AnshLabs

picoAMH ELISA

AL-124-i

MenoCheck®

CE

INTENDED USE

The picoAMH ELISA is an enzyme-linked immunosorbent assay (ELISA) for the quantitative measurement of anti-Müllerian hormone (AMH), also known as Müllerian Inhibiting Substance (MIS), concentrations in human serum, Li-Heparin and K2 EDTA plasma. It is intended to be used as an aid in the determination of ovarian status in women in reproductive age and during menopausal transition. It is intended for *in vitro* diagnostic use.

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[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 Anti-Müllerian Hormone (AMH) have been published in premature ovarian insufficiency[3] ovarian tumors,[4,5] menopause[6-8] intersex disorders and many more.

Menopause is the cessation of menstrual cycling and fertility. Clinically it is recognized by a cessation of menstrual bleeding; menopause is a woman's status after her final menstrual period (FMP). Menstrual cycling and bleeding are driven by the dynamics of ovarian follicle maturation. Because a woman is born with a limited number of follicles (non-growing "primodial" follicles) that cannot be replaced and are slowly consumed by the monthly maturation of smaller groups of follicles (primary, secondary and antral growing follicles), the natural cause of menopause is the absence of follicles. However, many natural (e.g., certain diseases, starvation, etc.) or interventional processes (chemotherapy, surgical removal of the uterus, ovaries, or pituitary gland, etc.) can cause a cessation of menses that can be either irreversible (e.g., functionally the same as menopause) or reversible. Diagnosing menopause (e.g. the FMP) currently is only done unequivocally after cessation of menses for 12 months in a previously cycling woman.

Naturally occurring (i.e., as a result of aging) menopause is referred to as "spontaneous" and, on average, occurs at the age of 51 years, but there is a very large variance of approximately +/- 10 years with respect to the occurrence of menopause in healthy women. Ovarian function deteriorates gradually leading up to menopause and contributes to the variance observed among women with respect to menopausal status. Ovarian function affects, primarily via the steroid hormones produced by growing follicles, virtually every organ in a woman's body. Physiological responses to the gradual or abrupt loss of ovarian function include a multitude of menopausal "symptoms" and consequences that affect a woman's health and quality of life. [9-10]

During several years leading up to the FMP and several years immediately following the FMP is a time called the climacteric or menopausal transition, when a woman transitions from the reproductive to a non-reproductive state. The transition is highly variable among women with respect to duration and intensity of associated physiological changes, which affect her well-being and level of disease risk. Thus, determining where a woman is in this process must be individualized and is clinically important, particularly during the menopausal transition and the 12 months following the FMP. Quantitative measurement of blood levels of Anti-Müllerian Hormone using the picoAMH (MenoCheck®) assay can aid in determining a woman's menopausal status during the menopausal transition.

Blood levels of AMH represent one of the markers available to clinicians to determine where a woman is in her menopausal transition. Other clinical tests relevant in this context are estradiol which is produced only by follicles in their final stages of maturation, and thus only indirectly reflects the total number of follicles in the ovary, and FSH which reflects the negative feedback by estradiol on pituitary gland secretion (i.e., also an indirect marker of ovarian follicular pool based on large follicle function). In contrast AMH is produced by the majority of ovarian follicles (primary, secondary and antral).

Blood levels of AMH have been shown to be highly correlated with the number of primordial follicles in an ovary (i.e., true ovarian reserve).[11] The picoAMH assay was developed to allow more sensitive measurements. AMH measured using the picoAMH assay provides a significant new parameter to aid physicians in determining the status of women during the menopausal transition.

PRINCIPLE OF THE TEST

The picoAMH (MenoCheck®) ELISA is a quantitative three-step sandwich type immunoassay that is designed to measure human AMH. 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 AMH 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-124A picoAMH Calibrator A / Sample Diluent

One vial, 10 mL, labeled AMH Cal A/Sample Diluent, containing 0 pg/mL AMH in serum with non-mercury preservative. Store unopened at 2-8°C until the expiration date.

CAL-124B - CAL-124F picoAMH Calibrators B thru F (Lyophilized)

Five vials, labeled B-F, containing concentrations of approximately 10-1000 pg/mL AMH in serum with non-mercury preservative. 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 immediately for multiple use and discard after run. Avoid repeated freeze thaws.

Assay Calibration: The recombinant AMH concentrations in calibrators are standardized to purified recombinant mature AMH preparation that is characterized by mass spectroscopy and optical density at 280 nm. Values assigned by other methodologies may be different. Such differences, if present, may be caused by inter-method bias.

CTR-124-I & CTR-124-II picoAMH Controls I & II (Lyophilized)

Two vials, labeled Levels I and II containing low and high AMH concentrations in serum with non-mercury preservative. 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 immediately for multiple use and discard after run. Avoid repeated freeze thaws.

PLT-124 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-124 picoAMH 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-124 picoAMH 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. Microplate reader capable of absorbance measurement at 450 nm, 405 nm and 630 nm.
2. Microplate orbital shaker.
3. Microplate washer.
4. 96 well dilution plate or culture tubes for dilutions.
5. Semi-automated/manual precision pipette to deliver 10–250 µL.
6. Repeator pipette
7. Vortex mixer.
8. Deionized water.

WARNINGS AND PRECAUTIONS

For *in vitro*-diagnostic 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.

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 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.[12]

WARNING: Potential Chemical Hazard

Some reagents in this kit contain Pro-Clean 400 and Sodium azide[13] 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, Lithium heparin and K2 EDTA 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) Within two hours after centrifugation, transfer at least 500 µL of cell free sample to a storage tube, vortex and tightly stopper the tube immediately.

d) 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.

e) Avoid assaying lipemic, hemolyzed or icteric samples.

f) Avoid repeated freezing and thawing of samples. Thaw samples no more than 3 times.

g) 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.[14]

PROCEDURAL NOTES

1. A thorough understanding of this package insert is necessary for successful use of the picoAMH (MenoCheck®) 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 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. **picoAMH Calibrators B-F and picoAMH Controls I & II**: Tap and reconstitute picoAMH Calibrator B-F and picoAMH Controls I & II each with 1 mL deionized water. Solubilize, mix well and use after reconstitution.
   Note: In case sensitivity below calibrator B level is desired, dilute reconstituted calibrator B as below.
2. (a) CAL B/2: Mix 150 µL of reconstituted Cal B with 150 µL of Cal A/Sample diluent.
   (b) CAL B/3: Mix 100 µL of reconstituted Cal B with 200 µL 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. **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.

PREPARATION OF SAMPLES

1. Specimens producing absorbance readings above the measurable range (e.g., 6.0 to approximately 1,150 pg/mL) can be diluted with Calibrator A/Sample diluent prior to testing (maintaining a pipetting total volume of 100 µL for either neat specimen or diluted specimen).
2. The assay has been designed and validated for specimen dilutions of up to 20-fold. AMH concentrations of 6 to 23,000 pg/mL (0.006 to 23 ng/mL) to be measured quantitatively.
3. The read out (pg/mL) for diluted specimens must be corrected for the dilution factor. For example, a specimen that was diluted 10-fold prior to assay and reading 200 pg/mL will be reported as 2,000 pg/mL (i.e., 200 pg/mL x 10-fold dilution factor).
4. For specimens where the range of AMH concentrations can be estimated, an initial dilution protocol can be employed for efficient workflow and reagent use.

ASSAY PROCEDURE

Allow all specimens and reagents to reach room temperature and mix thoroughly by gentle inversion before use. Calibrators, controls, and unknowns should be assayed in duplicate.

Protocol-1 (Female of Reproductive age ≤ 40yrs)

1. Label the microtitration strips to be used.
2. Add **50 µL** of the **AMH/MIS Assay Buffer** to each well using a repeater pipette.
3. Pipette **100 µL** of the reconstituted **Calibrator and Controls** to the appropriate wells.
4. Pipette **10 µL** of **samples** using precision pipette to the sample designated wells.
5. Pipette **90 µL of Cal-124A/Sample-diluent** to the sample added wells.
   Note: Dilution levels higher than 10 folds can be achieved in a 96 well dilution plate or in culture tubes. Example: For a 15-fold dilution level add 10 ul of sample and 140ul of calibrator A in dilution plate well or culture tubes. After addition, gently shake the plate for 10 minutes and transfer 100µl of the diluted sample to the designated wells on the antibody coated plate.
6. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **3 hrs** at room temperature (23 ± 2°C).
7. Aspirate and wash each strip **5 times** with Wash Solution **(350 µL/per well)** using an automatic microplate washer.
8. Add **100 µL** of the **Antibody-Biotin Conjugate RTU** to each well using a repeater pipette.
9. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **1 hr** at room temperature (23 ± 2°C).
10. Aspirate and wash each strip **5 times** with the Wash Solution **(350 µL/per well)** using an automatic microplate washer.
11. Add **100 µL** of the **Streptavidin-Enzyme Conjugate-RTU** to each well using a repeater pipette.
12. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **30 mins** at room temperature (23 ± 2°C).
13. Aspirate and wash each strip **5 times** with the Wash Solution **(350 µL/per well)** using an automatic microplate washer.
14. Add **100 µL** of the **TMB chromogen solution** to each well using a repeater pipette. Avoid exposure to direct sunlight.
15. 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.*
16. Add **100 µL** of the **Stopping solution** to each well using a repeater pipette. Read the absorbance of the solution in the wells within **10 minutes**, using a microplate reader set to **(1) 450 nm, (2) 405 nm and 630 nm (machine blank).**
17. **IMPORTANT:** All diluted specimens should be multiplied by the appropriate **dilution factor** (**i.e. 10 or higher**) for the final concentration. Alternatively, multiply the calibrators by the **dilution factor (e,g. 10 or higher)** and input the corrected calibrator values in the data reduction software of the spectrophotometer prior to reading the assay.

***NOTE**: a) While reading the absorbance of the microtitration well, it is necessary to program the zero calibrator as a "**Blank**".*
*b) Each lab should establish their extrapolation criteria 1) **450 nm-630 nm** at the low end and **405 nm-630 nm** at the high end of the curve if needed.*
c) All diluted samples reading lower than the limit of detection should be run following Protocol-2.

Protocol-2 (Female of Advanced Reproductive age > 40yrs) *Females of all ages with diminished ovarian reserve*

Allow all specimens and reagents to reach room temperature and mix thoroughly by gentle inversion before use. Calibrators, controls, and unknowns should be assayed in duplicate.

***NOTE:** All serum/plasma samples reading higher than the highest calibrator should be diluted as appropriate in the 0 pg/mL Calibrator A/Sample diluent prior to assay.*

1. Label the microtitration strips to be used.
2. Add **50 µL** of the **AMH/MIS Assay Buffer** to each well using a repeater pipette.
3. Pipette **100 µL** of the reconstituted **Calibrator and Controls** to the appropriate wells.
4. Pipette **50 µL of samples** using precision pipette to the sample designated wells.
5. Pipette **50 µL of Cal-124A/Sample-diluent** to the sample added wells.
6. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **3 hrs** at room temperature (23 ± 2°C).
7. Aspirate and wash each strip **5 times** with Wash Solution **(350 µL/per well)** using an automatic microplate washer.
8. Add **100 µL** of the **Antibody-Biotin Conjugate RTU** to each well using a repeater pipette.
9. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **1 hr**. at room temperature (23 ± 2°C).
10. Aspirate and wash each strip **5 times** with the Wash Solution **(350 µL/per well)** using an automatic microplate washer.
11. Add **100 µL** of the **Streptavidin-Enzyme Conjugate-RTU** to each well using a repeater pipette.
12. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **30 mins** at room temperature (23 ± 2°C).
13. Aspirate and wash each strip **5 times** with the Wash Solution **(350 µL/per well)** using an automatic microplate washer.
14. Add **100 µL** of the **TMB chromogen solution** to each well using a repeater pipette. Avoid exposure to direct sunlight.
15. 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.*
16. Add 100 µL of the Stopping solution to each well using a repeater pipette. Read the absorbance of the solution in the wells within 10 minutes, using a microplate reader set to to (1) 450 nm, (2) 405 nm and 630nm (machine blank).
17. **IMPORTANT:** All diluted specimens should be multiplied by the appropriate **dilution factor** (**i.e. 2**) for the final concentration. Alternatively, multiply the calibrators by **a factor of 2** and input the corrected calibrator values in the data reduction software of the spectrophotometer prior to reading the assay.

***NOTE**: a) When reading the absorbance of the microtitration well, it is necessary to program the zero calibrator as a "**Blank**".*
*b) Each lab should establish their extrapolation criteria 1) **450 nm-630 nm** at the low end and **405 nm-630 nm** at the high end of the curve if needed.*
c) All diluted samples reading lower than the limit of detection can be run neat (100 ul sample in step-4 and skip step-5) in protocol-2. No calibration factor is required if run neat.

Combination Protocol

NOTE: Protocol-1 and Protocol-2 can also be performed simultaneously in the same run by marking the sample wells as per the protocol used. The sample results then should be processed as per the protocol by applying the correct **dilution factor**.

RESULTS

1. Calculate the mean optical density (OD) for each calibrator, control, or unknown specimen.
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 pg/mL along the x-axis.
3. Determine the AMH concentrations of the Controls and Unknown specimens from the calibration curve by matching their mean OD readings with the corresponding AMH concentrations.

LIMITATIONS

1. AMH results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings when being interpreted for diagnostic purposes.
2. AMH test results < 10 pg/mL should be carefully evaluated in the context of a full clinical work up to ensure that the use of contraceptives is not discontinued in women who have not yet reached menopause.
3. AMH test results > 10 pg/mL should be carefully evaluated in the context of a full clinical work up to ensure that uterine bleeding due to endometrial cancer is not dismissed as a potential diagnosis.
4. The assay is unaffected by icterus (bilirubin ≤ 66mg/dL), hemolysis (Hb ≤ 1000mg/dL), lipemia (Triglyceride ≤2000 mg/dL) and biotin (200 ng/mL to ≤10,000 ng/mL). Criterion: Recovery within ± 10 % of initial value.
5. If there is evidence of microbial contamination or excessive turbidity in a reagent, discard the vial.
6. As for any assay employing antibodies, the possibility exists for interference by heterophile antibodies in the samples.[15] Interference from heterophile antibodies has not been evaluated for this assay.

QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to assure proper performance.
- picoAMH ELISA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for picoAMH 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 (pg/mL)	Conc (pMol/L)
A1, A2	Calibrator A	0.022 (**Blank**)	0	0
B1, B2	B/2	0.015	3.8	0.027
C1, C2	B	0.03	7.6	0.054
D1, D2	C	0.111	31	0.221
E1, E2	D	0.375	104.7	0.748
F1, F2	E	1.148	360.2	2.572
G1, G2	F	2.917	1091	7.789

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

PERFORMANCE CHARACTERISTICS

The performance characteristic results are reported in pg/mL and can be converted to pmol/L using the conversion factor below.

1pg/mL = 0.00714pMol/L

Limit of Blank, Limit of Detection and Limit of Quantitation:
The Limit of Blank, Limit of Detection and Limit of Quantitation were determined in accordance with the CLSI EP17-A2 guidelines.[16]

Limit of Blank (LOB): The Limit of Blank was 0.5 pg/mL, calculated as the 95th percentile value from a minimum of n = 324 measurements of 4-6 analyte-free samples in each of 4 reagent lots. The Limit of Blank corresponds to the concentration below which analyte-free samples are found with a probability of 95 %.

Limit of Detection (LoD):
The LOD was 1.3 pg/mL, calculated as the [LOB + (1.645 * SD)] of measurements of twelve (12) specimens containing AMH in the range of 2.8 to 10.0 pg/mL, 3 replicates each, in six independent runs and in 4 lots of reagents (n=862). The LOD is the lowest amount of AMH in a sample that can be detected (value above the LOB) with a probability of 90%.

Limit of Quantitation (LoQ):
In the absence of a reference method, the LOQ determination was based on the CV of replicate testing of the 12 human serum pools evaluated to calculate the LOD. Serum was assayed in triplicate in each of 6 runs in each of 4 reagent lots. The LOQ of 3.2 pg/mL was calculated at an imprecision of 20% CV based on precision profiling.

Imprecision:
Precision was determined using picoAMH reagents, using human specimens according to guidance from CLSI EP05-A3.[17] Each of 3 lots were tested using a protocol in which specimens were tested in quadruplicate (2 sets of duplicates in each assay run) in 2 runs per day over 20 days (n = 160 per sample per reagent lot) The following table summarizes results from all 3 reagent lots (n = 480 specimens).

Sample	Lot	Mean pg/mL	Repeatability		Intermediate Precision	
			SD (pg/mL)	% CV	SD (pg/mL)	% CV
Serum 1	Lot1	14.6	0.8	5.5%	1.2	8.1%
	Lot2	14.2	0.6	4.2%	0.8	5.9%
	Lot3	15.5	0.7	4.5%	1.0	6.7%
Serum 2	Lot1	80.1	2.2	2.8%	3.6	4.5%
	Lot2	80.0	2.0	2.5%	3.4	4.2%
	Lot3	80.8	2.8	3.5%	3.4	4.2%
Serum 3	Lot1	620.3	17.4	2.8%	22.9	3.7%
	Lot2	609.6	16.8	2.8%	24.2	4.0%
	Lot3	643.2	19.2	3.0%	24.0	3.7%
Serum 4	Lot1	942.8	28.9	3.1%	51.3	5.4%
	Lot2	924.1	23.7	2.6%	48.5	5.3%
	Lot3	935.2	36.8	3.9%	47.4	5.1%

Recovery
Known amounts of recombinant human AMH were added to low analyte serum samples pool to generate fifteen specimens. The concentration of AMH was determined before and after the addition of exogenous AMH and the percent recovery was calculated on 3 lots of reagents run in triplicates. A representative data is summarized below.

Specimen Tested	Target AMH (pg/mL)	Mean AMH (pg/mL)	% Difference	Mean Recovery (%)
Specimen 1	6.75	7.4	106	110.0
Specimen 2	12.5	12.8	100	102.1
Specimen 3	24	21.7	88	90.6
Specimen 4	36	32.9	89	91.4
Specimen 5	60	58.2	99	97.0
Specimen 6	120	119.7	98	99.7
Specimen 7	240	259.4	108	108.1
Specimen 8	360	377.0	105	104.7
Specimen 9	480	494.2	100	103.0
Specimen 10	600	641.6	107	106.9
Specimen 11	720	739.5	103	102.7
Specimen 12	840	862.5	103	102.7
Specimen 13	960	981.8	102	102.3
Specimen 14	1080	1108.8	101	102.7
Specimen 15	1200	1164.1	100	97.0

Hook Effect:
There is no high-dose hook effect at AMH concentrations up to 256,000 pg/mL.

Linearity:
Linearity of picoAMH ELISA was evaluated according to CLSI EP6-A using 16 specimens in the measuring interval of 1-1200 pg/mL in replicates of 3 for each specimen. The allowable nonlinearity was calculated on 3 lots of reagents run. The results are summarized below.

Parameter	Reagent Lot		
	Lot 1	Lot 2	Lot 3
N	16	16	16
Linear Range (pg/mL)	6-1095	6-1091	7-1110
Slope	1.01	1.01	1.03
X- Intercept	5.1	2.8	-1.2
SE (least square linear regression)	19.7	16.9	27.0

Analytical Specificity:

The monoclonal antibody pair used in the assay is specific for human AMH. The table below summarizes results of cross-reactivity of proteins or compounds structurally and functionally related to AMH in the picoAMH assay.

Cross-Reactant	Concentration of Cross-Reactant Tested (pg/mL)	AMH Reported Value (pg/mL)	% Cross-reactivity
Activin B	50000	< 2	0.003%
Inhibin A	100000	< 2	0.001%
Inhibin B	100000	< 2	0.001%
alpha-2 Macroglobulin	65000	< 2	0.002%
Follistatin-288	50000	< 2	0.003%
Follistatin-315	50000	< 2	0.003%
hAMH, ProMature*	600	627.8	104.641%
hAMH, Mature	600	< 2	0.217%
Myostatin	50000	< 2	0.003%
FSH**	39683	< 2	0.003%
TSH***	869565	< 2	0.000%
LH****	9312	< 2	0.014%
Prolactin	211000	< 2	0.001%
Testosterone	100000	< 2	0.001%
Estrone Sulphate	100000	< 2	0.001%
DHEA	100000	< 2	0.001%
Progesterone	100000	< 2	0.001%
Estradiol	50000	< 2	0.003%

*Positive Control

**Based on 2nd International standard, human, bioassay: 08/282

***Based on 3rd international standard for immunoassay: 81/565

****Based on 1st international standard, human, recombinant: 96/602

Interference:

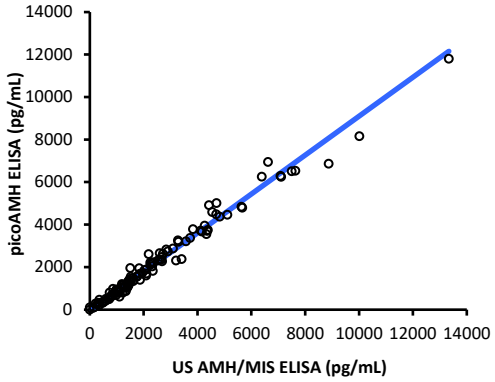
Interference was tested according to CLSI EP7-A2. Serum samples with AMH concentrations at 27 pg/mL and 300 pg/mL were evaluated as controls and tests with the doses of interferents specified in the table below. Interference was considered significant if the analyte recovery is ± 10% of the value of AMH measured. At the concentrations tested, none of the potential interferents tested showed more than ± 10% difference effect on picoAMH measurement in human sera.

Interferent	Highest Concentration Tested with no Significant Interference
Hemoglobin	1000 mg/dL
Ascorbic Acid	0.3 mg/mL
Bazedoxifene	1 µg/mL
Cefoxitin Na	2.5 mg/mL
Metformin	2 mg/mL
Triptorelin Acetate	15 µg/mL
Estradiol (beta)	1 ng/mL
Estrone Sulphate	1 ng/mL
Folic Acid	0.4 µg/mL
Levothyroxine	0.2 µg/mL
Medroxyprogesterone Acetate	1 µg/mL
Escitalopram Oxalate	0.1 mg/mL
Venlafaxine HCL	15 µg/mL
Doxycycline Hyclate	50 µg/mL
Bilirubin	0.66 mg/mL
Levodopa	30 µg/mL
Rifampicin	60 µg/mL
Heparin, Sodium Salt	30 U/mL
Intralipid	20 mg/mL
Acetaminophen	0.2 mg/mL
Ampicillin Na	1 mg/mL
Bisphosphonate	0.02 mg/mL
Cyclosporine	5 ug/mL
Estropipate	0.015 mg/mL
Fluoxetine HCl	3.5 µg/mL
Levonorgestrel	3 µg/mL
Metronidazole	0.2 mg/mL
Theophylline	0.1 mg/mL
Biotin	10,000 ng/mL
Acetylsalicyclic Acid	1 mg/mL
Citalopram	0.01 mg/mL
Ibuprofen	0.5 mg/mL
Phenylbutazone	0.1 mg/mL
Pregabalin	0.01 mg/mL
Raloxifene HCl	0.12 mg/mL
Paroxetine HCl	1 µg/mL
Gabapentin	0.09 mg/mL
Norethindrone	0.03 mg/mL
Cholesterol	5 mg/mL
Progesterone	0.4 mg/mL
Acetylcysteine	0.15 mg/mL
Cyproterone Acetate	0.3 mg/mL
Methyldopa	0.02 mg/mL

Method Comparison:

The picoAMH (MenoCheck®) ELISA has been compared to Ultra-Sensitive AMH/MIS ELISA (AL-105) using 115 serum samples in the range of 0 - 13300 pg/mL. Passing & Bablok fit analysis of the results yielded the following Regression: picoAMH (MenoCheck®) ELISA (AL-124-i) = 0.92 US AMH/MIS ELISA (AL-105) – 50.66

(rs=0.99 P<0.0001). *Note that the LoQ for AL.105 is 60 pg/mL.*



EXPECTED RESULTS

The expected results were determined by testing 20,738 specimens from a total of 13,555 healthy subjects whose blood was collected at multiple study sites in the US and Europe. Because AMH declines as ovarian reserve diminishes with age the values obtained using the picoAMH (MenoCheck®) ELISA are age stratified.

Age Range	Specimens Tested*	Mean	Mean SE	Lower Limit (2.5%)	Upper Limit (97.5%)
20-25	234	5018	331	740	17356
26-30	542	4218	151	444	12774
31-35	985	3424	106	249	12213
36-40	1436	2375	67	36	8874
41-45	2723	881	21	<2	3866
46-50	5931	258	6	<2	1718
51-55	4546	46	2	<2	390
56-60	1972	6	3	<2	12
61-65	2369	2	<2	<2	3
*	These 20,738 specimens were from a total of 13,555 individual women				

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.*

INTERPRETATION OF SERUM AMH VALUES

While healthy women become menopausal at an average age of 51 there is considerable individual variation and the age at menopause can range from approximately 40 to 60 years of age for an individual. This age based AMH results are only a rough indicator of menopausal status. In order to provide more detailed interpretative support, AMH levels were determined in approximately 1500 specimens from women participating in a study of the menopausal transition[25]. The final menstrual period (FMP) for each woman were assigned retrospectively after 12 months of amenorrhea (the clinical definition of natural menopause). Menopausal categories for assigning status were based on the approximate time to the final menstrual period (FMP). Three menopausal categories were defined based on the time to final menstrual period in order to calculate AMH cut-off values relative to greater than or less than 5 years to the FMP.

Years from FMP menopausal categories and AMH concentration.

Menopausal Category	picoAMH (pg/mL)
>5 years from FMP	≥100 pg/mL
< 5 years from FMP	10-99.9 pg/mL
at FMP or later	<10 pg/mL

Sensitivity (Detection Rate) and specificity (1-False positive rate) for the cutoffs at <10 pg/mL and ≥100 pg/mL are shown in the Table below.

Clinical performance of picoAMH ELISA to identify menopausal category.

picoAMH cutoff level	Menopausal Category	Detection Rate (%) (95% CI)[1]
≥100 pg/mL	>5 years from FMP	82.2 (76.6 – 86.9)
<10 pg/mL	at FMP or later	86.1 (80.4 – 89.9)
10-99.9 pg/mL	<5 years from FMP	34.8 (28.6 – 41.3)

[1] 95% confidence interval in parentheses.

False positive rate of picoAMH ELISA to identify menopausal category.

picoAMH cutoff level	Classified Menopausal Category	True Menopausal Status	False Positive Rate (%) (95% CI)[1]
≥100 pg/mL	>5 years from FMP	<5 years from FMP	26.5 (20.9-32.7)
		at FMP or later	1.7 (0.4 – 4.4)
<10 pg/mL	at FMP or later	>5 years from FMP	8.2 (5.0-12.6)
		<5 years from FMP	38.7 (32.4 – 45.3)
10-99.9 pg/mL	<5 years from FMP	>5 years from FMP	9.6 (6.1-14.1)
		at FMP or later	12.2 (8.2-17.1)

[1] 95% confidence interval in parentheses.

The picoAMH (MenoCheck®) ELISA performs reasonably well in distinguishing women at FMP or later and women >5 years from FMP. 86% of women who were at FMP or later also had a picoAMH level <10 pg/mL and 82% of women who were >5 years from FMP also had a picoAMH level > 100 pg/mL. However, the picoAMH (MenoCheck®) ELISA test result has limited deterministic value when AMH values fall between 10 and 99.9 pg/mL. Test results falling into this range should be interpreted with caution.

To aid in the interpretation of AMH levels between 10 and 99.9 pg/mL, the following table provides the likelihood ratios (LR) for distinguishing 1) women < 5 years away from their FMP vs women who are past their FMP, or 2) women who are less than 5 years away from their FMP vs women who are > 5 years from their FMP.

Likelihood ratios associated with menopausal categories.

Menopausal Category	Positive Likelihood Ratio (95% CI)	Negative Likelihood Ratio (95% CI)
<5 years from FMP vs. at FMP or later	2.86 (1.94-4.22)	0.74 (0.67-0.83)
<5 years from FMP vs. >5 years from FMP	3.64 (2.35-5.62)	0.72 (0.65-0.80)

Thus, a woman with an AMH test result between 10 and 99.9 pg/mL is 2.86 times more likely to be < 5 years away from her FMP than to be post-menopausal. Similarly, a woman with an AMH result between 10 and 99.9 pg/mL is 3.64 times more likely to be < 5 years away from her FMP than to be > 5 years away from her FMP.

Serum AMH levels can also provide a useful rule-out for post-menopausal status. Approximately 12% of post-menopausal women presented with an AMH test result higher than 10 pg/mL, and 2% present with an AMH test result higher than 100 pg/mL. During the peri-menopausal transition, pituitary, gonadal, and sex steroid hormone levels will vary considerably. However, an AMH result that is high or low relative to the patient clinical presentation is recommended to be repeated.

In some women, an unexpectedly high AMH relative to the woman's age or clinical presentation can represent residual ovarian activity or presence of antral follicles despite the self-reported absence of menstrual bleeding or due to secondary (hypothalamic) amenorrhea.[18] The absence of a menstrual cycling per se does not unequivocally indicated insufficient ovarian reserve. AMH-producing follicles have been documented despite their failure to ovulate and/or support menstruation.[19-21] Thus, AMH results must always be interpreted in the context of other clinical findings and laboratory results, such as: patient age, self-reported

menstrual history, vasomotor symptoms, family history (i.e., mother's reported age at menopause), history of fertility/infertility ovary or other reproductive surgery, FSH and estradiol determinations. AMH is not cycle day dependent.

The menstrual cycle is expected to be irregular in women for whom there is a clinical need to assess menopausal status. Samples may be drawn at any time for assessment of AMH.[22-24]

RISKS ASSOCIATED WITH FALSELY LOW OR FALSELY HIGH picoAMH TEST RESULTS

An inaccurate high AMH result could be wrongly suggest that a woman is not post-menopausal leading the physician to counsel a patient to continue hormonal contraceptives when it is no longer necessary. An inaccurate low AMH result may lead a physician to recommend 1) discontinuing contraceptives when it is still necessary to prevent pregnancy, or 2) evaluating bone mineral density or 3) consider prescribing hormone replacement therapy.

It should be noted that abnormal bleeding episodes may be independent of an AMH test result. Irregular or abnormal bleeding or vaginal discharge that is increasing in amount, occurring between anticipated periods, or occurring after the clinical assessment of menopause (i.e., > 12 consecutive months of amenorrhea) may indicate the presence of endometrial cancer. Physicians should carefully evaluate patients that report abnormal bleeding or unexpectedly high AMH test results. In this setting the evaluation should include a consideration of risk factors of endometrial cancer and gynecological cancers. Physicians should counsel these patients regarding their options for diagnosis by endometrial biopsy and transvaginal ultrasound accordingly.

As with any single assay determination, AMH testing should be used in conjunction with other clinical and/or laboratory findings. Appropriate mitigations include obtaining a detailed clinical history and additional laboratory testing. Risk is mitigated by other clinical features which if disparate with a high AMH would indicate the need to re-examine the patient and re-test AMH after several months.

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This assay is intended for *in vitro* diagnostic use.

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FOR EXPORT ONLY

picoAMH (MenoCheck®) ELISA PROTOCOLS

Steps	Procedure	Protocol-1 (Female ≤ 40yrs)	Protocol-2 (Female > 40yrs)* **Females all ages with diminished ovarian reserve*
1.	a.	Label Microtitration strips to be used.	
	b.	Add **50µL** of **AMH/MIS Assay Buffer** to each well.	
	c.	Add **100 µL** of the reconstituted **Calibrator, Controls** to the designated wells.	
	d.	Add **10 µL** of the **samples** to the designated wells.	Add **50 µL** of the **samples** to the designated wells.
	e.	Add **90 µL** of **Cal-124A/Sample-diluent** to the sample added wells.	Add **50 µL** of the **Cal-124A/Sample-diluent to** the sample added wells.
	f.	Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **3 hrs** at room temperature (23 ± 2°C).	
2.		Aspirate and wash each strip **5 times** with Wash Solution **(350 µL/per well)** using an automatic microplate washer.	
3.	a.	Add **100 µL** of the **Antibody-Biotin Conjugate RTU** to each well using a repeater pipette.	
	b.	Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **1 hr** at room temperature.	
4.		Aspirate and wash each strip **5 times** with the Wash Solution **(350 µL/per well)** using an automatic microplate washer.	
5.	a.	Add **100 µL** of the **Streptavidin-Enzyme Conjugate-RTU** to each well using a repeater pipette.	
	b.	Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **30 minutes** at room temperature.	
6.		Aspirate and wash each strip **5 times** with the Wash Solution **(350 µL/per well)** using an automatic microplate washer.	
7.	a.	Add **100 µL** of the **TMB chromogen solution** to each well using a repeater pipette. Avoid exposure to direct sunlight.	
	b.	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.*	
8.	a.	Add **100 µL** of the S**topping solution** to each well using a repeater pipette. Read the absorbance of the solution in the wells within **10 minutes**, using a microplate reader set to (1) **450 nm, (2) 405 nm and 630nm (machine blank).**	
9.	a.	Multiply the calibrators by **a factor of 10** prior to data reduction.	Multiply the calibrators by **a factor of 2** prior to data reduction.

NOTE:

Protocol-1 and Protocol-2 can also be performed simultaneously in the same run by marking the sample wells as per the protocol used. The sample results then should be processed as per the protocol by applying the protocol calibration factor.

Symbols used with Ansh Labs Assays

Symbol	English	Deutsch	Français	Español	Italiano
	Biohazard	Biogefahr	danger biologique	Riesgo biológico	rischio biologico
	Caution	Vorsicht	mise en garde	precaución	attenzione
	Consult instructions for use	Gebrauchsanweisung beachten	Consulter les instructions d'utilisation	Consulte las instrucciones de uso	Consultare le istruzioni per l'uso
	In vitro diagnostic device	In-vitro-Diagnostikum	Usage Diagnostic in vitro	Para uso Diagnóstico in vitro	Per uso Diagnostica in vitro
	Catalogue number	Katalog-Nr.	Numéro de catalogue	Número de catálogo	Numero di Catalogo
	Lot. No. / Batch code	Chargen-Nr.	Numéro de lot	Número de lote	Numero di lotto
	Storage Temperature	Lagerungstemperatur	Température de conservation	Temperatura de conservación	Temperatura di conservazione
	Expiration Date	Mindesthaltbarkeits-datum	Date limite d'utilisation	Fecha de caducidad	Data di scadenza
	Date of Manufacture	Herstellungsdatum	date de fabrication	fecha de manufactura	Data di produzione
	Legal Manufacturer	Hersteller	Fabricant	Fabricante	Fabbricante
Content	Content	Inhalt	Contionnement	Contenido	Contenuto
Volume/No.	Volume / No.	Volumen/Anzahl	Volume/Quantité	Volumen/Número	Volume/Quantità
	96-well plate	Platte mit 96 Vertiefungen	Plaque à 96 puits	placa de 96 pocillos	piastra a 96 pozzetti