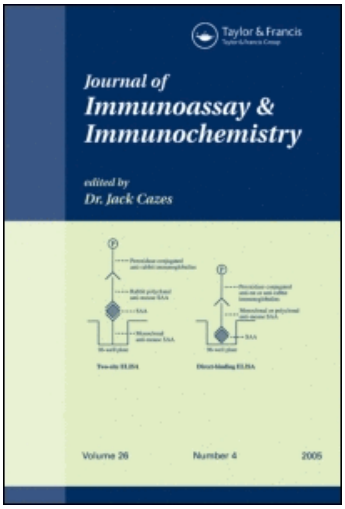

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CHARACTERIZATION OF A RAPID AND SENSITIVE ENZYME IMMUNOASSAY (EIA) FOR PROGESTERONE APPLIED TO CONDITIONED CELL CULTURE MEDIA

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ABSTRACT

Progesterone accumulation in conditioned media is a frequently employed endpoint for *in vitro* cell culture of steroidogenic cells. Although radioimmunoassay (RIA) has been the predominant method for measurement of progesterone, a number of nonradiometric immunoassays have been described but they have not been applied to conditioned media. Here, we report the characterization of a microtitre plate enzyme immunoassay (EIA) for determination of progesterone in conditioned media. The EIA has a sensitivity of 0.3 pg per well with intraassay and interassay coefficients of variation of 7.3 and 10.2%, respectively. The specificity of the EIA is no different than that of a comparable RIA showing crossreactivities of less than 0.1% for other steroids except 5α-pregnan-3,20-dione (47%) and 11α-hydroxyprogesterone (18%). Progesterone levels from conditioned cell culture media of either rat or human granulosa cell cultures measured by both EIA and RIA were in close agreement ($r = 0.96$) and serial dilutions of culture samples in the EIA were parallel to those of the standards. Also, extraction of culture media prior to EIA was found not to be necessary. Thus, this EIA is a highly sensitive and specific assay that provides a rapid, simple, inexpensive, and non-radiometric alternative to radioimmunoassay for measurement of progesterone in conditioned cell culture media. (KEY WORDS: immunoassay, progesterone, steroids, culture media)

INTRODUCTION

One of the most widely used techniques for measuring hormones is radioimmunoassay (RIA). This technique was developed over 30 years ago (1) and has revolutionized a number of fields by permitting the specific measurement of minute amounts of substances in complex biological samples. RIA has several advantages over previously used biological and chemical assays including measurement of large numbers of samples with very high sensitivity. With the advent of conjugated antigens, antibodies have now been developed for many nonimmunogenic substances leading to even wider use of RIA for measurement of a variety of compounds in biological samples. However, with concern over the use and disposal of radioactive materials, alternative techniques to RIA which do not use radiolabels have been developed.

Over the last several years, alternative techniques to RIA including enzyme immunoassay (EIA) and fluorescence immunoassay (FIA) have developed to the point where they are as sensitive and as rapid as RIA (2). EIA is becoming one of the most commonly used alternatives to RIA and has previously been adapted for measurement of steroids in serum and urine samples (3). However, use of this technique for measurement of steroids in cell culture samples has not yet been reported. In this study, we sought to determine whether EIA could be adapted for measurement of progesterone in cell culture samples and report herein an easy, rapid and nonradiometric method to measure progesterone levels in cell culture samples.

MATERIALS AND METHODS

Materials

Progesterone-3-CMO-horseradish peroxidase conjugate was obtained from Sigma Chemical Co. (St. Louis, MO). EIA plates (96 well, flat bottom, high binding, #3590) were purchased from Costar (Cambridge, MA). [1,2,6,7-^{3}H (N)]progesterone was purchased from New England Nuclear (Boston, MA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Conditioned media was prepared from either rat, human or porcine granulosa cell cultures or human term trophoblast cultures all done without serum as previously described (4,5).

Partial Antibody Purification

The gamma globulin fraction was isolated by repeated precipitation with ammonium sulfate from antiprogesterone antiserum prepared using a progesterone 11α-hemisuccinate-bovine serum albumin conjugate as the immunogen. Antiserum was diluted with an equal volume of phosphate buffered saline, 50 mmol/L, pH 7.4, (PBS) and two original volumes of saturated ammonium sulfate were slowly added. The mixture was incubated for 30 min at 4°C, centrifuged at 1000 x g for 15 min and the pellet resuspended in one original volume of PBS. The precipitation procedure was repeated two more times and the final pellet was resuspended in five original volumes of PBS. To eliminate anti-BSA antibodies from the gamma globulin fraction, BSA, 10 μg/400 μl, was added to the partially purified

antiserum and incubated overnight at 4°C. The antiserum was then centrifuged at 1000 x g for 15 min and the supernatant was dialyzed overnight against PBS.

Enzyme Immunoassay

Assays were conducted essentially as described by Munro and Lasley (3). Initially, EIA plates were coated with 50 μl of appropriately diluted (usually 1:24,000), partially purified antibody solution in coating buffer (sodium carbonate, 50 mmol/L, pH 9.6) and incubated overnight at 4°C. Plates were then washed three times with 0.2 ml of washing solution (NaCl, 150 mmol/L, Tween 20, 0.5 ml/L), inverted and dried for 2 min. The competition reaction was conducted by adding 50 μl of assay buffer (sodium phosphate, 100 mmol/L, pH 7.0, with sodium chloride, 8.7 g/L, BSA, 1 g/L), 25 μl of standard or sample and 25 μl of an appropriate concentration of progesterone-horseradish peroxidase conjugate (P-HRP, usually 6.7 ng/well). Following a 2 hr incubation at room temperature, bound and free hormone were separated by washing the plates three times with 0.2 ml of washing buffer, then inverting and drying for 2 min. The color reaction was then initiated by adding 0.1 ml of substrate solution (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid, 400 μmol/L) (ABTS), H_2O_2, 1.6 mmol/L, sodium citrate, 50 mmol/L, pH 4.0) to each well. This reaction was stopped after 30 min by adding 0.1 ml of stop reagent (hydrofluoric acid, 150 mmol/L). Absorbance of the wells was read at 405 nm using a 96 well microplate reader and the optical densities were entered into a four parameter

curvefitting program (from D. Rodbard, NIH) for calculation of results.

Radioimmunoassay

Assays were performed as previously described utilizing the same antiprogesterone antisera used for preparation of the EIA antibody (4). The competition assay was conducted by adding 25 μl of sample or standard, 375 μl of assay buffer (KH_2PO_4, 40 mmol/L, NaH_2PO_4, 100 mmol/L, NaCl, 170 mmol/L, Na azide, 10 g/L, gelatin, 10 g/L), 100 μl of [^{3}H] progesterone and 100 μl of appropriately diluted antiserum (1:30,000). Following a 16 hr incubation at 4°C, 100 μl of charcoal dextran (charcoal, 12.5 g/L, dextran T-70, 1.25 g/L) was added and the incubation continued for 15 min at 4°C. To separate charcoal-bound from antibody-bound steroid, tubes were centrifuged at 1000 x g for 15 min and the supernatants counted by scintillation spectrometry. Counts were then entered into the four parameter curvefitting program for calculation of results.

RESULTS

To initially characterize the EIA for progesterone, decreasing amounts of partially purified antibody were adsorbed onto EIA plates and incubated with a constant amount of progesterone-horseradish peroxidase (P-HRP) conjugate (10 ng/well). As shown in Figure 1, color development by antibody-bound HRP, as reflected by absorbance at the end of the substrate reaction, was dependent upon and inversely correlated with antibody concentration through a wide

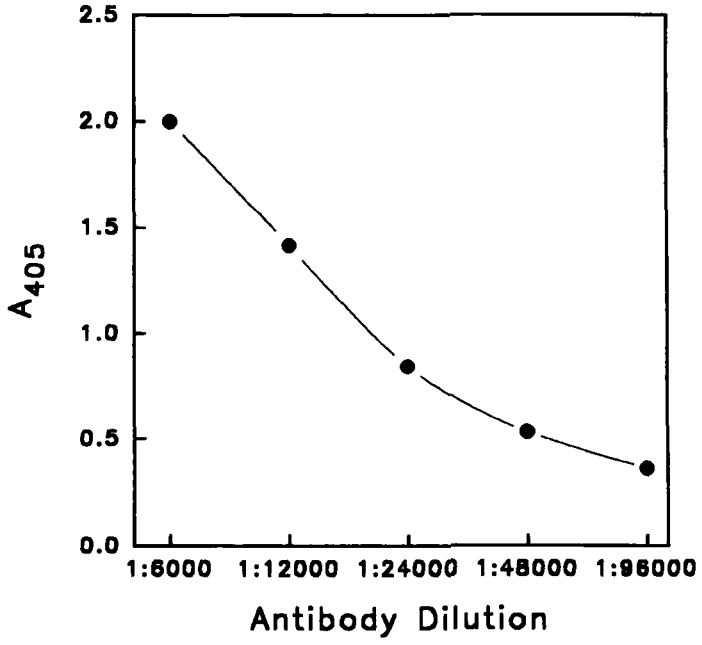

FIGURE 1. Maximal binding of progesterone-horseradish conjugate as a function of antibody dilution. A_{405} represents absorbance at 405 nm.

range of concentrations. Since an absorbance of 0.8 to 1.0 was considered optimal for maximal activity (Eo, no competing progesterone) wells, a 1:24,000 dilution of antibody was employed in further experiments.

The effect of varying the P-HRP conjugate concentration on color development was next evaluated. As shown in Figure 2, the color reaction was also dependent upon the concentration of P-HRP conjugate. Increasing the amount of conjugate increased the color

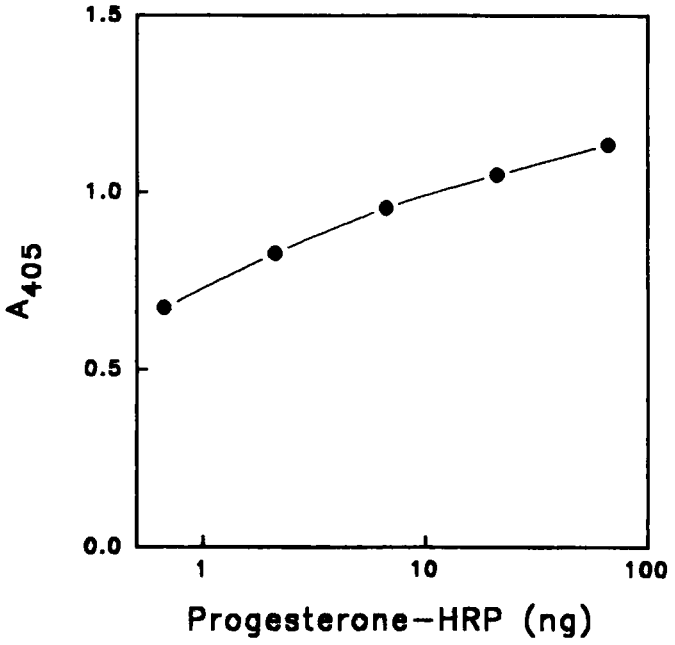

FIGURE 2. Color development as a function of progesterone–horseradish peroxidase conjugate concentration. A_{405} represents absorbance at 405 nm.

development in a range from 0.6 to 600 ng of P–HRP. Again, since an absorbance of 0.8 to 1.0 was considered optimal, a P–HRP concentration of 6.7 ng/well was employed in further experiments.

Figure 3 shows standard curves for both EIA and RIA plotted as fraction bound (E/Eo or B/Bo) versus concentration of competing unlabeled progesterone. For EIA, standard curves were run in EIA buffer or nonconditioned culture media and compared with an RIA standard curve run in RIA buffer. No differences were noted between

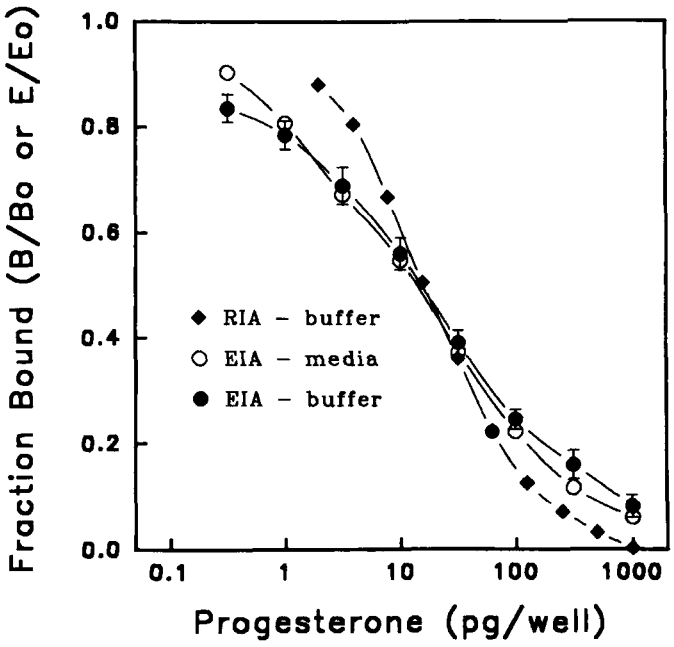

FIGURE 3. Comparison of standard curves for progesterone EIA and RIA. Standard competition curves were constructed with increasing amounts of unlabeled progesterone in either RIA or EIA with assay buffer (solid symbols) or nonconditioned culture media (open symbols). Each data point represents the mean ± standard error of the mean from three experiments. Error bars not shown are contained within the symbol.

EIA curves run in buffer or in culture media. However, both EIA curves had a slightly lower slope and a slightly greater range than the RIA curve. The midrange, slope and sensitivity (90% B/Bo) for the RIA (n=3) were 16.4 ± 1.2, 0.85 ± 0.04 and 1.9 pg, respectively,

TABLE 1

Comparison of Steroid Crossreactivities in EIA and RIA

Steroid	Crossreactivity (%) EIA	RIA
Androstenedione	0.03	0.01
Cortisol	<0.01	<0.01
17β-Estradiol	<0.01	<0.01
11α-hydroxyprogesterone	18	6.0
17α-hydroxyprogesterone	0.2	0.25
5α-pregnan-3,20-dione	45	12
4-pregnen-20α-ol-3-one	0.1	0.1
4-pregnen 20β-ol-3-one	0.2	0.1
Testosterone	<0.01	<0.01

whereas those for the EIA (n=3) were 15.9 ± 2.1, 0.61 ± 0.06 and 0.3 pg, respectively. Both types of assays had similar intraassay and interassay coefficients of variation (7.9 and 8.5%, respectively, for RIA and 7.3% and 10.2%, respectively, for EIA). In order to assess the accuracy of measurement of a known amount of progesterone in the EIA, either 1.95 or 7.8 pg of progesterone standard was added to nonconditioned media and measured in the EIA. Measured amounts (n=6 in each case) were 1.87 ± 0.06 pg (SEM) and 7.9 ± 0.2 pg (SEM), respectively.

Next, the crossreactivity of various steroids in the EIA was measured and compared with RIA (Table 1). Neither androstenedione, cortisol, 17β-estradiol, 17α-hydroxyprogesterone, 4-pregnen-20α-ol-3-one (20α-dihydroprogesterone), 4-pregnen-20β-ol-3-one (20β-dihydroprogesterone) nor testosterone showed significant

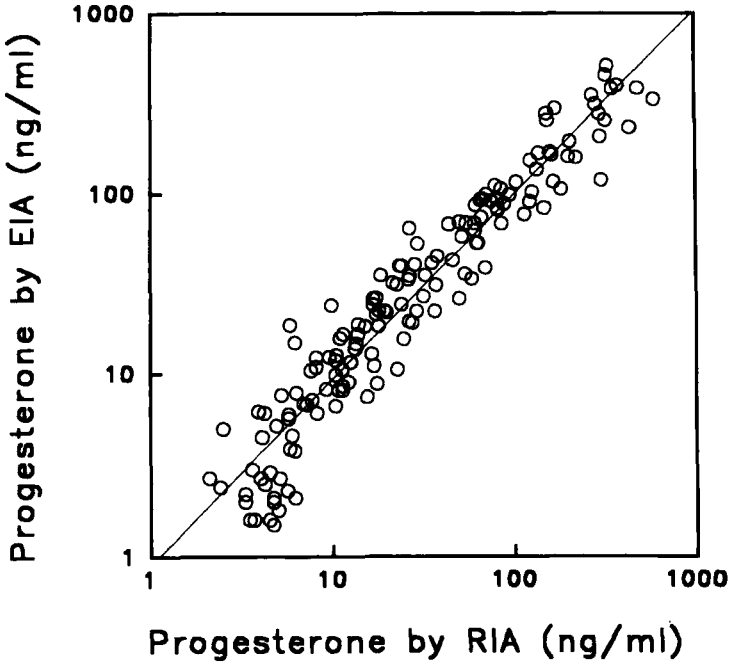

FIGURE 4. Correlation of progesterone levels in conditioned media from rat granulosa cell culture samples measured by EIA and RIA. One hundred and fifty individual culture samples were assayed by both EIA and RIA as described in the Experimental section. The regression line is also shown (r = 0.96).

crossreactivity in either assay. However, 11α-hydroxyprogesterone and 5α-pregnan-3,20-dione both showed significant but comparable crossreactivity in both assays.

Finally, measurement of progesterone in conditioned cell culture media was compared and correlated in EIA and RIA. Figure 4 shows a correlation plot of progesterone levels from rat granulosa

cell culture samples measured by EIA against measurements of the same samples by RIA. The regression equation was y= 1.02 x - 0.05 (r=0.96) (standard error of the intercept, 1.4, standard error of the slope, 0.037). Most of the samples which gave discrepant results in the two assays were at the low end of the RIA standard curve (B/Bo>0.85). A similar plot of progesterone levels from human IVF granulosa cell culture samples measured by both EIA and RIA gave a regression equation of y= 1.01 x + 0.03 (r=0.96) (standard error of the intercept, 1.6, standard error of the slope, 0.062). This EIA has also been used for measurement of progesterone in conditioned media from cultured porcine granulosa cells and cultured term human trophoblasts with comparable results (data not shown).

Parallelism of serial dilution curves for culture samples was assessed. Figure 5 shows dilution curves of 5 separate samples (three from cultured rat cells and two from cultured human cells) and clearly demonstrates that these dilution curves were parallel with the standard curve. Next, rat granulosa cell culture samples from a typical dose response experiment were measured for progesterone by both EIA and RIA (Figure 6). The results are similar both quantitatively and qualitatively when measured by either assay.

DISCUSSION

Progesterone accumulation in conditioned media is a common endpoint of steroidogenic capacity for *in vitro* cell culture of numerous cell types. Although most previous studies have utilized radioimmunoassay for measurement of progesterone, a number of

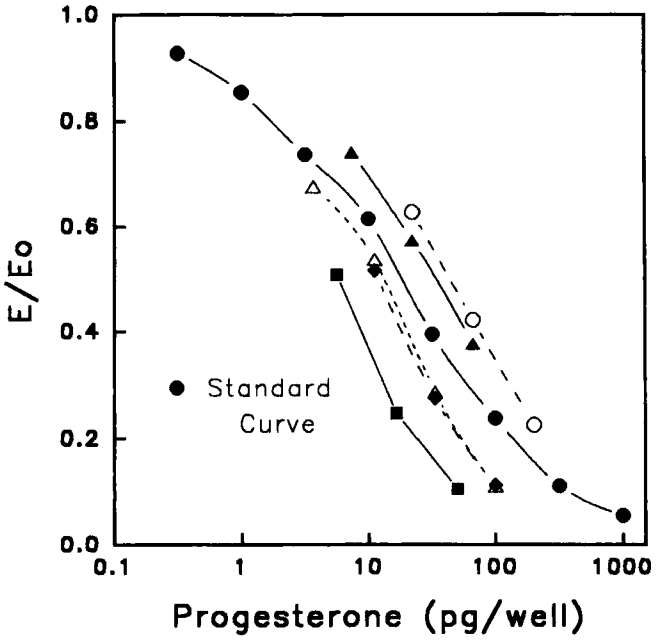

FIGURE 5. Parallelism of serial dilutions of conditioned media samples from rat or human granulosa cell cultures with progesterone standards in EIA. Serial dilutions of three conditioned media samples from rat granulosa cell cultures (solid symbols) and two from human granulosa cell cultures (open symbols) were compared with serial dilutions of progesterone standards (solid circles).

nonradiometric assays have been described (2). These nonradiometric assays are being increasingly used for measurement of progesterone in plasma, milk, saliva, and urine; however, little attention has gone toward measurement of conditioned culture media. The present studies report the development of an EIA method for measurement of progesterone in conditioned cell culture media. This assay is based

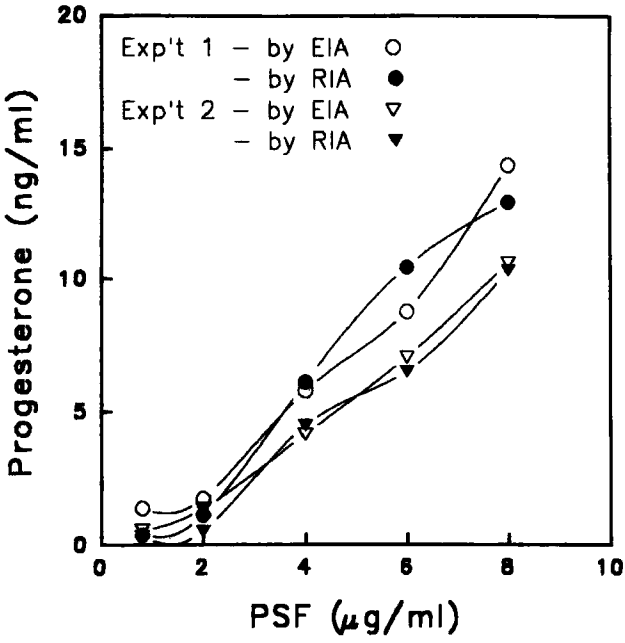

FIGURE 6. Comparison of progesterone levels in typical dose-response experiments measured by either EIA or RIA. Cultured rat granulosa cells were treated with increasing doses of Progesterone Stimulating Factor, PSF, (a previously described lymphocyte secretory product, see ref. 4) for 2 days in two separate experiments. The media from each experiment was then measured for progesterone content by either EIA (open symbols) or RIA (closed symbols).

upon the microtitre plate assay of Munro and Stabenfeldt (6) with some modifications. We have shown that the sensitivity, specificity and precision of this EIA are comparable with an analogous RIA and that the correlation between these two assays is excellent. We have

also shown that this assay is applicable to measurement of progesterone from conditioned culture media from several species (rat, pig and human) as well as several tissues (granulosa cell and trophoblast), and that the results obtained utilizing either assay are qualitatively and quantitatively similar.

A new assay must meet several criteria which we have addressed in these studies. First, both the intraassay and interassay coefficients of variation for the EIA are well within acceptable limits and are similar to those obtained with a more traditional RIA. Second, the characteristics of the standard curve, slope, midrange and sensitivity, are comparable with those of the RIA. Third, the specificity of the EIA is no different from that of the RIA with respect to the variety of steroids tested. Fourth, serial dilutions of culture samples were parallel to the standard curve indicating that determination of sample values is independent of amount measured and consistent with the assay measuring authentic progesterone. Fifth, the addition of known amounts of progesterone to the EIA produced expected values in the assay; thus, recovery of steroid and accuracy of measurement are excellent.

Unlike previous EIAs for measurement of progesterone in biological fluids, we found that extraction of culture samples with organic solvents was not required prior to sample measurement, thus considerably simplifying the assay procedure. However, since all culture samples used in these studies were obtained from serum-free cultures, extraction requirements should be reevaluated prior to using EIA with serum-containing media. Also, in this assay, we have utilized an antiserum generated against a progesterone-11α-

hemisuccinate–BSA conjugate and a "tracer" of progesterone–3–CMO–horseradish peroxidase conjugate. This combination of antiserum and "tracer" produced from structurally different conjugates is termed a heterologous assay. This type of assay system was chosen over a homologous system since Munro and Lasley (3) have reported previously that heterologous assay systems are more sensitive than homologous systems. While we have observed a greater sensitivity in the EIA than the RIA, it is unclear from these studies whether this results from use of a heterologous EIA system or from differences in antibody dilution and binding site availability since lower antibody dilutions in the EIA produced sensitivities closer to that of the RIA. Also, the greater sensitivity of heterologous EIA systems observed by Munro and Lasley has not been substantiated by others (7,8).

In conclusion, we describe the characterization of a nonradiometric EIA for measurement of progesterone in conditioned culture media samples. This EIA does not require extraction of samples prior to assay and is a simple, inexpensive, rapid and sensitive alternative to the RIA for measurement of progesterone levels in conditioned culture media. Since horseradish peroxidase conjugates of testosterone and estradiol are also commercially available, this technique could easily be applied to assay of these other steroids.

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Reprint Requests

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