



**AssayMax™**  
**Cortisol ELISA Kit**

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Thank you for choosing Assaypro.

## Assay Summary

**Step 1.** Add 25  $\mu$ l of Standard or Sample and 25  $\mu$ l of Biotinylated Steroid per well.  
Incubate 2 hours.

**Step 2.** Wash, then add 50  $\mu$ l of SP Conjugate per well.  
Incubate 30 minutes.

**Step 3.** Wash, then add 50  $\mu$ l of Chromogen Substrate per well.  
Incubate 15 minutes.

**Step 4.** Add 50  $\mu$ l of Stop Solution per well.  
Read at 450 nm immediately.

## Symbol Key



Consult instructions for use.





# AssayMax™ Cortisol ELISA Kit

Catalog No. EC3021-1

*Sample insert for reference use only*

## Introduction

Cortisol is a steroid hormone which belongs to the glucocorticoid class of hormones. When used as a medication, it is known as hydrocortisone. It is the main glucocorticoid secreted by the adrenal cortex, within the adrenal gland. Cortisol is released in response to stress and low blood-glucose concentration. Cortisol binds to the cytosolic glucocorticoid receptor and translocates into the cell nucleus, where it binds to many glucocorticoid response elements in the promoter region of the target genes. Cortisol promotes protein catabolism, gluconeogenesis, capillary wall stability, and renal excretion of calcium. It also suppresses immune and inflammatory responses. Cortisol regulates or supports a variety of important cardiovascular, metabolic, immunologic, and homeostatic functions (1-2).

## Principle of the Assay

The AssayMax™ Cortisol ELISA (Enzyme-Linked Immunosorbent Assay) Kit is designed for detection of cortisol in **plasma, serum, milk, urine, saliva, CSF, and cell culture samples**. This assay employs a quantitative **competitive enzyme immunoassay** technique that measures cortisol in approximately 3 hours. A polyclonal antibody specific for cortisol has been pre-coated onto a 96-well microplate with removable strips. Cortisol in standards and samples is competed with a biotinylated cortisol steroid sandwiched by the immobilized antibody and streptavidin-peroxidase (SP) conjugate. All unbound material is washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

## Caution and Warning

- This product is for **Research Use Only** and is not intended for use in diagnostic procedures.
- Prepare all reagents (diluent buffer, wash buffer, standard, biotinylated steroid, and SP conjugate) as instructed, prior to running the assay.
- Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this insert. However, the user should determine the optimal dilution factor.
- Spin down the standard vial and SP conjugate vial before opening and using contents.
- The Stop Solution is an acidic solution.

- The kit should not be used beyond the expiration date.

## Reagents

- **Cortisol Microplate:** A 96-well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against cortisol.
- **Sealing Tapes:** Each kit contains 3 pre-cut, pressure sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **Cortisol Standard:** Cortisol in a buffered protein base (800 ng/ml, 0.5 ml liquid).
- **Biotinylated Cortisol Steroid (2x):** Lyophilized.
- **EIA Diluent Concentrate (10x):** A 10-fold concentrated buffered protein base (20 ml).
- **Wash Buffer Concentrate (20x):** A 20-fold concentrated buffered surfactant (30 ml).
- **SP Conjugate (100x):** A 100-fold concentrate (80  $\mu$ l).
- **Chromogen Substrate (1x):** A stabilized peroxidase chromogen substrate tetramethylbenzidine (7 ml).
- **Stop Solution (1x):** A 0.5 N hydrochloric acid solution to stop the chromogen substrate reaction (11 ml).

## Storage Condition

- Upon arrival, immediately store components of the kit at recommended temperatures up to the expiration date.
- Store Standard and SP Conjugate at -20°C.
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8°C.
- Unused microplate wells may be returned to the foil pouch with the desiccant packs and resealed. May be stored for up to 30 days in a vacuum desiccator.
- Store Biotinylated Steroid at 2-8°C before reconstituting with Diluent and at -20°C after reconstituting with Diluent.

## Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm
- Pipettes (1-20  $\mu$ l, 20-200  $\mu$ l, 200-1000  $\mu$ l, and multiple channel)
- Deionized or distilled reagent grade water

## Sample Collection, Preparation, and Storage

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 3000 x g for 10 minutes and collect plasma. A 10-fold sample dilution is suggested into EIA Diluent; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles (EDTA or Heparin can also be used as an anticoagulant).
- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3000 x g for 10 minutes and remove serum. A 10-fold sample dilution is suggested into EIA Diluent; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Milk:** Collect milk using sample tube. Centrifuge samples at 800 x g for 10 minutes. An 8-fold sample dilution is suggested into EIA Diluent; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Urine:** Collect urine using sample pot. Centrifuge samples at 800 x g for 10 minutes. A 2-fold sample dilution is suggested into EIA Diluent; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Saliva:** Collect saliva using sample tube. Centrifuge samples at 800 x g for 10 minutes. An 8-fold sample dilution is suggested into EIA Diluent; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **CSF:** Collect cerebrospinal fluid (CSF) using sample pot. Centrifuge samples at 3000 x g for 10 minutes. A 5-fold sample dilution is suggested into EIA Diluent; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -80°C for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Cell Culture Supernatant:** Centrifuge cell culture media at 1500 rpm for 10 minutes at 4°C to remove debris and collect supernatant. Samples can be stored at -80°C. Avoid repeated freeze-thaw cycles.

*Applicable samples may also include biofluids, cell culture, and tissue homogenates. If necessary, user should determine optimal dilution factor depending on application needs.*

***Refer to Dilution Guidelines for further instruction.***

<b>Guidelines for Dilutions of 100-fold or Greater</b> <i>(for reference only; please follow the insert for specific dilution suggested)</i>	
<b>100x</b>	<b>10000x</b>
A) 4 µl sample : 396 µl buffer (100x) = 100-fold dilution  <i>Assuming the needed volume is less than or equal to 400 µl.</i>	A) 4 µl sample : 396 µl buffer (100x) B) 4 µl of A : 396 µl buffer (100x) = 10000-fold dilution  <i>Assuming the needed volume is less than or equal to 400 µl.</i>
<b>1000x</b>	<b>100000x</b>
A) 4 µl sample : 396 µl buffer (100x) B) 24 µl of A : 216 µl buffer (10x) = 1000-fold dilution  <i>Assuming the needed volume is less than or equal to 240 µl.</i>	A) 4 µl sample : 396 µl buffer (100x) B) 4 µl of A : 396 µl buffer (100x) C) 24 µl of B : 216 µl buffer (10x) = 100000-fold dilution  <i>Assuming the needed volume is less than or equal to 240 µl.</i>

## Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- **EIA Diluent Concentrate (10x):** Dilute the EIA Diluent Concentrate 10-fold with reagent grade water to produce a 1x solution. When diluting the concentrate, make sure to rinse the bottle thoroughly to extract any precipitates left in the bottle. Mix the 1x solution gently until the crystals have completely dissolved. Store for up to 30 days at 2-8°C.
- **Cortisol Standard:** Allow the vial to warm to room temperature prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting from the standard stock solution (800 ng/ml) 4-fold with EIA Diluent to produce 200, 50, 12.5, 3.125, and 0.781 ng/ml solutions. EIA Diluent serves as the zero standard (0 ng/ml). Aliquot remaining stock solution to limit repeated freeze-thaw cycles. This solution should be stored at -20°C.

<b>Standard Point</b>	<b>Dilution</b>	<b>[Cortisol] (ng/ml)</b>
P1	1 part Standard (800 ng/ml)	800
P2	1 part P1 + 3 parts EIA Diluent	200
P3	1 part P2 + 3 parts EIA Diluent	50
P4	1 part P3 + 3 parts EIA Diluent	12.5
P5	1 part P4 + 3 parts EIA Diluent	3.125
P6	1 part P5 + 3 parts EIA Diluent	0.781
P7	EIA Diluent	0.0



- **Biotinylated Cortisol Steroid (2x):** Reconstitute the Biotinylated Cortisol Steroid with 3.5 ml of EIA Diluent to generate a stock solution. Allow the vial to sit for 10 minutes with gentle agitation prior to dilution. From the stock solution, dilute 2-fold with EIA Diluent to produce a 1x working solution. Aliquot remaining stock solution to limit repeated freeze-thaw cycles. This solution should be stored at -20°C and **used within 30 days**.
- **Wash Buffer Concentrate (20x):** Dilute the Wash Buffer Concentrate 20-fold with reagent grade water to produce a 1x solution. When diluting the concentrate, make sure to rinse the bottle thoroughly to extract any precipitates left in the bottle. Mix the 1x solution gently until the crystals have completely dissolved.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 100-fold with EIA Diluent to produce a 1x solution. The undiluted conjugate should be stored at -20°C.

## Assay Procedure

- Prepare all reagents, standard solutions, and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-25°C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccants inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 25 µl of Cortisol Standard or sample to each well, and immediately add 25 µl of Biotinylated Cortisol Steroid to each well (on top of the standard or sample). Gently tap plate to ensure thorough mixing. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for 2 hours. Start the timer after the last addition.
- Wash the microplate manually or automatically using a microplate washer. Invert the plate and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If washing manually, wash five times with 200 µl of Wash Buffer per well. Invert the plate each time and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If using a microplate washer, wash six times with 300 µl of Wash Buffer per well; invert the plate and hit 4-5 times on absorbent material to completely remove the liquid.
- Add 50 µl of SP Conjugate to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash the microplate as described above.
- Add 50 µl of Chromogen Substrate to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed.

Incubate in ambient light for 15 minutes or until the optimal blue color density develops.

- Add 50  $\mu$ l of Stop Solution to each well. The color will change from blue to yellow. Gently tap plate to ensure thorough mixing. Break any bubbles that may have formed.
- Read the absorbance on a microplate reader at a wavelength of 450 nm **immediately**. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at low concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

## Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample.
- To generate a standard curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance (OD) on the y-axis. The best fit line can be determined by regression analysis using log-log or four-parameter logistic curve fit.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

## Typical Data

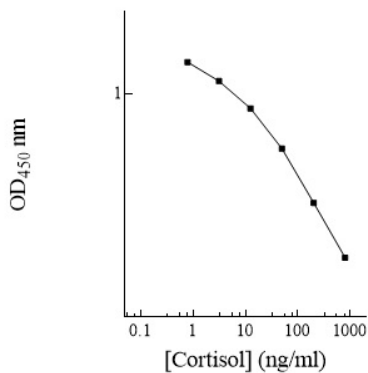
- The typical data is provided for reference only. Individual laboratory means may vary from the values listed. Variations between laboratories may be caused by technique differences.

Standard Point	ng/ml	OD	Average OD
P1	800	0.114 0.108	0.111
P2	200	0.236 0.226	0.231
P3	50	0.486 0.468	0.477
P4	12.5	0.832 0.806	0.819
P5	3.125	1.202 1.160	1.181
P6	0.781	1.551 1.493	1.522
P7	0.0	1.871 1.797	1.834
<b>Sample: Pooled Normal Human Sodium Citrate Plasma (10x)</b>		0.955 0.947	0.951

## Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.

Cortisol Standard Curve



## Performance Characteristics

- The minimum detectable dose of cortisol as calculated by 2SD from the mean of a zero standard was established to be 0.65 ng/ml.
- Intra-assay precision was determined by testing three human plasma samples twenty times in one assay.

- Inter-assay precision was determined by testing three human plasma samples in twenty assays.

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	20	20	20	20	20	20
CV (%)	6.8%	5.8%	6.2%	12.4%	11.3%	11.5%
Average CV (%)	6.3%			11.7%		

## Recovery

Standard Added Value	3.125 – 50 ng/ml
Recovery %	89 – 111%
<b>Average Recovery %</b>	<b>96%</b>

## Linearity

- Human plasma and serum samples were serially diluted to test for linearity.

Average Percentage of Expected Value (%)		
Sample Dilution	Plasma	Serum
5x	84%	95%
10x	101%	102%
20x	115%	110%

## Cross-Reactivity

Steroid	Cross-Reactivity (%)
PROGESTERONE	None
ALLOPREGNANOLONE	None
CORTEXOLONE	None
DEOXYCORTICOSTERONE	None
CORTISONE	None
CORTEXOLONE HEMISUCCINATE	None
CORTICOSTERONE	None
6-KETO-17 $\beta$ -ESTRADIOL	None
5-ANDROSTEN-3 $\beta$ -OL-7, 17-DIONE	None
6-KETO-17 $\alpha$ -ESTRADIOL	None
3-KETO-5 $\alpha$ , 16-ANDROSTENE	None
4-ANDROSTEN-17 $\alpha$ -OL-3-ONE	None
ALDOSTERONE	<1%

ETHYNYLESTRADIOL	None
6-KETOESTRIOL	None
6-KETOESTRONE	None
17 $\beta$ -HYDROXY-4-ANDROSTENE-3, 11-DIONE	None
CORTISONE Acetate	None
ALDOSTERONE 21-HEMISUCCINATE	None
4-PREGNEN-17, 20 $\beta$ - DIOL-3-ONE	None
11 $\alpha$ -HYDROXYTESTOSTERONE	None
20 $\alpha$ -HYDROXYPROGESTERONE	None
6 $\beta$ -HYDROXYPROGESTERONE	None
HYDROCORTISONE	None
17-HYDROXYPROGESTERONE	None

## Troubleshooting

Issue	Causes	Course of Action
Low Precision	Use of improper components	<ul style="list-style-type: none"> <li>• Check the expiration date listed before use.</li> <li>• Do not interchange components from different lots.</li> </ul>
	Improper wash step	<ul style="list-style-type: none"> <li>• Check that the correct wash buffer is being used.</li> <li>• Check that all wells are empty after aspiration.</li> <li>• Check that the microplate washer is dispensing properly.</li> <li>• If washing by pipette, check for proper pipetting technique.</li> </ul>
	Splashing of reagents while loading wells	<ul style="list-style-type: none"> <li>• Pipette properly in a controlled and careful manner.</li> </ul>
	Inconsistent volumes loaded into wells	<ul style="list-style-type: none"> <li>• Pipette properly in a controlled and careful manner.</li> <li>• Check pipette calibration.</li> <li>• Check pipette for proper performance.</li> </ul>
	Insufficient mixing of reagent dilutions	<ul style="list-style-type: none"> <li>• Thoroughly agitate the lyophilized components after reconstitution.</li> <li>• Thoroughly mix dilutions.</li> </ul>
	Improperly sealed microplate	<ul style="list-style-type: none"> <li>• Check the microplate pouch for proper sealing.</li> <li>• Check that the microplate pouch has no punctures.</li> <li>• Check that three desiccants are inside the microplate pouch prior to sealing.</li> </ul>
Unexpectedly Low or High Signal Intensity	Microplate was left unattended between steps	<ul style="list-style-type: none"> <li>• Each step of the procedure should be performed uninterrupted.</li> </ul>
	Omission of step	<ul style="list-style-type: none"> <li>• Consult the provided procedure for complete list of steps.</li> </ul>
	Steps performed in incorrect order	<ul style="list-style-type: none"> <li>• Consult the provided procedure for the correct order.</li> </ul>
	Insufficient amount of reagents added to wells	<ul style="list-style-type: none"> <li>• Check pipette calibration.</li> <li>• Check pipette for proper performance.</li> </ul>
	Wash step was skipped	<ul style="list-style-type: none"> <li>• Consult the provided procedure for all wash steps.</li> </ul>
	Improper wash buffer	<ul style="list-style-type: none"> <li>• Check that the correct wash buffer is being used.</li> </ul>
	Improper reagent preparation	<ul style="list-style-type: none"> <li>• Consult reagent preparation section for the correct dilutions of all reagents.</li> </ul>
	Insufficient or prolonged incubation periods	<ul style="list-style-type: none"> <li>• Consult the provided procedure for correct incubation time.</li> </ul>

<b>Deficient Standard Curve Fit</b>	Non-optimal sample dilution	<ul style="list-style-type: none"> <li>• Sandwich ELISA: If samples generate OD values higher than the highest standard point (P1), dilute samples further and repeat the assay.</li> <li>• Competitive ELISA: If samples generate OD values lower than the highest standard point (P1), dilute samples further and repeat the assay.</li> <li>• User should determine the optimal dilution factor for samples.</li> </ul>
	Contamination of reagents	<ul style="list-style-type: none"> <li>• A new tip must be used for each addition of different samples or reagents during the assay procedure.</li> </ul>
	Contents of wells evaporate	<ul style="list-style-type: none"> <li>• Verify that the sealing film is firmly in place before placing the assay in the incubator or at room temperature.</li> </ul>
	Improper pipetting	<ul style="list-style-type: none"> <li>• Pipette properly in a controlled and careful manner.</li> <li>• Check pipette calibration.</li> <li>• Check pipette for proper performance.</li> </ul>
	Insufficient mixing of reagent dilutions	<ul style="list-style-type: none"> <li>• Thoroughly agitate the lyophilized components after reconstitution.</li> <li>• Thoroughly mix dilutions.</li> </ul>

## References

- (1) PubChem CID: 5754.
- (2) DrugBank: DB00741.

Version 1.2R