Cat. # MK103

For Research Use

TaKaRa

Human Vitronectin EIA Kit

Product Manual

v201905Da

Table of Contents

I. Description

Vitronectin (VN) is a major adhesive protein in blood. It is also known as a complement-binding protein (S-protein), and is present at high concentrations in blood. VN is thought to adhere to cells with vitronectin receptors, and is involved in the immune system and blood coagulation-fibrinolytic system in blood vessels by binding to various blood coagulation factors (e.g., collagen, heparin, plasminogen activator inhibitor-1 (PAI-1), thrombin, antithrombin III complex, etc.). VN has a single polypeptide chain structure consisting of 459 amino acids (processed from a 478-amino acid precursor), and its molecular weight is 75 kD. It has an RGD (Arg-Gly-Asp) sequence at its cell-binding region, and a sequence similar to hemopexin (a heme-binding protein in blood) is repeated at the center of the region. The C-terminus has a heparin-binding domain that is hidden under native conditions, but can be exposed by treatment with denaturing reagents or by heating. Therefore, VN can be isolated with high purity by binding to a heparin column in the presence of a denaturing agent, such as urea. In some cases, the C-terminus is cleaved between amino acids 398 and 399 due to an amino acid mutation, and the resulting 65-kD protein is often present in blood in addition to the 75 kD form. However, the reason for the existence of these two structures and their physiological functions are not known.

The Human Vitronectin EIA Kit is a quantification kit that can detect two types of VN protein (65 and 75 kD) in blood using monoclonal antibodies. It can be also used for VN detection in urine and cell culture supernatant. In addition, this kit can also detect vitronectin in rabbit. Because the antibody in this kit does not cross-react with bovine vitronectin antigens, cell culture medium containing fetal bovine serum can be measured directly.

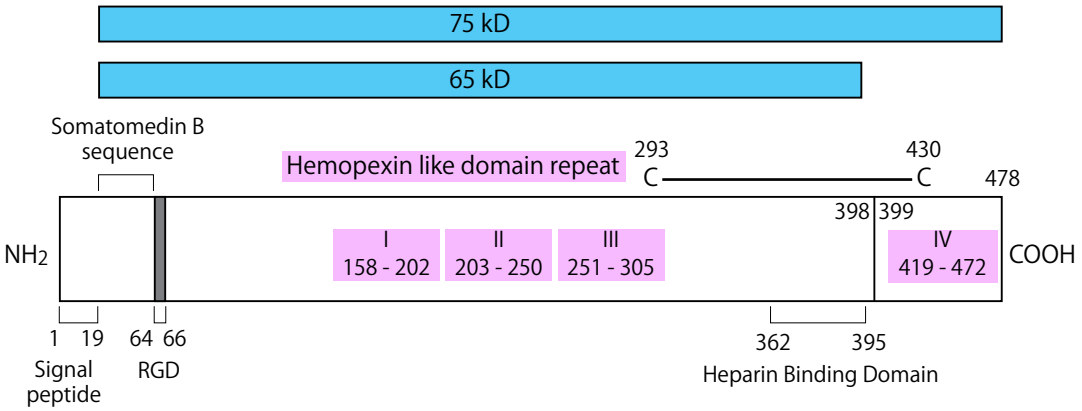


Figure 1. The structure of human vitronectin.

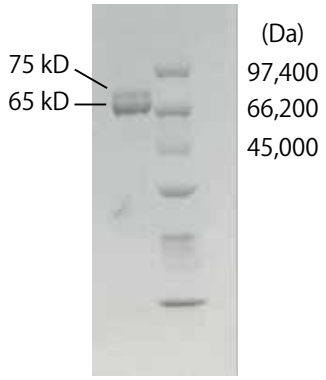
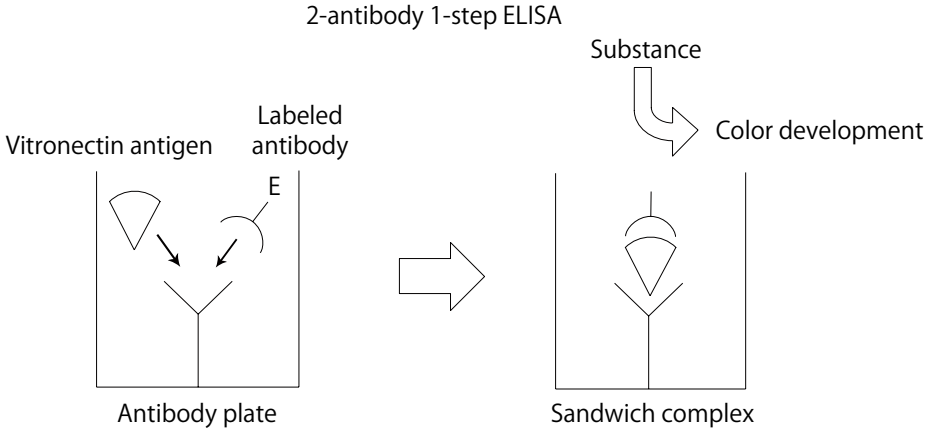


Figure 2. SDS-PAGE of vitronectin. Vitronectin was purified from human plasma using a heparin column in the presence of 8 M urea. Samples were heat treated, and SDS-PAGE was performed using a 4 - 20% gradient gel under reducing conditions.

II. Principles



III. Components

(1) Antibody Coated Microtiterplate — 1 plate
Anti-human VN monoclonal antibody coated plate
(96 wells: 8 wells x 12 strips)

(2) Antibody-POD Conjugate (lyophilized) — For 11 ml
Peroxidase-labeled anti-human VN monoclonal antibody

(3) Standard Vitronectin (lyophilized) — For 1 ml
Recombinant human VN (320 ng)
Purified VN derived from HEK293 cells (75 kDa)

(4) Sample Diluent — 11 ml x 2
PBS containing 1% bovine serum albumin and preservative

(5) Substrate Solution (TMBZ) — 12 ml
3,3′ ,5,5′ -tetramethylbenzidine solution

IV. Storage 4℃

V. Materials Required but not Provided

- Wash and Stop solution for ELISA without Sulfuric Acid (Cat. #MK021)
  Contains wash solution (10X PBS, 50 ml x 5 tubes; Tween 20, 3 ml) and reaction stop solution (60 ml).
  ＊ This product is a stop solution for peroxidase reactions without 1N sulfuric acid.
  ＊ 1N sulfuric acid can be used as a stop solution. Handle 1N sulfuric acid with caution.
- Pipette, micropipette, and tips
- Microplate reader (capable of measuring absorbance of up to 3.5 when set to 450 nm)

VI. Intended Use

Measurement of human or rabbit VN in plasma (EDTA-plasma or citrated plasma), serum, cell culture supernatant, or cultured cell extract.

<Caution>

1. It is not recommended to use heparin as an anticoagulant because VN binds to heparin.
2. VN can adhere to labware such as glass containers, etc. when stored in dilute solutions without a protective protein.
3. This kit is for research use only. It cannot be used for diagnostic purposes in humans or animals.

VII. Protocol

1. Samples

- Store specimens at 2 - 10℃; freeze samples if measurements will take place more than 12 hours after sample preparation.
- Determine the sample dilution ratio by preliminary testing. When a high value is predicted from the sample, dilute it using the provided (4) Sample Diluent.
- A normal serum sample is typically used after 500 - 1,000-fold dilution. We recommend performing a stepwise dilution. For example, first perform a 10-fold dilution and then further dilute by 50 - 100 fold.

  Do not use a sample that has hemolysis or chylopoiesis, since a low value is expected from this type of sample.
- Labeled antibodies can be affected by components in the sample, due to this product's a 1-Step method.
- VN concentration in urine samples is expected to be low, and we therefore recommend performing measurements using an undiluted sample. Confirm that the sample is not hematuric before using it for measurement.
- Avoid using samples containing sodium azide; sodium azide inhibits the POD activity of the labeled antibody. If you do use a sample containing sodium azide, use a 2-Step method for measurement (refer to Section IX-5).
- Because this kit does not cross-react with bovine antigens, samples of cell culture supernatant can be directly measured without being affected by serum in cell culture medium.
- Thaw frozen specimens at room temperature before measurement and mix gently by inversion.
- Avoid multiple freeze-thaw cycles of samples.
- When you prepare cell extracts, the following extraction solution is recommended:
  A neutral buffer (e.g., PBS, pH 7.4) containing 1% NP-40, 1 mM EDTA, and 1 mM PMSF (water soluble)

2. Reagent preparation

- Antibody plate [(1) Antibody Coated Microtiter plate]
  Before use, return to room temperature.
- Labeled antibody solution
  Dissolve (2) Antibody-POD Conjugate in 11 ml distilled water.
  The reagent is stable for 1 week at 4℃ after preparation. When storing for longer periods of time, freeze at -20℃. It is stable for 1 month in this condition. However, limit freeze-thawing to one time.

- Human VN standard solution
  Add 1 ml of distilled water to (3) Standard Vitronectin and dissolve to prepare human VN standard solution at 320 ng/ml. Then dilute stepwise with (4) Sample Diluent and prepare standard solutions at each concentration (160, 80, 40, 20, 10, and 5 ng/ml). Use (4) Sample Diluent as the zero-concentration control. Prepared VN standard solution (320 ng/ml) is stable for 1 week at 4℃ and for 1 month at -20℃. However, limit freeze-thawing to one time.
- (5) Substrate Solution (TMBZ)
  Before use, return this solution to room temperature and use as is. Confirm that the color of the solution has not changed to strong blue. Be careful not to contaminate with tap water, as coloring may occur in the presence of metal ions. If the solution will be used several times, aliquot the required amount beforehand.
- Stop solution
  Use the Stop solution included in Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021) directly.
  ＊ Because this is highly viscous, mix well using a plate mixer after its introduction.
- PBS with 0.1% Tween 20 for washing
  Dilute the 10X PBS included in Wash and Stop solution for ELISA without Sulfuric Acid (Cat. #MK021) 10 fold with distilled water, and then add Tween 20 to a final concentration of 0.1%.
  For 96 reactions performed with this kit, 300 ml of washing solution is required.

3. Procedure

Assay samples in duplicate.
Before use, allow your samples and each reagent in the kit to warm up to room tempearture, and make sure the solutions are mixed uniformly without creating bubbles.

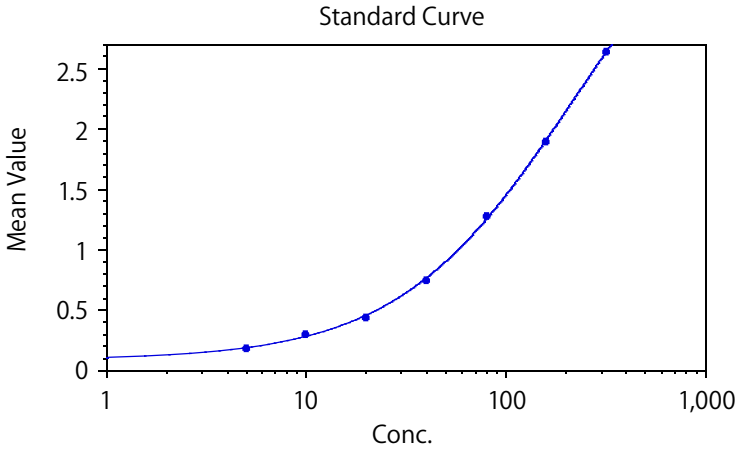
1. Dispense 100 μl of the labeled antibody solution into each well of the antibody plate with a micropipette. Add 50 μl each of VN standard solution prepared for the specimen in duplicate to each well, and agitate with a plate mixer for 5 seconds. Cover the plate with a film to avoid evaporation of the solution, and incubate at room temperature (20 - 30℃) for 1 hour [first reaction].
   ＊ Prepare samples and serial dilutions of standard solution using a separate 96-well plate in advance, and add to the antibody plate with an 8-channel pipette, etc. We recommend assaying a standard dilution series in the 1st and 12th rows. Perform the incubation at room temperature (20 - 30℃); heating at 37℃ may reduce antigenicity.
2. Discard the reaction solution and wash each well 4 times with PBS with 0.1% Tween 20. Then add 100 μl of (5) Substrate Solution (TMBZ) to each well using an 8-channel pipette and incubate at room temperature (20 - 30℃) for 15 min [second reaction].
3. Add 100 μl of stop solution＊ to each well in the same order in which the (5) substrate solution (TMBZ) was added, and agitate well using a plate mixer.
   ＊ Because the stop solution is highly viscous, agitate well using a plate mixer, etc., after its addition.
4. Measure the absorbance at a wavelength of 450 nm following calibration, using distilled water as a background. Coloration is stable for at least 1 hour after the reaction is stopped.
5. Prepare a standard curve by plotting the concentration of each standard solution on the horizontal axis and the corresponding absorbance on the vertical axis. Use the absorbance of the sample to calculate the corresponding concentration of VN.

VIII. Performance

1. Standard curve

The following example shows a typical standard curve generated using this kit. A new standard curve needs to be generated for each assay.

Limit of detection: 5 ng/ml
Curve fit: 4-Parameter



4-P Fit : $y = (A - D)/(1+(x/C)^B)+D$:

A	B	C	D	R^2
0.0851	0.991	217	4.37	1

Vitronectin (ng/ml)	320	160	80	40	20	10	5	0
A_{450}	2.640	1.896	1.273	0.747	0.438	0.302	0.182	0.082

(Time for color development 15 min)

2. Reproducibility

<Intra-assay precision (n=8)>

Reproducibility testing was performed with three concentrations of VN solution as samples.

Sample	Mean value (ng/ml)	Standard deviation (ng/ml)	CV (%)
Control A	148.4	7.027	4.7
Control B	33.5	1.430	4.3
Control C	12.4	0.530	4.3

<Inter-assay precision (n=3)>

Quantification of three concentrations of a sample was carried out over 3 days.

Sample	Mean value (ng/ml)	Standard deviation (ng/ml)	CV (%)
Control A	155.4	9.18	5.9
Control B	35.3	1.62	4.6
Control C	12.7	0.95	7.5

3. Recovery test

Equal amounts of various concentrations of samples were mixed. The recovery rate was determined by comparing the anticipated theoretical value with the actual measurement.

Sample A	Sample B	Theoretical value (A+B)/2	Measured value	Recovery rate (%)
320.0	160.0	240.0	229.1	95.5
320.0	80.0	200.0	165.7	82.9
160.0	80.0	120.0	107.1	89.3
80.0	40.0	60.0	67.5	112.5
80.0	20.0	50.0	43.6	87.2
29.0	11.1	20.1	20.9	104.2
118.4	11.1	64.8	55.4	85.6
118.4	5.0	61.7	55.8	90.4
29.0	5.0	17.0	16.2	95.3
11.1	5.0	8.1	8.5	105.6

(Unit: ng/ml)

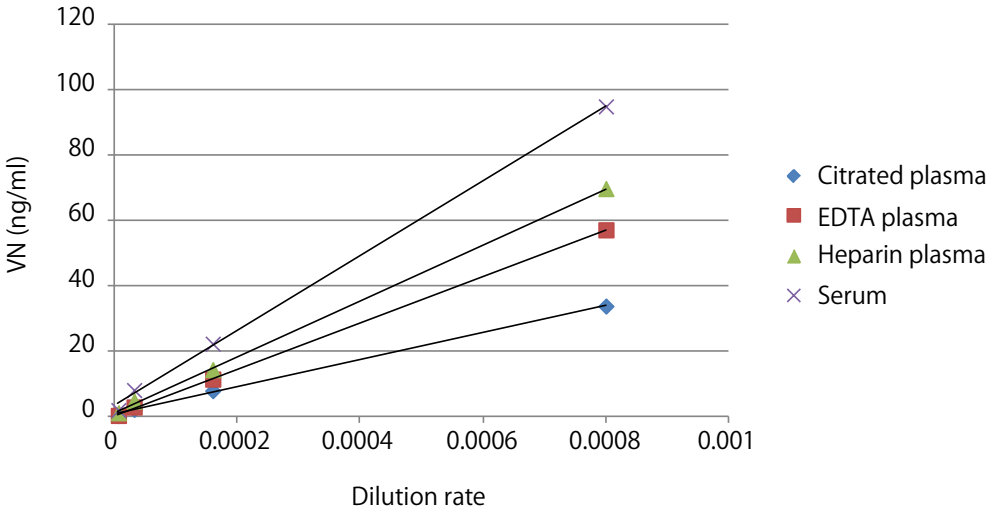
Result: The recovery rate was 82.9 - 112.5%.

IX. Experimental Examples

1. Effects of blood collection

The effects of anticoagulant in blood collection were examined in rabbit blood. Blood was collected from one rabbit using three different anticoagulants almost simultaneously, and the plasmas were prepared. The three plasmas and sera were measured, and VN concentrations were compared.

Dilution rate		Rabbit VN (ng/ml)			
		Citrated plasma	EDTA plasma	Heparin plasma	Serum
x 1,250	(0.0008)	33.9	57.3	69.9	95.1
x 6,250	(0.00016)	8.0	11.5	14.5	22.4
x 31,250	(0.000032)	2.3	3.0	4.8	8.2
x 156,250	(0.0000064)	0.4	0.4	1.2	2.1



<Result>
The blood samples must be collected by the same methods, since measurement values vary largely depending on the anticoagulant.

2. Effects of freeze-thaw on samples

The effects of repeated freeze-thaw cycles were examined using VN standard solution. Samples A and B were frozen and thawed repeatedly between -80℃ and 37℃ seven times. Samples aliquoted at the first thawing, and after 3 and 7 freeze-thaw cycles were analyzed at the same time.

	First thawing	After 3 freeze-thaw cycles	After 7 freeze-thaw cycles
Sample A	149.3	134.0	143.3
Sample B	34.9	29.9	25.0

(Unit: ng/ml)

<Result>
Seven freeze-thaw cycles did not affect the stability of VN. However, the antigen tended to decrease its concentration in low-concentration samples.

3. Monitoring VN plate coating

VN is used for the coating of cell culture dishes. In this example, VN was coated onto various cell culture dishes, and the efficiency of adherence to the dish surface was examined with this kit.

<Methods>

Recombinant VN produced from *E. coli* (20-398) (WAKO Code. 220-02041) and native VN prepared from human plasma (65 kD + 75 kD) were used in this experiment. The concentration of VN in these preparations was measured with this kit.
One milliliter of VN solution was added to each 60-mm cell culture dish, and evenly spread over the dish. Then the VN solution was recovered, and the concentration of VN in solution was measured.
The amount of residual VN was compared between two surface coating conditions: 37℃ for 3 hours, and 4℃ for 16 hours.

VN in solution recovered after coating (ng/ml)	Recombinant VN from *E. coli* (5,752 ng/ml)			VN from human plasma (562 ng/ml)		
Coating condition	37℃, 3 hr	4℃, 16 hr	Dilution ratio	37℃, 3 hr	4℃, 16 hr	Dilution ratio
60-mm dish						
BD/PRIMARIA (Code. 3082)	13.4	1.1	x 1	1.8	0.0	x 1
	9.0	0.7	x 2	1.2	0.0	x 2
BD/for Cell Culture (Code. 3002)	32.0	2.5	x 1	1.3	0.9	x 1
	17.3	1.6	x 2	0.7	1.2	x 2
IWAKI/non-Coated (Code. 1010-060)	46.8	41.6	x 1	5.0	3.6	x 1
	21.2	20.5	x 2	3.0	1.0	x 2
IWAKI/for Cell Culture (Code. 3010-060)	23.3	0.0	x 1	0.8	0.0	x 1
	11.1	0.0	x 2	0.0	0.0	x 2

<Results>

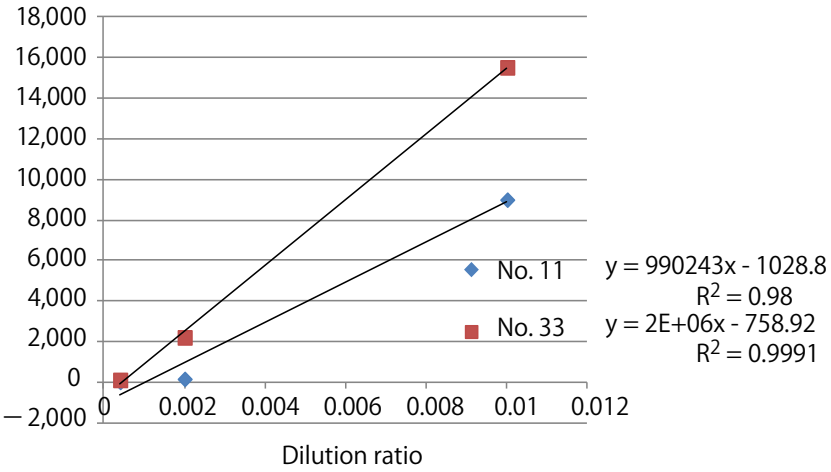
After incubation at 37℃ for 3 hours, 99% of VN had adhered to the dish surface. Furthermore, coating efficiency was about 100% with incubation at 4℃ for 16 hours. A difference was observed in coating efficiency depending on the characteristics of individual commercially available dishes.

4. Measurement of various blood

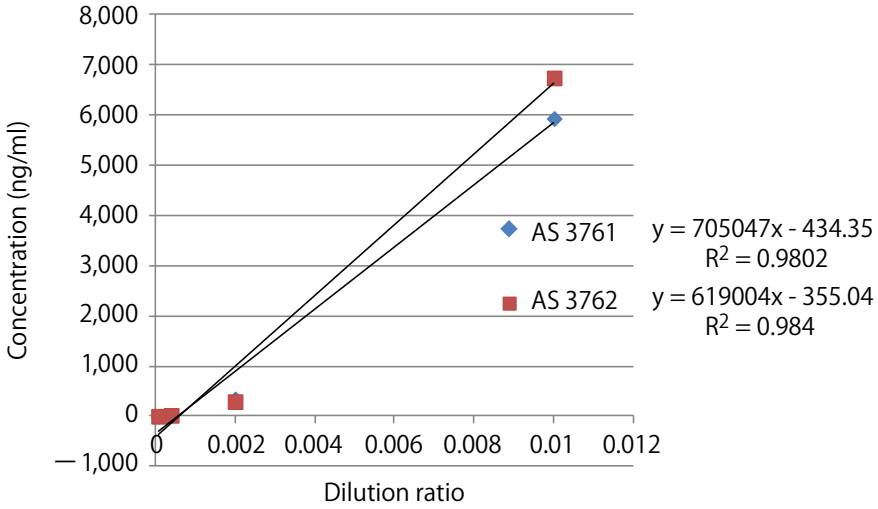
		Rabbit (ng/ml)		Human (ng/ml)	
		Plasma	Serum	Plasma	Serum
Dilution ratio		AS 3761	AS 3762	No.11	No.33
x 100	(0.01)	5934.2	6742.3	9004.2	15525.5
x 500	(0.002)	349.3	295.0	168.1	2214.5
x 2,500	(0.0004)	19.0	21.3	20.3	119.2
x 12,500	(0.00008)	2.5	3.0	2.8	23.9

We recommend dilution in a range between 500 and 2,500 fold in blood samples.

Human blood



Rabbit blood



5. Sensitivity improvement using a 2-Step method

It is possible to perform a 2-Step measurement using the reagents of this kit. This is an effective method when the detected antigen is in very small amounts or when the sample contains a substance that might inhibit the (2) Antibody-POD Conjugate, such as sodium azide.

<Method>

1) Dilute the (3) Standard Vitronectin (320 ng) with (4) Sample Diluent and adjust the concentration to 80 ng/ml. Prepare standard solutions from this 80-ng/ml solution through stepwise dilution as shown below.

2-Step vitronectin concentration (ng/ml)	80	40	20	10	5	2.5	1.25	0

2) Dispense 100 μl each of VN standard solution and the sample at each concentration into the wells of the antibody plate; incubate at 20 - 30℃ for 1 hour. It is preferable to complete sample addition within 5 minutes.
3) After discarding the reaction solution, wash 3 times with 0.1% Tween 20/PBS and remove it completely.
4) Add 100 μl of (2) Antibody-POD Conjugate to each well and incubate at 20 - 30℃ for 1 hour.
5) After discarding the reaction solution, wash 4 times with 0.1% Tween 20/PBS and remove it completely.
6) Add 100 μl of (5) Substrate Solution (TMBZ) to each well and incubate the coloring reaction at room temperature (20 - 30℃) for 10 to 15 minutes.
7) Add 100 μl of the stop solution to each well in the same order that the substrate solution (TMBZ) was added, and agitate well after the reaction has been stopped.
8) Measure absorbance at a wavelength of 450 nm following calibration, using distilled water as a background. Coloration is stable up to 1 hr after the reaction has stopped.
9) Prepare a standard curve by plotting the concentration of each VN standard solution on the horizontal axis and the corresponding absorbance on the vertical axis. Use the absorbance of the sample to calculate the corresponding VN concentration.

X. References

1) Hayashi, M. *et al*. (1985) *J Biochem.* **98**:1135-1138.
2) Suzuki, S. *et al*. (1985) *EMBO J*. **4**(10):2519-2524.

XI. Related Products

Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021)
Anti Human Vitronectin, Monoclonal (Clone : VN58-1) (Cat. #M017)

XII. Precautions

1. Do not mix reagents from kits with different lot numbers.
2. Do not expose reagents to strong light during storage or reaction.
3. The pipettes, etc., used for the substrate solution (TMBZ) and stop solution should contain no metal.
4. Take care to prevent the substrate solution (TMBZ) and stop solution from coming into contact with hands or mucous membranes.
5. Do not use the substrate solution (TMBZ) if it has changed color.
6. Each reaction can be affected by time and temperature, so prepare a standard curve for each measurement.
7. Handle blood specimens with sufficient care.