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TITLE: TRI-ELISA FOR SIMULTANEOUS ANALYSIS OF THREE ANALYTES

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13. ABSTRACT (Maximum 200 words)

The goal of this research effort was to develop a three-enzyme ELISA system in which three analytes can be quantitated concurrently from a single biological or environmental sample. Three Plasmodium (malaria) antigens and anti-strain specific antibodies previously identified and optimized at the Walter Reed Army Institute of Research were used as a model system. During this Phase I effort, we identified three enzymes and a common substrate solution that will work simultaneously, providing three discernable colors as perceived by visual observation. We then conjugated each enzyme to a separate antibody, developed individual ELISA's for each analyte, and tested the three analyte systems concurrently. The resulting tri-ELISA system is a logical, efficient and potentially productive approach for improving an existing diagnostic assay for malarial sporozoites in mosquitos. The improved assay can be used by the World Health Organization for epidemiological studies designed to monitor, and through preventative mechanisms, potentially eradicate this disease which currently threatens approximately 400 million of the world's population. The results of this Phase I project demonstrate feasibility and provide the basis for continuation into a Phase II project.

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U.S. DEPARTMENT OF DEFENSE

SMALL BUSINESS INNOVATION RESEARCH (SBIR) PROGRAM
PHASE 1—FY 1990
PROJECT SUMMARY

Topic No. A90-181   Military Department/Agency Army .

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Proposal Title

TRI-ELISA FOR SIMULTANEOUS ANALYSIS OF THREE ANALYTES

Technical Abstract (Limit your abstract to 200 words with no classified or proprietary information/data.)
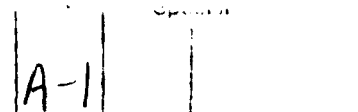
The goal of this research effort was to develop a three-enzyme ELISA system in which three analytes can be quantitated concurrently from a single biological or environmental sample. Three *Plasmodium* (malaria) antigens and anti-strain specific antibodies previously identified and optimized at the Walter Reed Army Institute of Research were used as a model system. During this Phase I effort, we identified three enzymes and a common substrate solution that will work simultaneously, providing three discernable colors as perceived by visual observation. We then conjugated each enzyme to a separate antibody, developed individual ELISA's for each analyte, and tested the three analyte systems concurrently. The resulting tri-ELISA system is a logical, efficient and potentially productive approach for improving an existing diagnostic assay for malarial sporozoites in mosquitos. The improved assay can be used by the World Health Organization for epidemiological studies designed to monitor, and through preventative mechanisms, potentially eradicate this disease which currently threatens approximately 400 million of the world's population. The results of this Phase I project demonstrate feasibility and provide the basis for continuation into a Phase II project.

Anticipated Benefits/Potential Commercial Applications of the Research or Development

A tri-ELISA system would greatly increase the ability of the U.S. Army, as well as other branches of the military and civilian medicine to screen personnel (or donor blood) for multiple infectious agents. The development of the system that we propose would have widespread applicability and could reduce assay time, required supplies and reagents, and associated costs by as much as 60%.

List a maximum of 8 Key Words that describe the Project.

ELISA, chromogenic compounds, *Plasmodium*, malaria

A-1

1. Purpose and Scope of Research Effort.

The goal of this research effort was to develop a three-enzyme ELISA system in which three analytes can be quantitated concurrently from a single biological or environmental sample. During this Phase I effort, we identified three enzymes and a common substrate solution that will work simultaneously, providing three readily discernable colors as perceived by visual observation. We then conjugated each enzyme to a separate antibody, developed individual ELISA's for each analyte, and tested the three analyte systems concurrently. In this Phase I effort, three *Plasmodium* antigens/antibodies previously identified and optimized by Dr. Robert Wirtz at the Walter Reed Army Institute of Research were used as a model system. The results of this Phase I project demonstrated feasibility and provide the basis for continuation into a Phase II project.

2. Technical Results.

Summary. The components of the final tri-ELISA is shown in Table 1.

TABLE 1 - TRI-ELISA COMPONENTS

ENZYME	ANTIBODY	SUBSTRATE	COLOR
β-galactosidase	anti-*P. falciparum*	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-GAL)	Blue
Peroxidase	anti-*P. malariae*	3-amino-9-ethyl carbazole (AEC)	Red/Brown
Alkaline Phosphatase	anti-*P. vivax*	p-nitrophenyl phosphate (PNPP)	Yellow

The Phase I proposal asked the following questions:

1) Can the three enzymes which we propose work concurrently at a sufficient activity level to be sensitive in the proposed format?

   We have identified three enzymes/substrates that will work concurrently in a tri-ELISA format. The results must be read by visual observation since two of the three chromogens yield insoluble products (use of a microplate reader provides inconclusive data). This assay is therefore qualitative rather than quantitative.

2) Can we successfully conjugate each of the enzymes to antibodies, while retaining sufficient activity of the proteins?

   The enzymes were successfully conjugated to the antibodies. Optimization of conjugation methodologies during a Phase II effort should improve activity.

3) Can we develop ELISA's for each of the three analytes having adequate sensitivity?

   The ELISA's for each of the three malarial analytes are sensitive to 0.39 ng BSA-P_f, 0.098 ng BSA-P_m, and less than 0.31 ng BSA-P_v conjugates. The desired sensitivity for the P_f and P_v systems was set at 50-100 sporozoites; the sensitivities achieved were 250 for P_v and 800 for the P_f systems (P_m sporozoites not available). The substrates used for this Phase I effort were not as sensitive as that currently used, however, optimization of the system is expected to improve sensitivity.

Task 1. Procurement of Reagents and Supplies

Each enzyme was procured from various sources. The enzymes yielding the greatest activity when conjugated included: α-glucosidase from Boehringer Mannheim (50 U/mg); β-galactosidase from Boehringer Mannheim (900 U/mg); alkaline phosphatase from Sigma (1200 U/mg); and Peroxidase from Sigma (300 U/mg). Substrates, as well as other needed chemical reagents were obtained from commercial suppliers.

At the onset of the Phase I project, we were contacted by Mr. Craig Lebo concerning the substitution of analytes for the tri-ELISA model system. Dr. Robert Wirtz at the Walter Reed Army Institute of Research then kindly provided us with three *Plasmodium* (malaria) antigens/antibodies: *P. falciparum* (P$_f$) peptide antigen (R32tet32 lot JFM14176-165), sporozoites, and monoclonal antibodies (MoAb) specific to P$_f$ (P$_f$ ascitic fluid lot 729-08, P$_f$ capture MoAb KF03-5, Peroxidase labeled P$_f$ MoAb KE36-25); *P. vivax* (P$_v$) peptide antigen (NS1V20 lot VJC-13528-247), sporozoites, and MoAb specific to P$_v$ (Protein A purified P$_v$ MoAb NSV#3 lot 85-2808, P$_v$ capture MoAb HL19-1, P$_v$ peroxidase labeled HK22-2); and, *P. malariae* (P$_m$) peptide antigen (AAGN$_5$ lot 019090), MoAb specific to P$_m$ (Protein A purified MoAb 453 lot 88-44-47, peroxidase labeled P$_m$ MoAb lot JC08-25).

ELISA plates (polyvinyl chloride and polystyrene) were purchased from Dynatech.

Task 2. Determination of Enzyme/Substrate Combination

Peroxidase, α-glucosidase, and β-galactosidase were incubated individually with their respective substrate solutions and absorbances were scanned between wavelengths of 320 and 750nm. The reagents were then combined and again scanned using the spectrophotometer. As can be seen in Figure 1, the peak at A$_{626}$ splits into two peaks (A$_{612}$ and A$_{652}$) when the substrates are added in combination. Evaluation of this phenomenon revealed that the problem occurred when the BCI formed by β-galactosidase reacting with BCIG was in a minimum of 40% H$_2$O *versus* N,N-dimethylformamide (DMF).

The kinetics of enzyme activities in the common substrate solution were evaluated for the initial 5 minutes of reaction (Figure 2). The initial common substrate solution was comprised of the following:

Solution A (Total 5 ml):
  2 ml 0.2 M K phosphate
  1 ml 10 mM 4-amino antipyrine (AA) in H$_2$O
  1 ml 10 mM p-nitrophenyl-α-D-glucopyranoside (PNAG) in H$_2$O
  0.1 ml 100 mM MgCl$_2$· 6 H$_2$O
  0.9 ml H$_2$O
Solution B (Total 5 ml):
  3 ml 0.2 M K phosphate
  1 ml 10 mM 2 hydroxy-3,5-dichlorobenzene sulfonic acid (HDCBS) in H$_2$O
  0.1 ml 100 mM H$_2$O$_2$
  0.9 ml H$_2$O
Solid C: 4.08 mg 5-Br-4-Cl-3-indolyl-β-D galactoside (BCIG)
To prepare, solid C was dissolved in 0.1 ml N,N-dimethylformamide, then mixed with solutions A and B immediately prior to use.

The β-galactosidase reaction showed an initial lag phase of 1-2 minutes, due to the two-step reaction process leading to chromophore formation: 1) enzyme-catalyzed hydrolysis of the galactoside and 2) oxidation of the resulting chromogen (5-Br-4-Cl-3-indole alcohol) to form the dye product.
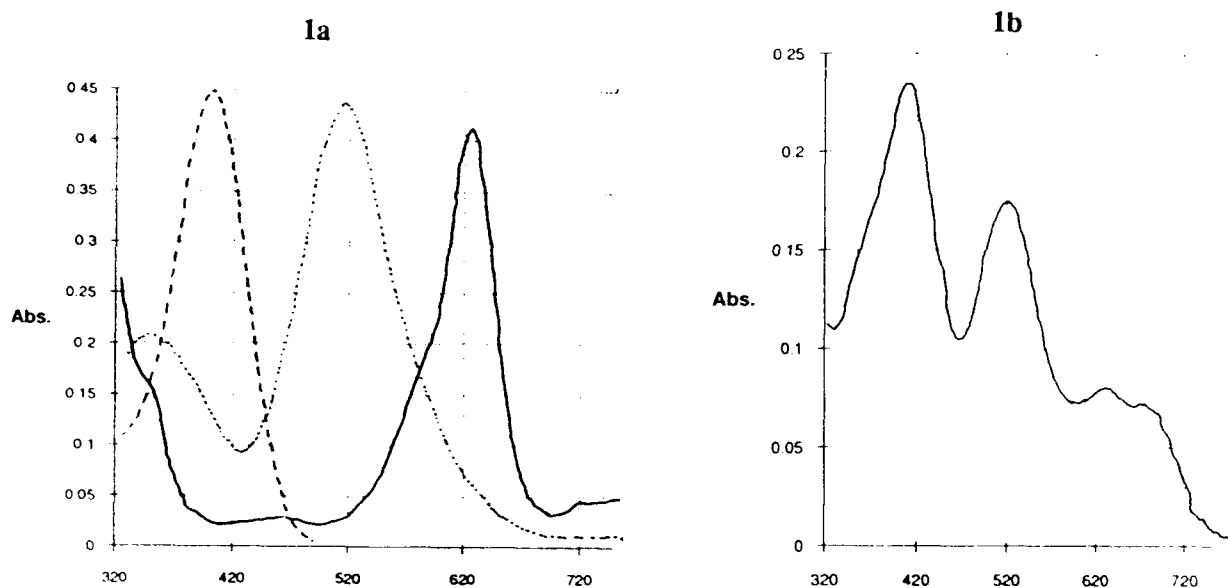
1a

1b

FIGURE 1. Spectrophotometric Analysis of the Three Enzyme/Substrates. Fig. 1a is a composite drawing of the three individual systems scanned from 320 to 750 nm. α-Glucosidase (- -), Peroxidase (····), β-galactosidase (—). For this experiment, the β-galactosidase reaction was in 99% DMF. In Fig. 1b, all three systems are combined before scanning. For this experiment, all three samples were mixed, so that the final DMF concentration was ~33%, causing the splitting of the 626 nm peak.

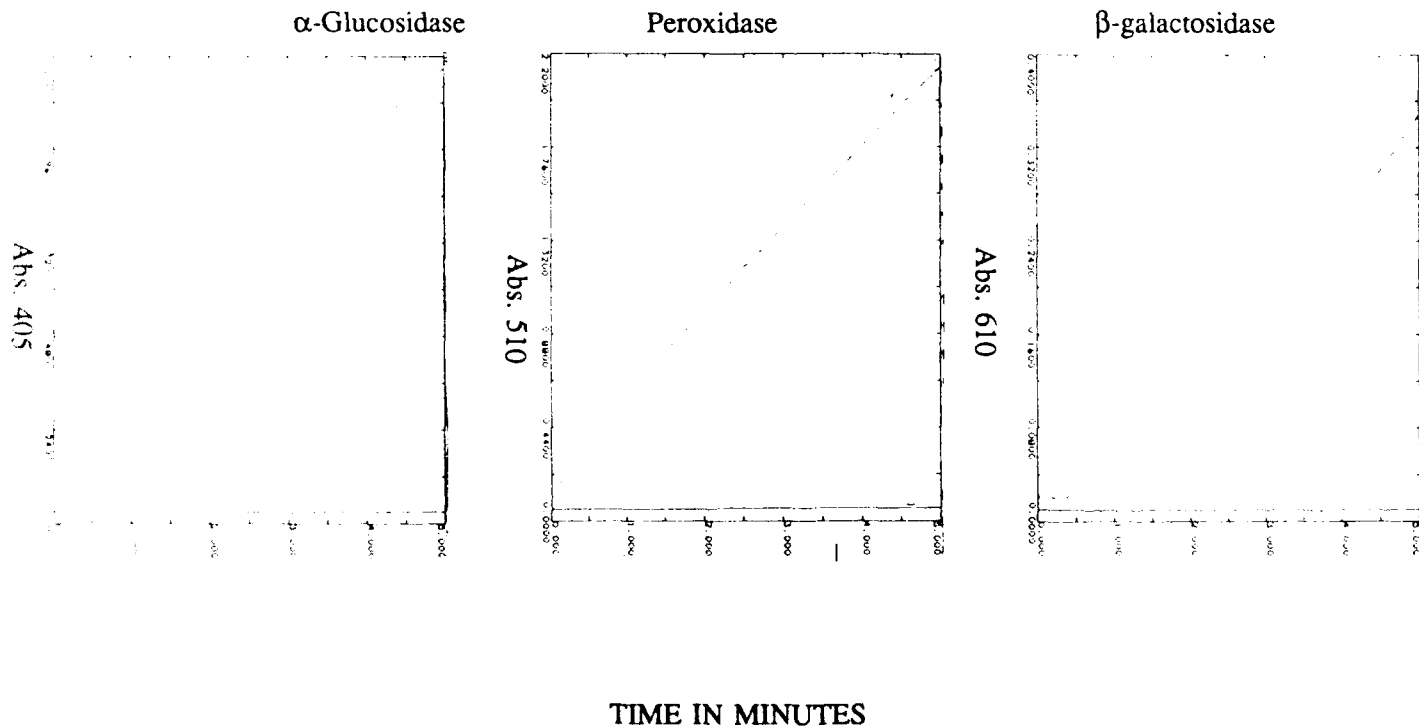## α-Glucosidase   Peroxidase   β-galactosidase

TIME IN MINUTES

FIGURE 2. Kinetics of Enzyme Activity Using the Common Substrate Developing Solution. Each enzyme was reacted with the three substrate solution and the known maximum absorbance was read for a period of 5 minutes.

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2 hydroxy-3,5-dichlorobenzene sulfonic acid (HDCBS) *versus* Phenol. A comparative study was performed to test the use of HDCBS or phenol (with 4-amino antipyrine, AA) as the peroxidase substrate. Initially, a standard quantity of peroxidase (313 ng) was tested against each substrate and absorbance was read at 510 nm over a period of 5 minutes. No difference was seen in reaction rates. It was previously noted that upon storage the HDCBS generated a background color. Therefore, another assay was run comparing the two substrate systems over a 1 hour incubation time at enzyme levels of 0, 12.5, 25, and 37 ng. The results clearly demonstrate that throughout the 1 hour incubation, phenol yielded superior signal *vs.* noise (HDCBS = 19.5; phenol = 55.9; 37 ng level results at 60 minutes). HDCBS, however, gave an overall greater signal (absorbance of 1.95 *vs.* 1.679 for phenol at 60 minutes). We then substituted phenol for HDCBS in the common substrate solution (stock solution of phenol at 10 mM).

Combinations of Enzymes with Common Substrate Solution. Peroxidase, α-glucosidase, and β-galactosidase were dissolved in PBS at 0.1 mg/ml. Samples were serially diluted in microtiter plates in a "checkerboard" format in order to determine the range of colors possible in a double positive system (see Photograph 1). The plates were incubated with common substrate solution and read at 405, 515 and 650 nm at 10 and 30 minutes, and 1, 2, and 6 hours. The results indicated that absorbance values did not correspond with the wide variety of colors, as shown for three combinations in Figure 3. With these results, and after discussion with Dr. Wirtz, it was decided to focus on the tri-ELISA as qualitative rather than a quantitative screening test.

Task 3. ELISA Testing Using Established Methods

System Optimization. While the enzyme/substrate studies were proceeding, ELISA's for P_f and P_v were evaluated using procedures established by Dr. Wirtz. Comparisons were made of various microtiter plates (Nunc, Immulon 4, Corning ELISA) with the round bottom PVC plates currently used; the PVC plates proved superior in both systems. Comparisons were made using PBS pH 7.2 *vs.* Carbonate bicarbonate buffer pH 9.6 (CBC) for adsorption of the capture antibody; use of CBC buffer resulted in greater absorbance values for the P_f system, but little improvement was seen using CBC buffer in the P_v system. The use of 3,3'5,5' tetramethyl benzidine (TMB) substrate/chromogen *vs.* the currently used 2,2'-Azino-di(3-ethyl benzthiazoline-6-sulfonic acid) (ABTS) in the P_v system resulted in superior sensitivity (Figure 4). For subsequent studies, TMB was used until the three enzyme common substrate was developed.

Multiple Capture Antibodies. The ELISA's were tested using combinations of anti-P_f and anti-P_v capture antibodies; results using P_f antigen and anti-P_f-HRP conjugate demonstrated no effect or deterioration of signal. Surprisingly, although the results demonstrate no crossreactivity of anti-P_f with P_v antigen, there is a definite enhancement of signal when the capture antibodies are combined in the P_v system. In this experiment, monoclonal antibodies were co-adsorbed to PVC plates, followed by addition of P_v antigen (40 pg/well) and peroxidase-labeled anti-P_v. TMB substrate was added, and the plates were read at A_{620} after a 30 minute incubation. With one antibody, absorbance at A_{620} was 1.211 *vs.* 1.629 with both antibodies.

ELISA's were also performed using various combinations of capture antibodies. In separate assays, signal was enhanced using doubles or triples of capture antibodies; results shown in Figures 5 and 6.

Testing with Alternative Substrates. Other substrates were tested using the peroxidase conjugates, including HDCBS *vs.* phenol and AA, 5-amino-salicylic acid, and AEC. AEC was chosen as the optimum substrate based on its signal generation as determined by visual inspection and its rust color.

GREEN 3a

ORANGE 3b

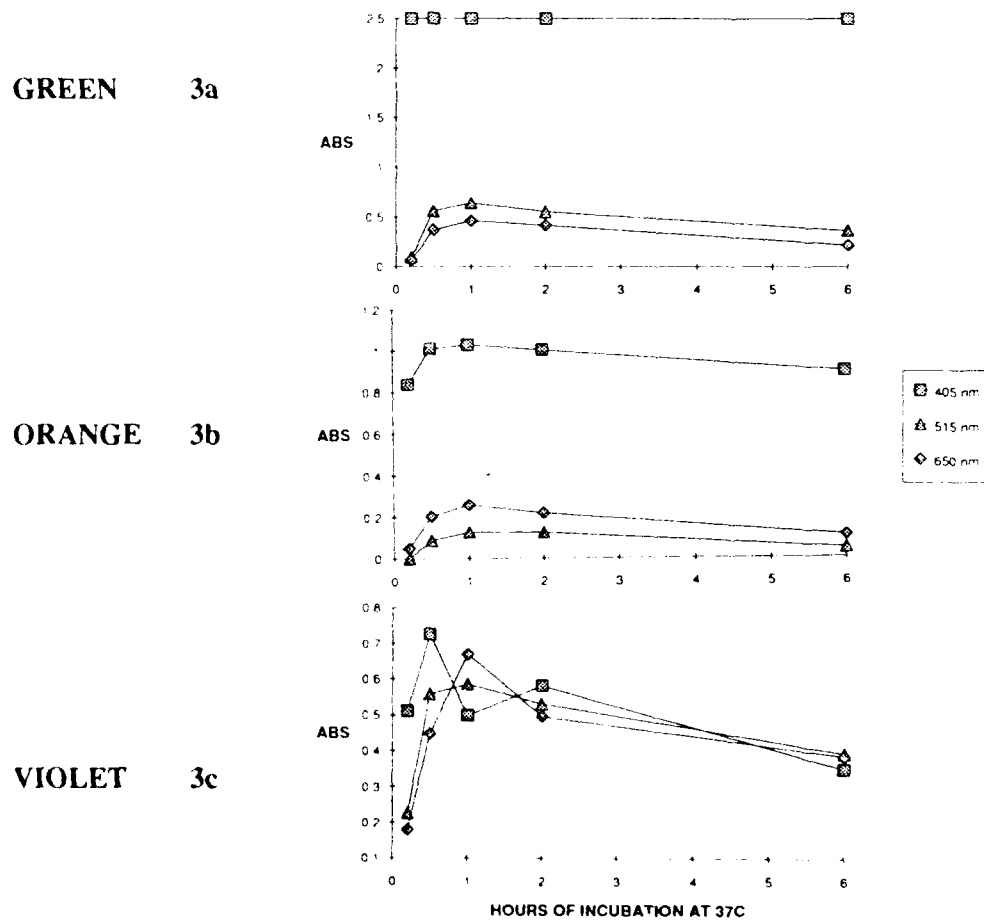VIOLET 3c

HOURS OF INCUBATION AT 37C

FIGURE 3. SPECTROPHOTOMETRIC ANALYSIS OF DUAL ENZYME SYSTEMS.
Checkerboard analysis of dual enzyme systems were conducted as described in the text. After addition of the common substrate solution, the plates were read at the three wavelength maxima for all three enzymes. Figure 3a shows absorbance values of 0.15 μg β-galactosidase and 0.15 μg α-glucosidase over a 6 hour incubation period, corresponding to a green color development. Figure 3b shows 0.023 μg peroxidase and 0.3 μg α-glucosidase, corresponding to an orange color. Figure 3c demonstrates the interference caused by precipitating substrates when 0.01 μg of peroxidase and 0.15 μg β-galactosidase are combined (violet color development).
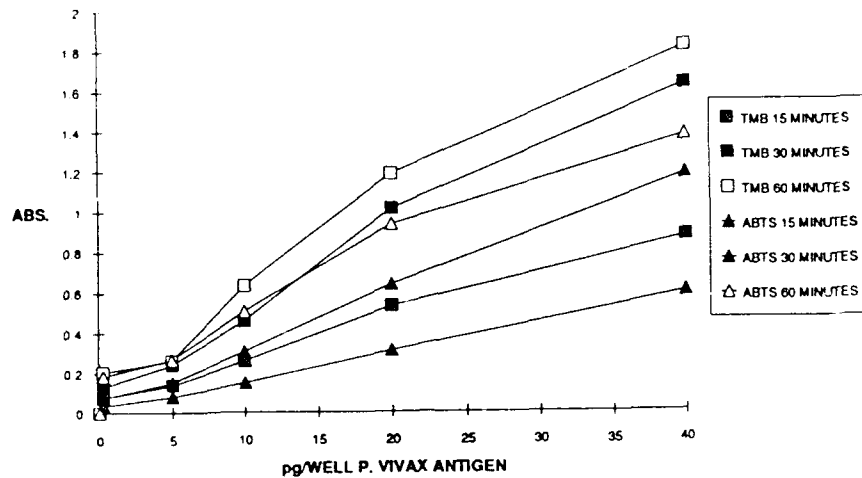
7

FIGURE 4. Use of TMB Substrate *versus* ABTS Substrate. An ELISA was run using the *P. vivax* system to determine optimal signal generation.
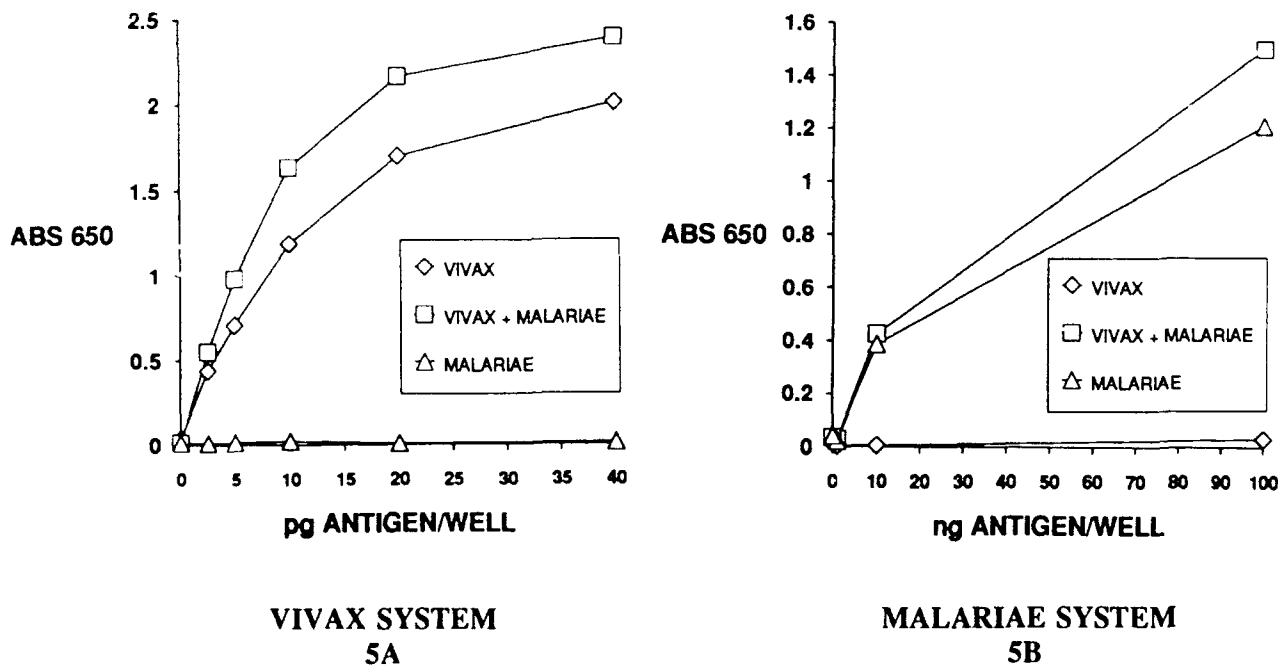

VIVAX SYSTEM
5A

MALARIAE SYSTEM
5B

FIGURE 5. ENHANCEMENT OF SIGNAL WITH TWO CAPTURE ANTIBODIES PER WELL.
ELISA's were performed using both P_v (0.5 μg/well) and P_m (5 μg/well) as capture antibodies. The P_v antigen and P_v-peroxidase ELISA (5A) demonstrated enhanced signal when P_m antibody was included in the adsorption step. The P_m antigen and P_m-peroxidase ELISA system (5B) also demonstrated enhancement of signal. In both cases, this enhancement was not due to cross-reactivity. In addition, BSA controls were run for the P_m system (since the antigen was coupled to BSA) and showed no undesired binding of antibodies to BSA.
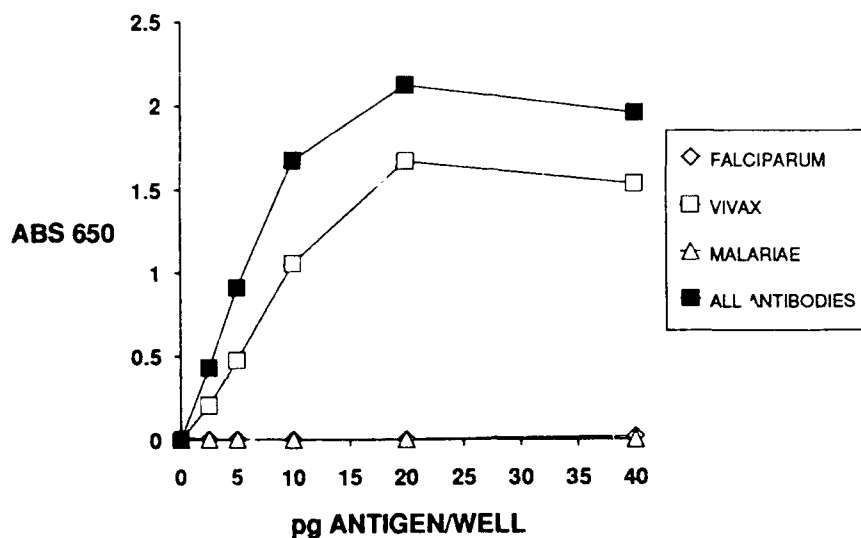
8

FIGURE 6. ENHANCEMENT USING COMBINATION OF CAPTURE ANTIBODIES.
Antibodies were adsorbed to PVC plates at 0.5 μg/ml P_v, 5 μg/ml P_m, and 2 μg/ml P_f using 50 μl per well. The ELISA was performed using P_v antigen and P_v-peroxidase conjugate. Enhancement of signal was not due to crossreactivity of antibodies.

Task 4. Immobilization of Capture Antibodies

Preliminary Studies on Antibody Immobilization. Prior to the start date of this feasibility study, experiments relevant to this project were performed. BSI was previously involved in a project to immobilize biomolecules via carbohydrate moieties to matrices such as polystyrene ELISA plates. As mentioned in the Tri-enzyme ELISA proposal to the U.S. Army, it has been well documented that adsorption of biologically active molecules results in a reduction in the specific activities of such immobilized moieties. Part of this loss of activity may be caused by the hostile environment at the support:aqueous boundary. Since non-specific adsorption of antibody to polymeric surfaces results in steric hindrance of the binding sites in at least a portion of the antibodies, it follows that specific attachment of antibody to the surface via the Fc portion of the molecules should result in superior activity. In addition, it is known that the use of spacer molecules between biomolecules and solid matrix improves their activities. Thus, we at BSI have developed a chemistry by which antibodies can be immobilized to polystyrene (and other polymeric matrices) via the carbohydrate moieties found predominantly on the Fc portion using a spacer molecule. This chemistry can be used to tether the molecules to the surface with the proteins juxtaposed in the aqueous environment such that they can bind antigen as efficiently as possible.

Results of previous studies ([³H] human gamma globulin (HGG) and anti-*Candida* sandwich assays) using this immobilization technology have shown improved antibody loading, improved specific activity, reduced background, greater signal:noise ratios, and improved storage stability of antibody-coated plates.

Plasmodium Antibody Immobilization. Microtiter 96-well plates were coated with a heterobifunctional reagent comprised of a photoactivatable compound, 4-benzoylbenzoyl-ε amino caproyl (BBA-EAC), and hydrazine, which can be coupled to periodate activated carbohydrate groups on the F_c portion of antibodies. Previous results have shown improved antibody activity (and stability) when the antibodies are immobilized such that their active sites are held away from the substrate surface. For these experiments, 12.5 nmole of BBA-EAC-hydrazide was coated on each well. The plates were then illuminated with 320 nm high intensity light to covalently bond the photogroup to the substrate surface.

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P_v antibody oxidized by 20 mM $NaIO_4$ and dialyzed against PBS (for storage) was coupled to hydrazide-coated and unmodified plates (PVC and Corning ELISA) in 0.1 M sodium acetate buffer, pH 5.5 (1 hr, 37°C) at 0.5, 2.5 and 5.0 µg/ml; antigen concentrations tested were 0, 10, and 40 pg/well. Controls included native antibody at the same concentrations. For P_v, using peroxidase conjugate and TMB substrate, hydrazide coating improved signal on the PVC plates when capture antibody was immobilized at a concentration of 0.5 µg/ml at both 10 pg/well (absorbance of 0.473 native antibody on non-modified PVC vs. 0.498 oxidized antibody on hydrazine PVC) and 40 pg/well (absorbance minus background of 1.312 native antibody on non-modified PVC vs. 1.557 oxidized antibody on hydrazide coated PVC) antigen concentrations. The absorbance value of 1.557 was the highest value of any of the concentrations or plates tested. At higher capture antibody concentrations, non-modified plates generated greater signal than hydrazide plates; however, BBA-EAC-hydrazide and capture antibody concentrations were not optimized.

In a separate system, P_m ELISA was tested using hydrazide-coated plates. At 1 µg/well BSA-antigen and 0.25 µg/well antibody, oxidized antibody yielded an absorbance (minus background) of 1.454 while native antibody yielded 0.949. Other concentrations of capture antibodies gave essentially comparable results, i.e., no significant differences between oxidized/hydrazide coupling and adsorbed antibodies. A separate study was performed at lower antigen concentrations; results again showed some improvement in signal with the immobilization of antibody via carbohydrate moieties (at 0.1 µg/well BSA-P_m antigen, A_{650} minus background was 1.388 for native antibody on non-modified PVC vs. 1.557 for oxidized antibody on hydrazide-coated PVC; at 0.01 µg/well, 0.767 for native antibody on non-modified PVC vs. 0.882 for oxidized antibody on hydrazide-coated PVC).

Task 5. Conjugate Preparation

MoAb Purification. P_f MoAb was purified from ascites (2A10-1D6 MAF 12-11-89, lot 729-08 5 ml) by precipitation with SAS, followed by DE52 ion-exchange chromatography. The purified antibody (20.33 mg) was dialyzed against PBS and stored at -20°C until further use. Use in ELISA's showed activity similar to purified MoAb obtained from Dr. Wirtz. In addition, some P_f antibody was purified using a Protein A column (Bio-Rad) according to the package insert. Again, the activity of the antibody in an ELISA showed good activity.

Preparation and Evaluation of Peroxidase Conjugates.

MoAb anti-P_f and anti-P_v were conjugated to peroxidase as follows: Peroxidase (4 mg) was dissolved in H_2O (1 ml) in an amber vial, and 200 µl of 0.1 M $NaIO_4$ was added and stirred at RT for 20 minutes. The enzyme was then dialyzed against 0.05 M CO_3, pH 9.5. The solution was equally split and each half was added to anti-P_f or anti-P_v. The solutions were stirred for 2 hours at RT, followed by addition of 100 µl of a 4 mg/ml $NaBH_4$ solution to each reaction mixture. The conjugates were purified on a P-200 column equilibrated in PBS, with the fractions being monitored at 280 and 403 nm. Those fraction with an A_{403}/A_{280} values between 0.3 and 0.6 were evaluated for activity. This procedure resulted in a 68% yield for each antibody. Evaluation by ELISA showed activity comparable to previously prepared conjugates

Preparation and Evaluation of β-galactosidase Conjugates.

Sulfosuccinimidyl 4-(N-maleimidomethyl) Cyclohexane 1-carboxylate (Sulfo-SMCC). Conjugation of anti-P_v with β-D-galactosidase was performed as described by Ishikawa et al. (Ishikawa, E. M. Imagawa, S. Hashida, S. Yoshitake, Y. Hamaguchi and T. Ueno, J. Immunoassay 4: 209, 1983).

Briefly, 2.8 mg of anti-P_v MoAb was reacted with sulfo-SMCC in 0.1 M PO_4, pH 7.0 for 30 minutes at 30°C. The mixture was applied to a G-25 column equilibrated with 0.1M PO_4, pH 6.5, and eluted with that same buffer. The IgG-containing fractions were collected and pooled. β-D-galactosidase was then added to the IgG-maleimide and incubated overnight at 4°C. The conjugate was then purified using a Sepharose 6B column equilibrated with 0.01 M PO_4, pH 6.5 containing 0.1 M NaCl and 1 mM $MgCl_2$. Two peaks were collected, one corresponding with the conjugate, and the other containing unreacted antibody.

Profusion β-Gal/G. Conjugation was attempted using a new chemical conjugate from Pierce, "Profusion β-Gal/G". This reagent is a recombinant fusion product of β-galactosidase and Protein G. The protein G reacts with the Fc portion of the IgG, and should not interfere with antibody activity. The enzyme was conjugated according to the package insert.

EDC. A conjugation was attempted using EDC coupling. For this procedure, 10 mg of β-galactosidase was dissolved in 1 ml 0.2 M PO_4, pH 7.1. Succinic anhydride (3.3 mg) was added in 0.5 mg portions over a two hour period while stirring at 0°C. The mixture continued to stir for an additional hour at 0°C. The material was purified on a G-25 column equilibrated with 0.1 M PO_4, pH 5.5. IgG P_f (2 ml, 3 mg) was dialyzed against 0.1 M PO_4, pH 5.5, transferred to an amber 4 dram vial. The enzyme solution was added to the antibody solution stirred at RT. EDC (5 mg) was added to the solution every 20 minutes to a total of 30 mg EDC. The resulting reagent was purified on a G-25 column equilibrated with PBS. The resulting conjugate had no enzymatic activity.

Sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate (Sulfo-SMPB). P_f MoAb was conjugated to β-galactosidase using sulfo-SMPB. The procedure was conducted as follows: P_f IgG was dialyzed against 0.1 M PO_4, pH 7.0. Sulfo-SMPB was dissolved at 1 mg/ml in H_2O and 65 μl of the maleimide solution was added to the IgG (1 ml at 1.5 mg/ml). The mixture was stirred for 30 minutes at 30°C, then applied to a G-25 column equilibrated in 0.1 M PO_4, pH 6.5. The material was eluted with the same buffer, and the IgG maleimide fraction (1.5 ml) was collected. β-galactosidase was then added (1.5 mg of protein) to the IgG-maleimide and stirred for 20 hours at 4°C. The conjugate was purified on a G-25 column equilibrated with 0.01 M PO_4, pH 6.5 containing 0.1 N NaCl and 1 mM $MgCl_2$.

ELISA's were conducted to compare the separate lots of P_f-β-galactosidase conjugates. The SMPB maleimide methodology proved to result in highly active conjugates. Conjugates were prepared using this procedure as the need arose.

α-Glucosidase Conjugation and Evaluation.

Glutaraldehyde. α-Glucosidase was coupled to anti-P_v antibody using glutaraldehyde. Conjugation proceeded as follows: a 1.5 ml column of BioRad Affi-Gel Blue was equilibrated with PBS for 45 minutes at 0.5 ml/minute. α-Glucosidase (Sigma, 1.5 ml) was applied to the column to remove BSA (stabilizing protein) and was eluted with PBS. The enzyme eluted in 3 fractions and the second fraction was chosen for coupling to the P_v antibody. Approximately 5 mg of enzyme was mixed with 1.5 ml of P_v MoAb (10 mg) in an 8 ml reaction vial with stirring. An aliquot of 50 μl of 25% glutaraldehyde was added and the mixture stirred for 1 hour at room temperature. The material was quickly desalted over a G-25 column and the first ml was evaluated in an ELISA. The remaining material was dialyzed overnight *versus* PBS at 4°C. BSA was added back (at 0.5 mg/ml) to stabilize the conjugate which was then stored at 4°C. ELISA testing in the P_v system showed color development, but 4 hours of incubation at 37°C was required before signal was strong enough to read by visual inspection. Several attempts were made to optimize this system, including incubation a 45°C. This system was not developing as quickly as the other enzyme systems, and with extended incubation (more than 10 hours) the common substrate began to develop a background brownish color. For this reason, a different enzyme system was

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chosen for further development.

Sulfo-SMCC. The Sulfo-SMCC procedure used to prepare β-galactosidase was repeated using α-glucosidase. The resulting conjugate showed no activity when tested in an ELISA.

Alkaline Phosphatase Conjugation and Evaluation.

Conjugation of this enzyme was conducted using glutaraldehyde coupling. P_v IgG, 4 mg in 2 ml of PBS, was mixed with 2 mg alkaline phosphatase. An aliquot of 8 μl of 25% glutaraldehyde solution was added with stirring to a final glutaraldehyde concentration of 0.1%. The mixture was stirred at room temperature for 2 hours, and dialyzed against 0.01 M PO_4, pH 6.5 containing 0.1 M NaCl and 1 mM $MgCl_2$.

Preparation of BSA-antigens.

With the difficulties involved in developing the enzyme assays, it was necessary to improve the signal to identify the effect of our modifications to the established procedures. These conjugates were prepared according to the protocol provided by Dr. Wirtz. In addition, a glutaraldehyde exposed BSA control was prepared using the same procedure. It was determined early in the project that little or no absorbance above background was seen with the BSA control at concentrations less than or equal to 0.1 μg/well, and therefore blocking buffer was substituted as the no antigen control in the later experiments.

An ELISA was run to compare the results of the alkaline phosphatase system *versus* the α-glucosidase system (Figure 7). These results demonstrate that the kinetics of the alkaline phosphatase system is more conducive to the tri-ELISA system than the α-glucosidase system.

Task 6. Tri-ELISA Testing

Di-ELISA's.

Di-ELISA's were conducted with the P_m-peroxidase and P_f-β-galactosidase systems. The plates were read at 20 minutes, 1 hour, and 7 hours at 515 and 650 nm wavelengths. Observations were made as to color development by visual inspection. Results showed that significant color had developed by 20 minutes (blue observed in wells containing the lowest level tested, 6.25 ng of P_f-BSA; red observed in wells containing 6.25 ng of P_m-BSA; violet-grey observed in wells containing 6.25 ng of each antigen). In one assay, a comparison was made between hydrazide-coated and uncoated PVC plates. Although the absorbances were higher using hydrazide-coated plates (e.g., at 1 hour and P_f antigen of 100 ng/well, A_{650} of 0.130 on raw PVC, and 0.219 for hydrazide coated plate), the color formation was essentially equal upon visual inspection.

Tri-ELISA's.

Initial Results. Tri-ELISA's have been successful when conducted much as for the di-ELISA system, but including alkaline phosphatase-P_v conjugate. In one assay, a raw PVC plate was coated with all three capture antibodies at 0.5 μg/well. The plate was incubated with 100, 25 or 6.25 ng/well of antigen-BSA conjugates. Enzyme conjugates were mixed to the appropriate working dilutions and 50 μl was added per well. Common substrate was added following a wash step. The plate was read at 405, 515, and 650 nm wavelengths at 15 and 45 minutes. In addition, visual observations were noted. Color

in the 100 ng wells was visible within 5 minutes. By 15 minutes, light color had developed down to the 6.25 ng/well concentration. By 45 minutes, colors were observed in wells down to the 6.25 ng level. Wells incubated with P_v-BSA were yellow, those with P_f-BSA were blue, and those with P_m were pink. Wells incubated with P_v plus P_m were orange, those with P_v plus P_f were green, those with P_m plus P_f were violet/grey, and finally, those with all three antigens were brownish-green (see Photograph 2). Again, the absorbance values obtained by reading the plate on the microtiter plate reader proved difficult to interpret.

Antibody Immobilization. A tri-ELISA was performed to compare the method of capture antibody immobilization (adsorbed from PBS or covalently immobilized using hydrazide chemistry). The concentration of capture antibody was varied from 2.5 to 20 µg/ml. The results showed that at higher antibody concentrations, the signal was more evident. In addition, the hydrazide chemistry provided a more sensitive assay.

Signal Enhancement With Multiple Capture Antibodies. The early signal enhancement results with multiple capture antibodies were obtained using the initial antibody loading levels suggested by Dr. Wirtz. However, in the actual tri-ELISA development, the optimal loading levels of the antibodies were increased to 0.25 and 0.5 µg/well. Because the antibody levels had been increased, a P_f ELISA with anti-P_f-peroxidase was run to see if the same trend was observed. The results obtained show that signal enhancement was seen using BSA-P_f, P_f sporozoites, and P_f CS protein when the three capture antibodies were used vs. only one antibody.

4 HOURS ABS 405

ng OF ANTIGEN

18 HOURS ABS 405
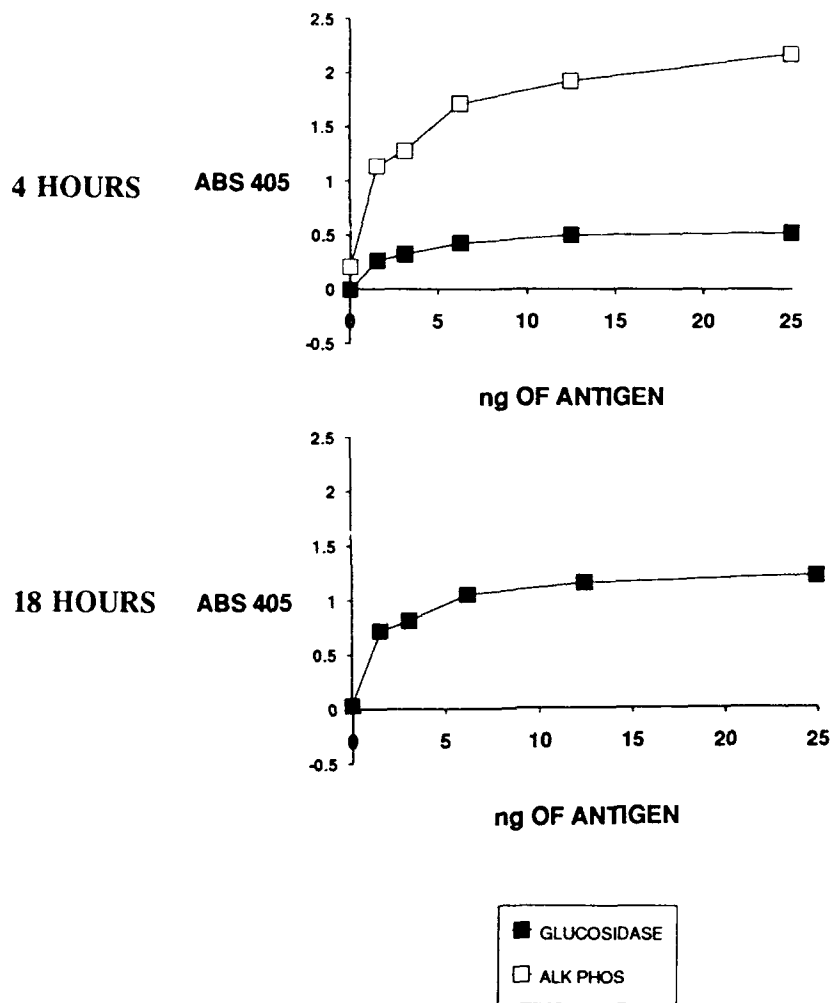
ng OF ANTIGEN

■ GLUCOSIDASE
□ ALK PHOS

FIGURE 7. COMPARISON OF α-GLUCOSIDASE AND ALKALINE PHOSPHATASE P_v CONJUGATES

For this assay, all three capture antibodies were immobilized to hydrazide-coated plates (P_v at 5 μg/ml, P_f and P_m at 10 μg/ml, 50μl/well). P_v-BSA antigen was tested at a range of 1.56-25 ng/well. The α-glucosidase (1:10 dilution) and alkaline phosphatase (1:20 dilution) conjugates were added to the separate wells. A common substrate solution consisting of PNPP and PNAG was used for developing the plates. The plates were read at 405 nm over a period of 18 hours. Two representative graphs are shown here; at 4 hours, the alkaline phosphatase system shows strong color development. After 18 hours of incubation at 37°C, the alkaline phosphatase wells were off-scale using the microtiter plate reader, while the α-glucosidase wells show some color. These results clearly demonstrate the superiority of alkaline phosphatase for sensitivity in the tri-ELISA format.

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The general protocol used at the completion of this Phase I effort for the tri-ELISA was as follows:

1. Periodate-activated capture antibodies were immobilized to PVC plates pre-coated with 12.5 nmoles of BBA-EAC-hydrazide/well. Aliquots of 50 μl of MoAb's were added to each well; 10 μg/ml of anti-P_f and anti-P_m, and 5 μg/ml of anti-P_v in 0.1M Na Acetate buffer, pH 5.5. Plates are incubated at 37°C for 1 hour.

2. Plates were washed three times with 0.05% Tween-20 in PBS.

3. Blocking buffer (BB, 1% BSA, 0.5% Casein, 0.5 mg/ml thimerosal, 0.02 mg/ml phenol red, in PBS pH 7.4) was added at 150 μl/well and allowed to incubated at RT for 1 hour.

4. Plates were washed three times with 0.05% Tween-20 in PBS.

5. Antigen was added at the appropriate dilutions in BB at 50 μl/well (sporozoites were added in BB-NP40 solution). Plates were then incubated for 1 hour at RT.

6. Plates were washed three times with 0.05% Tween-20 in PBS.

7. MoAb-enzyme conjugates were added at the appropriate concentrations in BB at 50 μl/well and allowed to incubate at RT for 1 hour.

8. Just prior to completion of this incubation (step 7), common substrate was prepared:

Stock Buffer Solution, kept at 4°C:
      200 ml 0.2 M K phosphate, pH 7.5
      120 ml diH$_2$O
      4 ml 100 mM MgCl$_2$
AEC Solution (Total 20 ml):
      0.8 ml 4 mg/ml 3-amino-9-ethyl carbazole in N,N-dimethylformamide (DMF)
      19.2 ml Stock Buffer Solution
      Mixed and filtered through Whatman #1 filter paper
Add just prior to use:
      20 mg of ρ-nitrophenyl phosphate* (PNPP, for alkaline phosphatase) OR 6 mg ρ-
            nitrophenyl-α-D-glucopyranoside (PNAG, for α-glucosidase)
      8 mg 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) in 200 μl DMF
      200 μl 3% H$_2$O$_2$

9. Plates were washed three times with 0.05% Tween-20 in PBS.

10. Common substrate was added to plates at 100 μl/well. Plates were incubated at 37°C for 30 minutes and 1 hour, then read and/or visually inspected.

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* Alkaline Phosphatase/PNPP performance was superior when added in Tris or HEPES buffer rather than phosphate buffer, however, the β-galactosidase/X-GAL kinetics was attenuated by the presence of either Tris or HEPES.

P_v Sporozoite Testing. P_v sporozoites were shipped from Walter Reed Army Institute by Dr. Wirtz. Initial testing of the alkaline phosphatase-conjugated anti-P_v using sporozoites and the PBS-based common substrate showed poor sensitivity (2500 sporozoites at 18 hours of incubation). After some discussion, an ELISA was tried using 0.1 M Tris, pH 7.5. For this assay, hydrazide/antibody coated plates were incubated with three separate antigens: 1) NP-40 extracted sporozoites from 1000 to 62/well; 2) BSA-P_v antigen conjugates from 10 ng to 0.63 ng/well; and, 3) circumsporozoite (CS) protein from 100 pg to 6.25 pg/well. Following an incubation step with the alkaline phosphatase anti-P_v, the plate was washed and incubated with PNPP at 1 mg/ml in 0.1 M Tris, pH 7.5. The alkaline phosphatase system showed color development within 45 minutes down to 0.63 ng/well of BSA-P_v. The color development was slower with the other antigens, however, by the end of two hours, the system was sensitive to 250 sporozoites and 25 pg/well CS protein. A peroxidase anti-P_v conjugate plus TMB was also used for all three antigens as a control; sensitivity using this system was less than 62 sporozoites.

Comparison of Phosphate vs. HEPES in Common Substrate. A typical tri-ELISA was performed using the three capture antibodies and BSA-antigens as well as sporozoites (see Photograph 3). The HEPES buffer yielded superior signal relative to PBS for the alkaline phosphatase conjugate. However, the β-galactosidase conjugate was attenuated by the use of HEPES rather than PBS. This issue could not be resolved within the time constraints of this Phase I project.

Blind Study. PVC plates were coated with 10 μg/ml capture antibody in PBS. Nine "unknowns" were prepared in blocking buffer by one scientist; another individual added both standards and unknowns to the plate, and proceeded with the tri-ELISA format (using PBS-based common substrate). Eight of the nine unknowns were easily discernable while one sample was borderline (see Photograph 4). The samples and results are shown in Table 2.

TABLE 2 - BLIND STUDY RESULTS

SAMPLE	DESCRIPTION	COLOR AT 15 MIN.	COLOR AT 1 HOUR	IDENTIFIED AS
BSA-P_f	25 ng/well 2.5 ng/well 0.25 ng/well	medium blue light blue no color	medium dark blue medium light blue very light blue	
BSA-P_m	25 ng/well 2.5 ng/well 0.25 ng/well	medium light pink very light pink no color	medium pink very light pink faint pink	
BSA-P_v	25 ng/well 2.5 ng/well 0.25 ng/well	medium light yellow light yellow no color	medium yellow medium light yellow very light yellow	
1	BSA-P_m 0.5 ng/well	faint pink	very light pink	P_m
3	BSA-P_v 5 ng/well	light yellow	medium light yellow	P_v
4	BSA-P_v 12.5 ng/well	light yellow	medium light yellow	P_v
5	P_f sporozoite 5000/well	very light blue	light blue	P_f
6	BSA-P_m 20 ng/well	medium light pink	medium dark pink	P_m
7	BSA-P_f 10 ng/well	medium light blue	medium dark blue	P_f
8	P_f sporozoites 2500/well	no color	no color or faint blue?	P_f or none
9	BSA-P_f 25 ng/well	medium blue	medium dark blue	P_f
10	BSA-P_v and BSA-P_m 10 ng/well	medium yellow	orange-yellow	P_m and P_v

PHOTOGRAPH 1

DUAL ENZYME SYSTEM
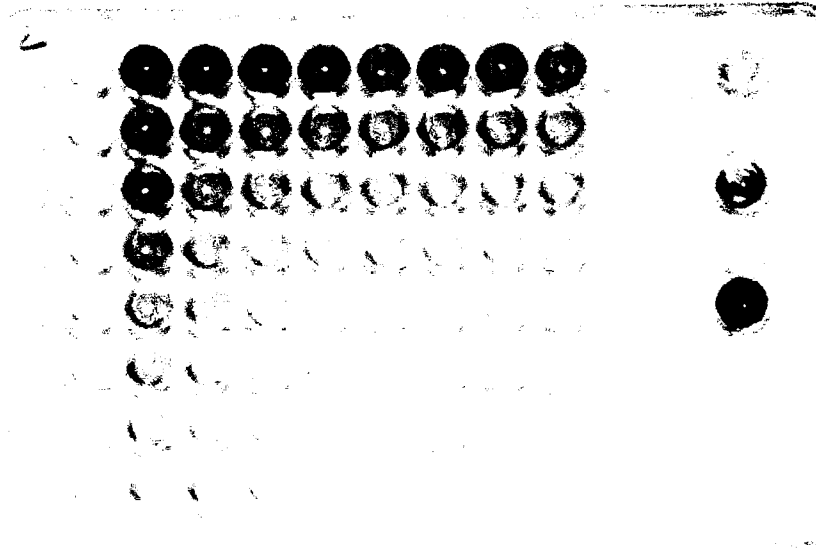
Checkerboard Study of α-Glucosidase and β-galactosidase with Common Substrate Solution. Samples were serially diluted in microtiter plates in a "checkerboard" format in order to determine the range of colors possible in a double positive. In this assay, α-glucosidase and β-galactosidase were dissolved in PBS. Row A contained 10 μg/well of β-galactosidase, and was then serially diluted down the plate (row B contains 5 μg/well, row C is 2.5 μg/well, etc.). Column 2 contained 10 μg/well and was serially diluted across the plate (column 3 has 5 μg/well, etc.). Well A12 contains α-glucosidase, C12 has peroxidase, and E12 is β-galactosidase with the common substrate solution. The spectrophotometric analysis of well B3 is shown in Figure 3a.

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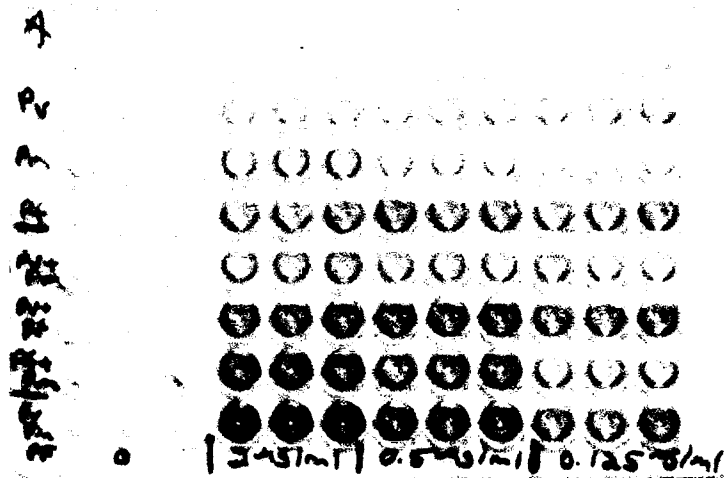# TRI-ELISA WITH ALKALINE PHOSPHATASE, PEROXIDASE, AND β-GALACTOSIDASE

In this assay, a raw PVC plate was coated with all three capture antibodies at 0.5 μg/well. The plate was incubated with 100, 25 or 6.25 ng/well of antigen-BSA conjugates. Enzyme conjugates were mixed to the appropriate working dilutions and 50 μl was added per well. Common substrate was added following a wash step. The plate was read at 405, 515, and 650 nm wavelengths at 15 and 45 minutes. In addition, visual observations were noted. Color in the 100 ng wells was visible within 5 minutes. By 15 minutes, light color had developed down to the 6.25 ng/well concentration. By 45 minutes, colors were observed in wells down to the 6.25 ng level. Wells incubated with BSA-P$_v$ were yellow, those with BSA-P$_f$ were blue, and those with BSA-P$_m$ were pink. Wells incubated with BSA-P$_v$ plus BSA-P$_m$ were orange, those with BSA-P$_v$ plus BSA-P$_f$ were green, those with BSA-P$_m$ plus BSA-P$_f$ were violet/grey, and finally, those with all three antigens were brownish-green (photograph color is off-hue).
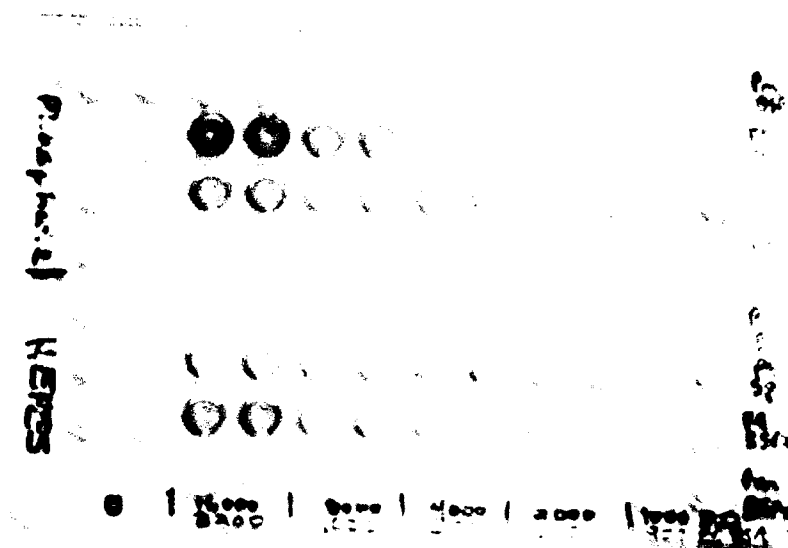
TRI-ELISA COMPARING PHOSPHATE AND HEPES BUFFERS

A typical tri-ELISA was performed using the three capture antibodies and BSA-antigens as well as sporozoites. The HEPES buffer yielded superior signal relative to PBS for the alkaline phosphatase conjugate. However, the β-galactosidase conjugate was attenuated by the use of HEPES rather than PBS. This issue could not be resolved within the time constraints of this Phase I project.
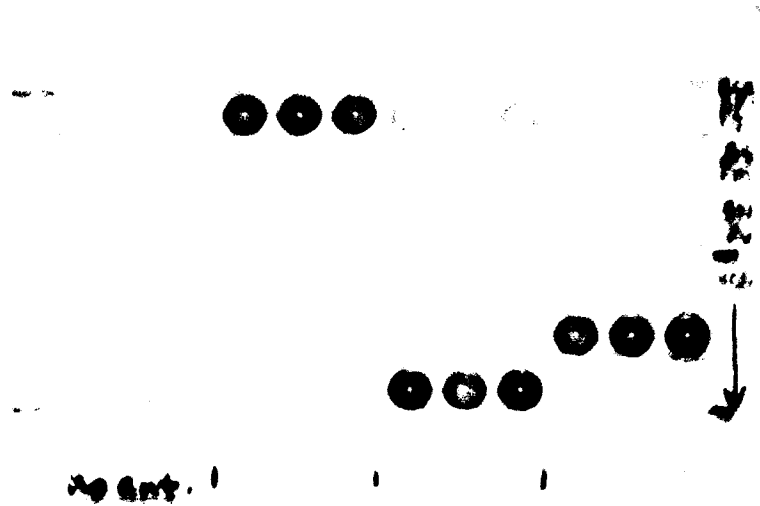
BLIND STUDY TRI-ELISA

PLATE PVC   NOTEBOOK 876   Tri-ELISA Unknowns

	1	2	3	4	5	6	7	8	9	10	11	12	
A													
B				25 ng/well ——→			2.5 ng/well ——→			0.25 ng/well ——→			BSA·P$_f$
C				25 ng/well ——→			2.5 ng/well ——→			0.25 ng/well ——→			BSA·P$_m$
D				25 ng/well ——→			2.5 ng/well ——→			0.25 ng/well ——→			BSA·P$_v$
E				#1 ————→			#3 ————→			#4 ————→			
F				#5 ————→			#6 ————→			#7 ————→			unknowns
G				#8 ————→			#9 ————→			#10 ————→			
H													

Capture Ab 10 µg/ml: α P$_f$, α P$_m$, α P$_v$

no Ag

PVC plates were coated with 10 µg/ml capture antibody in PBS. Nine "unknowns" were prepared in blocking buffer by one scientist; another individual added both standards and unknowns to the plate, and proceeded with the tri-ELISA format (using PBS-based common substrate). Eight of the nine unknowns were easily discernable while one sample was borderline (see results listed in Table 2).