



Human BD-4 Colorimetric ELISA Kit

NB-06-0653

Detection and Quantification of Human BD-4 Concentrations in Cell Lysates, Sera and Plasma.

Please read the provided manual as suggested experimental protocols may have changed.

Research Purposes Only. Not Intended for Diagnostic or Clinical Procedures.

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Human BD-4 Colorimetric ELISA Kit

Cat# NB-06-0653

INTRODUCTION

Human BD-4, also known as Beta-Defensin 4, is a 72 amino acid cytokine protein, encoded by the DEFB104AB gene, which is commonly known for its antimicrobial activity via synergistic effects with lysozymes and Beta-Defensin 3. The antimicrobial activity displayed by BD-4 is commonly dependent upon the sodium chloride concentration in the vicinity of the cell such that the antibiotic capability is decreased as NaCl concentration increases and vice versa. The BD-4 peptide undergoes proteolytic processing further into its mature form via cleavage of its 22 residue signal sequence, resulting in the mature 50 residue BD-4 cytokine. The mature forms of all 4 types of Beta-Defensin cytokines are distinguished from their Alpha-Defensin counterparts such that they contain the characteristic 6 cysteine structural motifs via 3 disulfide linkages. Generally, defensins form a family of microbicidal and cytotoxic peptides made by neutrophils. Moreover, members of the defensin family are highly similar in protein sequence. In terms of tissue specificity, Human BD-4 is highly expressed in male testes while expressed much less in the female uterus. Furthermore, the cytokine is highly prevalent on the plasma membranes of gastric antrum while lower in neutrophils, thyroid glands, lungs and kidneys. Aside from its antimicrobial functions, human Beta- Defensin 4 is attracted via chemical stimuli towards immature dendritic cells and memory T-cells.

Source: Entrez Gene: DEFB4AB, defensin, beta 4AB [Homo sapiens]; Swiss- Prot:Q8WTQ1

ASSAY PRINCIPLES

The Neo Biotech Human BD-4 ELISA Kit contains the components necessary for quantitative determination of natural or recombinant Human BD-4 concentrations within any experimental sample including cell lysates, serum and plasma. This particular immunoassay utilizes the quantitative technique of a “Sandwich” Enzyme-Linked Immunosorbent Assay (ELISA) where the target protein (antigen) is bound in a “sandwich” format by the primary capture antibodies coated to each well-bottom and the secondary detection antibodies added subsequently by the investigator. The capture antibodies coated to the bottom of each well are specific for a particular epitope on Human BD-4 while the user-added detection antibodies bind to epitopes on the captured target protein. Amid each step of the procedure, a series of wash steps must be performed to ensure the elimination of non-specific binding between proteins to other proteins or to the solid phase. After incubation and “sandwiching” of the target antigen, a peroxidase enzyme is conjugated to the constant heavy chain of the secondary antibody (either covalently or via Avidin/Streptavidin-Biotin interactions), allowing for a colorimetric reaction to ensue upon substrate addition. When the substrate TMB (3, 3', 5, 5'-Tetramethylbenzidine) is added, the reaction catalyzed by peroxidase yields a blue color that is representative of the antigen concentration. Upon sufficient color development, the reaction can be terminated through addition of Stop Solution (2 N Sulfuric Acid) where the color of the solution will turn yellow. The absorbance of each well can then be read by a spectrophotometer, allowing for generation of a standard curve and subsequent determination of protein concentration.

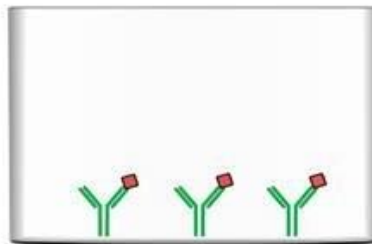
ASSAY FORMAT


Capture Antibody



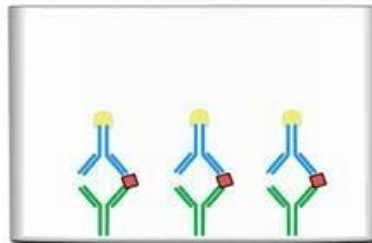
Capture antibodies specific for the target are coated to the plate. Additional binding sites on the plate are blocked.


Target Antigen



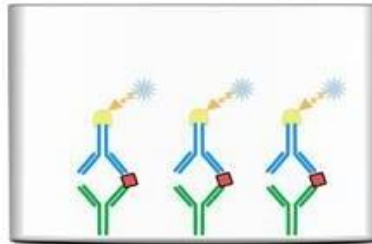
Target antigen present in standard or sample is bound by capture antibodies on the solid-phase.


Biotinylated Detection Antibody



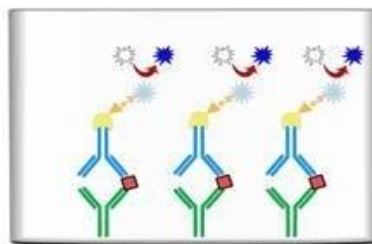
Biotinylated detection antibodies specific for the target are added to bind another epitope on the target antigen.


Streptavidin-HRP



Streptavidin-HRP attaches to detection antibody via high affinity streptavidin-biotin interaction.


Unreacted TMB



TMB substrate is converted to the blue TMB diimine product via the HRP enzyme. Upon addition of acid, the reaction terminates and the wells can be read at 450 nm.


Blue TMB Diimine Product

ASSAY RESTRICTIONS

- This ELISA kit is intended for research purposes only, NOT diagnostic or clinical procedures of any kind.
- Materials included in this kit should NOT be used past the expiration date on the kit label.
- Reagents or substrates included in this kit should NOT be mixed or substituted with reagents or substrates from any other kits.
- Variations in pipetting technique, washing technique, operator laboratory technique, kit age, incubation time or temperature may cause differences in binding affinity of the materials provided.
- The assay is designed to eliminate interference and background by other cellular macromolecules or factors present within any biological samples. However, the possibility of background noise cannot be fully excluded until all factors have been tested using the assay kit.

MATERIALS INCLUDED

| Component | Quantity Per Plate | Container |
|---|-------------------------|---------------|
| Microstrips Coated w/ Capture Antibody | 12 x 8-Well Microstrips | - |
| Protein Standard | Lyophilized | Red |
| Biotinylated Detection Antibody | Lyophilized | Yellow |
| 400x Streptavidin-HRP | 30 µl | Blue |
| Wash Buffer (15x) | 50 ml | Clear |
| Assay Diluent | 50 ml | Clear |
| Ready-to-Use Substrate | 12 ml | Brown |
| Stop Solution | 12 ml | Clear |
| Adhesive Plate Sealers | 2 Sheets | - |
| Technical Manual | 1 Manual | - |

ADDITIONAL MATERIALS REQUIRED

The following materials and/or equipment are NOT provided in this kit but are necessary to successfully conduct the experiment:

- Microplate reader able to measure absorbance at 450 nm (with correction wavelength set to 540 nm or 570 nm)
- Micropipettes with capability of measuring volumes ranging from 1 µl to 1 ml
- Deionized or sterile water
- Squirt bottle, manifold dispenser, multichannel pipette reservoir or automated microplate washer
- Graph paper or computer software capable of generating or displaying logarithmic functions
- Absorbent paper or vacuum aspirator
- Test tubes or microfuge tubes capable of storing ≥1 ml
- Bench-top centrifuge (optional)
- Bench-top vortex (optional)
- Orbital shaker (optional)

HEALTH AND SAFETY PRECAUTIONS

- Reagents provided in this kit may be harmful if ingested, inhaled or absorbed through the skin. Please carefully review the MSDS for each reagent before conducting the experiment.
- Stop Solution contains 2 N Sulfuric Acid (H_2SO_4) and is an extremely corrosive agent. Please wear proper eye, hand and face protection when handling this material. When the experiment is finished, be sure to rinse the plate with copious amounts of running water to dilute the Stop Solution prior to disposing the plate.

STORAGE INFORMATION

Note: If used frequently, reagents may be stored at 4°C.

Unopened Kits: Store at 4°C for 6 months.

| Component | Storage Time | Storage Information |
|---|---|---------------------|
| Microstrips Coated w/ Capture Antibody | 6 Months | 4°C |
| 400x Streptavidin-HRP | | |
| Wash Buffer (15x) | | |
| Assay Diluent | | |
| Ready-to-Use Substrate | | |
| Stop Solution | | |
| Protein Standard | Lyophilized: 6 Months Reconstituted: 1 Month | 4°C |
| Biotinylated Detection Antibody | | |
| Adhesive Plate Sealers | - | - |
| Technical Manual | - | - |

SAMPLE PREPARATION AND STORAGE

If samples are to be used within 24 hours, aliquot and store at 4°C. If samples are to be used over a long period of time, aliquot and store between -20°C and -80°C, depending on the duration of storage.

Note: Samples containing a visible precipitate or pellet must be clarified prior to use in the assay.

Caution: Avoid repeated freeze/thaw cycles to prevent loss of biological activity of proteins in experimental samples.

Cell Lysate and Supernatants

Remove large cell components via centrifugation and perform the assay. Cell lysates and supernatants require a dilution using Assay Diluent. A serial dilution may be performed to determine a suitable dilution factor for the sample. For future use of the sample, follow the sample storage guidelines stated above.

Serum

Allow samples to clot in a serum separator tube (SST) for 30 minutes. After sufficient clotting, centrifuge at 1000 x g for 15 minutes and remove serum from SST in preparation for the assay. A serial dilution may be performed to determine a suitable dilution factor for the sample. For future use of the sample, follow the storage guidelines above.

Plasma

Use heparin, citrate or EDTA as an anticoagulant to gather plasma from original biological sample. After collection of the plasma, centrifuge for 15 minutes at 1000 x g. This step must be performed within 30 minutes of plasma collection. A serial dilution may be performed to determine a suitable dilution factor for the sample. For future use of the sample, follow the storage guidelines above.

SAMPLE EXPERIMENT LAYOUT

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|-----------------------------|-----------------------------|-----------------------------|---------------|---------------|---------------|
| A | Standard (High Point) | Standard (High Point) | Standard (High Point) | Sample | Sample | Sample |
| B | Standard (1:2) | Standard (1:2) | Standard (1:2) | Sample | Sample | Sample |
| C | Standard (1:4) | Standard (1:4) | Standard (1:4) | Sample | Sample | Sample |
| D | Standard (1:8) | Standard (1:8) | Standard (1:8) | Sample | Sample | Sample |
| E | Standard (1:16) | Standard (1:16) | Standard (1:16) | Sample | Sample | Sample |
| F | Standard (1:32) | Standard (1:32) | Standard (1:32) | Sample | Sample | Sample |
| G | Standard (1:64) | Standard (1:64) | Standard (1:64) | Sample | Sample | Sample |
| H | Negative Control | Negative Control | Negative Control | Sample | Sample | Sample |

IMMUNOASSAY PROTOCOL

Note: If possible, all incubation steps should be performed on an orbital shaker to equilibrate solutions when added to the microplate wells. Also, all provided solutions should be at ambient temperature prior to use.

Note: Avoid adding solutions into wells at an angle, always keep pipette tip perpendicular to plate bottom.

Reconstitution of Provided Materials

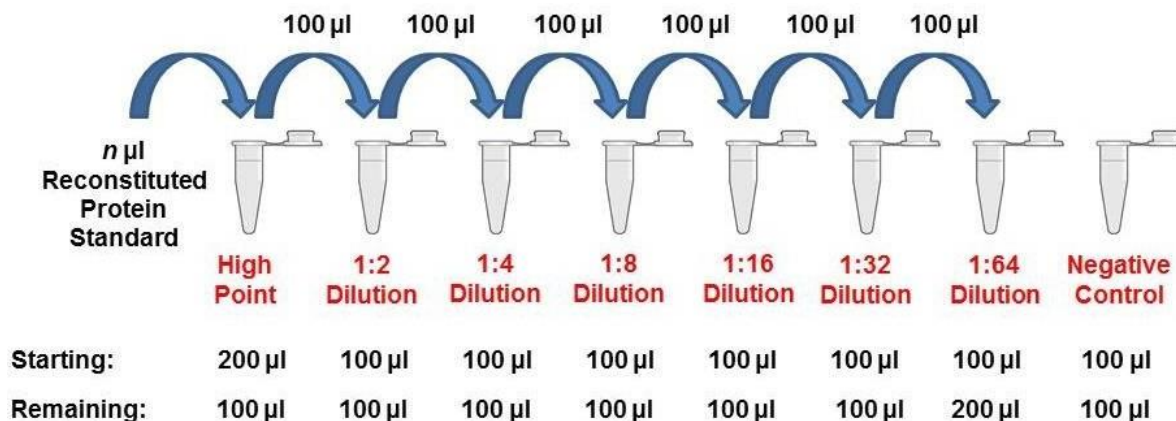
1. Reconstitute the Biotin-Conjugated Detection Antibody in 50 μ l of ddH₂O for a concentration of 83 μ g/ml.
2. Reconstitute the Protein Standard in 83 μ l of ddH₂O for a concentration of 1 μ g/ml.
3. Dilute the 15x Wash Buffer to 1x Wash Buffer using 14 volumes of ddH₂O and 1 volume of 15x Wash Buffer. Use as necessary.

Addition of Known Standard and Unknown Sample to Immunoassay

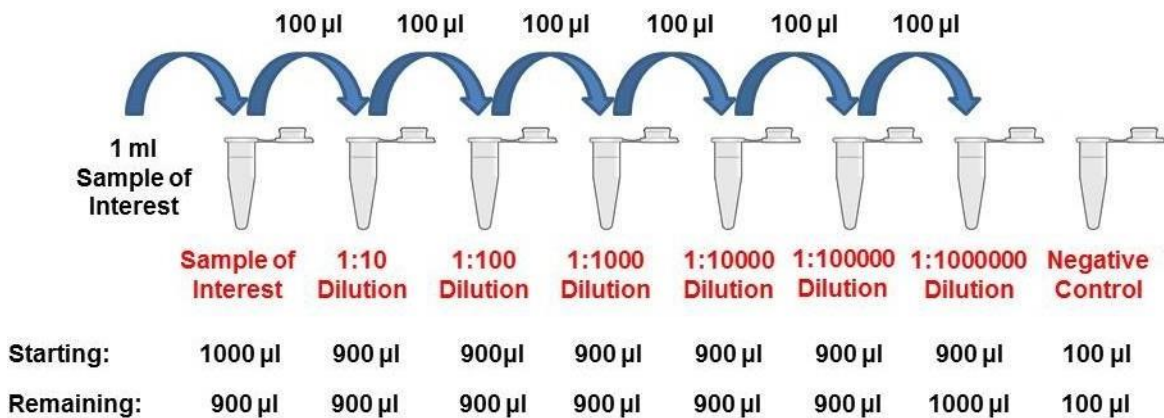
The Neo Biotech Human BD-4 ELISA Kit allows for the detection and quantification of endogenous levels of natural and/or recombinant Human BD-4 proteins within the range of 8-1000 pg/ml.

1. Dilute the known standard sample from 1000 pg/ml to 0 pg/ml in a series of microfuge tubes. Mix each tube thoroughly by inverting several times or by vortexing lightly to ensure proper equilibration. Add 100 μ l of each serial dilution step into the wells of a specified row or column of the 96-well microtiter plate in duplicate or triplicate and incubate at room temperature for 2 hours. Unknown samples of interest can be serially diluted with Assay Diluent to concentrations within the detection range of this assay kit and added to the plate at 100 μ l per well. Seal the microplate air-tight using one of the microplate adhesive seals provided in this kit or Parafilm if readily available. See Appendix for serial dilution diagram.

To obtain serial dilution high point, dilute reconstituted Protein Standard to the maximum concentration for serial dilution by adding $n \mu\text{l}$ reconstituted Protein Standard to serial dilution high point tube and then raising the volume to $200 \mu\text{l}$. Shown below is a diagram illustrating a hypothetical 2-fold serial dilution on a given reconstituted Protein Standard.



For samples of unknown protein concentrations, serial dilute the experimental sample using Assay Diluent to determine range of detection and acceptable dilutions. Shown below is a diagram illustrating a 10-fold serial dilution on a given Sample of Interest.



Addition of Detection Antibody to Capture Antibody-Bound Samples

1. Aspirate the protein standard solution out of the microplate wells. If your lab does not have a vacuum-based aspirator, you may dump the solutions from the microplate into a waste container and blot 3-4 times on a stack of paper towels until most or all of the liquid is removed from the wells. Dilute the 15x Wash Buffer to 1x using pure H₂O. Add 300-400 µl of 1x Wash Buffer to each well being used and gently shake for 5-7 minutes on an orbital shaker. Perform this wash step 4 times consecutively.
2. After the 4th wash step, dilute the detection antibody solution 1:200 in Assay Diluent to 0.4µg/ml. Mix the test tube either by inverting several times or vortexing to ensure proper equilibration. Ensure that there is enough detection antibody solution for all wells being used. Add 100 µl of the diluted detection antibody solution into each well, seal the plate and incubate at room temperature for 2 hours.

Conjugation of Streptavidin-HRP to Biotinylated Detection Antibody

1. Remove the detection antibody solution out of the microplate wells by either vacuum-based aspirator or paper towel blotting. Perform 4 consecutive wash steps with gentle shaking between each wash.
2. Dilute the 400x Streptavidin-HRP by 1:400 using Assay Diluent to a 1x Streptavidin-HRP solution.
3. After the 4th wash step, add 100 µl of 1x Streptavidin-HRP solution into each well and incubate at room temperature for 30 minutes.

Application of Liquid Substrate for Colorimetric Reaction

1. Remove the 1x Streptavidin-HRP solution out of the microplate wells by either vacuum-based aspirator or paper towel blotting. Prepare the Ready-to-Use Substrate by bringing it to room temperature without exposure to fluorescent or UV light as these may degrade the substrate. Perform 4 consecutive wash steps with gentle shaking between each wash.

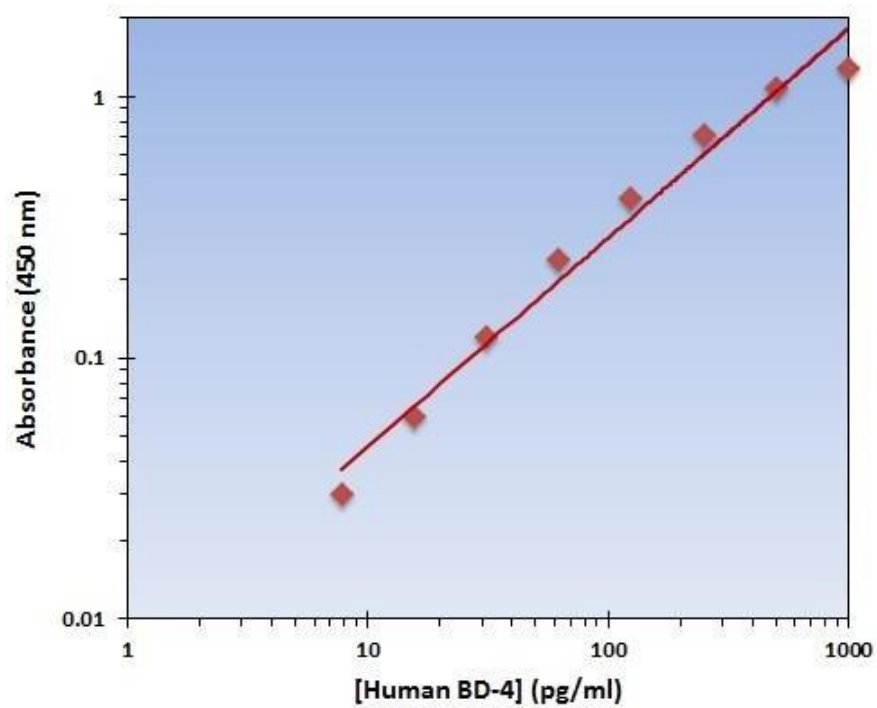
2. After the 4th wash step, add 100 μ l of Ready-to-Use Substrate solution into each well and incubate at room temperature for color development. The microplate should be kept out of direct light by either covering with an opaque object or putting it into a dark room. Closely monitor the color development as some wells may turn blue very quickly depending on analyte and/or detection antibody-HRP concentrations. Once the blue color has ceased to develop further, immediately add 100 μ l of Stop Solution to each well being used. The color in the wells should immediately change from blue to yellow.
3. The microplate is now ready to be read by a microplate reader. Within 30 minutes of adding the Stop Solution, determine the optical density (absorbance) of each well by reading the plate with the 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. **Caution:** Readings made directly at 450 nm without correction may be higher and less accurate.

Generation of Standard Curve and Interpretation of Data

1. Average the duplicate or triplicate readings for each standard, control and sample and subtract the average zero standard optical density.
2. Generate a standard curve by using Microsoft Excel or other computer software capable of establishing a 4-Parameter Logistic (4- PL) curve fit. If using Excel or an alternative graphing tool, plot the average optical density values in absorbance units (y-axis) against the known standard concentrations in pg/ml (x-axis). **Note:** Only use the values in which a noticeable gradient can be established. Afterwards, generate a best fit curve or “trend-line” through the plotted points via regression analysis. **Note:** Shown on the next page is an example of typical data produced by analysis of the standard sample.

The data and subsequent graph was obtained after performing a cytokine ELISA for Human BD-4. Each known sample concentration was assayed in triplicate.

| Human BD-4 Standard Curve | |
|------------------------------|---------------------|
| Concentration (pg/ml) | Average OD 450nm |
| 1000 | 1.2 9 |
| 500 | 1.0 8 |
| 250 | 0.7 2 |
| 125 | 0.4 1 |
| 62.5 | 0.2 4 |
| 31.25 | 0.1 2 |
| 15.625 | 0.0 6 |
| 7.8125 | 0.0 3 |



SUMMARIZED PROTOCOL

Reconstitute Biotinylated Detection Antibody and Protein Standard and dilute the 15x Wash Buffer as specified.



Perform serial dilution of Protein Standard and prepare samples as desired. See sample preparation section for instructions to dilute serum and plasma samples.



Add 100ul of Protein Standard, sample or control to each well and incubate for 2 hours at room temperature.



Aspirate Protein Standards, samples or controls out and wash plate 4 times.



Dilute Biotinylated Detection Antibody as specified. Add 100ul to each well and incubate for 2 hours at room temperature.



Aspirate Biotinylated Detection Antibody out and wash plate 4 times.



Dilute 400x Streptavidin-HRP as specified. Add 100ul of 1x Streptavidin-HRP to each well and incubate at room temperature for 30 minutes.



Aspirate 1x Streptavidin-HRP out and wash plate 4 times.



Add 100ul of Ready-to-Use Substrate to each well and incubate at room temperature for color development.



Add 100ul of Stop Solution and read plate at 450nm.

SENSITIVITY

The Human BD-4 ELISA Kit allows for the detection and quantification of endogenous levels of natural and/or recombinant Human BD-4 proteins within the range of 8-1000 pg/ml.

CROSS REACTIVITY AND SPECIFICITY

The Neo Biotech Human BD-4 ELISA is capable of recognizing both recombinant and naturally produced Human BD-4 proteins. The antigens listed below were tested at 50 ng/ml and did not exhibit significant cross reactivity or interference.

- Human: BD-1 (36 aa), BD-1 (47 aa), BD-2, BD-3

TECHNICAL SUPPORT

For troubleshooting, information or assistance, please go online to:

www.neo-biotech.com

or contact us at:

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Manual Version 1.2

ELISA PLATE TEMPLATE

| | A | B | C | D | E | F | G | H |
|----|---|---|---|---|---|---|---|---|
| 1 | | | | | | | | |
| 2 | | | | | | | | |
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NOTES