



# ZYMUTEST HIA MonoStrip IgGAM

## Screening assay (# ARK041D)

Qualitative screening assay for the detection of heparin-dependant antibodies (IgG, IgM, and IgA isotypes) by ELISA



Manufactured By: HYPHEN BioMed

For in vitro diagnostic use only

Last revision : 29/09/2009

### INTENDED USE:

The ZYMUTEST HIA MonoStrip, IgGAM ELISA kit, is a qualitative screening assay intended for the global detection of heparin-dependent antibodies, whether the isotype is: IgG, IgM and IgA, in human plasma, by clinical laboratories. It is intended for in vitro diagnostic use. Each kit allows running 4 series of 8 tests (ie C+, C-, sample and blank in duplicate), and offers the possibility of a unit test..

### ASSAY PRINCIPLE:

The diluted assayed plasma sample is introduced into one of the microwells of the coated plate, and supplemented with a platelet lysate. When present, heparin-dependent antibodies, of the IgG or IgM or 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 3 goat polyclonal antibodies anti-human IgG (Fcγ specific)- IgM (μ specific)- IgA (α specific)-peroxidase (HRP) conjugate. This immunoconjugate mixture reacts specifically and homogeneously with IgG, IgM and IgA isotypes. Following a new washing step, the peroxidase substrate, Tetramethylbenzidine (TMB) in presence of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), 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 IgG, IgM or IgA isotype, present in the tested sample.

### TESTED SAMPLES:

– Trisodium citrate anticoagulated human plasma.

### REAGENTS:

- COAT:** 4 strips of 8 wells (Micro ELISA), coated with unfractionated heparin, biologically available, saturated, then stabilized; each strip is individually packed in an aluminium pouch hermetically sealed in presence of a desiccant.
- SD:** 2 vials containing 12 ml of HIA Sample Diluent, ready to use. Contains Sodium Azide
- C+:** 4 vials of HIA Positive control, lyophilised. When restored with 0.5 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.
- C-:** 4 vials of negative control, lyophilised (diluted normal human plasma). When restored with 0.5 ml of HIA Sample Diluent, the ready to use negative control is obtained (already diluted 1:100).
- CLY:** 4 vials of cell lysate, lyophilised (diluted normal human plasma). When restored with 0.5ml of distilled water, the ready to use solution is obtained.
- IC:** 4 vials of immunoconjugate (Anti-IgG (Fcγ)- IgM (μ)- IgA(α)-HRP immunoconjugate), goat antibodies specific for human IgG (Fcγ), IgM (μ), IgA(α)- coupled to HRP, lyophilised. When restored with 2ml of Conjugate Diluent (CD), the ready to use immunoconjugate is obtained.
- CD:** 1 vial of 10 ml of conjugate diluent, ready to use.
- WS:** 2 vials of 12 ml of 20 fold concentrated Wash Solution.
- TMB:** 1 vial of 10 ml peroxidase substrate: 3,3',5,5' – Tetramethylbenzidine containing hydrogen peroxide, ready to use.
- SA:** 1 vial of 3 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 µl.
- 1-channel pipettes at variable volumes from 0 to 20 µl, 20 to 200 µl and 200 to 1000 µl.
- 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.

- Micro ELISA strip:** open the aluminium pouch and take off the strip for the test series. When out of the pouch, the strip must be used within 30 minutes.
- 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.

- HIA Positive Control:** restore each vial with 0.5 ml HIA sample diluent in order to obtain the ready to use positive control. It corresponds to a plasma containing IgG-IgM-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.
- Negative control:** restore each vial with 0.5 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.
- CLY:** restore each vial with 0.5 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.
- Anti-IgG (Fcγ)-IgM (μ)- IgA(α)-HRP immunoconjugate:** each vial must be restored with 2 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.
- 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.
- 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 12 ml contained in the vial allow to prepare 240 ml 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.
- 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.
- Stop solution:** It is ready to use.

**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.

**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.

### 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.

#### Tested plasma or sample or control:

Plasma 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).

#### Assay procedure:

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



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Reagent	Volume	Procedure
<b>CLy</b>	<b>50µl</b>	Introduce the <b>CLy</b> into the micro ELISA strip wells (a)
<b>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>Positive control</b> or – <b>negative control</b> or – <b>diluted sample</b> or – <b>sample diluent</b> into the micro ELISA strip wells (a)
<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 (c).
<b>Conjugate</b> (anti-IgG (Fc $\gamma$ )-IgM (Fc $\mu$ ), IgA(Fc $\alpha$ )-HRP immunoconjugate, restored with 2 ml of conjugate diluent)	<b>200 µl</b>	Immediately after the washing, introduce the immunoconjugate in the micro ELISA strip wells. (c)
<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 (c).
<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, well by well, must be accurate and at exact time intervals (c,d)
Let the colour develop for exactly <b>5 min.</b> at room temperature (18-25 °C) (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> (c,d)
Wait for <b>10 minutes</b> in order to allow the colour to stabilize and measure absorbance at <b>450 nm (A450)</b> (e). 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 strip 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 strip wells 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 wells 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 well 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:

$$P = A450 \text{ for positive control} \geq 1.0$$

$$N = A450 \text{ for negative control} \leq 0.30$$

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.

#### INTERPRETATION OF RESULTS:

When the assay is run at 20±1°C, the results are as follows:

$$\text{Positive A450} > 0.50 \quad ; \quad \text{Negative A450} \leq 0.50$$

**Note:** When the room temperature is out of the recommended range, absorbance values can be affected. The positive control can then be used for adjusting the cut-off value. The flyer provided in the kit indicates the A450 value obtained for the positive control of the ZYMUTEST HIA lot used, and the value in % of this A450 corresponding to the cut-off. The adjusted cut-off value is then the corresponding % of the absorbance measured for the positive control in your series of measurements.

#### 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. / As for any autoantibody assay, clinical situation such as presence of inflammation, infectious diseases, auto-immune diseases, immuno-complexes, can induce a high background, which can be within the grey zone or in the weak positive range. Check then for the possible presence of antibodies on another specimen collected later. / Erroneous results can occur from bacterial contamination of test materials, inadequate incubation periods, inadequate washing or decanting of test wells, exposure of substrate to stray light, omission of test reagents, exposure to higher or lower than prescribed temperature requirements or omission of steps. / The results of this assay should not be used as the sole basis for a clinical decision. / Although a positive reaction obtained using this assay may indicate the presence of a heparin-associated antibody, the detection of such antibodies, however, DOES NOT CONFIRM the diagnosis of heparin-induced thrombocytopenia (HIT). Some patients may have naturally occurring antibodies to PF4 or other chemokines.

#### PATHOLOGICAL VARIATIONS:

Heparin dependent antibodies are immunoglobulins present in plasma of patients with suspicion of Heparin-Induced Thrombocytopenia (HIT) type II. Type II HIT, the immunological type, occurs during heparin treatment [1-2] and remains a major complication of this therapy. It is caused by the development of antibodies to Heparin-Protein (usually Platelet Factor 4) macromolecular complexes [3-4]. In addition to antibodies to PF4-Heparin, antibodies to other chemokines such as Neutrophil-Activating Peptide or NAP2 and Interleukin-8 or IL8 have also been evidenced in some patients [5]. Development of pathology is mainly associated with heparin-dependent antibodies of the IgG isotype. However, when the test is used for assessing the risk of developing a clinical complication of HIT, the assay of the global IgG isotypes is useful as a prognostic factor for this complication. When HIT occurs first, inflammation and/or platelet activation mechanisms, associated with various medical or surgical contexts, develop and lead to an increased release of chemokines and then promote formation of heparin complexes with chemokines (usually PF4). These multimolecular complexes can become antigenic and induce the generation of heparin-dependent antibodies. Heterogeneity of these antibodies could partly explain some discrepancies between the clinical suspicion of HIT and biological tests [6]. Frequently, heparin dependent antibodies can be asymptomatic, especially when they are of the IgM isotype. The clinical association is higher with elevated antibody concentrations and with the IgG isotype.

#### RELATED ASSAYS:

Heparin-dependent antibodies of the IgG isotype are better associated with the clinical diagnosis of HIT. The ZYMUTEST HIA IgG assay (# ARK040A/ARK041A MonoStrip) offers then a better specificity for the clinical complication of HIT, but it has less sensitivity as cases associated with only IgM and/or IgA isotypes are missed.

#### COMPLEMENTARY CHARACTERIZATION OF POSITIVE SAMPLES (IF REQUIRED):

If required, positive samples can be further characterized by their binding inhibition in presence of heparin. For this confirmation, to 500µl of the 1:100 diluted tested specimen, 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 inhibited (decrease in absorbance more than 50%) in almost all the cases. This inhibition confirms the heparin dependent binding of antibodies. In very rare specimen, already positive in the absence of platelet lysate, this inhibition is not observed, and the assay remains positive without or with heparin in the diluent: the result (which remains unclear from the present knowledge) must then be considered as inconclusive, and interpreted along with other assays or criteria for the diagnosis of HIT

#### ASSAY SPECIFICITY AND CHARACTERISTICS:

The ZYMUTEST HIA (MonoStrip) IgGAM Kit, is a screening assay for use in unit testing that measures globally human heparin-dependent antibodies of the IgG, IgM or IgA isotypes. These isotypes can be specifically assayed with ZYMUTEST HIA IgG assay (#ARK040A/ARK041A). 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, by identifying all 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.

**INTERFERENCE:** No interference of Heparin up to 1 IU/ml.

#### PERFORMANCE EVALUATION:

- In house study: Zymutest IgGAM versus Asserachrom for n=44 plasma samples.

		Asserachrom	
		Positive	Negative
Zymutest IgGAM	Positive	28	2
	Negative	0	14
	Agreement	100%	88%

- Two site external study: Zymutest IgGAM versus Asserachrom for n=243 plasma samples.

		Asserachrom	
		Positive	Negative
Zymutest IgGAM	Positive	48	32
	Negative	27	136
Agreement		76%	
Co-positivity		64%	
Co-negativity		81%	
Sample Size		243	

- Three site external study: Zymutest IgGAM versus GTI PF4 Enhanced for n=345 plasma samples.

Combined Sites 1,2,3		GTI PF4-Enhanced	
		Positive	Negative
Zymutest IgGAM	Positive	101	17
	Negative	74	153
Agreement		73,62%	
Co-positivity		57,71%	
Co-negativity		90,00%	
Sample Size		345	

- Example of reproducibility data:

Sample:	N	Intra assay		Inter assay		
		A450	CV%	N	A450	CV%
IgGAM Positive control	9	1.74	4.75	7	1.84	7.50

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