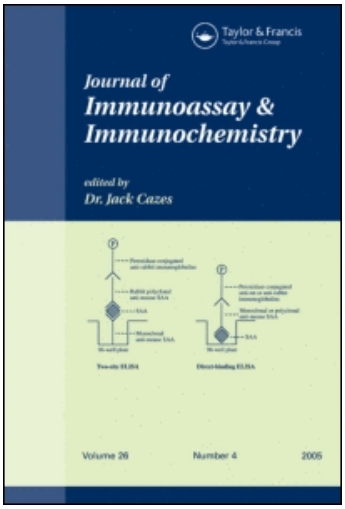

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SENSITIVITY OF IMMUNOENZYMOMETRIC ASSAY AND DETECTION METHOD OF ENZYME

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ABSTRACT

The effect of the detection limit of an enzyme measurement on the detection limit of the immunoenzymometric assay (IEMA) was investigated. Using a biotin-labelled antibody and avidin-biotin alkaline phosphatase complex (ABC enzyme) reagent, three IEMA systems for interferon-γ with different enzyme substrates for colorimetric, fluorometric, or chemiluminometric detection were developed. The optimum amounts of the reagents, the non-specific binding (NSB) level, and the detection limit of the IEMA were estimated. The results of this study suggest that the biotin-labelled antibody and ABC enzyme reagent should not decrease to less than 20 times the concentration of its K_d value and to less than 250 times the enzyme activity of the NSB, respectively. The detection limit of IEMA did not decrease as much as that of enzyme measurement because of lack of proportionate decrease of the NSB level. These findings should be very useful not only for IEMA but also for immunoblotting and immunocytochemistry research.

(KEY WORDS: Immunoenzymometric assay, colorimetric detection, fluorometric detection, chemiluminometric detection, detection limit, non-specific binding)

INTRODUCTION

Improvement in the sensitivity of immunoenzymometric assay (IEMA) has been generally expected with more sensitive detection methods for enzymes, such as fluorometry, luminometry, or enzyme-cycling, rather than the usual colorimetry (1-6). Many researchers have tried to increase the specific signal or to decrease the non-specific binding (NSB) by changing the amounts of reagents for IEMA when a more sensitive method of enzyme measurement was used. However, some papers have reported that the sensitivity of IEMA did not depend very much on the sensitivity of the detection system (7-10). Few reports have discussed these relationships in detail. The detection limit of an assay, defined as the minimum amount of an analyte for which the intensity of the signal exceeds the blank or NSB by twice standard deviation of it, is explored here as a function of sensitivity.

In this report, the relationship between the detection limit of IEMA and that of the enzyme measurement was investigated for IEMA systems of interferon-γ (IFN-γ, molecular weight: 17,000) and the effect of NSB was also considered. Alkaline phosphatase (AP) was used as the enzyme with three substrates for colorimetric, fluorometric, and chemiluminometric detection. The substrate chosen for colorimetry was p-nitrophenylphosphate (PNPP) and for fluorometry, 4-methylumbelliferyl phosphate (4MUP). For chemiluminometry, the substrate selected was 3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)phenyl-1,2-dioxetane (AMPPD) because it has been used for immunoassays and DNA probe assays (5, 6, 11, 12). In all these IEMA systems, the antibody (Ab) was labelled with biotin, and avidin-biotin AP complex (ABC enzyme) reagent was used with the biotin label (13).

The findings on the sensitivity of IEMA using ABC enzyme reagent and various substrates may also apply to immunoblotting and immunocytochemistry

studies, where problems caused by high non-specific binding (background) are predominant.

MATERIALS AND METHODS

Reagents

Recombinant IFN-γ was obtained from Shionogi (Osaka, Japan). Rabbit antiserum against IFN-γ, K36, was prepared by the usual method (14). Anti-IFN-γ monoclonal Ab, M30, was a gift from Dr. M. Ide of Shionogi Research Laboratories. All chemicals were of analytical grade, unless otherwise specified.

A 0.1 mol/l phosphate buffer, pH 7.4, containing 0.05% (w/v) sodium azide and 0.05 mol/l sodium chloride was used as a coating buffer. The coating buffer containing 0.5% (w/v) casein (according to Hammarsten, Merck, Darmstadt, Germany) was used as an assay buffer.

Ab-Immobilized Balls

Polystyrene balls (1/8 inch in diameter, Wako, Osaka, Japan) were immersed in the coating buffer solution of the Ab, IgG fraction of K36, (10 mg/ml) and left standing for 16 h at 15-25°C. The balls were then washed twice with the assay buffer and immersed again in the assay buffer for 2 h at 15-25°C. The Ab-immobilized balls were kept in the assay buffer at 4°C until use.

Biotin-Labelled Ab

Mouse Ab (IgG fraction of M30, K_d=2.1x10^{-9} mol/l), 2 mg (13 nmol) in 36 µl, and 64 µl of 50 mmol/l carbonate buffer, pH 8.5, were added to a small reaction tube which contained ca 80 µg (140 nmol) of sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-biotin, Pierce, Rockford, IL, U.S.A.) and mixed well. The mixture was left standing for 2 h at 4°C and then centrifuged to remove the

precipitate after addition of 900 μl of the coating buffer. Biotin-labelled Ab in the supernatant was further purified by ultrafiltration (Centricon-30, Amicon, Beverly, MA, U.S.A.). The final yield was 13 nmoles of the biotin-labelled Ab in 1 ml of the coating buffer. The binding activity of the biotin-labelled Ab was checked by surface plasmon resonance of the biospecific interaction analysis (BIA, Pharmacia, Uppsala, Sweden) (15, 16).

Immunoenzymometric Assay

A series of standard solutions, 50 μl, were added to each well of the microtiter plates (flexible polyvinyl chloride plate, conical "V" wells, Dynatech Lab., Chantilly, VA, U.S.A.) which contained an Ab-immobilized ball that had been washed with the assay buffer once. The plate was incubated for 5 h at 15-25°C and then, after three washings with 100 μl of the assay buffer, 50 μl of the biotin-labelled Ab (diluted with the assay buffer) was allowed to react with the balls for 16 h at 15-25°C. ABC enzyme reagent (prepared according to the protocol of the ABC-AP kit from Pierce, Rockford, IL, U.S.A. and diluted with the assay buffer), 50 μl, was added to the ball in the well after three washings and the mixture was incubated for 3 h at 15-25°C. The balls were washed 3 times with the assay buffer and then twice with saline. Enzyme activities on the balls which were transferred into polystyrene tubes were measured by the methods described in the following sections.

Colorimetric Determination for AP Activity

PNPP solution, 0.01 mol/l, was prepared with PNPP tablet (Boehringer Manheim GmbH, Mannheim, Germany) and AP assay buffer (Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturer's directions. The PNPP solution, 250 μl, was added to the ball of the IEMA (or

standard solution of AP, 10 μl), and the mixture was allowed to react for 60 min at 20°C. Next, 1 mol/l solution of sodium hydroxide, 3 ml, was added to stop the reaction and the absorbance at 405 nm was measured with a spectrophotometer (UV-160, Shimadzu, Kyoto, Japan).

Fluorometric Determination for AP Activity

4MUP (Sigma, St. Louis, MO, U.S.A.) solution (0.3 mmol/l, prepared with the AP assay buffer) (14), 250 μl, was added to the ball of the IEMA (or standard solution of AP, 10 μl) and the mixture was allowed to react for 60 min at 20°C. Next, 0.5 mol/l solution of phosphate-potassium hydroxide buffer, pH 10.4, containing 0.01 mol/l ethylenediaminetetraacetic acid, 3 ml, was added to stop the reaction, and the fluorescence intensity at 446 nm with excitation at 356 nm was measured with a fluorescence spectrophotometer (MPF-4, Hitachi, Tokyo, Japan).

Chemiluminometric Determination of AP Activity

AMPPD (Tropix, Bedford, MA, U.S.A.) solution, 0.4 mmol/l, was prepared with the AP assay buffer containing 10% (v/v) of chemiluminescence enhance reagent, Emerald (Tropix, Bedford, MA, U.S.A.), according to the manufacturer's directions. The AMPPD solution, 200 μl, was added to the ball of the IEMA (or standard solution of AP, 10 μl) and allowed to react with it for 40 min at 20°C. The chemiluminescence intensity was measured with a chemiluminescence reader (BLR-201, Aloka, Tokyo, Japan).

RESULTS

Determination of AP by Various Substrates

Calibration curves of AP measurement by three substrates, PNPP, 4MUP, and AMPPD, displayed good linearity (data not shown), and their detection limits ($p <$

TABLE 1

Comparison with Detection Limit of Enzyme, Optimum Amount of Reagents, NSB Level, and Detection Limit of IEMA.

Substrate for AP	PNPP	4MUP	AMPPD
Detection Limit for AP Assay[a]			
AP, fmole per assay[b]	0.11[c]	0.0027[c]	0.00024[d]
Optimum Amount of Reagents			
Labelled Ab, fmol per well (nmol/l)	6,600(132)	2,200(44)	2,200(44)
ABC reagent, fmol per well (nmol/l)[b]	600(12)	20(0.4)	6(0.12)
NSB of AP Activity			
AP, fmol per well (%CV)[b]	0.2 (7.2%)	0.08 (34%)	0.02 (11%)
Detection Limit for IEMA[a]			
IFN-g ,fmol per well	0.6	0.2	0.08

[a]Significantly different from blank or NSB ($p < 0.05$). [b]For calculation of the molar amount from enzyme activity, the molecular weight of AP was taken as 100,000 and the specific activity as 2,500 U/mg. Enzyme activity was calibrated from color development of PNPP (ratio method). [c]60-min incubation. [d]40-min incubation.

0.05) are shown in Table 1. The detection limit of AP by the 4MUP method was good but contamination by 4-methylumbelliferone in one batch of 4MUP reagent greatly affected its detection limit. The detection limit of the enzyme by the chemiluminometric assay with AMPPD was extremely low but depended very much on the performance of luminescence readers.

Detection of Ag-Ab and Biotin-Avidin Binding by BIA

Increases in the base lines of the BIA sensorgram of Fig. 1 after injection of rabbit Ab, IFN-γ, and mouse Ab show that these proteins were bound sequentially

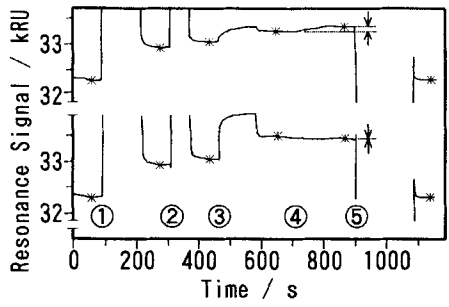

FIGURE 1. BIA sensorgram of antigen-antibody and avidin-biotin binding. Anti-rabbit IgG immobilized on the surface of the sensor was used according to the manufacturer's instructions. ①: Injection of rabbit anti-IFN-γ Ab (K36), 43 pmol in 10 μl. ②: Injection of IFN-γ, 53 pmol in 5 μl. ③: Injection of biotin-labelled mouse anti-IFN-γ Ab (M30), 6.7 pmol in 10 μl (upper chart) or unlabelled mouse anti-IFN-γ Ab (M30), 18 pmol in 10 μl (lower chart). ④: Injection of ABC enzyme reagent, 1.2 pmol in 10 μl of AP. ⑤: Injection of 0.1 mol/l hydrochloric acid, 15 μl.

to the anti-rabbit IgG immobilized on the sensor. The sensorgram after injection of ABC enzyme reagent show that ABC enzyme did bind to the biotin-labelled Ab but not to the unlabelled Ab, although the distance between the sensor surface and the bound ABC enzyme molecule was so long that the response was not very prominent. The binding activity of the biotin-labelled Ab was confirmed by the real time sensorgram of the BIA, which is very useful for developing IEMA systems without the need to any label for the sensor.

Optimum Amounts of Reagents

The ratios of the IEMA response at 2.9 fmol (50 pg) of IFN-γ to NSB were observed with various amounts of the biotin-labelled Ab and ABC enzyme reagent, where NSB was the IEMA response at 0 fmol of IFN-γ. The optimum amounts of the reagents which gave maximum ratio were estimated as shown in the upper

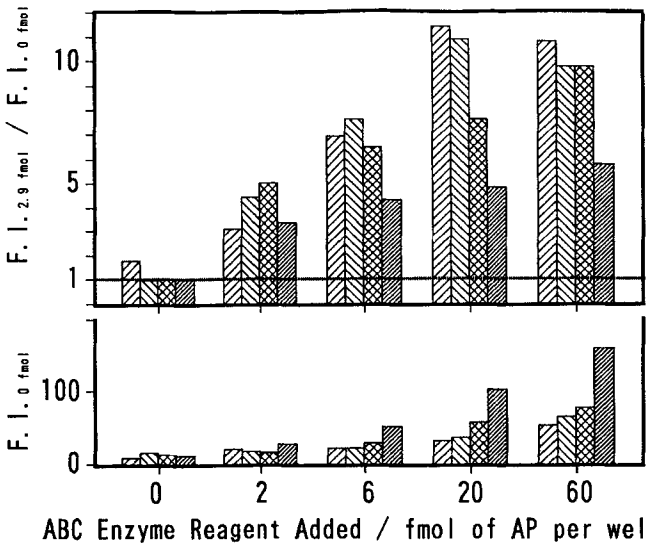

FIGURE 2. Estimation of optimum amount of reagents for the 4MUP system. Fluorescence intensities (F.I.) on 0 fmol of IFN-γ (NSB) with various amounts of ABC enzyme reagent (lower panel) and the ratio of F.I. of 2.9 fmol (50 pg) of IFN-γ to the F.I. of NSB (upper panel). Added amount of biotin-labelled Ab: 660 (▨), 2,200 (▧), 6,600 (▩), and 22,000 (▨) fmol per well.

panel of Fig. 2. The optimum amounts of the biotin-labelled Ab and ABC enzyme reagent for 4MUP were 2,200 fmol per well and 20 fmol per well, respectively. The lower panel of Fig. 2 shows the NSB levels and suggests that the maximum ratio was caused by a steeper increase of the NSB at high concentration of the reagents, compared to the IEMA response in the presence of IFN-γ. The optimum amounts for PNPP and AMPPD in Table 1 were also obtained from similar experiments (data not shown).

Estimation of NSB

Changes of the NSB level which greatly affected the ratios were investigated at various amounts of the biotin-labelled Ab and ABC enzyme reagent. Almost

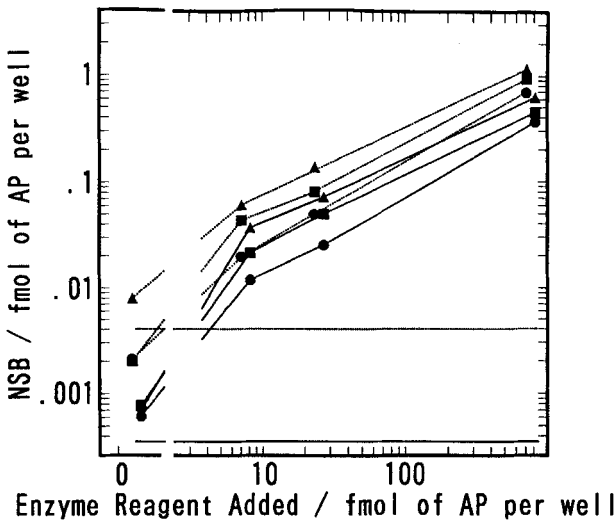

FIGURE 3. Effect of amounts of the biotin-labelled Ab and ABC enzyme reagent on NSB.
Added amount of biotin-labelled Ab: 0 (●), 2,200 (■), 6,600 (▲) fmol per well (n = 6). Detection system: 4MUP(....), and AMPPD(----). Detection limits of each detection system are shown as horizontal lines. Points under the detection limit were plotted as half of the detection limit.

NSBs for the colorimetric assay were below the detection limit of the enzyme. For fluorometric and chemiluminometric assays, NSB increased as the ABC enzyme reagent increased as shown in Fig. 3.

Enzyme activity of NSB was calculated from calibration curves using aqueous standard solutions of AP, and not by immobilized AP on the ball. Enzyme activities on the balls of the identical NSB samples were not equal among the substrates. For example, the NSB value of 0.19 fmol/well with PNPP at 6,600 fmoles of the biotin-labelled Ab and 600 fmoles of ABC enzyme reagent was much lower than the corresponding NSB values with other substrates. Significant chemiluminescence of AMPPD was observed without any IEMA reagents except the polystyrene ball.

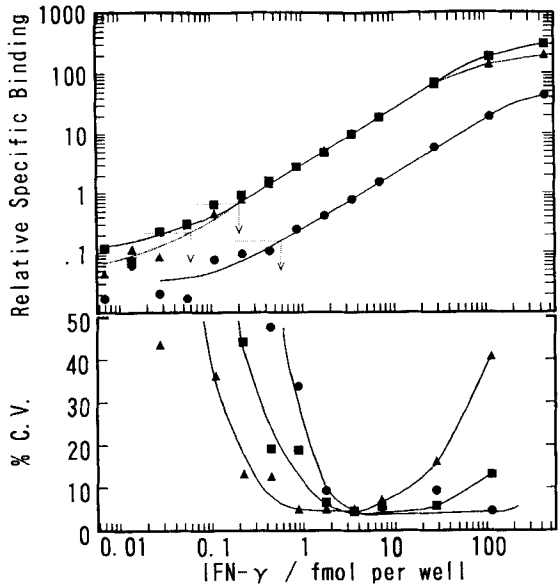

FIGURE 4. Standard curves of IEMA using various detection systems and their precision profiles.
Detection system: PNPP (●), 4MUP (■), and AMPPD (▲). Ordinate of standard curve (upper panel) represents a relative specific binding, (B - NSB)/ NSB, where B and NSB are the binding at each standard solution and at zero concentration, respectively. NSB levels were 0.0486 ± 0.0035 (CV: 7.2%) of absorbance by PNPP, 14.9 ± 5.0 (CV: 33.6%) of fluorescence intensity by 4MUP, and 414 ± 47 (CV: 11.4%) counts of chemiluminescence in 6 s by AMPPD (n = 12). Broken lines indicate 2CV levels from respective NSB.
Each %CV of the precision profile (lower panel) was obtained from 6 observations.

Standard Curve of IEMA

The upper panel of Fig. 4 shows standard curves of the IEMAs using the three substrates with the optimum amounts of the reagents. Detection limits of these IEMAs are shown in Table 1. The slope of the IEMA standard curve was similar for all substrates but displaced one order of magnitude to the left for PNPP. The lower panel of Fig. 4 shows the precision profiles of these standard curves.

DISCUSSION

We explored the effect of the detection limit of enzyme measurement, an optimum amounts of the biotin-labelled Ab and ABC enzyme reagent, the NSB level, and the detection limit of IEMA as shown in Table 1. Some of the detection limits of AP did not agree well with values reported in the literature (17) but these detection limits were at commonly acceptable levels for routine IEMAs. Optimum amounts of the biotin-labelled Ab and ABC enzyme reagent decreased with the decreasing detection limit of enzyme measurement but not proportionally. In particular, the biotin-labelled Ab did not decrease much. The results suggest that the minimum concentration of the biotin-labelled Ab should be more than 20 times its K_d value, 2.1×10^{-9} mol/l. Under these conditions, the biotin-labelled Ab theoretically binds more than 95% of the immobilized analyte.

The optimum amount of ABC enzyme reagent decreased much more than that of the biotin-labelled Ab, as shown in Table 1. The ratio of the IEMA response could not be improved below a certain amount of ABC enzyme reagent that was approximately 250 times the NSB. An NSB of less than 0.4% of total enzyme activity may be a minimum for a practical assay.

Fig. 3 indicates that it is not easy to reduce the NSB level by varying the amounts of the reagents. The differences among observed AP activities of the identical NSB samples by three methods are possibly due to differences in the accessibility of the three substrates to the enzyme caught on the solid phase. In addition, AMPPD may be slightly excited by the presence of the polystyrene ball.

Comparing the IEMA standard curves in the upper panel of Fig. 4 using the three substrates with the optimum amounts of the reagents, the curves on the 4MUP and AMPPD systems have almost the same profile, when expressed as the ratio to NSB.

Detection limits of these IEMA systems in Fig. 1 decreased as those of enzyme measurement decreased. The detection limit with AMPPD was lower than that with 4MUP in spite of the identical standard curve profile because of the improved precision of NSB on enzyme measurement. It is difficult to improve the detection limit of an IEMA by more than 10 times only using a more sensitive method for enzyme measurement because of an insufficient decrease of the NSB level.

Recently, some ultra-sensitive immunometric assays have been reported, such as the immune complex transfer immunoassay method with a very low NSB (18) or the immuno-polymerase chain reaction method using an extremely specific label (19). For smallest detection limits, immunometric assays should use a very small amount of labelled Ab with high affinity or react in a very small volume. The findings described here should be applicable also to immunoblotting, immunostaining and immunocytochemistry.

REFERENCES

1. Shalev, A., Greenberg, A. H., and McAlpine, P. J. Detection of attograms of antigen by a high sensitivity enzyme-linked immunoabsorbent assay (HS-ELISA) using a fluorogenic substrate. J. Immunol. Methods 1980; 38: 125-39.

2. Ali, A. and Ali, R. Enzyme-linked immunosorbent assay for anti-DNA antibodies using fluorogenic and colorigenic substrates. J. Immunol. Methods 1983; 56: 341-46.

3. Schmeer, N., Mueller, H-P., Baumgaertner, W., Wieda, J., and Krauss, H. Enzyme-linked immunosorbent fluorescence assay and high-pressure liquid chromatography for analysis of humoral immune responses to Coxiella-burnetii proteins. J. Clin. Microbiol. 1988; 26: 2520-25.

4. Bronstein, I., Voyta, J. C., Thorpe, G. H. G., Kricka, L. J., and Armstrong, G. Chemiluminescent assay of alkaline phosphatase applied in an ultrasensitive enzyme immunoassay of thyrotropin. Clin. Chem. 1989; 35: 1441-46.

5. Thorpe, G. H. G., Bronstein, I., Kricka, L. J., Edwards, B., and Voyta, J. C. Chemiluminescent enzyme immunoassay of alpha-fetoprotein based on an adamantyl dioxetane phenyl phosphate substrate. Clin. Chem. 1989; 35: 2319-21.

6. Beck, S. and Koester, H. Applications of dioxetane chemiluminescent probes to molecular biology. Anal. Chem. 1990; 62: 2258-70.

7. Torrance, L. and Jones, R. A. C. Increased sensitivity of detection of plant viruses obtained by using a fluorogenic substrate in enzyme-linked immunosorbent assay. Ann. Appl. Biol. 1982; 101: 501-09.

8. Yolken, R. H. and Leister, F. Comparison of fluorescent and colorigenic substrates for enzyme immunoassays. J. Clin. Microbiol. 1982; 15: 757-60.

9. Shekarchi, I. C., Sever, J. L., Nerurker, L., and Fuccillo, D. Comparison of enzyme-linked immunosorbent assay with enzyme-linked fluorescence assay with automated readers for detection of rubella virus antibody and herpes simplex virus. J. Clin. Microbiol. 1985; 21: 92-96.

10. Vilja, P. Flexibility of noncompetitive avidin-biotin immunoassay: Immunoassay of lutropin applied to different signal detection systems. Clin. Chem. 1990; 36: 1897-1901.

11. Bronstein, I., Edwards, B., and Voyta, J. C. 1,2-Dioxetanes: Novel chemiluminescent enzyme substrates. Applications to immunoassays J. Biolumin. and Chemilumin. 1989; 4: 99-111.

12. Schaap, A. P., Akhaven, H., and Romano, L. J. Chemiluminescent substrates for alkaline phosphatase: Application to ultrasensitive enzyme-linked immunoassays and DNA probes. Clin. Chem. 1989; 35: 1863-64.

13. Diamandis, E. P., and Christopoulos, T. K. The biotin-(strept) avidin system: Principles and applications in biotechnology. Clin. Chem. 1991; 37: 625-36.

14. Kominami, G. Practice of immunoassays. In: Tanaka, H. and Yokoyama, A., eds. Pharmaceutical Research and Development Vol. 10. Tokyo: Hirokawa, 1990: 92-146. [in Japanese]

15. Sjölander, S. and Urbaniczky, C. Integrated fluid handling system for biomolecular interaction analysis. Anal. Chem. 1991; 63: 2338-45.

16. Johnsson, B., Löfas, S., and Lindquist, G. Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance. Anal. Biochem. 1991; 198: 268-77.

17. Kricka, L. Chemiluminescent techniques. Clin. Chem. 1991; 37: 1472-81.

18. Ishikawa, E., Hashida, S., Tanaka, K., and Kohno, T. Development and applications of ultrasensitive enzyme immunoassays for antigens and antibodies. Clin. Chim. Acta 1989; 185: 223-30.

19. Sano, T., Smith, C. L., and Cantor, C. R. Immuno-PCR: Very sensitive antigen detection by means of specific antibody-DNA conjugates. Science 1992; 258: 120-22.