Progesterone Immunoassay

Catalog Number DE2200

For the quantitative determination of progesterone concentrations in cell culture supernates, saliva, and serum.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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INTRODUCTION

Progesterone is the major female sex hormone and is responsible for reproductive-related activities such as breast glandular development, the endometrial aspects of the menstrual cycle, and the establishment and maintenance of pregnancy. In addition, progesterone directs pregnancy-support physiology including changes in carbohydrate, protein and lipid metabolism, thermoregulation, sodium reabsorption in renal tubules and the reduction of alveolar and atrial carbon dioxide partial pressures (1 - 3). Progesterone is involved in cell cycle progression, acts as a neurosteroid to promote remyelination of nerve axons and is used therapeutically to treat menopause-related symptoms in women (4 - 6). It is secreted in large amounts by the corpus luteum and by the extracellular conversion of cholesterol, cholestryl esters, adrenal steroids, pregnenolone and pregnenolone sulfate. Small quantities are also secreted directly from the adrenal glands. Since sex steroids are not stored, progesterone is quickly cleared from circulation by extracellular conversion to androgens, estrogen, or to pregnanediol, which is conjugated to glucuronic acid in the liver and excreted in urine. Only a small portion of circulating plasma progesterone is free (2.4%), with the remaining steroid bound to serum proteins (7).

R&D Systems' Progesterone Immunoassay is a 3 hour competitive enzyme immunoassay designed to measure progesterone in cell culture supernates, saliva and serum.

PRINCIPLE OF THE ASSAY

This assay is based on the competitive binding technique in which progesterone present in a sample competes with a fixed amount of alkaline phosphatase-labeled progesterone for sites on a mouse monoclonal antibody. During the incubation, the monoclonal antibody becomes bound to the goat anti-mouse antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. Immediately following color development, the absorbance is read at 405 nm. The intensity of the color is inversely proportional to the concentration of progesterone in the sample.

TECHNICAL HINTS

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- Allow reagents to warm to room temperature before use.
- The unopened bottle of pNPP Substrate should be colorless to pale yellow.
- Stop Solution should be added to the plate in the same order as the pNPP Substrate. The color developed in the wells will remain yellow upon the addition of Stop Solution.
- When mixing or reconstituting protein solutions, always avoid foaming.
- Pre-rinse the pipette tips when pipetting standards.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- Pipette standards and samples to the bottom of the wells.
- Add all other reagents to the side of the wells to avoid contamination.
- If samples generate values higher than the highest standard, further dilute the samples with Assay Buffer ED1 and repeat the assay.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in this assay, the possibility of interference cannot be excluded.
- Alkaline Phosphatase is a temperature sensitive enzyme. Optical Density (OD) units may vary with temperature changes.

REAGENTS

Microplate (Part R80-0050) - 96 well microplate coated with a goat antibody specific for mouse IgG.

Progesterone Conjugate (Part R80-0115) - 6 mL of progesterone conjugated to alkaline phosphatase, with blue dye and preservatives.

Progesterone Standard (Part R80-0117) - 0.5 mL of progesterone (100,000 pg/mL) in buffer.

Progesterone Antibody Solution (Part R80-0116) - 6 mL of mouse monoclonal antibody to progesterone, with yellow dye and preservatives.

Assay Buffer ED1 (Part R80-0010) - 30 mL of a buffered protein base with preservative.

Steroid Displacement Reagent (R80-0120) - 1 mL of a specially formulated buffer to release bound progesterone from binding proteins.

Wash Buffer Concentrate (Part R80-1286) - 30 mL of a 20-fold concentrated solution of a buffered surfactant with preservative.

pNPP Substrate (Part R80-0075) - 20 mL of p-nitrophenyl phosphate in a buffered solution.

Stop Solution (Part R80-0247) - 6 mL of a trisodium phosphate (TSP) solution. **Keep tightly capped. Caution:** *caustic.*

Plate Cover - 1 adhesive strip.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8° C. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="9">Opened Reagents</td><td>Diluted Wash Buffer</td><td>May be stored at room temperature for up to 3 months.*</td></tr>
<tr><td>Stop Solution</td><td rowspan="8">May be stored at 2 - 8° C until the expiration date of the kit.</td></tr>
<tr><td>Assay Buffer ED1</td></tr>
<tr><td>Steroid Displacement Reagent</td></tr>
<tr><td>pNPP Substrate</td></tr>
<tr><td>Conjugate</td></tr>
<tr><td>Antibody Solution</td></tr>
<tr><td>Standard</td></tr>
<tr><td>Microplate Wells</td><td>Return unused wells to the foil pouch containing the desiccant pack, reseal along the entire edge of the zip-seal. May be stored at 2 - 8° C until the expiration date of the kit.</td></tr>
</table>

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 405 nm with wavelength correction set between 570 nm and 590 nm.
- Pipettes and pipette tips.
- 1000 mL graduated cylinder for preparation of Wash Buffer.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or autowasher.
- Horizontal orbital microplate shaker (0.12” orbit) capable of maintaining a speed of 500 $\pm$ 50 rpm.
- Diethyl ether (ACS Grade) (for extracting progesterone).

PRECAUTIONS

Some components of this kit contain sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

The Stop Solution provided with the kit is a caustic material. Wear eye, hand, face, and clothing protection when using this material.

The Progesterone Standard provided with this kit is supplied in ethanolic buffer at a pH optimized to maintain progesterone integrity. Care should be taken when handling this material because of the known and unknown effects of steroids.

The activity of the Progesterone Conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} ions and is affected by high concentrations of chelators, such as EDTA and EGTA. Samples that contain < 10 mM EDTA or EGTA can be assayed without interference. Samples containing higher concentrations of chelators must be diluted prior to assay.

Inorganic phosphate is a strong competitive inhibitor of alkaline phosphatase; avoid the use of PBS based wash buffers and other sources of inorganic phosphate contamination. Prior to addition of Substrate, ensure that there is no residual wash buffer in the wells.

SAMPLE COLLECTION AND STORAGE

Samples containing mouse IgG may interfere with this assay.

Cell Culture Supernate - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Saliva - Collect 2 - 3 mL of saliva in a conical plastic tube. Vortex briefly and centrifuge for 6 minutes at 14,000 rpm. Collect the aqueous layer (no pellet) and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST). Allow samples to clot for 30 minutes before centrifugation at 1600 x g for 15 minutes. Assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Samples containing steroid binding proteins (such as serum) need to be treated with Steroid Displacement Reagent. Add 5 µL of Displacement Reagent to 495 µL of sample.

Saliva, serum and cell culture supernate samples require at least a 10-fold dilution. A suggested 10-fold dilution is 25 µL sample + 225 µL Assay Buffer ED1.

Note: *After the initial 10-fold dilution, use fresh culture media instead of Assay Buffer ED1 for cell culture supernate samples.*

If diluted samples measure below the lowest standard, then it is recommended that the samples be extracted for accurate measurement of progesterone. The following protocol is a general extraction method for steroids.

SAMPLE EXTRACTION

1. Add sufficient progesterone to a typical sample for determination of extraction efficiency.
2. In a fume hood, add 1 mL of diethyl ether for every 1 mL of sample. Stopper and shake sample. **Caution: Diethyl ether is extremely flammable.**
3. Allow layers to separate. Carefully pipette off the top (ether) layer and place in a clean test tube.
4. Repeat steps 1 - 3 two more times, combining the ether layers.
5. Evaporate under nitrogen until dry.
6. Dissolve the extracted progesterone with at least 250 µL of Assay Buffer ED1. Vortex, allow to sit for 5 minutes at room temperature and repeat two more times.
7. Run the reconstituted samples in the assay immediately. If analysis is delayed, store samples evaporated and desiccated at ≤ -20° C.

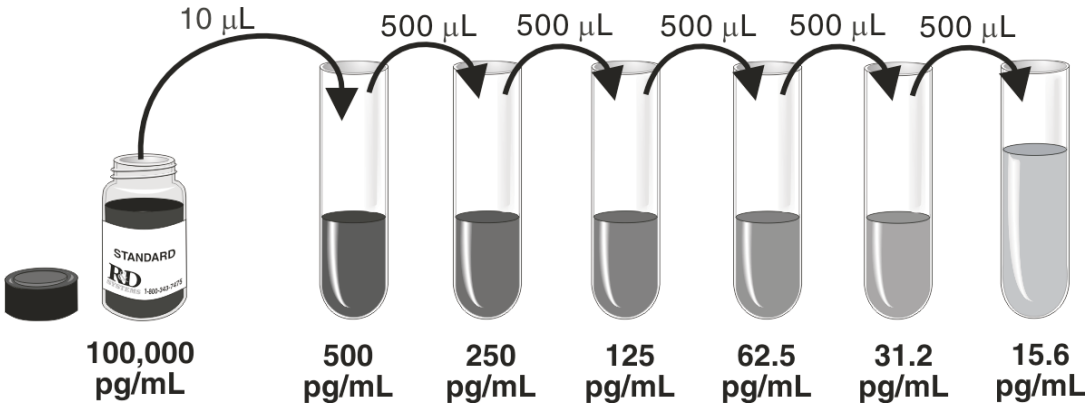
REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer - Dilute 30 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 600 mL of Wash Buffer (1X).

Progesterone Standard - Pipette 1.99 mL of Assay Buffer ED1 into the 500 pg/mL tube. Pipette 500 µL of Assay Buffer ED1 into the remaining tubes. *When pipetting standards, it is important to pre-rinse the pipette tips.* Use the 100,000 pg/mL standard stock to produce a dilution series (below). Vortex each tube thoroughly and change pipette tips between each transfer. The 500 pg/mL standard serves as the high standard and the Assay Buffer ED1 serves as the zero standard (B_0) (0 pg/mL).

When running cell culture supernate samples, use cell culture media (CCM) instead of Assay Buffer ED1 to produce the standard dilution series and as the zero standard. Changes in binding have been associated with running the standards and samples in media.



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

Total Activity (TA), Non-Specific Binding (NSB), Maximum Binding (B_0), and Substrate Blank wells should be included in the assay as a means of quality control for each assay.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate wells from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.
3. Reserve wells for TA and Substrate Blank.
4. Add 150 µL of Assay Buffer (or CCM) to the NSB wells.
5. Add 100 µL of Assay Buffer (or CCM) to the zero standard (B_0) wells.
6. Add 100 µL of Standard or sample* to the remaining wells.
7. Add 50 µL of Progesterone Conjugate to each well (excluding the TA and Substrate Blank wells).
8. Add 50 µL of Progesterone Antibody Solution to each well (excluding the NSB, TA, and Substrate Blank wells). Tap the plate gently to mix. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.

   Note: *The TA and Substrate Blank wells are empty at this point. The NSB wells should be blue and all other wells should be green.*
9. Aspirate or decant each well and wash, repeating the process twice for a total of three washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, multi-channel pipette, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels to remove any remaining Wash Buffer.
10. Add 5 µL of Progesterone Conjugate to the TA wells.
11. Add 200 µL of pNPP Substrate to **all wells.** Incubate for 45 minutes at room temperature **on the benchtop.**
12. Add 50 µL of Stop Solution to each well.
13. Determine the optical density of each well **immediately** using a microplate reader set to 405 nm with wavelength correction set between 570 nm and 590 nm.

*Samples require dilution as directed in the Sample Preparation Section.

CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample and subtract the average NSB optical density.

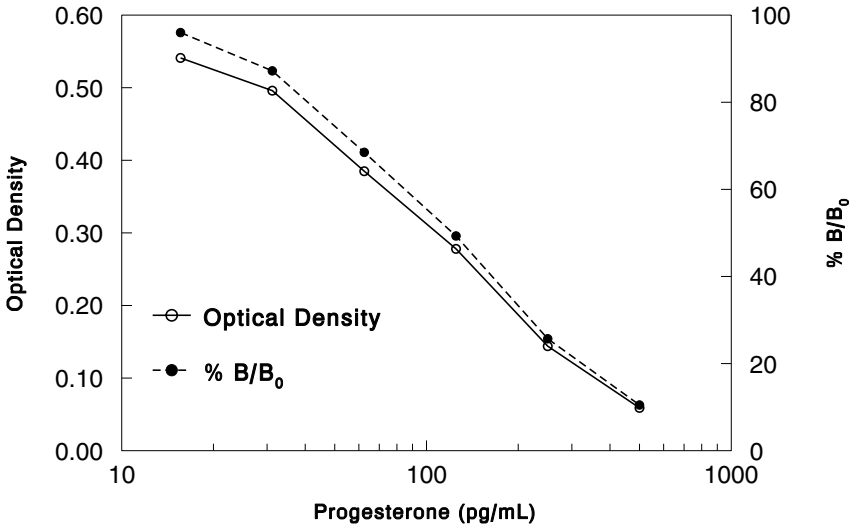
Create a standard curve by reducing the data using computer software capable of generating a four paramater logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on a linear y-axis against the concentration on a logarithmic x-axis and draw the best fit curve through the points on the graph.

% B/B_0 can be calculated by dividing the corrected OD for each standard or sample by the corrected B_0 OD and multiplying by 100.

Calculate the concentration of progesterone corresponding to the mean absorbance or % B/B_0 from the standard curve. Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



pg/mL	OD	Average	Corrected	% B/B_0
NSB	0.077 0.079	0.078	——	——
0 (B_0)	0.638 0.644	0.641	0.563	100.0
15.6	0.604 0.634	0.619	0.541	96.0
31.2	0.570 0.579	0.574	0.496	87.2
62.5	0.459 0.467	0.463	0.385	68.5
125	0.352 0.361	0.356	0.278	49.3
250	0.216 0.228	0.222	0.144	25.7
500	0.136 0.139	0.137	0.059	10.5

QUALITY CONTROL

Each laboratory should establish a quality control program to monitor the performance of the Progesterone Immunoassay. As a part of this program, TA, NSB, B_0, and Substrate Blank wells should be run in each assay.

Typical Quality Control Parameters

Substrate Blank (O.D.)	=	0.080
TA (TA x 10)	=	4.10
%NSB (NSB/TA x 100)	=	0.0%
% B_0 (B_0/TA x 100)	=	13.8%
Quality of Fit	=	0.999

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twelve times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in eight separate assays to assess inter-assay precision.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	12	12	12	8	8	8
Mean (pg/mL)	23.1	118	326	20.4	108	314
Standard deviation	1.75	6.4	16	1.39	9.0	8.5
CV (%)	7.6	5.4	4.9	6.8	8.3	2.7

RECOVERY

The recovery of progesterone spiked into samples in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell Culture Media*	105	102 - 108%
Human Serum*	106	97 - 115%
Human Saliva*	98	93 - 103%

*Samples were diluted prior to assay as directed in Sample Preparation.

LINEARITY

To assess the linearity of the assay, Assay Buffer ED1 spiked with progesterone was assayed using serial 2-fold dilutions.

Dilution	Observed (pg/mL)	Expected (pg/mL)	% Observed / Expected
1:2	341	—	—
1:4	176.6	170.5	104%
1:8	76.1	85.2	89.3%
1:16	46.5	42.6	109%
1:32	24.2	21.3	114%

SENSITIVITY

The sensitivity of the Progesterone assay is typically less than 8.57 pg/mL.

Sensitivity was determined by subtracting two standard deviations from the mean optical density value of twenty zero standard (B_0) replicates and calculating the corresponding concentration.

CROSS-REACTIVITY

Cross-reactivity for the following compounds was determined by adding the cross-reactant to Assay Buffer ED1 at concentrations ranging from 10 pg/mL to 10,000,000 pg/mL. The cross-reactivity was calculated at 50% B/B_0.

Compound	% Cross-reactivity
5α-Pregnane-3, 20-dione	100
17-OH-Progesterone	3.46
5-Pregnen-3β-ol-20-one	1.43
Corticosterone	0.77
4-Androstene-3, 17-dione	0.28
Deoxycorticosterone	0.056
DHEA	0.013
17β-Estradiol	< 0.001
Estrone	< 0.001
Estriol	< 0.001
Testosterone	< 0.001
Hydrocortisone	< 0.001
5α-Pregnane-3α, 20α-diol	< 0.001
Danazol	< 0.001

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