

Cortisol EIA Kit

Cat #: orb1822515 (manual)

Precautions

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete booklet should be read and understood before attempting to use the product.

The antibody coated plate needs to be stored desiccated. The silica gel pack included in the foil ziploc bag will keep the plate dry.

This kit utilizes a peroxidase-based readout system. Buffers, including other manufacturers Wash Buffers, containing sodium azide will inhibit color production from the enzyme. Make sure all buffers used for samples are azide free. Ensure that any plate washing system is rinsed well with deionized water prior to using the supplied Wash Buffer as prepared on page 9.

The Stop Solution is acid. The solution should not come in contact with skin or eyes. Take appropriate precautions when handling this reagent.

Storage

All components of this kit should be stored at 4°C until the expiration date of this kit.

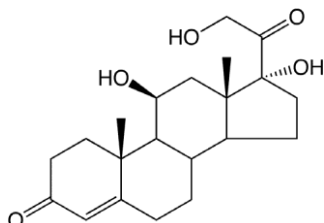
Materials Needed But Not Supplied

- Distilled or deionized water.
- Repeater pipet with disposable tips capable of dispensing 25 μ L, 50 μ L and 100 μ L.
- Colorimetric 96 well microplate reader capable of reading optical density at 450 nm.
- Software for converting raw relative optical density readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details.
- Orbital micro-plate shaker

Background

Cortisol, C₂₁H₃₀O₅, (hydrocortisone) is the primary glucocorticoid produced and secreted by the adrenal cortex. It is often referred to as the “stress hormone” as it is involved in the response to stress and it affects blood pressure, blood sugar levels, and other actions of stress adaptation. Immunologically, cortisol functions as an important anti-inflammatory and plays a role in hypersensitivity, immunosuppression, and disease resistance¹. In the metabolic aspect, cortisol promotes gluconeogenesis, liver glycogen deposition, and the reduction of glucose utilization². Production of cortisol follows an ACTH-dependent circadian rhythm, with a peak level in the morning and decreasing levels throughout the day. Most serum cortisol, all but about 4%, is bound to proteins including corticosteroid binding globulin and serum albumin^{1,3}. Only free cortisol is available to most receptors and it is through these receptors that physiological processes are

modulated. Abnormal cortisol levels are being evaluated for correlation with a variety of different conditions, such as prostate cancer⁴, depression⁵, and schizophrenia⁶. It is already known that abnormal levels of cortisol are involved in Cushing's Syndrome and Addison's disease⁷.



Assay Overview

The Cortisol EIA kit is designed to quantitatively measure cortisol present in dried fecal extracts, saliva, urine, serum, plasma and tissue culture media samples. Please read the complete kit booklet before performing this assay. This kit measures total cortisol in extracted samples and in serum and plasma and free cortisol in saliva and urine. A cortisol standard is provided to generate a standard curve for the assay and all samples should be read off the standard curve. Standards or diluted samples are pipetted into a clear microtiter plate coated with an antibody to capture mouse antibodies. A cortisol-peroxidase conjugate is added to the standards and samples in the wells. The binding reaction is initiated by the addition of a monoclonal antibody to cortisol to each well. After an 1 hour incubation the plate is washed and substrate is added. The substrate reacts with the bound cortisol- peroxidase conjugate. After a short incubation, the reaction is stopped and the intensity of the generated color is detected in a microtiter plate reader capable of measuring 450 nm wavelength. The concentration of the cortisol in the sample is calculated, after making suitable correction for the dilution of the sample, using software available with most plate readers.

Sample Types

Sample Types Validated: Dried Fecal Extracts, Saliva, Urine, Serum, EDTA and Heparin Plasma and Tissue Culture Media

Cortisol is identical across all species and we expect this kit may measure cortisol from sources other than human. The end user should evaluate recoveries of cortisol in other samples being tested.

Please note that samples containing mouse IgG may interfere with this assay.

This assay has been validated for saliva, urine, serum and EDTA and heparin plasma samples and for tissue culture samples. It has also been validated for dried fecal extract samples. Samples containing visible particulate should be centrifuged prior to using. Moderate to severely hemolyzed samples should not be used in this kit.

Sample Preparation

Serum and plasma samples need to be treated with the supplied Dissociation Reagent. Addition of this reagent will yield the total cortisol concentration in serum or plasma. Dissociation Reagent is to be used only with Serum and Plasma samples. Free cortisol can be measured in saliva and urine samples as directed below.

Dried Fecal Samples

We have a detailed Extraction Protocol available on this product's website page. The ethanol concentration in the final Assay Buffer dilution added to the well should be <5%.

Serum and Plasma Samples

Allow the Dissociation Reagent (DR) to warm completely to Room Temperature before use. We suggest pipetting 5 μ L of DR into 1 mL Eppendorf tubes. Add 5 μ L of serum or plasma to the DR in the tube, vortex gently and incubate at room temperature for 5 minutes or longer. Dilute with 490 μ L of supplied Assay Buffer. This 1:100 dilution can be diluted further with Assay Buffer. Final serum and plasma dilutions should be \geq 1:100.

NOTE: Dissociation Reagent is to be used only with Serum and Plasma samples.

Saliva Samples

Saliva samples should be diluted \geq 1:4 or greater with the supplied Assay Buffer prior running in the assay. See our Saliva Sample Handling Instructions available on this product's website page.

Urine Samples

Urine samples should be diluted \geq 1:8 with the supplied Assay Buffer prior running in the assay. Urinary cortisol normally ranges from 0.7-119 μ g/gram⁸ of creatinine or approximately 100,000 to 1,000,000 pg/mL⁹ in 24-hour urine samples. Samples may need to be diluted substantially to read within the standard curve range.

Tissue Culture Media

For measuring cortisol in tissue culture media (TCM), samples should be read off a standard curve generated in TCM. Samples may need to be diluted further in TCM. We have validated the assay using RPMI-1640.

Use all Samples within 2 Hours of preparation, or stored at \leq -20°C until assaying.

Reagent Preparation

Allow the kit reagents to come to room temperature for 30 minutes. We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine cortisol concentrations. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

Cortisol Conjugate

1. Determine the amount of Cortisol Conjugate preparation required. For every immunoassay plate strip used (8 wells), prepare 300 μ L of Cortisol Conjugate preparation.
2. Prepare Cortisol Conjugate preparation by diluting the Cortisol Conjugate 1/100 with Cortisol Conjugate Diluent (Purple). Mix well prior to use. For example, if 3mL of Cortisol Conjugate preparation is required, dilute 30 μ L of Cortisol Conjugate in 3mL of Cortisol Conjugate Diluent.
*Store the reconstituted Cortisol Conjugate on ice and use within 2 hours. Store any unused Cortisol Conjugate (Concentrate) at -20°C.

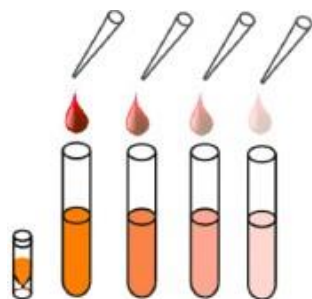
Wash Buffer

Dilute Wash Buffer Concentrate 1:10 by adding one part of the concentrate to nine parts of deionized water. Once diluted this is stable at room temperature for 3 months at room temperature.

Standard Preparation

Label six test tubes as #1 through #6. Pipet 450 μL of Assay Buffer into tube #1 and 250 μL into tubes #2 to #6. The cortisol stock solution contains an organic solvent. Prerinse the pipet tip several times to ensure accurate delivery. Carefully add 50 μL of the cortisol stock solution to tube #1 and vortex completely. Take 250 μL of the cortisol solution in tube #1 and add it to tube #2 and vortex completely. Repeat the serial dilutions for tubes #3 through #6. The concentration of cortisol in tubes 1 through 6 will be 3,200, 1,600, 800, 400, 200, and 100 pg/mL .

Use all Standards within 2 hours of preparation.



| | Standard 1 | Standard 2 | Standard 3 | Standard 4 | Standard 5 | Standard 6 |
|----------------------------------------|------------|------------|------------|------------|------------|------------|
| Assay Buffer Volume (μL) | 450 | 250 | 250 | 250 | 250 | 250 |
| Addition | Stock | Standard 1 | Standard 2 | Standard 3 | Standard 4 | Standard 5 |
| Volume of Addition (μL) | 50 | 250 | 250 | 250 | 250 | 250 |
| Final Concentration (pg/mL) | 3,200 | 1,600 | 800 | 400 | 200 | 100 |

Assay Protocol

1. Use the plate layout sheet on page 19 to aid in proper sample and standard identification. Determine the number of wells to be used and return unused wells to foil pouch with desiccant. Seal the ziploc plate bag and store at 4°C .
2. Pipet 50 μL of samples or standards into wells in the plate.
3. Pipet 75 μL of Assay Buffer into the non-specific binding (NSB) wells.
4. Pipet 50 μL of Assay Buffer into wells to act as maximum binding wells (Bo or 0 pg/mL).
5. Add 25 μL of the Cortisol Conjugate to each well using a repeater pipet.
6. Add 25 μL of the Cortisol Antibody to each well, except the NSB wells, using a repeater pipet.
7. Gently tap the sides of the plate to ensure adequate mixing of the reagents. Cover the plate with the plate sealer and shake at room temperature for 1 hour at 600rpm* critical step for proper assay function *

8. Aspirate the plate and wash each well 4 times with 300 μ L wash buffer. Tap the plate dry on clean absorbent towels.
9. Add 100 μ L of the TMB Substrate to each well, using a repeater pipet.
10. Incubate the plate at room temperature for 30 minutes with shaking.
11. Add 100 μ L of the Stop Solution to each well, using a repeater pipet.
12. Read the optical density generated from each well in a plate reader capable of reading at 450 nm.
13. Use the plate reader's built-in 4PLC software capabilities to calculate cortisol concentration for each sample.

Calculation of Results

Average the duplicate OD readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean OD's for the NSB. The sample concentrations obtained, calculated from the %B/B0 curve, should be multiplied by the dilution factor to obtain neat sample values.

Typical Data

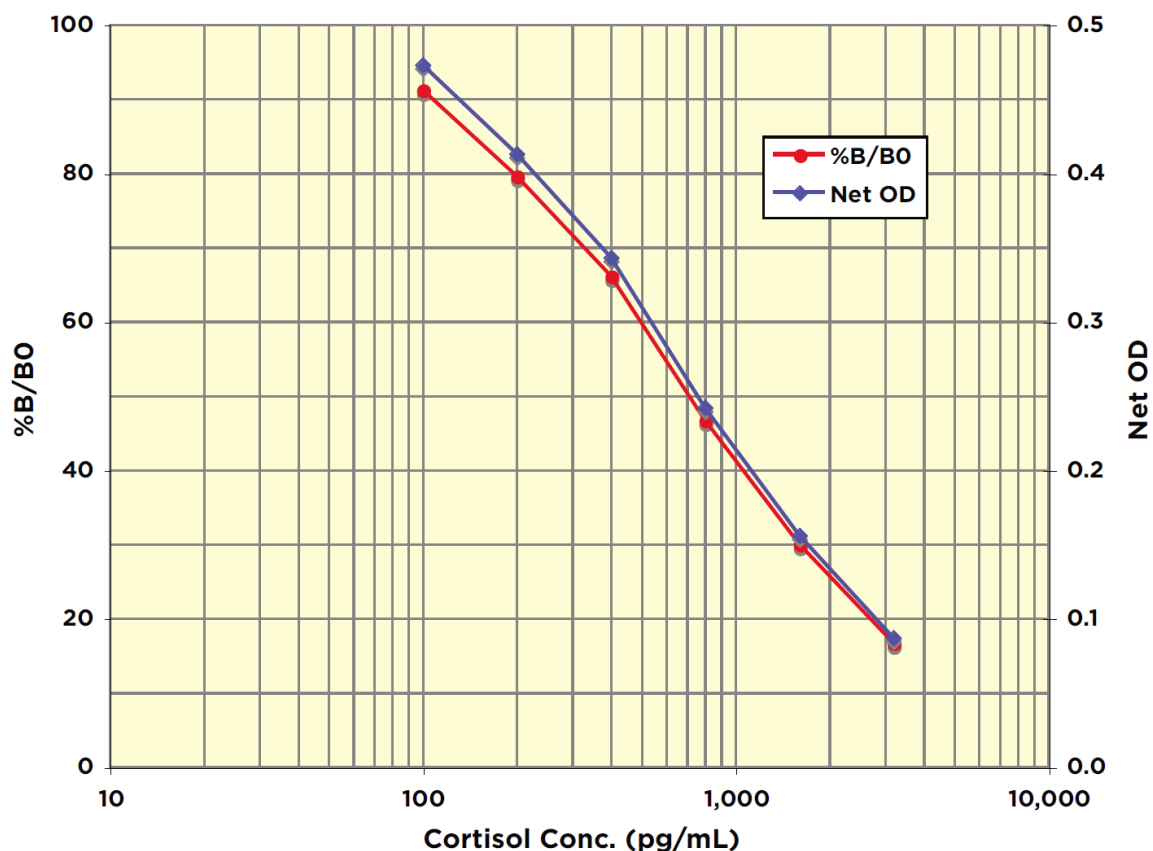
| <u>Sample</u> | <u>Mean OD</u> | <u>Net OD</u> | <u>% B/B0</u> | <u>Cortisol Concentration (pg/mL)</u> |
|-------------------|----------------|---------------|---------------|---------------------------------------|
| <u>NSB</u> | <u>0.044</u> | <u>0</u> | <u>-</u> | <u>-</u> |
| <u>Standard 1</u> | <u>0.131</u> | <u>0.087</u> | <u>16.7</u> | <u>3,200</u> |
| <u>Standard 2</u> | <u>0.200</u> | <u>0.156</u> | <u>30.1</u> | <u>1,600</u> |
| <u>Standard 3</u> | <u>0.286</u> | <u>0.242</u> | <u>46.7</u> | <u>800</u> |
| <u>Standard 4</u> | <u>0.387</u> | <u>0.343</u> | <u>66.2</u> | <u>400</u> |
| <u>Standard 5</u> | <u>0.457</u> | <u>0.413</u> | <u>79.7</u> | <u>200</u> |
| <u>Standard 6</u> | <u>0.517</u> | <u>0.473</u> | <u>91.3</u> | <u>100</u> |
| <u>B0</u> | <u>0.562</u> | <u>0.518</u> | <u>100.0</u> | <u>0</u> |
| <u>Sample 1</u> | <u>0.137</u> | <u>0.093</u> | <u>18.0</u> | <u>2974.9</u> |
| <u>Sample 2</u> | <u>0.478</u> | <u>0.434</u> | <u>83.8</u> | <u>163.9</u> |

Always run your own standard curve for calculation of results.

Do not use this data.

Conversion Factor: 100 pg/mL of cortisol is equivalent to 275.9 pM.

Typical Standard Curves



Validation Data

Sensitivity and Limit of Detection

Sensitivity was calculated by comparing the OD’s for nineteen wells run for each of the B0 and standard #6. The detection limit was determined at two (2) standard deviations from the B0 along the standard curve.

Sensitivity was determined as 30.3 pg/mL.

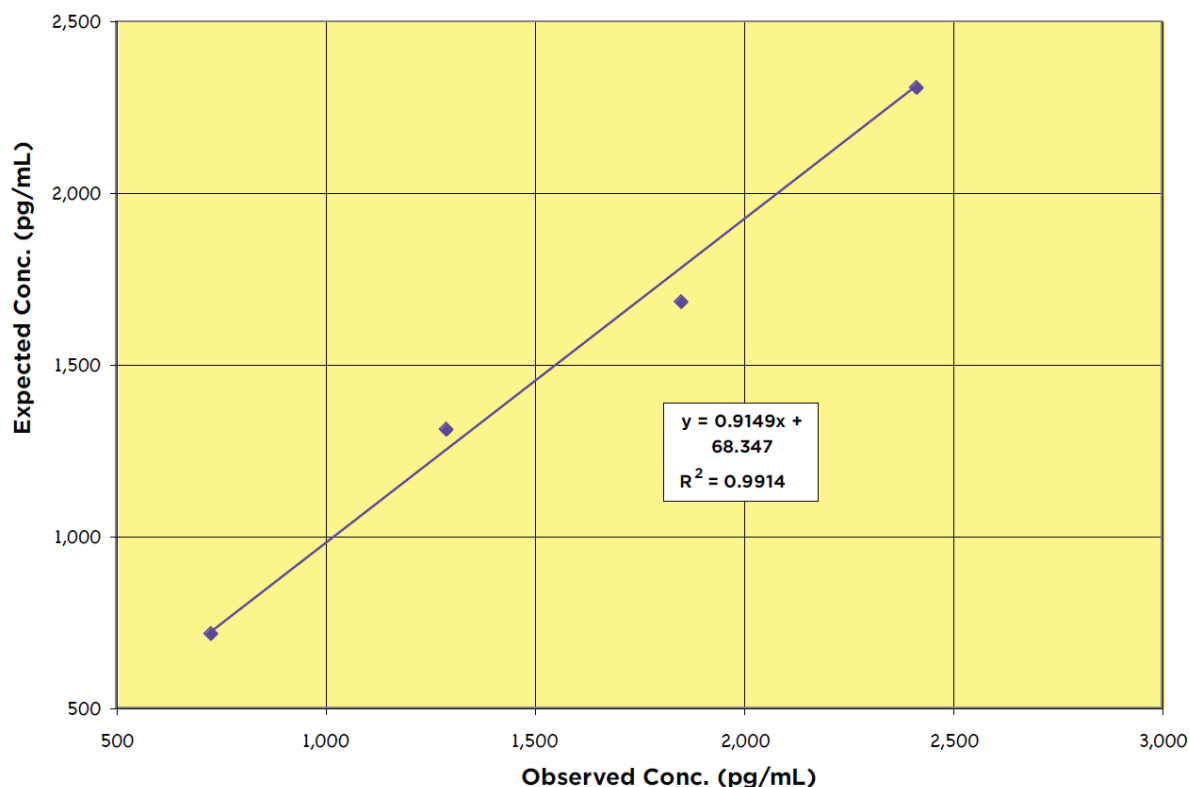
The Limit of Detection for the assay was determined in a similar manner by comparing the OD’s for twenty runs for each of the zero standard and a low concentration human sample. Limit of Detection was determined as 55.4 pg/mL

Linearity

Linearity was determined by taking two human urine samples diluted 1:140, one with a low diluted cortisol level of 163.9 pg/mL and one with a higher diluted level of 2,974.9 pg/mL and mixing them in the ratios given below. The measured concentrations were compared to the expected values based on the ratios used.

| Low Urine | High Urine | Observed Concentration (pg/mL) | Expected Concentration (pg/mL) | % Recovery |
|-----------|------------|--------------------------------|--------------------------------|------------|
| 100% | 0% | 163.9 | -- | -- |
| 80% | 20% | 715.7 | 726.1 | 98.6 |
| 60% | 40% | 1,311.5 | 1,288.3 | 101.8 |

| | | | | |
|-----|------|---------|---------------|-------|
| 40% | 60% | 1,683.3 | 1,850.5 | 91.0 |
| 20% | 80% | 2,306.3 | 2,412.7 | 95.6 |
| 0% | 100% | 2,974.9 | -- | -- |
| | | | Mean Recovery | 96.7% |



Intra Assay Precision

Three human samples were diluted with Assay Buffer and run in replicates of 20 in an assay. The mean and precision of the calculated Cortisol concentrations were:

| Sample | Cortisol Concentration (pg/ mL) | %CV |
|--------|---------------------------------|------|
| 1 | 1,174.3 | 6.0 |
| 2 | 475.9 | 5.6 |
| 3 | 177.4 | 14.7 |

Inter Assay Precision

Three human samples were diluted with Assay Buffer and run in duplicates in ten assays run over multiple days by four operators. The mean and precision of the calculated Cortisol concentrations were:

| Sample | Cortisol Concentration (pg/ mL) | %CV |
|--------|---------------------------------|------|
| 1 | 1,188.1 | 7.2 |
| 2 | 508.7 | 6.3 |
| 3 | 199.7 | 10.9 |

Sample Values

Six random human serum and plasma samples were tested in the assay. Neat sample values ranged from 8.5 to 23.8 µg/dL with an average of 12.2 µg/dL. The normal reference range for serum cortisol is 3-23 µg/dL⁹. Four random human urine samples were tested in the assay. Neat sample values ranged from 98.1 to 304.9 µg/g creatinine with an average of 159.8 µg/g creatinine.

Dried fecal samples were processed as described on page 7 and run in the assay. Samples kindly donated by Dr. J. Williams at the Indianapolis Zoo, which included Amur Tiger, Giraffe, Kudu, Lion, Reeves Muntjac, White Handed Gibbon, White Rhino, and Zebra, were tested and cortisol values obtained ranged from 2.48 to 27.22 pg/mg dried fecal material.

Palme and Möestl and colleagues have shown that radiolabeled administered cortisol is excreted in differing amounts in urine and feces¹⁰ across species, with fecal excretion ranging from 7% of administered cortisol in the pig to 82% in the cat^{11,12, 13}. Palme has also shown that the peak of fecal cortisol concentrations occur at 12 hours for sheep, but takes 48 hours to peak in pigs. It is therefore necessary to evaluate the ²⁰timing and relative fecal or urine excretion of glucocorticoids for each species.

Cross Reactivity

The following cross reactants were tested in the assay and calculated at the 50% binding point.

| Steroid | Cross Reactivity (%) |
|----------------------------------|----------------------|
| Cortisol | 100% |
| 11-Deoxycorticosterone | <0.1% |
| Prednisolone (1-Dehydrocortisol) | 5.6% |
| Corticosterone | 0.6% |
| 11-Hydroxyprogesterone | <0.1% |
| Progesterone | <0.1% |
| Estradiol | <0.1% |
| Danazol | <0.01% |

Warranty and Limitation of Remedy

For further details, please refer to our Warranty and Refund Policy located on our website and in our catalog.