PRODUCT INFORMATION

Annexin V (Human) ELISA

Enzyme-linked immunosorbent assay for

detection of human Annexin V.

For research use only, not for use in diagnostic procedures.

REF ***IB49646***

Manufactured for:
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1. Intended Use

The human Annexin V ELISA is an enzyme-linked immunosorbent assay for the detection of human Annexin V. **For research use only, not for use in diagnostic procedures.**

2. Principle of the Test

An anti-human Annexin V coating antibody is adsorbed onto microwells.

Figure 1 **Coated Microwell**

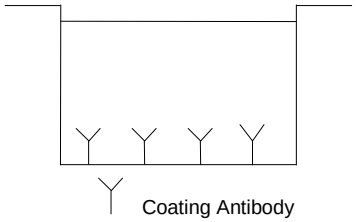


Figure 2 **First Incubation**

Human Annexin V present in the unknown or calibrator binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human Annexin V antibody is added and binds to human Annexin V captured by the first antibody.

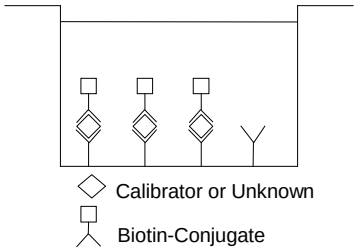


Figure 3 **Second Incubation**

Following incubation unbound biotin-conjugated anti-human Annexin V antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human Annexin V antibody.

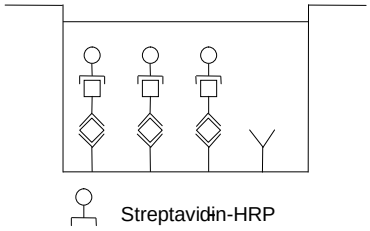


Figure 4 **Third Incubation**

Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

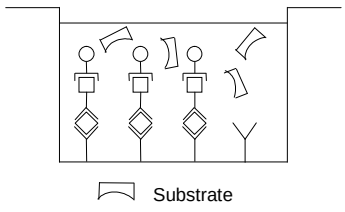
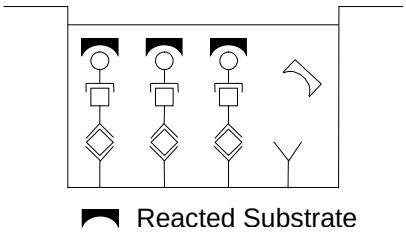


Figure 5

A coloured product is formed in proportion to the amount of human Annexin V present in the unknown or calibrator. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A calibrator curve is prepared from 7 human Annexin V calibrator dilutions and human Annexin V unknown concentration determined.



3. Reagents Provided

1 aluminum pouch with a **Microwell Plate coated** with monoclonal antibody to human Annexin V

1 vial (100 µl) **Biotin-Conjugate** anti-human Annexin V monoclonal antibody

1 vial (150 µl) **Streptavidin-HRP**

2 vials human Annexin V **Calibrator** lyophilized, 100 ng/ml upon reconstitution

1 vial (12 ml) **Sample Diluent**

1 vial (5 ml) **Assay Buffer Concentrate** 20x (PBS with 1% Tween 20 and 10% BSA)

1 bottle (50 ml) **Wash Buffer Concentrate** 20x (PBS with 1% Tween 20)

1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)

1 vial (12 ml) **Stop Solution** (1M Phosphoric acid)

1 vial (0.4 ml) **Blue-Dye**

1 vial (0.4 ml) **Green-Dye**

1 vial (0.4 ml) **Red-Dye**

4 **Adhesive Films**

4. Storage Instructions – ELISA Kit

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on labels. Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, this reagent is not contaminated by the first handling.

5. Collection and Handling of Unknowns

Cell culture supernatant and serum were tested with this assay. Other biologicals might be suitable for use in the assay. Remove serum from the clot as soon as possible after clotting.

Pay attention to a possible **“Hook Effect”** due to high unknown concentrations (see chapter 10).

Unknowns containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic unknowns. Unknowns should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human Annexin V. If unknowns are to be run within 24 hours, they may be stored at 2° to 8°C. Avoid repeated freeze-thaw cycles. Prior to assay, the frozen unknown should be brought to room temperature slowly and mixed gently.

6. Materials Required But Not Provided

- 5 ml and 10 ml graduated pipettes
- 5 µl to 1000 µl adjustable single channel micropipettes with disposable tips
- 50 µl to 300 µl adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

7. Precautions for Use

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or unknowns are handled.
- Avoid contact of skin or mucous membranes with kit reagents or unknowns.
- Rubber or disposable latex gloves should be worn while handling kit reagents or unknowns.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or unknowns which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.

- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose unknowns and all potentially contaminated materials as they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

8. Preparation of Reagents

Buffer concentrates should be brought to room temperature and should be diluted before starting the test procedure.

8.1 Wash Buffer

If crystals have formed in the **Wash Buffer Concentrate**, warm it gently until they have completely dissolved. Pour entire contents (50 ml) of the Wash Buffer Concentrate into a clean 1000 ml graduated cylinder. Bring to final volume of 1000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. The pH of the final solution should adjust to 7.4. Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer is stable for 30 days.

Wash Buffer may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

8.2 Assay Buffer (1x)

Pour the entire contents (5 ml) of the **Assay Buffer Concentrate** (20x) into a clean 100 ml graduated cylinder. Bring to final volume of 100 ml with distilled water. Mix gently to avoid foaming. Store at 2° to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.

Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

8.3 Biotin-Conjugate

Please note that the Biotin-Conjugate should be used within 30 minutes after dilution.
Make a 1:100 dilution of the concentrated **Biotin-Conjugate** solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

8.4 Streptavidin-HRP

Please note that the Streptavidin-HRP should be used within 30 minutes after dilution.
Make a 1:200 dilution of the concentrated **Streptavidin-HRP** solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

8.5 Human Annexin V Calibrator

Reconstitute **human Annexin V calibrator** by addition of Assay Buffer (1x). Reconstitution volume is stated on the label of the calibrator vial. Swirl or mix gently to insure complete and homogeneous solubilisation (concentration of reconstituted calibrator = 100 ng/ml). The calibrator has to be used immediately after reconstitution and cannot be stored.

8.5.1 Calibrator Dilution

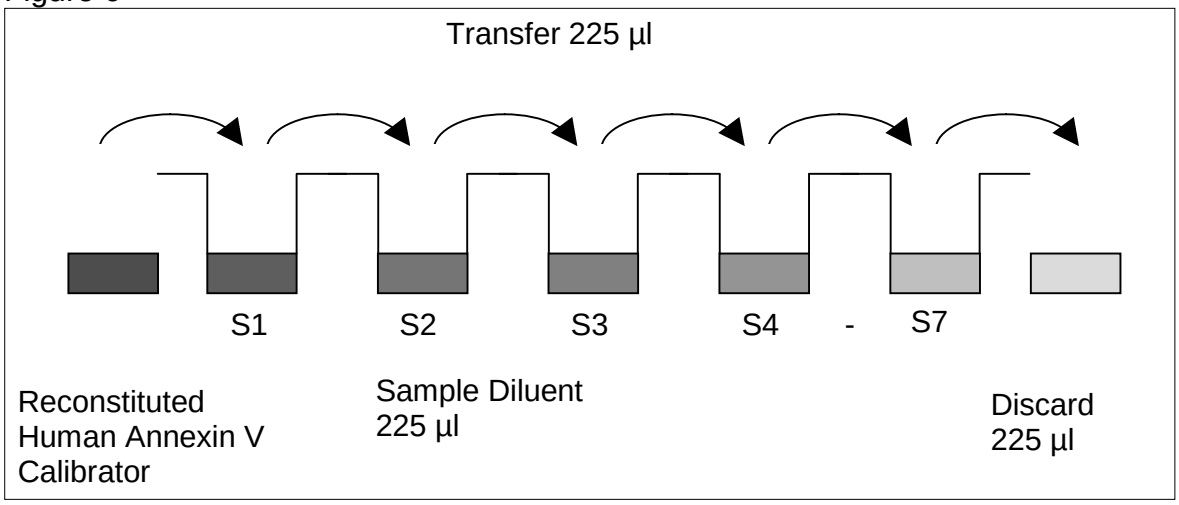
Label 7 tubes, one for each calibrator point.

S1, S2, S3, S4, S5, S6, S7

Then prepare 1:2 serial dilutions for the calibration curve as follows:
Pipette 225 µl of Sample Diluent into each tube.

Pipette 225 µl of reconstituted calibrator (concentration = 100 ng/ml) into the first tube, labelled S1, and mix (concentration of calibrator 1 = 50 ng/ml).
Pipette 225 µl of this dilution into the second tube, labelled S2, and mix thoroughly before the next transfer.
Repeat serial dilutions 5 more times thus creating the points of the calibrator curve (see Figure 6).
Sample Diluent serves as blank.

Figure 6



8.6 Addition of Colour-giving Reagents: Blue-Dye, Green-Dye, Red-Dye

In order to help our customers to avoid any mistakes in pipetting the IBL-America ELISAs, IBL-America offers a tool that helps to monitor the addition of even very small volumes of a solution to the reaction well by giving distinctive colours to each step of the ELISA procedure. **This procedure is optional**, does not in any way interfere with the test results, and is designed to help the customer with the performance of the test, but can also be omitted, just following the instruction booklet.

Alternatively, the dye solutions from the stocks provided (***Blue-Dye, Green-Dye, Red-Dye***) can be added to the reagents according to the following guidelines:

1. Diluent: Before calibrator and unknown dilution add the ***Blue-Dye*** at a dilution of 1:250 (see table below) to the appropriate diluent (1x) according to the test protocol. After addition of ***Blue-Dye***, proceed according to the instruction booklet.

5 ml Sample Diluent	20 µl ***Blue-Dye***
12 ml Sample Diluent	48 µl ***Blue-Dye***
50 ml Sample Diluent	200 µl ***Blue-Dye***

2. Biotin-Conjugate: Before dilution of the concentrated Biotin-Conjugate, add the ***Green-Dye*** at a dilution of 1:100 (see table below) to the Assay Buffer (1x) used for the final conjugate dilution. Proceed after addition of ***Green-Dye*** according to the instruction booklet: Preparation of Biotin-Conjugate.

3 ml Assay Buffer (1x)	30 µl ***Green-Dye***
6 ml Assay Buffer (1x)	60 µl ***Green-Dye***

3. Streptavidin-HRP: Before dilution of the concentrated Streptavidin-HRP, add the ***Red-Dye*** at a dilution of 1:250 (see table below) to the Assay Buffer (1x) used for the final Streptavidin-HRP dilution. Proceed after addition of ***Red-Dye*** according to the instruction booklet: Preparation of Streptavidin-HRP.

6 ml Assay Buffer (1x)	24 µl ***Red-Dye***
12 ml Assay Buffer (1x)	48 µl ***Red-Dye***

9. Test Protocol

1. Determine the number of microwell strips required to test the desired number of unknowns plus appropriate number of wells needed for running blanks and calibrators. Each unknown, calibrator, blank and optional control should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.

2. Wash the microwell strips twice with approximately 400 µl **Wash** Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about **10 – 15 seconds** before aspiration. Take care not to scratch the surface of the microwells. After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. **Do not allow wells to dry**.

3. Pipette 100 µl of calibrator dilutions (see section 8.5.1) in the calibrator wells according to Table 1.

   Table 1
   Table depicting an example of the arrangement of blanks, calibrators and unknowns in the microwell strips:

	1	**2**	**3**	**4**
A	Calibrator 1 (50.00 ng/ml)	Calibrator 1 (50.00 ng/ml)	Unknown 1	Unknown 1
B	Calibrator 2 (25.00 ng/ml)	Calibrator 2 (25.00 ng/ml)	Unknown 2	Unknown 2
C	Calibrator 3 (12.50 ng/ml)	Calibrator 3 (12.50 ng/ml)	Unknown 3	Unknown 3
D	Calibrator 4 (6.25 ng/ml)	Calibrator 4 (6.25 ng/ml)	Unknown 4	Unknown 4
E	Calibrator 5 (3.13 ng/ml)	Calibrator 5 (3.13 ng/ml)	Unknown 5	Unknown 5
F	Calibrator 6 (1.56 ng/ml)	Calibrator 6 (1.56 ng/ml)	Unknown 6	Unknown 6
G	Calibrator 7 (0.78 ng/ml)	Calibrator 7 (0.78 ng/ml)	Unknown 7	Unknown 7
H	Blank	Blank	Unknown 8	Unknown 8

4. Add 100 µl of **Sample Diluent** in duplicate to the **blank wells**.

5. Add 50 µl of **Sample Diluent** to the wells designated for unknowns being tested.

6. Add 50 µl of each **unknown** in duplicate to the appropriately assigned wells.

7. Prepare **Biotin-Conjugate** (see Preparation of Biotin-Conjugate 8.3).

8. Add 50 µl of **Biotin-Conjugate** to all wells.

9. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 2 hours, if available on a microplate shaker set at 100 rpm.

10. Prepare **Streptavidin-HRP** (refer to Preparation of Streptavidin-HRP 8.4).

11. Remove adhesive film and empty wells. **Wash** microwell strips 4 times according to point 2. of the test protocol. Proceed immediately to the next step.

12. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the blank wells.

13. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour, if available on a microplate shaker set at 100 rpm.

14. Remove adhesive film and empty wells. **Wash** microwell strips 4 times according to point 2. of the test protocol. Proceed immediately to the next step.

15. Pipette 100 µl of **TMB Substrate Solution** to all wells.

16. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 min. Avoid direct exposure to intense light.

    The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for colour development has to be done individually for each assay.

    It is recommended to add the stop solution when the highest calibrator has developed a dark blue colour. Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Calibrator 1 has reached an OD of 0.6 – 0.65.

17. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.

18. Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the unknowns and the calibrators.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

10. Results

- Calculate the average absorbance values for each set of duplicate calibrators and unknowns. Duplicates should be within 20 percent of the mean value.

- Create a calibration curve by plotting the mean absorbance for each calibrator concentration on the ordinate against the human Annexin V concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).

- To determine the concentration of circulating human Annexin V for each unknown, first find the mean absorbance value on the ordinate and extend a horizontal line to the calibration curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding human Annexin V concentration.

- **If instructions in this protocol have been followed unknowns have been diluted 1:2 (50 µl unknown + 50 µl Sample Diluent), the concentration read from the calibration curve must be multiplied by the dilution factor (x 2).**

- **Calculation of unknowns with a concentration exceeding calibrator 1 may result in incorrect, low human Annexin V levels (Hook Effect). Such unknowns require further external predilution according to expected human Annexin V values with Sample Diluent in order to precisely determine the actual human Annexin V level.**

- It is suggested that each testing facility establishes a control of known human Annexin V concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.

11. Limitations

- Since exact conditions may vary from assay to assay, a calibration curve must be established for every run.

- Bacterial or fungal contamination of either screen unknowns or reagents or cross-contamination between reagents may cause erroneous results.

- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

- Improper or insufficient washing at any stage of the procedure may result in inaccurate results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

- Human anti-mouse IgG antibodies (HAMA) may interfere with assays utilizing murine monoclonal antibodies leading to inaccurate results. Serum unknowns containing antibodies to murine immunoglobulins can still be analyzed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the unknown.

12. Performance Characteristics

12.1 Spike Recovery

The spike recovery was evaluated by spiking 4 levels of human Annexin V into different unknowns. Recoveries were determined in 3 independent experiments with 4 replicates each. The unspiked unknown was used as blank in these experiments. The overall mean recovery was 85%.

12.2 Dilution Parallelism

4 unknowns with different levels of human Annexin V were analyzed at serial 2 fold dilutions with 4 replicates each. The overall mean recovery was 89%.

12.3 Stability of Unknowns

12.3.1 Freeze-Thaw Stability

Aliquots of unknown (spiked or unspiked) were stored at -20°C and thawed 5 times, and the human Annexin V levels determined. A significant decrease of human Annexin V immunoreactivity was detected. Therefore unknowns should be stored in aliquots at -20°C and thawed only once.

12.3.2 Storage Stability

Aliquots of unknown (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the human Annexin V level determined after 24 h. There was no significant loss of human Annexin V immunoreactivity detected during storage under above conditions.

12.4 Specificity

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human Annexin V positive serum. There was no cross-reactivity detected, notably not with Annexin VIII (Vac beta).

13. Ordering Information

This kit is manufactured for Immuno-Biological Laboratories, Inc. (IBL-America). For ordering information, please contact:

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