The ZYMUTEST anti-β2GPI, IgG ELISA kit, is an optimized enzyme immuno-assay designed for measuring auto-antibodies to β2GPI of the IgG isotype, in human plasma or serum in any biological fluid where auto-antibodies to β2GPI must be measured.

**SUMMARY AND EXPLANATION:**

The ZYMUTEST anti-β2GPI, IgG Kit, specifically measures human auto and alloantibodies to β2GPI of the IgG isotype, reactive with immobilized β2GPI. IgM or IgA isotypes are not measured. This assay is designed with native uncleaved and non-altered, highly purified human β2GPI, which has then a preserved structure. This method then provides high reproducibility, high sensitivity and high specificity.

**ASSAY PRINCIPLE:**

Search of anti-β2GPI antibodies, with ZYMUTEST anti-β2GPI kit, is performed using an ELISA plate, sensitized by the native human β2GPI then stabilized.

The diluted plasma or serum sample or biological fluid is introduced into one of the microwells of the β2GPI coated plate. When present, anti-β2GPI auto-antibodies bind to immobilized β2GPI. Following a washing step, bound auto-antibodies, of the IgG isotype, are revealed by an immunocoujugate, goat anti-human IgG (Fc: 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-β2GPI 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 β2GPI, of the IgG isotype, must be assayed.

**REAGENTS:**

1. **COAT:** Micro ELISA plate: containing 12 strips of 8 wells, coated with highly purified human β2GPI, then stabilized; the plate is packed in an aluminum pouch hermetically sealed in presence of a desiccant.

2. **Anti-β2GPI**
   - 3 vials containing 50 mL of Autoimmunity Sample Diluent, ready to use. Contains Sodium Azide.

3. **CAL:** Anti-β2GPI 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 immunocoujugate (Anti-IgG [Fcγ]-HRP immunocoujugate), goat polyclonal antibodies specific for human IgG-Fcγ coupled to HRP, lyophilized.

6. **CD:** Conjugate Diluent: 1 vial of 25 mL of Immunocoujugate 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-β2GPI concentrations for the calibrators are 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.

**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 stored in original pouch. Remove carefully the stopper, in order to avoid any loss of powder when opening the vials.
- **COAT:** Open the aluminum 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 aluminum pouch, in presence of the desiccant, hermetically closed and protected from any moisture, and stored in the provided plastic microplate storage bag (minigrip).
- **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**

- **CAL:** Reconstitute each vial with 1 mL of Autoimmunity Sample Diluent, shake thoroughly for complete dissolution. The obtained calibrator is ready to use. It corresponds to a plasma containing IgG isotype auto-antibodies to β2GPI, 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**

- **C:** 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 vials 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)**

- **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**

- **WS:** Stable, 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:
  - **4 weeks at 2-8°C**

**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 allowing dispensing 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.

**SPECMEN 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).

- **Specimen:**
  - 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 β2GPI can also be assayed on serum. However it is better to perform the assays on plasma.
  - **Collection:**
    - Blood (9 mL) 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.
        - 4 weeks 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.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>1:100</td>
</tr>
<tr>
<td>Serum</td>
<td>1:100</td>
</tr>
<tr>
<td>Biological fluid</td>
<td>1:100</td>
</tr>
</tbody>
</table>

When high amounts of auto-antibodies to IgG 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 aluminum 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:

```markdown
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-β2GPI IgG Calibrator</td>
<td>200 µL</td>
<td>Introduce the : Calibrator or control or concentrated sample or sample diluent into the micro ELISA plate wells.</td>
</tr>
<tr>
<td>Anti-β2GPI IgG Calibrator</td>
<td></td>
<td>Incubate for 30 minutes at room temperature (18-25 °C) (a) (b)</td>
</tr>
<tr>
<td>or 1:100 diluted sample</td>
<td></td>
<td>(20 fold diluted in distilled water)</td>
</tr>
<tr>
<td>or Sample diluent (blank)</td>
<td></td>
<td>(20 fold diluted in distilled water)</td>
</tr>
</tbody>
</table>

**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, when the assay is performed at room temperature, between 18-25°C, they always are:
  - ODCAL for 1:1 calibrator ≥ 1.8
  - ODcal for negative control ≤ 0.25

**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 ODcal 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 abscissa the anti-IgG concentration (AU) and on ordinates the corresponding ODcal. The anti-IgG, 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) ; 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, gamopathy, elderly people...) can produce higher backgrounds, in auto-immune assays, than the normal individuals although these subjects have not anti-IgG 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 IgG of the IgG isotype.

- Grey Zone: ≥ 10 AU/mL to ≤ 20 AU/mL

**Positive range:** The positive range concerns the following anti-β2GPI 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, gamopathy, 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 IgG 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 IgG 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 IgG, increases with the IgG isotype, the antibody concentration and its affinity, and the time of exposure. IgG is the most pathogenic.

**APPLICATIONS:**

Assay of auto-antibodies to β2GPI 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-IgG 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 GP4-A4. "Procedures for the handling and processing of blood specimens for common laboratory tests".

**SYMBOLS:**

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