**EDI™ HAHA ELISA Kit**

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

**Catalog Number:** EPI-KT 806  
**Store at 2 – 8°C Upon Receipt**

For Research Use Only. Not for Use in Diagnostic Procedures

**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 present 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 use 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. Regents 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 20 mL of 30 fold concentrate. Before use the contents must be diluted with 580 mL of distilled water and mix 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.
SAFTY PRECAUTIONS

The reagents must be used in a professional laboratory environment and are for research use only. 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 donor health 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 potential 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 – 1500g 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 – 8°C if the assay is to be performed within 72 hours. Otherwise, patient samples should be stored at -20°C 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 must be diluted to working solution prior use. Please see REAGENTS section for details.

2. Test Configuration
   (1) Place a sufficient number of human IgG coated microwell strips/wells in a holder to run HAHA standards, controls and unknown samples in duplicate.
   (2) Measurements
      (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 (30329) with the tracer Antibody Diluant (30052). For each strip, it is required to mix 1 mL of the tracer antibody diluted 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 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 into each of the wells. Mix gently.
      (15) Read the absorbance at 450 nm within 10 minutes in a microplate reader.

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 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 murine 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 Quadratic curve fit.

The HAHA concentrations for the controls and patient samples are
read directly from the standard curve using their respective corrected
absorbance. If log-log graphic paper or computer assisted data
reduction program utilizing logarithmic transformation are used,
sample having corrected absorbance between the 2nd standard and
the next highest standard should be calculated by the formula:

\[
\text{Corrected absorbance (unknown) = \frac{\text{Corrected Absorbance}}{\text{STD (2nd STD)}} \times \text{Value of the 2nd STD}}
\]

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.

<table>
<thead>
<tr>
<th>Well I.D.</th>
<th>OD 450 nm Absorbance</th>
<th>Results µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Readings</td>
<td>Average</td>
</tr>
<tr>
<td>0 µg/mL</td>
<td>0.047</td>
<td>0.047</td>
</tr>
<tr>
<td>1 µg/mL</td>
<td>0.342</td>
<td>0.338</td>
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<tr>
<td>3 µg/mL</td>
<td>1.178</td>
<td>1.136</td>
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<tr>
<td>9 µg/mL</td>
<td>2.501</td>
<td>2.418</td>
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<tr>
<td>27 µg/mL</td>
<td>3.224</td>
<td>3.214</td>
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<tr>
<td>Control 1</td>
<td>0.598</td>
<td>0.620</td>
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<tr>
<td></td>
<td>0.643</td>
<td>0.620</td>
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<tr>
<td>Control 2</td>
<td>1.988</td>
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<td></td>
<td>2.013</td>
<td>2.000</td>
</tr>
</tbody>
</table>

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 250 µg/mL , it is recommend to
measure 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 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 (ng/mL)   CV (%)
16.2   5.1

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

Mean HAHA Value (ng/mL)   CV (%)
69.3   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

This product is developed and manufactured by
Epitope Diagnostics, Inc.
San Diego, CA 92126, USA

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