Pantex
Division of Bio-Analysis, Inc.

Providing Diagnostic Technology for a Better Tomorrow

AM/PM SALIVARY CORTISOL

Enzyme Immunoassay Kit

For in-vitro diagnostic use (IVD)

Catalog Number: 631

510(k) FDA Approved

Rev 16 12/2020

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I. Intended Use and Description

The Pantex Cortisol Kit is designed and validated for the quantitative measurement of salivary Cortisol by EIA. This kit features one 60-minute incubation for maximum assay sensitivity and precision as well as a saliva-like matrix assay buffer system containing a neutralizing agent for acidic or basic saliva samples. For further information about this kit, its application or the procedures in this insert, please contact the Technical Service Team at Pantex.

II. Assay Background

In 1966, Katz and Shannon (1,2,3) using the Porter-Silber method were able to determine corticosteroid concentrations in saliva and were able to show that concentrations of corticosteroids in saliva, were related to blood concentrations. The advent of immunoassay made it possible to measure minute amounts of steroid hormones in blood. Subsequent modification of those assays allowed their measurement in saliva as well. These early assays, however, lacked validity due to matrix differences between serum and saliva, poor sensitivity and cumbersome extraction methods. Recently, several papers have been published on the determination of salivary Cortisol under varying physiological conditions using more specific and sensitive EIA and ELISA methods (18,20).

Cortisol (hydrocortisone, compound F) is the principle glucocorticoid secreted by the adrenal cortex. Adrenal secretion of cortisol is modulated by a complex negative feedback mechanism involving the central nervous system, hypothalamus, pituitary and adrenals. ACTH released from the pituitary augment's adrenal secretion of cortisol. In turn, increased levels of cortisol suppress pituitary secretion of ACTH while falling levels of cortisol are associated with rising levels of ACTH. Normally there is diurnal variation of cortisol with highest values measurable in the morning samples and lowest values obtained in the late afternoon. Cortisol levels rise independently of this circadian rhythm in response to stress or depression. Increased cortisol production is associated with Cushing's Syndrome and adrenal tumors while decreased production of cortisol is associated with adrenal insufficiency (Addison's disease) and adrenocorticotropic hormone (ACTH) deficiency (21, 22, 23, 24).

In blood 90% of the circulating cortisol is firmly bound to cortisol binding globulin (CBG), 7% is weakly bound to albumin and only 1-3% is free or unbound. In saliva the majority of cortisol occurs in the free or unbound form and enters the saliva via intracellular mechanisms (25). Numerous studies consistently report a high correlation between serum and saliva cortisol indicating that salivary cortisol levels clinically confirm levels of cortisol in serum (26, 27, 28).

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III. Assay Principle

The Pantex AM/PM Salivary Cortisol EIA Kit, Cat #631 is based on the competition principal and microplate separation. Cortisol calibrators of known concentration, unknown amounts of cortisol in saliva samples and a fixed amount of cortisol (analog) conjugated to horse radish peroxidase (Cortisol-HRP) compete for binding sites with a rabbit polyclonal antiserum bound to GARGG (goat anti-rabbit gamma globulin) coated wells of a microplate. After incubation, unbound components are washed away, enzyme substrate solution is added, and a blue color formed. This reaction is stopped with an acid solution to produce a yellow color. The optical density is then read 450 nm. The amount of Cortisol-HRP detected is inversely proportional to the amount of cortisol in a sample.

IV. Reagents Provided and Reagent Preparation

1. GARGG Plate: One 96 well microplate (12x8 breakable strip wells) coated with goat anti-rabbit gamma globulin placed in a resealable foil bag with desiccant. One (1) 96 well kit is sufficient for 78 singlicate or 39 duplicate patient measurements.
2. Salivary Cortisol EIA Calibrators 7 bottles. 5.0 ml of 0 calibrator, 1.0 mL of 0.1, 0.3, 1, 3, 10 and 30 ng/mL.
3. Salivary Cortisol EIA Control #1(Low): 1 bottle, 1.0 mL. Concentration is on the label.
4. Salivary EIA Control #2 (High): 1 bottle, 1.0 mL. Concentration is on the label.
5. Salivary Cortisol EIA Antibody produced (Pantex) in rabbit. Diluted in phosphate buffer base. Contains animal protein and a binding protein blocker: 1 bottle, 6 ml of anti-Cortisol. The solution is blue.
6. Cortisol-Horse radish peroxidase (HRP) **concentrate (10X):** 1 amber glass bottle 0.7 mL. The solution is light brown and light sensitive.
7. Cortisol-Horse radish peroxidase (HRP) **conjugate buffer** 6.3 mL. The solution is yellow. **To be used for working reagent preparation only.**
8. Cortisol-Horse radish peroxidase (HRP) **working reagent.** Preparation: Determine the amount of working cortisol-HRP needed and dilute 1:10 in conjugate buffer (number 7). For example, mix 0.5 mL of Cortisol-HRP **concentrate** (number 6) + 4.5 mL of Cortisol-HRP conjugate buffer (number 7). This is sufficient for 100 wells. Immediately after use, store the unused portion of the Cortisol-HRP working solution at 2° - 8°C. Discard if not used within 4 (four) weeks of mixing.
9. Wash solution (10X concentrated) EIA #1: 1 bottle, 50 mL Prior to use dilute 1:10 with deionized water.

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10. Color Development Reagent EIA #1: 1 amber plastic bottle, 12 mL of Tetramethylbenzidine plus hydrogen peroxide. Light sensitive.

11. Stopping Solution EIA #1: 1 bottle, 12 mL acid solution.

*Concentration of cortisol calibrators and controls are actual and traceable to NIST Cortisol Catalog # SRM921 Lot# 921.

V. Storage and Stability

- When stored at 2° - 8°C, unopened reagents will retain activity until the expiration date. Do not use reagents beyond this date.
- Use only reagents supplied with this kit. Do not interchange reagents with different lot numbers.
- Opened reagents must be stored at 2° - 8°C.
- Microtiter wells must be stored at 2° - 8°C. Once the foil bag has been opened, care should be taken to reseal tightly.
- Opened kits retain activity for one (1) month if stored as described above.
- Expiration dates and lot numbers are printed on the labels.

VI. Materials Needed but Not Supplied

- Device to dispense very accurately 25 µL of saliva.
- Multichannel pipettors.
- Microplate or orbital shaker
- Vortex Mixer
- Microplate washer (not required, plates can be washed manually).
- Microplate reader capable of reading 450 nm with 4 parameter data reduction or comparable software.
- Plate sealers.
- Collection device, VWR Sample Mailing Tubes, Cat #16465-260. (VWR International, 975 Overland Crt, San Dimas, CA 91773)

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VII. Sample Collection and Processing

1. ***Collection:*** This sample collection and processing procedure must be followed.

- Avoid food consumption, drinking coffee or alcohol, smoking or chewing gum one (1) hour prior to sample collection.
- Rinse mouth thoroughly with water 15 minutes prior to collection.
- Collect whole saliva by unstimulated passive drool by allowing saliva to drip off the lower lip into a graduated plastic test tube (Pantex recommends VWR Sample Mailing Tubes Cat #16465-260) or by allowing saliva to accumulate in the floor of the mouth and spitting it into the recommended collection device.
- Time and date specimen. Refrigerate then freeze (-20°C or below) samples until day of assay. On day of assay, thaw samples to facilitate precipitation of mucins. Centrifuge at 1500xg for ten minutes. Bring samples to room temperature and assay.

2. Sample stability:

Storage	Room Temperature 20 – 30 °C	37 °C	2 – 8 °C	≤ -15 °C (7 freeze / thaw cycles)	≤ - 15 ° C (Long term)
Stability	Up to 7 days	Up to 7 days	Up to 7 days	Up to 7 days	Up to 180 days

3. Interferences:

An in-vitro experiment was performed by spiking three (3) levels of Cortisol with high concentrations (>1000 fold those of cortisol) of five (5) commonly consumed products: alcohol, coffee (as caffeine), cigarette (as nicotine) and food and gum as extracts. The results obtained demonstrate no significant differences between the controls and the spiked samples. However, as a precautionary measure, follow the instructions stated in Step 1, under X. Samples 1. Collection of samples.

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VIII. Assay Procedure Summary Flow Sheet

Calibrator Control Sample (I.D.)	Calibrator Control Sample (µL)	Cortisol-HRP Working Reagent (µL)	Anti- Cortisol (µL)	Mix. Incubate for 60 min. at room temp. with shaking.	Diluted 10X Wash Solution (µL)	Wash 3X	Color Developer (µL)	Mix. Incubate 30 min. at room temperature	Stopping Solution (µL)	Mix. Read at 450 nm
0	25	50	50		300		100		100	
0.1	25	50	50		300		100		100	
0.3	25	50	50		300		100		100	
1	25	50	50		300		100		100	
3	25	50	50		300		100		100	
10	25	50	50		300		100		100	
30	25	50	50		300		100		100	
Control #1	25	50	50		300		100		100	
Control #2	25	50	50		300		100		100	
Sample #1	25	50	50		300		100		100	

IX. Assay Procedure

1. To a 96 well GARGG microplate dispense 25 µL of ready-to-use **Salivary Cortisol EIA calibrators** (0, 0.1, 0.3, 1.0, 3.0, 10.0 and 30 ng/mL), controls, and saliva samples.

2. Add 50 µL of **Cortisol-HRP Working Reagent** to all wells (see **Reagent Preparation Section Page 4, number 8**).

3. Add 50 µL of **Anti-Cortisol EIA antibody**.

4. Cover microplate with plastic sealer. Incubate by shaking on a microplate orbital shaker set at 500-900 rpm for **60** minutes at room temperature.

5. After incubation, decant the contents of the wells. Wash 3 times with 300 ul of diluted wash solution (10 mL of 10XWash solution EIA #1 diluted with 90 mL of D.I. water). After the 3rd wash, invert GARGG microplate on an absorbent paper and tap dry.

6. Pipet 100 µL of **Color Development** reagent EIA #1 into each well. Shake briefly (manual). Cover microplate with plastic sealer. Incubate for 30 minutes at room temperature.

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7. Pipet 100 µL of **Stopping Solution** EIA #1 into each microtiter well of the GARGG plate. Shake briefly (manual). Color changes from blue to yellow.

8. Read at 450 nm on a microplate reader within 30 minutes.

   Note: If samples exceed the highest calibrator, dilute with zero calibrator and make appropriate concentration correction.

X. Typical Results

Typical Calibration Curve for 25 µL Sample Size			
Calibrators (ng/mL)	**Mean Absorbance (450 nM)**	**% B/Bo**	**Value (ng/mL)**
0	2.231	100.0	0.0
0.1	2.056	92.2	0.1
0.3	1.717	77.0	0.3
1	1.155	51.8	1.0
3	0.614	27.5	3.0
10	0.246	11.0	10
30	0.130	5.8	30
Control # 1	1.130	50.6	1.04
Control # 2	0.159	7.1	20.14
Sample # 1	0.970	43.5	1.40
Sample # 2	0.235	10.5	11.26

Conversion factor ng/mL to nmol/L = 2.76

XI. Calculation

1. Compute the average optical density (OD) for the zero (Bo) calibrator.

2. Calculate the percent bound (B/Bo) for each calibrator, control and unknown by dividing the average OD (B) by the average OD for the zero (Bo) x 100.

3. Plot percent bound (B/Bo) versus the calibrator concentrations and draw the best fit for the curve.

4. Plot percent bound (B/Bo) of the controls and unknowns to determine saliva cortisol concentrations.

5. Alternately, determine the concentrations of the controls and unknowns by interpolation using software capable of logistics using a 4-parameter sigmoid minus curve fit.

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Analytical measuring range (AMR)	0.1 ng/ml - 30.0 ng/ml

Samples with cortisol values greater than 30 ng/ml (82.77 nmol/L) should be diluted 1:10 with zero (0) calibrator and re-run for accuracy. Obtain the final Cortisol concentration by multiplying the diluted sample by the dilution factor.

XII. Quality Control

The expected values for the controls are stated on the label of each control which are included in the kit. The results can only be accepted if the expected values are met.

XIII. Expected Values

AM Expected Values:

Subjects (Number)	Subjects (Gender)	Age (Years)	AM Median (ng/mL)	AM Range (ng/mL)
152	76 Males 76 Females	23-68	6.70	2.58 - 12.69

PM Expected Values:

Subjects (Number)	Subjects (Gender)	Age (Years)	PM Median (ng/mL)	PM Range (ng/mL)
152	76 Males 76 Females	23-68	0.58	0.25 - 2.96

*It is recommended that each laboratory establishes its own range of normal values.

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XIV.Performance Characteristics

Antiserum Cross Reactivities are expressed as the ratios of the concentrations of unlabeled cortisol over the compound that displaces 50% of cortisol-enzyme conjugate from the antiserum. All the saliva samples were collected using the collection device, VWR Sample Mailing Tubes, Cat #16465-260. (VWR International, 975 Overland Crt, San Dimas, CA 91773).

A. Specificity of Antiserum

Compounds		
C-21 Steroids:	**Spiked Concentration**	**% Cross-reactivity**
Cortisol	10,000 ng/mL	100.00
17-OH-Progesterone	10,000 ng/mL	0.0284
Pregnenolone	10,000 ng/mL	0.0038
17-OH-Pregnenolone	10,000 ng/mL	0.0066
Progesterone	10,000 ng/mL	0.0079
Desoxycorticosterone	10,000 ng/mL	0.0517
11-Desoxycortisol	10,000 ng/mL	1.8133
Dexamethasone	10,000 ng/mL	0.0164
Cortisone	10,000 ng/mL	0.7600
Corticosterone	10,000 ng/mL	1.0847
Aldosterone	10,000 ng/mL	0.0070
C-19 Steroids:	**Spiked Concentration**	**% Cross-reactivity**
Androstenedione	10,000 ng/mL	0.0038
Testosterone	10,000 ng/mL	0.0042
5αDHT	10,000 ng/mL	0.0019
DHEA-SO4	10,000 ng/mL	0.0031
Androstanedione	10,000 ng/mL	0.0028
C-18 Steroids:	**Spiked Concentration**	**% Cross-reactivity**
Estradiol 17ß	10,000 ng/mL	0.0024
Estradiol 17α	10,000 ng/mL	0.0003
Estrone	10,000 ng/mL	0.0010
Estriol	10,000 ng/mL	0.0015
Other structurally related steroids:	**Spiked Concentration**	**% Cross-reactivity**
Dehydroisoandrosterone	1000 ng/mL	0.0076
6αmethyl-17-Hydroxyprogesterone	1000 ng/mL	0.1427
6ß-Hydroxycortisol	1000 ng/mL	1.7177
Prednisone	1000 ng/mL	1.0874
Prednisolone	1000 ng/mL	25.9001

At >10% cross reaction prednisolone is a potential interfering substance

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B. Detection Limits:

The LOB (limit of the blank), the LOD (limit of detection) and the LOQ (limit of quantitation), were determined by generating one hundred twenty (120) measurements each of "cortisol free saliva" and low level (<0.1 ng/mL) cortisol samples (Reference, CLSI EP17-A, protocols for Determination of Limits of Detection and Limits of Quantitation).

Limit of the Blank (LoB) ng/mL	Limit of Detection (LoD) ng/mL	Limit of Quantitation (LoQ) ng/mL
0.0392	0.0519	0.0519

C. Precision and Reproducibility:

Intra-assay

The intra-assay precision was determined from the mean of 20 replicates of low, medium and high samples.

Sample	N	Mean (ng/mL)	Standard Deviation (ng/mL)	%CV
Low	20	0.627	0.034	5.4
Medium	20	3.995	0.266	6.7
High	20	25.232	1.579	6.3

Inter-assay

The inter-assay precision was determined from the mean of the average duplicates of 12 separate assays with low, medium and high samples.

Sample	N	Mean (ng/mL)	Standard Deviation (ng/mL)	%CV
Low	12	0.587	0.037	6.3
Medium	12	4.163	0.301	7.2
High	12	25.126	0.712	2.8

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Repeatability

This study was conducted during 4 days of a familiarization period and 20 days of testing. Two assays were performed daily with a minimum of 2 hours between assays. Three (3) different reagents lots and three (3) saliva pools were used for the study (Low, medium and high). The pools were aliquoted and frozen until day of assay.

Precision Low Concentration Pool

	Standard Deviation, (SD)	% Coefficient of Variation (CV)
Within Run	0.0224	3.79
Between Run	0.0462	7.80
Repeatability	0.0162	2.73
Total Device Precision	0.0538	9.09

Precision Medium Concentration Pool

	Standard Deviation, (SD)	% Coefficient of Variation (CV)
Within Run	0.1475	3.60
Between Run	0.0514	1.26
Repeatability	0.1025	2.50
Total Device Precision	0.1869	4.56

Precision High Concentration Pool

	Standard Deviation, (SD)	% Coefficient of Variation (CV)
Within Run	0.4442	1.76
Between Run	0.2915	1.15
Repeatability	0.62276	2.46
Total Device Precision	0.8185	3.24

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Inter-lot Variation

The inter-lot (between lot) precision was determined by duplicate measurements of five (5) saliva pools and three (3) spiked controls in saliva like matrix, using flow sheet stated on the Pantex AM/PM Salivary Cortisol EIA Kit, Cat #631 section VII insert.

Saliva samples ID	Lot #012 Mean (ng/mL)	Lot #013 Mean (ng/mL)	Lot #014 Mean (ng/mL)	Inter-lot Mean (ng/mL)	Inter-lot SD (ng/mL)	Inter-lot CV (%)
20	4.65	4.45	4.79	4.64	0.164	3.5
21	0.67	0.61	0.71	0.67	0.049	7.4
22	2.02	1.95	2.09	2.02	0.069	3.4
23	4.75	4.69	4.76	4.73	0.041	0.9
24	2.01	1.99	2.04	2.01	0.026	1.3
25	3.64	3.67	3.71	3.68	0.036	1.0
LC	0.98	0.94	1.01	0.98	0.036	3.7
MC	5.21	5.31	5.49	5.34	0.140	2.6
HC	10.79	10.13	10.52	10.48	0.329	3.1

D. Linearity Study:

Ten (10) sample concentrations that span the assay measuring range were performed Per EP6-A, Evaluation of the Linearity of Quantitative Measurement Procedures.

S=10 samples (dilutions)
Concentration = (C1*V1 + C10*V10)/(V1+V10)

	C1 (ng/mL)	V1 (ng/mL)	C10 (ng/mL)	V10 (ng/mL)	Calculated Concentration (ng/mL)	Obtained Concentration (ng/mL)	Recovery (%)
1	0.093			*	0.100	0.093	93
2	0.093	0.889	33.788	0.111	3.833	3.729	97.3
3	0.093	0.778	33.788	0.222	7.573	7.620	100.6
4	0.093	0.667	33.788	0.333	11.313	10.842	95.8
5	0.093	0.556	33.788	0.444	15.054	14.350	95.3
6	0.093	0.444	33.788	0.556	18.827	18.313	97.3
7	0.093	0.333	33.788	0.667	22.568	21.547	95.5
8	0.093	0.222	33.788	0.778	26.308	24.694	93.9
9	0.093	0.111	33.788	0.889	30.048	30.459	101.4
10				*	35.000	33.788	96.6

*****Targets of low and high concentration

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E. Recovery:

Ten (10) saliva samples containing different levels of endogenous cortisol were spiked with known quantities of cortisol and assayed.

Sample	Endogenous (ng/mL)	Added (ng/mL)	Expected (ng/mL)	Observed (ng/mL)	Recovery (%)
1	0.493	0.250	0.743	0.739	99.5
2	0.878	0.500	1.378	1.291	93.7
3	1.551	1.000	2.551	2.641	103.5
4	1.850	2.000	3.850	3.958	102.8
5	0.936	4.000	4.936	4.951	100.3
6	1.042	8.000	9.042	9.394	103.9
7	0.691	16.000	16.691	17.165	102.8
8	0.622	20.000	20.622	19.997	97.0
9	2.057	24.000	26.057	24.938	95.7
10	0.348	28.000	28.348	28.943	102.1

F. Method Comparison:

A comparative study was performed between the Pantex Salivary AM PM Cortisol EIA Kit Cat #631 and a FDA cleared predicate device. A total of 160 samples were used for the study of which 6 samples were spiked representing 3.75 % of the total number of samples. The results show the following regression and correlation statistics.

Linear Regression equation	Y = 1.0269X + 0.0994
Correlation	R^2 = 0.989

XV. Limitations

- The reagents are optimized to measure cortisol directly in saliva.
- Cortisol levels are elevated during the later stages of pregnancy and in women on contraceptives or after long-term use of contraceptives (28, 29).
- Elevated cortisol levels can be found in conditions of sepsis, infection, chronic liver disease, and renal failure. Low cortisol levels result from liver disease, pituitary hyposecretion, hypothyroidism or steroid therapy.
- Note that an in-vitro study to identify potential interfering substances in the measurement of salivary cortisol, may not identify some interferents and the form(s) of potential interferents being tested may not represent the naturally occurring forms.

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- The use of topical creams or ointments containing hydrocortisone (true cortisol) should be avoided as they can cause preanalytical contamination of the saliva sample indistinguishable from endogenous cortisol as measured by Immunoassay or LC-MS/MS (30).

XVI. Precautions

- Only physician, clinical labs, research labs and hospital labs may acquire, possess and use the kit.
- Compare contents and packing list, if there is breakage or shortage, notify Pantex immediately.
- Do not pipet reagents by mouth.
- Do not smoke, eat or drink while performing assay.
- Wear disposable rubber gloves.
- Treat all saliva samples as potentially infectious.
- Do not mix reagent lot numbers or alter in any way the reagents in this kit. If this is done, Pantex will not be responsible for the performance of the assay.
- Avoid contact with Color Development Reagent (TMB). It contains solvents that can irritate skin and mucus membranes. If contact is made, wash thoroughly with water.
- Avoid contact with stopping solution. It contains acid. If contact is made, rinse thoroughly with water.

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