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ZYMUTEST™ Anti-VIII MonoStrip IgG

Ref RK039A-RUO

ELISA assay of antibodies to Factor VIII (FVIII), IgG isotype.

FOR RESEARCH USE ONLY.

DO NOT USE IN DIAGNOSTIC PROCEDURES.

English, last revision: 12-2021

INTENDED USE:

The ZYMUTEST™ Anti-VIII MonoStrip IgG kit is a qualitative or quantitative assay of ELISA sandwich type for measuring auto and allo-antibodies to Factor VIII, of the IgG isotype, in human plasma where auto-antibodies to FVIII must be sought.

This kit is for research use only and must not be used for patient diagnosis or treatment.

PRINCIPLE:

The diluted plasma sample is introduced into one of the microwells of the Recombinant FVIII coated strip. When present, anti-VIII auto and allo-antibodies bind to immobilized FVIII. Following a washing step, bound antibodies, of the IgG isotype, are revealed with a polyclonal goat anti-human IgG (Fcγ specific)-peroxidase conjugate (HRP), 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 color develops. The color turns yellow when the reaction is stopped with sulfuric acid. The color developed is directly proportional to the amount of anti-VIII antibodies, of the IgG isotype, present in the tested sample.

REAGENTS:

1. **COAT : ELISA Strips :** containing 4 strips of 8 wells, coated with Factor VIII, human, then stabilized and each strip is packed individually in presence of a desiccant.
2. **SD-Anti-VIII : Sample Diluent :** 2 vials of 12 mL of sample diluent for auto-immune test (Anti-FVIII Sample Diluent), ready to use. Contains goat serum.
3. **C+ : Positive control :** 4 vials of positive control (Anti-VIII, IgG control), lyophilised. After reconstitution with 0.5 mL of Sample Diluent (SD-Anti-VIII), the ready to use positive control is obtained (already diluted 1:100). Contains BSA.
4. **C- : Negative Control :** 4 vials of negative control, lyophilised. After reconstitution with 0.5 mL of sample diluent, the negative control is ready to use (already diluted 1:100). Contains human normal plasma diluted.
5. **IC : Immunoconjugate (Anti-IgG (Fcγ)-HRP immunoconjugate):** 4 vials of immunoconjugate, lyophilised. Affinity purified polyclonal goat antibodies specific for human IgG-Fcγ coupled to HRP. After reconstitution with 2 mL of Conjugate Diluent (CD), the immunoconjugate is ready to use. Contains BSA.
6. **CD : Conjugate Diluent :** 1 vial of 10 mL of diluent, ready to use. Contains BSA.
7. **WS : Wash Solution :** 2 vials of 12 mL, 20 fold concentrated.
8. **TMB : 3,3', 5,5'-Tetramethylbenzidine :** 1 vial of 10 mL of diluent, ready to use. Contains hydrogen peroxide.
9. **SA : 0.45M Sulfuric Acid :** 1 vial of 3 mL of diluent, ready to use.

WARNINGS AND PRECAUTIONS:

- Any product of biological origin must be handled carefully, as being potentially infectious.
- If the TMB 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.
- 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.
- The bovine plasma used to prepare the BSA has been tested by recorded methods and is certified free of infectious agents, in particular the causative agent of bovine spongiform encephalitis.
- For *in vitro* use.

CD : H317: May cause an allergic skin reaction.
WS : H317: May cause an allergic skin reaction.

REAGENT PREPARATION AND STABILITY:

Bring the kit at room temperature, at least 30 min before the assay. Store the unused reagents at 2-8°C. Remove carefully the stopper for lyophilized products, in order to avoid any loss of powder when opening the vials.

1. **COAT :** Open the container and take off the strip for the test series. After opening, the strips must be used within 30 minutes.
2. **SD-Anti-VIII :** Ready to use. Stability reagent after opening, provided that any contamination or evaporation is avoided, kept in its original vial is:
   - **4 weeks** at 2-8°C.
3. **C+ :** Restore each vial with exactly **0.5 mL** of **Sample Diluent (SD-Anti-VIII)**, shake thoroughly for complete dissolution. The reconstituted control is ready to use and corresponds to a plasma containing IgG isotype auto-antibodies to Factor VIII, already diluted 1:100.
   Stability reagent after reconstitution, provided that any contamination or evaporation is avoided, kept in its original vial is:
   - **2 weeks** at 2-8°C.
4. **C- :** Restore each vial with exactly **0.5 mL** of **Sample Diluent (SD-Anti-VIII),** shake thoroughly for complete dissolution. The reconstituted control is ready to use and corresponds to negative plasma, already diluted 1:100.
   Stability reagent after reconstitution, provided that any contamination or evaporation is avoided, kept in its original vial is:
   - **2 weeks** at 2-8°C.
5. **IC :** Restored each vial with exactly **2 mL** of **Conjugate Diluent (CD) at least 15 min before use**, shake thoroughly for complete dissolution.
   Stability reagent after reconstitution, provided that any contamination or evaporation is avoided, kept in its original vial is:
   - **4 weeks** at 2-8°C.
   - **24h** at room temperature 18-25°C.
6. **CD :** Ready to use.
   Stability reagent after opening, provided that any contamination or evaporation is avoided, kept in its original vial is:
   - **4 weeks** at 2-8°C.
7. **WS :** Incubate, if necessary, the vial in a water bath at 37°C, until complete dissolution of solids. Shake the vial and dilute the wash solution **1:20** in distilled water (the 12 mL of concentrated solution allow to prepare 240 mL of wash solution after dilution).
   Stability of wash solution, provided that any contamination or evaporation is avoided, kept in its original vial is:
   - **4 weeks** at 2-8°C.

   Stability of diluted wash solution, provided that any contamination or evaporation is avoided, kept in its original vial is:
   - **Once opened, 7 days** at 2-8°C.
8. **TMB :** Ready to use. This reagent contains oxygenated water.
   Stability reagent after opening, provided that any contamination or evaporation is avoided, kept in its original vial is:
   - **4 weeks** at 2-8°C.
9. **SA :** Stop Solution contains 0,45M sulfuric acid, ready to use.
   Stability reagent after opening, provided that any contamination or evaporation is avoided, kept in its original vial is:
   - **4 weeks** at 2-8°C.

STORAGE CONDITIONS:

Unopened reagents must be stored at 2-8°C, in their original packaging box. They are then usable until the expiration date printed on the kit.

REAGENTS AND MATERIAL REQUIRED BUT NOT PROVIDED:

Reagents:

- Distilled water.

Materials:

- **8-channel pipettes** allowing dispensing 50-300 µL**.**
- **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** (optional).
- **Micro ELISA plate reader** with a wavelength set up at 450 nm.

SPECIMEN COLLECTION AND PREPARATION:

Preparation and storage of specimens must be performed according to the current local regulations.

- 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.

- Collection:

Blood (9 volume) must be collected on trisodium citrate anticoagulant (1 volume) (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:
  - 24 hours at room temperature (18-25°C)
  - 6 months 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.

PROCEDURE:

Assay method:

1. Controls are ready to use **(already diluted at 1/100).**

2. Dilute the samples using Sample Diluent (SD) buffer as described in the table below:

Sample	Dilution
Plasma	1:100

In presence of sample with high amounts of antibodies anti-VIII, dilute at **1:200** or **1:400**. The obtained results should be multiplied by **2** or **4**.

3. Remove the strip from the package and put the strip 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- VIII IgG **Positive control** or **Negative control** or **1:100 diluted sample** or **sample diluent** (blank)	**200 µL**	Introduce the : • **Positive control** or • **Negative control** or • **Diluted sample** or • **Sample diluent** into the micro ELISA plate wells.
Incubate for 60 minutes at room temperature (18-25°C) (a)		
Wash Solution (20 fold diluted in distilled water)	**300 µL**	Proceed to 5 successive washings **(b)**
Conjugate **anti-IgG (Fcγ)-HRP** immunoconjugate, restored with 2 mL of conjugate diluent	**200 µL**	Immediately after the washing, Introduce the anti-IgG (Fcγ)-HRP **immunoconjugate** in the wells.
Incubate for 60 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, well by well, 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. Substract 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. The next reagent must be added within 3 minutes, in order to prevent the plate from drying, which could damage the immobilized components and reduce the reactivity plate. 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.

QUANTITATIVE METHOD:

Antibodies anti-VIII IgG can be quantified using the positive control as a calibrator. Concentration of Positive control is determined in AU/mL and is specific to each lot. The concentration of Positive control is indicated on the flyer provided in the kit with each lot. Standard solutions are obtained in tubes by doing a serial two-step dilution with 200 µL of positive control and 200 µL of Sample Diluent from C:1 to C:16. The other steps of the method are similar to the qualitative method.

Calibration curve: Plot the Factor anti-VIII antibody (concentration in **AU/mL**) on abscissa and the corresponding absorbance (**OD 450**) on ordinates, draw the "best fit" calibration curve.

For the measurement of anti-VIII antibodies concentration, only the calibration curve generated for this assay series has to be used. From the curve obtained, deduce directly the Factor anti-VIII antibody concentration for the tested sample. For obtaining the Factor anti-VIII antibody concentration in a sample tested at a higher dilution, this value must be **multiplied by the dilution factor used**.

Alternatively, an ELISA software (i.e., Dynex, Biolise, etc...) can be used for establishing the "best fit" curve and the calculation of concentrations.

VALIDATION:

- Positive and negative controls provided in the kit, allow validating the right performance of the assay.
- Expected OD values for positive and negative controls can vary from lot to lot but, when the assay is run at room temperature, between 18 and 25°C, OD are always of :
  - Qualitative method:

    OD_{450} for positive control ≥ 1.0 | **OD_{450} for negative control: ≤ 0.15**

  - Quantitative method:

    Concentration for negative control: ≤ 12AU/mL

Obtained values for positive and negative controls at 20±1°C, are indicated for each batch of reagents in the flyer provided in the kit.

Obtained OD_{450} can vary according to the effective temperature during the assay run.

QUALITY CONTROL:

Using quality controls allows validating the method compliance, as well as the homogeneous of assays for a same lot of reagents.

Quality control plasmas 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 for each test series.

Each laboratory can establish acceptance ranges and verify expected performances in its analytical system.

RESULTS:

- Results on qualitative method are expressed according to the **OD_{450}** obtains values for the positive and/or negative controls.
- Results are on quantitative method expressed according to the Anti-VIII concentration of the positive control.
- For higher dilutions, the complementary dilution factor must be considered.

The results obtained should be for research use only and must not be used for patient diagnosis or treatment.

RESULTS INTERPRETATION:

Qualitative method:

When the assay is run at 20±1°C, the results are as follows:

Positive: OD_{450} > 0.30 | **OD_{450}: 0.15 < Grey zone ≤ 0.30** | **Negative: OD_{450}≤ 0.15**

Quantitative method:

When the assay is run at 20±1°C, the results are as follows:

Positive: ≥ 24 AU/mL | **12 AU/mL < Grey zone < 24 AU/mL** | **Negative: ≤ 12 AU/mL**

When the room temperature is out of the recommended range, absorbance values can be affected. The positive control can then be used for adjusting the cut-off value. The flyer provided in the kit indicates the OD_{450} value obtained for the positive control of the ZYMUTEST™ Anti-VIII MonoStrip IgG lot used, and the value in % of this OD_{450} corresponding to the cut-off. The adjusted cut-off value is then the corresponding % of the absorbance measured for the positive control in your series of measurements.

LIMITATIONS:

- In order to get the optimal assay performances and adhere to specifications, the procedural instructions validated by HYPHEN BioMed must be strictly respected. It is responsibility of the user laboratory to validate any modification to those instructions for use.
- Any reagent presenting an unusual aspect or contamination signs must be rejected.
- Any suspect specimen collection or presenting contamination signs must be rejected.
- Any plasma containing a coagulum or contamination signs must be rejected.
- 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 autoantibody 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.

ASSAY SPECIFICITY AND CHARACTERISTICS:

The assay detects both neutralizing and non-neutralizing anti-VIII antibodies; therefore a neutralizing effect must be confirmed through functional tests.

Difficulties in identifying the coexistence of neutralizing anti-VIII antibodies (anti-VIII) and lupus anticoagulant (LA) are mainly due to the interference of LA on anti-VIII assays. We developed an enzyme-linked immunosorbent assay (ELISA) method that uses phospholipid-free recombinant Factor VIII as the antigen. Our aim was to reveal the presence of anti-VIII antibodies using a system that is not affected by LA.

PERFORMANCES:

Dynamic range: 0 to 300 AU/mL.
Detection threshold ≤ 5 AU/mL.
Intra-assay CV: ≤10%.
Inter-assay CV: ≤10%.

SYMBOLS:

Used symbols and signs listed in the ISO standard 15223-1, refer to the Definition of Symbols document.

Changes compared to the previous version.