Cat. # MK114

For Research Use

TaKaRa

Human/Pig Osteonectin EIA Kit

Product Manual

v201905Da

Table of Contents

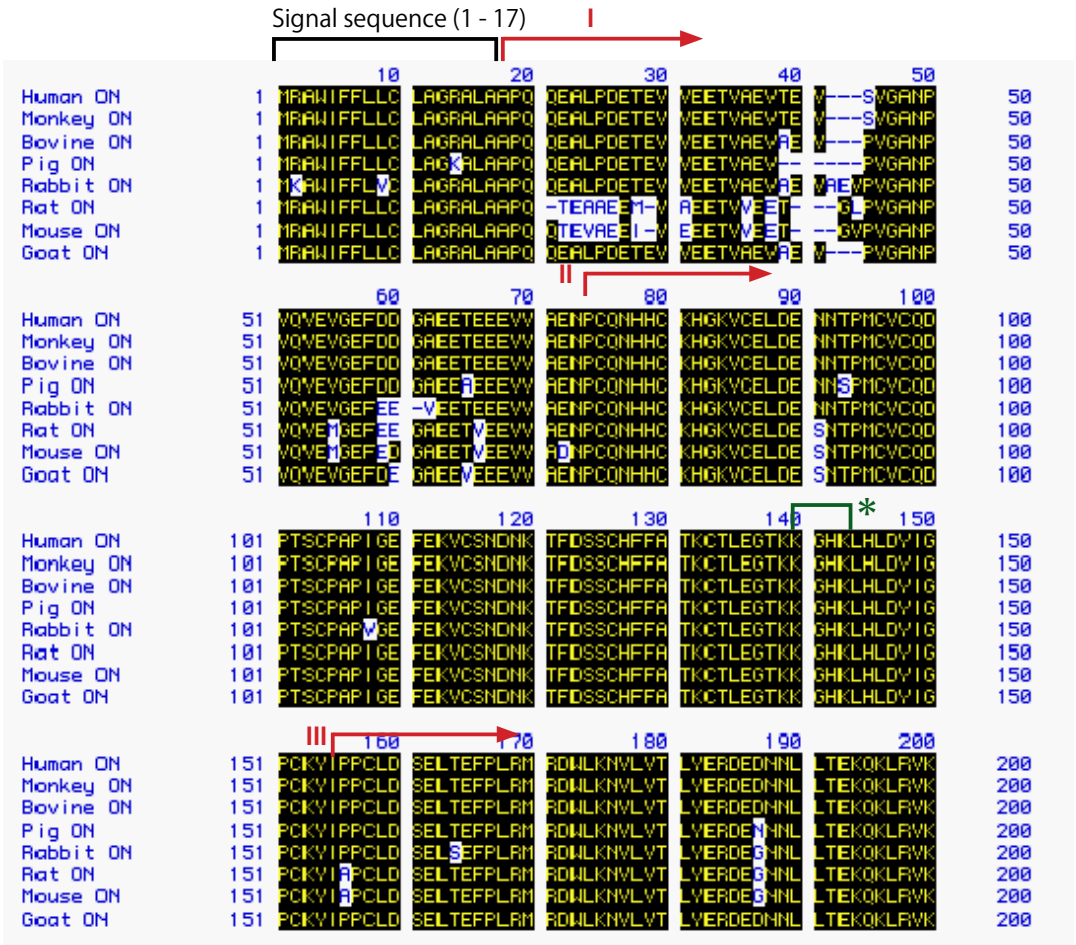
I. Description

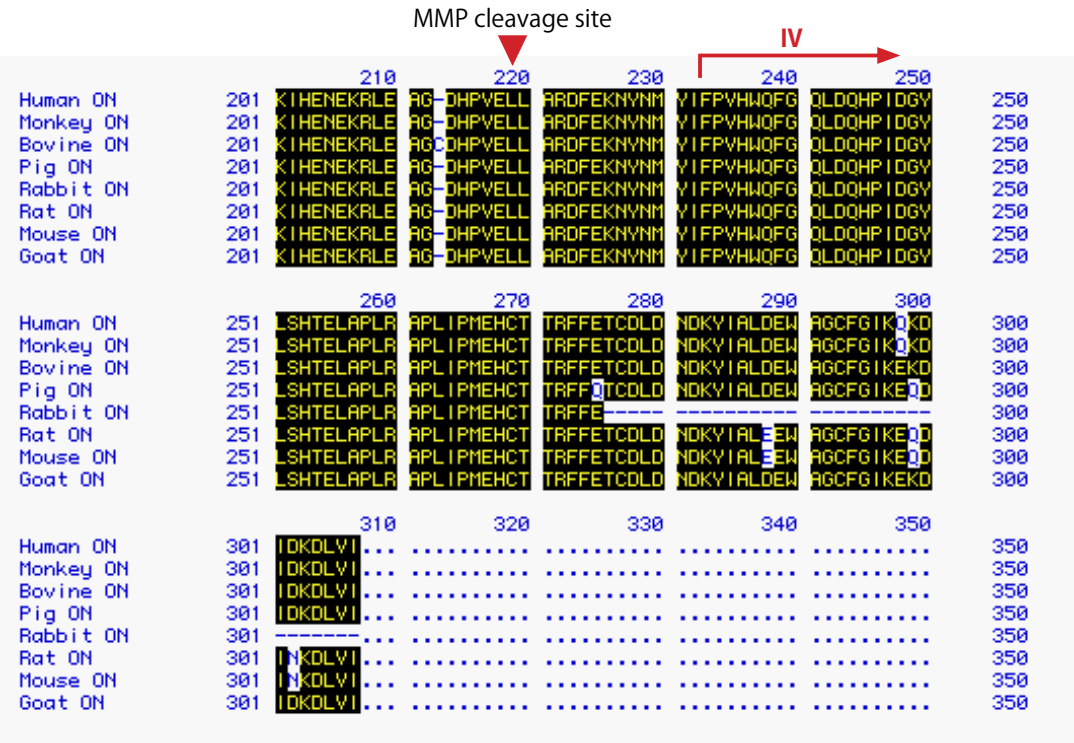
Osteonectin is an acidic phosphorylated glycoprotein with a molecular weight of 43 kD. It consists of a single polypeptide with a signal sequence of 17 amino acids and 283 - 287 amino acids which constitute osteonectin and differ between mammalian species. Osteonectin consists of four functional domains (I-IV). Domain I contains many acidic amino acids and several low-affinity calcium-binding domains; Domain II contains 10 cysteine residues; Domain III contains a protease-sensitive region; and Domain IV contains one low-affinity calcium-binding domain.

Osteonectin is also known as SPARC (Secreted Protein Acidic and Rich in Cysteine) or BM-40 (Basement Membrane 40 kD Molecule). Osteonectin was originally identified as a non-collagenous protein present in bone tissue. Later analysis indicated that it also exists in cartilaginous tissue, blood platelets, and vascular endothelial cells, and is expressed in several cell lines. It is now thought to be a multifunctional protein involved in cell proliferation and cell-matrix interactions. Moreover, because the synovial fluid of patients with rheumatism contains a high level of osteonectin, it may be useful as a diagnostic marker for arthritis.

This kit is a sandwich-type osteonectin EIA assay based on two monoclonal antibodies, which are derived from bovine and human osteonectin antigens. It enables simple quantification of human, pig, bovine, and rabbit osteonectin.

[Amino acid sequences of osteonectin in various species]

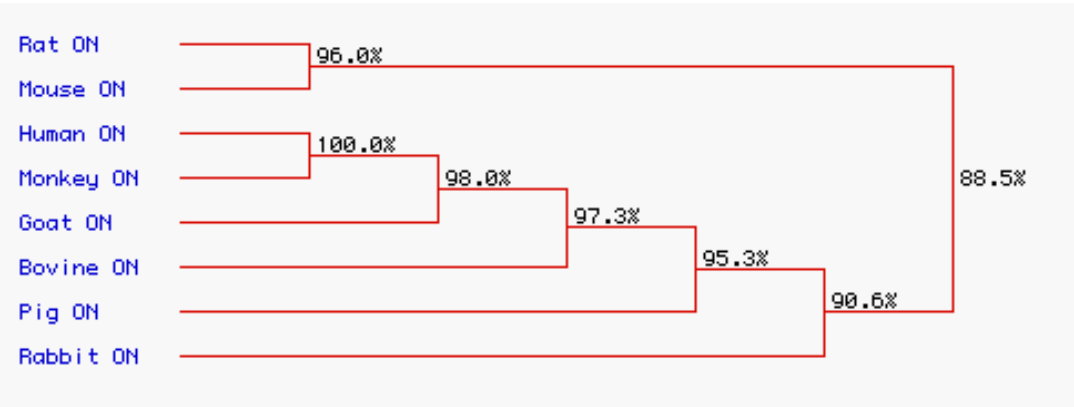




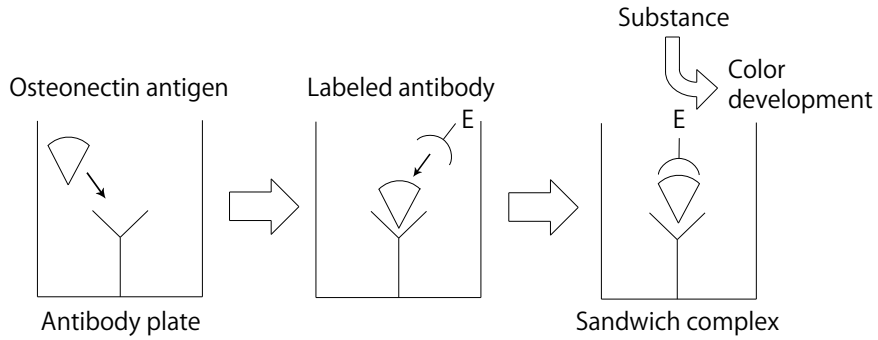
Domain I	: Acidic amino acid region
Domain II	: Cysteine-rich region
Domain III	: Contains an EF-hand motif, an α -helical domain, and an enzyme-sensitive region
Domain IV	: Contains an EF-hand motif and an α -helical domain

＊: Copper ion binding domain (KGHK)

[Amino acid sequence homology of osteonectin in various mammals]



II. Principle



III. Components

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

(2) Antibody-POD Conjugate (lyophilized) — For 11 ml
Peroxidase-labeled anti-ON monoclonal antibody

(3) Standard Osteonectin (lyophilized) — For 1 ml
Recombinant human osteonectin (derived from *E. coli*) 160 ng

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

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

IV. Materials Required but not Provided

- Wash and Stop solution for ELISA without Sulfuric Acid (Cat. #MK021)
  Contains wash solution (10X PBS; 50 ml x 5 tubes, Tween 20; 3 ml) and reaction stop solution (60 ml).
  - ＊ This product is a stop solution for peroxidase reactions without 1N sulfuric acid.
  - ＊ 1N sulfuric acid can be used as a stop solution. Handle 1N sulfuric acid with caution.
- Pipette, micropipette and tips
- Microplate reader (capable of measuring absorbance of up to 3.5 when set to 450 nm)

V. Storage

4℃

VI. Intended Use

Measurement of osteonectin in cultured cell extract, cell culture medium, or body fluids.

Note: This kit is for research use. It cannot be used for diagnostic purposes in humans and animals.

VII. Protocol

1. Samples

- This product can be used with cultured cell extracts, cell culture medium, plasma (citrated plasma and heparin plasma), or serum.
- Human, pig, bovine, and rabbit antigens can be measured using this kit.
- Based on sequence homology, it may be possible to measure monkey osteonectin with this kit, but reactivity has not been confirmed.
- This kit does not cross-react with mouse and rat antigens.
- Store samples at 2 - 10℃ before performing the assay, but freeze samples if measurements will take place >12 hours after sample collection.
- If using an anticoagulant for plasma sample preparation, do not use EDTA because it tends to yield low values.
- Due to a matrix effect, antigens tend to show low values when assaying samples containing high levels of protein, such as blood. Therefore, we recommend assaying samples that have been diluted 2-fold or more using (4) Sample Diluent.
- If dilution is necessary, dilute using (4) Sample Diluent.
- Thaw frozen samples at room temperature before measurement, and mix gently by inversion.
- Avoid subjecting samples to multiple freeze-thawing cycles.
- We recommend the following extraction buffer for preparing cell extracts:
  A neutral buffer (e.g., PBS, pH 7.4), containing 1% NP-40, 1 mM EDTA, and 1 mM PMSF (water soluble)

2. Reagent Preparation

- Antibody plate [(1) Antibody coated microtiter plate]
  Before use, return to room temperature.
- Labeled antibody solution
  Dissolve (2) Antibody-POD conjugate in 11 ml distilled water.
  The reagent is stable for 1 week at 4℃ after preparation. When storing it for longer periods of time, freeze it at -20℃. It is stable for 1 month under these conditions. However, avoid freeze-thawing this reagent more than once.
- Osteonectin standard solution
  Add 1 ml of distilled water to (3) Standard Osteonectin and dissolve completely to prepare osteonectin standard solution at 160 ng/ml. Then dilute stepwise using (4) Sample Diluent and prepare standard solutions at each concentration (160.0, 80.0, 40.0, 20.0, 10.0, 5.0 and 2.5 ng/ml). Use (4) Sample Diluent as the zero-concentration control. Prepared osteonectin standard solution (160 ng/ml) is stable for 1 week at 4℃ and for 1 month at -20℃. However, avoid freeze-thawing more than once.
- (5) Substrate solution (TMBZ)
  Before use, return this solution to room temperature and use as is. Confirm that the color of substrate solution has not changed to strong blue. Be careful not to contaminate the solution with tap water, as color development may occur in the presence of metal ions. If this solution will be used several times, aliquot the required amount beforehand.

- Stop solution
  Use the Stop Solution included in Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021) directly.
  * Because this solution is highly viscous, mix well using a plate mixer after it is added.
- PBS with 0.1% Tween 20 for washing
  Dilute one 50 ml tube of 10X PBS [included in Wash and Stop solution for ELISA without Sulfuric Acid (Cat. #MK021)] 10-fold with distilled water, add Tween 20 to a final concentration of 0.1%, and mix well. Use this diluted solution as a wash buffer.

3. Procedure

Perform measurements in duplicate.
Before use, bring the kit reagents and samples to room temperature and mix well (without creating bubbles).

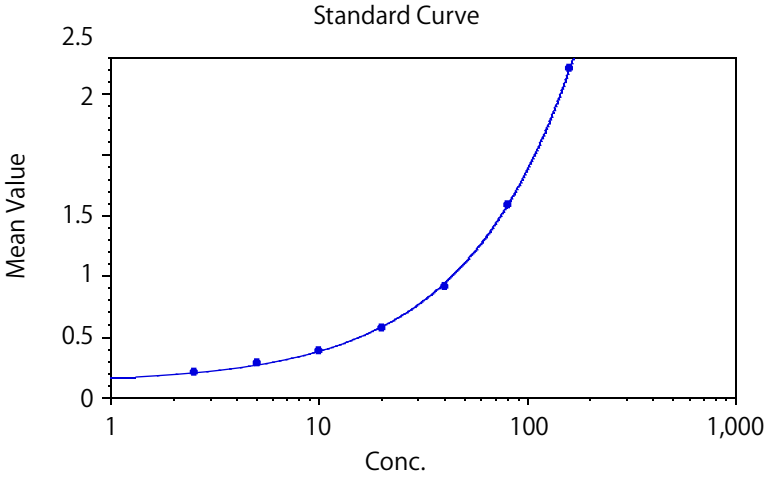
1. To each well, add 100 µl aliquots of samples or osteonectin standard solution (prepared ahead of time), using a micropipette, and mix with a plate mixer for 5 sec. Cover the plate with a film to avoid evaporation of the solution, and incubate the reaction at room temperature (20 - 30℃) for 1 hour (first reaction).
   * Prepare the standard solution and sample using a separate 96-well plate beforehand, and add promptly (within 5 minutes) to the antibody plate with an 8-channel pipette, etc. We r ecommend assaying a standard dilution series in the 1st and 12th rows. Perform the reaction at room temperature (20 - 30℃); heating at 37℃ may reduce antigenicity.
2. Discard the reaction solution and wash each well 3 times with PBS containing 0.1% Tween 20. Then add 100 µl of labeled antibody solution to each well using an 8-channel pipette and incubate the reaction at room temperature (20 - 30℃) for 1 hour (second reaction).
3. Discard the reaction solution and wash each well 4 times with PBS containing 0.1% Tween 20. Then add 100 µl of (5) Substrate Solution (TMBZ) to each well using an 8-channel pipette and incubate the reaction at room temperature (20 - 30℃) for approximately 15 minutes (third reaction).
4. Add 100 µl of Stop Solution * to each well in the same order in which the (5) Substrate Solution (TMBZ) was added, and mix well after the reaction has been stopped.
   * Because Stop Solution is highly viscous, mix well using a plate mixer, etc. after its addition.
5. Measure the absorbance at a wavelength of 450 nm following calibration, using distilled water as a background. Coloration is stable for at least 1 hour after the reaction is stopped.
6. Prepare a standard curve by plotting the concentration of each standard solution on the horizontal axis and the corresponding absorbance on the vertical axis. Use the absorbance of the sample to calculate the corresponding concentration of osteonectin.

VIII. Performance

1. Standard curve

The following is a representative standard curve. To obtain accurate results, prepare a standard curve for every measurement.

Limit of detection: 2.5 ng/ml



4-P Fit : $y = (A - D)/(1+(x/C)^B)+D$:

A	B	C	D	R^2
0.117	0.832	1.08e+09	1.24e+06	1

Osteonectin (ng/ml)	160.0	80.0	40.0	20.0	10.0	5.0	2.5	0.0
A_{450}	2.703	1.585	0.911	0.571	0.387	0.284	0.208	0.094

(Time for color development: 15 minutes)

2. Reproducibility

<Intra-assay precision (n=19)>

Reproducibility testing was performed with three concentrations of osteonectin solution.

Sample	Mean value (ng/ml)	CV (%)
Control A	72.34	4.2
Control B	21.99	7.6
Control C	5.37	8.7

<Inter-assay precision test (n=3)>

Quantification of three sample concentrations was performed over 3 days.

Sample	Mean value (ng/ml)	CV (%)
Control A	70.47	2.5
Control B	22.61	2.1
Control C	5.92	8.5

<Recovery test>

Equal amounts of various concentrations of samples were mixed. The recovery rate was determined by comparing the anticipated theoretical value with the actual measurement.

Sample A	Sample B	Theoretical value (A+B)/2	Measured value	Recovery rate (%)
72.3	22.0	47.2	43.8	92.9
72.3	5.4	38.9	36.9	95.0
22.0	5.4	13.7	13.0	94.9
160.0	80.0	120.0	108.5	90.4
80.0	40.0	60.0	54.3	90.5
40.0	20.0	30.0	27.2	90.6
72.3	10.0	41.2	39.0	94.8
5.4	5.0	5.2	5.4	103.6

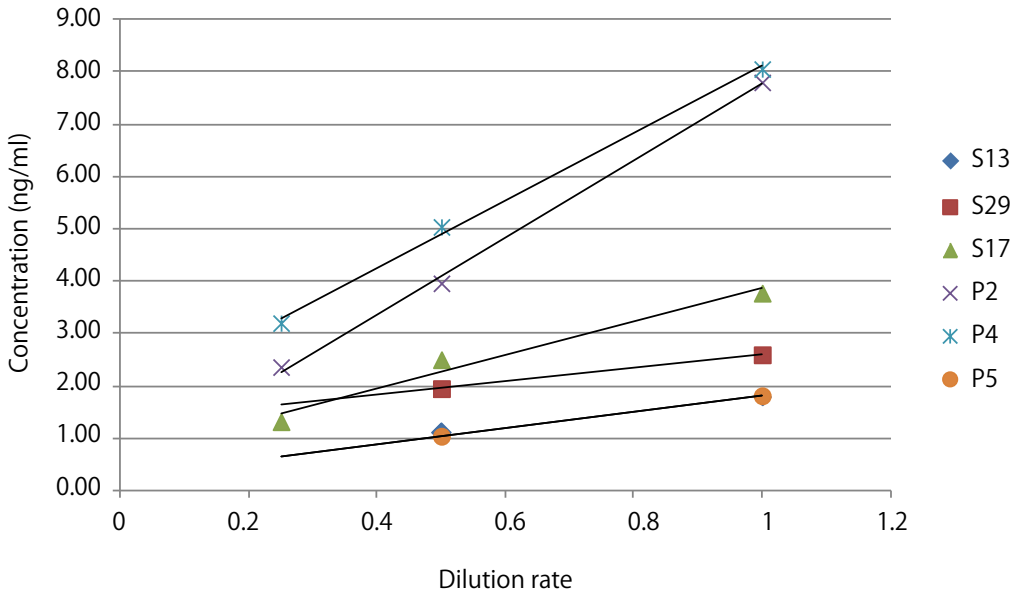
(Unit: ng/ml)

Result: The recovery rate was 90.5-103.6%.

3. Linearity of blood sample assay results

<Human blood>

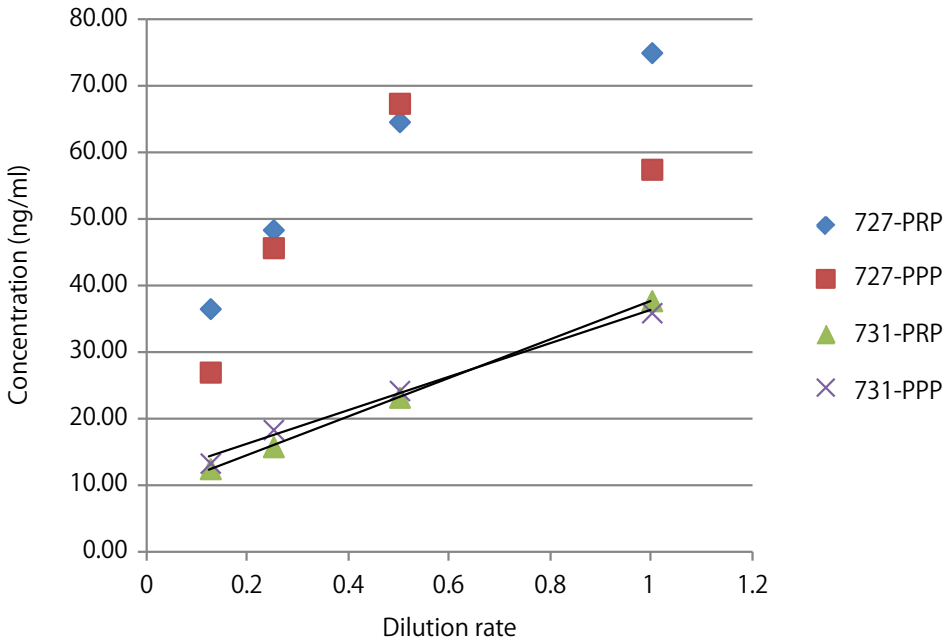
		Serum (ng/ml)			Citrated plasma (ng/ml)		
Dilution rate		S13	S29	S17	P2	P4	P5
x 1	1	1.80	2.60	3.78	7.81	8.06	1.81
x 2	0.5	1.13	1.95	2.50	3.96	5.04	1.04
x 4	0.25	-	-	1.32	2.36	3.20	-



<Miniature pig blood>

		Citrated plasma (ng/ml)			
		No. 727		No. 731	
Dilution rate		727-PRP	727-PPP	731-PRP	731-PPP
x 1	1	75.16	57.62	37.83	36.03
x 2	0.5	64.77	67.54	23.30	24.31
x 4	0.25	48.51	45.81	15.86	18.44
x 8	0.125	36.64	27.09	12.55	13.39

PRP: Platelet-rich plasma
PPP: Platelet-poor plasma

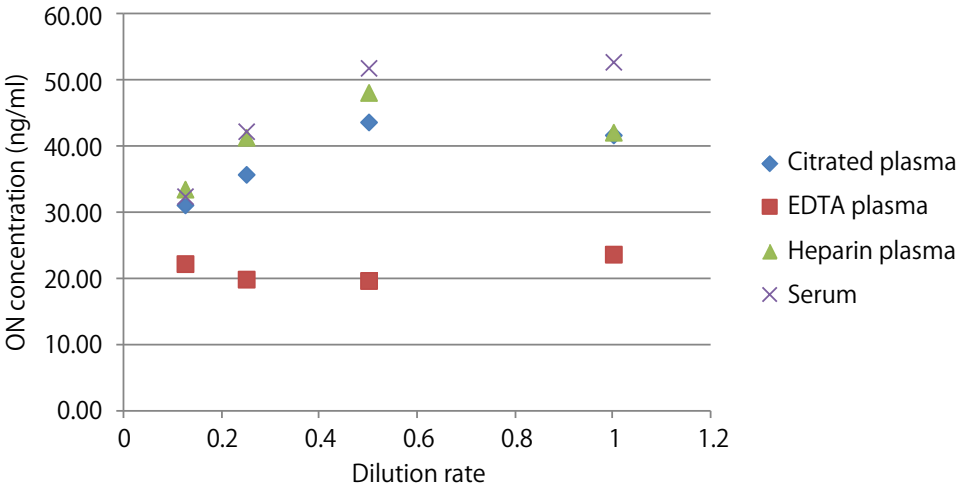


Result: We recommend measuring osteonectin concentrations in blood samples diluted 2-fold or more, since undiluted blood samples (x 1) can sometimes yield values that are lower than the estimated values.

4. Effect of anticoagulants

The effect of three different anticoagulants was evaluated by comparing the osteonectin concentrations of serial dilutions of rabbit blood treated with various anticoagulants.

		Rabbit osteonectin (ng/ml)			
Dilution rate		Citrated plasma	EDTA plasma	Heparin plasma	Serum
x 1	1	41.77	23.73	42.18	52.83
x 2	0.5	43.73	19.75	48.20	51.91
x 4	0.25	35.80	19.95	41.42	42.33
x 8	0.125	31.19	22.30	33.57	32.51



Result: Undiluted blood samples tended to show low osteonectin concentration values. The results also indicated that use of EDTA as an anticoagulant is not advisable when assaying plasma samples.

5. Effects of freeze-thawing on samples

The effect of repeated freeze-thawing on osteonectin concentration measurements was tested. Samples containing three different concentrations of fetal bovine serum were frozen and thawed repeatedly (seven times) between 25℃ and -80℃. Aliquots were collected after each thawing, and osteonectin concentrations of all the samples were measured simultaneously.

	Osteonectin (ng/ml)		
# of freeze-thawing cycles	High	Middle	Low
1	84.95	37.22	17.14
2	89.11	38.71	17.06
3	91.76	40.87	17.90
4	89.21	40.51	18.07
5	87.65	38.98	17.39
6	85.41	39.35	17.26
7	84.32	35.78	17.20
CV (%)	2.89	4.27	2.10

Result: Osteonectin concentrations are unlikely to be affected by freezing-thawing.

IX. Experimental Examples

1. Measurement of osteonectin and Gla-type osteocalcin in rabbit serum

Serum was collected sequentially from a rabbit bred for antibody production, and stored at -20℃. Osteonectin and Gla-type osteocalcin in these samples were simultaneously measured using this kit and the Gla-Type Osteocalcin (Gla-OC) EIA Kit (Precoated) (Cat. #MK111), respectively.

Age (day)	Osteonectin (ng/ml)	Gla-Type Osteocalcin (ng/ml)	
63	17.5	46.5	
73	17.0	34.5	
80	14.4	44.0	
87	-	28.5	
93	11.6	24.5	
101	15.7	21.0	
108	16.0	27.5	
115	11.7	20.5	
122	15.9	13.0	
129	12.1	7.5	
136	10.2	8.0	
143	14.5	8.5	
150	10.1	5.5	
157	10.4	4.0	
164	12.4	8.5	
199	42.9	8.5	← Immunization
220	164.7	15.5	← Immunization
241	246.9	18.4	← Immunization
262	92.6	13.5	← Immunization
283	21.2	10.3	← Immunization
293	13.1	8.0	

Result: Gla-type osteocalcin concentrations were higher at younger ages, and decreased with aging. However, osteonectin did not show the same pattern, with levels remaining nearly constant irrespective of age. When antigen was immunized with Freund's complete adjuvant, the amount of osteonectin in serum dramatically increased. This phenomenon may be the result of inflammation due to the process of immunization.

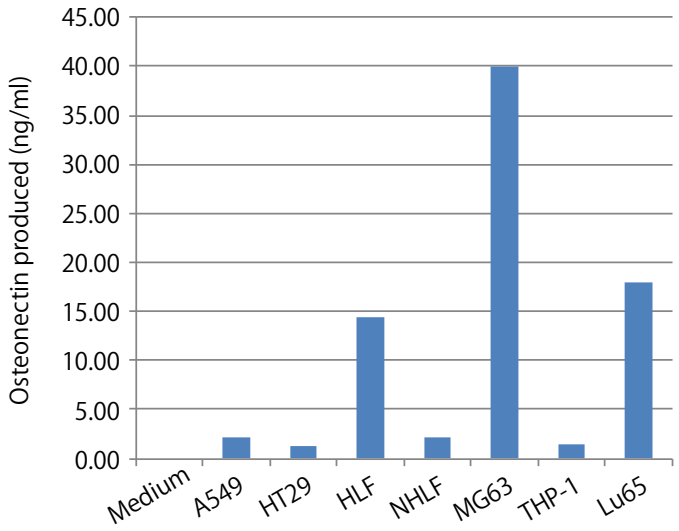
2. Measurement of osteonectin in human cell culture supernatants

Osteonectin concentrations were measured in cell culture supernatants from several human cell types. Each cell line was grown to confluence in a 10-cm cell culture dish using 30 ml of medium containing 10% fetal bovine serum. Osteonectin was also measured in the medium alone. The cell lines shown below were assayed:

Cell type	Origin	Culture method
A549	Human lung cancer cell	Adhesion
HT29	Human colonic gland cancer cell	Adhesion
HLF	Human hepatoma-derived cell	Adhesion
NHLF	Human normal lung fibroblast cell	Adhesion
MG63	Human osteosarcoma	Adhesion
THP-1	Human monocyte (acute leukemia)	Suspension
Lu65	Human lung cancer cell	Suspension
(Medium)	10% FCS/DMEM	

		Osteonectin in each supernatant (ng/ml)							
Dilution rate		Medium	A549	HT29	HLF	NHLF	MG63	THP-1	Lu65
x 1	1	2.73	4.85	4.05	17.08	4.78	42.77	4.18	20.66
x 2	0.5	0.00	3.21	2.35	12.53	2.91	21.65	2.76	8.11
x 4	0.25	0.00	1.36	0.46	8.25	1.01	11.83	1.54	3.34

		Osteonectin produced by cells (ng/ml): (concentration in each supernatant)-(concentration in culture medium)							
Dilution rate		Medium	A549	HT29	HLF	NHLF	MG63	THP-1	Lu65
x 1	1	0.00	2.12	1.32	14.35	2.05	40.04	1.45	17.93



Result: The osteonectin concentration in the medium was 2.73 ng/ml, low enough to avoid interfering with the measurement of osteonectin in the cell culture supernatants. The osteosarcoma cell line MG63 displayed an exceptionally high osteonectin concentration.

X. References

1) Termine JD. *et al*. (1981) *Cell.* **26**:99-105.
2) Romberg RW. *et al*. (1985) *J Biol Chem.* **260**(5):2728-2736.
3) Bolander ME. *et al*. (1988) *Proc Natl Acad Sci USA.* **85**(9):2919-2923.
4) Shiba H. *et al*. (1995) *Dev Biol.* **170**:457-466.
5) Nakamura S. *et al*. (1996) *Arthritis & Rheum*. **39**(4):539-551.

XI. Related products

Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021)
Anti-Osteonectin/SPARC, Monoclonal (OSN4-2) (Cat. #M124)
Anti-Osteonectin/SPARC, Monoclonal (ON1-1) (Cat. #M125)
Gla-Type Osteocalcin (Gla-OC) EIA Kit (Cat. #MK111)

XII. Precautions

1. Do not mix kits and reagents with different lot numbers.
2. Do not expose reagents to strong light during storage or reaction.
3. The pipettes, etc. used for Substrate Solution (TMBZ) and Stop Solution should contain no metal.
4. Take care to prevent Substrate Solution (TMBZ) and Stop Solution from coming into contact with hands or mucous membranes.
5. Do not use Substrate Solution (TMBZ) if it has changed color.
6. Each reaction can be affected by time and temperature, so prepare a standard curve for each measurement.
7. Handle blood samples with sufficient care.