11-Oxoetiocholanolone ELISA Kit

Item No. 501420

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size
401422	11-Oxoetiocholanolone ELISA Antiserum	1 vial/100 dtn
401420	11-Oxoetiocholanolone AP Tracer	1 vial/100 dtn
401424	11-Oxoetiocholanolone ELISA Standard	1 vial
400080	Tris Buffer Concentrate (10X)	1 vial/10 ml
411007	AP Wash Buffer Concentrate (150X)	1 vial/5 ml
400004/400006	Mouse Anti-Rabbit IgG Coated Plate	1 plate
400012	96-Well Cover Sheet	1 cover
400089	*p*NPP Substrate Solution	2 vials/12 ml
400040	ELISA Tracer Dye	1 vial
400042	ELISA Antiserum Dye	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 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: 734-971-3640

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 at 405 nm.
2. Adjustable pipettes and a repeating pipettor.
3. A source of pure water; glass distilled water or deionized water is acceptable. *NOTE: UltraPure water is available for purchase from Cayman (Item No. 400000).*
4. Materials used for **Sample Preparation** (see page 11).

INTRODUCTION

Background

11-Oxoetiocholanolone is a metabolite of cortisol that is found in the feces of ungulates and other animals. Stress-induced glucocorticoids (cortisol, corticosterone) traditionally have been measured through blood sampling, but the collection process is known to cause stress in animals, interfering with experimental results.[1] Urine, saliva, and fecal sampling have been explored as alternative non-invasive methods.[2] Fecal sampling is the most widely used for the analysis of glucocorticoid metabolites as it eliminates the need for direct contact with animals.[1] Fecal sample analysis provides more accurate detection of stress hormones, and it can be used to measure stress in both laboratory and free-ranging animals.[1] The levels of glucocorticoid metabolites can vary between species and between sexes, due to differences in metabolism and excretion, so it is necessary to determine baseline levels for each species and sex before using in a study.[1] Glucocorticoid metabolites in feces have been analyzed in ungulates and other animals, including the Syrian hamster, brown lemur and black howler monkey.[1,3,4]

11-Oxoetiocholanolone is also found in human urine. Increased levels of urinary 11-oxoetiocholanolone have been observed in the latter part of pregnancy and in patients with uterine leiomyomas.[5,6] A study of postmenopausal women determined that 11-oxoetiocholanolone was indicative of stress-induced urinary incontinence.[7]

About This Assay

Cayman's 11-Oxoetiocholanolone ELISA Kit is a competitive assay that can be used for quantification of 11-oxoetiocholanolone in ungulate feces and human urine. The ELISA typically displays a 50% B/B_0 of approximately 1.8 ng/ml and sensitivity (80% B/B_0) of approximately 0.2 ng/ml.

Principle of the Assay

This assay is based on the competition between 11-oxoetiocholanolone and a 11-oxoetiocholanolone alkaline phosphatase (AP) conjugate (11-oxoetiocholanolone tracer) for a limited amount of 11-oxoetiocholanolone polyclonal antibody. The concentration of the 11-oxoetiocholanolone tracer is held constant while the concentration(s) of free 11-oxoetiocholanolone (standard or samples) varies. Thus, the amount of 11-oxoetiocholanolone tracer that is bound to the 11-oxoetiocholanolone polyclonal antibody will be inversely proportional to the concentration of free 11-oxoetiocholanolone in the standard or sample well. This antibody-11-oxoetiocholanolone (either free or tracer) complex binds to mouse monoclonal anti-rabbit IgG that has been previously attached to the well. After incubation the plate is washed to remove any unbound reagents and pNPP substrate solution is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 405 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of 11-oxoetiocholanolone tracer bound to the well, which is inversely proportional to the amount of free 11-oxoetiocholanolone present in the well during the incubation; or

$$\text{Absorbance} \propto [\text{Bound 11-oxoetiocholanolone Tracer}] \propto 1/[\text{11-oxoetiocholanolone}]$$

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

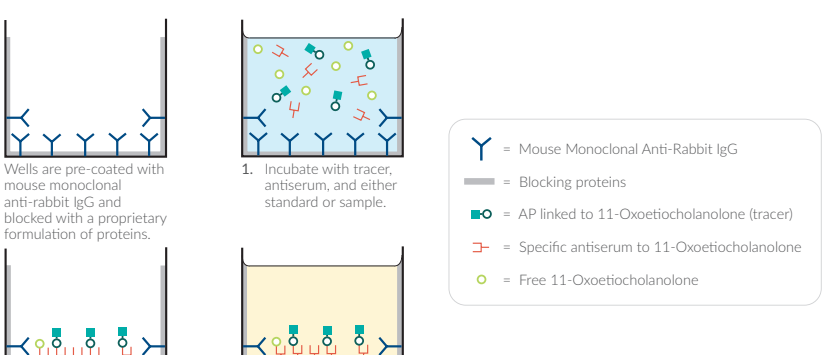


Figure 1. Schematic of the ELISA

Definition of Key Terms

Blank: background absorbance caused by pNPP Substrate Solution. The blank absorbance should be subtracted from the absorbance readings of all the other wells.

Total Activity (TA): total enzymatic activity of the alkaline phosphatase-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 particular sample or standard well to that of the maximum binding (B_0) well.

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:

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

Lower Limit of Detection (LLOD): the smallest amount of analyte that can be detected with reasonable certainty for a given analytical procedure. The LLOD is defined as a point two standard deviations away from the mean zero value.

PRE-ASSAY PREPARATION

Buffer Preparation

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

1. **Tris Assay Buffer Preparation**

   Dilute the contents of one vial of Tris Buffer Concentrate (10X) (Item No. 400080) with 90 ml of pure 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. **AP Wash Buffer (1X) Preparation**

   Dilute 5 ml of AP Wash Buffer Concentrate (150X) (Item No. 411007) to a total volume of 750 ml with pure water. Smaller volumes of AP Wash Buffer (1X) can be prepared by diluting the AP Wash Buffer Concentrate 1:150 in pure water.

Sample Preparation

We recommend purification of urine and fecal samples prior to the assay. Below is our recommended purification protocol. You may choose a different method based on your own requirements and experimental design.

Urine

1. Prepare 1.74M acetic acid by adding 1 ml of glacial acetic acid to 9 ml of water.
2. Add 4 µl of 1.74M acetic acid to 500 µl of human urine and vortex gently.
3. Add 1.5 ml of ethyl acetate to the urine sample, vortex and incubate at room temperature for 10 minutes.
4. Transfer upper (ethyl acetate) layer into a clean test tube.
5. Repeat steps 3 and 4 twice, combining all ethyl acetate fractions into one tube.
6. Evaporate ethyl acetate under a gentle stream of nitrogen, heating sample to 37°C or without heat.
7. Reconstitute in 500 µl of assay buffer. Centrifuge at 3000 x g for 5 min if necessary, to remove particles from the solution. Transfer buffer fraction into a clean test tube and analyze in the assay.

Feces

1. Lyophilize feces to remove water.
2. Place 50 mg of lyophilized feces into a clean centrifuge tube, add 500 µl of water and vortex thoroughly.
3. Prepare 1.74M acetic acid by adding 1 ml of glacial acetic acid to 9 ml of water.
4. Add 4 µl of 1.74M acetic acid to the fecal sample and vortex gently.
5. Add 800 µl of ethyl acetate, vortex and incubate at room temperature for 10 minutes.
6. Centrifuge at 3000 x g for 5 minutes.
7. Transfer upper (ethyl acetate) layer into a clean test tube.
8. Repeat steps 5 and 6 four times, combining all ethyl acetate fractions into one tube.
9. Evaporate ethyl acetate under a gentle stream of nitrogen, heating sample to 37°C or without heat.
10. Reconstitute in 500 µl of assay buffer. Centrifuge at 3000 x g for 5 min if necessary, to remove particles from the solution. Transfer buffer fraction into a clean test tube and analyze in the assay.

Testing for Interference

This assay has been tested using ungulate feces and human urine. Other sample types will need to be assessed for interference by the end user. To test for interference, dilute one or two test samples to obtain at least two different dilutions of each sample between approximately 0.3 and 15 ng/ml (i.e., between 25-75% B/B_0, which is the linear portion of the standard curve). If the two different dilutions of the sample show good correlation (differ by 20% or less) in the final calculated 11-oxoetiocholanolone concentration, extraction is not required. If you do not see good correlation of the different dilutions, sample extraction is advised. Extraction methods will need to be determined by the end user and tested for compatibility in the assay.

Sample Matrix Properties

Linearity

Sheep feces were spiked at 1500 ng/ml, extracted as described in the **Sample Preparation** section, on Page 12, then diluted with Tris Assay buffer and analyzed in the assay. A recovery calculated for each dilution is shown in the table below.

Dilution Factor	Measured 11-Oxoetiocholanolone (ng/ml), dilution adjusted	% Recovery
400	1,618.4	108
800	1,692.9	113
1600	1,566.9	104
3200	1,691.8	113

Table 1. Dilutional linearity in sheep feces

Spike and Recovery

Sheep feces and human urine were spiked with 11-oxoetiocholanolone, then extracted as described in the **Sample Preparation** section, on pages 11 and 12. Each spiked sample was tested in four serial dilutions and analyzed using the 11-Oxoetiocholanolone ELISA Kit. The results are shown on page 16 and 17.

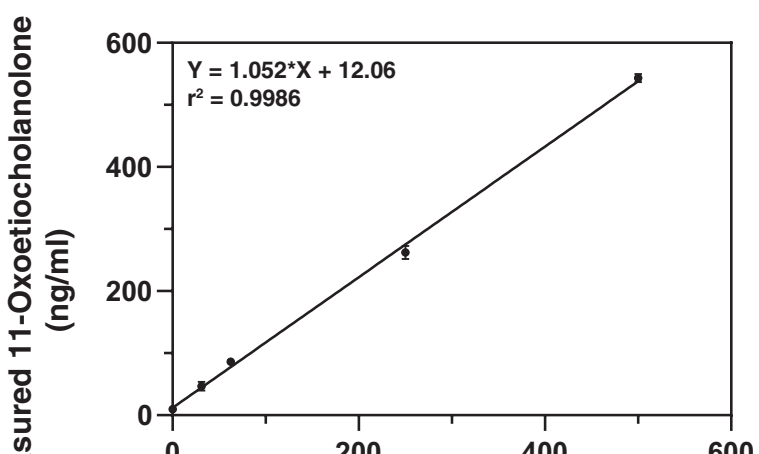


Figure 2. Spike and Recovery of 11-oxoetiocholanolone from human urine. Error bars represent standard deviations obtained from multiple dilutions of each sample

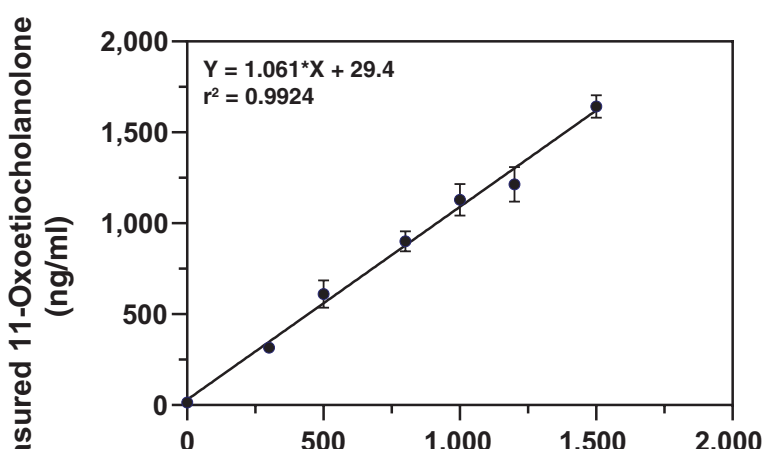


Figure 3. Spike and Recovery of 11-oxoetiocholanolone from sheep feces. Error bars represent standard deviations obtained from multiple dilutions of each sample

Parallelism

To assess parallelism, purified sheep feces and human urine were measured at multiple dilutions with the 11-Oxoetiocholanolone ELISA Kit. Concentrations were plotted as a function of sample dilution factor. The results are shown below.

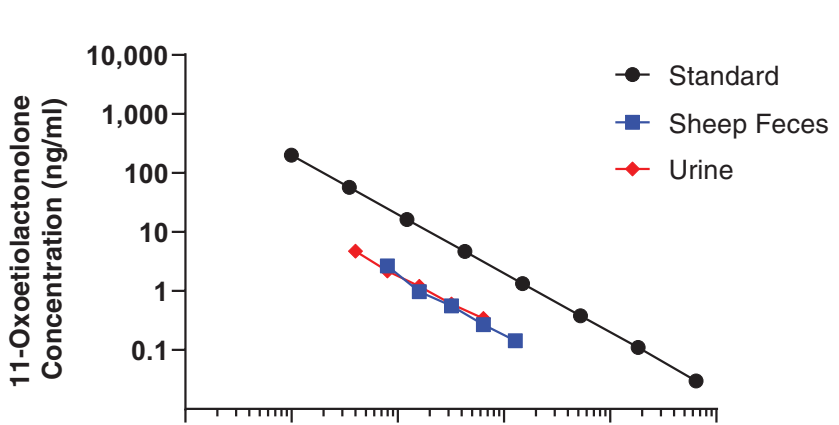


Figure 4. Parallelism of sample matrices in the 11-Oxoetiocholanolone ELISA Kit

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

11-Oxoetiocholanolone ELISA Standard

The concentration of the 11-Oxoetiocholanolone ELISA Standard (Item No. 401424) is 20 µg/ml in methanol.

To prepare the standard for use in ELISA: Obtain eight clean test tubes and number them #1 through #8. Aliquot 1.98 ml Tris Buffer to tube #1 and 500 µl Tris Buffer to tubes #2-8. Using the equilibrated pipette tip, transfer 20 µl of the standard (20 µg/ml) to tube #1 and mix thoroughly. The concentration of this standard, the first point on the standard curve, will be 200 ng/ml. Serially dilute the standard by removing 200 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 200 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.

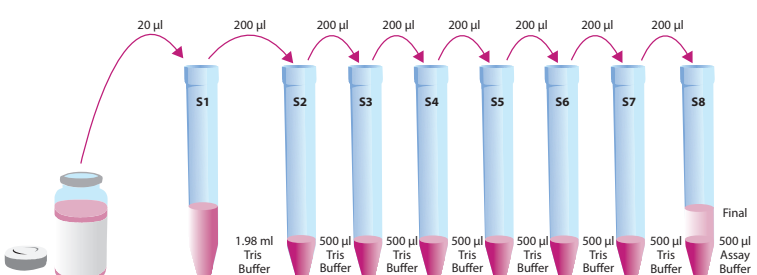


Figure 5. Preparation of the 11-oxoetiocholanolone standards

11-Oxoetiocholanolone AP Tracer (Item No. 401420)

Reconstitute 100 dtn vial with 6 ml Tris Buffer

Store the reconstituted 11-Oxoetiocholanolone AP Tracer at 4°C *(do not freeze!)* and use within two 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). *NOTE: Do not store tracer with dye for more than 24 hours.*

11-Oxoetiocholanolone ELISA Antibody (item No. 401422)

Reconstitute 100 dtn vial with 6 ml Tris Buffer.

Store the reconstituted 11-Oxoetiocholanolone ELISA Antibody at 4°C. It should be stable for at least two weeks. A 20% surplus of antibody has been included to account for any incidental losses.

Antibody Dye Instructions (optional)

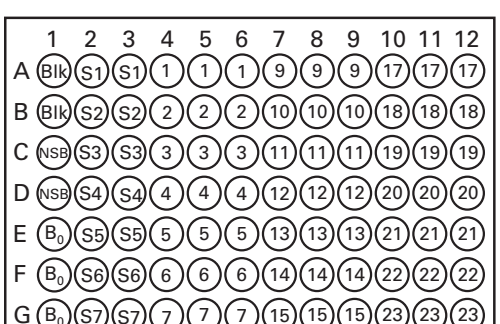
This dye may be added to the antibody, if desired, to aid in visualization of antibody-containing wells. Add the dye to the reconstituted antibody at a final dilution of 1:100 (add 60 µl of dye to 6 ml antibody).

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 below in Figure 5. 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 Chemical (see **Analysis**, page 26, for more details). We suggest you record the contents of each well on the template sheet provided (see page 34).



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.
- Do not expose the pipette tip to the reagent(s) already in the well.

Addition of the Reagents

1. **Tris Buffer**

   Add 100 µl Tris Buffer to Non-Specific Binding (NSB) wells. Add 50 µl Tris Buffer to Maximum Binding (B_0) wells.

2. **11-Oxoetiocholanolone 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.

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. **11-Oxoetiocholanolone AP Tracer**

   Add 50 µl to each well *except* the Total Activity (TA) and the Blank (Blk) wells.

5. **11-Oxoetiocholanolone ELISA Antibody**

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

Incubation of the Plate

Cover each plate with plastic film (Item No. 400012) and incubate overnight (18-24 hours) at 4°C.

Development of the Plate

1. Empty the wells and rinse five times with Wash Buffer.
2. Add 200 µl of *p*NPP substrate Solution to each well.
3. Add 5 µl of tracer to the TA well.
4. Cover the plate with plate cover (Item No. 400012) and place on an orbital shaker. Optimum development is obtained 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 *p*NPP Substrate Solution from splashing on the cover. *NOTE: Any loss of pNPP Substrate Solution will affect the absorbance readings.*
3. Read the plate at a wavelength of 405 nm.

ANALYSIS

Many plate readers come with data reduction software that plots 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.

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.)

Plot the Standard Curve

Plot $\%B/B_0$ for standards S1-S8 *versus* 11-oxoetiocholanolone 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 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 sample prior to the addition to the well.* Samples with $\%B/B_0$ values greater than 75% or less than 25% 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 standard curve.

Performance Characteristics

Representative Data

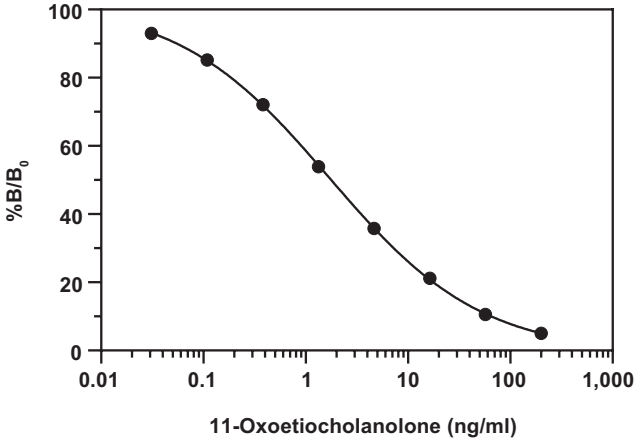
The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the value of your samples.

Absorbance at 405 nm at 90 minutes

11-Oxoetiochlanolone Standards (ng/ml)	Blank Corrected Absorbance	NSB Corrected Absorbance	%B/B_0	%CV Intra-assay Variance	%CV Inter-assay Variance
NSB	0.003	--	--	--	--
B_0	0.773	0.769	--	--	--
Total Activity	2.607	--	--	--	--
200	0.042	0.039	5.0	13.6	13.8
57.14	0.084	0.081	10.6	8.6	7.7
16.33	0.166	0.163	21.2	7.4	7.6
4.67	0.279	0.276	35.8	8.4	5.4
1.33	0.418	0.415	53.9	11.4	5.4
0.38	0.557	0.554	72.1	15.0	9.5
0.11	0.655	0.655	85.2	†	7.9
0.03	0.717	0.714	93.0	†	9.2

Table 2. Typical results

†Outside of the recommended usable range of the assay



Assay Range = 0.031-200 ng/ml
Sensitivity (defined as 80% B/B_0) = 0.186 ng/ml
Mid-point (defined as 50% B/B_0) = 1.778 ng/ml
Lower Limit of Detection (LLOD) = 0.046 ng/ml

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

Figure 7. Typical standard curve

Precision:

Intra-assay precision was determined by analyzing 24 replicates of three urine controls (extracted as described in the **Sample Preparation** section, on page 11) in a single assay.

Matrix Control (ng/ml)	%CV
1,496.0	17.6
627.7	11.8
100.9	11.4

Table 3. Intra-assay precision

Inter-assay precision was determined by analyzing replicates of three urine controls (extracted as described in the **Sample Preparation** section, on page 11) in separate assays on different days.

Matrix Control (ng/ml)	%CV
1,376.3	10.9
619.0	9.3
111.9	9.4

Table 4. Inter-assay precision

Cross Reactivity:

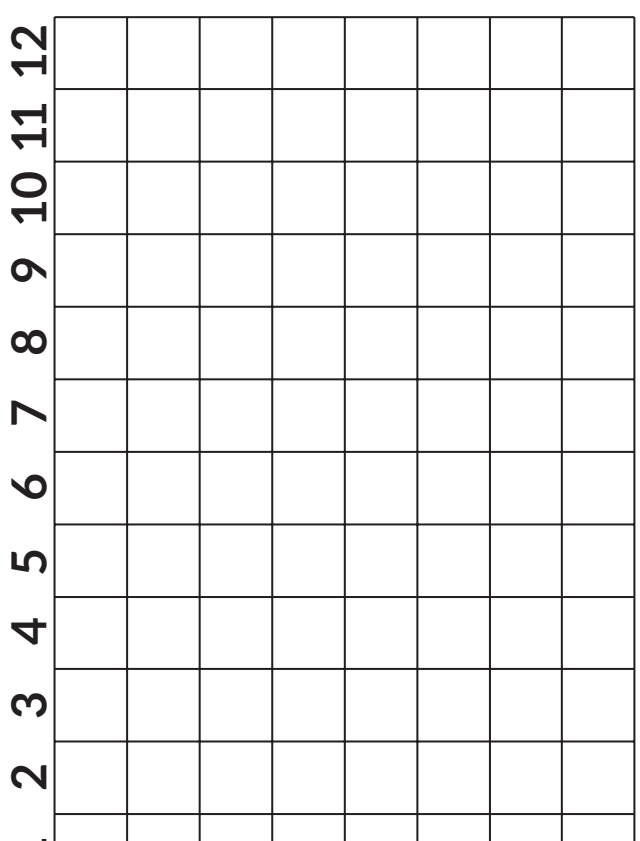
Compound	Common Name	Cross Reactivity
5β-Androstan-3α-ol-11,17-dione	11-keto Etiocholanolone	100%
5β-Androstan-3,11,17-trione		54.7%
5α-Androstan-3α-ol-11,17-dione	11-keto Androsterone	2.56%
5α-Androstan-3,11,17-trione		2.49%
5α-Androstan-3β-ol-11,17-dione	11-keto Epiandrosterone	1.4%
5β-Androstan-3α,11β-diol-17-one	11β-hydroxy Etiocholanolone	0.12%
5α-Androstan-3α-17β-diol	17β-dihydro Androsterone	<0.01%
5α-Androstan-3β-17β-diol	17β-dihydro Epiandrosterone	<0.01%

Table 5. Cross reactivity of the 11-Oxoetiocholanolone ELISA

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)	Standard is degraded or contaminated
Analyses of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference)	Interfering substances are present - consider sample purification prior to analysis



Procedure	Blk	TA	NSB	B_0	Standards/ Samples
Reconstitute and Mix	Mix all reagents gently				
Tris Buffer (1X)	--	--	100 µl	50 µl	--
Standards/Samples	--	--	--	--	50 µl
11-Oxoetiocholanolone AP Tracer	--	--	50 µl	50 µl	50 µl
11-Oxoetiocholanolone ELISA Antibody	--	--	--	50 µl	50 µl
Seal	Seal the plate and tap gently to mix				
Incubate	Incubate plate overnight at 4C				
Aspirate	Aspirate wells and wash 5 x ~300 µl with 1X AP Wash Buffer				
Apply *p*NPP Substrate	200 µl	200 µl	200 µl	200 µl	200 µl
TA - Apply Tracer	--	5 µl	--	--	--
Seal	Seal plate and incubate for 90 minutes at room temperature on orbital shaker, protect from light				
Read	Read O.D. at 405 nm				

References

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NOTES

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