Development and Validation of Rat Muscle Injury Biomarkers

Meso Scale Discovery (MSD) has developed a multiplexed panel and two singleplex assays for both traditional and emerging biomarkers of muscle injury. Assays were developed with design controls through phase-gated processes and follow "fit-for-purpose" principles, FDA Bioanalytical Method Validation guidance, and CLSI documents. The assays were validated for sensitivity, specificity, dilution linearity, spike recovery, precision, accuracy, robustness and sample handling. Antibodies to the following biomarkers used in the assays were well-characterized by analytical methods:

- Muscle Injury Panel 2 (rat): Parvalbumin, TIMP-1 and Creatine Kinase (CK)
- Rat sTnI
- Rat TNNI1

Description of Markers

Troponin is a heterotrimer that regulates muscle contraction in skeletal and cardiac muscle (but not in smooth muscle). Troponin acts with intracellular calcium to control the interaction of actin and myosin filaments in striated muscle fibers. There are three subunits (TnT, TnI, and TnC) of troponin. Troponin I is an inhibitory subunit that prevents muscle contraction in the absence of calcium. It is responsible for the binding of the troponin-tropomyosin complex to actin. Troponin I exists in three isoforms: slow-twitch (striated) skeletal muscle (TNNI1), fast-twitch (striated) skeletal muscle (TNNI2), and cardiac muscle (TNNI3).

When muscle tissue is damaged, the troponin-tropomyosin complex breaks down and troponin I and troponin T are released into the blood. Cardiac troponin T (cTnT) and cardiac troponin I (cTnI) can be readily distinguished from their skeletal muscle analogs allowing confirmation of cardiac muscle tissue damage over skeletal muscle tissue damage. Troponins are excellent biomarkers for myocardial injury in cardiotoxicity because of the demonstrated tissue-specificity of cardiac and skeletal troponins.

Parvalbumin is expressed in extrafusal and intrafusal skeletal muscle fibers and acts in the decay of Ca^{2+} in the contraction/relaxation cycle of fast twitch muscles. Studies have shown a positive correlation between the rate of relaxation and the concentration of parvalbumin. Therefore, parvalbumin could be used as a sensitive marker for early stages of neuromuscular disturbances and muscle transformations.

Tissue Inhibitors of Metalloproteinase-1 (TIMP-1) is a zinc and calcium-dependent protease responsible for the degradation of extracellular matrix components, such as collagen, laminin and proteoglycans during tumor progression and metastasis, and may play a direct role in pathologic processes such as arthritis, cardiovascular disease and muscle toxicity through key binding partners.

Creatine Kinase (CK) is an enzyme that catalyzes the rapid regeneration of ATP. It allows muscle contraction by transferring the phosphoryl group from phosphocreatine to ADP, resulting in creatine and ATP. In rat, CK activity is present in the heart, brain, skeletal and smooth muscle. Increased CK concentration is indicative of muscle injury.

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.
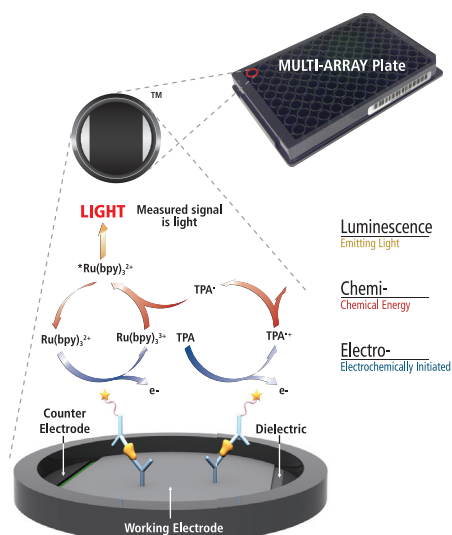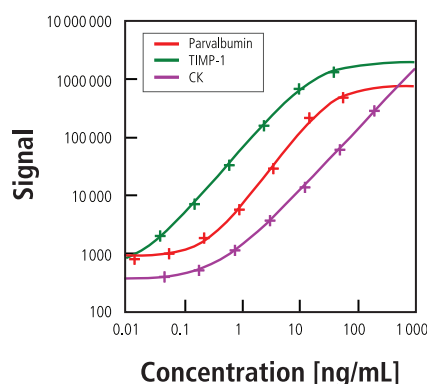

Electrochemiluminescence Features

- 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

Muscle Injury Panel 2 (rat) Assay: Parvalbumin, TIMP-1, CK

Standard Curve

The following standard curves show the dynamic range of the Muscle Injury Panel 2 multiplex panel for rat parvalbumin, TIMP-1 and CK.

A 4-parameter logistic model with $1/Y^2$ weighting function was used as the curve fitting model.

Protocol

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

2. Wash plates with PBS-T. Add 25 µL of standard or sample (MSD recommends a 1:20 dilution. Actual sample volume needed is less than 5 µL.). 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 on MSD SECTOR® Imager.

Parvalbumin		
Concentration (ng/mL)	Average Signal	% CV
0	723	4.3
0.0146	826	0.9
0.0586	1049	0.9
0.234	1919	3.6
0.938	5870	4
3.75	29 562	5.4
15	229 291	6.2
60	485 701	0.9

TIMP-1		
Concentration (ng/mL)	Average Signal	% CV
0	342	4.3
0.00977	799	2.9
0.0391	2112	3.4
0.156	7310	2.7
0.625	34 295	1.9
2.5	166 070	0.6
10	699 702	5.4
40	1 344 226	1.6

CK		
Concentration (ng/mL)	Average Signal	% CV
0	302	1.6
0.0488	399	6.6
0.195	547	0
0.781	1194	2.8
3.13	3852	3.5
12.5	14 128	0.7
50	63 614	0
200	290 284	1.6

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). LLOD is calculated based on 20 tests.

	Parvalbumin	TIMP-1	CK
Average LLOD (ng/mL)	0.019	0.0135	0.0926
LLOD Range (ng/mL)	0.00571-0.0407	0.0106-0.0236	0.0557-0.164

Specificity

To assess specificity of the detection antibodies, the Muscle Injury Panel 2 (rat) was run using blended calibrators with individual detection antibodies and using blended detection antibodies with individual calibrators (3.8 ng/mL parvalbumin; 2.5 ng/mL TIMP-1; 13 ng/mL CK). No significant cross-reactivity (< 0.5%) was observed.

In most cells, the CK enzyme consists of two subunits, which can be either B (brain type) or M (muscle type). This results in three isoenzymes: CK-MM, CK-BB and CK-MB. The CK assay recognizes recombinant rat CK-MM, recombinant human CK-MM, and recombinant human CK-MB. It does not cross-react with recombinant human CK-BB. The assay measures 100-fold higher CK concentration in rat quadriceps tissue homogenate than in rat cardiac tissue homogenate.

Precision: Multi-Day Study

Controls, spanning the dynamic range of the assays, were made using diluted rat quadricep lysates and spiked with calibrator to achieve desired levels of analytes.

The controls were tested over multiple days (n>3) across multiple plates. The panel demonstrated both good precision and accuracy.

Control		Plates	Average Conc. (ng/mL)	Average Intra-plate % CV	Inter-plate % CV
Parvalbumin	High	5	46.2	5.8	7.2
	Mid	5	2.90	2.9	7.7
	Low	5	0.365	2.9	8.3
TIMP-1	High	5	10.9	3.4	8.9
	Mid	5	0.942	2.6	9.8
	Low	5	0.0957	2.9	9.7
CK	High	5	99.9	3.3	7.4
	Mid	5	4.16	3.6	8.9
	Low	5	0.313	6.4	17.5

Dilution Linearity

Rat serum and plasma samples were diluted at multiple levels to assess dilution linearity.

Percent recovery is calculated as the measured concentration multiplied by the dilution factor and divided by the concentration measured for the previous dilution (expected).

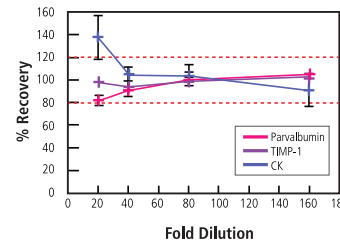% Recovery=(measured*dilution factor)/expected*100

Error bars represent standard deviations from 4 samples.

Acceptable dilutional linearity was observed for rat serum samples, and good linearity was seen with plasma samples at higher dilutions.

Dilutional Linearity in Serum
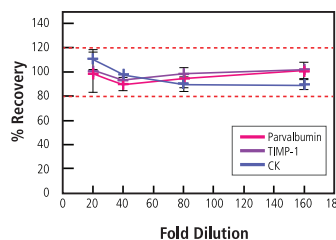
Dilutional Linearity in Plasma

Muscle Injury Panel 2 (rat) Assay: Parvalbumin, TIMP-1, CK

Spike Recovery

Rat serum and EDTA plasma samples were diluted 100-fold then spiked with calibrators at multiple levels as indicated in the table.
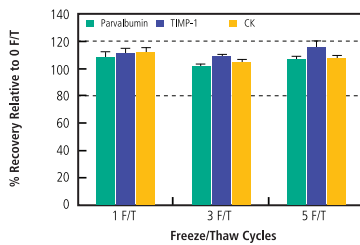
Recovery=measured/expected*100

Good recoveries were observed in both serum and plasma samples at all spike levels.

Spike Recovery in Serum

Spike Recovery in Plasma
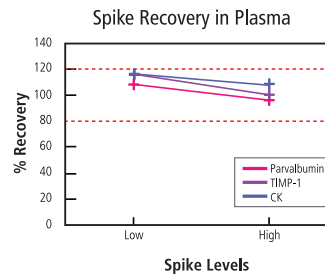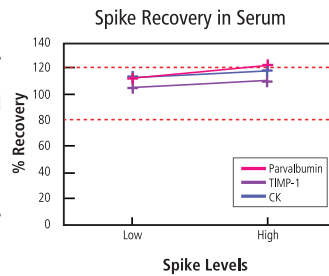
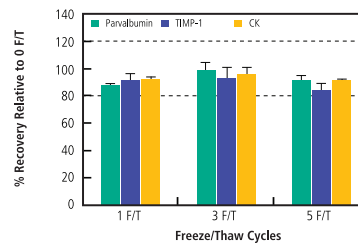|	Spike Conc. (ng/mL)	
	Low	High
Parvalbumin	4.44	13.3
TIMP-1	3.00	8.90
CK	14.8	44.4

Robustness
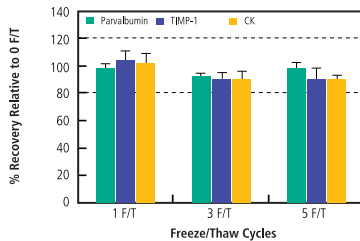
Calibrator Freeze/Thaw Stability

The calibrators were stable up to 5 freeze/thaw cycles.
Error bars represent standard deviations from 8 samples.
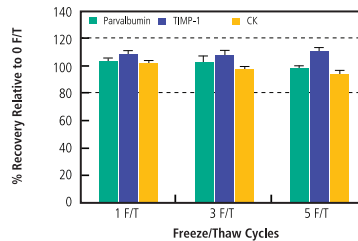
Control Freeze/Thaw Stability

Controls were stable up to 5 freeze/thaw cycles.
Error bars represent standard deviations from 3 samples.

Assay Diluent Freeze/Thaw Stability

Assay diluent was stable up to 5 freeze/thaw cycles.
Error bars represent standard deviations from 3 samples.

### Detection Antibody Diluent Freeze/Thaw Stability

Detection antibody diluent was stable up to 5 freeze/thaw cycles.
Error bars represent standard deviations from 3 samples.

Rat Skeletal Troponin I (sTnI) Assay

MSD's skeletal troponin I (sTnI) assay measures fast and slow twitch skeletal muscle injury. The sample data shown is from a qualification of a singleplex assay. The assay is quantitative over a 100- to 1000-fold range; we assigned an LLOQ of 0.781 ng/mL and a ULOQ of 160 ng/mL. In qualification, the assay passed dilution-linearity and spike recovery specifications (not shown). The assay is functional over a range of sample dilutions. We ran twenty serum and plasma samples at 4-fold dilution; thirteen were control samples and seven were samples from treatment with a variety of skeletal-injury inducing drugs. All control samples were below LLOQ. The treated samples had elevated levels of sTnI (from 42.5 to 1350 ng/mL). We also ran samples from both skeletal and cardiac muscle in a multiplex panel of sTnI and cTnI. These experiments showed that sTnI and cTnI do not cross-react and each is specific for their appropriate muscle type (skeletal and cardiac muscle, respectively). These data indicate that sTnI can be multiplexed with other cardiac biomarkers to produce a panel that interrogates injury to both skeletal muscle and cardiac muscle.

Standard Curve
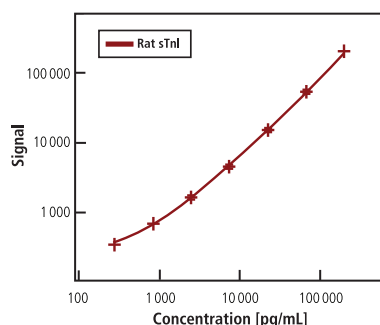
Rat sTnI		
Conc. (pg/mL)	Average Counts	% CV
0	213	1.77
274	358	2.94
823	704	4.11
2469	1672	8.51
7407	4568	7.94
22 222	14 847	7.78
66 667	53 441	6.91
200 000	206 052	3.35

	Rat sTnI (pg/mL)
LLOD	374
LLOQ	781
ULOQ	160 000

Spike Recovery

Individual, normal rat serum, heparin and EDTA plasma samples were spiked with the rat skeletal troponin I calibrator at multiple concentrations throughout the range of the assay. Spikes were made into samples diluted 2-fold prior to measurement in the assay and show good recovery.

% Recovery=measured/expected*100

	Spike Level. (pg/mL)	Conc. (pg/mL)	Conc % CV	% Recovery
Spiked Serum	20 000	21 184	4.0	108
	6667	7220	4.6	106
	2222	2506	3.7	106
	0	<LLOD	-	-
Spiked EDTA Plasma	20 000	21 703	2.1	100
	6667	6718	3.7	96
	2222	2186	5.3	91
	0	<LLOD		
Spiked Heparin Plasma	20 000	21 797	3.8	109
	6667	7139	3.9	101
	2222	2414	3.0	96
	0	<LLOD	-	-

Precision: Multi-Day Study

High, mid, and low control samples were measured on 21 plates across 7 days. The controls were run in triplicate or quadruplicate on each plate. The controls are a mix of normal rat serum, rat muscle homogenate and calibrators. The average intra-plate %CV and inter-plate %CV of the concentrations are shown below.

				Intra-plate	Inter-plate
	Control	Plates	Ave. Conc. (pg/mL)	Average % CV	% CV
Rat sTnI	High	21	100 445	3.6	6.2
	Mid	21	15 824	3.6	5.6
	Low	21	2797	4.5	7.2

Dilutional Linearity

To assess linearity, rat serum, EDTA and heparin plasma samples were spiked with known levels and further diluted 10-fold, 50-fold, and 250-fold. The concentrations shown below have been corrected for dilution. Percent recovery is calculated as the measured concentration divided by the concentration measured from the previous dilution (expected).

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

	Rat sTnI			
	Dilution Factor	Dilution Corrected Conc. (ng/mL)	Conc. CV	% Recovery
Serum	Spiked	90 222	4.0	
	10	81 150	3.5	90
	50	82 650	5.4	102
	250	61 000	13.0	74
EDTA Plasma	Spiked	83 170	3.9	
	10	74 270	4.7	89
	50	74 100	6.5	100
	250	53 000	11.7	71
Heparin Plasma	Spiked	84 358	5.8	
	10	74 820	6.3	87
	50	75 350	5.5	101
	250	<LLOD	-	-

Samples

Serum, heparin plasma, and EDTA plasma samples collected from normal, Sprague-Dawley rats were measured neat on the rat sTnI assay. Shown below are the median and range of concentrations for each sample set. Median levels of skeletal troponin I were below the quantitative range for all samples.

Sample	Statistic	Rat sTnI
Serum	Median (pg/mL)	336
	Range (pg/mL)	<LLOD-1878
	N	21
EDTA Plasma	Median (pg/mL)	378
	Range (pg/mL)	<LLOD-936
	N	10
Heparin Plasma	Median (pg/mL)	27
	Range (pg/mL)	<LLOD-514
	N	10

Rat Slow-Twitch Skeletal Troponin I (TNNI1) Assay

Standard Curve

MSD's TNNI1 assay measures the slow twitch skeletal muscle injury.

A 4-parameter logistic model with $1/Y^2$ weighting function was used in the curve fitting model.
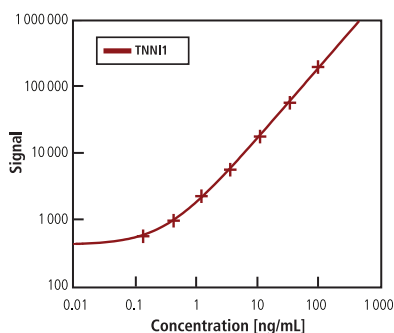
TNNI1		
Conc. (ng/mL)	Average Counts	% CV
0	360	4.3
0.14	581	0.4
0.41	993	15.1
1.2	2334	0.7
3.7	5782	3.3
11	18 101	3.5
33	58 259	3.7
100	193 341	7.6

Protocol

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

2   Wash plates with PBS-T. Add 50 µL of standard/sample (MSD recommends a 1:4 dilution. Actual sample volume needed is less than 15 µL.). 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 on MSD SECTOR® Imager.

	TNNI1 (ng/mL)
Average LLOD	0.241
LLOD Range	0.165-0.301

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). LLOD was calculated based on 10 experimental tests.

Specificity

The assay is specific to TNNI1. Signals from TNNI2 and sTnI were close to the blank.

Tissue homogenates from quadriceps (fast twitch), soleus (slow twitch) and vastus intermedium (slow twitch) were tested with the TNNI1 Assay. High concentrations of TNNI1 were detected in soleus and vastus intermedius.
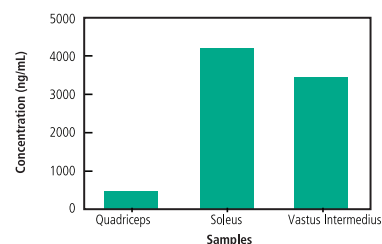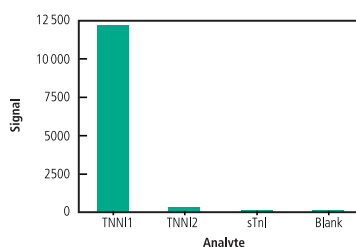
Conclusions

- MSD has developed high performance assays to measure both traditional and emerging biomarkers of muscle injury.

- These panels can identify and stratify injury to different muscle types (cardiac and skeletal muscle tissues) and between different muscle classes (fast-twitch and slow-twitch).

- The combination of multiplexing, wide dynamic range and increased throughput enables the user to interrogate damage in animal models of muscle injury which could improve our understanding of the mechanism of action of critical therapeutics as well as substantiate these biomarkers and their benefits in preclinical studies.

- Custom assay kits are available upon request.