



# Cortisol ELISA Kit

Catalog Number KA1901

96 assays

Version: 05

Intended for research use only

[www.abnova.com](http://www.abnova.com)

## Table of Contents

<b>Introduction .....</b>	<b>3</b>
Intended Use .....	3
Principle of the Assay .....	3
<b>General Information .....</b>	<b>4</b>
Materials Supplied .....	4
Storage Instruction .....	4
Materials Required but Not Supplied .....	5
Precautions for Use .....	5
<b>Assay Protocol .....</b>	<b>7</b>
Reagent Preparation .....	7
Sample Preparation.....	7
Assay Procedure .....	8
<b>Data Analysis.....</b>	<b>9</b>
Calculation of Results.....	9
Performance Characteristics .....	9
<b>Resources.....</b>	<b>11</b>
References .....	11
Troubleshooting.....	11
Plate Layout .....	12

## **Introduction**

### **Intended Use**

Competitive immunoenzymatic colorimetric method for quantitative determination of free cortisol concentration in urine. This kit is intended for research use only.

### **Principle of the Assay**

The Cortisol (antigen) in the sample competes with the antigenic Cortisol conjugated with horseradish peroxidase (HRP) for binding to the limited number of antibodies anti Cortisol coated on the microplate (solid phase).

After incubation, the bound/free separation is performed by a simple solid-phase washing.


Then, the enzyme HRP in the bound-fraction reacts with the Substrate ( $H_2O_2$ ) and the TMB Substrate and develops a blue color that changes into yellow when the Stop Solution ( $H_2SO_4$ ) is added.

The colour intensity is inversely proportional to the Cortisol concentration of in the sample. Cortisol concentration in the sample is calculated through a standard curve.

## General Information

### Materials Supplied

List of component

Component	Detail	Amount
Conjugate	Cortisol conjugated to horseradish peroxidase (HRP)	33 mL
Microtiterwells	Anti-Cortisol-antibody adsorbed on the microplate.	1 breakable microplate
Wash Solution (10x)	10x concentrated, Phosphate buffer 0.2 M, Proclin < 0.0015%	50 mL
Substrate Solution	H <sub>2</sub> O <sub>2</sub> -TMB 0.26 g/L (avoid any skin contact).	15 mL
Stop Solution	Sulphuric acid, 0.15 mol/L (avoid any skin contact).  Hazards identification: H290 May be corrosive to metals. H314 Causes severe skin burns and eye damage.	15 mL

Standards and Controls

Component	Concentration (ng/mL)	Amount
Standard A	0	4 mL
Standard B	1	1 mL
Standard C	5	1 mL
Standard D	30	1 mL
Standard E	200	1 mL
Control 1*	Refer to vial labels or QC-Report for expected value and acceptable range.	1 mL
Control 2*		1 mL

\* Control: ready to use

### Storage Instruction

- Store all reagents at 2°C -8°C in the dark.
- Open the bag of Coated Microplate only when it is at room temperature and close immediately after use. Once opened, the microplate is stable until expiry date of the kit.
- The kit is stable until the expiry date claimed on the kit label and the QC-Report. Do not use the kit or its components after the expiry date.

### **Materials Required but Not Supplied**

- Distilled water.
- Automatic dispenser.
- Microplate reader (450 nm, 620-630 nm)

### **Precautions for Use**

#### ✓ Warnings

- This kit is intended for research use only.
- Use appropriate personal protective equipment while working with the reagents provided.
- Follow Good Laboratory Practice (GLP) for handling blood products.
- Material of animal origin used in the preparation of the kit has been obtained from animal certified as healthy and the bovine protein has been obtained from countries not infected by BSE, but these materials should be handled as potentially infectious.
- Some reagents contain small amounts of Proclin 300 as preservative. Avoid the contact with skin or mucosa.
- The TMB Substrate contains an irritant, which may be harmful if inhaled, ingested or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes.
- The Stop Solution consists of a diluted sulphuric acid solution. Sulphuric acid is poisonous and corrosive and can be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes.
- Avoid the exposure of reagent TMB/H<sub>2</sub>O<sub>2</sub> to directed sunlight, metals or oxidants. Do not freeze the solution.
- This method allows the determination of Cortisol from 0.47 ng/mL to 200 ng/mL.

#### ✓ Precautions

- Please adhere strictly to the sequence of pipetting steps provided in this protocol.
- The performance data represented here were obtained using specific reagents listed in this instruction for use.
- All reagents should be stored refrigerated at 2 °C - 8 °C in their original container. Any exceptions are clearly indicated. The reagents are stable until the expiry date when stored and handled as indicated.
- Allow all kit components and specimens to reach room temperature (22 °C - 28 °C) and mix well prior to use.
- Do not interchange kit components from different lots. The expiry date printed on box and vials labels must be observed. Do not use any kit component beyond their expiry date.
- If you use automated equipment, the user has the responsibility to make sure that the kit has been appropriately tested.
- The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or

increase the background.

- To improve the performance of the kit on automatic systems it is recommended to increase the number of washes.
  - It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate
  - Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction.
  - Observe the guidelines for performing quality control in medical laboratories by assaying controls samples.
  - Maximum precision is required for reconstitution and dispensation of reagents.
  - Plate readers measure vertically. Do not touch the bottom of the wells.
- ✓ Waste management  
Reagents must be disposed off in accordance with local regulations

## Assay Protocol

### Reagent Preparation

- ✓ Preparation of the Standard and Controls

Before use, leave 5 minutes on a rotating mixer.

The standards are ready to use and have the following concentration of Cortisol:

	Standard A	Standard B	Standard C	Standard D	Standard E
ng/mL	0	1	5	30	200

The Controls are ready to use.

Once opened the standards and controls are stable 6 months at 2°C-8°C.

- ✓ Preparation of Conjugate

The Conjugate is ready to use.

Once opened, is stable 6 months at 2°C-8°C.

- ✓ Preparation of Wash Solution

Dilute the content of each vial of the “Wash Solution” 10x concentrate with distilled water to a final volume of 500 mL prior to use.

For smaller volumes respect the 1:10 dilution ratio.

The diluted wash solution is stable for 30 days at 2°C-8°C.

In concentrated wash solution it is possible to observe the presence of crystals; in this case mix at room temperature until the complete dissolution of crystals; for greater accuracy dilute the whole bottle of concentrated wash solution to 500 mL, taking care to transfer completely the crystals, then mix until crystals are completely dissolved.

### Sample Preparation

The determination of Cortisol with this kit should be performed in urine samples.

*Important note: The kit has been designed to be used on untreated urine samples; acidification treatments of the urine that lead the pH to values below 5.0 could interfere with the assay and produce aberrant results.*

It is not necessary to dilute the sample.

The total volume of urine excreted during 24 hours should be collected and mixed in a single container.

Urine samples which are not to be assayed immediately should be stored at 2°C-8°C or at -20°C for longer periods (maximum 6 months).

Samples with concentration greater than 200 ng/L have not to be diluted; such samples has to be reported as “> 200 ng/mL”.

## Assay Procedure

Allow all reagents to reach room temperature (22°C-28°C) for at least 30 minutes.

Unused coated microwell strips should be released securely in the foil pouch containing desiccant and stored at 2°C-8°C.

To avoid potential microbial and/or chemical contamination, unused reagents should never be transferred into the original vials.

As it is necessary to perform the determination in duplicate in order to improve accuracy of the test results, prepare two wells for each point of the Standard curve (A-E), two for each Control, two for each sample, one for Blank.

Reagent	Standards	Samples/Controls	Blank
Standard A-E	10 µL		
Samples/Controls		10 µL	
Conjugate	300 µL	300 µL	
Incubate at 37°C for 1 hour. Remove the contents from each well; wash the wells 3 times with 350 µL of diluted wash solution. <i>Important note: during each washing step, gently shake the plate for 5 seconds and remove excess solution by tapping the inverted plate on an absorbent paper towel.</i> Automatic washer: in case you use an automatic washer, it is advised to do 6 washing steps.			
Substrate Solution	100 µL	100 µL	100 µL
Incubate at room temperature (22-28°C) for 15 minutes in the dark			
Stop solution	100 µL	100 µL	100 µL
Shake the microplate gently. Read the absorbance (E) at 450 nm against reference wavelength of 620-630 nm or against Blank within 5 minutes.			

### ✓ Quality Control

Each laboratory should assay controls at normal, high and low levels range of Urinary Cortisol for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance limits. Other parameters that should be monitored include the 80, 50 and 20% intercepts of the standard curve for run-to-run reproducibility. In addition, maximum absorbance should be consistent with past experience. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.



## Data Analysis

### Calculation of Results

Interpolate the values of the samples on the standard curve to obtain the corresponding values of the concentrations expressed in ng/mL.

To calculate the cortisol concentration in urine, calculate as above and correct for total volume of volume of urine collected in 24 hours:

$$\text{ng/mL} \times \text{Vol (mL) urine 24 h} / 1000 = \mu\text{g Cortisol}/24$$

✓ Mean Absorbance

Calculate the mean of the absorbance (Em) for each point of the standard curve and of each sample.

✓ Standard Curve

Plot the values of absorbance (Em) of the standards (A-E) against concentration. Draw the best-fit curve through the plotted points (es: Four Parameter Logistic).

✓ Reference Values

To determine the normal range for urine sample, 128 apparently healthy male and female adults were tested.

Result:

Normal range urine (24h)
1.5 µg/24h – 63 µg/24h

### Performance Characteristics

✓ Analytical Sensitivity

Analytical Sensitivity was investigated through the LOB (white limit) the LOD (detection limit), the LOQ (quantification limit) and the anal sensitivity (A.S.).

The following table shows the criteria of the study and the results obtained.

	Criteria	Results (ng/mL)
LOB	60 replicates of Standard A, used as "Blank" have been investigated in 5 different sessions over 3 days.	0.28
LOD	6 urine samples with low cortisol concentration have been investigate over 10 assays in duplicate, performed in 5 days.	0.47
LOQ	6 urine samples with low cortisol concentration have been investigate over 10 assays in duplicate, performed in 5 days.	0.56
A.S	20 replicates of Standard A and replicates Standard B have been assayed. A.S has been calculated by linear regression.	0.22

✓ Precision and reproducibility (complex precision)

Precision and reproducibility have been assessed through 6 different urine samples with different concentration of Cortisol.

The table below shows the Within Run and Total CV%.

Sample	n	Mean (ng/mL)	Within Run CV%	Total CV%
PS 2	20	112.141	6.6%	12%
PS4	20	64.563	8.1%	12%
CT High	20	50.577	7.3%	11%
PS 5	20	25.878	7.6%	10%
PS 6	20	9.269	7.6%	11%
CT Low	20	3.438	7.0%	9%

✓ Analytical specificity

Interference for Albumin, Acetylsalicylic Acid, Ibuprofen and Ascorbic Acid were studied by adding the interfering substance to the urine sample with a low and high Cortisol concentration, and by comparing its concentration to the unspiked sample.

The interference has been evaluated as "significant" if it causes a concentration bias > 10% between spiked and unspiked sample.

The following table shows the results obtained:

Substance	Concentration	Interference
Albumin	5 mg/dL	No
Acetylsalicylic acid	3.62 mmol/L	No
Ibuprofen	2.42 mmol/L	No
Ascorbic Acid	5 mg/L	No

Conclusion: no interference has been found for Albumin, Acetylsalicylic Acid, Ibuprofen and Ascorbic Acid.

✓ Correlation

137 urine samples were tested with the Cortisol ELISA Kit and with a LC-MS method (reference).

The linear regression curve is:

$$y = 1,008x - 0.5019$$

$$r^2 = 0.83$$

## Resources

### References

1. Foster, L. B. and Dunn, R. T. Clin. Chem. 20/3 365 (1974)
2. De lacerda, et al J. Clin. Endocr. and Metab. 6, 227 (1973)
3. Rolleri, E., et al Clin. Chim. Acta 66 319 (1976)
4. Kobayashi, Y., et al Steroids, 32 no 1 (1978)
5. Akarawa, et al Anal. Biochem. 97 248 (1979)

### Troubleshooting

#### Errors/Possible Causes/Suggestions

- ✓ No colorimetric reaction
  - no conjugate pipetted
  - contamination of conjugates and/or of substrate
  - errors in performing the assay procedure (e.g. accidental pipetting of reagents in a wrong sequence or from the wrong vial, etc.)
  
- ✓ Too low reaction (too low ODs)
  - incorrect conjugate (e.g. not from original kit)
  - incubation time too short, incubation temperature too low
  
- ✓ Too high reaction (too high ODs)
  - incorrect conjugate (e.g. not from original kit)
  - incubation time too long, incubation temperature too high
  - water quality for wash buffer insufficient (low grade of deionization)
  - insufficient washing (conjugates not properly removed)
  
- ✓ Unexplainable outliers
  - contamination of pipettes, tips or containers
  - insufficient washing (conjugates not properly removed)
- 1. too high within-run CV%
  - reagents and/or strips not pre-warmed to room temperature prior to use
  - plate water is not washing correctly (suggestion: clean washer head)
- 2. too high between-run CV%
  - incubation conditions not constant (time, temperature)
  - controls and samples not dispensed at the same time (with the same intervals) (check pipetting order)
  - person-related variation

**Plate Layout**

12								
11								
10								
9								
8								
7								
6								
5								
4								
3								
2								
1								
	A	B	C	D	E	F	G	H