TWIST BIOPHARMA

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From Target to Molecule

A Comprehensive Guide to Antibody Discovery and Development

CONSIDERATIONS AND APPROACHES FOR THE BIOPHARMA INDUSTRY

Before We Get Started...

Welcome! You're about to embark on a journey of discovery. And like all journeys that are worthwhile, it's far from linear. The process of antibody discovery and development into a clinical candidate is one full of twists, turns, roundabouts and offshoots, yet one that an increasing number of researchers and companies, both small and large, have successfully navigated.

At least 570 mAbs have already been progressed to the clinic by commercial companies and, similarly, your goal is to advance your mAb candidates to clinical trials and obtain regulatory approval.[1] This journey is long, with most companies taking approximately 12 years to move from the first patent filing to FDA approval.[2] The multi-year journey may feel overwhelming, but it can be broken up into short-term goals and milestones that allow you to stay present for the full exhilarating experience.

This guide is here to help you as you begin your journey. As recently as the past decade, much has changed in antibody discovery, optimization and development. New techniques have made this entire process more rapid, cost-effective and targeted.

What follows is your handbook for the modern antibody development process.

Catch you on the flip side.

The History of mAbs and Their Current Therapeutic Importance

Since the first approval of a monoclonal antibody (mAb), Orthoclone OKT3 (muromonab-CD3), by the FDA in 1986, 570 different therapeutic mAbs have been clinically tested worldwide and 79 have been FDA-approved for a variety of indications.[1] The power of these therapeutics are well established, making the treatment of previously untreatable autoimmune, metabolic, neurologic, infectious diseases, and cancer possible. This has fueled enormous growth and investment in the related R&D and clinical development. In 2018 and 2019, the combined number of mAbs approved by the FDA totaled an impressive 18, with a further 12 mAbs approved in 2020 and an additional 16 mAbs in regulatory review.[1]

In part, the increasing number of approved mAbs with enhanced specificity, potency, efficacy, and safety is driven by a solid foundation of cutting-edge molecular biology techniques. For instance, bispecific monoclonal antibodies target both cancerous cells and T-cells. By binding both, T-cells are brought into close proximity with the cancer cells so they are destroyed. In the case of antibody-drug conjugates, antibodies specific to the cancer cells for instance, bring a conjugated chemotherapeutic drug to target those specific cells while preserving healthy cells. Novel analytical and synthetic chemistry, and automated instrumentation are also important contributors to the increasing number of approved mAbs. These advances have helped grow the global therapeutic mAb market to expected revenues of $143.5 billion in 2020 and will surpass $368.8 billion by 2027.[1]

Using the technical foundation above, the process for antibody discovery and pre-clinical development of therapeutic antibodies has changed dramatically. Orthoclone OKT3, for example, was FDA-approved for kidney transplant rejection, but post-market clinical challenges minimized its commercial success.[3] This was largely due to the use of hybridoma methods for generating therapeutic mAbs. At the time, this method was cutting edge, using antibody-producing cells from mice to test murine mAb candidates and scale up production of leads. At the time, it was a major success. But compared to current methods of discovery, this approach generates antibodies with an increased risk of immunogenicity due to the use of non-human antibody sequences. It also suffers from a number of manufacturing challenges, including low production yields and genetic instability, which make downstream production a challenge.[3]

Today, there are still challenges to face, they're just different. The problems that plagued Orthoclone OKT3 more than three decades ago have largely been solved through the use of humanized mice, synthetic antibody libraries, display technologies, and mammalian and prokaryotic expression systems in pre-clinical development.[1] But antibody discovery is still labor intensive, complex and costly. Many targets like G protein-coupled receptors (GPCRs), ion channels, carbohydrates, phosphatases and transcription factors have been deemed "undruggable" using traditional methodologies.[4]

However, something is only "impossible," until it's proven otherwise. As we'll see in the pages that follow, overcoming the undruggable moniker simply requires an updated approach.

The Molecular Structure of Antibodies

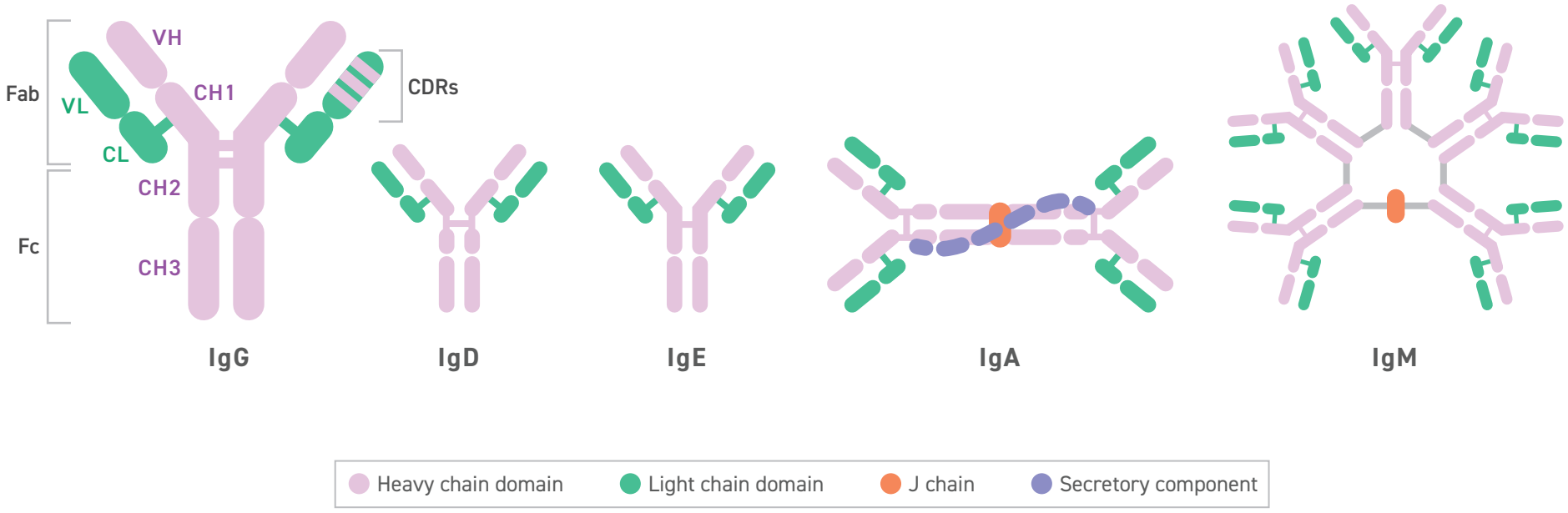
The success of monoclonal antibodies as a therapeutic class would not be possible without a strong understanding of antibody structure. This knowledge fuels the modern engineering of antibody and antibody-related therapeutics with the desired antigen affinity, effector function, mechanism of action, and biophysical properties.

TAILS AND ARMS

In simple terms, antibodies are Y-shaped quaternary proteins. The base or "tail" is called the fragment crystallizable (Fc) region.[5] The two "arms" extending outwards are known as the fragment antigen-binding (Fab) region. At the very tip of each arm is the paratope, which allows the antibody to bind a specific antigen epitope via the complementarity determining regions or CDRs.[5]

HEAVY AND LIGHT CHAINS

Zooming in, the critical Fragment Antigen Binding domains, e.g. Fab regions, comprise four polypeptide chains; two identical light chains and two identical heavy chains. Both light and heavy chains have a constant region and a variable region.[5] As the names suggest, the constant region remains the same across all antibodies in a given isotype. The variable region, also known as Fv, differs from antibody to antibody (unless they're produced by a single B cell or B cell clone).[5]



HYPERVARIABLE REGIONS

Complementarity-determining regions (CDRs; see image above and on page 15) are responsible for most of the sequence variation that occurs in antibodies and, in turn, the impressive scope these proteins have for binding different antigens.[5] For this reason, they are also referred to as hypervariable regions. Each of the four peptide chains (heavy and light) have three CDRs on their variable domains, for a total of 12 per IgG antibody (see above).

ANTIBODY CLASSES

Heavy chains also determine which of the main classes or isotypes the antibody fits into. In humans, there are five known isotypes: IgA, IgD, IgE, IgG, and IgM.[5] Each has a distinct role, with different binding characteristics and distinct biological properties. In the world of therapeutics, the vast majority of monoclonal antibodies and Fc fusion proteins are part of the IgG class, which has four different subclasses within it namely, IgG1, IgG2, IgG3 and IgG4.[5]

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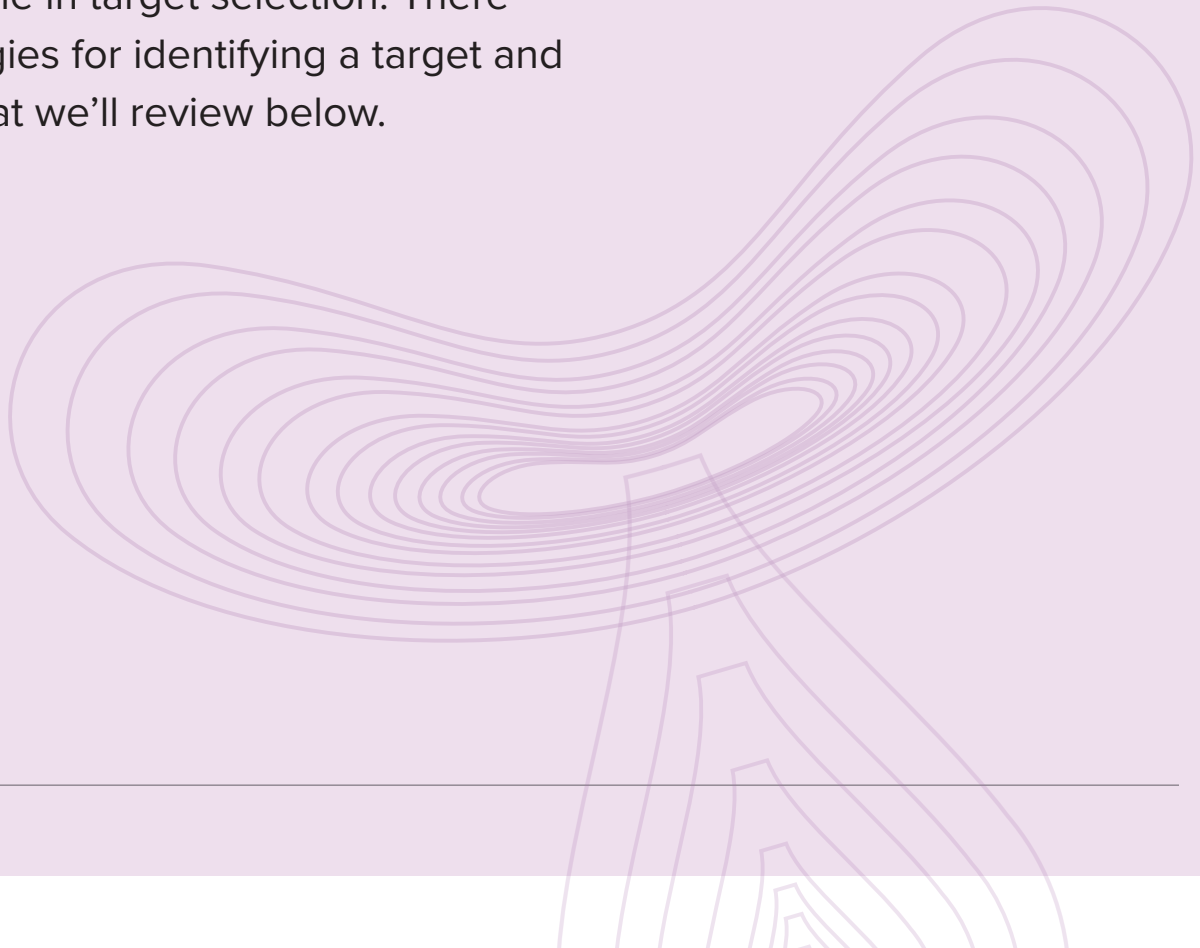
Target ID and Validation

A target is considered “druggable” when a pharmaceutical, such as a small molecule or monoclonal antibody, can be used to modulate the target’s activity.[4] But just because a proven or potential target is druggable, doesn’t mean you should start your antibody development adventure there. There really is no “tried and true” method for identifying and validating a target.[7,8] Serendipity, as in many aspects of research, can play a significant role in target selection. There are, however, a few common strategies for identifying a target and subsequent criteria for validation that we’ll review below.



Phenotypic Targeting

Phenotypic targeting, also known as target deconvolution, is the process of working backwards from a phenotype to a specific molecular target.[7,8] Through large, high-volume cell-based or animal screens of small or large molecules for a target phenotype, efficacious drugs can be identified without any knowledge or bias toward a specific molecular target.[6] This strategy has been broadly applied to identifying novel antibiotic classes.[8]

ADVANTAGES

- Efficacy is initially demonstrated, making translatability to later preclinical testing more probable

DISADVANTAGES

- Can be lower throughput depending on the phenotypic model
- Typically higher cost
- It often requires specialist instrumentation and expertise (eg., LC-MS)
- May be difficult to identify a single, definitive molecular target

Target-Based Discovery

Target-based discovery is widely used and has been applied to a much broader class of therapeutics, including not only small molecules and peptides, but newer classes of biologics, like mAbs, gene therapies, and nucleic acid therapeutics.[8] This approach has been largely successful, facilitated by the development and current ubiquity of recombinant technology, genomics, bioinformatics, and molecular biology. Generally, in a target-based approach, a defined molecular target is chosen based on genetic, genomic, molecular biology, and/or biochemical studies.[8] Once selected, recombinant technology, genomics, and cell-based experiments are used to judge if the target would be suitable.

ADVANTAGES

- Simple workflows that are less laborious and costly to execute
- Genomics, structural, biochemistry, biophysics, mutational, or pharmacological studies, whether published or unpublished, enable a broad knowledge base for target identification and validation and downstream drug optimization

DISADVANTAGES

- Failure to translate to the more complex, *in vivo* systems in later preclinical development

Reviewing Scientific Literature

There's a saying that "a day in the library will save you a week in the lab" (though many like to jokingly reverse it, much to the chagrin of librarians). Whether you're taking a phenotypic targeting or target-based strategy towards target identification and validation, a lot of information about the disease state, phenotype or specific molecular target can be gleaned from a thorough review of the scientific literature. In identifying and analyzing key findings from animal studies, *in vitro* experiments, or the increasing number of 'omics' studies, drug discovery groups can synthesize solid hypotheses around what might make a good target.[7,8]

While the literature can be the basis for solid target identification, key published findings may need to be repeated and validated before you commit to a given target, especially if it's novel and has never been drugged before. In addition, scientific literature that is in the public domain can be analyzed by any public or private research group, creating potential competition in drug discovery.[8]

DID YOU KNOW?

Of 113 first-in-class drugs approved by the FDA between 1999 and 2013, 70% were identified through target-based drug discovery.[8]

What Makes a Good Antibody Drug Target?

The criteria that make a drug target “good” are somewhat subjective and take a holistic view of the available evidence.

Broadly, successful target identification results from a thorough understanding of the pathophysiology of a disease, the underlying molecular pathways and the presence of predictive models and supporting technologies.[9] Analysis of gene expression, proteomics, molecular pathway databases, phenotype databases, knockout/knockdown screening, genetic association data, translational studies, and published scientific literature can all illuminate the journey ahead.

Here's a more defined list of what makes an ideal drug target and the pieces of evidence that should be available, either in-house or through a thorough literature review:[6,9]

1. UNDERSTANDING OF PATHOPHYSIOLOGICAL ROLE
The target's role in the disease is well backed-up with relevant animal models through knock-out/in or overexpression studies

2. INDIRECT THERAPEUTIC VALIDATION
The target is part of a signalling pathway that has been therapeutically manipulated before or clinically validated

3. STRUCTURAL DATA
The structure of the target has been solved to assess "druggability"

4. ASSAYABILITY
Target can be assayed easily with high-throughput screening

5. TISSUE-SPECIFIC EXPRESSION
Target is not uniformly distributed throughout the body and is localized to a specific tissue in the periphery that is accessible to a therapeutic

6. COMPANION BIOMARKER
A biomarker exists to monitor clinical efficacy

7. SAFETY AND TOXICITY
The target has a promising toxicity profile and adverse effects can be predicted from phenotypic data

8. FAVORABLE IP STATUS
The therapeutic will eventually need to be patented. At the target identification stage, the emphasis should be on determining if modulating the specific target you have in mind is patentable

Validating Your Target

Before you move forward to the antibody discovery phase, validation of a molecular target is necessary. Validation is really just a fancy way of saying that there is sufficient scientific evidence, either from the literature or from in-house studies, to suggest a role in the pathology of a disease and its therapeutic potential.[6]

IN GENERAL, THE CRITERIA BELOW CAN BE USED FOR VALIDATION OF A TARGET:[6]

- The target's biological and pathophysiological function are well understood
- Pathology has been validated in an animal model
- Antibody-based treatment in an animal model of the disease results in the desired outcome
- Genetic data establishes the target as being involved in the human disease
- The pathway in which the target lies has been therapeutically manipulated before

While many successful target choices meet these criteria, it’s not 100% essential to check every box to move forward. In addition, if one or multiple criteria are missing, it may be necessary to try to collect that data within your research group, before moving forward with antibody discovery. Completely meeting the criteria however, does help with de-risking a potential target before initiating a discovery program.

Identification and validation are two early pieces of the antibody discovery puzzle and the interplay between both may be a steady give and take: One target may be identified as another is being validated. The extent of target identification and validation will depend on the size, scope and budget of your own R&D group.

Antibody Discovery

Now that you've got a solid handle on what molecular target you're going after, let's move on to the fun part: Discovering an antibody that specifically binds to that target and picking antibodies.

Methods for discovering antibodies have gone through a massive transformation over the past few decades, streamlining the quality with which fully humanized monoclonal antibodies are discovered and tested. The now "traditional" approaches to antibody discovery relied on the tedious generation of hybridomas; a hybrid cell type formed when a primary antibody-producing cell is isolated from an animal and fused with a myeloma cell.[1]

The process involved immunization of a model organism (usually a rodent), with the target recombinant antigen. Antibody-producing splenocytes are then harvested from immunized mice, and single splenocytes producing a single antibody species are then fused with a continuously proliferating myeloma cell line to generate a hybridoma that secretes the antibody candidate.

The first hybridomas were developed in 1975[42] to suggest a role in the pathology of using mice, and the traditional process can lead to the identification of specific, high-affinity murine antibodies.[6] The first approved monoclonal antibody, Orthoclone OKT3, was made using this method. Since then, only three fully murine antibodies have been FDA approved, all of which have been discovered using the hybridoma method. The reason for this low number of approved murine antibodies is that they face a number of challenges, including the induction of a human anti-mouse antibody response (HAMA), which leads to rapid clearance of the mAb and can lead to allergic response and reduced efficacy.[1]

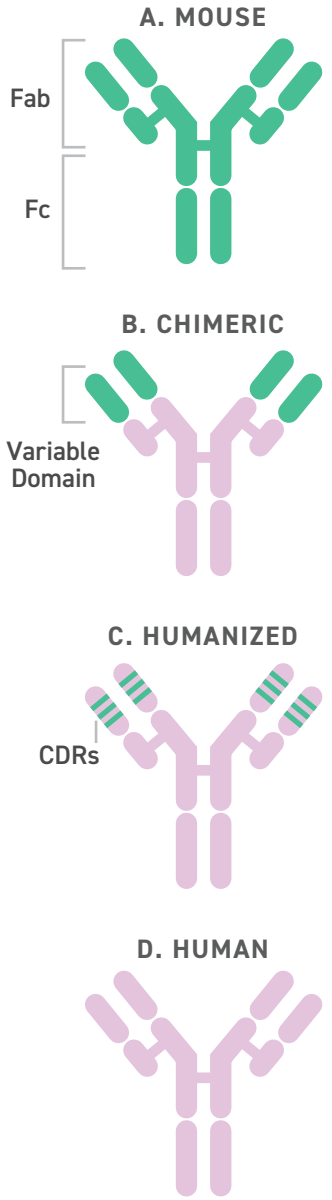
Humanizing the Mouse

Hybridoma technology has yielded the majority of FDA-approved therapeutic mAbs—49 out of 79—but only three are fully murine reflecting the need to overcome decreased efficacy due to immunogenicity caused by the human anti-murine antibody (HAMA) response.[1] To deal with the challenges of HAMA, several strategies and technologies have been developed to transition murine mAbs into more humanized versions of themselves. Traditional hybridoma technology can take anywhere from 3–6 months to generate a stable hybridoma cell line. It may then take at least another 2 months to run functional assays. This adds to development costs and lengthens timelines. At Twist Biopharma, we complete the discovery process with our proprietary processes in 8 weeks.

Chimeric and Humanized mAbs

High-affinity murine mAbs can be made more human using relatively straightforward cloning techniques.[1,6] It involves taking the DNA sequence from the murine heavy and light chain variable regions (VH and VL, respectively) and fusing them to the human heavy and light chain constant regions. Essentially, this creates a mAb with a murine Fab fragment and a human Fc region—a chimera. The first chimeric antibody Reopro (abciximab) an anti-GPIIb/IIIa antigen-binding fragment (Fab), was approved by the FDA in 1994 and there have been several other successful chimeric antibodies, including MabThera/Rituxan (rituximab), approved by the FDA in 1997 for the treatment of non-Hodgkin's lymphoma (other indications have since been added to the label).[1]

Another similar strategy for humanizing murine mAbs is by grafting murine CDRs into a human framework. The strategy is similar to creating chimeric mAbs, but is more specific, using only the murine regions that are involved in antigen binding. These humanized antibodies typically retain antigen binding function, without the immunogenicity issues seen with fully murine or chimeric mAbs. There may be a need for downstream engineering following CDR grafting of the human framework as other regions of the murine mAb, outside of the CDR, are involved in antigen binding.[1,6] This can typically be discovered through structural studies using X-ray crystallography or cryo-EM. Zinbryta (daclizumab), an anti-IL-2 receptor therapeutic, became the first humanized mAb to be approved by the FDA in 1997.[1]



Fully Human mAbs From Transgenic Mice

With the success of chimeric and humanized mAbs, techniques for developing fully human mAbs soon emerged.[1] In 1994, two transgenic mouse strains, the HuMabMouse and XenoMouse were created and used as the earliest *in vivo*-based technologies for developing fully human mAbs.[1] These strains were genetically modified to replace the endogenous mouse Ig genes with the human Ig genes.[1] These transgenic mice are capable of synthesizing fully human mAbs following immunization and, using the hybridoma technique, researchers are able to isolate target-specific mAbs. An anti-epidermal growth factor receptor (EGFR) antibody Vectibix (panitumumab), became the first mAb purified from transgenic mice to be FDA-approved in 2006. However, due to the presence of the human constant region, B cells were found not to compartmentalize efficiently in the natural B cell maturation process, therefore the next generation of transgenic mice using the murine constant region rectified this.

There are also now transgenic mice that produce chimeric antibodies, following immunization, with murine Fc and human Fab.[1] It therefore follows that the more efficient strains of transgenic mice generate chimeric antibodies that can be fully humanized in a straightforward manner following identification of antigen-positive B cells. These mice were created to overcome a specific challenge: HuMabMouse and XenoMouse exhibit only very low levels of somatic hypermutation and human antibody generation, due to the lack of the murine constant region.[1] There are now several transgenic chimeric mouse lines available, but only one, the VelocImmune mouse, has yielded FDA-approved mAbs—seven to be exact.[1] The first therapeutic mAb isolated using the VelocImmune mouse was Praluent (alirocumab), which gained FDA approval in 2015.[1]

DID YOU KNOW?

Over half of all FDA-approved mAbs are chimeric or humanized. This includes the blockbuster humanized mAb, Herceptin (trastuzumab), used for the treatment of HER2-positive metastatic breast cancer and other forms of cancer.[1]

What is Somatic Hypermutation?

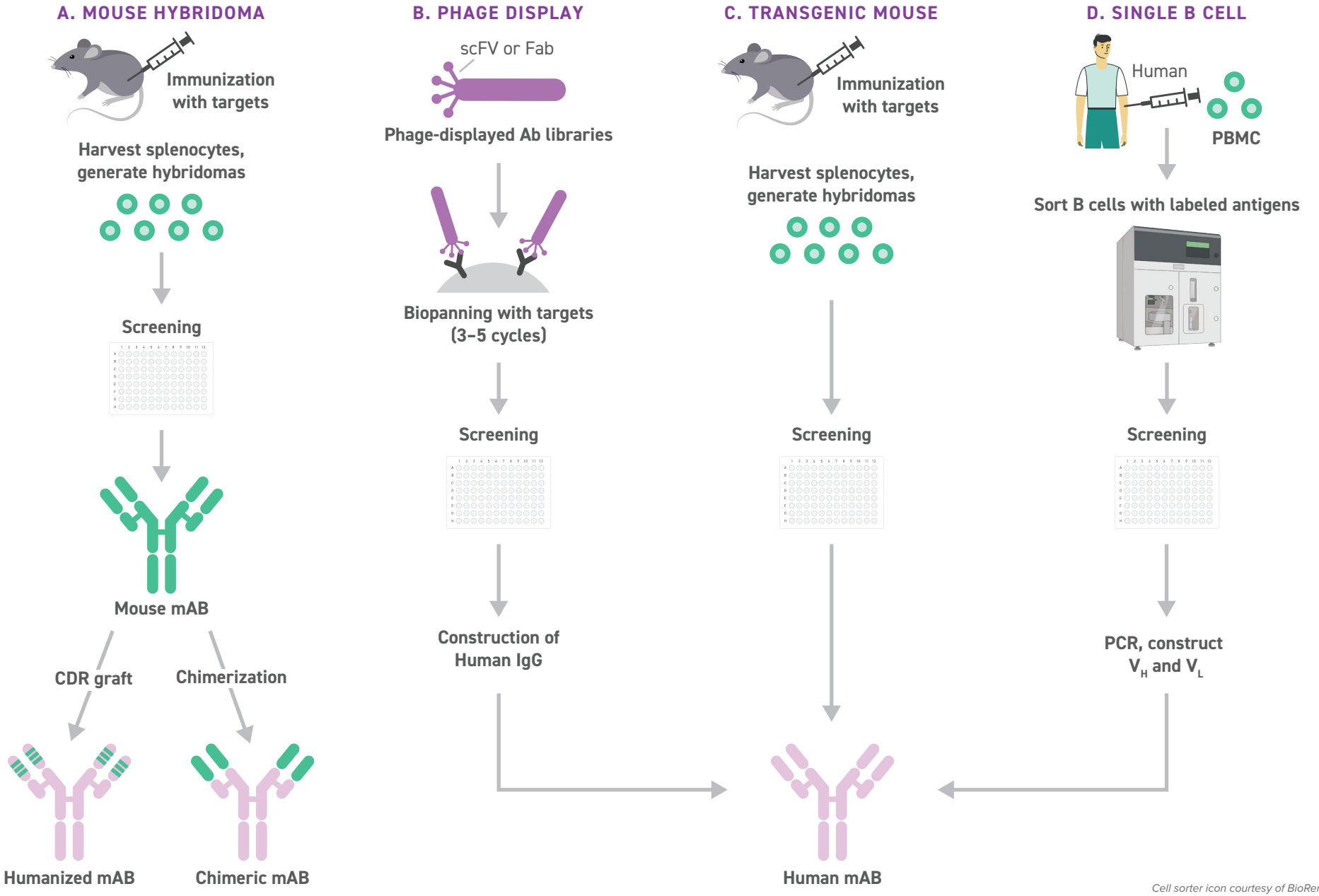
Somatic hypermutation is a process by which mutations are introduced into CDRs to expand or enhance the ability of antibody-producing B-cells to bind to specific antigens.

Fully Human mAbs Straight From the Source: Single Human B Cells

One promising and potentially rapid method for developing fully human mAb therapeutics, especially for emerging pathogens, is isolating and sorting single B cells from humans that have been infected with a bacterial, viral, or eukaryotic pathogen. Individual antigen-positive B cells can be sorted by their ability to bind to labeled antigens. Positive binders are then sequenced, and heavy and light chain genes can be cloned for downstream optimization.

This method is incredibly exciting and many research groups have reported the successful isolation of mAbs that target a number of pathogens.[1] Human mAbs that target Ebolavirus, HIV-1, influenza, and respiratory syncytial virus are all being evaluated clinically, however, no FDA-approved therapeutics have made it to market yet.[1]

While exciting, this methodology can be challenging in practice: Without specialized instrumentation, isolating single cells and rapidly assaying for specific binding can be imperfect and labor intensive. Thus, FACS, laser-capture microdissection, micromanipulator, and microfluidic techniques and instrumentation have been developed to assist research groups taking this approach.



Cell sorter icon courtesy of BioRender

What is in a Name?

Adalimumab, raxibacumab, and belimumab:

There's a method to this naming madness. The International Nonproprietary Name (INN)—an expert group within the World Health Organization (WHO)—oversees the official naming formula, which calls out specific mAb features including the intended target, original host, modifications, and conjugations.[10]

Here's a quick overview of the classification system, which was updated in 2017:[10]

- **Prefix:** This "random" section is your chance to add some personal flair and to differentiate your drug from other similar mAbs
- **Infixes:** Substem A refers to the intended target (e.g., "ci" for cardiovascular)
- **Suffix:** All mAbs end with the suffix "mab," for obvious reasons

New mAb Nomenclature Scheme

PREFIX	SUBSTEM A*: TARGET CLASS	STEM
Random	*ba* • bacterial *ami* • serum amyloid protein (SAP)/ amyloidosis (pre-substem) *ci* • cardiovascular *fung* • fungal *gros* • skeletal muscle mass related growth factors and receptors (pre-substem) *ki* • interleukin *li* • immunomodulating *ne* • neural *os* • bone *toxa* • toxin *ta* • tumour *vet* • veterinary use (pre-stem) *vi* • vitral	***-mAb***

** The substem A is currently under revision*

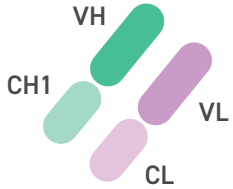
Monoclonal antibodies named prior to 2017 included two other sections. A second substem or infix was used to denote the source or host organism that the antibody was originally produced in (most commonly "u" for human or "o" for mouse). An additional modification was also used to indicate chimeric ("-xi-") or humanized ("-zu-") engineering. Due in part to ambiguities and concerns that the names were being manipulated for commercial reasons, these two features were scrapped when the new INN guidelines were introduced.

Rodents, Llamas, Sharks, Chickens, and Cows, Oh My!

Biomedical research and drug discovery have relied pretty heavily on mouse, rat and rabbit model systems, both for pre-clinical research and drug discovery. But with the recent approval of Cablivi (caplacizumab), a heavy-chain only, single-variable domain "nanobody" initially found in llamas and other camelids, many companies and researchers are focusing on discovery and development of these "small" biologics.[11] Nanobodies are 12–30 kDa, more than five times smaller than full-length murine and human monoclonal antibodies.[11] Like other antibodies, nanobodies are modular, yet their size gives them greater stability, flexibility in the routes of administration, less potential for immunogenicity and greater manufacturability.[11]

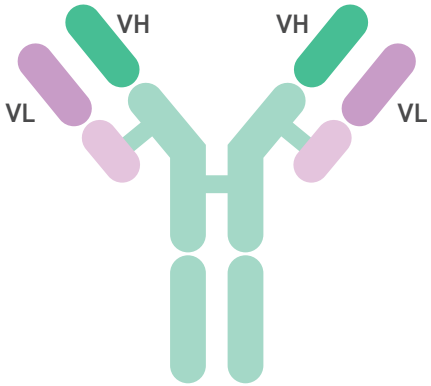
Their size also gives them greater accessibility to different antigens, unlocking the door to potential new targets. For example, Cablivi is the first approved drug to target von Willebrand factor, a protein that plays a critical factor in blood coagulation.[11] The approval is the first to demonstrate definitive efficacy and safety for the nanobody field, encouraging other developers to explore this newer approach.[11] Another advantage is the ability to use these small biologics to create conjugated, multidomain, and multivalent therapeutics.[11]

Another non-traditional format that is swimming onto the single domain antibody scene, is the variable new antigen receptor (VNAR) from sharks. Like VHH antibodies, they are small (~12 kDa) yet have larger CDRs than human antibodies and may hold promise for targeting GPCRs and ion channels, which have traditionally been difficult to drug. The uptick in interest in single domain antibodies is sure to bring new tools to the antibody development field, and with the increasing number of small biologics entering the clinic, there will no doubt be additional approvals in the coming years.

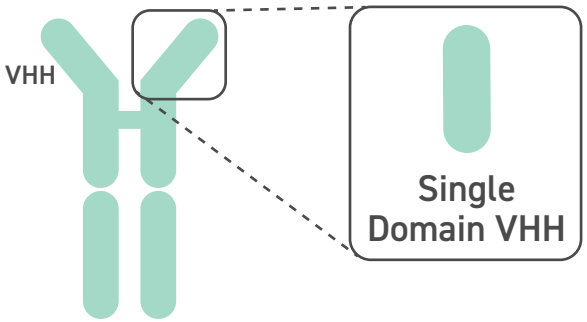


Fab

scFv Single chain
Fv Fragment



IgG Antibody



Camelid Antibody

Display Based Technologies and Synthetic Antibody Libraries

As molecular biology techniques got more sophisticated and cloning became commonplace, it became almost effortless to combine heterologous DNA sequences together to create antibody chimeras and do the CDR grafting described above. And as commercially-available DNA oligonucleotide synthesis became more widespread, site-directed mutagenesis for engineering mAbs with improved binding affinity, stability, or specificity became possible.

The availability of randomized oligo pools enabled researchers to mimic the natural process of somatic hypermutation, achieving some of the sequence diversity seen in natural CDRs. It also allowed them to focus entirely on human sequences, without the need for transgenic mice or primary B-cell isolation from infected or immunized humans or transgenic mice. However, to capitalize on this diversity, an efficient technique that mimics the process of affinity maturation was necessary.

Enter phage display (see page 19 for illustration), a revolutionary new technique that allows scientists to incorporate an exogenous gene, in this case a Fab or Fv region of an antibody, into a filamentous phage coat protein.[12] This construct can be used to incorporate a Fab or Fv library of high mutational diversity, often greater than 10^{10} and because it is surface exposed, it could be screened for binding to a specific antigen. With its “designer” approach, phage display unlocks new opportunities in antibody discovery that couldn’t be accessed with traditional immunization-based techniques, because of poor immune response or cytotoxicity. The technique is undertaken entirely in vitro, allowing careful control over binding conditions to select for higher or lower affinity clones.[12] Screening can be done iteratively to successively rid the library of unwanted binders, while enriching for those with specific binding properties. Any hits can be analyzed early and the diversity of the library can be continuously monitored using next-generation sequencing (NGS).[12] The selection process, often called biopanning, can also be automated and because it’s high-throughput, a variety of antigens can be targeted in parallel workflows.[12]

In addition, CDR libraries can be constructed using any number of fixed frameworks that are typically used for display techniques, including Fab, single chain Fv (scFv) or single-domain VHH frameworks.[12]

What is Affinity Maturation?

Affinity maturation is a process in adaptive immunity where B-cells produce antibodies with increased affinity for a specific antigen.[13]

What is NGS?

Next-generation sequencing is a fast, high-throughput and accurate method for sequencing a heterogeneous mixture of different DNA or RNA sequences.[14]

A First

The Blockbuster Humira

The first FDA-approved antibody isolated through phage display was Humira (adalimumab), in 2002. Humira brought in $19.73 billion in sales for Abbvie in 2019. Biosimilars for the drug have already emerged in Europe and the USA. This is likely to reduce Humira sales by the middle of the decade.[15] The parental antibody, D2E7, that would become Humira was initially discovered using murine hybridomas.[16] The clone that would eventually become Humira was discovered through the use of hybrid phage display libraries, using a technique called guided selection. One with murine heavy chain combined with human light chain variable domain and a second with murine light chain sequences with human heavy chain variable domains. Each library was selected against TNF-α and human sequences from selected clones were combined. With additional CDR mutagenesis and optimization, a high-affinity clone was isolated, which would eventually become the multi-billion-dollar biologic, Humira.

The success of Humira was major validation for phage display as a methodology for antibody discovery and development. Benlysta (belimumab) represents another success story for phage display, though unlike Humira, Benlysta was developed using a commercially-available, naive phage display library from Cambridge Antibody Therapeutics (CAT; acquired by MedImmune, now AstraZeneca).[16] The antibody was approved in 2011 for the treatment of systemic lupus erythematosus (SLE) and binds to B-lymphocyte stimulator (BLyS), which is elevated in patients with SLE.[16]

Sources of Phage Display Library Diversity

Fundamental to the success of a display-based approach is how diversity is introduced into the library being screened. If your library contains variants that have previously been shown to cause downstream liabilities or sequence space that will result in a non-functional antibody (i.e. stop codon), then the output of selection may not be desirable.

Three Central Strategies for Constructing Display Libraries Have Emerged Over the Years[12]

NAIVE LIBRARIES

Using naive libraries, where heavy and light chain genes are cloned from humans (or other species) that have not been purposefully exposed to any antigen, can offer high diversity as they include the repertoire from multiple donors. Due to the labor and cost of developing and validating a library, several commercially-available libraries have been developed, used and yielded clinical candidates and FDA-approved therapeutics.

IMMUNE LIBRARIES

Using immune libraries, where donors have been immunized, infected, or chronically diseased can yield high affinity antibodies for specific antigens but can be time and cost prohibitive to do for every disease.

SYNTHETIC LIBRARIES

Using synthetic or semi-synthetic libraries, randomized or purposefully-chosen sequences can be incorporated at specific sites within the CDRs. These libraries can achieve high diversity and access sequence space that may not be achieved using naive libraries. These directed or randomized sequences can be incorporated into a variety of scaffolds. The major limitation for synthetic libraries is the ability to synthesize DNA libraries that are long enough and sophisticated enough to focus on the appropriate sequence space, without introducing downstream liabilities. The number of companies that can provide such DNA is limited.

Synthesizing Synthetic Libraries

As the mAb market continues to grow and DNA synthesis gets more streamlined, many companies are designing new libraries to tackle hard-to-drug targets. As with the synthetic libraries that preceded them, they can use a variety of different germline, known, or consensus framework sequences to carry synthetic CDR regions.[12]

Traditionally, site saturation mutagenesis techniques have been used to randomize specific amino acid sequences and NNK/NNS degenerate oligos are typically used to sample all 20 amino acids. This technique presents significant disadvantages as it still allows stop codons to be incorporated 3% of the time and there is no way to remove downstream manufacturing liabilities, such as isomerization, cleavage, deamidation, or glycosylation sites.[12]

Techniques like TRIM, which use defined trinucleotide phosphoramidites during the oligo synthesis process, are also used for introducing diversity into display libraries.[12, 17] By using these during the synthesis process, the amino acid distribution can be carefully controlled, stop codons excluded and frame shifts avoided.

Identifying Antibodies from Libraries

Phage expressing these libraries are screened for binding to a target antigen and only those phage that bind are amplified. One round is typically not enough to enrich for high-affinity clones and multiple rounds of biopanning, usually two to five, is typical. Antigens can be presented in any number of ways, for example via coupling chemistries or terminally attached tags for immobilization to microtitre plates or resins/beads, captured from solution (eg., via antigen biotinylation) or bound to receptor-expressing cells.

DID YOU KNOW?

Thus far, the FDA has approved more than eight mAbs isolated from phage display.[1] The majority of them have come from two different commercially-available libraries: One scFv library from CAT and the other from a Fab library from Dyax (acquired by Shire, now Takeda).[12] MorphoSys has also developed fully synthetic human scFv and Fab antibody libraries—HuCAL, HuCAL Gold and HuCAL Platinum. HuCAL was used to generate Tremfya (guselkumab), which was approved in 2017 for the treatment of plaque psoriasis.[1]

Display Technologies Abound

In addition to phage display, a number of additional display-based technologies have been developed that have their own advantages and disadvantages.

Yeast Display

Much like phage display, the Fab, VHH, scFv, or IgG antibody library, is transformed into yeast and displayed on the cell surface. The α-agglutinin adhesion receptor is typically used for displaying the antibody fragments. Typically, flow cytometry is used to screen the library using fluorescently-labeled antigen, a significant advantage as this technique is high-throughput.[12]

ADVANTAGES

Cells can be sorted based on ability to bind antigen or by display level of antibody fragments (as each fragment is typically hard-coded with a common epitope tag). Defined parameters can be used to select clones. Antigens can be presented in any number of ways, for example via coupling chemistries or terminally attached tags for immobilization to microtitre plates or resins/beads, captured from solution (eg., via antigen biotinylation) or bound to receptor-expressing cells. Yeast are also eukaryotic and include the post-translational modification (PTM) pathways (i.e. glycosylation, ER trafficking) that exist in mammalian cells. Thus, they can be good surrogates for how candidates will behave when expressed in mammalian cells.[12]

DISADVANTAGES

Yeast transformation efficiency is lower than that seen in bacteria. As a result, this limits library diversity.[12] However, in recent times, there has been improvement in transformation efficiency with some groups (eg. Adimab) now routinely generating 10^9 libraries[18]

Mammalian Display

Conceptually, mammalian display is similar to yeast display, as antibody libraries are expressed on the cell surface via fusion to a transmembrane domain and panned using any number of methods that use purified recombinant target antigen.[12] More recently, sophisticated methods of mammalian display, where single cells express both antigen and a secreted full-length IgG variant have been developed. This method, called self-labelling integral membrane mammalian display (SLIM), presents a number of advantages over traditional mammalian display, including improved library diversity.

ADVANTAGES

Because libraries are expressed in mammalian cell lines, such as HEK293 or CHO cells, any difficulties with downstream expression are addressed as part of the library selection process. In addition, membrane targets are expressed in their native conformation. It's also easy to move directly from candidate selection to expression pilot studies.

DISADVANTAGES

Library size is limited by transduction efficiency into mammalian cells and workflows like SLIM need to parse apart non-specific binders.

In Vitro Transcription/Translation Display

Transformation efficiency, into either bacteria or yeast, can limit the diversity of a library. Purified *in vitro* transcription/translation systems that are totally cell-free can get around these complications. Fab or scFv libraries can be transcribed into mRNA and translated using purified components (i.e., ribosomes, tRNAs, translation factors, etc.). In ribosome display[19], the mRNAs are engineered so that they don't have a stop codon and ribosomes stall at the end of the mRNA, with nascent peptide containing the Fab or scFv variant displayed out of the ribosome exit tunnel. mRNA display[20] is conceptually similar, but puromycin (which essentially acts as an amino acyl-acceptor stem) is covalently attached to the 3' end of the mRNA, terminating translation and leaving the nascent peptide covalently attached to the mRNA encoding it.

CIS display is another variation on both ribosome and mRNA display that also uses an *in vitro* transcription/translation system for library expression. Template DNA is constructed such that when translated, scFv and Fab library sequences are N-terminally fused to the RepA protein, which is involved in DNA replication initiation of R1 plasmids.[21] Downstream of the RepA coding sequence lies a *CIS* DNA sequence, which triggers transcription termination and binds transiently to RepA. Downstream of the CIS sequence is the ori DNA sequence, which is stably bound by the RepA protein following transient interaction with the CIS sequence. Thus, *in vitro* transcription/translation results in the translation of scFv or Fab libraries fused to RepA which stays bound to its encoding DNA.

Covalent antibody display (CAD)[22] and *in vitro* compartmentalization[23] also offer advantages over phage display in library size, speed and the display of unnatural amino acids and nucleotides. These approaches avoid the library size limitation present in phage and yeast display technologies that are dependent on cell transformation efficiency.

As in other display methods, the ribosome, mRNA and CIS display libraries are exposed to antigen and rounds of biopanning occur, leading to the isolation of high-affinity clones. Each round of biopanning is cyclical. It involves the isolation of positive binders and mRNA from the ribosome. The mRNA is then reverse transcribed and amplified by PCR for the next round of selection. CIS display has the added benefit of not requiring these additional reverse transcription steps, as DNA can be used directly for future rounds of *in vitro* transcription/translation.

ADVANTAGES

No need for transformation into bacteria or yeast.

DISADVANTAGES

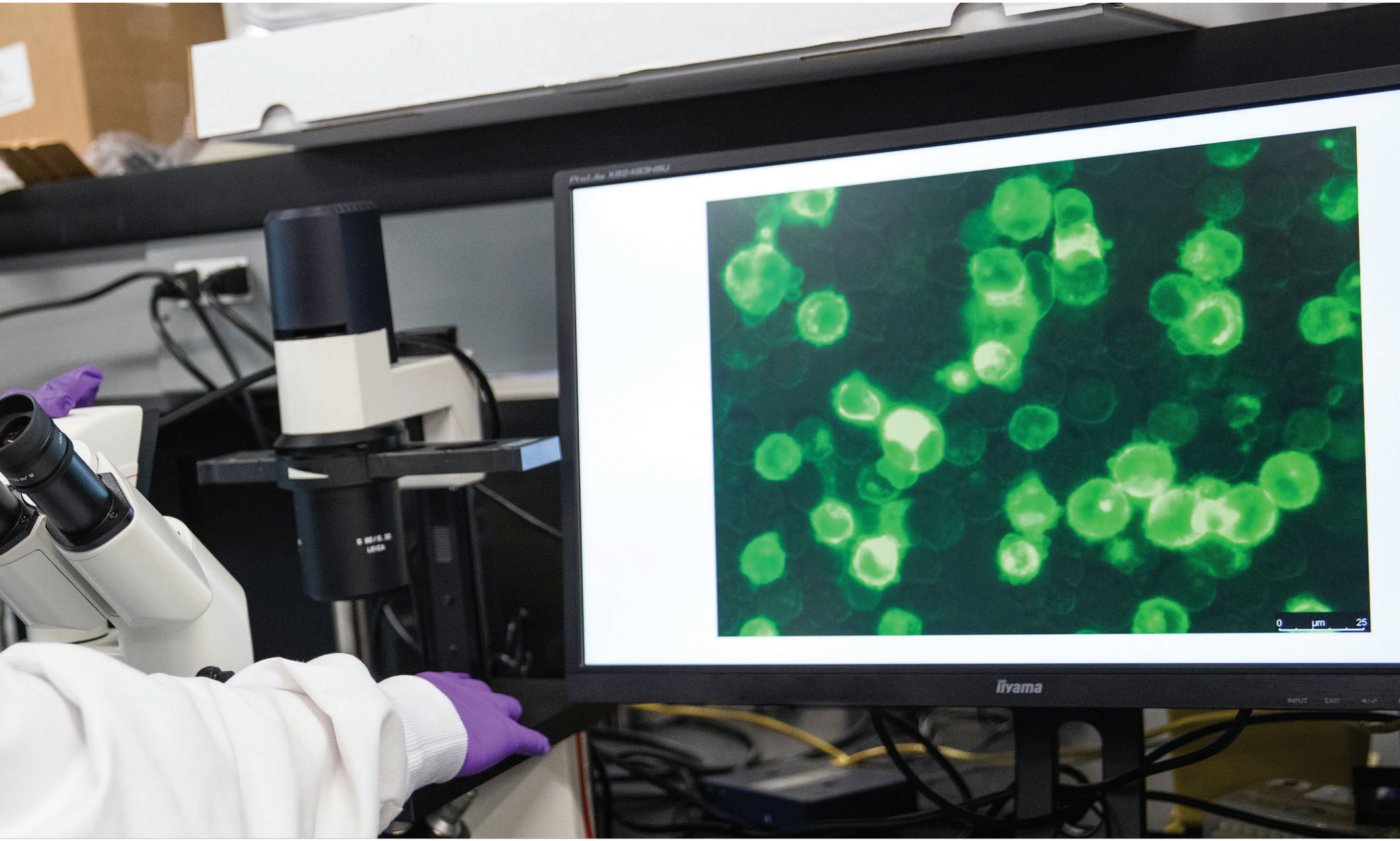
Because each round of selection involves PCR, additional library diversity can be introduced during each round of panning.

While most of these cell-free systems are still under investigation, the discovery and optimization of MedImmune's anti-IL-13 (tralokinumab) which used the ribosome display system is evidence of the power of cell-free systems. The FDA is currently reviewing tralokinumab with approval imminent.[43]

Selecting Antibodies

Identifying leads that you want to move ahead with involves a predetermined screening process that further characterizes leads for properties that are important for downstream clinical applications. Primary screening typically includes binding and specificity confirmation for the target antigen. This also involves characterizing species cross-reactivity which is important for studies in animal models as well as for safety studies.[6] Secondary and tertiary screens should include functional assays.[6]

Together, this screening process can whittle hundreds or thousands of antibodies down to tens. This makes the next step, IgG reformatting from Fab, scFv, or VHH formats much more manageable. IgG reformatting is accomplished by using standard molecular biology techniques, though with the growing availability of gene synthesis services, more antibody discovery programs are making use of these capabilities, as they enable additional engineering. After IgG reformatting, small scale expressions and purifications of the mAb are performed and biochemical or biophysical characterization is assessed.[6]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Antibody Optimization

Following the identification of antibodies from antibody discovery, optimization of these antibodies may be necessary to explore, in a more targeted way, if increases in affinity and potency can be achieved. A similar process, called affinity maturation, happens naturally during an immune response.

The immune system uses somatic hypermutation (as well as class switching, for example, from IgM to IgG) in a cellular process to increase the diversity of antigen-specific antibody CDRs and increase the immune response thereby adapting to new foreign threats during the lifetime of an organism, eg., microbial infection. This is then followed by antigen binding, similar to that used in the display technologies described in previous sections.[1] The process occurs over weeks following an infection, either acute or chronic, or vaccination. In the germinal centers, antibody-producing B cells are selected based on affinity for antigen, presented primarily by dendritic cells.[13] They can undergo additional rounds of selection or become memory B cells, which circulate in the blood as active sentinels for any invading, foreign intruder.[13] Due to the rounds of somatic hypermutation and rounds of selection in the germinal centers, the antibodies expressed by memory B cells bear little resemblance to their germline-encoded counterparts.[13]

For mAb development, optimization can involve antibodies modification to address several important considerations. The obvious one is improved affinity, which can be achieved through additional mutagenesis of antibody sequences and further rounds of biopanning using a display technology of your choosing.[1,6] Typically, phage, yeast, ribosome and mammalian displays are used and mutagenesis takes one of these two strategies:[1]

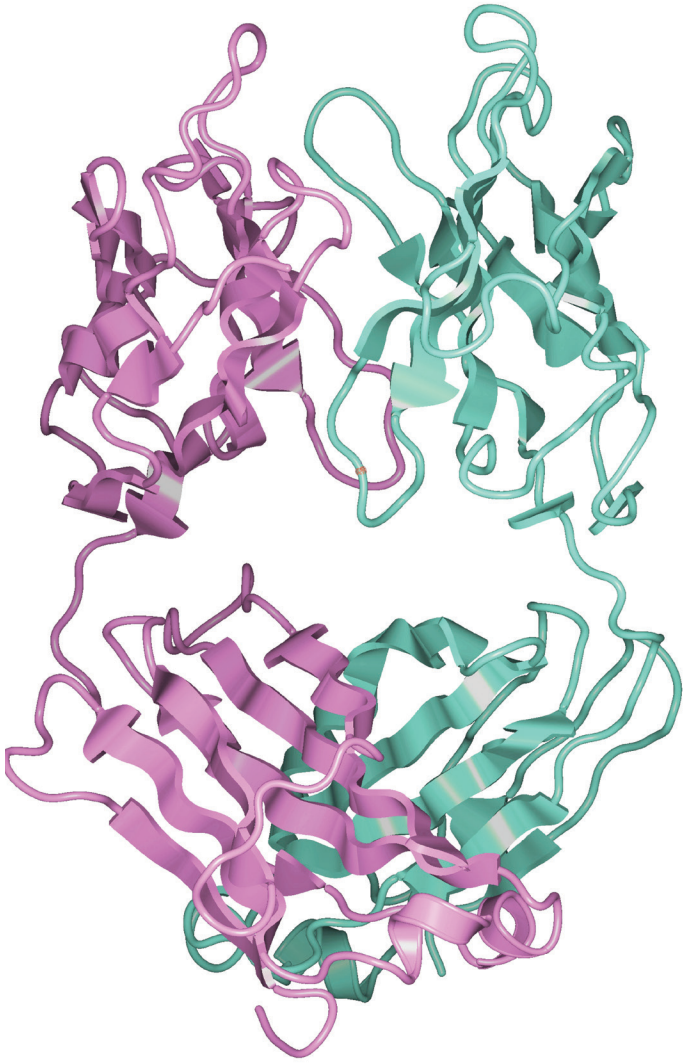
GOING BIG

This strategy takes a “go big or go home” approach with random or directed mutagenesis of CDR or variable regions, followed by selection.

STAYING SMALL

This strategy focuses on randomizing or directing specific mutations to key hotspots, whether it be a position in the variable region or a specific CDR.

Following mutagenesis, biopanning is performed under more stringent conditions, with decreased antigen concentrations, competition with soluble antigen, or extensive washing. Often, increases in affinity of 10 to 100 fold are seen, but some have reported 1200-fold increases after optimization.[6]



TWIST ANTIBODY OPTIMIZATION

QUICKLY GENERATE HIGH-DIVERSITY, HIGH QUALITY MOLECULES INSPIRED BY THE HUMAN REPERTOIRE

Twist's Antibody Optimization uses bioinformatics and a proprietary software platform to create an optimization library with natural human heavy and light chain CDR sequences. This "human repertoire inspired" phage library is selected for multiple rounds, enriching for high-affinity binding clones.

LEARN MORE

twistbiopharma.com/antibody-optimization

TWIST BIOPHARMA

Eliminating Downstream Liabilities

Another key part of optimization is adjusting for downstream issues such as immunogenicity or downstream liabilities, such as isomerization, cleavage, deamidation, or glycosylation. The former is typically addressed through conversion of murine or other animal sequences to chimeric, humanized or fully human mAbs.[6] As for the other downstream liabilities, addressing all of these can be a challenging molecular biology exercise, but can save significant time and money down the road to commercialization. Alternatively, these can be addressed using gene synthesis technology to eliminate any potential liabilities, in one fell swoop.

Optimizing the Constant Region

Optimization doesn't only focus on variable regions: The Fc region is involved in mediating a number of functions, including antibody-dependent cellular cytotoxicity (ADCC), a key activity for many successful immuno-oncology therapeutics.[6] Investigating mutation libraries in the Fc region can lead to an enhancement of ADCC and/or antibody-dependent cellular phagocytosis (ADCP), and/or complement-dependent cytotoxicity (CDC), and increases in half-life.[6]

Antibody Glycosylation

A Critical PTM with Enormous Drug DevelopmentSignificance

Antibody glycosylation is another critical consideration for developing and optimizing a therapeutic candidate, and one that can lead to downstream consternation if overlooked. Glycans, or carbohydrate molecules covalently appended to other biomolecules, are a common class of protein PTM, which have a wide variety of important roles in biological systems and mechanisms.[24,25]

The main challenge with glycan structures is that their biosynthesis is not template driven, like it is with DNA, RNA, and proteins. This results in high heterogeneity in glycan structures. An individual protein can have a wide number of glycoforms—varying only by glycosylation—when expressed in the *same cellular system at the same time*. Glycoforms can differ from one another with respect to different glycan structures occurring on the same sequence location, differential occupancy of glycosylation sites, and total number of glycans on a given protein.It is well established that glycosylation patterns and specific glycan structures impact antibody function and efficacy.[26] The glycans found on antibodies can have far reaching effects on a given antibody, impacting its half-life/clearance rate, ADCC, complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP), and immunogenicity—all of which play a key role in the development of safe and effective antibody therapies.[27]

To combat this variability, researchers have employed strategies to modify antibody glycosylation patterns, a process known as glycoengineering.[28] While many complicating issues with glycosylation can be eliminated with genetic removal of identified glycosylation sites, some glycan structures are essential or highly

advantageous to antibody efficacy. Collectively, there is demand to both eliminate glycosylation where it negatively affects the candidate and control glycosylation where it is needed or beneficial for instance, for enhanced tumor killing by ADCC.

The explosion of glycoscience methods and technologies in recent decades has brought some high-profile examples of therapeutic antibody glycoengineering. As one important example, Genentech employed glycoengineering to help develop Gazyva (obinutuzumab) improving upon the clinical success of its CD20-targeting antibody therapy, Rituxan (rituximab).[28] More recently, research groups have worked to make antibody glycoengineering more economical for large scale production by adopting different host expression systems and identifying new glycan modifying enzymes. In one such approach, researchers used a combination of genetically engineered *Pichia pastoris* yeast and newly identified endoglycosidase enzymes to generate homogenous populations of Herceptin, optimized to include a complex glycan structure known to elicit a strong ADCC response.[29]

Given the growing understanding of glycans, it has become critical that researchers identify various glycoforms in their antibody panels, understand their influence on therapeutic function, and then ultimately optimize and engineer their drug candidate to carry a consistent glycosylation pattern that offers ideal effector function.

Antibody Production and Predevelopment

Moving forward with a series of optimized leads represents a key milestone in the development process. Now, your focus can shift away from identifying leads to preliminary manufacturability evaluation and pre-clinical testing.

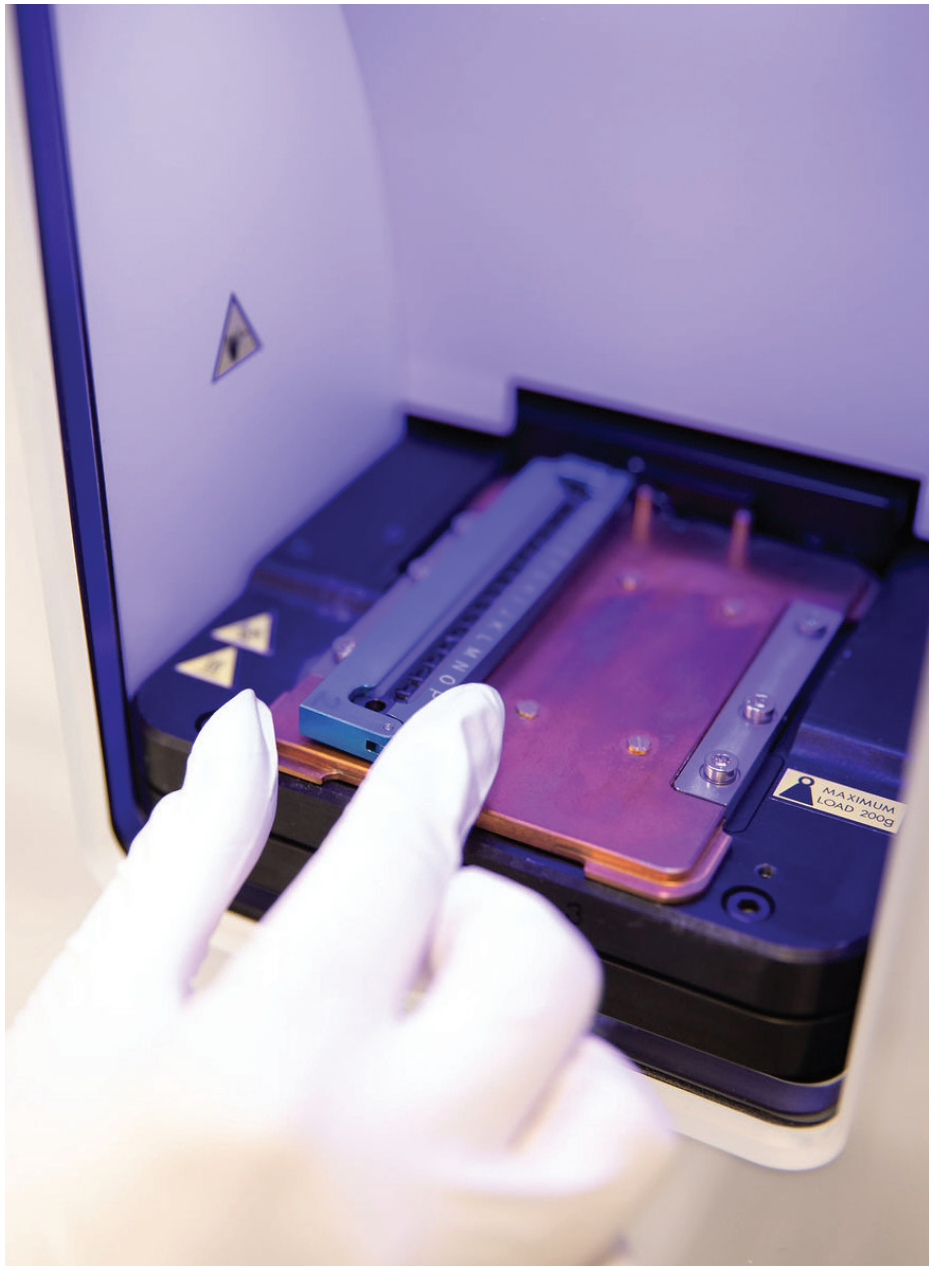
In this section we'll focus on conversion from antibody fragments to full-length IgG, expression systems for pilot and scale-up expression studies and biochemical or biophysical characterization.

As mentioned in the beginning of this book, the antibody development process is far from linear. So many groups may have completed IgG reformatting, pilot expression, and biochemical and biophysical characterization as part of the discovery and optimization stages and are coming back to it now to experiment with different expression systems or scale up for pre-clinical studies. Either way, the considerations discussed in the next section will likely be applicable at some point in the preclinical development of your lead candidate.

Full-Length IgG Reformatting

If ever there was a bottleneck in the pre-clinical development of antibodies, it comes when reformatting the output of *in vitro* display workflows, lead Fab, scFv, or VHH candidates, to full-length IgGs. This conversion is necessary as most of the successful antibodies that have achieved FDA approval are IgG isotypes (74 out of 79).[1] Cloning of IgG recombinant antibodies also allows for (a) reproducible expression across a number of platforms, (b) the ability of a therapeutic to induce complement activity, such as ADCC, and (c) bivalent binding of antigen.[30] To test these activities *in vitro*, requires IgG conversion. Assays of scFvs can often lead to aggregation and false positives.[31]

While necessary, IgG conversion is full of challenges. Cloning into full-length IgG expression vectors is executed on an individual basis and is time-consuming, laborious, and low-throughput. There is also a significant loss of positive leads during this process and reformatting to IgG can significantly change affinity and activity of Fab, scFv or VHH formats. Ultimately, this means additional lost time. Higher-throughput methods have been developed, but they rely on the use of restriction cloning, engineered into specific display frameworks, which limits their application to other libraries.[31] Gene synthesis of full-length IgG is an attractive option as it's the most straightforward and scalable process for converting Fab, scFv, or VHH leads to full-length IgGs.



Expression of Full-Length IgGs

There are a number of model expression systems used for the production of recombinant proteins, including IgG mAbs. There are pros and cons to each system:[32]

E. COLI EXPRESSION

This system is tried and true for recombinant protein expression, but has only been used for the production of antibody fragments, such as Fab and scFv. Quality and yield of recombinant antibody fragment production has been improved through secretion and co-expression with chaperones.[32] *E. coli* also lacks a glycosylation system.

YEAST EXPRESSION

Unlike bacterial expression systems, yeast have post-translational modification pathways, folding chaperones, and secretion pathways that model those used in mammalian cells. Several reports of functional IgG expression at yields as high as 1.4g/L have been published using *P. pastoris*.[32] Recently, glyco-engineered *P. pastoris* have been generated, that produce humanized glycosylation patterns, IgGs with effector functions.

MAMMALIAN EXPRESSION

Mammalian cells have become the gold standard for expression of recombinant IgGs. While costly and difficult to produce using these methods, folding, secretion, and post-translational modification pathways lead to the production of high-quality IgG, at yields approaching 12 g/L. In addition, Good Manufacturing Practices (GMP) have ensured that contamination of cell cultures doesn't happen. Expression in mammalian cell lines, such as HEK293 or CHO cells, can be undertaken transiently, which is typically used for pilot expression of a large number of leads in low volumes, or stably, through genomic integration.[32] Stable expression is required for GMP compliance to ensure long-term production stability.[32]

Recently, high-throughput workflows have been developed for mammalian transient transfection, accommodating handling of hundreds of IgG variants in a 96-well plate format.[33] Without a high-throughput way of doing this, in a plate-based, automated way, transformation and transfection remains another major bottleneck.

Purification of Antibodies

Another major bottleneck for moving preclinical candidates forward is managing the purification of IgG variants in a high-throughput manner. Several microscale approaches to purification have been published, including pipette tip columns, batch purification, and miniaturized column purification. Some of these are favorable for high-throughput and automated workflows, yet they can be costly and complex. More recently, groups have reported workflows that are able to express and purify approximately 2,000 IgG variants and automated instrumentation has made this process less of a bottleneck.[34]

Biochemical and Biophysical Characterization

Downstream of IgG purification, a number of biochemical and biophysical characteristics are assayed to ensure candidates are adequate for preclinical testing. As with the other steps in this process, these are traditionally not high-throughput and can be tedious and laborious to do manually on the scale of hundreds of IgG variants.

In general, the biochemical and biophysical assays include:

CONFIRMATION OF BINDING ACTIVITY

As binding to target antigen is a critical part of a mechanism of action, measuring IgG binding affinity is an important step. There are numerous high-throughput workflows for measuring binding kinetics with purified antigen in an automated way.

SIZE-EXCLUSION CHROMATOGRAPHY

This is widely used to analyze IgG aggregation in a high-throughput manner through HPLC.

THERMOSTABILITY

Unfavorable biophysical characteristics can lead to poor expression, aggregation and low stability *in vivo*. High-throughput thermostability screening of IgG variants can quickly identify which variants may have trouble in preclinical animal models or require additional optimization.

FUNCTIONAL ASSAYS

Assaying the functional outcome of IgG variant binding using a cell-based assay is the closest thing to conducting preclinical animal experiments. Many “off-the-shelf” validated fluorescent or luminescent assays are available to screen any number of drug targets in a high-throughput format. These are an essential step that should precede preclinical testing.

NON-SPECIFIC BINDING

While assaying to see if the Fab region binds non-specifically to an antigen other than the target typically happens in earlier steps when the initial hits have been identified, IgGs do need to be checked to make sure that the required specificity (and species cross-reactivity and affinity) are preserved following conversion. Many non-specific reagents can be used to assess for non-specific binding in ELISA or other SPR techniques, e.g. baculovirus particles and CHO cell lysate.[35]

TRANSFORMING BIOLOGICS DISCOVERY

Alongside our antibody discovery and Twist Antibody Optimization (TAO) core services, Twist Bioscience offers additional development services, including high-throughput full-length IgG conversion, expression, purification, biochemical characterization, biophysical characterization, and functional characterization.

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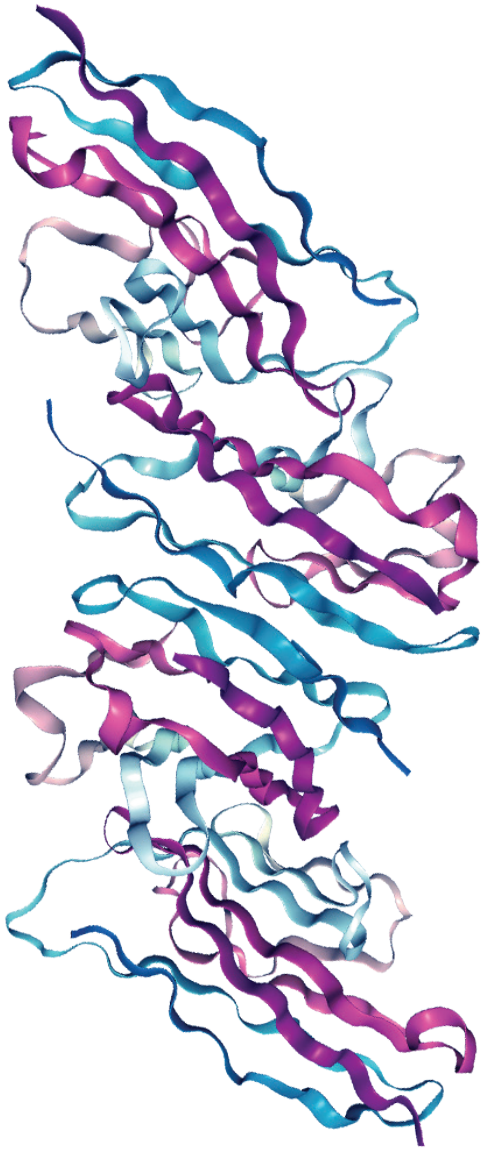


Antibody Development

Once the developability assessment hurdles are cleared, your antibody candidate moves into broader development. The emphasis during this phase is on understanding “the good and the bad” of how it will behave in the human body.

Clearly, the pharmacological profile of antibody therapeutics differs significantly from that of small molecules. A lot of this stems from the profound difference in molecular weight: antibodies are approximately 300 times larger, with a molecular weight of ~150 kDa compared to less than 500 Da for small molecules.[36]

Another important difference is the connection between pharmacokinetics (how an organism affects a drug) and pharmacodynamics (how the drug affects the organism). With small molecule drugs, these two phenomena can be discrete. With mAbs, they’re interconnected.

Pharmacokinetics (PK) and Pharmacodynamics (PD)

As protein therapeutics, mAbs have extremely limited oral bioavailability, typically less than 1–2%.[37] To combat this, they are predominantly administered intravenously (IV) or via subcutaneous (SC) injections. With IV dosing, they tend to demonstrate a biphasic PK profile in circulation, with a relatively fast distribution phase as the drug disperses through the vascular system and interstitial spaces, followed by a more gradual elimination.[36] The size and polarity of mAbs limit broader distribution throughout the body i.e., limited to the periphery and unable to cross the blood-brain-barrier to any significant extent. With SC injections, the antibodies disperse more slowly via the lymphatic channels, often taking several days to reach maximum plasma concentration.

A proven phenomenon to note here is target-mediated drug disposition (TMDD). When a mAb binds its target with high affinity, the interaction can alter the kinetics of its distribution and clearance. Due to this high affinity, the binding to the target and subsequent turnover of the drug-target complex can contribute significantly to the disposition of biologics. However, this elimination by binding to a target is saturable because of the finite number of targets on the cell surface. In other words, the antibody target biology impacts the PK profile of the drug.[38] Factors such as binding affinity, antigen density, turnover rate, internalization rate, and dose levels can all play a role.[39] For accurate PK/PD analysis, TMDD modeling should be factored in. Non-specific binding or non-specific clearance through pinocytosis or proteolysis can have an additional effect on PK of mAbs.[36]

When it comes to clearance, mAbs exceed the glomerular filtration cut-off threshold needed for elimination via the kidneys.[38] Instead, they are broken down into smaller peptides and amino acids through a process known as proteolytic catabolism.

DID YOU KNOW?

PK and PD can be often mixed up and the definitions can be confusing. One way to think about them is, PK is what the body does to your antibody or small molecule and PD is what your antibody or small molecule does to the body.

Route of Administration Preferences

Antibodies can't survive the enzymes and acidic pH of the gastrointestinal tract, which leaves two main routes of administration: intravenous (IV) and subcutaneous (SC). Though less common, intramuscular injection has also been reported.[40] Antibody fragment formats, such as VHH or nanobodies, are also being developed for the inhalation route (eg., ALX-0171 for treatment of RSV). During early development, the emphasis is on efficacy and safety, but it's worth considering how the delivery mechanisms may impact patient adoption and compliance later on. In a 2014 review of six relevant publications, two-thirds demonstrated a clear patient preference for subcutaneous injections due to the convenience and time-savings associated with treatment at home. The authors concluded; "If the safety and efficacy of both administration routes are equivalent, then the most important factor should be patient preference as this will ensure optimal treatment adherence and ultimately improve patient experience or satisfaction."[41]

Efficacy and Toxicology

Well-planned efficacy and toxicity studies are a critical part of the development process, helping scientists anticipate how the drug will perform in first-in-human studies. Along with determining efficacy and safety, these studies also generate information around the optimal dose and treatment regimen.

One of the big decisions for preclinical efficacy studies is determining which animal models to use. The key factors to consider are the ability of the model to accurately recreate the disease, such as a tumor, with similar vasculature and growth rates. The model also has to facilitate the same mAb mechanism of action including target binding and downstream pharmacology.[38] While transgenic mice expressing human neonatal Fc receptor (hFcRn) have shown early promise, the gold-standard remains non-human primates, such as the Cynomolgus macaque.[42] With the right animal models, a series of toxicology studies can be performed, including histological organ assessment and analysis of serum, urine, blood, and general health status.

It's worth noting that these early toxicology studies don't typically adhere to Good Laboratory Practice (GLP) guidelines. The more in-depth GLP studies come later.

Developing Stable Cell Lines

Monoclonal antibody engineering has advanced significantly over the years, helping optimize traits such as bioavailability. But it's not only the end product that has received a scientific boost; mAb production has also benefited from a range of new developments, bringing down costs and streamlining scale-up manufacturing.[43]

As noted above, the commercial production of mAbs typically begins with animal immunization, phage display technology, or a synthetic antibody library. Once a candidate is chosen, groups need to invest in a robust system for generating mAbs via cloning through stable cell line generation. Selecting the right cell line is one of the most critical steps, initially for producing a clinical supply and, longer-term, for efficient large scale manufacturing.

The most popular cell line for mAb production is derived from Chinese hamster ovary (CHO) cells, which can be genetically modified to produce large volumes of high-quality mAbs.[43] However, as is a general theme in this field, there is a lot of diversity and innovation. Genetically modified cells derived from plants, insects, and microorganisms (including yeast) are all carving out their own niche in the field of mAb production, overcoming some of the limitations of mammalian cell-mediated systems.[43] For example, plant cells can be easily propagated and are often high-yield, enabling low-cost scale-up production.[44]

As with other bioprocessing applications, mAb production has benefited from the emergence of single-use systems and continuous upstream bioprocessing. This has been complemented by significant increases in expression yield, driven by progress in cell lines, expression systems, and cell culture.

Following cell culture, the mAbs are recovered with the help of a centrifuge and then moved into filtration and downstream processing. The purification trains often include multiple chromatography steps, along with viral inactivation. While slow, affinity chromatography has established itself as the purification method of choice for antibody developers due to the highly specific binding between Protein A ligands and the Fc region of immunoglobulins.[43]

DID YOU KNOW?

Advanced single-use technologies, including perfusion bioreactors, are now generating yields that are more than 25 times higher than those achieved with batch culture.[45]

GLP Toxicology

Good Laboratory Practice (GLP) toxicology studies are performed to determine the therapeutic index of a therapy. This includes information on the highest tolerated dose to inform the final dosing regimen. The studies are also intended to rule out potential issues, such as carcinogenicity, and to identify any issues that should be monitored closely in subsequent human trials.[46]

According to the FDA, key toxicity studies include:[46]

- **Safety Pharmacology:** Identifies potential adverse pharmacological effects of the drug *in vitro* and/or *in vivo*
- **General Toxicity:** Single-dose and repeated-dose toxicity studies that evaluate drug safety from a systems-biology perspective
- **Carcinogenicity:** Predicts long-term risks that are difficult to assess or are unethical to assess in humans
- **Developmental and Reproductive Toxicity:** Identifies risks for special populations
- **Special Toxicity:** Identifies specific parameters to monitor more closely in clinical trials

As noted by the FDA's Center for Drug Evaluation and Research (CDER), there is no universal rule for the number and types of preclinical safety testing that need to be completed prior to a Biologics License Application (BLA) submission.[46] Broadly speaking the various FDA and International Council for Harmonization (ICH) guidance documents recommend a series of *in vitro* assays and whole animal testing methods.

COMPARISON OF HETEROLOGOUS BIOEXPRESSION SYSTEMS

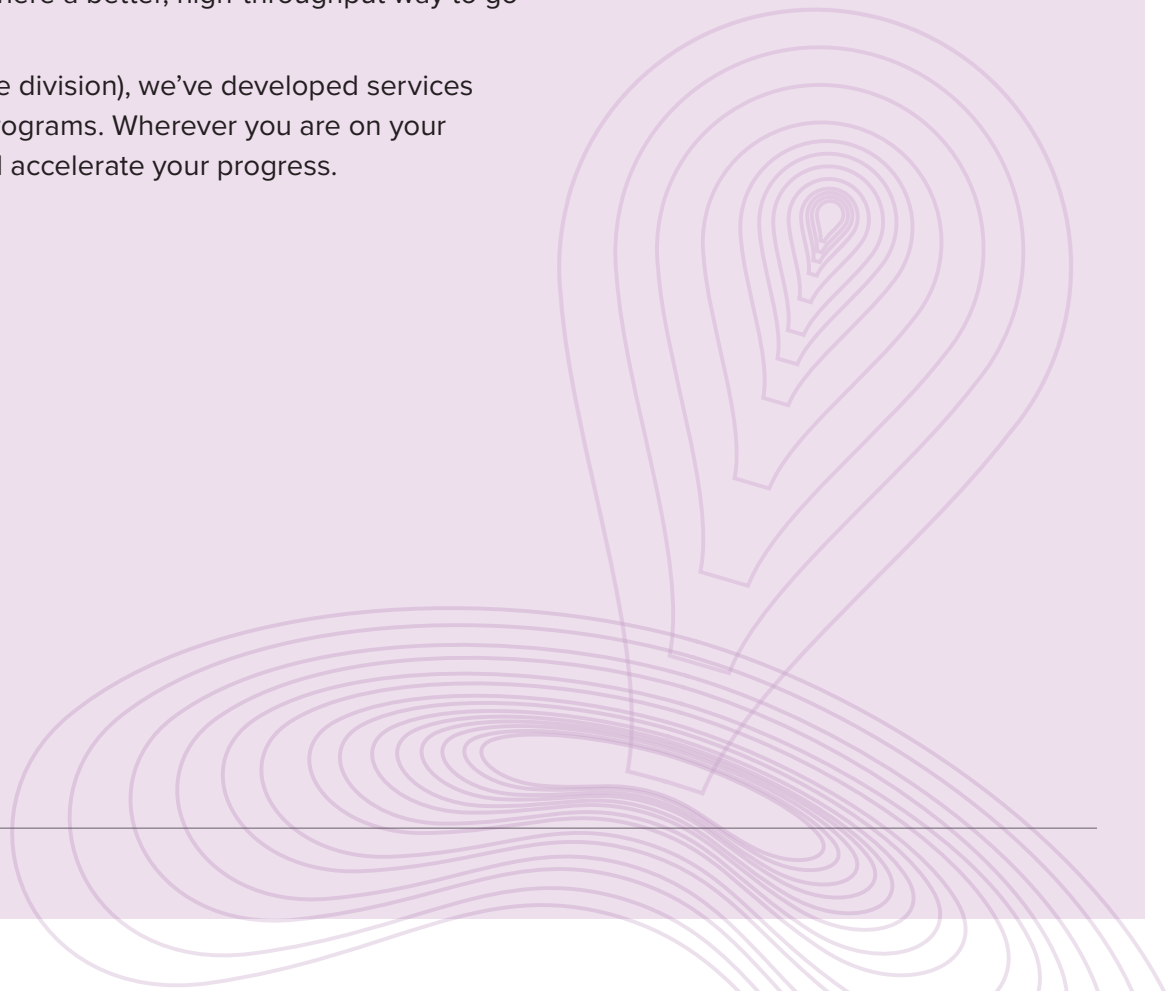
EXPRESSION SYSTEMS	YEAST	INSECTS	MAMMALIAN CELLS	PLANTS
Production cost	Medium	Medium to high	High	Low
Maintaining cost	Cheap	Expensive	Expensive	Cheap
Protein yield	High	Medium to high	Medium to high	High
Gene size restriction	Unknown	Unknown	Limited	Not limited
Therapeutic risk	Unknown	Unknown	Yes	Unknown
Glycosylation	High mannose	Mannose terminal	Correct	Plant Specific
Safety risk (for patient)	Unknown	Medium	Medium	High
Time required	Medium	Medium	High	Medium

Candidate Selection for Clinical Development

Here you are! You've made it through the twists and turns, the ups and downs, and the ins and outs of target validation, antibody discovery, and antibody development.

Along the way, you may have found yourself asking "Isn't there a better, high-throughput way to go about doing this?"

It turns out, there is. At Twist Biopharma (a Twist Bioscience division), we've developed services to help you navigate your biggest biologic development programs. Wherever you are on your antibody development journey, we have a solution that will accelerate your progress.



1. TARGET ID AND VALIDATION

Identify and functionally validate what proteins or biological targets are key to a specific disease.

2. ANTIBODY DISCOVERY AND ANTIBODY PANEL IDENTIFICATION

Discover an antibody that binds to and modulates the function of your target.

- **"Library of Libraries" Licensing:** Our team has pre-constructed and validated phage display libraries with a variety of antibody frameworks, including VHH, Fab, scFv. Select from libraries containing naive human diversity, rationally designed with antibody structural considerations, or with diversity computationally tailored to high-value targets, including: carbohydrates, hyperimmune, and structural motifs
- **Custom Library Construction:** We can work with you to construct a library in a Fab, scFv, or single domain VHH scaffold of your choosing against your target of-choice
- **Discovery Partnership:** Our team works with you to discuss your goals and library specifications. We then use our Library of Libraries or create a custom library in a vector and scaffold of your choosing

3. ANTIBODY OPTIMIZATION

Optimize antibodies for greater affinity, potency and developability.[6]

Twist Antibody Optimization (TAO) uses bioinformatics and our proprietary software platform to create an optimization library with natural human heavy and light chain CDR sequences. Simply input your murine, chimeric, humanized, or fully human antibody sequence into our software and our algorithm will determine which natural variants you should focus on. This "human repertoire inspired" phage library is selected for multiple rounds, enriching for high-affinity binding clones using bead- or cell-based assays.

4. ANTIBODY PANEL PRODUCTION AND DEVELOPABILITY EVALUATION

Express and purify adequate amounts of antibody for biochemical/biophysical characterization and exploratory pre-clinical studies.

Alongside our antibody discovery and TAO core services, Twist offers additional development services, following the identification and/or optimization of antibody candidates.

- **IgG Conversion, Expression & Purification:** Our gene synthesis capabilities quickly and easily convert 100 to 200 antibody candidates into full-length, codon-optimized IgG genes, cloned into our high-copy, custom expression vectors. We use a high throughput automated workflow to express and purify IgG antibodies on small and large scales—in as little as two weeks
- **IgG Characterization:** Our experts can assess yield and purity of IgG proteins and do rapid biophysical characterization, including thermostability (Tm and Tagg). We can also assess binding affinities and polyspecificity in a high throughput way using the Carterra LSA system and BVP binding ELISA, respectively
- **Functional Assays:** Our scientific experts use off-the-shelf or custom assays to readily assess the antagonist, agonist, or allosteric effects of your purified antibodies

5. ANTIBODY DEVELOPMENT

Finalize preclinical studies and collect supporting data for IND submission and clinical advancement.

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