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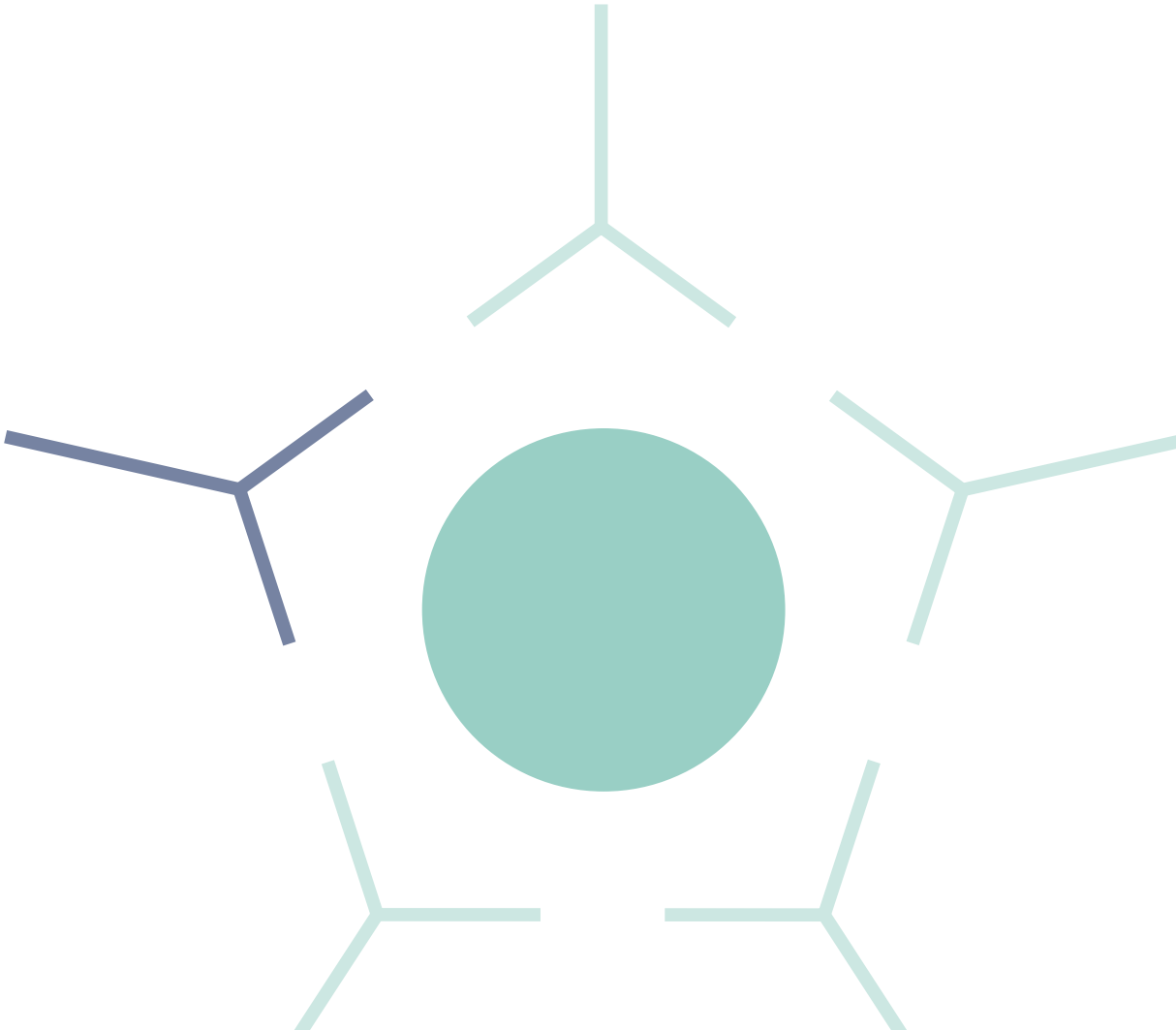
Biacore systems for immunogenicity testing



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Introduction

Immunogenicity is caused when biotherapeutics result in unwanted immune responses when administered to the patient. Immunogenicity is an important aspect to consider in drug development of new protein biotherapeutics as it can affect drug safety and efficacy.

Rigorous assays are needed to rapidly and accurately detect antibodies against the drug in question in patient samples. According to the FDA, the immunogenicity assay should detect antibodies of IgM and IgG with a recommended sensitivity of 100 ng/mL (1). The cutoff point of the initial screening should be set to allow for 5% false positives and should be followed by a confirmatory assay.

After identification and confirmation of positive samples, a full characterization of anti-drug antibodies (ADAs) should be performed. This should include:

- assessment of isotype (class or subclass)
- binding stability
- epitope specificity
- neutralizing capacity

These steps in characterization together give valuable information of the nature of the studied immune response.

This article provides examples of how label-free biophysical binding assays are successfully employed in all steps in the immunogenicity workflow to ensure confident detection, confirmation, and comprehensive characterization of immune responses (Fig 1). The benefits of using Biacore™ T200 or Biacore 8K series systems and surface plasmon resonance (SPR) technology compared to equilibrium-based assays is discussed.

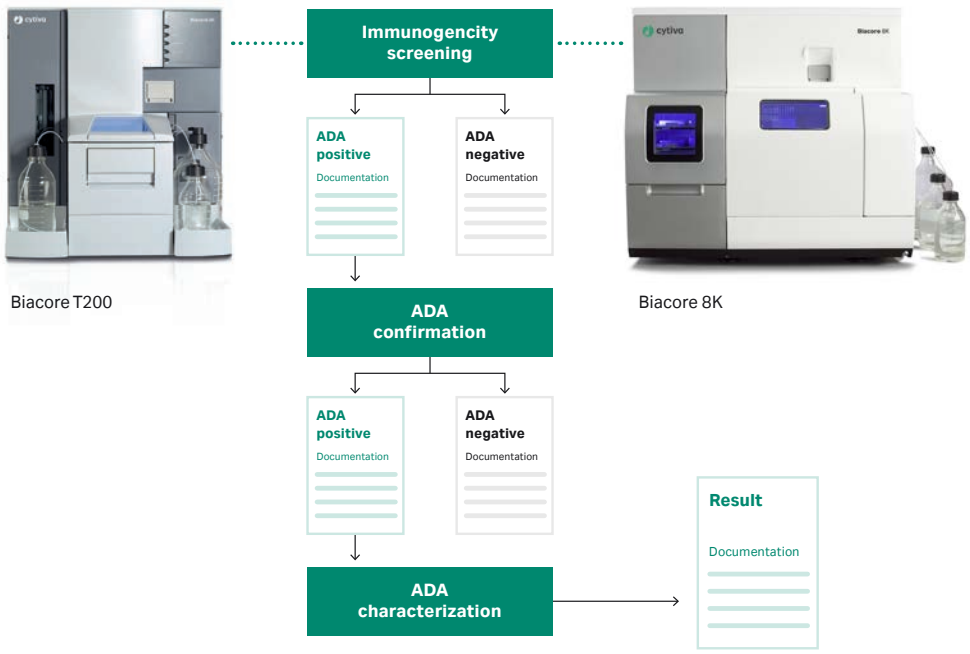

Fig 1. A typical multistep process for immunogenicity testing comprises a screening assay for identification of positive samples, confirmation of any positive samples, and sometimes further characterization of confirmed positives. The extent of testing varies with indication, type of drug, and preclinical/clinical phase. Biacore systems give valuable information throughout the entire workflow.

Proven sensitivity and detection of both low- and high-affinity antibodies

The usefulness of Biacore systems as a screening tool for ADA in patient samples has been demonstrated in several studies (2–5), each reporting levels of detection on par with the FDA-recommended sensitivity. Mytych *et al.* validated a Biacore 3000 screening assay that detects ADAs against darbepoetin alfa and epoetin alfa down to 100 ng/mL and 80 ng/mL, respectively (2, 4).

The strength of Biacore assays to detect low-affinity ADAs was shown by Lofgren *et al.* (6). They compared a bridging enzyme-linked immunosorbent assay (ELISA) to a Biacore assay for immunogenicity evaluation of the fully human panitumumab, a monoclonal antibody (mAb) that binds to the epidermal growth factor receptor (EGFR). The Biacore assay was considerably more sensitive for detection of low-affinity ADAs. In samples from the clinical trial, more positives were detected with the Biacore assay as compared to the ELISA assay. The Biacore assay identified eight samples with neutralizing antibodies (NAbs); these positive samples were missed with the ELISA assay.

The performance of SPR has also been compared to a bridging electrochemiluminescence (ECL) assay, where some antibodies were only detectable using the direct-binding approach used by SPR and biolayer interferometry (BLI). The improved performance of the Biacore assays was possibly due to the necessity of the ADA to bind two different molecules of the drug simultaneously to generate a signal (7–8). In addition, the low-affinity ADA might be more easily detectable in a direct binding assay due to avidity.

Boehringer Ingelheim compared a Biacore assay to a bridging ELISA assay in a clinical phase I multidose study in patients with a therapeutic humanized Ab. The results showed that Biacore T100 (now replaced by Biacore T200) assays detected positive samples much earlier than the ELISA assay (Fig 2). These early immune responses typically involve ADAs with low affinity for the drug with fast on/off binding kinetics. Although the ELISA assay had higher sensitivity, the importance of detection of both early and mature immune response made Boehringer Ingelheim implement Biacore assays as their immunogenicity screening method.

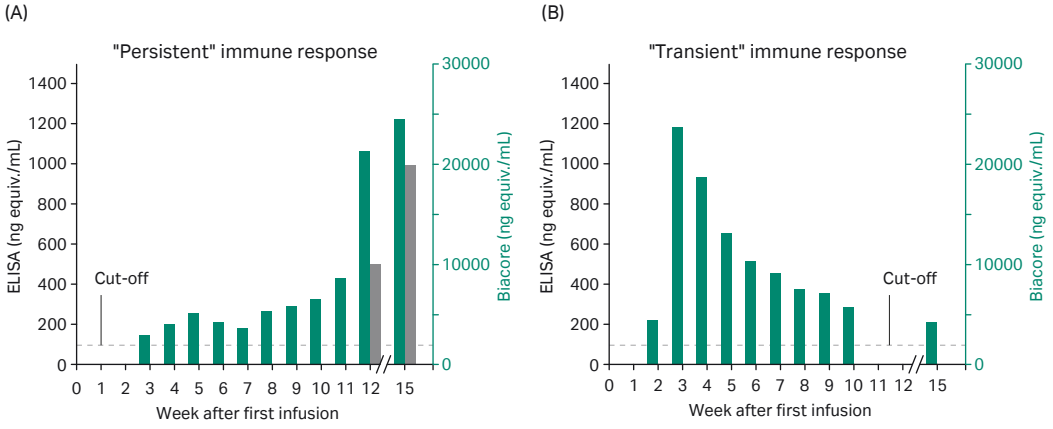

Fig 2. The optimized Biacore assay detected both “persistent” and”transient” responses 2–3 wk after the first infusion (green bars), while the bridging ELISA assay detected “persistent” responses after 12 wk (gray bars). “Transient” immune response was not detected at all by the bridging ELISA assay.

Table 1. Number of positive samples analyzed by ELISA and Biacore assays from a variety of therapeutics administered in clinical studies

Drug	No. of positives (ELISA assay)	No. of positives (Biacore assay)
Iodine 131 chimeric tumor necrosis mAb (11)	4 of 78	7 of 78
Biotherapeutic drug, Merck Serono (Presentation, Biologics Munich 2011)	19 of 62	25 of 62
Panitimumab (6)	2 of 612 (1 neutralizing)	25 of 604 (8 neutralizing)
Recombinant human erythropoietin (9)	6 of 8	8 of 8

Comparisons between bridging ELISA assays and Biacore immunogenicity assays in clinical samples are summarized in Table 1. In all cases, Biacore assays detected more positive samples than the ELISA assays. A likely reason for this could be that low-affinity, fast-dissociating ADAs and IgG4 are detected using Biacore assays.

The clinical relevance of ADAs detected with Biacore assays but missed with ELISA is shown in a study by Swanson *et al.* (9). Samples from eight patients with antibody (Ab)-mediated pure red-cell aplasia were detected positive with a Biacore assay while the ELISA failed to detect two of them.

Nechansky *et al.* also observed that Biacore assays detected significantly higher number of ADA cases and concluded that SPR is the method of choice, mainly due to the ability to detect low-affinity ADAs that risk maturing into higher affinities, but also for quantitative data such as on and off-rates and isotype determination (10).

Automated screening of ADAs in presence of drug

Drug interference is a major challenge for all immunogenicity assays, especially those for therapeutic mAbs, which are often administered at rather high doses and possess a long half-life. Drug present in samples binds to ADAs and prevents them from binding to the immobilized drug, thereby generating false negatives. Biacore T200 addresses this issue by enabling automated acidification and measurement of ADAs in the presence of excess amounts of drug. The samples are acidified to allow drug-ADA complexes to dissociate, and then neutralized using the Merge inject just before measurement to avoid reforming of complexes. The advantages with automated acidification are that the samples need to be acidic for a short time only and that the acidification time is constant for all samples.

The acid-dissociation strategy enables assays with recommended sensitivity also in the presence of drug. The effectiveness of this strategy to mitigate drug interference was demonstrated by a research group in Birmingham (12). A fixed concentration of anti-rituximab Abs was mixed with increasing amounts of drug (rituximab). The samples were analyzed with and without acid treatment. A concentration of 0.5 µg/mL of anti-rituximab was detected in the presence of a 100-fold molar excess of rituximab with acid treatment (Fig 3). In contrast, the binding signal was completely abolished for the corresponding sample without acid dissociation.

Implementation of the acid-dissociation strategy in a Biacore T100 instrument equipped with Immunogenicity Package revealed anti-rituximab in samples that had earlier been masked by rituximab. The acidification also showed the presence of rituximab in samples which was not detected without acidification.

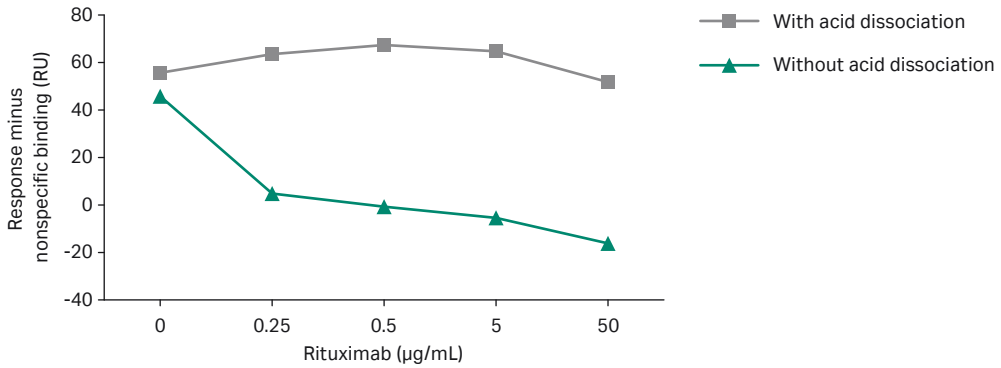

Fig 3. Detection of 0.5 µg/mL of anti-rituximab in the presence of different concentrations of rituximab with (gray) and without (green) acid dissociation strategy.

At Immunogenicity for Biologics in Munich 2011, Dr. Kramer from Merck Serono presented results from an automated Biacore assay based on the acid-dissociation strategy and compared with acid-dissociation strategy assays based on ELISA. Clinical samples were analyzed using the two methods and compared—the same positives were generally found in both assays (Table 2). The Biacore T100 assay, however, found more positives and the likely explanation for this behavior is that the Biacore assay detects also ADAs with lower affinities that are lost in the ELISA washing steps.

Table 2. Detection of ADAs in patient samples

	Patient A		Patient B		Patient C		Patient F	
Sampling time	**Biacore assay**	**ELISA**	**Biacore assay**	**ELISA**	**Biacore assay**	**ELISA**	**Biacore assay**	**ELISA**
Before								
168 h							•	
240 h							•	
312 h					•		•	
480 h							•	
648 h	•	•		•	•	•	•	•
816 h	•	•	•	•	•	•	•	•
984 h	•	•	•	•	•	•	•	•

Merck Serono found that the Biacore T100 assay offers valuable automation, reducing labor costs and risk for errors which is a great advantage when assays are transferred to contract research organization (CROs). The ELISA assay used was cumbersome with many manual pipetting steps and required three days to generate results (Fig 4). The Biacore T100 assay on the other hand required minimum sample preparation. The fewer manual steps gave the Biacore assay better precision compared to the ELISA assays (Table 3). The conclusion from Dr. Kramer was that SPR using Biacore systems is an excellent technology for acid-dissociation assays, and screening assays using acid dissociation on Biacore systems were being implemented in several projects at Merck Serono.

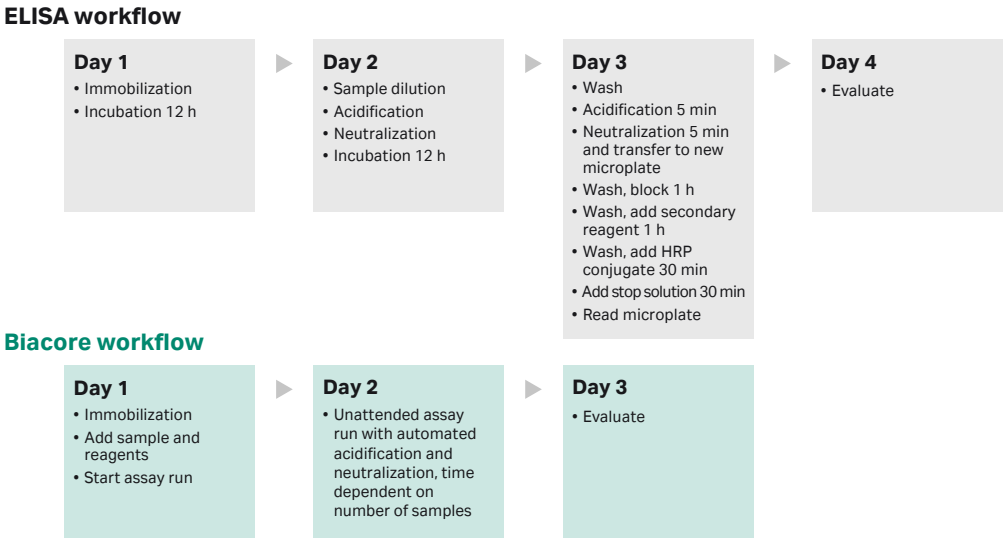

Fig 4. Workflows for Biacore and ELISA ADA assays.

Table 3. Interbatch precision comparison between assays with acid dissociation. Courtesy of Merck Serono

	ELISA, variation in percent	Biacore assay, variation in percent
Low positive control	26.4	11.5
High positive control	26.0	14.7

Elimination of false positives with a confirmation assay

A drug-depletion assay is often used to confirm that the positive response comes from ADAs that specifically bind to the drug and not from interactions with other serum components. Confirmation assays are easily set up in Biacore systems. Inhibition of the response by adding excess of the drug to the sample confirms that the response derives from specific binding to the drug on the sensor surface (Fig 5). The whole procedure can be automated.

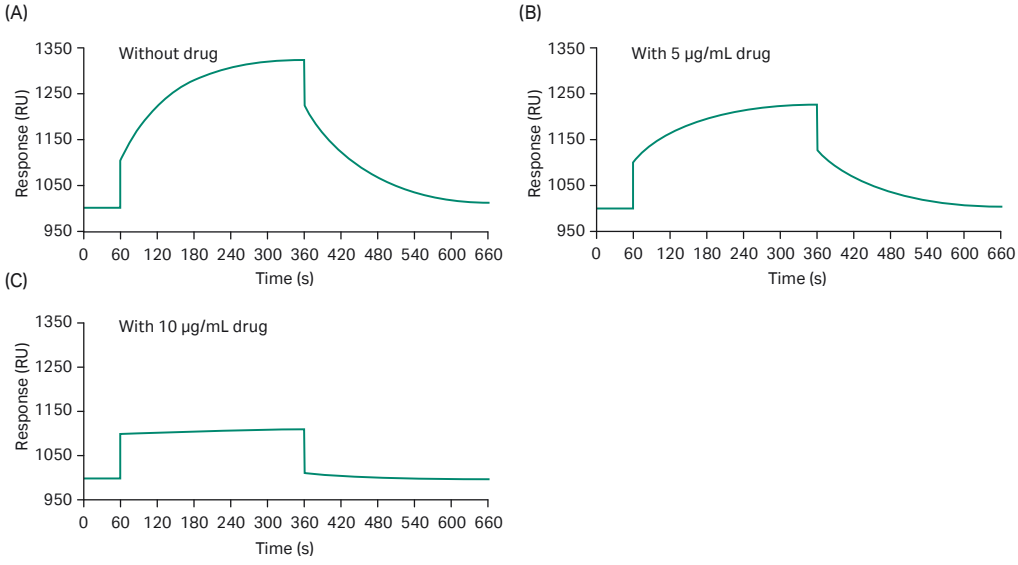

Fig 5. Confirmatory assays using Biacore systems: (A) without drug; (B) with 5 µg/mL drug; and (C) with 10 µg/mL drug.

Detection of IgG4

As stated by the FDA, an immunogenicity assay should be able to detect all IgG isotypes (1). IgG4, a major isotype of ADAs developed to therapeutic mAbs (9, 13) undergoes random exchange of half Ab (Fig 6A). Bispecific ADAs cannot be detected in bridging or homogenous assay formats (Fig 6B) that are often used in ELISA and ECL assays (14). Direct-binding assays such as Biacore assays are therefore more suitable for detecting IgG4, which can constitute a significant portion of the immune response.

Aarden *et al.* have observed that IgG4 is second to IgG1 as the major isotype in ADAs developed for therapeutic mAbs (13). IgG4 have been associated with immune responses generated under conditions of high doses and prolonged exposure to therapeutic proteins (15).

The prevalence of IgG4 has also been demonstrated by Lewis *et al.* to be common in patients generating antibodies against FVIII (16).

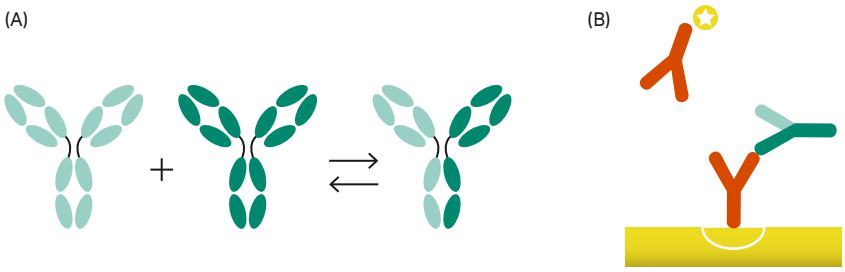

Fig 6. Monovalent ADAs are not detected in bridging ELISA assays. (A) Spontaneous and random exchange between Abs occurs in the human Ab where some have bispecific specificity. (B) Monovalent IgG4 will not be seen in bridging assays, since they depend on the binding of two drug molecules to one ADA.

Determination of ADA isotype

Isotype determination of the ADAs gives information about the immunobiological functions of the ADAs such as antibody Fc receptor binding. In a study by Mytych *et al.*, 12 clinical samples containing serum ADAs against darbepoetin alfa were analyzed for isotypes (4). All samples confirmed positive for a particular Ab isotype. All four major Ab isotypes were detected. The majority of the ADA positive samples were of IgG and IgM type (four subjects each). In addition, three IgA positives and one IgE positive were identified.

Lewis *et al.* showed the possibility of simultaneously characterizing the antibody response in terms of IgG-subclasses in a single experiment (Fig 7) by serial injection of various anti-subclass detection reagents (16). Using multiple anti-subtype detection Abs, the authors managed to characterize the anti-FVIII response in patients and stratify them based on the subtype content of the detected Abs. Since the method detects all anti-FVIII antibodies it can be used to detect an early—but not yet neutralizing—immune response in patients. Biacore 8K series instruments have eight analysis channels that operate in parallel. This enables a separate approach in which separate channels can be used for the different anti-isotype antibodies, which reduces the risk of interference. This also enables easier data interpretation.

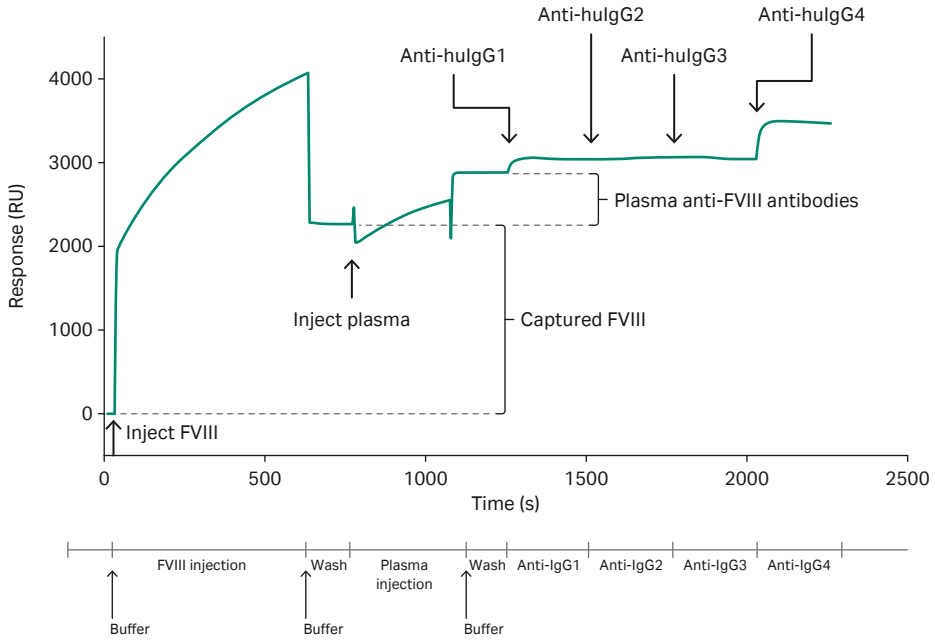

Fig 7. Representative binding curve (sensorgram) characterizing anti-FVIII antibodies in a human plasma sample. From Lewis *et al.* (2013) (16).

Determination of epitope specificity

The determination of epitope specificity of ADAs is important when studying immunogenicity. According to FDA recommendations, the applicant should investigate to which regions the immune responses are generated: "FDA recommends that sponsors direct initial screening and confirmatory tests against the whole therapeutic protein product. For multidomain therapeutic protein products, the sponsor may need to investigate whether the ADA binds to specific clinically relevant domains in the protein. A convenient setup is to immobilize the full-length drug in one flow cell/channel and domains of the drug in the other flow cells/channels (Fig 8). Cross-reactivity can also be checked using the same approach but with different drugs in each of the flow cells."

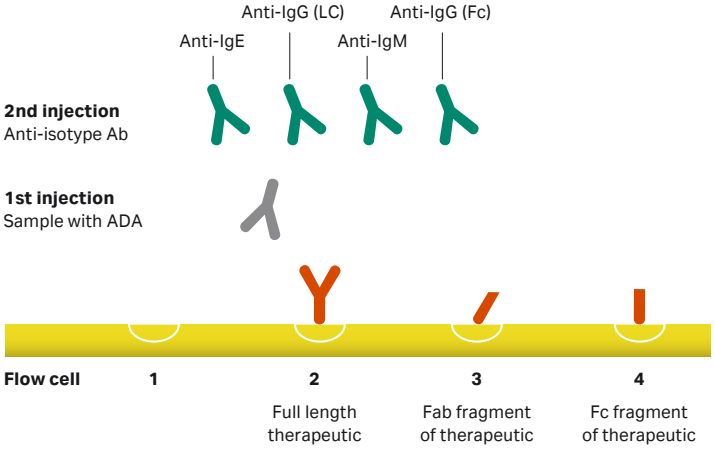

Fig 8. Assay setup for a combined epitope mapping and isotyping experiment.

The work carried out by Stubenrauch *et al*. demonstrates the wealth of information that can be obtained from a few Biacore experiments (17). Utilizing the four flow cells of Biacore 2000 efficiently, a combination of 11 measurements per sample provided a complete immunogenicity profile with response, isotype, specificity, and binding stability of ADAs in clinical samples (Fig 8). This setup can differentiate drug-specific responses from other responses such as IgM rheumatoid factor response against Fc fragments. The time course of specific ADA formation can be followed, which enables patient-specific determination of ADA responses and correlation with clinical events.

Biacore 8K series features eight channels (flow cells) in which separate samples can be analyzed. This is beneficial for all stages of immunogenicity testing where different experimental conditions can be run in parallel. It also saves time as it greatly increases the sample throughput. Several immobilization levels can be assessed simultaneously to aid in assay optimization, saving valuable time for analysis. Multiple drugs or drug domains can be immobilized on the same sensor chip to address antibody epitope specificity.

Assessing ADA binding stability

Generally, in biomolecular interaction experiments, the dissociation rate is an approximate indicator of the binding affinity, and Ab maturation towards higher binding affinity is often reflected in slower dissociation rates. Biacore systems enable monitoring of ADA maturation via assessment of Ab isotype and binding stability. The ADA population in positive clinical samples can be characterized in terms of the stability of binding to the drug on the surface (Fig 9). Assessing affinity or dissociation rates for ADAs is difficult since the Ab populations are bivalent and heterogeneous. Biacore T200 software offers tools that can be used to characterize the immune response in terms of fractions or populations with rapid and slow dissociation rates.

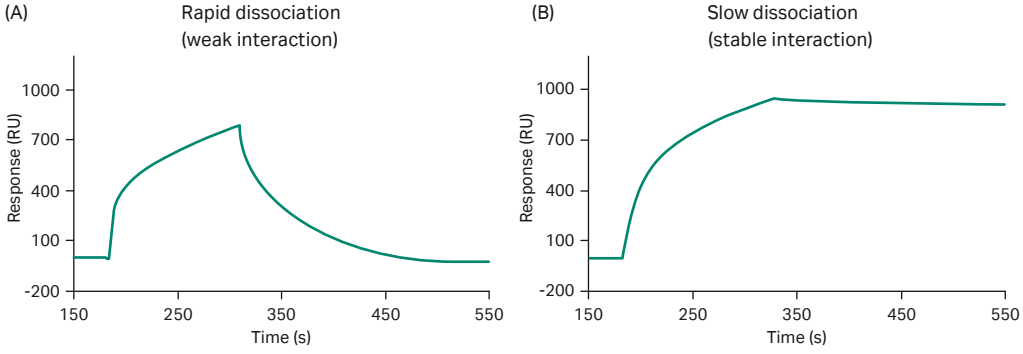

Fig 9. Biacore characterization of binding stability of ADAs from positive clinical samples to immobilized drug. Immune response maturation often leads to slower dissociation rates (lower picture).

Mytych *et al.* (4) described an alternative approach to assess the dissociation rates of ADAs in clinical samples. The response of the ADAs before and after 40 min dissociation was determined and the percentage loss calculated. The six clinical samples lost between 68% and 89% of binding after 40 min, whereas the high-affinity positive control had little dissociation. Gibbs *et al.* used a similar approach and found that patients with sustained antibody responses also had the slowest dissociation kinetics (18). These observations could only have been made with real-time monitoring of binding responses.

Competitive ligand-binding assays without labeling

As part of the characterization of ADAs, samples confirmed as positives in immunogenicity testing can be further tested for neutralizing antibodies (NAbs). NAbs have a neutralizing effect on the therapeutic biological drug and competitive ligand-binding (CLB) assays can often be used to detect them. SPR assays can easily be adapted to allow for detection of NAbs. Mojtahed *et al.* immobilized the drug target Interleukin 12 (IL-12) on a sensor chip and identified NAbs by pre-incubating ADA with Ustekinumab (UST) (19).

Labeling of reagents for such assays in ELISA or ECL formats can potentially influence the detection of NAbs negatively. CLB assays developed for Biacore systems do not require labeling and can be fully automated. The principle for a Biacore CLB assay is shown in Figure 10.

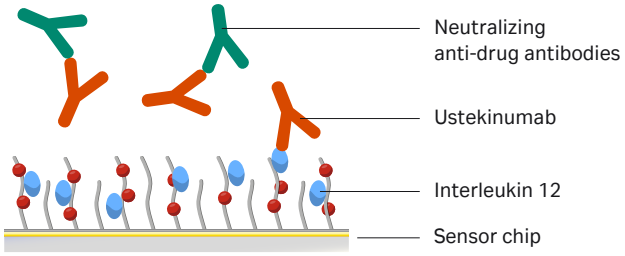

Fig 10. Detection of neutralizing anti-drug antibodies using Biacore. IL-12 was immobilized on the sensor surface, to which binding of UST can be detected. If a sample containing NAbs is added, the UST will be unable to bind, proving that the sample contains NAbs.

Reliable characterization and validation of reagents

Validated and well-characterized reagents are prerequisites for robust assays. Biacore systems provide detailed information on reagent properties such as antibody-antigen binding specificity, kinetics, and affinity, which is important for selecting optimal reagents in assay development. For methods requiring a secondary detection reagent, it is important to identify reagents that bind to the target antigen simultaneously and independently of each other. Such identification is easily done using sandwich epitope binning. The additional binding kinetics and affinity information obtained from a Biacore assay can also assist in optimization of assay performance without increasing demands on costs or other resources.

Examples of this kind of applications come from Merck Serono, who used a Biacore system to select optimal Abs for phosphokinase assays, and to check the potential impact of biotinylation of Ab reagents for use in bridging immunoassays.

Acknowledgement

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