

# Quantikine<sup>®</sup>

## BMP-2 Immunoassay

Catalog Number DBP200

SBP200

PDBP200

**For the quantitative determination of bone morphogenetic protein 2 (BMP-2) 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-2 (BMP-2, previously known as BMP-2A) is a member of the transforming growth factor beta (TGF- $\beta$ ) superfamily, based on amino acid (aa) sequence homology (1). 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-2 has pleiotropic functions including organogenesis, bone formation and regeneration, and regulation of pattern formation in the developing limb bud (1 - 7). Recombinant human BMP-2 has been shown to possess potent ectopic bone forming activity in a variety of experimental systems (6, 7).

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 (8, 9). BMP-2 is a 396 amino acid (aa) glycosylated polypeptide composed of a 19 aa signal sequence, a 263 aa pro-region, and a 114 aa mature segment (1). The mature region of BMP-2 has seven cysteines and one N-linked glycosylation site. Although the predicted mass of BMP-2 is 14 kDa, the mature segment is actually 18 kDa and is assumed to be glycosylated. The mature regions of human, mouse and rat BMP-2 are identical. With respect to other BMPs, BMP-2 and BMP-4 are 92% identical at the aa level and are therefore considered a subgroup within the BMP family (5). The human gene for BMP-2 maps to chromosome 20p12 (10, 11).

BMPs signal via different hetero-oligomeric complexes of type I and type II serine/threonine kinase receptors (for reviews, see references 12 and 13). BMP-2 receptors include the type I receptors, ALK-6/BMPRII, ALK-2/ActRI and ALK-3/BMPRI, and the type II receptors, BMPRII and ActRII (14 - 18). Endoglin, an accessory protein that interacts with the signaling receptor complex of TGF- $\beta$  superfamily members, can bind BMP-2 through interaction with the type I receptors, ALK-3/BMPRI and ALK-6/BMPRII (19). 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 20). The Smad1 pathway, for example, is involved in BMP-2 signaling (21, 22).

BMPs are important signaling molecules for embryonic development processes. During endochondral development, cartilage and bone differentiation involve a series of events that are directly influenced by BMPs. BMP-2, for example, obviously plays a critical role for development in mice, as BMP-2 gene knockout by homologous recombination results in embryonic lethality (2). Endochondral bone formation is not only necessary for limb formation in embryogenesis, but is also required for longitudinal bone growth in postnatal life and bone regeneration following injury. BMP-2 is expressed in the growth plate and regulates growth plate chondrogenesis by inducing chondrocyte proliferation and hypertrophy (23, 24). In addition to promoting bone formation during embryonic development, BMP-2 is also involved in dorsal-ventral pattern formation. BMP-2 influences patterning within mouse, zebrafish and *Xenopus* embryos (25 - 27).

The Quantikine BMP-2 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human BMP-2 in bone tissue extracts and cell culture supernates. It contains CHO cell-expressed recombinant human BMP-2 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural mouse BMP-2 showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that the Quantikine BMP-2 kit can be used to determine relative mass values for naturally occurring BMP-2.

## **PRINCIPLE OF THE ASSAY**

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for BMP-2 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any BMP-2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for BMP-2 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-2 bound in the initial step. 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.
- If samples generate values higher than the highest standard, dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

## MATERIALS PROVIDED

Description	Part #	Cat. # DBP200	Cat. # SBP200
<b>BMP-2 Microplate</b> - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against BMP-2.	892141	1 plate	6 plates
<b>BMP-2 Conjugate</b> - 21 mL/vial of monoclonal antibody against BMP-2 conjugated to horseradish peroxidase with preservative.	892142	1 vial	6 vials
<b>BMP-2 Standard</b> - 20 ng/vial of recombinant human BMP-2 in a buffered protein base with preservative; lyophilized.	892143	1 vial	6 vials
<b>Assay Diluent RD1-19</b> - 11 mL/vial of a buffered protein base with preservative.	895467	1 vial	6 vials
<b>Calibrator Diluent RD5P</b> - 21 mL/vial of a concentrated buffered protein base with preservative.	895151	1 vial	6 vials
<b>Wash Buffer Concentrate</b> - 21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives.	895003	1 vial	6 vials
<b>Color Reagent A</b> - 12.5 mL/vial of stabilized hydrogen peroxide.	895000	1 vial	6 vials
<b>Color Reagent B</b> - 12.5 mL/vial of stabilized chromogen (tetramethylbenzidine).	895001	1 vial	6 vials
<b>Stop Solution</b> - 6 mL/vial of 2 N sulfuric acid.	895032	1 vial	6 vials
<b>Plate Covers</b> - Adhesive strips.	—	4 strips	24 strips

DBP200 contains sufficient materials to run an ELISA on one 96 well plate.

SBP200 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PDBP200). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

## STORAGE

<b>Unopened Kit</b>	Store at 2 - 8° C. Do not use past kit expiration date.	
<b>Opened/ Reconstituted Reagents</b>	Diluted Wash Buffer	May be stored for up to 1 month at 2 - 8° C.*
	Stop Solution	
	Assay Diluent RD1-19	
	Calibrator Diluent RD5P (1X)	
	Conjugate	
	Unmixed Color Reagent A	
	Unmixed Color Reagent B	
	Standard	Aliquot and freeze at $\leq -20^{\circ}$ C for up to 1 month.*
Microplate Wells	Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*	

\*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.
- 250 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 rpm  $\pm$  50 rpm.
- Test tubes for dilution.
- Human BMP-2 Controls (optional; available from R&D Systems).

## PRECAUTION

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

## SAMPLE COLLECTION AND STORAGE

**Cell Culture Supernates** - Remove particulates by centrifugation and assay immediately or aliquot and store samples at  $\leq -20^{\circ}$  C. Avoid repeated freeze-thaw cycles.

**Bone** - Extract demineralized bone samples in 4 M Guanidine-HCl and protease inhibitors (28, 29). Dissolve the final sample in 2 M Guanidine-HCl.

**Note:** *Extractions can also be done in urea (29, 30).*

Bone extract samples must be diluted in Calibrator Diluent RD5P (1X) prior to assay so that the final concentration of Guanidine-HCl is  $\leq 0.06$  M, and the final concentration of Urea is  $\leq 0.25$  M.

## REAGENT PREPARATION

**Bring all reagents to room temperature before use.**

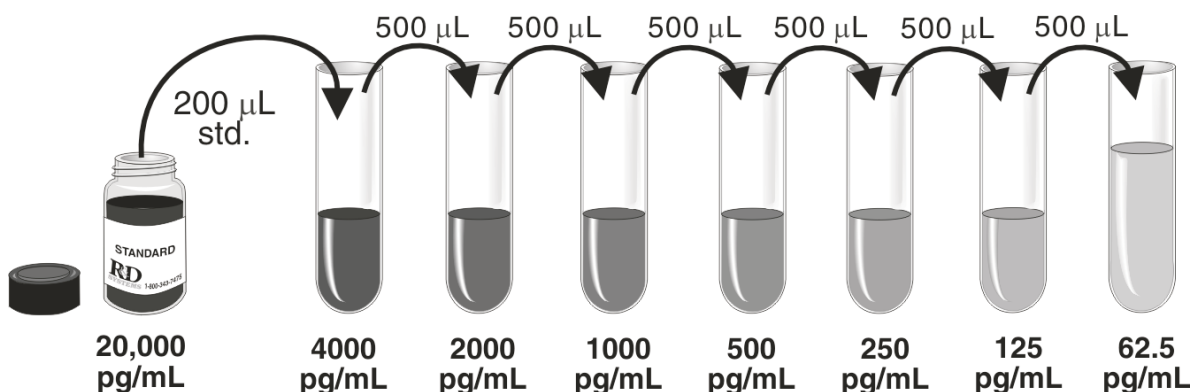
**Wash Buffer** - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

**Calibrator Diluent RD5P (1X)** - Add 20 mL of Calibrator Diluent RD5P Concentrate into deionized or distilled water to yield 200 mL of Calibrator Diluent RD5P (1X). Allow to mix for at least 15 minutes prior to use.

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

**BMP-2 Standard** - Reconstitute the BMP-2 Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 20,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 800  $\mu$ L of Calibrator Diluent RD5P (1X) into the 4000 pg/mL tube. Pipette 500  $\mu$ L of Calibrator Diluent RD5P (1X) into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 4000 pg/mL standard serves as the high standard. Calibrator Diluent RD5P (1X) 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 samples and standards 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  $\mu$ L of Assay Diluent RD1-19 to each well.
4. Add 50  $\mu$ L of Standard, control, or sample\* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 rpm  $\pm$  50 rpm. A plate layout is provided to record the standards and samples assayed.
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  $\mu$ L) 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 decanting. Invert the plate and blot it against clean paper towels.
6. Add 200  $\mu$ L of BMP-2 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200  $\mu$ L of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50  $\mu$ L 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.

## ASSAY PROCEDURE SUMMARY

1. Prepare reagents, samples, and standards as instructed.



2. Add 100  $\mu\text{L}$  Assay Diluent RD1-19 to each well.



3. Add 50  $\mu\text{L}$  Standard, control, or sample\* to each well. Incubate 2 hours on the shaker at RT.



4. Aspirate and wash 4 times.



5. Add 200  $\mu\text{L}$  Conjugate to each well. Incubate 2 hours on the shaker at RT.



6. Aspirate and wash 4 times.



7. Add 200  $\mu\text{L}$  Substrate Solution to each well. Incubate 30 minutes **on the benchtop. Protect from light.**



8. Add 50  $\mu\text{L}$  Stop Solution to each well. Read at 450 nm within 30 minutes.  
 $\lambda$  correction 540 or 570 nm

\*Bone samples require extraction and dilution.

## CALCULATION OF RESULTS

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

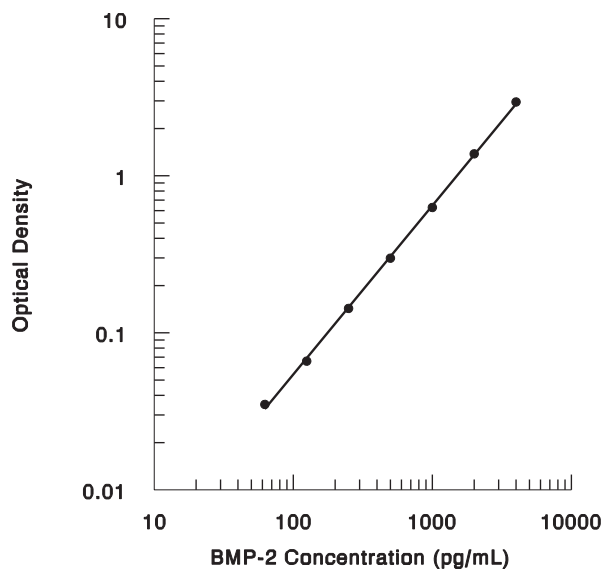
Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation.

To determine the BMP-2 concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding BMP-2 concentration.

If the 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	0.027 0.027 0.061	0.027	-
62.5	0.063 0.092	0.062	0.035
125	0.094 0.169	0.093	0.066
250	0.170 0.324	0.170	0.143
500	0.328 0.653	0.326	0.299
1000	0.656 1.405	0.655	0.628
2000	1.406 2.958	1.406	1.379
4000	2.999	2.979	2.952

## 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.
- 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.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- 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.

## 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.

Sample	Intra-assay Precision			Inter-assay Precision		
	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	323	1010	2313	344	1043	2213
Standard deviation	8.3	28.3	56.0	25.2	55.4	140.2
CV (%)	2.6	2.8	2.4	7.3	5.3	6.3

## RECOVERY

The recovery of recombinant human BMP-2 spiked to levels throughout the range of the assay in various matrices was evaluated.

Diluent	Average % Recovery	Range
Cell culture media (n=4)	102	94 - 110%
2 M Guanidine-HCl* (n=1)	98	94 - 101%
2 M Urea* (n=1)	101	92 - 106%

\*Samples were diluted prior to assay.

## LINEARITY

The following samples were spiked with rhBMP-2 and then diluted with Calibrator Diluent RD5P (1X). The samples were then serially diluted and tested to assess the linearity of the assay.

		Cell culture media (n=4)	2 M Guanidine-HCl* (n=1)	2 M Urea* (n=1)
1:2	Average % of Expected	103	100	103
	Range (%)	97 - 109	-	-
1:4	Average % of Expected	103	103	106
	Range (%)	98 - 110	-	-
1:8	Average % of Expected	109	112	114
	Range (%)	104 - 113	-	-
1:16	Average % of Expected	108	114	113
	Range (%)	102 - 112	-	-

\*The final Guanidine-HCl concentration was adjusted to 0.067 M (30-fold dilution) and the final Urea concentration was adjusted to 0.25 M (4-fold dilution).

## SENSITIVITY

Thirty-five assays were evaluated and the minimum detectable dose (MDD) of BMP-2 ranged from 4.3 - 29 pg/mL. The mean MDD was 11 pg/mL.

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

## CALIBRATION

This immunoassay is calibrated against a highly purified CHO cell-expressed recombinant human BMP-2 produced at R&D Systems.

The NIBSC/WHO Primary Biological recombinant human BMP-2 Standard 93/574 was evaluated in this assay.

The dose response curve of the NIBSC Standard 93/574 parallels the Quantikine standard curve. To convert sample values obtained with the Quantikine BMP-2 kit to approximate NIBSC Units, use the equation below.

NIBSC (93/574) approximate value (U/mL) = 0.0006 x Quantikine BMP-2 value (pg/mL).

## SAMPLE VALUES

**Cell Culture Supernates** - Cell culture supernates from the following unstimulated cell lines were tested for natural BMP-2. No BMP-2 was detected.

Cell Lines	Type	Growth Conditions
MDA-MB-453	Human breast adenocarcinoma.	RPMI + 10% FBS, L-glutamine, penicillin and streptomycin.
MCF-7	Human breast adenocarcinoma, from pleural effusion.	1:1 mixture high glucose DMEM and F-12 + 10% FBS, L-glutamine, penicillin and streptomycin.
ATDC5	Mouse teratocarcinoma AT805 derived, differentiates to chondrocytes.	1:1 mixture of F-12 and DMEM + 5% FBS, L-glutamine, penicillin and streptomycin.
ST-2	Mouse-bone marrow stroma-cell derived, fibroblast-like.	RPMI + 10% FBS, L-glutamine, penicillin and streptomycin.
U-2OS	Human osteogenic sarcoma, attached epithelial-like.	Grown to confluency in McCoy's 5a media + 15% FBS, L-glutamine, penicillin and streptomycin.

## SPECIFICITY

This assay recognizes recombinant and natural BMP-2. The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD5P (1X) and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant BMP-2 standard were assayed for interference. No significant cross-reactivity or interference was observed.

### **Recombinant human:**

Activin A	Inhibin A
Activin RIA	Inhibin B
Activin RIIA	LAP
Activin RIIB	TGF- $\alpha$
BMPR-IB	TGF- $\beta$ sRII
BMP-5	TGF- $\beta$ sRIII
BMP-6	TGF- $\beta$ 1
BMP-7	TGF- $\beta$ 1.2
Follistatin 288	TGF- $\beta$ 2
Follistatin 300	TGF- $\beta$ 3
Follistatin 315	

### **Other recombinants:**

rat Agrin  
mouse BMPR-IB  
mouse Follistatin  
porcine TGF- $\beta$ 2  
amphibian TGF- $\beta$ 5

### **Natural**

#### **proteins:**

human TGF- $\beta$ 1  
porcine TGF- $\beta$ 1

Recombinant human BMPR-IA and recombinant mouse BMPR-IA were found to interfere at concentrations > 10 ng/mL. Recombinant mouse Noggin was found to interfere at concentrations > 5 ng/mL.

Cross-reactivity was observed to be 1.2% with 50 ng/mL of recombinant human BMP-4.

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# PLATE LAYOUT

Use this plate layout as a record of standards and samples assayed.

1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
	A	B	C	D	E	F	G	H