

User's Manual

Thrombin-IgA ELISA

Enzyme Immunoassay for the determination of Thrombin-IgA antibodies in human serum

REF AE29007

 96

RUO

For Research Use Only – Not for Use in Diagnostic Procedures

1 INTENDED USE

This is a solid phase enzyme immunoassay employing native human thrombin for the quantitative and qualitative detection of IgA antibodies against thrombin in human serum.

For research use only, not for use in diagnostic procedures.

2 INTRODUCTION

Thrombin is not a normal constituent of the circulating blood. It is generated by the catalytic cleavage of its plasma precursor, prothrombin (factor II), by the activated Stuart factor (factor Xa). This is the final step of the intrinsic and extrinsic pathways of coagulation. The transformation requires the presence of an activated cofactor, factor Va, released from factor V by thrombin itself, and whose binding to prothrombin accelerates the activity of factor Xa in a non-enzymatic manner.

Thrombin is a glycoprotein formed by two peptides chains of 36 and 259 amino-acids linked by disulfure bonds. Three important sites have been identified on the surface of the enzyme: The catalytic site that confers to the molecule its serine protease activity, the exosite one responsible for the binding of the substrate (fibrinogen or thrombin receptor) and the exosite two responsible for the binding of antithrombin III and inactivation of thrombin. Thrombin is, however, more than a simple plasma enzyme. Its properties to stimulate platelets and cause them to expand aggregate and release components of the alpha and dense granules were recognized earlier on.

3 PRINCIPLE OF THE TEST

Serum samples diluted 1:101 are incubated in the microplates coated with the specific antigen. The antibodies, if present in the specimen, bind to the antigen. The unbound fraction is washed off in the following step. Afterwards anti-human immunoglobulins conjugated to horseradish peroxidase (conjugate) are incubated and react with the antigen-antibody complex of the samples in the microplates. Unbound conjugate is washed off in the following step. Addition of TMB-substrate generates an enzymatic colorimetric (blue) reaction, which is stopped by diluted acid (color changes to yellow). The intensity of color formation from the chromogen is a function of the amount of conjugate bound to the antigen-antibody complex and this is proportional to the initial concentration of the respective antibodies in the sample.

4 REAGENTS

| TO BE RECONSTITUTED | | | | |
|---|--------------------|-----------|----------------|--|
| Item | Quantity | Cap color | Solution color | Description / Contents |
| Sample Buffer (5x) | 1 x 20ml | White | Yellow | 5 x concentrated Tris, sodium chloride (NaCl), bovine serum albumin (BSA), sodium azide < 0.1% (preservative) |
| Wash Buffer (50x) | 1 X 20ml | White | Green | 50 x concentrated Tris, NaCl, Tween 20, sodium azide < 0.1% (preservative) |
| READY TO USE | | | | |
| Item | Quantity | Cap color | Solution color | Description / Contents |
| Negative Control | 1 x 1.5ml | Green | Colorless | Human serum (diluted), bovine serum albumin (BSA), sodium azide < 0.1% (preservative) |
| Positive Control | 1 x 1.5ml | Red | Yellow | Human serum (diluted), bovine serum albumin (BSA), sodium azide < 0.1% (preservative) |
| Cut-off Calibrator | 1 x 1.5ml | Blue | Yellow | Human serum (diluted), bovine serum albumin (BSA), sodium azide < 0.1% (preservative) |
| Calibrators | 6 x 1.5ml | White | Yellow * | Concentration of each calibrator: 0, 3, 10, 30, 100, 300 U/ml. Human serum (diluted), bovine serum albumin (BSA), sodium azide < 0.1% (preservative) |
| Conjugate, IgA | 1 x 15ml | Red | Red | Anti-human immunoglobulins conjugated to horseradish peroxidase, bovine serum albumin (BSA) |
| TMB Substrate | 1 x 15ml | Black | Colorless | Stabilized tetramethylbenzidine and hydrogen peroxide (TMB/H ₂ O ₂) |
| Stop Solution | 1 x 15ml | White | Colorless | 1M Hydrochloric Acid |
| Microtiter plate | 12 x 8 well strips | N/A | N/A | With breakaway microwells. Refer to paragraph 1 for coating. |
| * Color increasing with concentration | | | | |
| MATERIALS REQUIRED, BUT NOT PROVIDED | | | | |
| Microtiter plate reader 450 nm reading filter and recommended 620 nm reference filter (600-690 nm). Glass ware (cylinder 100-1000ml), test tubes for dilutions. Vortex mixer, precision pipettes (10, 100, 200, 500, 1000 µl) or adjustable multipipette (100-1000µl). Microplate washing device (300 µl repeating or multichannel pipette or automated system), adsorbent paper. Our tests are designed to be used with purified water according to the definition of the United States Pharmacopeia (USP 26 - NF 21) and the European Pharmacopeia (Eur.Ph. 4th ed.). | | | | |

5 STORAGE CONDITIONS

Store all reagents and the microplate at 2-8°C/35-46°F, in their original containers. Once prepared, reconstituted solutions are stable at 2-8°C/35-46°F for 1 month. Reagents and the microplate shall be used within the expiry date indicated on each component, only. Avoid intense exposure of TMB solution to light. Store microplates in designated foil, including the desiccant, and seal tightly.

6 WARNINGS AND PRECAUTIONS

1. **CAUTION:** This kit contains human material. The source material used for manufacture of this component tested negative for HBsAg, HIV 1/2 and HCV by FDA-approved methods. However, no method can completely assure absence of these agents. Therefore, all human blood products, including serum samples, should be considered potentially infectious. Handling should be as defined by an appropriate national biohazard safety guideline or regulation, where it exists.²⁵
2. Avoid contact with 1N HCl. It may cause skin irritation and burns. If contact occurs, wash with copious amounts of water and seek medical attention if irritation persists.
3. Do not use reagents after expiration date and do not mix or use components from kits with different lot numbers.
4. Replace caps on reagents immediately. Do not switch caps.
5. Do not pipette reagents by mouth.
6. For research use only, not for use in diagnostic procedures.

7 INSTRUMENTATION

A microtiter well reader with a bandwidth of 10 nm or less and an optical density range of 0 to 3 OD or greater at 450 nm wavelength is acceptable for absorbance measurement.

8 SAMPLE COLLECTION AND PREPARATION

1. The use of SERUM samples is required for this test.
2. Samples should be collected using standard venipuncture techniques. Remove serum from the coagulated or packed cells within 60 minutes after collection.
3. Samples which cannot be assayed within 24 hours of collection should be frozen at -20°C or lower, and will be stable for up to six months.
4. Avoid grossly hemolytic (bright red), lipemic (milky), or turbid samples (after centrifugation).
5. Samples should not be repeatedly frozen and thawed prior to testing. DO NOT store in "frost free" freezers, which may cause occasional thawing. Samples which have been frozen, and those which are turbid and/or contain particulate matter, must be centrifuged prior to use.

9 PROCEDURAL NOTES

1. Pipetting Recommendations (single and multi-channel). Pipetting of all standards, samples, and controls should be completed within 3 minutes.
2. All standards, samples, and controls should be run in duplicate concurrently so that all conditions of testing are the same.
3. It is recommended that the wells be read within 15 minutes following addition of Stop Solution.

10 PREPARATION OF REAGENTS AND SAMPLES

All reagents should be brought to room temperature (18°C - 25°C) before use.

Dilute concentrated reagents:

Dilute the concentrated sample buffer 1:5 with distilled water (e.g. 20 ml plus 80 ml).

Dilute the concentrated wash buffer 1:50 with distilled water (e.g. 20 ml plus 980 ml).

To avoid mistakes we suggest to mark the cap of the different calibrators.

Samples:

Dilute serum samples 1:101 with sample buffer (1x)

e.g. 1000 μl sample buffer (1x) + 10 μl serum. Mix well !

Washing:

Prepare 20 ml of diluted wash buffer (1x) per 8 wells or 200 ml for 96 wells

e.g. 4 ml concentrate plus 196 ml distilled water.

Automated washing:

Consider excess volumes required for setting up the instrument and dead volume of robot pipette.

Manual washing:

Discard liquid from wells by inverting the plate. Knock the microwell frame with wells downside vigorously on clean adsorbent paper. Pipette 300 μl of diluted wash buffer into each well, wait for 20 seconds. Repeat the whole procedure twice again.

Microplates:

Calculate the number of wells required for the test. Remove unused wells from the frame, replace and store in the provided plastic bag, together with desiccant, seal tightly ($2-8^{\circ}\text{C}/35-46^{\circ}\text{F}$).

11 ASSAY PROCEDURE

We suggest pipetting calibrators, controls and samples as follows:



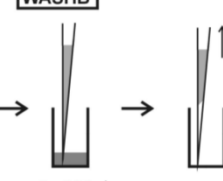
| For <i>QUANTITATIVE</i> interpretation | | | | | For <i>QUALITATIVE</i> interpretation | | | | |
|--|-------|-------|-----|------|---------------------------------------|----|-----|---|------|
| | 1 | 2 | 3 | 4... | | 1 | 2 | 3 | 4... |
| A | Cal A | Cal E | P1 | | A | NC | P2 | | |
| B | Cal A | Cal E | P1 | | B | NC | P2 | | |
| C | Cal B | Cal F | P2 | | C | CC | P3 | | |
| D | Cal B | Cal F | P2 | | D | CC | P3 | | |
| E | Cal C | PC | P3 | | E | PC | ... | | |
| F | Cal C | PC | P3 | | F | PC | ... | | |
| G | Cal D | NC | ... | | G | P1 | ... | | |
| H | Cal D | NC | ... | | H | P1 | ... | | |

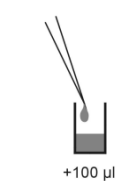
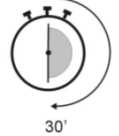
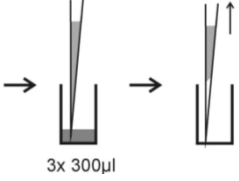
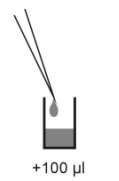

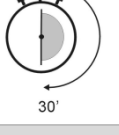
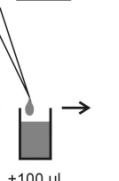


Cal.A: calibrator A
 Cal.B: calibrator B
 Cal.C: calibrator C

Cal.D: calibrator D
 Cal.E: calibrator E
 Cal.F: calibrator F

PC: positive control
 NC: negative control
 CC: cut-off calibrator

P1: sample 1
 P2: sample 2
 P3: sample 3

| Step | Description |
|-------------------------------|---|
| 1. | Ensure preparations from step 10 above have been carried out prior to pipetting. |
| 2. | Use the following steps in accordance with quantitative/ qualitative interpretation results desired: |
| CONTROLS & SAMPLES | |
| 3. |  <p>Pipette into the designated wells as described above, 100 µl of either:</p> <ul style="list-style-type: none"> a. Calibrators (CAL.A to CAL.F) for <i>QUANTITATIVE</i> or b. Cut-off Calibrator (CC) for <i>QUALITATIVE</i> interp. <p>and 100 µl of each of the following:</p> <ul style="list-style-type: none"> • Negative control (NC) and Positive control (PC), and • Sample diluted serum (P1, P2...) |
| 4. |  <p>Incubate for 30 minutes at 20-32°C/68-89.6°F.</p> |
| 5. |  <p>Wash 3x with 300 µl washing buffer (diluted 1:50).</p> |

| CONJUGATE | |
|-----------|---|
| 6. | <div style="display: flex; align-items: center;"> <div style="border: 1px solid black; padding: 2px; margin-right: 10px;">CONJ</div>  </div> <p>Pipette 100 µl conjugate into each well.</p> |
| 7. |  <p>Incubate for 30 minutes at 20-32°C/68-89.6°F.</p> |
| 8. | <div style="display: flex; align-items: center;"> <div style="border: 1px solid black; padding: 2px; margin-right: 10px;">WASHB</div>  </div> <p>Wash 3x with 300 µl washing buffer (diluted 1:50).</p> |
| SUBSTRATE | |
| 9. | <div style="display: flex; align-items: center;"> <div style="border: 1px solid black; padding: 2px; margin-right: 10px;">SUB</div>  </div> <p>Pipette 100 µl TMB substrate into each well.</p> |
| 10. | <div style="display: flex; align-items: center;">   </div> <p>Incubate for 30 minutes at 20-32°C/68-89.6°F, protected from intense light.</p> |
| STOP | |
| 11. | <div style="display: flex; align-items: center;"> <div style="border: 1px solid black; padding: 2px; margin-right: 10px;">STOP</div>  </div> <p>Pipette 100 µl stop solution into each well, using the same order as pipetting the substrate.</p> |
| 12. |  <p>Incubate 5 minutes minimum.</p> |
| 13. | <p>Agitate plate carefully for 5 sec.</p> |
| 14. | <div style="display: flex; align-items: center;"> <div style="border: 1px solid black; padding: 2px; margin-right: 10px;"> OD₄₅₀ - OD₆₂₀  </div> <p>450/620 nm</p> </div> <p>Read absorbance at 450 nm (recommended 450/620 nm) within 30 minutes.</p> |

12 INTERPRETATION

For **quantitative interpretation** establish the standard curve by plotting the optical density (OD) of each calibrator (y-axis) with respect to the corresponding concentration values in U/ml (x-axis). For best results we recommend log/lin coordinates and 4-Parameter Fit. From the OD of each sample, read the corresponding antibody concentrations expressed in U/ml.

| Normal Range | Equivocal Range | Positive Results |
|--------------|-----------------|------------------|
| < 12 U/ml | 12 - 18 U/ml | >18 U/ml |

Example of a standard curve

Do not use this example for interpreting your results!

| Calibrators | OD 450/620 nm | CV % (Variation) |
|-------------|---------------|------------------|
| 0 U/ml | 0,029 | 2,3 |
| 3 U/ml | 0,145 | 3,1 |
| 10 U/ml | 0,266 | 1,3 |
| 30 U/ml | 0,556 | 7,1 |
| 100 U/ml | 1,145 | 2,8 |
| 300 U/ml | 2,025 | 0,1 |

Example of calculation

| Sample | Replicate (OD) | Mean (OD) | Result (U/ml) |
|--------|----------------|-----------|---------------|
| P 01 | 1,360/1,328 | 1,344 | 129,0 |
| P 02 | 0,782/0,853 | 0,818 | 54,9 |

Samples above the highest calibrator range should be reported as >Max. They should be diluted as appropriate and re-assayed. Samples below calibrator range should be reported as < Min. For lot specific data, see enclosed quality control leaflet. Medical laboratories might perform an in-house quality control by using own controls and/or internal pooled sera, as foreseen by national regulations.

Each laboratory should establish its own normal range based upon its own techniques, controls, equipment and population according to their own established procedures.

In case that the values of the controls do not meet the criteria the test is invalid and has to be repeated.

The following technical issues should be verified: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, photometer, incubation conditions and washing methods.

If the items tested show aberrant values or any kind of deviation or that the validation criteria are not met without explicable cause please contact the manufacturer or the supplier of the test kit.

For **qualitative interpretation** read the optical density of the cut-off calibrator and the samples. Compare samples OD with the OD of the cut-off calibrator. For qualitative interpretation we recommend to consider sera within a range of 20% around the cut-off value as equivocal. All samples with higher ODs are considered positive, samples with lower ODs are considered negative.

| | | | | | |
|-------------------|--------------|-------------------|-------------|-------------------------|---------------------------|
| Negative: | | OD sample | < | 0.8 x OD cut-off | |
| Equivocal: | 0.8 x | OD cut-off | ≤ | OD sample | ≤ 1.2 x OD cut-off |
| Positive: | | OD sample | > | 1.2 x OD cut-off | |

13 TECHNICAL DATA

| | |
|---------------------------|---|
| Sample material: | serum |
| Sample volume: | 10 µl of sample diluted 1:101 with 1x sample buffer |
| Total incubation time: | 90 minutes at 20-32°C/68-89.6°F |
| Calibration range: | 0-300 U/ml |
| Analytical sensitivity: | 1.0 U/ml |
| Storage: | at 2-8°C/35-46°F use original vials only. |
| Number of determinations: | 96 tests |

14 PERFORMANCE DATA

14.1 Analytical Sensitivity

Testing sample buffer 30 times with this ELISA gave an analytical sensitivity of 1.0 U/ml.

14.2 Specificity and Sensitivity

The microplate is coated with native human thrombin. No cross reactivities to other autoantigens have been found. This ELISA exhibits a specificity of 100% and a sensitivity of 90%.

14.3 Linearity

Chosen sera have been tested with this kit and found to dilute linearly. However, due to the heterogeneous nature of human autoantibodies there might be samples that do not follow this rule.

| Sample No. | Dilution Factor | Measured (U/ml) | Expected (U/ml) | Recovery (%) |
|------------|-----------------|-----------------|-----------------|--------------|
| 1 | 1 / 100 | 202,3 | 200,0 | 101,0 |
| | 1 / 200 | 98,4 | 100,0 | 98,4 |
| | 1 / 400 | 52,3 | 50,0 | 104,6 |
| | 1 / 800 | 23,4 | 25,0 | 93,6 |
| 2 | 1 / 100 | 85,0 | 86,0 | 98,8 |
| | 1 / 200 | 41,4 | 43,0 | 96,3 |
| | 1 / 400 | 19,6 | 21,5 | 91,2 |
| | 1 / 800 | 10,1 | 10,8 | 93,5 |

14.4 Precision

To determine the precision of the assay, the variability (intra and inter-assay) was assessed by examining its reproducibility on three serum samples selected to represent a range over the standard curve.

| Intra-assay | | |
|-------------|-------------|--------|
| Sample No. | Mean (U/ml) | CV (%) |
| 1 | 20,7 | 1,2 |
| 2 | 45,2 | 3,1 |
| 3 | 114,5 | 6,8 |

| Inter-assay | | |
|-------------|-------------|--------|
| Sample No. | Mean (U/ml) | CV (%) |
| 1 | 18,4 | 1,8 |
| 2 | 47,5 | 2,6 |
| 3 | 126,4 | 6,5 |

14.5 Calibration

Due to the lack of international reference calibration this assay is calibrated in arbitrary units (U/ml).

15 LITERATURE

Jungermann K, Möhler H. Biochemie. Springer Verlag Berlin Heidelberg New York.

Manufactured for :

Immuno-Biological Laboratories, Inc. (IBL-America)
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Email: ibl@ibl-america.com Web: www.ibl-america.com

SYMBOLS USED WITH IBL-AMERICA ASSAYS

| Symbol | English | Deutsch | Français | Español | Italiano |
|---|------------------------------------|-------------------------------|--|---------------------------------------|-------------------------------------|
|  | Consult instructions for use | Gebrauchsanweisung beachten | Consulter les instructions d'utilisation | Consulte las instrucciones de uso | Consultare le istruzioni per l'uso |
|  | European Conformity | CE-Konfirmationskennzeichnung | Conformité aux normes européennes | Conformidad europea | Conformità europea |
|  | In vitro diagnostic device | In-vitro-Diagnostikum | Usage Diagnostic in vitro | Para uso Diagnóstico in vitro | Per uso Diagnostica in vitro |
|  | For research use only | Nur für Forschungszwecke | Seulement dans le cadre de recherches | Sólo para uso en investigación | Solo a scopo di ricerca |
|  | Catalogue number | Katalog-Nr. | Numéro de catalogue | Número de catálogo | Numero di Catalogo |
|  | Lot. No. / Batch code | Chargen-Nr. | Numéro de lot | Número de lote | Numero di lotto |
|  | Contains sufficient for <n> tests/ | Ausreichend für "n" Ansätze | Contenu suffisant pour "n" tests | Contenido suficiente para <n> ensayos | Contenuto sufficiente per "n" saggi |
|  | Storage Temperature | Lagerungstemperatur | Température de conservation | Temperatura de conservación | Temperatura di conservazione |
|  | Expiration Date | Mindesthaltbarkeitsdatum | Date limite d'utilisation | Fecha de caducidad | Data di scadenza |
|  | Legal Manufacturer | Hersteller | Fabricant | Fabricante | Fabbricante |
| Distributed by | Distributor | Vertreiber | Distributeur | Distribuidor | Distributore |
| Content | Content | Inhalt | Conditionnement | Contenido | Contenuto |
| Volume/No. | Volume / No. | Volumen/Anzahl | Volume/Quantité | Volumen/Número | Volume/Quantità |