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(57) Abstract

A method and apparatus optimized as an autoimmune diagnostic sandwich assay test kit, which is capable of immobilizing antigen onto precoated support wells for the purpose of detection of autoimmune antibodies specific to such compounds by either ELISA (Enzyme Linked Immunosorbent Assay) or FIA (Fluorescent Immunoassay) techniques.

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METHOD AND DIAGNOSTIC TEST KIT FOR DETECTION OF AUTOIMMUNE ANTIBODY

BACKGROUND OF THE INVENTION

The immune system is the body's defense mechanism against foreign substances and invading microorganisms. The underlying operating principle of the immune system is a self/nonself recognition pattern. If the invader organism is recognized as not being part of the "self", then a defensive immune response is mounted against it. In the case of autoimmune diseases (See Figure 7) the immune system fails to properly recognize "self" and mounts a defense immune response against its own normal body components. Figure 7 is a list of autoimmune diseases and the antigens associated with them.

Antibodies generated by the immune system to diverse tissue and cellular components have been used to diagnose and monitor autoimmune disease activity. These antibodies include anti-dsDNA, anti-RNP (ribonucleoprotein), anti-DNP (Deoxynucleoprotein), anti-Cardiolipin, anti-histones (symptomatic of drug induced lupus), anti-Sm (Smith), anti-ENA (extractable nuclear antigen), which are often found in patients with Systemic Lupus Erythematosus (SLE), anti-RNP, and anti-ENA are also symptomatic of Mixed Connective Tissue Disease (MCTD). Other antibodies, such as anti-RNA (ribonucleic acid), anti-Scl-70 (Scleroderma-70) are often found in patients with Progressive Systemic Sclerosis (PSS); and anti-SS-A and B (Sjogren's Syndrome Antibody A and B) which are frequently found in patients with Sjogren's Syndrome.

In SLE (a type of autoimmune disease) one of the antibodies produced reacts with DNA that is found widely distributed in cell nuclei in a multitude of body tissues. Formation of antibodies to double-stranded or native deoxyribonucleic acid (anti-dsDNA), is relatively specific to SLE. Although other disorders, such as Mixed Connective Tissue Disease (MCTD), Drug Induced Lupus (DIL), Rheumatoid Arthritis, Scleroderma, PSS, and Sjogren Syndrome, produce similar clinical manifestations as SLE high levels of anti-dsDNA are seldom associated with these disorders. Therefore, detecting anti-dsDNA is useful in specifically diagnosing SLE. Anti-dsDNA levels correlate well with the disease activity of the patient; thus making it a good monitoring tool.

Also in SLE one of the antibodies produced reacts with Sm antigen that is found widely distributed in cell nuclei in a multitude of body tissues. Formation of antibodies to Sm antigen is relatively specific to SLE. Detecting anti-Sm antibodies which are specific for SLE is useful in diagnosing SLE. Anti-Sm antibodies are frequently accompanied by another antibody anti-RNP. Antibodies directed against the RNP antigen are found in the absence

of anti-Sm antibodies in MCTD, which is an "overlap syndrome" that combines features of SLE, PSS, and Polymyositis. Therefore, detecting anti-Sm/RNP antibodies is useful in diagnosing two different autoimmune diseases, SLE and MCTD.

Many different techniques and different diagnostic kits have been developed in the search for a standardized, accurate, rapid, and stable method for the detection of anti-dsDNA, anti-Sm, and anti-Sm/RNP antibodies. Most of the methods have been somewhat successful, but due to unacceptably high levels of cross-reactivity (such as with single-stranded DNA [ssDNA]), the slow run time of the assays, and the short shelf life of the kit components, no method is fully adequate. Examples of the limitations of the prior methods follow.

A number of techniques have been developed to detect antibodies to dsDNA, including immunofluorescent assays (FIA), radioimmunoassay (RIA), and enzyme-linked immunosorbent assays (ELISA).

Firstly, the RIA technique has been developed in a variety of formats to measure levels of anti-dsDNA in sera. A RIA diagnostic test kit (using the Farr technique) has been developed for commercial sale to clinical laboratories. This test precipitates the bound labelled DNA which is then retained for counting. This test is sensitive to high levels of anti-DNA but has less specificity in lower levels of anti-dsDNA activity, thus resulting in false negatives. Unfortunately, this test has a run time of approximately two hours and fifteen minutes, and a limited stable shelf life. Furthermore, this test kit has the same draw-backs which are inherent in all RIAs; the expense associated with radioactive material, which also have a limited shelf life and can be potentially dangerous.

Secondly, Crithidia luciliae immunofluorescence tests have been developed as diagnostic test kits for the detection of anti-dsDNA. This test kit is based on a staining method associated with the kinetoplast of the protozoan. A positive reaction is demonstrated by detection of specific fluorescence in the kinetoplast of most cells. The kinetoplast, within the protozoan, contains many other components besides dsDNA which can cross react with anti-dsDNA or other antibodies, thus rendering false positive results. This test is extremely subjective, as it is based on an individual's ability to recognize a positive fluorescent pattern. Furthermore, this method, which has a run time of approximately two hours, has a lack of sensitivity in low levels of concentration of anti-dsDNA resulting in false negatives. The false negative and false positive results make this method more useful when used together with other tests in clinical practice.

Thirdly, the immunological community has developed diagnostic test kits for the determination of anti-dsDNA levels in the standard ELISA format. Although a variety of these kits have been developed, the same limitations of stability, cross reactivity with ssDNA, and length of run time plague each assay. Many of these kits claim a low level of cross reactivity with ssDNA which should render good sensitivity to low, medium, and high levels of anti-dsDNA; however, as evidenced by the kit instructions, there is a lack of sensitivity in the sera sample which are borderline positives. As a consequence, these kits cannot be used to accurately monitor patients who are borderline positives. In fact, most kits require that low positives must be retested to assure confidence in the results, thereby increasing the cost to the patient. Development of an assay which is sensitive to all levels of anti-dsDNA can only be achieved by reducing non-specific binding by such components as ssDNA.

A number of techniques including immunodiffusion or Ouchterlony analysis have been developed to detect antibodies to Sm antigen and Sm/RNP antigen often called ENA (Extractable nuclear antigens). Examples of the limitations of the prior methods follow.

Firstly, the immunodiffusion technique has a long incubation time, typically 24 hours or more. This time span is of course inconvenient for both the patient and the lab personnel. Secondly, the results gathered from Ouchterlony analysis are subjective. This results in different lab technicians reporting varied results based on individual data interpretation.

The immunological community has developed diagnostic test kits for the determination of anti-Sm antibody levels in the standard ELISA format. These ELISAs have shown certain limitations in stability, cross reactivity, and length of run time.

It is therefore an objective of the present invention to provide compositions, methods, articles, and a diagnostic test kit for the selective adsorption, or affixing of dsDNA, Sm antigen, or Sm/RNP antigen, or any of the various antigens to the autoimmune antibodies previously listed, to a pre-coated plate for the effective detection of the specific autoimmune antibodies present in sera or plasma. It is a further objective of this invention to overcome the aforementioned sensitivity, subjectivity, stability, and shelf life disadvantages inherent in many of the previously described assays. It is, moreover, also an objective of this invention to provide in the form of a kit a novel, readily utilizable means for quantitative and qualitative detection of anti-dsDNA, anti-Sm or anti-Sm/RNP. This will provide a method and apparatus for the purpose of clinical detection of anti-dsDNA, or the anti-Sm antibody, or the anti-

Sm/RNP antibody, or any of the listed autoimmune antibodies listed, or the detection of the specific antibody for any other purpose associated with human or animal medical testing. It is believed that with little modification the present invention can be used to detect anti-histone, anti-RNA, anti-SS-A, anti-SS-B, anti-Scl-70, and anti-DNP.

BRIEF SUMMARY OF THE INVENTION

The present invention includes novel compositions, novel methods and articles for the direct selective absorption, adsorption or attachment, by whatever mechanism, of the anti-dsDNA, or the anti-Sm or the anti-Sm/RNP antibody, or any of the listed autoimmune antibodies, from the body fluid sample to the dsDNA, the Sm-antigen, or the Sm-RNP antigen, or possibly for any of the autoimmune antigens coated onto any suitable solid support, such as test tubes, plates or wells (hereinafter referred to as wells or microwells), for the purpose of quantitative and qualitative identification. The present invention utilizes immobilized native DNA, Sm, Sm-RNP, or possibly for any listed autoimmune antigen from whatever source, which has an affinity for attachment of anti-dsDNA, anti-Sm, or anti-Sm/RNP, or the other antibodies of the listed autoimmune antibodies.

This invention utilizes a sandwich ELISA format which includes pre-coated wells made of any suitable material such as plastic, glass, etc., and the various reagents and antibodies necessary to run a highly sensitive, 45 minute assay for the presence of anti-dsDNA, or for the presence of anti-Sm, or for the presence of anti-Sm/RNP.

The various reagents and the method of coating these reagents to the microwells have been employed advantageously in the practice of the present invention to formulate a test which has minimized or eliminated the problems associated with previous assays. The coating protocol has advantageously utilized methylated Bovine Serum Albumin (mBSA) for two main purposes; namely, the mBSA provides a positively charged surface which enhances the adherence of the antigen such as dsDNA, Sm, and Sm/RNP to the polystyrene wells, and second to eliminate the binding of anti-histone antibodies, which can create false positive results.

The use of mBSA as a coating prior to the application of DNA antigen for use in solid phase assays for anti-dsDNA was first explored by R. Rubin (J. Immunology 63, 359-366 [1983]). In the practice of this invention, protamine sulfate as well as other functionally equivalent substitutes have been found to be capable of forming a positively charged surface; however,

due to the reactivity of these coatings with other components such as ssDNA and anti-histone antibodies, mBSA is preferred. It was highly surprising to find that a coating of mBSA on a solid support underlying the DNA antigen, or the Sm antigen, or the Sm-RNP antigen provides the pre-coated wells with a consistently high level of reproducibility even over a period of one year.

Methylated Bovine Serum Albumin is utilized for the previously mentioned reasons, the second of which is directly related to the specificity and sensitivity of these various assays. The lack of good correlation between ELISAs, RIAs and Crithidia luciliae staining procedures and other tests in some cases appears to be due to binding by anti-histone antibodies which gives the ELISA an elevated antibody reading. The mBSA pre-coating appears to have alleviated this area of non-specific binding.

A second factor which contributes to the lack of sensitivity and non-specificity is the cross reactivity. This is particularly a problem when detecting antibodies to dsDNA, because of the cross reactivity between ssDNA and anti-dsDNA. To eliminate the potential binding of the anti-dsDNA antibodies to ssDNA the microwells which have been coated with dsDNA were treated with an endonuclease, S_1 nuclease, which is an enzyme specific for the breakdown degradation of ssDNA and has no affect on dsDNA. Although other anti-dsDNA tests utilize S_1 nuclease (for example see A.G. Tzioufas, Clinical and Experimental Rheumatology 5, 247-253 [1987]) their sensitivity levels are still substantially lower than acceptable levels for patients with borderline positives. The use of S_1 nuclease in a digestion buffer with an acid pH, combined with the use of a mBSA pre-coating eliminates ssDNA activity and anti-histone activity providing a highly sensitive assay which renders few false positives. The S_1 nuclease is of course only necessary in the detection of anti-dsDNA antibodies and not in anti-Sm or anti-Sm/RNP antibody detection.

The shelf life and the reproducibility of results from the ELISA is associated not only with the mBSA pre-coating, and the S_1 nuclease treatment (in the anti-dsDNA kit), but also with the next two steps of forming the pre-treated wells; and the addition of a hydrolyzed casein blocker and the drying.

A casein-type blocker has been used in various ELISA techniques (Robert F. Bogt; J. Immunological Methods, 101, 43-50 [1987]) to block non-specific binding to plastic through a protein-plastic interaction. It is an unexpected realization that a coating of hydrolyzed casein blocker maintained a consistent inhibition of non-specific binding over an extended

period of time, when the wells were stored at 4 degrees C in a sealed plastic bag.

Although the direct mechanisms by which the drying process and the blocker increases the stability and shelf life of the wells is not fully understood, it is obvious that the storage time is increased by these processes. Little to no ssDNA, or loss of Sm or Sm-RNP antigen activity is generated by this method during the storage conditions in a period of up to one year.

A variety of differing blocking agents could be utilized which are functionally equivalent to or chemically related to the casein blocking agent. For example, BSA and porcine thyroglobulin, dried milk, whole goat serum, etc., however the most preferable is the hydrolyzed casein (commercially available from Sigma) due to its high level of inhibition of non-specific binding and its storage stability.

This invention's use of mBSA, the S_1 nuclease treatment (in the anti-dsDNA kit), combined with the casein blocker and the drying process have unexpectedly resulted in novel assays which are characterized by a low level of cross-reactivity, a high level of inhibition of non-specific binding, and a long shelf life.

The pre-coated wells are then used to detect the presence of anti-dsDNA antibody, anti-Sm antibody, or the anti-Sm/RNP antibody in the sample. The plasma or serum samples are prepared with a sample diluent and are then assayed for their components by an immunoassay technique, the ELISA and the fluorescent immunoassay (FIA) formats being the preferred methods, though it is possible to perform a RIA or a luminescent assay with little modification.

The assays depicted in the following examples have an approximate run time of 45 minutes. The wells, when exposed to the samples, are provided with approximately 15 minutes at room temperature to allow the binding of the antibody to the antigen to go to completion. Then the labelled goat anti-human antibodies are exposed to the wells and a similar 15 minute incubation at room temperature is provided for. If the enzyme is utilized a substrate can be added (although this is not necessary) and 10 minutes is allotted for the production of the color. If a fluorescent marker is used on the goat anti-human antibody then no substrate is needed, therefore the run time is shortened by 10 minutes reducing it to 35 minutes.

Subsequent qualitative and quantitative detection of the antibody is relatively simple if the format is either an ELISA or a FIA. Numerous enzyme-conjugated antibodies and fluorescent-labelled antibodies specific for any of the immunoglobulin classes can be utilized in this invention. The quantitation of the antibodies present is accomplished by the related instruments. The ELISA technique utilizes a spectrophotometer, and the FIA technique utilizes a microfluorometer. Use of the ELISA techniques were first described by Engvall and Perlman ([1971] Immunochemistry 8, 871-874 and [1972] J. Immunology 109, 129-135), and The Enzyme Linked Immunosorbent Assay (ELISA) by Voller, A., Bidwell, D.E., and Bartlett, A., (1979) Dynatech Laboratories, Inc., Alexandria, Virginia, both of which are, in their totality, incorporated herein by reference.

BRIEF DESCRIPTION OF THE GRAPH

Figure 1 - This graph depicts a standardized curve based on arbitrary units/ml. This curve was generated by using the ELISA format of this invention. The Center for Disease Control (CDC) ANA Human Reference Serum #1 has an antigen binding capacity of .59 micro grams DNA bound per ml of serum. This is equivalent to 100 AU per ml.

Figure 2 - This graph depicts a standardized curve obtained from the data in Example 3.

Figure 3 - This graph depicts a standardized curve based on arbitrary READS® units/ml. This curve was generated by using the ELISA format of this invention. The Center for Disease Control (CDC) ANA Human Reference Lot #82-001 #5 Serum (for Sm antigen) was run on an immunodiffusion assay with median titer of 1:64. This standard was serially diluted with normal sera and graphed. The 1:1 or neat dilution was assigned arbitrarily a value of 400 READS units.

Figure 4 - This graph depicts the range of negative and positive human sera control values expected for the anti-Sm test kit.

Figure 5 - This graph depicts a standardized curve based on arbitrary READS® units/ml. This curve was generated by using the ELISA format of this invention. The Center for Disease Control (CDC) ANA Human Reference Lot #82-001 #5 Serum (for Sm antigen) and the ANA Human Reference Lot #82-011 #4 Serum (for RNP antigen) was run on an immunodiffusion assay with median titer of 1:64. An equal volume of reference sera #4 and #5 are combined to form the standard. This standard was serially diluted with normal sera and graphed. The

1:1 or neat dilution was assigned arbitrarily a value of 1400 READS units.

Figure 6 - This graph depicts the range of negative and positive human sera control values expected for the anti-Sm/RNP test kit.

Figure 7 - This chart shows autoimmune disease and their associated antigens which could possibly be detected by the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The following definitions are supplied for the purpose of clarifying the invention and are not intended to limit the scope of the invention:

Methylated Bovine Serum Albumin Solution: Unless otherwise specified, is intended to mean a solution of 1 milliliter of water or PBS with 20 micrograms of methylated bovine serum Albumin (mBSA) dissolved in it. A substitute for mBSA is protamine sulfate or any other chemical or chemical process capable of producing a slightly positively charged coating which is evenly distributed over the surface of the microtitre well. (It is believed that the anti-histone test may not employ methylated bovine serum coating on the microtiter plate.)

PBS Solution: A .01 molar solution of buffer containing 1.43 g potassium phosphate, dibasic, .25 g potassium phosphate, monobasic, and 8.5 g sodium chloride in one liter of water. The pH is 7.3 +/- .1.

dsDNA Solution: Purified dsDNA solution from calif thymus was utilized. Alternative sources of DNA include, but are not limited to, native linear DNA from E. Coli, and circular DNA from plasmid, virus, crithidia, and synthetic polynucleotides (poly (dA.dT)).

Sm Solution: Purified Sm antigen solution from calf thymus was utilized. Alternative source can be employed.

Sm/RNP Solution: Purified Sm/RNP antigen solution from calf thymus was utilized. Alternative source can be employed.

(dsDNA) Casein Blocker Solution: 15 milligrams of hydrolyzed casein blocker (Sigma), 2 ml glyercol, 10 grams sucrose was dissolved in TEN buffer sufficient to bring the final volume

to 100 mls. The solution is adjusted to pH 7.3 +/- .1.

(Sm and Sm/RNP) Casein Blocker Solution: 25 milligrams of hydrolyzed casein blocker (Sigma), 2 ml glyercol, 10 grams sucrose was dissolved in TEN buffer sufficient to bring the final volume to 100 mls. The solution is adjusted to pH 7.3 +/- .1.

TEN Buffer: Is made by adding 6.1 g TRIS, .38 g EDTA, 8.8 g NaCL, 3.8 mL of concentrated HCL to 900 mL deionized water. Adjust pH to 7.3 and add deionized water sufficient to give 1000 milliliter total volume.

Anti-dsDNA Antibody: Circulating autoantibodies directed against dsDNA.

Anti-Smith Antibody: Circulating autoantibodies directed against Sm antigen.

Anti-Sm/RNP Antibody: Circulating autoantibodies directed against Sm/RNP antigen.

Anti-histone Antibody: Circulating autoantibodies directed against histone antigen.

Anti-DNP Antibody: Circulating autoantibodies directed against DNP antigen.

Anti-ENA: Circulating autoantibodies directed against ENA antigen.

Anti-RNA: Circulating autoantibodies directed against RNA antigen.

Anti-Scl-70: Circulating autoantibodies directed against Scl-70 antigen.

Anti-SS-A: Circulating autoantibodies directed against SS-A antigen.

Anti-SS-B: Circulating autoantibodies directed against SS-B antigen.

S_1 Nuclease: An endonuclease (enzyme) which is capable of digestion of ssDNA antigen.

Double Antibody Sandwich ELISA or FIA: A solid support is coated with material which detects and binds the antibody of interest to the coated surface. To render a signal, a second conjugated antibody with an affinity for the previously bound antibody is exposed to the coated surface. This antibody binding to the original antibody makes the sandwich. If

the sandwich assay is an ELISA then the second antibody is conjugated with an enzyme and substrate is used to produce a color. If the assay is an FIA then the second antibody is marked with a fluorescent tag and a substrate is unnecessary.

Buffer for S_1 Nuclease Digestion: 95 mls of acetate/acetic acid buffer, pH 4.6, is mixed with 5 mls glycerol, .29 g NaCL, .029g ZnSO4, for a final volume of 100 mls.

S_1 Nuclease Buffer Solution: Buffer for S_1 nuclease digestion plus 100 units (Sigma) S1 nuclease per ml of buffer. One unit of S_1 nuclease is defined as: Causing 1.0 microgram of ssDNA per minute to become perchloric acid soluble at pH 4.6 and 37 degrees C.

Serum: Is intended to mean the fluid component of any body fluid remaining after cells and coagulable proteins such as fibrin which may be present in such body fluidic components have been removed by appropriate physical, chemical, or physicochemical means. Typically, this term refers to the residual watery fluid remaining after clotting of blood and removal of the clot, but in its broad sense is intended to include the fluidic component of cerebrospinal fluid, urine, interstitial fluid, cellular cytoplasm, and the like.

Sample Diluent: A 1 liter solution has 100 mls of native bovine serum, 1.42 grams of potassium phosphate (dibasic), .26 g of potassium phosphate (monobasic), 1 gram of sodium azide, and 8.6 grams of sodium chloride dissolved in 900 mls of water. If an ELISA is run, then 1 ml of stock green dye is added to the solution. If the FIA format is used then the dye is unnecessary. The solution is then filtered through a .2 micron filter and stored at 4 degrees C.

Diluted (dsDNA) Sample Solution: 10 microliters of sera dissolved in 500 microliters of sample diluent (in dsDNA).

Diluted (Sm and Sm/RNP) Sample Solution: 10 microliters of sera dissolved in 490 microliters of sample diluent.

Conjugate Diluent: A phosphate buffer, and protein stabilizer, plus .02% thimerosal adjusted to a pH of 7.5 (commercially available from Medix Biotech Inc.) into which is added a protease inhibitor, aprotinin, (commercially available from Miles Pentex) at .01% of the volume of the buffer.

(dsDNA) Working Conjugated Antibody Solution: 1 volume of concentrated conjugated antibodies/3000 volumes of conjugate diluent. The dilution is subject to change based on the concentration level of the conjugated antibody (dsDNA).

(Sm and Sm/RNP) Working Conjugated Antibody Solution: 1 volume of concentrated conjugated IgG antibodies/4000 volumes of conjugate diluent, and 1 volume IgM to 1500 vol. The dilution is subject to change based on the concentration level of the conjugated antibody.

Conjugated Antibodies: For an ELISA, antibodies were chemically conjugated with horseradish peroxidase. For FIA, antibodies were chemically conjugated with Fluorescein Isothiocyanate.

Immunoglobulin: Any member of the gammaglobulin fraction of serum possessing the ability to bind another agent.

Antigen: Molecules (from whatever source, nature or man-made) which induce an immune reaction when recognized by the host's immune system.

Antibody: A class of serum proteins which specifically bind to an antigen which induced the formation of the antibody.

Immunoglobulin Classes: Antibodies separated by electrophorectic mobility specifically, IgG and IgM.

Substrate Solution: To quantitate the horseradish peroxidase, 100 microliters of buffered (3,3',5,5') Tetramethylbenzidine/ hydrogen peroxide (commercially available from Kirkegaard Perry) was used.

Labelled Antibodies: Any antibody substance which has been covalently or otherwise combined with a molecule or ion for the purpose of selectively identifying that group of antibodies. Such adduct molecules or ions include enzymes, fluorescent substances, radionuclides, and the like.

Labelled Antigens: Any antigen substance which has been covalently or otherwise combined with a molecule or ion for the purpose of selectively identifying that group of antigens. Such adduct molecules or ions include enzymes, fluorescent substances, radionuclides, and the

like.

Optical Density (OD) or Absorbance: A number which refers to the color absorbance of a sample. Optical density is related to the percent of light transmitted through the sample by the following formula: OD = 2-log(percent transmittance).

Test Kit for Anti-dsDNA

The preferred embodiment of the method and apparatus for the detection of anti-dsDNA in sera is a diagnostic test kit. The optimized kit contains:

5 vials (200 microliters) Assay Calibrators for anti-DNA containing these levels of anti-dsDNA activity: 120, 60, 30, 15, 7.5, 3.8 A.U./ml (quantitative format only)

1 vial (30 ml) Sample Diluent - green solution: contains 0.1% sodium azide.

1 vial Human Negative Serum Control (about 3 A.U./ml).

1 vial High Human Serum Positive Controls - about 100 A.U./ml with antigen binding capacity of 59 x 10-4 microgram of DNA.

1 vial Moderate Human Serum Positive Control - about 45 A.U./ml.

12 coated 8-well Microwell Strips with frame holder.

1 vial (15 ml) Conjugated Antibody Working Solution - containing horseradish peroxidase conjugated anti-human IgG, and IgM.

1 bottle (8 ml) TMB Substrate Solution A - contains 3,3',5,5' Tetramethylbenzidine.

1 bottle (8 ml) TMB Substrate Solution B - contains hydrogen peroxide.

1 bottle (12 ml) Stop Reagent: contains 2.5 N H2SO4. (1.O N HC1 can be used as a substitute)

1 packet Phosphate Buffered Saline (PBS) - reconstitutes to 2 liters of 0.01 M PBS, pH 7.4.

Plate Template

This kit for the measurement of anti-dsDNA in serum samples has been designed for use in a clinical laboratory. To determine the AU/ml of the anti-dsDNA present in the sample the kit includes calibrators and controls for generating a standard curve. The selection of the levels of anti-dsDNA activity in these controls and calibrators can be varied without affecting performance of the assay.

Optimization of the process has yielded kits with pre-coated test wells and reagents, which produce low levels of variation between assays and within assays. Furthermore, these reagents and test wells are stable for extended periods of time. This kit has been optimized to have a 45 minute run time at room temperature, which is significantly shorter than other test kits, presently marketed for anti-dsDNA detection.

What follows is a description of a preferred embodiment of the pre-coated wells, and the method of coating the wells of the present invention, along with the preferred method for preparation of and utilization of the various elements of an anti-dsDNA diagnostic test kit.

Step 1 - Affixation of the Coating on the Microwells: Methylated Bovine Serum Albumin (mBSA) is dissolved in distilled water or PBS at a ratio of 20 ug/ml. An aliquot of 100 micrograms of this prepared solution is placed in each microwell, to produce a surface which is slightly positively charged. The mBSA serves two important functions; one is to provide a stable evenly coated microwell surface, and second is to inhibit non-specific binding such as anti-histone activity. Ten mls of mBSA solution will coat 96 microtiter wells, such as a Dynatech Immulon 2, Dynatech Immulon 4, or Nunc Maxisorp. The coated wells are incubated overnight at 4 degrees C.

Unbound mBSA solution is shaken from the wells, and they are rinsed with PBS solution, pH 7.3, and drained thoroughly.

Next, the ligand or antigen is prepared and exposed to the receiving surfaces of the microwells. Purified dsDNA (calf thymus) is dissolved into .01M PBS, pH 7.4 in a ratio of 5 micrograms/ml (weight per volume). The buffered ligand is then dispensed into each microwell, 100 microliters of buffered ligand per well. The binding is enhanced by an incubation period of 18-24 hours at 4 degrees C. The excess solution is shaken from the

wells.

The next coating is applied to eliminate the cross reactivity with ssDNA. A buffer is prepared for S_1 nuclease digestion. To prepare the buffer .03M acetic acid was titrated together with .03M sodium acetate in a ratio of 1.1 part acetic acid to 1 part sodium acetate to a final pH of 4.6. 95 mls of the prepared acetate/acetic acid buffer is mixed with 5 mls of glycerol, .29 g NaC1, .029 g ZnS04, for a final volume of 100 mls. Added to this buffer was 100 units of S_1 nuclease (Sigma) per ml of digestion buffer solution to form a S_1 nuclease buffered solution which is dispensed in 100 microliter increments into each individual receiving well. Thus, 10 units of S_1 nuclease is contacted with each individual well. This solution is of sufficient concentration to digest the unwanted ssDNA in the previously coated ligand material after a two hour incubation period at 37 degrees C. S_1 nuclease eliminates ssDNA which is a source of false positives in many assays. After the incubation period the wells are inverted to remove excess solution, then the wells are thoroughly rinsed twice with PBS solution. The wells are again inverted on a paper towel and allowed to drain.

The next coating step is contacting casein blocker (pH of 7.3) with the microwells in aliquots of 200 microliters. This step decreases the non-specific binding that can occur due to protein-plastic interaction. The hydrolyzed casein used in the blocker solution can be commercially obtained from Sigma. The casein blocker solution is prepared by mixing 2 ml glycerol, 10 g sucrose, and 15 mg of hydrolyzed casein, and adding sufficient TEN buffer to make 100 ml of solution. The wells containing casein blocker solution are again incubated at 4 degrees C overnight, after which time the wells are inverted and allowed to drain for 15 minutes. Then the wells are uprighted and allowed to dry at room temperature for at least 24 hours.

This completes the process of coating the wells and each diagnostic kit is then supplied with 96 coated wells. The shelf life of coated microwells when stored at 4 degrees C in a sealed plastic bag is up to one year.

Step 2 - Adhering Anti-dsDNA to the Prepared Wells From Step 1: The sample diluent is supplied in the kit as a 30 ml green solution. To prepare a 1000 ml solution of Sample Diluent, 100 milliliters of native bovine serum, 1.42 g of Potassium Phosphate (dibasic), .26 g of Potassium Phosphate (monobasic), 1 gram of sodium azide, and 8.6 g of sodium chloride, and 1 ml of stock green dye are dissolved in deionized water sufficient to make 1000 milliliters of solution. This solution is then filtered through a .2 micron filter.

The Sample Diluent acts as a blocking agent similar to the casein blocker which was previously coated on the wells. The principle blocking component in the Sample Diluent is the native bovine serum which acts to inhibit the binding of any BSA-reactive antibodies to the mBSA coating on the surface of the well.

Prior to contacting the body fluid with the prepared plate, the serum is diluted by adding aliquots of sera to the sample diluent in a 1:50 ratio (volume of serum:volume of sample diluent), although this exact dilution is not critical and depends upon the nature of the body fluid and the assay techniques employed. To form the diluted sample solution the body fluid is aliquoted in 10 microliter proportions into 500 microliters of Sample Diluent. In an individual well, 100 microliters of the diluted sample is dispensed, and the affixation of the anti-dsDNA is enhanced by 15 minutes incubation at room temperature.

Following the affixation of the anti-dsDNA to the coated wells, the wells are thoroughly washed four times with PBS to remove the free unbound antibodies that are present in the sample, which if allowed to remain, would elevate the background absorbance.

Step 3 - Assay for the Anti-dsDNA Affixed to the Wells: Standard enzyme-linked immunoassay techniques, previously described, are used for this assay, although any suitable means of detection such as radioactive labeling, fluorescence, or the like can be employed. For the examples described hereinafter, anti-human IgG and anti-human IgM induced in goats were used to ascertain whether anti-dsDNA IgG and IgM antibodies were present. Please note that other species of animal can be used to produce anti-human antibodies. These antisera were linked to horseradish peroxidase, an enzyme which yields a colored product whenever one of its substrates is present together with hydrogen peroxide. The substrate should be chosen to be consistent with the enzyme conjugated to the antibody. For the examples described hereinafter, the substrate was (3,3',5,5') Tetramethylbenzidine and hydrogen peroxide.

The kit contains one 15 ml vial of conjugated antibody solution with anti-human IgM and IgG antibody conjugated to horseradish peroxidase. To prepare a working conjugated antibody solution, a phosphate buffer with protein stabilizer and .02% thimerosal solution at pH 7.4 (commercially available from Medix) was mixed with aprotinin, a protease inhibitor (commercially available from Miles Pentex) at a .01% ratio of inhibitor to volume of buffer. This diluent enhances the stability of the conjugated antibody. The solution is mixed at a

ratio of 1/3000; one part of concentrated conjugated IgM and IgG antibodies is aliquoted into 3000 parts conjugate diluent. The ratio of concentrated conjugated antibody to conjugate diluent is subject to wide latitudes of dilutions based on the manufacturer's concentration of conjugated antibody used in the assay.

Next 100 microliters of the enzyme conjugated goat anti-human antibody working solution, (prepared as described) is added to each microtiter well. Binding of these antibodies to the anti-dsDNA is permitted for at least 15 minutes at room temperature, then the microtiter wells are emptied of their contents, washed four times with PBS, and allowed to drain before the next step.

The presence of a label and antibody, as previously described, is determined by incubating the wells with a solution of buffered (3,3',5,5') Tetramethylbenzidine and hydrogen peroxide. This solution is supplied in the kit in two 8 ml vials; one contains (3,3',5,5') Tetramethylbenzidine; the other vial contains hydrogen peroxide. The separate vials are necessary due to the interaction between the two solutions. The two solutions are mixed in a one to one ratio just before use, and 100 microliters of the mixed solution is dispensed into each microwell. The reaction is permitted to continue for 10 minutes at room temperature, or until sufficient color appears to be read on the spectrophotometric device used. The reaction is subsequently stopped through the addition of an equal volume of 2.5 normal sulfuric acid, and the intensity of color (the optical density, "OD", or absorbance) is read by a spectrophotometric device such as a Dynatech MR600 or the like.

As with any enzyme-linked immune assay, the resultant color of the reaction product is proportional to the number of conjugated antibodies which have bound to the anti-dsDNA. For most cases, the number of bound conjugated antibodies is linearly related to the number of anti-dsDNA antibodies. Hence, as the amount of anti-dsDNA bound to the wells increases, so does the optical density, or absorbance of the enzyme reaction.

Test Kit for Anti-Sm or Anti-Sm/RNP

This preferred embodiment of the method and apparatus for the detection of anti-Sm antibodies in a sera is a diagnostic test kit. Similarly the preferred embodiment of the method and apparatus for the detection of anti-Sm/RNP antibodies in sera is a diagnostic test kit, with the following components (qualitative format).

1 vial (40 ml) Sample Diluent - green solution: contains 0.1% sodium azide.

1 vial Human Negative Serum Control (about 100 microliters).

1 vial High Human Serum Positive Controls (about 100 microliters).

12 coated 8-well Microwell Strips with frame holer.

1 vial (12 ml) Conjugated Antibody Working Solution - containing horseradish peroxidase conjugated anti-human IgG, and IgM.

1 bottle (8 ml) TMB Substrate Solution A - contains 3,3',5,5' Tetramethylbenzidine.

1 bottle (8 ml) TMB Substrate Solution B - contains hydrogen peroxide.

1 bottle (12 ml) Stop Reagent: contains 2.5 N H2SO4. (1.0 N HC1 can be used as a substitute) (not supplied).

1 packet Phosphate Buffered Saline (PBS) - reconstitutes to 2 liters of 0.01 M PBS, pH 7.4.

Plate Template

This kit for the measurement of anti-Sm antibodies in serum samples has been designed for use in a clinical laboratory. To determine the READS units/ml of the anti-Sm antibodies or the anti-Sm/RNP antibodies present in the sample kit can include calibrators and controls for generating a standard curve. The selection of the levels of anti-Sm or anti-Sm/RNP antibody activity in these controls and calibrators can be varied without affecting performance of the assay.

What follows is a description of a preferred embodiment of the pre-coated wells, and the method of coating the wells of the present invention, along with the preferred method for preparation of and utilization of the various elements of the anti-Sm or the anti-Sm/RNP antibody diagnostic test kit. The difference between the anti-Sm and the anti-Sm/RNP kits is only the concentration of antigen coated to the microwells.

Step 1: Affixation of the Coating on the Microwells: Methylated Bovine Serum Albumin

(mBSA) is dissolved in distilled water or PBS at a ratio of 20 ug/ml. An aliquot of 100 microliters of this prepared solution is placed in each microwell, to produce a surface which is slightly positively charged. The mBSA serves two important functions; one is to provide a stable evenly coated microwell surface, and second is to inhibit non-specific binding such as anti-histone activity. Ten mls of mBSA solution will coat 96 microtiter wells, such as a Dynatech Immulon 2, Dynatech Immulon 4, or Nunc Maxisorp. The coated wells are incubated overnight at 4 degrees C.

Unbound mBSA solution is shaken from the wells and drained thoroughly.

Next, the ligand or antigen is prepared and exposed to the receiving surfaces of the microwells. Purified Sm antigen or the purified Sm/RNP antigen (calf thymus) is dissolved into .01M PBS, pH 7.4 in a ratio of 5 units/ml, and for Sm/RNP 1 unit/ml. The buffered ligand is then dispensed into each microwell, 100 microliters of buffered ligand per well. The binding is enhanced by an incubation period of 18-24 hours at 4 degrees C.

The next coating step is contacting casein blocker (pH of 7.3) with the microwells in aliquots of 200 microliters. This step decreases the non-specific binding that can occur due to protein-plastic interaction. The hydrolyzed casein used in the blocker solution can be commercially obtained from Sigma. The casein blocker solution is prepared by mixing 2 ml glycerol, 10 g sucrose, and 25 mg of hydrolyzed casein, and adding sufficient TEN buffer to make 100 ml of solution. The wells containing casein blocker solution are again incubated at 4 degrees C overnight, after which time the wells are inverted and allowed to drain for 15 minutes. Then the wells are uprighted and allowed to dry at room temperature for at least 24 hours.

This completes the process of coating the wells and each diagnostic kit is then supplied with 96 coated wells. The shelf life of coated microwells when stored at 4 degrees C in a sealed plastic bag is up to one year.

Step 2: Adhering Anti-Sm or Anti-Sm/RNP antibodies to the Prepared Wells From Step 1: The sample diluent is supplied in the kit as a 40 ml green solution. To prepare a 1000 ml solution of Sample Diluent, 100 milliliters of native bovine serum, 1.42 g of Potassium Phosphate (dibasic), .26 g of Potassium Phosphate (monobasic), 1 gram of sodium azide, and 8.6 g of sodium chloride, and 1 ml of stock green dye are dissolved in deionized water sufficient to make 1000 milliliters of solution. This solution is then filtered through a .2 micron

filter.

The Sample Diluent acts as a blocking agent similar to the casein blocker which was previously coated on the wells. The principle blocking component in the Sample Diluent is the native bovine serum which acts to inhibit the binding of any BSA-reactive antibodies to the mBSA coating on the surface of the well.

Prior to contacting the body fluid with the prepared plate, the serum is diluted by adding aliquots of sera to the sample diluent in a 1:50 ratio (volume of serum:volume of sample diluent), although this exact dilution is not critical and depends upon the nature of the body fluid and the assay techniques employed. To form the diluted sample solution the body fluid is aliquoted in 10 microliter proportions into 490 microliters of Sample Diluent. In an individual well, 100 microliters of the diluted sample is dispensed, and the affixation of the anti-Sm or anti-Sm/RNP antibody is enhanced by 15 minutes incubation at room temperature.

Following the affixation of the anti-Sm or anti-Sm/RNP antibody to the coated wells, the wells are thoroughly washed four times with PBS to remove the free unbound antibodies that are present in the sample, which if allowed to remain, would elevate the background absorbance.

Step 3: Assay for the Anti-Sm or Anti-Sm/RNP antibody Affixed to the Wells: Standard enzyme-linked immunoassay techniques, previously described, are used for this assay, although any suitable means of detection such as radioactive labeling, fluorescence, or the like can be employed. For the examples described hereinafter, anti-human IgG and anti-human IgM induced in goats were used to ascertain whether anti-Sm IgG and IgM antibodies or anti-Sm/RNP IgG and IgM antibodies were present. Please note that other species of animal can be used to produce anti-human antibodies. These antisera were linked to horseradish peroxidase, an enzyme which yields a colored product whenever one of its substrates is present together with hydrogen peroxide. The substrate should be chosen to be consistent with the enzyme conjugated to the antibody. For the examples described hereinafter, the substrate was (3,3',5,5') Tetramethylbenzidine and hydrogen peroxide.

The kit contains one 12 ml vial of conjugated antibody solution with anti-human IgM and IgG antibody conjugated to horseradish peroxidase. To prepare a working conjugated antibody solution, a phosphate buffer with protein stabilizer and .02% thimerosal solution at pH 7.4 (commercially available from Medix) was mixed with aprotinin, a protease inhibitor

(commercially available from Miles Pentex) at a .01% ratio of inhibitor to volume of buffer. This diluent enhances the stability of the conjugated antibody. The solution is mixed at a ratio of 1/4000 for IgG and 1/1500 for IgM; one part of concentrated conjugated IgM and IgG antibodies is aliquoted into 4000 and 1500 parts conjugate diluent, respectively. The ratio of concentrated conjugated antibody to conjugate diluent is subject to wide latitudes of dilutions based on the manufacturer's concentration of conjugated antibody used in the assay.

Next 100 microliters of the enzyme conjugated goat anti-human antibody working solution, (prepared as described) is added to each microtiter well. Binding of these antibodies to the anti-Sm or anti-Sm/RNP antibody is permitted for at least 15 minutes at room temperature, then the microtiter wells are emptied of their contents, washed four times with PBS, and allowed to drain before the next step.

The presence of a label and antibody, as previously described, is determined by incubating the wells with a solution of buffered (3,3',5,5') Tetramethylbenzidine and hydrogen peroxide. This solution is supplied in the kit in two 8 ml vials; one contains (3,3',5,5') Tetramethylbenzidine; the other vial contains hydrogen peroxide. The separate vials are necessary due to the interaction between the two solutions. The two solutions are mixed in a one to one ratio just before use, and 100 microliters of the mixed solution is dispensed into each microwell. The reaction is permitted to continue for 10 minutes at room temperature, or until sufficient color appears to be read on the spectrophotometric device used. The reaction is subsequently stopped through the addition of an equal volume of 2.5 normal sulfuric acid, and the intensity of color (the optical density, "OD", or absorbance) is read by a spectrophotometric device such as a Dynatech MR600 or the like.

As with any enzyme-linked immune assay, the resultant color of the reaction product is proportional to the number of conjugated antibodies which have bound to the antibody. For most cases, the number of bound conjugated antibodies is linearly related to the number of bound antibodies. Hence, as the amount of anti-Sm or anti-Sm/RNP antibody bound to the wells increases, so does the optical density, or absorbance of the enzyme reaction.

EXAMPLE 1
Protocol for Pre-Coating Microwells with Purified dsDNA

Polystyrene wells were coated with (native) double stranded deoxyribonucleic acid (dsDNA) by the following procedure:

1. Methylated Bovine Serum Albumin (Sigma) (hereinafter designated as mBSA) was dissolved at a ratio of 20 micrograms/ml in distilled water or PBS.

2. 100 microliters of the mBSA solution was placed in each microwell, and incubated overnight at 4 degrees C.

3. The excess coating solution was shaken from the plate after incubation. The plate was rinsed once with a solution of .01M PBS, pH 7.3 to remove unbound mBSA. The wells were inverted to drain thoroughly.

4. 5 ug/ml of purified dsDNA was diluted in PBS, .01M, pH 7.3, and 100 microliters of the diluted dsDNA solution was dispensed into each microwell and allowed to incubate for 18-24 hours at 4 degrees C.

5. After incubation, the excess dsDNA solution was shaken from the wells and 100 microliters of S-1 nuclease in digestion buffer was added to each well.

6. To form S-1 nuclease buffer solution, .03M sodium acetate and .03M acetic acid in a 1.1 to 1 ratio at pH 4.6 was prepared. Then 95 mls of this buffer was mixed with 5 mls of glycerol, .29 g NaC1 and .029 g $ZnSO_4$ for a final volume of 100 mls at pH 4.6. S-1 nuclease (Sigma) was dissolved into this solution at a concentration of 100 units/ml buffer. (One unit of S-1 nuclease is defined as causing 1.0 microgram of ssDNA per minute to become perchloric acid soluble at pH 4.6 and 37 degrees C.)

7. 100 microliters of S-1 nuclease buffer solution was contacted with each well for two hours at 37 degrees C. After incubation, the wells were emptied, rinsed twice with PBS, and drained thoroughly.

8. Casein blocker solution (pH 7.3) was dispensed in 200 microliter increments into each

well. The casein blocker solution consists of 2 ml glycerol, 10 g sucrose, 15 mg casein and sufficient TEN buffer to bring the volume to 100 ml.

9. The wells coated with the blocker solution were incubated overnight at 4 degrees C. After incubation, the solution was shaken from the wells and the wells were inverted for 15 minutes. Then the wells were uprighted and allowed to dry 24 hours at room temperature.

The coated dsDNA wells were then used to determine the presence of anti-dsDNA in serum samples obtained from individuals with:

1. no apparent pathology (normal);

2. Systemic Lupus Erythematosus.

These sera were drawn from patients with known pathological status. Described hereinafter, is a method of performing an anti-dsDNA assay, using the dsDNA coated wells in a sandwich ELISA format.

1. Standards, Sera from patients with no apparent pathology and patients with SLE, a high positive human serum control (100 AU/ml), a moderate positive human serum control (45 AU/ml), and a negative human serum control (3 AU/ml) were diluted with Sample Diluent in a 1:50 ratio; 1 part serum to 50 parts Sample Diluent. The Sample Diluent was a solution consisting of 100 mls of native bovine serum in a phosphate buffered saline consisting of 1.42 g of potassium phosphate (dibasic), .26 g potassium phosphate (monobasic), 8.6 g sodium chloride, .1% sodium azide, 1 ml stock green dye, and distilled water added to bring the volume to 1000 mls. The Sample Diluent was then filtered through a .2 micron filter.

2. 100 microliters of the Sample Diluent solutions were placed in the designated wells at room temperature for 15 minutes to allow completion of the antigen-antibody binding process. Following incubation, the wells were emptied and washed four times with PBS solution.

3. The wells were exposed to a working conjugated antibody solution consisting of 1 part per volume horseradish peroxidase conjugated IgG and IgM specific antibodies, and

3000 part per volume conjugate diluent (Medix) consisting of phosphate buffer, protein stabilizer, and .02% thimerosal, to which was added .01% by volume a protease inhibitor (commercially available from Miles Pentex).

4. 100 microliter of the working conjugated antibody solution was placed in each microtiter well and allowed to incubate at room temperature for 15 minutes. After incubation, the wells were rinsed four times with PBS solution to remove unbound conjugated antibodies.

5. Each well was assayed for horseradish peroxidase activity by mixing equal volumes (3,3',5,5') Tetramethylbenzidine (Kirkegaard Perry) and hydrogen peroxide solution and dispensing 100 microliters of this substrate solution into each well. The presence of the anti-dsDNA was detected by a blue color appearing after the 10 minute room temperature incubation. 100 microliters per well of 2.5 N sulfuric acid terminated the reaction producing a yellow color. The yellow color was quantitated at 450 nm using a Dynatech MR600 plate reading spectrophotometer.

Detection of anti-dsDNA (IgG & IgM)

Source	Mean O.D.
CDC Ref Std.	1.08
anti DNA pos	1.13
pos SLE #1	.54
pos SLE #2	1.75
pos anti nucleolar	.10
normal serum	.09

EXAMPLE 2
Adsorption of anti-dsDNA From Serum by Uncoated Polystyrene Wells

To demonstrate the beneficial effect of the coating treatment, polystyrene wells were left totally uncoated but exposed to the sera and the standards as described in the previous example. The wells were then exposed to horseradish peroxide conjugated IgG and IgM antibodies, and assayed for peroxidase activity. As expected, only slight selectivity was exhibited by untreated wells and high amounts of non-specific binding led to very high background absorbance.

Source	AU/ML*	O.D. Coated as in Example 1	O.D. Uncoated Wells
Calibrator			
1	120.0	1.38	1.28
2	60.0	1.05	1.08
3	30.0	.55	.77
4	15.0	.37	.57
5	7.5	.29	.67
6	3.8	.22	.50
Normal Serum 1	8.0	.28	.84
Normal Serum 2	9.0	.29	.97
Normal Serum 3	3.0	.19	.65
High Positive SLE			
1	112.0	1.34	.54
2	50.0	.89	.51
3	120.0	1.96	1.67
Low Positive SLE			
1	13.0	.35	1.76

*AU/ml values calculated in previous experiment.

EXAMPLE 3
A Diagnostic Test Kit Assay Procedure

The kit contains pre-coated microwells and,

1 vial (30 ml) of Sample Diluent (green solution containing 0.1% sodium azide);

6 vials (200 ul each) of Assay Standards for anti-dsDNA, containing these levels of anti-dsDNA activity 120, 60, 30, 15, 7.5, 3.8 AU/ml (anti-dsDNA activity 100 AU/ml of this assay calibrator has an antigen binding capacity of 59 x 10-4 ug of DNA),

1 vial of high positive human serum control (about 100 AU/ml),

1 vial of moderate positive human serum control (about 25 AU/ml),

1 vial of negative human serum control (about 3 AU/ml). Also included in the diagnostic kit was a vial (15 ml) of conjugated antibody working solution containing horseradish peroxidase conjugated antihuman IgG, and IgM

1 bottle (8 ml) of TMB Substrate; solution A containing 3,3',5,5', tetramethylbenzidine,

1 bottle (8 ml) of TMB Substrate; Solution B containing hydrogen peroxide. When mixed with equal parts Solution A will form a substrate capable of generating a colored product.

1 bottle (12 ml) of Stop Reagent containing 2.5 N H2SO4 (1 N HCL can be substituted),

1 packet of Phosphate Buffered Saline (PBS) which reconstitutes to 2 liters of 0.01 M PBS, pH 7.3, which is utilized as a wash solution

Plate Template.

The various reagents of the kit were utilized to perform the assay. The directions for the method of assaying for anti-dsDNA were included in the kit and these directions were followed precisely during this experiment.

The plate templates were labelled for sample placement in the microwells. A 1:50 dilution of

the standards, controls and patient samples was prepared in Sample Diluent (green solution). 10 ul of sample was added to 500 ul sample diluent in a one volume to 50 volume sample dilution. 1:50 dilutions of the assay standards and controls were diluted in the same manner.

100 ul of each diluted sample, control or standard was added to the appropriate microwell(s). The wells were allowed to incubate for 15 minutes at room temperature. To perform the rinse step the contents of PBS packet was added to 2 liters of reagent grade water, and the solution was mixed well until all the crystals were dissolved. Then the PBS buffer was used to wash the wells four times. The microwells were inverted between each wash to empty the fluid, after which the wells were drained and blotted on absorbent paper to remove residual wash fluid. Per the kit instructions, the wells were not allowed to dry-out between washes.

100 ul of working conjugated antibody (red solution) was added to each well, incubated for 15 minutes at room temperature, and then the wells were again washed four times with PBS solution.

The working substrate solution was prepared just before use, according to the kit instructions. Equal volumes of TMB Substrate Solution A and TMB Substrate Solution B were combined to form the color generating substrate. The kit instructed that if properly combined this substrate solution would be colorless and it was colorless. Next a 100 ul of the colorless working substrate solution was added to each well and the wells were incubated for ten minutes at room temperature.

Next 100 ul stop reagent was added to each well to end the enzyme reaction, and the O.D. of each well was read at 450 nm against a water blank. A standard curve graph was generated by plotting AU/ml of standards against O.D. of standards, and sample results were obtained from this standard curve.

Source	O.D.	AU/ml
Calibrator 1	.2	3.8
Calibrator 2	.3	7.5
Calibrator 3	.4	15.0
Calibrator 4	.57	30.0
Calibrator 5	.88	60.0
Calibrator 6	1.24	120.0

Refer to Graph 2

Controls	O.D.	AU/ml
high positive	1.11	104.0
moderate positive	.55	24.0
negative	.14	2.0

	O.D.	AU/ml
normal human sera #1	.14	2.0
normal human sera #2	.04	1.0
normal human sera #3	.18	3.0
SLE positive #1	.53	23.0
SLE positive #2	1.36	130.0
SLE positive #3	1.48	142.0

EXAMPLE 4
Accelerated Stability Study

DNA coated microwells were treated with S_1-nuclease using the optimized protocol, then duplicate microwells were stored at 37 degrees C and 30 degrees C, and each set of wells was tested at various time intervals using anti-dsDNA and anti-ssDNA monoclonal antibodies. The results of this experiment are shown in the following table:

Real Time In Days*	Equivalent Time In Months**	% ssDNA Activity
0	0.0	3-4%
1	1.6	3-4%
2	3.0	2-3%
5	7.5	3-4%
7	10.5	4-5%
9	13.5	3-4%
13	19.5	3-4%

*incubated at 37 degrees C

**if incubated at 4 degrees C

The dsDNA activity in these microwells remained unchanged during the accelerated stability study. The results obtained with the microwells stored at 30 degrees C were similar to those presented above and confirmed that the stability of the dsDNA coated on the microwells using this protocol was equivalent to more than one year without the generation of significant amounts of ssDNA.

EXAMPLE 5
Protocol for Pre-Coating Microwells with Purified Smith Antigen

Polystyrene wells were coated with Sm antigen by the following procedure:

1. Methylated Bovine Serum Albumin (Sigma) (hereinafter designated as mBSA) was dissolved at a ratio of 20 micrograms/ml in distilled water or PBS.

2. 100 microliters of the mBSA solution was placed in each microwell, and incubated overnight at 4 degrees C.

3. The excess coating solution was shaken from the plate after incubation. The wells were inverted to drain thoroughly.

4. 5 units/ml of purified Sm antigen was diluted in PBS, .01M, pH 7.3, and 100 microliters of the diluted Sm antigen solution was dispensed into each microwell and allowed to incubate for 18-24 hours at 4 degrees C.

5. Casein blocker solution (pH 7.3) was dispensed in 200 microliter increments into each well. The casein blocker solution consists of 2 ml glycerol, 10 g sucrose, 25 mg casein and sufficient TEN buffer to bring the volume to 100 ml.

6. The wells coated with the blocker solution were incubated overnight at 4 degrees C. After incubation, the solution was shaken from the wells and the wells were inverted for 15 minutes. Then the wells were uprighted and allowed to dry 24 hours at room temperature.

The coated Sm antigen wells were then used to determine the presence of anti-Sm antibody in seven CDC reference sera for anti-nuclear antibodies. The results show that only anti-Sm antibodies react in the assay.

Described hereinafter, is a method of performing an anti-Sm antibody assay, using the Sm antigen coated wells in a sandwich ELISA format.

1. Standards, seven reference sera samples, a negative human serum control

READS/ml, a high positive human serum control (READS/ml), a moderate positive human serum control (READS/ml), and a low positive human serum control (READS/ml) were diluted with Sample Diluent in a 1:50 ratio; 1 part serum to 50 parts Sample Diluent. The Sample Diluent was a solution consisting of 100 mls of native bovine serum in a phosphate buffered saline consisting of 1.42 g of potassium phosphate (dibasic), .26 g potassium phosphate (monobasic), 8.6 g sodium chloride, .1% sodium azide, 1 ml stock green dye, and distilled water added to bring the volume to 1000 mls. The Sample Diluent was then filtered through a .2 micron filter.

2. 100 microliters of the Sample Diluent solutions were placed in the designated wells at room temperature for 15 minutes to allow completion of the antigen-antibody binding process. Following incubation, the wells were emptied and washed four times with PBS solution.

3. The wells were exposed to a working conjugated antibody solution consisting of 1 part per volume horseradish peroxidase conjugated IgG and IgM specific antibodies, and 4000 and 1500 part per volume conjugate diluent, respectively, (Medix) consisting of phosphate buffer, protein stabilizer, and .02% thimerosal, to which was added .01% by volume a protease inhibitor (commercially available from Miles Pentex).

4. 100 microliter of the working conjugated antibody solution was placed in each microtiter well and allowed to incubate at room temperature for 15 minutes. After incubation, the wells were rinsed four times with PBS solution to remove unbound conjugated antibodies.

5. Each well was assayed for horseradish peroxidase activity by mixing equal volumes (3,3',5,5') Tetramethylbenzidine (Kirkegaard Perry) and hydrogen peroxide solution and dispensing 100 microliters of this substrate solution into each well. The presence of the anti-Sm antibodies was detected by a blue color appearing after the 10 minute room temperature incubation. 100 microliters per well of 2.5 N sulfuric acid terminated the reaction producing a yellow color. The yellow color was quantitated at 450 nm using a Dynatech MR600 plate reading spectrophotometer.

Results are calculated by the following procedure:

1. Calculate the mean absorbance values for the duplicates of the controls. The duplicate absorbance values of the positive control should be within 10% of the mean

absorbance value.

2. The mean absorbance reading of the reagent blank should be less than 0.05. Readings greater than 0.05 may indicate possible contaminated of the Substrate Solution.

3. Calculate the cut-off absorbance values for the assay, and write them in the appropriate blanks on the results graph.

   a. The mean absorbance (O.D.) of negative control x 3.2 = negative cut-off absorbance, which is equal to 20 RE^ADS units of anti-Sm activity. Samples with O.D. values below the cut-off have less than 20 RE^ADS units of anti-Sm activity, and should be scored as "negative". Samples with O.D. values equal to or higher than the cut-off have greater than 20 RE^ADS units anti-Sm activity, and should be scored as "positive". Samples with O.D. values close to the cut-off may warrant further testing (see Figure 4).

The results were calculated by determining a cut-off O.D. The cut-off O.D. was determined by running 200 normal sera samples and taking the mean of the O.D.s plus three standard deviations. This cut-off was equal to 3.2 x the O.D. of the standardized negative control.

Detection of anti-Sm antibodies (IgG & IgM)

Sample	O.D.	Status
normal cut-off	0.112	
low positive serum	0.21	positive
medium positive serum	0.40	positive
high positive serum	0.89	positive
anti-Sm	1.22	positive
anti-RNP	0.112	negative
anti-SS-A	0.02	negative
anti-SS-B	0.04	negative
anti-Scl-70	0.06	negative
anti-nucleolus	0.04	negative

EXAMPLE 6
A Diagnostic Test Kit Assay Procedure

The kit contains pre-coated microwells (qualitative format) and,

1 vial (40 ml) of Sample Diluent (green solution containing 0.1% sodium azide);

1 vial (100 ul) of high positive human serum control (about 20-28 READS/ml),

1 vial (100 ul) of calibrator (optional),

1 vial (100 ul) of negative human serum control (about 6.2 READS/ml). The negative cut-off value is 20 READS units. To calculate O.D.s into READS units, the negative value 6.2 is divided by the mean value O.D. it produces; the resultant number is multiplied by the O.D.s of the samples to give READS units.

1 vial (12 ml) Conjugated Antibody Working Solution - containing horseradish peroxidase conjugated anti-human IgG and IgM,

1 bottle (8 ml) of TMB Substrate; solution A containing 3,3',5,5', tetramethylbenzidine,

1 bottle (8 ml) of TMB Substrate; Solution B containing hydrogen peroxide. When mixed with equal parts Solution A will form a substrate capable of generating a colored product.

1 bottle (12 ml) of Stop Reagent containing 2.5 N H_2SO_4 (1 N HCL can be substituted) (not necessarily supplied in kit),

1 packet of Phosphate Buffered Saline (PBS) which reconstitutes to 2 liters of 0.01 M PBS, pH 7.3, which is utilized as a wash solution

Plate Template.

The various reagents of the kit were utilized to perform the assay. The directions for the method of assaying for anti-Sm antibodies were included in the kit and these directions were followed precisely during this experiment.

The plate templates were labelled for sample placement in the microwells. A 1:50 dilution of the controls and patient samples was prepared in Sample Diluent (green solution) (no calibrators were used in this example). 10 ul of sample was added to 490 ul sample diluent in a one volume to 50 volume sample dilution.

100 ul of each diluted sample and control was added to the appropriate microwell(s). The wells were allowed to incubate for 15 minutes at room temperature. To perform the rinse step the contents of PBS packet was added to 2 liters of reagent grade water, and the solution was mixed well until all the crystals were dissolved. Then the PBS buffer was used to wash the wells four times. The microwells were inverted between each wash to empty the fluid, after which the wells were drained and blotted on absorbent paper to remove residual wash fluid. Per the kit instructions, the wells were not allowed to dry-out between washes.

100 ul of working conjugated antibody (red solution) was added to each well, incubated for 15 minutes at room temperature, and then the wells were again washed four times with PBS solution.

The working substrate solution was prepared just before use, according to the kit instructions. Equal volumes of TMB Substrate Solution A and TMB Substrate Solution B were combined to form the color generating substrate. The kit instructed that if properly combined this substrate solution would be colorless and it was colorless. Next a 100 ul of the colorless working substrate solution was added to each well and the wells were incubated for ten minutes at room temperature.

Next 100 ul stop reagent was added to each well to end the enzyme reaction, and the O.D. of each well was read at 450 nm against a water blank. The final resulting data can be read as O.D.s. To determine if the sample is positive for anti-Sm antibodies, the sample O.D. is compared to the cut-off O.D. value calculated for the assay (see Figure 4). For semi-quantitative results the O.D. value can be converted to RE&DS units by multiplying the sample O.D. by the quantity (6.2 RE&DS units/negative control O.D.). 6.2 RE&DS units is the anti-Sm activity of the negative control, which was standardized against the CDC reference serum. The cut-off is 20 RE&DS units.

SLE Sera Samples	O.D.	READS Units
1	0.16	16.5
2	0.10	10.3
3	0.07	7.2
4	0.09	9.3
5	0.12	12.4
6	0.10	10.3
7	0.12	12.4
8	0.08	8.2
9	0.09	9.3
10	0.12	12.4
11	0.19	19.6
12	0.18	18.5
13	0.27	27.8*
14	0.35	36.0*
15	0.29	29.9*
16	1.17	121.0*
17	0.15	15.4
18	0.35	36.0*
19	0.13	13.4

*positive result

EXAMPLE 7

Adsorption of anti-Sm antibodies From Serum by the Test Kit Invention and a Competitive Kit

To demonstrate the sensitivity of the anti-Sm test kit samples from SLE patients were run in a competitive kit and were run in the test kit in accordance with the present invention under the protocol demonstrated in Example 6, with the exception that only the negative serum was used to produce a cut-off level. The test was run as a qualitative test showing only negative or positive results. 19%-40% of SLE patients are expected to have anti-Sm antibodies present. With all the included samples (the data is only representative of a portion of the data), approximately 28% of the sera samples were positive according to the present invention and only 21% were positive according to the competitive kit. Note the present invention detects both IgG and IgM and the competitive kit only detects IgG.

The resulting O.D.s were obtained. The cut-off value for the present invention was 0.33 O.D. The cut-off value of the competitive kit was the given by the kit instructions.

SLE Sera Samples	O.D. From Invention	Status	O.D. From Competitive Kit	Status
1	0.59	positive	.94	positive
2	0.15	negative	.05	negative
3	0.36	positive	.12	negative
4	0.19	negative	.08	negative
5	0.48	positive	.61	positive
6	0.29	negative	.13	negative
7	0.24	negative	.07	negative
8	0.21	negative	.08	negative
9	0.21	negative	.22	negative
10	0.19	negative	.08	negative
11	0.25	negative	.10	negative
12	0.19	negative	.13	negative
13	0.28	negative	.14	negative
14	0.29	negative	.16	negative
15	0.08	negative	.05	negative
16	0.86	positive	1.10	positive

EXAMPLE 8
Accelerated Stability Study

Sm antigen coated microwells were treated with the optimized protocol, then duplicate microwells were stored at 37 degrees C and 4 degrees C, and each set of wells was tested on days 1, 4, 6, 8, and 12, using a high positive anti-Sm sera, a moderate positive anti-Sm sera, and a negative anti-Sm sera. The results of this experiment are shown in the following table:

	O.D.s Day 1	O.D.s Day 4	O.D.s Day 6	O.D.s Day 8	O.D.s Day 12
High anti-Sm activity					
4°	1.12	1.14	1.12	1.23	1.00
37°C (high)	1.15	1.05	1.08	1.09	1.12
Moderate* anti-Sm activity					
4°	0.92	0.93	0.78	0.93	0.95
37°	0.94	0.92	0.86	0.90	0.80
Low anti-Sm activity					
4° (negative)	0.07	0.07	0.05	0.06	0.05
37°	0.06	0.09	0.05	0.05	0.05

*(1:2 dilution of the high anti-Sm sera)

The Sm antigen activity in these microwells remained unchanged during the accelerated stability study. The results obtained with the microwells stored at 37 degrees C were similar to those presented above and confirmed that the stability of the Sm antigen coated on the microwells using this protocol was equivalent to more than one year shelf life.

EXAMPLE 9

Protocol for Pre-Coating Microwells with Purified Sm/RNP antigen

Polystyrene wells were coated with Sm/RNP antigen by the following procedure:

1. Methylated Bovine Serum Albumin (Sigma) (hereinafter designated as mBSA) was dissolved at a ratio of 20 micrograms/ml in distilled water or PBS.

2. 100 microliters of the mBSA solution was placed in each microwell, and incubated overnight at 4 degrees C.

3. The excess coating solution was shaken from the plate after incubation. The wells were inverted to drain thoroughly.

4. 1 unit/ml of purified Sm/RNP antigen was diluted in PBS, .01M, pH 7.3, and 100 microliters of the diluted Sm/RNP antigen solution was dispensed into each microwell and allowed to incubate for 18-24 hours at 4 degrees C.

5. Casein blocker solution (pH 7.3) was dispensed in 200 microliter increments into each well. The casein blocker solution consists of 2 ml glycerol, 10 g sucrose, 25 mg casein and sufficient TEN buffer to bring the volume to 100 ml.

6. The wells coated with the blocker solution were incubated overnight at 4 degrees C. After incubation, the solution was shaken from the wells and the wells were inverted for 15 minutes. Then the wells were uprighted and allowed to dry 24 hours at room temperature.

The coated Sm/RNP antigen wells were then used to determine the presence of anti-Sm/RNP antibody in serum samples obtained from individuals with Progressive Systemic Sclerosis (PSS).

Described hereinafter, is a method of performing an anti-Sm/RNP antibody assay, using the Sm/RNP antigen coated wells in a sandwich ELISA format.

1. Sera from patients with PSS, a high positive human serum control (READS/ml), and a negative human serum control (READS/ml) were diluted with Sample Diluent in a 1:50 ratio; 1 part serum to 50 parts Sample Diluent. The Sample Diluent was a solution consisting of 100 mls of native bovine serum in a phosphate buffered saline consisting of 1.42 g of potassium phosphate (dibasic), .26 g potassium phosphate (monobasic), 8.6 g sodium chloride, .1% sodium azide, 1 ml stock green dye, and distilled water added to bring the volume to 1000 mls. The Sample Diluent was then filtered through a .2 micron filter.

2. 100 microliters of the Sample Diluent solutions were placed in the designated wells at room temperature for 15 minutes to allow completion of the antigen-antibody binding process. Following incubation, the wells were emptied and washed four times with PBS solution.

3. The wells were exposed to a working conjugated antibody solution consisting of 1 part per volume horseradish peroxidase conjugated IgG and IgM specific antibodies, and 4000 and 1500 part per volume conjugate diluent, respectively, (Medix) consisting of phosphate buffer, protein stabilizer, and .02% thimerosal, to which was added .01% by volume a protease inhibitor (commercially available from Miles Pentex).

4. 100 microliter of the working conjugated antibody solution was placed in each microtiter well and allowed to incubate at room temperature for 15 minutes. After incubation, the wells were rinsed four times with PBS solution to remove unbound conjugated antibodies.

5. Each well was assayed for horseradish peroxidase activity by mixing equal volumes (3,3',5,5') Tetramethylbenzidine (Kirkegaard Perry) and hydrogen peroxide solution and dispensing 100 microliters of this substrate solution into each well. The presence of the anti-Sm/RNP antibodies was detected by a blue color appearing after the 10 minute room temperature incubation. 100 microliters per well of 2.5 N sulfuric acid terminated the reaction producing a yellow color. The yellow color was quantitated at 450 nm using a Dynatech MR600 plate reading spectrophotometer.

Results

1. Calculate the mean absorbance values for the duplicates of the controls. The

duplicate absorbance values of the positive control should be within 10% of the mean absorbance value.

2. The mean absorbance reading of the reagent blank should be less than 0.05. Readings greater than 0.05 may indicate possible contamination of the substrate solution.

3. Calculate the cut-off absorbance values for the assay:

   a. The mean absorbance (O.D.) of negative control x 2.8 = negative cut-off absorbance, which is equal to 17.5 RE^DS units of anti-SM/RNP activity. Samples with O.D. values below the cut-off have less than 17.5 RE^DS units of anti-SM/RNP activity, and should be scored as "negative". Samples with O.D. values equal to or higher than the cut-off have greater than 17.5 RE^DS units anti-SM/RNP activity, and should be scored as "positive". Samples with O.D. values very close to the cut-off may warrant further testing (see Figure 6).

The results were calculated by determining a cut-off O.D. The cut-off O.D. was determined by running 200 normal sera samples and taking the mean of the O.D.s plus three standard deviations. This cut-off was equal to 2.8 x the O.D. of the standardized negative control. The cut-off for the first 40 samples was 0.11 negative O.D. x 2.8 = 0.31 O.D. The cut-off for the second 40 samples was 0.09 x 2.8 = 0.25 O.D. See Figure 6.

Detection of anti-Sm/RNP antibodies (IgG & IgM)

(PSS) Sera Sample	O.D.	Status
1	0.14	negative
2	0.16	negative
3	0.17	negative
4	0.17	negative
5	0.09	negative
6	0.10	negative
7	0.15	negative
8	0.11	negative
9	0.19	negative
10	0.22	negative
11	0.17	negative
12	0.19	negative
13	0.18	negative
14	0.13	negative
15	0.17	negative
16	0.11	negative
17	0.15	negative
18	0.13	negative
19	0.15	negative
20	0.15	negative
21	0.22	negative
22	0.18	negative
23	0.15	negative
24	0.15	negative
25	0.60	positive
26	0.11	negative
27	0.16	negative
28	0.17	negative
29	0.13	negative
30	0.16	negative
31	0.18	negative
32	0.13	negative

(PSS) Sera Sample	O.D.	Status
33	0.20	negative
34	0.24	negative
35	0.24	negative
36	0.11	negative
37	0.19	negative
38	0.28	negative
39	0.12	negative
40	0.10	negative
41	0.18	negative
42	0.19	negative
43	0.14	negative
44	0.16	negative
45	0.08	negative
46	0.18	negative
47	0.11	negative
48	0.38	positive
49	0.09	negative
50	0.25	positive
51	0.23	negative
52	0.19	negative
53	0.21	negative
54	0.27	positive
55	0.17	negative
56	0.15	negative
57	0.21	negative
58	1.23	positive
59	0.16	negative
60	0.33	positive
61	0.12	negative
62	0.11	negative
63	0.24	negative
64	0.11	negative
65	0.09	negative
66	0.22	negative
67	0.18	negative

(PSS) Sera Sample	O.D.	Status
68	0.47	positive
69	0.09	negative
70	0.14	negative
71	0.18	negative
72	0.13	negative
73	0.20	negative
74	0.10	negative
75	0.17	negative
76	0.22	negative
77	0.07	negative
78	0.10	negative
79	0.17	negative
80	0.15	negative

EXAMPLE 10
A Diagnostic Test Kit Assay Procedure
(Qualitative Format)

The kit contains pre-coated microwells and,

1 vial (40 ml) of Sample Diluent (green solution containing 0.1% sodium azide);

1 vial (100 ul) of high positive human serum control (about 100 REâDS/ml),

1 vial (100 ul) of calibrator (optional),

1 vial (100 ul) of negative human serum control (about 6.3 REâDS/ml). The negative cut-off value is 17.5 REâDS units. To calculate O.D.s into REâDS units, the negative value 6.3 is divided by the negative O.D. mean value. The resultant number is multiplied by the O.D.s of the samples to give REâDS units.

1 vial (12 ml) Conjugated Antibody Working Solution - containing horseradish peroxidase conjugated anti-human IgG and IgM,

1 bottle (8 ml) of TMB Substrate; solution A containing 3,3',5,5', tetramethylbenzidine,

1 bottle (8 ml) of TMB Substrate; Solution B containing hydrogen peroxide. When mixed with equal parts Solution A will form a substrate capable of generating a colored product.

1 bottle (12 ml) of Stop Reagent containing 2.5 N H2SO4 (1 N HCL can be substituted),

1 packet of Phosphate Buffered Saline (PBS) which reconstitutes to 2 liters of 0.01 M PBS, pH 7.3, which is utilized as a wash solution

Plate Template.

The various reagents of the kit were utilized to perform the assay. The directions for the method of assaying for anti-Sm/RNP antibodies were included in the kit and these directions were followed precisely during this experiment.

The plate templates were labelled for sample placement in the microwells. A 1:50 dilution of the controls and SLE patient samples was prepared in Sample Diluent (green solution). 10 ul of sample was added to 500 ul sample diluent in a one volume to 50 volume sample dilution.

100 ul of each diluted sample and controls was added to the appropriate microwell(s). The wells were allowed to incubate for 15 minutes at room temperature. To perform the rinse step the contents of PBS packet was added to 2 liters of reagent grade water, and the solution was mixed well until all the crystals were dissolved. Then the PBS buffer was used to wash the wells four times. The microwells were inverted between each wash to empty the fluid, after which the wells were drained and blotted on absorbent paper to remove residual wash fluid. Per the kit instructions, the wells were not allowed to dry-out between washes.

100 ul of working conjugated antibody (red solution) was added to each well, incubated for 15 minutes at room temperature, and then the wells were again washed four times with PBS solution.

The working substrate solution was prepared just before use, according to the kit instructions. Equal volumes of TMB Substrate Solution A and TMB Substrate Solution B were combined to form the color generating substrate. The kit instructed that if properly combined this substrate solution would be colorless and it was colorless. Next a 100 ul of the colorless working substrate solution was added to each well and the wells were incubated for ten minutes at room temperature.

Next 100 ul stop reagent was added to each well to end the enzyme reaction, and the O.D. of each well was read at 450 nm against a water blank. For semi-quantitative results the conversion factor to convert O.D. of the sample to RE&DS units was the quantity (6.3/mean O.D. of negative control). 6.3 RE&DS units is the anti-Sm/RNP activity of the negative control, which was standardized against the CDC reference serum. In this example the conversion factor was 6.3/.0875 = 72.

Controls	RE&DS/ml
cut-off	17.5
negative	6.3

SLE Patient Sera	O.D.	READS Units
1	0.24	17.3
2	0.18	12.6
3	0.19	13.7
4	0.11	7.9
5	0.66	48.2*
6	0.15	11.2
7	0.16	11.5
8	0.84	60.5*
9	0.01	0.36
10	0.44	32.0*
11	0.31	22.3*
12	0.53	38.2*
13	1.03	74.2*
14	0.31	22.3*
15	1.49	107.0*
16	0.52	37.1*
17	1.42	102.0*
18	0.53	38.2*

*positive results

EXAMPLE 11
Adsorption of anti-Sm/RNP antibodies From Serum

To demonstrate the sensitivity of this assay for anti-SM and anti-RNP, the test kit was used in accordance with the protocol shown in Example 10. Nine CDC reference for nuclear antigen were tested. All were negative except for the anti-Sm and anti-RNP sera which were positive.

Sample	O.D.	Status
normal cut-off	0.12	
anti-Sm	0.98	positive
anti-RNP	0.56	positive
anti-SS-A	0.03	negative
anti-SS-B	0.06	negative
anti-Scl-70	0.08	negative
anti-nucleolus	0.06	negative
anti-centromere	0.06	negative
anti-dsDNA monoclonal	0.01	negative
anti-ssDNA monoclonal	0.01	negative

EXAMPLE 12
Accelerated Stability Study

Sm/RNP antigen coated microwells were treated with the optimized protocol, then duplicate microwells were stored at 37 degrees C and 4 degrees C, and each set of wells was tested at on day 1, 4, 6, 8, and 12, using a high anti-Sm/RNP sera sample, a moderate anti-Sm/RNP sera sample, and a negative anti-Sm/RNP sera sample. The results of this experiment are shown in the following table:

		O.D.s Day 1	O.D.s Day 4	O.D.s Day 6	O.D.s Day 8	O.D.s Day 12
ES 22	4°	1.39	1.26	1.35	1.42	1.33
(high)	37°	1.36	1.20	1.34	1.40	1.26
ES 22 (1:2)	4°	1.09	1.08	1.13	1.17	1.00
(moderate)	37°	1.08	1.01	1.07	1.16	1.06
negative	4°	0.10	0.10	0.07	0.07	0.09
(low)	37°	0.09	0.08	0.08	0.08	0.08

The significant Sm/RNP antigen activity in these microwells remained unchanged during the accelerated stability study. The Sm/RNP antigen coated on the microwells using this protocol was equivalent to more than one year shelf life.

The foregoing examples serve to illustrate the efficiency and utility of the methylated BSA to provide a coating which inhibits non-specific binding and provides a coating capable of affixing antigen evenly over the solid support. Without being bound to the specific quantities given in the definitional section, it is possible to utilize a wide latitude of concentrations of mBSA or other similar, functionally equivalent substitutes which have the capability to evenly attach antigen to the support solid by providing a charged surface or by any other like mechanism to affix the and to continue to inhibit non-specific binding.

Likewise, the blocker utilized in the preferred embodiment of this invention to stabilize the shelf life and eliminate non-specific binding cannot be limited to the compounds or the

ingredients or the concentrations thereof listed in the definitional section. A variety of functional equivalent blocking agents are known to those skilled in the art. A partial listing of some materials which could be utilized to perform a similar function is found in (Robert F. Bogt; J. Immunological Methods, 101, 43-50 [1987]) and is hereby incorporated herein by reference. A third element in the anti-dsDNA test kit which plays a function in eliminating non-specific binding is the S_1 nuclease used to degrade the ssDNA and thus to avoid any cross reactivity between the anti-dsDNA and the undesired ssDNA. The described method of limiting cross reactivity is not limited to the definition given but the method can be performed with varying concentrations of S_1 nuclease or other similar, endonuclease enzymes, or similar functional equivalents which are capable of eliminating problems of cross reactivity without adversely affecting the antigen present in the invention.

The treatment of the solid support which can be any of a variety of formats, i.e. test tubes, plates, wells, etc., made of various suitable materials, i.e. glass, plastics, etc. with the aforementioned technology affords many important and useful approaches to the detection of the specific antibodies. The detection of antibodies need not be limited to the conjugation of enzymes. Addition of fluorescent chemicals such as fluorescence or the like to the antibody will impart fluorescence to the assay if the antibody is present. Similarly, conjugation of the antibody with a radionuclide will impart radioactivity to the assay if the antibodies are present in the assay. Many other methods of detection of antibodies also exist, and these methods will yield positive results provided that the antibody exists in the assayed sera and is affixed according to the methods described herein.

The test kit and the underlying coating and detection methods herebefore described are not intended to be limited by the assay format described or by the volumes or the concentrations or specific ingredients given for the various reagents, controls, and calibrators. It should be understood that similar chemical equivalents or other functional equivalents of the components found in the coatings, or in any of the various reagents, controls, and calibrators can be utilized within the scope of this invention.

It is contemplated that the inventive concepts herein described may have differing embodiments and it is intended that the appended claims be construed to include all such alternative embodiments of the invention except insofar as they are limited by the prior art.

CLAIMS

1. A method for the detection of anti-double strand DNA in sera or plasma comprising the steps of:
providing a solid support capable of receiving a coating thereon,
coating said solid support with methylated Bovine serum albumin,
attaching native DNA to said coated support which native DNA has an affinity for the attachment thereto of specific anti-dsDNA,
contacting said coated support having native DNA attached thereto with sera or plasma to be tested for the presence therein of anti-dsDNA and subsequently detecting the attachment of anti-dsDNA to the said coating on said support after contacting said support with sera or plasma to be tested.

2. The method of Claim 1 wherein said coated support containing native DNA or antigen is contacted with an endonuclease prior to being contacted with sera or plasma.

3. The method of Claim 2 wherein said endonuclease is S_1 nuclease.

4. The method of Claim 3 wherein said coated support after being contacted with S_1 nuclease is then contacted with a hydrolyzed casein.

5. A diagnostic test kit comprising a coated solid support capable of selectively attaching and immobilizing anti-dsDNA present in sera or plasma for subsequent detection containing:
solid support means, coating means for forming a coating on said solid support means, said coating means including methylated Bovine serum albumin having adherent thereon a DNA antigen specific for binding an anti-dsDNA, said coating means in addition being treated after dsDNA attachment with an enzyme capable of degrading any ssDNA attached to said coated support without affecting the attached dsDNA, and indication means for providing an indication of the presence of attached anti-dsDNA on said coating.

6. A method for the detection of anti double strand DNA, or anti-Sm antibodies or anti-Sm/RNP antibodies in sera or plasma comprising the steps of:
providing a solid support capable of receiving a coating thereon,
coating said solid support with methylated Bovine serum albumin,
attaching native DNA antigen or anti-Sm antibodies, or anti-Sm/RNP antibodies to said

coated support which native DNA antigen or Sm antigen or Sm/RNP antigen has an affinity for the attachment thereto of specific anti-dsDNA antibodies, or anti-Sm antibodies, or anti-Sm/RNP antibodies contacting said coated support having native DNA or anti-Sm antibodies or anti-Sm/RNP antibodies attached thereto with sera or plasma to be tested for the presence therein of anti-dsDNA and subsequently detecting the attachment of anti-dsDNA to the said coating on said support after contacting said support with sera or plasma to be tested.

7. A diagnostic test kit comprising a coated solid support capable of selectively attaching and immobilizing a specific autoimmune antibody present in sera or plasma for subsequent detection containing:
solid support means, coating means for forming a coating on said solid support means, said coating means including methylated Bovine serum albumin having attached thereon an autoimmune antigen specific for binding an autoimmune antibody, said coating means in addition being treated after autoimmune antibody attachment with a casein blocker capable of reducing non-specific binding to said solid support without adversely affecting the attached autoimmune antigen, and indication means for providing an indication of the presence of attached autoimmune antibody on said coating.

8. A method for the detection of anti-Sm/RNP antibodies in sera or plasma comprising the steps of:
- providing a solid support capable of receiving a coating thereon,
- coating said solid support with methylated Bovine serum albumin,
- attached Sm/RNP antigen to said coated support with Sm/RNP antigen has an affinity for the attachment thereto of specific anti-Sm/RNP antibody,
- contacting said coated support having Sm/RNP antigen attached thereto with sera or plasma to be tested for the presence therein of anti-Sm/RNP antibody and subsequently detecting the attachment of anti-Sm/RNP antibody to said coating on said support.

9. The method of Claim 8 wherein said coated support is contacted with a hydrolyzed casein.

10. A diagnostic assay kit comprising a coated solid support capable of selectively attaching and immobilizing IgG and IgM anti-Sm/RNP present in sera or plasma for subsequent detection containing:

solid support means, coating means for forming a coating on said solid support means, said coating means including methylated Bovine serum albumin having attached thereon an Sm/RNP antigen specific for binding an anti-Sm/RNP antibody, said coating means further being treated after Sm/RNP attachment with rinsing procedure capable of removing any unattached Sm/RNP antigen without affecting the attached Sm/RNP antigen and indication means for providing an indication of the presence of attached anti-Sm/RNP antigen said coating.

11. A method for the detection of anti-Sm antibodies in sera or plasma comprising the steps of:

- providing a solid support capable of receiving a coating thereon,
- coating said solid support with methylated Bovine serum albumin,
- attaching Sm antigen to said coated support which Sm antigen has an affinity for the attachment thereto of specific anti-Sm antibody,
- contacting said coated support having Sm antigen attached thereto with sera or plasma to be tested for the presence therein of anti-Sm antibody and subsequently detecting the attachment of anti-Sm antibody to the said coating on said support.

12. The method of Claim 11 wherein said coated support is further coated with a hydrolyzed casein.

13. A diagnostic assay kit comprising a coated solid support capable of selectively attaching and immobilizing IgG and IgM anti-Sm present in sera or plasma for subsequent detection containing:
solid support means, coating means for forming a coating on said solid support means, said coating means including methylated Bovine serum albumin having attached thereon an Sm antigen specific for binding an anti-Sm antibody, said coating means further to being treated after Sm attachment with rinsing procedure capable of removing any unattached Sm antigen without affecting the attached Sm antigen, and indication means for providing an indication of the presence of attached anti-Sm antigen on said coating.

14. A method for the detection of an antibody generated by the human immune system in sera or plasma comprising the steps of:

- providing a solid support capable of receiving a coating thereon,
- coating said solid support with methylated Bovine serum albumin,
- attaching an antigen specific for a said antibody to said coated support which antigen has an affinity for the attachment thereto of specific human antibody,
- contacting said coated support having a said antigen attached thereto with sera or plasma to be tested for the presence therein of an antibody specific for a said antigen and subsequently detecting the attachment of a said antibody to said coating on the support.

15. A method according to Claim 14 wherein the antibody for whose presence is tested is a member selected from the group consisting of anti-dsDNA, anti-RNP, anti-DNP, anti-cardiolipin, anti-hesbone, anti-Sm, anti-ENA, anti-RNA, anti-Scl-70 and anti-SS-A and B.

FIG. 1.

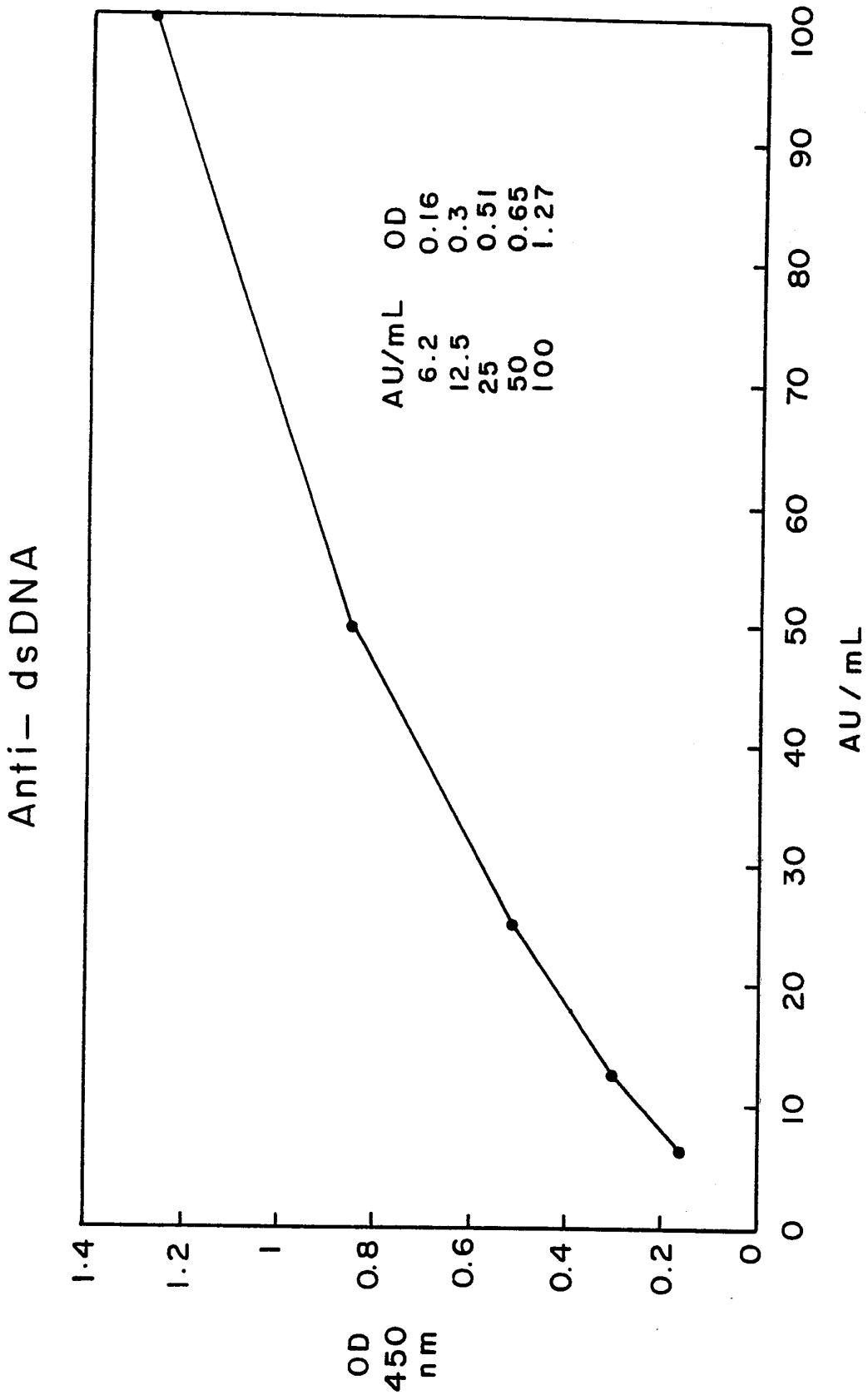
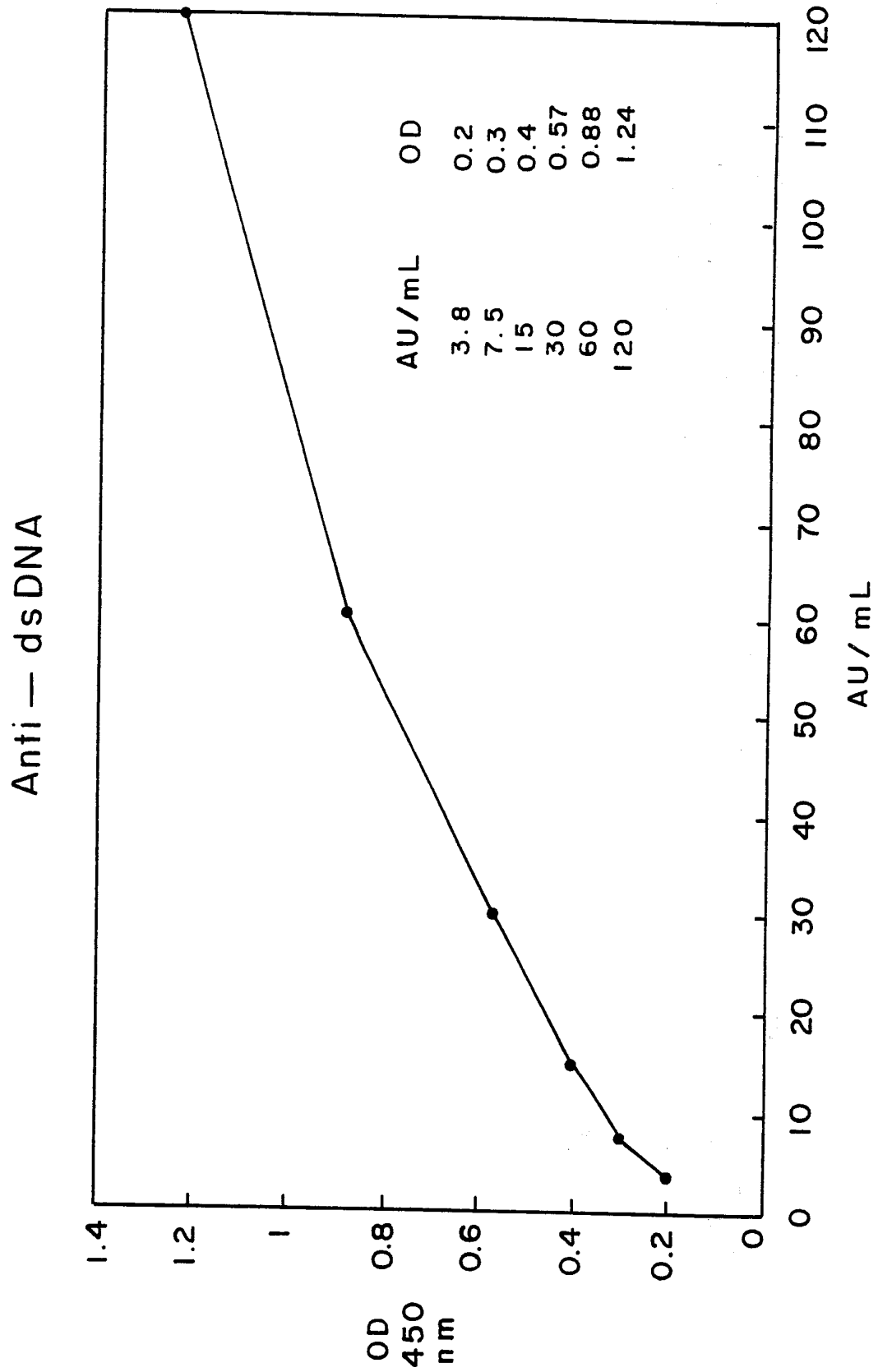

Anti- dsDNA
AU/mL
6.2
12.5
25
50
100
OD
0.16
0.3
0.51
0.65
1.27
OD 450 nm
0
0.2
0.4
0.6
0.8
1
1.2
1.4
0
10
20
30
40
50
60
70
80
90
100
AU / mL

FIG. 2.


Anti — dsDNA
OD 450 nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
AU/mL
0
10
20
30
40
50
60
70
80
90
100
110
120
AU/mL
3.8
7.5
15
30
60
120
OD
0.2
0.3
0.4
0.57
0.88
1.24

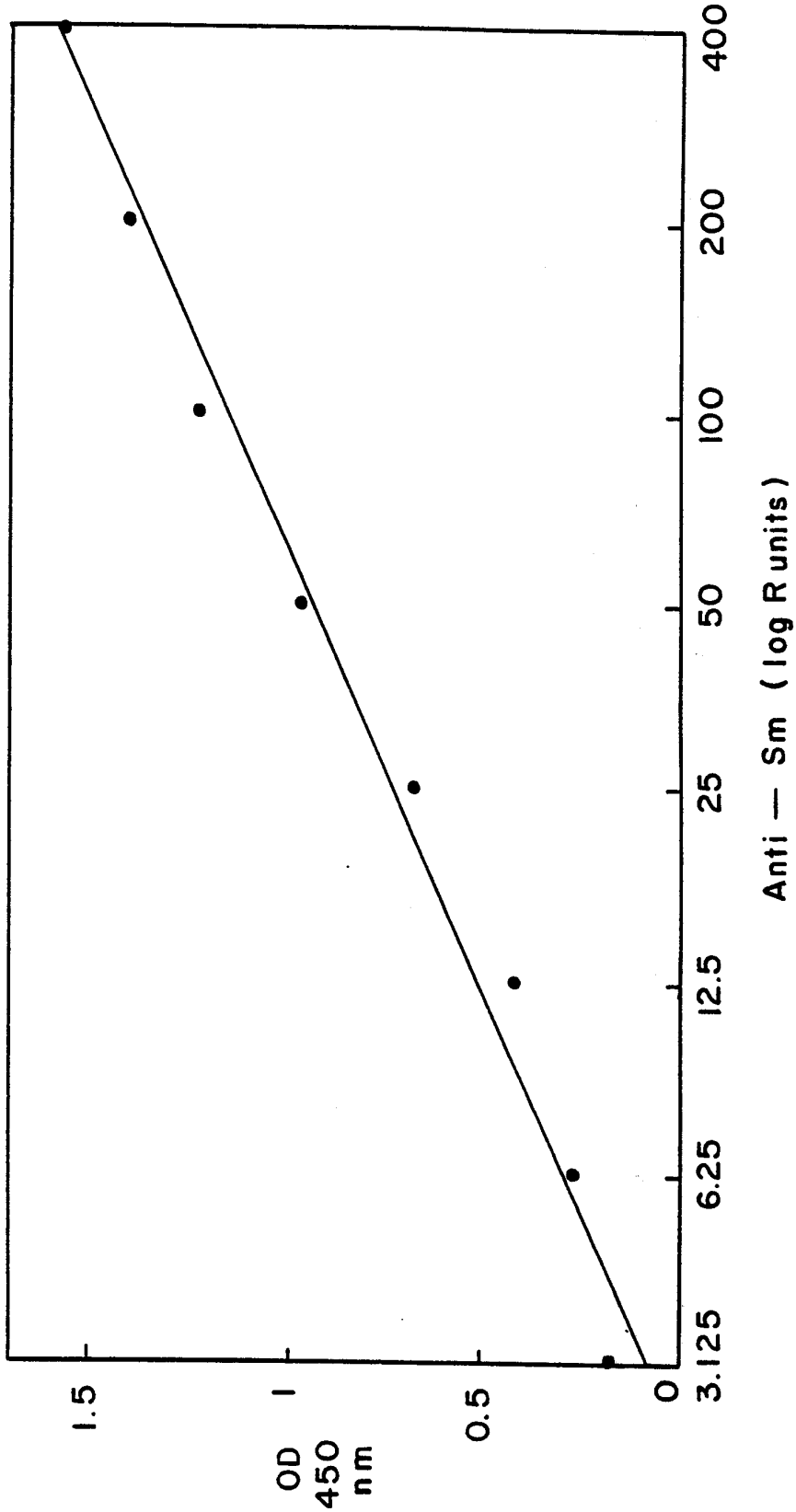
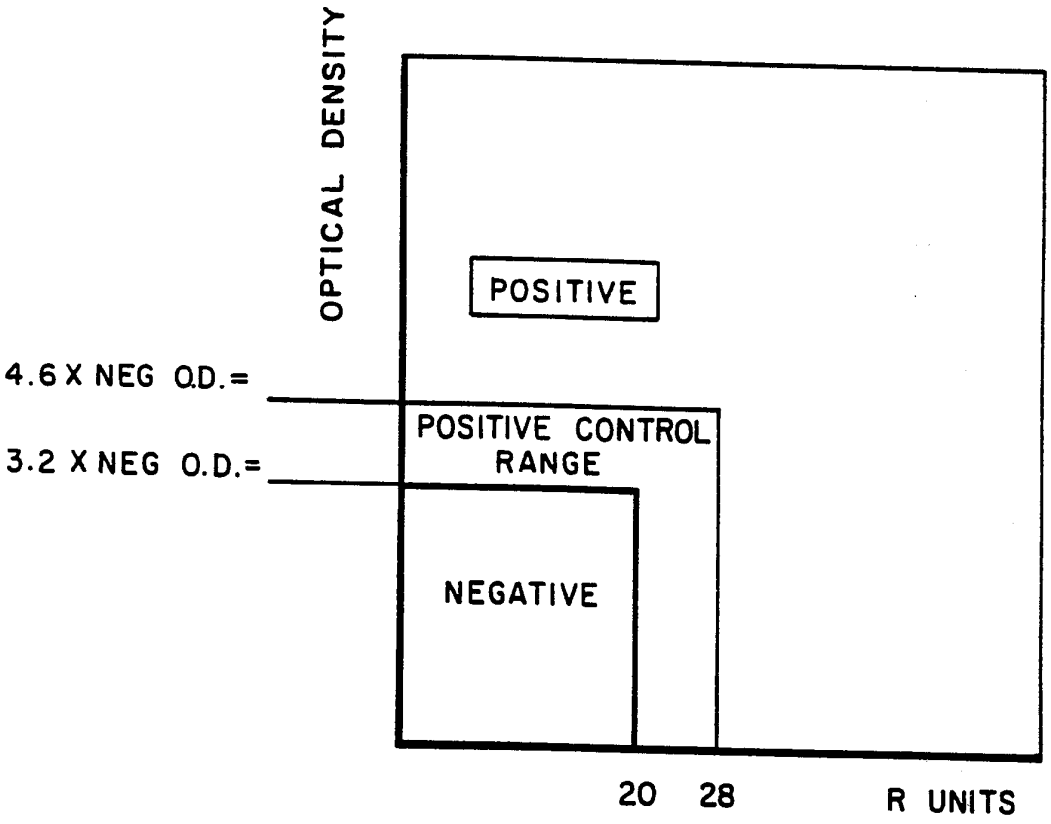

CDC STANDARD DILUTION CURVE
Anti — Sm
OD 450 nm
1.5
1
0.5
0
3.125
6.25
12.5
25
50
100
200
400
Anti — Sm (log R units)

FIG. 3.

FIG. 4.


OPTICAL DENSITY
POSITIVE
4.6 X NEG O.D.=
3.2 X NEG O.D.=
POSITIVE CONTROL RANGE
NEGATIVE
20
28
R UNITS

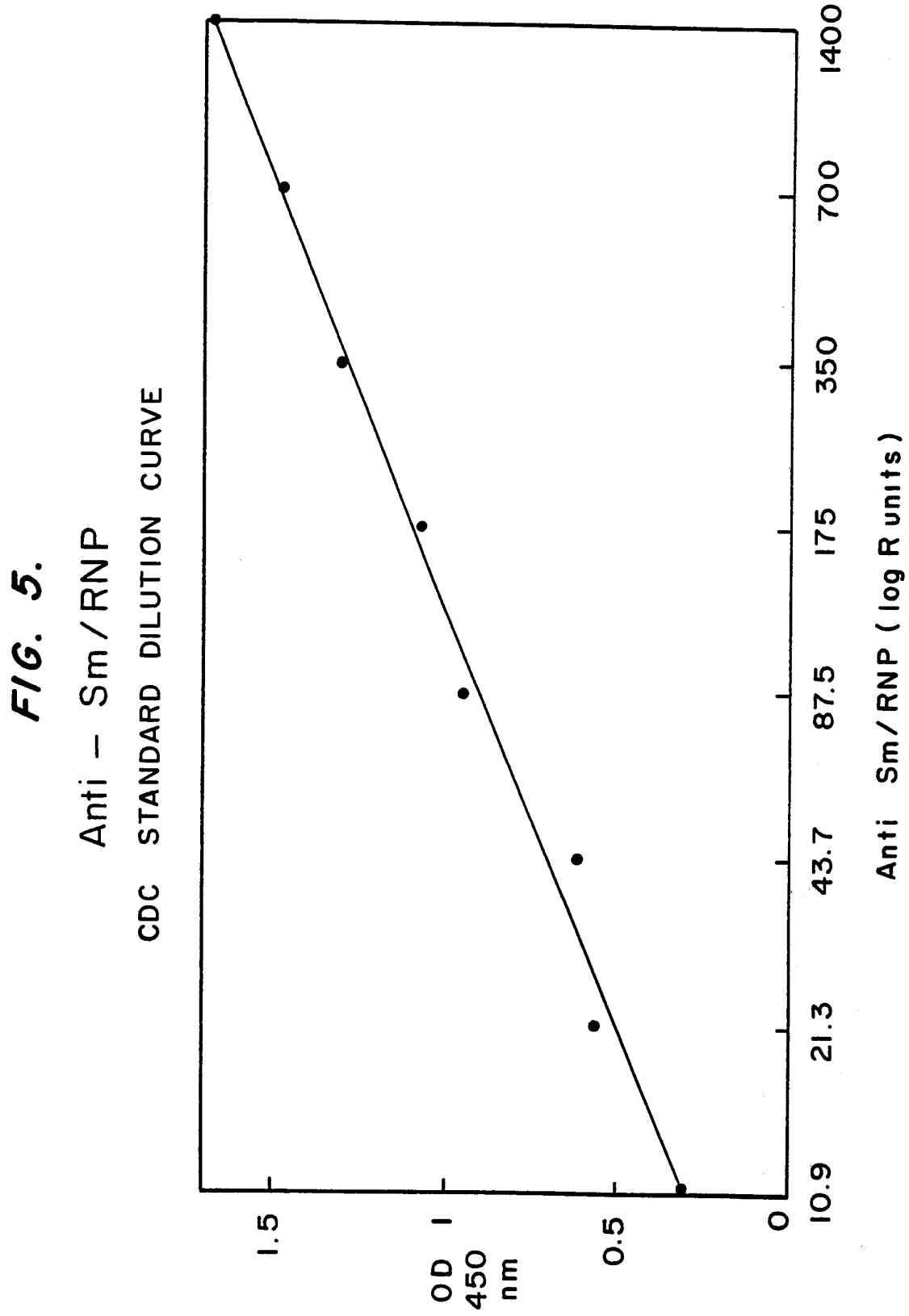
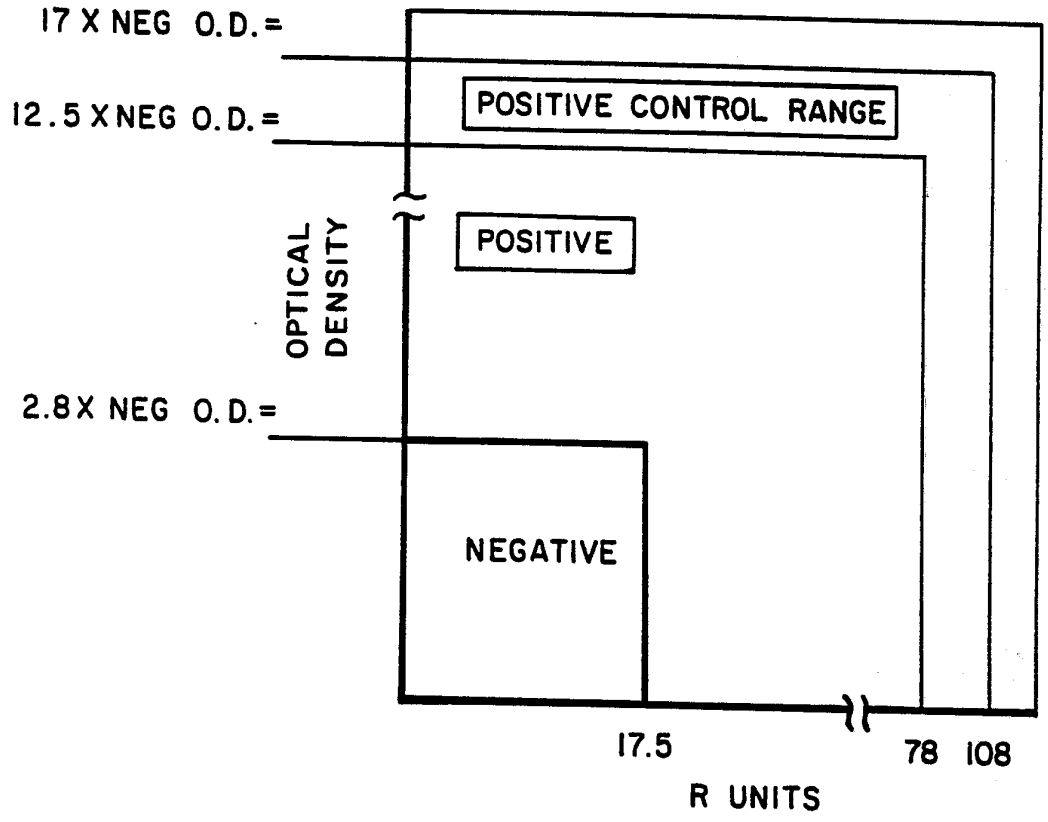


FIG. 5.
Anti – Sm/RNP
CDC STANDARD DILUTION CURVE
OD 1 450 nm
1.5
0.5
0
10.9
21.3
43.7
87.5
175
350
700
1400
Anti Sm/RNP (log R units)

FIG. 6.


17 X NEG O.D. =
12.5 X NEG O.D. =
2.8 X NEG O.D. =
OPTICAL DENSITY
POSITIVE CONTROL RANGE
POSITIVE
NEGATIVE
17.5
78
108
R UNITS

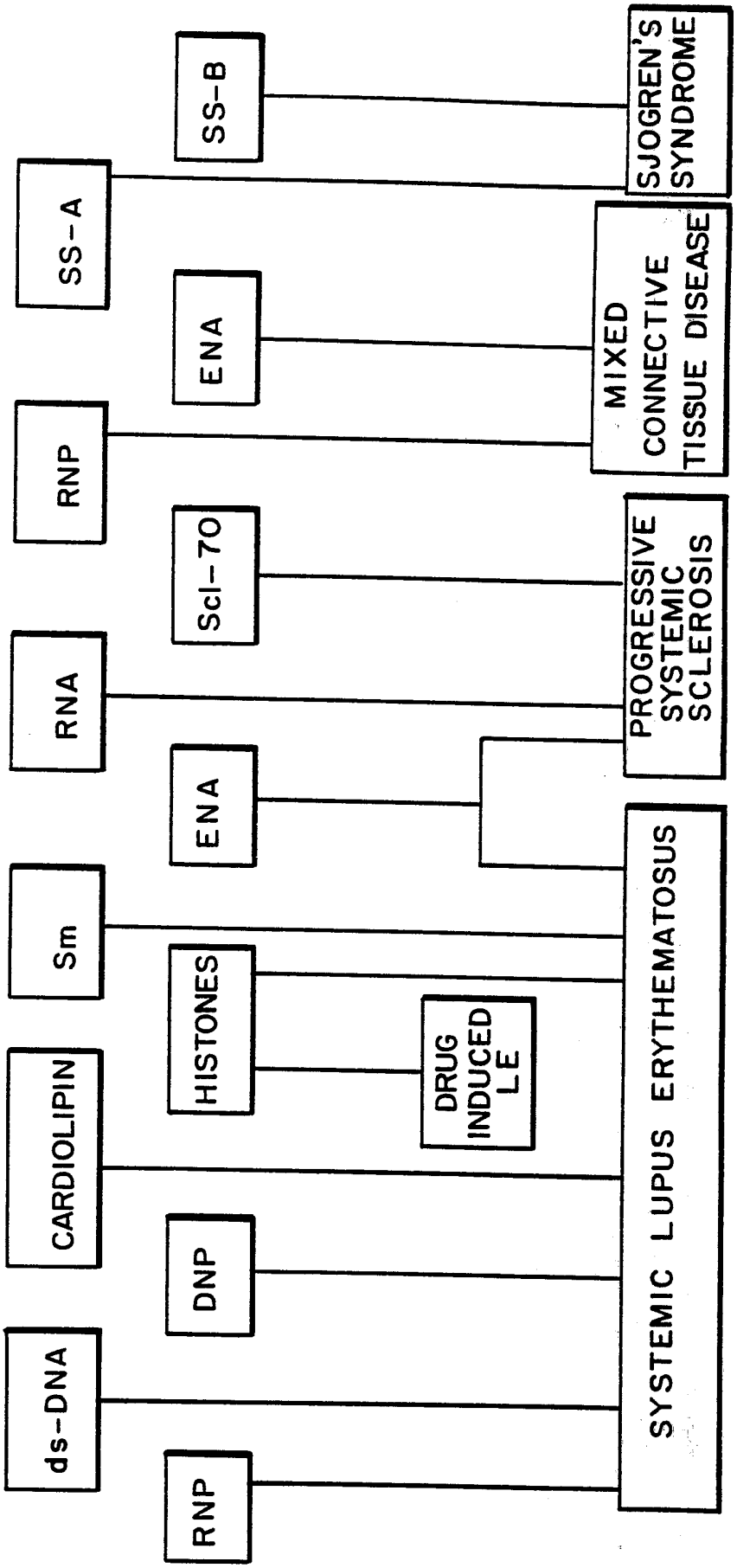

SS-B
SS-A
RNP
RNA
Sm
CARDIOLIPIN
ds-DNA
RNP
ENA
Scl-70
ENA
HISTONES
DNP
SJOGREN'S SYNDROME
MIXED CONNECTIVE TISSUE DISEASE
PROGRESSIVE SYSTEMIC SCLEROSIS
DRUG INDUCED LE
SYSTEMIC LUPUS ERYTHEMATOSUS

FIG. 7.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/01029

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): G01N 33/564, 33/543, 33/53, 5/00; C12Q, 1/68,
U.S.CL.: 436/506, 508, 518, 808, 811; 435/6,7, 240.27, 810; 530/387

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	436/506, 508, 518, 808, 811, 435/6,7, 240.27, 810 530/387

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [5]

Automated Patent Systems
Chemical Abstract Society

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	Journal of Immunological Methods, Volume 101 issued 1987, R.F. VOGT, JR. ET AL., "Quantitative differences among various proteins as blocking agents for ELISA microtier plates", 43-50. See the ABSTRACT.	1-15
Y	Journal of Immunological Methods, Volume 63, issued 1983, R.L. RUBIN ET AL. "An improved ELISA for anti-native DNA by elimination of interference by anti-histone antibodies" 359-366, see materials and methods section.	1-15
Y	Clinical and Experimental Rheumatology, Volume 5, issued 1987, A.G. TZIOUFAS, ET AL. "Enzyme immunoassays for the detection of Ig G and Ig M anti-ds DNA antibodies: clinical significance and specificity", 247-253, see entire document.	1-15
Y	Clinical and Experimental Immunology, Volume 62 issued 1985, S. LOIZOU, ET AL. "Measurement of anti-cardiolipin antibidies by an enzyme-linked immunosorbent assay (ELISA): standardization and quantitation of results", 738-745, see the abstract.	15

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
29 JUNE 1990	24 JUL 1990
International Searching Authority [1]	**Signature of Authorized Officer** [20]
ISA/US	KAREN I. KRUPEN

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	US, A, 4,738,932 (YABUSAKI, K.K.) 19 April 1988, see abstract and column 2, line 31 to column 4, line 25.	15
Y	US, A, 4,564,597 (LERNER, E.A. ET AL.) 14 January 1986, see the abstract.	1-15
Y	US, A, 4,784,942 (HARLEY, J.B.) 15 November 1988, see the abstract and column 9, line 30 through column 10, line 63.	1-15

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers....., because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.