DIRECT SALIVA CORTISOL

ELISA

For research use only (RUO)
Not for use in diagnostic procedures

CRTN-96-U 96 tests

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ENGLISH

INTENDED USE

The Direct Saliva Cortisol ELISA (CRTN-96-U) is intended for the quantitative determination of Cortisol in human saliva.

For research use only. Not intended for use in diagnostic procedures.

PRINCIPLE OF THE ASSAY

The Direct Saliva Cortisol ELISA is a competitive immunoassay. Competition occurs between cortisol present in calibrators, controls, and samples and an enzyme-labelled antigen (HRP conjugate) for a limited number of anti-cortisol antibody binding sites on the microplate during a first 60-minutes incubation step. Unbound enzyme label is then removed by a washing step, and TMB (tetramethylbenzidine) substrate is added to the wells. In a further 30-minutes incubation step a chromophore is formed (turns from colorless to blue) in inverse proportion to the amount of Cortisol originally present in the samples, controls and calibrators. The color turns from blue to yellow after the addition of an acidic stop solution and can be measured at 450 nm.

REAGENTS SUPPLIED AND PREPARATION

Reagents	Quantity	Code	Reconstitution
Microtiter Plate precoated with anti-Cortisol antibody	12x8 wells	CRTN-MP	Stored under 250 µL of protection buffer; wash 2x before use
Plate Sealer	3 pieces	-	
Wash Buffer Concentrate (10x) with preservatives	1 bottle 100 mL	B-WB	Dilute with 900 mL of deionized water
Zero Calibrator with preservatives	1 vial 5 mL	CRTN-CAL0	Ready to use
Blanking Reagent[1)] with preservatives	1 vial 0.5 mL	CRTN-BR	Ready to use
Calibrators with preservatives	5 vials 0.5 mL	CRTN-CASET	Ready to use
Control low / high[2)] with preservatives	2 vials 0.5 mL	CRTN-CONSET	Ready to use
Enzyme Label concentrate (100x) Cortisol conjugated to HRP	1 vial 120 µL	CRTN-EL	Dilute with Dilution Buffer just before use
Dilution Buffer with preservatives	1 vial 12 mL	CRTN-DB	Ready to use; yellow color
TMB Substrate buffered with citrate	1 vial 11 mL	B-TMBF	Ready to use
Stop Solution 0.25 M sulfuric acid (H_2SO_4)	1 vial 11 mL	B-STS	Ready to use **Irritant**

Table 1

[1)] The Blanking reagent contains a saturated cortisol solution. Prevent any contamination of other kit reagents.

[2)] Lot specific amount of cortisol (see QC data sheet added to the kit).

STORAGE AND SHELF LIFE OF REAGENTS

Sealed / Unopened Reagents	
Store at 2-8°C until expiration date. Do not use past expiration date.	
Opened / Reconstituted Reagents	
Microtiter Plate	Cover unused strips with a plate sealer, return them to the aluminium/plastic pouch and reseal along the entire edge of zip-seal. Store for up to 6 months at 2-8°C
Wash Buffer diluted	Store at 2-8°C up to 6 months.
Enzyme Label diluted	Store at 2-8°C up to 7 days
Calibrators	Store at 2-8°C until expiration date.
Controls	
Zero Calibrator	
Blanking Reagent	
Dilution Buffer	
TMB Substrate	
Stop Solution	

Table 2

MATERIALS REQUIRED BUT NOT PROVIDED

- 10-100 µL and 25 µL precision pipettes with disposable tips.
- Repeater or multichannel pipette for 100 µL.
- Disposable polypropylene tube for the dilution of the Enzyme Label.
- 1000 mL cylinder for the dilution of the Wash Buffer Concentrate.
- Microtiter plate washer or squeeze bottle for diluted Wash Buffer.
- Blotting paper.
- Microtiter plate orbital shaker.
- Microtiter plate reader for measurement of absorbance at 450 nm.
- **Optional:** Saliva Collection Devices (with cotton roll) can be ordered with NovoLytiX (order code: **B-SVC50-U).**

PRECAUTIONS

Safety precautions

- This test is for research use only, not intended for use in diagnostic procedures and must be handled by qualified personnel, in accordance with good laboratory practices (GLP).
- The Stop Solution (B-STS) contains 0.25 M sulfuric acid. It is irritant to eyes, skin and mucous membranes. Avoid contact with eyes, skin and clothes. Wear suitable protective clothing, gloves and eye protection. After contact with eyes or skin, wash immediately with plenty of water.
- Unused above solutions should be disposed of according to local state and federal regulations.

Technical precautions

Kit components

- Read this instruction for use (IFU) carefully before carrying out the test. Test performance will be adversely affected, if reagents are incorrectly diluted,

modified or stored under conditions other than those as detailed in this IFU.

- Components must not be used after the expiry date printed on the labels.
- Do not mix different lots of reagents.
- Every effort should be made to ensure that no cross contamination occurs between reagents, samples or between wells.
- Microtiter plate wells cannot be re-used.
- Mix the reagents well before use.

Assay procedure

- It is highly recommended that Calibrators and Controls are assayed in duplicates, preferably the samples as well.
- Change disposable tips after each pipetting step.
- The measuring range of the standard curve covers 0.5 to 50 ng/mL. If the expected Cortisol concentrations of the samples exceed 50 ng/mL, the samples should be diluted with Zero Calibrator accordingly.

Saliva sampling

- NovoLytiX **strongly recommends** to use **Salivettes with white caps** (e.g. Sarstedt, order code: 51.1534) containing cotton swabs. These Salivettes can also be ordered with NovoLytiX (order code: **B-SVC50-U**).
- The use of **Salivettes with blue caps** (e.g. Sarstedt order code: 51.1534.500) containing polystyrene swabs is **not recommended** although they are specifically designed by Sarstedt for Cortisol determination. They seem to contain constituents which interfere with the ELISA procedure. This is also reported by other Saliva Cortisol ELISA manufacturers. The lower the sample volume collected the more the interference can be observed (up to 1.5 - 2 ng/mL of unspecific response). Only if the collected saliva volume is higher than 2 mL the interference is neglectable in NovoLytiX's Direct Saliva Cortisol ELISA.
- The use of cotton swabs containing citric acid (e.g. **Salivettes with green caps** from Sarstedt) leads to wrong and irreproducible results. **Do not use them at all**.

SPECIMEN COLLECTION (SALIVA)

Saliva can be collected by passive drooling, spitting or using saliva collection devices (e.g. Salivettes). The Salivettes can absorb up to 3 mL of saliva. This ELISA procedure calls for <0.1 mL of saliva.

- Do not stimulate saliva flow by chewing gums or eating lemons.
- Individuals should perform the saliva collection on a day without sporting activities and any intense efforts, unless the study protocol forces to do it.
- The last meal and drinks except water must be taken at least 30 minutes before starting a saliva collection. Rinse the mouth with water 15 minutes before each collection time point.
- Individuals should avoid brushing their teeth, with or without toothpaste, during sampling periods. It is likely that individuals with gingivitis will contaminate the saliva with blood leading to unknown consequences.
- On the collection day, if possible, no medicines should be taken.

SPECIMEN STORAGE AND SHIPMENT

Storage: The saliva samples absorbed in the cotton swabs may be stored in the saliva collection device for up to 7 days at 2-8°C. If not assayed within one week after collection, samples should be frozen and may be stored for at least 12 months at ≤ -20°C. Do not add biocides to the saliva samples as this may lead to false results.

Shipment: Home- or outpatient-collected saliva samples can be shipped at ambient temperatures with a duration for up to three days. Before shipment such collected saliva samples can be kept in the refrigerator at 2-8°C for several days. There is no deterioration of Cortisol, however bacterial and fungal growth may happen.

ASSAY PROCEDURE

1. Dilute Enzyme Label concentrate with Dilution Buffer: 10 µL of concentrate per 1 mL of Dilution Buffer. Mix well. The diluted Enzyme label can be stored at 2-8°C for up to 2 weeks.
2. Use a plate with enough 8-well strips to test the desired number of Calibrators, Controls and samples. Remove excess strips from the holder, cover them again with the attached Plate Sealer and re-seal them in the aluminium/plastic foil pouch. Store refrigerated.

   Empty the wells and wash the strips twice using at least 300 µL of Wash Buffer per well. Empty the wells and strike the plate firmly onto blotting paper.

3a. Pipet 25 µL of Blanking Reagent in duplicate into wells A1+A2.

3b. Pipet 25 µL of Zero Calibrator in duplicate into wells B1+B2.

Pipet 25 µL of Calibrator A in duplicate into wells C1+C2.

Pipet 25 µL of Calibrator B in duplicate into wells D1+D2.

Pipet 25 µL of Calibrator C in duplicate into wells E1+E2.

Pipet 25 µL of Calibrator D in duplicate into wells F1+F2.

Pipet 25 µL of Calibrator E in duplicate into wells G1+G2.

3c. Pipet 25 µL of Low Control in duplicate into wells H1+H2.

Pipet 25 µL of High Control in duplicate into wells A3+B3.

3d. Pipet 25 µL of each sample into the subsequent wells.

4. Add 100 µL of diluted Enzyme Label (yellow solution) to all wells.
5. Cover the plate with the Plate Sealer, place it on a plate orbital shaker set at 600 rpm and incubate for 60 ± 5 minutes at 18-28°C.

Important: *Allow the TMB Substrate to equilibrate to 18-28°C prior to use in step 7.*

6. Remove and discard the Plate Sealer. Aspirate or invert the plate to empty the solution from each well and wash five times using at least 300 µL of Wash Buffer per well. Empty the wells and strike the plate firmly onto blotting paper.
7. Add 100 µL of TMB Substrate to all wells.
8. Cover the plate to protect it from direct light, place it on a plate orbital shaker set at 600 rpm and incubate for 30 ± 2 minutes at 18-28°C.
9. Add 100 µL of Stop Solution to all wells. Place the plate for 10 seconds on a plate orbital shaker set at 600 rpm and proceed to step 10 within 15 minutes.
10. Read the absorbance at 450 nm in a microtiter plate reader.

QUALITY CONTROL

A thorough understanding of this IFU is necessary for the successful use of the product. Reliable results will be obtained only by precise laboratory techniques (current GLP guidelines) and accurately following this IFU.
Since there are no controls for Cortisol commercially available, we recommend using internal saliva or plasma/serum pools containing different levels of Cortisol as internal quality controls. The reproducibility of standard curve parameters and control values should be within established limits of laboratory acceptability. The confidence limits for the Controls are lot-specific and are printed on the QC data sheet delivered with each test kit.
If the performance of the assay does not meet the established limits and repetition has excluded errors in technique, check the following issues: i) pipetting, temperature controlling and timing devices; ii) ELISA reader settings; iii) expiration dates of reagents; iv) storage and incubation conditions; v) TMB Substrate solution should be colorless before use; and vi) purity of water.

STANDARDIZATION

The Calibrators of the Direct Saliva Cortisol ELISA are standardized with Supelco's certified reference Cortisol material (CAS 50-32-7), Cerilliant® (Merck, order code: C-106).

RESULTS

Standard Curve

Record the absorbance at 450 nm for each calibrator and blank well. Average the duplicate values, subtract the average of the blank wells and record averages (=corrected average absorbance). Calculate the binding (B) of each pair of calibrator wells as a percent of Zero Calibrator (B_0 or Total, T), with the blank-corrected absorbance of the Zero Calibrator taken as 100 %.

$$B/B_0\ (\%) = \quad B/T\ (\%) = \frac{\text{net absorbance}}{\text{net absorbance of Zero Calibrator}} \times 100$$

Plot the percent bound (B/B_0 or B/T, vertical axis) versus the concentration of Cortisol in ng/mL (horizontal axis) using a lin/log graph paper. Draw the best fitting curve or calculate the standard curve using a four-parameter logistic (4-PL) or similar algorithm.

Samples and controls

- Record the absorbance at 450 nm for each control and each sample wells. Subtract the average of the blank wells and record the absorbance (=corrected average absorbance). Calculate, as described above, the binding of each pair of sample wells as a percent of Zero
- Calibrator (B_0 or T), with the blank-corrected absorbance of the Zero calibrator taken as 100%.
- Locate the B/B_0 (B/T) value of the samples on the vertical axis, draw a horizontal line intersecting the standard curve and read the Cortisol concentration (ng/mL) from the horizontal axis.
- If samples have been diluted before assaying them, correct the measured Cortisol concentration of the samples by multiplying with the respective sample dilution applied.

See Table 3 and Figure 1 for examples of results and standard curve. ***Results and standard curves are for demonstration purposes only. A standard curve must be generated for each set of samples to be assayed.***

PERFORMANCE CHARACTERISTICS

Limit of Blank (LoB): The LoB was established with 40 replicates of the Zero Calibrator according to the CLSI guideline EP17-A2 with the definition $LoB = \mu_B + 1.645\sigma_B$ and resulted in a value of **0.05 ng/mL** of Cortisol.

Limit of Detection (LoD): 0.17 ng/mL. The LoD was established according to the CLSI guideline EP17-A2 and with proportions of false positives (α) less than 5% and false negatives (β) less than 5% based on 80 determinations, with 40 blank (Zero Calibrator) and 40 low level replicates (<0.5 ng/mL); and a LoB of 0.05 ng/mL.

Limit of Quantitation (LoQ): 0.35 ng/mL
The LoQ was established using data obtained in the precision studies outlined below. The LoQ was determined as the melatonin concentration at which the non-linear fit of total precision data intersected the precision goal of 20% CV.

Linearity: <0.42 to >53.0 ng/mL. The linear range of the NovoLytiX Direct Saliva Cortisol ELISA was determined according to the CLSI guideline EP06-A. Three saliva samples with high amounts of Cortisol were sequentially diluted with a saliva pool containing less than 0.1 ng/mL of Cortisol and assayed according to the assay procedure. A maximum deviation from linearity of ±20% was allowed (see Table 4).

Specificity: The 50% binding (cross-reactivity) of the anti-Cortisol monoclonal antibody with different compounds (serially diluted with Zero Calibrator) were tested according to the assay procedure and are presented in Table 5.

Repeatability: 2.9 – 7.4 % CV
Within-Laboratory (Inter-Assay): 5.4 - 11.9 % CV
Precision was established using a 20 days x 2 runs x 2 replicates study design. 13 saliva samples with Cortisol concentrations ranging from 0.62 – 45.1 ng/mL were tested (see Table 6).

Spiking Recovery: 100.0 %. Three saliva pools with very low amounts of Cortisol have been spiked with increasing amounts of Cortisol solutions derived from the reference Cortisol material as specified in the *Standardization* section above. 5 to 7 different spiking concentrations were tested per specimen. The saliva pools were spiked with corresponding dilutions in a ratio 1 in 20 and analyzed according to the assay procedure. The results are presented in Table 7.

Method Comparison: The comparison was done with 81 saliva samples from 41 different donors collected over the entire 24-hours span. The samples were analyzed using this Direct Saliva Cortisol ELISA (CRTN-96-U) and compared to the results obtained by an LC-MS/MS method as described in Buning et al. (Metabolism 71, 7-16, 2017). The subsequent comparison by Passing Bablok plot resulted in a correlation factor of $R^2 = 0.945$, an intercept of +0.08 ng/mL and a slope of 1.03. The comparison graph is presented in Figure 2.

Expected Normal Values: 928 data points from apparently healthy adults (26 subjects) were collected at different day times in different research studies (see Table 8). **Important notice:** The majority of the samples were collected using blue-capped Salivettes (specifically designed by Sarstedt for Cortisol analysis). In a series of independent experiments, NovoLytiX GmbH could show that saliva samples collected with these blue-capped Salivettes can show a volume-dependent unspecific response of up to 1.5 ng/mL in the ELISA procedure (this seems to be the case for most commercial ELISAs), while white-capped Salivettes may show an unspecific response of maximum 0.4 ng/mL (see also chapter PRECAUTIONS on page 3/8). Please refer to Table 8 to see the differences of evening results among samples collected with the two different Salivettes.

Moreover, such results alone should not be the only reason for any therapeutic consequences. The results should be correlated to other clinical observations and diagnostic tests.

Cortisol levels show diurnal cycles, so that it is recommended to always collect a series of samples in the morning and another sample(s) in the evening. The difference between morning and evening seems to be an important parameter.

It is recommended that each laboratory generates its own reference ranges.

APPENDIX I

TABLES AND FIGURES

Examples of Results and Standard Curve (OD_{450})

	Conc. (ng/mL)	Absorbance (OD)	B/B0 (B/T) (%)	CV Conc. (%)	Calc. Conc. (ng/mL)
Blank Blank **Avg.**		0.052 0.056 **0.054**			
Zero Calibrator Zero Calibrator **Avg.**	**0**	1.826 1.844 **1.860**	99.4 100.6 **100.0**	**0.7**	
Cal A Cal A **Avg.**	**0.5**	1.474 1.500 **1.487**	80.3 81.7 **81.0**	**1.2**	
Cal B Cal B **Avg.**	**1.5**	1.111 1.141 **1.126**	60.9 62.4 **61.7**	**1.9**	
Cal C Cal C **Avg.**	**5**	0.605 0.600 **0.603**	33.5 32.7 **32.8**	**0.5**	
Cal D Cal D **Avg.**	**15**	0.246 0.255 **0.251**	13.4 13.9 **13.7**	**2.8**	
Cal E Cal E **Avg.**	**50**	0.091 0.088 **0.089**	5.0 4.8 **4.9**	**2.8**	
Ctrl. High Ctrl. High **Avg.**		0.357 0.361 **0.359**	19.5 19.7 **19.6**	**0.9**	10.3 10.1 **10.2**
Ctrl. Low Ctrl. Low **Avg.**		0.961 0.972 **0.966**	52.4 53.0 **52.7**	**1.8**	2.2 2.1 **2.1**

ED80 = 0.56 ng/mL ED50 = 2.37 ng/mL ED20 = 9.92 ng/mL

Table 3

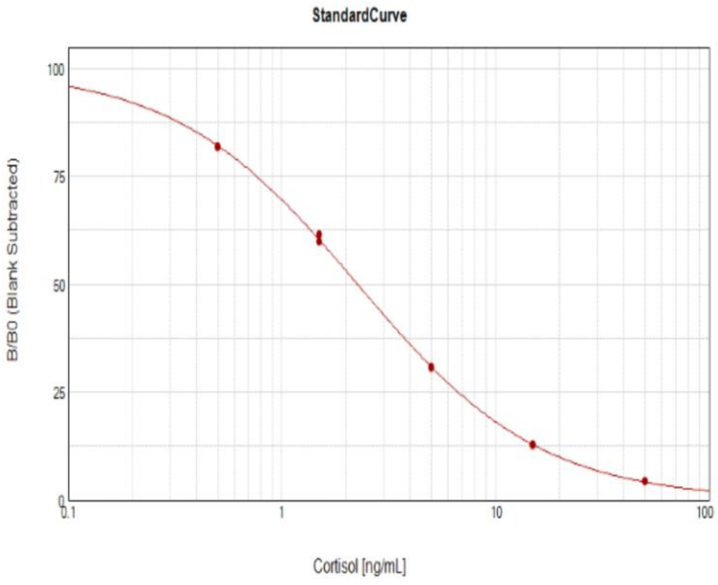


Figure 1

Dilution Linearity/ Parallelism

ID	Measuring range tested [ng/mL]	Pearson's r	p-value for non-linear coefficient	Linear range [ng/mL]
HP1	0.45 - 42.8	0.997	p >0.05	<0.45 - >42.8
HP2	0.42 - 43.5	0.996	p >0.05	<0.42 - >43.5
HP3	0.68 - 53.0	0.999	p >0.05	<0.68 - >53.0

Table 4

Specificity

Compound	Crossreactivity [%]
Cortisol	100
Cortisone	< 0.01
Corticosterone	45.0
11-Deoxycortisol	< 2.0
11-Deoxycorticosterone	3.7
Progesterone	9.0
17α-Hydroxyprogesterone	18.9
Pregrenolone	3.4
17α-Hydroxypregnenolone	10.1
Androstenedione	1.3
Aldosterone	< 0.01
Testosterone	< 0.01
Estrone	< 0.01
Estriol	< 0.01
Cholesterol	< 0.01
Prednisolone	126.0
Prednisone	0.01
Dexamethasone	< 2.0

Table 5

Precision

ID	Mean [ng/mL]	n	Repeata-bility		Between-run		Between-day		Within-laboratory	
			SD	CV	SD	%CV	SD	%CV	SD	CV
A	0.62	80	0.05	7.4%	0.04	6.6%	0.04	6.4%	0.07	11.9%
B	0.77	80	0.06	7.2%	0.06	8.4%	0.03	4.5%	0.09	12.0%
C	1.32	80	0.07	5.2%	0.10	7.2%	0.00	0.0%	0.12	8.9%
D	1.49	80	0.06	4.2%	0.06	4.1%	0.05	3.0%	0.10	6.7%
E	2.92	80	0.20	6.7%	0.14	4.7%	0.00	0.0%	0.24	8.2%
F	4.38	80	0.13	2.9%	0.12	2.7%	0.16	3.6%	0.23	5.4%
G	10.5	80	0.57	5.4%	0.30	2.9%	0.49	4.7%	0.81	7.7%
H	16.2	80	0.79	4.9%	0.25	1.6%	0.57	3.5%	1.01	6.2%
I	21.2	80	1.39	6.6%	0.00	0.0%	0.65	3.1%	1.54	7.2%
J	30.2	80	1.48	4.9%	0.70	2.3%	0.00	0.0%	1.64	5.4%
K	37.7	80	1.90	5.1%	0.00	0.0%	0.82	2.2%	2.07	5.5%
L	45.1	76	2.55	5.7%	0.00	0.0%	0.39	0.9%	2.58	5.7%

Table 6

Method Comparison

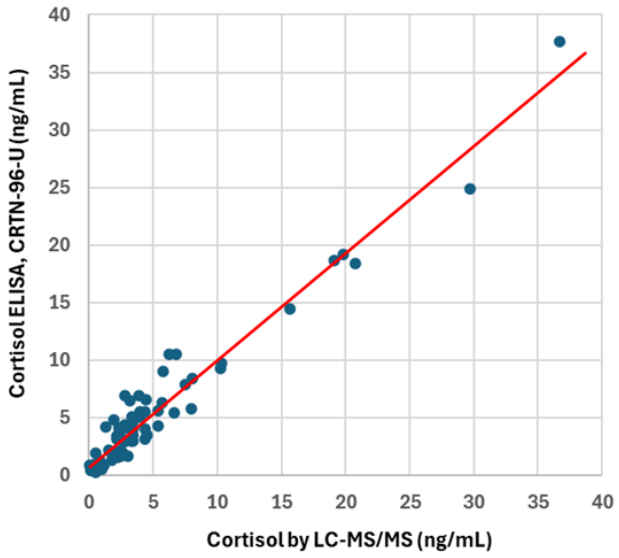


Figure 2

Spiking Recovery

Sample	Spiking (ng/mL)	Expected (ng/mL)	Observed (ng/mL)	Recovery O/E (%)
Pool1	-	-	1.16	-
	2	3.16	2.64	83.5
	4	5.16	4.78	92.6
	8	9.16	9.70	105.9
	16	17.16	17.56	102.3
	32	33.16	34.46	103.9
	64	65.16	69.63	106.9
Pool2	-	-	0.00	-
	2	2.00	1.85	92.5
	4	4.00	4.57	114.3
	8	8.00	9.1	113.1
	16	16.00	17.41	108.8
	32	32.00	33.37	104.3
Pool3	-	-	0.01	-
	1	1.01	0.94	93.1
	2	2.01	1.87	93.0
	4	4.01	3.70	92.3
	8	8.01	7.43	92.8
	16	16.01	15.77	98.5
	32	32.01	32.04	100.1
	64	64.01	64.98	101.5
Mean				**100.0**

Table 7

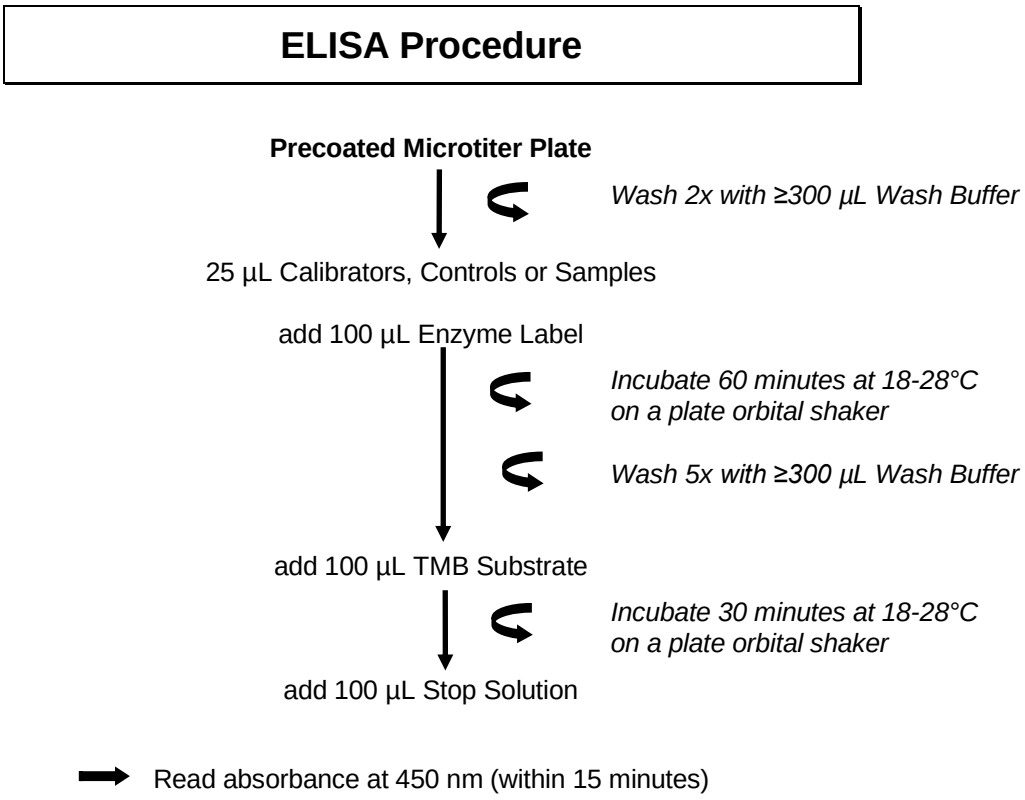
Expected Normal Values, Reference Ranges

Day time	n	Mean (ng/mL)	Median (ng/mL)	5 - 95% per-centile (ng/mL)
05:00 - 10:00	414	4.68	4.31	1.20 - 9.82
10:00 - 15:00	131	3.24	3.00	1.50 - 5.82
15:00 - 20:00	148	2.08	1.91	0.51 - 4.43
20:00 - 01:00	233	1.66	1.50	0.30 - 3.45
17:00 - 01:00 collected with white-capped Salivettes	42	0.98	0.90	0.09 - 2.37

Table 8

APPENDIX II

PIPETTING PROTOCOL



APPENDIX III

SYMBOLS

Symbol	Explanation
	Use By
REF	Order Code
LOT	Batch Code
	Contains sufficient for <n> tests
	Consult Instructions for Use
	Temperature Limitation
MP	Microtiter Plate coated with anti-Cortisol antibody
BUF WASH 10X	Wash Buffer Concentrate (10x)
BR	Blanking Reagent

Symbol	Explanation
CAL0	Zero Calibrator
CAL A - CAL E	Calibrator A - E
CONTROL L	Control Low
CONTROL H	Control High
EL	Enzyme Label concentrate (100x)
DB	Enzyme Label Dilution Buffer
SUBS TMB	TMB Substrate
SOLN STOP	Stop Solution

Change Log

Date	Version	Reason for change
2024-10-08	0.1	Preliminary version for evaluation testing
2025-03-17	01	First version