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Enzyme Immunoassay for Cortisol

Product No. EA 65

For Research Use Only
Store Kit at 4°C at all times

INTRODUCTION

Cortisol or hydrocortisone, is the primary corticosteroid secreted by the adrenal cortex. Cortisol is synthesized from cholesterol and may be found in the blood as free Cortisol or bound to corticosteroid-binding globulin. The release of Cortisol is controlled by ACTH which is produced in the anterior pituitary. Plasma Cortisol levels are highest in the morning and decrease throughout the day. Cortisol concentration in plasma also elevates in response to stress. Cortisol has an anti-inflammatory effect and aids in carbohydrate metabolism, renal function and the promotion of glucogenesis.

Measurement of plasma Cortisol levels is useful in diagnosing conditions related to functions of the adrenal cortex, including Cushing's syndrome (hypercortisolism), Addison's disease (hypocortisolism) and adrenal tumors. Abnormal Cortisol levels may also possibly be linked to prostate cancer, depression and schizophrenia.

Principles of the Procedure

This is an ELISA (Enzyme-Linked Immunosorbent Assay) for the quantitative analysis of Cortisol levels in biological fluid. This test kit operates on the basis of competition between the enzyme conjugate and the Cortisol in the sample for a limited number of binding sites on the antibody coated plate.

The sample or standard solution is first added to the micro-plate. Next, the diluted enzyme conjugate is added and the mixture is shaken and incubated at room temperature for one hour. During the incubation, competition for binding sites is taking place. The plate is then washed removing all the unbound material. The bound enzyme conjugate is detected by the addition of substrate which generates an optimal color after 30 minutes. Quantitative test results may be obtained by measuring and comparing the absorbance reading of the wells of the samples against the standards with a microplate reader at 650 nm. The extent of color development is inversely proportional to the amount of Cortisol in the sample or standard. For example, the absence of Cortisol in the sample will result in a bright blue color, whereas the presence of Cortisol will result in decreased or no color development.

REAGENTS

Materials Provided

1. **EIA BUFFER:** 30 mL. Provided to dilute enzyme conjugate and Cortisol standards.
2. **WASH BUFFER 10x:** 20 mL. Dilute 10 fold with deionized water. Diluted wash buffer is used to wash all unbound enzyme conjugate, samples and standards from the plate after the one hour incubation.
3. **SUBSTRATE:** 20 mL. Stabilized 3,3', 5,5' Tetramethylbenzidine (TMB) plus Hydrogen Peroxide (H₂O₂) in a single bottle. It is used to develop the color in the wells after they have been washed. Keep substrate refrigerated. It is LIGHT SENSITIVE.
4. **EXTRACTION BUFFER 5x:** 30 mL. Dilute 5 fold with deionized water. This buffer is used for diluting extracted and non-extracted samples.
5. **CORTISOL ENZYME CONJUGATE:** 150 µL. Cortisol horseradish peroxidase concentrate. Blue capped vial.
6. **CORTISOL STANDARD:** 100 µL. Cortisol standard provided at the concentration of 1 µg/mL in methanol. Green capped vial.
7. **CORTISOL ANTIBODY COATED PLATE:** A 96 well Costar™ microplate with anti-Cortisol rabbit antibody precoated on each well. The plate is ready for use as is. **DO NOT WASH!**

Materials Needed but not Provided

1. 300 mL deionized water for diluting wash buffer and extraction buffer.
 2. Precision pipettes that range from 10 µL-1000 µL and disposable tips.
- NOTE: If all or several strips are to be used at one time, it is suggested that a multichannel pipette be used.**
3. Clean test tubes used to dilute the standards and conjugate.
 4. Graduated cylinders to dilute and mix wash buffer and extraction buffer.
 5. Microplate reader with 650 nm filter.
 6. Plastic film or plate cover to cover plate during incubation.

Optional Materials

7. 1 N HCl.
8. Microplate shaker.

If performing an extraction on samples, the following will be required:

9. Ethyl ether
10. Nitrogen gas
11. Vortex

WARNINGS AND PRECAUTIONS

1. **DO NOT** use components beyond expiration date.
2. **DO NOT** mix any reagents or components of this kit with any reagents or components of any other kit. This kit is designed to work properly as provided.
3. **DO NOT** pipette reagents by mouth.

4. Always pour substrate out of the bottle into a clean test tube - **DO NOT** pipette out of the bottle (if your tip is unclean you could contaminate your substrate).
5. All specimens should be considered potentially infectious. Exercise proper handling precautions.
6. **DO NOT** smoke, eat or drink in areas where specimens or reagents are being handled.
7. Use aseptic technique when opening and removing reagents from vials and bottles.
8. Keep plate covered except when adding reagents, washing or reading.
9. Kit components should be refrigerated at all times when not in use.

PROCEDURAL NOTES

1. It is not necessary to allow reagents to warm to room temperature before use.
2. Desiccant bag must remain in foil pouch with unused strips. Keep pouch sealed when not in use to maintain a dry environment. Seal with a heat sealer. If a heat sealer is not available, thoroughly close the open end with tape. Try to remove excess air before sealing.
3. Always use different pipette tips for the buffer, enzyme conjugate, standards and samples.
4. Before pipetting a reagent, rinse the pipette tip three times with that reagent (i.e. fill the tip with the desired amount of reagent and dispense back into the same vial - repeat 2 times). Now the tip is properly rinsed and ready to dispense the reagent into your well or test tube.
5. When pipetting into the wells, **DO NOT** allow the pipette tip to touch the inside of the well, or any of the reagents already in the well - this can cause cross contamination.
6. Standards and samples should be assayed in duplicate.
7. To quantitate, always run a standard curve when testing samples. If testing a sample that is not extracted, dilute standards in the same type of medium being tested, which is known to be negative.
8. Gently mix specimens and reagents before use. Avoid vigorous agitation.
9. When using only partial amounts of a kit, it is recommended to transfer the appropriate volume of each reagent to a clean vessel for repeated dispensing. This will reduce reagent contamination by repeated sampling from the original container.
10. The enzyme conjugate is most stable in its concentrated form. Dilute only the volume necessary for the amount of strips currently being used.
11. Before taking an absorbance reading wipe the outside bottom of the wells with a lint-free wiper to remove dust and fingerprints.
12. Before opening the enzyme conjugate and standard vial, tap vial in an upright position to remove any liquid in the cap.

SAMPLE PREPARATION

Usually, urine and tissue culture supernatant can be assayed directly by diluting them with the diluted extraction buffer. Plasma and most other mediums will need to be extracted.

Extraction of Cortisol

1. Pipette 100 μL of plasma into a glass tube (10x75 mm) and add 1 mL of ethyl ether.
2. Vortex the tube for 30 seconds and then allow the phases to separate.
3. Transfer the organic phase into a clean glass tube and evaporate the solvent with a stream of N_2 .
4. Dissolve the residue in 100 μL of diluted extraction buffer.
5. Dilute the extract 100 fold by adding 10 μL of the above extract into 990 μL of diluted extraction buffer.
6. Vortex and assay 50 μL in duplicates.

7. The values obtained 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 samples in #6 need to be further diluted and reassayed.

NOTE: Extraction buffer must be diluted 5 fold with deionized water before use. Any precipitant present must be brought into solution before dilution.

TEST PROCEDURES

1. Prepare standards as follows:

STANDARD	PREPARATION
A	stock solution 1 µg/mL (this is provided)
B	take 20 µL of A, add to 980 µL of EIA buffer and mix=20 ng/mL
C	take 200 µL of B, add to 1.8 mL of EIA buffer and mix=2 ng/mL
D	take 200 µL of C, add to 1.8 mL of EIA buffer and mix=0.2 ng/mL

Continue standard preparation following Scheme I

Scheme I

Standard	ng/mL	EIA buffer (µL added)	B Standard (µL)	C Standard (µL)	D Standard (µL)
S ₀	0	as is	-	-	-
S ₁	0.04	800	-	-	200
S ₂	0.1	500	-	-	500
S ₃	0.2	-	-	-	as is
S ₄	0.4	800	-	200	-
S ₅	1	500	-	500	-
S ₆	2	-	-	as is	-
S ₇	10	500	500	-	-

2. Determine the number of wells to be used.

NOTE: Allow for extra wells when calculating amount of conjugate to dilute to allow for loss during pipetting (i.e. 4 extra wells if using a single pipette; 10 extra wells if using a multichannel pipette).

3. Dilute the Cortisol enzyme conjugate. Add 1 µL of enzyme conjugate into 50 µL total volume of EIA buffer for each well assayed. For the whole plate, add 110 µL of the enzyme conjugate into 5.5 mL total volume of EIA buffer. Mix the solution thoroughly.

- Add 50 μL of standards (S) or unknown (U) (some samples may require diluting) to the appropriate wells in duplicate.

See Scheme II for suggested template design.

Scheme II

	1	2	3	4	5	6	7	8	9	10	11	12
A	s_0	s_0	u_1	u_1	u_9	u_9	u_{17}	u_{17}	u_{25}	u_{25}	u_{33}	u_{33}
B	s_1	s_1	u_2	u_2	u_{10}	u_{10}	u_{18}	u_{18}	u_{26}	u_{26}	u_{34}	u_{34}
C	s_2	s_2	u_3	u_3	u_{11}	u_{11}	u_{19}	u_{19}	u_{27}	u_{27}	u_{35}	u_{35}
D	s_3	s_3	u_4	u_4	u_{12}	u_{12}	u_{20}	u_{20}	u_{28}	u_{28}	u_{36}	u_{36}
E	s_4	s_4	u_5	u_5	u_{13}	u_{13}	u_{21}	u_{21}	u_{29}	u_{29}	u_{37}	u_{37}
F	s_5	s_5	u_6	u_6	u_{14}	u_{14}	u_{22}	u_{22}	u_{30}	u_{30}	u_{38}	u_{38}
G	s_6	s_6	u_7	u_7	u_{15}	u_{15}	u_{23}	u_{23}	u_{31}	u_{31}	u_{39}	u_{39}
H	s_7	s_7	u_8	u_8	u_{16}	u_{16}	u_{24}	u_{24}	u_{32}	u_{32}	u_{40}	u_{40}

- Add 50 μL of the diluted enzyme conjugate to each well. (Use 8-channel pipette or 12-channel pipette for rapid addition.)
- Mix by shaking plate gently. (A microplate shaker may be used.)
- Cover plate with plastic film or plate cover and incubate at room temperature for one hour. **Note:** Keep plate away from drafts and temperature fluctuations.
- Dilute concentrated wash buffer with deionized water (i.e. 20 mL of wash buffer plus 180 mL of deionized water). Mix thoroughly.
- After incubation, dump out the contents of the plate. Tap out contents thoroughly on a clean lint-free towel.
- Wash each well with 300 μL of the washing buffer. Repeat for a total of three washings. (An automated plate washer can be used.)
- Add 150 μL of substrate to each well. (Use multichannel pipette for best results.) Mix by shaking plate gently.
- Allow to stand at room temperature for 30 minutes.
- Gently shake plate before taking a reading to insure uniform color throughout each well.
- Plate is read in a microplate reader at 650 nm. If a dual wavelength is used, set W_1 at 650 nm and W_2 at 490 nm.
- If accounting for substrate background, use 2 to 8 wells as blanks with only substrate in the wells (150 μL /well). Subtract the average of these absorbance values from the absorbance values of the wells being assayed.

NOTE: Some microplate readers can be programmed to do these subtractions automatically when reading the plate. Consult your instrument manual.

Optional Test Procedures

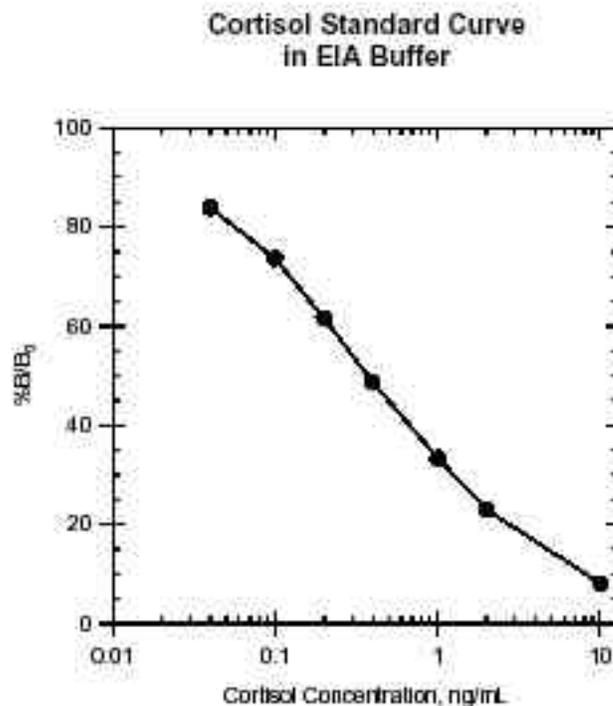
- Add 50-100 μL of 1 N HCl to each well to stop enzyme reaction.
- Read plate at 450 nm 1N HCl.
- Plot the standard curve and estimate the concentrations of the samples from the curve. See "CALCULATIONS."

Note: Absorbance readings will approximately double when stopped with acid. If absorbance readings are too high for measuring with your microplate reader, decrease the substrate incubation approximately 10 minutes but no more than 15 minutes.

CALCULATIONS

1. After the substrate background has been subtracted from all absorbance values, average all of your duplicate well absorbance values.
2. The average of your two S_0 values is now your B_0 value. (S_1 now becomes B_1 , etc.)
3. Next, find the percent of maximal binding ($\%B/B_0$ value). To do this, divide the averages of each standard absorbance value (now known as B_1 through B_7) by the B_0 absorbance value and multiply by 100 to achieve percentages.
4. Graph your standard curve by plotting the $\%B/B_0$ for each standard concentration on the ordinate (y) axis against concentration on the abscissa (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 B_0 value and multiply by 100 to achieve percentages.
6. Using the standard curve, the concentration of each sample can be determined by comparing the $\%B/B_0$ of each sample to the corresponding concentration of Cortisol standard.
7. If the samples were diluted, the concentration determined from the standard curve must be multiplied by the dilution factor.

TYPICAL STANDARD CURVE



TYPICAL DATA

Note: "Typical data" is a representation. Variances in data will occur. Optical density readings may fluctuate during the shelf-life of the kit, but the %B/B₀ should remain comparable.
Measuring wavelength: 650 nm

Standard	Standard Concentration (ng/mL)	Optical Density (Absorbance Value)	%B/B ₀
S ₀ (B ₀)	0	1.102	100
S ₁ (B ₁)	0.04	0.925	84
S ₂ (B ₂)	0.1	0.812	74
S ₃ (B ₃)	0.2	0.679	62
S ₄ (B ₄)	0.4	0.539	49
S ₅ (B ₅)	1	0.361	33
S ₆ (B ₆)	2	0.259	23
S ₇ (B ₇)	10	0.087	8

CROSS REACTIVITY

CORTISOL	100.00%
PREDNISOLONE	47.42%
CORTISONE	15.77%
11-DEOXYCORTISOL	15.00%
PREDNISON	7.83%
CORTICOSTERONE	4.81%
6-BETA-HYDROXYCORTISOL	1.37%
17-HYDROXYPROGESTERONE	1.36%
DEOXYCORTICOSTERONE	0.94%
PROGESTERONE	0.06%
BETAMETHASONE	0.05%
DEHYDROEPIANDROSTERONE	0.03%
DEXAMETHAZONE	0.03%
BECLMETHASONE	0.01%
d-ALDOSTERONE	0.01%
TESTOSTERONE	0.01%
17 -HYDROXYPREGNENOLONE	<0.01%
ANDROSTENEDIONE	<0.01%
CHOLESTEROL	<0.01%
ESTRADIOL	<0.01%
ESTRIOL	<0.01%
ESTRONE	<0.01%
PREGNENOLONE	<0.01%

MATERIAL SAFETY DATA SHEET

Physical and Chemical Data: Components are stable in closed containers under normal temperatures and pressures.

Precautions:

1. Safety glasses, gloves and lab coat should be worn at all times while performing this assay. Contents may be harmful if swallowed, inhaled or absorbed through the skin.
2. Do not pipette solutions by mouth.
3. Reagents may contain sodium azide. Sodium azide may react with lead and copper plumbing to form explosive metal azides. On disposal of reagents, flush with large volumes of water to prevent azide accumulation.
4. Do not eat or smoke in areas where specimens or kit reagents are being handled.

Health Hazards: Individual components may cause skin irritation or be harmful if swallowed. Avoid contact with skin and eyes.

First Aid: Call a physician. If swallowed give water or milk to dilute and induce vomiting. In case of contact with eyes, flush with copious amounts of water for at least 15 minutes, assure adequate flushing by separating eyelids with fingers. In case of skin contact, wash with soap or mild detergent and large amounts of water.

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