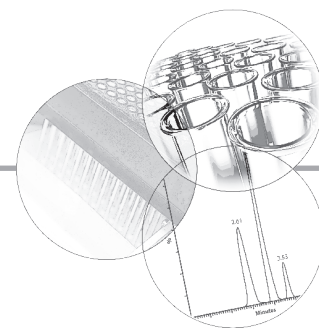




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Unwanted immunogenicity: lessons learned and future challenges

All biological therapeutics have the potential to induce an immune response in recipients of these products. Elicitation of an immune response can result in variable clinical impact, ranging from benign to severe adverse effects, a diminution in clinical efficacy or, in some cases, hypersensitivity or allergic reactions. Consequently, assessment of unwanted immunogenicity is an important element of the data required for regulatory submission for product approval. However, issues relating to immunogenicity occur throughout the life-cycle of a biotherapeutic and need to be considered appropriately when introducing any product change(s). Evaluation of immunogenicity of a product requires a well-considered strategy and a panel of appropriately validated (or 'fit-for-purpose') assays for antibody detection and characterization in clinical samples. An overview of the bioanalytical methods that are currently being used for assessment of immunogenicity of biotherapeutics and the guidance available along with some of the challenges facing the industry are discussed in this review.

Biological medicines are being increasingly used for the therapy of various diseases, including rheumatoid arthritis (RA), Crohn's disease, psoriasis and cancers. In 1982, human insulin became the first recombinant protein approved by the US FDA. Since then, many biotherapeutic products encompassing several product classes, such as enzymes, cytokines and monoclonal antibodies, have gained regulatory approval in the USA and Europe, providing benefit to the patients and, in some cases, generating huge revenues for the manufacturer. However, the success of new biological medicines has in recent years been dogged with unexpected safety issues following their administration. For example, the withdrawal of Tysabri® (a humanized antibody against $\alpha 4$ -integrin) due to the risk of occurrence of progressive multifocal leukoencephalopathy and its subsequent re-launch in the USA. Other examples are the two separate incidents relating to the induction of neutralizing antibodies in recipients of a pegylated truncated thrombopoietin product in development [1] and an approved erythropoietin product, Eprex®, which caused severe thrombocytopenia and pure red cell aplasia (PRCA) respectively [2]. Consequently, immunogenicity assessment has received significant attention, both from regulatory bodies and industry. The need for a risk-based approach and a risk-management plan for every biotherapeutic (including those in development) has also been emphasized.

From the perspective of unwanted immunogenicity, most biotherapeutic products induce immune responses, either due to their foreign nature (if they are of exogenous origin) or are administered as replacement therapy to patients with gene defects who lack immune tolerance (e.g., enzymes and coagulation factor concentrates) or because of their nonsimilarity to self-molecules (due to the presence of neo-antigens as a result of altered endogenous sequence) in other aspects. Two different mechanisms to explain the immune responses have been proposed; the classical immune response (non-human proteins and no immune tolerance) and the breaking of B-cell tolerance (human proteins). While this seems very simplistic, the issue of immunogenicity is multifactorial and, in fact, complex. It is clear however that in all instances, unwanted immunogenicity tends to manifest clinically through the activation of antibody-secreting B cells and, consequently, generation of antibodies against the therapeutic. These antibody responses are often benign but can cause hypersensitivity or allergic reactions. In certain instances, alteration in the pharmacokinetics and/or a diminution of the efficacy of the therapeutic becomes evident [3–5]. Examples include neutralizing antibodies seen with factor VIII concentrates and enzyme-replacement therapies [3,4]. In some cases, however, neutralization of the endogenous counterpart results in adverse clinical sequelae [2,6]. It is not surprising, therefore, that immune responses against

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KEY TERMS

Antibody: Globular protein made by cells of the B lymphocyte lineage in response to a foreign substance (antigen)

Immunogenicity: Ability of a substance to induce an immune response. Although undesirable for biotherapeutics, it is favourable to provoke an immune response for vaccines

a biotherapeutic continue to be a major safety and efficacy concern for regulatory agencies, manufacturers, clinicians and patients. As such, a guideline on immunogenicity assessment has been provided by the European Medicines Agency (EMA) and a draft guidance recently produced by the FDA, as well as several industry White Papers on the clinical assessment of unwanted immunogenicity [101,102]. Another EMA guideline on immunogenicity assessment of monoclonal antibodies intended for *in vivo* clinical use is expected soon, following consultation on a concept paper published earlier this year [103].

This article provides an overview of the current status, strategies and challenges related to the immunogenicity assessment of protein therapeutics in humans. Since the main focus is bioanalytical methods required for evaluation of immunogenicity of biotherapeutics, evaluation of nonclinical immunogenicity is only briefly considered.

Factors contributing to immunogenicity

As stated earlier, the issue of immunogenicity is highly complex and governed by a multitude of factors, which are not only related to the product but also dependent on the type of disease and the patient group being treated. These are summarized briefly in **Box 1**. The PRCA incident that occurred due to the formation of neutralizing antibodies, which crossreacted with endogenous erythropoietin in patients treated with Eprex, provides a striking example of the complexity of immunogenicity [2]. Despite

extensive research into the underlying cause of the upsurge in cases of PRCA in patients, it has been difficult to identify the predominant trigger factor from the numerous potential factors that have been postulated. These include change of formulation and issues relating to breakdown of cold chain, route of administration, rubber stoppers, leachates and presence of aggregates. In another example, 12% of patients with Crohn's disease treated with certolizumab as maintenance therapy developed antibodies, but the incidence of antibody formation decreased to 2% in patients treated with a combination of the antibody and immunosuppressive agents [7]. In addition, Rituximab®, which depletes B cells (and should reduce antibody formation), when administered to patients with systemic lupus erythematosus (SLE), induces antibodies in 65% of the patients but is totally non-immunogenic in patients with non-Hodgkin's lymphoma [8].

Risk-based approach for assessment of immunogenicity

The need for a risk-assessment analysis for each product in order to develop an appropriate strategy for immunogenicity assessment has been suggested [9–11]. In this assessment, it is critical to consider the risk of generating an immune response, the potential severity of a response and the overall risk/benefit to the target population. Other issues that need to be considered and have implications for the study include timing and frequency of sample collection and antibody assessment and measurement. The mode of action of the therapeutic also needs

Box 1. Some factors that influence the unwanted immunogenicity of therapeutic proteins.

- Product characteristics, molecular structure, variation in amino acid sequence (relative to native protein), novel epitopes, aggregates, degradation products, oxidation and deamidation
- Process related, such as host cell proteins, other contaminants and process change
- Formulation
- Biological properties such as immunostimulatory or immunosuppressive, use as replacement therapy or only therapy
- Dosing: single or multiple injections, high or low dose, short or long period
- Route of administration
- Genetic factors (HLA class II and gene deficiencies)
- Immune status: competent or immunocompromised
- Patient age
- Any concomitant medication
- Disease state: acute or chronic, inflammatory or oncology
- Target: endogenous counterpart, redundant or unique

to be considered, as this may impact on the choice of assay that is adopted for assessment of neutralizing antibodies.

A strategy for assigning risk levels to a product and risk-level-based bioanalytical testing schemes for evaluation of immunogenicity of a product in nonclinical and clinical studies has also been proposed [12]. While this approach is pertinent and may be justified and can influence decisions regarding the requirement of a **neutralizing antibody** assay for nonclinical and clinical programs, adoption of a pragmatic approach on a case-by-case basis is generally needed for assessing the immunogenicity of a product. Needless to say, it is important to consider the available regulatory guidance on immunogenicity assessment of biotechnology products.

Can immunogenicity be predicted?

Despite advances in our understanding and assessment of immunogenicity, the prediction of immunogenicity (both incidence and clinical significance) remains difficult, although much effort is being made to resolve this issue. Current efforts are primarily focused at selecting and optimizing lead candidates that pose a minimal risk of immunogenicity in clinical trials. Consequently, diverse methods are in development or have been developed. Determination of T-cell epitopes in T-cell assays (using T-cells from naive or previously exposed individuals), coupled with *in silico* approaches for sequence analysis and prediction of T-cell epitopes, appear to be useful for identifying immunogenic proteins and selecting those with a minimal risk of immunogenicity [8]. A phenotypic evaluation of the type of T-cells responding to the study protein is also warranted, as these T-cells may be effector or regulatory in nature, thus leading to divergent responses [13]. It is also important to understand that, although a protein that is immunogenic contains T-helper-cell epitopes, various other factors – product, patient and disease related (**Box 1**) – also have a very significant effect on immunogenicity and may even outweigh the inherent antigenicity of the protein. However, understanding the antigenic properties of the product is equally important in defining an optimal strategy for the development of proteins that are optimized for the avoidance of unwanted immunogenicity. Some retrospective studies advocate the use of certain *in silico* and *in vitro* methodologies for identification of T-cell epitopes [14,15]. Barbosa and co-workers demonstrated a relationship between the

immunogenicity of IFN- β and HLA-associated T-cell-dependent immune response [16]. However, a thorough evaluation of these methods is still needed. Towards this goal, an analysis of these methods with antibody assessment data and clinical outcomes in patients in prospective clinical trials should prove useful in addressing the utility of these tools as predictors of immunogenicity of a product and its relevance in a clinical setting.

Clinical assessment of immunogenicity

Currently, the most technically feasible approach for evaluation of unwanted immunogenicity is the detection and measurement of antibodies generated against the product. New platforms for immunogenicity assessment are continually being exploited to enhance the ability to detect therapeutic-induced antibodies in treated patients. To date, no single assay can provide all the necessary information on the immunogenicity profile of a biotherapeutic. Therefore, a state-of-the-art testing program for antibody detection that comprises a bioanalytical testing strategy involving a panel of assays, is a requirement in product development. Furthermore, an evaluation of antibody responses and assessment of whether it correlates with data on pharmacokinetics and/or pharmacodynamics and clinical effects in patients is required for assessing the clinical impact, if any, of the induced antibodies.

Studies on unwanted immunogenicity need to be designed prospectively and ensure that all the necessary protocols are implemented at study initiation. In the study strategy, it is critical to consider the risk of generating an immune response, the potential severity of the induced response, and the risk/benefit to the target population. This assessment will impact on issues such as the timing and frequency of sample collection and selection of assay strategies and assay types.

Assays for antibody detection

For most biotherapeutics, a multi-tiered approach is typically used for antibody testing. This consists of a screening assay complemented with a confirmatory step (e.g., antigen adsorption) and a neutralizing antibody assay for assessing the neutralizing capacity of the induced antibodies [7,16]. Further characterizations, such as antibody isotype, class and subclass, may be conducted as necessary. It is important to ensure that the assay platform

KEY TERM

Neutralizing antibody:
Antibody that inhibits or neutralizes the biological effect of an antigen

and parameters selected are satisfactory for the particular product. Moreover, all assays should be suitable for their intended purpose and capable of providing reliable quantitative, sensitive and specific data that can be appropriately interpreted and analyzed [17]. Assay validation and the inclusion of relevant antibody controls also need to be considered and implemented appropriately [18].

■ Screening assays

Several immunochemical procedures can be used for screening antibodies if induced in patients following treatment. These include conventional approaches such as ELISAs, radioimmuno-precipitation assays (RIPA) and other technologies such as surface plasmon resonance (SPR), time-resolved fluorescence resonance energy transfer and electrochemiluminescence (ECL). However, each of these methods has benefits and limitations that need to be considered prior to implementation of a testing strategy (TABLE 1). In our experience, the format of the screening assay

is very much dependent on the therapeutic in question. Since all antibodies need to be detected by the screening assays used, various procedures that are described here may need to be considered or even developed prior to selecting an assay for use in clinical trials. In some situations, the therapeutic may persist in the circulation and, consequently, may be present in the sample. The ability of the assay to detect antibodies in the presence of the therapeutic needs to be assessed and strategies to allow this, such as acid dissociation or variations of this, implemented if necessary [19,20].

ELISAs

Since ELISAs are very robust, reliable, highly sensitive, amenable to automation and cheap for routine use, they are often used in clinical immunology laboratories for antibody detection. ELISAs can be conducted using different assay principles, such as direct, indirect, bridging or using competitive ELISA formats that are described in detail elsewhere [16,18,21,22].

Table 1. Examples of screening assays for detection of antibodies.

Assay type	Pros	Cons	Example of therapeutic	Ref.
Direct ELISA	Rapid High throughput Easy to use Very good sensitivity	Prone to spurious binding, 'matrix effects' May fail to detect 'rapidly dissociating' antibodies Antigen immobilization may alter protein conformation Requires species specific secondary reagent	Granulocyte-macrophage colony stimulating factor	[51,53]
Bridging ELISA	High throughput Acceptable sensitivity No requirement for species-specific reagents	May require two antigen conjugates Antigen labeling may alter antigen & obscure epitopes Highly susceptible to interference from antigen May not detect IgG4 antibodies	Erythropoietin Natalizumab	[25,51]
Electrochemiluminescence assay	High throughput. Excellent sensitivity. Large dynamic range. Minimal influence of sample matrix, tolerant of residual therapeutic	Labeling may alter antigen and obscure epitopes Requires electrochemiluminescence instrument, vendor-specific reagents	Five different monoclonal antibodies	[33]
Radioimmunoprecipitation assay	Moderate-to-high throughput Solution-based Good sensitivity Can be specific	Can be isotype specific. May fail to detect rapidly dissociating antibodies Requires radiolabeled antigen. Radiolabel may alter/denature antigen. Decay of radiolabel may affect antigen stability	Erythropoietin Humira	[26,27]
Surface plasmon resonance assay	Automated Enables detection of both low- and high-affinity antibodies. Allows isotype determination Detection reagent not required	Antigen immobilization may alter protein conformation Regeneration step may degrade antigen. Sensitivity often less than binding assay Expensive. Requires dedicated equipment, reagents are costly and vendor specific	Erythropoietin Panitumumab Glial-derived neurotrophic factor IFN- β	[28,30,54,55]

Owing to its simplicity, a direct ELISA is often considered for antibody detection. Despite their high sensitivity, these assays can be confounded by nonspecific binding effects and other limitations [16,23]. For example, for measuring antibodies against a therapeutic antibody, the lack of secondary reagents that can discriminate effectively between a therapeutic antibody and induced antibodies presents a technical problem. Although this issue can be resolved by adopting other alternative approaches [24], the bridging assay (BA) has become a platform of choice for most biotherapeutic products, including monoclonal antibodies such as natalizumab (**Box 1**). In the BA, the antibody is captured by an immobilized antigen and detected using a labeled antigen. Albeit less sensitive than direct ELISAs, the BA potentially provides an acceptable sensitivity and a higher specificity (the antigen must be recognized twice by the antibody for the antibody to be detected). Use of streptavidin-coated plates for capturing biotinylated antigen and a differently labeled antigen (e.g., digoxigenin) for antibody detection has been shown to enhance sensitivity of the assay for detecting antibodies against erythropoietin [25].

While the BA overcomes the requirement for species-specific reagents associated with direct ELISAs, it has some caveats that are similar to direct ELISAs. For example, the BA may not detect rapidly dissociating low-affinity antibodies that are washed off during the ELISA procedure. Moreover such assays, such as direct ELISAs, are susceptible to significant inhibition because of soluble/circulating therapeutic antibodies present in samples. Unlike direct ELISAs, however, the BA may not detect IgG4 antibodies (if these are induced) which can be bispecific and unable to crosslink the same antigen due to the exchange of half-molecules [24]. In certain situations, the BA may provide false positive signals by crosslinking other serum factors.

Radioimmunoprecipitation assays

These assays provide a solution-based approach (as opposed to the use of immobilized antigen) and can be more sensitive than ELISAs due to a higher protein-binding capacity. However, the undesirable need for radioactivity and low throughput limits the utility of these assays. In these assays, serum is incubated with a radio-labeled antigen and the resulting antigen–antibody complex is precipitated using immobilized protein A/G or antiglobulin and the precipitated radioactivity assessed. The technique has been

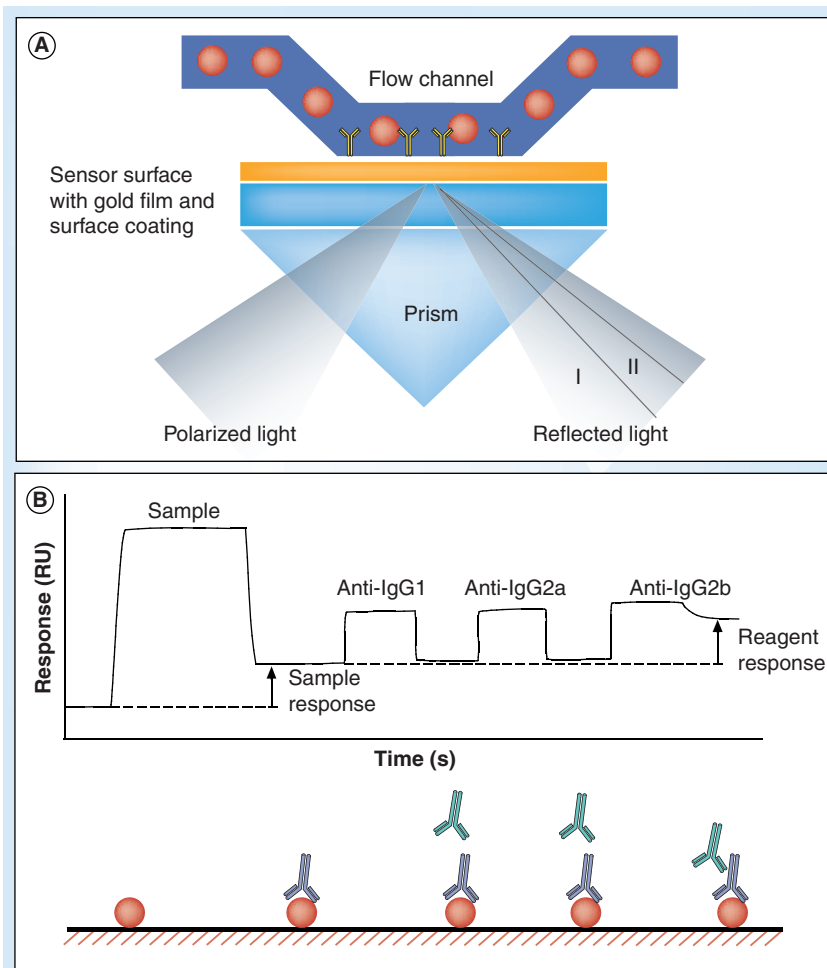


Figure 1. Surface plasmon resonance. (A) Principle of the Biacore platform. **(B)** Identification of the IgG2b subclass of an antibody evaluation of the binding profile using surface plasmon resonance. For this, the antibody sample was injected over an antigen immobilized sensor chip followed by sequential injections of specific isotype antibodies. Reproduced with permission from Biacore™, GE Healthcare. © Biacore™, GE Healthcare. All rights reserved.

shown to be useful for the detection of antibodies against erythropoietin [2,26] and, more recently, for detecting antibodies against the TNF antibody, adalimumab in the sera of RA patients [27]. A point to note is that these assays cannot detect certain antibody isotypes and low-affinity antibodies that can, in some cases, be clinically significant. However, these assays are able to detect clinically relevant IgG4 antibodies that may not be detected by other assays [24].

Surface plasmon resonance assays

This platform is popular for analysis of protein–protein and protein–small molecule interactions as, unlike other methods, it is rapid, sensitive, versatile, label free and requires small amounts of

KEY TERM

Binding antibody: Antibody capable of binding to an antigen but which may or may not neutralize the biological effect of the antigen to which it binds

sample. It provides real-time measurements and generates data on binding affinity, binding kinetics (both formation and dissociation of antigen–antibody complexes), concentration determinations, antibody isotyping and binding specificity.

Surface plasmon resonance (SPR) is an optical phenomenon that occurs when polarized light is reflected by a surface coated with a thin film of metal (typically gold and silver). Under certain conditions (e.g., wavelength, polarization and angle of incidence), the free electrons at the surface of the gold sensor chip absorb the incident photons during the SPR phenomenon, resulting in a dip in the intensity of the light in a defined direction (**FIGURE 1A**). Any change in the refractive index at the surface, caused by a difference in mass when an analyte binds to a ligand previously immobilized is then measured using an SPR detection system. Therefore, the change in refractive index induced by an antigen–antibody interaction is directly proportional to the amount (mass) of **binding antibody** in the sample being tested.

The Biacore technology (Biacore™, GE Healthcare) is based on SPR and uses sophisticated microfluidics to flow analyte over a ligand-coated channel (flow cell), injecting a single analyte over up to four serially addressed flow cells, which can be either coated with ligand or used as a reference surface. The instrument monitors the interaction in ‘real-time’ and provides a continuous signal of the events that occur on the sensor surface. Confirmation of specificity can be obtained by sequential injection of a species-specific antibody and by using other strategies, such as antigen adsorption. Antibody isotyping can also be performed, as shown in **FIGURE 1B**.

The Biacore 2000 and Biacore 3000 instruments are used widely for the evaluation of immunogenicity of vaccines and biotherapeutic products. This has resulted in a significant amount of literature on the attributes and successes in the area of unwanted immunogenicity [28–31]. More recently, the Biacore T100, which has dedicated tools that cater specifically for immunogenicity testing, has become available. This instrument not only supports screening, confirmation and characterization of induced antibodies but also addresses the significant issue of interference caused by excess amounts of residual therapeutic in the sample (e.g., therapeutic antibodies). Samples are acidified to allow dissociation of immune complexes and then neutralized just before measurement; it is claimed that this improves

the assay sensitivity in the presence of rapidly associating immune complexes and allows antibody detection in the presence of excess therapeutic. Despite their attributes, Biacore assays are low throughput compared with other platforms and require extensive development and optimization for evaluation of suitable immobilization conditions and regeneration strategies, which are critical parameters for assay performance.

From the assay perspective, SPR can detect early immune responses that are characterized by low affinity antibodies with rapid off-rates that may be clinically significant, but are easily missed by alternative techniques such as ELISAs, due to antibody losses during washing procedures [28,29]. Lofgren and co workers reported that the Biacore assay, despite its lower tolerance (to competing therapeutic) and lower sensitivity (detection limit: 1 µg/ml of positive control antibody) than a BA (detection limit: 10 ng/ml of positive control antibody), detected antibodies against panitumumab in a higher percentage of patients than the BA (4.1% by SPR vs 0.3% by BA) [30]. In addition, the SPR assay identified patients who developed neutralizing antibodies but were not recognised in the BA. Further evidence from studies in which sequential assessment of samples has been undertaken suggests that in some cases antibodies may be detected at earlier time-points than ELISAs [31].

Several SPR instruments, including those with refinements on the existing technology, are now available [32]. For example, the ProteOn XPR36 (Bio-Rad Laboratories) is a multiplexed SPR device that integrates microfluidics with a novel optical design to create an array system that enables multiple quantitative protein binding experiments in parallel. Consequently, a complete kinetic profile of a biomolecular interaction can be obtained by conducting a kinetic analysis of a concentration series of an analyte in one experiment, without the need for regeneration, using a single sensor chip. This renders it highly appealing for sequential assessment of clinical trial samples for studies on unwanted immunogenicity.

Other technologies complementary to SPR that differ in detection, sample delivery and/or throughput have also emerged. The Octet, commercialized by ForteBio and based on the principle of biolayer interferometry, offers label-free quantitation and kinetic analysis of antibodies and other proteins in real time. It uses disposable optical fiber biosensors and allows eight-channel parallel analysis of interactions at the surface of a 96-well plate eliminating microfluidics.

Despite its extensive use in determining kinetics and affinities of protein interactions, its use in immunogenicity applications has been scarce.

Electrochemiluminescence assays

Technological advances have led to the availability of electrochemiluminescence (ECL) reporter assays provided currently by Meso Scale Discovery (Meso Scale Diagnostics, LLC). ECL detection uses ruthenium labels that emit light when electrochemically stimulated. Background signals are minimal because the stimulation mechanism (electricity) is decoupled from the signal (light). MSD's two ruthenium labels, SULFO-TAG™ and MSD TAG, are stable, nonradioactive and offer a choice of convenient coupling chemistries. They emit light at approximately 620 nm, eliminating problems with color quenching. Further more, multiple excitation cycles of each label amplify the signal to enhance light levels and improve sensitivity. The ECL instrument uses carbon electrode plates (claimed as having a higher binding capacity over ELISA plates), which are available in a variety of

formats (either as standard, high bind or pre-coated with either streptavidin or avidin) and an ultralow noise charge-coupled device camera with custom-designed telecentric lenses for rapid signal detection.

In the ECL assays, antibodies are detected through simultaneous binding to either unlabeled or biotinylated therapeutic and ruthenium-conjugated therapeutic and the antigen–antibody complex formed is detected by measuring light emission following the application of a voltage. The emission of light involves an oxidation–reduction reaction with ruthenium ions and excess tripropylamine, resulting in multiple cycles of signal generation and increased sensitivity (**FIGURE 2**). Different formats of the types of assays that can be conducted using MSD technology are shown in **FIGURE 3**.

This technology has excellent sensitivity, good reproducibility and minimal interference from components in complex matrices. In addition, these assays can provide greater dynamic assay range (measuring range of five orders of magnitude and useful for some applications, including pharmacokinetics), which

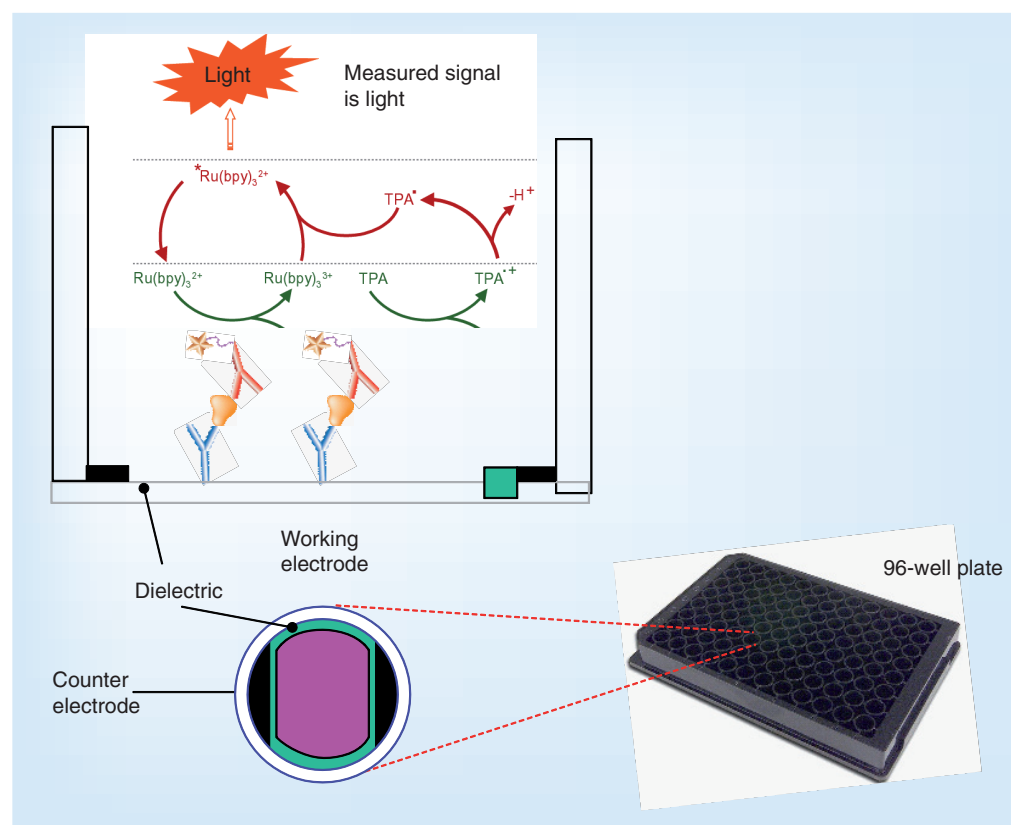


Figure 2. Electrochemiluminescence principle of the Meso Scale Diagnostics platform. Reproduced with permission from Meso Scale Diagnostics, LLC © 2010 Meso Scale Diagnostics, LLC. All rights reserved.

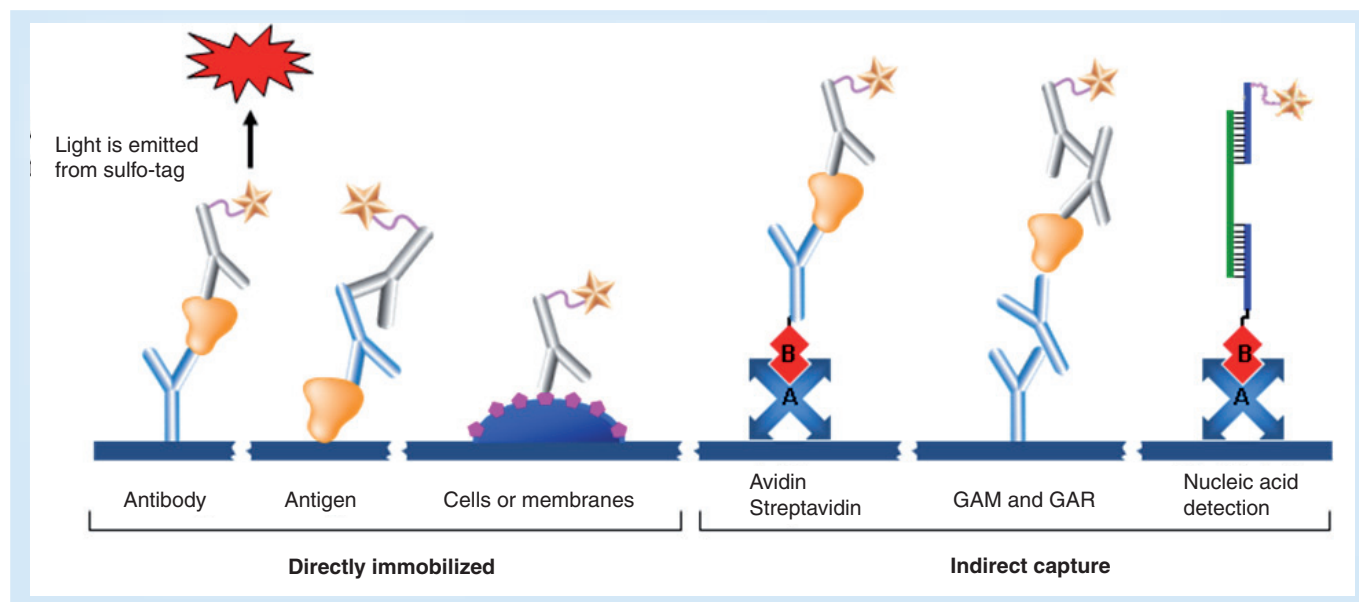


Figure 3. Different formats that can be used for developing assays using the Meso Scale Diagnostics platform.

Applications include binding assays, cell-based and noncell-based neutralizing antibody assays for evaluation of immunogenicity of biotherapeutics.

GAM: Goat antimouse antibody; GAR: Goat antirabbit antibody.

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minimizes sample dilutions and repeats, reducing handling time and repeat costs [33]. The assays are simple, rapid and easy to perform and are now commonly used for evaluating immunogenicity of vaccines and various biotherapeutic products [34,35]. The technology is particularly suitable for detecting antibodies generated against biotherapeutics as these assays seem to have a higher tolerance to the presence of circulating antigen and are therefore able to detect induced antibodies even if the therapeutic persists in the serum sample. Although these assays may in certain instances be challenged to detect low-affinity antibodies, a recent study to evaluate the presence of antibodies induced against an IL-8 human monoclonal antibody claimed the superiority of an ECL method in detecting antibodies of both high and low affinities in comparison with ELISA and SPR methods [35]. This, however, may need to be evaluated on a case-by-case basis due to the inclusion of wash steps in the procedure.

Other technologies

Besides the previous examples, a range of other instruments including the Axela dot lab system (Axela Inc.), a Gyrolab system (Gyros AB) and other technologies are also available for use in immunogenicity testing.

■ Neutralizing antibody assays

Neutralizing antibody (NAb) assays determine the neutralizing potential of the induced antibodies and are an essential element of immunogenicity evaluation. For detecting NABs, the sample is preincubated with the therapeutic and if NABs are present, the therapeutic is unable to bind to its target resulting in an inhibition of assay response. Two types of NAB assays are mainly used: a cell-based bioassay or a noncell-based competitive ligand-binding (CLB) assay. The former are more commonly used and, although they may be difficult to establish, their use is generally recommended by regulators as they are reflective of the *in vivo* situation, in contrast to noncell-based CLB assays. The latter are rapidly gaining momentum as a platform to use within the biotechnology industry at least for some products.

■ Cell-based bioassay

Since bioassays assess the cellular response of a therapeutic *in vitro*, they are ideal for development of a cell-based NAb bioassay. In such assays, treated cells respond directly or indirectly to the biotherapeutic in a concentration-dependent manner. Different biological responses of the cells to the therapeutic form the basis of a NAb assay. These include cell proliferation, apoptosis, phosphorylation of

intracellular substrates, chemokine release and expression of proteins or genes that may be quantitated using simple readouts, such as colorimetric/fluorescent dyes or by using techniques such as immunoassay, multiplex assays, ECL-based assays, flow cytometry and gene expression [31,36–40].

In general, biological therapeutics function either as agonists or antagonists. Agonistic biologicals such as cytokines, growth factors, hormones or monoclonal antibodies (mAbs) induce a functional response by binding directly to target cell-surface receptors, while antagonistic biologicals such as soluble receptors and mAbs function by blocking or inhibiting receptor–ligand binding. Understanding the mode of action and the target and effector pathways of the therapeutic is critical for identification of a suitable cell-based assay and end point. Often a potency assay serves as the basis of a NAb assay, however, this needs to be tailored prior to its use as a NAb assay. Continuously growing cell lines that bear receptors for the therapeutic or those that have been transfected with an appropriate receptor construct rather than primary cells are generally preferred [41,42].

Cell-based bioassays are complex, time consuming and can be lengthy, cumbersome and frequently variable. Although use of novel approaches, such as employing cell-based quantitative PCR assays [43] or chemically treated division-arrested cells transfected with an enzyme-linked reporter gene controlled by a cytokine-responsive promoter [44] overcomes some of the issues, cell-based assays are often influenced by the sample matrix and require significant validation. Consequently, these assays present several inherent technical challenges that need to be considered when developing and designing a NAb assay. For example, the assay should be capable of distinguishing the antibody from other sample components, such as complement, coagulation factors, soluble receptors, lipids, concomitant medications and administered product. Where feasible, implementation of approaches for overcoming problems, such as matrix effects and for assessing specificity of the antibodies are justified and important for providing sound scientific data. A specificity step has been described for the detection of neutralizing erythropoietin antibodies [37]; but other strategies may also be used [36,45]. However, it is important to implement this strategy simultaneously with the neutralizing antibody assay.

Gupta *et al.* have published recommendations for the design, optimization and qualification of NAb bioassays that provide a good starting point for assay development and understanding the potential challenges that may be encountered [36]. The draft FDA guidance covers validation aspects of NAb bioassays [8]. In addition, several validated cell-based methods, which may prove to be useful for validating NAb assays for use in clinical studies, have been published [37,42,46].

■ Competitive ligand-binding assays

Several laboratories are focusing their efforts in developing reliable and reproducible noncell-based competitive ligand-binding assays (that functionally mimic cell-based neutralization assays) for detection of neutralizing antibodies. In a CLB-based NAb assay, a microtiter plate or tube containing target (receptor or ligand) is incubated with a defined concentration of the therapeutic. The binding of the therapeutic to the target is then assessed directly (labeled therapeutic) or indirectly (detection reagent to therapeutic) by using any of the available detection systems (e.g., enzymatic, radiochemical, fluorometric, chemiluminescence, electrochemiluminescence or SPR technology) for the CLB assay. The CLB NAb assay provides a simple, sensitive approach for assessing the neutralizing potential of the antibodies, since it often employs a straightforward ELISA or the ECL platform described previously.

Competitive ligand-binding NAb assay formats are much easier to develop as compared with functional cell-based assays. Baltrukonis *et al.* compared NAb results from a 3-month toxicity study in monkeys treated with an agonistic CD40 drug and observed a significant correlation between the results of a CLB and cell-based assay [46]. A recent study also showed a significant correlation in the titres of neutralizing anti-IL-17 antibodies in autoimmune patients using an ECL-based CLB assay and a cell-based bioassay despite the higher sensitivity of the bioassay as opposed to the CLB NAb assay [47].

Although CLB assays appear to be useful and may be extendable to different biotherapeutics, the utility of these assays versus cell-based bioassays is currently being debated within the biotechnology industry and regulatory agencies. The EMA guideline primarily advocates the use of bioassays for all therapeutics and states that “if neutralizing cell-based assays are not feasible/available, CLB assays or other alternatives may be suitable. However, when these are used it must

KEY TERM

Biosimilar: Applies to a noninnovator product approved in Europe. This product, as per the requirements of the European Medicines Agency, has demonstrated similarity to the innovator (reference) product in terms of its quality, efficacy and safety

be demonstrated that they reflect neutralizing capacity/potential in an appropriate manner.” From this statement, the preference of the regulators for a cell-based NAb assay is evident [103].

A pragmatic approach is however needed, as in certain situations it may be preferable to use a CLB assay instead of a cell-based bioassay. This is particularly pertinent in cases where the therapeutic is an antagonist, since the mode of action involves inhibiting binding of the therapeutic to its target. Assessment of neutralizing antibodies in this scenario is complex, as the response will measure the abrogation of inhibition (or inhibition of inhibition). In some instances, it may not be possible to develop a cell-based NAb bioassay with the desired sensitivity and performance and so a CLB assay may be the only available option.

Much evidence regarding neutralizing antibodies and clinical impact has also been amassed from data derived from cell-based NAb assays. For example, in patients receiving IFN- β products for treatment of multiple sclerosis, the presence of neutralizing antibodies was correlated with a loss of efficacy and relapse in multiple sclerosis [48,49]. Similarly in patients treated with GM-CSF, results from neutralization assays correlated with the effect of antibodies on clinical response [19,50]. Data from clinical trials with natalizumab (Tysabri) also showed that neutralizing antibodies were associated with a loss of efficacy in multiple sclerosis patients [51]. However, there are also examples in which NABs appear to have no impact on drug efficacy, pharmacodynamics or adverse events. Therefore, when interpreting data from antibody assessments, it is important to ensure that the results of NAB assays are evaluated in the context of other clinical endpoints to determine their significance in terms of clinical outcome.

Relative immunogenicity

For products where relative immunogenicity needs to be considered, products should be evaluated during comparative clinical trials employing the same clinical protocols (e.g., routes of administration, treatment schedules, sampling procedures, time points including baseline/pre-treatment sample and storage conditions) and antibody assays for generation of valid comparative data. Inclusion of patients at the same disease stage for each arm of a study is also important [52]. Thus, clinical trials have to be designed, bearing in mind that relative immunogenicity needs to be assessed if valid conclusions are to be derived for a comparator product (e.g., a **biosimilar**) relative to the

reference product. Data should include antibody incidence, relative titers and characteristics of the antibodies induced with each product. Following this, a meaningful correlation with clinical data on safety and response can be undertaken with the products used in a study. If significant differences in immunogenicity profile of the two compared products are detected, it can be concluded that the products are significantly different.

Future perspective

Assessment of unwanted immunogenicity is an integral part of product development and needs to be conducted in a systematic manner. Issues relating to immunogenicity are likely to continue throughout the entire life-cycle of a biological product, for example, during product comparability following process/production changes if these are introduced. They are also very important during development of a biosimilar and should be included in postmarketing monitoring and considered in risk-management and risk-mitigation plans for all products. As more biotechnology products are licensed, our knowledge of issues that can result in unwanted immunogenicity is likely to increase. Undoubtedly, this will enable a better understanding of problems that must be avoided and solutions that can be implemented to avert the risk of immunogenicity. Moreover, continued attempts towards the ability to predict immunogenicity are likely to result in methods and strategies aimed at successfully achieving this goal. An analysis of different prediction methods with antibody assessment data and clinical outcomes in patients in prospective clinical trials should prove useful in addressing the utility of these tools as predictors of immunogenicity of a product and its relevance in a clinical setting. With such safeguards and pharmacovigilance in place, the risk of immunogenicity of a biotherapeutic should be minimized, resulting in better and safer products.

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Executive summary

- This article provides a brief background on issues relating to immunogenicity and the guidance available.
- Factors and risks that need to be considered prior to developing a strategy for assessment of immunogenicity have been discussed and the need for a case-by-case rather than a generic approach is emphasized.
- Approaches that are being used for the prediction of immunogenicity are considered very briefly but the reader is also directed to further recent reviews on the subject.
- The methodologies that are being used for antibody detection and characterization in clinical assessment of immunogenicity have been discussed.

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