

# Human Cortisol ELISA

Cat No: K12-1004

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**Principle:**

The Human Cortisol ELISA is competitive enzyme-linked immunosorbent assay (ELISA) to assay the level of Cortisol in samples. Standards or Samples competes with the Cortisol antigen coated microtiter well to form a complex with the biotinylated Anti-Cortisol. Wells are washed to remove the excess conjugate and Streptavidin:HRP Conjugate is added to the microplate and incubated. After incubation and a washing step TMB Substrate, are added. Blue color develops on incubation and the reaction is stopped with a Stop Solution to form a yellow color. The concentration of the Cortisol in the samples is inversely proportional to the yellow color developed (absorbance) in the wells

**Intended Use:**

This Kit is used to assay the level of Human Cortisol in Human serum, plasma and other biological samples. The Kit is For Laboratory / Research Use Only.

**Materials provided in the Kit:**

1. Human Cortisol Coated Microtiter Plate (96 wells) - 1 no
2. Human Cortisol Standard (lyophilized, concentrated, 25 ng/ml) - 2 vials
3. Biotinylated Anti- Cortisol (concentrated) - 60 ul
4. Streptavidin-HRP Conjugate - 120 ul
5. Sample Diluent - 20 ml
6. Biotin Antibody Dilution Buffer - 10 ml
7. HRP Conjugate Dilution Buffer - 10 ml
8. (25X) Wash Buffer - 30 ml
9. TMB Substrate - 10 ml
10. Stop Solution - 10 ml
11. Instruction Manual

**Materials to be provided by the End-User:**

1. Microplate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes to measure volumes ranging from 50 ul to 1000 ul.
3. Deionized (DI) water.
4. Wash bottle or automated microplate washer.
5. Clean tubes and Eppendorf tubes
6. Precision single and multi-channel pipette and disposable tips.
7. 37°C incubator
8. Timer.

**Storage Information:**

1. All reagents should be stored at 2°C to 8°C. For long term storage, store the biotin antibody and standards (preferably aliquoted) at -20°C. Avoid multiple freeze-thaws as it leads to loss of activity of the Components.
2. All the reagents and wash solutions are stable until the expiration date of the kit.
3. 20 minutes prior before use, bring all Components to room temperature (18-25°C). Store all the Components of the kit at its appropriate storage condition after use.
4. The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

**Health Hazard Warnings:**

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online for details.
2. To reduce the likelihood of blood-borne transmission of infectious agents, handle all samples in accordance with NCCLS regulations.

**Specimen Collection and Handling:**

1. **Serum-** Place whole blood sample at room temperature for 2 hours or put it at 2-8°C overnight and centrifugation for 20 minutes at approximately 1000×g, Collect the supernatant and carry out the assay immediately. Blood collection tubes should be disposable, non-pyrogenic, and non-endotoxin.

2. **Plasma-** Collect plasma using EDTA-Na<sub>2</sub> or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g at 2-8°C within 30 minutes of collection. Collect the supernatant and carry out the assay immediately. Avoid hemolysis, high cholesterol samples.
3. **Tissue Homogenates-** As hemolysis blood has relation to the assay results, it is necessary to remove residual blood by washing tissue with pre-coating PBS buffer (0.01M, pH=7.4). Mince tissue after weighing it and get it homogenized in PBS (the volume depends on the weight of the tissue Normal 9 ml PBS would be appropriate to 1 gram tissue pieces. Some protease inhibitors are recommended to add into the PBS) with a glass homogenizer or ice. To further break the cells, you can sonicate the suspension with an ultrasonic cell disruptor or subject it to freeze-thaw cycles. The homogenates are then centrifuged for 5 minutes at 5000×g to get the supernatant. The total protein concentration was determined by BCA kit and the total protein concentration of each pore sample should not exceed 0.3 mg,
4. **Cell Culture Supernatant-**Centrifuge supernatant for 20 minutes at 1000×g at 2-8°C to remove insoluble impurity and cell debris. Collect the clear supernatant and carry out the assay immediately.
5. **Cell Culture Lysate-** Commercial RIPA kits are recommended to follow the instructions provided. Generally 0.5 ml RIPA lysis buffer would be appropriate to 2×10<sup>6</sup> cells, DNA must be removed. The total protein concentration was determined by BCA kit and the total protein concentration of each pore sample should not exceed 0.3 mg.
6. **Other Biological Fluids-** Centrifuge samples for 20 minutes are 1000×g at 2-8°C. Collect supernatant and carry out the assay immediately.

**Note:** Samples to be used within 5 days can be stored at 2-8°C, besides that, samples must be stored at -20°C (assay ≤1 month) or -80°C(assay≤2 months) to avoid loss of bioactivity and contamination. Avoid multiple freeze-thaw cycles. The hemolytic samples are not suitable for this assay.

#### Sample Dilution

The user should estimate the concentration of target protein in the test sample, and select a proper dilution factor to make the diluted target protein concentration fall in the optimal detection range of the kit. Dilute the sample with the provided diluent, and several trials may be necessary. The test sample must be well mixed with the diluent. And also standard curves and sample should be making in pre-experiment. If samples with very high concentrations, dilute samples with PBS first and then dilute the samples with Sample Dilution.

#### Reagent Preparation (all reagents should be diluted immediately prior to use):

1. Label any aliquots made with the kit Lot No and Expiration date and store it at appropriate conditions mentioned.
2. Bring all reagents to Room temperature before use.
3. To make **Wash Buffer (1X); dilute 30 ml of (25X) Wash Buffer in 720 ml of DI water.**
4. **Biotinylated Cortisol Antibody Working Solution:** Prepare it within 1 hour before experiment. Calculate required total volume of the working solution: 0.1 ml / well x quantity of wells. (Allow 0.1-0.2 ml more than the total volume. Dilute the Biotinylated I Cortisol Antibody (concentrated) with Biotin Antibody Dilution Buffer at 1:100 and mix them thoroughly. (i.e. Add 1 ul Biotinylated I CORTISOL Antibody into 99 ul Biotin Antibody Dilution Buffer.)
5. **Streptavidin:HRP Conjugate Working Solution:** Prepare it within 30 minutes before experiment. Calculate required total volume of the working solution: 0.1ml / well x quantity of wells. (Allow 0.1-0.2 ml more than the total volume. Dilute the Streptavidin:HRP Conjugate with Streptavidin:HRP Conjugate Dilution Buffer at 1:100 and mix them thoroughly. (i.e. Add 1 ul of Streptavidin:HRP Conjugate into 99 ul of Streptavidin:HRP Conjugate Dilution Buffer.)
6. **Standards Preparation:** Reconstitute original Cortisol Standard with 1 ml of Sample Diluent. Keep the standard for 15 mins with gentle agitation before making further dilutions. Prepare the additional Standards by serially diluting the standard stock solution as per the below table.

Standard Concentration	Standard Vial	Dilution Particulars
25 ng/ml	Standard No.8	Reconstitute with 1ml Sample Diluent
12.5 ng/ml	Standard No.7	300 ul Standard No.8 + 300 ul Sample Diluent
6.25 ng/ml	Standard No.6	300 ul Standard No.7 + 300 ul Sample Diluent
3.125 ng/ml	Standard No.5	300 ul Standard No.6 + 300 ul Sample Diluent
1.562 ng/ml	Standard No.4	300 ul Standard No.5 + 300 ul Sample Diluent
0.781 ng/ml	Standard No.3	300 ul Standard No.4 + 300 ul Sample Diluent
0.391 ng/ml	Standard No.2	300 ul Standard No.3 + 300 ul Sample Diluent
0 ng/ml	Standard No.1	300 ul Sample Diluent only

**Procedural Notes:**

1. In order to achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess un-reacted reagents is essential.
2. High Dose Hook Effect may be observed in samples with very high concentrations of Human Cortisol. High Dose Hook Effect is due to excess of antibody for very high concentrations of Human Cortisol present in the sample. High Dose Hook effect is most likely encountered from samples early in the purification process. If Hook Effect is possible, the samples to be assayed should be diluted with a Compatible diluent. Thus if the Human Cortisol concentration of the undiluted sample is less than the diluted sample, this may be indicative of the Hook Effect.
3. Avoid assay of Samples containing Sodium Azide ( $\text{NaN}_3$ ), as it could destroy the HRP activity resulting in under-estimation of the amount of Human Cortisol.
4. It is recommended that all Standards and Samples be assayed in duplicates.
5. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
6. If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to poor sensitivity of the assay.
7. The plates should be read within 30 minutes after adding the Stop Solution.
8. Make a work list in order to identify the location of Standards and Samples.

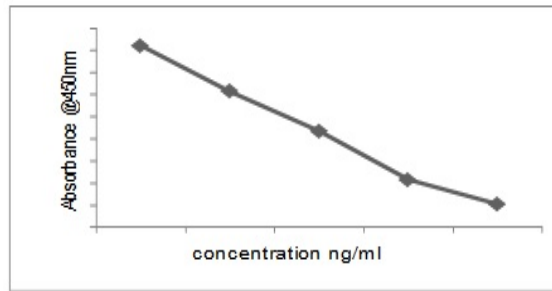
**Assay Procedure:**

1. It is strongly recommended that all Standards and Samples be run in duplicates or triplicates. A standard curve is required for each assay.
2. Add **50 ul prepared Standards and diluted Samples** to respective wells.
3. Add **50 ul Biotinylated Cortisol Antibody Working Solution** to all wells.
4. Cover the plate with a sealer and incubate for 45 minutes at 37°C.
5. Aspirate and wash plate 4 times with diluted Wash Buffer (1X) and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.
6. Pipette **100 ul Streptavidin:HRP Conjugate Working Solution** to all wells. Mix well.
7. Cover the plate with a sealer and incubate for 30 minutes at 37°C.
8. Aspirate and wash as per Step (5) above.
9. Pipette **90 ul TMB Substrate** in all the wells.
10. Incubate the plate at **37°C for 10 minutes**. DO NOT SHAKE or else it may result in higher backgrounds and worse precision. Positive wells should turn bluish in color.
11. Pipette **50 ul of Stop Solution** to all wells. The wells should turn from blue to yellow in color.
12. Read the absorbance at 450 nm with a microplate within 10-15 minutes after addition of Stop solution.

**Calculation of Results:**

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Use the Net Absorbance (Absorbance of Standard/Sample - Absorbance of Blank) to calculate the Mean Absorbances. Using standard graph paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the concentration. If samples were diluted, multiply by the appropriate dilution factor.

Software which is able to generate a cubic spline curve-fit, 4PL or a polynomial regression to the 2<sup>nd</sup> order is best recommended for automated results.

**Typical Graph**

**Stability**

The stability of ELISA kit is determined by the loss rate of activity. The loss rate of this kit is less than 10% within the expiration date under appropriate storage condition.

Standard(n=5)	37°C for 1 month	2-8°C for 6 months
Average (%)	80	95-100

**Precautions:**

Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this set.

**Performance Characteristics:**

Please note that this validation is performed in our laboratory and will not necessarily be duplicated in your laboratory. This data has been generated to enable the user to get a preview of the assay and the characteristics of the kit and is generic in nature. We recommend that the user performs at the minimum; the spike and recovery assay and the dilutional linearity assay to assure quality results. For a more Comprehensive validation, the user may run the protocols as suggested by us herein below to develop the parameters for quality control to be used with the kit.

**Sensitivity:**

Limit Of Detection: It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2\* SD. 10 replicates of '0' standards were evaluated and the LOD was found to **0.234 ng/ml**.

**Specificity:**

This assay has high sensitivity and excellent specificity for detection of Cortisol. No significant cross-reactivity or interference between Cortisol and analogues was observed.

**Recovery**

Matrices listed below were spiked with certain level of Cortisol and the recovery rates were calculated by Comparing the measured value to the expected amount of Cortisol in samples.

Matrix	Recovery Range (%)	Average (%)
Serum(n=5)	90-104	95
EDTA Plasma(n=5)	90-103	98
Heparin Plasma(n=5)	87-104	95

**Assay Range:**

**0.391 ng/ml – 25 ng/ml**

**Precision:**

Intra-Assay: CV<8%

Inter-Assay: CV<10%

**Linearity:**

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of Cortisol and their serial dilutions. The results were demonstrated by percentage of calculated concentration to the expectation.

<b>Sample</b>	<b>1:2</b>	<b>1:4</b>	<b>1:8</b>
serum (n=5)	88-104%	87-105%	86-104%
EDTA plasma (n=5)	85-98%	88-92%	83-101%
heparin plasma (n=5)	83-96%	84-100%	80-99%

**LIMITED WARRANTY**

KinesisDx does not warrant against damages or defects arising in shipping or handling, or out of accident or improper or abnormal use of the product; against defects in products or Components not manufactured by KinesisDx, or against damages resulting from such non-KinesisDx made products or Components. KinesisDx passes on to customer the warranty it received (if any) from the maker thereof of such non- KinesisDx made products or Components. This warranty also does not apply to product to which changes or modifications have been made or attempted by persons other than pursuant to written authorization by KinesisDx.

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## Human Cortisol ELISA

### ASSAY PROCEDURE

1	Bring all reagents to room temperature before use.		
2	Pipette Standards 1 - 8 Samples	50 ul	50 ul
3	Pipette Biotinylated Anti-Cortisol	50ul	50ul
4	Incubate 45 minutes (37°C)		
5	Wash 1X Wash Buffer Decant, 4 x 300 ul		
6	Pipette Streptavidin: HRP Conjugate	100 ul	100 ul
7	Incubate 30 minutes (37°C)		
8	Wash 1X Wash Buffer Decant, 4 x 300 ul		
9	TMB Substrate	90 ul	90 ul
10	Incubate in the dark 10 minutes (37°C)		
11	Pipette Stop Solution	50 ul	50 ul
12	Measure 450 within 15 mins		