Cat. # MK107

For Research Use

TaKaRa

Laminin EIA Kit

Product Manual

v201607Da

Table of Contents

I. Description

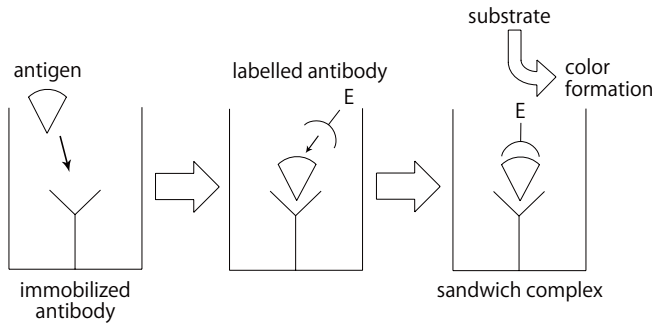
Laminins, which are large multidomain glycoproteins of the extracellular matrix, have attracted much attention because of their importance in cellular functions including induction of cell adhesion, growth promotion, mediation of cell communication, and enhancement of the metastatic phenotype of tumor cells (1). Laminins (LN) consist of three different polypeptide chains, α, β, and γ chain types, which replaced the previous designation of A, B1, and B2 (2). These chains are linked by disulfide bridges to form a characteristic asymmetric cross-structure as seen by electron microscopy (3). LN binds to various components of the basement membrane and probably links these to one another (4). Cell surface receptors that may play a role in LN-mediated cell adhesion have been isolated from metastatic tumor cells (5) and platelets (6). Most of the studies on LN have focused on the protein isolated from tumors (7), placenta (8), or products from cultured cell lines (9). Serum levels of LN fragments have been reported to be elevated in patients with hepatic fibrosis (10), alcholic liver (11), hypertension (12), and several kinds of tumors (13). Since LN was isolated also from urine and urinary LN was identified to be a fragmented γ 1 chain (14) recently, the detection system having higher sensitivity has been required. The enzyme immunoassay system was developed for quantitative detection of LN by using domain-specific monoclonal antibodies. This kit uses γ 1-specific monoclonal antibodies and can be used to detect quantitatively both serum and urinary LN. By usage of this kit, it has been shown that the levels of urinary LN fragments become elevated in some tumors (14).

II. Intended Use

The Laminin EIA Kit is an in vitro enzyme immunoassay (EIA) kit for quantitative determination of human LN in plasma, serum, urine, 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.

III. Principle

The Laminin EIA Kit is a solid phase EIA based on a sandwich method that utilizes two mouse monoclonal anti-LN antibodies to detect LN by two-step procedure. One of the 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. After washing the plate, the second anti-LN which is labelled with peroxidase (POD) is added to the wells and incubated. During these steps, LN is captured onto the solid support on one side and tagged on the other by POD-anti-LN. The reaction between POD and substrate (H_2O_2, and tetramethylbenzidine) results in colour development with intensities proportional to the amount of LN present in samples and standards. The amount of LN can be quantitatively determined by measuring the absorbance using an EIA plate reader. Accurate sample concentrations of LN can be determined by comparing their specific absorbance with those obtained for the standards plotted on a standard curve.



IV. Reagents and Materials

Each Laminin 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 Microtiterplate Anti-HLN monoclonal antibody-coated plate (96 wells: 8 wells x 12 strips)	1 plate
(2)	Antibody-POD Conjugate Peroxidase-labeled anti-HLN monoclonal antibody	For 11 ml
(3)	Standard Lyophilized human Laminin	For 1 ml
(4)	Sample Diluent PBS containing 25% Block Ace and 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 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 meta 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.

VI. Specimen Collection and Handling

Cell or tissue extract is suitable for use in the assay, however, plasma, serum, urine, or cell culture supernatant can be also used. PBS containing 0.1% NP40, 10 mM EDTA and 2 mM Phenylmethylsulfonyl fluoride (pH 7.2) should be used for preparation of cell extracts. Venous blood samples are collected aseptically.
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℃. If the length of time between sample collection and assay is to exceed 24 hours, samples should be stored frozen
at -80℃ 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 of human, dilute the samples with Sample Diluent (4) by 6 - 10 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 6 - 10 folds as the middle concentration.

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 min 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 min or let vials stand and sporadically mix gently.

The Standard Solution contains 320 ng human Laminin/ml.
Prepare dilution series of 160, 80, 40, 20, 10, and 5 ng/ml by diluting the Standard Solution with Sample Diluent (4).

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 1 month 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.

1. Sample incubation: Pipette 100 µl of sample or Standard Solution (Solution 2) into one well within 5 min Mix, seal the microtiter plate (e.g. with a foil) and incubate for 1 hour at 25℃.
2. Remove sample or Standard 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.
3. Antibody-POD conjugate incubation: Add 100 µl of Antibody-POD Conjugate Solution (Solution 1) into each well, mix and seal the microtiter plate (e.g. with a foil) and incubate 1 hour at 25℃.
4. Remove the reaction 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).
5. Substrate incubation: Add 100 µl of Substrate Solution (5) into each well and incubate at room temperature (20 - 30℃) for 15 min.
6. Add 100 µl of Stop Solution into each well in the same order as fo substrate. Tap plate gently to mix.
7. 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 LN concentration in ng/ml (horizontal axis) for the standards.

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

XII. Performance Characteristics

1. **Range of standard curve:** 5 - 320 ng/ml.

2. **Specificity:**
   This kit specifically measures human LN with no detectable cross reaction with human fibronectin, vitronectin, fibrinogen, collagen type I, or collagen type III. This kit can be also used to measure rabbit LN. The application of this kit for quantitative detection of LN from other sources has not been tested.

3. **Assay duration:** Two and a half hours 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 and rabbit serum, plasma, or urine; culture supernatants, cell extracts.

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 LN concentration from the curve.

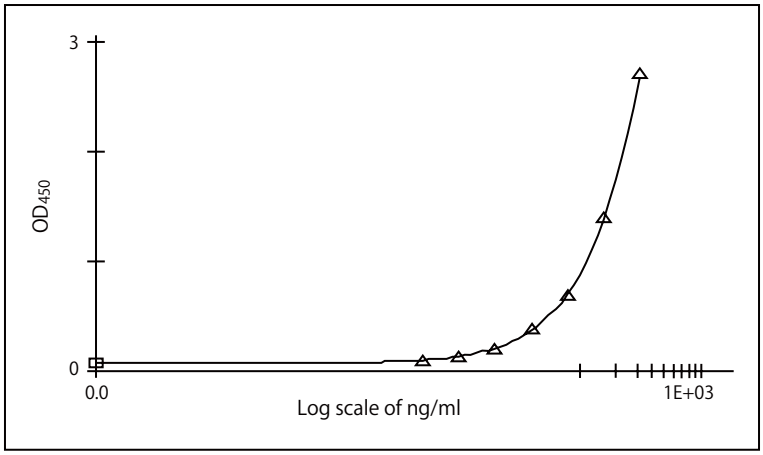
9. **Notes:**
   According to the assay results using control serum or urine, it could be possible to determine the concentration of antigen present in a biological.
   However, the measurement may be potentially disturbed by the unknown organic factors in serum, plasma or urine samples in patients with 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.

XIII. 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 = 0.0787 B = 01.15 C = 01.10E + 03 D = 013.6



Laminin (ng/ml)	320	160	80	40	20	10	5	0
A_{450} nm	2.712	1.418	0.700	0.385	0.215	0.145	0.104	0.075

2. Reproducibility

<Intra-assay precision (n=16)>

Assay was carried out with 16 replicates of 3 samples containing different concentration of Laminin.

Sample	Ave. (ng/ml)	S.D.	CV (%)
Control A	121.3	6.3	5.2
Control B	36.6	1.5	4.0
Control C	11.0	0.6	5.7

<Inter-assay precision (n=3)>

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	121.3	6.3	5.2
Control B	36.6	1.5	4.0
Control C	11.0	0.6	5.7

3. Recovery test

The recovery of Laminin was tested by adding 2 samples out of 4 different level in various matrices.

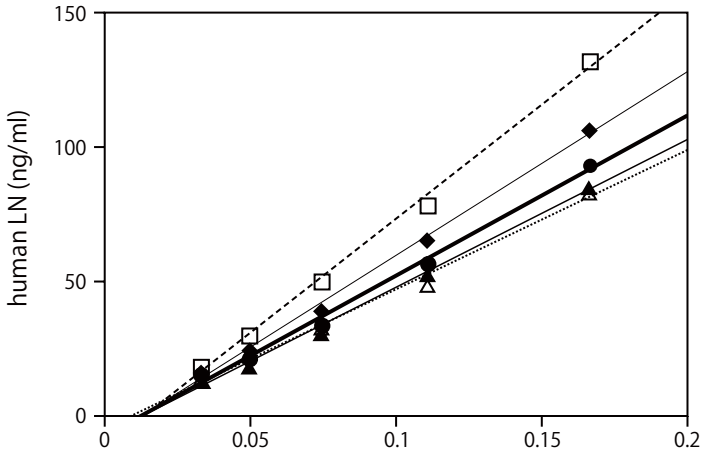
Sample A	Sample B	A+B Measured	A+B Calculated	Recovery (%) *
198.6	0.0	103.0	99.3	103.7
198.6	198.6	200.0	198.6	100.7
198.6	97.3	145.4	148.0	98.3
198.6	45.6	121.8	122.1	99.8
198.6	26.9	110.8	112.8	98.3
198.6	9.8	104.1	104.2	99.9
97.3	0.0	50.7	48.7	104.2
97.3	97.3	96.9	97.3	99.6
97.3	45.6	71.4	71.5	99.9
97.3	26.9	56.4	62.1	90.8
97.3	9.8	51.4	53.6	96.0
45.6	0.0	25.1	22.8	110.1
45.6	45.6	46.1	45.6	101.1
45.6	26.9	35.8	36.3	98.8
45.6	9.8	30.7	27.7	110.8
26.9	0.0	13.3	13.5	98.9
26.9	26.9	24.4	26.9	90.7
26.9	9.8	18.1	18.4	98.6

(unit: ng/ml)

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

4. Dilution curves of serum samples

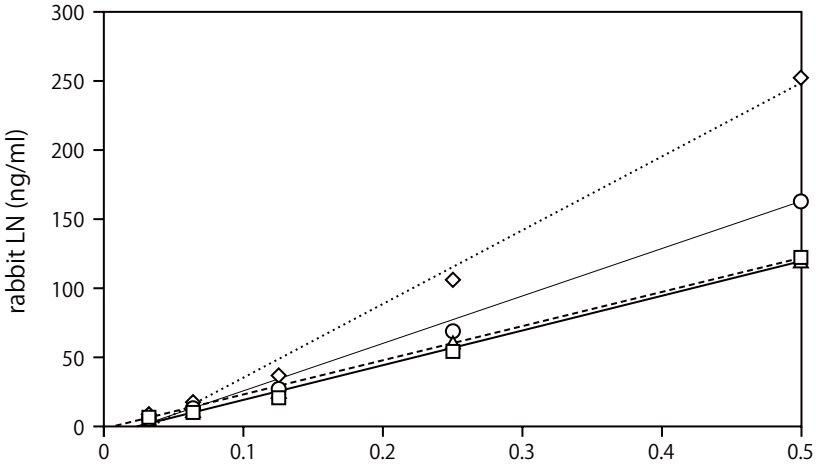
Each assay was performed using 5 kinds of samples which were prepared as dilution series starting from 6-fold.



●	no.1	y = 593.187x - 7.681	r = 0.997
◆	no.2	y = 682.517x - 9.374	r = 0.998
△	no.3	y = 515.298x - 5.146	r = 0.994
▲	no.4	y = 545.773x - 7.669	r = 0.998
□	no.5	y = 851.807x - 12.321	r = 0.998

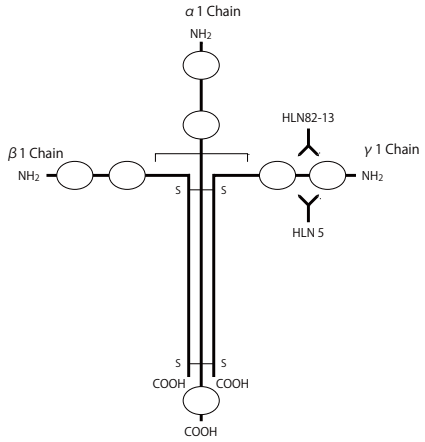
Effect of anticoagulants:

Effect of anticoagulants was evaluated by comparing the dilution curve of the samples which were simultaneonsly treated with different anticoagulants (normal rabbit; Jw, 10 weeks sample)



□	citrate	y = 248.571x - 4.988	r = 0.997
◇	EDTA.2Na	y = 530.374x - 19.094	r = 0.996
○	heparin	y = 339.691x - 9.358	r = 0.997
△	serum	y = 244.382x - 2.177	r = 0.999

5. Epitope of the antibodies supplied in this kit



Solid antibody (Antibody coated on the plate):
HLN82-13 (Cat. #M020)

Labelled antibody (Antibody-POD conjugate):
HLN5 (not provided)

6. Daily variation of human urinary Laminin and other proteins

The daily variation of urinary Laminin, Fibronectin and E-cadherin was measured using the samples from 3 individuals. For Laminin (ULN) and Fibronectin (UFN) assay, the samples were used directly. For E-cadherin (UEcad) 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 the former kits Cat. #MK007＊, MK015＊ and MK017＊. The value of creatinine was also measured.)

＊：Cat. #MK007, MK015, and MK017 are already discontinued.

		ULN (ng/ml)	Cr (g/l)	ULN/Cr (μg/g.Cr)	UFN/Cr (μg/g.Cr)	UEcad (/Cr) (mg/g.Cr)
1. female	day 1	75.6	1.264	59.8	76.8	2.82
	day 2	42.5	1.004	42.3	66.2	3.66
	day 3	36.1	0.605	59.7	75.4	4.33
	day 4	94.8	1.514	62.6	134.4	2.69
	day 5	81.6	2.346	34.8	57.5	3.42
	day 6	93.1	1.915	48.6	48.9	4.20
	day 7	87.3	1.313	66.5	99.8	3.89
	day 8	61.4	0.483	127.2	106.6	6.08
2. female	day 1	41.0	1.182	34.7	19.7	1.11
	day 2	64.2	2.045	31.4	17.6	0.59
	day 3	30.3	1.594	19.0	10.7	0.49
	day 4	66.8	1.565	42.7	21.4	0.20
	day 5	48.4	1.814	26.7	9.1	0.40
	day 6	63.5	2.562	24.8	20.1	0.22
	day 7	55.6	2.031	27.4	7.4	0.23
	day 8	60.8	1.579	38.5	14.8	0.33
3. male	day 1	54.3	1.195	45.4	58.5	0.73
	day 2	9.6	0.403	23.9	0.0	0.17
	day 3	61.5	1.016	60.5	56.4	0.86
	day 4	64.0	1.397	45.8	78.9	0.93
	day 5	16.8	0.422	39.9	5.7	1.37
	day 6	43.3	0.561	77.2	18.2	0.14
	day 7	53.4	1.466	36.4	21.6	1.17
	day 8	52.5	0.645	81.4	42.9	1.61

7. Urinary Laminin excretion in a day

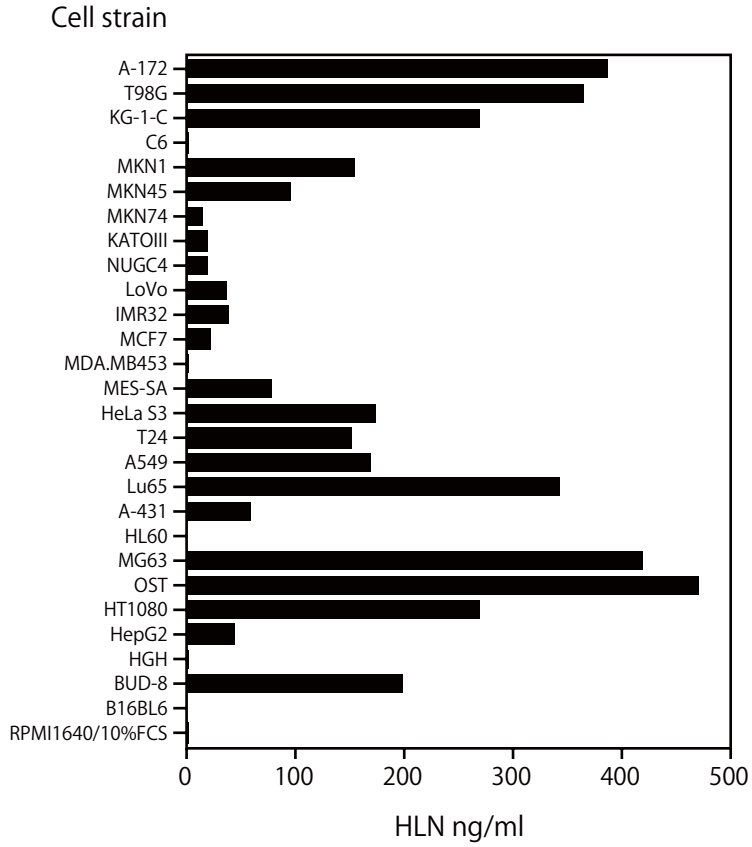
The amount of urinary Laminin and other proteins (FN and E-cadherin) excretion in a day was measured with the samples collected from 4 individuals.
For ULN and UFN assay, the samples were used directly. For UE-cadherin assay, the samples were used after 9-fold dilution. In the following table, the estimated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

＊：Cat. #MK007, MK015, and MK017 are already final sales.

	Urine (ml)	ULN (ng/ml)	Cr (g/l)	ULN/Cr (μg/g.Cr)	UFN/Cr (μg/g.Cr)	UEcad (/Cr) (mg/g.Cr)
4. male	270	114.8	1.760	65.2	113.4	0.40
	525	54.2	1.100	49.3	172.6	0.26
	70	101.7	1.489	68.3	44.7	1.04
	570	36.0	0.897	40.1	12.5	0.47
5. female	80	83.3	1.331	62.6	13.1	0.94
	100	135.0	1.441	93.7	28.0	0.80
	180	59.7	1.478	40.4	15.1	0.58
	200	52.8	0.974	54.2	46.8	1.52
	180	77.8	1.466	53.1	26.2	0.63
6. male	350	22.6	0.584	38.7	40.8	0.85
	470	11.8	0.481	24.6	24.3	1.32
	370	27.7	0.676	41.0	17.9	0.53
	180	62.5	1.548	40.4	49.5	0.50
	130	79.2	1.890	41.9	15.9	0.31
7. male	250	94.7	1.718	55.1	35.3	1.04
	150	117.8	2.759	42.7	53.9	1.47
	160	89.2	1.579	56.5	72.6	2.43
	175	163.9	2.400	68.3	59.1	1.43
	175	118.6	1.844	64.3	51.6	1.33

8. Laminin in cell culture supernatant

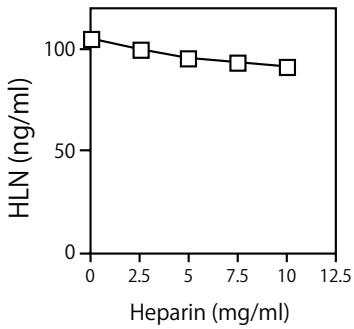
The amount of Laminin in the supernatant of various cells cultured in 10% FCS/ RPMI1640 was measured. The supernatant was applied to assay without dilution. Fetal Calf Serum does not inhibit this assay system.
(This data was obtained by using this precoated kit Cat. #MK107.)

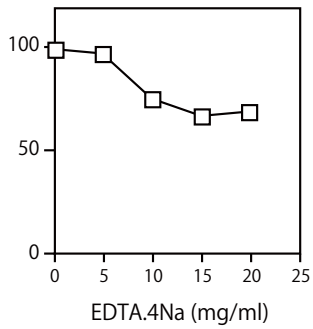


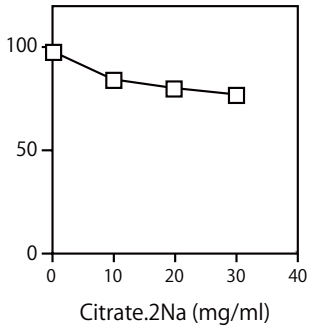
A172:	human glioblastoma	HeLa S3:	human uterus sarcoma
T98G:	human glioblastoma	T24:	human bladder carcinoma
KG-1-C:	human glioma	A549:	human lung carcinoma
C6:	rat glioma	Lu65:	human lung carcinoma
MKN1:	human gastric carcinoma	A-431:	human epidermoid carcinoma
MKN45:	human gastric carcinoma	HL60:	human promyelocytic leukemia
MKN74:	human gastric carcinoma	MG63:	human osteosarcoma
KATOIII:	human gastric carcinoma	OST:	human osteosarcoma
HUGC4:	human gastric carcinoma	HT1080:	human fibrosarcoma
LoVo:	human colon adenocarcinoma	HepG2:	human hepatocellular carcinoma
IMR32:	human neuro blastoma	HGH:	human girardi heart
MCF7:	human breast cancer	BUD-8:	human normal skin bibroblast
MDA.MB453:	human breast cancer	B16BL6:	mouse melanoma
MES-SA:	human uterus sarcoma	RPMI1640/10%FCS:	medium

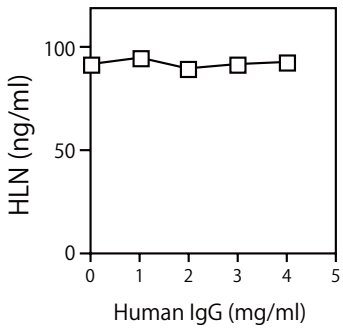
9. Influence of coexistence

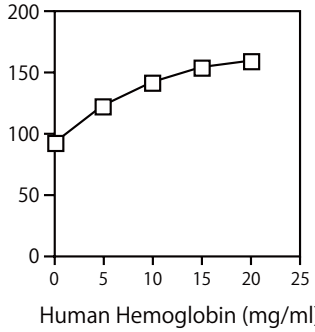
The volume ratio of sample to co-existing substance is 4:1. Co-existing substance is shown in its final concentration.

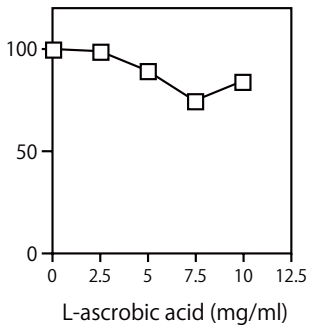


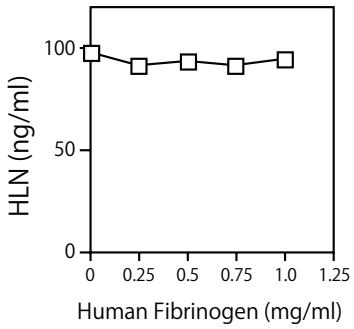


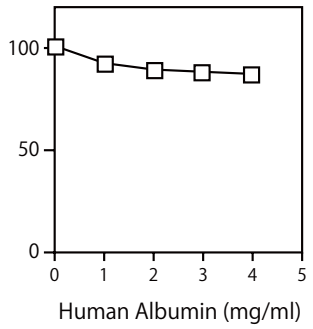


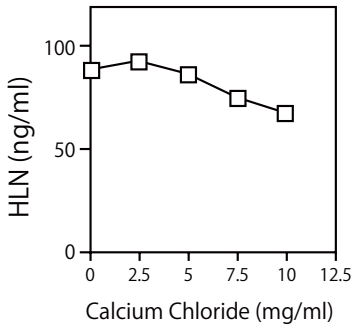


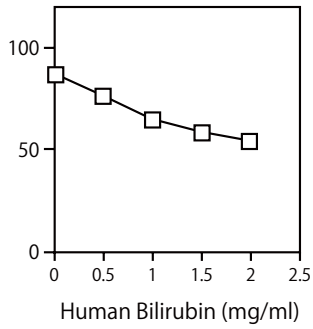






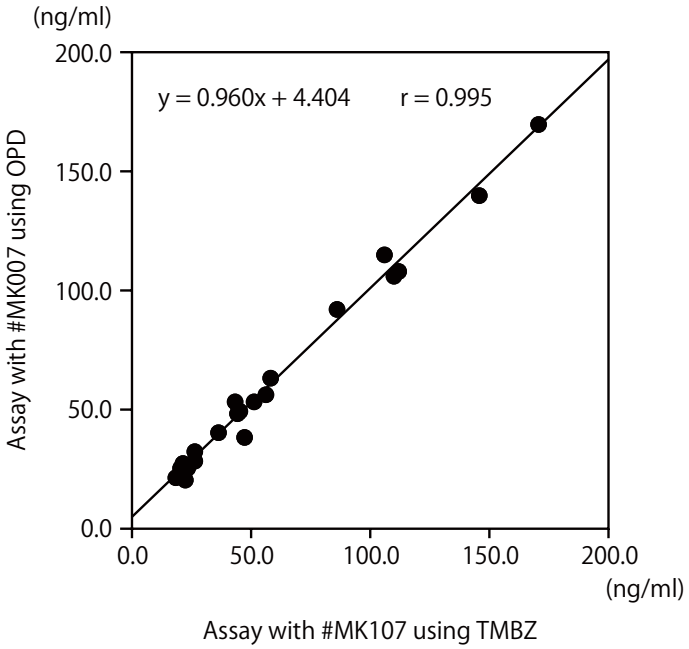






10. Correlation with the former kit (Cat. #MK007)＊

Correlation of precoated type kit (Cat. #MK107) 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. (n=22)
＊：Cat. #MK007 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

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2) Burgeson, R. *et al.* (1994) *Matrix Biology.* **14**, 209-211.
3) Beck, K. *et al.* (1990) *FASEB J*. **4**, 148.
4) Timpl, R. (1989) *Eur J Biochem*. **180**, 487.
5) Rao, C. N. *et al.* (1989) *Biochemistry*. **28**, 7476-7486.
6) Sonnenberg, A. *et al.* (1988) *Nature.* **336**, 487.
7) Timpl, R. *et al.* (1979) *J Biol Chem*. **254**, 9933.
8) Wewer, U. *et al.* (1983) *J Biol Chem*. **258**, 12654.
9) Alitalo, K. *et al*. (1980) *Cell.* **19**, 1053.
10) Kropf, J. *et al.* (1988) *Clin Chem*. **34**, 2026.
11) Niemelä, O. *et al.* (1985) *Eur J Clin Invest*. **15**, 132.
12) Kropf, J. *et al.* (1991) *Clin Chem*. **37**, 30.
13) Brocks, D. G. *et al.* (1986) *Clin Chem*. **32**, 787.
14) Katayama, M. *et al.* (1992) *Br J Cancer.* **65**, 509-514.

XVI. Protocol Summary

1. Prepare all reagents as directed in the Package Insert.
2. Bring all reagents to room temperature.
3. Add 100 μl of Standard or sample to appropriate wells, and incubate 1 hour at 25℃.
4. Remove Standard or sample and wash the wells 3 times with 400 μl of PBS + 0.1% Tween 20.
5. Add 100 μl of Antibody-POD Conjugate Solution into wells and incubate at 25℃ for 1 hour.
6. Remove the solution from wells. Wash the wells 4 times with 400 μl of PBS + 0.1% Tween 20, aspirating thoroughly between washes.
7. Add 100 μl of Substrate Solution to each well. Incubate 15 min at room temperature.
8. Add 100 μl of Stop Solution to each well. Mix gently.
9. Read at 450 nm as soon as possible.