COSMO BIO CO., LTD.
Inspiration for Life Science

YK241 Cortisol (Saliva) EIA
Product Instructions

FOR LABORATORY USE ONLY

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Contents

– Please read all the package insert carefully before beginning the assay –

YK241 Cortisol (Saliva) EIA Kit

I. Introduction

Cortisol (also called hydrocortisone, compound F) is a primary glucocorticoid hormone secreted from the cortex of adrenal gland. The adrenal corticosteroids are apparently involved in the regulation of a large number of biological activities, including carbohydrate, protein, and lipid metabolism; water and electrolyte balance; bone formation, etc. Cortisol also functions as an important anti-inflammatory and plays a role in hypersensitivity, immunosuppression, and disease resistance. Production of cortisol follows an ACTH-dependent circadian rhythm, with a peak level in the morning and decreasing levels throughout the day [(1)]. Cortisol is often referred to as the "stress hormone" as it is involved in the response to stress sensitively and the levels rise independently of circadian rhythm in response to stress [(2)]

In blood, over than 90% of cortisol is bound to a plasma protein called corticosteroid-binding globulin (CBG) and plasma albumin, some parts of unbound free cortisol are secreted to saliva. Majority of cortisol in saliva remains unbound to protein. The levels of saliva cortisol are not affected with saliva secretion flow rate, and have good correlation between cortisol measurements in saliva and serum. Furthermore, saliva cortisol is relatively stable to the degradation by enzymes and freezing-thawing cycles [(3)].

The newly developed cortisol (saliva) EIA kit by our laboratory provides a high sensitivity, quantitative tool for direct determination of cortisol in saliva without pre-treatment for sample. Furthermore, assays using this kit can be completed within a short period. The cortisol (saliva) EIA kit newly developed will be a quite useful tool for further development in cortisol research.

YK241 Cortisol (Saliva) EIA Kit	**Contents**
▼ The kit assay range: 0.012-3.000μg/dL.	1) Antibody Coated Plate
▼ The assay running time: 1h. + 0.5 h.	2) Cortisol Standard
▼ Maximum measurable samples: 41 in duplicate	3) HRP-Labeled Cortisol
▼ Test sample: saliva.	4) Buffer Solution
▼ The 96-wells plate in kit is consisted by 8-wells strips, and the strips can be used separately.	5) TMB Substrate 6) Concentrated Wash Solution
▼ Intra-assay %CV: 3.9~4.6	7) Reaction Stopping Solution
▼ Inter-assay %CV: 4.3~5.9	8) Adhesive Foil
▼ Store all the components in the kit at 2-8℃.	
▼ The expiry date is stated on the package.	

II. Characteristics

This EIA kit is used for quantitative determination of cortisol in saliva. It has various advantages, such as no extraction procedure of samples, short assay time, practically no influences of other body fluids or physiological active substances coexisting in samples assayed.

< Specificity **>**

The specificity of this EIA kit is shown on page 9.

< Assay Principle **>**

This EIA kit for determination of cortisol is based on a competitive enzyme immunoassay using combination of specific antibody to cortisol and cortisol-horseradish peroxidase (HRP) conjugate (HRP-labeled cortisol) system. The 96 wells plate is coated with cortisol specific antibody, to which cortisol standard or samples, HRP-labeled cortisol are added for competitive immunoreaction. After incubation and plate washing, HRP enzyme activity is determined by 3,3',5,5'-tetramethylbenzidine (TMB) and the concentration of cortisol is calculated.

III. Composition

	Component	Form	Quantity	Main Ingredient
1	Antibody Coated Plate	microtiter plate	1 plate (96 wells)	Monoclonal anti-cortisol antibodies
2	Cortisol Standard	lyophilized powder	1 vial (0.12μg)	Synthetic cortisol
3	HRP-Labeled Cortisol	liquid	1 vial (0.15mL)	HRP conjugated cortisol
4	Buffer Solution	liquid	1 bottle (50 mL)	BSA-containing PBS buffer
5	TMB Substrate	liquid	1 bottle (12 mL)	3,3',5,5'-Tetramethylbenzidine (TMB)
6	Concentrated Wash Solution	liquid	2 bottles (25 mL x 2)	1% tween20 concentrated saline
7	Reaction Stopping Solution	liquid	1 bottle (12 mL)	1M Sulfuric acid
8	Adhesive Foil		2 sheets	

IV. Method

< Equipment required **>**

1. Photometer for microtiter plate (plate reader), which can read extinction 2.5 at 450 nm
2. Washing device for microtiter plate and dispenser with aspiration system (optional)
3. Micropipettes for volumes between 50 μL –1000 μL
4. Multi-channel pipettes for 8 or 12 wells and the tips
5. Polypropylene tubes for preparation of standard solutions
6. A microplate shaker (210-220 rpm)
7. Graduated cylinder (1,000 mL)
8. Distilled or deionized water

< Preparatory work **>**

1. Preparation of cortisol standard solution: Reconstitute lyophilized **Cortisol Standard** (0.12μg /vial) with 1mL of **Buffer Solution** which affords 12μg/dL standard solution. The reconstituted cortisol standard solution (0.2mL) is diluted with 0.6 mL of **Buffer Solution** that yields 3μg/dL standard solution. The standard solution (3μg/dL) (0.2mL) is diluted with 0.4mL of **Buffer Solution** to yield 1μg/dL of standard solution. Repeat the same dilution to make standard solution of 0.333, 0.111, 0.037, and 0.012μg/dL, respectively. **Buffer Solution** is used as 0μg/dL.
2. Preparation of HRP-labeled cortisol solution: Take 0.085mL of **HRP-Labeled Cortisol** from the labeled vial to dilute with 17mL of **Buffer Solution** completely.
3. Preparation of wash solution: Dilute two bottles of **Concentrated Wash Solution** (25 mL) to 1,000 mL with distilled or deionized water.
4. Other reagents are ready for use.

< Assay procedure **>**

1. Before starting assay, bring all the reagents to room temperature (22-25°C).
2. Add 200 μL of **Buffer Solution** to each well and keep it for about 10 minutes, and then aspirate or decant the **Buffer Solution** in the wells. Invert the plate and tap it onto an absorbent surface, such as paper toweling, to ensure blotting free of most residual **Buffer Solution**.
3. Pipet 50 μL of cortisol standard solutions (0, 0.012, 0.037, 0.111, 0.333, 1, and 3μg/dL) or samples, after vortexed, into appropriate wells. Add 150μL of HRP-labeled cortisol solution into each well.
4. Cover the plate with adhesive foil and incubate it on a shaker at 210-220 rpm at room temperature for 60 minutes.
5. After incubation, take off the adhesive foil, aspirate or decant the solutions in the wells. Add 350 μL of diluted wash solution to each well and keep it for about 30 seconds, and then aspirate or decant the wash solution in the wells. Repeat this wash process 6 times (total 7

times). Finally, invert the plate and tap it onto an absorbent surface, such as paper toweling, to ensure blotting free of most residual wash solution.

6. Add 100 µL of **TMB Substrate** into each well.
7. Cover the plate with adhesive foil and incubate it on a shaker at 210-220 rpm at room temperature for 30 minutes.
8. Add 100 µL of **Reaction Stopping Solution** into each well to stop color reaction.
9. Read the optical absorbance of the wells at 450 nm (if possible, read 620nm for correction).
10. The assay fits best to a 4 (or 5)-parameter logistic equation, Y= (a-d)/(1+(x/c)^b)+d; here a,b,c,d represent constant parameter. Alternatively, calculate mean optical density values of wells containing standard solutions or their percent bound to maximum binding wells (0 µg/dL) and plot a standard curve on a semi-logarithmic graph paper (abscissa: concentrations of standard; ordinate: optical density or bound%). Use the average optical density or bound% of each sample to determine the corresponding value by simple interpolation from the standard curve. The results should be multiplied by the diluting factor to obtain the actually concentrations for undiluted unknown samples.

V. Notes

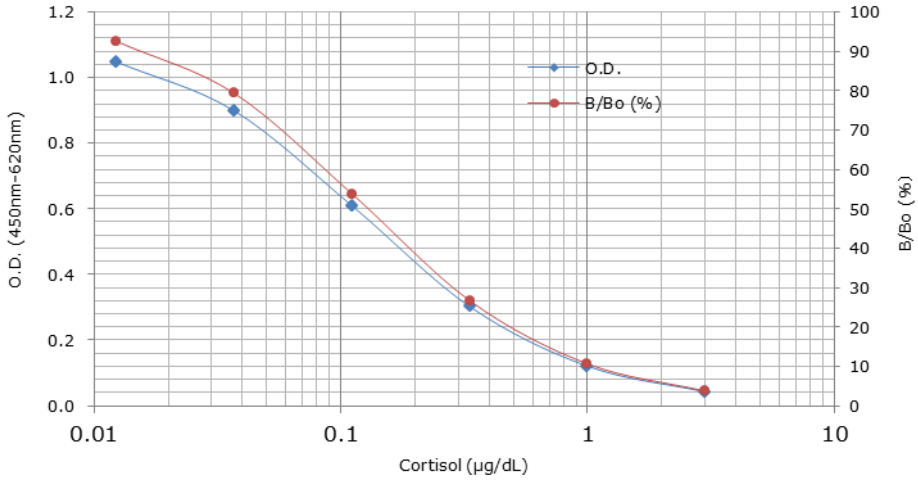
1. It is recommended that saliva samples should be collected by using special collecting tubes such as Saliva Bio Oral Swab (Item No. 5001.02 Salimetrics) and Swab strage tube (Item No. 5001.05 Salimetrics). The collected saliva samples should be centrifuged at 3,000 X g for 15 minutes. If the sample is tested later, they should be frozen below –30°C. Avoid repeated freezing and thawing of samples.
2. Avoid sample collection within 60 minutes after eating a meal or within 12 hours after consuming alcohol.
3. Avoid taking acidic or high sugar foods and caffeine drinks before collection.
4. To avoid the influences of food ingredients, rinse mouth thoroughly with water, then collected after an interval for at least 10 minutes before collection. Tooth brushing is not recommended.
5. Do not use samples contaminated with blood.
6. Do not use sodium azide as a preservative for collected samples.
7. Cortisol standard solutions and HRP-labeled cortisol solution should be prepared immediately before use. If the kit used dividedly, the rest of the reconstituted cortisol standard solution (12 µg/dL), **HRP-Labeled Cortisol** and **TMB substrate** except wash solution and **Reaction Stopping Solution** should be stored at 4°C and used within 2 weeks. Diluted standard solutions, except 12µg/dL of standard solution, and diluted HRP-labeled cortisol solution should not be reused for another assay.
8. Samples which values over than upper detection limit should be diluted with **Buffer Solution** and measured once more.
9. Because prednisolone, dexamethasone and 21-deoxycortisol have a relatively high cross-reactivities with cortisol antibody in the kit, please take care when measuring such samples collected from who are treated with prednisolone or dexamethasone; or from who is 21-hydroxylase deficiency (21OHD) patient.
10. Incomplete washing of the microplate will interfere with assay precision. If a microplate

washer is not available, completely aspirate the solutions in the wells of assay plate to be removed or decant them by inverting the plate and tapping it onto absorbent tissue in each wash cycle. Ensure that there is no residual wash solution in the wells after final wash.

11. As pipetting operations may affect precision of the assay, pipet cortisol standard solutions or samples precisely into the wells of assay plate. In addition, use clean test tubes or vessels in assay and a new tip for each standard diluting process and for each sample or standard solution pipetting to avoid cross contamination.
12. Perform all the determination in duplicate.
13. To quantitate accurately, always run a standard curve for each assay.
14. Color reaction should be carried out under the light proof condition.
15. Read optical absorbance of reaction solution in wells as soon as possible after stopping the color reaction.
16. Protect the reagents from strong light (e.g. direct sunlight) during storage and assay.
17. Satisfactory performance of the assay will be guaranteed only when reagents are used from combination pack with identical lot number.

VI. Performance Characteristics

Typical standard curves of cortisol (saliva) EIA



< Assay range **>** 0.012– 3.000μg/dL

<Sensitivity**>**

Sensitivity can be calculated using the following formula under the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols [4].

$$\text{Sensitivity (μg/dL)} = \frac{2\times\text{SD of the Zero Standard}\times 0.012\text{μg/dL}}{(\text{Optical Density of } 0\text{μg/dL} - \text{Optical Density of } 0.012\text{μg/dL})}$$

< Precision and reproducibility **>**

Saliva sample	Intra-assay variation (mean±SD, n=10)		Inter-assay variation (mean±SD, n=8)	
	Measured (μg/dL)	%CV	Measured (μg/dL)	%CV
1	0.246±0.010	4.1	0.241±0.014	5.9
2	0.671±0.031	3.9	0.653±0.028	4.3
3	1.033±0.041	4.6	0.982±0.058	5.9

< Analytical recovery **>**

Saliva sample	Cortisol added (μg/dL)	Observed (μg/dL)	Expected (μg/dL)	Recovery (%)
A	0	0.225		
	0.067	0.282	0.292	96.6
	0.2	0.411	0.425	96.7
	0.8	0.964	1.025	94.1
B	0	0.232		
	0.067	0.296	0.299	99.0
	0.2	0.397	0.432	91.9
	0.8	0.984	1.032	95.3
C	0	0.253		
	0.067	0.339	0.320	105.9
	0.2	0.469	0.453	103.5
	0.8	1.017	1.053	96.6
D	0	0.460		
	0.067	0.514	0.527	97.5
	0.2	0.625	0.660	94.7
	0.8	1.138	1.260	90.3
E	0	0.282		
	0.067	0.356	0.349	102.0
	0.2	0.482	0.482	100.0
	0.8	1.054	1.082	97.4
F	0	0.262		
	0.067	0.329	0.329	100.0
	0.2	0.462	0.462	100.0
	0.8	0.968	1.062	91.1

<Dilution test**>**

Saliva sample	Dilution ratio (1X)	Observed (μg/dL)	Expected (μg/dL)	% Of expected
No.1	1	0.237		
	2	0.122	0.119	102.5
	4	0.065	0.059	110.2
	8	0.036	0.030	120.0
No.2	1	0.250		
	2	0.123	0.125	98.4
	4	0.061	0.063	96.8
	8	0.031	0.031	100.0
No.3	1	0.287		
	2	0.141	0.144	97.9
	4	0.074	0.072	102.8
	8	0.043	0.036	119.4
No.4	1	0.464		
	2	0.236	0.232	101.7
	4	0.122	0.116	105.2
	8	0.063	0.058	108.6
No.5	1	0.324		
	2	0.164	0.162	101.2
	4	0.085	0.081	104.9
	8	0.050	0.041	122.0
No.6	1	0.279		
	2	0.127	0.140	90.7
	4	0.059	0.070	84.3
	8	0.026	0.035	74.3

<Cross reactivity**>**

Cross reactivities of the antibody used in the kit.

Compound	Spiked Concentration (μg/dL)	% Cross-reactivity
Prednisolone	10	11.159
Prednisone	100	0.301
Cortisone	100	1.980
Corticosterone	100	3.563
21-Deoxycortisol	10	11.436
11-Deoxycortisol	100	2.949
11-Dehydrocorticosterone	100	2.649
11-Deoxycorticosterone	100	0.704
Progesterone	1000	0.044
17α-Hydroxyprogesterone	100	0.602
11α-Hydroxyprogesterone	1000	0.007
Testosterone	1000	0.012
Aldosterone	1000	0.084
Betamethasone	100	0.393
Dexamethasone	10	24.045
DHEA	1000	0.026
4-Androstene-3,17-dione	1000	0.005
17β-Estradiol	1000	0.005
Triamcinolone	1000	0.014
Cholesterol	1000	Not detected

VII. Stability and Storage

< Storage **>** Store all the components in the kit at 2°C - 8°C.

< Shelf Life **>** The Kit is stable under the storage condition for 6 months from the date of manufacture.
The expiry date is stated on the label of package.

< Package **>** For 96 tests per one kit including standards.

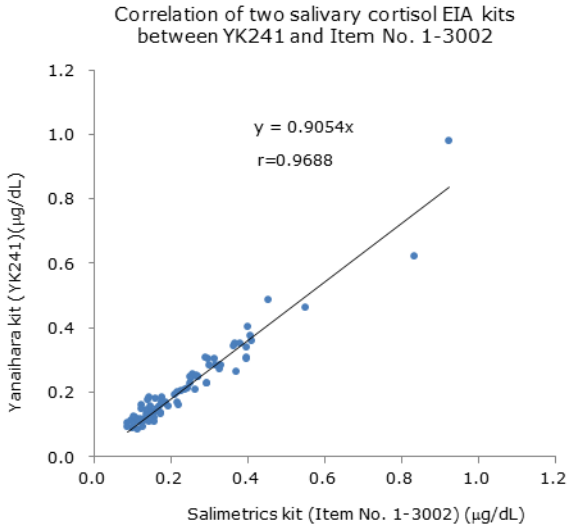
VIII. References

1. Knutsson U, Dahlgren J et al: **Circadian cortisol rhythms in heathy boys and girls: Relationship with age, growth, body composition, and pubertal development.** *J Clin Endocrinol Metab* 82:536-40, 1997

2. Miller GE, Chen E et al: **If it goes up, must it come down? Chronic stress and the hypothalamic-pituitary adrenocortical axis in humans .** *Psychol Bull* 133:25-45, 2007
3. Carde AH, and Hansen AM: **Long-term stability of salivary cortisol.** *Scan J Clin Lab Invest* 65:433-6, 2005
4. National Committee for Clinical Laboratory Standards Evaluation Protocols, SC1 (1989), Vallanova, PA: NCCLS

IX. Appendix

The YK241 Cortisol (Saliva) EIA kit has been compared to Salimetrics High Sensitivity Salivary Cortisol Enzyme Immunoassay Kit（Item No. 1-3002）. Ninety one samples of saliva from normal volunteers were assayed and linear regression analysis of the results yielded as shown in the graph.



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