

**ZYMUTEST HIA IgA**  
**# ARK040C-RUO**  
Assay of heparin-dependent antibodies  
of the IgA isotype by ELISA

**FOR RESEARCH USE ONLY.**  
**NOT FOR USE IN DIAGNOSTIC PROCEDURES.**



Last revision : 30/03/2011

**INTENDED USE:**

The ZYMUTEST HIA, IgA ELISA kit, is a standardised and optimised enzyme immuno-assay designed for measuring heparin-dependent antibodies of the IgA isotype, in human plasma or serum, or in any biological fluid where these antibodies must be measured. **This assay is for research use only and should not be used for patient diagnosis or treatment.**

**ASSAY PRINCIPLE:**

The diluted assayed plasma sample or biological fluid is introduced into one of the microwells of the coated plate, and supplemented with a platelet lysate. When present, heparin-dependent antibodies, of the IgA isotype, form complexes onto the biologically available unfractionated heparin, immobilised and saturated. Following a washing step, bound antibodies are revealed with the immunoconjugate, which is made of goat polyclonal antibodies anti-human IgA ( $\alpha$  specific)-peroxidase (HRP) conjugate. This immunoconjugate reacts specifically with IgA isotypes. Following a new washing step, the peroxidase substrate, Tetramethylbenzidine (TMB) in presence of hydrogen peroxide ( $H_2O_2$ ), is introduced and a blue colour develops. The colour turns yellow when the reaction is stopped with sulfuric acid. The colour developed is directly proportional to the amount of heparin-dependent antibodies, of the IgA isotype, present in the tested sample.

**TESTED SAMPLES:**

- Trisodium citrate or  $Na_2$  EDTA anticoagulated human plasma or human serum.
- Any biological fluid, where human heparin-dependent antibodies, of the IgA isotype, must be assayed.

**REAGENTS:**

1. **COAT:** Micro ELISA plate, containing 12 strips of 8 wells, coated with unfractionated heparin, biologically available, saturated, then stabilized; the plate is packed in an aluminium pouch hermetically sealed in presence of a desiccant.
2. **SD:** 2 vials containing 50 ml of HIA Sample Diluent, ready to use. Contains Sodium Azide
3. **C+**: 3 vials of HIA IgA Positive control, lyophilised. When restored with 1 ml of HIA Sample Diluent, the ready to use positive control is obtained (already diluted 1:100). The expected reactivity is indicated on the flyer provided with the kit.
4. **C-**: 3 vials of negative control, lyophilised (diluted normal human plasma). When restored with 1 ml of HIA Sample Diluent, the ready to use negative control is obtained (already diluted 1:100).
5. **CLy:** 3 vials of cell lysate, lyophilised (diluted normal human plasma). When restored with 2 ml of distilled water, the ready to use solution is obtained.
6. **IC:** 3 vials of immunoconjugate (Anti-IgA ( $\alpha$ )-HRP immunoconjugate), goat antibodies specific for human IgA ( $\alpha$ )- coupled to HRP, lyophilised. When restored with 7.5 ml of Conjugate Diluent (CD), the ready to use immunoconjugate is obtained.
7. **CD:** 1 vial of 25 ml of conjugate diluent, ready to use.
8. **WS:** 1 vial of 50 ml of 20 fold concentrated Wash Solution.
9. **TMB:** 1 vial of 25 ml peroxidase substrate: 3,3',5,5' - Tetramethylbenzidine containing hydrogen peroxide, ready to use.
10. **SA:** 1 vial of 6 ml of 0.45M Sulfuric Acid (Stop Solution), ready to use.

**Note:** Use only components from a same kit lot number. Do not mix components from different lots when running the assay.

**REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED:**

- 8-channel or repeating pipette allowing dispensing 50-300  $\mu$ l.
- 1-channel pipettes at variable volumes from 0 to 20  $\mu$ l, 20 to 200  $\mu$ l and 200 to 1000  $\mu$ l.
- Micro ELISA plate washing equipment (and shaker).
- Micro ELISA plate reader with a wavelength set up at 450 nm.
- Distilled water.

**REAGENTS PREPARATION, STORAGE AND STABILITY:**

In their original packaging box, before use, when stored at 2-8°C, the unopened reagents are stable until the expiration date printed on the box.

1. **Micro ELISA plate:** open the plastic pouch and take off the required amount of 8 well strips for the test series. When out of the pouch, the strips must be used within 30 minutes. Unused strips can be stored at 2-8°C for 8 weeks in their original aluminium pouch, in presence of the

desiccant, hermetically closed and protected from any moisture, and stored in the provided microplate storage bag (minigrip).

2. **HIA Sample Diluent:** It is ready to use. When open, it can be used for 8 weeks, stored at 2-8°C, and provided that it remains protected from any bacterial contamination. This reagent contains sodium azide.

**Warning:** The HIA Sample Diluent contains sodium azide, which may react with lead and copper plumbing to form highly explosive metal azides. Flush with large volumes of water when discarding into a sink.

3. **HIA IgA Positive Control:** restore each vial with 1 ml HIA sample diluent in order to obtain the ready to use positive control. It corresponds to a plasma containing IgA isotype heparin-dependent antibodies, already diluted 1:100. Following reconstitution, the positive control is stable for 2 weeks at 2-8°C, provided that it remains protected from any bacterial contamination, or 2 months at -20°C or below.
4. **Negative control:** restore each vial with 1 ml HIA sample diluent in order to obtain the ready to use negative control. It corresponds to a normal human plasma, already diluted 1:100. Following reconstitution, the negative control is stable for 2 weeks at 2-8°C, provided that it remains protected from any bacterial contamination, or 2 months at -20°C or below.
5. **CLy:** restore each vial with 2 ml distilled water in order to obtain the ready to use reagent. Following reconstitution, the reagent is stable for 2 weeks at 2-8°C, provided that it remains protected from any bacterial contamination, or 2 months at -20°C or below.

**Warning:** The CLy used for the assay is extracted from fresh human platelet concentrates. The negative control is also prepared with human plasma, tested with registered methods and found negative for HIV antibodies, HBs Ag and HCV antibodies. However, no assay may warrant the total absence of infectious agents. Any product of human origin must then be handled with all the required cautions, as being potentially infectious.

6. **Anti-IgA ( $\alpha$ )-HRP immunoconjugate:** each vial must be restored with 7.5 ml of conjugate diluent. Let the pellet to be completely dissolved before use, and shake the vial gently in order to homogenize the content. The restored conjugate is stable for at least 24 hours at room temperature or for at least 4 weeks at 2-8°C, or 2 months at -20°C or below.
7. **Conjugate diluent:** It is ready to use. When open, it can be used for 8 weeks, stored at 2-8°C, and provided that it remains protected from any bacterial contamination. This reagent contains 0.05% Kathon CG.
8. **Wash Solution:** Incubate the vial for 15-30 minutes in a water bath at 37°C until complete dissolution of solids, when present. Shake the vial and dilute the amount required 1:20 in distilled water (the 50 ml contained in the vial allow to prepare 1 liter of Wash Solution). The Wash Solution must be stored at 2-8°C in its original vial and used within 8 weeks following opening. The diluted Wash Solution must be used within 7 days, when protected from any contamination. This reagent contains 0.05% Kathon CG.
9. **TMB substrate:** It is ready to use. When open, it can be used for 8 weeks, stored at 2-8°C, and provided that it remains protected from any bacterial contamination.
10. **Stop solution:** It is ready to use.

**Cautions:** Sulfuric Acid, although diluted to 0.45M, is caustic. As for any similar chemical, handle Sulfuric Acid with great care. Avoid any skin and eye contact. Wear protection glasses and gloves when handling.

**Note:** Bring the kit at room temperature, at least 30 min. before use. Store the unused reagents at 2-8°C.

The stability studies performed at 30°C show that the reagents keep their performances and can be shipped at room temperature without any damage.

When appropriately used and stored, according to the recommended protocol and cautions, the kit can be used over a two month period, and strip by strip, if required.

**PROCEDURE:**

**Sample collection:**

Blood plasma (9 vol.) must be collected on 0.109M (or 0.129M) citrate anticoagulant (1 vol.); plasma supernatant is decanted following a 20 min. centrifugation at 2,500 g; citrated plasma should be tested within 24 hours or stored frozen at -20°C or below for up to 6 months, and thawed for 15 min. at 37°C just before use. Thawed specimen must be tested within 2 hours. EDTA collected human plasma may also be used. Conditions of storage are the same than those for citrated plasma.

Heparin-dependent antibodies can also be assayed on serum. Serum is then prepared according to the usual conditions for laboratory testing. Serum must be decanted from the clot before being used or stored frozen. Conditions of storage are the same than those for citrated plasma.

**Tested plasma or sample or control:**

Plasma or serum is tested at 1:100 dilution in HIA Sample Diluent (SD). When high amounts of heparin-dependent antibodies are expected, samples must be assayed at 1:200 or 1:400 dilution, etc.... Results (corresponding absorbance) must then be multiplied by 2 or 4, etc....

Controls are ready to use (already diluted 1:100).



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**Assay procedure:**

Remove the required number of strips from the aluminium pouch, for the series of measures to be performed. Then put the strips in the frame provided. In the different wells of the micro ELISA plate introduce the reagents and perform the various assay steps as indicated on the following table:

Reagent	Volume	Procedure
<b>CLy</b>	<b>50µl</b>	Introduce the <b>CLy</b> into the micro ELISA plate wells <b>(a)</b>
<b>IgA Positive control</b> or <b>Negative control</b>  or <b>1:100 diluted sample</b> or <b>sample diluent</b> (blank)	<b>200 µl</b>	Introduce the : – <b>IgA Positive control</b> or – <b>negative control</b> or – <b>diluted sample</b> or – <b>sample diluent</b> into the micro ELISA plate wells <b>(a)</b>
<b>Incubate for 60 minutes at room temperature (18-25 °C) (b)</b>		
<b>Wash Solution</b> (20 fold diluted in distilled water)	<b>300 µl</b>	Proceed to 5 successive washings using the washing instrument <b>(c)</b> .
<b>Conjugate</b> (anti-IgA (α)-HRP immunoconjugate, restored with 7.5 ml of conjugate diluent)	<b>200 µl</b>	Immediately after the washing, introduce the immunoconjugate in the micro ELISA plate wells. <b>(c)</b>
<b>Incubate for 60 minutes at room temperature (18-25 °C) (b)</b>		
<b>Wash Solution</b> (20 fold diluted in distilled water)	<b>300 µl</b>	Proceed to 5 successive washings using the washing instrument <b>(c)</b> .
<b>TMB/H<sub>2</sub>O<sub>2</sub> Substrate</b>	<b>200 µl</b>	Immediately after the washing, introduce the substrate into the wells. <b>Note:</b> The substrate distribution, row by row, must be accurate and at exact time intervals <b>(c,d)</b>
Let the colour develop for exactly <b>5 min.</b> at room temperature (18-25 °C) <b>(b)</b>		
<b>0.45M Sulfuric Acid</b>	<b>50 µl</b>	Following exactly the same time intervals than for the addition of substrate, stop the colour development by introducing the <b>0.45M Sulfuric Acid</b> <b>(c,d)</b>
Wait for <b>10 minutes</b> in order to allow the colour to stabilize and measure absorbance at <b>450 nm (A450)</b> <b>(e)</b> . Subtract the blank value.		

**Note:**

- Distribute controls and tested specimen as rapidly as possible (within 10 minutes), in order to obtain an homogeneous immunological kinetics for antibodies binding. A too long delay between the distribution of the first and the last wells may induce an influence of immunological kinetics and produce wrong results.
- Avoid letting the plate in the bright sunlight during incubations and more particularly during colour development. A micro-ELISA plate shaker can be used. An incubation temperature of 18-25°C must be respected. Results are affected by a too high (>25°C) or too low (<18°C) temperature, and measured A450 are then too high or too low. It has to be considered when analyzing the results. In the same way, if a microplate shaker is used, it should be used only at the beginning of each step (sample introduction, immunoconjugate introduction, stop solution introduction), for 1 to 2 minutes, in order to obtain a good homogeneity. A450 values generated in the assay are significantly increased if shaking is used throughout the incubation steps.
- Never let the plates empty between the addition of the reagents or following the washing step. The next reagent must be added within 3 minutes, in order to prevent the plate from drying, which could damage the immobilized components. If necessary, keep the plate filled with Wash Solution and empty it just before the introduction of the next reagent. The washing instrument must be adjusted in order to wash the plates gently, and to avoid a too drastic emptying, which could lower plate reactivity.
- For addition of the TMB substrate, the time interval between each row must be accurate and exactly determined. It must be the same when stopping the reaction.
- For bichromatic readings, a reference wavelength at 690 nm or at 620 nm can be used.

**QUALITY CONTROL:**

- Controls provided in the kit allow validating the right performance of the assay.
- Expected A450 values for positive and negative controls can present variations from lot to lot but, when the assay is run at room temperature, between 18 and 25°C, they always are:

**P = A450 for positive control ≥ 1.0**

**N = A450 for negative control: ≤ 0.25**

Obtained values for P and N, at 20±1°C, are indicated on the flyer provided in the kit.

Obtained A450 can vary according to the effective temperature during the assay run.

**EXPRESSION OF RESULTS:**

- Results are expressed according to the A450 values, as positive or negative.
- When higher dilutions are used, (i.e. D), the complementary dilution factor must be considered.
- The results obtained should be for research purposes only and not used for patient diagnosis or treatment.

**LIMITATIONS OF THE ASSAY:**

If the washing step is not correctly performed, the negative control can produce a high absorbance value. In order to avoid non-specific colour development, check that the washing step is performed efficiently.

**CONFIRMATION OF POSITIVE SAMPLES (IF REQUIRED):**

If required, positive samples can be confirmed by their binding inhibition in presence of heparin. For this confirmation, to 500µl of the 1:100 diluted tested specimen (plasma or serum) add 10µl of a 100 IU/ml Unfractionated heparin solution and mix homogeneously. This heparinized solution (2 IU/ml final) must then be tested in the assay. Heparin dependent antibody binding to the plate is then highly inhibited. This inhibition confirms the heparin dependent binding of antibodies.

**ASSAY SPECIFICITY AND CHARACTERISTICS:**

This optimised assay is designed with biologically available and immobilized heparin, then stabilized and saturated, which allows reacting fully with heparin binding proteins and antibodies. This reliable method then provides high reproducibility, high sensitivity and high specificity, by identifying IgA isotype heparin-dependent antibodies, and by mimicking the binding mechanism of antibodies in vivo, on heparin present at the cell surface, especially on platelets or endothelial cells.

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