Noncompetitive Enzyme Immunoassay for α-Fetoprotein Using Flow Injection Chemiluminescence

JIEHUA LIN,[1] FENG YAN,[2] AND HUANGXIAN JU*,[1]

[1]*State Key Laboratory of Coordination Chemistry, Department of Chemistry, Institute of Chemical Biology, Nanjing University, Nanjing 210093, P. R. China, E-mail: hxju@nju.edu.cn; and [2]Jiangsu Institute of Cancer Prevention and Cure, Nanjing 210009, P. R. China*

Abstract

A novel, direct noncompetitive flow injection enzyme immunoassay for α-fetoprotein (AFP) was developed by enhanced chemiluminescence detection. The method was based on off-line incubation of AFP and horseradish peroxidase (HRP)-labeled anti-AFP, and then trapping of the unbound enzyme conjugate by an immunoaffinity column filled with AFP-modified Sepharose. The immunocomplex formed in incubation passed through the column and then was directly detected by a postcolumn chemiluminescence technique. The optimal conditions for the immunoassay procedure and chemiluminescence detection were established. At a 1:10 dilution of enzyme conjugate solution, the linear range for chemiluminescence detection of AFP was from 2.0 to 75 ng/mL with a correlation coefficient of 0.993 and a coefficient of variation of 2.67% at 30 ng/mL. The detection limit was 0.5 ng/mL. This method was flexible, sensitive, and rapid. The immunoaffinity column of 200 μL could be repeatedly used 100 times without a single decrease. The whole assay time including the preincubation step was only 30 min for one sample.

Index Entries: Flow injection analysis; enzyme immunoassay; noncompetitive immunoassay; immunoaffinity; α-fetoprotein; horseradish peroxidase; chemiluminescence.

*Author to whom all correspondence and reprint requests should be addressed.

Introduction

α-Fetoprotein (AFP), as an oncofetal glycoprotein, has been well known as a tumor marker. The concentration of serum AFP in healthy adults is typically in the range of 4–13 ng/mL, but it could attain 10 µg/mL or more in patients with liver cancer *(1)*. The increased AFP level has been considered an early indication of hepatocellular carcinoma *(2)*, liver metastasis from gastric cancer *(3)*, lung cancer *(4)*, premature rupture of the membranes in preterm patients *(5)*, and nasopharyngeal carcinoma *(6)*. Recently, it has been found that AFP is associated with tumor cell growth and may serve as an important target of tumor therapy *(7)*. Thus, the detection of AFP plays an important role in the initial diagnostic evaluation as well as in the follow-up examination during therapy. It is necessary to develop a highly sensitive and selective rapid determination method of AFP in serum or other biofluids.

Numerous immunologic methods for determination of AFP have been exploited owing to the high specificity of immunoreaction *(8–12)*. Among the various immunoassay formats, the most common is the enzyme-linked immunosorbent assay (ELISA). Sandwich ELISA is usually performed in microtiter plates, which involves a washing and separation step to remove the free antibody from the bound antibody. Rapid and easy operations are attractive in the field of clinical immunoassay especially for tumor markers. Various techniques have been adopted for determination of AFP, such as liquid-phase binding assay *(1,13)*, micellar electrokinetic capillary chromatography *(14)*, and flow injection amperometric enzyme immunoassay *(15)*, to overcome the limitations of conventional approaches. Flow injection immunoassay (FIIA) has been shown to be useful for improving cumbersome, time-consuming, and labor-intensive traditional immunoassay *(16,17)*, and it has been applied in many assays, such as food, pharmaceutical, environmental, and clinical *(15,18–21)*, owing to the small volume required, reduced handling of samples, good reproducibility, and easy automatization for high sample throughput. In the present work, we developed a novel flow injection chemiluminescent enzyme immunoassay method for AFP using an immunoaffinity column filled with AFP-modified Sepharose and a noncompetitive immunoassay mechanism.

The noncompetitive immunoassay technique, compared to competitive immunoassay and sandwich ELISA, not only offers a higher sensitivity and precision, but also is convenient and less susceptible to interfering factors. It has been extensively adopted in flow injection enzyme immunoassay (EIA) *(17,22–25)*. Its sensitivity depends on not only the sensitivity of the enzyme label and the extent of nonspecific interactions, but also on the method of detection *(17)*. Enzymes such as horseradish peroxidase (HRP) and alkaline phosphatase have been used widely as labels in EIA *(26)*. HRP, which catalyzes the luminescent luminol oxidation in the presence of hydrogen peroxide (H_2O_2), is one of the most commonly used enzymes in chemiluminescence for antibody and antigen labeling. To detect HRP at a

very low level, an enhanced luminol chemiluminescence detection method has been widely used in HRP-labeled EIA *(27)*. Several compounds, such as phenol derivatives *(28)*, carbon dioxide *(29)*, fluorescein *(30)*, and phenylboronic acid *(31)*, have been used to enhance the intensity of the light emitted during the course of the peroxidase-catalyzed luminol oxidation. The *p*-iodophenol (PIP)-enhanced chemiluminescence system has also been used for the study of real-time antigen–antibody interaction *(32)* and immunoassay for dichlorprop methyl ester *(33)* and DDT 1,1,1-trichloro-2,2-bis (*p*-chlorophenyl ethane) *(34)*.

The aim of the present work was to develop an easily automated, simple, and rapid noncompetitive FIIA for AFP by PIP-enhanced luminol-H_2O_2-HRP chemiluminescence. This developed assay for AFP is much faster than a conventional ELISA. The entire assay time including the pre-incubation step for one sample is only 30 min. It can be satisfactorily used to determine AFP in human serum.

Materials and Methods

Reagents and Standard Solutions

HRP (EC 1.11.1.7; *RZ* > 3.0, >250 U/mg) was purchased from Sigma (St. Louis, MO) and used as received. AFP-EIA kits, including a series of AFP standard solutions with different concentrations from 0 to 1000 ng/mL and a bottle of HRP-labeled anti-AFP solution, were purchased from Everlong. CNBr-activated Sepharose™ 6MB was purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Luminol (5'-amino-2,3-dihydro-phthalazine-1,4-dione, >95%) was purchased from Shanxi Normal University (Xian, China). PIP (>99.9%) was purchased from Weihai Newera Chemical (Weihai, China). All other reagents were of analytical reagent grade and used without further purification.

Luminol stock solution (0.01*M*) was prepared with 0.1*M* NaOH solution. PIP stock solution (0.01*M*) was prepared by dissolving 0.1103 g of PIP in 5 mL of dimethyl sulfoxide and then diluting with water to 50 mL and kept in the dark. H_2O_2 solution was prepared by appropriate dilution of a 30% solution (Shanghai Chemical, Shanghai, China) with water daily. The working solution was prepared with 0.1 *M* Tris-HCl buffer solution (pH 8.5). Double distilled water was used in all experiments.

Preparation of Immunoaffinity Columns

The following buffers were used to prepare antigen-modified Sepharose for preparation of immunoaffinity column: coupling buffer (0.1*M* NaHCO₃/0.5*M* NaCl, pH 8.0), washing buffer (0.1*M* CH₃COONa-CH₃COOH/0.5*M* NaCl, pH 4.5; and 0.1*M* Tris-HCl/0.5*M* NaCl, pH 8.0), and blocking buffer (0.1*M* NaHCO₃/0.2*M* glycine, pH 8.5). Preparation was performed according to instruction's provided by Amersham Pharmacia Biotech AB. Freeze-dried powder of CNBr-activated Sepharose 6MB (0.1 g) was suspended in 1 m*M* HCl. The swollen Sepharose was then washed with 1 m*M* HCl on a
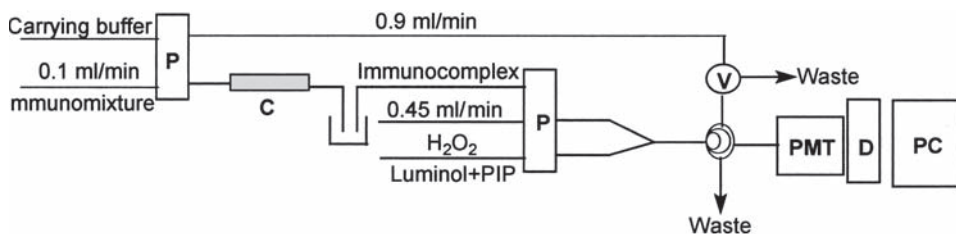
Fig. 1. Schematic diagram of chemiluminescent noncompetitive FIIA system: P, peristaltic pump; V, eight-way valve; C, immunoaffinity column; PMT, photomultiplier; D, detector; PC, computer.

sintered glass filter for 15 min. After it was further washed with the coupling buffer, 300 µL of 1000 ng/mL AFP was added to the Sepharose gel. The mixture was incubated for 24 h at 4°C and then mixed with the blocking buffer for another 24 h at 4°C. Finally, it was washed with pH 4.5 and 8.0 washing solutions for three cycles and filled a glassy tube to about 200 µL (1.6 mm id and 100 mm length). After use, the filled AFP-modified gel was regenerated by passing a solution of $0.1M$ glycine-HCl (pH 2.2) through the column and equilibrated with $0.1M$ phosphate-buffered saline (PBS) (pH 7.0). When not in use, the columns were stored in $0.1M$ pH 7.0 PBS at 4°C.

Instrumentation

The immunoaffinity column was connected to a flow system as illustrated in Fig. 1. Two peristaltic pumps of Luminescence Analyzer (IFFM-D; Remex Electronic, Xi'an, China) were used to deliver flow streams. Polytetrafluoroethylene tubing (0.8 mm id) was used to connect all components in the flow system. A colorless spiral glass tubing (10 cm length and 2.0 mm id) was used as the flow cell. Chemiluminescence substrates were mixed with a Y-shaped element and reacted with the immunocomplex from a merging flow outside of the affinity column. The chemiluminescence emission was detected by a photomultiplier tube (PMT) placed near the flow cell and was recorded with a computer via an A/D convert card. PBS ($0.1M$, pH 7.0) was used as the carrier.

Noncompetitive FIIA

A diagram of the noncompetitive EIA is shown in Fig. 2. HRP-labeled anti-AFP solution was first diluted with $0.1M$ PBS (pH 7.0) at different dilutions. Fifty microliters of HRP-anti-AFP solution was then mixed with 50 µL of AFP standard solution. After an incubation time of 25 min at room temperature, the mixture was injected into the flow system at a flow rate of 0.1 mL/min. AFP-modified Sepharose filled in the immunoaffinity column was used to trap the unbound enzyme conjugate, whereas the immunocomplex passed through the column and was collected in a 0.5-mL vessel.
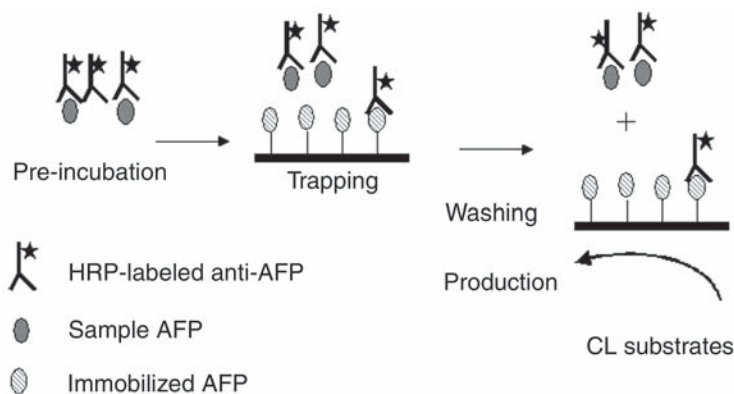
Fig. 2. Schematic diagram of noncompetitive EIA procedure for AFP. CL, chemiluminescence.

The collection was injected by the valve as a merging flow to react with the chemiluminescence substrates. The generated signal was detected by PMT.

Results and Discussion

Optimization of Chemiluminescence Detection System

FIIA performance depends not only on the immunoreagents and assay procedure, but also on the characteristics of the substrates. Chemiluminescence emission intensity is sensitive to a variety of environmental factors such as temperature, solvent, ionic strength, pH, and other species present in the system. Our work examined the effects of the concentrations of luminol, H_2O_2, and PIP; pH value and kind of chemiluminescence detection buffer solution; and flow rate on chemiluminescence intensity. The results showed that the optimal concentrations of luminol, H_2O_2, and PIP were $5.0 \times 10^{-4} M$, $1.0 \times 10^{-3} M$, and $4.0 \times 10^{-4} M$, respectively. These results correspond with those reported previously *(33)*. Tris-HCl buffer (0.1*M*, pH 8.5) was selected as the chemiluminescence detection solution. The carrier flow rate was selected at 0.9 mL/min.

Optimization of Immunoassay Procedure

The performance evaluation of the immunoassay system included the optimization of preincubation time for the immunoreaction between analyte AFP and enzyme conjugate, the working concentration of HRP-anti-AFP, and the residence time of the reaction mixture in the immunoaffinity column.

The effect of preincubation time on the immunoreaction between 10 ng/mL of AFP and enzyme conjugate at a dilution of 1:10 on the response is shown in Fig. 3. With an increasing preincubation time, the chemiluminescence signal increased and reached a maximum value after a preincubation time of 25 min. Ninety percent of the binding occurred within 25 min
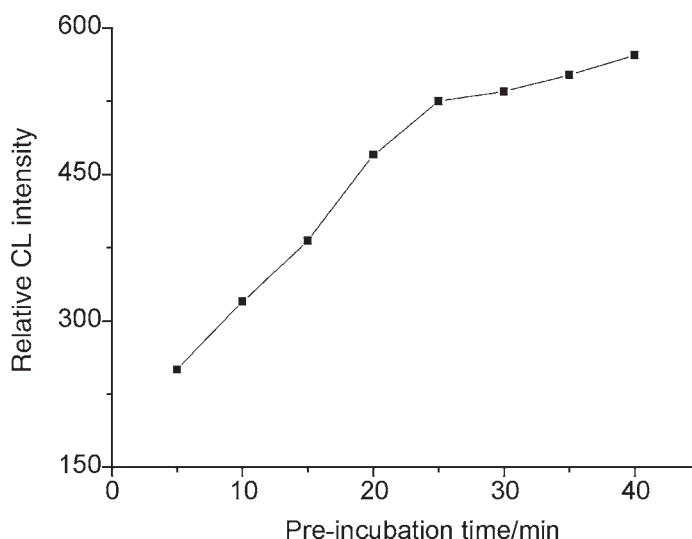
Fig. 3. Effect of preincubation time on chemiluminescence (CL) intensity at concentration of 10 ng/mL of AFP with 1:10 dilution of enzyme conjugate.

at room temperature (27°C); thus, a preincubation time of 25 min was chosen for AFP detection throughout the experiment.

The anti-AFP working concentration should be selected to give a compromise between a good sensitivity and a wide assay range. The optimal dilution of HRP-anti-AFP was established by using different diluted anti-AFP solutions (the volume ratios of 1:5, 1:10, 1:15, and 1:20 for HRP-labeled anti-AFP solution provided in the AFP-EIA kits to 0.1M PBS, pH 7.0) as the working solutions in AFP immunoassay to examine the signal change with an increasing AFP concentration; the results are shown in Fig. 4. The signal change was defined as $(I_s - I_0)/I_0$, in which I_0 is the blank signal obtained in the absence of AFP analyte, and I_s is the signal obtained in the presence of the analyte antigen. In the AFP concentration range of 1.0–20 ng/mL, a dilution of 1:10 gave the best sensitivity and the widest linear range, whereas the linear ranges at 1:15 and 1:20 were narrower. The immunoassay at all three dilutions gave a detection limit of 0.5 ng/mL. At a dilution of 1:5, the signal change showed a small slope, indicating a low sensitivity. This phenomenon was possibly owing to the fact that the amount of unbound HRP-anti-AFP exceeded the trapping ability of the immunoaffinity column at high enzyme conjugate concentration. At low enzyme conjugate concentrations such as those at 1:15 and 1:20 dilutions, most of the labeled antibody reacted with the antigen to form immunocomplex at low antigen concentrations. The immune immobilization of the enzyme conjugate by the immunoaffinity column showed a smaller effect on the chemiluminescence intensity when the mixture passed through the column, which resulted in a narrow linear range. Thus, the HRP-anti-AFP solution with a dilution of 1:10 was selected as the working solution.
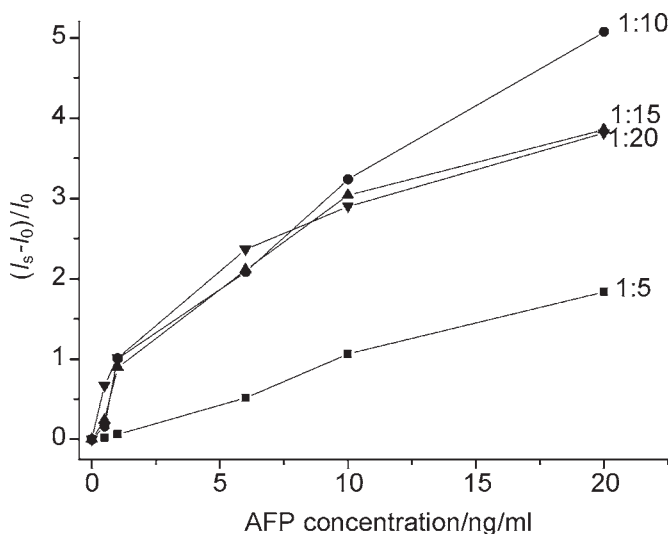
Fig. 4. Effect of dilution of enzyme conjugate on response signals on increasing AFP concentration at preincubation time of 25 min.

The residence time of the reaction mixture in the immunoaffinity column depended on the flow rate of the mixture through the column. We wished to determine the binding efficiency of the unbound enzyme conjugate to the immobilized AFP on the gel surface. Partial removal of the unbound enzyme conjugate from the mixture owing to a short residence time or high flow rate resulted in a high background chemiluminescence signal. However, the complete capture of the unbound enzyme conjugate required a long time, which was disadvantageous to practical application of this method. At the same time, the column capacity also played an important role in the capture of unbound enzyme conjugate. To improve the trapping ability of this column, 1000 ng/mL of AFP was used to prepare the column. Figure 5 shows the dependence of chemiluminescence intensity on flow rate. At a flow rate of 0 mL/min, the stream of the mixture was retained in the column for 30 min, giving a minimum chemiluminescence intensity. At a flow rate of 0.1 mL/min, the capture of unbound enzyme conjugate could reach 70% of that at 0 mL/min. Considering the practical application, 0.1 mL/min was selected for injection immunoassay of AFP.

Noncompetitive FIIA for AFP

The AFP standard solutions of different concentrations were mixed with a 1:10 diluted HRP-anti-AFP solution to perform the preincubation step. As shown in Fig. 6, the linear range for chemiluminescence detection of AFP was from 2.0 to 75 ng/mL with a correlation coefficient of 0.993. Using this standard curve, the coefficients of variation (CVs) for five determinations of AFP at 30 and 2.0 ng/mL were 2.67 and 7.04%, respectively.
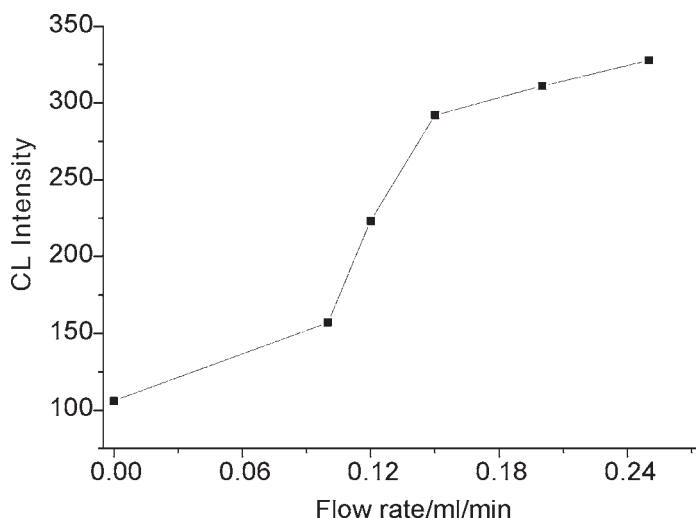
Fig. 5. Effect of flow rate of immunomixture through immunoaffinity column at 1:10 dilution of enzyme conjugate and 25-min preincubation time. CL, chemiluminescence.
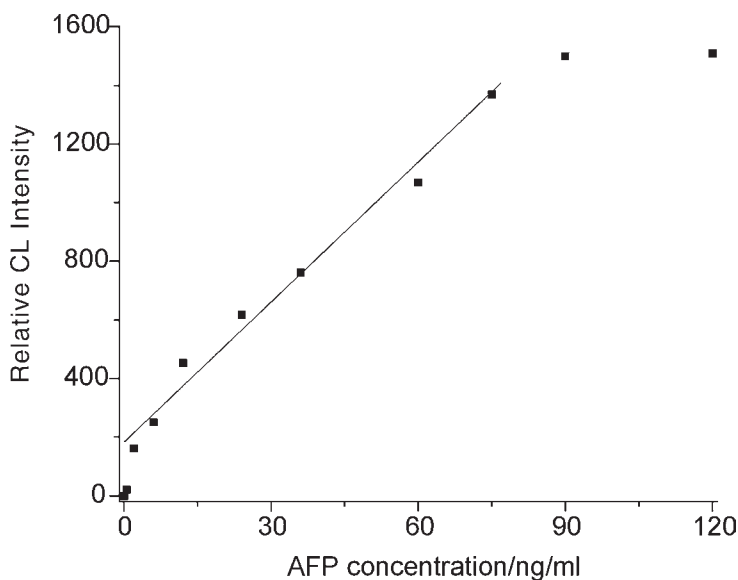

Fig. 6. AFP calibration curve at 1:10 dilution of enzyme conjugate. CL, chemiluminescence.

This method was flexible and rapid. The entire assay time including the preincubation step was only 30 min for one sample.

It also can be seen from the calibration plot shown in Fig. 6 that the detected antigen bound most of HRP-anti-AFP in the preincubation solution when the AFP concentration was as high as 120 ng/mL. The detection of AFP at concentrations higher than 75 ng/mL required a dilution of sample or higher concentration of HRP-anti-AFP used in the preincubation

solution. The latter needed to obtain a new calibration curve at the HRP-anti-AFP concentration higher than that at a 1:10 dilution.

Accuracy and Clinical Application

The accuracy of the AFP determination was examined by comparing the results obtained from this method and immunoradiometric (IRMA). In both techniques, the AFP contents in two sera were quantified using a related calibration plot for three replicates at each concentration. The mean AFP concentrations in two serum samples determined with IRMA were 37.03 and 74.07 ng/mL, respectively. This method gave values of 34.72 and 68.22 ng/mL, respectively. The relative errors of the two methods were 6.24 and 7.90%, respectively; thus, they were in good agreement.

Stability of Immunoaffinity Columns

The stability of the immunoaffinity columns was assessed by intra- and interassay CVs. The intraassay CV was the difference among three determinations of one sample on the same column. The interassay CV was the difference among the measurements of one same sample on three different columns. The intra- and interassay CVs obtained at an AFP concentration of 6.0 ng/mL were 2.7 and 10% respectively. The column could be reused at least 100 times before the signals reached 90% of the preliminary one. These results demonstrated that the affinity column possessed good stability and that the preparation of affinity columns from the same immobilization batch possessed good reproducibility.

Conclusion

A new noncompetitive FIIA for AFP by postcolumn enhanced chemiluminescence detection was described. In this postcolumn mode, the free antibody molecules after the preincubation procedure were separated with an immunoaffinity column, and the immunocomplexes flowed through the column to catalyze the chemiluminescence reaction of luminol and were detected with a chemiluminescence analytical system. In comparison with the conventional immunoassay approaches, which are performed in microtiter plates or other solid matrix, this proposed method is more flexible and rapid. It shortens the analysis time to 30 min. Although the detection limit obtained with the present system is not as low as 0.07 ng/mL, as found by Della Ciana et al. *(15)*, the immunoassay format is quicker, simpler, and more easily automated, and, thus, the system can be further developed for practical clinical detection of serum AFP level.

Acknowledgments

References

1. Yamagata, Y., Katoh, H., Nakamura, K., Tanaka, T., Satomura, S., and Matsuura, S. (1998), *J. Immunol. Methods* **212,** 161–168.
2. Yusof, Y. A. M., Salleh, M. N., and Abdullah, N. A. (1997), *J. Biochem. Mol. Biol.* **2,** 18–22.
3. Webb, A., Scott-Mackie, P., Cunningham, D., Norman, A., Andreyev, J., O'Brien, M., and Bensted, J. (1996), *Eur. J. Cancer* **32,** 63–68.
4. Kubota, M., Tsubota, N., Yoshimura, M., Murotani, A., and Sakamoto, T. (1995), *Lung Cancer* **12,** 290.
5. Kishida, T., Yamada, H., Negishi, H., Sagawa, T., Makinoda, S., and Fujimoto, S. (1996), *Eur. J. Obstet. Gynecol. R. B.* **69,** 77–82.
6. Wang, C. C., Hong, J. H., Hsu, K. H., Tsang, N. M., Leung, W. M., and Simon, G. T. (1996), *Int. J. Radia. Oncol. Biol. Phys.* **36,** 163.
7. Wang, X. W. and Xu, B. (1998), *Int. J. Cancer* **75,** 596–599.
8. Kirkpatrick, A. and Nakamura, H. T. (1977), *Clin. Chem.* **23,** 50–59.
9. Xue, M., Haruyama, T., Kobatake, E., and Aizawa, M. (1996), *Sens. Actuators B* **36,** 458–462.
10. Matsumoto, K., Yuan, J. L., Wang, G. L., and Kimura, H. (1999), *Anal. Biochem.* **276,** 81–87.
11. Ito, K., Oda, M., Tsuji, A., and Maeda, M. (1999), *J. Pharm. Biomed.* **20,** 169–178.
12. Ci, Y. X., Qin, Y., Chang, W. B., and Li, Y. Z. (1995), *Anal. Chim. Acta* **300,** 273–276.
13. Nakamura, K., Imajo, N., Yamagata, Y., Katoh, H., Fujio, K., and Tanaka, T. (1998), *Anal. Chem.* **70,** 954–957.
14. Wang, R. Y., Lu, X. M., and Ma, W. Y. (2002), *J. Chromatogr. B* **779,** 157–162.
15. Della Ciana, L., Bernacca, G., De Nitti, C., and Massaglia, A. (1996), *J. Immunol. Methods* **193,** 51–62.
16. Pollema, C. H., Ruzicka, J., and Christian, G. D. (1992), *Anal. Chem.* **64,** 1356–1361.
17. Gunaratna, P. C. and Wilson, G. S. (1993), *Anal. Chem.* **65,** 1152–1157.
18. Nandakumar, R., Nandakumar, M. P., and Mattiasson, B. (2000), *Biosens. Bioelectron.* **15,** 241–247.
19. Bereczki, A. and Horváth, V. (1999), *Anal. Chim. Acta* **391,** 9–17.
20. Bjarnason, B., Bousios, N., Eremin, S., and Johansson, G. (1997), *Anal. Chim. Acta* **347,** 111–120.
21. Burestedt, E., Kjellström, S., Emnéus, J., and Marko-Varga, G. (2000), *Anal. Biochem.* **279,** 46–54.
22. Lövgren, U., Kronkvist, K., Bäckström, B., Edholm, L. E., and Johansson, G. (1997), *J. Immunol. Methods* **200,** 159–168.
23. Önnerfjord, P., Eremin, S. A., Emnéus, J., and Marko-Varga, G. (1998), *J. Chromatogr. A* **800,** 219–230.
24. Piran, U., Riordan, W. J., and Livshin, L. A. (1996), *Biotechnol. Adv.* **14,** 519.
25. Lim, T. K., Nakamura, N., and Matsunaga, T. (1998), *Anal. Chim. Acta* **370,** 207–214.
26. Zhu, Q. Z., Liu, F. H., Li, D. H., Xu, J. G., Su, W. J., and Huang, J. W. (1998), *Anal. Chim. Acta* **375,** 177–185.
27. Dodeigne, C., Thunus, L., and Lejeune, R. (2000), *Talanta* **51,** 415–439.
28. Thorpe, G. H. G., Kricka, L. J., Gillespie, E., Moseley, S., Amess, R., Bagget, N., and Whitehead, T. P. (1985), *Anal. Biochem.* **145,** 96–100.
29. Zhang, H. L. and Horacio, A. M. (1996), *Analyst* **121,** 211–218.
30. Navas Diaz, A., Gonaalez Garcia, J. A., and Lovillo, J. (1997), *J. Biolumin. Chemilumin.* **12,** 199–205.
31. Kricka, L. J. and Ji, X. Y. (1997), *Talanta* **44,** 1073–1079.
32. Osipov, A. P., Zaitseva, N. V., and Egorov, A. M. (1996), *Biosens. Bioelectron.* **11,** 881–887.
33. Díaz, A. N., Sánchez, F. G., Lovillo, J., and García, J. A. G. (1996), *Anal. Chim. Acta* **321,** 219–224.
34. Botchkareva, A. E., Fini, F., Eremin, S., Mercader, J. V., Montoya, A., and Girotti, S. (2002), *Anal. Chim. Acta* **453,** 43–52.