ZYMUTEST Annexin V

 # RK004A

Annexin V antigen

(Complete ELISA kit for the assay of human Annexin V)

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

HYPHEN BioMed

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INTENDED USE:

The ZYMUTEST Annexin V kit is a two-site enzyme immuno-assay for measuring human Annexin V in plasma, or in any fluid where Annexin V can be present.
This kit is for research use only and should not be used for patient diagnosis or treatment.

ASSAY PRINCIPLE:

ZYMUTEST Annexin V is a sandwich ELISA designed with affinity purified rabbit polyclonal antibodies specific for human Annexin V.
The diluted tested plasma or biological fluid is introduced in a microwell, coated with affinity purified rabbit polyclonal antibodies (F(ab')2 fragments) specific for human Annexin V*. When present, this protein is captured onto the solid phase. Following a washing step, the immunoconjugate, which is an affinity purified rabbit polyclonal antibody coupled to horse radish peroxidase (HRP), is introduced, and binds to the free epitopes of immobilized Annexin V. Following a new washing step, the peroxidase substrate, Tetramethylbenzidine (TMB) in presence of Hydrogen Peroxide (H_2O_2), is introduced and a blue colour develops. The colour turns yellow when the reaction is stopped with Sulfuric Acid. The amount of colour developed is directly proportional to the concentration of human Annexin V in the tested sample.

***NOTE: Use of anti-Annexin V F(ab')2 fragments for the pre-coated plates allows to avoid the interference of Rheumatoid Factor.**

TEST SAMPLE:

- Trisodium Citrate or Na_2 EDTA anticoagulated human plasma.
- Any biological fluid where Annexin V must be measured.
- Quality control of platelet concentrates in order to evaluate the cell storage lesion, by measuring released Annexin V in supernatants.

REAGENTS:

1. **COAT: Micro ELISA plate,** containing 12 strips of 8 wells, coated with affinity purified rabbit polyclonal antibodies (F(ab')2 fragments) specific for human Annexin V*, then stabilised ; the plate is packed in an aluminium pouch hermetically sealed in presence of a desiccant.
2. **SD:** 2 vials containing 50 ml of **Sample Diluent**, ready to use.
3. **Std:** 3 vials of **human Annexin V (recombinant) Standard**, lyophilised. When restored with **2 ml of Sample Diluent**, a solution containing a concentration **"C"** of human Annexin V is obtained. The exact Annexin V-Ag concentration is indicated on the flyer provided in the kit. Usually about 10 ng/ml.
4. **CI:** 1 vial containing 1 ml of lyophilised **Plasma Control I** (human plasma, **UTA, high**).
5. **CII:** 1 vial containing 1 ml of lyophilised **Plasma Control II** (human plasma, **UTA, low**).

   The Annexin V concentrations and acceptance ranges for controls are indicated on the flyer provided in the kit.
6. **IC:** 3 vials of **Anti-Annexin V-HRP immunoconjugate**, an affinity purified rabbit polyclonal antibody coupled to HRP, lyophilised.
7. **CD:** 1 vial of 25 ml of **Conjugate Diluent**, ready to use.
8. **WS:** 1 vial of 50 ml of 20 fold concentrated **Wash Solution**.
9. **TMB:** 1 vial of 25 ml peroxidase substrate: 3,3',5,5' **– Tetramethylbenzidine** containing hydrogen peroxide. Ready to use.
10. **SA:** 1 vial of 6 ml of **0.45 M Sulfuric acid (Stop solution)**. Ready to use.

Note: Use only components from kits with the same lot number. Do not mix components from different lots of kits when running the assay.

REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED:

- **8-channel** or repeating **pipette** allowing dispensing 50-300 µl.
- **1-channel 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.
- Micro ELISA plate **reader** with a wavelength set up at 450 nm.
- Distilled water.

REAGENT 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 (minigrip).
2. **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. This reagent contains 0.05% Kathon CG.
3. **Annexin V Standard:** restore each vial with **2 ml** Sample Diluent. This solution is stable for at least **4 hours** at room temperature.
4. **Plasma Control I** (human plasma, **UTA, high**): restore with **1 ml** distilled water.
5. **Plasma Control II** (human plasma, **UTA, low**): restore with **1 ml** distilled water.

   Note: when restored, Annexin V controls are stable for **8 hours** at room temperature, **24 hours at 2-8°C** or **2 months** frozen at **–20°C** or below.

Warning: Controls (4&5) are prepared with normal human plasma. This latter was tested with registered methods and found negative for HIV antibodies, HBs Ag and HVC 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.

6. **Anti-Annexin V-HRP immunoconjugate**: each vial must be restored with **7.5 ml of Conjugate Diluent**. Let the pellet completely dissolve before use, and shake the vial gently in order to homogenize the contents. The restored conjugate is stable for at least **24 hours** at room temperature or for at least **4 weeks at 2-8°C**.
7. **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.
8. **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.
9. **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.
10. **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.

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

PROCEDURE:

Specimen 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 4 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.
Use of EDTA anticoagulant, or supplementation of citrated blood with 0.025M Na_2 EDTA (at pH 7.5) final concentration, allows obtaining a better recovery of Annexin V, a calcium dependent protein, in plasma (ref. 6).

Tested plasma or sample:
The sample must be tested diluted **two fold** (**1:2**) in the Sample Diluent. For expected Annexin V concentrations >20 ng/ml, plasma or samples can be diluted **1:5, or 1:10, or 1:20.**
Controls I and II must be tested diluted **two fold (1:2)** as for plasmas.

Calibration:
Using the Annexin V standard provided in the kit, (Annexin V Ag concentration "**C**" indicated, for each lot of reagents, on the flyer provided in the kit), prepare the following standard solutions:

Annexin V concentration (ng/ml)	C	C/2	C/4	C/10	C/20	0
Vol. of Annexin V Std at "C" ng/ml	1 ml	0.5 ml	0.25 ml	0.1 ml	0.05 ml	0 ml
Vol. of Sample Diluent	0 ml	0.5 ml	0.75 ml	0.9 ml	0.95 ml	1 ml

Mix gently for a complete homogenization.
The standard dilutions are stable for at least **4 hours** at room temperature.

ASSAY PROCEDURE:

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:

Reagent	Volume	Procedure
Annexin V standard solution or **tested sample or controls**	**200 µl**	Introduce the standard solutions or the tested samples in the corresponding micro ELISA plate well **(a)**.
Incubate for 1 hour at room temperature (18-25 °C) (b)		
Wash Solution (20 fold diluted in distilled water)	**300 µl**	Proceed to 5 successive washings using the washing instrument **(c)**.
Conjugate (anti Annexin V polyclonal antibody coupled with peroxidase. Restored with 7.5 ml of Conjugate Diluent)	**200 µl**	Introduce the Anti-Annexin V HRP immunoconjugate in the micro ELISA plate wells **(c)**.
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 **(c)**.
TMB / H_2O_2 Substrate	**200 µl**	Immediately after the washing, introduce the substrate into the wells. ***Note:*** The substrate distribution, row by row, must be accurate and at exact time intervals **(c,d)**.
Incubate for exactly 5 minutes at room temperature (18-25 °C) (b)		
0.45 M 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
Wait for 10 minutes in order to allow the colour to stabilize and measure absorbance at **450 nm (A450) (e)**.		

Note:

a) The two fold dilutions can be performed directly into the reactive well by introducing 100 µl of Sample Diluent and 100 µl of tested plasma sample or control. Calibrators, controls and tested specimen must be distributed as rapidly as possible on the micro Elisa plate (within 10 min.) in order to allow obtaining homogeneous immunological kinetics for immunocapture.

b) Avoid letting the plate in the bright sunlight during incubations and more particularly during colour development. A micro-ELISA plate shaker can be used. In the same way, if a microplate shaker is used, it should be used only at the beginning of each step (sample introduction, immunoconjugate introduction, stop solution introduction), for 1 to 2 minutes, in order to obtain a good homogeneity. A450 values generated in the assay are significantly increased if shaking is used throughout the incubation steps.

c) 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.

d) 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.

e) For bichromatic readings, a reference wavelength at 690 nm or at 620 nm can be used.

RESULTS:

Users must construct their own calibration curve obtained using their standard dilutions (see flyer).

- On a linear graph paper plot the **Annexin V concentration** (ng/ml) on abscissae and the corresponding absorbance on ordinates.
- From the curve obtained, deduce the Annexin V concentration for the tested dilution. For obtaining the Annexin V concentration in the tested sample, this value must be multiplied by the dilution factor **(i.e., 2, 5, 10, or 20).**

Alternatively, an ELISA software (i.e., Dynex, Biolise, etc.) can be used for the calculation of concentrations.

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

ASSAY REACTIVITY:

The ZYMUTEST Annexin V test measures recombinant Annexin V as well as native Annexin V in plasma.

BIOCHEMISTRY OF ANNEXIN V:

Annexin V is a calcium dependent protein with a MW of 35 K. daltons, present in many tissues and mainly in endothelial cells and in placenta. It is present at low concentrations in platelets and at higher concentrations in red blood cells and leukocytes. It binds to anionic phospholipids and to activated cell membranes in a calcium dependent manner (ref. 1,2). It inhibits procoagulant and pro-inflammatory activities of dying cells during apoptosis (ref. 4). Annexin V concentration in normal human plasma is <10 ng/ml (ref. 3).

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