
# Salivary Cortisol Ultrasensitive ELISA

Catalog Number:
COR32-K01 ( 1 x 96 Wells )

*For Research Use Only. Not for use in diagnostic procedures*

*v. 16 (31 DEC 24)*

EAGLE BIOSCIENCES, INC.
20A NW Blvd., Suite 112, Nashua, NH 03063
Phone: 617-419-2019 Fax: 617-419-1110
WWW.EAGLEBIO.COM

## INTENDED USE

The Eagle Biosciences Salivary Cortisol ELISA Assay kit is designed and validated for the quantitative measurement of salivary Cortisol by ELISA. 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 Eagle Biosciences, Inc at www.EagleBio.com or at 866-411-8023.*

## ASSAY BACKGROUND

In 1966, Katz and Shannon 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.

Cortisol (hydrocortisone, compound F) is the principal 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 augments 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 Cushings Syndrome and adrenal tumors while decreased production of cortisol is associated with adrenal insufficiency (Addison's disease) and adrenocorticotropic hormone (ACTH) deficiency.

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. Numerous studies consistently report a high correlation between serum and saliva cortisol indicating that salivary cortisol levels clinically confirm levels of cortisol in serum.

## ASSAY PRINCIPLES

This ultrasensitive cortisol saliva ELSIA 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 the 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 is formed. This reaction is stopped with an acid solution to produce a yellow color. The optical density is then read at 450 nm. The amount of cortisol-HRP detected is inversely proportional to the amount of cortisol in a sample.

## REAGENTS PROVIDED AND REAGENT PREPARATION

1. **GARGG Plate**- 1, 96-well plate (12x8 breakable strips) coated with goat anti-rabbit gamma globulin placed in a resealable foil bag. Sufficient for 78 singlicate or 39 duplicate sample measurements
2. **CALIBRATORS** – 7 vials, ready-to-use vials

   **CAL 0:** 0 ng/mL.....5.0 mL vial
   **CAL 1:** 0.1 ng/mL.....1.0 mL vial
   **CAL 2:** 0.3 ng/mL.....1.0 mL vial
   **CAL 3:** 1.0 ng/mL.....1.0 mL vial
   **CAL 4:** 3.0 ng/mL.....1.0 mL vial
   **CAL 5:** 10.0 ng/mL.....1.0 mL vial
   **CAL 6:** 30.0 ng/mL.....1.0 mL vial
3. **CONTROL 1 (LOW)** – 1 vial of 1.0 mL, concentration on vial, Range on QC
4. **CONTROL 2 (HIGH)** – 1 vial of 1.0 mL, concentration on vial, Range on QC
5. **ANTI-CORTISOL ANTIBODY** – 1 vial of 6 ml of antibody produced in rabbit. Diluted in phosphate buffer base. Contains animal protein and a binding protein blocker in blue solution.
6. **HRP CONCENTRATE (10X)** – 1 amber glass vial of 0.7 mL
7. **HRP CONJ BUFFER** – 6.3 mL of yellow solution to be used for working reagent preparation only
8. **HRP WORKING REAGENT** – Preparation;
   Determine the amount of working cortisol-HRP needed and dilute 1:10 in conjugate buffer (# 7). For example, mix 0.5 mL of cortisol-HRP concentrate ( #6) + 4.5 mL of Cortisol-HRP conjugate buffer (#7). This is sufficient for 100 wells. Immediately after use, store the unused portion of the Cortisol-HRP working solution at 2-8ºC. Discard if not used within 4 (four) weeks of mixing.
9. **WASH SOLUTION (10x)** – 1 vial of 50 mL. Dilute 1:10 with DI Water
10. **COLOR DEVELOPMENT REAGENT (TMB)** – 1 vial of 12 mL of TMB plus H2O2. Light sensitive
11. **STOP SOLUTION** – 1 vial of 12 mL acid solution

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

## STORAGE AND STABILITY

- When stored at 2-8ºC, 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 2-8ºC
- Microtiter wells must be stored at 2-8ºC. Once foil bag has been opened, care should be taken to reseal tightly.
- Opened kits retain activity one (1) month if stored properly
- Expiration dates and lot numbers are printed on the labels

## MATERIALS NEEDED BUT NOT SUPPLIED

1. Device to dispense very accurately 25 µl of saliva.
2. Multichannel pipettors.
3. Microplate or orbital shaker
4. Vortex Mixer
5. Microplate washer (not required, plates can be washed manually).
6. Microplate reader capable of reading 450 nm with 4 parameter data reduction or comparable software.
7. Plate sealer
8. Collection device

## SAMPLE COLLECTION AND PREPERATION

Sample collection and processing procedure must be followed.

1. Avoid food consumption, drinking coffee or alcohol, smoking, or chewing gum one (1) hour prior to sample collection.
2. Rinse mouth thoroughly with water 15 minutes prior to collection
3. Collect whole saliva by unstimulated passive drool by allowing saliva to drip off the lower lip into a graduated plastic tube or by allowing saliva to accumulate in the floor of the mouth and spitting it into the recommended collection device.
4. Time and date specimen. Refrigerate then freeze (-20C or below) samples until day of assay. On day of assay, thaw samples to facilitate precipitation of mucins. Centrifuge at 1500xg for ten (10) minutes. Bring samples to room temperature and assay

### Sample Stability

| **STORAGE** | Room Temperature 20-30ºC | 37ºC | 2-8ºC | ≤ -15ºC (7 freeze / thaw cycles) | ≤ -15ºC (Long term) |
|---|---|---|---|---|---|
| **STABILITY** | Up to 7 days | Up to 7 Days | Up to 7 Days | Up to 7 Days | Up to 180 Days |

### 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 difference between the controls and the spiked samples.

| Calibrator Control Sample (I.D.) | Calibrator control (µL) | Cortisol-HRP Working Reagent (µL) | Anti-Cortisol (µL) | **Mix. Incubate 60 min. at room temperature. 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 450nm** |
|---|---|---|---|---|---|---|---|---|---|---|
| 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 | |

## ASSAY PROCEDURE

1. To GARGG microplate pipet **25 µL** of ready-to-use Salivary Cortisol ELISA 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-number 8).
3. Add **50 µL** of Cortisol ELISA 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 µL** of diluted wash solution (10 mL of 10X Wash solution ELISA #1 diluted with 90 mL of D.I. water). After the 3rd wash, invert GARGG microplate on an absorbent paper and tap dry.
6. Pipette **100 µL** of TMB into each well. Shake briefly (manual). **Incubate** for 30 **minutes** at room temperature.
7. Pipette **100 µL** of Stopping Solution ELISA #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.

## TYPICAL RESULTS

| Typical Calibration Curve for 25 µL Sample Size | | | |
|---|---|---|---|
| **Calibrators (ng/L)** | **Mean Absorbance (450 nm)** | **%B/B0** | **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(low) | 1.130 | 50.6 | 1.04 |
| Control #2 (high) | 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**

## CALCULATION

1. Compute the average optical density (OD) for the zero (B0) calibrator
2. Calculate the percent bound (B/B0) for each calibrator, control and unknown by dividing the average OD (B) by the average OD for the zero (B0) x 100
3. Plot percent bound (B/B0) versus the calibrator concentrations and draw the best fit for the curve.
4. Plot percent bound (B/B0) of the controls and unknowns to determine saliva cortisol concentrations.
5. Alternatively, determine the concentrations of the controls and unknowns by interpolation using software capable of logistics using a 4-parametrer sigmoid minus curve fit.

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

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

## 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.

## EXPECTED VALUES

| AM Expected Values | | | | |
|---|---|---|---|---|
| Subjects (Number) | Subjects (Gender) | Age (Years) | AM Median (ng/mL) | AM Range (ng/mL) |
| 152 | 76 Males<br>76 Females | 23-68 | 6.70 | 2.58-12.69 |

| PM Expected Values | | | | |
|---|---|---|---|---|
| Subjects (Number) | Subjects (Gender) | Age (Years) | AM Median (ng/mL) | AM Range (ng/mL) |
| 152 | 76 Males<br>76 Females | 23-68 | 0.58 | 0.25-2.96 |

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

## PERFORMANCE CHARACTERISTICS

### Specificity of Antiserum

| **Compounds** | | |
|---|---|---|
| **C-21 Steroids** | **Spiked Concentration (ng/mL)** | **% Cross-reactivity** |
| Cortisol | 10,000 | 100.0 |
| 17-OH-Progesterone | 10,000 | 0.0284 |
| Pregnenolone | 10,000 | 0.0038 |
| 17-OH-Pregnenolone | 10,000 | 0.0066 |
| Progesterone | 10,000 | 0.0079 |
| Deoxycorticosterone | 10,000 | 0.0517 |
| 11-Desoxycortisol | 10,000 | 1.8133 |
| Dexamethasone | 10,000 | 0.0164 |
| Cortisone | 10,000 | 0.7600 |
| Corticosterone | 10,000 | 1.0847 |
| Aldosterone | 10,000 | 0.0070 |
| **C-19 Steroids** | | |
| Androstenedione | 10,000 | 0.0038 |
| Testosterone | 10,000 | 0.0042 |
| 5aDHT | 10,000 | 0.0019 |
| DHES-SO4 | 10,000 | 0.0031 |
| Androstanedione | 10,000 | 0.0028 |
| **C-18 Steroids** | | |
| Estradiol 17ß | 10,000 | 0.0024 |

| | | |
|---|---|---|
| Estradiol 17a | 10,000 | 0.0003 |
| Estrone | 10,000 | 0.0010 |
| Estriol | 10,000 | 0.0015 |
| **Other structurally related steroids** | | |
| Dehydroisoandrosterone | 1000 | 0.0076 |
| 6amethyl-17-hydroxyprogesterone | 1000 | 0.1427 |
| 6ß-Hydroxycortisol | 1000 | 1.7177 |
| Prednisone | 1000 | 1.0874 |
| Prednisolone | 1000 | 25.9001 |

At >10% cross reaction prednisolone is a potential substance

**Detection Limit**

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 of cortisol free saliva and low level (<0.1 ng/mL) cortisol samples

| 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 |

**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 |

*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 reagent lots and three (3) saliva pools were used for the study (low, medium, and high). The pools were aliquoted and frozen until day of assays.

*Precision Low Concentration Pool*

| | Standard Deviation, (SD) | % Coefficient of Variation (CV) |
|---|---|---|
| Within Run | 0.0224 | 3.79 |
| Between Run | 0.0426 | 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 |

*Inter-lot Variation*

The inter-lot precision was determined by duplicate measurements of five (5) saliva pools and three (3) spiked controls in saliva like matrix.

| Saliva Samples | Lot #012 | Lot #013 | Lot #014 | Inter-lot | Inter-lot | Inter-lot |
|---|---|---|---|---|---|---|
| ID | Mean (ng/ml) | Mean (ng/ml) | Mean (ng/ml) | Mean (ng/ml) | SD (ng/ml) | 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 |
| LC | 0.98 | 0.94 | 1.01 | 0.98 | 0.036 | 3.7 |
| MC | 5.21 | 5.31 | 5.49 | 5.34 | 0.14 | 2.6 |
| HC | 10.79 | 10.13 | 10.52 | 10.48 | 0.329 | 3.1 |

*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 |

### *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 |

### *Method Comparison*

A comparative study was performed between this assay and an FDA cleared predicted 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

## 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.

- 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).

**PRECAUTIONS**

- This kit is for research use only
- Compare contents and packing list, if there is breakage or shortage, notify Eagle Biosciences immediately
- Do not pipette reagents by mouth
- Do not smoke, eat or drink while performing assay
- Wear disposable gloves and proper lab protection and attire
- Treat all samples as potentially infectious
- Do not mix reagents from other lots
- Avoid contact with TMB and Stop solutions. If contact occurs, what thoroughly with water
- Eagle Biosciences is not responsible for outcomes as results of tampering with the reagents or using them unconventionally

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