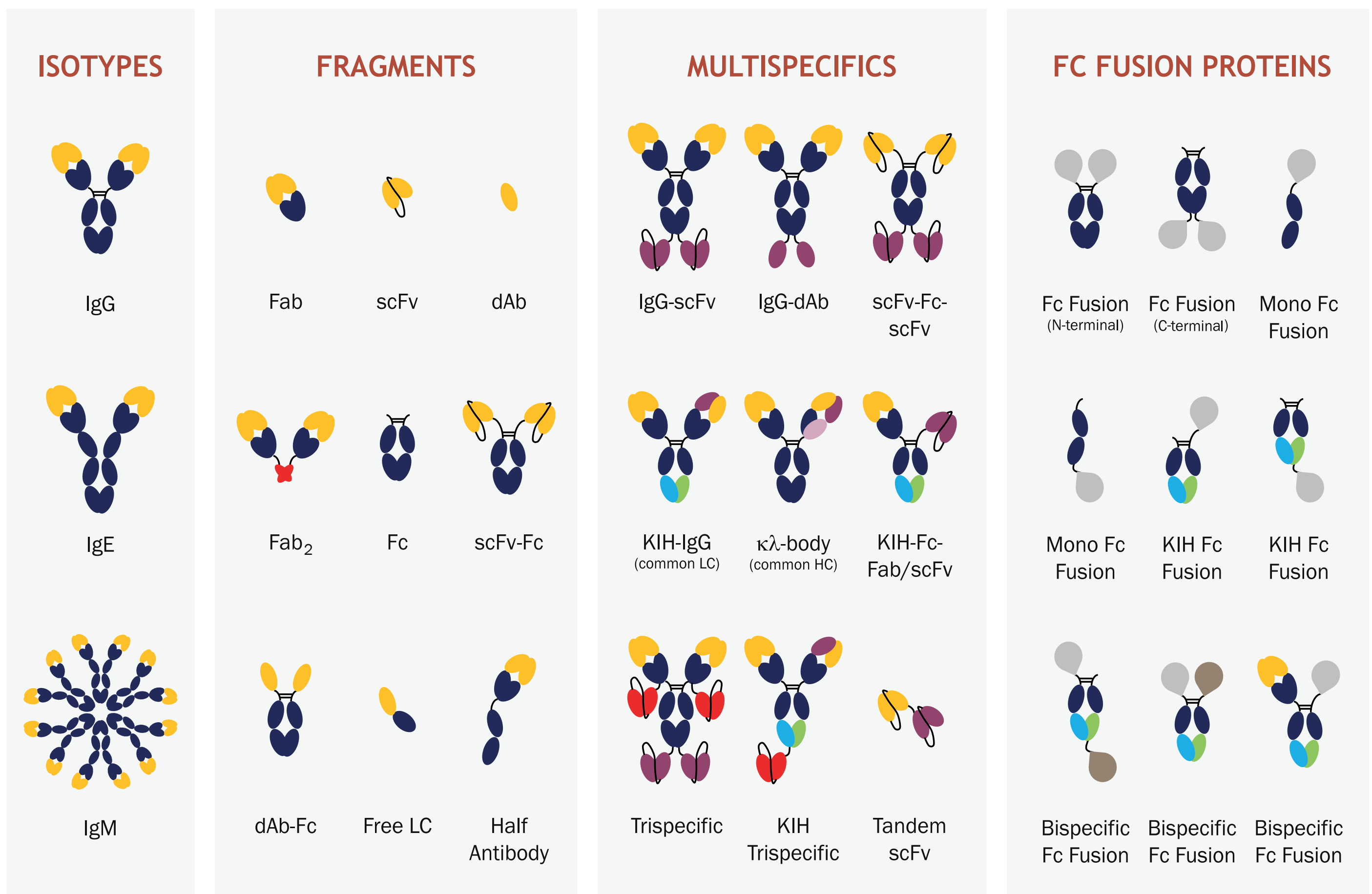
Guide to Recombinant Antibody Engineering

White Paper



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This white paper will provide an overview of recombinant antibody engineering. It will cover key antibody background, then describe the different design parameters to consider when engineering an antibody. We will then outline different antibody engineering options, including humanization, fragments, multispecifics, and Fc fusion proteins. Finally, we will look at recent industry developments and the future of antibody engineering.

Antibody Background

A Brief History of Antibodies

The earliest reference to antibodies came from Emil von Behring and Shibasabura Kitasato in 1890. In a landmark publication, they showed that the transfer of serum from animals immunized against diptheria to animals suffering from it could cure the infected animals (1). The potential for treatment in humans was immediately apparent and Behring was later awarded the Nobel Prize for this work in 1901.

In 1900, Paul Ehrlich, who is regarded as one of the fathers of modern immunology, proposed the side-chain theory, which hypothesized that side chain receptors on cells bind to a given pathogen. He was the first to propose a model for an antibody molecule in which the antibody was branched and consisted of multiple sites for binding to foreign material, known as the antigen, and for the activation of the complement pathway (2).

By 1959, Gerald Edelman and Rodney Porter independently published the molecular structure of antibodies, for which they were later jointly awarded the Nobel Prize in 1972 (3,4). The first atomic resolution structure of an antibody fragment was published in 1973, and this was quickly followed by the invention of monoclonal antibodies in 1975 by Georges Köhler and César Milstein (5,6).

Monoclonal antibodies were initially produced from hybridomas, created by fusing antibody-producing B-cells with immortal myeloma cells. However, hybridomas produce antibodies of mouse origin, and despite the initial promise of mouse-derived antibodies in the clinic, it soon became apparent that patients were raising a human anti-mouse antibody (HAMA) response, which led to faster clearance of the therapeutic antibody and an increased safety risk.

Recombinant protein production offered a potential solution to the problem, with the first recombinant protein, human somatostatin, produced in *E. coli* in 1977 (7). Combining the burgeoning fields of molecular biology and recombinant protein production would create a new area of biotechnology: antibody engineering.

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Antibody Structure and Isotypes

Antibodies consist of the antigen binding fragment (Fab), which recognizes the antigen, and the crystallizable fragment (Fc), which interacts with other immune system elements to promote removal of the antigen. The basic structure consists of two heavy and two light chains, folded into constant and variable domains (Figure 1).

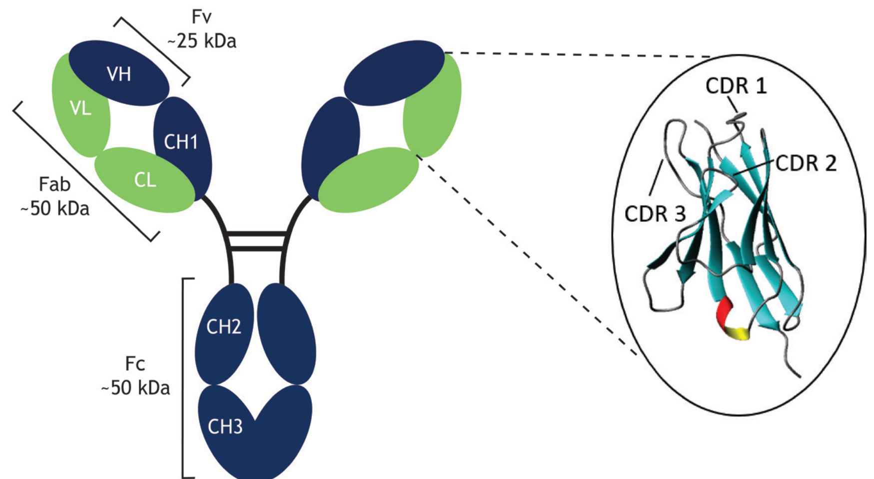

Figure 1. Schematic representation of an IgG. An antibody consists of two heavy chains (blue) and two light chains (green) folded into constant and variable domains. The enlargement of the variable domain shows a ribbon representation of the β-sheet framework and CDR loops.

The variable domains (Fv) provide the antigen specificity of the antibody, while the constant domains act as a structural framework (5). Each variable domain contains three hypervariable loops, known as complementarity determining regions (CDRs), evenly distributed between four less variable framework regions. It is the CDRs that provide a specific antigen recognition site on the surface of the antibody and the hypervariability of these regions enables antibodies to recognize an almost unlimited number of antigens (8).

In mammals, antibodies are classified into five main classes or isotypes – IgA, IgD, IgE, IgG and IgM – which differ in the sequence and number of constant domains, hinge structure and valency of the antibody. IgG is the most abundant antibody in normal human serum, and the most relevant for therapeutic development.

Antibody Applications

Antibodies have always had the potential to be significant biochemical tools for a range of applications due to their high specificity and selectivity, and they have truly revolutionized the field of biological science over the past 40 years. For research applications, they are excellent tools for the selective detection and/or isolation of antigens and are commonly used in techniques such as western blots, flow cytometry, enzyme-linked immunosorbent assays (ELISA), and immunohistochemistry (IHC).

Antibodies have also become a critical component of many diagnostic assays, used to detect infections, allergies, and other biological markers in the blood. Finally, the power of antibodies has been harnessed for the treatment of cancer, autoimmune diseases, and other conditions. Since the mid-90s, therapeutic antibodies have become one of the fastest growing classes of therapeutics, with nearly 100 antibody-based drugs currently on the market.

Antibody Engineering Design Parameters

Before engineering any therapeutic protein, careful consideration must be taken to the desired properties of the resulting molecule and the pharmacological effects.

Of course, specificity and affinity are the primary parameters by which an antibody is typically selected. Although these can also be altered, for example through affinity maturation, here we will focus on other design considerations. It is worth considering, though, that higher affinity is not always better and avidity, which is impacted by the valency of the antibody, as well as affinity play an important role in biological activity.

Half-Life and Molecular Size

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, stability, glycosylation, FcRn recycling and receptor mediated endocytosis.

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. If a rapid clearance from the circulation is required, then the Fc domain must either be removed from the molecule or mutated to prevent interaction with FcRn. IgG-like antibodies have a longer half-life, typically weeks in humans, while antibody fragments have a much shorter half-life, typically hours in humans. Any molecules smaller than the renal filtration limit of approximately 70 kDa will be filtered through the kidneys and cleared in a matter of hours.

Different strategies can enhance the half-life of a biologic, in order to decrease its administration frequency while maintaining or improving efficacy. For example, the antibody can be fused to albumin or conjugated to polymers (e.g. PEGylation) to increase the size of the protein. Introducing mutations at the interface between the CH2 and CH3 domains of the Fc can also extend the plasma half-life of an IgG antibody. In addition to affecting half-life, however, the size of a therapeutic protein correlates with its ability to penetrate solid tumors (9). Determining the appropriate size of a therapeutic protein can therefore be a fine balance between optimum penetration and optimum half-life.

Fc Effector Function

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 (10-13).

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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 due to the potential for Fab-arm exchange, where heavy chains can be swapped between IgG4 *in vivo* (14,15). Instead, various mutations to human IgG1 have been reported that result in a loss of binding to Fc receptors (16-18). Absolute Antibody's Fc Silent™ mutation abolishes binding to Fc receptors, which in turn abolishes ADCC effector function. Figure 2 shows a selection of other amino acids that have been targeted for increasing or decreasing effector function.

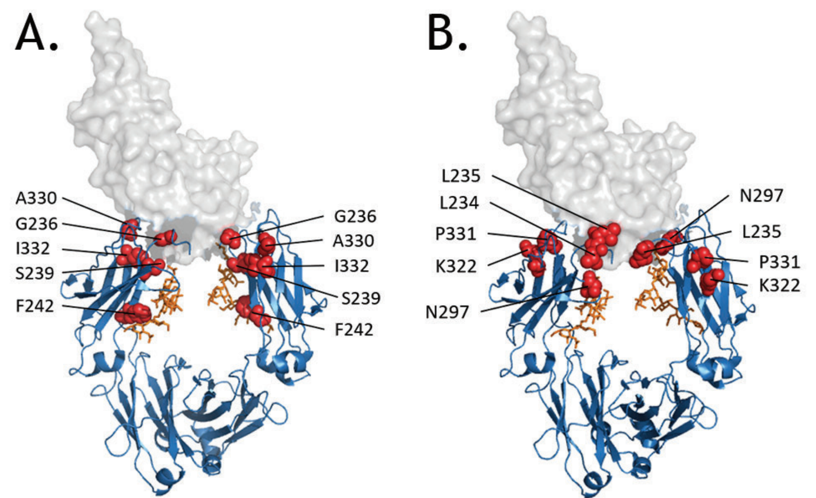

Figure 2. Engineering Fc effector functions. Structural representation of a human IgG1 Fc domain in complex with FcγRIII (PDB 1T83). The Fc domain is shown as a ribbon representation in blue, glycosylation in orange and FcγRIII as a transparent space filled model. The red shows a selection of the amino acids that have been targeted for enhancing Fc effector functions (A) and for abolishing interaction with Fc receptors (B).

Manufacturability

Manufacturability encompasses a range of properties such as expression titer, aggregation, long-term stability and solubility. 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.

Traditionally, these concerns would be addressed in the later stages of projects, sometimes resulting in projects being cancelled for cost of goods and lack of manufacturability. More recently, however, efforts are being made to consider developability concerns as early on as possible, in order to potentially engineer out these problems during the early-stage research phase of a therapeutic project.

Antibody Engineering Options

The dawn of the recombinant DNA revolution in the 1970s and 1980s led to the production and engineering of recombinant monoclonal antibodies. Early efforts in the 1980s and 1990s focused on the humanization and fragmentation of antibodies. Into the 21st century, the appetite to engineer antibodies with a myriad of improved functionalities has continued to grow, resulting in the creation of second and third generation antibodies. This section aims to summarize some of the efforts in this ever-expanding field.

Antibody Humanization

The early success of mouse monoclonal antibodies led to the FDA approval of the first therapeutic antibody OKT3 (muromonab) in 1986, as a treatment of kidney transplant rejection. However, most mouse antibodies were shown to have limited use as therapeutic agents because of a short serum half-life, an inability to trigger human effector functions, and their recognition by patients' immune systems as foreign proteins, which triggered a human anti-mouse antibody (HAMA) response.

In an attempt to reduce the immunogenicity of the mouse antibodies, genetic engineering was used to generate chimeric antibodies containing human constant domains and mouse variable domains to retain the original specificity (19,20). This was then taken a step further by grafting the CDRs from a mouse antibody onto a human variable region framework, creating humanized antibodies (21).

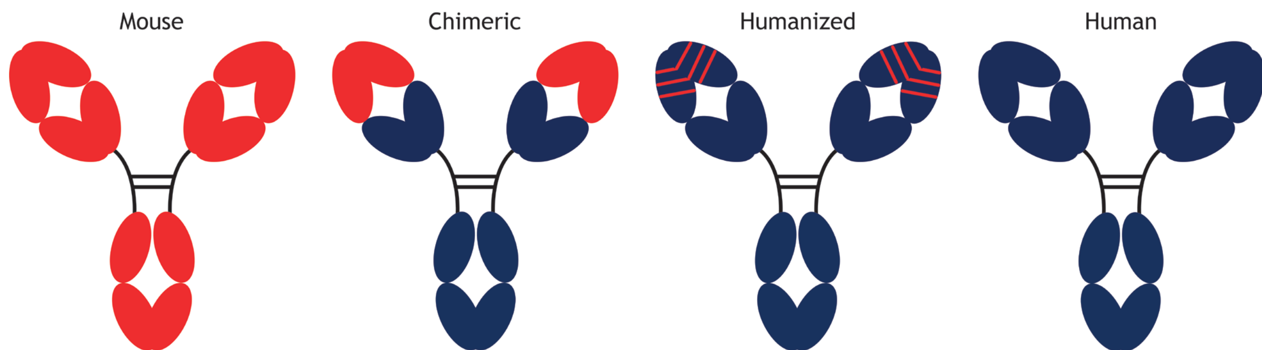

Figure 3. Progressive humanization of antibodies. A schematic representation of the advancement from fully mouse antibodies, represented by red domains, to fully human antibodies, represented by blue domains. Note these images are somewhat misrepresentative, as immunoglobulin sequences are highly homologous across species, meaning a so-called fully mouse antibody is still relatively close in sequence to a fully human one.

In 1996, the FDA approved the first chimeric antibody ReoPro (abciximab), which lessens the risk of blood clots in patients with cardiovascular disease by binding to a receptor on platelets (22). In 1997, the FDA approved the first humanized antibody Zenapax (daclizumab), which is an anti-CD25 antibody used to prevent organ transplant rejection (23).

The drive to reduce immunogenicity by decreasing the mouse content of monoclonal antibodies inevitably culminated in the creation of so-called fully human antibodies, which was made possible by the expression of isolated human variable domain genes in *E. coli* (24,25). Two of the most widely used techniques developed for the production of fully human monoclonal antibodies are phage display, where a library of human antibodies is expressed on the surface of phage and subsequently selected and amplified in *E. coli* (26), and transgenic mice expressing a human antibody repertoire (27).

Figure 3 above shows a graphical depiction of mouse, chimeric, humanized and human antibodies.

Antibody Fragments

Antibody fragments with no Fc region can offer several advantages compared to full-length antibodies. Their smaller size may enable better tissue penetration into solid tumors (9), and their shorter half-life is ideal if using an antibody as a radioactive imaging agent (28). Antibody fragments also have no Fc effector function, making them useful for applications in which the antibody doesn't need to engage with the immune system, such as the blocking of a signaling molecule or receptor (29).

Enzymatic digestion of IgG has been reported since the early 20th century with two proteases in particular. Pepsin cleaves in the hinge region after the disulphide bonds, creating a dimeric Fab fragment known as a $F(ab')_2$. Papain cleaves above the disulphide bonds, thus creating a monomeric Fab fragment. Although these processes work on a lab scale, they aren't ideal for large-scale therapeutic production due to the cost and complexity of the purification.

Following the advent of antibody engineering techniques, researchers began to generate and produce antibody fragments recombinantly. Three main fragments were developed that became the building blocks for many alternative formats. First, recombinant Fab is a clean and defined alternative to the Fab produced by enzymatic digestion. Second, the single chain variable fragment (scFv) is the smallest stable and fully functional form of an IgG. It consists of the variable heavy domain and variable light domain with a flexible linker between the two. Finally, single domain antibodies (dAb) lack a light chain and represent a minimal binding domain of about 15 kDa in size. Single domain antibodies are found in camelids and cartilaginous fish such as sharks, and they contain longer CDR loops compared to conventional murine and human antibodies.

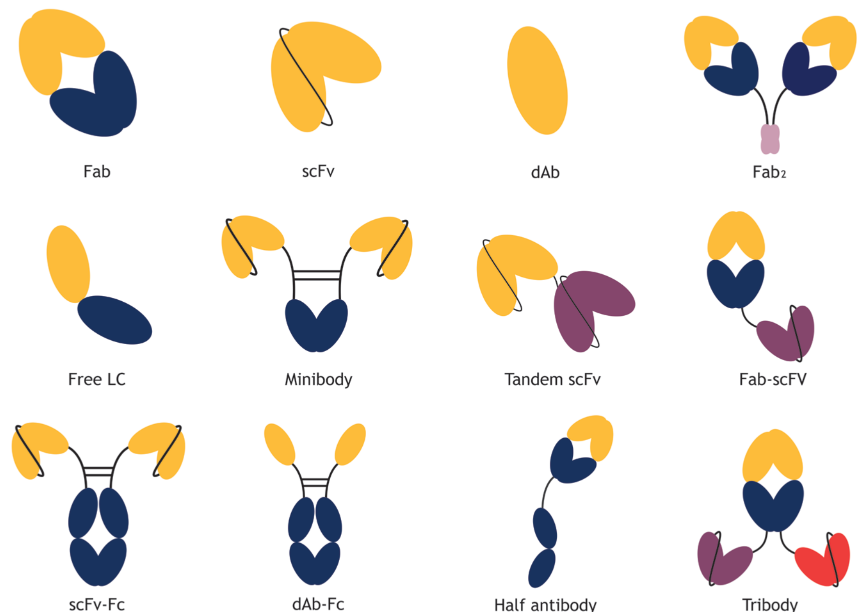

Figure 4. A selection of engineered antibody fragments.

Fabs, scFvs and dAbs can be combined in a number of different ways, with or without Fc domains, to create a variety of antibody fragments. Figure 4 shows a sample of the different antibody fragments that have been reported to date.

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Multispecific Antibodies

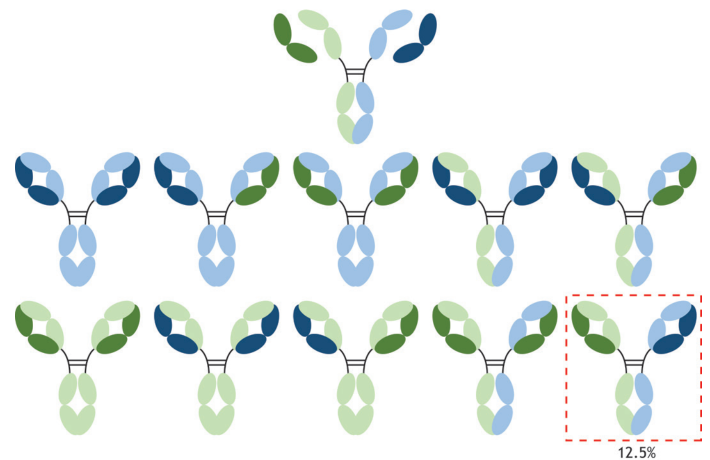

Figure 5. The chain association problem. Representation of the antibody combinations that can be produced by two antibodies assuming random chain association. In total 16 formats are possible, of which six are identical. Six tetramers, including the desired bispecific antibody, occur twice (each with a yield of 12.5%) and four tetramers occur once (each with a yield of 6.25%).

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 with 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.

Since bispecific antibodies do not typically occur in nature, they are constructed either chemically or biologically, using techniques such as cell fusion or recombinant DNA technologies. When people think of a bispecific antibody, they typically think of a Y-shaped IgG molecule with one arm binding one antigen and the other binding a second antigen. Unfortunately, the production of such a bispecific molecule is not simple. Co-production of two antibodies can result in a large number of potential arrangements, only a small proportion of which are the desired bispecific. This is often referred to as the chain association problem, and it is a significant challenge to yields and purification (Figure 5).

In the last 20 years, efforts have focused on recombinant DNA technology to enable engineering of antibody formats that minimize or completely avoid this chain association problem. These multispecific antibodies have been a key growth area in the antibody field, and below we describe various antibody formats that have been developed as a result.

Heterodimeric bispecific antibodies

When people refer to bispecific antibodies, they are typically referring to heterodimeric antibodies, or a traditional IgG molecule with one arm targeting one antigen and the other targeting a second antigen. These molecules are thus bispecific and monovalent. The first engineered bispecific antibody of this kind was described by Genentech (30). They created a "knob-into-hole" antibody (often abbreviated as KIH), whereby a large amino acid on one chain was replaced with a small amino acid (mutation Y407T) and on the second chain a small amino acid was replaced with a large amino acid (mutation T366Y). This resolved the heavy chain pairing problem and resulted in preferential formation of heterodimer. Subsequent attempts to generate monovalent bispecific antibodies have followed a similar approach.

These approaches suffer from two main problems: light chain shuffling and homodimer formation. The light chain shuffling problem can be resolved by using antibodies that have been developed to have a common light chain (31), but in most situations this option is not available. The problem can be removed completely, however, by switching to using a single chain Fc (scFv) for one or both of the specificities (Figure 6). All techniques still result in varying amounts of homodimer being present, which must be removed during purification.

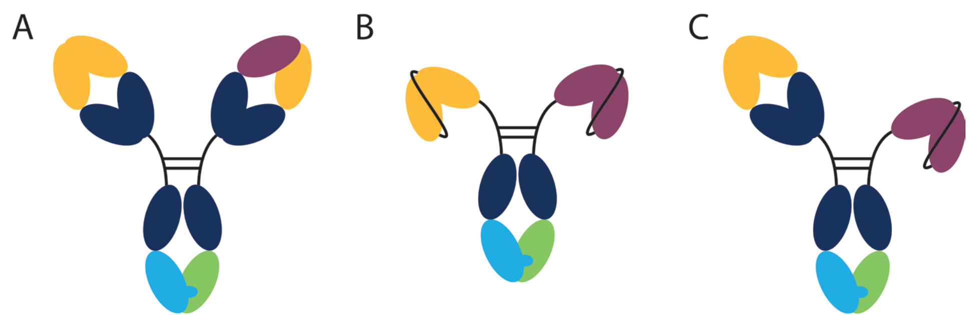

Figure 6. Knob-into-hole (KIH) antibodies. Variable domains for one specificity are shown in yellow with the second specificity in purple. Antibody constant domains are shown in dark blue apart from the mutated CH3 domains containing the knob and hole mutations which are shown in light blue and green. Panel A shows a classical KIH antibody as an IgG with a common light chain. The use of two (B) or one (C) scFvs removes the light chain shuffling problem to enable two different light chains to be utilized.

Bispecific antibody fusions

To avoid the main manufacturing issues associated with heterodimeric bispecific antibodies (i.e., light chain shuffling and homodimer formation), groups have explored IgG fusions. The direct genetic coupling of the desired variable domains in these formats avoids production of alternative shuffled variants, but results in a non-standard IgG molecule, which itself may cause manufacturing problems such as reduced expression levels or increased aggregation tendency.

Abbott has developed dual variable domain antibodies (DVD-Ig) (32), in which an IgG is elongated at its N-terminus on the corresponding heavy and light chains by an additional variable domain of a second antibody (Figure 7A). An alternative approach is the fusion of a scFv to an IgG. This can be done via the attachment of the scFv to the N or C-terminus of the heavy or light chain, giving a total of four possible formats (Figure 7B depicts one such format). In a similar manner, a scFv-Fc-scFv format could also be engineered (Figure 7C). These formats are all bispecific and bivalent.

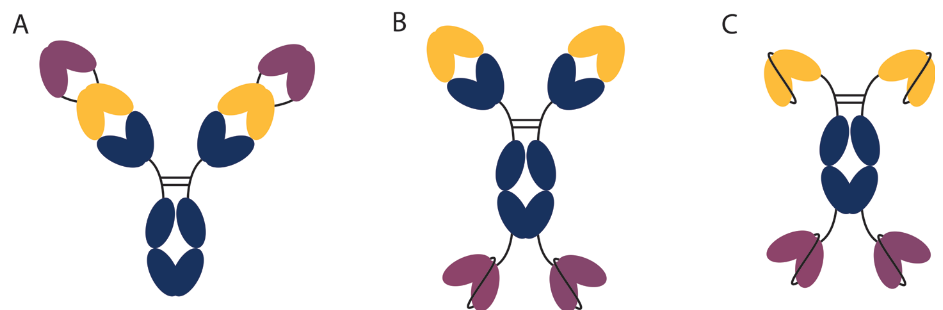

Figure 7. Bispecific antibody fusions. Representations of a dual variable domain antibody (A), an IgG-scFv (B) and a scFv-Fc-scFv (C). In each format, one pair of variable domains is shown in yellow and the second pair in purple.

Bispecific antibody fragments

A whole variety of multi-specific antibody fragments have been developed over the last 30 years. Although numerous formats are currently in clinical development, the only approved bispecific antibody fragment to date is blinatumomab (trade name Blincyto®) from Amgen. This is a bispecific T-cell engager, or BiTE, which is constructed by genetic fusion of two scFvs. One scFv binds to CD3ε on T-cells and the other binds to a surface molecule on the specific target cancer cells. Once bound to the target antigen on the cancer cell, binding of CD3ε leads to activation and polyclonal expansion of cytotoxic T-cells, ultimately resulting in lysis of tumor cells (33). Please see Figure 4 above for more examples of bispecific fragments.

Trispecific Antibodies

The same technologies used to generate bispecific antibodies can also be combined to generate trispecific antibodies with varying valencies. For example, MedImmune has described an IgG with scFvs at the C-terminus of both the heavy and light chain (34). Figure 8 shows a sample of trispecific antibody formats under exploration.

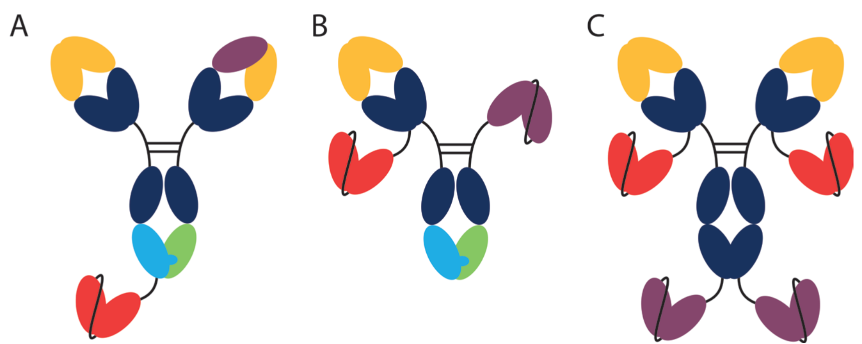

Figure 8. A selection of trispecific antibodies. Variable domains for one specificity are shown in yellow, the second specificity in purple and the third specificity in red. A and B are monovalent trispecifics containing a knob-into-hole heterodimeric Fc domain. A is a KIH IgG with a common LC and scFv fused at the C-terminus of the heavy chain. B represents a format with one Fab and two scFvs to avoid light chain shuffling. C is an example of a bivalent trispecific.

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.

Although Fc fusions have typically been produced as homodimeric constructs, a wide variety of different formats can be prepared using many of the same technologies previously described for antibodies (Figure 9). Monomeric Fc (35) or knob-into-hole Fc technology can be used to produce monomeric formats (30). Alternatively, constructs can be produced that consist of multiple different proteins and/or antibody biding portions such as scFvs or Fabs.

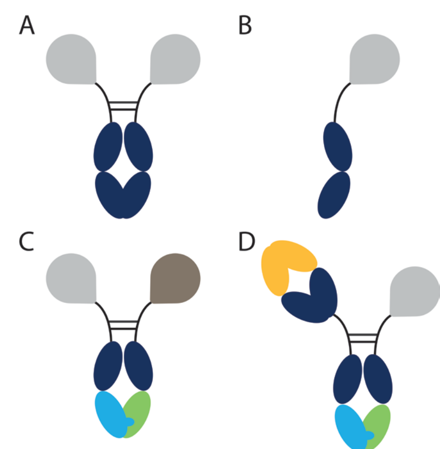

Figure 9. A selection of Fc fusion proteins. A shows a classical homodimeric Fc fusion; B shows a monomeric Fc protein; C and D show bispecific constructs with either two proteins (C) or one antibody and one protein (D).

Further Antibody Developments

An approach starting to show clinical promise after many years of research is the use of antibody drug conjugates (ADCs). The concept is simple: combine the selectivity of an antibody with the cytotoxic potency of a small molecule. The implementation, however, is very challenging. Many different factors need to be optimized, including the choice of small molecule, often known as the payload; the method of conjugation; and the location and number of conjugation sites on the antibody. Although the first ADC to be approved by the FDA was gemtuzumab ozogamicin in 2000, this was withdrawn in 2011. Two further ADCs have been approved more recently, with many also in the latter stages of clinical trials.

Another antibody area nearing clinical use is immunokines, a specific class of Fc fusion protein that combines an antibody with a cytokine. As cytokines act as modulators of the immune system, this approach can create a very potent molecule when combined with the tumor-targeting ability of an antibody. A variety of immunokines are in late-stage clinical trials, in particular using IL-2, IL-12 and GM-CSF, with other targets currently in preclinical development.

Other longer-term developments in the pipeline include targeting antibody delivery to areas of the body that have been inaccessible, such as the brain or intracellular targets, as well as developing masked or slow release antibodies to remove the need for frequent injections. In addition, novel approaches are being taken to develop antibodies that have been traditionally difficult to manufacture, such as IgM antibodies, recombinant polyclonals, or antibodies to complex membrane proteins such as GPCRs and ion channels. Finally, the number of engineered antibody formats should continue to increase. The Periodic Table of Antibodies on the next page illustrates the 140+ different antibody formats Absolute Antibody has engineered and manufactured to date, and we only expect this antibody innovation to continue (Figure 10).

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 on 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. This is due to the significant benefits recombinant antibodies offer over traditional hybridoma-produced monoclonal antibodies, such as batch-to-batch reproducibility, guaranteed long-term supply, and opening up new experimental possibilities with engineered formats.

This white paper only covered a selection of antibody formats that have been generated to date, and we expect innovation in recombinant antibody engineering to continue for therapeutic, diagnostic and research applications.

Periodic Table of Antibodies

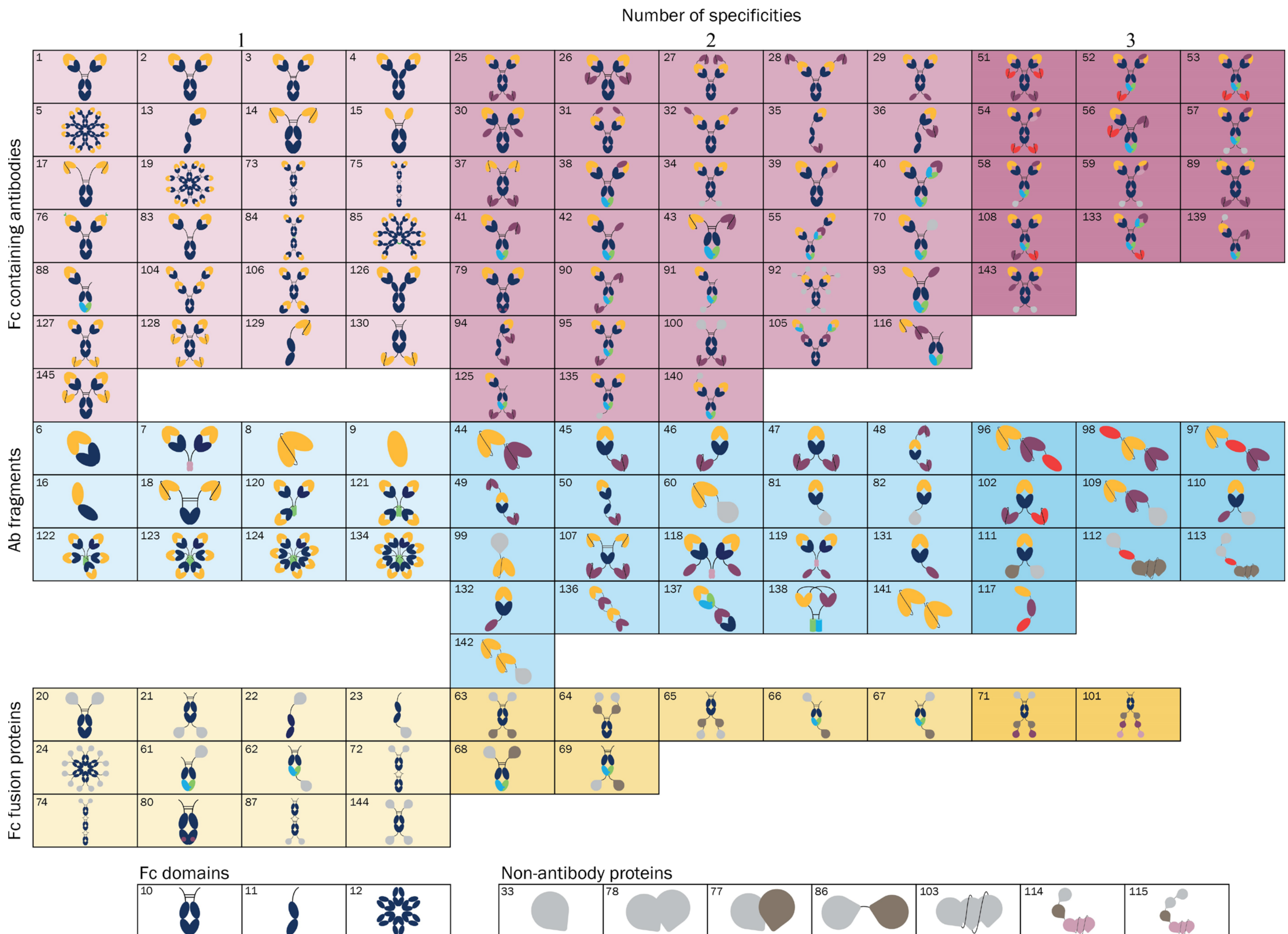

Figure 10. Periodic Table of Antibodies. A selection of antibody formats manufactured by Absolute Antibody, organized by number of specificities and further characterized as Fc containing antibodies, antibody fragments or Fc fusion proteins. View an interactive version and the full table legend at AbsoluteAntibody.com/PeriodicTable.

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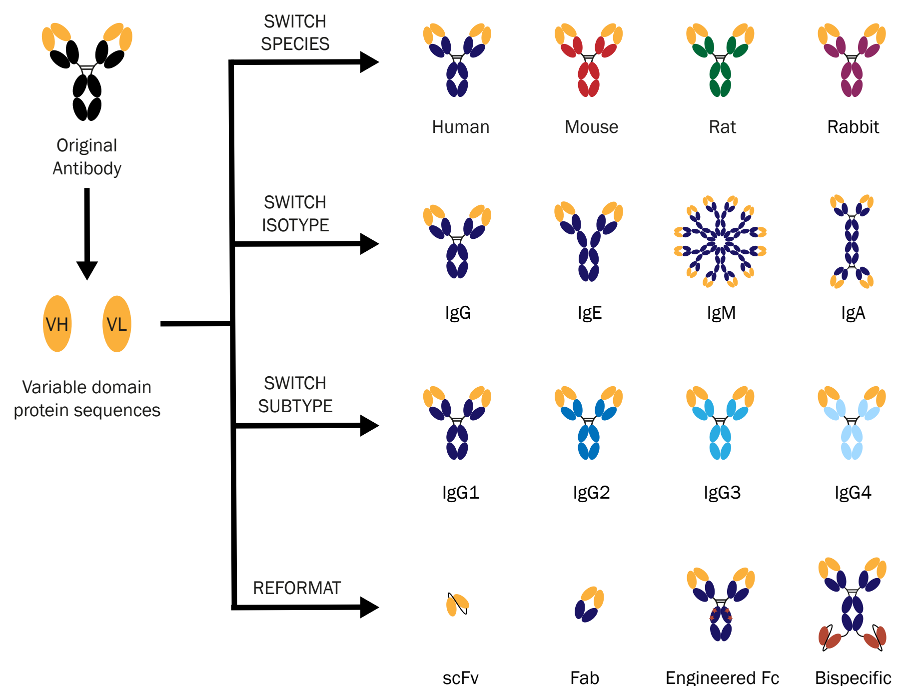
Absolute Antibody was founded in 2012 with a vision to make recombinant antibody technology accessible to all. We offer antibody sequencing, engineering and recombinant production as royalty-free custom services, as well as a unique catalog of engineered recombinant antibodies and Fc Fusion proteins.

Our Antibody Engineering Services

Our proprietary cloning system enables rapid reformatting of antibodies into any format. Since our founding in 2012, we have engineered more than 140 different antibody formats for customers across pharmaceuticals, biotechnology, diagnostics and academia.

We can begin engineering from an electronic antibody sequence, hybridoma cell line or purified antibody protein. Our antibody engineering services include:

- Species, isotype and subtype switching
- Antibody fragments, such as Fab, dAb and scFv
- Multispecific antibodies (bispecific and trispecific)
- Antibody chimerization
- Antibody humanization
- Engineered Fc domains
- Fc fusion proteins



All production occurs via our HEXpress™ antibody expression platform, which rapidly produces high-quality, animal-free recombinant antibodies at milligram-to-gram scale. This serum-free mammalian transient expression system offers a faster, more affordable alternative to stable cell line generation.

- Manufacture any antibody species, isotype or format
- High purity and low endotoxin levels guaranteed
- All production occurs in our ISO 9001:2015-certified facility

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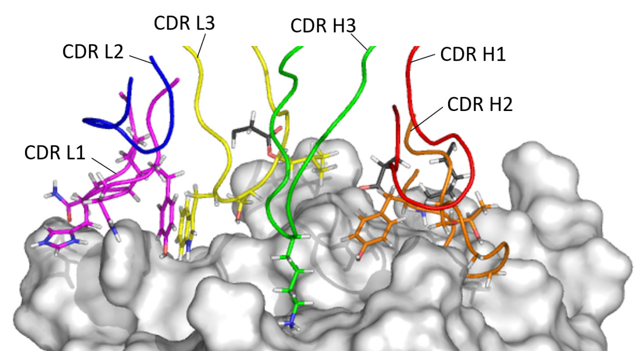
Below is more detail about several of our engineering offerings. Looking for something else? Get in touch — if you can describe it, we can make it!

Antibody Humanization

Our Prometheus™ platform delivers a panel of humanized variants from your clone, guaranteeing to produce a variant with comparable activity to the parent antibody. Our process focuses on enhancing antibody manufacturability, leading to increased expression, lower aggregation, and long-term stability and solubility.

- Royalty-free with a no-success-no-fee guarantee
- Four levels of service, ranging from only *in silico* humanization to full expression and analytics
- Structure-guided CDR grafting onto preferred germline backbones selected for preferential manufacturability properties
- Minimum of 12 humanized variants created
- Deep humanization expertise, dating back to the first humanized antibody in 1988
- 80+ successful humanizations completed



Model of antibody-antigen interface

Engineered Reagents Catalog

We've applied our antibody engineering expertise to offer a unique catalog of engineered recombinant reagents. We build the catalog by sequencing existing monoclonal antibodies, producing recombinant versions, and engineering the antibodies into new formats to increase experimental flexibility. One proven clone thus becomes available in many unique formats unavailable in any other reagents catalog.

Novel catalog formats enabled by antibody engineering include:

- Fc Silent™ antibodies: contain a genetically engineered Fc domain with key point mutations that abrogate binding of Fc receptors and abolish ADCC effector function
- Species-matched chimeric antibodies: consist of a clone's original antigen-binding variable domains with the constant domains of different species to reduce immunogenicity
- Murine bispecific antibodies: fully murine knob-into-hole (KIH) bispecific antibodies that enable easier evaluations of potential bispecific combinations in mouse models
- Antibody fragments: off-the-shelf fragment formats, including our proprietary Fab2 antibodies with a dimerization motif in place of the hinge region to improve stability

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