

ZYMUTEST Anti-Prothrombin IgM

ARK007B

Auto-antibodies to Prothrombin, IgM isotypes



Manufactured By: HYPHEN BioMed

For in vitro use only

For research use only

Last revision: 28/08/2006

INTENDED USE:

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

ASSAY PRINCIPLE:

The assay of human auto-antibodies to Prothrombin with the ZYMUTEST anti-Prothrombin, IgM kit, is designed with highly purified human native Prothrombin, coated onto a micro ELISA plate.

The diluted plasma sample or biological fluid is introduced into one of the microwells of the coated plate. When present, anti-Prothrombin auto-antibodies bind to immobilised Prothrombin. Following a washing step, bound auto-antibodies of the IgM isotype are revealed with a goat anti-human IgM (Fc μ specific)-peroxidase conjugate, which reacts specifically with IgM isotypes. Following a new washing step, the peroxidase substrate, 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-Prothrombin auto-antibodies, of the IgM isotype, present in the tested sample.

TESTED SAMPLES:

- Trisodium citrate or Na₂ EDTA anticoagulated human plasma or human serum.
- Any biological fluid where human auto-antibodies to Prothrombin, of the IgM isotype, must be assayed.

REAGENTS:

1. **COAT:** Micro ELISA plate, containing 12 strips of 8 wells, coated with highly purified human native Prothrombin, 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.
3. **Cal:** 3 vials of anti-Prothrombin IgM Calibrator, lyophilised. When restored with 1 ml of Autoimmunity-Sample Diluent, the ready to use calibrator is obtained (already diluted 1:100).
This calibrator has a defined anti-Prothrombin 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-(h)-IgM (μ)-HRP immunoconjugate, PAV), affinity purified goat antibodies specific for human IgM (heavy chain- μ) 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:** 1 vial of 6 ml of 0.45M Sulfuric acid (Stop Solution), ready to use.

Nota: Use only components from a same kit lot number. Do not mix components from different lots when running the assay.

REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED:

- 8-channel or repeating pipette allowing dispensing 50-300 μ l.
- 1-channel pipettes at variable volumes from 0 to 20 μ l, 20 to 200 μ l and 200 to 1000 μ l.
- 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 (minigrp).

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.

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. **Anti Prothrombin IgM Calibrator:** restore each vial with 1 ml Autoimmunity-Sample Diluent in order to obtain the ready to use calibrator. It corresponds to a normal plasma, already diluted 1:100. Following reconstitution the calibrator is stable for 5 days at 2-8°C, provided that 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 plasma, already diluted 1:100. Following reconstitution the positive control is stable for 2 weeks at 2-8°C, provided that any bacterial contamination is avoided during use.

Warning: Human Prothrombin used for coating the plates is extracted from human plasma. The calibrator and negative control are prepared with human plasma. Plasma used was 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-(h)-IgM (μ)-HRP immunoconjugate (PAV):** 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 preparing 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 and stored at 2-8°C. 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.

Nota: Bring the kit at room temperature, at least 30 min. before use. Store the unused reagents at 2-8°C.

PROCEDURE:

Sample collection:

Blood plasma (9 vol.) must be collected on 0.109M citrate anticoagulant (1 vol.); plasma supernatant is decanted following a 20 min. centrifugation at 2,500 g; citrated plasma should be tested within 8 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 4 hours. EDTA collected human plasma may also be used. Conditions of storage are the same than those for citrated plasma.

Auto-antibodies to Prothrombin can also be assayed on serum. However, it is better to measure these antibodies on human plasma in order to avoid contact with activated blood cell surfaces.

Assay preparation:

Plasma or serum is tested at 1:100 dilution in Autoimmunity-Sample Diluent. When high amounts of auto-antibodies to Prothrombin are expected, samples must be assayed at 1:200 or 1:400 dilution. Results must then be multiplied by 2 or 4.

The calibrator and negative control are ready to use and correspond to plasma already diluted 1:100.

Assay procedure:

Calibration curve: The assay can be calibrated with the calibrator included 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:



8580 Gove Court - Mason, OH 45040

Phone: 513.770.1991

Toll Free: 866.783.3797

Fax: 513.573.9241

Email: info@aniara.com

www.aniara.com

D.750.02/ZY/007B

Reagent	Volume	Procedure
Anti-Prothrombin IgM 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 in the micro ELISA plate wells.
Incubate for 1 hour 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-IgM (µ)-HRP immunoconjugate, restored with 7.5 ml of conjugate diluent)	200 µl	Introduce the anti-IgM (µ)-HRP immunoconjugate in the micro ELISA plate wells.
Incubate for 1 hour 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 ₂ O 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). Subtract the blank value		

Nota:

- Avoid letting the plate in the bright sunlight during incubations and more particularly during colour development. A micro ELISA plate shaker can be used.
- 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.
- 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.
- For bichromatic readings, a reference wavelength at 690 nm or at 620 nm can be used.

QUALITY CONTROL:

- The calibrator and negative control provided in the kit allow validating the right performance of the assay.
- Expected A450 values for undiluted calibrator and negative control can present variations from lot to lot but, when the assay is performed at room temperature (18-25°C), they always are:

$$P = A_{450} \text{ for 1:1 Calibrator: } \geq 1.5 \quad N = A_{450} \text{ for negative control: } \leq 0.25$$

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 A₄₅₀ values obtained for samples, negative control and using the calibration curve.
- The calibration curve is obtained by plotting the anti-Prothrombin concentrations expressed in AU on the abscissae and the corresponding A₄₅₀ on the ordinates (see model on the flyer). The anti-Prothrombin, autoantibody concentration, of the IgM 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, gammopathy, ...) 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 Prothrombin of the IgM isotype.

Grey Zone: ≥ 10 AU/ml to < 20 AU/ml

Positive range: The positive range concerns the following anti-Prothrombin 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, presence of inflammation, infectious diseases, auto-immune diseases, immun-complexes, high concentrations of IgM, can induce a high background A450 which is within the grey zone or in the weak reactive range.

PATHOLOGICAL VARIATIONS:

Auto-antibodies to Prothrombin are usually not found in normal population.

Their presence at high concentrations can be associated with recurrent abortions, miscarriages or with some types of the anti-phospholipid syndrome.

Auto-antibodies to Prothrombin have also been reported in young children, associated with lupus anticoagulant activity and infectious diseases. These antibodies are often transitory and disappear in few months.

Some studies have reported that presence of anti-Prothrombin auto-antibodies can be associated with thrombotic tendencies and myocardial infarction. This association still needs to be confirmed.

Up to now, only poor correlation has been established between the presence of a Prothrombin dependent lupus anticoagulant activity and auto-antibodies to Prothrombin, excepted during some infections.

APPLICATIONS :

Assays of auto-antibodies to Prothrombin of the IgM isotype in plasma or serum, in the following clinical situations:

- Pregnancies with miscarriage.
- Lupus anticoagulant associated with infectious diseases.
- Anti-phospholipid syndrome.
- Any clinical situation where the assay of anti-Prothrombin auto-antibodies is required. This assay is usually associated to the assay of the IgM isotype auto-antibodies.

ASSAY SPECIFICITY:

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

REFERENCES:

- Forastiero RR, Martinuzzo ME, Cerrato GS, Kordich LC, Carreras LO. Relationship of antiB2-glycoprotein I and anti prothrombin antibodies to thrombosis and pregnancy loss in patients with antiphospholipid antibodies. *Thromb Haemost* 78: 1008-1014; 1997.
- Vaara O, Puurunen M, Mänttari M, Manninen V, Aho K, Palosuo T. Antibodies to prothrombin imply a risk of myocardial infarction in middle-aged men. *Thromb Haemost* 75(3): 456-459; 1996.
- Arvieux J, Darnige L, Caron C, Reber G, Bensa JC, Colomb MG. Development of an ELISA for autoantibodies to prothrombin showing their prevalence in patients with lupus anticoagulants. *Thromb Haemost* 74(4): 1120-1125; 1995.
- Palosuo T, Virtamo J, Haukka J, Taylor PR, Aho K, Puurunen M, Vaara O. High antibody levels to prothrombin imply a risk of deep venous thrombosis and pulmonary embolism in middle-aged men. *Thromb Haemost* 78: 1178-1182; 1997.
- Permpikul P, Rao LVM, Rapaport SI. Functional and binding studies of the roles of prothrombin and B2-glycoproteins I in the expression of lupus anticoagulant activity. *Blood* 83: 2878-2892; 1994.
- Gris JC, Quéré I, Sanmarco M, Boutière B, Mercier E, Amiral J, Hubert AM, Ripart-Neveu S, Hoeffet M, Tailland ML, Rousseau O, Monpeyroux F, Douzat M, Sampol J, Daures JP, Berlan J, Marès P. Antiphospholipid and anti-protein Syndromes in non-thrombotic, non autoimmune women with unexplained recurrent primary early foetal loss. *Thromb Haemost* 84: 228-236; 2000.