

Cat. # **MK101**

For Research Use

TAKARA

**Procollagen Type I C-Peptide
(PIP) EIA Kit**

Product Manual

v201607Da

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I. Description

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 carboxy-terminal ends. The function of these propeptides is to facilitate the winding of procollagen molecules into a triple-helix conformation within the endoplasmic reticulum. These propeptides are then cleaved from the triple helix collagen molecule during secretion, and the triple helix collagens polymerize into extracellular fibrils. Thus, the amount of free propeptide stoichiometrically reflects the amount of synthesized collagen, a relationship analogous to that of the carboxy-terminal proinsulin peptide and 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 a reference have correlated collagen levels with certain health disorders, including bone disease,²⁾ alcoholic liver disease,³⁾ liver cirrhosis⁴⁾ and Borrmann type IV cirrhosis, as well as adenocarcinoma of the stomach.^{5,6)}

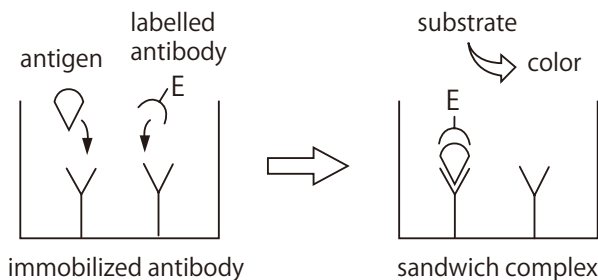
II. Intended Use

The Procollagen Type I C-peptide EIA Kit is an *in vitro* enzyme immunoassay (EIA) kit for the quantification of human, bovine, canine, horse, or monkey PIP in plasma, serum, cultured cell extracts, cell culture supernatants, or other biological fluids.

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

III. Principle

The PIP EIA Kit is a solid phase sandwich EIA that utilizes two mouse monoclonal anti-PIP antibodies to detect PIP using a one-step procedure. One of the monoclonal antibodies is pre-coated onto a microtiterplate and is blocked against non-specific binding. Samples, standard, and peroxidase (POD)-labelled anti-PIP antibody are simultaneously added to the wells of the plate and incubated. During incubation, PIP binds to anti-PIP on the plate (solid phase) and is recognized by POD-anti-PIP. The reaction between POD and substrate (H_2O_2 and tetramethylbenzidine) results in color development with intensity proportional to the amount of PIP present in the samples and standards. The amount of PIP can be quantified by measuring absorbance using a microplate reader. Accurate sample concentrations of PIP can be determined by comparing their specific absorbances with those obtained for standards plotted on a standard curve.



IV. Components

(1) Antibody Coated Microtiterplate Anti-PIP monoclonal antibody-coated plate (96 well : 8 wells x 12 strips)	1 plate
(2) Antibody-POD Conjugate (lyophilized) Peroxidase-labeled anti-PIP monoclonal antibody	for 11 ml
(3) Standard (lyophilized) Procollagen Type I (640 ng)	for 1 ml
(4) Sample Diluent 1% BSA containing PBS (with preservative)	11 ml x 2
(5) Substrate Solution (TMBZ) 3,3',5,5'-Tetramethylbenzidine solution	12 ml

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. Precautions

- Do not mix kit reagents from different lots.
- Do not use reagents past the expiration date on the label.
- Avoid reagent contamination by using 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 intense light during storage or incubation.
- Avoid skin or mucous membrane contact with the Substrate Solution and Stop Solution. 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 bodily fluids should be considered 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 color has changed to dark blue.

VII. Storage 4°C

VIII. Protocol

1. Specimen collection and handling

Collect venous blood samples aseptically. Serum can be used in this assay, as well as plasma and cell culture extracts and supernatants. Remove the clot or red blood cells from serum or plasma, respectively, promptly after clotting and separation. Samples containing a visible precipitate must be cleaned-up prior to use in the assay. Do not use overly hemolyzed or lipidemic specimens. Samples may be stored up to 12 hours at 2 - 10°C. When stored for more than 12 hours, samples should be stored frozen at -20°C for optimal results. Excessive freezing-thawing should be avoided. Prior to assay, frozen samples should be thawed at room temperature and slowly and gently mixed completely. Do not thaw samples quickly in a hot water bath. Do not vortex or agitate the samples. The cell extract should be prepared using PBS containing 0.5% Triton X-100, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (pH 7.2). If it is necessary to dilute human serum samples, refer to the results for sample dilution in X-4. Effect of anticoagulants. Samples diluted 5 - 10 fold are typically used with this kit.

2. Preparation of solutions

Note: The following solutions should be prepared just before use.

Solution 1. Antibody-POD Conjugate Solution

Dissolve (2) Antibody-POD Conjugate in 11 ml of distilled water and mix gently. Continue mixing for 10 minutes by slowly rolling or occasionally mixing, avoiding foam formation. Once reconstituted, the solution is stable for 1 week at 4°C, or for 1 month at -20°C. Do not repeatedly freeze and thaw.

Solution 2. Standard Solution

Dissolve (3) Standard in 1 ml of distilled water. Slowly roll for approximately 10 minutes or let vials stand and occasionally mix gently. Once reconstituted, the solution is stable for 1 week at 4°C, or for 1 month at -20°C. Do not repeatedly freeze and thaw.

The Standard Solution contains 640 ng PIP/ml. A dilution series can be prepared by mixing the Standard Solution and (4) Sample Diluent while establishing the calibration curve (see below for an example).

Final conc. (ng/ml)	0	10	20	40	80	160	320	640
Sample Diluent (Vial 4)	400 μ l	393.75 μ l	387.5 μ l	375 μ l	350 μ l	300 μ l	200 μ l	—
Standard Solution (Vial 3; 640 ng/ml)	—	6.25 μ l	12.5 μ l	25 μ l	50 μ l	100 μ l	200 μ l	400 μ l

Wash Buffer :

Wash buffer is prepared by diluting 50 ml of 10X PBS in Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021) with 450 ml of distilled water.

Stop Solution :

Stop Solution in Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021) can be used directly. Alternatively, 1 N H₂SO₄ can also be used for Stop Solution.

3. Procedure

Assay samples in duplicate.

Place all reagents in the kit and samples at room temperature. Ensure that all solutions are mixed uniformly before use, being careful to avoid creating bubbles.

1. Immunological reaction: Transfer 100 μ l of Antibody-POD Conjugate Solution (Solution 1) into one well, and subsequently add 20 μ l of sample or Standard Solution (Solution 2). Mix, seal the microtiter plate (e.g. with foil), and allow to incubate for 3 hours at 37°C.

All samples and the Standard Solution should be added within 5 minutes.

2. Discard well contents using suction and wash wells 4 times with 400 μ l of Wash Buffer. After each washing step, empty the microplate completely by inverting and tapping lightly onto a paper towel. This is especially important for the last wash.
3. Substrate incubation: Add 100 μ l of (5) Substrate Solution (TMBZ) into each well and incubate at room temperature (20 - 30°C) for 15 minutes.
4. Add 100 μ l of Stop Solution to each well in the same order as (5) Substrate Solution (TMBZ). Tap the plate gently to mix.
5. Measure the absorbance at 450 nm using 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 the addition of Stop Solution if wells are protected from light at room temperature.

Note: In the first step of the Procedure, it is recommended to mix the Antibody-POD Conjugate Solution and sample or standard by shaking. However, for the subsequent 3 hour incubation, do not shake the plate. During incubation at 37°C, the microtiter plate should be covered with an appropriate film to prevent evaporation of the solution.

4. Results analysis

1. Standard curve

- Record the absorbance at 450 nm for each well containing Standard Solution.
- Average the duplicate values.
- Plot the absorbance (vertical axis) versus the PIP concentration in ng/ml (horizontal axis) for the standards using an optimal fitting curve.

2. Samples

- Record the absorbance at 450 nm for each sample well.
- Average the duplicate values.
- Locate the average absorbance value on the vertical axis and find where it intersects the standard curve. At the point of intersection, read the PIP concentration (ng/ml) from the horizontal axis.

IX. Performance

1. Range of standard curve : 10 - 640 ng/ml.

2. Specificity :

This kit 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 PIP.

Note :

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

Measurement values may be altered when samples containing animal serum (e.g., fetal bovine serum (FBS) or horse serum) are measured. Use samples that have been grown in serum-free media. If using serum-containing media is unavoidable, refer to section XII-3. Measurement of bovine PIP in cell culture medium containing FCS.

3. Assay time :

3.5 hours

4. Total assay capacity : 96 assays.

5. Assay capacity for test samples :

If all assays (including standards and test samples) are performed in duplicate, 40 samples can be tested in duplicate per kit.

6. Specimen type :

Human, bovine, horse, canine, or monkey serum or plasma; culture supernatants, cell extracts.

7. Specimen volume required :

If each sample is run in duplicate, approximately 50 μ l (i.e., 20 μ l per assay well plus-10 μ l for each sample transfer) is required.

8. Limitations :

Because conditions may vary from assay to assay, a standard curve must be generated for every run. Because cross contamination between reagents will invalidate the test, disposable pipette tips should be used.

Thorough washing of the wells between incubation steps is required:

- 1) Completely empty out the remaining fluid from the well before dispensing fresh wash solution.
- 2) Use sufficient volume of Wash Buffer for each wash (approximately 400 μ l).
- 3) Do not allow plates to remain uncovered for extended periods of time between incubation steps.

Only samples with absorbance values falling within the range of the standard curve should be assigned a PIP concentration.

Notes :

According to the assay results using controls, it is possible to determine the concentration of antigen present in a biological sample. However, the measurement may be affected by unknown organic factors in serum or plasma samples in patients with specific diseases. Similarly, a specimen obtained from an apparent healthy subject might also be affected. When an antigen level in an unknown organic specimen is high as compared to the calibration range of the standard curve, dilute the specimens with Sample Diluent and assay again.

X. Basic Data

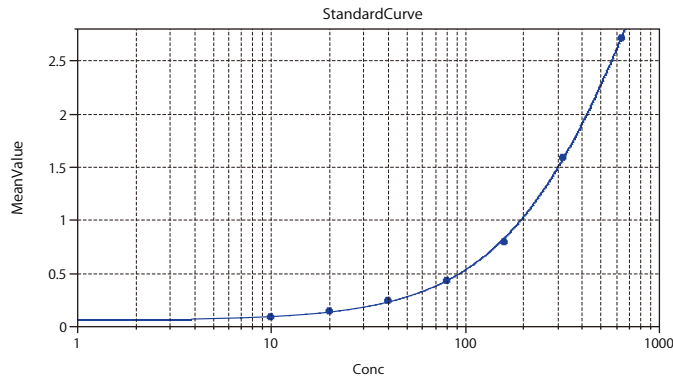
1. Standard curve

Shown below is an example standard curve for PIP.
A standard curve for calculation needs to be produced for each assay.

Limit of detection: 10 ng/ml.

Note: Shown as an example only; do not use to calculate experimental samples.

Curve Fit: 4-Parameter Corr. Coeff: -1.00
 $y=(A - D) / (1+(x/C)^B)+D$
 A=0.0491 B=0.933 C=542. D=4.34



PIP (ng/ml)	640	320	160	80	40	20	10	0
A ₄₅₀	2.709	1.593	0.797	0.435	0.240	0.141	0.087	0.040

2. Reproducibility

< Intra-assay variation (n=16) >

Assay was carried out using 16 replicates of 3 samples containing different concentrations of PIP.

	Ave. (ng/ml)	S.D. (ng/ml)	CV (%)
Sample A	484.8	35.64	7.4
Sample B	87.3	6.282	7.2
Sample C	31.7	1.411	4.5

< Inter-assay variation (n=3) >

Assay to assay variation from one laboratory was evaluated by three independent experiments.

	Ave. (ng/ml)	S.D. (ng/ml)	CV (%)
Sample A	466.1	20.25	4.3
Sample B	90.7	4.349	4.8
Sample C	29.6	1.873	6.3

3. Recovery test

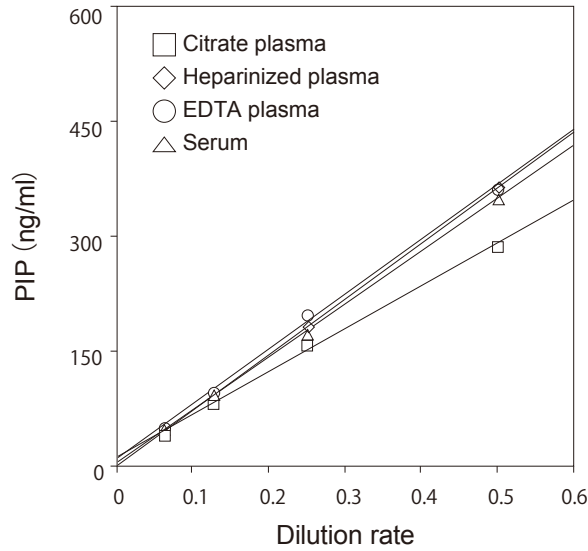
The recovery of PIP was tested by adding two samples at five different concentrations and comparing their percent recovery against the calculated amount.

(ng/ml)

Sample A	Sample B	A+B Measured	A+B Calculated	Recovery (%)
390.8	0.0	217.3	195.4	111
390.8	390.8	410.0	390.8	105
390.8	219.8	328.7	305.3	108
390.8	110.8	272.8	250.8	109
390.8	55.9	245.7	223.3	110
390.8	31.4	238.8	211.1	113
219.8	0.0	118.1	109.9	107
219.8	219.8	272.5	219.8	124
219.8	110.8	179.6	165.3	109
219.8	55.9	145.0	137.8	105
219.8	31.4	126.2	125.6	100
110.8	0.0	57.5	55.4	104
110.8	110.8	111.6	110.8	101
110.8	55.9	99.5	83.3	119
110.8	31.4	83.7	71.1	118
55.9	0.0	25.6	27.9	92
55.9	55.9	56.6	55.9	101
55.9	31.4	41.4	43.6	95
31.4	0.0	14.3	15.7	91
31.4	31.4	30.4	31.4	97

4. Effect of anticoagulants

The effect of anticoagulant treatment on healthy samples was examined. Different anticoagulants were tested and human blood samples were diluted accordingly.



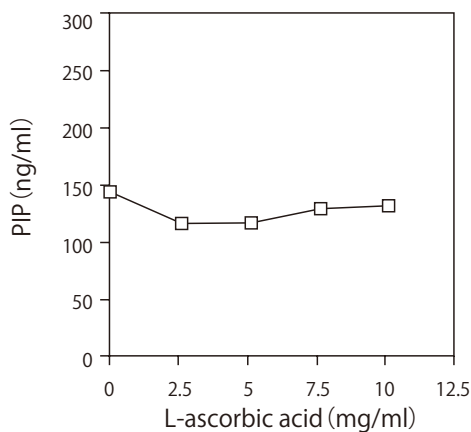
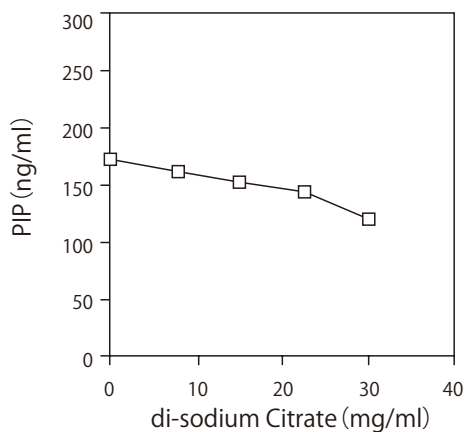
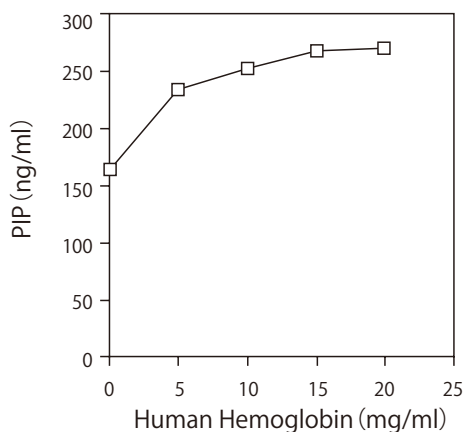
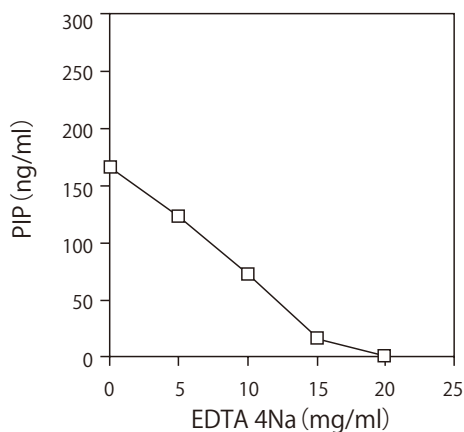
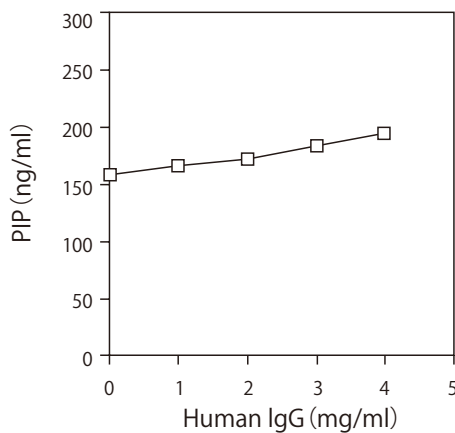
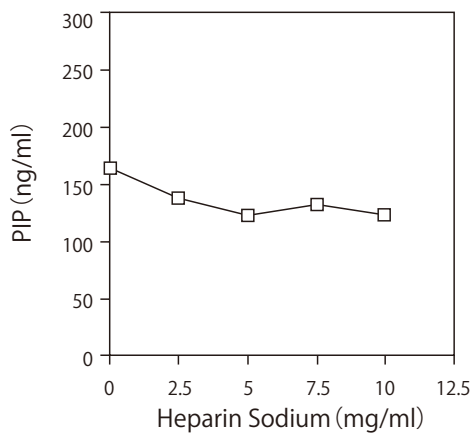
$y=562.087x+10.361$
 $y=737.475x - 1.196$
 $y=715.951x+8.274$
 $y=692.856x+4.487$

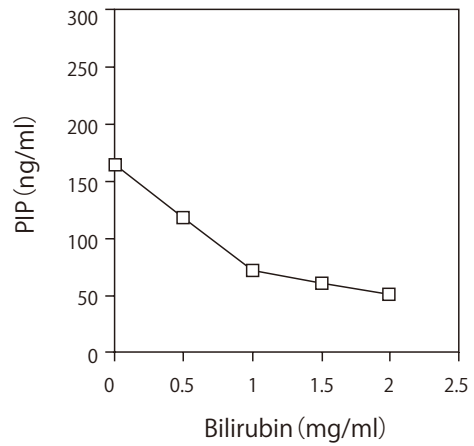
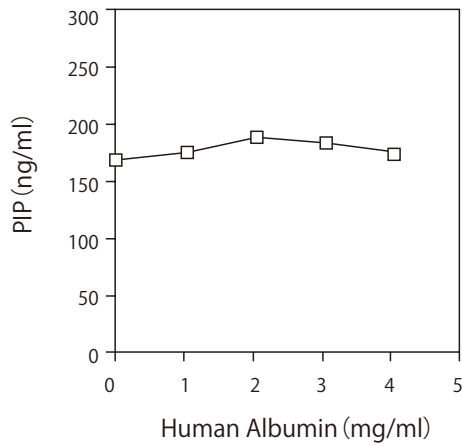
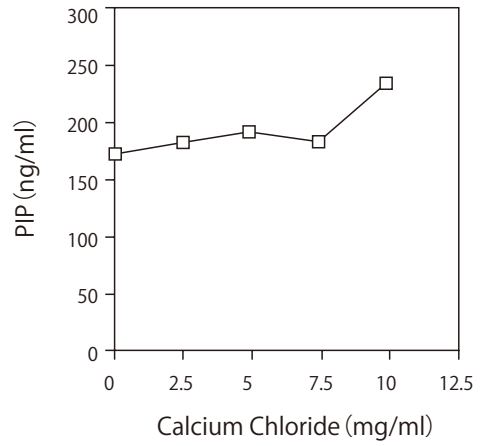
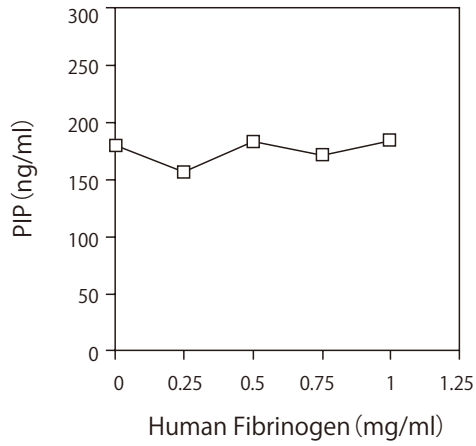
$r=0.998$
 $r=1.000$
 $r=0.998$
 $r=1.000$

Citrate plasma
 Heparinized plasma
 EDTA plasma
 Serum

5. Influence of coexisting substances

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





XI. High Sensitive Assay Using a Two-step Procedure

This kit can also be used with a two step procedure, which is useful for samples containing trace amounts of antigen or other contaminants. The two step procedure is also useful when the samples contain sodium azide, which inhibits the Antibody-POD Conjugate.

Procedure:

1. Dilute the Standard Solution (640 ng/ml) to a concentration of 160 ng/ml with Sample Diluent. Using 160 ng/ml as the highest concentration, prepare serial dilutions of the Standard Solution.
2. Add each 100 μ l of the prepared Standard Solution and sample to appropriate wells of a microtiter plate and incubate at 37°C for 2 hours. All Samples and Standard Solution should be added within 5 minutes.
3. Aspirate or decant well contents. Wash 3 times with 400 μ l of Wash Buffer per well.
4. Add 100 μ l of Antibody-POD Conjugate Solution (Solution 1)* to each well. Incubate at 37°C for 1 hour.
5. Aspirate or decant well contents. Wash 4 times with 400 μ l of Wash Buffer per well.
6. Add 100 μ l of (5) Substrate Solution (TMBZ) into each well and incubate 15 minutes at room temperature (20 - 30°C).
7. Add 100 μ l of Stop Solution into each well in the same order as (5) Substrate Solution (TMBZ) was added, and mix well.
8. Read at 450 nm after zeroing with distilled water. Color remains stable for 1 hour after stopping the reaction.

*: Please refer to section VIII-2 "Preparation of Solutions" (page 4) for preparing Solutions 1 and 2.

XII. Experimental Examples

1. Monitoring PIP during osteoblast differentiation

Human PIP was measured in culture supernatants from human mesenchymal stem cell (hMSC; Lonza Cat. #PT-2501) during osteoblast differentiation. The culture supernatant was diluted 3^6 - 3^7 -fold with Sample Diluent.

Because bovine fetal serum in the culture medium contains bovine PIP, it was necessary to subtract the culture medium signal (B) from that of the sample (A).

Sample	Dilution	PIP Concentration		(A) - (B)
		Sample (A)	Medium (B)	
hMSC 3 day Control	$\times 3^6$	16.67	2.72	13.95
	$\times 3^7$	4.73	0.00	4.73
hMSC 3 day Induction	$\times 3^6$	19.24	2.72	16.52
	$\times 3^7$	8.56	0.00	8.56
Medium change				
hMSC 7 day Induction	$\times 3^6$	82.18	2.72	79.46
	$\times 3^7$	35.57	0.00	35.57
hMSC 10 day Induction	$\times 3^6$	63.67	2.72	60.95
	$\times 3^7$	31.74	0.00	31.74
Medium change				
hMSC 14 day Induction	$\times 3^6$	111.50	2.72	108.78
	$\times 3^7$	54.51	0.00	54.51
Medium change				
hMSC 21 day Induction	$\times 3^6$	161.10	2.72	158.38
	$\times 3^7$	74.44	0.00	74.44

(ng/ml)

<Result>

During osteoblast differentiation, the amount of human PIP in the culture supernatant increased dramatically.

2. Monitoring PIP in human osteoblast culture supernatant

Human Osteoblast Cells (NHOst; Lonza Cat. #CC-2538) were cultured in growth medium containing 10% FCS. The amount of human PIP in the culture supernatant was monitored.

The supernatant was diluted 20-fold or 40 - fold with Sample Diluent.

Sample	Dilution	PIP Concentration		(A) - (B)
		Sample (A)	Medium (B)	
NHOst day 10	x 20	111.50	41.66	69.84
	x 40	74.88	20.76	54.12
NHOst day 14	x 20	181.80	41.66	140.14
	x 40	108.10	20.76	87.34
NHOst day 35	x 20	393.40	41.66	351.74
	x 40	201.60	20.76	180.84

(ng/ml)

<Result>

Human PIP concentration increased as a function of NHOst cell growth. Medium (B) indicates endogenous bovine PIP concentration in the FCS.

3. Measurement of bovine PIP in cell culture medium containing FCS (Fetal Calf Serum)

There are lot-to-lot variations in PIP concentration in commercially available FCS. PIP concentration was measured in several different commercially available media containing 10% FCS. Supernatants were diluted 20 - or 40 - fold in sample diluent.

PIP concentration in 10% FCS/RPMI 1640 medium

FCS	Dilution	PIP Concentration
A company	x 3 ³	69.61
	x 3 ⁴	21.14
B company	x 3 ³	88.04
	x 3 ⁴	29.67
C company	x 3 ³	73.39
	x 3 ⁴	29.04
D company	x 3 ³	109.40
	x 3 ⁴	50.50

(ng/ml)

PIP concentration in cell culture supernatant (10% FCS/RPMI 1640 medium)

Sample	Dilution	PIP Concentration
Medium only (10% FCS)	x 3 ³	70.93
	x 3 ⁴	25.49
MES.SA Human uterus sarcoma	x 3 ³	143.60
	x 3 ⁴	73.92

(ng/ml)

<Result>

Human PIP concentration can be detected in 10% FCS/RPMI 1640 medium by dilution the cell culture supernatant x 3³ or x 3⁴ fold.

XIII. References

- 1) Taubman, M. B., *et al.* (1974) *Science*. **186**:1115.
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- 7) Kanayama, N. and Terao, T. (1992) *Gynecol Obste Invest.* **34**:24-26.

XIV. Protocol Summary

1. Transfer 100 μ l of Antibody-POD Conjugate Solution into appropriate wells.
2. Add 20 μ l of Standard or sample to the wells within 5 minutes. Incubate 3 hours at 37°C.
3. Remove sample solution and wash the wells 4 times with 400 μ l of Washing Buffer.
4. Add 100 μ l of Substrate Solution to each well. Incubate at room temperature for 15 minutes.
5. Add 100 μ l of Stop Solution to all wells. Mix gently.
6. Read at 450 nm as soon as possible.

NOTE: This product is for research use only. It is not intended for use in therapeutic or diagnostic procedures for humans or animals. Also, do not use this product as food, cosmetic, or household item, etc.

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