



## IMUCLONE® Anti-Annexin V IgM ELISA

Product No. 652M

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### INTENDED USE

The IMUCLONE® Anti-Annexin V IgM ELISA is an enzyme-linked immunosorbent assay for measuring the IgM isotypes of auto-antibodies to Annexin V in human plasma and in any biological fluid in which auto-antibodies to Annexin V must be measured. The kit is limited to "Research Use Only" in the United States.

### PRINCIPLE OF THE METHOD

The IMUCLONE® Anti-Annexin V IgM ELISA uses a highly purified human recombinant Annexin V for isolating the autoantibody to Annexin V. A diluted plasma sample or biological fluid is added to an Annexin V coated microtest well. If present, anti-Annexin V autoantibodies bind to the immobilized Annexin V. Following a wash step, bound autoantibodies of the IgM isotype are detected using an affinity purified, IgM specific goat anti-human IgM (Fcγ specific)-peroxidase conjugate. Following another wash step, a peroxidase substrate, 3,3',5,5' - Tetramethylbenzidine (TMB), in the presence of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), is added to the microwell and the subsequent enzymatic reaction yields a solution with a blue color. Addition of sulphuric acid stops the reaction and turns the solution color to yellow. The amount of color developed is directly proportional to the amount of IgM isotype anti-Annexin V autoantibodies in the sample.

### REAGENTS

- 12 strips of 8 Annexin V coated microwells (total of 96 wells) in frame holder.
- 2 vials of Sample Diluent, ready to use (50 mL)
- 3 vials of Anti-Annexin V IgM Calibrator (lyophilized)
- 3 vials of Anti-Annexin V IgM Negative Control (lyophilized)
- 3 vials of Anti-IgM (Fcγ)-HRP Immunoconjugate (lyophilized)
- 1 vial of Conjugate Diluent, ready to use (25 mL)
- 1 vial of Wash Solution, 20 fold concentrate (50 mL)
- 1 vial of Substrate, TMB (25 mL)
- 1 vial of Stop Solution, 0.45M H<sub>2</sub>SO<sub>4</sub> (6 mL)

### ADDITIONAL MATERIALS REQUIRED BUT NOT PROVIDED

- 0.22 μm filtered deionized or distilled water
- 50- 300 μL 8-channel or repeating pipette
- 0-20 μL, 20-200 μL and 100-1000 μL single channel pipettes
- ELISA plate washing equipment
- ELISA plate reader with a wavelength set at 450 nm

### PREPARATION OF THE REAGENTS, STORAGE AND STABILITY

Intact, unopened reagents are stable until the expiration date printed on the box when stored in their original packaging at 2-8°C.

- Annexin V Coated Microwells** - Open the aluminum pouch and remove from the plate frame those 16 well strips not to be used. Once removed from the pouch, strips should be used within 30 minutes. Unused strips can be stored up to 4 weeks at 2-8°C in their original aluminum pouch, in presence of the desiccant, hermetically closed and protected from any moisture, and stored in the provided storage bag.
- Sample Diluent** - Supplied ready to use. Once opened, the Sample Diluent may be stored for up to 4 weeks at 2-8 °C provided bacterial contamination is avoided during use.

**Warning:** The **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.

- IgM Calibrator** – Reconstitute each vial with 1 mL of Sample Diluent. This control is equivalent to plasma containing IgM isotype autoantibodies to Annexin V at a 1:100 dilution. Reconstituted Positive Control is stable for 5 days at 2 – 8° C providing bacterial contamination is avoided.
- Negative Control** - Reconstitute each vial with 1 mL of Sample Diluent. This control is equivalent to normal plasma at a 1:100 dilution. Reconstituted Negative Control is stable for 2 weeks at 2-8°C providing bacterial contamination is avoided.
- Conjugate Diluent** - Supplied ready to use. Once opened, the Conjugate Diluent is stable for up to 4 weeks at 2-8°C, provided any bacterial contamination is avoided during use.
- Anti-IgM (Fcγ)-HRP Immunoconjugate** - Reconstitute each vial with 7.5 mL of Conjugate Diluent. Allow the pellet to completely dissolve and shake the vial gently in order to homogenize the contents before use. Reconstituted conjugate is stable for at least 24 hours at room temperature or for at least 7 days at 2-8°C.
- Wash Solution** - Incubate the vial for 15-30 minutes in a 37°C water bath for complete dissolution. Shake the vial and dilute the amount required 1:20 in distilled water (the 50 mL provided is sufficient to prepare 1 Liter of Wash Solution). The concentrated Wash Solution must be stored at 2-8°C in its original vial and used within 4 weeks once opened. Diluted Wash Solution must be used within 7 days, stored at 2-8°C and protected from any contamination.
- Substrate** – Supplied ready to use. Once opened, the Sample Diluent may be stored for up to 4 weeks at 2 – 8° C provided bacterial contamination is avoided during use.
- Stop Solution** – Supplied ready to use.

### WARNING

The source material for the controls in this kit are of human origin and have been found to be non-reactive for Hepatitis B Surface Antigen (HBsAg), Hepatitis C Virus (HCV) and Human Immunodeficiency Virus Type 1 and Type 2 (HIV-1, HIV-2) using FDA Approved methods. As no known test method can provide complete assurance that products derived from human blood will not transmit HBsAg, HCV, HIV-1, HIV-2 or other blood-borne pathogens, this reagent should be handled as recommended for any potentially infectious human specimen.

### SPECIMEN COLLECTION

Nine volumes of blood is collected in 1 volume of 0.109M trisodium citrate and centrifuged at 2,500 x g for 20 minutes. Plasma supernatant containing the PAI-1 is decanted. Citrated plasma should be tested within 8 hours, stored frozen at –20°C for up to 6 months or at –70°C for up to 2 years and thawed once for 15 minutes at 37°C just prior to use. EDTA collected human plasma may also be used.

Autoantibodies to Annexin V in serum can also be assayed. However, it is better to measure these antibodies from human plasma in order to avoid inhibition by Annexin V released from blood cells during clotting.

### ASSAY PROCEDURE

Plasma or serum samples are diluted 1:100 with Sample Diluent and tested. If high amounts of autoantibodies to Annexin V are expected, samples must be assayed at a 1:200 or a 1:400 dilution. Results must then be multiplied by 2 or 4.

### Calibration Curve

The assay is calibrated with the supplied Anti-Annexin V IgM Calibrator whose concentration (C) is indicated in arbitrary units (AU) on the flyer

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provided. Prepare additional calibrators via a serial two-step dilution from 1:1 to 1:323 using the Sample Diluent. Calibrators in the range of C:1 to C:32 are obtained.

Remove the required number of strips from the aluminum pouch, for the number of assays to be performed. Place the strips in the frame provided. To the appropriate wells, add the reagents and perform the various assay steps as indicated on the following table:

Reagent	Volume	Procedure
Anti Annexin V IgM Calibrator, Negative Control or 1:100 diluted sample	200 µL	Add the Calibrator, Negative control or diluted sample into the microwells.
Incubate for 1 hour at room temperature (18-25 °C)		
Wash Solution	300 µL	Wash the wells 5 times.
Anti-IgM (Fc $\gamma$ )-HRP Immunoconjugate	200 µL	Add the Anti-IgM (Fc $\gamma$ )-HRP immunoconjugate to the microtest wells.
Incubate for 1 hour at room temperature (18-25 °C)		
Wash Solution	300 µL	Wash the wells 5 times.
TMB/H $_2$ O $_2$ Substrate	200 µL	Add the Substrate immediately after washing the microwells
Incubate for exactly 5 minutes at room temperature (18-25 °C)		
Stop Solution	50 µL	Following exactly the same time intervals used for adding the substrate, stop the reaction by adding 0.45M H $_2$ SO $_4$
Wait for 10 minutes for the color to stabilize and measure the absorbance at 450 nm (A $_{450}$ ).		

#### PROCEDURAL NOTES

- Never let the plates empty between the addition of the reagents or following the washing step. Always add the next reagent within 3 minutes in order to prevent the plate from drying, which might damage the immobilized components. If necessary, keep the plate filled with Wash Solution in between steps.
- For addition of the TMB/H $_2$ O $_2$  substrate, the time interval between each microwell must be accurate and exactly determined. It must be the same when stopping the reaction.
- Avoid exposing the plate to bright sunlight during incubation and color development.

#### QUALITY CONTROL

The Calibrator and Negative Controls provided in this kit permit assay validation. Expected A $_{450}$  values for the Calibrator and negative controls can present variations from lot to lot but they are always:

$$A_{450} \text{ for the undiluted Calibrator } \geq 1.5$$

$$A_{450} \text{ for the Negative Control (N)} \leq 0.25$$

#### EXPRESSION OF RESULTS

Results are expressed according to the A $_{450}$  values obtained for samples and controls using the calibration curve. The calibration curve is obtained by plotting the Anti-Annexin V IgM in AU versus the corresponding A $_{450}$  (see the sample on the enclosed flyer). The Anti Annexin V IgM concentration of the undiluted test sample is read directly from the calibration curve. If higher sample dilutions were used, the sample concentration must be multiplied by the complementary dilution factor (i.e. the concentration for samples diluted 1:200 are to multiplied by 2). Alternately, an ELISA software package (i.e. Dynex) may be used to calculate the concentration of the test sample.

#### INTERPRETATION OF RESULTS

##### Negative Range

The calibrator expressed in Arbitrary Units (AU) is defined respective to the upper limit of the normal range that corresponds to the mean value obtained in a normal population plus 2 standard deviation (SD). This corresponds to 10 (AU). Therefore,

##### Negative Range < 10 AU/mL

##### Grey Zone

An "intermediate zone" is defined because some pathological samples (inflammation, infectious diseases, auto-immune disease, gammopathy etc.) can produce a high background. This can mimic or mask a low reactivity. When a patient is in the intermediate zone, it is recommended to repeat the test using a new sample, in order to follow the generation of autoantibodies to Annexin V of the IgM isotype.

##### Grey Zone $\geq 10$ AU/ mL to < 20 AU/ mL

##### Positive Range

##### Positive Range $\geq 20$ AU/mL

The positive range can be further classified as follows:

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

Improper washing steps can create a negative control with a high absorbance value. In order to avoid non-specific color development, check that the washing step is performed efficiently. As with any auto-immune assay, presence of inflammation, infectious diseases, auto-immune diseases, immune complexes and high concentrations of IgM, can induce a high absorbance background that is within the intermediate zone. The possible presence of antibodies should be checked on another specimen collected at a later time.

#### PATHOLOGICAL VARIATIONS

Autoantibodies to Annexin V are usually not found in the normal population. They can be present at low concentrations during pregnancy. Their presence at high concentrations can be associated with recurrent abortion, miscarriage or some types of anti-phospholipid syndrome (APS). The pathological effect of autoantibodies to Annexin V remains uncertain. Pathogenicity of the various isotypes remains to be completely understood.

This assay is useful for measuring autoantibodies to Annexin V of the IgM isotype in plasma or serum in the following clinical situations: pregnancy resulting in miscarriage, excess of apoptosis, anti-phospholipid syndrome in the absence of a typical profiling, and any clinical situation in which the assay of anti Annexin V autoantibodies is required.

#### SPECIFICITY

The IMUCLONE® Anti Annexin V IgM ELISA measures only human autoantibodies to Annexin V of the IgM isotype that are reactive with immobilized Annexin V. IgG or IgA isotypes are not measured with this kit.

#### REFERENCES

- Gris, J. C., *et al.* Antiphospholipid and antiprotein Syndromes in non-thrombotic, non autoimmune women with unexplained recurrent primary early foetal loss. *Thrombosis and Haemostasis* 2000, **84**: 228-236.
- Kaburaki, J., *et al.* Clinical significance of anti Annexin V antibodies in patients with systemic lupus erythematosus. *American Journal of Hematology* 1997, **54**: 209-213.
- Matsuda, J., *et al.* Anti-Annexin antibody in the sera of patients with habitual fetal loss or preeclampsia. *Thrombosis Research* 1994, **75** (1): 105-106.