ELISA PRODUCT INFORMATION & MANUAL

ESTRIOL

NBP2-61289

Enzyme-linked Immunosorbent Assay for quantitative detection of ESTRIOL.
For research use only. Not for diagnostic or therapeutic procedures.

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Estriol ELISA kit

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Description

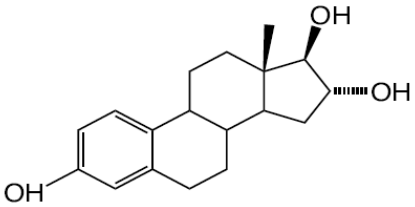
The Estriol ELISA kit is a competitive immunoassay for the quantitative determination of free Estriol in biological fluids. Please read the complete kit insert before performing this assay. The kit for the quantitative measurement of Estriol uses a polyclonal antibody to Estriol to bind, in a competitive manner, Estriol in the standard or sample or an alkaline phosphatase molecule which has Estriol covalently attached to it. After a simultaneous incubation at room temperature the excess reagents are washed away and substrate is added. After a short incubation time the enzyme reaction is stopped and the yellow color generated is read on a microplate reader at 405nm. The intensity of the bound yellow color is inversely proportional to the concentration of Estriol in either standards or samples. The measured optical density is used to calculate the concentration of Estriol . For further explanation of the principles and practice of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

This kit measures the concentration of free, conjugated and unconjugated Estriol in biological matrices. The polyclonal antibody used in the kit also binds the sulfate and glucuronide conjugates of Estriol. However, the absence of detergents or steroid analogs in the kit reagents means that protein- bound Estriol is not displaced, resulting in the measurement of free steroid only.

Introduction

Estriol is a female sex steroid hormone produced by the placenta during pregnancy and is the major estrogen produced in the normal fetus. In non-pregnant circumstances, estriol is derived almost exclusively from 17 ß-Estradiol[3]. Estriol exists in biological matrix in unconjugated (~9%) and conjugated forms (~91%). Conjugation with glucuronic or sulfuric acid of estriol occurs in the liver, which aides in steroid solubility, therefore allowing rapid elimination via the kidney[3].

Estriol



Precautions

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1. Some kit components contain azide, which may react with lead or copper plumbing. When disposing of reagents always flush with large volumes of water to prevent azide build-up.
2. Stop Solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in use.
3. The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} ions. The activity of the conjugate is affected by concentrations of chelators (>10 mM) such as EDTA and EGTA.
4. We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results.
5. The Estriol Standard provided is supplied in ethanolic buffer at a pH optimized to maintain Estriol integrity. Care should be taken handling this material because of the known and unknown effects of steroids.

Materials Supplied

1. **Goat anti-Rabbit IgG Microtiter Plate, One Plate of 96 Wells.**
   A plate using break-apart strips coated with goat antibody specific to rabbit IgG.
2. **Estriol ELISA Conjugate, 5 mL.**
   A blue solution of alkaline phosphatase conjugated with Estriol.
3. **Estriol ELISA Antibody, 5 mL.**
   A yellow solution of a rabbit polyclonal antibody to Estriol.
4. **Assay Buffer Concentrate, 27 mL.**
   Tris buffered saline containing proteins, detergents and sodium azide as a preservative.
5. **Wash Buffer Concentrate, 27 mL.**
   Tris buffered saline containing detergents.
6. **Estriol Standard, 0.5 mL.**
   A solution of 5,000,000 pg/mL Estriol.
7. **p-Npp Substrate, 20 mL.**
   A solution of p-nitrophenyl phosphate in buffer. Ready to use.
8. **Stop Solution, 5 mL.**
   A solution of trisodium phosphate in water. Keep tightly capped. Caution: **Caustic.**
9. **Estriol Assay Layout Sheet, 1 each.**
10. **Plate Sealer, 1 each.**

Storage

All components of this kit are stable at 4°C until the kit's expiration date.

Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Precision pipets for volumes between 5 µL and 1,000 µL.
3. Repeater pipets for dispensing 50 µL and 200 µL.
4. Disposable beaker for diluting buffer concentrates.
5. Graduated cylinders.
6. A microplate shaker.
7. Adsorbent paper for blotting.
8. Microplate reader capable of reading at 405 nm, preferably with correction between 570 and 590 nm.

Sample Handling

The Estriol ELISA kit is compatible with Estriol samples in a wide range of matrices. Samples diluted sufficiently into Assay Buffer can be read directly from the standard curve. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions. However, the end user **must verify** that the recommended dilutions are appropriate for their samples. **Samples containing rabbit IgG may interfere with the assay.**

Samples in the majority of Tissue Culture Media can also be read in the assay, provided the standards have been diluted into the Tissue Culture Media instead of Assay Buffer. There will be a small change in the binding associated with running the standards and samples in media. Users should only use standard curves generated in media or buffer to calculate concentrations of Estriol in the appropriate matrix. Some samples may have very low levels of Estriol present and extraction may be necessary for accurate measurement. **This extraction may yield both free and bound Estriol**. A suitable extraction procedure is outlined below:

Materials Needed

1. Estriol Standard to allow extraction efficiency to be accurately determined.
2. ACS Grade Diethyl Ether.
3. Glass test tubes.

Procedure

1. Add sufficient Estriol to a typical sample for determination of extraction efficiency.
2. In a fume hood add 1 mL of Diethyl Ether for every mL of sample. Stopper and shake sample.
3. Allow layers to separate. Carefully pipet off the top ether layer and place in a clean test tube.
4. Repeat steps 1 and 2 twice more, combining the ether layers.
5. Evaporate the ether to dryness under nitrogen.
6. Dissolve the extracted Estriol with at least 250 µL of Assay Buffer. Vortex well then allow to sit for five minutes at room temperature. Repeat twice more.
7. Run the reconstituted samples in the assay immediately or keep the dried samples frozen below -20°C in desiccation.

Procedural Notes

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed foil bag. The wells should be used in the frame provided.
8. **Care must be taken to minimize contamination by endogenous alkaline phosphatase.** Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high

blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands.

9. **Prior to addition of substrate, ensure that there is no residual wash buffer in wells. Any remaining wash buffer may cause variation in assay results.**

Reagent Preparation

1. **Assay Buffer**

   Just before use, prepare the Assay Buffer by diluting 10 mL of the supplied Assay Buffer Concentrate with 90 mL of deionized water. Discard unused buffer or add up to 0.09% sodium azide (w/v) for storage.

2. **Estriol Standard**

   Allow the 5,000,000 pg/mL Estriol standard solution to warm to room temperature. Label seven 12 x 75 mm glass tubes #1 through #7. Pipet 1,000 µL of standard diluent (Assay Buffer or Tissue Culture Media) into tube #1. Pipet 750 µL of standard diluent into tubes #2 through #7. Remove 100 µL of diluent from tube #1. Add 100 µL of the 5,000,000 pg/mL standard to tube #1. Vortex thoroughly. Add 250 µL of tube #1 to tube #2 and vortex thoroughly. Add 250 µL of tube #2 to tube #3 and vortex. Continue this for tubes #4 through #7.

   The concentration of Estriol in tubes #1 through #7 will be 500,000, 125,000, 31,250, 7,812, 1,953, 488 and 122 pg/mL respectively. See the Estriol Assay Layout Sheet for dilution details.

   Diluted standards should be used within 60 minutes of preparation.

3. **Wash Buffer**

   Prepare the Wash Buffer by diluting 5 mL of the supplied concentrate with 95 mL of deionized water. This can be stored at room temperature until the kit expiration date, or for 3 months, whichever is earlier.

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the foil pouch and seal the ziploc. Store unused wells at 4°C.

2. Pipet 100 µL of standard diluent (Assay Buffer or Tissue Culture Media) into the NSB and the Bo (0 pg/mL Standard) wells.

3. Pipet 100 µL of Standards #1 through #7 into the appropriate wells.

4. Pipet 100 µL of the Samples into the appropriate wells.

5. Pipet 50 µL of Assay Buffer into the NSB wells.

6. Pipet 50 µL of blue Conjugate into each well, except the Total Activity (TA) and Blank wells.

7. Pipet 50 µL of yellow Antibody into each well, except the Blank, TA and NSB wells.

NOTE: Every well used should be **Green** in color except the NSB wells which should be **Blue**. The Blank and TA wells are empty at this point and have no color.

8. Incubate the plate at room temperature on a plate shaker for 2 hours at ~500 rpm. The plate may be covered with the plate sealer provided, if so desired.

9. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 2 more times for a total of **3 washes.**

10. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint Fr paper towel to remove any remaining wash buffer.

11. Add 5 µL of the blue Conjugate to the TA wells.

12. Add 200 µL of the pNpp Substrate solution to every well. Incubate at room temperature for 1 hour without shaking.

13. Add 50 µL of Stop Solution to every well. This stops the reaction and the plate should be read immediately.

14. Blank the plate reader against the Blank wells, read the optical density at 405nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all readings.

Calculation of Results

Several options are available for the calculation of the concentration of Estriol in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If this sort of data reduction software is not readily available, the concentration of Estriol can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:
   Average Net OD = Average Bound OD - Average NSB OD
2. Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:
   $$\text{Percent Bound} = \frac{\text{Net OD}}{\text{Net Bo OD}} \times 100$$
3. Using Logit-Log paper, plot Percent Bound versus Concentration of Estriol for the standards. Approximate a straight line through the points. The concentration of Estriol in the unknowns can be determined by interpolation.

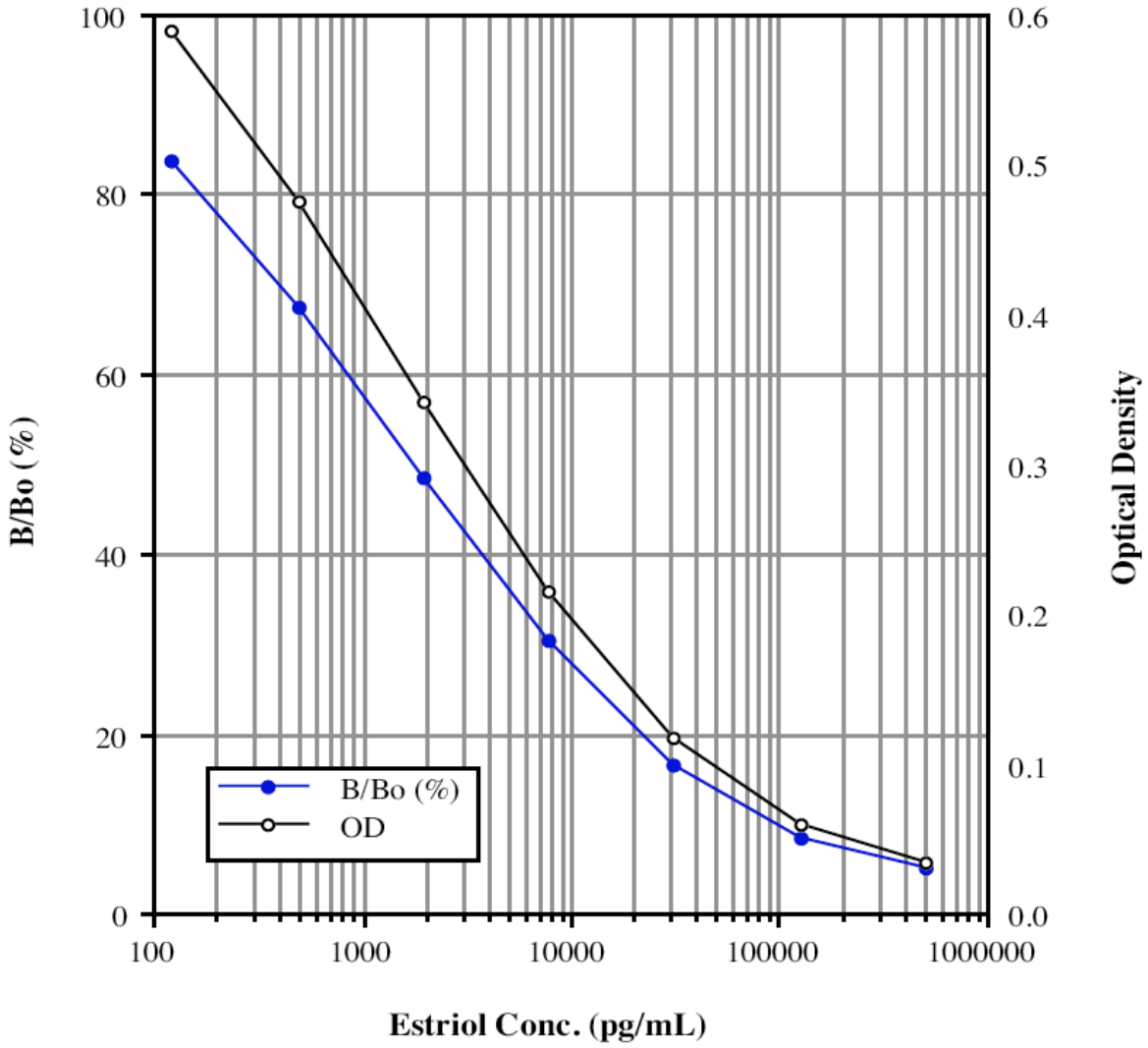
Typical Results

The results shown below are for illustration only and **should not** be used to calculate results from another assay.

Sample	Mean OD (-Blank)	Average Net OD	Percent Bound	**Estriol (pg/mL)**
Blank OD	(0.123)			
TA	0.369			
NSB	0.003	0.000	0.0%	
Bo	0.707	0.704	100%	**0**
S1	0.038	0.035	5.0%	**500,000**
S2	0.063	0.060	8.5%	**125,000**
S3	0.119	0.116	16.5%	**31,250**
S4	0.218	0.215	30.5%	**7,812**
S5	0.344	0.341	48.4%	**1,953**
S6	0.478	0.475	67.5%	**488**
S7	0.593	0.590	83.8%	**122**
Unknown 1	0.206	0.203	28.8%	**8,762**
Unknown 2	0.251	0.249	35.3%	**5,117**

Typical Standard Curve

A typical standard curve is shown below. This curve **must not** be used to calculate Estriol concentrations; each user must run a standard curve for each assay.



Typical Quality Control Parameters

Total Activity Added	=	0.369 x 10 = 3.69
%NSB	=	0.81%
%Bo/TA	=	22.49%
Quality of Fit	=	1.000 (Calculated from 4 parameter logistic curve fit)

20% Intercept	=	21,184 pg/mL
50% Intercept	=	1,739 pg/mL
80% Intercept	=	175 pg/mL

Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols[4].

Sensitivity

Sensitivity was calculated in Assay Buffer by determining the average optical density bound for sixteen (16) wells run as Bo, and comparing to the average optical density for sixteen (16) wells run with Standard #7. The detection limit was determined as the concentration of Estriol measured at two (2) standard deviations from the zero along the standard curve.

Average Optical Density for the Bo = 0.578 ± 0.022 (3.8%)
Average Optical Density for Standard #7 = 0.488 ± 0.013 (2.8%)

Delta Optical Density (0-122 pg/mL) = 0.090

2 SD's of the Zero Standard = 2 x 0.022 = 0.044

Sensitivity = $\frac{0.044}{0.090}$ x 122 pg/mL = **59.64 pg/mL**

Linearity

A sample containing 177,611 pg/mL Estriol was diluted 7 times 1:2 in the kit Assay Buffer and measured in the assay. The data was plotted graphically as actual Estriol concentration versus measured Estriol concentration.

The line obtained had a slope of 1.089 and a correlation coefficient of 0.999.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of Estriol and running these samples multiple times (n=16) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of Estriol in multiple assays (n=8).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of Estriol determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	Estriol (pg/mL)	Intra-assay %CV	Inter-assay %CV
Low	2,271	8.90	
Medium	5,750	8.42	
High	11,047	8.64	
Low	1,963		3.0
Medium	4,833		7.0
High	9,143		10.3

Cross Reactivity

The cross reactivities for a number of related steroid compounds was determined by dissolving the cross reactant (purity checked by N.M.R. and other analytical methods) in Assay Buffer at concentrations from 10,000,000 to 10 pg/mL. These samples were then measured in the Estriol assay, and the measured Estriol concentration at 50% B/Bo was calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

Compound	Cross Reactivity	Compound	Cross Reactivity
Estriol	100%	Dehydroisoandrosterone	<0.01%
Estriol-3-Sulfate	105%	Dehydroisoandrosterone3-Sulfate	<0.01%
Estriol-3-Glucuronide	100%	Deoxycorticosterone	<0.01%
17 ß-Estradiol	2.0%	Estrone	<0.01%
17-Epiestriol	1.7%	Hydrocortisone	<0.01%
4-Hydroxyestrone	<0.01%	Pregesterone	<0.01%
Androstenedione	<0.01%	Pregnenolone	<0.01%
Androsterone	<0.01%	Prednisolone	<0.01%
Corticosterone	<0.01%	Testosterone	<0.01%
Cortisone	<0.01%		

Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard preparation.

Estriol concentrations were measured in a variety of different samples including tissue culture media, mouse serum, sheep heparin plasma, human urine and human saliva. Estriol was spiked into the undiluted samples of these matrices which were then diluted with the appropriate diluent and then assayed in the kit. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
Tissue Culture Media	101.6	≥1:16
mouse Serum	101.4	1:40
sheep heparin Plasma	106.3	≥1:40
human Urine	107.2	≥1:200
human Saliva	108.9	≥1:64

* See Sample Handling instructions on page 4 for details.

References

1. T. Chard,"An Introduction to Radioimmunoassay & Related Techniques 4th Ed", (1990) Amsterdam: Elsevier.
2. P. Tijssen,"Practice & Theory of Enzyme Immunoassays", (1985) Amsterdam: Elsevier.
3. N.W. Tietz, "Textbook of Clinical Chemistry", (1986) Philadelphia, PA: W. B. Saunders Co.
4. National Committee for Clinical Laboratory Standards Evaluation Protocols, SC1, (1989) Villanova, PA: NCCLS.

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