EPITOPE DIAGNOSTICS, INC.

Serolisa™ Human Anti-Müllerian Hormone ELISA Kit

Enzyme Linked ImmunoSorbent Assay (ELISA) for the Quantitative Measurement of Human Anti-müllerian Hormone (AMH) Level in Serum or Plasma

REF KT-804 CE IVD 96

INTENDED USE

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

SUMMARY OF PHYSIOLOGY

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.

ASSAY PRINCIPLE

This ELISA is designed, developed and produced for the quantitative measurement of human Anti-Müllerian 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 wells of a microtiter plate that is coated with antibody to N-terminal AMH along with another AMH specific antibody labeled with horseradish peroxidase (HRP). After an initial incubation period, the plate is washed 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 calibration curve is generated by plotting the absorbance versus the respective human AMH concentration for each calibrator on a Cubic or point-to-point curve fitting. The concentration of human AMH in test samples is determined directly from this calibration curve.

REAGENTS: PREPARATION AND STORAGE

This test kit must be stored at 2 – 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 (31049)

Microplate coated with anti-AMH antibody.

Qty: 1 x 96 well microplate
Storage: 2 – 8°C
Preparation: Ready to Use.

2. AMH Tracer Antibody (31050)

HRP-labeled anti-human AMH antibody in a stabilized protein matrix.

Qty: 1 x 0.35 mL
Storage: 2 – 8°C
Preparation: 21X Concentrate. The contents must be diluted with tracer antibody diluent (30017) and mixed well before use.

3. ELISA Wash Concentrate (10010)

Surfactant in a phosphate buffered saline with non-azide preservative.

Qty: 1 x 30 mL
Storage: 2 – 25°C
Preparation: 30X Concentrate. The contents must be diluted with 870 mL distilled water and mixed well before use.

4. ELISA HRP Substrate (10020)

Tetramethylbenzidine (TMB) with stabilized hydrogen peroxide.

Qty: 1 x 12 mL
Storage: 2 – 8°C
Preparation: Ready to Use.

5. ELISA Stop Solution (10030)

0.5 M sulfuric acid

Qty: 1 x 12 mL
Storage: 2 – 25°C
Preparation: Ready to Use.

6. AMH Calibrators Levels 1 to 6 (31061 - 31066)

Human AMH in a lyophilized bovine serum-based matrix with Proclin-300 as preservative. Refer to vials for exact concentration.

Qty: 6 x vials
Storage: 2 – 8°C , <-20°C for long term storage
Do not exceed 3 freeze-thaw cycles.
Preparation: Must be reconstituted with 0.5 mL of demineralized water, allowed to sit for 10 minutes, and then mixed by inversions or gentle vortexing. Make sure that all solids are dissolved completely prior to use.

For Reference Use

7. AMH Control I, II (31067, 31068)
Human AMH in a lyophilized bovine serum-based matrix with Proclin-300 as preservative. Refer to vials for exact concentration.

Qty: 2 x vials
Storage: 2 – 8°C , <-20°C for long term storage
Do not exceed 3 freeze-thaw cycles.
Preparation: Must be reconstituted with 0.5 mL of demineralized water, allowed to sit for 10 minutes, and then mixed by inversions or gentle vortexing. Make sure that all solids are dissolved completely prior to use.

8. Tracer Antibody Diluent (31059)
Buffer for tracer antibody dilution according to the assay procedures.

Qty: 1 x 7 mL
Storage: 2 – 8°C
Preparation: Ready to use.

SAFETY PRECAUTIONS

The reagents are for in vitro diagnostic use only. Source material which contains reagents of bovine serum albumin 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 were potentially infectious. Avoid contact with reagents containing hydrogen peroxide, or sulfuric acid. 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.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Precision single channel pipettes capable of delivering 50 µL, 100 µL, 1,000 µL, etc.
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

SPECIMEN COLLECTION & STORAGE

Serum or heparin plasma are acceptable samples. Collect whole venous blood into serum collection tubes (such as BD 366430) or tubes containing lithium heparin (such as BD 367880). 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).

ASSAY PROCEDURE

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 (10010) must be diluted to working solution prior to use. Please see REAGENTS section for details.
3. Reconstitute all assay calibrators level 1 to level 6 (31061 - 31066) and controls (31067, 31068) by adding 0.5 mL of deminerialized water to each vial. Allow the calibrators and controls to sit undisturbed for 10 minutes, and then mix well by inversions or gentle vortexing. One must make sure that all solid is dissolved completely prior to use. These reconstituted calibrators and controls may be stored at 2 – 8°C for up to 3 days or at –20°C or below for long-term storage. Do not exceed 3 freeze-thaw cycles.

2. Assay Procedure

1. Place a sufficient number of microwell strips (31049) in a holder to run calibrators (31061 – 31066), controls (31067, 30168), and samples in duplicate.

2. Test Configuration

Row	Strip 1	Strip 2	Strip 3
A	Calibrator Level 1	Calibrator Level 5	SAMPLE 1
B	Calibrator Level 1	Calibrator Level 5	SAMPLE 1
C	Calibrator Level 2	Calibrator Level 6	SAMPLE 2
D	Calibrator Level 2	Calibrator Level 6	SAMPLE 2
E	Calibrator Level 3	Control 1	SAMPLE 3
F	Calibrator Level 3	Control 1	SAMPLE 3
G	Calibrator Level 4	Control 2	SAMPLE 4
H	Calibrator Level 4	Control 2	SAMPLE 4

3. Prepare the antibody working solution by 1:21 fold dilution of the tracer antibody (31050) with the diluent (31059). For each strip, it is required to mix **0.5 mL** of the diluent (31059) with **25 µL** of the tracer antibody (31050) in a clean test tube. *Note: This antibody working solution should be freshly prepared.*
4. Add **50 µL** of calibrators (31061 - 31066), controls (31067, 31068), and samples into the designated microwells. Mix by gently tapping the plate.
5. Add **50 µL** of antibody working solution to each microwell.
6. Cover the plate with one plate sealer and aluminum foil. Incubate at **2-8 ºC** for **18 to 24 hours, static.** It is optional to incubate the plate at room temperature **(20-25 ºC)** 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).
7. Remove the plate sealer. Aspirate the contents of each microwell. Wash each well **5 times** by dispensing **350 µL** of diluted wash solution (10010) into each well and aspirate the contents completely. Alternatively, an automated microplate washer can be used.
8. Add **100 µL** of substrate (10020) into each microwell. Mix by gently tapping the plate.
9. Cover the plate with one plate sealer and aluminum foil. Incubate at **room temperature (20-25 ºC)** for **20 minutes.**
10. Remove the aluminum foil and plate sealer and add **100 µL** of Stop Solution (10030) into each of the wells. Mix by gently tapping the plate.
11. Read the absorbance at **450/(620, 630, or 650) nm** within **10 minutes** with a microplate reader.

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.

For Reference Use

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 automated ELISA system (such as DS-2 or EuChrom DUO) 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 as: **AMH Sample Diluent (31060).**

INTERPRETION OF RESULTS

1. It is recommended to use a cubic plot calibration curve fitting.
2. Calculate the average absorbance for each pair of duplicate test results.
3. Subtract the average absorbance of the level 1 calibrator (0 ng/mL) from the average absorbance of all other readings to obtain corrected absorbance or use 0 calibrator for blank in computer program.
4. 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.
5. The AMH concentrations for the controls and the patient samples are read directly from the calibration curve using their respective corrected absorbance.

LIMITATIONS 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 calibrator, it is recommended to re-assay samples with dilution (i.e. 1:5 or 1:10) with AMH Sample Diluent (Cat. No. 31060).
3. Water deionized with polyester resins may inactivate the horseradish peroxidase enzyme.

QUALITY CONTROL

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

EXPECTED VALUES

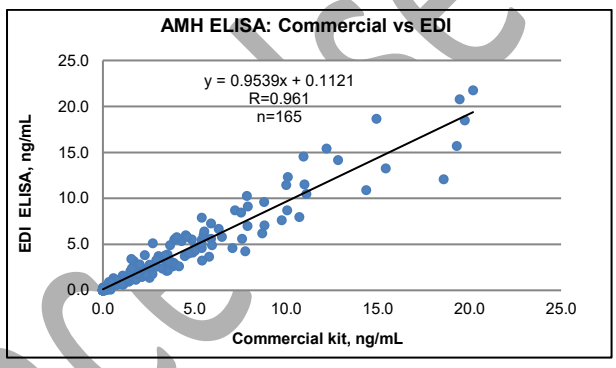
Mayo Clinic has suggested the following normal range for AMH and is summarized in the table below.

Reference Values	
Males	
< 24 months:	14.0 – 466 ng/mL
> 12 years	0.70 – 19.0 ng/mL
Females	
< 24 months	< 4.70 ng/mL
24 months – 12 years	< 8.80 ng/mL
13 – 45 years	0.90 – 9.50 ng/mL
> 45 years	< 1.00 ng/mL

It is important that each laboratory should establish its own normal range based on gender and age. Epitope Diagnostics has validated the above reference range from Mayo Clinic with 133 apparently healthy individuals. Donor samples were collected and tested with Epitope Diagnostics' Serolisa™ human AMH ELISA kit. The data obtained is summarized below.

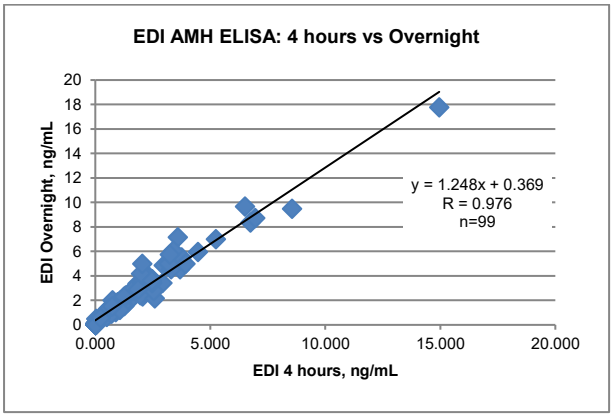
	Males, Ages 14 – 68	Females, Ages 18 - 43	Females, Ages > 45
Number	45	64	24
Average	4.83	3.67	0.03
Standard Deviation	4.08	2.40	0.07
Minimum	0.73	0.95	0.00
Maximum	18.47	9.12	0.32

Commercial Kit vs. EDI Kit Comparison: 165 serum and Lithium Heparin samples, age ranging from 14 to 81 years old, with AMH concentration range of 0ng/mL – 20ng/mL were tested side-by-side using Serolisa™ human AMH ELISA and a well-known commercial human AMH ELISA. The data obtained is summarized below.



Male and Female, 14 - 81		
N=165	**Commercial kit**	**EDI kit**
Average	3.62	3.57
Standard Deviation	4.48	4.45
Minimum	0.00	0.00
Maximum	20.19	21.75

Overnight vs. 4 Hours Comparison: Total of 99 samples were tested side-by-side using Serolisa™ human AMH ELISA with two different incubation conditions, (a) Overnight at 2-8ºC, static and (b) 4 hours at room temperature, shaking. The summary of the data is shown below.



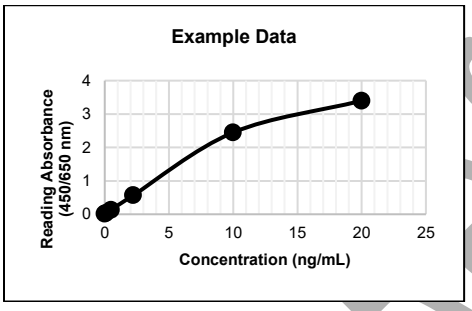
For Reference Use

EXAMPLE DATA

A typical absorbance data and the resulting calibration curves from are represented.

Note: This curve should not be used in lieu of calibration curve run with each assay.

Well ID	Reading Absorbance (450/650 nm)			Concentration ng/mL
	Readings	Average	Corrected	
Calibrator Level 1: 0 ng/mL	0.014	0.014	0.000	
	0.014			
Calibrator Level 2: 0.11 ng/mL	0.037	0.037	0.023	
	0.037			
Calibrator Level 3: 0.49 ng/mL	0.128	0.132	0.118	
	0.135			
Calibrator Level 4: 2.22 ng/mL	0.574	0.568	0.540	
	0.562			
Calibrator Level 5: 10 ng/mL	2.420	2.450	2.436	
	2.481			
Calibrator Level 6: 20 ng/mL	3.451	3.398	3.384	
	3.346			
Control 1	0.335	0.0337	0.0323	1.32
	0.339			
Control 2	1.576	1.586	1.572	6.12
	1.596			



PERFORMANCE CHARACTERISTICS

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.

Hook Effect

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

Reproducibility and Precision

The intra-assay precision was validated by measuring two serum samples in a single assay with 16 replicate determinations. The inter-assay precision was validated by measuring two control samples in duplicate in 9 individual assays over an 18 day period. The results are as follows:

	Intra-Assay		Inter-Assay	
Sample	**1**	**2**	**1**	**2**
Mean (ng/mL)	4.50	10.50	1.38	6.03
CV (%)	5.6	3.9	8.1	2.1

Linearity

Two serum samples were diluted with AMH Sample Diluent (31060) and tested. The results are as follows:

Sample	Observed (ng/mL)	Expected (ng/mL)	Recovery (%)
Sample 1	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	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%

Spike Recovery

Two serum samples were spiked together in varying volumes and tested. The results are as follows:

Samples	Observed (ng/mL)	Expected (ng/mL)	Recovery (%)
Sample 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%
Sample 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%

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:

	Test AMH (ng/ml)	Control AMH (ng/ml)	Bias (ng/ml)	Bias (%) (dobs)
Hemoglobin 50 mg/dL	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%

	Test AMH (ng/ml)	Control AMH (ng/ml)	Bias (ng/ml)	Bias (%) (dobs)
Lipid 100 mg/dL	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%

For Reference Use

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%

WARRANTY

This product is warranted to perform as described in its labeling and literature when used in accordance with all instructions. Epitope Diagnostics, Inc. DISCLAIMS ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, and in no event shall Epitope Diagnostics, Inc. be liable for consequential damages. Replacement of the product or refund of the purchase price is the exclusive remedy for the purchaser. This warranty gives you specific legal rights and you may have other rights, which vary from state to state.

REFERENCES

1. Lee, M. et al (1993); Müllerian-inhibiting substance: A gonadal hormone with multiple functions. *Endocrine Reviews,* 142, 152-164.
2. Hudson, et al (1990); An immunoassay to detect human Müllerian inhibiting substance in males and females during normal development. *Journal of Clinical Endocrinology and Metabolism, 70, 16-22.*
3. Lee, M et al (1996); Müllerian Inhibiting Substance in human: normal levels from infancy to adulthood. Journal of Clinical Endocrinology and Metabolism 81, 571 – 575.
4. Mayo Clinic ; Test ID: AMH; Antimullerian Hormone (AMH), Serum Clinical and Interpretive. https://www.mayomedicallaboratories.com/test-catalog/Clinical+and+Interpretive/89711

TECHNICAL ASSISTANCE AND CUSTOMER SERVICE

For technical assistance or place an order, please contact Epitope Diagnostics, Inc. at (858) 693-7877 or fax to (858) 693-7678.

This product is developed and manufactured by

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Please visit our website at www.epitopediagnostics.com to learn more about our products and services.

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GLOSSARY OF SYMBOLS (EN 980/ISO 15223)

IVD In Vitro Diagnostic Device	RUO For Research Use Only	LOT Lot Number
REF Catalog Number	Read instructions before use	Number of Tests
Store at	Use by	Keep away from heat and direct sun light
Manufacturer	EC REP Authorized Representative in Europe	CE European Conformity

SHORT ASSAY PROCEDURE

1. Add **50 µL** of calibrators, controls, and samples into the designated microwells.
2. Add **50 µL** of antibody working solution into the designated microwells.
3. Mix, cover, and incubate at **2-8 ºC** for **18 to 24 hours static,** or **room temperature (20-25 ºC)** with **shaking at 400 to 450 rpm** or **180 rpm** for **4 hours.**
4. Wash each well five times.
5. Add **100 µL** of substrate to each well.
6. Cover and incubate at **room temperature (20-25 ºC)** for **20 minutes**.
7. Add **100 µL** of the stop solution to each well.
8. Read the absorbance at **450/(620, 630, or 650) nm**.

For Reference Use