# Qualification of Multiplexed Cardiac Injury Markers for Preclinical Studies



Traditional clinical biomarkers such as ALT, AST, and CK are not sensitive enough to detect subtle drug induced muscle injury and often do not correlate to results from immunohistopathology. The volume of sample required for these traditional assays is often larger than is feasible for rodent models in preclinical studies. This poster describes a multiplex panel of traditional and novel biomarkers for muscle injury that overcome these limitations. Our Cardiac Injury Panel 3 (rat) includes cardiac Troponin I, cardiac Troponin T, FABP3, and Myl3. Troponins are widely accepted biomarkers for cardiac muscle toxicity; FABP3 and Myl3 are emerging biomarkers for muscle injury.1 This panel has advantages that are typical of assays from Meso Scale Discovery (MSD): greater sensitivity, reduced sample volume, a greater dynamic range (both endogenous and elevated levels can be measured at a single dilution factor) and improved throughput. The Cardiac Injury Panel 3 (rat) is now available for purchase from MSD as a qualified kit.

[1] Smith H, Sun D, Pritt M, Schultze E, Watson D. Development and Characterization of Serological Markers for Detection of Drug-Induced Muscle Injury in Rats, Presentation at the World Biomarker Congress, Philadelphia, PA. May 2007.

# Description of Markers

**Troponin** is a protein that acts with intracellular calcium to control the interaction of actin and myosin filaments of striated muscle fibers, thus regulating muscle contraction.

Troponin is made of the following 3 subunits:

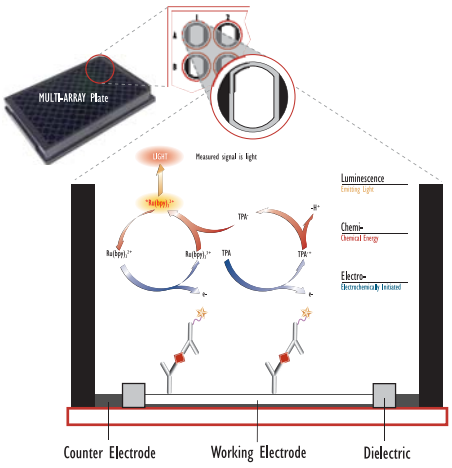
a) Troponin T: a cardiac muscle regulatory protein
b) Troponin I: an inhibitory subunit of the complex that prevents muscle contraction in the absence of calcium.
   It exists in 3 isoforms:
   i. Slow-twitch (striated) skeletal muscle
   ii. Fast- twitch (striated) skeletal muscle
   iii. Cardiac muscle
c) Troponin C: the subunit that binds calcium

**Myosin light chain 3 (MyI3)** is the light chain of the myosin protein molecule. The myosin molecule consists of 2 heavy chains and 4 light chains. The two essential myosin light chains are encoded by the MyI3 genes. The function of this protein is not fully understood, yet considered important to the heart's ventricular contractibility and relaxation.

**Fatty acid binding protein 3 (FABP3)** is a protein that modulates the uptake of fatty acids in cells. Heart-type fatty acid-binding protein is released into circulation after myocardial ischemia and necrosis. FABP3 is mostly present in heart and skeletal muscle but can also be found in brain, liver and small intestine.

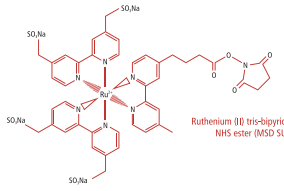
# The MSD® Platform

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 background signals and high signal to background ratios - the stimulation mechanism (electricity) is decoupled from the signal (light)
- Proximity - only labels bound near the electrode surface are detected, enabling non-washed assays
- Flexibility - labels are stable, non-radioactive, and are conveniently conjugated to biological molecules
- Emission at ~620 nm - eliminating problems with color quenching
- Signal amplification - multiple excitation cycles of each label enhance light levels and improve sensitivity

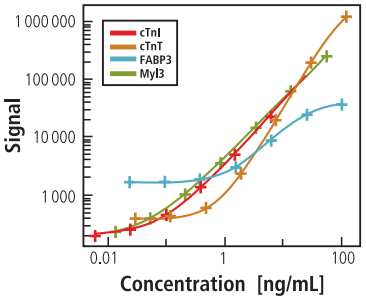


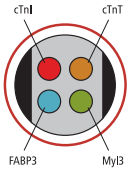
# Cardiac Injury Panel 3 (rat): cTnI, cTnT, FABP3, and MyI3

Our Cardiac Injury Panel 3 measures cardiac troponin I (cTnI), cardiac troponin T (cTnT), fatty acid binding protein 3 (FABP3), and myosin light chain 3 (MyI3) in serum or plasma. The analytes cTnI and cTnT are widely accepted as cardiac injury markers; FABP3 and MyI3 are emerging biomarkers. We qualified this panel according to typical practices for pre-clinical biomarkers. The qualification procedure involved multi-day controls, establishment of limits of quantitation, spike recovery, dilutional linearity, and measurement of control and treated samples.

## Standard Curve

The following standard curve is an example of the dynamic range of the assay. The lower limit of detection (LLOD) is determined by calculating 2.5 standard deviations above the average background signal (no analyte). The lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) were assigned following a multi-day study. We assigned the LLOQ (or ULOQ) as the lowest (or highest) concentration where the %CV of the calculated concentration was less than 20% and the percent recovery of the calculated concentration of the standard was between 80% and 120%.




| | cTnI | cTnT | FABP3 | MyL3 |
|---|---|---|---|---|
| LLOD (ng/mL) | 0.012 | 0.170 | 0.225 | 0.008 |
| LLOQ (ng/mL) | 0.098 | 0.488 | 0.781 | 0.054 |
| ULOQ (ng/mL) | 20 | 100 | 25 | 44 |

### Protocol:

1 Add 25 µL Assay Diluent GF1, incubate 30 min at RT.

2 Add 25 µL of standard/sample, incubate 2 hours at RT.

3 Wash with PBS-T. Add 25 µL of Detection Antibody, incubate 2 hours at RT.

4 Wash with PBS-T. Add 150 µL of Read Buffer T, read.

| cTnI | | |
|---|---|---|
| Concentration (ng/mL) | Average Counts | % CV |
| 0.00 | 179 | 2.5 |
| 0.01 | 201 | 4.1 |
| 0.02 | 255 | 5.0 |
| 0.10 | 468 | 3.1 |
| 0.39 | 1377 | 4.1 |
| 1.56 | 5038 | 5.8 |
| 6.25 | 22933 | 5.5 |
| 25.0 | 118131 | 1.9 |

| cTnT | | |
|---|---|---|
| Concentration (ng/mL) | Average Counts | % CV |
| 0.00 | 347 | 4.0 |
| 0.03 | 376 | 4.9 |
| 0.12 | 420 | 6.0 |
| 0.49 | 605 | 4.0 |
| 1.95 | 2338 | 6.2 |
| 7.81 | 19344 | 5.6 |
| 31.3 | 196824 | 1.7 |
| 125 | 1203689 | 3.6 |

| FABP3 | | |
|---|---|---|
| Concentration (ng/mL) | Average Counts | % CV |
| 0.00 | 1600 | 2.6 |
| 0.02 | 1681 | 2.4 |
| 0.10 | 1695 | 3.2 |
| 0.39 | 1840 | 2.5 |
| 1.56 | 3013 | 4.5 |
| 6.25 | 8986 | 7.1 |
| 25.0 | 24969 | 5.3 |
| 100 | 37087 | 2.1 |

| MyI3 | | |
|---|---|---|
| Concentration (ng/mL) | Average Counts | % CV |
| 0.00 | 181 | 6.5 |
| 0.01 | 228 | 7.0 |
| 0.05 | 418 | 7.5 |
| 0.21 | 1052 | 4.0 |
| 0.86 | 3774 | 6.1 |
| 3.44 | 15302 | 4.8 |
| 13.8 | 65811 | 6.0 |
| 55.0 | 255189 | 1.5 |

## Precision: Multi-Day Study

Control samples were measured on 14 plates across four days. The controls were run in triplicate or quadruplicate on each plate. Control 4 is a skeletal muscle control.

| | | | | Intra-plate | | | Inter-plate |
|---|---|---|---|---|---|---|---|
| | Control | Plates | Ave. Conc. (ng/mL) | Average % CV | Max % CV | Min % CV | % CV |
| cTnI | Control 1 | 14 | 7.44 | 3.8 | 10.4 | 0.4 | 6.7 |
| | Control 2 | 14 | 1.20 | 4.1 | 11.0 | 0.8 | 7.4 |
| | Control 3 | 14 | 0.28 | 5.0 | 14.3 | 1.3 | 9.9 |
| | Control 4 | 14 | <LLOQ | | | | |
| cTnT | Control 1 | 14 | 79.71 | 3.3 | 5.5 | 1.0 | 6.1 |
| | Control 2 | 14 | 5.61 | 3.5 | 7.3 | 1.0 | 7.8 |
| | Control 3 | 14 | 0.61 | 6.7 | 21.3 | 0.9 | 12.3 |
| | Control 4 | 14 | <LLOQ | | | | |
| FABP3 | Control 1 | 14 | 4.53 | 5.0 | 11.0 | 3.2 | 8.7 |
| | Control 2 | 14 | 3.71 | 6.3 | 16.6 | 0.2 | 9.7 |
| | Control 3 | 14 | <LLOQ | | | | |
| | Control 4 | 14 | 11.81 | 5.9 | 11.0 | 1.5 | 10.2 |
| MyI3 | Control 1 | 14 | 44.31 | 5.7 | 10.4 | 1.6 | 7.7 |
| | Control 2 | 14 | 1.61 | 4.6 | 13.1 | 0.4 | 8.3 |
| | Control 3 | 14 | <LLOQ | | | | |
| | Control 4 | 14 | 0.50 | 6.2 | 16.5 | 2.1 | 9.1 |

## Spike Recovery

Pooled, normal Serum, Heparin Plasma, and EDTA Plasma were spiked with the calibrators at multiple values throughout the range of the assay. Spikes were made into neat samples. Values in italics for FABP3 were slightly above the ULOQ of 25 ng/mL. % Recovery = measured / expected * 100

| | cTnI | | | | | FABP3 | | | | | cTnT | | | | | MyI3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Spike Conc. (ng/mL) | Expected Conc. (ng/mL) | Measured Conc. (ng/mL) | % CV | % Recovery | Spike Conc. (ng/mL) | Expected Conc. (ng/mL) | Measured Conc. (ng/mL) | % CV | % Recovery | Spike Conc. (ng/mL) | Expected Conc. (ng/mL) | Measured Conc. (ng/mL) | % CV | % Recovery | Spike Conc. (ng/mL) | Expected Conc. (ng/mL) | Measured Conc. (ng/mL) | % CV | % Recovery |
| Spiked Serum | 2.5 | 3.27 | 3.26 | 5.17 | 100 | 10 | *24.92* | *26.97* | *13.64* | *108* | 12.5 | 13.17 | 11.56 | 1.66 | 88 | 5.5 | 5.87 | 6.45 | 4.79 | 110 |
| | 0.625 | 1.40 | 1.48 | 3.29 | 106 | 2.5 | 17.42 | 16.74 | 7.44 | 96 | 3.125 | 3.79 | 3.39 | 5.45 | 89 | 1.375 | 1.74 | 1.84 | 3.16 | 106 |
| | 0.156 | 0.93 | 0.97 | 1.06 | 105 | 0.625 | 15.54 | 16.25 | 3.63 | 105 | 0.781 | 1.45 | 1.29 | 1.27 | 89 | 0.344 | 0.71 | 0.77 | 2.26 | 108 |
| | 0 | | 0.77 | 2.40 | | 0 | | 14.92 | 3.29 | | 0 | | 0.67 | 4.69 | | 0 | | 0.37 | 4.78 | |
| Spiked Heparin Plasma | 2.5 | 3.58 | 3.70 | 5.65 | 103 | 10 | *28.15* | *28.86* | *8.52* | *103* | 12.5 | 13.51 | 15.75 | 1.75 | 113 | 5.5 | 5.90 | 6.60 | 1.68 | 112 |
| | 0.625 | 1.71 | 1.78 | 8.02 | 104 | 2.5 | 20.65 | 20.08 | 4.68 | 97 | 3.125 | 4.13 | 4.64 | 3.84 | 112 | 1.375 | 1.78 | 1.87 | 6.51 | 105 |
| | 0.156 | 1.24 | 1.16 | 3.13 | 93 | 0.625 | 18.77 | 21.67 | 7.24 | 115 | 0.781 | 1.79 | 1.90 | 1.95 | 106 | 0.344 | 0.75 | 0.72 | 4.65 | 96 |
| | 0 | | 1.08 | 4.30 | | 0 | | 18.15 | 4.09 | | 0 | | 1.01 | 8.22 | | 0 | | 0.40 | 4.30 | |
| Spiked EDTA Plasma | 2.5 | 3.49 | 3.33 | 1.96 | 96 | 10 | *27.68* | *27.70* | *7.07* | *100* | 12.5 | 13.08 | 10.89 | 1.39 | 83 | 5.5 | 5.97 | 7.19 | 3.23 | 120 |
| | 0.625 | 1.62 | 1.79 | 8.38 | 111 | 2.5 | 20.18 | 21.81 | 2.81 | 108 | 3.125 | 3.70 | 3.33 | 1.29 | 90 | 1.375 | 1.85 | 2.11 | 6.49 | 114 |
| | 0.156 | 1.15 | 1.11 | 0.69 | 97 | 0.625 | 18.31 | 19.01 | 3.07 | 104 | 0.781 | 1.36 | 1.35 | 3.30 | 99 | 0.344 | 0.82 | 0.85 | 2.37 | 104 |
| | 0 | | 0.99 | 7.55 | | 0 | | 17.68 | 2.99 | | 0 | | 0.58 | 6.66 | | 0 | | 0.47 | 6.25 | |

## Dilutional Linearity

Multiple samples were tested for linearity of dilution. Recovery is calculated as the ratio of the adjusted concentration to the adjusted concentration of the previous dilution. Measurements that were outside of the quantitative range are shown in italics. For all measurements within the quantitative range, all the CV's on concentration were less than 15%.

% Recovery = (measured * dilution factor) / expected * 100

| | | cTnI | | cTnT | | FABP3 | | MyI3 | |
|---|---|---|---|---|---|---|---|---|---|
| Sample | Fold Dilution | Adjusted Conc. (ng/mL) | % Recovery | Adjusted Conc. (ng/mL) | % Recovery | Adjusted Conc. (ng/mL) | % Recovery | Adjusted Conc. (ng/mL) | % Recovery |
| Pooled EDTA Plasma, Normal | Neat | 3.71 | | 2.85 | | *79.76* | | 1.53 | |
| | 2 | 3.35 | 90.1 | 2.97 | 104.3 | *66.89* | > ULOQ | 1.26 | 82.3 |
| | 4 | 3.03 | 90.6 | 2.62 | 88.3 | 55.33 | NA | 1.05 | 83.1 |
| | 8 | 2.83 | 93.4 | *2.38* | < LLOQ | 44.78 | 80.9 | 0.95 | 90.8 |
| Pooled Heparin Plasma, Normal | Neat | 3.55 | | 2.60 | | *49.53* | | 1.22 | |
| | 2 | 3.44 | 96.8 | 2.40 | 92.4 | *52.13* | > ULOQ | 1.21 | 98.8 |
| | 4 | 3.10 | 90.1 | 2.24 | 93.2 | 49.93 | NA | 1.10 | 90.8 |
| | 8 | 2.92 | 94.1 | *1.99* | < LLOQ | 42.36 | 84.8 | 0.98 | 89.6 |
| Serum from Normal Rat 1 | Neat | 1.29 | | 0.86 | | 22.33 | | 0.41 | |
| | 2 | 1.25 | 96.4 | *0.86* | < LLOQ | 21.54 | 96.5 | 0.41 | 100.2 |
| | 4 | 1.07 | 86.1 | *0.72* | < LLOQ | 18.87 | 87.6 | 0.34 | 82.0 |
| | 8 | 0.89 | 82.9 | *0.22* | < LLOQ | 13.54 | 71.7 | *0.27* | < LLOQ |
| Serum from Normal Rat 2 | Neat | 0.72 | | 0.60 | | 19.16 | | 0.54 | |
| | 2 | 0.69 | 96.0 | *0.54* | < LLOQ | 16.87 | 88.1 | 0.45 | 84.1 |
| | 4 | 0.65 | 94.5 | *0.43* | < LLOQ | 14.88 | 88.2 | 0.37 | 81.5 |
| | 8 | *0.62* | < LLOQ | *0.64* | < LLOQ | 13.08 | 87.9 | *0.38* | < LLOQ |
| Serum from Isoproterenol treated Rat 1 | Neat | 14.58 | | 7.32 | | *141.36* | | 6.73 | |
| | 2 | 11.97 | 82.1 | 7.25 | 99.1 | 82.73 | > ULOQ | 6.12 | 91.0 |
| | 4 | 10.10 | 84.4 | 6.04 | 83.2 | 87.49 | NA | 5.29 | 86.4 |
| | 8 | 8.74 | 86.5 | 4.62 | 76.6 | 93.31 | 106.7 | 4.33 | 81.9 |
| Serum from Isoproterenol treated Rat 2 | Neat | 20.43 | | 11.28 | | *68.88* | | 8.98 | |
| | 2 | 15.97 | 78.2 | 10.64 | 94.3 | 75.31 | > ULOQ | 7.46 | 83.1 |
| | 4 | 13.28 | 83.2 | 9.28 | 87.2 | 95.74 | NA | 6.84 | 91.7 |
| | 8 | 10.72 | 80.7 | 7.05 | 76.0 | 90.49 | 94.5 | 5.56 | 81.3 |

## Samples

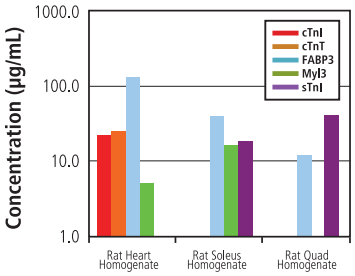
Serum, EDTA Plasma, and Heparin Plasma samples collected from normal, Sprague-Dawley rats were tested at 2-fold dilution on the Cardiac Injury Panel 3 (rat). Shown below are the median and range of concentrations for each sample set.

| Sample | Statistic | cTnI | cTnT | FABP3 | MyI3 |
|---|---|---|---|---|---|
| EDTA Plasma | Median (ng/mL) | 1.85 | 1.21 | 35.01 | 1.06 |
| | Range (ng/mL) | 1.85 - 6.48 | < 0.98 - 5.43 | 27.6 - > 50.0 | 0.63 - 2.68 |
| | N | 6 | 6 | 6 | 6 |
| Heparin Plasma | Median (ng/mL) | 1.89 | 1.38 | 26.57 | 0.83 |
| | Range (ng/mL) | 0.56 - 3.87 | < 0.98 - 3.37 | 10.9 - 42.5 | 0.34 - 1.94 |
| | N | 10 | 10 | 10 | 10 |
| Serum | Median (ng/mL) | 1.19 | 0.73 | 19.63 | 0.60 |
| | Range (ng/mL) | 0.26 - 2.49 | < 0.98 - 2.19 | 5.14 - 27.0 | 0.17 - 0.99 |
| | N | 10 | 10 | 10 | 10 |

## Specificity of Binding

Tissue homogenates from heart, fast twitch, and slow twitch muscle were tested at 100X, 1000X and 10000X sample dilution. The assay for skeletal troponin was positive for muscle homogenates and negative for other cardiac homogenates, demonstrating specificity for muscle tissue. The assay for skeletal Troponin I was specific for fast and slow twitch muscle and is the same assay used for the MSD Rat sTnI Assay Kit.

| Sample Group | cTnI | | cTnT | | FABP3 | | MyI3 | | Skeletal TnI | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Sample Dilution | Conc. (µg/mL) | Sample Dilution | Conc. (µg/mL) | Sample Dilution | Conc. (µg/mL) | Sample Dilution | Conc. (µg/mL) | Sample Dilution | Conc. (µg/mL) |
| Rat Heart Homogenate | 1000 | 22.6 | 1000 | 25.1 | 10000 | 125.2 | 1000 | 5.0 | 100 | < LLOD |
| Rat Soleus Homogenate (slow twitch) | 100 | < LLOD | 100 | < LLOD | 10000 | 38.8 | 1000 | 16.4 | 1000 | 18.1 |
| Rat Quad Homogenate (fast twitch) | 100 | < LLOD | 100 | < LLOD | 1000 | 12.2 | 100 | 0.08 | 1000 | 40.9 |




## Conclusions

MSD has developed high performance, multiplex assays to measure biomarkers of cardiac injury. Composed of both traditional and emerging biomarkers, these panels can identify and stratify injury to different muscle types (cardiac and muscle tissues) and between different muscle classes (fast-twitch and slow-twitch muscles). The combination of multiplexing, wide dynamic range, and increased throughput enables studies that measure many analytes from a small volume of pre-clinical samples. Although it is not the subject of this poster, the analytes presented here have been studied by others to verify large fold changes in analyte concentration upon exposure to muscle toxicants. The Cardiac Injury Panel 3 (rat) is now available for purchase from MSD as a qualified kit.