Human BD3 immunoassay is a 3.5 - 4.5 hour solid phase ELISA designed to measure human BD3 in SALIVA, cell culture supernates, and EDTA plasma. It contains recombinant human BD3 and antibodies raised against this protein. It has been shown to accurately quantify recombinant human BD3. Results obtained with naturally occurring BD3 samples showed linear curves that were parallel to the standard curves obtained using the kit standards. These results indicate that the Immunoassay kit can be used to determine relative mass values for natural human BD3.

This assay employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for BD3 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any BD3 present is bound by the immobilized antibody. After washing away any unbound substances, a biotinylated antibody specific for BD3 is added to the wells. Following a wash to remove any unbound antibody-biotin reagent, HRP link Streptavidin is added to the wells. After washing away any unbound enzyme, a substrate solution is added to the wells and color develops in proportion to the amount of BD3 bound in the initial step. The color development is stopped and the intensity of the color is measured.

_ FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
_ The kit should not be used beyond the expiration date on the kit label.
_ Do not mix or substitute reagents with those from other lots or sources.
_ It is important that the **SAMPLE SOLUTION** selected for the standard curve be consistent with the samples being assayed.
_ If samples generate values higher than the highest standard, dilute the samples with the appropriate **SAMPLE SOLUTION** and repeat the assay.
_ Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
_ This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Immunoassay, the possibility of interference cannot be excluded.

Unopened Kit: Unopened Kit: Store at 2 - 8° C for up to 6 months. For longer storage, unopened Standard, Detection Antibody Concentrated should be stored at -20 or -70 °C. Do not use past kit expiration date.
Opened / Reconstituted Reagents: Reconstituted Standard, Detection Antibody Solution SHOULD BE STORED at -20 °C or – 70°C for up to one month. Streptavidin - HRP Conjugate 100-fold concentrated and other components may be stored at 2 - 8°C for up to 6 months.
Microplate Wells: Return unused wells to the plastic bag containing the desiccant pack, reseal along entire edge of zip-seal. Microplate may be stored for up to 6 months at 2 - 8° C.

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Microplate shaker (250-300rpm).
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 500 mL graduated cylinders.

Saliva – Saliva samples were centrifuged at 10, 000*g* at 4 °C for 20 min, Collect supernatants and were stored at –70 °C until use. Note: 1. Saliva has high concentrations of BD3, wash hand and wear mask to perform standard dilution and or sample dilution as well as assay. 2. Saliva collector must not have any protein binding or filtering capabilities.

Serum Free Cell Culture Supernates - Remove particulates by centrifugation immediately aliquot and store samples at -20 °C ~-70 °C. Avoid repeated freeze-thaw cycles. Cell Culture Supernates require peptide extraction by C18 column as well as other suitable peptide extraction protocol due serum and proteins interface BD3 sample assay. Note. Samples contain serum are not suitable for BD3 assay. Reconstitute lyophilized peptide extractions with 0.22 µm filtered 18.2 mΩ deionized water (without any proteins) in small volume and may require dilution with **Sample Solution** (DB30) to perform assay.

Plasma - Collect plasma using EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Aliquot and store samples at -20 °C ~-70 °C. Avoid repeated freeze-thaw cycles. EDTA plasma samples require peptide extraction.

Saliva samples require a 2 or 4-fold dilution. A suggested 2-fold dilution is 150 µL sample + 150 µL Sample Solution (DB30). A suggested 4-fold dilution is 80 µL sample + 240 µL Sample Solution (DB30). Reconstitute lyophilized peptide extractions (from EDTA plasma, Cell Cultures) with 0.22 µm filtered 18.2 mΩ deionized water (without any proteins) in small volume. That should be diluted with **Sample Solution** (DB30) to perform assay. Optimal dilutions should be determined by each laboratory for each application.
***Use polypropylene test tubes*.**

Bring all reagents to room temperature before use.
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 50 mL of Wash Buffer Concentrate into deionized or distilled water (450 mL) to prepare 500 mL of Wash Buffer.
BD3 Standard - Refer to vial label for reconstitution volume. Reconstitute the **BD3** Standard with 1mL of **Sample Solution (DB30)**. This reconstitution produces a stock solution of **2000 pg/mL**. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Pipette 250 µL of the appropriate Sample Solution into the tube #1 and #6. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 2000 pg/mL standard serves as the high standard. The appropriate Sample Solution serves as the zero standard (0 pg/mL). *Note: Antibody & HRP Diluent Solution (DB06) as well as Wash Buffer (WB01) can not be used to dilute Standard or saliva samples.*

250 μl 2 μl 2 μl 2 μl 2 μl 250 μl

1 mL

Standard		1	2	3	4	5	6	
Concen	20	1000		2	1	62	3	pg/ml

Detection Antibody- Reconstitute the **Detection Antibody** with 105 μl of Antibody & HRP Diluent Solution to produce a 100-fold concentrated stock solution. Pipette 10.395 mL of the appropriate Antibody & HRP Diluent Solution into the 15 ml centrifuge tube and transfer 105 μl of 100-fold concentrated stock solution to prepare working solution.

Streptavidin-HRP Conjugate - Pipette 11.88 mL of Antibody & HRP Diluent Solution into a 15 ml centrifuge tube and transfer 120 μl of 100-fold concentrated stock solution to prepare working solution. *Note: 1 x working solution of Streptavidin-HRP Conjugate should be used within a few days.*

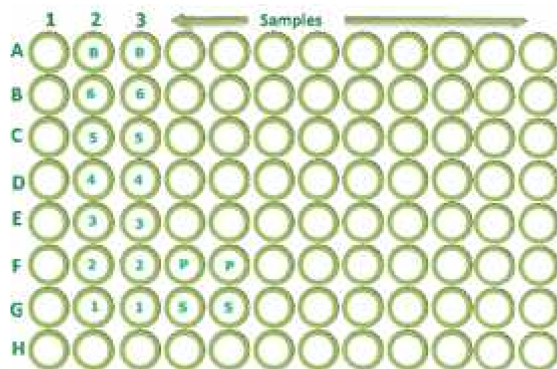
Positive Control- Reconstitute the positive control with 2 mL of **Sample Solution DB30** to make Positive Control working solution.

Bring all reagents and samples to room temperature before use. It is recommended that standards be assayed in duplicate.

1. Prepare all reagents and working standards as directed in the previous sections.
2. Remove excess micro-plate strips from the plate frame, return them to the plastic bag containing the desiccant pack, reseal.
3. Add 100 μL of **Sample Solution** to Blank well (A2, A3).
4. Add 100 μL of Standard (from B2 to G3, G4 to G5), samples, or positive control (F4, F5) per well. Cover with the Sealer. Incubate for 2 hours on micro-plate shaker at room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (300 μL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 100 μL of Detection Antibody working solution to each well. Cover with sealer. Incubate for 2 hours on micro-plate shaker at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 μL of **Streptavidin-HRP Conjugate** working solution to each well. Incubate for 60 minutes on micro-plate shaker at room temperature. **Protect from light.**
9. Repeat the aspiration/wash as in step 5.
10. Add 100 μL of Substrate Solution to each well. Incubate for 12-15 minutes at room temperature. **Protect from light.**
11. Add 100 μL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green, or if the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
12. Determine the optical density of each well within 15 minutes, using a micro-plate reader set to 450 nm.

Average the duplicate readings for each standard, positive control, and sample and subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a log-log curve fit. As an alternative, construct a standard curve by plotting

the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the BD3 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.
If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.



This immunoassay is calibrated against a highly purified E. Coli-expressed recombinant human BD3.

Twenty-five assays were evaluated and the minimum detectable dose (MDD) of BD3 was 15 pg/mL.

To assess the linearity of the assay, pooled human Saliva samples were diluted with Sample Solution DB30 and assayed.

This assay recognizes both natural and recombinant human BD3. The factors listed below were prepared at 50 ng/mL in Dilution Buffer, and assayed for cross reactivity. Preparations of the following factors at 50 ng/mL in a mid-range rh BD3 control were assayed for interference. No significant cross-reactivity or interference was observed.

This standard curve* is provided for demonstration only. A standard curve should be generated for each set of samples assayed.

SUMMARY OF ASSAY PROCEDURE