ZYMUTEST ANTI β_2-GLYCOPROTEIN I IgG - Isotype

Ref: RK014A

CE IVD

Auto-antibodies to β_2-Glycoprotein I (β_2GPI), IgG isotype

HYPHEN BioMed

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INTENDED USE:

The ZYMUTEST anti-β_2GPI, IgG ELISA kit, is an optimized enzyme immuno-assay designed for measuring auto-antibodies to β_2GPI of the IgG isotype, in human plasma or serum or in any biological fluid where auto-antibodies to β_2GPI must be measured.

SUMMARY AND EXPLANATION:

The ZYMUTEST anti-β_2GPI, IgG Kit, specifically measures human auto and alloantibodies to β_2GPI of the IgG isotype, reactive with immobilized β_2GPI. IgM or IgA isotypes are not measured. This assay is designed with native uncleaved and non-altered, highly purified human β_2GPI, which has then a preserved structure. This method then provides high reproducibility, high sensitivity and high specificity.

ASSAY PRINCIPLE:

Search of anti-β_2GPI antibody, with ZYMUTEST anti-β_2GPI kit, is performed using an ELISA plate, sensitized by the native human -β_2GPI then stabilized.
The diluted plasma or serum sample or biological fluid is introduced into one of the microwells of the β_2GPI coated plate. When present, anti-β_2GPI auto-antibodies bind to immobilized β_2GPI. Following a washing step, bound auto-antibodies, of the IgG isotype, are revealed by an immunoconjugate, goat anti-human IgG (Fcγ specific)-peroxidase conjugate, which reacts specifically with IgG isotypes. Following a new washing step, the peroxidase substrate, 3,3',5,5' – 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 anti-β_2GPI auto-antibodies, of the IgG isotype, present in the tested sample.
Tested samples:

- Trisodium citrate or EDTA anticoagulated human plasma or human serum.
- Any biological fluid, where human auto-antibodies to β_2GPI, of the IgG isotype, must be assayed.

REAGENTS:

1. **COAT: Micro ELISA plate:** containing 12 strips of 8 wells, coated with highly purified human β_2GPI, then stabilized; the plate is packed in an aluminium pouch hermetically sealed in presence of a desiccant.
2. **SD: Autoimmunity Sample Diluent:** 2 vials containing 50 mL of Autoimmunity Sample Diluent, ready to use. Contains Sodium Azide.
3. **CAL: Anti-β_2GP1 IgG Calibrator:** 3 vials of calibrator, lyophilized. After reconstitution with 1 mL of Autoimmunity Sample Diluent, the calibrator is ready to use (already diluted 1:100).
4. **C-: Negative Control:** 3 vials of negative control, lyophilized containing diluted normal human plasma. After reconstitution with 1 mL of Autoimmunity Sample Diluent, the negative control is ready to use (already diluted 1:100).
5. **IC: Anti-IgG-Peroxidase:** 3 vials of immunoconjugate (Anti-IgG (Fcγ)-HRP immunoconjugate), goat polyclonal antibodies specific for human IgG-Fcγ coupled to HRP, lyophilized.
6. **CD: Conjugate Diluent:** 1 vial of 25 mL of immunoconjugate diluent, ready to use.
7. **WS: Wash Solution:** 1 vial of 50 mL of 20 fold concentrated Wash Solution**.**
8. **TMB: Tetramethylbenzidine:** 1 vial of 25 mL peroxidase substrate (3,3',5,5' – Tetramethylbenzidine) containing hydrogen peroxide, ready to use.
9. **SA: Stop Solution:** 1 vial of 6 mL of **0.45M Sulfuric acid**, ready to use.

The exact concentration of calibrator and the concentration's acceptance interval for control is indicated on the flyer provided in the kit. The anti-β_2GPI concentrations for the calibrators, expressed in Arbitrary Units (AU), vary from lot to lot. For the assay, refer to the concentration indicated on the flyer provided in the kit used.
Reagent 2 contains low concentration of Sodium azide (0.9 g/L), 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.
- If the substrate becomes yellow, this indicates the presence of a contaminant. It must be rejected, and a new vial must be used.
- 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. Evaporation reduces reagent stability on instrument board.
- 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, in order to avoid any loss of powder when opening the vials.

1. **COAT:** Open the aluminium pouch and take off the required amounts 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 4 weeks** in their original aluminium pouch, in presence of the desiccant, hermetically closed and protected from any moisture, and stored in the provided plastic microplate storage bag (minigrip).
2. **SD:** Ready to use. This reagent contains sodium azide. Stability of reagent, provided that any contamination or evaporation is avoided, kept in its original vial:
   - **4 weeks** at 2-8°C
3. **CAL:** Reconstitute each vial with **1 mL** of **Autoimmunity Sample Diluent**, shake thoroughly for complete dissolution. The obtained calibrator is ready to use calibrator. It corresponds to a plasma containing IgG isotype auto-antibodies to β_2GPI, already **diluted 1:100**.
   Stability of reconstituted reagent, provided that any contamination or evaporation is avoided, kept in its original vial:
   - **5 days** at 2-8°C
4. **C-:** Reconstitute each vial with **1 mL** of **Autoimmunity Sample Diluent**, shake thoroughly for complete dissolution. The obtained negative control is ready to use. 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
5. **IC:** Reconstitute each vial with **7.5 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)
6. **CD:** 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:
   - **4 weeks** at 2-8°C
7. **WS:** 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 50 mL contained in the vial allow to prepare 1 liter of Wash Solution).
   Stability of the wash solution, provided that any contamination or evaporation is avoided, kept in its original vial:
   - **4 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.
8. **TMB:** Ready to use. Stability of reagent, provided that any contamination or evaporation is avoided, kept in its original vial:
   - **4 weeks** at 2-8°C
9. **SA:** Stop solution containing 0.45M sulfuric acid, ready to use.

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 label.

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 (In the USA, refer to CLSI Document GP44-A4 for further instructions on specimen collection, handling and storage).

- Specimens:

Human plasma obtained from trisodium citrate anticoagulated blood. EDTA collected human plasma may also be used. The storage conditions are the same with citrated plasma. Auto-antibodies to β_2GPI can also be assayed on serum. However it is better to perform the assays on plasma.

- 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:
  - 8 hours at room temperature (18-25°C)
  - 48 hours at 2-8°C.
  - 1 month at -20°C.

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

TEST PROCEDURE:

Assay procedure:

1. Calibrator and negative control 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
Serum	1:100
Biological fluid	1/100

When high amounts of auto-antibodies to β_2GPI are expected, dilute at **1:200** or **1:400** dilutions. Results must then be multiplied by **2** or **4**.

3. Remove the required number of strips from the aluminium pouch and 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
Anti-β_2GPI IgG Calibrator or **Negative control** or **1:100 diluted sample** or **Sample diluent** (blank)	**200 µL**	Introduce the : – **Calibrator** or – **negative control** or – **diluted sample** or – **sample diluent** into the micro ELISA plate wells.
Incubate for 30 minutes at room temperature (18-25 °C) (a) (b)		
Wash Solution (20 fold diluted in distilled water)	**300 µL**	Proceed to 5 successive washings. **(b)**
Conjugate anti-IgG (Fcγ)-HRP immunoconjugate, restored with 7.5 mL of conjugate diluent	**200 µL**	Immediately after the washing, Introduce the anti-IgG (Fcγ)-HRP **immunoconjugate** in the micro ELISA plate wells.
Incubate for 30 minutes at room temperature (18-25 °C) (a)		
Wash Solution (20 fold diluted in distilled water)	**300 µL**	Proceed to 5 successive washings **(b)**
TMB/H_2O_2 Substrate	**200 µL**	Immediately after the washing, introduce the substrate into the wells. **Nota**: The substrate distribution, row by row, must be accurate and at exact time intervals **(c)**.
Let the colour develop for **5 min**. at room temperature (18-25 °C) **(a)**		
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)**
Wait for 10 minutes in order to allow the colour to stabilize and measure absorbance at 450 nm. Subtract the blank value (d).		

Note:

a) Avoid letting the plate in the bright sunlight during incubations and more particularly during colour development. A micro ELISA plate shaker can be used.

b) Never let the plates empty between the addition of the reagents or following the washing step to preserve the immobilized proteins. The next reagent must be added within 3 minutes, in order to prevent the plate from drying, which could damage the immobilized proteins and reduce plate reactivity. 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.

c) 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 by sulfuric acid.

d) For a bichromatic reading, the reference wavelength at 690 nm or at 620 nm can be used.

VALIDATION:

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

OD_{450} for 1:1 calibrator : ≥ 1.5 **OD_{450} for negative control : ≤ 0.25**

Concentrations obtained for calibrator and negative control, at 18-25°C, are indicated on the flyer provided in the kit for each reagent lot.

QUALITY CONTROL:

Using quality controls, allows validating the calibration curve, as well as the homogeneous reactivity from run to run, when using a same lot of reagents.

Quality control must be included in each series, as per good laboratory practice, in order to validate test results. A new calibration curve must be carried out preferentially for each test series, and at least for each new lot of reagents or, after each important analyzer's maintenance, or when quality controls values are measured outside the acceptance range determined for the method.

Each laboratory should establish and verify its own target values, acceptance ranges and expected performances, according to the instruments and protocols used.

RESULTS:

- Results are expressed according to the **OD_{450}** values obtained for samples and control using the calibration curve.
- For the manual method, draw the calibration curve on a bi-logarithmic graph paper plot, with on abscissae the anti-β_2GPI concentration (**AU)** and on ordinates the corresponding **OD_{450}**. The anti-β_2GPI, autoantibody concentration, of the IgG isotype, for the sample, tested at the standard **1:100** dilution, and expressed in **AU**, is directly deduced from the calibration curve.
- When higher dilutions are used, (**i.e. D**), the concentration measured must be multiplied by the complementary dilution factor (i.e. **D:100** ; for example **x2** for **1:200** or **x4** for **1:400**).
- Alternatively, specific software (i.e. Dynex, Biolise, etc…), can be used for the calculation of concentrations.

INTERPRETATION OF RESULTS:

A calibration curve is realized using a serial two-fold dilution. This ensures a higher reliability of the assay, and a higher accuracy and reproducibility from lot to lot, and run to run, for the cut-off.

Negative range: The calibrator expressed in Arbitrary Unit (**AU**), is defined respectively to the upper limit of the normal range, which corresponds to the mean value obtained in a normal population plus 2 standard deviations (SD). By definition, this corresponds to **10 AU**. Therefore, normal values are:

Negative range: < 10 AU/mL

Grey zone: A "grey zone" is defined because some pathological samples (inflammation, infectious diseases, autoimmune diseases, gammopathy, elderly people…) can produce higher backgrounds, in auto-immune assays, than the normal individuals although these subjects have not anti-β_2GPI antibodies. This can mimic or mask a low reactivity. When patients are in the grey zone, it is recommended to perform a new testing on another sample, later, in order to follow a possible ongoing generation of autoantibodies to β_2GPI of the IgG isotype.

Grey Zone: ≥ 10 AU/mL to < 20 AU/mL

Positive range: The positive range concerns the following anti-β_2GPI autoantibody concentrations:

Positive range: :≥ 20 Au/mL

The positive range can be classified as follows:

Low positive:	**≥ 20 to < 50 AU/mL**
Moderate positive:	**≥ 50 to < 100 Au/mL**
High positive:	**≥ 100 AU/mL**

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 a coagulum or 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.

PATHOLOGICAL VARIATIONS:

- Auto-antibodies to β_2GPI are usually absent in normal population.
- Their presence at moderate or high concentrations can be associated with recurrent abortions, miscarriages or with the anti-phospholipid syndrome (APS), sometimes associated with thrombotic diseases.
- The pathological effect of auto-antibodies to β2GPI is still discussed, but these latter are thought to contribute to trigger hypercoagulability. Pathogenicity of the various isotypes is still not completely understood. Severity of clinical complications associated with the presence of autoantibodies to β2GPI, increases with the IgG isotype, the antibody concentration and its affinity, and the time of exposure. IgG isotype is the most pathogenic.

APPLICATIONS:

Assay of auto-antibodies to β_2GPI of the IgG isotype, in the following clinical situations:

- Anti-phospholipid syndrome.
- Recurrent unexplained miscarriages.
- Unexplained lupus anticoagulant, without or with thrombosis.
- Any clinical situation where the assay of anti-β2GPI autoantibodies is required. This assay is usually associated to the assay of the IgM isotype autoantibodies.

PERFORMANCE:

- The lower limit of detection is ≤ 5 AU/mL.
- Inter assay: ≤ 10%
- Intra assay: ≤ 10%

REFERENCES:

1. CLSI Document GP44-A4: "Procedures for the handling and processing of blood specimens for common laboratory tests".
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4. Martinuzzo M.E., *et al.* Anti-β2-Glycoprotein I antibodies : detection and association with Thrombosis. Brit. J. haemat. 1995.
5. Sanmarco M. and Soler C. Heterogeneity of β2-Glycoprotein I antibodies. Nouv. Rev. Fr. Haemat. 1995.
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SYMBOLS:

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