

Meso Scale Discovery[®]

MULTI-SPOT[®] Assay System

Muscle Injury Panel 1 (rat) Assay Kit

1-Plate Kit

K15181C-1

5-Plate Kit

K15181C-2

25-Plate Kit

K15181C-4

This product insert should be used with kit lots: K0040062
K0040075

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MSD Toxicology Assays

Muscle Injury Panel 1 (rat) Assay Kit

cTnl, cTnT, sTnl, FABP3, Myl3

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Meso Scale Discovery

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Ordering Information

ordering information

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MSD Advantage

MSD advantage

MESO SCALE DISCOVERY'S MULTI-ARRAY[®] Technology is a multiplex immunoassay system that enables the measurement of biomarkers utilizing the next generation of electrochemiluminescent detection. In an MSD[®] assay, specific capture antibodies for the analytes are coated in arrays in each well of a 96-well carbon electrode plate surface. The detection system uses patented SULFO-TAG[™] labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of the MULTI-ARRAY and MULTI-SPOT[®] plates. The electrical stimulation is decoupled from the output signal, which is light, and this generates assays with minimal background. MSD labels are stable, non-radioactive, and can be conveniently conjugated to biological molecules. Additionally, only labels near the electrode surface are detected, enabling non-washed assays.

One of the advantages of MSD assays is the minimal sample volume required as compared to traditional ELISA, which is also limited by its ability to measure only a single analyte. With an MSD assay, ten different biomarkers can be analyzed simultaneously using as little as 10-25 μ L of sample. These assays have high sensitivity, up to five logs of linear dynamic range, and excellent performance in complex biological matrices. Combined, these advantages enable the measurement of native levels of biomarkers in normal and diseased samples without multiple dilutions. Further, the simple and rapid protocols of MSD assays provide a powerful tool to generate reproducible and reliable results. The MSD product line offers a diverse menu of assay kits for profiling biomarkers, cell signaling pathways, and other applications, as well as a variety of plates and reagents for assay development.



Introduction

i n t r o d u c t i o n

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:

- **Troponin T** is the subunit that interacts with tropomyosin to form the troponin-tropomyosin complex.
- **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.
- **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.



Principle of the Assay

principle of the assay

MSD[®] toxicology assays provide a rapid and convenient method for measuring the levels of protein targets within a single small-volume sample. The assays are available in both singleplex and multiplex formats. In a singleplex assay, an antibody for a specific protein target is coated on one electrode (or “spot”) per well. In a multiplex assay, an array of capture antibodies against different targets is patterned on distinct spots in the same well. Our Muscle Injury Panel 1 (rat) Assay is a sandwich immunoassay (Figure 1). MSD provides a plate that has been pre-coated with capture antibodies for cTnI, cTnT, sTnI, FABP3, and MyI3 on spatially distinct spots. The user adds the sample and a solution containing the labeled detection antibodies—anti-cTnI, anti-cTnT, anti-sTnI, anti-FABP3, and anti-MyI3 labeled with an electrochemiluminescent compound, MSD SULFO-TAG[™] label—over the course of one or more incubation periods. Analytes in the sample bind to capture antibodies immobilized on the working electrode surface; recruitment of the labeled detection antibodies by bound analytes completes the sandwich. The user adds an MSD read buffer that provides the appropriate chemical environment for electrochemiluminescence and loads the plate into an MSD SECTOR[®] instrument for analysis. Inside the SECTOR instrument, a voltage applied to the plate electrodes causes the labels bound to the electrode surface to emit light. The instrument measures intensity of emitted light to afford a quantitative measure of cTnI, cTnT, sTnI, FABP3 and MyI3 present in the sample.

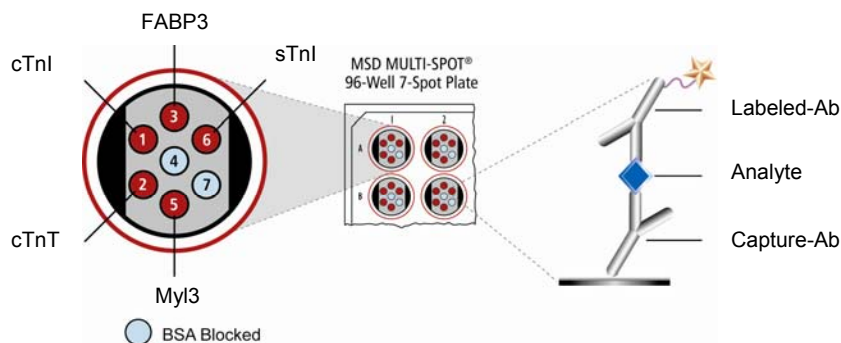


Figure 1. Spot diagram showing placement of analyte capture antibody. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files. A unique bar code label on each plate allows complete traceability back to MSD manufacturing records.

IV

Reagents Supplied

reagents supplied

Product Description	Storage	Quantity per Kit		
		K15181C-1	K15181C-2	K15181C-4
MULTI-SPOT® 96-well Muscle Injury Panel 1 (rat) Plate N75181A-1	2–8°C	1 plate	5 plates	25 plates
SULFO-TAG™ Anti-rat cTnI Antibody (50X) ¹	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
SULFO-TAG Anti-rat cTnT Antibody (50X) ¹	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
SULFO-TAG Anti-rat FABP3 Antibody (50X) ¹	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
SULFO-TAG Anti-rat Myl3 Antibody (50X) ¹	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
SULFO-TAG Anti-rat sTnI Antibody (50X) ¹	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
Muscle Injury Panel 1 (rat) Calibrator Blend (20X) Lot A0040030 cTnI: 468 ng/mL cTnT: 940 ng/mL sTnI: 4180 ng/mL FABP3: 1904 ng/mL Myl3: 994 ng/mL	≤ -70°C	1 vial (20 µL)	5 vials (20 µL ea)	25 vials (20 µL ea)
Diluent 7 R54BB-4 (5 mL) R54BB-3 (50 mL)	≤ -10°C	2 bottles (5 mL ea)	1 bottle (50 mL)	5 bottles (50 mL ea)
Diluent 30 R50AB-4 (25 mL)	≤ -10°C	1 bottle (25 mL)	1 bottle (25 mL)	5 bottles (25 mL ea)
25 mM DTT	≤ -10°C	1 vial (1 mL)	1 vial (1 mL)	5 vials (1 mL ea)
0.5 M EDTA pH 8.0	RT	1 bottle (4 mL)	1 bottle (4 mL)	5 bottles (4 mL ea)
Read Buffer T (4X) R92TC-3 (50 mL)	RT	1 bottle (50 mL)	1 bottle (50 mL)	5 bottles (50 mL ea)

¹ Some SULFO-TAG labeled detection antibodies may be light-sensitive, so they should be stored in the dark.

V

Required Materials and Equipment - not supplied

required materials and equipment — not supplied

- Deionized water for diluting concentrated buffers
- 50 mL tubes for reagent preparation
- 15 mL tubes for reagent preparation
- Microcentrifuge tubes for preparing serial dilutions
- Phosphate buffered saline plus 0.05% Tween-20 (PBS-T) for plate washing
- Appropriate liquid handling equipment for desired throughput, capable of dispensing 10 to 150 μ L into a 96-well microtiter plate
- Plate washing equipment: automated plate washer or multichannel pipette
- Adhesive plate seals
- Microtiter plate shaker

VI

Safety

safety

Safe laboratory practices and personal protective equipment such as gloves, safety glasses, and lab coats should be used at all times during the handling of all kit components. All hazardous samples should be handled and disposed of properly, in accordance with local, state, and federal guidelines. Material Safety Data Sheets are available upon request.

VII

Reagent Preparation

reagent preparation

Bring all plates and diluents to room temperature. *This is especially important for the Diluent 7, as some components are not soluble below room temperature.* Thaw the stock Calibrator blend on ice.

Important: Upon first thaw, separate Diluent 7 and Diluent 30 into aliquots appropriate to the size of your assay needs. These diluents can go through up to three freeze-thaw cycles without significantly affecting the performance of the assay.

Prepare Diluent 7 + Additives

For Muscle Injury Panel 1, samples and Calibrators are diluted in Diluent 7 that contains EDTA and DTT. These two Additives must be added into the diluent by the user before each assay is carried out. EDTA and DTT additive stocks are provided at the concentrations in the table below.

For one plate combine:

- 540 μ L of EDTA stock solution
- 90 μ L of DTT stock solution
- 8370 μ L of Diluent 7

If sample dilution is not required, then a smaller volume of this reagent can be prepared.

Additive	Stock Conc.	Final Conc.
EDTA	500 mM (16.7X)	30 mM (1X)
DTT	25 mM (100X)	0.25 mM (1X)

Prepare Calibrator Solutions

Calibrators for the Muscle Injury Panel 1 (rat) are supplied at 20-fold higher concentration than the recommended highest Calibrator. For each assay, an 8-point standard curve is recommended with 3-fold serial dilution steps and a zero Calibrator. The Calibrators are supplied as a blend. The concentrations provided are for Calibrator Lot A0040030. The stock Calibrator blend should be thawed and kept on ice, but should be added into diluent at room temperature to make the standard curve solutions (STD-01 through STD-08 below). The table below shows the concentrations of the 8-point standard curve:

Standard	cTnI (ng/mL)	cTnT (ng/mL)	FABP3 (ng/mL)	MyI3 (ng/mL)	sTnI (ng/mL)	Dilution Factor
Stock Cal. Vial	468	940	1904	994	4180	
STD-01	23.4	47.0	95.2	49.7	209	20
STD-02	7.80	15.7	31.7	16.6	69.7	3
STD-03	2.60	5.22	10.6	5.52	23.2	3
STD-04	0.87	1.74	3.53	1.84	7.74	3
STD-05	0.29	0.58	1.18	0.61	2.58	3
STD-06	0.10	0.19	0.39	0.20	0.86	3
STD-07	0.032	0.064	0.13	0.068	0.29	3
STD-08	0	0	0	0	0	n/a

To prepare this 8-point standard curve for up to 5 replicates:

- 1) Prepare the highest Calibrator by adding 12 μ L of the Calibrator stock vial to 228 μ L of Diluent 7 + Additives.
- 2) Prepare the next Calibrator by transferring 80 μ L of the diluted Calibrator to 160 μ L of Diluent 7 + Additives. Repeat 3-fold serial dilutions 5 additional times to generate 7 Calibrators.
- 3) The recommended 8th Standard is Diluent 7 + Additives alone (i.e. zero Calibrator).

After preparation of the Calibrators at the concentrations above, incubate the Calibrator solutions without shaking for 30 minutes at room temperature prior to addition to the plate.

Dilution of Samples

Rat serum and plasma samples should be diluted prior to the assay. A 4-fold dilution of serum and plasma samples into Diluent 7 + Additives is recommended for this multiplex panel. Depending on the sample set under investigation, higher or lower dilution factors may be necessary. To perform sample dilution, add 25 μ L sample to 75 μ L of Diluent 7 + Additives.

Diluted samples should be incubated without shaking at room temperature for 30 minutes prior to addition to the plate.

Prepare Detection Antibody Solution

The Detection Antibodies are provided as a 50X stock solution. The working Detection Antibody Solution should contain 1X as final concentration of each antibody.

In a 15 mL tube combine (per plate):

- 60 μ L of 50X SULFO-TAG Anti-rat cTnI Antibody
- 60 μ L of 50X SULFO-TAG Anti-rat cTnT Antibody
- 60 μ L of 50X SULFO-TAG Anti-rat FABP3 Antibody
- 60 μ L of 50X SULFO-TAG Anti-rat MyI3 Antibody
- 60 μ L of 50X SULFO-TAG Anti-rat sTnI Antibody
- 2700 μ L of Diluent 30

Prepare Read Buffer

The Read Buffer should be diluted 4-fold in deionized water to make a final concentration of 1X Read Buffer T. Add 5 mL of 4X Read Buffer T to 15 mL of deionized water for each plate.

Prepare MSD Plate

This plate has been pre-coated with antibody for the analytes shown in Figure 1. The plate can be used as delivered; no additional preparation (e.g., pre-wetting) is required. The plate has also been exposed to a proprietary stabilizing treatment to ensure the integrity and stability of the immobilized antibodies.

VIII Assay Protocol

assay protocol

(Dilution of samples/Calibrators should be completed prior to Step 1)

- 1. Addition of Diluent 7 + Additives:** Dispense 25 μ L of Diluent 7 + Additives into each well. Seal the plate with an adhesive plate seal and incubate for 30 min with vigorous shaking (300–1000 rpm) at room temperature.
- 2. Addition of the Sample or Calibrator:** Dispense 25 μ L of sample or Calibrator (which has been pre-incubated for 30 min following dilution with Diluent 7 + Additives) into separate wells of the MSD plate. Seal the plate with an adhesive plate seal and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.
- 3. Wash and Addition of the Detection Antibody Solution:** Wash the plate 3 times with PBS-T. Dispense 25 μ L of the 1X Detection Antibody Solution into each well of the MSD plate. Seal the plate and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.
- 4. Wash and Read:** Wash the plate 3 times with PBS-T. Add 150 μ L of 1X Read Buffer T to each well of the MSD plate. Analyze the plate on the SECTOR Imager. Plates should be read immediately after the addition of Read Buffer.

Notes

Shaking a 96-well MSD MULTI-SPOT plate typically accelerates capture at the working electrode.

Bubbles in the fluid will interfere with reliable reading of MULTI-SPOT plate. Use reverse pipetting techniques to insure bubbles are not created when dispensing the Read Buffer.

IX

Analysis of Results

analysis of results

The Calibrators should be run in duplicate to generate a standard curve. The standard curve is modeled using least squares fitting algorithms so that signals from samples with known levels of the analyte of interest can be used to calculate the concentration of analyte in the sample. The assays have a wide dynamic range (3-4 logs) which allows accurate quantitation in many samples without the need for dilution. The MSD DISCOVERY WORKBENCH[®] analysis software utilizes a 4-parameter logistic model (or sigmoidal dose-response) and includes a $1/Y^2$ weighting function. The weighting function is important because it provides a better fit of data over a wide dynamic range, particularly at the low end of the standard curve.

For the best fit of the data, the zero Calibrator should typically be excluded from the fit model. The MyI3 assay is an exception to this rule because the LLOQ is below the lowest standard. If measurements below the STD-07 concentration are required, the zero calibrator should be included in the fit. In the MSD DISCOVERY WORKBENCH analysis software, this can be accomplished by setting up the standard curve in the plate layout in two steps:

- 1) Select the wells for STD-01 through STD-07, and define the concentrations for all assays. The "Apply To All Assays" checkbox should be unchecked so that different concentrations can be defined for each assay. After entering the concentrations, click "OK" to close out the "Assign Standard To Wells" window.
- 2) Select the wells for STD-08 (zero Calibrator), and define only the MyI3 concentration to be zero. Concentrations for the other assays should be left blank. Click "OK" to close out the "Assign Standard To Wells" window.

X

Assay Qualification and Verification

assay qualification and verification

The performance of this MSD Qualified Kit meets levels of consistency and robustness as determined by methods based on the principles outlined in “Fit -for-Purpose Method Development and Validation for Successful Biomarker Measurement” by Lee, J.W. et al.¹

Bioanalytical and functional characterizations of calibrators, antibodies and assay components are completed to allow for bridging of reagents between lots. This includes plate coating uniformity and reagent and component specificity testing for individual kit lots.

Control samples for specific matrices are designed and tested to meet the accuracy, precision and sensitivity criteria for a MSD Qualified Kit. Spike recovery and dilution linearity of endogenous samples, pooled and individual matrices are tested across the assay range.

➤ **Sensitivity, Range and Curve Fitting**

- Sample range and assay sensitivity are established from 4-PL fitted calibration curves with $1/Y^2$ weighting. Percent recovery of calibrators and controls between the upper limit of quantification (ULOQ) and lower limit of quantification (LLOQ) must have calculated concentration %CV of less than 20% and accuracy within 20% of the expected concentration.
- The limits of quantification defined in the product insert are verified for each lot as part of the lot verification and quality control release.

➤ **Accuracy and Precision**

High, mid, and low controls made in matrix (need to be defined on a kit by kit basis) are run to measure accuracy and precision.

- Qualification – Testing on multiple days (>6 days) and multiple runs per day for a total of 15-20 runs of complete kits. Precision is measured for the standard curve for intra- and intra-day CVs of less than 20%. CVs and accuracy of the controls are measured on all runs and must meet the kit specification as defined in the Certificate of Analysis (C of A). The typical calculated concentration CV specification is less than 20% and accuracy with 20% of expected concentration and a total error of less than 30%.
- Verification – A multi-day (2-3 days), multiple runs per day for a total of 6-12 plates is performed as part of the release testing for each lot. The specifications for release are provided in the C of A.

➤ **Robustness and Stability**

Freeze-thaw testing and accelerated stability studies performed during assay development (calibrators, antibodies, controls) are augmented with real-time stability studies on complete kits out to 18 months from the date of manufacture.

All acceptance criteria and verification conformance are defined in the C of A for all kit lots. Presented below are representative data from the assay qualification for this assay that meets the criteria described above. The actual kit-specific standard curve and measured limits of quantification can be found in the C of A enclosed with the kit.

XI

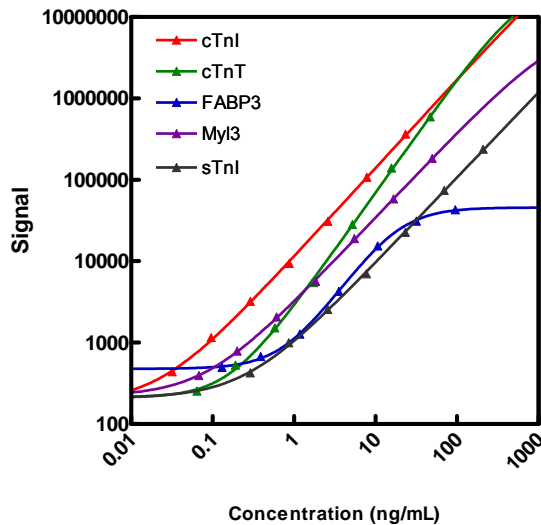
Typical Standard Curve

typical 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.

cTnl			cTnT		
Conc. (ng/mL)	Average Signal	%CV	Conc. (ng/mL)	Average Signal	%CV
0	107	12.4	0	120	8.5
0.032	440	6.2	0.064	254	6.8
0.096	1155	7.0	0.19	529	6.7
0.29	3214	6.4	0.58	1519	5.6
0.87	9433	5.5	1.74	5468	5.1
2.6	31018	4.5	5.2	28325	4.1
7.8	107673	4.0	15.7	139526	5.9
23.4	365820	7.0	47.0	594838	5.6

FABP3			MyI3		
Conc. (ng/mL)	Average Signal	%CV	Conc. (ng/mL)	Average Signal	%CV
0	421	6.8	0	167	10.8
0.13	496	7.4	0.068	394	4.8
0.39	675	4.9	0.20	785	6.6
1.18	1270	7.1	0.61	2066	5.1
3.53	4257	5.2	1.84	5645	6.4
10.6	15362	5.7	5.5	19059	3.9
31.7	31031	6.0	16.6	58134	3.0
95.2	42881	6.4	49.7	182582	3.5



sTnl		
Conc. (ng/mL)	Average Signal	%CV
0	165	9.6
0.29	427	5.8
0.86	996	3.7
2.58	2556	3.0
7.74	7019	3.8
23.2	22614	3.5
69.7	74092	4.5
209	237149	6.1

XII

Sensitivity

sensitivity

The lower limit of detection (LLOD) is measured as the concentration at 2.5 standard deviations over the zero Calibrator.

A multi-plate, multi-day study was performed to measure the reproducibility of the assay. The lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) were established from the multiple plate run.

The LLOQ is determined as the lowest concentration where the %CV of the calculated concentration is less than 20% and the percent recovery of the standard is between 80% and 120%. For the FABP3 and Myl3 assay's LLOQ, the %CV of the calculated concentration is less than 25% and the percent recovery of the standard is between 75% and 125%.

The ULOQ is determined as the highest concentration where the %CV of the calculated concentration is less than 20% and the percent recovery of the standard is between 80% and 120%. For the FABP3 assay's ULOQ, the %CV of the calculated concentration is less than 25% and the percent recovery of the standard is between 75% and 125%.

	cTnl (ng/mL)	cTnT (ng/mL)	FABP3 (ng/mL)	Myl3 (ng/mL)	sTnl (ng/mL)
LLOD	0.0040	0.017	0.16	0.015	0.047
LLOQ	0.098	0.488	0.391	0.054	0.781
ULOQ	20.0	35	15.0	44.0	160

XIII

Precision

precision

The controls were run in triplicate or quadruplicate on each plate. Normal rat serum, rat soleus homogenate, and assay Calibrators are used to make control samples. The high control contains 25% normal rat serum and Calibrators. The mid control contains rat soleus homogenate and Calibrators. The low control contains only the assay Calibrators.

Average Intra-plate % CV is the average percent CV of the control replicates within an individual plate. Inter-plate %CV is the variability of controls across 9 plates over 3 days.

	Control	Plates	Average Conc. (ng/mL)	Average Intra-plate %CV	Inter-plate %CV
cTnl	High	9	9.36	3.8	5.9
	Mid	9	1.31	3.9	6.0
	Low	9	0.28	4.0	5.1
cTnT	High	9	35.4	3.9	5.0
	Mid	9	6.46	2.5	4.0
	Low	9	1.06	3.3	4.6
FABP3	High	9	13.9	5.9	7.5
	Mid	9	8.44	5.5	7.6
	Low	9	2.85	2.8	4.8
Myl3	High	9	30.3	5.9	7.0
	Mid	9	3.01	3.8	6.9
	Low	9	0.32	6.0	7.1
sTnl	High	9	108	4.3	5.8
	Mid	9	17.4	2.4	3.1
	Low	9	2.88	3.5	10.5

XIV

Spike Recovery

spike recovery

Normal rat serum, EDTA plasma, and heparin plasma were diluted 4-fold and spiked with the Calibrators at multiple values throughout the range of the assay. Values in italics are outside of the range of quantitation.

% Recovery = measured / expected x 100

cTnI	Spike Conc. (ng/mL)	Measured Conc. (ng/mL)	Measured Conc. %CV	% Recovery
Serum #1	2.34	3.90	8.3	102
	0.78	2.37	3.8	104
	0.26	1.80	5.2	102
	0	1.50	2.6	
Serum #2	2.34	2.67	1.9	114
	0.78	0.93	3.9	119
	0.26	0.32	2.5	124
	0	0		
Serum #3	2.34	2.80	5.6	120
	0.78	0.98	2.3	126
	0.26	0.33	7.0	128
	0	0		
EDTA Plasma #1	2.34	2.74	3.3	115
	0.78	1.04	2.6	127
	0.26	0.37	4.0	125
	0	0.04	1.5	
EDTA Plasma #2	2.34	2.74	3.7	116
	0.78	0.98	4.1	121
	0.26	0.37	5.5	130
	0	0.03	5.1	
EDTA Plasma #3	2.34	2.77	2.4	118
	0.78	1.00	2.0	128
	0.26	0.35	1.1	135
	0	0		
Heparin Plasma #1	2.34	2.99	3.6	115
	0.78	1.20	3.9	114
	0.26	0.59	5.9	111
	0	0.27	1.8	
Heparin Plasma #2	2.34	2.82	8.4	108
	0.78	1.27	0.2	120
	0.26	0.61	3.2	114
	0	0.28	6.4	
Heparin Plasma #3	2.34	2.71	2.2	102
	0.78	1.10	1.4	100
	0.26	0.61	2.2	105
	0	0.32	3.1	

cTnT	Spike Conc. (ng/mL)	Measured Conc. (ng/mL)	Measured Conc. %CV	% Recovery
Serum #1	4.70	5.84	3.8	103
	1.57	2.53	5.4	99
	0.52	1.55	2.4	103
	0	0.99	5.2	
Serum #2	4.70	4.20	2.1	89
	1.57	1.27	3.8	81
	0.52	0.40	4.3	76
	0	0		
Serum #3	4.70	4.31	4.0	92
	1.57	1.37	0.9	88
	0.52	0.44	6.0	85
	0	0		
EDTA Plasma #1	4.70	4.56	1.9	97
	1.57	1.44	1.2	92
	0.52	0.46	3.7	88
	0	0		
EDTA Plasma #2	4.70	4.42	3.1	94
	1.57	1.35	1.0	86
	0.52	0.46	8.8	87
	0	0		
EDTA Plasma #3	4.70	4.32	0.7	92
	1.57	1.34	1.1	86
	0.52	0.44	5.7	85
	0	0		
Heparin Plasma #1	4.70	4.70	3.3	99
	1.57	1.54	3.1	95
	0.52	0.58	3.6	101
	0	0.05	35.0	
Heparin Plasma #2	4.70	4.40	9.4	92
	1.57	1.61	0.9	99
	0.52	0.59	3.7	101
	0	0.06	86.7	
Heparin Plasma #3	4.70	4.84	3.7	100
	1.57	1.58	3.9	93
	0.52	0.66	2.2	102
	0	0.13	10.4	

FABP3	Spike Conc. (ng/mL)	Measured Conc. (ng/mL)	Measured Conc. %CV	% Recovery
Serum #1	9.52	32.0	21.7	84
	3.17	30.6	8.8	97
	1.06	26.9	7.6	91
	0	28.4	10.1	
Serum #2	9.52	12.1	10.8	119
	3.17	4.51	4.6	118
	1.06	1.84	2.2	108
	0	0.64	4.8	
Serum #3	9.52	12.6	2.3	119
	3.17	4.86	6.1	115
	1.06	2.19	4.8	104
	0	1.05	10.2	
EDTA Plasma #1	9.52	19.0	2.5	91
	3.17	14.2	1.2	98
	1.06	12.5	11.4	101
	0	11.3	5.9	
EDTA Plasma #2	9.52	17.4	5.6	96
	3.17	12.7	4.1	107
	1.06	10.1	6.6	104
	0	8.66	4.6	
EDTA Plasma #3	9.52	12.1	6.5	111
	3.17	5.15	1.1	114
	1.06	2.51	4.0	105
	0	1.33	8.3	
Heparin Plasma #1	9.52	22.4	8.0	103
	3.17	16.9	12.0	110
	1.06	13.8	4.6	105
	0	12.1	7.4	
Heparin Plasma #2	9.52	20.6	8.3	91
	3.17	14.8	6.9	91
	1.06	12.4	4.1	88
	0	13.1	30.7	
Heparin Plasma #3	9.52	23.8	9.2	107
	3.17	16.4	1.2	103
	1.06	14.6	8.4	106
	0	12.8	0.9	

Myl3	Spike Conc. (ng/mL)	Measured Conc. (ng/mL)	Measured Conc. %CV	% Recovery
Serum #1	4.97	6.81	6.1	105
	1.66	3.47	4.2	110
	0.55	2.28	3.5	111
	0	1.51	4.2	
Serum #2	4.97	6.14	6.5	122
	1.66	2.05	5.2	120
	0.55	0.69	6.9	115
	0	0.05	40.1	
Serum #3	4.97	6.14	4.4	122
	1.66	2.06	4.3	120
	0.55	0.73	4.4	121
	0	0.06	8.5	
EDTA Plasma #1	4.97	6.29	2.5	117
	1.66	2.39	0.6	116
	0.55	1.08	6.4	114
	0	0.39	2.9	
EDTA Plasma #2	4.97	6.21	3.6	114
	1.66	2.46	3.2	114
	0.55	1.15	8.1	110
	0	0.50	6.2	
EDTA Plasma #3	4.97	6.28	3.6	124
	1.66	2.16	2.6	124
	0.55	0.77	5.6	122
	0	0.08	19.2	
Heparin Plasma #1	23.52	6.28	3.7	115
	9.22	2.53	7.9	116
	4.50	1.24	11.3	116
	1.92	0.52	11.4	
Heparin Plasma #2	24.26	6.48	2.8	118
	9.42	2.58	3.6	118
	4.48	1.24	1.5	114
	1.98	0.53	5.5	
Heparin Plasma #3	4.97	6.69	0.5	119
	1.66	2.66	4.3	114
	0.55	1.43	3.2	117
	0	0.67	3.4	

sTnl	Spike Conc. (ng/mL)	Measured Conc. (ng/mL)	Measured Conc. %CV	% Recovery
Serum #1	20.86	26.1	3.2	107
	6.95	11.0	3.2	105
	2.32	6.14	2.8	106
	0	3.50	1.6	
Serum #2	20.86	20.6	4.3	98
	6.95	7.20	5.9	103
	2.32	2.48	5.3	106
	0	0.03	64.0	
Serum #3	20.86	21.7	3.6	104
	6.95	7.25	2.4	104
	2.32	2.44	1.5	105
	0	0.01	71.4	
EDTA Plasma #1	20.86	25.4	2.5	101
	6.95	12.0	5.1	106
	2.32	6.92	6.1	103
	0	4.40	2.7	
EDTA Plasma #2	20.86	24.3	3.3	101
	6.95	11.1	1.7	109
	2.32	5.79	4.1	105
	0	3.19	5.6	
EDTA Plasma #3	20.86	20.2	3.1	96
	6.95	7.26	1.5	103
	2.32	2.59	3.0	108
	0	0.08	63.1	
Heparin Plasma #1	20.86	24.3	6.2	101
	6.95	10.8	5.4	106
	2.32	5.70	7.6	103
	0	3.23	8.2	
Heparin Plasma #2	20.86	26.0	5.4	109
	6.95	11.3	6.2	114
	2.32	5.72	3.0	108
	0	3.00	3.3	
Heparin Plasma #3	20.86	24.3	2.4	101
	6.95	10.2	3.3	100
	2.32	5.79	3.5	103
	0	3.28	0.4	

To assess linearity, rat serum, EDTA plasma, and heparin plasma samples from Sprague-Dawley rats were tested at 2-fold, 4-fold, 8-fold, and 16-fold dilution. All samples were taken from normal rats. The concentrations shown below have been corrected for dilution (concentration = measured x dilution factor). Percent recovery is calculated as the measured concentration divided by the concentration for the previous dilution (expected).

$$\% \text{ Recovery} = (\text{measured} \times \text{dilution factor}) / \text{expected} \times 100$$

cTnl	Fold Dilution	Conc. (ng/mL)	Conc. %CV	% Recovery
Serum #1	2	19.4	0.2	-
	4	17.3	3.8	89
	8	16.8	7.1	97
	16	15.7	6.2	94
Serum #2	2	5.82	7.6	-
	4	5.99	4.2	103
	8	6.32	1.4	105
	16	5.72	2.4	91
Serum #3	2	< LLOQ	-	-
	4	< LLOQ	-	-
	8	< LLOQ	-	-
	16	< LLOQ	-	-
EDTA Plasma #1	2	< LLOQ	-	-
	4	< LLOQ	-	-
	8	< LLOQ	-	-
	16	< LLOQ	-	-
EDTA Plasma #2	2	< LLOQ	-	-
	4	< LLOQ	-	-
	8	< LLOQ	-	-
	16	< LLOQ	-	-
EDTA Plasma #3	2	< LLOQ	-	-
	4	< LLOQ	-	-
	8	< LLOQ	-	-
	16	< LLOQ	-	-
Heparin Plasma #1	2	1.62	2.0	-
	4	1.57	2.8	97
	8	1.52	2.6	97
	16	< LLOQ	-	-
Heparin Plasma #2	2	1.30	4.2	-
	4	1.25	5.1	96
	8	1.29	1.2	103
	16	< LLOQ	-	-
Heparin Plasma #3	2	1.30	5.0	-
	4	1.27	5.7	98
	8	1.24	2.7	97
	16	< LLOQ	-	-

cTnT	Fold Dilution	Conc. (ng/mL)	Conc. %CV	% Recovery
Serum #1	2	13.2	2.9	-
	4	12.5	1.4	95
	8	12.4	2.2	99
	16	11.4	5.1	93
Serum #2	2	13.2	2.9	-
	4	12.5	1.4	95
	8	12.4	2.2	99
	16	11.4	5.1	93
Serum #3	2	< LLOQ	-	-
	4	< LLOQ	-	-
	8	< LLOQ	-	-
	16	< LLOQ	-	-
EDTA Plasma #1	2	< LLOQ	-	-
	4	< LLOQ	-	-
	8	< LLOQ	-	-
	16	< LLOQ	-	-
EDTA Plasma #2	2	< LLOQ	-	-
	4	< LLOQ	-	-
	8	< LLOQ	-	-
	16	< LLOQ	-	-
EDTA Plasma #3	2	< LLOQ	-	-
	4	< LLOQ	-	-
	8	< LLOQ	-	-
	16	< LLOQ	-	-
Heparin Plasma #1	2	< LLOQ	-	-
	4	< LLOQ	-	-
	8	< LLOQ	-	-
	16	< LLOQ	-	-
Heparin Plasma #2	2	< LLOQ	-	-
	4	< LLOQ	-	-
	8	< LLOQ	-	-
	16	< LLOQ	-	-
Heparin Plasma #3	2	< LLOQ	-	-
	4	< LLOQ	-	-
	8	< LLOQ	-	-
	16	< LLOQ	-	-

FABP3	Fold Dilution	Conc. (ng/mL)	Conc. %CV	% Recovery
Serum #1	2	> ULOQ	-	-
	4	> ULOQ	-	-
	8	> ULOQ	-	-
	16	> ULOQ	-	-
Serum #2	2	> ULOQ	-	-
	4	> ULOQ	-	-
	8	> ULOQ	-	-
	16	124	10.9	-
Serum #3	2	3.24	6.0	-
	4	3.05	3.7	94
	8	3.28	6.3	108
	16	< LLOQ	-	-
EDTA Plasma #1	2	> ULOQ	-	-
	4	54.7	10.4	111
	8	55.6	3.7	102
	16	52.1	6.5	94
EDTA Plasma #2	2	> ULOQ	-	-
	4	40.8	13.8	100
	8	38.1	10.3	93
	16	37.7	4.4	99
EDTA Plasma #3	2	6.00	2.8	-
	4	5.47	5.1	91
	8	5.29	6.5	97
	16	< LLOQ	-	-
Heparin Plasma #1	2	> ULOQ	-	-
	4	> ULOQ	-	-
	8	66.8	6.3	-
	16	67.2	7.2	101
Heparin Plasma #2	2	> ULOQ	-	-
	4	56.1	5.4	-
	8	55.7	1.0	99
	16	50.6	6.5	91
Heparin Plasma #3	2	> ULOQ	-	-
	4	59.4	3.2	-
	8	57.9	2.9	98
	16	51.8	1.6	89

MyI3	Fold Dilution	Conc. (ng/mL)	Conc. %CV	% Recovery
Serum #1	2	15.5	5.5	-
	4	13.4	0.5	86
	8	13.8	4.6	103
	16	12.9	2.2	93
Serum #2	2	8.32	11.3	-
	4	7.53	2.5	90
	8	7.23	3.5	96
	16	6.57	4.7	91
Serum #3	2	0.35	7.0	-
	4	0.31	5.1	88
	8	< LLOQ	-	-
	16	< LLOQ	-	-
EDTA Plasma #1	2	1.93	1.5	-
	4	1.88	3.5	98
	8	1.87	3.9	99
	16	1.47	5.1	79
EDTA Plasma #2	2	2.94	7.2	-
	4	2.67	3.5	91
	8	2.56	6.2	96
	16	1.91	8.9	74
EDTA Plasma #3	2	0.54	8.5	-
	4	0.48	8.5	89
	8	0.51	12.4	106
	16	< LLOQ	-	-
Heparin Plasma #1	2	3.08	3.7	-
	4	2.84	3.3	92
	8	2.58	8.9	91
	16	2.10	8.3	82
Heparin Plasma #2	2	3.43	3.1	-
	4	3.00	4.4	87
	8	2.90	5.9	97
	16	2.48	8.8	86
Heparin Plasma #3	2	3.48	5.0	-
	4	3.12	3.1	90
	8	3.10	3.4	100
	16	2.60	0.4	84

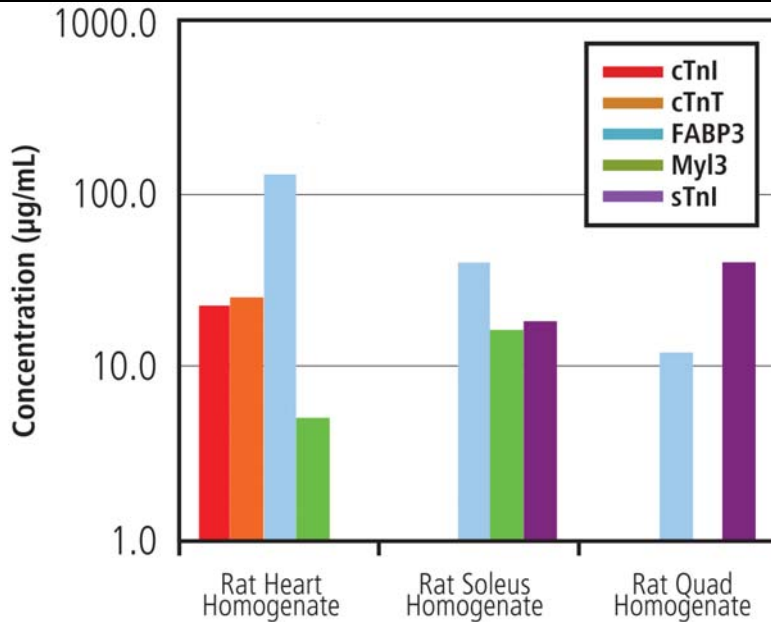
sTnl	Fold Dilution	Conc. (ng/mL)	Conc. %CV	% Recovery
Serum #1	2	23.1	3.4	-
	4	22.4	5.4	97
	8	22.0	3.9	98
	16	21.5	3.3	98
Serum #2	2	14.9	7.8	-
	4	14.2	3.7	95
	8	13.9	6.1	98
	16	13.1	1.1	95
Serum #3	2	< LLOQ	-	-
	4	< LLOQ	-	-
	8	< LLOQ	-	-
	16	< LLOQ	-	-
EDTA Plasma #1	2	20.6	6.1	-
	4	20.4	2.8	99
	8	19.5	6.8	95
	16	19.3	9.0	99
EDTA Plasma #2	2	13.0	11.7	-
	4	13.3	6.5	102
	8	13.1	7.5	98
	16	< LLOQ	-	-
EDTA Plasma #3	2	< LLOQ	-	-
	4	< LLOQ	-	-
	8	< LLOQ	-	-
	16	< LLOQ	-	-
Heparin Plasma #1	2	16.4	2.7	-
	4	16.0	2.6	97
	8	15.3	2.4	96
	16	15.3	6.4	100
Heparin Plasma #2	2	14.1	8.1	-
	4	13.8	2.3	98
	8	13.3	2.3	97
	16	< LLOQ	-	-
Heparin Plasma #3	2	13.9	3.9	-
	4	14.0	5.8	101
	8	13.7	2.0	98
	16	12.7	4.9	93

The assays on the Muscle Injury Panel 1 (rat) are all highly specific for their respective analytes. Specificity was demonstrated by testing rat muscle homogenates, and by running the assay with single Calibrators and single detection antibodies. Results are detailed in the sections below.

Testing of Rat Muscle Homogenates:

Tissue homogenates from heart, fast twitch, and slow twitch muscle were tested at 100-fold, 1000-fold, and 10000-fold sample dilution. The assays for cardiac troponins were positive for cardiac homogenates and negative for other muscle homogenates, demonstrating specificity for cardiac tissue. The assay for skeletal Troponin I was specific for fast and slow twitch skeletal muscle. The assay measured FABP3 in cardiac muscle and skeletal muscle. The slow twitch muscle was positive for Myl3, while approximately 200-fold less Myl3 was measured in fast twitch.

Sample Group	cTnl		cTnT		FABP3		Myl3		sTnl	
	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



Specificity of the assays for individual Calibrators:

The Muscle Injury Panel 1 assay was run with each single Calibrator at a high level in order to assess specificity. The table below shows the % cross-reactivity of each assay for each Calibrator. The % cross-reactivity is calculated as the ratio of the non-specific signal to the specific signal.

Blended Detection Antibodies and Single Calibrator % Cross-Reactivity					
Spot	cTnl	cTnT	FABP3	MyI3	sTnl
cTnl	100	0.55	< 0.1	< 0.1	< 0.1
cTnT	0.19	100	< 0.1	< 0.1	1.93
FABP3	< 0.1	< 0.1	100	< 0.1	< 0.1
MyI3	< 0.1	< 0.1	< 0.1	100	< 0.1
sTnl	< 0.1	< 0.1	< 0.1	< 0.1	100

Specificity of the Detection Antibodies:

In order to assess specificity of the detection antibodies, the Muscle Injury Panel 1 panel was run with blended Calibrator diluted to STD-02, and single detection antibodies. The table below shows the % cross-reactivity for each individual detection antibody. The data shows that the presence of cTnl Calibrator on the cTnl spot is reported out by the cTnl, cTnT, and sTnl detection antibodies.

Blended Calibrator and Single Detection Antibody % Cross-Reactivity					
Spot	cTnl	cTnT	FABP3	MyI3	sTnl
cTnl	100	< 0.1	< 0.1	< 0.1	50.9
cTnT	0.44	100	< 0.1	0.17	7.89
FABP3	< 0.1	< 0.1	100	< 0.1	0.11
MyI3	< 0.1	< 0.1	< 0.1	100	0.11
sTnl	0.24	< 0.1	< 0.1	< 0.1	100

While these detection antibodies are not highly specific, the assays themselves are highly specific due to the specificity of the capture antibodies.

XVII

Samples

s a m p l e s

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.

		cTnI	cTnT	FABP3	Myl3	sTnI
Serum	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
EDTA Plasma	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

XVIII

Calibrators

c a l i b r a t o r s

Rat cardiac troponin I (cTnI), rat cardiac troponin T (cTnT), and rat fatty acid binding protein 3 (FABP3) were purified from rat heart tissue. Rat skeletal troponin I was purified from rat skeletal muscle. Full-length recombinant rat myosin light chain 3 (Myl3) with an N-terminal 10xHis tag was expressed in *E. coli*. These analytes were calibrated against internal controls, diluted, and pooled to make the Muscle Injury Panel 1 (rat) Calibrator Blend.

XIX

References

r e f e r e n c e s

Babuin L and Jaffe A S. Troponin: the biomarker of choice for the detection of cardiac injury. CMAJ.173(10): 1191-1202, 2005.

Summary Protocol

MSD 96-well MULTI-SPOT Muscle Injury Panel 1 (rat) Assay Kit

MSD provides this summary protocol for your convenience.
Please read the entire detailed protocol prior to performing the Muscle Injury Panel 1 Assay.

Step 1 : Sample and Reagent Preparation

Bring all reagents to room temperature and thaw the Calibrator on ice.

Diluent 7 + Additives should be prepared by diluting the provided DTT (100X) and EDTA (16.7X) stock solutions to 1X concentration in Diluent 7.

Prepare an 8-point standard curve using supplied Calibrators:

- The Calibrator Blend should be diluted in Diluent 7 + Additives.
- Dilute the stock Calibrator Blend 1:20 in Diluent 7 + Additives then perform a series of 3-fold dilution steps and a no Calibrator blank.
- Dilute samples by 4-fold into Diluent 7 + Additives prior to addition to the plate.

Incubate the diluted Calibrators and diluted samples for 30 minutes without shaking at room temperature prior to addition to the plate.

Prepare Detection Antibody Solution by diluting the 50X Detection Antibodies to a 1X final concentration of each antibody. The Detection Antibodies should be diluted in a final volume of 3.0 mL Diluent 30 per plate.

Prepare 20 mL of 1X Read Buffer T by diluting Read Buffer T with deionized water.

Step 2 : Add Diluent 7 + Additives

Dispense 25 μ L/well Diluent 7 + Additives.

Incubate at room temperature with vigorous shaking (300–1000 rpm) for 30 min.

Step 3 : Add Sample or Calibrator

Dispense 25 μ L/well Calibrator or Sample.

Incubate at room temperature with vigorous shaking (300–1000 rpm) for 2 hours.

Step 4 : Wash and Add Detection Antibody Solution

Wash plate 3X with PBS-T.

Dispense 25 μ L/well 1X Detection Antibody Solution.

Incubate at room temperature with vigorous shaking (300–1000 rpm) for 2 hours.

Step 5 : Wash and Read Plate

Wash plate 3X with PBS-T.

Dispense 150 μ L/well 1X Read Buffer T.

Immediately analyze plate on SECTOR instrument.

	1	2	3	4	5	6	7	8	9	10	11	12
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	1	2	3	4	5	6	7	8	9	10	11	12
A	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
B	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
C	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
D	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
E	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
F	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
G	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
H	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>