Bovine AMH ELISA

RUO

AL-114

INTENDED USE

The Bovine Anti-Müllerian hormone (AMH) enzyme-linked immunosorbent assay (ELISA) kit provides materials for the quantitative measurement of AMH in bovine EDTA plasma and other biological fluids. This kit is intended for laboratory research use only and is not for use in diagnostic or therapeutic procedures.

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.

Based on the physiology of AMH, the veterinary and management utility of AMH levels in female cattle is increasingly being assessed, particularly as reagents for quantitative measurement of AMH in peripheral circulation are becoming more widely available. Even at this early stage of investigation, AMH clearly has utility. AMH levels are directly related to the size of the antral follicular pool and is a powerful predictor of response to ovarian stimulation. AMH levels prior to ovarian stimulation are directly correlated with the resultant number of ovulations.(1-4)

AMH is also a marker of the quality of developing follicles. Healthy follicles at all stages of secondary and early antral development produce more AMH than unhealthy follicles. AMH has been shown to be a useful predictor of embryo yield. AMH concentration, measured in the plasma of donor dairy cows during first lactation and several months before the start of the embryo production campaigns, is highly correlated with the maximal number of recovered and transferable embryos per cow.(5)

Neoplastic granulosa cells in surgical biopsy of granulosa-thecal cell tumors and processed for immunohistochemistry are clearly labeled with AMH antibody and plasma AMH levels are significantly higher in cows with granulosa-thecal cell tumors than healthy cows with a functional corpus luteum, cows with ovarian cysts or super-ovulated cows.(6)

Castrate bull (veterinary use) and Freemartin (research use) AMH testing is a cost-effective substitute for expensive surgical or imagining assessments.(7-8)

PRINCIPLE OF THE TEST

The Bovine 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 Calibrators 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 / Pro-Clean™ 400. Store unopened at 2-8°C until the expiration date.

CAL-114B – CAL-114F Bovine AMH Calibrators B - F (Lyophilized)

Five vials, labeled B-F, containing concentrations of approximately 13.0 – 2200.0 pg/mL Bovine AMH in protein-based buffer and ProClin™ 300 / Pro-Clean™ 400. Refer to the **calibration card** for exact concentration. Store unopened at 2-8°C until the expiration date. Reconstitute the calibrators B-F with **1 mL** of deionized water. Solubilize, mix well, and use after reconstitution. Aliquot and freeze immediately for multiple use and discard after run. Avoid repeated freeze thaws.

CTR-114-I & CTR-114-II Bovine AMH Controls I & II (Lyophilized)

Two vials, labeled Levels I and II containing low and high Bovine AMH concentrations in protein-based buffer and ProClin™ 300 Pro-Clean™ 400. Refer to the **calibration card** for exact concentrations. Store unopened at 2 to 8°C until the expiration date. Reconstitute control Levels I and II with **1 mL** deionized water. Solubilize, mix well, and use after reconstitution. Aliquot and freeze immediately for multiple use and discard after run. Avoid repeated freeze thaws.

PLT-114 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-114 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-114 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-114 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, 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. 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 and human source material (e.g. serum or plasma) 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[9].

WARNING: Potential Chemical Hazard

Some reagents in this kit contain ProClin™ 300 / Pro-Clean™ 400 and Sodium azide[10] as a preservative. ProClin™ 300 / Pro-Clean™ 400 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) **EDTA Plasma and serum** are the recommended sample types. Other biological fluids may produce different results.

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 EDTA plasma 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.[11]

PROCEDURAL NOTES

1. A thorough understanding of this package insert is necessary for successful use of the Bovine 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. **Bovine AMH Calibrators B-F:** Tap and reconstitute Bovine AMH Calibrators B-F with 1 mL deionized water. Solubilize for ten minutes, mix well before use.
2. **Bovine AMH Controls I and II:** Tap and reconstitute Bovine AMH Controls I and II with 1 mL deionized water. Solubilize for ten minutes, mix well before use.
3. **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.
4. **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:

a) All male samples should be diluted 1:20 in Calibrator A/Sample diluent prior to assay.

b) All samples reading higher than the highest calibrator should be mixed and diluted in the 0 pg/mL Calibrator A/Sample diluent prior to assay.

1. Label the microtitration strips to be used.
2. Pipette **50 µL** of the **Calibrators**, **Controls and Unknown sample** 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 **(350 µL/per well)** using an automatic microplate washer.
6. Add **100 µL** of the **AMH 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 **(350 µL/per well)** using an automatic microplate washer.
9. Add **100 µL** of the **AMH 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 **(350 µL/per well)** 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 30 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 **log optical density (OD) data on the y-axis and log AMH concentration on X-axis** using a cubic regression curve-fit. Alternatively, log vs. log quadratic 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 pg/mL along the x-axis, using a cubic 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 pg/mL (CAL A / Sample Diluent) and re-assayed.
6. Any sample reading lower than the analytical sensitivity should be reported as such.
7. Multiply the value by a dilution factor, if required.

LIMITATIONS

The reagents supplied in this kit are optimized to measure AMH levels in bovine EDTA plasma and 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[12].

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 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 (pg/mL)
A1, A2	A	0.034 (*Blank*)	0
B1, B2	B	0.032	13.5
C1, C2	C	0.149	63.4
D1, D2	D	0.461	206.0
E1, E2	E	1.406	710.0
F1, F2	F	3.463	2240.0

***NOTE:** CAL B – F mean absorbance data listed above is after blank subtraction.*
***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 pg/mL (1 pg/mL AMH = 0.00714 pM)

Analytical Sensitivity

The analytical sensitivity in the assay as calculated by the interpolation of mean plus two standard deviation of 14 replicates of calibrator A (0 pg/mL) and low calibrator (39 pg/mL) is 11 pg/mL.

Imprecision

Three control samples were assayed in 24 replicates to determine intra-assay precision. Values obtained are shown below.

Sample	Mean Conc. (pg/mL)	SD	% CV
Pool-1	621	0.018	2.92
Pool-2	1759	0.032	2.54

Linearity

Multiple dilutions of five bovine plasma samples containing various AMH levels were performed in Calibrator A/sample diluent. The samples were assayed and the % recovery was calculated. The % recovery is represented in the following table.

Sample	Dilution Factor	Expected Conc. (pg/mL)	Observed Conc. (pg/mL)	% Recovery
1	Neat	7468	NA	NA
	1:2	3734	3731	100%
	1:4	1867	1973	106%
	1:8	934	980	105%
	1:16	467	511	109%
2	Neat	5010	NA	NA
	1:2	2505	2450	98%
	1:4	1253	1261	101%
	1:8	626	665	106%
	1:16	313	334	107%
3	Neat	4530	NA	NA
	1:2	2265	2199	97%
	1:4	1133	1154	102%
	1:8	566	588	104%
	1:16	283	297	105%
Male	1:4	1913	NA	NA

	1:8	956	940	98%
	1:16	478	471	99%
	1:32	239	254	106%
Female	Neat	1655	NA	NA
	1:2	828	837	101%
	1:4	414	430	104%
	1:8	207	224	108%

Recovery

A high reading neat female serum sample was spiked into a low reading neat female serum sample at two different levels and assayed. The spike recovery is shown below.

Sample	Endogenous Conc.(ng/mL)	Expected Conc. (ng/mL)	Observed Conc. (ng/mL)	% Recovery
1	421.393	478.144	483.655	101%
		534.896	552.673	103%

Analytical Specificity:

This monoclonal antibody pair used in the Bovine AMH assay detects human AMH and does not detect FSH, LH, Inhibin A, Inhibin B, Activin A, Activin B, Activin AB. The antibody pair detects caprine 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

ND= Non-Detectable

Interference

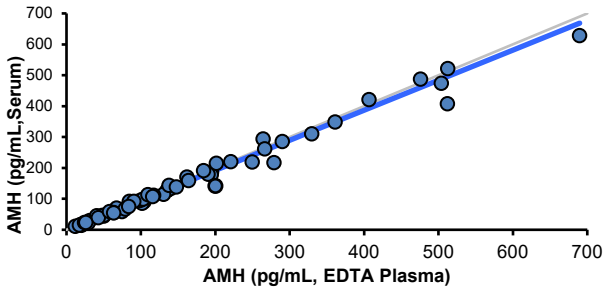
When hemoglobin, biotin, bilirubin and intralipids were added at a concentration greater than two folds of their physiological concentration to control sample, average Bovine AMH concentration were within ±15% of the control as represented in the following table.

Interferent	Interferent Dose	Analyte Conc. (pg/mL)	Spiked Sample Value (pg/mL)	% Difference
Hemoglobin	1 mg/mL	2917.1	3007.2	3.1
	0.5 mg/mL	3037.4	3059.7	0.7
	0.1 mg/mL	3049.1	3159.8	3.6
Hemoglobin	1 mg/mL	2466.7	2575.6	4.4
	0.5 mg/mL	2620.4	2538.8	-3.1
	0.1 mg/mL	2694.8	2729.7	1.3
Biotin	1200 ng/mL	2956.8	3034.3	2.6
	600 ng/mL	3077.3	3210.8	4.3
Biotin	1200 ng/mL	2585.9	2573.6	-0.5
	600 ng/mL	2655.2	2696.3	1.5
Bilirubin	0.66 mg/mL	2574.6	2616.8	1.6
	0.2 mg/mL	3014.6	3090.5	2.5
Bilirubin	0.66 mg/mL	2000.1	2032.2	1.6
	0.2 mg/mL	2632.7	2676.1	1.6
Intralipids	20 mg/mL	1438.6	1265.4	-12.0
	10 mg/mL	1512.4	1330.6	-12.0
	5 mg/mL	1549.4	1525.1	-1.6

Sample Type

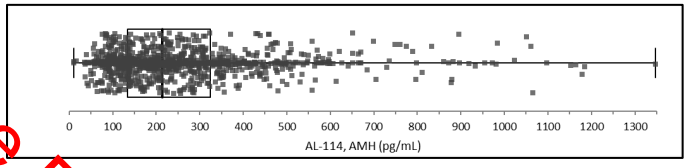
Sixty matched serum and EDTA plasma specimens in the range of 11.54 – 689.79 pg/mL were compared in the assay. Passing Bablok analysis of the results yielded the following Regression:

Serum = 0.97 (EDTA Plasma) – 2.15, (r=0.99; P<0.0001)



Expected Values:

Expected Bovine AMH concentration was calculated by evaluating 1084 Holstein cow samples in Ansh Labs Bovine AMH ELISA. The frequency distribution was calculated using Analyse-It® for Microsoft Excel and is shown below.



	Bovine AMH (pg/mL)		
	Mean	Median	Range
1084	258.9	213.4	10.8 – 1346.6

Quantile	Bovine AMH (pg/mL)
0.100	92.10
0.200	121.54
0.300	145.57
0.400	180.95
0.500	213.37
0.600	252.66
0.700	297.86
0.800	355.73
0.900	477.64

REFERENCES

1. Kitahara G, Kamata R, Sasaki Y, El-Shaikh Ali H, Mido S, Kobayashi I, Hemmi K, Osawa T 2016 Changes in peripheral anti-Mullerian hormone concentration and their relationship with testicular structure in beef bull calves. Domestic Animal Endocrinology 57:127-132
2. Scarlet D, Aurich C, Ille N, Walter I, Weber C, Pieler D, Peinhopf W, Wohlsein P, Aurich J+ 2017 Anti-Muellerian hormone, inhibin A, gonadotropins, and gonadotropin receptors in bull calves after partial scrotal resection, orchidectomy, and Burdizzo castration. Theriogenology 87:242-249
3. Souza AH, Carvalho PD, Rozner AE, Vieira LM, Hackbart KS, Bender RW, Dresch AR, Verstegen JP, Shaver RD, Wiltbank MC 2015 Relationship between circulating anti-Mullerian hormone (AMH) and superovulatory response of high-producing dairy cows. Journal of Dairy Science 98:169-178
4. Rico C, Drouilhet L, Salvetti P, Dalbies-Tran R, Jarrier P, Touze J-L, Pillet E, Ponsart C, Fabre S, Monniaux D 2012 Determination of anti-Mullerian hormone concentrations in blood as a tool to select Holstein donor cows

for embryo production: from the laboratory to the farm. Reprod Fertil Dev 24:932-944

5. Abdel Aziz RL, Khalil AAY, Abdel-Wahab A, Hassan NY, Abdel-Hamied E, Kasimanickam RK 2017 Relationship among circulating anti-Mullerian hormone, insulin like growth factor 1, cadmium and superovulatory response in dairy cows. Theriogenology 100:72-79
6. Rico C, Medigue C, Fabre S, Jarrier P, Bontoux M, Clement F, Monniaux D 2011 Regulation of Anti-Mulerian Hormone Production in the Cow: A Multiscale Study at Endocrine, Ovarian, Follicular, and Granulosa Cell Levels. Biology of Reproduction 84:560-571
7. Monniaux D, Barbey S, Rico C, Fabre S, Gallard Y, Larroque H 2010 Anti-Mllerian hormone: a predictive marker of embryo production in cattle. Reprod Fertil Dev 22:1083-1091
8. Kitahara G, Nambo Y, El-Sheikh Ali H, Kajisa M, Tani M, N K, Kamimura S 2012 Anti-Mullerian Hormone Profiles as a Novel Biomarker to Diagnose Granulosa-theca Cell Tumors in Cattle. Journal of Reproduction and Development 58:98-104
9. HHS Publication, 65th ed., 2020. Biosafety in Microbiological and Biomedical Laboratories. Available https://www.cdc.gov/labs/pdf/SF__19_308133-A_BMBL6_00-BOOK-WEB-final-3.pdf
10. DHHS (NIOSH) Publication No. 78-127, August 1976. Current Intelligence Bulletin 13 - Explosive Azide Hazard. Available http://www.cdc.gov/niosh.
11. Clinical and Laboratory Standards Institute (CLSI). Procedures for the Handling and Processing of Blood Specimens for Common Laboratory Tests; Approved Guideline—Fourth Edition. CLSI document H18-A4. Wayne, PA: Clinical and Laboratory Standards Institute; 2010.Kricka L. Interferences in immunoassays – still a threat. Clin Chem 2000; 46: 1037–1038.

Manufactured by:
Ansh Labs
445 Medical Center Blvd.
Webster, TX 77598-4217, U.S.A.