

IMUCLONE® Annexin V ELISA

Product No. 650

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INTENDED USE

The IMUCLONE® Annexin V ELISA is an enzyme-linked immunosorbent assay for the measurement of human Annexin V in plasma⁵ (citrate or EDTA collected), platelet and blood cell concentrates⁴, other biological fluid or cell culture supernatant in which Annexin V may be present. This assay recognizes both native and recombinant Annexin V. The kit is limited to "Research Use Only" in the United States.

EXPLANATION OF THE TEST

Annexin V is a calcium dependent protein (MW = 35,000 D) found in endothelial cells, red blood cells, leukocytes, placenta and most human tissues. It is present at low concentrations in human platelets. It binds to anionic phospholipids and to activated cell membranes in a calcium dependent manner^{1,2}. It inhibits procoagulant and pro-inflammatory activities of dying cells during apoptosis⁵.

PRINCIPLE OF THE METHOD

The IMUCLONE Annexin V ELISA is a two-site sandwich ELISA utilizing affinity purified rabbit polyclonal antibodies specific for human Annexin V. A diluted plasma or biological fluid (the test sample) is added to a microtest well coated with affinity purified rabbit anti-human Annexin V ((Fab')₂ fragments), capturing the protein to the solid phase. After the well is washed, an affinity purified rabbit polyclonal antibody coupled to horseradish peroxidase (HRP) is added and binds to its corresponding free epitope of the immobilized Annexin V. Following another washing step, the substrate, Ortho-Phenylene-Diamine (OPD) in the presence of hydrogen peroxide, is added to the well. The subsequent enzyme reaction yields an orange-brown colored solution. The addition of sulfuric acid stops the reaction and turns the solution color to red. The amount of color (i.e. the absorbance of the solution at 492 nm) is directly proportional to the concentration of human Annexin V present in the tested sample.

REAGENTS

- 1 microtest plate, 96 wells (6 strips of 16 antibody coated wells)
- 2 vials of Sample Diluent, ready to use (50 mL)
- 3 vials of Human Annexin V Standard, recombinant (lyophilized)
- 3 vials of Anti-Annexin V HRP Conjugate (lyophilized)
- 1 vial of Conjugate Diluent, ready to use (25 mL)
- 1 vial of Wash Solution, 20-fold concentrate (50 mL)
- 2 sets of Substrate Tablets: 3 x OPD, 3 x Urea Peroxide

ADDITIONAL MATERIALS REQUIRED BUT NOT PROVIDED

- 0.22 µm filtered deionized or distilled water
- 2M sulfuric acid H₂SO₄
- 50-300 µL eight channel or repeating pipette
- 0-20 µL, 20-200 µL and 100-1000 µL single channel pipettes
- Microtest plate reader with a wavelength setting of 492 nm

Caution: Always add sulfuric acid into water to avoid any risk of splashing. Avoid any skin and eye contact. Wear protection glasses and gloves when handling.

PREPARATION OF THE REAGENTS

A. Microtest Plate

Open the aluminum pouch and remove from the plate frame those 16 well strips not to be used. When removed from the pouch, strips should be used within 30 minutes. Unused strips can be stored for up to 4 weeks 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 microplate storage bag.

B. 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.

C. Human Annexin V Standard

1. Add 2 mL of Sample Diluent to the vial and mix gently. The standard is a solution containing 20 ng/mL of human Annexin V (recombinant).
2. Prepare additional standards for the standard curve by adding the 20 ng/mL standard to Sample Diluent in the following volumes. Mix each standard for a few seconds. The standards are stable for at least 6 hours at room temperature.

Human Annexin V Standard Concentration	Volume of Sample Diluent	Volume of 20 ng/mL Standard
0 ng/mL	1.00 mL	0.00 mL
1 ng/mL	0.95 mL	0.05 mL
2 ng/mL	0.90 mL	0.10 mL
5 ng/mL	0.75 mL	0.25 mL
10 ng/mL	0.50 mL	0.50 mL
20 ng/mL	0.00 mL	1.00 mL

D. Conjugate Diluent

Supplied ready-to-use. Once opened, the Conjugate Diluent may be stored for up to 4 weeks at 2-8°C, provided bacterial contamination is avoided during use.

E. Anti-Annexin V HRP Conjugate

Add 7.5 mL of Conjugate Diluent to the vial and gently mix. Allow the pellet to be completely dissolved before use. The conjugate is stable for at least 8 hours when stored at room temperature or for 72 hours when stored at 2-8°C.

F. Wash Solution

Incubate the vial for 15-30 minutes in a 37°C water bath to ensure the complete dissolution of solids. Shake the vial and dilute the amount required 1:20 in filtered deionized/distilled water (the 50 mL provided is sufficient to prepare 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.

G. Substrate

Thirty (30) minutes before use, add one tablet of urea peroxide in 20 mL of filtered deionized/distilled water. Immediately prior to use, add one tablet of OPD and shake gently until complete dissolution. The substrate must be used within 1 hour following preparation and it must be protected from bright sunlight. Unused substrate must be discarded.

Caution: Handle OPD and urea peroxide with the usual precautions required for these chemicals. Store tablets in the dark. Avoid any metal contact.

Warning: OPD is toxic. Handle with care. Avoid any skin and eye contact. Wear protection glasses and gloves.

REAGENT STORAGE AND STABILITY

Unopened reagents are stable until the expiration date printed on their vials when stored at 2-8°C.

SPECIMEN COLLECTION

Nine volumes of blood is collected in 1 volume of 0.109M trisodium citrate and centrifuged at 2,500 x g for 15 minutes. Plasma supernatant containing the Annexin V is decanted. Citrated plasma should be tested within 4 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 plasma may also be used.

Use of EDTA anticoagulant, or supplementation of citrated blood with 0.025M Na₂ EDTA (final concentration) allows for a better recovery of Annexin V, a calcium dependent protein, in plasma.

PROCEDURE

Plasma samples must be tested diluted two fold (1:2) in Sample Diluent. Samples with expected Annexin V concentrations >40 ng/ml should be diluted 1:5, 1:10 or 1:20 with Sample Diluent.

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 dictated in the following table:

Reagent	Volume	Procedure
Annexin V Standard or diluted test sample	200 µL	Add the standard or diluted test samples to appropriate microtest well.
Incubate for 1 hour at room temperature (18-25°C)		
Wash Solution	300 µL	Wash the wells 5 times.
Anti-Human Annexin V HRP Conjugate	200 µL	Add the conjugate to each microtest well.
Incubate for 1 hour at room temperature (18-25°C)		
Wash Solution	300 µL	Wash the wells 5 times.
OPD / H ₂ O ₂ Substrate	200 µL	Add the substrate Immediately after washing the wells.
Incubate for exactly 5 minutes at room temperature (18-25°C)		
2M H ₂ SO ₄	50 µL	Following exactly the same time intervals used for adding the substrate, stop the reaction by adding 2M H ₂ SO ₄ .
Wait for 10 minutes in order to allow the color to stabilize and measure absorbance at 492 nm.		

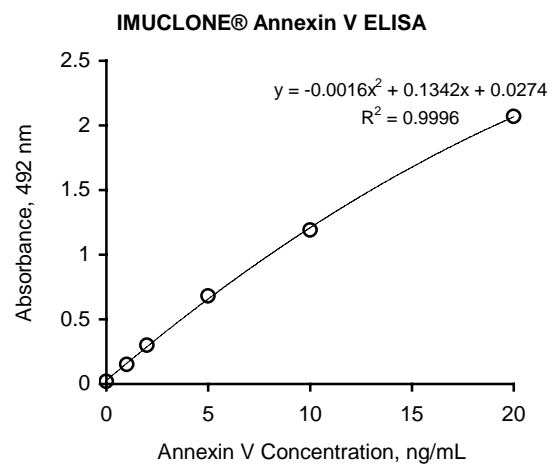
PROCEDURAL NOTES

- For addition of the OPD/H₂O₂ substrate, the time interval between each row must be accurate and exactly determined. It must be the same when stopping the reaction.

- Never let the plate 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.
- Do not place the plate in bright sunlight during incubations and more so during color development.
- Alternatively, the enzyme reaction may be stopped by using 100 µL of 1M HCl.

REPRESENTATIVE STANDARD CURVE

The standard curve is constructed by plotting the mean absorbance value measured for each Annexin V standard (background subtracted) versus its corresponding concentration. Interpolate the Annexin V concentration of the diluted plasma sample directly from the standard curve. The following curve is for demonstration purposes only. A standard curve should be constructed each time the assay is performed.



CALCULATION OF RESULTS

From the curve obtained, interpolate the Annexin V concentration for the diluted sample tested. To obtain the concentration of the collected sample, multiplied by the dilution factor (i.e. 2, 5, 10, or 20).

EXPECTED RANGE

The Annexin V concentration in normal human plasma is <10 ng/mL^{3,4}. Annexin V concentrations can be elevated in autoimmune disease states such as Systemic Lupus Erythematosus (SLE).

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