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(54) **METHOD OF ALTERING THE BINDING SPECIFICITY OF PLASMA PROTEINS BY OXIDATION-REDUCTION REACTIONS**

VERFAHREN ZUR ÄNDERUNG DER BINDUNGSSPEZIFITÄT VON PLASMAPROTEINEN DURCH OXIDATIONS-REDUKTIONSDREAKTIONEN

PROCEDE PERMETTANT DE MODIFIER LA SPECIFICITE DE LIAISON DES PROTEINES PLASMIQUES AU MOYEN DE REACTIONS D'OXYDATION-REDUCTION

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(73) Proprietor: **Redox-Reactive Reagents L.L.C.**  
**Beech Grove, IN 46107 (US)**

- **NAGABABU ET AL: "Formation of Fluorescent Heme Degradation Products during the Oxidation of Hemoglobin by Hydrogen Peroxide" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, ACADEMIC PRESS INC. ORLANDO, FL, US, vol. 247, no. 3, 29 June 1998 (1998-06-29), pages 592-596, XP005300947 ISSN: 0006-291X**
- **CABIEDES JAVIER ET AL: "Hidden anti-phospholipid antibodies in normal human sera circulate as immune complexes whose antigen can be removed by heat, acid, hypermolar buffers or phospholipase treatments" EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 28, no. 7, July 1998 (1998-07), pages 2108-2114, XP002385020 ISSN: 0014-2980**
- **MCINTYRE, J.A.: 'The appearance and disappearance of antiphospholipid antibodies subsequent to oxidation-reduction reactions' THROMBOSIS RESEARCH vol. 114, no. 5-6, 2004, pages 579 - 587, XP004613204**

(72) Inventor: **MCINTYRE, John A.**  
**Indianapolis, IN 46220-4006 (US)**

(74) Representative: **Hutter, Anton et al**  
**Venner Shipley LLP**  
**20 Little Britain**  
**London**  
**EC1A 7DH (GB)**

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## Description

[0001] The present application claims the benefit of the filing date of United States Provisional Application No. 60/476,607, filed June 9, 2003.

## TECHNICAL FIELD

[0002] The present invention relates to a method of altering a binding specificity of a plasma protein that has a binding specificity that can be altered by oxidation-reduction reactions. The present invention further relates to a method of obtaining autoantibodies by unmasking autoantibodies naturally present in the blood, plasma or serum of normal subjects.

## BACKGROUND OF THE INVENTION

[0003] The term "autoimmune disease" refers to a group of diseases wherein the immune system mistakenly attacks cells, tissues and organs of a person's own body. Typically, autoimmune diseases involve antibody binding of the body's own components, such as common proteins and lipids. Antibodies that bind to self-compounds (or, more typically, to compounds that are so common that they are found in every organism) are referred to as autoantibodies. As an example, autoantibody binding of phospholipids and/or phospholipid-binding plasma proteins is associated with diseases such as systemic lupus erythematosus (SLE), deep vein and recurrent arterial thrombosis, pulmonary embolisms, recurrent spontaneous abortion, thrombocytopenia, chorea, epilepsy, livedo, idiopathic pulmonary hypertension, rheumatological conditions and a host of collagenous diseases. Other diseases associated with autoantibodies include multiple sclerosis, Crohn's disease, discoid lupus erythematosus, Hashimoto's thyroiditis, psoriasis, diabetes and rheumatoid arthritis. There are about 80 different autoimmune diseases, and as a group, these diseases affect millions of people.

[0004] A conventional theory regarding the etiology of autoimmune diseases has been that these diseases are caused by an overproduction of autoantibodies in the diseased individual, possibly due to an overexpression of a gene encoding such autoantibodies. According to this theory, the blood of an affected individual contains an elevated level of the particular autoantibody causing the disease, while the blood of a normal individual contains none of the autoantibody or only a trivial amount. This theory is seemingly supported by conventional assays, in which abundant autoantibodies can be detected in blood, or blood products such as plasma or serum, from subjects having an autoimmune disease, whereas only a zero or minimal amount of autoantibodies can be detected in blood or blood products from subjects that do not have an autoimmune disease.

[0005] The present invention is based on the remarkable discovery, reported herein, that blood from normal

individuals in fact contains a significant number of autoantibodies, in a wide variety of types and specificities. It is possible to detect and isolate these autoantibodies from blood or a blood product of a normal individual if the blood or blood product is treated by oxidation, by, for example with an oxidizing agent or electric current, according to a method described herein. This discovery of autoantibodies in significant quantities in normal blood is previously unreported and, to the best of the inventor's knowledge, the existence of such autoantibodies in significant quantities in normal blood was completely unknown prior to the present invention.

[0006] Without being held to any particular theory, it is evident that if autoantibodies may be obtained by manipulating normal blood taken from persons who do not have any symptoms of autoimmune disease, then it must be that the immune system of normal persons routinely creates and circulates these autoantibodies, but in some form wherein they are masked or blocked, or otherwise prevented from having any harmful effects.

[0007] The discovery of autoantibodies in significant quantities in normal individuals raises the question of why the autoantibodies are not detected in a standard assay (typically based on the binding of an antibody to its corresponding antigen) and why the autoantibodies do not cause disease symptoms in normal individuals.

[0008] Based on earlier experiments described herein, a initial tentative explanation for how normal blood could contain autoantibodies without such antibodies being detected through ordinary screening procedures and without such antibodies causing disease, was that autoantibodies in normal individuals were somehow sequestered after they are produced. For example, the sequestration could be in the form of macromolecules such as a low or high-density lipoproteins (LDL, HDL) or some other type of microparticles, vesicles or micelles that could have the ability to keep autoantibodies cordoned off and separated from other components of the bloodstream. Under this theory, autoimmune disease could be triggered, not by the production of autoantibodies per se, but by the breakdown, disruption or lack of formation of the macromolecules, microparticles, vesicles or micelles sequestering the autoantibodies. This theory seemed supported by the initial experiments wherein autoantibodies were obtained from blood or serum samples after fairly drastic manipulation of the samples including shaking and heating.

[0009] In later experiments, described herein, however, it was shown that simpler methods of the invention, such as exposing blood or a blood product to an oxidizing agent or to a DC electric current, can be enough to obtain autoantibodies from normal blood, and that the process is reversible. Further, it was found that autoantibodies could be obtained by treating commercial IVIg products, which would be free of any type of macromolecular sequestering entity. Based on these experiments, a more likely theory for how normal blood could contain autoantibodies without such antibodies being detected through ordinary screening procedures and without such antibod-

ies causing disease, is that the autoantibodies freely circulate along with other antibodies but that the antigen binding site of autoantibodies is somehow blocked or inactivated in normal individuals. Under this theory, autoimmune disease could be triggered by oxidation to unblock the antigen-binding site of autoantibodies. Further, this theory suggests a more general mechanism by which the binding specificity of certain plasma proteins may be altered.

**[0010]** An immediate practical use of the discovery that forms the basis of the present invention is that it allows for an almost unlimited supply of autoantibodies to be obtained, which autoantibodies can be used as standards in diagnostic kits for the laboratory diagnosis of autoimmune and other aPL-related diseases. Previously, collection of large amounts of autoantibodies for commercial use has been difficult because it was thought that the autoantibodies had to be obtained from individuals having an autoimmune disease or testing positive for autoantibodies in standard assays. The amount of such blood that can be obtained from phlebotomy of individual patients or by pooling blood from a group of patients known to test positive for autoantibodies is limited. Other methods of obtaining autoantibodies, such as screening phage libraries as described in U.S. Patent No. 5,885,793, may be difficult and time-consuming.

**[0011]** US 4,950,612 discloses a method for releasing vitamin B12 bound to endogenous serum binding proteins. This method uses an oxidising agent (peroxy acid) and enables serum vitamin B12 concentrations to be determined.

**[0012]** Nagababu et al, "Formation of fluorescent Heme degradation products during the oxidation of Hemoglobin by hydrogen peroxide", Biochemical or Biophysical Research Communications, Vol 247, No. 3, 29th June (1998), discloses that haemoglobin and methemoglobin oxidised by hydrogen peroxide generate ferrylhemoglobin and oxoferrylhemoglobin, respectively. Two fluorescent compounds were found to be produced during the reaction of oxyhemoglobin, but not methemoglobin with H<sub>2</sub>O<sub>2</sub>.

**[0013]** Cabiedes et al, "Hidden anti-phospholipid antibodies in normal human sera circulate as immune complexes whose antigen can be removed by heat, acid, hypermolar buffers or phospholipase treatments", Biosciences Information Service, July 1998, refers to "hidden" antibodies that circulate in a bound state or in a complex. The heat or acid treatment merely releases the antibodies from this bound state.

**[0014]** US 5,939,394 discloses the administration of magnesium gluconate for the treatment or prevention of allergic diseases, autoimmune diseases, septic shock or infectious diseases. It is thought that the magnesium gluconate acts by preventing or inhibiting abnormal production of lipid peroxides or cytokines.

**[0015]** EP 0 778 025 discloses a method of treating or inhibiting free radical induced disease states by administering an antioxidant amount of 8,9-dehydroestrone or

its pharmaceutically acceptable salt of the 3-sulfate ester, to a mammal in need thereof.

**[0016]** Testing blood samples for the presence or absence of masked antibodies may have important diagnostic value as it might presage or predict what antibodies could appear subsequent to oxidative stress in particular individuals

#### DISCLOSURE OF INVENTION

**[0017]** The invention provides a method for obtaining or isolating an autoantibody from an antibody-containing biological fluid comprising diluted whole blood, serum or plasma from an antibody-containing extract of a biological fluid comprising diluted whole blood, said biological fluid or extract containing masked autoantibodies that, before the method is carried out, are not capable of binding to a self antigen and therefore are not detectable by an assay based on receptor-ligand binding, the method comprising the steps of exposing the biological fluid or extract to an oxidising agent or to a 6-24 Volt DC electric current for a few seconds to a few minutes sufficient to alter a binding specificity of the masked autoantibody so that the said autoantibody becomes capable of binding to an antigen, thereby becoming detectable and recoverable from the biological fluid or extract by receptor-ligand binding separation method, and recovering the autoantibody from the biological fluid.

**[0018]** The antibody-containing extract of a biological fluid may be intravenous immunoglobulin (Ivlg); or the oxidizing agent may be hemin.

**[0019]** Another method of the invention comprises screening a normal individual's biological fluid or extract to determine an amount of autoantibodies that are masked, comprising the steps of assaying a diluted whole blood, serum or plasma sample from a subject to determine an amount and/or type of masked autoantibodies detectable in the sample, treating the sample from the subject by exposing the sample to an oxidising agent or a 6-24 Volt DC electric current for a few seconds to a few minutes, assaying the sample from the subject to determine an amount and/or type of autoantibodies detectable in the treated sample, and comparing the amount and/or type of masked autoantibodies detectable in the untreated sample with the amount and/or type of autoantibodies detectable in the treated sample.

**[0020]** Another method of the invention comprises effecting an alteration of the binding specificity of a masked autoantibody comprising exposing a composition comprising at least one masked antibody suspended or dissolved in a liquid medium comprising diluted whole blood, serum or plasma or diluted placental cord blood to an oxidising agent or a 6-24 Volt DC current for a few seconds to a few minutes sufficient to effect the alteration of the binding specificity of said masked autoantibody, wherein the binding specificity is altered from the masked autoantibody not having binding capability with respect to a specific antigen or ligand to an autoantibody having

binding capability with respect to the specific antigen or ligand.

**[0021]** The method comprises isolating and recovering the autoantibody having an altered binding specificity.

**[0022]** The composition, biological fluid, antibody-containing extract of a biological fluid, diluted whole blood, serum or plasma or diluted placental cord blood may be exposed to hemin for a period of time sufficient to alter the binding specificity of the antibody.

**[0023]** The composition, biological fluid, antibody-containing extract of a biological fluid, whole blood, serum or plasma may be exposed to a 6-24 volt DC electric current for a few seconds to a few minutes.

**[0024]** The biological fluid may comprise diluted serum or plasma.

**[0025]** The liquid medium may comprise diluted serum or plasma.

**[0026]** These and other objectives are achieved by a method of altering a binding specificity of at least one circulating protein in a biological fluid or in a protein-containing extract of a biological fluid, the circulating protein having a binding site with a binding specificity that can be altered by a change in a redox state of the protein, by exposing the protein in the biological fluid or extract to an oxidizing agent or to a direct electric current (DC) to effect the alteration of the binding specificity of the circulating protein.

**[0027]** The objects are further achieved by a method comprising the steps of providing a composition comprising at least one plasma protein suspended or dissolved in a liquid medium, the plasma protein having a binding specificity that can be altered by a change in its redox state, and exposing the composition to an oxidizing agent or a DC electric potential sufficient to effect the alteration of the binding specificity of the plasma protein.

**[0028]** In another embodiment, the invention relates to a method of obtaining autoantibodies or other masked circulating proteins from a biological fluid or from an extract of a biological fluid by exposing the autoantibody or other masked circulating protein in the biological fluid or extract to an oxidizing agent or to a DC electric current sufficient to alter the binding specificity of the autoantibody or other masked circulating protein so that the autoantibody or other masked circulating protein becomes capable of binding to an antigen or ligand, thereby becoming detectable and recoverable from the biological fluid or extract, and recovering the autoantibody or other masked circulating protein from the biological fluid.

**[0029]** As a particular example, blood, plasma or serum, or a blood extract such as an immunoglobulin mixture, may be exposed to an oxidizing agent or to a DC electric current to effect the alteration of the binding specificity of at least one autoantibody contained in the blood, plasma, serum or extract, so that the autoantibody becomes detectable in and recoverable from the blood, plasma, serum or extract.

## BRIEF DESCRIPTION OF THE DRAWINGS

### **[0030]**

5 Figure 1 is a table listing the particular antiphospholipid antibodies (aPL) that were assayed by the in-house enzyme-linked immunosorbent assay (ELISA) format used in many of the Examples, described below.

10 Figure 2 is a table summarizing aPL assay results for a blood sample from a normal aPL-negative subject, incubated according to the method described in the opening section of the Examples.

15 Figure 3 is a composite table summarizing aPL assay results of blood samples from seven aPL-negative normal individuals, incubated according to the method described in the opening section of the Examples.

20 Figure 4 is a table summarizing aPL assay results for a serum sample from a normal aPL-negative subject, incubated according to the method described in the opening section of the Examples, with the feature that horse red blood cells (RBC) were substituted for human RBC in the procedure.

25 Figure 5 is a table summarizing aPL assay results of an incubation of a serum sample carried out according to a method described in the opening section of the Examples, except that horse serum was substituted for human serum.

30 Figure 6 is a table summarizing aPL assay results for a blood sample from a normal aPL-negative subject, incubated according to the method described in the opening section of the Examples, except that the incubation was carried out at room temperature (22 °C).

35 Figure 7 is a table summarizing aPL assay results for a blood sample from a normal aPL-negative subject, incubated according to the method described in the opening section of the Examples, with the feature that 0.7 mm Degalan (plastic) beads were used as the particulate solid in the incubation mixture.

40 Figure 8 is a table summarizing aPL assay results for a blood sample from a normal aPL-negative subject, incubated according to the method described in the opening section of the Examples, except that the incubation mixture was kept stationary, instead of being shaken or rocked.

45 Figure 9 is a table summarizing aPL assay results for a blood sample from a normal aPL-negative subject, incubated according to the method described in the opening section of the Examples, with the added feature that the incubation mixture was heated to 56° C for 30 minutes.

50 Figure 10 is a table summarizing aPL assay results for a blood sample from a normal aPL-negative subject, incubated according to the method described in the opening section of the Examples, with the feature that a bacterial culture growth medium from a

different supplier (Becton Dickinson, Sparks, Md) was used in place of the bacterial culture growth medium from Biomerieux.

Figure 11 is a table summarizing aPL assay results for a blood sample from a normal aPL-negative subject, incubated according to the method described in the opening section of the Examples, with the feature that the incubation occurred under anaerobic conditions.

Figure 12 is a table summarizing aPL assay results for a blood sample from a normal aPL-negative subject, incubated according to the method described in the opening section of the Examples, with the feature that K562 cells (a human tumor cell line) were used instead of RBC.

Figure 13 is a table summarizing aPL assay results for a blood sample from a normal aPL-negative subject, except that the bacteria culture growth medium was replaced with a cell culture medium used for growing human cells.

Figure 14 is a table summarizing aPL assay results for a cord blood sample from a normal aPL-negative mother and baby.

Figure 15 is a table summarizing aPL assay results for a blood sample from a normal aPL-negative subject, incubated according to the method described in the opening section of the Examples, with the feature that sodium nitroprusside (SNP) was used in place of RBC in the incubation mixture.

Figure 16 is a table summarizing results of a lupus anticoagulant activity assay for a blood sample incubated according to the method described in the opening section of the Examples. The blood sample was obtained from a subject whose blood is lupus anticoagulant negative prior to seroconversion by the present invention process.

Figure 17 is a table listing othertypes of autoantibodies that have been identified in blood samples that are incubated according to the method described in the opening section of the Examples. The listed antibodies were identified by immunofluorescence microscopy.

Figure 18 is a graph showing the forward scatter (size) and side scatter (granularity) profile of the monocyte population of cells as defined for density gradient isolated human white blood cells by flow cytometry.

Figures 19 A - D are flow cytometry histograms showing monocyte activity of various sera. In the histograms, antibody activity, if present, is measured by shifts in the median channel values (log scale) along the horizontal axis. Figure 19A shows monocyte reactivity of pooled normal human sera (NHS). Figure 19B shows the monocyte activity of a serum from a single normal subject. Figure 19C shows the monocyte activity of a blood sample from the subject shown in Figure 19B that was treated according to the method described in the opening section of the

Examples. Figure 19D shows the monocyte activity of positive control sera.

Figure 20 is a table summarizing the results of anti-nuclear-antibody (ANA) testing of various samples using a RELISA® screening assay.

Figure 21 is a table summarizing aPL assay results for a sample of Ivlg that was incubated with hemin in a tris buffer.

Figure 22 is a graph showing the amount of aPS, aCL, aPE, and aPC (as measured by optical density, OD) detected in a series of Ivlg preparations that were incubated with hemin, as a function of the amount of human serum (in  $\mu$ l) added to the preparations.

Figure 23 is a graph showing the amount of aPS, aCL, aPE, and aPC (as measured by optical density, OD) detected in a series of diluted human serum preparations that were incubated with hemin, as a function of the amount of hemin (in  $\mu$ l) added to the preparations.

Figure 24 is a graph showing the amount of aPS, aCL, aPE, and aPC (as measured by multiples of the mean, MoMs) detected in a series of Ivlg preparations that were incubated with hemin and Vitamin C, as a function of the amount of Vitamin C (in  $\mu$ g) added to the preparations.

Figure 25 is a graph showing the amount of aPS, aCL, aPE, and aPC (as measured by multiples of the mean, MoMs) detected in a series of Ivlg preparations that were incubated with NaOH solubilized hemin, DMSO-solubilized hematoporphyrin IX (hpIX), NaOH-solubilized hpIX, NaOH alone, DMSO alone, and DMSO-solubilized hemin.

Figure 26 is a graph showing the amount of aPS (as measured by optical density, OD) detected in a series of Ivlg preparations that were incubated with increasing amounts of hemin and increasing amounts of hemin and hemopexin (hpx).

Figure 27 shows the Western blots obtained for three cell lysates with hemin-treated Ivlg and untreated Ivlg used as primary antibodies, along with a blot wherein anti-human HRP-tagged conjugate was used as a control.

Figures 28A and 28B are graphs showing the amount of aPL dependent and aPL independent aPS, aCL, aPE, and aPC (as measured by multiples of the mean, MoMs) detected in a series of Ivlg preparations in which electrodes connected to a 9 volt battery were immersed in a phosphate buffered saline solution containing the Ivlg for 2 minutes.

Figure 29 is a graph showing the amount of aPS, aCL, aPE, and aPC (as measured by multiples of the mean, MoMs) detected in a series of Ivlg preparations in which electrodes connected to a 6 volt battery were immersed in a phosphate buffered saline solution containing the Ivlg for 60 seconds.

Figure 30 is a graph showing the amount of aPS, aCL, aPE, and aPC (as measured by multiples of

the mean, MoMs) detected in a series of Ivlg preparations in which electrodes connected to a 6 volt battery were immersed in a phosphate buffered saline solution containing the Ivlg, as a function of the time of immersion.

Figures 31A, 31B and 31C are graphs showing the amount of aCL, aPE, and aPS, respectively (as measured by multiples of the mean, MoMs), detected in control solutions before and after exposure for 240 seconds to electrodes connected to a 6 volt battery.

Figure 32 is a graph showing the amount of aPS and aCL, respectively (as measured by multiples of the mean, MoMs), detected in the PBS-diluted serum of an aPS and aCL-positive patient. In the experiment, graphite electrodes connected to a 6 volt battery were immersed in the diluted serum for a variable amount of time.

Figure 33 is a graph showing the amount of aPS, aCL, aPE, and aPC (as measured by multiples of the mean, MoMs), respectively, detected in the PBS-diluted serum of an aPE-positive patient. In the experiment, graphite electrodes connected to a 6-volt battery were immersed in the diluted serum for a variable amount of time.

Figure 34 is a graph showing the amount of aPS, aCL and aPE, (as measured by optical density, OD), respectively, detected in the PBS-diluted serum of an aPE-positive patient. In the experiment, the 10% adult bovine plasma (ABP) used in the determination of protein-dependent aPL binding was treated by immersing graphite electrodes connected to a 6-volt battery in the ABP for a variable amount of time.

### **BEST MODE FOR CARRYING OUT THE INVENTION**

**[0031]** The present invention relates to a method of altering the binding specificity of at least one plasma protein or circulating protein in a biological fluid or extract of a biological fluid.

**[0032]** As used herein, the terms "circulating protein" and "plasma protein" are used to refer to a protein naturally found in the circulation system of animals. Examples of circulating proteins include antibodies and other plasma proteins. It is to be understood that the method of the invention is not meant to apply universally to all plasma proteins or circulating proteins, but rather applies to any plasma protein or circulating protein that has the property of having a binding specificity that can be altered by a change in the redox state of the protein. The discovery by the inventor that there are circulating proteins, such as autoantibodies, that have this property forms a basis of the invention. Examples of non-antibody proteins that have been found to have a binding specificity that can be altered by a change in the redox state include kininogen and prothrombin and/or beta2 glycoprotein.

**[0033]** The term "masked circulating protein" is newly coined for the present invention to designate and de-

scribe a circulating protein that, in normal individuals, is present in the blood, but is not detectable by conventional binding assays based on receptor-ligand binding because its binding site is, in the normal individual or in a sample taken from the normal individual, masked or blocked or otherwise prevented from binding an antigen, and that, when a sample containing the masked circulating protein is treated by changing its redox state, such as by exposure to an oxidizing agent or electric current according to a method of the present invention, becomes capable of binding an antigen and thereby becomes detectable in a sample. An example of a masked circulating protein is an autoantibody. As discovered by the present inventor, autoantibodies circulate in significant quantities in normal blood, but they are not detectable in conventional assays based on antibody-antigen binding. As discussed herein, an autoantibody becomes detectable and recoverable when the autoantibody is subjected to oxidation-reduction conditions sufficient to alter its binding specificity. Autoantibodies that have been unmasked by oxidation include anti-phospholipid, anti-nucleolar (scleroderma associated), anti-lamins (very bright at nuclear pores), anti-mitochondrial (cytoplasmic), and anti-centriole antibodies. Further, it has also been found that blood, serum or Ivlg samples that initially test negative for HCV (hepatitis C virus) tests positive for HCV after a treatment according to the present invention, suggesting that normal individuals have masked anti-HCV antibodies in their circulation.

**[0034]** The term "altering the binding specificity" of a protein refers to a process whereby a protein is changed or altered, such as by oxidation or reduction, so that it becomes capable of specific binding of an antigen or ligand that it had not previously been capable of specifically binding or becomes incapable of specific binding of an antigen or ligand that it had previously been capable of specifically binding. The term "unmasking" refers to a process wherein the binding specificity of a masked circulating protein is altered so that the protein becomes detectable by a binding assay based on the altered binding specificity.

**[0035]** The term "autoantibody" refers to any naturally occurring antibody produced by the immune system of an animal and that binds to a self-antigen, that is, to a compound or antigen produced by the animal itself.

**[0036]** The term "biological fluid" includes any bodily fluid that contains circulating proteins, including plasma, serum and whole blood, saliva, urine, lactation fluids and other secretions. The term "protein-containing extract of a biological fluid" refers to any preparation that is collected or separated from a biological fluid, such as immunoglobulin fractions. Blood, serum or plasma that may be used in the present invention may be freshly obtained from an individual, or it may be obtained from such sources as pooled blood or plasma preparations obtained from blood banks or other blood collection facilities. For the purposes of the present invention, the blood, serum or plasma may also be from collections that are out-of-date

or otherwise found to be substandard by blood banks or blood collection facilities. Although this description focuses upon human blood, plasma and serum, the identical process of this invention can be applied to animal blood and should result in obtaining analogous animal antibodies for purposes relating to veterinary medicine. Preferably, blood or serum used in the method of the invention is diluted to reduce the effect of any antioxidants that may be contained in the blood, plasma or serum.

**[0037]** In the method of the present invention, the binding specificity of at least one circulating protein or plasma protein in a biological fluid is altered by exposing the protein to an oxidant or to an electric current. For example, the binding specificity of a masked circulating protein can be altered so that the protein is unmasked, that is, so that it is able to bind an antigen that it was not able to bind before the method was carried out. A protein that has had its binding specificity altered may then be isolated and recovered by any separation method based on specific binding.

**[0038]** If an oxidizing agent is used to carry out the method of the invention, the oxidizing agent can be any compound that is capable of altering the redox state of a biological molecule. More specifically, the oxidizing agent is a molecule that has the ability to be reduced by acting as an electron acceptor for other molecules that act as electron donors. Examples of oxidizing agents include, but are not limited to hemin, chlorophyll, or other ring compounds containing a strong oxidizing metal, and  $\text{KMnO}_4$ . Typically, when an oxidizing agent is used, a mixture of the biological fluid or extract and the oxidizing agent must be incubated for a period of time, typically for about a day or overnight. The oxidizing agent should be used at a concentration sufficient enough to alter the binding specificity of a protein having an alterable binding specificity, but not at a concentration that might destroy the protein. In the case of autoantibodies, it has been found that different types of autoantibodies can interact differently with different antioxidants. For example, for the unmasking of aPC autoantibodies, the results are poor with hemin and very good with  $\text{KMnO}_4$ .

**[0039]** If a DC electric current is used to carry out the method of the invention, the method may be carried out by any means of delivering an electric current, such as by immersing positive and negative electrodes into a conductive solution containing the sample to be treated. Typically, a solution containing a biological fluid may be exposed to an electric potential of a sufficient magnitude and of a sufficient duration to alter the binding specificity of a protein having an alterable binding specificity. It has been found that positive results may be obtained by exposing a solution to an electric potential of 6 - 24 volts for a few seconds to a few minutes. As discussed in the examples, an extended exposure to an electric current may result in reversibility of the alteration of the binding specificity.

**[0040]** Attempts to produce positive results using an AC current have been unsuccessful.

**[0041]** Without being bound to a specific theory, it is preferred, in the case of an autoantibody, that the autoantibody be exposed to the oxidizing agent or electric current in an amount or for a time sufficient to oxidize an antigen binding site in a Fab portion of the autoantibody.

**[0042]** Whether a particular protein of interest is one that has a binding specificity that can be altered by changing its redox state and the effectiveness of any set of conditions for altering the binding specificity of the particular protein of interest may be readily determined by ELISA or other ligand-receptor assays. Such assays can be carried out before and after a protein is subjected to redox conditions to see whether the process has altered the binding specificity of the protein. For example, the best oxidizing agent to recover a specific autoantibody can be readily determined by simple experimentation.

**[0043]** A further aspect of the present invention is the possibility of treating a subject having an autoimmune disease, either by administering to the subject an amount of an antioxidant sufficient to inactivate autoantibodies in the subject or by taking a blood sample from the subject, exposing the blood sample to an antioxidant or electric current sufficient to inactivate autoantibodies in said blood sample, and returning the blood sample to the subject.

**[0044]** A further aspect of the present invention is a method of screening a normal individual's biological fluid or extract to determine which autoantibodies are masked and thus construct a potential antibody profile of autoantibodies that could cause autoimmune disease in that individual if exposed or unmasked by oxidation or an electromotive force. For example, in general terms, a blood, plasma or serum sample from a subject can be assayed to determine an amount and/or type of autoantibodies detectable in the sample. Thereafter, a blood, plasma or serum sample from the subject can be treated by exposing the sample to an oxidizing agent or a DC electric current, and the treated blood, plasma or serum sample from the subject can be assayed to determine an amount and/or type of autoantibodies detectable in the treated sample. Thereafter, the amount and/or type of autoantibodies detectable in the sample before the treating step can be compared with the amount and/or type of autoantibodies detectable in the sample after the treating step.

**[0045]** It has been found that untreated blood, plasma, serum or Ivlg samples and blood, plasma or serum or Ivlg samples treated according to the method of the present invention can be lyophilized and shipped or stored. When the samples are reconstituted, they retain their respective activity.

## EXAMPLES

**[0046]** Having described the invention, the following examples are given to illustrate specific applications of the invention, including the best mode now known to perform the invention. The examples are presented in approximate chronological order and thus show a progres-

sion in the understanding of components and procedures required to achieve the effects of the invention. These specific examples are not intended to limit the scope of the invention described in this application.

**[0047]** Regarding each of the Examples 1 - 17 described herein, unless otherwise noted, the following procedure was typically used: A 10 ml sample of whole blood or 5 ml of serum or plasma from a normal aPL-negative subject and 4-5 ml of packed mammalian red blood cells were added to a vial containing 30 ml of Biomerieux brand bacterial culture growth medium (containing at least the following ingredients: distilled water, soybean-casein digest broth, yeast extract; dextrose; sucrose; hemin; menadione (vitamin K3); pyridoxal HCl (vitamin B6); and sodium polyanetholesulfonate (SPS) and charcoal. Then, the mixture was incubated, with rocking or shaking, at 37 °C for a period of 18 - 22 hours. Following the incubation and centrifugation, a sample of the incubated blood or serum/RBC was tested for the presence of antiphospholipid antibodies (aPL) using a comprehensive in-house ELISA aPL format that provides 24 separate aPL test results. The testing procedure is described in greater detail in the following publications: Wagenknecht DR, et al., The Evolution, Evaluation and Interpretation of Antiphospholipid Antibody Assays, Clinical Immunology Newsletter, Vol. 15, No. 2/3 (1995) pp. 28 - 38 and McIntyre JA, et al., Frequency and Specificities of Antiphospholipid Antibodies (aPL) in Volunteer Blood Donors, Immunobiology 207(1): 59-63, 2003.

**[0048]** Figure 1 shows the 24 separate aPL specificities that were tested for by using the comprehensive in-house ELISA aPL format. Four specificities were assessed, 1) aPS = antiphosphatidylserine, 2) aCL = anti-cardiolipin, 3) aPE = antiphosphatidylethanolamine, and 4) aPC = antiphosphatidylcholine. For each of these aPL specificities, three immunoglobulin isotypes were sought, IgG, IgA and IgM. Each specificity and each isotype were assessed in the presence (dependent) and absence (independent) of a buffer diluent supplement, 10% adult bovine plasma (ABP), which contains the phospholipid-binding plasma proteins) or 1 % bovine serum albumin, (BSA, which is devoid of phospholipid-binding plasma proteins), respectively. The final dilution of the subjects' blood samples was between 1/50 and 1/100.

**[0049]** The results in the 24 aPL specificities obtained for the various experiments described herein are given in the accompanying figures. The positive/negative findings are expressed in multiples of the means (MoM) based on testing plasma samples from 775 normal blood donors, as described in McIntyre JA, Immunobiology, above. The presence of +++ indicates strong antibody activity. The markers of + and ++ indicate low and intermediate antibody activity, respectively. The figures also provide the normal range values for each aPL specificity and isotype combination.

**[0050]** A positive result in the column indicated as PL binding protein "dependent" means that the antiphos-

pholipid antibody (aPL) is actually binding to a plasma protein that initially has bound to the particular phospholipid indicated. Plasma proteins that typically can be bound by PS and CL include the following: beta<sub>2</sub>-glycoprotein I, prothrombin, protein C, protein S, annexin V, and complement components Factor H and C4 (see, for example, McIntyre, J.A., Wagenknecht, D.R. and Faulk, W.P. Antiphospholipid antibodies: Discovery, definition, detection and disease. Prog. Lipid Res. 42(3): 176-237, at page 182). The physiological nature of the plasma protein binding is not known precisely for all of the phospholipids, but such binding is thought to induce conformational changes in the plasma protein structure, thereby exposing novel or cryptic epitopes that then are targeted by the individuals' autoantibodies. Plasma proteins that typically can be bound by phospholipid PE include the following: high and low molecular weight kininogens, and factor XI and prekallikrein. The latter two proteins can be detected by virtue of their fidelity in binding to high molecular weight kininogen. The plasma proteins that bind to PC have not yet been defined. In certain experiments, plasma-protein independent aPL are observed (see Figure 3). A possible explanation for this activity is that it represents the presence of residual phospholipid-binding plasma proteins that are present in the original blood sample.

#### Example 1.

**[0051]** A sample of blood from a normal subject was incubated and tested according to the procedure described above. The results of the aPL ELISA are shown in Figure 2. As shown in Figure 2, the incubated blood sample shows a dramatic presence of autoantibody activity, in comparison to the normal, untreated blood shown in the Normal ranges column. In particular, strong autoantibody activity is shown in the protein-dependent category for aPS (IgG), aCL (all isotypes), and aPE (IgG). The low or absent IgG aPC autoantibody activity was a characteristic finding in the early examples and in procedures in which hemin was used as the oxidizing agent. This result indicates that autoantibodies to PC, especially of the IgG isotype are different and perhaps do not become activated in the same way as do the others. In later experiments, it was found that significant levels of aPC can be detected in samples that were treated with KMnO<sub>4</sub> (data not shown).

#### Example 2.

**[0052]** Blood samples drawn from seven healthy subjects were incubated and tested according to the procedure described above. In particular, all seven subjects' bloods were drawn within a 20 minute period and incubated for 20 hours in identical conditions. Figure 3 is a composite table showing the range of aPL seroconversion for these seven samples. These results show that there are variations in the aPL levels detected as well as

the isotypes present among different individuals. Nevertheless, as shown by the invention, each individual had aPL antibodies that could be detected after incubation.

#### Example 3.

**[0053]** In a first experiment, a serum sample from a normal subject was incubated and tested according to the basic procedure described above. In the incubation mixture, horse red blood cells (RBC) were used instead of human RBC. The results of the aPL ELISA are shown in Figure 4. As shown in Figure 4, significant aPL activity was obtained, particularly with respect to aPS (IgG and IgM) and aCL (IgA and IgM).

**[0054]** In a second experiment, a horse serum, instead of human serum, was incubated with human RBC and tested according to the basic procedure described above. The results of the aPL ELISA are shown in Figure 5. As shown in Figure 5, aPL activity was not obtained. (The ELISA assay used in this experiment utilized human-antibody-specific alkaline phosphatase labeled antibody probes to detect aPL, so whether the incubated sample contained horse aPL is unknown.)

**[0055]** The results shown summarized in Figures 4 and 5 unequivocally demonstrate that all aPL that are obtained during the seroconversion process of the present invention originate from the human serum and are not released from the human RBC, since the first experiment uses horse RBC, which are devoid of human antibodies, in place of human RBC and still shows positive results, whereas the second experiment uses horse serum in the presence of human RBC and shows negative results.

#### Example 4.

**[0056]** A blood sample from a normal subject was incubated and tested according to the basic procedure described above, except that the incubation was carried out at room temperature (22°C), instead of at an elevated temperature. Figure 6 shows that the sample did not undergo seroconversion when incubated at room temperature. These results suggest that the process of seroconversion may be temperature sensitive.

#### Example 5.

**[0057]** A blood sample from a normal subject was incubated and tested according to the basic procedure described above, with the feature that 0.7 mm Degalan (plastic) beads were used as the particulate solid in the incubation mixture instead of charcoal. Since charcoal was used in initial experiments showing seroconversion, this experiment was carried out to determine whether charcoal plays a specific role in the seroconversion. Figure 7 shows that the sample exhibited seroconversion even when plastic beads were used in place of charcoal. These results suggest that the role of charcoal is mechanical, rather than chemical, in nature, and that any

particulate solid, such as plastic, resin or glass beads, can be used. Without being limited to any particular theory, it can be theorized that the particulate component acts as an abrasive upon the RBC membrane, probably causing release of the NO ion from the RBC, either by interacting with the RBC AE1/Band 3 protein or with the SNO-hemoglobin transition molecules or both. The possibility of mechanical abrasion is supported by the observation in Example 6, wherein negative assay results are shown for an incubation mixture that is not rocked or shaken. The particulate solids may also serve a mechanical function of assisting autoantibody release.

#### Example 6.

**[0058]** A blood sample from a normal subject was incubated and tested according to the basic procedure described above except that the incubation mixture was kept stationary, instead of being shaken or rocked. Figure 8 shows that the sample did not undergo seroconversion when it was kept stationary. These results suggest that movement may facilitate interaction between solid particles and RBC. Stationary incubation conditions did not facilitate aPL release, although a small amount of movement such as produced by transport of the samples to the incubator may produce small amounts of aPL release.

#### Example 7.

**[0059]** A blood sample from a normal subject was incubated and tested according to the basic procedure described above, with the added feature that after incubation and removal of RBC and charcoal by centrifugation, the incubation mixture was heated to 56° C for 30 minutes. Figure 9 shows that the amount of detected aPL was significantly increased by this procedure.

#### Example 8.

**[0060]** A blood sample from a normal subject was incubated and tested according to the basic procedure described above, with the feature that a bacterial culture growth medium from a different supplier (Becton Dickinson, Sparks, MD) was used in place of the bacterial culture growth medium from Biomerieux. Figure 10 shows that the sample exhibited seroconversion in the Becton Dickinson medium, indicating that the method of the present invention is not dependent upon a bacterial culture growth medium from a particular source.

#### Example 9.

**[0061]** A blood sample from a normal subject was incubated and tested according to the basic procedure described above, with the feature that the incubation occurred under anaerobic conditions (under nitrogen) instead of under aerobic conditions (in the presence of ox-

xygen and CO<sub>2</sub>). Figure 11 shows that the sample exhibited seroconversion even under anaerobic conditions and that the method of the present invention is not dependent upon an aerobic environment.

#### Example 10.

**[0062]** A blood sample from a normal subject was incubated and tested according to the basic procedure described above, with the feature that K562 cells (a human hematopoietic tumor cell line) were used instead of red blood cells. Further, only 11.3 million K562 cells were present in the culture media, compared to 3-4 mls of packed RBC typically used in the method of the invention. Figure 12 shows that the sample exhibited seroconversion.

**[0063]** Other experiments have shown that samples that are incubated with other isolated cell types, lymphocytes, monocytes and neutrophils typically do not exhibit aPL seroconversion. In particular, white blood cells of the lymphoid and myeloid series did not support aPL release, nor did a cell line of porcine B lymphocytes designated as L14 (data not shown). These results suggest that hemoglobin may be a key component in the incubation mixture, since K562 cells and RBC contain hemoglobin, and lymphocytes, monocytes and neutrophils do not.

#### Example 11.

**[0064]** A blood sample from a normal subject was incubated and tested according to the basic procedure described above, except that the bacteria culture growth medium was replaced with a cell culture medium used for growing human cells: RPMI. Figure 13 shows that seroconversion did not occur. This experiment shows the importance of some ingredient in the bacterial culture media for the purpose of this invention. While RPMI is a culture media designed for human cells, it does not support aPL release when substituted for vial broth. Listings and comparisons of the ingredients in the two different microbiology vial broths with RPMI show that RPMI lacks hemin and menadione (a man-made provitamin K) called vitamin K3. It is known that hemin is a porphyrin chelater of iron (Fe<sup>+++</sup>) derived from RBC, and menadione is a fat-soluble vitamin. This indicates that redox reactions may play a role in autoantibody release

#### Example 12.

**[0065]** A placental cord blood sample incubated and tested according to the basic procedure described above. The placental cord blood was drawn after the birth of the baby, but before the placenta was detached from the uterine wall. Neither the mother's blood nor the baby's cord blood showed the presence of aPL in conventional laboratory assays. When processed according to the invention described herein, strong aPL antibody was dem-

onstrated present in the cord blood samples, as shown in Figure 14. The antibodies were IgG only, an observation that is compatible with antibodies of maternal origin. Since the mother transports IgG to the fetus prior to birth, this experiment seems to indicate that the masked maternal autoantibodies transported to the fetus by way of specialized Fc $\gamma$  receptors on the trophoblast (Fc $\gamma$ Rn) remain masked by the fetus in the fetal blood. Since the mother's blood and the cord blood were shown to be aPL-negative prior to seroconversion by the method of the invention, and since there were no IgM or IgA immunoglobulins detected, these findings support the contention that the IgG aPL observed in the cord blood subsequent to seroconversion are maternal in origin. It also is of interest that the trophoblast that expresses the Fc $\gamma$ Rn does not express HLA antigens.

#### Example 13.

**[0066]** A plasma sample from a normal subject was incubated and tested according to the basic procedure described above; with the feature that sodium nitroprusside (SNP, 200 micromolar) was used in place of RBC in the incubation mixture. Figure 15 shows that the sample exhibited seroconversion.

**[0067]** Since SNP is a potent nitric oxide (NO) donor, these results provide supportive evidence that the NO radical is involved in the autoantibody release and further support a theory that RBC and solid particulates fulfill a role of providing NO- donation from the RBC. Other free radical mediated reactions apart from sodium nitroprusside may also cause autoantibody release.

#### Example 14.

**[0068]** A blood sample from a normal subject was incubated according to the basic procedure described above and was tested for lupus anticoagulant activity. Lupus anticoagulant or inhibitor is another type of aPL and is typically detectable only by functional laboratory assays. The results in Figure 16 show a strong lupus anticoagulant (LA) in the seroconverted blood taken from a lupus inhibitor negative individual and processed by the method of this invention. While initially corrected by adding normal plasma to the seroconverted broth in the dRVVT assay, incubation for 1-2 hours resulted in the reappearance of the inhibitor. This time frame is proposed as the time it takes for the LA or unmasked antibodies to bind the relevant phospholipid-binding plasma proteins introduced by the mixing study. It also rules out the possibility of clotting factor deficiencies since a 1:1 mix provides sufficient levels of clotting factors to correct clotting times in a factor deficient sample. The dilute prothrombin time (dPT) did not correct in the presence of normal plasma and increased prolongation of clotting times was observed after incubation with normal plasma, which is indicative of a strong lupus inhibitor.

**Example 15.**

**[0069]** Blood samples from five normal subjects were incubated according to the basic procedure described above and were tested by fluorescence microscopy for the presence of other types of autoantibodies. Sera and plasma samples from these five individuals were negative prior to processing according to the teachings of the invention.

**[0070]** Figure 17 lists additional autoantibody specificities identified by using the Hep-2 cell line. Identified were anti-nucleolar (scleroderma associated), anti-lamins (very bright at nuclear pores), anti-mitochondrial (cytoplasmic), and anti-centriole. The results show that autoantibodies released by the method of the present invention can also be detected by a different methodology of detection, fluorescence microscopy, as opposed to ELISA-based testing. The results confirm that many types of autoantibodies besides aPL are masked in the blood of individuals whose serum and plasma test negative for these antibodies in routine laboratory analyses.

**[0071]** From these results, it can be expected that many more autoantibody specificities await to be found by testing bloods processed by this invention.

**Example 16.**

**[0072]** A blood sample from a normal subject was incubated according to the basic procedure described above and was tested for reactivity with monocytes using flow cytometry and fluorescent conjugated antihuman IgG antibodies. Comparative testing was done with untreated-pooled normal human sera (NHS), with serum from the same normal subject used with the invention and with positive control human sera. (The treated blood showed no auto reactivity with lymphocytes and neutrophils; these data are not shown.) Figure 18 depicts the forward scatter (size) and side scatter (granularity) profile of the normal subject's monocyte population of cells as defined by flow cytometry. This monocyte population of cells was confirmed by showing reactivity with CD 14 monoclonal antibodies. Figure 19A shows anti-monocyte reactivity with NHS. The median reactivity shown is 743.50 on a linear scale. Figure 19B shows the auto-anti-monocyte activity of the normal subject's serum; this subject does not have antibody activity to autologous monocytes. The median reactivity shown is 737.00. Figure 19C shows the auto-anti-monocyte activity of a blood sample from the subject shown in Figure 19B after it is treated according to the method of the invention. The median value is shown is 864.00, indicating strong auto-anti-monocyte activity. Despite the fact that the plasma processed according to the teachings of the invention were used at a dilution of 1/8, it showed more reactivity with monocytes than did the undiluted positive control sera. Thus, this example shows that blood or serum samples processed according to the method of this invention release autoantibodies that specifically target monocytes.

The same results were documented for four additional samples from other individuals when processed according to the teachings of the invention.

**Example 17.**

**[0073]** Comparative tests for the presence of anti-nuclear-antibodies (ANA) using a RELISA® screening assay were carried out on untreated cord blood serum; cord blood incubated according to the method of the present invention, with no rocking; cord blood treated according to the method of the present invention, with rocking; untreated serum from an ANA-negative healthy donor (identified as ACS) and serum from the same ANA-negative healthy donor that was incubated according to the method of the present invention. As shown in Figure 20, a significant amount of ANA was identified in cord blood and serum samples that were treated by the method of the present invention. From the results in Figures 16 and 17, it can be expected that many more autoantibody specificities wait to be found by testing bloods processed by this invention.

**Example 18**

**[0074]** To understand the role of red blood cells in the phenomenon of autoantibody release, experiments were designed to replace the red blood cells with simpler ingredients that might mimic the action of the red blood cells. In the present experiment, the red blood cells and charcoal were replaced with sodium nitroprusside (SNP) and ferric chloride. This substitution was made because sodium nitroprusside is a powerful nitric oxide producer, and it is known that the RBC are carriers of NO<sup>-</sup>. Ferric chloride (FeCl<sub>3</sub> stock solution, 25 μM), was added as a substitute for the iron in hemoglobin.

**[0075]** Culture bottles containing the bacterial culture growth medium and 5 ml of human plasma or serum and varying concentrations of sodium nitroprusside (SNP, 200 μM) and exogenous ferric chloride (4.1 μM final concentration) was used in place of red blood cells and charcoal, were incubated at 37 °C and then heated to 56 °C for 30 minutes. The samples showed seroconversion of aPL, but only IgG (data not shown).

**[0076]** The results suggest that NO<sup>-</sup> may be involved in antibody unmasking, and suggest that the mechanical action of a solid phase material in the culture bottle disrupts the red blood cells and releases NO<sup>-</sup>. Alternatively, the release or modification of NO may enable the hemoglobin molecule to participate in redox reactions.

**Example 19**

**[0077]** In an effort to determine whether the effect of unmasking autoantibodies was due to the breakdown of autoantibody-containing macromolecular structures within serum or blood or whether it was due to direct changes in the binding specificity of antibodies them-

selves, a series of experiments were carried out in which commercial intravenous immunoglobulin (Ivlg) was substituted for human plasma or serum. Commercial Ivlg is an alcohol precipitate fraction of pooled plasma from multiple donors, typically from 1,000 - 10,000 donors. Typically, Ivlg contains primarily IgG, and is mostly devoid of IgA, IgM and other plasma proteins. When untreated Ivlg is tested for the presence of autoantibodies by ELISA testing, the test results are negative. Because of its manner of preparation, Ivlg is also free of lipoprotein micelles, vesicles or other macromolecular structures. Therefore, if Ivlg were to test positive for the presence of autoantibodies after an incubation treatment, it would have to be that the autoantibodies were obtained by an alteration of IgG antibodies already present in the Ivlg preparation and not by a breakdown of structures or vesicles concealing the autoantibodies.

**[0078]** In the examples that follow, the commercial preparation of Ivlg used was lyophilized Ivlg (Immune Globulin Intravenous (Human) Gammar- PL.V., Aventis Behring, Kankakee, Illinois).

**[0079]** A 5 gram commercial preparation of lyophilized Ivlg was reconstituted in sterile phosphate buffered saline (PBS, 100mg/ml). 1.7 ml of the reconstituted Ivlg solution was added to a culture bottle containing the bacterial culture growth medium (without red blood cells or charcoal) and was incubated at 37 °C for 20 hours. The incubated mixture showed seroconversion and the presence of aPL IgG (data not shown). (As expected, only IgG was detected, not IgA or IgM.)

**[0080]** In similar experiments, autoantibodies were detected in a mixture that was incubated at room temperature in a shaking vial, but the results were not as good as at 37 degrees (results not shown).

**[0081]** Heating the Ivlg-bacterial growth medium mixture above 37 °C did not result in further increases in autoantibodies.

**[0082]** As a control, Ivlg straight out of the bottle was tested for aPL and other autoantibodies, and the results were negative.

#### Example 20

**[0083]** In Example 19, it is shown that autoantibodies can be obtained by incubating a commercial Ivlg preparation in a bacterial growth medium. The next step was to try to determine which ingredients in the bacterial culture growth medium play a role in producing detectable autoantibodies.

**[0084]** First, Ivlg in 2% tryptic soy broth (TSB), (which contains peptones in a 17 to 3 ratio of pancreatic digest of casein to papaya digest of soy, respectively) (the remainder being water) was incubated at 37°C for 20 hours with shaking. The incubated mixture was tested for the presence of aPL, and the result was negative.

**[0085]** Next, Ivlg was incubated in a test tube in soy broth, sodium nitroprusside (SNP) and hemin (an iron (ferric) containing protoporphyrin) at 37°C for 20 hours

with shaking. The amounts used were 60 microliters of Ivlg, 5 microliters of SNP and 5 microliters of hemin in a total of 1 ml of soy broth. The incubated mixture tested positive for the presence of aPL, particularly aPS (15 MoM) and aPE (41 MoM). (data not shown)

#### Example 21

**[0086]** A series of experiments was conducted to determine if incubation with hemin alone would be sufficient to cause the appearance of autoantibodies in Ivlg or in plasma or serum.

**[0087]** Reconstituted lyophilized Ivlg (at a concentration of 100 mg/ml) was added to and incubated in a phosphate buffered saline (PBS) solution with hemin for 20 hours at 37 °C. The amounts used were 300 µl of Ivlg solution and 5 µl of a hemin solution (75 µg) in a total volume 1 ml.

**[0088]** As shown in Figure 21, the incubated mixture showed significant amounts of aPS and aPE IgG, and, to a lesser extent, aCL IgG.

**[0089]** When serum or plasma were incubated with hemin under similar conditions, no autoantibodies were detected.

#### Example 22

**[0090]** The fact that positive results for the presence of autoantibodies could be obtained when Ivlg was incubated with hemin, whereas negative results were obtained when serum or plasma were incubated with hemin suggested that serum or plasma could contain substances that inhibit or interfere with the process of obtaining autoantibodies.

**[0091]** In a series of experiments, Ivlg was incubated in a Tris buffer with hemin, for 20 hours at 37 °C, similar to the process of Example 21, with the added feature that an increasing amount of human serum (the inventor's) was added to the batches before incubation. Each separate batch was tested for the presence of aPS, aCL, aPE and aPC autoantibodies, and the results are shown in Figure 22. The results shown in Figure 22 demonstrate that increasing amounts of serum did have an inhibitory effect on the release of antiphospholipid antibodies. Similar results were shown with substituting plasma for serum (data not shown). A possible explanation for these results is that hemin, which contains an iron molecule in the ferric state and which is known as an active oxidizing agent, may act to oxidize a binding site of certain immunoglobulin molecules so that the altered binding site is able to bind self antigens. This process may be inhibited by substances, perhaps antioxidants, in the blood.

#### Example 23

**[0092]** Human serum (the inventor's) was diluted 1/10 in Tris buffer. In a series of experiments, this diluted serum, in 1 ml batches, was incubated with an increasing

amounts of hemin, specifically, 0  $\mu$ l, 10  $\mu$ l, 25  $\mu$ l and 50  $\mu$ l. (Previously, it had been found that hemin by itself was not sufficient to cause the release of autoantibodies from blood or serum, although it was sufficient to cause such release from Ivlg. Therefore, the purpose of diluting the serum was to dilute the effect of any interfering substances found in the blood, such as antioxidants.) The batches were tested for the presence of aPS, aCL, aPE and aPC autoantibodies, and the results are shown in Figure 23. The results shown in Figure 23 show that while no significant amounts of autoantibodies were detected in diluted serum when 0 or 10  $\mu$ l of hemin is added, significant amounts are detected with 25  $\mu$ l of hemin. For an unknown reason, the amounts of detected autoantibodies were less with 50  $\mu$ l of hemin.

#### Example 24

[0093] The next series of experiments was designed to determine if an antioxidant such as vitamin C, which is present in blood, would inhibit the release of autoantibodies. In a series of experiments, Ivlg was incubated in a Tris buffer with hemin, with the added feature that an increasing amount of ascorbic acid (Vitamin C) was added to the hemin-containing buffer and allowed to mix for 30 minutes before adding the Ivlg and before incubation. As shown in Figure 24, there was about 78% inhibition of hemin-induced aPE release with 1 mg of Vitamin C, an amount that represents a physiological concentration of Vitamin C. There is a biphasic curve with aPS release, which raises the possibility that Vitamin C at low concentrations can act as an oxidizing agent, but becomes an antioxidant (reducing) agent at higher concentrations.

#### Example 25

[0094] The next series of experiments was designed to determine whether the vehicle that hemin is dissolved in has an impact on the results obtained and whether the iron atom in hemin is necessary. In a series of experiments, Ivlg was incubated in a Tris buffer with hemin, or with other additives. In particular, in one instance, hemin was solubilized with NaOH. In another instance, it was solubilized with DMSO. In other instances, hematoporphyrin IX (hpIX), which is the same molecule as hemin, but without the iron ( $Fe^{+++}$ ), was used in place of hemin and was solubilized with NaOH or DMSO. In other instances, NaOH and DMSO were tested as controls (without hemin or hpIX). As shown in Figure 25, the use of NaOH solubilized hemin produced positive results for the presence of autoantibodies, whereas hemin + DMSO, hpIX + NaOH, hpIX + DMSO, NaOH alone, and DMSO alone did not produce positive results.

#### Example 26

[0095] To further establish that hemin was causing oxidation of antibodies, equimolar amounts of hemopexin

(Hpx) were added to the Ivlg PBS hemin mixture. Hpx is an antioxidant molecule with an extraordinarily high binding affinity for heme iron. Lyophilized Hpx purchased from SciPac (Kent, England) was reconstituted in PBS at 10mg/ml. Shown in Fig. 26 are the aPS redox data resulting from adding increasing concentrations of hemin to the Ivlg countered with adding equimolar concentrations of Hpx. Because there is an 1:1 binding interaction between hemin and Hpx, the Hpx was able to negate the redox capability of the ferric iron present in hemin.

#### Example 27

[0096] To illustrate the broad range and activity of autoantibodies that can be obtained by an oxidation treatment of Ivlg, a series of Western blots were set up using cell lysates from 3 different cell lines using hemin-treated Ivlg or untreated Ivlg as primary antibodies and using anti-human HRP-tagged conjugate as a control (HRP = horseradish peroxidase). The blots are shown in Figure 27. The "B" lysate is a B lymphocyte cell line called Raja from a patient with a lymphoma. The "T" lysate is a T-lymphocyte-derived cell line called Jurkat again from a leukemic patient. The U87MG lysate is a glioblastoma blast cell line (brain cancer). The reduced lysates were run into the gel at 50 mg/ml concentration. To obtain the hemin-treated Ivlg preparation, 75  $\mu$ g of hemin was combined with 1 ml of PBS containing 6 mg of IvlgG. Incubation was for 20 hours at 37 degrees. In Figure 27, the blot wherein hemin-treated Ivlg was used as the primary antibodies is labeled "Test IgG; the blot wherein untreated Ivlg was used as the primary antibodies is labeled "Control", and the blot to which anti-human HRP-tagged conjugate was applied without primary antibodies is labeled "Secondary". The hemin-treated and untreated IgG preparations were diluted 1/1000 respectively. The anti-human HRP-tagged conjugate was used at a dilution of 1/5000.

[0097] These data clearly show that the hemin-treated Ivlg has abundant activity towards human cellular components in comparison to untreated IvlgG and the conjugate control, which do not.

#### Example 28

[0098] The next experiment was carried out to determine whether oxidizing agents other than hemin, and in particular, oxidizing agents that do not contain iron, would be effective to unmask autoantibodies. A mixture of 25  $\mu$ g of potassium permanganate ( $KMnO_4$ ) at a concentration of 100  $\mu$ M, and 2 mg of Ivlg in a total volume of 1 ml of phosphate buffered saline was incubated overnight at 37  $^{\circ}$ C. In the incubated mixture, aPC and aPS could be detected. aCL was usually detected, but not aPE (data not shown). It was later determined that a reason why aPE is not detected is because  $KMnO_4$  alters the PE phospholipid antigen used in the ELISA testing.

**Example 29**

**[0099]** After it was shown that autoantibodies could be unmasked by oxidation reactions, the next question was whether electrochemical methods, such as an electro-motive force from a battery, could achieve the same effect.

**[0100]** Ivlg was dissolved in a phosphate buffered saline solution, and, in separate experiments, galvanized steel, copper, or stainless steel electrodes were connected to the positive and negative terminals of a 9-volt battery and were submersed into the solution for 1 - 2 minutes. During this period, bubbling was noticed in the solution and the PBS solution changed color (blue when copper wires were used, brown when stainless steel wires were used and green when galvanized steel wires were used). As shown in Figures 28A and 28B, the treated solution tested positive for the presence of aPS, aCL, aPE and aPC autoantibodies, in aPL dependent testing, and positive for the presence of aPS, aPE and aPC autoantibodies in aPL independent testing.

**Example 30**

**[0101]** To avoid the interaction of metals with the solution and thereby determine the effect only of an electric current, graphite electrodes were used in place of the metal electrodes. Graphite is inert, but is able to pass electrons into conducting solutions without participating in reactions.

**[0102]** Ivlg was dissolved in a phosphate buffered saline solution, and graphite electrodes connected to the positive and negative terminals of a 6-volt battery were submersed into the solution for 60 seconds. As shown in Figure 29, the treated solution tested positive for the presence of aPS, aPE and aPC autoantibodies.

**Example 31**

**[0103]** In the experiments involving applying electric current to solutions of Ivlg in phosphate buffered saline, a significant increase in pH was noticed, possibly due to the formation of NaOH. In order to keep the reactions at physiological pH levels, a cell culture medium, RMPI, was substituted for the phosphate buffered saline.

**[0104]** The next series of experiments was carried out to determine the effects of the time of exposure to the electric current on the unmasking of autoantibodies. Ivlg was dissolved in RMPI, a cell culture medium and graphite electrodes connected to the positive and negative terminals of a 6-volt battery were submersed into the solution for a variable amount of time. As shown in Figure 30, the maximum release of dependent aPL was obtained after 60 seconds of exposure to the current. Curiously, between 2 minutes and 4 minutes, the amount of aPL declined or disappeared.

**Example 32**

**[0105]** Since the previous experiment had shown that aPL antibodies could be obtained from Ivlg after exposure to an electric current, but that the aPL antibodies disappeared after further exposure to the current, the next question was whether the unmasking of autoantibodies could be reversed by an electric current. That is, could a positive control serum be treated so that autoantibodies were no longer detectable?

**[0106]** In separate experiments, aCL positive control serum, at a dilution of 1:400, aPE positive control serum at a dilution of 1:75, and aPS at a dilution of 1:400 were exposed to an electric current by immersing graphite electrodes connected to the positive and negative terminals of a 6-volt battery for up to 240 seconds. As shown in Figures 31 A - 31C, each control sera became negative for its respective specificity.

**Example 33**

**[0107]** Based on the results in Example 32, the next question that was asked was whether autoantibodies of a patient having an autoimmune disease could be re-masked if the patient serum was exposed to an electric current. Serum from a patient having elevated levels of aPS and aCL was diluted 1/400 with phosphate-buffered saline (the dilution in PBS was in an amount that would achieve an OD value of 1.000 in 10-15 minutes) and graphite electrodes connected to the positive and negative terminals of a 6-volt battery were submersed into the solution for a variable amount of time. As shown in Figure 32, the amount of aCL and aPS detectable in the samples of the autoimmune patient's serum declined significantly after 30 seconds and was no longer detectable after 2 minutes. These experiments were repeated for other patients' antibodies and the same result was obtained (data not shown).

**Example 34**

**[0108]** In an early experiment, a blood sample from a patient who had a very specific and high titer IgA aPE was exposed to hemin in a routine microbiology culture bottle. It was observed that after exposure to hemin her IgA aPE disappeared, and the emergence of IgG aPS, aCL and most spectacularly, IgG aPE was detected in the aPL ELISA. At the time, an explanation for this phenomenon was not readily apparent.

**[0109]** With the discovery of a faster unmasking process using electric current, it became possible to confirm the earlier results with another patient having a high aPE. In this experiment, serum from a patient having a high aPE was diluted in PBS by 1/75 and graphite electrodes connected to the positive and negative terminals of a 6-volt battery were submersed into the solution for a variable amount of time. As shown in Fig. 33, the aPE became undetectable (masked) within 30 seconds of a 6-

volt DC current application, with a concomitant unmasking and detection of aPS and aCL IgG. The newly unmasked aPL peaked around 30 seconds only to become masked again after 2-4 minutes of exposure.

**[0110]** An important technical aspect addressed by the above experiment was that the patient aPE was treated apart from the plasma protein diluent used in the assay, in the present case, 10% adult bovine plasma (ABP). In other experiments not shown, the diluted patient sera were exposed to 6-volt EMF conditions before adding the plasma proteins used in the ELISA diluent. The important aspect of these experiments was to show that the EMF effects were being applied to the patients' antibodies and not to EMF changes in the plasma proteins used in the diluent.

**[0111]** These experimental data support the observations that redox reactions are determining the appearance and disappearance of different antibody specificities. What is also learned from these experiments is that the redox effects appear to be limited to the antibody binding site(s), the Fab portion of the antibody molecule. This is because the heterologous antihuman antibody-labeled conjugates used in the ELISA are not affected as the conjugates continue to recognize the different antibody heavy chain targets (Fc portions) of the antibody molecules. Thus, as the human antibody is not consumed or destroyed by redox, the most plausible explanation is that the antibody-binding site in the Fab portion of the antibody molecule contains accessible electrons that can participate in the oxidation/reduction process.

### Example 35

**[0112]** The next experiments were carried out to see whether plasma proteins other than autoantibodies can have their binding specificity altered by oxidation-reduction. In these experiments, a 10% adult bovine plasma (ABP) solution, the same solution containing phospholipid-binding proteins that had been used to determine protein-dependent aPL binding, was exposed to an electric current from a 6-volt battery for a variable period of time. The treated ABP samples were then used in ELISA assays with aPS-, aCL- and aPE-positive patient sera to see if the treatment of the ABP would affect the outcome of the ELISA. As shown in Figure 34, at time zero (untreated ABP), the positive patient sera give the aPL response in ABP that is routinely seen. As the 10% ABP is exposed to oxidation-reduction (EMF) over time, amount of aPL detected diminishes and after 2 minutes, the aPE positive serum is no longer positive. These results indicate that the plasma proteins that are responsible for the patients' aPL reactivity are altered by the exposure to the electric current. For example, as kininogen is the plasma protein responsible for providing a positive ELISA signal for aPE dependent reactions (the kininogen binds to PE, then the antibody binds to the kininogen, the aPE however will not bind to either PE or kininogen independently), this shows that the kininogen in the ABP

sample is being altered by the redox exposure. aCL is also negative at 240 seconds exposure and since this patient serum requires either prothrombin and/or beta2 glycoprotein (or both could be involved) for producing a positive signal in the aPL ELISA, these two proteins must also be altered by the redox reactions. The same two plasma proteins are involved in the aPS example.

### 10 Claims

1. A method of obtaining and isolating an autoantibody from an antibody-containing biological fluid comprising diluted whole blood, serum or plasma or from an antibody-containing extract of a biological fluid comprising diluted whole blood, said biological fluid or extract containing masked autoantibodies that, before the method is carried out, are not capable of binding to a self antigen and therefore are not detectable by an assay based on receptor-ligand binding, the method comprising the steps of:

exposing the biological fluid or extract to an oxidising agent or to a 6-24 Volt DC electric current for a few seconds to a few minutes sufficient to alter a binding specificity of the masked autoantibody so that the said autoantibody becomes capable of binding to an antigen, thereby becoming detectable and recoverable from the biological fluid or extract by a receptor-ligand binding separation method, and recovering the autoantibody from the biological fluid.

2. The method of claim 1, wherein:

- a) the antibody-containing extract of a biological fluid is intravenous immunoglobulin (Ivlg); or
- b) the oxidizing agent is hemin.

3. A method of screening a normal individual's biological fluid or extract to determine an amount of autoantibodies that are masked, comprising the steps of:

assaying a whole blood, serum or plasma sample from a subject to determine an amount and/or type of masked autoantibodies detectable in the sample, treating the sample from the subject by exposing the sample to an oxidising agent or a 6-24 Volt DC electric current for a few seconds to a few minutes, assaying the sample from the subject to determine an amount and/or type of autoantibodies detectable in the treated sample, and comparing the amount and/or type of masked autoantibodies detectable in the untreated sam-

ple with the amount and/or type of autoantibodies detectable in the treated sample.

4. A method of effecting an alteration of the binding specificity of a masked autoantibody comprising exposing a composition comprising at least one masked antibody suspended or dissolved in a liquid medium comprising whole blood, serum or plasma or diluted placental cord blood to an oxidising agent or a 6-24 Volt DC current for a few seconds to a few minutes sufficient to effect the alteration of the binding specificity of said masked autoantibody, wherein the binding specificity is altered from the masked autoantibody not having binding capability with respect to a specific antigen or ligand to an autoantibody having binding capability with respect to the specific antigen or ligand. 5
5. The method of claim 4, comprising isolating and recovering the autoantibody having an altered binding specificity. 10
6. The method of claims 1, 3 or 4, wherein the composition, biological fluid, antibody-containing extract of a biological fluid, whole blood, serum or plasma is exposed to hemin for a period of time sufficient to alter the binding specificity of the antibody. 15
7. The method of claims 1, 3 or 4, wherein the composition, biological fluid, antibody-containing extract of a biological fluid, whole blood, serum or plasma is exposed to a 6-24 volt DC electric current for a few seconds to a few minutes. 20
8. The method of either claim 1 or claim 3, wherein the biological fluid comprises diluted serum or plasma. 25
9. The method of claim 4, wherein the liquid medium comprises diluted serum or plasma. 30

#### Patentansprüche

1. Verfahren zum Erlangen und Isolieren eines Autoantikörpers aus einer Antikörper enthaltenden biologischen Flüssigkeit, die verdünntes Vollblut, Serum oder Plasma umfasst, oder aus einem Antikörper enthaltenden Extrakt einer biologischen Flüssigkeit, der verdünntes Vollblut umfasst, wobei die biologische Flüssigkeit oder der Extrakt maskierte Autoantikörper enthält, die vor dem Ausführen des Verfahrens nicht imstande sind, an ein Eigenantigen zu binden, und daher durch eine Prüfung, die auf Rezeptor-Ligand-Bindung basiert, nicht detektierbar sind, das Verfahren die folgenden Schritte umfassend: 35

Aussetzen der biologischen Flüssigkeit oder

des Extrakts einem Oxidationsmittel oder einem elektrischen Strom von 6 - 24 Volt Gleichstrom für einige Sekunden bis einige Minuten, die ausreichend sind, um eine Bindungsspezifität des maskierten Autoantikörpers zu ändern, so dass der Autoantikörper fähig wird, an ein Antigen zu binden, **dadurch** detektierbar und aus der biologischen Flüssigkeit oder dem Extrakt durch ein Rezeptor-Ligand-Bindungs-Trennverfahren wiedergewinnbar wird, und Wiedergewinnen des Autoantikörpers aus der biologischen Flüssigkeit. 40

2. Verfahren nach Anspruch 1, wobei:
  - a) der Antikörper enthaltende Extrakt einer biologischen Flüssigkeit intravenöses Immunglobulin (Ivlg) ist; oder
  - b) das Oxidationsmittel Häm in ist.
3. Verfahren zum Untersuchen der biologischen Flüssigkeit oder des Extrakts eines normalen Individuums zum Bestimmen einer Menge von Autoantikörpern, die maskiert sind, die folgenden Schritte umfassend: 45

Prüfen einer Vollblut-, Serum- oder Plasmaprobe von einer Person, um eine Menge und/oder einen Typ von maskierten Autoantikörpern, die in der Probe detektierbar sind, zu bestimmen, Behandeln der Probe von der Person, indem die Probe einem Oxidationsmittel oder einem elektrischen Strom von 6 - 24 Volt Gleichstrom für einige Sekunden bis einige Minuten ausgesetzt wird, 50

Prüfen der Probe von der Person, um eine Menge und/oder einen Typ von Autoantikörpern, die in der behandelten Probe detektierbar sind, zu bestimmen, und

Vergleichen der Menge und/oder des Typs von maskierten Autoantikörpern, die in der nicht behandelten Probe detektierbar sind, mit der Menge und/oder dem Typ von Autoantikörpern, die in der behandelten Probe detektierbar sind. 55

4. Verfahren zum Bewirken einer Änderung der Bindungsspezifität eines maskierten Autoantikörpers, umfassend, eine Zusammensetzung, die mindestens einen maskierten Antikörper umfasst, der in einem flüssigen Medium, das Vollblut, Serum oder Plasma oder verdünntes plazentares Nabelschnurblut umfasst, suspendiert oder gelöst ist, einem Oxidationsmittel oder einem Strom von 6 - 24 Volt Gleichstrom für einige Sekunden bis einige Minuten auszusetzen, die ausreichend sind, um die Änderung der Bindungsspezifität des maskierten Autoantikörpers zu bewirken, wobei die Bindungsspezifität von dem maskierten Autoantikörper, der in Bezug

auf ein spezifisches Antigen oder einen spezifischen Ligand keine Bindungsfähigkeit aufweist, zu einem Autoantikörper, der in Bezug auf das spezifische Antigen oder den spezifischen Ligand Bindungsfähigkeit aufweist, geändert wird.

5. Verfahren nach Anspruch 4, umfassend, den Autoantikörper mit einer geänderten Bindungsspezifität zu isolieren und wiederzugewinnen.
6. Verfahren nach Anspruch 1, 3 oder 4, wobei die Zusammensetzung, die biologische Flüssigkeit, der Antikörper enthaltende Extrakt einer biologischen Flüssigkeit, das Vollblut, das Serum oder das Plasma für eine Zeitperiode, die ausreichend ist, um die Bindungsspezifität des Antikörpers zu ändern, Hämin ausgesetzt wird.
7. Verfahren nach Anspruch 1, 3 oder 4, wobei die Zusammensetzung, die biologische Flüssigkeit, der Antikörper enthaltende Extrakt einer biologischen Flüssigkeit, das Vollblut, das Serum oder das Plasma für einige Sekunden bis einige Minuten einem elektrischen Strom von 6 - 24 Volt Gleichstrom ausgesetzt wird.
8. Verfahren nach entweder Anspruch 1 oder Anspruch 3, wobei die biologische Flüssigkeit verdünntes Serum oder Plasma umfasst.
9. Verfahren nach Anspruch 4, wobei das flüssige Medium verdünntes Serum oder Plasma umfasst.

#### Revendications

1. Procédé pour obtenir et isoler un autoanticorps à partir d'un fluide biologique contenant des anticorps comprenant du sang total, du sérum ou du plasma dilués ou à partir d'un extrait contenant des anticorps d'un fluide biologique comprenant du sang total dilué, ledit fluide biologique ou extrait contenant des autoanticorps masqués qui, avant que le procédé ne soit exécuté, ne sont pas capables de se lier à un autoantigène et ne sont donc pas détectables par un dosage basé sur la liaison ligand-récepteur, ce procédé comprenant les étapes consistant à :

exposer le fluide biologique ou l'extrait à un agent oxydant ou à un courant électrique c.c. à 6 - 24 volts pendant quelques secondes à quelques minutes suffisantes pour modifier une spécificité de liaison de l'autoanticorps masqué de manière à ce que ledit autoanticorps devienne capable de se lier à un antigène, devenant ainsi détectable et récupérable du fluide biologique ou de l'extrait par un procédé de séparation de liaison ligand-récepteur, et à

récupérer l'autoanticorps du fluide biologique.

2. Procédé selon la revendication 1, dans lequel :

- a) l'extrait contenant des anticorps d'un fluide biologique est de l'immunoglobuline intraveineuse (Ivlg) ; ou
- b) l'agent oxydant est de l'hémine.

3. Procédé pour passer au crible le fluide biologique d'un individu normal ou un extrait de celui-ci pour déterminer une quantité d'autoanticorps qui sont masqués, comprenant les étapes consistant à :

doser un échantillon de sang total, de sérum ou de plasma pris sur un sujet afin de déterminer une quantité et/ou un type d'autoanticorps masqués détectables dans l'échantillon, traiter l'échantillon pris sur le sujet en exposant l'échantillon à un agent oxydant ou à un courant électrique c.c. à 6-24 volts pendant quelques secondes à quelques minutes, doser l'échantillon pris sur le sujet afin de déterminer une quantité et/ou un type d'autoanticorps détectables dans l'échantillon traité, et à comparer la quantité et/ou le type d'autoanticorps masqués détectables dans l'échantillon non traité avec la quantité et/ou le type d'autoanticorps détectables dans l'échantillon traité.

4. Procédé pour accomplir une modification de la spécificité de liaison d'un autoanticorps masqué comprenant l'exposition d'une composition comprenant au moins un anticorps masqué suspendu ou dissous dans un milieu liquide comprenant du sang total, du sérum ou du plasma ou du sang de cordon ombilical dilué à un agent oxydant ou à un courant c.c. à 6 - 24 volts pendant quelques secondes à quelques minutes suffisantes pour accomplir la modification de la spécificité de liaison dudit autoanticorps masqué, dans lequel la spécificité de liaison est modifiée, ce qui fait que l'autoanticorps masqué n'ayant initialement pas de capacité de liaison par rapport à un antigène ou à un ligand spécifique a alors une capacité de liaison par rapport à cet antigène ou à ce ligand spécifique.

5. Procédé selon la revendication 4, comprenant l'isolation et la récupération de l'autoanticorps ayant une spécificité de liaison modifiée.

6. Procédé selon les revendications 1, 3 ou 4, dans lequel la composition, fluide biologique, extrait contenant des anticorps d'un fluide biologique, sang total, sérum ou plasma, est exposée à de l'hémine pendant une période de temps suffisante pour modifier la spécificité de liaison de l'anticorps.

7. Procédé selon les revendications 1, 3 ou 4, dans lequel la composition, fluide biologique, extrait contenant des anticorps d'un fluide biologique, sang total, sérum ou plasma, est exposée à un courant électrique c.c. à 6 - 24 volts pendant quelques secondes à quelques minutes. 5
8. Procédé selon soit la revendication 1, soit la revendication 3, dans lequel le fluide biologique comprend du sérum ou du plasma dilués. 10
9. Procédé selon la revendication 4, dans lequel le milieu liquide comprend du sérum ou du plasma dilués. 15

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## In-house aPL ELISA\*

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<b><u>PS</u></b>	<b><u>CL</u></b>	<b><u>PE</u></b>	<b><u>PC</u></b>
<b>IgG</b>	<b>IgG</b>	<b>IgG</b>	<b>IgG</b>
<b>IgA</b>	<b>IgA</b>	<b>IgA</b>	<b>IgA</b>
<b>IgM</b>	<b>IgM</b>	<b>IgM</b>	<b>IgM</b>

\* In the presence (dependent) and absence (independent) of phospholipid-binding plasma proteins

Figure 1

## Unconditioned broth + blood

### Antiphospholipid Antibody (aPL) Results

		<u>PL binding protein</u>		<u>Normal ranges</u>
		<u>dependent</u>	<u>Independent</u>	
<b>aPS</b>	IgG	24 MoM +++	3 MoM	≤ 4 MoM
	IgA	8 MoM +	2 MoM	≤ 3 MoM
	IgM	8 MoM +	1 MoM	≤ 5 MoM
<b>aCL</b>	IgG	12 MoM +++	2 MoM	≤ 4 MoM
	IgA	12 MoM +++	2 MoM	≤ 4 MoM
	IgM	15 MoM +++	1 MoM	≤ 6 MoM
<b>aPE</b>	IgG	36 MoM +++	7 MoM +	≤ 4 MoM
	IgA	7 MoM +	2 MoM	≤ 3 MoM
	IgM	8 MoM +	3 MoM	≤ 5 MoM
<b>aPC</b>	IgG	2 MoM	4 MoM	≤ 4 MoM
	IgA	1 MoM	3 MoM	≤ 3 MoM
	IgM	6 MoM +	5 MoM +	≤ 4 MoM

Figure 2

<b>aPL range for 7 healthy persons</b>					
<b>Antiphospholipid Antibody (aPL) Results</b>					
		<u>PL binding protein</u>		<u>Normal ranges</u>	
		<u>dependent</u>	<u>Independent</u>		
<b>aPS</b>	IgG	17 - 25 +++	5 - 14 ++	≤	4 MoM
	IgA	4 - 14 ++	4 - 8 +	≤	3 MoM
	IgM	5 - 16 ++	3 - 13 ++	≤	5 MoM
<b>aCL</b>	IgG	7 - 10 ++	1 - 3	≤	4 MoM
	IgA	6 - 19 ++	2 - 7 +	≤	4 MoM
	IgM	6 - 17 ++	2 - 13 ++	≤	6 MoM
<b>aPE</b>	IgG	22 - 33 +++	7 - 20 ++	≤	4 MoM
	IgA	3 - 7 +	1 - 5 +	≤	3 MoM
	IgM	2 - 7 +	2 - 8 +	≤	5 MoM
<b>aPC</b>	IgG	1 - 2	1 - 4	≤	4 MoM
	IgA	1 - 4+	5 - 20 ++	≤	3 MoM
	IgM	1 - 8 +	4 - 17 ++	≤	4 MoM

Figure 3

<b>Horse RBC, human serum, broth</b>					
<b>Antiphospholipid Antibody (aPL) Results</b>					
		<u>PL binding protein</u>		<u>Normal ranges</u>	
		<u>dependent</u>	<u>independent</u>		
<b>aPS</b>	IgG	14 MoM +++	1 MoM	≤	4 MoM
	IgA	5 MoM +	1 MoM	≤	3 MoM
	IgM	19 MoM +++	1 MoM	≤	5 MoM
<b>aCL</b>	IgG	2 MoM	1 MoM	≤	4 MoM
	IgA	13 MoM +++	1 MoM	≤	4 MoM
	IgM	27 MoM +++	1 MoM	≤	6 MoM
<b>aPE</b>	IgG	1 MoM	2 MoM	≤	4 MoM
	IgA	1 MoM	1 MoM	≤	3 MoM
	IgM	1 MoM	4 MoM	≤	5 MoM
<b>aPC</b>	IgG	1 MoM	1 MoM	≤	4 MoM
	IgA	1 MoM	1 MoM	≤	3 MoM
	IgM	1 MoM	1 MoM	≤	4 MoM

Figure 4

<b>Human RBC, horse serum, broth</b>				
<b>Antiphospholipid Antibody (aPL) Results</b>				
		<u>PL binding protein</u>		<u>Normal ranges</u>
		<u>dependent</u>	<u>independent</u>	
aPS	IgG	1 MoM	1 MoM	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 3 MoM
	IgM	1 MoM	1 MoM	≤ 5 MoM
aCL	IgG	1 MoM	1 MoM	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 4 MoM
	IgM	1 MoM	1 MoM	≤ 6 MoM
aPE	IgG	1 MoM	1 MoM	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 3 MoM
	IgM	1 MoM	1 MoM	≤ 5 MoM
aPC	IgG	1 MoM	1 MoM	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 3 MoM
	IgM	1 MoM	1 MoM	≤ 4 MoM

Figure 5

<b>Blood in rocking vial at 22° C</b>				
<b>Antiphospholipid Antibody (aPL) Results</b>				
		<u>PL binding protein</u>		<u>Normal ranges</u>
		<u>dependent</u>	<u>independent</u>	
aPS	IgG	6 MoM +	3 MoM	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 3 MoM
	IgM	1 MoM	1 MoM	≤ 5 MoM
aCL	IgG	2 MoM	2 MoM	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 4 MoM
	IgM	1 MoM	1 MoM	≤ 6 MoM
aPE	IgG	4 MoM	1 MoM	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 3 MoM
	IgM	1 MoM	1 MoM	≤ 5 MoM
aPC	IgG	2 MoM	2 MoM	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 3 MoM
	IgM	1 MoM	6 MoM +	≤ 4 MoM

Figure 6

<b>Replace charcoal: 0.7mm Degalan</b>				
<b>Antiphospholipid Antibody (aPL) Results</b>				
		<u>PL binding protein</u>		<u>Normal ranges</u>
		<u>dependent</u>	<u>independent</u>	
<b>aPS</b>	IgG	31 MoM +++	3 MoM	≤ 4 MoM
	IgA	3 MoM	1 MoM	≤ 3 MoM
	IgM	2 MoM	1 MoM	≤ 5 MoM
<b>aCL</b>	IgG	7 MoM +	2 MoM	≤ 4 MoM
	IgA	5 MoM +	2 MoM	≤ 4 MoM
	IgM	10 MoM +++	1 MoM	≤ 6 MoM
<b>aPE</b>	IgG	8 MoM +	1 MoM	≤ 4 MoM
	IgA	2 MoM	1 MoM	≤ 3 MoM
	IgM	1 MoM	1 MoM	≤ 5 MoM
<b>aPC</b>	IgG	2 MoM	1 MoM	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 3 MoM
	IgM	1 MoM	4 MoM	≤ 4 MoM

Figure 7

<b>Blood in stationary vial at 37° C</b>				
<b>Antiphospholipid Antibody (aPL) Results</b>				
		<u>PL binding protein</u>		<u>Normal ranges</u>
		<u>dependent</u>	<u>independent</u>	
<b>aPS</b>	IgG	1 MoM	1 MoM	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 3 MoM
	IgM	2 MoM	1 MoM	≤ 5 MoM
<b>aCL</b>	IgG	1 MoM	1 MoM	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 4 MoM
	IgM	7 MoM +	1 MoM	≤ 6 MoM
<b>aPE</b>	IgG	1 MoM	1 MoM	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 3 MoM
	IgM	4 MoM	3 MoM	≤ 5 MoM
<b>aPC</b>	IgG	1 MoM	3 MoM	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 3 MoM
	IgM	3 MoM	7 MoM +	≤ 4 MoM

Figure 8

### Effect of heating at 56° C

Antiphospholipid Antibody (aPL) Results

		<u>PL binding protein</u>		<u>Normal ranges</u>
		<u>preheat</u>	<u>heated</u>	
aPS	IgG	47 MoM +++	69 MoM +++	≤ 4 MoM
	IgA	12 MoM +++	18 MoM +++	≤ 3 MoM
	IgM	3 MoM	1 MoM	≤ 5 MoM
aCL	IgG	13 MoM +++	16 MoM +++	≤ 4 MoM
	IgA	16 MoM +++	22 MoM +++	≤ 4 MoM
	IgM	12 MoM +++	16 MoM +++	≤ 6 MoM
aPE	IgG	48 MoM +++	44 MoM +++	≤ 4 MoM
	IgA	8 MoM +	8 MoM +	≤ 3 MoM
	IgM	2 MoM	1 MoM	≤ 5 MoM
aPC	IgG	5 MoM +	8 MoM +	≤ 4 MoM
	IgA	1 MoM	2 MoM	≤ 3 MoM
	IgM	1 MoM	3 MoM	≤ 4 MoM

Figure 9

### Becton Dickinson\* culture vial

Antiphospholipid Antibody (aPL) Results

		<u>PL binding protein</u>		<u>Normal ranges</u>
		<u>dependent</u>	<u>Independent</u>	
aPS	IgG	11 MoM +++	3 MoM	≤ 4 MoM
	IgA	failure	failure	≤ 3 MoM
	IgM	4 MoM	1 MoM	≤ 5 MoM
aCL	IgG	6 MoM +	4 MoM	≤ 4 MoM
	IgA	5 MoM +	5 MoM +	≤ 4 MoM
	IgM	9 MoM +	1 MoM	≤ 6 MoM
aPE	IgG	32 MoM +++	1 MoM	≤ 4 MoM
	IgA	2 MoM	1 MoM	≤ 3 MoM
	IgM	10 MoM +++	1 MoM	≤ 5 MoM
aPC	IgG	1 MoM	1 MoM	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 3 MoM
	IgM	1 MoM	1 MoM	≤ 4 MoM

\*Replacing Biomerieux

Figure 10

<b>Anaerobic culture vial broth + blood</b>				
<b>Antiphospholipid Antibody (aPL) Results</b>				
		<u>PL binding protein</u>		<u>Normal ranges</u>
		<u>dependent</u>	<u>independent</u>	
<b>aPS</b>	IgG	16 MoM +++	10 MoM +++	≤ 4 MoM
	IgA	3 MoM	2 MoM	≤ 3 MoM
	IgM	9 MoM +	5 MoM	≤ 5 MoM
<b>aCL</b>	IgG	8 MoM +	3 MoM	≤ 4 MoM
	IgA	4 MoM	1 MoM	≤ 4 MoM
	IgM	17 MoM +++	3 MoM	≤ 6 MoM
<b>aPE</b>	IgG	33 MoM +++	10 MoM +++	≤ 4 MoM
	IgA	4 MoM +	1 MoM	≤ 3 MoM
	IgM	9 MoM +	3 MoM	≤ 5 MoM
<b>aPC</b>	IgG	1 MoM	2 MoM	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 3 MoM
	IgM	3 MoM	5 MoM +	≤ 4 MoM

Figure 11

<b>Replace RBC with K562*</b>				
<b>Antiphospholipid Antibody (aPL) Results</b>				
		<u>PL binding protein</u>		<u>Normal ranges</u>
		<u>dependent</u>	<u>independent</u>	
<b>aPS</b>	IgG	10 MoM +++	6 MoM +	≤ 4 MoM
	IgA	5 MoM +	1 MoM	≤ 3 MoM
	IgM	1 MoM	1 MoM	≤ 5 MoM
<b>aCL</b>	IgG	3 MoM	1 MoM	≤ 4 MoM
	IgA	7 MoM +	1 MoM	≤ 4 MoM
	IgM	2 MoM	1 MoM	≤ 6 MoM
<b>aPE</b>	IgG	14 MoM +++	4 MoM	≤ 4 MoM
	IgA	7 MoM +	2 MoM	≤ 3 MoM
	IgM	1 MoM	1 MoM	≤ 5 MoM
<b>aPC</b>	IgG	1 MoM	2 MoM	≤ 4 MoM
	IgA	1 MoM	3 MoM	≤ 3 MoM
	IgM	1 MoM	3 MoM	≤ 4 MoM

Total cell count  $11.3 \times 10^6$

Figure 12

**Replace broth with RPMI and beads**

**Antiphospholipid Antibody (aPL) Results**

		<u>PL binding protein</u>		<u>Normal ranges</u>
		<u>dependent</u>	<u>Independent</u>	
<b>aPS</b>	IgG	1 MoM	1 MoM	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 3 MoM
	IgM	2 MoM	2 MoM	≤ 5 MoM
<b>aCL</b>	IgG	1 MoM	2 MoM	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 4 MoM
	IgM	2 MoM	2 MoM	≤ 6 MoM
<b>aPE</b>	IgG	1 MoM	1 MoM	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 3 MoM
	IgM	2 MoM	4 MoM	≤ 5 MoM
<b>aPC</b>	IgG	1 MoM	1 MoM	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 3 MoM
	IgM	3 MoM	5 MoM +	≤ 4 MoM

Figure 13

**Placental cord blood + broth**

**Antiphospholipid Antibody (aPL) Results**

		<u>PL binding protein</u>		<u>Normal ranges</u>
		<u>dependent</u>	<u>Independent</u>	
<b>aPS</b>	IgG	42 MoM +++	27 MoM +++	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 3 MoM
	IgM	1 MoM	1 MoM	≤ 5 MoM
<b>aCL</b>	IgG	14 MoM +++	6 MoM +	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 4 MoM
	IgM	2 MoM	1 MoM	≤ 6 MoM
<b>aPE</b>	IgG	38 MoM +++	23 MoM +++	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 3 MoM
	IgM	1 MoM	1 MoM	≤ 5 MoM
<b>aPC</b>	IgG	1 MoM	6 MoM +	≤ 4 MoM
	IgA	1 MoM	1 MoM	≤ 3 MoM
	IgM	1 MoM	1 MoM	≤ 4 MoM

Figure 14

**Replace RBC with sodium nitroprusside\***

**Antiphospholipid Antibody (aPL) Results**

		<u>PL binding protein</u>		<u>Normal ranges</u>
		<u>dependent</u>	<u>independent</u>	
<b>aPS</b>	IgG	18 MoM +++	5 MoM +	≤ 4 MoM
	IgA	8 MoM +	4 MoM +	≤ 3 MoM
	IgM	1 MoM	1 MoM	≤ 5 MoM
<b>aCL</b>	IgG	7 MoM +	1 MoM	≤ 4 MoM
	IgA	7 MoM	1 MoM	≤ 4 MoM
	IgM	1 MoM	1 MoM	≤ 6 MoM
<b>aPE</b>	IgG	23 MoM +++	9 MoM +	≤ 4 MoM
	IgA	3 MoM	4 MoM +	≤ 3 MoM
	IgM	1 MoM	1 MoM	≤ 5 MoM
<b>aPC</b>	IgG	1 MoM	2 MoM	≤ 4 MoM
	IgA	2 MoM	1 MoM	≤ 3 MoM
	IgM	1 MoM	1 MoM	≤ 4 MoM

\*200µM

Figure 15

**Lupus anticoagulant activity**  
Whole blood broth

	<u>Immediate Mix</u> 1:1	<u>Incubated Mix</u> 1:1 1-2 hours
<b>dRVVT</b>	46.7 sec*	104.4 sec
<b>dPT</b>	42.5 sec†	48.7 sec

\* normal = 28 - 49 sec † normal = 29.6 - 41.8

Figure 16

## Autoantibodies identified by Immunofluorescence

Observed fluorescence patterns on Hep-2 cell line

- Anti-nucleolar (scleroderma associated)
- Anti-lamins (very bright at nuclear pores)
- Anti-mitochondrial (cytoplasmic)
- Anti-centriole (unknown significance)

Figure 17

## Flow cytometry gated monocytes

Forward Scatter by Side Scatter Dot Plot

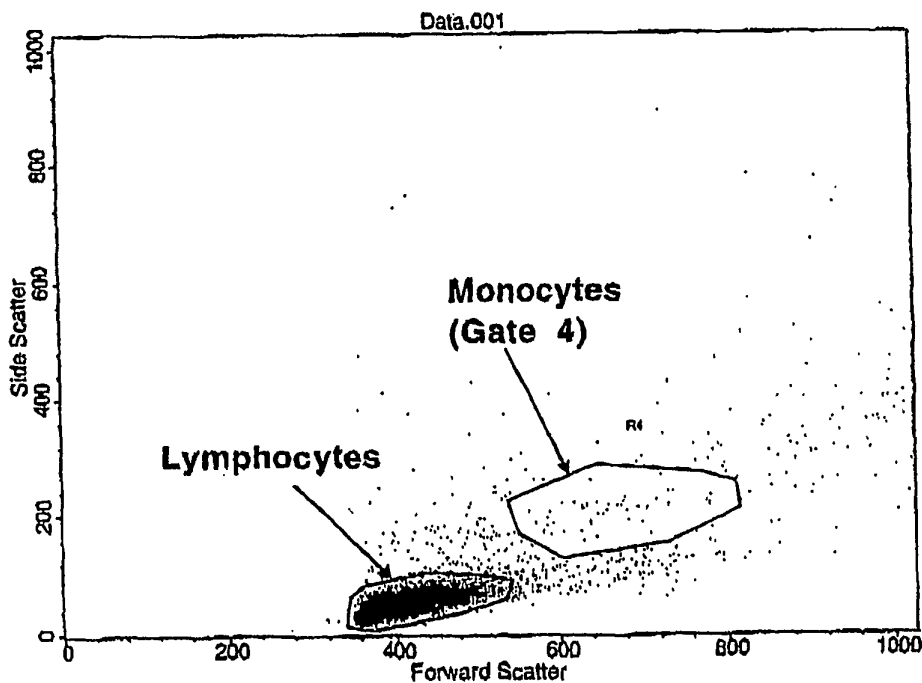


Figure 18

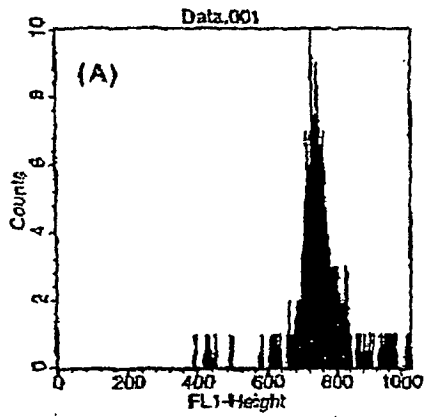
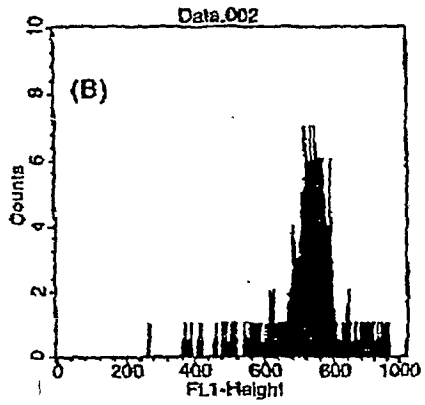


Figure 19A

Histogram Statistics

Log Data Units: Channel Values  
 Patient ID: XXXX CELLS  
 Gate: G4 (Pooled NHS)  
 Total Events: 10000

% Total	Mean	Geo Mean	CV	Median
3.78	748.20	744.62	9.60	743.50



Histogram Statistics

Log Data Units: Channel Values  
 Patient ID: XXXX CELLS (Normal person's serum)  
 Gate: G4  
 Total Events: 10000

% Total	Mean	Geo Mean	CV	Median
4.13	732.09	726.99	10.96	737.00

Figure 19B

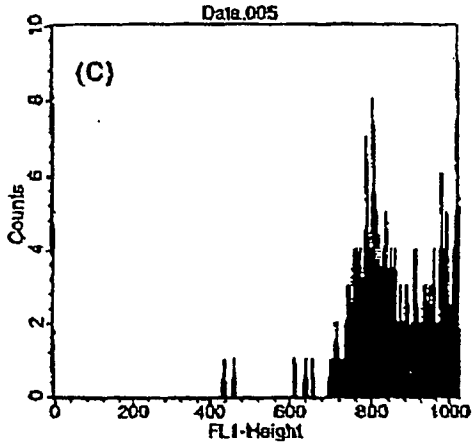


Figure 19C

Histogram Statistics

Log Data Units: Channel Values  
 Patient ID: xxxx CELLS (Normal person's  
 Gate: G4 serum treated by  
 Total Events: 10000 invention)

% Total	Mean	Geo Mean	CV	Median
4.72	872.61	866.72	11.38	864.00

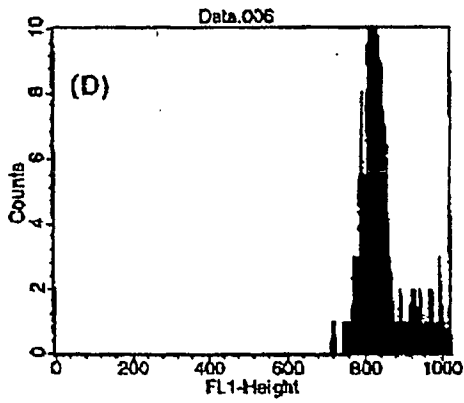


Figure 19D

Histogram Statistics

Log Data Units: Channel Values  
 Patient ID: .xxxx CELLS  
 Gate: G4 (Positive control)  
 Total Events: 10000

% Total	Mean	Geo Mean	CV	Median
5.18	834.75	833.53	5.54	827.50

<b>ANA Antibodies Identified by ImmunoConcepts Laboratories*</b>	
<b>Using the RELISA® screening assay†</b>	
<b><u>Sample</u></b>	<b><u>Units</u></b>
<b>Cord serum</b>	<b>0</b>
<b>Cord stationary</b>	<b>27</b>
<b>Cord shake</b>	<b>75</b>
<b>ACS serum</b>	<b>0</b>
<b>ACS shake + heat</b>	<b>90</b>

**\*Sacramento, California 1 < 10 units = neg 10-15 units = borderline**

Figure 20

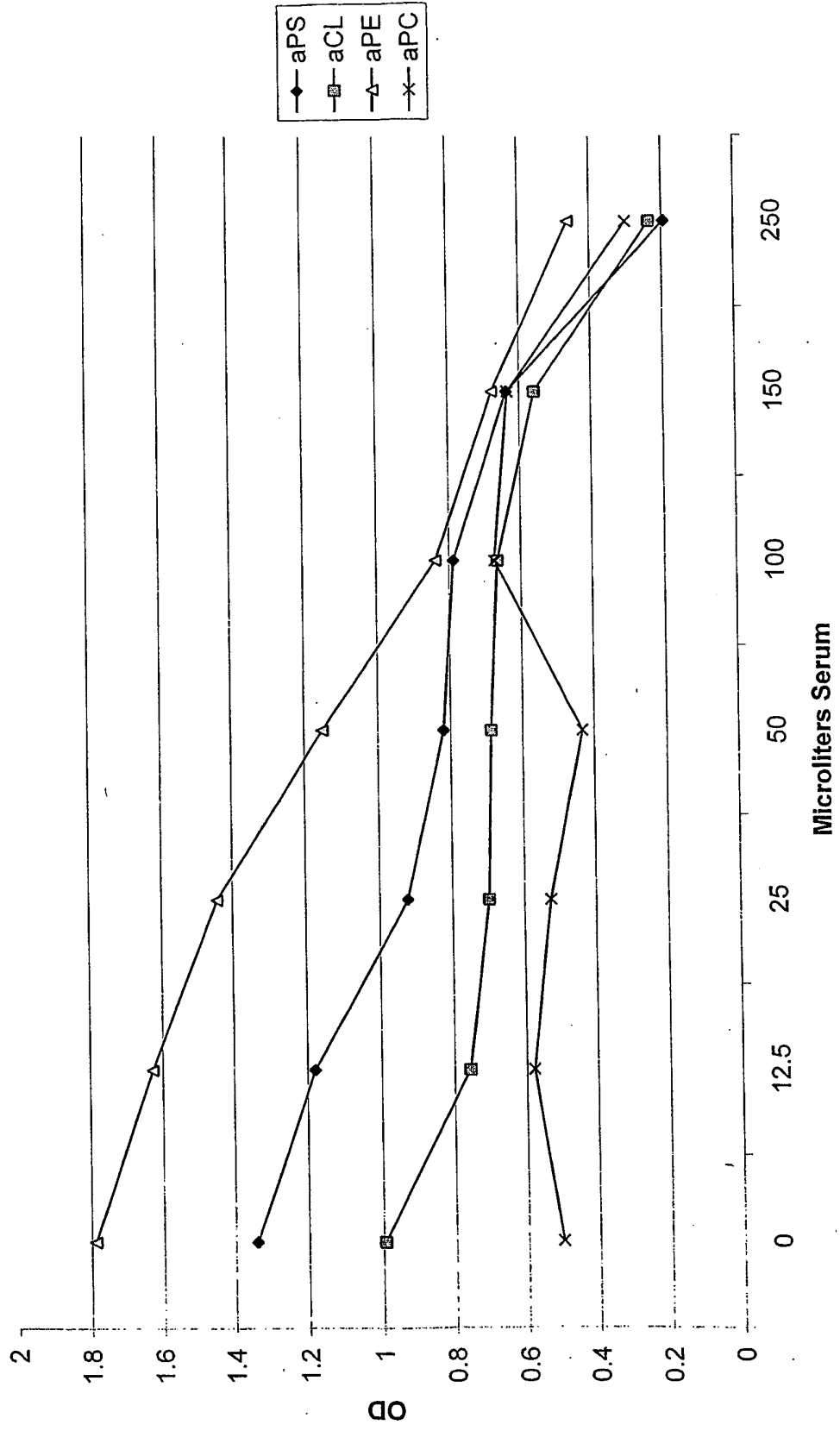
Figure 21

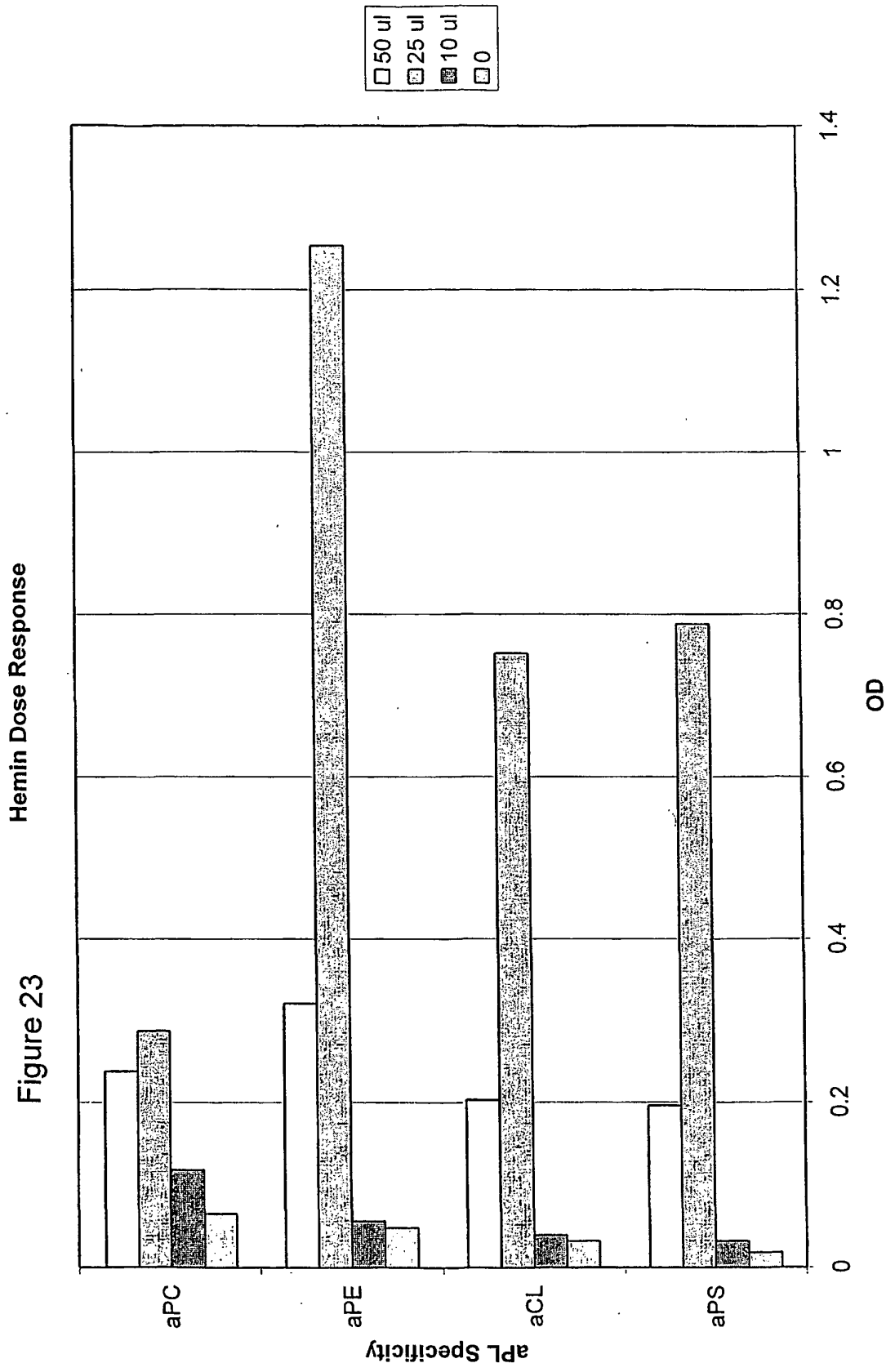
## Antiphospholipid Antibody (aPL) Results

### PL binding protein      Normal ranges

		<u>dependent</u>	<u>independent</u>		
<b>aPS</b>	IgG	31 MoM +++	4 MoM	VI	4 MoM
	IgA	1 MoM	1 MoM	VI	3 MoM
	IgM	1 MoM	1 MoM	VI	5 MoM
<b>aCL</b>	IgG	7 MoM +	1 MoM	VI	4 MoM
	IgA	1 MoM	1 MoM	VI	4 MoM
	IgM	1 MoM	1 MoM	VI	6 MoM
<b>aPE</b>	IgG	32 MoM +++	3 MoM	VI	4 MoM
	IgA	1 MoM	1 MoM	VI	3 MoM
	IgM	1 MoM	1 MoM	VI	5 MoM
<b>aPC</b>	IgG	5 MoM +	1 MoM	VI	4 MoM
	IgA	1 MoM	1 MoM	VI	3 MoM
	IgM	1 MoM	1 MoM	VI	4 MoM

Figure 22





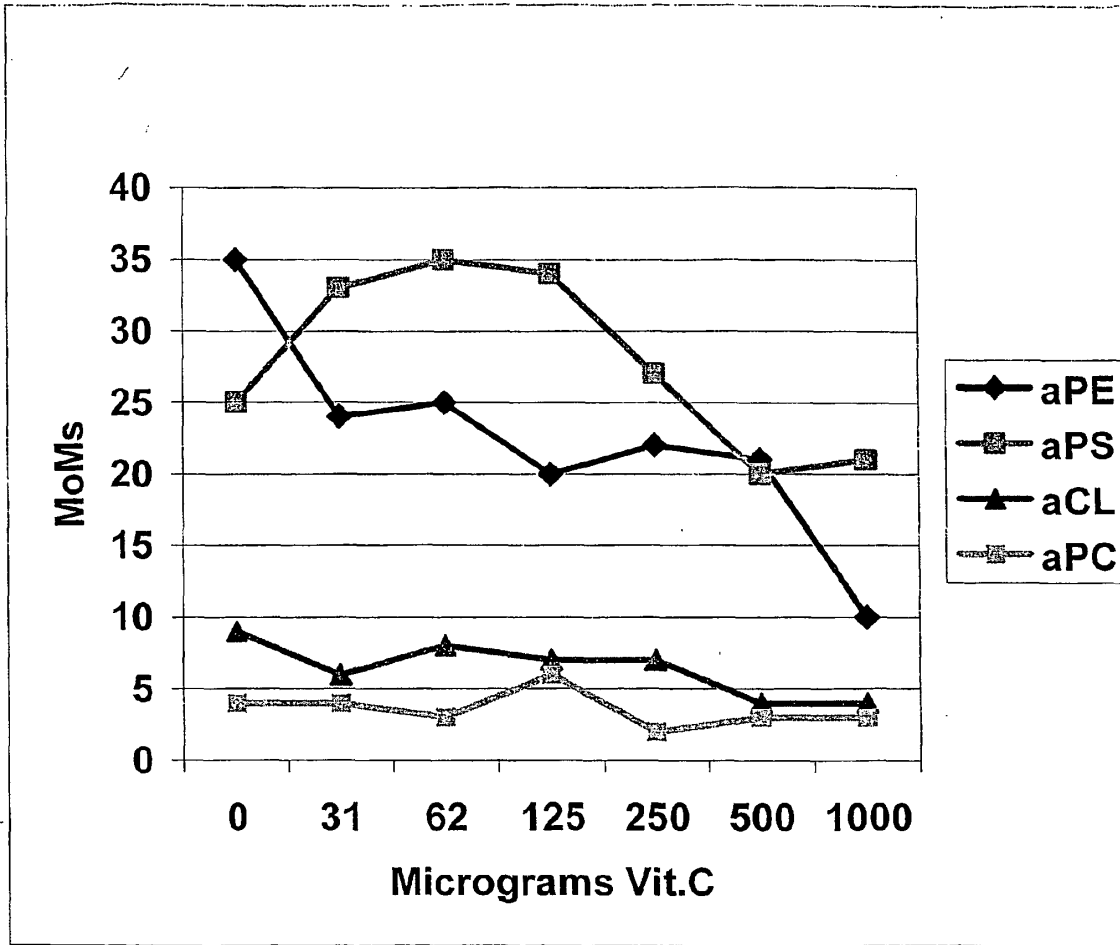


Figure 24

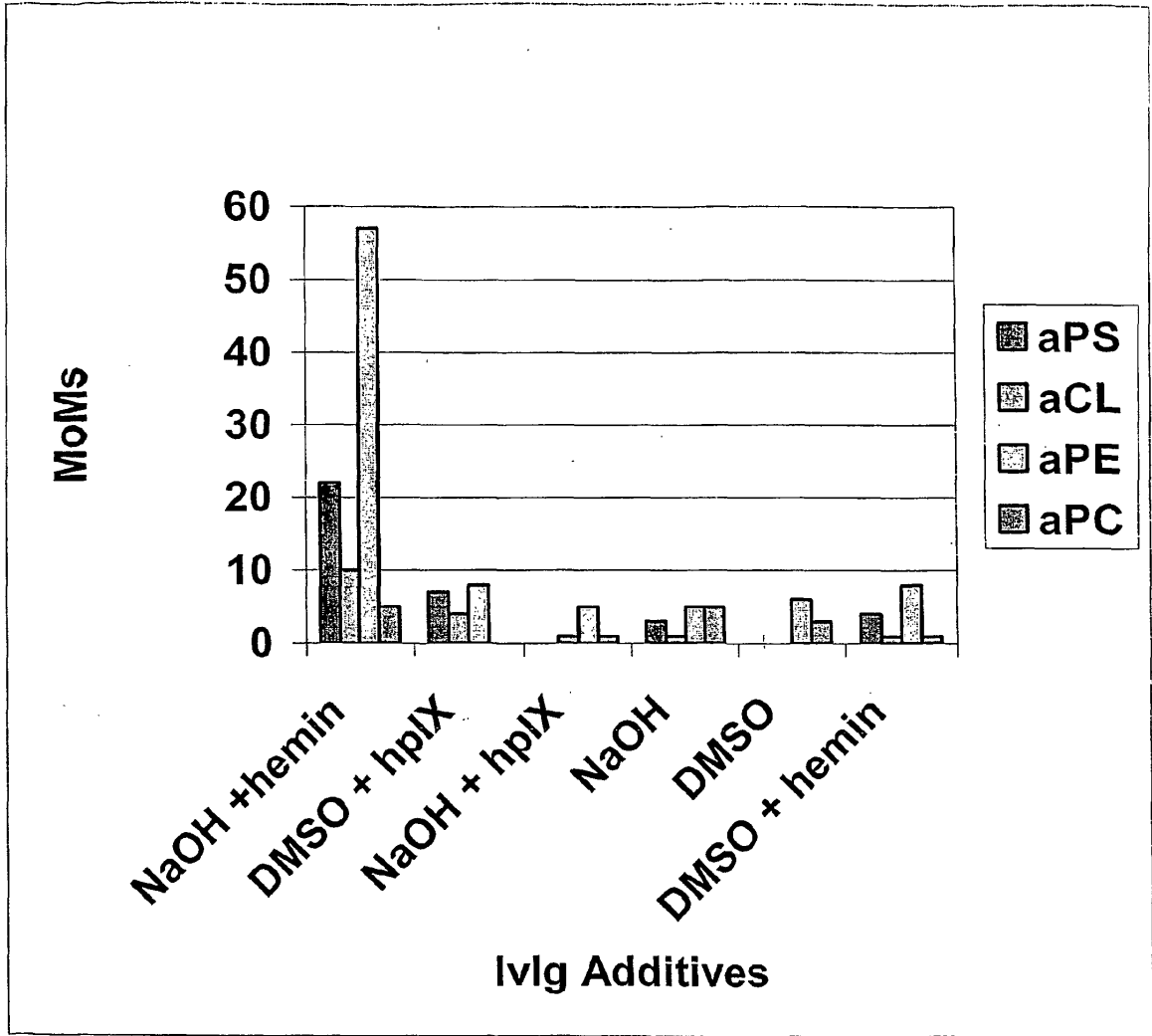


Figure 25

# Hemin-hemopexin Effects on Hemin Ivlg aPS Conversion

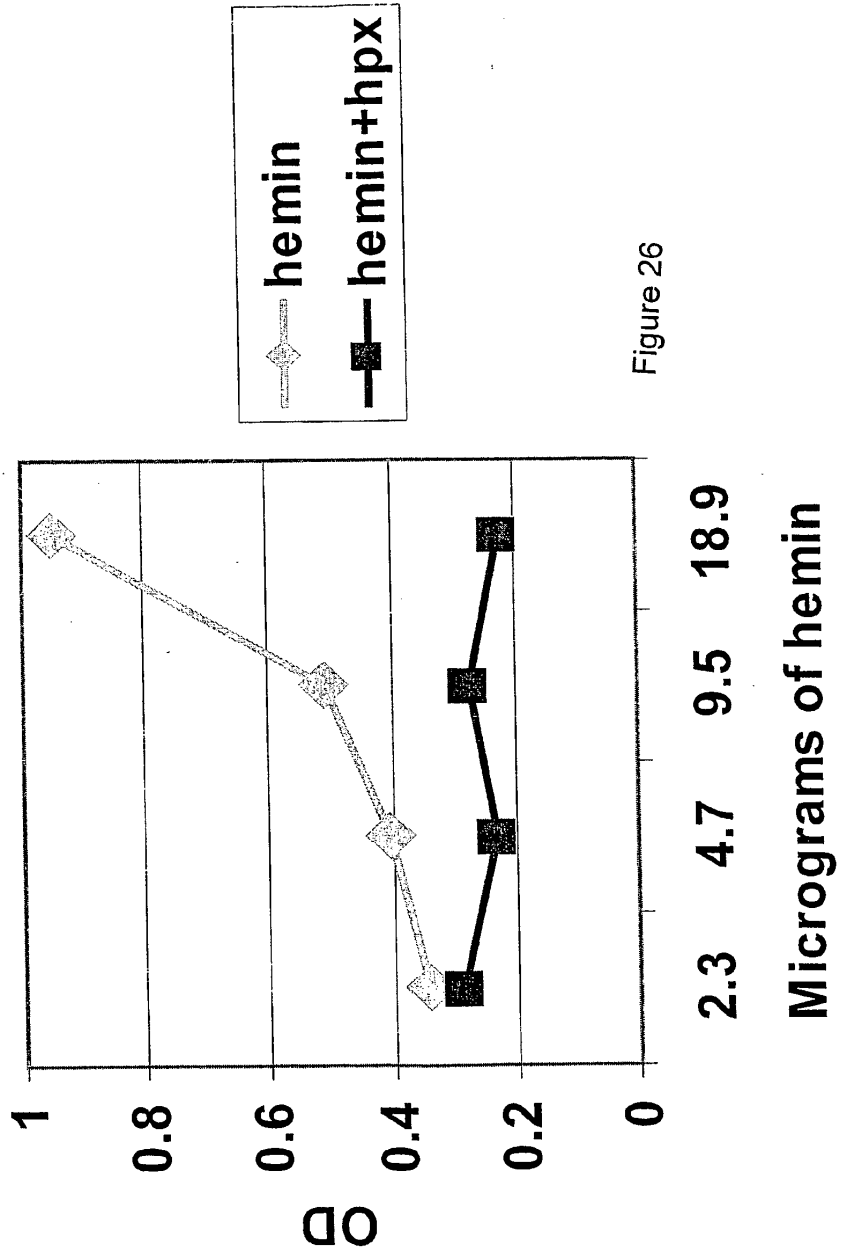


Figure 26

Figure 27  
**Test IgG      Control      Secondary**

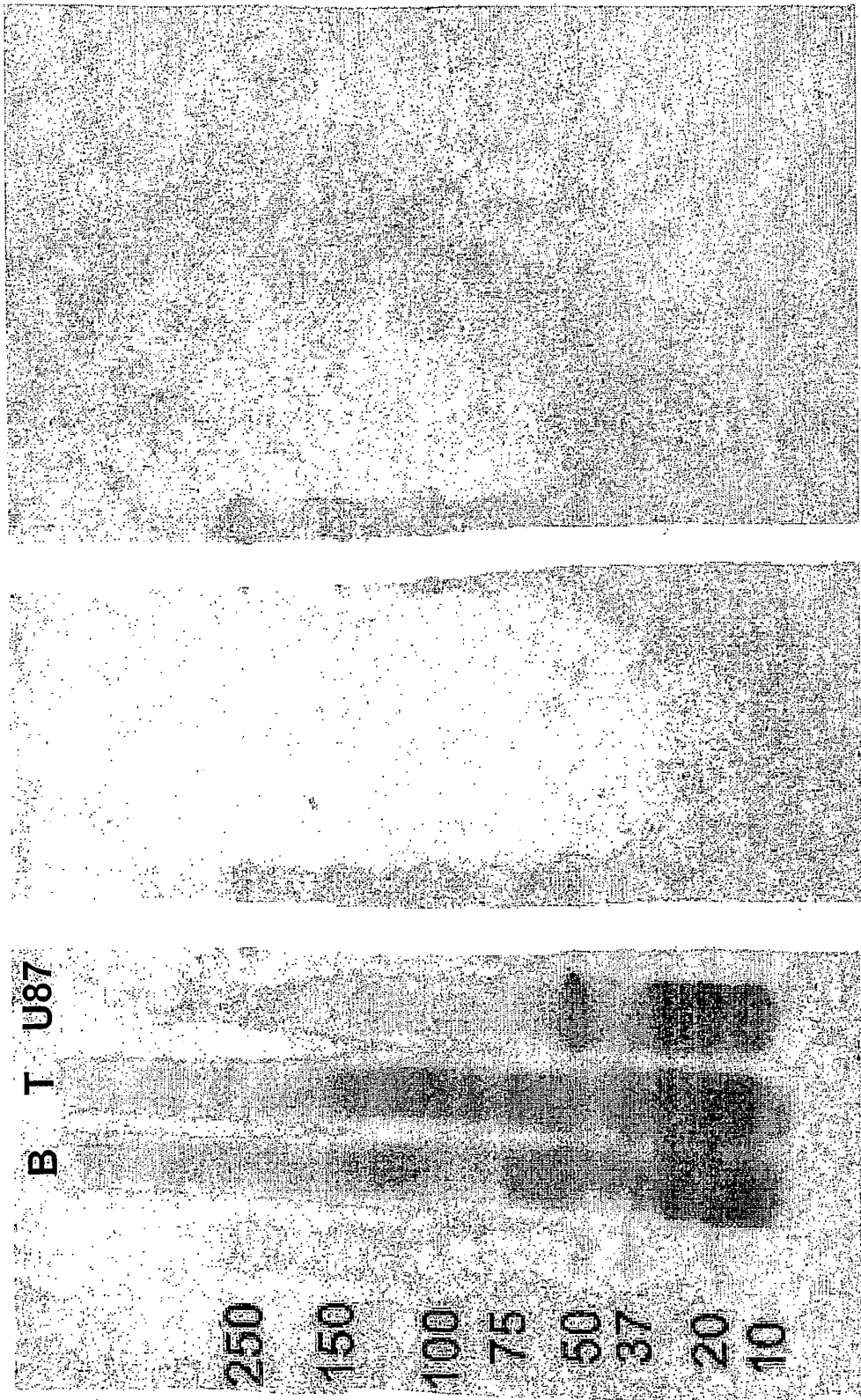


Figure 28A Ivlg in PBS @ 9 volts 2 min

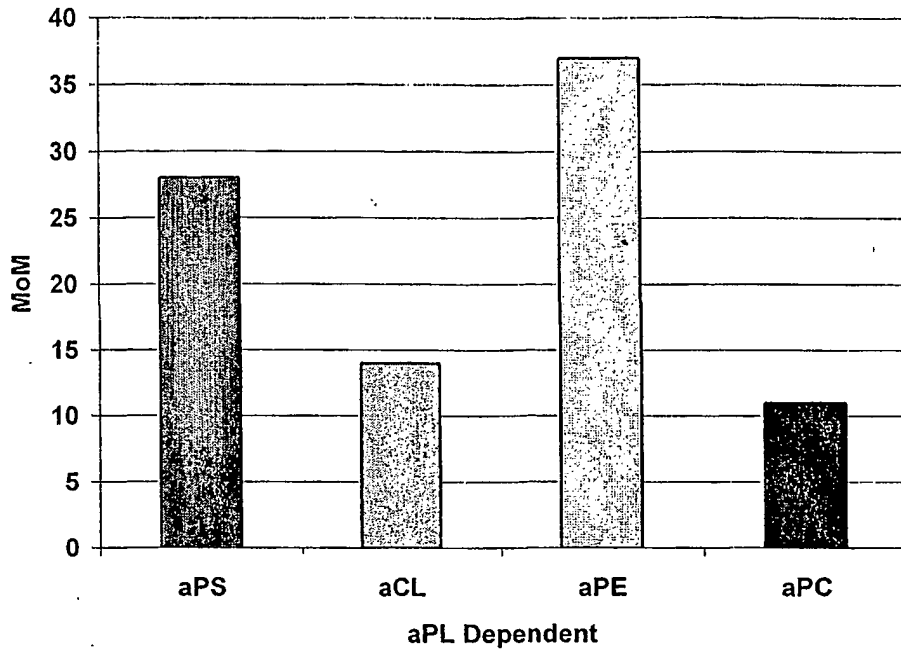
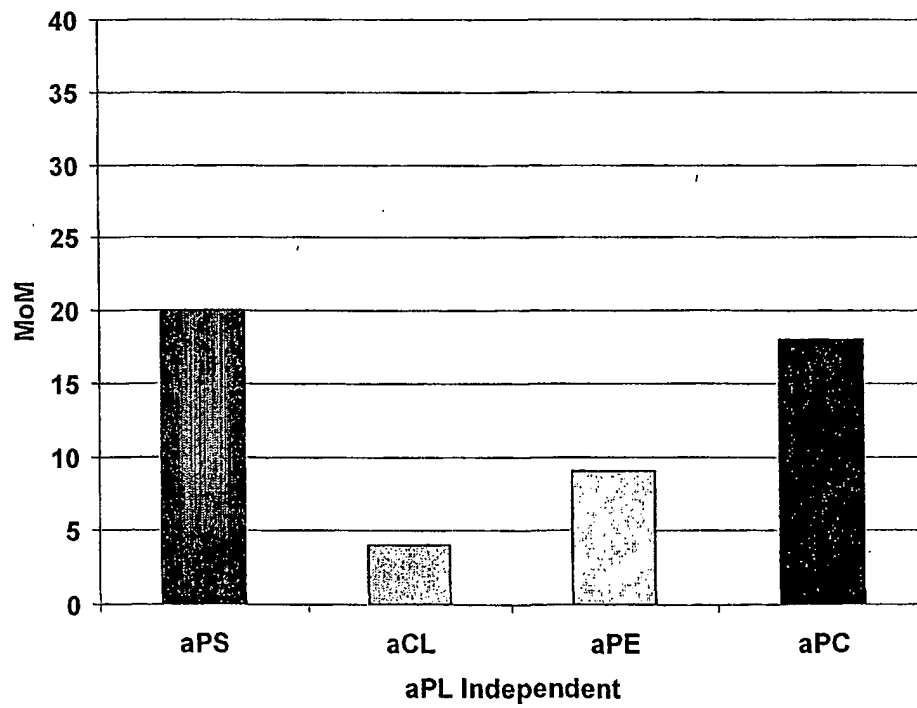


Figure 28B Ivlg in PBS @ 9 Volts 2 min



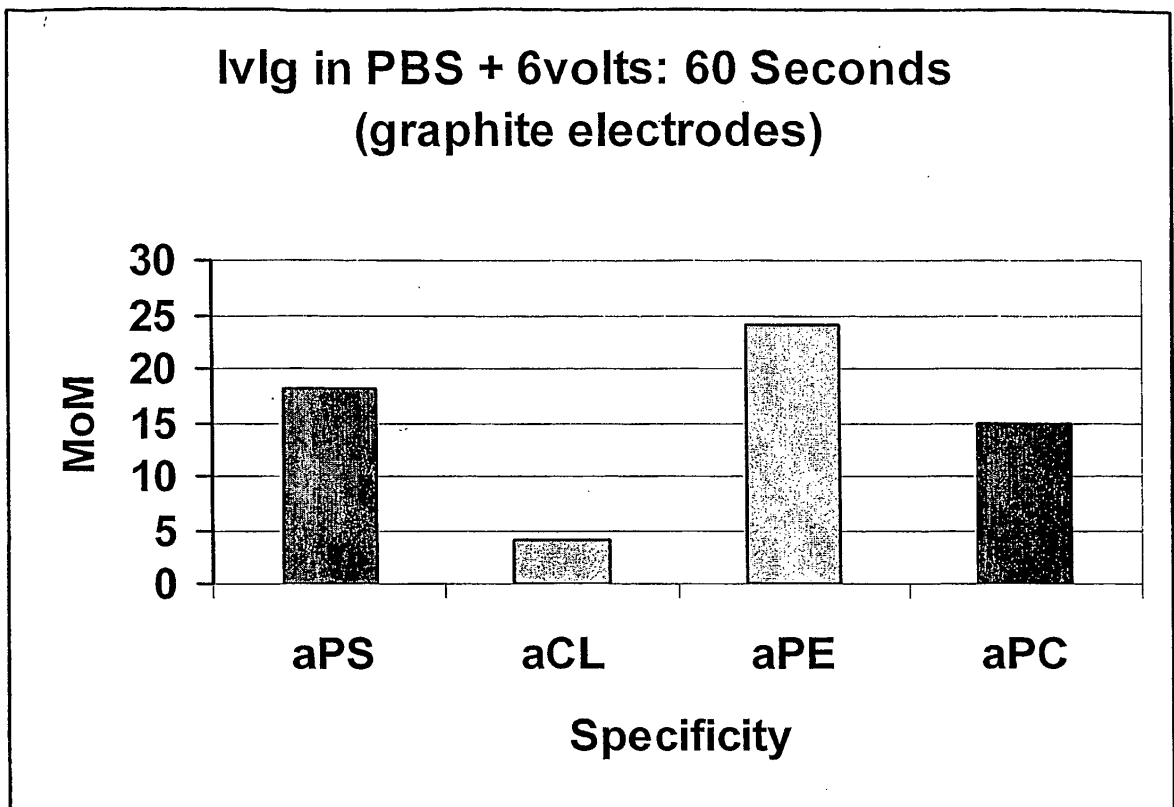


Figure 29

# Ivlg\* 6-Volt Time Release (Dependent aPL)

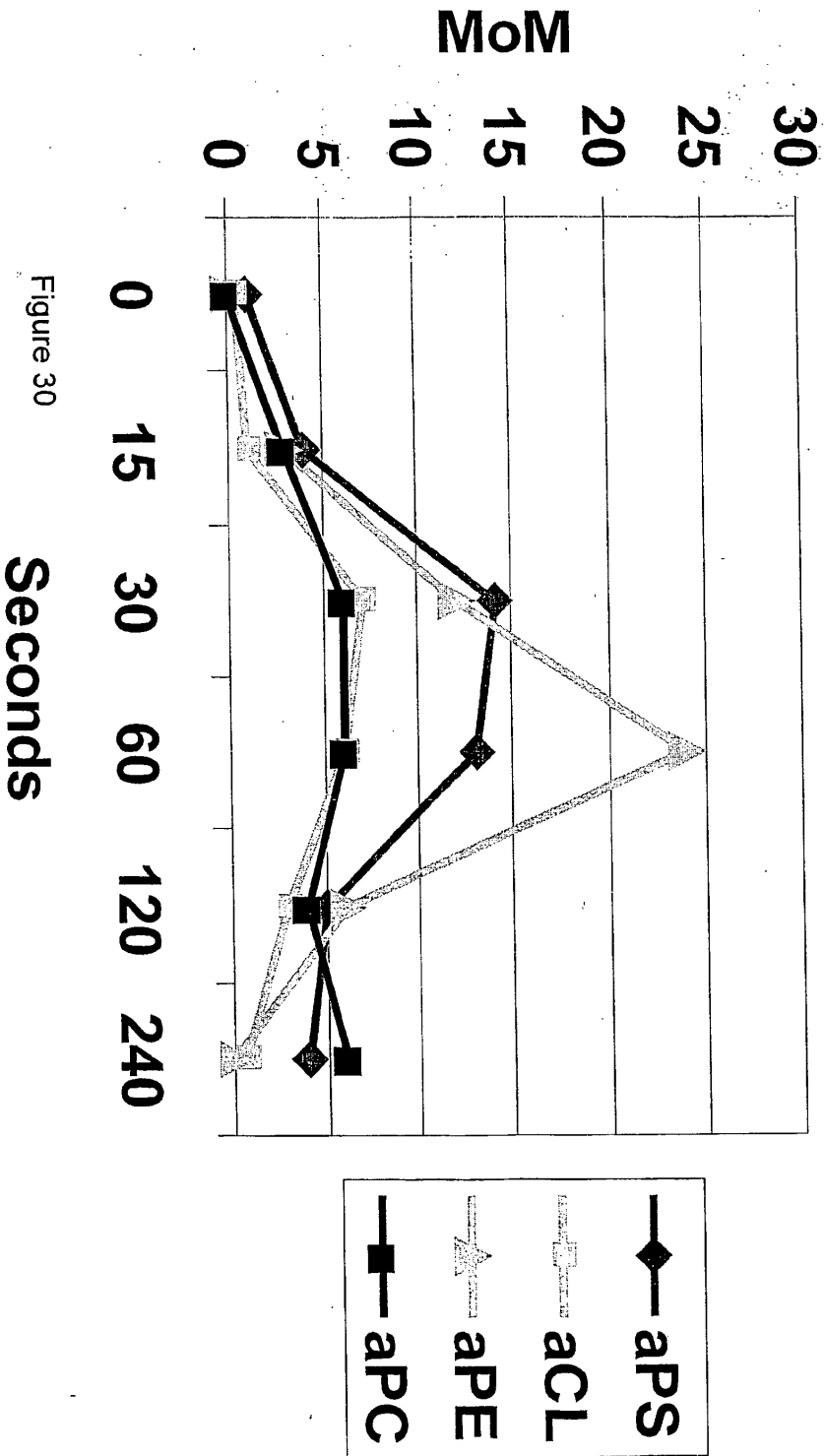
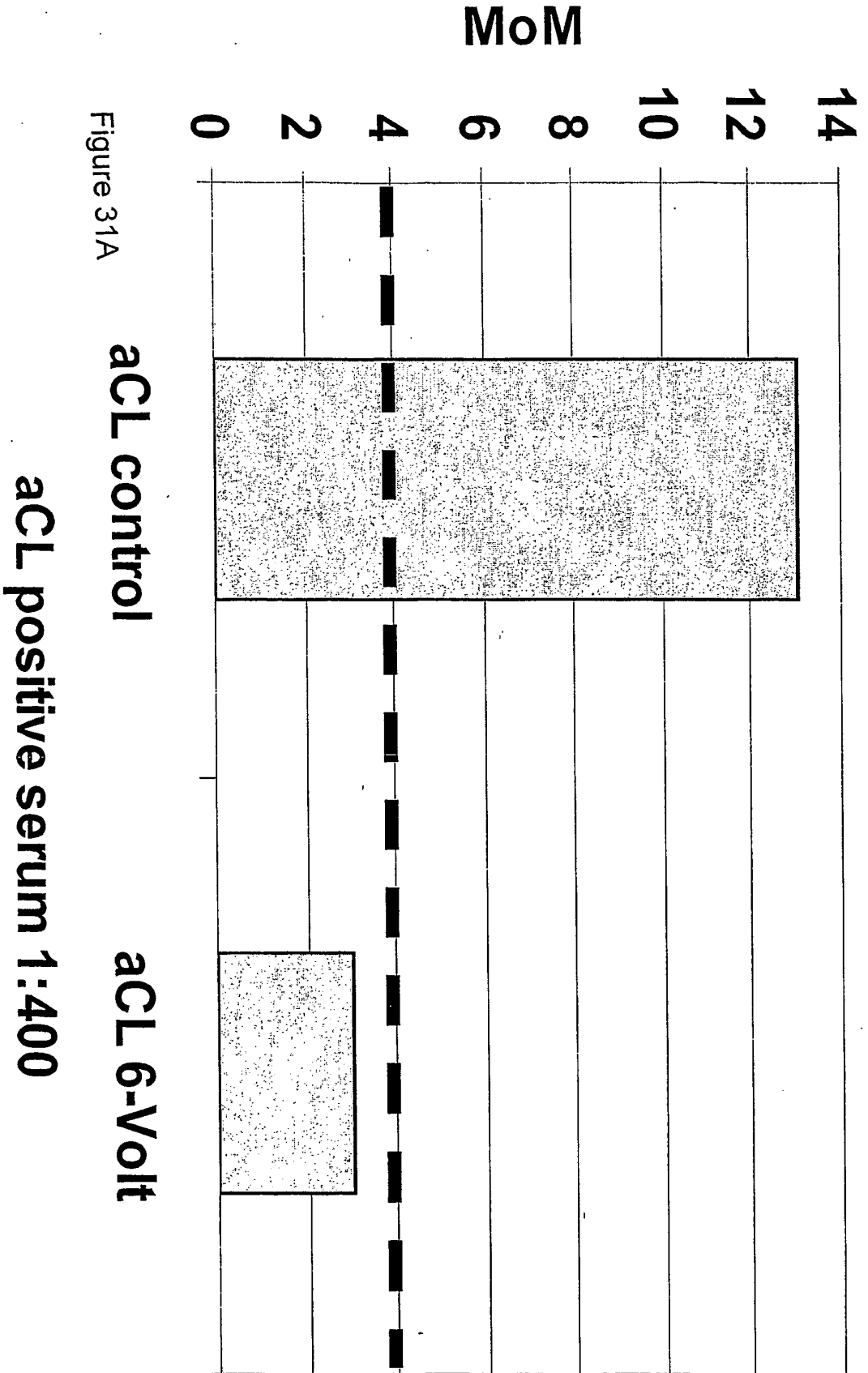


Figure 30

Seconds



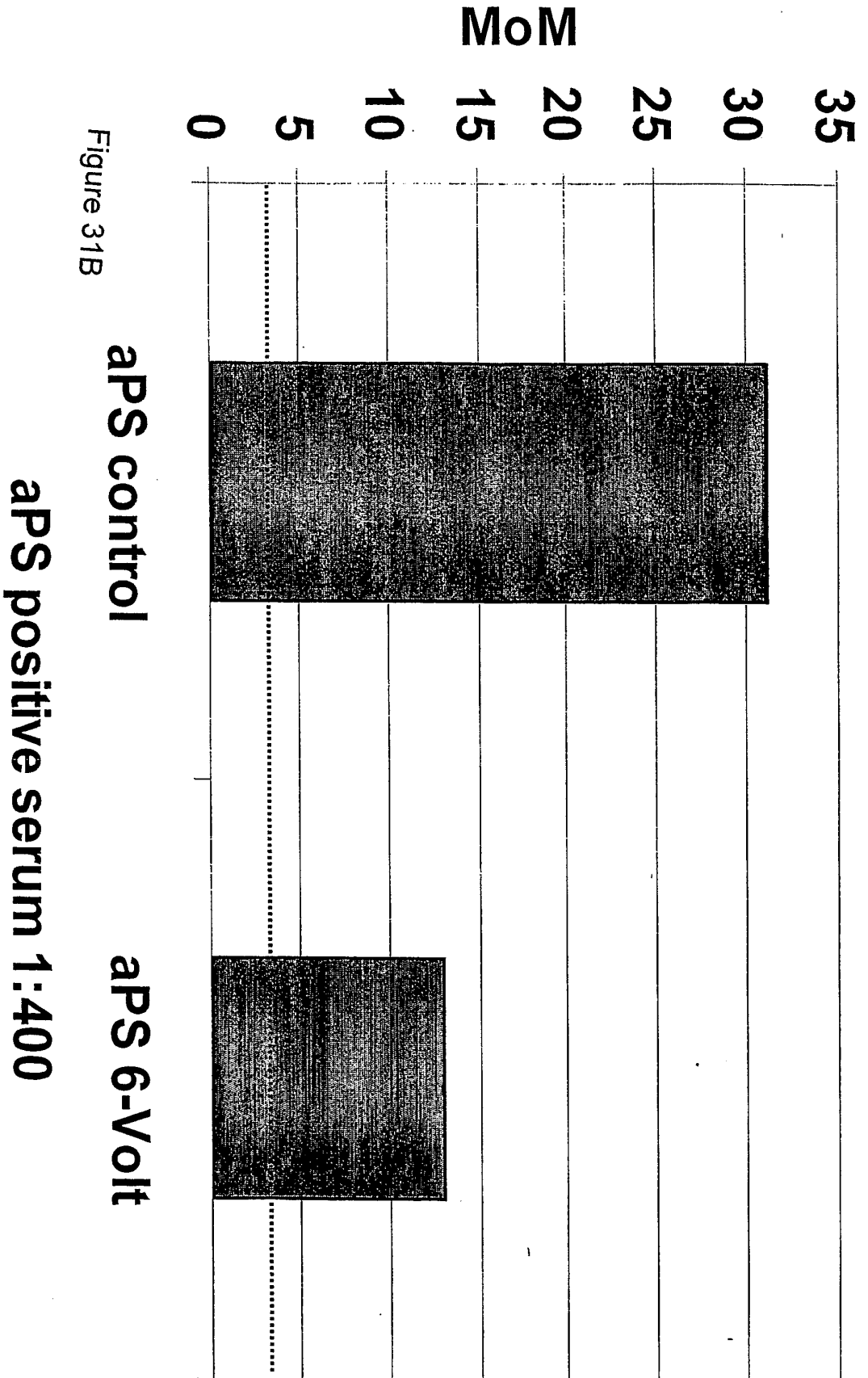


Figure 31B

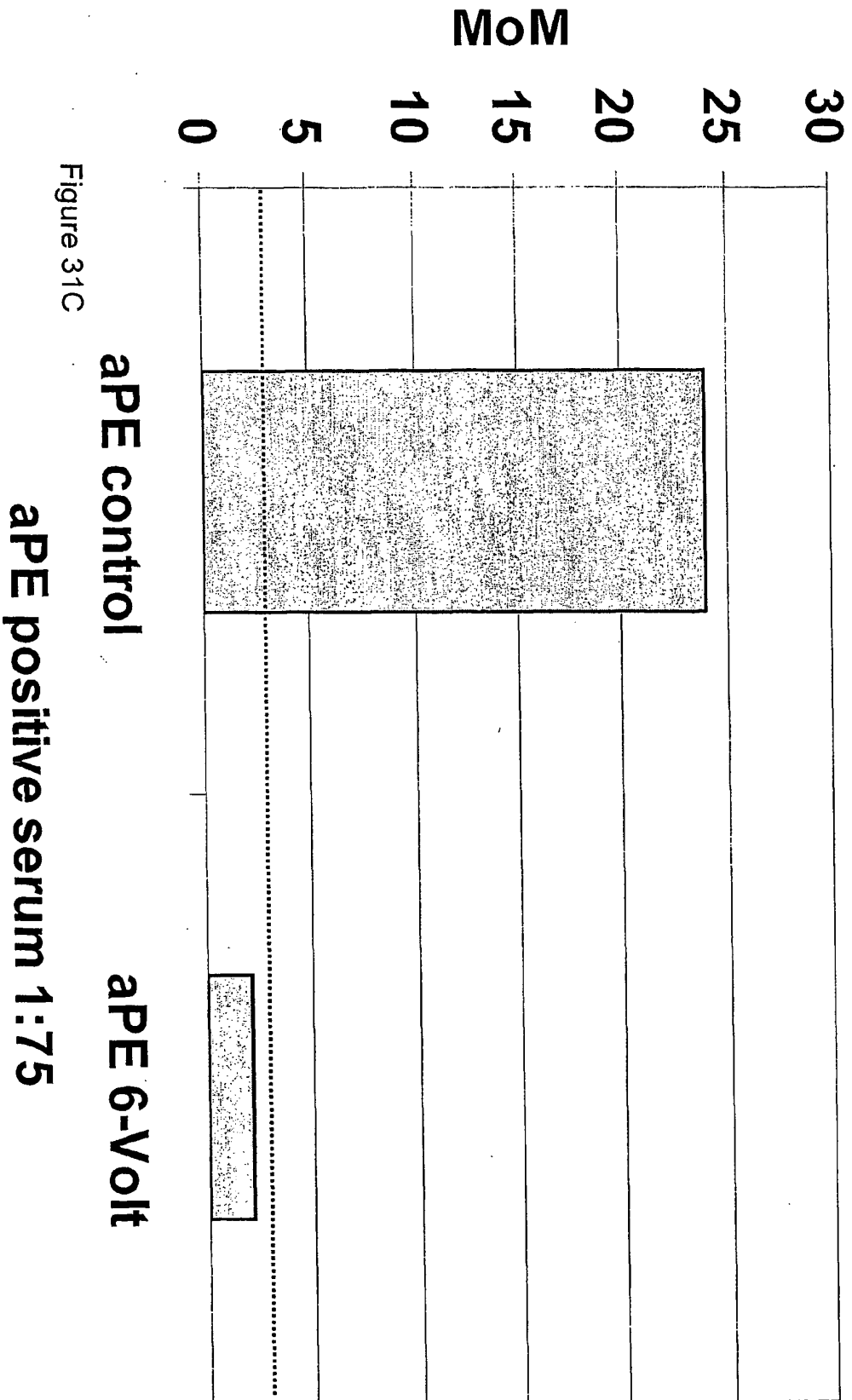


Figure 31C

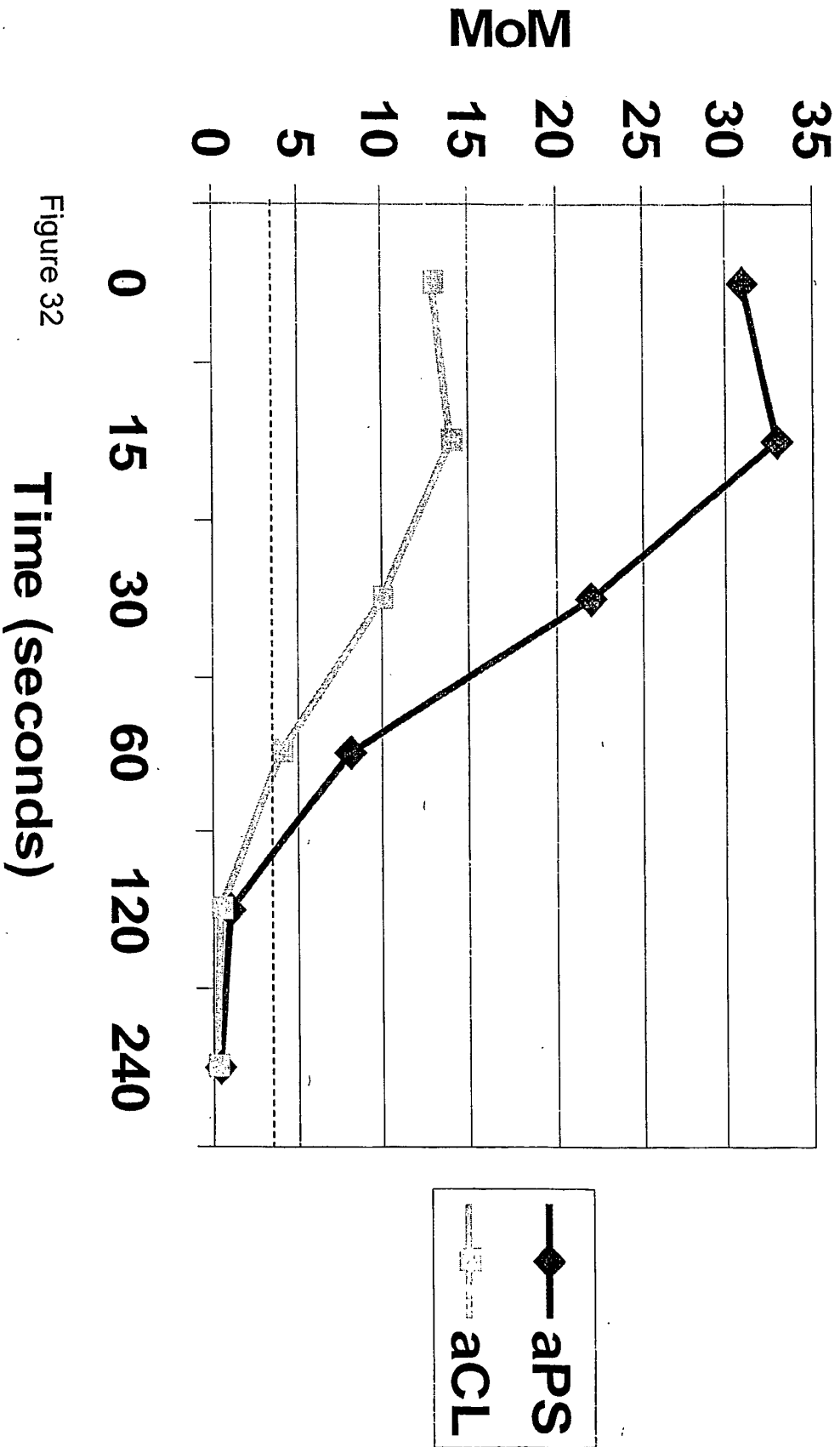


Figure 32

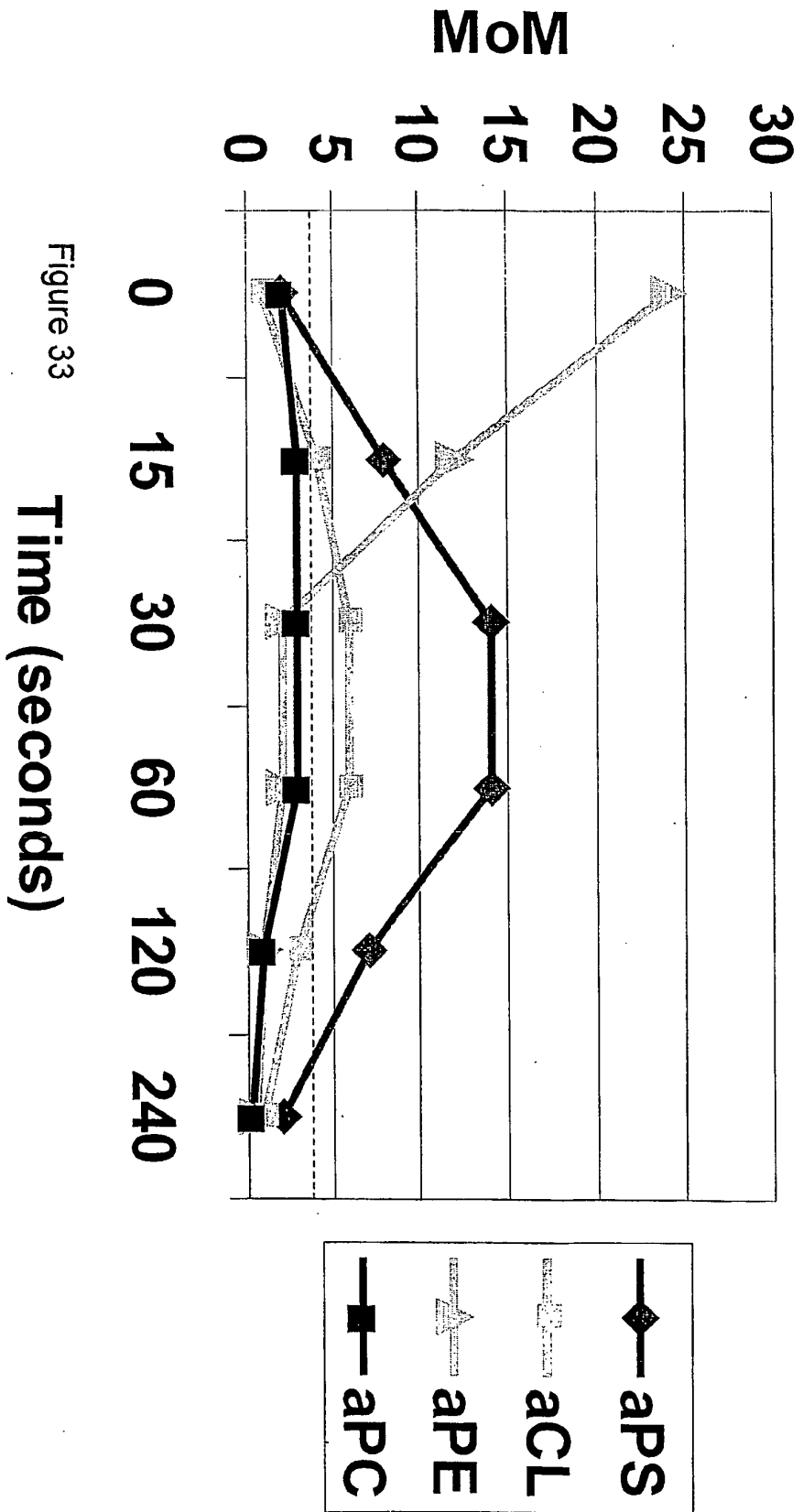


Figure 33

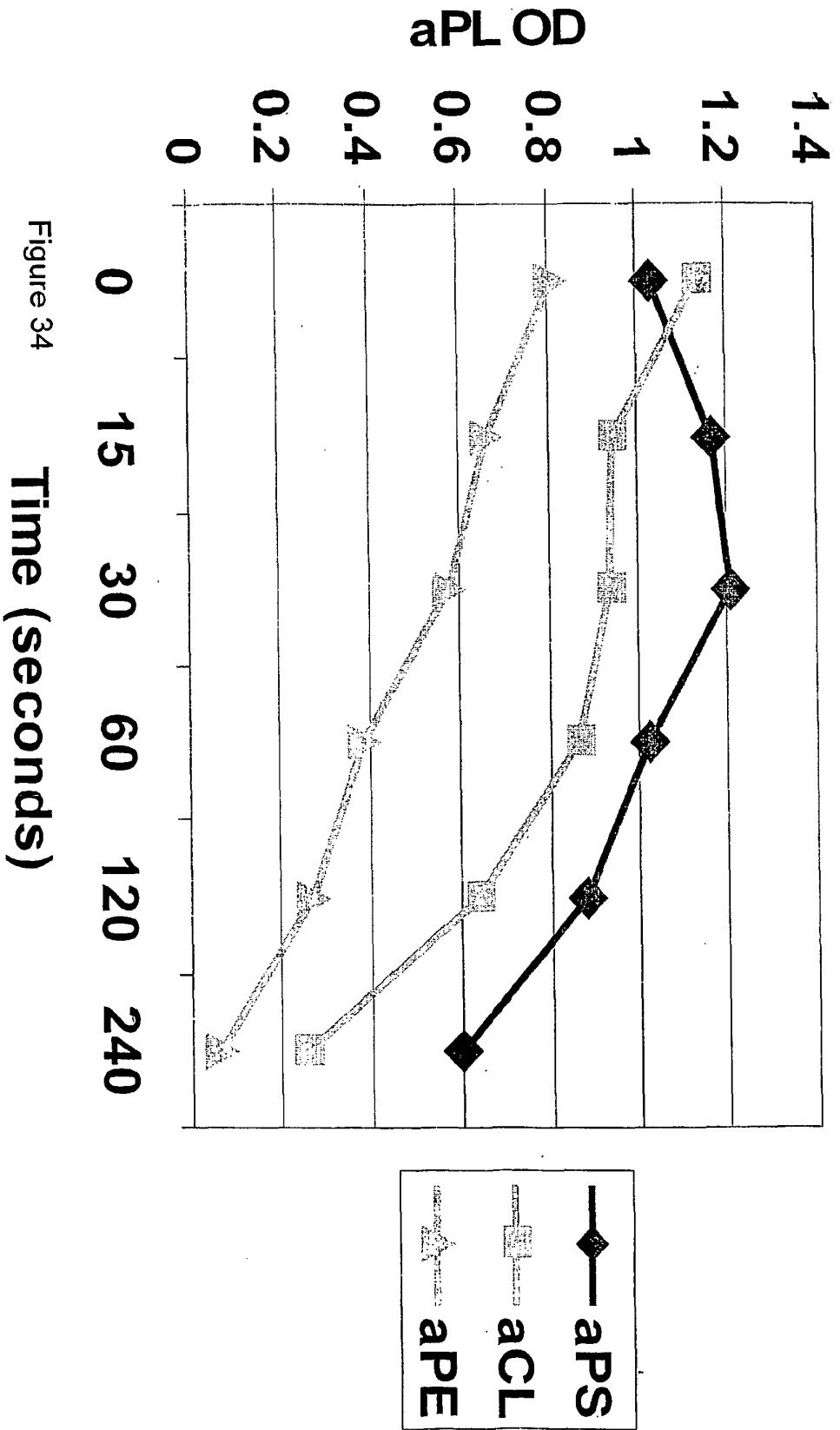


Figure 34

Time (seconds)

## REFERENCES CITED IN THE DESCRIPTION

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### Patent documents cited in the description

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- **McIntyre, J.A. ; Wagenknecht, D.R. ; Faulk, W.P.** Antiphospholipid antibodies: Discovery, definition, detection and disease. *Prog. Lipid Res.*, vol. 42 (3), 176-237 [0050]

专利名称(译)	通过氧化还原反应改变血浆蛋白结合特异性的方法		
公开(公告)号	<a href="#">EP1631314A4</a>	公开(公告)日	2006-08-16
申请号	EP2004754487	申请日	2004-06-09
申请(专利权)人(译)	麦金太尔约翰A.		
当前申请(专利权)人(译)	麦金太尔约翰A.		
[标]发明人	THE DESIGNATION OF THE INVENTOR HAS NOT YET BEEN FILED		
发明人	THE DESIGNATION OF THE INVENTOR HAS NOT YET BEEN FILED		
IPC分类号	A61K39/395 C07K16/00 B01D57/02 C07K1/113 C07K1/24 C07K1/26 C07K14/475 C07K14/765 C07K16/18 C12N15/01 G01N G01N33/53 G01N33/561 G01N33/564		
CPC分类号	A61P1/04 A61P11/00 A61P15/06 A61P17/00 A61P17/06 A61P19/02 A61P19/04 A61P25/00 A61P25/10 A61P25/14 A61P29/00 C07K16/00 C07K16/18 C07K2317/21 G01N33/5306 G01N33/5375 G01N33/564 G01N33/6854 Y10S424/81 Y10S436/825 Y10S530/868		
代理机构(译)	killin詹姆斯, 史蒂芬		
优先权	60/476607 2003-06-09 US		
其他公开文献	EP1631314B1 EP1631314A2		
外部链接	<a href="#">Espacenet</a>		

摘要(译)

通过将蛋白质暴露于足以改变其结合特异性的氧化剂或电流中，可以改变至少一种悬浮或溶解在液体介质中的血浆蛋白的结合特异性。通过氧化蛋白质以改变其结合特异性，可以从血液或血液制品或提取物中回收被掩盖的蛋白质，例如自身抗体。

Unconditioned broth + blood				
Antiphospholipid Antibody (aPL) Results				
		PL binding protein		Normal ranges
		dependent	independent	
aPS	IgG	24 MoM +++	3 MoM	≤ 4 MoM
	IgA	8 MoM +	2 MoM	≤ 3 MoM
	IgM	8 MoM +	1 MoM	≤ 5 MoM
aCL	IgG	12 MoM +++	2 MoM	≤ 4 MoM
	IgA	12 MoM +++	2 MoM	≤ 4 MoM
	IgM	15 MoM +++	1 MoM	≤ 6 MoM
aPE	IgG	36 MoM +++	7 MoM +	≤ 4 MoM
	IgA	7 MoM +	2 MoM	≤ 3 MoM
	IgM	8 MoM +	3 MoM	≤ 5 MoM
aPC	IgG	2 MoM	4 MoM	≤ 4 MoM
	IgA	1 MoM	3 MoM	≤ 3 MoM
	IgM	6 MoM +	5 MoM +	≤ 4 MoM