Developing high affinity antibodies as diagnostics

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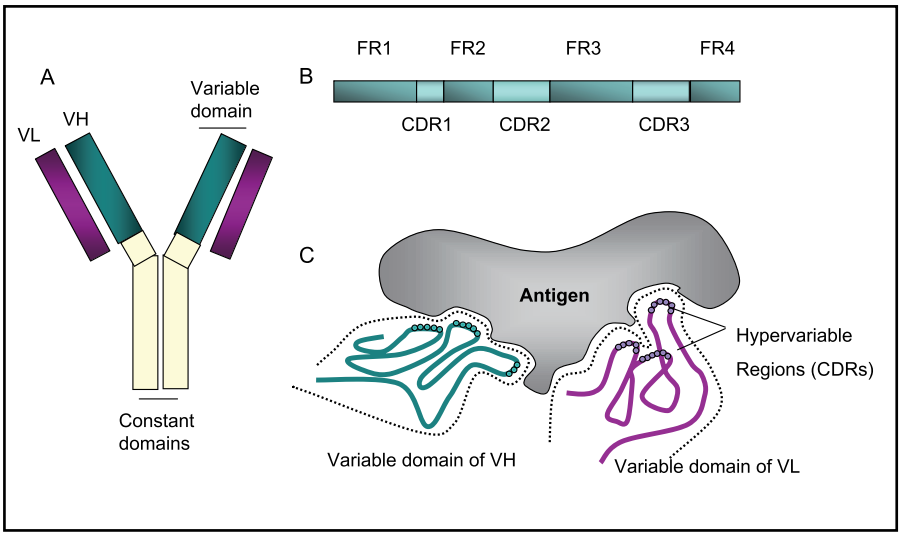
The success of diagnostic reagents in the market today is measured by their level of specificity, affinity and stability. In the case of antibodies which represent approximately 30% of the global diagnostic market, the majority of molecules isolated from naïve or synthetic libraries have low affinities in the micromolar range [1] which are not sufficient for diagnostic applications.

Antibody affinity may be significantly improved by applying directed evolution technologies commonly referred to as affinity maturation. These methodologies are all characterised by several rounds of mutation, display, selection and amplification steps. By increasing affinities into the nanomolar and sub nanomolar range, the reagent becomes a more robust, valuable diagnostic.

Antibody affinity refers to the binding strength of one molecule to another at a single site. A high affinity antibody will either bind its ligand faster, remain bound longer or exhibit both these features. These properties are determined by the association (k_{on} or k_a) and dissociation (k_{off} or k_d) rate constants, which can be measured using a range of technologies including surface plasmon resonance (Biacore), resonant mirror (IAsys), solution based kinetics exclusion assay (KinExA), isothermal titration calorimetry or fluorescence-polarisation. The equilibrium constant, KD, is then calculated as a function of k_{on} and k_{off}.

Figure 1. Schematic representation of an antibody (A) consisting of two heavy (VH) and light (VL) chains called the variable domain and a constant domain. Each variable domain comprises framework regions (FR) and hypervariable loops (CDRs) (B). One of the antigen binding sites (C). CDRs denoted as circles have the majority of contact with the antigen surface.



Antibodies are typically depicted as 'Y' shaped molecules, consisting of two identical heavy (VH) chains and two light (VL) chains which are joined by disulfide bonds. The forked part of the Y shape is involved in binding the antigen. These protein domains are characterised by great sequence variability in complementary determining regions (CDRs) flanked by more constrained framework regions (FR). The stem of the Y comprises the two heavy chains and is referred to as the Fc or constant region as the sequence is conserved. This part of the molecule binds to complement proteins and other receptors but is not involved in binding the antigen.

Altering the structure of the antigen binding site can affect the antibody affinity by changing the number and nature of physical contact points on the antigen (Figure 1). This can be achieved by introducing nucleotide acid changes into the antibody sequence in one or more of the three hypervariable loops (CDRs) of the light (VL) and or heavy chain (VH). The most significant affinity gains often result from mutations in the CDR3 of the heavy chain, whereas mutations in framework residues mostly cause subtle changes in affinity and or antibody solubility and stability [2-5]. However, it must be noted that framework changes in critical regions flanking the CDRs can also affect affinity [6, 7].

A range of *in vitro* mutagenesis strategies can be applied to generate large libraries containing more than 10^{10} different antibody variants. Typically, a random approach is initially used to create a library and subsequently combined with

MICRO-FACT

The leading cause of peptic and gastic ulcers is a microbe, Helicobacter pylori.

other targeted mutagenesis strategies. Many of these technologies rely on PCR based methodologies which introduce either random point mutations into DNA sequences using a low-fidelity DNA polymerase (error prone PCR) or defined mutations at specific locations using pools of engineered primers (site-directed mutagenesis) during the amplification process.

A variation of these technologies, DNA shuffling (sexual PCR), involves digesting mutant libraries of closely related fragments with DNase I and performing a PCR reaction in the absence of primers. An increase in diversity is achieved by hetero-duplexes from different parental genes forming and assembling into full length gene products. Although the probability of DNA fragments from the same parental gene annealing is high, sufficient chimeric forms are generated such that this has become a very widely accepted technology in the field. Furthermore, reaction conditions can be optimised to decrease the formation of useless 'junk' DNA sequences or multimeric sequences containing multiple copies of the reassembled product.

Mutagenesis can be performed on the entire VH or VL sequence, CDRs, frameworks or on specific residues. For example, DNA shuffling can occur between libraries of VH genes or VL genes, or between VH and VL libraries. Similarly, entire heavy and light chains can be sequentially replaced in a process called chain shuffling. In contrast, CDR walking focuses on sequentially modifying individual CDRs of the light and heavy chain and subsequently combines the best candidates. Likewise, window mutagenesis can replace several short contiguous DNA sequences in the CDRs or frameworks either simultaneously or through multiple rounds of mutation using PCR based techniques.

Although such approaches do not require any functional knowledge of individual amino acids in the wild type, specific residues affecting affinity can be determined experimentally using techniques such as alanine scanning or they may be inferred from structural knowledge of the antibody or fragment. Substituting such key residues as well as those naturally occurring in germline hotspots sequences can significantly enhance antibody affinity, with increases of up to ten fold in recombinant antibodies.

Mutant libraries can be displayed in a number of different formats including on the surface of cells (bacteriophage, bacteria, yeast) or *in vitro* (ribosome display, DNA display, mRNA display, *in vitro* compartmentalisation). By performing the mutagenesis and display steps in the same reaction vessel, the diversity generated is retained and large antibody libraries (10^{12} to 10^{13} mutant clones) can be screened. However, selection processes on solid supports (either well or bead formats) can become laborious and intensive unless automated. In addition, selection processes must be stringent to exclude misfolded and non-functional molecules which arise from a cell-free system.

Conversely, using cell surface display methods, the DNA is transferred into cells; consequently, some genetic diversity is lost due to limitations in current transformation and transfection protocols. Nevertheless, whilst library sizes are restricted 10^{6}-10^{8} clones, screening methods are more amenable to high through-put processes such as flow cytometry. Furthermore, yeast produce correctly folded, modified antibody molecules. This is particularly relevant for a diagnostic that relies on such features for accurate recognition.

Developing antibodies with high affinity is crucial for diagnostic purposes. More specifically, an increase in affinity can potentially translate to higher sensitivity as well as decreasing false positives. In addition, antibody stability and solubility which define a robust, commercially viable *in vitro* diagnostic may also be altered by applying the processes discussed here.

References

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Some microbes can grow on refrigerator walls at concentration greater than 10 million per square centimetre.