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Approaches for the Detection and Analysis of Anti-Drug Antibodies to Biopharmaceuticals: A Review

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Abstract

Antibody-based therapeutic agents and other biopharmaceuticals are now used in the treatment of many diseases. However, when these biopharmaceuticals are administrated to patients, an immune reaction may occur that can reduce the drug's efficacy and lead to adverse side effects. The immunogenicity of biopharmaceuticals can be evaluated by detecting and measuring antibodies that have been produced against these drugs, or anti-drug antibodies (ADAs). Methods for ADA detection and analysis can be important during the selection of a therapeutic approach based on such drugs and is crucial when developing and testing new biopharmaceuticals. This review examines approaches that have been used for ADA detection, measurement, and characterization. Many of these approaches are based on immunoassays and antigen binding tests, including homogeneous mobility shift assays. Other techniques that have been used for the analysis of ADAs are capillary electrophoresis, reporter gene assays, surface plasmon resonance spectroscopy, and liquid chromatography-mass spectrometry. The general principles of each approach will be discussed, along with their recent applications with regards to ADA analysis.

Keywords: Anti-drug antibodies; Biopharmaceuticals; Monoclonal antibodies; Immunogenicity; Binding assay

Abbreviations

ABT: Antigen binding test

ACE: Affinity capture elution

ADA: Anti-drug antibody

ADL: Adalimumab

bt: Biotin

CDR: Complementary determining region

CE: Capillary electrophoresis

cIEF: Capillary isoelectric focusing

ECL: Electrochemiluminescence

ELISA: Enzyme-linked immunosorbent assay

F_{ab}: Fragment antigen-binding

F_c: Fragment crystallizable

HMSA: Homogenous mobility shift assay

HPLC: High-performance liquid chromatography

HRP: Horseradish peroxide

IFX: Infliximab

Ig: Immunoglobulin

IgA: Immunoglobulin A

IgD: Immunoglobulin D

IgE: Immunoglobulin E

IgG: Immunoglobulin G

IgM: Immunoglobulin M

LC-MS: Liquid chromatography-mass spectrometry

mAb: Monoclonal antibody

PIA: pH-shift-anti-idiotype antigen binding test

Prot. A: Protein A

RGA: Reporter gene assay

RIA: Radioimmunoassay

SEC: Size exclusion chromatography

SPR: Surface plasmon resonance

TMB: 3,3',5,5'-tetramethylbenzidine

TNF-α: Tumor necrosis factor alpha

1 Introduction

1.1 Biopharmaceuticals and Therapeutic Monoclonal Antibodies

Biopharmaceuticals that are based on antibodies, proteins, or related biological agents have become common in recent years. For instance, the 100th monoclonal antibody (mAb) product has recently been approved by U.S. Food and Drug Administration (FDA) [1]. In 2020 alone, the U.S. FDA approved 13 mAbs for use as therapeutic agents [2]. Antibody-based biopharmaceuticals now make up an important component of the therapeutic market, with most of the top-selling pharmaceuticals in recent years being based on mAbs [3-7]. Adalimumab (Humira, from Abbvie), infliximab (Remicade, Johnson & Johnson), rituximab (Rituxan, Roche), and pembrolizumab (Keytruda, Merck) are all examples of mAbs that are used for this purpose [5,6,8-13].

The target specificity of mAbs [4,6,7,9,12-14] has made these agents of great interest as therapies for otherwise intractable diseases, such as various forms of cancer and autoimmune disease [3,8]. It is further possible through recombinant techniques to tailor many of the properties of mAbs, such as their affinity, size, and general structure [5,7-9,11,13]. However, because mAbs and other biopharmaceuticals are human-made and foreign to the body, there is a possibility that these agents may elicit an immune response when they are administered to a patient [9,11,14-18]. Although not all cases of an immune response may lead to adverse effects, the development of immunogenicity can lead to a loss of efficacy for a biopharmaceutical [14-18]. Over time, this can mean that an increase in dose is needed for the biopharmaceutical or that this drug may have to be discontinued if a more severe immune response occurs [11,14,15,17,18].

The immune response to a biopharmaceutical, such as an mAb-based drug, can be detected through the analysis of anti-drug antibodies (ADAs) [9,11,14,17,18]. As an example, the occurrence of ADAs is a known factor in the therapeutic failure of mAbs (e.g., adalimumab) that

bind to and inhibit tumor necrosis factor alpha (TNF-α), as can occur through the formation of drug-ADA complexes and neutralization of the drug's activity [15,16,17]. These effects have resulted in an increasing need for sensitive, precise, and reliable ADA assays [14,17,18]. Many approaches are now available to detect, measure, and characterize ADAs. This review will describe the various techniques that have been used in ADA detection or analysis and discuss the applications of each approach.

1.2 General Properties of Antibodies and Monoclonal Antibodies

Antibodies are glycoproteins that are produced by the immune system in response to foreign agents, or antigens [4,9,19]. The basic structure of a typical antibody, as represented by immunoglobulin G (IgG), consists of two identical long (or "heavy") polypeptide chains and two shorter ("light") polypeptide chains that are joined through disulfide bonds into a "Y"-shaped form (see Figure 1). The two upper arms of this structure contain the fragment antigen-binding (F_{ab}) regions of the antibody, which is where the antibody has sites that can selectively bind to their given target. The lower stem of the antibody is known as the fragment crystallizable (F_c) region. The function of this region is to mediate interactions between the antibody and other components of the immune system [4,5,9].

Antibodies belong to the immunoglobulin (Ig) superfamily, which can be divided into five common classes (or isotypes): IgA, IgD, IgE, IgG, and IgM (see Figure 1 for examples). The IgD, IgE, and IgG classes of antibodies are expressed as monomers, while IgM presents in the body as a pentamer of the basic Ig structure, and IgA is commonly found as a dimer [5,6,9,12,19]. Each of these classes has different functions. For example, IgM is the most abundant type of antibody that is produced in blood at the early stages of an immune response, with IgG being the dominant Ig class in the circulatory system as immunity to a foreign agent becomes established [12,19].

mAbs are antibodies that are produced by a single clonal line of cells. This group of antibodies is valuable as both reagents for antibody-based assays and as biotherapeutic agents because of their well-defined binding strengths and specificities for their target antigen [5,6,8,19,20]. In contrast to this, antibodies that are normally produced by the immune system are highly heterogeneous and are produced by many cell lines in the immune system, giving a mixture referred to as "polyclonal antibodies" [9]. During the development of mAbs for therapeutic applications, the immunoglobulin isotype is carefully selected, such as to provide relatively long half-lives for the drug during its use in treatment [12,17]. In practice, IgG-class antibodies are the most frequently used form of immunoglobulins employed to produce therapeutic mAbs [4,6].

The level of immunogenicity of mAbs in humans is related to the antibody's level of modification [8]. The first generation of mAbs was created through the hybridoma technique of Kohler and Milstein, which involved the fusion of mouse myeloma cells with B lymphocytes to produce murine mAbs [5,9,11,12]. Although murine mAbs are valuable as reagents in biochemical research, their immunogenicity in humans has limited their therapeutic utility [5,8]. It was later found that human-like or humanized mAbs, which contain fewer structural differences from human antibodies, induced a lower immune response than murine mAbs [9]. Several types of human-like or humanized mAbs have now been developed [9,11]. For instance, combining murine-derived variable regions from a mouse with the constant region from a human antibody will result in a "chimeric" mAb. In a "humanized" mAb, all sections of the murine antibody have been replaced with human counterparts except the antigen recognition regions. In a "fully-humanized" mAb, all parts of the antibody are of human origin and do not contain any part from another species [8].

1.3 Anti-Drug Antibodies

Immunogenicity refers to the immune response of a host against a foreign agent, such as a therapeutic mAb [11,14,17]. For a biotherapeutic, this response is reflected by the generation of ADAs by the immune system [14,17,21]. The formation of ADAs against a biotherapeutic can result in adverse effects during a patient's treatment, such as an increased clearance rate and hypersensitivity to the drug [14]. A low success rate (~3%) for the murine mAbs that were used in the first-generation of biopharmaceuticals demonstrated the effects that could be caused by immunogenicity [5]. However, it was also found this immune response could be mediated by designing mAbs with more human-like structures through recombinant DNA techniques and phage display methods [11,12]. The use of humanized mAbs that have been made through these approaches has increased the average success rate of these biotherapeutic agents to 25% [5].

The presence and levels of ADAs are now considered measures of immunogenicity by biotherapeutic agents such as mAbs [21]. In addition, ADAs can impact the pharmacodynamics and pharmacokinetics of their target drug, thereby reducing this drug's efficacy [11,12,14,17]. In some cases, ADAs can cause severe adverse effects in patients with chronic conditions [22]. ADAs can be divided into two major categories: neutralizing and non-neutralizing [11,14,17,23,24]. Neutralizing ADAs can directly block and interfere with the functional activity of a biotherapeutic agent to bind to its desired target. Non-neutralizing ADAs (or "binding" ADAs) bind to the biotherapeutic agent but do so at a region that does not affect the drug's ability to bind to its target [23]. Neutralizing ADAs are generally considered more important in a clinical setting because they directly affect a drug's biological activity, but non-neutralizing ADAs may also indirectly reduce a drug's efficacy [24].

As a specific example, it is known that ADAs can be produced following the treatment of a patient with biotherapeutics that are TNF-α antagonists. This group of drugs includes mAbs such as infliximab and adalimumab that are used to treat chronic inflammatory disease (e.g., rheumatoid arthritis, psoriatic arthritis, and Crohn's disease) [8,25]. TNF-α antagonists reduce the inflammatory response by targeting TNF-α and inhibiting its binding to the TNF-α receptor [9,17,23,24]. Neutralizing ADAs can directly interfere with the binding of the TNF-α antagonists to their target molecule (i.e., TNF-α), thus directly preventing the drug's biological activity [26]. Non-neutralizing ADAs can indirectly reduce the same drug's efficacy by compromising its bioavailability and alter the drug's clearance from the circulation [14,17,22], such as through the formation of ADA-drug immune complexes [24].

2 Immunoassays and Binding Assays for Anti-drug Antibodies

The development of assays with high sensitivity, precision, and specificity for ADAs has become critical for the quality control of biopharmaceuticals and in examining the treatment of patients with these drugs [27-34]. Many of these methods are based on immunoassays [33,34]. Immunoassays are analytical techniques that utilize an antibody or antibody-related reagent to identify or measure a target analyte [35]. The strong binding of antibodies with their antigens allows immunoassays to be used for the selective detection of their target even in complex biological samples such as serum, plasma, urine, tissue, and blood [35,36].

Detection and quantification of an analyte by an immunoassay is accomplished by using a label that can be conjugated to one of the assay components and monitored at a suitable level for measurement of the target [35,36]. The most common labels that are employed in immunoassays are enzymes, fluorescent tags, chemiluminescent labels, and radioactive isotopes. These labels can be used with antibody-antigen binding in competitive or non-competitive and homogeneous

or heterogeneous formats [35,36]. If a biological agent other than an antibody or antigen is used in one of these formats, the result is sometimes given the more general name "binding assay". Specific examples of immunoassays and binding assays that have been employed in the measurement or detection ADAs are discussed in this section.

2.1 Antigen Binding Tests

An antigen binding test (ABT) is one type of binding assay that has been used to assess the immunogenicity of biopharmaceuticals [27,29,37-44]. In an ABT, antibodies or their corresponding antigens are immobilized and used to capture their corresponding binding partner, or target analyte. Various types of labels can be incorporated within this assay to generate a signal for detecting or determining the amount of the captured analyte [38-40].

One format for such an assay is a pH-shift-anti-idiotype antigen binding test (or PIA), as illustrated in Figure 2 [39,40]. This method first makes use of an acid dissociation step to release ADAs from any drug that is bound to these antibodies; this solution is then neutralized and $F_{(ab)}$ fragments that can bind to the drug are added to prevent rebinding by the drug with the ADAs. The ADAs and drug are then both adsorbed to a support containing immobilized protein A, which can bind to the ADAs (e.g., from various subclasses of IgG) and to the drug through their F_c regions. Labeled $F_{(ab)2}$ portions of the drug are then added to bind to and detect the captured ADAs [39]. This method has been used with ^{125}I-labeled $F_{(ab)2}$ portions of adalimumab to measure ADAs in the presence of this mAb-based drug [39-41].

2.2 Electrochemiluminescence Assays

In a binding assay that is based on electrochemiluminescence (ECL), a signal is produced by using an applied potential to produce light from a chemical tag [37,44-46]. The tag reaches an

excited state through an oxidation/reduction reaction, followed by relaxation of the excited state into a lower energy state through light emission. This type of detection has been employed in assays for ADA detection by using a ruthenium label (e.g., *tris*(bipyridine)ruthenium(II), or $[Ru(bpy)_3]^{2+}$) [44-46]. In this method, ADAs from a sample are allowed to bind with a form of the target mAb drug that contains two labels: biotin and ruthenium. The bridged complexes of ADAs with the labeled drug are then allowed to adsorb to a gold plate that contains immobilized streptavidin, which can bind with the biotin tag. The signal due to ECL for the adsorbed complexes and their associated ruthenium tags is then measured [37,44-46].

An ECL binding assay was used in one study in which polyclonal and affinity-purified antibodies against five mAb-based drugs were used as model ADAs. Samples of these antibodies were incubated with biotin- and ruthenium-conjugated forms of the drugs, followed by measurement of luminescence by the ruthenium tag [44]. The general scheme for such an approach, with the use of an acid dissociation step, is shown in Figure 2 [37]. ADAs for the given drugs were detected at levels down to 5-64 µg/L and over a wide dynamic range (i.e., 10-10,000 µg/L) [44]. Ruthenium conjugates in a sulfonated form (i.e., a sulfo-tag, which helps enhance water solubility) have also been employed in ADA assays with ECL detection [37,46].

A strategy using both precipitation and acid dissociation of drug-ADA complexes has been explored for ADA detection in assays with ECL detection [46]. In this method, ADAs in samples were saturated by adding an excess of the corresponding mAb-based drug to form immune complexes, followed by precipitation of these complexes using polyethylene glycol. The drugs and ADAs were then released from the complexes under acidic conditions and adsorbed to a plate for detection. Sulfo-tagged forms of the drugs were next applied to bind with immobilized ADAs and to generate a signal due to ECL. An improvement in tolerance of the assay to high drug

concentrations and a high recovery of ADAs were found in this format when compared with ECL bridging immunoassays that used only acid dissociation [46].

Domain-specific detection has been carried out in an immunoassay with ECL detection for ADAs to moxetumomab pasudotox (i.e., a recombinant anti-CD 22 immunotoxin with two specific domains) [47]. This assay was based on the competitive binding of domain-specific ADAs with and without domain-containing molecules in the presence of biotinylated forms of the drug (i.e., for capturing ADAs) and a ruthenylated tagged form of the drug (for ECL detection). Signal inhibition occurred as a result of the binding by domain-specific ADAs with domain-containing molecules. This assay allowed for the detection of lower abundance domain-specific ADAs in presence of more dominant forms of domain-specific ADAs [47].

2.3 Radioimmunoassays

A radioimmunoassay (RIA) is a type of immunoassay that uses a radioactive isotope as the label for the detection of an analyte [48]. Iodine-125 is a common radioisotope that is employed as a label in RIAs [35,36,38]. Although the use of such a label can provide low detection limits, there are also issues with safety, handling, and label storage that need to be considered when using a radioisotope as a tag. Other items to consider are the possible denaturation of the labeled binding agent and eventual loss of signal over time when radiolabels are employed [48].

A few studies have used RIAs for the analysis of ADAs [37,49,50]. Acid dissociation of drug-ADA immune complexes has been utilized with an RIA to overcome the underestimation of ADAs that can occur when such immune complexes are present. This approach has been called an acid-dissociation radioimmunoassay (ARIA) [37,49]. As is shown in Figure 2, in an ARIA unbound ADAs against the target biopharmaceutical are allowed to bind to biotinylated $F_{(ab')2}$ fragments against such antibodies, followed by acid dissociation of immune complexes is the

sample and later neutralization of the solution. The $F_{(ab')2}$-biotin-ADA complexes that are produced in this mixture are then captured by using a support containing protein A, which is used bind to the F_c regions of the ADAs or an mAb-based drug. The captured ADAs that are also bound to the biotinylated $F_{(ab')2}$ fragments are then detected by adding ^{125}I-labeled streptavidin, which combines with the biotin tag [37,49].

Other forms of the RIA have been used in the analysis of ADAs [37,50]. For instance, a similar approach to the PIA was used in a temperature-shift RIA (TRIA) for ADAs against adalimumab [37]. This method (see Figure 2) used biotinylated $F_{(ab')2}$ fragments that could bind to ADAs to replace the need for an acidification step in an ADA assay that was to be conducted in the presence of the drug of interest (i.e., adalimumab, in this case) [37]. In another report, a fluid-phase RIA that was combined with affinity chromatography was used to determine the levels of ADAs against infliximab and adalimumab in patient samples [50]. Free and antibody-bound forms of radiolabeled infliximab were separated in this work through the use of a support that contained anti-human immunoglobulin λ-chain antibodies [50].

2.4 Enzyme-linked Immunosorbent Assays

Enzyme-linked immunosorbent assays (ELISAs) are the most common type of immunoassays that are used to detect and measure ADAs [51-53]. Advantages of employing ELISAs for this purpose include their high sensitivity, low cost, ease-of-use, and relatively high throughput [52,53]. There are various ELISA formats that have been used for ADA detection, such as direct, indirect, and bridging (or sandwich) methods [51,52,54].

2.4.1 Direct and Indirect ELISAs

The direct assay format is the simplest type of ELISA. When used for the analysis of ADAs, this format involves the immobilization (e.g., through adsorption or covalent attachment) of ADAs in a sample onto an ELISA plate [52]. Next, secondary antibodies that contain an enzyme label such as horseradish peroxidase (HRP) or alkaline phosphatase are added to the plate and allowed to bind the immobilized ADAs. A suitable substrate for the enzyme label is then added to produce a signal that is proportional to the amount of ADAs that are present. As an alternative approach, biotin can be used to label the captured ADAs and then allowed to bind to a streptavidin-conjugated enzyme; this enzyme label is then later combined with its substrate to produce a signal that is related to the amount of captured ADAs [52].

One example of this approach is an affinity capture elution (ACE)-based direct ELISA that was developed to detect ADAs against mAb-based drug bamlanivimab [55]. An acid dissociation step was used to break up ADA-drug complexes in the samples, followed by use of the target drug on a solid-phase support to capture the free or released ADAs. The captured ADAs were then eluted from the support by employing a second acid dissociation step. The eluted ADAs were then allowed to adsorb to an ELISA support surface and detected after the addition of a biotinylated form of the drug, HRP-labeled streptavidin, and a substrate for HRP. It was found that this direct ELISA format could detect as low as 500 ng/mL of ADAs in the presence of a 1000-fold excess of the mAb-based drug [52].

Although a direct ELISA can be simple and easy to perform, the use of this method for the detection and measurement of ADAs does have several disadvantages. First, immobilization of the ADAs onto an ELISA plate can cause changes in the conformation of these antibodies and mask certain regions in their structures; this masking may hinder binding by these captured agents

with secondary binding agents [52,56]. In addition, because the immobilization of ADAs onto an ELISA support or plate is usually not specific, other proteins in the sample can bind to this support/plate and possibly lead to a high background signal [21,29].

In an indirect ELISA for ADAs, the original biopharmaceutical agent is added to the ELISA plate after the immobilization of the ADAs. An enzyme-labeled form of a secondary form of this antigen is then added to the plate. This forms a labeled immune complex that can generate a signal that is directly proportional to the amount of ADAs on the plate. A key feature of the indirect ELISA is the requirement for a species-specific secondary antigen for detection, an item that can be an issue when animal serum is used as a positive control for the detection of ADAs in human serum [52]. However, a bridging ELISA (as discussed in the next section) can be used to overcome this problem and allow the detection of antibodies for any isotype or species [52,56].

2.4.2 Bridging ELISAs

A bridging ELISA uses two binding agents, such as the target drug, to interact with different regions on an ADA (see Figure 3) [29,52,53,57]. One of these binding agents is coated on the ELISA plate and used to capture the ADA. The second binding agent, which contains an enzyme label, is then added to the mixture, allowed to bind to the captured ADA, and to produce a signal for detection [29].

The use of the two binding agents that are specific to different regions on an ADA makes the bridging ELISA more specific than the direct and indirect ELISA formats, a feature that is useful when working with complex samples. Other advantages of a bridging ELISA over direct and indirect ELISAs are that it is highly sensitive and does not require species-specific antibodies as reagents [55]. Moreover, this method is more tolerant to the concentration of the circulating

drug than the direct and indirect assay formats [52]. Due to these advantages, the bridging ELISA is currently the most common ELISA format that is used for the detection of ADAs [51,55,58,59].

However, the bridging ELISA also has disadvantages [29]. For instance, this type of assay is more complex than direct or indirect ELISA methods due to its use of two binding agents for separate regions on the ADAs. Furthermore, the bridging ELISA format may be unable to detect low affinity ADAs and monovalent antibodies [51,52,55,60]. Although this format is highly tolerant to the presence of circulating drugs, it is still susceptible to interferences from high concentrations of these drugs [55,60,61]. The issue of drug interference can be minimized to some extent when this method is combined with solid-phase extraction and acid dissociation [24,55,57,62]. This latter approach relies on the fact that complexes formed between antibodies and immobilized antigens are generally more stable than complexes that form between antibodies and soluble antigens or that occur in antibody-antibody complexes [57,63-69].

Bridging ELISAs have been used to detect ADAs against various biopharmaceuticals and mAb-based drugs; examples include ADA assays for erythropoietin, interferon beta, metuzumab, adalimumab, infliximab, and eternacept [43,57,62,70-76]. A bridging ELISA assay was used to detect antibodies against metuzumab by placing some of this drug on a microtiter plate to capture ADAs against this drug [53]. HRP-labeled metuzumab was then added and used with a chromogenic substrate (3,3',5,5'-tetramethylbenzidine, or TMB) to provide a limit of detection of 0.39 ng/mL and a linear range of 0.39-50 ng/mL. In another study, a bridging ELISA was used to detect IgE-class ADAs against the mAb-based drug MAB072 [77]. This work employed plates that were coated with the human IgE receptor FcεRIα to capture IgE-class ADAs from serum samples. A biotinylated form of MAB072 was then added and allowed to bind to the captured IgE-class ADAs, followed by the addition of streptavidin-labeled HRP and TMB as a chromogenic

substrate for detection. Another study used a bridging ELISA that combined a covalently coupled high-density antigen surface with acid dissociation to minimize interferences from high drug concentration during the analysis of ADAs against an mAb-based chemotherapeutic drug [57]. In addition, a bridging ELISA has been combined with solid-phase extraction based on the biotin-avidin system and acid dissociation pretreatment to separate and measure, ADAs and drug-bound ADAs from an mAb-based drug [62].

A growing number of biotherapeutic agents contain engineered sequences or several functional domains [78,79]. Work in one study used an ELISA-based assay for the detection ADAs and characterization of the immunogenic parts of a bispecific F_{ab} fragment [80]. This method combined an indirect bridging ELISA with a molecular engineering approach that used several domain detection assays based on variants of the drug. Ranibizumab, an anti-VEGF F_{ab} agent which has no mutations in the constant region, was used as a control for the assay. The results showed that ADAs were mainly directed against both antigen-binding sites of the drug. Moreover, this method could distinguish between antibodies against one or both antigen-binding sites and the constant domain region of the mAb-based drug [80].

In recent years, bridging ELISA formats have been developed for the measurement of immune complexes rather than just free ADAs, or for the analysis of both ADAs and immune complexes [81-86]. An example is work in which a bridging ELISA was used to measure circulating ADA-human IgG immune complexes that were found in the plasma of mice that had been treated with adalimumab [81]. In this assay, polyclonal goat anti-human IgG was immobilized onto an ELISA plate and used to capture ADA-human IgG complexes that were present in the samples. Next, HRP-labeled goat anti-mouse IgG and a substrate for HRP were added for detection [81]. In a similar study, acid dissociation was used with a bridging ELISA and

chromatography employing a size exclusion support to quantify and characterize drug-ADA immune complexes [82]. A biotinylated mAb against the complementary determining region (CDR) of the target drug was immobilized onto a streptavidin-coated plate and was used to capture the drug-ADA immune complexes from samples. This was followed by the addition of a digoxigenin-labeled mAb against the CDR of the drug, followed by the addition of HRP-labeled anti-digoxigenin F_{ab} fragments and the use of 3-(4-hydroxyphenyl)propionic acid as an HRP substrate for fluorescence-based detection [82].

2.5 Homogeneous Mobility Shift Assays

The homogenous mobility shift assay (HMSA) is a method that combines a ligand binding assay with size exclusion chromatography (SEC) [87-93]. SEC is a chromatographic technique that can separate target compounds based on their relative size and shape [87,90,92,94]. This separation is carried out by using a column that contains a porous support. Because smaller targets will be able to access more of the pore volume of the support, they will elute later from the column than larger compounds. The use of SEC in an HMSA method makes it possible to discriminate between isotypes of ADA with different sizes, such as those based on IgG vs IgM or IgA [87,92,94].

The general approach in an HMSA is illustrated in Figure 4, as shown for the application of this method in the detection and size-based separation of ADAs against infliximab in human serum [87]. In this report, an acid dissociation step was used to release ADAs from ADA-drug complexes. The solution containing the released ADAs was then neutralized and incubated with a mixture of fluorescent-labeled infliximab (i.e., using an Alexa Fluor 488 tag) and a form of the drug with its active-site blocked, with the latter being used as an internal standard. To measure concentration of infliximab in the samples, the same procedure was followed but now with the addition of Alexa Fluor 488-labeled TNF-α and no acid association step. The results of this method

were compared to those of a bridging ELISA. It was found this method could detect down to 0.036 ug/ml ADAs in the presence of a high concentration (60 ug/ml) of infliximab in serum [87].

The same approach has been used with other types of ADAs and biopharmaceuticals (e.g., adalimumab) [40,87-93]. For instance, dimeric and multimeric immune complexes of adalimumab and 11 different ADAs were identified and separated by using a HMSA [40]. It was reported that neutralizing ADAs could be discriminated from other components in adalimumab- and infliximab-treated samples by using HMSAs [90]. A correlation was found in the detection of neutralizing antibodies from HMSA and another assay based on affinity capture and elution [90]. A similar comparison was made with an ELISA [90,91]. HMSA has also been employed to identify ADAs to the mAb-based drugs natalizumab and ustkinumab [92,93].

A modified, competitive binding form of HMSA was developed to measure a protein-based therapeutic in the presence of another drug in the same class [94]. Adalimumab was used as a model drug and the serum concentration of adalimumab was quantified in this work. Serum samples that contained infliximab, as a related drug, were mixed with fluorescent-labeled adalimumab, followed by the addition of ADAs to adalimumab and use of a 1 h incubation step to allow the formation of immune complexes. The SEC component of the HMSA was used to discriminate the free drug (molar mass, ~150 kDa) from the adalimumab-ADA dimer (~300 kDa), and an ELISA was used to validate the HMSA method [94].

3 Other Methods for the Analysis of ADAs

Several other approaches besides immunoassays and binding assays have been employed for the analysis of ADAs. These other techniques have included capillary electrophoresis, reporter gene assays, surface plasmon resonance spectroscopy, and liquid chromatography-mass

spectrometry. This section will examine the general principles and applications for each of these approaches as related to the detection, measurement, or identification of ADAs.

3.1 Capillary Electrophoresis

Capillary electrophoresis (CE) is a separation technique that is based on the differential migration of analytes through a capillary and in the presence of an applied potential [95-98]. Factors that affect the rate of migration and mobility of an analyte in CE include the size and charge of the analyte as well as the pH, temperature, and composition of the background electrolyte within the capillary. Several modes of CE have been used to examine antibody-based drugs and antibody-drug conjugates. These modes have included capillary zone electrophoresis, capillary isoelectric focusing (cIEF), and capillary gel electrophoresis [95,98].

CE has been used to screen ADAs against antibody-related biotherapeutics, including nanobodies [96]. Nanobodies are recombinant VHH fragments derived from the unique heavy chain antibodies that are produced by animals belonging to the camelids family [96]. Each VHH fragment can be linked by short peptide sequences to construct a nanobody that is equipped with specific binding properties. Nanobodies, which have a molar mass of around 15 kDa, are much smaller than traditional polyclonal or monoclonal antibodies (typical molar mass, ~150 kDa for IgG-class antibodies). The smaller size of nanobodies, along with the specificity and strong binding they exhibit for their targets, have made these agents appealing as platforms for biotherapeutics [99].

One report described an automated CE method that was a modified version of a capillary Western blotting system [96]. This method was used to detect ADAs to three distinct multi-modular nanobodies. In this approach, nanobodies were immobilized onto the surfaces of capillaries to analyze the levels of ADAs in ADA-positive mice samples. CE was first used to

separate ADAs from other sample components based on differences in their sizes and migration times. The separated ADAs were then immobilized within the capillary through photoactivated capture chemistry. HRP-labeled secondary antibodies where then added to bind to and measure the immobilized ADAs through the production of light in the presence of luminol and peroxide. Induced immune responses from various regions in multi-modular nanobodies were examined through the approach [96].

Another CE-based approach for ADA detection against nanobodies was created that used the Peggy Sue system to reduce the impact of a denaturation step on the sample [98]. The Peggy Sue system is a charge and size-based separation and quantification system which combines features of immunoassays with a Western blot and cIEF. In this technique, a pH gradient is generated in a capillary and then used in cIEF to separate proteins based on their isoelectric points, or pI values. Samples that may contain ADAs are incubated within the capillary and with the separated proteins to form immune complexes. Detection of these immune complexes can then be accomplished by using enzyme-labeled secondary antibodies to the given species of ADAs [98].

3.2 Reporter Gene Assays

A reporter gene assay (RGA) is another approach that has been used to examine ADAs [100-103]. The RGA is a cell-based assay that uses a signal based on light, such as bioluminescence, to investigate a given gene in cells. This is accomplished by measuring the light emission that results from the interactions between a promoter and reporter genes for enzymes or proteins that can be linked to a bioluminescent signal, such as luciferase, green fluorescent protein, chloramphenicol acetyltransferase, aequorin, or β-galactosidase [102,103]. For instance, luciferase can produce bioluminescence through its enzymatic activity, while green fluorescent protein can directly produce a signal due to its inherent fluorescence.

RGA has been applied in studies of protein-based biotherapeutics and related ADAs (see Figure 5) [100,101]. A TNF-α responsive reporter gene cell line using a NFkB regulated firefly luciferase and Renilla luciferase reporter genes was used to examine the activities of TNF-α and three TNF-α antagonists: infliximab, adalimumab, and etanercept. The TNF-α antagonists were monitored by using TNF-α-induced firefly luciferase activity in the assay. This response was later normalized that produced by the Renilla luciferase activity. The levels of neutralizing antibodies to the TNF-α antagonists were estimated by using the signal ratio for these two types of luciferase activities [100]. The same assay format was employed to screen infliximab activity and the activity of neutralizing ADAs in a large set of clinical samples. This demonstrated the potential use of RGAs in clinical studies for screening ADAs to TNF-α antagonists and the neutralizing activity of ADAs [101].

3.3 Surface Plasmon Resonance Spectroscopy

Surface plasmon resonance (SPR) spectroscopy is an optic-based measurement system that is often used for the study of biological interactions and as a biosensor platform [103-108]. This detection approach makes use of delocalized electrons between adjacent two interfaces, such as a dielectric material and a metal (e.g., gold or silver). In SPR, a light source is used to excite these delocalized electrons to produce a surface plasmon resonance wave. A dielectric, such as a prism, splits the incident light into the two sections that either resonate with this wave or that does not resonate. Without the resonance with the wave, the light is reflected; in the presence of resonance, the light excites the wave and results in a shift of refractive index of the incident light at the surface of the metal. This size of this effect can be altered by binding events that involve substances adsorbed on the metal surface, allowing SPR to be used as a tool for examining the extent and rate of biological interactions.

The detection and measurement of both ADAs and ADA-drug conjugates has been carried out by using SPR [103-109]. As an example, SPR was utilized to assess the immunogenicity of panitumumab, an mAb-based drug that binds to epidermal growth factor receptor [105]. In an SPR-based immunoassay, a sample of panitumumab was covalently immobilized on the sensor chip, and used to detect ADAs at levels down to 1 μg/ml. The SPR assay also made it possible to identify neutralizing antibodies in some samples [105]. SPR was used as alternative to bridging assay with ECL detection for ADAs to trebananib [106]. In another report, anti-adalimumab antibodies with dissociation equilibrium constants between 10^{-6} M and 10^{-9} M were found in ADAs that had been isolated from pediatric patient serum samples [107].

Several other applications have been reported for SPR as related to ADAs, such as the simultaneous measurement of biopharmaceuticals and their ADAs. One such application used a sensor array for SPR with six parallel strips, in which TNF-α, infliximab, and IgG-class antibodies were immobilized; this array made it possible to measure both infliximab and its ADAs within a few minutes in serum [104]. In addition, an SPR-based immunoassay with calibration-free concentration analysis was used to estimate the levels of active antibodies in a control sample that were known to bind human insulin, insulin degludec (Tresiba), or turoctocogalfa (NovoEight) [109].

3.4 Liquid Chromatography-Mass Spectrometry

The limitations associated with ELISAs and other ligand binding assays, such as interferences from circulating drugs and the inability to identify isotypes, have led to the development of approaches for ADA detection based liquid chromatography-mass spectrometry (LC-MS) [110]. LC-MS methods for ADA usually start with an immunocapture step to bind ADAs or related targets in a sample (see Figure 6). For example, a biotinylated form of an mAb-

based drug can be added to a sample to bind ADAs for that drug or associated ADA-drug immune complexes. In this case, magnetic beads that contain immobilized streptavidin could then also be added to capture the biotinylated form of the drug and any ADAs or ADA-drug complexes that are bound to this drug analog. An acid dissociation step can be used to release the target drug and the remaining ADAs can be digested using trypsin, giving peptides from the ADAs that can be quantified and characterized by LC-MS [110,111]. Some advantages of using immunocapture with LC-MS are that it allows for antibody isotyping and analysis of multiple classes of antibodies in a single assay [112-115].

Several studies in recent years have used immunocapture with LC-MS for the detection of ADAs to mAb-based therapeutics. One way this can be accomplished is through the indirect measurement of ADAs [110,116]. For example, immunoprecipitation has been used with LC-MS to provide an indirect assay for ADAs in serum that can also tolerate the presence of high drug concentrations [110]. This method involved the saturation of all available binding sites on the ADAs with the use of a high concentration of their target mAb drug. This step was followed by use of protein G (i.e., immobilized on magnetic beads) to isolate IgG-class ADAs that were bound to the added mAb-based drugs. After several washing steps and elution of the isolated ADA-drug complexes, stable isotope-labeled peptide standards were added. After a digestion step, several peptides from the mAb drug were quantified by using matrix-assisted laser desorption/ionization time-of-flight MS. The ADAs in the sample were indirectly measured by correlating the amount of a given peptide with the concentration of antibodies in positive control samples [110]. Another report used an immunocapture-LC-MS assay with indirect ADA detection to simultaneously quantitate the residual amount of an mAb drug, the IgG-class antibodies present in residual serum components, and neutralizing ADAs positive controls by using bead extraction and acid

dissociation [116]. This assay also used an indirect approach to quantitate neutralizing antibodies in human serum. It was found that bead extraction and acid dissociation effectively removed high concentrations of the mAb drug and interfering serum components from the samples [116].

A major limitation of ADA detection with an indirect immunocapture-LC-MS method is the inability to perform ADA isotyping [111]. This issue can be overcome by using immunocapture with LC-MS for direct ADA detection [114]. For instance, an immunocapture LC-MS assay was employed to simultaneously give semiquantitative information and to isotype ADAs in human plasma [113]. The assay uses a biotinylated form of the target drug or of an ADA that is bound to streptavidin-coated magnetic beads and used to capture ADAs or ADA-drug complexes from samples. This is followed by removal of the beads from the sample and use of an acid dissociation step with the beads to release the ADAs. Next, the ADAs were digested using trypsin. LC-MS was then utilized to detect and measure several, universal peptides that occur in the heavy chains of antibodies for the semi-quantitation and isotyping of the ADAs [113]. A similar scheme has been evaluated for use in the analysis of antibodies against biotherapeutics [117]. A related strategy for direct ADA analysis has used an immunocapture step that is based on the F_{ab} region of a mAb that is coated on a magnetic bead [118]. This technique has been used with LC-MS for the isotyping and semi-quantitation of ADAs against fully-humanized mAb-based drugs in monkey serum [118].

Although assays that combine immunocapture with LC-MS have shown great promise for the analysis of ADAs, they do have several limitations when compared with the more common ligand binding assays [112]. These limitations include some issues with reliability, sensitivity, and quantitative accuracy that have been reported with the use of selected reaction monitoring in LC-MS for the measurement of mAb-based drugs in pharmaceutical matrices. In addition, it can

be challenging to select an optimum peptide for use in quantification methods that are based on the signature peptides for ADA analysis [112].

4 Concluding Remarks

Biopharmaceuticals, such as those based on mAbs, have seen great growth and interest for use as drugs in recent years [1-7]. However, concerns about the immunogenicity of mAbs and other biopharmaceuticals, as reflected by the production of ADAs, have led to the need for arrays that can detect, measure, and characterize ADAs to these drugs [14,17,18]. This review discussed several assays and analytical approaches that have been created for this purpose and their applications. Many ADA assays depend on reactions that involve antigen-antibody binding or related biospecific interactions. These methods include various forms of immunoassays and antigen binding tests, such as several forms of ELISAs, RIAs, ECL-based methods, and newer HMSA methods. Other methods that have also been used for the analysis of ADAs involve the use of CE, reporter gene assays, SPR, or LC-MS.

This current set of methods has made it possible to determine ADA concentration with higher accuracy and good limits of detection. A number of schemes have further been employed in these methods to allow the analysis of ADAs in the presence of high doses of the corresponding biopharmaceutical or to identify different antibody classes of ADAs [37-39,41,71,78,84,88,91]. However, further work is still needed in this area. For example, several studies have shown inconsistent values across these methods when used with false positive or false negative samples and when determining absolute ADA levels in the same sample [17,34,41,91,92,94,103], a fact that indicates the need for more standardization of methods in this area. Other issues that have been reported include the presence of non-parallel titrations for samples in binding assays for ADAs, the tendency for some of these formats to underestimate ADA concentrations in the

presence of high biopharmaceutical levels, and the need to determine a standard set of cut-off points for ADA detection in these methods [32,34,43,52,102]. Thus, it is expected that efforts in this field will continue with the goal of producing even more robust and consistent assays for the analysis of ADAs for use in clinical and pharmaceutical testing.

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Figure Legends

Figure 1. General structure of an antibody, as illustrated using IgG, and examples of major classes, or isotypes, of antibodies (e.g., IgG, IgM, and IgA).

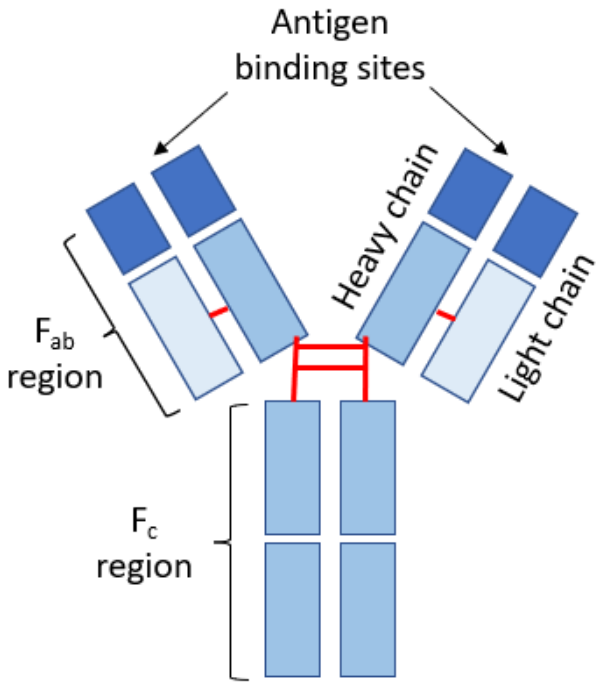
Figure 2. Examples of four types of immunoassays or binding assays that have been used for the analysis of ADAs to mAb-based biopharmaceuticals. These methods are illustrated by using adalimumab as the drug of interest. Abbreviations: ADA, anti-drug antibody; ADL, adalimumab; bt, biotin; prot. A, protein A. Reproduced and adapted from Ref. [37] with permission from Elsevier.

Figure 3. General scheme in a bridging ELISA for ADA detection.

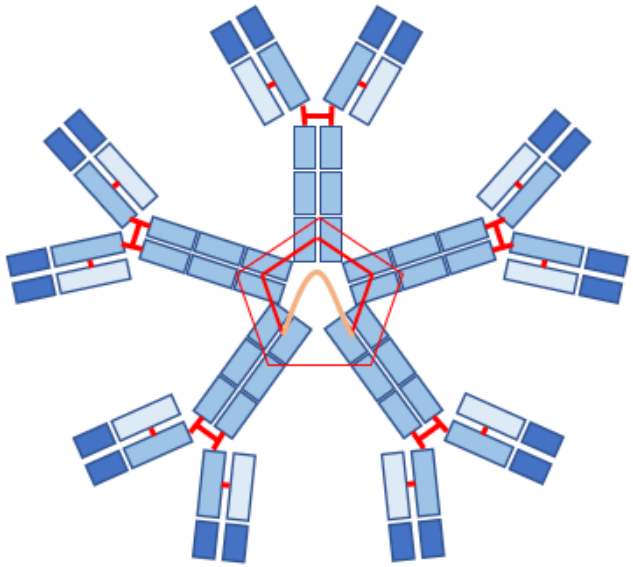
Figure 4. Scheme for a homogeneous mobility shift assay (HMSA) when used for (a) the detection of ADAs to a given drug or (b) the parent drug, using infliximab (IFX) and Alexa Fluor 488-labeled agents (e.g., IFX-488) in this example. The TNF-488 represents Alexa Fluor 488-labeled TNF-α. Terms: HPLC, high-performance liquid chromatography; SEC, size exclusion chromatography. Reproduced from Ref. [87] with permission from Elsevier.

Figure 5. TNF-α responsive reporter gene construction for use in measuring ADAs to TNF-α antagonists. In this figure, κB is the NFκB recognition sequence; the coding regions for firefly luciferase and Renilla luciferase are both shown. The intron is from the human β-globulin gene, SV40 Poly A is the SV40 polyadenylation site, and SV40 Min. prom. represents the SV40 minimal promoter. This figure is adapted from Ref. [100] with permission from Elsevier.

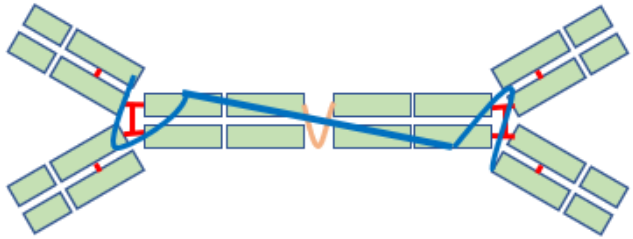
Figure 6. General scheme for the use of immunocapture with LC-MS for ADA detection.



Immunoglobulin G
(IgG, ~150 kDa)



Immunoglobulin M
(IgM, ~900 kDa)



Immunoglobulin A
(IgA, ~320 kDa)

Figure 1

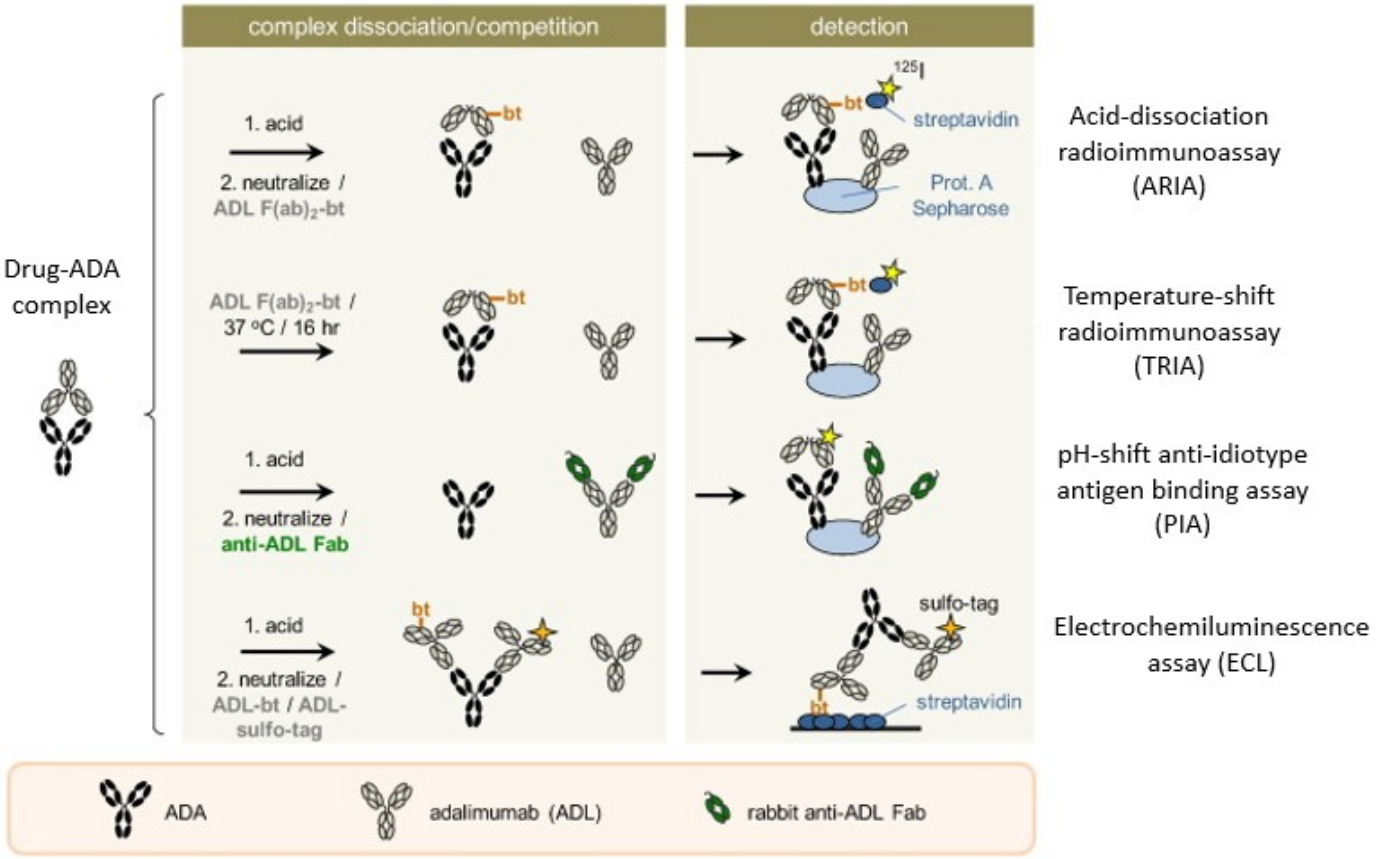

complex dissociation/competition
detection
Drug-ADA complex
1. acid
2. neutralize / ADL F(ab)2-bt
bt
125I
streptavidin
Prot. A Sepharose
Acid-dissociation radioimmunoassay (ARIA)
ADL F(ab)2-bt / 37 °C / 16 hr
Temperature-shift radioimmunoassay (TRIA)
2. neutralize / anti-ADL Fab
pH-shift anti-idiotype antigen binding assay (PIA)
2. neutralize / ADL-bt / ADL-sulfo-tag
sulfo-tag
Electrochemiluminescence assay (ECL)
ADA
adalimumab (ADL)
rabbit anti-ADL Fab

Figure 2

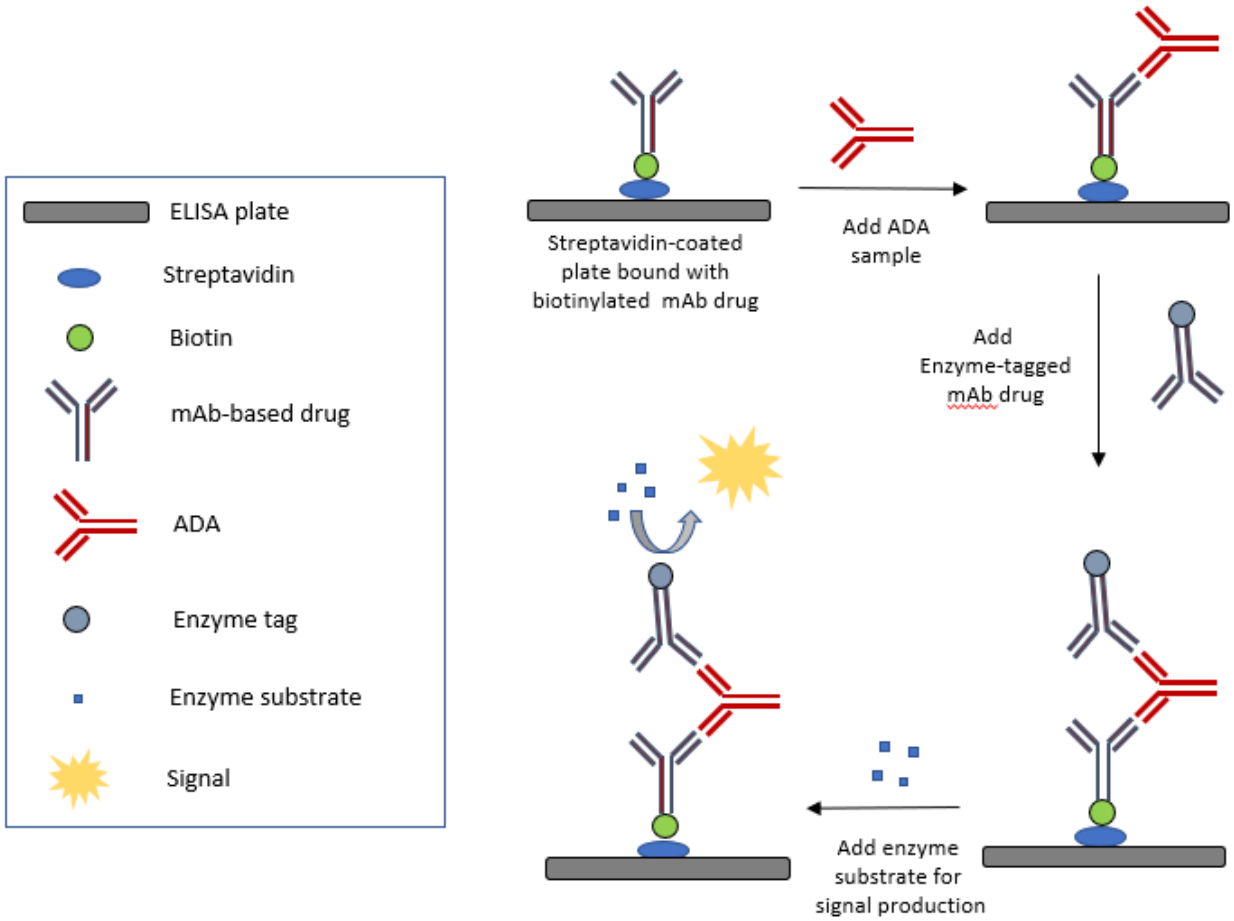

ELISA plate
Streptavidin
Biotin
mAb-based drug
ADA
Enzyme tag
Enzyme substrate
Signal
Streptavidin-coated plate bound with biotinylated mAb drug
Add ADA sample
Add Enzyme-tagged mAb drug
Add enzyme substrate for signal production

Figure 3

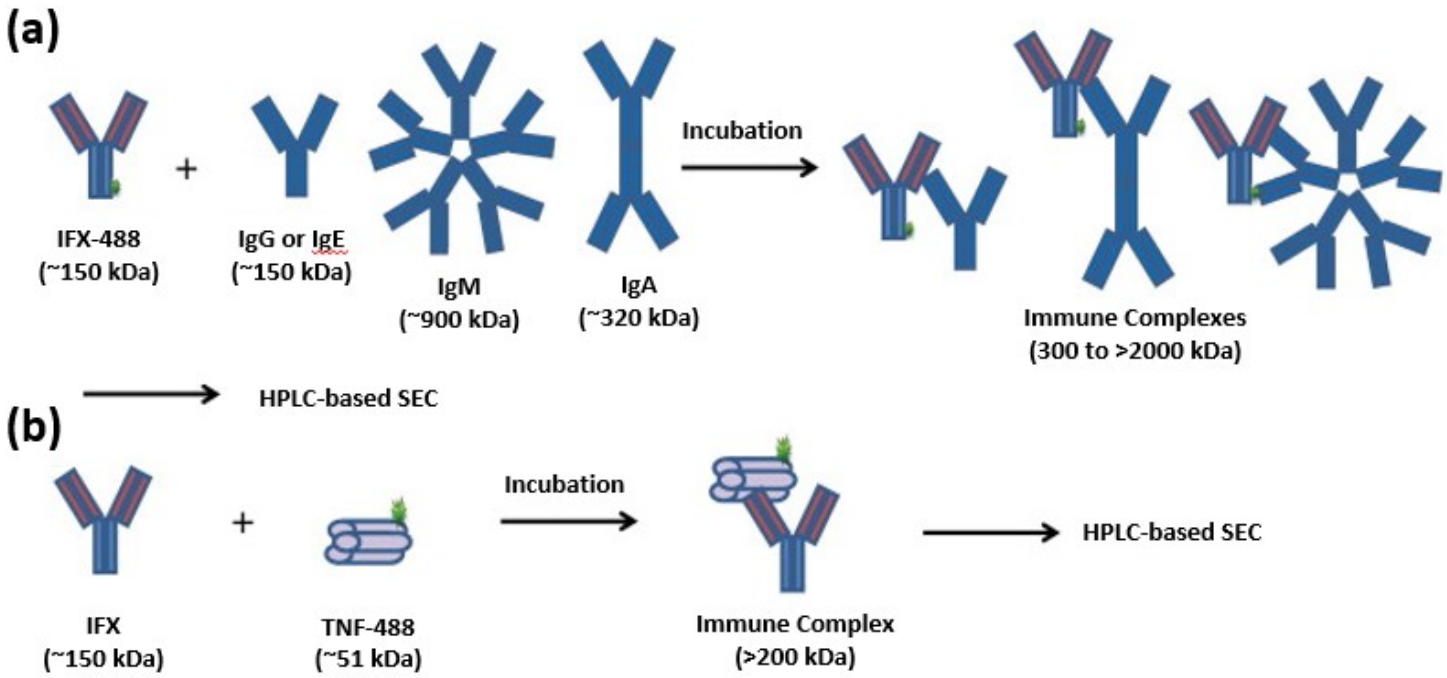

(a)
IFX-488
(~150 kDa)
+
IgG or IgE
(~150 kDa)
IgM
(~900 kDa)
IgA
(~320 kDa)
Incubation
Immune Complexes
(300 to >2000 kDa)
HPLC-based SEC
(b)
IFX
(~150 kDa)
+
TNF-488
(~51 kDa)
Incubation
Immune Complex
(>200 kDa)
HPLC-based SEC

Figure 4

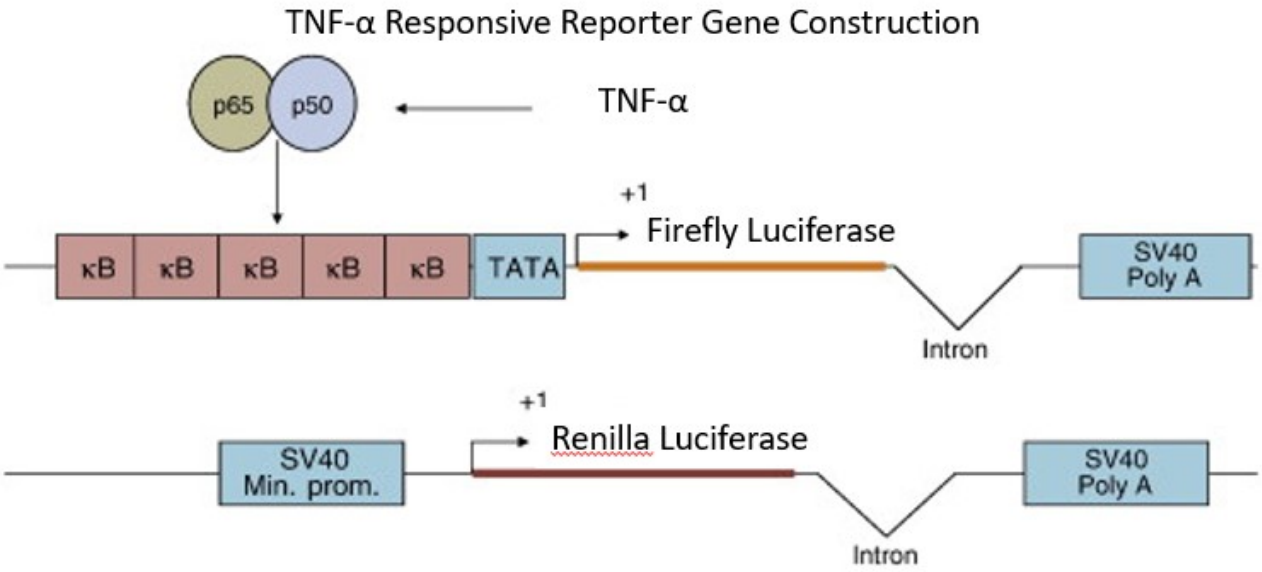

TNF-α Responsive Reporter Gene Construction
p65
p50
TNF-α
+1
Firefly Luciferase
κB
κB
κB
κB
κB
TATA
SV40
Poly A
Intron
+1
Renilla Luciferase
SV40
Min. prom.
SV40
Poly A
Intron

Figure 5

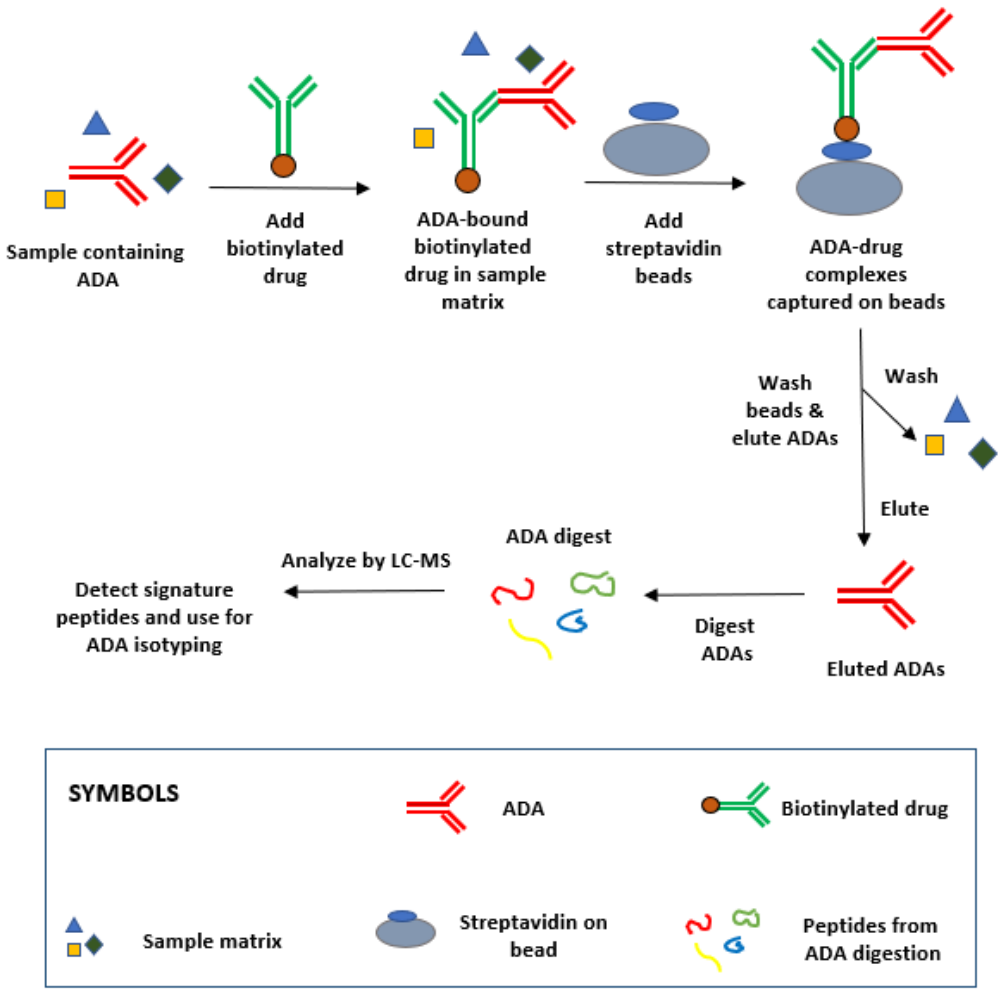

Sample containing ADA
Add biotinylated drug
ADA-bound biotinylated drug in sample matrix
Add streptavidin beads
ADA-drug complexes captured on beads
Wash beads & elute ADAs
Wash
Elute
Eluted ADAs
Digest ADAs
ADA digest
Analyze by LC-MS
Detect signature peptides and use for ADA isotyping
SYMBOLS
ADA
Biotinylated drug
Sample matrix
Streptavidin on bead
Peptides from ADA digestion

Figure 6