INTENDED USE:
The ZYMUTEST anti-β2-GPI ELISA kit is a standardised and optimised enzyme immuno-assay designed for measuring auto-antibodies to β2-GPI of the IgG isotype, in human plasma or serum or in any biological fluid where auto-antibodies to β2-GPI must be measured.

ASSAY PRINCIPLE:
The diluted plasma sample or biological fluid is introduced into one of the microwells of the [β2-GPI] coated plate. When present, anti-β2-GPI auto-antibodies bind to immobilised β2-GPI. 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, 3,3',5,5'-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 sulfuric acid. The colour developed is directly proportional to the amount of anti-β2-GPI auto-antibodies, of the IgG isotype, present in the tested sample.

TESTED SAMPLES:
- Blood plasma (9 vol.) must be collected on 0.109 M citrate anticoagulant (1 vol.); plasma supernatant or for at least 30 min. at 2-8°C just before use. Thawed specimen must be tested within 2-8°C for 4 weeks following a 20 min. centrifugation at 2,500 g; citrated plasma should be tested within 2-8°C for 4 weeks, provided any bacterial contamination is avoided during use. This reagent contains sodium azide.
- 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.
- Calibrator: store at 2-8°C for 4 weeks, provided any bacterial contamination is avoided during use.
- Negative control: store at 2-8°C for 4 weeks, provided any bacterial contamination is avoided during use.

REAGENTS PREPARATION, STORAGE AND STABILITY:
- All reagents must be mixed well before use. Thawed specimen must be tested within 2-8°C just before use. Beware that reagents may have ethanol added.
- Store at 2-8°C. Protein stability studies at 30°C show that the reagents can be shipped at room temperature without damage.
- Anti-β2-GPI (Fcγ) have a similar stability profile. Store at 2-8°C. Use only components from a same kit lot. Do not mix components from different lots of kits.
- Include a negative control and a calibrator for each assay.

REAGENTS:
- Micro ELISA plate: containing 12 strips of 8 wells, coated with highly purified human β2-GPI, already diluted 1:100. Following reconstitution, the calibrator is stable for at least 4 weeks at 2-8°C, provided any bacterial contamination is avoided during 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.
- Calibrators: 3 vials of anti-β2-GPI, IgG, calibrator, lyophilised. When restored with 1 ml of Autoimmunity Sample Diluent, the ready to use calibrator is obtained (already diluted 1:100).
- Negative control: 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).
- Wash Solution: 1 vial of 20 fold concentrated Wash Solution. The diluted Wash Solution must be used within 2-8°C, provided any bacterial contamination is avoided during use.

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 8 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.

Tested plasma or serum:
Samples are tested at 1:100 dilution in Autoimmunity Sample Diluent. When high amounts of auto-antibodies to β2-GPI 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 controls are ready to use (already diluted 1:100).
**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 Autobody 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:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-β2GPI IgG Calibrator or Negative control</td>
<td>200 µl</td>
<td>Introduce the:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>– Calibrator or – negative control</td>
</tr>
<tr>
<td>or 1:100 diluted sample or sample diluent (blank)</td>
<td></td>
<td>– diluted sample or – sample diluent</td>
</tr>
<tr>
<td></td>
<td></td>
<td>into the micro ELISA plate wells.</td>
</tr>
</tbody>
</table>

**Incubate for 30 minutes at room temperature (18-25 °C) (a) (b)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wash Solution (20 fold diluted in distilled water)</td>
<td>300 µl</td>
<td>Proceed to 5 successive washings using the washing instrument. (b)</td>
</tr>
<tr>
<td>Conjugate anti-β2GPI (Fcγ)/HRP immunocompact, reconstituted with 7.5 ml of conjugate diluent</td>
<td>200 µl</td>
<td>Immediately after the washing, introduce the anti-β2GPI (Fcγ)/HRP immunocompact in the micro ELISA plate wells.</td>
</tr>
</tbody>
</table>

**Incubate for 30 minutes at room temperature (18-25 °C) (a)**

**Wash Solution (20 fold diluted in distilled water)**

**TMB/H₂O₂ Substrate**

**0.45M Sulfuric Acid**

Follow exactly the same time intervals than for the addition of substrate, stop the colour development by introducing the 0.45M sulfuric acid (c).

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

Wait for 10 minutes in order to allow the colour to stabilize and measure absorbance at 450 nm (A450) (d). Subtract 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:

  \[ P = A_{nm}/A_{nm} = 1.5 \]

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.

**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.

Positive range: The positive range concerns the following anti-β2GPI autoantibody concentrations:

- Low positive: \( \geq 20 \) to \( < 50 \) AU/ml
- Moderate positive: \( \geq 50 \) to \( < 100 \) AU/ml
- High positive: \( \geq 100 \) AU/ml

**LIMITATIONS OF THE ASSAY:**

- 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 isoantibody, the antibody concentration and its affinity, and the time of exposure. IgG isotype is the most pathogenic.

**APPLICATIONS:**

- Assay of auto-antibodies to β2GPI is 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 isoantibody, the antibody concentration and its affinity, and the time of exposure. IgG isotype is the most pathogenic.

**ASSAY SPECIFICITY AND CHARACTERISTICS:**

The ZYMUTEST anti-β2GPI, IgG Kit, specifically measures human autoantibodies to β2GPI of the IgG isotype, reactive with immobilised β2GPI. IgM or IgA isotypes are not measured.

This assay is designed with native uncleaved and non-altered, highly purified human IgG, which has then a preserved structure. This method then provides high reproducibility, high sensitivity and high specificity.

**REFERENCES:**