Cortisol ELISA Kit

Item No. 500360

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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	1 vial
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 cover	5 covers
400050	Ellman's Reagent	3 vials/100 dtn	6 vials/250 dtn
400040	ELISA Tracer Dye	1 vial	1 vial
400042	ELISA Antiserum Dye	1 vial	1 vial

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.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's AChE ELISA Kits. 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
Fax:	734-971-3641
Email:	techserv@caymanchem.com
Hours:	M-F 8:00 AM to 5:30 PM EST

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. Adjustable pipettes and a repeating pipettor.
3. A source of 'UltraPure' water. Water used to prepare all ELISA reagents and buffers must be deionized and free of trace organic contaminants ('UltraPure'). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for ELISA. *NOTE: UltraPure water is available for purchase from Cayman (Item No. 400000).*
4. 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). Cortisol is secreted with a circadian periodicity and peaks just prior to waking in the morning.[1] The production of glucocorticoids is increased by stress, therefore, cortisol can be used as a biomarker of stress. Cortisol levels increase with age, and are often elevated in major depressive disorder, certain forms of hypertension, and AIDS.[2-4] Pharmacological treatment with glucocorticoids can result in cognitive impairment, decreased bone density, hypertension, and an increased risk of development of type II diabetes.[5]

Cortisol binds to two intracellular receptors, the mineralocorticoid receptor (MR), and the glucocorticoid receptor (GR). 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.[3] 11β-Hydroxysteroid dehydrogenase (Type 2) (11β-HSD2) converts cortisol to inactive cortisone. This enzyme is expressed predominantly in mineralocorticoid target tissues including kidney, colon, and salivary gland where it serves to protect the MR from glucocorticoid excess. Individuals lacking this enzyme exhibit a syndrome known as apparent mineralocorticoid excess which features hypertension and hypokalemia.[2]

The enzyme 11β-HSD1 is a key regulator of intracellular glucocorticoid levels, catalyzing the regeneration of cortisol from cortisone.[6,7] Visceral adipose tissue from obese humans has increased 11β-HSD1 activity compared to adipose tissue obtained from normal individuals.[6,8] Cortisol strongly promotes adipocyte differentiation; mature visceral adipocytes express high levels of the glucocorticoid receptor.[6,8]

Cortisol can be measured in many matrices including blood, feces, urine, and saliva. Serum cortisol concentrations range from about 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.[9]

About This Assay

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

Description of AChE Competitive ELISAs[10,11]

This assay is based on the competition between cortisol and cortisol-acetylcholinesterase (AChE) conjugate (cortisol tracer) for a limited number of cortisol-specific mouse monoclonal antibody binding sites. Because the concentration of the cortisol tracer is held constant while the concentration of cortisol varies, the amount of cortisol tracer that is able to bind to the Cortisol monoclonal antibody will be inversely proportional to the concentration of cortisol in the well. This antibody-cortisol (either free or tracer) complex binds to the goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then 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 tracer bound to the well, which is inversely proportional to the amount of free cortisol present in the well during the incubation; or

$$\text{Absorbance} \propto [\text{Bound Cortisol Tracer}] \propto 1/[\text{Cortisol}]$$

A schematic of this process is shown in Figure 1, on page 8.

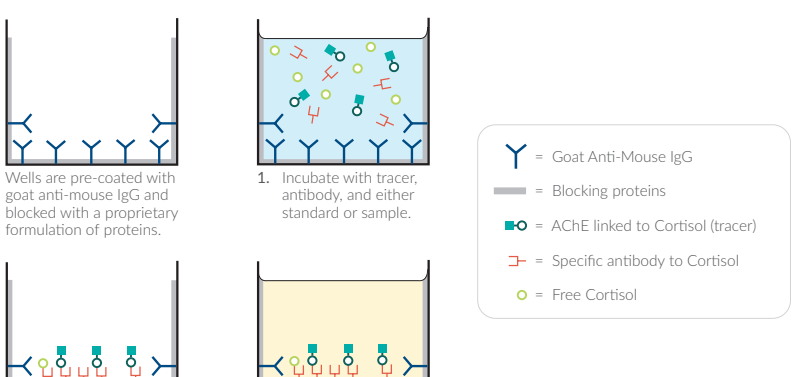


Figure 1. Schematic of the AChE ELISA

Biochemistry of Acetylcholinesterase

The electric organ of the electric eel, *E. electricus*, contains an avid AChE capable of massive catalytic turnover during the generation of its electrochemical discharges. The electric eel AChE has a clover leaf-shaped tertiary structure consisting of a triad of tetramers attached to a collagen-like structural fibril. This stable enzyme is capable of high turnover (64,000 s^{-1}) for the hydrolysis of acetylthiocholine.

A molecule of the analyte covalently attached to a molecule of AChE serves as the tracer in AChE enzyme immunoassays. Quantification of the tracer is achieved by measuring its AChE activity with Ellman's Reagent. This reagent consists of acetylthiocholine and 5,5'-dithio-*bis*-(2-nitrobenzoic acid). Hydrolysis of acetylthiocholine by AChE produces thiocholine (see Figure 2, on page 10). The non-enzymatic reaction of thiocholine with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) produces 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm (ε = 13,600).

AChE has several advantages over other enzymes commonly used for enzyme immunoassays. Unlike horseradish peroxidase, AChE does not self-inactivate during turnover. This property of AChE also allows redevelopment of the assay if it is accidentally splashed or spilled. In addition, the enzyme is highly stable under the assay conditions, has a wide pH range (pH 5-10), and is not inhibited by common buffer salts or preservatives. Since AChE is stable during the development step, it is unnecessary to use a 'stop' reagent, and the plate may be read whenever it is convenient.

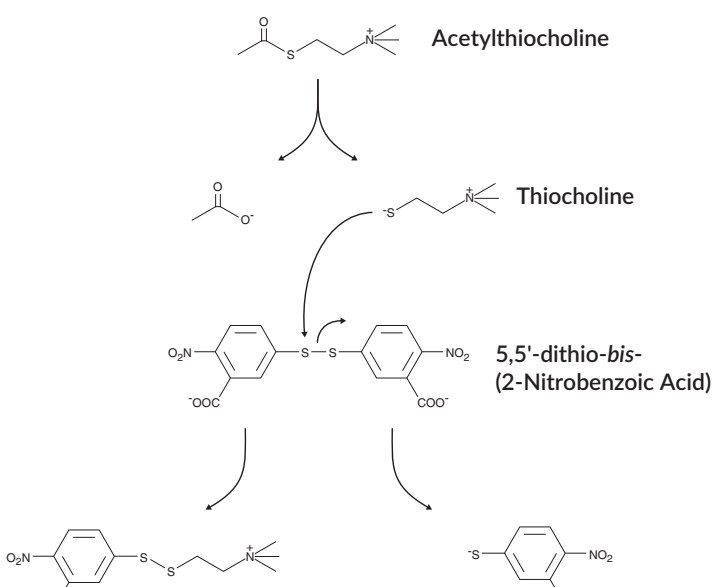


Figure 2. Reaction catalyzed by acetylcholinesterase

Definition of Key Terms

Blank: background absorbance caused by Ellman's Reagent. The blank absorbance should be subtracted from the absorbance readings of all the other wells, including NSB wells.

Total Activity: total enzymatic activity of the AChE-linked tracer. This is analogous to the specific activity of a radioactive 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. Do not forget to subtract the Blank absorbance values.

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 particular sample or standard well to that of the maximum binding (B_0) well.

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, where 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:

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

PRE-ASSAY PREPARATION

NOTE: Water used to prepare all ELISA reagents and buffers must be deionized and free of trace organic contaminants ('UltraPure'). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for ELISA. UltraPure water may be purchased from Cayman (Item No. 400000).

Buffer Preparation

Store all diluted buffers at 4°C; they will be stable for about two months.

1. **ELISA Buffer 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. *NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with water.*

2. **Wash Buffer Preparation**

   5 ml vial Wash Buffer concentrate (400X) (96-well kit; Item No. 400062): Dilute to a total volume of 2 liters 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; Item No. 400062): Dilute to a total volume of 5 liters with UltraPure water and add 2.5 ml of Polysorbate 20 (Item No. 400035).

Smaller volumes of Wash Buffer can be prepared by diluting the Wash Buffer concentrate 1:400 and adding Polysorbate 20 (0.5 ml/liter of Wash Buffer).

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

This assay has been validated for urine and plasma. Proper sample storage and preparation are essential for consistent and accurate results. Please read this section thoroughly before beginning 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.

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 -80°C.
- Samples of mouse origin may contain antibodies which 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.

Urine

Urine samples should be stored at -20°C immediately after collection. Interference in urine is infrequent; dilutions appropriate for this assay (*i.e.*, dilutions falling between 20-80% B/B_0) show a direct linear correlation between cortisol immunoreactivity and cortisol concentration (see Figure 3, on page 15). As with any urinary marker, we recommend standardizing the values obtained by ELISA to creatinine levels. Creatinine (urinary) Assay Kit may be purchased from Cayman (Item No. 500701).

Plasma

Collect blood in tubes containing either heparin or sodium citrate. 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. Process following the **Plasma Extraction Protocol** beginning on page 16.

Sample Purification

Urine may be diluted with ELISA Buffer and added directly to the assay well. Heterogenous mixtures such as lavage fluids and aspirates often contain contaminants which interfere in the assay. The presence of mouse IgG in the sample may cause interference in the assay. It is best to check for interference embarking on a large number of sample measurements. To test for interference, dilute one or two test samples to obtain at least two different dilutions of each sample between approximately 35 and 1,000 pg/ml (*i.e.*, between ~20-80% B/B_0). If the two dilutions of each sample show good correlation (differ by 20% or less) in the final calculated cortisol concentration, purification is not required. If you do not see good correlation of the different dilutions, purification is advised.

Urine

Generally speaking, urine can be used in this assay without prior purification. If purification is desired, the protocol described for plasma, on page 16, may be used.

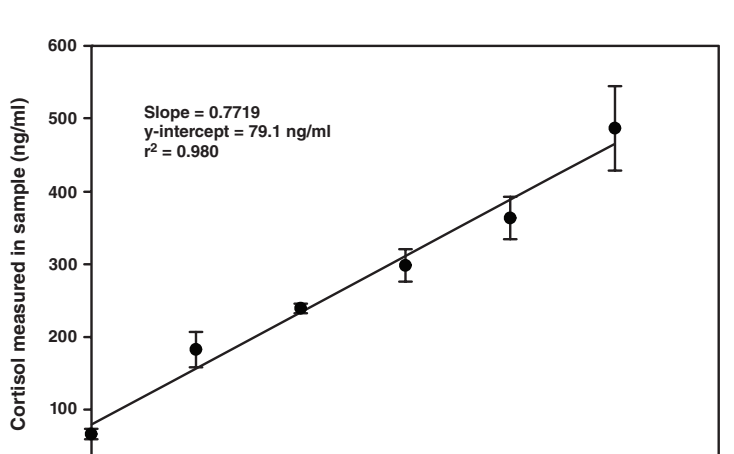


Figure 3. Recovery of cortisol from urine
Urine samples were spiked with cortisol, diluted as described in the **Sample Preparation** section, on page 13, and analyzed using the Cortisol ELISA Kit. The y-intercept corresponds to the amount of cortisol is unspiked urine. Error bars represent standard deviations obtained from multiple dilutions of each sample.

Plasma Extraction Protocol

The following protocol is a suggestion only. You may choose a different protocol based on your own requirements, sample type, and expertise. If desired, recovery may be tracked by spiking samples with tritium-labeled cortisol ([^{3}H]-cortisol) and follow the spiked-sample recovery calculations in the **Analysis** section, on page 27. Otherwise, omit steps 2 and 7.

Materials Needed

1. Tritium-labeled cortisol (optional)
2. 3 M hydrochloric acid and methylene chloride

NOTE: We do not recommend the use of plastic vials or caps for this procedure. The methylene chloride may extract interfering compounds from the plastic.

1. Aliquot a known amount of each sample into a clean test tube (500 µl is recommended). If your samples need to be concentrated, a larger volume should be used (*e.g.*, a 5 ml sample will be concentrated by a factor of 10, a 10 ml sample will be concentrated by a factor of 20, etc.).
2. Add 10,000 cpm of tritium-labeled cortisol ([^{3}H]-cortisol). Use a high specific activity tracer to minimize the amount of radioactive cortisol as the ELISA will be able to detect the added cortisol.
3. Adjust the pH of the samples to 1.5-2 by the addition of a few drops of 3 M HCl.
4. Add 4X the sample volume of methylene chloride and mix thoroughly with a vortexer. Allow layers to separate. Transfer the methylene chloride (lower) layer to a clean tube using a transfer pipette. Repeat this extraction procedure three times.*
5. Evaporate the methylene chloride by heating to 30°C under a gentle stream of nitrogen.
6. Dissolve the extract in 0.5 ml of ELISA Buffer. Use this for ELISA analysis.
7. Use 50 µl of this reconstituted sample for scintillation counting. The remainder is available for use in the ELISA.

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

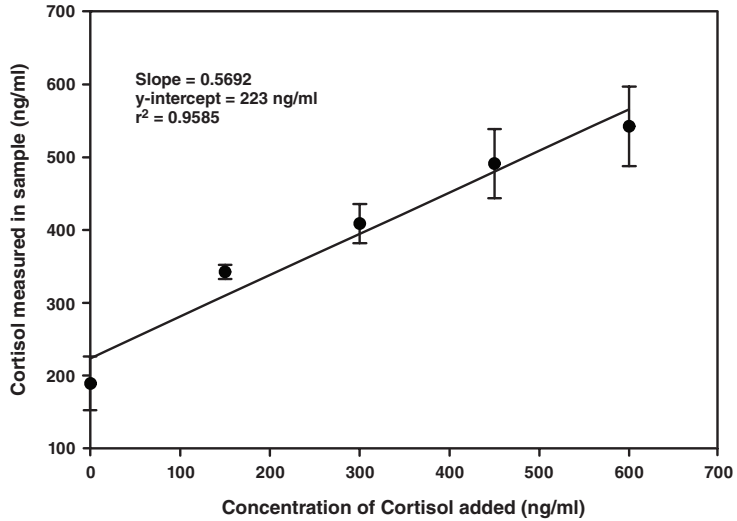


Figure 4. Recovery of cortisol from plasma
Plasma samples were spiked with cortisol, purified as described in the **Sample Purification** section, on page 13, and analyzed using the Cortisol ELISA Kit. The y-intercept corresponds to the amount of cortisol is unspiked plasma. Error bars represent standard deviations obtained from multiple dilutions of each sample.

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

Cortisol ELISA Standard

Equilibrate a pipette tip in ethanol by repeatedly filling and expelling the tip with ethanol several times. Using the equilibrated pipette tip, transfer 100 µl of the Cortisol ELISA Standard (Item No. 400364) 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. It will be stable for at least six weeks.

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

To prepare the standard for use in ELISA: Obtain eight clean test tubes and number them #1 through #8. Aliquot 900 µl ELISA Buffer to tube #1 and 600 µl ELISA Buffer to tubes #2-8. Transfer 100 µl of the bulk standard (40 ng/ml) to tube #1 and mix thoroughly. The concentration of this standard, the first point on the standard curve, will be 4 ng/ml (4,000 pg/ml). Serially dilute the standard by removing 400 µl from tube #1 and placing 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.

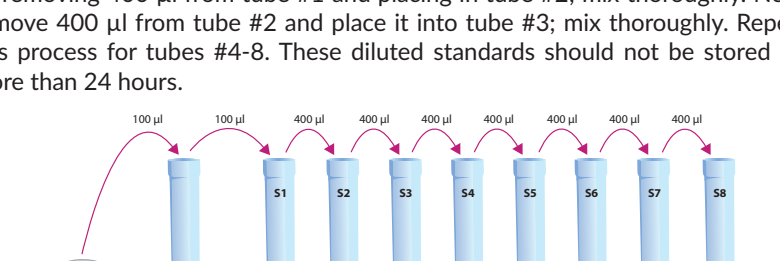


Figure 5. Preparation of the cortisol standards

Cortisol AChE Tracer

Reconstitute the Cortisol AChE Tracer as follows:

100 dtn Cortisol AChE Tracer (96-well kit; Item No. 10005272): Reconstitute with 6 ml ELISA Buffer.

OR

500 dtn Cortisol AChE Tracer (480-well kit; Item No. 10005272): Reconstitute with 30 ml ELISA Buffer.

Store the reconstituted Cortisol AChE Tracer at 4°C *(do not freeze!)* and use within 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 as follows:

100 dtn Cortisol ELISA Monoclonal Antibody (96-well kit; Item No. 400362): Reconstitute with 6 ml ELISA Buffer.

OR

500 dtn Cortisol ELISA Monoclonal Antibody (480-well kit; Item No. 400362): Reconstitute with 30 ml ELISA Buffer.

Store the reconstituted Cortisol ELISA Monoclonal Antibody at 4°C. 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 blanks (Blk), two non-specific binding wells (NSB), two maximum binding wells (B_0), 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 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, below. 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 25, for more details). We suggest you record the contents of each well on the template sheet provided (see page 34).

	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 to NSB wells. Add 50 µl ELISA Buffer to B_0 wells. If culture medium was used to dilute the standard curve, substitute 50 µl of culture medium for ELISA Buffer 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 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 two 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, the NSB, and the Blk wells.

Well	ELISA Buffer	Standard/Sample	Tracer	Antibody
Blk	-	-	-	-
TA	-	-	5 µl (at devl. step)	-
NSB	100 µl	-	50 µl	-
B_0	50 µl	-	50 µl	50 µl
Std/Sample	-	50 µl	50 µl	50 µl

Table 1. Pipetting summary

Incubation of the Plate

Cover each plate with plastic film (Item No. 400012) and incubate overnight at 4°C.

Development of the Plate

1. Reconstitute Ellman's Reagent immediately before use (20 ml of reagent is sufficient to develop 100 wells):

   100 dtn vial Ellman's Reagent (96-well kit; Item No. 400050): Reconstitute with 20 ml of UltraPure water.

   OR

   250 dtn vial Ellman's Reagent (480-well kit; Item No. 400050): Reconstitute 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 Wash Buffer.
3. Add 200 µl of Ellman's Reagent to each well.
4. Add 5 µl of tracer to the TA wells.
5. Cover the plate with plastic film. 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. This assay typically develops (*i.e.*, B_0 wells ≥0.3 A.U. (blank 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 plate cover being careful to keep Ellman's Reagent from splashing on the cover. *NOTE: Any loss of Ellman's Reagent will affect the absorbance readings. If Ellman's Reagent is present on the cover, use a pipette to transfer the Ellman's Reagent into the well. If too much Ellman's Reagent has splashed on the cover to easily redistribute back into the wells, wash the plate three times with Wash Buffer and repeat the development with fresh Ellman's Reagent.*
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. (blank subtracted). The plate should be read when the absorbance of the B_0 wells are in the range of 0.3-1.5 A.U. (blank subtracted). If the absorbance of the wells exceeds 2.0, 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/analysis/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 blank 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; the corrected B_0 divided by the actual TA (10X measured absorbance) will give the %Bound. This value should closely approximate the %Bound that can be calculated from the ***Sample Data*** *(see page 28). Erratic absorbance values and a low (or no) %Bound could indicate the presence of organic solvents in the buffer or other technical problems (see page 32 for* ***Troubleshooting****).*

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 4-parameter logistic fit.

Alternative Plot - The data can also be lineraized 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 using the equation obtained from the standard curve plot. *NOTE: Remember to account for any concentration or dilution of the sample prior to the 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.

Spiked-Sample Recovery Calculation

$$\text{Recovery Factor} = \frac{\text{10X cpm of sample}}{[^3\text{H}]\text{-Cortisol added to sample}}$$

$$\text{Cortisol (pg) in purified sample} = \left[\frac{\text{Value from ELISA (pg/ml)}}{\text{Recovery Factor}}\right] \times 0.5 \text{ ml}^* - \text{added } [^3\text{H}]\text{-cortisol (pg)}$$

For liquid samples:

$$\text{Cortisol in sample (pg/ml)} = \frac{\text{Cortisol (pg) in purified sample}}{\text{Volume of sample used for purification (ml)}}$$

For solid samples:

$$\text{Cortisol in sample (pg/g)} = \frac{\text{Cortisol (pg) in purified sample}}{\text{Weight of sample (g)}}$$

*Volume of reconstituted sample after purification; adjust this number accordingly if a different volume of ELISA Buffer was used to reconstitute the sample.

Performance Characteristics

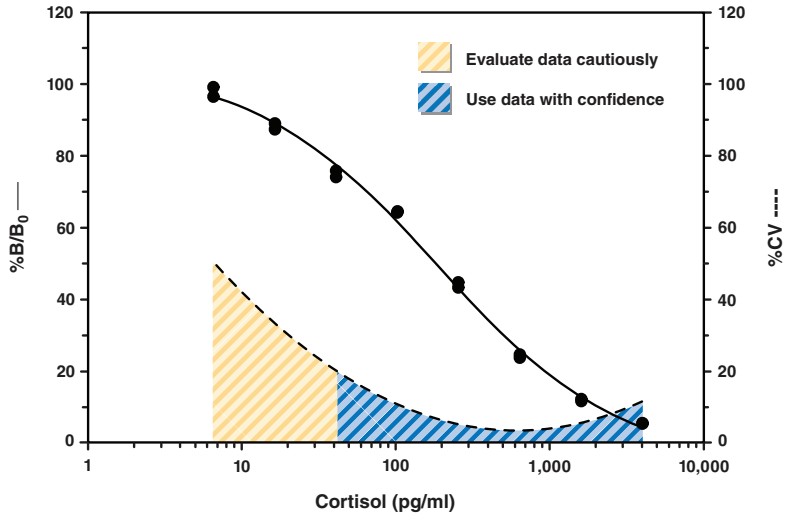
Sample Data

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. Your results could differ substantially.

	Raw Data		Average	Corrected
Total Activity	0.935	0.911	0.923	
NSB	0.000	0.000	0.000	
B_0	1.104	1.077		
	1.038	1.159	1.095	1.095

Dose (pg/ml)	Raw Data		Corrected		%B/B_0	
4,000	0.060	0.061	0.060	0.061	5.5	5.5
1,600	0.129	0.134	0.129	0.134	11.7	12.2
640	0.271	0.261	0.271	0.261	24.7	23.8
256	0.491	0.474	0.491	0.474	44.8	43.2
102.4	0.703	0.705	0.703	0.705	64.2	64.4
41.0	0.831	0.811	0.831	0.811	75.9	74.1
16.4	0.957	0.974	0.957	0.974	87.4	88.9
6.6	1.085	1.056	1.085	1.056	99.1	96.4

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) = 150-210 pg/ml

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

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 29 and in the table below.

Dose (pg/ml)	%CV* Intra-assay variation	%CV* Inter-assay variation
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	†	20.1
6.6	†	†

Table 3. Intra- and inter-assay variation

*%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-Deoxycorticosterone	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-Hydroxypregnenolone	<0.01%
Pregnenolone	<0.01%
Testosterone	<0.01%

Table 4. Cross Reactivity of the Cortisol ELISA

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique	A. Replace activated carbon filter or change source of UltraPure water
High NSB (>10% of B_0)	A. Poor washing B. Exposure of NSB wells to specific antibody	A. Re-wash plate and redevelop
Very low B_0	A. Trace organic contaminants in the water source B. Plate requires additional development time C. Dilution error in preparing reagents	A. Replace activated carbon filter or change source of UltraPure water B. Return plate to shaker and re-read later
Low sensitivity (shift in dose response curve)	Standard is degraded	Replace standard
Analyses of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference)	Interfering substances are present	Purify sample prior to analysis by ELISA[12]
Only Total Activity (TA) wells develop	Trace organic contaminants in the water source	Replace activated carbon filter or change source of UltraPure water

Additional Reading

Go to **www.caymanchem.com/500360/references** for a list of publications citing the use of Cayman's Cortisol ELISA Kit.

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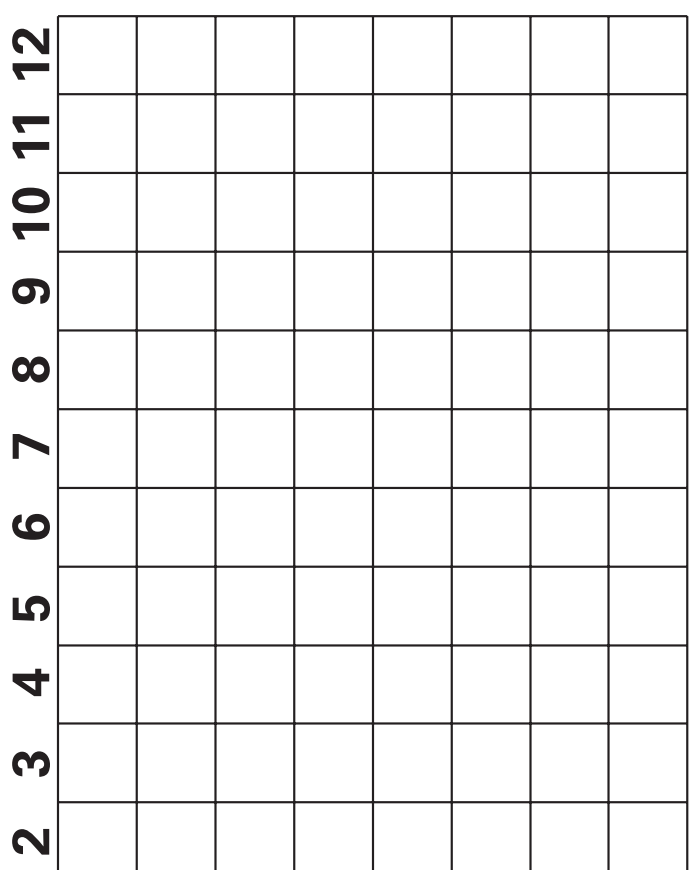

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.