Quantikine™ ELISA

Human NT-ProANP Immunoassay

Catalog Number DANP00

For the quantitative determination of human N-Terminal ProAtrial Natriuretic Peptide (NT-ProANP) concentrations in cell culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Atrial Natriuretic Peptide (ANP), also known as gamma-ANP, CDP/cardiodilatin-related peptide, and CDD-ANF, is a secreted member of the natriuretic peptide family of molecules (1, 2). It is synthesized as a preproprecursor that contains a signal sequence, cardiodilatin-related peptide, a propeptide, and the C-terminal atrial natriuretic factor (ANF) (3, 4). ANP is expressed in multiple cell types including atrial myocardiocytes, macrophages, and select hypothalamic neurons (2). It is stored intracellularly in this form and is proteolytically processed to active ANF by the transmembrane enzyme Corin (5-7). Following its release, ANF can be inactivated by Neprilysin/CD10-mediated cleavage at various sites (8, 9). Elevated circulating levels of ANF are associated with heart failure resulting from increased mechanical stress in the heart during hypertension. The propeptide, known as NT-ProANP, circulates at reduced levels during the early stages of hypertension (10) but is elevated during chronic heart failure (11). Multiple polymorphisms in ANP are associated with variability in blood pressure regulation and hypertension (12). An alternative isoform of ANF associated with early onset atrial fibrillation is resistant to proteolytic degradation and circulates at elevated levels (13, 14). Human NT-ProANP shares 83% amino acid sequence identity with mouse and rat NT-ProANP, respectively.

Bioactive ANF regulates blood pressure by promoting sodium and water excretion. It also exerts multiple endocrine and metabolic effects including inhibition of aldosterone, vasopressin, ACTH, CRH, and cortisol secretion as well as the enhancement of lipolysis and the release of testosterone, LH, and insulin (1, 15, 16). ANF is upregulated in activated macrophages where it promotes microbial clearance and dampens local inflammatory reactions (17). ANF binds to the receptors NPR-A and NPR-C, although it signals primarily through NPR-A (14, 18).

The Quantikine™ Human NT-ProANP Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human NT-ProANP in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant human NT-ProANP and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human NT-ProANP showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for naturally occurring human NT-ProANP.

PRINCIPLE OF THE ASSAY

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

LIMITATIONS OF THE PROCEDURE

- 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.
- If samples generate values higher than the highest standard, further dilute the samples with calibrator diluent and repeat the assay.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine™ Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human NT-ProANP Microplate	898396	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human NT-ProANP.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human NT-ProANP Standard	898398	2 vials of recombinant human NT-ProANP in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Use a new standard for each assay. Discard after use.
Human NT-ProANP Conjugate	898397	21 mL of a monoclonal antibody specific for human NT-ProANP conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1W	895117	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-3	895436	21 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm
- Pipettes and pipette tips
- Deionized or distilled water
- Squirt bottle, manifold dispenser, or automated microplate washer
- 500 mL graduated cylinder
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- Test tubes for dilution of standards and samples
- Human NT-ProANP Controls (optional; R&D Systems®, Catalog # QC227)

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*
Grossly hemolyzed samples are not suitable for use in this assay.

SAMPLE PREPARATION

Serum and plasma samples require at least a 2-fold dilution due to high endogenous levels. A suggested 2-fold dilution is 100 µL of sample + 100 µL of Calibrator Diluent RD5-3.

REAGENT PREPARATION

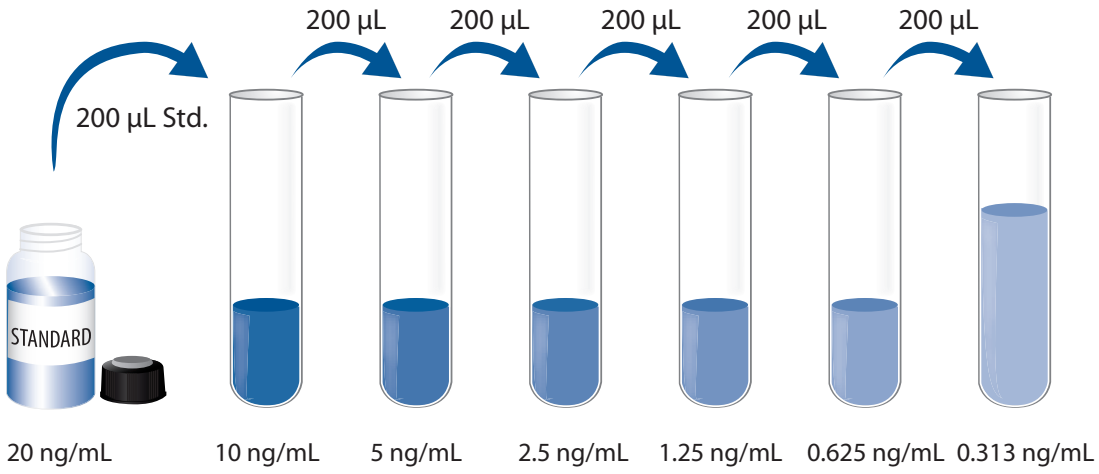
The Human NT-ProANP Conjugate must remain at 2-8 °C during use. Bring all other reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Human NT-ProANP Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human NT-ProANP Standard with Calibrator Diluent RD5-3. This reconstitution produces a stock solution of 20 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. **Note:** *Do not use on a rocker.*

Pipette 200 µL of Calibrator Diluent RD5-3 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Human NT-ProANP Standard (20 ng/mL) serves as the high standard. Calibrator Diluent RD5-3 serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

The Human NT-ProANP Conjugate must remain at 2-8 °C during use. Bring all other reagents and samples to room temperature before use. It is recommended that all standards and samples be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL of Assay Diluent RD1W to each well.
4. Add 50 µL of standard, control, or sample* per well. Cover with the adhesive strip provided. **Incubate for 2 hours at room temperature** on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
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 (400 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 200 µL of **cold** Human NT-ProANP Conjugate to each well. Cover with a new adhesive strip. **Incubate for 2 hours at 2-8 °C on the benchtop.**
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 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 the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See the Sample Preparation section.

CALCULATION OF RESULTS

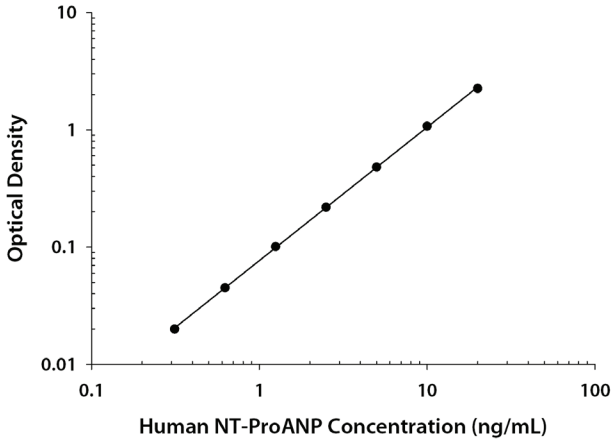
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 a log/log graph. The data may be linearized by plotting the log of the human NT-ProANP concentrations versus the log of the O.D. on a linear scale, and the best fit line 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.

TYPICAL DATA

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



(ng/mL)	O.D.	Average	Corrected
0	0.017 0.017	0.017	—
0.313	0.036 0.038	0.037	0.020
0.625	0.062 0.062	0.062	0.045
1.25	0.118 0.118	0.118	0.101
2.5	0.235 0.237	0.236	0.219
5	0.494 0.501	0.498	0.481
10	1.068 1.117	1.093	1.076
20	2.257 2.281	2.269	2.252

PRECISION

Intra-Assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (ng/mL)	1.71	6.04	11.1	1.81	5.95	11.5
Standard deviation	0.069	0.224	0.583	0.129	0.426	0.842
CV (%)	4.0	3.7	5.3	7.1	7.2	7.3

RECOVERY

The recovery of human NT-ProANP spiked to levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	104	87-114%
Serum* (n=4)	108	95-122%
EDTA plasma* (n=4)	102	84-120%
Heparin plasma* (n=4)	98	84-111%

*Samples were diluted prior to assay as directed in the Sample Preparation section.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human NT-ProANP were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)
1:2	Average % of Expected	93	99	100	96
	Range (%)	89-98	91-109	93-109	89-104
1:4	Average % of Expected	86	98	100	96
	Range (%)	80-90	92-113	89-114	91-100
1:8	Average % of Expected	89	93	95	94
	Range (%)	85-91	82-112	81-117	87-110
1:16	Average % of Expected	91	92	94	92
	Range (%)	85-98	79-117	81-116	83-112

*Samples were diluted prior to assay as directed in the Sample Preparation section.

SENSITIVITY

Twenty-seven assays were evaluated and the minimum detectable dose (MDD) of human NT-ProANP ranged from 0.013-0.146 ng/mL. The mean MDD was 0.037 ng/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human NT-ProANP manufactured at R&D Systems®.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human NT-ProANP in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=30)	9.75	0.964-38.3	7.10
EDTA plasma (n=30)	10.2	1.07-42.1	7.87
Heparin plasma (n=30)	10.1	1.13-35.9	6.78

Cell Culture Supernates - Human cardiomyocytes were obtained from iPSC, differentiated using StemXVivo™ Cardiomyocyte Differentiation Kit (R&D Systems, Catalog # SC032) and maintained in the Cardiomyocyte Maintenance Media Supplement (R&D Systems, Catalog # AR011). Cells were cultured unstimulated or stimulated with 5 nM Endothelin-1 (Tocris™, Catalog # 1160) for 1 day. Aliquots of the cell culture supernates were removed and assayed for human NT-ProANP.

Condition	(ng/mL)
Unstimulated	251
Stimulated	442

SPECIFICITY

This assay recognizes natural and recombinant human NT-ProANP, Pro-ANP (aa 26-151) and NT-ANP (aa 26-123).

The factors listed below were prepared at 200 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 200 ng/mL in a mid-range recombinant human NT-ProANP control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

BNP
CNP
Corin
Neprilysin (aa 45-743)
Neprilysin (aa 52-750)
NPR-1
NPR-2

Peptide:

human/porcine mature ANF

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