Development of Rat Natriuretic Peptide Biomarker Assays

Heart failure (HF) is a clinical syndrome associated with progressive cardiac, vascular, and renal dysfunction that affects more than 23 million people annually worldwide. Despite medical advances, an aging population and new therapies that prolong the lives of diagnosed patients continue to increase the prevalence of HF. Early identification of susceptible persons may save lives and ultimately reduce the overall prevalence of the disease.

The measurement of protein biomarkers presents possible solutions for screening, diagnosis, prognosis, and therapeutic management of the disease with downstream benefits of improved clinical decision making and more effective patient care. Natriuretic peptides have been identified as potential biomarkers for cardiac injury that precedes heart failure 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. Additionally, 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) has developed and characterized immuno-assays for 3 rat natriuretic peptide biomarkers (BNP, NT-proBNP, and NT-proANP). These assays offer high sensitivity, reduced sample volume, and wide dynamic range. Collectively, these advantages enable endogenous and elevated levels to be measured at a single dilution factor and provide improved assay throughput (over ELISA and bead-based assays).

Description of Markers

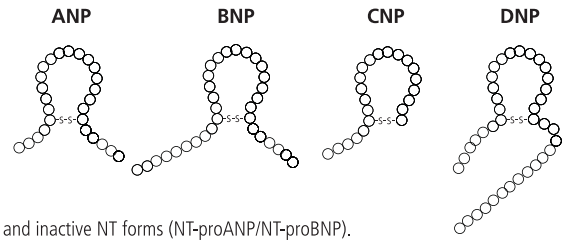
- Natriuretic peptides play a key role in antagonizing the actions of the renin-angiotensin-aldosterone system, thus promoting vasodilatation and natriuresis. Natriuretic peptides include 4 family members that share a common 17-amino acid ring structure.

  ANP: Atrial natriuretic peptide, 28 amino acids

  BNP: Brain natriuretic peptide, 32 amino acids

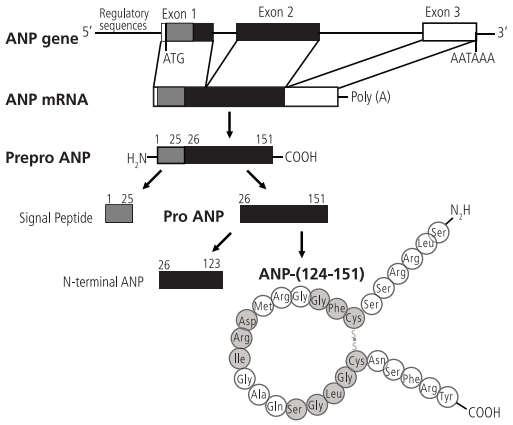
  CNP: C-type natriuretic pepide, 22 amino acids

  DNP: Dendroaspis natriuretic peptide

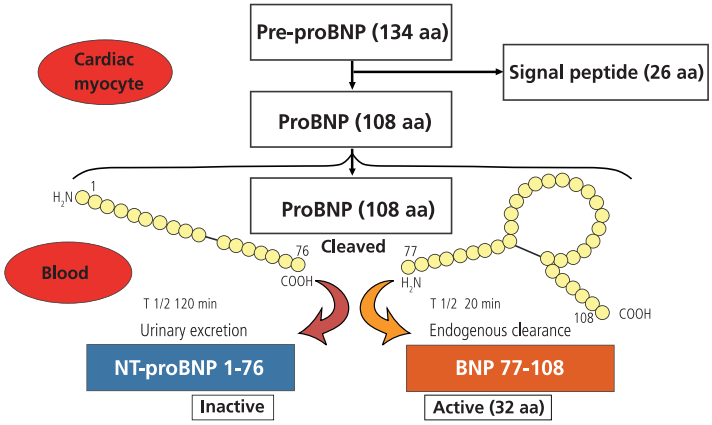


- Natriuretic peptides are produced as prohormones and cleaved to active (ANP/BNP) and inactive NT forms (NT-proANP/NT-proBNP).

ANP Transcription and Translation



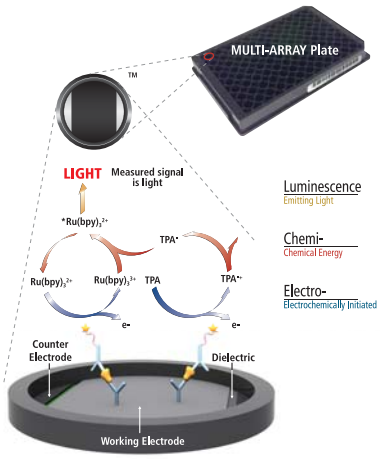
BNP Transcription and Translation



- 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

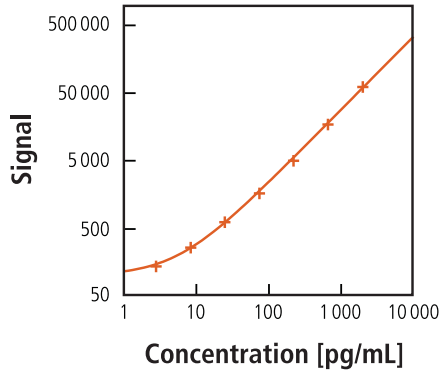
MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.



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	79	5.2
2.74	138	4.3
8.23	271	3.8
24.7	620	3.3
74.1	1692	4.5
222	5187	4.6
667	17085	4.3
2000	63192	4.1

Protocol:

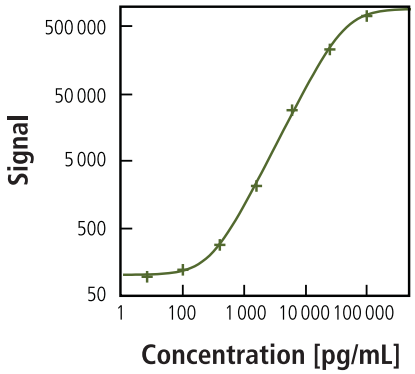
1 Add 150 µL blocking solution. Incubate for 1 hour at RT.

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

3 Wash with PBS-T. Add 25 µL of standard or diluted sample. 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 and then read on a SECTOR® Imager.



NT-proANP		
Concentration (pg/mL)	Average Signal	% CV
0	89	8.2
24.4	102	12.4
97.7	131	6.6
391	300	4.5
1563	2321	5.8
6250	31406	3.0
25000	249481	6.0
100000	811537	5.3

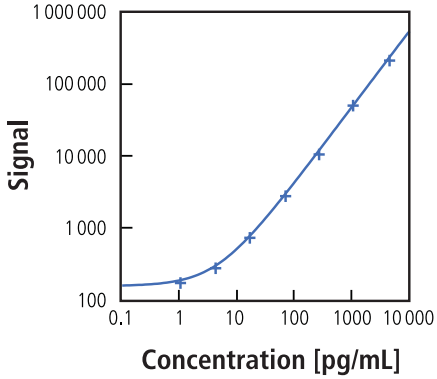
Protocol:

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

2 Wash with PBS-T. Add 25 µL of standard or sample. Incubate for 2 hours at RT.

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

4 Wash with PBS-T. Add 150 µL of Read Buffer T and then read on a SECTOR® Imager.



NT-proBNP		
Concentration (pg/mL)	Average Signal	% CV
0	146	7.7
1.10	184	4.8
4.30	315	3.1
17.4	782	5.3
69.5	2708	5.2
278	11300	5.1
1111	51081	4.0
4445	223091	3.9

Protocol:

1 Wash the plate with PBS-T. Add 25 µL assay diluent followed by 25 µL standard or sample. Incubate for1 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 and then read on a SECTOR® Imager.

Assay Sensitivity

A multi-plate, multi-day study was performed to measure the reproducibility of the assay. The lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) were proposed from the multiple plate run and determined as the standard point where the % CV of the calculated concentration was less than 20% and the percent recovery of the standard was between 80% and 120%.

The lower limit of detection (LLOD) is the calculated concentration of the signal that is 2.5 standard deviations over the blank (zero calibrator).

	BNP	NT-proANP	NT-proBNP
LLOD (pg/mL)	1.47	142	0.817
Proposed LLOQ (pg/mL)	10.0	400	5.0
Proposed ULOQ (pg/mL)	800	100000	4000

Precision: Multi-Day Study

The controls were made by spiking calibrators into rat EDTA plasma and tested in triplicate or quadruplicate on each of 9 plates across multiple days (n>3). Average Intra-plate % CV is the average % CV of the control replicates within 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	7.0
	Low	9	24.0	4.0	9.0
NT-proBNP	High	9	2268	4.6	7.4
	Mid	9	243	3.7	6.6
	Low	9	18.4	4.2	15

Spike Recovery

Rat EDTA and heparin plasma samples were spiked with calibrator at multiple values throughout the range of the assay. Results of spike recovery may vary based on the individual samples.
% Recovery = measured / expected x 100

	BNP			
Samples	Spike Conc. (pg/mL)	Measured Conc. (pg/mL)	Measured Conc. % CV	% Recovery
EDTA Plasma 1	800	811	2.7	100
	400	429	2.2	105
	200	211	1.5	102
	100	119	2.2	110
	0	7.78		
EDTA Plasma 2	800	881	1.9	107
	400	428	11.3	100
	200	231	8.0	102
	100	131	1.4	104
	0	26.4		
EDTA Plasma 3	800	932	1.0	108
	400	478	8.9	103
	200	260	7.1	98
	100	165	5.1	100
	0	64.7		
Heparin Plasma	800	846	0.6	106
	400	387	3.3	97
	200	194	9.1	97
	100	105	10.3	104
	0	1.07		

	NT-proANP			
Samples	Spike Conc. (pg/mL)	Measured Conc. (pg/mL)	Measured Conc. % CV	% Recovery
EDTA Plasma	50000	54774	3.7	99
	12500	19270	5.8	108
	3130	9172	1.2	109
	0	5289		
Heparin Plasma	50000	48806	1.9	87
	12500	18098	0.7	98
	3130	9206	2.0	101
	0	5957		

	NT-proBNP			
Samples	Spike Conc. (pg/mL)	Measured Conc. (pg/mL)	Measured Conc. % CV	% Recovery
EDTA Plasma	4000	4023	1.8	100
	1000	1036	2.6	102
	250	281	1.1	105
	62.5	83.0	2.4	106
	15.6	33.6	1.2	109
	0	15.6		
Heparin Plasma	4000	3777	1.1	94
	1000	991	4.3	98
	250	269	1.5	103
	62.5	77.2	4.8	104
	15.6	30.0	3.3	110
	0	11.9		

Dilution Linearity

Rat EDTA and heparin plasma were tested and data are shown below. The concentrations shown below have been corrected for dilution (concentration = measured x dilution factor). Percent recovery is calculated as the measured concentration divided by the concentration of the previous dilution (expected).
% Recovery = (measured x dilution factor) / expected x 100.

	BNP			
Samples	Fold Dilution	Conc. (pg/mL)	Conc. % CV	% Recovery
EDTA Plasma	Neat	701	10.4	
	2	650	9.1	93
	4	589	5.9	91
	8	575	3.1	98
	16	581	1.3	101
	32	589	2.3	101
	64	< LLOQ	17.2	97
	128	< LLOQ	-	-
Heparin Plasma	Neat	561	6.3	
	2	558	10.6	99
	4	553	8.3	99
	8	542	3.0	98
	16	537	1.6	99
	32	569	5.8	106
	64	552	3.6	97
	128	< LLOQ	-	-

	NT-proANP			
Samples	Fold Dilution	Conc. (pg/mL)	Conc. % CV	% Recovery
EDTA Plasma	Neat	21343	4.7	
	2	23213	0.3	109
	4	24628	0.2	106
	8	25194	0.5	102
	16	25703	2.3	102
Heparin Plasma	Neat	8432	4.5	
	2	9498	6.1	113
	4	10515	2.0	111
	8	12822	2.3	122
	16	16322	4.6	127

	NT-proBNP			
Samples	Fold Dilution	Conc. (pg/mL)	Conc. % CV	% Recovery
EDTA Plasma	2	96.8	5.6	
	5	78	3.6	81
	10	< LLOQ	-	-
	20	< LLOQ	-	-
	40	< LLOQ	-	-
Heparin Plasma	2	137.9	5.7	
	5	108.6	0.9	79
	10	127.4	4.2	117
	20	< LLOQ	-	-
	40	< LLOQ	-	-

Samples

Rat samples were assayed for NT-proBNP, BNP, and NT-proANP. Samples were measured neat for NT-proBNP, but were diluted at 1:2 for BNP and NT-proANP measurement. Concentrations in gray were below proposed LLOQ for the analyte designated.

		BNP			
Samples	Fold Dilution	Average Signal	% CV	Calc. Conc. (pg/mL)	Measured Conc. % CV
Serum-1	2	100	4.2	2.21	22.6
Serum -2	2	103	1.4	2.56	6.5
Serum -3	2	101	3.5	2.27	18.3
Serum -4	2	99	7.1	2.09	39.8
EDTA plasma-1	2	308	10.8	25.4	14.3
EDTA plasma-2	2	283	7.8	22.7	10.6
EDTA plasma-3	2	157	10.8	8.74	21.9
Heparin plasma-1	2	113	0	3.73	0
Heparin plasma-2	2	170	0	10.2	0

		NT-proANP			
Samples	Fold Dilution	Average Signal	% CV	Calc. Conc. (pg/mL)	Measured Conc. % CV
Serum-1	2	17567	7.1	21502	4.4
Serum -2	2	20474	4.3	23678	2.7
Serum -3	2	35699	2.8	34099	1.9
Serum -4	2	14730	2.3	19281	1.4
EDTA plasma-1	2	18305	1.6	22067	1.0
EDTA plasma-2	2	20264	8.8	23518	5.6
EDTA plasma-3	2	40687	1.8	37342	1.3
Heparin plasma-1	2	22853	9.0	25389	5.7
Heparin plasma-2	2	16214	10.8	20449	6.7

		NT-proBNP			
Samples	Fold Dilution	Average Signal	% CV	Calc. Conc. (pg/mL)	Measured Conc. % CV
Serum-1	Neat	387	0.7	6.8	1.1
Serum-2	Neat	199	5.7	1.9	16.6
Serum-3	Neat	144	3.4	0.3	50.3
Serum-4	Neat	168	1.3	1.0	6.0
Serum-5	Neat	340	9.4	5.6	14.8
Serum-6	Neat	383	5.0	6.7	7.3
Serum-7	Neat	505	5.5	9.8	7.1
Serum-8	Neat	256	2.2	3.4	4.4
EDTA plasma-1	Neat	5717	3.5	130	3.4
EDTA plasma-2	Neat	2487	2.5	57.1	2.5
EDTA plasma-3	Neat	1366	2.3	30.8	2.5
EDTA plasma-4	Neat	1897	0.6	43.4	0.6
EDTA plasma-5	Neat	2474	3.3	56.8	3.3
EDTA plasma-6	Neat	1728	15.7	39.4	16.2
EDTA plasma-7	Neat	3,941	14.7	90.2	14.5
EDTA plasma-8	Neat	2,037	7.4	46.6	7.5
Heparin plasma-1	Neat	5816	2.5	132	2.5
Heparin plasma-2	Neat	1970	5.0	45.1	5.1
Heparin plasma-3	Neat	889	2.9	19.3	3.2
Heparin plasma-4	Neat	874	0.2	19.0	0.3
Heparin plasma-5	Neat	2712	8.1	62.3	8.1
Heparin plasma-6	Neat	1679	8.8	38.3	9.2
Heparin plasma-7	Neat	2856	24.6	65.5	24.6
Heparin plasma-8	Neat	1918	2.5	43.9	2.6

Specificity

In order to assess assay specificity, NT-proANP, BNP, and NT-proBNP assays were run with single NT-proANP, BNP, and NT-proBNP calibrators and single detection antibodies. The table below 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

Conclusions

Biomarker measurement is emerging as a powerful tool for diagnosis, prognostic stratification, and administration of personalize medical treatment. MSD has developed ultra-sensitive assays to measure rat natriuretic peptides to support research into the pathophysiology of heart failure. Through diligent characterization, the following advantages have been verified:

- Simple protocols that require minimal sample volumes
- Peptide specificity (no cross-reactivity between the peptides)
- Wide dynamic range
- High precision (average intra-plate % CV<5 and average inter-plate % CV <10)