Version 22-04/24

User's Manual

Salivary Cortisol ELISA Kit

REF **DEIA-JY2313**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

Address: 45-1 Ramsey Road, Shirley, NY 11967, USA

Tel: 1-631-624-4882 (USA) 44-161-818-6441 (Europe) **Fax: 1-631-938-8221**

Email: info@creative-diagnostics.com **Web: www.creative-diagnostics.com**

PRODUCT INFORMATION

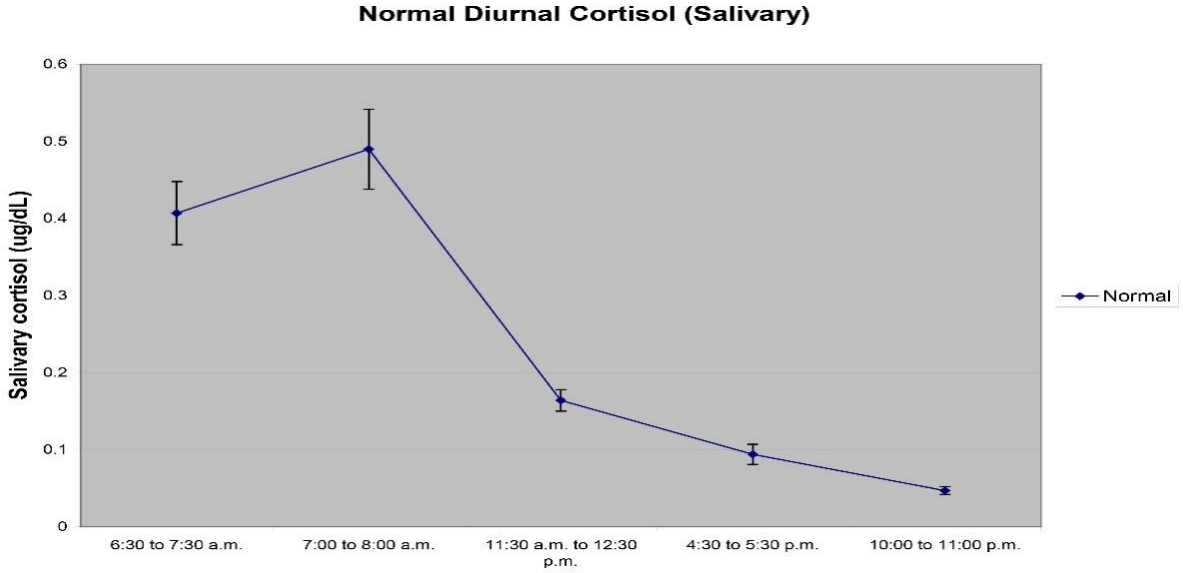
Intended Use

The Cortisol Enzyme Immunoassay Kit is a competitive immunoassay specifically designed and validated for the quantitative measurement of salivary Cortisol. It is not intended for diagnostic use. It is intended only for research use in humans and some animals. CD has not validated this kit for serum or plasma samples.

Note: Please read the complete kit insert before performing this assay. Failure to follow kit procedure and recommendations for saliva collection and sample handling may result in unreliable values.

General Description

Cortisol (hydrocortisone, Compound F) is the major glucocorticoid produced in the adrenal cortex. Cortisol production has a circadian rhythm, with levels peaking in the early morning and dropping to lowest values at night. Levels rise independently of circadian rhythm in response to stress. In blood, only about 5-10% of Cortisol is in its unbound or biologically active form. The remaining Cortisol is bound to serum proteins. Unbound serum Cortisol enters saliva via intracellular mechanisms; in saliva, the majority of Cortisol remains unbound to protein. Salivary Cortisol levels are unaffected by salivary flow rate and are relatively resistant to degradation from enzymes or freeze-thaw cycles. Studies consistently report high correlations between serum and salivary Cortisol, indicating that salivary Cortisol levels reliably estimate serum Cortisol levels).



Internal CD Data, n=26. Time of Cortisol peak will vary in individuals relative to their normal wake-up time.

Principles of Testing

This is a competitive immunoassay kit. Cortisol in standards and samples compete with Cortisol conjugated to horseradish peroxidase for the antibody binding sites on a microtitre plate. After incubation, unbound components are washed away. Bound Cortisol Enzyme Conjugate is measured by the reaction of the horseradish peroxidase enzyme to the substrate tetramethylbenzidine (TMB). This reaction produces a blue color. A yellow color is formed after stopping the reaction with an acidic solution. The optical density is read

on a standard plate reader at 450 nm. The amount of Cortisol Enzyme Conjugate detected is inversely proportional to the amount of Cortisol present in the sample.

General Kit Use Advice

1. This kit uses break-apart microtitre strips. You may run less than a full plate. Unused wells must be stored at 2-8°C in the foil pouch with desiccant and used in the frame provided.
2. Avoid microbial contamination of opened reagents. CD recommends using opened reagents within one month. Store all reagents at 2-8°C.
3. The quantity of reagent provided with a single kit is sufficient for three partial runs. The volumes of wash buffer and enzyme conjugate prepared for assays using less than a full plate should be scaled down accordingly, keeping the same dilution ratio.
4. Do not mix components from different lots of kits.
5. To ensure highest quality assay results, pipetting of samples and reagents must be done as quickly as possible (without interruption) across the plate. Ideally, the process should be completed within 20 minutes or less.
6. When using a multichannel pipette to add reagents, always follow the same sequence when adding all reagents so that the incubation time is the same for all wells.
7. When running multiple plates, or multiple sets of strips, a standard curve must be run with each individual plate and/or set of strips.
8. The temperature of the laboratory may affect assays. CD' kits have been validated at 68-74°F (20-23.3°C). Higher or lower temperatures may affect OD values.
9. Routine calibration of pipettes and other equipment is critical for the best possible assay performance.
10. When mixing plates during assay procedures, avoid speeds that spill the contents of the wells.

pH Indicator

Cortisol values from samples with a pH < 3.5 or > 9.0 may be inaccurate. A pH indicator in the Assay Diluent alerts the user to samples with high or low pH values. Upon addition of the Assay Diluent, acidic samples will turn yellow and alkaline samples will turn purple. Dark yellow or purple wells indicate that a pH value for that sample should be obtained using pH strips. Samples with a pH < 3.5 or > 9.0 should be recollected.

Reagents And Materials Provided

1. **Microtitre Plate** Coated with monoclonal anti-Cortisol antibodies. 1/96 well
2. **Cortisol Standard** In a saliva-like matrix. Ready to use, traceable to NIST standard: 3.0, 1.0, 0.333, 0.111, 0.037, 0.012 µg/dL (82.77, 27.59, 9.19, 3.06, 1.02, 0.33 nmol/L). Contains: Cortisol, buffer, preservative. 6 vials / 500 µL each
3. **Cortisol Controls** High, Low, in a saliva-like matrix. Ready to use. Contain: Cortisol, buffer, preservative. 2 vials / 500 µL each
4. **Cortisol Enzyme Conjugate** Concentrate. Dilute before use with Assay Diluent. (See step 5 of Assay Procedure.) Contains: Cortisol conjugated to HRP, preservative. 1 vial / 50 µL
5. **Assay Diluent** Contains: phosphate buffer, pH indicator, preservative. 1 bottle / 60 mL
6. **Wash Buffer Concentrate (10×)** Dilute before use according to Reagent Preparation. Contains: phosphate buffer, detergent, preservative. 1 bottle / 100 mL
7. **TMB Substrate Solution** Non-toxic, ready to use. 1 bottle / 25 mL

8. **Stop Solution** 1 bottle / 12.5 mL
9. **Non-Specific Binding (NSB) Wells** Do not contain anti-Cortisol antibody. Break off and insert as blanks (optional) where needed. 1 strip

Materials Required But Not Supplied

1. Precision pipette to deliver 15 and 25 µL
2. Precision multichannel pipette to deliver 50 µL and 200 µL
3. Vortex
4. Plate rotator with 0.08-0.17 inch orbit capable of 500 rpm
5. Plate reader with 450 nm and 490 to 492 nm reference filters
6. Computer software for data reduction
7. Deionized water
8. Reagent reservoirs
9. One disposable polypropylene tube to hold at least 24 mL
10. Pipette tips
11. Serological pipette to deliver up to 24 mL
12. Centrifuge capable of 1500 × g

Storage

All unopened components of this kit are stable at 2-8°C until the kit's expiration date.

Specimen Collection And Preparation

Specimen Collection

Avoid sample collection within 60 minutes after eating a major meal or within 12 hours after consuming alcohol. Acidic or high sugar foods can compromise assay performance by lowering sample pH and influencing bacterial growth. To minimize these factors, rinse mouth thoroughly with water 10 minutes before sample is collected.

Collect whole saliva by unstimulated passive drool. Donors may tilt the head forward, allowing the saliva to pool on the floor of the mouth, then pass the saliva through the SalivaBio Collection Aid (SCA) into a polypropylene vial.

Samples visibly contaminated with blood should be recollected. Do not use dipsticks, which result in false positive values due to salivary enzymes.

It is important to record the time and date of specimen collection when samples are obtained due to the diurnal variation in Cortisol levels.

Sample Handling and Preparation

After collection, it is important to keep samples cold in order to avoid bacterial growth in the specimen. Refrigerate sample within 30 minutes, and freeze at or below -20°C within 4 hours of collection. (Samples

may be stored at -20°C for up to 6 months.)

Note: Do not add sodium azide to saliva samples as a preservative, as it may cause interference in the immunoassay.

On day of assay, thaw the saliva samples completely, vortex, and centrifuge at 1500 × g for 15 minutes. Freezing saliva samples will precipitate mucins. Centrifuging removes mucins and other particulate matter which may interfere with antibody binding and affect results. Samples should be at room temperature before adding to assay plate. Pipette clear sample into appropriate wells. Re-freeze saliva samples as soon as possible after adding to the assay plate. Re-centrifuge saliva samples each time that they are thawed. Avoid multiple freeze-thaw cycles.

Reagent Preparation

1. Bring all reagents to room temperature and mix before use. A minimum of 1.5 hours is recommended for the 24 mL of Assay Diluent used in Step 5 (conjugate dilution) to come to room temperature.
2. Bring Microtitre Plate to room temperature before use. **It is important to keep the foil pouch with the plate strips closed until warmed to room temperature, as humidity may have an effect on the coated wells**.
3. Prepare 1× wash buffer by diluting Wash Buffer Concentrate (10×) 10-fold with roomtemperature deionized water (100 mL of Wash Buffer Concentrate (10×) to 900 mL of deionized H2O). **Dilute only enough for current day's use and discard any leftover reagent**. (If precipitate has formed in the concentrated wash buffer, it may be heated to 40°C for 15 minutes. Cool to room temperature before use in assay.)

Assay Procedure

1. **Step 1**: Read and prepare reagents according to the Reagent Preparation section before beginning assay. Determine your plate layout. Here is a suggested layout. (Standards, controls, and saliva samples should be assayed in duplicate.)

	1	2	3	4	5	6	7	8	9	10	11	12
A	3.000 Std	3.000 Std	Ctrl-H	Ctrl-H								
B	1.000 Std	1.000 Std	Ctrl-L	Ctrl-L								
C	0.333 Std	0.333 Std	SMP-1	SMP-1								
D	0.111 Std	0.111 Std	SMP-2	SMP-2								
E	0.037 Std	0.037 Std	SMP-3	SMP-3								
F	0.012 Std	0.012 Std	SMP-4	SMP-4								
G	Zero	Zero	SMP-5	SMP-5								
H	NSB*	NSB*	SMP-6	SMP-6								

*NSB = Non-specific binding wells. These may serve as blanks. Use is optional.

2. **Step 2**: Keep the desired number of strips in the strip holder and place the remaining strips back in the foil

pouch. If you choose to place non-specific binding wells in H-1, 2, remove strips 1 and 2 from the strip holder and break off the bottom wells. Place the strips back into the strip holder leaving H-1, 2 blank. Break off 2 NSB wells from the strip of NSB wells included in the foil pouch. Place in H-1, 2. Alternatively, NSBs may be placed wherever you choose on the plate. Reseal the foil pouch with unused wells and desiccant. Store at 2-8°C.

Cautions: 1. Extra NSB wells should not be used for determination of standards, controls, or unknowns.

2. Do not insert wells from one plate into a different plate

3. **Step 3:** Pipette 24 mL of Assay Diluent into the disposable tube. (Scale down proportionally if using less than the entire plate.) Set aside for Step 5.
4. **Step 4:**

   • Pipette 25 µL of standards, controls, and saliva samples into appropriate wells.

   • Pipette 25 µL of Assay Diluent into 2 wells to serve as the zero.

   • Pipette 25 µL of Assay Diluent into each NSB well.
5. **Step 5**: Dilute the Enzyme Conjugate 1:1600 by adding 15 µL of the conjugate to the 24 mL tube of Assay Diluent. (Scale down proportionally if not using the entire plate.) Conjugate tube may be centrifuged for a few minutes to bring the liquid down to the tube bottom. Immediately mix the diluted conjugate solution and add 200 µL to each well using a multichannel pipette.
6. **Step 6**: Mix plate on a plate rotator for 5 minutes at 500 rpm and incubate at room temperature for a total of 1 hour.
7. **Step 7**: Wash the plate 4 times with 1X wash buffer. A plate washer is recommended. However, washing may be done by gently squirting wash buffer into each well with a squirt bottle, or by pipetting 300 µL of wash buffer into each well and then discarding the liquid over a sink. After each wash, the plate should be thoroughly blotted on paper towels before turning upright. If using a plate washer, blotting is still recommended after the last wash.
8. **Step 8**: Add 200 µL of TMB Substrate Solution to each well with a multichannel pipette.
9. **Step 9**: Mix on a plate rotator for 5 minutes at 500 rpm and incubate the plate in the dark (covered) at room temperature for an additional 25 minutes.
10. **Step 10**: Add 50 µL of Stop Solution with a multichannel pipette.
11. **Step 11:**

    • Mix on a plate rotator for 3 minutes at 500 rpm. If green color remains, continue mixing until green color turns to yellow. Be sure all wells have turned yellow. Caution: Spillage may occur if mixing speed exceeds 600 rpm.

    • Wipe off bottom of plate with a water-moistened, lint-free cloth and wipe dry.

    • Read in a plate reader at 450 nm. Read plate within 10 minutes of adding Stop Solution. (For best results, a secondary filter correction at 490 to 492 nm is recommended.)

Quality Control

The High and Low Cortisol Controls should be run with each assay. The control ranges established are to be used as a guide. Each laboratory should establish its own range. Variations between laboratories may be

caused by differences in techniques and instrumentation.

Calculation

1. Compute the average optical density (OD) for all duplicate wells.
2. Subtract the average OD for the NSB wells (if used) from the OD of the zero, standards, controls, and saliva samples.
3. Calculate the percent bound (B/Bo) for each standard, control, and saliva sample by dividing the OD of each well (B) by the average OD for the zero (Bo). (The zero is not a point on the standard curve.)
4. Determine the concentrations of the controls and saliva samples by interpolation using data reduction software. We recommend using a 4-parameter non-linear regression curve fit.
5. Samples with Cortisol values greater than 3.0 µg/dL (82.77 nmol/L) should be diluted with Assay Diluent and rerun for accurate results. If a dilution of the sample is used, multiply the assay results by the dilution factor.

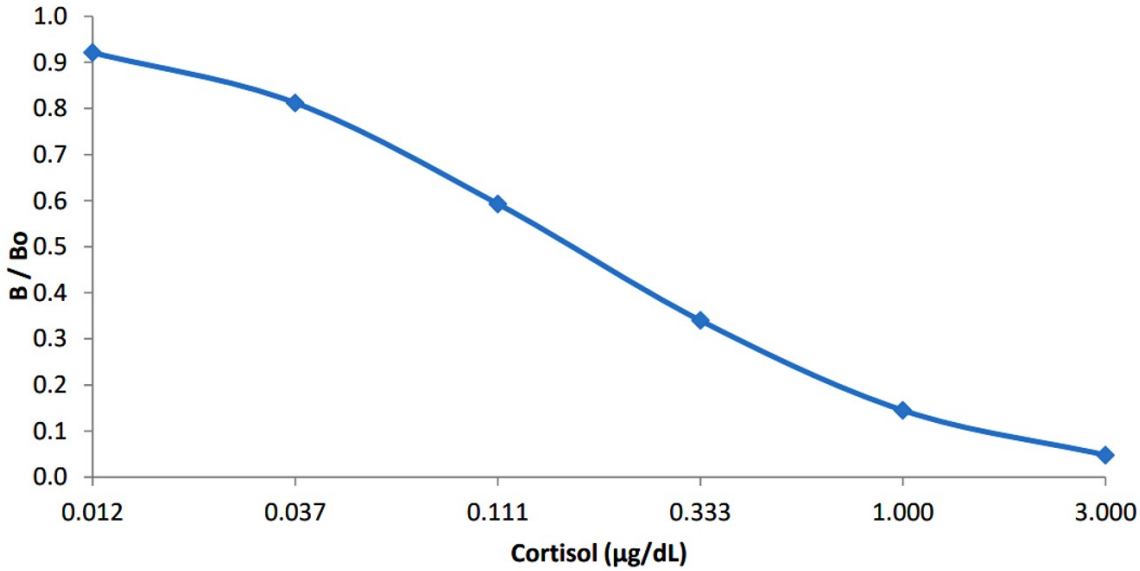
A new Standard Curve must be run with each full or partial plate.

Typical Standard Curve

The results shown below are for illustration only and should not be used to calculate results from another assay.

Well	Standard	Average OD	B	B/Bo	Cortisol (µg/dL)
A1,A2	S1	0.094	0.071	0.048	3.000
B1,B2	S2	0.236	0.213	0.145	1.000
C1,C2	S3	0.524	0.501	0.340	0.333
D1,D2	S4	0.897	0.874	0.593	0.111
E1,E2	S5	1.219	1.196	0.812	0.037
F1,F2	S6	1.379	1.356	0.921	0.012
G1,G2	Bo	1.496	1.473	NA	NA
H1,H2	NSB	0.023	NA	NA	NA

Example: HS Cortisol 4-Parameter Curve Fit



Salivary Cortisol Example Ranges*

Group	Number	Overall Range (µg/dL)
Children, neonatal	275	ND - 3.417
Children, age 6 months	165	ND - 2.734

Group	Number	23:00 hrs. (µg/dL)
Normal subjects	19	0.007 – 0.115
Cushing's subjects	21	0.130 – 2.972

Group	Number	AM Range (μg/dL)	PM Range (μg/dL)
Children, ages 2.5-5.5	112	0.034 - 0.645	0.053 - 0.607
Children, ages 8-11	285	0.084 - 0.839	ND - 0.215
Adolescents, ages 12-18	403	0.021 - 0.883	ND - 0.259
Adult males, ages 21-30	26	0.112 - 0.743	ND - 0.308
Adult females, ages 21-30	20	0.272 - 1.348	ND - 0.359
Adult males, ages 31-50	67	0.122 - 1.551	ND - 0.359
Adult females, ages 31-50	31	0.094 - 1.515	ND - 0.181
Adult males, ages 51-70	28	0.112 - 0.812	ND - 0.228
Adult females, ages 51-70	23	0.149 - 0.739	0.022 - 0.254
All adults	192	0.094 - 1.551	ND - 0.359

*To be used as a guide only. Each laboratory should establish its own range.

ND = None detected Expected ranges for neonates to 5.5 years were derived using the CD Salivary Cortisol Immunoassay Kit. Expected ranges for 8 to 18 years were reported from an unpublished manuscript, Pennsylvania State University's Behavioral Endocrinology Laboratory. Adult ranges were obtained from published literature.

Precision

The intra-assay precision was determined from the mean of 20 replicates each.

Saliva Sample	N	Mean (μg/dL)	Standard Deviation (μg/dL)	Coefficient of Variation (%)
1	20	2.07	0.08	4
2	20	1.14	0.05	4
3	20	0.42	0.01	3
4	20	0.16	0.01	5
5	20	0.06	0.00	7

The inter-assay precision was determined from the mean of average duplicates for 20 separate runs.

Saliva Sample	N	Mean (µg/dL)	Standard Deviation (µg/dL)	Coefficient of Variation (%)
1	20	1.99	0.05	3
2	20	1.16	0.05	4
3	20	0.43	0.01	3
4	20	0.18	0.01	9
5	20	0.06	0.01	11

Sensitivity

Analytical Sensitivity

The lower limit of sensitivity was determined by interpolating the mean optical density minus 2 SDs of 10 sets of duplicates at the 0 µg/dL level. The minimal concentration of Cortisol that can be distinguished from 0 is 0.007 µg/dL.

Functional Sensitivity

The functional sensitivity was determined by assaying 60 saliva samples at a concentration level resulting in a CV of approximately 20%. The functional sensitivity of the salivary Cortisol ELISA is 0.018 µg/dL.

Correlation with Serum

The correlation between serum and saliva Cortisol was determined by assaying 49 matched samples using the Diagnostic Systems Laboratories Serum Cortisol EIA and the CD HS Salivary Cortisol EIA.

The correlation between saliva and serum was highly significant, $r(47) = 0.91$, $p < 0.0001$.

Specificity

Compound	Spiked Concentration (ng/mL)	% Cross-reactivity in HS Salivary Cortisol EIA
Prednisolone	100	0.568
Prednisone	1000	ND
Cortisone	1000	0.130
11-Deoxycortisol	500	0.156
21-Deoxycortisol	1000	0.041
17α-Hydroxyprogesterone	1000	ND
Dexamethasone	1000	19.2
Triamcinolone	1000	0.086
Corticosterone	10,000	0.214
Progesterone	1000	0.015
17β-Estradiol	10	ND
DHEA	10,000	ND
Testosterone	10,000	0.006
Transferrin	66,000	ND
Aldosterone	10,000	ND

ND = None detected (<0.004)

Linearity

Saliva Sample	Samples		Avg Observed (µg/dL)	Expected (µg/dL)	Recovery (%)
	Low	High			
a (Low)	100%	0%	0.07	0.07	N/A
b	90%	10%	0.36	0.34	108
c	80%	20%	0.63	0.61	104
d	70%	30%	0.93	0.88	106
e	60%	40%	1.13	1.15	98
f	50%	50%	1.45	1.42	102
g	40%	60%	1.64	1.69	97
h	30%	70%	1.88	1.96	96
i	20%	80%	2.27	2.23	102
j	10%	90%	2.49	2.50	99
k (High)	0%	100%	2.77	2.77	N/A

Recovery

Five saliva samples containing different levels of an endogenous Cortisol were spiked with known quantities of Cortisol and assayed.

Saliva Sample	Endogenous (µg/dL)	Added (µg/dL)	Expected (µg/dL)	Observed (µg/dL)	Recovery (%)
1	0.071	2.00	2.07	2.20	106
2	0.071	0.20	0.27	0.28	104
3	0.071	0.04	0.11	0.11	98
4	0.078	2.33	2.41	2.33	97
5	0.078	0.20	0.28	0.31	113
6	0.080	0.04	0.12	0.12	103
7	0.860	0.20	1.06	1.16	109
8	0.890	0.04	0.93	1.02	109

Sample Dilution Recovery

Saliva Sample	Dilution Factor	Expected (µg/dL)	Observed (µg/dL)	Recovery (%)
1	undiluted	N/A	0.73	N/A
	1:2	0.37	0.39	107
	1:4	0.18	0.20	111
	1:8	0.09	0.10	111
	1:16	0.05	0.05	105
2	undiluted	N/A	0.80	N/A
	1:2	0.40	0.40	101
	1:4	0.20	0.19	97
	1:8	0.10	0.09	94
	1:16	0.05	0.05	110

Precautions

Liquid Stop Solution is caustic; use with care. We recommend the procedures listed below for all kit reagents.

Handling

Follow good laboratory practices when handling kit reagents. Laboratory coats, gloves, and safety goggles are recommended. Wipe up spills using appropriate absorbent materials while wearing protective clothing. Follow local regulations for disposal.

Emergency Exposure Measures

In case of contact, immediately wash skin or flush eyes with water for 15 minutes. Remove contaminated clothing. If inhaled, remove individual to fresh air. If individual experiences difficulty breathing call a physician.

The above information is believed to be accurate but is not all-inclusive. This information should be used only as a guide.

Limitations

1. Samples with Cortisol values greater than 3.0 µg/dL (82.77 nmol/L) should be diluted with Assay Diluent and rerun for accurate results. To obtain the final Cortisol concentration, multiply the concentration of the diluted sample by the dilution factor.
2. A pH value should be obtained on samples that appear yellow or purple after the diluted conjugate solution is added and the plate is mixed (Step 6). Samples with pH values ≤ 3.5 or ≥ 9.0 should be recollected.
3. See "Specimen Collection" recommendations to ensure proper collection of saliva specimens and to avoid interfering substances.

4. Samples collected with sodium azide are unsuitable for this assay.
5. Any quantitative results indicating abnormal Cortisol levels should be followed by additional testing and evaluation.