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ELISA **E**NZYME **L**INKED **I**MMUNO**S**ORBENT **A**SSAY

Microwell Method

AMH

REF: Z17301

For in vitro Diagnostic Use

P r o d u c t I n s e r t

Enzyme Linked Immunosorbent Assay for the **quantitative** determination of Anti-Müllerian Hormone in human serum or plasma.

CE IVD

Microwell Method - 96 wells

(12 x 8-well Antibody coated strips)

Individual breakaway

GENERAL INFORMATION

- **Wavelength**

  Measurement Filter: 450 nm

  Reference Filter: 630 nm

- **Incubation Time**

  160 minutes (90/30/30/10)

- **Enzyme Conjugate**

  Biotin Conjugate

  Streptavidin Conjugate

- **Substrate**

  TMB (3,3´,5,5´-Tetramethyl-benzidine)

- **Sample**

  Serum or Plasma

- **Calibration Range**

  0 – approx. 15.0 ng/mL

INTENDED USE

The Anti-Müllerian Hormone ELISA kit provides materials for the quantitative measurement of AMH in human serum or plasma. This assay is intended for *in vitro* diagnostic use only. **This assay is not intended for the prediction of the ovarian response to follicle stimulation protocols**.

MATERIALS SUPPLIED

Microplate	12x8 wells coated with AMH antibody immobilised 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.
Calibrator A/ Sample Diluent	1x 11 mL, containing 0 ng/mL AMH in protein based buffer and Pro-Clean 400. Store unopened at 2-8 °C until the expiration date.
Calibrators B – F	lyophilised, containing concentrations of approximately 0.09 – 15.0 ng/mL AMH in protein based buffer and Pro-Clean 400. Refer to labels for exact concentrations. Reconstitute calibrators with 1 mL deionised water, mix well and use after reconstitution. The AMH concentration in the 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.
Control High **Control Low**	lyophilised, one vial for each level, containing low and high AMH concentrations in protein based buffer and pro-clean 400, exact concentration levels are mentioned on the label. Reconstitute controls with 1 mL deionised water, mix well and use after reconstitution.
Assay buffer	1x 12 mL, containing a protein-based (BSA)-buffer with a non-mercury preservative. Store at 2-8°C until expiration date. Ready to use.
Biotin Conjugate	1x 12 mL, containing biotinylated anti-AMH antibody in protein-based buffer with a non-mercury preservative. Store at 2-8°C until expiration date. Ready to use.
Enzyme Conjugate	1x 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. Ready to use.
Substrate Solution	1x 12 mL, containing a solution of tetramethylbenzidine (TMB) in buffer with hydrogen peroxide. Store at 2-8°C until expiration date. Ready to use.
Stop Solution	1x 12 mL, containing 0.2 M sulfuric acid. Store at 2 to 30°C until expiration date.
Wash Solution	1x 60 mL, containing buffered saline with a non-ionic detergent. Store at 2-30 °C until expiration date. Dilute 25-fold with deionised water prior to use.

REAGENT STABILITY AND STORAGE

Microplate	Store at 2-8°C until expiration date in the resealable pouch with a desiccant to protect from moisture.
Calibrators B – F	Store unopened at 2-8 °C until the expiration date. Aliquot and freeze in plastic vials for multiple use. Alternatively, freeze in the same vial

	within 2 hours of reconstitution. Avoid repeated freeze thaws.
Control High **Control Low**	Store unopened at 2-8 °C until the expiration date. Aliquot and freeze in plastic vials for multiple use. Alternatively, freeze in the same vial within 2 hours of reconstitution. Avoid repeated freeze thaws.
Assay Buffer **Biotin Conjugate** **Enzyme Conjugate** **Substrate Solution**	Store at 2-8 °C until expiration date.
Stop Solution **Wash Solution**	Store at 2-30 °C until expiration date.

Materials required but not provided:

- Microtiter plate reader capable of absorbance measurements at 450 and 630 nm
- Microplate orbital shaker
- Microplate washer
- Pipettes to deliver 10-250 µL
- Stepper
- Vortex mixer
- Deionised water

CLINICAL SIGNIFICANCE

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 disulphide 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 signalling, 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

The Dialab Anti-Müllerian Hormone 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.

WARNINGS AND PRECAUTIONS

- For in vitro diagnostic use only.
- Follow good laboratory practice.
- Use personal protective equipment. Wear lab coats and disposable gloves when handling immunoassay materials.
- Handle and dispose of all reagents and material in compliance with applicable regulations.
- Warning: Potential Biohazardous Material
  This reagent may contain some human source materials. Handle all reagents and patient samples as hazardous material.
- Warning: Potential Chemical Hazard
  Some reagents in this kit contain Pro-Clean 400 and sodium azide as a preservative. Pro-Clean 400 and sodium azide[4] in concentrated amounts are irritant to skin and mucous membranes.

SAMPLE COLLECTION AND PREPARATION

- Serum and lithium-heparin plasma is the recommended sample type.
- 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.
- 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.
- Avoid assaying lipemic, hemolyzed or icteric samples.
- Avoid repeated freezing and thawing of samples. Thaw samples no more than 3 times.
- 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 Dialab AMH 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.

REAGENT PREPARATION

Microplate	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.
Calibrators B – F	Tap and reconstitute AMH Calibrators B-F each with 1 mL deionised water. Solubilise, 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.
Control High **Control Low**	Tap and reconstitute AMH Controls each with 1 mL deionised water. Solubilise, 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.
Wash Solution	Dilute the 25x concentrated Wash solution with deionised water. The diluted wash solution is stable for 1 month at room temperature (23 ± 2°C) when stored in a tightly sealed bottle.

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 Calibrators B-F and Controls High and Low each with 1 mL deionised water. Solubilise for 10 minutes and 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 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 Biotin Conjugate 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 Enzyme Conjugate 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 Substrate 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 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 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.

Representative Calibration Curve:

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

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

QUALITY CONTROL

Each laboratory should establish mean values and acceptable ranges to assure proper performance.

- AMH ELISA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for AMH 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 colourless. Development of any colour may indicate reagent contamination or instability

EXPECTED VALUES

These samples were analysed using Dialab AMH ELISA test. The expected ranges for AMH were calculated on serum samples using 90-95% non-parametric estimation using Analyse-It® for Microsoft Excel.

Sample	Number of Specimens	Median Age	Median AMH (ng/mL)	AMH Range (ng/mL)
Females < 8 weeks	33	3 weeks	0.00	<0.02 – 0.49
Females < 10 years	23	5 years	1.69	0.05 – 10.40
Females 11 – 20 years	35	17 years	3.25	0.62 – 11.0
Females 21 – 30 years	33	26 years	3.78	<0.02 – 10.39
Females 31 – 40 years	56	35 years	2.39	0.14 – 10.40
Females 41 – 50 years	79	44 years	0.42	<0.01 – 6.35
Females > 51 years	94	59 years	0.00	<0.02 – 0.39
Males < 3 days	15	NA	50.84	25.9 – 69.1
Males < 3 months	52	5 days	83.39	24.22 – 275.46
Males 1 - 11 years	45	7 years	122.40	38.25 – 322.40

Males 12 – 20 years	23	14 years	6.47	1.12 – 143.64
Males > 20 years	83	47 years	4.90	0.59 – 17.71

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.

PERFORMANCE 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 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 guidelines. Twelve assay runs were performed over two days with samples run in duplicate per run.

Limit of Quantification (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 guidelines.

Imprecision:
Reproducibility of the AMH 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 guidelines and are presented in the following table.

Sample	Mean conc. (ng/mL)	Within run		Between run		Total	
		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, multiple dilutions of the three serum samples containing various AMH 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
	1:2	3.69	3.85	104%
	1:4	1.85	1.87	101%
	1:8	0.92	0.94	102%
	1:16	0.46	0.46	99%
2	Neat	4.44	Neat	NA
	1:2	2.22	2.26	102%
	1:4	1.11	1.20	108%
	1:8	0.56	0.61	109%
	1:16	0.28	0.29	105%
3	Neat	7.11	Neat	NA
	1:2	3.55	3.89	109%
	1:4	1.78	1.90	107%
	1:8	0.89	0.99	111%
	1:16	0.44	0.48	107%

Recovery:
Known amounts of AMH were added to three serum samples containing different levels of endogenous AMH. The concentration of AMH was determined before and after the addition of exogenous AMH 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 and does not cross react to other species (bovine, equine, ovine, canine, rat and mouse).

Cross Reactant	Concentration	% Cross-reactivity
Inhibin A	100 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%

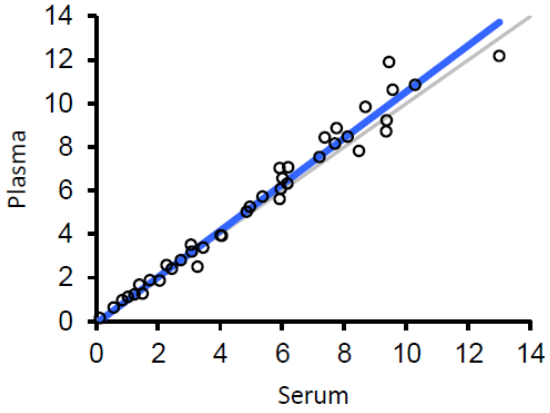
Interference:

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

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

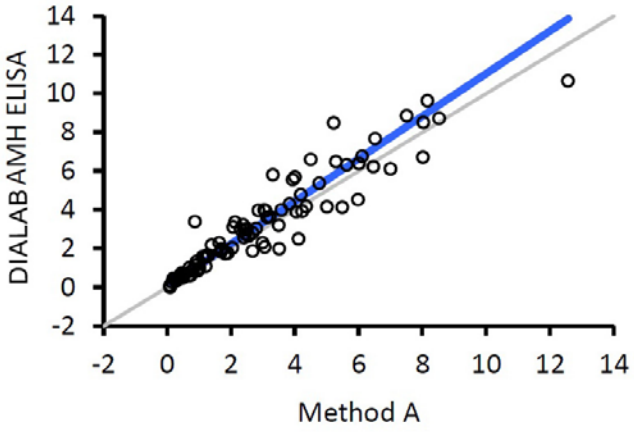
Sample Type:
Forty matched serum and Lithium heparin plasma specimens in the range of 0.13-13.01 ng/mL were compared in Dialab AMH ELISA assay.
Passing Bablok analysis of the results yielded the following Regression:

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



Method Comparison:
The Dialab AMH 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:

Dialab AMH ELISA (AL-105) = 1.10 (Method A) + 0.06, (r=0.98; P<0.0001)



WASTE MANAGEMENT

Reagents must be disposed of in accordance with local regulations.

REFERENCES

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Symbol	English
	Consult instructions for use
	European Conformity
	In vitro diagnostic device
	Catalogue number
	Lot. No. / Batch code
	Storage Temperature
	Expiration Date
	Legal Manufacturer
Distributed by	Distributor
Content	Content
Volume/No.	Volume / No.

ELISA **E**nzyme **L**inked **I**mmuno**s**orbent **A**ssay

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