Human BMP-4 immunoassay is a 3.5 - 4.5 hour solid phase ELISA designed to measure Human BMP-4 in cell culture supernates, serum and EDTA plasma. It contains recombinant Human BMP-4 and antibodies raised against this protein. It has been shown to accurately quantitie recombinant Human BMP-4. Results obtained with naturally occurring BMP-4 samples showed linear curves that were parallel to the standard curves obtained using the kit standards. These results indicate that the Immunoassay kit can be used to determine relative mass values for natural Human BMP-4.

This assay employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for 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, a biotinylated antibody specific for BMP-4 is added to the wells. Following a wash to remove any unbound antibody reagent, A Streptavidin HRP conjugate is add to the wells. After washing away any unbound enzyme, a substrate solution is added to the wells and color develops in proportion to the amount of BMP-4 bound in the initial step. The color development is stopped and the intensity of the color is measured.

_ 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.
_ It is important that the DILUTION BUFFER selected for the standard curve be consistent with the samples being assayed.
_ If samples generate values higher than the highest standard, dilute the samples with the appropriate DILUTION BUFFER 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 Immunoassay, the possibility of interference cannot be excluded.

Unopened Kit: Unopened Kit: Store at 2 - 8° C for up to 6 months. For longer storage, unopened Standard, Detection Antibody Concentrated should be stored at -20 or -70 °C. Do not use past kit expiration date.
Opened / Reconstituted Reagents: Reconstituted Standard, Detection Antibody Solution SHOULD BE STORED at -20 °C or – 70°C for up to one months. Streptavidin - HRP Conjugate 100-fold concentrated and other components may be stored at 2 - 8°C for up to 6 months.
Microplate Wells: Return unused wells to the plastic bag containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 6 months at 2 - 8° C.

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Microplate shaker (250-300rpm).
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 500 mL graduated cylinders.

SAMPLE COLLECTION AND STORAGE

Plasma - Collect plasma using EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Aliquot and store samples at -20 °C ~-70 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Optimal dilutions should be determined by each laboratory for each application. ***Use polypropylene test tubes*.**

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 50 mL of Wash Buffer Concentrate into deionized or distilled water (450 mL) to prepare 500 mL of Wash Buffer.
BMP-4 Standard - Refer to vial label for reconstitution volume. Reconstitute the **BMP-4** Standard with 1 mL of **Dilution Buffer**. This reconstitution produces a stock solution **of 2000 pg/mL**. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Pipette 375 µL of the appropriate Dilution Buffer into the tube #1. Pipette 250 µL of the appropriate Dilution Buffer into the tube #2 to #6. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 500 pg/mL standard serves as the high standard. The appropriate Sample Solution serves as the zero standard (0 pg/mL).

		125 µl	2 µl	25 µl	2 µl	25 µl	250 µl	50 µl
1 mL								
Standard	ock	1	2	3	4	5	6	7
Concen	200	500	2	1	6	3	15	7.8

Detection Antibody- Reconstitute the **Detection Antibody** with 105 µl of Dilution Buffer to produce a 100-fold concentrated stock solution. Pipette 10.395 mL of the appropriate Dilution Buffer into the 15 ml centrifuge tube and transfer 105 µl of 100-fold concentrated stock solution to prepare working solution.

Streptavidin HRP Conjugate - Pipette 11.925 mL of Dilution Buffer into a 15 ml centrifuge tube and transfer 60 µl of 200-fold concentrated stock solution to prepare working solution. *Note: 1 x working solution of Streptavidin-HRP Conjugate should be used within a few days.*

Positive Control- Reconstitute the positive control with 1mL of **Dilution Buffer** to make positive control solution.

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

1. Prepare all reagents and working standards as directed in the previous sections.
2. Remove excess micro-plate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.
3. Add 100 µL of **Dilution Buffer** to Blank well (B2, B3).
4. Add 100 µL of Standard (from C2 to G3, G4 to F5), samples, or control per well (E4, E5). Cover with the Sealer. Incubate for 2 hours on micro-plate shaker at room temperature. A plate layout is provided to record 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 (300 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 decanting. Invert the plate and blot it against clean paper towels.
6. Add 100 µL of Detection Antibody working solution to each well. Cover with sealer. Incubate for 2 hours on micro-plate shaker at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 µL of **Streptavidin HRP Conjugate** working solution to each well. Incubate for 60 minutes on micro-plate shaker at room temperature.
9. Repeat the aspiration/wash as in step 5.
10. Add 100 µL of Substrate Solution to each well. Incubate for 15-20 minutes at room temperature. **Protect from light.**
11. Add 100 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 if the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
12. Determine the optical density of each well within 15 minutes, using a micro-plate reader set to 450 nm.

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a log-log 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 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.

This immunoassay is calibrated against a highly purified recombinant Human BMP-4.

Twenty-five assays were evaluated and the minimum detectable dose (MDD) of BMP-4 was 3 pg/mL.

These standard curves* are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

This assay recognizes both natural and recombinant Human BMP-4. The factors listed below were prepared at 50 ng/mL in Dilution Buffer, and assayed for cross reactivity. No significant cross-reactivity or interference was observed.