FOR RESEARCH USE ONLY

NOT FOR IN VITRO CLINICAL DIAGNOSIS

Rat NT-proANP(N-Terminal Pro Atrial Natriuretic Peptide) ELISA Kit

Catalog NO.FY-ER4521 size: 96T/48T

This manual must be read attentively and completely before using this product.
If you have any problems, please contact us.

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Intended Use

The kit is intended for use in quantitative determination of Rat NT-proANP concentrations in serum, plasma, tissue homogenates, cell lysates, cell culture supernates and other biological fluids.

Specification

★ Sensitivity:0.19 ng/mL.
★ Detection Range:0.32-20ng/mL.
★ No significant cross-reactivity or interference between Rat NT-proANP and analogues was observed.
★ Repeatability: Coefficient of variation is 8%.

Principle of the Procedure

This kit uses the Sandwich-ELISA principle. The microtiter plate strips has been pre-coated with an affinity purified antibody to Rat NT-proANP. Standards or samples containing Rat NT-proANP are added to the plate and reacted with capture antibody. A second anti-Rat NT-proANP antibody labeled biotin is then added and binds to Rat NT-proANP captured on the plate. After that, Streptavidin-Horseradish Peroxidase(SA-HRP) is added to form a sandwich complex of solid phase antibody-Rat NT-proANP-biotin labeled antibody-SA-HRP. And then, TMB substrate solution is added to all wells and incubated. An enzyme-catalyzed reaction generates a blue color in the solution, thereafter, stop solution is added to stop the substrate reaction and the color turns yellow. The yellow solution is read at a wavelength of 450nm. The concentration of Rat NT-proANP in the samples is then calculated from the OD value by establishing a standard curve.

Limitations of the Procedure

1. This kit is for laboratory scientific research only, we will not be responsible for any consequences if this kit is used for clinical diagnosis or any other procedures.
2. Due to the uncertainty of its validity, this kit may not be suitable for testing some special experimental samples, such as gene knockout experiments.
3. This kit should be used before its expiration date, and please strictly follow the instructions for storage.
4. Different manufacturers' kits or testing the same analyte by other methods may produce inconsistent results because we do not compare our products with those of other manufacturers.
5. Since the antibodies used in the kit are usually prepared from recombinant proteins as immunogen, and recombinant proteins can be limited by different fragmentation, expression and purification systems, it is not recommended to

use this kit to detect recombinant proteins

6. In order to get the best experimental results, please use only the reagents provided by the manufacturer, and do not mix reagents from different batches.
7. Due to the existing conditions and the limitations of science and technology, we cannot fully identify and analyze the raw materials provided by the supplier comprehensively. Therefore, the kit may have some quality and technical risks.
8. The possibility of interference cannot be excluded before all factors are tested in the ELISA immunoassay.
9. In order to obtain reproducible results, each step in the experiment should be controlled and variations in sample collection, handling and storage may also lead to differences in sample measurements.
10. Although each kit passes rigorous quality testing, differences in measured values between batches of kits can still be caused by factors such as shipping conditions and different laboratory equipment.

Reagents&Materials Provided

Item	Specifications	Storage
Micro ELISA Plate (Dismountable)	96T: 8 wells ×12 strips 48T: 8 wells ×6 strips	-20℃, 6 months
Reference Standard	96T: 2 vials 48T: 1 vial	
Concentrated Biotinylated Detection Ab (100×)	96T: 1 vial,120μL 48T: 1 vial, 60μL	
Concentrated HRP Conjugate (100×)	96T: 1 vial, 120μL 48T: 1 vial, 60μL	-20℃, 6 months
Reference Standard & Sample Diluent	1 vial, 20 mL	2-8℃, 6 months
Biotinylated Detection Ab Diluent	1 vial, 13 mL	
HRP Conjugate Diluent	1 vial, 13mL	
Concentrated Wash Buffer (25×)	1 vial, 30 mL	

Substrate Reagent	1 vial, 10 mL	2-8°C (Protect from light)
Stop Solution	1 vial, 10 mL	2-8°C, 6 months
Plate Sealer	5 pieces	RT
Product Description	1 copy	
Certificate of Analysis	1 copy	

Materials & Equipment Required But Not Provided

Microplate reader with 450nm wavelength filter
Incubator capable of maintaining 37°C
Single or multi-channel pipettes with high precision
disposable pipette tips
EP tubes
Container for Wash Solution
Squirt bottle, manifold dispenser, or automated microplate washer
Deionized or distilled water
Absorbent paper for blotting the microplate

Sample collection

Serum: Allow blood samples to clot for 2 hours at room temperature or overnight at 2-8°C before centrifugation for 15 min at 2000×g at 2-8°C. Supernatants should be taken for assay testing immediately or stored at -20°C or -80°C for later use.
Plasma: Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 min at 2000×g at 2-8°C within 30 min of collection. Supernatants

should be taken for assay testing immediately or stored at -20℃ or -80℃ for later use.

Tissue homogenates: Tissues should be rinsed in ice-cold PBS to remove excess blood thoroughly, weighed, minced into small pieces. then Tissue pieces omogenized in PBS (tissue weight (g): PBS (mL) volume=1:9) with a glass homogenizer on ice. The resulting suspension is sonicated with an ultrasonic cell disrupter till the solution is clarified. The homogenates are then centrifuged for 5 min at 10000×g. Supernatants should be taken for assay testing immediately or stored at -20℃ or -80℃ for later use.

Cell lysates: For adherent cells, gently wash the cells with moderate amount of cool PBS and detach the cells with trypsin. Collect cells by centrifugation for 5 min at 1500×g(suspension cells can be collected by centrifugation directly). Discard the supernate and wash cells 3 times with cool PBS. Resuspend cells in cool PBS with concentration of 5×10^6 cells/mL. Repeat the freeze-thaw process several times until the cells are fully lysed. Centrifuge for 15min at 2000×g at 2-8℃. Supernatants should be taken for assay testing immediately or stored at -20℃ or -80℃ for later use.

Cell culture supernatant and other biological fluids: Centrifuge samples for 15 min at 1500× g at 2-8℃. Supernatants should be taken for assay testing immediately or stored at -20℃ or -80℃ for later use.

Note for sample

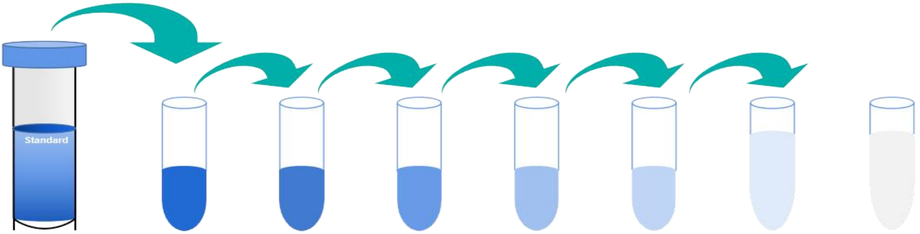
1. Samples should be used within 6 days when stored at 2-8℃, otherwise samples must be stored at -20℃ (≤1month) or -80℃ (≤2months). Avoid repeated freeze-thaw cycles.
2. Please predict the concentration before assaying. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
3. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
4. If a lysis buffer is used to prepare tissue homogenates or cell culture supernatant, there is a possibility to cause a deviation due to the introduced chemical substance.

5. Some recombinant protein may not be detected due to a mismatching with the coated antibody or detection antibody.
6. Please do not use hemolyzed samples for ELISA as it will affect the test results.
7. Fresh samples without long time storage is recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong result.

Reagent Preparation

1. Allow all reagents to return to room temperature (18-25℃) before use.
2. **Wash Buffer:** Dilute 20 mL of Concentrated Wash Buffer with 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.
3. **Standard working solution:** First, centrifuge the standard at 1000×g for 1 min, add 1mL of standard sample dilution, let it stand for 10min and then mix gently, that is 20ng/mL of standard working solution.

Second, take 7 EP tubes, add 500μL of Standard & Sample Diluent to each tube. Pipette 500μL of the 20ng/mL working solution to the first tube and mix up to produce a 10ng/mL working solution. Pipette 500μL of the solution from the former tube into the latter one according to the picture shown below. Mix each tube thoroughly before the next transfer. Set up 7 points of diluted standard such as 20ng/mL,10ng/mL,5ng/mL,2.5ng/mL,1.25ng/mL,0.63ng/mL,0.32ng/mL and the last EP tubes with Standard & Sample Diluent is the blank as 0ng/mL.



20	10	5	2.5	1.25	0.63	0.32	0
ng/mL	ng/mL	ng/mL	ng/mL	ng/mL	ng/mL	ng/mL	ng/mL

4. **Biotinylated Detection Ab working solution**: Calculate the required amount before the experiment (100 μL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the stock tube before use, dilute the 100× Concentrated Biotinylated Detection Ab to 1×working solution with Biotinylated Detection Ab Diluent.
5. **HRP Conjugate working solution**: Calculate the required amount before the experiment (100 μL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the stock tube before use, dilute the 100× Concentrated HRP Conjugate to 1× working solution with HRP Conjugate Diluent.

Assay Protocol

1. The Micro ELISA Plate slats to be used were removed from the plate frame and the remaining slats were returned to the aluminum foil bag containing the desiccants and then resealed for storage.
2. Determine wells for diluted standard, blank and sample. Add 100μL each dilution of standard, blank and sample into the appropriate wells (It is recommended that all samples and standards be assayed in duplicate). Cover the plate with the sealer provided in the kit. Incubate for 90 min at 37℃. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.
3. Aspirate and wash plate for 3 times. Immediately add 100μL of Biotinylated Detection Ab working solution to each well. Cover the plate with a new sealer. Incubate for 1 hour at 37°C.
4. Decant the solution from each well add 300μL of wash buffer to each well. Soak for 0.5 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. Note: a microplate washer can be used in this step and other wash steps. Make the tested strips in use immediately after the wash step. Do not allow wells to be dry.
5. Add 100μL of HRP Conjugate working solution to each well. Cover the plate with a new sealer. Incubate for 30 min at 37℃.
6. Decant the solution from each well, repeat the wash process for 3 times as conducted in step 4.
7. Add 100μL of Substrate Reagent to each well. Cover the plate with a new sealer. Incubate for about 15 min at 37°C. Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30 min. Preheat the Microplate Reader for about 15 min before OD measurement.
8. Add 50μL of Stop Solution to each well. Note: adding the stop solution should be done in the same order as the substrate solution.
9. Determine the optical density (OD value) of each well at once with a micro plate reader set to 450 nm.

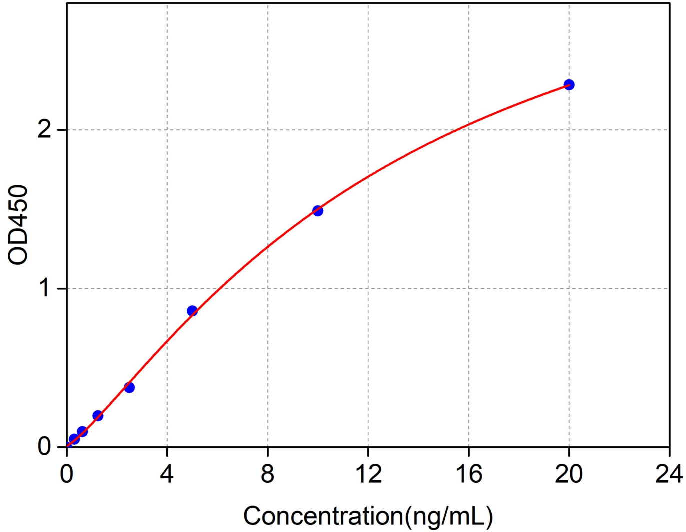
Example Data

The OD values of the standard curve may vary according to the conditions of the actual assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), so plotting log of the data to establish a standard curve for each test is strongly recommended. Typical standard curve is provided below for reference only.

Concentration (ng/mL)	OD450nm		Mean OD	Corrected OD
	1	2		
20	2.313	2.315	2.314	2.284
10	1.519	1.529	1.524	1.49
5	0.888	0.89	0.889	0.859
2.5	0.405	0.408	0.407	0.376
1.25	0.226	0.228	0.227	0.197
0.63	0.126	0.126	0.126	0.097
0.32	0.079	0.079	0.079	0.05
0	0.029	0.029	0.029	0

Calculation of Results

Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density. Create a standard curve with standard concentration on the x-axis and OD values on the y-axis. Draw a best fit curve through the points and it can be determined by regression analysis. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor



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Sensitivity

The minimum detectable dose of Rat NT-proANP is typically less than 0.19 ng/mL.

The sensitivity of this assay, or Limit of Detection (LOD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined by adding three standard deviations to the mean OD value of twenty zero standard replicates and calculating the corresponding concentration.

Specificity

This assay has high sensitivity and excellent specificity for detection of Rat NT-proANP. No significant cross-reactivity or interference between Rat NT-proANP and analogues was observed. Limited by current skills and knowledge, it is impossible for us to complete the cross- reactivity detection between Rat NT-proANP and all the analogues, therefore, cross reaction may still exist.

Precision

Mean coefficient of variation for Intra-Assay and Inter-Assay: 3 samples with low, middle and high level concentration were tested for repeat multiple times, respectively.

Item	Intra-Assay	Inter-Assay
Sample number	3	3
Replicate	9	18
CV(%)	5	8

Recovery

Three matrices listed below were spiked with certain level of Rat NT-proANP, The recovery rates of Rat NT-proANP were calculated by comparing the measured value to the expected amount of Rat NT-proANP in samples.

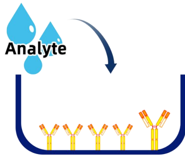
Matrix type	Recovery Range (%)	Average (%)
Serum (n=5)	82-94	88
EDTA plasma (n=5)	85-97	91
Cell culture media (n=5)	83-95	89

Linearity

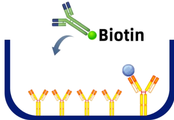
Three types of Sample were spiked with appropriate concentrations of Rat NT-proANP and diluted into a series of concentration gradients, then the linearity of the assay was demonstrated by the percentage of comparing calculated concentrations and expected values.

Dilution Factor	Recovery Range (%)		
	Serum (n=5)	EDTA plasma (n=5)	Cell culture media (n=5)
1:2	95-104	80-92	82-93
1:4	82-93	86-102	96-105
1:8	81-96	87-103	87-98
1:16	83-94	85-101	85-97

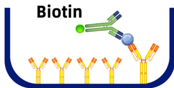
Summary for Procedure



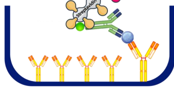
1、Add 100μL standard or sample to the wells. Incubate for 90 min at 37°C, Aspirate and wash plate for 3 times



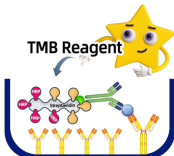
2、Immediately add 100μL Biotinylated Detecting Ab working solution to each well.Incubate for 60 min at 37°C



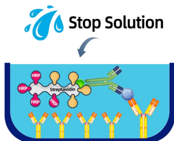
3、Aspirate and wash the plate for 3times



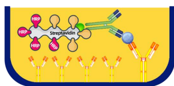
4、Add 100μL HRP conjugate working solution.incubate for 30 min at 37°C



5、Aspire and wash the plate for 3times. Add 100uL Substrate Reagent. Incubate for 15 min at 37°C



6、Add 50μL Stop Solution



7、Read at 450nm immediately and calculate of results

Troubleshooting

Problem	Possible Causes	Subsequent Actions
Poor standard curve	Improper standard dilution	Ensure that standards are dissolved and diluted in the recommended manner
	Inaccurate pipetting	Periodically calibrate pipettes and check the pipette tips
	Evaporate the reaction solution	Seal the enzyme plate with plate sealer
	Incomplete plate washing	Adequate washing times and the amount of washing solution added
	Foreign matter in the bottom of wells	Clean the bottom of the plate before reading
Weak or no color development	Insufficient reaction of reagents	Ensure incubation time and incubate at the recommended temperature
	Inadequate reagent volumes	Check the pipette and Follow the steps strictly to operate
	Improper dilution	Check the reagent dilution process
	Inactivation of enzyme conjugate	Mix conjugate and substrate, check by color development
Low OD value	Incorrect setting of microplate reader	Check the wavelength of reader
	No stop solution added	Add appropriate amount of stop solution
	Waiting too long time to read	Read the plate in time
High background	Contaminated chromogenic solution	Replace chromogenic solution
	Coloring time is too long	Control the coloring time
	Wrong dilution of reaction reagent	Use the recommended dilution
	Inadequate washing of the plate	Adequate washing times and the amount of washing solution added