LDN Labor Diagnostika Nord GmbH & Co. KG
Am Eichenhain 1, 48531 Nordhorn
Telefon: +49-5921-8197 0
Telefax: +49-5921-8197 222
e-mail: info@ldn.de
Internet: http://www.ldn.de

Instructions for use

Estrone-3-Sulfate equine ELISA

REF **AR E-8300**

Σ 96

+2 +8 °C

RUO For Research use only- Not for use in diagnostic procedures

Estrone-3-Sulfate equine ELISA

INTRODUCTION

INTENDED USE

The Estrone-3-Sulfate equine is a competitive enzyme immunoassay for the quantitative measurement of estrone-3-sulfate in mare serum.

SUMMARY AND EXPLANATION

Estrone-3-Sulfate (E3S) is the predominant conjugated estrogen during pregnancy. It is produced by the fetus, possibly in association with the endometrium in the pregnant mare.
Different hormones are important for the complex events that occur during pregnancy in all mammals. In the mare these events include the maintenance of the corpus luteum function, formation of endometrial cups and development of secundary corpora lutea. Progesterone and PMSG (Pregnant Mare Serum Gonadotropine, eCG) and also free Estrogens, e.g. Estrone, are associated with these processes. It has been shown, that Estrone is rapidly conjugated after secretion and the ratio between conjugated and unconjugated estrogens is 100:1 in mare serum.
The conjugated estrogenes, especially Estrone-3-sulfate, provide the opportunity to improve the accuracy of pregnancy diagnosis, to monitor the pregnancy and to distinguish whether the fetal development is normal or impaired. The diagnosis of embryonic death is usually made by using techniques of palpation of the uterus per rectum or ultrasound echography. The determination of Estrone-3-sulfate is an aid in the non-invasive diagnosis which allows a monitoring of the feto-placental unit during pregnancy. Only in mares with normal fetal development the values of Estrone-3-sulfate show a tremendous increase between day 75 and 100 of gestation.

PRINCIPLE

The Estrone-3-Sulfate equine ELISA test kit is a solid phase enzyme immunoassay (ELISA) in the microplate format, designed for the quantitative measurement of estrone-3-sulfate. The microplate is coated with a polyclonal antibody specific for estrone-3-sulfate.
Calibrators and samples as well as a biotin-labeled estrone-3-sulfate are pipetted into the antibody coated microplate. During one hour incubation the estrone-3-sulfate of the calibrator/sample competes with the biotin-labeled estrone-sulfate for the binding sites of the antibody fixed on the inner surface of the wells.
Afterwards, horseradish peroxidase-labeled streptavidine is added. During an 30 minutes incubation, this enzyme-labeled streptavidine binds to the biotinylated estrone-3-sulfate. All wells are washed to remove excess of non-bound enzyme-labeled streptavidine.
A chromogenic substrate, TMB (3,3',5,5'-Tetra-Methyl-Benzidine), is added to all wells. During a 30 minutes incubation, the substrate is converted to a colored end product (blue) by the fixed enzyme. Enzyme reaction is stopped by dispensing of hydrochloric acid as stop solution (change from blue to yellow). The color intensity is inversely related to the concentration of estrone-3-sulfate present in the sample.
The optical density of the color solution is measured with a microplate reader at 450 nm. Bi-chromatic measurement with a 600 - 690 nm reference filter is recommended.

WARNINGS AND PRECAUTIONS

All reagents of this test kit are strictly intended for **veterinary use** only. Use by staff, who is specially informed and trained in methods which are carried out by use of immunoassays.

Please adhere strictly to the sequence of pipetting steps provided in this protocol.

All reagents should be stored refrigerated at 2 - 8 °C in their original container. Do not interchange kit components from different lots and assays. The expiration dates stated on the labels of the shipping container and all vials have to be observed. Do not use kit components beyond their expiration dates.

Allow all kit components and specimen to reach room temperature (18 – 28 °C) prior to use and mix well. During handling of all kit reagents, control and serum samples observe the existing legal regulations handling potentially infectious materials. Especially the following precautions should be taken:
- do not eat, drink or smoke, - do not pipette by mouth, use safety pipettes, - wear disposable gloves and avoid contact with kit reagents, control and sample material.

The test kit contains components of human origin which were found negative for Hepatitis B surface antigen and HIV (Human Immunodeficiency Virus). Nevertheless, for products derived from human or animal source it cannot be completely guaranteed, that they don't contain the above mentioned, others and not yet known or not diagnosticable pathogens. Sample material of patients (for example serum or plasma) normally used in laboratory determinations are always classified as potentially infectious. According to the same safety guides, kit reagents and control material are to be used. Samples of risk patients should be specially labeled and if necessary be handled in safety work benches (lamina flow bench).

The assay reagents contain against microbial growth preservation substances, avoid contact with skin and/or mucous membranes.

Avoid contact with the TMB (3,3',5,5'-Tetra-Methyl-Benzidine) substrate solution containing peroxide. If it comes into contact with skin, wash thoroughly with water. Avoid contact with any easily oxidized materials. Extreme temperature changes may cause spontaneous decay of the peroxide. Avoid the contact with the stop solution containing acid. By skin contact, wash thoroughly with water. All instrumentation employed to dispense the stop solution should be thoroughly cleaned after use.

REAGENTS

Reagents provided

AR E-8331 96 **Microtiterplate**, 12 x 8 (break apart) strips with 96 wells;
Wells coated with a polyclonal anti-equine E3S antibody.

Calibrators - lyophilized, reconstitution required

Reconstitute lyophilized Calibrator A with 2.0 ml dist. water and Calibrator B through Calibrator G with 1.0 ml dist. water 30 min. before use.

Listed below are approximate concentrations, please refer to vial labels for exact concentrations:

Cat. no.	Symbol	Calibrator	Concentration	Volume/Vial
AR E-8301	STANDARD A	**Calibrator 0**	0 ng/ml	2 mL
AR E-8302	STANDARD B	**Calibrator 1**	5 ng/ml	1 mL
AR E-8303	STANDARD C	**Calibrator 2**	10 ng/ml	1 mL
AR E-8304	STANDARD D	**Calibrator 3**	50 ng/ml	1 mL
AR E-8305	STANDARD E	**Calibrator 4**	100 ng/ml	1 mL
AR E-8306	STANDARD F	**Calibrator 5**	300 ng/ml	1 mL
AR E-8307	STANDARD G	**Calibrator 6**	1000 ng/ml	1 mL

AR E-8370 SAMPLE-BUFF **Sample Buffer**, 1 vial 11 ml, brown, ready to use

AR E-8310 BIOTIN-AB **Biotin Antibody**, 1 vial, 11 ml, ready to use;
in a buffered solution with preservative

AR E-8340 CONJUGATE **Streptavidine Conjugate**, 1 vial, 3 ml, blue, ready to use;
containing horseradish-peroxidase-labeled streptavidine

AR E-0055 SUBSTRATE **Substrate Solution**, 1 vial, 22 ml each, ready to use;
contains tetramethylbenzidine (TMB) and hydrogen peroxide in a buffered matrix.

AR E-0080 STOP-SOLN **Stop Solution**, 1 vial, 7 ml, ready to use;
contains 2 N Hydrochloric Acid solution.

AR E-0030 WASH-CONC 10x **Wash Solution**, 1 vial, 50 ml (10X concentrated);
Dilute with 450 ml dist. water to a final volume of 500 ml.

Note: Additional Calibrator 0 for sample dilution is available upon request.

Materials required but not provided

- Microplate reader capable for endpoint measurements at 450 nm (optional reference filter in the range of 600 - 690 nm)
- Vortex mixer
- Microplate mixer operating at 350 - 400 rpm
- Distilled or deionized water
- Graduated cylinders for 500 ml
- Plastic container for storage of the wash solution
- Adjustable pipette for up to 1000 µl
- Dispenser or repeatable pipet for 20 µl, 100 µl and 200 µl.

STORAGE CONDITIONS

When stored at 2°C to 8°C all reagents are stable until expiration date or 30 days after opening.

The Stop Solution is stable up to 2 months after opening or until the expiration date.

The Wash Buffer is stable for 3 months after dilution or until the expiration date.

Store Calibrators refrigerated, they will be stable at 2°C to 8°C for 7 days after reconstitution or until expiration date. For longer storage freeze at -20°C.

Protect Divisible Microplate from moisture. Store together with desiccant and carefully sealed in the plastic bag.

Protect TMB-Substrate Solution from light.

SPECIMEN

For determination of Estrone-3-Sulfate equine serum is the preferred sample matrix. The procedure calls for 20 µl matrix per well.

Blood collection should be as stress-free as possible.

The samples may be stored refrigerated at 2 - 8°C for one week, or up to 6 months frozen at -20°C. To avoid repeated thawing and freezing the samples should be aliquoted.
Samples expected to contain estrone-3-sulfate concentrations higher than the highest calibrator (1000 ng/ml) should be diluted with the Zero Calibrator before assay. The additional dilution step has to be taken into account for the calculation of the results.

ASSAY PROCEDURE

GENERAL REMARKS

- Do not interchange components of different lots.
- All components should be at room temperature (18 – 28 °C) before use.
- All components of these test kits, supplied as concentrate should be diluted to their final concentration at least 30 minutes prior to use. Mix well, but prevent of foam formation.
- Use a disposable-tip micropipette to dispense serum samples. Pipet directly to the bottom of the wells. Change the tip between samples, to avoid carryover contamination.

ASSAY PROCEDURE

1. Preparation of calibrators:
The calibrators are supplied lyophilized. At least 30 minutes before use, reconstitute the calibrator A with 2.0 ml of distilled water, and each of the remaining calibrators B through G with 1.0 ml of distilled water. Use volumetric pipettes and mix by gentle inversion.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
a	A	E	P2	P..								
b	A	E	P2	P..								
c	B	F	P3									
d	B	F	P3									
e	C	G	P4									
f	C	G	P4									
g	D	P1	P5									
h	D	P1	P5									

2. Pipet **20 µl** of each calibrator and equine sample into the wells prepared.

3. Add **100 µl** of **Biotin-Labeled Estrone-3-Sulfate** to every well.

4. Add **100 µl** of **Sample Buffer** to every well.

5. Rotate for **1 hour** at room temperature (18 - 28 °C) on a plate mixer (350-400 rpm). **Afterwards do not wash or discard!**

6. Add **25 µl** of **Enzyme-Labeled Streptavidine** to every well.

7. Shake again for **30 minutes.**

8. Discard the content of the wells and wash **4 times** with **300 µl** buffered **wash solution.**
Remove wash solution as much as possible by beating the microplate carefully.

9. Add **200 µl** of liquid **TMB/Substrate Solution** to all wells.

10. Incubate without shaking for **30 minutes** in the dark.

11. Add **50 µl** of **Stop Solution** to each well and mix carefully.

12. Read the optical density at **450 nm**. Bi-chromatic measurement with a reference at 600 - 690 nm is recommended.

The developed color is stable for at least 15 minutes. Read optical densities during this time-frame.

CALCULATION OF RESULTS

For evaluation of Estrone-3-Sulfate equine ELISA a 4-Parameter-Fit with lin-log coordinates for optical density (linear scale) and concentration (logarithmic scale) is recommended.

Spline approximation with lin-log coordinates and log-log coordinates are also suitable.

EXAMPLE OF TYPICAL CALIBRATOR CURVE

The figure below shows typical results for Estrone-3-Sulfate equine ELISA tests. These data are intended for illustration only and should not be used to calculate results from another run.

Estrone-3-Sulfate equine ELISA

	Replicate (OD)	Mean (OD)	Binding (%)	Equine E3S (ng/ml)
Calibrators				
A	2.702 ---------- 2.571	2.637	**100**	**0**
B	1.933 ---------- 1.941	1.937	**73**	**5**
C	1.706 ---------- 1.725	1.716	**65**	**10**
D	0.870 ---------- 0.884	0.877	**33**	**50**
E	0.671 ---------- 0.604	0.638	**24**	**100**
F	0.258 ---------- 0.252	0.255	**9.7**	**300**
G	0.078 ---------- 0.096	0.087	**3.3**	**1000**
Unknown Samples				
X 001	0.589 ---------- 0.571	0.580	22	105
X 002	0.323 ---------- 0.322	0.323	12	242
X 003	0.227 ---------- 0.236	0.232	8.8	366

EXPECTED NORMAL VALUES

In a reference range study equine serum samples were collected in the morning between 8 and 9 a.m. and in the evening between 5 and 6 p.m. Diurnal variations have not been observed. Analysis by the Estrone-3-Sulfate equine test kit yielded the following results:

Group	**Absolute Range (ng/ml)**	**n**
Normal mares	nondetectable -10	22

During the course of pregnancy in 26 mares the estrone-3-sulfate serum concentrations were measured using the Estrone-3-Sulfate equine test procedure.

Gestation Week	N	mean ng/ml	min ng/ml	max ng/ml
3	12	2.6	0.4	9.2
4	20	1.8	0.4	4.9
5	21	2.1	0.2	6.5
6	18	2.8	0.4	7.1
7	17	3.2	0.4	9.1
8	16	4.5	0.9	14.0
9	19	5.1	0.9	14.1
10	11	9.7	2.8	34.7
11	18	11	2.1	54.9
12	13	23	2.7	109
13	20	34	3.1	161
14	9	94	7.8	232
15	17	80	4.8	262
16	10	185	30.9	340
17	16	186	11.6	421
18	11	367	67.5	577
19	16	374	22.2	863
20	10	575	289	905
21	12	516	71.5	1233
22	9	497	247	889
23	11	571	106	861
24	12	634	263	1621
25	13	577	184	1229
26	9	528	255	949
27	12	523	213	1258
28	12	464	267	795
29	11	473	245	778
30	7	336	209	590
31	5	385	206	478
32	4	349	184	504
33	11	302	180	510
34	10	316	131	523
35	7	215	84	506
36	8	233	113	349
37	10	189	85	358
38	11	207	85	305
39	6	93	77	123
40	7	157	52	252
41	5	145	88	227
42	8	147	58	239
43	2	106	86	126
44	6	168	101	210
45	4	80	74	85
46	9	112	32	192
47	5	101	43	214
48	5	94	66	129

Because of differences which may exist between laboratories with respect of population, laboratory technique and selection of reference groups, it is recommended that each laboratory establishes its own normal and pathological ranges of equine E3S. The reference ranges should be regarded as guidelines only.

PERFORMANCE CHARACTERISTICS

SENSITIVITY

The detection limit of the assay, defined as the concentration three standard deviation above the response at zero dose, is approximately 0.14 ng/ml.

SPECIFICITY

The antibodies in the Estrone-3-Sulfate equine ELISA procedure are highly specific for estrone-3-sulfate, with low cross-reactivities to other steroids as listed below

Compound	**Amount added ng/ml**	**Apparent Conc. ng/ml**	**Percent crossreactivity**
Estrone	1000	78	7.8%
Estradiol	1000	2.2	0.22%
Estradiol-sulfate	1000	4.5	0.45 %
Equilin	1000	8.8	0.88%
Equilenin	1000	2.3	0.23%
Equilin-sulfate	1000	27.2	2.7%
Equilenin-Sulfate	1000	217	21.7%
Androstendione	1000	1.0	0.1%
Dehydro-iso-androsterone-3-sulfate	1000	5.2	0.5%
Dihydrotestosterone	1000	2.0	0.2%
Testosterone	1000	1.4	0.14%
Progesterone	1000	2.3	0.23%
Androsterone	1000	1.5	0.15%

REPRODUCIBILITY

Statistics for Coefficients of variation (CV) were calculated for each of three samples from the results of 12 pairs of wells in a single run for Intra-Assay precision and the Inter-Assay precision was calculated from the results of 10 different runs of three samples:

Estrone-3-Sulfate equine ELISA

Intra-Assay		
Sample	**Mean**	**CV %**
1	13.3	6.2
2	27.7	7.6
3	70.6	7.8

Inter-Assay		
Sample	**Mean**	**CV %**
1	31.1	9.6
2	89.5	5.3
3	241	7.0

RECOVERY

Three spiking solutions were prepared using the equine E3S zero calibrator, to represent 1500, 4000 and 6000 ng/ml, respectively. A 50 µl aliquot of each solution (A, B, C) was spiked into 950 µl aliquots of three different patient serum samples, for a spiking ratio of 1 to 20, leaving the serum matrix of the spiked samples relatively intact. All samples were then assayed by the Estrone-3-Sulfate equine test procedure.

Sample	Diluted Solution	measured Concentration [ng/ml]	expected Concentration [ng/ml]	Recovery [%]
1	-	1.0	-	-
	A	79.4	76	104
	B	200	201	100
	C	324	301	108
2	-	0.0	-	-
	A	67.8	75	90
	B	176	200	88
	C	274	300	91
3	-	1.7	-	-
	A	81.9	76.7	107
	B	216	201.7	107
	C	298	301.7	99

LINEARITY

Three serum samples were assayed both undiluted and diluted with E3S zero calibrator. The observed and expected values are presented below in ng/ml

Estrone-3-Sulfate equine ELISA

Sample	Dilution Factor	measured Concentration	expected Concentration	Recovery [%]
		[ng/ml]	**[ng/ml]**	
1	8 in 8	235	-	-
	4 in 8	107	118	91
	2 in 8	53	59	90
	1 in 8	27	29	93
2	8 in 8	322	-	-
	4 in 8	155	161	96
	2 in 8	74	81	91
	1 in 8	38	40	95
3	8 in 8	470	-	-
	4 in 8	215	235	91
	2 in 8	107	118	91
	1 in 8	50	59	85

LIMITATIONS OF PROCEDURE

Samples expected to contain estrone-3-sulfate concentrations greater than the highest calibrator (1000 ng/ml) should be diluted with Zero Calibrator.

Symbols:

	Storage temperature		Manufacturer		Contains sufficient for <n> tests
	Expiry date	LOT	Batch code	I V D	For in-vitro diagnostic use only!
	Consult instructions for use	CONT	Content	CE	CE labelled
	Caution	REF	Catalogue number	RUO	For research use only!