Cat. #MK115

For Research Use

TaKaRa

Fibronectin EIA Kit

Product Manual

v201910Da

Table of contents

I. Description

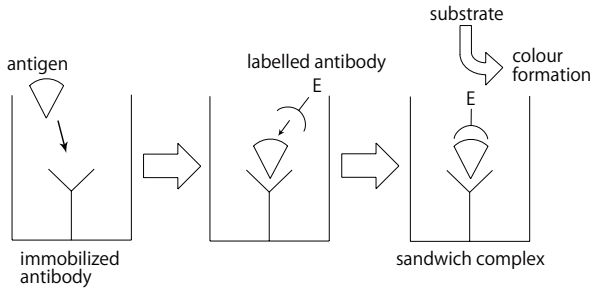
The adhesive glycoprotein fibronectin (FN), Mr440,000, is widely distributed on cell surfaces, in the extracellular matrix, and in plasma. FN is involved in a variety of cellular processes, including cell-to-substrate adhesion, cell migration, and regulation of cell morphology (1). Webb *et al.* first reported in 1980 (2) that the amount of fragmented FN in the urine of patients with prostatic cancer was increased compared to normal subjects. Another group has reported that an FN-like protein, with a low molecular mass than plasma FN, was increased in sera from patients with malignant disease (3). In particular, many examinations have been done to evaluate the clinical usefulness of measurements of plasma FN (4 - 7). Recently, Katayama *et al.* reported that urinary FN (UFN) in cancer patients was almost always present as fragments of different sizes containing the central "cell-binding domain", and that a polyclonal antibody raised against native plasma FN could not react well with these fragmented UFN. They demonstrated that two monoclonal antibodies, reactive with the "cell-binding domain" (8) are suitable for in vitro assays of FN in biological fluids and FN-fragments excreted in the urine of cancer patients, and that significantly higher levels of UFN were observed in many kinds of cancer (9, 10).

II. Intended Use

The human Fibronectin EIA Kit is an in vitro enzyme immunoassay (EIA) kit for the specific quantitative determination of human Fibronectin (hFN) in serum, urine, cell culture supernatants, and other biological fluids. This kit is suitable for quantitation of soluble human Fibronectin.
This kit is for research use only. It is not for use in diagnostic or therapeutic procedures.

III. Principle

The human Fibronectin EIA Kit is a solid phase EIA based on a sandwich method that utilizes two mouse monoclonal anti-human Fibronectin antibodies to detect Fibronectin by two-step procedure. One of the mouse monoclonal anti-human Ffibronectin antibodies is bound to a microtiter plate to create the solid phase. Non-specific binding is blocked by a blocking buffer. Samples and standards are incubated in microtiter-plate wells. The second step is to wash the plate and add a second anti-Fibronectin antibody labelled with peroxidase (POD). During the incubation, human Fibronectin, bound to anti-human Fibronectin antibody (solid phase) on one side, is tagged on the other by POD-anti-human Fibronectin antibody. The reaction between POD and substrate (H_2O_2 and tetramethylbenzidine) results in colour development with intensities proportional to the amount of human Fibronectin present in samples and standards. The amount of human Fibronectin can be quantitated by measuring the absorbance using an EIA plate reader. Accurate sample concentrations of human Fibronectin can be determined by comparing their specific absorbances with those obtained for the standards plotted on a standard curve.



IV. Reagents and Materials

Each human Fibronectin 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℃.

A. Materials Provided

(1)	Antibody Coated Microtiter plate Anti-human Fibronectin monoclonal antibody (96 wells: 8 wells x 12 strips)	1 plate
(2)	Antibody - POD Conjugate (lyophilized) Peroxidase-labeled anti-human Fibronectin monoclonal antibody	For 11 ml
(3)	Standard (lyophilized) Purified human Fibronectin 800 ng	For 1 ml
(4)	Sample Diluent 25% Block Ace in PBS (with preservative)	11 ml x 2
(5)	Substrate Solution (TMBZ) 3, 3', 5, 5'-Tetramethylbenzidine solution	12 ml

B. 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. 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 Substate Solution to strong light during storage or incubation.
- Avoid contact of Substrate 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 potentiallyinfectious.
- Avoid contact of Substrate Solution and Stop Solution with any metal surface. Disposable glassware or test tubes are recommended for handling the Substrate Solution. If non-disposable glassware 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.

VI. Specimen Collection and Handling

Plasma, serum, urine or cell culture supernatant is suitable for use in the assay, however, cell or tissue extract can be also used. PBS containing 0.1% NaN_3, 5 mM EDTA and 0.3 mM Phenylmethylsulfonyl fluoride (pH 7.2) should be used for preparation of cell extracts. Remove the serum 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. Plasma and serum samples must be diluted several hundred fold. Urine samples can be used directly, but it is necessary to be adjusted by urine creatinine as ng FN/mg creatinine for the data comparison. Samples may be stored up to 24 hours at 4℃. If the length of time between sample collection and assay is to exceed 24 hours, samples should be stored frozen under-20℃ 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.

VII. Recommended Sample Dilution

In case of using plasma or serum, dilute the samples by 100 - 250 folds before assay. In case of using urine, no need to dilute the sample before 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 100 - 250 folds as the middle concentration. In case of using urine samples, they can be used directly.

VIII. 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 of distilled water and mix gently followed by 10 minutes slowly rolling or occasional mixing, avoiding foam formation.

Solution 2. Standard Solution
Rehydrate Standard (3) with 1 ml of distilled water. Slowly roll for approximately 10 minutes or let the vial stand and sporadically mix gently. The standard solution contains 800 ng human Fibronectin/ml.
Prepare dilution series of 400, 200, 100, 50, 25, and 12.5 ng/ml by diluting the Standard Solution with Sample Diluent.

IX. Stability of Solutions

Solution 1. Antibody-POD Conjugate Solution
The reconstituted lyophilisate is stable for 1 week at 4℃ and for 1 month when stored at -20℃. Do not repeat freeze-thaw cycles.

Solution 2. Standard Solution
The reconstituted lyophilisate is stable for 2 weeks stored at -80℃.
Do not repeat freeze-thaw cycles.

X. 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.

[Enzyme Immunoassay]

- Sample incubation: Pipette 100 μl of sample or standard (Solution 2) into one well within 5 minutes. Mix, seal the microtiter plate (e.g. with a foil) and incubate for 1 hour at 37℃.
- Remove sample solution and wash the wells 3 times with ca. 400 μl of Washing Buffer; between the separate washing steps, empty out the microtiter plate and vigorously tap onto paper towel, especially after the last washing.
- Antibody-POD conjugate incubation: Pipette 100 μl of Antibody-POD conjugate Solution (Solution 1) into one well. Mix, seal the microtiter plate (e.g. with a foil) and incubate 1 hour at 37℃.
- Remove the sample solution and wash the wells 4 times as described above (It is specially important after this step to thoroughly empty out the remaining fluid before adding the substrate).
- Substrate incubation: Add 100 μl of Substrate Solution (5) into each well and incubate at room temperature (20 - 30℃) for 15 minutes.
- Add 100 μl of Stop Solution into each well in the 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.

  Note: It is important that Stop Solution is added to wells prior to reading at 450 nm. Addition of Stop Solution causes an increase in absorbance of the Substrate Solution and shift in absorbance spectrum.

XI. 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 Fibronectin concentration in ng/ml (horizontal axis) for the standards using optimal fitting curve.

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 Fibronectin concentration (ng/ml) from the horizontal axis.

XII. Performance Characteristics

1. **Range of standard curve:** 12.5 - 800 ng/ml.
2. **Specificity:**
   This kit specifically measures human Fibronectin. This kit cannot be used to measure mouse, bovine, rabbit, porcine Fibronectin. The application of this kit for quantitating Fibronectin from other sources has not been tested.
3. **Assay duration:** Two and a half hour after sample incubation
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 plasma, serum and urine ; culture supernatants,
7. **Specimen volume required:**
   If each test sample is run in duplicate, approximately 220 μl (i.e., 100 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.

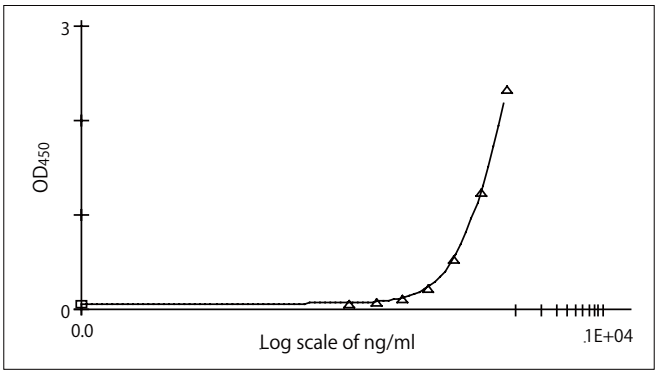
   Only samples with absorbance values falling within the range of the standard curve should be assigned a human Fibronectin concentration from the curve.
9. **Notes :**
   According to the assay results using control serum, it could be possible to determine the concentration of antigen present in biological fluids. However, the measurement may be potentially disturbed by the unknown organic factors in serum 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 Sample Diluent (4) included in the kit and assay them again in another run.

VIII. Basal Data

1. Typical 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.

Curve Fit: 4-Parameter Corr. Coeff: -1.00
$y = (A-D)/\{1 + (x/C)^B\} + D$
A = 0.0536 B = 1.61 C = 7070. D = 4.21



Fibronectin (ng/ml)	800	400	200	100	50	25	12.5	0
A_{450}	2.336	1.242	0.530	0.226	0.115	0.079	0.058	0.046

2. Intra-assay precision (n=16)

Assay was carried out with 16 replicates of 3 samples containing different concentration of control Fibronectin. All samples was diluted 100-fold for assay.

	Sample Ave. (ng/ml)	S.D.	CV (%)
Control A	244.4	9.49	3.9
Control B	61.4	2.87	4.7
Control C	22.7	1.61	7.1

Inter-assay precision (performance 3 times)

Assay to assay precision with one laboratory was evaluated in 3 independent experiments over 3 days. All samples was diluted 100-fold for assay.

	Sample Ave. (ng/ml)	S.D.	CV (%)
Control A	412.9	12.91	3.1
Control B	124.0	7.43	6.0
Control C	51.3	1.87	3.6

3. Recovery test

The recovery of Fibronectin was tested by adding 2 samples out of 5 different level in various matrices.

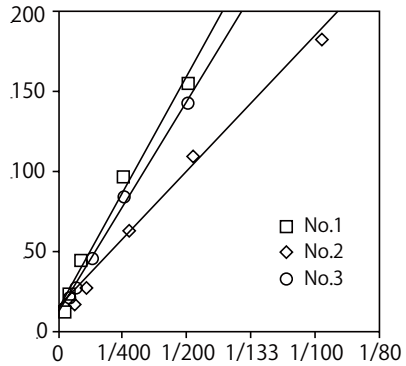
Sample A	Sample B	A+B Measured	A+B Calculated	Recovery (%)*
589.0	589.0	600.4	589.0	101.9
589.0	316.7	445.4	452.9	98.4
589.0	161.8	395.2	375.4	105.3
589.0	85.6	338.8	337.3	100.4
589.0	39.5	331.3	314.2	105.4
589.0	0.0	255.1	294.5	86.6
316.7	316.7	309.2	316.7	97.6
316.7	161.8	244.5	239.3	102.2
316.7	85.6	200.2	201.2	99.5
316.7	39.5	175.0	178.1	98.3
316.7	0.0	151.0	158.4	95.4
161.8	161.8	151.7	161.8	93.8
161.8	85.6	119.8	123.7	96.8
161.8	39.5	109.0	100.6	108.3
161.8	0.0	80.2	80.9	99.1
85.6	85.6	76.4	85.6	89.3
85.6	39.5	58.6	62.5	93.7
85.6	0.0	37.7	42.8	88.0
39.5	39.5	38.6	39.5	97.7
39.5	0.0	19.8	19.7	100.5

(unit: ng/ml)

* Recovery (%) = (A+B measured) / (A+B calculated) x 100

4. Dilution curves of serum samples

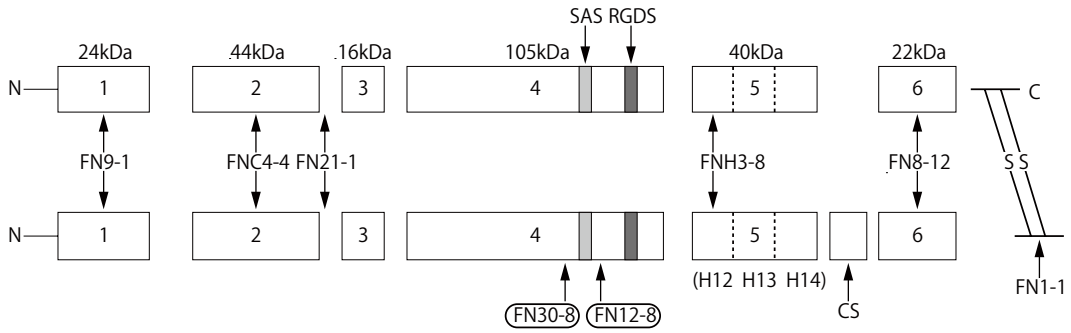
Each assay was performed using 3 kinds of samples which were prepared as dilution series starting from 100-fold.



No. 1 : $y = 2.91 \times 10^4 x + 14.2$ $r = 0.992$
No. 2 : $y = 1.71 \times 10^4 x + 14.8$ $r = 0.996$
No. 3 : $y = 2.65 \times 10^4 x + 11.8$ $r = 0.998$

5. Epitope of the antibodies supplied in this kit

Solid antibody (Antibody coated on the plate): FN30-8 (Cat. #M010)
Labelled antibody (Antibody-POD conjugate): FN12-8 (Cat. #M002)



Domain Structure

1: Fibrin-heparin
2: Collagen
3: Heparin
4: Cell
5: Heparin
6: Fibrin

SAS: Synergistic adhesion site
RGDS: Arg-Gly-Asp-Ser-peptide
CS: Connecting segment

Speicifity

Clone No.	Domain Specifity*	Inhibition of Cell adhesion	Cross with bovine FN	Cross with porcine FN	Cross with rabbit FN	Cross with rat FN
FN12-8	cell	Yes	Yes	None	None	None
FN30-8	cell	Yes	None	None	None	None

(* Please refer to the above figure for Domain Specifity.)

6. Daily variation of urinary FN and other proteins

The daily variation of urinary Fibronectin, Laminin and E-cadherin was measured using the samples from 3 individuals. For Fibronectin(FN) and Laminin(LN) assay, the samples were used directly. For E-cadherin assay, the samples were used after 9-fold dilution. The following table shows the estimated E-cadherin values at the original concentration. The samples were collected freely during daytime.
(This data was obtained by using Cat. #MK007＊ and the former kits Cat. #MK015＊ and MK017＊. The value of creatinine was also measured.)
＊: Cat. #MK007, MK015, and MK017 are already final sales.

		FN (ng/ml)	Cr (g/l)	UFN (FN/Cr) (μg/g.Cr)	ULN (LN/Cr) (μg/g.Cr)	UEcad (Ecad/Cr) (mg/g.Cr)
1. female	day 1	97.1	1.264	76.8	59.8	2.82
	day 2	66.5	1.004	66.2	42.3	3.66
	day 3	45.6	0.605	75.4	59.7	4.33
	day 4	203.5	1.514	134.4	62.6	2.69
	day 5	135.0	2.346	57.5	34.8	3.42
	day 6	93.7	1.915	48.9	48.6	4.20
	day 7	131.1	1.313	99.8	66.5	3.89
	day 8	51.5	0.483	106.6	127.2	6.08
2. female	day 1	23.3	1.182	19.7	34.7	1.11
	day 2	35.9	2.045	17.6	31.4	0.59
	day 3	17.0	1.594	10.7	19.0	0.49
	day 4	33.5	1.565	21.4	42.7	0.20
	day 5	16.5	1.814	9.1	26.7	0.40
	day 6	51.5	2.562	20.1	24.8	0.22
	day 7	15.1	2.031	7.4	27.4	0.23
	day 8	23.3	1.579	14.8	38.5	0.33
3. male	day 1	69.9	1.195	58.5	45.4	0.73
	day 2	0.0	0.403	0.0	23.9	0.17
	day 3	57.3	1.016	56.4	60.5	0.86
	day 4	110.2	1.397	78.9	45.8	0.93
	day 5	2.4	0.422	5.7	39.9	1.37
	day 6	10.2	0.561	18.2	77.2	0.14
	day 7	31.6	1.466	21.6	36.4	1.17
	day 8	27.7	0.645	42.9	81.4	1.61

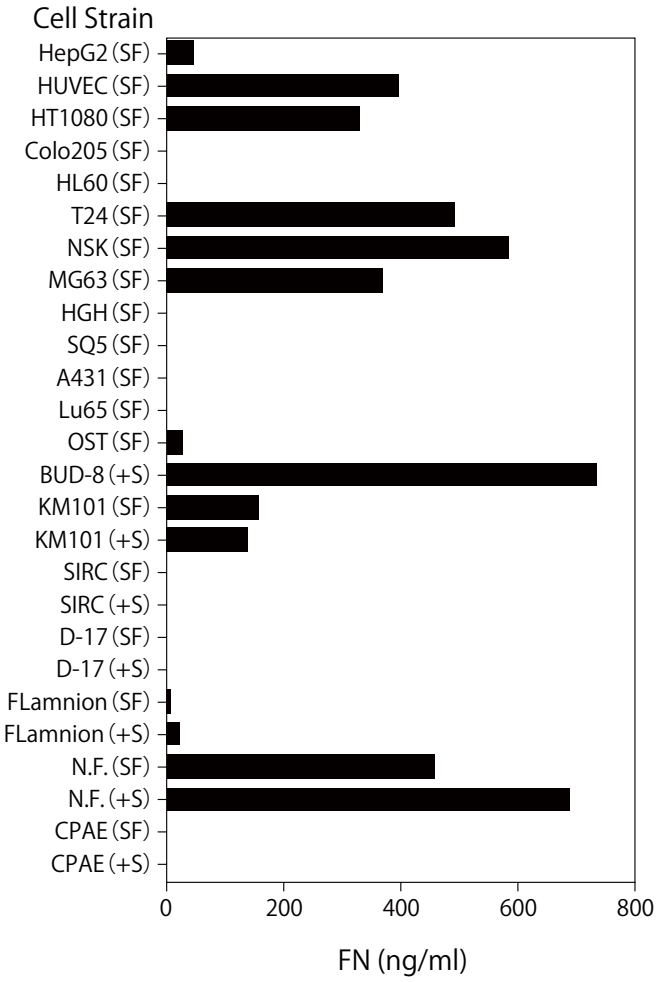
7. Urinary Fibronectin excretion in a day

The amount of urinary Fibronectin and other proteins (LN and E-cadherin) excretion in a day was measured with the samples collected from 4 individuals.
For FN and LN assay, the samples were used directly. For E-cadherin assay, the samples were used after 9-fold dilution. In the following table, the estimated E-cadherin values at the original concentration are shown. The samples were collected freely during a day.
(This data was obtained by using Cat. #MK007＊ and the former kits Cat. #MK015＊ and MK017＊. The value of creatinine was also measured.)
＊ Cat. #MK007, MK015, and MK017 are already final sales.

	Urine (ml)	FN (ng/ml)	Cr (g/l)	UFN (FN/Cr) (μg/g.Cr)	ULN (LN/Cr) (μg/g.Cr)	UEcad (/Cr) (mg/g.Cr)
4. male	270	199.6	1.760	113.4	65.2	0.40
	525	189.9	1.100	172.6	49.3	0.26
	70	66.5	1.489	44.7	68.3	1.04
	570	11.2	0.897	12.5	40.1	0.47
5. female	80	17.5	1.331	13.1	62.6	0.94
	100	40.3	1.441	28.0	93.7	0.80
	180	22.3	1.478	15.1	40.4	0.58
	200	45.6	0.974	46.8	54.2	1.52
	180	38.4	1.466	26.2	53.1	0.63
6. female	350	23.8	0.584	40.8	38.7	0.85
	470	11.7	0.481	24.3	24.6	1.32
	370	12.1	0.676	17.9	41.0	0.53
	180	76.7	1.548	49.5	40.4	0.50
	130	30.1	1.890	15.9	41.9	0.31
7. male	250	60.7	1.718	35.3	55.1	1.04
	150	148.6	2.759	53.9	42.7	1.47
	160	114.6	1.579	72.6	56.5	2.43
	175	141.8	2.400	59.1	68.3	1.43
	175	95.2	1.844	51.6	64.3	1.33

8. Fibronectin in cell culture supernatant

The amount of Fibronectin in the supernatant of various cells cultured in 10% FCS/ RPMI1640) or serum free/Ultradoma PF was measured. The supernatant was applied to assay without dilution. Fetal Calf Serum does not inhibit this assay system. (This data was obtained by using this precoated kit Cat. #MK115.)

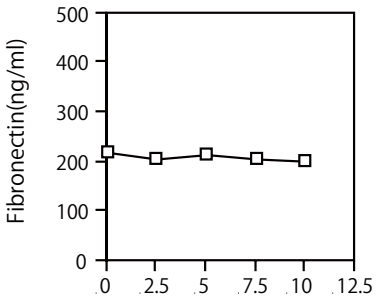


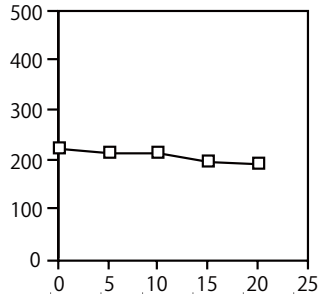
+S : 10% FCS/RPMI1640
SF : serum free medium (2 days after the change from the medium containing serum)

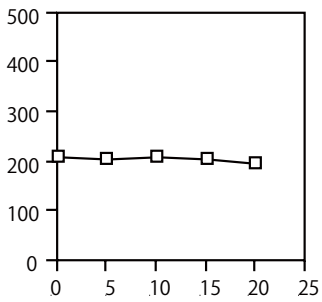
HepG2:	human hepatocellular carcinoma
HUVEC:	human umbilical vein endotherial cell
HT1080:	human fibrosarcoma
Colo205:	human adeno carcinoma
HL60:	human promyelocytic leukemia
T24:	human bladder transitional-cell carcinoma
NSK:	human normal skin cell
MG63:	human osteosarcoma
HGH:	human girardi heart
SQ5:	human lung squamos-cell carcinoma
A431:	human epidermoid carcinoma
Lu65:	human lung carcinoma
OST:	human osteosarcoma
KM101:	human bone marrow stroma cell
SIRC:	rabbit cornea cell
D-17:	canine osteogenic sarcoma
FLamnion:	human amnion cell
N.K.:	human normal fibroblast cell
CPAE:	bovine vein endotherial cell

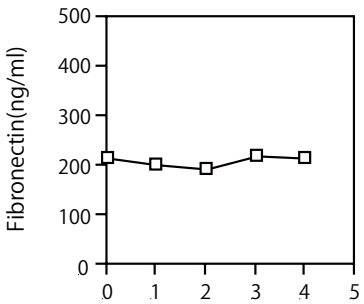
9. Influence of coexistence

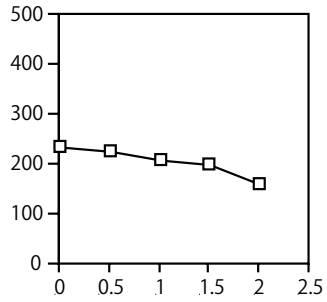
The volume ratio of sample to co-existing substance is 4:1. Co-existing substance is shown in its final concentration.

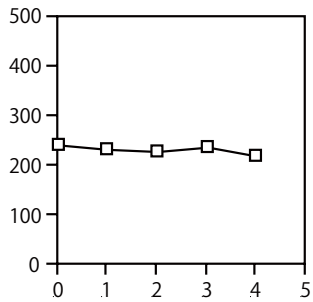


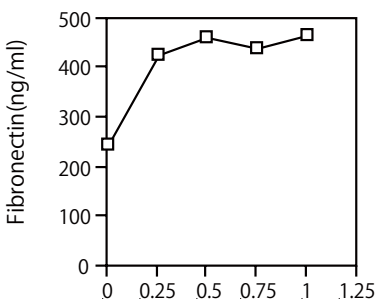


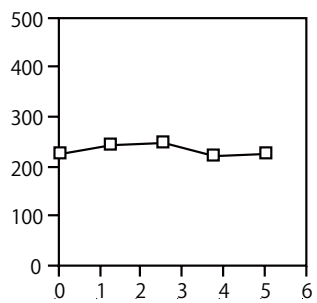


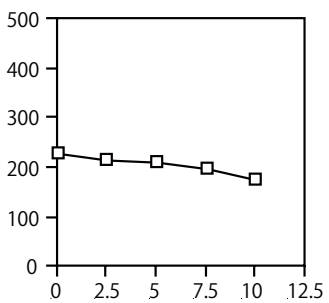


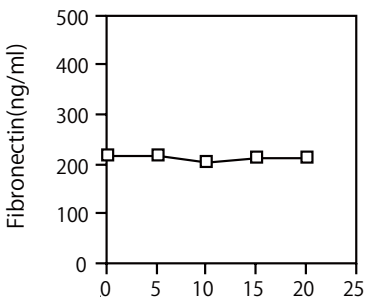








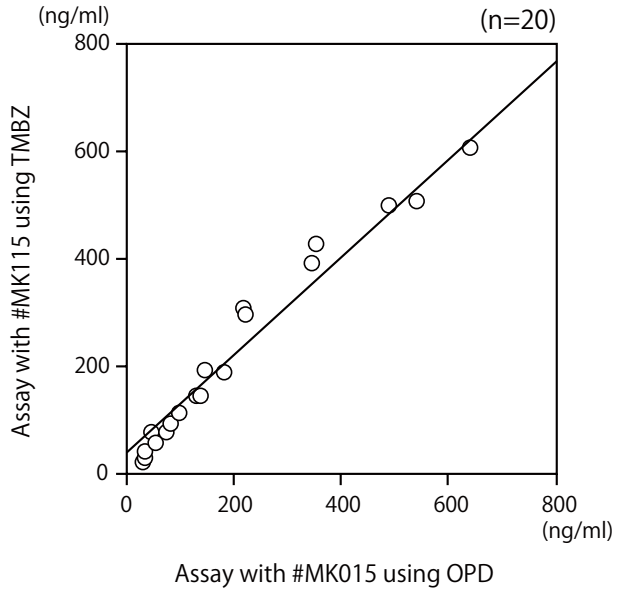




10. Correlation with the former kit (Cat. #MK015)＊

Correlation of precoated type kit (Cat. #MK115) assay to that of the former kit. The former kit employed O-phenylendediamine (OPD) as the substrate, and the precoated one employs 3, 3', 5, 5'-tetramethylbenzidine (TMBZ) as the substrate. Assay was performed using 100-fold diluted serum samples. (n=20)

＊: Cat. #MK015 is already final sales.



XIV. Storage and Stability

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

XV. References

1. Hynes, R.O. *et al*. *J Cell Biol*. (1982) **95**: 369.
2. Webb, K.S. *et al*. *Invest Urol*. (1980) **17**: 401.
3. Parsons, R.G. *et al*. *Cancer Res*. (1979) **39**: 4341.
4. Choate, J.J. *et al*. *Cancer*. (1983) **51**: 1142.
5. Boccard, A. *et al*. *Cancer Lett*. (1986) **33**: 317.
6. Ruelland, A. *et al*. *Clin Chem Acta*. (1988)**178**: 283.
7. Stathakis, N.E. *et al*. *J Clin Pathol*. (1981) **34**: 504.
8. Katayama, M. *et al*. *Exp Cell Res*. (1989) **185**: 229.
9. Katayama, M. *et al*. *Clin Chem*. (1991) **37**: 466.
10. Katayama, M. *et al*. *Clin Chem Acta*. (1993) **217**: 115.

XVI. Protocol Summary

1. Prepare all reagents as directed in this Package Insert.
2. Bring all reagents to room temperature and prepare the solutions.
3. Add 100 μl of Standard or sample to appropriate wells, and incubate for 1 hour at 37℃.
4. Remove sample solution and wash the wells 3 times with 400 μl of Washing Buffer.
5. Add 100 μl of antibody-POD conjugate solution into wells and incubate at 37℃ for 1 hour.
6. Aspirate solution from wells. Wash 4 times with ca. 400 μl of Washing Buffer per wells, aspirating thoroughly between washes.
7. Add 100 μl of Substrate Solution to each well. Incubate 15 minutes at room temperature.
8. Add 100 μl of Stop Solution to all wells. Mix gently.
9. Read at 450 nm as soon as possible.