

**Human BMP-4 ELISA Kit
(hBMP-4-ELISA)****Cat. No. EK0314***96 Tests in 8 x 12 divisible strips***Background**

Human bone morphogenetic protein 4 is encoded by the BMP4 gene. It is a member of the bone morphogenetic protein family which is part of the transforming growth factor-beta superfamily. It is involved in bone and cartilage development, specifically tooth and limb development and fracture repair. It is involved in muscle development, bone mineralization, and uterine bud development. BMP4 has also been implicated in Fibrodysplasia Ossificans Progressiva, in which it is under expressed. In human embryonic development, BMP4 is a critical signaling molecule required for the early differentiation of the embryo and establishing of a dorsal-ventral axis. BMP4 is secreted from the dorsal portion of the notochord, and it acts in concert with sonic hedgehog (released from the ventral portion of the notochord) to establish a dorsal-ventral axis for the differentiation of later structures. BMP4 stimulates differentiation of overlying ectodermal tissue. Inhibition of the BMP4 signal (by chordin, noggin, or follistatin) causes the ectoderm to differentiate into the neural plate. The BMP4 gene is assigned to the chromosomal location 14q22-q23. The standard product used in this kit is recombinant BMP-4 with the molecular mass of 26KDa.

ScienCell's human BMP-4 ELISA Kit is based on standard sandwich enzyme-linked immunosorbent assay technology. Human BMP-4 specific monoclonal antibodies are precoated onto 8 x 12 divisible strips. The human specific detection monoclonal antibodies are biotinylated. The test samples and biotinylated detection antibodies are subsequently added to the wells and then washed with PBS or TBS buffer. Avidin-Biotin-Peroxidase Complex is added, and unbound conjugates are washed away with PBS or TBS buffer. HRP substrate TMB is used to visualize HRP enzymatic reaction. TMB is catalyzed by HRP to produce a blue color product that changes into yellow after adding acidic stop solution. The intensity of yellow is proportional to the amount of human BMP-4 that is captured in the strips.

Size	96 Tests in 8 x 12 divisible strips
Assay type	Sandwich ELISA
Range	62.5 pg/ml-4000 pg/ml
Sensitivity	< 4 pg/ml
Specificity	No detectable cross-reactivity with any other cytokine.
Storage	Store at 4°C for frequent use, at -20°C for infrequent use. Avoid multiple freeze-thaw cycles

Shipping	Shipped on gel ice.
Expiration	Four months at 4°C and eight months at -20°C.
Application	For quantitative detection of human BMP-4 in bone tissue and cell culture supernatants.
Kit components	1. Lyophilized recombinant human BMP-4 standard: 10ng/tube×2. 2. 8 x 12 divisible strips pre-coated with anti- human BMP-4 antibody. 3. Sample diluent buffer: 30 ml 4. Biotinylated anti- human BMP-4 antibody: 130μl, dilution 1:100. 5. Antibody diluent buffer: 12ml. 6. Avidin-Biotin-Peroxidase Complex (ABC): 130μl, dilution 1:100. 7. ABC diluent buffer: 12ml. 8. TMB color developing agent: 10ml. 9. TMB stop solution: 10ml.
Materials	1. Microplate reader.
Required But	2. Automated plate washer.
Not Provided	3. Adjustable pipettes and pipette tips. Multichannel pipettes are recommended for large number of samples. 4. Clean tubes and Eppendorf tubes. 5. Washing buffer (neutral PBS or TBS). Preparation of 0.01M TBS: Add 1.2g Tris, 8.5g NaCl; 450μl of purified acetic acid or 700μl of concentrated hydrochloric acid to 1000ml H₂O and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1L. Preparation of 0.01 M PBS: Add 8.5g sodium chloride, 1.4g Na₂HPO₄ and 0.2g NaH₂PO₄ to 1000ml distilled water and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1L.
Usage	This product is for research use only. It is not approved for use in humans, animals, or <i>in vitro</i> diagnostic procedures.

Reference

1. Van den Wijngaard A, Weghuis DO, Boersma CJ, van Zoelen EJ, Geurts van Kessel A, Olijve W (Nov 1995). "Fine mapping of the human bone morphogenetic protein-4 gene (BMP4) to chromosome 14q22-q23 by in situ hybridization". *Genomics* 27 (3): 559-60.
2. Oida S, Iimura T, Maruoka Y, Takeda K, Sasaki S (Nov 1995). "Cloning and sequence of bone morphogenetic protein 4 (BMP-4) from a human placental cDNA library". *DNA Seq* 5 (5): 273-5.

Protocol for Human BMP-4 ELISA (96 well format)

Notes before you begin

1. To inspect the validity of experiment operation and the appropriateness of sample dilution proportion, a pilot experiment using standards and a small number of samples is recommended.
2. The TMB Color developing agent should be colorless and transparent before using.
3. Before using the kit, spin tubes and bring down all components to the bottom of tubes.
4. Duplicate well assay is recommended for both standard and samples.

5. Do not let strips dry, as this will inactivate active components in wells.
6. Do not reuse tips and tubes to avoid cross contamination.
7. Avoid using reagents from different batches.
8. In order to avoid marginal effect of plate incubation due to temperature difference (reaction may be stronger in the marginal wells), it is suggested that the diluted ABC and TMB solution be pre-warmed in 37°C for 30 minutes before use.

Preparation

Sample Preparation and Storage

Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C.

Avoid repeated freeze-thaw cycles.

- **Bone Tissue:** Extract demineralized bone samples in 4 M Guanidine-HCl and protease inhibitors. Dissolve the final sample in 2 M Guanidine-HCl.
- **Cell culture supernatants:** Remove particulates by centrifugation, assay immediately or aliquot and store samples at -20°C.

Sample Dilution Guideline

The user needs to estimate the concentration of the target protein in the sample and select a proper dilution factor so that the diluted target protein concentration falls near the middle of the linear regime in the standard curve. Dilute the sample using the provided diluent buffer. The following is a guideline for sample dilution. Several trials may be necessary in practice. **The sample must be well mixed with the diluent buffer.**

- **High target protein concentration (40-400ng/ml).** The working dilution is 1:100. i.e. Add 1 µl sample into 99 µl sample diluent buffer
- **Medium target protein concentration (4-40 ng/ml).** The working dilution is 1:10. i.e. Add 10 µl sample into 90 µl sample diluent buffer.
- **Low target protein concentration (62.5-4000 pg/ml).** The working dilution is 1:2. i.e. Add 50 µl sample to 50 µl sample diluent buffer.
- **Very Low target protein concentration (≤62.5 pg/ml).** No dilution necessary, or the working dilution is 1:2.

Reagent Preparation and Storage

A. Reconstitution of the human BMP-4 standard: BMP-4 standard solution should be prepared no more than 2 hours prior to the experiment. Two tubes of BMP-4 standard (10ng per tube) are included in each kit. Use one tube for each experiment.

- 10,000 pg/ml of human BMP-4 standard solution: Add 1 ml sample diluent buffer into one tube, keep the tube at room temperature for 10 minutes and mix thoroughly.
- 4000 pg/ml of human BMP-4 standard solution: Add 0.4 ml of the above 10ng/ml BMP-4 standard solution into 0.6 ml sample diluent buffer and mix thoroughly.
- 2000 pg/ml→62.5 pg/ml of human BMP-4 standard solutions: Label 6 Eppendorf tubes with 2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, respectively. Aliquot 0.3 ml of the sample diluent buffer into each tube. Add 0.3 ml of the above 4000pg/ml BMP-4 standard solution into 1st tube and mix. Transfer 0.3 ml from 1st tube to 2nd tube and mix. Transfer 0.3 ml from 2nd tube to 3rd tube and mix, and so on.

Note: The standard solutions are best used within 2 hours. The 10ng/ml standard solution should be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.

B. Preparation of biotinylated anti-human BMP-4 antibody working solution: The solution should be prepared no more than 2 hours prior to the experiment.

- The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
- Biotinylated anti-human BMP-4 antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly.

C. Preparation of Avidin-Biotin-Peroxidase Complex (ABC) working solution: The solution should be prepared no more than 1 hour prior to the experiment.

- The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
- Avidin-Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with the ABC dilution buffer and mixed thoroughly.

Assay Procedure

The ABC working solution and TMB color developing agent must be kept warm at 37°C for 30 minutes before use. When diluting samples and reagents, they must be mixed completely and evenly. Standard BMP-4 detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of BMP-4 amount in samples.

1. Aliquot 0.1ml per well of the 4000 pg/ml, 2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, human BMP-4 standard solutions into the precoated strips. Add 0.1ml of the sample diluent buffer into the control well (**blank well**). Add 0.1ml of each properly diluted sample of human bone tissue, cell culture supernatants or serum to each empty well. See “**Sample Dilution Guideline**” above for details. We recommend that each human BMP-4 standard solution and each sample is measured in duplicate.
2. Seal the strips with the cover and incubate at 37°C for 90 minutes.
3. Remove the cover, discard strips’ contents, and blot the strips onto paper towels or other absorbent material. **Do NOT** let the wells completely dry at any time.
4. Add 0.1ml of biotinylated anti-human BMP-4 antibody working solution into each well and incubate the strips at 37°C for 60 minutes.
5. Wash strips 3 times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1 minutes. Discard the washing buffer and blot the strips onto paper towels or other absorbent material. (**Strips Washing Method:** Discard the solution in the strips without touching the side walls. Blot the strips onto paper towels or other absorbent material. Soak each well with at least 0.3 ml PBS or TBS buffer for 1~2 minutes. Repeat this process two additional times for a total of THREE washes. Note: For automated washing, aspirate all wells and wash THREE times with PBS or TBS buffer, overfilling wells with PBS or TBS buffer. Blot the strips onto paper towels or other absorbent material).
6. Add 0.1ml of prepared ABC working solution into each well and incubate the strips at 37°C for 30 minutes.
7. Wash strips 5 times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1-2 minutes. Discard the washing buffer and blot the strips onto paper towels or other absorbent material. (See Step 5 for strips washing method).
8. Add 90 µl of prepared TMB color developing agent into each well and incubate strips at 37°C in dark for 25-30 minutes (**Note:** For reference only, the optimal incubation time should be determined by end user. And the shades of blue can be seen in the wells with the four most concentrated human BMP-4 standard solutions; the other wells show no obvious color).
9. Add 0.1ml of prepared TMB stop solution into each well. The color changes into yellow immediately.
10. Read the O.D. absorbance at 450 nm in a microplate reader within 30 minutes after adding the stop solution.

For calculation, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of blank well). The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The human BMP-4 concentration of the samples can be interpolated from the standard curve.

Note: if the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

Summary

1. Add samples and standards and incubate the strips at 37°C for 90 minutes. Do not wash.
2. Add biotinylated antibodies and incubate the strips at 37°C for 60 minutes. Wash strips 3 times with 0.01M TBS.
3. Add ABC working solution and incubate the strips at 37°C for 30 minutes. Wash strips 5 times with 0.01M TBS.
4. Add TMB color developing agent and incubate the strips at 37°C in dark for 25-30 minutes.
5. Add TMB stop solution and read.

Typical Data Obtained from Human BMP-4

(TMB reaction incubate at 37°C for 27 min)

Concentration (pg/ml)	0.0	62.5	125	250	500	1000	2000	4000
Absorbance (450 nm)	0.007	0.030	0.092	0.164	0.404	0.729	1.346	1.880

Typical Human BMP-4 ELISA Kit Standard Curve

This standard curve was generated for demonstration purpose only. A standard curve must be run with each assay.

