Instructions for Use

Human Anti-Mullerian Hormone ELISA

IVD

CE

REF EIA-6053

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Please use only the valid version of the Instructions for Use provided with the kit.

Table of Contents

1 INTENDED USE

This test kit is intended for use in the quantitative determination of human Anti-Mullerian Hormone (AMH) levels in serum lithium heparin plasma samples.

It is for in-vitro diagnostic use only.

2 INTRODUCTION

Anti-Müllerian Hormone or Müllerian-inhibiting hormone (MIH) is a glycoprotein hormone structurally related to inhibin and activin from the transforming growth factor beta superfamily, whose key roles are in growth differentiation and folliculogenesis. AMH expression is critical to sex differentiation at a specific time during fetal development, and appears to be tightly regulated by nuclear receptor SF1, transcription GATA factors, sex-reversal gene DAX1, and follicle-stimulating hormone (FSH). AMH is activated by SOX9 in the Sertoli cells of the male fetus thereby arresting the development of fallopian tubes, uterus, and upper vagina. AMH is also a product of granulosa cells of the preantral and small antral follicles in women. As such, AMH is only present in the ovary until menopause. AMH level is also lower and even below the detection limit if women with premature ovarian failure of any cause, including after cancer chemotherapy, etc.

3 ASSAY PRINCIPLE

This ELISA is designed, developed and produced for the quantitative measurement of human Anti-Mullerian Hormone in serum or heparin plasma samples. The assay utilizes the two-site “sandwich” technique with two selected antibodies that bind to different epitopes of human AMH.

Assay calibrators, controls and patient samples are added directly to microtiter wells of a microplate that is coated with antibody to N-terminal AMH along with another AMH specific antibody labelled with horseradish peroxidase (HRP). After an initial incubation period, the plate is washed and a “sandwich” of solid-phase antibody – human AMH – HRP-conjugated monoclonal antibody is formed. The unbound monoclonal antibodies and buffer matrix are removed in the subsequent washing step. For the detection of this immunocomplex, the well is then incubated with a substrate solution in a timed reaction and then measured in a spectrophotometric microplate reader. The enzymatic activity of the immunocomplex bound to the wall of each microtiter well is directly proportional to the amount of human AMH in the test sample. A standard curve is generated by plotting the absorbance versus the respective human AMH concentration for each standard on a Cubic or point-to-point curve fitting. The concentration of human AMH in test samples is determined directly from this calibration curve.

4 REAGENTS: PREPARATION AND STORAGE

This test kit must be stored at 2 °C - 8 °C upon receipt. For the expiration date of the kit refer to the label on the kit box. All components are stable until this expiration date.

Prior to use allow all reagents to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.

1. **AMH Antibody Coated Microplate**
   One microplate with 12 x 8 strips (96 wells total) coated with anti-AMH antibody. The plate is framed and sealed in a foil zipper foil pouch with a desiccant.
   This reagent should be stored at 2 °C - 8 °C and is stable until the expiration date on the kit box.
2. **AMH Tracer Antibody**
   One vial contains 0.35 mL HRP-labeled anti-human AMH antibody in a stabilized protein matrix.
   This reagent should be stored at 2 °C - 8 °C and is stable until the expiration date on the kit box.
3. **ELISA Wash Concentrate**
   One bottle contains 30 mL of 30-fold concentrate.
   Before use the contents must be diluted with **870 mL** of demineralized water and mixed well. Upon dilution, this yields a working wash solution containing a surfactant in phosphate-buffered saline with a non-azide, non-mercury preservative.
   The diluted wash solution may be stored at room temperature and is stable until the expiration date on the kit box.
4. **ELISA HRP Substrate**
   One bottle contains 12 mL of ready-to-use tetramethylbenzidine (TMB) with hydrogen peroxide.
   This reagent should be stored at 2 °C - 8 °C and is stable until the expiration date on the kit box.
5. **ELISA Stop Solution**
   One bottle contains 12 mL of 0.6 N sulfuric acid.
   This reagent may be stored at 2 °C - 8 °C or room temperature and is stable until the expiration date on the kit box.
   Caution: this component contains potentially hazardous material.
6. **AMH Calibrators**
   Six vials containing human AMH in a lyophilized bovine serum-based matrix with Proclin-300 as a preservative.
   Refer to vials for exact concentration for each standard.
   These reagents should be stored at 2 °C – 8 °C prior to reconstitution and are stable until the expiration date on the kit box.
7. **AMH Controls**
   Two vials containing human AMH in a lyophilized bovine serum-based matrix with Proclin-300 as preservative.
   Refer to vials for exact concentration range for each control.
   Both controls should be stored at 2 °C – 8 °C prior to reconstitution and are stable until the expiration date on the kit box.
8. **Tracer Antibody Diluent**
   One vial containing 7 mL ready-to-use buffer. It should be only used for tracer antibody dilution according to the assay procedures.
   This reagent should be stored at 2 °C - 8 °C and is stable until the expiration date on the kit box.

5 SAFETY PRECAUTIONS

The reagents must be used in a professional setting by trained personnel. The source material for reagents containing bovine serum was derived in the contiguous 48 United States. It was obtained only from healthy donor animals maintained under veterinary supervision and found free of contagious diseases. Wear gloves while performing this assay and handle these reagents as if they are potentially infectious. Avoid contact with reagents containing TMB, hydrogen peroxide, or hydrochloric acid. TMB may cause irritation to skin and mucous membranes and cause an allergic skin reaction. TMB is a suspected carcinogen. Hydrochloric acid may cause severe irritation on contact with skin. Provide good ventilation in process area to prevent formation of vapor. Do not breathe mist, vapors, spray. Do not get in eyes, on skin, or on clothing. Do not ingest or inhale fumes. On contact, flush with copious amounts of water for at least 15 minutes. Use Good Laboratory Practices.

6 MATERIALS REQUIRED BUT NOT PROVIDED

1. Precision single channel pipettes capable of delivering 50 µL, 100 µL, and 1000 µL
2. Disposable pipette tips suitable for above volume dispensing
3. Disposable plastic 100 mL and 1000 mL bottle with caps
4. Aluminum foil
5. Deionized or distilled water
6. Plastic microtiter well cover or polyethylene film
7. ELISA multichannel wash bottle or automatic (semi-automatic) washing system
8. Spectrophotometric microplate reader capable of reading absorbance at 450 nm and 650 or 630 nm

7 SPECIMEN COLLECTION

Serum or heparin plasma are acceptable samples.

Collect whole venous blood into serum collection tubes or tubes containing lithium heparin. Gently invert tube 3-4 times according to manufacturer's directions. Centrifuge tubes at 1500 RCF for 15 minutes. Carefully pipette off the serum or plasma and transfer to a clean test tube or vial. It is recommended to store samples at 2-8 ºC if tested within one week of collection or aliquot samples and store at ≤ - 20 ºC for future testing (within 2 weeks).

8 ASSAY PROCEDURE

8.1 Reagent Preparation

1. Prior to use allow all reagents to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.
2. ELISA **Wash Concentrate** must be diluted to working solution prior use. Please see REAGENTS section for details.
3. Reconstitute all assay calibrators level 1 to level 6 and controls by adding **0.5 mL** of demineralized water to the vial.
   Allow the standards and controls to sit undisturbed for 5 minutes, and then mix well by gentle vortexing.
   Make sure that all solid is dissolved completely prior to use. These reconstituted standards and controls may be stored at 2 °C – 8 °C for up to 6 hours or at ≤ - 20 °C for long term storage. Do not exceed 3 freeze-thaw cycles
4. Test Configuration

ROW	STRIP 1	STRIP 2	STRIP 3	STRIP 4
A	CAL 1	CAL 5	SAMPLE 1	SAMPLE 5
B	CAL 1	CAL 5	SAMPLE 1	SAMPLE 5
C	CAL 2	CAL 6	SAMPLE 2	SAMPLE 6
D	CAL 2	CAL 6	SAMPLE 2	SAMPLE 6
E	CAL 3	C 1	SAMPLE 3	Etc.
F	CAL 3	C 1	SAMPLE 3	
G	CAL 4	C 2	SAMPLE 4	
H	CAL 4	C 2	SAMPLE 4	

5. Place a sufficient number of AMH-coated microwell strips in a holder to run human AMH calibrators, controls and unknown samples in duplicate.
6. Prepare Tracer Antibody by diluting with Tracer Antibody Diluent at 1:21 prior to use according to the table below. Once diluted, the reagent is stable for up to 3 hours at 25 ºC or up to 20 hours at 2 – 8 ºC. **Caution: some of the reagent may become trapped in the vial cap. To ensure complete recovery of the reagent, vial should be centrifuged briefly or shaken down prior to opening cap. Open cap carefully to avoid spillage.**

# of Strip	Tracer Antibody Diluent	Tracer Antibody
1	0.5 mL	25 µL
2	1 mL	50 µL
3	1.5 mL	75 µL
4	2 mL	100 µL
5	2.5 mL	125 µL
6	3 mL	150 µL
7	3.5 mL	175 µL
8	4 mL	200 µL
9	4.5 mL	225 µL
10	5 mL	250 µL
11	5.5 mL	275 µL
12	6 mL	300 µL

8.2 Assay Procedure

1. Add **50 µL** of Calibrators, Controls and patient samples into the designated microwells.
2. Add **50 µL** of above diluted tracer antibody into the designated microwells.
3. Seal the plate securely, cover with foil or other material to protect from light, and ***incubate the plate at 2 ℃ – 8 ℃ for 18 to 20 hours, static***. It is optional to incubate the plate at room temperature by rotating on an ELISA plate shaker for **4 hours ± 15 minutes** at 400 to 450 rpm (small orbit radius) or at 180 rpm (large orbit radius).
4. Remove the aluminum foil and plate sealer. Aspirate the contents of each well. Wash each well 5 times by dispensing **350 µL** of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
5. Immediately add **100 µL** of ELISA HRP Substrate into each of well.
6. Cover with foil or other material to protect from light, and incubate static at room temperature for 20 ± 2 minutes.
7. Remove the aluminum foil and add **100 µL** of ELISA Stop Solution into each of the wells. Mix gently.
8. Read the absorbance at **450 nm** with reference filter at 620, 630 or 650 nm immediately.

9 PROCEDURAL NOTES

1. It is recommended that all calibrators, controls and unknown samples be assayed in duplicate. The average absorbance reading of each duplicate should be used for data reduction and the calculation of results.
2. Keep light sensitive reagents in the original amber bottles.
3. Store any unused antibody-coated strips in the foil zipper bag with desiccant to protect from moisture.
4. Careful technique and use of properly calibrated pipetting devices are necessary to ensure reproducibility of the test.
5. Incubation times or temperatures other than those stated in this insert may affect the results.
6. Avoid introducing air bubbles into the microwells as this could result in lower binding efficiency and higher CV% of duplicate reading.
7. All reagents should be mixed gently and thoroughly prior to use. Avoid foaming.
8. If adapting this assay to an automated ELISA system a procedural validation is necessary if there is any modification of the assay procedure.
9. To ensure the accuracy of samples that test above the dynamic range of the assay (around 20 ng/mL), a special diluent is required and can be purchased separately.

10 INTERPRETATION OF RESULTS

It is recommended to use a cubic plot calibration curve fitting.

1. Calculate the average absorbance for each pair of duplicate test results.
2. Subtract the average absorbance of the level 1 standard (0 ng/mL) from the average absorbance of all other readings to obtain corrected absorbance or use 0 calibrator for blank in computer program.
3. The calibration curve is generated by the **corrected absorbance** of all calibrator levels on the ordinate against the calibrator concentration on the abscissa using a **cubic plot.** Appropriate computer assisted data reduction programs may also be used for the calculation of results.

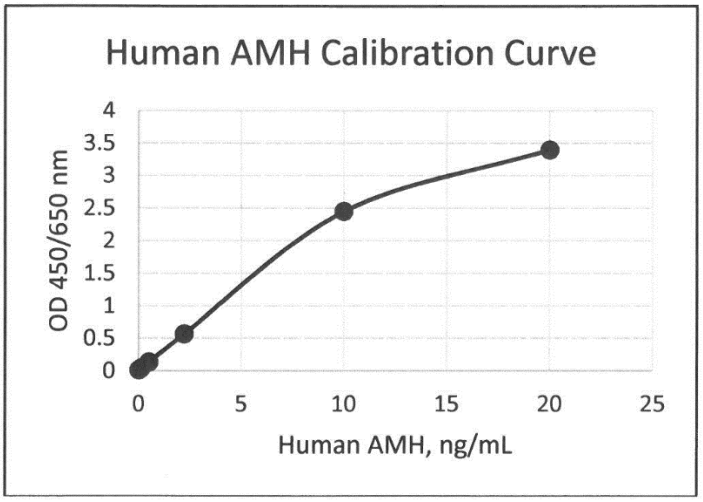
The AMH concentrations for the controls and the patient samples are read directly from the calibration curve using their respective corrected absorbance.

11 EXAMPLE DATA AND STANDARD CURVE

A typical absorbance data and the resulting standard curve from this AMH ELISA are represented.

This curve should not be used in lieu of standard curve generated with each assay.

Well I.D.	OD 450/650 nm Absorbance			Results ng/mL
	Readings	Average	Corrected	
Cal-1 0 ng/mL	0.014 0.014	0.014	0.000	
Cal-2 0.11 ng/mL	0.037 0.037	0.037	0.023	
Cal-3 0.49 ng/mL	0.128 0.135	0.132	0.118	
Cal-4 2.22 ng/mL	0.574 0.562	0.568	0.540	
Cal-5 10 ng/mL	2.420 2.481	2.450	2.436	
Cal-6 20 ng/mL	3.451 3.346	3.398	3.384	
Control 1	0.335 0.339	0.337	0.323	1.32 ng/mL
Control 2	1.576 1.596	1.586	1.572	6.12 ng/mL

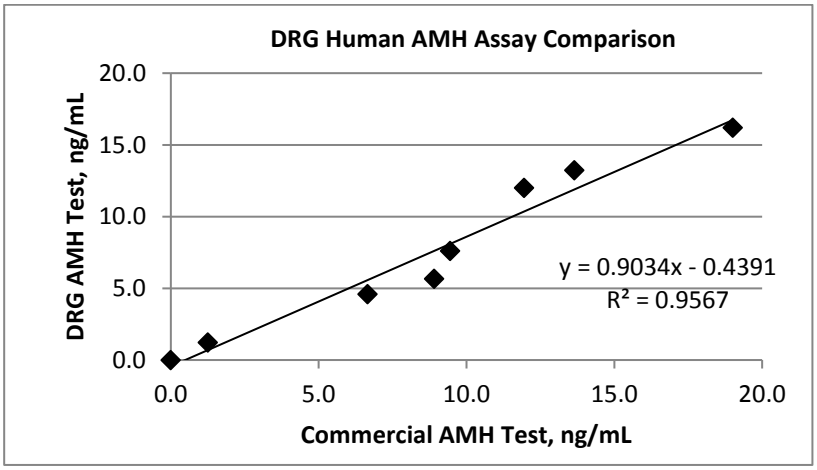


12 EXPECTED VALUES

Each laboratory should establish its own normal range based on gender and age.

Eight fresh serum samples were tested side-by-side using DRG human AMH ELISA and a well-known commercial human AMH ELISA. A satisfactory correlation result was obtained as showed in the chart below.

Serum	Age	Gender	Commercial	DRG
1	26	F	9.45	7.60
2	37	M	8.91	5.65
3	51	F	0.00	0.00
4	24	F	13.65	13.23
5	31	M	19.00	16.21
6	35	M	6.66	4.60
7	24	F	1.25	1.22
8	38	F	11.95	12.00



13 LIMITATION OF THE PROCEDURE

1. In view of complicated AMH range which is strongly related to age and gender, each laboratory should establish its own normal range for the application of AMH test.
2. For sample values reading greater than the highest standard, it is recommended to re-assay samples with dilution (i.e. 1:5 or 1:10) with AMH Sample Diluent.
3. Water deionized with polyester resins may inactive the horseradish peroxidase enzyme.

14 QUALITY CONTROL

To assure the validity of the results each assay should include adequate controls.

15 PERFORMANCE CHARACTERISTICS

15.1 Sensitivity

The analytical sensitivity (LLOD) of the human AMH ELISA as determined by two standard deviations above the average absorbance of 20 replicate determinations of zero calibrator is approximately 0.02 ng/mL.

15.2 High Dose "hook" effect

This assay showed no high dose "hook" effect for AMH level up to 1,000 ng/mL.

15.3 Precision

The intra-assay precision was validated by measuring two serum samples in a single assay with 16-replicate determinations.

Mean AMH Value (ng/mL)	CV (%)
4.50	5.6
10.50	3.9

The inter-assay precision was validated by measuring two control samples in duplicate in 9 individual assays over an 18 day period.

Mean AMH Value (ng/mL)	CV (%)
1.38	8.1
6.03	2.1

15.4 Linearity

Two serum samples were diluted with AMH Sample Diluent and tested. The results of AMH concentration in the value of ng/mL are as follows:

Sample 1 Dilution	Observed Value	Expected Value	Recovery %
Neat	19.28	-	-
80%	15.25	15.42	99%
60%	11.63	11.57	101%
40%	8.24	7.71	107%
20%	4.17	3.86	107%

Sample 2 Dilution	Observed Value	Expected Value	Recovery %
Neat	15.52	-	-
80%	12.07	12.42	97%
60%	9.25	9.31	99%
40%	5.90	6.21	95%
20%	3.08	3.10	99%

15.5 Spike Recovery

Two serum samples were spiked together in varying volumes and tested. The results of AMH concentration in the value of ng/mL are as follows:

#	Orig. Value	Amount Spiked	Observed Value	Expected Value	Recovery %
1	4.08	1.95	5.58	6.03	93%
		3.89	6.73	7.97	84%
		5.83	8.12	9.91	82%
		7.78	10.93	11.86	92%
2	9.07	0.21	8.88	9.28	96%
		0.42	9.40	9.49	99%
		0.62	9.84	9.69	102%
		0.80	10.98	9.90	111%

15.6 Interference

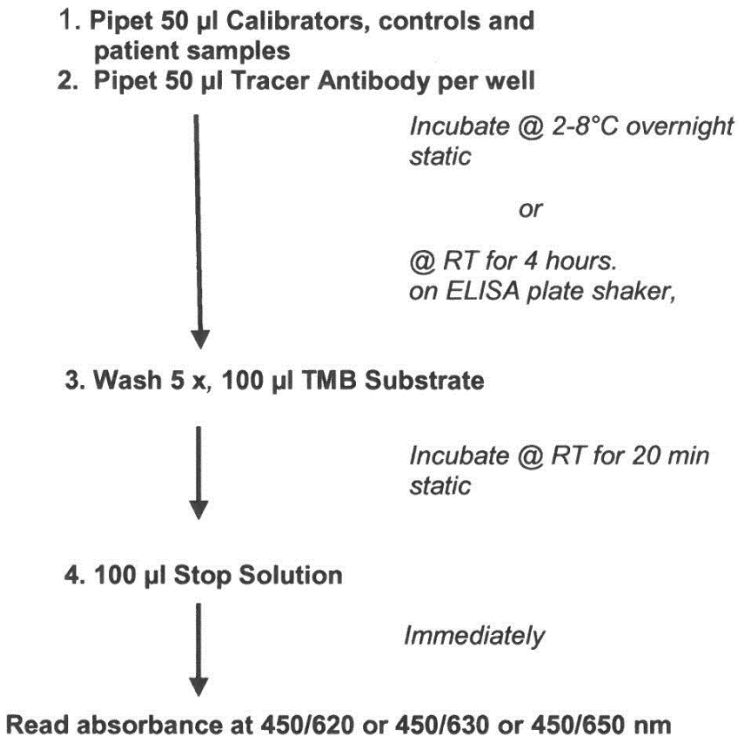
Interference was tested by spiking potential interferents into a sample at various concentrations along with a control serum, which was spiked with solvent without an interferent. The samples were then tested in the assay. The results are as follows:

Hemoglobin 50 mg/dL	Test AMH (ng/mL)	Control AMH (ng/mL)	Bias (ng/mL)	Bias (%) (dobs)
	1.25	1.25	0.00	0.4%
	4.85	4.96	0.12	2.3%
Hemoglobin 100 mg/dL	Test AMH (ng/mL)	Control AMH (ng/mL)	Bias (ng/mL)	Bias (%) (dobs)
	1.19	1.25	0.06	5.2%
	4.47	4.96	0.49	9.8%
Hemoglobin 200 mg/dL	Test AMH (ng/mL)	Control AMH (ng/mL)	Bias (ng/mL)	Bias (%) (dobs)
	1.51	1.25	0.26	21.0%
	5.25	4.96	0.29	5.7%

Lipid 100 mg/dL	Test AMH (ng/mL)	Control AMH (ng/mL)	Bias (ng/mL)	Bias (%) (dobs)
	1.18	1.05	0.13	-11.9%
	3.82	4.91	1.10	22.3%
Lipid 200 mg/dL	Test AMH (ng/mL)	Control AMH (ng/mL)	Bias (ng/mL)	Bias (%) (dobs)
	1.11	1.05	0.06	-5.5%
	3.64	4.91	1.27	25.8%
Lipid 400 mg/dL	Test AMH (ng/mL)	Control AMH (ng/mL)	Bias (ng/mL)	Bias (%) (dobs)
	1.23	1.05	0.18	17.2%
	4.31	4.91	0.60	-12.3%

Bilirubin 10 mg/dL	Test AMH (ng/mL)	Control AMH (ng/mL)	Bias (ng/mL)	Bias (%) (dobs)
	2.10	2.15	0.05	2.1%
	12.09	11.36	0.73	-6.4%
Bilirubin 20 mg/dL	Test AMH (ng/mL)	Control AMH (ng/mL)	Bias (ng/mL)	Bias (%) (dobs)
	2.14	2.15	0.00	0.2%
	11.11	11.36	0.24	2.2%
Bilirubin 40 mg/dL	Test AMH (ng/mL)	Control AMH (ng/mL)	Bias (ng/mL)	Bias (%) (dobs)
	1.93	2.15	0.22	-10.1%
	12.21	11.36	0.85	7.5%

16 AMH ELISA: CONDENSED ASSAY PROTOCOL



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SYMBOLS USED

Symbol	English
CE	European Conformity
	Consult instructions for use
IVD	*In vitro* diagnostic medical device
REF	Catalogue number
LOT	Batch code
	Contains sufficient for <n> tests
	Temperature limit
	Use-by date
	Manufacturer
	Caution
RUO	For research use only
Distributed by	Distributed by
Content	Content
Volume/No.	Volume / No.

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