The Canine Anti-Müllerian hormone (AMH) enzyme-linked immunosorbent assay (ELISA) kit provides materials for the quantitative measurement of AMH in Canine serum and other biological fluids.

**INTENDED USE**

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**SUMMARY AND EXPLANATION**

The anti-Müllerian hormone (AMH) is a member of the transforming growth factor β family of growth and differentiation factors. AMH is produced exclusively in testicular Sertoli cells and ovarian granulosa cells. The physiological role of AMH in the two sexes is quite distinct. In the male AMH has an essential role in sex differentiation. Fetal Sertoli cells secrete AMH which signals the active removal of the Müllerian ducts, the anlagen of the oviducts, uterus and the upper part of the vagina in females, thereby preventing the formation of these structures in the male. The fetal and postnatal AMH production in males is used as a diagnostic marker to determine the presence of testicular tissue in the clinic. In post-pubertal males AMH has a role in the regulation of testosterone production by the Leydig cells. A negative correlation between testosterone and AMH has been found in human males and it appears that testosterone suppresses Sertoli cell AMH production.

In females, AMH is not produced by the fetal ovary, ensuring that the Müllerian ducts stay intact in developing females. AMH appears to have two roles in ovarian physiology. Firstly, AMH has a negative feedback effect on primordial follicles, i.e., it suppresses recruitment thereby signaling the presence of enough small growing follicles to the primordial follicle pool. Secondly, AMH suppresses the sensitivity of the follicle to FSH in an autocrine manner, preventing selection. As small follicles grow and differentiate AMH starts to decrease when the differentiation state reaches the point when successful FSH selection is imminent, FSH sensitivity increases and the follicle is selected.

In veterinary practice the presence or absence of functional gonadal tissue in dogs is a recurrent challenge. In particular when the reproductive history is not known, it may be difficult to determine whether a female animal has been spayed. In female dogs the presence of remaining functional ovarian tissue after spaying is relevant when a presumably spayed animal is presented with a history of bitches or queens. In these cases serum AMH measurement can thus be used as a diagnostic tool in the determination of functional gonadal status in dogs.

**PRINCIPLE OF THE TEST**

The Canine AMH ELISA is a quantitative three-step sandwich type immunoassay. In the first step Calibrators, Controls 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-avidin 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 Calibrator 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-116B – CAL-116F** Canine AMH Calibrators B thru F (Lyophilized)
  - Five vials, labeled B-F, containing concentrations of approximately 0.3 – 15 ng/mL native Canine AMH in protein based buffer and Pro-Clean 400. Refer to calibration card for exact concentrations. Store unopened at 2 to 8°C until the expiration date. Reconstitute calibrators B-F with 1 mL deionized water. Solubilize, mix well and use after reconstitution. Aliquot and Freeze in plastic vials for multiple use. Alternatively, freeze in the same vial within 2 hours of reconstitution. Avoid repeated freeze thaws.

- **CTR-116-I & CTR-116-II** Canine AMH Controls I & II (Lyophilized)
  - Two vials, labeled Levels I and II containing low and high native Canine AMH concentrations in protein based buffer and Pro-Clean 400. Refer to 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 in plastic vials for multiple use. Alternatively, freeze in the same vial within 2 hours of reconstitution. Avoid repeated freeze thaws.

- **PLT-113** AMH Coated Microtiteration 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-116** 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-116** 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 Chromom 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. Microtiterplate reader capable of absorbance measurement at 450 nm, 405 nm 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 immunoreagents.
c) Handle and dispose of all reagents and material in compliance with applicable regulations.
d) If external package is damaged, inspect the components inside for any other damage. Do not use if the components are damaged.

**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 AND PREPARATION**

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 Canine 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 (23 ± 2°C) 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. Canine AMH Calibrators B-F and Canine AMH Controls I and II: Tap and reconstitute Canine AMH Calibrators B-F and Controls I and II with 1 mL deionized water. Solubilize for ten minutes, mix well before use.
2. Calibrator B/2: Mix 150 ul of reconstituted Cal B with 150 ul of Cal A/Sample diluent.
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. Microtiteration 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 samples: Dilute 1:10 into Calibrator A/Sample Diluent. (1 part unknown sample into 9 parts Calibrator A/Sample Diluent).
2. Female samples: Dilute 1:2 into Calibrator A/Sample Diluent. (1 part unknown sample into 1 part Calibrator A/Sample Diluent).

Note: This can be achieved by adding 25ul of sample and 25ul of Cal A/Sample diluent directly to the microwell in step 3 of the assay procedure.

**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.

1. Label the microtiteration strips to be used.
2. Add 50 μL of the AMH Assay Buffer to each well using a repeater pipette.
3. Pipette 50 μL of the Calibrators, controls and diluted unknowns (See “Sample Preparation” section) to the appropriate wells.
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 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 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 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 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.
5. Any sample reading higher than the highest calibrator should be appropriately diluted with the 0 ng/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 Canine 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 controls, 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>A</td>
<td>0.027 (Blank)</td>
<td>0</td>
</tr>
<tr>
<td>B1, B2</td>
<td>B</td>
<td>0.047</td>
<td>0.11</td>
</tr>
<tr>
<td>C1, C2</td>
<td>C</td>
<td>0.089</td>
<td>0.22</td>
</tr>
<tr>
<td>D1, D2</td>
<td>D</td>
<td>0.285</td>
<td>0.77</td>
</tr>
<tr>
<td>E1, E2</td>
<td>E</td>
<td>0.835</td>
<td>2.58</td>
</tr>
<tr>
<td>F1, F2</td>
<td>F</td>
<td>1.785</td>
<td>6.2</td>
</tr>
<tr>
<td>G1, G2</td>
<td>G</td>
<td>3.421</td>
<td>13.2</td>
</tr>
</tbody>
</table>

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)

Analytical Sensitivity:
The analytical sensitivity in the Canine AMH ELISA assay, as calculated by the interpolation of mean plus two standard deviations of 16 replicates of Calibrator A (0 ng/mL) and Calibrator B (0.28 ng/mL), is 0.055 ng/mL.

Linearity:
Multiple dilutions of the three canine serum samples containing various AMH 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 Conc. (ng/mL)</th>
<th>Observed Conc. (ng/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Neat</td>
<td>9.55</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>4.77</td>
<td>4.27</td>
<td>90%</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>2.39</td>
<td>2.05</td>
<td>86%</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>1.19</td>
<td>1.10</td>
<td>92%</td>
</tr>
<tr>
<td></td>
<td>1:16</td>
<td>0.597</td>
<td>0.60</td>
<td>101%</td>
</tr>
<tr>
<td></td>
<td>1:32</td>
<td>0.30</td>
<td>2.00</td>
<td>99%</td>
</tr>
<tr>
<td>2</td>
<td>1:16</td>
<td>1.51</td>
<td>1.467</td>
<td>97%</td>
</tr>
<tr>
<td></td>
<td>1:32</td>
<td>0.755</td>
<td>0.753</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>1:64</td>
<td>0.377</td>
<td>0.392</td>
<td>104%</td>
</tr>
<tr>
<td></td>
<td>1:128</td>
<td>0.187</td>
<td>0.187</td>
<td>100%</td>
</tr>
<tr>
<td>3</td>
<td>1:16</td>
<td>2.15</td>
<td>1.85</td>
<td>86%</td>
</tr>
<tr>
<td></td>
<td>1:32</td>
<td>1.07</td>
<td>1.03</td>
<td>96%</td>
</tr>
<tr>
<td></td>
<td>1:64</td>
<td>0.536</td>
<td>0.567</td>
<td>106%</td>
</tr>
</tbody>
</table>

[Note: This is an example of data from a package insert. The actual content may vary.]
Imprecision:
Reproducibility of the Canine AMH ELISA assay was determined in a study using four samples over 12 runs. The study included two kit controls (n=48) and two samples (n=40, n=48 respectively). Representative data were calculated and are presented in the following table.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean conc. (ng/mL)</th>
<th>SD</th>
<th>%CV</th>
<th>SD</th>
<th>%CV</th>
<th>SD</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>CI</td>
<td>0.791</td>
<td>0.040</td>
<td>5.01</td>
<td>0.034</td>
<td>4.38</td>
<td>0.052</td>
<td>6.59</td>
</tr>
<tr>
<td>C II</td>
<td>2.396</td>
<td>0.051</td>
<td>2.14</td>
<td>0.082</td>
<td>3.43</td>
<td>0.097</td>
<td>4.04</td>
</tr>
<tr>
<td>S 1</td>
<td>8.248</td>
<td>0.284</td>
<td>3.45</td>
<td>0.086</td>
<td>1.07</td>
<td>0.298</td>
<td>3.61</td>
</tr>
<tr>
<td>S 2</td>
<td>3.158</td>
<td>0.102</td>
<td>3.23</td>
<td>0.072</td>
<td>2.32</td>
<td>0.125</td>
<td>3.95</td>
</tr>
</tbody>
</table>

Reference Ranges:
These average AMH concentration and range were calculated on canine samples using Ansh Canine AMH ELISA.

<table>
<thead>
<tr>
<th>ID</th>
<th>Age (Years)</th>
<th>Weight (lbs)</th>
<th>Average AMH (ng/mL)</th>
<th>Range (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact Females</td>
<td>3.5</td>
<td>62</td>
<td>3.63</td>
<td>0.2 – 15</td>
</tr>
<tr>
<td>Spayed Females</td>
<td>10.3</td>
<td>41.91</td>
<td>&lt; 0.2</td>
<td>ND – 0.2</td>
</tr>
<tr>
<td>Castrated Males</td>
<td>7.3</td>
<td>48.54</td>
<td>&lt; 0.2</td>
<td>ND – 0.2</td>
</tr>
<tr>
<td>Intact Males</td>
<td>6.69</td>
<td>53.31</td>
<td>20.9</td>
<td>0.52 – 98</td>
</tr>
</tbody>
</table>

Note: It is recommended that each laboratory should determine the reference range(s) for its own patient population. The results of this assay should be used in conjunction with other relevant and applicable clinical information.

REFERENCES

The Ansh Labs logo is a trademark of Ansh Labs.

Manufactured by:
Ansh Labs
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Refer to package insert included with the product for exact specifications.