ELISA Kit Components	Amount	Cat/Part No.
Anti-BD-3 Microwell Strip Plate	8-well strips (12)	100-261
BD-3 Standard, lyophilized	2 vials	100-262
Anti-BD-3 Detection Antibody (100X)	0.15 ml	100-263
Streptavidin HRP Conjugate (100X)	0.15 ml	S-HRP100
Sample Diluent Concentrate (10X)	10 ml	SD-10TC
Conjugate Diluent	22 ml	TBT
Wash Solution Concentrate (100X)	10 ml	WB-100
TMB Substrate	12 ml	80091
Stop Solution	12 ml	80101
Product Manual	1 ea	100-260-BD3

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. 100-260-BD3

Human Beta Defensin 3

ELISA Kit Cat. No. 100-260-BD3

For Quantitation of BD-3 in Biological Solutions

ALPHA DIAGNOSTIC INTERNATIONAL

India Contact:

Life Technologies (India) Pvt. Ltd.
306, Aggarwal City Mall, Opposite M2K Pitampura, Delhi – 110034
Ph: +91-11-42208000, 42208111, 42208222

Mobile: +91-9810521400
Fax: +91-11-42208444
Email: customerservice@atzlabs.com
Web: www.atzlabs.com

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INTENDED USE

The Human Beta Defensin 3 (BD-3) ELISA Kit is an in vitro immunoassay for research use in the quantification of BD-3 in cultures of human cells and in appropriately qualified samples from serum, saliva, or other tissue fluids.

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 have been described in humans, the alpha- and beta-defensins, which 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 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 3 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 parallelism and recovery.

PRINCIPLE OF THE TEST

The Human Beta Defensin 3 ELISA kit is based on the binding of Human Beta Defensin 3 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-3 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-3 in samples is calculated from a standard curve of purified recombinant human BD-3 of designated concentration.

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 box label. Stabilities of the working solutions are indicated under Reagent Preparation.

PERFORMANCE CHARACTERISTICS & EXPECTED RESULTS

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

Human Serum
BD-3 Levels
Assay of stored, frozen sera from six individual humans and two human serum pools, ranged from 0 to 3.2 ng/ml. Fresh sera may contain higher quantities.

Recovery
Purified BD-3 was spiked into each of 8 stored serum samples diluted 1:56. Observed assay values compared to expected values ranged from 0 to 113%. This high variation amongst sera is unexplained and may not be an issue with fresh samples. Investigators should perform recovery studies with fresh samples with and without added BD3 Standard.

Sample	Initial pg/ml	+ 267 pg/ml BD-3	% Recovery
Female serum 1	88	389	**113**
Female serum 2	87	277	**71**
Female serum 3	88	382	**110**
Male serum 1	107	256	**56**
Male serum 2	114	318	**77**
Male serum 3	108	263	**58**
Serum pool 1	0	0	**0**
Serum pool 2	0	0	**0**

Human Saliva
BD-3 Levels
Assay of 2 freshly collected samples ranged from 3.2 to 9.1 ng/ml.

Parallelism and Recovery
BD-3 was spiked into 5% saliva at 4 levels, 100-800 pg/ml. The mean recovery ranged from 82 to 108%, demonstrating linear dilution and equivalent quantification across the standard range.

Culture Medium
Parallelism and Recovery
BD-3 was spiked into 10% Neonatal Bovine Serum in Sample Diluent at 4 levels, 100-800 pg/ml. The mean recovery ranged from 97 to 104%, demonstrating linear dilution and equivalent quantification across the standard range.

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-3 concentrations may be determined as follows:

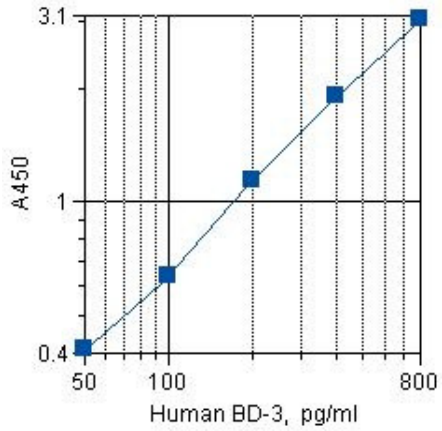
1. Calculate the mean OD of duplicate samples.
2. On graph paper plot the mean OD of the standards (y-axis) against the concentration (pg/ml) of BD-3 (x-axis). Draw the best fit curve through these points to construct the standard curve. A point-to-point construction is most common and reliable.
3. The BD-3 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-3 pg/ml
A1, A2	**Negative Diluent Control**	0.19	0
B1, B2	50 pg/ml **Standard**	0.41	50
C1, C2	100 pg/ml **Standard**	0.64	100
D1, D2	200 pg/ml **Standard**	1.14	200
E1, E2	400 pg/ml **Standard**	1.91	400
F1, F2	800 pg/ml **Standard**	3.02	800
G1, G2	**Sample** [Diluted 1:20]	0.75	419
	Calculated: 20-fold dilution x 118 pg/ml = **2.36** 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 3 Standard Part No. 100-262	Two (2) vials, each containing rBD-3 lyophilized in buffer with protein as stabilizers. Keep lyophilized vials refrigerated until used or kit lot expires.

Reconstitute 1 vial with the volume of **Working Sample Diluent** indicated on the Standard label to provide a 800 pg/ml Top Standard. Prepare 2-fold dilutions, as follows; sufficient for at least one curve:

Standard	**+ Diluent =**	**Final Concn**
Reconstituted Standard	None	800 pg/ml
225 ul of 800pg/ml	225ul	400 pg/ml
225 ul of 400pg/ml	225ul	200 pg/ml
225 ul of 200pg/ml	225ul	100 pg/ml
225 ul of 100pg/ml	225ul	50 pg/ml

Use within 5 days of preparation; store @ 2-8° C.

Sample Diluent Concentrate (10x) Part SD-10TC, 10ml	Dilute the entire volume, 10ml + 90ml 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. Note: The Sample Diluent is required for proper performance; do not substitute. Samples should be diluted at least 2 to 5-fold for optimal recovery and assay sensitivity.
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 3 Detection Antibody Concentrate (100x) Part No. 100-263, 0.15ml	Biotinylated anti-human BD-3 in buffer with protein, detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **Conjugate 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 antimicrobial as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **Conjugate 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	Amt	Contents
Anti-Human Beta Defensin 3 Microwell Strip Plate	100-261	8-well strips (12)	Coated with purified anti-Human BD-3 antibodies. Return unused strips to the pouch with desiccant; re-seal and store refrigerated.
Conjugate Diluent	TBT	22 ml	Buffer with protein and antimicrobials as stabilizers. Use as is for dilution of HRP and Detection conjugates.
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; 100ml 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 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 bromo-nitro-dioxane (BND: 0.05%, 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.

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, store refrigerated for up to two weeks, or frozen for long-term storage. Avoid freeze-thaw cycles.

ASSAY PROCEDURE

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

Use freshly diluted Standards as described on page 2. Dilute samples in Working Sample Diluent according to expected BD-3 concentrations. Dilute serum and other body fluids at least 5-fold to avoid sample matrix issues; dilute culture medium at least 2-fold.

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. 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 about 5 minutes before sample addition.
- 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 may be used. 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, or read OD at 405-410 nm (results are valid).

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.