LDN®
IMMUNOASSAYS AND SERVICES
BIOGENIC AMINES & NEUROSCIENCE | ENDOCRINOLOGY | FOOD SAFETY
LABOR DIAGNOSTIKA NORD GmbH & Co.KG | Am Eichenhain 1 | 48531 Nordhorn | Germany | Tel. +49 5921 8197-0 | Fax +49 5921 8197-222 | info@ldn.de | www.ldn.de

Instructions for use

TSH canine ELISA

REF **AR E-8500**

Σ 96

+2 +8 °C

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

TSH canine ELISA

INTRODUCTION

INTENDED USE

The TSH canine ELISA is an enzyme immunoassay for the quantitative measurement of canine TSH (thyrotropin).

SUMMARY AND EXPLANATION

Thyroid stimulating hormone (TSH, thyrotropin) in dogs is similar in function to TSH found in other mammalian species, including humans. It is a glycoprotein produced by the anterior pituitary gland. Through its action on the thyroid gland, it plays a major role in maintaining normal circulating levels of the iodothyronines, T4 and T3. The production and secretion of TSH is controlled by negative feedback from circulating T4 and T3, and by the hypothalamic hormone TRH (thyrotropin releasing hormone). The TSH molecule is composed of two nonidentical subunits, α and β, that are bound together in a noncovalent manner. Within a species, the TSH α subunit is structurally identical to the α subunits of the related glycoprotein hormones (LH, FSH and chorionic gonadotropin). The β subunit of TSH and the β subunits of the related hormones are structurally hormone-specific, and confer upon them their unique biological activities.
Hypothyroidism is considered to be a common endocrine disorder in dogs, whereas hyperthyroidism in this species is nearly unknown. Most cases of canine hypothyroidism are primary in nature, involving impaired production of the thyroid hormones, T4 and T3. In this condition, elevated TSH levels are expected. Secondary or tertiary hypothyroidism, where thyroid hormone production is low as a consequence of hypothalamic or pituitary disease, is believed to account for less than 5% of canine hypothyroidism cases. In the latter conditions, lowered levels of TSH would be expected. Usually, hypothyroidism in dogs is suspected on the basis of clinical history and the presence of lowered levels of thyroid hormones. However, suppressed thyroid hormone levels are nonspecific indicators of the disease, since they are often observed in nonthyroid illnesses. The evaluation of thyroid function and the diagnosis of hypothyroidism in dogs can be greatly improved through the use of the valid assay for the determination of canine TSH.

PRINCIPLE

The test kit is a solid phase enzyme immunometric assay (ELISA) in the microplate format, designed for the quantitative measurement of canine TSH. The microplate is coated with a monoclonal antibody specific for canine TSH.
Calibrators and samples are pipetted into the antibody coated microplate. During an one-hour incubation TSH of the sample binds to the antibodies immobilized to the surface of the wells. Non-reactive sample components are removed by a washing step.
Afterwards, a polyclonal horseradish peroxidase-labeled antibody is added. During an one-hour incubation, a sandwich complex consisting of the two antibodies and the canine TSH is formed. An excess of enzyme conjugate is washed out.
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 direct proportional to the concentration of canine TSH 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 research** 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 veterinary 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 do not 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-8531 96 **Microtiterplate**, 12 x 8 (break apart) strips with 96 wells; ready to use
Wells coated with an anti-canine monoclonal TSH antibody.

Calibrators - lyophilized, reconstitution required

Reconstitute lyophilized Calibrators A through F with **1.0 ml dist. water** 30 minutes before use.

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

Cat. no.	Symbol	Calibrator	Concentration	Volume/Vial
AR E-8501	STANDARD A	**Calibrator 0**	0 ng/ml	1 ml
AR E-8502	STANDARD B	**Calibrator 1**	0.2 ng/ml	1 ml
AR E-8503	STANDARD C	**Calibrator 2**	0.46 ng/ml	1 ml
AR E-8504	STANDARD D	**Calibrator 3**	1.05 ng/ml	1 ml
AR E-8505	STANDARD E	**Calibrator 4**	2.2 ng/ml	1 ml
AR E-8506	STANDARD F	**Calibrator 5**	5.2 ng/ml	1 ml

AR E-8540 CONJUGATE **Enzyme-Labeled anti canine TSH Antibody**, 1 vial, 11 ml, red, ready to use; contains a horseradish peroxidase-labeled polyclonal anti canine TSH antibody (rabbit), in a buffered solution with preservative.

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 M 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.

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 600 – 900 rpm
- Distilled water
- Graduated cylinders for 500 ml
- Plastic containers for storage of the wash solution
- Adjustable pipette for up to 1000 µl
- Dispenser or repeatable pipet for 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 Solution is stable for 3 months after dilution or until the expiration date.
Store Calibrators refrigerated, it 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 canine TSH serum and EDTA plasma are the preferred sample matrices. The procedure calls for 100 µl sample per well. The samples may be stored refrigerated at 2 - 8 °C for 1 week, or up to 2 months at –20 °C. To avoid repeated thawing and freezing the samples should be aliquoted.

Samples expected to contain canine TSH concentrations higher than the highest calibrator 6 should be diluted in the Canine TSH 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 plasma samples. Pipet directly to the bottom of the wells. Change the tip between samples, to avoid carryover contamination.

ASSAY PROCEDURE

1. Prepare a sufficient number of microplate wells to accommodate calibrators (A through F) and patient samples in duplicates.

	1	2	3	4	5	6	7	8	9	10	11	12
a	A	E	P3	P..								
b	A	E	P3	P..								
c	B	F	P4									
d	B	F	P4									
e	C	P1	P5									
f	C	P1	P5									
g	D	P2	P6									
h	D	P2	P6									

2. Pipet **100 µl** of each **calibrator** and **patient sample** into the wells prepared.

3. Add **100 µl** of **Enzyme-Labeled Anti-Canine TSH antibody** to every well.

4. Rotate for **2 hours** on a plate mixer (600 – 900 rpm).

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

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

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

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

9. 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.

CALCULATION OF RESULTS

For evaluation of TSH canine 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.

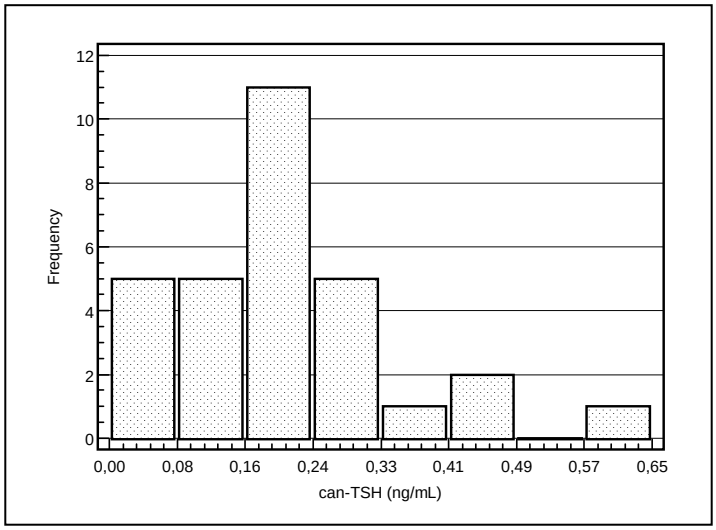
EXAMPLE OF TYPICAL CALIBRATOR CURVE

The figure below shows typical results for TSH canine test kits. These data are intended for illustration only and should not be used to calculate results from another run.

	Replicate (OD)	Mean (OD)	Binding (%)	canine TSH (ng/ml)
Calibrators				
A (NSB)	0.085 0.078	0.082	**-**	**0**
B	0.151 0.151	0.151	**6.6%**	**0.2**
C	0.278 0.282	0.280	**12.3%**	**0.46**
D	0.551 0.549	0.550	**24.1%**	**1.05**
E	1.067 1.076	1.072	**47%**	**2.2**
F ("MB")	2.301 2.262	2.282	**100%**	**5.2**

EXPECTED NORMAL VALUES

Blood was collected from 30 apparently healthy dogs. A mean canine TSH value of 0.216 ng/ml was found, with an absolute range of 0.017 to 0.591 ng/ml.



Frequency distribution of canine TSH in serum of healthy blood donors (median = 0,198 ng/ml, 95 % Percentile = 0,48 ng/ml)

Laboratories should consider reference range limits *as guidelines only*. Because of differences which may exist between laboratories and locales with respect to population, laboratory technique and selection of reference groups, it is important for each laboratory to establish by similar means the appropriateness of adopting the reference range suggested here.

PERFORMANCE CHARACTERISTICS

ANALYTICAL SENSITIVITY

The assay's detection limit, defined as the concentration two standard deviations above the response at zero dose, is approximately 0.01 ng/ml.

SPECIFICITY
The antibodies used in the TSH canine kit are highly specific for canine TSH, with no detectable crossreactivity to other hormones that may be present in canine serum samples.

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

Intra-Assay		
Sample No	Mean $\bar{x}$ (ng/ml)	CV (%)
1	0.53	5.7
2	0.96	3.1
3	1.9	6.8
4	2.8	5.7

Inter-Assay		
Sample No	Mean $\bar{x}$ (ng/ml)	CV (%)
1	0.54	5.6
2	0.88	8.0
3	1.9	6.8
4	2.6	5.4

RECOVERY

Three spiking solutions were prepared using the Zero Calibrator (8, 16, and 32 ng/ml, respectively). A 50 µl aliquot of each solution (A, B, C) was spiked into 950 µl aliquots of three different low concentration serum samples, for a spiking ratio of 1 to 19, leaving the serum matrix of the spiked samples relatively intact. All samples were then assayed by the TSH canine procedure.

Sample	Diluted Solution	measured Concentration	expected Concentration	Recovery [%]
		[ng/ml]	**[ng/ml]**	
1	-	0.1	-	-
	A	0.49	0.5	98
	B	0.91	0.9	101
	C	1.7	1.7	100
2	-	0.45	-	-
	A	0.81	0.85	95
	B	1.22	1.25	98
	C	1.94	2.05	95
3	-	0.83	-	-
	A	1.20	1.23	98
	B	1.60	1.63	98
	C	2.30	2.43	95

The results confirm that the TSH canine kit yields an accurate measure of canine TSH throughout its working range.

LINEARITY

In dilution experiments sera with high antibody concentrations were diluted with the zero calibrator and assayed in the TSH canine kit. The assay showed linearity over the full measuring range.

Sample	Dilution Factor	measured Concentration	expected Concentration	Recovery [%]
		[ng/ml]	**[ng/ml]**	
1	8 in 8	3.4	-	-
	4 in 8	1.8	1,7	106
	2 in 8	0.8	0.85	94
	1 in 8	0.4	0.43	93
2	8 in 8	3.0	-	-
	4 in 8	1.6	1.5	107
	2 in 8	0.72	0.75	96
	1 in 8	0.38	0.38	100
3	8 in 8	3.0	-	-
	4 in 8	1.7	1.5	113
	2 in 8	0.8	0.75	107
	1 in 8	0.38	0.38	100

EFFECT OF ANTICOAGULANTS

To determine whether anticoagulants interfere with the assay, five sera/plasma pairs were collected at the same time. All samples were assayed by the TSH canine procedure, with the following results.

	Serum	EDTA plasma
1	0.13	0.09
2	ND	ND
3	0.08	0.07
4	0.10	0.09
5	0.20	0.20

ND denotes non-detectable

REFERENCES

1. Ruschig, S., Kraft, W.
Bestimmung von caninem Thyroidea-stimulierendem Hormon (cTSH) im Blutserum des Hundes und seine Reaktion im TRH-Stimulationstest.
Tierärztl Prax 1996; 24: 479-483.

2. Iversen, L., Hoier, R., Jensen, A.L. , Skydsgaard, M., Koch, J.
Evaluation of the analytical performance on an enzyme immunometric assay (EIA) designed to measure endogenous thyroid-stimulating hormone (TSH) in canine serum samples.
J. Vet. Med. A 45 (1998): 93-98.

3. Ramsey, I.K., Evans, H., Herritage, M.E.
Thyroid-stimulating hormone and total thyroxine concentrations in euthyroid, sick euthyroid and hypothyroid dogs.
Small Animal Practice 38 (1997): 540-545.

4. Cortese, L., Oliva, G., Verstegen, J., Ciaramella, P., Persechino, A.
Hyperprolactinaemia and galactorrhoea associated with primary hypothyroidism in a bitch.
Small Animal Practice 38 (1997): 572-575.

Short instruction

(all sample sizes given in µl)

MP Well		A	B	C	D	E	F	Sample
	ng/ml	0	0.20	0.46	1.05	2.20	5.20	
Steps	**Solution**							
Pipet	Calibrator	**100**	**100**	**100**	**100**	**100**	**100**	**-**
Pipet	Sample	**-**	**-**	**-**	**-**	**-**	**-**	**100**
Pipet	Enzyme-labeled Canine TSH antibody	**100**	**100**	**100**	**100**	**100**	**100**	**100**
Incubate for **2h** at RT on a shaker								
Decant Wash **4x with 300 µL** of buffered wash solution								
Pipet	Substrate Solution	**200**	**200**	**200**	**200**	**200**	**200**	**200**
Incubate for **30 min** at RT **in the dark**								
Pipet	Stop Solution	**50**	**50**	**50**	**50**	**50**	**50**	**50**
Read at **λ = 450 nm**								

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!