Equine AMH ELISA

AL-115

INTENDED USE

The Equine Anti-Müllerian hormone (AMH) enzyme-linked immunosorbent assay (ELISA) kit provides materials for the quantitative measurement of AMH in equine serum and other biological fluids.

SUMMARY AND EXPLANATION

Anti-Müllerian hormone is a 140 kDa glycoprotein that is produced during normal embryogenesis by the Sertoli cells of the embryonic testis, causes the involution of the Müllerian duct, and inhibits female gonadogenesis by inducing apoptosis of target gonadal cells. It belongs to the transforming growth factor-β super family. AMH causes apoptosis of specific Anti-Müllerian inhibiting substance (MIS) receptor-bearing cells, while having no effect on cells without receptors. AMH is also expressed in granulosa cells of preantral and small antral follicles in the ovary, and AMH inhibits recruitment of primordial follicles into the pool of growing follicles and decreases responsiveness of growing follicles to FSH.

A quantitative testing for equine Anti-Mullerian hormone (AMH) can be used for diagnosis granulosa cell tumors in mares.[1] The panel of tests consisting of AMH/inhibin/testosterone is 98% accurate in detecting tumors. Unlike inhibin and testosterone, AMH level is not affected by pregnancy, and so is the most useful diagnostic analyte in these situations.[2] AMH is highly precise test for the determination of cryptorchidism in geldings, especially in prepubertal colts, before testosterone levels have begun to rise.[3] AMH has a long half-life (1.5-2 days) and therefore concentrations to reach baseline after castration takes about 10-14 days[1, 3].

PRINCIPLE OF THE TEST

The Equine AMH ELISA is a quantitative three-step sandwich type immunoassay. In the first step serially diluted Calibrators 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-115G Equine AMH Calibrator G (Lyophilized)

Reconstitute the Equine AMH Calibrator G with 1 mL of deionized water. Solubilize, mix well, and use after reconstitution. The concentration of Calibrator G in the stock solution is approximately 15 ng/mL. Refer to the **vial label** for exact concentration. Store unopened at 2-8°C until the expiration date. Reconstitute calibrator G with 1 mL deionized water. Solubilize, mix well, and use after reconstitution. Aliquot and freeze in eppendorf vials for multiple use. Alternatively, freeze in the same vial within 2 hours of reconstitution. Avoid repeated freeze thaws.

CTR-115 Equine AMH Control (Lyophilized)

Reconstitute the Equine AMH Control with 1 mL of deionized water. Solubilize, mix well, and use after reconstitution. The concentration of the Control in the stock solution is approximately 7 ng/mL. Refer to the **vial label** for exact concentration. Store unopened at 2-8°C until the expiration date. Reconstitute control with 1 mL deionized water. Solubilize, mix well, and use after reconstitution. Aliquot and freeze in eppendorf 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 stripholder, 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-113 AMH Assay Buffer

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

BCR-115 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-115 AMH Streptavidin-Enzyme Conjugate-Ready-to-Use (RTU)

One amber 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, 11 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, 11 mL, containing 0.2 M sulfuric acid. Store at 2°C - 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. Vortex mixer.
6. Deionized water.

WARNINGS AND PRECAUTIONS

For *in-vitro research* 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.

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," 5th Edition, 2007[4].

WARNING: Potential Chemical Hazard

Some reagents in this kit contain ProClin 300 and Sodium azide[5] 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[6].

PROCEDURAL NOTES

1. A thorough understanding of this package insert is necessary for successful use of the Equine 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 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. **Equine AMH Calibrators B-G:**

a) Tap and reconstitute Equine AMH Calibrator G with 1 mL deionized water. Solubilize for ten minutes and mix well before use.

b) Prepare five eppendorf tubes and label them as Cal A, Cal B, Cal C, Cal D, Cal E and Cal F.

c) Add 1 mL of AMH Calibrator A/Sample Diluent to each polystyrene tube labeled Cal A-F.

d) Add 500 µl of reconstituted AMH Calibrator G (from step a) to the tube labeled Cal F. Vortex and mix the content in the tube thoroughly before the next dilution transfer.

e) Add 500 µl of Cal F (from step d) to the tube labeled Cal E. Vortex and mix the content in the tube thoroughly before the next dilution transfer.

f) Add 500 µl of Cal E (from step e) to the tube labeled Cal D. Vortex and mix the content in the tube thoroughly before the next dilution transfer.

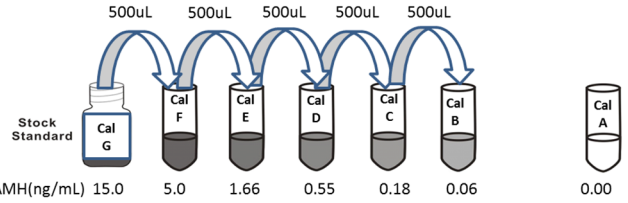
g) Add 500 µl of Cal D (from step f) to the tube labeled Cal C. Vortex and mix the content in the tube thoroughly before use.

h) Add 500 µl of Cal C (from step g) to the tube labeled Cal B. Vortex and mix the content in the tube thoroughly before use.

i) The tube labeled Cal A contains 1 mL AMH Calibrator A/Sample Diluent and has 0 AMH concentrations and should be used as Blank.

j) The **Equine** Calibrators A-G for instance should read as 0.0 ng/mL, 0.06 ng/mL, 0.18 ng/mL, 0.55 ng/mL, 1.66 ng/mL, 5.0 ng/mL and 15 ng/mL. Aliquot and freeze immediately for multiple uses. Avoid repeated freeze thaws. Frozen aliquots at -20°C are good for one year.

k) The AMH concentration in the **Equine** AMH calibrator G is traceable to the manufacturer's working calibrators. Values assigned by other methodologies may be different. Such differences, if present, may be caused by inter-method bias.



2. **Wash Solution:** Dilute wash concentrate 25-fold with deionized water. The wash solution is stable for one month at room temperature (23±2°C) 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.

NOTE: All serum samples reading higher than the highest calibrator should be mixed and diluted in the 0 ng/mL Calibrator A/Sample diluent prior to assay. Granulosa Cell Tumor (GCT) cyst fluid may be diluted 1:250 or higher. Serum specimens from Stallion may be diluted 1:5 or higher.

1. Label the microtitration strips to be used.
2. Pipette **50 µL** of the Calibrators (Cal A-G), Control and Unknowns to the appropriate wells.
3. Add **50 µL** of the AMH Assay Buffer to each well using a repeater pipette.
4. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **120 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 precision pipette. Avoid exposure to direct sunlight.
13. Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **8-10 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 precision 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.*

RESULTS

***NOTE:** The results in this package insert were calculated by plotting the data on a log vs. log scale and using a cubic regression curve fit. Other data reduction methods may give slightly different results.*

1. Calculate the mean optical density (OD) for each calibrator, Control, or Unknown.
2. 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 cubic regression curve fit.
3. Determine the AMH concentrations of the unknowns from the calibration curve by matching their mean OD readings with the corresponding AMH concentrations.
4. Any sample reading higher than the highest Calibrator should be appropriately diluted with the 0 ng/mL (CAL A / Sample Diluent) and re-assayed.
5. Any sample reading lower than the analytical sensitivity should be reported as such.
6. Multiply the value by a dilution factor, if required.

LIMITATIONS

The reagents supplied in this kit are optimized to measure AMH levels in equine serum, plasma, and cyst fluid. 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.

QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to assure proper performance.
- Each laboratory should establish internal AMH controls ranges. The results should fall within established confidence limits.
- A full calibration curve, and control, 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	0.004 (Blank)	0
B1, B2	B	0.026	0.058
C1, C2	C	0.074	0.17
D1, D2	D	0.213	0.52
E1, E2	E	0.594	1.56
F1, F2	F	1.552	4.66
G1, G2	G	3.228	14.0

***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/L).

Analytical Sensitivity:

The analytical sensitivity in the assay as calculated by the interpolation of mean plus two standard deviation of 16 replicates of calibrator A (0 ng/mL) and low calibrator (0.056 ng/mL) is 0.009 ng/mL.

Imprecision:

Reproducibility of the Equine AMH ELISA assay was determined in a study using kit control and 2 samples pools. The study included a total of 6 assays, four replicates of each per assay (n=24). Representative data were calculated and are presented in the following table.

Sample	Mean Conc. (ng/mL)	Within run		Between run		Total	
		SD	CV	SD	CV	SD	CV
Control	6.94	0.21	2.97%	0.14	2.00%	0.25	3.58%
Pool-1	1.47	0.04	2.57%	0.03	1.80%	0.05	3.14%
Pool-2	3.61	0.1	2.89%	0.09	2.53%	0.14	3.84%

Recovery:

Equine AMH Calibrator A (0 ng/mL) was spiked with high reading equine samples at three different levels and assayed. The spike recovery is shown below.

Spike Sample ID	Cal. A Conc. (ng/mL)	% Spike	Expected Conc. (ng/mL)	Observed Conc. (ng/mL)	% Recovery	Average % Recovery
S1	0	5	0.74	0.78	105%	104%
		10	1.39	1.45	104%	
		15	2.14	2.18	102%	
S2	0	5	1.28	1.40	109%	105%
		10	2.41	2.56	106%	
		15	3.69	3.67	100%	

Linearity:

Multiple dilutions of equine serum samples and equine follicular fluid samples containing various AMH levels were performed in Calibrator A/Sample diluent. The % recovery is represented in the following table.

Sample ID	Dilution factor	Expected (ng/mL)	Observed (ng/mL)	% Recovery	Average % Recovery
S1	1:5	13.93			101%
	1:10	6.97	6.98	100%	
	1:20	3.48	3.45	99%	
	1:40	1.74	1.76	101%	
	1:80	0.87	0.89	103%	
	1:160	0.44	0.45	103%	
S2	1:10	12.03			107%
	1:20	6.02	6.28	104%	
	1:40	3.01	3.18	106%	
	1:80	1.50	1.62	108%	
	1:160	0.75	0.84	111%	
	1:320	0.38	0.40	106%	
FF-1	1:40	8.91			102%
	1:80	4.46	4.54	102%	
	1:160	2.23	2.23	100%	
	1:320	1.11	1.17	105%	
	1:640	0.56	0.57	102%	
	1:1280	0.28	0.28	101%	
FF-2	1:40	14.81			104%
	1:80	7.40	7.46	101%	
	1:160	3.70	3.78	102%	
	1:320	1.85	1.94	105%	
	1:640	0.93	0.99	106%	
	1:1280	0.46	0.50	108%	

Interference:

When potential interferents (hemoglobin, triglycerides, and bilirubin) were added at two times their physiological concentration to control sample, Equine AMH concentration were within ± 7% of control as represented in the following table.

Interferent	Interferent Dose	Sample#	Unspiked sample (ng/mL)	Spiked sample (ng/mL)	Difference (ng/mL)	% Difference
Hemoglobin	1.35 mg/mL	1	3.21	3.00	-0.21	-6.4
		2	3.06	3.03	-0.03	-1.1
		3	13.70	14.22	0.52	3.8
Triglycerides	5.0 mg/mL	1	3.21	3.13	-0.07	-2.3
		2	3.06	3.12	0.06	2.0
		3	13.70	14.35	0.65	4.8
Bilirubin	0.6 mg/mL	1	3.07	2.86	-0.21	-6.7
		2	3.12	3.05	-0.07	-2.2
		3	13.66	13.58	-0.08	-0.6

Analytical Specificity:

The monoclonal antibody pair used in the Equine AMH assay does not detect FSH, LH, Inhibin A, Inhibin B, Activin A, Activin B, and Activin AB.

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

ND= Non-Detectable

Reference Range:

Expected Equine AMH concentration was calculated by evaluating 35 equine intact female samples, 14 equine intact male samples and 16 equine castrated male samples in Ansh Labs Equine AMH ELISA. The expected ranges were calculated using 95% non-parametric estimation using Analyse-It® for Microsoft Excel.

Sample	n	Median (ng/mL)	95% CI (ng/mL)
Equine Intact Female	35	3.2	0.3 – 11.6
Equine Intact Male	14	166.2	4.0 – 255.9
Equine Castrated Male	16	0.11	0.05 – 0.26

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.

REFERENCES

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