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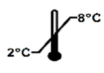
LABOR DIAGNOSTIKA NORD GmbH & Co.KG | Am Eichenhain 1 | 48531 Nordhorn | Germany | Tel. +49 5921 8197-0 | Fax +49 5921 8197-222 | info@ldn.de | www.ldn.de

Instructions for use

AMH ELISA

REF **FR E-2900R**



RUO For research use only – Not for use in diagnostic procedures

AMH ELISA

1 INTENDED USE

The **AMH ELISA** is a manual enzyme immunoassay for the **quantitative** measurement of Anti-Müllerian Hormone (AMH) in human serum or plasma (EDTA or Li-heparin plasma).
For *reserach* use. For laboratory professional use.

1.1 Scientific Validity Report

Anti-Müllerian Hormone (AMH), a dimeric 140 KDa glycoprotein, is a member of the transforming growth factor-β (TGF-β) family of cytokines which plays an essential role in the normal differentiation of reproductive structures. (1)
AMH is secreted by Sertoli cells of the testes during embryogenesis of the fetal male, preventing the development of the Müllerian ducts to the uterus and other Müllerian structures. In females, AMH is secreted by the granulosa cells of ovarian follicles. (2, 3)

2 PRINCIPLE OF THE TEST

The AMH ELISA is a solid phase enzyme-linked immunosorbent assay (ELISA) based on the **sandwich principle.**
The microtiter wells are coated with a monoclonal antibody (mouse) directed towards a unique antigenic site of the AMH molecule.
During the first incubation, AMH in the added sample binds to the immobilized antibody. The simultaneously added enzyme conjugate, which contains an anti-AMH antibody conjugated to horseradish peroxidase, binds to the AMH forming a sandwich complex.
After a washing step to remove all unbound substances, the solid phase is incubated with the substrate solution. The colorimetric reaction is stopped by addition of stop solution, and optical density (OD) of the resulting yellow product is measured. The intensity of color is proportional to the concentration of the analyte in the sample.
A standard curve is constructed by plotting OD values against concentrations of standards, and concentrations of unknown samples are determined using this standard curve.

3 WARNINGS AND PRECAUTIONS

- This kit is for *research* use only.
- Before starting the assay, read the instructions for use completely and carefully. Use the valid version of instructions for use provided with the kit. Be sure that everything is understood.
- Do not mix or use components from kits with different lot numbers. It is advised not to interchange wells of different plates even of the same lot. The kits may have been shipped or stored under different conditions and the binding characteristics of the plates may result slightly different.
- Do not use reagents beyond expiry date as shown on the kit labels.
- Do not reuse microtiter wells.
- Reagents of other manufacturers must not be used together with the reagents of this test kit.
- All reagents in this kit are clear liquids, substrate solution is clear and colorless. Changes in its appearance may affect the performance of the test. In that case, contact the manufacturer.
- Microbial contamination of reagents or samples may give false results.
- Allow the reagents to reach room temperature (20 °C to 26 °C) before starting the test. Temperature will affect the optical density readings of the assay. However, values for the samples will not be affected.
- All indicated volumes must be performed according to the protocol. Optimal test results are only obtained when using calibrated pipettes and microtiter plate readers.
- Use reservoirs only for single reagents. This especially applies to the substrate reservoirs. Using a reservoir for dispensing a substrate solution that had previously been used for the conjugate solution may turn solution coloured. Do not pour reagents back into original vials as reagent contamination may occur.

General precautions

- Follow laboratory quality assurance and laboratory safety guidelines.
- Never pipet by mouth and avoid contact of reagents and samples with skin and mucous membranes.
- Do not smoke, eat, drink, or apply cosmetics in areas where samples or kit reagents are handled.
- Wear lab coats and disposable latex gloves when handling samples and reagents and where necessary safety glasses.

Biohazard information

- All reagents of this test kit which contain human serum or plasma have been tested and confirmed negative for HIV I/II, HBsAg and HCV by FDA approved procedures. However, no known test method can offer total assurance that no infectious agent is present.
- The device contains material of animal origin, which is certified apparently free of infectious or contagious diseases and injurious parasites.

- Bovine components originate from countries where BSE (Bovine spongiform encephalopathy) has not been reported.
- All materials and samples of human or animal origin must be handled as if capable of transmitting infectious diseases.
- Handling must be done in accordance with the procedures defined by appropriate national biohazard and safety guideline or regulation. Waste must be discarded according to local rules and regulations.

Information to chemical hazards and hazard classification

- Some reagents contain preservatives in non-declarable concentrations. Nevertheless, in case of contact with eyes or skin, flush immediately with water.
- Substrate Solution contains an ingredient in non-declarable concentrations which causes serious eye irritation. In case of possible contact with eyes, rinse immediately carefully and thoroughly with eye wash or water. After contact with skin, wash with plenty of water. Take-off contaminated clothing and wash it before reuse.
- Avoid contact with Stop Solution containing < 5% H_2SO_4. It may cause skin irritation and burns.
- Chemicals and prepared or used reagents must be treated as hazardous waste according to the national safety guideline or regulation.
- This product does not contain substances which have carcinogenic, mutagenic or toxic for reproduction (CMR) properties.

All reagents of this test kit do NOT contain hazardous substances in concentrations to be declared, a classification and labelling is not required.

For detailed information, please refer to the Safety Data Sheet, which is available upon request directly from the manufacturer.

4 MATERIALS

4.1 Materials provided with the kit

FR E-2931 96 **Microtiterwells** – Ready to use

Content: 12 x 8 wells (break apart);
Coated with anti-AMH antibody (monoclonal).

Standards and **Controls** – Ready to use

Cat. no.	Component	Standard	Concentration ng/ml	Volume / vial
FR E-2901	STANDARD A	Standard A	0.0	1 ml
FR E-2902	STANDARD B	Standard B	0.4	1 ml
FR E-2903	STANDARD C	Standard C	1.0	1 ml
FR E-2904	STANDARD D	Standard D	4.0	1 ml
FR E-2905	STANDARD E	Standard E	10	1 ml
FR E-2906	STANDARD F	Standard F	20	1 ml
FR E-2951	CONTROL 1	Control 1	For control values and ranges please refer to vial label or QC-Report	1 ml
FR E-2952	CONTROL 2	Control 2		1 ml

Conversion: 1 ng/ml = 7.14 pmol/l

The Standard are calibrated against the following reference material:
1st WHO International Reference Reagent, Mullerian Inhibiting Substance/Anti-Mullerian Hormone, NIBSC code: 16/190

Contains non-mercury preservative.

FR E-2940 CONJUGATE **Enzyme Conjugate** – Ready to use

Content: Anti-AMH antibody conjugated to horseradish peroxidase; colored red.
Contains non-mercury preservative.

Volume: 1 x 14 ml

FR E-0055 SUBSTRATE **Substrate Solution** – Ready to use

Content: Contains 3,3‘,5,5‘-tetramethylbenzidine (TMB).
Keep away from direct sun light.

Volume: 1 x 14 ml

FR E-0080	STOP-SOLN	**Stop Solution** – Ready to use
Content:	Contains < 5% H_2SO_4. *Avoid contact with the stop solution. It may cause skin irritations and burns.*	
Volume:	1 x 14 ml	
Hazards identification:		
	H290 May be corrosive to metals. H314 Causes severe skin burns and eye damage.	
FR E-0030	WASH- CONC 40x	**Wash Solution** – 40X concentrate
Volume:	1 x 30 ml See "*Reagent Preparation*" Contains non-mercury preservative.	

1x Instructions for Use
1x Certificate of Analysis (CoA)

4.2 Materials required but not provided

- A calibrated microtiter plate reader (450 nm, with reference wavelength at 620 nm to 630 nm)
- Calibrated variable precision micropipettes
- Manual or automatic equipment for rinsing microtiter plate wells
- Absorbent paper
- Distilled water
- Timer
- Graph paper or software for data reduction

4.3 Storage and Stability of the Kit

Unopened kits and reagents as well as **opened reagents** must be stored at 2 °C to 8 °C.

The microplate must always be stored in the resealable aluminum pouch containing a desiccant. Do not open the pouch until it has reached room temperature. The microtiter plate consists of 12 individual strips. Each strip can be divided into 8 individual wells.
Unused wells must be immediately returned to the aluminum pouch with the desiccant and stored again tightly resealed at 2 °C to 8 °C.

Once opened, reagent vials must be closed tightly again.

	Storage Temperature	**Stability**
Unopened kits and unopened reagents	2 °C to 8 °C	Until the expiration date printed on the label. Do not use reagents beyond this date!
Opened kit	2 °C to 8 °C	8 weeks

4.4 Reagent Preparation

Bring all reagents and required number of strips to room temperature (20 °C to 26 °C) prior to use.

Wash Solution
Add distilled water to the 40X concentrated Wash Solution.
Dilute 30 ml of concentrated *Wash Solution* with 1170 ml distilled water to a final volume of 1200 ml.

Stability after dilution:	at 20 °C to 26 °C	1 week

4.5 Disposal of the Kit

The disposal of the kit and all used materials/reagents must be performed according to the national regulations. Special information for this product is given in the Safety Data Sheet, section 13.

4.6 Damaged Test Kits

In case of any damage to the test kit or components, the manufacturer must be informed in writing, at the latest one week after receiving the kit. Damaged single components must not be used for a test run. They have to be stored until a final solution has been found. After this, they must be disposed of according to the official regulations.

5 SAMPLE COLLECTION, STORAGE AND PREPARATION

The following sample material can be used in this test:

Human serum or plasma (EDTA plasma or lithium heparin plasma)

Samples containing sodium azide should not be used in the assay.
In general, it should be avoided to use hemolytic, icteric, or lipemic samples. For further information refer to chapter "*Interfering Substances*".

5.1 Sample Collection

Serum: Collect blood by venipuncture (e.g. Sarstedt Monovette for serum), allow to clot, and separate serum by centrifugation at room temperature. Do not centrifuge before complete clotting has occurred. Donors receiving anticoagulant therapy may require increased clotting time.

Plasma: Whole blood should be collected into centrifuge tubes containing anticoagulant (e.g. Sarstedt Monovette with the appropriate plasma preparation) and centrifuged immediately after collection.

Whole blood should not be frozen before centrifugation.

5.2 Samples Storage

Samples must be stored tightly capped prior to performing the assay. If stored frozen, freeze only once. Thawed samples must be inverted several times prior to testing.

Stability	at 2 °C to 8 °C	7 days
	at -20 °C (in aliquots)	up to 3 months

5.3 Sample Preparation

Samples can be assayed without additional preparation.

6 ASSAY PROCEDURE

6.1 Procedural Notes

- All reagents and samples must be allowed to come to room temperature (20 °C to 26 °C) before use.
- All reagents must be mixed without foaming.
- Do not interchange caps of reagent vials to avoid cross-contamination.
- Use new disposal plastic pipette tips for each standard, control, or sample in order to avoid carry-over.
- To avoid cross-contamination and falsely elevated results pipette samples and dispense conjugate without splashing accurately to the bottom of wells.
- Mix the contents of the microtiter plate wells thoroughly to ensure good test results.
- Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.
- Once the test has been started, all steps must be completed without interruption and in the same sequence for each step.
- The enzymatic reaction is linearly proportional to time and temperature.
- Optical density is a function of the incubation time and temperature. Respect the incubations times and temperatures as given in chapter "Test Procedure".
- Before starting the assay, it is recommended that all reagents are ready, caps removed, all needed wells secured in holder, etc. This will ensure equal elapsed time for each pipetting step without interruption.
- **Important note to wash procedure:**
  Washing is critical. Improperly washed wells will give erroneous results. The sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure!
- **Test performance using fully automated analysis devices:**
  Automated test performance using fully automated, open-system analysis devices is possible. However, the combination must be validated by the user.

6.2 Test Procedure

Each run must include a standard curve.

The controls serve as internal controls for the reliability of the test procedure. They must be assayed with each test run.

The given test procedure describes manual processing.

1.	Secure the desired number of microtiter wells in the frame holder.
2.	Pipette **25 µl** of each ***Standard***, ***Control,*** and **sample** with new disposable tips into appropriate wells.
3.	Add **100 µl *Enzyme Conjugate*** into each well. Thoroughly mix for 10 seconds. It is important to have a complete mixing in this step.
4.	Incubate for **60 minutes** at room temperature.
5.	Wash the wells as follows: If the wash step is performed manually: Briskly shake out the contents of the wells. Rinse the wells **3 times** with **300 µl** diluted *Wash Solution* per well. If an automated plate washer is used: Rinse the wells **3 times** with **400 µl** diluted *Wash Solution* per well. At the end of the washing step, always strike the wells sharply on absorbent paper to remove residual droplets!
6.	Pipette **100 µl** of ***Substrate Solution*** to each well.
7.	Incubate for **15 minutes** at room temperature.
8.	Stop the enzymatic reaction by adding **50 µl** of ***Stop Solution*** to each well.
9.	Measure the optical density (OD) of the solution in each well at **450 nm (reading) and at 620 nm to 630 nm (background subtraction, recommended)** with a microtiter plate reader. It is recommended that the wells be read **within 10 minutes** after adding the *Stop Solution*.

6.3 Calculation of Results

1. The concentration of the samples can be read directly from the standard curve.
2. For duplicate determinations, the mean of the two optical density (OD) values for each standard, control, and sample must be taken. If the two values deviate substantially from one another, the manufacturer recommends retesting the samples.
3. Samples with concentrations exceeding the highest standard can be further diluted with *Standard 0* and re-assayed as described in "Test Procedure", or must be reported as > 20 ng/ml. For the calculation of the concentrations, this dilution factor must be considered.
   (*Example*: dilution 1:10: 10 µl sample + 90 µl *Standard A*)
4. Automated method:
   The results in the instructions for use have been calculated automatically using a four-parameter logistic (4PL) curve fit. (4PL Rodbard or 4PL Marquardt are the preferred methods.) Other data reduction functions may give slightly different results.
5. Manual method:
   Using linear or semi-logarithmic graph paper, construct a standard curve by plotting the (mean) OD obtained from each standard against its concentration with OD value on the vertical (Y) axis and concentration on the horizontal (X) axis.
   Determine the corresponding sample concentration from the standard curve by using the (mean) OD value for each sample.

6.3.1 Example of Typical Standard Curve

The following data is for demonstration only and **cannot** be used in place of data generations at the time of assay.

Standard	Optical Density (450 nm)
Standard A (0.0 ng/ml)	0.03
Standard B (0.4 ng/ml)	0.08
Standard C (1.0 ng/ml)	0.16
Standard D (4.0 ng/ml)	0.55
Standard E (10 ng/ml)	1.27
Standard F (20 ng/ml)	2.28

7 REFERENCE VALUES

It is strongly recommended that each laboratory determine its own reference values.

In a study conducted with apparently healthy adults, using the AMH ELISA the following data were observed:

Population	n	Mean (ng/ml)	Median (ng/ml)	2.5th – 97.5th Percentile (ng/ml)	Range (min. – max.) (ng/ml)
Males	30	3.99	3.69	0.32 – 7.36	0.06 – 7.75
Females (20 – 29 years)	30	2.71	2.38	0.69 – 6.23	0.66 – 6.37
Females (30 – 39 years)	30	2.42	1.85	0.51 – 6.72	0.48 – 8.39
Females (40 – 49 years)	30	0.61	0.29	< 0.06 – 4.09	< 0.06 – 4.37

Values above or below reference range should be considered as suspicious and require additional testing.

8 QUALITY CONTROL

Good quality assurance in the laboratory requires that controls be run with each standard curve. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance.

It is recommended to use control samples according to state and federal regulations. The use of control samples is advised to assure the day-to-day validity of results. Use controls at both normal and pathological levels.

The controls and the corresponding results of the Quality Control Laboratory are stated in the Certificate of Analyses (CoA) added to the kit. The values and ranges stated on the CoA always refer to the current kit lot and must be used for direct comparison of the results.

If available, it is also recommended to make use of national or international Quality Assessment programs in order to ensure the accuracy of the results.

Apply appropriate statistical methods for analyzing control values and trends. If the results of the assay do not agree with the established acceptable ranges of control materials, results should be considered invalid.

In this case, please check the following technical areas: Pipetting and timing devices; photometer, expiration dates of reagents, storage and incubation conditions, aspiration and washing methods.

After checking the above-mentioned items without finding any error contact your distributor or the manufacturer directly.

9 PERFORMANCE CHARACTERISTICS

9.1 Specificity of Antibodies (Cross-Reactivity)

The following substances were tested for cross-reactivity of the assay:

Substance	Concentration Range of Spiked Substance (ng/ml)	Mean cross-reactivity (%)
AMH	0.40 – 10	100
Inhibin A	2.0 – 2000	0.03
Actvin AB	2.0 – 2000	0.27
LH	2.0 – 2000	0.18
FSH	2.0 – 2000	0.26
HCG	2.0 – 2000	0.12
TSH	2.0 – 2000	0.39
TGF-β1	2.0 – 2000	0.18
TGF-β2	2.0 – 2000	0.18
Prolactin	2.0 – 2000	0.38

9.2 Sensitivity

Limit of Blank (LoB)	0.044 ng/ml
Limit of Detection (LoD)	0.052 ng/ml
Limit of Quantification (LoQ)	0.062 ng/ml
Measuring range	0.052 ng/ml – 20.0 ng/ml
Linear range	0.19 ng/ml – 20 ng/ml

9.3 Reproducibility

9.3.1 Within-run Precision

The within-run precision was determined with 4 samples covering the complete measuring range in 1 run with 10 replicates. CV was calculated as mean CV of 10 replicates.

Sample	n	Mean (ng/ml)	CV (%)
1	10	0.22	7.3
2	10	0.67	3.1
3	10	5.76	2.4
4	10	15.88	2.6

9.3.2 Between-run Precision

The between-run precision was determined for 4 samples covering the measuring range in 3 independent runs on 3 days with 10 determinations. CV was calculated from 30 determinations.

Sample	n	Mean (ng/ml)	CV (%)
1	30	0.23	6.0
2	30	0.69	5.4
3	30	5.73	3.1
4	30	15.90	4.1

9.3.3 Between-lot Precision

The between-lot variation was determined by 6 measurements of different samples with 3 different kit lots.

Sample	n	Mean (ng/ml)	CV (%)
1	18	0.33	7.82
2	18	1.04	5.59
3	18	6.63	1.99
4	18	12.87	0.27

9.4 Recovery

Recovery was determined by adding increasing amounts of the analyte to different samples containing different amounts of endogenous analyte. The percentage recoveries were determined by comparing expected and measured values of the samples.

		Sample 1	Sample 2	Sample 3	Sample 4
Concentration (ng/ml)		0.20	0.70	5.85	15.89
Average Recovery (%)		98.8	97.5	97.0	98.5
Range of Recovery (%)	**from**	94.2	95.4	94.1	95.9
	to	101.8	100.3	101.1	100.8

9.5 Linearity

Samples containing different amounts of analyte were serially diluted with *Standard A*. The percentage recovery was calculated by comparing the expected and measured values for the analyte.

		Sample 1	Sample 2	Sample 3	Sample 4
Concentration (ng/ml)		3.01	5.99	9.75	16.29
Average Recovery (%)		105.2	106.8	95.2	110.3
Range of Recovery (%)	**from**	94.7	102.4	94.5	106.6
	to	111.9	110.9	95.9	113.8

9.6 Method Comparison

A comparison of AMH ELISA (EIA-6141) (y) and the reference method Roche Elecsys® AMH Plus (x) using samples gave the following correlation:

$y = -0.1419 + 0.905x$; $n = 52$, $r = 0.980$

10 LIMITATIONS OF THE PROCEDURE

Reliable and reproducible results will be obtained when the assay procedure is performed with a complete understanding of the instructions for use and in compliance with the laboratory quality assurance guidelines.
Any improper handling of samples or modification of this test might influence the results.

10.1 Interfering Substances

Hemoglobin (up to 4 mg/ml), bilirubin (up to 0.5 mg/ml) and triglyceride (up to 7.5 mg/ml) have no influence on the assay results.

10.2 Drug Interferences

Until today no substances (drugs) are known to us, which have an influence on the measurement of AMH in a sample.

10.3 High-Dose Hook Effect

"High-Dose Hook Effect" is not detected up to 400 ng/ml of AMH.

11 LEGAL ASPECTS

11.1 Reliability of Results

The test must be performed exactly as per the manufacturer's instructions for use. Moreover, the user must strictly adhere to the laboratory quality assurance guidelines and applicable national standards and/or laws. This is especially relevant for the use of control reagents. It is important to always include, within the test procedure, a sufficient number of controls for validating the accuracy and precision of the test.
The test results are valid only if all controls are within the specified ranges and if all other test parameters are also within the given assay specifications. If there is any doubt or concern regarding a result, please contact the manufacturer.

11.2 Liability

Any modification of the test kit and/or exchange or mixture of any components of different lots from one test kit to another could negatively affect the intended results and validity of the overall test. Such modification and/or exchanges invalidate any claim for replacement.
Claims submitted due to customer misinterpretation of laboratory results subject to point 11.2 are also invalid. Regardless, in the event of any claim, the manufacturer's liability is not to exceed the value of the test kit. Any damage caused to the test kit during transportation is not subject to the liability of the manufacturer.

12 LITERATURE

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2. Hampl R, Šnajderová, M, Mardešić T. Antimullerian Hormone (AMH) Not Only a Marker for Prediction of Ovarian Reserve.
   Physiol. Res. 60: 217-223, 2011.
3. Matuszczak E, Hermanowicz A, Komarowska M, Debek W. Serum AMH in Physiology and Pathology of Male Gonads.
   Int J Endocrinol. 2013:128907.

Symbols:

	Storage temperature		Manufacturer		Contains sufficient for <n> tests
	Use-by date	LOT	Batch code		
	Consult instructions for use	CONT	Content		
	Caution	REF	Catalogue number		Distributor
	Date of manufacture			RUO	For research use only!