17α-Hydroxyprogesterone Enzyme Immunoassay Kit

Catalog No. 900-112

96 Well Kit

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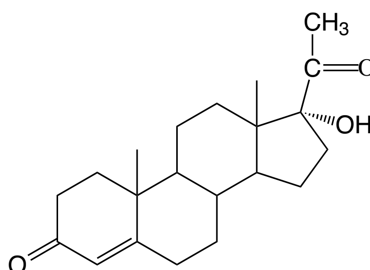
Description

Assay Designs' 17α-Hydroxyprogesterone (17-OHP) kit is a competitive immunoassay for the quantitative determination of 17-OHP in biological fluids. Please read the complete kit insert before performing this assay. The kit uses a polyclonal antibody to 17-OHP to bind, in a competitive manner, the 17-OHP in the sample, or an alkaline phosphatase molecule which has 17-OHP covalently attached to it. After a simultaneous incubation at room temperature the excess reagents are washed away and substrate is added. After a short incubation time the enzyme reaction is stopped and the yellow color generated read on a microplate reader at 405 nm. The intensity of the bound yellow color is inversely proportional to the concentration of 17-OHP in either standards or samples. The measured optical density for the samples is used to calculate the concentration of 17-OHP in the sample. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

Introduction

The progestin 17α-Hydroxyprogesterone (17α-Hydroxypregn-4-ene-3,20-dione; 17-OHP) is the product of steroid α-hydroxylation of Progesterone by steroid 17α-hydroxylase[3]. 17-OHP can be converted into 11-Deoxycortisol (by hydroxylation at C_{21}) or Androstenedione and acetate (by cleavage of steroid C_{17}-C_{20} bond)[3]. Serum levels of 17-OHP have primarily been used in the clinical setting as a means of diagnosis for 21-hyrdroxylase deficiency, a congenital adrenal hyperplasia[4-8].

17α-Hydroxyprogesterone



Precautions

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1. Some kit components contain azide, which may react with lead or copper plumbing. When disposing of reagents always flush with large volumes of water to prevent azide build-up.
2. Stop solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in use.
3. The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} ions. The activity of the conjugate is affected by concentrations of chelators (>10 mM) such as EDTA and EGTA.
4. The 17-OHP Standard provided, Catalog No. 80-1038, is supplied in ethanolic buffer at a pH optimized to maintain 17-OHP integrity. Care should be taken handling this material because of the known and unknown effects of steroids.

Materials Supplied

1. **Goat anti-Rabbit IgG Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0060**
   A plate using break-apart strips coated with goat antibody specific to the Fc portion of rabbit IgG.
2. **17-OHP EIA Conjugate, 5 mL, Catalog No. 80-1040**
   A blue solution of alkaline phosphatase conjugated with 17-OHP.
3. **17-OHP EIA Antibody, 5 mL, Catalog No. 80-1039**
   A yellow solution of a rabbit polyclonal antibody to 17-OHP.
4. **Assay Buffer, 27 mL, Catalog No. 80-1591**
   Tris buffered saline containing proteins and sodium azide as preservative.
5. **Wash Buffer Concentrate, 27 mL, Catalog No. 80-1286**
   Tris buffered saline containing detergents.
6. **17-OHP Standard, 0.5 mL, Catalog No. 80-1038**
   A solution of 17-OHP in buffer containing 10,000 pg/mL.
7. **pNpp Substrate, 20 mL, Catalog No. 80-0075**
   A solution of p-nitrophenyl phosphate in buffer.
8. **Stop Solution, 5 mL, Catalog No. 80-0247**
   A solution of trisodium phosphate in water. Keep tightly capped. Caution: **Caustic.**
9. **17-OHP Assay Layout Sheet, 1 each, Catalog No. 30-0185**
10. **Plate Sealer, 1 each, Catalog No. 30-0012**

Storage

All components of this kit are stable at 4°C, except the standard, until the kit's expiration date. The 17-OHP Standard must be stored at -20°C.

Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Precision pipets for volumes between 5 µL and 1,000 µL.
3. Repeater pipets for dispensing 50 µL and 200 µL.
4. Disposable beaker for diluting Wash Buffer.
5. Graduated cylinders.
6. A microplate shaker.
7. Adsorbent paper for blotting.
8. Microplate reader capable of reading at 405 nm, preferably with correction at between 570 and 590 nm.

Sample Handling

Assay Designs' 17α-Hydroxyprogesterone (17-OHP) kit is compatible with 17-OHP samples in a wide range of matrices. Samples diluted sufficiently in Assay Buffer can be read directly from the standard curve. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions. However, the end user **must verify** that the recommended dilutions are appropriate for their samples. **Samples containing rabbit IgG may interfere with the assay.**

Samples in the majority of Tissue Culture Media, including those containing fetal bovine serum, can also be read in the assay, provided the standards have been diluted into the Tissue Culture Media instead of Assay Buffer. There will be small change in binding associated with running the standards and samples in media. Users should only use standard curves generated in media or buffer to calculate concentrations of 17-OHP in the appropriate matrix.

Plasma samples should only be collected in tubes containing heparin. Other anticoagulants will yield poor sample recoveries.

Due to the presence of steroid sulfates, umbilical cord plasma must be extracted.

Some samples may require extraction for accurate measurement. A suitable extraction procedure is outlined below:

Materials Needed

1. 17-OHP Standard to allow extraction efficiency to be accurately determined.
2. ACS Grade Diethyl Ether.

Procedure

1. Add sufficient 17-OHP Standard to a typical sample.
2. In a fume hood add 1 mL of Diethyl Ether for every mL of sample. Stopper and shake sample.
3. Allow layers to separate. Carefully pipet off the top ether layer and place in a clean test tube.
4. Repeat, combining the ether layers.
5. Evaporate the ether to dryness under nitrogen.
6. Dissolve the extracted 17-OHP with at least 250 μL Assay Buffer by vortexing well. Allow to sit 5 minutes at room temperature. Repeate twice more.
7. Run reconstituted samples in the assay immediately or keep frozen below -20 °C.

Procedural Notes

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4 °C in the sealed bag provided. The wells should be used in the frame provided.
8. **Care must be taken to minimize contamination by endogenous alkaline phosphatase.** Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands.
9. **Prior to the addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.**

Reagent Preparation

1. 17-OHP Standard

Allow the 10,000 pg/mL 17-OHP standard solution to warm to room temperature. Label five 12 x 75 mm glass tubes #1 through #5. Pipet 1,000 µL of standard diluent (Assay Buffer or Tissue Culture Media) into tube #1. Pipet 750 µL of standard diluent into tubes #2 through #6. Remove 100 µL of diluent from tube #1. Add 100 µL of the 10,000 pg/mL standard to tube #1. Vortex thoroughly. Add 250 µL of tube #1 to tube #2 and vortex thoroughly. Add 250 µL of tube #2 to tube #3 and vortex. Continue this for tubes #4 through #5.

The concentration of 17-OHP in tubes #1 through #5 will be 1,000, 250, 62.5, 15.6 and 3.91 pg/mL respectively. See 17-OHP Assay Layout Sheet for dilution details.

Diluted Standard should be used within 60 minutes of preparation.

2. Wash Buffer

Prepare the Wash Buffer by diluting 5 mL of the supplied concentrate with 95 mL of deionized water. This can be stored at room temperature until the kit expiration date, or for 3 months, whichever is earlier.

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4 °C.

2. Pipet 100 μL of standard diluent (Assay Buffer or Tissue Culture Media) into the NSB and the Bo (0 pg/mL Standard) wells.

3. Pipet 100 μL of Standards #1 through #5 into the appropriate wells.

4. Pipet 100 μL of the Samples into the appropriate wells.

5. Pipet 50 μL of Assay Buffer into the NSB wells.

6. Pipet 50 μL of blue Conjugate into each well, except the Total Activity (TA) and Blank wells.

7. Pipet 50 μL of yellow Antibody Solution into each well, except the Blank, TA and NSB wells.

NOTE: Every well used should be **Green** in color except the NSB wells which should be **Blue**. The Blank and TA wells are empty at this point and have no color.

8. Incubate the plate at room temperature on a plate shaker for 2 hours at ~500 rpm. The plate may be covered with the plate sealer provided, if so desired.

9. Empty the contents of the wells and wash by adding 400 μL of wash solution to every well. Repeat the wash 2 more times for a total of **3 Washes.**

10. After the final wash, empty or aspirate the wells and firmly tap the plate dry on a lint free paper towel to remove any remaining wash buffer.

11. Add 5 μL of the blue Conjugate to the TA wells.

12. Add 200 μL of the pNpp Substrate solution to every well. Incubate at room temperature for 1 hour without shaking.

13. Add 50 μL of Stop Solution to every well. This stops the reaction and the plate should be read immediately.

14. Blank the plate reader against the Blank wells, read the optical density at 405 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all readings.

Calculation of Results

Several options are available for the calculation of the concentration of 17-OHP in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of 17-OHP can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:

   Average Net OD = Average OD - NSB OD

2. Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:

   $$\text{Percent Bound} = \frac{\text{Net OD}}{\text{Net Bo OD}} \times 100$$

3. Using Logit-Log paper plot Percent Bound versus Concentration of 17-OHP for the standards. Approximate a straight line through the points. The concentration of 17-OHP in the unknowns can be determined by interpolation.

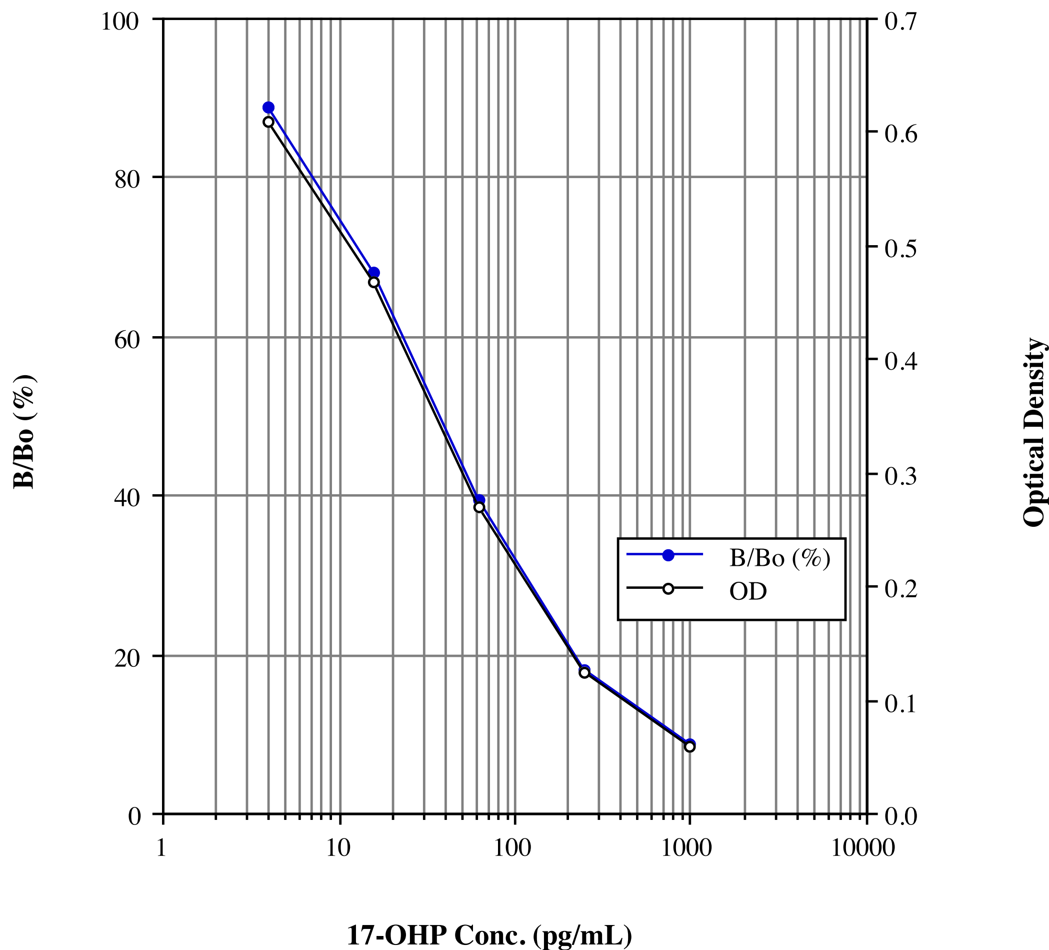
Typical Results

The results shown below are for illustration only and **should not** be used to calculate results from another assay.

Sample	Mean OD(-Blank)	Average Net OD	Percent Bound	**17-OHP (pg/mL)**
Blank OD	(0.180)			
TA	0.269			
NSB	0.007	0.000		
Bo	0.693	0.686	100%	
S1	0.066	0.059	8.6%	**1,000**
S2	0.131	0.124	18.1%	**250**
S3	0.277	0.270	39.4%	**62.5**
S4	0.475	0.468	68.2%	**15.6**
S5	0.617	0.610	88.9%	**3.91**
Unknown 1	0.128	0.121	17.6%	**260**
Unknown 2	0.369	0.362	52.8%	**32.9**

Typical Standard Curve

A typical standard curve is shown below. This curve **must not** be used to calculate 17-OHP concentrations; each user must run a standard curve for each assay.



Typical Quality Control Parameters

Total Activity Added	=	0.269 x10 = 2.69
%NSB	=	0.3%
%Bo/TA	=	25.5%
Quality of Fit	=	1.000 (Calculated from 4 parameter logistic curve fit)
20% Intercept	=	210.8 pg/mL
50% Intercept	=	37.4 pg/mL
80% Intercept	=	7.9 pg/mL

Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols[9].

Sensitivity

Sensitivity was calculated by determining the average optical density bound for sixteen (16) wells run as Bo, and comparing to the average optical density for sixteen (16) wells run with Standard #5. The detection limit was determined as the concentration of 17-OHP measured at two (2) standard deviations from the zero along the standard curve.

Average Optical Density for the Bo =	0.623 ± 0.020 (3.1%)
Average Optical Density for Standard #5 =	0.564 ± 0.026 (4.5%)
Delta Optical Density (0-3.91 pg/mL) =	0.059
2 SD's of the Zero Standard =	2 x 0.020 = 0.040
Sensitivity $= \frac{0.040}{0.059} \times 3.91 =$	**2.65 pg/mL**

Linearity

A sample containing 269 pg/mL 17-OHP was diluted 6 times 1:2 in the kit Assay Buffer and measured in the assay. The data was plotted graphically as actual 17-OHP concentration versus measured 17-OHP concentration.

The line obtained had a slope of 0.976 with a correlation coefficient of 0.999.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of 17-OHP and running these samples multiple times (n=16) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of 17-OHP in multiple assays (n=8) over multiple days (n=5).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of 17-OHP determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	17-OHP (pg/mL)	Intra-assay %CV	Inter-assay %CV
Low	3.58	16.5	
Medium	33.3	8.3	
High	220	4.3	
Low	4.9		13.9
Medium	32.2		5.9
High	225		11.3

Cross Reactivities

The cross reactivities for a number of related steroid compounds were determined by dissolving the cross reactant (purity checked by N.M.R. and other analytical methods) in Assay Buffer at concentrations from 10,000 to 0.1 pg/mL. These samples were then measured in the 17-OHP assay, and the measured 17-OHP concentration at 50% B/Bo calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

Compound	Cross Reactivity
17α-Hydroxyprogesterone	100%
17-Hydroxypregnenolone	1.69%
Progesterone	1.93%
17α-Hydroxypregnenolone sulfate	0.55%
16-Hydroxypregnenolone	<0.01%
21-Desoxycortisol	<0.01%
11-Desoxycortisol	<0.01%
Pregnanolone	<0.01%
4-Androsten-3,17-dione	<0.01%
Pregnenolone	<0.01%
DHEA	<0.01%
Cortisol	<0.01%

Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard preparation.

17-OHP concentrations were measured in a variety of different samples including Tissue Culture Media, human saliva, human serum, bovine heparin plasma and human amniotic fluid. For serum, plasma and amniotic fluid, the endogenous levels of 17-OHP in the samples were used. For Tissue Culture Media and saliva, 17-OHP was spiked into the undiluted samples of these matrices. The samples were diluted into the appropriate diluent and then assayed in the kit. The following results were obtained.:

Sample	% Recovery	Recommended Dilution
Tissue Culture Media	103.4	None
human Saliva	101.1	≥1:2
human Serum	101.5	≥1:4
bovine heparin Plasma	100.9	≥1:16
human Amniotic Fluid	106.3	≥1:16

References

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LIMITED WARRANTY

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For more details concerning the information within this kit insert, or to order any of Assay Designs' products, please call (734) 668-6113 between 8:30 a.m. and 5:30 p.m. EST. Orders or technical questions can also be transmitted by fax or e-mail 24 hours a day.

Material Safety Data Sheet (MSDS) available on our website or by fax.

Assay Designs, Inc.
5777 Hines Drive
Ann Arbor, MI 48108
U.S.A.

Telephone: (734) 668-6113
(800) 833-8651 (USA & Canada only)
Fax: (734) 668-2793
e-mail: info@assaydesigns.com
website: www.assaydesigns.com

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