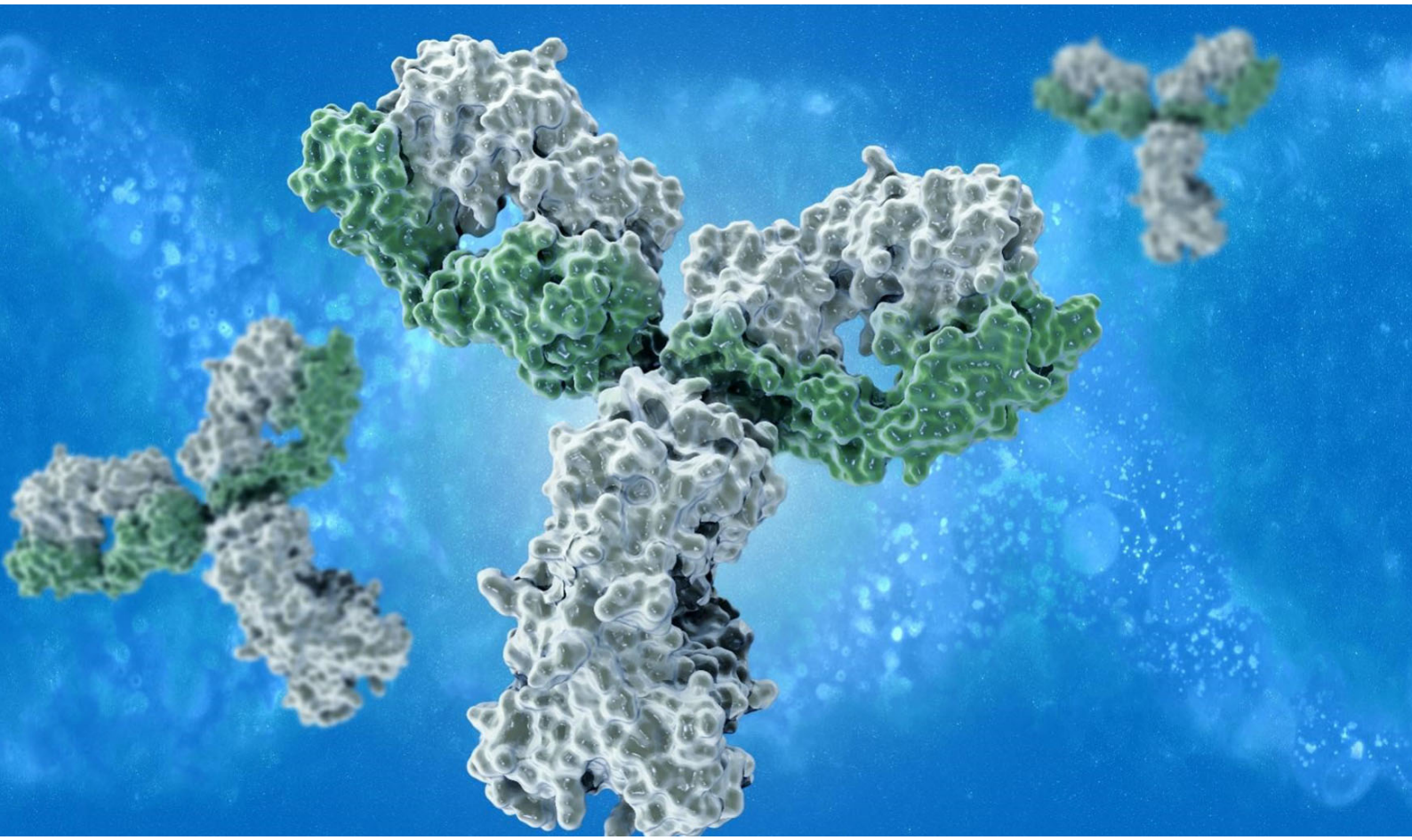
Antibody Basics Technical Guide



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Antibody Basics Technical Guide

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01 Introduction to Antibody Structure and Isotypes

Antibodies, also known as immunoglobulins (Ig), are glycoproteins synthesized and secreted by B cells. They have a unique Y-shaped structure and are the main immune defense tools. Antibodies can specifically bind to pathogen molecules called antigens. Each antibody contains one or more Y-shaped units composed of four polypeptide chains. Each Y-shaped unit contains two identical heavy (H) and light (L) chains that vary in sequence and length. The top of the Y-shaped unit has a variable region (V), also called the fragment antigen-binding region (F(ab) region). This region binds tightly to a specific part of the antigen, also known as the antigen's epitope. The Y-shaped base of the antibody is made up of the constant domain (C) and forms the fragment crystallizable region (Fc).

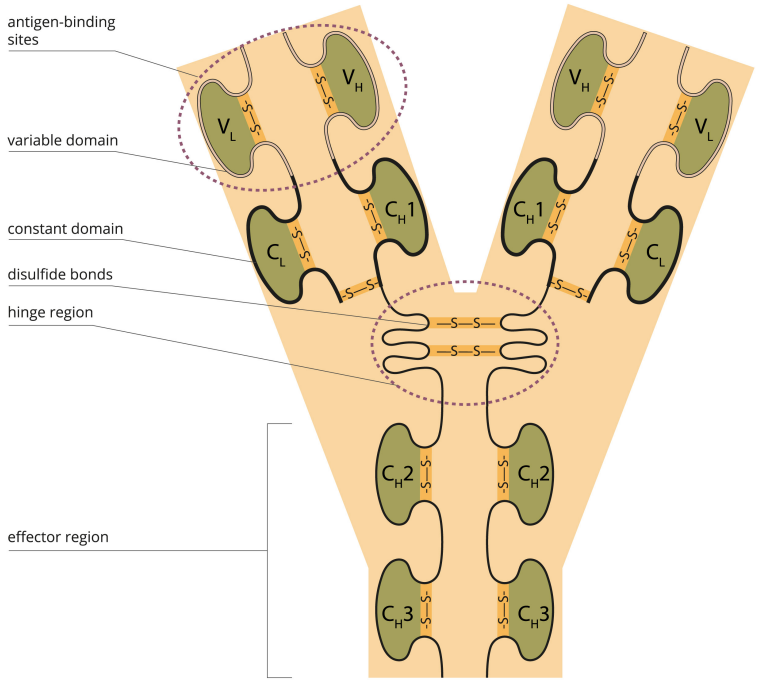

Figure 1. Antibody structure and isotypes.

F(ab) and Fc Regions of Antibodies

The Y-shaped structure of the antibody can be broken down by pepsin into three distinct parts: two F(ab) regions and one Fc region. The F(ab) region contains a variable domain that can specifically bind to homologous antigens. The Fc fragment provides the antibody with binding sites for endogenous Fc receptors on the surface of lymphocytes and secondary antibodies. In addition, the Fc part can also be covalently linked to dyes and enzymes for visualization of antibody-related experiments. Antibody fragments also play other roles in specific immunochemical techniques. For example, fragmented IgG antibodies play an important role in functional studies. Furthermore, because the F(ab) fragment is small and does not contain the Fc region, it will not precipitate after binding to the antigen, nor will it interact with immune cells. The distribution and function of the antibody can be observed and tracked after radioactive labeling; the Fc fragment is usually used as an Fc receptor blocker in immunohistochemical staining and has completely different applications.

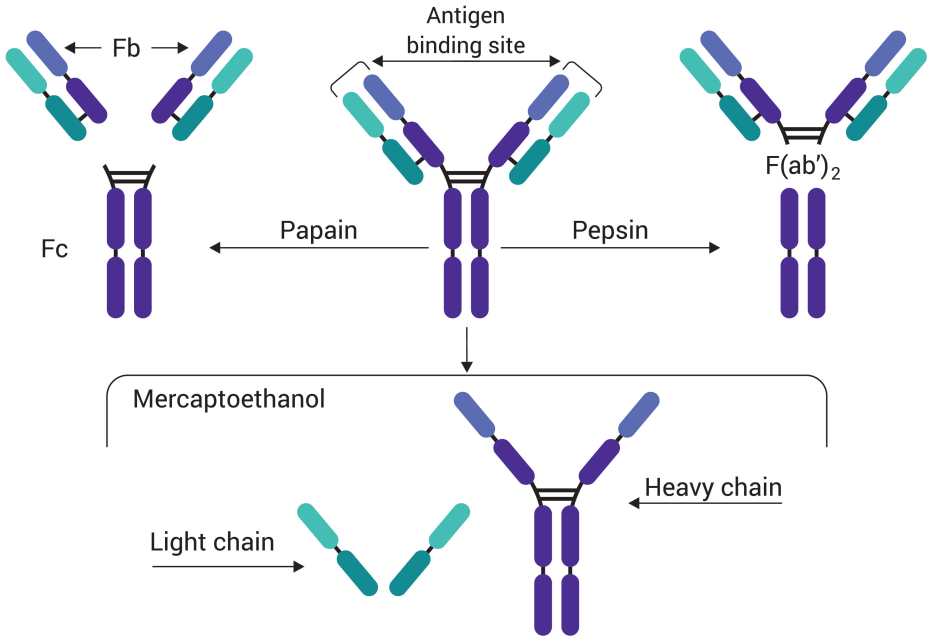

Figure 2. F(ab) and Fc regions of antibody.

Antibody Heavy Chain

The type of antibody heavy chain determines the class or isotype of the antibody. Studies have found that there are five types of mammal Ig heavy chains, usually represented by the Greek letters α, δ, ε, γ and μ. These chains make up the IgA, IgD, IgE, IgG and IgM types of antibodies. Additionally, there are differences in the size and composition of the heavy chains. The α and γ heavy chains contain approximately 450 amino acids, while the μ and ε heavy chains contain approximately 550 amino acids. Each heavy chain consists of a constant region (CH) and a variable region (VH). The constant regions remain consistent across antibody isotypes, but differ between antibodies of different subtypes. The gamma, alpha, and delta heavy chains contain a constant region composed of three connected Ig domains (CH1, CH2, CH3), with a hinge region to increase their flexibility. The mu and epsilon heavy chains include a constant region composed of four immunoglobulin domains. As for the variable region (VH), it varies depending on the B cell that produces it, but is consistent for all antibodies produced by the same B cell or B cell clone. Studies have shown that the variable region of each heavy chain consists of approximately 110 amino acids and contains a separate Ig domain.

Antibody Light Chain

Mammals have only two types of light chains, lambda (λ) and kappa (κ), and their polypeptide sequences are extremely similar. Among them, the light chain is composed of a continuous constant domain (CL) and a variable domain (VL). Typically, light chains are approximately 211-217 amino acids in length. Each antibody contains two identical light chains. Light chains of the iota (ι) type are found in lower vertebrates, such as cartilaginous fish and teleosts.

Antibody Light Chain

In mammals, antibodies are divided into five different isotypes, namely IgG, IgM, IgA, IgD and IgE. Each isoform has unique structural features determined by the number of Y units and the type of heavy chain. They have obvious differences in biological properties, functional localization, and ability to process different antigens.

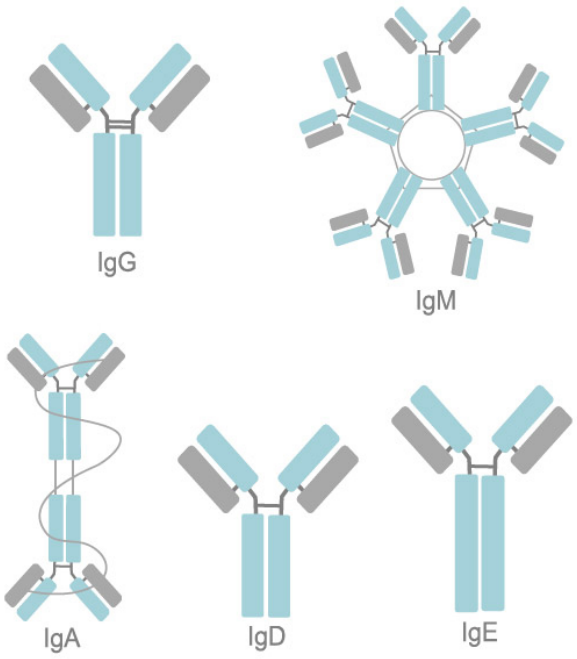

Figure 3. Antibodies are divided into five different isotypes.

Table 1. Structure and function of various antibody isotypes.

Isotype	Heavy chain	Light chain	MW (kDa)	Structure	Function
IgA1 IgA2	α1 α2	λ or κ	150–600	Monomer-tetramer	This isotype of antibody is the most diverse antibody isotype produced by mice and humans. These antibodies are mainly distributed in mucosal areas such as the intestines, respiratory tract, and urogenital tract, where they play an important role in preventing colonization by pathogens. Additionally, these antibodies have anti-digestive properties and can be passed on to offspring through milk secretion.
IgD	δ	λ or κ	150	Monomer	Its function is not yet clear, but it usually cooperates with IgM in B cells to promote development and mainly binds to B cells.

IgE	ε	λ or κ	190	Monomer	It binds to allergens and triggers mast cells to release histamine, thereby participating in allergic reactions. Additionally, it has the ability to protect against parasitic infections.
IgG1 IgG2a IgG2b IgG3 IgG4	γ1, γ2, γ3, γ4	λ or κ	150	Monomer	It is the most abundant immunoglobulin (Ig) in serum, accounting for approximately 75% of total human serum antibodies. It provides most antibody immunity against invading pathogens. At the same time, it activates the complement system to a moderate extent.
IgM	μ	λ or κ	900	Pentamer	This is a typical primary immune response antibody that is expressed on the surface of B cells and exists in a high-affinity secreted form. In the early stages of the immune response, especially before sufficient IgG is produced, it is able to eliminate pathogens as part of the B cell-mediated immune process.

How and Where Antibodies Bind to Antigens

The F(ab) region of an antibody contains a binding site, called a paratope, for interaction with the antigen. A paratope binds to a specific part of an antigen, also called an epitope. These epitopes are usually a small portion of the antigen, sometimes consisting of only a few amino acids. The binding between paratopes and epitopes is achieved through their shapes and interactions between molecules, including van der Waals forces, hydrogen bonds, electrostatic attractions, and hydrophobic interactions. The strength of these interactions determines the affinity between the antibody and the antigen.

Antigens

The core principle of immunoassay is that a specific antibody interacts with its specific antigen to form a unique antibody-antigen complex.

Antigen definition: Antigens are substances that trigger the immune system to produce a specific antibody response. They can be bound by specific antibodies. Typically, antigens have a higher molecular mass and are usually proteins or polysaccharides, but can also include peptides, lipids, nucleic acids, and a variety of other substances.

Hapten: Haptens refer to relatively small substances that are unable to induce an immune response on their own. However, when bound to a large carrier protein, they can induce an immune response. Common carrier proteins include bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), or other synthetic carriers.

Many molecules can serve as haptens, including drugs, simple sugars, amino acids, small peptides, phospholipids, or triglycerides. Therefore, the immune system is able to recognize almost any foreign substance and induce a specific antibody response within a certain period of time. However, the intensity

of this specific immune response varies depending on the size, structure, and composition of the antigen. Antigens that elicit a strong immune response are said to be highly immunogenic.

Antigen-antibody Binding

Antibodies recognize and bind to specific parts of an antigen, called epitopes. For efficient antigen-antibody interaction, the epitope must bind easily. If the antigen molecule is denatured, the structure of the epitope may change, which may affect how well the antibody interacts with it. For example, certain antibodies may be ineffective in Western blotting but highly effective in immunohistochemistry (IHC), where the tissue may retain more intact antigenic sites. In contrast, in Western blotting, the sample preparation process can significantly change the conformation of the protein, resulting in damage to the antigenic site and a reduction in antibody binding ability.

Therefore, the epitope may be present in the native cellular environment of the antigen or may be exposed only when the antigen is denatured. In their native form, epitopes can be located in the cytoplasm (soluble), coupled to the cell membrane, or secreted. The amount of antigen used in the antibody preparation process determines the number, location and size of epitopes.

Good Antigens Include the Following Characteristics:

- Antigen molecules contain regions of stability and chemical complexity with intramolecular structure.
- They do not have a large number of repeating units.
- Molecular weight usually ranges from 8,000 to 10,000 Da. In the presence of a carrier protein, the molecular weight of the hapten can be as low as 200 Da.
- Ability to be recognized by the immune system.
- There are immunogenic regions that can be recognized by the antibody-forming machinery.
- Structural elements differ significantly from the main body.
- For peptide antigens, it contains at least 30% immunogenic amino acids such as lysine, arginine, glutamic acid, aspartic acid, glutamine and asparagine.
- Peptide antigens should have distinct hydrophilic or charged residues.

The Immune System and Its Role in Producing Specific Antibodies

The main task of the immune system is to protect animals from invasion by foreign pathogens and infectious microorganisms. It responds to pathogens in a specific way while also exhibiting long-term memory of infectious agents to which it has been exposed.

The immune system mainly consists of two functional parts:

The innate immune system, also known as the non-specific immune system.

The adaptive immune system, also called the specific immune system.

- Innate Immune System

Components of the innate immune system form the first line of defense against infection. This physical barrier includes skin, which blocks the entry of pathogens, and body fluids (such as mucus), which collect

and remove pathogens. The innate immune system consists of numerous cellular and biochemical components, including complement proteins, innate immune cells, and phagocytes, which enable the recognition and elimination of pathogens in the body. The function and efficiency of the innate immune system are not altered by repeated exposure to foreign pathogens.

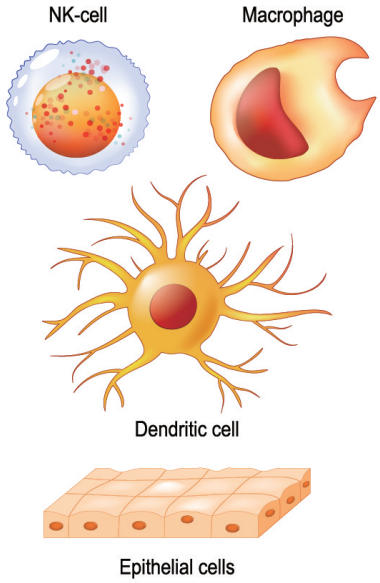

Figure 4. Innate Immune System.

- Adaptive Immune System

When an antigen invades the body, if the innate immune system cannot effectively eliminate the pathogen, the adaptive immune system will be activated. This system is composed of many different types of cells and molecules, of which lymphocytes and antibodies play an important role in this process. Lymphocytes are generated from progenitor cells in the bone marrow. These lymphocytes synthesize specific cell surface receptors or secrete antibodies that specifically bind foreign molecules. Pathogens that bind to the antibodies are marked for elimination or destruction. The main functions of the adaptive immune system are performed by three types of cells, namely:

- B cells Cytotoxic
- Cytotoxic T cells (TC cells)
- Helper T cells (Th cells)

Adaptive immune responses can be divided into two types: humoral-mediated and cell-mediated. B lymphocytes mediate humoral responses by releasing specific antibodies that target foreign infectious agents. In cell-mediated responses, TC cells bind to foreign or infected cells and then lyse these cells. Th cells participate in both responses by releasing cytokine proteins. All three types of lymphocytes carry cell surface receptors that can bind antigens. These antigen receptors are all glycoproteins, and only one type of receptor is synthesized in each cell. This fact affects the specificity of the immune system, allowing one cell to recognize only one antigen.

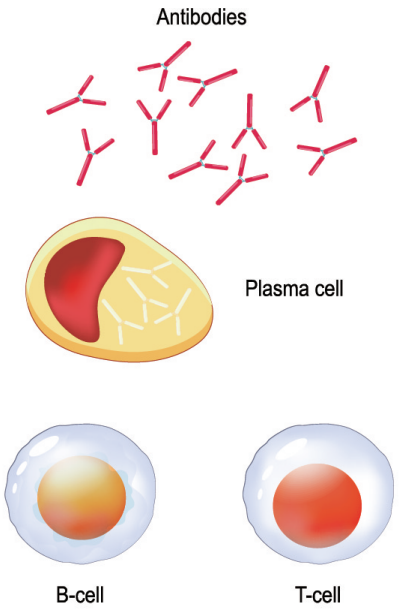

Figure 5. Adaptive Immune System.

- Antibody- Antigen Response

Specific recognition and binding between antibodies and antigens form the basis of all immunoassays and are the core of immune responses.

The region of interaction between the antibody and the antigen is called the paratope. The specific antigen region to which the antigen binds is called the epitope. Affinity is used to measure the binding strength between the epitope and the antibody, and also reflects the overall stability of the antibody-antigen complex, usually expressed by the dissociation constant KD.

02 Antibody Production

Preparation of Polyclonal Antibodies

Polyclonal antibodies are made up of multiple different antibodies that each recognize different sites on a specific antigen.

The preparation of polyclonal antibodies usually begins with immunization of an animal to elicit an immune response that prompts the animal's B cells to produce specific antibodies. The animal is then immunized repeatedly every few weeks to increase the number and affinity of specific antibodies in the animal. The obtained immune serum (the part of blood containing antibodies) can be used as a crude product to detect antigens, or it can be used to isolate higher-purity antibodies through affinity purification.

Because polyclonal antibodies are composed of a mixture of naturally immunoreactive antibodies, they generate a strong signal against the target antigen in relevant applications without being biased toward a single site. However, they also have some disadvantages, including limited supply and higher batch-to-batch variability than monoclonal antibodies. In addition, polyclonal antibodies may exhibit cross-reactivity and lack specificity, so there is a risk of binding to other proteins with similar sequences. These problems can often be solved by cross-adsorbing (i.e., further purifying) the polyclonal antibody mixture

to remove antibodies with undesirable binding properties, which often cross-react to similar antigens in specific species.

Preparation of Monoclonal Antibodies Using Hybridoma Technology

Compared with polyclonal antibodies, monoclonal antibodies are cloned from a single B cell parent and can only recognize a single site of each antigen. In the hybridoma preparation method, B cells are fused with hybridoma cells to immortalize them so that they can produce immunoglobulin (Ig) for a long time.

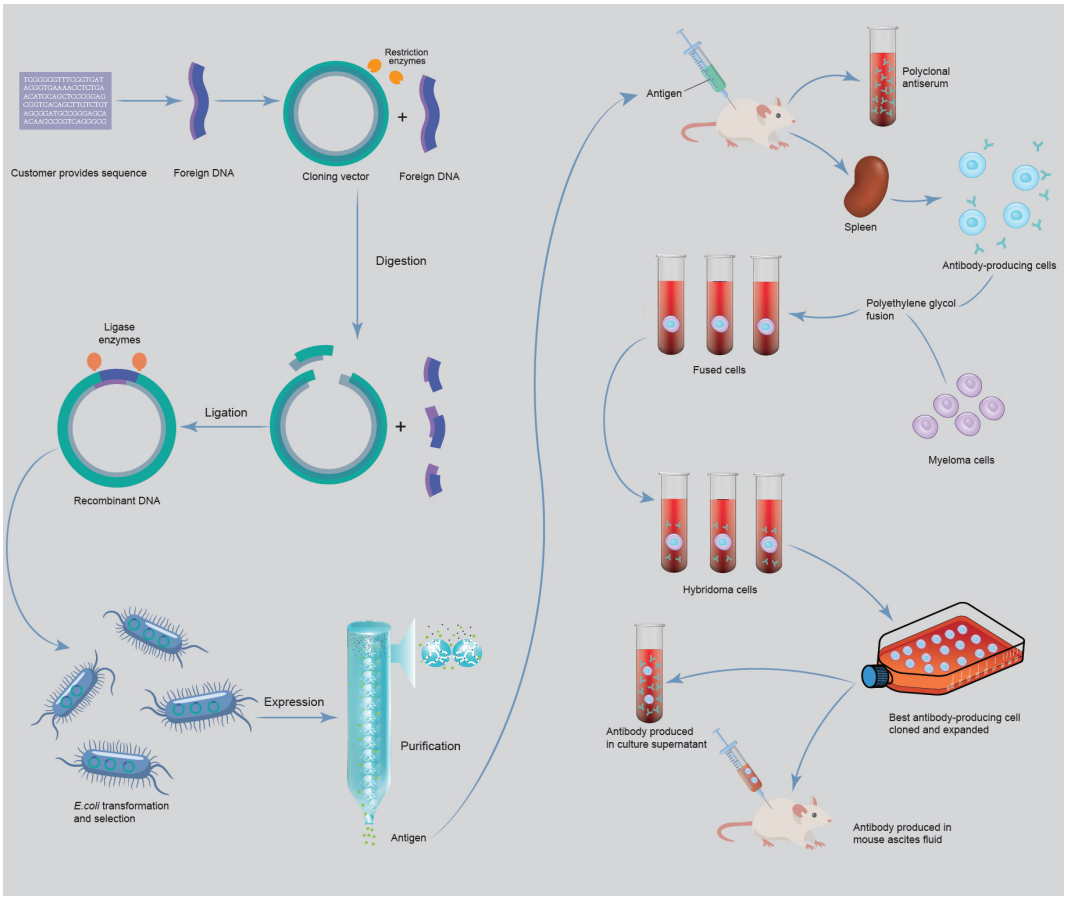

Figure 6. The process of monoclonal antibodies production.

The initial stages of preparing monoclonal antibodies are similar to those for polyclonal antibodies, starting with the same immunization protocol. After immunization, antibody-producing cells are collected from the spleen and then fused with immortal tumor cells to form hybridoma cells. These are finally screened to select hybridomas with better antibody production and performance.

When the isolated hybridoma cells are cultured, the cells secrete antibodies into the culture medium. These antibodies can be collected and used in crude form or purified through affinity purification. Unlike polyclonal antibodies, monoclonal antibodies are homogeneous and have clear specificity for a specific site.

Monoclonal antibodies uniformly recognize a single site on an antigen and therefore have less potential for cross-reaction with other proteins and less batch-to-batch variation than polyclonal antibodies.

Monoclonal antibodies produced using hybridoma cell lines have the potential for genetic drift over time. Thus, an antibody produced years later using the same cell line may differ somewhat from the original version. Therefore, in order to maintain a long-term supply of antibodies with stable product quality,

hybridoma-derived antibodies must be engineered into recombinant forms.

Recombinant Monoclonal Antibody Preparation

Recombinant antibodies are antibodies produced using genes synthesized *in vitro*. This allows precise control of coding sequences, thereby optimizing expression (increasing antibody yield) and increasing the reproducibility of antibodies produced by hybridomas. Recombinant antibodies offer long-term, reliable supply with minimal batch-to-batch variation compared to traditional monoclonal and polyclonal antibodies. Additionally, because the antibody coding sequence is known and well-defined, further engineering and manipulation can be performed to meet the needs of specific uses. For example, tags can be added or FC fragments from other species can be inserted. The process of preparing recombinant antibodies involves cloning the antibody-encoding gene into a high-yield mammalian expression vector and then introducing the vector into an expression host to produce functional antibodies. HEK 293 or CHO-K1 mammalian cell lines are often used as expression hosts to ensure correct post-translational modifications.

A variety of methods are used to obtain antibody-encoding genes, including hybridoma technology, phage display, B-cell cloning, and next-generation sequencing (NGS). Here, we focus on describing the recombinant antibody preparation process based on hybridoma and phage display technology.

- Preparation of Recombinant Monoclonal Antibodies Using Existing Hybridoma Technology

  Existing hybridoma-based monoclonal antibodies can be engineered to ensure enhanced consistency and specificity. The process involves extracting the sequences of antibody-producing genes from hybridomas and expressing them in mammalian cell lines. Here's a brief overview of the process:

  1. Hybridomas are prepared using the same procedure as described previously.
  2. The mRNA of a specific hybridoma is isolated, transcribed into cDNA, and then amplified by PCR.
  3. The antibody coding sequence is sequenced to determine the antibody coding sequence and cloned into an expression vector.
  4. These genes are then expressed in mammalian cell lines.
  5. The resulting recombinant antibody is validated to ensure that its performance matches that of the original hybridoma version.

- Preparation of Recombinant Monoclonal Antibodies Using Phage Display Technology

  1. *In vitro* phage display technology allows faster discovery of recombinant monoclonal antibodies against targets without the need for animal immunization (in the case of natural libraries). The technology involves constructing an antibody library, screening antibodies that bind to the target/antigen of interest, and increasing their affinity.
  2. The phage display process includes the following steps:
  3. The protein or peptide of interest is captured on an ELISA plate or attached to magnetic beads.

4. These antigen-coated surfaces are used to screen phage display libraries. The plate is washed to remove non-specific binding.

5. Next, the specific phage display conjugates are eluted from the vector and directed into bacterial cells for amplification.

6. This is followed by multiple screens to increase the number of antigen-specific phages. Typically, 2-3 rounds of screening are performed.

7. Specific binding of the target is confirmed through various detection methods, such as ELISA. DNA from phages associated with positive binding is then isolated and sequenced.

8. Antibody coding sequences are engineered for insertion into mammalian or IgG expression vectors for large-scale recombinant monoclonal antibody production.

9. Finally, the antibodies are validated.

References

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