Procollagen Type I C-Peptide EIA KIT (precoated) ver. 384 wells format

A high throughput enzyme immunoassay kit
for the quantitative determination of
Procollagen Type I C-Peptide (PIP)

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

Code No. MK110
For 384 assays

Table of contents

Introduction

Collagens (types I, II, III, IV and V) are synthesized as precursor molecules called procollagens. These contain additional peptide sequences, usually called "propeptides", at both the amino-terminal and the carboxy-terminal ends. The function of these propeptides is to facilitate the winding of procollagen molecules into a triple-helical conformation within the endoplasmic reticulum. The propeptides are cleaved off from the collagen triple helix molecule during its secretion, after which, the triple helix collagens polymerize into extracellular collagen fibrils. Thus, the amount of the free propeptides reflects stoichiometrically the amount of collagen molecules synthesized (a relationship analogous to that between the carboxy-terminal peptide of proinsulin and the endogenously produced insulin). Quantitative detection of collagen synthesis was first reported by Taubman et al(1) who performed competitive radioimmunoassays for procollagen type I carboxy-terminal peptide (PIP) using polyclonal antibodies. Similar methods using PIP as reference have been used in studying the correlation of collagen levels with certain health disorders, ie. bone diseases(2), alcoholic liver diseases(3), liver cirrhosis (4) and scirrhous (Borrmann type IV) adenocarcinoma of the stomach(5,6).

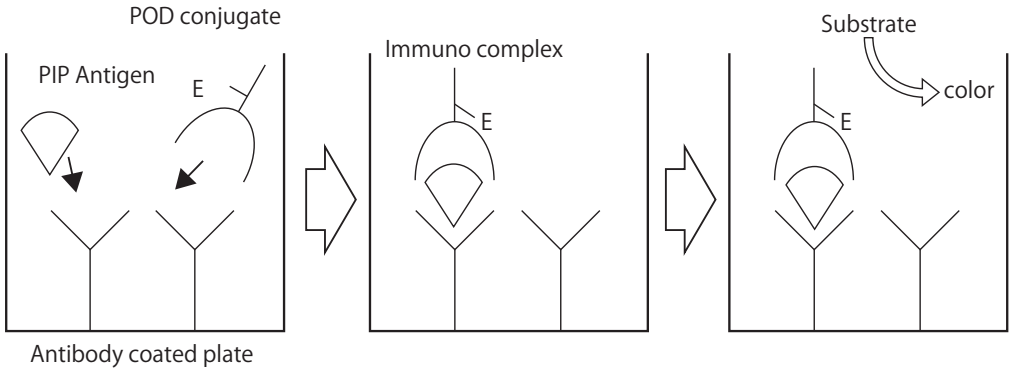
Intended use

The Procollagen Type I C-peptide EIA Kit ver.384 wells format is an *in vitro* enzyme immunoassay (EIA) kit for quantitative and high throughput determination of human, bovine or canine PIP in plasma, serum, cultured cell extracts, cell culture supernatants, or other biological fluids. This kit can be used not only with a dispenser machine but also with a single handy pipet.

This kit is for research use only. It is not for use in diagnostic or therapeutic procedures.

Principle

The PIP EIA Kit is a solid phase EIA based on a sandwich method that utilizes two mouse monoclonal anti-PIP antibodies to detect PIP by one-step procedure. One of the monoclonal antibodies has been pre-coated onto a microtiter-384 wells plate and blocked against non-specific binding. Samples, standard, and peroxidase(POD)-labelled anti-PIP antibody are simultaneously added to the wells of plates, and then incubated. During the incubation, PIP is bound to anti-PIP (solid phase) on one side and tagged by POD-anti-PIP on the other. The reaction between POD and substrate (H_2O_2 and tetramethylbenzidine) results in colour development with intensities proportional to the amount of PIP present in samples and standards. The amount of PIP can be quantitated by measuring the absorbance using an EIA plate reader for 384 multiwell. Accurate sample concentrations of PIP can be determined by comparing their specific absorbances with those obtained for the standards plotted on a standard curve.

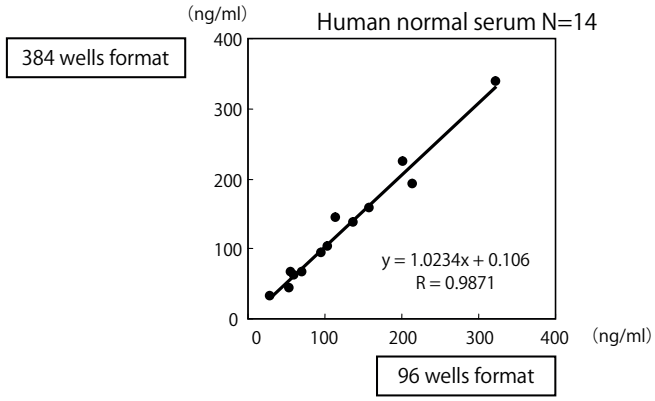


This 384 wells format kit keeps good correlation to 96 wells format kit (MK101).

Correlation with 96 wells format kit (Cat.#MK101)

Correlation with this kit (Cat.#MK110) between 96 wells format kit (Cat.#MK101) was examined to measure PIP amount of various concentrations of human serum.

The data showed good correlation between 96 wells format kit and 384 wells format kit. 384 wells format kit is useful for high-throughput assay.



Compare sample volume size	96 well	384 well
Standard and samples	20 μl/well	5 μl/well
Conjugate	100 μl/well	25 μl/well
Washing	400 μl/well	100 μl/well
Total assay capacity	96 assays	384 assays

The amount of the sample for 384 wells format kit needs only 1/4 for 96 wells format kit, but total assay capacity of 384 wells format kit is four times of 96 wells format kit.

Reagents and materials

Each PIP EIA Kit includes reagents sufficient for 384 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

Plate 1. Antibody Coated **Multi-Microtiterplate** - 1 plate (384 well x 1) **with plate-seal**
The plate contains murine monoclonal antibody to PIP and blocking material. Store at 2 - 8℃ .

Vial 2. Antibody-POD Conjugate - 1 vial (**for 12 ml** x 1)
The vial contains lyophilized horseradish peroxidase (POD) conjugated murine monoclonal antibody to PIP. Store at 2 - 8℃ . Avoid prolonged exposure to light.

Vial 3. Standard - 1 vial (640 ng x 1)
The vial contains lyophilized PIP 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℃ .

Vial 5. Substrate Solution - 1 vial (**7.5 ml x 2**)
Each vial contains hydrogen peroxide and tetramethylbenzidine in a buffered solution. Store at 2 - 8℃ .

Note: The parts of **Bold-faced** type are the different point from 96 wells format type(Cat.#MK101).

B. Materials required but not provided

1. Reagents
   - Washing Buffer: Phosphate-buffered Saline (PBS)
     (Dissolve 8.0 grams of NaCl, 0.2 grams of KCl, 2.9 grams of $Na_2HPO_4 \cdot 12H_2O$ and 0.2 grams of KH_2PO_4 in 1000 ml of distilled water.)
     PBS tablet (Cat.# T900) is useful to prepare washing buffer.
   - Stop solution: 1 N H_2SO_4

2. Materials
   - Pipet Station and Washer system for 384 wells plate (recommend)
   - Precision pipettes with disposable tips: 5 and 25 µl micropipettes, 5 - 100 µl adjustable multiwell pipetter or 5 µl and 25 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 in 384 well
   - Graph paper

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 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 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.

Specimen collection and handling

Venous blood samples are collected aseptically. Serum is suitable for use in the assay, however, plasma, 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 12 hours at 2 - 10℃. If the length of time between sample collection and assay is to exceed 12 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. PBS containing 0.5% Triton X-100, 1mM EDTA and 1mM Phenylmethylsulfonyl fluoride (pH7.2) should be used for preparation of cell extracts.
It is necessary to dilute human blood sample refer to sample dilution curve in basal data. (probably 5-10 folds)

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 **12 ml distilled water** and mix gently followed by 10 minutes 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 minutes or let stand and sporadically mix gently. The standard solution contains 640 ng PIP/ml. A dilution series can be formed by mixing the standard solution and Sample Diluent (Vial 4) for establishing the calibration curve, e.g.:

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 2 - 26℃ for up to 6 months.

Stability of solutions

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

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

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. During incubation, plate is kept on quiet.

1. Enzyme immunoassay

- Immunological reaction: Transfer 25 µl of antibody-POD conjugate solution (Solution 1) into one well, and subsequently add 5 µl sample or standard (Solution 2). Mix, seal the microtiter plate (e.g. with a foil) and incubate 3 hours at 37℃ .
  A sample and standard solution should be added within 15-20 minutes per plate.
  Unused well are covered with attached plate-seal.
  Standard well positions are (for example) A1-P1 and A24-P24 (32 point double file position)
- Remove contents by suction and wash the wells 4 times with ca.100 µl of PBS; between the separate washing steps empty out the microtiter 384 well plate and vigorously tap onto paper towel, especially after the last washing.
- Substrate incubation: Add 30 µl of Substrate Solution (vial 5) into each well and incubate at room temperature (20 – 30℃) for 15 minutes.
- Add 30 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 allowed to 384 wells format. 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 :

At the first step of the Procedure in Enzyme Immunoassay on page 5, it is recommended to mix the antibody-POD conjugate solution and sample or standard by shaking.
But, during the subsequent 3-hour incubation, the plate should not be shaken. Just leave it still. During the incubation at 37℃ or room temperature, the microtiter plate should be covered with an appropriate film to prevent the evaporation of the solution.

(Figure 1) 96-wells plate

	1	2	3	4	5	6
A	640	640	a	a	i	i
B	320	320	b	b	j	j
C	160	160	c	c	k	k
D	80	80	d	d	l	l
E	40	40	e	e	m	m
F	20	20	f	f	n	n
G	10	10	g	g	o	o
H	0	0	h	h	p	p

(ng/ml) sample

Plate format design

(Figure 2) 384-wells plate

	1	2	3	...	24
A	640	a	i		640
B	640	a	i		640
C	320	b	j		320
D	320	b	j		320
E	160	c	k		160
F	160	c	k		160
G	80	d	l		80
H	80	d	l		80
I	40	e	m		40
J	40	e	m		40
K	20	f	n		20
L	20	f	n		20
M	10	g	o		10
N	10	g	o		10
O	0	h	p		0
P	0	h	p		0

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 PIP concentration in ng/ml (horizontal axis) for the standards using optimal fitting curve.
- Linear curve fitting is under 320 ng/ml concentration.

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

Performance characteristics

1. **Range of standard curve:** 10 - 640 ng/ml. Linear fitting: 10-320 ng/ml
2. **Specificity:** This kit specifically measures human PIP with no detectable cross reaction with human fibronectin, vitronectin, laminin, collagen type I, or collagen type III. This kit cannot be used to measure mouse or rat PIP.

Note

The antibodies used in this kit cross react with procollagen type I C-peptides from bovine, horse and dog.

The measurement may be disturbed, when it is performed with samples that includes animal serum (ex. Fetal Bovine Serum (FBS) and Horse Serum). It is recommended to perform the measurement with the kit under the serum-free condition.

3. **Assay duration:** Three and a half hour.
4. **Total assay capacity:** 384 assays.
5. **Assay capacity for test samples:** If all assay wells (including standards and test samples) are run in duplicate, 184 test samples can be run in duplicate per kit.
6. **Test specimen type:** human bovine or canine serum or plasma, culture supernatants, cell extracts.
7. **Specimen volume required:** If each test sample is run in duplicate, approximately 20 µl (i.e., 5 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 100 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 PIP concentration from the curve.

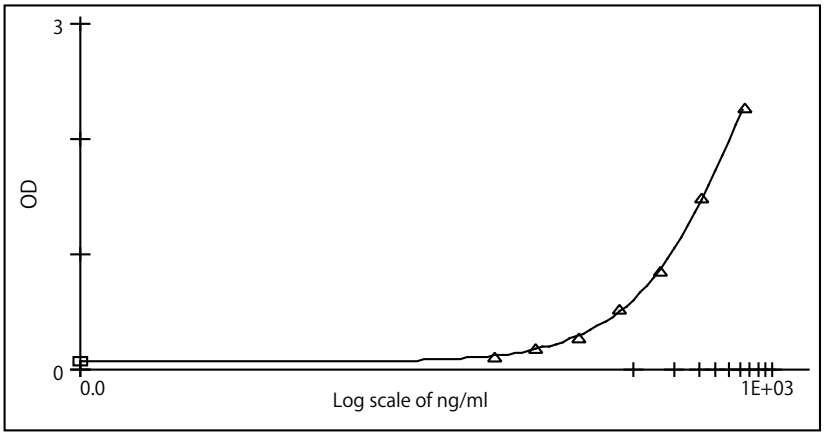
9. **Notes :** According to the assay results using control serum or urine, it could be possible to determine the concentration of antigen present in a biological.
However, the measurement may be potentially disturbed by the unknown organic factors in serum, plasma or urine samples in patients with specific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

Basal data

1. Typical standard curve

Detection limit is 10 ng/ml.

Curve Fit : 4-Parameter Corr. Coeff : -1.00
y = (A-D)/(1 + (x/C)^B) + D
A = 0.0761 B = 1.04 C= 710. D = 4.75



PIPconc. (ng/mi)	640.0	320.0	160.0	80.0	40.0	20.0	10.0	0.0
Absorbance A450	2.286	1.503	0.871	0.543	0.283	0.187	0.127	0.080

2. Intra-assay precision (n=32)

Assay was carried out with 32 replicates of 3 samples containing different concentrations of PIP.

sample(n=32)	average(ng/ml)	SD	CV(%)
Control A	109.91	10.8	9.8
Control B	56.02	5.6	10.0
Control C	23.33	2.7	11.5

Intra-assay precision (n=384)
Assay was carried out with 384 replicates of same concentration of PIP

total intra-assay precision (n=384)

Absorbance average	1.3135
SD	0.1264
CV (%)	9.63

column intra-assay precision (n=16)

	1	2	3	4	5	6	7	8
Absorbance average	1.3408	1.4091	1.2282	1.2817	1.3423	1.3789	1.2472	1.2639
SD	0.1248	0.1081	0.1039	0.0849	0.1391	0.0806	0.1179	0.0892
CV (%)	9.31	7.67	8.46	6.62	10.36	5.85	9.45	7.06
	9	10	11	12	13	14	15	16
Absorbance average	1.3312	1.3723	1.2375	1.2512	1.3008	1.3756	1.2450	1.2396
SD	0.1255	0.0814	0.1231	0.1065	0.1533	0.0826	0.1316	0.0578
CV (%)	9.43	5.93	9.95	8.51	11.78	6.01	10.57	4.66
	17	18	19	20	21	22	23	24
Absorbance average	1.3191	1.3682	1.2588	1.2672	1.3382	1.3556	1.3826	1.3899
SD	0.1214	0.1011	0.1666	0.0721	0.1414	0.0880	0.1408	0.0804
CV (%)	9.20	7.39	13.23	5.69	10.57	6.49	10.18	5.78

row intra-assay precision (n=24)

	A	B	C	D	E	F	G	H
Absorbance average	1.1190	1.1581	1.3492	1.3075	1.3567	1.3365	1.3704	1.3220
SD	0.1423	0.1453	0.1006	0.0676	0.0754	0.0714	0.0868	0.0746
CV (%)	12.72	12.54	7.46	5.17	5.56	5.34	6.33	5.64
	I	J	K	L	M	N	O	P
Absorbance average	1.3816	1.3558	1.4175	1.3897	1.3711	1.2928	1.2928	1.1961
SD	0.0983	0.0973	0.0764	0.0846	0.0703	0.0787	0.1160	0.0954
CV (%)	7.12	7.17	5.39	6.09	5.12	6.09	8.98	7.97

Inter-assay precision (performance 3 times)
Assay to assay precision with one laboratory was evaluated in three independent ex periments.

sample(n=3 times)	average(ng/ml)	SD	CV(%)
Control A	118.22	11.7	9.9
Control B	52.44	3.8	7.2
Control C	22.28	2.0	9.0

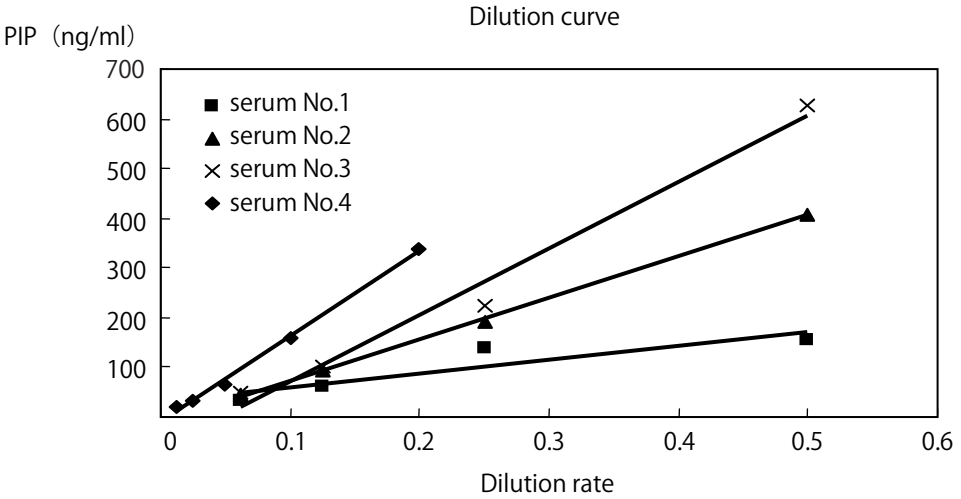
3. Recovery test

The recovery of PIP was tested by adding two samples out of five different level in various matrices.

Sample A	Sample B	A+B (result)	A+B (theoretical)	recovery %
126.2	0.0	57.3	63.1	91
126.2	67.7	87.2	96.9	90
126.2	30.3	78.2	78.2	100
126.2	299.7	225.3	213.0	106
126.2	256.9	195.8	191.6	102
67.7	0.0	36.9	33.8	109
67.7	30.3	48.6	49.0	99
67.7	299.7	172.1	183.7	94
67.7	256.9	172.9	162.3	107
30.3	0.0	13.3	15.2	88
30.3	299.7	190.9	165.0	116
30.3	256.9	182.7	143.6	127
299.7	0.0	173.6	149.9	116
640.0	0.0	367.1	320.0	115

4. Dilution curve

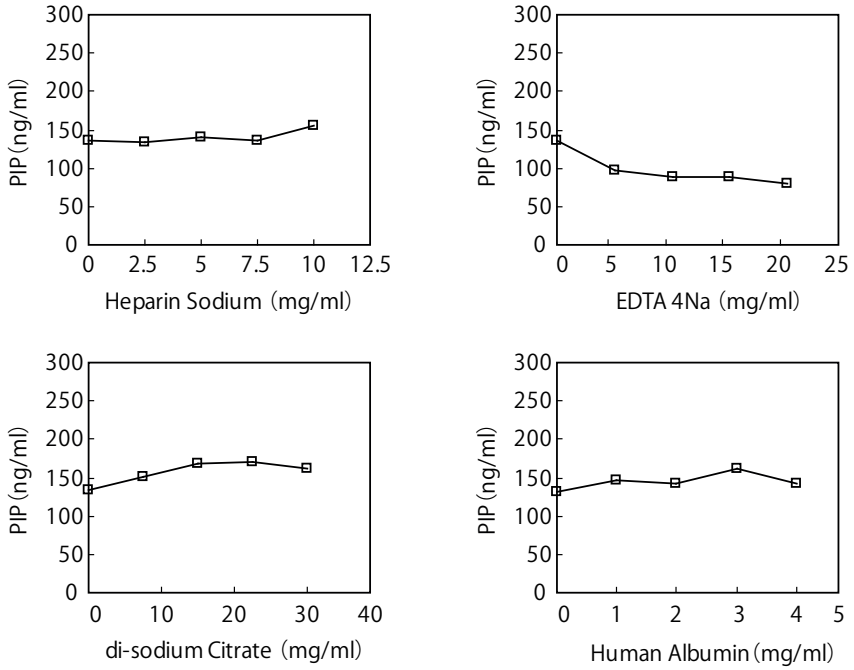
Healthy four samples are comparing the dilution curve. It is necessary to dilute human blood sample appropriately.



serum No.1	y = 277.5x + 31.223	R = 0.9093
serum No.2	y = 833.19x - 11.029	R = 0.9997
serum No.3	y = 1337.5x - 63.103	R = 0.9914
serum No.4	y = 1727.1x - 11.153	R = 0.9986

5. Influence of coextence

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



Application 1: Monitoring of PIP during differentiation into osteoblast

A monitoring of PIP in culture supernatant of human Mesenchymal Stem Cell (hMSC Lonza code. PT-2501) was monitored during differentiation to osteoblast.
The dupernatant was 3^6- fold and 3^7- fold diluted by sample diluent as a result of the preliminary experiment.
As the bovine fetal serum that contains bovine PIP is in basal medium, it is necessary to subtract basal medium concentration(B) from sample concentration.

Concentration ng/ml

Dilution	Medium Blank (B)		hMSC Day 3 Induction	Medium Change	hMSC Day 7 Induction	hMSC Day 10 Induction	Medium Change	hMSC Day 14 Induction	Medium Change	hMSC Day 21 Induction
× 3	169.84		over*		over*	over*		over*		over*
× 3^2	133.94		over*		over*	over*		over*		over*
× 3^3	54.68		419.97*		over*	over*		over*		over*
× 3^4	18.08		143.08*		564.73*	over*		over*		over*
× 3^5	7.24		56.66*		194.13*	328.58*		over*		470.71*
× 3^6	3.83		23.72*		87.46*	124.86*		244.57*		116.79*
× 3^7	2.40		12.45*		40.31*	70.41*		88.16*		60.76*
× 3^8	3.82		5.57*		21.69*	33.06*		46.46*		32.39*

* subtract blank from sample conc.

<Result>
During the differentiation to osteoblast , the production of procollagen increases dramatically in culture supernatant.

Application 2: Monitoring of PIP in culture supernatant in human osteoblast

Normal human osteoblast（NHOst Lonza code. CC-2538）were cultured in the growth medium containing 10% FCS. The amount of human PIP in culture supernatant was monitored.
The supernatant was 20-fold or 40 fold diluted by the sample diluent.

PIP conc.in NHOst culture supernatant ng/ml

Dilution	Medium Blank		NHOst Day 10	NHOst Day 14	NHOst Day 35
× 20	43.07		452.62*	over*	over*
× 40	22.12		274.94*	562.87*	over*
× 80	12.83		120.47*	210.14*	463.85*
× 160	8.22		57.63*	95.99*	203.41*

* subtract blank from sample conc.

<Result>
Hight concentration of human PIP is detected in NHOst culture supernatant.
Medium blank concentration means bovine PIP antigen conc.

Application 3: Measurement of a culture supernatant containing FCS(Fetal Calf Serum)

PIP concentration in culture supernatant of one cell line ng/ml

Dilution	Medium	MES.SA Human uterine sarcoma
× 3	242.17	354.36
× 3^2	99.87	148.75
× 3^3	33.41	57.47

<Result>
Human PIP concentration can be detected in 10% FCS/RPMI1640 medium by dilution the cell culture supernatant x 3 to x 3^3 fold to subtract the amount of medium from total value.

Application 4: Plate format design for high through-put assay

Application 1-3 can be assayed simultaneously using only half assay plate.

<Plate format design>

	1. 12	2～7	8. 9	10
A	640	× 3	× 20	× 3
B	640	× 3	× 20	× 3
C	320	× 3^2	× 40	× 3^2
D	320	× 3^2	× 40	× 3^2
E	160	× 3^3	× 80	× 3^3
F	160	× 3^3	× 80	× 3^3
G	80	× 3^4	× 160	× 3
H	80	× 3^4	× 160	× 3
I	40	× 3^5	× 20	× 3^2
J	40	× 3^5	× 20	× 3^2
K	20	× 3^6	× 40	× 3^3
L	20	× 3^6	× 40	× 3^3
M	10	× 3^7	× 80	
N	10	× 3^7	× 80	
O	0	× 3^8	× 160	
P	0	× 3^8	× 160	

(ng/mL)

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD	hMSC Medium Blank	hMSC Day 3	hMSC Day 7	hMSC Day 10	hMSC Day 14	hMSC Day 21	NHOst Medium Blank	NHOst Day 14	Mes. SA Medium Blank	control	STD
B												
C												
D												
E												
F												
G												
H												
I										Mes. SA sup		
J												
K												
L								NHOst Day 10	NHOst Day 35			
M												
N												
O										control		
P												

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.

References

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Protocol summary

1. Transfer 25 µl of antibody-POD conjugate solution into appropriate wells.
2. Add 5 µl of Standard or sample to the wells within 5 minutes, and incubate 3 hours at 37℃ .
3. Remove sample solution and wash the wells 4 times with 100 µl of PBS.
4. Add 30 µl of Substrate Solution to each well. Incubate at room temperature for 15 minutes.
5. Add 30 µl of Stop Solution to all wells. Mix gently.
6. Read at 450 nm as soon as possible.