Quantikine® ELISA

Human BMP-4 Immunoassay

Catalog Number DBP400

For the quantitative determination of human Bone Morphogenetic Protein 4 (BMP-4) concentrations in bone tissue extracts and cell culture supernates.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Bone morphogenetic protein-4 (BMP-4, previously known as BMP-2b) is a member of the transforming growth factor beta (TGF-β) superfamily. BMPs were originally identified as protein regulators of cartilage and bone formation. They have also been implicated in embryogenesis and morphogenesis of various tissues and organs. They can regulate growth, differentiation, chemotaxis and apoptosis of a variety of cell types, including mesenchymal, epithelial, hematopoietic and neuronal cells. BMP-4 plays an important role in the onset of endochondral bone formation, dorsal/ventral patterning and has also been implicated in the commitment of embryonic mesodermal cells to a hematopoietic fate in a number of systems (for reviews, see references 1-5).

Each BMP is synthesized as a precursor peptide, processed to a mature form, and subsequently secreted as a dimer. Although homodimers are considered the standard form, there are natural heterodimers with equal, if not increased, bioactivity (6, 7). BMP-4 is a 408 amino acid (aa) prepropeptide composed of a 19 aa signal sequence, a 273 aa pro-region, and a 116 aa mature segment (8). Both the pro-region and mature segment contain two potential N-linked glycosylation sites. The mature region contains seven highly conserved cysteine aa residues that form the characteristic cysteine knot found within TGF-β superfamily members. The mature regions of human, mouse and rat BMP-4 share 98% aa sequence identity.

BMPs signal via different hetero-oligomeric complexes of type I and type II serine/threonine kinase receptors (for reviews, see references 9-10). BMP-4 receptors include the type I receptor, ALK-6/BMP RIB (11), and the type II receptor, BMP RII (12, 13). Signals from activated BMP receptors are directly transduced to the cell nucleus by Smad proteins that then become incorporated into transcriptional complexes (for a review, see reference 14). Smad1 and Smad4, for example, are components of the BMP-4-induced transcription complex that is essential for dorsoventral patterning in Xenopus embryos (15).

In addition to promoting bone formation, BMP-4 is involved in other aspects of development. BMP-4, produced by the dorsal aorta, can direct sympathetic neuron differentiation (16). It may also influence somite development by inhibiting the process of myogenesis (17). BMP-4 plays a central role in dorsal/ventral patterning (for a review, see reference 18). It specifies the development of ventral structures (*e.g.* skin from ectoderm and connective tissue/blood from mesoderm). The distribution of BMP-4 expression suggests a direct role in the specification of human hematopoietic cells from embryonic mesoderm *in vivo* (19). Dorsal structures (nervous systems and muscle) appear when BMP-4 signals are interrupted through the activities of binding proteins, such as Noggin.

Variable expression levels of BMP-4 have been linked to different pathological states. In mice, BMP-4 gene knockout by homologous recombination results in embryonic lethality (1). Cases of patients displaying an interstitial deletion of chromosome 14 suggest that the 14q22 region *(i.e.* BMP-4 gene location) is important for human eye and pituitary development (20). BMP-4 is overexpressed in patients suffering from fibrodysplasia ossificans progressiva (FOP, an extremely rare, inherited disorder that is associated with abnormal skeletal patterning) (21, 22).

The Quantikine® Human BMP-4 Immunoassay is a 4.5 hour solid phase ELISA designed to measure human BMP-4 levels in bone tissue extracts and cell culture supernates. It contains NS0-expressed recombinant human BMP-4 and antibodies raised against the recombinant factor.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human BMP-4 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any BMP-4 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human BMP-4 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of BMP-4 bound. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with calibrator diluent and repeat the assay.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine® Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human BMP-4 Microplate	890828	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human BMP-4.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human BMP-4 Conjugate	890829	21 mL of a monoclonal antibody specific for human BMP-4 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human BMP-4 Standard	890830	Recombinant human BMP-4 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-61	895329	11 mL of a buffered protein base with preservatives. *May contain crystals. Warm to room temperature and mix gently to dissolve.*	
Calibrator Diluent RD5-13	895309	21 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- **Polypropylene** test tubes for dilution of standards.
- Human BMP-4 Controls (optional; R&D Systems®, Catalog # QC50).

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Note: *Polypropylene tubes must be used. Do not use glass.*

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Bone - Extract demineralized bone samples in 4 M Guanidine-HCl and protease inhibitors (23, 24). Dissolve the final sample in 2 M Guanidine-HCl.

Note: *Extractions can also be done in Urea (24, 25).*

Bone extract samples must be diluted in Calibrator Diluent RD5-13 prior to assay so that the concentration of Guanidine-HCl is ≤ 0.5 M, or the concentration of Urea is ≤ 1 M.

REAGENT PREPARATION

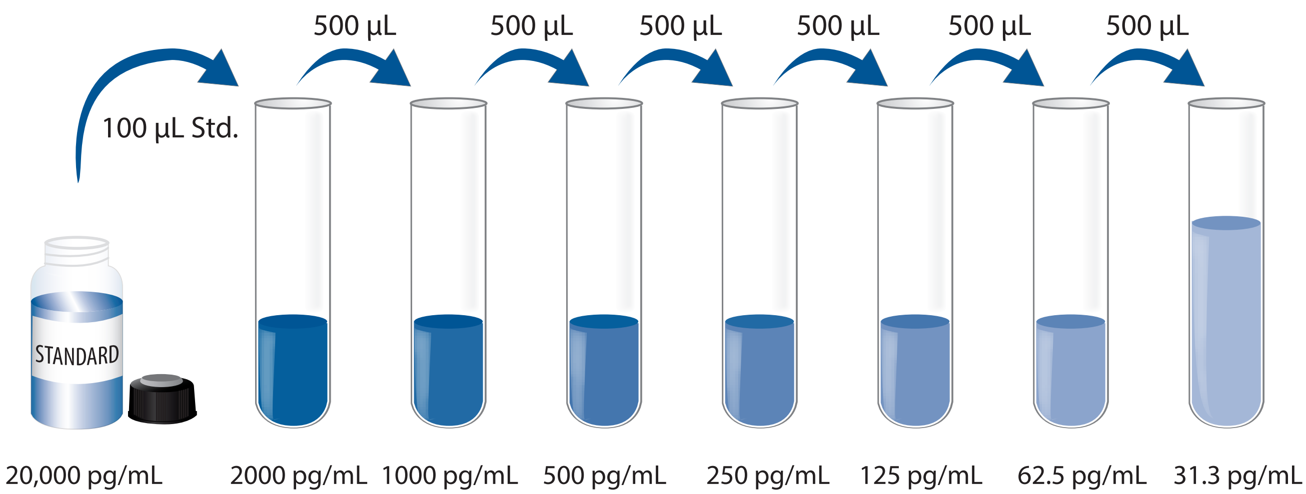
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μL of the resultant mixture is required per well.

Human BMP-4 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human BMP-4 Standard with deionized water. This reconstitution produces a stock solution of 20,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 900 μL of Calibrator Diluent RD5-13 into the 2000 pg/mL tube. Pipette 500 μL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 2000 pg/mL standard serves as the high standard. Calibrator Diluent RD5-13 serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Assay Diluent RD1-61 to each well. *Assay Diluent RD1-61 may contain crystals. Warm to room temperature and mix gently to dissolve before use.*
4. Add 50 µL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of Human BMP-4 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature. Protect from light.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Bone samples require extraction and dilution. See Sample Collection section.

CALCULATION OF RESULTS

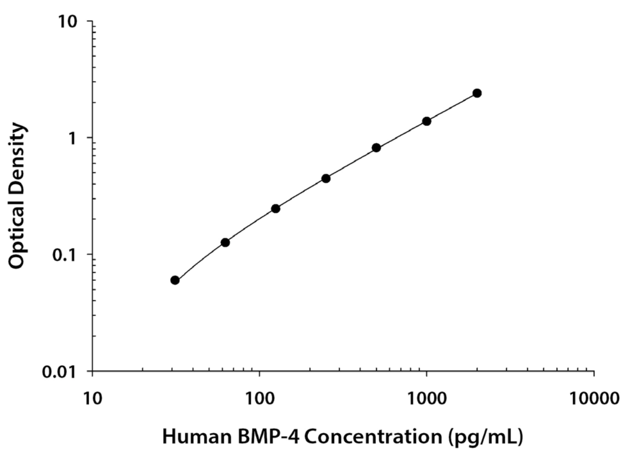
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human BMP-4 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.008 0.008	0.008	—
31.3	0.072 0.065	0.068	0.060
62.5	0.140 0.128	0.134	0.126
125	0.263 0.243	0.253	0.245
250	0.454 0.449	0.452	0.444
500	0.851 0.793	0.822	0.814
1000	1.409 1.364	1.386	1.378
2000	2.366 2.435	2.400	2.392

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in forty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	40	40	40
Mean (pg/mL)	301	719	1351	289	734	1429
Standard deviation	15.9	22.5	60.1	16.9	42.7	75.4
CV (%)	5.3	3.1	4.4	5.8	5.8	5.3

RECOVERY

The recovery of human BMP-4 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
2 M Guanidine-HCl* (n=2)	96	93-103%
2 M Urea* (n=2)	97	91-105%
Cell culture media (n=8)	100	91-109%

*Samples were diluted prior to assay.

LINEARITY

To assess the linearity of the assay, samples spiked with high concentrations of human BMP-4 were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=8)	2 M Guanidine-HCl* (n=2)	2 M Urea* (n=2)
1:2	Average % of Expected	104	102	101
	Range (%)	101-105	98-105	99-103
1:4	Average % of Expected	109	101	104
	Range (%)	105-111	100-103	101-107
1:8	Average % of Expected	110	101	98
	Range (%)	107-115	100-103	97-98
1:16	Average % of Expected	108	98	91
	Range (%)	103-111	94-101	90-92

*Samples were diluted prior to assay.

SENSITIVITY

Thirty-six assays were evaluated and the minimum detectable dose (MDD) of human BMP-4 ranged from 0.43-3.68 pg/mL. The mean MDD was 1.04 pg/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against highly purified NS0-expressed recombinant human BMP-4 produced at R&D Systems®.

SPECIFICITY

This assay recognizes natural and recombinant human BMP-4.

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human BMP-4 control were also assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Activin A
Activin RI
Activin RIIa
Activin RIIb
BMP-2
BMP-5
BMP-6
BMP-7
BMPR-1A
BMPR-1B
IL-3 Rα
Inhibin A
Inhibin B
Lefty
$Follistatin_{288}$
$Follistatin_{300}$
$Follistatin_{315}$
TGF-α
TGF-β1
TGF-β2
TGF-β3
TGF-β5
TGF-β RI
TGF-β RII

Recombinant mouse:

BMP-4
Follistatin
SCF
TNF-α

Other recombinants:

rat Agrin
zebrafish BMP-4

Natural proteins:

human α_2-macroglobulin
human TGF-β1
porcine TGF-β1

Recombinant mouse Noggin interferes at concentrations > 1.0 ng/mL in this assay.

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