Rat and Mouse AMH ELISA

AL-113

INTENDED USE

The Rat and Mouse Anti-Müllerian hormone (AMH) enzyme-linked immunosorbent assay (ELISA) kit provides materials for the quantitative measurement of AMH in rat and mouse 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 Rat and Mouse 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-113H Rat and Mouse AMH Calibrator H (Lyophilized)

Reconstitute the Rat and Mouse AMH Calibrator H with 1 mL of deionized water. Solubilize, mix well, and use after reconstitution. The concentration of the calibrator H in the stock solution is approximately 20 ng/mL. Refer to the **vial label** for exact concentration. Store unopened at 2-8°C until the expiration date.

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-113 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-113 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 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.

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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

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.

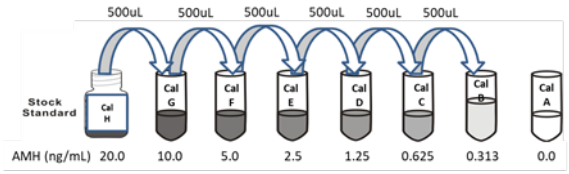
f) For shipping, place specimens in leak proof containers in biohazard specimen bags with appropriate specimen identification and test requisition information in the outside pocket of the biohazard specimen bag. Follow DOT and IATA requirements when shipping specimens.

PROCEDURAL NOTES

1. A thorough understanding of this package insert is necessary for successful use of the Rat and Mouse 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 H:**
   a. Tap and reconstitute Rat and Mouse AMH Calibrator H with 1 **mL** deionized water. Solubilize for ten minutes, mix well before use.
   b. Prepare seven polystyrene tubes and label them as Cal A, Cal B, Cal C, Cal D, Cal E, Cal F and Cal G.
   c. Add **500 µl** of AMH/MIS Calibrator A/Sample Diluent to each polystyrene tube labeled Cal A-G.
   d. Add **500 µl** of reconstituted AMH Calibrator H (from step a) to the tube labeled Cal G. Vortex and mix the content in the tube thoroughly before the next dilution transfer.
   e. Add **500 µl** of Cal G (from step d) to the tube labeled Cal F. Vortex and mix the content in the tube thoroughly before the next dilution transfer.
   f. Add **500 µl** of Cal F (from step e) to the tube labeled Cal E. Vortex and mix the content in the tube thoroughly before the next dilution transfer.
   g. Add **500 µl** of Cal E (from step f) to the tube labeled Cal D. Vortex and mix the content in the tube thoroughly before use.
   h. Add **500 µl** of Cal D (from step g) to the tube labeled Cal C. Vortex and mix the content in the tube thoroughly before use.
   i. Add **500 µl** of Cal C (from step h) to the tube labeled Cal B. Vortex and mix the content in the tube thoroughly before use.
   j. The tube labeled Cal A contains **500 µl** AMH Calibrator A/Sample Diluent and has 0 AMH concentrations and should be used as Blank. ***NOTE:*** *If greater sensitivity is desired, Cal B can be further diluted to B/2 using Cal A.*
   k. The Calibrators A-H for instance should read as 0.0 ng/mL, 0.313 ng/mL, 0.625 ng/mL, 1.25 ng/mL, 2.5 ng/mL, 5 ng/mL, 10 ng/mL and 20 ng/mL. Aliquot and freeze the Rat and Mouse AMH Cal H Stock immediately for multiple uses. Avoid repeated freeze thaws. Frozen aliquots at -20°C are good for one year.
   l. The AMH concentration in the Rat and Mouse AMH calibrators H 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.

SAMPLE PREPARATION

1. **Male rat and mouse samples:** Dilute **1:50** into Calibrator A/Sample Diluent. (**1 part unknown sample** into **49 parts Calibrator A/Sample Diluent**).
2. **Female rat and mouse samples:** Dilute **1:10** into Calibrator A/Sample Diluent. (**1 part unknown sample** into **9 parts Calibrator A/Sample Diluent**).

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3. **Rat and Mouse tissue extract:** Dilute tissue extract 1:5 into Calibrator A/Sample Diluent. (1 part extract into 4 parts Calibrator A/Sample Diluent).
   NOTE: *AMH values can vary from one breed to another. You may need to establish your dilution factor accordingly to ensure the diluted samples fall within the measurable range of the assay.*

ASSAY PROCEDURE

Allow all specimens and reagents to reach room temperature (23 ± 2˚C) and mix thoroughly by gentle inversion before use.

NOTE: *All samples should be diluted as per the "Sample Preparation" section.*

NOTE: *Calibrators and diluted unknowns should be assayed in duplicate.*

NOTE: *All diluted samples reading higher than the highest calibrator should be further 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-H)** and **diluted unknowns** (See "Sample Preparation" section) 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/well)** 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 **(350 µL/well)** 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 **(350 µL/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 **8-12 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**.*

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.* Optimum results can be obtained at incubation temperature of **23 ± 2°C**.

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 recovered unknown values by the appropriate dilution factor.

LIMITATIONS

The reagents supplied in this kit are optimized to measure AMH levels in rat and mouse 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[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	0.060 (*Blank*)	0
B1, B2	B	0.06	0.336
C1, C2	C	0.136	0.672
D1, D2	D	0.252	1.344
E1, E2	E	0.511	2.687
F1, F2	F	0.988	5.375
G1, G2	G	1.934	10.75
H1, H2	H	3.419	21.5

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

NOTE: *The OD values listed above for calibrators B-H are blank subtracted.*

ANALYTICAL CHARACTERISTICS

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

Specificity:

The Ansh Labs Rat and Mouse AMH assay has been screened for cross reactivity with other species AMH, including rabbit, avian, hamster, beluga whale, and bottlenose dolphin. The antibodies exhibit high affinity to AMH of these species. Tests for parallelism by linearly diluting specimens from these species and assaying the diluted serum demonstrate the ELISA kit accuracy for determining AMH in these animals

Linearity:

Multiple dilutions of samples containing various AMH/MIS levels were diluted with Calibrator A/sample diluent. The % recovery on individual samples is represented in the following table.

Sample	Dilution Factor	Expected Conc. (ng/mL)	Observed Conc. (ng/mL)	% Recovery
Hamster	1:10	2.2	NA	NA
	1:20	1.1	1.1	100%
	1:40	0.55	0.68	124%

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	1:80	0.27	0.31	115%
Mouse 1	1:20	20.665	NA	NA
	1:40	10.333	9.865	95%
	1:80	5.166	4.771	92%
	1:160	2.583	2.403	93%
Mouse 2	1:10	21.696	NA	NA
	1:20	10.848	11.171	103%
	1:40	5.424	5.710	105%
Mouse 3	1:10	19.739	NA	NA
	1:20	9.870	10.328	105%
	1:40	4.935	5.206	105%
Recombinant Rat AMH	Neat	9.93	NA	NA
	1:02	4.97	4.78	96%
	1:04	2.48	2.21	89%
	1:08	1.24	1.04	84%

Expected Values:

Expected AMH concentrations in male and female mice were calculated by evaluating 26 female and 29 male mouse samples in Ansh Labs Rat and Mouse AMH ELISA. AMH distribution by age in days was calculated using Analyse-It® for Microsoft Excel and is shown below.

Gender	Age (Days)	No of specimens (n)	Median Conc. (ng/mL)	Range AMH (ng/mL)
Female	20 - 50	8	211.0	95.7 - 532.5
	100 - 240	9	127.3	67.7 - 246.5
	380 - 540	9	61.4	10.7 - 140.8
Male	8	8	811.6	506.7 - 1801.5
	9	7	965.8	596.0 - 1164.5
	10 - 12	7	238.7	207.4 - 1347.0
	20	3	49.1	44.8 - 132.9
	50 - 60	4	15.9	14.3 - 21.0

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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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