CE ZYMUTEST Anti-β_2-GLYCOPROTEIN I IgG - Isotype
(# ARK014A)

ANIARA
Manufactured by Hyphen BioMed.

Auto-antibodies to β_2GlycoProtein I (β_2GPI), IgG isotype

For in vitro diagnostic use only

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INTENDED USE:

The ZYMUTEST anti-β_2GPI, IgG ELISA kit, is a standardised and optimised 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.

ASSAY PRINCIPLE:

The diluted plasma 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 immobilised β_2GPI. Following a washing step, bound auto-antibodies, of the IgG isotype, are revealed with a goat anti-human IgG (Fcγ specific)-peroxidase conjugate, which 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 anti-β_2GPI auto-antibodies, of the IgG isotype, present in the tested sample.

TESTED SAMPLES:

- Trisodium citrate or Na_2 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: 2 vials containing 50 ml of **Autoimmunity Sample Diluent**, ready to use. Contains Sodium Azide.
3. CAL: 3 vials of **anti-β_2GPI, IgG, calibrator**, lyophilised. When restored with **1 ml** of **Autoimmunity Sample Diluent**, the ready to use calibrator is obtained (already diluted 1:100).

Note: This calibrator has a defined anti-β_2GPI concentration, expressed in **Arbitrary Units (AU)** and indicated on the flyer provided with the kit.

4. C-: 3 vials of **negative control**, lyophilised (diluted normal human plasma). When restored with **1 ml** of **Autoimmunity Sample Diluent**, the ready to use negative control is obtained (already diluted 1:100).
5. IC: 3 vials of **immunoconjugate (Anti-IgG (Fcγ)-HRP immunoconjugate)**, affinity purified goat antibodies specific for human IgG-Fcγ coupled to HRP, lyophilised.
6. CD: 1 vial of 25 ml of **Conjugate Diluent**, ready to use.
7. WS: 1 vial of 50 ml of 20 fold concentrated **Wash Solution**.
8. TMB: 1 vial of 25 ml peroxidase substrate: 3,3',5,5' **– Tetramethylbenzidine (TMB)** containing hydrogen peroxide, ready to use.
9. SA: One vial of 6 ml of **0.45M Sulfuric acid (Stop Solution)**, ready to use.

Note: *Use only components from a same kit lot. Do not mix components from different lots of kits, 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.

1. **Micro ELISA plate:** open the plastic 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 microplate storage bag (minigrip).
2. **Autoimmunity Sample Diluent:** It is ready to use. When open, it can be used for **4 weeks**, stored **at 2-8°C**, and provided that any bacterial contamination is avoided during use. This reagent contains sodium azide.

Warning: The **Autoimmunity 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.

3. **Calibrator:** restore each vial with **1 ml** Autoimmunity Sample Diluent in order to obtain the ready to use calibrator. It corresponds to a plasma containing IgG isotype auto-antibodies to β_2GPI, already **diluted 1:100**. Following reconstitution, the calibrator is stable for **5 days at 2-8°C**, provided any bacterial contamination is avoided during use.
4. **Negative control:** restore each vial with **1 ml** Autoimmunity 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 any bacterial contamination is avoided during use.

Warning: β2GPI used for coating the plates is extracted from human plasma. Negative control is also prepared with human plasma. Any human plasma used is 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.

5. **Anti-IgG (Fcγ)-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. The restored conjugate is stable for at least **24 hours at room temperature** or for at least **4 weeks at 2-8°C**.
6. **Conjugate Diluent**: It is ready to use. When open, it can be used for **4 weeks**, stored at **2-8 °C**, and provided that any bacterial contamination is avoided during use. This reagent contains 0.05% Kathon CG.
7. **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 **4 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.
8. **TMB substrate:** It is ready to use. When open, it can be used for **4 weeks**, stored **at 2-8°C**, and provided that any bacterial contamination is avoided during use.
9. **Stop solution:** It is ready to use.

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 at 30°C show that the reagents can be shipped at room temperature without damage.

PROCEDURE:

Sample collection:

Blood plasma (9 vol.) must be collected on 0.109 M 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 **12 hours**. EDTA collected human plasma may also be used.
Auto-antibodies to β_2GPI can also be assayed on serum.

Tested plasma or sample or control:

Plasma or serum is tested at **1:100** dilution in Autoimmunity Sample Diluent. When high amounts of auto-antibodies to β_2GPI are expected, samples must be assayed at **1:200** or **1:400** dilutions. Results must then be multiplied by **2** or **4**.
Calibrator and negative control are ready to use (**already diluted 1:100**).

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Assay procedure:

Calibration curve: The assay can be calibrated with the calibrator provided in the kit, and which concentration **(C)** is indicated in arbitrary units, **(AU)** on the flyer provided. Prepare the standard solutions for calibration by doing a serial two-step dilution of the calibrator in Autoimmunity Sample Diluent, from **1:1** to **1:32**. A concentration range from **C:1** to **C:32** is obtained.

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:

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 using the washing instrument. **(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 using the washing instrument **(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 (A450) (d)**. Substract the blank value		

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

QUALITY CONTROL:

– Calibrator and controls provided in the kit allow validating the right performance of the assay.
– Expected A450 values for undiluted calibrator and the negative control can present variations from lot to lot but they always are:

P = A_{450} for 1:1 calibrator : ≥ 1.5 N = A_{450} for negative control : ≤ 0.25

In addition, concentrations obtained for negative control must be within the acceptance range indicated on the flyer provided in the kit. If negative control is out of this range check carefully the assay conditions and re-run the assay, if required.

EXPRESSION OF RESULTS:

– Results are expressed according to the **A_{450}** values obtained for samples, controls and using the calibration curve.
– The calibration curve is obtained by plotting the anti-β_2GPI concentrations expressed in **AU** on the abscissae and the corresponding **A_{450}** on the ordinates (see model on the flyer). 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 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, an ELISA software (i.e. Dynex, Biolise, etc…), can be used for the calculation of concentrations.

INTERPRETATION OF RESULTS:

A single and standardised calibrator is used for the assay calibration and the calibration range is prepared using a serial two-step 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:

Negative range: < 10 AU/ml

Grey zone: A "grey zone" is defined because some pathological samples (inflammation, infectious diseases, autoimmune diseases, gammaphathy, elderly people,…) can produce higher backgrounds, in auto-immune assays, than the normal individuals. This can mimic or mask a low reactivity. When a patient is 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 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 auto-antibody assay, clinical situation such as presence of inflammation, infectious diseases, auto-immune diseases, immun-complexes, high concentrations of IgG in the tested sample, 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.

PATHOLOGICAL VARIATIONS:

- Auto-antibodies to β2GPI 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 manifestations 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:

– Recurrent unexplained miscarriages.
– Unexplained lupus anticoagulant, without or with thrombosis.
– Anti-phospholipid syndrome.

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.

ASSAY SPECIFICITY AND CHARACTERISTICS:

The ZYMUTEST anti-β_2GPI, IgG Kit, specifically measures human autoantibodies to β_2GPI of the IgG isotype, reactive with immobilised 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.

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