments are converted into full antibodies, their binding ability may sometimes change (Chan et al., 2017).

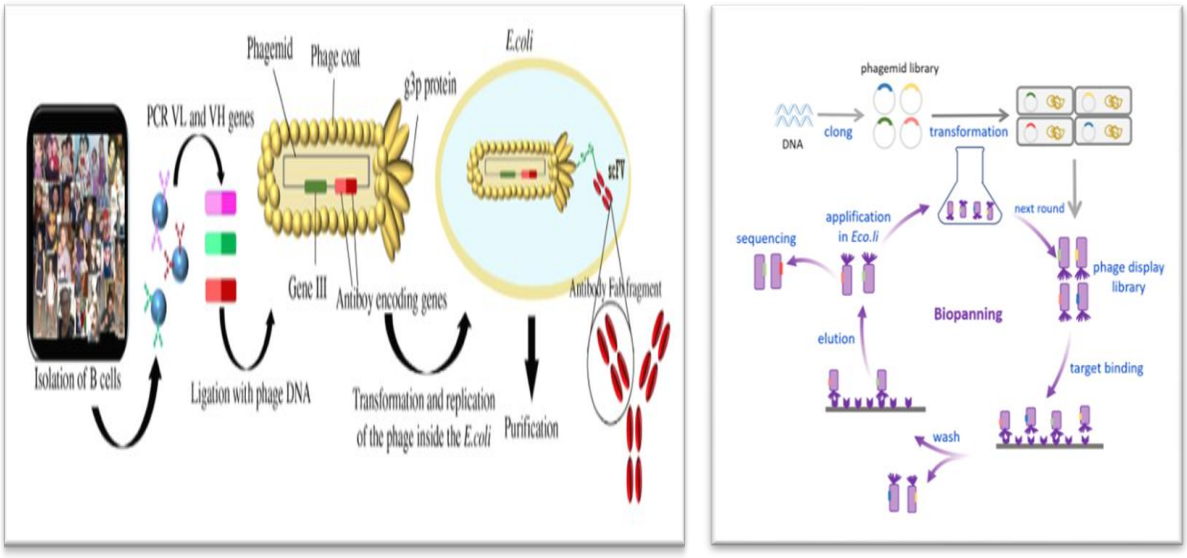


Figure 28 : Schematic and Diagram Representation of the Phage Display Technique (Büyükköroğlu & Şenel, 2018) & (Wu et al., 2025)

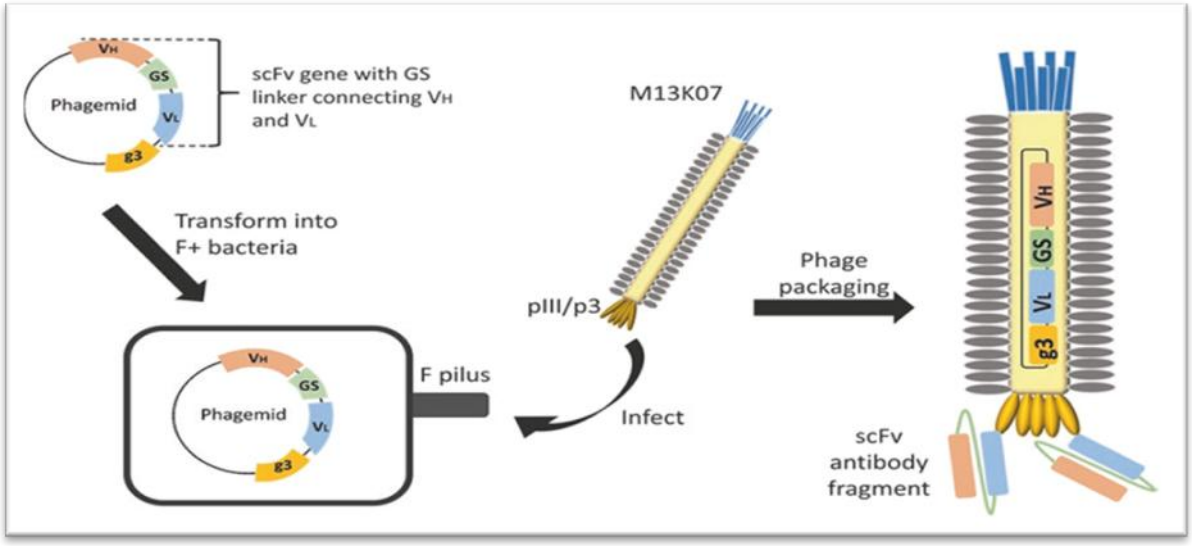


Figure 29 : Phage Display of scFv Antibodies Using Phagemid and Helper Phage (Chan et al., 2017).

The scFv gene is fused to geneIII in a phagemid, introduced into E. coli with the F pilus, and packaged with the assistance of a helper phage (M13K07), which provides all the proteins necessary for phage assembly and display.

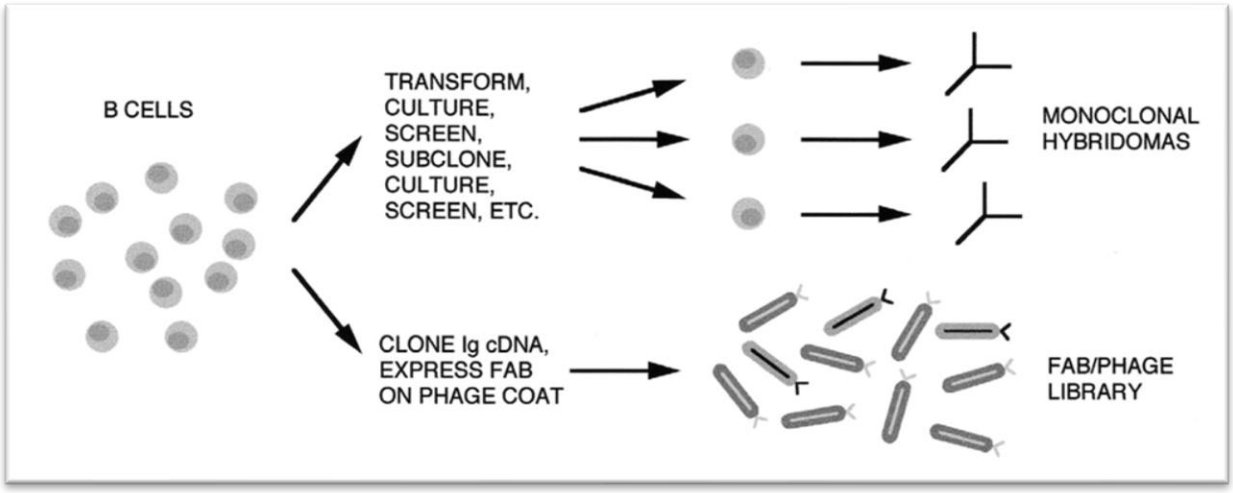


Figure 30 : Schematic Comparison of Cellular and Phage Display Technologies in Monoclonal Antibody Generation (Siegel, 2002)

II.2.3. Production of Antibodies in Transgenic Animals

The production of human antibodies in transgenic animals represents an advanced biotechnological approach. According to Saleem et al. (2025), this process involves several key steps including gene inactivation, gene insertion, immunization, and antibody purification.

First, the endogenous immunoglobulin genes of the animal are inactivated to prevent interference with human antibody expression. Then, human immunoglobulin genes are introduced into the animal genome using genetic engineering techniques.

After that, the transgenic animals are immunized to stimulate antibody production. The antibodies are collected from blood and purified using standard purification methods. Finally, these antibodies are validated and can be used for therapeutic and commercial applications (Saleem et al., 2025).

Importance of Animals Used in Antibody Production:

- Cows are used for large-scale production of antibodies.
- Rabbits are preferred for their low cost and high efficiency.
- Chickens are useful for conserved antigens.
- Pigs are less commonly used due to ethical limitations. (Saleem et al., 2025)

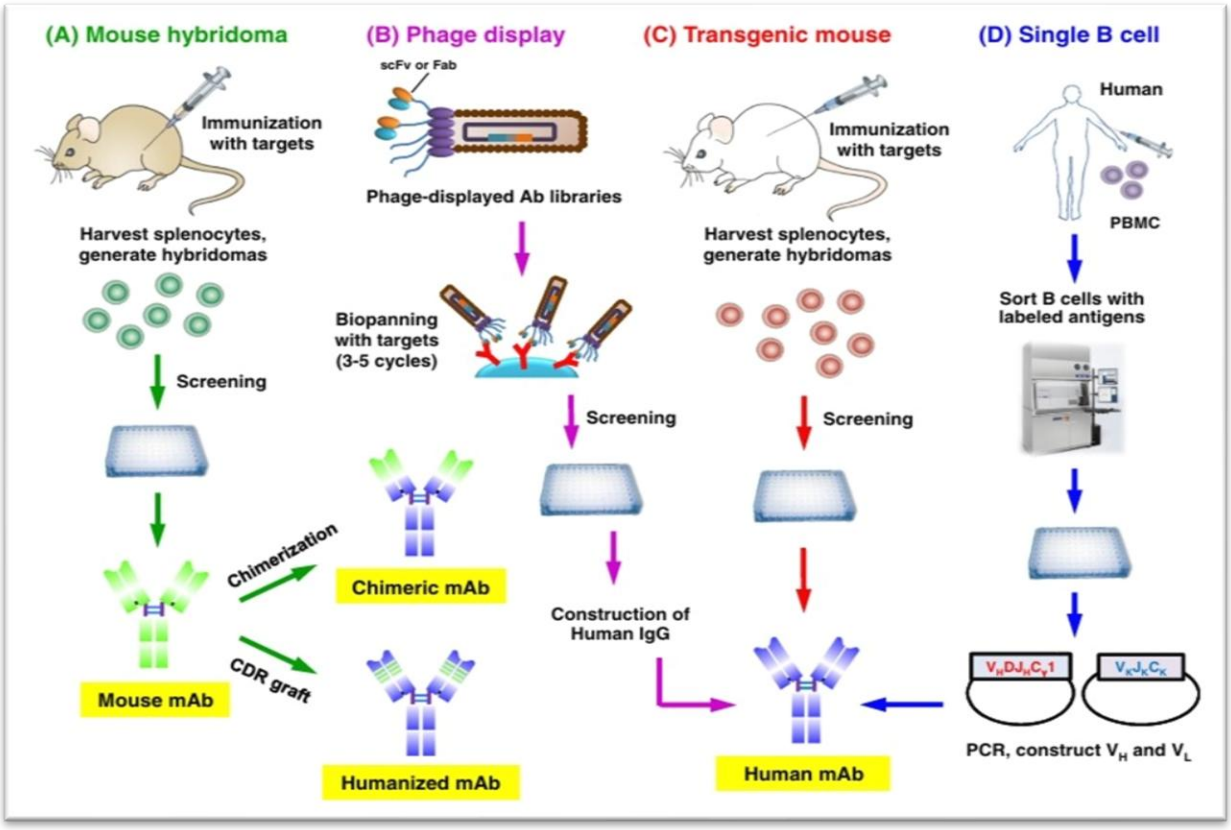


Figure 31 : Schematic Representation of Different Approaches for Therapeutic Antibody Development (Lu et al., 2020)

II.3. Recombinant Antibodies and Molecular Engineering

II.3.1. Definition and Fundamental Concepts

Recombinant antibodies (rAbs) are advanced immunoglobulin proteins synthesized by cloning specific antibody genes into highly efficient expression vectors (Trimmer, 2020). Unlike traditional monoclonal antibodies derived from hybridomas, rAbs are produced in various host cell systems, ensuring a reliable and unlimited supply (Saleem et al., 2025). This genetic approach allows researchers to digitally archive antibody DNA sequences, making global distribution easy and removing the risks of hybridoma cell line instability or chromosome loss over time (Trimmer, 2020). Furthermore, the recombinant nature of these molecules provides a broad platform for molecular engineering, allowing scientists to customize their physical and pharmacological properties for specific diagnostic or therapeutic uses (Basu et al., 2019).

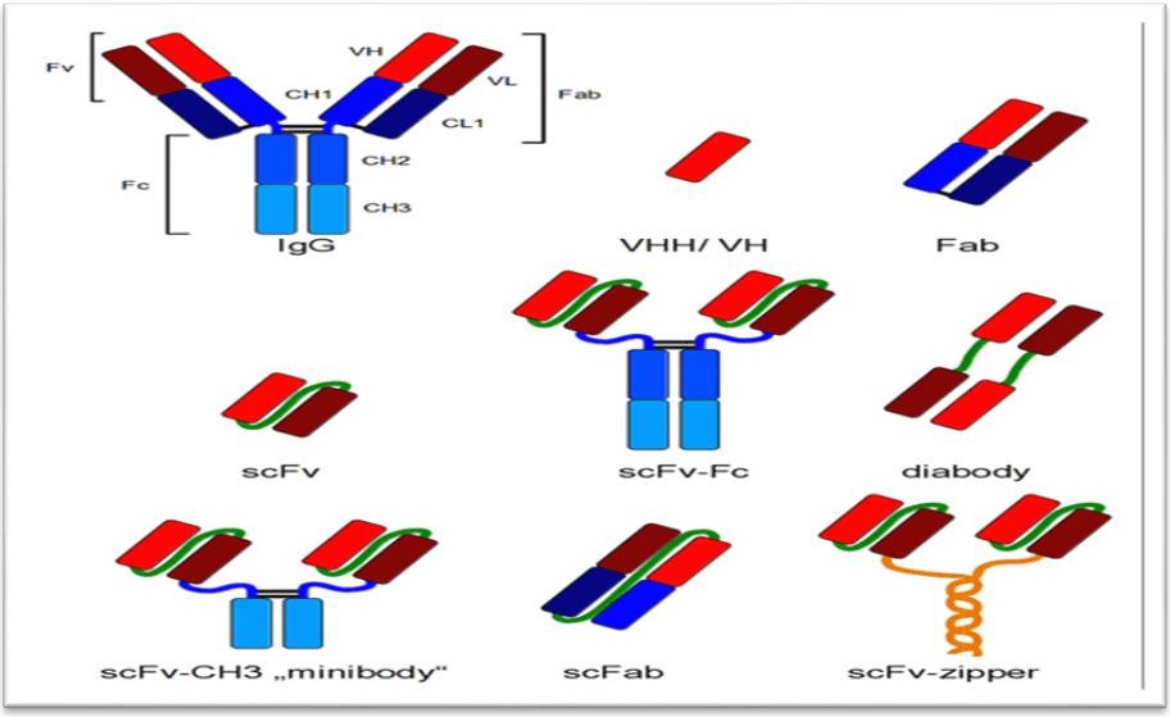


Figure 32 : Advancements in Recombinant Antibody Technology for Targeted Applications in Neuroscience. (Trimmer, 2020)

II.3.2. The Production Pipeline:

From Gene Isolation to Host Expression

Generating recombinant antibodies follows a strict molecular process that starts with isolating the genes that code for the antibodies (Trimmer, 2020). This involves extracting mRNA and turning it into cDNA, followed by amplifying the variable heavy (VH) and light (VL) chain domains using the polymerase chain reaction (PCR) (Siegel, 2002).

These genetic fragments are then inserted into display platforms, most commonly phage display (Kuhn et al., 2016).

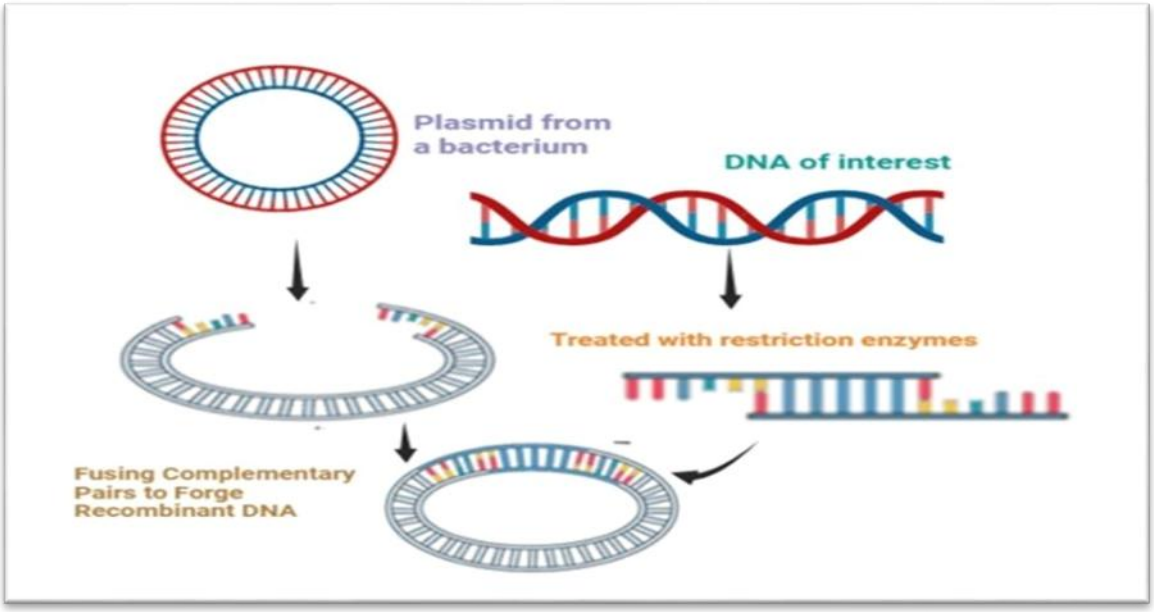


Figure 33 : Recombinant antibody formats and their structural variations. (Saleem et al., 2025)

In this method, antibody fragments are displayed on the surface of bacteriophages to quickly screen and select them (a process called "bio-panning") against specific target antigens (Basu et al., 2019).

This lab-based (in vitro) selection bypasses the limits of animal immune systems, making it possible to isolate antibodies against toxic, non-immunogenic, or highly conserved targets (Kuhn et al., 2016). Once selected, the optimized gene sequences are put into expression hosts (Frenzel et al., 2013).

These range from simple prokaryotic systems like *Escherichia coli* for smaller antibody fragments, to complex eukaryotic mammalian cells (like CHO or HEK293) for full-length IgGs that require precise post-translational modifications (Saleem et al., 2025; Frenzel et al., 2013).

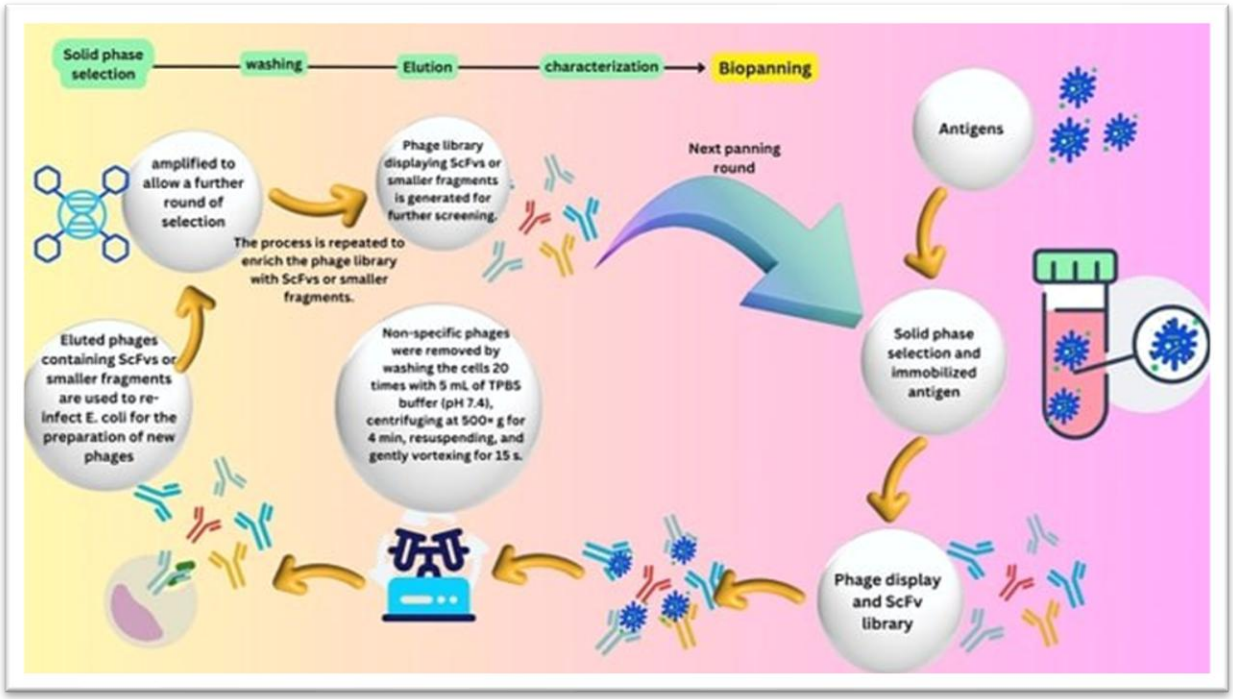


Figure 34 : From library to ligand: Navigating the iterative bio-panning rounds for scFv selection (Saleem et al., 2025)

II.3.3. Molecular Engineering Strategies and Structural Formatting

A primary advantage of recombinant technology is its inherent modular flexibility, which enables the precise modification of antibody architecture to suit specific research or clinical needs (Trimmer, 2020). Through molecular engineering, full-length antibodies can be "reformatted" into significantly smaller constructs, such as single-chain variable fragments (scFv), (Fab) fragments, or single-domain antibodies (nanobodies) (Saleem et al., 2025).

The reduced molecular weight of these fragments enhances their pharmacokinetic profile, allowing for superior tissue penetration and the ability to access cryptic epitopes that are often sterically hindered from larger IgG molecules (Carter & Lazar, 2018).

Beyond size reduction, sophisticated design strategies such as the "knobs-into-holes" (KiH) model are employed to construct bispecific antibodies (bsAbs). These engineered proteins possess the dual capacity to engage two distinct targets or facilitate cellular cross-linking simultaneously (Borrelli et al., 2025).

Furthermore, Fc-engineering serves as a critical tool for modulating effector functions. By introducing specific mutations in the Fc region, researchers can either amplify or silence immune

responses—such as antibody-dependent cellular cytotoxicity (ADCC)—or extend the molecule's serum half-life by optimizing its interaction with the neonatal Fc receptor (FcRn) (Jefferis, 2009).

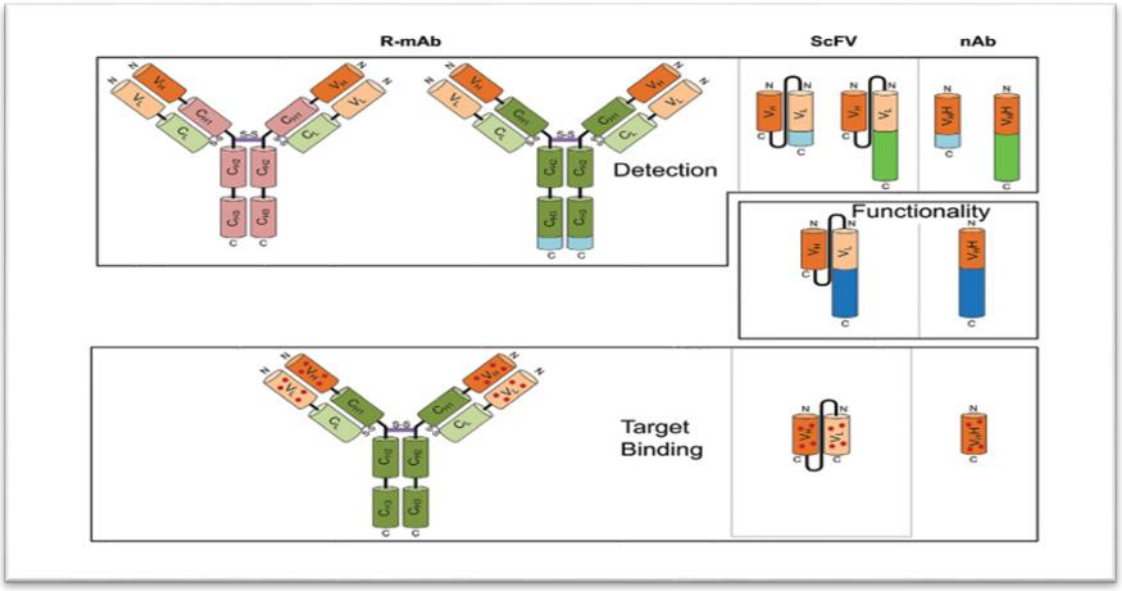


Figure 35 : Modular Engineering Strategies for Recombinant Antibody Formats to Optimize Detection, Functionality, and Antigen Binding. (Trimmer, 2020)

II.3.4. Advantages and Clinical Utility

- Recombinant antibody platforms provide significant advantages over traditional production methods, particularly in terms of molecular precision and reproducibility. (Wei et al., 2026; Trimmer, 2020).
- Their sequences are fully defined at the DNA level, enabling accurate identification and characterization (Wei et al., 2026; Trimmer, 2020).
- This eliminates variability associated with animal-derived antibody production systems. (Basu et al., 2019).
- They enable in vitro affinity maturation, where targeted mutations are introduced to improve antigen-binding strength. (Basu et al., 2019).
- This process can generate antibodies with affinities exceeding those produced by the natural immune system (Basu et al., 2019).

- Recombinant antibodies can be engineered for multifunctional applications, enhancing their clinical utility:

- Conjugation with cytotoxic agents leads to the development of antibody-drug conjugates (ADCs) for targeted therapy. (Carter & Lazar, 2018).
- Fusion with detection elements enables their use in high-sensitivity diagnostic platforms (Carter & Lazar, 2018).

- The digital nature of antibody sequences ensures long-term reliability and scalability:
  - Once a high-affinity antibody is identified, it can be reproduced globally with consistent quality. (Trimmer, 2020).
  - This avoids issues such as genetic drift or loss of production efficiency seen in traditional systems (Trimmer, 2020).

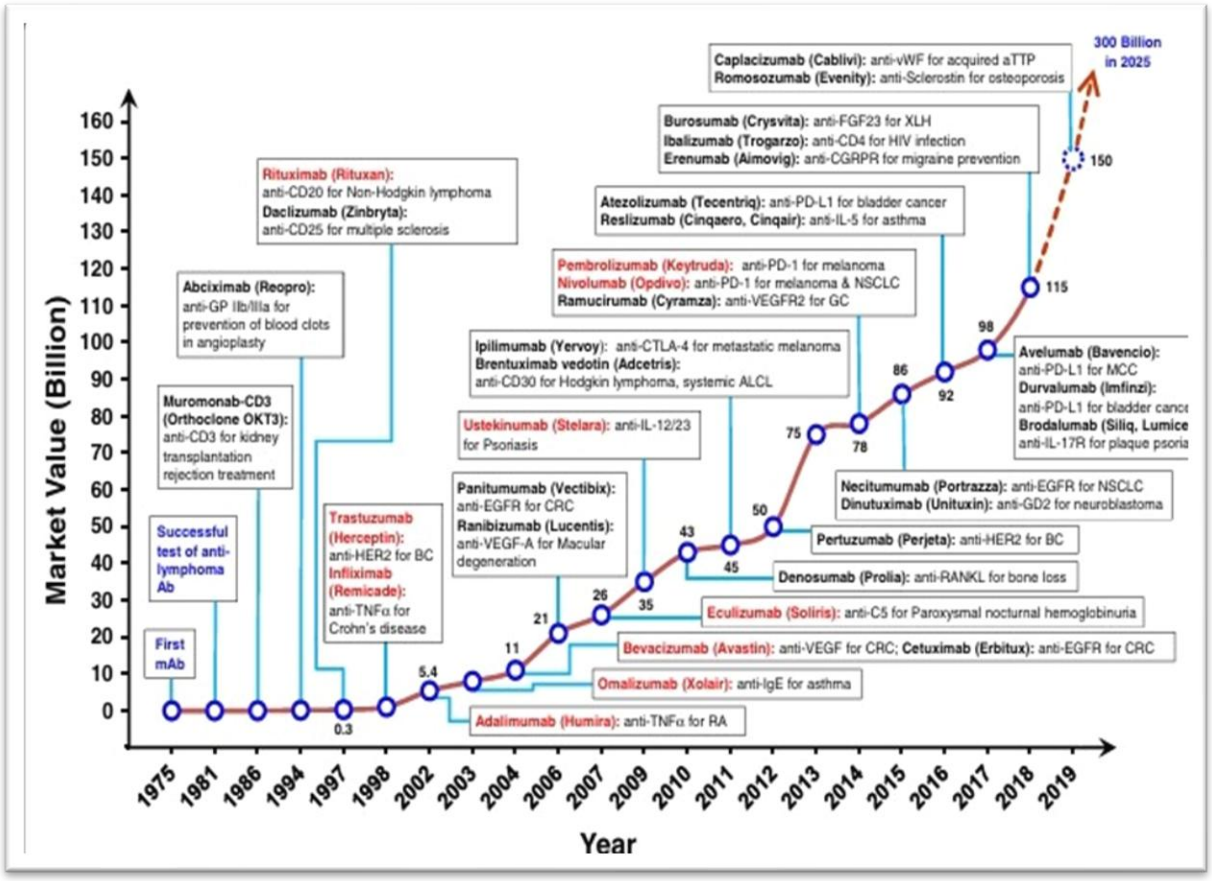


Figure 36 : The trajectory of therapeutic antibody development, illustrating the transition from early production to advanced engineered formats (e.g., ADCs, Bispecifics) and their subsequent clinical and economic impact. **(**Lu et al., 2020).

Table 3: Biotechnological and Therapeutic Applications of Diverse Recombinant Antibody Formats (Saleem et al., 2025) .

Antibody Format	Clinical and Biotechnological Applications	References
ScFv	Hepatocellular carcinoma therapy; Anti-BCL1 lymphoma DNA vaccination; Structural and functional proteomics; Development of self-assembling monolayer antibody arrays.	Petersson et al. (2023) Petersson et al. (2023)
Multivalent scFv	Generation of carbohydrate-specific antibodies.	Ahamadi-Fesharaki et al. (2019)
Fab	Inhibition of platelet aggregation (anti-platelet IIb/IIIa); Neutralization of Shiga Toxin 1; Catalytic antibody exploration in retro-Diels–Alder reactions.	Schneider (2011); Yang et al. (2010)
DsFv	Production of recombinant immunotoxins targeting mesothelin; Treatment of drug-resistant hairy-cell leukemia via CD22 targeting.	Kreitman and Pastan (2020); Kreitman et al. (2005)
Minibody	Anti-idiotypic antibody development for cancer immunotherapy.	Shi et al. (2007)
Bispecific minibody	Therapeutic intervention for thrombocytopenia; Induction of tumor lysis.	Brinkmann and Kontermann (2017)
IgG	Neutralizing activity against Hepatitis B surface antigen (HbsAg).	Shiraishi et al. (2023)
IgG, IgE	Isolation of allergen-specific Fab fragments (e.g., birch pollen) from combinatorial libraries.	Eckl-Dorna et al. (2019)

Nanobodies (VHHs)	Advanced diagnostic assays; Therapeutic interventions; Specialized research reagents.	Muyldermans (2021a)
sdAb (Nb V565)	Mitigation of gastrointestinal (GI) infections (bacterial, viral, and parasitic).	Petersson et al. (2023)
M1095	Targeting tumor necrosis factor (TNF) and neutralizing inflammatory cytokines in psoriasis management.	Jin et al. (2023); Svecova et al. (2019)

II.3.5. Challenges and Technical Limitations

Despite their advantages, recombinant antibody production presents several technical and biological challenges, particularly in the selection of an appropriate expression system (Frenzel et al., 2013).

Prokaryotic expression systems (e.g., E. coli):

- **Advantages:** rapid growth and cost-effectiveness. (Saleem et al., 2025).
- **Limitations:** inability to perform complex post-translational modifications, especially glycosylation (Saleem et al., 2025).

This is a critical drawback, as glycosylation is essential for the stability and effector functions of full-length IgG antibodies (Jefferis, 2009).

Mammalian expression systems:

- **Advantages:** ability to produce antibodies with human-like post-translational modifications, including proper glycosylation. (Borrelli et al., 2025; Frenzel et al., 2013).
- **Limitations:** higher production costs and increased risk of viral contamination (Borrelli et al., 2025; Frenzel et al., 2013).

Engineered antibody fragments (e.g., scFvs):

- Offer improved tissue penetration and ease of production.
- However, they suffer from reduced in vivo stability and are rapidly cleared via renal filtration.

- As a result, additional modifications (e.g., PEGylation or Fc fusion) are often required to extend their half-life and therapeutic efficacy

(Carter & Lazar, 2018; Borrelli et al., 2025).

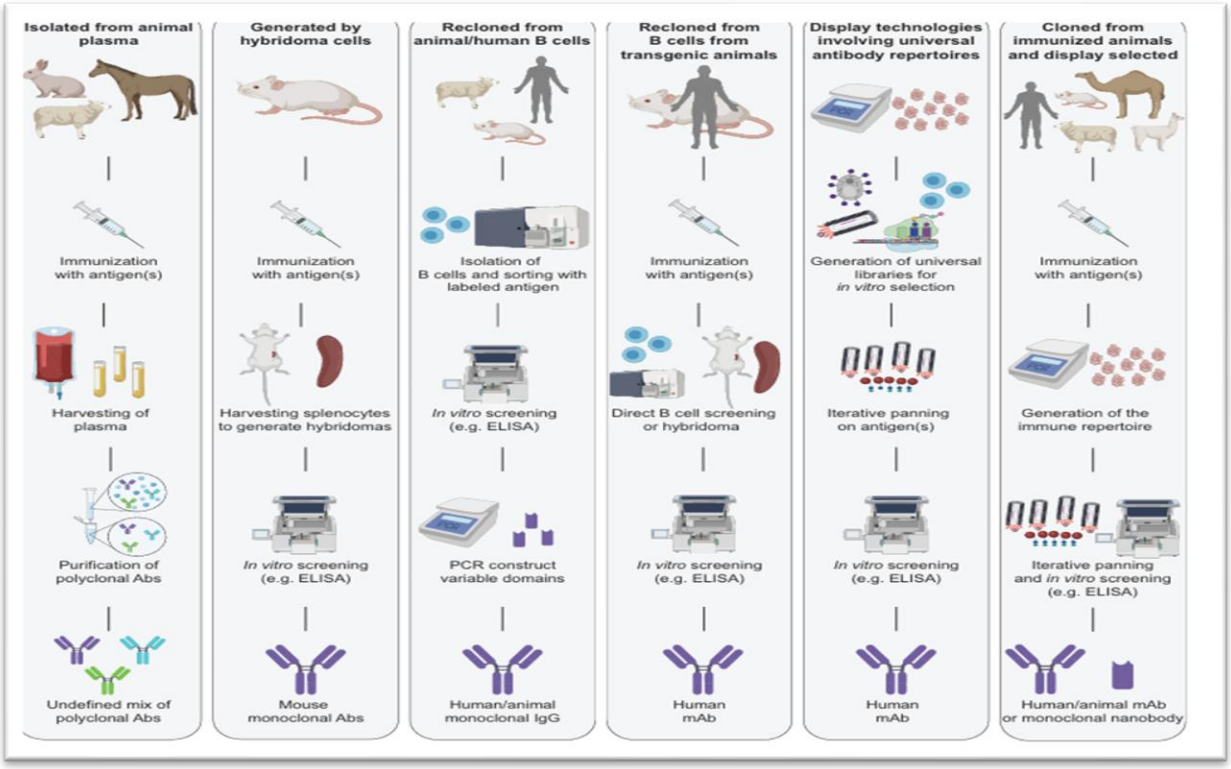


Figure 37 : Methodological Pathways in Antibody Engineering: Comparative Workflows for Polyclonal, Monoclonal, and Recombinant Fragments. (Laustsen et al. (2021)

Table 4: Comparison of Polyclonal, Monoclonal, and Recombinant Antibody Characteristics (Sophia Learning ,2026) and (A. H. Laustsen et al,2021), and (Saleem et al,2025).

Characteristic	**Polyclonal Antibodies (pAbs)**	**Monoclonal Antibodies (mAbs)**	**Recombinant Antibodies (rAbs)**
Production Method	Produced in vivo via animal immunization (e.g., rabbits, goats).	Produced in vitro using hybridoma technology (mouse-derived).	Produced in vitro using display technologies (e.g., Phage Display) and microbial hosts.

Specificity	Recognition of multiple epitopes on a single antigen.	High specificity; targets a single, defined epitope.	Highly programmable; targets specific epitopes with engineered precision.
Cost & Time	Inexpensive and rapid production.	Expensive and time-consuming (hybridoma screening).	Moderate to high initial cost; rapid once the sequence is identified.
Consistency	Batch-to-batch variability in composition and titer.	Continuous and uniform production from stable hybridomas.	Absolute consistency; based on defined genetic sequences.
Cross-Reactivity	High risk of cross-reactivity and false-positive results.	Minimal cross-reactivity due to single-epitope targeting.	Significantly reduced through negative selection during biopanning.
Applications	General diagnostic assays and secondary detection.	Therapeutic interventions and high-precision diagnostics.	Specialized therapies (e.g., scFv, Nanobodies) and advanced bioengineering.

II.4. Expression Systems and Production Optimization

II.4.1. Bacterial and Yeast Expression Systems

Particularly *Escherichia coli*, are widely used for the production of recombinant antibody fragments such as single-chain variable fragments (scFv) and antigen-binding fragments (Fab) (Sandomenico et al., 2020; Pirkalkhoran et al., 2023). The widespread use of *E. coli* is mainly due to its rapid growth rate, well-characterized genetics, and low production cost (Lee & Jeong, 2015).

Despite these advantages, bacterial systems have important limitations. They lack the cellular machinery required for complex post-translational modifications, especially glycosylation, which is essential for the full biological activity of complete IgG antibodies (Yeoh et al., 2026;

Pirkalkhoran et al., 2023). For this reason, *E. coli* is mainly suitable for producing smaller antibody fragments rather than full-length antibodies.

In contrast, yeast expression systems represent an intermediate solution between prokaryotic and eukaryotic systems. They combine the ease of microbial cultivation with the ability to perform certain post-translational modifications, including proper protein folding and secretion. This makes yeast a more suitable and cost-effective platform for producing more complex recombinant antibodies compared to bacterial systems (Meskova et al., 2023).

II.4.1.1. Production in Escherichia coli

The production of recombinant antibodies in *E. coli* involves several key steps.

First, the antibody gene is cloned into an expression vector containing an inducible promoter such as lac or T7. Signal peptides (e.g., pelB) are often included to direct the protein to the periplasm, where proper folding occurs (Frenzel et al., 2013).

The bacterial cells are then cultured in suitable media such as LB or TB. Protein expression is induced by adding IPTG during the exponential growth phase. Lower temperatures are often used to reduce protein aggregation and improve solubility (Sandomenico et al., 2020).

To enhance correct folding, molecular chaperones may be co-expressed. Finally, the recombinant antibodies are extracted and purified using affinity chromatography techniques (Lee & Jeong, 2015).

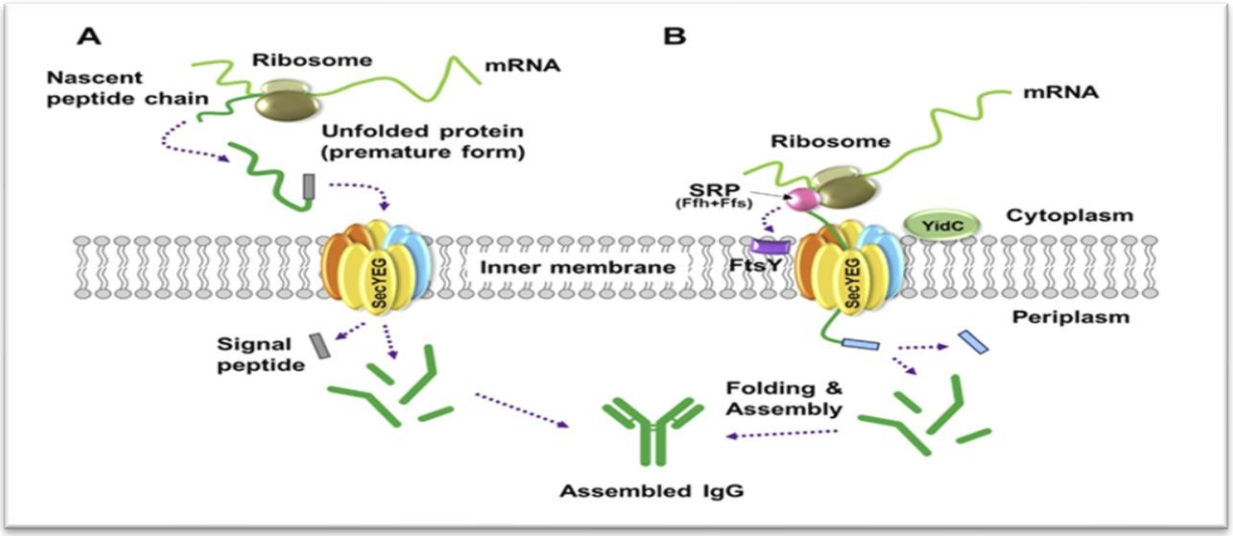


Figure 38 : Antibody secretion and folding strategies in E. coli. (A) Sec-dependent secretion pathway; (B) signal recognition particle (SRP)-dependent secretion pathway (Lee & Jeong, 2015)

II.4.1.2. Production in Yeast

Yeast systems such as *Pichia pastoris* and *Saccharomyces cerevisiae* are widely used for recombinant antibody production due to their ability to perform post-translational modifications. The antibody gene is integrated into the yeast genome under strong promoters such as AOX1 (Lee & Jeong, 2015).

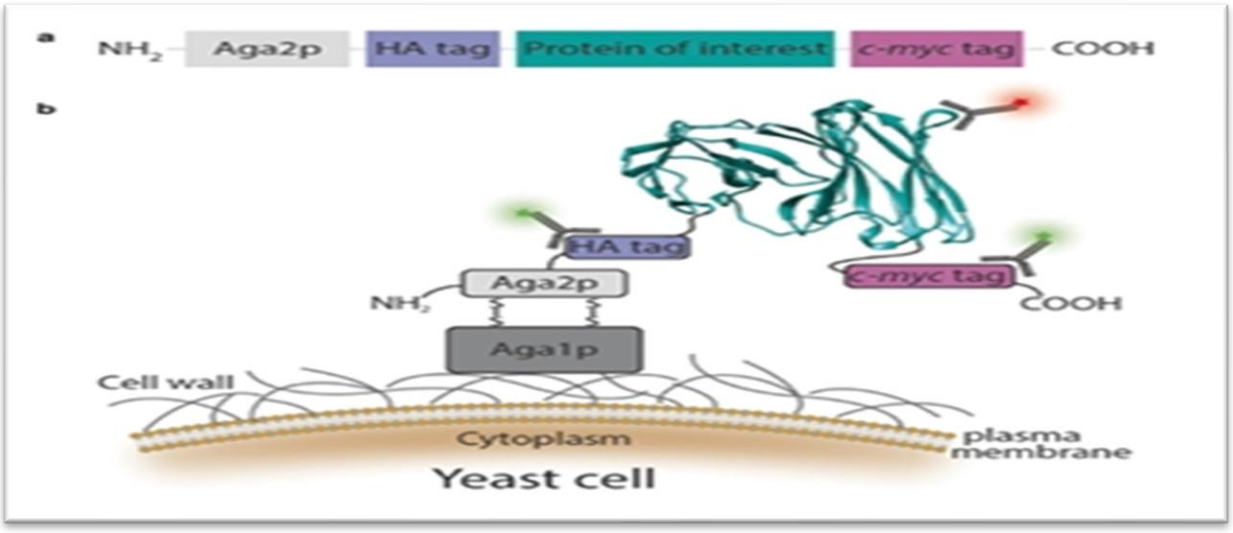


Figure 39 : Schematic representation of the yeast surface display selection cycle (Mustafa et al., 2024).

In some cases, yeast surface display is used to screen antibody libraries and select high-affinity variants. Antibodies are secreted into the culture medium, which simplifies purification. However, yeast glycosylation patterns may differ from human ones, although engineered strains can improve this limitation (Mustafa et al., 2024; Yeoh et al., 2026).

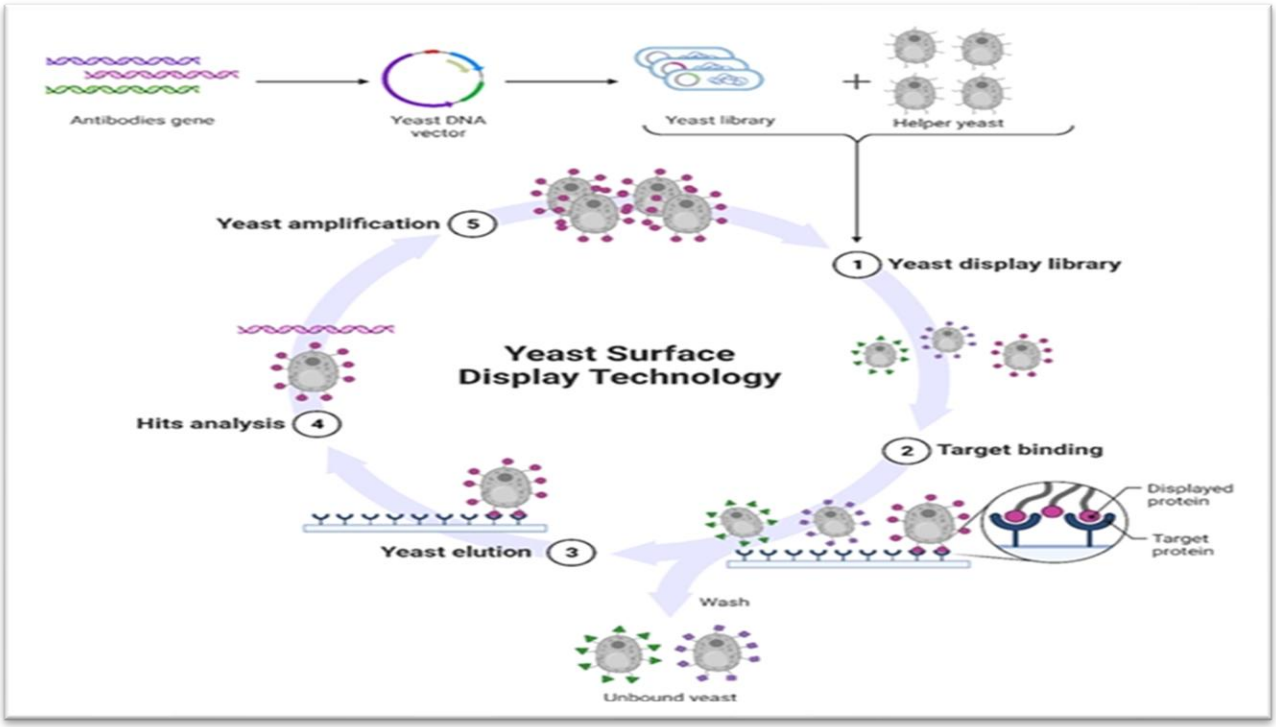


Figure 40 : Molecular architecture of the Aga1p-Aga2p anchoring system on the yeast cell wall (Mustafa et al., 2024).

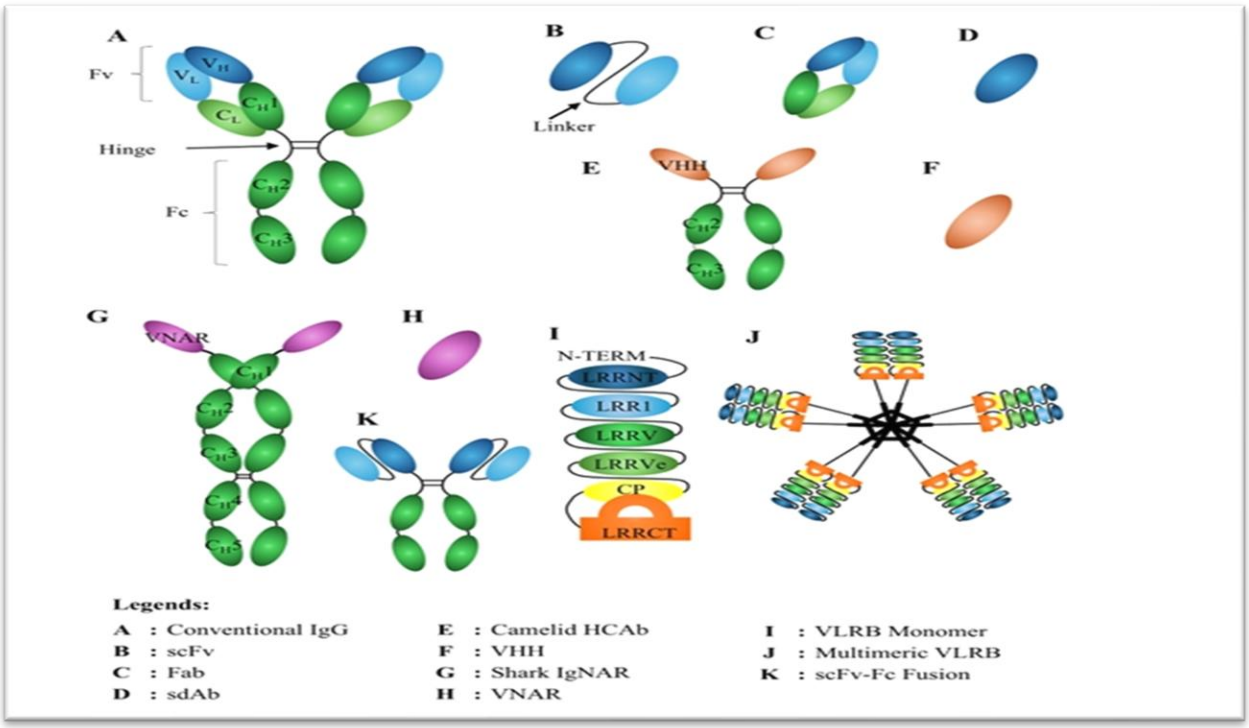


Figure 41 : Comparison of recombinant antibody production and processing between Escherichia coli and yeast expression systems (Yeoh et al., 2025)

II.4.2. Mammalian Cell Systems (CHO, HEK293)

Chinese Hamster Ovary (CHO) cells are considered the "gold standard" in the biopharmaceutical industry, accounting for the majority of therapeutic antibodies approved for clinical use. Their primary advantage lies in their ability to perform human-like **glycosylation**, which ensures the stability of the drug *in vivo* and minimizes the risk of immunogenic rejection (Szkodny & Lee, 2025). Meanwhile, **HEK293** cells are widely utilized in research settings for transient protein expression, allowing for the rapid generation of analytical quantities of proteins. (Szkodny & Lee, 2025)

II.4.3. Comparison of Expression Platforms

The selection of an expression platform is dictated by the final application. While bacterial systems are superior for producing fragments for diagnostic purposes due to their speed, mammalian cells (particularly CHO) remain the nearly exclusive choice for producing full-length **Immunoglobulins (IgGs)** intended for therapy. This ensures biological efficacy, proper effector functions, and immunological safety (Zheng, 2024).

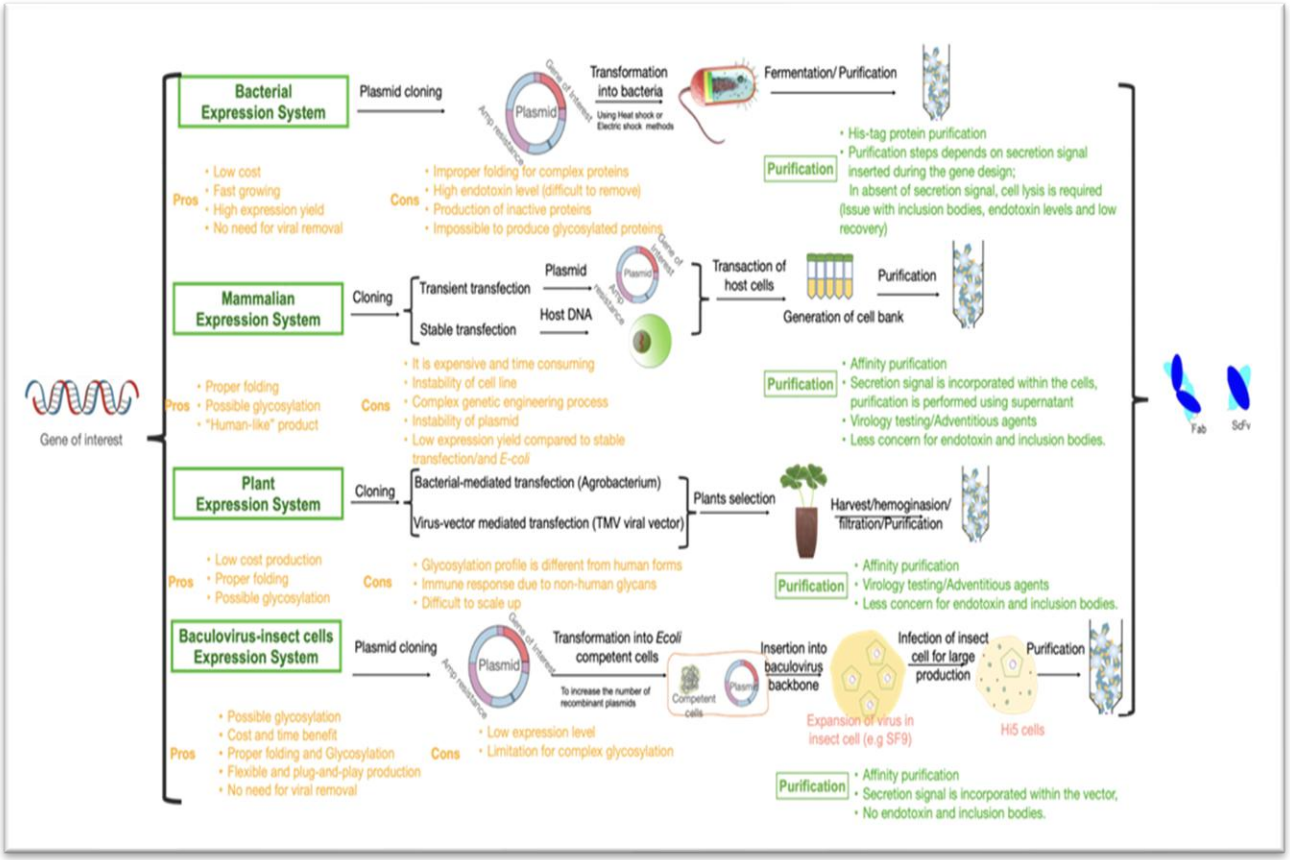


Figure 42 : Challenges and Opportunities Associated with Different Expression Systems for the Bioengineering of Fab and scFv Antibody Fragments. (Pirkalkhoran et al., 2023)

II.5. Antibody Purification Techniques :

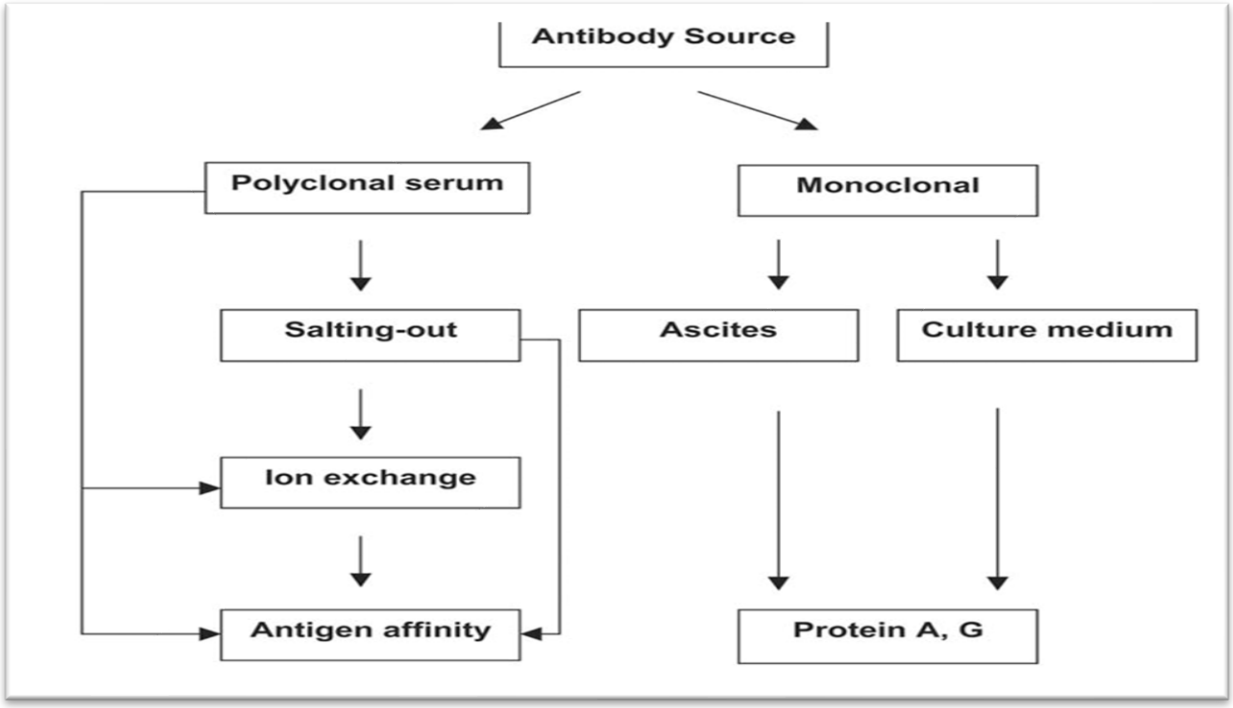


Figure 43 : Schematic Representation of Antibody Purification Procedures. (Larsson, 2002)

II.5.1. Affinity Chromatography

Affinity chromatography is the most common method used to separate antibodies from complex mixtures (Murphy et al., 2016). This technique works like a "lock and key" system, where a specific molecule called a ligand is used to catch the target antibody while allowing other impurities to wash away (Darcy et al., 2017). The most widely used ligands are bacterial proteins such as Protein A, which is the gold standard for industrial manufacturing, and Protein G or Protein L, which are used for different antibody types and animal species (Grodzki & Berenstein, 2010).

The process of affinity chromatography involves two main stages: the preparation of the functionalized matrix through ligand immobilization, followed by the purification cycle which includes loading the sample, washing non-specific proteins, and eluting the pure antibody, as illustrated in **Figure 44** (Mustafa et al., 2024).

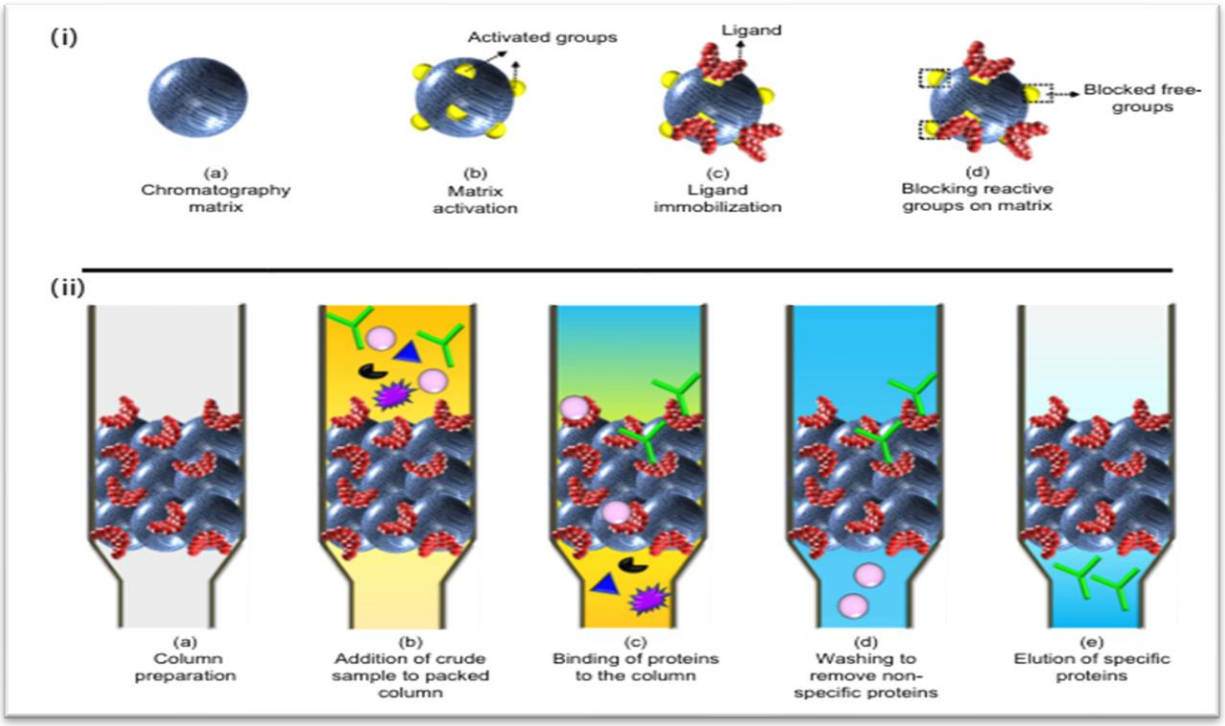


Figure 44 : 4 Steps involved in antibody purification using affinity chromatography**.** (Ayyar et al., 2012).

Affinity chromatography is a multistep procedure for separating specific antibodies from a mixture of proteins. It involves the following steps: Preparation of absorbent medium: The matrix (a) is prepared by chemically activating it resulting in the generation of reactive functional groups on the surface (b). The chemistry can be selected based on the choice of ligand to be immobilized. Once the ligand is immobilized (c), unoccupied reactive groups are blocked (d), to prevent them from binding with the contaminations in the sample matrix. Purification procedure: A typical purification procedure consists of (a) column preparation, which involves the packing and equilibration of the ligand–immobilized matrix. Crude sample (containing a mixture of proteins and other biomolecules) is passed through the packed column (b). The specificity of the ligand causes the retention of specific proteins from the mixture (c). However, there is a possibility of some non-specific binding of proteins due to their charge. This can be removed by stringently washing the column prior to final elution (d). Specific protein can be eluted either competitively or by altering the pH that breaks the interaction between the protein and the ligand (e)**.** (Ayyar et al., 2012).

II.5.2. Ion-Exchange Chromatography (IEX)

This method separates antibodies based on their net electrical charge (Fishman & Berg, 2019). It is frequently used as a secondary "polishing" step to remove any remaining small contaminants after the primary purification (Murphy et al., 2016). This technique is favored because it is fast, easy to scale up, and relatively inexpensive (Fishman & Berg, 2019).

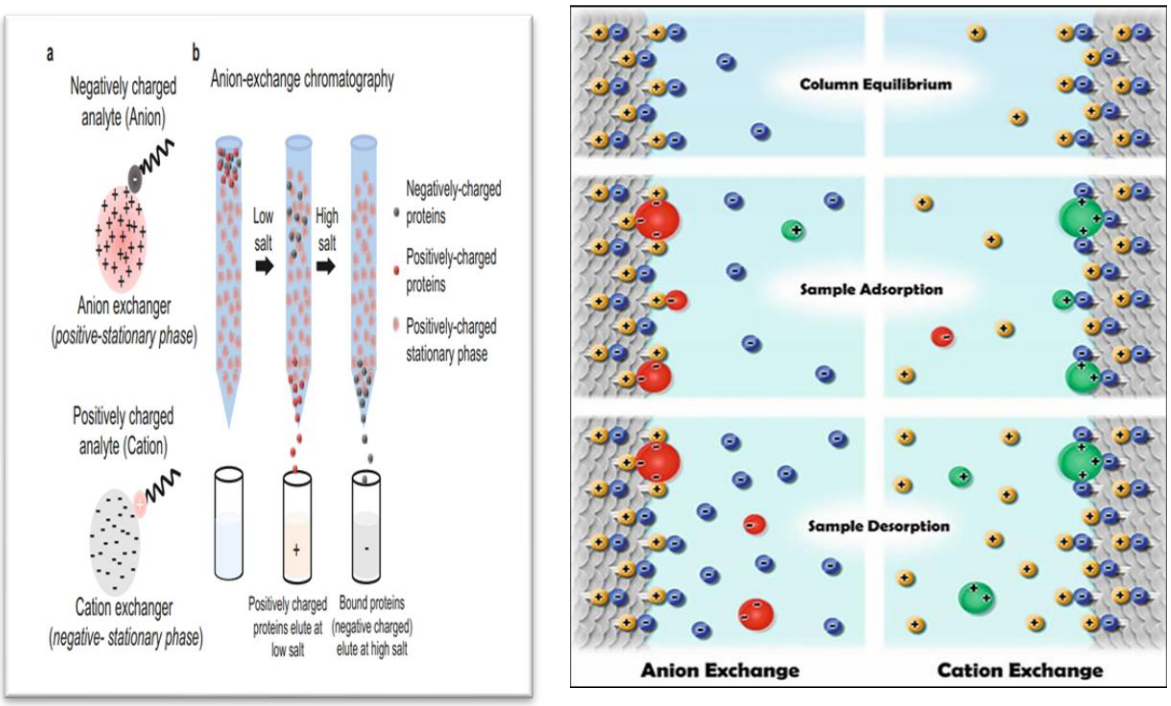


Figure 45 : Ion-exchange chromatography Advanced Strategies in Antibody Purification: From Chromatographic Techniques to Physical Separation Methods (Bose, 2022) and (Asare et al., 2025)

II.5.3. Precipitation and Filtration

These methods leverage the distinct physical and chemical properties of antibodies for separation. Traditional precipitation involves the addition of salts, such as ammonium sulfate (AMS), or acids to reduce solubility, causing antibodies to aggregate and settle (Arakawa et al., 2023). more advanced strategies like non-chromatographic affinity precipitation utilize specific trivalent haptens to induce highly selective antibody complexation and subsequent precipitation. Following this**, filtration and centrifugation** are employed to physically partition the targeted antibodies from cellular debris and contaminants based on molecular weight and size (Murphy et al., 2016).

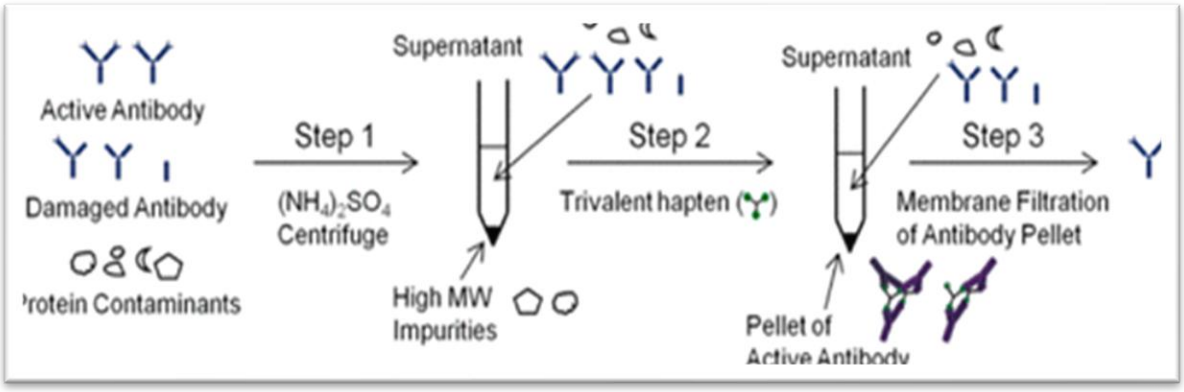


Figure 46 : Schematic of the three-step purification process for active mAb recovery via selective precipitation and membrane filtration "This procedure involves (1) precipitation of proteins heavier than immunoglobulins with ammonium sulfate; (2) formation and selective precipitation of cyclic antibody complexes created by binding to trivalent haptens specific for the antibody; and (3) membrane filtration of the solubilized antibody pellet to remove the trivalent hapten from the purified antibody. (Handlogten et al., 2013)

II.5.4. Production and Initial Purification using Dialysis Tubing

The efficiency of the purification process begins with the optimization of the initial antibody production step. Cultivating hybridoma cells inside dialysis tubing offers a highly effective method that yields immunoglobulin concentrations 10 to 50 times higher compared to conventional methods (Sjögren-Jansson et al., 1991).

As illustrated in **Figure 47,** the selective permeability of the membrane ensures that immunoglobulins are retained within the tubing, while a state of equilibrium is established for smaller solutes. This molecular confinement facilitates the continuous removal of inhibitory metabolic by-products while simultaneously promoting significant antibody enrichment. Consequently, this technique produces antibodies with a high level of initial purity, thereby resulting in the observed 50-fold concentration increase and significantly facilitating subsequent downstream purification steps.

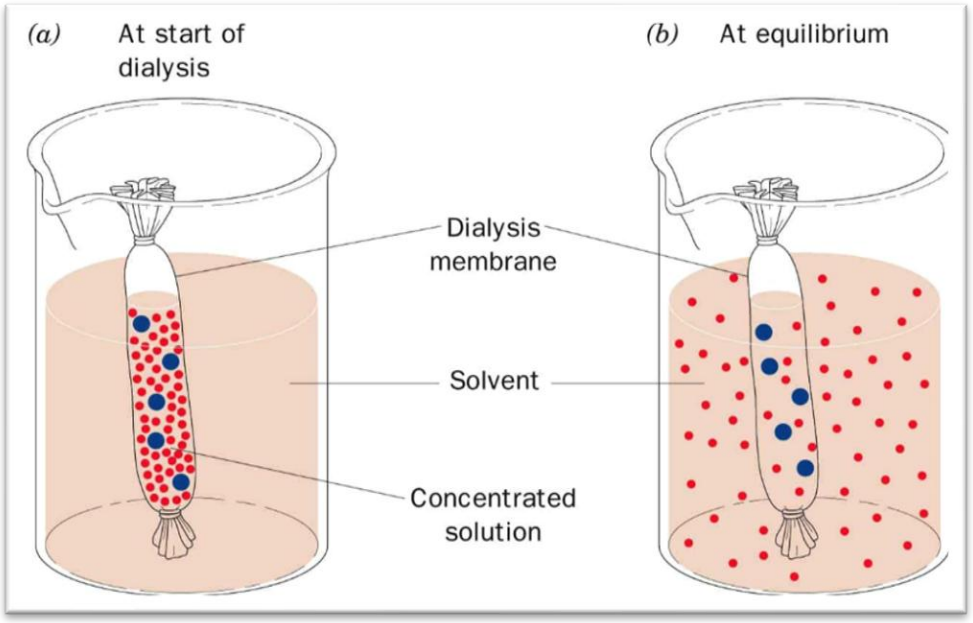


Figure 47: Principles of dialysis for protein purification, showing the diffusion of small molecules across a semi-permeable membrane until equilibrium is reached. (Berg et al,2019)

II.5.5. Hydrophobic Interaction Chromatography (HIC)

Hydrophobic interaction chromatography is widely utilized as a final "polishing" step to purify recombinant antibodies and eliminate product-related impurities, such as protein aggregates and host cell proteins. This technique relies on the interactions between non-polar regions present on the surface of the antibody and hydrophobic ligands, achieving a purity of 99.9% and a recovery yield of 96.2% when high concentrations of salts are applied (Bresolin et al., 2020). The fundamental thermodynamic principle behind this interaction—driven by the displacement of water molecules and the resulting increase in system entropy—is schematically detailed in Figure48 Beyond preparative polishing, HIC-based HPLC analyses are heavily utilized to characterize the structural properties and hydrophobicity of antibodies (Deol et al., 2025).

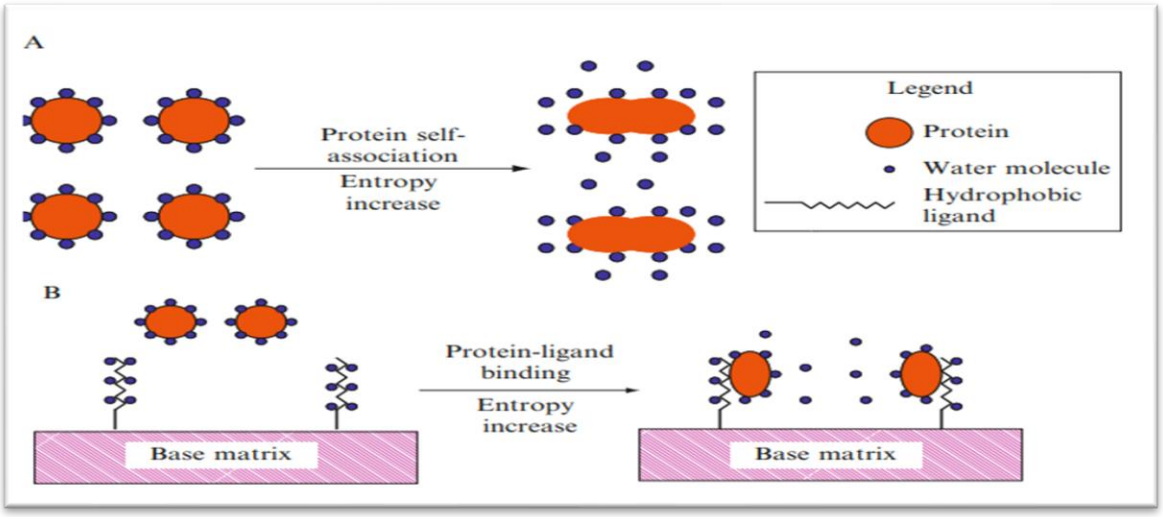


Figure 48 : Molecular mechanisms of Hydrophobic Interaction Chromatography (HIC). **(A)** Protein self-association in aqueous environments. **(B)** Selective binding of proteins to hydrophobic ligands facilitated by the entropy-driven exclusion of water molecules. (McCue, 2009)

II.5.6. High-Performance Liquid Chromatography (HPLC) Analysis

While traditional chromatography focuses on bulk purification, HPLC is a cornerstone technology for the precise separation and characterization of antibodies. Analytical techniques, including Size Exclusion Chromatography (SEC) and Reversed-Phase Liquid Chromatography (RPLC), are indispensable for evaluating antibody size, aggregation, and chemical modifications, ensuring that therapeutic products comply with stringent regulatory standards (Deol et al., 2025). The instrumental configuration required for such high-resolution separations, involving sophisticated solvent delivery and detection systems, is schematically represented in Figure 52(Ahmed, 2024).

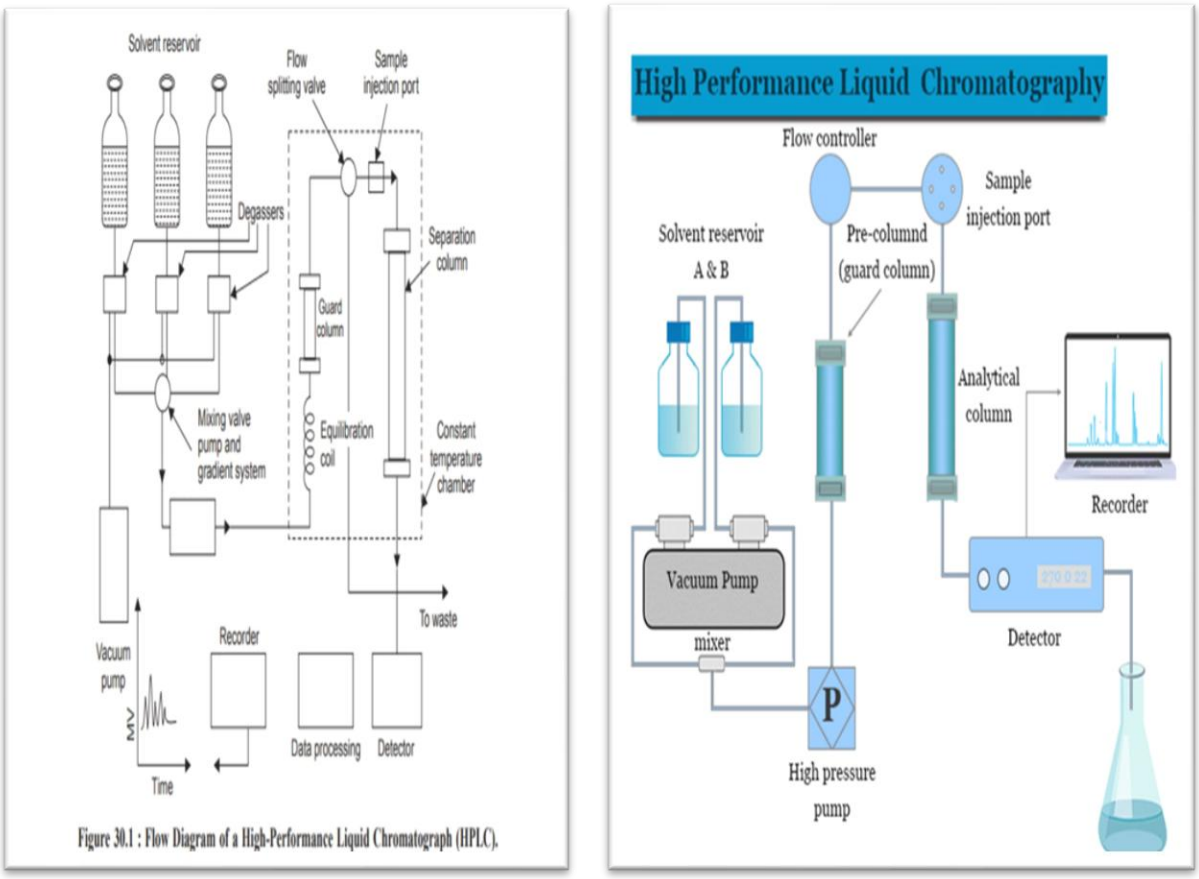


Figure 49 : Structural and functional architecture of High-Performance Liquid Chromatography (HPLC). **(Left)** Detailed flow diagram illustrating the solvent delivery system and gradient mixing. **(Right)** Modern HPLC instrumentation setup including the analytical column and data processing unit. (Ahmed,2024)

II.5.7. Liquid Chromatography-Mass Spectrometry (LC-MS): Analytical Principles and Applications in Antibody Purification and Quality Control

Liquid Chromatography–Mass Spectrometry (LC–MS) has become one of the most important analytical techniques in modern biotechnology due to its ability to combine the high separation efficiency of liquid chromatography with the sensitivity and structural analysis capabilities of mass spectrometry (Alanazi, 2025). In the field of biopharmaceuticals, LC–MS plays a crucial role in the purification and characterization of monoclonal antibodies (mAbs). It is widely used for the verification of primary amino acid sequences and the identification of post-translational modifications (PTMs), which are essential for maintaining the structural integrity and biological activity of therapeutic proteins (Camperi et al., 2021). In addition, advanced LC–MS systems allow the evaluation of difficult-to-express antibodies in CHO cell expression platforms,

providing important information about protein stability and developability under industrial production conditions (Szkodny & Lee, 2025).

In quality control (QC) and analytical characterization, LC–MS has evolved into an effective Multi-Attribute Method (MAM) capable of simultaneously identifying and quantifying several critical quality attributes, including glycosylation patterns, oxidation, and deamidation (Beccaria & Cabooter, 2020). Furthermore, for complex biotherapeutic products such as Antibody–Drug Conjugates (ADCs), LC–MS is considered the reference analytical technique for determining the Drug-to-Antibody Ratio (DAR), which is essential for ensuring therapeutic efficacy and product safety (Zhu et al., 2020).

From an operational and clinical perspective, the application of LC–MS requires rigorous validation and quality assurance (QA) procedures in order to reduce matrix effects and maintain high analytical accuracy and reproducibility (Rappold, 2022). Moreover, the development of ultra-high-resolution mass analyzers has significantly improved the precise measurement of intact antibodies and their engineered fragments, allowing a comprehensive evaluation of molecular heterogeneity and binding efficiency (Holčapek et al., 2012; Pirkalkhoran et al., 2023). Compared with conventional immunoassay-based techniques, LC–MS demonstrates superior analytical specificity and reduced susceptibility to cross-reactivity, thereby improving the accuracy and reliability of clinical and biopharmaceutical analyses. (Remane et al., 2016).

Overall, LC–MS analytical platforms have become indispensable tools for the characterization, purification, and quality assessment of monoclonal antibodies and other complex biopharmaceutical products.

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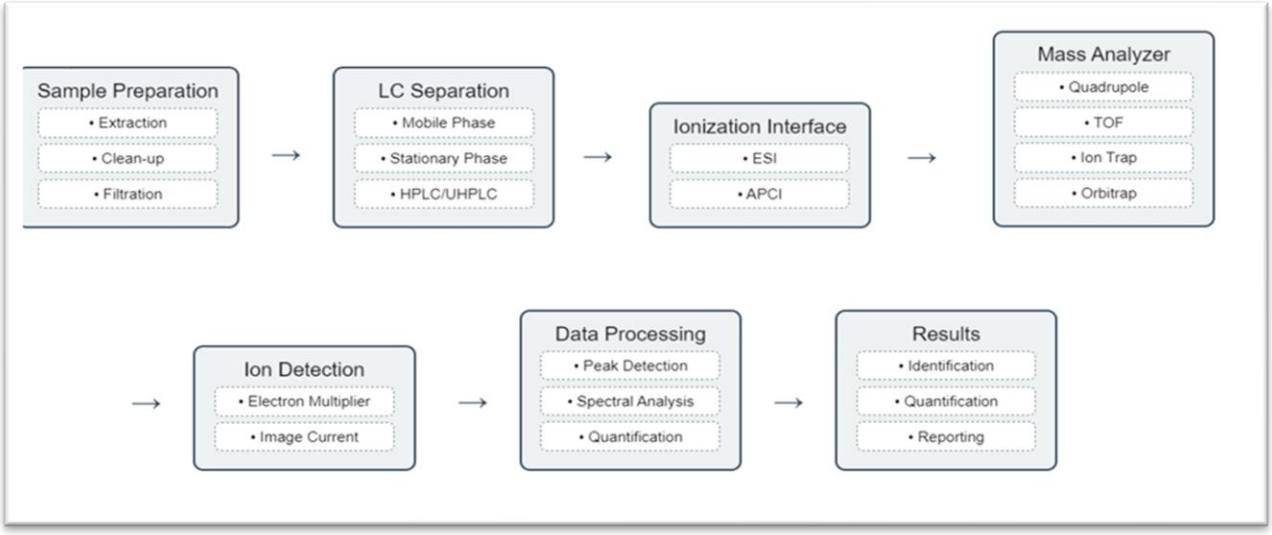


Figure 50:LC-MS Workflow (Madhuri et al., 2024)

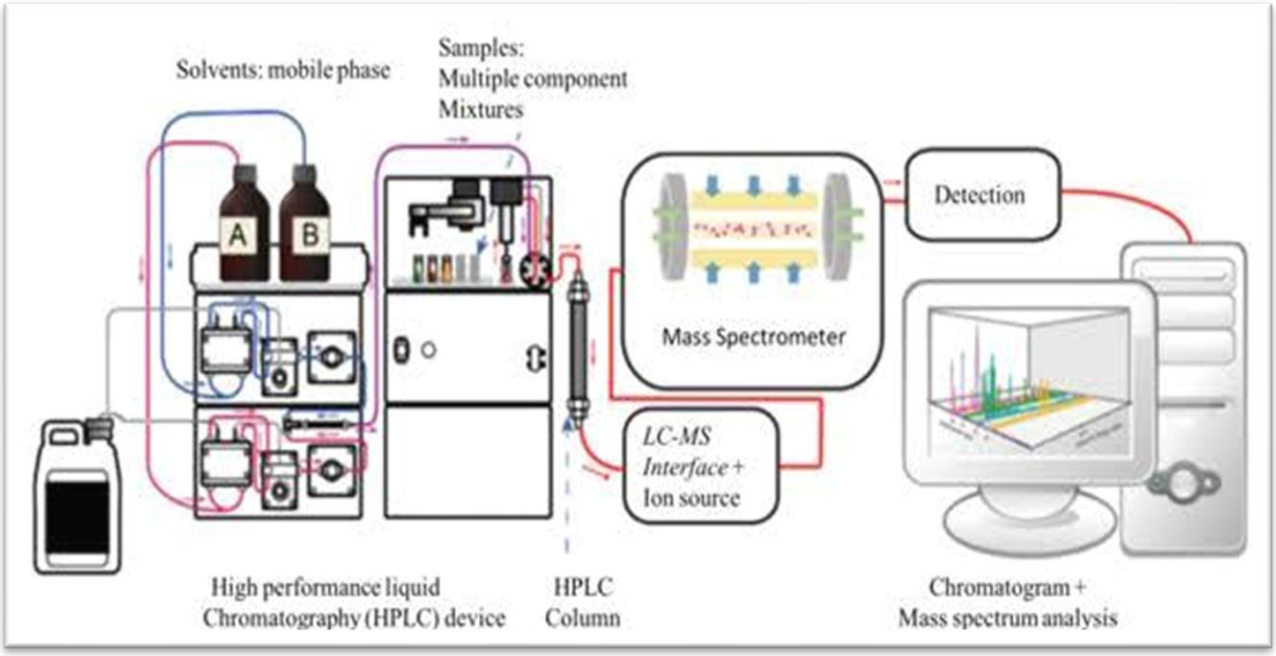


Figure 51: Direct electron ionisation Liquid Chromatography–Mass Spectroscopy (LC–MS) system illustrating chromatographic separation, ionization, mass analysis, and detection processes. (Renuka et al., 2025).

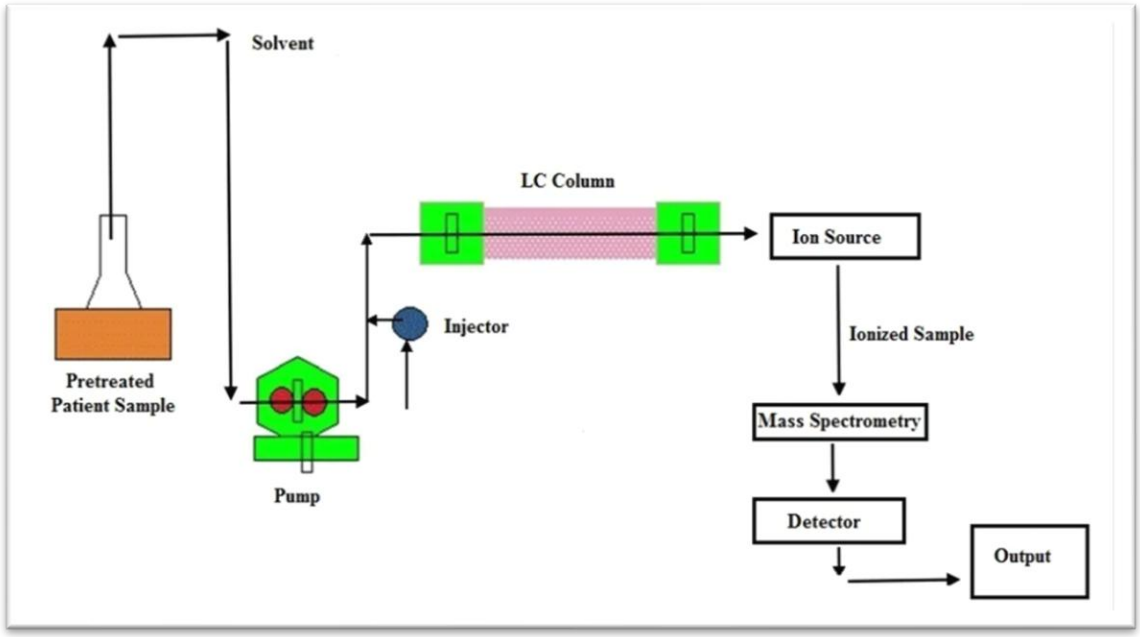


Figure 52: Schematic representation of LC-MS (Source: https://b2capi.thyrocare.com/Liquid-Chromatography.html)

II.6. Quality Control of Antibodies

II.6.1. Specificity and Affinity Assessment

Accurately measuring the **specificity** (the precision of antigen recognition) and **affinity** (the strength of the binding) is a fundamental requirement. In both therapeutic and diagnostic contexts, advanced techniques such as **Surface Plasmon Resonance (SPR)** are employed to ensure the antibody hits the intended target without cross-reacting with other proteins, thereby reducing potential off-target toxicity (Szkodny & Lee, 2025).

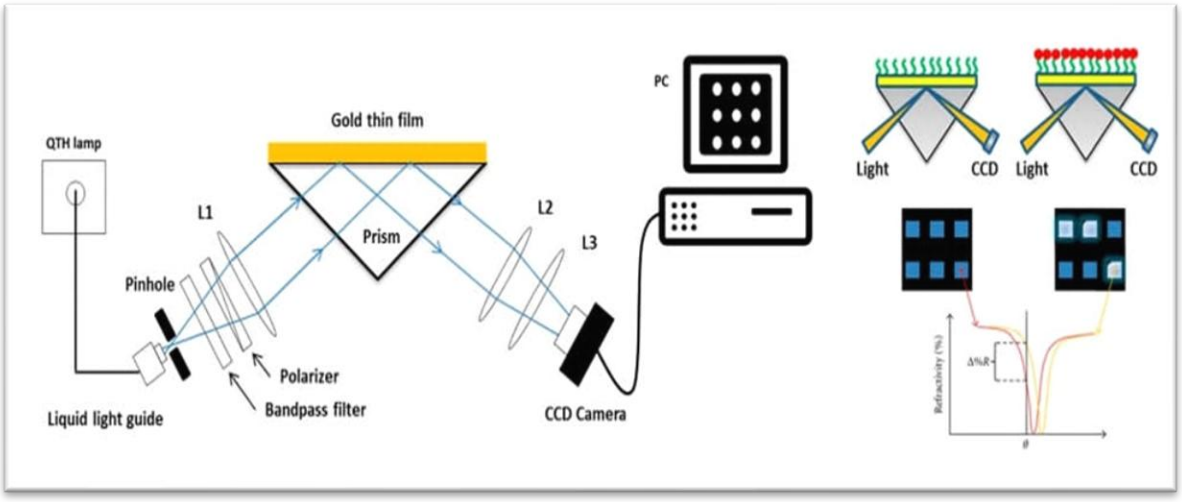


Figure 53 : General principle of surface plasmon resonance imaging (SPRI). (Left) The instrumentation of an SPR imaging system: The light source is a quartz tungsten-halogen lamp; the light is delivered through a liquid light guide to a goniometer arm, collimated by lenses, and passed through a narrow interference filter and a polarizer. A p-polarized and monochromatic light beam is then focused directly onto a prism coupler. The reflected light from the gold surface is captured by a monochromatic CCD camera. L2, L3 are lenses positioned in front of CCD for higher quality images. The images could be digitally stored using a B/W frame grabber and further analyzed using photography software; (Right) The analyte-ligand interaction shifts the SPR curve towards a higher angle (red to orange). Due to the measurement confinements (fixed wavelength and angle of incidence θ), changes in the reflectivity (Δ%R) at a single spot of the array can be simultaneously detected. (Nguyen et al., 2015)

II.6.2. Purity and Stability Evaluation

Regulatory standards demand exceptionally high levels of purity. It is mandatory to remove impurities such as **Host Cell Proteins (HCPs)** and residual DNA. Furthermore, the stability of the antibody must be evaluated to prevent aggregation or degradation; the presence of protein aggregates is a critical concern as they can trigger severe adverse immune responses in patients (Bouzid et al., 2012).

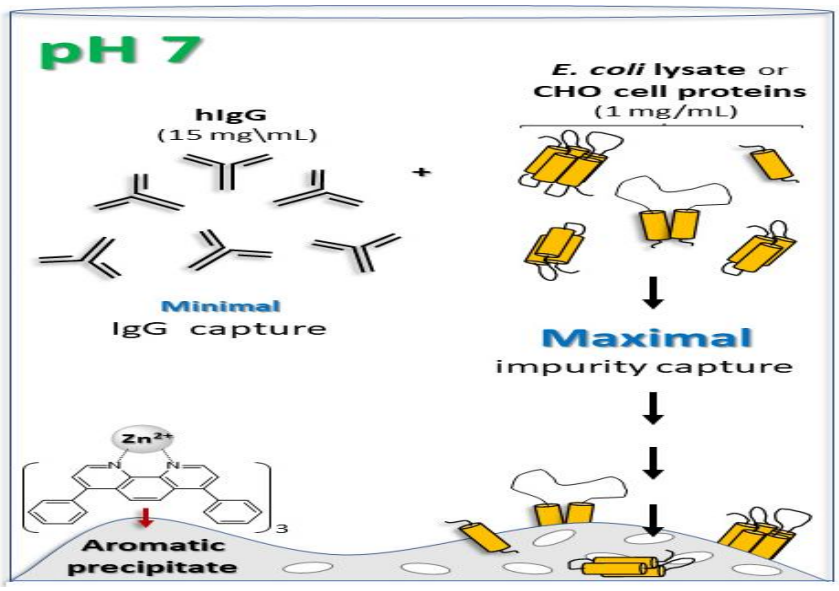


Figure 54: Schematic illustration of the purification protocol for concentrated (15 mg/mL) human IgG (hIgG). (Withanage et al ,2025) A 30 min incubation in 50 mM NaPi, pH 7 at 10 ◦C for polyclonal hIgG plus E. coli lysate or proteins secreted from CHO cells (1 mg/mL) and mixed with the washed, porous, aromatic Figure 1. Schematic illustration of the purification protocol for concentrated (15 mg/mL) human IgG (hIgG). A 30 min incubation in 50 mM NaPi, pH 7 at 10 °C for polyclonal hIgG plus E. coli lysate or proteins secreted from CHO cells (1 mg/mL) and mixed with the washed, porous, aromatic precip itate leads to the capture of the majority of the protein impurities. These impurities serve to mimic the expression of the cells' proteinaceous content. Most of the hIgG molecules remain in the super natant. The schematic illustration is not drawn to scale. precipitate leads to the capture of the majority of the protein impurities. These impurities serve to mimic the expression of the cells' proteinaceous content. Most of the hIgG molecules remain in the supernatant. The schematic illustration is not drawn to scale

II.6.3. Quality Criteria for Therapeutic and Diagnostic Use

Antibodies destined for clinical use must adhere to rigorous quality benchmarks. These include verifying the **genetic stability** of the production cell line, ensuring the final product is free from pathogens, and maintaining **batch-to-batch consistency**. Such measures are essential to guarantee patient safety and product efficacy, in alignment with international regulatory frameworks (Bouzid et al., 2012).

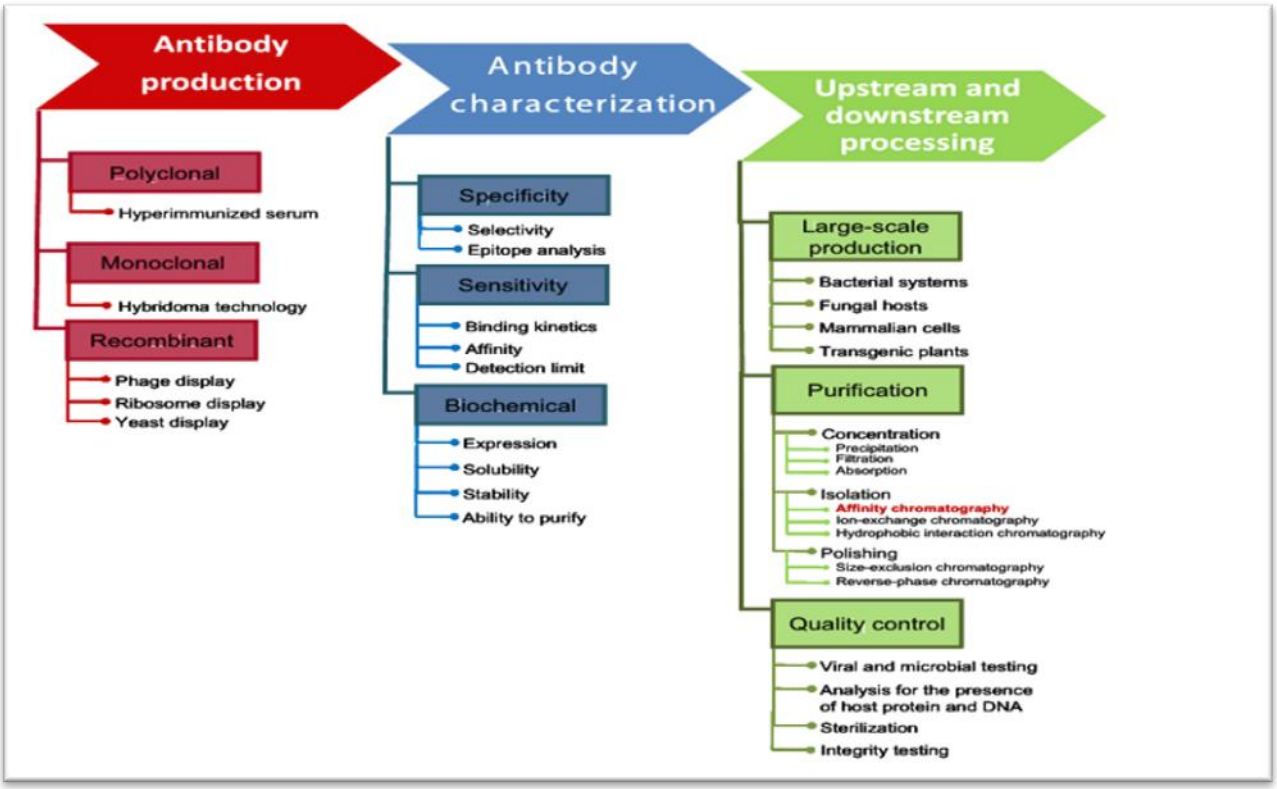


Figure 55: Production, Characterization and Purification of Antibodies (Ayyar et al., 2012)

Antibodies represent a large market sector owing to their application in various fields including diagnostics and therapy. A brief overview of the steps involved in antibody production including scale-up and purification is shown. Careful selection and optimization of each step is necessary depending on the intended use of the product. The quality of the final product is a crucial aspect. Thus, it is regularly checked to ensure compliance with the relevant standards before the product is used**.**

Chapter III.

Therapeutic Applications of Antibodies

III.1. Antibodies in Immunotherapy

Immunotherapy is a treatment approach that works by modifying or supporting the immune system to fight different diseases, including allergies, cancer, and autoimmune disorders. Antibodies play an important role in this field in two main ways.

First, in allergen immunotherapy (AIT), the treatment helps the body produce protective antibodies such as IgG, which reduce allergic reactions (Till et al., 2004).

Second, monoclonal antibodies (mAbs), which are produced in the laboratory, are used as targeted treatments. They bind to specific antigens on diseased or immune cells and help control the progression of the disease (Redman et al., 2015; Shakir et al., 2010). Today, mAbs are widely used in cancer treatment because they can target tumor cells with high precision while causing less damage to healthy tissues (Justiz-Vaillant et al., 2025; Chiavenna et al., 2017).

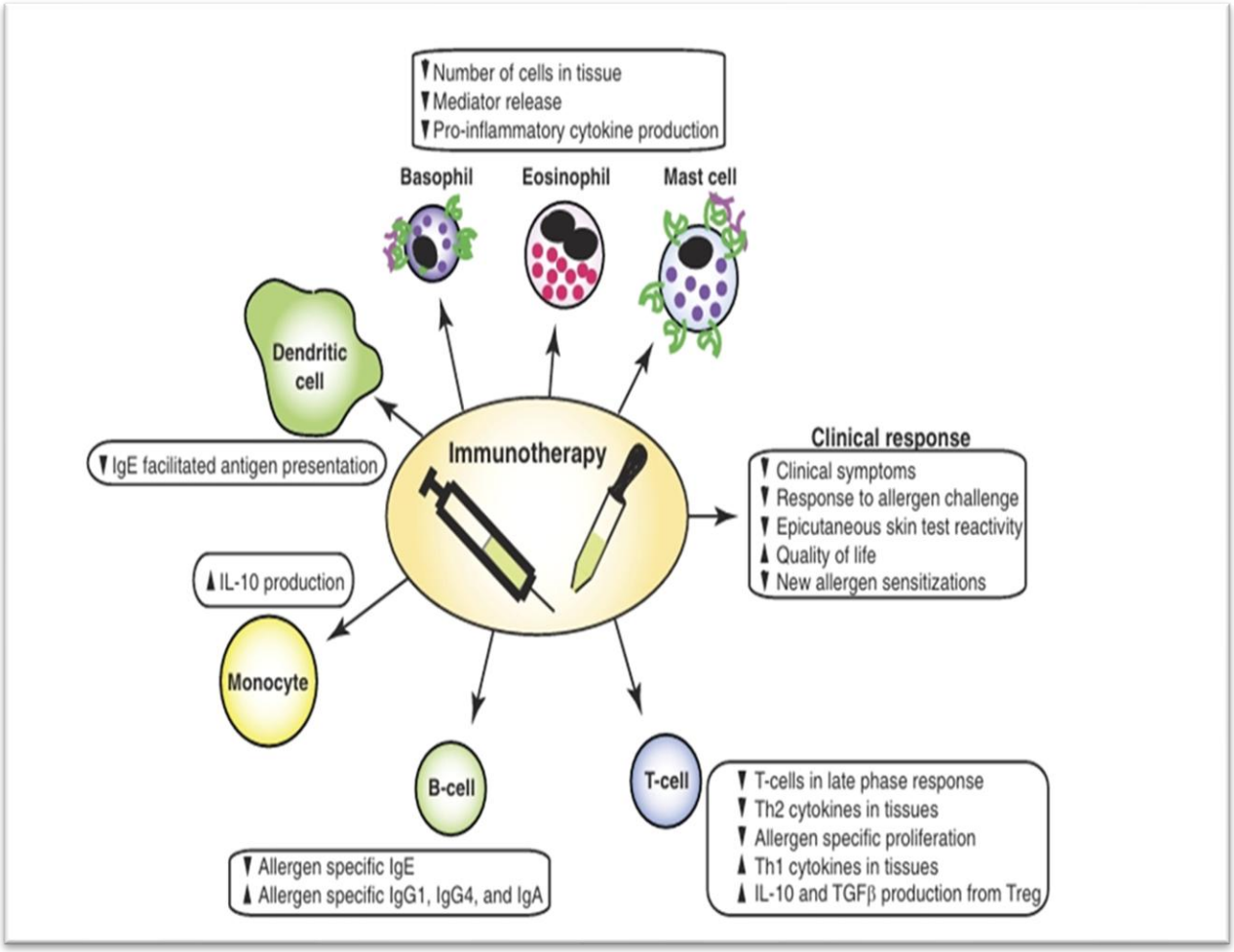


Figure 56: Cellular and molecular mechanisms underlying allergen-specific immunotherapy. (Shakir et al., 2010)

This schematic representation illustrates the currently accepted immunological and clinical effects of subcutaneous (SCIT) and sublingual immunotherapy (SLIT) on distinct cell populations. Upward and downward arrows denote the upregulation (increase) or downregulation (decrease) of respective inflammatory mediators, cellular responses, and clinical symptoms. Abbreviations: IL, Interleukin; TGF-β, Transforming Growth Factor-beta; Treg, Regulatory T cells. (Shakir et al., 2010)

III.1.1. Mechanisms of Action

Antibodies can work in different ways depending on the disease and type of treatment:

A. Blocking Antibodies in Allergy:

In allergy immunotherapy, repeated exposure to small amounts of allergens increases the level of allergen-specific IgG, especially IgG4, sometimes by 10- to 100-fold (Greenberger, 2002). These antibodies act as "blocking antibodies" by competing with IgE, which prevents the activation of mast cells and basophils and reduces the release of substances like histamine (Till et al., 2004; Wachholz & Durham, 2004). This effect is also supported by the production of IL-10 from regulatory immune cells, which helps reduce the allergic response (Aalberse, 2011; Till et al., 2004).

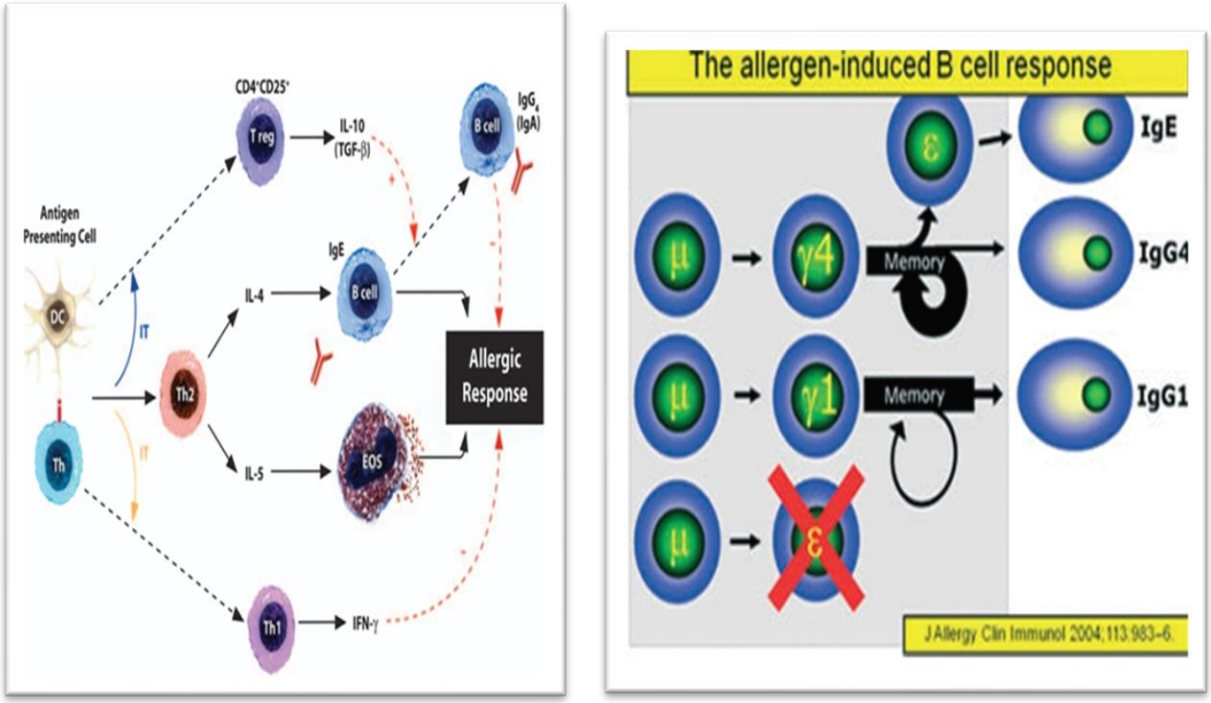


Figure 57: Cellular and Humoral Pathways in Allergic Response and Immunotherapy Modulation. (Till et al., 2004; Aalberse, 2011)

B. Cell Destruction (ADCC & CDC):

In cancer treatment, monoclonal antibodies can destroy target cells in more than one way. In antibody-dependent cellular cytotoxicity (ADCC), antibodies connect the target cell with immune cells such as natural killer cells, leading to its destruction (Tsao et al., 2021).

In complement-dependent cytotoxicity (CDC), antibodies activate the complement system, which forms pores in the cell membrane and causes cell death (White et al., 2001; Park & Kim, 2024).

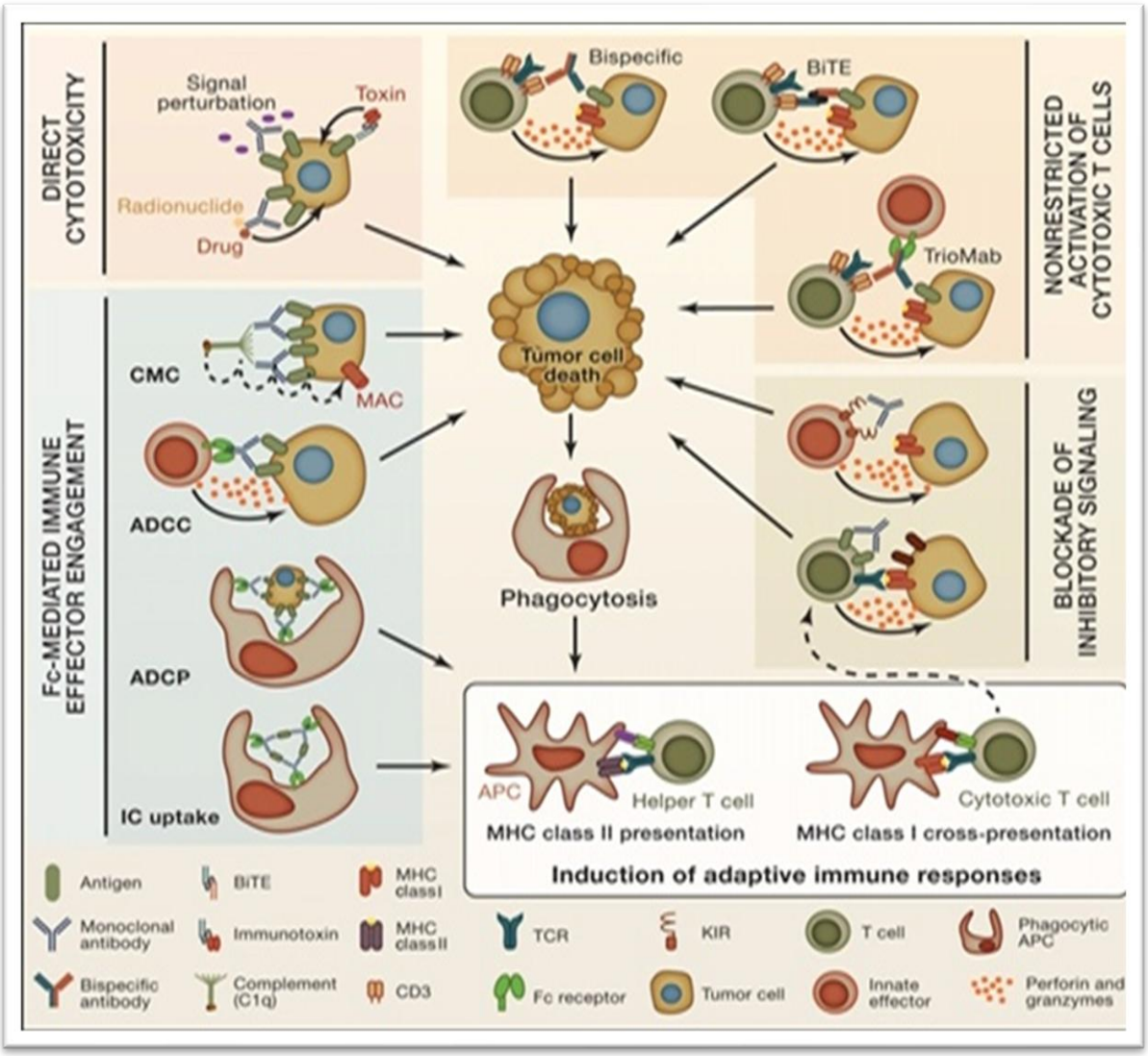


Figure 58: Diverse mechanisms of action in anticancer antibody-based immunotherapy, including direct cytotoxicity, Fc-mediated effector engagement, and modulation of T-cell activation (Weiner et al., 2012)

C. Immune Checkpoint Inhibition:

Some antibodies, called checkpoint inhibitors, target molecules such as PD-1 and CTLA-4. These molecules normally reduce immune activity, but tumors use them to escape the immune system. By blocking them, antibodies help reactivate T cells so they can attack cancer cells (Disis, 2014; Wei et al., 2018).

III.1.2. Receptor-Targeting Antibodies

Targeting cell surface receptors is one of the most effective uses of monoclonal antibodies in modern immunotherapy:

•**Blocking Growth Signals:** Some antibodies block receptors that cancer cells need to grow. For example, trastuzumab targets HER2 in breast cancer, while others target EGFR in colorectal , stopping signals that support tumor growth (White et al., 2001; Justiz-Vaillant et al., 2025).

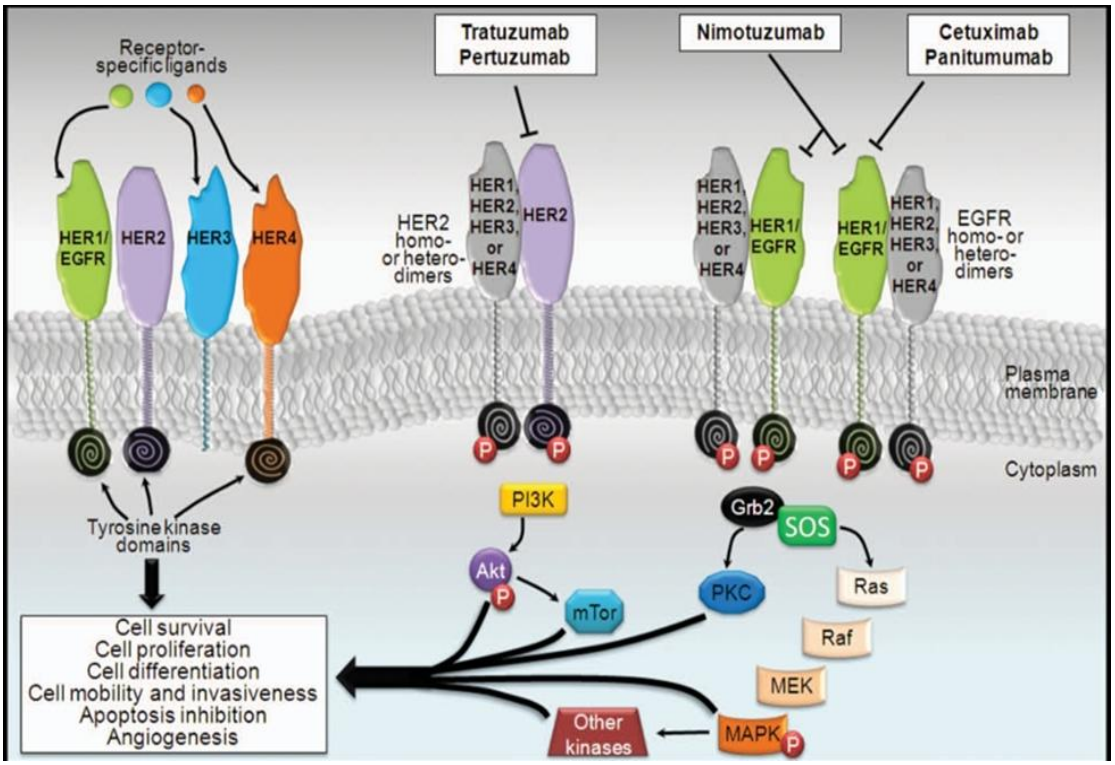


Figure 59: Schematic representation of HER family signaling pathways and their targeted inhibition by monoclonal antibodies in oncogenic contexts (Fauvel & Yasri, 2014).

• **Anti-Angiogenesis:** Antibodies such as bevacizumab target VEGF or its receptor, preventing the formation of new blood vessels and limiting tumor growth (Seebacher et al., 2019).

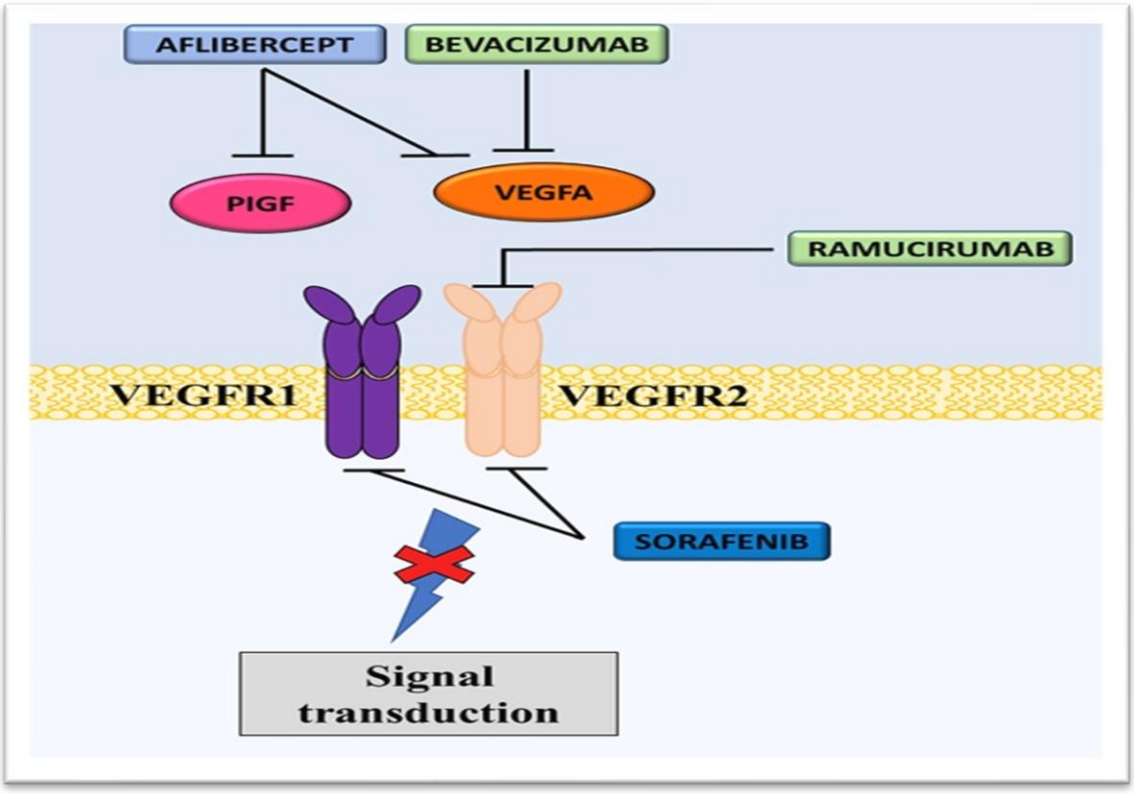


Figure 60: Mechanism of action of anti-VEGF/VEGFR drugs. Due to activation of VEGF signaling pathways in various cancers, several anti-cancer drugs have been developed to target the VEGF pathway. Aflibercept is a peptide-antibody directed at PIGF and VEGFA, while bevacizumab is a mAb specific for VEGFA. Ramucirumab is a mAb that targets the VEGFA receptor (VEGFR2). On the other hand, sorafenib is a tyrosine kinase inhibitor for VEGFR, primarily VEGFR2. Each of these drugs prevent oncogenic signaling by VEGF overexpression (Seebacher et al., 2019)

•**Targeted Drug Delivery:** New approaches use antibodies to deliver drugs directly to cancer cells. For example, therapies targeting Trop-2 use antibody-drug conjugates (ADCs) to reduce side effects on healthy cells (Park & Kim, 2024)

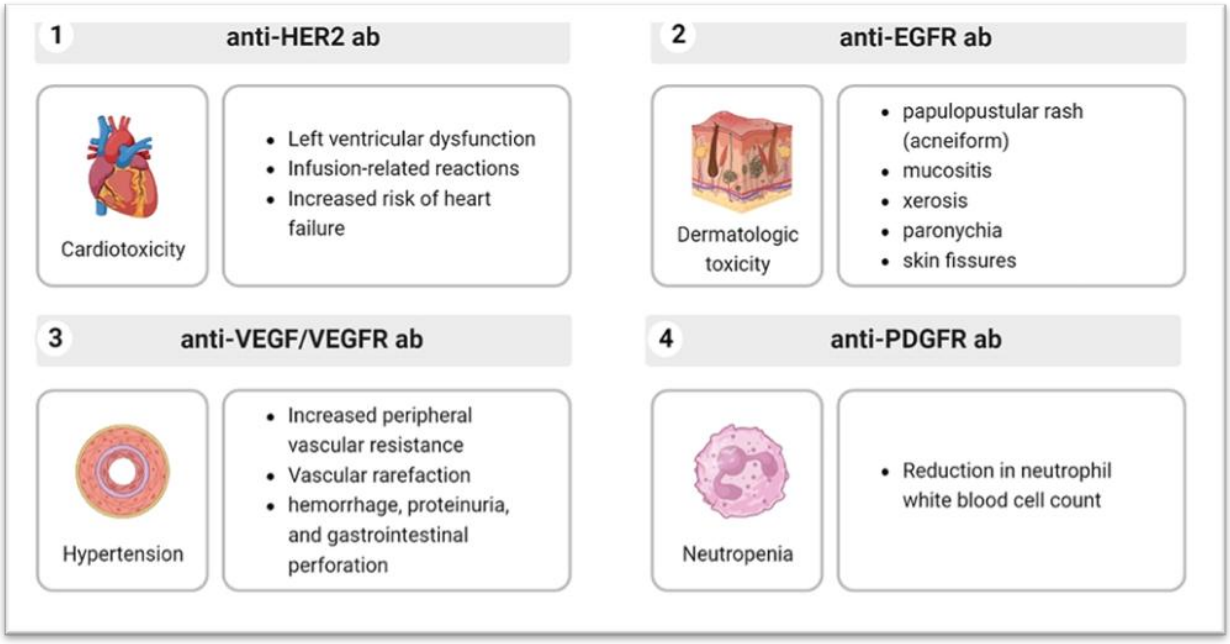


Figure 61: Toxicities of mAbs targeting growth factor signaling. Schematic representation of the four antibodies targeting growth factor signaling (Park & Kim, 2024).

III.2. Antibodies in Autoimmune and Infectious Diseases

Antibodies play a fundamental role in host defense against pathogens by recognizing microorganisms and infected cells. Beyond their ability to bind and directly neutralize pathogens, they contribute to the clearance of bacteria, viruses, fungi, and parasites through interactions with both the innate and adaptive immune systems (Lu et al., 2017). They also form immune complexes that facilitate pathogen sequestration and uptake, promote toxin clearance, eliminate infected cells, and regulate inflammatory responses (Lu et al., 2017).

Furthermore, antibody-based therapies, including convalescent plasma and monoclonal antibodies, have emerged as important strategies for reducing disease severity, as clearly demonstrated during the COVID-19 pandemic (Casadevall et al., 2021).

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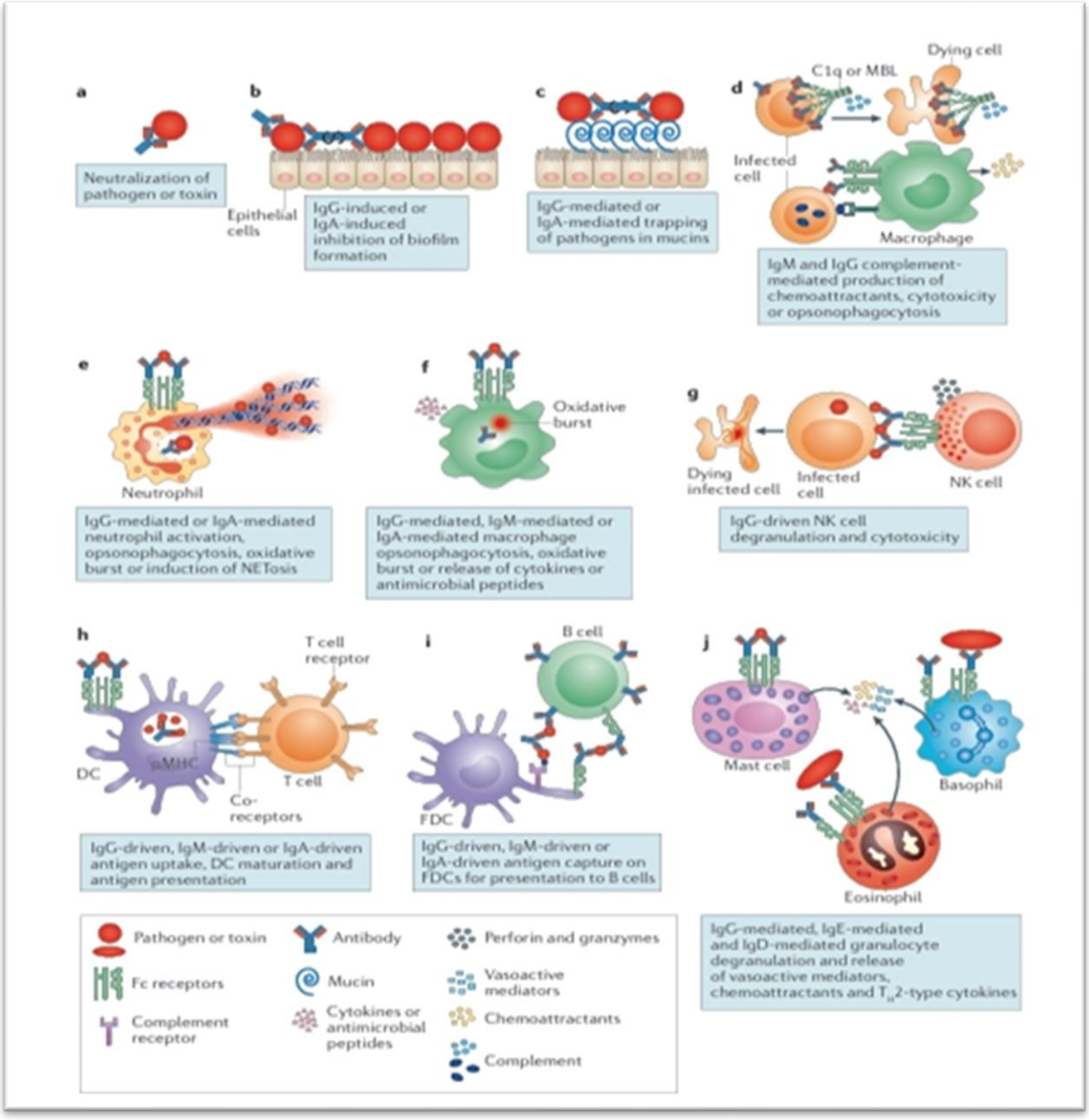


Figure 62: Comprehensive overview of antibody effector functions in infectious diseases, illustrating neutralization, complement activation, and the modulation of innate and adaptive immune cell responses (Lu et al., 2017).

In addition to their protective roles, infections can influence the development and progression of autoimmune diseases. In humans, infections are known to trigger disease flares, while in animal models they can act as initiating factors of autoimmunity. These effects are largely attributed to mechanisms such as molecular mimicry and epitope spreading (Johnson & Jiang, 2022). Moreover, infections may induce bystander polyclonal activation of autoreactive T and B cells, contributing to both pathogenic and non-pathogenic autoimmune responses (Johnson & Jiang, 2022). Anti-microbial antibodies themselves can also modulate host immune responses and have been implicated in the initiation of autoimmune diseases (Zhang et al., 2018).

Autoimmunity has been reported in several globally significant infectious diseases, including malaria and COVID-19 (Rivera-Correa & Rodriguez, 2022). These conditions, along with other hyperinflammatory states, are frequently associated with the presence of autoantibodies. For example, systematic reviews have demonstrated a significantly higher prevalence of autoantibody responses in the serum of COVID-19 patients compared to healthy individuals (Xu et al., 2026).

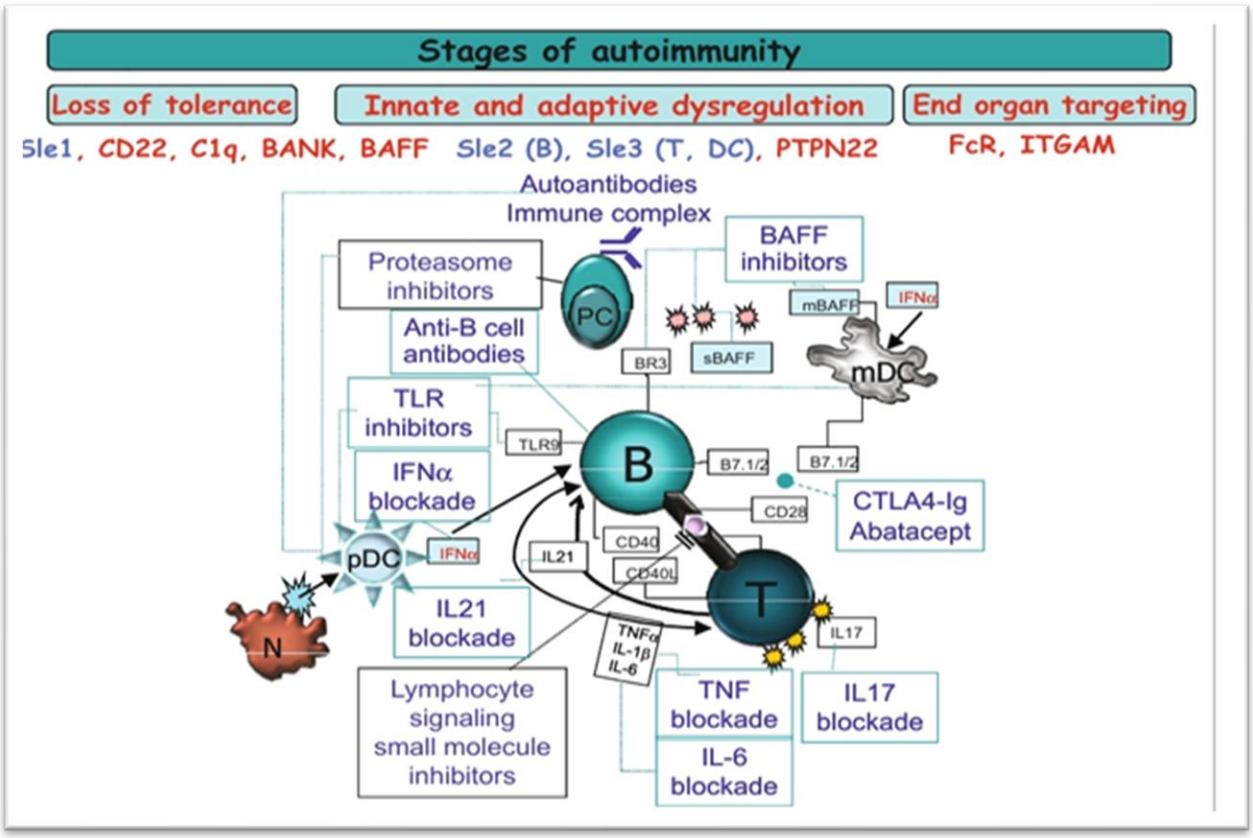


Figure 63 : Pathophysiological stages of Systemic Lupus Erythematosus (SLE) and corresponding therapeutic targets within the innate and adaptive immune pathways (Marian & Anolik, 2012).

III.2.1. Anti-TNF Antibodies

Anti–tumor necrosis factor-alpha (anti–TNF-α) antibodies represent a key therapeutic approach in the management of autoimmune diseases, particularly rheumatoid arthritis (RA). RA is a systemic inflammatory disorder characterized by chronic inflammation of the synovial joints, leading to cartilage degradation and bone erosion (Feldmann & Maini, 2001). Pro-inflammatory cytokines, especially TNF-α, play a central role in driving the inflammatory processes observed in affected joints (Feldmann & Maini, 2001).

The clinical use of anti–TNF-α agents—including monoclonal antibodies such as infliximab and adalimumab, as well as receptor fusion proteins like etanercept—is widely recommended for patients who exhibit an inadequate response to conventional disease-modifying anti-rheumatic drugs (DMARDs) (Jin et al., 2010; Seymour et al., 2001). The therapeutic efficacy of these agents is primarily attributed to their ability to inhibit TNF-α activity, which subsequently reduces the production of other pro-inflammatory cytokines, such as interleukin-1 (IL-1), thereby limiting inflammation and preventing joint damage (Feldmann & Maini, 2001).

However, treatment responses vary among patients. A significant limitation of anti–TNF-α therapy is the development of anti-drug antibodies (ADAbs), which can reduce drug efficacy, alter pharmacokinetics, and ultimately compromise clinical outcomes (Wu et al., 2016).

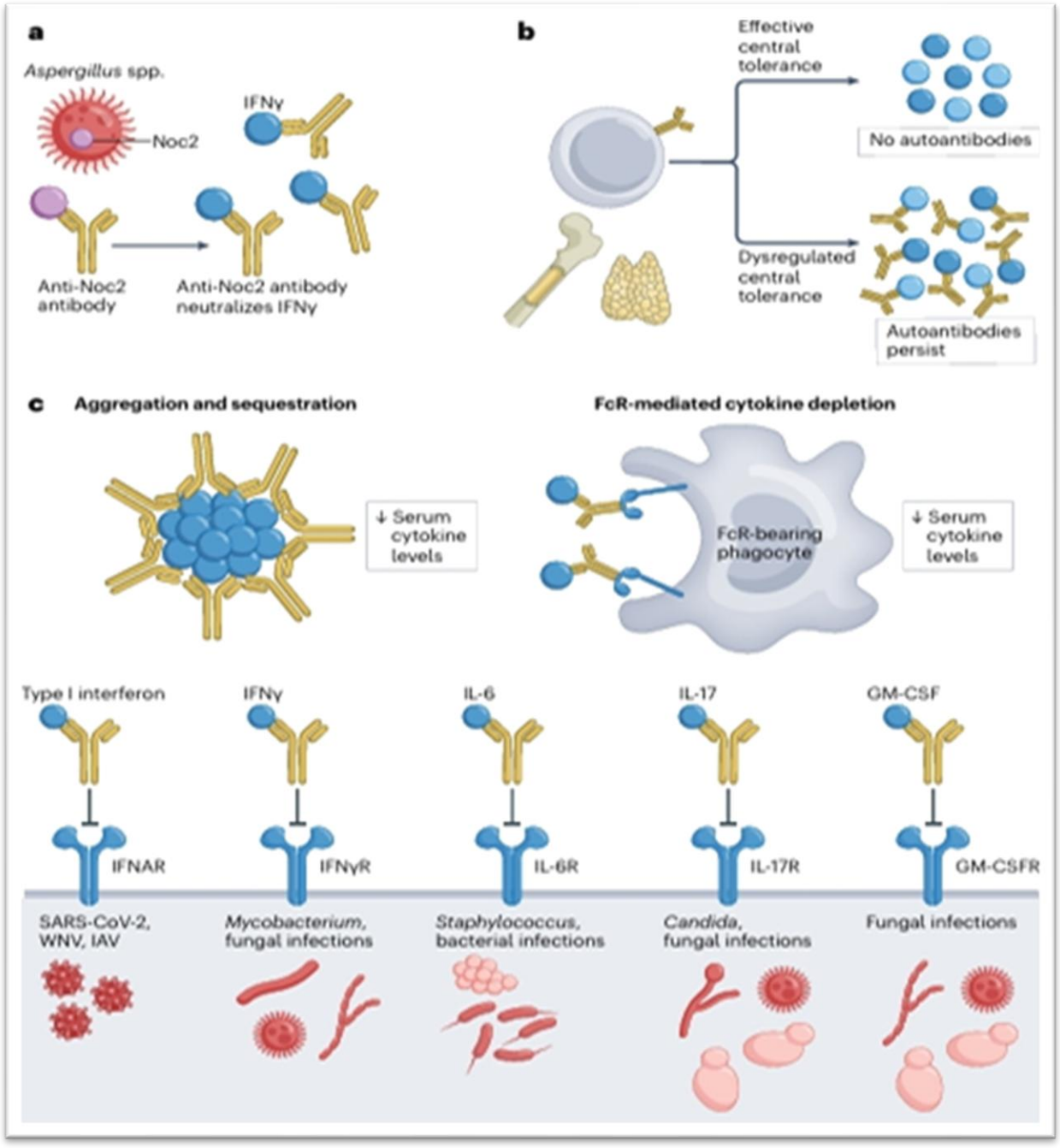


Figure 64 : Mechanism of anti-cytokine autoantibodies enhancing infection. a, Molecular mimicry: IFNγ autoantibodies arise after Aspergillus infection, as an epitope in the Noc2 protein is similar to an epitope in IFNγ. b, Individuals with dysfunctional autoimmune regulator protein (AIRE) acquire autoantibodies to type I interferon. c, Anti-cytokine autoantibodies disrupt protective cytokine function by depleting the serum cytokine pool or preventing the interaction of the cytokine with its cognate receptor. FcR, crystallizable fragment (Fc) receptor; GM-CSF, granulocyte–monocyte colony-stimulating factor; IAV, influenza A virus; WNV, West Nile virus (Wells et al., 2025).

III.2.2. Neutralizing Antibodies

Neutralizing antibodies constitute a specialized class of immunoglobulins that protect against infection by directly binding to pathogens and preventing their entry into host cells (Lu et al., 2017). Their importance was prominently highlighted during the COVID-19 pandemic. The entry of SARS-CoV-2 into host cells depends on the interaction between the receptor-binding domain (RBD) of the viral spike protein and the host cell receptor angiotensin-converting enzyme 2 (ACE2) (Ju et al., 2020).

Neutralizing monoclonal antibodies generated in response to SARS-CoV-2 infection have demonstrated potent antiviral activity. This effectiveness is largely due to their ability to compete with ACE2 for binding to the RBD, thereby blocking viral entry into host cells (Ju et al., 2020).

In contrast, antibodies that bind to pathogens without effectively neutralizing them may contribute to a harmful phenomenon known as antibody-dependent enhancement (ADE). In this process, non-neutralizing antibodies interact with Fc receptors on immune cells, facilitating viral entry or enhancing replication within these cells. This mechanism can lead to increased pathogen load and exacerbation of disease severity (Wells et al., 2025).

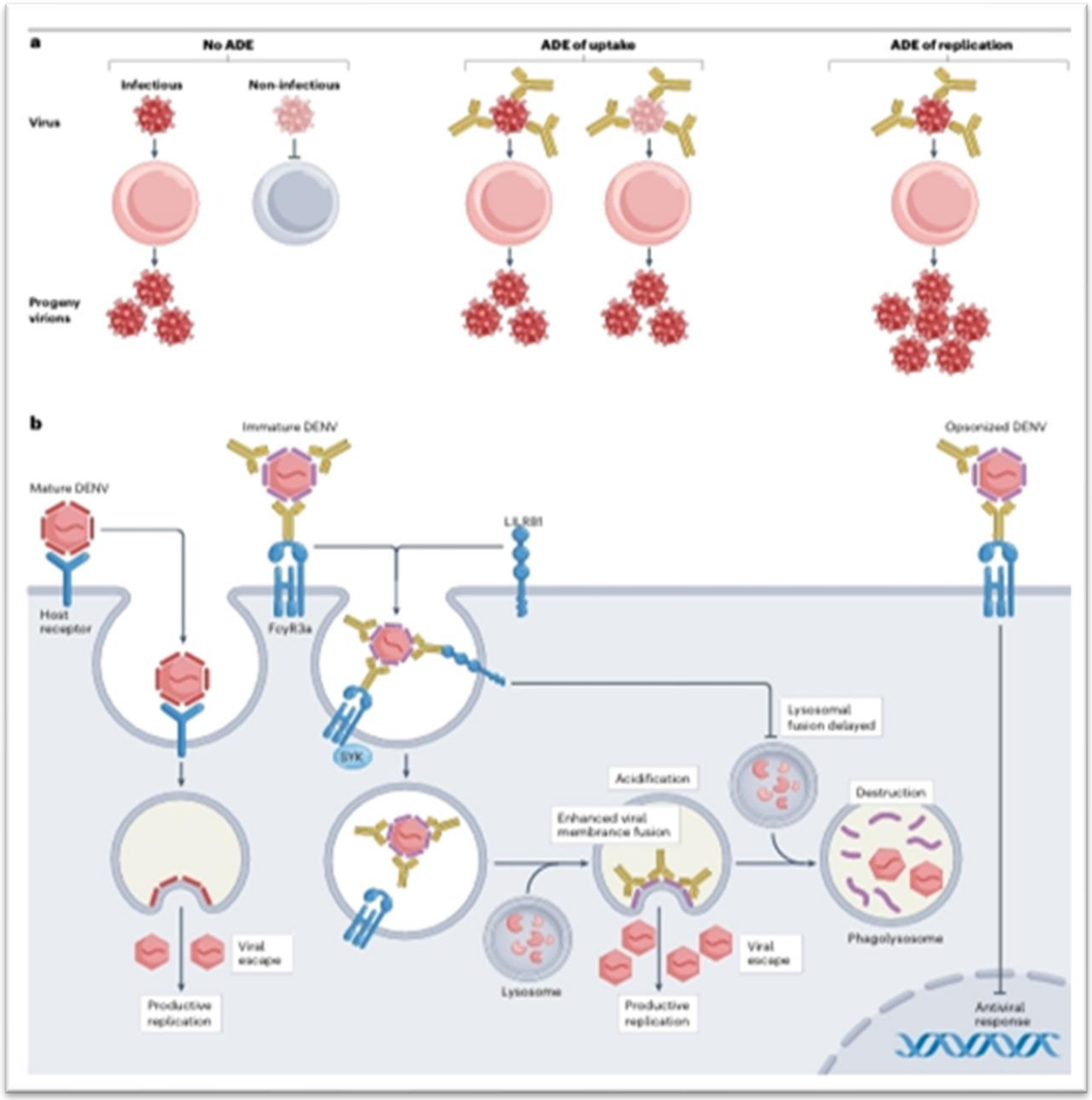


Figure 65 : Mechanisms of antibody-dependent enhancement of pathogen uptake and replication. (Wells et al., 2025). a, Antibodies can increase the total pathogen load. This may occur by increasing the number of susceptible cells through crystallizable fragment (Fc) receptor (FcR)-dependent binding or uptake, increasing the number of infected cells. Alternatively, antibodies may increase pathogen burst size, increasing the progeny release per infected cell. Both scenarios may occur during antibody-dependent increase in pathogen load (ADIPL). b, Molecular mechanisms for how antibodies increase dengue virus entry into FcγR-expressing cells. During infection in the absence of antibodies, the virus is taken up into endosomes where acidification promotes fusion of the dengue virus envelope protein with the endosomal membrane, facilitating cytosolic escape of the dengue virus nucleocapsid and genome into the

cytosol for further replication. Sub-neutralizing titres of antibodies directed against the envelope, the precursor membrane or the envelope–precursor membrane interface on both mature or partially immature dengue virions promote entry into cells via FcγRs. When the FcγR recognizes the opsonized virion, the FcγR recruits and activates spleen tyrosine kinase (Syk), initiating phagocytosis of the opsonized virus and trafficking into the phagolysosomal pathway, for eventual destruction in the lysosome. However, the acidifying environment within the phagolysosome activates the dengue envelope fusion protein on both mature and partially immature virions, allowing the virus to escape into the cytosol. Dengue virus also co-ligates leukocyte immunoglobulin-like receptor B1 (LILRB1), which slows Syk-mediated phagolysosomal fusion, affording the virus enough time to escape into the cytosol. Thus, antibodies can enhance the uptake and internalization of wholly or partially immature virions, which would otherwise have reduced infectivity. For non-myeloid tropic pathogens, antibody opsonization may present a unique entry route into otherwise non-susceptible cells. Additionally, specific antibodies may also promote viral membrane fusion or reduce cellular antiviral responses, enhancing viral survival within the cell. ADE, antibody-dependent enhancement (Wells et al., 2025).

III.3. Antibodies in oncology

Monoclonal antibodies (mAbs) targeting various pathways in cancer therapy play crucial roles in enhancing the immune system's ability to recognize and eliminate tumor cells (Justiz-Vaillant et al., 2025). The biotechnological development of monoclonal antibodies and their immunotherapeutic use in oncology have grown exponentially in the last decade, becoming the first-line therapy for some types of cancer (Toledo-Stuardo et al., 2024).

III.3.1. Mechanism of action of anticancer monoclonal antibodies

- **Direct Interaction and Inhibition**: The mechanism of action of therapeutic antibodies is based on their ability to regulate the immune system or interact with targets that are either overexpressed in tumor cells, released into the extracellular milieu, or involved in processes that favor tumor growth (Toledo-Stuardo et al., 2024). These therapies are specifically designed to target tumor cell markers for direct destruction (Justiz-Vaillant et al., 2025). For example, trastuzumab binds to the extracellular juxtamembrane domain of HER2 and inhibits the proliferation and survival of HER2-dependent tumors (Hudis, 2007).

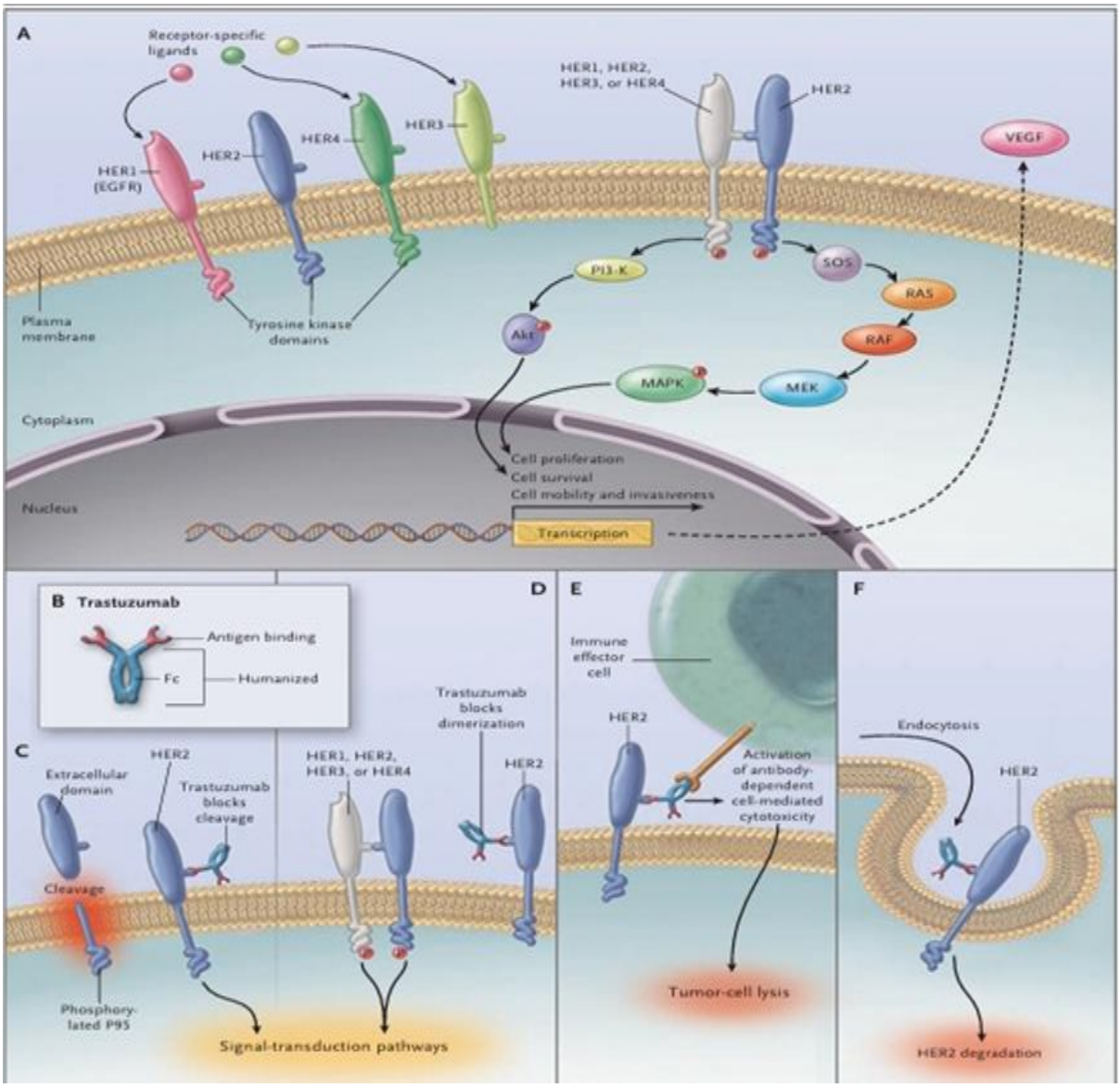


Figure 66 : Molecular mechanisms of action of anti-HER2 monoclonal antibodies, illustrating the inhibition of signal transduction pathways and the induction of tumor-cell lysis (Hudis, 2007).

- **Immune Effector Functions:** The intrinsic characteristics of each subclass of antibodies provide specific effector functions against the tumor (Toledo-Stuardo et al., 2024). These mechanisms include activating antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and antibody-dependent cellular phagocytosis (ADCP) (Toledo-Stuardo et al., 2024).
- **Modulation of the Tumor Microenvironment:** Furthermore, mAbs can modulate the tumour microenvironment and inhibit angiogenesis, further amplifying their therapeutic impact (Justiz-Vaillant et al., 2025).

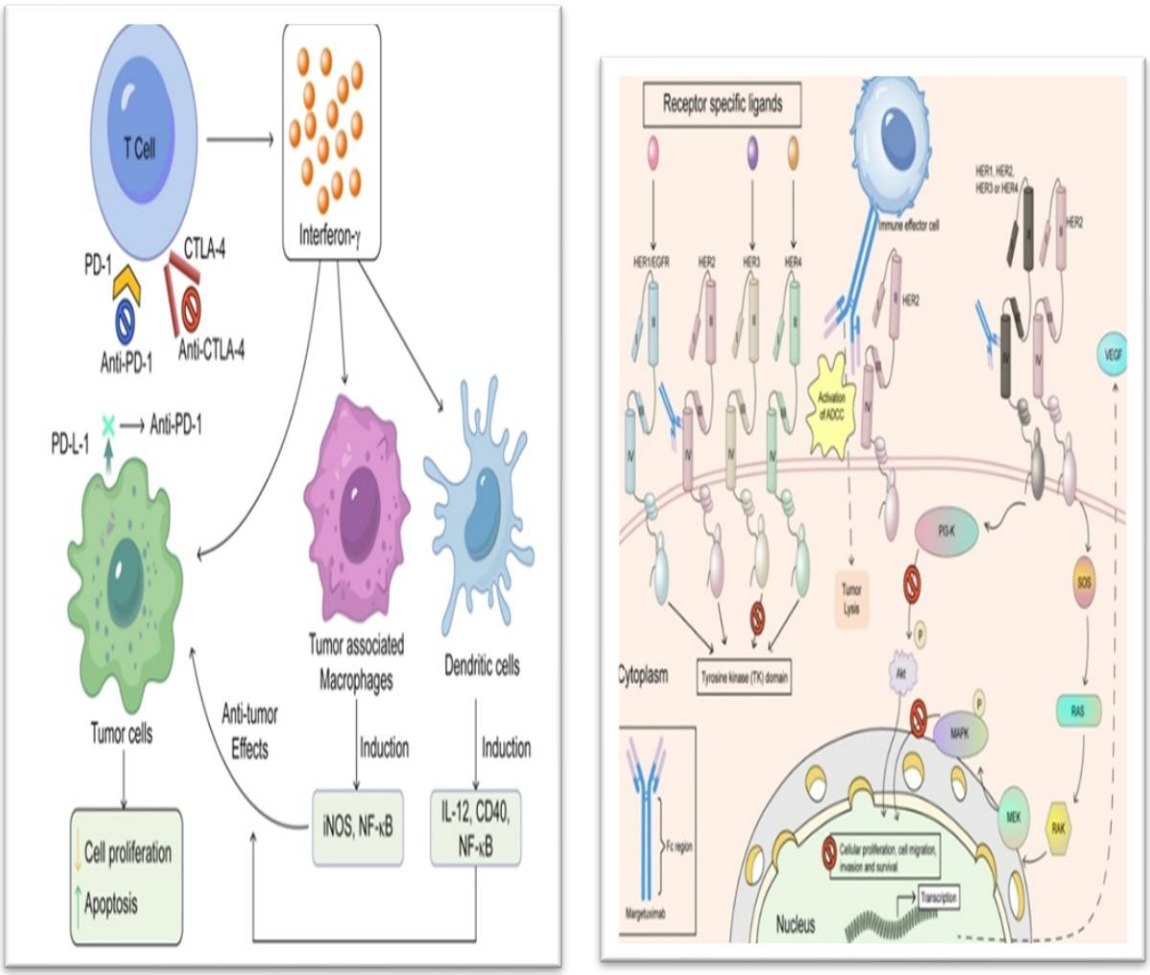


Figure 67 : Integrated mechanisms of therapeutic monoclonal antibodies: (A) Action of immune checkpoint inhibitors on T-cell activation; (B) Targeted inhibition of HER2 signaling and ADCC enhancement by Margetuximab (Justiz-Vaillant et al., 2025).

III.3.2. Mechanism of action of immune checkpoint inhibitors

- **Blocking Inhibitory Pathways**: Immune checkpoints are critical in modulating immune responses and maintaining self-tolerance, and cancer cells can exploit these mechanisms to evade immune detection (Zamani & Šácha, 2025). Immune checkpoint modulators are designed to block these inhibitory immune checkpoint pathways (Justiz-Vaillant et al., 2025).
- **Targeting the CTLA-4 and PD-1/PD-L1 Pathways:** The cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4)/B7 and programmed death 1 (PD-1)/programmed cell death-ligand 1 (PD-L1) are the two most representative immune checkpoint pathways,

which negatively regulate T cell immune function during different phases of T-cell activation (Zhang et al., 2021). The emergence of immune checkpoint inhibitors (ICIs), primarily including anti-PD-1, anti-PD-L1, and anti-CTLA-4 monoclonal antibodies, has significantly shaped the therapeutic landscape of several types of cancers (Qin et al., 2019). For instance, ipilimumab is an antibody blocking the immune checkpoint CTLA-4, while pembrolizumab and nivolumab target PD-1, and atezolizumab and durvalumab target PD-L1 (Robert, 2020). Inhibitors targeting the CTLA-4/B7 and PD1/PD-L1 pathways have revolutionized immunotherapies for numerous cancer types (Zhang et al., 2021).

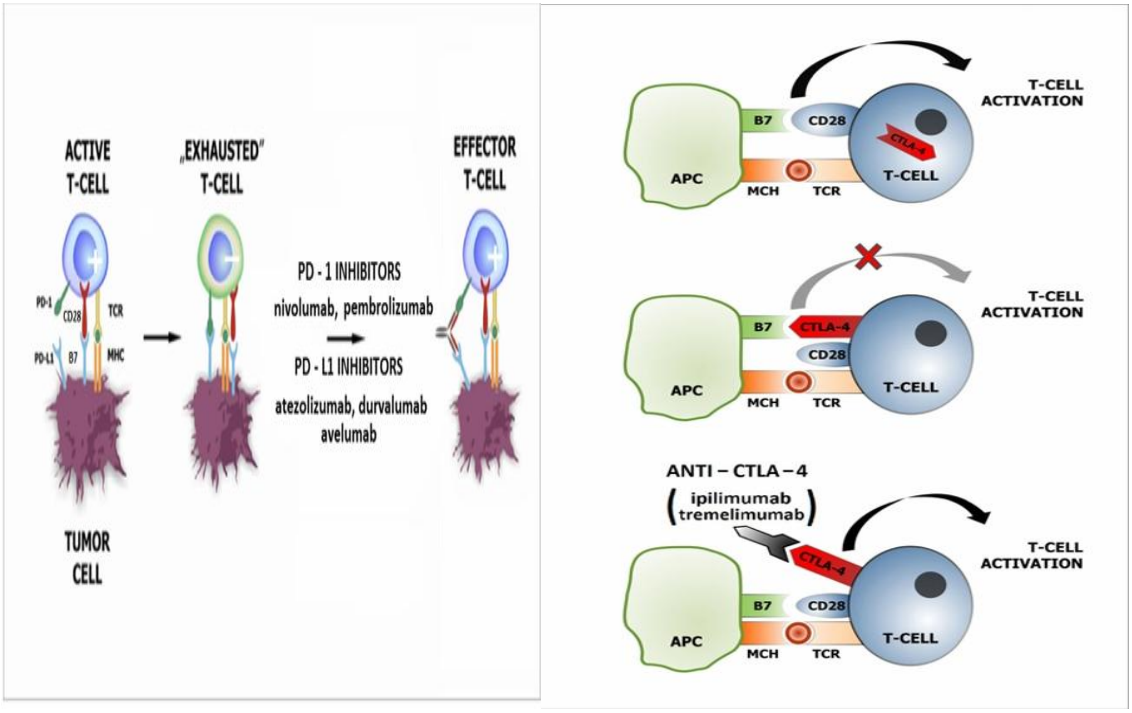


Figure 68 : Mechanism of T-cell modulation via immune checkpoint pathways: Activation through costimulatory signals and reactivation using anti-CTLA-4 and anti-PD-1/PD-L1 monoclonal antibodies (Wojtukiewicz et al., 2021)

- **Overcoming Resistance and Emerging Targets**: Overall, these inhibitors have contributed to a significant improvement in the outcome of treatment and prognosis of patients with different types of malignancy (Wojtukiewicz et al., 2021). However, because the majority of patients still showed de novo or adaptive resistance, exploring additional immune checkpoint molecules is a hot research topic (Qin et al., 2019). Recent studies have identified several new immune checkpoint targets, like lymphocyte activation gene-3 (LAG-3), T cell immunoglobulin and mucin-domain containing-3 (TIM-3), T cell

immunoglobulin and ITIM domain (TIGIT), and V-domain Ig suppressor of T cell activation (VISTA) (Qin et al., 2019).

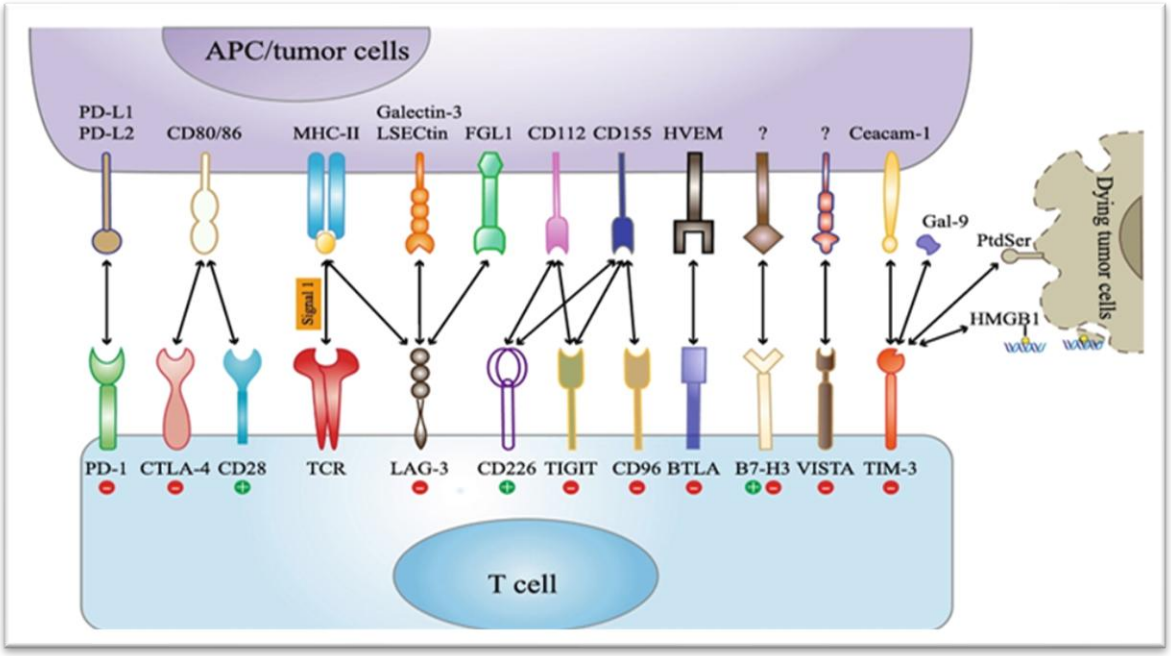


Figure 69 : Current and emerging immune checkpoint receptors and their corresponding ligands: A focus on novel therapeutic targets to overcome immunotherapy resistance (Qin et al,2019)

III.3.3. Advantages of Therapeutic Antibodies

Monoclonal antibodies (mAbs) offer several distinct clinical and pharmacological benefits over traditional therapies:

- **High Target Specificity:** Due to their ability to recognize specific antigens, mAbs exhibit minimal cross-reactivity with non-target tissues, which significantly reduces the adverse effects typically associated with conventional chemotherapy (Lu et al., 2020; Sapra & Shor, 2013).
- **Enhanced Therapeutic Index:** By utilizing antibodies as delivery vehicles—such as in Antibody-Drug Conjugates (ADCs)—potent cytotoxic agents can be delivered directly to the site of the disease, increasing efficacy while sparing healthy cells (Sapra & Shor, 2013).
- **Immune System Recruitment:** Therapeutic antibodies can trigger the host's own immune response to destroy target cells through specialized mechanisms, including Antibody-

dependent Cellular Cytotoxicity (ADCC) and Complement-Dependent Cytotoxicity (CDC) (Chames et al., 2009).

- **Prolonged Serum Half-life:** The structural stability of full-length IgG antibodies prevents rapid renal clearance, allowing for a long half-life in the bloodstream and reducing the frequency of patient dosing (Chames et al., 2009).
- **Broad Therapeutic Versatility:** Antibody engineering allows for the treatment of a diverse range of conditions, including solid tumors, hematological malignancies, autoimmune disorders, and emerging infectious diseases such as COVID-19 (Lu et al., 2020; Marzouni et al., 2022).

III.3.4. Limitations of Therapeutic Antibodies

Despite their advantages, the clinical use of traditional antibodies faces several functional and economic constraints:

- **Restricted Tissue Penetration:** The large molecular weight of full-length antibodies (approx. 150 kDa) hinders their ability to penetrate deeply into solid tumors or cross the blood-brain barrier (Chames et al., 2009; Kholodenko et al., 2019).
- **Risk of Immunogenicity:** Patients may develop an immune response against the therapeutic antibody itself (Anti-Drug Antibodies or ADAs), which can neutralize the treatment's efficacy or lead to severe hypersensitivity reactions (Chames et al., 2009; Marzouni et al., 2022).
- **Unintended Off-target Toxicities:** Some antibodies may cause adverse effects through interactions with Fc receptors on non-target cells or via non-specific uptake in organs like the liver (Chames et al., 2009).
- **High Manufacturing Costs:** The production of mAbs requires complex recombinant DNA technology, specialized cell culture systems, and rigorous purification processes, making them significantly more expensive than small-molecule drugs (Lu et al., 2020; Kholodenko et al., 2019).
- **Pharmacokinetic Limitations:** In some therapeutic contexts, the long half-life of mAbs may be a disadvantage, as it prevents rapid clearance of the drug once its clinical objective is met or if toxicity occurs (Chames et al., 2009).

Chapter IV. Diagnostic Applications and Test Analysis

IV.1. Role of Antibodies in Biomedical Diagnosis

- **Dual Role of Antibodies:** Antibodies are the foundation of modern diagnostics because they can act both as natural indicators of disease inside the body and as highly specific tools in the laboratory (Ng et al., 2025).
- **High Specificity:** In clinical microbiology, the reliability of pathogen identification comes down to the precise fit between the antibody's paratope and the antigen's epitope (Anochie et al., 2023).
- **Standard Lab Probes:** Because of this precise targeting, antibodies are widely used as diagnostic probes in routine laboratory tests, such as ELISA and immunofluorescence (Jones et al., 2022).
- **Detecting Autoimmune Diseases:** From a clinical standpoint, testing for specific autoantibodies in the blood is essential for early diagnosis, classification, and assessing the risk of autoimmune disorders (Pisetsky, 2023).
- **Neurological Strategy:** In neurology, finding antibodies against GAD or other surface proteins on neurons helps doctors choose the right treatment strategy and predict how a patient will recover (Brown et al., 2025; Gilligan et al., 2024).
- **Early Cancer Markers:** Recent progress in mapping the viral proteome has helped discover new biomarkers for cancer, often making it possible to detect malignancies before the patient shows any symptoms (Liang et al., 2024; Prüss, 2021).
- **Advantages of Nanobodies (VHH):** Single-domain antibodies, or nanobodies, provide major practical advantages over traditional antibodies, including much better heat stability and the ability to penetrate tissues more easily (Pillay & Muyldermans, 2021).
- **Improving ELISA Sensitivity:** Using these small VHH fragments in competitive ELISA (cELISA) setups significantly lowers the detection limit, making the tests much more sensitive than standard formats (Alexander & Leong, 2024; Duan et al., 2021).
- **Recombinant Antibody Production:** Phage display remains the primary method for isolating and screening high-affinity recombinant antibody fragments against new pathogens and biological toxins (Roth et al., 2021).
- **Point-of-Care (POC) Tests:** Monoclonal antibodies are the core component of lateral flow immunoassays (LFAs), which have greatly improved rapid, on-site testing for infectious diseases and drug abuse (Kasana et al., 2025; Parolo et al., 2020; Qriouet et al., 2021).

- **Next-Generation Biosensors:** Linking nanobodies directly to fluorescent proteins is a promising strategy to boost signal strength and accuracy in modern optical biosensors (Chen et al., 2023).

Building on the central role of antibodies in biomedical diagnostics, a wide range of antibody-based immunological tests have been developed for practical applications in clinical and research settings.

IV.2. Comparison Between Therapeutic and Diagnostic Antibodies

The advancement of antibody engineering has led to the development of different strategies for designing antibodies, depending on their therapeutic or diagnostic use. The Following table presents the main differences between them.

Table 5: Comparison Between Therapeutic and Diagnostic Antibodies (Lu et al., 2020), (Brady et al., 1987), (Ducancel & Muller, 2012). (Byrne et al., 2013).(Chakhtoura & Abdelnoor, 2010) (Kumar et al., 2024). (Shim, 2011).

Feature	Therapeutic Antibodies	Diagnostic Antibodies
Primary Objective	To neutralize pathogens, inhibit pathological signaling pathways, or eradicate malignant cells in diseases such as cancer and autoimmune disorders (Lu et al., 2020).	To localize lesions, characterize disease states through radio imaging, or quantify biomarkers in clinical samples (Brady et al., 1987).
Molecular Structure	Predominantly utilize full-length IgG molecules (chimeric, humanized, or fully human) to maintain stability and immune effector functions (Ducancel & Muller, 2012).	Often employ smaller antibody fragments (e.g., Fab, scFv) or bispecific scaffolds to facilitate superior tissue penetration and rapid clearance (Byrne et al., 2013).
Half-life	Engineered for a prolonged half-life to ensure sustained pharmacological activity and reduce dosing frequency (Chakhtoura & Abdelnoor, 2010).	Designed with a short half-life to minimize patient exposure to radiation and ensure rapid systemic elimination (Brady et al., 1987).

Conjugation	Conjugated with potent cytotoxic payloads (Antibody-Drug Conjugates - ADCs) or therapeutic radionuclides to induce cell death (Kumar et al., 2024).	Coupled with diagnostic radioisotopes (e.g., In-111), fluorophores, or enzymes to enable signal detection and visualization (Ducancel & Muller, 2012).
Mechanism of Action	Operate through effector-mediated mechanisms like ADCC and CDC, or by blocking ligand-receptor interactions to modulate biological responses (Shim, 2011).	Function as highly specific molecular probes that rely on high-affinity antigen binding to identify and signal the presence of a target (Byrne et al., 2013).
Pharmacokinetics	Characterized by high stability and slow clearance to maintain therapeutic concentrations within the target tissue over time (Lu et al., 2020).	Prioritize rapid distribution and fast blood clearance to optimize the signal-to-noise ratio for accurate imaging or sensing (Brady et al., 1987).

IV.3. Antibody-Based Immunological Tests

These diagnostic platforms are designed to detect specific antibodies or antigens within biological matrices, such as serum or other physiological fluids. The fundamental mechanism underlying these assays is the high-affinity biochemical interaction between a paratope and its corresponding epitope. These methodologies are indispensable in clinical diagnostics, therapeutic drug monitoring (TDM), and large-scale epidemiological surveillance.

IV.3.1. ELISA

The Enzyme-Linked Immunosorbent Assay (ELISA) is a widely utilized immunological tool in basic science research, clinical applications, and diagnostics (Hayrapetyan et al., 2023). It serves as an analytical method for the detection and quantification of specific antigens or antibodies within a given biological sample (Gan & Patel, 2013). The technique fundamentally relies on the specific interaction between an antigen and its corresponding antibody, where the presence of the target molecule is confirmed through enzymatic catalysis of an added substrate, producing measurable signals via spectrophotometry or luminometry (Hayrapetyan et al., 2023).

IV.3.1.1. Historical Development

The conceptual origins of ELISA are derived from the Radioimmunoassay (RIA) first described by Berson and Yalow in 1960 (Gan & Patel, 2013). Due to safety concerns regarding radioactivity, RIA was modified by replacing radioisotopes with enzymes as reporter labels (Gan & Patel, 2013). The modern ELISA was developed independently and simultaneously in the early 1970s by two research groups: Peter Perlmann and Eva Engvall at Stockholm University in Sweden, and Anton Schuurs and Bauke van Weemen in the Netherlands (Lequin, 2005).

IV.3.1.2. Classification of ELISA Techniques

The basic ELISA procedure sequentially involves plate coating, blocking, washing, signal generation, and plate reading (Gao et al., 2018). Methodologies are categorized into four primary iterations based on the arrangement of reagents:

- **Direct ELISA:** This method involves the binding of enzyme-conjugated primary antibodies directly to the antigen-coated plate (Hayrapetyan et al., 2023).

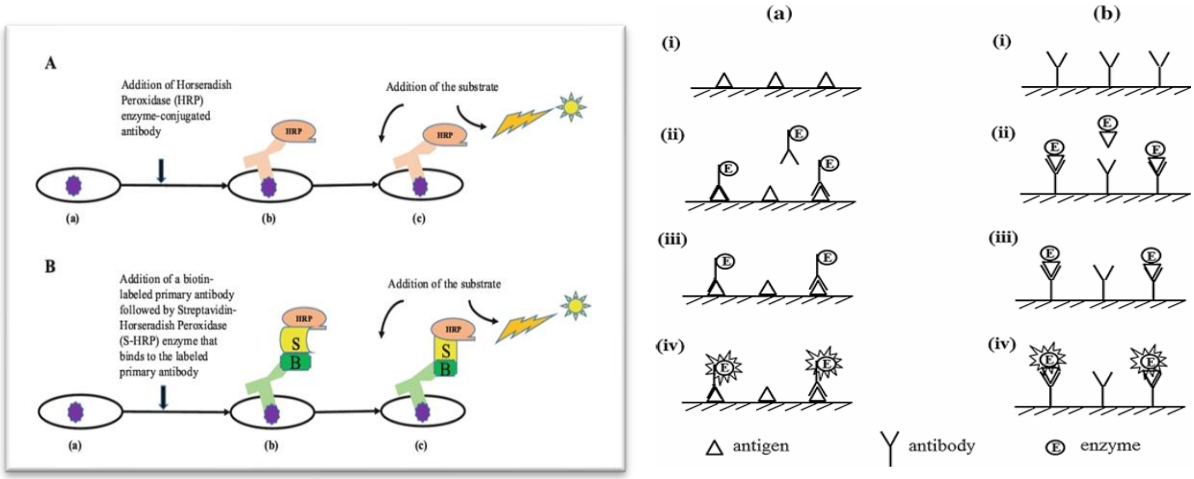


Figure 70: Schematic representation of Direct ELISA. (Reprinted from Hayrapetyan et al.,2023).

- **Indirect ELISA:** It introduces an enzyme-linked secondary antibody specific to a primary antibody that has already bound to the antigen-coated plate (Hayrapetyan et al., 2023).

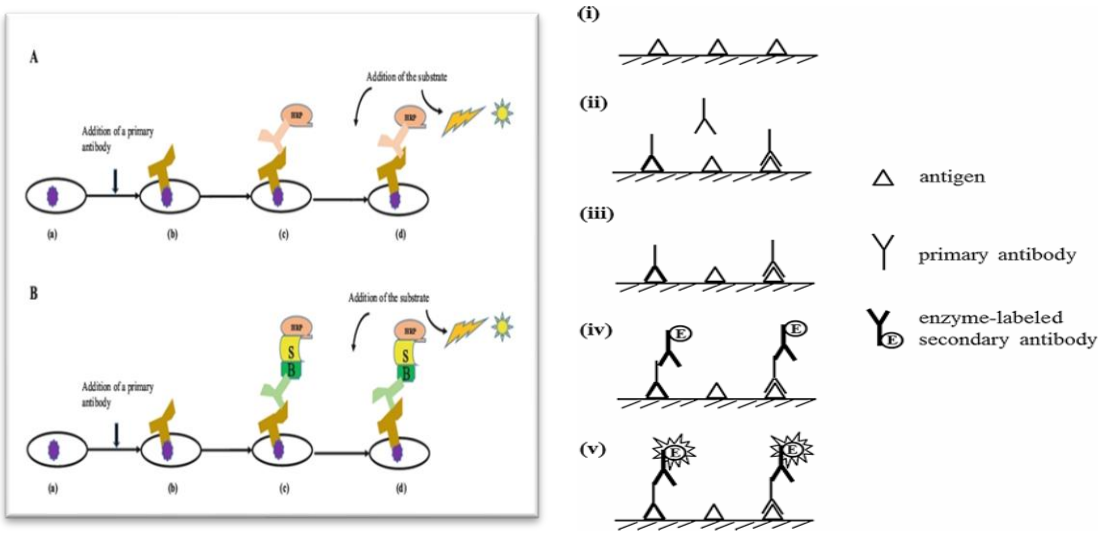


Figure 71 : Schematic Representation of Indirect ELISA for Antibody Detection. The procedure includes: (i) immobilization of antigen on a solid phase, (ii) incubation with primary antibody, (iii) washing to remove unbound primary antibody, (iv) incubation with enzyme-labeled secondary antibody, and (v) color development upon substrate addition. (Reprinted from Hayrapetyan et al., 2023; Sakamoto et al., 2018).

- **Sandwich ELISA:** This technique utilizes a capture antibody pre-coated on the plate to bound the sample antigen, followed by the addition of a detection antibody (Hayrapetyan et al., 2023). This format is noted for its high sensitivity and has been recently validated for measuring Factor XI in intravenous immunoglobulin products (Kim et al., 2026).

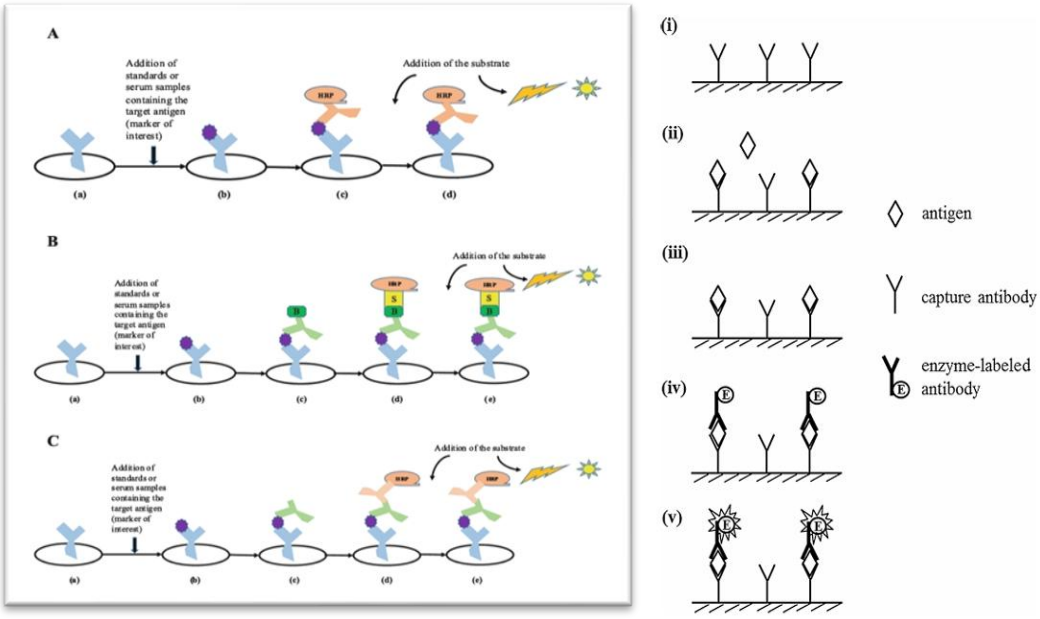


Figure 72: Schematic Representation of Sandwich ELISA for Specific Antigen Detection. The procedure includes: (i) immobilization of capture antibody on a solid phase, (ii) incubation with the target antigen, (iii) washing to remove unbound antigen, (iv) incubation with enzyme-labeled detection antibody, and (v) color development upon substrate addition. (Reprinted from Hayrapetyan et al., 2023; Sakamoto et al., 2018)

- **Competitive ELISA:** This involves competition between the sample antigen and a plate-coated antigen for a limited amount of primary antibody; consequently, a weak color signal represents a higher concentration of antigen in the sample (Gan & Patel, 2013).

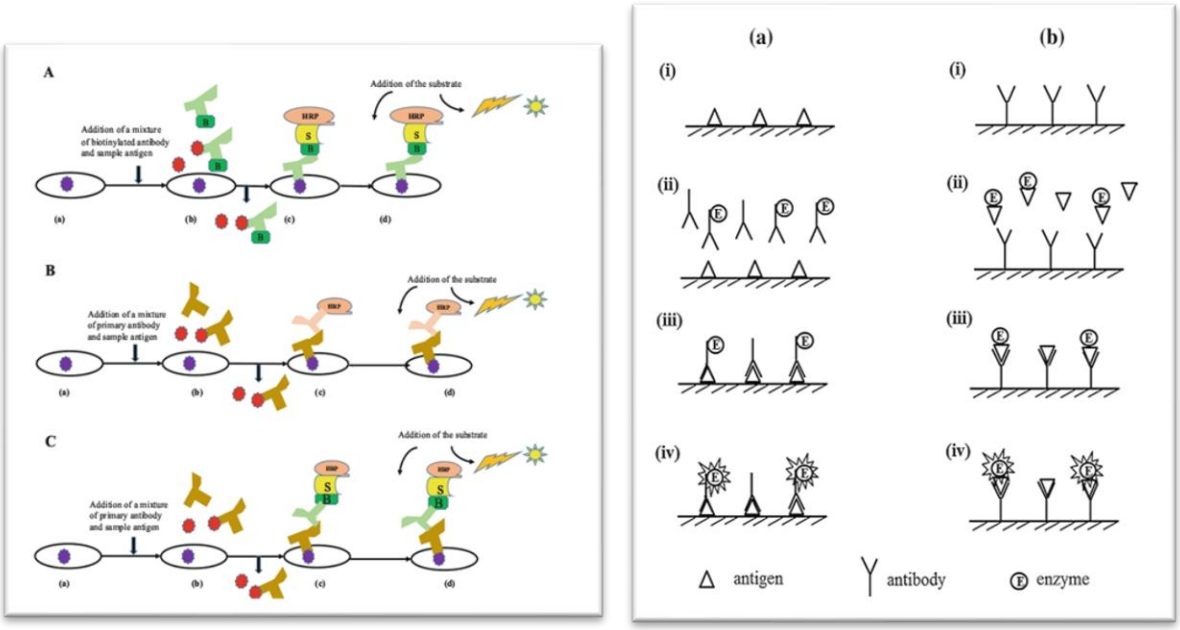


Figure 73: Schematic Representation of Direct ELISA for Antigen (a) and Antibody (b) Detection. The procedure involves: (i) immobilization of antigen/antibody on a solid phase, (ii) incubation with enzyme-labeled antibody/antigen, (iii) washing to remove unbound conjugates, and (iv) color development upon substrate addition. (Hayrapetyan et al., 2023; Sakamoto et al., 2018).

	Direct ELISA	Competitive ELISA	Indirect ELISA	Indirect competitive ELISA	Sandwich ELISA
Advantage	Simple because only one antibody is used		Higher sensitivity and versatility than direct methods owing to usage of PAb that recognizes different epitopes of primary antibody		High specificity as two antibodies possessing different epitopes are used
Disadvantage	Labeling antibody is necessary for each ELISA, which may result in inactivation of antibody		Nonspecific signal is induced through cross-reactivity of secondary antibody		To prepare two different antibodies is labor-intensive and expensive
Target	Macromolecules	Macromolecules (Hapten)	Macromolecules	Macromolecules (Hapten)	Generally macromolecules
Signal (as target antigen increase)	Increase	Decrease	Increase	Decrease	Increase

Figure 74 Analytical Characteristics of Various ELISA Techniques for Quantitative and Qualitative Applications (Sakamoto et al., 2018)

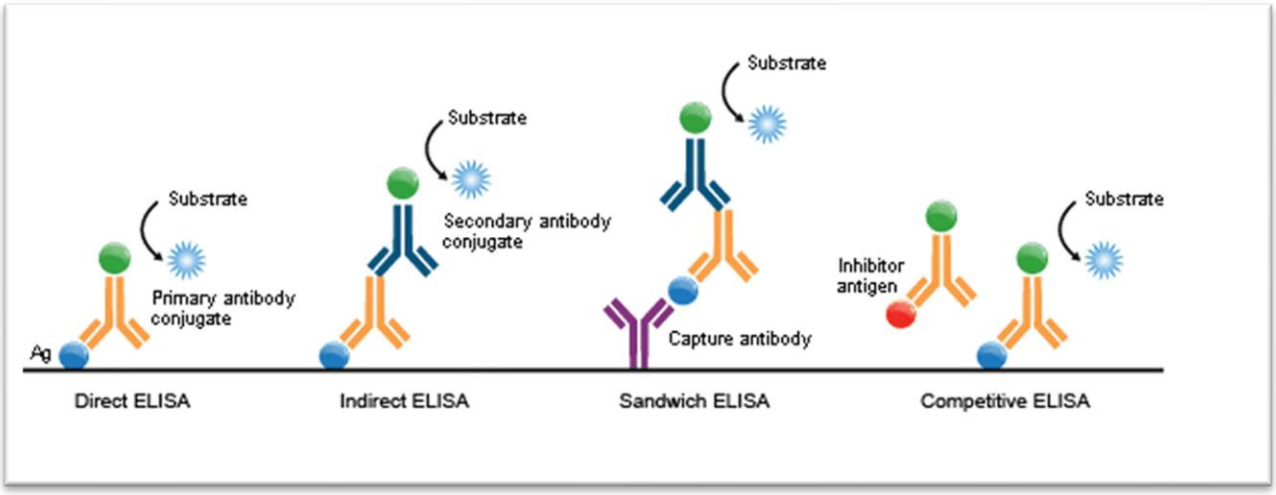


Figure 75 Comparison between ELISA types (Direct, Indirect, Sandwich, and Competitive). (Boster Biological Technology, 2022)

IV.3.1.3. Advanced Signal Generation Methods

Beyond traditional enzyme-conjugation, novel methods have been developed to enhance sensitivity:

- **Immuno-PCR:** Replaces the enzyme with a DNA sequence that is replicated into millions of copies via polymerase chain reaction for massive signal amplification (Gao et al., 2018).
- **Fluorophore-DNA Barcodes:** A system that conjugates detection antibodies with labeled nucleic acids on microbeads. Upon the formation of immune complexes on magnetic particles, fluorescent nucleic acids are released and quantified (Gao et al., 2018).

IV.3.1.4. Research and Clinical Significance

ELISA is a highly adaptable tool essential for studying humoral and cellular immune responses, a role that became particularly prominent during the SARS-CoV-2 pandemic (Portilho et al., 2022). Its utility extends to the precise detection of various biological molecules, including proteins, peptides, and hormones, even at low concentrations (Gan & Patel, 2013). While it remains a standard for quality control in the biotechnology industry (Gan & Patel, 2013), commercial kits sometimes fail to meet the specific requirements of specialized proteome research (Gao et al., 2018). As a result, researchers often develop "in-house" ELISA methods; however, this remains a complex, multidisciplinary task requiring extensive optimization over months or years (Gao et al., 2018)

IV.3.2. The Western Blotting Technique: Based on Antigen-Antibody Interaction

Western blotting, also referred to as protein immunoblotting, is a cornerstone laboratory technique utilized for the identification and quantification of specific proteins within complex biological mixtures. Originally described by Towbin et al. (1979) and subsequently named by Burnette (1981), this method relies fundamentally on the highly specific antigen-antibody interaction to isolate target molecules from cellular or tissue extracts (Gwozdz & Dorey, 2017; Mahmood & Yang, 2012). It remains an indispensable staple in contemporary biomedical research and diagnostics (Begum et al., 2022).

IV.3.2.1. Methodological Procedures

The execution of a Western blot involves a standardized sequence of technical phases:

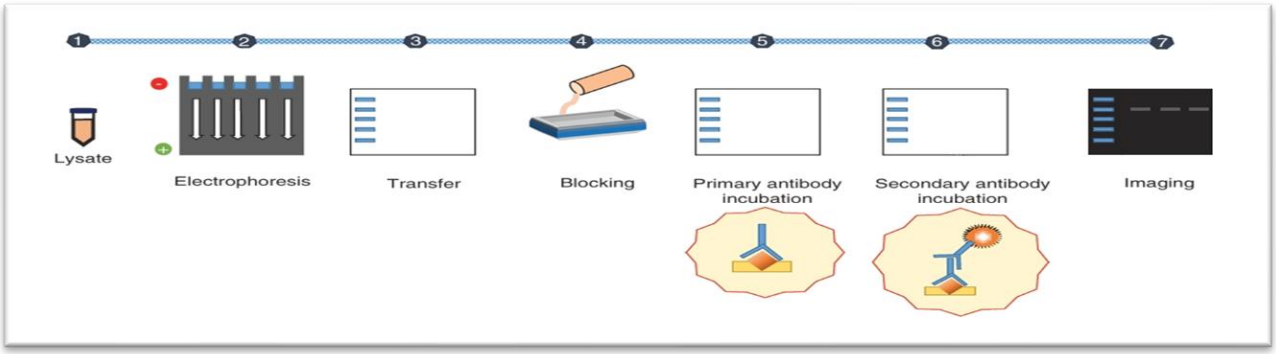


Figure 76: Schematic representation of the standard Western blotting workflow .(Begum et al., 2022)

- **Sample Preparation:** Proteins are extracted via cellular lysis using specialized chemical buffers. This process requires the inclusion of protease inhibitors to prevent enzymatic degradation and maintain the integrity of the protein samples (Gwozdz & Dorey, 2017).
- **Electrophoresis:** The extracted proteins are separated based on their molecular weight using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). During this stage, proteins migrate through the gel matrix under the influence of an electromagnetic field (Mahmood & Yang, 2012).

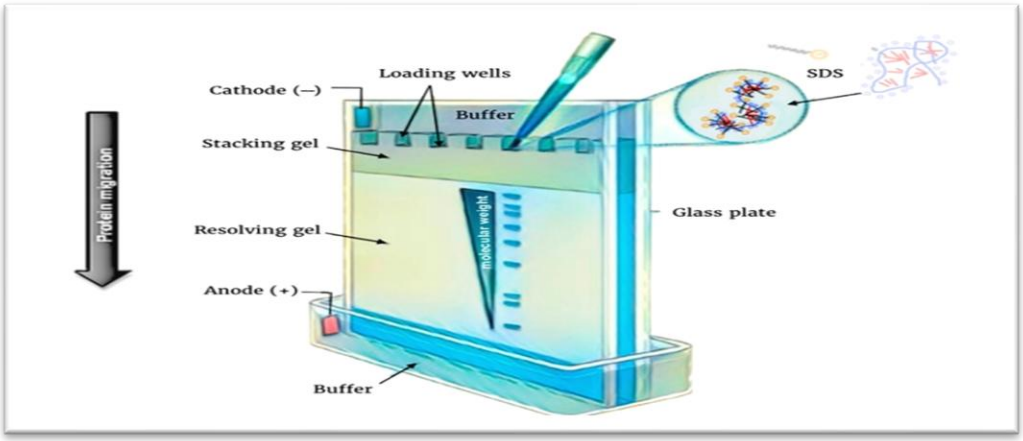


Figure 77: Illustration of protein separation using sodium dodecyl sulfate-polyacrylamidegel electrophoresis (SDS-PAGE).(Gwozdz & Dorey,2017)

- **Electro transfer:** Following separation, proteins are transferred from the gel onto a solid support membrane, typically composed of nitrocellulose or polyvinylidene difluoride (PVDF). This immobilizes the proteins for subsequent analytical steps (Gwozdz & Dorey, 2017).

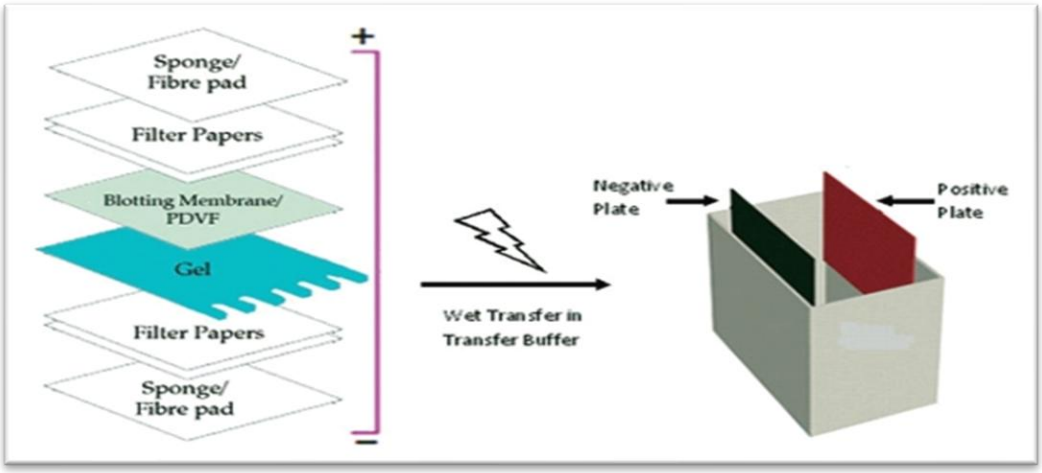


Figure 78: Electrotransfer setup for Western blotting, illustrating the standard "sandwich" assembly**.** (Mahmood & Yang, 2012)

- **Blocking:** The membrane is incubated in a blocking solution (such as non-fat dry milk or BSA) to saturate unoccupied binding sites on the membrane surface. This stage is crucial for minimizing non-specific binding and reducing background noise (Mahmood & Yang, 2012).
- **Antibody Incubation:** The membrane is first treated with a primary antibody designed to recognize the target protein. After washing, a secondary antibody conjugated to a reporter

enzyme is applied, which binds specifically to the primary antibody (Gwozdz & Dorey, 2017).

- **Detection:** The final phase involves the visualization of the protein bands through chemiluminescence or other imaging techniques. The signal intensity and band thickness are directly proportional to the concentration of the target protein in the sample (Mahmood & Yang, 2012).

IV.3.2.2. Scientific Applications

Western blotting is extensively employed to evaluate relative levels of protein expression and to determine the molecular weight of specific proteins (Mahmood & Yang, 2012). It is also an essential tool for investigating post-translational modifications, including phosphorylation and ubiquitination (Gwozdz & Dorey, 2017). To ensure the acquisition of reliable quantitative data, the implementation of standardized protocols and optimized analytical controls is mandatory (Taylor et al., 2013).

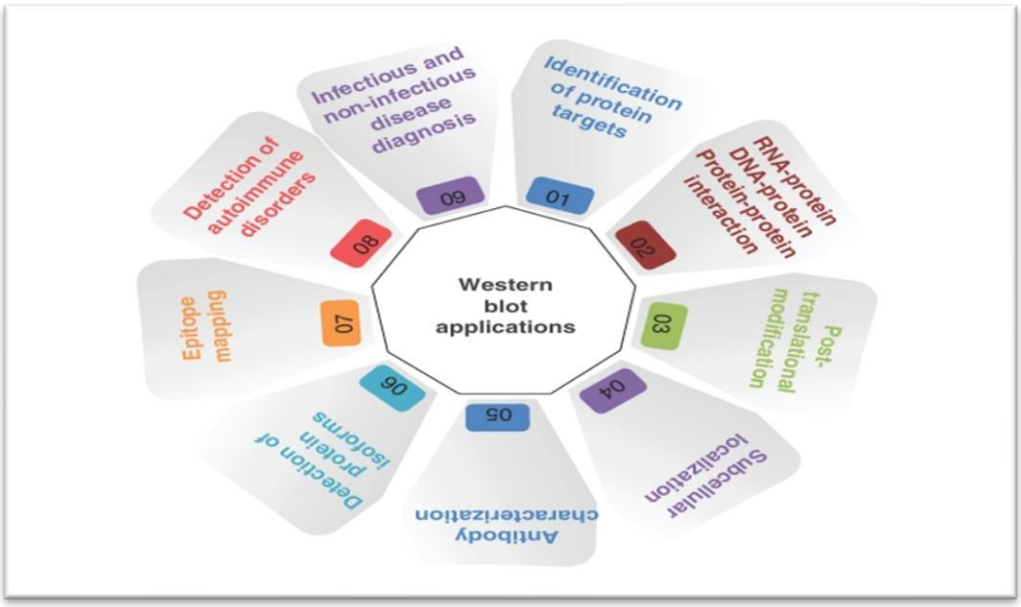


Figure 79: Diverse scientific, clinical, and diagnostic applications of the Western blot technique. (Begum et al., 2022)

IV.3.2.3. Clinical and Diagnostic Applications

Beyond its fundamental role in proteomics research, Western blotting is recognized as a robust tool in clinical diagnostics. The high specificity of the antigen-antibody interaction allows this technique to be utilized in the immunodiagnosis of various pathological conditions. In the field of oncology, it is employed to identify tumor-associated antigens and potential diagnostic

biomarkers for diseases such as laryngeal carcinoma and hepatocellular carcinoma (Begum et al., 2022). Furthermore, Western blotting serves as a critical confirmatory assay in clinical settings to verify the presence of specific proteins associated with infectious agents and stress-related disorders. Its ability to provide precise qualitative data regarding the presence or absence of a target antigen makes it an essential method for clinical researchers seeking to validate preliminary screening results (Gwozdz & Dorey, 2017).

Table 6: Comparative Analysis of Analytical Performance and Operational Parameters of ELISA and Western Blotting. (Kasana et al., 2025)

Detection Method	Sensitivity	Specificity	Ease of Use	Advantages	Disadvantages
ELISA	High (Typically, >10-100 pg; depends on substrate and antibodies used)	Highly specific	Requires trained manpower and is labor intensive	Highly sensitive and specific, faster than Western blot	Time consuming (at least 2-3 h), requires specialized equipment, expensive
Western blot	High (Typically, >100 pg; depends on substrate, membrane, and antibodies used)	Highly specific	Requires trained manpower and is labor intensive	Highly sensitive and specific	Time consuming (at least 4-6 h), requires specialized equipment, expensive

IV.3.3. Immunohistochemistry (IHC)

IV.3.3.1. Theoretical Foundation and Antigen–Antibody Recognition

Immunohistochemistry (IHC) is a fundamental immunolabeling technique that combines classical histopathology with molecular detection through highly specific antigen–antibody interactions (Duraiyan et al., 2012; Kabir et al., 2024). The method relies on the identification of

tissue antigens using antibodies conjugated either directly or indirectly to enzymatic or fluorescent detection systems. Following antigen recognition, chromogenic substrates such as 3,3′-Diaminobenzidine (DAB) generate an insoluble colored precipitate at the antigenic site, thereby enabling microscopic visualization while preserving tissue morphology (Schimanski et al., 2025; Kim et al., 2016). Consequently, IHC allows the precise spatial localization of proteins within intact tissue architecture and provides critical information regarding cellular differentiation, pathological alterations, and biomarker expression patterns (Schacht & Kern, 2015).

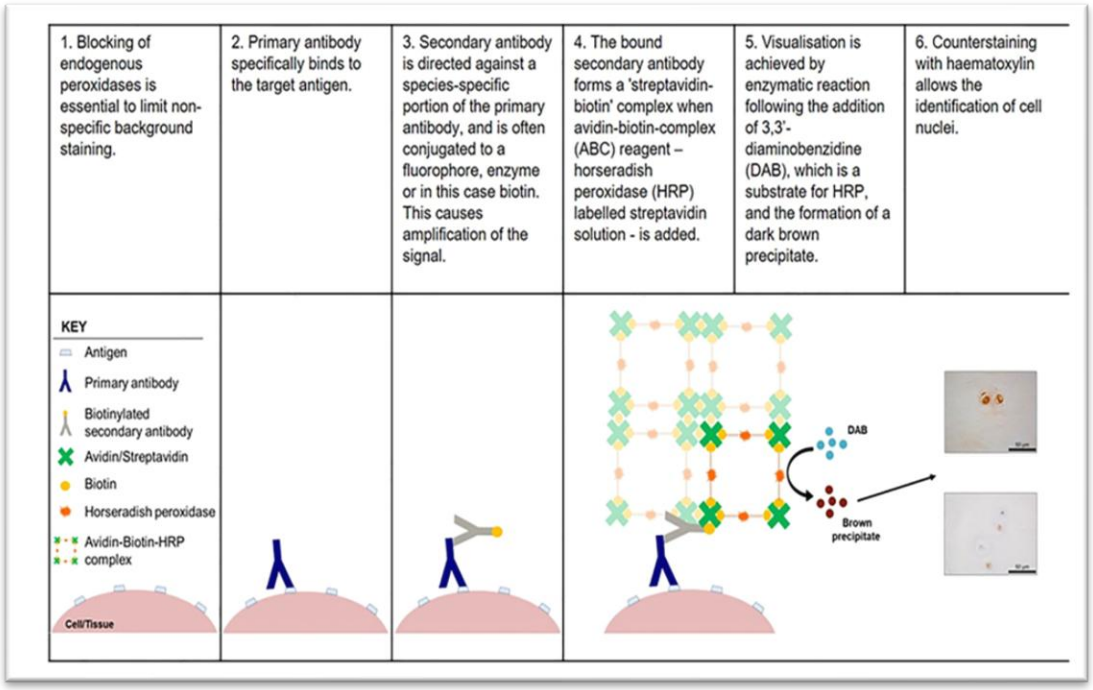


Figure 80 : Principle of Immunohistochemistry. Target antigens are detected by the application of specific primary antibodies, followed by the addition of a biotin conjugated secondary antibody which recognizes a specific sequence on the primary antibody. Localisation of bound biotinylated secondary antibody is detected by the addition of streptavidin HRP labeled ABC solution which binds to the biotin on the secondary. Enzymatic color changes occur when DAB reacts with the HRP bound to the secondary antibody, thus resulting in the detection of the target antigen (Binch et al., 2021)

IV.3.3.2. Detection Strategies

The selection of an appropriate IHC detection system depends primarily on antigen abundance, tissue quality, and the degree of analytical sensitivity required (Kabir et al., 2024; Duraiyan et al., 2012)

• **Direct Immunohistochemistry:** This simplified approach utilizes a primary antibody directly conjugated to a detectable label such as horseradish peroxidase (HRP) or a fluorophore (Duraiyan

et al., 2012). Although this method minimizes procedural time and reduces no-specific interactions associated with secondary antibodies, its limited signal amplification restricts its application to highly expressed antigens (Kabir et al., 2024).

• **Indirect Immunohistochemistry:** In this widely adopted method, an unlabeled primary antibody is detected using a labeled secondary antibody directed against the host species of the primary antibody (Kim et al., 2016). Due to the ability of multiple secondary antibodies to bind a single primary antibody, indirect IHC provides substantial signal amplification and improved sensitivity, making it the gold standard in diagnostic pathology and research applications (Duraiyan et al., 2012).

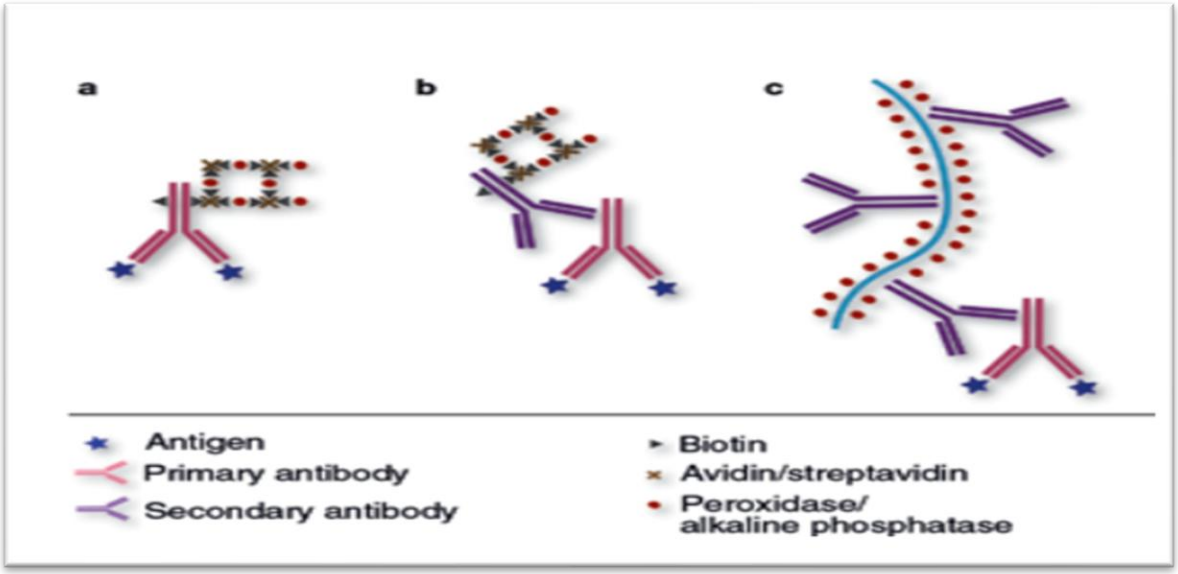


Figure 81 : Schematic diagram of immunohistochemical techniques. (a) Direct method: the antigen-specific primary antibody is biotin labeled. Biotin binds to avidin/streptavidin. Color visualization is achieved through enzymatic reaction of horseradish peroxidase/alkaline phosphatase. (b) Indirect method: the antigen-specific primary antibody is unlabeled. The secondary, biotin-labeled antibody binds to primary antibody. Visualization is achieved accordingly through avidin/streptavidin and peroxidase/alkaline phosphatase complexes. The indirect method increases versatility because unlabeled primary antibodies can be used. (c) Indirect method with polymer chain detection system. Biotin and avidin/streptavidin are replaced by a labeled polymer chain, allowing for increased sensitivity and specificity (Schacht & Kern, 2015).

• **Advanced Detection Systems (ABC, LSAB, and Polymer-Based Methods):**

To further enhance sensitivity, advanced amplification systems have been developed. The Avidin–Biotin Complex (ABC) and Labeled Streptavidin–Biotin (LSAB) methods exploit the high affinity between biotin and streptavidin to intensify chromogenic signals (Duraiyan et al., 2012). More recently, polymer-based non-biotin systems have gained prominence due to their superior sensitivity and reduced background staining caused by endogenous biotin activity (Kabir et al., 2024).

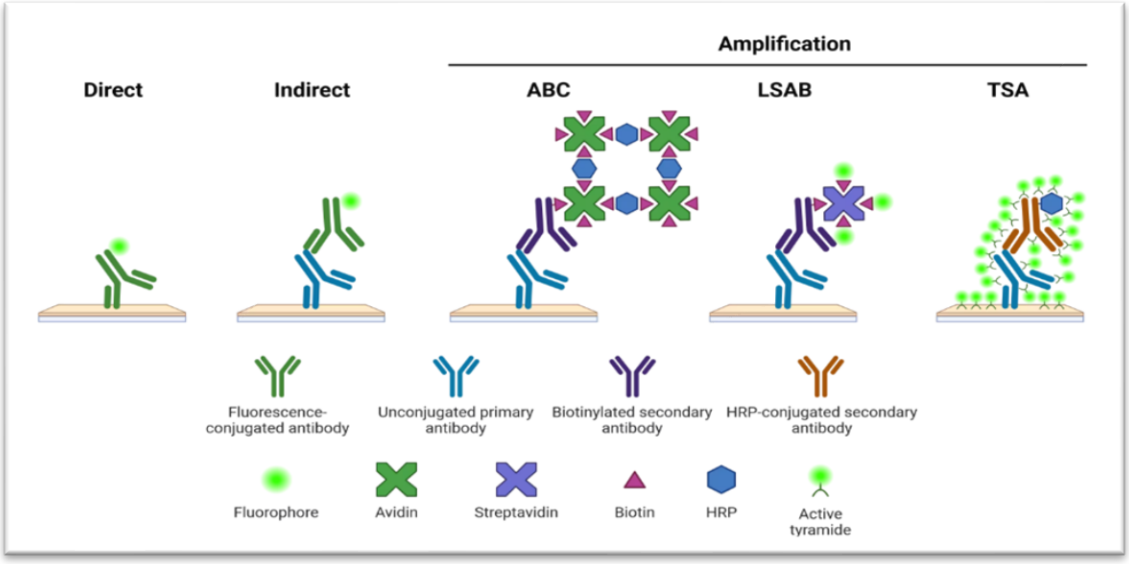


Figure 82: Schematic representation of different methods of target detection. Methods of amplification include the avidin-biotin complex (ABC), labeled streptavidin-biotin (LSAB) and tyramide signal amplification (TSA) systems. Note that direct, indirect and LSAB methods can be either chromogenic or fluorescent. ABC is typically chromogenic, and TSA is always fluorescent. (Stewart & Hamnett, 2025).

IV.3.3.3. Optimization of Critical Experimental Parameters

Obtaining reproducible and high-specificity IHC staining requires rigorous optimization of both pre-analytical and analytical parameters. The principal experimental stages are summarized below:

▪ **Fixation and Tissue Processing:** Proper tissue fixation is essential for preserving both cellular morphology and antigenicity. According to CAP recommendations, specimens are commonly fixed in 10% neutral buffered formalin for 12–48 hours (Kabir et al., 2024). Following paraffin embedding, tissue sections are generally cut at 4–5 µm thickness and mounted on positively charged slides to ensure adequate adhesion during staining procedures (Magaki et al., 2018).

▪ **Antigen Retrieval:** Formalin fixation induces methylene bridge cross-linking that may mask antigenic epitopes. Therefore, antigen retrieval is necessary to restore epitope accessibility. This is achieved either through Heat-Induced Epitope Retrieval (HIER) using buffered heating systems or Proteolytic-Induced Epitope Retrieval (PIER) employing enzymatic digestion methods (Yigzaw & Dagnaw, 2024).

▪ **Blocking of Non-Specific Reactions:** To improve the signal-to-noise ratio, endogenous enzymatic activity and non-specific protein interactions must be blocked prior to antibody incubation. Endogenous peroxidase activity is commonly quenched using hydrogen peroxide (H2O2), while non-specific binding sites are saturated using bovine serum albumin (BSA) or normal serum solutions (Kim et al., 2016).

▪ **Experimental Controls and Validation:** The reliability of immunohistochemical staining depends on the implementation of rigorous quality controls. Positive controls confirm antibody reactivity, whereas negative controls and isotype controls help identify non-specific staining or technical artifacts (Kabir et al., 2024). Standardization of staining protocols is therefore essential for accurate interpretation and diagnostic reproducibility.

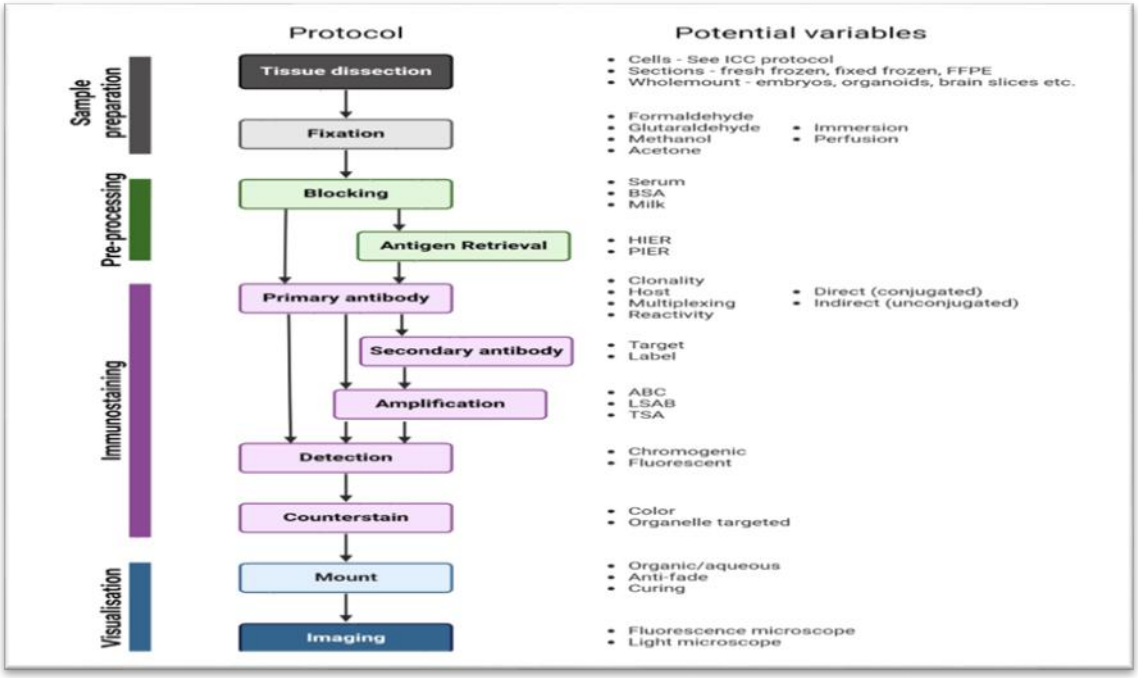


Figure 83: Generalized protocol for IHC (Stewart & Hamnett, 2025).

IV.3.3.4. Advanced Applications and Digital Pathology

Recent advances in IHC have expanded its applications beyond conventional single-marker analysis. Multiplex Immunohistochemistry (mIHC) now enables the simultaneous visualization of multiple biomarkers within the same tissue section, facilitating comprehensive characterization of the tumor microenvironment and cellular interactions (Harms et al., 2023; Sun et al., 2025).

In parallel, the integration of artificial intelligence (AI) and digital pathology platforms has significantly improved quantitative interpretation. Deep-learning algorithms applied to Whole Slide Images (WSI) can automate biomarker scoring, reduce observer variability, and even generate virtual IHC predictions directly from routine H&E sections (Bai et al., 2021; Ghahremani et al., 2022). Despite these technological advances, challenges such as tissue autofluorescence, fixation variability, antigen degradation, and subjective interpretation continue to affect analytical accuracy. Consequently, strict quality assurance protocols remain indispensable for maintaining diagnostic reliability (Kabir et al., 2024)

Table 7: Key applications and differences between common biochemical techniques for protein detection. (Stewart & Hamnett, 2025).

	ICC/IHC	Wes tern Blot	ELISA
Sample Preparation	Fixed cells on coverslip (ICC) Fixed tissue sectioned or wholemount (IHC)	Lysed cells Denatured protein	Lysed cells or tissue Biological fluids (plasma, urine, cell culture supernatant)
Protein State	In situ, but fixed	Denatured	Native, unfixed
Multiplex	Easily up to 4 targets; more are possible through unmixing or sequential probing	Fluorescent multiplexing is possible, as is sequential blotting (stripping and re-blotting the same membrane	No, typically requires bead-based immunoassays for multiplexing
Can be high throughput?	Yes	Rarely	Commonly

Sensitivity * ability to detect low levels of target	Medium	High	High
Specificity * antibody binding only the target protein	Medium	High	High, particularly sandwich ELISAs
Subcellular compartmentalization of proteins	Highly suitable	Limited to subcellular fractionation before application	Limited to subcellular fractionation before application
Expression in mixed cell populations	Highly suitable	Limited to cell sorting before application	Limited to cell sorting before application
Protein-protein interactions	Limited to co-localization in cells or subcellular compartments	Yes, through far-western blotting[1]	Yes[2]
Protein weight or size	None	Highly suitable	None

IV.3.4. Immunofluorescence (IF):

IV.3.4.1. . Theoretical Foundation and Molecular Recognition

Immunofluorescence (IF) represents a cornerstone of modern bio-imaging, combining the high specificity and affinity of antigen–antibody interactions with the exceptional sensitivity of fluorescence microscopy (Abbas et al., 2021; CST, 2024). This technique is based on the conjugation of fluorophores to antibodies, which emit photons upon excitation at a defined wavelength. This process follows the Stokes shift principle, where emitted light exhibits a longer wavelength and lower energy than the absorbed light (Murphy & Davidson, 2012; Thermo Fisher, 2024). As a result, IF enables high-resolution visualization and precise spatial localization of target biomolecules while preserving their structural organization within the native cellular environment (Abbas et al., 2021; Murphy & Davidson, 2012).

IV.3.4.2. Detection Strategies

The selection of an appropriate IF strategy is primarily dictated by the relative abundance of the target antigen and the experimental sensitivity thresholds required (Harlow & Lane, 2014; CST, 2024):

- **Direct Immunofluorescence:** This streamlined technique employs a primary antibody directly labeled with a fluorophore (Abbas et al., 2021). While it significantly reduces procedural time and minimizes potential cross-reactivity, its lack of inherent signal amplification restricts its utility to the detection of highly expressed antigens (Abbas et al., 2021; CST, 2024).

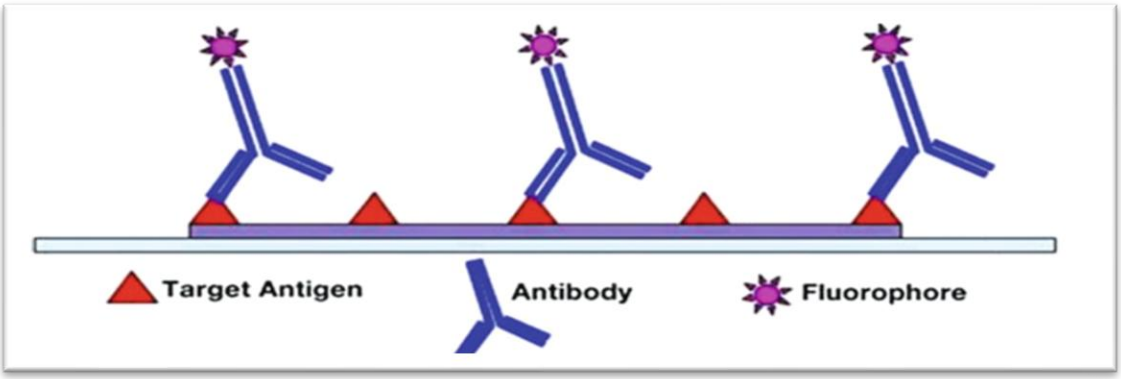


Figure 84: Schematic Representation of the Direct Immunofluorescence (DIF) Technique for Antigen Detection (Im et al., 2019).

- **Indirect Immunofluorescence:** This multi-step approach involves the sequential application of an unlabeled primary antibody followed by a fluorophore-conjugated secondary antibody (Wheatley & Wang, 1998). It remains the gold standard in research due to its enhanced sensitivity; multiple secondary antibodies can bind to various epitopes on a single primary antibody, effectively amplifying the fluorescent signal (Harlow & Lane, 2014; CST, 2024).

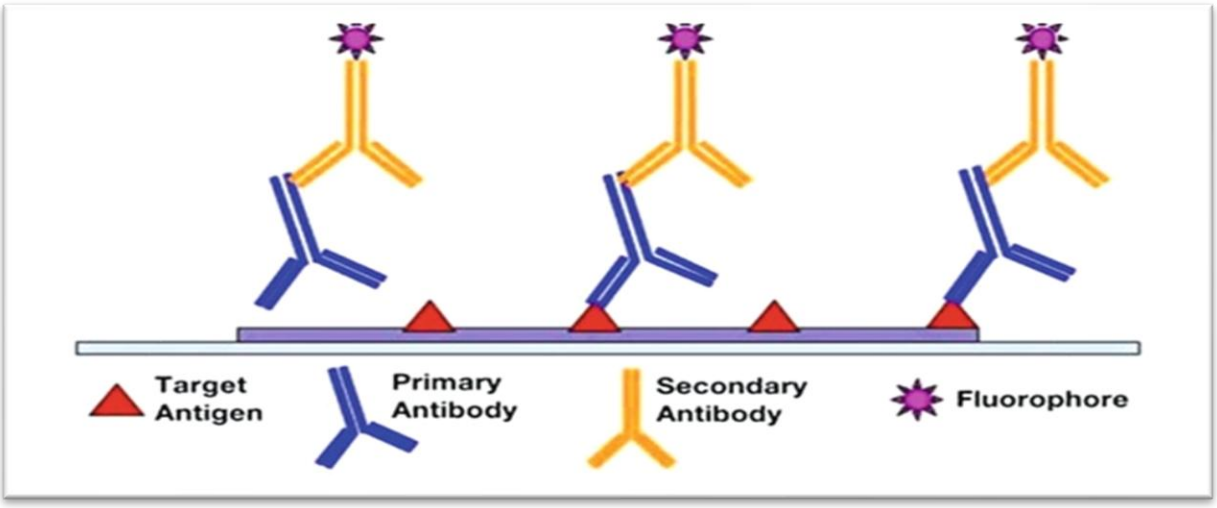


Figure 85: Indirect Immunofluorescence Technique (Im et al., 2019).

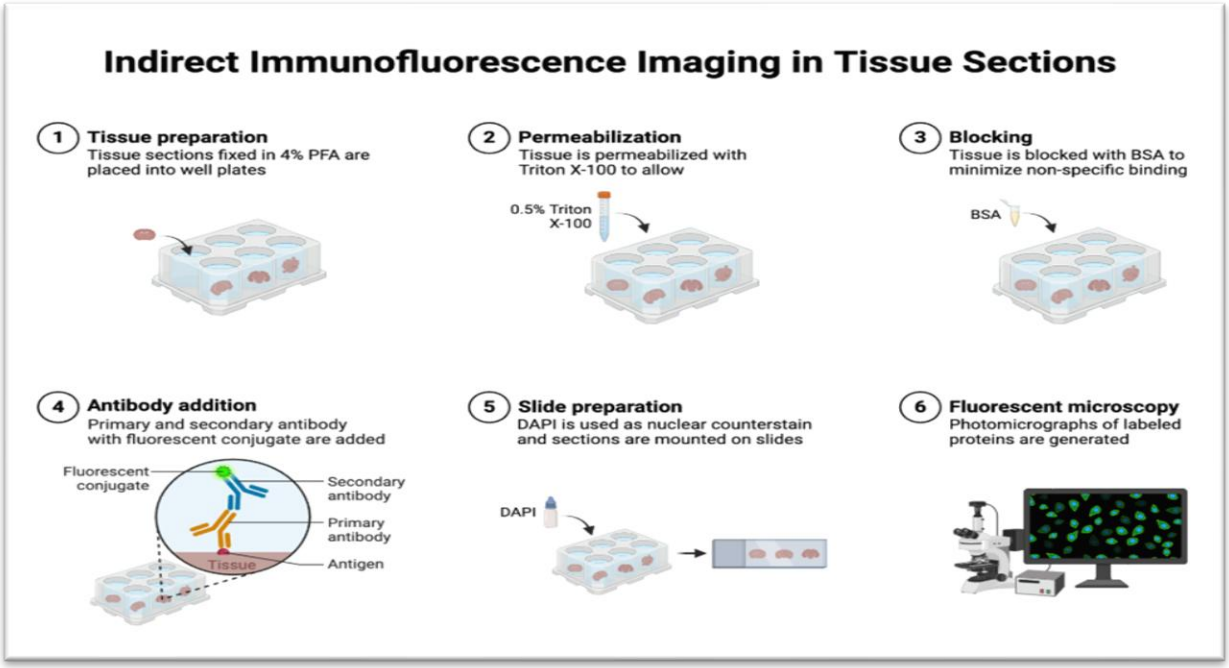


Figure 86 : Indirect Immunofluorescence Imaging in Tissue Sections (Kelly et al., 2022).

- **Advanced Amplification (Biotin-Avidin & TSA):** For low-abundance targets, signal intensity can be further augmented using biotinylated secondary antibodies paired with streptavidin complexes or through Tyramide Signal Amplification (TSA), which utilizes enzymatic reactions to deposit fluorophores locally (CST, 2024; Thermo Fisher, 2024).

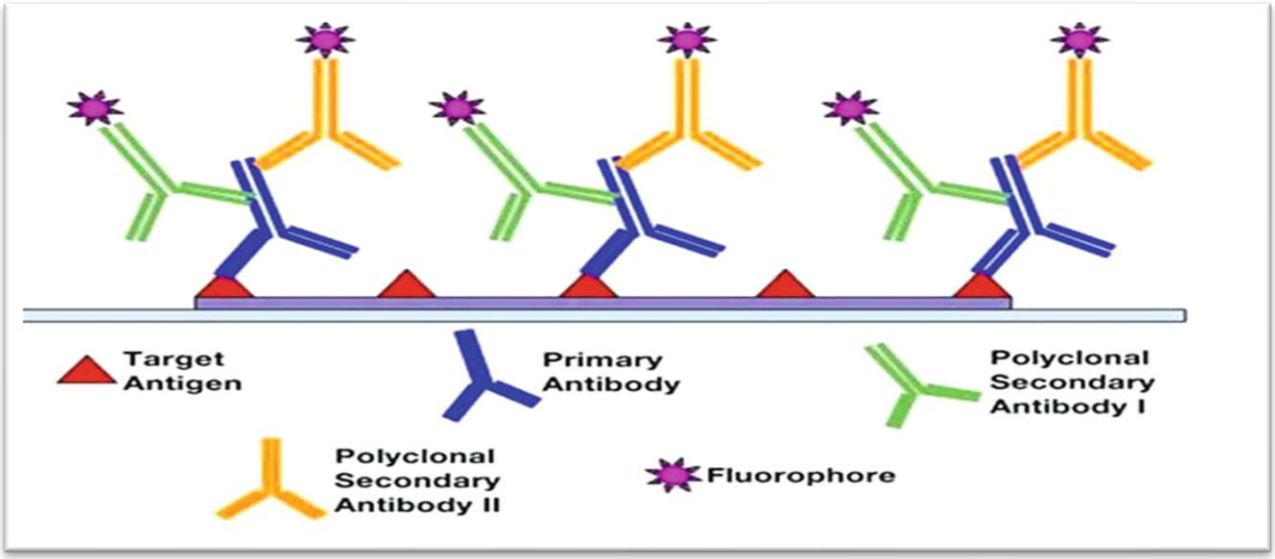
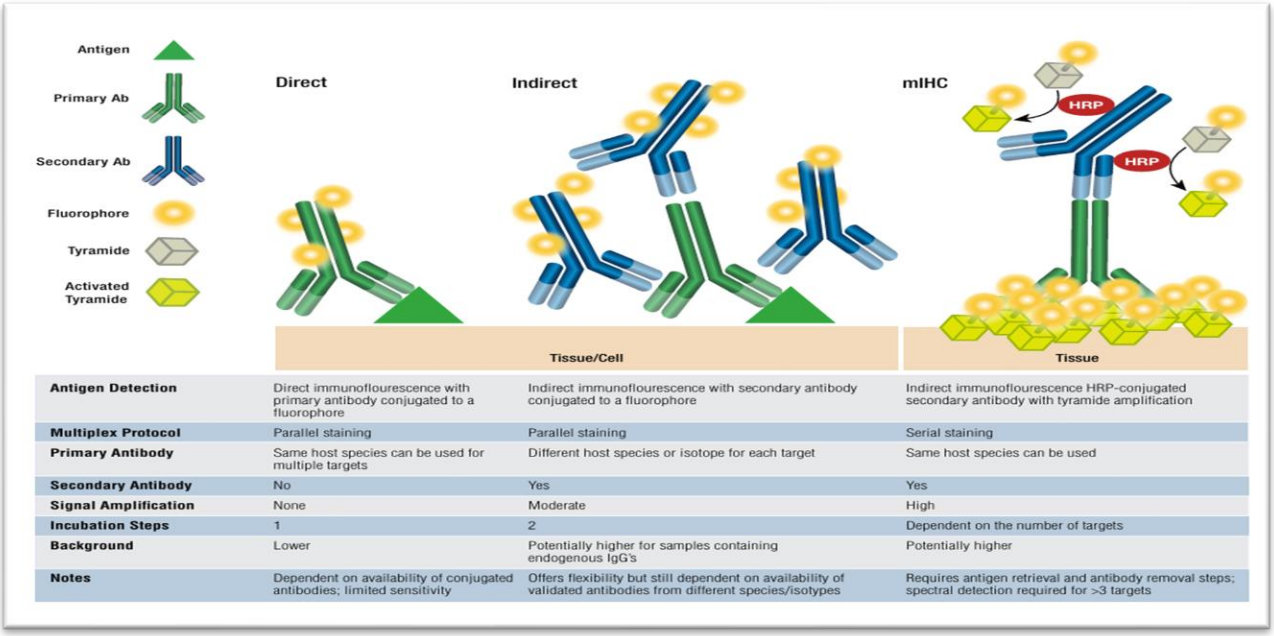


Figure 87: Signal Amplification Strategies utilizing Polyclonal Secondary Antibodies and Biotin-Streptavidin Complexes. (Im et al., 2019).



Antigen Detection	Direct immunoflourescence with primary antibody conjugated to a fluorophore	Indirect immunoflourescence with secondary antibody conjugated to a fluorophore	Indirect immunoflourescence HRP-conjugated secondary antibody with tyramide amplification
Multiplex Protocol	Parallel staining	Parallel staining	Serial staining
Primary Antibody	Same host species can be used for multiple targets	Different host species or isotope for each target	Same host species can be used
Secondary Antibody	No	Yes	Yes
Signal Amplification	None	Moderate	High
Incubation Steps	1	2	Dependent on the number of targets
Background	Lower	Potentially higher for samples containing endogenous IgG's	Potentially higher
Notes	Dependent on availability of conjugated antibodies; limited sensitivity	Offers flexibility but still dependent on availability of validated antibodies from different species/isotypes	Requires antigen retrieval and antibody removal steps; spectral detection required for >3 targets

Figure 88: Technical Principles and Optimization Strategies for Successful Immunofluorescence Assays (Cell Signaling Technology, 2017)

IV.3.4.3. Optimization of Critical Experimental Parameter

Achieving high-fidelity and reproducible immunofluorescence results necessitates a rigorous optimization of the experimental workflow to ensure both signal specificity and structural integrity. The comprehensive technical stages, ranging from systemic tissue stabilization to final microscopic acquisition, are systematically delineated in **Figure 89**

- **Fixation and Permeabilization:** As illustrated in the initial stages of the protocol (**Figure 89**Steps 1-2), tissue stabilization is a critical prerequisite. This is typically achieved via systemic perfusion and subsequent immersion in 4% Paraformaldehyde (PFA), which preserves cellular architecture by inducing protein cross-linking (Wheatley & Wang, 1998; CST, 2024).

Following tissue slicing, permeabilization is performed using non-ionic detergents such as Triton X-100 (**Figure89**, Step 4). This step is essential to disrupt lipid bilayers, thereby facilitating the penetration of bulky antibody complexes into the intracellular compartment (Wheatley & Wang, 1998).

- **Blocking of Non-Specific Binding:** To optimize the signal-to-noise ratio, blocking is an indispensable step (**Figure 89**, Step 4). By employing agents such as Bovine Serum Albumin (BSA) or normal serum, potential non-specific binding sites are saturated, preventing the primary and secondary antibodies from adhering to off-target epitopes or the mounting substrate (Abbas et al., 2021).
- **Experimental Controls and Validation:** The reliability of the fluorescent signal is verified through stringent controls. As depicted in the labeling phases (**Figure89** Steps 5-6), the precise application of primary and secondary antibodies must be validated using negative and isotype controls. These measures are crucial to confirm that the observed fluorescence reflects true antigen localization rather than non-specific IgG interactions or reagent artifacts (Hewitt et al., 2014).

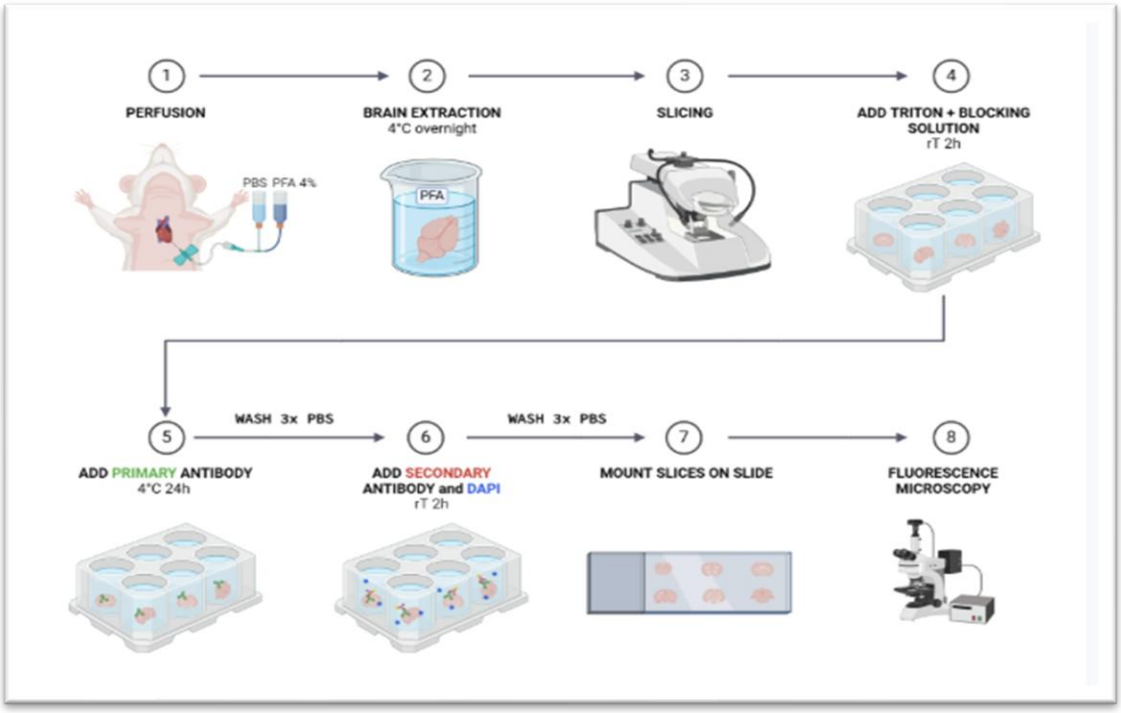


Figure 89: Schematic workflow of the experimental protocol for immunofluorescence in mouse brain tissue (De amici ,2026).

IV.3.4.4. Advanced Imaging Considerations

Modern immunofluorescence techniques often involve multiplexing, allowing the simultaneous detection of multiple targets using fluorophores with distinct emission spectra (Murphy & Davidson, 2012). However, image quality can be affected by photobleaching and autofluorescence, which may interfere with signal detection (Hewitt et al., 2014).

To minimize these effects, several strategies are applied, including the use of anti-fade mounting media, optimized optical filters, and more stable fluorescent dyes. These approaches help improve signal stability and ensure accurate imaging results (Thermo Fisher, 2024).

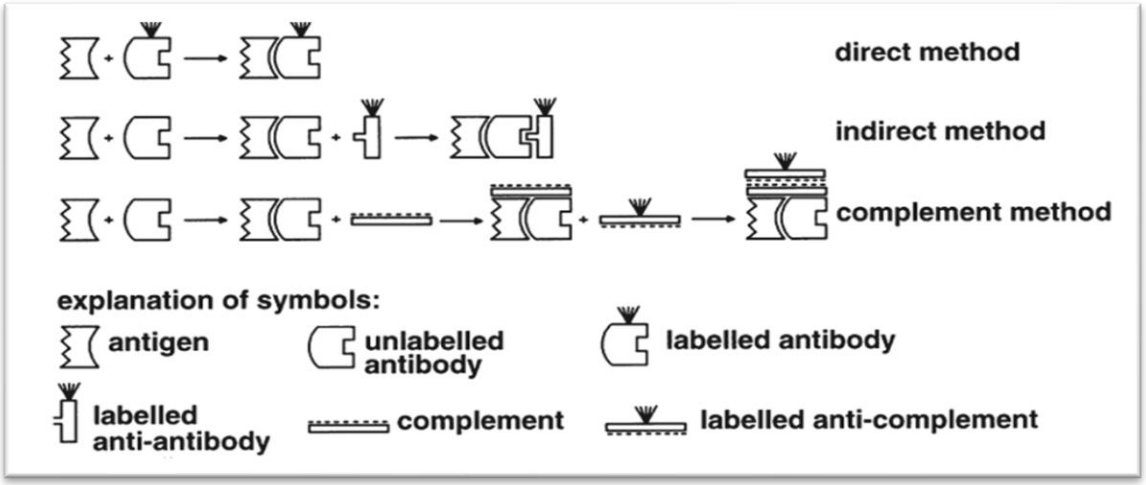


Figure 90 : Schematic representation of immunofluorescence techniques. (Storch, 2000, p. 2)

IV.3.5. Rapid Diagnostic Tests (RDTs)

Rapid diagnostic tests (RDTs) are simple, fast tests that give results in minutes to under an hour, often at the bedside or in the community. They are widely used for infectious diseases (like COVID 19, HIV, meningitis, sepsis) and for biomarkers such as cardiac markers and inflammation

IV.3.5.1. Introduction and Strategic Significance

Rapid Diagnostic Tests (RDTs) have fundamentally transformed the management of infectious diseases by providing immediate, decentralized diagnostic solutions (Majima, 2024). According to the World Health Organization (WHO), an ideal RDT must fulfill the "ASSURED" criteria: Affordable, Sensitive, Specific, User-friendly, Rapid, and Robust (Nabil et al., 2025). The clinical necessity for such tools was critically highlighted during the COVID-19 pandemic, where RDTs played a dual role in individual patient management and large-scale epidemiological surveillance (Jegerlehner et al., 2021; Nabil et al., 2025).

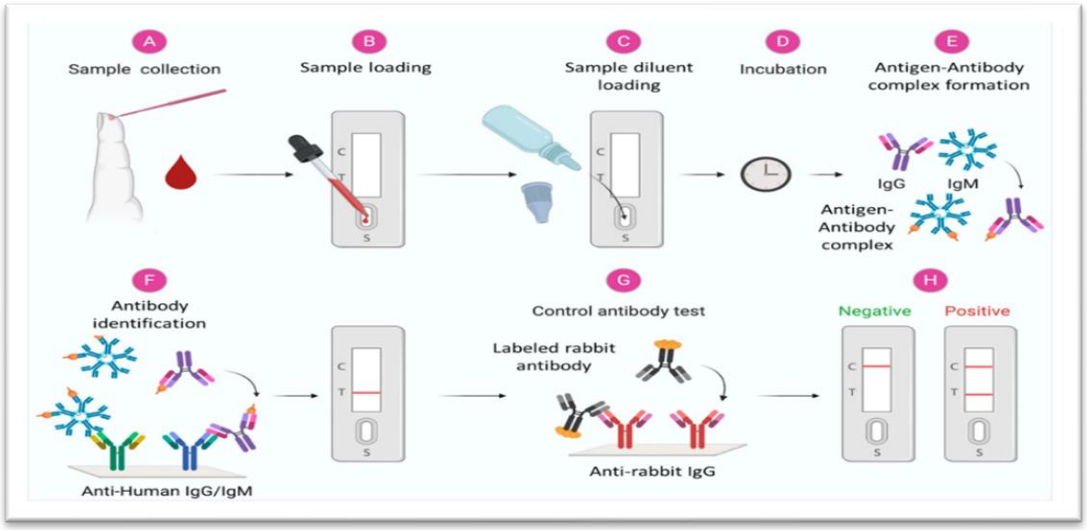


Figure 91 : general Procedure of Antibody-Based Rapid Diagnostic Testing for Viral Infections Using Lateral Flow Immunoassay (Augustine et al., 2020)

IV.3.5.2. Biochemical Principles and Analytical Rigor

Most RDTs are engineered using immunochromatographic techniques, which utilize the high affinity of antigen-antibody interactions to produce visual results (Nabil et al., 2025). In the context of viral diagnostics, the development of these assays relies heavily on the specificity of monoclonal antibodies, which serve as precise detection reagents for viral proteins (Kasana et al., 2025). The analytical performance of these assays is rigorously measured through sensitivity,

specificity, and predictive values, often interpreted via the Fagan nomogram to integrate pre-test probability into the diagnostic outcome (Nabil et al., 2025).

IV.3.5.3. . Multidisciplinary Clinical Applications

Rapid Diagnostic Tests (RDTs) are currently used in several microbiological and clinical fields due to their speed, simplicity, and ability to provide early diagnostic information.

- **Parasitology and Mycology:** RDTs are widely employed for malaria diagnosis through the detection of HRP2 antigens. They also play an important role in the early detection of cryptococcal infection in HIV-positive patients by identifying cryptococcal antigen (CrAg), which has contributed to reducing mortality rates (Nabil et al., 2025).

- **Virology:** Besides their major contribution during the COVID-19 pandemic, RDTs are commonly used for respiratory viral infections such as Influenza and Respiratory Syncytial Virus (RSV), as well as arboviral diseases including Dengue fever (Kasana et al., 2025; Nabil et al., 2025).

- **Bacteriology and Zoonotic Diseases:** Rapid serological techniques are widely applied in the diagnosis of bacterial zoonoses, particularly brucellosis. As reported in "*Précis de biopathologie: Analyses médicales spécialisées* (Biomnis, 2016)", several rapid assays are routinely used for the detection of *Brucella* infection. The Rose Bengal test is primarily employed as a screening tool due to its simplicity, low cost, and good sensitivity. Wright's Sero agglutination test is commonly used to detect anti-*Brucella* antibodies, especially during the acute phase of infection. More advanced methods, such as ELISA and indirect immunofluorescence, allow the identification of specific immunoglobulin classes, including IgM and IgG, and can help differentiate between acute and chronic forms of the disease. Overall, these serological approaches offer faster diagnostic support compared to conventional culture methods (Biomnis, 2016, pp. 262–263).

- **Meningitis and Clinical Bacteriology:** New generations of RDTs are being developed to improve the diagnosis of bacterial meningitis and other severe infections directly at the point of care. These approaches aim to increase diagnostic accuracy while reducing the delay associated with conventional laboratory techniques (Feagins et al., 2020).

- **Global Health and Antimicrobial Stewardship:** Rapid diagnostic tests are considered valuable tools for supporting antimicrobial stewardship by enabling earlier and more

targeted therapeutic decisions, although their implementation varies considerably across healthcare settings (Poole et al., 2021).

IV.3.5.4. Technical Challenges and Future Paradigms

Despite their utility, RDTs face limitations, particularly regarding lower sensitivity compared to molecular reference methods and susceptibility to environmental conditions (Nabil et al., 2025; Poole et al., 2021). To mitigate human error in interpretation, recent innovations have integrated Deep Learning Algorithms and mobile applications to automate the reading of RDT results, achieving sensitivity levels as high as 97.8% (Turbé et al., 2021).

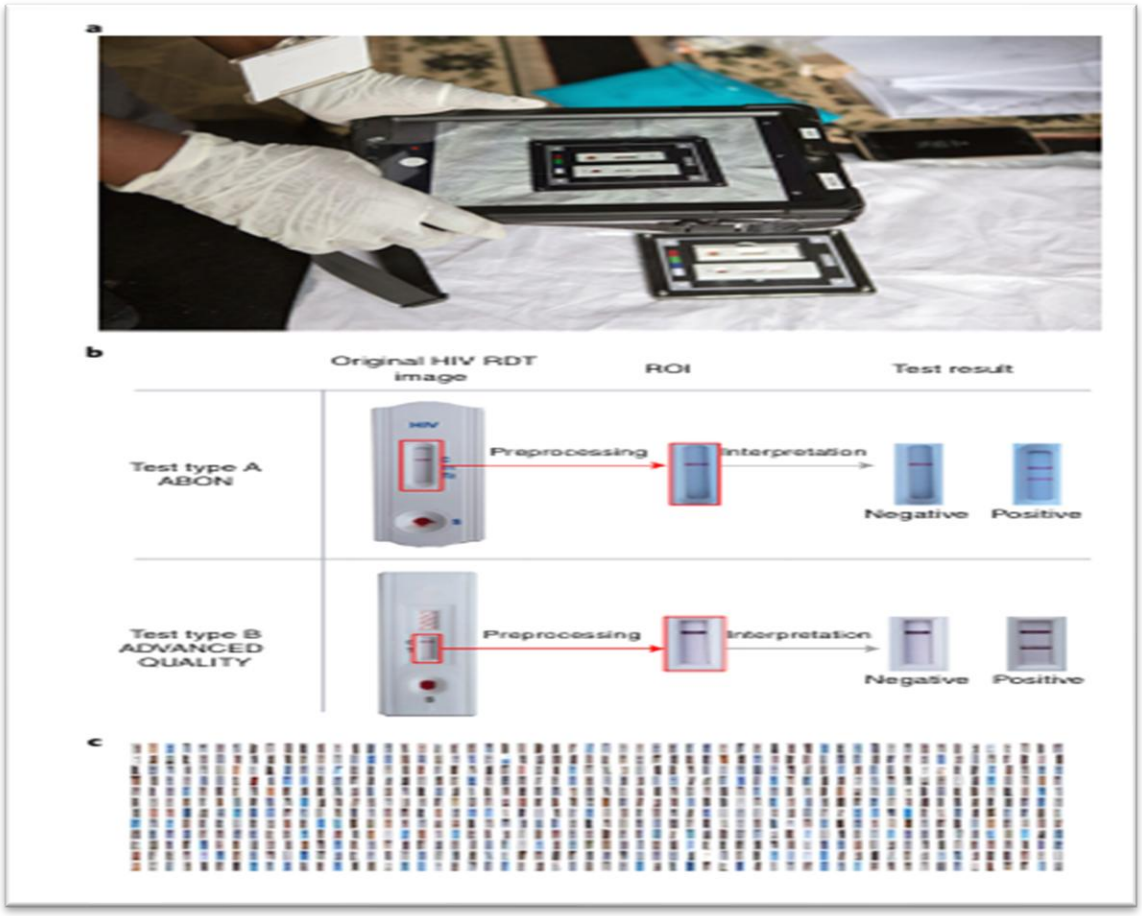


Figure 92 : Standardized protocol for digital image capture, preprocessing, and deep learning-based interpretation of HIV rapid diagnostic tests (RDTs) in field settings. (Turbé et al,2021).

Furthermore, the transition toward molecular RDTs and digital biosensors is bridging the gap between rapid testing and high-precision laboratory diagnostics. The widespread implementation of these tests also enhances antimicrobial stewardship, particularly in resource-limited settings, by guiding rapid therapeutic decisions and reducing inappropriate antibiotic use.s (Nabil et al., 2025; Majima, 2024).

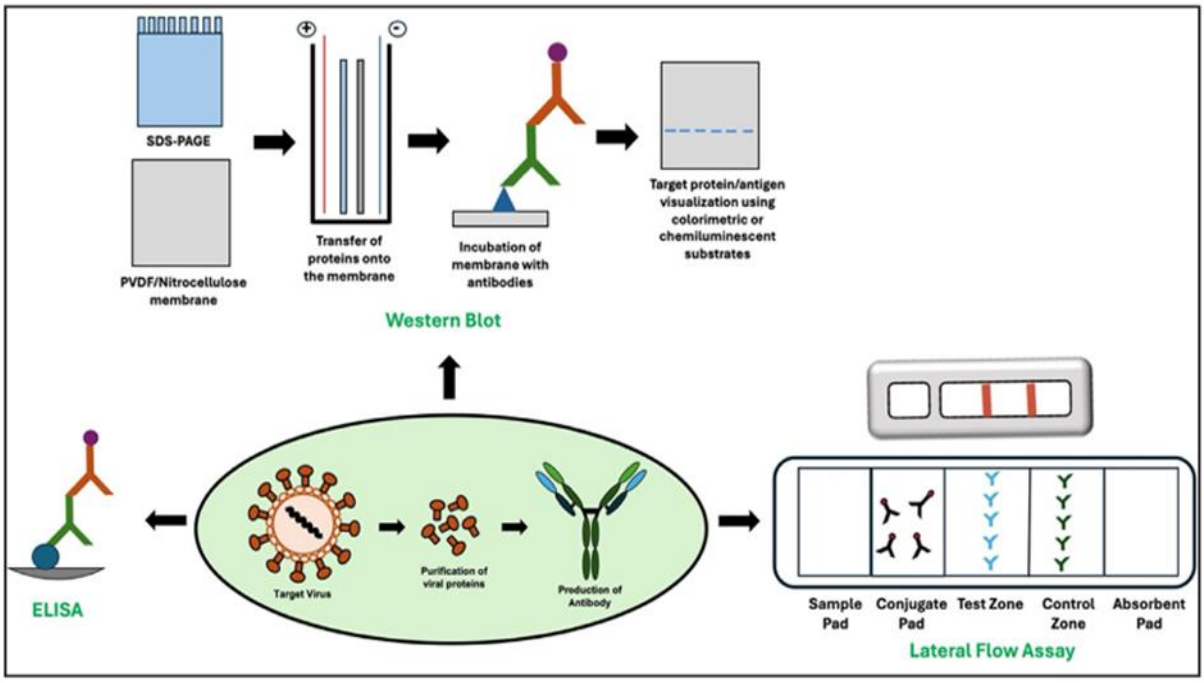


Figure 93 : Comparative schematic of immunodiagnostic platforms utilizing specific antibodies for viral target detection, highlighting the mechanisms of ELISA, Western Blot, and Lateral Flow Immunoassays (Kasana et al., 2025).

IV.4. Performance Analysis of Diagnostic Tests

The evaluation of diagnostic test performance is a fundamental pillar in medical research and clinical practice. It aims to assess the test's ability to discriminate between individuals with a specific condition and those without it. This process necessitates comparing the results of the index test against a "Reference Standard" (Gold Standard), which is the most accurate method available for determining the true disease status (van Stralen et al., 2009).

IV.4.1. Sensitivity:

- **Definition**: Sensitivity, also referred to as the "True Positive Rate," represents the test's ability to correctly identify individuals who have the disease (Altman & Bland, 1994).

- **Statistical Interpretation**: It is the probability that a diagnostic test will yield a positive result in a patient who truly has the condition; thus, it reflects the test's capacity to minimize false negatives (Drake & Levine, 2005).
- **Calculation:** Calculated as the number of True Positives (TP) divided by the total number of individuals with the disease (TP + False Negatives) (Altman & Bland, 1994).
- **Clinical Utility:** A highly sensitive test is primarily used for screening and "ruling out" a disease (SnNout). If a highly sensitive test is negative, clinicians can be confident that the patient does not have the condition (van Stralen et al., 2009).

IV.4.2. Specificity:

- **Definition:** Specificity is the test's ability to correctly identify individuals who do not have the disease (Altman & Bland, 1994).
- **Statistical Interpretation:** It represents the probability of a negative test result in a healthy individual (one without the disease), indicating the test's capacity to avoid false positive results (Drake & Levine, 2005).
- **Calculation**: Calculated as the number of True Negatives (TN) divided by the total number of individuals without the disease (TN + False Positives) (Altman & Bland, 1994).
- **Clinical Utility**: High specificity is essential for confirming a diagnosis (SpPIn). If a highly specific test yields a positive result, it is highly probable that the person truly has the disease (van Stralen et al., 2009).

IV.4.3. Accuracy:

- **Definition**: Diagnostic accuracy refers to the overall level of agreement between the results of the index test and the reference standard (van Stralen et al., 2009).
- **Statistical Interpretation:** It is the percentage of all tested individuals (both diseased and healthy) who were correctly classified by the test (Smith, 2012).
- **Calculation:** (TP + TN) / Total number of individuals tested (Smith, 2012).
- **Note:** While accuracy provides a general overview, sensitivity and specificity offer more nuanced details regarding the test’s performance in specific subgroups (van Stralen et al., 2009).

IV.4.4. Predictive Values:

Unlike sensitivity and specificity, which are considered inherent characteristics of the test, predictive values are heavily influenced by the prevalence of the disease in the studied population (Franco & Di Napoli, 2016; van Stralen et al., 2009).

- Positive Predictive Value (PPV): The probability that an individual truly has the disease given a positive test result. This value increases as the disease prevalence in the population rises (Franco & Di Napoli, 2016).
- Negative Predictive Value (NPV): The probability that an individual is truly free of the disease given a negative test result. This value increases when the disease prevalence is low (Franco & Di Napoli, 2016).

IV.5. Limitations of Diagnostic Tests and Recent Innovations

IV.5.1. Analytical and Operational Constraints in Modern Diagnostics

Diagnostic platforms are fundamentally governed by a complex multi-parameter trade-off involving accuracy, velocity, cost-effectiveness, and ease of implementation (Augustine et al., 2025). These constraints manifest significantly in both centralized laboratory settings and decentralized Point-of-Care (POC) environments.

A primary concern is the sub-optimal sensitivity of rapid antigen-based assays for respiratory pathogens, such as Influenza and RSV, which often leads to missed diagnoses in POC settings (Cheng et al., 2020). For instance, in pediatric SARS-CoV-2 testing, antigen-based POC tests demonstrated a pooled sensitivity of only 64.2%, illustrating a high rate of false negatives (Gentilotti et al., 2021). Furthermore, traditional Lateral Flow Assays (LFAs) have historically exhibited lower sensitivity and specificity compared to gold-standard methods like ELISA or PCR (Liu et al., 2021). Even when utilizing molecular diagnostics, the results remain heavily influenced by pre-analytical variables, such as the timing of sample collection and the specific site of the specimen (Plebani et al., 2024).

In addition to accuracy, operational limitations restrict the applicability of molecular techniques, as they often require advanced laboratory infrastructure and specialized personnel (Kardjadj, 2025). Conversely, POC testing faces challenges related to operator dependency and the potential for undetected pre-analytical errors like hemolysis or clotting (Plebani et al., 2024). Moreover, certain established tests like HbA1c may have reduced clinical sensitivity at diagnostic thresholds and are susceptible to influence by demographic factors such as age and ethnicity

(Augustine et al., 2025). Taken together, these limitations underscore the urgent need for transformative diagnostic solutions that can overcome existing analytical and operational constraints while maintaining efficiency and accessibility across diverse healthcare settings.

IV.5.2. Frontier Innovations: Overcoming Traditional Barriers

In response to the aforementioned diagnostic limitations, recent advancements have focused on integrating emerging technologies such as nanotechnology, microfluidics, and artificial intelligence into next-generation diagnostic platforms. These innovations aim to enhance analytical performance while improving accessibility and usability in point-of-care (POC) settings.

- **Nanotechnology and advanced biosensors:**Nanotechnology and advanced biosensors have improved diagnostic sensitivity and reduced the limits of detection (LoD), enabling rapid and sometimes non-invasive biomarker detection (Kim et al., 2024).

- **Advanced analytical techniques:** Techniques such as surface-enhanced Raman scattering (SERS) and surface plasmon resonance (SPR) have enhanced analytical sensitivity and portability of diagnostic devices (Wang et al., 2021).
- **Microfluidics and Lab-on-a-Chip systems:** Lab-on-a-chip technologies integrate several analytical steps into compact platforms, reducing reagent Consumption, minimizing sample volume, and shortening turnaround time (TAT) (Mejía-Salazar et al., 2020 ; Sachdeva et al., 2021).

- **Next-generation molecular tools:** Isothermal amplification methods such as loop-mediated isothermal amplification (LAMP) provide faster and simpler alternatives to conventional PCR with good sensitivity and specificity (Kardjadj, 2025).
- **CRISPR-based diagnostics:** CRISPR-based diagnostic tools enable rapid and highly specific pathogen detection in decentralized settings (Hassan et al., 2025).
- **Artificial intelligence (AI):** AI and machine learning improve result interpretation, reduce user-dependent variability, and support real-time quality control (Han et al., 2025).
- **Smartphone-integrated diagnostics:** Smartphone-based microfluidic platforms allow portable and accessible detection of viruses, parasites, and other biological targets (Wang et al., 2023).

- **Wearable diagnostic sensors:** AI-enabled wearable sensors support continuous and non-invasive health monitoring outside clinical environments (Yammouri & Lahcen, 2024)

IV.5.3. Summary of Diagnostic Limitations and Innovative Solutions

The following table synthesizes the relationship between current diagnostic bottlenecks and the technological advancements designed to mitigate them.

Table 8: Overview of Analytical and Operational Limitations in Modern Diagnostic Systems ((Kim et al., 2024&Kardjadj, 2025&Sachdeva et al., 2021&Han et al., 2025&Wang et al., 2023).

Limitation Category	**Clinical/Practical Impact**	**Innovative Mitigation Strategy**	**Key Citation**
Analytical Sensitivity	Sub-optimal detection in early infection.	Nanomaterial-enhanced biosensors (LoD reduction).	(Kim et al., 2024)
Infrastructure Needs	Reliance on heavy laboratory hardware.	Isothermal NAATs (LAMP) and CRISPR.	(Kardjadj, 2025)
Sample Processing	Large volume requirements and slow TAT.	Microfluidic Lab-on-a-Chip integration.	(Sachdeva et al., 2021)
Operational Bias	User-dependent interpretation errors.	AI/ML-driven automated image analysis.	(Han et al., 2025)
Accessibility	High cost and limited remote monitoring.	Smartphone platforms and wearable sensors.	(Wang et al., 2023)

Chapter V.

General Discussion and Future Perspectives

V.1. Critical Synthesis of Bibliographic Data (Discussion and Critical Analysis)

The comparative evaluation of antibody production technologies constitutes a fundamental aspect in understanding their efficiency, specificity, reproducibility, and suitability for modern biomedical applications. In contemporary immunological and biotechnological research, the selection of polyclonal, monoclonal, or recombinant antibodies depends primarily on the objectives of the study, the required analytical precision, production feasibility, and regulatory considerations (Leenaars & Hendriksen, 2005). While earlier studies focused mainly on antibody production efficiency, recent literature increasingly emphasizes issues related to structural characterization, reproducibility, batch consistency, and quality-control requirements for therapeutic and diagnostic applications (Kumar et al., 2026). Consequently, a critical comparative analysis of these technologies requires evaluating the immunization strategies, purification methods, screening systems, production hosts, and the final biological performance of the generated antibodies (Ascoli & Aggeler, 2018; Dunbar & Schwoebel, 1990).

Looking closely at the first generation, polyclonal antibodies (pAbs) are a complex mix of complete Immunoglobulin G (IgG) molecules secreted by different B-lymphocyte clones. Because of this, they can bind to multiple targets (epitopes) on the same antigen, giving them a unique structural advantage.

Dunbar and Schwoebel (1990) argue that pAbs remain highly attractive for routine lab work because this multi-binding capability provides excellent sensitivity and amplifies signals beautifully in tests like ELISA, Western blotting, and tissue staining (immunohistochemistry).

Tabll et al. (2024) outline the simplicity of making them: you inject lab animals (like rabbits or goats) with the target antigen, give them booster shots, and then collect the antibody-rich blood serum. This method is fast and requires very little upfront money compared to complex molecular systems.

However, Lieners and Hendriksen (2005) strongly criticize this approach. They warn that relying on live animals creates massive variation from one batch to another (batch-to-batch variability), making it hard to get consistent results over time. This unrefined mix also increases the risk of cross-reactivity (binding to the wrong proteins), which causes messy background signals and ruins diagnostic accuracy.

On the other side, Ascoli and Aggarwal (2018) defend pAbs, arguing that batch variation can be managed and that their ability to detect damaged or rare proteins is worth the trouble. Kumar et al. (2026) offer a middle ground: instead of abandoning the technology, we can control this variation by using advanced purification like antigen-affinity chromatography, followed by high-speed spinning (ultracenntrifugation) and fine filtering to remove serum impurities. This is crucial

since pAbs are still vital for making snakebite antivenoms and running rapid food safety tests in poor regions. To solve the ethical issues of animal testing raised by Lieners and Hendriksen (2005), Pelletier et al. (2020) introduced a smart genetic method using DNA immunization. This reduces animal suffering while still producing highly specific antibodies that recognize the natural shapes of mammalian proteins.

To fix the biological flaws of the first generation, hybridoma technology for making monoclonal antibodies (mAbs) was a massive leap forward. Barari et al. (2020) explain that this method solved the variation problem by fusing B-cells from an immunized animal's spleen with cancerous myeloma cells. This creates a single, immortalized hybrid cell line that guarantees identical IgG molecules targeting just one specific epitope.

Khalaf et al. (2025) emphasize that this absolute consistency made mAbs the gold standard for clinical diagnostic kits and targeted medical treatments.

Even so, Carvalho et al. (2016) push back against over-optimism, highlighting the extreme complexity and high costs of this technology. It requires tedious sorting using a special selection liquid (HAT medium) to find successful cells, followed by complex culturing in massive factory tanks (bioreactors). This shift was necessary to stop using live animal bellies to grow antibodies (ascites), a practice criticized by Lieners and Hendriksen (2005) and strictly banned by regulators like the European Medicines Agency (2016) for animal welfare reasons.

The criticism doesn't stop at production; it also targets the cleaning and isolation phase (downstream processing). Cavallo and Zúñiga (2012) point out a major headache: it is incredibly difficult to control how cells add sugars to proteins after making them (post-translational modifications, or PTMs). Any tiny change in the sugars on the antibody's tail (Fc region) can ruin the treatment or trigger dangerous immune reactions in patients.

Baran and Podgórski (2026) prove that standard factory cleaning—which uses salt precipitation followed by Protein A or G affinity chromatography and ion exchange—puts the antibodies under violent physical and chemical stress (shear stress). This makes the antibodies clump together (aggregate) or lose their proper shape. Because of this, Jafarzadeh Chirani et al. (2025) call this purification phase a "financial graveyard," as it devours over 60% of the total manufacturing budget.

From a real-world economic view, Werner (2004) and Mota-Santos et al. (2026) criticize these inflated production and regulatory costs. They see them as a social barrier that keeps poor communities in developing countries from accessing life-saving treatments, such as targeted cancer therapies (like Trastuzumab and Rituximab) and rheumatoid arthritis drugs. Gieber et al. (2023) back this up by showing how unfairly clinical trials are distributed globally, while Sewell

et al. (2017) argue that international agencies must make it easier to approve generic versions of these drugs (biosimilars) to break monopolies and lower prices.

To break free from the limits of hybridoma technology, the third generation emerged: recombinant and engineered antibodies (rAbs), which completely eliminate the need for animal cells. Kunert and Reinhart (2016) view this as the peak of genetic engineering. Scientists isolate the genes for the antibody's heavy and light chains, insert them into molecular vehicles (expression vectors), and grow them using techniques like phage display. This allows them to "humanize" the antibodies completely, so the patient's body doesn't reject them as foreign objects.

Becker et al. (2025) praise the absolute molecular freedom of this platform. Designers are no longer stuck with the traditional, heavy IgG shape. Instead, they can build tiny, customized antibody fragments.

Mamidi et al. (2024) showcase how these new engineered formats—like single-chain fragments (scFv), antigen-binding fragments (Fab), and double-targeting antibodies (bsAb)—are much better at penetrating deep into solid tumor tissues than traditional antibodies. However, DeLuca et al. (2021) note a technical downside: it is very difficult to keep these engineered fragments stable and dissolved properly in production lines without them breaking down.

There is also a fierce debate about which cell systems are best for growing these recombinant antibodies. Hing et al. (2022) and Hussain et al. (2021) insist that mammalian cells—like Chinese Hamster Ovary (CHO) and human embryonic kidney (HEK293) cells—are considered the gold standard because they support correct protein folding and generate glycosylation patterns that are compatible with human biological systems.

On the other hand, Juliani et al. (2022) criticize these cells for growing slowly and requiring expensive food, championing *E. coli* bacteria as a lightning-fast, cheap alternative.

Yet, Becker et al. (2025) counter this defense, pointing out that bacteria cannot add necessary sugars and often produce clumped up, useless proteins trapped inside dark cell pockets called "inclusion bodies." Cleaning these requires an arduous process of washing them with harsh chemicals like urea or guanidine hydrochloride to dissolve them, and then slowly trying to refold them—a tricky process that often fails at an industrial scale.

To solve the mammalian cell efficiency problem, Carrara et al. (2021) suggest an innovative strategy using a single DNA ring with two starters (dual-promoter single plasmid) to simplify genetic engineering and balance the heavy-to-light chain ratio, reducing protein clumping.

Looking past both animals and bacteria, Damos et al. (2020) highlight the high efficiency and low cost of growing antibodies in plants (like tobacco) using modified plant viruses.

Mukai et al. (2025) support a different alternative: harvesting recombinant monoclonal antibodies directly from the yolks of genetically modified chicken eggs. Cleaning them involves separating egg fats using simple polymers (like PEG) before running standard chromatography. This simplifies the workflow and opens the door for massive use in epidemic prevention, rapid testing, and immune supplements.

Based on this deep analysis, the future of antibody technology is being shaped by a fierce rejection of current manufacturing bottlenecks. Shukla and Wolfe (2017), along with Pons Royo and Jungbauer (2025), argue that the next big breakthrough requires completely ditching traditional Protein A chromatography columns, because the harsh acids used to wash them actually damage the antibodies. They suggest switching to continuous manufacturing systems and smart precipitation techniques.

Meanwhile, Strazza et al. (2024) criticize how slow traditional cell sorting is. They present a revolutionary fix using magnetic fluids (ferrofluid technology) to grab antibody genes directly from single secreting B-cells, cutting development time from months to just days.

In the final twist of this technological race, Swartz (2024) and Yu et al. (2025) offer a bold vision that bypasses factories, cleaning steps, and high costs entirely: nucleic acid-encoded antibodies (DNA/RNA antibodies). They propose tearing down manufacturing plants altogether. Instead, the genetic blueprint of the antibody is injected directly into the patient (*in vivo* technology). The patient's own cells become the factory, churning out their own medicine. This pivots the future of science away from cold, sterile lab manufacturing and places it squarely on efficient gene delivery, shaping a new generation of immunotherapy that is affordable and accessible to everyone on earth.

As summary, **Verification of Antibody Production Technologies in Polyclonal Antibodies (pAbs)**

- Advanced purification strategies, including antigen-affinity chromatography followed by ultracentrifugation and fine filtration, can reduce batch-to-batch variability and improve antibody quality by eliminating serum impurities (Kumar et al., 2026).

In Monoclonal Antibodies (mAbs)

- Downstream processing represents a major economic challenge, accounting for more than 60% of the total manufacturing cost (Jafarzadeh Chirani et al., 2025).

- Conventional purification methods based on salt precipitation, Protein A/G affinity chromatography, and ion-exchange chromatography may expose antibodies to shear stress, leading to aggregation and structural alterations (Baran & Podgórski, 2026).
- Inadequate control of post-translational modifications, particularly Fc glycosylation, may compromise therapeutic efficacy and increase the risk of adverse immune reactions (Cavallo & Zúñiga, 2012).

In Recombinant and Engineered Antibodies (rAbs)

- Recombinant antibodies produced in bacterial systems such as *E. coli* often require complex purification and refolding procedures because proteins accumulate in inclusion bodies and must be solubilized using agents such as urea or guanidine hydrochloride (Becker et al., 2025).
- Engineered antibody fragments, including scFv, Fab, and bispecific antibodies, may present stability challenges during production and purification processes (De Luca et al., 2021).
- The use of dual-promoter single-plasmid systems can improve heavy- and light-chain expression balance and reduce protein aggregation during production (Carrara et al., 2021).

As Future manufacturing strategies; may replace conventional Protein A chromatography with continuous processing and advanced precipitation technologies to minimize antibody damage and reduce production costs (Shukla & Wolfe, 2017; Pons Royo & Jungbauer, 2025).

- Nucleic acid-encoded antibodies (DNA/RNA antibodies) represent a promising approach that could bypass conventional manufacturing and purification processes by enabling in vivo antibody production directly within the patient (Swartz, 2024; Yu et al., 2025).

the comparative analysis of antibody production technologies demonstrates that no single platform can be considered universally superior. Rather, each technology possesses distinct advantages and limitations depending on the intended biomedical application, analytical requirements, production scale, and regulatory context.

Consequently, the choice of the optimal platform requires a multi-dimensional balance between technical precision, economic constraints, and regulatory requirements. To provide a comprehensive overview of these parameters, a systematic comparative evaluation is synthesized in Table 9.

Table 9: Comparative Evaluation of Antibody Production Technologies: Strengths, Limitations, and Applications

Antibody Type	Strengths & Advantages	Key Limitations & Challenges	Biomedical Applications
Polyclonal Antibodies (pAbs)	• Broad antigen recognition via multi-epitope binding (Dunbar & Schwoebel, 1990). • High signal intensity and assay sensitivity (Ascoli & Aggeler, 2018). • Rapid, cost-effective, and simpler production workflow (Leenaars & Hendriksen, 2005).	• High batch-to-batch variability and composition inconsistency (Kumar et al., 2026). • Risk of cross-reactivity due to trace impurities in the immunogen (Dunbar & Schwoebel, 1990). • Represents a finite biological resource, lacking long-term standardization (Ascoli & Aggeler, 2018).	• Classical western blotting and immunolocalization (Dunbar & Schwoebel, 1990). • Routine and multi-site ELISA development (Ascoli & Aggeler, 2018). • Exploratory academic research and primary antibody screening (Leenaars & Hendriksen, 2005).

Monoclonal Antibodies (mAbs)	• Homogeneous specificity targeted against a single epitope (Büyükköroğlu & Şenel, 2016). • Exceptional batch-to-batch consistency and definition (EMA, 2016). • Indefinite, sustained production via secure hybridoma cell banks (Khalaf et al., 2025).	• High initial development costs and prolonged production timelines (Khalaf et al., 2025). • Risk of genetic and biological instability during prolonged cell passage (EMA, 2016). • Traditional *in vivo* ascites method is ethically restricted (Leenaars & Hendriksen, 2005; EMA, 1994).	• Clinical oncology and targeted cancer immunotherapies (Bouzid et al., 2012). • High-throughput immunophenotyping and flow cytometry (Shukla & Wolfe, 2017). • Standardized, commercial-grade *in vitro* diagnostics (Büyükköroğlu & Şenel, 2016).
Recombinant Antibodies (rAbs)	• Complete genetic sequence definition, eliminating biological drift (Kunert & Reinhart, 2016). • Unmatched engineering flexibility (e.g., chimeric, humanized, scFv, Fab formats) (Hussain et al., 2021).	• Requires highly sophisticated molecular biology and genetic infrastructure (Heng et al., 2022). • Demands substantial initial financial and technology investment (Huleani et al., 2022). • Bacterial expression systems lack critical post-translational	• Next-generation biopharmaceuticals and engineered clinical therapeutics (Kunert & Reinhart, 2016). • Tissue-penetrating diagnostics and miniaturized biosensors (Huleani et al., 2022). • Emerging platform for specialized or highly regulated antibody

	• Completely animal-free selection via *in vitro* phage display technologies (Baker et al., 2025).	glycosylation (Hussain et al., 2021; Baker et al., 2025).	fragments (Hussain et al., 2021).

Polyclonal antibodies remain appropriate for rapid and cost-effective laboratory detection due to their high sensitivity, while monoclonal antibodies are optimal for highly specific analytical and standardized therapeutic applications. Meanwhile, recombinant antibodies constitute the preferred, future-proof platform for modern clinical biotechnology and next-generation immunotherapies. Ultimately, the selection of the antibody technology must be directly dictated by the specific scientific objective and the available infrastructure.

V.2. Future Perspectives on Polyclonal Antibody Production and Local Biotechnological Applications

Polyclonal antibodies (pAbs) continue to represent essential tools in biotechnology due to their broad epitope recognition, high analytical sensitivity, and relatively accessible production process. In the future, the development of local polyclonal antibody production may offer promising opportunities for university laboratories and research centers, particularly in developing countries where access to advanced recombinant technologies remains limited.

Future progress in this field may focus on the establishment of standardized production workflows, beginning with antigen selection and purification, followed by antigen–adjuvant formulation and controlled animal immunization protocols. Subsequent experimental stages may include booster administration, blood collection, serum separation, antibody purification, quality control analysis, and long-term preservation of purified antibodies.

Optimization of these production steps could improve antibody specificity, affinity, stability, and reproducibility while reducing experimental variability between animal models. In addition, alternative production systems such as avian IgY technology may provide cost-effective and ethically favorable approaches for future antibody generation.

Locally produced polyclonal antibodies may also be integrated into several immunodiagnostic applications, including ELISA, Western blotting, immunofluorescence assays, and rapid diagnostic tests. The implementation of such local biotechnological platforms could

reduce dependence on imported reagents, lower laboratory costs, and support the development of regional biomedical research and diagnostic capacities.

Overall, polyclonal antibodies remain promising candidates for future biotechnological and diagnostic development due to their practical accessibility, versatility, and significant scientific value in both research and clinical applications. A proposed standardized workflow for future local polyclonal antibody production is illustrated in **Figure 94**



Figure 94 : Standardized workflow proposed for future local polyclonal antibody production platforms. (Source: Created by the author)

Conclusion

Conclusion

Antibodies have become essential tools in modern biotechnology and biomedical sciences because of their high specificity and their important role in both diagnosis and treatment. This bibliographic review has highlighted the main aspects of antibodies, including their biological properties, production methods, engineering approaches, and their various applications in research, diagnostics, and medicine.

The development of antibody production technologies, from polyclonal antibodies to monoclonal and recombinant antibodies, has significantly improved their quality and effectiveness. Today, antibodies are widely used in the treatment of several diseases, especially cancer, autoimmune disorders, and infectious diseases. They are also important components of many diagnostic techniques, including ELISA, Western blotting, immunofluorescence, immunohistochemistry, and rapid diagnostic tests.

Despite these advances, several challenges still need to be addressed. Antibody production remains expensive and requires complex manufacturing and purification processes. Other important issues include reproducibility between production batches, large-scale manufacturing, immunogenicity, and product stability. Improving antibody stability is particularly important to maintain biological activity during storage, transport, and clinical use, while reducing immunogenicity is necessary to improve treatment safety and effectiveness.

Future research is expected to focus on the development of more efficient and safer antibody-based products. Recent studies have highlighted the potential of innovative antibody formats such as antibody-drug conjugates (ADCs), bispecific antibodies, and mRNA-based delivery systems, which may improve target specificity and therapeutic outcomes (Lu, 2025; Alradwan, 2025). In addition, considerable efforts are being made to develop strategies that enhance antibody stability and reduce unwanted immune responses, including the use of advanced formulations and nanotechnology-based approaches (Wang, 2023; Alanazi, 2025).

Another important research axis is the integration of artificial intelligence, bioinformatics, and computational tools into antibody development. These technologies can help predict stability, aggregation, and immunogenicity at early stages, thereby reducing development time and improving production efficiency (Khetan, 2022; Makowski, 2021).

In conclusion, continuous progress in immunology, biotechnology, and antibody engineering confirms the growing importance of antibodies in modern healthcare. Further research

and technological innovation will contribute to the development of safer, more stable, and more effective antibody-based products, while expanding their accessibility for medical and scientific applications worldwide.

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