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(54) Title: IMMUNOASSAY OF PROTEIN A UNDER ACIDIC CONDITIONS

(57) Abstract

The present invention features an improvement in a method for detecting protein A in an IgG-containing sample in a liquid medium, which method utilizes anti-protein A antibody. The improvement involves buffering the liquid medium, when the sample is contacted with the anti-protein A antibody for the purpose of promoting binding between the protein A in the sample and the protein A antibody, with a buffer to a pH at which protein A dissociates from the F_c region of the IgG.

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IMMUNOASSAY OF PROTEIN A UNDER ACIDIC CONDITIONS

Background of the Invention

The invention relates to immunoassays for protein A.

Assays capable of accurately measuring small amounts of protein A are useful in a number of contexts, including the measurement of protein A contaminants in immunoglobulin preparations. Accurate measurements are particularly crucial in the latter context because certain immunoglobulin preparations are useful as in vivo diagnostic or therapeutic agents, and any contaminating protein A may have undesirable effects in humans. Therefore, it may be important to test purified immunoglobulin preparations, particularly those purified with protein A as discussed below, for the presence of protein A using an assay that is sensitive to parts-per-million levels of contamination.

One class of immunoglobulins, immunoglobulin G (IgG) including such molecules as murine monoclonal antibodies, may be effectively isolated by affinity chromatography on immobilized protein A resins. This procedure, however, often results in contamination of the purified immunoglobulin by small amounts of protein A that have leached from the resin. Assays for protein A contaminants in these preparations may be complicated by the binding of protein A to the F_c region of mammalian IgG. This binding adversely affects conventional assays which are typically done using neutral to slightly basic pH values.

Conventional assays, such as those described in Fey et al. (1981) J. Immunological Methods, 47: 99-107, and Warnes et al. (1986) J. Immunological Methods, 93: 63-70, rely on solid-phase human IgG to capture soluble protein A, which binds to the antibody F_c domains. Unfortunately, excess antibody in the protein A sample seriously suppresses the response of these tests by competing with the solid-phase capture antibodies for binding to the F_c receptor domains of protein

A. These assays are therefore unsuitable for the measurement of protein A contamination of immunoglobulin preparations.

Other assays, described in Dertzbaugh et al. (1985) J. Immunological Methods, 83: 169-177, Lucas et al. (1988) J. Immunological Methods, 113: 113-122, and Bloom et al. (1989) J. Immunological Methods, 117: 83-89, utilize rabbit anti-protein A antiserum as the solid-phase capture reagent, and are more effective because a fraction of the capture antibodies bind to regions of protein A that lie outside the F_c receptor domains. Signal suppression can be a problem in such assays, lowering sensitivity, and, in some cases, for the accurate measurement of protein A in an immunoglobulin of interest, necessitating the inclusion of that immunoglobulin in the protein A solutions used to construct the assay standard curves. This process is inconvenient and subject to inaccuracy if the concentration of IgG in the standard solutions is not exactly the same as that of the IgG in the test sample.

Other assays for protein A, such as those described in Langone et al., J. Immunological Methods, (1983) 63: 145-157, utilize chicken anti-protein A, in which the F_c region does not bind significantly to protein A.

Summary of the Invention

The present invention features an improvement in a method for detecting protein A in an IgG-containing sample in a liquid medium, which method utilizes anti-protein A antibody. The improvement involves buffering the liquid medium, when the sample is contacted with the anti-protein A antibody for the purpose of promoting binding between the protein A in the sample and the anti-protein A antibody, with a buffer to a pH at which protein A dissociates from the F_c region of the IgG. In preferred embodiments, the pH is between 3.4 and 6.0, inclusive. In other preferred embodiments the buffer is an acetate buffer, a formate buffer or a citrate buffer. In preferred embodiments, the anti-protein A antibody is chicken anti-protein A antibody. In particular embodiments in which the sample contains murine immunoglobulin which is exclusively,

or nearly exclusively, of the subtype IgG_1, IgG_{2a}, IgG_{2b} or IgG_3, the liquid medium is buffered with an acetate buffer to a pH which is, respectively, within the range of about 3.4 to 6.0, 3.4 to 4.5, 3.4 to 4.5 and 3.4 to 4.5.

The invention also features a kit for assaying the quantity of protein A in a sample, containing a buffer solution at a pH at which protein A dissociates from the F_c region of IgG, and anti-protein A antibody. In preferred embodiments, the buffer is an acetate buffer. In particular embodiments the anti-protein A antibody is unlabelled chicken anti-protein A antibody, which may be attached to a solid phase. In particular embodiments the kit may also include labelled anti-protein A antibody.

The assay of the present invention allows accurate measurements of the quantity of protein A in the presense of immunoglobulin, and, in particular, in the presence of mammalian IgG. At the buffered pH values used in the method of the present invention, the binding of chicken anti-protein A antibody to protein A is preserved despite the acidic conditions. The method of the present invention is well-suited for the measurement of protein A contamination of immunoglobulin preparations at parts-per-million levels.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

We now turn to a description of preferred embodiments of the invention, after first describing the drawings.

Drawings

Fig. 1 is a schematic diagram of a microtiter plate with labelled wells for sample dilutions.

Fig. 2 is a graph of data points from ELISA assays in acetate buffer of protein A standards containing murine monoclonal antibodies, with the concentration of protein A plotted on the X axis and the absorbance measurements at 630nm wavelength on the y axis.

Fig. 3 is a graph of data points from ELISA assays in acetate buffer of protein A standards, with the concentration of protein A plotted on the X axis and the absorbance measurements at 630nm wavelength on the y axis.

Fig. 4a - 4d are graphs of data points from ELISA assays of protein A standards containing polyclonal human IgG, with the concentration of protein A plotted on the x axis and the absorbance measurements at 410 nm wavelength on the y axis.

Fig. 5 is a graph of data points from ELISA assays in PBS-T (PBS containing 0.50 ml Tween-20 per 500 ml PBS, pH 7.2 - 7.4) of protein A standards containing mouse monoclonal antibody (MOPC141), with the concentration of protein A plotted on the x axis and the absorbance measurements at 630 nm wavelength on the y axis.

Fig. 6a - 6f are graphs of data points from ELISA assays in acetate buffer of protein A standards in the presence and absence of mouse monoclonal antibody (MOPC141), with the concentration of protein A plotted on the x axis and the absorbance measurements at 630 nm wavelength on the y axis.

Fig. 7a - 7d are graphs of data points from ELISA assays in formate buffer of protein A standards in the presence and absence of mouse monoclonal antibody (MOPC141), with the concentration of protein A plotted on the x axis and the absorbance measurements at 630 nm wavelength on the y axis.

Fig. 8a - 8g are graphs of data points from ELISA assays in citrate buffer of protein A standards in the presence and absence of mouse monoclonal antibody (MOPC141), with the concentration of protein A plotted on the x axis and the absorbance measurements at 630 nm wavelength on the y axis.

Fig. 9a - 9b are graphs of data points from ELISA assays in lactate buffer of protein A standards in the presence and absence of mouse monoclonal antibody (MOPC141), with the concentration of protein A plotted on the x axis and the absorbance measurements at 630 nm wavelength on the y axis.

Fig. 10a - 10 b are graphs of data points from ELISA assays in glycine buffer of protein A standards in the presence

and absence of mouse monoclonal antibody (MOPC141), with the concentration of protein A plotted on the x axis and the absorbance measurements at 630 nm wavelength on the y axis.

Methodologies

The buffer of the present invention may be used in any of a wide range of standard immunoassay formats for protein A in liquid media which utilize anti-protein A antibody. Such assays include sandwich assays, competitive binding assays, direct binding assays, double antibody assays, and precipitation assays of antigen-antibody complexes.

In a method of the invention for detecting protein A in a liquid medium, described in detail below, an acetate buffer is employed in a type of ELISA assay known as a sandwich ELISA assay.

Materials

Reagent A is a concentrated solution of the acetate buffer of the invention, which is 5X the final concentration used in the assay. Reagent A consists of 2.5 M acetic acid (pH 3.30), 0.5 M sodium chloride, and 0.5% Tween-20. The reagent is corrosive, and should be stored at 2-8 degrees C.

Reagent B is a protein A standard solution containing 1.0 mg/ml staphylococcal protein A (for example Ultrapure rProtein A™, available from Repligen Corporation, Cambridge, MA, USA) in sterile water. It should be stored at -10 degrees C or below.

Reagent C is a rabbit anti-protein A:Biotin probe. It may be prepared as follows: 50 mg of ammonium sulfate-precipitated rabbit anti-protein A antisera (Sigma Chemical Company, St. Louis, MO, USA) is reconstituted in 2.0 ml distilled water. In a glass vessel, 1.0 ml of this solution is diluted with 4.0 ml of 0.1 M sodium bicarbonate (pH 8.5), and treated with 0.17 ml of 10 mg/ml NHS-x-Biotin (Calbiochem Biochemicals, La Jolla, CA, USA) in dimethylformamide. The solution is then rotated for 1-2 hours at room temperature and then diafiltered, using a Centricon 30 cartridge (Amicon Division of W.R. Grace, Danvers, MA, USA), against PBS

containing 0.2% sodium azide. The final concentration is 3.0 mg/ml, and should be stored at -10 degrees C or below.

Reagent D is a streptavidin:peroxidase conjugate, which is available from Calbiochem Biochemicals (La Jolla, CA, USA). It should be stored at -10 degrees C or below.

Reagent E is a TMB peroxidase substrate, which is available from Kirkegaard and Perry Laboratories (Gaithersburg, MD, USA). It contains 0.4 g/liter 3,3',5,5' - tetramethylbenzidine in an organic base, and should be stored at 2-8 degrees C.

Reagent F is a peroxidase substrate solution, which is available as Peroxidase Solution B from Kirkegaard and Perry Laboratories (Gaithersburg, MD, USA). It contains 0.02% hydrogen peroxide in citric acid buffer, and should be stored at 2-8 degrees C.

Chicken anti-Protein A coated plates are used. Affinity purified chicken anti-protein A antibodies are available from Immunosystem AB (Uppsala, Sweden). Alternatively, chicken anti-protein A antibodies may be raised against natural or recombinant protein A, and isolated according to well-known procedures. The chicken anti-protein A antibodies are dissolved in 0.05M sodium carbonate (pH 9.6) at a concentration of 3 µg/ml, and 100 µl of this solution is added to each well (except 1A to 3A) of a polystyrene 96-well microtiter plate (Immulon II, Dynatech Inc., Chantilly, VA, USA). After incubation for 12 hours at room temperature, the antibody solution is removed and 200 µl of 10 mg/ml ovalbumin in PBS containing 0.02% sodium azide is added to each well. After standing for 1 hour at room temperature, the ovalbumin solution is removed and the plate washed two times with PBS containing 0.1% Tween 20, and washed one time with distilled water. It should be stored at -10 degrees C or below.

Phosphate buffered saline (PBS) consists of 0.010 M phosphate buffer (pH 7.2-7.4), containing 0.14 M sodium chloride, and 0.003 M potassium chloride.

All reagents should be allowed to reach room temperature before performing the assay.

Procedure

Preparation of the Protein A Standard Curve

Prepare 15 ml of sample buffer by diluting 3.0 ml of Reagent A with 12 ml of distilled water in a 50-ml plastic centrifuge tube. Vortex for 5-10 seconds, or invert 10-15 times, to ensure thorough mixing. The resulting Sample Buffer Diluent is stable for 2 weeks at room temperature.

Obtain an uncoated, polystyrene 96-well microtiter plate and label this "Dilutions". Add 150 µl of prepared Sample Buffer Diluent to each well in columns 1 through 3 of the Dilution plate, except row A.

Label three (3) 1.5-ml Eppendorf tubes "Tube No. 1", "Tube No. 2", and "Tube No. 3". Prepare a 1.0 ng/ml protein A solution by diluting Reagent B with Sample Buffer Diluent, as set forth in Table 1 below.

Table 1

Tube No. Diluent	ml Protein A Standard	ml Sample Buffer
1	10 of Reagent B	990
2	10 of Tube No. 1	990
3	10 of Tube No. 2	990

Vortex each tube thoroughly between dilutions.

Distribute the protein A Standard to the wells on the microtiter plate as follows. Using a P200 micropipet, transfer 150 µl from Tube No. 3 to wells 1H, 2H and 3H of the Dilution Plate. Pump the pipet 5-7 times in the wells to ensure adequate mixing of the protein A standard. Using a multichannel pipet, transfer 150 µl of the solution in wells 1H through 3H to wells 1G through 3G. Pump the pipet 5-7 times in the wells to ensure adequate mixing. Referring to Fig. 1 as a guide, continue to use the multichannel pipet to transfer 150 µl volumes of the solution between appropriate wells, pumping the pipet 5-7 times in the wells to ensure adequate mixing

after transfer, to sequentially transfer the protein A standard until a 0.016 ng/ml standard solution is obtained in wells 1C through 3C of the Dilution plate.

Preparation of Antibody Samples

Dialyze all antibody samples thoroughly against PBS, until the samples are fully equilabrated in PBS.

Up to six (6) samples can be assayed. Prepare the sample solutions for testing as follows. For each sample to be tested, add 200 µl of Reagent A to a labelled, 1.5-ml Eppendorf tube. Then, to each tube containing 200 µl Reagent A, add 300 µl distilled water. Vortex each tube for 5-10 seconds to ensure thorough mixing. To each labelled tube containing diluted Reagent A, add 500 µl of the dialyzed sample to be assayed. Vortex each tube for 5-10 seconds to ensure thorough mixing. Allow tubes to stand for 5 to 10 minutes at room temperature. This is the 1:2 sample dilution shown in Fig. 1. The pH should be measured and confirmed to be 3.4 to 3.5. In other assays, specific pH values within the range 3.4 to 6.0 may be chosen.

Referring to Fig. 1, add 300 µl of each diluted sample to 3 (triplicate) wells on the Dilution plate, leaving three (3) sets of triplicate wells empty between each sample to be assayed. For example, add 300 µl of diluted Sample #1 to wells 4A-6A on the Dilution plate. Then, add 300 µl of diluted Sample #2 to wells 4E-6E on the Dilution plate, and so on until all samples have been placed in the appropriate wells, as shown in Fig. 1. Add 150 µl of 1X Sample Buffer Diluent to the remaining empty wells on the Dilution plate, excluding those wells previously used for the protein A standard solutions.

Using a multichannel pipet, transfer 150 µl of diluted Sample #1 (wells 4A-6A) to the 1X Sample Buffer Diluent in wells 4B - 6B of the Dilution plate. Pump the pipet 5-7 times in the wells to ensure adequate mixing.

Immediately transfer 150 µl of the solution in wells 4B-6B to wells 4C-6C, pumping the pipet 5 times to mix. Repeat

this transfer, adding 150 µl of the solution in wells 4C-6C to wells 4D-6D. Change pipet tips and repeat this procedure for each sample to be assayed. Samples #1 and #2, and their corresponding dilutions, should now be in the positions on the Dilution plate set forth in Table 2 below.

Table 2

Sample No.	Sample Dilution	Wells on Dilution plate
1	1:2	4A-6A
	1:4	4B-6B
	1:8	4C-6C
	1:16	4D-6D
2	1:2	4E-6E
	1:4	4F-6F
	1:8	4G-6G
	1:16	4H-6H

The remaining samples, and their corresponding dilutions, should be in positions consistent with the pattern set forth in Table 2.

It should be noted that the dilutions set forth in the foregoing protocol for the samples and the standards are suggested dilutions only. Given the high sensitivity of this assay, it may be necessary to retest samples at higher dilutions. Therefore, if the amount of protein A contamination cannot be estimated before testing, it is recommended that one-half of the ELISA plate be left sealed, and saved for retesting.

ELISA Testing

Prepare the chicken anti-protein A coated plate (ELISA plate) as follows. Allow the ELISA plate to come to room temperature. Using a wash bottle or automated plate washing system (recommended), wash the ELISA plate by filling each well to be used with distilled water. Dump or aspirate the fluid from the wells and pound to dry.

Add the standard and samples to the ELISA plate as follows.

Referring to Fig. 1, using a multichannel pipet, transfer 100 µl of the 0 ng/ml protein A standard solution from wells 1B through 3B of the Dilution plate to wells 1B through 3B of the washed ELISA plate. Repeat the foregoing procedure to transfer 100 µl of each of the remaining protein A standard solutions from the wells on the Dilution plate to the appropriate wells on the ELISA plate, as set forth in Table 3 below, changing pipet tips between each transfer of protein A standard.

Table 3

Protein A Concentration, ng/ml	Wells on Dilution/ELISA plates
0.016	1C-3C
0.031	1D-3D
0.063	1E-3E
0.125	1F-3F
0.25	1G-3G
0.50	1H-3H

Transfer 100 µl of each sample dilution to the corresponding wells on the ELISA plate, changing pipet tips between each transfer. When all samples and standards have been transferred, discard the Dilution plate.

Cover the ELISA plate with parafilm and incubate at room temperature for 90 minutes. At the end of the 90 minute incubation, empty the wells of sample, pound to dry, and wash the ELISA plate with PBS-Tween 20. Pound the plate to dry, and repeat the wash three times, for a total of four (4) washes as follows. Using a wash bottle or automated plate washing system (recommended), wash the ELISA plate by filling each well used with PBS-Tween 20. Dump or aspirate the fluid from the wells.

The probe solution is made and added to the ELISA plate as follows. Prepare 12 ml of rabbit anti-protein A:biotin probe solution by adding 20 µl of Reagent C to 12 ml of PBS-Tween 20 in a 15-ml conical centrifuge tube. Mix solution thoroughly by vortex or inversion. Using a multichannel pipet, add 100 µl of the probe solution to each well containing sample or standard on the ELISA plate. Leave wells 1A-3A empty.

Cover the plate with parafilm and incubate at room temperature for 90 minutes. Wash the plate four (4) times with PBS-Tween 20, as previously described.

Prepare 12 ml of streptavidin:peroxidase conjugate solution by adding 12ul of Reagent D to 12 ml of PBS-Tween 20 in a 15-ml conical centrifuge tube. Add 100 µl of the conjugate solution to each well containing sample or standard on the ELISA plate.

Cover the plate with parafilm and incubate at room temperature for 90 minutes.

During the conjugate incubation step, prepare the TMB substrate solution by mixing 6.0 ml of Reagent E with 6.0 ml of Reagent F in a glass test tube (15-20 ml). Mix the substrate solution by vortex or inversion, and allow it to come to room temperature in the dark.

After the 90 minute incubation period, remove the conjugate solution from the wells and pound to dry. Wash the plate twice with PBS-Tween 20 as previously described. Repeat the washing procedure two (2) more times using PBS, for a total of four (4) washes. Dry the plate thoroughly by pounding over paper towels.

Perform the color development reaction as follows, making sure that the TMB substrate solution previously prepared is at room temperature before use since a cold solution will dramatically increase color development time. Using a multichannel pipet, add 100 µl of TMB substrate to each of the eight (8) wells in Column 1 of the ELISA plate. Wait 8 seconds, then add 100 µl TMB to each well in Column 2. Continue to add 100 µl TMB to each column of the ELISA plate at 8 second intervals until all wells are filled. Incubate the plate for 5 to 10 minutes, or until well 1H reaches an absorbance of 1.0 +- 0.1 AU against a substrate blank (wells 1A-3A). It is important to tap the bottom right- or left-hand corner of the plate gently against the palm of the hand to distribute any precipitate which has formed in the bottom of the wells.

Read the absorbance at 630 nm using an ELISA plate reader (Dynatech MR6000 or equivalant).

As an optional step, the color development reaction can be stopped at this point, if desired, by addition of 100 µl of 1M phosphoric acid to the well columns at 8 second intervals. The yellow color produced can be read at 410 nm.

Calculations

The parts per million (ppm) protein A contained in each sample can be calculated as follows.

Average the absorbance values for the triplicates of each standard curve concentration and each unknown sample. This is the mean absorbance value (Mean A).

Construct a standard curve as follows. Using linear graph paper, plot each standard curve concentration (ng/ml Protein A) on the abscissa (x-axis) versus the corresponding absorbance value on the ordinate (y-axis). Using linear regression, calculate the best straight line through the points of the standard curve, excluding the 0 ng/ml value.

Determine the concentration of protein A in the sample dilutions as follows. For each sample dilution, use the regression line to determine the protein A concentration [PA] for that dilution. This can be done graphically or analytically, using the slope and intercept of the best straight line through the points.

Determine the contamination in ng/ml of protein A (represented by C in the equation below) in each of the monoclonal antibody (MAb) samples as follows:

[PA], ng/ml X Sample Dilution = C, ng/ml

It is important to determine this value only for those dilutions whose absorbance values fall within the linear range of the protein A Standard Curve. Average the ng/ml values for each sample. This is the mean concentration value (Mean C).

Determine the ppm protein A contamination in each MAb sample using the following formula:

$$\text{ppm protein A} = \frac{\text{Mean Concentration, ng/ml}}{\text{Protein Concentration of antibody sample, mg/ml*}}$$

* The initial (i.e., undiluted) concentration of the sample should be less than or equal to 1.0 mg/ml total protein.

Experimental Data

To demonstrate that the presence of murine monoclonal antibodies do not interfere with the response of the assay, protein A standard curves were constructed as described in the above procedure, except that murine monoclonal antibodies were added to the sample diluent at a concentration of 0.25 mg/ml. In these experiments, the protein A concentrations used were 0.05, 0.10, 0.20, 0.40, 0.80, and 1.60 ng/ml (illustrating that different concentrations can be used). The antibodies were obtained from Sigma Chemical Company (St. Louis, MO, USA) in clarified ascites, and are designated MOPC21 (subclass IgG1), UPC10 (subclass IgG2a), MOPC195 (subclass IgG2b), and Y5606 (subclass IgG3). The immunoassay was performed using the above procedure.

Referring to Fig. 2, the absorbance data obtained at 630 nm is shown plotted against the known standard concentrations of protein A for samples which contained no added murine monoclonal antibodies and for samples containing the antibodies described above. As seen in Fig. 2, the curves obtained in the presence of antibody are close to the curve obtained in sample diluent above. As the data in Fig. 2 indicates, the foregoing procedure is insensitive to interference by murine immunoglobulin concentrations up to 0.25 mg/ml. For this reason the inclusion of IgG in the standard solutions is not required for accurate protein A concentration

meaurements, and the procedure is well-suited to measuring the protein A contamination of immunoglobulin samples at parts-per-million levels.

To further illustrate the use of the subject invention, two of the monoclonal antibodies described above (MOPC21 and MOPC195) were purified using immobilized protein A and tested for protein A contamination. Each antibody was purified on Immobilized rProtein A™ (IPA-300) and Fast Flow Immobilized rProtein A™ (IPA-400), obtained from Repligen Corporation (Cambridge, MA, USA). The manufacturer's recommended procedures were followed for the purifications. The purified antibodies were dialyzed against PBS and their concentrations were determined from their absorbance at 280 nm (a 1.0 mg/ml solution gives an abosorbance of 1.4). The concentrations determined are set forth below in Table 4.

Table 4

Antibody	Resin	Concentration (mg/ml)
MOPC21	IPA-300	0.33
	IPA-400	0.36
MOPC195	IPA-300	0.65
	IPA-400	0.66

The data obtained for the standard curve are set forth in Table 5.

Table 5

ABSORBANCE 630 nm

ng/ml rPA		Rep #1	Rep #2	Rep #3	Mean	STD DEV	%CV
Standard Curve, pH 3.50							
0		0.143	0.145	0.149	0.146	0.0031	2.10%
0.015625		0.191	0.231	0.188	0.203	0.0240	11.81%
0.03125		0.400	0.268	0.243	0.304	0.0844	22.78%
0.0625		0.343	0.328	0.315	0.329	0.0140	4.26%
0.125		0.442	0.418	0.475	0.445	0.0288	6.43%
0.25		0.675	0.709	0.623	0.669	0.0433	6.47%
0.5		0.913	1.127	1.046	1.029	0.1080	10.50%
IPA300 Samples							
Mo1	1:2	1.966	1.867	1.902	1.912	0.0502	2.63%
MoPC21	1:4	1.473	1.542	1.593	1.536	0.0602	3.92%
	1:8	1.098	1.073	1.029	1.067	0.0349	3.27%
	1:16	0.732	0.690	0.702	0.708	0.0216	3.08%
Mo2b	1:2	1.926	1.966	1.980	1.957	0.0280	1.43%
	1:4	1.455	1.511	1.532	1.499	0.0398	2.65%
MoPC195	1:8	0.919	0.985	1.053	0.986	0.0670	6.80%
	1:16	0.645	0.669	0.681	0.665	0.0183	2.76%
IPA400 Samples							
Mo1	1:2	0.560	0.597	0.522	0.560	0.0375	6.70%
MoPC21	1:4	0.358	0.337	0.391	0.362	0.0272	7.52%
	1:8	0.290	0.294	0.271	0.285	0.0123	.31%
	1:16	0.221	0.214	0.212	0.216	0.0047	2.19%
Mo2b	1:2	0.622	0.593	0.585	0.600	0.0195	3.24%
MoPC195	1:4	0.375	0.344	0.380	0.366	0.0195	5.32%
	1:8	0.245	0.228	0.250	0.241	0.0115	4.79%
	1:16	0.223	0.204	0.229	0.219	0.0131	5.97%

Referring to Fig. 3, the data points were plotted to generate a standard curve, at pH 3.50.

Based on this data and the standard curve, the ppm contamination of protein A in the antibody samples, resulting from leaching from the affinity columns, is set forth in Table 6.

Table 6

Sample		Mean A630	ng/ml Leaching	ppm Leaching
IPA 300				
Mouse IgG1	1:2	1.912	----	
	1:4	1.536	----	
	1:8	1.067	4.090	
	1:16	0.708	4.7000	
			Avg. = 4.4	15 (0.3 mg/ml IgG)
Mouse IgG2b	1:2	1.957	----	
	1:4	1.499	----	
	1:8	0.986	3.700	
	1:16	0.665	4.300	
			Avg. = 4.0	7 (0.6 mg/ml IgG)
IPA 400 Fast Flow				
Mouse IgG1	1:2	0.560	0.406	
	1:4	0.362	0.328	
	1:8	0.285	----	
	1:16	0.216	----	
			Avg. = 0.37	1 (0.3 mg/ml IgG)
Mouse IgG2b	1:2	0.600	0.454	
	1:4	0.366	0.338	
	1:8	0.241	----	
	1:16	0.219	----	
			Avg. = 0.40	1 (0.6 mg/ml IgG)

Buffer pH

The pH of the buffered medium in which binding is promoted between protein A and anti-protein A antibody must be at or below the pH at which protein A--F_c complexes dissociate, but above a pH at which protein A and the anti-protein A antibody used in the assay dissociate.

Referring to Fig. 4a-d, in a set of initial experiments, data was generated at a range of pH values for protein A standard solutions to which human polyclonal IgG was added. Still referring to Fig. 4a-d, ELISA assays for protein A were performed in four experiments in which the pH was

buffered to 2.00, 3.00, 4.00, and 5.00, respectively. Two sets of standard solutions containing between 1 and 32 ng/ml protein A were used in each experiment. One set of standard solutions contained 0.05 mg/ml human polyclonal IgG, and the second set contained 1.0 mg/ml human polyclonal IgG. As indicated in Fig. 4a-d, the buffer used was either a citrate or an acetate buffer. The initial experiments indicated that, while some binding of protein A to chicken anti-protein A antibody took place at pH values between 3.00 and 5.00, inclusive, virtually no binding between protein A and chicken anti-protein A antibody was detected at a pH value of 2.00. The experimental data also indicated that the system buffered with acetate to a pH of 4.00 gave the most consistent results, i.e. the corresponding standard solutions containing 0.05 mg/ml human polyclonal IgG yielded absorbance readings that were close to the absorbance readings for standards containing 1.0 mg/ml human polyclonal IgG. Based upon this data and the results of further experiments, depicted in Fig. 5 - Fig. 10b, it was determined that in preferred embodiments the assay should be carried out at a pH of about 3.4 or higher, and below the pH at which protein A--F_c complexes dissociate.

In assays for protein A contaminants in murine IgG preparations of specific subclasses of IgG, an appropriate buffered pH may be selected. For assays of protein A in samples in which the IgG present is exclusively, or nearly exclusively, murine IgG of the subtype IgG_1, a pH within the range of about 3.4 to 6.0 is appropriate. When the IgG present in a sample is exclusively, or nearly exclusively, murine IgG of the subtype IgG_{2a}, a pH within the range of about 3.4 to 4.5 is appropriate. When the IgG present is exclusively, or nearly exclusively, murine IgG of the subtype IgG_{2b}, a pH of about 3.4 to 4.5 is appropriate. When the IgG present is exclusively, or nearly exclusively, IgG of the subtype IgG_3, a pH within the range of about 3.4 to 4.5 is appropriate.

The appropriate pH for determining protein A contaminant in any particular IgG preparation may be determined

by elution studies. If the IgG of interest is bound to a protein A affinity column, the pH at which the IgG is released from the column can be determined. The pH of the buffer of the invention in an assay for protein A contamination in a preparation of a particular IgG should be set at or below the pH at which the IgG is eluted, and at or above a pH of about 3.4.

A series of experiments were carried out to detect protein A in an ELISA assay using a variety of buffers. Referring to Fig. 5 - 10b, Protein A standard solutions were run in the ELISA assay in the absence of IgG and in the presence of mouse monoclonal antibody MOPC141 (which is of the subtype IgG_{2b}), referred to in Fig. 5 - 10b as Mo2b. The ELISA assays were carried out for each buffer system at a variety of pH values.

Referring to Fig. 5, protein A standards (rPA) were run in ELISA assays in PBS-T (PBS containing 0.50 ml Tween-20 per 500 ml PBS, pH 7.2-7.4). Still referring to Fig. 5, the data points demonstrate suppression of the assay results in the presence of 100µg/ml MOPC141 when the buffer of the invention is not used.

Referring to Fig. 6a-6f, the data points indicate that acetate buffer provides a highly effective ELISA assay system. As demonstrated in Fig. 6a-6f, in acetate buffer at pH values ranging from 3.50 to 4.50, the problem of suppression of the ELISA results in the presence of MOPC141 is virtually eliminated.

Referring to Fig. 7a-7d, in the presence of formate buffer at pH values between 3.50 and 4.50, inclusive, the suppression of results from a ELISA assay in the presence of MOPC141 appears to be somewhat ameliorated at pH values between 3.50 and 4.50, inclusive, but not at a pH value of 3.00.

Referring to Fig. 8a-g, Fig. 9a-b, and Fig. 10a-b, in which citrate, lactate, and glycine were used to buffer the conditions of ELISA assays, respectively, the absorbance data obtained are shown graphed as a function of the protein A

standard concentrations. These preliminary results indicate that there may be some artifact or specific interaction in these systems which interferes with the ELISA assays.

What is claimed is:

1. In a method for detecting protein A in an IgG-containing sample in a liquid medium, which method utilizes anti-protein A antibody, the improvement which comprises buffering the liquid medium, when the sample is contacted with said anti-protein A antibody for the purpose of promoting binding between said protein A in the sample and said anti-protein A antibody, with a buffer to a pH at which protein A dissociates from the F_c region of said IgG.

2. The method of claim 1 in which said pH is between 3.4 and 6.0, inclusive.

3. The method of claim 1 in which said buffer is an acetate buffer.

4. The method of claim 1 in which said buffer is a formate buffer.

5. The method of claim 1 in which said anti-protein A antibody is chicken anti-protein A antibody.

6. The method of claim 1 in which said IgG is exclusively, or nearly exclusively, murine IgG of the subtype IgG_1, and said pH is within the range of about 3.4 to 6.0.

7. The method of claim 1 in which said IgG is exclusively, or nearly exclusively, murine IgG of the subtype IgG_{2a}, and said pH is within the range of about 3.4 to 4.5.

8. The method of claim 1 in which said IgG is exclusively, or nearly exclusively, murine IgG of the subtype IgG_{2b}, and said pH is within the range of about 3.4 to 4.5.

9. The method of claim 1 in which said IgG is exclusively, or nearly exclusively, murine IgG of the subtype IgG_3, and said pH is within the range of about 3.4 to 4.5.

10. A kit for assaying the quantity of protein A in a sample, comprising a) a buffer solution which comprises a buffer at a pH at which protein A dissociates from the F_c region of IgG, and b) anti-protein A antibody.

11. The kit of claim 10 in which said buffer solution comprises an acetate buffer.

12. The kit of claim 10 in which said anti-protein A antibody comprises unlabelled chicken anti-protein A antibody.

13. The kit of claim 12 in which said unlabelled chicken anti-protein A antibody is attached to a solid phase.

14. The kit of claim 12 or 13 further comprising labelled chicken anti-protein A antibody.

FIG. 1

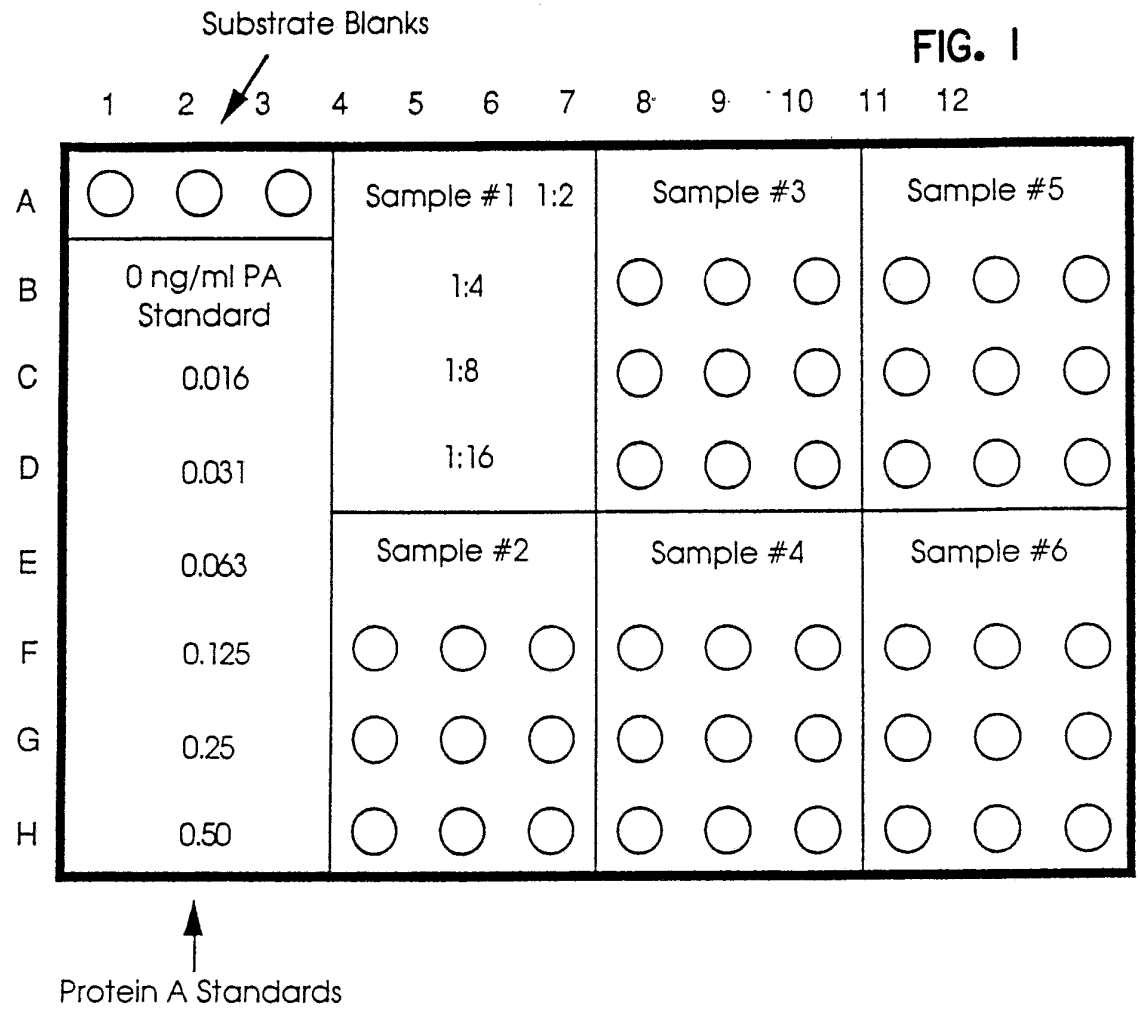

Substrate Blanks
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
0 ng/ml PA Standard
0.016
0.031
0.063
0.125
0.25
0.50
Sample #1 1:2
1:4
1:8
1:16
Sample #2
Sample #3
Sample #4
Sample #5
Sample #6
Protein A Standards

FIG. 3

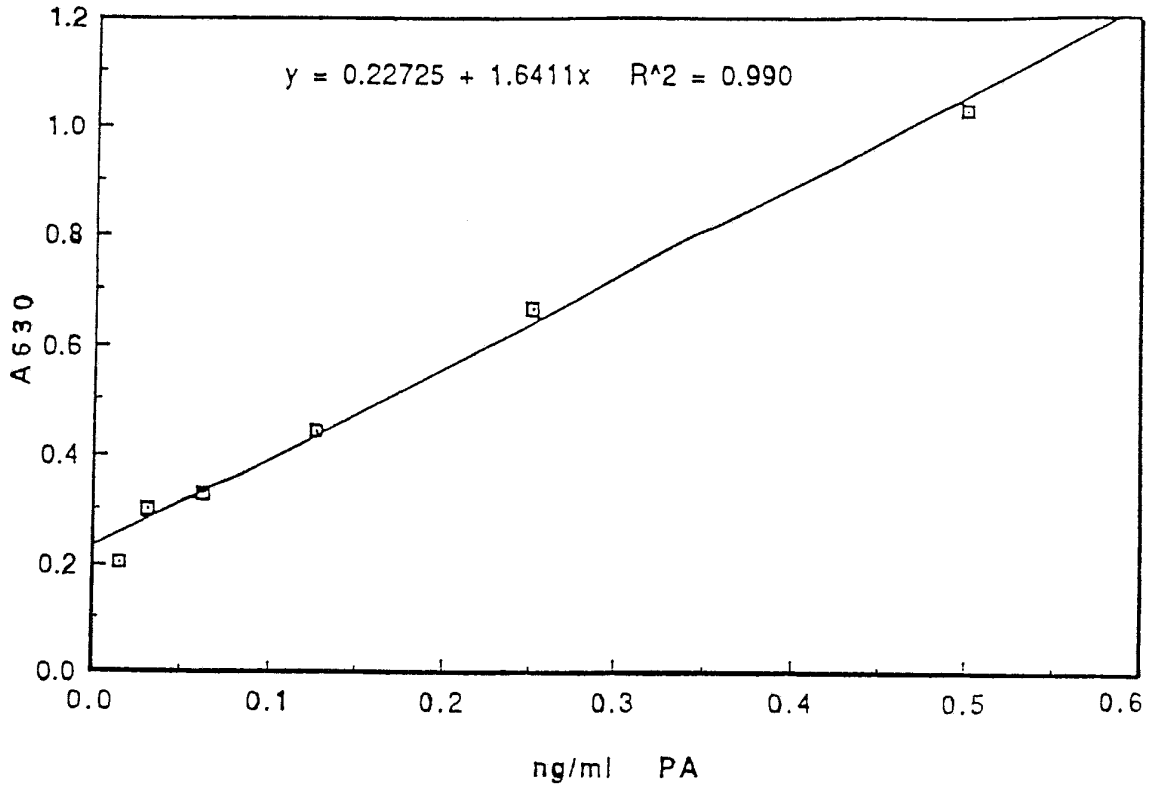
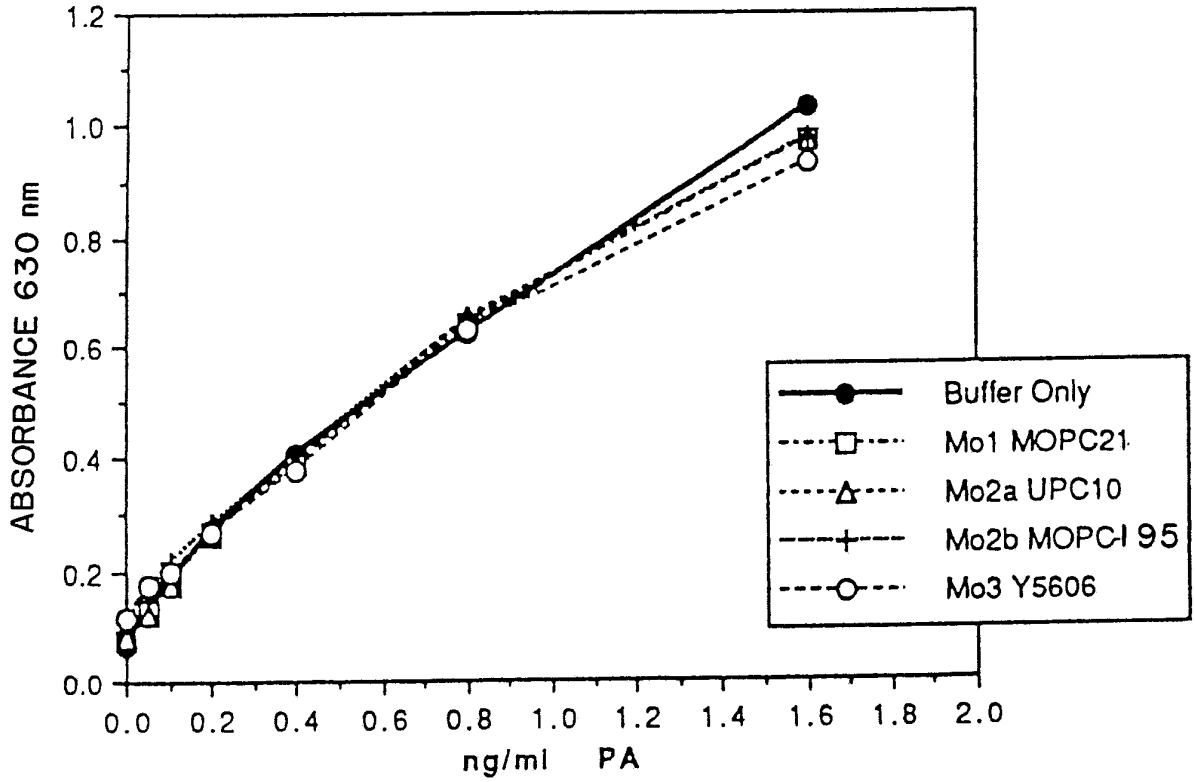

y = 0.22725 + 1.6411x R^2 = 0.990
A630
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0 0.1 0.2 0.3 0.4 0.5 0.6
ng/ml PA

FIG. 2


ABSORBANCE 630 nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0
ng/ml PA
Buffer Only
Mo1 MOPC21
Mo2a UPC10
Mo2b MOPC-I 95
Mo3 Y5606

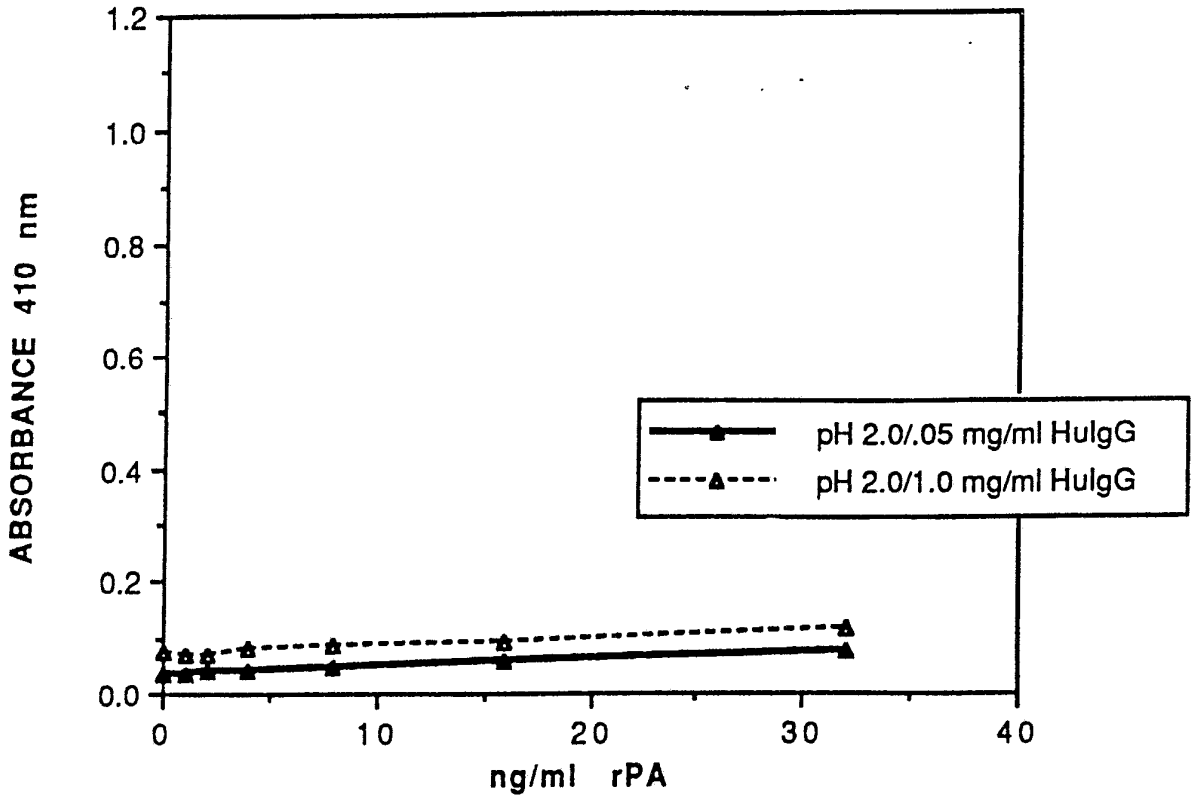

ABSORBANCE 410 nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
ng/ml rPA
pH 2.0/.05 mg/ml HulgG
pH 2.0/1.0 mg/ml HulgG

FIG. 4A

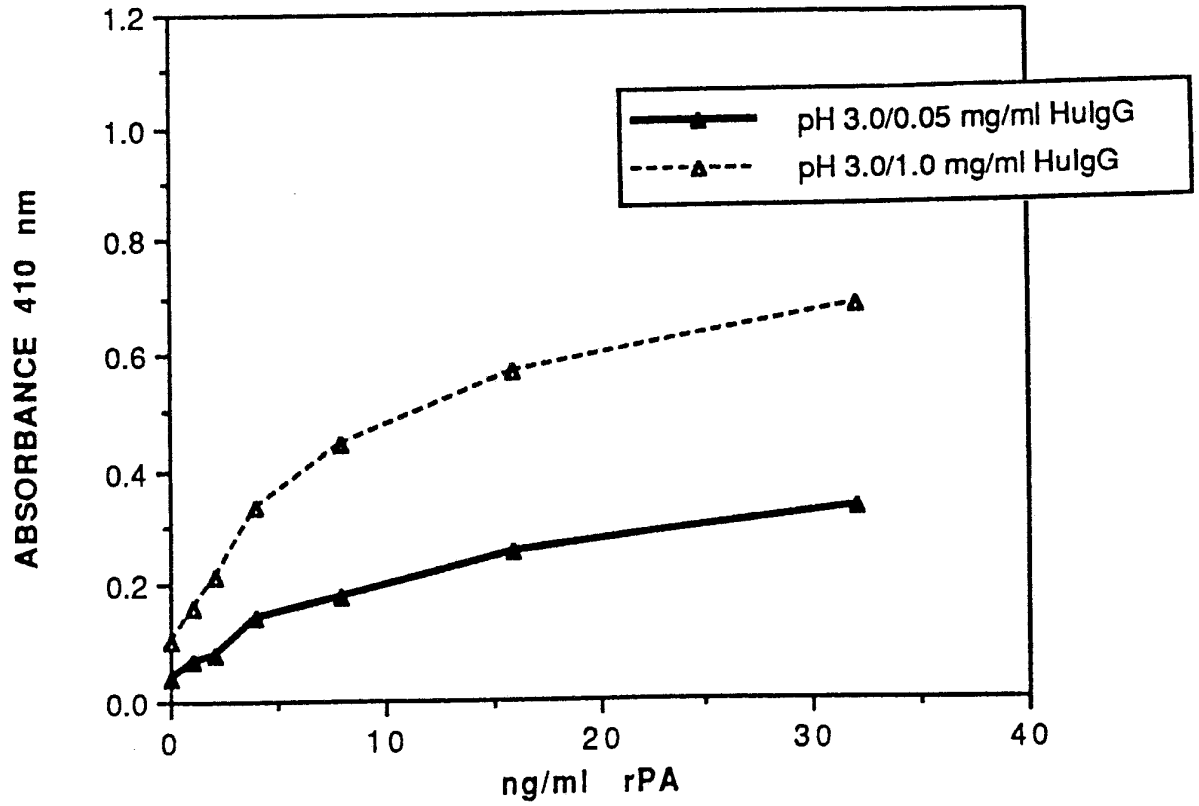

ABSORBANCE 410 nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
ng/ml rPA
pH 3.0/0.05 mg/ml HulgG
pH 3.0/1.0 mg/ml HulgG

FIG. 4B

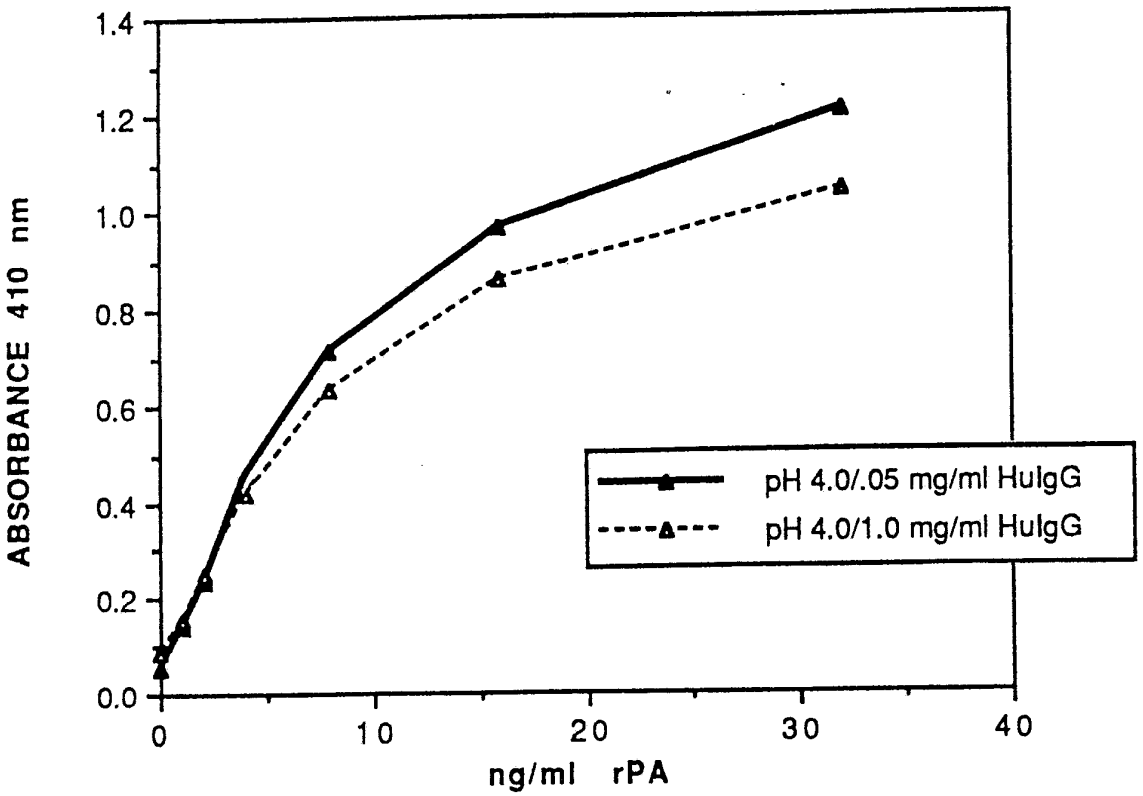

ABSORBANCE 410 nm
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
ng/ml rPA
pH 4.0/.05 mg/ml HulgG
pH 4.0/1.0 mg/ml HulgG

FIG. 4C

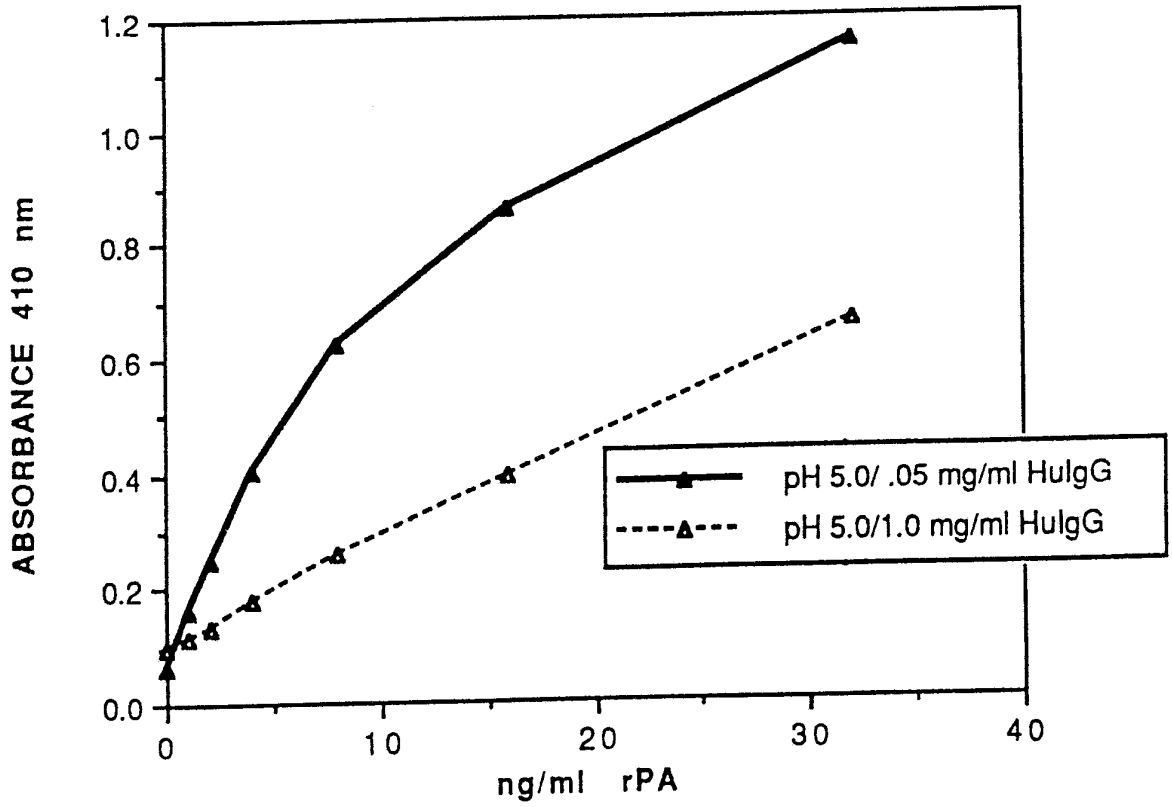

ABSORBANCE 410 nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
ng/ml rPA
pH 5.0/ .05 mg/ml HulgG
pH 5.0/1.0 mg/ml HulgG

FIG. 4D

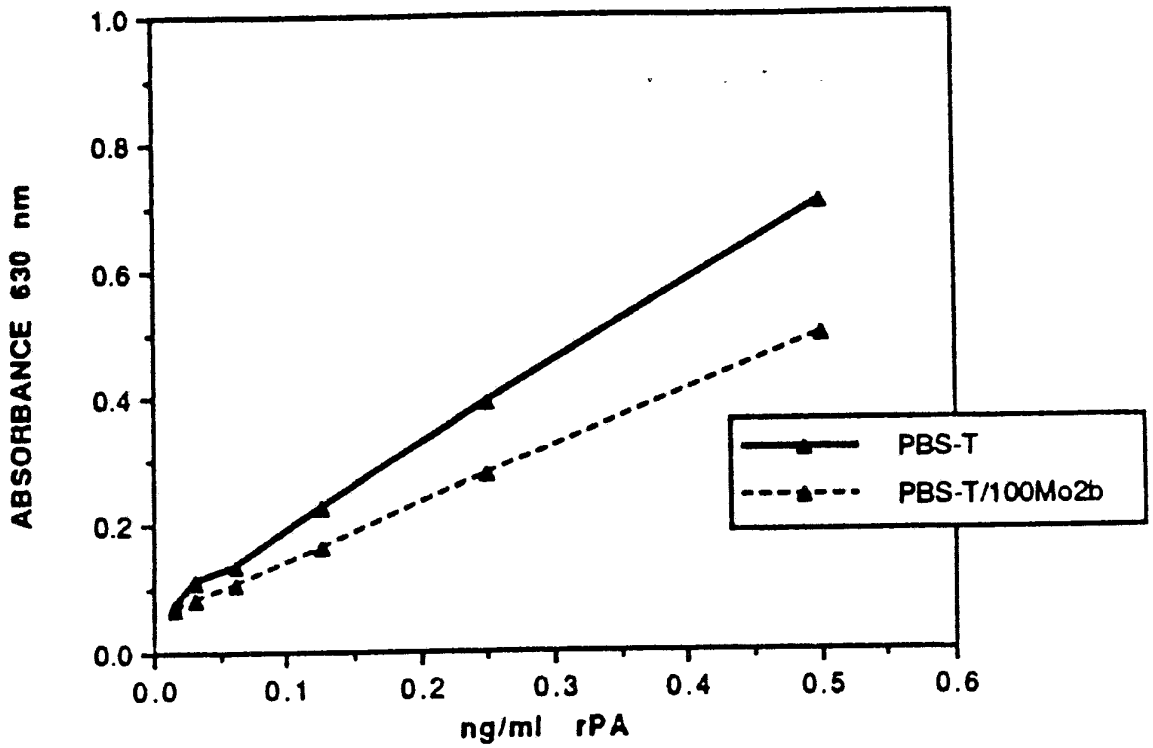

ABSORBANCE 630 nm
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ng/ml rPA
PBS-T
PBS-T/100Mo2b

FIG. 5

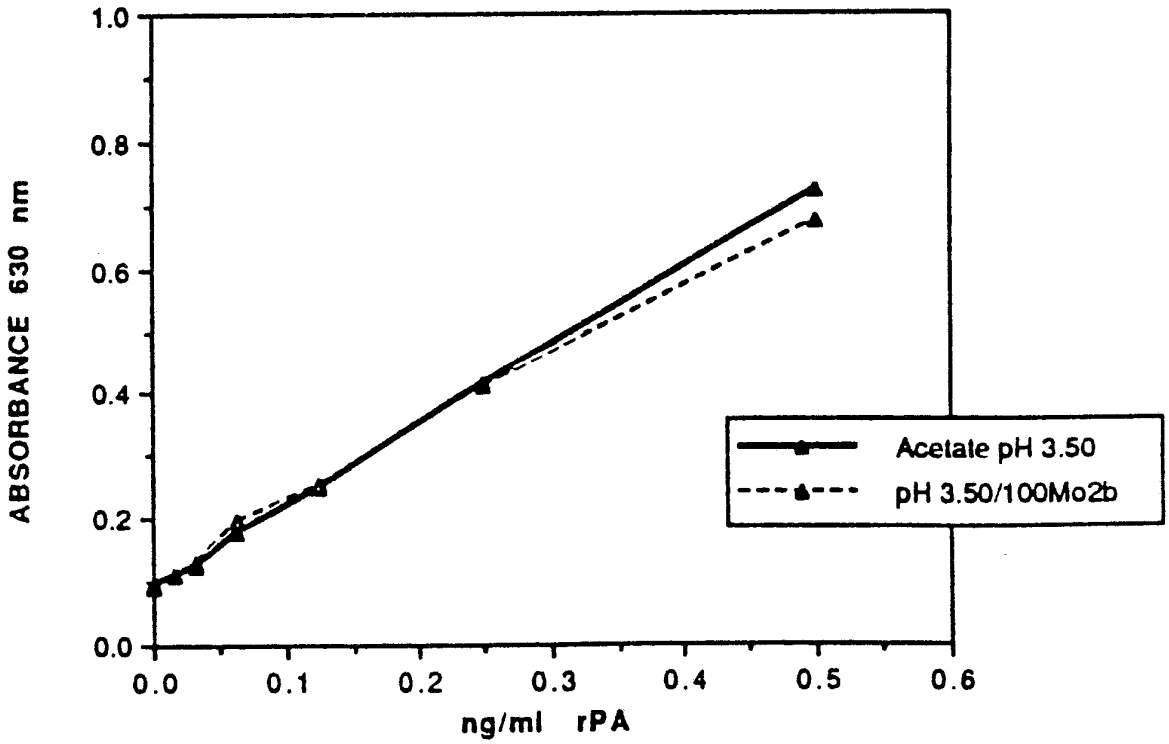

ABSORBANCE 630 nm
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ng/ml rPA
Acetate pH 3.50
pH 3.50/100Mo2b

FIG. 6A

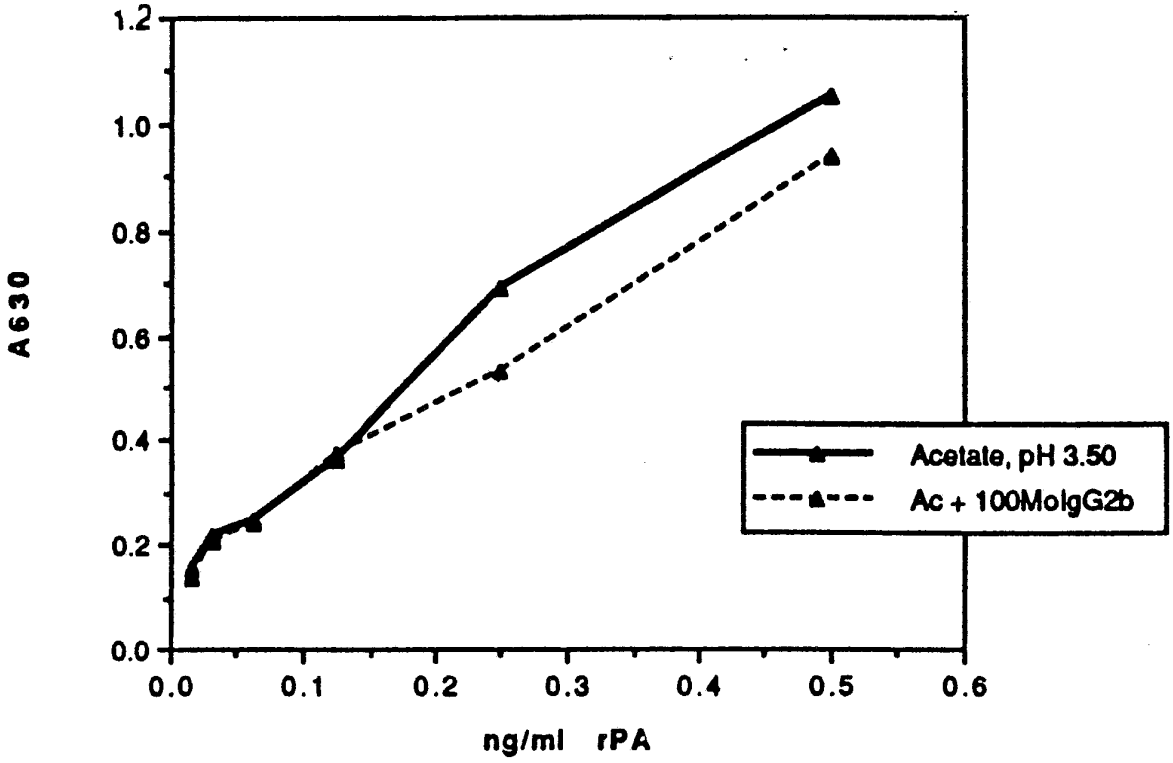

A630
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ng/ml rPA
Acetate, pH 3.50
Ac + 100MoIgG2b

FIG. 6B

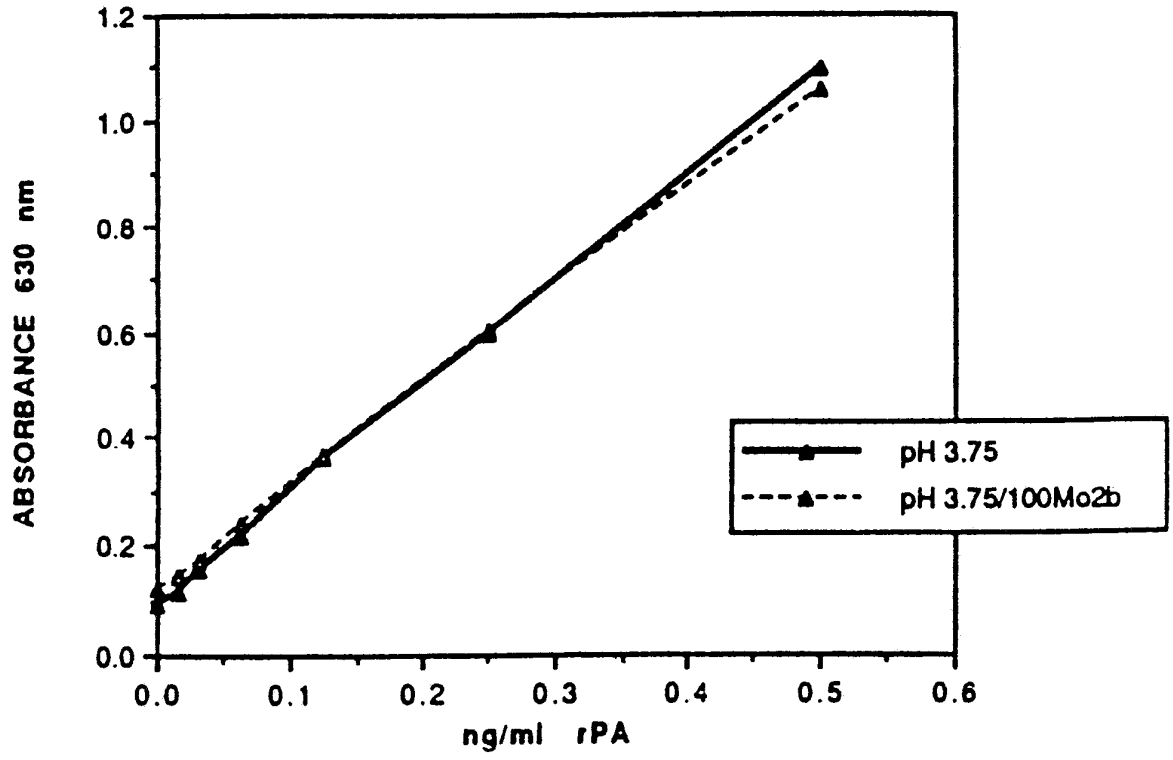

ABSORBANCE 630 nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ng/ml rPA
pH 3.75
pH 3.75/100Mo2b

FIG. 6C

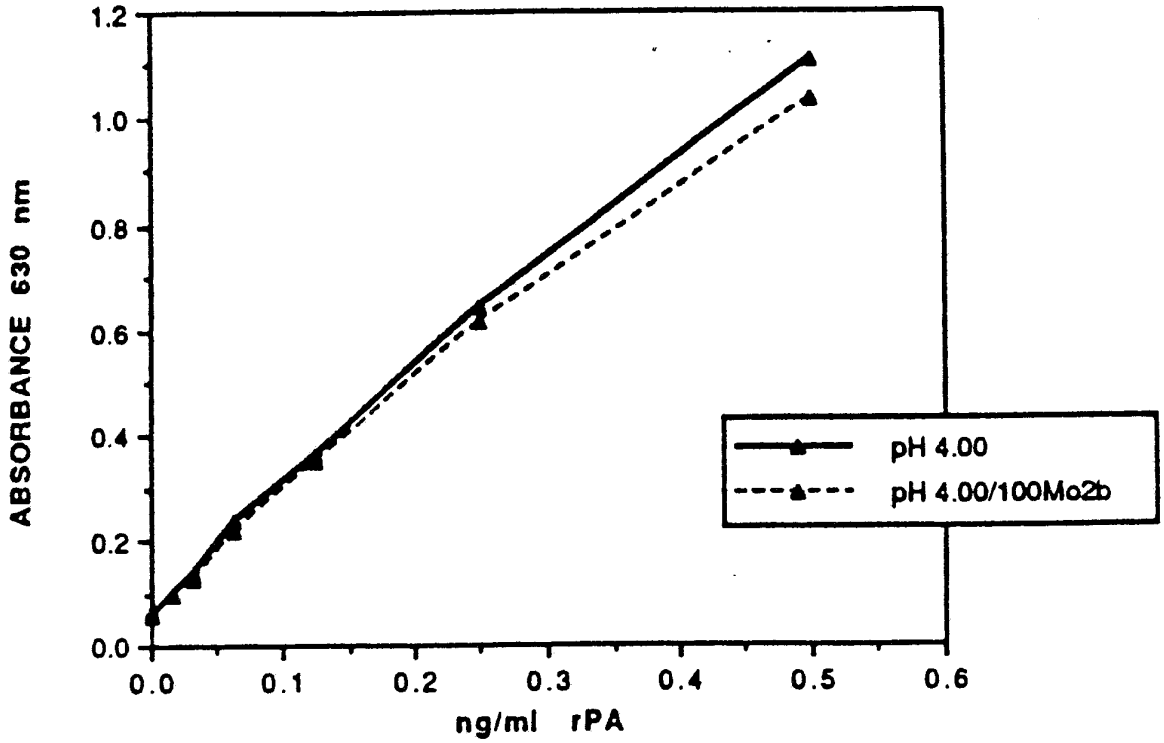

ABSORBANCE 630 nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ng/ml rPA
pH 4.00
pH 4.00/100Mo2b

FIG. 6D

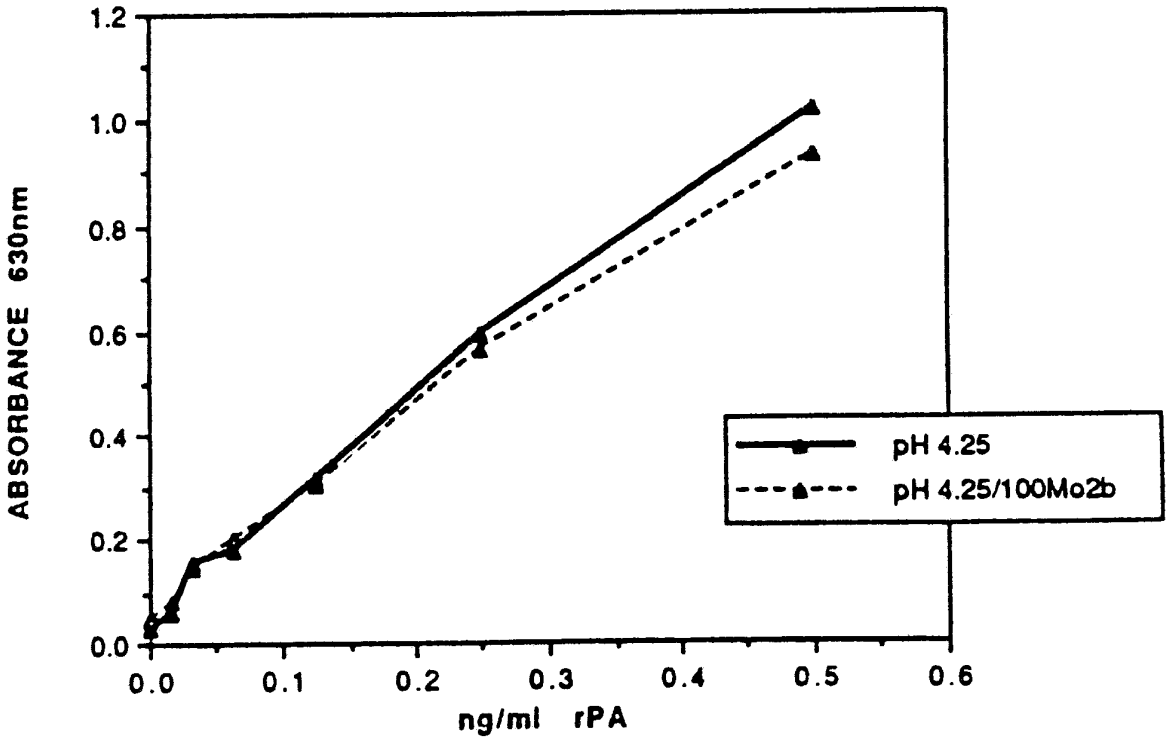

ABSORBANCE 630nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ng/ml rPA
pH 4.25
pH 4.25/100Mo2b

FIG. 6E

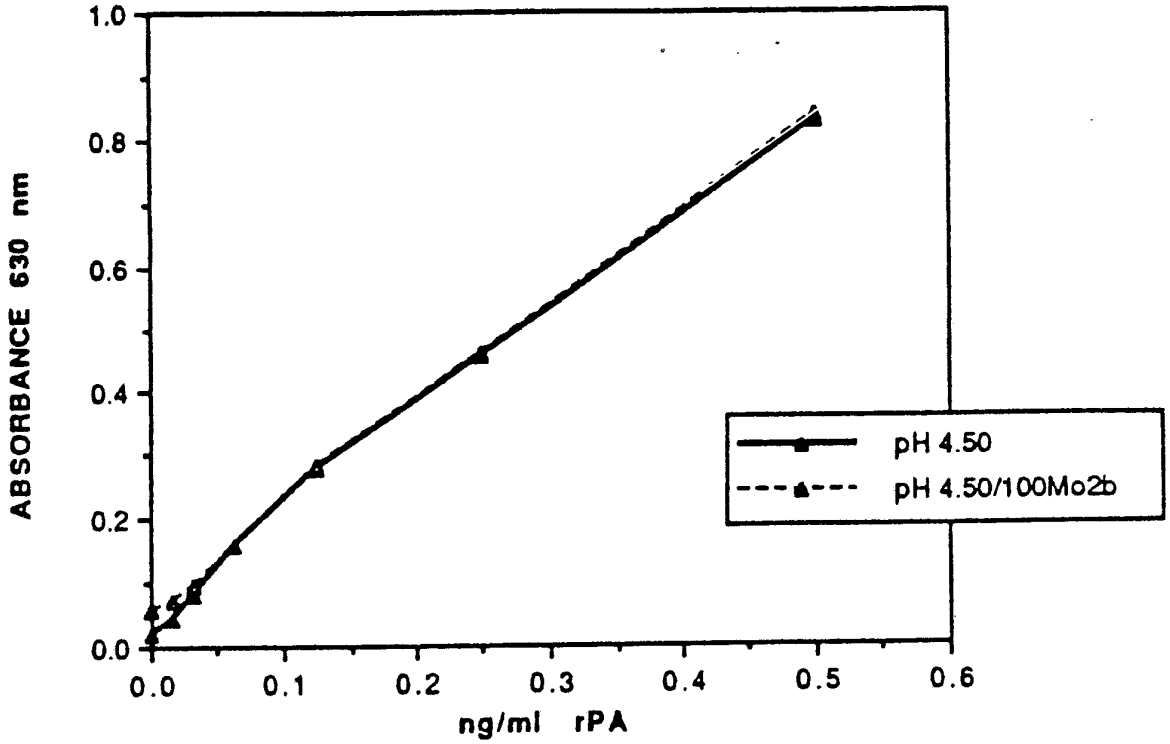

ABSORBANCE 630 nm
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ng/ml rPA
pH 4.50
pH 4.50/100Mo2b

FIG. 6F

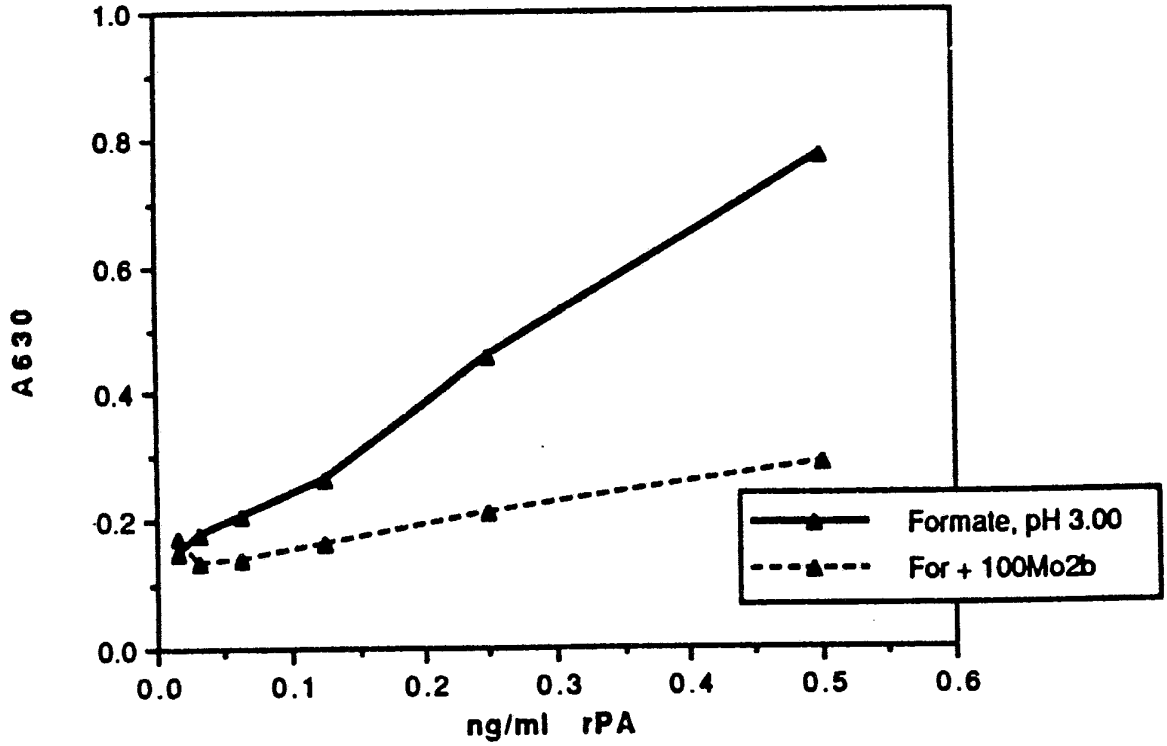

A630
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ng/ml rPA
Formate, pH 3.00
For + 100Mo2b

FIG. 7A

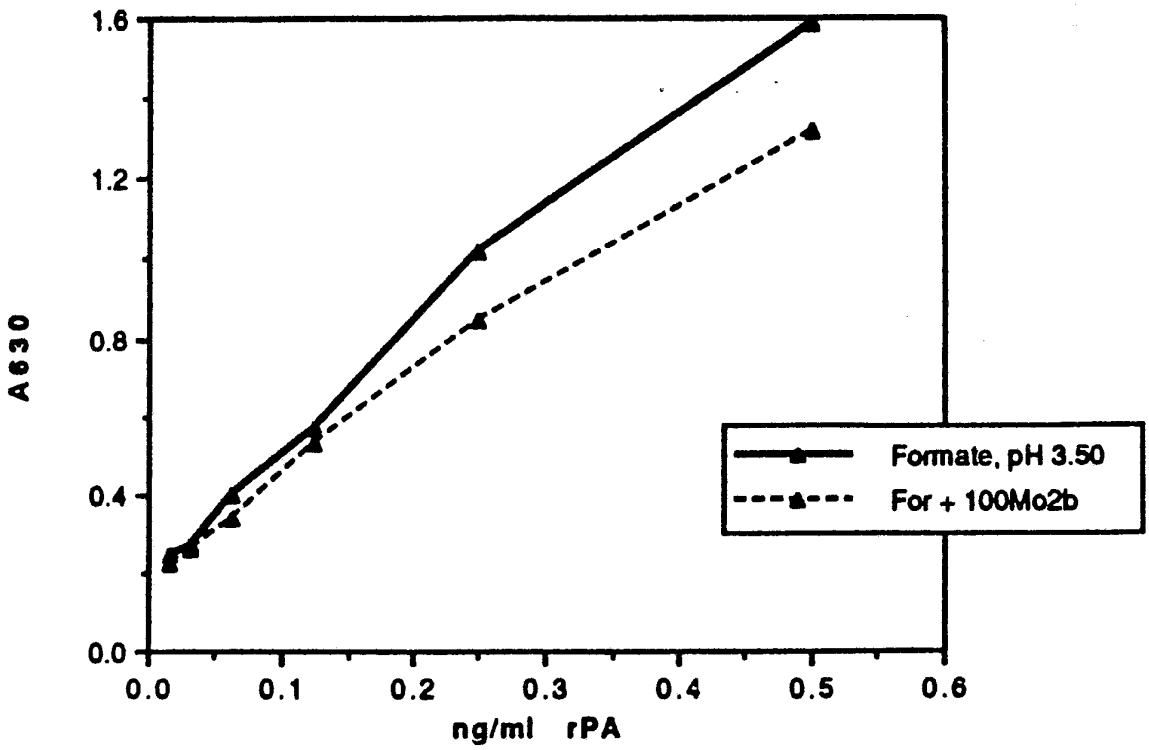

A630
1.6
1.2
0.8
0.4
0.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ng/ml rPA
Formate, pH 3.50
For + 100Mo2b

FIG. 7B

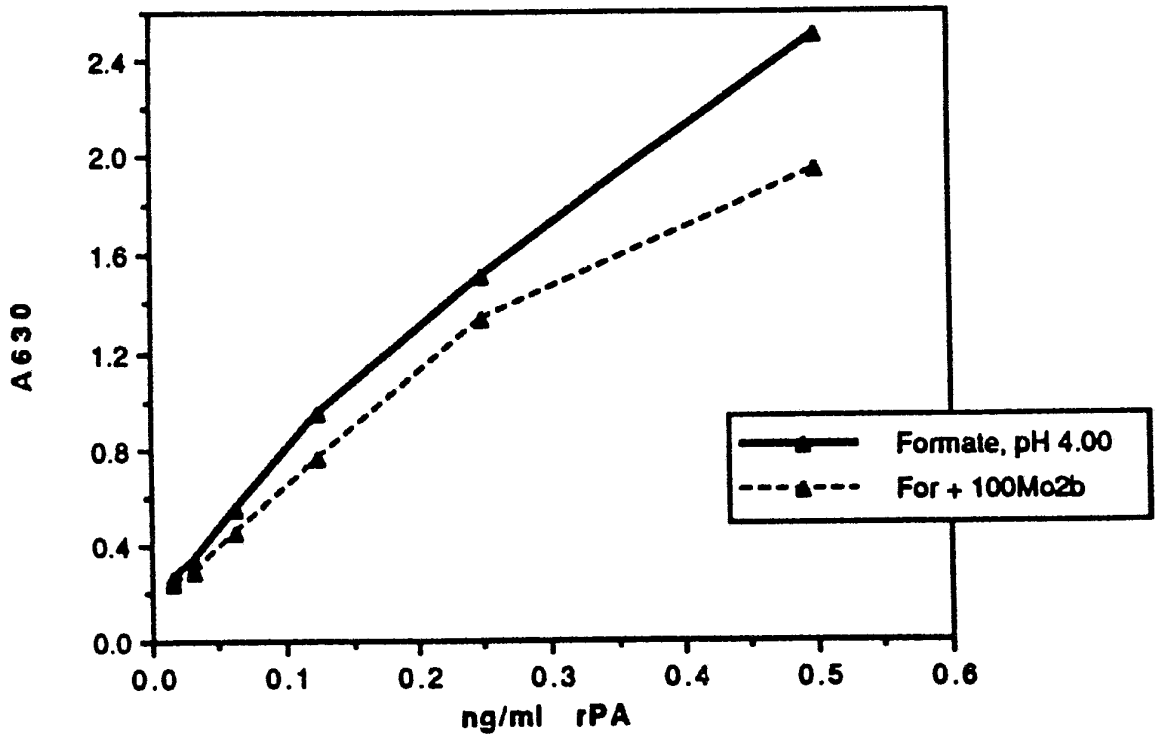

A630
2.4
2.0
1.6
1.2
0.8
0.4
0.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ng/ml rPA
Formate, pH 4.00
For + 100Mo2b

FIG. 7C

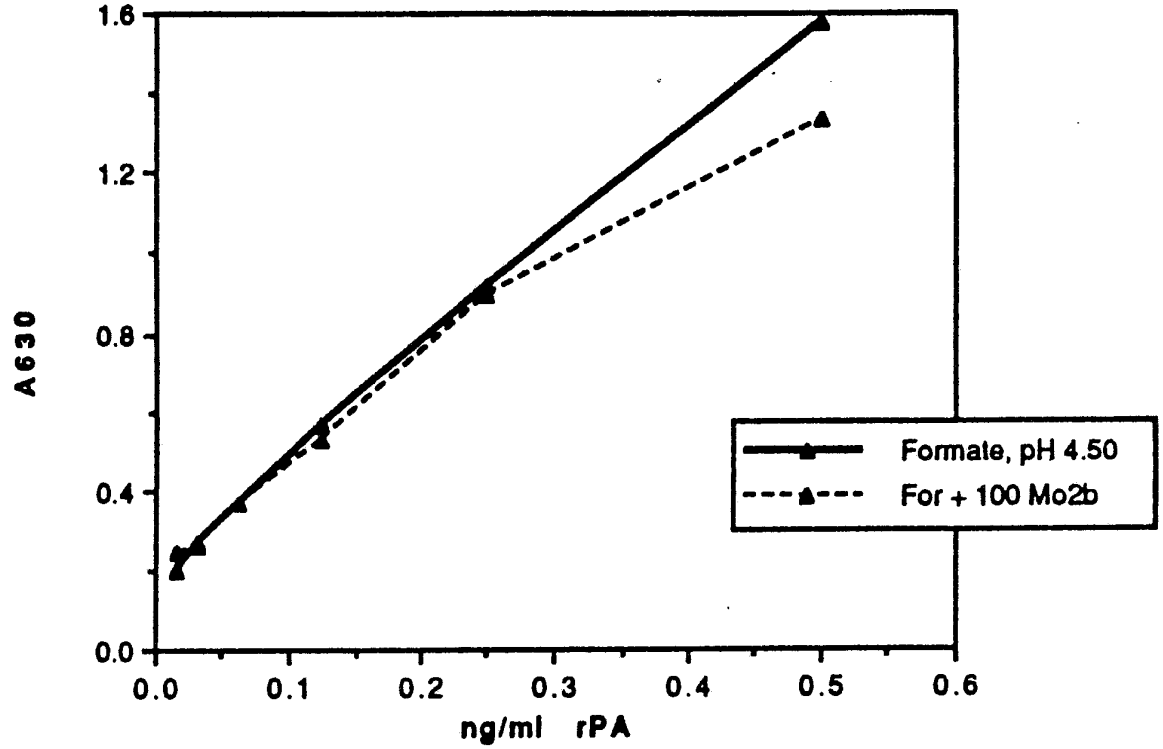

A630
1.6
1.2
0.8
0.4
0.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ng/ml rPA
Formate, pH 4.50
For + 100 Mo2b

FIG. 7D

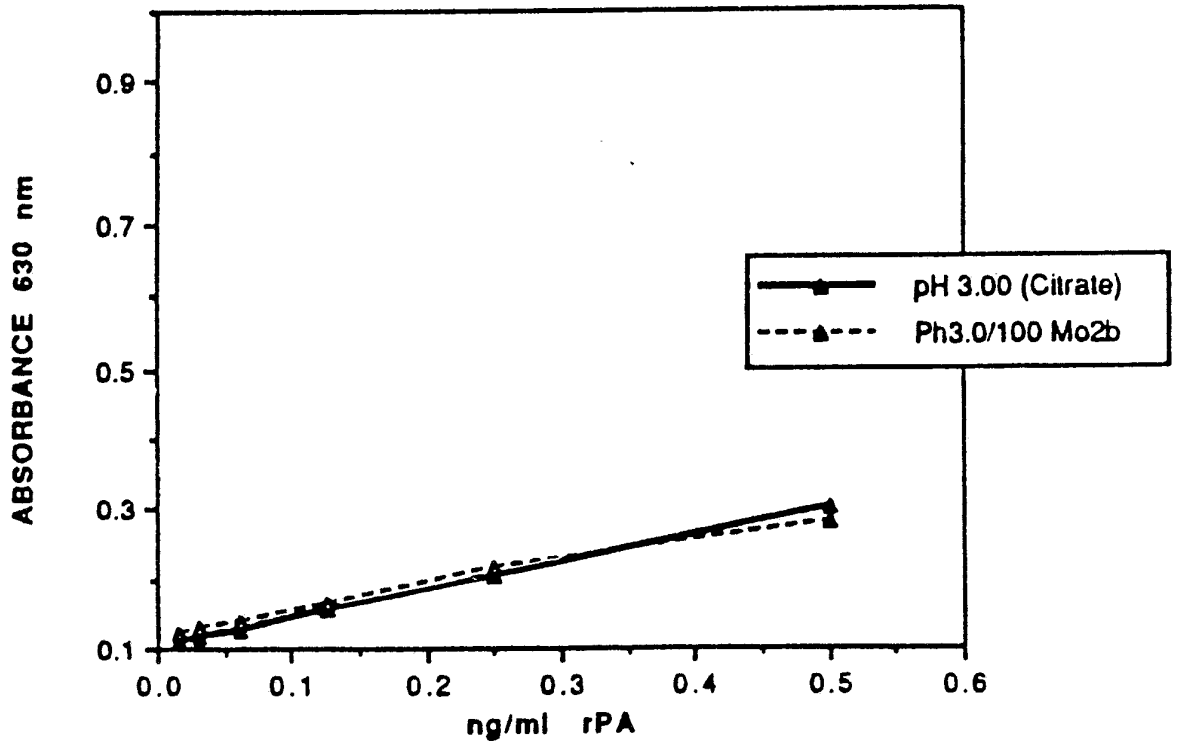

ABSORBANCE 630 nm
0.9
0.7
0.5
0.3
0.1
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ng/ml rPA
pH 3.00 (Citrate)
Ph3.0/100 Mo2b

FIG. 8A

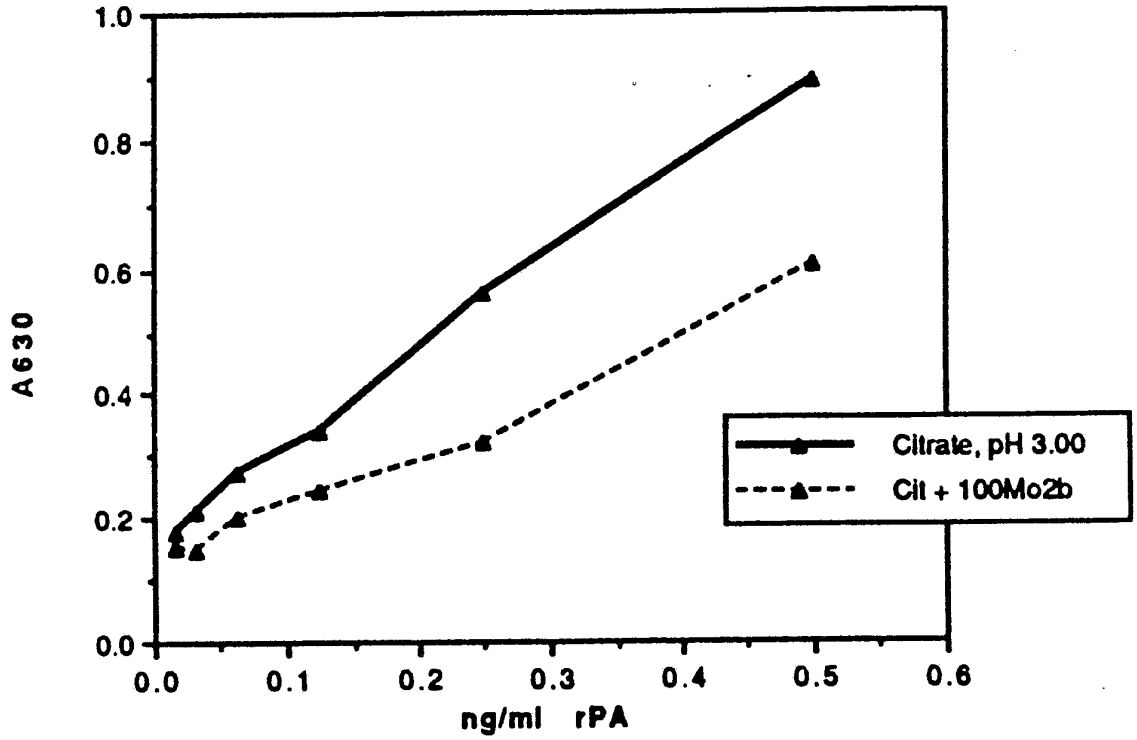

1.0
0.8
0.6
0.4
0.2
0.0
A630
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ng/ml rPA
Citrate, pH 3.00
Cit + 100Mo2b

FIG. 8B

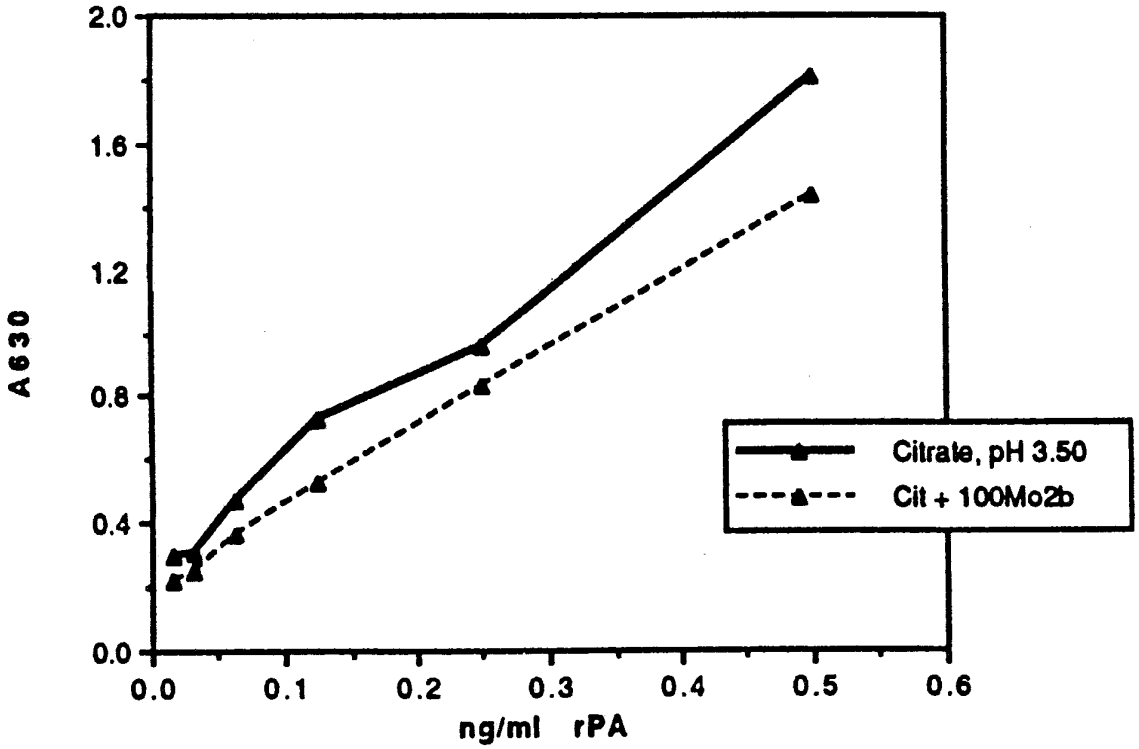

2.0
1.6
1.2
0.8
0.4
0.0
A630
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ng/ml rPA
Citrate, pH 3.50
Cit + 100Mo2b

FIG. 8C

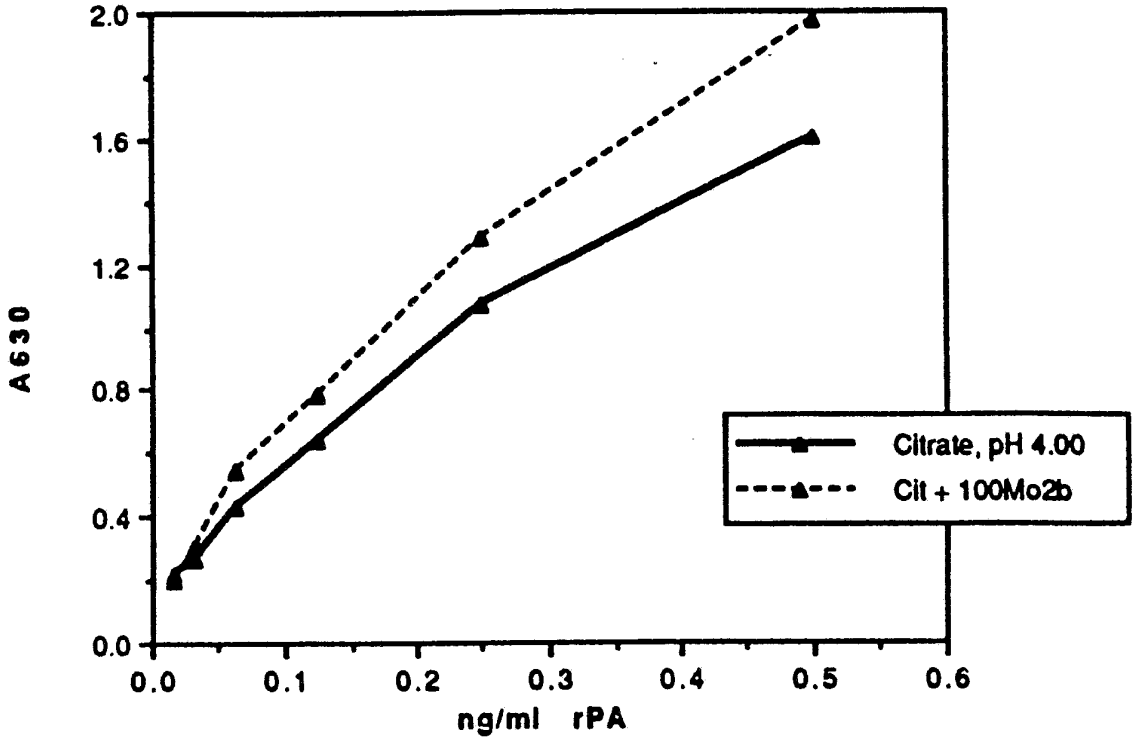

2.0
1.6
1.2
0.8
0.4
0.0
A630
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ng/ml rPA
Citrate, pH 4.00
Cit + 100Mo2b

FIG. 8D

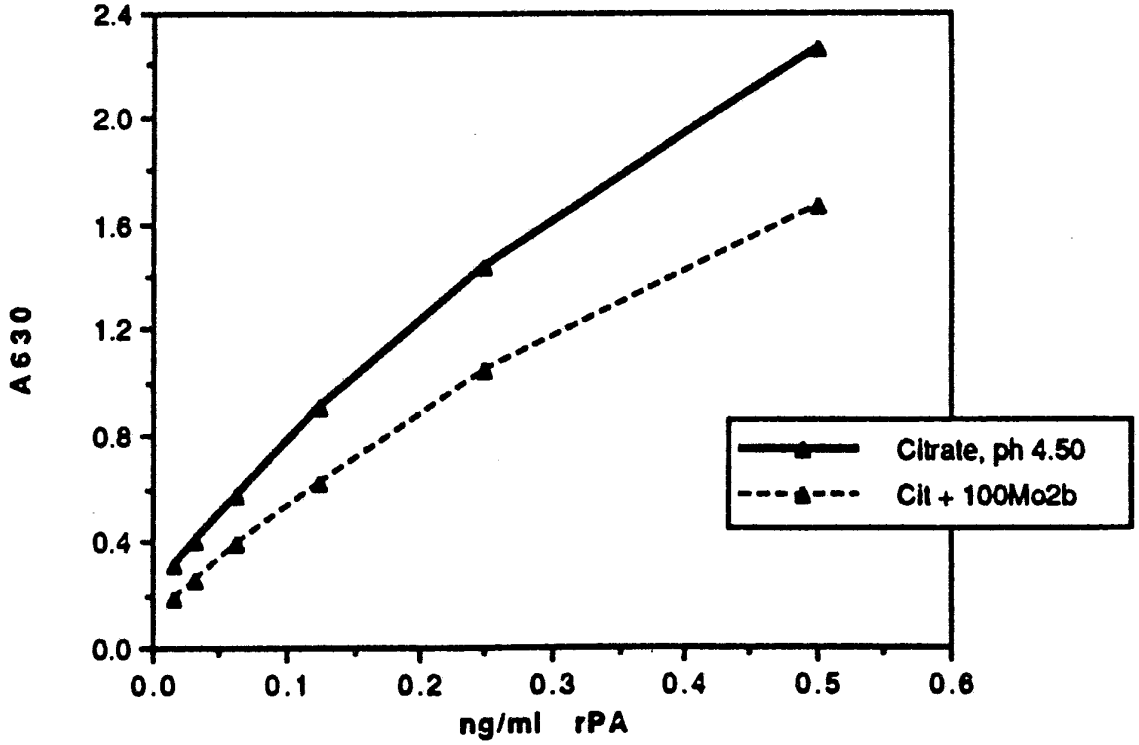

2.4
2.0
1.6
1.2
0.8
0.4
0.0
A630
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ng/ml rPA
Citrate, ph 4.50
Cit + 100Mo2b

FIG. 8E

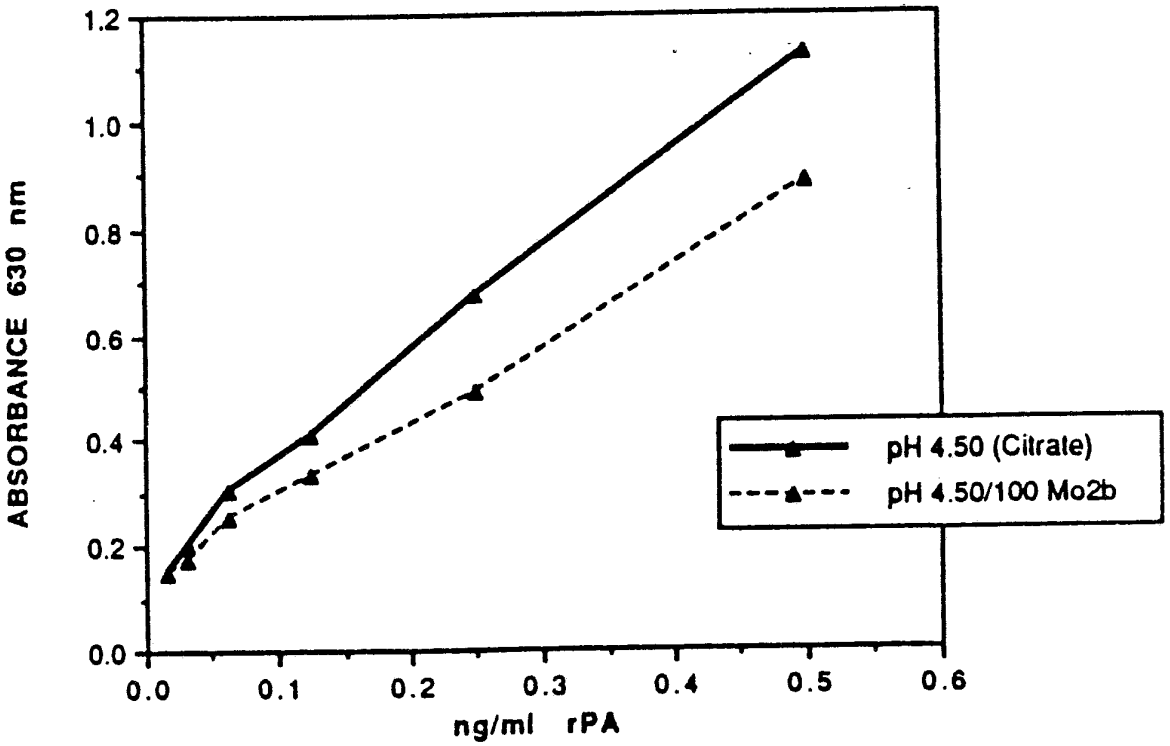

ABSORBANCE 630 nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ng/ml rPA
pH 4.50 (Citrate)
pH 4.50/100 Mo2b

FIG. 8F

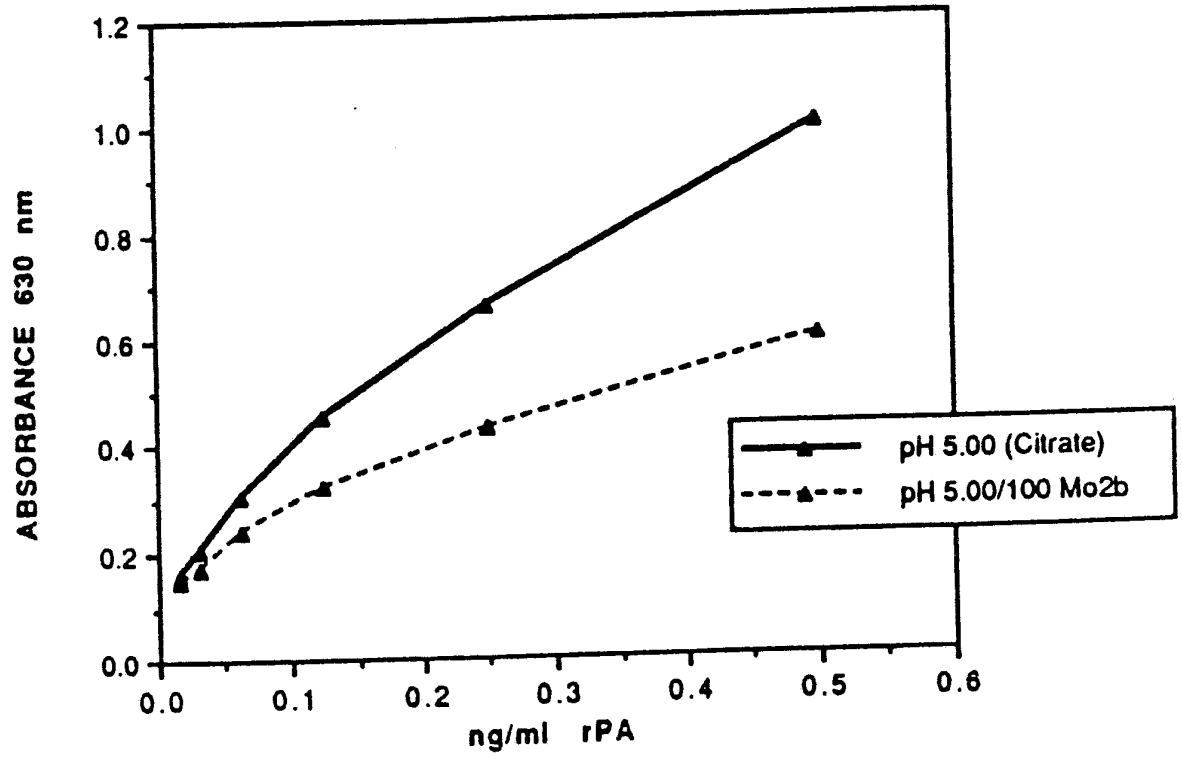

ABSORBANCE 630 nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ng/ml rPA
pH 5.00 (Citrate)
pH 5.00/100 Mo2b

FIG. 8G

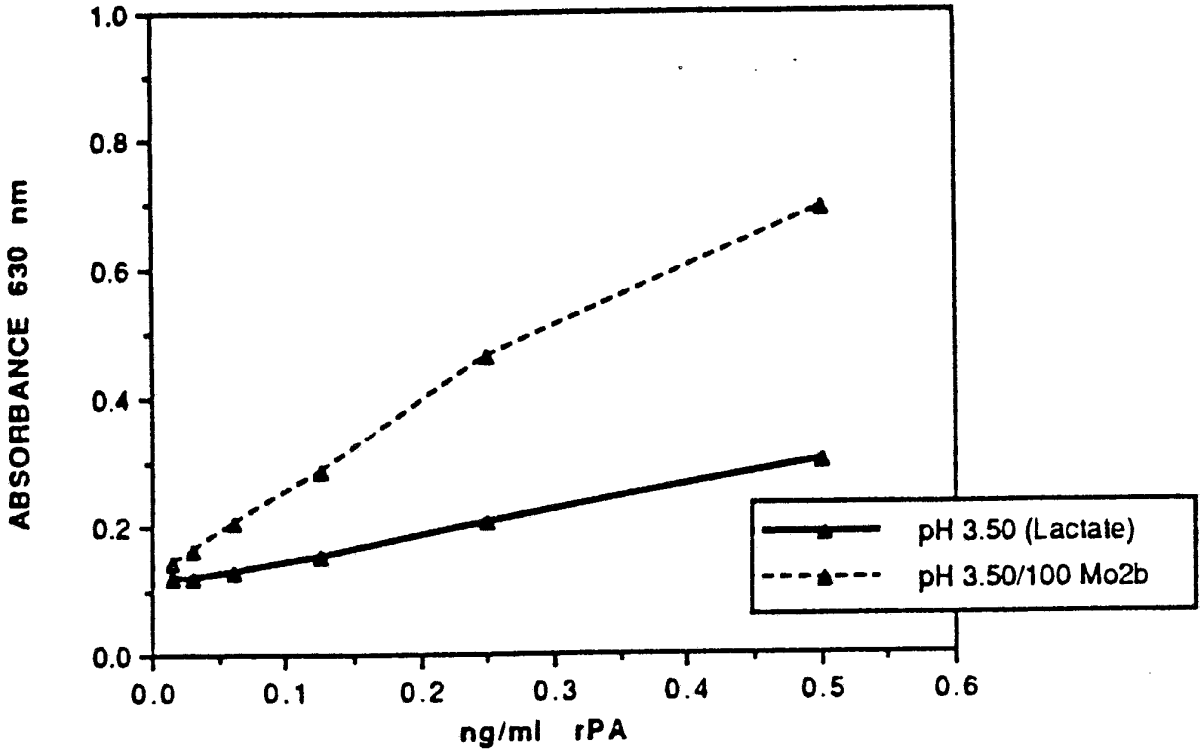

ABSORBANCE 630 nm
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ng/ml rPA
pH 3.50 (Lactate)
pH 3.50/100 Mo2b

FIG. 9A

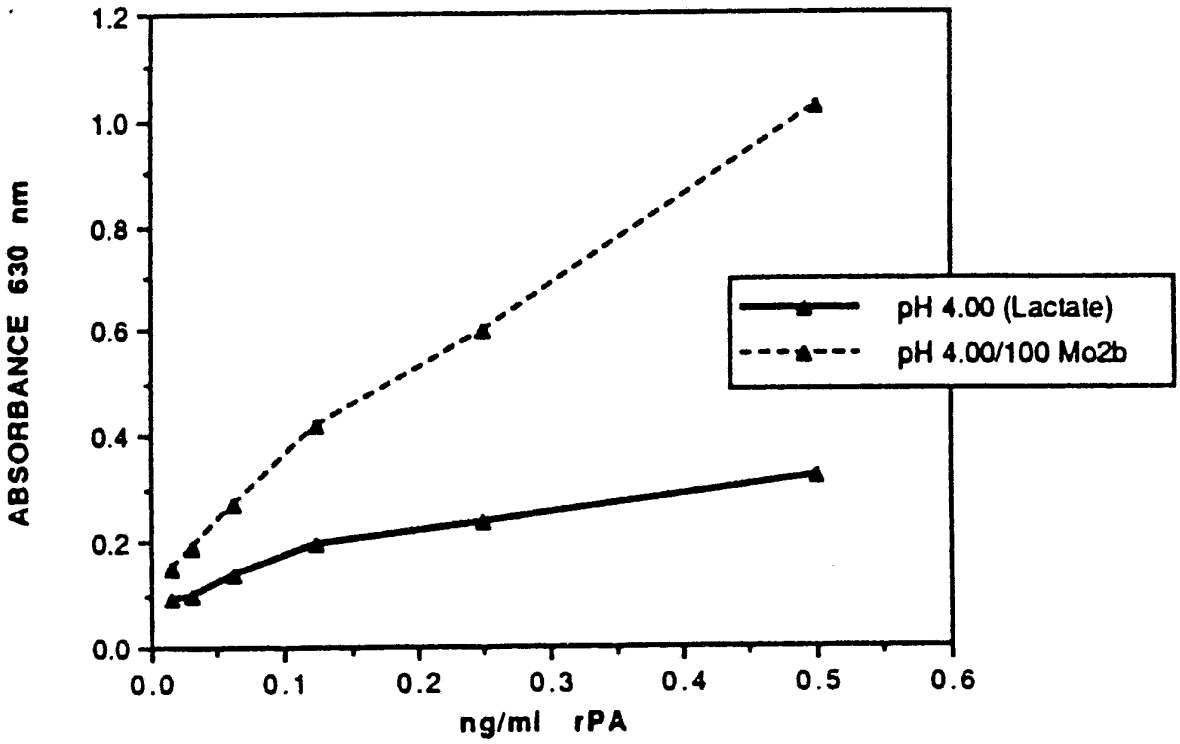

ABSORBANCE 630 nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ng/ml rPA
pH 4.00 (Lactate)
pH 4.00/100 Mo2b

FIG. 9B

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/00438

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

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

IPC(5): G01N 33/543, 33/53, 33/68

US.CL.: 436/518

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S. Cl.	436/501, 518,529,531; 435/7.33,7.92; 530/413; 435/7.94

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS DIALOG, BIOSIS, Medline, search terms: affinity chromatography or purif?, protein A, mouse or murine 5a monoclonal antibod ?, (formate or acetate)(w)(buffer#)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	European Journal of Clinical Investigation, Vol. 16, issued 1986, Kinet et al., "Ex vivo perfusion of plasma over protein A columns in human mammary adenocarcinoma. Evidence for a protein A leaking by radioimmunoassays" see pages 43-49, especially see Abstract.	1,10,12-14
Y	R.M.C. Dawson et al., "DATA for BIOCHEMICAL RESEARCH" published 1969 by Oxford University Press (Oxford), see page 481.	2-4,6-9,11
Y	JOURNAL OF IMMUNOLOGY, Volume 133,No6, issued December 1984, Young et al, "Staphylococcal Protein A Binding to the to the Fab Fragments of mouse monoclonal Antibodies," pages 3163-3166, especially see Abstract and first paragraph on page 3163.	1, 6-10

* Special categories of cited documents: [10]

"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

20 March 1991

Date of Mailing of this International Search Report

02 MAY 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

Carol E. Bidwell

Form PCT/ISA/210 (second sheet) (rev. 11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US, A, 4,752,571 (BERGLUND ET AL.) 21 June 1988, see entire document, especially column 2, lines 31-51.	1-14
Y	Journal of Immunological Methods, Vol. 83, No.1, issued 24 October 1985, Dertzbaugh et al. "An Enzyme Immunoassay for the Detection of Staphylococcal Protein A in Affinity-Purified Products," see pages 169-177, especially see "Introduction" pages 169-177.	1,6-10 12-14
Y	Journal of Immunological Methods, Volume 117, No. 1, issued 08 February 1989, Bloom et al. "Detection and reduction of Protein A contamination in immobilized Protein A purified monoclonal antibody preparations", see pages 83-89, especially see abstract and page 87 last paragraph continuing on page 88.	1,10-14
Y	JOURNAL OF CLINICAL MICROBIOLOGY, volume 27, No. 12, issued December 1989, Larsson et al., "Novel Latex Agglutination Method with Chicken Anti-Protein A for Detection of Staphylococcus aureus Infections", see pages 2856-2857, especially page 2856, column 1, third paragraph.	1, 5,10, 12-14