For in vitro diagnostic use only

INTENDED USE:
The ZYMUTEST HIA IgG, IgA, IgM ELISA kit is a standardised and optimised enzyme immunoassay designed for specifically measuring heparin-dependent antibodies of IgG, or IgM, or IgA isotype, in human plasma or serum, or in any biological fluid where these antibodies must be measured.

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 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 specific immunoenzyme reactant, which is made of goat polyclonal antibodies anti-human IgG (Fcγ specific) or anti-human IgM (μ specific) or anti-human IgA (α specific)-peroxidase (HRP) conjugate. Each immunoenzyme reactant reacts specifically with IgG, or IgM, or IgA isotypes. Following a new washing step, the peroxidase substrate, Tetramethylbenzidine (TMB) in presence of hydrogen peroxide (H₂O₂), is introduced and a blue colour develops. The colour turns yellow when the reaction is stopped with sulphuric acid. The colour developed is directly proportional to the amount of heparin-dependent antibodies of the IgG or IgM or IgA isotype, present in the tested sample.

TESTED SAMPLES:
- Tryptidic citrate or Na EDTA anticoagulated human plasma or human serum.
- Any biological fluid, where human heparin-dependent antibodies, of the IgG or IgM or 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. CD: 1 vial of HIA Positive control (IgG), 1 vial of HIA Positive control (IgM), and 1 vial of HIA Positive control (IgA).
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 specific positive 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: 1 vial of specific immunoenzyme reactant (Anti-IgG (Fcγ)-HRP immunoconjugate), 1 vial of specific immunoenzyme reactant (Anti-IgM (μ)-HRP immunoconjugate), and 1 vial of specific immunoenzyme reactant (Anti-IgA (α)-HRP immunoconjugate), goat antibodies specific for human IgG (Fcγ), or IgM (μ), or IgA(α)- coupled to HRP, lyophilised. When restored with 7.5 ml of Conjugate Diluent (CD), the ready to use immunoenzyme reactant 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 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 50-300μl.
- 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.

1. Micro ELISA plate: open the plastic pouch and take off the required amount of 8 wells 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.

3. HIA Positive Control IgG or IgM or IgA: restore each vial with 1 ml HIA sample diluent in order to obtain each ready to use specific positive control. They correspond to a plasma containing IgG or IgM or IgA isotype heparin-dependent antibodies, already diluted 1:100. Following reconstitution, each specific 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.

6. Anti-IgG (Fcγ)- or Anti-IgM (μ)- or Anti-IgA (α)-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. Each specific 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.

Caution:
- 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:
The kit allows running 32 tests for each specific isotype.

PROCEDURE:
Sample collection:
Blood plasma (8 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 just before use. Thawed specimen must be tested within 8 weeks.

Warning: The kit for the assay of heparin-dependent antibodies of IgG, or IgM, or IgA isotype, is designed for specifically measuring heparin-dependent antibodies of IgG, or IgM, or IgA isotype, in human plasma or serum, or in any biological fluid where these antibodies must be measured.
**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:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLy</td>
<td>50µl</td>
<td>Introduce the CLy into the micro ELISA plate wells (a)</td>
</tr>
<tr>
<td>Positive control IgG or IgA or IgM or IgG or IgM (Fc&lt;sub&gt;d&lt;/sub&gt;) (anti-e)</td>
<td>200 µl</td>
<td>Introduce the – Positive control or negative control – diluted sample or sample diluent (blank) into the micro ELISA plate wells (a)</td>
</tr>
</tbody>
</table>

**Incubate for 60 minutes at room temperature (18-25 °C) (b):**

<table>
<thead>
<tr>
<th>Wash Solution (20 fold diluted in distilled water)</th>
<th>300 µl</th>
<th>Proceed to 5 successive washings using the washing instrument (c).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjugate (anti-IgG (Fc&lt;sub&gt;d&lt;/sub&gt;) or anti-IgM (Fo&lt;sub&gt;d&lt;/sub&gt;) or anti-IgA/Fo&lt;sub&gt;d&lt;/sub&gt; )&lt;sub&gt;HRP&lt;/sub&gt; immunocoujugate, reconstituted with 7.5 ml of conjugate diluent)</td>
<td>200 µl</td>
<td>Immediately after the washing, introduce the specific immunocoujugate in the micro ELISA plate wells. (c)</td>
</tr>
</tbody>
</table>

**Incubate for 60 minutes at room temperature (18-25 °C) (b):**

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<th>Wash Solution (20 fold diluted in distilled water)</th>
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<tbody>
<tr>
<td>TMB&lt;sub&gt;4&lt;/sub&gt;H&lt;sub&gt;2&lt;/sub&gt;O Substrate</td>
<td>200 µl</td>
<td>Immediately after the washing, introduce the substrate into the wells. (c) The substrate distribution, row by row, must be accurate and at exact time intervals (c.d)</td>
</tr>
</tbody>
</table>

Let the colour develop for exactly 5 min. at room temperature (18-25 °C) (b)

0.45M Sulfuric Acid | 50 µl | Following exactly the same time intervals than for the addition of substrate, stop the colour development by introducing the 0.45M Sulfuric Acid (c.d) |

Wait for 10 minutes in order to allow the colour to stabilize and measure absorbance at 450 nm (A<sub>450</sub>) (e). Subtract the blank value.

**QUALITY CONTROL:**

- Controls provided in the kit allow validating the right performance of the assay.
- Expected A<sub>450</sub> values for each specific positive control, 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:

  \[ P = A_{450} \text{ for positive control } \geq 1.0 \]
  \[ N = A_{450} \text{ for negative control } \leq 0.25 \]

Obtained values for P and N, at 20±1°C, are indicated on the flyer provided in the kit. Obtained A<sub>450</sub> can vary according to the effective temperature during the assay run.

**EXPRESSION OF RESULTS:**

- For each specific isotype, results are expressed according to the A<sub>450</sub> 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 for each specific isotype are as follows:

- Positive A<sub>450</sub> > 0.50
- Weakly Positive A<sub>450</sub> 0.30 to 0.50
- Negative A<sub>450</sub> ≤ 0.30

Note: When the room temperature is out of the recommended range, absorbance values can be affected. Each specific positive control can then be used for adjusting the cut-off value. The flyer provided in the kit indicates the A<sub>450</sub> value obtained for each positive control of the ZYMUTEST HIA kit lot used, and the value in % of this A<sub>450</sub> corresponding to each cut-off. The adjusted cut-off value is then the corresponding % of absorbance measured for each positive control in your series of measures.

**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, immunocomplexes, can induce a high background, which can be within the grey scale in the weak positive range. Check then for the possible presence of antibodies on another specimen collected later.

**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 immunoglobulin 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 IgGAM 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.

**APPLICATIONS:**

- Complete isolopy of Heparin dependent antibodies, as a primary approach in research applications or as a second indication assay for characterizing patients measured positive for the ZYMUTEST HIA IgGAM, screening assay, (# RK040D).
- The various isotypes can be specifically measured with the ZYMUTEST HIA IgG, IgM, IgA kit (# RK040E), which allows a full isolation of heparin dependent antibodies. This assay is of special relevance for all research or prospective studies on development of HIT during heparin therapy.

**RELATED ASSAYS:**

- **ZYMUTEST HIA IgG Kit (RK040D),** is a global screening assay that measures globally human heparin-dependent antibodies of the IgG, IgM or IgA isotypes, for the following applications:
  - Assessment of the risk to develop HIT, in patients treated with heparins (Unfractionated or LMWH): presence of antibodies is a risk indicator for development of HIT.
  - Clinical suspicion of HIT during heparin therapy (skin necrosis, drop of platelet count to <100.10<sup>9</sup> G/L or decrease > 30% on two successive counts:...). Other possible causes of thrombocytopenia should be investigated and ruled out. In presence of thrombocytopenia, a positive ZYMUTEST HIA IgGAM test allows confirming heparin therapy as allergen.
- **Heparin-dependent antibodies of the IgG isotype** are better associated with the clinical diagnosis of HIT. The ZYMUTEST HIA IgG assay (# RK040A) 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.

**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 50µl of the 1:10 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 concentration) is then added to the well containing the antibody and the ZYMUTEST HIA IgGAM test is performed. If the antibody is inhibited in presence of heparin, the sample is considered as positive.

**ASSAY SPECIFICITY AND CHARACTERISTICS:**

The various isotypes can be specifically measured with the ZYMUTEST HIA IgG, IgM, IgA kit (# RK040E), which allows a full isolation of heparin dependent antibodies.

This optimised assay is designed with biologically available and immobilized heparin, then stabilized in inhibited. This inhibition confirms the heparin dependent binding of antibodies.

**REFERENCES:**