



ELISA PRODUCT INFORMATION & MANUAL

Human Annexin A5 ELISA Kit *NBP2-60643*

Enzyme-linked Immunosorbent Assay for quantitative detection of Human Annexin A5. For research use only.
Not for diagnostic or therapeutic procedures.

Assay Summary

Step 1. Add 50 μ l of Standard or Sample per well.
Incubate 2 hours.

Step 2. Wash, then add 50 μ l of Biotinylated Antibody per well.
Incubate 1 hour.

Step 3. Wash, then add 50 μ l of SP Conjugate per well.
Incubate 30 minutes.

Step 4. Wash, then add 50 μ l of Chromogen Substrate per well.
Incubate 15 minutes.

Step 5. Add 50 μ l of Stop Solution per well.
Read at 450 nm immediately.

Human Annexin A5 ELISA Kit

Catalog No. **NBP2-60643**

Sample insert for reference use only

Introduction

Annexin V (Annexin A5, ANXA5) is a calcium dependent phospholipid binding protein of the annexin group. Although little is known about the function of this protein, it has been proposed that calcium-specific adsorption of annexin A5 to phospholipid monolayers induces polymerization of the protein in the plane of adsorption, potentially exerting a biological effect on the membrane itself or upon membrane-bound proteins. Electron image analysis has shown that annexin A5 molecules form trimers in a triskelion pattern on lipid monolayers (1). Annexin A5 also displays upregulation and has been shown to interact with kinase insert domain receptor and integrin beta 5 (2-4).

Principle of the Assay

The Human Annexin A5 ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for detection of annexin A5 in human **plasma, serum, saliva, and cell culture samples**. This assay employs a quantitative **sandwich enzyme immunoassay** technique that measures human annexin A5 in approximately 4 hours. A polyclonal antibody specific for human annexin A5 has been pre-coated onto a 96-well microplate with removable strips. Annexin A5 in standards and samples is sandwiched by the immobilized antibody and a biotinylated polyclonal antibody specific for human annexin A5, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Caution and Warning

- This product is for **Research Use Only** and is not intended for use in diagnostic procedures.
- Prepare all reagents (diluent buffer, wash buffer, standard, biotinylated antibody, and SP conjugate) as instructed, prior to running the assay.
- Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this insert. However, the user should determine the optimal dilution factor.
- Spin down the SP conjugate vial and the biotinylated antibody vial before opening and using contents.
- The Stop Solution is an acidic solution.

- The kit should not be used beyond the expiration date.

Reagents

- **Human Annexin A5 Microplate:** A 96-well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against human annexin A5.
- **Sealing Tapes:** Each kit contains 3 precut, pressure sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **Human Annexin A5 Standard:** Human annexin A5 in a buffered protein base (192 ng, lyophilized, 2 vials).
- **Biotinylated Human Annexin A5 Antibody (50x):** A 50-fold biotinylated polyclonal antibody against human annexin A5 (120 μ l).
- **EIA Diluent Concentrate (10x):** A 10-fold concentrated buffered protein base (20 ml).
- **Wash Buffer Concentrate (20x):** A 20-fold concentrated buffered surfactant (30 ml, 2 bottles).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (80 μ l).
- **Chromogen Substrate:** A ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).
- **Stop Solution:** A 0.5 N hydrochloric acid to stop the chromogen substrate reaction (12 ml).

Storage Condition

- Upon arrival, immediately store components of the kit at recommended temperatures up to the expiration date.
- Store Standard, SP Conjugate, and Biotinylated Antibody at -20°C.
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8°C.
- Unused microplate wells may be returned to the foil pouch with the desiccant packs and resealed. May be stored for up to 30 days in a vacuum desiccator.
- Diluent (1x) may be stored for up to 30 days at 2-8°C.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm.
- Pipettes (1-20 μ l, 20-200 μ l, 200-1000 μ l, and multiple channel).
- Deionized or distilled reagent grade water.

Sample Collection, Preparation, and Storage

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 3000 x *g* for 10 minutes and collect plasma. The sample is suggested for use at 1x; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles (EDTA or Heparin can also be used as an anticoagulant).
- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3000 x *g* for 10 minutes and remove serum. The sample is suggested for use at 1x; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Saliva:** Collect saliva using sample tube. Centrifuge samples at 800 x *g* for 10 minutes. The sample is suggested for use at 1x; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Cell Culture Supernatants:** Centrifuge cell culture media at 3000 x *g* for 10 minutes at 4°C to remove debris and collect supernatants. Samples can be stored at -20°C or below. Avoid repeated freeze-thaw cycles.

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- **EIA Diluent Concentrate (10x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the EIA Diluent Concentrate 10-fold with reagent grade water. Store for up to 30 days at 2-8°C.
- **Human Annexin A5 Standard:** Reconstitute the Human Annexin A5 Standard (192 ng) with 1.5 ml of EIA Diluent to generate a 128 ng/ml standard stock solution. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. The standard stock solution (128 ng/ml) should be further diluted 4-fold with EIA Diluent to produce a 32 ng/ml standard working solution. Prepare duplicate or triplicate standard points by serially diluting the standard working solution (32 ng/ml) 2-fold with EIA Diluent to produce 16, 8, 4, 2, 1, and 0.5 ng/ml solutions. EIA Diluent serves as the zero standard (0 ng/ml). Aliquot remaining stock solution to limit repeated freeze-thaw cycles. This solution should be stored at -20°C and **used within 48 hours**.

Standard Point	Dilution	[ANXA5] (ng/ml)
P1	1 part Standard (128 ng/ml) + 3 parts EIA Diluent	32
P2	1 part P1 + 1 part EIA Diluent	16
P3	1 part P2 + 1 part EIA Diluent	8.0
P4	1 part P3 + 1 part EIA Diluent	4.0
P5	1 part P4 + 1 part EIA Diluent	2.0
P6	1 part P5 + 1 part EIA Diluent	1.0
P7	1 part P6 + 1 part EIA Diluent	0.5
P8	EIA Diluent	0.0

- **Biotinylated Human Annexin A5 Antibody (50x):** Spin down the antibody briefly and dilute the desired amount of the antibody 50-fold with EIA Diluent. The undiluted antibody should be stored at -20°C.
- **Wash Buffer Concentrate (20x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the Wash Buffer Concentrate 20-fold with reagent grade water.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 100-fold with EIA Diluent. The undiluted conjugate should be stored at -20°C.

Assay Procedure

- Prepare all reagents, standard solutions, and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-25°C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccants inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 50 µl of Human Annexin A5 Standard or sample to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for 2 hours. Start the timer after the last addition.
- Wash five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If using a machine, wash six times with 300 µl of Wash Buffer and then invert the plate, decanting the contents; hit 4-5 times on absorbent material to completely remove the liquid.
- Add 50 µl of Biotinylated Human Annexin A5 Antibody to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for 1 hour.
- Wash the microplate as described above.

- Add 50 μ l of Streptavidin-Peroxidase Conjugate to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash the microplate as described above.
- Add 50 μ l of Chromogen Substrate to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Incubate for 15 minutes or until the optimal blue color density develops.
- Add 50 μ l of Stop Solution to each well. The color will change from blue to yellow. Gently tap plate to ensure thorough mixing. Break any bubbles that may have formed.
- Read the absorbance on a microplate reader at a wavelength of 450 nm **immediately**. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample.
- To generate a standard curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance (OD) on the y-axis. The best-fit line can be determined by regression analysis using log-log or four-parameter logistic curve-fit.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

Typical Data

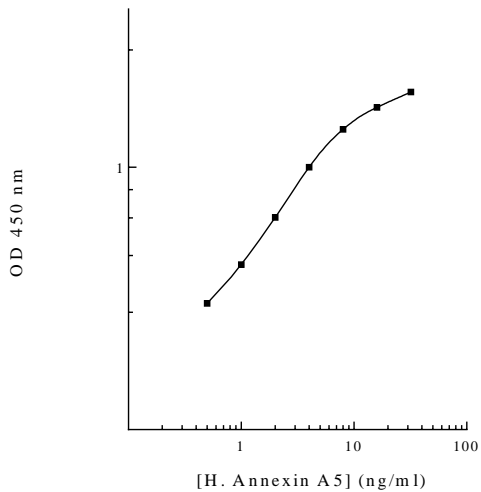
- The typical data is provided for reference only. Individual laboratory means may vary from the values listed. Variations between laboratories may be caused by technique differences.

Standard Point	ng/ml	OD	Average OD
P1	32	1.942 1.923	1.933
P2	16	1.700 1.679	1.690
P3	8.0	1.399 1.387	1.393
P4	4.0	1.010 0.990	1.000
P5	2.0	0.649 0.637	0.643
P6	1.0	0.431 0.419	0.425
P7	0.5	0.308 0.298	0.303
P8	0.0	0.166 0.165	0.166
Sample: Pooled Normal Sodium Citrate Plasma (1x)		0.496 0.517	0.507

Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.

Human Annexin A5 Standard Curve



Reference Value

- Human plasma and serum samples from healthy adults were tested (n=40). On average, annexin A5 level was 1.6 ng/ml.

Performance Characteristics

- The minimum detectable dose of annexin A5 as calculated by 2SD from the mean of a zero standard was established to be 0.15 ng/ml.
- Intra-assay precision was determined by testing three plasma samples twenty times in one assay.
- Inter-assay precision was determined by testing three plasma samples in twenty assays.

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	20	20	20	20	20	20
CV (%)	3.2%	3.2%	3.5%	8.7%	9.0%	9.7%
Average CV (%)	3.3%			9.1%		

Recovery

Standard Added Value	1 – 10 ng/ml
Recovery %	89 – 114%
Average Recovery %	97%

Linearity

- Plasma and serum samples were serially-diluted to test for linearity.

Average Percentage of Expected Value (%)		
Sample Dilution	Plasma	Serum
1x	101%	99%
2x	104%	105%
4x	105%	107%

Cross-Reactivity

Species	Cross Reactivity (%)
Bovine	None
Monkey	>80%
Mouse	<10%
Rat	<30%
Swine	>80%
Rabbit	None
Canine	>80%

- No significant cross reactivity observed with annexin A1, A2, A3, A4, A10, and A13.

Troubleshooting

Issue	Causes	Course of Action
Low Precision	Use of expired components	<ul style="list-style-type: none"> Check the expiration date listed before use. Do not interchange components from different lots.
	Improper wash step	<ul style="list-style-type: none"> Check that the correct wash buffer is being used. Check that all wells are empty after aspiration. Check that the microplate washer is dispensing properly. If washing by pipette, check for proper pipetting technique.
	Splashing of reagents while loading wells	<ul style="list-style-type: none"> Pipette properly in a controlled and careful manner.
	Inconsistent volumes loaded into wells	<ul style="list-style-type: none"> Pipette properly in a controlled and careful manner. Check pipette calibration. Check pipette for proper performance.
	Insufficient mixing of reagent dilutions	<ul style="list-style-type: none"> Thoroughly agitate the lyophilized components after reconstitution. Thoroughly mix dilutions.
	Improperly sealed microplate	<ul style="list-style-type: none"> Check the microplate pouch for proper sealing. Check that the microplate pouch has no punctures. Check that three desiccants are inside the microplate pouch prior to sealing.
Unexpectedly Low or High Signal Intensity	Microplate was left unattended between steps	<ul style="list-style-type: none"> Each step of the procedure should be performed uninterrupted.
	Omission of step	<ul style="list-style-type: none"> Consult the provided procedure for complete list of steps.
	Steps performed in incorrect order	<ul style="list-style-type: none"> Consult the provided procedure for the correct order.
	Insufficient amount of reagents added to wells	<ul style="list-style-type: none"> Check pipette calibration. Check pipette for proper performance.
	Wash step was skipped	<ul style="list-style-type: none"> Consult the provided procedure for all wash steps.
	Improper wash buffer	<ul style="list-style-type: none"> Check that the correct wash buffer is being used.
Improper reagent preparation	<ul style="list-style-type: none"> Consult reagent preparation section for the correct dilutions of all reagents. 	

	Insufficient or prolonged incubation periods	<ul style="list-style-type: none"> Consult the provided procedure for correct incubation time.
Deficient Standard Curve Fit	Non-optimal sample dilution	<ul style="list-style-type: none"> Sandwich ELISA: If samples generate OD values higher than the highest standard point (P1), dilute samples further and repeat the assay. Competitive ELISA: If samples generate OD values lower than the highest standard point (P1), dilute samples further and repeat the assay. User should determine the optimal dilution factor for samples.
	Contamination of reagents	<ul style="list-style-type: none"> A new tip must be used for each addition of different samples or reagents during the assay procedure.
	Contents of wells evaporate	<ul style="list-style-type: none"> Verify that the sealing film is firmly in place before placing the assay in the incubator or at room temperature.
	Improper pipetting	<ul style="list-style-type: none"> Pipette properly in a controlled and careful manner. Check pipette calibration. Check pipette for proper performance.
	Insufficient mixing of reagent dilutions	<ul style="list-style-type: none"> Thoroughly agitate the lyophilized components after reconstitution. Thoroughly mix dilutions.

Version 1.2