Canine/Feline AMH ELISA

AL-116

INTENDED USE

The Canine/Feline Anti-Müllerian hormone (AMH) enzyme-linked immunosorbent assay (ELISA) kit provides materials for the quantitative measurement of AMH in Canine and Feline serum. The test is intended to be used as a diagnostic tool in the determination of functional gonadal (spayed, neutered, or intact) status in dogs and cats.

SUMMARY AND EXPLANATION

The anti-Müllerian hormone (AMH) is a member of the transforming growth factor β family of growth and differentiation factors[1]. AMH is produced exclusively in testicular Sertoli cells and ovarian granulosa cells. The physiological role of AMH in the two sexes is quite distinct[2,3]. In the male AMH has an essential role in sex differentiation. Fetal Sertoli cells secrete AMH which signals the active removal of the Müllerian ducts, the anlagen of the oviducts, uterus, and the upper part of the vagina in females, thereby preventing the formation of these structures in the male. The fetal and postnatal AMH production in males is used as a diagnostic marker to determine the presence of testicular tissue in the clinic. In post-pubertal males AMH has a role in the regulation of testosterone production by the Leydig cells. A negative correlation between testosterone and AMH has been found in human males[5] and it appears that testosterone suppresses Sertoli cell AMH production[6,7].

In females, AMH is not produced by the fetal ovary, ensuring that the Müllerian ducts stay intact in developing females. AMH appears to have two roles in ovarian physiology. Firstly, AMH has a negative feedback effect on primordial follicles, i.e., it suppresses recruitment thereby signaling the presence of enough small growing follicles to the primordial follicle pool[3,8]. Secondly, AMH suppresses the sensitivity of the follicle to FSH in an autocrine manner, preventing selection[9]. As small follicles grow and differentiate AMH starts to decrease when the differentiation state reaches the point when successful FSH selection is imminent, FSH sensitivity increases, and the follicle is selected.

In veterinary practice the presence or absence of functional gonadal tissue in dogs is a recurrent challenge. In particular, when the reproductive history is not known, it may be difficult to determine whether a female animal has been spayed. In female dogs the presence of remaining functional ovarian tissue after spaying is relevant when a presumably spayed animal is presented with clinical signs of gonadal hormone activity. In addition, surgery performed at a young age renders the visibility of surgical scar much more difficult. Serum AMH measurement can thus be used as a diagnostic tool in the determination of functional gonadal status in dogs[10].

There is significant value in determining the gonadal status of unowned or stray feline queens surrendered to shelters (and others encountered in practice). The establishment of reference ranges in intact and gonadectomized cats using canine-equivalent AMH, progesterone, and inhibin-B determinations provides clarity to how reliably these biomarkers can be used in clinical diagnosis. Less variability in AMH than either inhibin-B or progesterone suggests it is the more reliable, diagnostic indicator of gonadal status in queens[11].

PRINCIPLE OF THE TEST

The Canine/Feline AMH ELISA is a quantitative three-step sandwich type immunoassay. In the first step Calibrators, Controls and unknown samples are added to AMH antibody coated micro titer wells and incubated. After the first incubation and washing, the wells are incubated with biotinylated AMH antibody solution. After second incubation and washing, the wells are incubated with streptavidin horseradish peroxidase conjugate (SHRP) solution. After the third incubation and washing step, the wells are incubated with substrate solution (TMB) followed by an acidic stopping solution. In principle, the antibody-biotin conjugate binds to the solid phase antibody-antigen complex which in turn binds to the streptavidin-enzyme conjugate. The antibody-antigen-biotin conjugate-SHRP complex bound to the well is detected by enzyme-substrate reaction. The degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 nm as primary test filter and 630 nm as reference filter. The absorbance measured is directly proportional to the concentration of AMH in the samples and calibrators.

MATERIALS SUPPLIED

CAL-105A AMH/MIS Calibrator A / Sample Diluent

One bottle, 11 mL, labeled AMH/MIS Cal A/Sample Diluent, containing 0 ng/mL AMH in protein-based buffer and ProClin™ 300. Store unopened at 2-8°C until the expiration date.

CAL-116B – CAL-116D Canine AMH Calibrators B thru D (Lyophilized)

Three vials, labeled B-D, containing concentrations of approximately 0.3 – 11.9 ng/mL native canine AMH in protein-based buffer and ProClin™ 300. Refer to calibration card for exact concentration. Store unopened at 2 to 8°C until the expiration date. Reconstitute calibrators B-D with **1 mL** deionized water. Solubilize, mix well, and use after reconstitution. Aliquot and freeze in plastic vials for multiple use. Alternatively, freeze in the same vial within 2 hours of reconstitution. Avoid repeated freeze thaws.

CTR-116 -ve Control Canine AMH -ve Control (Lyophilized)

One vial, labeled -ve Control containing low native Canine AMH concentrations in protein-based buffer and ProClin™ 300. Refer to the calibration card for exact concentrations. Store unopened at 2 to 8°C until the expiration date. Reconstitute Control with **1 mL** deionized water. Solubilize, mix well and use after reconstitution. Aliquot and freeze in plastic vials for multiple use. Alternatively, freeze in the same vial within 2 hours of reconstitution. Avoid repeated freeze thaws.

CTR-116 +ve Control Canine AMH +ve Control (Lyophilized)

One vial, labeled +ve Control containing high native Canine AMH concentrations in protein-based buffer and ProClin™ 300. Refer to the calibration card for exact concentrations. Store unopened at 2 to 8°C until the expiration date. Reconstitute Control with **1 mL** deionized water. Solubilize, mix well, and use after reconstitution. Aliquot and freeze in plastic vials for multiple use. Alternatively, freeze in the same vial within 2 hours of reconstitution. Avoid repeated freeze thaws.

PLT-113 AMH Coated Microtitration strips

One strip holder, containing 12 strips and 96 microtitration wells with AMH antibody immobilized to the inside wall of each well. Store at 2-8°C until expiration date in the resealable pouch with a desiccant to protect from moisture.

ASB-116 Canine AMH Assay Buffer

One bottle, 10 mL, containing a protein-based (BSA)-buffer with a non-mercury preservative. Store at 2-8°C until expiration date.

BCR-116 AMH Biotin Conjugate Ready-To-Use (RTU)

One bottle, 12 mL, containing biotinylated anti-AMH antibody in protein-based buffer with a non-mercury preservative. Store at 2-8°C until expiration date.

SAR-116 AMH Streptavidin-Enzyme Conjugate Ready-to-Use (RTU)

One bottle, 12 mL, containing Streptavidin-HRP (horseradish peroxidase) in a protein-based buffer and a non-mercury preservative. Store undiluted at 2-8°C until expiration date.

TMB-100 TMB Chromogen Solution

One bottle, 12 mL, containing a solution of tetramethylbenzidine (TMB) in buffer with hydrogen peroxide. Store at 2-8°C until expiration date.

STP-100 Stopping Solution

One bottle, 12 mL, containing 0.2 M sulfuric acid. Store at 2 to 30°C until expiration date.

WSH-100 Wash Concentrate A

One bottle, 60 mL, containing buffered saline with a nonionic detergent. Store at 2-30°C until expiration date. Dilute 25-fold with deionized water prior to use.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Microtitration plate reader capable of absorbance measurement at 450 nm, 405nm and 630 nm.
2. Microplate shaker.
3. Microplate washer.
4. Semi-automated/manual precision pipette to deliver 10–250 µL.
5. Repeater pipette.
6. Vortex mixer.
7. Deionized water.

WARNINGS AND PRECAUTIONS

For *in-vitro* use.

The following precautions should be observed:

a) Follow good laboratory practice.
b) Use personal protective equipment. Wear lab coats and disposable gloves when handling immunoassay materials.
c) Handle and dispose of all reagents and material in compliance with applicable regulations.
d) If external package is damaged, inspect the components inside for any other damage. Do not use if the components are damaged.

WARNING: Potential Biohazardous Material

This reagent may contain some animal and/or human source material (e.g. serum) or materials used in conjunction with human source materials. Handle all reagents and patient samples at a Biosafety Level 2, as recommended for any potentially infectious human material in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories," 6th Edition, 2020[12].

WARNING: Potential Chemical Hazard

Some reagents in this kit contain ProClin™ 300 and Sodium azide[13] as a preservative. ProClin™ 300 and Sodium azide in concentrated amounts are irritants to skin and mucous membranes.

For further information regarding hazardous substances in the kit, please refer to the MSDS, either at AnshLabs.com or by request.

SAMPLE COLLECTION AND PREPARATION

a) Serum is the recommended sample type.
b) Sample handling, processing, and storage requirements depend on the brand of blood collection tube that you use. Please reference the manufacturer's instructions for guidance. Each laboratory should determine the acceptability of its own blood collection tubes and serum separation products.
c) Samples may be stored at 4°C if assayed within 24 hours; otherwise, samples must be stored at -20°C or -80°C to avoid loss of bioactivity and contamination.
d) Avoid assaying lipemic, hemolyzed or icteric samples.
e) Avoid repeated freezing and thawing of samples. Thaw samples no more than 3 times.
f) For shipping, place specimens in leak proof containers in biohazard specimen bags with appropriate specimen identification and test requisition information in the outside pocket of the biohazard specimen bag. Follow DOT and IATA requirements when shipping specimens[14].

PROCEDURAL NOTES

1. A thorough understanding of this package insert is necessary for successful use of the Canine/Feline AMH ELISA assay. It is the laboratory's responsibility to validate the assay for their use. Accurate results will only be obtained by using precise laboratory techniques and following the package insert.
2. A calibration curve must be included with each assay.
3. Bring all kit reagents to room temperature (23 ± 2°C) before use. Thoroughly mix the reagents before use by gentle inversion. Do not mix various lots of any kit component and do not use any component beyond the expiration date.
4. Use a clean disposable pipette tip for each reagent, calibrator, control, or sample. Avoid microbial contamination of reagents, contamination of the substrate solutions with the HRP conjugates. The enzyme used as the label is inactivated by oxygen, and is highly sensitive to microbial contamination, sodium azide, hypochlorous acid and aromatic chlorohydrocarbons often found in laboratory water supplies. Use deionized water.
5. Incomplete washing will adversely affect the outcome and assay precision. Care should be taken to add TMB into the wells to minimize potential assay drift due to variation in the TMB incubation time. Avoid exposure of the reagents to excessive heat or direct sunlight.

PREPARATION OF REAGENTS

1. **Canine AMH Calibrators B-D and Canine AMH Controls I and II:** Tap and reconstitute Canine AMH Calibrators B-D and Canine AMH -ve Control and +ve Controls with **1 mL** deionized water. Solubilize for ten minutes, mix well before use.

2. **Wash Solution:** Dilute wash concentrate 25-fold with deionized water. The wash solution is stable for one month at room temperature when stored in a tightly sealed bottle.
3. **Microtitration Wells:** Select the number of coated wells required for the assay. The remaining unused wells should be placed in the resealable pouch with a desiccant. The pouch must be resealed to protect from moisture.

ASSAY PROCEDURE

Allow all specimens and reagents to reach room temperature and mix thoroughly by gentle inversion before use. Calibrators and unknowns should be assayed in duplicate.

1. Label the microtitration strips to be used.
2. Add **75 µL** of the **Canine AMH Assay Buffer** to each well using a repeater pipette.
3. Pipette **25 µL** of the Calibrators, controls, and unknowns to the appropriate wells.
4. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **60 minutes** at room temperature (23 ± 2°C).
5. Aspirate and wash each strip **5 times** with Wash Solution using an automatic microplate washer.
6. Add **100 µL** of the **Antibody-Biotin Conjugate RTU** to each well using a repeater pipette.
7. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **60 minutes** at room temperature (23 ± 2°C).
8. Aspirate and wash each strip **5 times** with the Wash Solution using an automatic microplate washer.
9. Add **100 µL** of the **Streptavidin-Enzyme Conjugate-RTU** to each well using a repeater pipette.
10. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **30 minutes** at room temperature (23 ± 2°C).
11. Aspirate and wash each strip **5 times** with the Wash Solution using an automatic microplate washer.
12. Add **100 µL** of the **TMB chromogen solution** to each well using a repeater pipette. Avoid exposure to direct sunlight.
13. Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **10-12 min** at room temperature (23 ± 2°C).
    NOTE: Visually monitor the color development to optimize the incubation time.
14. Add **100 µL** of the **Stopping solution** to each well using a repeater pipette. Read the absorbance of the solution in the wells **within 20 minutes,** using a microplate reader set to **450 nm.**
    ***NOTE**: Zero calibrator should be programmed as "**Blank**" while reading the optical density. If instrument has a wavelength correction, set the instrument to dual wavelength measurement at **450 nm** with background wavelength correction at **630 nm**. Extrapolate the calibration curve by a factor of 1.5.*

RESULTS

***NOTE:** The results in this package insert were calculated by plotting the **log optical density (OD) data on the y-axis and log AMH concentration on X-axis** using a linear regression curve fit. Alternatively, log vs. log cubic regression curve-fit can be used. Other data reduction methods may give slightly different results.*

1. Optimum results can be obtained at incubation temperature of **(23 ± 2°C)**.
2. Calculate the mean optical density (OD) for each calibrator, control, or Unknown.
3. Plot the log of the mean OD readings for each of the calibrators along the y-axis versus log of the AMH concentrations in ng/mL along the x-axis, using a linear regression curve-fit.
4. Determine the AMH concentrations of the unknowns from the calibration curve by matching their mean OD readings with the corresponding AMH concentrations.
5. Any sample reading higher than the highest calibrator should be appropriately diluted with the 0 ng/mL (CAL A / Sample Diluent) and re-assayed.
6. Any sample reading lower than the analytical sensitivity should be reported as such.

LIMITATIONS

The reagents supplied in this kit are optimized to measure AMH levels in Canine serum. If there is evidence of microbial contamination or excessive turbidity in a reagent, discard the vial. For assays employing antibodies, the possibility exists for interference by heterophile antibodies in the samples[15].

QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to ensure proper performance.
- Each laboratory should establish internal AMH controls ranges. The results should fall within established confidence limits.
- A full calibration curve, and controls, should be included in each assay.
- TMB should be colorless. Development of any color may indicate reagent contamination or instability.

REPRESENTATIVE CALIBRATION CURVE DATA

Well Number	Well Contents Calibrators	Mean Absorbance	Conc. (ng/mL)
A1, A2	A	*0.029 (Blank)*	0
B1, B2	B	0.14	0.3
C1, C2	C	0.55	1.2
D1, D2	D	3.7	11.9

***CAUTION:** The above data must not be employed in lieu of data obtained by the user in the laboratory.*

ANALYTICAL CHARACTERISTICS

All analytical characteristics are stated in ng/mL (1 ng/mL AMH = 7.14 pM)

Analytical Sensitivity:

The analytical sensitivity in the Canine/Feline AMH ELISA assay, as calculated by the interpolation of mean plus two standard deviations of 16 replicates of calibrator A (0 ng/mL) and calibrator B (0.3 ng/mL), is **0.015 ng/mL**.

Imprecision:

Reproducibility of the Canine/Feline AMH ELISA assay was determined in a study using four samples. The study included two controls (n=48), sample-1 (n=48) and sample-2 (n=40) over 12 runs. Representative data were calculated and are presented in the following table.

Sample	Mean (ng/mL)	Within run		Between run		Total	
		SD	CV	SD	CV	SD	CV
Control I	0.8	0.04	4.9%	0.03	4.2%	0.05	6.4%
Control II	2.4	0.07	2.8%	0.07	2.9%	0.10	4.0%
Sample-1	3.2	0.08	2.7%	0.06	1.9%	0.10	3.3%
Sample-2	8.2	0.43	5.2%	0.00	0.0%	0.43	5.2%

Linearity:

Multiple dilutions of the two canine samples and Calibrator D containing various canine AMH levels were diluted with calibrator A. The % recovery on individual samples is represented in the following table.

Sample ID	Dilution factor (1 in X)	Expected Value (ng/mL)	Observed Value (ng/mL)	%Recovery	Average %Recovery
Calibrator D	Neat	11.90			91%
	2	5.95	6.44	108%	
	4	2.98	2.83	95%	
	8	1.49	1.26	85%	
	16	0.74	0.60	81%	
	32	0.37	0.32	85%	
Sample-1	50	10.76			95%
	100	5.38	5.30	98%	
	200	2.69	2.65	99%	
	400	1.35	1.23	92%	
	800	0.67	0.64	94%	
	1600	0.34	0.32	94%	
Sample -2	8	9.48			95%
	16	4.74	4.86	103%	
	32	2.37	2.33	98%	
	64	1.19	1.16	98%	
	128	0.59	0.55	92%	
	256	0.30	0.25	86%	

Analytical Specificity:

This monoclonal antibody pair used in the Canine/Feline AMH assay detects mature and pro+mature AMH and does not detect FSH, LH, Inhibin A, Inhibin B, Activin A, Activin B, Activin AB and pro AMH. The antibody pair detects feline and exotic feline samples.

Cross-reactant	Concentration	% Cross-reactivity
FSH	50 ng/mL	ND
LH	50 ng/mL	ND
Inhibin A	50 ng/mL	ND
Inhibin B	50 ng/mL	ND
Activin A	58 ng/mL	ND
Activin B	50 ng/mL	ND
Activin AB	10 ng/mL	ND
hAMH, Mature	25 ng/ml	100%
hAMH, Pro+Mature	33 ng/mL	100%
hAMH, Pro	31 ng/mL	ND

ND= Non-Detectable

Interference:

When hemoglobin, biotin, intralipids and bilirubin were added at a concentration greater than two folds of their physiological concentration to control sample, average AMH concentration were within ± 20% of the control as represented in the following table.

Interferent	Interferent Dose	Analyte Conc. (ng/mL)	Spiked Sample Value (ng/mL)	% Difference
Hemoglobin	1mg/mL	1.54	1.21	-21.4
	0.5mg/mL	1.71	1.62	-5.3
	0.1mg/mL	1.66	1.67	0.6
Biotin	1200 ng/mL	1.52	1.44	-5.3
	600 ng/mL	1.53	1.48	-3.3
	200 ng/mL	1.64	1.63	-0.6
Intralipids	20 mg/mL	1.42	1.56	9.9
	10 mg/mL	1.55	1.63	5.2
	5 mg/mL	1.66	1.69	1.8
Bilirubin	0.66 mg/mL	1.03	1.18	14.6
	0.2 mg/mL	1.51	1.55	2.6

Reference Ranges: Canine

Canine samples were analyzed using Ansh Labs Canine/Feline AMH ELISA. The expected ranges were calculated using 95% non-parametric estimation in the Canine AMH ELISA kit using Analyse-It® for Microsoft Excel and should be used as guidance only.

ID	n	Mean Age (Years)	Mean AMH (ng/mL)	Range (ng/mL)
Intact Males	32	6.7	8.25	0.2 – 73.4
Castrated Males	29	7.2	0.15	<0.15
Intact Females	30	3.5	1.22	0.2 – 5.0
Spayed Females	30	10.3	0.15	<0.15

Note: *It is recommended that each laboratory should determine the reference range(s) for its own patient population. The results of this assay should be used in conjunction with other relevant and applicable clinical information.*

Reference Ranges: Feline

Feline intact queens, ovariohysterectomized queens and neutered tomcat samples were tested using Ansh Labs Canine/Feline AMH ELISA. Reference ranges based on nonparametric (intact queens) or robust parametric (ovariohysterectomized queens) 90% CIs for AMH (ng/mL), were assessed using the Reference Value Advisor macro in Excel. Intact queens ranged from 2 to 72 months in age. The ages of ovariohysterectomized queens were unknown. The number of samples from individual cats (n) used are shown along with each of the respective ranges as described by Johnson et al [11].

ID	n	AMH Range (ng/mL)
Intact queens	205	0.27 – 18.9
Ovariohysterectomized queens	49	0.01 – 0.16
Neutered tomcats	17	0.01 – 0.23

Note: *It is recommended that each laboratory should determine the reference range(s) for its own patient population. The results of this assay should be used in conjunction with other relevant and applicable clinical information.*

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FOR RESEARCH USE ONLY

This assay is intended for in-vitro research use only.

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