Multianalyte Immunoassay Based on Spatially Distinct Fluorescent Areas Quantified by Laser-Excited Solid-Phase Time-Resolved Fluorometry

Sotiris E. Kakabakos,[1] Theodore K. Christopoulos,[1] and Eleftherios P. Diamandis[1,2,3]

We describe a new multianalyte immunoassay principle and apply it to the simultaneous immunoassay of lutropin, follitropin, choriogonadotropin, and prolactin in serum. The method is based on the coating of distinct areas of polystyrene with analyte-specific antibodies. These antibodies react with the analyte and immobilize it in a specific area while another biotinylated antibody also reacts with the analyte to form a sandwich. After addition of streptavidin labeled with the fluorescent europium chelate of 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid, fluorescent areas are formed, the intensity of which is related to the amount of each analyte present in the sample. The fluorescent areas are quantified on the dry solid phase with laser-excited time-resolved fluorometric measurements. The assays developed are highly sensitive, precise, and accurate. We believe that this system shows potential for multianalyte immunoassay of diverse groups of compounds in disciplines such as endocrinology, infectious disease, hematology, and oncology.

Additional Keyphrases: *europium chelates · streptavidin · panel testing · lutropin · follitropin · choriogonadotropin · prolactin*

Multiple testing is frequently requested by physicians as an aid to diagnosing diverse diseases. Examples of such panel testing include the simultaneous analysis of free thyroxin and thyrotropin (TSH); of lutropin (LH), follitropin (FSH), and prolactin (PRL); and of vitamin B_{12} and folate for the investigation of thyroid, gonadal, and hematopoietic disorders, respectively.[4] In immunology, therapeutic drug monitoring, or oncology, screening of patients' sera for infectious agents, antibodies, drugs, or tumor markers is also of interest. Although multiple testing is very frequently requested by physicians, the clinical laboratory, with some exceptions, analyzes the samples separately for each analyte, mainly because the technology needed for multianalyte assays is not yet available.

A few dual assays have proved feasible and are even used routinely. For example, vitamin B_{12} and folate are assayed simultaneously by using ^{125}I-labeled folate and ^{57}Co-labeled cobalamin as tracers (*1–4*); however, this requires use of a gamma scintillation counter that discriminates between the disintegration produced by ^{57}Co and ^{125}I tracers. Similarly, simultaneous assays for LH and FSH (*5*), for TSH and free thyroxin (*6, 7*), and for TSH and total thyroxin (*8*) also exist, based on ^{57}Co and ^{125}I tracers.

Recently, dual-label immunoassays involving time-resolved fluorometry and either europium–terbium (*9, 10*) or europium–samarium (*11, 12*) labels have been proposed. These label combinations take advantage of the narrow fluorescence emission bands of the lanthanide chelates and the lack of overlap between their emission spectra (*13*). Thus, two lanthanides can be quantified in the presence of each other.

True multianalyte immunoassay, however, is not feasible with either radionuclides or lanthanides as labels because there are no known combinations of more than two or three radionuclides or lanthanides that can be quantified individually in a mixture. Moreover, because of the different detectabilities of these labels, the detection limits of the analytes of interest are different and in many instances are compromised because of the suboptimal label used (*9*).

Ekins et al. (*14–16*) have repeatedly proposed interesting ideas for devising novel multianalyte immunoassays. Their method is based on the use of a very small amount of antibody molecules immobilized on a microspot on a solid phase. These antibodies will bind a tiny fraction of the analyte of interest and the fractional occupancy of antibody binding sites will be proportional to the analyte concentration. One version of this principle also uses a labeled detection antibody in a manner similar to the conventional noncompetitive immunoassay. This principle can be used for multianalyte immunoassay if the binding antibodies are coated to defined areas of the solid phase, which are then scanned for signal quantification with a proper instrument. Practical examples of this assay principle have been published (*14–16*).

A practical, true multianalyte immunoassay system recently became available commercially (*17*). In this system, nitrocellulose is the solid-phase matrix that contains distinct regions coated with various antigens of infectious agents. When serum is added, any antigen-specific antibodies present bind to the specific antigen regions. This binding step is followed by addition of

[1] Department of Clinical Biochemistry, Toronto Western Hospital, 399 Bathurst St., Toronto, Ontario M5T 2S8, Canada.
[2] Department of Clinical Biochemistry, University of Toronto, 100 College St., Toronto, Ontario M5G 1L5, Canada.
[3] To whom correspondence should be addressed, at the Toronto Western Hospital.

[4] Nonstandard abbreviations: SBMC, streptavidin-based macromolecular complex; BCPDA, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid; IRP, International Reference Preparation; LH, lutropin (luteinizing hormone); FSH, follitropin (follicle-stimulating hormone); hCG, choriogonadotropin (chorionic gonadotropin); TSH, thyrotropin (thyroid-stimulating hormone); PRL, prolactin; and BSA, bovine serum albumin.

Received July 26, 1991; accepted January 7, 1992.

anti-human antibody conjugated to horseradish peroxidase (EC 1.11.1.7). A substrate is then added that forms a colored precipitate at the regions of enzyme localization. The color in the regions is quantified by reflectance spectroscopy. This assay format is successful for analytes present in serum at relatively high concentrations.

We recently described a streptavidin-based macromolecular complex (SBMC) multiply labeled with the europium chelate of 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) (*18*).[4] This reagent was successfully used for devising highly sensitive time-resolved fluorometric immunoassays (*19, 20*) in combination with biotinylated antibodies. The final immunocomplex can be quantified either in solution or on the dry solid phase. We here report multianalyte assay designs based on this reagent and biotinylated detection antibodies and applied to the simultaneous quantification of LH, FSH, choriogonadotropin (hCG), and PRL.

Materials and Methods

Instrumentation

For solid-phase time-resolved fluorometric measurements, we used the CyberFluor 615™ Immunoanalyzer (CyberFluor Inc., Toronto, Canada).

Materials

The coating buffer was a 50 mmol/L Tris solution, pH 7.80. The blocking buffer was a 50 mmol/L sodium phosphate solution, pH 7.4, containing 9 g of NaCl and 10 g of bovine serum albumin (BSA) per liter. The assay buffer was a 50 mmol/L Tris solution, pH 7.8, containing 9 g of NaCl, 0.5 g of sodium azide, 5 g of BSA, 0.5 g of bovine globulin, and 0.1 mL of polyoxyethylenesorbitan monopalmitate (Tween 40) surfactant per liter. The streptavidin dilution buffer was a 50 mmol/L Tris solution, pH 7.8, containing 58.5 g of NaCl, 40 g of BSA, 40 μmol of Eu^{3+}, and 0.5 g of sodium azide per liter. The wash solution was a 9 g/L NaCl solution containing 0.5 mL of polyoxyethylenesorbitan monolaurate (Tween 20) per liter.

Standards. Purified LH [6000 kilo-int. units/g in terms of the First International Reference Preparation (IRP), 68/40, for immunoassay] and purified FSH [2150 kilo-int. units/g in terms of the World Health Organization (WHO) Second IRP-HMG, for immunoassay] were obtained from Scripps Labs., San Diego, CA. Purified hCG (specific activity, 3310 kilo-int. units/g in terms of the First IRP 75/537, for immunoassay) and purified PRL (320 ng/vial by RIA, standardized with WHO IRP 75/504) were obtained from Calbiochem, Behring Diagnostics, La Jolla, CA. The preparations were reconstituted according to the manufacturers' recommendations and diluted in 50 mmol/L Tris buffer, pH 7.4, containing 60 g of BSA per liter (standards diluent buffer) to give the desired standard concentrations. The BSA was RIA-grade and was obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals were from Sigma.

Monoclonal antibodies. The monoclonal antibodies used are available from CyberFluor Inc. and have the following characteristics: (*a*) PRL: coating antibody code 5602; detection antibody code 5601. These antibodies do not cross-react with any whole molecule or subunit of the glycoprotein hormones LH, FSH, hCG, or TSH. (*b*) LH: coating antibody code M1L 0102. This antibody recognizes the whole LH molecule and has the following cross-reactivities—LH, 100%; FSH, 0.02%; TSH, 0.05%; hCG, 0.5%; alpha hCG, 0.2%; beta hCG, 0.6%. (*c*) FSH: coating antibody code 5000. This antibody recognizes specifically the beta subunit of FSH. (*d*) hCG: coating antibody code M1H 9812. This antibody recognizes the beta subunit of hCG and has the following cross-reactivities—hCG, 100%; beta hCG, 100%; alpha hCG, 0.0%; LH, 0.3%; and TSH, 0.02%.

The detection antibody for LH, FSH, and hCG was an alpha subunit monoclonal antibody (code no. 094-10725) recognizing equally the alpha subunits of LH, FSH, hCG, and TSH. Cross-reactivity with the respective beta subunits was <1.4%.

Specimens. Human serum samples containing various concentrations of the analytes were obtained from the clinical laboratory of the Toronto Western Hospital and stored at −20 °C until analysis. Lyphocheck immunoassay control sera (human) Levels I, II, and III were purchased from Bio-Rad Labs., Clinical Division, Richmond, CA 94801.

Procedures

Comparison methods. We compared the results of the method developed with those obtained with the FIAgen™ kits (CyberFluor Inc.) for each separate analyte.

Biotinylation of antibodies. One of the monoclonal antibodies to PRL and the monoclonal antibody to the alpha subunit of FSH, LH, and hCG were biotinylated and separated from the unreacted biotin as previously described (*20*). The biotinylated antibodies were then titrated to determine the optimal concentration for the assay and were stored at 4 °C. Before use, the antibodies to PRL and to the alpha subunit of FSH, LH, and hCG were diluted in the same tube at final concentrations of 0.89 and 3.33 mg/L, respectively, with the assay buffer.

Preparation of labeled streptavidin. The procedure has been described in detail previously (*18*). SBMC labeled with BCPDA (Eurofluor S™) is commercially available from CyberFluor Inc. as a 15 mg/L streptavidin stock solution; we diluted it 50-fold in the streptavidin dilution buffer just before use.

Preparation of the solid-phase antibodies. The coating solutions of the different antibodies were prepared by diluting separately the monoclonal antibodies to the intact LH, to the beta subunit of FSH, to the beta subunit of hCG, and to PRL in the coating buffer to a final concentration of 5 mg/L. We then added 100 μL of these solutions per well in white microtiter strips (Dynatech Labs., Alexandria, VA 22314) and allowed the antibody to adsorb to the wells at room temperature overnight. The wells were then washed twice with the wash solution, and 200 μL of the blocking buffer was

added per well. After a 1-h incubation, the wells were washed briefly with isotonic saline, blotted dry, and lyophilized. The wells coated with the specific antibody for each analyte were then marked at 2.5 mm from the outer surface of the bottom of the wells and were cut at that position with a blade. The resulting well bottoms were mounted in a fixed order onto a plastic stick, with chloroform or epoxy glue as the fixative. The configuration is shown in Figure 1; two well bottoms were fixed on one side of the stick and two were fixed on the opposite side. The distance between the two adjacent well bottoms was carefully chosen so that they could fit into holes of a plastic microtiter-well strip holder and their fluorescence be read with the 615 Immunoanalyzer. Sticks with the affixed well bottoms were stored dry under reduced pressure until use. Before use, they were incubated for 1 h in the blocking solution to saturate any uncoated surfaces of the sticks.

Assay procedure. Pipet 150 μL of standards or samples into 12 × 75 mm glass tubes (in duplicate or triplicate), and add 1 mL of biotinylated antibodies working mixture solution and one stick to each tube. Vortex-mix the tubes' contents briefly and incubate with shaking for 2 h on the platform of an orbital shaker at room temperature. Aspirate the contents of each tube and wash the sticks in the tubes three times with the wash solution. With a semiautomatic dispenser, pipet 1 mL of the streptavidin tracer working solution into each tube. After incubation for 30 min with shaking, wash the sticks three times with the wash solution, pull them out of the tubes, and blot them briefly on paper towels. Assemble the sticks in the appropriate order, place them into 96-well strip holders, dry them for 10 min at 50 °C, and measure the fluorescence on the dried surface of the two discs with the 615 Immunoanalyzer. Then, turn the sticks around and read the fluorescence of the other two discs. Each row of the printout corresponds to one analyte. The fluorescence readings can be interpreted manually or by using the built-in computer of the Immunoanalyzer.

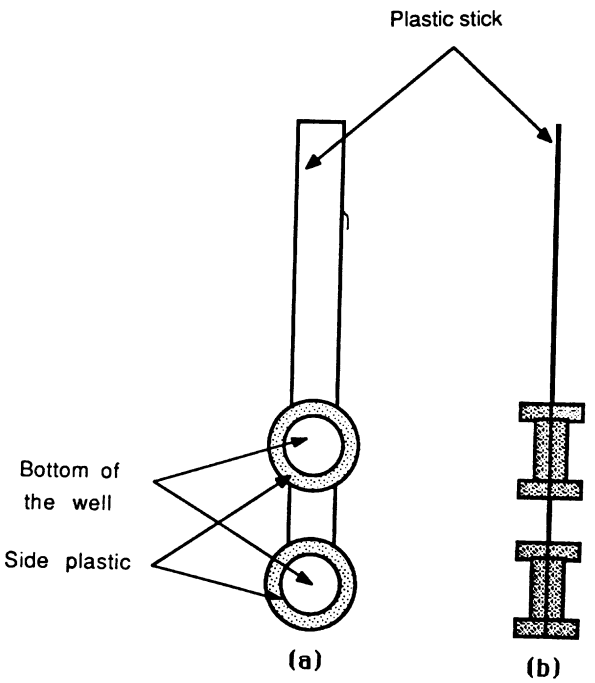

Fig. 1. Configuration of the solid phase used for the multianalyte immunoassay

(*a*) Front view showing one side of the plastic stick containing two microtiter well bottoms coated with capture antibodies. The *spotted area* represents uncoated regions. The distance between the centers of the well bottoms is exactly the same as that in microtiter well strips so that the stick can be placed on microtiter well-strip holders for measurement. (*b*) Side view showing the four microtiter well bottoms

Results

Calibration curves and detection limits. Figure 2 shows typical assay calibration curves (log–log plots) for LH, FSH, hCG, and PRL. The response (mean fluorescence intensity of duplicate measurements after subtraction of the zero-standard signal) was nearly linear over a concentration range of 5.0–250 int. units/L for LH and 5.0–150 int. units/L for FSH. Although the calibration curves for hCG and PRL change slope when >100 int. units/L and >50 μg/L, respectively, they are still useful up to the highest standard used, i.e., 500 int. units/L and 200 μg/L, respectively.

The limit of detection, defined as the concentration corresponding to the mean + 3 SD of the zero-standard signal for 12 replicates, was 1.2 int. units/L for LH, 0.9 int. units/L for FSH, 1.5 int. units/L for hCG, and 0.5 μg/L for PRL.

Precision. To evaluate the precision of the developed assays, we assayed pooled human sera with three different concentrations of each of the analytes. Within-run precision was determined by analyzing eight replicates of each sample in the same run (Table 1). The CVs obtained were between 4.3% and 10.8% in all cases.

Recovery. For recovery experiments, various amounts of the different analytes were added to two serum samples. Analytical recovery, assessed by analyzing the samples before and after the additions and subtracting

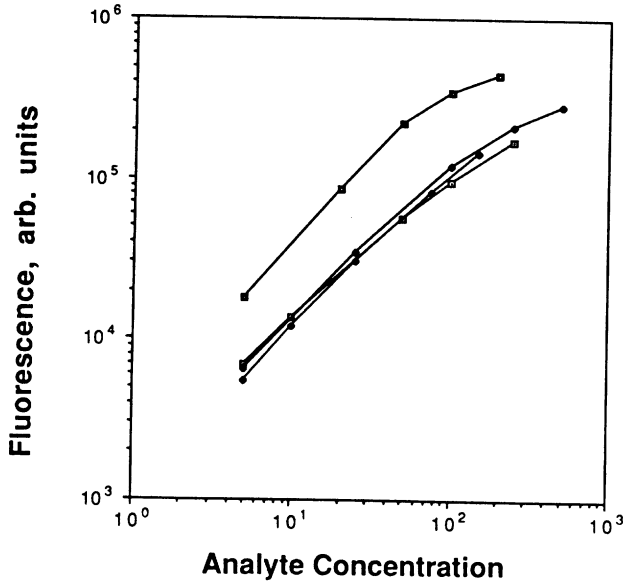

Fig. 2. Calibration curves of the developed assays: (□) prolactin, in μg/L; (◇) hCG, (◆) FSH, and (⊡) LH, in int. units/L

Table 1. Within-Run Precision of the Developed Assays

Mean	SD	CV, %
LH, int. units/L		
4.2	0.3	7.1
24.4	2.5	10.1
87.2	4.9	5.6
FSH, int. units/L		
6.3	0.7	10.7
13.4	1.1	8.2
33.6	1.4	4.3
hCG, int. units/L		
7.4	0.8	10.8
26.2	1.6	6.2
126.9	12.5	9.9
PRL, μg/L		
11.4	0.8	7.0
22.1	1.9	8.6
46.6	2.1	4.5

n = 8 each.

the estimated endogenous concentration of each analyte, was close to 100% for each analyte (Table 2).

Specificity. Each of the four assays was tested for cross-reactivity with the other three analytes. Standards diluent buffer was supplemented with various amounts of each analyte, and the equivalent concentration of each hormone was then calculated from the corresponding dose–response curve.

We found that the cross-reactivity of FSH in the LH assay was 5.5% for FSH ≤500 int. units/L, whereas the cross-reactivity of LH in the FSH assay was 0.2% for LH ≤500 int. units/L. PRL and hCG did not interfere in the measurement of these two hormones at concentrations ≤500 μg/L and ≤1000 int. units/L, respectively. The cross-reactivities of LH and FSH in the hCG assay were 3.1% and 0.7%, respectively, for concentrations ≤500 int. units/L of each hormone, whereas PRL did not interfere in the measurement of hCG at concentrations ≤500 μg/L. We could not detect any interference in the measurement of PRL for concentrations of LH and FSH ≤500 int. units/L and of hCG ≤1000 int. units/L.

Table 2. Analytical Recovery in the Developed Assays

Initially present	Added	Recovered	Recovery, %
LH, int. units/L			
4.8	17.2	15.4	89.5
	51.4	54.9	106.8
24.6	17.6	19.3	109.6
	54.8	52.3	95.4
			Mean (SD) 100.3 (9.5)
FSH, int. units/L			
5.0	16.0	16.3	101.9
	49.0	43.7	89.2
17.2	16.0	17.3	108.1
	49.0	53.7	109.6
			Mean (SD) 102.2 (9.3)
hCG, int. units/L			
6.3	12.3	14.0	113.8
	55.5	52.8	96.0
25.2	12.3	11.4	92.7
	55.5	58.9	107.1
			Mean (SD) 102.4 (9.8)
PRL, μg/L			
8.3	12.0	11.3	94.2
	32.1	31.2	97.5
17.2	12.0	12.2	101.7
	32.1	34.5	107.8
			Mean (SD) 100.3 (5.9)

Correlation with other methods. We analyzed 31 serum samples with the developed multianalyte procedure and with the FIAgen kits for each separate analyte. Table 3 summarizes the statistical parameters obtained by linear-regression analysis of the results.

Discussion

There is increased interest for the simultaneous assay of more than two analytes in the same cuvet in areas such as endocrinology, oncology, infectious disease, and therapeutic drug monitoring. Systems that rely on the differential assay of labels in a liquid mixture or on radionuclides are not likely to quantify more than two or three analytes at a time. There are no practical radionuclide-label combinations for such assays and, for liquid-based chemistry tests, no more than three lanthanides could form fluorescent complexes that could be individually quantified in each other's presence. Use of conventional fluorophores, which have broad emission spectra, would make simultaneous quantification even more difficult.

One practical approach for true multianalyte immunoassays involves the spatial distribution of immunospots, the responses of which are then quantified with a suitable instrument (*14–17*). The immunospots could carry a detectable moiety, i.e., an insoluble colored precipitate generated by an enzyme (*17*) or a fluorescent tag. For highly sensitive assays, the fluorescent europium chelates are the labels of choice (*13*). In this study we took advantage of a highly sensitive detection reagent based on streptavidin multiply labeled with the fluorescent europium chelate of BCPDA (*18*). This reagent can fluorescently label areas containing biotinylated antibodies; the fluorescent areas can be quantified on the dry solid phase by using laser excitation. Multianalyte immunoassay with high sensitivity can thus be achieved by individually quantifying closely spaced fluorescent areas, each one representing one analyte.

Although in theory an unlimited number of analytes can be assayed by such principles (*14–16*), several practical problems must be considered. For these assays to

Table 3. Linear-Regression Comparison of Multianalyte Immunoassay and FIAgen Results

Analyte	Slope	Intercept	S_{yx}
LH, int. units/L	0.995	0.19	7.97
FSH, int. units/L	1.040	0.32	3.77
hCG, int. units/L	0.991	−0.91	9.19
PRL, μg/L	0.950	−0.95	5.35

n = 31. Multianalyte immunoassay (*y*); FIAgen assay (*x*).

be successful, the coating antibody must be very specific; otherwise, cross-reacting moieties will also be immobilized, leading to either negative errors (by preventing analyte binding) or positive errors (if the interferent also cross-reacts with the detection antibody). This problem is more pronounced in comparison with single-analyte assays because more than one detection antibody is present in the reaction mixture. In multianalyte immunoassay designs, it is more difficult to optimize the assay range for all analytes because of the fixed sample volume that must be used. Thus, a larger sample volume can improve the detection limit of one analyte but restrict the dynamic range of another analyte that is present in serum at higher concentrations. The fixed sample volume may also affect assay specificity in some cases.

In conclusion, we present a practical method for multianalyte immunoassay that is sensitive, precise, and accurate and that gives results comparable with those obtained in individual assays. We believe that this principle warrants further investigation and may prove to be a practical way to simultaneously assay different analytes, especially in areas where panel testing is frequently performed.

This work was partially supported by a grant from the Medical Research Council of Canada.

References

1. Fairbanks VF, Klee GG. Biochemical aspects of hematology. In: Tietz NW, ed. Fundamentals of clinical chemistry. Philadelphia: WB Saunders, 1987:789–824.
2. Challand GS. Immunoassays in hematology. In: Voller A, Bartlett A, Bidwell D, eds. Immunoassays for the 80s. Baltimore: University Park Press, 1981:325–33.
3. Gutcho S, Mansbach L. Simultaneous radioassay of vitamin B_{12} and folic acid. Clin Chem 1977;23:1609–15.
4. Perlstein Rock MT, Schneider MS, Fialkowski DM. Monoclonal antibody-based radioimmunoassay for vitamin B_{12} (^{57}Co)/folate (^{125}I) determinations [Abstract]. Clin Chem 1987;33:922–3.
5. Wians FH, Dev J, Powell MM, Heald JI. Evaluation of simultaneous measurement of lutropin and follitropin with the SimulTROPIN™ radioimmunoassay kit. Clin Chem 1986;32:887–90.
6. Gow SM, Caldwell G, Toft AD, Beckett GJ. SimulTRAC™ simultaneous radioimmunoassay of thyrotropin and free thyroxin evaluated. Clin Chem 1986;32:2191–4.
7. Chaudhuri J, Amirkhan N. Evaluation of a simultaneous radioimmunoassay for free T_4 and TSH [Abstract]. Clin Chem 1986;32:1155.
8. Groman EV, Havelick J, Markinac J. Development of a simultaneous 57-Co total T_4/125-I TSH assay using a magnetic solid phase [Abstract]. Clin Chem 1986;32:1159.
9. Hemmilä I, Holttinen S, Petterson K, Lövgren T. Double-label time-resolved immunofluorometry of lutropin and follitropin in serum. Clin Chem 1987;33:2281–3.
10. Siitari H. Dual label time-resolved fluoroimmunoassay for the simultaneous detection of adenovirus and rotavirus in faeces. J Virol Methods 1990;28:179–88.
11. Bador R, Dechaud H, Claustrat F, Desuzinges C. Europium and samarium as labels in time-resolved immunofluorometric assay of follitropin. Clin Chem 1987;33:48–51.
12. Saarma M, Jarvekulg L, Hemmilä I, Siitari H, Sinijarv R. Simultaneous quantification of two plant viruses by double-label time-resolved immunofluorometric assay. J Virol Methods 1989; 23:47–54.
13. Diamandis EP. Immunoassays with time-resolved fluorescence spectroscopy. Principles and applications [Review]. Clin Biochem 1988;21:139–50.
14. Ekins R, Chu F, Biggart E. Fluorescence spectroscopy and its application to a new generation of high sensitivity, multi-microspot, multianalyte immunoassay [Review]. Clin Chim Acta 1990; 194:91–114.
15. Ekins R, Chu F, Biggart E. Multispot, multianalyte, immunoassay [Review]. Ann Biol Clin (Paris) 1990;48:655–66.
16. Ekins R, Chu F, Biggart E. Multianalyte immunoassay: the immunological "compact disc" of the future [Review]. J Clin Immunoassay 1990;13:169–81.
17. Donohue J, Bailey M, Grey R, et al. Enzyme immunoassay system for panel testing. Clin Chem 1989;35:1874–7.
18. Morton RC, Diamandis EP. Streptavidin-based macromolecular complex labeled with a europium chelator suitable for time-resolved fluorescence immunoassay applications. Anal Chem 1990; 62:1841–5.
19. Diamandis EP, Christopoulos TK. Europium chelate labels in time-resolved fluorescence immunoassays and DNA hybridization assays [Review]. Anal Chem 1990;62:1149A–57A.
20. Christopoulos TK, Lianidou ES, Diamandis EP. Ultrasensitive time-resolved fluorescence method for α-fetoprotein. Clin Chem 1990;36:1497–502.