Ultra-Sensitive AMH/MIS ELISA RUO

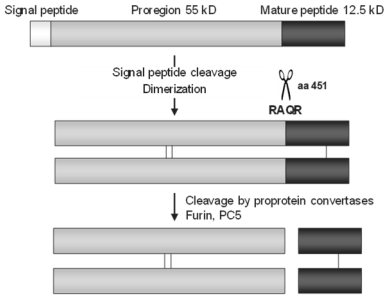
AL-105

INTENDED USE

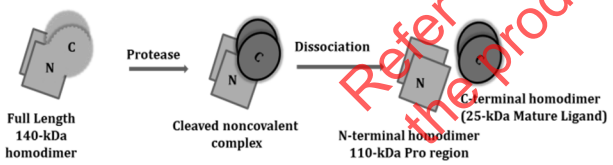
The Ultra-Sensitive Anti-Müllerian hormone/Müllerian inhibiting substance (US AMH/MIS) enzyme linked immunuosorbent assay (ELISA) kit provides materials for the quantitative measurement of AMH/MIS in human serum, plasma and other biological fluids. This kit is intended for laboratory research use only and is not for use in diagnostic or therapeutic procedures.

SUMMARY AND EXPLANATION

Anti-Müllerian hormone (AMH), a member of the TGFβ superfamily, is a homodimeric glycoprotein composed of two 55 kDa N-terminal and two 12.5 kDa C-terminal homodimers, non-covalently linked by disulfide bridges. Processing of AMH is shown below.[1]



Recent studies have shown that the AMH C-terminal homodimer is much less active than the noncovalent complex, but almost all activity can be restored by associating with the N-terminal pro-region, which reforms a complex with the mature C-terminal homodimer. This finding raises the possibility that the AMH noncovalent complex is the active form of protein. It was reported that the cleaved AMH noncovalent complex binds to AMHRII and stimulates intracellular signaling, whereas full-length AMH shows only minimal activity.[2]



AMH is secreted by the Sertoli cells in males. During embryonic development, AMH is responsible for Müllerian duct regression. AMH continues to be produced by the testes until puberty and then decreases slowly to residual post-puberty values. In females, AMH is produced by the granulosa cells of small growing follicles from the 36th week of gestation onwards until menopause when levels become undetectable. Potential clinical applications of low end anti-müllerian hormone (AMH) have been published in premature ovarian insufficiency, ovarian tumors, menopause and many more.

PRINCIPLE OF THE TEST

The US AMH/MIS ELISA is a quantitative three-step sandwich type immunoassay. In the first step Calibrators, Controls and unknown samples are added to AMH antibody coated microtiter wells and incubated. After the first incubation and washing, the wells are incubated with biotinylated AMH antibody solution. After the 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/MIS 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-105B – CAL-105F AMH/MIS Calibrators B thru F (Lyophilized)

Five vials, labeled B-F, containing concentrations of approximately 0.09 – 15.0 ng/mL 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. The AMH/MIS concentration in the AMH/MIS calibrators 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.

CTR-105-I & CTR-105-II AMH/MIS Controls I & II (Lyophilized)

Two vials, labeled Levels I and II containing low and high 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-105 AMH/MIS 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-205 AMH/MIS Assay Buffer

One bottle, 12 mL, containing a protein-based (BSA)-buffer with a non-mercury preservative. Store at 2-8°C until expiration date.

BCR-105 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-105 AMH/MIS 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, 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 orbital shaker.
3. Microplate washer.
4. Semi-automated/manual precision pipette to deliver 10–250 µL.
5. Repeator pipette.
6. Vortex mixer.
7. Deionized water.

WARNINGS AND PRECAUTIONS

For Research Use Only. Not for use in diagnostic procedures.
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 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[3].

WARNING: Potential Chemical Hazard

Some reagents in this kit contain Pro-Clean 400 and Sodium azide[4] 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 and lithium-heparin plasma 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.[5]

PROCEDURAL NOTES

1. A thorough understanding of this package insert is necessary for successful use of the US AMH/MIS ELISA assay. It is the user's responsibility to validate the assay for their purpose. 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. **AMH/MIS Calibrators B-F and AMH/MIS Controls I & II**: Tap and reconstitute AMH/MIS Calibrator B-F and AMH/MIS Controls I & II each with 1 mL deionized water. Solubilize, mix well and use after reconstitution.
2. **Wash Solution:** Dilute wash concentrate 25-fold with deionized water. The wash solution is stable for one month at room temperature (23 ± 2°C) 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 (23 ± 2°C) and mix thoroughly by gentle inversion before use. Calibrators, controls, 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. Reconstitute AMH/MIS Calibrator B-F and AMH/MIS Controls I & II each with 1 mL deionized water. Solubilize for **10 minutes**, Mix well by gentle vortex.
2. Label the microtitration strips to be used.

3. Pipette **25 µL** of the Calibrator, Controls and Unknowns to the appropriate wells.
4. Add **100 µL** of the AMH/MIS Assay Buffer to each well using a repeater pipette.
5. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **90 minutes** at room temperature (23 ± 2°C).
6. Aspirate and wash each strip **5 times (350 µL/per well)** with Wash Solution using an automatic microplate washer.
7. Add **100 µL** of the Antibody-Biotin Conjugate RTU to each well using a repeater pipette.
8. 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).
9. Aspirate and wash each strip **5 times** with the Wash Solution **(350 µL/per well)** using an automatic microplate washer.
10. Add **100 µL** of the Streptavidin-Enzyme Conjugate-RTU to each well using a repeater pipette.
11. 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).
12. Aspirate and wash each strip **5 times** with the Wash Solution **(350 µL/per well)** using an automatic microplate washer.
13. Add **100 µL** of the TMB chromogen solution to each well using a repeater pipette. Avoid exposure to direct sunlight.
14. 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.*
15. 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/MIS concentrations in ng/mL along the x-axis, using a cubic regression curve-fit.
4. Determine the AMH/MIS concentrations of the Controls and unknowns from the calibration curve by matching their mean OD readings with the corresponding AMH/MIS 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/MIS levels in human serum and lithium heparin plasma. 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[6].

QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to assure proper performance.
- AMH/MIS ELISA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for AMH/MIS controls are printed on the **Calibration card**.
- A full calibration curve, low and high level 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

Well Number	Well Contents	Mean Absorbance	Conc (ng/mL)
A1, A2	Calibrators A	0.04 (Blank)	0
B1, B2	B	0.04	0.08
C1, C2	C	0.09	0.30
D1, D2	D	0.31	1.03
E1, E2	E	1.07	3.96
F1, F2	F	2.86	14.2

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 = 7.14 pmol/L)

Limit of Detection (LoD):

The lowest amount of AMH/MIS in a sample that can be detected with a 95% probability (n=24) is 0.023 ng/mL. The value was determined by processing five serum samples in the range of 0.03 to 0.346 ng/mL following CLSI EP17 guidelines. Twelve assay runs were performed over two days with samples run in duplicate per run.

Limit of Quantitation (LoQ):

The estimated minimum dose achieved at 20% total imprecision is 0.06 ng/mL. The value was determined by processing eight samples in the range of 0.03-2.85 ng/mL over twelve runs and two days in duplicates (n=24) following CLSI EP17 guidelines.

Imprecision:

Reproducibility of the US AMH/MIS ELISA assay was determined in a study using three serum pools. The study included a total of 12 assays, four replicates of each per assay (n=48). Representative data were calculated based on NCCLS EP5-A guidelines and are presented in the following table.

Sample	Mean conc.	Within run		Between run		Total	
	(ng/mL)	SD	%CV	SD	%CV	SD	%CV
Pool-1	0.35	0.01	1.97%	0.02	4.63%	0.02	5.13%
Pool-2	0.72	0.03	3.66%	0.03	4.79%	0.04	6.03%
Pool-3	1.85	0.07	4.00%	0.04	1.98%	0.08	4.46%

Linearity:

Based on NCCLS EP-6-P multiple dilutions of the three serum 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
1	Neat	7.39	Neat	NA
	1:02	3.69	3.85	104%
	1:04	1.85	1.87	101%
	1:08	0.92	0.94	102%
	1:16	0.46	0.46	99%
2	Neat	4.44	Neat	NA
	1:02	2.22	2.26	102%
	1:04	1.11	1.20	108%
	1:08	0.56	0.61	109%
	1:16	0.28	0.29	105%
3	Neat	7.11	Neat	NA
	1:02	3.55	3.89	109%
	1:04	1.78	1.90	107%
	1:08	0.89	0.99	111%
	1:16	0.44	0.48	107%

Recovery:

Known amounts of AMH/MIS were added to three serum samples containing different levels of endogenous AMH/MIS. The concentration of AMH/MIS was determined before and after the addition of exogenous AMH/MIS and the percent recovery was calculated.

Sample	Endogenous Conc.(ng/mL)	Expected Conc. (ng/mL)	Observed Conc. (ng/mL)	% Recovery
1	1.56	1.92	1.78	93%
		2.27	2.18	96%
		2.63	2.52	96%
2	1.13	1.51	1.42	94%
		1.89	1.69	89%
		2.27	1.96	86%
3	1.20	1.58	1.41	89%
		1.95	1.78	91%
		2.33	2.11	91%

Analytical Specificity:

This monoclonal antibody pair used in the assay is specific to human AMH/MIS and does not cross react to other species (bovine, equine, ovine, canine, rat and mouse).

Cross-Reactant	Concentration	% Cross-reactivity
Inhibin A	I00 ng/mL	ND
Inhibin B	100 ng/mL	ND
Activin A	50 ng/mL	ND
Activin B	50 ng/mL	ND
Activin AB	50 ng/mL	ND
Full Length AMH dimer	1000 ng/mL	100
rAMH	130 ng/mL	ND
Mature AMH	120 ng/mL	1.33
hAMH(Pro)	300 ng/mL	0.23
ProMature hAMH	110 ng/Ml	100

Interference:

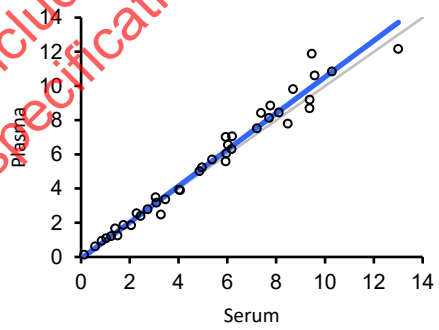
When potential interferents (hemoglobin, triglycerides, biotin, and bilirubin) were added at least at two times their physiological concentration to control sample, AMH/MIS concentration were within ± 10% of the control as represented in the following table. This study was based on NCCLS EP7-P to serum matrix added.

Interferents	Analyte Conc.	Unspiked Sample Value (ng/mL)	Spiked Sample Value (ng/mL)	% Difference
Hemoglobin	1.35 mg/mL	6.15	6.21	1.01
		4.67	4.62	-0.88
Triglycerides	5.00 mg/mL	6.15	6.33	2.98
		4.67	4.51	-3.37
Biotin	1200 ng/mL	4.219	4.217	-0.1
		7.806	7.316	-6.3
Bilirubin	0.60 mg/mL	4.86	4.80	-1.23
		3.11	3.08	-0.77

Sample Type:

Forty matched serum and Lithium heparin plasma specimens in the range of 0.13-13.01 ng/mL were compared in Ansh US AMH/MIS ELISA assay.
Passing Bablok analysis of the results yielded the following Regression:

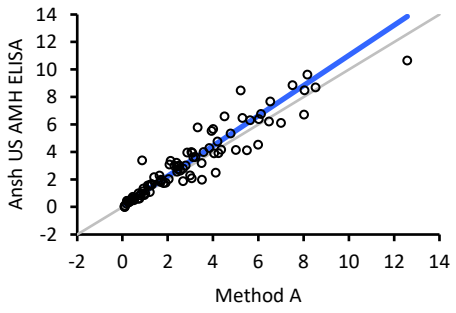
Plasma=1.06 (serum)-0.10, (r=0.995; P<0.0001)



Method Comparison:

The Ultra-Sensitive AMH/MIS ELISA has been compared to Commercial AMH assay (Method A) using 90 serum samples in the range of 0.1-12.58 ng/mL.
Passing Bablok analysis of the results yielded the following Regression:

Ultra-Sensitive AMH/MIS ELISA (AL-105) = 1.10 (Method A) + 0.06
(r=0.98; P<0.0001)



REFERENCES

1. Pepinski, R.B., et al. (1988) J. Biol. Chem., 263, 18961-18964.
2. di Clemente et al. Mol Endocrinol, November 2010, 24 (11): 2193-2206.

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

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