PERFORMANCE CHARACTERISTICS (continued)

Human Saliva

BD-1 Levels**:** Assay of 3 freshly collected samples, ranged from 9.4 to 23.4 ng/ml.

Linearity Dilution: Saliva was diluted to 5 levels for testing, and concordance of the assay values were compared. The mean recovery ranged from 93 to 104%, demonstrating linear dilution and equivalent quantification across the standard range.

Sample	Dilution	ng/ml	Mean	% Recovery
Saliva	1:5	9.73		104
	1:10	9.49	9.4	101
	1:20	8.77		93
	1:40	9.59		102
	1:80	9.44		100

Culture Medium

Linearity of Dilution and Recovery: BD-1 was spiked into Sample Diluent with 10% Neonatal Bovine Serum at 4 levels, 100-800pg/ml. The mean recovery ranged from 105 to 118%, demonstrating linear dilution and equivalent quantification across the standard range.

STORAGE AND STABILITY

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

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.

Sample Controls Each lab should assay internal control samples, which represent the lab's expected sample population and that are maintained stabilized. Reproducible control values indicate proper assay performance. A Negative Diluent Control should also be run.

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-continuously increasing or low signals may indicate problems with technique, protocol directions and/or reagent preparation, use or stability. A Negative Diluent Control should be of lower signal than the lowest standard. 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.

Instruction Manual No. 100-240-BD1

Human Beta Defensin 1

ELISA Kit Cat. No. 100-240-BD1

For Quantitative Determination of BD-1 in Biological Solution

For research use only (RUO), not for diagnosis, cure or prevention of the disease.

ALPHA DIAGNOSTIC INTERNATIONAL

6203 Woodlake Center Drive • San Antonio• Texas 78244 • USA.

Phone (210) 500-9515 • Fax (210) 500-9544

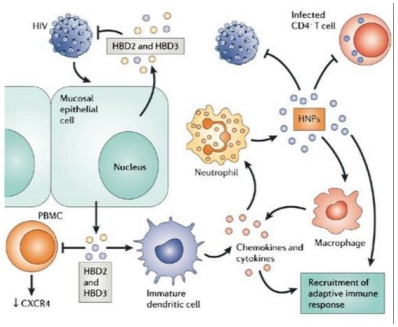
Toll Free (800) 786-5777

Email: service@4adi.com

INTENDED USE

The Human Beta Defensin 1 (BD-1) ELISA Kit is a sandwich ELISA for the quantification of BD-1 in cultures of human cells and in appropriately qualified samples from serum, saliva, or other tissue fluids. For research use only (RUO), not for diagnosis, cure or prevention of the disease.

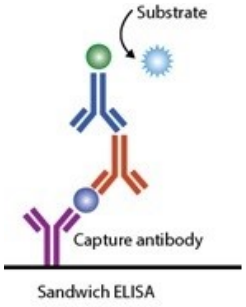
RESEARCH USE OF THE TEST



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 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 inflammatory responses, 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 1 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 1 ELISA kit is based on the binding of Human Beta Defensin 1 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-1 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-1 in samples is calculated from a standard curve of purified recombinant human BD-1 of designated concentration.

PERFORMANCE CHARACTERISTICS & EXPECTED RESULTS

Specificity

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

Sera from rhesus and cynomolgous monkeys show reactivity in the ELISA; thus, the assay could be suitable for studies in primates.

Human Serum

BD-1 Levels**:** Assay of stored, frozen sera from six individual humans and two human serum pools, ranged from 0 to 22.4 ng/ml. Fresh sera may contain higher quantities.

Recovery**:** Purified BD-1 was spiked into each of 6 serum samples and diluted 1/40. Observed assay values compared to expected values ranged from 81 to 132%, indicating reasonable quantification of BD-1 in human serum.

Sample	Initial pg/ml	+ 400 pg/ml BD-1	% Recovery
Serum F1	588	916	**93**
Serum F2	340	709	**96**
Serum F3	347	873	**117**
Serum M1	441	967	**115**
Serum M2	325	650	**90**
Serum M3	570	1028	**106**

Linearity of Dilution: The 6 sera were diluted to 2 levels for testing, and concordance of the assay values were compared. The mean recovery ranged from 90 to 98%, demonstrating linear dilution and equivalent quantification across the standard range.

Sample	Dilution	Assay Value pg/ml	Serum Value ng/ml	Recovery %
Serum F1	1:20 1:80	978 217	19.6 17.4	**94**
Serum F2	1:20 1:80	989 219	19.8 17.5	**94**
Serum F3	1:20 1:160	919 94.4	18.4 15.1	**90**
Serum M1	1:20 1:160	1046 144	20.9 23.0	**95**
Serum M2	1:20 1:160	711 77.1	14.2 12.3	**93**
Serum M3	1:20 1:80	1093 286	21.9 22.9	**98**

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-1 concentrations may be determined as follows:

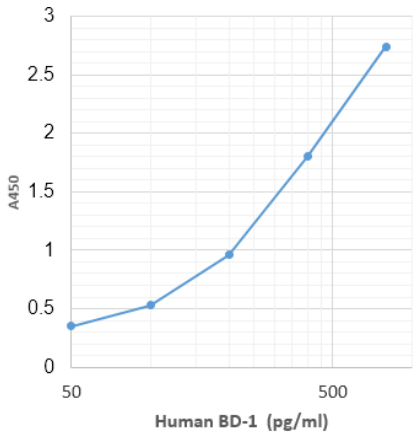
1. Calculate the mean OD of duplicate samples.
2. On graph paper plot the mean OD of the standards (y-axis) against the concentration (pg/ml) of BD-1 (x-axis). Draw the best fit curve through these points to construct the standard curve. A point-to-point construction is most common and reliable.
3. The BD-1 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 of each sample.
5. Samples producing signals higher than the 800 pg/ml standard should be further diluted and re-assayed.

TYPICAL RESULTS

The following data are for illustration purposes only. A complete standard curve should be run in every assay to determine sample values.

Wells	Standards, Control & Samples	A450 nm	BD-1 pg/ml
A1, A2	**Negative Diluent Control**	0.15	0
B1, B2	50 pg/ml **Standard**	0.35	50
C1, C2	100 pg/ml **Standard**	0.53	100
D1, D2	200 pg/ml **Standard**	0.96	200
E1, E2	400 pg/ml **Standard**	1.80	400
F1, F2	800 pg/ml **Standard**	2.74	800
G1, G2	**Sample** [Diluted 1:40]	1.84	419
	Calculated: 40-fold dilution x 419 pg/ml = **16.8** ng/ml in serum		

A typical assay Standard Curve (do not use for calculating sample values)



KIT CONTENTS

To Be Reconstituted: Store as indicated.

Component	Instructions for Use
Human Beta Defensin 1 Standard Part No. 100-242	Three (3) vials, each containing rBD-1 lyophilized in buffer with protein, detergents and ProClin 300 as stabilizers. Keep lyophilized vials refrigerated until used or kit lot expires.

Reconstitute 1 vial with 1.0 ml **Working Sample Diluent** to provide a 800 pg/ml Top Standard, sufficient for two entire curves. Prepare 2-fold dilutions, as follows:

Standard	**+ Diluent**	**= Final Concn**
Reconstituted Standard	None	800 pg/ml
250 ul of 800pg/ml	250ul	400 pg/ml
250 ul of 400pg/ml	250ul	200 pg/ml
250 ul of 200pg/ml	250ul	100 pg/ml
250 ul of 100pg/ml	250ul	50 pg/ml

Use within 2 weeks of preparation; store @ 4° C

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 Wash Solution** and store at ambient temperature until kit is used entirely.
Anti-Human Beta Defensin 1 Detection Antibody Concentrate (100x) Part No. 100-243, 0.15ml	Biotinylated anti-human BD-1 in buffer with protein, detergents and ProClin 300 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 ProClin 300 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 1 Microwell Strip Plate	100-241	8-well strips (12)	Coated with purified anti-Human BD-1 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, Detection Antibody Concentrate and Streptavidin-HRP Concentrate.
- Graduated cylinder to dilute Wash Concentrate and Sample Diluent concentrate; 200ml 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.

PRECAUTIONS AND SAFETY INSTRUCTIONS

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

Calibrators, Sample Diluent, and Antibody HRP contain bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains dilute 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 can be requested or obtained from the ADI website: Sample Diluent and anti-Protein G-HRP contain Proclin 300 (0.05%, v/v). http://4adi.com/objects/catalog/product/extras/ELISA-Kit-SDS-MSDS-Set-1.pdf

SPECIMEN COLLECTION AND HANDLING

Culture medium, serum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference. For serum, collect blood by venipuncture, allow clotting, and separate the serum by centrifugation at room temperature. For other samples, including tissue culture media, clarify the sample by centrifugation and/or filtration prior to dilution in Working Sample Diluent. If samples will not be assayed immediately, stored refrigerated for up to a few weeks, or frozen for long-term storage. Avoid freeze-thaw cycles.

ASSAY PROCEDURE

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

Use freshly diluted Standards as described on page 2. Dilute samples in Working Sample Diluent according to expected BD-1 concentrations. Dilutions of 20 to 50-fold are appropriate for most normal Human sera.

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

1. Set-up

- 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.
- Add 200-300ul Working Wash Solution to each well and let stand for 5 to 30 minutes before use.
- Aspirate or dump the liquid and pat the plate dry on a paper towel.

2. 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 is recommended. Improper washes may lead to falsely elevated signals and poor reproducibility.

3. 2nd Incubation [100ul – 60 min; 4 washes]

- Add 100ul of Working Detection Antibody to each well.
- Incubate for 60 minutes.
- Wash wells 4 times as in step 2.

4. 3rd Incubation [100ul – 30 min; 5 washes]

- Add 100ul of Working Streptavidin-HRP Conjugate to each well.
- Incubate for 30 minutes.
- Wash wells 5 times as in step 2.

5. 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, assuring the top standard does not surpass 2 OD.

6. 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.

7. 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.