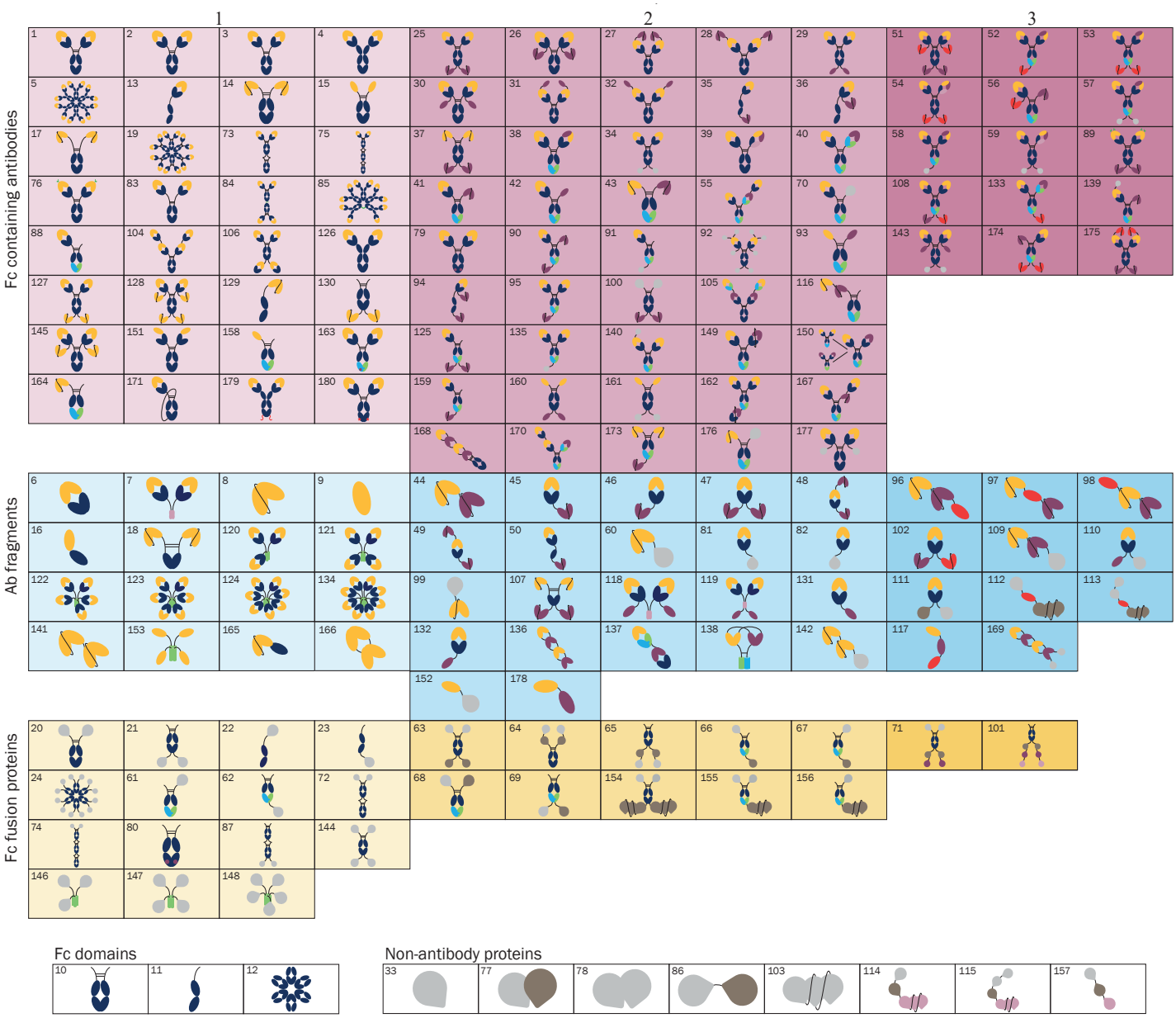
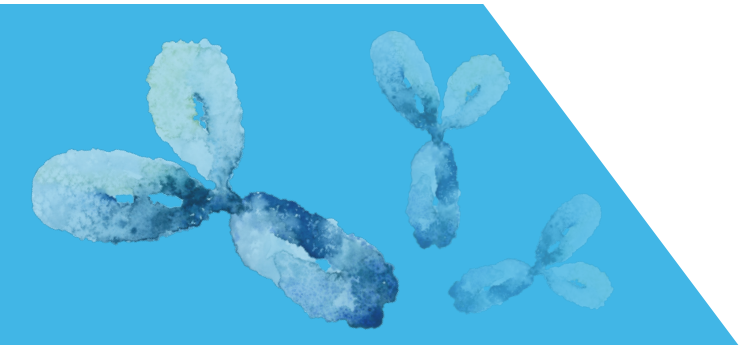
# 9 Questions to Consider Before Your Next Antibody Engineering Project

## White Paper



absolute antibody
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*The popularity and utilization of antibody engineering is a growing trend in the research, therapeutics, and diagnostics industries, as antibody engineering allows you to modify, and potentially even build, an antibody that is better suited for your specific needs. Absolute Antibody has engineered more than 180 antibody formats and continues to work with clients to design their custom antibodies. With infinite varieties of antibody constructs possible, how do you decide where to start and what options are best for your desired outcome?*

*As antibody engineering experts, we get asked a lot of questions about the various engineering options. Our consultative approach allows us to work one-on-one with clients to answer all project questions and advise the best solutions. The following list, while not exhaustive, discusses crucial components of the antibody engineering process and details key questions to ask when starting an antibody engineering project.*

# What are the Options for Antibody Species, Isotype, and Subtype?

The format of your antibody contributes greatly to its performance and manufacturability. One of the first antibody engineering options for researchers to consider is altering the structure and type of their antibody via species, isotype and/or subtype switching.

## Species Switching

Species switching involves reformatting the variable regions to an antibody backbone of a different species. Species switching has grown in popularity for *in vitro* research due to its ability to increase compatibility with a secondary antibody, enable easier co-labeling studies and prevent unwanted antibody interactions in serological assays. For diagnostic applications, species switching allows the creation of human variants that avoid HAMA (human anti-mouse antibody) responses and the standardization of Fc domains to streamline conjugation and immobilization protocols.

Species switching is also especially important for *in vivo* research using animal models. Recombinantly produced, species-matched antibodies offer many advantages compared to the original antibody format, including reduced immunogenicity and increased potency. No neutralizing antibodies are induced in the host organism, meaning the antibody works for longer and cohorts respond more consistently, and less antibody is required to achieve the same results. For example, researchers in Switzerland used a species-swapped mouse-anti-mouse antibody to deplete CD8+ T-cells in mice more completely and for longer than the original rat antibody (Figure 1).

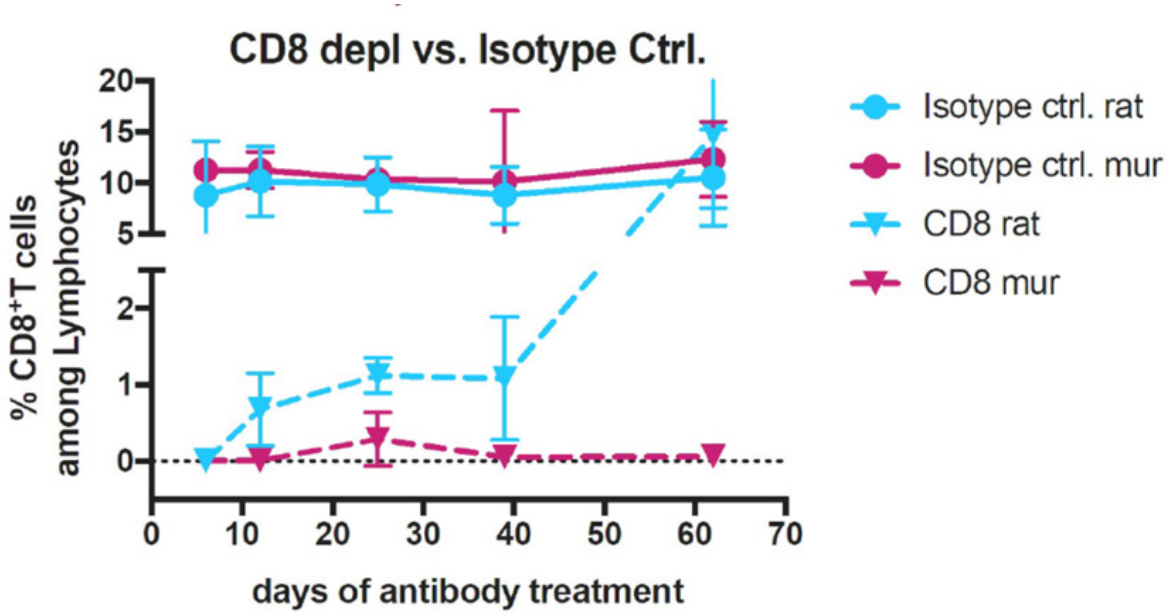



*Figure 1. CD8+ T-cell population in mice treated with anti-CD8 antibody clone YTS 169.4 for depletion and isotype controls. Mouse IgG2a format (Ab00166-2.0) is shown in purple, and Rat IgG2b (Ab00166-8.1) is shown in blue. Unpublished data, courtesy and property of the University of Basel, Switzerland.*

## Isotype and Subtype Switching

Isotype and subtype switching, also referred to as class switching, allows you to alter the isotype or subtype of any antibody. These engineered antibodies can be used to alter the *in vivo* effector function and stability of an antibody. Since some subtypes are prone to aggregation, class switching can help overcome this obstacle. It is also used to increase an antibody's avidity or reduce the number of needed controls in an experiment.

For example, an IgG antibody, the major antibody of the secondary immune response, can be reformatted to an IgM antibody, the predominant antibody of the primary immune response, to aid in infectious disease research and diagnostic assay development. During the early stages of the COVID-19 pandemic, Absolute Antibody was able to rapidly reformat our anti-coronavirus spike glycoprotein CR3022 antibody into human IgG, IgA, and IgM versions. These antibodies were widely used for research and as serological controls in diagnostic assays, leading to the WHO including them in their guidelines for secondary standards (1). In addition, altering the IgG subtype can have a significant impact on the anti-tumor activity of immunotherapy antibodies. For instance, converting an original mouse IgG2b subtype to a mouse IgG2a subtype greatly increased anti-tumor activity of an anti-CTLA-4 antibody in mouse models (2). Similarly, switching a mouse anti-TIGIT antibody from an IgG1 subtype to the IgG2a version boosted the antibody's anti-tumor potency (3).

# Which Format is Best Suited for My Needs?

There are infinite options when it comes to formatting an antibody, but depending on the desired outcome, not every format is required or feasible. Reformatting options include a variety of antibody fragments, bispecifics and trispecific antibodies, and Fc fusion proteins. Below we detail the various format options and describe the situations when each one should be considered.

## Antibody Fragments

Antibody fragments typically contain no Fc region and can offer several advantages compared to full-length antibodies.

- Smaller size may enable better tissue penetration into solid tumors (4)
- Shorter half-life is ideal if using an antibody as a radioactive imaging agent (5)
- No Fc effector function, making them useful for applications in which the antibody does not need to engage with the immune system (6)

There are many different antibody fragment formats to consider, but most are built on three main building blocks (Figure 2). These are recombinant Fab; single-chain variable fragment (scFv), the smallest stable and fully functional form of an IgG; and single domain antibodies (dAb), which lack a light chain and are found naturally in camelids and cartilaginous fish such as sharks.

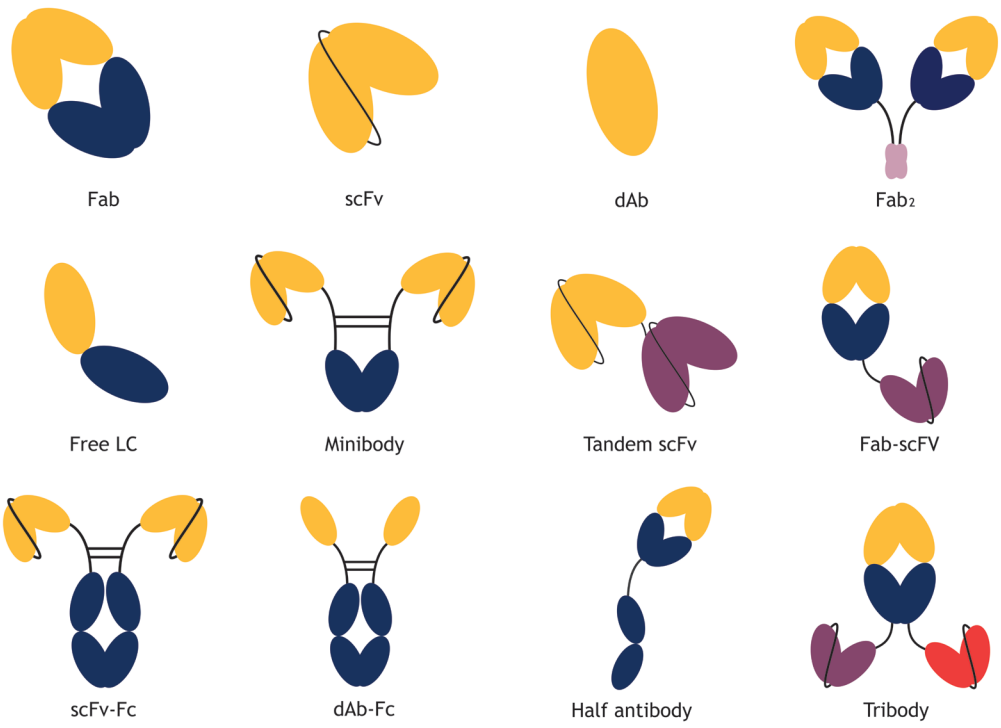



*Figure 2. A selection of engineered antibody fragments.*

## Bispecifics and Multispecifics

Bipecifics and multispecifics are a class of engineered antibody and antibody-like proteins that, in contrast to “regular” monospecific antibodies, combine multiple specific antigen-binding elements in a single construct. The ability to bind two or more different epitopes within a single molecule offers several potential advantages. One approach is to use the specificity of one arm to target an individual protein, marker or organism, and another arm to recruit effector cells or deliver molecular payloads to the target such as drugs, cytokines, or toxins. Alternatively, bispecifics can be used to dual target, allowing detection or binding of a target cell type with much higher specificity than monospecific antibodies.

Based on years of experience engineering bispecific antibodies, we have concluded that having a small panel of recommended designs is helpful for clients new to bispecifics. Figure 3 below represents the three “go to” format designs for IP-free bispecific antibodies. They utilize a single-chain Fv for one of the specificities to avoid issues associated with light chain shuffling, and two use a knob-into-hole platform to promote Fc heterodimerization. As a result, high titers of the desired bispecific can be obtained.

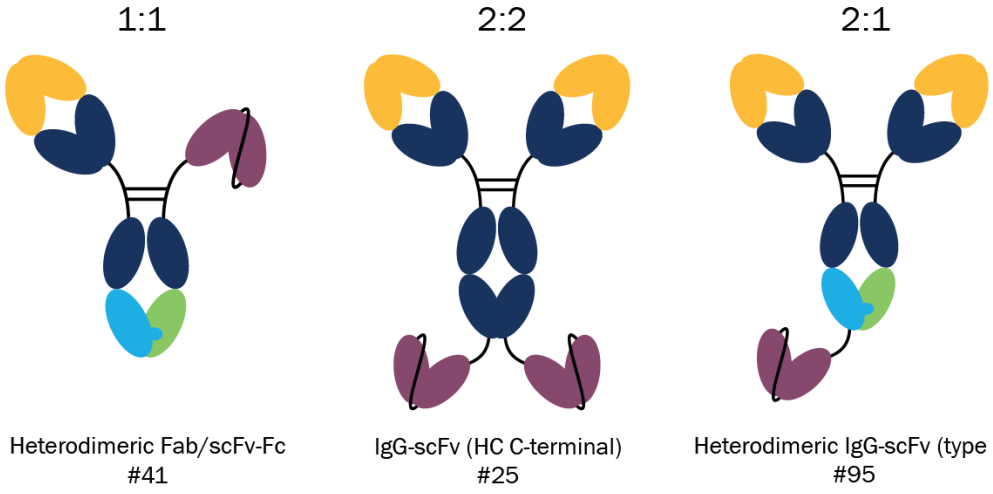



*Figure 3. Examples of “go to” format designs for IP-free bispecific antibodies.*

## Fc Fusion Proteins

Fc fusion proteins are composed of the Fc domain of IgG genetically linked to a peptide or protein of interest. Although antibodies are by far the most popular format of therapeutic protein, at least nine different Fc fusion proteins have also been approved by the FDA to date. In addition, they can be useful proof-of-concept tools for early-stage *in vitro* or *in vivo* studies before generating an antibody against a particular target.

Examples of Fc-fused binding partners include, but are not limited to:

- Single peptides
- Ligands activated upon cell-surface receptor binding
- Signaling molecules
- Extracellular domains of a receptor activated upon dimerization
- Bait proteins used to identify binding partners in protein microarrays

One of the most valuable characteristics conferred by the Fc domain *in vivo* is the dramatic prolonging of the plasma half-life (t1/2) of the protein of interest, which for biotherapeutic drugs can result in improved therapeutic efficacy. This attribute has made Fc fusion proteins attractive biotherapeutic agents. In addition, Fc fusion proteins can serve as immunogens with long half-lives; researchers can fuse an Fc region onto the protein for which they want to make an antibody against.

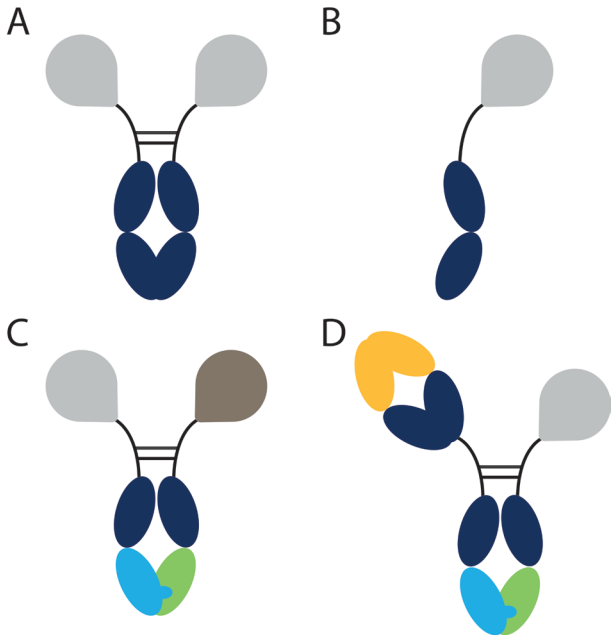



*Figure 4. A selection of Fc fusion proteins. A shows a classical homodimeric Fc fusion; B shows a monomeric protein; C and D show bispecific constructs with either two proteins (C) or one antibody and one protein (D).*

*In vitro* applications of Fc fusion proteins include, among others, ELISA, flow cytometry (FC), protein binding assays, and use as microarray baits. In these applications, the Fc domain behaves as a supporting module to which proteins can be attached while retaining their native biological activity. The Fc domain can also improve the *in vivo* and *in vitro* solubility and stability of some binding partners.

# What Size Antibody Do I Want?

Antibody size can be an important factor to consider and is dependent on intended use. For example, the size of a therapeutic protein correlates with its ability to penetrate solid tumors (4). Generally speaking, the larger the molecule, the worse the penetration. Additionally, antibody size affects half-life. The renal filtration limit is approximately 30-50 kDa and molecules smaller than this size will be filtered through the kidneys and cleared in a matter of hours. Molecules with a size above the renal filtration limit but without the ability to bind to FcRn have been defined as having a moderate half-life (typically days in humans). Molecules with a size above the renal filtration limit and the ability to bind to FcRn have been defined as having a long half-life (typically weeks in humans). Determining a suitable size for the antibody can be a fine balance between optimum penetration and optimum half-life.

# How Long of a Half-Life is Required?

In simple terms, the half-life of a drug is a pharmacokinetic parameter that is defined as the time it takes for the concentration of the drug in the plasma to decrease by 50%. The half-life of a biologic is affected by a number of factors, including molecular size, charge, FcRn recycling, receptor mediated endocytosis (RME), and stability. So, what is your desired half-life? To answer that question, again you need to consider the intended use and function of the antibody.

If a long half-life is required, then it is likely that the resulting molecule must contain an Fc domain to enable recirculation via FcRn binding. In this instance, utilizing the naturally long serum persistence of the Fc domain makes sense over other options such as albumin fusion, albumin binding domains or PEGylation. When rapid clearance from the circulation is required, shortening the half-life is possible by engineering the Fc region or by removing the Fc region all together and creating a Fab or scFv.

IgG-like antibodies have a longer half-life, typically greater than 10 days in humans, while antibody fragments have a much shorter half-life, typically hours in humans.

# What Do I Want for the Fc Effector Function?

The Fc domain is more than just a structural backbone. This region has proven to be critical to the antibody's function and is a focus of many engineering efforts, including increasing and decreasing effector functions.

## Increasing Effector Functions

If engagement with the immune system to activate ADCC and/or CDC is required, then an Fc domain capable of engaging with Fc receptors is required. Typically, this would be a human IgG1 Fc domain. In addition, Fc engineering approaches can be used to incorporate mutations into the Fc domain that enhance Fc receptor binding.

## Decreasing Effector Functions

Alternatively, if engagement with the immune system is undesirable, then the molecule must either contain no Fc domain or an Fc domain with minimal or no binding to Fc receptors. Human IgG4 was typically used for this purpose but has fallen out of favor more recently. Instead, various mutations to human IgG1 have been reported that result in a loss of binding to Fc receptors. Absolute Antibody's Fc Silent™ mutation abolishes binding to Fc receptors, which in turn abolishes ADCC effector function. The STR Fc silencing platform, developed by mAbsolve and former Absolute Antibody scientists, delivers the only truly silent Fc mutations described to date (7).

# How Strongly Should the Antibody Bind?

Avidity gives a measure of the overall strength of an antibody-antigen complex. It is dependent on three major parameters:

1. Affinity of the antibody for the epitope
2. Valency of both the antibody and the antigen
3. Structural arrangement of the parts that interact

Careful consideration to each of these components must be taken to achieve the optimal binding characteristics for any given antibody-antigen pairing. One aspect to consider is how many “arms” of the antibody you want binding to each antigen. When referring to bispecifics, the classic Y-shaped IgG, one Fab arm binding antigen A and the other binding antigen B, comes to mind. However, this does not have to be the case. This standard design is known as a 1:1 binder but you can also generate 2:1 and 2:2 binders (Figure 3).

For some targets, more binding arms may be better to increase avidity. In other instances, this can be detrimental. This is particularly true for CD3e where it is now widely accepted that you do not want to over-engage CD3e, as this leads to increased systemic toxicity. This moderate binding can be achieved by just having one binding arm or by using a CD3e-binding antibody with only a modest affinity. Therefore, in the literature you will often see researchers working with 1:1 or 2:1 bispecifics for T-cell recruitment.

# How Easy Will the Antibody Be to Manufacture?

Manufacturability of an antibody is an important component to consider early on in the project. In some instances, developability concerns that are identified early can potentially be engineered out during the early-stage research phase. Manufacturability encompasses a range of properties, such as expression titer, aggregation, long-term stability, and solubility. It is important to also note that the manufacturability of a format can change dramatically depending on the specific sequences (e.g., variable domains) being used.

In order for antibodies to make their way into clinical use, they must be optimized for strong manufacturability and large-scale production. It is therefore important to ensure an engineered antibody format can be produced with high expression and low aggregation levels. For example, to develop our antibody humanization approach, we analyzed manufacturability data for more than 100 antibodies that have reached at least late-stage clinical trials to create a set of criteria for selecting germline sequences that are associated with less potentially adverse events. View the case study below (Figure 5) for more information.

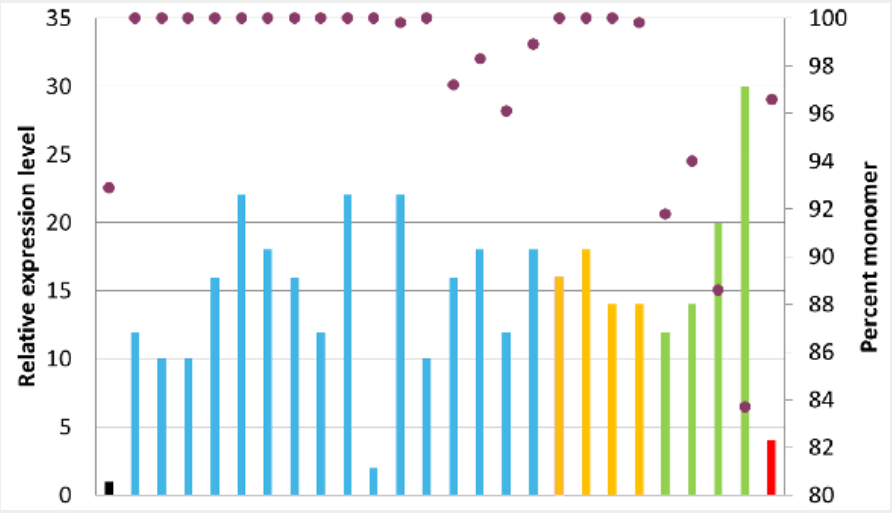
# Figure 5. Manufacturability Case Study

## Humanization

An antibody being developed for treatment of glioblastoma was selected as a case study. The chimeric antibody demonstrates poor manufacturability, in particular precipitation and weak expression, making it an excellent candidate for engineering onto a more favorable framework. The antibody was humanized onto two favorable VH and VL germline frameworks and one unfavorable VH and VL framework. From these, 25 humanized variants were created using Absolute Antibody's proprietary Prometheus™ humanization technology.

## Manufacturability Assessment

The chimeric antibody showed very poor expression (2.5 mg/L) and unsatisfactory monomer content (92%). By comparison, all humanized variants showed enhanced titers by as much as 30-fold. Antibodies containing an unfavorable VH framework (green bars) showed greater levels of aggregation than antibodies that contained a favorable VH. But of the 16 antibodies humanized to favorable VH and VL frameworks, 15 showed a 10-fold or greater increase in expression level and 12 showed minimal aggregation (>99.5% monomer).




Expression yields relative to the chimeric antibody shown as bars and monomer content shown as purple dots. Black bar is chimeric antibody, blue bars have favorable VH and VL, yellow bars have unfavorable VL only, green bars have unfavorable VH only and red bars have both unfavorable VH and VL.

# Is Chimerization or Humanization Required?

This section may not be relevant for all antibody engineering projects, but it is still important to consider. Will your engineered antibody be used *in vivo* or is it being developed for therapeutic purposes? Was the antibody developed from a murine or other animal origin species? If the answer is yes, it may be necessary to explore engineering a chimeric or humanized antibody (Figure 6).

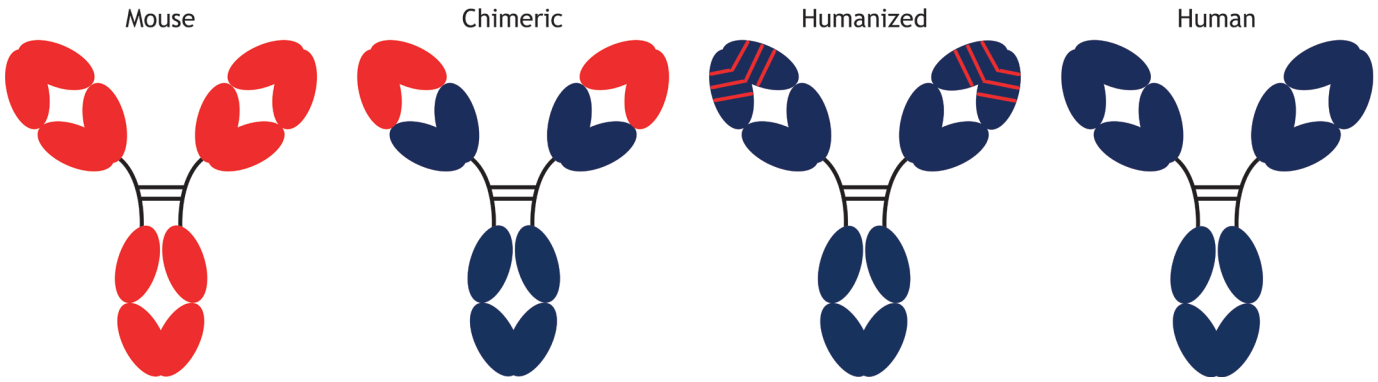



*Figure 6: Progressive humanization of antibodies. A schematic representation of the advancement from fully mouse antibodies, represented by red domains, to fully human antibodies, represented by blue domains. Note these images are somewhat misrepresentative, as immunoglobulin sequences are highly homologous across species, meaning a so-called fully mouse antibody is still relatively close in sequence to a fully human one.*

## Chimerization

Chimeric antibodies consist of an antibody's original antigen-binding variable domains, retaining the original specificity and affinity, and the constant domains from a different species. These antibodies have been shown to be a suitable option for *in vivo* research, *in vitro* research, and diagnostic assay development.

Prior to humanization being widely adopted, many therapeutic antibodies were originally generated in mice and chimerized to reduce immunogenicity in humans. Some examples of these antibodies in the clinic include infliximab, rituximab and abciximab. Chimeric antibodies containing human constant domains and mouse variable domains are substantially less expensive than fully humanized antibodies. This makes them useful at the early stages of biotherapeutics research. They have also proven useful for diagnostic assay development due to their batch-to-batch reproducibility and homogeneous specificity and affinity. Additionally, chimeric antibodies reduce the risk of non-specific binding to heterophilic antibodies, such as human anti-mouse antibodies (HAMA), that can cause false positive assay results.

## Humanization

Humanized antibodies are critical for the development of therapeutic antibodies derived from non-human sources. The humanization process involves the transfer of critical non-human amino acids onto a human antibody framework. This primarily includes the grafting of amino acids in the complementarity-determining regions (CDRs), but also other framework amino acids critical for the VH:VL interface and CDR orientation. It is a balance between introducing as much human content as possible to reduce the risk of immunogenicity, while retaining enough non-human content to maintain the original binding activity of the parent antibody.

As mentioned previously, antibody manufacturability is an important aspect of the engineering process. Occasionally chimeric antibodies demonstrate manufacturability properties unsuitable for large-scale therapeutic use, in particular precipitation and weak expression. When attempts to modify the backbone do not effectively solve the problem, antibody humanization is the next logical step. View the antibody humanization manufacturability case study (Figure 5) above for more information.

# Which Cell Line is Best to Express My Antibody?

Now that you have considered the engineering and formatting options for your antibody, it is time to think about how to express it.

HEK and CHO are two cell line options for serum-free mammalian transient expression. Transient expression is a more affordable and rapid alternative to stable cell line generation. While stable cell line generation typically takes six months to a year, transient expression platforms can produce high-quality recombinant antibodies in engineered formats within a month.

HEK293 (human embryonic kidney) cell lines are relatively easy to work with and have historically produced higher protein production yields than CHO. Expression in a HEK platform is a cost-effective option for early-stage antibody development, offering high-throughput recombinant production ideal for screening antibody candidates. It is also the expression system of choice for reagent and diagnostics antibodies. Additionally, HEK293 platform may provide improved expression for antibodies that are difficult to produce in CHO (8).

CHO (Chinese hamster ovary) cell lines are the preferred platform for therapeutic antibody expression. The risk of infection from human viruses is low, and the platform “enables efficient expression of proteins with the need for human-like-post-translational modifications” (8), including different glycan modifications. CHO expression is ideal for research projects in which minor differences between HEK and CHO-made antibodies may be significant, such as half-life, potency, and glycosylation studies. Transient CHO production, at milligram-to-gram scales, is particularly useful for bridging the gap to stable CHO production, or for tackling challenging constructs. It enables therapeutic antibody developers to quickly manufacture and screen a group of antibodies before taking the time to generate stable cell lines for their most promising candidates.

# Conclusion

Recombinant antibody engineering has been used to develop therapeutic antibodies in a wide variety of formats since the first approval in 1986, with nearly 100 antibody-based drugs currently in the market and many more in late-stage clinical trials. In addition, more recently, engineered recombinant antibodies have begun to be used for research and diagnostic applications.

The considerations described are not an exhaustive list of options, but rather a starting point to further explore antibody engineering capabilities. Every antibody is different, and what works well for one clone may not be as successful for the next. Trial and error during the engineering process still exists. However, we hope this overview can help streamline your next engineering project and further your antibody research.

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# About Us

## Absolute Antibody

The Absolute Antibody vision is to make recombinant antibody technology accessible to all researchers. We offer antibody sequencing, engineering, and recombinant production as royalty-free custom services, as well as a unique reagents catalog of recombinant antibodies and Fc Fusion proteins, engineered into new and useful formats.

## Absolute Biotech

Absolute Antibody is part of Absolute Biotech, a new company that unites multiple life science brands into one organization specializing in antibody reagents and services. Our mission is to serve as “antibody curators” for customers worldwide, treating each antibody like a work of art to deliver unique and absolutely defined reagents that empower scientists.

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