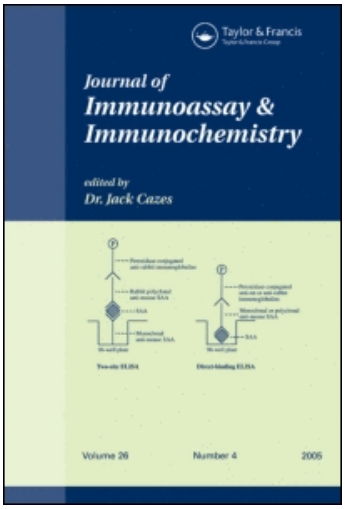

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AN ELISA FOR PGE_2 UTILIZING MONOCLONAL ANTIBODY

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ABSTRACT

An ELISA for PGE_2 has been developed which is sensitive to concentrations of 0.5 to 20.0 ng PGE_2/ml. Mouse monoclonal anti-PGE_2 ascites is utilized in a binding competition between the test sample and an adsorbed conjugate of PGE_2-BSA. The antibody which remains bound to the solid phase is quantitated colorimetrically by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG followed by incubation with p-nitro-phenylphosphate. PGE_1, PGA_1, PGA_2, PGB_2, 6-keto-$PGF_1\alpha$, $PGF_2\alpha$, 13,14-dihydro-15-keto-PGE_2, thromboxane B_2 and arachidonic acid showed minimal cross-reactivity with the anti-PGE_2.

The PGE_2 ELISA permits the quantitative analysis of large numbers of samples at a fraction of the cost and time required to process a commercial RIA kit. When linked to the appropriate computer software, data collection and analysis can be performed

in less than 10 minutes per 96-well plate. Furthermore, the use of an ELISA system eliminates the radioactive and toxic chemical waste generated by RIA methods.

INTRODUCTION

It is generally accepted that prostaglandin E_2 (PGE_2) is a potent mediator of numerous biological processes including inflammation, immunoregulation, bone resorption, renal function, and cytoprotection of the gastric mucosa (1,2). Both the therapeutic value and the adverse side effects of many non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to correlate with their potency as inhibitors of PGE_2 biosynthesis in vitro (3). The measurement of PGE_2 concentrations in biological fluids, tissue culture supernatants, or in cell-free enzyme systems is therefore a valuable tool for illucidating how various target cells and tissues are activated or for determining the potency of cyclooxygenase inhibitors.

The measurement of prostaglandins (PGs) has undergone considerable modification since their discovery in 1930 (4). The development of radioimmunoassays (RIAs), in use for nearly two decades, has allowed for rapid and quantitative determination of PGs. More recently, enzyme-linked immunosorbent assays (ELISAs) have proven to be valuable analytical tools which are comparable to RIAs in accuracy and sensitivity. In comparison to the RIA, the ELISA offers the advantage of lower expense, greater testing capacity, faster sample turnover, and elimination of radioactive

waste. Small haptens, such as PGE_2, contain only one epitope and therefore are unsuitable candidates for the original "sandwich" ELISAs, which required that two molecules of antibody recognize the same antigen. Furthermore, adsorption of a small hapten directly to the ELISA plate is usually unsuccessful either because the hapten fails to bind or because it loses antigenicity. These problems can be circumvented by adsorbing conjugates of hapten and protein onto the solid phase.

The development of monoclonal antibodies has resulted in increased sensitivity, specificity, and reproducibility of immunoassays. Ascites containing high titres of monoclonal antibody against PGE_2 have been raised in rats (5) and mice (6). Since these ascites are often active at dilutions of 1:5000, five milliliters raised in one mouse is sufficient to perform the analysis of 250,000 samples. The combination of monoclonal antibody technology, automatable ELISA technology and data reduction software is, therefore, an attractive route for assay development. We describe the development of an ELISA for PGE_2 that uses monoclonal antibody in an adaptation of the methods of Miller, et al. (7).

MATERIALS AND METHODS

Monoclonal Anti-PGE_2

The techniques used to generate mouse monoclonal antibodies have been described in detail elsewhere (8). PGE_2 conjugated

to keyhole limpet hemocyanin (PGE_2-KLH) was purchased from Seragen (Boston, MA). Balb/C mice (Jackson Laboraties) were immunized i.p. at multiple intervals with 10 µg of PGE_2-KLH emulsified in complete Freund's adjuvant. Animals which demonstrated a high serum titre against PGE_2 were boosted with 10 µg of PGE_2-KLH i.v. and i.p. Spleen cells from the selected animals were fused with Sp2/O-Ag14 myeloma cells in the presence of PEG M.W.1000 (J.T.Baker Chemical Co, Phillipsburg, NJ). The hybrids were cloned by limiting dilution and grown in media containing 0.04 µM aminopterin. Clones that were determined by RIA to produce high titres of anti-PGE_2 were introduced i.p. into Balb/C mice, and the ascites were harvested in two to three weeks. The anti-PGE_2 ascites were tested with an IgG subisotyping kit (Southern Biotechnology Associates, Birmingham, AL) and were shown to have antibodies of the IgG_3 subclass. The ascites were diluted twofold in phosphate buffer, pH 6.5, and stored at -80°C.

Eicosanoids

PGE_2, 13,14-dihyro-15-keto-PGE_2 (DHK-PGE_2), PGB_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$, were purchased from Upjohn Diagnostics (Kalamazoo, MI). PGE_1 was purchased from Sigma (St. Louis, MO); PGA_1, PGA_2 and thromboxane B_2 (TXB_2) were purchased from Advanced Magnetics, Inc. (Cambridge MA) and arachidonic acid (AA) was purchased from Nu-chek, Inc. (Elysan, MN).

Conjugation of PGE_2 to Bovine Serum Albumin

PGE_2 was conjugated to bovine serum albumin (BSA) using an amphoteric carbodiimide. First, 10 mg of PGE_2 were dissolved in 2 ml of N,N-dimethyl formamide (DMF, Fisher Scientific, Fair Lawn, NJ). Then 40 mg of 1-cyclohexyl-3- (2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate (Aldrich Chemical Co., No. C 10,640-2 Milwalkee, WI) were added gradually to the PGE_2-DMF solution while stirring.

The PGE_2-DMF-carbodiimide solution was then added dropwise into a separate solution of 40 mg bovine serum albumin (Pentex BSA, Miles Scientific, code 81-001-3, Naperville, IL) in 2 ml water. The reaction mixture was maintained pH 5.0-5.2 with dilute HCl and stirred continuously at room temperature for 24 hours. The conjugate was then dialyzed against one litre of 0.1 M phosphate buffer, pH 6.5, overnight at 4°C. The dialysis step was repeated, after which the conjugate was diluted in an equal volume of glycerol and stored in aliquots at -80°C.

Adsorption of Conjugate to Test Plate

PGE_2-BSA conjugate was diluted in adsorption buffer consisting of 54 mM NaCl, 45 mM glycine, and 0.05 % sodium azide, pH 8.2. Aliquots of 50 µl/well were adsorbed to 96-well test plates (Costar Serocluster Flat Bottom EIA plate, No.3590). After incubating for 2 hours at room temperature, the non-specific protein binding sites were blocked by the addition of 150 µl/well of adsorption buffer containing 0.1% ovalbumin

(Sigma). The plate was sealed (Linbro® plate sealers, Flow Laboratories, McLean, VA) and stored overnight at 4°C. Plates were stable at 4°C for up to two weeks.

PGE_2 Stock Solutions

PGE_2 stock solutions were prepared by dissolving one 10.0 mg vial of PGE_2 in 1.0 ml of ethanol. The 10 mg/ml standards were further diluted 1:1000 in 0.1 M phosphate buffer, pH 6.5, containing 0.1% ovalbumin to give a 10 µg/ml solution, which was stored at -80°C.

Assay Procedure

Using a Nunc Immunowash, the plates which had been coated with PGE_2-BSA and blocked as described above were washed six times with Dulbecco's Phosphate Buffered Saline, pH 7.5, containing 0.05% polyethylene sorbitan monolaurate (Tween 20, Sigma), leaving the wells full of wash buffer. The wells were aspirated and immediately refilled with 100 µl of the standard diluent. A standard PGE_2 solution was prepared in a separate tube by dilution of the PGE_2 stock solution into 0.1 M phosphate buffer, pH 7.5, supplemented with 0.1% ovalbumin (PB-OA). Alternately, a diluent of cell culture media containing fetal calf serum was used. Standard PGE_2 or samples were added to duplicate adjacent wells, and twofold serial dilutions were made directly in the ELISA plate progressing down the columns or across the rows. For comparison, serial dilutions were also made in a separate

plate containing diluent and were transferred onto the ELISA plate after aspirating the wash buffer.

Mouse monoclonal anti-PGE_2 ascites fluid was diluted in PB-OA, and 100 µl were added to the samples and standards in the ELISA plate. Wells that were designated for nonspecific binding received buffer only. The presence of 0.1 M phosphate in the antibody dilution buffer served to normalize any pH differences between samples. The presence of phenol red in the culture medium gave visual confirmation that sufficient mixing and buffering occurred upon addition of the antibody. Incubation with first antibody was for 2 hours at 4°C. The plate was then washed as described above and incubated for one hour at room temperature with 100 µl/well of alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson Immunoresearch, Avondale, PA, code 115-5503) diluted 1:3000 in PB-OA. Finally, the plate was washed and incubated at room temperature with 200 µl of 0.1% p-nitrophenyl phosphate (Sigma) in carbonate buffer consisting of 50 mM Na_2CO_3 and 1.0 mM $MgCl_2$, pH 9.8. Color development intensified with time and correlated inversely with the amount of PGE_2 contained in the standards and samples. Absorbance was quantitated at 405 nm on a Dynatech MR 580 automated ELISA reader.

Data Reduction

The most rapid and convenient derivation of unknown PGE_2 concentrations in test samples was accomplished by using an Apple

IIe or IBM PC computer programmed with ELISA data capture and reduction software (Immunosoft, Dynatech, Alexandria, VA). The program converted unknown absorbance data to raw PGE_2 concentration from a binomial expression of the absorbance versus the logarithm of the standard PGE_2 concentration. Values which fell outside the 15-85% inhibition range were generally disregarded.

RESULTS

Formation of the Solid Phase Antibody-Conjugate Complex

Initially, PGE_2-BSA conjugate was adsorbed at dilutions of 1:500, 1:1000, 1:2000, and 1:4000, using 50 µl/well, and the plate was blocked as described above. Anti-PGE_2 was added to the ELISA plate at dilutions of 1:3000, 1:6000, and 1:12000. Figure 1 shows the mean absorbance and standard errors from six replicate wells per point. Saturation of the conjugate-antibody complex was not observed at the highest titres tested.

Assay Standardization

The optimum conjugate titre was determined by running standard inhibition curves against several dilutions of conjugate and incubating with a 1:4000 dilution of anti-PGE_2 ascites, a titre that had been used previously for the RIA detection of PGE_2 (9) and that was predicted from Figure 1 to give satis-

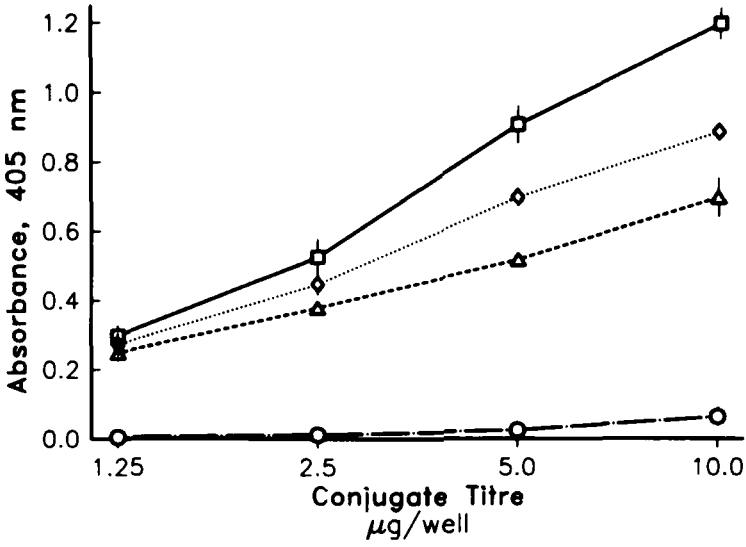

FIGURE 1: Increasing absorbance correlated with increasing concentration of conjugate (measured as µg BSA) at three anti-PGE_2 titres: 1:3000 -□-, 1:6000 -◇-, 1:12,000 -△- and nonspecific binding (no anti-PGE_2) -○-

factory results in the ELISA. A binomial regression of 1.000 was obtained when the standards were run against a 1:800 dilution of conjugate, which corresponds to 6.25 µg BSA/ml. A tenfold increase in absorbance was typically seen between the maximum and minimum standard PGE_2 concentrations. Inhibition was saturated at concentrations >20.0 ng/ml and was not detectable at <0.1 ng/ml (Figure 2). The presence of FCS at 5% did not appear to effect the sensitivity of the assay (data not shown). No difference in the standard inhibition curves was seen between standards that were diluted directly in the ELISA plate and those

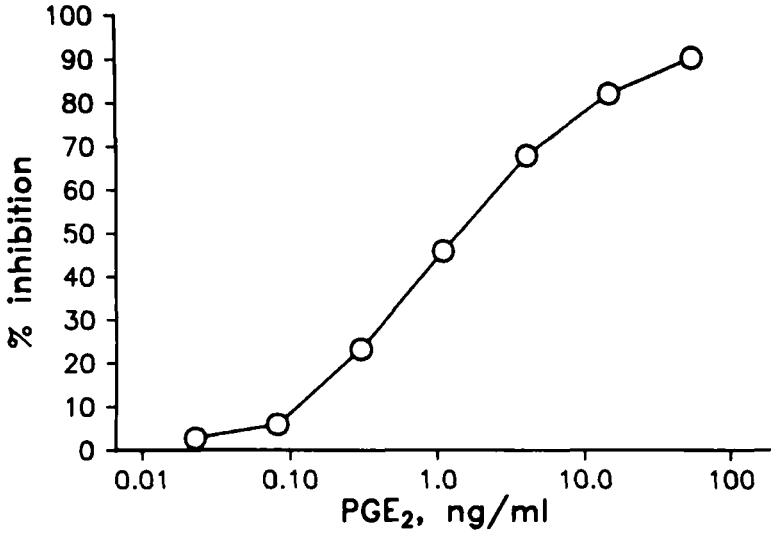

FIGURE 2: The presence of standard PGE_2 in the soluble phase produced dose-dependent inhibition of the binding of anti-PGE_2 to the solid-phase PGE_2. Concentrations refer to sample volumes of 100 µl and do not take into account the volume of the antibody.

diluted in a separate plate and then transferred onto the assay plate (data not shown).

Specificity of Monoclonal Anti-PGE_2

The concentrations of the other arachidonate metabolites required for 50% binding inhibition (IC_{50}) were determined from dose versus inhibition data, and cross reactivity was determined from the ratio:

$$(IC_{50}\ PGE_2/IC_{50}\ \text{test substance})\ X\ 100\%$$

TABLE 1
Cross-Reactivity of Arachidonate Metabolites

Metabolites	% ng PGE_2/ml
PGE_2	100.0%
PGA_1	0.24%
PGA_2	0.26%
13,14-dihydro-15-keto-PGE_2	0.01%
PGE_1	6.7%
PGB_2	0.04%
$PGF_{2\alpha}$	6.2%
6-keto-$PGF_{1\alpha}$	12.5%
Thromboxane B_2	< 0.1%
Arachidonic acid	< 0.005%

The percentages shown are equivalent to the ng PGE_2/ml that would be estimated by the assay if each substance listed were actually present at 100 ng/ml, i.e. (IC_{50} PGE_2/IC_{50} Test Substance) X 100.

PGA_1, PGA_2, PGB_2, DHK-PGE_2, TXB_2, and AA all showed minimal cross reactivity in the assay. $PGF_{2\alpha}$, PGE_1, and 6-keto-$PGF_{1\alpha}$ showed cross-reactivities of 6.2%, 6.7% and 12.5%, respectively (Table 1).

PGE_2 Determinations in Biological Fluids

The ELISA method has been used to quantitate PGE_2 in cell-conditioned media (10) and the results have been compared to the

same samples run concurrently in a commercial RIA kit (Seragen, Boston MA). There was no significant difference between the values generated by either method, although the total PGE_2 levels detected by the ELISA were often slightly lower than those detected by the RIA (data not shown). When PGE_2 biosynthesis was inhibited in cell cultures by the addition of non-steroidal anti-inflammatory drugs (NSAIDs), the ELISA and the RIA generated the same IC_{50} value for a given drug (10). The presence of NSAIDs in the standards, run as a control for the ELISA, had no effect on the standard inhibition curves (data not shown).

As a further test of the effects of cell-conditioned media on the detection of PGE_2 in the ELISA, supernatants from both unstimulated and cytokine-stimulated synoviocyte cultures (10) were divided into two groups, one of which was spiked with exogenous PGE_2. The samples were serially diluted directly in the ELISA plate. When corrected for dilution, good agreement was found for all the dilutions of a given sample provided that the values were within the range of the standards (i.e. within the range of 15-85% inhibition of binding). ELISA values for the spiked samples were in concurrence with the amounts expected to be present (Table 2). Furthermore, the ELISA has also been used in our laboratory to quantitate PGE_2 in rat urine, rat peritoneal exudates, human crevicular fluid, and in culture supernatants from numerous cell types.

Effect of Organic Solvents on Standard Inhibition Curves

Organic solvents may be present in samples that are to be

TABLE 2

PGE_2 Determinations of Synovial Cell Culture Supernatants

	Synoviocyte Conditioned Media[a]			Synoviocyte Conditioned Media + 150 ng/ml PGE_2[b]		
Dilution	%I	Raw	Corrected	%I	Raw	Corrected
Neat	90.5	31.91	31.91	93.22	38.88	38.88
1:2	80.3	16.23	32.46	93.52	39.77	79.54
1:4	68.9	8.48	33.92	91.18	33.46	133.8
1:8	54.6	4.12	33.96	85.71	22.95	183.6
1:16	35.4	1.77	28.32	76.59	13.04	208.5
1:32	18.6	0.91	29.02	63.03	6.24	199.7
1:64	7.3	0.60	38.28	45.38	2.71	173.4
1:128	3.6	0.52	66.56	28.99	1.36	174.0

Reproducibility of values derived from serially diluted samples was tested in a) bovine synoviocyte conditioned media and b) media "(a)" to which 150 ng/ml of exogenous PGE_2 was added. Reproducibility was demonstrated for samples that fell within the standard range of 15-85%. %I is the % inhibition of antibody binding.

assayed for prostaglandins, either as a solvent for the prostaglandin, a residual from extraction procedures, or as a vehicle for drug delivery in vitro. Accordingly, ethanol (EtOH), dimethyl sulfoxide (DMSO), dimethyl formamide (DMF) and methyl formate (MeF) were added in serial dilutions to an ELISA plate that contained standard PGE_2 at a fixed concentration of 2.0 ng/ml. The ability of standard PGE_2 to inhibit binding to the solid phase was markedly decreased by all of the organic solvents at concentrations greater than 0.2 % of the total sample volume (Figure 3), resulting in an underestimate of the soluble PGE_2.

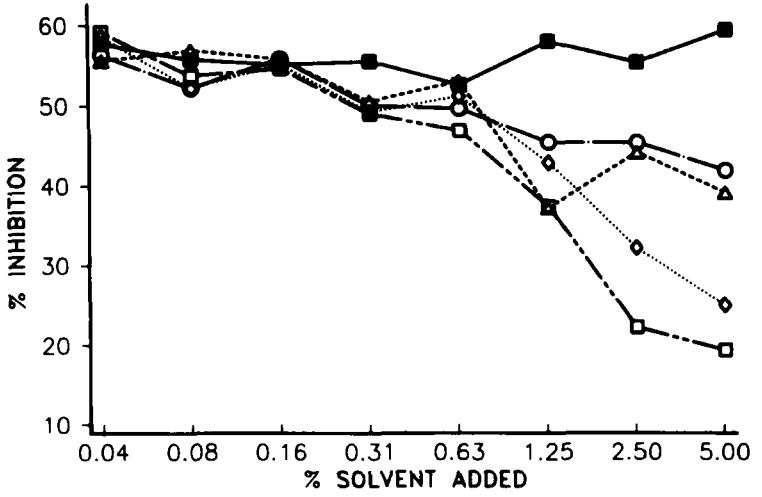

FIGURE 3: The presence of organic solvents were shown to reduce the amount of inhibition produced by 2.0 ng/ml PGE_2. Solvent, present in % v/v of sample, is shown as follows: Control -■-, DMSO -○-, DMF -△-, MeF -◇-, and EtOH -□-.

In an effort to determine whether the solvents were causing an increase in non-specific binding due to direct action on the antibody or the plastic, the solvents were added to mouse anti-PGE_2 and to goat anti-mouse-AP antisera in control plates that contained no conjugate. In order to test for solvent effects on the conjugate, the solvents were also added to a conjugate-adsorbed plate and incubated with goat anti-mouse-AP antisera. No absorbance was obtained by any of these methods. The results suggest that the organic solvents we tested cause a shift in the equilibrium of the antibody-hapten complex to favor the solid phase, and that the ELISA can withstand organic solvents in concentrations of 0.1% or less.

Statistical Evaluations

The degree of intraplate error that was attributable to the MR 580 was determined by reading a plate in which all the wells contained the same concentration of p-nitrophenol. At 405 nm, the standard deviation of a 0.61 mean absorbance was 0.012, giving a coefficient of variation of 2.1%.

The coefficient of variation for duplicate wells within a plate was highest for the samples containing the most PGE_2 (and therefore the lowest absorbance). Table 3 shows the mean coefficients of variation for duplicate wells, determined by analyzing the standards inhibition curves from 25 different plates. The percent inhibition observed at each standard PGE_2 concentration was seen to vary slightly from day to day. However, since there is considerable latitude in the parameters of the assay, no particular effort was made to keep them exact; incubation time and reagent titres were varied slightly when necessary or convenient, and the computer-generated regressions of all the standard inhibition curves were typically greater than 0.99. When standards were run repeatedly in the same plate, the values that were generated for a given standard concentration were within a 12% coefficient of variation (data not shown).

DISCUSSION

Prostaglandins are widely studied mediators of biological processes that are conventionally quantitated by radioimmuno-

TABLE 3
Coefficient of Variation for Duplicate Determinations

Concentration	Mean % C.V.*	Mean O.D., 405nm ± S.D.	% Inhibition** ± S.D.
20.0 ng/ml	17.2	0.10 ± 0.09	84.9 ± 12.0
10.0 ng/ml	16.1	0.17 ± 0.13	72.5 ± 18.3
5.0 ng/ml	11.6	0.23 ± 0.16	63.2 ± 21.4
2.5 ng/ml	10.9	0.32 ± 0.17	50.4 ± 20.8
1.25 ng/ml	10.5	0.40 ± 0.18	38.6 ± 21.1
0.63 ng/ml	8.4	0.48 ± 0.19	25.5 ± 17.1

* Well to well variability for duplicates within a plate were averaged from 25 different plates.

** Plate to plate variability in the % Inhibition of binding produced by standard PGE_2 concentrations (relative to 0 ng/ml) is represented as the mean and standard deviation from standard curves run on 25 different plates over a 30 day period.

assay. By modification of the procedures reported by Miller, et al. (7), we have developed a sensitive, accurate, rapid, and reproducible ELISA system for quantitating PGE_2. The monoclonal anti-PGE_2 utilized has an excellent cross-reactivity profile and can be used to measure prostaglandin E_2 concentrations in cell culture supernatants or biological fluids without a preliminary extraction of most samples.

A comparison of this ELISA with a commercially available RIA kit suggested that lessor total PGE_2 values may be derived by the ELISA. These differences, while not significant, may be

attributable to the different sources of anti-PGE_2 used in the two assays. When our monoclonal anti-PGE_2 was tested in the RIA, a lower cross reactivity for $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ was obtained in the RIA (9) than we have shown in the ELISA. This observation would suggest that differences in binding affinities may exist between the RIA and the ELISA. Finally, the observation that organic solvents can specifically enhance binding to the conjugated PGE_2 supports the idea that there are differences between the solid phase and the soluble phase in antibody-hapten complex formation.

In general, ELISA technology has numerous advantages over RIAs, such as a dramatic increase in testing capacity, a substantial monetary savings in both reagents and manhours, the elimination of radioactive waste, and an appreciable decrease in the time required for sample turnover. The use of an ELISA method such as the one we have described in this report could greatly facilitate research efforts which are focused on PGE_2-mediated processes.

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