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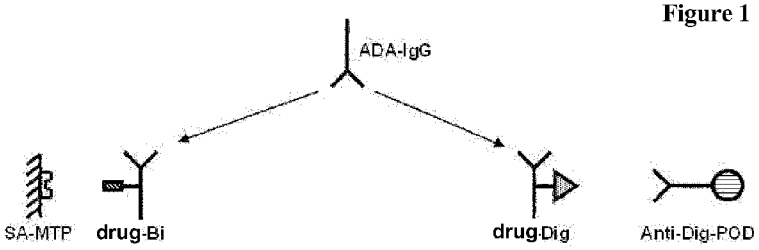
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(54) Title: INTERFERENCE-SUPPRESSED IMMUNOASSAY TO DETECT ANTI-DRUG ANTIBODIES IN SERUM SAMPLES

Figure 1



(57) Abstract: Herein is reported an enzyme linked immunosorbent assay for the detection of anti-drug antibodies against a drug antibody in a sample comprising a capture drug antibody and a tracer drug antibody, wherein the capture drug antibody and the tracer drug antibody are employed in a concentration of 0.5 µg/ml or more, the sample is incubated simultaneously with the capture drug antibody and the tracer drug antibody for 1 to 24 hours, the capture drug antibody and the tracer drug antibody are derivatized via a single lysine residue, the sample comprises 10 % serum, and oligomeric human IgG is added to the sample prior to the incubation with the capture drug antibody and the tracer drug antibody.

Interference-suppressed immunoassay to detect anti-drug antibodies in serum samples

Herein is reported an interference-suppressed immunoassay to detect anti-drug antibodies in serum samples from patients treated with an anti-inflammatory antibody and uses thereof.

Background of the Invention

The clinical development of novel therapeutic antibodies requires the evaluation of their potential immunogenicity by appropriate assays (Kaliyaperumal, A. and Jing, S., Curr. Pharm. Biotechnol. 10 (2009) 352-358). The anti-drug antibody (ADA) testing usually involves a two tier approach: (1) assays for ADA detection and (2) assays for ADA characterization. ADA detection assays include screening and specificity confirmation (confirmatory) assays. Microtiter plate-based enzyme-linked immunosorbent assays (ELISAs) are still the most widely used format to screen for ADAs due to their high-throughput efficiency, relative simplicity and high sensitivity (Geng, D., et al., J. Pharm. Biomed. Anal. 39 (2005) 364-375). ADA ELISAs are most often designed in a bridge format which provides high selectivity, detection of all isotypes and pan-species ADA detection capability (Mire-Sluis, A.R., et al., J. Immunol. Methods 289 (2004) 1-16).

A bridging ELSA has been developed and used as a screening and confirmation ADA assay for the anti-IL6R antibody tocilizumab (Stubenrauch, K., et al., Clin. Ther. 32 (2010) 1597-1609).

Stubenrauch, K., et al. report a generic anti-drug antibody assay with drug tolerance in serum samples from mice exposed to human antibodies (Anal. Biochem. 430 (2012) 193-199). Bourdage, J. S., et al. report the effect of double antigen bridging immunoassay format on antigen coating concentration dependence and implications for designing immunogenicity assays for monoclonal antibodies (J. Pharm. Biochem. Anal. 39 (2005) 685-690). Mikulskis, A., et al. report solution ELISA as a platform of choice for development of robust, drug tolerant immunogenicity assays in support of drug development (J. Immunol. Meth. 365 (2010) 38-49). Pan, J., et al. report the comparison of the NIDSA® rapid assay with ELISA methods in immunogenicity testing of two biotherapeutics (J. Pharm. Tox. Meth. 63 (2010) 150-159.

In WO 2009/077127 a distinguishing assay is reported.

Qiu, Z. J., et al. report a novel homogeneous biotin-digoxigenin based assay for the detection of human anti-therapeutic antibodies in autoimmune serum (J. Immunol. Meth. 362 (2010) 101-111).

Summary of the Invention

Herein is reported a bridging enzyme linked immunosorbent assay (bridging ELISA) that can be used as screening, confirmation and follow-up assay for the detection of anti-drug antibodies (ADA) in serum containing samples of patients treated with a therapeutic antibody. The assay as reported herein is especially useful if the serum containing sample is from a patient with an autoimmune diseases such as rheumatoid arthritis (RA).

The assay as reported herein shows an improved tolerance with respect to the amount of therapeutic antibody in the sample to be analyzed (increased drug tolerance of the ADA ELISA) and at the same time the number of false positive assay results is reduced.

It has been found that with the assay as reported herein interferences by free drug and by rheumatoid factors (RF) can be minimized.

This assay is especially useful if the sample contains antibodies other than the anti-drug antibody in question which can interfere in immunoassays for the detection of anti-drug antibodies and, thus, would account for a false positive immunoassay result.

In one embodiment the methods as reported herein are used for the determination of anti-drug antibodies of drug antibodies used for an anti-inflammatory therapy.

The increased drug tolerance was achieved by a synergistic interaction of 1) increasing the concentration of biotinylated and digoxigenylated capture and tracer reagents; 2) simultaneous, instead of sequential incubation of the serum sample with the capture and tracer reagents; 3) a prolonged incubation time; 4) use of homogenously mono-coupled capture and tracer reagents; and 5) use of an increased serum matrix content.

The interference from rheumatoid factors can be suppressed by addition of oligomeric human immunoglobulin G (IgG) as an additive.

The drug tolerance of the interference-suppressed ADA assay as reported herein is at least 10-fold higher than that of assays known in the art.

One aspect as reported herein is an enzyme linked immunosorbent assay for the detection of anti-drug antibodies against a drug antibody in a sample comprising a capture drug antibody and a tracer drug antibody, wherein

a) the capture drug antibody and the tracer drug antibody are employed in a concentration of more than 0.5 µg/ml,

b) the sample is incubated simultaneously with the capture drug antibody and the tracer drug antibody for 4 to 24 hours,

c) the capture drug antibody and the tracer drug antibody are derivatized via a single lysine residue,

d) the sample comprises 7.5 % serum or more, and

e) oligomeric human IgG is added to the sample prior to the incubation with the capture drug antibody and the tracer drug antibody.

One aspect as reported herein is an enzyme linked immunosorbent assay for the detection of anti-drug antibodies against a drug antibody in a sample of a rheumatoid arthritis patient comprising a capture drug antibody and a tracer drug antibody, wherein

a) the capture drug antibody and the tracer drug antibody have a concentration of 0.5 µg/ml or more in the enzyme linked immunosorbent assay,

b) the sample is incubated simultaneously with the capture drug antibody and the tracer drug antibody for 0.5 to 24 hours,

c) the capture drug antibody is a 1:1 conjugate of the capture drug antibody and a first component of a specific binding pair and the tracer drug antibody is a 1:1 conjugate of the tracer drug antibody and a detectable label,

d) the sample comprises 1 % serum or more, and

e) oligomeric human IgG is added to the sample prior to the incubation with the capture drug antibody and the tracer drug antibody.

In one embodiment the sample comprises 5 % serum or more. In one embodiment the sample comprises 7.5 % serum or more.

In one embodiment the sample comprises anti-drug antibodies and rheumatoid factors.

In one embodiment the drug antibody is an antibody for the treatment of an inflammatory disease. In one embodiment the antibody for the treatment of an inflammatory disease is an antibody for the treatment of an autoimmune disease. In one embodiment the autoimmune disease is rheumatoid arthritis or juvenile arthritis or osteoarthritis or Castleman's disease.

In one embodiment the drug antibody is an antibody for the treatment of cancer. In one embodiment the antibody is for the treatment of myeloma or plasmacytoma.

In one embodiment the drug antibody is an antibody against the IL-6 receptor (anti-IL6R antibody), or against the IGF-1 receptor (anti-IGF1R antibody), or the IL-13 receptor 1 alpha (anti-IL13R1alpha antibody), or against Ox40L (anti-Ox40L antibody), or against tumor necrosis factor alpha (anti-TNFalpha antibody). In one embodiment the drug antibody is an anti-IL6R antibody. In one embodiment the anti-IL6R antibody is tocilizumab.

In one embodiment the capture drug antibody is conjugated to a solid phase. In one embodiment the conjugation of the capture drug antibody to the solid phase is performed via a specific binding pair. In one embodiment the specific binding pair (first component/second component) is selected from Streptavidin or Avidin/biotin, or antibody/antigen (see, for example, Hermanson, G.T., et al., Bioconjugate Techniques, Academic Press, 1996), or lectin/polysaccharide, or steroid/steroid binding protein, or hormone/hormone receptor, or enzyme/substrate, or IgG/Protein A and/or G.

In one embodiment the capture drug antibody is conjugated to biotin (as first component of a specific binding pair). In this case the conjugation to the solid phase is performed via immobilized Avidin or Streptavidin.

In one embodiment the tracer drug antibody is conjugated to a detectable label. In one embodiment the tracer drug antibody is conjugated to the detectable label via a

specific binding pair. In one embodiment the specific binding pair (first component/second component) is selected from Streptavidin or Avidin/biotin, or antibody/antigen (see, for example, Hermanson, G.T., et al., Bioconjugate Techniques, Academic Press, 1996), or lectin/polysaccharide, or steroid/steroid binding protein, or hormone/hormone receptor, or enzyme/substrate, or IgG/Protein A and/or G.

In one embodiment the tracer drug antibody is conjugated to digoxigenin (as detectable label). In this case linking to the detectable label is performed via an antibody against digoxigenin.

In one embodiment the capture drug antibody and the tracer drug antibody have a concentration in the enzyme linked immunosorbent assay (ELISA) of about 0.5 µg/ml to about 10 µg/ml. In one embodiment the capture drug antibody and the tracer drug antibody have a concentration of more than 0.5 µg/ml to less than 10 µg/ml. In one embodiment the capture drug antibody and the tracer drug antibody have a concentration of about 1 µg/ml to about 5 µg/ml. In one embodiment the capture drug antibody and the tracer drug antibody have a concentration of about 1.4 µg/ml to about 1.8 µg/ml. In one embodiment the capture drug antibody and the tracer drug antibody have a concentration of about 1.45 µg/ml to about 1.6 µg/ml. In one preferred embodiment the capture drug antibody and the tracer drug antibody have a concentration of about 1.5 µg/ml.

In one embodiment the incubation time is at least 6 hours. In one embodiment the incubation time is at least 12 hours. In one embodiment the incubation time is at least 16 hours.

In one embodiment the incubation time is at most 24 hours.

In one embodiment the incubation time is between 4 hours and 24 hours. In one embodiment the incubation time is between 6 hours and 24 hours. In one embodiment the incubation time is between 12 hours and 24 hours. In one preferred embodiment the incubation time is between 12 hours and 20 hours. In one embodiment the incubation time is between 14 hours and 18 hours. In one embodiment the incubation time is about 16 hours.

In one embodiment the sample comprises 1 % to 20 % serum. In one embodiment the sample comprises about 10 % serum.

In one embodiment the oligomeric human IgG is added to a final concentration of 10 µg/mL to 1000 µg/mL. In one embodiment the oligomeric human IgG is added to a final concentration of 15 µg/mL to 500 µg/mL. In one embodiment the oligomeric human IgG is added to a final concentration of 20 µg/mL to 250 µg/mL. In one embodiment the oligomeric human IgG is added to a final concentration of 25 µg/mL to 100 µg/mL. In one preferred embodiment the oligomeric human IgG is added to a final concentration of about 50 µg/mL.

One aspect as reported herein is an enzyme linked immunosorbent assay for the detection of anti-drug antibodies against a drug antibody in a sample of a rheumatoid arthritis patient comprising a capture drug antibody and a tracer drug antibody, wherein

a) the capture drug antibody and the tracer drug antibody have a concentration of about 1.5 µg/ml in the enzyme linked immunosorbent assay,

b) the sample is incubated simultaneously with the capture drug antibody and the tracer drug antibody for 14 to 16 hours,

c) the capture drug antibody is a 1:1 conjugate of the capture drug antibody and biotin via a lysine residue of the capture drug antibody and the tracer drug antibody is a 1:1 conjugate of the tracer drug antibody and digoxigenin via a lysine residue of the tracer drug antibody,

d) the sample comprises 1 % to 20 % serum, and

e) oligomeric human IgG is added to the sample to a final concentration of 25 µg/mL to 100 µg/mL prior to the incubation with the capture drug antibody and the tracer drug antibody.

One aspect as reported herein is the use of oligomeric human IgG for the capture of rheumatoid factors in an anti-drug antibody ELISA.

One aspect as reported herein is a method of treating an individual having a disease comprising administering to the individual an effective amount of a therapeutic antibody (drug) and determining the presence of anti-drug antibodies with an assay as reported herein.

One aspect as reported herein is a method of treating an individual having an inflammatory disease comprising administering to the individual an effective amount of an anti-IL6R antibody (drug) and determining the presence of anti-anti-IL6R antibody antibodies (anti-drug antibodies) with an assay as reported herein.

In one embodiment the inflammatory disease is an autoimmune disease. In one embodiment the autoimmune disease is selected from rheumatoid arthritis, juvenile arthritis, osteoarthritis, or Castleman's disease.

In one embodiment the inflammatory disease is mesangial proliferative glomerulonephritis.

One aspect as reported herein is a method of treating an individual having plasmacytoma comprising administering to the individual an effective amount of an anti-IL6R antibody (drug) and determining the presence of anti-anti-IL6R antibody antibodies (anti-drug antibodies) with an assay as reported herein.

One aspect as reported herein is a method of treating an individual having myeloma comprising administering to the individual an effective amount of an anti-IL6R antibody (drug) and determining the presence of anti-anti-IL6R antibody antibodies (anti-drug antibodies) with an assay as reported herein.

One aspect as reported herein is a method of inhibiting IL6R activity in an individual comprising administering to the individual an effective amount of an anti-IL6R antibody to inhibit IL6R activity and determining the presence of anti-anti-IL6R antibody antibodies (anti-drug antibodies) with an assay as reported herein.

Summary of the Figures

Figure 1 Assay principle of the drug-tolerant anti-drug antibody assay exemplified for anti-IL6R antibody tocilizumab.

Figure 2 Assay principle of the interference-suppressed anti-drug antibody assay for anti-IL6R antibody tocilizumab.

Figure 3 Calibration curve obtained for the interference-suppressed anti-drug antibody assay for anti-IL6R antibody tocilizumab.

Figure 4 Comparison of drug tolerance in the two-step anti-drug antibody ELISA and in the interference-suppressed anti-drug antibody ELISA as reported herein for the anti-IL6R antibody tocilizumab; signal of 300 ng/mL anti-drug antibody in presence of increasing amounts of drug are shown; dotted line: CP conventional assay; solid line: CP interference-suppressed assay as reported herein; dotted line with circles: conventional assay; solid line with squares: interference-suppressed assay as reported herein.

Figure 5 Cut point determination with the interference-suppressed anti-drug antibody ELISA.

Figure 6 Cut point determination with the interference-suppressed anti-drug antibody ELISA with TCZ-Bi(mono) and TCZ-Dig(mono).

Figure 7 Signal variations using conventional ELISA in 77 different serum samples from TCZ-treated RA patients.

Figure 8 Signal variations using the interference suppressed ELISA as reported herein in 77 different serum samples from TCZ-treated RA patients.

Definitions

The term "1:1 conjugate" denotes a conjugate consisting of exactly two entities joined/conjugated to each other via a single covalent bond. For example the term "1:1 conjugate of the capture drug antibody and a first component of a specific binding pair" denotes a chemical conjugate consisting of exactly one molecule of the capture drug antibody covalently conjugated via a single chemical bond to exactly one molecule of the first component of a specific binding pair. Likewise the term "1:1 conjugate of the tracer drug antibody and a detectable label" denotes a chemical conjugate consisting of exactly one molecule of the tracer drug antibody covalently conjugated via a single chemical bond to exactly one detectable label molecule.

The term "drug antibody" according to the invention denotes an antibody which can be administered to an individual, so that a sample of said individual is suspected to comprise said drug antibody after administration. A drug antibody is an antibody that is intended to be administered to a human for a therapeutic purpose. Within one assay as reported herein the drug antibody, the capture drug antibody and the tracer drug antibody comprise the "same" antibody molecule, e.g. recombinantly produced with the same expression vector and comprising the same

amino acid sequence. Drug antibodies (therapeutic monoclonal antibodies) are being used widely for the treatment of various diseases such as oncological diseases (e.g. hematological and solid malignancies including non-Hodgkin's lymphoma, breast cancer, and colorectal cancer) or inflammatory diseases. Such antibodies are reported, for example, by Levene, A.P., et al., Journal of the Royal Society of Medicine 98 (2005) 145-152; Groner, B., et al., Curr. Mol. Meth. 4 (2004) 539-547; and Harris, M., Lancet Oncol. 5 (2004) 292-302.

In one embodiment the drug antibody is an antibody which is useful for the treatment of an inflammatory disease, i.e. an anti-inflammatory antibody, such as an anti-IL-6 receptor antibody, or an anti-IGF-1 receptor antibody, or an anti-IL-13 receptor 1 alpha antibody.

An example (preferably monoclonal) drug antibody is an antibody against the IL-6 receptor (anti IL6R antibody). Such an antibody is, for example, reported by Mihara, et al., Clin. Immunol. 98 (2001) 319-326; Nishimoto, N., et al, Blood 106 (2005) 2627-2632, in clinical trial NCT00046774, or in WO 2004/096274.

An example (preferably monoclonal) drug antibody is an antibody against the IGF-1 receptor (anti IGF1R antibody). Such an antibody is, for example, reported in WO 2004/087756 or in WO 2005/005635.

An example (preferably monoclonal) drug antibody is an antibody against the IL-13 receptor alpha (anti IL13R1alpha antibody). Antibodies against IL-13R1alpha are known from, e.g., WO 96/29417, WO 97/15663, WO 03/080675, Graber, P., et al., Eur. J. Immunol. 28 (1998) 4286-4298; Poudrier, J., et al., J. Immunol. 163 (1999) 1153-1161; Poudrier, J., et al., Eur. J. Immunol. 30 (2000) 3157-3164; Aikawa, M., et al., Cytokine 13 (2001) 75-84, and are commercially available from, e.g., R&D Systems Inc. USA. Further exemplary antibodies against IL-13R1alpha are reported in WO 2006/072564.

The term "drug antibody used for an anti-inflammatory therapy" as used herein denotes a drug antibody that is directed against a cell surface receptor that mediates inflammation. Such receptors are for example the IL-6 receptor, or the IGF-1 receptor, or the IL-13a receptor 1. If a sample from a patient, which is treated with such an anti-inflammatory drug antibody, is analyzed, it has to be determined, whether the positive result of the method is based on a true anti-drug antibody (true positive result) or on an antibody other than an anti-drug antibody of the sample (false positive result). An example of such a case is a sample from a patient, who

has an autoimmune disease such as rheumatism, and, thus, a sample obtained from said patient contains so called "rheumatoid factors". The term "rheumatoid factors" as used herein denotes antibodies binding to human IgG, to be more precisely to the Fc-region of human IgG. In most cases these "rheumatic factors" are oligomeric binding molecules.

The term "anti-drug antibody" as used herein denotes an antibody, which is directed against, i.e. binds to, an antigenic region of a drug antibody. This antigenic region may be the variable region, a CDR, the constant region, or the glycostructure of the drug antibody. In one embodiment the anti-drug antibody is directed against a CDR of the drug antibody or a secondary modification of the drug antibody resulting from the recombinant production of the drug antibody in recombinant cells, such as, CHO cells, HEK cells, Sp2/0 cells, or BHK cells. Generally anti-drug antibodies are directed against an antigenic region of a drug antibody that is recognized by the immune system of an animal to which the drug antibody is administered. The above described antibodies are termed "specific anti-drug antibodies".

Drug antibodies are designed to comprise as few as possible antigenic regions. For example, drug antibodies intended for the use in humans are humanized prior to the application to a human patient in order to minimize the generation of an immune response against the drug antibody. This immune response would be in the form of anti-drug antibodies (ADAs), which are directed against the non-human parts of such a humanized drug antibodies, such as e.g. the complementary determining regions in the variable domains (see e.g. Pan, Y., et al., FASEB J. 9 (1995) 43-49).

The term "hypervariable region" or "HVR" as used herein refers to each of the regions of an antibody variable domain which are hypervariable in sequence ("complementarity determining regions" or "CDRs") and/or form structurally defined loops ("hypervariable loops") and/or contain the antigen-contacting residues ("antigen contacts"). Generally, antibodies comprise six HVRs: three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). Exemplary HVRs herein include:

(a) hypervariable loops occurring at amino acid residues 26-32 (L1), 50-52 (L2), 91-96 (L3), 26-32 (H1), 53-55 (H2), and 96-101 (H3) (Chothia and Lesk, J. Mol. Biol. 196:901-917 (1987));

(b) CDRs occurring at amino acid residues 24-34 (L1), 50-56 (L2), 89-97 (L3), 31-35b (H1), 50-65 (H2), and 95-102 (H3) (Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991));

(c) antigen contacts occurring at amino acid residues 27c-36 (L1), 46-55 (L2), 89-96 (L3), 30-35b (H1), 47-58 (H2), and 93-101 (H3) (MacCallum et al. J. Mol. Biol. 262: 732-745 (1996)); and

(d) combinations of (a), (b), and/or (c), including HVR amino acid residues 46-56 (L2), 47-56 (L2), 48-56 (L2), 49-56 (L2), 26-35 (H1), 26-35b (H1), 49-65 (H2), 93-102 (H3), and 94-102 (H3).

Unless otherwise indicated, HVR residues and other residues in the variable domain (e.g., FR residues) are numbered herein according to Kabat.

Antibodies contain a number of reactive moieties, such as, for example, amino groups (lysins, alpha-amino groups), thiol groups (cystins, cysteine, and methionine), carboxylic acid groups (aspartic acid, glutamic acid) and sugar-alcoholic groups. These can be employed for coupling to a binding partner like a surface, a protein, a polymer (such as e.g. PEG, Cellulose or Polystyrol), an enzyme, or a member of a binding pair (see e.g. Aslam M. and Dent, A., Bioconjuation MacMillan Ref. Ltd. (1999) 50-100).

The term "anti-idiotypic antibody" denotes an antibody, which specifically binds to a binding specificity, such as e.g. a binding site, of a parent antibody, i.e. an anti-idiotypic antibody is directed e.g. against an antigen binding site of a parent antibody.

In one embodiment the anti-idiotypic antibody specifically binds to one or more of the CDRs of the parent antibody.

In one embodiment the parent antibody is a therapeutic antibody. In one embodiment the parent antibody is a multispecific antibody. In one embodiment the parent antibody is a bispecific antibody.

One of the most common reactive groups of proteins is the aliphatic ε-amine of the amino acid lysine. In general, nearly all antibodies contain abundant lysine residues. Lysine amines/amino groups are reasonably good nucleophiles above pH

8.0 (pK_a = 9.18) and therefore react easily and cleanly with a variety of reagents to form stable bonds.

Another common reactive group in antibodies is the thiol residue from the sulfur-containing amino acid cystine and its reduction product cysteine (or half cystine). Cysteine contains a free thiol group, which is more nucleophilic than amines and is generally the most reactive functional group in a protein. Thiols are generally reactive at neutral pH, and therefore can be coupled to other molecules selectively in the presence of amines. Since free sulfhydryl groups are relatively reactive, proteins with these groups often exist with them in their oxidized form as disulfide groups or disulfide bonds.

In addition to cystine and cysteine, some proteins also have the amino acid methionine, which is containing sulfur in a thioether linkage. The literature reports the use of several thiolating crosslinking reagents such as Traut's reagent (2-iminothiolane), succinimidyl (acetylthio) acetate (SATA), or sulfosuccinimidyl 6-[3-(2-pyridyldithio) propionamido] hexanoate (Sulfo-LC-SPDP) to provide efficient ways of introducing multiple sulfhydryl groups via reactive amino groups.

Reactive esters, particularly N-hydroxysuccinimide (NHS) esters, are among the most commonly employed reagents for modification of amine groups. The optimum pH for reaction in an aqueous environment is pH 8.0 to 9.0.

Isothiocyanates are amine-modification reagents and form thiourea bonds with proteins. They react with protein amines in aqueous solution (optimally at pH 9.0 to 9.5).

Aldehydes react under mild aqueous conditions with aliphatic and aromatic amines, hydrazines, and hydrazides to form an imine intermediate (Schiff's base). A Schiff's base can be selectively reduced with mild or strong reducing agents (such as sodium borohydride or sodium cyanoborohydride) to derive a stable alkyl amine bond.

Other reagents that have been used to modify amines are acid anhydrides. For example, diethylenetriaminepentaacetic anhydride (DTPA) is a bifunctional chelating agent that contains two amine-reactive anhydride groups. It can react with N-terminal and ε-amine groups of proteins to form amide linkages. The anhydride rings open to create multivalent, metal-chelating arms able to bind tightly to metals in a coordination complex.

The term "sample" includes, but is not limited to, any quantity of a substance from a living thing or formerly living thing. Such living things include, but are not limited to, humans, mice, monkeys, rats, rabbits, and other animals. In one embodiment the sample is obtained from a monkey, especially a cynomolgus monkey, or a rabbit, or a mouse or rat. Such substances include, but are not limited to, in one embodiment whole blood, serum, or plasma from an individual, which are the most widely used sources of sample in clinical routine.

The term "solid phase" denotes a non-fluid substance, and includes particles (including microparticles and beads) made from materials such as polymer, metal (paramagnetic, ferromagnetic particles), glass, and ceramic; gel substances such as silica, alumina, and polymer gels; capillaries, which may be made of polymer, metal, glass, and/or ceramic; zeolites and other porous substances; electrodes; microtiter plates; solid strips; and cuvettes, tubes or other spectrometer sample containers. A solid phase component is distinguished from inert solid surfaces in that a "solid phase" contains at least one moiety on its surface, which is intended to interact with a substance in a sample. A solid phase may be a stationary component, such as a tube, strip, cuvette or microtiter plate, or may be non-stationary components, such as beads and microparticles. A variety of microparticles that allow both non-covalent or covalent attachment of proteins and other substances can be used. Such particles include polymer particles such as polystyrene and poly (methylmethacrylate); gold particles such as gold nanoparticles and gold colloids; and ceramic particles such as silica, glass, and metal oxide particles. See for example Martin, C.R., et al., Analytical Chemistry-News & Features, 70 (1998) 322A-327A, or Butler, J.E., Methods 22 (2000) 4-23.

From chromogens (fluorescent or luminescent groups and dyes), enzymes, NMR-active groups, metal particles, or haptens, such as digoxigenin, the detectable label is selected in one embodiment. In one embodiment the detectable label is digoxigenin. The detectable label can also be a photoactivatable crosslinking group, e.g. an azido or an azirine group. Metal chelates which can be detected by electrochemiluminescense are also in one embodiment signal-emitting groups, with particular preference being given to ruthenium chelates, e.g. a ruthenium $(bispyridyl)_3^{2+}$ chelate. Suitable ruthenium labeling groups are described, for example, in EP 0 580 979, WO 90/05301, WO 90/11511, and WO 92/14138.

The principles of different immunoassays are described, for example, by Hage, D.S. (Anal. Chem. 71 (1999) 294R-304R). Lu, B., et al. (Analyst 121 (1996) 29R-

32R) report the orientated immobilization of antibodies for the use in immunoassays. Avidin-biotin-mediated immunoassays are reported, for example, by Wilchek, M., and Bayer, E.A., in Methods Enzymol. 184 (1990) 467-469.

Detailed Description of the Invention

Herein reported is an interference-suppressed anti-drug antibody assay using serum samples with increased tolerance to free therapeutic antibody and increased resistance to rheumatoid factor interference.

The principle of an anti-drug antibody assay is the capture of anti-drug antibodies (ADAs) in a complex with digoxigenylated drug (drug-Dig) and biotinylated drug (drug-Bi) (e.g. tocilizumab (TCZ-Dig and TCZ-Bi, respectively)), the latter one leading to immobilization onto a streptavidin-coated plate (SA-MTP). The ADA/drug-Dig complex bound to drug-Bi on the SA-MTP is detected by an anti-digoxigenin antibody horseradish peroxidase enzyme conjugate (anti-Dig-HRP). The horseradish peroxidase (HRP) catalyzes a color reaction of the substrate ABTS. The color intensity is proportional to the concentration of the analyte. The general principle of an anti-drug antibody assay is shown in Figure 1.

It has been found that without alteration of the general assay principle the drug and rheumatoid factor tolerance of a conventional anti-drug antibody assay could be increased by

1) increasing the concentration of biotinylated and digoxigenylated capture and tracer reagents;
2) simultaneous, instead of sequential incubation of the serum sample with the capture and tracer reagents;
3) prolonged incubation of the serum sample with the capture and tracer reagents;
4) use of homogenous capture and tracer reagents instead of a heterogeneously coupled mixture;
5) use of an increased serum matrix;
6) inclusion of oligomeric IgG as assay additive; and
7) use of mono biotinylated capture and mono digoxigenylated tracer antibody.

These measures provided for a synergistic effect.

The above measures lead to an interference-suppressed drug-tolerant anti-drug antibody assay for the detection of anti-drug antibodies against a therapeutic drug antibody.

The general principle of the interference-suppressed drug-tolerant anti-drug antibody assay as reported herein is shown in Figure 2 exemplified for the anti-IL6R antibody tocilizumab.

With the assay setup as reported herein drug tolerance of the ADA assay was increased at least 10-fold in serum samples from patients compared to a conventional anti-drug antibody assay. At the same time susceptibility to rheumatoid factors leading to false-positive assay results was also decreased.

The therapeutic anti-inflammatory antibody tocilizumab (TCZ) is a recombinant humanized monoclonal antibody directed against the interleukin-6 receptor. It has been shown to be effective in clinical studies of rheumatoid arthritis (Ohsugi, Y. and Kishimoto, T., Expert Opin. Biol. Ther. 8 (2008) 669-681). The ADA screening and confirmation assay used in these studies shows sufficient drug tolerance for typical TCZ serum concentrations reached at steady state using an intravenous dosing regimen.

But different routes of administration, such as subcutaneous administration, more frequent administration, and new indications in children might result in higher TCZ serum concentrations at steady state.

Additionally, for example, rheumatoid factors (RF) are often significantly increased in patients with autoimmune diseases, such as e.g. rheumatoid arthritis patients. RFs demonstrate a preferential binding to aggregated gamma globulins and are involved in the clearance mechanism of immune complexes in vivo (Tatarewicz, S., et al., J. Immunol. Methods. 357 (2010) 10-16). RFs are preferentially of the pentameric immunoglobulin M (IgM) isotype (Artandi, S.E., et al., Proc. Natl. Acad. Sci. USA 89 (1991) 94-98) and can non-specifically bind with multivalency and medium affinity to the constant part of the therapeutic antibody leading to a false positive result in an ADA assay. For example, affinity purified rabbit anti-human IgM antibody was included in the sample diluent to overcome the cross reactive IgM antibody interference in RA samples (see e.g. Araujo, J., et al., J. Pharm. Biomed. Anal., 55 (2011) 1041-1049).

It has been found that unspecific binding of RF present in the serum sample to the therapeutic antibody could be prevented by adding oligomeric human IgG to the sample prior to performing the ADA assay. The added oligomeric IgG provides for additional targets for the RF and at most eliminates the interference of RF in the ADA assay as reported herein.

In the following the interference-suppressed ADA assay as reported herein is exemplified by the analysis of serum samples of tocilizumab (TCZ) treated rheumatoid arthritis patients.

Measures to increase drug tolerance of the conventional anti-drug antibody assay for detecting anti-drug antibodies against the anti-IL6R antibody tocilizumab were

1) increasing the concentration of biotinylated and digoxigenylated TCZ (e.g. from 0.5 µg/mL to 1.5 µg/mL);

2) simultaneous incubation of the serum sample with TCZ-Bi and TCZ-Dig;

3) prolonged incubation of the serum sample with TCZ-Bi and TCZ-Dig (e.g. from 1 hour to 16 hours);

4) use of only lysine-coupled TCZ-Bi and TCZ-Dig reagents instead of a mixture of lysine- and carbohydrate-coupled reagents;

5) use of an increased serum matrix content; and

6) addition of oligomeric human IgG to the sample prior to incubation TCZ-Bi and TCZ-Dig.

Drug tolerance

Concentrations of the anti-IL6R antibody tocilizumab to be detected in clinical samples are 0.5 µg/mL or higher, often in the range of from 1 µg/mL to 10 µg/mL.

The drug tolerance of the conventional anti-drug antibody assay was evaluated by determining the highest TCZ concentration at which a given concentration of positive control ADA can be detected above the cut-point. Table 1 presents a summary of the results.

Table 1: Determination of drug (tocilizumab) tolerance in the conventional anti-drug antibody ELISA. Left-aligned signal values are below the plate-specific cut-point of 0.136 AU.

	tocilizumab [µg/mL]					
	6250	**1250**	**250**	**50**	**10**	**0**
ADA [ng/mL]	**signal mean [AU]**					
100000	0.049	0.109	0.949	3.094	3.169	3.144
10000	0.050	0.062	0.156	1.265	3.117	3.387
1000	0.052	0.058	0.071	0.189	0.865	2.141
500.0	0.051	0.057	0.065	0.120	0.448	1.282
250.0	0.055	0.060	0.065	0.092	0.255	0.703
125.0	0.056	0.060	0.064	0.076	0.157	0.385
62.5	0.056	0.060	0.062	0.067	0.108	0.226
0	0.059	0.061	0.062	0.062	0.060	0.071

An ADA concentration of 125 ng/mL was detected and tested positive in the presence of 10 µg/mL TCZ.

The drug tolerance of the interference-suppressed drug-tolerant anti-drug antibody assay as reported herein was evaluated by determining the highest TCZ concentration at which a given concentration of positive control ADA can be detected above the cut-point. Table 2 presents a summary of the results.

Table 2: Determination of drug (tocilizumab) tolerance in the interference-suppressed drug-tolerant anti-drug antibody ELISA as reported herein. Left-aligned signal values are below the plate-specific cut-point of 0.045 AU.

	tocilizumab [µg/mL]					
	100	**30**	**10**	**3**	**1**	**0**
ADA [ng/mL]	**signal mean [AU]**					
10,000	0.948	2.263	3.266	3.536	3.146	3.272
3,000	0.310	0.943	1.956	> 3.5	2.860	3.161
1,000	0.119	0.364	0.789	1.051	1.180	2.292
300	0.055	0.133	0.280	0.376	0.426	0.857
100	0.033	0.057	0.105	0.144	0.163	0.310

	tocilizumab [µg/mL]					
	100	**30**	**10**	**3**	**1**	**0**
ADA [ng/mL]	**signal mean [AU]**					
30	0.027	0.036	0.051	0.063	0.068	0.113
10	0.022	0.025	0.032	0.039	0.040	0.056
0	0.024	0.023	0.026	0.027	0.027	0.027

In the interference-suppressed drug-tolerant anti-drug antibody ELISA as reported herein a very low ADA concentration of 30 ng/mL was detected and tested positive in the presence of 10 µg/mL TCZ. Furthermore ADA concentrations of 100 ng/mL and 300 ng/mL show a drug antibody tolerance of 30 µg/ml and even 100 µg/mL, respectively.

In comparison with the same experiments conducted with the previously used, two-step conventional anti-drug antibody ELISA for tocilizumab revealed an at least 10-fold higher drug tolerance with the interference-suppressed assay (see also Figure 4 for 300 ng/mL ADA concentration).

The drug tolerance of the interference-suppressed drug-tolerant anti-drug antibody assay as reported herein with 1:1 conjugates of the monovalent bonded biotin and digoxygenin to the capture and tracer drug antibody, respectively, was evaluated by determining the highest TCZ concentration at which a given concentration of positive control ADA can be detected above the cut-point. Table 3 presents a summary of the results.

Table 3: Determination of drug (tocilizumab) tolerance in the interference-suppressed drug-tolerant anti-drug antibody ELISA with 1:1 conjugates of biotin and digoxygenin to the capture and tracer drug antibody as reported herein. Left-aligned signal values are below the plate-specific cut-point of 0.037 AU.

	tocilizumab [µg/mL]					
	80	**70**	**60**	**50**	**5**	**0**
ADA [ng/mL]	**signal mean [AU]**					
500	0.090	0.097	0.112	0.122	0.380	1.071
250	0.057	0.061	0.067	0.075	0.215	0.653
125	0.043	0.045	0.048	0.053	0.133	0.343

	tocilizumab [µg/mL]					
	80	**70**	**60**	**50**	**5**	**0**
ADA [ng/mL]	**signal mean [AU]**					
0.0	0.025	0.024	0.026	0.027	0.030	0.027

In the interference-suppressed drug-tolerant anti-drug antibody ELISA as reported herein an ADA concentration of 250 ng/mL was detected and tested positive in the presence of 80 µg/mL TCZ.

Interference suppression:

Sixteen clinical serum samples were analyzed by a conventional anti-drug antibody assay as described in Stubenrauch et al. (supra). The results are summarized in Table 4a.

Table 4a: Results of the analysis of 16 serum samples from rheumatoid arthritis patients treated with tocilizumab with the ADA assay according to Stubenrauch et al. (supra).

sample No.	conventional ADA assay
1	+
2	+
3	+
4	+
5	+
6	+
7	+
8	-
9	-
10	-
11	-
12	-
13	-
14	-
15	-
16	+

Without alteration of the assay principle, a series of measures were taken to obtain an interference-suppressed drug tolerant anti-drug antibody ELISA as reported herein.

These are:

1) increasing the concentration of biotinylated and digoxigenylated TCZ (e.g. from 0.5 µg/mL to 1.5 µg/mL);

2) simultaneous incubation of the serum sample with TCZ-Bi and TCZ-Dig;

3) prolonged incubation of the serum sample with TCZ-Bi and TCZ-Dig (e.g. from 1 hour to 16 hours);

4) use of only lysine-coupled TCZ-Bi and TCZ-Dig reagents instead of a mixture of lysine- and carbohydrate-coupled reagents;

5) use of an increased serum matrix content; and

6) addition of oligomeric human IgG to the sample prior to incubation TCZ-Bi and TCZ-Dig.

The results as obtained with the interference suppressed assay as reported herein is shown in the following Table 4b.

Table 4b: Comparative analysis of 16 serum samples from rheumatoid arthritis patients treated with tocilizumab with the conventional and the herein reported interference suppressed ADA assay.

sample No.	conventional ADA assay	interference suppressed ADA assay
1	+	+
2	+	+
3	+	+
4	+	+
5	+	+
6	+	+
7	+	+
8	-	-
9	-	-
10	-	-
11	-	-
12	-	-
13	-	-
14	-	-
15	-	+
16	+	-

It has been found that if only a part of the measures as outlined above were taken the reduction of interference was not sufficient and still a susceptibility to interference by rheumatoid factors existed resulting in false-positive ADA assay results.

If, for example, only the measures

1) increasing the concentration of biotinylated and digoxigenylated TCZ (e.g. from 0.5 µg/mL to 1.5 µg/mL);

2) simultaneous incubation of the serum sample with TCZ-Bi and TCZ-Dig;

3) prolonged incubation of the serum sample with TCZ-Bi and TCZ-Dig (e.g. from 1 hour to 16 hours);

4) use of only lysine-coupled TCZ-Bi and TCZ-Dig reagents instead of a mixture of lysine- and carbohydrate-coupled reagents; and

5) use of an increased serum matrix content

were taken not the full reduction of susceptibility to false positive assay results can be seen. The comparative data is shown in Table 4c.

Table 4c: Comparative analysis of 16 serum samples from rheumatoid arthritis patients treated with tocilizumab with the different formats of the ADA assay.

	interference-suppressed ADA assay as reported herein	
sample No.	**without additive**	**with additive**
1	+	+
2	+	+
3	+	+
4	+	+
5	+	+
6	+	+
7	+	+
8	+	-
9	-	-
10	+	-

	interference-suppressed ADA assay as reported herein	
sample No.	without additive	with additive
11	+	-
12	+	-
13	+	-
14	+	-
15	+	+
16	+	-

Comparative evaluation of 258 different serum samples from TCZ-treated RA patients with the conventional ADA assay and the interference-suppressed drug-tolerant ADA assay as reported herein showed the same positive results in 12 Samples. The conventional assay measured 27 placebo patients positive whereas the interference-suppressed drug-tolerant ADA assay as reported herein only 4. In conclusion, the set of measures as described herein and the addition of oligomeric human IgG as an ADA assay additive conferred increased drug tolerance and suppressed interference by RF compared to the conventional ADA assay.

A subset analysis of above-mentioned data is shown in Table 7 below.

Table 7.

Patient	conventional ELISA		interference-suppressed ELISA	
P1	+	0.812	+	0.110
P2	+	1.009	+	0.893
P2	-	0.097	+	0.088
P2	+	0.276	+	0.228
P3	+	0.349	+	0.566
P3	+	2.405	+	1.760
P3	+	1.307	+	1.001
P4	+	1.409	+	0.242
P5	+	0.219	+	0.060
P6	+	0.387	+	0.688
P6	+	3.689	+	3.721
P6	+	3.506	+	3.722
P7	+	0.771	+	0.058

study related CP: 0.215 (conventional ELISA); 0.058 (interference-suppressed ELISA); +: positive ELISA result; -: negative ELISA result

In Table 8a assay signals for 27 placebo patients not treated with TCZ are shown. Due to the absence of TCZ-treatment induced ADA, high assay signals in this group are not expected and would indicate a potential interference.

Table 8a.

Patient	conventional ELISA		interference-suppressed ELISA	
P8	+	0.270	-	0.032
P8	+	0.231	-	0.033
P9	+	3.736	-	0.055
P9	+	3.297	+	0.058
P9	+	2.863	-	0.046
P9	+	3.739	-	0.052
P1	+	0.812	+	0.110
P10	+	0.755	-	0.021
P10	+	0.635	-	0.021
P11	+	0.272	-	0.020
P11	+	0.271	-	0.021
P11	+	0.234	-	0.020
P12	+	1.157	-	0.027
P12	+	1.362	-	0.028
P12	+	1.149	-	0.029
P4	+	0.522	-	0.033
P4	+	1.409	+	0.242
P4	+	0.651	-	0.047
P13	+	0.349	-	0.023
P14	+	0.245	-	0.033
P14	+	0.276	-	0.035
P14	+	0.275	-	0.037
P15	+	0.580	-	0.049
P7	+	0.990	-	0.056
P7	+	0.822	-	0.052
P7	+	0.523	-	0.042
P7	+	0.771	+	0.058

study related cut-point (CP): 0.215 (conventional ELISA); 0.058 (interference-suppressed ELISA); +: positive ELISA result; -: negative ELISA result

Signal pattern of both assay are very different: whereas all 27 placebos samples were determined to be positive using the conventional ELISA, but only 4 out of the 27 sample were determined to be positive with the interference-suppressed ELISA as reported herein.

In addition to placebo-treated patients sample of TCZ-treated patients have been analyzed. In Table 8b results for the patients prior to TCZ-treatment are shown. In Table 8c results for TCZ-treated patients are shown.

Table 8b.

Patient	Time point	conventional ELISA		interference-suppressed ELISA		dosing
P2	baseline	+	1.009	+	0.893	4
P16	baseline	+	0.745	-	0.021	8
P17	baseline	+	0.281	-	0.020	8
P18	baseline	+	1.401	-	0.023	4

study related CP: 0.215 (conventional ELISA); 0.058 (interference-suppressed ELISA); +: positive ELISA result; -: negative ELISA result

Table 8c.

Patient	Time point	conventional ELISA		interference-suppressed ELISA		dosing
P19	week 24	+	0.281	-	0.030	4 mg/kg
P19	week 24	+	0.226	-	0.031	4 mg/kg
P19	week 4	+	0.293	-	0.030	4 mg/kg
P19	week 8	+	0.362	-	0.028	4 mg/kg
P2	week 4	-	0.097	+	0.088	4 mg/kg
P2	week 8	+	0.276	+	0.228	4 mg/kg
P16	week 24	+	0.416	-	0.030	8 mg/kg
P16	week 4	+	0.542	-	0.029	8 mg/kg
P16	week4	+	0.397	-	0.024	8 mg/kg
P3	week 24	+	2.405	+	1.760	4 mg/kg
P3	week 28	+	1.307	+	1.001	4 mg/kg
P3	week 4	-	0.181	+	0.120	4 mg/kg
P3	week 8	+	0.349	+	0.566	4 mg/kg
P20	week 8	+	0.818	-	0.034	4 mg/kg
P21	week 4	+	0.289	-	0.031	8 mg/kg
P17	week 12	+	0.369	-	0.023	8 mg/kg
P17	week 24	+	0.330	-	0.022	8 mg/kg
P17	week 4	+	0.488	-	0.022	8 mg/kg
P17	week 4	+	0.465	-	0.025	8 mg/kg
P17	week 8	+	0.409	-	0.026	8 mg/kg
P18	week 4	+	1.218	-	0.026	4 mg/kg
P18	week 4	+	1.041	-	0.028	4 mg/kg
P5	week 28	+	0.219	+	0.060	8 mg/kg
P6	week 12	+	3.689	+	3.721	4 mg/kg

Patient	Time point	conventional ELISA		interference-suppressed ELISA		dosing
P6	week 24	+	3.506	+	3.722	4 mg/kg
P6	week 8	+	0.387	+	0.688	4 mg/kg
P22	week24	+	0.423	-	0.020	4 mg/kg

study related CP: 0.215 (conventional ELISA); 0.058 (interference-suppressed ELISA); +: positive ELISA result; -: negative ELISA result

The assay as reported herein provides a benefit independent from the therapeutic antibody and the target employed. This is shown in the following Table for an anti-IL6R antibody, an anti-IGF-1R antibody, an anti-IL13Ralpha antibody, an anti-OX40L antibody and an anti-Abeta antibody using samples of patients being diagnosed positive for rheumatoid arthritis.

Table 9a: anti-IL6R antibody

Patient	conventional ELISA		interference-suppressed ELISA	
P23	+	0.169	-	0.079
P24	+	0.197	-	0.082
P25	+	0.240	-	0.110
P26	-	0.131	-	0.088
P27	+	0.215	-	0.111
P28	+	0.199	-	0.136
P29	-	0.135	-	0.085
P30	+	0.220	-	0.086
P31	+	0.158	-	0.100
P32	+	0.221	-	0.132
P33	-	0.110	-	0.081
P34	-	0.099	-	0.090
P35	-	0.100	-	0.082
P36	-	0.098	-	0.076
CP	0.157		0.140	

Table 9b: anti-IGF-1R antibody

Patient	conventional ELISA		interference-suppressed ELISA	
P23	-	0.153	-	0.127
P24	+	0.423	-	0.132
P25	+	0.266	-	0.145
P26	-	0.171	-	0.163

Patient	conventional ELISA		interference-suppressed ELISA	
P27	-	0.152	-	0.132
P28	-	0.133	-	0.120
P29	+	0.245	-	0.124
P30	-	0.173	-	0.142
P31	-	0.152	-	0.115
P32	-	0.172	-	0.131
P33	-	0.131	-	0.134
P34	-	0.124	-	0.115
P35	+	0.189	+	0.189
P36	-	0.157	-	0.154
CP	0.176		0.185	

Table 9c: anti-IL13Ralpha antibody

Patient	conventional ELISA		interference-suppressed ELISA	
P23	+	0.556	-	0.080
P24	+	0.881	+	0.198
P25	+	2.192	+	0.761
P26	+	0.674	+	0.235
P27	+	0.604	-	0.044
P28	+	0.177	-	0.000
P29	-	0.000	-	0.006
P30	+	0.424	-	0.091
P31	+	0.342	-	0.000
P32	+	0.353	-	0.092
P33	+	0.208	-	0.021
P34	-	0.000	-	0.000
P35	-	0.079	-	0.097
P36	+	0.238	+	0.235
CP	0.100		0.100	

Table 9d: anti-OX40L antibody

Patient	conventional ELISA		interference-suppressed ELISA	
P23	+	0.148	-	0.073
P24	+	0.477	-	0.066
P25	+	0.414	+	0.087
P26	-	0.103	-	0.067
P27	+	0.137	-	0.072
P28	+	0.211	+	0.126

Patient	conventional ELISA		interference-suppressed ELISA	
P29	-	0.116	-	0.074
P30	+	0.436	-	0.064
P32	+	0.198	-	0.079
P33	+	0.122	-	0.078
P34	-	0.096	-	0.070
P35	-	0.088	-	0.067
P36	+	0.121	-	0.076
CP	0.117		0.085	

Table 9e: anti-Abeta antibody

Patient	conventional ELISA		interference-suppressed ELISA	
P25	+	0.063	-	0.025
P26	-	0.025	-	0.026
P28	+	0.086	+	0.080
P29	-	0.020	-	0.021
P30	-	0.037	-	0.022
P31	-	0.036	-	0.024
P32	-	0.029	-	0.024
P33	-	0.027	-	0.026
P34	+	0.069	+	0.069
P35	-	0.019	-	0.021
P36	-	0.033	-	0.032
CP	0.044		0.042	

The following examples and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

EXAMPLES

Materials and Methods

Purified pooled human immunoglobulin class G (IgG) was prepared as described by Stubenrauch et al. (Anal. Biochem. 390 (2009) 189-196). Briefly, pooled human serum from healthy donors has been delipidated with Aerosil (silicon dioxide, 1.5% (w/v)) and precipitated with ammonium sulfate (ad 2.0 M). The pellet was homogenized in phosphate buffer and dialyzed against phosphate buffer, pH 7.0.

The mixture was separated by DEAE ion exchange chromatography at pH 7.0 and the IgG in the flow through was concentrated to 5.93 mg/mL and purified by gel filtration.

Polyclonal anti-digoxigenin-horse radish peroxidase (HRP) conjugate (Fab fragments) was obtained from Roche Diagnostics GmbH, Mannheim, Germany (cat. no. 11633716). Polyclonal rabbit anti-TCZ antibodies (0.5 mg-equivalent/mL) used as positive quality controls (QC) and calibration standards (CS) were prepared as described in Stubenrauch et al. (supra).

Individual human serum samples were provided by the serum bank of Roche Diagnostics GmbH, Penzberg, Germany. Pooled human serum matrix for negative control was supplied by TCS Biosciences Ltd., Buckingham, UK.

The following reagents were obtained from Roche Diagnostics GmbH, Mannheim, Germany: 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) substrate (cat. no. 11684302-001), the washing buffer for the ELISA: phosphate-buffered saline (PBS) (0.01 M KH2PO4, 0.1 M Na2HPO4, 1.37 M NaCl, 0.027 M KCl; pH 7.0)/ 0.05% polysorbate 20 (Tween 20) (cat. no. 11332465-001) and the ready-to-use Universal buffer (cat no.4742672) which was used as dilution buffer in the ELISA. All chemicals were of analytical grade.

Streptavidin-coated microtiter plates (SA-MTP) were obtained from MicroCoat Biotechnologie GmbH, Bernried. Uncoated Nunc 96-microwell plates were from Fisher Scientific GmbH, Schwerte, Germany (cat no. 442587) and used for pre-incubation.

Conventional anti-drug antibody assay:

The assay was performed at room temperature. In the first step, TCZ-Bi was bound to SA-MTPs at a concentration of 0.5 µg/ml by incubating 100 µL on a shaker at 400 rpm for 1 hour. Before adding the pre-incubation solution to the SA-MTPs, the excess unbound TCZ-Bi was removed by washing 3 times. In parallel with the coating procedure, pre-incubation of standards and samples was performed in duplicate in a separate uncoated 96-well plate. The samples and standards were diluted (1:10) in the wells with 10 % serum matrix to a volume of 75 µL and mixed with the same volume of TCZ-DIG, starting the one hour pre-incubation period. The TCZ-Bi-coated SA-MTPs were loaded with the pre-incubation solutions by transferring 100 µL from each well of the pre-incubation plate to the wells of the

coated MTP and incubated on a shaker at 400 rpm for one hour. After washing, polyclonal anti-Dig horseradish peroxidase (HRP) conjugate in a volume of 100 µL (100 mU/mL) was added to the wells and incubated on a shaker for one hour. After washing, the HRP-catalyzed color-generating reaction was initiated by adding 100 µL ABTS solution. When the maximum optical density (OD) was about 2.0, usually within 20 to 30 minutes, the signal of the color reaction was measured by an ELISA reader at a wave length of 405 nm (reference, 490 nm). The same assay was performed in the presence of the confirmation reagents, with simultaneous measurement without the confirmation reagents. The obtained OD data were used for generating the standard calibration curve by nonlinear 4-parameter regression curve fitting according to the Wiemer-Rodbard method for calculating the sample concentration.

The cutoff points for the test results were set at the 95 % CIs of the assay signals (OD) from multiple analyses of human blank serum samples from healthy volunteers and patients with RA. A screening test result was considered positive at a value above the cutoff. A decrease in absorbance of > 20 % relative to the non-spiked sample indicated a positive result. The screening cutoff was determined at 61.4 ng/mL of reference antibody. Intra-assay and inter-assay accuracy were 84.8 % to 93.1 % and 91.3 % to 92.2 %, respectively. The corresponding values for intra-assay and inter-assay precision were 1.8 % to 2.0 % and 6.8 % to 8.0 %. The accuracy of the ELISA was defined by the extent to which the results of the assay agreed with the true values. Accuracy was determined by comparing measured concentrations of the rabbit polyclonal anti-TCZ-positive control standard spiked into human serum with nominal concentrations of anti-TCZ. High-concentration (360 ng equivalents/mL) and low-concentration (60 ng equivalents/mL) positive control standards were analyzed in six aliquots of each positive control standard measured in duplicate to determine intra-assay accuracy and in three aliquots of each positive control standard measured in duplicate to determine inter-assay accuracy.

Example 1

Biotinylation of anti-IL6R antibody tocilizumab

a) preparation of conventionally biotinylated IgG

The anti-IL6R antibody tocilizumab has been dialyzed against buffer (100 mM potassium phosphate buffer (in the following denoted as K-PO4), pH 8.5).

Afterwards the solution was adjusted to a protein concentration of 5 mg/ml. D-biotinoyl-aminocaproic acid-N-hydroxysuccinimide ester was dissolved in dimethyl sulphoxide (DMSO) and added to the antibody solution in a molar ratio of 1:5. After 60 minutes the reaction was stopped by adding L-lysine. The surplus of the labeling reagent was removed by dialysis against 50 mM K-PO4 supplemented with 150 mM KCl, pH 7.5. Aliquots of TCZ-Bi were stored including 6.5 % sucrose at -80°C.

b) preparation of mono biotinylated IgG

The anti-IL6R antibody tocilizumab has been dialyzed against 100 mM K-PO4, pH 8.5 and afterwards the solution was adjusted to a protein concentration of 5 mg/ml. D-biotinoyl-aminocaproic acid-N-hydroxysuccinimide ester was dissolved in dimethyl sulphoxide (DMSO) and added to the antibody solution in a molar ratio of 1:1. After 60 minutes the reaction was stopped by adding L-lysine. The surplus of the labeling reagent was removed by dialysis against 25 mM K-PO4 supplemented with 150 mM KCl, pH 7.2. The mixture was transferred to a buffer with 100 mM K-PO4, 150 mM KCl, pH 7.2 including 1 M ammonium sulfate and applied to a column with streptavidin mutein sepharose. The non biotinylated IgG is in the flow through, the mono biotinylated IgG is eluted with 100 mM K-PO4, 150 mM KCl, 1.5 % DMSO, pH 7.2 and the biotinylated IgG including higher biotinylated populations is eluted with 100 mM K-PO4, 150 mM KCl, 2 mM D-biotin, pH 7.2. The mono biotinylated antibody was dialyzed against 50 mM K-PO4 supplemented with 150 mM KCl, pH 7.5. The aliquots were stored including 6.5 % sucrose at -80°C.

Example 2

Digoxigenylation of anti-IL6R antibody tocilizumab

a) preparation of conventionally digoxigenylated IgG

The anti-IL6R antibody tocilizumab has been dialyzed against buffer (100 mM potassium phosphate buffer (in the following denoted as K-PO4), pH 8.5). Afterwards the solution was adjusted to a protein concentration of 5 mg/ml. Digoxigenin 3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester was dissolved in DMSO and added to the antibody solution in a molar ratio of 1:4. After 60 minutes the reaction has been stopped by adding L-lysine. The surplus of labeling reagent was removed by dialysis against 50 mM K-PO4

supplemented with 150 mM NaCl, pH 7.5. Digoxigenylated TCZ (TCZ-Dig) was stored in aliquots including 6.5 % sucrose at -80°C.

b) preparation of mono digoxigenylated IgG

The anti-IL6R antibody tocilizumab has been dialyzed against 100 mM K-PO4, pH 8.5 and afterwards the solution was adjusted to a protein concentration of 5 mg/ml. Digoxigenin 3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester was dissolved in dimethyl sulphoxide (DMSO) and added to the antibody solution in a molar ratio of 1:1. After 60 minutes the reaction was stopped by adding L-lysine. The surplus of the labeling reagent was removed by dialysis against 50 mM K-PO4 supplemented with 150 mM KCl, pH 7.5. The mixture was applied to a sepharose column with immobilized monoclonal antibodies against digoxigenin. The non digoxigenylated antibody is in the flow through, the mono digoxigenylated IgG is eluted with gentle elution buffer (Thermo Scientific, # 21013) and the digoxigenylated antibody including higher digoxigenylated populations is eluted with 1 M propionic acid. The fraction with mono digoxigenylated antibody was dialyzed first against 20 mM TRIS, 20 mM NaCl, pH 7.5 and second against 50 mM K-PO4, 150 mM KCl, pH 7.5. The aliquots were stored including 6.5 % sucrose at -80°C.

Example 3

Generation of human IgG in oligomeric form

Human IgG purified from human serum by ion exchange chromatography was dialyzed against 150 mM potassium phosphate buffer containing 100 mM NaCl, pH 8.4, and the protein solution was concentrated to a protein concentration of 50 mg/ml. Disuccinimidyl suberate (DSS) was dissolved in DMSO and added to the antibody solution in a molar ration of 1:6 (IgG:DSS). The mixture was incubated at 25 °C and pH 8.4 with stirring and the reaction was analyzed with an analytical gel filtration column (e.g. using a TSK 4000 column). The polymerization was stopped after 140 min. by adding lysine to a final concentration of 20 mM. After 45 min. incubation at 25 °C the oligomeric human IgG was separated by gel filtration (e.g. using a Sephacryl S400 column) to remove low molecular fractions. The composition of the oligomers was characterized by UV spectroscopy, size exclusion chromatography and SDS-PAGE gel electrophoresis. The oligomeric human IgG was aliquoted (10.5 mg/mL) and stored at -65 °C until

it was freshly diluted with Universal buffer (cat no.4742672) to a concentration of 55.6 µg/mL for use as ADA assay additive (AAA) in the immunoassay.

Example 4

Preparation of calibration standards and quality control samples

Stock solutions for calibration standards (CS) and quality control samples (QC) were prepared separately. The CS samples were freshly prepared at the assay day using a 0.5 mg/mL stock solution of TCZ. After pre-dilution with human pooled serum (HPS), the resulting CS working solution was stepwise 1:1 diluted with 100 % HPS to yield calibrator concentrations of 1,000; 500; 250; 125; 62.5; 31.3; and 15.6 ng/mL before use in the assay. The negative control was 100 % HPS. For the pre-incubation step in the assay, the CS samples were diluted 1:10 to adjust to a serum concentration of 10 % and an assay concentration range of 100 ng/mL to 1.56 ng/mL.

The QC stock samples used were made in 100 % human pooled serum and stored as single use aliquots at -20 °C. Three separate QC samples were prepared and stored at stock concentrations representing high (750 ng/mL), medium (400 ng/mL) and low (50 ng/mL) undiluted serum concentrations. For the pre-incubation step in the assay the QC samples were freshly diluted 1:10 in the capture/detection solution to reach a serum concentration of 10 %. A fourth QC sample at the typical cut point of the assay at 25 ng/mL was further used.

Example 5

ADA screening and confirmation assay

A sandwich ELISA was used for both screening and confirmation of anti-drug antibodies (ADAs) against tocilizumab (TCZ) (see Stubenrauch, K., et al., Clin. Ther. 32 (2010) 1597-1609). The principle of the method is the capture of ADAs in complex with TCZ-Dig and TCZ-Bi, the latter one leading to immobilization onto a streptavidin-coated plate. The TCZ-Bi/ ADA/ TCZ-Dig complex bound to the SA-MTP was detected by an anti-Dig-HRP enzyme conjugated antibody. The principle of the drug-tolerant anti-drug antibody assay is shown in Figure 1. Inclusion of oligomeric IgG as ADA assay additive leads to an interference-suppressed anti-drug antibody assay as shown in Figure 2. The horseradish peroxidase (HRP) of the polyclonal antibody catalyzes a color reaction of the substrate ABTS. The color intensity is proportional to the concentration of the analyte.

The screening assay was performed at room temperature. Reagents and serum samples were diluted with Universal buffer (cat no.4742672), all washing steps were performed with the washing buffer (PBS, 0.05% polysorbate 20 (Tween 20) (cat. no. 11332465-001)) three times with 300 µL per well. Incubations were performed under shaking on a microtiter plate shaker (MTP shaker) at 500 rpm. Test samples, QC and CS samples were incubated overnight (up to 16 hours) with the capture antibody TCZ-Bi and the detection antibody TCZ-Dig. Each well of the pre-incubation microtiter plate (MTP) was loaded with 225 µL of the capture/detection solution containing 1.667 µg/mL TCZ-Bi and 1.667 µg/mL TCZ-Dig with oligomeric human IgG ADA assay additive (AAA) and thereafter 25 µL of the respective samples were added. The resulting concentrations of TCZ-Bi and TCZ-Dig were 1.5 µg/mL each and the oligomeric human IgG had a concentration of 50 µg/mL. The loaded MTP was covered to prevent evaporation and incubated overnight. Duplicates of 100 µL of each well of the pre-incubation plate were transferred to the wells of a streptavidin-coated microtiter plate (SA-MTP) which was covered and incubated for 1 h.

After washing, the polyclonal anti-Dig Fab-HRP conjugate with a concentration of 25 mU/mL was added in a volume of 100 µL to each well and incubated for 1 h. After washing, the ABTS ready-to-use solution was added in 100 µL aliquots to each well and incubated for about 10 to 15 min. while shaking. The signal of the color-generating reaction was measured by an ELISA reader at 405 nm wavelength (reference wavelength: 490 nm). Absorbance values of each serum sample were determined in triplicates. The highest standard should reach an optical density (OD) between 1.8 and 2.2 arbitrary units (AU). The obtained OD data was used for generating the standard calibration curve by non-linear 4-paramter fit "Wiemer Rodbard" for calculating the sample concentration. A sample was confirmed as positive to ADAs if the recovery of the concentration was less than the specificity cut-point.

Evaluation of the specificity cut-point was performed by analysis of 32 individual blank human serum samples of rheumatoid arthritis patients in duplicates on one MTP. The cut-point specifies the signal above which a sample is defined as potentially positive for the presence of ADAs in the ADA screening assay. Due to non-normality of the data, a non-parametric approach with a 95 % percentile was applied for cut-point calculation based on the mean of cut-points on replicate plates.

The experiments conducted to determine the cut-point of the ADA assay from duplicate measurements of 32 individual human blank serum samples of rheumatoid arthritis patients revealed a mean AU of about 0,026 on three different plates with a standard deviation (SD) of about 0.009. The corresponding coefficient of variation (CV) was 23.8 %; 20.0 %; and 19.0%, respectively. Based on these data sets, a normalization factor of NF=1.6905 was derived which was used throughout the assay qualification and applied to plate-specific cut-points (plate specific cut-point [AU] = Signal [AU] (negative control) x NF).

For qualification of the screening ADA assay, five independent calibration curve preparations with seven calibrator samples with an assay concentration range of 1.56 ng/mL to 100 ng/mL and a blank sample were measured in duplicates on one plate. The intra-assay qualification runs were performed with five replicates (five separate vials) with each of the four QC samples measured in duplicates on a single plate. Inter-assay qualification data for all QC samples measured in duplicates were obtained from seven independent test runs performed by at least two operators on four different days.

A typical calibration curve of the interference-suppressed ELISA is shown in Figure 3. The precision of duplicate measurements of samples was assessed during the qualification experiments and its CV did not exceed 15%. Intra-assay and inter-assay precision and accuracy values of the interference-suppressed ADA ELISA are summarized in Table 5.

Table 5: Determination of intra-assay and inter-assay precision and accuracy of the interference-suppressed ELISA using tocilizumab-specific ADAs spiked into human serum.

	ADA concentration in 100 % serum [ng/mL]					
	Intra-assay (n=5)			**Inter-assay (n=7)**		
	High QC	**Mid QC**	**Low QC**	**High QC**	**Mid QC**	**Low QC**
spiked (expected concentration)	750	400	50	750	400	50
mean of measured concentration	683	382	50.8	696	402	52.5
SD of measured concentration	15.6	15.8	1.33	21.2	13.6	2.8
precision (% CV)	2.28	4.14	2.62	3.04	3.38	5.33

	ADA concentration in 100 % serum [ng/mL]					
	Intra-assay (n=5)			Inter-assay (n=7)		
	High QC	Mid QC	Low QC	High QC	Mid QC	Low QC
accuracy (% recovery)	91.1	95.5	102	92.8	101	105

The determined intra-assay precision was <5 % for all QCs including the cut-point QC of 25 ng/mL. The determined intra-assay accuracy was in the range of 91.1 % to 102 % and all cut-point QC samples tested positive. The inter-assay precision for back-calculated QCs was < 6 % for all QCs. The determined inter-assay accuracy was 92.8 % to 105 % for the high, medium and low QCs. All cut-point QC measurements provided ADA-positivity.

A potential high-dose hook effect was assessed by serial titration (1:2) of a positive control sample within an assay concentration range of 25,000 ng/mL to 6.1 ng/mL. Recovery of ADA concentrations within the assay range was between 77.9 % and 98.9 %. For analysis of potential matrix effects, 11 individual normal human serum samples were spiked at high and low dose QC, i.e. 50 and 750 ng/mL in 100 % serum, with positive control ADA and were quantified. In addition, QC samples were also analyzed on the same plate. Recovery of the low and high ADA concentrations was 111 % (range: 104 to 117 %) and 111 % (range: 107 to 117 %), indicating that there was no matrix effect in the interference-suppressed ADA ELISA.

The drug tolerance was evaluated by determining the highest TCZ concentration at which a given concentration of positive control ADA can be detected above the cut-point. Table 2 presents a summary of a complete data set.

Table 2: Determination of drug (tocilizumab) tolerance in the interference-suppressed drug-tolerant anti-drug antibody ELISA as reported herein. Left-aligned signal values are above the plate-specific cut-point of 0.045 AU, right-aligned values are below the cut-point.

	TCZ [µg/mL]					
	100	30	10	3	1	0
ADA [ng/mL]	signal mean [AU]					
10,000	0.948	2.263	3.266	3.536	3.146	3.272
3,000	0.310	0.943	1.956	> 3.5	2.860	3.161

	TCZ [µg/mL]					
	100	**30**	**10**	**3**	**1**	**0**
ADA [ng/mL]	**signal mean [AU]**					
1,000	0.119	0.364	0.789	1.051	1.180	2.292
300	0.055	0.133	0.280	0.376	0.426	0.857
100	0.033	0.057	0.105	0.144	0.163	0.310
30	0.027	0.036	0.051	0.063	0.068	0.113
10	0.022	0.025	0.032	0.039	0.040	0.056
0	0.024	0.023	0.026	0.027	0.027	0.027

Concentrations of the anti-IL6R antibody tocilizumab to be detected in clinical samples are 0.5 µg/mL or higher, often in the range of from 1 µg/mL to 10 µg/mL. A very low ADA concentration of 30 ng/mL was detected and tested positive in the presence of 10 µg/mL TCZ. Furthermore ADA concentrations of 100 ng/mL and 300 ng/mL show a drug antibody tolerance of 30 µg/ml and even 100 µg/mL, respectively. The same experiments conducted with the previously used, two-step conventional ADA ELISA for tocilizumab revealed an at least 10-fold higher drug tolerance with the interference-suppressed assay (see Figure 4 for 300 ng/mL ADA concentration).

The concentration of TCZ to be used as the excess free drug in the confirmation assay was based on the data set obtained in the drug interference experiments. To evaluate the TCZ concentration that can inhibit high levels of ADAs in the sample, four different concentrations of positive control samples (1,000; 500; 250; 83.3 ng/mL in 100 % serum) were each incubated with increasing concentrations of TCZ (0; 16; 31; 63; 125; 250 µg/mL in 100 % serum). The TCZ concentration that inhibited at least 95 % (corresponding to less than 5% signal recovery) of the measured signal at high concentrations of the positive control ADA was determined to be 25 µg/mL of TCZ.

To reduce the likelihood of false-negatives due to affinity differences of ADAs in study samples during the later in-study testing phase, a two-fold higher excess free drug concentration of the determined value was used for further evaluation, i.e. 40 µg/mL assay concentration corresponding to 400 µg/mL in 100 % serum.

The minimal signal inhibition value needed for confirming specific ADAs was determined by pre-incubation of 16 individual blank human serum samples of rheumatoid arthritis patients with TCZ and analyzed in duplicates in one test run. The analysis was performed with and without 400 µg/mL of free TCZ. It has been

found that addition of free TCZ reduced the assay signal by a mean of 14.1 % ranging from -11.5 % to 34.8 %, and a SD of 11.2 %. Applying a 99.9 % confidence interval (mean + 3.09 SD) resulted in a minimal signal reduction of 49 % for blank serum samples. Based on this calculation, a sample was assessed confirmation positive if the signal decreased by more than 49% in presence of excess free drug when compared with that in absence of free drug. As reference samples, the corresponding samples without excess TCZ were used. To demonstrate the reproducibility of signal inhibition in the confirmation assay, serum samples with high, medium and low positive QC concentrations were analyzed three times with and without excess TCZ at the predefined concentration.

The interference-suppressed ADA assay for TCZ had a measurement range of from 1,000 ng/mL to 15.6 ng/mL of ADA calibrator in 100 % serum. Intra-assay precision was less than 5 % for all quality controls and the intra-assay accuracy was 91.1 % to 102 %. The inter-assay precision and accuracy were less than 6% and 92.8 % to 105 %, respectively.

Qualification of the assay did exclude a hook and matrix effect.

It can be seen that the drug tolerance of the interference-suppressed ADA assay was at least 10-fold higher than that of the previous version.

The confirmation assay was performed essentially as described before for the screening assay except that samples were analyzed in parallel without and with excess free drug, i.e. TCZ. The confirmation capture/detection solution contained the same volume of the capture/detection solution with additional excess TCZ (44.4 µg/mL) to achieve a final assay concentration of 40 µg/mL TCZ after addition of the samples. Calculation of the percent signal inhibition under confirmatory conditions with excess drug was done using the following equation:

$$\% \text{ signal inhibition} = 100 \times (1 - ([AU]\text{drug-pretreated sample} / [AU]\text{untreated sample})).$$

Example 6

Application of the interference-suppressed ADA assay to clinical samples

Measures to increase drug tolerance consisting in 1) increasing the concentration of biotinylated and digoxigenylated TCZ (e.g. to 1.5 µg/mL); 2) simultaneous incubation of the serum sample with TCZ-Bi and TCZ-Dig; 3) prolonged

incubation of the serum sample with TCZ-Bi and TCZ-Dig (e.g. overnight); 4) use of only lysine-coupled TCZ-Bi and TCZ-Dig reagents instead of a mixture of lysine- and carbohydrate-coupled reagents; and use of an increased serum matrix (e.g. 10 % instead of 5 %) resulted in an increase of ADA positives from 12/28 to 25/28.

Sixteen clinical serum samples were analyzed by a set of three different ADA assays: the conventional ADA assay, interference-suppressed ADA assay as reported herein without added oligomeric human IgG, and the interference-suppressed ADA assay as reported herein with the addition of oligomeric human IgG. The results are summarized in Table 6. Of the 16 samples, 15 were tested positive in the version of the ADA assay wherein only measures 1 to 5 have been taken whereas only 8/16 tested positive in the conventional as well as in the interference-suppressed drug-tolerant ADA assay as reported herein wherein measures 1 to 6 have been taken, with identical results in seven of the eight samples. These seven samples with identical results were characterized by low RF concentrations in the samples and/or at baseline. All seven samples contained ADAs of the IgG isotype which bound to the Fab part of tocilizumab indicative of true tocilizumab specific ADAs. Three of the seven samples also had IgM isotype ADAs, but which also bound to the Fab part. In contrast, the vast majority of the remaining samples had ADAs predominantly of the IgM isotype which bound to the constant Fc part of tocilizumab. These samples also contained a high, i.e. > 1,000 U/mL, concentration of RF.

Table 6: Comparative analysis of 16 serum samples from rheumatoid arthritis patients treated with tocilizumab with the different formats of the ADA assay, in the BIAcore assay and in the rheumatoid factor assay.

	interference-suppressed ADA assay as reported herein		**conventional ADA assay**	**BIAcore: isotype / epitope binding of**		**RF assay [U/ml]**	
sample No.	**without additive**	**with additive**		**IgG ADA**	**IgM ADA**	**study sample**	**baseline**
1	+	+	+	IgG / Fab	IgG	-	312
2	+	+	+	IgG / Fab	-	324	324
3	+	+	+	IgG / Fab	-	591	324

	interference-suppressed ADA assay as reported herein		conventional ADA assay	BIAcore: isotype / epitope binding of		RF assay [U/ml]	
sample No.	without additive	with additive		IgG ADA	IgM ADA	study sample	baseline
4	+	+	+	IgG / Fab	-	56	37
5	+	+	+	IgG / Fab	IgG / Fab	117	129
6	+	+	+	IgG / Fab	IgG / Fab	-	47
7	+	+	+	IgG / Fab	-	-	< 15
8	+	-	-	IgG / Fc	IgG / Fc	2,870	1,305
9	-	-	-	IgG / Fc	IgG / Fc	1,790	1,790
10	+	-	-	IgG / Fc	IgG / Fc	1,630	-
11	+	-	-	-	IgG / Fc	2,320	1,393
12	+	-	-	-	IgG / Fc	-	5,510
13	+	-	-	IgG / Fc	IgG / Fc	1,500	1,315
14	+	-	-	-	IgG / Fc; Fab	-	107
15	+	+	-	IgG / Fab; Fc	IgG / Fc	4,450	2,620
16	+	-	+	IgG	-	-	1,099

The ADA immunoassay as reported in Example 5 was used to analyze 148 different serum samples from rheumatoid arthritis patients taken at baseline and after administration of tocilizumab. The results of the analysis with the interference-suppressed ADA assay were compared with those obtained by analysis with the conventional ADA immunoassay. For more detailed analysis, a total of 92 serum samples (out of the 148) from 18 different patients with additional information on ADA isotype and binding region as well as on clinical events were selected. Patients were selected if they fulfilled at least one of the following criteria: 1) ADA positive immune response at any time point; 2) high TCZ serum concentration; 3) clinical reaction such as infusion-related, hypersensitivity or

anaphylaxis. Analysis of the binding region and the isotype of the ADAs were performed with a biosensor immunoassay as previously described (Stubenrauch, K., et al., Anal. Biochem. 390 (2009) 189-196). Briefly, the surface plasmon resonance (SPR) assay set up made use of the four parallel flow cells on a single biosensor chip by immobilization of full-length antibody and its constant (Fc) and antigen binding (Fab) fragments for differential binding analysis of ADAs. The positive control standard conjugates mimicking polyclonal human ADAs of different isotypes were obtained by conjugating polyclonal rabbit antibodies against TCZ to human immunoglobulin (Ig) M, IgG, or IgE (see WO 2008/061684). The Rheumatoid Factor (RF) assay was performed on the Siemens BN II Nephelometer using RF reagents from Siemens Healthcare Diagnostics (Newark, DE, USA). Briefly, polystyrene particles coated with an immune-complex consisting of human immunoglobulin and anti-human IgG from sheep are aggregated when mixed with samples containing RF. These aggregates scatter a beam of light passed through the sample. The intensity of the scattered light is proportional to the concentration of the respective protein in the sample. The result is evaluated by comparison with a standard of known concentration.

Comparative evaluation of 258 different serum samples from TCZ-treated RA patients with the conventional and interference-suppressed drug-tolerant ADA assay as reported herein showed the same positive results in 12 Samples. The conventional assay measured 27 placebo patients positive whereas the interference-suppressed drug-tolerant ADA assay as reported herein 4. In conclusion, the set of measures as described herein and the addition of oligomeric human IgG as an ADA assay additive conferred increased drug tolerance and suppressed interference by RF compared to the conventional ADA assay.

A subset analysis of above-mentioned data is shown in Table 7 below.

Table 7.

Patient	**Time point**	**conventional ELISA**		**interference-suppressed ELISA**		**dosing**
P1	week 4	+	0.812	+	0.110	placebo
P2	baseline	+	1.009	+	0.893	4 mg/kg
P2	week 4	-	0.097	+	0.088	4 mg/kg
P2	week 9	+	0.276	+	0.228	4 mg/kg
P3	week 8	+	0.349	+	0.566	4 mg/kg
P3	week 24	+	2.405	+	1.760	4 mg/kg

Patient	Time point	conventional ELISA		interference-suppressed ELISA		dosing
P3	week 28	+	1.307	+	1.001	4 mg/kg
P4	week 4	+	1.409	+	0.242	4 mg/kg
P5	week 28	+	0.219	+	0.060	8 mg/kg
P6	week 8	+	0.387	+	0.688	4 mg/kg
P6	week 12	+	3.689	+	3.721	4 mg/kg
P6	week 24	+	3.506	+	3.722	4 mg/kg
P7	week 4	+	0.771	+	0.058	placebo

study related CP: 0.215 (conventional ELISA); 0.058 (interference-suppressed ELISA); +: positive ELISA result; -: negative ELISA result

In Table 8a assay signals for 27 placebo patients not treated with TCZ are shown as bars and numbers. Due to the absence of TCZ-treatment induced ADA, high assay signals in this group are not expected and would indicate a potential interference.

Table 8a.

Patient	Time point	conventional ELISA		interference-suppressed ELISA		dosing
P8	baseline	+	0.270	-	0.032	placebo
P8	week 4	+	0.231	-	0.033	placebo
P9	week8	+	3.736	-	0.055	placebo
P9	baseline	+	3.297	+	0.058	placebo
P9	week 12	+	2.863	-	0.046	placebo
P9	week 4	+	3.739	-	0.052	placebo
P1	week 4	+	0.812	+	0.110	placebo
P10	week 4	+	0.755	-	0.021	placebo
P10	week 4	+	0.635	-	0.021	placebo
P11	baseline	+	0.272	-	0.020	placebo
P11	week 4	+	0.271	-	0.021	placebo
P11	week 4	+	0.234	-	0.020	placebo
P12	baseline	+	1.157	-	0.027	placebo
P12	baseline	+	1.362	-	0.028	placebo
P12	week 4	+	1.149	-	0.029	placebo
P4	baseline	+	0.522	-	0.033	placebo
P4	week 4	+	1.409	+	0.242	placebo
P4	week 4	+	0.651	-	0.047	placebo
P13	week 36	+	0.349	-	0.023	placebo
P14	week 4	+	0.245	-	0.033	placebo
P14	baseline	+	0.276	-	0.035	placebo
P14	week 4	+	0.275	-	0.037	placebo
P15	week 4	+	0.580	-	0.049	placebo
P7	baseline	+	0.990	-	0.056	placebo

Patient	Time point	conventional ELISA		interference-suppressed ELISA		dosing
P7	week 4	+	0.822	-	0.052	placebo
P7	week 4	+	0.523	-	0.042	placebo
P7	week 4	+	0.771	+	0.058	placebo

study related CP: 0.215 (conventional ELISA); 0.058 (interference-suppressed ELISA); +: positive ELISA result; -: negative ELISA result

Signal pattern of both assay are very different: whereas all 27 placebos samples were determined to be positive using the conventional ELISA, only 4 out of the 27 sample were determined to be positive with the interference-suppressed ELISA as reported herein.

In addition to placebo-treated patients sample of TCZ-treated patients have been analyzed. In Table 8b results for the patients prior to TCZ-treatment are shown. In Table 8c results for TCZ-treated patients are shown.

Table 8b.

Patient	Time point	conventional ELISA		interference-suppressed ELISA		dosing
P2	baseline	+	1.009	+	0.893	4
P16	baseline	+	0.745	-	0.021	8
P17	baseline	+	0.281	-	0.020	8
P18	baseline	+	1.401	-	0.023	4

study related CP: 0.215 (conventional ELISA); 0.058 (interference-suppressed ELISA); +: positive ELISA result; -: negative ELISA result

Table 8c.

Patient	Time point	conventional ELISA		interference-suppressed ELISA		dosing
P19	week 24	+	0.281	-	0.030	4 mg/kg
P19	week 24	+	0.226	-	0.031	4 mg/kg
P19	week 4	+	0.293	-	0.030	4 mg/kg
P19	week 8	+	0.362	-	0.028	4 mg/kg
P2	week 4	-	0.097	+	0.088	4 mg/kg
P2	week 8	+	0.276	+	0.228	4 mg/kg
P16	week 24	+	0.416	-	0.030	8 mg/kg
P16	week 4	+	0.542	-	0.029	8 mg/kg
P16	week4	+	0.397	-	0.024	8 mg/kg

Patient	Time point	conventional ELISA		interference-suppressed ELISA		dosing
P3	week 24	+	2.405	+	1.760	4 mg/kg
P3	week 28	+	1.307	+	1.001	4 mg/kg
P3	week 4	-	0.181	+	0.120	4 mg/kg
P3	week 8	+	0.349	+	0.566	4 mg/kg
P20	week 8	+	0.818	-	0.034	4 mg/kg
P21	week 4	+	0.289	-	0.031	8 mg/kg
P17	week 12	+	0.369	-	0.023	8 mg/kg
P17	week 24	+	0.330	-	0.022	8 mg/kg
P17	week 4	+	0.488	-	0.022	8 mg/kg
P17	week 4	+	0.465	-	0.025	8 mg/kg
P17	week 8	+	0.409	-	0.026	8 mg/kg
P18	week 4	+	1.218	-	0.026	4 mg/kg
P18	week 4	+	1.041	-	0.028	4 mg/kg
P5	week 28	+	0.219	+	0.060	8 mg/kg
P6	week 12	+	3.689	+	3.721	4 mg/kg
P6	week 24	+	3.506	+	3.722	4 mg/kg
P6	week 8	+	0.387	+	0.688	4 mg/kg
P22	week24	+	0.423	-	0.020	4 mg/kg

study related CP: 0.215 (conventional ELISA); 0.058 (interference-suppressed ELISA); +: positive ELISA result; -: negative ELISA result

The signal pattern with both assays is not similar: whereas 25 patients were determined to be positive using the conventional ELISA only 10 patients were determined to be positive using the interference-suppressed ELISA as reported herein.

Example 7

Influence of kind of derivatization of capture and tracer reagents

Mono- vs. Multi-labeling

Measures to increase drug tolerance consisting in increasing the concentration of biotinylated and digoxigenylated TCZ (e.g. to 1.5 µg/mL); this could cause higher background by sticky digoxigenin; To beware of worse drug tolerance due to higher cut-points, a mono biotinylation and mono digoxigenylation give lower background signals by presence of higher capture and tracer concentration.

A sandwich ELISA was used for both screening and confirmation of anti-drug antibodies (ADAs) against tocilizumab (TCZ) (see Stubenrauch, K., et al., Clin. Ther. 32 (2010) 1597-1609). The principle of the method is the capture of ADAs in

complex with TCZ-Dig(mono) and TCZ-Bi(mono), the latter one leading to immobilization onto a streptavidin-coated plate. The TCZ-Bi/ADA/TCZ-Dig complex (pre-incubation overnight) bound to the SA-MTP was detected by an anti-Dig-HRP enzyme conjugated antibody. The principle of the drug-tolerant anti-drug antibody assay is shown in Figure 1. Inclusion of oligomeric IgG as ADA assay additive leads to an interference-suppressed anti-drug antibody assay as shown in Figure 2. The horseradish peroxidase (HRP) of the polyclonal antibody catalyzes a color reaction of the substrate ABTS. The color intensity is proportional to the concentration of the analyte.

The screening assay was performed at room temperature. Reagents and serum samples were diluted with Universal buffer (cat no.4742672), all washing steps were performed with the washing buffer (PBS, 0.05% polysorbate 20 (Tween® 20) (cat. no. 11332465-001)) three times with 300 µL per well. Incubations were performed under shaking on a microtiter plate shaker (MTP shaker) at 500 rpm. Test samples, QC and CS samples were incubated overnight (16 hours) with the capture antibody TCZ-Bi and the detection antibody TCZ-Dig. Each well of the pre-incubation microtiter plate (MTP) was loaded with 225 µL of the capture/detection solution containing 1.667 µg/mL TCZ-Bi(mono) and 1.667 µg/mL TCZ-Dig (mono) with oligomeric human IgG ADA assay additive (AAA) and thereafter 25 µL of the respective samples were added. The resulting concentrations of TCZ-Bi(mono) and TCZ-Dig(mono) were 1.5 µg/mL each and the oligomeric human IgG had a concentration of 50 µg/mL. The loaded MTP was covered to prevent evaporation and incubated overnight. Duplicates of 100 µL of each well of the pre-incubation plate were transferred to the wells of a streptavidin-coated microtiter plate (SA-MTP) which was covered and incubated for 1 h.

After washing, the polyclonal anti-Dig Fab-HRP conjugate with a concentration of 25 mU/mL was added in a volume of 100 µL to each well and incubated for 1 h. After washing, the ABTS ready-to-use solution was added in 100 µL aliquots to each well and incubated for about 10 to 15 min. while shaking. The signal of the color-generating reaction was measured by an ELISA reader at 405 nm wavelength (reference wavelength: 490 nm). Absorbance values of each serum sample were determined in triplicates. The highest standard should reach an optical density (OD) between 1.8 and 2.2 arbitrary units (AU). The obtained OD data was used for generating the standard calibration curve by non-linear 4-paramter fit "Wiemer Rodbard" for calculating the sample concentration. A sample was confirmed as

positive to ADAs if the recovery of the concentration was less than the specificity cut-point.

To determine a cut point 35 native sera of patients with RD (rheumatic disease) were measured in both assays. As shown in Figure 5 and 6 the variances between the signals of the sera in the interference-suppressed anti-drug antibody ELISA compared to the interference-suppressed anti-drug antibody ELISA with TCZ-Bi(mono) and TCZ-Dig(mono) are high.

For further assessment 77 different serum samples from TCZ-treated RA patients were analyzed with both variants of interference-suppressed ADA-assay. Because all samples were taken before TCZ treatment (baseline), ADA against TCZ should be absent. Signals higher than cut point may indicated inference (false positives) not related with ADA against the treatment drug (TCZ),

The assay with multi-labeled TCZ (Figure 7) showed more signal variation than the assay with mono labeled TCZ (Figure 8). As can be seen this variation is not a systemic setup of the system, which would simply be a shift on the Y-axis, but it is an increase in the bandwidth of obtained signals. Using these assay-specific cut points nearly all samples in the interference-suppressed drug-tolerant ADA assay (mono) are below cut point. Mostly all samples measured in the multi assay are above the cut point.

Patent Claims

1. A method for the detection of anti-drug antibodies against a drug antibody in a sample of an rheumatoid arthritis patient comprising a capture drug antibody and a tracer drug antibody, wherein

   a) the capture drug antibody and the tracer drug antibody have a concentration of 0.5 µg/ml or more,
   b) the sample is incubated simultaneously with the capture drug antibody and the tracer drug antibody for 0.5 to 24 hours,
   c) the capture drug antibody and the tracer drug antibody are each derivatized only once via a single lysine residue,
   d) the sample comprises 1 % serum/plasma or more,
   e) oligomeric human IgG is added to the sample prior to the incubation with the capture drug antibody and the tracer drug antibody, and
   f) monomeric biotin and digoxygenin on capture and tracer.

2. The method according to claim 1, wherein the sample comprises anti-drug antibodies and rheumatoid factors.

3. The method according to any one of claims 1 to 2, wherein the antibody is an anti-inflammatory antibody.

4. The method according to claim 3, wherein the anti-inflammatory antibody is an antibody for the treatment of rheumatoid arthritis, or juvenile arthritis, or osteoarthritis, or Castleman's disease, or mesangial proliferative glomerulonephritis.

5. The method according to any one of claims 1 or 2, wherein the antibody is an anti-cancer antibody.

6. The method according to claim 5, wherein the anti-cancer antibody is an antibody for the treatment of myeloma or plasmacytoma.

7. The method according to any one of claims 1 to 6, wherein the antibody is an anti-IL6R antibody.

8. The method according to claim 7, wherein the anti-IL6R antibody is tocilizumab.

9. A method of treating an individual having plasmacytoma comprising administering to the individual an effective amount of an anti-IL6R antibody (drug) and determining the presence of anti-anti-IL6R antibody antibodies (anti-drug antibodies) with an assay according to any one of claims 1 to 8.

10. A method of treating an individual having myeloma comprising administering to the individual an effective amount of an anti-IL6R antibody (drug) and determining the presence of anti-anti-IL6R antibody antibodies (anti-drug antibodies) with an assay according to any one of claims 1 to 8.

11. A method of inhibiting IL6R activity in an individual comprising administering to the individual an effective amount of an anti-IL6R antibody to inhibit IL6R activity and determining the presence of anti-anti-IL6R antibody antibodies (anti-drug antibodies) with an assay according to any one of claims 1 to 8.

Figure 1

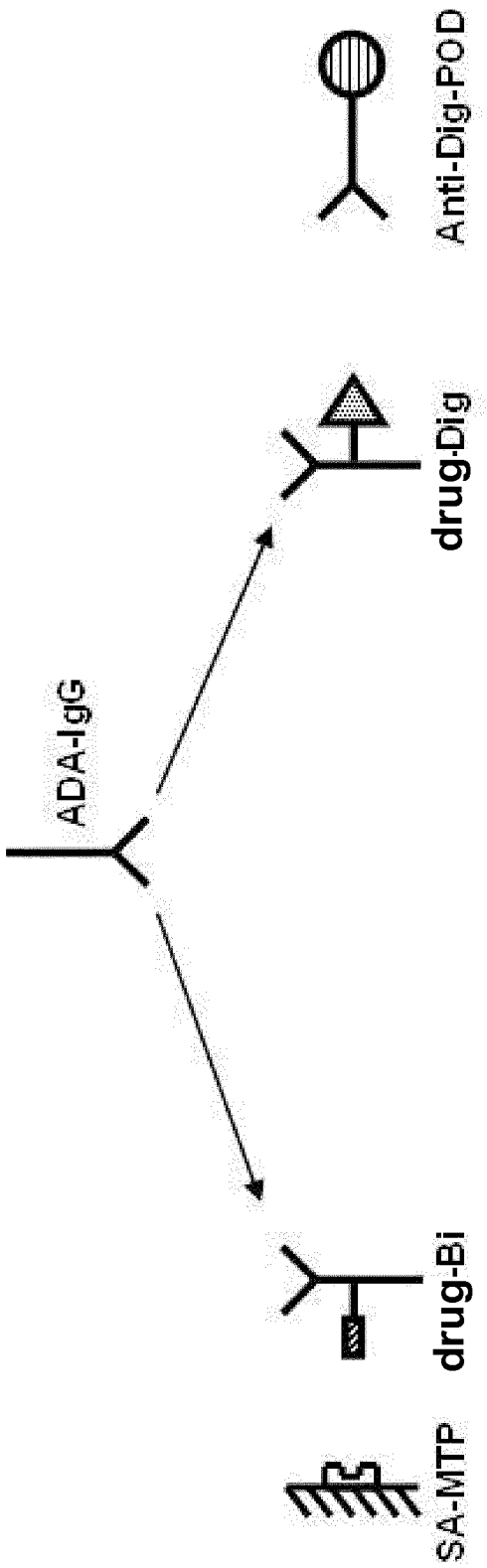


Figure 2

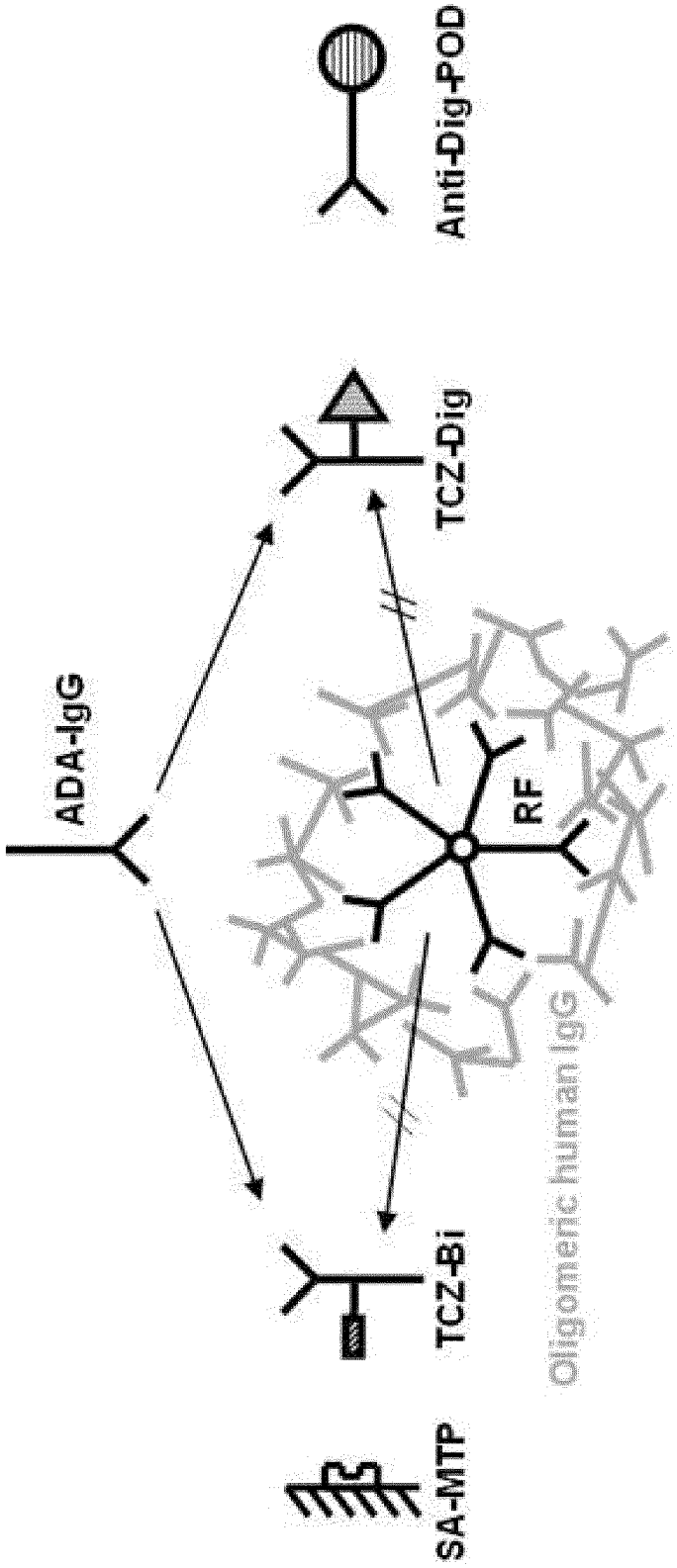

Anti-Dig-POD
TCZ-Dig
ADA-IgG
RF
Oligomeric human IgG
TCZ-Bi
SA-MTP

Figure 3

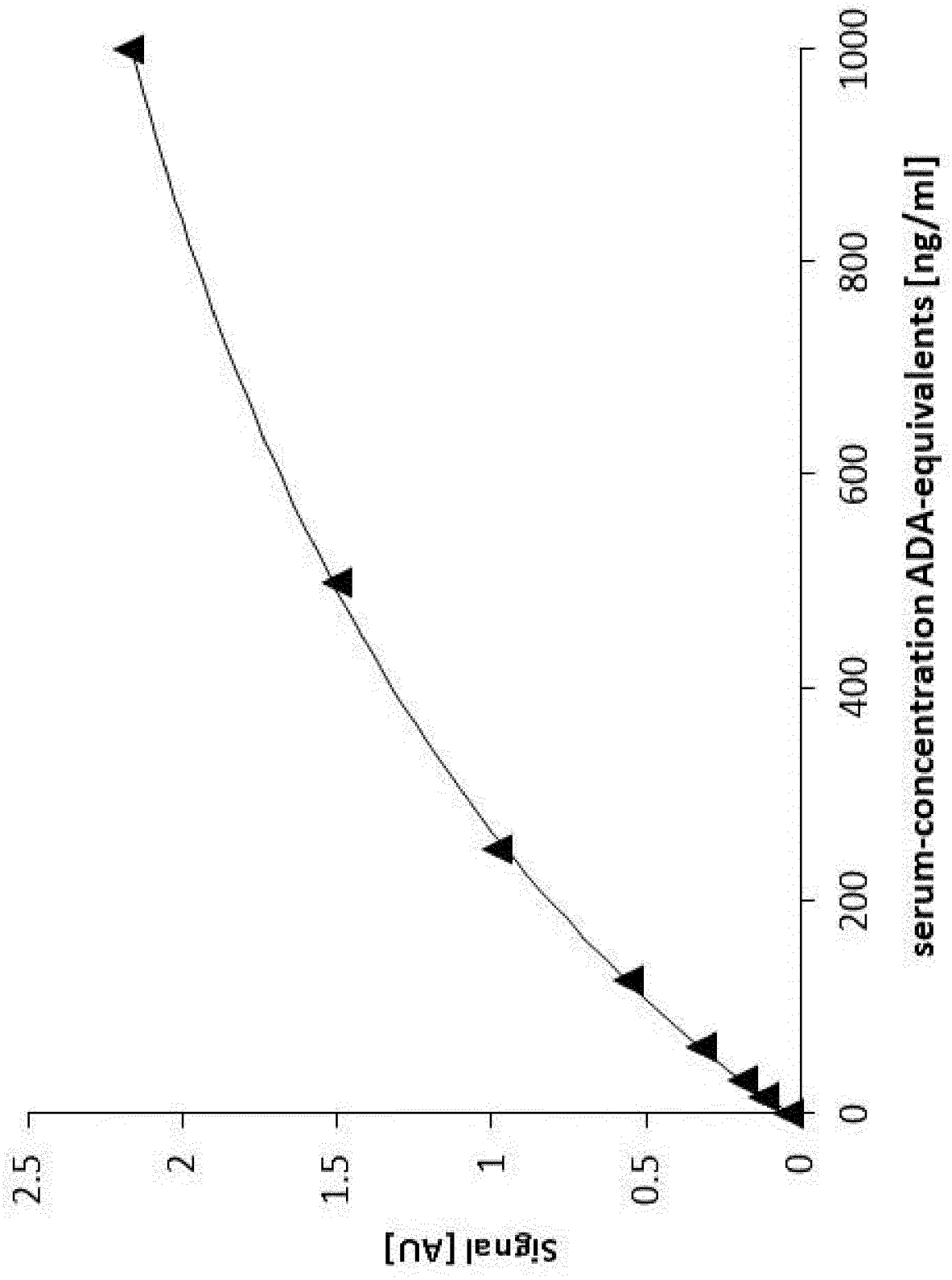

Signal [AU]
0
0.5
1
1.5
2
2.5
0
200
400
600
800
1000
serum-concentration ADA-equivalents [ng/ml]

Figure 4

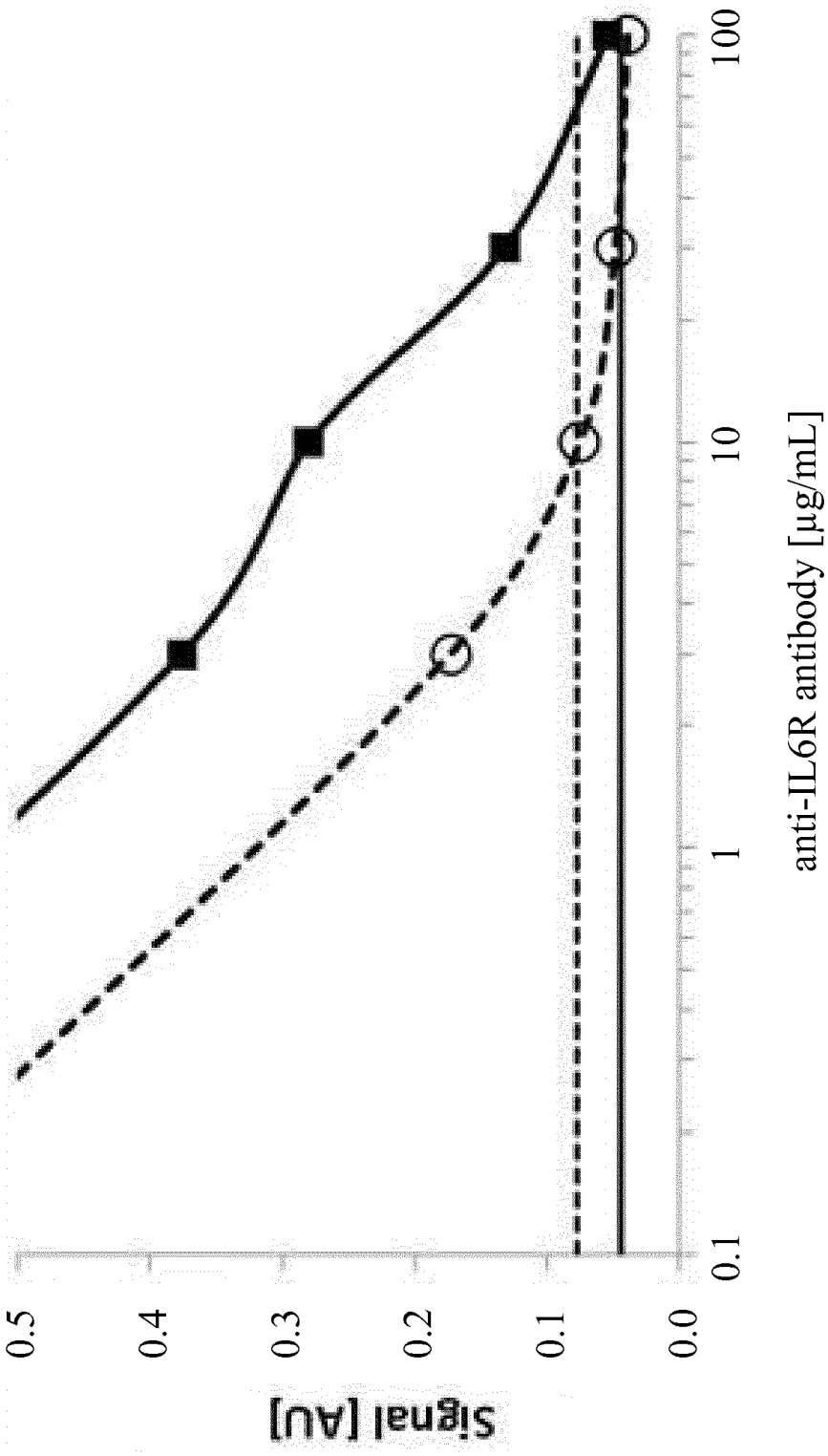


Figure 5

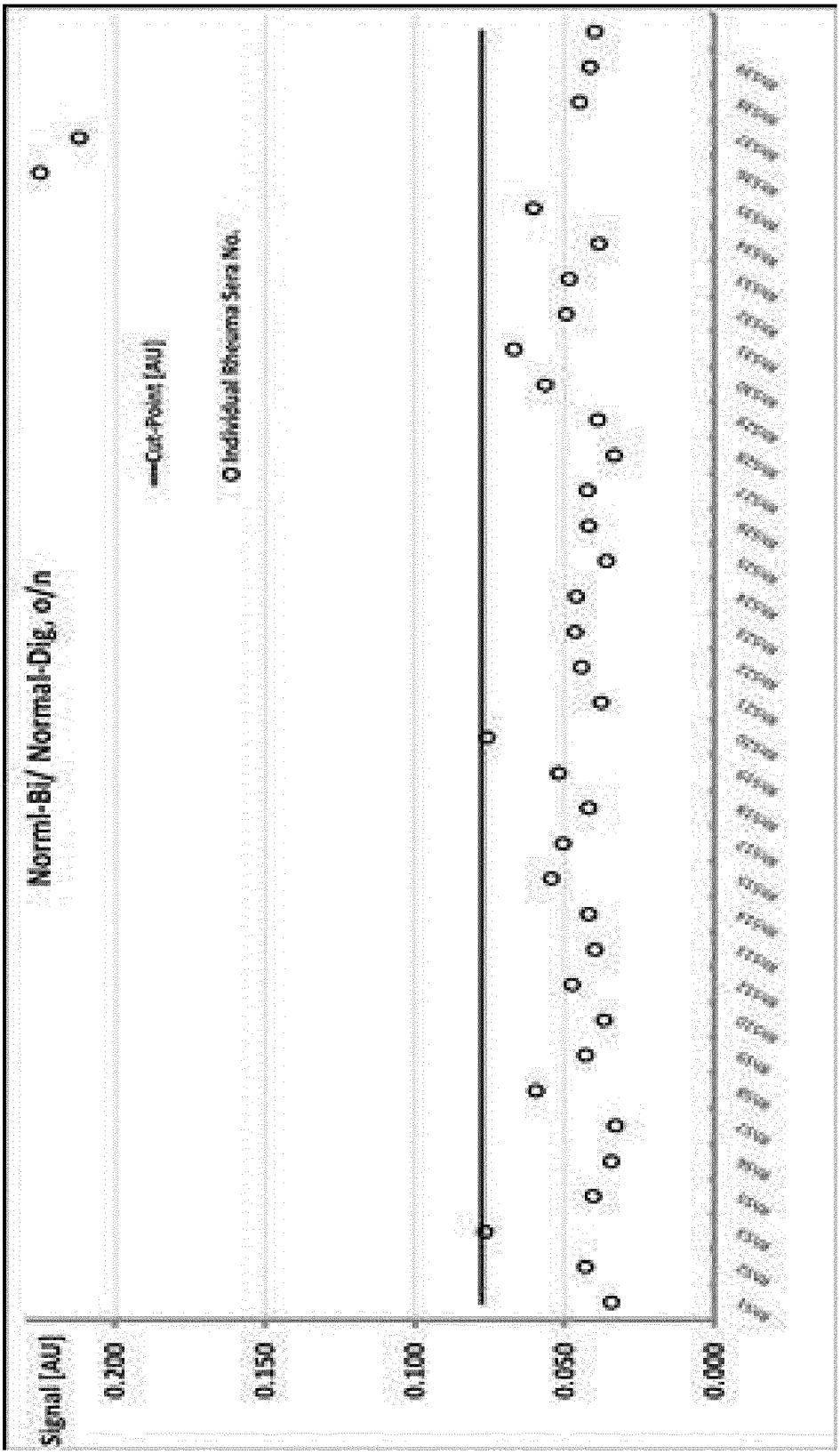

Norml-Bl/ Normal-Dig. o/n
Signal [AU]
0.200
0.150
0.100
0.050
0.000
Cut-Point [AU]
Individual Rheuma Sera No.

Figure 6

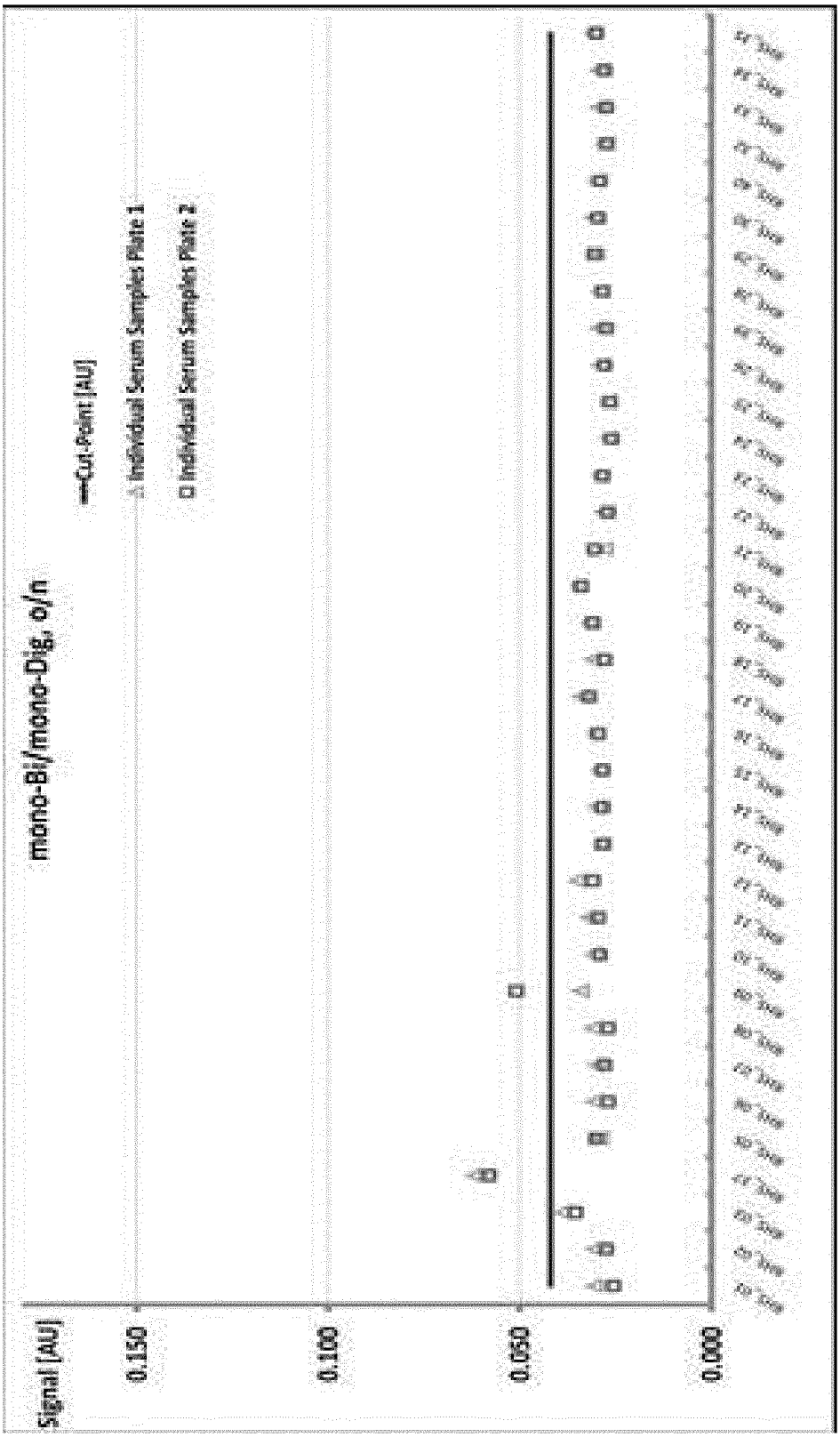

mono-Bi/mono-Dig, o/n
Cut-Point [AU]
Individual Serum Samples Plate 1
Individual Serum Samples Plate 2
Signal [AU]
0.150
0.100
0.050
0.000

Figure7

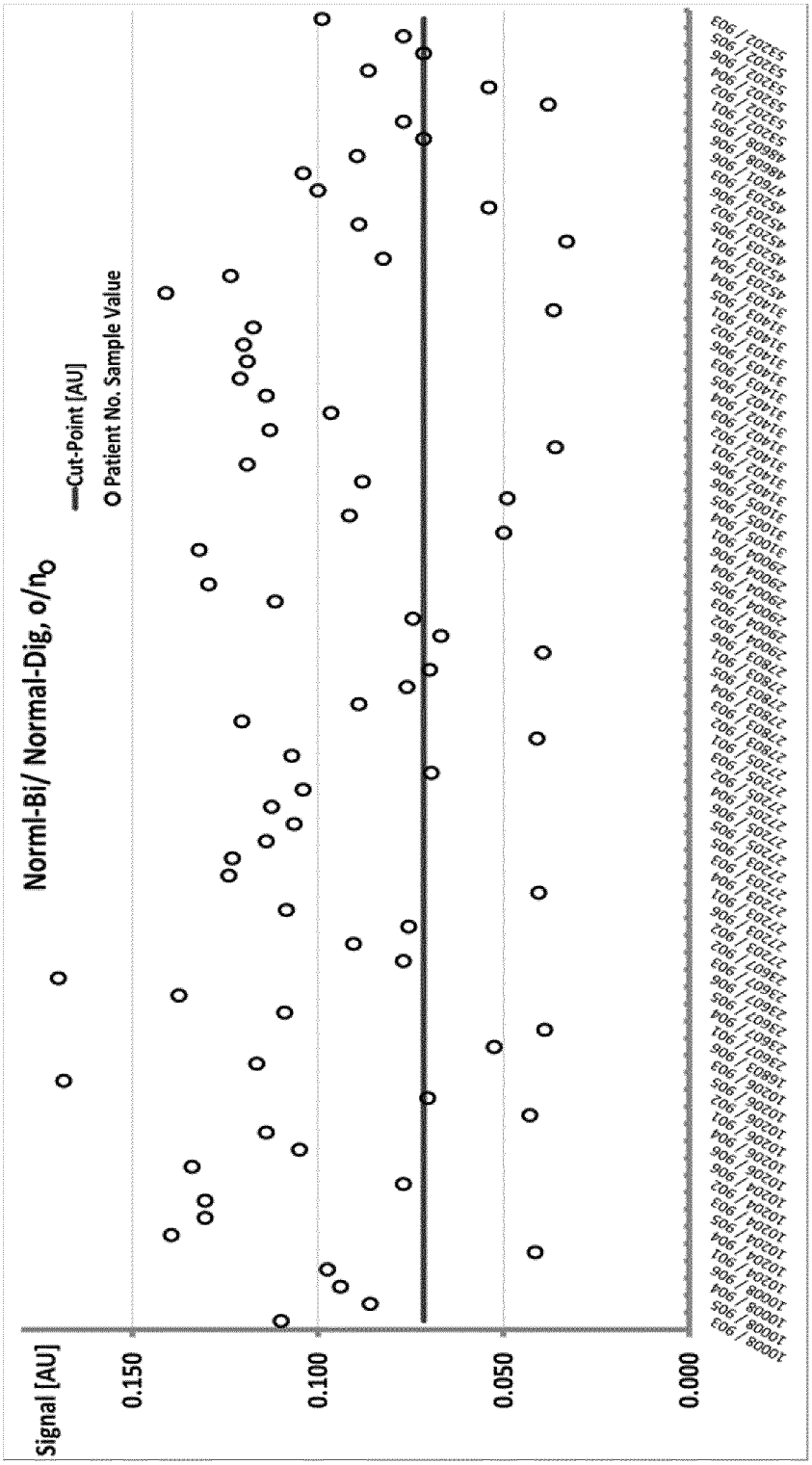
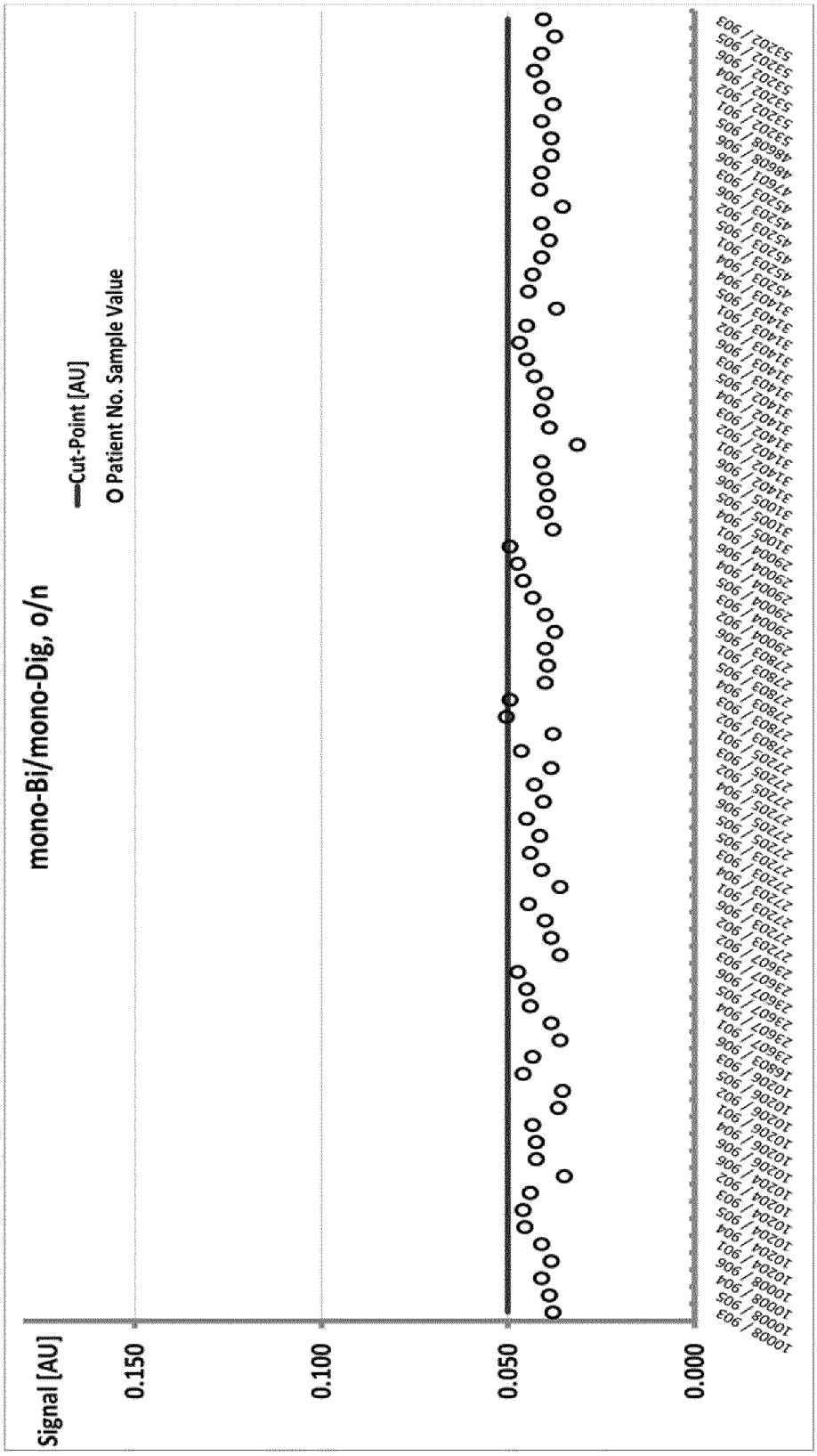

Norml-Bi/ Normal-Dig, o/no
Cut-Point [AU]
Patient No. Sample Value
Signal [AU]
0.150
0.100
0.050
0.000

Figure 8


mono-Bi/mono-Dig, o/n
Cut-Point [AU]
O Patient No. Sample Value
Signal [AU]
0.150
0.100
0.050
0.000

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/063891

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/543
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
22 September 2014	01/10/2014

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/063891

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/063891

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2014/063891

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