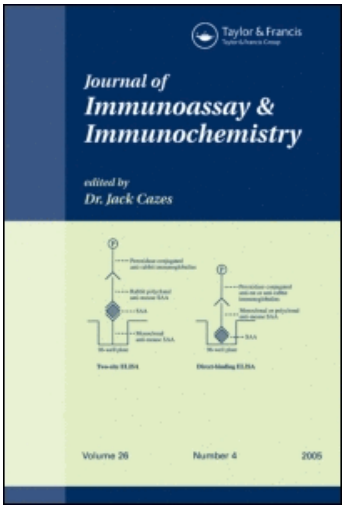


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# Optimization of the Covalent Conjugating Procedure ($NaIO_4$) of Horseradish Peroxidase to Antibodies for Use in Enzyme-Linked Immunosorbent Assay

Victor C. W. Tsang[a]; Ryan M. Greene[a]; Joy B. Pilcher[a]
[a] Immunology Branch, Division of Parasitic Diseases, Department of Health and Human Service, National Center for Infectious Disease, Centers for Disease Control and Prevention, Public Health Services, U.S., Atlanta, GA, U.S.A.

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# OPTIMIZATION OF THE COVALENT CONJUGATING PROCEDURE ($NaIO_4$) OF HORSERADISH PEROXIDASE TO ANTIBODIES FOR USE IN ENZYME-LINKED IMMUNOSORBENT ASSAY

Victor C. W. Tsang, Ryan M. Greene, and Joy B. Pilcher

Immunology Branch, Division of Parasitic Diseases, National Center for Infectious Disease, Centers for Disease Control and Prevention, Public Health Services, U.S. Department of Health and Human Service, Atlanta, GA 30341-3724, U.S.A.



## ABSTRACT

The procedure for covalent conjugation of horseradish peroxidase (POD) to goat anti-human IgG (GAHG) molecules was systematically optimized in terms of reactant molar ratio, time of reaction, pH, and temperature. The optimum conjugation procedure was defined by the conditions that produced an enzyme-labeled Ab with the highest specific activity in immunosorbent assays for normal human IgG (NHIgG). The best conditions are: Sodium *meta*-periodate (NaIO4) to Ab molar ratio during oxidation is 40:1; time of oxidation is 5 min at 37 °C; oxidation reaction is conducted at pH 5.0; the molar ratio of POD:GAHG is 6:1; the conjugation time is 24 h at 4 °C; and the optimal conjugation pH is 10.0. A conjugate constructed under these conditions is capable of generating 1.8 and 12.6 times more specific signals ($\Delta A_{650nm}$/min) than the best and worst commercial conjugates, respectively. This conjugate is also able to detect NHIgG at a concentration of 2.25 $x10^{-13}$ M, a sensitivity 25 times that achieved by most comparable commercial products in identical assays.

(KEY WORDS: Peroxidase, conjugate, immunoassay, ELISA, HIV, immunoblot)

## INTRODUCTION

The principal reaction in immunoassays occurs between antibody (Ab) and antigen (Ag), yielding an essentially invisible product, the Ag-Ab complex. To observe and measure this reaction, a label is usually introduced via a second Ab. In enzyme-linked immunosorbent assays (ELISA), the introduced label is in the form of an enzyme. Immunologic reactions are, therefore, measured indirectly through the enzymatic activity of the label, most often demonstrated by the change of absorbance of a chromogenic substrate. It is logical, therefore, that the enzyme-labeled second Ab, or conjugate, is the molecule most responsible for the sensitivity of the ELISA. Poorly-labeled or unlabeled second Abs occupy available binding sites but provide no signal, thus seriously compromising the sensitivity of the entire assay.



Horseradish peroxidase (POD, E.C. 1.11.1.7) and alkaline phosphatase (AP, E.C. 3.1.3.1) are commonly used enzymes in ELISA (1). These 2 enzymes owe their popularity as labels for ELISA conjugates to their low cost, availability, stability, low molecular weights, and high substrate turnover rates. The most common and convenient methods for coupling these enzymes to Ab or Ag are generally dependent on hetero-bifunctional reagents or dialdehydes that serve as covalent bridges between the 2 biological molecules (2). For POD, a glycoprotein with a carbohydrate shell unessential for enzymatic activity, we have the additional advantage of being able to oxidize adjacent vicinal hydroxyl (-OH) groups of this shell with $NaIO_4$ and create an active aldehyde. The POD-aldehyde can then be coupled directly to the $-NH_2$ groups of an Ab or Ag molecule (3). We chose to

optimize the $NaIO_4$-POD conjugate because of its proven superior efficacy in comparison with other enzyme labels and coupling methods (4,2,5,6).

## MATERIALS AND METHODS

POD was purchased from Sigma Chemical Co, St. Louis, MO (Type III, RZ≥3.0). Inorganic, analytical reagent grade chemicals were purchased from Mallinckrodt Chemical Co. St. Louis, MO. POD-labeled goat anti-human conjugates were purchased from commercial sources. The respective lot numbers and origins of these conjugates are listed in the *"Comparisons with commercial conjugates"* section to follow.



### Immunological reagents

Normal human IgG (NHIgG) was prepared by protein-G affinity chromatography from a serum pool donated by healthy persons (7). Goat anti-human IgG (GAHG) Abs from serum of hyper-immunized animals were prepared by immunoaffinity as described previously (8). Protein determinations were performed according to the method of Bradford (9).

### Preparation of POD-anti-human IgG conjugates

POD (20 mg/ml) was dissolved in buffer at specified pH, and stirred while $NaIO_4$ (30 mg/ml) in the same buffer was slowly added to achieve specified $NaIO_4$/POD molar ratios. For experiments involving the pH variable, the buffer used in this oxidation step was 0.1 M citric acid, 0.1 M $Na_2HPO_4$, titrated to specified pH.

For all other experiments, 0.1 M citric acid/sodium citrate, pH 5.0 was used as the buffer during oxidation of POD. The enzyme-oxidant mixture was incubated with stirring at specified temperatures in a water bath for specified times. Ethylene glycol was added to a final concentration of 0.01 M. The mixture was then immediately desalted with a PD-10 column (Pharmacia Biotech, Piscataway, New Jersey), equilibrated with 0.5 M NaCl, 0.001 M citric acid/sodium citrate, pH 5.0, and the greenish-brown POD-aldehyde was collected.

For experiments comparing the pH variable during coupling, a 1.0 M N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES), 1.0 M boric acid, 1.0 M $Na_2HPO_4$, titrated to specified pH was added to the GAHG at 0.1 x total volume, and pH stability was verified. For all other experiments a 1.0 M $Na_2CO_3/NaHCO_3$ buffer, pH 10.00 was used during the coupling step. The GAHG was then mixed with the oxidized POD at a given POD/IgG molar ratio, and the pH was again verified. A freshly prepared stock solution of $NaBH_4CN$ in water (10 mg/ml) was added to the POD-IgG mixture to specified final concentrations. The enzyme and Ab were then allowed to couple while being continuously mixed end-over-end.

After coupling, 0.1 x total reactant volume of 1.0 M $NaH_2PO_4.H_2O$ was added, followed by another addition of freshly prepared $NaBH_4CN$. The conjugate was stirred at 25°C for 15 min, and then cooled to 4°C in an ice bath. Labeled and unlabeled IgG was precipitated by the addition of an equal volume of 90% saturated $(NH_4)_2SO_4$ in phosphate buffered saline, 0.15 M NaCl, 0.05 M $Na_2HPO_4/NaH_2PO_4$, ph 7.2 (PBS). The 90% saturated $(NH_4)_2SO_4$ was at pH 6.5 - 6.8 as well as the final pH of the precipitated suspension. The IgG-POD suspension was mixed end-over-

end for 30 min at 4°C and centrifuged at 10,000 x G for 10 min to precipitate the conjugate. The pellet was dissolved in PBS, and glycerol was added at 0.77 x volume. The final protein concentration of the conjugates was maintained at 3 - 10 mg/ml. All reagent preparation and reactions were conducted in glass containers. All reagents were kept on ice until used.

Experimental variables

The following variables were investigated individually to arrive at the optimal conjugation conditions:

1. Oxidation temperature: 25, 37 °C
2. Oxidation time: 5, 10, 15, 30, 60 min
3. Oxidation pH: 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8
4. $NaIO_4$/POD molar ratio: 10, 20, 30, 40, 50, 60, 70, 80, 90, 100
5. Coupling temperature: 4, 25, 37 °C
6. Coupling time: 0.5, 1, 5, 22 h
7. Coupling pH: 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11
8. POD/IgG molar ratio: 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10
9. $(NH_4)_2SO_4$ precipitation vs. no treatment
10. Ethylene glycol treatment vs. no treatment
11. [$NaBH_3CN$]: 0.1, 0.01, 0.001 M

Quantitative measurements of conjugate activities (FAST-ELISA)

All quantitative measurements of conjugate activities were made with the Falcon assay screening test-enzyme linked immunosorbent assay (FAST-ELISA) as

previously described (10). Comparisons of the Specific Activity (SpAct) of the conjugates were made under Ag-limiting conjugate-excess conditions. SpAct was reported as change of absorbance at 650nm per minute ($\Delta A_{650nm}$/min). The Falcon Assay System Test (FAST™) from Becton/Dickinson (B/D, Falcon Division, Oxnard, Ca) immunoassay plate and stick system was used for all assays. Briefly, polystyrene sticks were sensitized with 0.3 $\mu$g/ml (Ag-limiting level) NHIgG in 0.05 M Tris-HCl, 0.3 M KCl, 0.002 M EDTA, pH 8.00 for 5 min. Unbound NHIgG on the sticks was removed by washing with PBS with 0.3% tween-20 (polyoxyethylenesorbitan monolaurate) (PBS/Tw) (Tw is from Sigma Chemical Co.). All conjugates were normalized to 0.05 mg/ml in PBS with 0.3% Tween 20, and added to 96-well polystyrene plates at 100 $\mu$l/well. An existing reference conjugate was used as a positive control, and PBS/Tw as a negative control. The sensitized sticks were incubated with the conjugates for 5 min, and unbound Abs were removed by washing. A commercial POD substrate solution (Kirkegaard & Perry Labs, cat # 50-76-00, Gaithersburg, MD) of $H_2O_2$ mixed with buffer and 3,3',5,5'-Tetramethylbenzidine (TMB) was added to another 96-well plate at 150 $\mu$l/well. Subsequent incubation of the sticks in TMB produced a blue color change in proportion to the amount of POD bound. The bound POD-anti-human IgG specific activities were then measured as $\Delta A_{650nm}$/min and plotted against the variables being investigated in the conjugation.



Comparing activities of conjugates

Commercial conjugates were obtained from Accurate Chemical & Scientific Corp. Westbury, NY (Cat# 3620, lot# I9R119), Bio-Rad Laboratories, Melville, NY

(Cat# 172-1050, lot # 74006A), Calbiochem-Novabiochem Corp. La Jolla, CA (Cat# 401455, lot# 545592), Cappel Research Products, Durham, NC (Cat# 55220, Lot# 35932), Kirkegaard & Perry Laboratories Inc. (Cat #074-1006, Lot# QH24-1), and Sigma Chemical Co. (Cat# A-8667, lot# 042H4863).

To establish the excess conjugate concentration used in our assays, a concentration (or dilution) series of each conjugate was prepared in PBS/Tw. All conjugates were diluted from 1:10, in 10-fold steps up to $1:10^7$. Sticks were sensitized with 5 $\mu$g/ml (Ag excess level) NHIgG in 0.05 M Tris-HCl, 0.3 M KCl, 0.002 M EDTA, pH 8.00 for at least 2 hours, and were washed with PBS/Tw. The conjugate dilutions were added in triplicate to 96-well plates (100 $\mu$l/well), and the sticks were incubated in the dilutions for 5 min. The sticks were again washed, and incubated for 1-5 min in TMB (150 $\mu$l/well). Bound POD-anti-human IgG activities were quantified as $\Delta A_{650nm}$/min and plotted against the concentrations of the conjugates. The concentration of conjugate at which the activity curve first developed a plateau was considered excess. A 10-fold higher concentration of conjugate excess was used in all subsequent assays. Whenever possible, a protein concentration for the active component [POD-Ab] was derived for the excess level of each conjugate. Derivation of protein concentrations for commercial conjugates was based on numbers supplied by the respective manufacturers.

To compare the efficacy of commercial conjugates against one prepared under our optimized conditions, FAST-ELISA sticks were sensitized with a dilution series of NHIgG, in 5-fold steps, from $2 \times 10^{-2}$ $\mu$g/ml ($1.41 \times 10^{-10}$ M) to $6.4 \times 10^{-6}$ $\mu$g/ml ($4.51 \times 10^{-14}$ M) . For the purpose of this experiment, the molecular weight

of NHIgG was determined by SDS-PAGE to be 142 KD. In many of the commercial conjugate preparations, bovine serum albumin (BSA) was added by the manufacturer as a preservative. To maintain equivalent conditions during our comparison, all conjugates were, therefore, diluted in 1% BSA in PBS. A 10-fold excess concentration was used for all conjugates. FAST-ELISA assays were performed as described above.

Enzyme-linked immuno-electrotransfer blot (Immunoblot, EITB) assays were performed as previously described (11,12). The Ag was heat-inactivated, and solubilized human immunodeficiency virus (HIV) whole viruses, loaded at 0.02 $\mu g/mm^2$ of gel. A reference HIV-positive serum (CDC Cat #VS2151) was 5-fold serially diluted in PBS/Tw and a 0.5 ml aliquot was incubated with each immunoblot strip for 1 h. Conjugates were diluted with 1% BSA in PBS and a 0.5 ml aliquot was incubated with each blot strip for 1 h. Because of the large volume and long incubation time, the concentration of the conjugates was 1/5 of that used in the FAST-ELISA. All strips were developed as described previously (11,12).



## RESULTS

Based on the initial POD-Ab protein concentrations supplied by the manufacture and titration of the conjugates under Ag saturating conditions, we found the 10-fold excess levels to be between 0.01 - 0.14 mg/ml. The 10-fold excess levels of our own conjugates were all approximately 0.05 mg/ml. All experiments comparing the specific activities of conjugate were performed at their respective 10-

fold excess level. All results are reported as the mean of at least 3 replicate determinations. Limit bars in figures represent the standard deviation of the mean.

Optimal conditions for POD oxidation

Figure 1 shows that the optimal temperature and time for oxidation of POD by $NaIO_4$ is 37 °C for 5 min. The optimal oxidation pH for POD by $NaIO_4$ is at pH 5.0 (Figure 2). The optimum temperature for oxidation is at 37 °C (Figure 2). We also observed that oxidation at higher temperatures, or for longer than optimal times resulted in conjugates that were insoluble and had lower activities. This, presumably, was due to excessive cross-linking between POD and Ab molecules and/or denaturation of the enzyme leading to loss of Ab activity and/or enzymatic activity.

Figure 3 shows the optimal $NaIO_4$/POD molar ratio during oxidation to be 40:1. There was an instantaneous change of color in the oxidation mixture from reddish-brown to green immediately after the addition of $NaIO_4$. In higher molar ratios this greenish color was maintained throughout the subsequent coupling reaction with Abs. At a molar ratio of 40:1, the redness returned after conjugation with Ab.

Optimal conditions for coupling POD to Ab

Figure 4 shows that the covalent coupling of POD-aldehyde to the $-NH_2$ of the Ab is pH dependant. The optimum for this reaction was at pH 10.00. Another critical factor for this reaction was the POD/Ab molar ratio. Figure 5 shows the

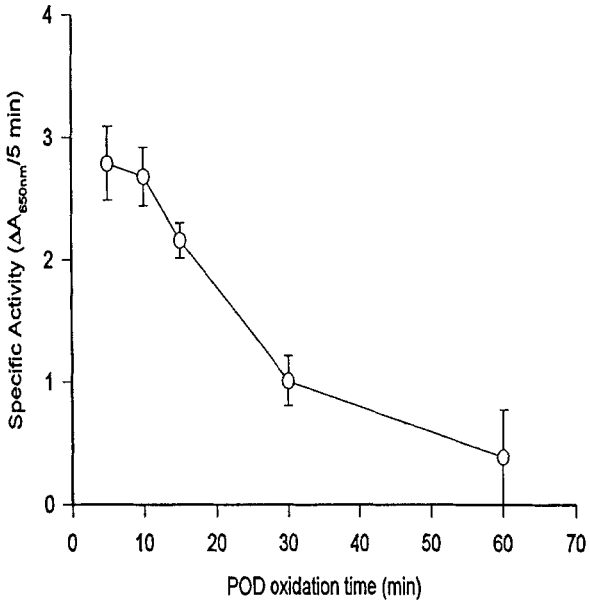


Fig. 1. Optimal oxidation time for POD. POD (20 mg/ml) dissolved in 0. 1 M citric acid/Na citrate at pH 5.0, was oxidized with $NaIO_4$ at a $NaIO_4$/POD molar ratio of 50:1, at 37 °C, for the times specified on the x-axis and conjugated to GAHG (10 mg/ml) in 1.0 M $NaCO_3$/$NaHCO_3$, pH 9.5 at a molar ratio of 4:1 (POD/GAHG). The activity of each conjugate (0.05 mg/ml) was measured by FAST-ELISA against 0.3 $\mu$g/ml NHIgG (Ag-limiting). Limit bars represent standard deviation of the mean from 3 replicate determinations.



optimum of this stage to be a POD/Ab molar ratio of 6. Coupling time and temperature for this reaction, on the other hand, was less critical (Figure 6). Coupling at 4 °C for 24 h seemed to produce the most active product.

Treatment with Ethylene glycol, $(NH_4)_2SO_4$, and $NaBH_3CN$

Whether or not ethylene glycol was added to stop the oxidative action of $NaIO_4$ made little difference in the SpAct of our small experimental lots of

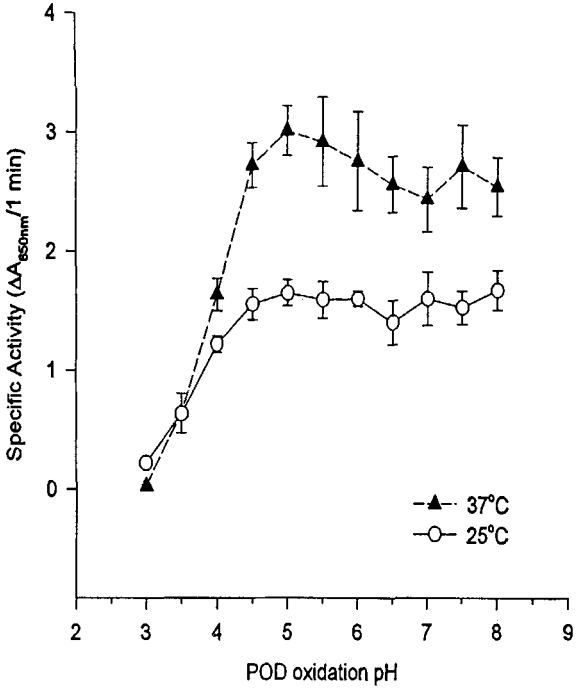


Fig. 2. Optimal oxidation pH for POD. POD (20 mg/ml) dissolved in 0.1 M citric acid, 0.1 M $NaH_2PO_4$, at the pH specified on the x-axis, was oxidized with $NaIO_4$ at a $NaIO_4$/POD molar ratio of 50:1, at 25 or 37 °C, for 5 min and conjugated to GAHG (10 mg/ml) in 1.0 M $NaCO_3$/$NaHCO_3$, pH 9.5, at a molar ratio of 4:1 (POD/GAHG). The activity of each conjugate was measured in Ag-limiting conditions as described in Fig 1.

conjugate. There was no significant difference between the conjugate lots that were further purified by $(NH_4)_2SO_4$ precipitation and those without the benefit of this treatment. Treatment with $NaBH_3CN$ at 0.1, 0.01, and 0.001 produced little difference in the SpAct of the final conjugate. However, conjugates constructed without the benefit of Schiff's base stabilization by $NaBH_3CN$ did not retain their initial activities (data not shown).

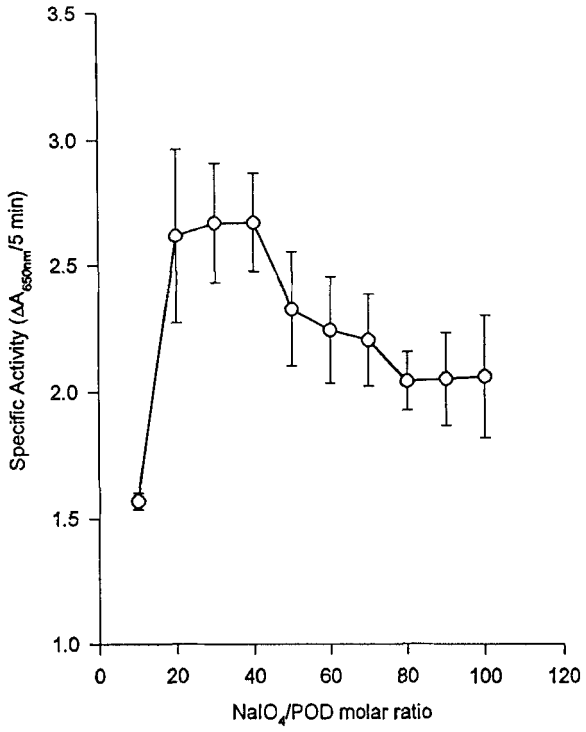


Fig. 3. Optimal $NaIO_4$/POD molar ratio. POD (20 mg/ml) dissolved in 0. 1 M citric acid/Na Citrate at pH 5.0, was oxidized with $NaIO_4$ at the $NaIO_4$/POD molar ratio specified on the x-axis, at 37 °C, for 5 min, and conjugated to GAHG (10 mg/ml) in 1.0 M $NaCO_3$/$NaHCO_3$, pH 9.5, at a molar ratio of 4:1 (POD/GAHG). The activity of each conjugate was measured in Ag-limiting conditions as described in Fig 1.



Comparing activities of conjugates

Figure 7 shows a comparison by FAST-ELISA of the SpAct of 6 commercial conjugates with one prepared by our optimized method. At $1.41 \times 10^{-10}$ M NHIgG concentration, the SpAct produced by our conjugate (1.062) is 1.8 times higher than that of the best commercial product tested (Calbiochem, SpAct = 0.594) and 12.6

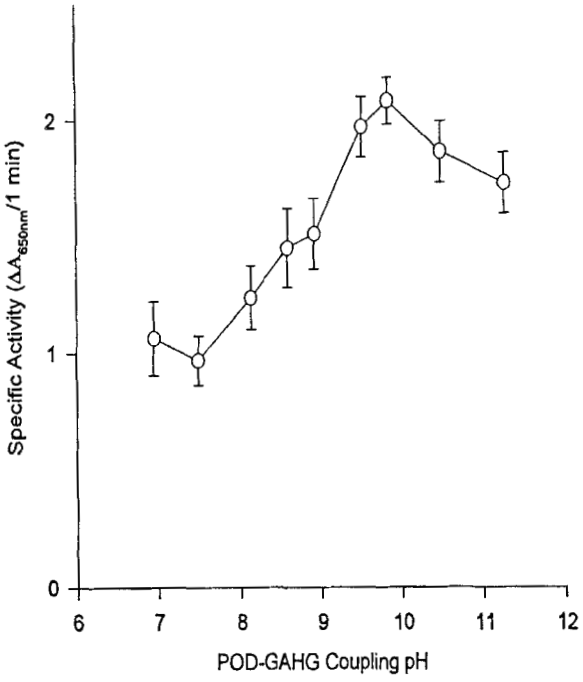


Fig. 4. Optimal coupling pH for POD-GAHG. POD (20 mg/ml) dissolved in 0. 1 M citric acid/Na citrate at pH 5.0, was oxidized with $NaIO_4$ at a $NaIO_4$/POD molar ratio of 50:1, at 37 °C, for 5 min. Goat anti-human IgG Abs (GAHG) (8 mg/ml) dissolved in 1.0 M HEPES, 1.0 M boric acid, 1.0 M $Na_2HPO_4$, titrated to the pH specified on the x-axis, and coupled to the oxidized POD at a molar ratio of 4:1 (POD/GAHG). The activity of each conjugate was measured in Ag-limiting conditions as described in Fig 1.

times that of the worst tested (Sigma, SpAct = 0.084). The signal to noise ratios at this concentration of NHIgG for these 3 conjugates were, 20.42, 18.00, and 2.55, respectively. Table I summarizes the detection limits of all conjugates for NHIgG, and shows the sensitivity of our conjugate to be superior to those of commercial preparations. The multiphasic response of the CDC conjugate at 2.25 x $10^{-13}$ M

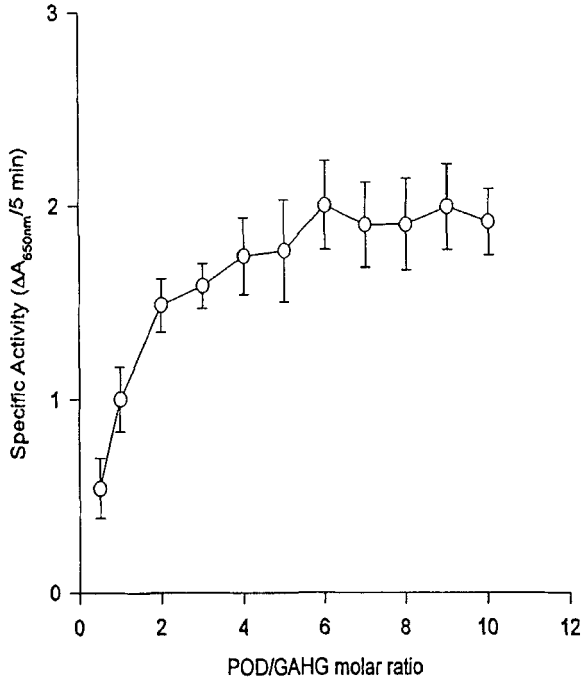


Fig. 5. Optimal POD/GAHG coupling molar ratio. Oxidized POD (see Fig. 4) was conjugated to Goat anti-human IgG Abs (GAHG) (10 mg/ml) in 1.0 M $NaCO_3/NaHCO_3$, pH 9.5, at the molar ratios specified on the x-axis (POD/GAHG). The activity of each conjugate was measured in Ag-limiting conditions as described in Fig 1.



NHIgG (Figure 7) is probably an aberration or experimental error and we have no explanation for it at this time.

Figure 8 shows a comparison by EITB of all conjugates. The detection limit for serum antibodies to the p24 core antigen of HIV with the CDC, Biorad, and KPL conjugates was at a serum dilution of $1{:}1.6 \times 10^{-5}$, while that of the Sigma conjugate was at $4 \times 10^{-4}$. The detection limits with the same conjugates for antibodies to the

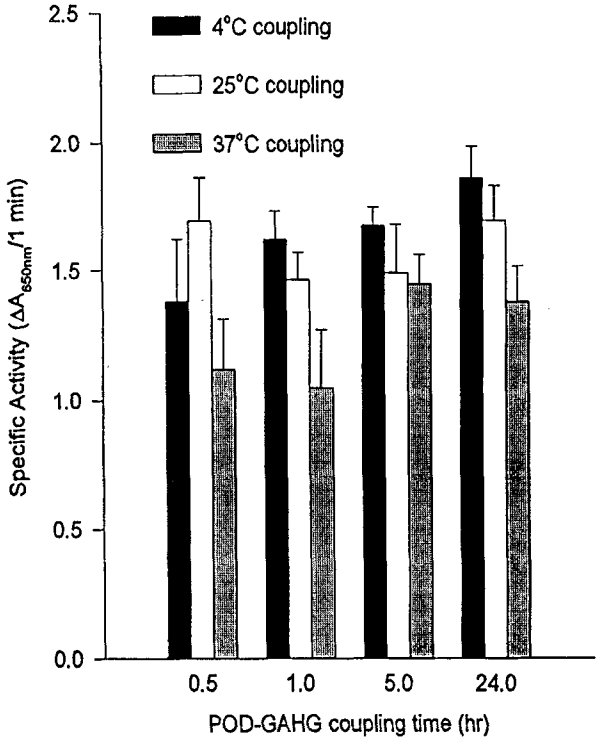


Fig. 6. Optimal POD/GAHG coupling time and temperature. Oxidized POD (see Fig. 4) was conjugated to Goat anti-human IgG Abs (GAHG) (10 mg/ml) in 1.0 M $NaCO_3/NaHCO_3$, pH 9.5, at a molar ratio of 4:1 (POD/GAHG). The coupling times and temperatures are listed in the figure. The activity of each conjugate was measured in Ag-limiting conditions as described in Fig 1.

gp41 envelope antigen were $2.0 \times 10^{-3}$ and $<1 \times 10^{-6}$. These 4 conjugates were chosen because their SpAct in the FAST-ELISA covered the top, middle, and low ranges of the sensitivity spectrum.

## DISCUSSION

Our goal in conducting these experiments was to define optimal and reproducible conditions for producing POD-Ab conjugates. We chose to optimize

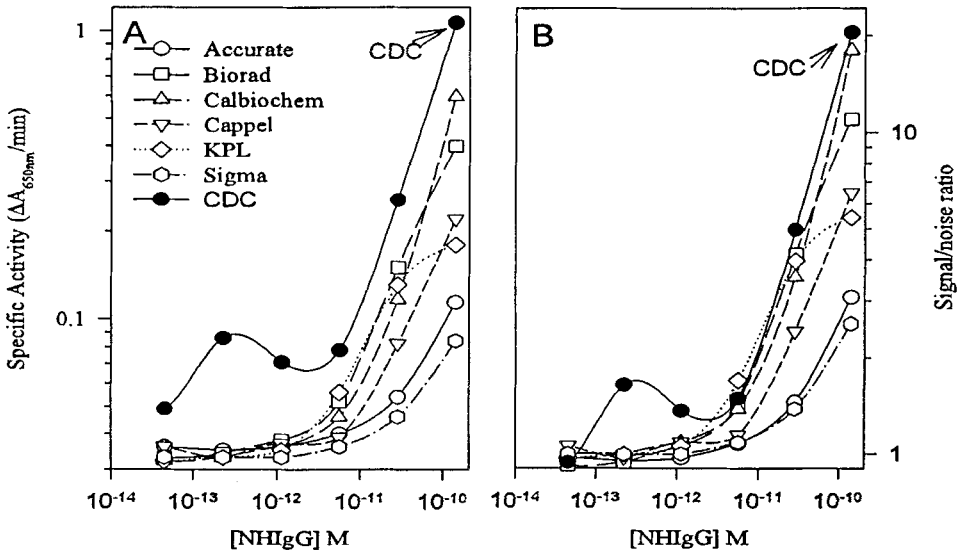


Fig. 7. Comparing specific activities of commercial and optimized CDC conjugates by a quantitative, kinetic assay. The specific activity of each conjugate was measured by FAST-ELISA against Ag-limiting concentrations of NHIgG specified on the x-axis. Limit bars for standard deviation were omitted for clarity. All conjugates were diluted in 1% BSA in PBS/Tw. Each conjugate was used at their respective 10-fold excess level. The respective lot numbers for the 6 commercial conjugates are listed in the text (see Methods and Materials section). Background (zero level [NHIgG] SpAct) values are available from Table I, but were not subtracted. Signal/noise ratios were computed as specified in Table I.



the procedure for coupling of POD to Ab because of the high efficiency of POD-conjugate compared to that of alkaline phosphatase (6). We prefer the oxidative modification of POD over that of other conjugation procedures because of the relative high efficiency of this system (3) and the high activity of the products (5). The introduction of conjugating oxidized POD to Ab by Nakane (3) represents an important advance to immunoassay. Subsequent modifications to their method,

## TABLE I

Comparing Detection Limits for Commercial and CDC Conjugates[1]

| Conjugates | Lowest detectable [NHIgG] M[2] | Signal/noise ratio[3] | SpAct at detection limit (±SD)[1] | SpAct with no antigen (±SD)[1] |
|---|---|---|---|---|
| CDC | $2.25 \times 10^{-13}$ | 1.65 | 0.086 ± 0.007 | 0.052 ± 0.0013 |
| Accurate | $2.82 \times 10^{-11}$ | 1.46 | 0.054 ± 0.006 | 0.037 ± 0.002 |
| Biorad | $5.63 \times 10^{-12}$ | 1.44 | 0.052 ± 0.006 | 0.036 ± 0.0006 |
| Calbiochem | $5.63 \times 10^{-12}$ | 1.39 | 0.046 ± 0.002 | 0.033 ± 0.002 |
| Cappel | $5.63 \times 10^{-12}$ | 1.15 | 0.039 ± 0.001 | 0.034 ± 0.002 |
| KPL | $5.63 \times 10^{-12}$ | 1.70 | 0.056 ± 0.002 | 0.033 ± 0.006 |
| Sigma | $2.82 \times 10^{-11}$ | 1.39 | 0.046 ± 0.008 | 0.033 ± 0.001 |

[1] SpAct was reported as change of absorbance at 650nm per minute ($\Delta A_{650nm}$/min). All data were based on 3 replicate experiments, ±SD = standard deviation.

[2] Lowest detectible [NHIgG] M was determined as the concentration that produced a signal/noise ratio≥ 1.10, and a SpAct of 10% above that of the zero [NHIgG] baseline.

[3] Signal/noise ratio derived from SpAct at lowest detection divided by SpAct with no antigen (NHIgG).

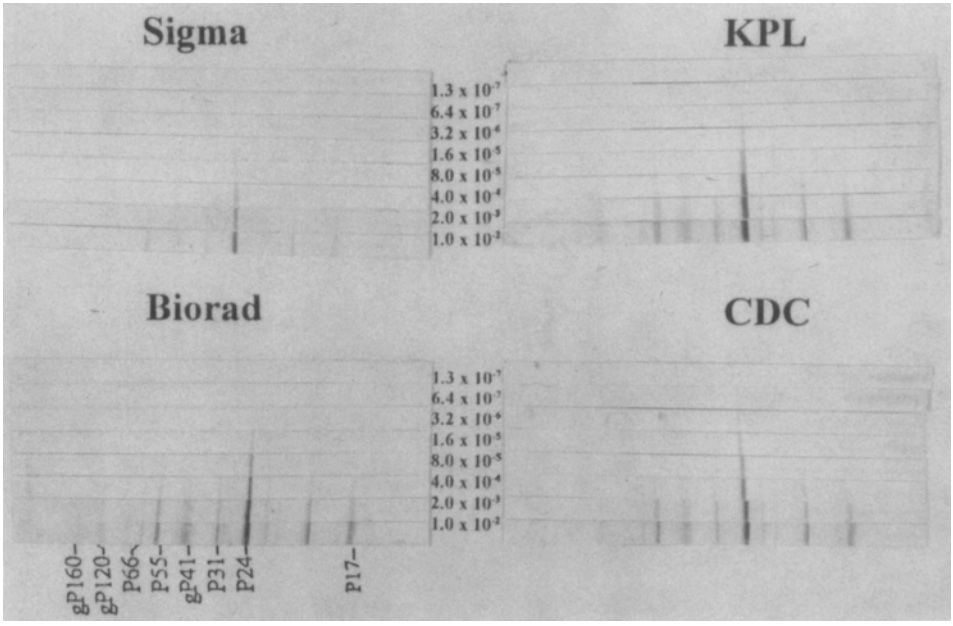


Fig. 8. Comparing specific activities of commercial and optimized CDC conjugates by immunoblot (EITB) assay. A dilution series (center numbers indicate dilution folds) of the CDC reference HIV+ serum (CDC Cat# VS2151) was incubated with immunoblot strips containing SDS-PAGE-resolved HIV antigens for 1 h, respectively. The strips were washed and exposed to diluted conjugate solutions for 1 h. All conjugates were diluted in 1% BSA in PBS/Tw. Each conjugate was used at their respective 10-fold excess level. Details are described in the Methods and Materials section.



however, failed to produce a method capable of generating consistent, high-activity conjugates. This may explain the inconsistency of commercial conjugates, the majority of which were manufactured by variations of the Nakane procedure.

Our data indicate the optimal conditions and procedure for conjugating POD to Ab are as follows:

1. Sufficient POD (20 mg/ml) was dissolved in 0.1 M Citric acid/Na Citrate,

pH 5.00 and warmed to 37 °C. Sodium *meta*-periodate ($NaIO_4$, 30 mg/ml in water) was added to a final $NaIO_4$/POD molar ratio of 40:1. Oxidation was allowed to proceed for 5 min at 37 °C with constant stirring.

2. At the end of 5 min, ethylene glycol was added to achieve a final concentration 0.01 M and the reaction mixture was immediately desalted with a PD-10 column equilibrated in 0.001 M Citric acid/Na Citrate, pH 5.00. The buffering capacity of this solution was deliberately kept low to allow easy shift of pH during subsequent procedures. For large production lots of POD, a Sephadex G-25M (Pharmacia) column of appropriate size was used instead of the PD-10.

3. The required volume of Ab, in our case, GAHG was mixed with 0.1 x volume of 1.0 M $Na_2CO_3$/$NaHCO_3$ buffer at pH 10.00, the desalted POD-aldehyde, and sufficient freshly prepared $NaBH_3CN$ (10 mg/ml) to achieve a final concentration of 0.001 M. The molar ratio of POD:GAHG was kept at 6:1. The conjugation between POD and Ab is allowed to proceed for 24 h at 4°C.

4. At end of the coupling period (24 h) the reaction mixture is adjusted to pH 6.0 with 1.0 M $NaH_2PO_4$. Usually, 0.1 x of the reaction volume is required to bring the pH to 6.0.

5. An identical volume of freshly-prepared $NaBH_3CN$ (10 mg/ml) as that used in step 3 above is again added to the coupling mixture. The mixture is incubated at room temperature for 30 min.

6. An equal volume of 90% saturated $(NH_4)_2SO_4$ in PBS is added to the coupling mixture and stirred in an ice bath for 30 min. The precipitated POD-Ab conjugate is separated by centrifugation at 10,000 g for 10 min.

7. The conjugate is dissolved in PBS and 0.77 x volume of glycerol is added. We generally store our conjugate bulk stocks at -85°C and keep the working aliquot at -20 °C. Tthe conjugate will not freeze at -20 °C because of glycerol,. The SpAct of our bulk stocks showed no detectable change for up to 2 years in storage under these conditions. The working aliquot (stored at -20 °C) remained unchanged for up to 1 year.



Although we detected no difference in SpAct between ethylene glycol-treated and untreated lots, our POD samples were desalted in small PD-10 columns immediately upon completion of oxidation, and they are of sufficiently small volumes that essentially all free $NaIO_4$ was separated from the POD almost immediately. This would not happen with large volume, production lots. Ethylene glycol would be important in this case to prevent over-oxidation of POD molecules by $NaIO_4$ while the reaction mixture is undergoing buffer exchange.

The traditional reducer used to stabilize the Schiff's base formed during conjugation was $NaBH_4$. This reducer, however, produced unstable conjugates (13). We substituted $NaBH_4CN$ for $NaBH_4$, which resulted in conjugates that are stable for 1 year or more at -20 °C (data not shown). Postconjugation purification by precipitation with $(NH_4)_2SO_4$ to rid the final product of free POD may not be necessary in most applications. Only those systems that have potential nonspecific

binding sites for POD will be affected by the presence of free enzyme. We further purified our conjugates simply as a precaution.

The most critical steps of the conjugation procedure appear to be those pertaining to the oxidation of POD with $NaIO_4$. The molar ratio of $NaIO_4$/POD, time, pH, and time for oxidation are all critical parameters. Slight deviations from the optimal conditions for these parameters will result in over- or under-oxidation of POD. This will translate into destruction of enzymatic activity, excessive cross-linking of POD with Ab, or inefficient labeling of Ab molecules. We also realize that the absolute concentration of the reactants are vital to the rate of the reaction. We have, therefore, maintained our stock solutions of [POD] at 20 mg/ml, [$NaIO_4$] at 30 mg/ml, [$NaBH_3CN$] at 10 mg/ml, and [GAHG] Ab at 3 - 10 mg/ml. With strict adherence to the above optimal conditions, we were able to produce over 25 lots of POD-Ab conjugates with highly predictable SpAct. Although we did not systematically optimize reagents from other animal species, we had, however, use the above procedure to produce conjugates from mouse monoclonal and rabbit polyclonal antibodies with very good SpAct.

When comparing our optimized conjugates to given lots of commercial products, we noted significant differences in our FAST-ELISA data. With the EITB data, however, the difference is only obvious between the best and the worst conjugates tested. The differences in sensitivity for detecting bound Ab in this test were less obvious between the top performing conjugates. The FAST-ELISA is a kinetic and quantitative assay, which is dependent on the rate-kinetic of the immunologic and enzymatic reactions (10,14). The EITB, however, is a qualitative

and steady-state assay. Furthermore, the 3-dimensional constraints to antibody binding imposed by the 2 different types of solid matrix in these assays may also contribute to steric hindrance and cause significant differences in the way conjugates of different molecular weights bind to Ab in these assays. The molecular weight-dependent kinetics of conjugate binding to Ab were described and discussed previously (5). However, precisely what properties of these 2 assays caused their different responses to the conjugates remains speculative at this time. The consequence of using a conjugate with low specific activity, is obvious; the lack of sensitivity of these conjugates will surely compromise our ability to detect disease-specific antibodies. In an infection such as HIV, the implication is serious.



We acknowledge that the SpAct of any given conjugate is a function of how well it was labeled, and the quality (affinity, avidity, and specificity) of the antibodies used. It is impossible to dissect the influence of these parameters on the overall specific activities of the various conjugates studied in this report. We submit, however, that by comparing their activities at conjugate-excess conditions, we were measuring their respective ultimate achievable sensitivity.

With this report we hope to raise the awareness of the pitfalls of using conjugates with low specific activities. By defining the optimal conditions for conjugation, we also hope to provide a means to ensure the quality of future conjugates.

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report is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services. Please direct correspondence to: Victor C.W. Tsang, Immunology Branch, Division of Parasitic Diseases, Mail Stop F-13, CDC, 4770 Buford Hwy., NE, Atlanta, GA 30341-3724, USA., E-mail: vct1@ciddpd1.em.cdc.gov



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