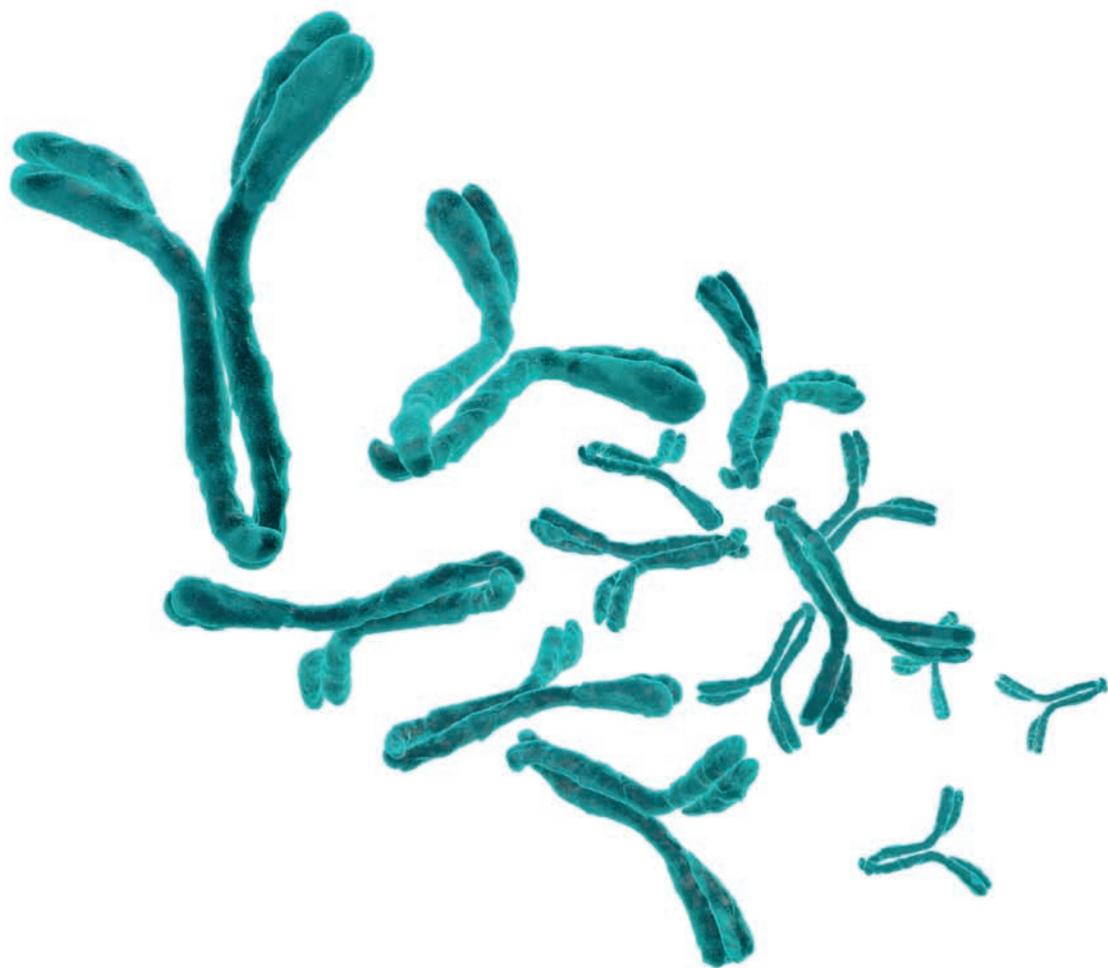


GE Healthcare
Life Sciences

Biacore™ systems for immunogenicity testing



Introduction

Immunogenicity is the propensity of biotherapeutics to induce unwanted immune responses when administered to the patient. Immunogenicity is an important aspect to consider when developing new protein therapeutics as it can affect both safety and efficacy.

Testing for immunogenicity is performed during the preclinical and clinical phases.

The US FDA "Guidance for Industry: Assay Development for Immunogenicity Testing of Therapeutic Proteins" (1) states that an immunogenicity assay should, in addition to being sensitive, also be able to detect all isotypes, in particular IgM and all IgG isotypes. The recommended sensitivity is 250 to 500 ng/ml. Studies are performed in three steps: Screening, confirmation, and characterization of positives.

Initial screening can result in false positives, and therefore, the initial screening assay is usually followed by a confirmatory assay. After identification and confirmation of positive samples, a full characterization of anti-drug antibodies (ADAs) in terms of assessment of isotype (class

or subclass), binding stability, epitope specificity, and neutralizing capacity give valuable information of the nature of the studied immune response.

Aarden *et al.* (2) have observed that IgG4 is second to IgG1 as the major isotype in ADAs developed for therapeutic monoclonal antibodies (MAbs). IgG4 have been associated with immune responses generated under conditions of high doses and prolonged exposure to therapeutic proteins. IgG4 ADAs can be difficult to detect in traditional bridging or homogenous enzyme-linked immunosorbent assay (ELISA) and enhanced chemiluminescent (ECL™) assays due to their bispecific nature.

GE Healthcare offers systems designed specifically for GLP/GMP-regulated working processes that are usually required for immunogenicity studies. This paper shows 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 benefit of using Biacore system and surface plasmon resonance (SPR) technology compared to traditional technologies is presented.

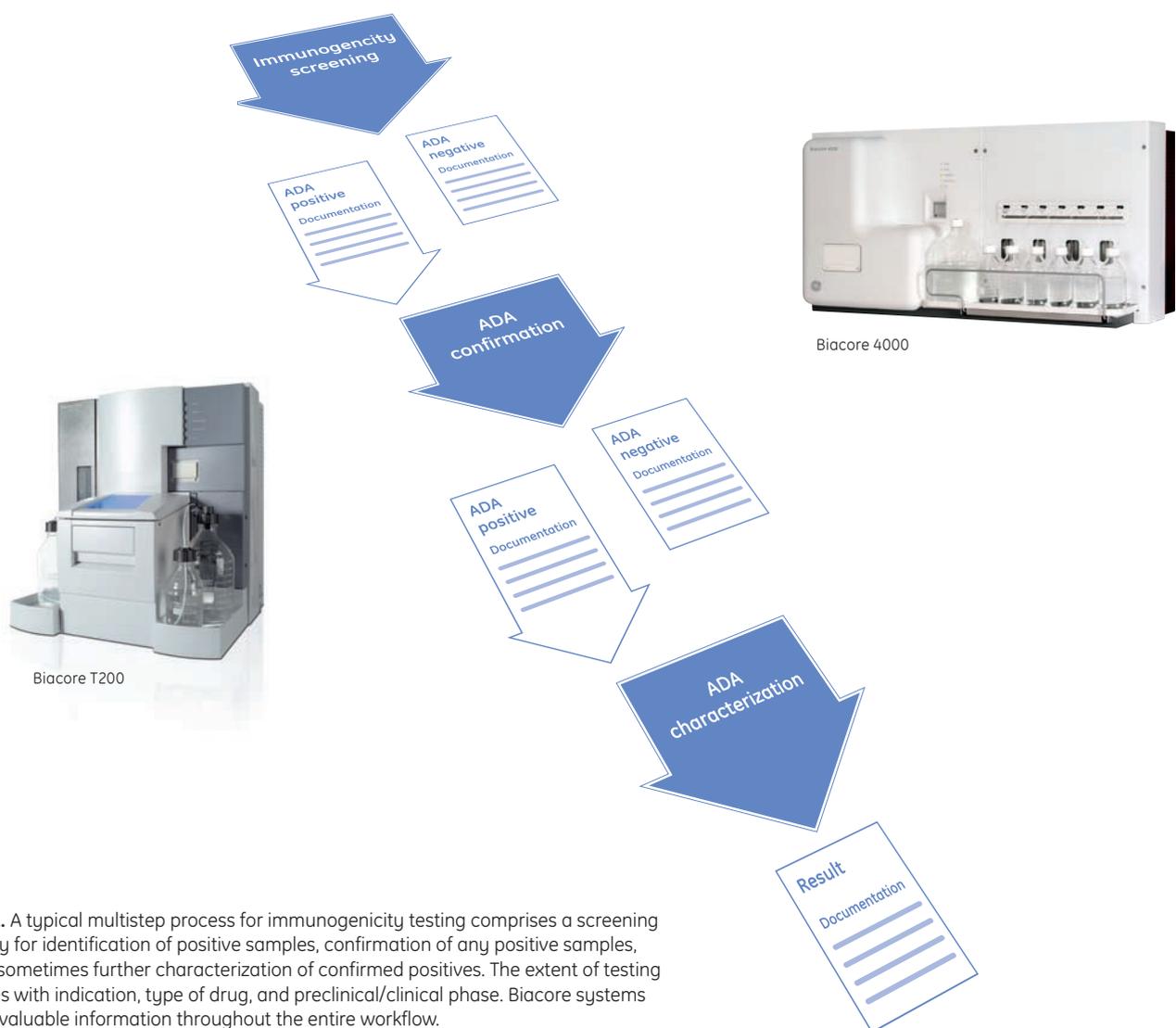


Fig 1. A typical multistep process for immunogenicity testing comprises a screening assay for identification of positive samples, confirmation of any positive samples, and sometimes further characterization of confirmed positives. The extent of testing varies with indication, type of drug, and preclinical/clinical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

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

A validated Biacore 3000 screening assay has been shown to detect ADAs against darbepoetin alfa and epoetin alfa down to 100 ng/ml and 80 ng/ml, respectively (3). In another study, sensitivities of 400 ng/ml and 200 ng/ml have been reported for ADAs against romiplostim and thrombopoietin, respectively (4). In addition to reaching the required sensitivity, the Biacore assays gave valuable kinetic information and detected both high- and low-affinity ADAs.

As an example, 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 T100¹ 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 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.

¹Now replaced with Biacore T200.

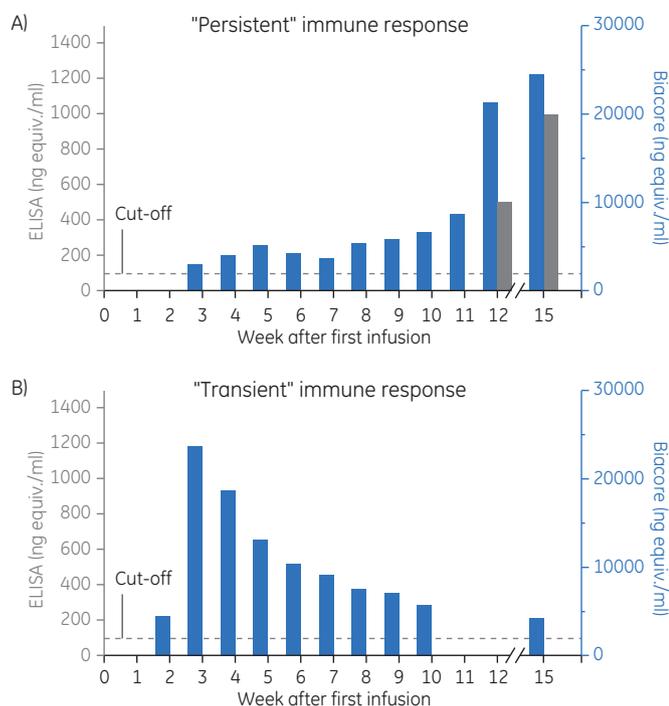


Fig 2. The optimized Biacore assay detected both "persistent" and "transient" responses 2 to 3 weeks after the first infusion (blue bars), while the bridging ELISA assay detected "persistent" responses after 12 weeks (gray bars) while "transient" immune response was not detected at all by the bridging ELISA assay.

The strength of Biacore assays to detect low-affinity ADAs was also shown by Lofgren *et al.*, (5). They compared a bridging ELISA to a Biacore assay for immunogenicity evaluation of the fully human panitumumab, a MAb that binds to the EGF receptor. The ELISA assay was more sensitive in detecting high-affinity ADAs but 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. Moreover, this assay identified eight samples with neutralizing antibodies (NABs); these positive samples were missed with the ELISA assay.

A number of comparisons between bridging ELISA assays and Biacore immunogenicity assays in clinical samples are summarized in Table 1. In all cases, Biacore assays detected a higher number of 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 (see below).

The clinical relevance of ADAs detected with Biacore assays but missed with ELISA is shown in a study by Swanson *et al.*, (6). 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.

Table 1. ADA-positive clinical samples detected with ELISA and Biacore assay

Drug	No. of positives (ELISA assay)	No. of positives (Biacore assay)
Iodine 131 chimeric tumor necrosis MAb (7)	4 of 78	7 of 78
Biotherapeutic drug, Merck Serono*	19 of 62	25 of 62
Panitumumab (5)	2 [†] of 612	25 [‡] of 604
Recombinant human erythropoietin (6)	6 of 8 [§]	8 of 8 [§]

* Acid-dissociation assay presented at Immunogenicity for Biologics, Munich 2011

[†] One of them found to be neutralizing in a cell-based assay

[‡] Eight of them found to be neutralizing in a cell-based assay

[§] All eight samples were from patients with antibody-mediated pure red-cell aplasia

Nechansky *et al.*, also observed that Biacore assays detected significantly higher number of ADA cases (8) 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.

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 thus prevents them from binding to the immobilized drug, thereby generating false negatives. Biacore T200 system 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 just before measurement to avoid re-forming of complexes. The advantages with

automated acidification is that the samples need to be acidic for a short time only and that the acidification time is constant for all samples.

This acid-dissociation strategy enables assays with recommended sensitivity also in the presence of drug. As an example, different concentrations of anti-rituximab Abs were mixed with increasing amounts of drug (rituximab). A total of 0.5 µg/ml anti-rituximab was detected in the presence of a 100-fold molar excess of rituximab (Fig 3).

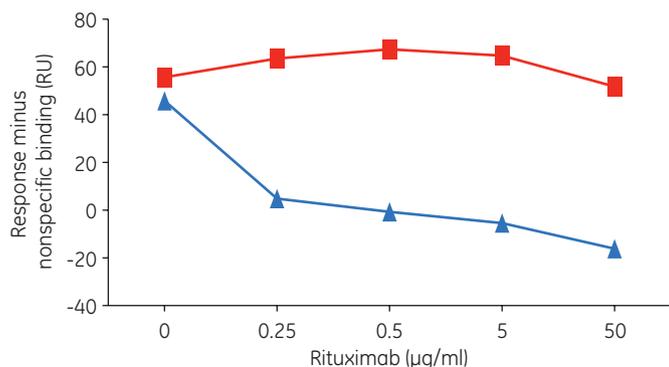


Fig 3. Detection of 0.5 µg/ml of anti-rituximab in the presence of different concentrations of rituximab with (red) and without (blue) acid dissociation strategy.

A research group at the University of Birmingham analyzed anti-rituximab and rituximab levels in clinical samples from SLE patients (9). 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.

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. When 69 clinical samples were analyzed using the two methods and compared, the same positives were generally found in both assays (partly shown in Fig 4). The Biacore T100 assay, however, found more positives and the

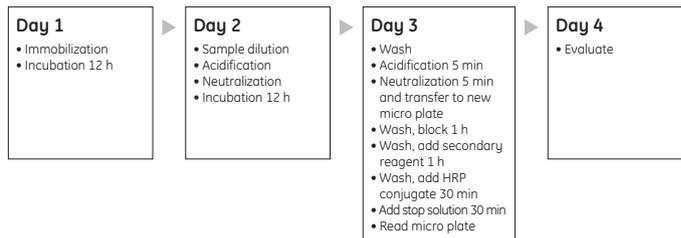
Sampling time	Patient A		Patient B		Patient C		Patient F	
	Biacore assay	ELISA						
Before								
168 h							•	
240 h							•	
312 h					•		•	
480 h							•	
648 h	•	•		•	•	•	•	•
816 h	•	•	•	•	•	•	•	•
984 h	•	•	•	•	•	•	•	•

Fig 4. Detection of ADAs in patient samples.

likely explanation for this behavior is that the Biacore assay detects also ADAs with lower affinities that are lost in the ELISA washing steps.

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 CROs. The ELISA assay used was cumbersome with many manual pipetting steps and required three days to generate results.

ELISA workflow



Biacore workflow

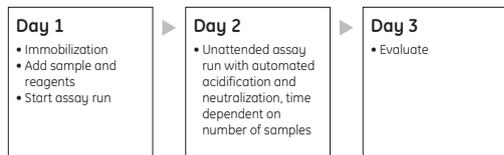


Fig 5. Workflows for Biacore and ELISA ADA assays.

The Biacore T100 assay required minimum sample preparation. The few steps with manual handling gave the Biacore assay better precision as compared to the ELISA assays (Table 2).

Dr Kramer concluded that "Biacore seems to be an ideal technology for acid dissociation assays", and Merck Serono is now implementing a screening assay using Biacore system in several projects using acid dissociation.

Table 2. Interbatch precision comparison between assays with acid dissociation. Courtesy of Merck Serono

	ELISA, variation in percent	Biacore, 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 6). The whole procedure can be automated.

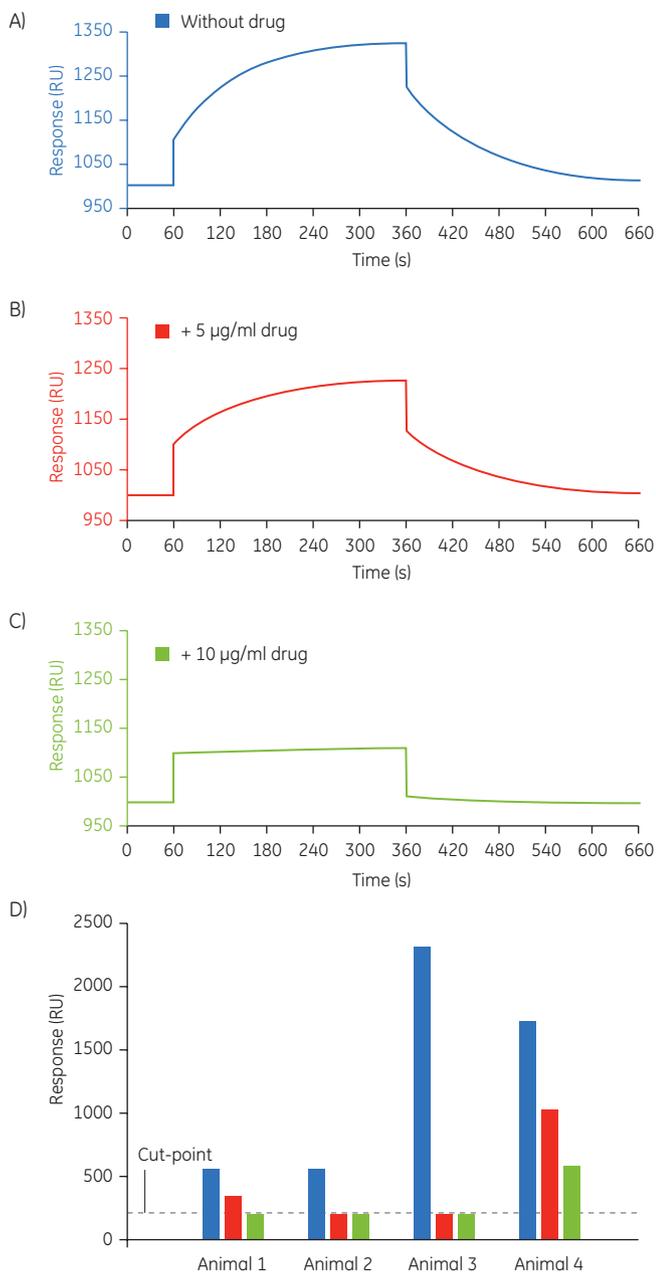


Fig 6. Confirmatory assay using Biacore system.

Comprehensive characterization of ADAs

After identification and confirmation of positive samples, a comprehensive characterization can be carried out using Biacore analysis. Competitive ligand binding assays, assessment of isotype (class or subclass), binding stability, epitope specificity, and neutralizing capacity give valuable information of the nature of the studied immune response. Biacore T200 offers dedicated software tools for isotype determination and binding stability.

Determination of ADA isotype

Isotype determination of the ADAs gives information about the immunobiological functions of the ADAs such as antibody Fc receptor binding. The principle for isotype determination is shown in Figure 7. In a study by Mytych *et al.*, 12 clinical samples containing serum ADAs against darbepoetin alfa were analyzed for isotypes (3). 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.

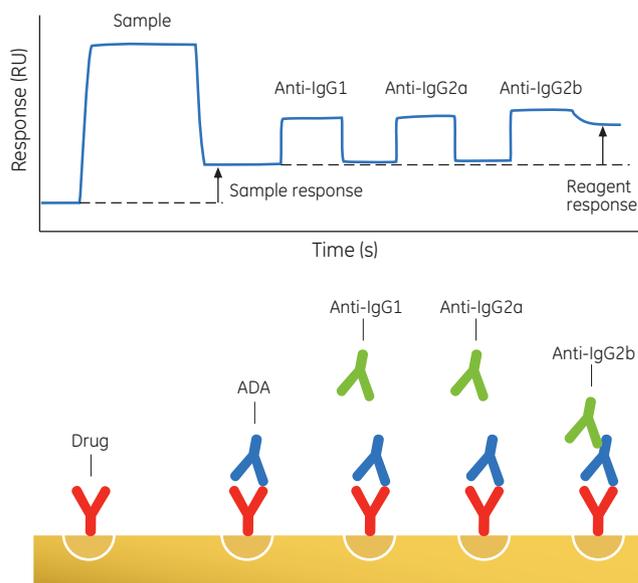


Fig 7. Isotype determination with a Biacore assay. ADAs from samples are allowed to bind to the drug, which is immobilized onto the surface. Then a series of anti-isotype Abs are passed over the bound ADA and the resulting response indicates the presence of different isotypes. In this illustration, reagent anti-IgG2b gives a significant response, identifying the bound antibody as subtype IgG2b.

Detection of IgG4

As stated by US Food and Drug Administration (FDA), an immunogenicity assay should be able to detect all IgG isotypes (1). IgG4, a major isotype of ADAs developed to therapeutic MAbs (2, 6), undergoes random exchange of half Ab (Fig 8A). Bispecific ADAs cannot be detected in bridging or homogenous assay formats (Fig 8B) that are often used in ELISA and ECL assays. IgG4 is however detected in the direct binding assays in Biacore systems.

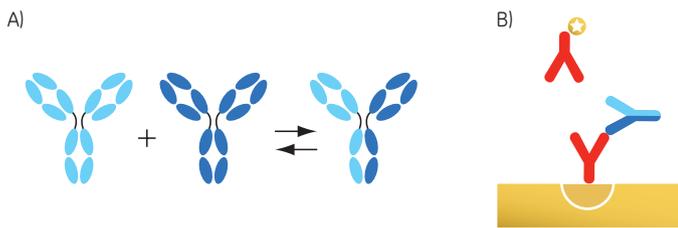


Fig 8. Monovalent ADAs are not detected in bridging ELISA assays. **(A)** Spontaneous and random exchange between Abs occurs in the human Ab whereof some have bispecific specificity. **(B)** Monovalent IgG4 will not be seen in bridging assays, since they depend on the binding of two drug molecules to one ADA.

Determination of epitope specificity

The determinations of epitope specificity of ADAs are important when studying immunogenicity. According to FDA recommendations (1), the applicant should investigate to which regions the immune responses are generated: "FDA recommends the applicant direct initial screening tests against the whole molecule and endogenous counterpart. However, for product development, the applicant should investigate the regions or 'epitopes' to which immune responses are specifically generated. This determination may be particularly important with fusion molecules in which two proteins are genetically or physically fused." Epitope specificity determination with Biacore systems can be performed as early as the screening stage. A convenient setup is to immobilize the full-length drug in one flow cell and domains of the drug in the other flow cells (Fig 9).

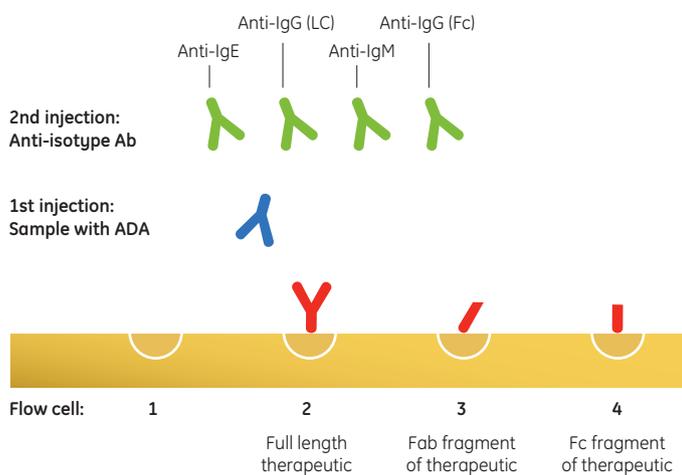


Fig 9. Assay setup for a combined epitope mapping and isotyping experiment.

Cross-reactivity can also be checked using the same approach but with different drugs in each of the flow cells. To assess the potential anti-darbepoetin alfa Ab cross-reactivity to epoetin alfa, Mytych *et al.* developed a Biacore dual-flow cell biosensor immunoassay including an epoetin alfa surface (3). This immunoassay is used by Amgen as the preferred method to detect and characterize anti-epoetin alfa and anti-darbepoetin alfa Abs in human serum.

The work carried out by Stubenrauch *et al.* demonstrates the wealth of information that can be obtained from a few Biacore experiments (10). Utilizing the four flow cells 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 9). 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.

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 10).

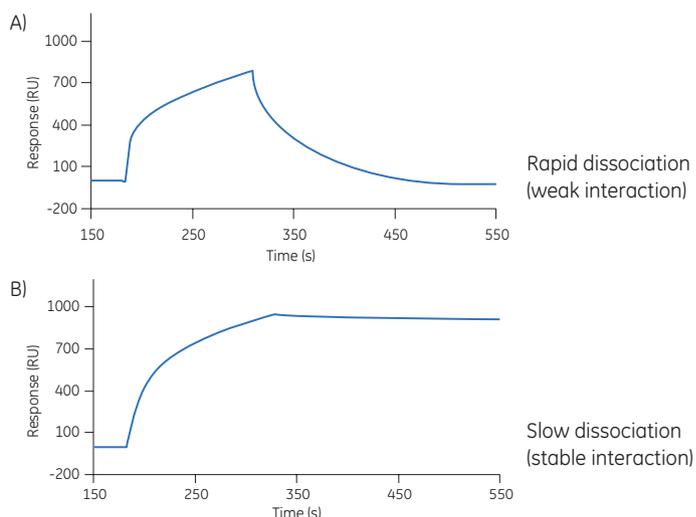


Fig 10. Biacore characterization of binding stability of ADAs from positive clinical samples to immobilized drug. Immune response maturation often leads to slower dissociation rates (lower picture).

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. Mytych *et al.*, (3) 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.

Competitive ligand-binding assays without need for labeling

As part of the characterization of ADAs, samples confirmed as positives in immunogenicity testing can be further tested for NABs. NABs have a neutralizing effect on the therapeutic biological drug. The conventional assays used for identification of NABs are cell-based assays, and these assays are often cumbersome with poor reproducibility. As an alternative, competitive ligand-binding (CLB) assays are sometimes used. For MAb drugs, CLB assays are often the preferred choice rather than bioassays according to the EMA draft guideline (11). Case studies from four companies have shown that bioassays and CLB assays give comparable detection of NABs (12). 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 11.

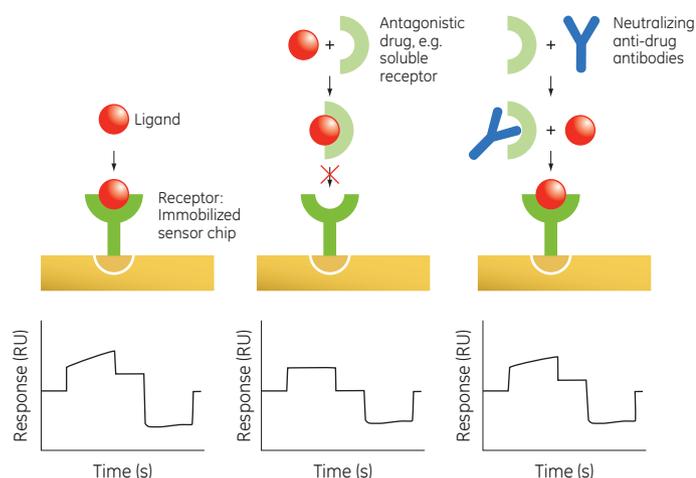


Fig 11. ADAs that neutralize the effect of the drug are detected in this CLB assay.

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 antigen simultaneously and independently of each other. Such identification is easily done using the pairwise epitope mapping tool in Biacore systems. The additional kinetic and affinity information obtained from the 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 Biacore system to select optimal Abs for phosphokinase assays, and also to check the potential impact of biotinylation of Ab reagents for use in bridging immunoassays.

Acknowledgement

Data from assay comparison, acid dissociation assays, and confirmation were kindly provided by Dr. Kramer, Merck Serono, Germany. Immunogenicity screening data were kindly provided by Boehringer Ingelheim.

Technology overview



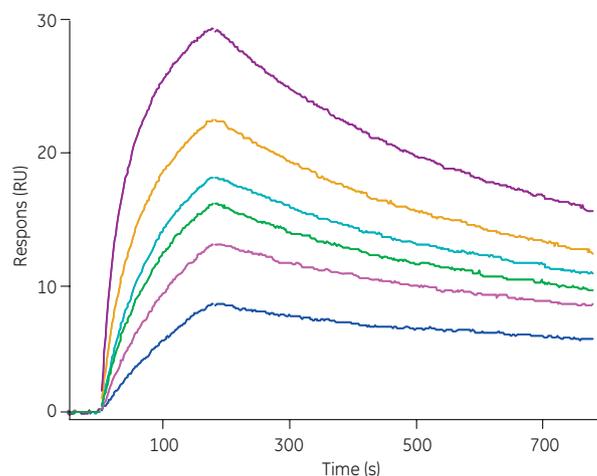
Binding strength and kinetics with Biacore systems

Biacore systems monitor molecular interactions in real-time using surface plasmon resonance (SPR). Without the need for labels, Biacore assays provide information on affinity, kinetics, and specificity of molecular interactions. The active concentration of biomolecules can also be determined.

One of the interacting molecules is immobilized onto a sensor surface, while the other molecule flows over the sensor surface in solution. Any interaction between the two is detected in real-time via changes in mass concentration close to the sensor surface and binding data is presented in a sensorgram where SPR responses in resonance units (RU) are plotted versus time.

The formation and dissociation of complexes are followed during the course of an interaction, with the binding kinetics (k_{on} , k_{off}) revealed by the shape of the binding curve.

More information can be found at www.gelifesciences.com/biacore



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