Homogeneous Immunofluorometric Assays of α-Fetoprotein with Macroporous, Monosized Particles and Flow Cytometry

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We evaluated two homogeneous immunofluorometric assays (IFMAs) of α-fetoprotein (AFP) based on new macroporous acrylate particles combined with flow cytometry. The standard IFMA, requiring 1 h of incubation, provided a working range from 1.8 to >900 kIU/L (CV <10%) and a detection limit of 0.6 kIU/L. Use of overnight incubation and a lower particle concentration extended the working range by 1 decade in the lower end. Analytical recoveries for the standard IFMA varied between 97% and 108%. The slope and y-intercept of the regression line correlating measurements by the standard IFMA and a routine immunoradiometric assay were not significantly different from 1 and 0, respectively (P >0.5), and the correlation coefficient was 0.996. High precision and warning of spurious high measurements were obtained by including in each sample separate particle types for detecting instrument instability and measuring nonspecific binding only.

Indexing Terms: biotin–streptavidin interaction · immunoradiometric assay compared · phycoerythrin

α-Fetoprotein (AFP), the fetal equivalent to albumin, is a 67-kDa glycoprotein produced during embryonic development and found in high concentrations in fetal serum and amniotic fluid (1). Maternal serum AFP is ordinarily moderately increased during pregnancy. Abnormal increases are seen if the fetus has open neural tube defects (2), whereas low maternal serum AFP has been used as a screening test for Down syndrome (3). In normal nonpregnant adults AFP is present in low concentrations in serum, but AFP can be markedly increased in patients with cancer of the liver, testis, or ovary (1, 4).

Several AFP assays have been reported since the presentation of a highly sensitive radioimmunoassay for AFP in 1971 (5). A now classical study by Hunter and Budd (6) used measurement of AFP to demonstrate the advantages of an immunoradiometric assay (IRMA) over a radioimmunoassay, e.g., a markedly lower detection limit, a wider working range, and improved assay robustness.

Radioimmunoassays and IRMAs are still widely used, but there has been a growing interest in the development of assays based on labels other than radioactivity. Thus, assays based on different enzyme labels (7–10), chemiluminescent labels (11), and fluorescent europium chelate labels (12–14) have been developed. In the present study we demonstrate that a homogeneous immunofluorometric assay (IFMA) of AFP, i.e., an assay not requiring any separation or washing steps, performs as well as a well-designed IRMA. The use of flow cytometry facilitates homogeneous particle-based assays because only negligible amounts of medium fluorescence are excited from the single-particle sensing volume of the flow cytometer.

Another important feature of flow cytometry is the ability to simultaneously detect single-particle light-scatter and fluorescence. Thus, particles of different sizes can be discriminated by the light-scatter measurement, making multiparticle analysis possible. In the present work, particles for monitoring fluorescence intensity were added to all samples; this improved precision by providing calibration of all fluorescence measurements to the intensity of these particles. A separate particle type coated with antibody of irrelevant specificity was included to identify sera that gave extraordinarily high nonspecific binding (NSB). This ability to monitor NSB of individual samples should eliminate falsely high assay results. Inclusion of other monosized particles coated with antibodies to other analytes opens the possibility to measure several analytes simultaneously.

Materials and Methods

Materials

Monoclonal antibodies. Two monoclonal antibodies (K57 and K52) against different AFP epitopes were produced by immunizing female BALB/c mice (Charles River U.K. Ltd., Kent, UK) with AFP purified from cord blood, essentially as described by Gold et al. (15). Both the fusion and the screening for clones producing antibodies against AFP were performed as described for antibodies to neuron-specific enolase (16). K52 and K57 were of subclass IgG2a and IgG1, respectively, as determined by agarose double-diffusion against subclass-specific antisera from Miles Scientific (Naperville, IL). The monoclonal antibodies were produced in ascites and purified by Protein A–Sepharose chromatography as previously described (16).

NSB was measured by using a third monoclonal antibody specific for an irrelevant epitope, i.e., a cell surface antigen on human B-cells (FN61, mouse IgG1,
CD69). Another B-cell-specific antibody, HH1 (mouse IgG1, CD37), was used with other inert proteins as buffer additives to avoid false-positive results from interfering substances (17). Both FN61 and HH1 were kindly provided by S. Funderud (18, 19).

**Standards, controls, and patients’ samples.** The primary AFP standard was the 1st International AFP Standard for Immunoassays, kindly provided by the World Health Organization, International Laboratories for Biological Standards (London, UK). Secondary standards were made by diluting cord serum AFP in heat-inactivated (at 56 °C for 1 h) normal human serum of low AFP concentration (<0.1 kIU/L). AFP controls were made from pooled human sera and normal heat-inactivated serum to give 9.7 and 100 kIU/L. Selected patients’ sera, with AFP contents ranging from undetectable to ~1000 kIU/L, were measured by the IRMA and two versions of the IFMA. All sera were stored frozen at −20 °C until use.

**Buffers.** Phosphate-buffered saline (PBS): 0.15 mol/L NaCl, 0.01 mol/L sodium phosphate buffer, pH 7.5. Stock solution buffer: 0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, 0.1 g/L thimerosal, pH 7.4. Blocking buffer: Stock solution buffer supplemented with (per liter) 5 g of bovine gamma globulin (cat. no. G7516; Sigma Chemical Co., Poole, UK), 20 mL of normal rat serum obtained by cardiac puncture of surplus conventional rats of various strains, 20 mL of normal sheep serum (cat. no. BP013; The Binding Site, Birmingham, UK), 0.01 g of mouse monoclonal IgG1 (HH1), and 2 g of casein hydrolysate (Merck, Darmstadt, Germany). Borate buffer: 0.1 mol/L boric acid/NaOH buffer, pH 9.5 or pH 8.0, containing 0.1 mol/L NaCl. IFMA-diluent: PBS supplemented with (per liter) 10 g of bovine serum albumin (BSA, cat. no. 10857; US Biochemical Corp., Cleveland, OH), 1 g of NaN₃, and 1 mL of Tween 20 (Merck).

**Microsphere particles.** Monodisperse (<1%) macroporous acrylate particles were especially designed for the present flow-cytometric assays. The particles were prepared at SINTEF (Trondheim, Norway) by the unique activated swelling method developed by Ugelsstad et al. (20, 21). The particles were highly macroporous with pore volumes of about 1.5 mL/g and a total surface area of ~33 m²/g (excluding pores of diameter <20 nm). This provides particles of high binding capacity and low sedimentation rate. The particle surface is covered by epoxy groups for covalent attachment of the antibody, and the particles are prepared in different sizes without essential changes in pore-size distribution, pore volume, and amount of surface active groups.

For fluorescence intensity monitoring we used 2.8-μm-diameter compact fluorescent polystyrene particles (cat. no. 19508; Polysciences, Warrington, PA).

For IRMA, we used monodisperse magnetizable Dynabeads M-280 particles (Dynal, Oslo, Norway) of 2.8 μm diameter. These particles have a rugged surface with a nominal surface area of 1.4 m²/g, but a measured effective area of 5 m²/g. The M-280 particles have primary hydroxyl groups, which are activated by p-toluene sulfonyl chloride (22).

**Procedures**

**Particle coating.** Antibody K57 was used to coat 7.5-μm particles at 150 μg of antibody per milligram of particles. The coating was performed in PBS at an antibody concentration of 0.3-1 g/L for 20 h, with use of end-over-end rotation at room temperature. The particles were washed once in PBS and then rotated for 2 h in stock solution buffer supplemented with 1 g of BSA and 1 mL of Tween 20 per liter. Finally, the particles were rotated overnight in stock solution buffer containing 1 g/L BSA, resuspended in the same buffer, and stored at 4 °C. On average, 120 μg of antibody was bound per milligram of particles as calculated either from the amount of antibody recovered in the primary PBS washing or from bound 125I-labeled K57 antibody added before the coating process. This relation was only slightly influenced by increasing the coupling pH from 7.5 to 9.5 or by coupling at 37 °C rather than room temperature.

To obtain particles for measurement of NSB, we coated the irrelevant antibody FN61 onto particles of 5.5 μm diameter, using the same procedure.

**AFP antibody K57 was also coated onto Dynabeads M-280, 20 μg of antibody per milligram of particles, in pH 9.5 borate buffer at 37 °C for 20 h. Removal of unbound antibody was performed as described above, and the amount of antibody bound was typically 8–14 μg/mg particles.**

**Antibody biotinylation.** The AFP antibody K52 in PBS was labeled with biotin according to the guidelines given by Bayer and Wilchek (23). Biotin-ε-aminocaproic acid-N-hydroxysuccinimide ester (Calbiochem, La Jolla, CA) freshly dissolved in 10 μL of dimethyl sulfoxide was added in 10× molar excess to K52 in pH 8.0 borate buffer and incubated by end-over-end rotation for 16–20 h at 4 °C. The labeled antibody was separated from free biotin by gel filtration on a 1.6 × 15 cm column of BioGel P30 (Bio-Rad Labs., Hercules, CA), equilibrated with PBS containing NaN₃, 0.1 g/L. The biotinylated antibody collected in the void volume was pooled, BSA (1 g/L) was added, and the sample was passed through a 0.22-μm (pore size) filter before storage at 4 °C.

**Protein iodination.** Monoclonal antibodies and AFP were iodinated by using Iodo-Gen (cat. no. 28600; Pierce Chemical Co., Rockford, IL) as an oxidant, at an equimolar ratio between protein and 125I, as described earlier (24). Labeled proteins were stored in 500 mL/L ethylene glycol at −20 °C before use.

**Assays**

**IRMA.** Labeled K52 was diluted in blocking buffer, and solid-phase K57 was diluted in stock solution buffer containing 1 g of BSA and 1 mL of Tween 20 per liter. AFP in 50-μL, serum-based standards was incubated with 100 μL of 125I-labeled K52 (250 000 counts/min; ~24 ng) for 5 min at room temperature. The complex formed was bound to antibody K57 immobilized on Dynabeads M-280 (0.5 mg of particles in 100 μL) by incubation for 15 min at room temperature in a total volume of 250 μL. Excess labeled K52 was removed by three wash-
ings, each with 0.7 mL of PBS containing Tween 20, 1 mL/L, and immobilizing the paramagnetic particles on a magnetic rack (Amersham, Arlington Heights, IL). All serum samples were analyzed at two different sample volumes, 25 and 50 µL. The bound radioactivity was counted with a Wallac 1277 Gammanastor (Wallac Oy, Turku, Finland), and the Wallac Multicalc software was used to compute assay results.

**IFMAs.** Biotinylated antibody K52 was diluted to 1.9 mg/L in blocking buffer (see Buffers) and mixed with one-sixth volume of streptavidin–R-phycocerythrin (SA-PE, cat. no. 9023; Becton Dickinson, San Jose, CA) at room temperature 5–10 min before use. For the standard IFMA, particles coated with antibody K57 were diluted to 930 µg of particles per milliliter (~14 000 particles per microliter) in IFMA diluent. To each assay tube we added 40 µL of labeled K52, 40 µL of K57-coated particles, 50 µL of NSB particles (500 mg/L in IFMA diluent), and 50 µL of fluorescent particles (~14 000 particles per microliter in IFMA diluent). Serum samples (10 µL) were added to each of two duplicate tubes and incubated for 1–1.5 h on a horizontal rotatory shaker at room temperature. Each sample was measured for 10 s at an injection rate of 2 µL/min.

For the optimal IFMA the particle concentration was decreased 20-fold, all reagent volumes were doubled, and the samples were incubated for 18–24 h before IFMA measurements for 30 s at an injection rate of 5 µL/min.

**Flow cytometry.** All fluorescence and light-scatter measurements were performed with a Skatron Argus Flow Cytometer (Skatron AS, Tranby, Norway) with a 75 W mercury–xenon lamp. A G1 filterblock provided excitation in the wavelength range 510–560 nm and fluorescence measurements in the range 590–640 nm. Particle-associated light scatter and fluorescence signals were measured simultaneously and presented in histograms as shown in Figure 1. The median channel of the logarithmic fluorescence histogram was taken as a measure of the particle-associated fluorescence. By using appropriate windows in the light-scatter histogram, we determined the fluorescence intensity from the particles of interest. All measurements of immunofluorescence were calibrated to the fluorescence intensity of the

![Histograms from samples containing (a) 7.5-µm-diameter specific-binding particles, (b) 5.5-µm-diameter nonspecific-binding particles, and (c) 2.8-µm-diameter intensity-monitoring particles.](image)

The histograms show logarithmic signal intensity over three decades on 256 channels (an interval of 25 channels corresponds to a doubling of the signal intensity). A: Light-scatter histogram showing the three particle populations and a gate set around the specific-binding particle. B: Histogram showing the fluorescence intensity of the gated specific-binding particle. C: Two-parameter plots of light-scatter vs fluorescence intensity for the results shown in A and B (AFP 850 kIU/L). D: Corresponding plot of a different sample containing AFP at 8.8 kIU/L.

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intensity-monitoring particles. Assay results were calculated by the Multicalc program.

Assay Evaluation

**Calculation of precision profiles and detection limits.** Intraassay precision profiles were obtained essentially as described by Ekins (25), from the assay results of duplicate serum samples processed by the Multicalc program. The lowest limit of detection, defined as three standard deviations of the zero dose (3σD0) (26), could not be determined directly in the present assay because the low concentrations of AFP (<0.1 kIU/L) in the serum used for standard dilutions made determination of the zero dose and standard curve for concentrations <0.1 kIU/L difficult. However, the standard deviation in the dose determination (σD) will approach σD0 as the standard curve flattens out at the lower end (25). Thus 3σD at the lower end of the working range (CV = 10%) provides a comparable, although less favorable, measure of the lowest limit of detection and has been used for the three assays presented here.

**Regression analyses.** These were performed based on the logarithm of the assayed AFP concentrations. By using this transform, the variance of y about the regression line was approximately the same throughout the concentration range of interest, thus fulfilling the conditions for making statistical inferences from the regression analysis (27).

Results

**IRMA**

Labeled K52 bound AFP in 5 min, and only 15 min was needed to obtain maximum binding of the complex to an excess of particles coated with K52 (results not shown). A high-dose "hook effect" was present at AFP concentrations >2000 kIU/L (results not shown), but could be reliably detected because two serum volumes were assayed, 25 and 50 µL.

The working range of the assay was from 0.5 to >900 kIU/L (CV <10%) (Figure 2), the lowest limit of detection was 0.15 kIU/L, and the two controls gave an interassay CV of 3.7% and 2.1% at 9.7 and 100 kIU/L, respectively (n = 20). The assay has been in routine use at the Norwegian Radium Hospital for 3 years.

**Designing IFMAs**

Figure 3A demonstrates how an optimal labeling of biotinylated antibody with SA-PE was obtained by mixing 40 µL each of SA-PE and biotinylated antibody together with 80 µL of particles in excess and 40 µL of AFP serum before incubating overnight. Keeping the concentration of biotinylated antibody constant at 1.15 mg/L, we serially diluted the SA-PE solution twofold. The optimal signal-to-noise ratio, i.e., the maximum difference between specific binding and NSB, was obtained with the SA-PE diluted 1:10. We maintained this molar ratio between antibody and SA-PE in the subsequent experiments.

The assay was intended to cover AFP concentrations up to ~800 kIU/L. The concentration of labeled antibody necessary to bind this amount of AFP was determined as shown in Figure 3B; 80 µL of solution with particles in excess (200 mg/L) was added to 40 µL of serum (containing 800 kIU/L AFP). We then added to the tubes 80 µL of serially twofold-diluted SA-PE-labeled antibody (initial K52 concentration 4.3 mg/L) and incubated overnight. At a K52 concentration of 0.54 mg/L, denoted CAb,eq, there was sufficient antibody to bind all the AFP in the sample.

The necessary minimum binding capacity for the particles was determined similarly (Figure 3C), keeping the concentration of labeled antibody K52 constant at 2.3 mg/L while adding serial twofold dilutions of particles (initially 124 mg/L), to AFP serum (800 kIU/L). This saturation curve indicates that a minimum particle concentration of 15.5 mg/L (Cp,eq) is necessary to provide binding capacity up to 800 kIU/L AFP.

The standard curve obtained for overnight incubation and reagent volumes as described for the optimal IFMA (Materials and Methods) increases with AFP concentrations up to 800 kIU/L when the particle concentration Cpb,eq and antibody concentration CAb,eq were used (Figure 3D). However, linear characteristics and measuring precision at concentrations up to 800 kIU/L AFP were obtained by increasing both the particle concentration and labeled antibody concentration threefold, constituting an assay subsequently referred to as the optimal IFMA.

For routine applications of the IFMA, reagent concentrations had to be modified to provide rapid kinetics and assay results within 1 h. The reaction between labeled antibody and AFP reached equilibrium within ~5 min when the concentrations used were 3 × Cpb,eq, 3 × CAb,eq (results not shown). Thus the particle concentration was assumed to be rate limiting, and the binding kinetics of the antigen–antibody complex to the solid-phase particles was determined by preincubating AFP and labeled

**Fig. 2.** Standard curve (left ordinate) for measurements of two duplicate standards (□, ○) and precision profile (●, right ordinate) of the routine IRMA +, lower limit of the working range (0.5 kIU/L)
antibody 15 min before adding particles. Assays were set up from the zero standard and two different patients’ sera containing AFP at 3.7 and 780 kIU/L, with the labeled antibody concentration being 3 × C_{\text{Ab,ref}}. The particle concentrations were varied: 3 × C_{\text{p,ref}} in one assay, 20 × and 150 × C_{\text{p,ref}} in two others. The incubation times needed to reach approximately constant fluorescence intensity were >16 h, 1.5 h, and 15 min, respectively (Figure 4). An increase in binding for the low-AFP sample at some time after the initial plateau was reached indicated that, to obtain consistent results, the measurement period should not be too long.

To obtain the desired incubation time of 1 h, we chose a particle concentration of 60 × C_{\text{p,ref}} and a labeled antibody concentration of 3 × C_{\text{Ab,ref}} as the standard IFMA. Preincubation of AFP and labeled antibody was unnecessary: assays with and without 15 min of preincubation both were at equilibrium after 1 h of incubation (Figure 5).

**IFMA Performance**

The working range of the standard IFMA was from 1.8 to 900 kIU/L (CV < 10%) (Figure 6), and the lowest antibody 15 min before adding particles. Assays were set up from the zero standard and two different patients’ sera containing AFP at 3.7 and 780 kIU/L, with the labeled antibody concentration being 3 × C_{\text{Ab,ref}}. The particle concentrations were varied: 3 × C_{\text{p,ref}} in one assay, 20 × and 150 × C_{\text{p,ref}} in two others. The incubation times needed to reach approximately constant fluorescence intensity were >16 h, 1.5 h, and 15 min, respectively (Figure 4). An increase in binding for the low-AFP sample at some time after the initial plateau was reached indicated that, to obtain consistent results, the measurement period should not be too long.

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**IFMA Performance**

The working range of the standard IFMA was from 1.8 to 900 kIU/L (CV < 10%) (Figure 6), and the lowest
I.100

Fig. 5. Assay kinetics with (○) or without (□) 15 min preincubation of AFP and labeled antibody (3 × C_{AFP,serum}) before particles (60 × C_{AFP,serum}) were added. AFP concentrations in serum as in Fig. 4.

Fig. 6. Standard curve (left ordinate) for measurements of two duplicate standards (○, □) and precision profile (◇, right ordinate) of the standard IFMA +, lower limit of the working range (1.8 kIU/L)

An average intermethod CV of 8.2% was observed, with no single measurement diverging by >45%.

By allowing overnight incubation, a superior working range of nearly 4 decades (0.11–900 kIU/L AFP) and a lowest limit of detection of 0.03 kIU/L AFP could be obtained by the optimal IFMA shown in Figure 3D. Measurements for 43 of 45 serum samples correlated well between this optimal IFMA (y) and the IRMA (x) (y = 0.980x + 0.134, r = 0.993, n = 43); the recoveries ranged from 101% to 145%. For one serum sample the optimal IFMA provided an erroneously low measurement, and for another an erroneous high (2.3×) value, compared with the IRMA results. However, in the latter case a warning against the high measurement was provided by a 3× increased signal for the NSB particle.

Discussion

Particle Characteristics

For the present IFMAs, macroporous acrylate particles were developed that allow chemical immobilization, have low inherent fluorescence, low sedimentation rate, and large surface area. The high monodispersity of the particles (CV <1%) makes them especially well suited for use in multianalyte analysis, where several particle sizes should be clearly differentiated in the size histogram. Given the surface area obtained when pore diameters <20 nm were excluded, we calculated that the binding of K57 was 0.36 μg/cm². This is close to that of a monolayer of IgG on a polymer surface, reported as 0.31 μg/cm² (28). The paramagnetic M-280 Dynabead particles, coated according to previous optimizations (16, 29–32), resulted in a K57 binding of 0.16–0.28 μg/cm², based on the surface area experimentally determined. However, measuring the binding capacities for both particle types demonstrated that antibody K57 was three times more capable of binding the 125I-labeled K52–AFP complex when immobilized on the M-280 particles than on the macroporous acrylate particles (results not shown). This difference is probably due to steric hindrance imposed on the binding of the complex to K57 residing in pores. For flow cytometry, however, the more important figure is the binding capacity per particle, which is 76 times higher per particle for the 7.5-μm macroporous particles than for the 2.8-μm Dynabead M-280 particles.

IRMA

This assay had rapid kinetics and a working range of >3 decades. Low serum interference from heterophile

<table>
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<tr>
<th>Serum</th>
<th>Original AFP conc, kIU/L</th>
<th>AFP added, kIU/L</th>
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<tr>
<td>Pool A</td>
<td>2.0</td>
<td>105</td>
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<tr>
<td>Pool B</td>
<td>6.6</td>
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<tr>
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<td>105</td>
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<tr>
<td>Pool D</td>
<td>44.3</td>
<td>106</td>
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Table 1. Analytical Recovery of AFP Added to Pooled Patients’ Sera, Measured by Standard IFMA.
antibodies and other serum constituents was secured by including a cocktail of animal serum proteins in the blocking buffer (17). Complement binding to the solid-phase antibody was reduced by choosing the mouse IgG1 (K57) rather than the mouse IgG2a (K52) for the solid-phase antibody, as previously shown by Børner for an assay of carcinoembryonic antigen (33).

Disadvantages of the IRMA were the tedious washing procedure, radiation safety considerations, and the limited stability of the $^{125}$I label.

IFMAs

The optimization of the IFMA protocols shows how the critical variables can be tailored to meet the user’s specifications in assays read by flow cytometry. The present aim, to develop an AFP assay covering concentrations from $\sim 2$ to $\sim 800$ kIU/L, was reached with a simple one-step assay requiring an incubation time of 1 h. Although the lowest limit of detection was slightly higher for this standard IFMA than for the routine IRMA, the precision within the working range 2–800 kIU/L was comparable for the two assays.

In addition to the robustness and satisfactory working range of the standard IFMA, an important feature of the flow-cytometric IFMAs is the ability to perform homogeneous assays (34–37). With no separation steps, automation could easily be implemented to provide constant incubation times. Measurements could then be performed reliably at semiequilibrium, providing either an even shorter incubation time or increased detectability by reducing the particle concentration. The results in Figure 4 (open and filled circles) show that the signal-to-noise ratio of the optimal IFMA with the particle concentration of $3 \times C_{\text{part}}$ would only be reduced from a factor of 750 (240 channels) to 500 (225 channels) if the incubation time were reduced from 18 to 2 h. Thus, most of the superior detection limit of 0.03 kIU/L and working range of 4 decades provided by the optimal IFMA would be maintained by an assay providing results within 2 h. We have also observed that the problem of nonspecific serum interferences may be reduced within this shorter incubation time.

Although lowering the detection limit might not be of clinical importance for AFP measurements, it could be crucial in development of assays for other analytes. As demonstrated in Figure 3D (squares), further improvements of detectability are feasible by lowering the concentrations of particles and labeled antibody. The detection limit could also be lowered by washing the particles before the flow-cytometric reading, reducing NSB by $\sim 20\%$ in the present assay. However, the advantages of a homogeneous assay would then be lost.

The present IFMAs include no warning against misclassification due to the hook effect, which would be present at 1500 and $>10,000$ kIU/L for the optimal and standard IFMAs, respectively. This problem could be eliminated as in the IRMA by using two different sample dilutions. However, in IFMAs read by flow cytometry, erroneous results due to the hook effect could be detected by a binary assay (34) utilizing immobilized AFP-specific antibodies of different affinities on particles of different sizes. Thus, the standard curve of the high-affinity particle would provide measuring precision for low-AFP concentrations, whereas the standard curve for the low-affinity particle would increase the working range at high concentrations.

The ability to identify different particle types offers several other advantages to flow-cytometric assays. In the present work we used this to eliminate possible instrument instability by calibrating fluorescence measurements against the intensity-monitoring particle. In addition, NSB was individually quantified by a separate particle. In the standard IFMA the specific AFP measurements showed no significant outliers in comparison with the IRMA results, and the absence of serum interference was reflected by a low variation in the NSB-particle measurements. However, the results of the optimal IFMA show that spuriously high measurements of AFP can be singled out by the NSB particle. If developed into a more systematic approach, the measurement of the NSB-particle could provide a means to correct for the sample-related NSB on the specific particle. This principle has been demonstrated in a difference-ELISA (39) involving microtiter plates with a separate measurement of NSB, which provides increased accuracy in the lower end of the working range.

The results of the standard IFMA, providing adequate working range and detectability, show that flow cytometry offers a basis for reliable and simple immunonassays for AFP. Nevertheless, the greater potential of the optimal IFMA, with respect to detectability and working range, point out the importance of developing an automated assay design that can read nonequilibrium assays.

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