



## HuCAL® Antibodies Technical Manual

### Introduction to Recombinant Antibodies

Over the last three decades the use of antibodies has increased greatly, both as tools for basic research and diagnostics and as therapeutic agents. This has been driven in part by advances in recombinant antibody technology. At the forefront of these advances is the Human Combinatorial Antibody Library (HuCAL®), one of the most powerful synthetic antibody libraries ever created. The HuCAL library is a highly sophisticated tool with features that make it the system of choice for basic research, target validation, drug screening and to study novel proteins created by the proteomics revolution. It has proved to be a direct source of antibodies for diagnostic and therapeutic applications and has been integrated into the research and development processes of many leading pharmaceutical and biotechnology companies. Bio-Rad uses HuCAL technology to provide a custom service for the development of novel antibody specificities for research, diagnostic, and other non-therapeutic commercial applications.

Our HuCAL Antibodies Technical Manual will enable you to discover the benefits of this sophisticated *in vitro* technology and is divided into easy-to-use chapters full of technical and application information. The first two chapters outline the concept of the HuCAL technology platform, and describe its many advantages over antibodies generated using traditional animal-based technologies. The next chapters explain the use of HuCAL antibodies in applications including ELISA, flow cytometry and immunofluorescence. These sections feature standard protocols which have been optimized for HuCAL monoclonals, as well as actual examples, hints and troubleshooting tips. The manual is a comprehensive technical resource for HuCAL recombinant antibody technology.

#### What are Antibodies?

Antibodies are glycoproteins that are naturally produced in response to invading foreign particles (antigens) such as microorganisms and viruses. They play a critical role in the immune system's defense against infection and disease.

Ideally, every antibody recognizes and binds to just one antigen. In reality, most antibodies are not fully monospecific and will also bind to other substances, albeit with lower affinity. Antigens can be proteins (for instance, receptors expressed on cancerous cells), sugars on bacterial and viral cell surfaces, hormones, chemical compounds, nucleic acid structures and so on. The region of an antigen that interacts with an antibody is termed the epitope. In the case of protein antigens, epitopes can be linear or three dimensional configurations of amino acids, or individual amino acids that have been modified (for example, by phosphorylation or oxidation). Typically, only foreign substances elicit an immune response.

Normally, an animal's immune system will generate a large group of antibodies that recognize several epitopes from a particular antigen. Each antibody is secreted by a different antibody-producing B cell and may be specific for a different epitope. Since the antibodies found in serum are collectively produced by many B cells (clones), they are described as polyclonal. While this is an advantage for fighting infections in nature, the heterogeneity of polyclonal antibodies limits their use as research tools.

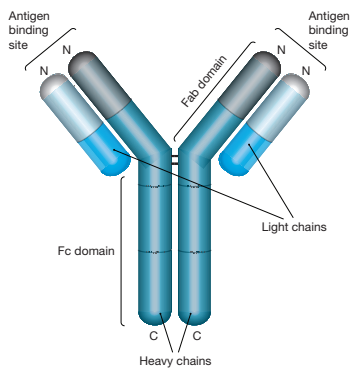
A major breakthrough in the development of antibodies for research applications was the production of monoclonal antibodies in 1975 by Köhler and Milstein.

Their technique involved removing B cells (plasma cell precursors) from the spleen of an animal that had been challenged with an antigen and subsequent fusing with an immortal myeloma tumor cell line. This resulted in a single-cell hybrid known as a hybridoma. The B cells confer antibody production capability, while the myeloma cells enable hybridomas to divide indefinitely and to grow well in cell culture. Hybridomas secrete only one antibody type, effectively ensuring an infinite supply of antibodies selective for a single epitope, which are also known as monoclonals.

#### Basic Antibody Structure

Many of the key structural features of antibodies can be considered using immunoglobulin G (IgG) as a model, since IgG is the most abundant antibody in human serum.

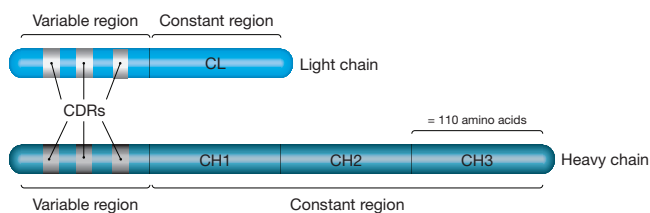
The classical representation of an antibody is as a Y-shaped molecule composed of four polypeptide subunits with two identical heavy and light chains (Figure 1). The N-terminal domains of each heavy chain associates with one of the light chains to create two antigen-binding regions; these are the arms of the Y shape and are termed Fab domains. The C-termini of the two heavy chains combine to form the Fc domain; this is the tail of the Y. The Fc domain is important for the antibody's interaction with macrophages and for activation of the complement cascade. The four polypeptide chains are held together by covalent disulfide bridges and non-covalent bonds.



**Fig. 1. Schematic Structure of an IgG Molecule.**

Sequence analysis of various light chains has revealed two distinct regions (Figure 2), a highly variable N-terminal half (VL) and a constant C-terminal half (CL). Similar studies on heavy chains have shown that they are also comprised of variable and constant regions (VH and CH, respectively). However, IgG heavy chains contain one variable region (found at the N-terminus) and three constant regions (Figure 2).

Unsurprisingly, the variable regions of both chains localize to the Fab domains, providing the structural basis for the antigen and epitope selectivity of antibodies.



**Fig. 2. Light and Heavy Chain Structure.** Light chains are approximately 220 amino acids in length and can be divided into a variable and a constant region. Heavy chains are approximately 440 amino acids in length and are subdivided into four segments, with one variable and three constant regions.

The sequence heterogeneity within the variable regions is most pronounced at three short segments (5-30 amino acids in length) named the hypervariable regions. Since the residues in hypervariable regions form the actual binding site for the antigen, they are also known as the complementarity determining regions (CDRs) (Figure 2).

### Advances in Antibody Technology

Although the development of hybridoma technology was a landmark event, the methodology still has a number of limitations. Developing hybridomas requires considerable time, expense and expertise, as well as specialized cell culture and animal facilities. Additionally, antigens can be toxic or poorly immunogenic in mammals and therefore, do not elicit an immune response. Moreover, mouse monoclonal antibodies are unsuitable for use as therapeutic agents because they are rejected by the human immune system. Various approaches to overcome this therapeutic limitation have been tried, such as:

- Combining mouse monoclonal binding sites with human antibody sequences to create chimeric or humanized antibodies

- Using genetically engineered mice to produce antibodies with human sequences
- Developing fully human antibodies directly with *in vitro* recombinant antibody technology

### Recombinant Antibodies

Recombinant antibody technology, such as HuCAL, is a rapidly evolving field with a number of major benefits over conventional antibody generation and production methods.

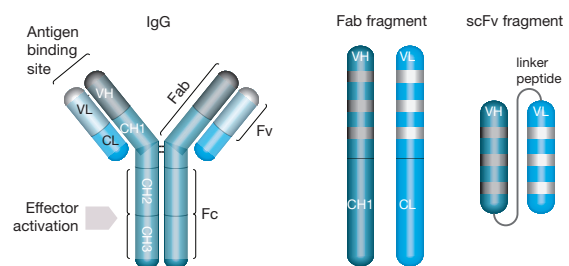
First, the immediate availability of gene sequences enables antibodies to be genetically engineered, allowing for the following:

- Study of the structural and functional properties of antibodies (binding, folding, stability, catalysis, etc.)
- Generation of smaller antibody fragments that retain the entire antigen binding site, such as the Fab and single-chain variable fragment (scFv) seen in Figure 3
- Enhancement of antibody affinity or specificity via mutagenesis
- Production of novel antibody fusion proteins, which couple the antibody binding capacity to additional properties (enzymes, toxins, multimerization modules and epitope tags)
- Direct creation of partially or fully human antibodies

Second, recombinant monoclonal antibody fragments can be produced in bacteria. This is easier, faster and less expensive than using animals (ascites) or mammalian cell culture techniques.

Third, the technology enables the use of *in vitro* selection steps that facilitate the isolation of antibodies with desired characteristics, e.g. antibodies that distinguish closely related antigens.

Finally, the method does not rely on the use of laboratory animals and does not require immunogenicity of the antigen. Antibodies can be produced from non-animal derived materials and therefore are free from animal pathogens.



**Fig. 3. Commonly Used Recombinant Antibody Molecules.** IgG: Full length molecules exactly matching the natural antibody composition. Fab fragment: The variable domain and the first constant domain of one heavy chain, plus one complete light chain of an antibody. The two chains can be covalently linked by a disulfide bond and are also linked by strong non-covalent interactions. scFv fragment: A single-chain antibody consisting of one VH and one VL chain expressed as a single polypeptide joined by a peptide linker. The polypeptide linker stabilizes the interaction between VH and VL chains.

## Methods for the Generation of Recombinant Antibodies

In the past, the initial step in generating recombinant antibodies was to isolate the relevant genetic material from a pre-selected hybridoma cell line and clone the antibody genes into an expression vector. Another option was to amplify the antibody repertoire of an immunized animal by polymerase chain reaction (PCR), using degenerate primers and to clone the sequences into a bacterial selection system such as phage display.

Modern techniques to generate recombinant antibodies start instead from very large (>1 billion) libraries of antibody genes. These libraries usually contain human antibody gene sequences since they were originally designed to produce human antibodies for therapeutic purposes. Such libraries can be derived from B cells taken from non-immunized donors, or they can be generated *de novo* by gene synthesis (such as with HuCAL). Therefore, they are called unselected or naïve libraries. If they contain a sufficient number of functional members, these libraries can be used to select specific antibodies against virtually any antigen.

Since large gene libraries cannot be directly screened for the binding properties of individual members, the antibodies that bind a given antigen are identified instead by selection. All selection methods to date physically link the antibody protein in the library (the phenotype) with the genetic information that encodes the given antibody (the genotype). There are a number of techniques, of which the most commonly used are phage display, ribosome display and yeast display. Phage display is the most popular and best established of the selection methods.

### Phage Display Selection

Phage display is used to select *E. coli* host cells that contain the desired antibody fragment, i.e. the antibody that binds a given antigen (Smith and Scott, 1993; Kretzschmar and von Rüden, 2002). Filamentous *E. coli* phages, such as M13 (which is non-lytic), are most commonly used (Figure 4). For phage display, the antibody library is cloned into a phagemid vector with the following properties:

- Has both a bacterial and a phage origin of replication (ORI)
- Contains an antibiotic resistance marker
- Allows expression of the encoded antibody
- Links the expressed antibody to a phage surface protein (typically protein III) by genetic fusion

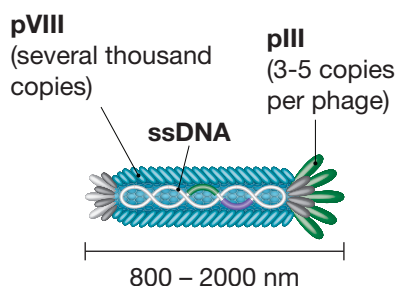


Fig. 4. Schematic Drawing of M13 Phage.

The process begins by using helper phages to infect *E. coli* host cells containing phagemid vectors encoding the antibody collection. On the phagemid the antibody genes are fused to the phage gIII gene encoding the pIII coat protein, which will later form part of the phage particle. The helper phage genome directs the *E. coli* cell to produce daughter phages that incorporate the antibody-pIII fusion protein into the phage coat. Since the helper phage genome contains a defective ORI, which does not replicate, the daughter phages incorporate the phagemid into their capsid. Hence, resulting phages will contain the antibody's genetic information and will simultaneously display the corresponding antibody binding sites on their surfaces. Such phage particles can be harvested from the culture supernatant. About one thousand phages per antibody ( $10^{13}$  phage particles for a  $10^{10}$  antibody library) are subsequently used for the antibody selection process to ensure full coverage of the antibody library.

Phage displaying the desired antibodies are selected by 'phage panning', which shares similarities with solid-phase immunoassays (Barbas and Lerner, 1991). In this process, the antigen of interest is immobilized on a solid support, such as microplate wells or magnetic beads. The phage particles are then added to allow binding of phages that display appropriate antibodies. After extensive washing to remove most non-specifically bound material, the bound phages are eluted and amplified by replication in new host cells. The selection procedure is repeated several times, thereby enriching a population that consists to a high degree of phages that express the desired antibodies (i.e. those that bind the antigen of interest). After this selection, the antibody genes can be purified from the enriched pool of phagemid DNA and ligated into a suitable *E. coli* expression vector, which usually contains appropriate sequences encoding short peptide tags for later purification and detection (see below).

### Expression and Screening of Antibody Fragments

*E. coli* cells transformed with the cloned pool of enriched antibody genes are grown on agar plates with appropriate antibiotics, forming colonies. Each colony contains the genetic information for one antibody (heavy and light chain) enriched by the previous phage display selection. Therefore, each colony contains the genetic information for one monoclonal antibody.

At this stage, colonies are picked and grown and antibody expression is induced. Cells are subsequently lysed and the antibody-containing lysate is tested with for instance ELISA for the presence of antigen-specific antibody material. The plasmid DNA from ELISA-positive colonies is then isolated and the antibody gene is sequenced. At this point, the panning method typically results in the isolation of a number of different antigen-specific monoclonal antibody fragments from the original large library of antibody genes. The overall procedure (from panning to antibody sequences) takes about 4 to 6 weeks.

## Purification of Antibody Fragments

Soluble antibody fragments (e.g. Fab or scFv) produced by bacterial cultures are usually purified by one-step affinity chromatography using epitope tags that have been genetically fused to the C-terminus of the antibody heavy chain. Epitope tags commonly used for this purpose are the Strep-tag®, a short peptide with 8 or 10 amino acids that binds to streptavidin (Strep-Tactin®, a mutated streptavidin with higher affinity to the Strep-tag is also used); or the Histidine tag (His-6), which is a series of six histidines that can be complexed by metal chelates such as nickel nitriloacetic acid (Ni-NTA). Since Strep and His-6 epitope tag antibodies are commercially available, the tags can also be used for highly specific immunodetection.

It should be noted that the antibody fragments that have been isolated will include the complete antigen binding site and as a result they usually have the same intrinsic antigen-binding affinity as the complete antibody. However, full length IgG antibodies contain two binding sites per molecule, which increases their avidity and therefore their apparent affinity in some assays. To match the affinity and avidity of natural immunoglobulins (Igs), recombinant antibody fragments can be dimerized or further multimerized by engineering.

**Affinity** is the strength of binding of an epitope to an antibody binding site.

**Avidity** is the overall stability of the antibody-antigen complex, which is dependent on affinity, valency and structural arrangements.

## Advantages of Recombinant Antibodies

Recombinant antibodies generated from *in vitro* antibody libraries offer many advantages over conventional monoclonals as described in the following sections. These stem from several properties inherent in the system:

- All stages of antibody selection occur *in vitro*
- Resulting antibodies can be engineered using the isolated plasmid DNA in combination with the known antibody DNA sequences
- Technology is based on a bacterial expression system

## In Vitro Selection

The ability to select antibodies *in vitro* is especially valuable for generating antibodies to non-immunogenic and toxic antigens which cannot be easily used in animals. Furthermore, it is an open system which allows modification of the selection conditions. Using guided selection, the enrichment process can for instance be pushed towards selection of epitope-specific antibodies, e.g. those that bind only to post-translationally modified epitopes like phosphorylated amino acid residues.

Once recombinant antibodies with desired specificities are found, they can be further enhanced by generating new combinations of heavy and light chains, or by mutating individual CDRs (Karu et al. 1995).

## Engineering

Peptides and proteins adding new functions can be linked by genetic fusion to the antibody. Examples include:

- Epitope tags for purification, immobilization and detection
- Enzymatic activities, such as alkaline phosphatase (AP), for direct detection
- Multimerization modules that increase avidity
- Heterodimerization modules that allow for the creation of bi-specific antibodies
- Toxins for the elimination of tumor cells or enzymes that convert a pro-drug into a drug at the site of a tumor (ADEPT)

## E. coli Production

Bacterial production is very fast (overnight expression), less expensive than mammalian cell culture and can be performed without the use of animal-derived components. In addition, the bacterial system is fully-defined and free from animal-based viruses, prions and contaminating immunoglobulins. High purity of antibodies purified from *E. coli* cultures is typically accomplished using one-step affinity chromatography. *E. coli* production of antibody fragments has been shown to result in high yields (Ge et al. 1995), with demonstrated production yields of more than 1 gram per liter *E. coli* fermentation culture (Carter et al. 1992).

## Antibody Fragments

Antibody fragments, such as Fabs, which lack the Fc domain, often show benefits over full-size IgGs, including:

- Elimination of non-specific binding to cellular Fc receptors leading to lower background and increased sensitivity in cell based assays and in immunohistochemistry (IHC)
- Avoidance of cytokine release in functional assays with immunocompetent cells, since complement cannot bind
- Increased diffusion through tissue with smaller Fab fragments, leading to faster and more efficient staining
- Creation of fewer artifacts in receptor studies because monovalent Fabs do not cross-link receptors
- Reduced non-specific binding to the solid phase in heterogeneous ELISA experiments due to lower hydrophobicity
- Improved stoichiometry with less interference from serum factors and macromolecules (which mostly bind to the Fc region) in fluorescent or enzyme conjugates



- Simplified co-crystallization of target proteins with antibodies
- Easier determination of true intrinsic affinities between antigen and antibody due to the monovalency of Fab

## Therapeutics

Antibodies selected from *in vitro* libraries of human antibody genes do not elicit the same immune responses in patients that are seen with non-human antibodies. Therefore, such antibodies can be used for therapeutic development. More and more fully human antibodies obtained from antibody libraries are entering clinical development and are reaching the market (Weiner, 2006).

## Speed and High-throughput

Antibody selection using phage display of antibody libraries is a process that takes around 4 to 6 weeks for antigen-specific members to be enriched, isolated and sequenced. Further production and characterization of the monoclonal antibodies is also rapid, due the fast growth rate of *E. coli* cultures. Nevertheless, there are attempts to further accelerate the process with the goal being to perform selections in just a few days (Hogan et al. 2005).

The phage display selection process can be automated to a large degree (Krebs et al. 2001) so that many target antigens can be processed in parallel, further increasing the efficiency of the process.

## Considerations When Using Recombinant Antibodies

Some reagents commonly used for immunoassays are not appropriate for protocols involving recombinant antibodies selected from human antibody libraries such as HuCAL. Anti-mouse and anti-rabbit IgG secondary reagents, for example, are unsuitable for detection. Instead, detection of recombinant antibody fragments is usually performed using antibodies directed against epitope tags, such as: Strep-tag, His-6, V5, c-myc or the FLAG®-tag (DYKDDDDK). These anti-tag antibodies are usually directly labeled with an enzyme such as horseradish peroxidase (HRP) or a fluorophore. They can also be used in combination with a labeled tertiary antibody. Alternatively, detection of human recombinant Fabs can be achieved using anti-human Fab antisera labeled with AP, HRP, or fluorescent dyes.

Further, pull-down experiments or immunoprecipitation using recombinant Fab cannot be performed using Protein A Sepharose® (which mostly relies on the presence of the Fc domain), but can be performed by coupling the recombinant antibody to magnetic beads.

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