ZYMUTEST HIA MonoStrip IgG

Ref RK041A (32 tests)

IVD

Qualitative assay for the detection of heparin-dependent antibodies of the IgG isotype by ELISA

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English, last revision: 02-2018

INTENDED USE:

The ZYMUTEST HIA MonoStrip, IgG ELISA kit, is a qualitative assay intended for the detection of heparin-dependent antibodies of the IgG isotype, 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.

SUMMARY AND EXPLANATION:

This assay for use in unit testing 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 IgG 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.

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 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 IgG (Fcγ specific)-peroxidase (HRP) conjugate. This immunoconjugate reacts specifically with IgG 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 IgG isotype, present in the tested sample.

REAGENTS:

1. **COAT: Micro ELISA plate,** containing 4 strips of 8 wells, coated with unfractionated heparin, biologically available, saturated, then stabilized; each strip is packed individually in an aluminium pouch hermetically sealed in presence of a desiccant.
2. **SD:** 2 vials containing 12 mL of **HIA Sample Diluent**, ready to use. Contains Sodium Azide
3. **C+:** 4 vials of **HIA IgG 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 (OD_{450nm}) is indicated on the flyer provided with the kit.
4. **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).
5. **CLy:** 4 vials of **cell lysate** lyophilised containing diluted normal human plasma. When restored with **0.5 mL** of **distilled water**, the ready to use solution is obtained.
6. **IC:** 4 vials of **immunoconjugate (Anti-IgG (Fcγ)-HRP immunoconjugate)**, goat polyclonal antibodies specific to the Fcγ coupled to HRP of human IgG, and lyophilised. When restored with 2 mL of Conjugate Diluent (**CD**), the ready to use immunoconjugate is obtained.
7. **CD:** 1 vial of 10 mL of **conjugate diluent**, ready to use.
8. **WS:** 2 vials of 12 mL of **Wash Solution**, 20 fold concentrated.
9. **TMB:** 1 vial of 10 mL peroxidase substrate: **3,3',5,5' – Tetramethylbenzidine** containing hydrogen peroxide, ready to use.
10. **SA:** 1 vial of 3 mL of **0.45M Sulfuric Acid (Stop Solution)**, ready to use.

Reagent SD contains low concentration of Sodium azide (0.9 g/L) and reagent SA contains sulfuric acid, see CAUTIONS AND WARNINGS

CAUTIONS AND WARNINGS:

- Any product of biological origin must then be handled carefully, as being potentially infectious.
- Sodium azide may react with lead and copper plumbing to form highly explosive metal azides.
- The disposal of waste materials must be carried out according to current local regulations
- Use only reagents from kits with the same lot number. Do not mix reagents from kits with different lots when running the assay; they are optimized for each lot of kits.
- Reagents must be handled with care, in order to avoid any contamination during use. Take care to limit as much as possible any evaporation of the reagents during use, by limiting the liquid-air surface exchange.
- In order to preserve the stability of the reagents, close the vials with their original screw cap following each use.
- Stability studies for 3 weeks at 30°C show that the reagents can be shipped at room temperature for a short period without damage.
- For in vitro diagnostic use.
- Sulfuric acid, although diluted to 0.45M is caustic. As for any similar chemical, handle Sulfuric acid with great care. Wear protection glasses and gloves when handling. Avoid any skin and eye contact.

PREPARATION AND STABILITY OF REAGENTS:

Bring the kit at room temperature, at least 30 min before use. Store the unused reagents at 2-8°C. Vials are closed under vacuum. Remove carefully the stopper of lyophilized reagents, in order to avoid any loss of powder when opening the vials.
When appropriately used and stored, according to the recommended protocol and cautions, the kit can be used over a two month period.

1. **COAT (Micro ELISA plate):** Open the aluminium pouch and take off the strip for the test series. When out of the pouch, the strips must be used within 30 minutes.
2. **SD (HIA Sample Diluent):** Ready to use. This reagent contains sodium azide. Stability of reagent, provided that any contamination or evaporation is avoided, kept in its original vial is:
   - **8 weeks** at 2-8°C
3. **C+ (HIA IgG Positive Control):** Reconstitute each vial with **0.5 mL** of **"HIA Sample Diluent"**, shake thoroughly for complete dissolution. The obtained control is ready to use and it corresponds to a plasma containing IgG isotype heparin dependant antibodies, already **diluted 1:100**.
   Stability of reconstituted reagent, provided that any contamination or evaporation is avoided, kept in its original vial is:
   - **2 weeks** at 2-8°C.
   - **2 months** frozen at -20°C or below.
4. **C- (Negative Control):** Reconstitute each vial with **0.5 mL** of **"HIA Sample Diluent"**, shake thoroughly for complete dissolution. The obtained negative control is ready to use and it corresponds to a normal human plasma, already **diluted 1:100**.
   Stability of reconstituted reagent, provided that any contamination or evaporation is avoided, kept in its original vial:
   - **2 weeks** at 2-8°C.
   - **2 months** frozen at -20°C or below.
5. **CLy (Cell lysate):** Reconstitute each vial with **0.5 mL** of distilled water, shake thoroughly for complete dissolution. The obtained reagent is ready to use.
   Stability of reconstituted reagent, provided that any contamination or evaporation is avoided, kept in its original vial is:
   - **2 weeks** at 2-8°C
   - **2 months** frozen at -20°C or below.
6. **IC (Anti-IgG(Fcγ)-HRP immunoconjugate):** Reconstitute each vial with **2 mL of Conjugate Diluent** at least 15 min before use. Let the pellet to be completely dissolved before use, and shake the vial gently in order to homogenize the content.
   Stability of reconstituted reagent, provided that any contamination or evaporation is avoided, kept in its original vial:
   - **4 weeks** at 2-8°C.
   - **24 hours** at room temperature (18-25°C).
   - **2 months** frozen at -20°C or below.
7. **CD (Conjugate Diluent):** Ready to use. This reagent contains 0.05% Kathon CG. Stability of reagent, provided that any contamination or evaporation is avoided, kept in its original vial:
   - **8 weeks** at 2-8°C
8. **WS (Wash Solution):** Incubate, if necessary, the vial in a water bath, at **37°C**, until complete dissolution of crystals. 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).
   Stability of the wash solution, provided that any contamination or evaporation is avoided, kept in its original vial:
   - **8 weeks** at 2-8°C

   Stability of the dilute wash solution, provided that any contamination or evaporation is avoided, kept in its original vial:
   - **When open, 7 days** at 2-8°C

   This reagent contains 0.05% Kathon CG.
9. **TMB:** Ready to use. Stability of reagent, provided that any contamination or evaporation is avoided, kept in its original vial:
   - **8 weeks** at 2-8°C
10. **SA (Stop Solution):** Stop solution containing 0.45M sulfuric acid, ready to use. See CAUTIONS AND WARNINGS

STORAGE CONDITIONS:

Unopened reagents must be stored at 2-8°C, in their original packaging box. They are usable until the expiration date printed on the kit.

REAGENTS AND MATERIAL REQUIRED BUT NOT PROVIDED:

Reagents:

- Distilled water.

Materials:

- **8-channel** or **repeating pipette** allowing dispensing volumes of 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.

SPECIMEN COLLECTION:

Preparation and storage of specimens must be performed according to the current local regulations.

- Specimens:

Human plasma obtained from trisodium citrate anticoagulated blood.

- Collection:

Blood (9 vol.) must be collected on trisodium citrate anticoagulant (1 vol.) (0.109M), with caution, through a net venipuncture. The first tube must be discarded.

- Centrifugation:

Within 2 hours, use a validated method in the laboratory to obtain a platelet-poor plasma, e.g., a minimum of 15 minutes at 2500 g at room temperature (18-25°C) and plasma must be decanted into a plastic tube.

- Storage of plasma:
  - 24 hours at room temperature (18-25°C).
  - 6 months at -20°C.

Frozen plasma specimens should be rapidly thawed at 37°C, then gently mixed and tested within 72 hours. Resuspend any precipitation by thorough mixing immediately after thawing and before testing.

TEST PROCEDURE:

Assay procedure:

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

2. The samples should be diluted using SD solution as described in the table below:

Samples	Dilution
Plasma	1:100

When high amounts of heparin dependent antibodies are expected, dilute at **1:200** or **1:400** dilutions. Results must then be multiplied by **2** or **4**.

3. Remove the strip from the aluminium pouch and put the strip in the frame provided. In the different strip of the micro ELISA plate, introduce the reagents and perform the various assay steps as indicated on the following table:

Reagent	Volume	Procedure
CLy	**50µL**	Introduce the CLy into the micro ELISA strip wells
IgG Positive control or **Negative control** or **1:100 diluted sample** or **sample diluent** (blank)	**200 µL**	Introduce immediately the dilutions : – **IgG Positive control** or – **Negative control** or – **Diluted sample** or – **Sample diluent** into the micro ELISA strip wells **(a)**
Incubate for 60 minutes at room temperature (18-25°C) (b)		
Wash Solution (20 fold diluted in distilled water)	**300 µL**	Proceed to 5 successive washings **(c).**
Immunconjugate (anti-IgG (Fcγ)-HRP immunoconjugate, reconstituted with 2 ml of conjugate diluent)	**200 µL**	Immediately after the washing, introduce the **immunoconjugate** in the micro ELISA strip wells**(c)**
Incubate for 60 minutes at room temperature (18-25 °C) (b)		
Wash Solution (20 fold diluted in distilled water)	**300 µL**	Proceed to 5 successive washings **(c)**.
TMB/H_2O_2 Substrate	**200 µL**	Immediately after the washing, introduce the substrate into the wells. **Nota**: The substrate distribution, well by well, must be accurate **(c,d)**
Let the colour develop for exactly **5 min**. at room temperature (18-25 °C) **(b)**		
0.45M Sulfuric Acid (Stop Solution)	**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 450nm. Substract the blank value (e).		

Remarks:

a) 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.

b) 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.

c) 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.

d) 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.

e) For bichromatic readings, a reference wavelength at 690 nm or at 620 nm can be used.

VALIDATION:

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

P =OD_{450} for C+ 1:1: ≥ 1.0 **N = OD_{450} 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.

RESULTS:

- Results are expressed according to the A450 values, as positive or negative.
- When higher dilutions are used, the complementary dilution factor must be considered.

INTERPRETATION OF RESULTS:

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

Positive: **A450 > 0.50**
Weakly Positive: **0.30 < A450 ≤ 0.50**
Negative: **A450 ≤ 0.30**

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:

- In order to get the optimal performances of the assay, the technical instructions must be strictly respected.
- Any reagent presenting an unusual aspect or contamination signs must be rejected.
- Any plasma containing contamination signs must be rejected.
- If washing steps are not correctly performed, it can induce high background and a high absorbance value of the negative control. In order to avoid non-specific colour development, check that the washing step is efficiently and correctly performed.
- As for any auto-antibody assay, the presence of inflammation, infectious diseases, circulating immune-complexes, gammopathy, auto-immune diseases can induce an low unspecific reactivity in the grey zone or weakly positive. Check for the possible presence of antibodies on a new specimen.
- 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 anti-PF4 or anti-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 immunoallergic 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:

- Clinical suspicion of HIT during a heparin treatment (skin necrosis, falling of platelet count < $100,10^9$ G/L or decrease more than 30% between successive counts ...). Other possible causes of thrombocytopenia should be sought and excluded. In the presence of thrombocytopenia, a positive test can confirm the diagnosis.
- The dependent IgG isotype heparin antibodies are associated with better clinical diagnosis of HIT. The ZYMUTEST HIA IgG (#RK040A) kit offers better specificity of clinical complication of HIT, but less sensitivity as cases associated with only IgM isotype and / or IgA are not detected.

RELATED ASSAYS:

The various isotypes can be detected globally, using the ZYMUTEST HIA IgGAM screening assay kit (#RK040D), for assessments of the risk to develop HIT, in patients treated with heparins: presence of antibodies is a risk indicator for development of HIT.

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 (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 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.

PERFORMANCES:

- No interference of Heparin up to 1 IU/mL.
- External study: Zymutest HIA IgG versus Serotonin Release Assay (SRA) for n=174 samples. Matches indicate that both were positive or both were negative.

Matches	131
% Matching	75.29

- Two-site external study: Zymutest HIA IgG versus Asserachrom® HPIA for n=243 samples:

		Asserachrom® HPIA	
		Positive	Negative
Zymutest HIA IgG	Positive	33	17
	Negative	42	151
Agreement		76%	
Co-positivity		44%	
Co-negativity		90%	
Sample Size		243	

- Example of reproducibility data:

	Intra assay			Inter assay		
Sample:	N	A450	CV%	N	A450	CV%
HIA IgG Positive control	6	1.31	3.07	7	1.34	7.11

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SYMBOLS:

Used symbols and signs listed in the ISO standard 15223-1.

Changes compared to the previous version.