



**Heparin Dependent Antibodies
Specific Isotyping Assay
IgG**

Clinical Significance: To measure heparin-dependent antibodies of IgG in human plasma or serum.

Principle: Heparin dependent antibodies are immunoglobulins present in plasma of patients with suspicion of heparin induced thrombocytopenia (HIT) type II. This immunoallergic type occurs during heparin treatment and may be a major complication of this therapy. It is caused by the development of antibodies to Platelet Factor 4 complexes and is mainly associated with antibodies of the IgG type. This assay is designed with biologically available and immobilized heparin, which is then stabilized and saturated allowing a reaction with heparin binding proteins and antibodies.

A diluted plasma sample is added to a microwell of an unfractionated heparin coated plate in the presence of platelet lysate. If heparin dependent antibodies of an IgG isotype are present, they will bind to biologically active unfractionated heparin. These bound antibodies are demonstrated with a specific immunoconjugate. IgG conjugate contains anti-human polyclonal goat antibodies which are Fcγ specific. The plate is washed with Tetramethylbenzidine (TMB) a peroxidase substrate in the presence of hydrogen peroxide resulting in a blue color development. This reaction is stopped with sulfuric acid. A yellow color is developed which is directly proportional to the amount of heparin-dependent antibodies of the IgG type.

INSTRUMENT/

Methodology: ELISA

MIN. VOLUME: 100ul

LIS CODE:

SPECIMEN:

1. Trisodium Citrate, EDTA anticoagulant, Serum
2. Sample is spun for 20 minutes at 2,500 g
3. Tested within 24 hours or stored frozen at -20 degrees C for up to 6 months.
4. Thaw sample for 15 minutes at 37 degrees C prior to use.
5. Standard precautions must be adhered to.

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REAGENTS AND EQUIPMENT:

EQUIPMENT:

1. 8 - channel pipette: Dispensing 50-300ul
2. Adjustable pipettes: 0-20ul, 20-200ul, 220-1000ul.
3. Micro Elisa plate shaker
4. Micro Elisa plate reader – wavelength set at 450nm.
5. Distilled water
6. Sample reservoirs

REAGENTS:

Bring the kit to room temperature for 30 minutes prior to use.

1. **Zymutest HIA IgG kits: (#ARK040E)** in original packing box, when stored at 2-8 degrees C, unopened reagents are stable until the expiration date printed on the box.
2. **Micro ELISA plate:** 12 strips of 8 wells coated with unfractionated heparin. When out of the pouch, strips must be used within 30 minutes. Unused strips can be stored at 2-8 degrees C for 4 weeks in the original pouch in the presence of the desiccant.
3. **HIA Sample diluent: READY TO USE.** Open vial stable for 4 weeks at 2-8 degrees C, protect from any possible bacterial contamination. **CAUTION: This diluent contains sodium azide which may react with copper plumbing to form highly explosive metal azides. Flush with copious amount of water when discarding.**
4. **Positive Control:** Lyophilized IgG heparin-dependent antibodies. Reconstitute with 1ml of HIA sample diluent. **NO FURTHER DILUTION IS REQUIRED.** (Sample already diluted at 1:100) Stable 2 weeks at 2-8 degrees C, protect from any possible Bacterial contamination.
5. **Negative Control:** Normal Human plasma. Reconstitute with 1ml of HIA sample diluent. **NO FURTHER DILUTION IS REQUIRED.** (Sample already diluted at 1:100) Stable 2 weeks at 2-8 degrees C, protect from any possible bacterial contamination.
6. **Lyophilized Platelet Lysate:** lyophilized, extracted from fresh human platelet concentrates. **Reconstitute with 2ml of DISTILLED WATER.**
7. **Conjugate Diluent:** 0.05% Kathon CG, 25mls, **ready to use.** Stable for 4 weeks at 2-8 degrees C, protect from any possible bacterial contamination
8. **Anti-IgG Fcy- HRP immunoconjugate:** **Reconstitute with 7.5ml of conjugate diluent.** Pellet must be completely dissolved prior to use, swirl to mix completely. Stable 24 hours at room temperature, or 4 weeks stored at 2-8 degrees C.
9. **Wash Solution:** 0.05 % CG, 50 mls. Incubate 15-30 minutes at 37 degrees C to dissolve any crystals present. Solution is 20 fold concentrate and must be **diluted 1:20 in distilled water.**

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- (50 mls QS to 1 liter with distilled water). Store at 2-8 degrees C and use within 4 weeks. Diluted wash solution must be used with 7 days.
10. **Tetramethylbenzidine**:(TMB) 25mls of a peroxidase substrate, **ready to use**. Store at 2-8 degrees C and use within 4 weeks.
 11. **Stop Solution**: 0.45M Sulfuric Acid, **ready to use**.
Caution: Although diluted, sulfuric acid is caustic. Handle with care. Wear protective gloves and glasses. Avoid any skin or eye contact.

QUALITY CONTROL

1. Run 2 levels of controls.
2. Controls should be run in the same manner as test samples
Positive control: OD value ≥ 1.0
Negative control: OD value ≤ 0.2

PROCEDURE:

1. Remove the required number of strips from the package needed to perform testing. Samples must be run in duplicate if performed manually.
2. Dilute patient samples at 1:100 in Sample Diluent (10ul of sample to 990ul of diluent.) Add 1 well of sample diluent as a blank. Control samples are already diluted 1:100, and are ready to use.
3. Add 200ul of controls, test samples and blank into the ELISA plate wells.
4. With a multichannel pipette, add 50ul of platelet lysate to each well.
5. Add controls and the 1:100 dilutions of patient samples. This should be completed within 10 minutes to obtain homogeneous kinetics for antibody binding.
6. Incubate for 60 minutes at room temperature (18-25 degrees C). A shaker may be used for the first 2 minutes of incubation. Try to maintain consistent temperature and minimal light exposure.
7. Empty the well, and with a multichannel pipette add 300ul of the diluted wash solution. This must be done 5 times and performed gently or the plates reactivity may be lowered. Washing should be done promptly so as to minimize amount of time the plate is left without reagents. It is better to let the wells contain wash solution than remaining dry. Never let a plate sit longer than 3 minutes.
8. Immediately add 200ul of the anti-IgG Conjugate to each well.
9. Incubate for 60 minutes at room temperature (18-25 degrees C).
10. Rewash the plate with 300ul of wash solution (Step 7)
11. Add 200ul of the TMB substrate. NOTE: The substrate distribution must be accurate and at exact time intervals.
12. Color should develop for exactly 5 minutes.
13. 50ul of Stopping Solution should be added at the exact same time intervals of the substrate.
14. Allow the plate to sit for 10 minutes for the color to stabilize.

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15. Measure the absorbance at 450nm making sure to subtract the value of the blank.

INTERPRETATION OF RESULTS:

When the assay is run at 20+/-1 degree C the results are as follows:

Positive = OD >0.500

Negative = OD <0.500

LIMITATIONS OF THE ASSAY:

1. If the washing step is not accurately performed, the negative control can produce high absorbance value.
2. Specimens must be added rapidly within 10 minutes, if too long of a delay occurs, it may induce an influence of immunological kinetics and produce wrong results.
3. The presence of inflammation, infectious disease, auto-immune diseases, immune-complexes can induce a high background which may produce a weak positive range. Another sample should be tested at a later date.

CONFIRMATION OF POSTIVE SAMPLES:

Positive samples may be confirmed by the inhibition of their binding in the presence of heparin. This may be performed simultaneously as an independent dilution initially, or later to confirm a positive result.

PROCEDURE:

1. To 500ul of the 1:100 diluted tested specimen, add 10ul of a 100IU/ml unfractionated heparin solution. (Spiked)
2. This sample is tested in the same manner.
3. If the result of an unspiked sample (no heparin) is positive and the spiked sample (2U/ml final concentration) is negative. This inhibition of heparin dependent binding antibodies is used as confirmation.

REFERENCES:

1. Gruel, Y., Thrombopenie induite par les heparines manifestations cliniques et physiopathologie. Presse Med 1998;27:S7-S12.
2. Warkentin, TE., Levine, MN., Hirsch, J., et al: Heparin induced thrombocytopenia in patient treated with low molecular weight heparin or unfractionated heparin, N Eng J Med 1995; 332:1330-1335.
3. Amiral J. Bridey, F., Wolf, M., et al, Platelet factor 4 complexed to heparin is the target for antibodies generated in heparin induced thrombocytopenia, Thrombo haemost, 1992;68:95-96.
4. Amiral J. Bridey, F., Wolf, M., et al, Antibodies to macromolecular platelet factor 4 heparin complexes in heparin induced thrombocytopenia, Thrombo haemost, 1995;73:21-28