Cortisol Immunoassay

Catalog Number DE2700

For the quantitative determination of Cortisol concentrations in cell culture supernates, serum, plasma, urine, saliva and other biological fluids.

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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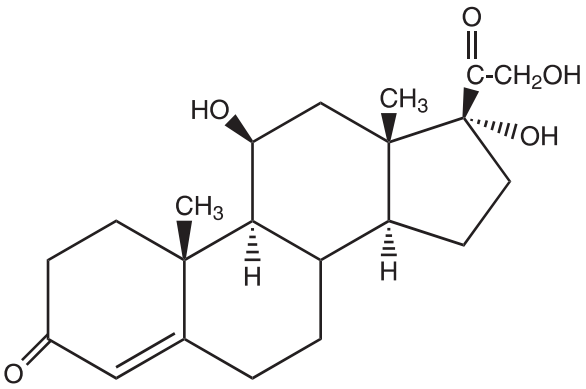
INTRODUCTION

Cortisol (also known as hydrocortisone or 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) (1, 2). Serum levels are highest in the early morning and decrease throughout the day (1). Cortisol is involved primarily in metabolic and immunological actions (1). In the metabolic aspect, it promotes gluconeogenesis, liver glycogen deposition, and the reduction of glucose utilization (1). Immunologically, cortisol functions as an important anti-inflammatory and plays a role in hypersensitivity, immunosuppression, and disease resistance (1). It has also been shown that plasma cortisol levels elevate in response to stress (1, 3). Abnormal cortisol levels are being tested for correlation with a variety of different conditions. These include prostate cancer (4), depression (5), and schizophrenia (6). An excess of cortisol in all bodily tissues is the cause of Cushing's Syndrome (7).

Cortisol



R&D Systems' Cortisol Immunoassay is a 3 hour competitive enzyme immunoassay designed to measure cortisol in cell culture supernates, urine, serum, plasma, saliva and other biological fluids.

PRINCIPLE OF THE ASSAY

This assay is based on the competitive binding technique in which cortisol present in a sample competes with a fixed amount of alkaline phosphatase-labeled cortisol for sites on a mouse monoclonal antibody. During the incubation, the 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 cortisol 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 reagents from different lots.
- Allow reagents to warm to room temperature before use.
- The unopened bottle of pNPP Substrate should be colorless to pale yellow.
- Care must be taken to minimize contamination by endogenous alkaline phosphatase. Contaminating alkaline phosphatase, especially in the substrate, may lead to high blanks. Precautions should be taken to avoid contamination from bare hands, saliva, and contaminated labware.
- 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 and repeat the assay.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Alkaline Phosphatase is a temperature sensitive enzyme. Optical Density (OD) units may vary with temperature changes.
- 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.

REAGENTS

Microplate (Part R80-0050) - 96 well microplate coated with a goat anti-mouse polyclonal antibody.

Cortisol Conjugate (Part R80-0680) - 6 mL of Cortisol conjugated to alkaline phosphatase, with blue dye and preservative.

Cortisol Standard (Part R80-0677) - 0.5 mL of Cortisol (100,000 pg/mL) in buffer, with preservative.

Cortisol Antibody Solution (Part R80-0678) - 6 mL of mouse monoclonal antibody to Cortisol, with yellow dye and preservative.

Steroid Displacement Reagent (Part R80-0120) - 1 mL of a specially formulated displacer to inhibit steroid binding to proteins.

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

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

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

Stop Solution (Part R80-0247) - 5 mL of a trisodium phosphate (TSP) solution. **Keep tightly capped. Caution: Caustic.**

Plate Covers - 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="7">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>pNPP Substrate</td></tr>
<tr><td>Steriod Displacement Reagent</td></tr>
<tr><td>Antibody Solution</td></tr>
<tr><td>Standard</td></tr>
<tr><td>Conjugate</td></tr>
<tr><td>Microplate Wells</td><td>Return unused wells to the pouch containing the desiccant pack, reseal along entire edge of 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.
- 500 mL graduated cylinder for preparation of Wash Buffer.
- Deionized or distilled water.
- Squirt bottle or manifold dispenser.
- Horizontal orbital microplate shaker (0.12” orbit) capable of maintaining a speed of 500 ± 50 rpm.

If extracting Cortisol from the sample matrix, the following supplies are also required:

- Glass test tubes.
- ASC grade diethyl ether.

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

The activity of the Cortisol 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 that contain higher concentrations of chelators must be diluted prior to assay.

This kit has been tested with a variety of samples; however, it is possible that high levels of interfering substances may cause variation in assay results.

SAMPLE COLLECTION AND STORAGE

Steroid Displacement Reagent should be added to serum, plasma, and other samples containing steroid binding proteins. Add 1 part of the Reagent for every 99 parts of sample.

Samples containing mouse IgG may interfere with this assay.

Cell Culture Supernates - Remove particulates by centrifugation. Assay immediately or freeze at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST). Allow samples to clot for 30 minutes. Centrifuge for 15 minutes at approximately 1000 x g. Assay immediately or freeze the serum at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or freeze the plasma at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Note: *Grossly hemolyzed or lipemic samples are not suitable for use in this assay.*

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter. Assay immediately or freeze the urine at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Saliva - Collect saliva using a SalivaSac® or equivalent. Assay immediately or aliquot and store samples at ≤ -70° C. Avoid repeated freeze-thaw cycles.

Other Biological Fluids - The recovery and linearity characteristics of this assay using other biological fluids has not been evaluated. Therefore, we recommend sample extraction before running the assay.

SAMPLE PREPARATION

All serum and plasma samples require a minimum 8-fold dilution.

All saliva samples require a minimum 4-fold dilution.

Note: *Samples containing low levels of cortisol may require extraction to measure within the dynamic range of the assay.*

SAMPLE EXTRACTION

1. Add sufficient Cortisol for determination of extraction efficiency.
2. Using a fume hood, add an equal amount of diethyl ether to sample. Insert stopper and shake. Allow layers to separate.
3. Pipette off the organic solvent (ether layer) and place in a clean test tube. Repeat this process two times combining the three ether layers.
4. Evaporate samples to dryness under a stream of nitrogen.
5. If analysis is to be carried out immediately, dissolve the extracted Cortisol with at least 250 µL of Assay Buffer and vortex well. Allow to sit for 5 minutes at room temperature. Repeat twice more.
6. Run the reconstituted samples in the assay immediately or keep the dried samples frozen at ≤ -20° C in dessication. Avoid repeated freeze-thaw cycles.

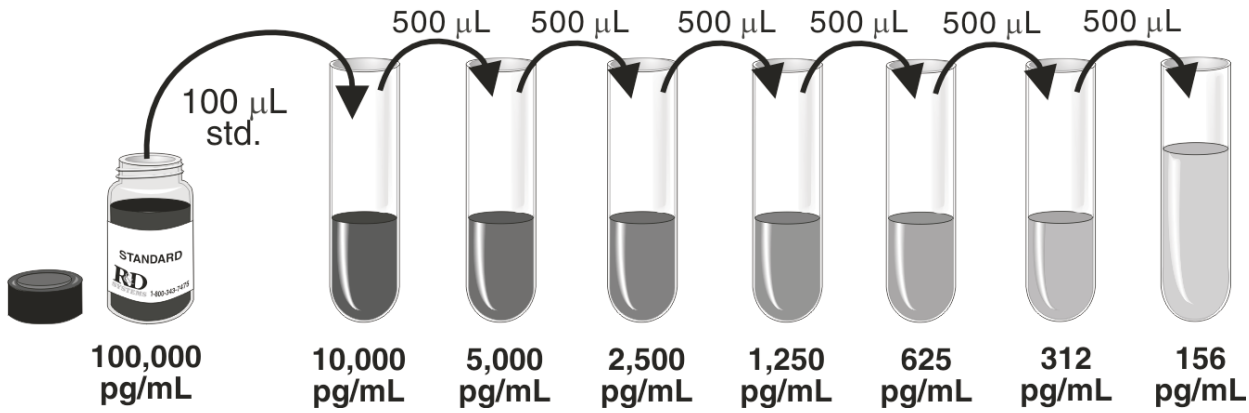
REAGENT PREPARATION

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).

Cortisol Standard - Pipette 900 µL of Assay Buffer ED1 into the 10,000 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). Mix each tube thoroughly and change pipette tips between each transfer. The 10,000 pg/mL standard serves as the high standard and the Assay Buffer ED1 serves as the zero standard (B_0) (0 pg/mL). **Use diluted standards within 60 minutes of preparation.**

Note: *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 run 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 strips from the plate frame, return them to the 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 Cortisol Conjugate to each well (excluding the TA and Substrate Blank wells).
8. Add 50 µL of Cortisol Antibody Solution to each well (excluding the NSB, TA, and Substrate Blank wells). Cover with the adhesive strip provided.

   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. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
10. 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 or manifold dispenser. 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.
11. Add 5 µL of Cortisol Conjugate to the TA wells.
12. Add 200 µL of pNPP Substrate to **all wells.** Incubate for 1 hour at room temperature on the benchtop (do not shake).
13. Add 50 µL of Stop Solution to each well.
14. 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. If wavelength correction is available, set to 570 nm or 590 nm. If wavelength correction is not available, subtract readings at 570 nm or 590 nm from the readings at 405 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 405 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

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

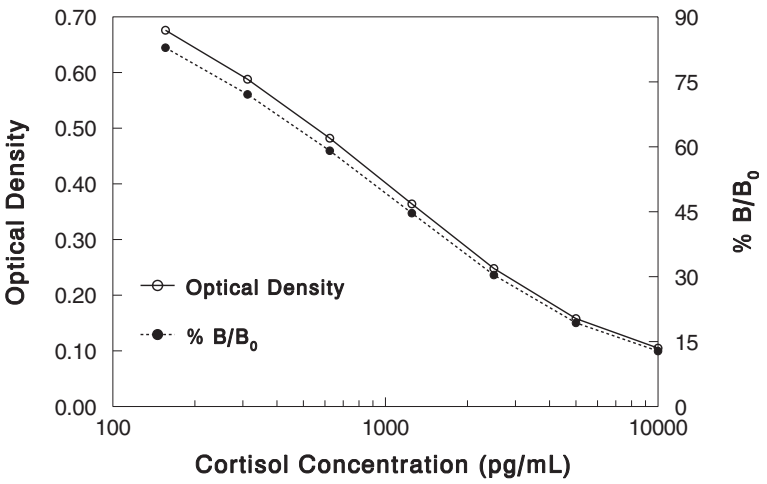
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on a linear y-axis against the concentration on a logarithmic x-axis and 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 Cortisol corresponding to the mean absorbance or the % B/B_0 from the standard curve. If samples have been diluted or extracted, the concentration read from the standard curve must be multiplied or divided 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	O.D.	Average	Corrected	% B/B_0
NSB	0.076 0.079	0.077	—	—
0(B_0)	0.888 0.897	0.893	0.816	100
156	0.750 0.756	0.753	0.676	82.9
312	0.661 0.669	0.665	0.588	72.1
625	0.544 0.574	0.559	0.482	59.1
1250	0.437 0.446	0.442	0.365	44.7
2,500	0.320 0.330	0.325	0.248	30.4
5,000	0.233 0.237	0.235	0.158	19.3
10,000	0.179 0.185	0.182	0.105	12.8

QUALITY CONTROL

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

Typical Quality Control Parameters

Substrate Blank (O.D.)	=	0.077
TA (TA x 10)	=	3.07
% NSB (NSB/TA x 100)	=	0.03%
% B_0 (B_0/TA x 100)	=	26.6%
Quality of Fit	=	1.00

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested sixteen 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	16	16	16	8	8	8
Mean (pg/mL)	333	1088	3155	451	969	3052
Standard deviation	35.0	71.8	230.3	60.4	75.6	262.5
CV (%)	10.5	6.6	7.3	13.4	7.8	8.6

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell Culture Media	107	97 - 113%
Porcine Serum*	104	102 - 105%
Porcine EDTA Plasma*	106	101 - 111%
Human Saliva*	97	91 - 111%
Human Urine	102	84 - 115%

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

LINEARITY

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

Dilution	Observed (pg/mL)	Expected (pg/mL)	% $\frac{\text{Observed}}{\text{Expected}}$
Neat	7789	—	—
1:2	4074	3894	105
1:4	1811	1947	93.0
1:8	1012	973.5	104
1:16	502.6	486.7	103

SENSITIVITY

The sensitivity of the Cortisol assay is typically less than 56.7 pg/mL.

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

CROSS-REACTIVITY

Cross-reactivity of the following compounds was determined by adding the cross-reactant to Assay Buffer ED1 at concentrations ranging from 10 pg/mL to 100,000 pg/mL. The cross-reactivity was calculated at 50% B/B_0.

Compound	% Cross-reactivity
Prednisolone	122
Corticosterone	27.7
11-deoxycortisol	4.0
Progesterone	3.6
Prednisone	0.85
Testosterone	0.12
Androstenedione	< 0.1
Cortisone	< 0.1
Estradiol	< 0.1

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