VITRONECTIN EIA Kit Manual

VITRONECTIN EIA KIT (precoated)

An enzyme immunoassay kit
for the quantitative determination of
Vitronectin (VN)

For research use only. Not for use in diagnostic or therapeutic procedures.

Catalog Number: 40-831-160002
For 96 assays

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Introduction

The adhesion protein vitronectin (VN), or serum spreading factor (identical to complement S-protein) is a major cell adhesion protein in plasma(1-7), usually present at the concentration of 200 - 300 mg/l. VN is also present in the extracellular matrix of tissues, and atherosclerotic plaques(4,5,8). VN contributes in various ways to the regulation of the immune and hemostatic systems, in particular at the blood-endothelium interphase(9). These functions include its role as complement inhibitor(10) and as “scavenger protein” for reaction products of the blood coagulation cascade such as the thrombin-antithrombin III complex(11-13). Moreover, VN constitutes the major binding and stabilizing protein for plasminogen activator inhibitor-1(14). Through binding to different cytoadhesion receptors, termed integrins(15), VN may directly interact with endothelial cells(16) and platelets(17) and thereby localize its diverse function at certain pericellular sites. In liver disorders with high fibroblastic activity such as liver cirrhosis, a significant diminution of plasma vitronectin levels was observed(18,19).

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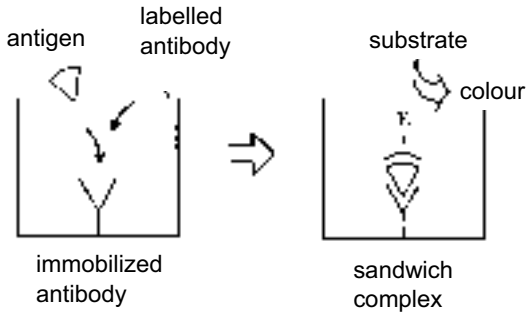
Intended use

The Vitronectin EIA Kit is an *in vitro* enzyme immunoassay (EIA) kit for quantitative determination of human vitronectin (VN) in plasma, serum, cultured cell extracts, cell culture supernatants, and other biological fluids.
This kit is for research use only. It is not for use in diagnostic or therapeutic procedures.

Principle

The VN EIA Kit is a solid phase EIA based on a sandwich method that utilizes two mouse monoclonal anti-VN antibodies to detect VN by one-step procedure. One of the antibodies has been precoated, and blocked against non-specific biuding. Then, samples, standards and peroxidase (POD)-labelled anti-VN antibody are simultaneously added to the wells of plates. During the incubation, VN is bound to anti-VN (solid phase) on one side and tagged by POD-anti-VN on the other. The reaction between POD and substrate (H_2O_2, and tetramethylbengidine) results in colour development with intensities proportional to the amount of VN present in samples and standards. The amount of VN can be quantitated by measuring the absorbance using an EIA plate reader. Accurate sample concentrations of VN can be determined by comparing their specific absorbances with those obtained for the standards plotted on a standard curve.



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Reagents and materials
Each VN EIA Kit includes reagents sufficient for 96 wells. The expiration date for the complete kit is stated on the outer box label and the recommended storage temperature is 2 - 8°C.

A. Materials provided

Plate 1. Antibody Coated Microtiterplate - 1 plate (8 well x 12 strips)
The plate contains murine monoclonal antibody to VN and blocking material. Store at 2 - 8°C.

Vial 2. Antibody-POD Conjugate - 1 vial (for 11 ml x 1)
The vial contains lyophilized horseradish peroxidase (POD) conjugated murine monoclonal antibody to VN. Store at 2 - 8°C. Avoid prolonged exposure to light.

Vial 3. Standard - 1 vial (320 ng x 1)
The vial contains lyophilized VN Standard.

Vial 4. Sample Diluent - 2 vials (11 ml x 2)
Each vial contains protein in a buffered solution. Use for Zero standard, and for dilution of the standard (vial 3) and samples which are above the calibration curve. Store at 2 - 8°C.

Vial 5. Substrate Solution - 1 vial (12 ml x 1)
Each vial contains hydrogen peroxide and tetramethylbenzidine in a buffered solution. Store at 2-8°C.

B. Materials required but not provided

1. Reagents
   - Washing Buffer: Phosphate-buffered Saline (PBS) containing 0.1% Tween 20
     (Dissolve 8.0 grams of NaCl, 0.2 grams of KCl, 2.9 grams of $Na_2HPO_4 12H_2O$ and 0.2 grams of KH_2PO_4 in 1000 ml of distilled water and add 1 ml Tween 20.)
     PBS tablet is useful to prepare washing buffer.
   - Stop solution: 1 N H_2SO_4

2. Materials
   - Precision pipettes with disposable tips: 20 and 100 µl micropipettes, 10 - 200 µl adjustable multiwell pipetter or 20 µl and 100 µl multiwell pipetters
   - Beakers, flasks, cylinders necessary for preparation of reagents
   - Disposable pipettes and test tubes
   - Microtiter plate reader for measurement of absorbance at 450 nm
   - Graph paper

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Precautions

- Do not mix reagents from different kit lots.
- Do not use reagents beyond expiration date on label.
- In order to avoid reagent contamination, use disposable pipette tips and/or pipettes.
- Sodium azide inactivates POD. Solutions containing sodium azide should not be used in this assay.
- Do not expose Substrate Solution to strong light during storage or incubation.
- Avoid contact of Substrate Solution and stop solution with skin or mucous membranes. If these reagents come into contact with skin, wash thoroughly with water. Do not pipette by mouth. Do not smoke, eat, or drink in area where specimens or kit reagents are handled. All blood fluids should be considered as potentially infectious.
- Avoid contact of, Substrate Solution and Stop Solution with any metal surfaces. Disposable glassware or test tubes are recommended for, handling the Substrate Diluent. If non-disposable glass ware is used it must be acid washed and thoroughly rinsed with distilled, deionized water.
- Do not use the Substrate Solution if its colour is changed to thick blue.

Specimen collection and handling

Venous blood samples are collected aseptically. EDTA, or citrated plasma is suitable for use in the assay, however, serum, cultured cell extracts or cell culture supernatant can be also used. Remove the serum or plasma from the clot or red cells, respectively, soon after clotting and separation. Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens. Samples may be stored up to 24 hours at 4°C. If the length of time between sample collection and assay is to exceed 24 hours, samples should be stored frozen under -20°C for optimal results. Excessive freeze-thaw cycles should be avoided. Prior to assay, frozen samples should be brought to room temperature slowly, and gently mixed by hand. Do not thaw samples in a hot bath. Do not vortex or sharply agitate. PBS containing 1% NP40, 1 mM EDTA and 1mM Phenylmethylsulfonyl fluoride (pH7.2) should be used for preparation of cell extracts.

Recommended Sample Dilution

In case of using serum or plasma, dilute the samples by 500-1000 folds before the assay. When the diluted samples generate values out of the standard range, dilute the samples with the different dilution rate referring to the first assay result, and repeat the assay. Or it is recommended to assay using three kinds of sample dilutions making the 500-1000 fold as the middle concentration.

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Preparation of solutions

Note: The following solutions should be prepared directly before use.

Solution 1. Antibody-POD Conjugate Solution
Dissolve the contents of Vial 2 in 11 ml distilled water and mix gently followed by 10 min slowly rolling or occasional mixing, avoiding foam formation.

Solution 2. Standard Solution
Rehydrate Standard (Vial 3) with 1 ml distilled water. Slowly roll for approximately 10 min or let stand and sporadically mix gently.

The standard solution contains 320 ng VN/ml. A dilution series can be formed by mixing the standard solution and Sample Diluent (Vial 4) for establishing the calibration curve, e.g.:

Final conc. (ng/ml)	0	5	10	20	40	80	160	320
Sample Diluent (Vial 4)	400 µl	393.75 µl	387.5 µl	375 µl	350 µl	300 µl	200 µl	-
Standard solution (Vial 3; 320 ng/ml)	-	6.25 µl	12.5 µl	25 µl	50 µl	100 µl	200 µl	400 µl

Solution 3 Stop Solution (1N H_2SO_4)
Add 5.8 ml concentrated H_2SO_4 carefully to approximately 180 ml of distilled water (acid MUST be added to water, not vice versa). Add distilled water to a final volume of 200 ml. Mix well.
Store at room temperature for up to 6 months.

Stability of solutions

Solution 1. The reconstituted lyophilisate is stable for 1 week stored at 4°C,or for 1 month stored at -20 °C. Do not repeat freeze-thaw cycle.

Solution 2. The reconstituted lyophilisate is stable for 1 week stored at 4°C,or for 1 month stored at -20 °C. Do not repeat freeze-thaw cycle.

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Procedure
Double determinations of all samples and standards should be performed.
All of the Kit's content should be brought to room temperature before use !
For thorough mixing, the microtiter plate can be gently agitated on a plate mixer or by mixing the plate sporadically by hand.

1. Enzyme immunoassay
- *Immunological reaction*: Transfer 100 µl of antibody-POD conjugate solution (Solution 1) into one well, and subsequently add 50 µl sample or standard (Solution 2). Mix, seal the microtiter plate (e.g. with a foil) and incubate 2 hours at room temperature (20-30°C). A sample and standard solution should be added within 5 minutes per well.
- Remove contents by suction and wash the wells 4 times with ca.400 µl of Washing Buffer Containing; between the separate washing steps empty out the microtiter plate and vigorously tap onto paper towel, especially after the last washing.
- *Substrate incubation*: Add 100 µl of Substrate Solution (vial 5) into each well and incubate at room temperature (20 - 30°C) for 15 minutes.
- Add 100 µl of Stop Solution (Solution 3) into each well in same order as for substrate. Tap plate gently to mix.
- Measure the absorbance at 450 nm with a plate reader. The absorbance should be read as soon as possible after the completion of the assay. It may be read up to 1 hour after addition of Stop Solution if wells are protected from light at room temperature.

Results
1. Standard curve
- Record the absorbance at 450 nm for each standard well.
- Average the duplicate values and record the averages.
- Plot the absorbance (vertical axis) versus the VN concentration in ng/ml (horizontal axis) for the standards using log-log scale.
2. Samples
- Record the absorbance at 450 nm for each sample well.
- Average the duplicate values and record the averages.
- Locate the average absorbance value on the vertical axis and follow a horizontal line intersecting the standard curve. At the point of intersection, read the VN concentration (ng/ml) from the horizontal axis.

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Performance characteristics

1. **Range of standard curve:** 5 - 320 ng/ml.
2. **Specificity:** This kit specifically measures VN with no detectable cross reaction with human fibronectin, laminin, collagen type I, or collagen type III. This kit cannot be used to measure mouse VN. The application of this kit for quantitating VN from other sources has not been tested.
3. **Assay duration:** Two and a half hours.
4. **Total assay capacity:** 96 assays.
5. **Assay capacity for test samples:** If all assay wells (including standards and test samples) are run in duplicate, 40 test samples can be run in duplicate per kit.
6. **Test specimen type**: Human serum or plasma. Rabbit serum or plasma.
7. **Specimen volume required:** If each test sample is run in duplicate, approximately 120 µl (i.e., 50 µl per assay well plus ~10 µl for each sample transfer) is required.
8. **Limitation:** Since conditions may vary from assay to assay, a standard curve must be established for every run. Since cross contamination between reagents will invalidate the test, disposable pipette tips should be used.

   Thorough washing of the wells between incubations is required:
   1) Completely empty out the remaining fluid from the well before dispensing fresh wash solution.
   2) Use sufficient wash solution for each wash cycle (approximately 400 µl).
   3) Do not allow wells to sit uncovered for extended periods between incubation steps.

   Only samples with absorbance values falling within the range of the standard curve should be assigned a VN concentration from the curve.

9. **Notes :** According to the assay results using control serum or urine, it could be possible to determine the concentration of antigen present in a biological. However, the measurement may be potentially disturbed by the unknown organic factors in serum, plasma or urine samples in patients with specific diseases. Similarly, a specimen obtained from an apparent healthy subject might also be interrupted. When an antigen level in an unknown organic specimen is observed to be elevated as compared to the calibration range of the standard curve, it is recommended to dilute the specimens properly with the dilution solution included in the kit and assay them again in another run.

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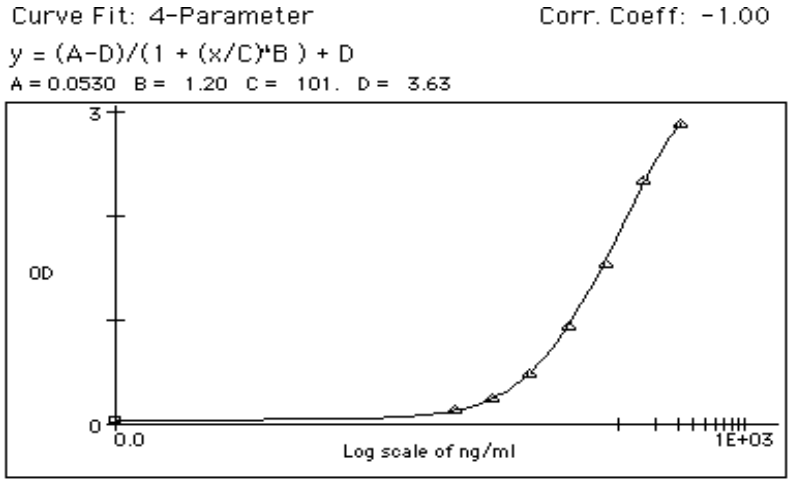
Basal data

1. Expected values (ref. 19)

The VN levels were determined in plasma of 43 healthy subjects. The mean level was 211 μg/ml (SD=42). Each laboratory should establish its own normal range for VN level.

2. Typical Standard Curve

(Do Not Use To Calculate Unknowns)



Vitronectin(ng/ml)	320	160	80	40	20	10	5	0
A.450nm	2.898	2.350	1.554	0.952	0.499	0.269	0.149	0.046

3. Intra-asay precision (n=8)

Sample	Ave. (ng/ml)	S.D.(ng/ml)	CV(%)
Control A	110.3	6.9	6.3
Control B	61.7	3.3	5.3
Control C	15.5	0.8	4.9

Intra-asay precision (performanu 3 times), (n=3)

Sample	Ave. (ng/ml)	S.D.(ng/ml)	CV(%)
Control A	100.3	9.7	9.6
Control B	61.3	1.7	2.8
Control C	15.8	0.3	2.2

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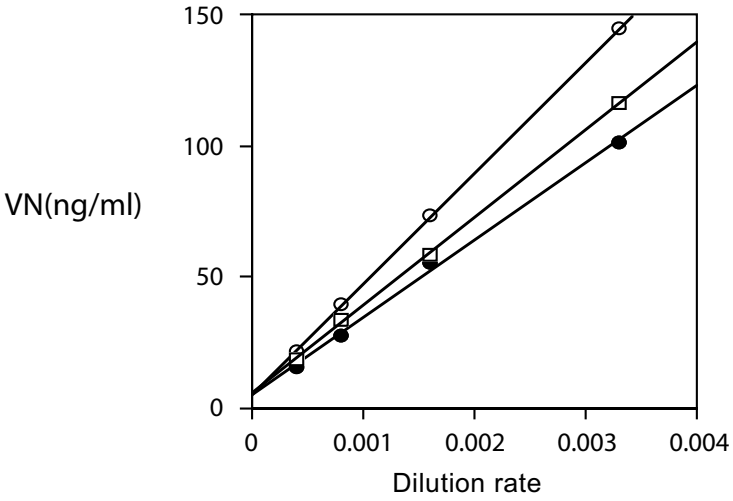
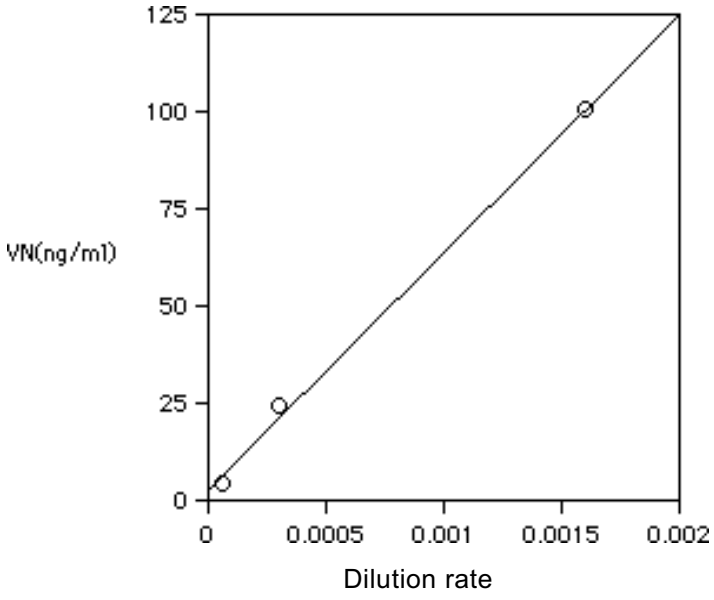
4. Dilution curves of three different human and rabbit serum samples.

Dilution curve of three different human



□	No.1	y = 33281.203x + 6.069
●	No.2	y = 29508.135x + 5.065
○	No.3	y = 42242.900x + 5.382

Dilution curve of a rabbit serum



○ y = 61031.768x + 3.290

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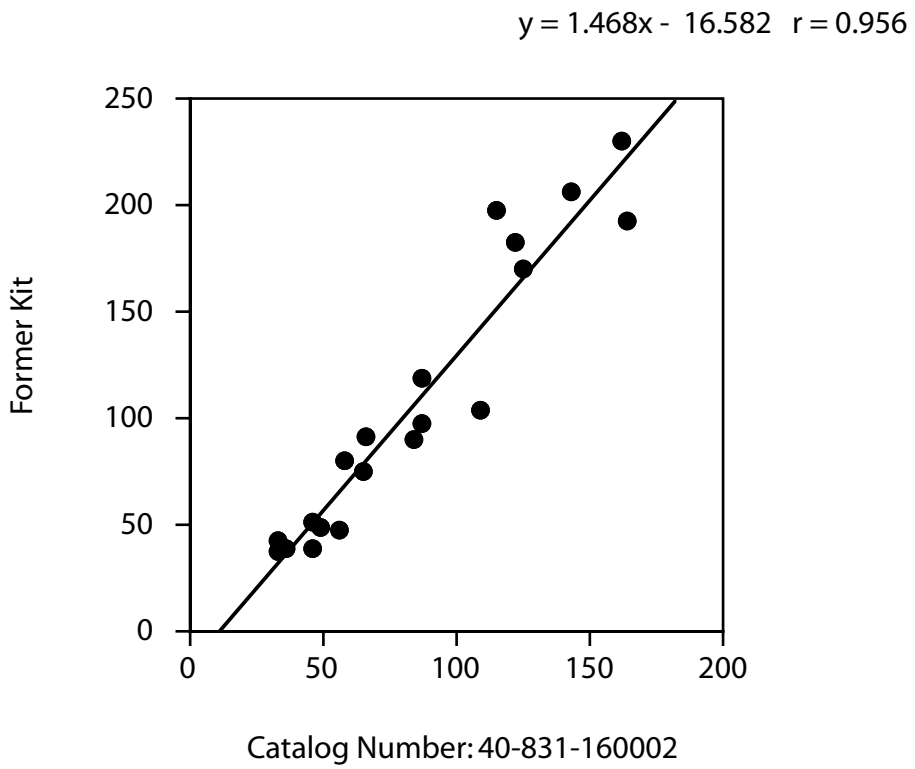
5. Correlation with a former kit
   Correlation of OPD to TMBZ in VN EIA in using them as the substrate.



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Storage and Stability

This kit is shipped at 2 - 8°C and should be stored at 2 - 8°C if not used.
Under this condition, the kit is stable until expiration date on label.

References

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Protocol summary

1. Transfer 100 µl of antibody-POD conjugate solution into appropriate wells.
2. Add 50 µl of Standard or sample to the wells within 5 minutes, and incubate 2 hours at 20-30°C.
3. Remove sample solution and wash the wells 4 times with 400 µl of PBS.
4. Add 100 µl of Substrate Solution to each well. Incubate at room temperature for 15 minutes.
5. Add 100 µl of Stop Solution to all wells. Mix gently.
6. Read at 450 nm as soon as possible.

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