Ultrasensitive Time-Resolved Fluorescence Method for α-Fetoprotein

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We have examined the maximum sensitivity of a newly developed and optimized time-resolved fluorescence immunoassay system. The system, originally described elsewhere (Clin Biochem 1988;21:139–50), has undergone significant improvements in sensitivity through improvements of the labeled reagent used. We have chosen an α-fetoprotein (AFP) assay as a model and used monoclonal "capture" antibodies and monoclonal or polyclonal biotinylated antibodies in "sandwich-type" assay configurations. Streptavidin labeled with the europium chelator 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid was used for detection. We can measure as few as 3×10^5 molecules of AFP with the optimized system. We have applied this assay to measure AFP in the serum of normal individuals after a 10-fold sample dilution. We conclude that this system is extremely sensitive and can be used in immunoassay or other applications where biotinylated reagents can be applied.

Additional Keyphrases: *nonisotopic immunoassay · europium chelates · attomole detection · reference interval*

Immunoassays, introduced about 30 years ago, have made an enormous impact on biomedical research and clinical practice because they combine specificity and sensitivity (*1*). Currently, there is considerable interest in further improving the sensitivity of this technique by using nonisotopic labeling systems (*2–4*). Improved sensitivity is desirable because of the potential for new applications, for example: (*a*) measurement of analytes at sub-normal concentration ranges (e.g., antidiuretic hormone and corticotropin); (*b*) measurement of new analytes in serum (e.g., the hypothalamic releasing hormones); (*c*) discovery of new analytes; (*d*) detection of very small amounts of tumor-

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related products; (e) measurement of receptor numbers on cell surfaces; and (*f*) measurement of antigens of and antibodies to infectious agents.

The most sensitive immunoassay systems rely on nonisotopic detection techniques, high-affinity antibodies, and "two-site, sandwich-type" assay formats (*5, 6*). The most promising nonisotopic labeling systems are based on fluorescent (*7, 8*) and chemiluminescent probes (*9, 10*) or enzymes (*11, 12*). Powerful combinations arise when enzymes are used with substrates that release either fluorescent (*13*) or chemiluminescent (*14, 15*) products. A recent review (*16*) summarizes the most promising techniques that have been used for both immunoassays and DNA probes.

The actual detection limits of many analytical techniques are difficult to deduce from literature reports because in many instances what is reported is the ultimate detectability of the label itself, e.g., an enzyme–substrate combination, rather than the analyte of interest. These detectabilities are not the same and in many instances the former may be much worse than the latter, because when the label is conjugated to an antibody or a DNA probe, the nonspecific binding of the labeled reagent is what determines the ultimate detection limit of the assay (*6*). In other words, in practice, the true detection limit of a system is not that which is calculated under conditions of zero nonspecific binding but that derived under the conditions of measuring the analyte of interest.

In other reports (not necessarily related to immunoassay), extremely low detection limits, in terms of mass of label measured, have been achieved by using very small volumes, sometimes in the submicroliter or subnanoliter range (*17*). These assays, although seemingly impressive in terms of sensitivity (detection limits of a few thousand molecules), are actually measuring substances not much lower than 10^{-12}–10^{-13} mol/L. Except for some specialized applications, techniques involving <1-μL samples are generally not practical for routine use.

Here, we report on the detection limits of a newly developed and optimized time-resolved fluorescence immunoassay system. This system was described earlier (*8*), but several significant improvements have dramatically improved its sensitivity. We have selected an α-fetoprotein (AFP) assay of the "sandwich" type as the model, with either monoclonal or polyclonal antibodies for detection.[5] Using this system, we could detect ~300 000 molecules of AFP. We have used this assay to measure AFP in the sera of normal individuals after a 10-fold sample dilution. The system, which is based on streptavidin, is extremely sensitive and can be used for any immunological assay or in other applications, e.g., DNA probes in which biotinylated reactants can be used as complementary reagents.

Materials and Methods

Materials

Solid-phase time-resolved fluorometric measurements of Eu^{3+} were carried out with the Model 615 Time-Resolved Fluorometer/Immunoanalyzer™ (CyberFluor Inc., Toronto, Canada); the excitation wavelength was 337.1 nm (nitrogen laser source), the emission wavelength 615 (±5) nm (interference filter).

Opaque, flat-bottom polystyrene microtiter-plate strips coated with a monoclonal anti-AFP antibody were purchased from CyberFluor Inc. and used for the analysis of all the serum samples. U-bottom opaque microtiter plates (Dynatech Labs., Alexandria VA 22314), coated with monoclonal anti-AFP antibodies as previously described (*18*), were used to reach the maximum sensitivity of the system (see below).

AFP standards. Human AFP (InterMedico, Toronto, Canada) was calibrated against the International Reference Standard (72/227) for AFP. AFP standards, at concentrations ranging from 0 to 10 μg/L, were prepared by appropriate dilutions in the standard's diluent, 50 mmol/L Tris buffer, pH 7.4, containing 60 g of bovine serum albumin (BSA, RIA grade; Sigma Chemical Co., St. Louis MO 63178) per liter.

Serum samples. Serum samples used in this study were obtained from healthy men and women, ages 20–50 years, and kept frozen at −20 °C. All serum samples were diluted 10-fold in the standard's diluent before measurement.

Wash solution. Wash solution was prepared by dissolving 9 g of NaCl and 0.5 mL of polyoxyethylene sorbitan monolaurate (Tween 20) in 1 L of distilled water.

Procedures

Preparation of biotinylated antibodies. The conjugation of biotin to monoclonal and polyclonal anti-AFP was accomplished by using sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-Biotin; Pierce Chemical Co., Rockford, IL 61105) as previously described (*18*). Working solutions of the biotinylated monoclonal and polyclonal anti-AFP antibodies were prepared by diluting them in the standard's diluent to 5 (monoclonal) or 2 mg/L (polyclonal).

Preparation of labeled streptavidin. We initially conjugated streptavidin (SA) to BCPDA-labeled thyroglobulin (TG) to produce SA[$TG(BCPDA)_{150}$] as described previously (*19*). This preparation also contains unconjugated $TG(BCPDA)_{150}$, which co-elutes with the conjugate during the Ultrogel A-34 gel-filtration chromatography purification used to remove the unconjugated streptavidin. The void-volume eluate of this column (approximately 26 mL, having a streptavidin concentration of ~0.35 g/L) is then diluted in a 75 mmol/L 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 6.0, to obtain 600 mL of solution having a streptavidin concentration of 15 mg/L (*20*). The BCPDA concentration in this solution, determined by absorbance measurements at 325 nm (*21*), is approximately 130 μmol/L. This mixture is then titrated with Eu^{3+} (1 mmol/L $EuCl_3$ solution, pH 5.0) to saturate the BCPDA with Eu^{3+} (1:1 stoichiometry) to about 80 (SD 5)%. The mixture is then incubated at 50 °C for 3 h and subsequently cooled to room temperature and filtered through a 0.45-μm (pore size) filter.

The final concentration of streptavidin in the streptavidin-based macromolecular complex (SBMC) solution was 15 mg/L, determined by using ^{125}I-labeled streptavidin as the internal standard. Labeled-streptavidin working solution was prepared by diluting the SBMC solution 80-fold in a 50 mmol/L Tris buffer, pH 7.20, containing, per liter, 40 g of BSA, 9 g of NaCl, 0.1 g of sodium azide, and 40 μmol of Eu^{3+}.

One-step assay with monoclonal anti-AFP detection anti-

[5] Nonstandard abbreviations: BSA, bovine serum albumin; AFP, alpha-fetoprotein; BCPDA, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid; SA, streptavidin; TG, bovine thyroglobulin; SBMC, streptavidin-based macromolecular complex; and RT, room temperature.

bodies. Pipet 50 μL of standards or samples into each flat-bottom well (in duplicate or triplicate), then add 10 μL of biotinylated monoclonal anti-AFP working solution (~0.3 pmol/well). After shaking the wells for 2 h at room temperature (RT), wash them three times with the wash solution. Add 100 μL of the labeled-streptavidin working solution (~0.4 pmol of streptavidin/well) and incubate for 30 min with shaking at RT. Wash the wells as above and dry them with a forced-air plate dryer (from CyberFluor Inc.) for 5 min. Measure the fluorescence of Eu^{3+} on the solid phase with the Model 615 Immunoanalyzer.

Two-step assay with polyclonal anti-AFP detection antibodies. Pipet 50 μL of standards or samples into each well as above. Incubate for 2 h with shaking at RT. Wash three times, add 100 μL of the biotinylated polyclonal anti-AFP working solution (~0.9 pmol/well), and then shake for another 2 h at RT. Wash three times, and then pipet 100 μL of the labeled-streptavidin working solution into each well (~0.4 pmol/well). Incubate for 30 min with shaking at RT. Wash four times, dry the wells as above, and measure the fluorescence of Eu^{3+} on the solid phase.

Procedure for maximum sensitivity. We used U-bottom white microtiter plates that had been coated by adsorption of the monoclonal anti-AFP on the polystyrene as previously described (*18*).

Carefully apply 5, 10, or 20 μL of the antibody-coating solution (5 mg/L) at the center of the bottom of each well (we used a Hamilton syringe). Cover the plate and incubate overnight at RT without shaking. Afterwards, wash the wells twice, add 200 μL of blocking solution (60 g/L BSA solution in 50 mmol/L Tris buffer, pH 7.4) per well, and incubate for 2 h at RT. Wash the wells twice and shake them dry. For the assay, pipet 5, 10, or 20 μL of AFP standards into each well and incubate by shaking for 4 h at RT. Wash the wells twice, pipet 20 μL of the biotinylated polyclonal anti-AFP working solution, and incubate another 2 h at RT. Again, wash the wells twice and add 50 μL of the labeled-streptavidin working solution. After 30 min of shaking at RT, wash the wells four times, dry them for 10 min, and measure the fluorescence of Eu^{3+} on the solid phase.

Calculation of detection limits. Detection limits, defined as the analyte concentrations that produce a signal twice the standard deviation of the background signal, were calculated as follows: detection limit (DL) = $2\ SD_0/S$, where SD_0 is the standard deviation of the raw fluorescence measurements for zero dose, and S is the slope of the calibration curve in the area close to the detection limit. $S = \Delta F/\Delta C$, where ΔF is the difference in fluorescence readings of the zero standard and the first standard, and ΔC the difference in concentration between the zero and the first standard (i.e., identical to the concentration of the first standard). The concentration of the first standard was 20 ng/L in all studies.

Results

Recovery. AFP-supplemented serum samples were prepared by adding known concentrations of exogenous AFP to five pooled serum samples. Analytical recovery was assessed by analyzing the samples before and after the additions (Table 1). The mean (±SD) analytical recoveries were 81.2 ± 4.6% for the one-step assay (monoclonal anti-AFP) and 103.7 ± 9.1% for the two-step assay (polyclonal anti-AFP).

Sensitivity. Typical calibration curves obtained with the monoclonal or polyclonal detection antibody are presented in Figure 1. The detection limit was calculated for each assay as the concentration of AFP that could be distinguished from zero with 95% confidence. Table 2 presents the calculated detection limits (expressed in ng/L, mol/L, and molecules/well) for the assay with monoclonal and polyclonal detection antibodies as well as for the procedures in which 20-, 10-, or 5-μL coated U-bottom microtiter wells were used in combination with the polyclonal detection antibody.

Precision. Samples at three concentrations of AFP were

Table 1. Analytical Recovery of α-Fetoprotein Added to Serum Samples[a]

	Alpha-fetoprotein, μg/L		
		Found	
Pool	Added	Monoclonal	Polyclonal
1	—	2.3	2.2
	4.2	5.6 (78.6)[b]	6.5 (102.4)
	8.3	8.9 (79.5)	11.5 (112.0)
2	—	2.4	2.8
	4.2	5.8 (80.9)	7.0 (100.0)
	8.3	8.7 (75.9)	12.7 (119.3)
3	—	1.4	1.4
	4.2	4.8 (81.0)	5.3 (92.9)
	8.3	8.5 (85.5)	10.3 (107.2)
4	—	2.2	1.9
	4.2	5.9 (88.1)	6.4 (107.1)
	8.3	8.3 (73.5)	10.9 (108.4)
5	—	1.2	1.5
	4.2	4.8 (85.7)	5.7 (100.0)
	8.3	8.1 (83.1)	8.8 (88.0)

[a] See text for details on procedures with biotinylated monoclonal or polyclonal detection antibodies.
[b] Analytical recoveries (%) are given in parentheses.

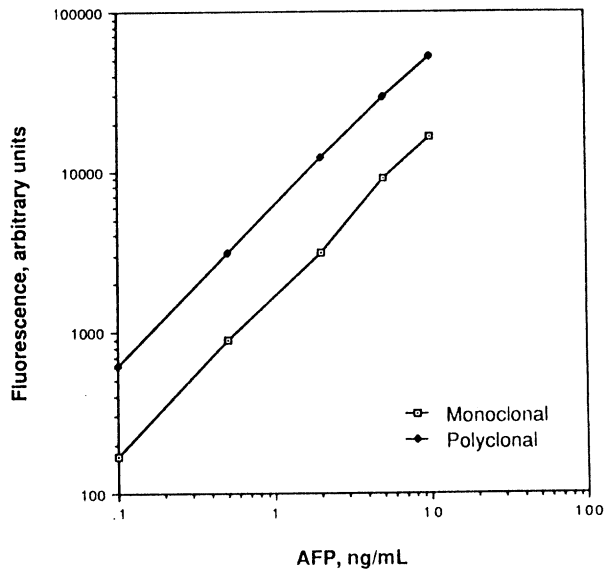

Fig. 1. Calibration curves for the AFP assay, with monoclonal or polyclonal detection antibodies

The fluorescence of the zero standard has been subtracted from all other fluorescence readings. The plots are double logarithmic; the one with the higher *y*-intercept corresponds to a more sensitive assay

Table 2. Detection Limits for AFP Assays in Different Protocols

Detection anti-AFP antibody	Coating anti-AFP, μL	Detection limit ng/L	mol/L, $\times 10^{-14}$	Molecules/well, $\times 10^3$
Flat bottom				
Monoclonal	100	12	20.2	6067
Polyclonal	100	4.6	7.7	2309
U-bottom				
Polyclonal	20	4.3	7.2	863
Polyclonal	10	5.5	9.2	552
Polyclonal	5	5.7	9.5	286

analyzed 12 times, with either monoclonal or polyclonal detection antibodies. The results are presented in Table 3.

Correlation studies. We analyzed 82 serum samples from apparently healthy subjects, using both the monoclonal (one-step) and the polyclonal (two-step) AFP assays. Comparison of the results (Figure 2) gave a correlation coefficient of 0.96 and a slope of 0.93. The histograms of the results with both antibodies are shown in Figure 3. Some statistical comparisons (monoclonal/polyclonal) are as follows (μg/L): minimum value 0.5/0.6, maximum 8.9/9.0, mean 3.0/2.9, median 2.7/2.5, SD 1.8/1.7, mean ± 2 SD 0–6.6/0–6.3, and 2.5th–97.5th percentile 0.7–7.5/0.8–8.0. These values closely agree with previously published data (*22, 23*).

Discussion

Highly sensitive immunoenzymometric assays of AFP have been reported (*24*). Time-resolved fluorometric assays of AFP have also already been published (*18, 25*). These "sandwich"-type immunoassays are generally performed with one of the following schemes:

- Detection antibodies are directly labeled with Eu^{3+} (*25*), and Eu^{3+} is quantified by dissociating it from the solid phase at low pH, complexing it with 2-naphthoyltrifluoroacetone and trioctylphosphine oxide, and measuring the fluorescence of the solution. The detection limit for this method was 0.1 μg/L, which is equivalent to 2.5×10^7 AFP molecules per well (for a 25-μL sample).
- In the biotin–streptavidin amplification system, the detection antibody is biotinylated, the streptavidin molecule is multiply labeled with a suitable Eu^{3+} chelator (BCPDA), and excess Eu^{3+} is added to form a fluorescent complex that is measured on the solid phase. With $SA(BCPDA)_{14}$ as the detection reagent and polyclonal biotinylated antibody, the detection limit was ~0.1 μg/L (2.0×10^7 molecules per well for a 20-μL sample) (*18*). With a monoclonal detection antibody, the detection limit was around 1 μg/L (*19*).

Table 3. Within-Run Precision of the Proposed Assay

Mean AFP, μg/L	CV, %[a] Monoclonal	Polyclonal
0.05	14.3	8.4
0.20	7.3	—
0.50	6.3	6.9
5.00	6.2	3.6

[a] The CVs shown are for the "dose" of AFP. n = 12.

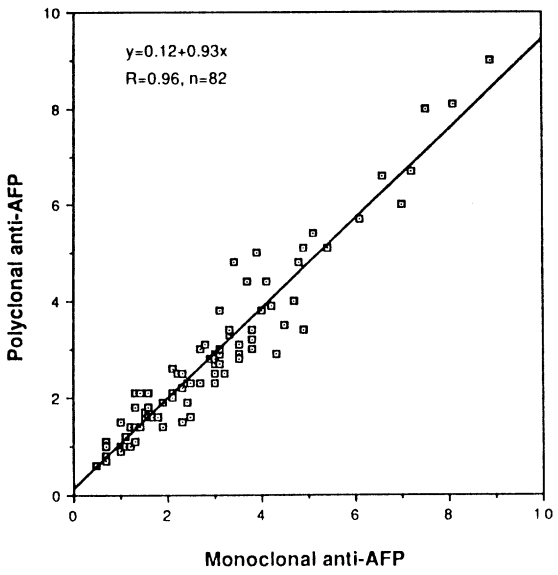

Fig. 2. Correlation between the results obtained with monoclonal and polyclonal detection antibodies for 82 serum samples

BCPDA, in contrast to other fluorescent probes (e.g., fluorescein or phycoerythrin), has the advantage of allowing multiple labeling of proteins without any quenching effects. Therefore, we developed a more-sensitive system, in which streptavidin was covalently coupled to BCPDA-labeled thyroglobulin to produce a novel reagent, $SA[TG(BCPDA)_{150}]$. The detection limit of the AFP assay with this reagent was 0.2 μg/L (4.0×10^7 molecules per well) when a monoclonal detection antibody was used (*19*). However, Morton and Diamandis (*20*) recently observed that when streptavidin coupled to BCPDA-labeled thyroglobulin, $SA[TG(BCPDA)_{150}]$, is incubated with free $TG(BCPDA)_{150}$ in the presence of a certain Eu^{3+} concentration, a new, stable macromolecular complex [postulated to be $SA(TG)_3(BCPDA)_{480}$] is formed, carrying more BCPDA molecules per molecule of streptavidin and thus being a more sensitive reagent.

We used this novel streptavidin-based detection system in the present work and obtained better detection limits, i.e., 12 ng/L (~6.1×10^6 molecules per well) with the monoclonal biotinylated detection anti-AFP antibody and 4.6 ng/L (~2.3×10^6 molecules per well) with the polyclonal biotinylated detection antibody (for a 50-μL sample). Therefore, a 15- or 40-fold improvement over the $SA[TG(BCPDA)_{150}]$ or $SA(BCPDA)_{14}$ assay is introduced by using this new reagent in combination with monoclonal or polyclonal detection antibodies, respectively. The proposed system is also eight (with monoclonal) or 20 times (with polyclonal) more sensitive than that in which monoclonal detecting antibodies are directly labeled with Eu^{3+}. When polyclonal detection antibody is used, the heterogeneity of the antibody population results in more than one detection antibody being bound to different epitopes of the same AFP molecule; therefore, the detection limits are better than those obtained with monoclonal detection antibodies.

The optics of the Model 615 Immunoanalyzer have been outlined elsewhere (*8*). The excitation laser beam hits the bottom of the microtiter well vertically, the same direction in which the fluorescence signal is collected.

Routinely, the whole bottom as well as part of the side of the microtiter well (corresponding to 100 μL of solution) is

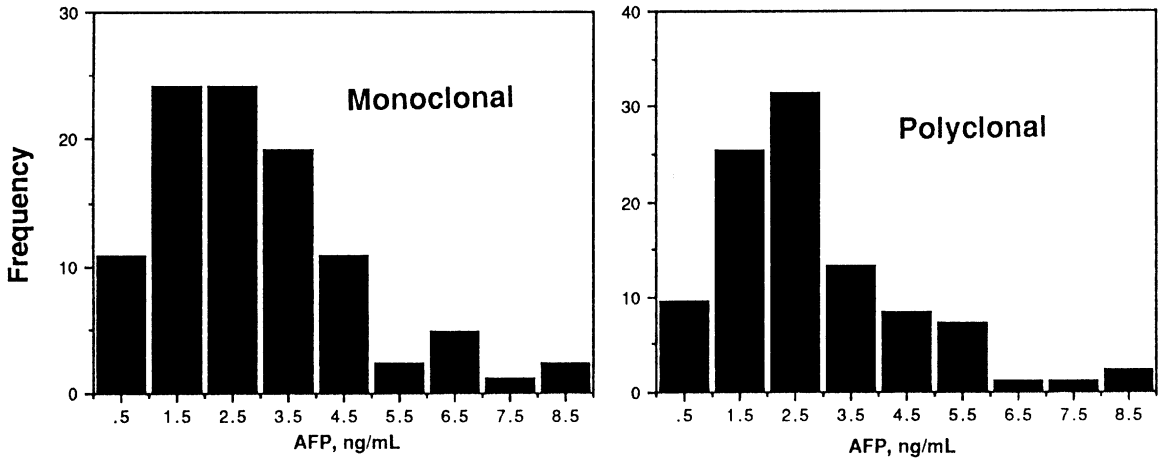

Fig. 3. Histograms of the results for 82 normal serum samples, obtained with monoclonal and polyclonal detection antibodies
Note difference in *ordinate* scales

coated with monoclonal anti-AFP antibody, all of which thus participate in the series of the assay reactions, i.e., binding AFP molecules from the sample, attracting detection antibodies, and finally binding fluorescent-labeled streptavidin. However, only a small surface around the center of the bottom of the well is struck by the excitation laser beam and the fluorescence of only this part is measured. The rest is potentially fluorescent but is not excited and thus does not contribute to the measurement. This results in a loss of sensitivity because a large percentage of the AFP molecules are dispersed and bound to coated areas that are outside of the excitation beam. In some of our experiments, we coated a limited surface area, to coincide with the excitation surface. To do this, we used U-bottom microtiter plates and coated only a small surface around the center of the bottom by applying small volumes (5 to 20 μL) of coating solution. After the coating, we added 200 μL of blocking solution per well so that all the surface of the well was blocked. After this point, any sample volume could be applied but the assay reactions would be restricted to the coated area. As Table 2 showed, detection limits <300 000 molecules (0.5 amol) per well can be achieved by using 5 μL of coating solution, with a precision the same as that of the opaque, flat-bottom wells. This modification, in comparison with the standard flat-bottom wells, is illustrated in Figure 4.

By using the above techniques, we extended the capabilities of the system to achieve the lowest detection limits reported so far for time-resolved fluorescence assay.

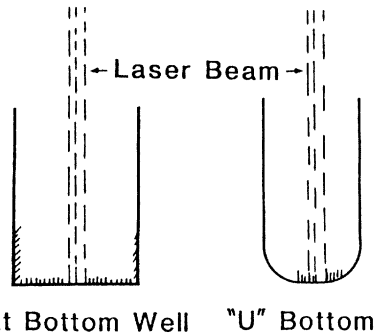

Fig. 4. Design for increasing sensitivity of the proposed system
By using U-bottom wells and small volumes of coating solution, all the assay reactions are confined in the excitation area and the detectability is increased more than fivefold (see Table 2)

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