

Enzyme Immunoassay for Cortisol

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INTRODUCTION

Cortisol is a glucocorticoid steroid hormone produced in the adrenal cortex of the adrenal gland. Cortisol is produced throughout the day; with very high levels in the early morning it quickly decreases to a low level in the early afternoon. After that, it continues to slowly decrease into the night quickly spiking before morning. Cortisol levels may also spike immediately after waking up, during periods of low blood glucose levels, and in response to stress.

Cortisol is also known as Hydrocortisone when it is used medicinally, usually as a supplement or for its anti-inflammatory properties. Knowing plasma Cortisol levels is useful in identifying a serious lack of cortisol usually found in Addison's disease, or a serious overproduction of cortisol as in Cushing's syndrome. Cortisol is also frequently used to evaluate the stress levels of a subject.

PRINCIPLES OF PROCEDURE

This kit is a competitive enzyme-linked immunoassay (ELISA) for determining levels of Cortisol in biological samples such as urine, saliva, and plasma. Briefly, cortisol in the samples or standards competes with cortisol conjugated to horseradish peroxidase (HRP) for binding to a polyclonal antibody specific for cortisol coated on the microplate. The HRP activity results in brilliant blue color development when the substrate is added, with the intensity of the color proportional to the amount of cortisol-HRP bound and inversely proportional to the amount of unconjugated cortisol in the samples or standards.

MATERIALS PROVIDED

Component	Description	Volume	Storage	Cat. No.
Coated Plate	96-well microplate coated with a rabbit anti-cortisol antibody.	1 plate	4°C	EA65a
Cortisol Standard	0.5 µg/mL Cortisol standard solution.	100 µL	4°C	EA65b
EIA Buffer	Buffer used to dilute the Conjugate and Cortisol Standards.	30 mL	4°C	EA65c
5x Extraction Buffer	Buffer used to dilute extracted and non-extracted samples.	30 mL	4°C	EA65d
10x Wash Buffer	Buffer used to wash the plate prior to color development.	20 mL	4°C	EA65e
Cortisol-HRP Conjugate	Cortisol horseradish peroxidase concentrated conjugate.	150 µL	4°C	EA65f
TMB Substrate	TMB substrate used for color development.	20 mL	4°C	EA65g

MATERIALS NEEDED BUT NOT PROVIDED

1. Microplate reader with a 450 nm or 650 nm filter
2. Adjustable micropipettes (10 – 1000 µL) and tips
3. Deionized water
4. Plate cover or plastic film
5. Test tubes
6. 1 N HCl (optional)

EXTRACTION MATERIALS

1. Ethyl Ether
2. Nitrogen Gas
3. Vortex

STORAGE

1. Store the components of this kit at the temperatures specified on the labels.
2. Unopened reagents are stable until the indicated kit expiration date.
3. Desiccant bag must remain in foil pouch with unused strips. Keep pouch sealed when not in use to maintain a dry environment. Remove excess air before sealing.

WARNINGS AND PRECAUTIONS

1. Use aseptic technique when opening and dispensing reagents.
2. This kit is designed to work properly as provided and instructed. Additions, deletions or substitutions to the procedure or reagents are not recommended, as they may be detrimental to the assay.

PROCEDURAL NOTES

1. The enzyme conjugate is most stable in its concentrated form. Dilute only the volume necessary for the amount of strips currently being used.
2. To minimize errors in absorbance measurements due to handling, wipe the exterior bottom of the microplate wells with a lint-free paper towel prior to inserting into the plate reader.

SAMPLE PREPARATION

1. Saliva, urine, and tissue culture supernatant can be assayed after diluting them with diluted Extraction Buffer.
2. Plasma and most other mediums will need to be extracted using the extraction protocol below.

EXTRACTION PROTOCOL

1. Pipette 100 μ L of plasma into a glass test tube and add 1 mL of Ethyl Ether.
2. Vortex the tube for 30 seconds and allows the phases to separate.
3. Transfer the upper organic phase into a clean glass test tube and evaporate the solvent using a stream of nitrogen gas. Discard the aqueous phase.
4. Dissolve the residue in 100 μ L of diluted Extraction Buffer.
5. Dilute 100 fold by adding 10 μ L of the above extract into 990 μ L of diluted Extraction Buffer. Store unused sample at -20°C.
6. Vortex the sample and proceed to the assay procedure.
7. The values obtained from the assay are multiplied by 100 to give final ng/mL concentrations. If additional dilution is necessary, values must be multiplied by the additional dilution factor in order to calculate final ng/mL concentration.
8. If the concentration is higher than the high range of the standard curve, the sample should be further diluted. If the concentration is undetectable, reduce the dilution in step 5 above.

REAGENT PREPARATION

1. **5x Extraction Buffer:** Dilute the appropriate amount to 1x with deionized water prior to use.
2. **10x Wash Buffer:** Add 20 mL of 10x Wash Buffer to 180 mL of deionized water prior to use.
3. **Cortisol-HRP Conjugate:** Dilute 110 μ L of Conjugate into 5.5 mL total volume of EIA Buffer.

STANDARD CURVE PREPARATION

The Cortisol Standard is provided as a 500 ng/mL stock solution. Use the following table to dilute a set of standard stock solutions and construct an eight-point standard curve.

Table 1: Standard Curve Preparation

Standard	Cortisol Conc. (ng/mL)	Vol. of EIA Buffer (μ L)	Transfer Volume (μ L)	Transfer Source	Final Volume (μ L)
S7	50	450	50	Cortisol Standard	400
S6	10	400	100	S7	400
S5	2	400	100	S6	375
S4	0.5	375	125	S5	400
S3	0.1	400	100	S4	400
S2	0.02	400	100	S3	375
S1	0.005	375	125	S2	500
S0	0.000	450	-	-	450

ASSAY PROCEDURE

1. Add 50 μ L of Standards or Samples (may require diluting) to the corresponding wells on the microplate in duplicate. See **Scheme I** for a sample plate layout.
2. Add 50 μ L of diluted Cortisol-HRP Conjugate to each well. Incubate at room temperature for one hour.
3. Wash the plate three times with 300 μ L of diluted Wash Buffer per well. Wash 5 times if using an automated plate washer.
4. Add 150 μ L of TMB Substrate to each well. Incubate at room temperature for 30 minutes.
5. Read the plate at 650 nm.

Alternately, the color reaction can be stopped after 30 minutes by adding 50 μ L of 1 N HCl and read at 450 nm.

NOTE: If accounting for substrate background, use 2 wells as blanks (BLK) with only 150 μ L TMB Substrate in the wells. Subtract the average of these absorbance values from the absorbance values of the wells being assayed.

Scheme I: Sample Plate Layout

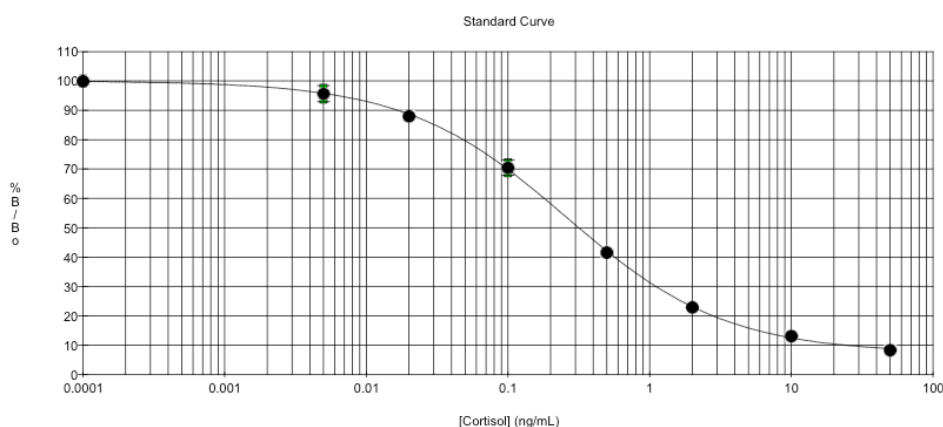
	1	2	3	4	5	6	7	8	9	10	11	12
A	S0	S0	U1	U1	U9	U9	U17	U17	U25	U25	U33	U33
B	S1	S1	U2	U2	U10	U10	U18	U18	U26	U26	U34	U34
C	S2	S2	U3	U3	U11	U11	U19	U19	U27	U27	U35	U35
D	S3	S3	U4	U4	U12	U12	U20	U20	U28	U28	U36	U36
E	S4	S4	U5	U5	U13	U13	U21	U21	U29	U29	U37	U37
F	S5	S5	U6	U6	U14	U14	U22	U22	U30	U30	U38	U38
G	S6	S6	U7	U7	U15	U15	U23	U23	U31	U31	U39	U39
H	S7	S7	U8	U8	U16	U16	U24	U24	U32	U32	BLK	BLK

CALCULATIONS

1. Subtract the substrate blank from all absorbance values and then average all duplicate wells for standards and unknown samples.
2. The average of your two S0 values is now your B0 value (S1 now becomes B1, etc.). B0 will be your highest OD and represents the standard without added cortisol.

3. Divide the averaged OD value of each standard (B₁ through B₇) by the B₀ to express it as a percent of maximum binding (%B/B₀).
4. Graph your standard curve by plotting the %B/B₀ for each standard concentration on the y-axis against concentration on the x-axis. Draw a curve by using a curve-fitting routine (i.e. 4-parameter or linear regression).
5. Divide the averages of each sample absorbance value by the B₀ value and multiply by 100 to achieve percentages (%B/B₀).
6. Determine the concentration of each sample by comparing to the standard curve. For best results, sample %B/B₀ should be between 20-80%. Samples falling outside 20-80% B/B₀ should be rediluted and rerun.
7. If the samples were diluted, the concentration determined from the standard curve must be multiplied by the dilution factor. If the samples were extracted, remember to multiply by that dilution value as well.

Figure 1: Typical Standard Curve



CROSS REACTIVITY

Cortisol	100.00%	Estrone	4.1%
Prednisolone	66.9%	d-Aldosterone	3.6%
11-Deoxycortisol	58.1%	Progesterone	3.5%
Cortisone	15.9%	6-β-Hydroxycortisol	3.4%
Prednisone	13.7%	trans Dehydroandrosterone	1.9%
17-Hydroxyprogesterone	5.4%	Testosterone	1.7%
Dexamethasone	4.6%	Corticosterone	1.4%
Estriol	4.5%	Pregnenolone	1.3%

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