THE DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY(ELISA) FOR THE DETECTION OF ANTIBODIES TO *TREPONEMA PALLIDUM* SUBSPECIES *PALLIDUM*

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DEDICATION

To my Father

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Abstract

An enzyme linked immunosorbent assay (ELISA) was developed for the detection of specific antibodies to *Treponema pallidum* subspecies *pallidum*, the causative agent of syphilis. When compared to a commercial (DCL) Syphilis G ELISA, the newly developed Syphilis ELISA was found to have a relative sensitivity of 98.7%, a relative specificity of 99.5%, and overall agreement of 99.3%. The assay was also shown to have a relative sensitivity of 92.7%, a relative specificity of 99.5%, and 97.5% agreement when compared to the FTA-ABS Assay, the current assay of choice for the confirmation of treponemal antibodies produced in response to syphilis infection.

The Syphilis ELISA showed excellent intra-assay and inter-assay reproducibility. There was < 10% intra-assay coefficient of variation when a set of six serum samples with different antibody levels were run on the Syphilis ELISA, and replicated at least eight times within each assay. There was < 20% inter-assay coefficient of variation when the same six samples were run on the Syphilis ELISA on three separate days.

Accelerated stability testing was initiated on the solid phase antigen coated plates used in the Syphilis ELISA. The data accumulated showed that the antigen coated plates were stable at least eight weeks when stored at 2-8^{0}C. Accelerated stability testing was also completed for eight weeks with plates stored at 22-25^{0}C, 37^{0}C, and 45^{0}C. The data generated

showed that there was acceptable stability at both 37^0C, and 45^0C for at least eight weeks.

Overall, it was shown that a sensitive, specific ELISA was developed for the detection of antitreponemal antibodies. The Syphilis ELISA demonstrated sensitivity and specificity comparable to that of the DCL Syphilis G Assay and the FTA-ABS test. The Syphilis ELISA will be a rapid, objective alternative for the confirmation of a syphilis infection.

INTRODUCTION

The disease syphilis was first reported in medical literature in 1495, however, it wasn't until 1905 that the causative agent, *Treponema pallidum* was discovered (44). *Treponema pallidum* is a microaerophilic, gram negative spirochete belonging to the Genus *Treponema*, Order *Spirochaetales*, and Family *Spirochaetaceae* (11,15,17,44). The genus Treponema contains at least four pathogenic bacteria and six non-pathogenic bacteria (15). Currently, treponemes are now divided into culturable organisms (non-pathogens), and non-culturable organisms (pathogens) (15).

The four pathogenic bacteria consist of *Treponema pallidum* subspecies *pallidum*, the causative agent of syphilis; *Treponema pallidum* subspecies *pertenue*, the causative agent of yaws; *Treponema pallidum* subspecies *endemicum*, the causative agent of endemic syphilis; and *Treponema carateum*, the causative agent of pinta (15,17). Currently, the four pathogenic bacteria can only be differentiated by epidemiology, clinical manifestation, and mode of transmission since a subspecies specific antigen has not yet been identified that would allow a true clinical or serological diagnosis to be made (15). A definitive diagnosis is made increasingly difficult due to the fact that all treponemes are morphologically identical in size and shape and that the

serological and biochemical reactions are the same for each subspecies (15,17). Similarily, disease differentiation is difficult because the four pathogenic treponemes are considered to be non-culturable, although maintenance *in vitro* for 1-6 days has been reported using a maintenance medium consisting of Nelson medium, rabbit serum, cysteine, thioglycollate, glutathione, vitamins, co-factors, amino acids and salts (15,44).

The six non-pathogenic treponemes have been found to make up part of the normal human flora, and can be found in the oral cavity and the genital and intestinal tracts. Some of the non-pathogenic treponemes may be associated with gingivitis and periodontal disease (15,17).

The disease syphilis is transmitted through direct sexual contact but can also be spread through contact with infectious secretions. Primary syphilis begins with a generalized bacteremia within approximately 30 hours after infection (24). The incubation period for syphilis generally lasts about three weeks but could also range between 10-90 days (4,11,31,44). At the end of the incubation period a primary inflammatory lesion or chancre appears at the site of initial contact and is the site at which the spirochetes proliferate. This lesion exists for 1-5 weeks, after which secondary syphilis develops. This stage of syphilis is characterized by a generalized illness with symptoms suggesting a viral infection, including headache, sore throat, low grade fever

and nasal discharge (4,44). The latent stage of syphilis follows secondary syphilis and it is during this non-infectious period that diagnosis can only be made serologically. Tertiary syphilis follows 3-10 years after the primary infection in untreated syphilis. It is during the tertiary stage of infection that numerous sequelae may develop including destructive granulomas, cardiovascular manifestations, and central nervous system involvement (4,44).

The first diagnostic blood test for syphilis, the Wassermann test, was developed in 1906. Previously, direct microscopic darkfield examination of lesion material was the only means of identifying a syphilis infection (44). Current serological tests for syphilis have been divided into two groups based on the type of antibody each detects. A non-treponemal test detects non-specific antibody (reagin) that is produced during syphilis infection against negatively charged phospholipids of the cellular mitochondrial inner membrane (11). This antibody reacts with cardiolipin which is the basis for several non-treponemal flocculation tests including the Rapid Plasmin Reagin (RPR) test and the Venereal Disease Research Laboratory (VDRL) assay (24,44). The non-treponemal tests are instrumental in the diagnosis of early stages of syphilis, as well as in the monitoring of the antibody response during treatment with antibiotics. The non-treponemal tests tend to parallel the course of infection with *Treponema pallidum* by showing increasing titers during

clinical infection and decreasing titers during latency (15,17). Patients with other infectious diseases such as measles, chicken pox, hepatitis, leprosy, tuberculosis, and malaria can also produce reagin or non-specific antibody, thus increasing the incidence of false positive results in a non-treponemal assay. Other conditions that may cause false positive non-treponemal test results include autoimmune disorders, drug addiction, old age, pregnancy, and recent immunization (44). Since the incidence of biological false positive reactions with non-treponemal assays may be as high as 70% in some populations, confirmation by a treponemal test is strongly recommended to establish or rule out a diagnosis of syphilis (24).

Treponemal tests, such as the Fluorescent Treponemal Antibody-Absorption (FTA-ABS), Hemagglutination Inhibition (HAI), or Microhemagglutination Assay for Antibodies to *Treponema pallidum* (MHA-TP), detect antibodies specific for treponemal antigens (15,17,24). The treponemal tests may not show increasing antibody titers until after the first signs of clinical manifestation. Likewise, antibody titers may remain high during latency and after treatment (15,17). The current recommendation for serological testing as set forth by the World Health Organization, consists of initial screening with the non-treponemal VDRL assay, followed by confirmation with the treponemal FTA-ABS assay (15).

The Enzyme-Linked Immunosorbent Assay (ELISA) offers a

sensitive and specific alternative to existing testing methods for syphilis and other infectious diseases (44). The ELISA has been shown to have several advantages over current test procedures. For example, the ELISA is simple, rapid, and results are read objectively. It also lends itself easily to increased automation (44). In 1984, the first commercially available ELISA test for syphilis was developed and received provisional status (24). This assay, the Syphilis Bio-Enzabead, was found to be comparable to the FTA-ABS and VDRL assays, however, the test was subsequently removed from the market (24). The purpose of this study was to develop an ELISA for the detection of *Treponema pallidum* antibodies, that is as specific and sensitive as the FTA-ABS test, which can be used as an alternative testing methodology for the confirmation of *Treponema pallidum* antibodies.

MATERIALS AND METHODS

Treponema pallidum, Nichols strain antigen (Catalog #M92725-50BC) was obtained from Lee Laboratories, Grayson, Georgia. The *Treponema pallidum* antigen was supplied as a suspension of intact organisms (approximately $6X10^8$ organisms/ml) that had been extracted from syphilis infected rabbit testicular tissue. Samples of each antigen preparation were evaluated by darkfield microscopy with a 40X objective, using oil immersion.

The protein concentration of each antigen was determined using the Bicinchoninic Acid (BCA) Protein Assay microtiter plate protocol supplied by Pierce (Rockford, Illinois). Briefly, a set of protein standards was prepared by diluting the Bovine Serum Albumin Standard (BSA) from 2 mg protein/ml to .0625 mg/ml. Working reagent was then prepared by combining 50 parts Reagent A with 1 part Reagent B. Ten microliters of each standard, and unknown sample was placed in appropriate wells of a microtiter plate. Two hundred microliters of working reagent was then added to each well and the samples were mixed on a microtiter plate shaker for 30 seconds. The microtiter plate was covered and incubated at 37^0 C for 30 minutes. After 30 minutes the absorbance of the plate was read on a microtiter plate reader at a wavelength of 562 nm. A standard curve was prepared by plotting the absorbance at 562 nm. vs. protein concentration. This standard curve was then used to determine the protein

concentration of the *Treponema pallidum* antigen sample.

Antigen Coating Procedure

The *Treponema pallidum* antigen was sonicated for 3 minutes using a VibraCell sonicator with a microtip and a setting of 3.5. Following sonication the antigen was again observed using darkfield microscopy to ensure adequate disruption of the treponemes. The sonicated antigen was diluted in Carbonate/Bicarbonate coating buffer (1.59 g Na_2CO_3 H_2O, 2.93 g $NaHCO_3$, 0.2 g Thimerasol, 1.0 L DiH_2O, pH 9.6), and used to coat microtiter plates (Nunc, Denmark) at antigen dilutions of 1:50,1:100,1:200,1:400,1:800, and 1:1600 (125 ul/well). Following an overnight incubation at 2-8^{0}C, the antigen containing solution was removed by aspiration and the wells were "blocked" using a solution of concentrated protein, Bovine Serum Albumin (BSA). The blocking solution was aspirated, the plates were dried at room temperature, and then packaged with dessicants for further testing.

Titration of *Treponema pallidum* Antigen

Using plates coated previously at antigen dilutions 1:50, 1:100,1:200,1:400,1:800, and 1:1600, an antigen block titration was set up using a known syphilis positive serum sample and a known syphilis negative serum sample. The assay was performed as follows:

1. The positive and negative serum samples were diluted 1:21 in sample diluent (Zeus Scientific, Cat. #007). One hundred microliters of diluted sample was added to wells of the

microtiter plate as follows: the positive to rows A,C,E,G and the negative to rows B,D,F, and H.

2. The plate was incubated for 20 minutes at room temperature. Goat anti-Human IgG Horseradish Peroxidase (HRP) conjugate (O.E.M Concepts, Toms River, NJ) was diluted 1:2,000,1:4,000,1:8,000, and 1:16,000. The plate was washed three times using a microplate washer with PBS-Tween 20 (Zeus Scientific, Cat. #0008L) as the wash buffer. One hundred microliters of the diluted HRP conjugate was added to the appropriate wells: 1:2,000 rows A&B, 1:4,000 rows C&D, 1:8,000 rows E&F, and 1:16,000 rows G&H. The plate was then incubated at room temperature for an additional 20 minutes.

3. After incubation the plate was again washed three times in a microplate washer using PBS-Tween 20. One hundred microliters of substrate solution, 5,5,5',5'-Tetramethylbenzidine Hydrochloride (TMB) (Zeus Scientific, Cat. #002) was added to each well of the microtiter plate and incubated at room temperature for 10 minutes.

4. The assay was stopped by the addition of 50ul stop reagent (4.0 N H_2SO_4), and read spectrophotometrically at 450nm.

5. The optimal antigen dilution chosen was that which demonstrated the best discrimination between positive and negative samples, as shown by the positive/negative ratio.

Antigen Processing

Several different antigen processing procedures were used in an attempt to reduce the overall background of the assay. Because the *Treponema pallidum* organism must be grown intratesticularly in rabbits, treponemal antigens are difficult to produce and are often contaminated with rabbit tissue components (4). The first antigen processing procedure used was to wash the antigen twice in 30 ml Phosphate Buffered Saline (PBS) by centrifugation (5000 X g, 10 min.), and resuspension. Following the last centrifugation, the pellet was resuspended to the starting volume with PBS + .01% NaN_3. A BCA protein assay was performed on both the washed and unwashed antigen preparations.

A second antigen purification method utilized was Percoll Density Gradient Centrifugation as described by Hanff, et al (19). Briefly, the procedure used was as follows:

1. Percoll (Pharmacia, LKB) was diluted 1:9 with 1.5M NaCl (10X FTA PBS Buffer, Zeus Scientific, Cat. #7008). This was considered to be the "working Percoll Solution".
2. The working Percoll solution was diluted to 43% Percoll with .15M NaCl (1X FTA PBS Buffer, Zeus Scientific).
3. *Treponema pallidum* antigen was diluted 1:2 in the 43% Percoll: 3ml *T.pallidum* to 6ml Percoll (43% Percoll).
4. The antigen, and a separate centrifuge tube containing Density Gradient Beads and Percoll, were centrifuged at 34,800 X g for 20-30 minutes.

5. Five separate bands could be easily distinguished. Each band was removed and tested separately.

6. The five fractions were diluted with 1X PBS and centrifuged at 100,000 X g for one hour to remove the remaining Percoll from the fractions (Depercollization).

7. The *Treponema pallidum* pellet was carefully removed from each fraction using a Pasteur pipette and observed using darkfield microscopy for the presence of viable treponemes.

8. The fraction with the greatest number of treponemes present in the most purified form, as identified microscopically, was BCA protein tested, sonicated, and an antigen titration was performed to determine an optimal coating concentration.

Sample Diluent Evaluation

A variety of different sample diluent configurations were evaluated in an attempt to reduce non-specific reactivity in the assay as well as improve the signal-to-noise ratio. Each diluent configuration was introduced into the assay independently.

The sample diluents tested included:

A. PBS + .05 % Tween 20

B. PBS + .05 % Tween 20 + 5 % Rabbit Serum

C. PBS + .05 % Tween 20 + 5 % Proprietary Additive

D. Spirolate Broth

E. Spirolate Broth + 5 % Rabbit Serum

F. Spirolate Broth + 5 % Proprietary Additive

G. Lyme ELISA Sample Diluent + 5 % Rabbit Serum

H. Lyme ELISA Sample Diluent + 5 % BSA

I. Sorbent

J. Sorbent + 5 % Proprietary Additive

K. Sorbent + 5 % Proprietary Additive + 5 % Rabbit Serum

PBS/Tween 20 is a standard starting point for the development of ELISA sample diluents. Rabbit serum and BSA are frequently used as sources of protein to reduce non-specific binding of antibodies (including conjugate) to an ELISA solid phase. The Lyme ELISA sample diluent contains a sonicated preparation of Reiter's treponemes and a proprietary additive which has been demonstrated to reduce the non-specific adsorption of immunoglobulins to a variety of immunoassay substrates and solid phases. Sorbent is prepared from cultures of non-pathogenic Reiter's treponemes and is used as sample diluent in the FTA-ABS test.

Each sample diluent configuration was tested using a set of reactive samples which were selected from a normal plasma donor population because of their reactivity in the Syphilis ELISA. In addition, a known positive and negative sample was included in each run. An attempt was made to identify the sample diluent that would give the lowest background using the normal population samples, reduce the activity of the non-specific samples to background levels, and maintain the activity of the positive samples.

RPR Assay Procedure

All of the samples used in the development of the

Syphilis ELISA were first screened using the RPR assay (Becton Dickinson, Cockeysville, MD), and the results confirmed using the FTA-ABS assay. The RPR antigen suspension used as the basis for this flocculation test consists of .003% cardiolipin, .020-.022% lecithin, .09% cholesterol, 10% choline chloride, .0125M EDTA, .01875% charcoal, .01M Na_2HPO_4, .01M KH_2PO_4, and .1% Thimerosal (24). In the RPR assay, the RPR antigen is mixed with patient or control serum on a plastic coated card. If antibodies are present, they combine with the lipid particles of the antigen, causing agglutination to occur. This agglutination shows up as black charcoal clumps against the white card. The results reported using the Qualitative RPR test procedure are either reactive (R), or non-reactive (NR). A sample is considered reactive if there is characteristic clumping present, ranging from marked and intensely reactive to slight but definitely reactive. A sample is non-reactive if there is slight roughness or no clumping present in the assay (24).

FTA-ABS Assay Procedure

In addition to the RPR assay, all samples used in this study were tested using the FTA-ABS assay (Zeus Scientific, Cat. #7001). In the FTA-ABS assay, non-viable *Treponema pallidum* (Nichols strain) antigen is used as the substrate. The substrate is reacted with patient samples and kit controls that have been previously heat-inactivated and treated with sorbent to reduce non-specific reactivity that might be

present. If the sample contains specific treponemal antibodies, an antigen-antibody reaction takes place between the cell substrate and the anti-treponemal antibodies in the patient samples. Goat anti-human IgG labeled with fluorescein isothiocyanate (FITC) is then added to the substrate as an indicator. When examined with a fluorescence microscope, the intensity of the fluorescence staining is graded on a scale of 1+ to 4+, or negative, indicating no fluorescence. The FTA-ABS assay is considered to be a treponemal test for syphilis, and is designed to confirm positive samples on non-treponemal tests such as the RPR or VDRL assays.

DCL Syphilis IgG Assay

The Diagnostic Chemicals Limited (DCL) Syphilis IgG assay, an enzyme immunoassay for the detection of IgG antibodies to *Treponema pallidum* in human serum, became available during the course of this study and was also used as a basis for sample comparison. The assay was performed according to the manufacturer's package insert. The assay procedure is as follows: wells of a microtiter plate are coated with *Treponema pallidum* antigen to which specific antibodies present in the test serum will bind. Any unbound serum components are then rinsed away during the wash procedure. Specifically bound IgG is traced during the second incubation with biotinylated anti-human IgG monoclonal antibody labeled with streptavidin-HRP. Following a second wash procedure any bound enzyme is detected by reaction with

a substrate. The intensity of the colored product is proportional to the serum concentration of *Treponema pallidum* specific IgG. The assay was used as an aid in the determination of the relative sensitivity and specificity of the Syphilis ELISA assay currently under development.

Serum Sample Selection

In order to develop this assay a wide variety of serum samples were required. These samples were obtained from several sources. Several samples used in the beginning of the developmental phase of the assay were obtained from Zeus Scientific serum sample inventory, and these samples were characterized in house by RPR and FTA-ABS. Other FTA-ABS and RPR positive samples were obtained from a variety of serum vendors, and the samples were then verified as positive or negative upon receipt. Two normal human serum panels were also obtained from serum vendors. These normal panels were tested, and used as the basis for the determination of assay cut-off, by providing a fair representation of the background reactivity in a normal population. Clinical serum samples (n=32) were obtained from a reference clinical laboratory that had been previously screened and found to be MHA-TP positive. Additional clinical samples (n=15) were obtained from a Public Health Department. These serum samples had been tested using the Unheated Serum Reagin (USR) test, a test similar to RPR and VDRL, and a Hemagglutination (HA) assay. One sample in the last group was obtained from a patient that was diagnosed

with primary syphilis. Therefore, the samples used in the development of the Syphilis ELISA reflect a variety of syphilis antibody specificities and provide an appropriate population of reactivities for setting a positive/negative cut-off.

Sensitivity and Specificity Testing

The "relative" sensitivity and "relative" specificity of the ELISA assay was determined by comparing results obtained on a series of both positive and negative samples. The samples were assayed using the FTA-ABS Procedure, the DCL Syphilis G assay and the newly developed Syphilis ELISA. Clinically, sensitivity is considered to be the percentage of reactive results obtained in a population of persons with syphilis. Likewise, specificity is defined as the percentage of non-reactive results obtained in a population of persons without syphilis (24). Thus, sensitivity is calculated from the number of true positives (TP) detected in a diseased population divided by the number TP plus the number of false-negatives (FN) results (24). The formula is as follows:

% SENSITIVITY = TP/TP+FN X 100

Specificity is determined in a population of people without syphilis (TN) and is calculated as follows:

% SPECIFICITY = TN/TN+FP X 100

Specificity refers to the possibility of obtaining false positive results using the assay in question (24). The "relative" sensitivity and "relative" specificity of the assay

was determined using both the FTA-ABS and DCL-ELISA assays as "gold standard" methodologies since the clinical diagnoses were not available for all specimens.

Precision and Intra\Inter Assay Testing

The precision of the Syphilis ELISA was determined by assaying a well characterized set of samples (n=6). A high positive, mid-positive, low positive, equivocal, negative and high negative sample was chosen to give a broad range of reactivity in the assay. Intra-assay reproducibility was determined by simultaneously assaying the six different samples 32 times. Inter-assay reproducibility was determined by assaying the same set of six samples in three separate assays on three different days. Each specimen was replicated at least eight times within each assay. The standard deviations and coefficients of variation were calculated and results recorded. The coefficient of variation (CV) is a measure of the precision of the assay and is derived by dividing the standard deviation (SD) by the mean value (X) for that sera and is expressed as a percentage.

Cross Reactivity Testing

Possible causes of biological false positives in specific serologic tests for syphilis include: Lupus Erythematosus, Rheumatoid arthritis, hyperglobulinemia, cirrhosis, non-syphilitic treponemal infections, infectious mononucleosis, lepromatous leprosy, scleroderma, mixed connective tissue diseases, and genital herpes (11). During

the course of assay development cross-reactivity testing was completed using 66 samples identified to have potentially cross-reacting antibodies. These samples were shown to be Anti-Nuclear Antibody (ANA) positive samples(n=36), Lyme IgG positive samples(n=8), and routine samples collected during pregnancy(n=22).

Stability Testing

Stability of a pharmaceutical or diagnostic product is an integral element of the production of a quality product. Actual elapsed time (or real time) stability studies are the most reliable demonstration of a product's usefullness or shelf-life. The length of time required for these stability studies, however, often makes them impractical, therefore, accelerated degradation testing have often been employed (10). An accelerated degradation test may be conducted by placing samples at a series of elevated storage temperatures and comparing their activities with those of samples stored at a suitable low temperature reference (10).

Accelerated stability testing was performed on the solid phase plate to determine the rate at which degradation of the syphilis antigen coated plate occurs. Microtiter plates coated with antigen #M28449 at a 1:100 dilution were placed at four different temperature conditions: 2-8^{0}C, 22-25^{0}C (Room Temperature), 37^{0}C, and 45^{0}C. The plates were tested on a weekly basis starting at week 0 and continuing through week 8. Each week the plates were removed from their respective

storage conditions, and three strips from each plate were used to complete the assay. The assay was standardized at week 0, with an optimal sample diluent and conjugate dilution chosen. Specific panel members were selected and run each week in an attempt to determine the point at which the degradation of the antigen affects the performance of the assay.

RESULTS

Comparison of Antigen Processing Procedures

In an effort to determine the optimal antigen for use in the Syphilis ELISA a variety of antigen processing procedures were used (Table 1). To assess improvements in specific positive signals as well as reduction in background noise, positive/negative (P/N) ratios were generated for each antigen treatment, using validated positive and negative samples. The first two antigens optimized, M27417 and M27269, were sonicated as part of the antigen processing procedure. Antigen M27417 appeared to give a P/N ratio twice as high as antigen M27269 (7.97 vs. 4.13), however, when the P/N ratios were normalized using the coating concentration of protein, the difference in antigen performance was minimal (1.53 vs. 1.12).

Antigen M27269 was further evaluated by a 2X wash in PBS. This procedure resulted in a decrease in the total protein concentration of the antigen, but also increased the P/N signal. The P/N ratio of washed and sonicated M27269 was 11.82 vs. 4.13 for sonication only. The difference was even more dramatic when the normalized values were used for comparison (7.48 vs. 1.12). The wash procedure may have decreased the amount of rabbit tissue components often found contaminating *T. pallidum* antigen preparations, which would account for the decrease in total protein concentration.

Percoll purification was performed using antigen M27267.

Table 1: COMPARISON OF *T. PALLIDUM* ANTIGEN PROCESSING PROCEDURES. The effect of antigen processing procedures on protein concentration, coating dilution, coating concentration, P/N ratio, and P/N ratio/protein concentration in the Syphilis ELISA.

TABLE 1: COMPARISON OF T. pallidum ANTIGEN PROCESSING PROCEDURES

ANTIGEN		TREATMENT	PROTEIN (mg/ml)	COATING DILUTION	COATING CONC. (ug/ml)	POSITIVE SAMPLE O.D.	NEGATIVE SAMPLE O.D.	P/N RATIO	P/N RATIO/ ug PROTEIN
1.	M27417	SONICATION	2.070	1:400	5.200	1.880	0.236	7.970	1.530
2.	M27269	SONICATION	1.470	1:400	3.700	1.136	0.275	4.130	1.120
3.	M27269	WASHED/SONICATED	0.630	1:400	1.580	1.549	0.131	11.820	7.480
4.	M27267	PERCOLL PURIFICATION SONICATED							
		BAND #1	0.245	1:70	3.500	1.220	0.234	5.210	1.490
		BAND #2	3.110	1:900	3.460	1.610	0.187	8.610	2.490
		BAND #3	3.210	1:900	3.570	1.270	0.200	6.350	1.780
		BAND #4	1.730	1:500	3.460	1.810	0.090	20.110	5.810
		BAND #5	0.540	1:150	3.600	0.663	0.083	7.980	2.220
5.	M27935*	PURIFIED/SONICATED	0.920	1:200	4.600	1.720	0.020	86.000	18.700

*PURIFIED AT LEE LABORATORIES USING A PROPRIETARY PROCESS

Five distinct bands were formed during the ultra-centrifugation step. When separated and viewed using darkfield microscopy Band #4 contained the highest density of treponemes, with the least amount of debris. Band #5 was shown to contain a large quantity of rabbit spermatozoa when observed using darkfield microscopy. The protein concentration of each band was determined using the BCA protein assay. The protein concentration of bands #2 and #3 were the highest with 3.11 mg/ml and 3.21 mg/ml respectively. Band #4 which contained the most purified treponemes had a protein concentration of 1.73 mg/ml. Each band was tested separately and the P/N ratios compared for each band. As anticipated from the microscopic evaluation, band #4 had the highest P/N ratio, 20.11. Band #2 and Band #3 had lower P/N ratios than Band #4 which could have been related to contaminating rabbit tissue components. Band #1 did not contain much material and Band #5 contained spermatozoa. The P/N ratios for Band #1 and #5 were also low. Since each band was evaluated at a comparable protein concentration, normalization to protein did not alter the relative differences between bands. Interestingly, once purified through Percoll and normalized for protein, Band #4/antigen M27267 was no better than antigen M27269 which had only been washed. Because of this phenomenon, the added complexity of the Percoll steps and the overall loss of material, Percoll density centrifugation was not considered a useful procedure

for antigen processing.

Antigen M27935 was received from Lee Laboratories in a purified form. The purification method is considered proprietary information. When optimized the P/N ratio was found to be 86.00, 18.70 after normalization. This ratio was a direct result of an increase in positive signal, with a concomitant decrease in the negative sample O.D. Therefore, this antigen as purified by Lee Laboratories resulted in a decrease in background reactivity (noise) while increasing the reactivity of specific positive samples. A coating dilution of 1:200, and good antigen availability makes this the antigen of choice for the Syphilis ELISA. In addition to antigen M27935, several other purified antigen preparations were procured from Lee Laboratories. Each antigen was shown to be comparable to antigen M27935 (data not shown).

Determination of Assay Cut-off

Three antigen preparations from Table 1 (M27269, M27267, and M27935) were further evaluated using additional serum samples to assess the performance of the antigens with a broader spectrum of samples. A 48 member normal serum panel was used for the determination of the assay cut-off, as well as providing additional evidence to support the choice of antigen treatment. The assay cut-off (C.O.) may be defined as an experimentally derived value that delineates the breakpoint between samples that are *T. pallidum* antibody positive and *T. pallidum* antibody negative. The normal panel was tested with

each antigen treatment, and the mean and standard deviation was then calculated. The mean of the normal samples plus three standard deviations was used as the basis for the assay "cut-off". A number of syphilis positive samples were also used in each assay to assess the validity of the assay "cut-off". The graphical representations of the effect of antigen processing and the assay "cut-off" are depicted in Figures 1-3. With each different antigen preparation, the graphs show the difference between assay "cut-off" and the distance between the positive and negative samples. When choosing an antigen concentration for use in the ELISA it is desirable to maximize the distance between the antibody positive samples and the antibody negative samples to show good separation and discrimination between both sample populations. In Figure 1 the separation between the positive and negative samples is less than it is in Figure 2 or 3. By using the mean plus three standard deviations as the "cut-off" it is evident that washing and sonicating the antigen is not an adequate method for antigen preparation as several positive samples are found below the "cut-off" value. In addition, the absolute O.D. values for this antigen are lower than the values for the other two antigens. In Figure 2 it is evident that the mean O.D. of the normal samples is much lower than it had been previously thus making the "cut-off" much lower as well. There is a significant increase in the separation between the positive and negative samples, and there are no positive

samples that are found below the assay "cut-off". In Figure 3, the representation of the assay "cut-off" for the purified antigen from Lee Laboratories is shown. The "cut-off" for this antigen preparation is higher than it had been with the Percoll purified antigen, however, the separation between the positive and negative samples is still significant. Only one positive sample was below the assay "cut-off". By comparing the data in Table 1, showing the positive/negative ratio for each antigen preparation, with the graphs shown in Figure 1-3, a definite correlation can be seen between positive/negative ratio,"cut-off" and the spread between the positive and negative samples. The data generated on each of the different antigen preparations shows that the Percoll purified antigen would be the best antigen to use in the development of the Syphilis ELISA, however, the low yield does not make this antigen a feasible, economical alternative. The antigen purified by Lee Laboratories gives excellent discrimination between the positive and negative samples, however, certain members of the "normal" panel gave elevated results. Because of the good availability of this antigen at a moderate cost, this antigen was chosen for use in the development of the Syphilis ELISA.

To be consistent with other Zeus ELISA products, the following ratio ranges were used in the determination of the assay "cut-off":

NEGATIVE $\leq$ **0.90**

Figure 1: *T. PALLIDUM* ANTIGEN M27269-ANTIGEN WASHED AND SONICATED. A graphical representation of the effect of antigen processing on the Positive/Negative Cut-off in the Syphilis ELISA, using *T. pallidum* antibody positive sera and *T. pallidum* antibody negative sera.

FIGURE 1: ANTIGEN M27269– ANTIGEN WASHED & SONICATED
ANTIGEN PROCESSING VS. ASSAY CUT–OFF

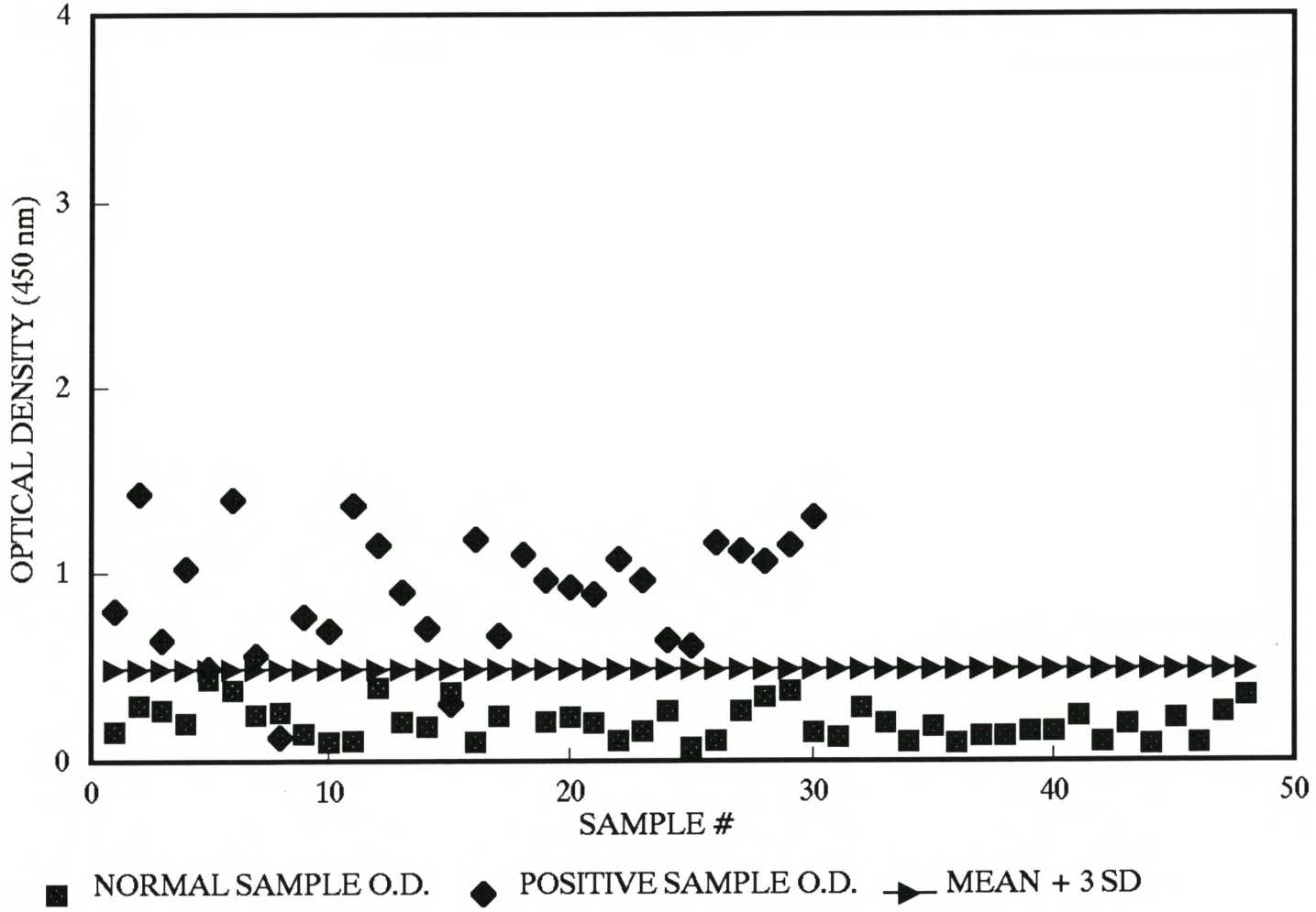


Figure 2: *T. PALLIDUM* ANTIGEN M27267-PERCOLL BAND 4/SONICATED. A graphical representation of the effect of antigen processing on the Positive/Negative Cut-off in the Syphilis ELISA using *T. pallidum* antibody positive sera and *T. pallidum* antibody negative sera.

FIGURE 2: ANTIGEN M27267–PERCOLL BAND 4/SONICATED
ANTIGEN PROCESSING VS. ASSAY CUT–OFF

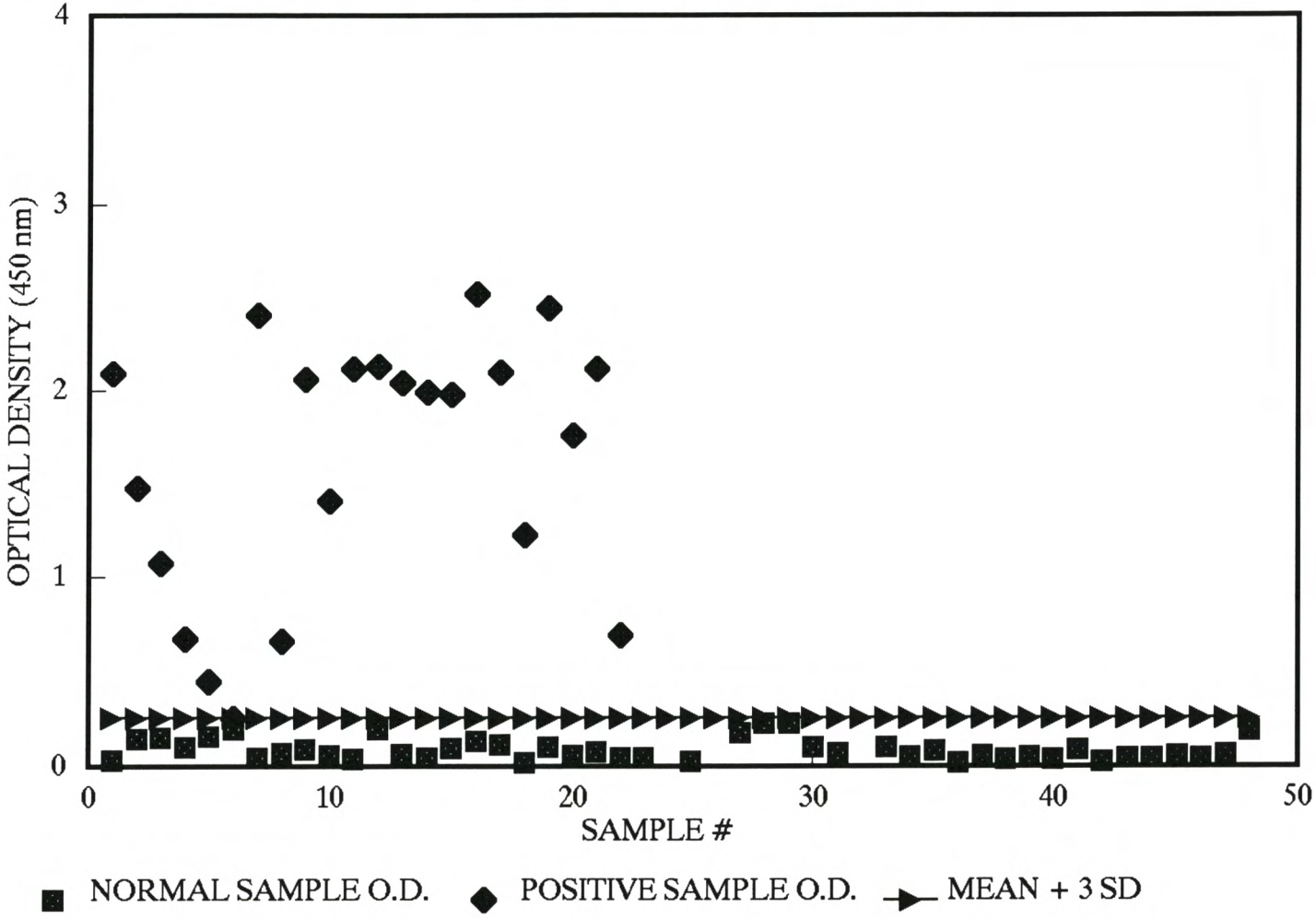
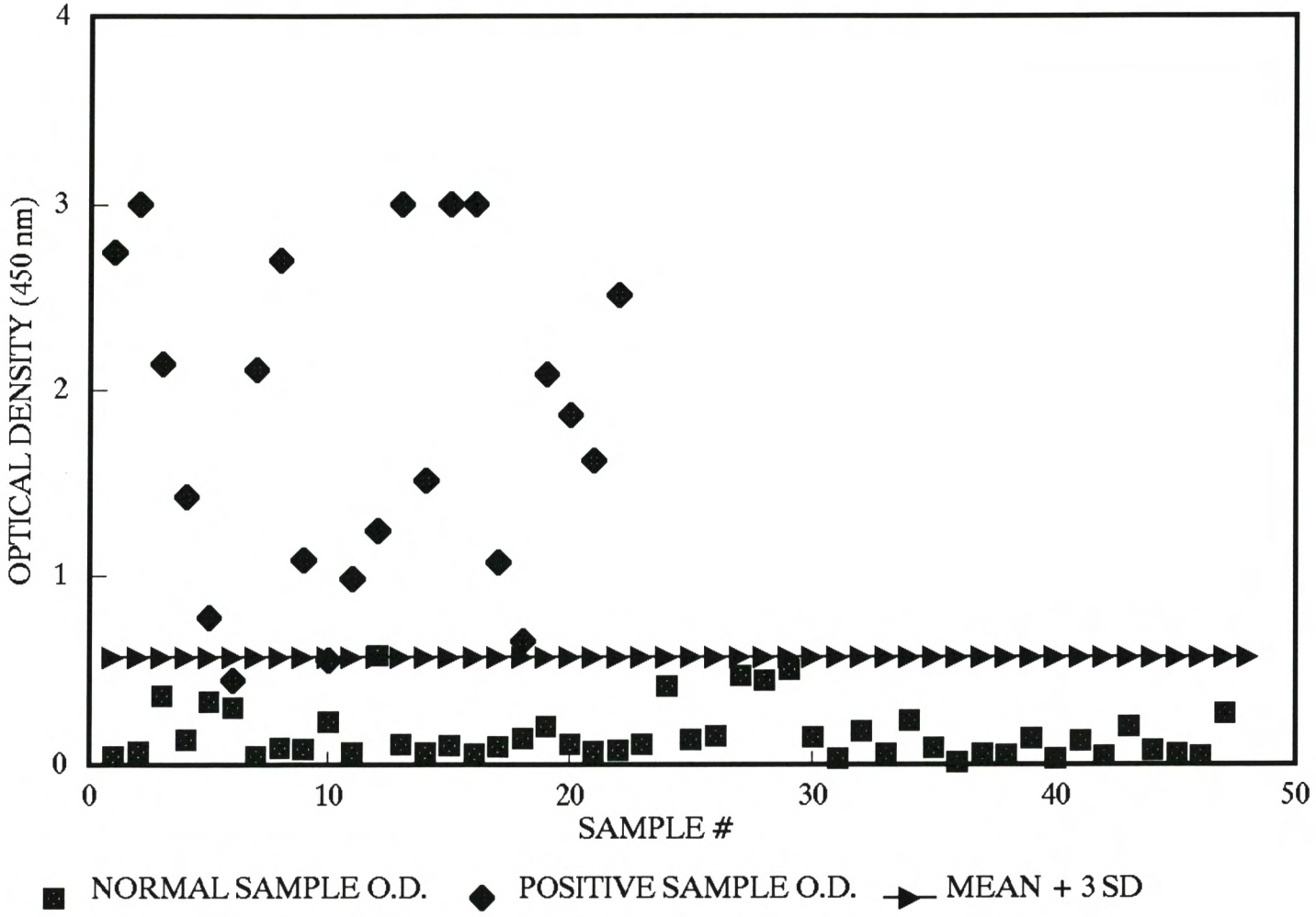


Figure 3: *T. PALLIDUM* ANTIGEN M27935-PURIFIED/SONICATED ANTIGEN FROM LEE LABS. A graphical representation of the effect of antigen processing on the Positive/Negative Cut-off in the Syphilis ELISA using *T. pallidum* antibody positive sera and *T. pallidum* antibody negative sera.

FIGURE 3: ANTIGEN M27935 PURIFIED/SONICATED–LEE LABS
ANTIGEN PROCESSING VS. ASSAY CUT–OFF



EQUVOCAL 0.91-1.09

POSITIVE ≥ 1.10

Serum sample controls (HPC, LPS, and NC) were chosen for the assay to fit certain Quality Control criteria. These criteria are as follows:

HP/LPS Ratio ≥ 1.25

NC O.D. ≤ 0.25

LPS O.D. ≥ 0.30

HPC O.D. ≥ 0.50

Once the control sera were established a correction factor was determined that gave the desired "cut-off". The "cut-off" was obtained by multiplying LPS O.D. X C.F. The "cut-off" was then used in the calculation of ratio ranges by dividing the mean sample O.D by the optimal "cut-off" value. This "cut-off" was shown to give optimal separation between the known syphilis positive and known negative samples. The use of a correction factor in the determination of an assay "cut-off" corrects for slight day to day variation in test results.

Sample Diluent Testing

To further reduce non-specific reactivity and improve the separation between positive and negative samples, a study was completed to evaluate different additives to the sample diluent. Ten serum samples from the normal plasma donor population were selected because these samples exhibited increased levels of reactivity in the Syphilis ELISA then was

expected for a normal population (all ten samples were negative by RPR and FTA-ABS). In fact, two of these samples had absorbances equal to or greater than the absorbances for the known positive sample (Table 2, A).

Ten different additive combinations (Table 2, B-K) were evaluated and compared to a diluent containing only PBS and 0.05% Tween 20; a basic formulation for sample diluents. The selected samples were diluted 1:21 in each diluent configuration and tested in the Syphilis ELISA. The optical density of each sample is shown in Table 2. In addition, a P/N ratio was calculated from the positive and negative samples included in the test. Finally, a cut-off value was calculated from the mean plus three standard deviations of the normal samples. The positive sample was also compared to this cut-off value.

As stated previously, the results from PBS/Tween diluent (Table 2,A) provide reference values for the rest of the study. The P/N ratio is 3.28 and the ratio of the positive to the cut-off (P/C.O.) is < 1.00 (0.616). Rabbit serum, an animal serum sometimes used as a protein additive to sample diluents, was added to the PBS/Tween (Table 2,B) with some improvement in results; raw absorbances were lower, the P/N ratio improved to 5.01 and the P/C.O. improved to 1.29.

A proprietary additive for sample diluents, developed and characterized at Zeus Scientific, was substituted for the rabbit serum (Table 2,C). An additional reduction in optical

density values for the normal samples was noted with a corresponding increase in the P/N and P/C.O. values. *T. phagedenis*, biotype Reiter is a treponeme that is cultured in Spirolate Broth (BBL Cat. #11636) or NIH Thioglycollate Broth (DIFCO Cat. # 0256-01-0) and used to prepare Sorbent for the FTA-ABS test. Spirolate broth alone, and in combination with rabbit serum or the proprietary additive were also evaluated as sample diluents for the Syphilis ELISA (Table 2, D-F). Spirolate broth was comparable to PBS/Tween and the Spirolate Broth plus additives exhibited little improvement.

Zeus Scientific, Inc. markets a Lyme ELISA kit that utilizes a sample diluent that contains PBS, Tween 20, proprietary additive and a sonicate of the Reiter's treponemes. This diluent was tested with the addition of rabbit serum or BSA (Table 2, G-H), and significant improvements in all the parameters(optical density value, P/N ratio, P/C.O. ratio) were evident.

Finally, the sorbent product used as a sample diluent for the FTA-ABS test was evaluated (Table 2, I-K). Sorbent alone appeared to have the most dramatic effect on raw absorbance reduction for the normal samples and improvement in the P/N and P/C.O. ratios.

The results of this evaluation indicate that sample diluents containing Reiter treponeme extracts are most useful for reducing nonspecific reactivity in the Syphilis ELISA. Therefore, sorbent was chosen as the sample diluent for

Table 2: SAMPLE DILUENT EVALUATION. The effect of sample diluent formulations on a selected set of *T. pallidum* antibody negative samples which have demonstrated elevated levels of reactivity on the Syphilis ELISA.

TABLE 2: SAMPLE DILUENT EVALUATION

SAMPLE	A	B	C	D	E	F	G	H	I	J	K
1	0.464	0.355	0.222	0.421	0.327	0.312	0.200	0.137	0.134	0.198	0.193
2	0.403	0.491	0.246	0.502	0.418	0.295	0.257	0.232	QNS	0.251	0.305
3	1.120	0.763	0.574	0.827	0.881	0.575	0.422	0.499	0.262	0.555	0.505
4	1.600	0.970	0.704	1.060	0.662	0.706	0.632	0.686	0.360	0.592	0.572
5	1.590	0.754	0.424	0.646	0.552	0.501	0.279	0.320	0.164	0.246	0.272
6	1.440	0.694	0.350	0.778	0.509	0.408	0.264	0.279	QNS	0.240	0.391
7	0.660	0.507	0.309	0.337	0.435	0.282	0.421	0.235	QNS	0.268	0.246
8	0.606	0.665	0.354	0.687	0.647	0.647	QNS	0.228	0.107	0.284	0.255
9	1.212	0.750	0.560	0.894	0.618	0.618	0.359	0.413	0.276	0.471	0.444
10	QNS	QNS	QNS	0.660	0.555	0.555	0.317	0.461	QNS	0.286	0.312
NEG	0.470	0.353	0.182	0.467	0.254	0.241	0.160	0.176	0.084	0.100	0.108
POS	1.540	1.770	1.517	1.190	1.047	1.150	1.830	1.910	1.525	1.360	1.410
P/N RATIO	3.280	5.014	8.335	2.548	4.122	4.772	11.438	10.852	18.150	13.600	13.056
MEAN-NORMALS	0.910	0.595	0.374	0.681	0.560	0.490	0.315	0.349	0.130	0.339	0.350
3X S.D.	1.587	0.771	0.576	0.630	0.441	0.444	0.468	0.468	0.381	0.408	0.351
C.O.	2.497	1.366	0.950	1.311	1.001	0.934	0.783	0.817	0.511	0.747	0.701
POS/C.O.	0.616	1.290	1.600	0.908	1.050	1.230	2.340	2.340	2.980	1.820	2.010

A PBS + .05 % TWEEN
B PBS + .05 % TWEEN + 5 % RABBIT SERUM
C PBS + .05 % TWEEN + 5% P/A
D SPIROLATE BROTH
E SPIROLATE BROTH + 5% RABBIT SERUM
F SPIROLATE BROTH + 5% P/A
G LYME DILUENT + 5% RABBIT SERUM
H LYME DILUENT + 5% BSA
I SORBENT
J SORBENT + 5% P/A
K SORBENT + 5% P/A + 5% RABBIT SERUM

NOTE: P/A REPRESENTS THE ADDITION OF A PROPRIETARY ADDITIVE

subsequent development.

Determination of Syphilis ELISA Sensitivity and Specificity

The "relative" sensitivity,"relative" specificity, and percent agreement of the Syphilis ELISA was determined in comparison to the DCL Syphilis G assay and the FTA-ABS assay.

A total of 293 serum samples were compared using the DCL Syphilis G assay and the Syphilis ELISA currently under development. The DCL Syphilis G testing was performed following the manufacturer's package insert. The results from the DCL Syphilis G assay were analyzed using the mean of the three Low Positive Control (LPC) values as the assay's "cut-off". Sample ratios for the DCL Syphilis G assay were calculated by dividing the sample O.D. by the mean LPC value. A sample was considered positive if the ratio was >1.1, equivocal if the ratio was .90-1.1, and negative if the ratio was <.90. There was total agreement between the two assays on 76 positive samples and 212 negative samples. One sample, negative on the DCL Syphilis G assay, was positive on the Syphilis ELISA. This sample was also found to be FTA-ABS and RPR negative. One sample, negative on the Syphilis ELISA was positive on the DCL Syphilis G assay. This sample was RPR negative but FTA-ABS positive. Three samples, equivocal on the Syphilis ELISA were not used in the calculation of "relative" sensitivity and "relative" specificity. The "relative" sensitivity of the Syphilis ELISA when compared to

the DCL Syphilis G assay was found to be 98.7 %. "Relative" specificity of the test was found to be 99.5 %, and percent agreement between the two assays was found to be 99.3 %. These data show excellent correlation between the DCL Syphilis G assay and the current Syphilis ELISA under development. The summary of these results can be found in Table 3.

A total of 293 samples were also tested on the FTA-ABS assay (Zeus Scientific). Samples were considered to be positive on the FTA-ABS assay if they exhibited greater than a 1+ fluorescence when viewed under a fluorescence microscope as described in the manufacturer's package insert. Nine samples which exhibited 1+ fluorescence by FTA-ABS were considered equivocal and were not used in the calculation of "relative" sensitivity and "relative" specificity. There were 76 positive samples and 202 negative samples that were in total agreement between the FTA-ABS assay and the Syphilis ELISA. One sample was negative on the FTA-ABS assay and positive on the Syphilis ELISA. This sample was also negative on RPR and the DCL Syphilis G assay. Six samples were positive on the FTA-ABS assay and negative on the Syphilis ELISA. These same six samples were negative on the DCL Syphilis G assay as well. Four of the six samples were found to be RPR positive. The "relative" sensitivity of the Syphilis ELISA as compared to FTA-ABS was found to be 92.7 %. The "relative" specificity of the assay was found to be 99.5 % and the percent agreement was found to be 97.5 %.

Table 3: THE DETERMINATION OF THE "RELATIVE" SENSITIVITY AND "RELATIVE" SPECIFICITY OF THE ZEUS SYPHILIS ELISA VS. DCL SYPHILIS G ELISA.

TABLE 3:

SENSITIVITY AND SPECIFICITY VS. DCL SYPHILIS G ELISA

ZEUS	DCL SYPHILIS G P	DCL SYPHILIS G N
P	76 (A)	1 (C)
N	1 (B)	212 (D)

SENSITIVITY(A/A+B)=98.7 %

SPECIFICITY(D/C+D)=99.5 %

PERCENT AGREEMENT=

(A+D/A+B+C+D)=99.3 %

Table 4: THE DETERMINATION OF THE "RELATIVE" SENSITIVITY AND "RELATIVE" SPECIFICITY OF THE ZEUS SYPHILIS ELISA VS. ZEUS FTA-ABS ASSAY.

TABLE 4:

SENSITIVITY AND SPECIFICITY vs. FTA-ABS

ZEUS	FTA-ABS P	FTA-ABS N
P	76 (A)	1 (C)
N	6 (B)	202 (D)

SENSITIVITY(A/A+B)=92.7 %

SPECIFICITY(D/C+D)=99.5 %

PERCENT AGREEMENT=

(A+D/A+B+C+D)=97.5 %

Table 5: SYPHILIS ASSAY COMPARISON. A comparison of samples tested by the Zeus Syphilis ELISA, RPR, Zeus FTA-ABS assay, and the DCL Syphilis G ELISA.

TABLE 5: SYPHILIS ASSAY COMPARISON
SYPHILIS ELISA, RPR, FTA-ABS, DCL SYPHILIS ELISA

	-----SYPHILIS ELISA-----						----------DCL SYPHILIS G-------			
SAMPLE	O.D.	C\O	RATIO	INT.	RPR	FTA	O.D.	C\O	RATIO	INT.
1088 1:1	2.082	0.539	3.863	+	3-4+	3+	1.343	0.398	3.374	+
1088 1:2	1.741	0.539	3.230	+	2+	3+	1.182	0.398	2.970	+
1088 1:4	1.547	0.539	2.870	+	+/-	3+	1.039	0.398	2.611	+
1088 1:8	1.161	0.539	2.154	+	-	2-3+	0.790	0.398	1.985	+
1088 1:16	0.831	0.539	1.542	+	-	2+	0.534	0.398	1.342	+
1088 1:32	0.539	0.539	1.000	+/-	-	2+	QNS	QNS	QNS	
1088 1:64	0.360	0.539	0.668	-	-	2+	0.257	0.398	0.646	-
1088 1:128	0.218	0.539	0.404	-	-	1+	0.205	0.398	0.515	-
1088 1:256	0.095	0.539	0.176	-	-	1+	0.166	0.398	0.417	-
1088 1:512	0.052	0.539	0.096	-	-	+/-	0.133	0.398	0.334	-
1088 1:1024	0.040	0.539	0.074	-	-	-	0.121	0.398	0.304	-
SY 1	1.824	0.539	3.384	+	1+	3+	0.998	0.378	2.640	+
SY 2	2.233	0.539	4.143	+	2+	2++	1.710	0.378	4.524	+
SY 4	1.738	0.539	3.224	+	2-3+	2++	1.081	0.378	2.860	+
SY 5	1.418	0.539	2.631	+	1+	3+	1.037	0.378	2.743	+
SY 6	2.675	0.539	4.963	+	QNS	3+	1.692	0.378	4.476	+
SY 8	0.225	0.507	0.444	-	3+	2+	0.145	0.378	0.384	-
SY 9	1.167	0.539	2.165	+	+/-	2++	0.667	0.378	1.765	+
SY 10	1.272	0.539	2.360	+	3-4+	3+	1.002	0.328	3.055	+
SY 12	2.144	0.539	3.978	+	4+	3+	1.574	0.378	4.164	+
SY 13	1.635	0.539	3.033	+	4+	2++	0.765	0.378	2.024	+
SY 14	0.357	0.539	0.662	-	3-4+	2+	0.127	0.378	0.336	-
SY 15	0.295	0.539	0.547	-	3-4+	3-4+	0.186	0.378	0.492	-
SY 16	1.776	0.539	3.295	+	4+	2++	1.650	0.378	4.365	+
SY 17	1.528	0.539	2.835	+	1-2+	2++	1.152	0.378	3.048	+
MHA 1	1.581	0.539	2.933	+	3+	3+	0.953	0.378	2.521	+
MHA 2	0.483	0.539	0.896	-	1+	1+	0.047	0.378	0.124	-
MHA 4	1.717	0.539	3.186	+	3+	3+	0.889	0.378	2.352	+
MHA 5	1.359	0.539	2.521	+	1+	2++	0.849	0.378	2.246	+
MHA 6	0.838	0.539	1.555	+	-	1+	0.612	0.378	1.619	+
MHA 7	1.126	0.539	2.089	+	2+	2++	0.597	0.378	1.579	+
MHA 9	1.111	0.539	2.061	+	-	3+	0.768	0.378	2.032	+
MHA 10	0.448	0.539	0.831	-	3+	2+	0.316	0.378	0.836	-
MHA 11	2.338	0.539	4.338	+	4+	4+	1.756	0.378	4.646	+

TABLE 5: SYPHILIS ASSAY COMPARISON

SYPHILIS ELISA, RPR, FTA-ABS, DCL SYPHILIS ELISA

	-----SYPHILIS ELISA-----						----------DCL SYPHILIS G-------			
SAMPLE	O.D.	C\O	RATIO	INT.	RPR	FTA	O.D.	C\O	RATIO	INT.
MHA 12	1.532	0.539	2.842	+	-	3+	0.853	0.378	2.257	+
MHA 13	2.077	0.539	3.853	+	3+	4+	1.259	0.378	3.331	+
MHA 14	2.200	0.539	4.082	+	3+	2-3+	1.644	0.378	4.349	+
MHA 15	1.374	0.539	2.549	+	-	2+	0.774	0.378	2.048	+
MHA 16	1.182	0.539	2.193	+	-	2+	0.619	0.378	1.638	+
MHA 17	1.616	0.539	2.998	+	2+	3-4+	1.071	0.378	2.833	+
MHA 18	1.575	0.539	2.922	+	3+	4+	0.948	0.378	2.508	+
MHA 19	0.556	0.539	1.032	+/-	-	1-2+	0.543	0.378	1.437	+
MHA 20	1.456	0.539	2.701	+	-	3+	1.009	0.378	2.669	+
MHA 21	1.835	0.539	3.404	+	1+	4+	1.161	0.378	3.071	+
MHA 22	1.154	0.539	2.141	+	+/-	3-4+	0.955	0.378	2.526	+
MHA 23	0.637	0.539	1.182	+	2+	2+	0.519	0.378	1.373	+
MHA 24	1.214	0.539	2.252	+	-	3+	0.991	0.378	2.622	+
MHA 25	1.385	0.539	2.570	+	-	3+	0.881	0.378	2.331	+
MHA 26	0.566	0.539	1.050	+	-	1-2+	0.468	0.378	1.238	+
MHA 27	0.831	0.539	1.542	+	-	2+	0.482	0.378	1.275	+
MHA 28	1.681	0.539	3.119	+	3+	4+	1.052	0.378	2.783	+
MHA 29	1.313	0.539	2.436	+	3+	4+	0.742	0.378	1.963	+
MHA 30	1.106	0.539	2.052	+	QNS	3+	0.707	0.378	1.870	+
MHA 31	0.060	0.539	0.111	-	-	-	0.133	0.378	0.352	-
MHA 32	1.442	0.539	2.675	+	1-2+	4+	0.833	0.378	2.204	+
BMR 1	1.749	0.539	3.245	+	3+	3+	1.169	0.378	3.093	+
BMR 2	1.517	0.539	2.814	+	2+	3+	1.270	0.378	3.360	+
BMR 3	2.271	0.539	4.213	+	2+	3-4+	1.486	0.378	3.931	+
BMR 4	2.048	0.539	3.800	+	2-3+	2+	1.511	0.378	3.997	+
BMR 5	1.462	0.539	2.712	+	1-2+	2-3+	0.838	0.378	2.217	+
BMR 6	1.998	0.539	3.707	+	1-2+	3+	1.206	0.378	3.190	+
BMR 7	1.153	0.539	2.139	+	4+	3+	0.557	0.378	1.474	+
BMR 8	0.180	0.539	0.334	-	-	1+/+/	0.162	0.378	0.429	-
BMR 9	1.340	0.539	2.486	+	3-4+	3+	0.609	0.378	1.611	+
BMR 10	0.465	0.539	0.863	-	2+	3+	0.221	0.378	0.585	-
BMR 11	0.092	0.539	0.171	-	+/-	-	0.124	0.378	0.328	-
12177	0.023	0.539	0.043	-	-	-		0.378	0.000	-
10	0.013	0.539	0.024	-	-	-	0.122	0.398	0.307	-
10195	0.015	0.539	0.028	-	-	-	0.105	0.398	0.264	-

TABLE 5: SYPHILIS ASSAY COMPARISON

SYPHILIS ELISA, RPR, FTA-ABS, DCL SYPHILIS ELISA

	-----SYPHILIS ELISA-----						----------DCL SYPHILIS G-------			
SAMPLE	O.D.	C\O	RATIO	INT.	RPR	FTA	O.D.	C\O	RATIO	INT.
12385	1.611	0.539	2.989	+	1+	2-3+	1.148	0.398	2.884	+
12426	1.598	0.539	2.965	+	+/-	3+	1.021	0.398	2.565	+
NABI A1	0.066	0.543	0.122	-	-	-	0.059	0.328	0.180	-
NABI A2	0.036	0.543	0.066	-	-	-	0.086	0.328	0.262	-
NABI A3	0.117	0.543	0.215	-	-	-	0.192	0.328	0.585	-
NABI A4	0.060	0.543	0.110	-	-	-	0.098	0.328	0.299	-
NABI A5	0.071	0.543	0.131	-	-	-	0.144	0.328	0.439	-
NABI A6	0.032	0.543	0.059	-	-	-	0.109	0.328	0.332	-
NABI A7	0.065	0.543	0.120	-	-	-	0.136	0.328	0.415	-
NABI A8	0.046	0.543	0.085	-	-	-	0.203	0.328	0.619	-
NABI A9	0.038	0.543	0.070	-	-	-	0.085	0.328	0.259	-
NABI A10	0.075	0.543	0.138	-	-	-	0.161	0.328	0.491	-
NABI A11	0.092	0.543	0.169	-	-	-	0.086	0.328	0.262	-
NABI A12	0.025	0.543	0.046	-	-	-	0.074	0.328	0.226	-
NABI B1	0.071	0.543	0.131	-	-	-	0.145	0.328	0.442	-
NABI B2	0.033	0.543	0.061	-	-	-	0.068	0.328	0.207	-
NABI B3	0.100	0.543	0.184	-	-	-	0.130	0.328	0.396	-
NABI B4	0.089	0.543	0.164	-	-	-	0.112	0.328	0.341	-
NABI B5	0.103	0.543	0.190	-	-	-	0.134	0.328	0.409	-
NABI B6	0.036	0.543	0.066	-	-	-	0.079	0.328	0.241	-
NABI B7	0.035	0.543	0.064	-	-	-	0.078	0.328	0.238	-
NABI B8	0.032	0.543	0.059	-	-	-	0.076	0.328	0.232	-
NABI B9	0.022	0.543	0.041	-	-	-	0.069	0.328	0.210	-
NABI B10	0.022	0.543	0.041	-	-	-	0.087	0.328	0.265	-
NABI B11	0.074	0.543	0.136	-	-	-	0.079	0.328	0.241	-
NABI B12	0.043	0.543	0.079	-	-	-	0.066	0.328	0.201	-
NABI C1	0.131	0.543	0.241	-	-	-	0.175	0.328	0.534	-
NABI C2	0.050	0.543	0.092	-	-	-	0.106	0.328	0.323	-
NABI C3	0.016	0.543	0.029	-	-	-	0.074	0.328	0.226	-
NABI C4	0.039	0.543	0.072	-	-	-	0.087	0.328	0.265	-
NABI C5	0.021	0.543	0.039	-	-	-	0.066	0.328	0.201	-
NABI C6	0.034	0.543	0.063	-	-	-	0.104	0.328	0.317	-
NABI C7	0.037	0.543	0.068	-	-	-	0.090	0.328	0.274	-

TABLE 5: SYPHILIS ASSAY COMPARISON
SYPHILIS ELISA, RPR, FTA-ABS, DCL SYPHILIS ELISA

	-----SYPHILIS ELISA-----						----------DCL SYPHILIS G-------			
SAMPLE	O.D.	C\O	RATIO	INT.	RPR	FTA	O.D.	C\O	RATIO	INT.
NABI C8	0.043	0.543	0.079	-	-	-	0.070	0.328	0.213	-
NABI C9	0.047	0.543	0.087	-	-	-	0.147	0.328	0.448	-
NABI C10	0.016	0.543	0.029	-	-	-	0.058	0.328	0.177	-
NABI C11	0.010	0.543	0.018	-	-	-	0.064	0.328	0.195	-
NABI C12	0.083	0.543	0.153	-	-	-	0.108	0.328	0.329	-
NABI D1	0.024	0.543	0.044	-	-	-	0.084	0.328	0.256	-
NABI D2	0.070	0.543	0.129	-	-	-	0.141	0.328	0.430	-
NABI D3	0.037	0.543	0.068	-	-	-	0.079	0.328	0.241	-
NABI D4	0.054	0.543	0.099	-	-	-	0.108	0.328	0.329	-
NABI D5	0.033	0.543	0.061	-	-	-	0.101	0.328	0.308	-
NABI D6	0.062	0.543	0.114	-	-	-	0.125	0.328	0.381	-
NABI D7	0.028	0.543	0.052	-	-	-	0.069	0.328	0.210	-
NABI D8	0.044	0.543	0.081	-	-	-	0.089	0.328	0.271	-
NABI D9	0.080	0.543	0.147	-	-	-	0.154	0.328	0.470	-
NABI D10	0.120	0.543	0.221	-	-	-	0.218	0.328	0.665	-
NABI D11	0.050	0.543	0.092	-	-	-	0.125	0.328	0.381	-
NABI D12	0.064	0.543	0.118	-	-	-	0.074	0.328	0.226	-
NABI E1	0.055	0.543	0.101	-	-	-	0.082	0.328	0.250	-
NABI E2	0.047	0.543	0.087	-	-	-	0.109	0.328	0.332	-
NABI E3	0.058	0.543	0.107	-	-	-	0.097	0.328	0.296	-
NABI E4	0.053	0.543	0.098	-	-	-	0.106	0.328	0.323	-
NABI E5	0.080	0.543	0.147	-	-	-	0.208	0.328	0.634	-
NABI E6	0.024	0.543	0.044	-	-	-	0.092	0.328	0.280	-
NABI E7	0.017	0.543	0.031	-	-	-	0.082	0.328	0.250	-
NABI E8	0.062	0.543	0.114	-	-	-	0.130	0.328	0.396	-
NABI E9	0.026	0.543	0.048	-	-	-	0.121	0.328	0.369	-
NABI E10	0.006	0.543	0.011	-	-	-	0.061	0.328	0.186	-
NABI E11	0.080	0.543	0.147	-	-	-	0.126	0.328	0.384	-
NABI E12	0.060	0.543	0.110	-	-	-	0.168	0.328	0.512	-
NABI F1	0.120	0.543	0.221	-	-	-	0.149	0.328	0.454	-
NABI F2	0.021	0.543	0.039	-	-	-	0.089	0.328	0.271	-
NABI F3	0.033	0.543	0.061	-	-	-	0.119	0.328	0.363	-
NABI F4	0.060	0.543	0.110	-	-	-	0.147	0.328	0.448	-
NABI F5	0.033	0.543	0.061	-	-	-	0.070	0.328	0.213	-

TABLE 5: SYPHILIS ASSAY COMPARISON
SYPHILIS ELISA, RPR, FTA-ABS, DCL SYPHILIS ELISA

	-----SYPHILIS ELISA-----						----------DCL SYPHILIS G-------			
SAMPLE	O.D.	C\O	RATIO	INT.	RPR	FTA	O.D.	C\O	RATIO	INT.
NABI F6	0.074	0.543	0.136	-	-	-	0.147	0.328	0.448	-
NABI F7	0.039	0.543	0.072	-	-	-	0.147	0.328	0.448	-
NABI F8	0.046	0.543	0.085	-	-	-	0.164	0.328	0.500	-
NABI F9	0.047	0.543	0.087	-	-	-	0.097	0.328	0.296	-
NABI F10	0.020	0.543	0.037	-	-	-	0.088	0.328	0.268	-
NABI F11	0.049	0.543	0.090	-	-	-	0.075	0.328	0.229	-
NABI F12	0.014	0.543	0.026	-	-	-	0.080	0.328	0.244	-
NABI G1	0.061	0.543	0.112	-	-	-	0.078	0.328	0.238	-
NABI G2	0.039	0.543	0.072	-	-	-	0.089	0.328	0.271	-
SER. 1	0.065	0.512	0.127	-	-	-	0.113	0.398	0.284	-
SER. 2	0.167	0.512	0.326	-	-	-	0.173	0.398	0.435	-
SER. 3	0.371	0.512	0.725	-	-	-	0.190	0.398	0.477	-
SER. 4	0.241	0.512	0.471	-	-	-	0.192	0.398	0.482	-
SER. 5	0.314	0.512	0.613	-	-	-	0.294	0.398	0.739	-
SER. 6	0.291	0.512	0.568	-	-	-	0.317	0.398	0.796	-
SER. 7	0.064	0.512	0.125	-	-	-	0.158	0.398	0.397	-
SER. 8	0.206	0.512	0.402	-	-	-	0.140	0.398	0.352	-
SER. 9	0.103	0.512	0.201	-	-	-	0.107	0.398	0.269	-
SER. 10	0.134	0.512	0.262	-	-	-	0.115	0.398	0.289	-
SER. 11	0.049	0.512	0.096	-	-	-	0.150	0.398	0.377	-
SER. 12	0.391	0.507	0.771	-	-	-	0.163	0.398	0.410	-
SER. 13	0.105	0.512	0.205	-	-	-	0.114	0.398	0.286	-
SER. 14	0.063	0.512	0.123	-	-	-	0.112	0.398	0.281	-
SER. 15	0.148	0.512	0.289	-	-	-	0.190	0.398	0.477	-
SER. 16	0.096	0.512	0.188	-	-	-	0.096	0.398	0.241	-
SER. 17	0.134	0.512	0.262	-	-	-	0.084	0.398	0.211	-
SER. 18	0.199	0.512	0.389	-	-	-	0.158	0.398	0.397	-
SER. 19	0.272	0.512	0.531	-	-	-	0.151	0.398	0.379	-
SER. 20	0.095	0.512	0.186	-	-	-	0.142	0.398	0.357	-
SER. 21	0.103	0.512	0.201	-	-	-	0.155	0.398	0.389	-
SER. 22	0.085	0.512	0.166	-	-	-	0.103	0.398	0.259	-
SER. 23	0.083	0.512	0.162	-	-	-	0.091	0.398	0.229	-
SER. 24	0.429	0.507	0.846	-	-	-	0.327	0.398	0.822	-

TABLE 5: SYPHILIS ASSAY COMPARISON
SYPHILIS ELISA, RPR, FTA-ABS, DCL SYPHILIS ELISA

	-----SYPHILIS ELISA-----						----------DCL SYPHILIS G-------			
SAMPLE	O.D.	C\O	RATIO	INT.	RPR	FTA	O.D.	C\O	RATIO	INT.
SER. 25	0.053	0.512	0.104	-	-	-	0.088	0.398	0.221	-
SER. 26	0.218	0.512	0.426	-	-	-	0.076	0.398	0.191	-
SER. 27	0.361	0.507	0.712	-	-	-	0.145	0.398	0.364	-
SER. 28	0.492	0.507	0.970	+/-	-	-	0.263	0.398	0.661	-
SER. 29	0.627	0.512	1.225	+	-	-	0.050	0.398	0.126	-
SER. 30	0.182	0.512	0.355	-	-	-	0.214	0.398	0.538	-
SER. 31	0.106	0.512	0.207	-	-	-	0.116	0.398	0.291	-
SER. 32	0.302	0.512	0.590	-	-	-	0.095	0.398	0.239	-
SER. 33	0.093	0.512	0.182	-	-	-	0.230	0.398	0.578	-
SER. 34	0.096	0.512	0.188	-	-	-	0.146	0.398	0.367	-
SER. 35	0.135	0.512	0.264	-	-	-	0.124	0.398	0.312	-
SER. 36	0.029	0.512	0.057	-	-	-	0.076	0.398	0.191	-
SER. 37	0.053	0.512	0.104	-	-	-	0.091	0.398	0.229	-
SER. 38	0.092	0.512	0.180	-	-	-	0.113	0.398	0.284	-
SER. 39	0.071	0.512	0.139	-	-	-	0.135	0.398	0.339	-
SER. 40	0.087	0.512	0.170	-	-	-	0.085	0.378	0.225	-
SER. 41	0.271	0.512	0.529	-	-	-	0.113	0.378	0.299	-
SER. 42	0.056	0.512	0.109	-	-	-	0.081	0.378	0.214	-
SER. 43	0.115	0.512	0.225	-	-	-	0.084	0.378	0.222	-
SER. 44	0.069	0.512	0.135	-	-	-	0.082	0.378	0.217	-
SER. 45	0.092	0.512	0.180	-	-	-	0.087	0.378	0.230	-
SER. 46	0.048	0.512	0.094	-	-	1+	0.065	0.378	0.172	-
5374	2.145	0.512	4.189	+	1-2+	2-3+	1.145	0.378	3.029	+
9159	2.390	0.512	4.668	+	2-3+	3+	1.534	0.378	4.058	+
90-69	2.625	0.512	5.127	+	4+	3+	1.299	0.398	3.264	+
90-146	2.329	0.512	4.549	+	4+	2+	1.474	0.398	3.704	+
91-64	2.360	0.512	4.609	+	3+	2-3+	1.548	0.398	3.889	+
90-68	1.989	0.512	3.885	+	3+	4+	0.868	0.398	2.181	+
90-147	2.514	0.512	4.910	+	4+	4+	1.456	0.398	3.658	+
90-67	1.375	0.512	2.686	+	4+	3+	0.637	0.398	1.601	+
91-63	2.265	0.512	4.424	+	4+	4+	1.392	0.398	3.497	+
11625	2.415	0.512	4.717	+	1-2+	3-4+	1.697	0.378	4.489	+
404 B	1.251	0.512	2.443	+	-	3+	0.500	0.378	1.323	+

TABLE 5: SYPHILIS ASSAY COMPARISON
SYPHILIS ELISA, RPR, FTA-ABS, DCL SYPHILIS ELISA

	-----SYPHILIS ELISA-----						----------DCL SYPHILIS G-------			
SAMPLE	O.D.	C\O	RATIO	INT.	RPR	FTA	O.D.	C\O	RATIO	INT.
460 B	0.252	0.512	0.492	-	-	1-2+	0.690	0.378	1.825	+
MDH 1*	1.146	0.512	2.238	+	+/-	1-2+	0.796	0.398	2.000	+
MDH 2	2.503	0.512	4.889	+	2+	4+	1.513	0.398	3.802	+
MDH 3	1.974	0.512	3.855	+	3+	3+	1.209	0.398	3.038	+
MDH 4	1.837	0.512	3.588	+	+/-	1-2+	1.208	0.398	3.035	+
MDH 5	1.916	0.512	3.742	+	2+	3+	1.248	0.398	3.136	+
MDH 6	1.744	0.512	3.406	+	3+	3+	1.129	0.398	2.837	+
MDH 7	2.502	0.512	4.887	+	1+	4+	2.030	0.398	5.101	+
MDH 8	0.317	0.512	0.619	-	-	-	0.227	0.398	0.570	-
MDH 9	0.116	0.512	0.227	-	-	-	0.145	0.398	0.364	-
MDH 10	1.253	0.512	2.447	+	3+	3+	0.730	0.398	1.834	+
MDH 11	2.202	0.512	4.301	+	3+	2+	1.522	0.398	3.824	+
MDH 12	2.432	0.512	4.750	+	3+	4+	1.495	0.398	3.756	+
MDH 13	2.115	0.512	4.131	+	3+	4+	1.537	0.398	3.862	+
MDH 14	2.185	0.512	4.268	+	+/-	3+	1.713	0.398	4.304	+
MDH 15	2.215	0.512	4.326	+	-	2+	1.545	0.398	3.882	+
SKP1	0.051	0.583	0.087	-	-	-	0.081	0.328	0.247	-
SKP2	0.011	0.583	0.019	-	-	-	0.069	0.328	0.210	-
SKP3	0.012	0.583	0.021	-	-	-	0.075	0.328	0.229	-
SKP4	0.020	0.583	0.034	-	-	-	0.076	0.328	0.232	-
SKP5	0.029	0.583	0.050	-	-	-	0.085	0.328	0.259	-
SKP6	0.073	0.583	0.125	-	-	-	0.094	0.328	0.287	-
SKP7	0.056	0.583	0.096	-	-	-	0.133	0.328	0.405	-
SKP8	0.080	0.583	0.137	-	-	-	0.082	0.328	0.250	-
SKP9	0.033	0.583	0.057	-	-	-	0.049	0.328	0.149	-
SKP10	0.029	0.583	0.050	-	-	-	0.085	0.328	0.259	-
SKP11	0.016	0.583	0.027	-	-	-	0.073	0.328	0.223	-
SKP12	0.003	0.583	0.005	-	-	-	0.084	0.328	0.256	-
SKP13	0.002	0.583	0.003	-	-	-	0.064	0.328	0.195	-
SKP14	0.035	0.583	0.060	-	-	-	0.066	0.343	0.192	-
SKP15	0.021	0.583	0.036	-	-	-	0.087	0.343	0.254	-
SKP16	0.030	0.583	0.051	-	-	-	QNS	QNS	QNS	
SKP17	0.000	0.583	0.000	-	-	-	0.051	0.343	0.149	-
SKP18	0.052	0.583	0.089	-	-	-	0.108	0.343	0.315	-
SKP19	0.026	0.583	0.045	-	-	-	0.065	0.343	0.190	-

TABLE 5: SYPHILIS ASSAY COMPARISON

SYPHILIS ELISA, RPR, FTA-ABS, DCL SYPHILIS ELISA

	-----SYPHILIS ELISA-----						----------DCL SYPHILIS G-------			
SAMPLE	O.D.	C\O	RATIO	INT.	RPR	FTA	O.D.	C\O	RATIO	INT.
SKP20	0.027	0.583	0.046	-	-	-	0.270	0.343	0.787	-
SKP21	0.287	0.583	0.492	-	-	-	0.114	0.343	0.332	-
SKP22	0.082	0.583	0.141	-	-	-	0.075	0.343	0.219	-
SKB-1A	0.044	0.512	0.086	-	-	-	0.072	0.378	0.190	-
SKB-1B	0.075	0.512	0.146	-	-	-	0.154	0.378	0.407	-
SKB-1C	0.080	0.512	0.156	-	-	-	0.086	0.378	0.228	-
SKB-1D	0.060	0.512	0.117	-	-	-	0.107	0.378	0.283	-
SKB-1E	0.065	0.512	0.127	-	-	-	0.109	0.378	0.288	-
SKB-1F	0.029	0.512	0.057	-	-	-	0.111	0.378	0.294	-
SKB-1G	0.041	0.512	0.080	-	-	-	0.096	0.378	0.254	-
SKB-1H	0.065	0.512	0.127	-	-	-	0.074	0.378	0.196	-
SKB-2A	0.163	0.583	0.280	-	-	-	0.103	0.378	0.272	-
SKB-2B	0.030	0.583	0.051	-	-	-	0.095	0.378	0.251	-
SKB-2C	0.034	0.583	0.058	-	-	-	0.131	0.378	0.347	-
SKB-2D	0.071	0.583	0.122	-	-	-	0.111	0.378	0.294	-
SKB-2E	0.057	0.583	0.098	-	-	-	0.153	0.378	0.405	-
SKB-2F	0.020	0.583	0.034	-	-	-	0.075	0.378	0.198	-
SKB-2G	0.043	0.583	0.074	-	-	-	0.080	0.378	0.212	-
SKB-2H	0.111	0.583	0.190	-	-	-	0.199	0.378	0.526	-
SKB-3A	0.249	0.583	0.427	-	-	-	0.253	0.343	0.738	-
SKB-3B	0.044	0.583	0.075	-	-	-	0.097	0.343	0.283	-
SKB-3C	0.022	0.583	0.038	-	-	-	0.110	0.343	0.321	-
SKB-3D	0.013	0.583	0.022	-	-	-	0.077	0.343	0.224	-
SKB-3E	0.076	0.583	0.130	-	-	-	0.163	0.343	0.475	-
SKB-3F	0.026	0.583	0.045	-	-	-	0.131	0.343	0.382	-
SKB-3G	0.021	0.583	0.036	-	-	-	0.072	0.343	0.210	-
SKB-3H	0.032	0.583	0.055	-	-	-	0.066	0.343	0.192	-
SKB-4A	0.026	0.583	0.045	-	-	-	0.146	0.343	0.426	-
SKB-4B	0.096	0.583	0.165	-	-	-	0.099	0.343	0.289	-
SKB-4C	0.014	0.583	0.024	-	-	-	0.174	0.343	0.507	-
SKB-4D	0.035	0.583	0.060	-	-	-	0.080	0.343	0.233	-
SKB-4E	QNS				-	-	0.059	0.343	0.172	-

TABLE 5: SYPHILIS ASSAY COMPARISON
SYPHILIS ELISA, RPR, FTA-ABS, DCL SYPHILIS ELISA

	-----SYPHILIS ELISA-----						----------DCL SYPHILIS G-------			
SAMPLE	O.D.	C\O	RATIO	INT.	RPR	FTA	O.D.	C\O	RATIO	INT.
SKB-4F	0.035	0.583	0.060	-	-	-	0.105	0.343	0.306	-
SKB-4G	0.013	0.583	0.022	-	-	-	0.168	0.343	0.490	-
SKB-4H	QNS				-	-	0.067	0.343	0.195	-
LYME 1	0.033	0.583	0.057	-	-	-	0.100	0.343	0.292	-
LYME 4	0.053	0.583	0.091	-	-	-	0.109	0.343	0.318	-
LYME 7	0.009	0.583	0.015	-	-	-	0.088	0.343	0.257	-
LYME 8	0.005	0.583	0.009	-	-	-	0.066	0.343	0.192	-
LYME 9	0.071	0.583	0.122	-	-	-	0.142	0.343	0.414	-
LYME 13	0.282	0.583	0.484	-	-	-	0.194	0.343	0.566	-
LYME 15	0.053	0.583	0.091	-	-	-	0.140	0.343	0.408	-
LYME 17	0.039	0.583	0.067	-	-	-	0.069	0.343	0.201	-
LYME 16	0.035	0.583	0.060	-	-	-	0.073	0.343	0.213	-
LYME 18	0.033	0.583	0.057	-	-	-	0.073	0.343	0.213	-
LYME 22	0.147	0.583	0.252	-	-	-	0.095	0.343	0.277	-
LYME 24	0.020	0.583	0.034	-	-	-	0.082	0.343	0.239	-
LYME 25	0.013	0.583	0.022	-	-	-	0.074	0.343	0.216	-
LYME 26	0.029	0.583	0.050	-	-	-	0.088	0.343	0.257	-
LYME 27	0.070	0.583	0.120	-	-	-	0.272	0.343	0.793	-
LYME 29	0.094	0.583	0.161	-	-	-	0.081	0.343	0.236	-
LYME 30	0.017	0.583	0.029	-	-	-	0.115	0.343	0.335	-
LYME 31	0.030	0.583	0.051	-	-	-	0.120	0.343	0.350	-
LYME 32	0.025	0.583	0.043	-	-	-	0.181	0.343	0.528	-
LYME 34	0.075	0.583	0.129	-	-	-	0.079	0.343	0.230	-
LYME 35	0.020	0.583	0.034	-	-	-	0.079	0.343	0.230	-
LYME 36	0.054	0.583	0.093	-	-	-	0.146	0.343	0.426	-
LYME 37	0.079	0.583	0.136	-	-	-	0.333	0.343	0.971	+/-
LYME 38	0.023	0.583	0.039	-	-	-	0.142	0.343	0.414	-
LYME 11	0.446	0.583	0.765	-	-	-	0.120	0.343	0.350	-
LYME 5	0.019	0.583	0.033	-	-	-	0.099	0.343	0.289	-

*CLINICALLY-DEFINED SYPHILIS SPECIMENS

The results of this study are summarized in Table 4. These data show that there is excellent correlation between the Syphilis ELISA and the FTA-ABS assay.

A tabulation of all the data used in the generation of Tables 3 and 4 is presented in Table 5.

Clinical Sample Testing

Clinically defined syphilis specimens were received from a Public Health Department during the development of the Syphilis ELISA. These samples had been previously tested at the Public Health Department using the Unheated Serum Reagin Test (USR), a non-treponemal test for the detection of antilipid (reagin) antibodies comparable to the RPR and VDRL assays. The samples were also tested by hemagglutination (HA), to verify the non-treponemal test result. Each of these samples (n=15) were then tested using the Syphilis ELISA currently being developed. The results can be found in Table 6. There was 100 % agreement between the HA testing performed at the Public Health Department and the results that were obtained using the Syphilis ELISA. Note that samples 8 and 9 were submitted as true syphilis negative specimens. One serum sample (No. 1) was known to be darkfield (+), and was a documented case of primary syphilis. This sample was positive on the Syphilis ELISA showing that this treponemal test was sensitive enough to detect specific syphilis antibody during a stage of syphilis when other treponemal tests might not have been able to. Often specific treponemal antibodies do not

Table 6: SYPHILIS CLINICAL SAMPLE TESTING. A comparison of data compiled on a set of 15 clinically defined Syphilis samples from a Public Health Department. Comparison of USR, HA, and Zeus Syphilis ELISA results.

TABLE 6:

SYPHILIS CLINICAL SAMPLE TESTING

SAMPLE	USR	HA	SYPHILIS ELISA RATIO	
1	1:2	+	2.238	+ *
2	1:2	+	4.889	+ **
3	1:32	+	3.855	+
4	1:256	+	3.588	+
5	1:256	+	3.742	+
6	1:64	+	3.406	+
7	1:64	+	4.887	+
8	-	-	0.619	-
9	-	-	0.227	-
10	1:32	+	2.447	+
11	1:64	+	4.301	+
12	1:128	+	4.750	+
13	1:16	+	4.131	+
14	1:32	+	4.268	+
15	1:32	+	4.326	+

* SAMPLE WAS + DARKFIELD, AND A DOCUMENTED CASE OF PRIMARY SYPHILIS
**SAMPLE FROM A PATIENT WHO HAD CONTACT WITH A SYPHILIS PATIENT

become apparent until after the first signs of clinical manifestation (17). Future studies will help to define the sensitivity and specificity of this assay during the different stages of syphilis.

Precision and Intra/Inter Assay Testing

Six serum samples representing different antibody levels, high positive, mid positive, equivocal, negative, and high negative samples were run on the Syphilis ELISA, three different times, on three separate days. Each sample was replicated at least eight times in each assay. The mean, standard deviation, and % CV were calculated for each sample on each different day. Inter-assay reproducibility was calculated by determining the mean, standard deviation, and % CV for each sample by combining the data calculated on each of the three separate days. The results are shown in Table 7. These results show that there is excellent reproducibility both within assay and between assays.

Cross Reactivity Testing

A total of 66 serum samples were tested that had potentially cross-reacting antibodies on the Syphilis ELISA. These samples represented ANA positive samples, Lyme IgG positive samples, and routine samples collected during pregnancy. All of these samples were tested on the prototype Syphilis ELISA, and the results recorded in Table 8. Only one sample was found to be positive on the Syphilis ELISA. This sample was found to be smooth muscle antibody positive,

Table 7: INTRA/INTER ASSAY PRECISION STUDIES. An analysis of Mean, Standard Deviation (STD) and Percent Coefficient of Variation (% CV) for a set of samples to complete Intra/Inter assay precision studies.

TABLE 7: INTRA/INTER ASSAY PRECISION STUDIES

INTRA ASSAY REPRODUCIBILITY

DAY #1

SAMPLE	MEAN*	STD	%CV	
1	3.430	0.219	6.38	Mid-Positive
2	0.762	0.021	2.76	High Negative/Equivocal
3	0.999	0.037	3.70	Equivocal
4	0.515	0.022	4.27	Negative
5	0.745	0.015	2.01	High Negative
6	5.390	0.128	2.37	High Positive

DAY #2

SAMPLE	MEAN	STD	%CV
1	4.200	0.088	2.09
2	0.875	0.025	2.86
3	0.999	0.030	3.00
4	0.251	0.124	4.94
5	0.595	0.050	8.40
6	5.440	0.440	8.09

DAY #3

SAMPLE	MEAN	STD	%CV
1	3.550	0.176	4.95
2	0.542	0.017	3.14
3	0.999	0.033	3.30
4	0.473	0.017	3.59
5	0.707	0.015	2.12
6	4.430	0.374	8.44

INTER ASSAY REPRODUCIBILITY

SAMPLE	MEAN	STD	%CV
1	3.570	0.298	8.34
2	0.788	0.104	13.19
3	0.999	0.034	3.40
4	0.467	0.081	17.34
5	0.711	0.050	7.03
6	4.970	0.568	11.43

*Note: All data reported as sample/cut-off ratios.

Table 8: CROSS-REACTIVITY TESTING. An analysis of 66 potentially cross-reacting samples as assayed on the Syphilis ELISA.

TABLE 8:
CROSS-REACTIVITY TESTING

SAMPLE	POSITIVE	NEGATIVE
PREG. +	0	22
LYME IgG+	0	8
ANA+	1	35

however, an additional smooth muscle antibody sample gave negative results on the Syphilis ELISA.

Stability Testing

Accelerated stability testing was initiated for the *Treponema pallidum* antigen coated plates. Antigen coated plates were stored at the following temperatures: 2-8^{0}C, 22-25^{0}C (Room Temperature), 37^{0}C, and 45^{0}C. On a weekly basis, beginning with Week 0 and ending with Week 8, strips were removed from each plate and tested using a predetermined set of samples and a specific conjugate dilution to determine the point of antigen degradation (low signal). The results of the stability testing from weeks 1, 2, 4, 6, and 8 are graphically depicted in Figures 4-8. This data shows that even after storage of the solid phase antigen coated plate at 45^{0}C for 8 weeks, no loss of signal could be detected. Therefore, we were unable to determine a definitive shelf-life for the antigen coated plates using the procedure as set forth by the Health Industry Manufacturers Association. A conservative 12 month expiration date may be assigned to the antigen-coated plates, stored at 4^0 C until real-time stability could be completed to confirm and/or extend the assigned expiration date.

Figure 4: ACCELERATED STABILITY TESTING
Accelerated stability testing completed on Syphilis antigen coated plates. Week # 1 of Stability Testing. Optical Density (O.D.) vs. Inverse Dilution of sample.

FIGURE 4: ACCELERATED STABILITY

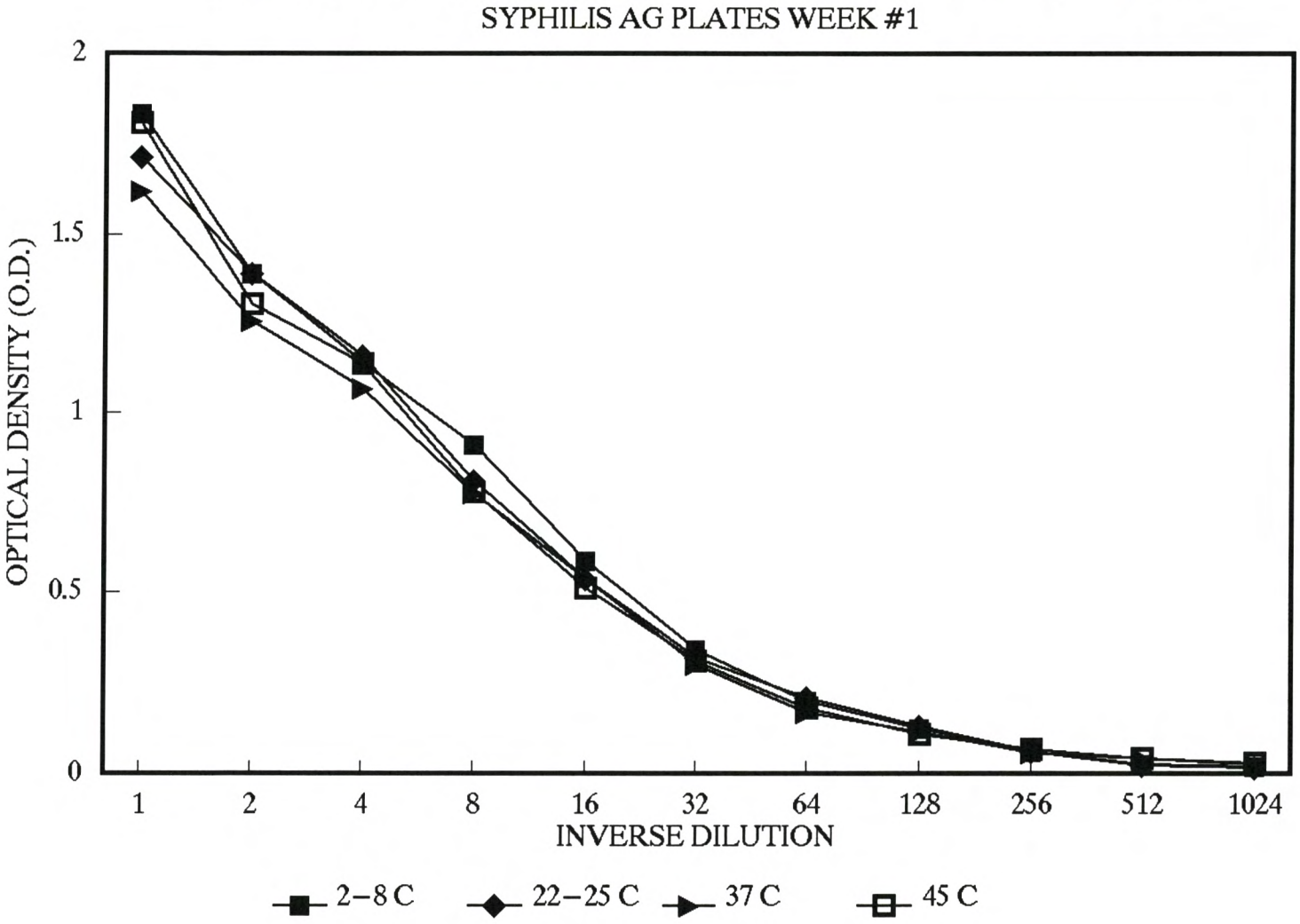


Figure 5: ACCELERATED STABILITY TESTING
Accelerated stability testing completed on Syphilis antigen coated plates. Week # 2 of Stability Testing. Optical Density (O.D.) vs. Inverse Dilution of sample.

FIGURE 5: ACCELERATED STABILITY

SYPHILIS AG PLATES WEEK #2

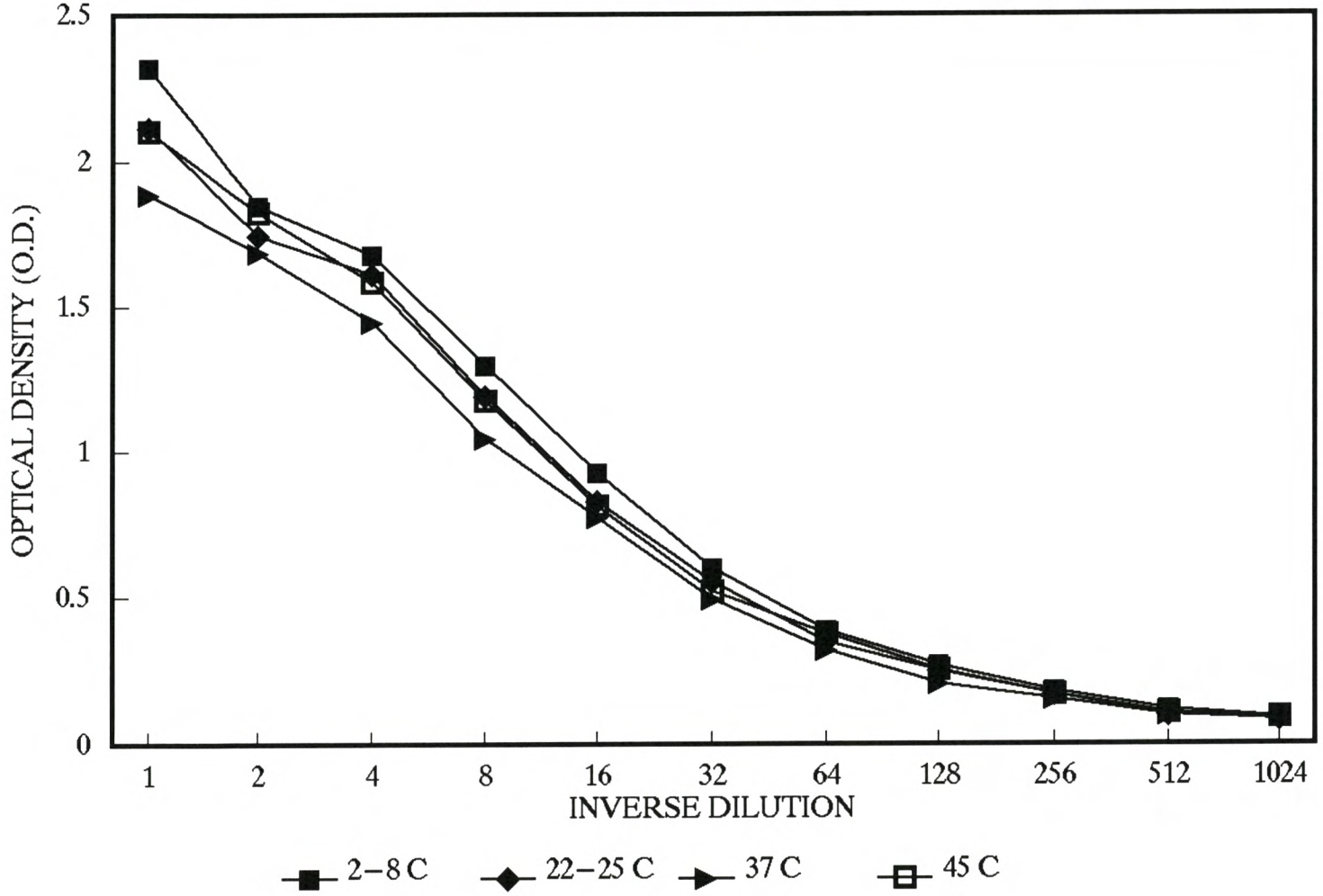


Figure 6: ACCELERATED STABILITY TESTING
Accelerated stability testing completed on Syphilis antigen coated plates. Week # 4 of Stability Testing. Optical Density (O.D.) vs. Inverse Dilution of sample.

FIGURE 6: ACCELERATED STABILITY

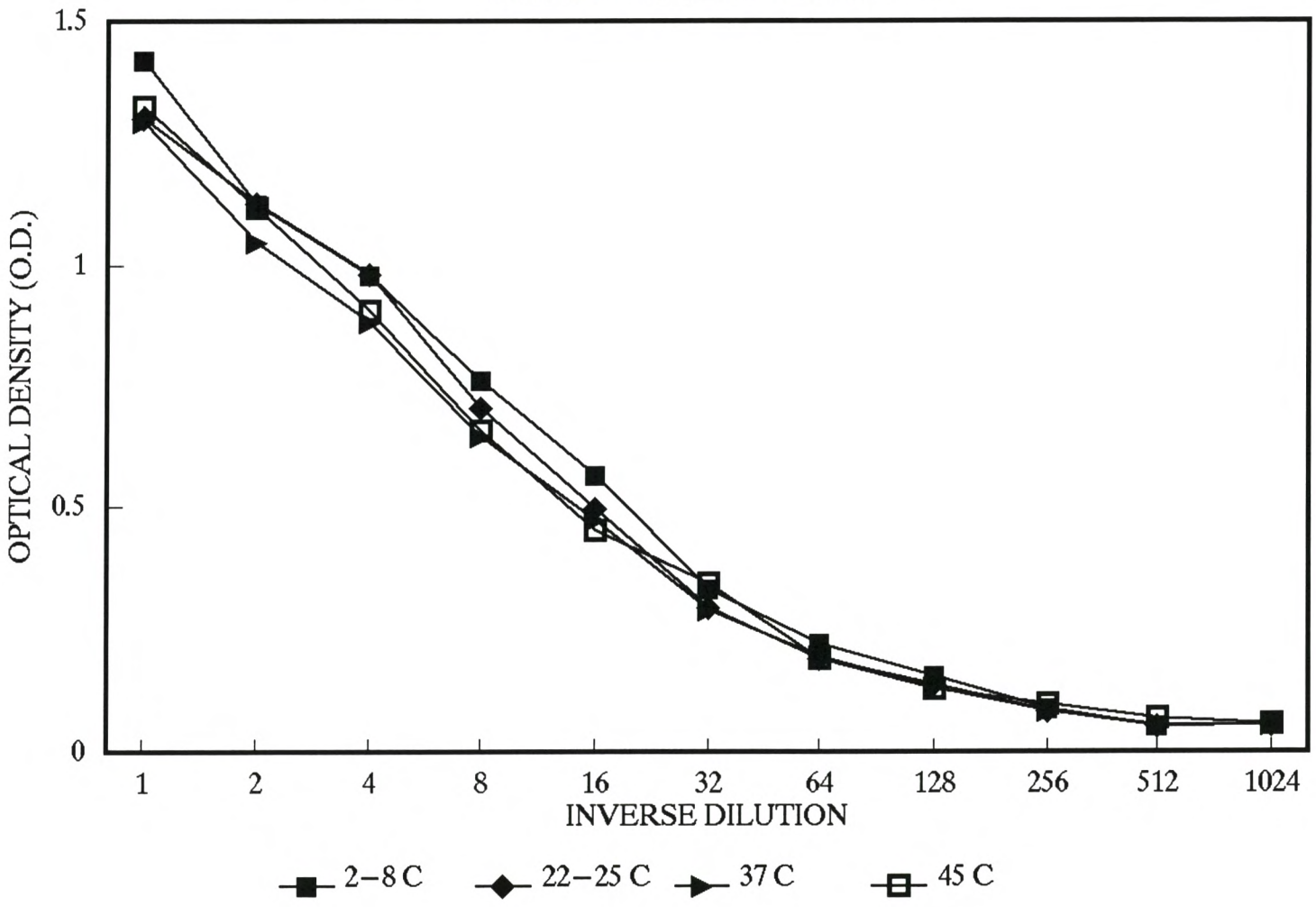


Figure 7: ACCELERATED STABILITY TESTING
Accelerated stability testing completed on Syphilis antigen coated plates. Week # 6 of Stability Testing. Optical Density (O.D.) vs. Inverse Dilution of sample.

FIGURE 7: ACCELERATED STABILITY

SYPHILIS AG PLATES WEEK #6

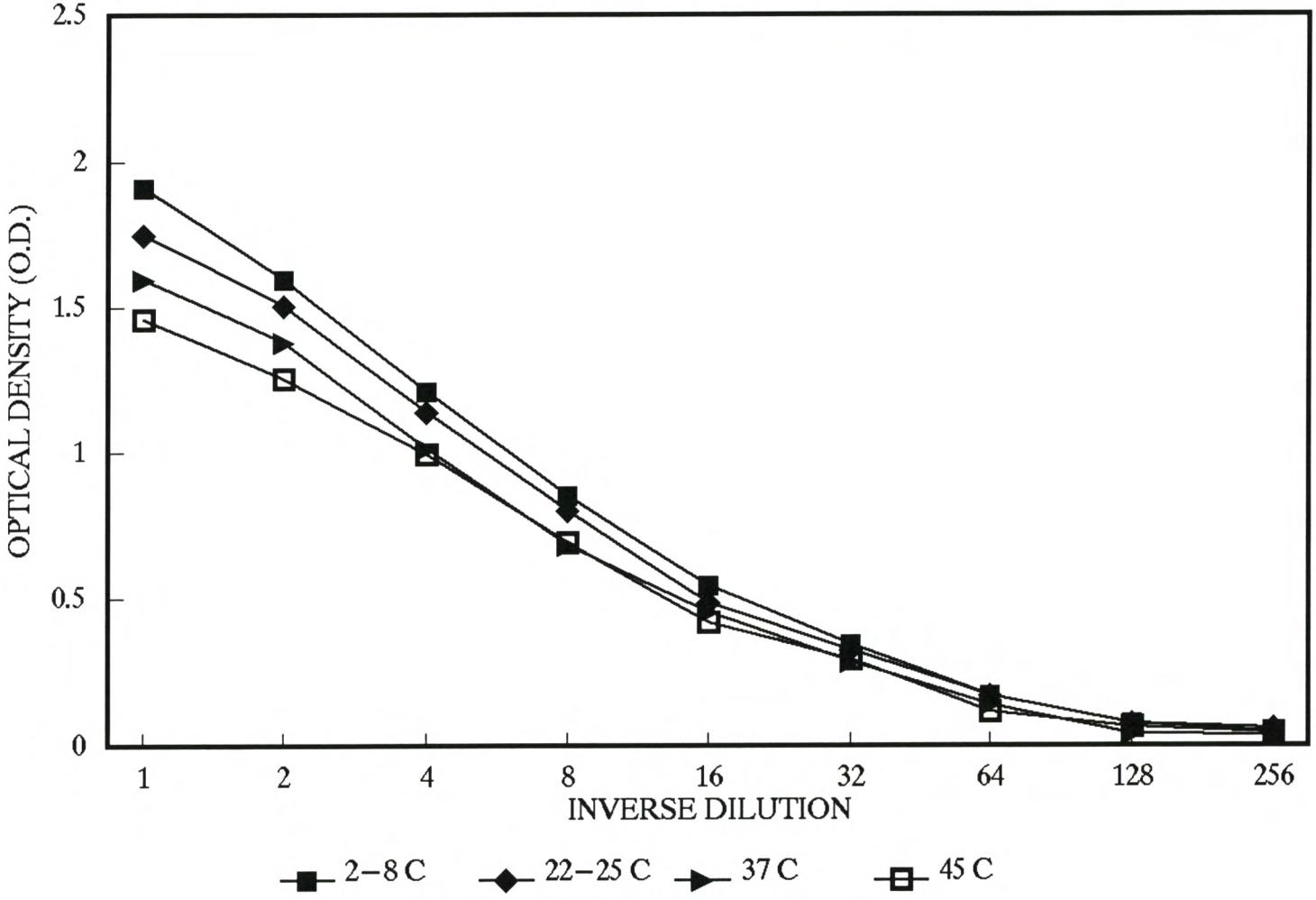
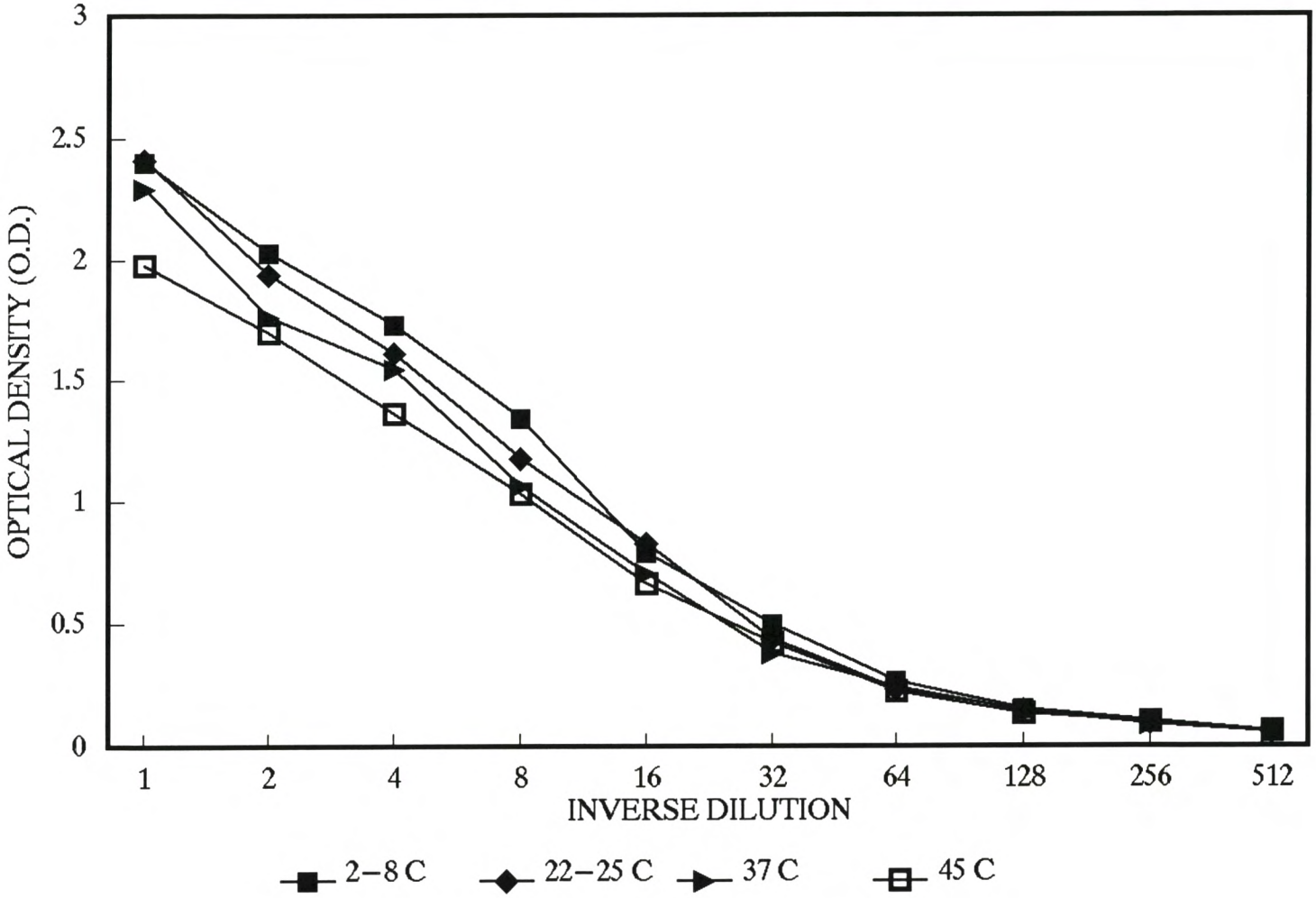


Figure 8: ACCELERATED STABILITY TESTING
Accelerated stability testing completed on Syphilis antigen coated plates. Week # 8 of Stability Testing. Optical Density (O.D.) vs. Inverse Dilution of sample.

FIGURE 8: ACCELERATED STABILITY

SYPHILIS AG PLATES WEEK #8



DISCUSSION

The development of an enzyme linked immunosorbent (ELISA) assay for the detection of antibodies to *Treponema pallidum* subspecies *pallidum* provides an alternate treponemal confirmatory test for aid in the diagnosis of syphilis. Currently the diagnosis of syphilis is made increasingly difficult due to the fact that the *T.pallidum* organism is considered to be non-culturable, and at this time cannot be distinguished morphologically, serologically, or chemically from other pathogenic treponemes (15,24). Serological tests used in the diagnosis of syphilis infections have been divided into two groups based on the type of antibody each detects. Non-treponemal tests, used for the detection of non-treponemal antibodies, measure antilipid antibody formed by the host in response to the release of lipid from the cell surface of the treponemes (24). Treponemal tests, on the other hand detect specific treponemal antibodies produced in response to *T. pallidum* infection. Non-treponemal tests are instrumental in the diagnosis of earlier stages of syphilis, as well as being useful in the monitoring of antibody response during treatment with antibiotics (24,44). Treponemal tests on the other hand may not show increasing titers until after the first signs of clinical manifestation and treponemal antibodies may remain apparent for months to years even after successful treatment of the disease (15,17). The current recommendation for

serological testing as set forth by the World Health Organization (WHO) consists of initial screening with the non-treponemal VDRL assay followed by confirmation with the treponemal FTA-ABS assay (15). The Syphilis ELISA offers several advantages over confirmation by FTA-ABS. The ELISA is simple, rapid, and results can be read objectively, thereby decreasing the incidence of technical error. The ELISA is also readily automated thus increasing the number of samples a lab can run in any one day while decreasing the need for additional hands-on technician time.

A purified *T. pallidum* antigen received from Lee Laboratories (Grayson, Georgia) was found to be the antigen of choice for use in the Syphilis ELISA. This antigen was found to give the lowest possible background noise along with the highest possible specific signal as demonstrated by a positive to negative ratio of 86.00. When normalized to account for the optimal coating concentration of 4.6 ug/ml, the P/N ratio was found to be 18.70. This P/N/ug protein ratio was 2.5X better than the next best antigen (No. 3) with no loss to purification procedures. The optimal coating concentration for this antigen was 4.6 ug/ml. This is consistent with studies of the microplate antigen binding characteristics as reported by Conroy, et al (9). Detailed studies of microplate binding capacities have determined that plastic microplate wells are saturated with protein coating concentrations of 4 ug/ml. Generally, the lowest concentration of coating

material is used that gives maximum antigen binding (9).

Using an optimal antigen processing procedure, and antigen dilution an assay was run using samples from a normal plasma donor population for the determination of the assay cut-off. The cut-off of the assay was calculated by determining the mean + 3SD of the normal sample population (n=48). Once a cut-off was determined a number of known syphilis positive samples were then tested to assess the validity of the assay cut-off. The optimal sample diluent for use with the Syphilis ELISA was found to be sorbent, which is prepared from cultures of non-pathogenic Reiter's treponemes. This sorbent was found to decrease the incidence of false positives, by absorbing any cross-reacting antibodies that might be present in each patient serum sample.

The "relative" sensitivity and "relative" specificity was determined for the Syphilis ELISA in comparison to the FTA-ABS (Zeus Scientific) and the DCL Syphilis G assay (Diagnostic Chemicals Limited). Both assays are treponemal assays for the detection of antibody to *T. pallidum*, and both are currently being marketed in the United States with FDA approval. The Syphilis ELISA shows a "relative" sensitivity of 98.7%, "relative" specificity of 99.5% and overall agreement of 99.3% when compared to the DCL Syphilis G assay. Likewise, the Syphilis ELISA shows excellent results when compared to the FTA-ABS assay. Compared to FTA-ABS, the Syphilis ELISA shows a "relative" sensitivity of 92.7%, "relative" specificity of

months may be assigned to the solid phase until real-time stability can be completed.

CONCLUSION

The Syphilis ELISA cumulative test performance data indicates that the assay compares favorably to the DCL Syphilis ELISA and the FTA-ABS assay, both of which are currently being marketed for the confirmation of treponemal antibodies in a syphilis infection. The assay shows excellent reproducibility, both intra-assay and inter-assay, and the accelerated stability shows that the antigen-coated plates are stable for an extended period of time at 2-8^{0}C. Altogether, the Syphilis ELISA will be a rapid, objective alternative for the confirmation of a syphilis infection.

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