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 (a & b) that have only 51% homology, Parvalbumin a is 110 aa and contains two EF-hand domains (aa 39-74 & 78-110) which bind calcium. In addition to the residue differences, parvalbumin b lacks the C-terminal amino acid 109 and is generally restricted in expression to the preterm placenta.

Parental parvalbumin a is expressed in extracellular and intracellular skeletal muscle fibers whereas in humans, parvalbumin a 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 45% 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 no glycolysis or 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-MB, CK-MM and CK-BB.

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 immunomesss.

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 (stallate) skeletal muscle, fast-twitch (stallate) 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 cardotoxicity 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-SLOT® microplates.

Electrochemiluminescence Features:

- Minimal background signal and high signal to background ratio - the stimulation mechanism (electricity) is decoupled from the signal (light)
- Proximity - only labels bound near the electrode surface are detected, enabling non-washed assays
- Reversibility - labels are stable, non-radioactive, and can be reversibly conjugated to biological molecules
- Emission at 620 nm - eliminating problems with color quenching
- Signal amplification - multiple excitation cycles of each label enhance light level 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 validated this panel according to typical practices for pre-clinical biomarkers. The validation procedure involved multi-day controls, establishment of limits of quantification, 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.

![Standard Curve Graph](image.png)

**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.

\[
\% \text{ Recovery} = \frac{\text{measured}}{\text{expected}} \times 100
\]

**Dilutional Linearity**

To assess linearity, rat EDTA plasma samples from Sprague-Dawley control rats 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 40-fold 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).

\[
\% \text{ Recovery} = \frac{\text{measured}}{\text{dilution factor}} \times \frac{\text{expected}}{100}
\]

**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.

**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 validation 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 validation, 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 muscle-injuring 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 cTnl. These experiments showed that sTnI and cTnl do not cross-react and each is specific for its 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 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 showed good recovery.

\[
\% \text{ Recovery} = \frac{\text{measured} \times 2 \text{-fold}}{\text{expected}} \times 100
\]

**Dilution 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).

\[
\% \text{ Recovery} = \frac{\text{measured} \times \text{dilution factor}}{\text{expected}} \times 100
\]

**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, MyB, 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 MyB and FABP3 in cardiac muscle and, to a lesser amount, in skeletal (slow twitch) muscle. Measurements of paraalbumin using another MSD assay determined that paraalbumin is present in bovine muscle (fast twitch). (Data not shown)

**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 changes in muscle 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 with full qualification.

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