

## Development of a novel diffraction-based immunoassay for characterizing the primary and ternary structure of the circulating form of cardiac troponin

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### Abstract

The detection of cardiac muscle specific troponins in blood is the current gold standard for the diagnosis of patients with acute myocardial infarction (AMI). In cardiac muscle, the troponin (cTn) complex comprises 3 tightly interacting subunits cTnI, cTnT, and cTnC. With AMI, cardiac troponin is released from the heart into the circulation where it can be detected using a variety of immunoassays that independently quantify cTnI or cTnT. cTnI is a complex analyte. Its circulating form has the potential of disease-induced, posttranslational modifications such as the specific and selective degradation of the N- and/or C-terminus and the possibility of presenting different ternary structures. There is indirect evidence suggesting that the dominant circulating form of cTnI is the cTnI-cTnC complex and that other potential complexes with cTnT are rare or nonexistent. However, there has been no study that has directly characterized the circulating ternary form of these biomarkers, in part, due to a limitation in technology.

Here we describe the development of a novel immunoassay to characterize the physical form of circulating cTnI using diffractive optics technology, or dot™. Core to this technology is a diffraction grating formed from affinity reagents such as antibodies. This grating is comprised of a repetitive sequence of lines and generates a specific, reflected diffraction pattern when interrogated with a laser. As molecules exhibit affinity for the capture molecules that make up the grating, the diffraction efficiency is improved. In this investigation, cTnI was captured using a biotinylated anti-cTnI antibody to the constant region (a.a residues 137-148) and was immobilized on a pre-patterned avidin sensor and then probed, i) with antibodies to either or both the N- and C-terminus to determine if cTnI was degraded or, ii) sequentially with anti-cTnT and/or anti-cTnC antibodies to determine whether cTnI existed as a monomer, dimer or trimer. The binding of the immobilized antibody, protein analyte and other antibodies were observed in real time as an increase in diffraction signal intensity. By continuously monitoring the intensity, we can characterize the differences in the circulating form of cTnI. Using this new method, we have now directly measured the circulating cTnI-cTnT complex in patients diagnosed with AMI.

### Background

Cardiac troponin I (cTnI) is the current gold standard for the diagnosis of acute myocardial infarction (AMI, heart attack). In the heart, cTnI is part of the troponin (Tn) complex comprising troponin I, troponin T (cTnT) and troponin C (TnC), and is released into the blood upon cardiac muscle necrosis and cell death. It is accepted dogma that the analyte circulates in the blood as a cTnI-cTnC complex, yet this is based primarily on indirect evidence. A further complexity of this analyte is that cTnI can specifically and selectively degrade at the C- and then N-terminus with increasing severity of AMI. Until now, the technology for direct detection of the cTnI primary sequence integrity and the ternary form of circulating cTnI has been lacking. Therefore, the question remains whether circulating cTnI is degraded or is bound to cTnC and/or cTnT and whether these various circulating forms of cTnI correlate to the long term patient outcomes.

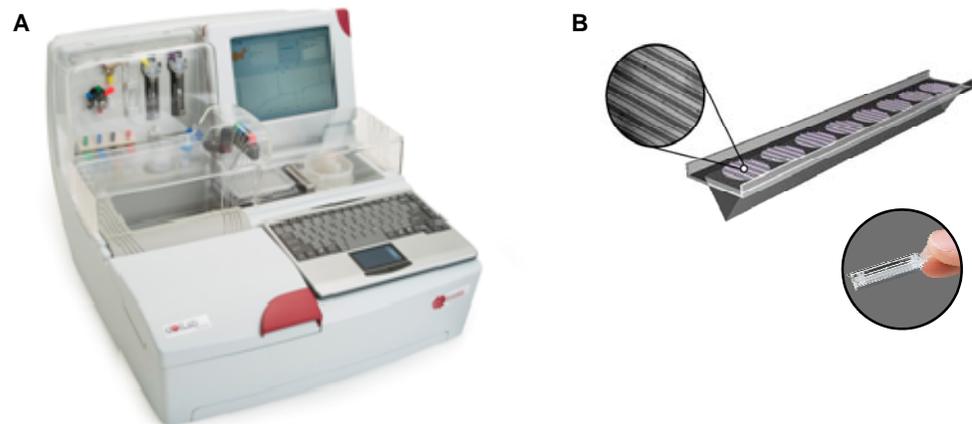
In this study, we show that by using diffractive optics technology (dot™), we have developed a sensitive and simple-to-use method to directly characterize the interactions between cTnI, cTnT and/or cTnC from clinical samples.

### The dotLab® mX System

The dotLab mX System utilizes diffraction-based optical sensing for the real time, label-free measurement of molecular interactions. The system uses inexpensive, disposable biosensors with coupling reagents (eg: avidin, amine reactive substrates or unique oligonucleotide-based addressing reagents to allow multiplexing) pre-patterned on the surface of 10 µL flow channels forming a diffraction grating (Figure 1). The dotLab mX instrument illuminates the grating with a

laser generating a diffraction image with is monitored by a photodiode detector. Diffractive efficiency increases as molecules bind to the surface resulting in an increase in image intensity. Conversely, molecular dissociation from the surface results in a decrease in image intensity. Therefore, the real time monitoring of molecular interactions through changes in diffractive efficiency provides information on the quantity and rate of binding and dissociation events. The dotLab System simplifies and automates this analysis using a fully integrated, easy to use, bench top instrument.

**Figure 1:** (A) The dotLab mX Instrument: a fully automated, bench-top instrument for real time molecular interaction analysis. (B) Schematic of a dotLab sensor with a contiguous array of capture surfaces (spots) with coupling reagent pre-patterned on the surface forming diffraction gratings.



#### **Materials and methods**

##### **REAGENTS:**

- 8I-7 (monoclonal mouse antibody against 137-148 a.a of cTnI) and 3E3 (monoclonal mouse antibody against 55-94 a.a of cTnI) were from Spectral Diagnostics Inc. (Toronto, Ontario, Canada).
- P3 (monoclonal goat antibody against 37-50 a.a of cTnI) was from BiosPacific (Emeryville, CA).
- MF4 (monoclonal mouse antibody against 190-196 a.a of cTnI), 7B9 (monoclonal mouse antibody against cTnC), and purified cTn and cTnI were from Hytest Ltd. (Turku, Finland).
- 1A11 (monoclonal mouse antibody against cTnT) was from Bidesign International (Saco, ME).
- TrueBlue™ Peroxidase Substrate, a precipitating form of TMB, was from KPL Inc. (Gaithersburg, MD).

##### **BIOTINYLATION AND HRP-CONJUGATION:**

- 8I-7 was biotinylated using Fluoreporter® Mini-Biotin-XX Protein Labeling Kit from Invitrogen (Carlsbad, CA) according to manufacturer's recommendations.
- 3E3, 1A11, and 7B9 were labeled with horseradish peroxidase (HRP) using SureLINK™ HRP conjugation kit from KPL according to manufacturer's recommendations.

##### **DIFFRACTION-BASED dot™ IMMUNOASSAY:**

- The assays were performed using dotLab sensors pre-patterned with avidin surface chemistry on the base of the flow cell. (Axela Inc., Toronto, Ontario, Canada). All experiments were carried out on the dotLab System (Axela Inc.) and real-time traces were recorded accordingly.
- Sequential sandwich immunoassay: Running buffer (PBS with 0.025% Tween 20, pH7.4) was introduced into the dry avidin sensors for 200 sec to stabilize the flow system and remove the preservatives from the sensor. BSA blocking buffer (5 mg/mL of BSA in Running buffer) was introduced and incubated for 5 min in mixing mode (repeatedly reversing flow directions within the sensor). This mode was used in all subsequent incubations. 10 µg/mL of biotinylated 8I-7 was introduced and incubated for 20 min. The sensor was washed with Running buffer. BSA-milk blocking buffer (4.5 mg/mL of BSA and 1% of nonfat milk in Running buffer) was introduced and incubated for 5 min. 1 µg/mL of purified analyte (cTn or cTnI) or a serum sample was

introduced and incubated for 10 min. The sensor was washed with Running buffer prior to a 5 min incubation of the BSA blocking buffer. A detector antibody (10 µg/mL) was introduced and incubated for 10 min. The sensor was washed at the end of run, or BSA and another detector antibody were introduced and the cycle above repeated.

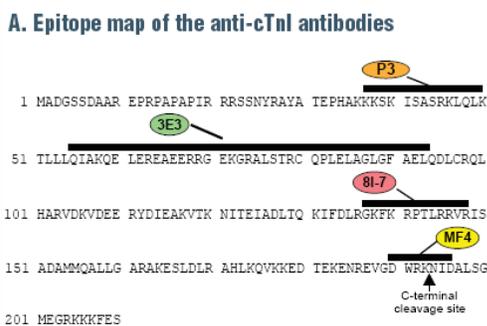
- TMB amplified assay for detection and characterization of cTn in serum from AMI patients: 4 µg/mL of HRP conjugated detector antibody and 10% (v/v) AMI patient serum were incubated offline at 4°C overnight. The sensor was washed, blocked and the capture antibody (bt-8I-7) (5 µg/mL, 10 min) was introduced to the sensor as described in the sequential sandwich immunoassay above. The premix (detector antibody and serum sample) was introduced into the sensor and incubated for 20 min. The sensor was washed with the Running buffer and PBS buffer. Finally TrueBlue™ TMB was introduced into the sensor and incubated for 10 min in static mode.

DATA ANALYSIS:

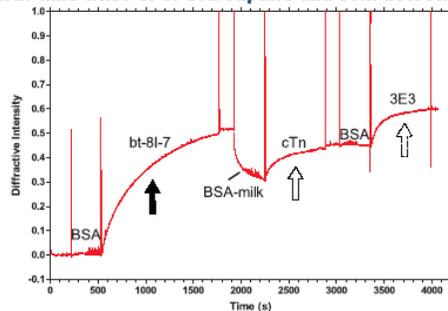
- All data recorded in dotLab™ software were exported as a csv file and analyzed in GraphPad Prism™ (GraphPad Software Inc., San Diego, CA.)

**Figure 2: Detection of cTnI by diffraction-based dot™ technology.** (A) Epitope map of the anti-cTnI antibodies. (B) A real-time trace of the capture of cTn and detection of cTnI. bt-8I-7 was immobilized on the avidin sensor surface (black arrow). The cTn complex was captured (empty arrow) and anti-cTnI (3E3) detected cTnI as part of the cTn complex (dashed arrow). All non-labeled portions are buffer wash. Spikes are air gaps separating reagents.

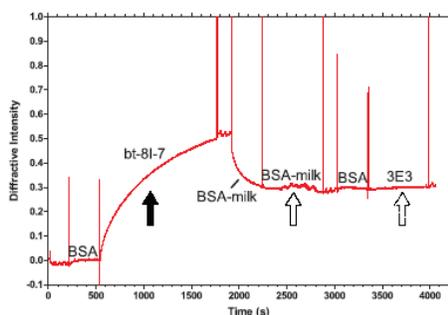
(C) Analyte negative control of (B). BSA-milk (empty arrow) instead of cTn was used in the otherwise identical experiment as (B).



**B. Real-time trace of of cTn capture and cTnI detection**



**C. Negative Control**

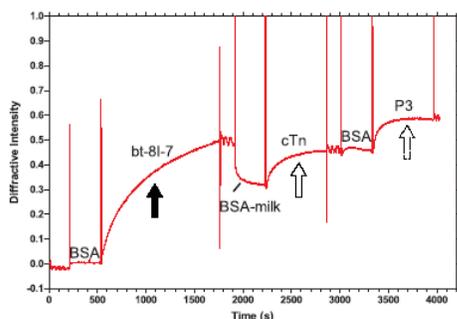


**Figure 3: Characterization of cTnI integrity.**

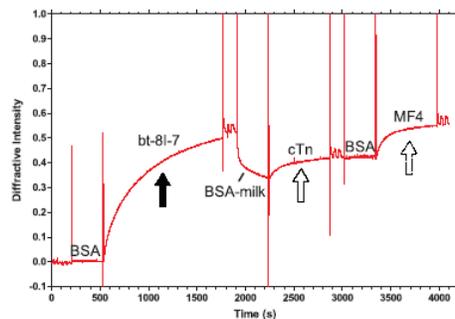
(A) Capture of cTn (empty arrow) by bt-8I-7 and detection of cTnI by antibody against N-terminus of cTnI (P3) (dashed arrow).

(B) Capture of cTn (empty arrow) by bt-8I-7 and detection of cTnI by antibody against C-terminus of cTnI (MF4) (dashed arrow).

**A. Detect N- terminus of cTnI**



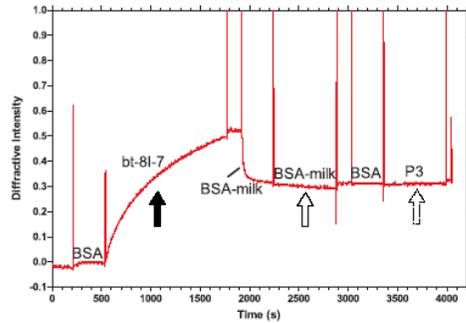
**B. Detect C- terminus of cTnI**



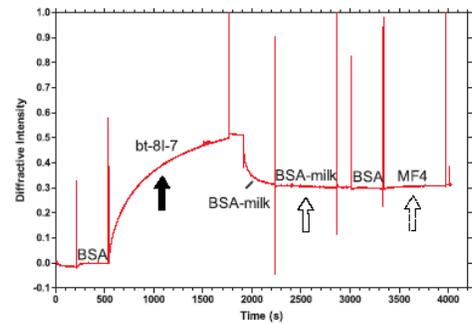
**Figure 3 (con't): Characterization of cTnI integrity.**

(C) and (D) Analyte negative control of (A) and (B) respectively. BSA-milk (empty arrow) instead of cTn was used.

**C. Negative control of (A)**



**D. Negative control of (B)**



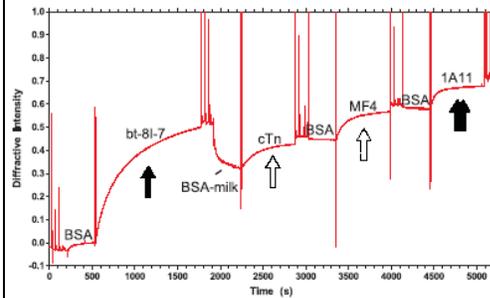
**Figure 4: Detection of the protein complex cTnI-cTnT.**

(A) Avidin immobilized bt-8I-7 (black arrow) captured the cTn complex (cTnI-cTnT-cTnC, empty arrow). cTnI was confirmed using anti-cTnI MF4 antibody (dashed arrow). cTnT was detected with anti-cTnT (1A11) (black double arrow).

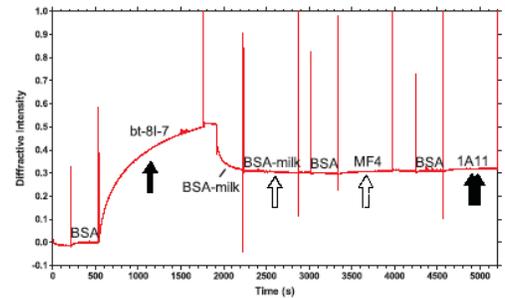
(B) Analyte negative control of (A). BSA-milk (empty arrow) instead of cTn was used.

(C) Analyte control of (A). cTnI (empty arrow) instead of cTn was used.

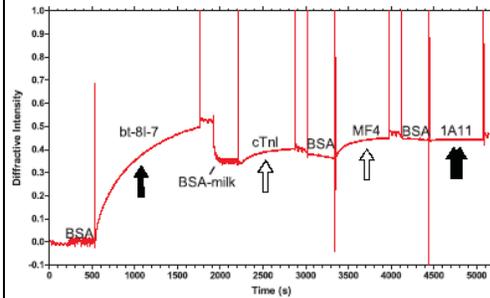
**A. Detection of the cTnI-cTnT complex**



**B. Negative control (no analyte)**



**C. Analyte control (cTnI analyte, no cTnT)**

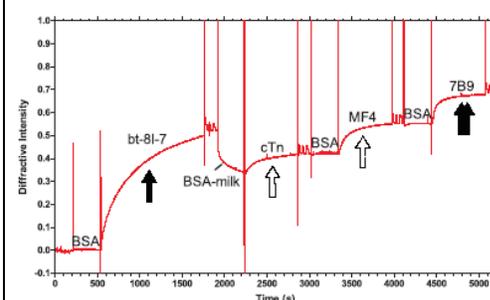


**Figure 5: Detection of the protein complex cTnI-cTnC.**

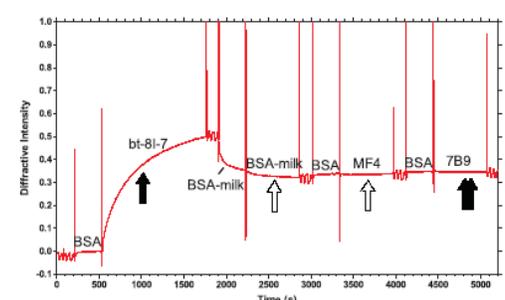
(A) Avidin immobilized bt-8I-7 (black arrow) captured the cTn complex (cTnI-cTnT-cTnC, empty arrow). cTnI was confirmed using anti-cTnI MF4 antibody (dashed arrow). cTnC was detected with anti-cTnC (7B9) (black double arrow).

(B) Analyte negative control of (A). BSA-milk (empty arrow) instead of cTn was used.

**A. Detection of the cTnI-cTnC complex**

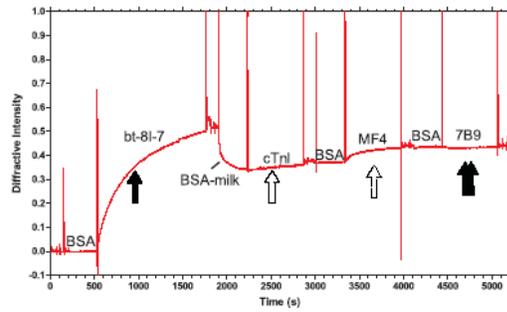


**B. Negative control (no analyte)**



**Figure 5 (con't):** (C) Analyte control of (A). cTnI (empty arrow) instead of cTn was used.

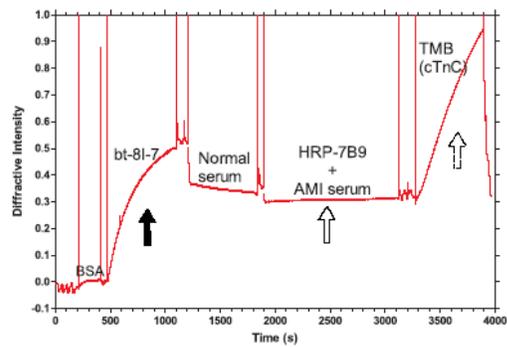
**C. Analyte control (cTnI analyte, no cTnC)**



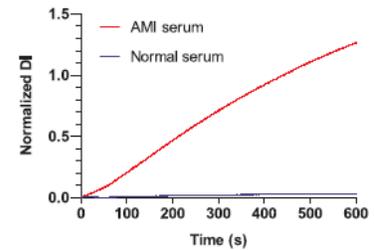
**Figure 6: Detection of the cTnI-cTnC and cTnI-cTnT complexes in serum from an AMI patient**

(A) Detection of the cTnI-cTnC complex. HRP-7B9 antibody (anti-cTnC) and the AMI serum (premix) was preincubated at 4°C overnight prior to adding to the sensor (empty arrow). Avidin immobilized bt-8I-7 antibody (anti-cTnI, black arrow) captured the “cTn” complex. TMB amplified the detection of cTnC (dashed arrow).

**A. Detection of the cTnI-cTnC complex**

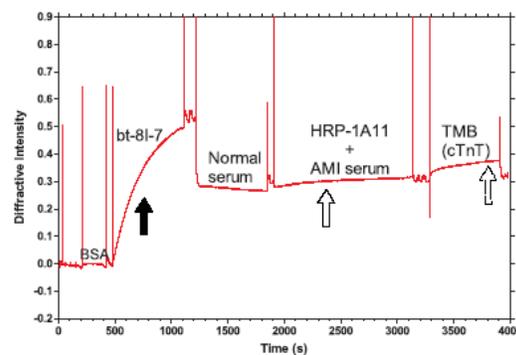


**B. Comparison of cTnC detection**

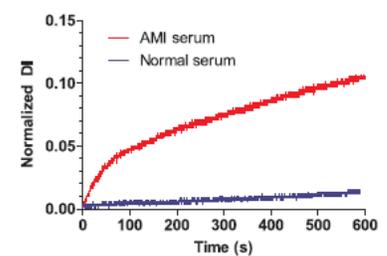


(B) Comparison of the TMB signals between the AMI serum and normal serum for cTnC detection. Note the “Normalized DI” is the ratio of diffractive intensity (DI) of TMB to maximal DI change of bt-8I-7.

**C. Detection of the cTnI-cTnT complex**



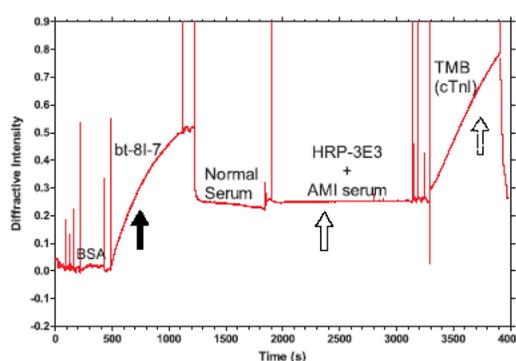
**D. Comparison of cTnT detection**



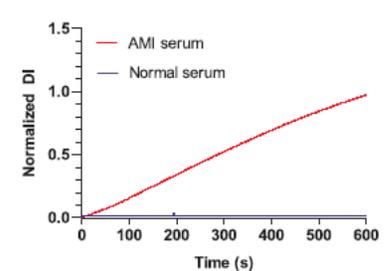
(C) Detection of a small amount of cTnT bound to cTnI (or cTnI-cTnC). HRP-1A11(anti-cTnT, empty arrow) instead of HRP-7B9 was used in the otherwise identical experiment as (A).

(D) Comparison of the TMB signals between the AMI serum and normal serum for cTnT detection.

**E. Confirmation of cTnI**



**F. Comparison of cTnI detection**



(E) Confirmation of the presence of cTnI in the “cTn” complex. HRP-3E3 (anti-cTnI, empty arrow) instead of HRP- 7B9 was used in the otherwise identical experiment as (A).

(F) Comparison of the TMB signals between the AMI serum and normal serum for cTnI detection.

### **Conclusion**

- A novel diffraction-based immunoassays using the dotLab System was developed that allows for characterizing the primary and ternary structure of the circulating form of cardiac troponin.
- The new assay was able to directly probe the integrity of cTnI and determine if the protein was degraded.
- The new assay was able to directly detect circulating cTnI bound to cTnC and cTnT from serum obtained from an AMI patient indicating the dimer or intact cTn (cTnI-cTnT-cTnC) was present.
- The dot immunoassay is the first clinical applicable and easy-to-use assay that can be used to address the direct interactions of the subunits within the cTn complex.
- The dotLab System allows the direct measure of protein interactions in complex samples. Real-time binding data allows a more thorough understanding of these complex interactions.

dotLab® System, dotReady™ Reagents and associated software are for Research Use Only.

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Axela's platforms provide powerful new approaches to multiplexed DNA, RNA and protein analysis designed to greatly simplify biomarker testing in clinical research and diagnostics. Axela's commercial research products significantly improve the amount and quality of information derived from traditional assays. This approach shortens time to result and provides access to unique categories of markers that form a pipeline of future diagnostic offerings.

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The logo for Axela Inc. features the word "axela" in a lowercase, sans-serif font. The letters "a", "x", and "e" are in blue, while "l", "a", and "x" are in black. A small superscripted "x" is positioned to the right of the final "a".