

Corticosterone Enzyme Immunoassay Kit

Catalog No. 901-097

480 Well (5 x 96 Well) Kit

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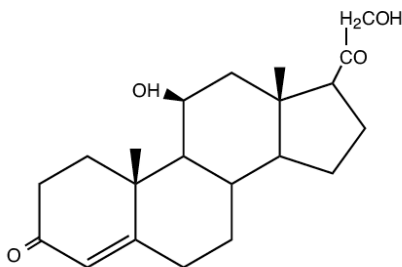
Description

The Assay Designs™ Corticosterone Enzyme Immunoassay (EIA) Kit is a competitive immunoassay for the quantitative determination of Corticosterone in biological fluids. Please read the complete kit insert before performing this assay. The kit uses a polyclonal antibody to Corticosterone to bind, in a competitive manner, Corticosterone in the standard or sample or an alkaline phosphatase molecule which has Corticosterone 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 Corticosterone in either standards or samples. The measured optical density is used to calculate the concentration of Corticosterone. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard¹ or Tijssen².

Introduction

Corticosterone (also referred to as Kendall's Compound B and Reichstein Substance B) is a glucocorticoid secreted by the cortex of the adrenal gland. Corticosterone is produced in response to the stimulation of the adrenal cortex by adrenocorticotrophic hormone (ACTH) and is the precursor of aldosterone. Corticosterone is a major indicator of stress since stress increases the production of corticosteroids. Studies involving corticosterone and levels of stress include impairment of long term memory retrieval³, chronic corticosterone elevation due to dietary restrictions⁴ and in response to burn injuries⁵. In addition to stress levels, corticosterone is believed to play a decisive role in sleep-wake patterns^{6,7}.

Corticosterone



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. 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 Corticosterone Standard provided, Catalog No. 80-0916, is supplied in ethanolic buffer at a pH optimized to maintain Corticosterone integrity. Care should be taken handling this material because of the known and unknown effects of steroids.

Materials Supplied

1. **Donkey anti-Sheep IgG Microtiter Plate, Five Plates of 96 Wells, Catalog No. 80-0045**
A plate using break-apart strips coated with donkey antibody specific to sheep IgG.
2. **Corticosterone EIA Conjugate, 25 mL, Catalog No. 80-0919**
A blue solution of alkaline phosphatase conjugated with Corticosterone.
3. **Corticosterone EIA Antibody, 25 mL, Catalog No. 80-0920**
A yellow solution of a sheep polyclonal antibody to Corticosterone.
4. **Assay Buffer 15 Concentrate, 100 mL, Catalog No. 80-1009**
Tris buffered saline containing proteins and sodium azide as preservative.
5. **Wash Buffer Concentrate, 100 mL, Catalog No. 80-1287**
Tris buffered saline containing detergents.
6. **Corticosterone Standard, 3 x 0.5 mL, Catalog No. 80-0916**
A solution of 200,000 pg/mL Corticosterone.
7. **Steroid Displacement Reagent, 2 mL, Catalog No. 80-0925**
A special formulated displacer to inhibit steroid binding to proteins.
8. **p-Npp Substrate, 100 mL, Catalog No. 80-0076**
A solution of p-nitrophenyl phosphate in buffer. Ready to use.
9. **Stop Solution, 30 mL, Catalog No. 80-0248**
A solution of trisodium phosphate in water. Keep tightly capped. Caution: **Caustic.**
10. **Corticosterone Assay Layout Sheet, 1 each, Catalog No. 30-0162**
11. **Plate Sealer, 5 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™ Corticosterone EIA Kit is compatible with Corticosterone samples in a wide range of matrices. Samples diluted sufficiently into Assay Buffer 15 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. 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 be diluted with 2.5 parts of the Reagent for every 97.5 parts of sample. **Samples containing sheep IgG may interfere with the assay.** Please contact our Technical Support department for an alternate procedure for small volume serum/plasma samples.

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 15. 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 Corticosterone in the appropriate matrix.

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

Materials Needed

1. Corticosterone Standard to allow extraction efficiency to be accurately determined.
2. ACS Grade Ethyl Acetate.
3. Glass test tubes.

Procedure

1. Add sufficient Corticosterone to a typical sample for determination of extraction efficiency.
2. In a fume hood, add 1 mL of Ethyl Acetate for every mL of sample. Stopper and shake sample.
3. Allow layers to separate. Carefully pipet off the top organic layer and place in a clean test tube.
4. Repeat steps 1 and 2 twice more, combining the organic layers.
5. Evaporate the Ethyl Acetate to dryness under nitrogen.
6. Dissolve the extracted Corticosterone with at least 250 μ L of Assay Buffer 15. Vortex well then 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. Assay Buffer 15

Just before use prepare the Assay Buffer 15 by diluting 10 mL of the supplied concentrate with 90 mL of deionized water. Discard unused buffer or add up to 0.09% sodium azide (w/v) for storage.

2. Corticosterone Standard

Allow the 200,000 pg/mL Corticosterone standard solution to warm to room temperature. Label five 12 x 75 mm glass tubes #1 through #5. Pipet 1,000 μ L of standard diluent (Assay Buffer 15 or Tissue Culture Media) into tube #1. Pipet 800 μ L of standard diluent (Assay Buffer 15 or Tissue Culture Media) into tubes #2 through #5. Remove 100 μ L of diluent from tube #1. Add 100 μ L of the 200,000 pg/mL standard to tube #1. Vortex thoroughly. Add 200 μ L of tube #1 to tube #2 and vortex thoroughly. Add 200 μ L of tube #2 to tube #3 and vortex. Continue this for tubes #4 and #5.

The concentration of Corticosterone in tubes #1 through #5 will be 20,000, 4,000, 800, 160 and 32 pg/mL respectively. See the Corticosterone Assay Layout Sheet for dilution details.

Diluted standards should be used within 60 minutes of preparation.

3. 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 15 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 15 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 Corticosterone 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 Corticosterone 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 Corticosterone for the standards. Approximate a straight line through the points. The concentration of Corticosterone in the unknowns can be determined by interpolation.

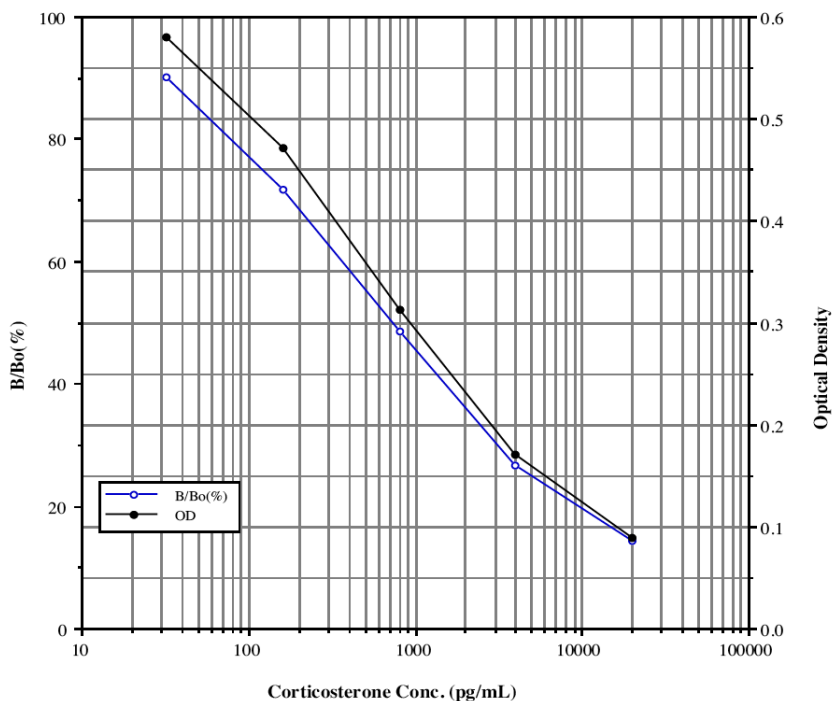
Typical Results

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>Corticosterone (pg/mL)</u>
Blank OD	0.087			
TA	0.263			
NSB	0.087	0.000	0.0	
Bo	0.761	0.674	100%	0
S1	0.176	0.089	12.6%	20,000
S2	0.258	0.171	24.8%	4,000
S3	0.400	0.313	48.1%	800
S4	0.558	0.471	78.4%	160
S5	0.667	0.580	92.0%	32
Unknown 1	0.639	0.552	81.9%	54
Unknown 2	0.460	0.372	55.2%	445

Typical Standard Curve

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



Typical Quality Control Parameters

Total Activity Added	=	$0.263 \times 10 = 2.63$
%NSB	=	0.015%
%Bo/TA	=	25.63%
Quality of Fit	=	1.00 (Calculated from 4 parameter Logistic Curve fit)
20% Intercept	=	7,073 pg/mL
50% Intercept	=	630 pg/mL
80% Intercept	=	66 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 Protocols⁸.

Sensitivity

Sensitivity was calculated in Assay Buffer 15 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 Corticosterone measured at two (2) standard deviations from the zero along the standard curve.

Average Optical Density for the Bo = 1.103 ± 0.035 (3.2%)

Average Optical Density for Standard #5 = 1.020 ± 0.022 (2.2%)

Delta Optical Density (0-32 pg/mL) = 0.083

2 SD's of the Zero Standard = 2 x 0.035 = 0.070

Sensitivity = $\frac{0.070}{0.083}$ x 32 pg/mL = **26.99 pg/mL**

Linearity

A sample containing 4,111 pg/mL Corticosterone was diluted 5 times 1:2 in the kit Assay Buffer 15 and measured in the assay. The data was plotted graphically as actual Corticosterone concentration versus measured Corticosterone concentration.

The line obtained had a slope of 0.931 and a correlation coefficient of 0.999.

Precision

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

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

	Corticosterone (pg/mL)	Intra-assay <u>%CV</u>	Inter-assay <u>%CV</u>
Low	171	8.0	
Medium	403	8.4	
High	780	6.6	
Low	174		13.1
Medium	415		8.2
High	780		7.8

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 15 at concentrations from 100,000 to 10 pg/mL. These samples were then measured in the Corticosterone assay, and the measured Corticosterone 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>
Corticosterone	100%
Deoxycorticosterone	28.6%
Progesterone	1.7%
Testosterone	0.13%
Tetrahydrocorticosterone	0.28%
Aldosterone	0.18%
Cortisol	0.046%
Pregnenolone	< 0.03%
β-Estradiol	< 0.03%
Cortisone	< 0.03%
11-dehydrocorticosterone acetate	< 0.03%

Sample Recoveries

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

Corticosterone concentrations were measured in a variety of different samples including tissue culture media, human saliva, and equine serum and plasma. Corticosterone was spiked into the undiluted samples of these matrices which were then diluted with the appropriate diluent and assayed in the kit. The following results were obtained:

<u>Sample</u>	<u>% Recovery*</u>	<u>Recommended Dilution*</u>
Tissue Culture Media	103.9	None
Human Saliva	105.9	≥1:16
Equine Serum	112.0	≥1:8
Equine Heparinized Plasma	99.7	≥1:50
Rhinoceros Feces	93.9	≥1:100
Mouse Feces	109.0	≥1:500

* See Sample Handling instructions on page 4 for details.

References

1. T. Chard, "An Introduction to Radioimmunoassay & Related Techniques 4th Ed.", (1990) Amsterdam: Elsevier.
2. P. Tijssen, "Practice & Theory of Enzyme Immunoassays", (1985) Amsterdam: Elsevier.
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5. O. Thellin, Shock, (2001) 16(5): 393-397.
6. K.M. Krame and R.B. Sothorn, Chronobiol. Int., (2001) 18(6): 933-945.
7. G. Vazquez-Palacios, *et al.*, Pharmacol. Biochem. Behavior, (2001) 70(2-3): 305-310.
8. 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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