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.

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 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 Pro-Clean 400. Store unopened at 2-8°C until the expiration date.

CAL-115F Equine AMH Calibrator F (Lyophilized)

Reconstitute the Equine AMH Calibrator F with 1 mL of deionized water. Solubilize, Mix well and use after reconstitution. The concentration of the calibrator F 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.

PLT-115 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 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 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[1].

WARNING: Potential Chemical Hazard

Some reagents in this kit contain Pro-Clean 400 and Sodium azide[2] as a preservative. 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) Serum is the recommended sample type.

b) Use the following recommendations for handling, processing and storing blood samples.[3]

- Allow samples to clot for two hours at room temperature or overnight at 4°C and follow blood collection tube manufacturer's recommendations for centrifugation. Keep tubes stoppered at all times. Within two hours after centrifugation, transfer at least 500 µL of cell free sample to a storage tube. Tightly stopper the tube immediately.
- Samples if used within 24 hours may be stored at 4°C, otherwise samples must be stored at -20°C or -80°C to avoid loss of bioactivity and contamination.
- Remove residual fibrin and cellular matter prior to analysis.

c) Avoid assaying lipemic, hemolyzed or icteric samples.

d) Each laboratory should determine the acceptability of its own blood collection tubes and serum separation products.

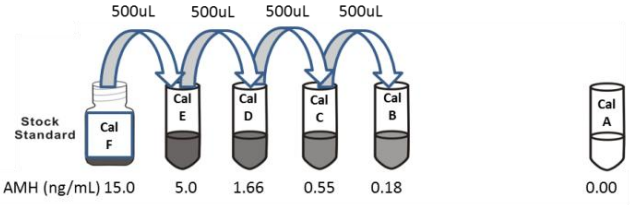
e) Avoid repeated freezing and thawing of samples. Thaw samples no more than 3 times.

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. **AMH Calibrators F:**
   a) Tap and reconstitute AMH Calibrator F with 1 mL deionized water. Solubilize for ten minutes, mix well before use.
   b) Prepare five polystyrene tubes and label them as Cal A, Cal B, Cal C, Cal D and Cal E.
   c) Add 1 mL of AMH Calibrator A/Sample Diluent to each polystyrene tube labeled Cal A-E.
   d) Add 500 µl of reconstituted AMH Calibrator F (from step a) to the tube labeled Cal E. Vortex and mix the content in the tube thoroughly before the next dilution transfer.
   e) Add 500 µl of Cal E (from step d) to the tube labeled Cal D. Vortex and mix the content in the tube thoroughly before the next dilution transfer.
   f) Add 500 µl of Cal D (from step e) to the tube labeled Cal C. Vortex and mix the content in the tube thoroughly before the next dilution transfer.
   g) Add 500 µl of Cal C (from step f) to the tube labeled Cal B. Vortex and mix the content in the tube thoroughly before use.
   h) The tube labeled Cal A contains 1 mL AMH Calibrator A/Sample Diluent and has 0 AMH concentrations and should be used as Blank.
   i) The Calibrators A-F for instance should read as 0.0 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.
   j) The Equine AMH concentration in the AMH calibrators F 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 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.

1. Label the microtitration strips to be used.
2. Pipette **50 µL** of the Calibrators (Cal A-F) 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.
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.
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.
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 **10-12 min** at room temperature.
    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: While reading the absorbance of the microtitration well, it is necessary to program the zero calibrator (Cal A) as a "Blank".

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 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[4].

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	Mean Absorbance	Conc (ng/mL)
A1, A2	**Calibrators** A	0.090 (*Blank*)	0
B1, B2	B	0.048	0.18
C1, C2	C	0.137	0.55
D1, D2	D	0.394	1.66
E1, E2	E	1.12	5
F1, F2	F	2.83	15

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)

REFERENCES

1. HHS Publication, 5th ed., 2007. Biosafety in Microbiological and Biomedical Laboratories. Available http://www.cdc.gov/biosafety/publications/bmbl5/BMBL5
2. DHHS (NIOSH) Publication No. 78–127, August 1976. Current Intelligence Bulletin 13 - Explosive Azide Hazard. Available http:// www.cdc.gov/niosh.
3. Approved Guideline – Procedures for the Handling and Processing of Blood Specimens, H18-A3. 2004. Clinical and Laboratory Standards Institute.
4. Kricka L. Interferences in immunoassays – still a threat. Clin Chem 2000; 46: 1037–1038.

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