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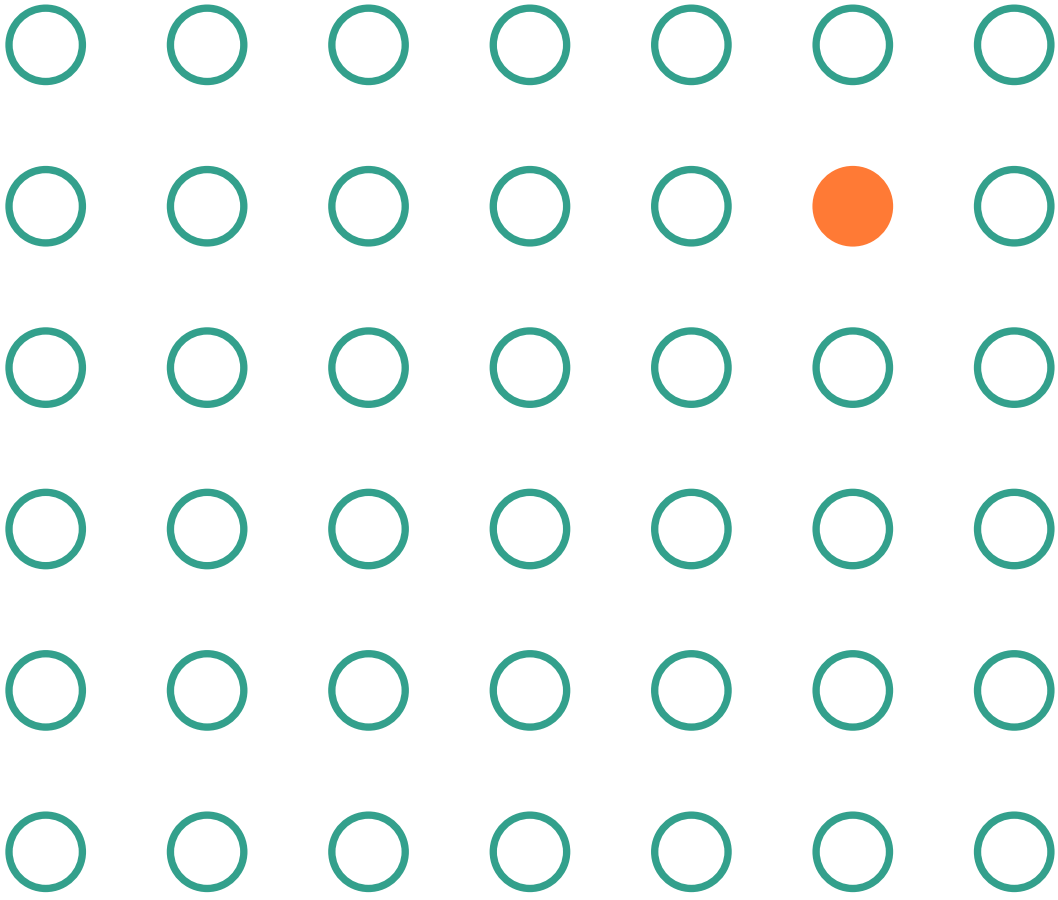
Biacore™ systems in discovery and early-stage development of biotherapeutic antibodies



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Abstract

Advances in antibody use for the treatment of disease has driven demand for new antibody formats designed to improve the efficacy of therapy and reach new targets. Biacore™ systems are extensively used in biotherapeutic antibody discovery and development. Here we discuss their utility at all stages of the process from selection of first candidates to clinical lead. We show that a combination of Biacore™ instruments, software, sensor chips, and kits support the setup of screening and characterization assays and reduces assay development efforts. In screening, antibody capture followed by an antigen injection permits the selection of monophasic and stable binders with preferred kinetics and stoichiometry. Standardized epitope binning procedures ensure reliable determination of epitope specificity. The effect of antibody engineering efforts can be investigated by analysis of antibody binding to antigen and Fcγ-receptors. Developability aspects are also addressed, for instance by ensuring critical binding properties remain unchanged in forced degradation studies. A further advantage of using Biacore™ systems is that antibody concentration and kinetics can be monitored in the presence of nonbinding unfolded fractions, host cell proteins, and other impurities.

Introduction

Over the last 30 years, recombinant proteins including hormones, cytokines, and therapeutic antibodies have been developed for treatment of a variety of diseases including diabetes, cancer, and rheumatic disorders. Recombinant insulin in various forms is probably the most prescribed biotherapeutic medicine. While hormones and cytokines represent an important class of biotherapeutics, antibodies are now more in focus and will eventually have a wider applicability. The number of FDA approved antibodies is steadily increasing (1). Several of these antibodies are directed against the same target molecule, but the target space is growing with approved antibodies now directed to over thirty different target molecules. In 2021 the FDA approved the 100th monoclonal antibody product. (Fig 1). Other modalities of biotherapeutics including antisense oligonucleotides, mRNA- based drugs, and targeted protein degraders are also emerging areas of medicine.

New antibody formats

A large majority of the approved antibodies are full-length antibodies and are of IgG1, IgG2, or IgG4 subclass. However, non-traditional antibody formats are slowly emerging. For example, blinatumomab (Blincyto™, approved 2014) is a bispecific antibody while brentuximab vedotin

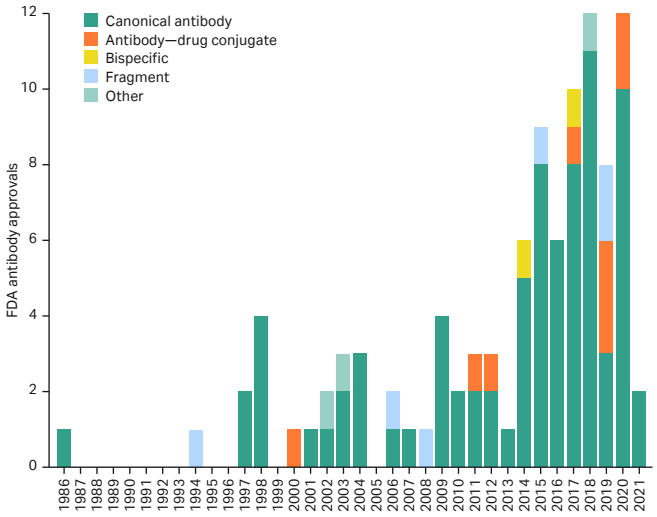


Fig 1. The FDA's first 100 antibody approvals, by year. Sources: The Antibody Society, Nature Reviews Drug Discovery.

(Adcetris™, approved 2011) and ado-trastuzomab emtansine (Kadcyla™, approved 2013) are based on conventional antibodies but are conjugated with cytotoxic agents and are so-called antibody-drug conjugates (ADCs). New formats are introduced to improve the efficacy (2) of therapies and to reach new targets, for instance by designing antibodies that have the capability to cross the blood-brain barrier (3). Antibody formats that retain the basic structure of IgGs may inherit their pharmacokinetic properties (4) but novel constructs that lack the Fc-part of the antibody may have reduced half-life (5). This can be an advantage if the antibody is used for imaging purposes (6). For therapeutic purposes, there needs to be a balance between efficacy and half-life for smaller antibody formats such as single chain Fvs and nanobodies or other scaffolds that have the potential to reach more hidden targets and even to act as intrabodies to target intracellular antigens (7). Several pharmaceutical companies now have bispecific antibodies (8) and antibody-drug conjugates in their clinical pipeline (9) while intracellular antibodies may still be in research phase (Fig 2).

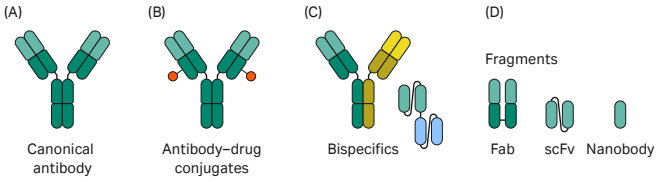


Fig 2. Antibody formats include (A) canonical, (B) antibody–drug conjugates, (C) bispecifics, and (D) fragments. Fragments include antigen- binding fragments (Fabs), single-chain variable region (scFv) constructs, and domain antibodies. Radiolabelled antibodies and antibody–immunotoxins are not shown. These formats can be further subcategorized, and antibodies can span classifications. There are at least 30 different bispecific formats, for example, some of which include fragments. Modified from *Nature Reviews Drug Discovery*.

From antibody generation to clinical lead

The antibody development workflow (Fig 3) has evolved, and early development is no longer focused entirely on potency and functional aspects such as specificity, affinity, and kinetics for their molecular targets. While these factors are crucial, developability aspects play an increasingly important role for reducing the risk of a later failure of the development program (10).

Developability aspects include studies on:

- The impact of post-translational modifications on stability and conformation (11, 12).
- Aggregation and fragmentation tendencies (13).
- Solubility and solution stability (14).
- Biological factors such as immunogenicity (15) and pharmacokinetic properties (16).

Developability studies try to answer questions such as:

- Can the antibody be manufactured?
- Is it safe?
- Will it have acceptable bioavailability and efficacy?

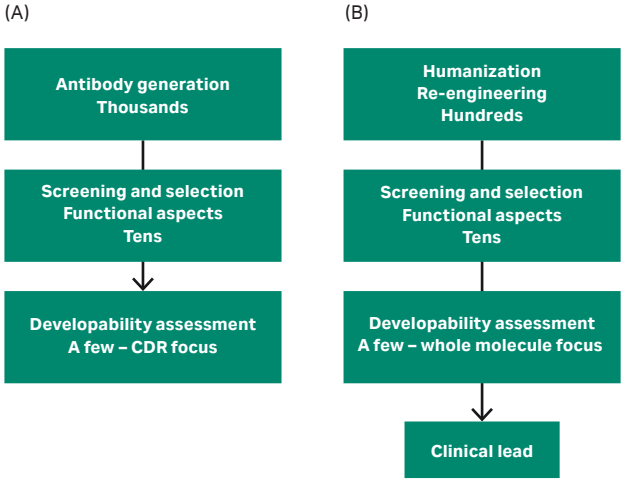


Fig 3. The antibody development workflow. (A) First selection round with focus on antigen binding, and (B) Re-engineering and selection of clinical lead.

Efficiently select and optimize antibody candidates

Biacore™ surface plasmon resonance (SPR) technology was incorporated in the antibody development workflow almost immediately after the launch of the first Biacore™ system in 1990 when kinetic analysis of antibody-antigen interactions (17) and epitope binning procedures (18) were described. Antibody D2E7, that was later to become Humira, the bestselling therapeutic antibody in 2021 (19), was selected from a Biacore™ screen in the mid-1990s (20). From screening of candidates to antibody engineering and final development, Biacore™ systems have consistently been used to determine specificity of binding to characterize antibody-antigen and antibody-Fc receptor interactions and to guide development towards a clinical lead. In developability studies, Biacore™ systems are typically used to monitor effects of forced degradation on antigen and Fc gamma receptor binding and for assessment of pharmacokinetic properties where binding to FcRn is related to antibody half-life. More recently the use of binding mode-specific reagents (21) has been described for detection of changes in antibody topography as a consequence of forced degradation.

Biacore™ systems in antibody development

Depending on the design of the flow system, direct interaction analysis with several target molecules can be performed with a single injection of sample. Typically one measuring spot is used for active analysis and one for referencing (Fig 4). Depending on Biacore™ system, from 1 to 8 active/reference pairs can be used. Independently of the number of active reference pairs, double referencing is typically performed to arrive at high quality data by subtracting blank cycles.

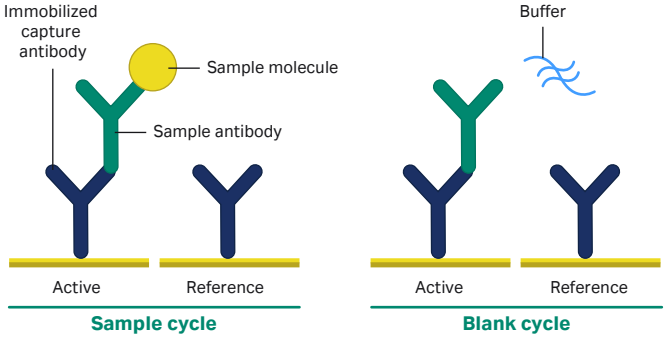


Fig 4. Active and reference measuring spots and their use in sample and blank cycles.

Sample throughput is linked to the number of microwell plates that can be handled in an automated run. Higher throughput systems are particularly well-suited for antibody screening and large-scale epitope binning experiments while other systems have functions that make them ideal for detailed characterization studies. There is a considerable overlap in the systems and those intended for screening are also used for characterization and vice versa.

Minimize time spent on assay development with dedicated sensor chips and reagents

Antibody applications are supported by several sensor chips and reagents provided with ready-to-use protocols enabling rapid assay development using well known and reversible capture formats.

Biacore™ Sensor Chip Protein A, Sensor Chip Protein G, or Sensor Chip Protein L (Fig 5A) can be used directly for antibody concentration measurements or for capture of antibodies and subsequent kinetic analysis of antibody–antigen interactions. Sensor Chip PrismA (Fig 5A) is suitable for antibody concentration measurements but not recommended for kinetic analysis of antibody–antigen interactions. Biacore™ Sensor Chip CM5 (Fig 5B) can be used for direct immobilization and be combined with several antibody capture kits. With the Mouse Antibody Capture Kit, all IgG subclasses, IgM and IgA can be captured to immobilized polyclonal rabbit anti-mouse immunoglobulin. The Human Antibody Capture Kit includes a monoclonal mouse anti-human IgG (Fc) antibody capable of capturing all IgG subclasses. The Human Fab Capture Kit features a mix of monoclonal antibodies and capture Fab through kappa and lambda chains. ScFv antibodies may be captured using protein L or variants thereof (22). In cases where a small antibody fragment, antigen or an Fc-receptor is captured on the sensor surface the His Capture Kit can be used to capture histidine-tagged molecules using a monoclonal anti-histidine antibody. Biotinylated reagents can be captured onto Biacore™ Sensor Chip SA, Sensor Chip NA, or for a reversible biotin-streptavidin interaction, Biotin CAPture Kit (Fig 5C) can be used.

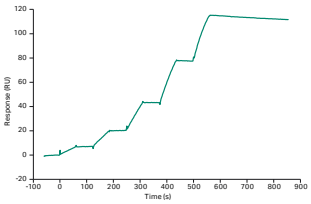
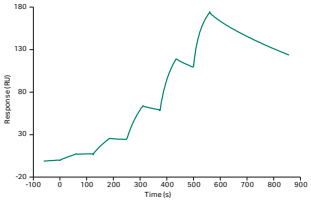
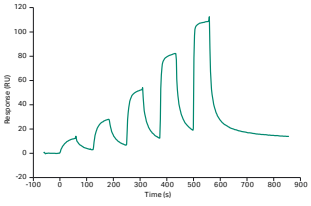
Products	Application example
Sensor Chip Protein A Sensor Chip Protein G Sensor Chip Protein L Sensor Chip PrismA	Antigen binding to captured antibody 
Capture reagents Histidine capture Anti mouse Fc Anti human Fc Anti human Fab Direct immobilization	Antibody binding to histidine captured FcgRI 
Reversible streptavidin Biotin CAPture kit	Antibody binding to biotin captured FcRn 

Fig 5. Biacore™ sensor chips and kits for reversible capture of biomolecules.

Deeper insight into biotherapeutic characteristics with improved efficiency

Early assessment of expression levels, target specificity, and binding stability is essential for clone selection. The number of samples from hybridoma cells or recombinant expression systems varies greatly but may range up to thousands. A multi-step approach is often applied as shown in Figure 6.

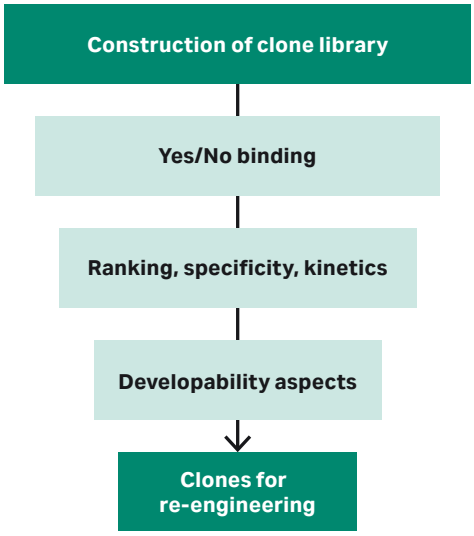


Fig 6. A stepwise approach to antibody selection. The clone library is analysed to allow selection of candidates for re-engineering.

During these stages, samples are often crude and of limited volume. ELISA based methods are often used in screening and provide end-point results. Biacore™ systems can monitor and quantitate the entire interaction and samples from cell culture supernatants can be directly injected (23). With the assay setup described in Figure 7, injections for capture of antibody and antigen binding are made in sequence (Fig 7A). The antibody capture step provides information related to the expression level/concentration of the antibody and the second injection provides information on the rate and stability of the interaction with antigen. The antigen binding level may provide stoichiometric information and a closer inspection of the buffer flow phase may reveal whether the interaction is monophasic or biphasic (Fig 7B).

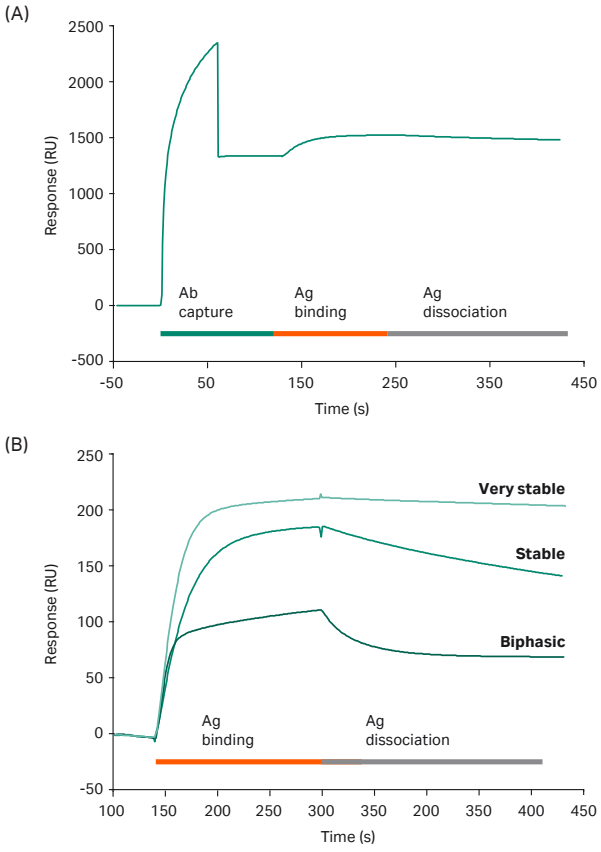


Fig 7. Screening data. (A) Assay setup with capture of antibody from media and injection of antigen, and (B) Comparison of different antigen binding profiles for identification of stable, very stable, and biphasic binding properties.

With hundreds to thousands of sensorgrams, it may be convenient to condense the result into plots of report points (Fig 8). In Figure 8A, the position of two regions: stability early and stability late are highlighted in the sensorgrams. In Figure 8B, a plot of the response levels associated with these regions are used to rapidly identify binders with slow off-rate. In this way, Biacore™ systems are capable of yielding high-content information with single injections of antibody and antigen. Data analysis is straightforward and can be focused directly to the desired binding properties.

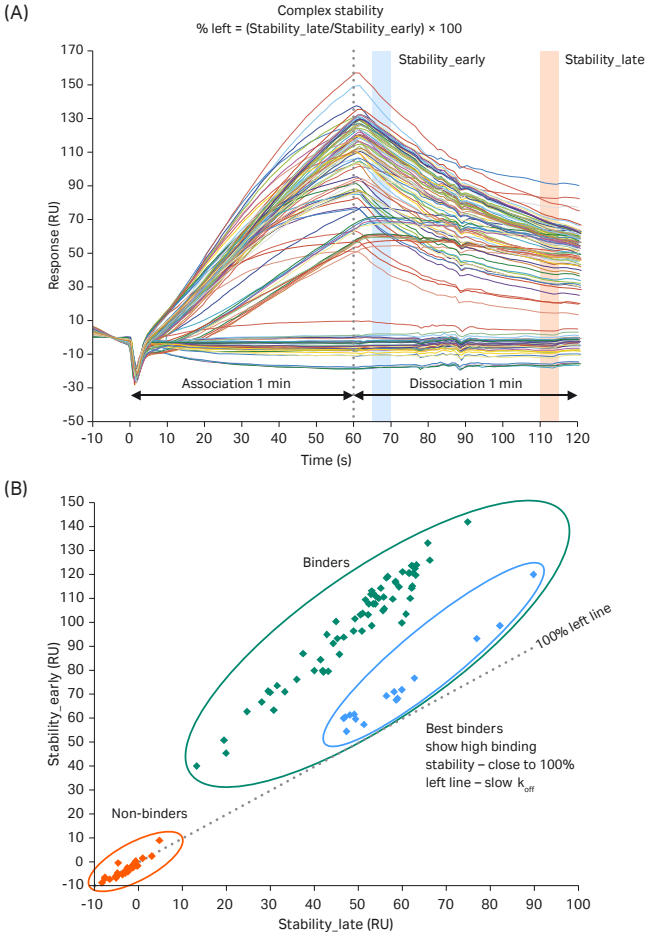


Fig 8. Sensorgram and report point analysis. (A) Antigen binding to antibody is presented in overlay plots. Two regions: stability early and stability late are indicated. (B) Response levels associated with the stability early region is plotted versus the response level from the stability late region. If the values are identical no dissociation has occurred, and the most stable binders are found close to the 100% left line.

Epitope binning for selection of appropriate specificities

Antigen binding may be the first selection criterion, but this has to be complemented with knowledge about epitope specificity (24) and whether the antibodies are capable of asserting the desired biological effect. Epitope binning experiments serve to identify and bin together antibodies with similar epitope specificity. Epitope binning experiments (25) are easily performed on Biacore™ systems by testing the selected antibodies against each other in a combinatorial setup. The experimental design includes capture of the first antibody, blocking of free antibody binding sites, injection of antigen, and finally injection of the second antibody. The assay setup and a corresponding sensorgram are schematically shown in Figures 8A and 8B and the result matrix from an eight-by-eight mapping experiment is shown in Figure 9C (39).

The result matrix is based on user-defined thresholds for antibody and antigen binding. When both antibodies can bind and no blocking occurs between them, the cells are white. Red cells indicate that antibodies share the same or have overlapping epitopes. In cases where the binding responses are too low or antigen dissociates too rapidly from the first antibody, mapping information may be uncertain and this is shown as yellow cells. These pairs can be revisited in a second experiment using longer injection times, or if antigen dissociates rapidly, the ***Dual*** command can be used to inject the second antibody directly after the antigen injection with zero dissociation time. When binding of an antibody is blocked only in one direction, i.e., when antibody A blocks binding of antibody B but not the other way around, their interaction is said to be uni-directional. Uni-directional blocking is illustrated with arrows in the affected cells in the heat map. In this way it is possible to obtain high-resolution maps. Antibodies with shared or overlapping epitopes may be candidates for therapeutic use provided they exhibit the expected biological effect while antibodies directed towards different epitopes may be candidates for a sandwich assay. To identify epitopes more precisely, inhibition mapping (18) can be performed by pre-incubating the antibody with antigen domains or antigen-derived peptides and observing whether these constructs inhibit antibody binding to the antigen.

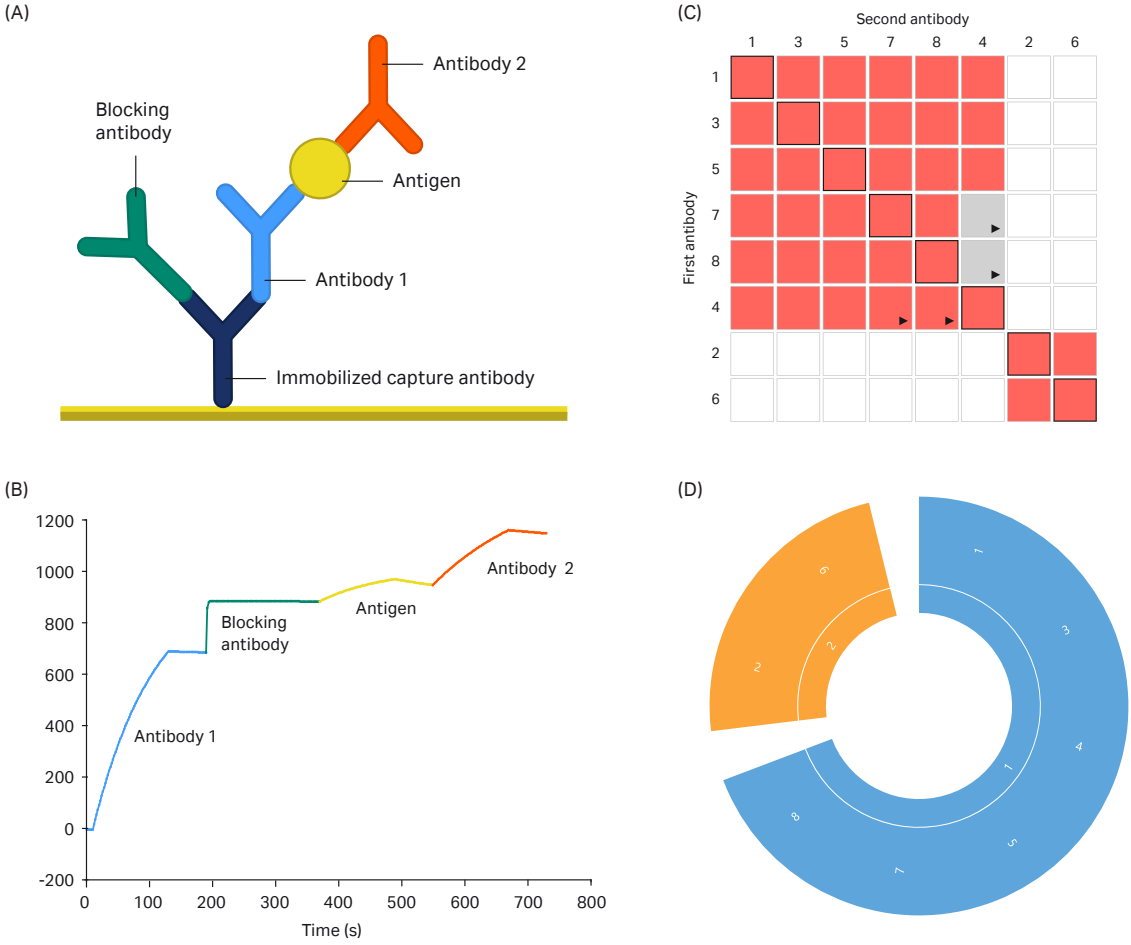


Fig 9. Epitope binning. (A) Binding sequence and molecular events, (B) sensorgram events, and (C, D) results from epitope binning experiment evaluated with Biacore™ Insight Software and Biacore™ Insight Epitope Binning Extension. The binning results from the heat map (C) is represented in a bin chart (D). Two well defined bins are identified for the eight antibodies.

Kinetic analysis for improved understanding of drug activity

Kinetic data resolves the affinity of an interaction and provides information about the rate and stability of binding. The affinity constant, K_D, is separated into an association rate constant, k_a, and a dissociation rate constant, k_d, where $K_D = k_d/k_a$

Kinetic data introduces the time domain and allows a better understanding of target occupancy and drug residence time (26, 27). It may also contribute to the understanding of off-target effects. During humanization and re-engineering efforts, it provides detailed information on how structural changes affect binding properties. Engineering efforts may involve antigen sites, Fc-receptor binding sites for improved (28–30) or reduced (31) receptor binding, T-cell epitopes (15), introduction of linker positions for antibody drug conjugates (32), and even more complex tasks if the antibody is to be developed into a bi- or multi-specific antibody (8). Kinetics is also used in developability assays where the effects of forced degradation on binding properties (11, 12, 20) can be studied and binding to the neonatal Fc-receptor can be used to estimate antibody half-life (16). Thermodynamic analysis has also been suggested as a tool to assess developability. A screening approach to thermodynamics has been described (33) where a favorable ratio of association rate constants determined at 13°C and 37°C was indicative of the binding specificity. Biacore™ 8K and Biacore™ 8K+, open up possibilities to perform kinetic and concentration analysis in parallel (Fig 10).

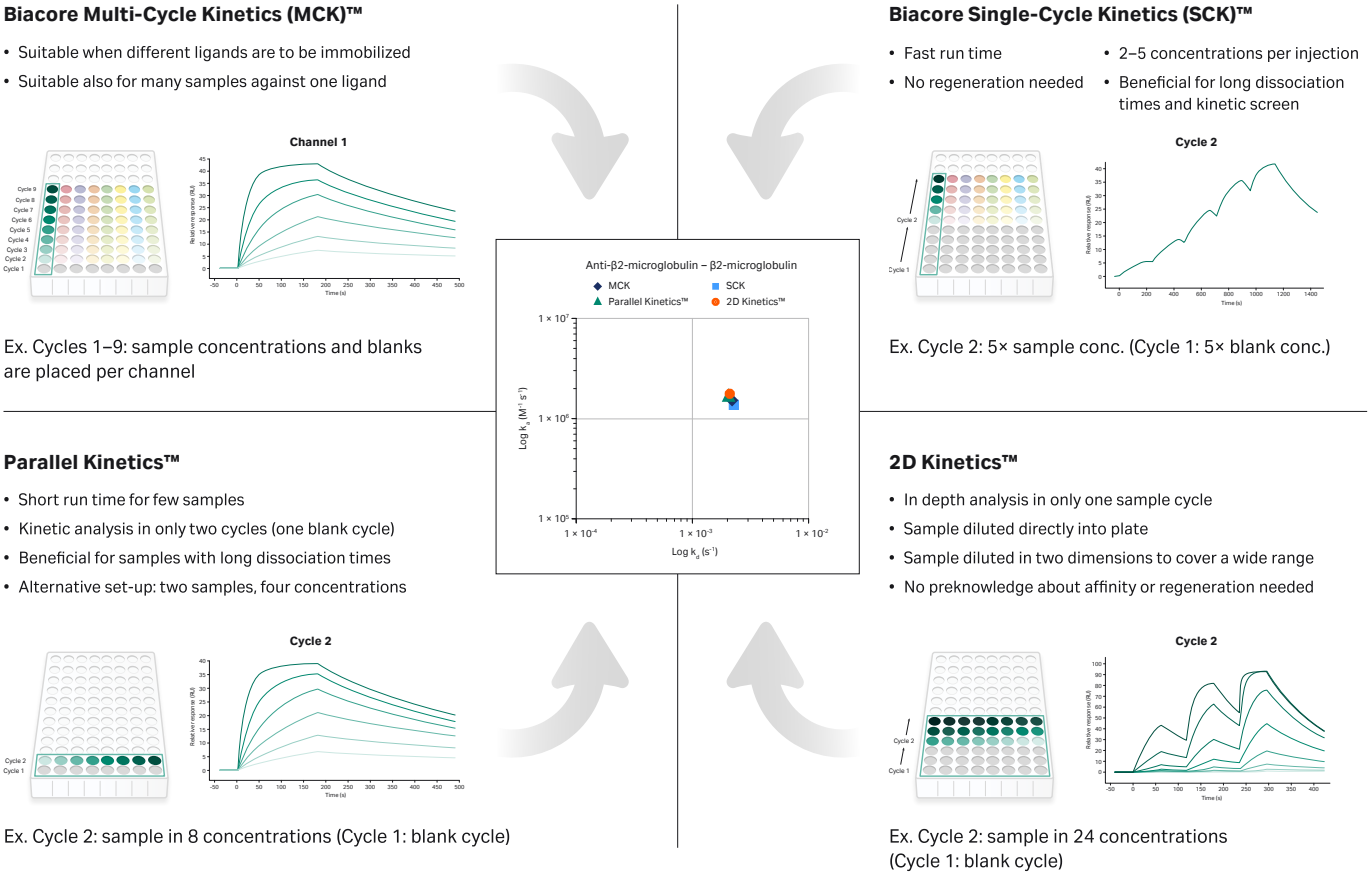


Fig 10. Depending on the preferences and circumstances of each assay, kinetic analysis can be performed in four different ways. The 2D Kinetics™ functionality offers detailed kinetics from only one sample cycle, thereby significantly reducing assay development time.

Kinetic analysis formats for rapid and informative selection of candidates

Antigen typically binds to antibodies with association rate constants in the range from 5×10^4 M^{-1} s^{-1} to 5×10^6 M^{-1} s^{-1} and with dissociation rate constants ranging from $1 \times 10^{-3} s^{-1}$ to $1 \times 10^{-5} s^{-1}$ as illustrated in the k_a vs k_d plot in Figure 11.

To illustrate possible outcomes of kinetic experiments, the rate constants associated with points A to E were used to simulate sensorgrams using varying antigen concentrations and varying injection and dissociation times. The antigen was assumed to bind antibody according to a 1:1 interaction model. The resulting scenarios illustrate different screening and characterization approaches (Fig 12).

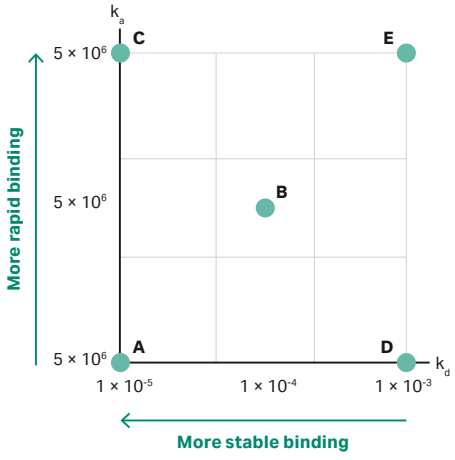


Fig 11. k_a vs k_d plot for typical antibody-antigen interactions. Response curves for conditions A to E are shown in Figure 12.

The top row shows sensorgrams based on a single injection of antigen at 10 nM concentration. Injection and dissociation times were 30 s and the maximum binding level is indicated by the red horizontal line. Binding is clearly observed for conditions B, C, and E but none of the sensorgrams reach the saturation level. The column to the right is based on normalized data and allows a direct comparison of relative dissociation rates. With a 30 s dissociation phase and low binding levels the resolution of off-rates is poor. As the concentration of antigen is increased tenfold to 100 nM binding associated with all conditions (A to E) is readily observed but again the resolution of off-rates is poor. With two injections of antigen at 10 and 100 nM performed in Biacore Single-Cycle Kinetics (SCK)™ mode the response levels for conditions A and D are getting higher and as the dissociation time is now 60 s, the resolution of off-rates is starting to improve. When the experiment is extended to four antigen injections and antigen is injected from 7.7 to 200 nM (3-fold dilutions) for 90 s, binding curves become more suitable for detailed kinetic analysis. Note that with a dissociation phase of 480 s, it is easy to differentiate between k_ds of $10^{-3} s^{-1}$ and $10^{-4} s^{-1}$ and it starts to be possible to differentiate kds between $10^{-4} s^{-1}$ and $10^{-5} s^{-1}$. By extending injection and dissociation times and increasing the concentration range, more detailed kinetic data is obtained. For practical reasons high-resolution k_d analysis will typically be restricted to a few selected samples. It will take at least 2.9 h to monitor a 10% decrease in the signal level for a dissociation rate constant of 1×10^{-5} s^{-1}. The corresponding kinetic half-life is 19.3 h.

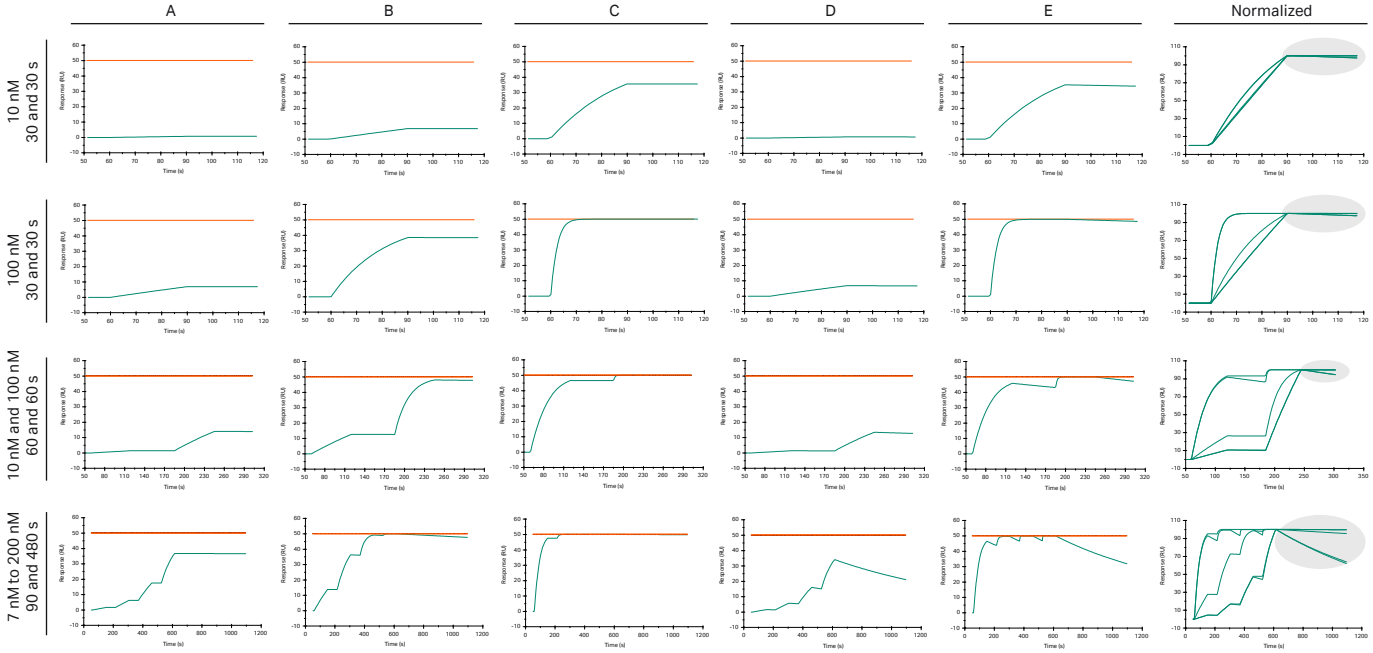


Fig 12. Antibody screening and characterization scenarios. Antigen is injected at various concentrations and for varying time as indicated to the left. Conditions A to E correspond to rate constants defined in Figure 11. The red horizontal line in A to E plots indicates the saturation response. The normalized plots were obtained by re-scaling sensorgrams on a % scale and are intended for comparison of off-rates as indicated by the gray ovals.

These scenarios demonstrate how screening with one low antigen concentration can be used to select rapid binders with slow off-rates. Using injection and dissociation times of 30 s will identify slow binders and antibodies from which antigen dissociates rapidly. These antibodies can be deselected. Remaining antibodies can be re-tested using the same antigen concentration injected for a longer time (90 s) and with a longer dissociation time (10 min) to allow better differentiation of off rates. A few remaining candidates may then be characterized in more detail using a series of antigen concentrations and a further extended dissociation time.

Early indication of cell- or antibody-mediated cytotoxicity effect

Fcγ-receptors interact with IgG to regulate the immune response with implications for several disease states (34). From a therapeutic antibody perspective, binding of IgG to Fcγ-receptors may be of particular interest for anti-cancer antibodies where FcγIII-receptors on natural killer cells have been implicated in antibody-dependent cell-mediated cytotoxicity (34). IgG binds to Fcγ-receptors with varying affinity. In contrast to antibody-antigen interactions the medium- to high-affinity interactions seen with Fcγ-receptors I and III are often complex and cannot readily be fitted to a simple interaction model (35).

The complexity most likely arises from heterogeneity in glycosylation that is found in both antibodies and receptors. Antibody binding to FcγIII-receptors is directly linked to efficacy and are important to characterize binding events. Two main approaches to Fcγ-receptor analysis have been established (35, 36). In one approach, histidine tagged Fcγ-receptor is captured by an anti-histidine antibody and antibody is injected (Fig 13A). In the second approach a Protein A surface is used. Antibody is captured and Fcγ receptor is injected (Fig 13B).

Both assay types are easy to set up, avoid covalent attachment of antibody or receptor, and regeneration of sensor surfaces is provided as part of the capture protocols. Note that the two approaches reflect heterogeneity in different ways as the orientation of molecules differ. The shape of the sensorgrams can therefore not be expected to be identical. Kinetic analyses of these interactions are challenging due to pronounced heterogeneity (35).

Better prediction of candidate efficacy

Antibody clearance and half-life is partly determined by its interactions with the neonatal Fc-receptor, FcRn. Antibodies that are taken up into the endosome and directed for degradation in lysozomes can be salvaged from degradation and recirculated by binding to FcRn. Binding to FcRn in the endosome takes place at pH 6.0 and the antibody is released at pH 7.4 when it is returned to the cell surface (37). Increased half-life is of interest from an efficacy and dosage perspective and part of antibody engineering projects.

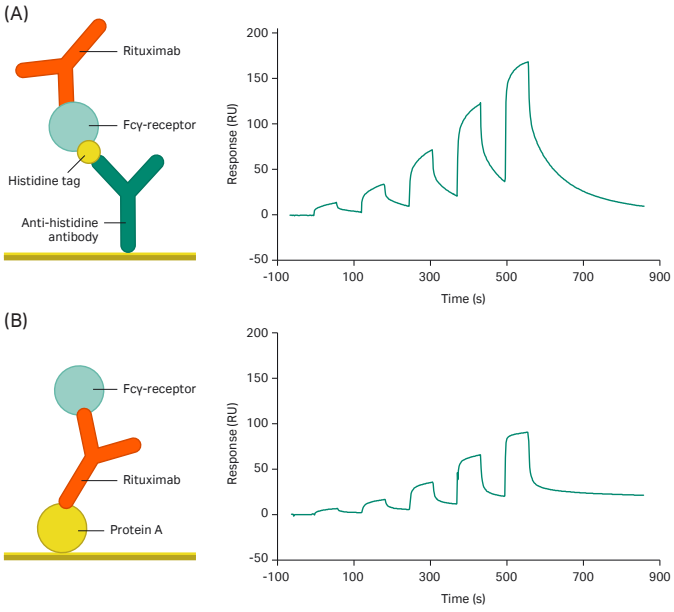


Fig 13. Antibody- Fcγ-receptor interactions are studied using either (A) histidine tagged receptor captured to an anti-histidine antibody or (B) antibody captured to protein A. The sensorgrams show the interaction between Rituximab and Fcγ-receptor IIIa$_{Val\ 158}$.

The pH-dependence of antibody-FcRn interactions is easily studied in Biacore™ systems (Fig 14). Biotinylated FcRn is captured to streptavidin using the Biotin CAPture Kit. The running buffer is maintained at pH 6.0. Antibody is injected at pH 6.0 using ***Dual*** command. It binds to the receptor and when the antibody injection stops it is immediately followed by injection of buffer at pH 7.4 to monitor dissociation at this pH. With this procedure it is possible to seamlessly establish relevant conditions for both binding and release of antibody from FcRn. Note that the solutions for ***Dual*** command can be selected and used to introduce new buffer conditions while the running buffer remains the same.

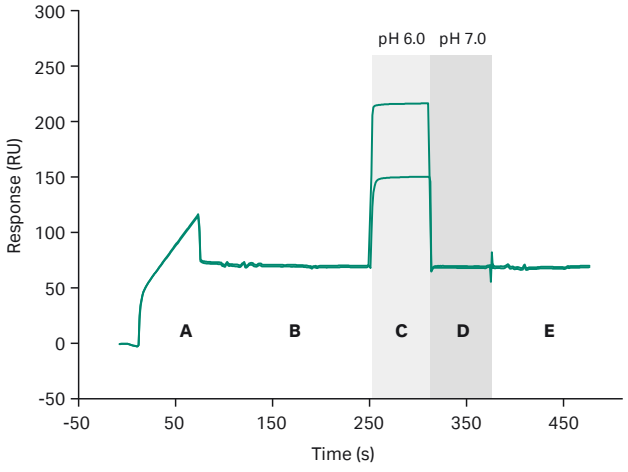


Fig 14. Binding of infliximab to hFcRn. (A) Capture of biotinylated hFcRn, (B) running buffer PBS 6.0, (C) and (D) ***Dual*** command phase. (C) Infliximab injected at 333 and 2000 nM at pH 6.0. (D) Injection of pH 7.4 buffer for dissociation of infliximab. (E) Return to running buffer conditions.

Conclusion

As early and late-stage antibody development processes become more integrated (38), analytical technologies that guide developers and support decision making become key tools in these workflows. Biacore™ systems deliver the resolution, sensitivity, precision, throughput, ease-of use, and assay versatility required to develop a first candidate into a clinical lead with the critical quality binding attributes required for approved biologics.

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