Rat BNP-45 (rBNP-45) ELISA Kit

Introduction

Natriuretic peptides (ANP, BNP and CNP) comprise a family of structurally related peptides, which are derived from three different genes and share a 17-amino acid internal ring (1). A high level of plasma BNP may have a strong, independent association with increased mortality rates in patients with primary pulmonary hypertension (PPH) (2), congestive heart failure and/or after acute myocardial infarction (3, 4).

Principal of the Assay

The rBNP-45 ELISA kit is designed for detection of rat BNP-45 in plasma, serum, tissue extract, and cell culture supernatants. This assay employs a quantitative sandwich enzyme immunoassay technique that measures rBNP45 in less than 5 hours. A polyclonal antibody specific for rBNP-45 has been pre-coated onto a microplate. The rBNP-45 in standards and samples is sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for rBNP-45, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Caution and Warning

- **Prepare all reagents (working diluent buffer, wash buffer, standards, biotinylated-antibody, and SP conjugate) as instructed, prior to running the assay.**
- **Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this protocol. However, the user should determine the optimal dilution factor.**
- **Spin down the SP conjugate vial and the biotinylated-antibody vial before opening and using contents.**
- This kit is for research use only.
- The kit should not be used beyond the expiration date.
- The Stop Solution is an acid solution

Reagents

- **rBNP-45 Microplate:** A 96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against rBNP-45.
- **Sealing Tapes:** Each kit contains 3 pre-cut, pressure-sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **rBNP45 Standard:** Rat BNP-45 in a buffered protein base (4 ng, lyophilized).

- **Biotinylated rBNP-45 Antibody (100x):** A 100-fold biotinylated polyclonal antibody against rBNP-45 (80 µl).
- **MIX Diluent Concentrate (10x):** A 10-fold concentrated buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x)**: A 20-fold concentrated buffered surfactant (30 ml, 2 bottles).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (80 µl).
- **Chromogen Substrate**: A ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).
- **Stop Solution**: A 0.5 N hydrochloric acid to stop the chromogen substrate reaction (12 ml).

Storage Condition

- Store components of the kit at 2-8^{0}C or -20^{0}C upon arrival up to the expiration date.
- Store SP Conjugate and Biotinylated Antibody at -20^{0}C.
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8^{0}C.
- Opened unused microplate wells may be returned to the foil pouch with the desiccant packs. Reseal along zip-seal. May be stored for up to 1 month in a vacuum desiccator.
- Diluent (1x) may be stored for up to 1 month at 2-8^{0}C.
- Store Standard at 2-8^{0}C before reconstituting with Diluent and at -20^{0}C after reconstituting with Diluent.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm.
- Pipettes (1-20 µl, 20-200 µl, 200-1000 µl and multiple channel).
- Deionized or distilled reagent grade water.

Sample Collection and Storage

- **Plasma:** Collect plasma using a final concentration of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 2000 x g for 10 minutes and assay undiluted plasma for medium and high level of BNP-45. Samples can be stored at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2000 x g for 10 minutes. Remove serum and perform the assay for medium and high level of BNP-45. Samples can be stored at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Cell Culture Supernatants:** Centrifuge cell culture media at 2000 x g for 10 minutes to remove debris. Collect supernatants and assay. Store samples at -20^{0}C or below. Avoid repeated freeze-thaw cycles.
- **Tissue:** Extract tissue samples with 0.1 M phosphate-buffered saline (pH7.4) containing 1% Triton X-100 and centrifuge at 14000 x g for 20 min. Collect the supernatant, measure the protein concentration and assay. Freeze the remaining extract at -20^{0}C or below.

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- **MIX Diluent Concentrate (10x)**: If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the MIX Diluent Concentrate 1:10 with reagent grade water. Store for up to 1 month at 2 - 8^0C.
- **rBNP-45 Standard:** Reconstitute the 4 ng of rat BNP-45 Standard with 4 ml of MIX Diluent to generate a stock solution of 1 ng/ml. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting the standard (1 ng/ml) 1:2 with MIX Diluent to generate 0.5, 0.25, 0.125, 0.0625, and 0.0313 ng/ml. MIX Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20^0C.

Standard Point	Dilution	[rBNP-45] (ng/ml)
P1	1 part Standard (1 ng/ml)	1.0000
P2	1 part P1 + 1 part MIX Diluent	0.5000
P3	1 part P2 + 1 part MIX Diluent	0.2500
P4	1 part P3 + 1 part MIX Diluent	0.1250
P5	1 part P4 + 1 part MIX Diluent	0.0625
P6	1 part P5 + 1 part MIX Diluent	0.0313
P7	MIX Diluent	0.0000

- **Biotinylated rBNP-45 Antibody (100x):** Spin down the antibody briefly and dilute the desired amount of the antibody 1:100 with MIX Diluent. Any remaining solution should be frozen at -20^0C.
- **Wash Buffer Concentrate (20x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the Wash Buffer Concentrate 1:20 with reagent grade water.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 1:100 with MIX Diluent. Any remaining solution should be frozen at -20^0C.

Assay Procedure

- Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-30^0C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 50 µl of Standard or sample per well. Cover wells with a sealing tape and incubate for two hours. Start the timer after the last sample addition.
- Wash five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid. If using a machine wash six times with 300 µl of Wash Buffer and then invert the plate, decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.
- Add 50 µl of Biotinylated rBNP-45 Antibody to each well and incubate for two hours.
- Wash the microplate as described above.
- Add 50 µl of Streptavidin-Peroxidase Conjugate per well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash the microplate as described above.

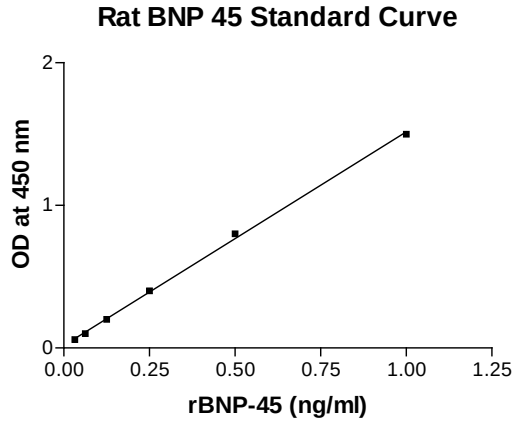
- Add 50 µl of Chromogen Substrate per well and incubate until the optimal color density develops, which usually takes about 10 minutes. Gently tap the plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
- Add 50 µl of Stop Solution to each well. The color will change from blue to yellow.
- Read the absorbance on a microplate reader at a wavelength of 450 nm **immediately**. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample.
- To generate a Standard Curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis and draw a best fit curve through the points on the graph. Plotting the 4 parameters or linear graph may linearize the data and the best-fit line can be determined by regression analysis of the linear portion of the curve.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.



Performance Characteristics

- The minimum detectable dose of rat BNP-45 is typically ~0.03 ng/ml.
- Intra-assay and inter-assay coefficients of variation were 4.3 % and 7.4% respectively.

References

(1) Wiedemann K, Jahn H, Kellner M *Exp Clin Endocrinol Diabetes* 2000; 108(1):5-13
(2) Nagaya N. et al. *Circulation* 2000 Aug 22; 102(8): 865-70
(3) Cheng V et. al. *J Am Coll Cardiol* 2001 Feb; 37(2): 386-91
(4) Bettencourt P. et. al. *Clin Cardiol* 2000 Dec; 23(12): 921-7

Version 6.4