

Enzyme-Linked Immunosorbent Assay of Progesterone in Serum Using Penicillinase as Label

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ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) for progesterone measurement in serum or plasma samples using penicillinase as label enzyme is reported. A C₃ and C₁₁ derivatives of progesterone were prepared and conjugated to bovin serum albumin (BSA). Polyclonal antibody against these two immunogens were prepared in New Zealand white rabbits. Purified Ig fractions of antibodies were immobilized onto the wells of microtiter plates. Progesterone 3-(O-carboxymethyl) oxime was linked to penicillinase and used as tracer (enzyme-conjugate). The standard assay completed within three hours had a low limit of detection, from 5 pg/well (50 pg/ml) covering up to 1 ng/well (10 ng/ml). For the first time in this assay the color development in case of penicillinase as enzyme label was measured in the wells directly. Recoveries were measured to be within the range of 95-98%. Percent Coefficient of variations (CV%) obtained between and within runs of several assays were 3% and 7% respectively. When compared between values of progesterone measured by radioimmunoassay (RIA) and present ELISA method, a correlation value of $r = 0.9$ was obtained. Freeze-dried progesterone-enzyme conjugate was found stable for at least three months at 4° C without using any other preservative. *Iran. Biomed. J. 2: 115-122, 1998*

Keywords: Progesterone, ELISA, Penicillinase

INTRODUCTION

Clinically, progesterone measurement is used to confer ovulation in assessing luteal phase function, to check the effectiveness of induction of ovulation [1-3], to assess the normal and ectopic pregnancy [46], and to determine the spontaneous abortion in early pregnancy [7-9]. For determination of progesterone in serum or plasma samples a number of methods have been described. The original progesterone radioassay in plasma was used to determine the affinity of progesterone for corticosteroid binding globulin [10-13]. Abraham *et al* [14] and a number of other workers [15-17] reported a sensitive and specific RIA for progesterone. However, due to many difficulties encountered in working with radioactive materials, alternative labels were used. Dray *et al* [18] introduced the first non-isotopic enzymatic assay for progesterone measurement. Since then a number of progesterone enzyme immunoassays (EIA) using

different enzymes as label, such as alkaline phosphatase [19], horseradish peroxidase [20], and P-galactosidase [21] have been reported. Penicillinase has been adopted as label by number of workers [22 - 27]. Sauer *et al* [28] showed that in addition to other suitability of this label, the detection limits were improved over other enzyme labels. In this paper we reported a sensitive and specific heterologous ELISA for progesterone measurement in serum using penicillinase as marker.

MATERIAL AND METHODS

Penicillinase (-lactamase, type I from bacillus cereus; EC 3.5.2.6) pyrolidin, N-hydroxy succinimide, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide-hydrochloride, bovine serum albumin (BSA), 8-anilino-1-naphthalene sulphonic acid (8-ANS), Sephadex G-25, Dexteran T-70,

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DEAE-cellulose, Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), phenoxy methyl penicillinic acid (penicillin V), 2,5-diphenyl oxazol (PPO), 1,4-bis-2(4-methyl-1,5 phenoxyazolyl) benzene (POPOP), progesterone and all other steroids used in this study were purchased from Sigma Chemical Company, St. Louis MO. USA. [1,2,6,7]-³H progesterone (specific activity, 85 Ci/mmol) was purchased from Amersham international place, Bueks, U. K. Microtiter plates (96 wells) were purchased from Nunc, Denmark. All other reagents and compounds were obtained from E. Merck, Germany.

Production of progesterone antibody. Progesterone-3-(O-carboxymethyl) oxime (P-3-O-CMO) was synthesized following the procedure of Janosky *et al* [29]. The derivative was characterized by TLC and conjugated to BSA according to the procedure of Axen *et al* [30]. New Zealand white male rabbits were immunized using two immunogens (P-3-O-CMO-BSA and progesterone 11 -hemisuccinate-BSA) following the low dose multi-intradermal procedure of Vaitukaitis *et al* [31]. Antibodies obtained in this way were characterized for titer, specificity and affinity by RIA and ELISA. Antibody against progesterone was purified by the method of Levy and Sober [32], freeze-dried and stored at 4 °C.

Preparation of progesterone-penicillinase conjugate. Progesterone-3-(O-carboxymethyl) oxime was conjugated to penicillinase following active ester method of Hosoda *et al* [33] with a minor modification as follow. Progesterone-3-O-CMO (1.5 mg or 4μM) was dissolved in 150 μl of 1,4-dioxane and added to 575 μg (5μM) of N-hydroxy succinimide and 1.148 mg (6μM) of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-hydrochloride. The mixture was stirred at room temperature for 4 h. The clear solvent was removed under stream of N₂ gas till the residue was dried. Penicillinase (250μg) was dissolved in 0.5 ml of 10 mmol /l phosphate buffer saline pH 7.0 (0.895 g/l Na₂ HPO₄.2H₂O and 0.39 g/l NaH₂PO₄.2H₂O containing 154 mmol/l sodium chloride) and added to the above mixture and incubated at 4°C for 2h. The reaction mixture was chromatographed on Sephadex G-25 column (12 mm × 15 mm) using the same buffer for equilibration and elution. Peak fractions containing highest optical density at 280 nm were checked for enzyme activity and were

pooled, added with 10 μg/1 BSA, 1 g/1 NaN₃ and stored at -20°C in 10 μl aliquots.

Radioimmunoassay (RIA). The RIA of progesterone was performed according to the method described by Abraham *et al* [14]. In this procedure plasma progesterone was extracted with diethyl ether.

Checkerboard titration assay in ELISA. Optimal titer of antibody and enzyme conjugate were obtained following a procedure previously explained [26]. In this study a homologous assay using anti-P-3-O-CMO-BSA and P-3-O-CMO- Enzyme and a heterologous combination using anti-P-11 -HS-BSA and P-3-O-CMO-Enzyme were tasted together. Furthermore, slight modification in color development was applied such that color monitoring were carried out in plate itself by adding 150μl of concentrated starch iodine reagent adjusted to optical density of 3.0 nm .

Enzyme-linked immunosorbent assay. Microtiter plates (96 wells) were coated with purified Ig fraction of anti-progesterone antibody (in 10 mmol/1 PB pH 7.2) over night at 37°C. Plates were washed with double distilled water × 4 and the remaining binding sites were blocked by coating each well with 300 μl of 0.3% gelatin in PBS at room temperature for 30 minutes. Wells were washed and could be stored at 4°C in air tight bags for at least 3 months at this condition. Standard doses of progesterone were prepared from an stock ethanolic solution (1 mg/10 ml) in 10 mmol/1 PBS from 100 pg to 4 ng / ml. These were added to the corresponding wells (50 μl / well), leaving at least four wells to stand for total and nonspecific absorption. The nonspecific bindings were assessed using 1:300 dilution of normal rabbit serum. Enzyme-steroid conjugate was diluted in 10 mmol/1 PBS, pH 7.0 containing 0.1% gelatin and 0.1% sodium azide and added to each well (50μl) except the two standing for total absorption. Plates were incubated at 37°C for 2 h. At the end of incubation time plates were washed with double distilled water ×4, tapped, dried and added with 100 μl of substrate solution (2.8 mg of penicillin V in 10 ml of a 0.2 mmol /l PB, pH 7.4) and incubated at 37°C for 1 h. Finally, 150 μl of starch iodine solution of suitable color concentration prepared as reported before [26] was added to each well (150 μl), incubated for 10 minutes and read out at 620 nm in a Multiscan Plus II ELISA reader.

Assay validity:

A) Cross reaction: Structurally related molecules to progesterone were prepared in PBSG (10 mmol, pH 7.4 containing 0.1% gelatin) from 1100 ng in concentration and assayed in parallel with the standard assay for progesterone. Results were calculated according to Abraham et al [14].

B) Recoveries: Known concentration of progesterone was added to stripped pooled plasma as reported before [26]. These were extracted using diethyl ether and reconstituted in PBSG. These samples were extracted six times in this way and assayed each time in 6 replicates.

C) Inter and intra assay variations : Samples of low, medium and high concentration of progesterone content were collected, assayed, pooled and repeatedly assayed in replicates.

D) Correlation of ELISA and RIA : Samples of various phases of mens and pregnant women cycles were collected and assayed by ELISA and RIA and their correlation coefficients were calculated.

RESULTS

Progesterone was successfully derivitized and conjugated to BSA. Number of moles of progesterone conjugated to each molecule of BSA was calculated by spectrophotometric procedure [26] and was 22. Antibodies obtained against two immunogens [namely progesterone-3-O-CMO-BSA and progesterone-11-HS-BSA] were obtained after six months of immunization (Figure 1). These antibodies were checked for affinity and specificity, None of them had any cross - reactivity with other similar steroids such as 11-(OH)-progesterone, 17-(OH)-progesterone, cortisol, cortisone, pregnantriol, 17-(OH)-pregnandiol, testosterone, dehydrotestosterone, 17-esteradiol, aldosterone and 8ANS. The standard assays performed by MA, developed in our laboratory, were sensitive and with this assay we could detect as low as 5 pg/ tube (50 pg/ml) of progesterone in a given sample.

Results of five experiments are shown in Figure 2 (A and B). High titer antibodies were purified and used in ELISA. Progesterone-3-(O-carboxymethyl) oxime was conjugated to penicillinase, purified and used in this procedure. Results of titer assays are shown in Figure 3. When anti-P-11 -HS-BSA and P-3-O-CMO-Enzyme were used the optimum titer was 2.5µg of antibody/well by using 1:100 dilution of enzyme-conjugate (Figure 3A). However when

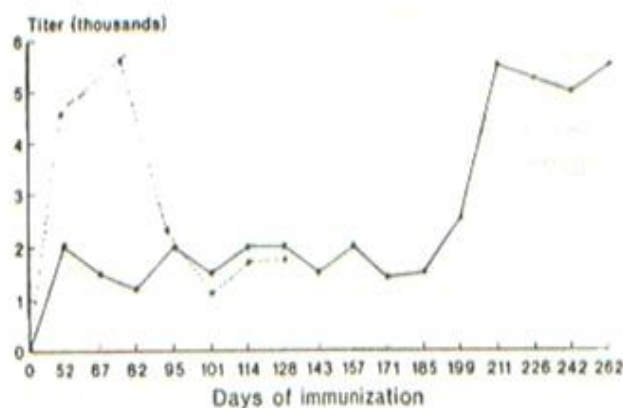


Fig. 1. Effect of duration of immunization an antibody titer anti-P-11 -HS-BSA (••), anti-P-3-O-CMO-BSA (o.....o).

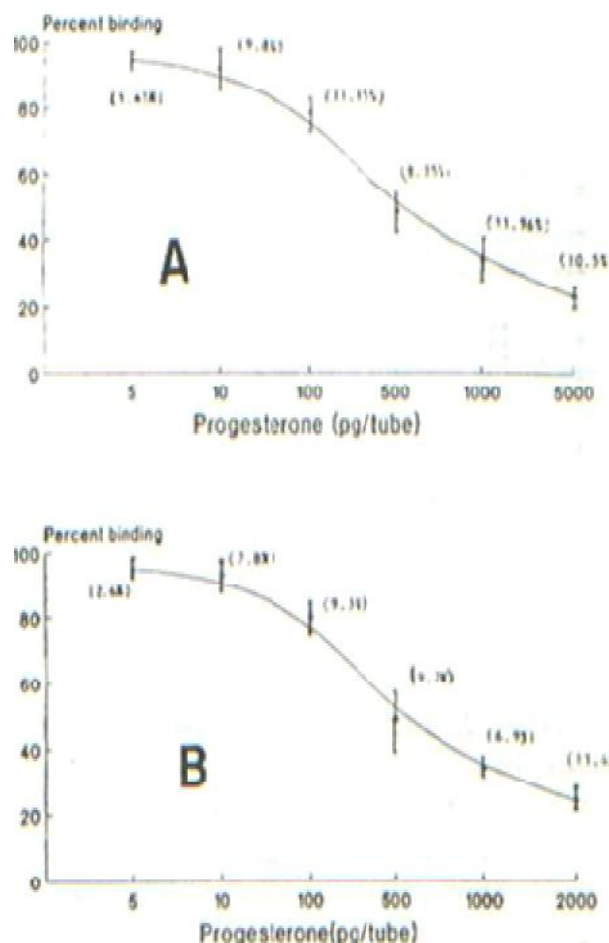


Fig. 2. Standard curve by RIA. (A) anti-P-11 -HSBSA (sensitivity from 5 pg/tube ranging up to 5 pg/tube). (B) anti-P-3-O-CMO-BSA (sensitivity from 5 pg/tube ranging up to 2 ng/tube). CV% (Value in parenthesis), SD (bars), each point on the curve result of five experiments.

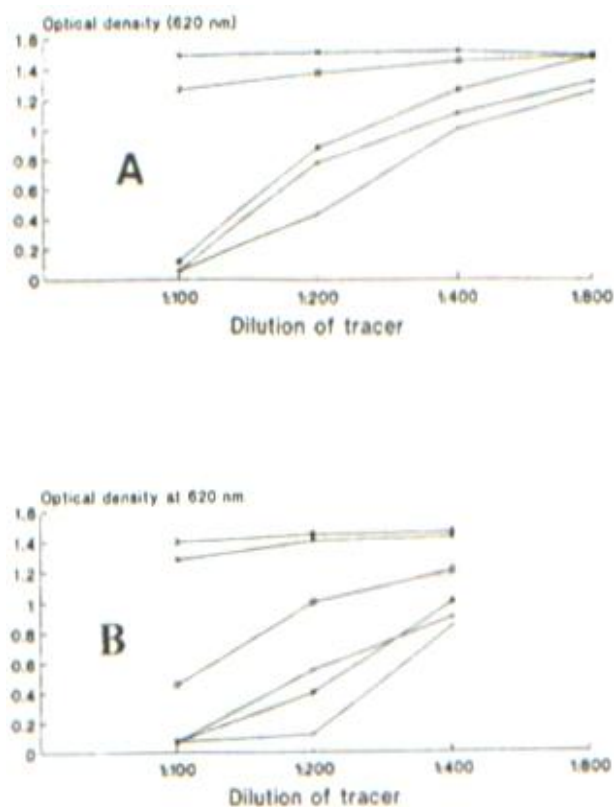


Fig. 3. Titer assay of two antibodies raised in this study and assayed with conjugate P-3-O-CMO-Enzyme by ELISA. **A)** anti-P-11 -HS-BSA (—●— 10 µg/well, —+— 5 µg/well, —*— 2.5 µg/well, —□— 1 µg/well, —x— NBS). **B)** anti P-3-O-CMO-BSA (—●— 12.5 µg/well, —+— 6.25 µg/well, —*— 3.12 µg/well, —□— 1.56 µg/well, —x— 0.78 µg/well, —◇— NBS). Best titer of antibody (2.5 µg/well) and best titer of enzyme conjugate (1:100) in both cases.

anti-P-3-O-CMO-BSA with P-3-O-CMO-enzyme, were used the antibody dilution was 1.5 µg/well and the enzyme conjugate dilution was still 1:100 (Figure 3B).

The standard curve in both cases was sensitive from 5 pg/well (50 pg/ml) up to 1000 pg/well (1000 pg/ml). We have observed the same curve, when lyophilized tracer was used after three months of storage at 4°C (Figure 4). The affinities of antibody against P-11 -HS-BSA and p-3-O-CMO-BSA obtained by ELISA were calculated to be 1.2×10^{-9} mol/l and 1×10^{-9} mol/l. These results were reported when the affinities were calculated assaying antibodies by RIA in the same respects.

Results of recovery are shown in (Table 1). These results indicated that between 90-116% of

progesterone added to the serum samples were recovered both in RIA and in ELISA with acceptable ranges (5.1-11.28%). In Table 2 results of inter and intra assay variations obtained by RIA and ELISA are shown. The coefficient of variation (CV) was 7.9-17.17 % within run assays and 5-15.73% for between runs which indicates consistency in the assay performance (reproducibility and accuracy).

Finally, samples obtained from Shariatee hospital were measured with our own ELISA and RIA and by RIA routinely used by the hospital (Table 3). A comparison between results obtained in these assay showed a correlation of $r = 0.99$ between the two first methods (RIA and ELISA) and $r = 0.95$ between our ELISA and RIA used by Shariatee hospital (Figures 5A and 5B).

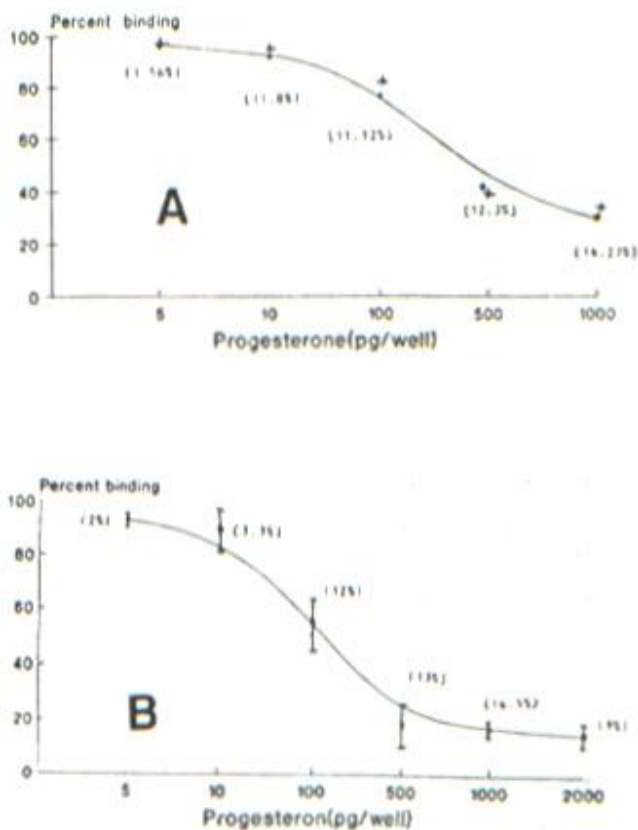


Fig. 4. Standard assay of antibodies obtained in this study with P-3-O-CMO-Penicillinase by ELISA. **A)** anti-P-3-O-CMO-BSA and P-3-O-CMO-Enzyme in buffer (Tracer was kept for three months at 4°C), —●— lyophilized tracer and + fresh tracer). **B)** anti-P-3-O-CMO-BSA and P-3-O-CMO-Enzyme (Results of six experiments, sensitivity from 5 pg/well ranging up to 1 ng/well). CV% (value in parenthesis) and SD (bars).

Table 1. Percent recoveries of progesterone added to charcoal stripped plasma.

Progesterone Conc (ng/ml)	No. of Assay	RIA			ELISA		
		% CV	Amount recovered	% RC	% CV	Amount recovered	% RC
Low (0.4)	6	5.1	0.39	102	7.2	0.42	105
Medium (4)	6	6.9	4.2	94	4.82	4.4	110
High (20)	6	11.28	20.4	116	6.82	18	90

Table 2. Intra and inter assay variations of pooled serum samples prepared and assayed in four replicates in four different occasions.

Interassay	Sn	n	Pool A				Pool B				Pool C			
			RIA		ELISA		RIA		ELISA		RIA		ELISA	
			Mean±SD (ng/ml)	% CV	Mean±SD (ng/ml)	% CV	Mean±SD (ng/ml)	% CV	Mean±SD (ng/ml)	% CV	Mean±SD (ng/ml)	% CV	Mean±SD (ng/ml)	% CV
	1	4	0.63±0.03	4.76	0.79±0.04	5.1	5.12±0.25	5	3.7±0.1	2.7	11.6±0.9	8.1	7.2±0.42	5.8
	2	4	0.66±0.04	7.43	0.72±0.06	8.9	4.22±0.55	13	3.44±0.29	8.4	12.8±1.4	11.11	7.4±0.7	9.46
	3	4	0.68±0.04	6.74	0.88±0.05	6.1	4.45±0.7	15.73	3.8±0.31	8.3	11.7±1.6	13.26	6.9±0.4	6.55
	4	4	0.56±0.05	8.6	0.91±0.1	10.98	4.22±0.23	5.4	3.8±0.18	4.7	12.62±1.3	10.24	7.5±1	13.27
Intraassay			---	---	---	---	---	---	---	---	---	---	---	---
	4 × 4		0.63±0.06	9.89	0.81±0.08	10.2	4.42±0.76	17.17	3.67±0.29	7.9	12.2±1.4	11.5	7.26±0.72	9.87

Table 3. Samples assayed by our methods (ELISA and RIA) and performed by Shariatee Hospital. FP (follicular phase) LP (luteal phase) P (pregnancy) PM (post menopause) M (mens) *RIA (developed by us) **RIA (performed by Shariatee Hospital).

Sn	FP (ng/ml)		LP (ng/ml)		P (ng/ml)		PM (ng/ml)		M (ng/ml)	
	*RIA	ELISA	*RIA	ELISA	*RIA	ELISA	*RIA	ELISA	*RIA	ELISA
1	1.5	1.88	17.5	19.0	21.0	22.0	1.9	1.78	2.0	2.2
2	2.0	1.8	16.0	16.6	20.0	19.6	1.5	1.02	1.9	2.3
3	2.1	2.0	18.5	18.0	23.0	22.0	1.7	1.8	0.5	0.4
4	1.9	1.9	17.0	16.0	21.7	23.0	1.56	1.51	1.8	1.8
5	3.5	3.8	18.1	18.0	42.0	44.0	1.2	1.05	1.56	1.8
6	2.4	2.5	17.0	17.21	19.7	24.0	1.3	1.23	1.1	1.2
7	2.0	1.98	18.3	18.5	32.1	30.0	1.8	1.7	1.9	2.3
8	2.1	1.67	17.2	16.9	-	-	-	-	-	-
9	1.9	2.3	17.9	18.3	-	-	-	-	-	-
10	2.2	2.17	18.0	18.25	-	-	-	-	-	-
range	1.5-3.5	1.67-3.8	16.0-18.5	16.0-19.0	19.7-42.0	19.6-46.0	1.2-1.9	1.02-1.8	0.5-2.0	0.4-2.3

Sn	**RIA (ng/ml)	ELISA (ng/ml)
1	0.71	0.96
2	14.2	17.0
3	2.51	6.8
4	0.1	2.3
5	0.3	0.9
6	7.8	10.3
7	6.0	7.6
8	0.79	1.2
9	0.4	1.7
10	10.9	14.0
11	15.6	17.0
12	12.5	14.0
13	0.16	0.3
14	3.2	4.0
15	29.4	32.0

DISCUSSION

RIA of progesterone, like all other RIAs, suffers from problems inherent in the use of radioisotope restricted shelf life of labeled reagents because of radioactive decay or radiolysis and also the problem of disposal of radioactive waste. These difficulties have been eliminated using nonisotopic labels among which enzymes have been the most successful [18]. Our previous experience with ELISA [26, 27] had led us to expect that the development of the ELISA for progesterone would be straight forward and simple, this was indeed the case.

In this paper we described a novel competitive, heterologous and simple enzyme immunoassay for progesterone using penicillinase as label enzyme. Penicillinase as a successful marker has been adopted by few groups [22-27] and found to be suitable in many respects such as ease of conjugation, color development and reagent stability [28, 29]. The original antibodies prepared in this experiment were against two well defined derivatives of progesterone (C_3 and C_{11}), both showing high titer, high specificity and high affinity. However our previous experience with heterologous enzyme immunoassay [26] proved to be successful for this report as well. The C_3 derivative conjugated with enzyme and a combination of anti- C_{11} with C_3 - Enzyme were used throughout the experiments reported thereafter.

When titration assays were performed by homologous combinations, we observed the color differences between low antibody dilution and enzyme conjugate with the corresponding higher dilution in heterologous combinations. These results were in agreement with previous reports by others [24, 25].

In the same way, sensitivity was around 5 pg/well which is about equal to the MA values. The steepness of the standard curve, up to 1000 pg/well was such that almost a linear curve was obtained when a log transformation was calculated. Finally, considering these results and results obtained on specificity of anti- C_{11} antibody in combination with P- C_3 -Enzyme, in the case of progesterone, we observed that a heterologous combination is much better than homologous combination.

Good correlation between RIA (developed and correlated with standard kit) and ELISA, both developed in our laboratory, also satisfied our prediction. In conclusion we report an effective EIA for the determination of progesterone. It is rapid to perform, requiring a 2 h competitive antibody-antigen reaction and 1 h enzyme-substrate reaction.

The separation of bound from free hormone requires about 30 seconds, the color development takes place inside the wells of microtiter plate and the determination of absorption takes about 10 seconds for a 96 well plate.

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