INTENDED USE

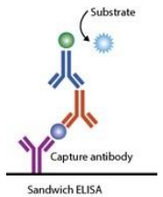
The Human Beta Defensin 2 (hBD-2) ELISA Kit is a sandwich ELISA kit for the quantification of BD-2 in cultures of human cells and in appropriately qualified samples from serum, saliva, or other tissue fluids. For research use only (RUO); not for therapeutic or diagnostic use.

GENERAL INFORMATION

Defensins are cationic, anti-microbial peptides, produced by many cell types (e.g., leukocytes, epithelium, dendrites), and which play prominent roles in the innate immune response of mammals. Two classes which have been described in humans, the alpha- and beta-defensins, range from 3.5 to 4.5 kDa and are stabilized by three intramolecular disulfide bonds differing by the ordering of the disulfide bonds in the mature peptides. To date, six alpha-defensins (also referred to as Human Neutrophil Peptides, HNP1-6) and six beta-defensins (HBD1-6) have been investigated. All have demonstrated broad-spectrum in vitro antimicrobial activity against bacteria, fungi and enveloped viruses – activities that are expected to be significant for *in vivo* protection against pathogens. Researchers have also investigated the ability of defensins to induce the release from tissues of cytokines and chemokines involved in inflammation and/or adaptive immunity, thereby providing a regulatory role that bridges innate and adaptive immunity. Investigative research has led to the measurement of defensins in serum, saliva, milk, amniotic fluid and lung and cervicovaginal lavage, and culture media of cells and tissue of blood, lung, skin, bowel, muscle, cartilage and kidney, among other sample types.

Alpha Diagnostics has developed an immunoassay for detection and quantification of beta defensin 2 in human samples used in the research of innate immunity. The kit is suitable for testing a variety of sample types, in accordance with appropriate validation of linearity and recovery.

PRINCIPLE OF THE TEST



The Human Beta Defensin 2 ELISA kit is based on the binding of Human Beta Defensin 2 in samples to two antibodies, one immobilized on the microtiter wells, and the other conjugated to biotin, which then binds to a streptavidin horseradish peroxidase (HRP) conjugate. After a washing step, chromogenic substrate is added and color is developed by the enzymatic reaction of HRP on the TMB substrate, which is directly proportional to the amount of BD-2 present in the sample. Stopping Solution is added to terminate the reaction, and absorbance at 450nm is then measured using an ELISA microtiter well reader. The concentration of BD-2 in samples is calculated from a standard curve of purified recombinant human BD-2 of designated concentration.

STORAGE AND STABILITY

The microtiter well plate and all other reagents, if unopened, are stable at 2-8°C until the expiration date printed on the label. Stabilities of the working solutions are indicated under Reagent Preparation.

KIT CONTENTS

To Be Reconstituted or Diluted: Store as indicated.

Component	Instructions for Use
Human Beta Defensin 2 Standard Part No. 100-252	Three (3) vials, each containing BD-2 lyophilized in buffer with proteins and antimicrobial. Refrigerate lyophilized vials until used or kit lot expires.

Reconstitute 1 vial with the volume of **Working Sample Diluent** indicated on the Standard label to provide a 200 pg/ml Top Standard. Prepare 2-fold dilutions, as follows; sufficient for one entire curve:

Standard	+ Diluent =	Final Concn
Reconstituted Standard	None	200 pg/ml
225 ul of 200 pg/ml	225ul	100 pg/ml
225 ul of 100 pg/ml	225ul	50 pg/ml
225 ul of 50 pg/ml	225ul	25 pg/ml
225 ul of 25 pg/ml	225ul	12.5 pg/ml
225 ul of 12.5 pg/ml	225ul	6.25 pg/ml
225 ul of 6.25 pg/ml	225ul	3.125 pg/ml

Use the same day of testing, and/or store frozen to 4 weeks.

Sample Diluent Concentrate (20x) Cat. No. SD-20T, 10ml	Dilute the entire volume, 10ml + 190ml with distilled or deionized water into a clean stock bottle. Label as **Working Sample Diluent** and store at 2-8°C until the kit lot expires or is used up.
Wash Solution Concentrate (100x) Cat. No. WB-100, 10ml	Dilute the entire volume, 10ml, to 1L with distilled or deionized water into a clean stock bottle. Label as **Working WashSolution** and store at ambient temperature until kit is used entirely.
Anti-Human Beta Defensin 2 Detection Antibody Concentrate (100x) Part No. 100-253, 0.15ml	Biotinylated anti-human BD-2 in buffer with protein, detergents and BND as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **Working Sample Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return concentrate to 2-8°C storage.
Streptavidin-HRP Conjugate Concentrate (100x) Part No. S-HRP100, 0.15ml	Peroxidase conjugated streptavidin in buffer with protein, detergents and BND as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **Working Sample Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return concentrate to 2-8°C storage.

Ready For Use: Store as indicated on labels.

Component	Part No.	Amt	Contents
Anti-Human Beta Defensin 2 Coated Strip Plate	100-251	8-well strips (12)	Coated with purified anti-Human BD-2 antibodies. Return unused strips to the pouch with desiccant; re-seal and store refrigerated.
TMB Substrate	80091	12 ml	Chromogenic substrate for HRP containing TMB and peroxide.
Stop Solution	80101	12 ml	1% sulfuric acid.

Materials Required But Not Provided:

- Pipettors and pipettes that deliver 100ul and 1-10ml. A multi-channel pipettor is recommended.
- Disposable glass or plastic 5-15ml tubes for diluting samples and Antibody HRP Concentrate.
- Graduated cylinder to dilute Wash Concentrate; 0.2 to 1L.
- Stock bottle to store diluted Wash Solution; 200ml to 1L.
- Distilled or deionized water to dilute reagent concentrates.
- Microwell plate reader at 450 nm wavelength.

ASSAY DESIGN AND SET-UP

Sample Collection and Handling
Culture medium, bioprocessingpreparations, serum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference (See Limits of the Assay, page 6). For **serum**, collect blood by venipuncture, allow clotting, and separate the serum by centrifugation at room temperature.

For all samples, clarify by centrifugation and/or filtration prior to dilution in Sample Diluent. If samples will not be assayed immediately, store refrigerated for up to a few weeks, or frozen for long-term storage.

Assay Validation
Validate the performance of the sample antigen and matrix in the assay system for recovery and parallelism (see Limits of the Assay, page 6), as follows:

Recovery – a measure of the interference of the sample matrix (diluent effect) in providing accurate quantitation of the sample Humira relative to the Standard curve.

Prepare and run a series of dilutions of the sample antigen (concentrations that will fall within the Standard range) in Working Sample Diluent to determine the dilutions that give consistent and accurate quantitation. For most buffer solutions a minimum 5-fold sample dilution is usually sufficient. Serum and plasma require at least a 10-fold dilution to obtain consistent quantitation or complete antigen recovery.

Parallelism – dilutions of the sample should read equivalent values from the top and bottom of the Standard curve to provide good assay precision.

Prepare a dilution series of the sample antigen that gives complete recovery and falls within the full range of the Standard curve. Sample readings from the upper and lower regions of the curve should differ by less than 25%.

Plate Set-up
Bring all reagents to room temperature (18-30° C) equilibration (at least 30 minutes).

- Determine the number of wells for the assay run. Duplicates are recommended, including 10 Standard wells and 2 wells for each sample and control to be assayed.
- Remove the appropriate number of microwell strips from the pouch and return unused strips to the pouch. Reseal the pouch and store refrigerated.
- Prior to sample addition, add 200-300ul Working Wash Solution to each well and let stand for about 5 minutes.
- Aspirate or dump the liquid and pat the plate dry on a paper towel.

Assay Procedure

ALL STEPS ARE PERFORMED AT ROOM TEMPERATURE. After each reagent addition, gently tap the plate to mix the well contents prior to beginning incubation.

1. 1st Incubation [100ul – 60 min; 4 washes]

- Add 100ul of standards, samples and controls each to pre-determined wells.
- Tap the plate gently to mix reagents and incubate for **60** minutes.
- Wash wells 4 times and pat dry on fresh paper towels. As an alternative, an automatic plate washer may be used. Improper washes may lead to falsely elevated signals and poor reproducibility.

2. 2nd Incubation [100ul – 60 min; 4 washes]

- Add 100ul of diluted Anti-BD-2 Detecting Antibody to each well.
- Incubate for **60** minutes.
- Wash wells 4 times as in step 1.

3. 3rd Incubation [100ul – 30 min; 5 washes]

- Add 100ul of diluted Streptavidin-HRP Conjugate to each well.
- Incubate for **30** minutes.
- Wash wells 5 times as in step 1.

4. Substrate Incubation [100ul – 15 min]

- Add 100ul TMB Substrate to each well. The liquid in the wells will begin to turn blue.
- Incubate for **15** minutes in the dark, e.g., place in a drawer or closet.

Note: If your microplate reader does not register optical density (OD) above 2.0, incubate for less time, or read OD at 405-410 nm (results are valid).

5. Stop Step [Stop: 100ul]

- Add 100ul of Stop Solution to each well.
- Tap gently to mix. The enzyme reaction will stop; liquid in the wells will turn yellow.

6. Absorbance Reading

- Use any commercially available microplate reader capable of reading at 450nm wavelength. Use a program suitable for obtaining OD readings, and data calculations if available.
- Read absorbance of the entire plate at 450nm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630nm to normalize well background.

CALCULATION OF RESULTS

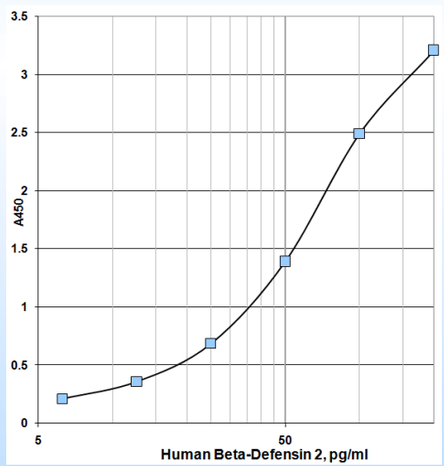
The results may be calculated using any immunoassay software package. The four-parameter curve-fit is recommended. If software is not available, BD-2 concentrations may be determined as follows:

1. Calculate the mean OD of duplicate samples.
2. On graph paper plot the mean OD of the standards (y-axis) against the concentration (pg/ml) of BD-2 (x-axis). Draw the best fit curve through these points to construct the standard curve. A point-to-point construction is most common and reliable.
3. The BD-2 concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
4. Multiply the values obtained for the samples by the dilution factor.
5. Samples producing signals higher than the 200 pg/ml standard should be further diluted and re-assayed.

Typical Results:

Wells	Standards, Control & Samples	A450	pg/ml
A1, A2	**Negative Diluent Control**	0.04	0
B1, B2	6.25 pg/ml **Standard**	0.21	6.25
C1, C2	12.5 pg/ml **Standard**	0.36	12.5
D1, D2	25 pg/ml **Standard**	0.69	25
E1, E2	50 pg/ml **Standard**	1.39	50
F1, F2	100 pg/ml **Standard**	2.49	100
G1, G2	200 pg/ml **Standard**	3.21	200
H1, H2	**Sample** [Diluted 1:10]	1.55	68

Calculated: 10-fold dilution x 68 pg/ml = **680** pg/ml in serum



PERFORMANCE CHARACTERISTICS

Specificity
The antibodies used in this kit have been affinity-purified using a purified recombinant human BD-2 immunosorbent and have been shown by ELISA to react specifically with hBD-2, and to have essentially no reactivity with recombinant hBD-1, hBD-3 nor hNP-1.

Human Serum

BD-2 Levels
Assay of stored, frozen sera from six individual humans and two human serum pools, ranged from 0 to 770 pg/ml. Fresh sera may contain higher quantities.

Recovery
Purified BD-2 was spiked into each of 8 serum samples diluted 1:10. Observed assay values compared to expected values ranged from 78 to 120%, indicating good quantification of BD-2 in human serum.

Sample	Initial pg/ml	+ 200pg/ml BD-2	% Recovery
Female serum 1	77	258	**90**
Female serum 2	0	218	**109**
Female serum 3	0	240	**120**
Male serum 1	24	258	**117**
Male serum 2	0	206	**103**
Male serum 3	0	168	**84**
Serum pool 1	0	166	**83**
Serum pool 2	0	155	**78**

Human Saliva

BD-2 Levels
Assay of a freshly collected sample =**705** pg/ml.

Linearity of Dilution and Recovery:
- 2 dilutions of the fresh saliva agreed at 98% concordance.
- BD-2 was spiked into 2 different saliva samples (1:10) at 4 levels, 50-400pg/ml. The mean recovery ranged from **111** to **133%**, demonstrating linear dilution and equivalent quantification across the standard range.

Culture Medium

Linearity of Dilution and Recovery
BD-2 was spiked into Sample Diluent with 10% Neonatal Bovine Serum at 4 levels, 50-400pg/ml. The mean recovery ranged from 103 to 133%, demonstrating linear dilution and equivalent quantification across the standard range.

LIMITS OF THE ASSAY

The **recovery,** or accuracy, of Human BD-2 measurement in stored samples may differ from that in fresh samples. Recovery in fresh, individual human serum or plasma samples has not been determined

QUALITY CONTROL

Reagents Accurate and reproducible assay results rely on proper storage, handling and control of reagent and sample temperature. Store all reagents as indicated, and warm to room temperature only those to be used in the assay. Shelf-life of the critical reagents and samples will diminish with extended exposure to non-refrigeration, resulting in inaccurate assay results. All solutions should be clear. Cloudiness or particulates are indications of reagent contamination or instability and may interfere with proper performance of the assay. Do not use.

Standard Curve The signal generated by the standards should be continuously increasing in OD from the lowest Standard to the highest Standard, with a difference greater than 1.2 OD. Non-uniform or low signals may indicate problems with technique, protocol directions and/or reagent preparation, use or stability. Do not rely on results generated from an assay with these issues.

Technique Accurate and reproducible assay results rely on good lab technique regarding pipetting, plate washing and handling of samples and reagents.

Equipment Precision of results relies on uniform and effective washing techniques; an automatic washer may be used. ELISA reader and pipettes should be properly calibrated.

PRECAUTIONS AND SAFETY INSTRUCTIONS

Human serum and other bodily fluids may contain infectious material. Always wear gloves when handling human samples, and dispose of these samples and containers as biohazard waste.

Standards, Controls, Sample Diluent, Detection Antibody and Streptravidin-HRP contain Bromonitrodioxane (BND: 0.02%, w/v). Stop Solution contains 1% sulfuric acid. Follow good laboratory practices, and avoid ingestion or contact of any reagent with skin, eyes or mucous membranes. All reagents may be disposed of down a drain with copious amounts of water.
MSDS for TMB, sulfuric acid and BND, if not already on file, can be requested or obtained from the ADI website.

Instruction Manual No. *M-100-250-BD2*

Human Beta Defensin 2 ELISA Kit

Cat. 100-250-BD2, 96 Tests

For Quantitation of BD-2 in serum, plasma or other biological samples

For research use only (RUO), not for diagnostic or therapeutic use.

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