Cat. # MK140

For Research Use

TaKaRa

RetroNectin® EIA Kit

Product Manual

v201905Da

Table of Contents

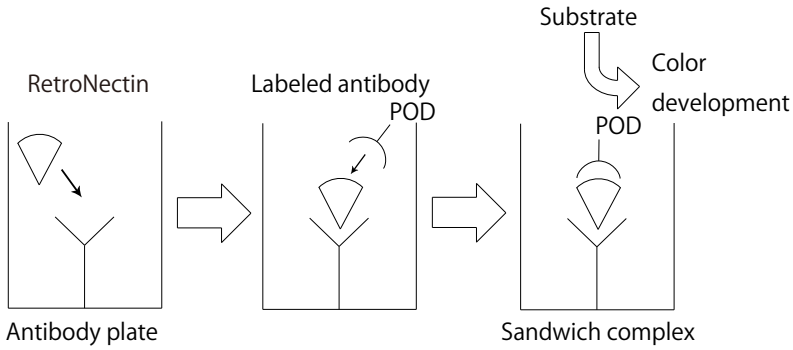
I. Description

This kit is a sandwich ELISA using as the capture antibody a plate-coated RetroNectin-specific monoclonal antibody that does not cross-react with human fibronectin. It is not subject to inhibition even in the presence of human blood components, allowing easy monitoring of the level of RetroNectin. This kit allows highly sensitive quantitative determination of release from RetroNectin-coated plates and residual RetroNectin levels in both cryopreservation solutions of cells cultured in a RetroNectin-added medium and cell washings.

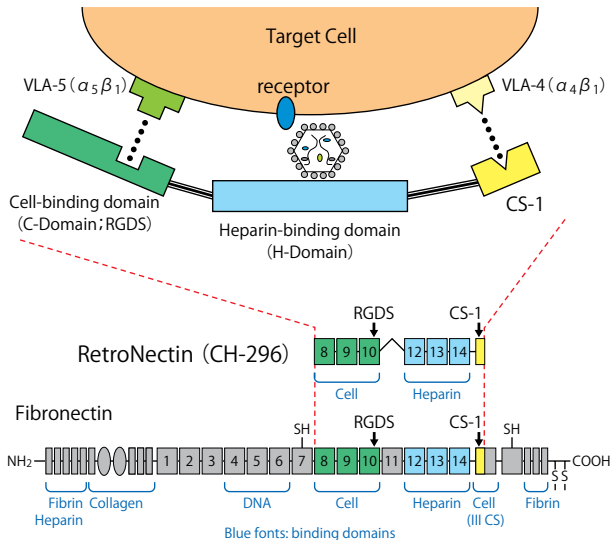
RetroNectin (recombinant human fibronectin CH-296), a recombinant protein, is composed of 3 functional domains: human fibronectin cell-binding (C-domain), heparin-binding domain (H-domain) and CS-1 sequence. A combination of a recombinant retroviral vector and RetroNectin is frequently used for gene transfer into mammalian cells. This protocol, in which the RetroNectin molecule brings the retroviral vector that binds to the H-domain into close proximity with the animal cell that has an affinity for the C-domain, can dramatically increase transduction efficiency.

In addition, RetroNectin has been used to aid lymphocyte expansion after it was shown to be an effective matrix for coating apparatuses used in ex vivo expansion of lymphocytes.

II. Principle



Illustrative diagram of RetroNectin and fibronectin structures and RetroNectin-mediated gene transduction.



III. Components

(1) Antibody Coated Microtiter Plate — 1 plate
Anti-RetroNectin monoclonal antibody plate (96 wells: 8 wells x 12 strips)

(2) Antibody-POD Conjugate (lyophilized) — for 11 ml
Peroxidase-labeled anti-RetroNectin antibody

(3) Standard (lyophilized) — for 1 ml
RetroNectin standard, 200 ng

(4) Sample Diluent — 11 ml x 2
PBS containing 25% Block Ace and preservative

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

IV. 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)

V. Storage 4℃

VI. Intended Use

- Detect residual RetroNectin in frozen preserved cell suspensions transformed with RetroNectin and in cell washings.
- Detect residual RetroNectin in samples from lymphocyte expansion cultures that used RetroNectin.

VII. Protocol

1. Sample

- Use cryopreservation or washing solution of cells cultured in RetroNectin-added medium as samples.
- Samples may be stored up to 12 hours at 4℃. If the assay will be performed longer than 12 hours after sample preparation, then store samples frozen at -20℃.
- Due to a matrix effect, antigens have a tendency to show a low value in sample solutions with a high protein level such as in blood. Therefore, it is recommended to assay concurrently the stock solution as well as dilutions of a sample. Prepare 2- to 8-fold serial dilutions using (4) Sample Diluent.

Note :

- RetroNectin is a protein with a cell-binding domain. The presence of cell components may slightly affect the antigen-antibody reaction.
- When determining the residual RetroNectin after washing cells with RetroNectin, it is recommended to use surfactant NP-40 (CAS No.9016-45-9) for assaying the cells and washing buffer together by ELISA.

  Prepare samples in the presence of NP-40 at a final concentration of 1%, or prepare assay samples by suspending cells in the (4) Sample Diluent containing NP-40 at 1%. Using up to 100 μl of a sample containing 1% NP-40 does not inhibit antigen-antibody reactions.

2. Reagent preparation

- (1) Antibody Coated Microtiter Plate

  Allow the unopened plate to reach room temperature in its package before use.

- POD-labeled Antibody Solution

  Reconstitute (2) Antibody - POD Conjugate with 11 ml of distilled water. Once reconstituted, it is stable for up to 1 week at 4℃. For longer storage, freeze at -20℃, at which it is stable for up to 1 month. However, no more than 1 cycle of freeze-thaw is allowed.

- RetroNectin Standard Solution

  Add 1 ml of distilled water to reconstitute (3) Standard, yielding a RetroNectin Standard Solution (200 ng/ml). Dilute with (4) Sample Diluent before use to prepare fresh serial dilutions (Standard Solutions at concentrations of 200, 100, 50, 25, 12.5, 6.25, and 3.125 ng/ml. Use (4) Sample Diluent as the 0-concentration standard.

  The RetroNectin Standard Solution (200 ng/ml) is stable for up to 1 week after preparation when stored at 4℃, or for up to 1 month at -20℃.

- Substrate solution (TMBZ)

  Return the (5) Substrate Solution to room temperature before use. It is supplied ready to use in reactions. Check before use that it has not developed a dark blue color. A reaction with metal ions will result in coloration; make sure it is not contaminated with any tap water.

  If the Substrate Solution will be used for several reactions, divide it into aliquots of the required volume in advance.

- Stop solution

  Use the Stop Solution included in Wash and Stop Solution for ELISA without

Sulfuric Acid (Cat. #MK021) directly.

＊ Because this solution is highly viscous, mix well using a plate mixer after it is added.

- PBS with 0.1% Tween 20 for washing

  Dilute one 50 ml tube of 10X PBS [included in Wash and Stop solution for ELISA without Sulfuric Acid (Cat. #MK021)] 10-fold with distilled water, add Tween 20 to a final concentration of 0.1%, and mix well. Use this diluted solution as a wash buffer.

3. Procedure

Assay samples in duplicate.
Return each reagent in the kit and samples to room temperature and make sure solutions are mixed uniformly without creating bubbles before use.

1. Prepare samples (100 μl/well) and the Standard Solution in a separate 96 well plate in advance so that they can be added to the antibody-plate quickly (within 5 minutes) using an 8-channel pipette or similar apparatus. In order to provide highly reliable results, it is recommended to place serial dilutions of the Standard Solution in the 1st and 12th rows. Add samples and Standard Solution to antibody-plate and let stand at room temperature (20 - 30℃) for 1 hour; incubation at 37℃ may compromise antigenicity. [First reaction]
2. Discard reaction mixtures, followed by 3 washes with Washing Buffer. Then add 100 μl of the POD-labeled antibody solution per well using an 8-channel pipette and allow to react for 1 hour at room temperature (20 - 30℃). [Second reaction]
3. Discard reaction mixtures, followed by 4 washes with Washing Buffer. Then add 100 μl of (5) Substrate Solution (TMBZ) per well using an 8-channel pipette and allow to react at room temperature (20 - 30℃) for 15 minutes. [Third reaction]
4. Add 100 μl of Stop Solution to each well to stop the reaction in the same order as for (5) Substrate Solution (TMBZ). Then mix well.
5. Use distilled water as a control to make zero adjustment and measure absorbance at 450 nm.

   The color is stable for up to 1 hour after reaction termination.
6. Plot a standard curve based on the results obtained from the Standard Solutions (with concentration as x-axis and absorbance as y-axis) and use it to determine the corresponding concentrations of RetroNectin based on the sample's absorbance.

Note :

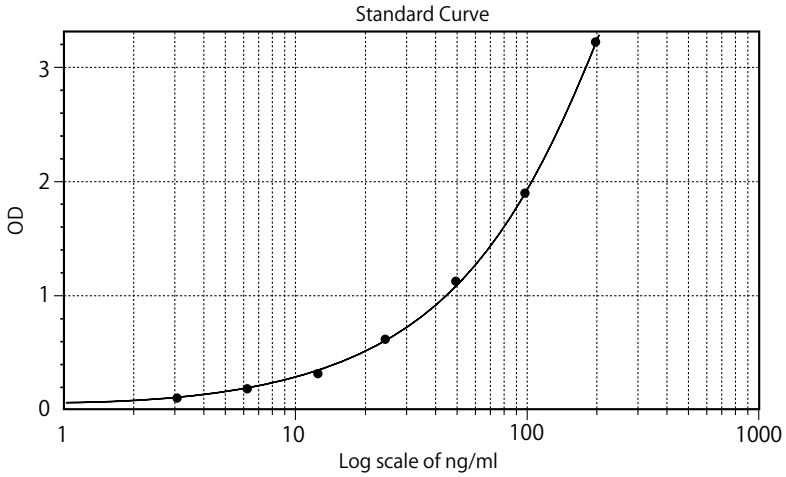
- Cover the plate with film or the like to prevent evaporation of solutions during reactions at room temperature or in an incubator.
- It is recommended that the Washing Buffer be completely discarded to get rid of the residual fluid.

VIII. Performance

1. Standard curve

The following shows a typical standard curve of this kit as an example.
The standard curve for calculation needs to be established in each assay.

Limit of detection : 3.125 ng/ml



RetroNectin concentration (ng/ml)	200	100	50	25	12.5	6.25	3.125	0.0
A_{450}	3.201	1.889	1.122	0.618	0.307	0.175	0.111	0.040

2. Reproducibility

<Intra-assay reproducibility test >

We conducted a within-run reproducibility test using controls at 3 different concentrations prepared by adding RetroNectin to human serum samples.

Sample (n = 16)	Mean (ng/ml)	SD	CV(%)
Control A	77.652	3.516	4.5
Control B	34.277	0.630	1.8
Control C	19.622	0.653	3.3

Result : Excellent within-run reproducibility was confirmed with CV = no more than 5%.

<Inter-assay precision test >

A reproducibility test was performed by assaying 3 different concentrations of control over 3 days.

Sample(n = 3)	Mean (ng/ml)	SD	CV(%)
Control A	72.756	3.339	4.9
Control B	32.996	1.240	1.9
Control C	18.516	0.787	3.4

Result : Excellent between-run reproducibility was demonstrated with CV = no more than 5%.

3. Recovery test

Equal volume of samples in different concentrations were combined and assayed. The assay result of each mixture was compared with the theoretical value to determine the recovery rate.

Sample A	Sample B	A+B/2 (theoretical value)	A+B/2 (assay result)	Recovery rate (%)
121.880	88.629	105.255	103.074	97.93
121.880	53.195	87.538	88.849	101.50
121.880	29.404	75.642	75.445	99.74
121.880	17.205	69.543	67.020	96.37
121.880	6.521	64.201	61.442	95.70
121.880	0.086	60.983	57.660	94.55
88.629	53.195	70.912	69.218	97.61
88.629	29.404	59.017	55.746	94.46
88.629	17.205	52.917	48.110	90.92
88.629	6.521	47.575	43.878	92.23
88.629	0.086	44.358	42.042	94.78
53.195	29.404	41.300	40.593	98.29
53.195	17.205	35.200	35.841	101.82
53.195	6.521	29.858	31.327	104.92
53.195	0.086	26.641	26.032	97.72
29.404	17.205	23.305	21.244	91.16
29.404	6.521	17.963	15.768	87.78
29.404	0.086	14.745	15.193	103.04
17.205	6.521	11.863	12.671	106.81
17.205	0.086	8.646	9.786	113.19
6.521	0.086	3.304	3.597	108.88

Unit: ng/ml

4. Effects of freeze-thawing or heating of samples

We studied the effects of freeze-thaws on the stability of RetroNectin Standard added to human serum samples. Samples that had undergone repeated cycles of freeze-thaw and samples thawed for the first time were assayed using this kit. In addition, to study the effect of incubation at 37℃, samples were incubated at 37℃ for 1 to 2 hours before assay.

Freeze-thaw cycle (n=2)	Sample A	Sample B
0	52.463	36.666
1	51.567	26.149
2	55.204	42.596
3	50.550	34.868
4	57.566	35.306
5	49.710	32.385

Incubation at 37℃ (n=2)	Sample A	Sample B
0 h	52.463	36.666
1 h	46.986	32.385
2 h	45.529	34.614

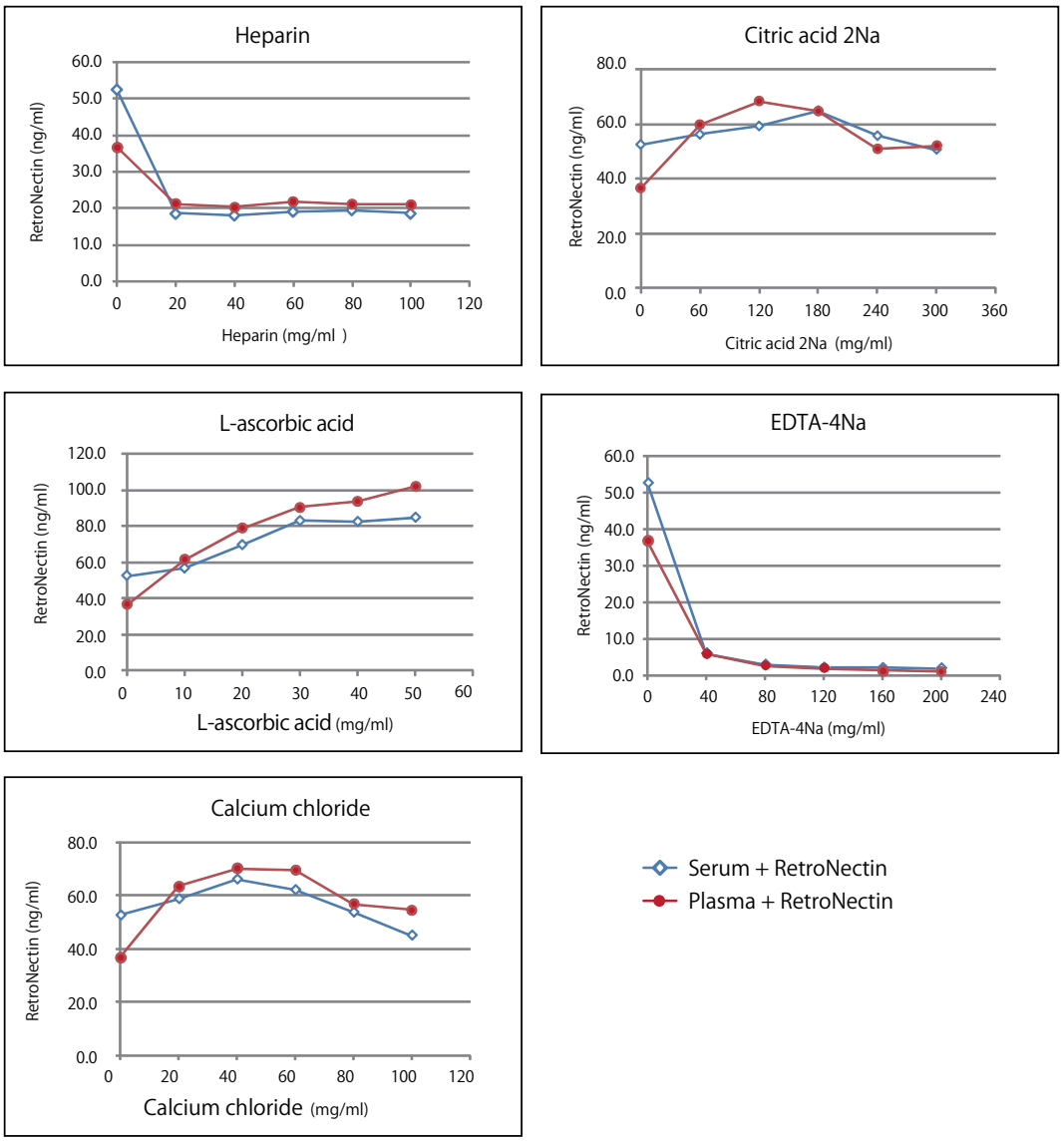
Unit : ng/ml

Result : The effect of freeze-thaw was minimal, but incubation at 37℃ led to a slight decrease in the RetroNectin level.

5. Effects of coexisting substances

Serum or plasma containing RetroNectin were combined with a test substance at a ratio of 9 to 1 to investigate the effect on RetroNectin measurement using this assay.

Test substance concentrations are the final concentration.



Result: Heparin and EDTA inhibit the immunoassay using this kit.

6. Assay of RetroNectin in cryopreservation solution

Cryopreservation solution was spiked with RetroNectin and then assayed.

- Cryopreservation solution constituents

  RPMI 1640 serum-free medium
  Human serum albumin (final concentration 8%)
  HES (Hydroxyethyl starch)
  DMSO (Dimethylsulfoxide)
  Saline

- High RetroNectin

  2 μl of a 1 mg/ml stock RetroNectin solution was added to 1 ml of (4) Sample Diluent to make a concentration of 2,000 ng/ml. 10 μl of the diluted stock solution was then added to 90 μl of cryopreservation solution.

  (Theoretical concentration 200 ng/ml)

- Low RetroNectin

  125 μl of diluted stock solution (2,000 ng/ml) was added to 875 μl of (4) Sample Diluent to make a concentration of 250 ng/ml. 10 μl of the 250-ng/ml diluted solution was then added to 90 μl of a preserved cell suspension.

  (Theoretical concentration 25 ng/ml)

Cryopreservation solution + High RetroNectin	Measurement	177.589 ng/ml
	Theoretical concentration	200 ng/ml
	Recovery rate	88.8%
Cryopreservation solution + Low RetroNectin	Measurement	23.182 ng/ml
	Theoretical concentration	25 ng/ml
	Recovery rate	92.7%

Recovery rate (%) = (theoretical concentration / measurement) x 100

Note :

RetroNectin is a functional protein that interacts with the cell surface and exhibits an affinity to heparin, an anticoagulant. The recovery rates were around 90% in the spike recovery test of cryopreservation solution. Recovery rates from blood samples (high-concentration plasma protein samples) spiked with RetroNectin may be less than 85% and unstable.

Result : Mearurements of RetroNectin in cryopreservation solution were around 90% of the theoretical concentration.

IX. Precautions

1. Do not mix/use kits or reagents from different lots.
2. Do not expose reagents to strong light during storage or reactions.
3. Use pipettes free of metal when handling (5) Substrate Solution (TMBZ).
4. Avoid contact of (5) Substrate Solution (TMBZ) with hands or mucous membranes.
5. Do not use (5) Substrate Solution (TMBZ) that has developed color.
6. Each reaction varies subject to length of time and temperature. Therefore, a new standard curve must be generated for each assay.
7. Handle blood samples with great care as they are potentially infectious.

X. Related products

RetroNectin® Recombinant Human Fibronectin Fragment (Cat. #T100A/B)
RetroNectin® Precoated Dish (Cat. #T110A)
Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021)
RetroNectin® GMP grade Recombinant Human Fibronectin Fragment CH-296 (Cat. #T201)