Development and Validation of Rat Natriuretic Peptide Biomarkers

Heart failure (HF) is a clinical syndrome associated with progressive cardiac, vascular, and renal dysfunction that affects more than 23 million people annually worldwide. For cardiac injury that precedes HF, natriuretic peptides have been identified as potential biomarkers for their role in vasodilation, anti-inflammation and natriuresis. In particular, it has been demonstrated that BNP and NT-proBNP levels can facilitate diagnosis and guide HF therapy. BNP and NT-proBNP were recently shown to be useful cardiac injury markers for risk assessment in non-Hodgkin lymphoma patients treated with chemotherapy.

MESO SCALE DISCOVERY (MSD) developed and characterized three single-plex immunoassays for rat natriuretic peptide biomarkers (BNP, NT-proBNP, and NT-proANP) following fit-for-purpose principles,1 FDA Bioanalytical Method Validation and CLSI guidance. These assays were validated for sensitivity, specificity, dilution linearity, spike recovery, precision, accuracy and robustness. The data indicates that these assays have high sensitivity and a wide dynamic range (both endogenous and elevated levels can be measured at a single dilution factor) and provide greater throughput than ELISA and bead-based assays.

Description of Markers

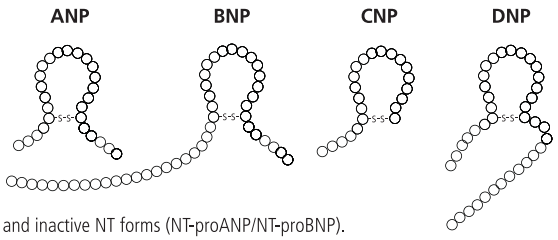
- Natriuretic peptides play a key role in antagonizing the actions of the renin-angiotensin-aldosterone system, thus promoting vasodilatation and natriuresis. Natriuretic peptides include 4 family members that share a common 17-amino acid ring structure.

  ANP: Atrial natriuretic peptide, 28 amino acids

  BNP: Brain natriuretic peptide, 45 amino acids

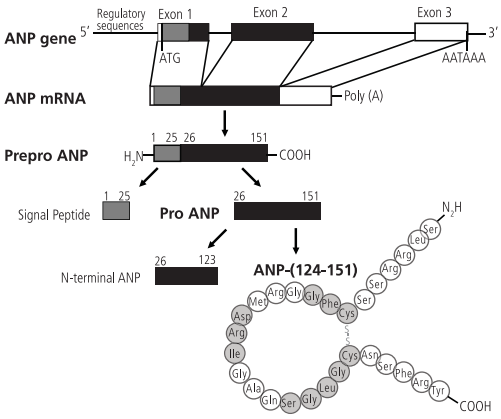
  CNP: C-type natriuretic pepide, 22 amino acids

  DNP: Dendroaspis natriuretic peptide

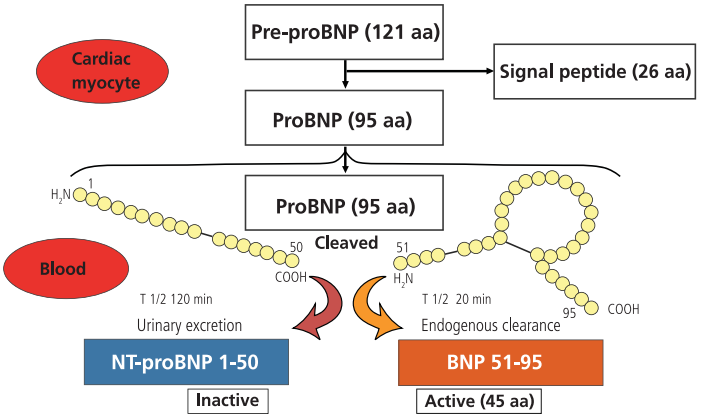


- Natriuretic peptides are produced as prohormones and cleaved to active (ANP/BNP) and inactive NT forms (NT-proANP/NT-proBNP).

ANP Transcription and Translation



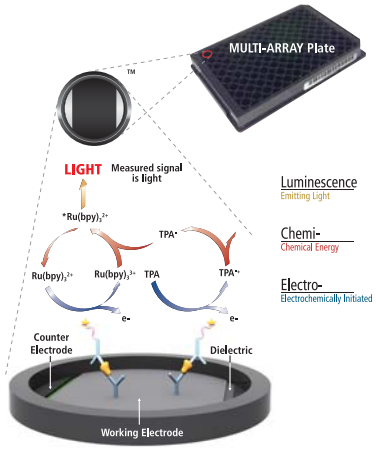
BNP Transcription and Translation



- Elevated levels of ANP/BNP have been associated with heart failure, systemic and pulmonary hypertension, hypertrophic and restrictive cardiomyopathy, pulmonary embolism, COPD, cor pulmonale, AMI cirrhosis and renal failure.

The MSD® Platform

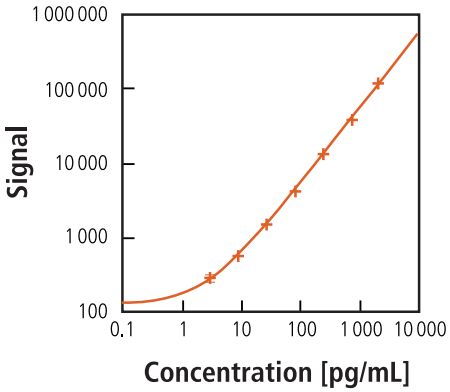
MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels which emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.



Electrochemiluminescence Features

- Minimal non-specific backgrounds and strong signal responses to analyte yield high signal to background ratios
- The stimulation mechanism (electricity) is decoupled from the response (light signal)
- Proximity assay - only labels bound near the electrode surface are excited, enabling non-washed assays
- Flexibility - labels are stable, non-radioactive, and directly conjugated to biological molecules
- Emission at ~620 nm - eliminating problems with color quenching
- Signal amplification - multiple rounds of excitation and emission of each label enhance light levels and improve sensitivity
- Carbon electrode surface has 10X greater binding capacity than polystyrene well
- Surface coatings can be customized

Standard Curve



BNP		
Concentration (pg/mL)	Average Signal	% CV
0	106	6.4
2.93	290	10.6
8.79	618	8.1
26.4	1606	5.3
79.1	4581	7.9
237	13 848	3.7
712	41 458	5.9
2136	126 004	4.8

Protocol

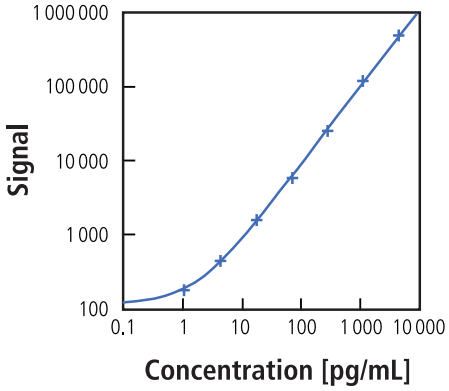
1 Add 150 µL blocking solution. Incubate for 1 hour at room temperature (RT).

2 Wash with PBS-T. Add 25 µL of capture antibody. Incubate for 1 hour at RT.

3 Wash with PBS-T. Add 50 µL of standard or diluted sample (MSD recommends a 1:2 dilution). Incubate for 2 hours at RT.

4 Wash with PBS-T. Add 25 µL of detection antibody. Incubate for 2 hours at RT.

5 Wash with PBS-T. Add 150 µL of Read Buffer T. Read on MSD SECTOR® Imager.



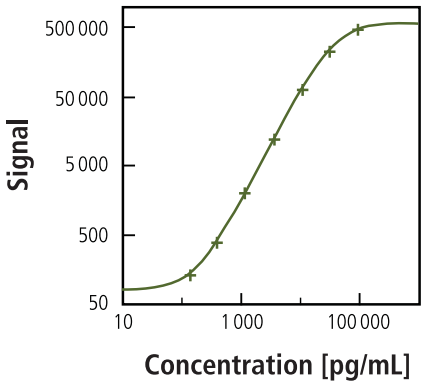
NT-proBNP		
Concentration (pg/mL)	Average Signal	% CV
0	97	6.0
1.09	193	8.0
4.34	462	5.2
17.4	1595	6.3
69.5	5889	4.4
278	26 446	6.0
1111	119 498	3.0
4445	492 745	2.8

Protocol

1 Wash the plate with PBS-T. Add 50 µL of standard or diluted sample (MSD recommends a 1:2 dilution). Incubate for 1 hour at RT.

2 Wash with PBS-T. Add 25 µL of detection antibody. Incubate for 2 hours at RT.

3 Wash with PBS-T. Add 150 µL of Read Buffer T. Read on MSD SECTOR Imager.



NT-proANP		
Concentration (pg/mL)	Average Signal	% CV
0	81	4.5
132	141	8.3
396	417	7.9
1188	2156	9.7
3563	12 568	7.2
10 689	68 143	7.6
32 067	238 190	5.3
96 200	491 473	5.6

Protocol

1 Add 25 µL MSD Diluent 30. Incubate for 30 min at RT.

2 Wash with PBS-T. Add 50 µL of standard or diluted sample (MSD recommends a 1:4 dilution). Incubate for 2 hours at RT.

3 Wash with PBS-T. Add 25 µL of detection antibody. Incubate for 1 hour at RT.

4 Wash with PBS-T. Add 150 µL of Read Buffer T. Read on MSD SECTOR Imager.

Assay Sensitivity

The lower limit of detection (LLOD) is a calculated concentration based on a signal 2.5 standard deviations above the average of multiple blanks (zero calibrator).

Multi-plate, multi-day runs (N=6) were conducted to establish the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) with acceptable precision (≤20%).

	BNP	NT-proBNP	NT-proANP
LLOD (pg/mL)	1.47	0.820	99.9
Proposed LLOQ (pg/mL)	10.0	5.00	200
Proposed ULOQ (pg/mL)	800	4000	50 000

Specificity

In order to assess assay specificity, NT-proANP, BNP and NT-proBNP assays were run with single BNP and NT-proBNP calibrators and single detection antibodies. The table to the right shows the % cross-reactivity for each individual detection antibody.

	% Cross-Reactivity	
Assay	BNP calibrator	NT-proBNP calibrator
BNP	100	<0.1
NT-proBNP	<0.1	100
NT-proANP	<0.1	<0.1

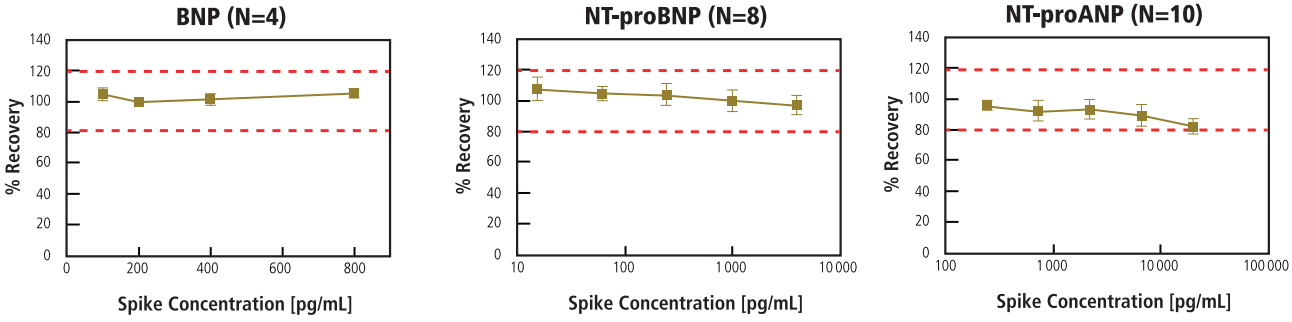
Precision: Multi-Day Study

The controls were prepared with or without spiking calibrator into matrices (serum or plasma) and run in triplicate or quadruplicate on 9 plates across multiple days (n≥3). Average Intra-plate % CV is the average percent CV of the control replicates on an individual plate. Inter-plate %CV is the variability of controls across 9 plates over 3 days.

	Control	Plates	Average Conc. (pg/mL)	Average Intra-plate % CV	Inter-plate % CV
BNP	High	9	425	3.6	7.6
	Mid	9	75.9	2.7	7
	Low	9	24.0	4	9
NT-proBNP	High	9	2268	4.6	7.4
	Mid	9	243	3.7	6.6
	Low	9	18.4	4.2	14.8
NT-proANP	High	9	77 700	15.3	3.4
	Mid	9	9770	4.7	2.8
	Low	9	855	6.2	2.7

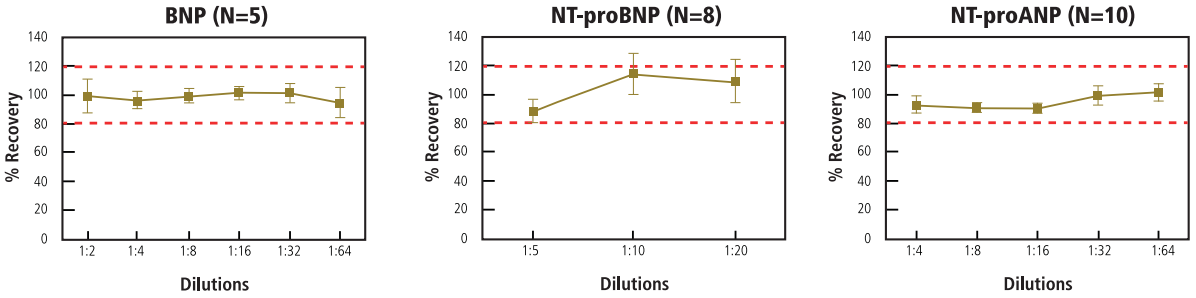
Spike Recovery

Serum, EDTA and heparin plasma samples were spiked with calibrator at multiple values throughout the range of the assay. Results of the spike-recovery may vary based on the individual samples. The data below shows spike recovery for all three assays was 80-120%. Error bars are standard deviation calculated from these samples.



Dilution Linearity

Rat serum, EDTA and heparin plasma were evaluated for dilution linearity; data are shown below. Percent recovery was calculated as the measured concentration times the dilution factor divided by the concentration of the previous dilution (expected).
Average recovery was calculated based the samples tested for each assay. The data below shows the recovery of dilution linearity for each assay was 80-120%. Error bars are standard deviation calculated from these samples.



Samples

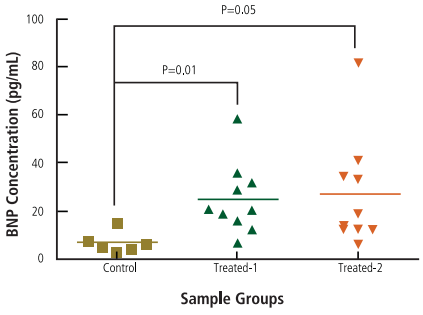
Normal rat samples (serum, EDTA and heparin plasma) were assayed for NT-proBNP, BNP and NT-proANP.

A normal sample range was established based on the data below. Results showed that our assays could detect the majority of normal samples.

Pre-clinical study showed the level of BNP was elevated by different compound treatments (treated-1 and treated-2 groups).

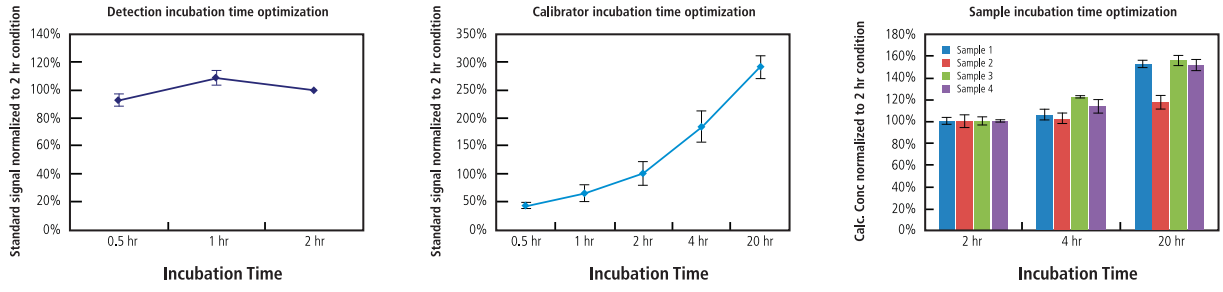
		BNP	NT-proBNP	NT-proANP
Serum	Median (pg/mL)	2.3	4.5	15121
	Range (pg/mL)	2.0-2.5	<LLOD-9.8	3891-21 332
	# of samples	4	8	15
	# of samples above LLOD	4	7	15
EDTA Plasma	Median (pg/mL)	9.0	51.7	15720
	Range (pg/mL)	4.4-65.3	30.8-130	10 118-39 046
	# of samples	11	8	27
	# of samples above LLOD	11	8	27
Heparin Plasma*	Median (pg/mL)	N/A	44.5	-
	Range (pg/mL)	3.7-10.2	19.0-132	-
	# of samples	2	8	-
	# of samples above LLOD	2	8	-

*Heparin plasma was not tested for NT-proANP



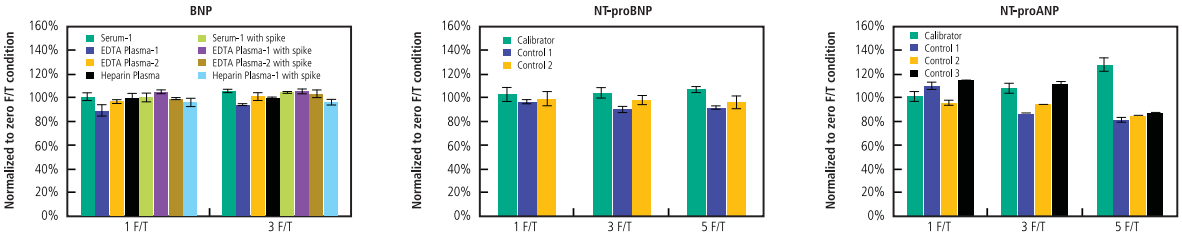
Robustness

Protocol Time for Rat NT-proANP



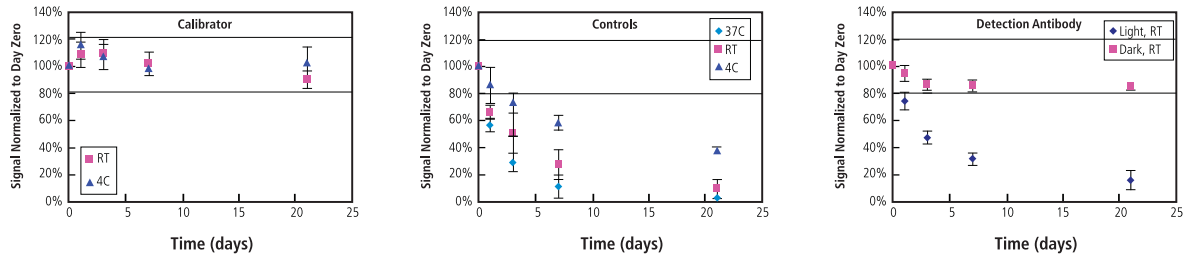
Calibrator/sample and detection antibody incubation time was optimized for all three assays. Rat NT-proANP data is shown in this set of graphs (BNP and NT-proBNP data not shown).
Standard signal was elevated with the increase of sample incubation time, but calculated sample concentration didn't change significantly between 2 and 4 hours incubation time, thus the 2 hour sample incubation time was selected. The data above shows 1 hour detection antibody incubation was optimal.

Calibrator and Control Freeze/Thaw Stability



Calibrator and control or samples were tested with multiple freeze/thaw cycles for all panels. The data showed calibrator and controls can go through up to five freeze-thaw cycles without affecting the performance of the assay.

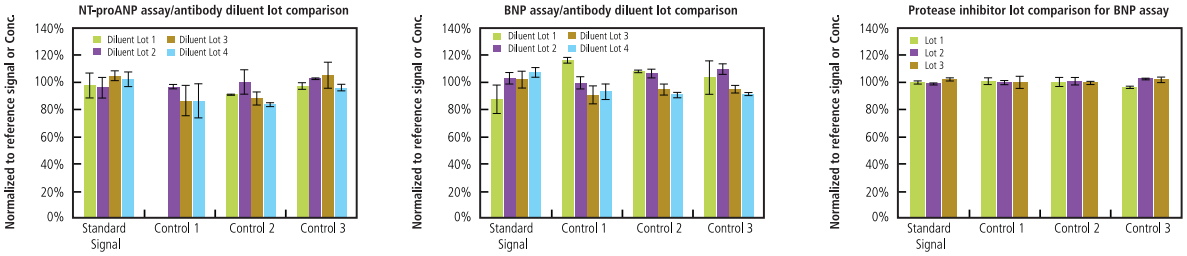
Accelerated Stability for Rat NT-proANP



Calibrator, detection antibody and controls were stored under different conditions (4°C, RT and 37°C). Data from rat NT-proANP assay showed that the detection antibody is sensitive to light and controls are not stable under these treatments. BNP and NT-proBNP have similar data for calibrator and detection antibodies, but controls are stable under these treatments (data not shown).

Typically MSD recommends storing calibrators and controls at ≤-70°C and detection antibodies at 4°C in the dark.

Reagent Lot Comparison



Multiple lots of reagent for diluent or critical assay components (i.e., protease inhibitor) were tested during the validation. The data show consistency across the reagent lots.

Conclusions

- MSD's ultra sensitive rat natriuretic peptide assays can measure NT-proANP, BNP and NT-proBNP in normal and treated samples.
- These assays are validated for accuracy, precision, sensitivity, reproducibility and stability.
- Data presented here is highly precise (average intra-plate %CV <5 and average inter-plate %CV <10) and accurate (spike recovery bias <15%).
- Preliminary data shows the level of BNP was significantly elevated with drug treatment.

[1] Lee JW, Devanarayan V, Barrett YC, Weiner R, Allinson J, Fountain S, Keller S, Weinryb I, Green M, Duan L, Rogers JA, Millham R, O'Brien PJ, Sailstad J, Khan M, Ray C, Wagner JA. Fit-for-purpose method development and validation for successful biomarker measurement. Pharm Res. 2006, 23(2):312-28.