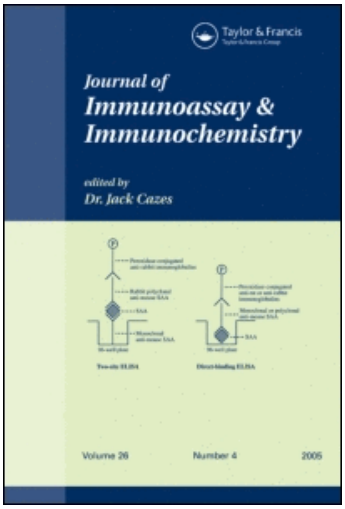

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Use of Progesterone-3(O-Carboxymethyl Oxime)-Horseradish Peroxidase in a Sensitive Microtitre-Plate EIA and Its Application to a Visual Membrane EIA of Progesterone

D. Y. Yoon[a]; E. Y. Song[a]; D. H. Kwon[a]; M. J. Choi[b]; S. M. Byun[c]; I. S. Choe[a]; T. W. Chung[a]
[a] Genetic Engineering Research Institute, Korea Institute of Science and Technology (KIST)., Taejon, Korea [b] Doping Control Center, Korea Institute of Science and Technology (KIST)., Seoul, Korea [c] Dept. of Life Science, Korea Advanced Institute of Science and Technology (KAIST)., Taejon, Korea

To cite this Article Yoon, D. Y. , Song, E. Y. , Kwon, D. H. , Choi, M. J. , Byun, S. M. , Choe, I. S. and Chung, T. W.(1995) 'Use of Progesterone-3(O-Carboxymethyl Oxime)-Horseradish Peroxidase in a Sensitive Microtitre-Plate EIA and Its Application to a Visual Membrane EIA of Progesterone', Journal of Immunoassay and Immunochemistry, 16: 2, 137 — 153

To link to this Article: DOI: 10.1080/15321819508013554

URL: http://dx.doi.org/10.1080/15321819508013554

JOURNAL OF IMMUNOASSAY, 16(2), 137-153 (1995)

USE OF PROGESTERONE-3(O-CARBOXYMETHYL OXIME)-HORSERADISH PEROXIDASE IN A SENSITIVE MICROTITRE-PLATE EIA AND ITS APPLICATION TO A VISUAL MEMBRANE EIA OF PROGESTERONE

D.Y. Yoon, E.Y. Song, D.H. Kwon, M.J. Choi[1], S.M. Byun[2], I.S. Choe, and T.W. Chung

Genetic Engineering Research Institute, Korea Institute of Science and Technology (KIST). P. O. Box Yuseong 115, Taejon 305-600, Korea
Doping Control Center[1], Korea Institute of Science and Technology (KIST). P.O.Box 131 Cheongryang, Seoul 130-650, Korea
Dept. of Life Science[2], Korea Advanced Institute of Science and Technology (KAIST). Kuseong Dong 373-1, Yuseong Gu, Taejon 305-701, Korea

ABSTRACT

A simple method of visual membrane enzyme-immunoassay (EIA) for the detection of progesterone is described. When two types of progesterone-horseradish peroxidase (HRP) tracers were challenged for binding, in the presence of progesterone, to the monoclonal anti-progesterone antibody, 15A, coated on the microtitre plate, the HRP conjugated at the C-3 position (A-ring) of progesterone competed more effectively with progesterone to the binding site of the monoclonal antibody (mAb) than HRP conjugated at the C-11 position of the C-ring. By using this combination of mAb, 15A, and progesterone-3(O-carboxymethyloxime)-HRP (P-3CMO-HRP), we developed a visual membrane EIA system in which free progesterone in the sample could be quantified by the degree of color development. In this system, free

progesterone competed with P-3CMO-HRP for binding sites of mAb immobilized on the nitrocellulose membrane. The stable grey color was formed on the surface of membrane for progesterone-negative and no color for progesterone-positive sample using 3,3'-diaminobenzidine (DAB) with Co^{2+} as an insoluble substrate solution. To examine whether tetramethylbenzidine (TMB) can substitute for DAB in membrane EIA, an experiment was conducted where TMB was used as an insoluble substrate.

KEY WORDS: progesterone; visual membrane immunoassay

INTRODUCTION

The female ovulatory cycle is characterized by a remarkable sequence of events, which ultimately culminates in the ovulation of a mature egg for fertilization. Brann concluded his recent review (1) by summarizing three important effects of progesterone on proestrus as follows: (a) initiation and potentiation of the surge of gonadotropins, (b) facilitation of the estrous follicle-stimulating hormone (FSH) surge, and (c) limitation of the LH surge to a single day. In addition, progesterone has been known to play a significant role in the facilitation of implantation and in the maintenance of pregnancy (2). This critical influence of progesterone is most likely due to the well-known ability of progesterone to inhibit uterine contractility and to suppress the immune response to the fetus (3-4). During pregnancy as well as the luteal phase of the menstrual cycle after ovulation, progesterone is essential for reproductive function. Therefore, progesterone can be utilized as a marker for the diagnosis of early pregnancy and antibodies which bind specifically progesterone are essential in developing immunoassay method to measure the progesterone level. There is

a trend that up-to-date techniques of immunochemical analysis oriented to a sensitive *on-site* detection method. *On-site* screening tests require a simple and rapid, instrument-independent procedure. These "express" tests detect drug levels qualitatively, semi-quantitatively, and utilize reagents immobilized on the membranes of porous carriers (5-7). Also they are usually ready-to-use analytical devices containing all the necessary reagents. The tests allow analyte determination by comparing the color intensity of the active zone with a reference color detected either visually or by using a reflectance spectrometer. Even though the homogeneous immunoassay (8-9) is more convenient than solid-phase immunoassay (10), it is not adequate as a field test because it needs an expensive and complicated instrument. The purpose of our visual membrane EIA for progesterone detection is to overcome such limitations and provide *on-site* screening. This method needs no instrument and the resulting color differences between positive and negative samples can be recognized with bare eyes.

MATERIALS AND METHODS

Reagents

Bovine serum albumin, horseradish peroxidase (EC 1.11.1.7), progesterone, dimethyl sulfoxide (DMSO), incomplete Freund's adjuvant, *o*-phenylenediamine (OPD), 3,3'-diaminobenzidine tetrahydrochloride (DAB), tetramethylbenzidine (TMB), 8-anilino-1-naphthalene sulfonic acid (ANS), 11-hydroxyprogesterone, and other steroid hormones were purchased form Sigma Chemical Co. (St.Louis, U.S.A). Progesterone-[^{125}I] radioimmunoassay kit was purchased from Diagnostic Products Co. (Los Angeles, U.S.A.). Fetal bovine serum (FBS) was from Gibco BRL

(Gaithersberg, U.S.A.), glucose oxidase from Toyobo Co. (Osaka, Japan), and polystyrene microtitre plates (high affinity binding capacity) from Nunc (Kamstrup, Denmark). The membrane matrixes used were Nitrocell, pore size 0.45 μm (Hoefer Scientific Instruments, Minnesota, U.S.A.); UltraBind US-450, pore size 0.45 μm (Gelman Science Inc., Ann Arbor, U.S.A.); pure nitrocellulose membrane AE 99, pore size 5.0 μm , and AE 100, pore size 12.0 μm (Schleicher & Schuell, Inc., Keene, U.S.A.); Nylaflo nylon membrane, pore size 0.45 μm (Gelman Science Inc., Ann Arbor, U.S.A.); and Nylon 66 plus (Hoefer Scientific Instruments, Minnesota, U.S.A.).

Buffers and Solutions

Phosphate buffered saline (PBS) contained 0.14M NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM $Na_2HPO_4 \cdot 12H_2O$, and 0.02% thimerosal, pH 7.4. PBST is PBS containing 0.05% Tween-20. Coating buffer is 50mM carbonate buffer, pH 9.6. The *o*-phenylenediamine (OPD) substrate solution for peroxidase determination contained 5.1mM H_2O_2 and 5.5mM OPD. This substrate was dissolved in 53mM sodium citrate buffer containing 10mM $Na_2HPO_4 \cdot 12H_2O$, pH 5.3. Sodium phosphate buffer (SPB), pH 7.4, consisted of 11.5g $Na_2HPO_4 \cdot 12H_2O$, 1.17g $NaH_2PO_4 \cdot 2H_2O$, 1g thimerosal, and 1g BSA per one liter of distilled water.

Antibody

The monoclonal antibody (mAb) clone, 15A (IgG_1, K_a = 2.3 X 10^8 M^{-1} for progesterone, binding capacity = 11.2 pmole progesterone/mg IgG) was prepared with a slight modification of the method of Kohler and Milstein (11) and characterized in our laboratory as described

previously (12). To collect ascitic fluids containing mAbs, BALB/c mice were preprimed by incomplete Freund's adjuvant and then intraperitoneally injected with hybridoma cells. Immunoglobulins were precipitated by adjusting the ammonium sulfate concentration to 40% saturation and then dialyzed against PBS containing 0.02% thimerosal at 4°C. Further purification was performed using protein A-Sepharose 4B affinity chromatography utilizing their subtype characteristics as previously described (13). The purity of the isolated immunoglobulins was measured using SDS-12% PAGE (14) and the amount of protein in ascitic fluids was determined by Bradford method (15). The antibody specificities and affinity constants (K_a) were determined by the method of Scatchard (16) as modified (12).

Tracers

(1) P-3CMO-HRP Tracer: Progesterone-3(O-carboxymethyl oxime)-HRP was prepared according to the N-hydroxysuccinimide ester method (17-18) with slight modifications (19). The RZ (Reinheitzahl), the absorbance ratio of A_{403}/A_{280}, was used as an indication of the uniformity of HRP tracer (20). The RZ of fractionated P-3CMO-HRP (0.84 mg/ml) was 1.12.

(2) Progesterone-11HS-HRP (P-11HS-HRP) Tracer: This tracer was prepared with a similar technique as described in preparation of P-3CMO -HRP tracer. The RZ of the fractionated P-11HS-HRP (1.0 mg/ml) was 2.86.

Progesterone Standards

Stock solution of progesterone (1 $\mu g/ml$) was stored in ethanol at

4°C. Standard solutions for the assays were freshly prepared by diluting this stock solution. Standard tubes containing various amount of progesterone were dried *in-vacuo* at 40°C. These *in-vacuo* dried progesterone standards were redissolved in 1ml of each SPB, or serum matrix and followed by its calibration using progesterone-[^{125}I] RIA Kit. Serum matrix was prepared in FBS treated with ANS (0.5 mg/ml) for 15 min at room temperature and dialyzed against PBS. ANS-treated serum showed no detectable progesterone and was used as a negative control. As standards, seven serial amounts of progesterone (0.1, 0.5, 2, 10, 20, 40, and 100 ng/ml) were used in the assay for the determination of progesterone content in the samples.

Enzyme Immunoassay Procedure

Fifty μl aliquot of sample or standard was mixed with 50 μl aliquot of the progesterone-HRP tracer in microtitre plate wells which were coated with 100 μl of monoclonal anti-progesterone antibody, 15A (10 $\mu g/ml$). The plate was incubated for 15 min at RT followed by three times washing with PBST buffer. One hundred μl aliquot of freshly prepared HRP substrate was incubated for 15 or 20 min at RT and absorbance was measured at 450 nm using a Titertek Multiscan spectrophotometer (EL Lab, Helsinki, Finland).

Visual Membrane Assay Procedure

The nitrocellulose membrane was placed into the solution of monoclonal anti-progesterone antibody, 15A (0.01 mg/ml), overnight at 4°C and washed with PBS. After the nonspecific binding was blocked by soaking the membrane into PBS containing 3% BSA, membrane

coated by mAb was washed and cut into small pieces (larger than 0.5 X 0.5 cm). The pieces were placed into the 24-well microtitre plate which contains mixture of antigen analyzed (0 - 100 ng/ml) and P-3CMO-HRP (0.5 - 1.5 $\mu g/ml$) in PBST. After 15 min incubation at RT, the membrane was washed 3 times with PBST, followed by soaking into the DAB substrate solution (5 mg of DAB, 0.02% Co^{2+}, 0.02 % Ni^{2+}, and 0.03% H_2O_2 per 10 ml of PBS). The membrane was washed 3 times with distilled water after 2-5 min. Then the color intensity of the membrane surface was measured using a reflectance spectro-colorimeter at 560 nm (Hunter Associates Laboratory, Inc., U.S.A.).

Tetramethylbenzidine (TMB) As An Alternative Substrate in Membrane Test

TMB was dissolved in DMSO to a final concentration of 42mM (10 mg/ml). Ten ml of TMB/DMSO was added drop-wise with mixing to 1 liter of 50 mM phosphate-citric acid buffer (PCB), pH 6.0. Peroxide and metal ion such as Co^{2+} or Ni^{2+} were added just prior to use. UltraBind US-450 nitrocellulose membrane was directly soaked in TMB solution for 10 min and then dried 30 min in air.

Pinch-test Membrane EIA of Progesterone

TMB was dissolved in acetone and diluted with 50 mM PCB buffer to a final concentration of 0.1 mg/ml. Nylon 66 plus was wetted in the TMB/ acetone solution and dried in air. The dried membrane was soaked in the solution of glucose oxidase (1,180 IU/ml PCB) and dried again in air. UltraBind US-450 nitrocellulose paper immobilized with mAb, 15A (10 $\mu g/ml$ PCB) was used in the visual membrane enzyme immuno

test. All steps of the immunoassay procedure can be made on the separate "immuno"-membrane. These two membranes were wetted in PCB buffer containing glucose (10 mg/ml) and then pinched to each other for 2-5 min. The nylon-66 plus membrane would change its color. The intensity of membrane was measured using a reflectance spectro-colorimeter at 560 nm.

RESULTS

Choice of Tracer

Effect of site-specific tracers on the performance of EIA was assessed. In this assay system, the two enzyme-labeled tracers (P-3CMO-HRP and P-11HS-HRP) were respectively added into microtitre plate wells coated with monoclonal anti-progesterone antibody, 15A, in order to compete with free progesterone to the binding sites of mAb. P-3CMO-HRP tracer gave a more sensitive standard curve for progesterone than P-11HS-HRP tracer (Fig. 1). The P-3CMO-HRP tracer was used because of the specificity of 15A, both in the performance of microtitre enzymeimmunoassay of progesterone (19) and in this visual membrane EIA of progesterone. Within-assay coefficient of variation and the detection limit of progesterone were 7-8% (n = 13) and 0.43 ng/ml, respectively (19).

Choice of Matrix

In order to select a type of matrix suitable for the visual membrane EIA of progesterone, six types of membranes were chosen for examination (Fig. 2). Since all were mechanically stable, the test focused

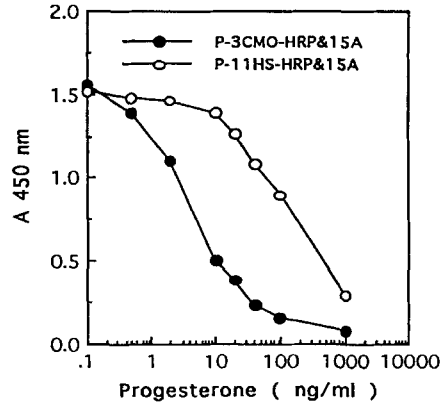

FIGURE 1. Comparison of response for progesterone determination between combination of [15A & P-3CMO-HRP] and [15A & P-11HS-HRP].

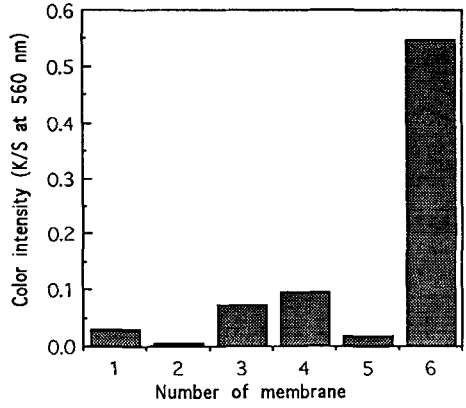

FIGURE 2. Non-specific binding of membranes to insoluble substrate (DAB/Co^{2+}) as measured by color intensity after 5 min of incubation. Types of membranes used in blank test are as follows: 1; Nitrocell (0.45μm), 2; UltraBind US450 (0.45μm), 3; Pure nitrocellulose AE99 (5.0μm), 4; Pure nitrocellulose AE100 (12μm), 5; Nylaflo nylon (0.45μm), 6; Nylon 66 plus (0.45μm).

on the degree of nonspecific interaction with the components of staining solution. Nonspecific binding was determined by the degree of color development upon incubation with insoluble substrate solution (DAB + H_2O_2 + Co^{2+}). For all membranes tested, the color developed within 5 min of incubation with a varying degree of intensity. UltraBind US-450 nitrocellulose paper turned out to be the best in terms of non-specific binding, whereas Nylon 66 plus was found to be the most nonspecific in its binding to insoluble substrates. Therefore, UltraBind paper was chosen to be used as a matrix in later experiments.

Optimal Conditions for Membrane EIA of Progesterone

The concentration of anti-progesterone mAb (15A) was varied from 0.1 $\mu g/ml$ to 100 $\mu g/ml$ in PBS solution and P-3CMO-HRP tracer from 0.1 $\mu g/ml$ to 10 $\mu g/ml$. The optimal concentration of antibody in soaking solution and P-3CMO-HRP tracer in the reaction mixture were determined to be 10 $\mu g/ml$ and 0.5 $\mu g/ml$, respectively. Matrixes were incubated in the mixture of free progesterone and P-3CMO-HRP from 5 to 30 min. The incubation time was optimized at 15 min. The optimal conditions for membrane test for progesterone showed that the standard curve covered the range from 0.1 to 500 ng/ml (Fig. 3).

Tetramethyl Benzidine (TMB) As An Alternative Substrate in Membrane EIA

To examine whether TMB can substitute for DAB in membrane EIA, an experiment was conducted where TMB was used as an insoluble substrate. The progesterone-HRP tracer was reacted with specific mAb (15A) preimmobilized on UltraBind US-450 membrane support. Then this

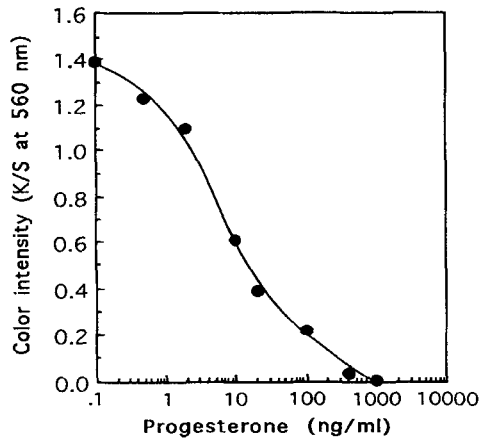

FIGURE 3. Visual membrane EIA of progesterone: The Ultrabind US-450 membrane was treated with an antibody solution, 15A. After blocking with BSA-PBS, 50 μl of progesterone in a concentration range between 0 and 1000 ng/ml, and 50 μl of progesterone-3CMO-HRP tracer were added. The solution was incubated for 15 min at RT. After washing with PBST 3 times, the membrane was incubated with diaminobenzidine/ Co^{2+}/Ni^{2+} substrate for 3 min and washed with water. The color intensity of the membrane was measured at 560 nm using a reflectance spectrometer. Each data point represents the mean from three experiments.

matrix was placed into substrate solution containing TMB, H_2O_2, Co^{2+}, or Ni^{2+}. Incubation time was about 5 min. As a result, deep blue color was obtained. The matrix color intensity for the TMB solution containing Co^{2+} or Ni^{2+} was 3 times more than in case of substrate solution containing only TMB (Table 1). The optimum conditions were found to be TMB (0.1 mg/ml), 0.03% H_2O_2, 0.02% Co^{2+}, or 0.02% Ni^{2+} in 50 mM phosphate-citric acid buffer, pH 6.0. The main disadvantage of

TABLE 1

The Use of TMB for Membrane Staining*.

	Matrix color intensity for different substrates				Ratio of color intensity			
	I	II	III	IV	I	II	III	IV
10 min substrate incubation	0.66	1.97	2.14	1.36	1	3	3.2	2
10 min substrate incubation & dry 30 min in air	0.13	1.31	1.72	1.53	1	9.8	12.9	11.4

I. TMB + H_2O_2
II. TMB + H_2O_2 + Ni^{2+}
III. TMB + H_2O_2 + Co^{2+}
IV. TMB + H_2O_2 + Co^{2+} + Ni^{2+}
*Two independent experiments.

the TMB substrate containing Co^{2+} or Ni^{2+} is that the insoluble blue product of this enzyme reaction can be so weakly absorbed on the membrane that the main part of this dye goes to the solution.

Pinch-Test Membrane EIA of Progesterone

The necessity of substrate (DAB or TMB) solution required in the final immunoassay kit isn't convenient. These solutions are not very stable and it should be made with some special preparation steps. It can be easy to have special membrane containing all (or main part) substances of substrate mixture. All steps of the immunoassay procedure was performed on the "immuno" membrane immobilized with mAb, 15A. When both the "immuno" membrane, and the nylon

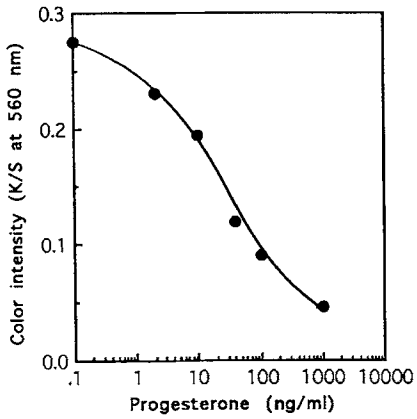

FIGURE 4. Pinch-test membrane EIA of progesterone. Each data point represents the mean from three experiments.

membrane treated with TMB and glucose oxidase, were pinched together in the PCB buffer containing glucose, the nylon membrane changed its color, indicating that progesterone could be semiquantified (Fig. 4). In this case we should need only the glucose solution that is dramatically more stable than H_2O_2 solution.

DISCUSSION

The results of visual enzyme immunoassay largely depend on the choice of matrix as solid support. The matrix should be a neutral polymer with a minimal nonspecific interaction with biomolecules and the components of the staining solution and be able to absorb quantitatively, the final insoluble product of enzyme reaction. Other requirements are mechanical strength and chemical stability during

immobilization and under reaction conditions (5). UltraBind US-450 nitrocellulose paper satisfied the above requirements and was selected for this visual membrane EIA. Although *o*-phenylene diamine (OPD) has been widely used as a hydrogen donor in colorimetric determination of HRP, it was found to be mutagenic (21-22). Benzidine also has long been used as a sensitive and specific reagent for the detection of a peroxidase activity. However, it has been reported that *o*-hydroxylation of aromatic amines is the main cause of carcinogenicity (23). TMB has been offered as an ideal alternative to DAB or OPD for its nonmutagenicity (24) or noncarcinogenicity (25) and its insensitivity to light when prepared in DMSO (26). The developed color is stable for at least 90 min (26). In this work, TMB was used in membrane assay. The final product of enzyme reaction (in the presence of Co^{2+} and Ni^{2+} ions) is intensely blue and stable in air. But it has low affinity to membrane. This disadvantage can be solved by introducing some additional components to the substrate buffer, changing the matrix nature, ionic strength, and pH, etc. TMB can be also used in the "pinch"-test. In this case, all products remain on the membrane. The main problem is uniform color of membranes. We tried to use glucose oxidase for producing H_2O_2 for peroxidase reaction so that it is not necessary to have a solution of this unstable compound in the final immunoassay kit.

ACKNOWLEDGEMENTS

This work was supported from the Ministry of Science and Technology, Korea. We would like to thank Dr. B.M. Gorovits of Institute of Biochemistry, Russian Academy of Sciences for his intensive

discussions and valuable suggestions. Reprints requests should be addressed to: Dr. Do-Young Yoon, Mol. and Cell. Res. Group, Genetic Engineering Research Institute, P.O.Box, Yuseong 115, Taejon 305-600, Korea.

REFERENCES

1. Brann, D.W. Progesterone : The forgotten hormone?. Perspect. Biol. Med. 1993; 36: 642-53.

2. Corner, G.W. The early history of progesterone. Gynec. Invest. 1974; 5: 106-12.

3. Csapo, A.I. and Resch, B.A. Induction of preterm labor in the rat by antiprogesterone. Am. J. Obstet. Gynecol. 1979; 134: 823-27.

4. Siiteri, P.K. and Stites, D.P. Immunologic and endocrine interrelationships in pregnancy. Biol. Reprod. 1982; 26: 1-14.

5. Gorovits, B.M., Osipov,A.P., Doseeva, V.V. and Egorov, A.M. New enzyme immunoassay with visual detection based on membrane photoimmobilized antibodies. Anal. Lett. 1991; 24: 1937-66.

6. Gorovits, B.M., Osipov, A.P. and Egorov, A. M. New immunoassay technique using antibody immobilized on a membrane and a flow cuvette as reaction vessel. J. Immunol. Methods 1993; 157: 11-17.

7. Osikowicz, G., Beggs, M., Brrokhart, P., et al. One-step chromatographic immunoassay for quantitative determination of gonadotropin in urine. Clin. Chem. 1990; 36: 1586-7.

8. Eremin, S.A., Gallacher, G., Lotey, H., Smith, D.S. and Landon, J. Single reagent polarization fluoroimmunoassay of methamphetamine in urine. Clin. Chem. 1987; 33: 1903-6.

9. Colbert, D.L., Eremin, S.A. and Landon, J. The effect of fluorescein labels on the affinity of antisera to small haptens. J. Immunol. Methods 1991; 140: 227-33.

10. Inoue, T., Kanda, Y., Kishi, T., et al. Studies on radioimmunoassay for methamphetamine excreted in human urine. Chem. Pharm. Bull. 1984; 32: 344-7.

11. Kohler, G. and Milstein, C. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. Eur. J. Immunol. 1976; 6: 511-9.

12. Yoon, D.Y., Choi, S.H., Choi, M.J., Choe, I.S. and Chung, T.W. Production, purification and characterization of monoclonal antibodies specific to progesterone. Kor. Biochem. J. 1991; 24: 71-8.

13. Ey, P.L., Prowse, S.J. and Jenkin, C.R. Isolation of pure IgG_1, IgG_{2a}, and IgG_{2b} immunoglobulins from mouse serum using protein A-Sepharose. Immunochemistry 1978; 15: 429-36.

14. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680-5.

15. Bradford, M.M. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976; 72: 248-54.

16. Scatchard, G. The attractions of proteins for small molecules and ions. Ann. N. Y. Acad. Sci. 1949; 51: 660-72.

17. Sauer, M.J., Foulkes, J.A., Worsford, A. and Morris, B.A. Use of progesterone11-glucuronide-alkaline phosphatase conjugate in a sensitive microtitre-plate enzyme immunoassay of progesterone in milk and its application to pregnancy testing in dairy cattle. J. Reprod. Fertil. 1986; 76: 375-91.

18. Hosoda,H., Sakai, Y., Yoshida, H., Miyairi, S., Ishii, K. and Nambara, T. The preparation of steroid N-hydroxysuccinimide esters and their reactivities with bovine serum albumin. Chem. Pharm. Bull. 1979; 27: 742-6.

19. Yoon, D.Y., Choi, M.J., Choe, I.S., Chung, T.W. and Buyn, S.M. Influence of conjugation site on the specificity of monoclonal antibodies specific to progesterone and on the performance of

direct enzyme immunoassay. Biochem. Mol. Biol. International. 1993; 33: 553-63.

20. Shannon, L.M., Kay,E. and Lew, T.Y. Peroxidase isozymes from horseradish roots. J. Biol. Chem. 1966; 241: 2166 -72.

21. Voogd, C.E., van der Stel, J.J. and Jacobs, J.J.J.A.A. On the mutagenic action of some enzyme immunoassay substrates. J. Immunol. Methods 1980; 36: 55-61.

22. Ames, B.N., Kammen, H.O. and Yamasaki, E. Hairdyes are mutagenic Identification of a variety of mutagenic ingredients. Proc. Natl. Acad. Sci. USA 1975; 72: 2423-27.

23. Miller, E.C., Miller, J.A. and Hatmann, H.A. N-hydroxy-2-acetylaminofluorene, a metabolite of 2-acetylaminofluorene with increased carcinogenic activity in the rat. Cancer Res. 1961; 21: 815-24.

24. Garner, R.C., Walpole, A.L. and Rose, F.L. Testing of some benzidine analogues for microsomal activation to bacterial mutagens. Cancer Lett. 1975; 1: 39-42.

25. Holland, V.R., Saunders, B.C., Rose, F.L. and Walpole, A. L. A safer substitute for benzidine in the detection of blood. Tetrahedron 1974; 30: 3299 -302.

26. Bos, E.S., van der Doelen, A.A., van Rooy, N. and Schuurs, A.H.W.M. 3,3',5,5'-Tetramethylbenzidine as an Ames test negative chromogen for horseradish peroxidase in enzymeimmunoassay. J. Immunoassay 1981; 2: 187-204.