Epitope Diagnostics, Inc.

EDI™ Human Anti-Human Antibody (HAHA) ELISA Kit

Enzyme Linked ImmunoSorbent Assay (ELISA) for the measurement of Human Anti-Human Antibody (HAHA) Level in Serum and Plasma

REF KT 806 EU: IVD CE Σ 96 +2 +8 °C

INTENDED USE

This ELISA (enzyme-linked immunosorbent assay) kit is produced for the quantitative determination of human anti-human (IgG) antibody (HAHA) levels in patient serum or plasma samples. It detects both HAHA-IgG and HAHA-IgM subtypes. The test might be used as an aid for detection of patients with positive HAHA that may affect prescribed diagnosis and treatment involving humanized monoclonal antibody.

SUMMARY OF PHYSIOLOGY

Clinically, humanized monoclonal antibodies (IgG) and their fragments are used in vivo diagnosis procedure (radionuclides) and treatment for patients with various diseases. In patients, even a single dose injection of a humanized monoclonal antibody or its fragment may induce immune response directed against this foreign protein (immunogen). Also, people with autoimmune diseases, such as rheumatoid arthritis, lupus, etc. produce autoantibody against human IgG. In the circulation, the presence of human antibody against human IgG would bind to the injected humanized antibody therapeutics or diagnosis and, therefore, diminish the efficacy of either in-vivo diagnosis or treatment. Especially, the HAHA would increase the risk of anaphylactic complications to subsequent administration of the humanized monoclonal antibody-based therapy.

The presence of HAHA in patient serum or plasma specimens may cause both false positive and false negative immunoassay test results depending on assay principles and monoclonal antibodies used in the assay system. This HAHA ELISA is a ready-to-use test kit with well-breakable microtiter plate and simple test procedures. It also provides a wide measurement range without high dose "hook" effect.

ASSAY PRINCIPLE

This ELISA is designed, developed and produced for the quantitative measurement of HAHA in serum and plasma samples. The assay utilizes the two-site "sandwich" technique with two selected antibodies that bind to HAHA.

Assay standards, controls and patient samples are directly added to wells of a microplate that is coated with highly purified human IgG. After the first incubation period, the HAHA binds to the human IgG on the wall of microtiter well and unbound proteins in each microtiter well are washed away. Then a horseradish peroxidase (HRP)-labeled human antibody is added to each microtiter well and a "sandwich" of "well coated human IgG – HAHA – HRP-Conjugated human antibody" is formed. The unbound HRP conjugated human antibody is 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 HAHA on the wall of the microtiter well is directly proportional to the amount of HAHA in the sample. A standard curve is generated by plotting the absorbance versus the respective HAHA concentration for each standard on point-to-point or 4 parameter curve fit. The concentration of HAHA in test samples is determined directly from this standard 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. **Human IgG Coated Microplate (Cat. No. 30328)**
One well-breakable microplate with 12 x eight strips (96 wells total) coated with highly purified human IgG. The plate is framed and sealed in a foil zipper bag with a desiccant. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

2. **HRP Conjugated Human Antibody (Cat. No. 30329)**
One vial containing 0.6 mL HRP-labeled human antibody in a stabilized protein matrix. This reagent must be diluted with Tracer Antibody Diluent before use. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

3. **Tracer Antibody Diluent (Cat. No. 30052)**
One vial containing 12 mL ready to use buffer. It should be used only for tracer antibody dilution according to the assay procedures. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

4. **Assay Buffer (Cat. No. 30074)**
One bottle containing 30 mL of ready-to-use phosphate buffered saline based assay buffer with bovine serum albumin added. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

5. **ELISA Wash Concentrate (Cat. No. 10010)**
One bottle contains 30 mL of 30-fold concentrate. Before use the contents must be diluted with 870 mL of distilled water and mixed well. Upon dilution this yields a working wash solution containing a surfactant in phosphate-buffered saline with a non-azide and non-mercury based preservative. The diluted wash buffer should be stored at room temperature and is stable until the expiration date on the kit box.

6. **ELISA HRP Substrate (Cat. No. 10020)**
One bottle contains 12 mL of tetramethylbenzidine (TMB) with stabilized hydrogen peroxide. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

7. ELISA Stop Solution (Cat. No. 10030)
One bottle contains 12 mL of 0.5 M sulfuric acid. This reagent should be stored at 2 – 8°C or room temperature and is stable until the expiration date on the kit box.

8. HAHA Standards (Cat. No. 30341 – 30345)
Five vials each containing 0.5mL of a different level of HAHA in a liquid protein matrix with a non-azide based preservative. **Refer to vials for exact concentration for each standard.** These reagents should be stored at 2 – 8°C and are stable until the expiration date on the kit box.

9. HAHA Controls (Cat. No. 30346 – 30347)
Two vials each containing 0.5mL of a different level of HAHA in a liquid protein matrix with a non-azide based preservative. **Refer to vials for exact concentration range for each control.** Both controls should be stored at 2 – 8°C and are stable until the expiration date on the kit box.

SAFETY PRECAUTIONS

The reagents must be used in a professional laboratory environment. Source material (e.g. highly purified bovine serum albumin) of 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 sulfuric acid. TMB may cause irritation to skin and mucous membranes and cause an allergic skin reaction. TMB is a suspected carcinogen. Sulfuric acid may cause severe irritation on contact with skin. 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 25 µL, 50 µL, 100 µL, and 1000 µL etc.
2. Repeating dispenser suitable for delivering 100 µL.
3. Disposable pipette tips suitable for above volume dispensing.
4. Disposable 12 x 75 mm or 13 x 100 glass tubes.
5. Disposable plastic 100 mL and 1000 mL bottle with caps.
6. Aluminum foil.
7. Deionized or distilled water.
8. Plastic microtiter well cover or polyethylene film.
9. ELISA multichannel wash bottle or automatic (semi-automatic) washing system.
10. Spectrophotometric microplate reader capable of reading absorbance at 450 nm.

SPECIMEN COLLECTION

Only 50 µL of human serum or plasma is required for HAHA measurement in duplicate. No special preparation of individual is necessary prior to specimen collection. In the case of serum, whole blood should be collected and must be allowed to clot for a minimum of 30 minutes at room temperature before the serum is separated by centrifugation (850 – 1500xg for 10 minutes). The serum should be separated from the clot within three hours of blood collection and transferred to a clean test tube. Serum or plasma samples should be stored at 2 - 8ºC if the assay is to be performed within 72 hours. Otherwise, patient samples should be stored at - 20ºC or below until measurement. Avoid repeated (more than three times) freezing and thawing of specimen.

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 (Cat. 10010) must be diluted to working solution prior use. Please see REAGENTS section for details.

2. Assay Procedure

(1) Place a sufficient number of human IgG coated microwell strips/wells (Cat. 30328) in a holder to run HAHA standards, controls and unknown samples in duplicate.
(2) Test Configuration

ROW	STRIP 1	STRIP 2	STRIP 3
A	STD 1	STD 5	SAMPLE 2
B	STD 1	STD 5	SAMPLE 2
C	STD 2	C 1	SAMPLE 3
D	STD 2	C 1	SAMPLE 3
E	STD 3	C 2	SAMPLE 4
F	STD 3	C 2	SAMPLE 4
G	STD 4	SAMPLE 1	
H	STD 4	SAMPLE 1	

(3) Add **25 µL** of standards, controls and patient samples into the designated microwell.
(4) Add **100 µL** of assay buffer (Cat. 30074) to each well
(5) Cover the plate with one plate sealer and incubate plate at room temperature, shaking for **45 minutes**.
(6) Prepare HAHA Tracer antibody working solution by **1:21** fold dilution of the HRP-conjugated human antibody (Cat. 30329) with the tracer Antibody Diluent (Cat. 30052). For each strip, it is required to mix 1 mL of the tracer antibody diluent with 50 µL of the tracer antibody in a clean test tube.
(7) Remove 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.
(8) Add **100 µL** of above diluted HRP-conjugated Human Antibody working solution to each of the wells.
(9) Cover the plate with a plate sealer and an aluminum foil to and incubate plate at room temperature, shaking for **45 minutes**.
(10) Remove 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.
(11) Add **100 µL** of ELISA HRP Substrate (Cat. 10020) into each of the wells.
(12) Cover the plate with a plate sealer and also with an aluminum foil to avoid exposure to light.
(13) Incubate plate at room temperature for **20 minutes**.
(14) Remove the aluminum foil and plate sealer. Add 100 µL of ELISA Stop Solution (Cat. 10030) into each of the wells. Mix gently.
(15) Read the absorbance at **450 nm** within 10 minutes in a microplate reader
NOTE: to reduce the background, one can set the instrument to dual wavelength measurement at 450 nm with background wavelength correction set at 595 nm or 620 nm or 630 nm.

PROCEDURAL NOTES

1. It is recommended that all standards, 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. For patient samples with concentration higher than level 5 standard, it is recommended to measure diluted the specimen with assay buffer at 1:10, 1:100, etc. for a more accurate report.
3. Keep light-sensitive reagents in the original amber bottles.
4. Store any unused human IgG coated strips in the foil Ziploc bag with desiccant to protect from moisture.
5. Careful technique and use of properly calibrated pipetting devices are necessary to ensure reproducibility of the test.
6. Incubation times or temperatures other than those stated in this insert may affect the results.
7. Avoid air bubbles in the microwell as this could result in lower binding efficiency and higher CV% of duplicate reading.
8. All reagents should be mixed gently and thoroughly prior use. Avoid foaming.

INTERPRETATION OF RESULTS

1. Calculate the average absorbance for each pair of duplicate test results.
2. Subtract the average absorbance of the STD 1 (0 µg/mL) from the average absorbance of all other readings to obtain corrected absorbance.
3. The standard curve is generated by the corrected absorbances of all standard levels on the ordinate against the standard concentration on the abscissa using point-to-point or log-log paper. Appropriate computer assisted data reduction programs may also be used for the calculation of results. We recommend using 4-parameter or **Point-to-Point** curve fit.

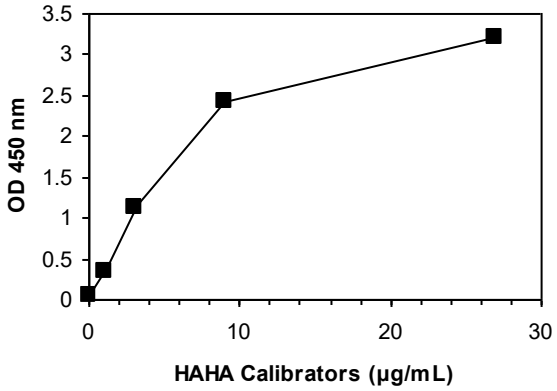
The HAHA concentrations for the controls and patient samples are read directly from the standard curve using their respective corrected absorbance.

EXAMPLE DATA AND STANDARD CURVE

A typical absorbance data and the resulting standard curve from this HAHA ELISA are represented. **This curve should not be used in lieu of standard curve run with each assay.**

Well I.D.	OD 450 nm Absorbance			Results µg/mL
	Readings	Average	Corrected	
0 µg/mL	0.047 0.046	0.047	0.000	
1 µg/mL	0.342 0.338	0.340	0.293	
3 µg/mL	1.178 1.094	1.136	1.089	
9 µg/mL	2.501 2.336	2.418	2.371	
27 µg/mL	3.224 3.205	3.214	3.167	
Control 1	0.598 0.643	0.620	0.573	1.71 µg/mL
Control 2	1.988 2.013	2.000	1.953	7.04 µg/mL

HAHA ELISA



EXPECTED VALUES

Eighty six normal adult sera were measured with this HAHA ELISA. One hundred sixty sera showed the OD reading very close to the zero calibrator. The 99% confidence normal cut-off is **0.5 µg/mL** .

It is highly recommend that each laboratory establish its own normal cut off level.

LIMITATION OF THE PROCEDURE

1. Since this is the first commercial assay of this kind and there is no Gold Standard concentration or international standard available for HAHA measurement, the values of assay standards were established and validated by Epitope Diagnostics. Results obtained with different assay methods or kits cannot be used interchangeably.
2. For unknown sample value read directly from the assay that is greater than the highest assay standard, it is recommend measuring a further diluted sample for more accurate measurement.
3. Bacterial or fungal contamination of serum specimens or reagents, or cross-contamination between reagents may cause erroneous results.
4. 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 with known HAHA levels. We recommend that all assays include the laboratory's own HAHA controls in addition to those provided with this kit.

PERFORMANCE CHARACTERISTICS

Sensitivity

The sensitivity of this HAHA ELISA as determined by the 95% confidence limit on 20 duplicate determination of zero standard is about 0.1 µg/mL.

High Dose "hook" effect

This assay has showed that it did not have any high dose "hook" effect up to 3,000 µg/mL.

Precision
The intra-assay precision was validated by measuring one control sample in a single assay with eight replicate determinations.

Mean HAHA Value (µg/mL)	CV (%)
1.62	5.1

The inter-assay precision is validated by measuring one control sample in duplicate in 6 individual assays.

Mean HAHA Value (µg/mL)	CV (%)
6.93	6.7

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. Szolar OH, Stranner S, Zinoecker I, Mudde GC, Himmler G, Waxenecker G, Nechansky A. Qualification and application of a surface plasmon resonance-based assay for monitoring potential HAHA responses induced after passive administration of a humanized anti Lewis-Y antibody. J Pharm Biomed Anal. 2006 Jun 16;41(4):1347-53. Epub 2006 Apr 27
2. Ritter G, Cohen LS, Williams C Jr, Richards EC, Old LJ, Welt S. Serological analysis of human anti-human antibody responses in colon cancer patients treated with repeated doses of humanized monoclonal antibody A33. Cancer Res. 2001 Sep 15;61(18):6851-9
3. Lofgren JA, Dhandapani S, Pennucci JJ, Abbott CM, Mytych DT, Kaliyaperumal A, Swanson SJ, Mullenix MC. Comparing ELISA and surface plasmon resonance for assessing clinical immunogenicity of panitumumab. J Immunol. 2007 Jun 1;178(11):7467-72

Short Assay Protocol:

- Add 25 µl of standards, control and patient sample to the plate
- Add 100 µl of assay buffer
- Incubate 45 min at RT, shaking
- Wash strips with diluted wash buffer
- Add 100 µl HAHA Tracer Antibody
- Incubate 45 min at RT, shaking
- Wash strips with diluted wash buffer
- Add 100 µl TMB substrate
- Incubate 20 min at RT
- Add 100 µl stop solution
- Read strips at OD 450 nm

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.
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Manufacturer	No. of tests
Catalog Number	Keep away from heat and direct sun light
Concentrate	Store at
In Vitro Diagnostic Device	Use by
Read instructions before use	Lot No.
Authorized Representative In Europe	