Development and preliminary clinical validation of a high sensitivity assay for cardiac troponin using a capillary flow (single molecule) fluorescence detector

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Background: The European and American Cardiology Societies (ESC/ACC) have established cardiac troponin (cTnI) as the gold standard for diagnosis of myocardial infarction (AMI) and risk stratification for adverse cardiac events. The ESC/ACC recommends a cTnI cutoff at the 99th% of the normal range with an assay imprecision (CV) of <10%. Currently none of the commercial assays can detect cTnI in healthy subjects with the requisite precision, therefore many have advocated a cTnI cutoff at the 10% CV value. We developed and evaluated a high-sensitivity cTnI assay and determined its analytical and preliminary clinical performance.

Methods: The Singulex cTnI assay utilizes a 384-well ELISA plate, and the ZeptX™ Digital Molecule Counting (DMC) System. This assay uses a monoclonal capture antibody and a fluorescently-tagged affinity-purified goat detecting antibody. After washing, the fluorescently- tagged antibody is chemically released into each well. An aliquot is pumped into the analyzer. Individually-labeled antibodies are measured during capillary flow by setting the interrogation volume such that the emission of only 1 fluorescent molecule is detected in a defined space following laser excitation. With each signal representing a digital event, this configuration enables extremely high analytical sensitivities. Total fluorescent signal is determined as a sum of the individual digital events. Each molecule counted is a positive data point with hundreds to thousands of DMC events/sample. The limit of detection the Singulex cTnI assay was determined by the mean +3 SD method. The normal range was determined on a population of 88 apparently healthy subjects. This assay was correlated to the Bayer Centaur on 130 samples from patients admitted with chest pain. We also examined 47 serial samples from 18 patients who presented to the ED with a diagnosis of AMI. In this latter group, all had initial Centaur cTnI results that were <0.35 ng/mL (10% CV cutpoint), and 12 were <0.1 ng/mL (99th%). The cTnI concentration was positive on all subsequent serial samples from these patients on the Centaur, establishing the diagnosis of AMI.

Results: The analytical sensitivity of the Singulex assay was 1 pg/mL. The precision was 10% at 4 and 12 pg/mL. The reference population exhibited a normal distribution. The 99th percentile was determined to be <7 pg/mL. The linear regression between Singulex (y) and Centaur (x) was: y= 0.113x + 0.048, r=0.937. In the 3 cases that had initial Centaur cTnI value between 0.1 and 0.35 ng/mL, all were positive for Singulex. In the 12 cases that had initial Centaur cTnI value <0.1 ng/mL, 5 of 12 cases were positive. The prospective use of the Singulex assay would have detected 53% more AMI cases than the Centaur when the admission sample was tested.

Conclusion: The use of a highly sensitive and precise cTnI assay will enable detection of AMI earlier than with existing cTnI assays. A higher number of patients at risk for adverse cardiac events may be detected. The increased sensitivity was achieved by counting individual fluorescent emission events by the ZeptX analyzer.

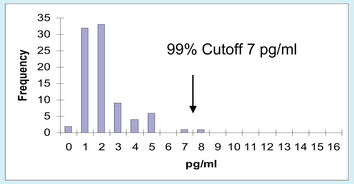
The ZeptX™ System technology

- 1-10 µl sample
- 384 well ELISA Plate sandwich assay
- Fluorescently-labeled detection antibody
- Capillary flow, laser-induced fluorescence, high-sensitivity detection
- Fluorescent detection antibody released from plate & sipped into instrument capillary
- Excitation and detection performed: Count individual labeled antibody peaks in capillary with S:N >30
- Total signal is the sum of antibody peaks
- Sensitivity routinely sub-fmol/l

Instrument sensitivity, n=15 runs

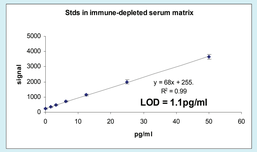
Calibrator fM	Signal counts	CV
0	11	
12	302	9
60	1341	8
300	4784	7

99% of the normal range for cTnI



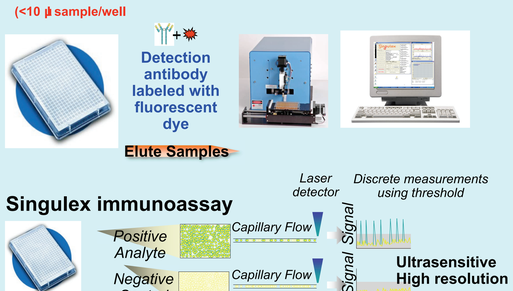
Serum from 88 normal random donors were tested (in quadruplicate). The 99% cutoff was 7 pg/ml (mean + 3 SD).

Analytical limit of detection for cTnI

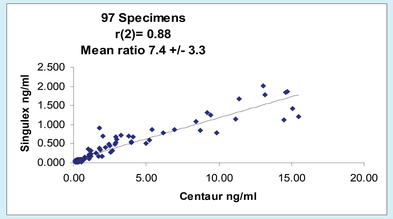


The analytical limit of detection (LoD) was determined across 15 sequential assays. The LoD was the mean of the 0 std + 3 SD (n=4) intra-assay determinations. The average LoD was 1.7 pg/ml (range 0.4-2.8 pg/ml)

Fluorescently labeled molecules are counted during capillary flow



Correlation between Singulex vs. Bayer Centaur cTnI



Clinical utility studies

Study 1:

- Retrospective study of 18 patients
- Subjects presented with chest pain in ED
- All had non-ST elevated ECG
- All subjects Troponin-I negative (<0.1 ng/ml) or equivocal (0.1-0.35 ng/ml) on Bayer Centaur at presentation
- All subjects subsequently became Troponin-I positive on Bayer Centaur
- Longitudinal specimens (4-8 r intervals between blood draws) provided to Singulex in blind manner for testing in triplicate
- Quantitative data Cross standardized using a factor of 7.4

Study 2:

- 50 additional single samples were tested with negative cTnI on Centaur.

Examples of assays developed on ZeptX System and sensitivity enhancements

Analyte	*ZeptX Detection Limit (pg/ml)*	*Improvement Over Standard Formats*	*Assay Format*
Prion	**1**	**100x**	Sandw
HBsAg	**2**	**20x**	Sandw
IL-6	**0.02**	**50x**	Sandw
Proprietary Pharma	**10**	**50x**	Single Ab
S100A8	**3**	**25x**	Sandw
Interferon Gamma	**0.5**	**25x**	Sandw
Proprietary (Wash U)	**10**	**25x**	Sandw

Representative data, study 1

Patient	ID-bleed #	Singulex ng/ml	Bayer ng/ml	Bayer ng/ml standardized
51 y/o male Chest pressure Dx, non-ST-elevation MI	B-1	**0.009**	<0.1	<0.014
	B-2	**0.015**	<0.1	<0.014
	B-3	**0.033**	<0.1	<0.014
	B-4	**0.009**	<0.1	<0.014
	B-5	**0.009**	<0.1	<0.014
	B-6	**0.034**	**0.33**	**0.045**
	B-7	**0.083**	**0.64**	**0.086**
66 y/o F Chest pain Dx, nSTEMI	F-1	**0.013**	<0.1	<0.014
	F-2	**>1**	**10.94**	**1.478**
	F-3	**>1**	**10.39**	**1.404**
70 y/o F General weakness Troponin leak due to intracranial hemorrhage	N-1	**0.016**	<0.1	<0.014
	N-2	**0.065**	**0.22**	**0.030**
	N-3	**0.054**	**0.27**	**0.036**
	N-4	**0.059**	**0.32**	**0.043**
81 y/o M, Presented after a fall Dx, nSTEMI	P-1	**0.013**	<0.1	<0.014
	P-2	**0.376**	**3.23**	**0.436**

Test Method	*# Detected*	*# Non-Detected*
Bayer Centaur	7	11
Singulex	13	5

Precision, recovery and linearity for cTnI

Precision –
Intraplate precision, n=20

Avg (pg/ml)	12	95
Std dev	1.4	10.1
%CV	12	11

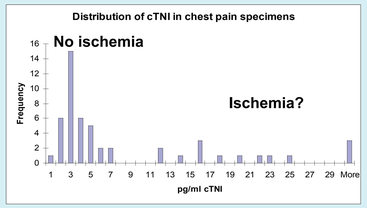
Recovery (serum)
Spiking 5, 15, 45, and 135 pg/ml TnI standard into pooled serum that was TnI immunodepleted

Spike (pg/ml)	%Recovery
5	114
15	91
45	97

Linearity
Pooled human serum was spiked with a moderate concentration of TnI and diluted with calibrator diluent.

Serum Dilution	% of expected
1:2	79
1:4	87
1:8	96

Study 2



Of the 50, 36 were within the 99th%. There were 14 samples which were abnormal by the ZeptX system and normal by the Centaur. Outcomes analysis is necessary to determine if these subjects had minor (ischemic) injury.

Conclusions

- The ZeptX system is a highly sensitive cappilary flow laser-induced fluorescence immunoassay analyzer
- The Singulex cTnI assay has 10-20 fold higher sensitivity than the Bayer Centaur
- The LoD was 1.7 pg/ml and the 99th% for a healthy population was 7 pg/ml
- For nSTEMI patients, ZeptX detected cTnI earlier than Centaur
- A high sensitivity cTroponin-I assay may enable more patients to be identified at high short-term cardiovascular risk (study to be conducted)