Rat and Mouse AMH ELISA

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-antigen 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 REQUIRED BUT NOT PROVIDED
- Microplate reader capable of absorbance measurement at 450 nm, 405nm and 630 nm.
- Microplate shaker.
- Microplate washer.
- Semi-automated/manual precision pipette to deliver 10–250 μL.
- Repeater pipette.
- Vortex mixer.
- Deionized water.

MATERIALS SUPPLIED

<table>
<thead>
<tr>
<th>CAL-105A</th>
<th>AMH/MIS Calibrators A / Sample Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>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.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CAL-113H</th>
<th>Rat and Mouse AMH Calibrator H (Lyophilized)</th>
</tr>
</thead>
<tbody>
<tr>
<td>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 μg/mL. Refer to the vial label for exact concentration. Store unopened at 2-8°C until the expiration date.</td>
<td></td>
</tr>
</tbody>
</table>

PLT-113 AMH Coated Microtitration strips
One stripholder, containing 12 strips and 96 microtiter 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 undiluted 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.

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.

**WARNING: Potential Chemical Hazard**

Some reagents in this kit contain Pro-Clean 400 and Sodium azide 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

1. Serum is the recommended sample type.
2. 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.
3. 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.
4. Avoid assaying lipemic, hemolyzed or icteric samples.
5. Avoid repeated freezing and thawing of samples. Thaw samples no more than 3 times.
6. 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. Add 500 µL of Cal B (from step i) to the tube labeled Cal A. Vortex and mix the content in the tube thoroughly before use.
   k. The tube labeled Cal A contains 500 µL AMH Calibrator A/Sample Diluent and has 0 AMH concentrations and should be used as Blank.
   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).
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 microwturbation 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 at 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 ng/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.

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9. Add 100 μL of the Streptavidin-Enzyme Conjugate-RTU to each well using a repeater pipette.
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4. Determine the AMH concentrations of the unknowns from the calibration curve by matching their mean OD readings with the corresponding AMH concentrations.

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.

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

<table>
<thead>
<tr>
<th>Well Number</th>
<th>Well Contents</th>
<th>Mean Absorbance</th>
<th>Conc (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1, A2</td>
<td>Cal A</td>
<td>0.060</td>
<td>0</td>
</tr>
<tr>
<td>B1, B2</td>
<td>Blank</td>
<td>0.06</td>
<td>0.336</td>
</tr>
<tr>
<td>C2</td>
<td>Cal B</td>
<td>0.136</td>
<td>0.672</td>
</tr>
<tr>
<td>D1, D2</td>
<td>Cal C</td>
<td>0.252</td>
<td>1.344</td>
</tr>
<tr>
<td>E1, E2</td>
<td>Cal D</td>
<td>0.511</td>
<td>2.687</td>
</tr>
<tr>
<td>F1, F2</td>
<td>Cal E</td>
<td>0.988</td>
<td>5.375</td>
</tr>
<tr>
<td>G1, G2</td>
<td>Cal F</td>
<td>1.934</td>
<td>10.75</td>
</tr>
<tr>
<td>H1, H2</td>
<td>Cal G</td>
<td>3.019</td>
<td>43.7</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution Factor</th>
<th>Expected (ng/mL)</th>
<th>Observed (ng/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamster</td>
<td>1:10</td>
<td>2.2</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>1.1</td>
<td>1.1</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>1:40</td>
<td>0.55</td>
<td>0.68</td>
<td>124%</td>
</tr>
<tr>
<td></td>
<td>1:80</td>
<td>0.27</td>
<td>0.31</td>
<td>115%</td>
</tr>
<tr>
<td>Mouse 1</td>
<td>1:10</td>
<td>20.663</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>1:40</td>
<td>10.333</td>
<td>9.865</td>
<td>95%</td>
</tr>
<tr>
<td></td>
<td>1:80</td>
<td>5.166</td>
<td>4.771</td>
<td>92%</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>1:20</td>
<td>1:40</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>--------</td>
</tr>
<tr>
<td>Mouse 2</td>
<td>21.686</td>
<td>10.848</td>
<td>5.424</td>
<td>NA</td>
</tr>
<tr>
<td>Mouse 3</td>
<td>19.739</td>
<td>9.870</td>
<td>4.935</td>
<td>NA</td>
</tr>
<tr>
<td>Recombinant</td>
<td>Neat</td>
<td>9.93</td>
<td>4.97</td>
<td>2.48</td>
</tr>
</tbody>
</table>

REFERENCES


The Ansh Labs logo is a trademark of Ansh Labs.

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