Bovine AMH ELISA
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.

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. 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 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 REQUIRED BUT NOT PROVIDED
1. Microtiter plate reader capable of absorbance measurement at 450 nm, 405 nm and 630 nm.
2. Microplate shaker.
3. Vortex mixer.
4. Semi-automated/manual precision pipette to deliver 10–250 μL.
5. Deionized water.

MATERIALS SUPPLIED

**CAL-105A  AMH/MIS Calibrators A / Sample Diluent**
One bottle, 11 mL, labeled AMH/MIS Cal A/Sample Diluent, containing 0 pg/mL AMH in protein based buffer and Pro-Clean 400. Store unopened at 2-8°C until the expiration date.

**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 Pro-Clean 400. Refer to the **calibration card** for exact concentrations. 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 thaw.

**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 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 thaw.

**PLT-114  AMH Coated Microtiter strips**
One stripholder, containing 12 strips and 96 microtitration wells with AMH antibody immobilized in 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 unidiluted 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 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," 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 AND PREPARATION**

1. **EDTA Plasma and serum** are the recommended sample types. Other biological fluids may produce different results.

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 EDTA plasma 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 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. **Microtiter 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 microtiter 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 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. 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.

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 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1, A2</td>
<td>A</td>
<td>0.0034 (Blank)</td>
<td>0</td>
</tr>
<tr>
<td>B1, B2</td>
<td>B</td>
<td>0.032</td>
<td>13.5</td>
</tr>
<tr>
<td>C1, C2</td>
<td>C</td>
<td>0.149</td>
<td>63.4</td>
</tr>
<tr>
<td>D1, D2</td>
<td>D</td>
<td>0.461</td>
<td>206.0</td>
</tr>
<tr>
<td>E1, E2</td>
<td>E</td>
<td>1.406</td>
<td>710.0</td>
</tr>
<tr>
<td>F1, F2</td>
<td>F</td>
<td>2.463</td>
<td>2240.0</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean Conc. (pg/mL)</th>
<th>SD</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool-1</td>
<td>611</td>
<td>0.018</td>
<td>2.92</td>
</tr>
<tr>
<td>Pool-2</td>
<td>1259</td>
<td>0.032</td>
<td>2.54</td>
</tr>
</tbody>
</table>

Linearity

Multiple dilutions of the 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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution Factor</th>
<th>Expected Conc. (pg/mL)</th>
<th>Observed Conc. (pg/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Neat</td>
<td>7468</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>1</td>
<td>1:2</td>
<td>3734</td>
<td>3731</td>
<td>100%</td>
</tr>
<tr>
<td>1</td>
<td>1:4</td>
<td>1867</td>
<td>1973</td>
<td>106%</td>
</tr>
<tr>
<td>1</td>
<td>1:8</td>
<td>934</td>
<td>980</td>
<td>105%</td>
</tr>
<tr>
<td>1</td>
<td>1:16</td>
<td>467</td>
<td>511</td>
<td>109%</td>
</tr>
<tr>
<td>2</td>
<td>Neat</td>
<td>5010</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>1:2</td>
<td>2505</td>
<td>2450</td>
<td>98%</td>
</tr>
<tr>
<td>2</td>
<td>1:4</td>
<td>1253</td>
<td>1261</td>
<td>101%</td>
</tr>
<tr>
<td>2</td>
<td>1:8</td>
<td>626</td>
<td>665</td>
<td>106%</td>
</tr>
<tr>
<td>2</td>
<td>1:16</td>
<td>313</td>
<td>334</td>
<td>107%</td>
</tr>
<tr>
<td>3</td>
<td>Neat</td>
<td>4530</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>1:2</td>
<td>2265</td>
<td>2199</td>
<td>97%</td>
</tr>
<tr>
<td>3</td>
<td>1:4</td>
<td>1133</td>
<td>1154</td>
<td>102%</td>
</tr>
<tr>
<td>3</td>
<td>1:8</td>
<td>566</td>
<td>588</td>
<td>104%</td>
</tr>
<tr>
<td>3</td>
<td>1:16</td>
<td>283</td>
<td>297</td>
<td>105%</td>
</tr>
<tr>
<td>Male</td>
<td>1:4</td>
<td>1913</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Male</td>
<td>1:8</td>
<td>956</td>
<td>940</td>
<td>98%</td>
</tr>
<tr>
<td>Male</td>
<td>1:16</td>
<td>478</td>
<td>471</td>
<td>99%</td>
</tr>
<tr>
<td>Male</td>
<td>1:32</td>
<td>239</td>
<td>254</td>
<td>106%</td>
</tr>
<tr>
<td>Female</td>
<td>Neat</td>
<td>1655</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Female</td>
<td>1:2</td>
<td>828</td>
<td>837</td>
<td>101%</td>
</tr>
<tr>
<td>Female</td>
<td>1:4</td>
<td>414</td>
<td>430</td>
<td>104%</td>
</tr>
<tr>
<td>Female</td>
<td>1:8</td>
<td>207</td>
<td>224</td>
<td>108%</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Endogenous Conc.(ng/mL)</th>
<th>Expected Conc. (ng/mL)</th>
<th>Observed Conc. (ng/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>421.393</td>
<td>478.144</td>
<td>483.655</td>
<td>101%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>534.894</td>
<td>552.673</td>
<td>103%</td>
</tr>
</tbody>
</table>

Refer to package insert included with the product for exact specifications.

For Illustrative Purposes Only

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Bovine AMH ELISA
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:

\[ \text{Serum} = 0.97 \times (\text{EDTA Plasma}) - 2.15, \quad (r=0.99; \ P<0.0001) \]

![Graph showing the regression between AMH (pg/mL, Serum) and AMH (pg/mL, EDTA Plasma).](image)

REFERENCES


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