Qualification of Muscle Injury Panel 2 and Rat Skeletal Troponin I Markers for Preclinical Studies

Assays of serum enzymes, such as aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) are widely performed in the early phase of suspected ischemic myocardial injury. However, these enzymes are not restricted to cardiac muscle tissue and increases in their serum concentrations have been observed in non-cardiac conditions. In addition, these measures correlate poorly with standard histochemical indices of muscle damage. 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 and singleplex assay for both traditional and novel biomarkers for muscle injury that overcome these limitations. Our Muscle Injury Panel 2 includes Parvalbumin, Creatine Kinase (CK), and Tissue Inhibitor of Metalloproteinase-1 (TIMP-1). This panel allows monitoring energy homeostasis, calcium-dependent protease action and neuromuscular disturbances that are often associated with muscle toxicity. Meso Scale Discovery (MSD) also offers a qualified assay for rat Skeletal Troponin I (sTnI). Troponins are widely accepted biomarkers for cardiac and striated muscle toxicity. Following muscle injury, sTnI is released into the blood and measurement of Troponin I in serum or plasma provides a good indicator of the extent of muscle injury. The combination of these biomarkers allows researchers to stratify drug-induced muscle injury between cardiac and skeletal muscle, and between fast and slow twitch skeletal muscle. These panels have advantages that are typical of assays from 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. These assays are now available for purchase from MSD.

Description of Markers

Parvalbumin is a 12 kDa Ca^{2+}-binding protein. There are two forms of parvalbumin (α & β) that have only 51% homology. Parvalbumin α is 110 aa and contains two EF-hand domains (aa 39-74 & 78-110) that bind calcium. In addition to the residue differences, parvalbumin β lacks the C-terminal amino acid 109 and is generally restricted in expression to the preterm placenta. Rat parvalbumin α is expressed in extrafusal and intrafusal skeletal muscle fibers whereas in humans, parvalbumin α is found mainly in muscle spindles. It 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 28 kDa glycoprotein that is expressed by a variety of cell types. The (TIMPs) were first identified as inhibitors of matrix metalloproteinases (MMPs), a family of zinc and calcium-dependent proteases responsible for the degradation of extracellular matrix components such as collagen, laminin and proteoglycans during cell growth and invasion during tumor progression and metastasis. Among the three known rat TIMPs, TIMP-1 shares 40% and 35% identity to TIMP-2 and TIMP -3, respectively. It is well established that TIMP-1 forms a non-covalent, stoichiometric complex with both latent and active MMPs, preferentially binding and inhibiting MMP-9 and MMP-1 protease function. More recently, TIMPs have been implicated in direct regulation of cell growth and apoptosis 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 responsible for regeneration of ATP within contractile and ion transport systems. In tissues that consume ATP rapidly, especially skeletal muscle, but also brain and smooth muscle, CK serves as an energy reservoir for the rapid regeneration of ATP. It allows for muscle contraction when neither glycolysis nor respiration is present by transferring a phosphate group from phosphocreatine to ADP to form ATP. CK is found in heart muscle, liver, skeletal muscle and in the brain. In most cells, the CK enzyme consists of two subunits, which can be either B (brain type) or M (muscle type). This, therefore, results in three different isoenzymes: CK-MM, CK-BB and CK-MB.

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. Though they perform similar functions, cardiac and skeletal troponins differ in sequence and can be differentiated in immunoassays.

The three subunits of troponin are:

a) Troponin T: is the subunit that interacts with tropomyosin to form the troponin-tropomyosin complex.

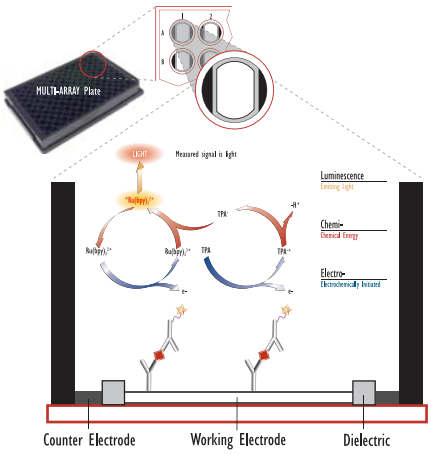
b) Troponin I: is an inhibitory subunit that prevents muscle contraction in the absence of calcium. It is responsible for the binding of the troponin-tropomyosin complex to actin. Troponin I exists in three isoforms: slow-twitch (striated) skeletal muscle, fast-twitch (striated) skeletal muscle, and cardiac muscle.

c) Troponin C: binds calcium, producing a conformational change in troponin I and activating the troponin-tropomyosin complex.

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.

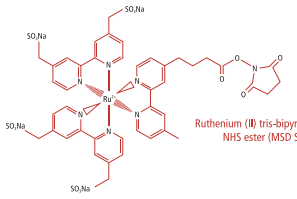
The MSD® Platform

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

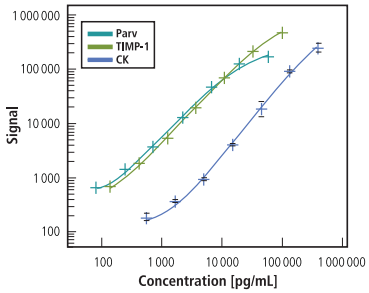


Muscle Injury Panel 2 (rat): Parvalbumin, TIMP-1, CK

Our Muscle Injury Panel 2 measures Parvalbumin, TIMP-1 and CK in rat serum and plasma. 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

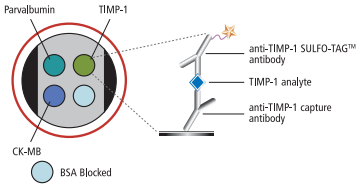
The following standard curve is an example of the dynamic range of the Muscle Injury Panel 2 multiplex panel that measures Parvalbumin, TIMP-1 and CK in rat serum and plasma. These markers are present at very high abundance in control samples and increase 100- to 1000-fold upon muscle injury. The actual signals may vary and a standard curve should be run for each set of samples on each plate for the best quantitation of unknown samples.



Parvalbumin		
Concentration (pg/mL)	Average Counts	% CV
0	314	1.7
82	680	8.5
247	1412	8.7
741	3593	5.3
2222	12364	2.8
6667	46917	3.6
20000	125562	2.7
60000	170688	0.8

TIMP-1		
Concentration (pg/mL)	Average Counts	% CV
0	199	4.6
55	690	4.6
165	1756	6.9
494	5338	0.5
1481	19336	4.0
4444	69963	5.1
13333	220947	1.3
40000	472678	1.8

CK-MB		
Concentration (pg/mL)	Average Counts	% CV
0	106	2.5
549	191	14.2
1646	362	7.0
4938	936	6.1
14815	3997	4.9
44444	19297	31.5
133333	90488	4.6
400000	251595	18.8



	Parvalbumin	TIMP-1	CK-MB
LLOD (pg/mL)	12	12	369
LLOQ (pg/mL)	200	60	1500
ULOQ (pg/mL)	48000	32000	320000

Protocol:

1 Add 25 µL Assay Diluent GF2, 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.

Spike Recovery

Pooled normal rat Serum, Heparin Plasma and EDTA Plasma were spiked with each calibrator at multiple values throughout the range of the assay. Spikes were made into neat samples.

% Recovery = measured / expected * 100

	Parvalbumin			
	Spike Level (ng/mL)	Conc. (ng/mL)	% CV	% Recovery
Spiked Serum	0	2.76	1.4	
	5.21	9.84	10	124
	10.41	15.6	9.5	119
Spiked Heparin Plasma	0	0.24	3.2	
	2.08	2.73	1.7	117
	5.21	6.62	0.1	121
	10.41	14.5	2.9	136
Spiked EDTA Plasma	0	0.24	1.0	
	2.08	2.60	8.3	112
	5.21	6.19	0.1	114
	10.41	13.0	4.0	122

	TIMP-1			
	Spike Level (ng/mL)	Conc. (ng/mL)	% CV	% Recovery
Spiked Serum	0	0.09	3.5	
	2.91	2.69	4.6	90
	7.30	7.16	3.1	97
	14.56	16.2	2.6	110
Spiked Heparin Plasma	0	0.52	2.2	
	2.91	3.34	3.7	98
	7.30	7.87	2.9	111
	14.56	18.6	2.7	124
Spiked EDTA Plasma	0	0.61	6.1	
	2.91	3.58	9.7	98
	7.30	8.27	1.3	105
	14.56	20.8	0.3	137

	CK-MB			
	Spike Level (ng/mL)	Conc. (ng/mL)	% CV	% Recovery
Spiked Serum	0	41.6	6.4	
	166.66	193	7.3	93
Spiked Heparin Plasma	0	22.6	5.1	
	8.33	31.1	3.5	106
	20.83	46.2	9.1	107
	166.66	181	3.7	94
Spiked EDTA Plasma	0	4.10	3.9	
	8.33	12.7	3.2	102
	20.83	24.8	8.8	100
	166.66	162	3.9	95

Dilutional Linearity

To assess linearity, rat EDTA Plasma samples from Sprague-Dawley normal controls were tested at multiple (10-fold thru 160-fold) dilutions for each analyte. The concentration shown below have been corrected for dilution. The control plasma was detectable at 40x dilution for all the assays, and we observe acceptable linearity. Percent recovery is calculated as the measured concentration divided by the concentration measured for the previous dilution (expected).

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

	Dilution Factor	Conc. (pg/mL)	Conc. % CV	% Recovery
Parvalbumin	10	13950	6.8	
	20	10490	4.3	75
	40	8560	5.8	82
	80	8340	2.7	97
	160	7740	2.7	93
TIMP-1	10	8970	6.6	
	20	9130	6.1	102
	40	7930	1.0	87
	80	9130	10.6	115
CK-MB	10	86070	1.7	
	20	70320	2.9	82
	40	70460	12.5	100

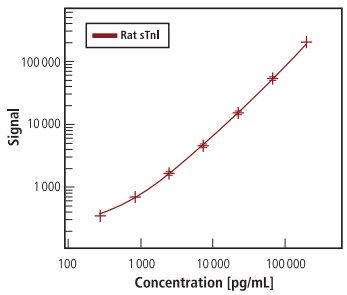
Samples

Ten Serum, Heparin and EDTA Plasma samples collected from normal, Sprague-Dawley rats were tested at 20-fold dilution on the Muscle Injury Panel – 2 (rat). Shown below are the median and range of concentrations for each sample set.

Sample	Statistic	Parvalbumin	TIMP-1	CK-MB
EDTA Plasma	Median (ng/mL)	4.90	8.30	442.0
	Range (ng/mL)	2.6 - 31	5.5 - 12	137 - 770
Heparin Plasma	Median (ng/mL)	5.50	10.10	81.0
	Range (ng/mL)	3.7 - 15	4.9 - 13	26 - 152
Serum	Median (ng/mL)	11.50	9.30	217.0
	Range (ng/mL)	6 - 27	6.1 - 22	70 - 834

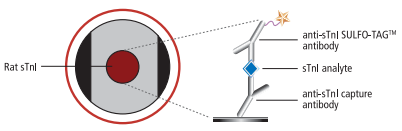
Rat Skeletal Troponin I (sTnI) Assay

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.



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
22222	14847	7.78
66667	53441	6.91
200000	206052	3.35

	Rat sTnI (pg/mL)
LLOD	374
LLOQ	781
ULOQ	160000



Rat Skeletal Troponin I (sTnI) Assay

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

Rat sTnI	Spike Level, (pg/mL)	Conc. (pg/mL)	Conc % CV	% Recovery
Spiked Serum	20000	21184	4.0	108
	6667	7220	4.6	106
	2222	2506	3.7	106
	0	<LLOD	-	-
Spiked EDTA Plasma	20000	21703	2.1	100
	6667	6718	3.7	96
	2222	2186	5.3	91
	0	<LLOD		
Spiked Heparin Plasma	20000	21797	3.8	109
	6667	7139	3.9	101
	2222	2414	3.0	96
	0	<LLOD	-	-

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	90222	4.0	
	10	81150	3.5	90
	50	82650	5.4	102
	250	61000	13.0	74
EDTA Plasma	Spiked	83170	3.9	
	10	74270	4.7	89
	50	74100	6.5	100
	250	53000	11.7	71
Heparin Plasma	Spiked	84358	5.8	
	10	74820	6.3	87
	50	75350	5.5	101
	250	54250	13.9	70

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	100445	3.6	6.2
	Mid	21	15824	3.6	5.6
	Low	21	2797	4.5	7.2

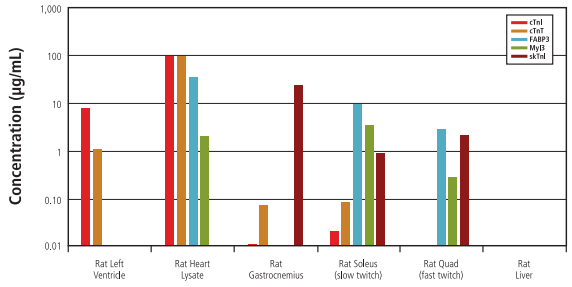
Samples

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 was below the quantitative range for all samples.

Sample	Statistic	Rat sTnI
Serum	Median (ng/mL)	336
	Range (ng/mL)	102 - 1878
	N	21
EDTA Plasma	Median (ng/mL)	378
	Range (ng/mL)	106 - 936
	N	10
Heparin Plasma	Median (ng/mL)	27
	Range (ng/mL)	< 0.374 - 514
	N	10

Specificity of Binding

Tissue homogenates from heart, fast twitch muscle, slow twitch muscle and liver were tested at multiple dilutions on Muscle Injury Panel I containing cTnI, cTnT, FABP3, MyI3 and sTnI. The assay for skeletal troponin I was specific for fast and slow twitch muscle. All markers were negative in liver homogenate. The assays for cardiac troponins were positive for cardiac homogenates and negative for other muscle homogenates, demonstrating specificity for cardiac tissue. The assay measured MyI3 and FABP3 in cardiac muscle and, to a lesser amount, in skeletal (slow twitch) muscle. Measurements of parvalbumin using another MSD assay determined that parvalbumin is present in biceps muscle (fast twitch). [1] (Data not shown)



Sample Group	cTnI (µg/mL)	cTnT (µg/mL)	FABP3 (µg/mL)	MyI3 (µg/mL)	sTnI (µg/mL)
Rat Left Ventricle	7.70	1.07	not tested	not tested	0.00
Rat Heart Lysate	101.00	95.00	34.50	2.08	0.00
Rat Gastrocnemius	0.01	0.07	not tested	not tested	22.94
Rat Soleus (slow twitch)	0.02	0.08	9.54	3.47	0.88
Rat Quad (fast twitch)	0.00	0.00	2.75	0.28	2.12
Rat Liver	0.00	0.00	0.00	0.00	0.00

Conclusions

In toxicity studies, compound- or pathologic-induced changes are typically evaluated using a combination of histochemical endpoints and often a number of potential biomarkers in biological fluids, each of which can indicate toxic change in tissues and organs. However, some biomarkers are not specific to the organ of injury and therefore there is a continuing search for more sensitive and specific indicators of target organ toxicity. MSD has developed high performance, multiplex panels and individual assays to measure both traditional and emerging biomarkers of muscle injury. 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).

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 novel biomarkers and their benefit to both preclinical and clinical studies. MSD has released certain panels as preconfigured, fully qualified kits. Other panels are available on a custom basis without full qualification.

[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.