Qualification of Muscle Injury Panel 1 Markers for Preclinical Studies

MESO SCALE DISCOVERY® Applications

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 Muscle Injury Panel 1 (rat) includes cardiac Troponin I, skeletal Troponin I, cardiac Troponin T, FABP3, and Myl3. Troponins are widely accepted biomarkers for cardiac and skeletal muscle toxicity; FABP3 and Myl3 are emerging biomarkers for muscle injury.[1] MSD also offers a Muscle Injury Panel that includes Parvalbumin, CK, and TIMP-1 and a separate assay for Skeletal Troponin I. 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. 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.

[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 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.

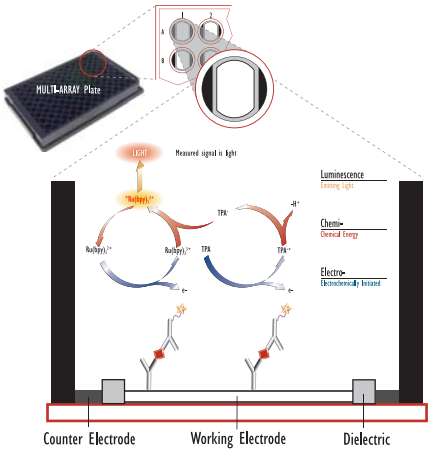
When muscle tissue is damaged, the troponin-tropomyosin complex breaks down and troponin I and troponin T are released into the blood. Cardiac troponin T (cTnT) and cardiac troponin I (cTnI) can be readily distinguished from their skeletal muscle analogs allowing confirmation of cardiac muscle tissue 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.

Myosin light chain 3 (Myl3) is an essential light chain of the myosin molecule found in cardiac and slow-twitch skeletal muscle. Myosin is a hexamer ATPase motor protein that is a major constituent of thick muscle filament. The myosin molecule consists of a head domain that "walks" along the actin chain to contract the muscle and a tail domain that is responsible for binding the myosin to its cargo. Two heavy chain subunits intertwine to form the head and tail domains and four light chain subunits—two regulatory light chains with phosphorylation sites (encoded by the MYL2 genes), and two essential light chains (encoded by the MYL3 genes). These light chains bind the heavy chains together in the neck region between the head and tail domains. After damage to muscle tissue, myosin breaks down and Myl3 becomes elevated in the blood. Myl3 can be used in conjunction with other toxicity biomarkers to confirm cardiac and slow twitch skeletal muscle injury.

Fatty acid binding protein 3 (FABP3) is a monomeric 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.

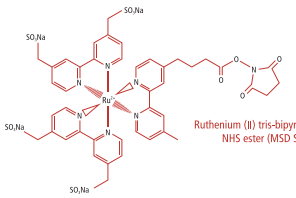
The MSD® Platform

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



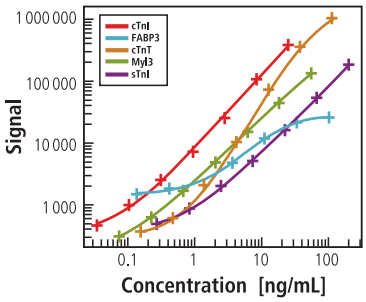
Ruthenium (II) tris-bipyridine-(4-methylsulfonate) NHS ester (MSD SULFO-TAG™ label)

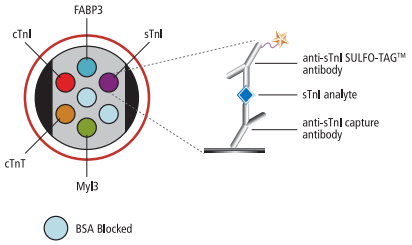
Muscle Injury Panel 1 (rat): cTnI, cTnT, FABP3, MyI3, and sTnI

Our Muscle Injury Panel 1 measures cardiac troponin I (cTnI), cardiac troponin T (cTnT), fatty acid binding protein 3 (FABP3), myosin light chain 3 (MyI3) and skeletal troponin I (sTnI) in serum or plasma. Troponins 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 actual signals may vary and a standard curve should be run for each set of samples and on each plate for the best quantitation of unknown samples.





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
0,000	232	13,6
0,034	476	2,4
0,103	984	12,3
0,309	2579	8,4
0,926	7253	6,0
2,78	26307	3,8
8,33	105531	3,8
25	385257	7,4

cTnT		
Concentration (ng/mL)	Average Counts	% CV
0,000	257	13,4
0,171	374	8,5
0,514	626	9,7
1,54	2086	7,1
4,63	10965	11,1
13,9	74886	3,5
41,7	363787	2,6
125	1058622	11,3

FABP3		
Concentration (ng/mL)	Average Counts	% CV
0,000	1301	5,1
0,137	1491	6,9
0,412	1812	10,7
1,24	2374	12,5
3,70	4802	4,6
11,1	11834	4,7
33,3	21333	6,6
100	26017	3,7

MyI3		
Concentration (ng/mL)	Average Counts	% CV
0,000	156	13,8
0,075	316	5,7
0,226	665	7,7
0,679	1709	7,3
2,04	4863	9,2
6,11	15912	6,6
18,3	46686	6,4
55,0	132236	11,8

sTnI		
Concentration (ng/mL)	Average Counts	% CV
0,000	289	12,1
0,274	496	6,6
0,823	870	11,9
2,47	2037	8,2
7,41	5150	6,0
22,2	16275	5,4
66,7	54028	5,9
200	187128	2,9

	cTnI	cTnT	FABP3	MyL3	sTnI
LLOD (ng/mL)	0.016	0.242	0.272	0.027	0.153
LLOQ (ng/mL)	0.098	0.488	0.781	0.054	0.781
ULOQ (ng/mL)	20	100	15	44	160

Precision: Multi-Day Study

Control samples were measured on 17 plates across five days. The controls were run in triplicate or quadruplicate on each plate. The control samples 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. (ng/mL)	Average % CV	% CV
cTnI	High	17	9,16	7,0	8,1
	Mid	17	1,24	7,5	12,0
	Low	17	0,26	5,1	7,0
cTnT	High	17	40,5	5,7	7,5
	Mid	17	7,12	4,4	6,1
	Low	17	1,21	4,3	9,0
FABP3	High	17	13,4	11,9	15,3
	Mid	17	8,24	10,5	12,5
	Low	17	2,75	9,5	13,0
MyI3	High	17	32,0	5,8	8,6
	Mid	17	2,90	5,3	8,9
	Low	17	0,31	7,2	10,2
sTnI	High	17	105	5,2	8,4
	Mid	17	16,8	5,0	7,1
	Low	17	2,71	5,8	9,1

Spike Recovery

Pooled normal Rat 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 are outside of the range of quantitation.

% Recovery = measured / expected * 100

	cTnI				cTnT				FABP3				MyI3				sTnI			
	Spike Level (ng/mL)	Conc. (ng/mL)	% CV	% Recovery	Spike Level (ng/mL)	Conc. (ng/mL)	% CV	% Recovery	Spike Level (ng/mL)	Conc. (ng/mL)	% CV	% Recovery	Spike Level (ng/mL)	Conc. (ng/mL)	% CV	% Recovery	Spike Level (ng/mL)	Conc. (ng/mL)	% CV	% Recovery
Spiked Serum	2,50	4,05	5,6	108	12,5	11,61	3,2	88	10,0	*16,0*	*12,9*	*114*	5,50	8,84	0,9	139	20,0	23,0	9,2	113
	0,83	2,24	3,0	107	4,17	4,73	4,5	96	3,33	8,28	3,29	113	1,83	3,74	4,0	139	6,67	8,38	6,5	120
	0,28	1,53	3,9	100	1,39	1,98	4,6	92	1,11	5,03	1,06	98	0,61	1,81	2,9	123	2,22	2,99	3,5	117
	0,00	1,26	5,0		0,00	0,76	3,4		0,00	4,01	2,40		0,00	0,85	5,7		0,00	*0,33*	*54*	
Spiked Heparin Plasma	2,50	4,20	3,2	110	12,5	12,39	0,7	91	10,0	*32,6*	*5,65*	*90*	5,50	8,49	3,2	137	20,0	27,4	1,8	133
	0,83	2,27	3,6	106	4,17	4,89	7,0	93	3,33	*29,2*	*8,02*	*99*	1,83	3,11	11,7	124	6,67	8,95	2,5	124
	0,28	1,57	17,4	99	1,39	2,32	8,7	94	1,11	*23,8*	*3,13*	*87*	0,61	1,51	10,6	117	2,22	3,66	8,0	132
	0,00	1,31	4,8		0,00	1,08	3,7		0,00	*26,2*	*4,30*		0,00	0,68	5,7		0,00	0,56	10,8	
Spiked EDTA Plasma	2,50	4,30	5,5	102	12,5	10,47	4,6	77	10,0	13,6	1,96	100	5,50	7,80	11,3	134	20,0	19,3	1,4	95
	0,83	2,62	5,2	102	4,17	4,47	4,8	84	3,33	6,54	8,38	93	1,83	2,95	2,2	136	6,67	6,50	11,9	94
	0,28	2,21	6,7	110	1,39	2,33	3,0	92	1,11	4,35	0,69	91	0,61	1,10	10,2	116	2,22	2,59	10,0	104
	0,00	1,73	18,2		0,00	1,15	10,7		0,00	3,66	7,55		0,00	0,33	7,8		0,00	*0,27*	*62*	

Muscle Injury Panel 1 (rat): cTnI, cTnT, FABP3, MyI3, and sTnI

Dilutional Linearity

To assess linearity, rat Serum, EDTA Plasma, and Heparin Plasma samples were tested undiluted (neat) and at 2-fold, 4-fold, and 8-fold dilution. The concentrations shown below have been corrected for dilution. Percent recovery is calculated as the measured concentration divided by the concentration measured for the previous dilution (expected). Values in italics are above the assay ULOQ.

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

		cTnI			cTnT			FABP3			MyI3			sTnI		
Sample	Fold Dilution	Conc. (ng/mL)	Conc. % CV	% Recovery	Conc. (ng/mL)	Conc. % CV	% Recovery	Conc. (ng/mL)	Conc. % CV	% Recovery	Conc. (ng/mL)	Conc. % CV	% Recovery	Conc. (ng/mL)	Conc. % CV	% Recovery
Serum	Neat	1.52	8.8		2.08	2.5		*16.9*	*28*		1.22	18.0		30.7	3.1	
	2	1.50	0.8	98	1.91	10.8	92	16.7	20	99	1.01	9.4	82	26.8	4.6	87
	4	1.35	9.3	90	1.98	12.2	103	14.3	11	86	0.86	17.7	85	26.9	6.9	101
	8	1.44	2.6	107	< LLOQ			11.8	16	82	0.85	6.5	100	35.7	3.0	132
Heparin Plasma	Neat	1.49	6.6		2.08	6.8		*21.9*	*13*		1.30	11.0		123	1.7	
	2	1.39	6.1	93	2.20	2.9	106	21.3	27	97	1.15	5.1	89	121	3.5	98
	4	1.55	5.5	111	2.04	7.4	93	19.7	12	93	1.02	8.5	89	134	7.2	111
	8	1.45	7.0	94	< LLOQ			20.5	4.4	104	0.86	16.5	85	131	5.2	97
EDTA Plasma	Neat	1.89	14.5		1.05	12.5		3.99	14		1.33	9.6		30.7	3.1	
	2	1.99	1.2	105	1.14	7.5	109	3.72	29	93	1.20	6.6	90	26.8	4.6	87
	4	1.80	6.7	91	< LLOQ			3.74	14	100	0.96	14.2	80	26.9	6.9	101
	8	2.03	4.9	113	< LLOQ			< LLOQ			0.94	4.9	98	35.7	3.0	132

Samples

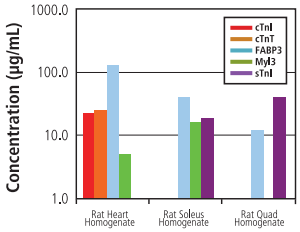
Serum, Heparin Plasma, and EDTA Plasma samples collected from normal, Sprague-Dawley rats were tested at 2-fold dilution on the Muscle Injury Panel 1 (rat). Shown below are the median and range of concentrations for each sample set. Skeletal Troponin I was below the quantitative range for all samples.

Sample	Statistic	cTnI	cTnT	FABP3	MyI3	sTnI
EDTA Plasma	Median (ng/mL)	1.59	0.83	19.84	0.61	< 1.56
	Range (ng/mL)	0.44 - 3.53	< 0.976 - 2.04	5.28 - > 30	0.25 - 1.05	< 1.56
	# of Samples	10	10	10	10	10
Heparin Plasma	Median (ng/mL)	1.81	1.06	28.35	0.55	< 1.56
	Range (ng/mL)	0.37 - 4.10	< 0.976 - 2.79	4.12 - > 30	0.16 - 1.16	< 1.56
	# of Samples	10	10	10	10	10
Serum	Median (ng/mL)	2.31	1.28	35.63	0.81	< 1.56
	Range (ng/mL)	1.45 - 4.05	< 0.976 - 2.80	19.6 - > 30	0.43 - 1.17	< 1.56
	# of Samples	10	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 muscle 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. MSD has released certain panels as preconfigured, fully qualified kits. Other panels are available on a custom basis, albeit without full qualification. The Muscle Injury Panel 1 (rat) is now available for purchase from MSD.