Estradiol ELISA Kit

Item No. 501890

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size	480 wells Quantity/Size
501891	Estradiol ELISA Antiserum	1 vial/100 dtn	1 vial/500 dtn
501892	Estradiol AChE Tracer	1 vial/100 dtn	1 vial/500 dtn
501893	Estradiol 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
400004/400006	Mouse Anti-Rabbit IgG-Coated Plate	1 plate	5 plates
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
400012	96-Well Cover Sheet	1 ea	5 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

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 414 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 10)

INTRODUCTION

Background

Estradiol is a steroid hormone produced from testosterone *via* the aromatase system in the granulosa cells of ovarian follicles.[1,2] It is instrumental in the development of secondary sex characteristics at puberty and in the menstrual cycle.[3-5] Plasma levels of estradiol peak during the follicular phase of the menstrual cycle to approximately 300 pg/ml.[1,4,5] During this time, it stimulates proliferation of granulosa cells, increases the size of uterine glands, and exerts positive feedback on the hypothalamus, leading to an increase in luteinizing hormone.[5,6] Blood levels of estradiol drop as luteinizing hormone levels increase and trigger ovulation, then rise again during the luteal phase to approximately 100 pg/ml.[4] Estradiol is metabolized into estrone by 17β-hydroxysteroid dehydrogenase 2 and hydroxylated metabolites such as estriol, as well as glucuronidated and sulfonated metabolites, which are excreted in the urine and feces.[7]

About This Assay

Cayman's Estradiol ELISA Kit is a competitive assay that can be used for quantification of estradiol in plasma and serum. The assay has a range of 0.61-10,000 pg/ml and a sensitivity (80% B/B_0) of approximately 20 pg/ml.

Principle of the Assay

This assay is based on the competition between native estradiol and an estradiol acetylcholinesterase (AChE) conjugate (Estradiol AChE Tracer) for a limited amount Estradiol Antiserum. Because the concentration of the Estradiol AChE Tracer is held constant while the concentration of native estradiol varies, the amount of Estradiol AChE Tracer that is able to bind to the Estradiol Antiserum will be inversely proportional to the concentration of native estradiol in the well. This antibody-estradiol complex binds to mouse monoclonal anti-rabbit 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 414 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of Estradiol AChE Tracer bound to the well, which is inversely proportional to the amount of native estradiol present in the well during the incubation, as described by the equation:

Absorbance ∝ [Bound Estradiol AChE Tracer] ∝ 1/[Estradiol]

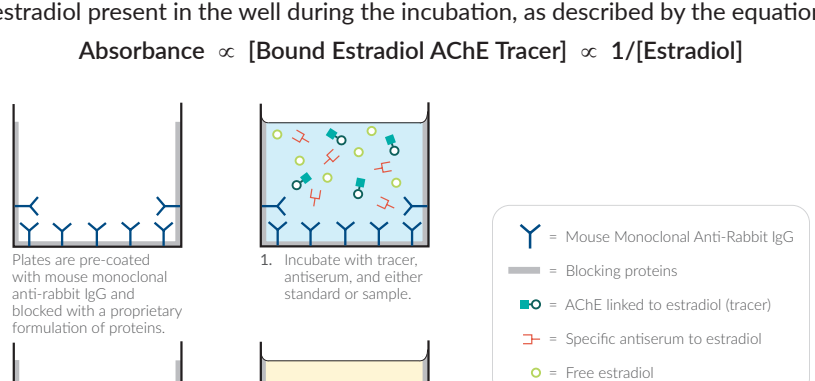


Figure 1. Schematic of the ELISA

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 the non-specific binding (NSB) wells.

Total Activity: total enzymatic activity of the 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 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:

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

Lower Limit of Detection (LLOD): the smallest measure 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 should be stable for approximately two months.

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

   Dilute the contents of one vial of ELISA Buffer Concentrate (10X) (Item No. 400060) 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 pure water.*

2. **Wash Buffer (1X) Preparation**

   Dilute the contents of one vial of Wash Buffer Concentrate (400X) (Item No. 400062) with pure water to a total volume of 2 L and add 1 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 with pure water and adding Polysorbate 20 to an end concentration of 0.5 ml/L.

   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 tested using human plasma and serum. Other samples types should be checked 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 test samples to obtain at least two different dilutions of each sample between approximately 900 and 20 pg/ml (*i.e.*, between 30-80% B/B_0, which is the linear portion of the standard curve). The two different dilutions of the sample should show good correlation (differ by 20% or less) in the final calculated concentration.

Plasma and Serum

Plasma and serum samples may require extraction prior to quantification in the assay. Sample extraction should be performed using a method similar to the following protocol.

1. To a 300 µl sample add 1,200 µl of methanol. Mix and incubate at room temperature for 10 minutes.
2. Centrifuge at 2,000 x g for 10 minutes.
3. Carefully transfer supernatant to a clean tube and evaporate to dryness under inert gas.
4. Reconstitute with 300 µl of the assay buffer and measure immediately. *NOTE: If samples cannot be measured immediately, store at -80°C.*

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 rabbit origin may contain antibodies that interfere with the assay by binding to the mouse anti-rabbit plate. We recommend that all rabbit samples be purified prior to use in the assay.

Sample Matrix Properties

Linearity

Human plasma and serum samples were extracted, serially diluted, and evaluated for linearity using the Estradiol ELISA Kit. The results are shown in the table below.

Sample	Dilution	Measured Concentration (pg/ml)	% Recovery
Serum	1:1	435	100
	1:2	475	109
	1:4	421	97
	1:8	409	94
Plasma	1:1	805	100
	1:2	819	102
	1:4	910	113
	1:8	1,079	134

Table 1. Dilutional linearity of human plasma and serum samples

Spike and Recovery

Human plasma samples were spiked with 17β-estradiol, then extracted and diluted as described in the **Sample Preparation** section and analyzed using the Estradiol ELISA Kit. The results are shown below.

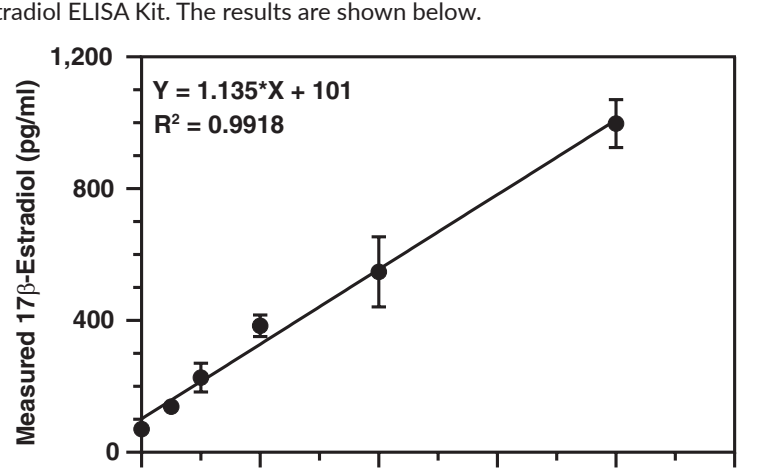


Figure 2. Spike and recovery in human plasma

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

Estradiol ELISA Standard

Obtain eight clean test tubes and label them #1-8. Aliquot 1,980 µl ELISA Buffer (1X) to tube #1 and 750 µl ELISA Buffer (1X) to tubes #2-8. Equilibrate a pipette tip by repeatedly filling and expelling the tip with Estradiol ELISA Standard (Item No. 501893) several times. Transfer 20 µl of the Estradiol ELISA standard into tube #1 and mix thoroughly. Serially dilute the standard by removing 250 µl from tube #1 and placing in tube #2. Next, remove 250 µl from tube #2 and place into tube #3, mix thoroughly. Repeat this process for tubes #4-8. These diluted standards should not be stored for more than two hours.

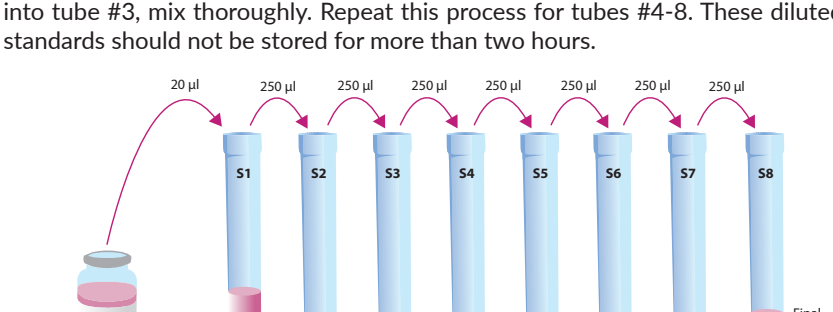


Figure 3. Preparation of the estradiol standards

Estradiol AChE Tracer

Reconstitute the 100 dtn Estradiol AChE Tracer (Item No. 501892) with 6 ml of ELISA Buffer (1X) and the 500 dtn Estradiol AChE Tracer with 30 ml ELISA Buffer.

Store the reconstituted Estradiol 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). Reconstituted tracer with added dye can be stored at 4°C for up to two weeks.

Estradiol Antiserum

Reconstitute the 100 dtn Estradiol Antiserum (Item No. 501891) with 6 ml of ELISA Buffer (1X) and the 500 dtn Estradiol Antiserum with 30 ml ELISA Buffer.

Store the reconstituted Estradiol Antiserum 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.

Antiserum Dye Instructions (optional)

This dye may be added to the antiserum, if desired, to aid in visualization of antiserum-containing wells. Add the dye to the reconstituted antiserum at a final dilution of 1:100 (add 60 µl of dye to 6 ml antiserum or add 300 µl of dye to 30 ml of antiserum). Reconstituted tracer with added dye can be stored at 4°C for up to two weeks.

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 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 4. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided on page 17 has been designed to allow for easy data analysis using a convenient spreadsheet offered by Cayman (see page 20 for more details). We suggest you record the contents of each well on the template sheet provided (see page 27).

	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 4. 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.

2. **Estradiol 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. **Estradiol AChE Tracer**

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

5. **Estradiol ELISA Antiserum**

   Add 50 µl to each well *except* the TA, NSB, and Blk wells immediately after addition of the tracer.

Incubation of the Plate

Cover each plate with a 96-Well Plate Cover Sheet (Item No. 400012) and incubate 2 hours at room temperature on an orbital shaker.

Development of the Plate

1. Reconstitute Ellman's Reagent (Item No. 400050) immediately before use. Reconstitute the 100 dtn Ellman's Reagent with 20 ml of pure water and the 250 dtn Ellman's Reagent with 50 ml pure water.
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 a 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 for 60 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.*
3. Read the plate at a wavelength of 414 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. *NOTE: Cayman Chemical 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.)

Plot the Standard Curve

Plot %B/B_0 for standards S1-S8 *versus* estradiol concentration using linear (y) and log (x) axes and perform a four-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 the concentration of the sample prior to the addition to the well.* Samples with %B/B_0 values outside of the linear portion of the standard curve should be re-assayed results. A 20% or greater disparity between the results 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

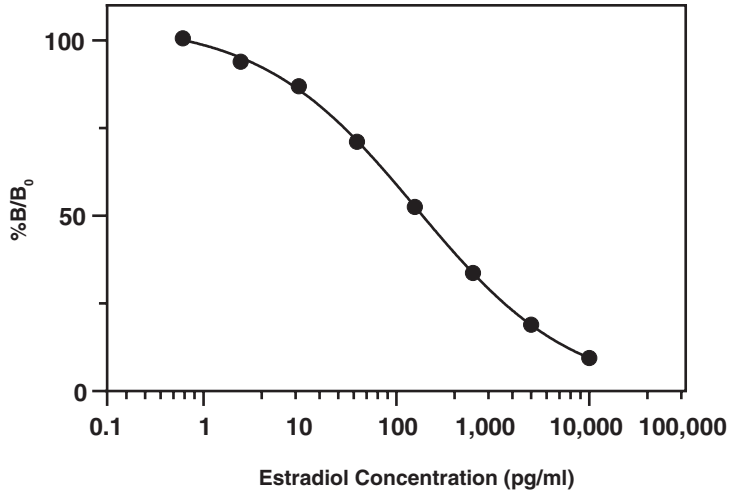
The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You **must** run a new standard curve. Do not use the data below to determine the values of your samples. **Absorbance at 414 nm (60 minutes)**

Estradiol Standards (pg/ml)	Blank-Subtracted Absorbance	NSB-Corrected Absorbance	%B/B_0	%CV Intra-Assay Precision*	%CV Inter-Assay Precision*
NSB	0.002	--	--	--	--
B_0	0.923	0.921	--	--	--
10,000.000	0.087	0.085	9.4	6.6	8.1
2,500.000	0.172	0.170	19.0	6.8	4.4
625.000	0.303	0.301	33.7	7.2	4.7
156.250	0.471	0.469	52.6	12.1	7.1
39.063	0.638	0.636	71.2	12.5	10.9
9.766	0.779	0.776	87.0	23.0†	17.5
2.441	0.840	0.838	94.0	33.8†	27.8†
0.610	0.899	0.897	100.6	60.0†	39.3†
TA	1.562	--	--	--	--

Table 3. Typical results

*%CV represents the variation in concentration (not absorbance) as determined using a reference standard curve

†Evaluate data in this range with caution



Assay Range = 0.61-10,000 pg/ml
Sensitivity (defined as 80% B/B_0) = 20 pg/ml
Mid-point (defined as 50% B/B_0) = 188 pg/ml
Lower Limit of Detection (LLOD) = 6 pg/ml

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

Figure 5. Typical standard curve for estradiol

Precision:

Intra-assay precision was determined by analyzing 24 replicates of three plasma controls in a single assay.

Plasma Control (pg/ml)	%CV
972	6.5
535	12.1
64.8	10.8

Table 4. Sample Intra-assay precision

Inter-assay precision was determined by analyzing replicates of three plasma controls in eight separate assays on different days.

Plasma Control (pg/ml)	%CV
963	12.3
429	8.4
69.1	8.0

Table 5. Sample Inter-assay precision

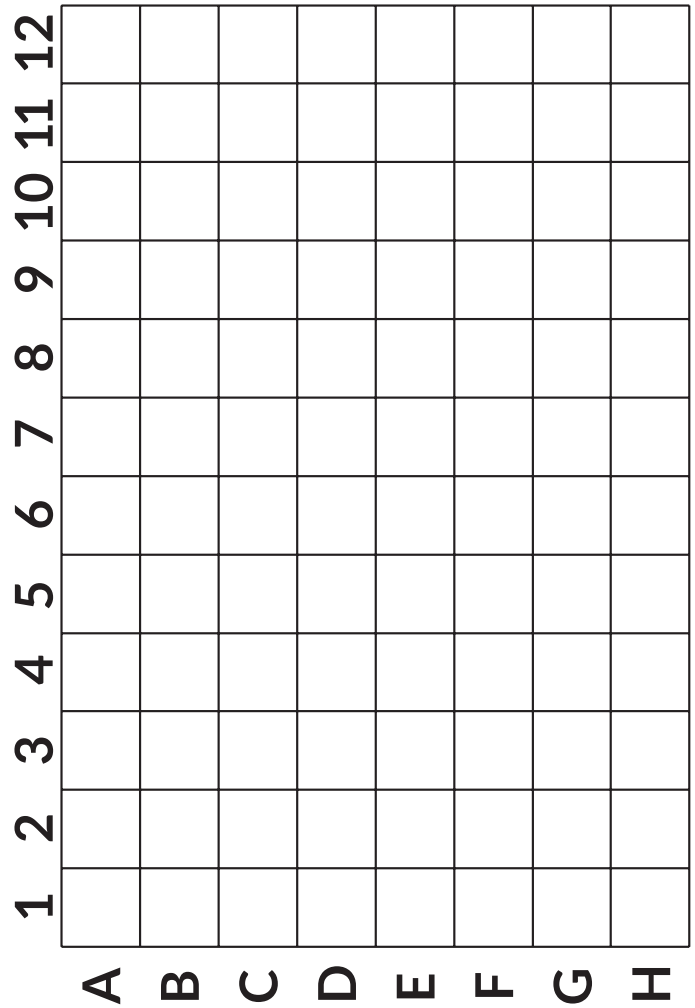
Cross Reactivity:

Compound	Cross Reactivity
Estradiol	100%
Methoxyestradiol	2.5%
Estradiol 3-(β-D-Glucuronide)	2.3%
Estrone	1.38%
2-Hydroxyestradiol	1.3%
Estriol	1.0%
Estradiol Benzoate	0.7%
Estradiol 3-sulfate	0.53%
Ethynyl Estradiol	0.14%
5-Androstan-17β-ol-3-one	0.06%
17α-Estradiol	0.04%
5α-dihydro Testosterone	0.04%
Androstenediol	0.03%
Testosterone	0.03%
Estradiol 17-sulfate	0.02%
Estradiol 17-(β-D-Glucuronide)	0.02%
Aldosterone	<0.01%
Cortisol	<0.01%
DHEA	<0.01%
DHEA Sulfate	<0.01%
Maturation-Inducing Steroid (salmonid)	<0.01%
Progesterone	<0.01%

Table 6. Cross Reactivity of the Estradiol Antiserum

Estradiol Assay Summary					
Procedure	Blk	TA	NSB	B_0	Standards/ Samples
Reconstitute and Mix	Mix all reagents gently				
ELISA Buffer (1X)	-	-	100 µl	50 µl	-
Standards/Samples	-	-	-	-	50 µl
Estradiol AChE Tracer	-	-	50 µl	50 µl	50 µl
Estradiol Antiserum	-	-	-	50 µl	50 µl
Seal	Seal the plate				
Incubate	Incubate for 2 hours at room temperature on an orbital shaker				
Aspirate	Aspirate wells and wash 5 x ~300 µl with Wash Buffer (1X)				
Ellman's Reagent	200 µl	200 µl	200 µl	200 µl	200 µl
Estradiol Tracer	-	5 µl	-	-	-
Seal	Seal plate and incubate for 60 minutes at room temperature on orbital shaker, protected from light				
Read	Remove plastic seal and read absorbance at 414 nm				

Table 7. Assay Summary



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

References

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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.