LSBio
an Absolute Biotech Company

Human/Mouse/Rat BNP ELISA (Competitive EIA)

User Manual

Catalog No. LS-F3

It is important that you read this entire manual carefully before starting your experiment.

SAMPLE ONLY

This kit is for **Research Use Only**. **Not for Diagnostic Use.**
This kit is not approved for use in humans or for clinical diagnosis.

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ASSAY SPECIFICATIONS

Target: BNP

Synonyms: BNP, Natriuretic peptides B, Natriuretic peptide B, Natriuretic protein, Brain type natriuretic peptide, NPPB, NPPB / BNP

Specificity: Recombinant and natural Mouse/Human/Rat BNP. This kit detects BNP. This ELISA kit shows no cross-reactivity with any of the adipokines tested: Ghrelin, Nesfatin, Angiotensin II, NPY and APC. This claim is limited by existing techniques therefore cross-reactivity may exist with untested analogs.

Sample Types: This kit is recommended for use with human, mouse and rat serum and cell culture supernatants. Use with other sample types is not supported.

Detection Range: 0.1-1,000 pg/ml

Sensitivity: Typically less than 1.66 pg/ml

Performance: Intra-Assay CV (<10%); Inter-Assay CV (<15%)

Limitations: This kit is for **Research Use Only** and is not intended for diagnostic use. This kit is not approved for use in humans or for clinical diagnosis.

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ASSAY PRINCIPLE

This assay is based on the Competitive EIA principle. Each well of the supplied microtiter plate has been pre-coated with an anti-rabbit antibody. An antibody specific to the target antigen (the capture antibody) is then added to each well and binds to the anti-rabbit antibody. Next a biotinylated synthetic antigen peptide is added to each well along with either the non-biotinylated standard peptide or the test sample. The biotinylated synthetic antigen peptide competes with the non-biotinylated standard peptide or native antigen in the sample to bind with the capture antibody. Unbound antigen and peptide is washed away. An Avidin-Horseradish Peroxidase (HRP) conjugate is then added which binds to the biotin. Unbound HRP-conjugate is washed away. A TMB substrate is then added which reacts with the HRP enzyme resulting in color development. A sulfuric acid stop solution is added to terminate color development reaction and then the optical density (OD) of the well is measured at a wavelength of 450 nm ± 2 nm. The OD of an unknown sample can then be compared to an OD standard curve generated using known antigen concentrations in order to determine its antigen concentration. In contract to typical Sandwich ELISA assays, in competition assays the greater the amount of antigen in the sample, the lower the color development and optical density reading.

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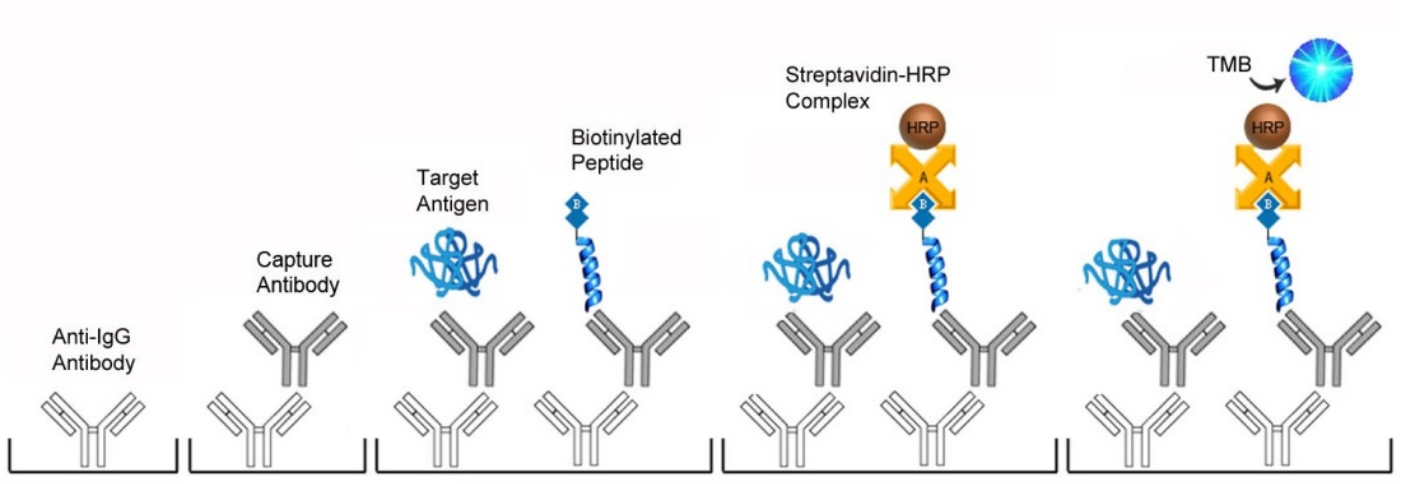
Assay Principle Image



KIT COMPONENTS

Component	Quantity
Coated 96-well Strip Plate	1
Standard (lyophilized)	2 vials
Assay Diluent B (5x)	1 vial x 15 ml
Capture Antibody (lyophilized)	2 vials
Biotinylated Peptide (lyophilized)	2 vials
HRP-Streptavidin Conjugate (200x)	1 vial x 600 µl
Positive Control (lyophilized)	1 vial
Wash Buffer (20x)	1 vial x 25 ml
TMB Substrate	1 vial x 12 ml
Stop Solution	1 vial x 8 ml
Plate Sealers	4
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KIT STORAGE

The Standard, Biotinylated Peptide, and Positive Control should be stored at -20°C/-80°C upon arrival. Avoid freeze thaw cycles. Store all other kit components at -20°C. Once individual reagents are opened it is recommended that the kit be used within 1 month. Unused Strip Plate wells should be stored at 2°-8°C for 1 month in a sealed bag containing desiccant in order to minimize exposure to moisture. Do not use the kit beyond its expiration date.

OTHER REQUIRED SUPPLIES

- Microplate reader with 450nm wavelength filter
- High-precision pipette and sterile pipette tips
- Eppendorf tubes
- 37°C incubator
- Deionized or distilled water
- Absorbent paper

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SAMPLE COLLECTION

This assay is recommended for use with Mouse/Human/Rat serum and cell culture supernatants. Use with other sample types is not supported. The sample collection protocols below have been provided for your reference.

Breast Milk - Centrifuge samples for 20 minutes at 1000×g to remove particulates. Collect the supernatant for assaying.

Cell Lysates - Collect and pellet the cells by centrifugation and remove the supernatant. Wash the cells 3 times with PBS* then resuspend in PBS*. Lyse the cells by ultrasonication 4 times. Alternatively freeze the cells to -20°C and thaw to room temperature 3 times. Centrifuge at 1500×g for 10 minutes at 2 - 8°C to remove cellular debris. Collect the supernatant for assaying.

Erythrocyte Lysates - Centrifuge whole blood for 20 minutes at 1000×g to pellet the cells and remove the supernatant. Wash the cells 3 times with PBS* then resuspend in PBS*. Freeze (-20°C)/thaw (room temperature) the cells 3 times. Centrifuge at 5,000×g for 10 minutes at 2-8°C to remove cellular debris. Collect the supernatant for assaying. Erythrocyte lysates must be diluted with Sample Diluent before running.

Plasma - Collect plasma using Heparin, EDTA, or Citrate as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g at 2–8°C within 30 minutes of collection. Collect the supernatant for assaying.

Platelet-Poor Plasma - Collect plasma using Heparin, EDTA, or Citrate as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g at 2–8°C within 30 minutes of collection. It is recommended that samples should be centrifuged for 10 minutes at 10,000×g for complete platelet removal. Collect the supernatant for assaying.

Sperm and Seminal Plasma – Allow semen to liquefy at room temperature or 37°C. After liquefaction, centrifuge at 2,000×g for 10-15 minutes. Collect seminal plasma supernatant for assaying. Wash the precipitated protein 3 times with PBS* then resuspend in PBS*. Lyse the cells by ultrasonication then centrifuge at 2,000×g for 10-15 minutes. Collect the supernatant for assaying.

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Serum - Use a serum separator tube and allow samples to clot for 2 hours at room temperature or overnight at 4°C before centrifugation for 20 minutes at approximately 1000×g. Collect the supernatant for assaying.

Tissue Homogenates – Because preparation methods for tissue homogenates vary depending upon tissue type, users should research tissue specific conditions independently. The following is one example only. Rinse tissues in PBS* to remove excess blood and weigh before homogenization. Finely mince tissues and homogenize them in 5-10mL of PBS*with a glass homogenizer on ice. Lyse the cells by ultrasonication or freeze (-20°C)/thaw (room temperature) 3 times. Centrifuge homogenate at 5000×g for 5 minutes. Collect the supernatant for assaying.

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter and collect the supernatant for assaying.

Cell culture supernatants, cerebrospinal, follicular, and lung lavage fluids, saliva, sweat, tears, and other biological fluids - Centrifuge samples for 20 minutes at 1000×g to remove particulates. Collect the supernatant for assaying.

* 1xPBS (0.02mol/L pH7.0-7.2)

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SAMPLE COLLECTION NOTES

1. LSBio recommends that samples are used immediately upon preparation. Alternatively, samples stored at 2-8°C should be used within 5 days. For long-term storage sample aliquots should be prepared and stored at -20°C if used within 1 month, or -80°C if used within 6 months. Long term storage can result in protein degradation and denaturation, which may result in inaccurate results.
2. Avoid repeated freeze/thaw cycles for all samples.
3. In the event that a sample type not listed above is intended to be used with the kit, it is recommended that the customer conduct validation experiments in order to be confident in the results.
4. Due to chemical interference, the use of tissue or cell extraction samples prepared by chemical lysis buffers may result in inaccurate results.
5. Due to factors including cell viability, cell number, or sampling time, samples from cell culture supernatant may not be detected by the kit.
6. Samples should be brought to room temperature (18-25°C) before performing the assay without the use of extra heating.
7. Sample concentrations should be predicted before being used in the assay. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
8. LSBio is responsible for the quality and performance of the kit components but is NOT responsible for the performance of customer supplied samples used with the kit.

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REAGENT PREPARATION

Keep kit reagents on ice during reagent preparation steps. Equilibrate plate to room temperature before opening the sealed pouch.

1x Wash Buffer *(for use in Assay Procedure (page 15))*: If crystals have formed in the concentrate, warm to room temperature and mix it gently until crystals have completely dissolved. Prepare 400 ml of Working Wash Buffer by diluting the supplied 20 ml of 20x Wash Buffer Concentrate with 380 ml of deionized or distilled water. Wash Buffer can be stored at 4°C once prepared.

1x Assay Diluent B: Prepare 75 ml of Assay Diluent by diluting the supplied 15 ml of 5x Assay Diluent Concentrate with 60 ml of deionized or distilled water. Assay Diluent can be stored at 4°C once prepared.

100x Capture Antibody Concentrate: Briefly spin down the Capture Antibody and add 55 µl of 1x Assay Diluent B. Do not store or reuse.

1x Capture Antibody Solution: *(for use in Assay Procedure (page 15))***:** Calculate the volume of Capture Antibody Solution needed for your particular experiment and prepare that volume by diluting the Capture Antibody Concentrate 100-fold (1:100) with 1x Assay Diluent B. Capture Antibody Solution must be prepared fresh for each experiment and cannot be stored.

Working Biotinylated Peptide Solution (20 pg/ml): *(for use in Control, Sample, and Standard preparation (pages 11, 12, 13))* Briefly spin down the Biotinylated Peptide and reconstitute with 20µl ddH_20. Combine this entire volume with 10 ml of 1x Assay Diluent. The final concentration of the Biotinylated Peptide in this solution is 20 pg/ml.

HRP-Streptavidin Working Solution *(for use in Assay Procedure (page 15))*: Gently mix the stock solution before use. Calculate the volume of HRP-Streptavidin Working Solution needed for your particular experiment and prepare that volume by diluting the HRP-Streptavidin Conjugate Solution 200-fold (1:200) with Assay Diluent B. HRP-Streptavidin working solution must be prepared fresh for each experiment and cannot be stored.

TMB Substrate *(for use in Assay Procedure (page 15))*: Using sterile techniques remove the needed volume of TMB Substrate Solution for

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the number of wells you are planning to run. Dispose of unused TMB Substrate Solution rather than returning it to the stock container.

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POSITIVE CONTROL PREPARATION

The Positive Control is a cell culture medium sample that is meant to be a system control to verify that the detection and kit components are working. The resulting OD will not be used in any calculations.

Briefly spin down the Positive Control before opening. Reconstitute the positive control with 100µl of ddH_20. Next, combine this 100 µl of Positive Control with 100 µl of Working Biotinylated Peptide Solution (20 pg/ml) and mix thoroughly. This is a 2-fold dilution of the Biotinylated Peptide and its final concertation in this solution is 10 pg/ml.

If this positive control does not produce a positive signal in the assay please contact LifeSpan Technical Support.

The positive control may be diluted further if desired, but be sure that the final concentration of Biotinylated Peptide is 10 pg/ml.

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SAMPLE PREPARATION

Please predict the concentration of your samples before assaying. The resulting optical density (OD) values of your sample must fall within the OD readings of the standard curve in order for the calculated antigen concentration to be accurate. A dilution series of each sample may be necessary. The following dilutions will provide you with a 2-fold to 100-fold range.

Dilution	Sample	1x Assay Diluent	Working Biotinylated Peptide Solution (20 pg/ml)	Final Volume
2-fold	125 µl	0 µl	125 µl	250 µl
4-fold	65 µl	65 µl	130 µl	260 µl
10-fold	25 µl	100 µl	125 µl	250 µl
100-fold	10 µl	490 µl	500 µl	1000 µl

It is recommended that all samples are run in duplicate (100 µl/well).

When samples are added to the wells, they must contain Biotinylated Peptide at a concertation of **10 pg/ml**.

Serum typically require a 2-fold dilution in order for their OD readings to fall within the Standard Curve of this assay. However, due to sample variation, it is recommended that researchers determine the optimal dilution for their samples as described above.

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STANDARD PREPARATION

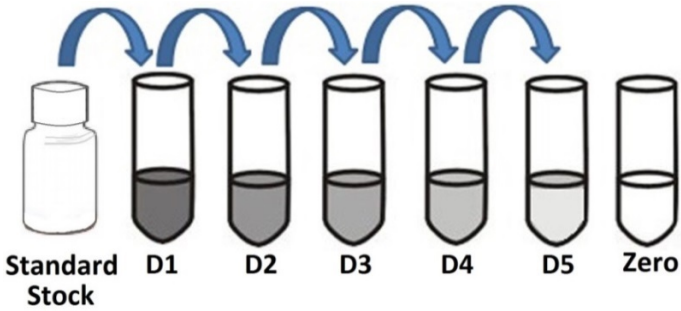
The following are instructions for the preparation of a Standard dilution series which will be used to generate the standard curve. The standard curve is then used to determine the concentration of target antigen in unknown samples (see the **Calculation of Results** section). The following will prepare sufficient volumes to run the Standard dilution series in duplicate. Reconstituted Standard and prepared standard dilutions should be used immediately and not stored for future use.

10 pg/ml Biotinylated Peptide Solution: Combine 2.0 ml of Working Biotinylated Peptide Solution (20 pg/ml) with 2.0 ml of 1x Assay Diluent and mix thoroughly.

Standard Stock Solution: Briefly spin down the Standard and reconstitute in 8 µl of ddH2O.

Preparation of Standard Dilutions: Prepare the following 10-fold serial dilutions.

D1 (1,000 pg/ml):	Combine 8 µl of the Standard Stock Solution with 792 µl of 10 pg/ml Biotinylated Peptide Solution.
D2 (100 pg/ml):	Pipette 50µl of D1 into 450µl of 10 pg/ml Biotinylated Peptide Solution
D3 (10 pg/ml):	Pipette 50µl of D2 into 450µl of 10 pg/ml Biotinylated Peptide Solution
D4 (1 pg/ml):	Pipette 50µl of D3 into 450µl of 10 pg/ml Biotinylated Peptide Solution
D5 (0.1 pg/ml):	Pipette 50µl of D4 into 450µl of 10 pg/ml Biotinylated Peptide Solution
Blank (0 pg/ml):	Use 500µl of 10 pg/ml Biotinylated Peptide Solution alone.



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REAGENT PREPARATION NOTES

1. It is highly recommended that standard curves and samples are run in duplicate within each experiment.
2. Once prepared, standards should be used immediately, and used only once. Long-term storage of reconstituted standards is NOT recommended.
3. All solutions prepared from concentrates are intended for one-time use. Do not reuse solutions.
4. Do not prepare Standard dilutions directly in wells.
5. Prepared Reagents may adhere to the tube wall or cap during transport; centrifuge tubes briefly before opening.
6. All solutions should be gently mixed prior to use.
7. Reconstitute stock reagents in strict accordance with the instructions provided.
8. To minimize imprecision caused by pipetting, ensure that pipettes are calibrated. Pipetting volumes of less than 10µL is not recommended.
9. Substrate Solution is easily contaminated; sterility precautions should be taken. Substrate Solution should also be protected from light.
10. Do not substitute reagents from one kit lot to another. Use only those reagents supplied within this kit.
11. Due to the antigen specificity of the antibodies used in this assay, native or recombinant proteins from other manufacturers may not be detected by this kit.

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ASSAY PROCEDURE

Keep kit reagents on ice during reagent preparation steps. Prepare all reagents, working standards, and samples as directed in the previous sections.

1. Add 100 µl of **1x Capture Antibody Solution** to each well, cover with a plate sealer, and incubate for 1.5 hours at room temperature, or overnight at 4°C, with gentle shaking (1-2 cycles/sec).
2. Aspirate the liquid from each well and wash 4 times. Wash by adding approximately 200-300 µl of Wash Buffer using a squirt bottle, multi-channel pipette, manifold dispenser or automated washer. Allow each wash to sit for 1-2 minutes before completely aspirating. After the last wash, aspirate to remove any remaining Wash Buffer then invert the plate and tap against clean absorbent paper.
3. Add 100 µl of **Standard, Blank, Positive Control, or Sample** per well, cover with a plate sealer, and incubate for 2.5 hours at room temperature, or overnight at 4°C, with gentle shaking (1-2 cycles/sec).
4. Aspirate and wash the wells 4 times as outlined in step 2.
5. Add 100 µl of **HRP-Streptavidin Working Solution** to each well and incubate for 45 minutes at room temperature with gentle shaking (1-2 cycles/sec).
6. Aspirate and wash the wells 4 times as outlined in step 2.
7. Add 100 µl of **TMB Substrate** to each well and incubate for 30 minutes at room temperature in the dark with gentle shaking (1-2 cycles/sec). Monitor periodically until optimal color development has been achieved.
8. Add 50 µl of **Stop Solution** to each well and record the total development time. The blue color will change to yellow. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. The Stop Solution should be added to wells in the same order and timing as the substrate solution.
9. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm.

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ASSAY PROCEDURE NOTES

1. **ELISA Plate**: Keep appropriate numbers of strips for 1 experiment and remove extra strips from microtiter plate. Removed strips should be placed in a sealed bag containing desiccant and stored at 2-8°C.
2. **Solutions**: To avoid cross-contamination, change pipette tips between additions of each standard, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
3. **Applying Solutions:** All solutions should be added to the bottom of the ELISA plate well. Avoid touching the inside wall of the well. Avoid foaming when possible.
4. **Assay Timing**: The interval between adding sample to the first and last wells should be minimized. Delays will increase the incubation time differential between wells, which will significantly affect the experimental accuracy and repeatability. For each step in the procedure, total dispensing time for addition of reagents or samples should not exceed 10 minutes.
5. **Incubation**: To prevent evaporation and ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods of time between incubation steps. Do not let wells dry out at any time during the assay. Strictly observe the recommended incubation times and temperatures.
6. **Washing**: Proper washing procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings. Residual liquid in the reaction wells should be patted dry against absorbent paper during the washing process. Do not put absorbent paper directly into the reaction wells.
7. **Controlling Substrate Reaction Time**: After the addition of the TMB Substrate, periodically monitor the color development. Stop color development before the color becomes too deep by adding Stop Solution. Excessively strong color will result in inaccurate absorbance reading.

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8. **Reading**: The microplate reader should be preheated and programmed prior to use. Prior to taking OD readings, remove any residual liquid or fingerprints from the underside of the plate and confirm that there are no bubbles in the wells.
9. **Reaction Time Control**: Control reaction time should be strictly followed as outlined.
10. **Stop Solution**: The Stop Solution contains an acid, therefore proper precautions should be taken during its use, such as protection of the eyes, hands, face, and clothing.
11. **Mixing**: During incubation times, the use of a micro-oscillator at low frequency is recommended. Sufficient and gentle mixing is particularly important in producing reliable results.
12. Kits from different batches may be a little different in detection range, sensitivity, and color developing time. Please perform the experiment exactly according to the supplied instructions.
13. Due to inter- and intra-assay variability, it is recommended that appropriate carry-over controls be included between assays.
14. Prior to running valuable samples, LSBio recommends that the user run a preliminary experiment using the supplied controls in order to validate the assay.
15. To minimize external influence on the assay performance, operational procedures and lab conditions (such as room temperature, humidity, incubator temperature) should be strictly controlled. It is also strongly suggested that the whole assay is performed by the same operator from the beginning to the end.
16. The kit should not be used beyond the expiration date on the kit label.

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Assay Procedure Summary

Prepare all reagents, samples and standards.

Add 100 µl of **Capture Antibody** to each well and incubate for 1.5 hours at room temperature or overnight at 4°C.

Aspirate and wash 4 times.

Add 100 µl of **Standard, Blank, Positive Control,** or **Sample** to each well and incubate for 2.5 hours at room temperature or overnight at 4°C.

Aspirate and wash 4 times.

Add 100 µl of **HRP-Streptavidin Solution** and incubate for 45 minutes at room temperature.

Aspirate and wash 4 times.

Add 100 µl of **TMB Substrate** and incubate for 30 minutes at room temperature.

Add 50 µl of **Stop Solution**.

Read immediately at 450nm.

CALCULATION OF RESULTS

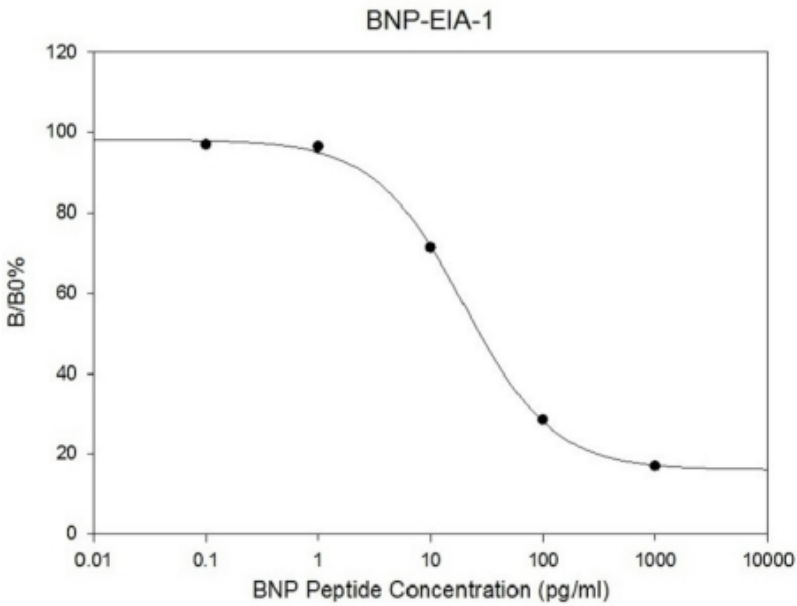
Calculate the mean absorbance for each set of duplicate stands, controls, and samples and subtract the blank optical density. Plot the standard curve using SigmaPlot software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis and percentage of absorbance (see calculation below) on the y-axis. Draw the best-fit curve through the standard points.

Percentage absorbance = (B – blank OD)/(B_0 – blank OD) where

B = OD of sample or standard and

B_0 = OD of zero standard (total binding)

Typical Data: The following standard curve is an example only and should not be used to calculate results for tested samples. A new standard curve must be generated for each set of samples tested.



TROUBLESHOOTING GUIDE

Problem	Possible Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standard dilution	Briefly spin the vial of standard and dissolve the powder thoroughly by a gentle mix.
	Wells not completely aspirated	Completely aspirate wells between steps.
Low signal	Too brief incubation times	Ensure sufficient incubation time.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrate to room temperature before use.
	Inadequate reagent volumes	Check pipettes and ensure correct preparation.
	Improper dilution	
Deep color but low value	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
		Open the Plate Reader ahead to pre-heat.

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Troubleshooting Guide (continued)

Problem	Possible Cause	Solution
Large CV	Inaccurate pipetting	Check pipettes
High background	Concentration of detector too high	Use recommended dilution factor.
	Plate is insufficiently washed	Review the manual for proper washing instructions. If using a plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Make fresh wash buffer.
Low sensitivity	Improper storage of the ELISA kit	All the reagents should be stored according to the instructions.
	Stop solution not added	Stop solution should be added to each well before measurement.

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Important Note: During shipment, small volumes of product will occasionally become entrapped in the seal of the product vial. We recommend briefly centrifuging the vial to dislodge any liquid in the container's cap prior to opening.

Warning: This reagent may contain sodium azide and sulfuric acid. The chemical, physical, and toxicological properties of these materials have not been thoroughly investigated. Standard Laboratory Practices should be followed. Avoid skin and eye contact, inhalation, and ingestion. Sodium azide forms hydrazoic acid under acidic conditions and may react with lead or copper plumbing to form highly explosive metal azides. On disposal, flush with large volumes of water to prevent accumulation.

Returns, Refunds, Cancelations: Any problems with LifeSpan products must be reported to LifeSpan within 10 days of product receipt. The customer must obtain written authorization from LifeSpan before returning items. To request that goods be returned, please contact LifeSpan Technical Support. If an error by LifeSpan BioSciences results in shipment of an incorrect order, LifeSpan will, at its option, either ship a replacement order at no charge, or credit the customer's account for the original product shipped in error. Returns and cancelations may be subject to a 30% restocking fee. Conditions & Warranty: All LifeSpan products are intended for Research Use Only and are not for use in Human therapeutic or diagnostic applications. The information supplied with each product is believed to be accurate, but no warranty or guarantee is offered for the products, because the ultimate conditions of use are beyond LifeSpan's control. The information supplied with each product is not to be construed as a recommendation to use this product in violation of any patent, and LifeSpan will not be held responsible for any infringement or other violation that may occur with the use of its products. Under no event will LifeSpan be responsible for any loss of profit or indirect consequential damage, including, but not limited to, personal injuries resulting from use of these products. LifeSpan's liability to any user of Products for damages that do not result from any fault of the user, will be limited to replacement of the Product(s) only, and in no event shall LifeSpan's liability exceed the actual price received by LifeSpan for the Product(s) at issue. LifeSpan shall not be liable for any indirect, special, incidental or consequential damages. LIFESPAN FURTHER DISCLAIMS ANY AND ALL EXPRESS AND IMPLIED OR STATUTORY WARRANTIES WITH RESPECT TO THE PRODUCTS, INCLUDING BUT NOT LIMITED TO ANY IMPLIED WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE. LifeSpan disclaims any and all responsibility for any injury or damage which may be caused by the fault of the user.

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