Cortisol ELISA Kit

Item No. 500360

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size	480 wells Quantity/Size
400362	Cortisol ELISA Monoclonal Antibody	1 vial/100 dtn	1 vial/500 dtn
10005272	Cortisol-AChE Tracer	1 vial/100 dtn	1 vial/500 dtn
400364	Cortisol ELISA Standard	1 vial/0.5 ml	1 vial/0.5 ml
400060	ELISA Buffer Concentrate (10X)	2 vials/10 ml	4 vials/10 ml
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml	1 vial/12.5 ml
400035	Polysorbate 20	1 vial/3 ml	1 vial/3 ml
400008/400009	Goat Anti-Mouse IgG-Coated Plate	1 plate	5 plates
400012	96-Well Cover Sheet	1 ea	5 ea
400050	Ellman's Reagent	3 vials/100 dtn	6 vials/250 dtn
400040	ELISA Tracer Dye	1 ea	1 ea
400042	ELISA Antiserum Dye	1 ea	1 ea

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

This kit may not perform as described if any reagent or procedure is replaced or modified.

When compared to quantification by LC/MS or GC/MS, it is not uncommon for immunoassays to report higher analyte concentrations. While LC/MS or GC/MS analyses typically measure only a single compound, antibodies used in immunoassays sometimes recognize not only the target molecule, but also structurally related molecules, including biologically relevant metabolites. In many cases, measurement of both the parent molecule and metabolites is more representative of the overall biological response than is the measurement of a short-lived parent molecule. It is the responsibility of the researcher to understand the limits of both assay systems and to interpret their data accordingly.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance between 405-420 nm
2. An orbital microplate shaker
3. Adjustable pipettes; multichannel or repeating pipettor recommended
4. A source of ultrapure water, with a resistivity of 18.2 MΩ·cm and total organic carbon (TOC) levels of <10 ppb, is recommended. Pure water - glass-distilled or deionized - may not be acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
5. Materials used for **Sample Preparation** (see page 13)

INTRODUCTION

Background

Cortisol is a glucocorticoid produced by the adrenal cortex in response to adrenocorticotropic hormone (ACTH).[1] Cortisol is secreted with a circadian periodicity and peaks just prior to waking in the morning. The production of glucocorticoids is increased by stress and can be used as a biomarker of stress. Cortisol levels increase with age and are often elevated in various disease states, including major depressive disorder, certain forms of hypertension, and AIDS.[2-4] Cortisol also strongly promotes adipocyte differentiation.[5,6] Pharmacological intervention with glucocorticoids is associated with cognitive impairment, decreased bone density, hypertension, and an increased risk of type 2 diabetes.[7]

Cortisol binds to two intracellular receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR), which is highly expressed on mature visceral adipoctyes.[3] Of the two receptors, the MR has the higher affinity for cortisol. This receptor will be almost completely occupied by cortisol at levels too low to activate the GR. 11β-Hydroxysteroid dehydrogenase 2 (11β-HSD2) and 11β-HSD1 are regulators of intracellular glucocorticoid levels, catalyzing the conversion of cortisol to inactive cortisone and the regeneration of cortisol from cortisone, respectively.[4,5,8] 11β-HSD2 is expressed predominantly in mineralocorticoid target tissues including the kidney, colon, and salivary gland, to protect the MR from glucocorticoid excess. Individuals lacking 11β-HSD2 exhibit a syndrome known as apparent mineralocorticoid excess, which features hypertension and hypokalemia.[2] 11β-HSD1 cortisol regeneration is increased in visceral adipose tissue isolated from obese individuals compared with adipose tissue isolated from non-obese individuals.[5,6]

Cortisol can be measured in many matrices including plasma, serum, urine, saliva, feces, and hair. Serum cortisol concentrations range from 25-800 nM (9-300 ng/ml) and approximately 90-95% of the cortisol is bound to proteins.[9] Urinary cortisol is not bound to proteins, but its levels are dependent on glomerular and tubular function. In saliva, approximately 67% of cortisol is unbound. There is generally good correlation between cortisol measurements in saliva and serum.

About This Assay

Cayman's Cortisol ELISA Kit is a competitive assay that can be used for the quantification of cortisol in plasma, urine, saliva, feces, hair, and other sample matrices. The assay has a range of 6.6-4,000 pg/ml, with a midpoint (50% B/B_0) of 150-250 pg/ml, and a sensitivity (80% B/B_0) of approximately 35 pg/ml.

Principle of This Assay

This assay is based on the competition between free cortisol and a cortisol-acetylcholinesterase (AChE) conjugate (Cortisol-AChE Tracer) for a limited number of cortisol monoclonal antibody binding sites. Because the concentration of the Cortisol-AChE Tracer is held constant while the concentration of free cortisol varies, the amount of Cortisol-AChE Tracer that is able to bind to the cortisol monoclonal antibody will be inversely proportional to the concentration of free cortisol in the well. This antibody-cortisol complex binds to the polyclonal goat anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of Cortisol-AChE Tracer bound to the well, which is inversely proportional to the amount of free cortisol present in the well during the incubation, as described in the equation:

$$\text{Absorbance} \propto \text{[bound cortisol-AChE tracer]} \propto 1/\text{[cortisol]}$$

A schematic of this process is shown in Figure 1, on page 9.

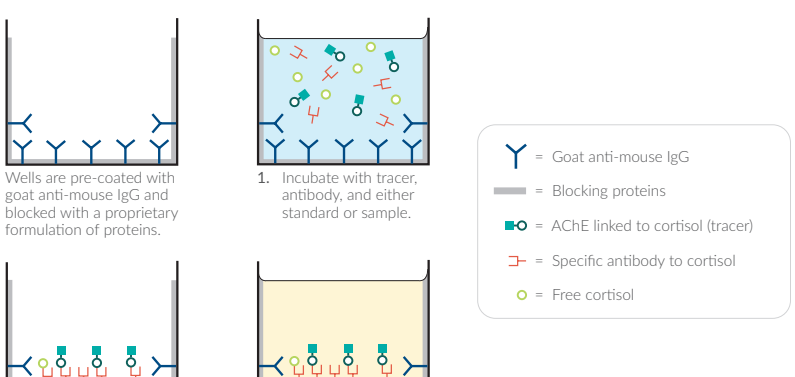


Figure 1. Schematic of the ELISA

Definition of Key Terms

Blk (Blank): background absorbance caused by Ellman's Reagent. The blank absorbance should be subtracted from the absorbance readings of all the other wells, including the non-specific binding (NSB) wells.

TA (Total Activity): total enzymatic activity of the cortisol AChE-linked tracer.

NSB (Non-Specific Binding): non-immunological binding of the tracer to the well. Even in the absence of specific antibody a very small amount of tracer still binds to the well; the NSB is a measure of this low binding.

B_0 (Maximum Binding): maximum amount of the tracer that the antibody can bind in the absence of free analyte.

%B/B_0 (%Bound/Maximum Bound): ratio of the absorbance of a sample or standard well to the average absorbance of the maximum binding (B_0) wells.

Standard Curve: a plot of the %B/B_0 values *versus* concentration of a series of wells containing various known amounts of analyte.

Dtn (Determination): one dtn is the amount of reagent used per well.

Cross Reactivity: numerical representation of the relative reactivity of this assay towards structurally related molecules as compared to the primary analyte of interest. Biomolecules that possess similar epitopes to the analyte can compete with the assay tracer for binding to the primary antibody. Substances that are superior to the analyte in displacing the tracer result in a cross reactivity that is greater than 100%. Substances that are inferior to the primary analyte in displacing the tracer result in a cross reactivity that is less than 100%. Cross reactivity is calculated by comparing the mid-point (50% B/B_0) value of the tested molecule to the mid-point (50% B/B_0) value of the primary analyte when each is measured in assay buffer using the following formula:

$$\text{\% Cross Reactivity} = \left[\frac{\text{50\% } B/B_0 \text{ value for the primary analyte}}{\text{50\% } B/B_0 \text{ value for the potential cross reactant}} \right] \text{x } 100\%$$

LLOD (Lower Limit of Detection): the smallest measure that can be detected with reasonable certainty for a given analytical procedure. The LLOD is defined as a concentration two standard deviations higher than the mean zero value.

PRE-ASSAY PREPARATION

Buffer Preparation

Store all diluted buffers at 4°C; they will be stable for approximately two months. NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with ultrapure water.

1. **ELISA Buffer (1X) Preparation**

   Dilute the contents of one vial of ELISA Buffer Concentrate (10X) (Item No. 400060) with 90 ml of ultrapure water. Be certain to rinse the vial to remove any salts that may have precipitated.

2. **Wash Buffer (1X) Preparation (Item No. 400062)**

   5 ml vial Wash Buffer Concentrate (400X) (96-well kit): Dilute to a total volume of 2 L with ultrapure water and add 1 ml of Polysorbate 20 (Item No. 400035).

   OR

   12.5 ml vial Wash Buffer Concentrate (400X) (480-well kit): Dilute to a total volume of 5 L with ultrapure water and add 2.5 ml of Polysorbate 20 (Item No. 400035).

Smaller volumes of Wash Buffer (1X) can be prepared by diluting the Wash Buffer Concentrate (400X) 1:400 and adding 0.5 ml of Polysorbate 20 per 1 L of Wash Buffer (1X).

NOTE: Polysorbate 20 is a viscous liquid and cannot be measured by a regular pipette. A positive displacement pipette or a syringe should be used to deliver small quantities accurately.

Sample Preparation

Testing for Interference

This assay has been validated in plasma, urine, saliva, feces, and hair. Other sample types should be tested for interference to evaluate the need for sample purification before embarking on a large number of sample measurements. To test for interference, dilute one or two samples to obtain at least two different dilutions of each sample within the linear portion of the standard curve. If two different dilutions of the same sample show good correlation (differ by 20% or less) in the final calculated cortisol concentration, sample purification is not required. If you do not see good correlation of the different dilutions, purification is advised. Purification methods will be determined by the end user and tested for compatibility in the assay.

NOTE: Due to an approximate 15% cross reactivity with dexamethasone, this kit may not be suitable for use with samples that contain high concentrations of dexamethasone, such as those collected during a dexamethasone suppression test.

Plasma, urine, saliva, feces, and hair were processed using the following protocols. Alternative protocols may be used based on the experimental requirements, sample type, and the end user's expertise.

Plasma

Collect blood in vacutainers containing heparin, EDTA, or sodium citrate for plasma samples. To obtain plasma, spin samples at 1,000 x g for 15 minutes. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -80°C.

1. Aliquot a known amount of each sample into a clean test tube (500 µl is recommended). If the samples need to be concentrated, a larger volume should be used (*e.g.*, a 1 ml sample will be concentrated by a factor of 2, a 2 ml sample will be concentrated by a factor of 4, *etc.*).
2. Adjust the pH of the samples to 1.5-2 by adding a few drops of 3 M HCl.
3. Add 4X the sample volume of ethyl acetate and vortex thoroughly. Allow layers to separate. Transfer the ethyl acetate (upper) layer to a clean tube using a transfer pipette. Repeat this extraction procedure three times.*
4. Evaporate the ethyl acetate under a gentle stream of nitrogen without heat or by heating the sample to 37°C.
5. Dissolve the extract in 500 µl of ELISA Buffer (1X), and use this for ELISA analysis.

*If it is necessary to stop during this purification, samples may be stored in the ethyl acetate solution at -20°C or -80°C.

Urine

Urine samples should be assayed immediately or stored at -20°C immediately after collection. Interference in urine is infrequent. Dilute urine samples with ELISA Buffer (1X) to fall within the range of the standard curve. It is recommended that the values obtained from urine samples be standardized to creatinine levels using Cayman's Creatinine ELISA Kit (Item No. 502330), Creatinine (urinary) Colorimetric Assay Kit (Item No. 500701), or a similar assay.

Saliva

Centrifuge saliva at 5,000 x g for 20 minutes and use the supernatant for the assay.

Feces

1. Lyophilize feces to remove excess water.
2. Place 50 mg of lyophilized feces into a clean centrifuge tube, add 500 µl of ultrapure water, and vortex thoroughly.
3. Add 900 µl of ethyl acetate, vortex, and rock on a shaker for 30 minutes at room temperature.
4. Centrifuge at 2,000 x g for 5 minutes.
5. Transfer upper (ethyl acetate) layer into a clean test tube.
6. Evaporate ethyl acetate under a gentle stream of nitrogen without heat or by heating the sample to 37°C.
7. Reconstitute in 400 µl of ELISA Buffer (1X). If necessary, centrifuge at 2,000 x g for 5 minutes to remove particles from the solution. Transfer buffer fraction into a clean test tube and use for ELISA analysis.

Hair

1. Place 35 mg of hair into a clean Precellys® tissue homogenizer tube or any similar tissue homogenizer, add 1.5 ml of hexane, and incubate at room temperature for 2 minutes. Remove hexane from the tube and dry the hair under a gentle stream of nitrogen.
2. Cap the Precellys® tube and flash freeze the hair sample using liquid nitrogen. Grind the hair sample using a Precellys® homogenizer at 6,800 rpm, 5x20s cycles, pausing for 30 seconds between the cycles.
3. Add 1.8 ml of methanol, vortex, and rock overnight on a shaker at room temperature.
4. Collect methanol into a clean test tube.
5. Evaporate methanol under a gentle stream of nitrogen at 37°C.
6. Reconstitute in 400 µl of ELISA Buffer (1X) and analyze in the assay.

General Precautions

- All samples must be free of organic solvents prior to assay.
- Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -20 or -80°C.
- Samples of mouse origin may contain antibodies that interfere with the assay by binding to the goat anti-mouse plate. We recommend that all mouse samples be purified prior to use in this assay.
- AEBSF (Pefabloc SC®) and PMSF inhibit AChE. Samples containing these protease inhibitors should not be used in this assay.

Sample Matrix Properties

Parallelism

To assess parallelism, human plasma, urine, and saliva, sheep feces, and dog and cat hair were processed as described in the Sample Preparation section (see page 13), serially diluted with ELISA Buffer (1X), and evaluated using the Cortisol ELISA Kit. Measured concentrations were plotted as a function of the sample dilution. The results are shown below.

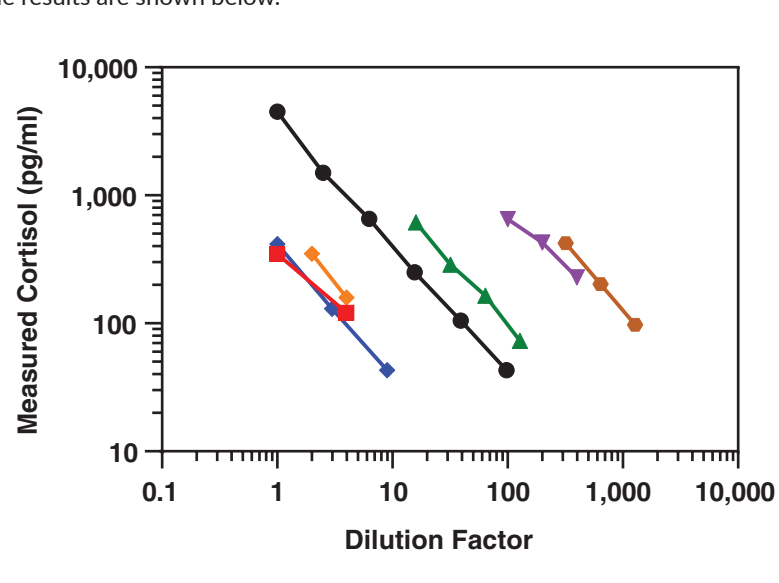


Figure 2. Parallelism of various matrices in the Cortisol ELISA

Spike and Recovery

Human plasma, urine, and saliva were spiked with different amounts of cortisol, processed as described in the Sample Preparation section, serially diluted with ELISA Buffer (1X), and evaluated using the Cortisol ELISA Kit. The results are shown below. The error bars represent standard deviations obtained from multiple dilutions of each sample.

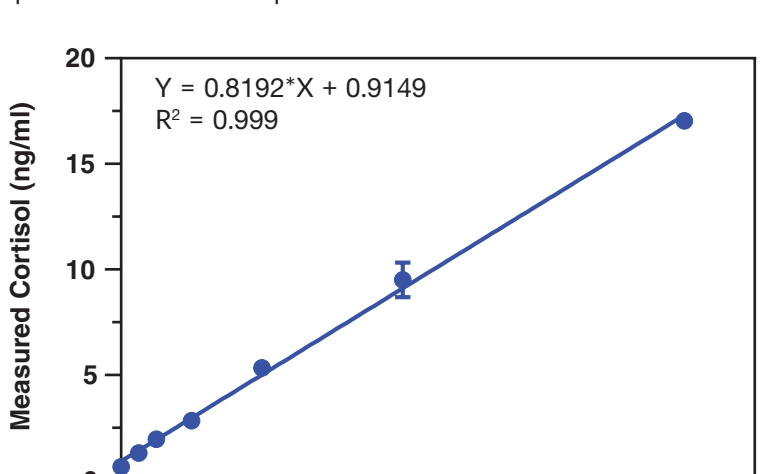


Figure 3. Spike and recovery of cortisol in saliva

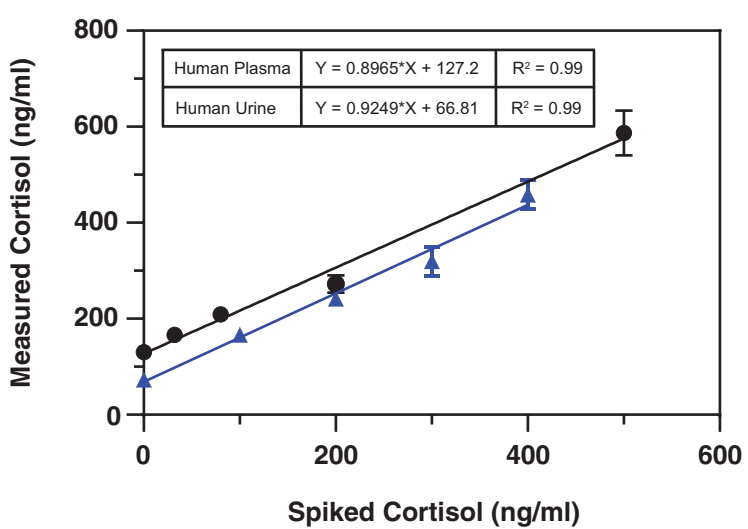


Figure 4. Spike and recovery of cortisol in plasma and urine

Linearity

Human plasma, urine, and saliva and dog hair were spiked with cortisol, processed as described in the Sample Preparation section, serially diluted with ELISA Buffer (1X), and evaluated for linearity using the Cortisol ELISA Kit. The results are shown in Table 1, on page 20.

Dilution Factor	Measured Concentration	Linearity (%)
Human plasma spiked with 500 ng/ml cortisol		
1,000	524	100
2,000	603	115
4,000	634	121
8,000	586	112
Human urine spiked with 400 ng/ml cortisol		
500	438	100
1,000	448	102
2,000	503	115
Human saliva spiked with 5 ng/ml cortisol		
10	5.4	100
20	5.4	100
40	5.3	98
Dog hair spiked with 1,142 pg/mg cortisol		
160	1,086	100
320	1,044	96
640	949	87

Table 1. Linearity in various matrices

*NOTE: Linearity has been calculated using the following formula: %Linearity = (Observed concentration value, dilution adjusted / First observed concentration value in the dilution series, dilution adjusted)*100*

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

Cortisol ELISA Standard

Equilibrate a pipette tip by repeatedly filling and expelling the tip with the Cortisol ELISA Standard (Item No. 400364) several times. Using the equilibrated pipette tip, transfer 100 µl of the standard into a clean test tube, then dilute with 900 µl ultrapure water. The concentration of this solution (the bulk standard) will be 40 ng/ml.

NOTE: If assaying culture medium samples that have not been diluted with ELISA Buffer (1X), culture medium should be used in place of ELISA Buffer (1X) for dilution of the standard curve.

To prepare the standard for use in ELISA: Obtain eight clean test tubes and label them #1-8. Aliquot 900 µl ELISA Buffer (1X) to tube #1 and 600 µl ELISA Buffer (1X) to tubes #2-8. Transfer 100 µl of the bulk standard (40 ng/ml) to tube #1 and mix thoroughly. Serially dilute the standard by removing 400 µl from tube #1 and placing it in tube #2; mix thoroughly. Next, remove 400 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. These diluted standards should not be stored for more than 24 hours.

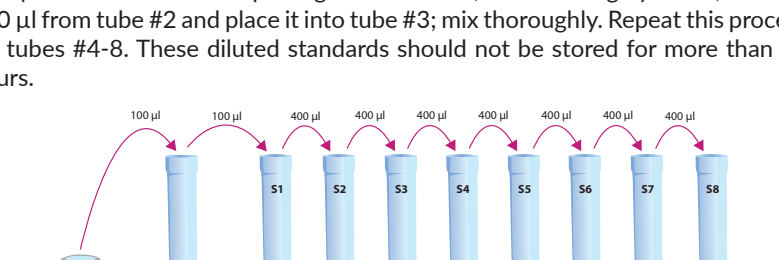


Figure 5. Preparation of the cortisol standards

Cortisol-AChE Tracer

Reconstitute the Cortisol-AChE Tracer (Item No. 10005272) as follows:

100 dtn Cortisol-AChE Tracer (96-well kit): Reconstitute with 6 ml of ELISA Buffer (1X).

OR

500 dtn Cortisol-AChE Tracer (480-well kit): Reconstitute with 30 ml of ELISA Buffer (1X).

Store the reconstituted Cortisol-AChE Tracer at 4°C *(do not freeze!)*. It will be stable for at least four weeks. A 20% surplus of tracer has been included to account for any incidental losses.

Tracer Dye Instructions (optional)

This dye may be added to the tracer, if desired, to aid in visualization of tracer-containing wells. Add the dye to the reconstituted tracer at a final dilution of 1:100 (add 60 µl of dye to 6 ml tracer or add 300 µl of dye to 30 ml of tracer).

Cortisol ELISA Monoclonal Antibody

Reconstitute the Cortisol ELISA Monoclonal Antibody (Item No. 400362) as follows:

100 dtn Cortisol ELISA Monoclonal Antibody (96-well kit): Reconstitute with 6 ml of ELISA Buffer (1X).

OR

500 dtn Cortisol ELISA Monoclonal Antibody (480-well kit): Reconstitute with 30 ml of ELISA Buffer (1X).

Store the reconstituted Cortisol ELISA Monoclonal Antibody at 4°C (*do not freeze!*). It will be stable for at least four weeks. A 20% surplus of antibody has been included to account for any incidental losses.

Antiserum Dye Instructions (optional)

This dye may be added to the antibody, if desired, to aid in visualization of antibody-containing wells. Add the dye to the reconstituted antibody at a final dilution of 1:100 (add 60 µl of dye to 6 ml antibody or add 300 µl of dye to 30 ml of antibody).

Plate Set Up

The 96-well plate(s) included with this kit is supplied ready to use. It is not necessary to rinse the plate(s) prior to adding the reagents. *NOTE: If you do not need to use all the strips at once, place the unused strips back in the plate packet and store at 4°C. Be sure the packet is sealed with the desiccant inside.*

Each plate or set of strips must contain a minimum of two Blk, two NSB, and two B_0 wells, and an eight-point standard curve run in duplicate. *NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results.* Each sample should be assayed at a minimum of two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying samples in triplicate.

A suggested plate format is shown in Figure 6, on page 25. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided below has been designed to allow for easy data analysis using a convenient spreadsheet offered by Cayman (see page 29 for more details). We suggest recording the contents of each well on the template sheet provided (see page 37).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	S1	S1	1	1	1	9	9	9	17	17	17
B	Blk	S2	S2	2	2	2	10	10	10	18	18	18
C	NSB	S3	S3	3	3	3	11	11	11	19	19	19
D	NSB	S4	S4	4	4	4	12	12	12	20	20	20
E	B_0	S5	S5	5	5	5	13	13	13	21	21	21
F	B_0	S6	S6	6	6	6	14	14	14	22	22	22
G	B_0	S7	S7	7	7	7	15	15	15	23	23	23
H	TA	S8	S8	8	8	8	16	16	16	24	24	24

Blk - Blank
TA - Total Activity
NSB - Non-Specific Binding
B_0 - Maximum Binding
S1-S8 - Standards 1-8
1-24 - Samples

Figure 6. Sample plate format

Performing the Assay

Pipetting Hints

- Use different tips to pipette each reagent.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

Addition of the Reagents

1. **ELISA Buffer**

   Add 100 µl ELISA Buffer (1X) to NSB wells. Add 50 µl ELISA Buffer (1X) to B_0 wells. If culture medium was used to dilute the standard curve, substitute 50 µl of culture medium for ELISA Buffer (1X) in the NSB and B_0 wells (*i.e.*, add 50 µl culture medium to NSB and B_0 wells and 50 µl ELISA Buffer (1X) to NSB wells).

2. **Cortisol ELISA Standard**

   Add 50 µl from tube #8 to both of the lowest standard wells (S8). Add 50 µl from tube #7 to each of the next standard wells (S7). Continue with this procedure until all the standards are aliquoted. The same pipette tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipette tip in that standard.

3. **Samples**

   Add 50 µl of sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

4. **Cortisol-AChE Tracer**

   Add 50 µl to each well *except* the TA and the Blk wells.

5. **Cortisol ELISA Monoclonal Antibody**

   Add 50 µl to each well *except* the TA, NSB, and Blk wells, within 15 minutes of addition of the tracer.

Incubation of the Plate

Cover each plate with a 96-Well Cover Sheet (Item No. 400012) and incubate overnight at 4°C.

Development of the Plate

1. Reconstitute Ellman's Reagent (Item No. 400050) immediately before use. Reconstitute 100 dtn vial with 20 ml of ultrapure water. Reconstitute 250 dtn vial with 50 ml of ultrapure water.

NOTE: Reconstituted Ellman's Reagent is unstable and should be used the same day it is prepared; protect the Ellman's Reagent from light when not in use. Extra vials of the reagent have been provided should a plate need to be re-developed or multiple assays run on different days.

2. Empty the wells and rinse five times with ~300 µl Wash Buffer (1X).
3. Add 200 µl of Ellman's Reagent to each well.
4. Add 5 µl of the reconstituted tracer to the TA wells.
5. Cover the plate with the 96-Well Cover Sheet. Optimum development is obtained by using an orbital shaker equipped with a large, flat cover to allow the plate(s) to develop in the dark at room temperature. This assay typically develops (*i.e.*, B_0 wells ≥0.6 A.U. (Blk subtracted)) in 90-120 minutes.

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, *etc.*
2. Remove the cover sheet being careful to keep Ellman's Reagent from splashing on the cover. *NOTE: Any loss of Ellman's Reagent will affect the absorbance readings.*
3. Read the plate at a wavelength between 405 and 420 nm. The absorbance may be checked periodically until the B_0 wells have reached a minimum of 0.3 A.U. (Blk subtracted). The plate should be read when the absorbance of the B_0 wells is in the range of 0.3-1.0 A.U. (Blk subtracted). If the absorbance of the wells exceeds 1.5 A.U., wash the plate, add fresh Ellman's Reagent, and let it develop again.

ANALYSIS

Many plate readers come with data reduction software that plot data automatically. Alternatively, a spreadsheet program can be used. The data should be plotted as either $\%B/B_0$ *versus* log concentration using a four-parameter logistic fit or as logit B/B_0 *versus* log concentration using a linear fit. *NOTE: Cayman has a computer spreadsheet available for data analysis. Please contact Technical Service or visit our website (www.caymanchem.com/analysisTools/ELISA) to obtain a free copy of this convenient data analysis tool.*

Calculations

Preparation of the Data

The following procedure is recommended for preparation of the data prior to graphical analysis.

NOTE: If the plate reader has not subtracted the absorbance readings of the Blk wells from the absorbance readings of the rest of the plate, be sure to do that now.

1. Average the absorbance readings from the NSB wells.
2. Average the absorbance readings from the B_0 wells.
3. Subtract the NSB average from the B_0 average. This is the corrected B_0 or corrected maximum binding.
4. Calculate the B/B_0 (Sample or Standard Bound/Maximum Bound) for the remaining wells. To do this, subtract the average NSB absorbance from the S1 absorbance and divide by the corrected B_0 (from Step 3). Repeat for S2-S8 and all sample wells. (To obtain $\%B/B_0$ for a logistic four-parameter fit, multiply these values by 100.)

NOTE: The TA values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool. Low or no absorbance from a TA well could indicate a dysfunction in the enzyme-substrate system.

Plot the Standard Curve

Plot %B/B_0 for standards S1-S8 *versus* cortisol concentration using linear (y) and log (x) axes and perform a four-parameter logistic fit.

Alternative Plot - The data can also be linearized using a logit transformation. The equation for this conversion is shown below. *NOTE: Do not use %B/B_0 in this calculation.*

$$\text{logit } (B/B_0) = \ln [B/B_0/(1 - B/B_0)]$$

Plot the data as logit (B/B_0) *versus* log concentrations and perform a linear regression fit.

Determine the Sample Concentration

Calculate the B/B_0 (or %B/B_0) value for each sample. Determine the concentration of each sample by identifying the %B/B_0 on the standard curve and reading the corresponding values on the x-axis. *NOTE: Remember to account for any dilution of the original sample concentration prior to its addition to the well.* Samples with %B/B_0 values greater than 80% or less than 20% should be re-assayed as they generally fall out of the linear range of the standard curve. A 20% or greater disparity between the apparent concentration of two different dilutions of the same sample indicates interference which could be eliminated by purification.

NOTE: If there is an error in the B_0 wells it is possible to calculate sample concentrations by plotting the absorbance values and back calculating sample absorbance off the standard curve.

Performance Characteristics

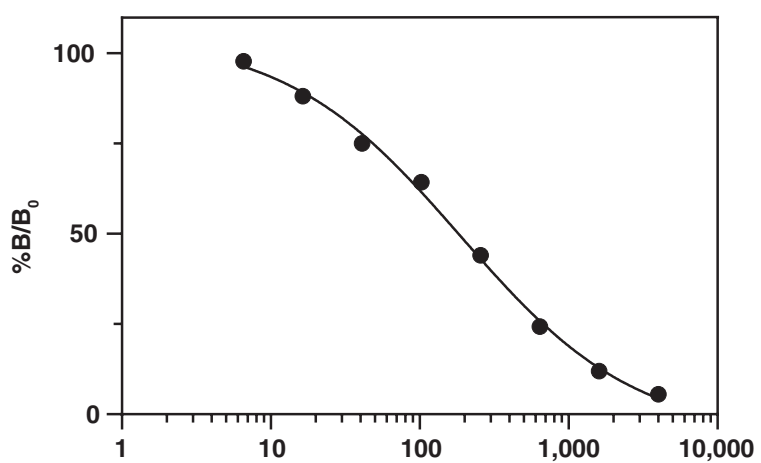
Representative Data

The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the values of your samples.

Absorbance at 414 nm

Cortisol Standards (pg/ml) and Controls	Blk-subtracted Absorbance	NSB-corrected Absorbance	%B/B_0
NSB	0		
B_0	1.095	1.095	
TA	0.923		
4,000	0.060	0.060	5.500
1,600	0.132	0.132	11.950
640	0.266	0.266	24.250
256	0.483	0.483	44.000
102.4	0.704	0.704	64.300
41.0	0.821	0.821	75.000
16.4	0.966	0.966	88.150
6.6	1.071	1.071	97.750

Table 2. Typical results



Assay Range = 6.6-4,000 pg/ml
Sensitivity (defined as 80% B/B_0) = 35 pg/ml
Mid-point (defined as 50% B/B_0) = 190 pg/ml
LLOD = 26 pg/ml

The sensitivity and mid-point were derived from the standard curve shown above. The standard was diluted with ELISA Buffer (1X).

Figure 7. Typical standard curve

Precision:

The intra- and inter-assay CVs have been determined at multiple points on the standard curve. These data are summarized in the graph on page 32 and in the table below.

Dose (pg/ml)	%CV* Intra-assay Precision	%CV* Inter-assay Precision
4,000	10.1	6.7
1,600	7.4	6.7
640	5.1	6.7
256	6.7	9.0
102.4	8.2	20.1
41.0	13.4	25.8
16.4	42.3**	20.1
6.6	47.7**	54.4**

Table 3. Intra- and inter-assay precision
*%CV represents the variation in concentration (not absorbance) as determined using a reference standard curve.
**Outside of the recommended usable range of the assay.

Cross Reactivity:

Compound	Cross Reactivity
Cortisol	100%
Prednisolone	4.0%
Cortexolone	1.6%
11-deoxy Corticosterone	0.23%
Dexamethasone	15%
17-Hydroxyprogesterone	0.23%
Cortisol Glucuronide	0.15%
Corticosterone	0.14%
Cortisone	0.13%
Androstenedione	<0.01%
Enterolactone	<0.01%
Estrone	<0.01%
17-hydroxy Pregnenolone	<0.01%
Pregnenolone	<0.01%
Testosterone	<0.01%

Table 4. Cross reactivity of the Cortisol ELISA Kit

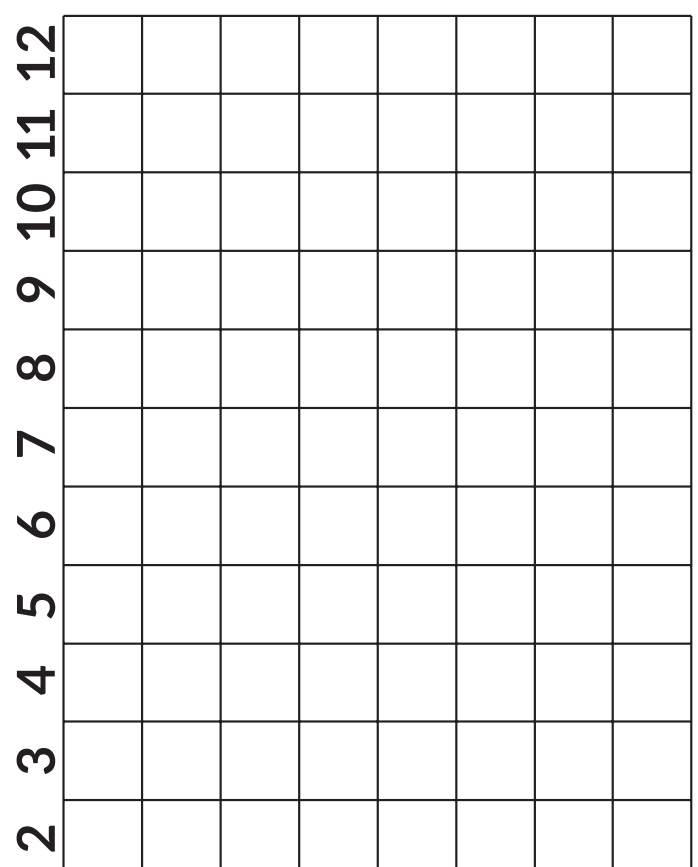
RESOURCES

Troubleshooting

Problem	Possible Causes
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique
High NSB (>10% of B_0)	A. Poor washing B. Exposure of NSB wells to specific antibody
Very low B_0	A. Trace organic contaminants in the water source B. Dilution error in preparing reagents
Low sensitivity (shift in dose-response curve)	A. Standard is degraded or contaminated B. Dilution error in preparing standards
Analyses of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference)	Interfering substances are present
Low signal in the sample wells (below the range of the standard curve)	A. AChE inhibitors are present; ensure that the samples and buffers are free of AChE inhibitors B. Sample requires further dilution
Only TA wells develop	A. Trace organic contaminants in the water source B. The tracer was not added to the wells

Procedure	Blk	TA	NSB	B_0	Standards/ Samples
Reconstitute and mix	Mix all reagents gently				
ELISA Buffer (1X)	--	--	100 µl	50 µl	--
Cortisol Standards/ Samples	--	--	--	--	50 µl
Cortisol-AChE Tracer	--	--	50 µl	50 µl	50 µl
Cortisol Monoclonal Antibody	--	--	--	50 µl	50 µl
Incubate	Seal the plate and incubate overnight at 4°C				
Aspirate	Aspirate wells and wash 5 x ~300 µl with Wash Buffer (1X)				
Apply Ellman's Reagent	200 µl	200 µl	200 µl	200 µl	200 µl
TA - Apply Tracer	--	5 µl	--	--	--
Develop	Seal plate and incubate for 90-120 minutes at room temperature on an orbital shaker protected from light				
Read	Read absorbance at 405-420 nm				

Table 5. Assay summary



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NOTES

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