

Assay Designs, Inc.

Correlate-EIA™

Cortisol

Enzyme Immunoassay Kit

Catalog No. 900-071

96 Well Kit

Table of Contents

Description	2
Introduction	2
Precautions	2
Materials Supplied	3
Storage	3
Materials Needed but Not Supplied	3
Sample Handling	4
Procedural Notes	5
Reagent Preparation	5
Assay Procedure	6
Calculation of Results	7
Typical Results	7
Typical Standard Curve	8
Typical Quality Control Parameters	8
Performance Characteristics	9
Sample Dilution Recommendations	11
References	11
Limited Warranty	12



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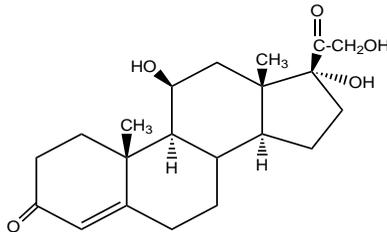
Description

The Assay Designs' Correlate-EIA™ Cortisol kit is a competitive immunoassay for the quantitative determination of Cortisol in biological fluids. Please read the complete kit insert before performing this assay. The kit for the quantitative measurement of Cortisol uses a monoclonal antibody to Cortisol to bind, in a competitive manner, Cortisol in a sample or an alkaline phosphatase molecule which has Cortisol 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 is read on a microplate reader at 405 nm. The intensity of the bound yellow color is inversely proportional to the concentration of Cortisol in either standards or samples. The measured optical density is used to calculate the concentration of Cortisol. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard¹ or Tijssen².

Introduction

Cortisol (hydrocortisone, compound F) is a steroid hormone synthesized from cholesterol. It is the primary glucocorticoid produced and secreted by the adrenal cortex. Cortisol is found in the blood either as free Cortisol, or bound to corticosteroid-binding globulin (CBG)^{3,4}. Serum levels are highest in the early morning and decrease throughout the day³. Cortisol is involved primarily in metabolic and immunological actions³. In the metabolic aspect, it promotes gluconeogenesis, liver glycogen deposition, and the reduction of glucose utilization³. Immunologically, Cortisol functions as an important anti-inflammatory, and plays a role in hypersensitivity, immunosuppression, and disease resistance³. It has also been shown that plasma Cortisol levels elevate in response to stress^{3,9}. Abnormal Cortisol levels are being tested for correlation with a variety of different conditions, these include: Prostate cancer⁵, depression⁶, and schizophrenia⁷. It is already known that an excess of Cortisol in all bodily tissues is the cause of Cushing's Syndrome⁸.

Cortisol



Precautions

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

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 Mg²⁺ and Zn²⁺ ions. The activity of the conjugate is affected by concentrations of chelators (>10 mM) such as EDTA and EGTA.

4. We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results.
5. The Cortisol Standard provided, Catalog No. 80-0677, is supplied in ethanolic buffer at a pH optimized to maintain Cortisol integrity. Care should be taken handling this material because of the known and unknown effects of steroids.

Materials Supplied

1. **Goat anti-Mouse IgG Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0050**
A plate using break-apart strips coated with goat antibody specific to mouse IgG.
2. **Cortisol EIA Conjugate, 6 mL, Catalog No. 80-0680**
A blue solution of alkaline phosphatase conjugated with Cortisol.
3. **Cortisol EIA Antibody, 6 mL, Catalog No. 80-0678**
A yellow solution of a mouse monoclonal antibody to Cortisol.
4. **Assay Buffer, 30 mL, Catalog No. 80-0010**
Tris buffered saline, containing proteins and sodium azide as a preservative.
5. **Wash Buffer Concentrate, 30 mL, Catalog No. 80-1286**
Tris buffered saline containing detergents.
6. **Cortisol Standard, 0.5 mL, Catalog No. 80-0677**
A solution of 100,000 pg/mL Cortisol.
7. **Steroid Displacement Reagent, 1 mL, Catalog No. 80-0120**
A special formulated displacer to inhibit steroid binding to proteins.
8. **pNpp Substrate, 20 mL, Catalog No. 80-0075**
A solution of p-nitrophenyl phosphate in buffer. Ready to use.
9. **Stop Solution, 5 mL, Catalog No. 80-0247**
A solution of trisodium phosphate in water. Keep tightly capped. Caution: **Caustic.**
10. **Cortisol Assay Layout Sheet, 1 each, Catalog No. 30-0145**
11. **Plate Sealer, 1 each, Catalog No. 30-0012**

Storage

All components of this kit are stable at 4 °C until the kit's expiration date.

Materials Needed but Not Supplied

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

Sample Handling

The Assay Designs' Correlate-EIA™ Cortisol enzyme immunoassay is compatible with Cortisol samples in a wide range of matrices. Samples diluted sufficiently into Assay Buffer can be read directly from the standard curve. Please refer to recovery data on page 11 for suitable dilutions for samples. However, the end user **must verify** that the recommended dilutions are appropriate for their samples. Included with the kit is the Steroid Displacement Reagent which should be added to serum, plasma and other samples containing steroid binding proteins. Samples should then be diluted with 1 part of the Reagent for every 99 parts of sample. **Samples containing mouse IgG may interfere with the assay.**

Samples in the majority of Tissue Culture Media 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 a small change in the 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 Cortisol in the appropriate matrix.

Some samples may have very low levels of Cortisol present and extraction may be necessary for accurate measurement. A suitable extraction procedure is outlined below:

Materials Needed

1. Cortisol Standard to allow extraction efficiency to be accurately determined.
2. ACS Grade Diethyl Ether.
3. Glass test tubes.

Procedure

1. Add sufficient Cortisol to a typical sample for determination of extraction efficiency.
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 steps 1 and 2 twice more, combining the ether layers.
5. Evaporate the ether to dryness under nitrogen.
6. Dissolve the extracted Cortisol with at least 250 μ L of Assay Buffer and vortex well. Allow to sit for five minutes at room temperature. Repeat twice more.
7. Run the reconstituted samples in the assay immediately or keep the dried samples frozen below -20 °C in desiccation.

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 addition of substrate, ensure that there is no residual wash buffer in wells. Any remaining wash buffer may cause variation in assay results.**

Reagent Preparation

1. Cortisol Standard

Allow the 100,000 pg/mL Cortisol standard solution to warm to room temperature. Label seven 12 x 75 mm glass tubes #1 through #7. Pipet 1,000 µL of standard diluent (Assay Buffer or Tissue Culture Media) into tube #1. Pipet 500 µL of standard diluent into tubes #2 through #7. Remove 100 µL of diluent from tube #1. Add 100 µL of the 100,000 pg/mL standard to tube #1. Vortex thoroughly. Add 500 µL of tube #1 to tube #2 and vortex thoroughly. Add 500 µL of tube #2 to tube #3 and vortex. Continue this for tubes #4 through #7.

The concentration of Cortisol in tubes #1 through #7 will be 10,000, 5,000, 2,500, 1,250, 625, 313 and 156 pg/mL respectively. See the Cortisol Assay Layout Sheet for dilution details.

Diluted standards 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 #7 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 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 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 Cortisol in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If this sort of data reduction software is not readily available, the concentration of Cortisol 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:

$$\text{Average Net OD} = \text{Average Bound OD} - \text{Average NSB OD}$$
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 Cortisol for the standards. Approximate a straight line through the points. The concentration of Cortisol in the unknowns can be determined by interpolation.

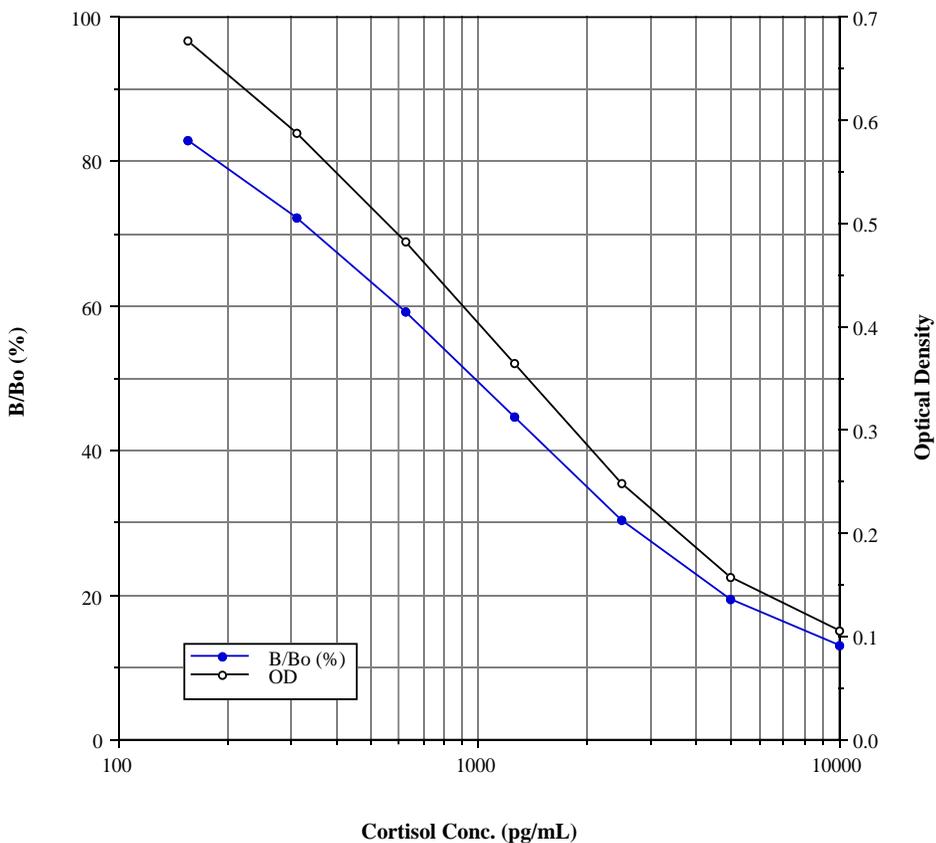
Typical Results

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

<u>Sample</u>	<u>Mean OD (-Blank)</u>	<u>Average Net OD</u>	<u>Percent Bound</u>	<u>Cortisol (pg/mL)</u>
Blank OD	(0.077)			
TA	0.307			
NSB	0.001	0.000	0.0	
Bo	0.816	0.815	100%	0
S1	0.105	0.104	12.9%	10,000
S2	0.158	0.157	19.3%	5,000
S3	0.248	0.247	30.4%	2,500
S4	0.365	0.364	44.7%	1,250
S5	0.482	0.481	59.1%	625
S6	0.588	0.587	72.1%	313
S7	0.676	0.675	82.9%	156
Unknown 1	0.260	0.259	31.8%	2,311
Unknown 2	0.462	0.461	56.6%	702

Typical Standard Curve

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



Typical Quality Control Parameters

Total Activity Added = $0.307 \times 10 = 3.07$

%NSB = 0.03%

%Bo/TA = 26.58%

Quality of Fit = 1.00

20% Intercept = 4919 pg/mL

50% Intercept = 957 pg/mL

80% Intercept = 192 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 Protocols¹⁰.

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 #7. The detection limit was determined as the concentration of Cortisol measured at two (2) standard deviations from the zero along the standard curve.

Average Optical Density for the Bo = 0.791 ± 0.020 (2.3%)

Average Optical Density for Standard #7 = 0.681 ± 0.024 (3.1%)

Delta Optical Density (0-156 pg/mL) = 0.110

2 SD's of the Zero Standard = 2 x 0.020 = 0.040

Sensitivity = $\frac{0.040}{0.110} \times 156.0 \text{ pg/mL} = \mathbf{56.72 \text{ pg/mL}}$

Linearity

A sample containing 7,789 pg/mL Cortisol was diluted 5 times 1:2 in the kit Assay Buffer and measured in the assay. The data was plotted graphically as actual Cortisol concentration versus measured Cortisol concentration.

The line obtained had a slope of 1.046 and a correlation coefficient of 0.999.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of Cortisol 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 Cortisol in multiple assays (n=8).

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

	<u>Cortisol</u> (pg/mL)	<u>Intra-Assay</u> %CV	<u>Inter-Assay</u> %CV
Low	333	10.5	
Medium	1,088	6.6	
High	3,155	7.3	
Low	451		13.4
Medium	969		7.8
High	3,052		8.6

Cross Reactivity

The cross reactivities for a number of related steroid compounds was determined by dissolving the cross reactant (purity checked by N.M.R. and other analytical methods) in Assay Buffer at concentrations from 100,000 to 10 pg/mL. These samples were then measured in the Cortisol assay, and the measured Cortisol concentration at 50% B/Bo was calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

<u>Compound</u>	<u>Cross Reactivity</u>
Cortisol	100%
Prednisolone	122.35%
Corticosterone	27.68%
11-deoxycortisol	4.0%
Progesterone	3.64%
Prednisone	0.85%
Testosterone	0.12%
Androstenedione	<0.1%
Cortisone	<0.1%
Estradiol	<0.1%

Sample Recoveries

Cortisol concentrations were measured in a variety of different samples including tissue culture media, human saliva and urine, and porcine serum and plasma. For samples in tissue culture media, ensure that the standards have been diluted into the same media (refer to page 4). Cortisol was spiked into the undiluted samples of these media which were then diluted with the kit Assay Buffer and then assayed in the kit. The following results were obtained:

<u>Sample</u>	<u>% Recovery*</u>	<u>Recommended Dilution*</u>
Tissue Culture Media	106.6	Neat
Human Saliva	97.4	≥1:4
Human Urine	102.2	None
Porcine Serum	103.8	≥1:8
Porcine Plasma	106.1	≥1:8

* See Sample Handling instructions on page 4 for details.

References

1. T. Chard, "An Introduction to Radioimmunoassay & Related Techniques 4th Ed", (1990) Amsterdam: Elsevier.
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7. C.R. Dequet and D.J. Wallace, Current Opin Invest Drugs, (2001) 8: 1045-53.
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9. National Committee for Clinical Laboratory Standards Evaluation Protocols, SC1, (1989) Villanova, PA: NCCLS.

LIMITED WARRANTY

Assay Designs, Inc. warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

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

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