A General Procedure for Optimizing Concentrations of Capture Antibody, Biotinylated Detecting Antibody, and Enzyme-Labeled Avidin in ELISAs: Application to Assays for α-Fetoprotein, Prolactin, FSH and LH in Serum

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The purpose of this study was to develop a procedure for optimizing concentrations of capture antibody, biotinylated detecting antibody, and enzyme-labeled avidin in enzyme-linked immunosorbent assays (ELISA). This procedure was used to establish analyses for α-fetoprotein (AFP), prolactin (PRL), follicle stimulating hormone (FSH), and luteinizing hormone (LH). Consecutive incubations with standard or sample, biotinylated detecting antibody, peroxidase-conjugated avidin, and enzyme substrate in wells coated with capture antibody were employed. To optimize each step, the standards were measured at various concentrations of reagent, and the logarithm of the measurements was plotted against the logarithm of the reagent concentration. The optimal concentration of reagent was taken as the concentration where the distance from the response of the zero standard (the background) to any of the other standards was longest, that is, where the ratio of the response from any standard to the background is greatest. The smallest detectable concentration is obtained (minimal $SD_{background}$/signal ratio) when the optimal concentration of detecting antibody is used (maximal signal/background ratio). For the four optimized analyses, between-run coefficients of variation (CVs) were 4.9–7.9%, and within-run CVs were 1.9–4.2%. Minimal detectable concentrations were <1 μg/L for AFP, 0.014 int. units/L for PRL, <1 int. unit/L for LH, and 2 units/L for FSH. Recoveries for the assays were 90–100%. In the PRL assay, no interference from placental lactogen or growth hormone was found. In the assays for FSH and LH, no interferences from the other glycoprotein hormones were found. When compared with commercial two-site immunometric assays, the intercept was not significantly different from zero, and the correlation coefficients were 0.97–0.994. Thus, a practical approach for choosing the optimal concentrations of reagents was determined, and data provided to document this approach.

Keywords: POD-avidin; Two-site immunometric assay.
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Introduction

Two-site immunometric methods are sensitive and specific methods for measuring proteins (*1–5*). One antibody is bound to a solid surface to capture the analyte and quantitation is achieved through a label attached to the detecting antibody. In enzyme-linked immunosorbent assays (ELISA) using biotin-avidin, the detecting antibody is labeled with biotin, which in turn is quantitated by a reaction with an enzyme-labeled avidin. The kinetics (*6*) and the minimal detectable doses for different error models (*7–9*) have been described for immunoradiometric assays. In some cases, the concentration of labeled antibody is selected as a compromise between conflicting requirements for detection limit, maximal absolute signal, and width of working range (*10,11*). However, these theoretical models have not been theoretically applied or practically tested in ELISA systems. Although the avidin-biotin method has been in use for several years (*12,13*), the procedure for selecting the optimal concentration of enzyme-labeled avidin has not been described previously.

Traditionally, the concentrations of reagents are determined by checkerboard titrations in which the concentrations of two reagents are varied (*1,14*). It is possible to increase the signals of the standards by

increasing the concentrations of detecting antibody, the enzyme-labeled avidin, and/or by increasing the incubation times. Therefore, "infinite" numbers of combinations of both reagent concentrations and incubation times can give the same signal of the standards. Since the minimal detectable concentration is dependent upon the precision of the response of the zero standard and the slope of the standard curve, the signal of the zero standard, i. e. the background, should also be considered.

The purpose of this study was to develop and describe the strategy for experimentally selecting the optimal concentrations of the capture and detecting antibodies and the enzyme-labeled avidin. The concentration of only one reagent is varied at a time. The concentration that gives the highest signal/background ratio is considered the optimal concentration because this concentration gives the lowest minimal detectable concentration. The data supporting this assumption are presented for the PRL assay (Table 3). The data for the other assays are not shown.

The strategy has been applied to develop methods for measuring α-fetoprotein (AFP), prolactin (PRL), follicle stimulating hormone (FSH), and luteinizing hormone (LH) in serum.

Materials and methods

Reference population: Blood was sampled from 106 male blood donors (ages 20–40 yr, median 33 yr) after they had donated 450 ml of blood at 9.00–12.00 a.m.

Antibodies and standards: The concentrations of capture and biotinylated antibodies are those used in the optimized assays.

AFP: Polyclonal rabbit anti-AFP (*15*) (A008, batch no. 097), 1.9 g/L purified immunoglobulin fraction, was from DAKO A/S, Glostrup, Denmark, 3 mL/L in coating buffer. Monoclonal anti-AFP (MCA320), 30 g/L ascitic fluid Ig fraction, was from Serotec, Oxford, U.K. Biotinylated MCA320 was 1 mL/L in detection buffer. AFP-Standard-Serum (OTOD 03) was from Behringwerke AG, Marburg, Germany. One int. unit is equivalent to 1.6 ng, using the AFP Reference standard of the World Health Organization (WHO), no. 72/225.

PRL: Monoclonal anti-PRLs (*16*) (MCA714, INN-hPRL-9 and MCA712, INN-hPRL-1), 4 g/L purified ascites fluid, were from Serotec, Oxford, U.K. For coating MCA714 was 1 mL/L in coating buffer. Biotinylated MCA 712 was 1 mL/L in detection buffer. The third International standard (IS) 84/500 from WHO (*17*) was the primary standard. PRL (i017) from UCB-Bioproducts, Brussels, Belgium was the working standard.

FSH: Monoclonal anti-β-chain FSH (MCA338), and monoclonal anti-holo FSH (MCA337), both 4 g/L purified ascites fluid, were from Serotec, Oxford, U.K. For coating 2 mL/L MCA338 in coating buffer was used. Biotinylated MCA337 was 0.5 mL/L in detection buffer. The first IS 83/875 from WHO (*18*) was the primary standard. FSH (i007) (UCB-Bioproducts, Brussels, Belgium) was the working standard.

LH: Monoclonal anti-LH (A2543), 7.7 g/L purified immunoglobulin, was from Ventrex Laboratories Inc., Portland, ME, 200 μL/L in coating buffer, and monoclonal anti-LH (03-1600), 1 g/L purified immunoglobulin from ascites fluid, was from Zymed Laboratories Inc., So. San Francisco, CA. Biotinylated 03-1600 was 0.5 mL/L in detection buffer. The second IS 80/552 from WHO was the primary standard (*19*). Human pituitary LH (*20*) from Porton Products Ltd., Salisbury, U.K. was the working standard.

Other reagents: High capacity polystyrene microwell plates (Immuno Plate MaxiSorp F-96; Life Technologies A/S, Roskilde, Denmark) were used. Other reagents were as follows: biotinamidocaproate N-hydroxysuccinimide ester, bovine serum albumin (A4378), bovine γ-globulins (G5009), and lysozyme (L6876) (Sigma, St. Louis, MO); rabbit γ-globulin (Calbiochem, San Diego, CA); TMB Microwell Peroxidase Substrate System (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD); horseradish peroxidase avidin (POD-avidin, P364) and mouse serum (DAKO A/S, Glostrup, Denmark); human urinary chorionic gonadotropin (iodination grade) (UCB Bioproducts S.A., Braine-L'Alleud, Belgium); purified placental lactogen (Immunotech A/S, Odense, Denmark); and human growth hormone (Norditropin) (Novo Nordisk A/S, Bagsvaerd, Denmark). Visking dialysis tubing was from Medicell International Ltd, London, UK. Analytical grade chemicals and glass-distilled water were used throughout.

Coating buffer: 15 mM Na_2CO_3, and 35 mM $NaHCO_3$, pH 9.6.

ELISA buffer: 1.5 mM $NaH_2PO_4 \cdot H_2O$, 8.5 mM $Na_2HPO_4 \cdot 2H_2O$, 145 mM NaCl, and 0.1% Tween 20, pH 7.4.

Diluent: 1.5 mM $NaH_2PO_4 \cdot H_2O$, 8.5 mM $Na_2HPO_4 \cdot 2H_2O$, 400 mM NaCl, and 0.1% Tween 20, pH 7.4.

Diluent for standards: 1.5 mM $NaH_2PO_4 \cdot H_2O$, 8.5 mM $Na_2HPO_4 \cdot 2H_2O$, 145 mM NaCl, 1 g/L bovine serum albumin, 400 mg/L bovine γ-globulins, and 200 mg/L rabbit γ-globulins, pH 7.4.

Carrier proteins: per liter of diluent, 4 mL mouse serum, 2 g bovine serum albumin, 800 mg bovine γ-globulins, and 400 mg rabbit γ-globulins.

Detection buffer: per liter of ELISA buffer, 2 mL mouse serum, 1 g bovine serum albumin, 400 mg bovine γ-globulins, and 200 mg rabbit γ-globulins.

POD-avidin solution: 1.5 mM $NaH_2PO_4 \cdot H_2O$,

8.5 mM $Na_2HPO_4 \cdot 2H_2O$, 400 mM NaCl, 200 mg/L lysozyme, and 500 μL/L POD-avidin.

Procedures: Blood samples: Blood samples were collected by venipuncture in glass tubes without additive. Serum was stored at 4°C for 1 week before analysis or frozen immediately at −20°C.

Preparation of Standards: Dilute the standard in diluent for standards to give the appropriate concentrations. Store the standards from WHO at −80°C in aliquot. Store the other standards at −20°C in aliquot.

Preparation of controls: Either pool sera with the appropriate concentration of analyte or dilute a serum with a high concentration of analyte into sera from blood donors to give the desired concentration of analyte. Store in aliquot at −20°C.

Biotinylation of antibody (2,21): Dilute 1 mg of antibody with 100 mmol/L $NaHCO_3$ pH 8.3 to a volume of 1 mL. Dialyse against two changes of 1 liter 100 mmol/L $NaHCO_3$ pH 8.3 for 20 hr at 4°C. Add 50 μL of freshly prepared 4 mmol/L biotinamidocaproate N-hydroxysuccinimide ester in anhydrous dimethyl sulphoxide, and incubate at room temperature in the dark for 4 hr. Add 50 μl of 100 mmol/L lysine · HCl and 50 μl of a solution containing 100 mg/L of bovine γ-globulins, and 50 mg/L of rabbit γ-globulins in diluent without Tween. Dialyse against two changes of 1 liter ELISA buffer without Tween for 20 hr at 4°C, giving the stock solution of biotinylated antibody. Store at −20°C or at 4°C for a maximum of 1 month.

Washing of microtiterplates: Wash each well three times with 300 μL of ELISA buffer without soaking using a Titertek Washer S8/12.

The optimized ELISA procedure: Coat each well with 100 μL of capture antibody in coating buffer. Cover with adhesive foil and store at 4°C without agitation for 16 hr–1 month. Empty the wells and wash. Add 10 μL of standard or serum and 40 μL of diluent to each well using a Hamilton Microlab M diluter, and then add 50 μL of carrier proteins to each well. Plate duplicate wells for all standards and samples. Incubate 30 min for AFP and LH assays, and 60 min for the PRL and FSH assays. All incubations in the microtiter plate, covered with adhesive foil, are at room temperature with agitation. Empty the wells and wash. Add 100 μL of biotinylated antibody in detection buffer to each well. Incubate plate for 30–45 min. Empty the wells and wash. Add 100 μL of the POD-avidin solution to each well. Incubate for 30 min. Empty the wells and wash. Add 100 μL of TMB Microwell Peroxidase Substrate to each well. Develop the color for 5–30 min and stop the reaction by adding 100 μL of 1 M phosphoric acid to each well. Read the absorbances at 450 and 620 nm. Use a cubic spline curve-fitting procedure (*22*) for calculating the results.

Minimal detectable concentration. Analyze the zero standard in 72 wells in one run. The minimal detectable concentration for duplicate measurements is SD multiplied by $t_{0.95;df=1}$ value (6.3138) divided by the slope of the standard curve and the square root of 2 (*2,23*).

Recovery. Measure diluent and samples with low concentrations of analyte as described above and with the addition of 50 ml of a standard per liter of carrier proteins. The variance of recovery is calculated as described (*24*).

Comparison of methods. The following samples and methods were used to validate the present methods:

AFP: Sera from pregnant women in the second trimester were analyzed by the immunoluminometric kit BeriLux AFP (Behringwerke AG, Marburg, Germany) or by Amerlex-M AFP 2nd Trimester radioimmunoassay kit (Amerlite Diagnostics Ltd., Amersham, Buckinghamshire, England). In both cases the calibrators were calibrated against AFP WHO 72/225 (*25*). The unit was kiloint. units/L. For the Amerlex-M kit 1 int. unit is equivalent to 0.96 ng.

PRL: Sera from the routine analyses were analyzed by the PRL Fenzia kit (Orion Diagnostica, Espoo, Finland), whose standards were calibrated against WHO third IS 84/500.

FSH: Sera from the routine analyses were analyzed by the Amerlex FSH radioimmunoassay kit (Amerlite Diagnostics Ltd., Amersham, Buckinghamshire, England), whose standards were calibrated against the second International Reference Preparation- (IRP-HMG). Furthermore, serum samples from the routine analyses were analyzed by the Delfia FSH kit (Wallac OY, Turku, Finland), whose standards have been calibrated against the second International Reference Preparation (IRP) of Pituitary FSH/LH (ICSH) human for bioassay (78/549).

LH: Serum samples from the routine analyses were analyzed by the Amerlex LH radioimmunoassay kit (Amerlite Diagnostics Ltd., Amersham, Buckinghamshire, England), whose standards were calibrated against the second IRP-HMG and by the LH Fenzia kit (Orion Diagnostica, Espoo, Finland), whose standards were calibrated against WHO second IS 80/552. Furthermore, serum samples from the routine analyses were analyzed by the Delfia hLH Spec kit (Wallac Oy, Turku, Finland), whose standards have been calibrated against WHO IRP 68/40, human pituitary LH for immunoassay.

Statistical methods. Between-run SD is calculated from the average of the results of duplicate measurements.

Within-run SD for duplicate results is calculated as the SD of the results of single measurements divided by the square root of 2.

Reference intervals given here are nonparametric central 0.95-interfractile intervals.

Regression analysis was performed as described by Altman and Gardner (*26*). Intervals in parentheses are 95% confidence intervals.

Results

Selection of antibody pairs. Two different antibodies were needed to develop specific ELISA methods. The antibodies were selected in the following way (data not shown).

AFP: The polyclonal rabbit anti-AFP, A008, was used previously in this laboratory, and the monoclonal anti-AFP, MCA320, was chosen as the antibody that gave the highest signal among two monoclonal antibodies tested.

PRL: Three monoclonal antibodies from Serotec were tested. The results obtained by the chosen antibody pair showed the best correlation with the Amersham PRL radioimmunoassay kit.

FSH: The antibody pair suggested by the supplier was used.

LH: Seven monoclonal antibodies from various suppliers were tested for the ability to form pairs and for specificity against other glycoprotein hormones. The selected antibody pair gave a sensitive and specific assay, *vide infra*.

Kinetics of the binding reaction between analyte and capture antibody: Kinetic experiments have shown that the reactions between the capture antibody and standards were completed within 30 min for assays for AFP and LH, but the reaction times had to be extended to 60 min in the PRL and FSH assays (unpublished experiments).

Optimal concentration of capture antibody: Figure 1 shows the results from four experiments in which the concentrations of capture antibodies in the coating buffer were varied. For each analyte, the signals of the standards reach a plateau without any major change in the background.

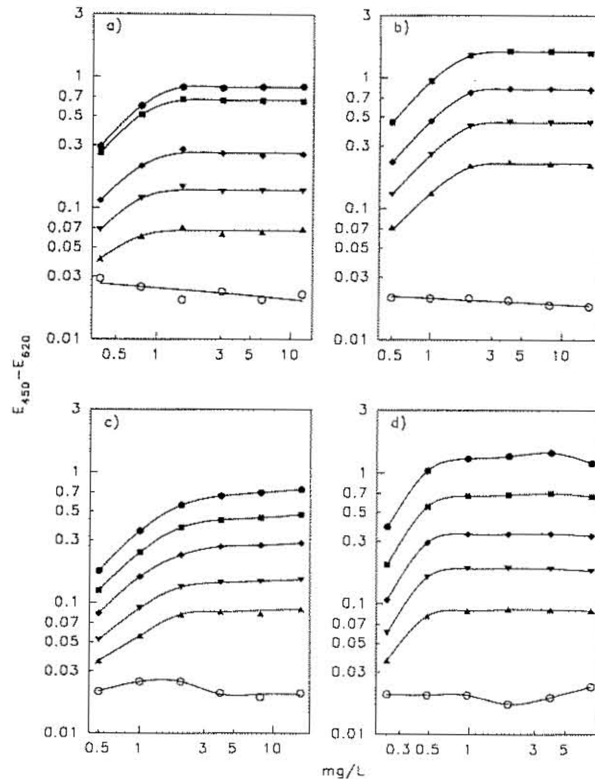

Fig. 1 *The effect of varying concentrations of the capture antibodies in the coating buffer. The antibody concentrations are given in mg per liter of coating buffer.* ***(A)*** *AFP: The concentrations of the standards were 0 (○), 10 (▲), 25 (▼), 50 (◆), 150 (■), and 200 (●) µg/L. The enzyme reactions were developed for 10 min.* ***(B)*** *PRL: The concentrations of the standards were 0 (○), 0.14 (▲), 0.28 (▼), 0.55 (◆), and 1.11 (■) int. units/L. The enzyme reactions were developed for 7 min.* ***(C)*** *FSH: The concentrations of the standards were 0 (○), 30 (▲), 60 (▼), 120 (◆), 180 (■), and 300 (■) int. units/L. The enzyme reactions were developed for 30 min.* ***(D)*** *LH: The concentration of the standards were 0 (○), 6 (▲), 15 (▼), 30 (◆), 60 (■), and 120 (●) int. units/L. The enzyme reactions were developed for 10 min.*

Optimal concentration of detecting antibody: Figure 2 shows the results from four experiments in which the concentrations of the biotinylated antibodies in detection buffer were varied. For all of the assays, the signals of the standards and the background kept increasing with increasing concentrations of the biotinylated antibody in the detection buffer. For the AFP and LH assays the optimal concentrations of biotinylated antibodies were 1 and 0.5 mL/L, respectively. For the PRL assay the signal/background ratio was 10% higher at 2 mL/L than at 1 mL/L, but a concentration of 1 mL/L was chosen, because the signal and minimal detectable concentration were sufficient at this concentration. Although the signal/background ratio is 5% higher at 0.25 mL/L than at 0.5 mL/L for the FSH assay, a concentration of 0.5 mL/L was chosen to allow for some deterioration of activity.

Optimal concentration of POD-avidin: Figure 3 shows the results from four experiments in which the concentrations of POD-avidin were varied. For each analyte the optimal POD-avidin concentration was 500 µL per liter POD-avidin solution.

Precision: Controls were analyzed in duplicates in 14–25 runs to determine the between-run coefficient of variation (CV) (Table 1). Between-run CVs were 4.9–7.9%.

Within-run CVs were measured by assaying fresh samples or in-house controls in 40–76 wells in one run each (Table 2). The within-run CVs of duplicate measurements were 1.9–4.2%.

Minimal detectable concentration: The minimal detectable concentrations were <1 µg/L for AFP, 0.014 int. unit/L for PRL, <1 int. unit/L for LH, and 2.4 int. units/L for FSH (Table 3). The minimal detectable concentrations were determined using detecting

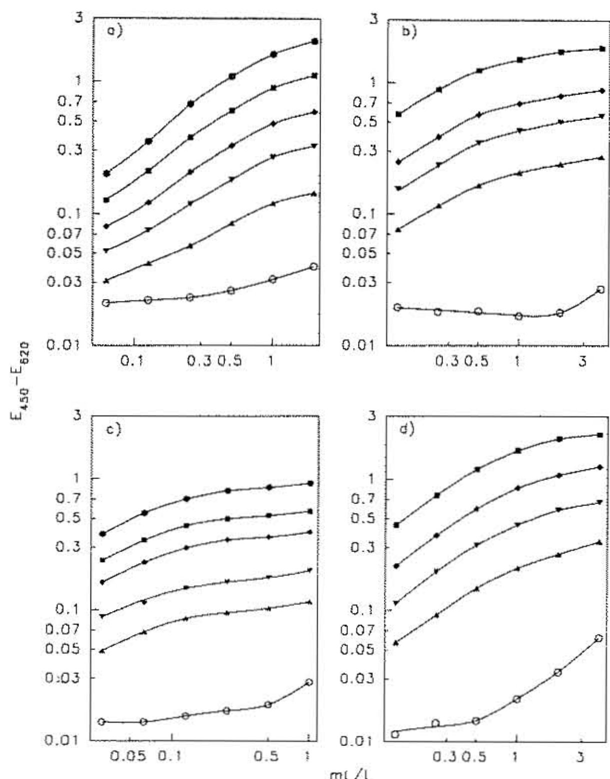

Fig. 2 *The effect of varying concentrations of the detecting antibodies. The antibody concentrations are given in mL of biotinylated antibody per liter of detection buffer. (A) AFP: The concentrations of the standards were 0 (○), 10 (▲), 25 (▼), 50 (♦), 100 (■), and 200 (●) μg/L. The enzyme reactions were developed for 12 min. (B) PRL: The concentrations of the standards were 0 (○), 0.14 (▲), 0.28 (▼), 0.55 (♦), and 1.11 (■) int. units/L. The enzyme reactions were developed for 9 min. (C) FSH: The concentrations of the standards were 0 (○), 30 (▲), 60 (▼), 120 (♦), 180 (■), and 300 (■) int. units/L. The enzyme reactions were developed for 30 min. (D) LH: The concentrations of the standards were 0 (○), 6 (▲), 15 (▼), 30 (♦), 60 (■), and 120 (●) int. units/L. The enzyme reactions were developed for 10 min.*

antibody concentrations of 0.25-fold, 1-fold, and 4-fold the optimal concentration. For each analyte, the lowest minimal detectable concentration was measured with the optimal concentration of detecting antibody. The results for the analysis for PRL are also shown in Table 3 (The results for the other analyses are not shown).

Recovery: Recoveries were 90–100% (Table 4).

Specificity: The specificity of the analysis for PRL was measured by analyzing solutions of 21 milli-int. units/L of placental lactogen and 2.4 int. units/L of human growth hormone in the PRL assay. The measured concentrations were less than 0.014 int. unit/L. The specificities of the analyses for FSH and LH were measured by analyzing the working standards and purified chorionic gonadotropin (Table 5). To test the assays for FSH and LH toward samples with very high concentrations of chorionic gonadotropin, sera from nine pregnant women in the second trimester were also measured.

Comparisons of methods: To evaluate the present methods, selected serum samples were analyzed by commercial methods and by the present method. The results were analyzed by linear least-squares regression analysis (Table 6). Visual inspection of the results obtained from the comparison between the Amerlex method and the present method for the analysis for LH showed that the calculated intercept was caused by a slightly curved correlation. The remainder of the intercepts were not significantly different from zero.

Reference intervals: The reference interval was determined for male blood donors 20–40 yr of age (Table 7).

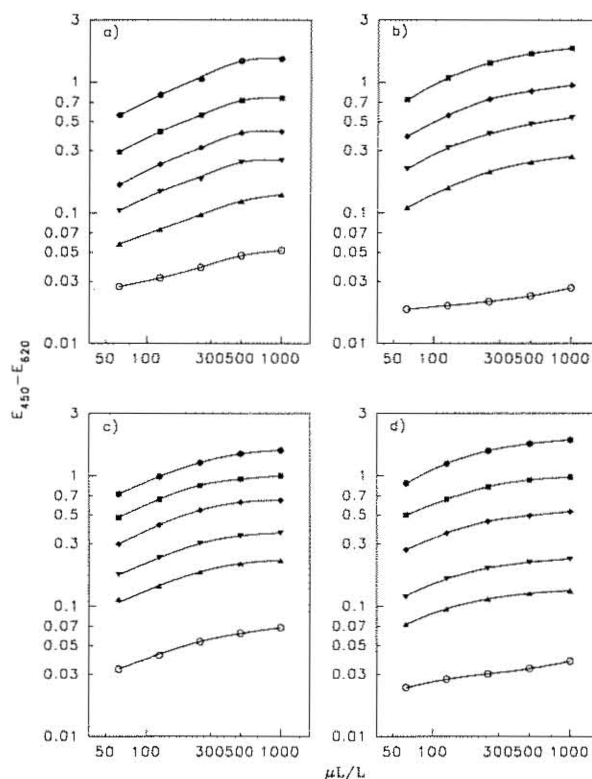

Fig. 3 *The effect of varying concentrations of POD-avidin. The POD-avidin concentrations are given in μL of POD-avidin liter of POD-avidin solution. (A) AFP: The concentrations of the standards were 0 (○), 10 (▲), 25 (▼), 50 (♦), 100 (■), and 200 (●) μg/L. The enzyme reactions were developed for 15 min. (B) PRL: The concentrations of the standards were 0 (○), 0.14 (▲), 0.28 (▼), 0.55 (♦), and 1.11 (■) int. units/L. The enzyme reactions were developed for 7 min. (C) FSH: The concentrations of the standards were 0 (○), 30 (▲), 60 (▼), 120 (♦), 180 (■), and 300 (■) int. units/L. The enzyme reactions were developed for 22 min. (D) LH: The concentrations of the standards were 0 (○), 4.5 (▲), 9 (▼), 22 (♦), 45 (■), and 89 (●) int. units/L. The enzyme reactions were developed for 13 min.*

TABLE 1 Between-run precision of duplicate measurements of the analyses for AFP (μg/L), PRL (int. units/L), FSH (int. units/L), and LH (int. units/L)

Analysis	AFP		PRL		FSH		LH	
Mean concentration	91	15.8	1.03	0.34	176	29	21.8	7.3
No. of determinations	22	21	14	14	25	25	21	21
CV, %	6.0	7.3	6.2	7.2	6.1	7.9	4.9	5.1

TABLE 2 Within-run precision of the analyses for AFP (μg/L), PRL (int. units/L), FSH (int. units/L), and LH (int. units/L)

Analysis	AFP		PRL		FSH		LH	
Mean concentration	12	94	0.18	0.34	127	176	13.2	35.5
No. of determinations	40	40	72	72	66	76	71	72
Within-run CV duplicate measurements, %	3.2	2.3	4.2	2.4	2.2	1.9	3.1	4.2

TABLE 3 Minimal detectable concentrations of the analyses for AFP (μg/L), PRL (int. units/L), FSH (int. units/L), and LH (int. units/L)

Analysis	AFP	PRL			FSH	LH
Concentration of detecting antibody, mL/L	1	0.25	1	4	0.5	0.5
No. of determinations	72	72	72	72	72	72
Mean absorbance of background, mA	32	24	28	38	34	25
SD of background, mA	1.8	2.2	2.2	4.1	3.3	2.7
Slope of standard curve, mA/(unit/L)	17.5	437	717	948	6.7	27.9
Minimal detectable concentration	0.5	0.023	0.014	0.019	2.4	0.4

Discussion

An ELISA using biotin-avidin technique is constructed with a hierarchy of levels. In level 1, the capture antibody is bound to the plastic surface. In level 2, the analyte from the sample is bound to the capture antibody. In level 3, the biotinylated detecting antibody is bound to the analyte. In level 4, POD-avidin is bound to the biotin-residues, and in level 5, the peroxidase is measured by its reaction with substrate. For practical purposes, the complexes formed at each level are stable at the conditions used at the higher levels, and the reaction at each level can be optimized independently of the reactions at the other levels.

The information flows from level 2 to level 5. If some information is lost at a lower level, this information cannot be regained at a higher level. Information can be lost if some analyte is not adsorbed to the capture antibody, or if some bound analyte does not react with the biotinylated detecting antibody. If the information is distorted, the reactions at the higher levels cannot distinguish how the information was generated. Information can be distorted if the biotinylated antibody is adsorbed to the solid phase or to the capture antibody, either directly or through an anti-animal IgG from the sample. Information can also be distorted by nonspecific binding of POD-avidin to the solid phase or capture antibody. Nonspecific variations in the measurement of POD are small (Tables 1 and 2).

In the present study, the concentration of only one reagent is varied in each experiment to determine the optimal concentration of reagent. The logarithm of the responses of the standards is plotted against the logarithm of the concentrations of the reagent (Figures 1–3). Although the responses of several standards are measured, the experiments show that the logarithms of responses of the standards are equidistant and that all of the standards have the same optimal concentration of reagent. The optimal concentration of reagent is chosen as the concentration where the distance from the background to any other standard is longest, i.e., maximal signal/background ratio. This approach is appropriate 1) where the imprecision of the background is proportional to the magnitude of the background, and 2) where the background introduced by the other steps in the procedure is low and reproducible.

Maximal signal is obtained when all of the analyte has reacted with the coating antibody within the chosen reaction time. The reaction time is chosen so that the reaction has reached equilibrium. The concentration of the coating antibody is chosen so that no further increase in the reaction is obtained when the concentration of the coating antibody is increased. The optimal concentration of coating antibody is limited by the concentration that saturates the surface. DAKO A/S recommends a protein concentration of 10 mg/L in the coating solution. This concentration is used in the AFP and FSH assays, but lower protein concentrations are optimal for the PRL and LH assays.

TABLE 4 Recoveries of the analyses for AFP (µg/L), PRL (int. units/L), FSH (int. units/L), and LH (int. units/L)

	AFP	PRL	FSH	LH
No. of determinations	12	8	8	8
Recovered from diluent	50.4 ± 1.7	0.62 ± 0.02	38.3 ± 2.7	23.2 ± 1.4
No. of sera	36	24	24	24
Recovered from serum	50.4 ± 3.1	0.61 ± 0.01	36.2 ± 2.9	20.8 ± 1.5
Recovery, %	99.9 ± 7.1	98.1 ± 3.9	94.6 ± 10.2	89.8 ± 8.5

TABLE 5 Specificity for the analyses for FSH (int. units/L) and LH (int. units/L)

	Analysis	
Test solution	FSH	LH
Working standard for FSH	300	1
Working standard for LH	<1	89
Purified urinary chorionic gonadotropin (500 int. units/L)	<1	<1
Nine sera from pregnant women (28,356 ± 9,642 int. units/L of chorionic gonadotropin)	<1	<1

For a given reaction time, the concentration of the detecting antibody is selected so that as much signal as possible is obtained without introducing too much noise. At higher concentrations of detecting antibody, the gain in signal is superseded by the increase in background. At lower concentrations of detecting antibody the lower background is accompanied by much lower signals. The strategy in this experiment finds the balance between these effects.

The minimal detectable concentration is proportional to the ratio of the standard deviation of the background to the signal. The minimal detectable concentrations have been measured for varying concentrations of detecting antibody, and the smallest detectable concentration was obtained (minimal $SD_{background}$/signal ratio) when the optimal concentration of detecting antibody was used (maximal signal/background ratio).

It has been suggested that the least detectable concentration can be measured with "infinite" concentrations of antibodies (*6,27*). However, Figure 2 shows that the nonspecific binding at high concentrations of the detecting antibody limits the sensitivity for some assays.

The conditions for the reactions between POD-avidin and the biotinylated antibodies are the same for the four analyses (Figure 3). The most likely explanation is that the reactions between the biotin-residues and POD-avidin are independent of the antibody to which the biotin-residues are attached. Thus, the conditions for these reactions are common for the four methods. This simplifies the development of further assays. In the four assays the different reactions observed with the background are caused by the different reaction times for the enzyme reactions and by the different backgrounds introduced by the detecting antibodies.

The performance of an ELISA is influenced by a multitude of factors, e. g., buffer composition, incubation time, agitation, temperature, and volume. The optimal reagent concentration is only valid for the conditions under which it was determined.

The measuring range for an ELISA is limited by the background and the linear range of the photometer. Since the background for the four assays described in this study was 0.025–0.034 A and the photometer is linear up to 2.2 A, the measuring range is 65–88 fold, because the standard curves are linear.

Heterophilic antibodies are human anti-animal IgGs that can link the detecting antibody to the capture antibody, generating a false response (*28–30*). Since the heterophilic antibodies can react with antibodies from various animals (*31,32*), immunoglobulins from cow, rabbit, and mouse are present in both the carrier proteins and detection buffers to neutralize the heterophilic antibodies. Mouse serum is used as a source of mouse immunoglobulins. Under these conditions no interference from heterophilic antibodies has been observed.

Comparison experiments show that methods developed employing the presented approach correlate well with the commercial methods. The existence of two or more standards for each analyte (*33*) and several isoforms of each analyte complicates the assays for these analytes. The conversion factor between ng and int. unit for AFP varies from company to company, and the commercial methods for FSH and LH use different standards from WHO. Therefore, it is not surprising that most of the slopes from the comparison experiments are significantly different from 1 (Table 6). Exceptions are the comparisons of the methods for measuring LH by two-site immunometric assays. The accuracy of an immunometric assay is dependent, among other things, upon the ability of the antibodies to react with the molecular forms of the analyte in the standard and the specimens.

It has been reported that LH from some individuals shows impaired immunoreactivity with certain antibody pairs (*34,35*). This has not been observed with this assay for LH.

TABLE 6 Results of the comparison experiments*

Analysis	Comparative method	No.	Slope	Intercept	r
AFP	Berilux	94	2.47 (2.34–2.61)	−0.9 (−4.0–2.1)	0.97
AFP	Amerlex-M	93	1.69 (1.61–1.77)	0.6 (−2.0–3.2)	0.98
PRL	Fenzia	102	1.09 (1.06–1.12)	−0.01 (−0.03–0.01)	0.993
FSH	Amerlex	103	1.69 (1.66–1.74)	1.2 (−3.2–0.8)	0.993
FSH	Delfia	108	3.57 (3.50–3.65)	−1.1 (−3.6–1.3)	0.994
LH	Amerlex	107	0.33 (0.32–0.35)	1.6 (0.6–2.6)	0.97
LH	Delfia	99	1.01 (0.96–1.06)	−0.02 (−0.97–0.94)	0.97
LH	Fenzia	93	1.01 (0.95–1.06)	0.93 (−0.27–2.13)	0.97

* *Selected samples of serum were analyzed by a commercial method (x) and by the present methods (y).* The results with confidence intervals obtained by linear least-squares regression analysis are shown.

TABLE 7 Reference intervals for AFP (μg/L), PRL (int. units/L), FSH (int. units/L), and LH (int. units/L) for men, ages 20–40 yr

	AFP	PRl	FSH	LH
No.	105	105	106	106
Mean concentration	2.2	0.24	14.7	3.3
SD	1.6	0.11	8.4	1.5
Reference interval	≤6	0.11–0.59	5–35	1–7

Only a few experiments are needed to determine the concentrations of reagents in an ELISA when the practical approach outlined in this article is followed. Data have been provided to prove the suitability of the approach and that the qualities of the assays are adequate for clinical use.

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