

MSD[®] MULTI-SPOT Assay System

Rat Skeletal Troponin I (sTnI) Assay Kit

1-Plate Kit
5-Plate Kit
25-Plate Kit

K151IMC-1
K151IMC-2
K151IMC-4



MSD Toxicology Assays

Rat Skeletal Troponin I Assay Kit

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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MSD Advantage

MESO SCALE DISCOVERY'S unique spot patterns are a hallmark of our MULTI-ARRAY[®] technology, which 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, to generate assays with minimal background. MSD labels can be conveniently conjugated to biological molecules, are stable, and are non-radioactive. 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 a traditional ELISA, which is also limited by its inability to measure more than a single analyte. With an MSD assay, up to 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

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.

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. These assays have been qualified according to the principles outlined in “Fit-for-Purpose Method Development and Validation for Successful Biomarker Measurement” by Lee, J.W. et al.¹ 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. The Rat Skeletal Troponin I (sTnI) Assay is a sandwich immunoassay (Figure 1). MSD provides a plate that has been pre-coated with capture antibody for sTnI. The user adds the sample and a solution containing the detection antibody—anti-sTnI conjugated with an electrochemiluminescent compound, MSD SULFO-TAG label—over the course of one or more incubation periods. Analyte in the sample binds to the capture antibody immobilized on the working electrode surface; recruitment of the conjugated detection antibody 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[®] Imager for analysis. Inside the SECTOR Imager, 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 provide a quantitative measure of sTnI present in the sample.

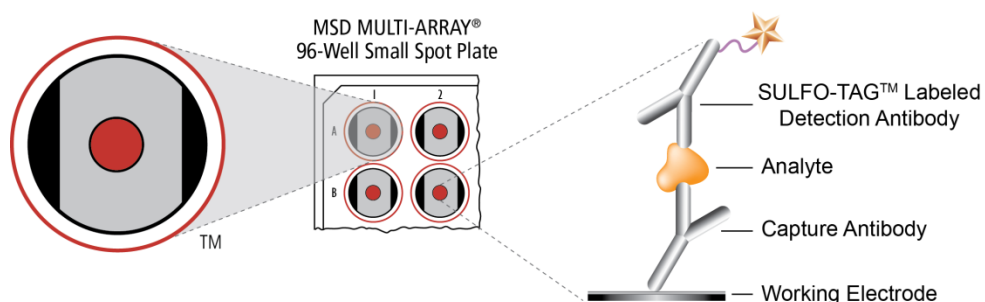


Figure 1. Spot diagram showing placement of analyte capture antibody. A unique bar code label on each plate allows complete traceability back to MSD manufacturing records.

Reagents Supplied

Product Description	Storage	Quantity per Kit		
		K153IMC-1	K153IMC-2	K153IMC-4
MULTI-ARRAY 96-Well Small Spot Rat Skeletal Troponin I Plate L453IMA-1	2–8°C	1 plate	5 plates	25 plates
SULFO-TAG Anti-rat sTnI Antibody ¹ (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
Rat Skeletal Troponin I Calibrator (20X)	≤-70°C	1 vial (15 µL)	5 vials (15 µL ea)	25 vials (15 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)

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 µL into a 96-well microtiter plate
- Plate washing equipment: automated plate washer or multichannel pipette
- Adhesive plate seals
- Microtiter plate shaker

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

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.

Reagent Preparation

Bring all reagents 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 the Rat Skeletal Troponin I Assay, 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 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 and Control Solutions

Calibrator for the Rat Skeletal Troponin I Assay is supplied at 20-fold higher concentration than the recommended highest calibrator. An 8-point standard curve is recommended with 3-fold serial dilution steps and a zero calibrator. The stock calibrator should be thawed and kept on ice and then should be added into diluent at room temperature to make the standard curve solutions. For the actual concentration of the calibrator, refer to the certificate of analysis (C of A) supplied with the kit. A copy of the kit-specific C of A can also be found at www.mesoscale.com

To prepare an 8-point standard curve for up to 4 replicates:

- 1) Prepare the highest calibrator by adding 12 μL of the calibrator stock vial to 228 μL of Diluent 7 + additives.
- 2) Prepare the next calibrator by transferring 80 μL of the diluted calibrator to 160 μL 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 (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

For serum and plasma from normal rats, no dilution is necessary. However, for rats that have suffered a muscle injury, 2 – 20-fold dilution of serum or plasma samples is sometimes necessary. If sample dilution is required to get the analyte levels into the detection range, Diluent 7 + additives should be used to dilute the samples.

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 antibody is provided as a 50X stock solution. The final concentration of the working detection antibody solution should be at 1X. For each plate used, dilute a 60 μL aliquot of the stock detection antibody into 2940 μL of Diluent 30.

Prepare Read Buffer

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

Prepare MSD Plate

This plate has been pre-coated with the antibody for the analyte 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.

Assay Protocol

Notes

(Dilution of samples/calibrators should be completed prior to step 1)

- 1. Addition of Diluent 7 + Additives:** Dispense 25 μL of Diluent 7 + additives into each well. Seal the plate with an adhesive plate seal and incubate for 30 minutes with vigorous shaking (300–1000 rpm) at room temperature.
- 2. Addition of the Sample or Calibrator:** Dispense 25 μL of sample or calibrator (which has been pre-incubated for 30 minutes 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 300 μL of PBS-T. Dispense 25 μL of 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 300 μL of PBS-T. Add 150 μL of 1X Read Buffer T to each well of the MSD plate. Analyze the plate on the SECTOR Imager. No incubation in read buffer is required before reading the plate.

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

Bubbles in the fluid will interfere with reliable reading of MULTI-ARRAY plate. Use reverse pipetting techniques to ensure bubbles are not created when dispensing the read buffer.

Analysis of Results

The calibrators should be run minimally 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 quantification 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.

Assay Validation and Verification

The performance of this 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 kit that has completed the validation process. 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 (defined on a kit-by-kit basis) are run to measure accuracy and precision.

- Validation – The assay is tested over 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 inter-day coefficients of variance (CVs) of less than 20%. The typical specification includes a calculated concentration CV of less than 20%, accuracy within 20% of expected concentration, and a total error of less than 30%. The kit specifications for this lot are provided in the enclosed Certificate of Analysis (C of A).
- Verification – A multi-day (2-3 days) analysis with multiple runs per day of 6-12 total 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

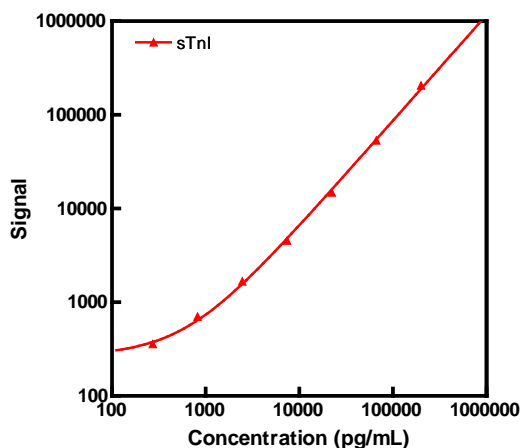
Freeze-thaw testing and accelerated stability studies performed during assay development (calibrators, antibodies, controls) are augmented with real-time stability studies on complete kits out to 18 months from the date of manufacture.

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 validation for this assay that meets the criteria described above. The kit lot-specific standard curve and measured limits of quantification can be found in the C of A enclosed with the kit.

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 quantification of unknown samples.

Some variation in the concentration of the highest calibrator is permissible between kit lots. For each individual kit lot, the calibrator concentrations are shown in the C of A.



Skeletal Troponin I		
Conc. (pg/mL)	Average Signal	%CV
0	213	1.8
274	358	2.9
823	704	4.1
2469	1672	8.5
7407	4568	7.9
22222	14847	7.8
66667	53441	6.9
200000	206052	3.4

Sensitivity

The lower limit of detection (LLOD) is the calculated concentration of the signal that is 2.5 standard deviations over the blank (zero calibrator).

A multi-plate, multi-day study was performed to measure the reproducibility of the assay. The lower limit of quantification (LLOQ) and upper limit of quantification (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%.

The ULOQ is determined as the highest concentration where the %CV of the calculated concentration is less than 25% and the percent recovery of the standard is between 80% and 120%.

	Skeletal Troponin I (pg/mL)
LLOD	374
LLOQ	781
ULOQ	160000

Precision

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. 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. Controls are run neat. The average intra-plate %CV and inter-plate %CV of the concentrations are shown below.

	Control	Plates	Average Conc. (pg/mL)	Average Intra-plate %CV	Inter-plate %CV
Skeletal Troponin I	High	21	100445	3.6	6.2
	Mid	21	15824	3.6	5.6
	Low	21	2797	4.5	7.2

Spike Recovery

Normal serum, EDTA plasma, and heparin plasma were spiked with the calibrators at multiple levels throughout the range of the assay. The samples were diluted 2-fold and then spiked with calibrator at the levels indicated in the table below.

% Recovery = measured / expected x 100

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

Linearity

To assess linearity, rat serum, EDTA plasma, and heparin plasma samples were spiked with Rat Skeletal Troponin I calibrator and further diluted 10-fold, 50-fold, and 250-fold. 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 measured from the previous dilution (expected)

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

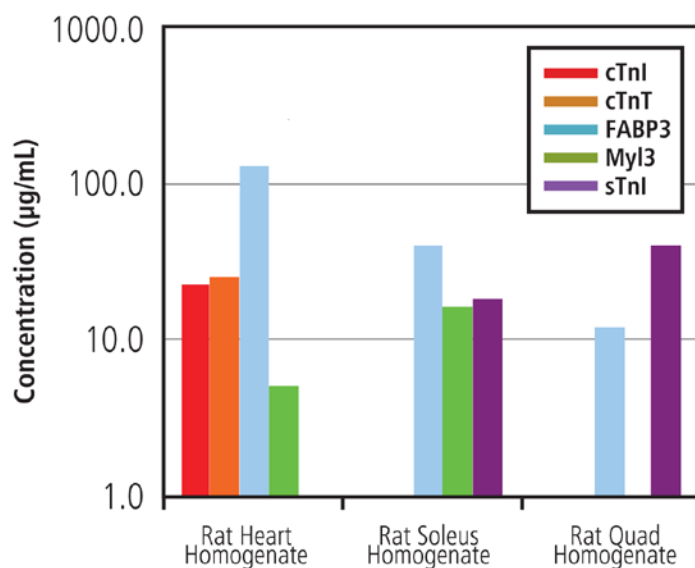
Sample	Fold Dilution	Conc. (pg/mL)	Conc. %CV	% Recovery
Serum	Spiked	90222	4.0	
	10	81150	3.5	90
	50	82650	5.4	102
	250	61000	13.0	74
EDTA Plasma	Spiked	83170	3.9	
	10	74270	4.7	89
	50	74100	6.5	100
	250	53000	11.7	71
Heparin Plasma	Spiked	84358	5.8	
	10	74820	6.3	87
	50	75350	5.5	101
	250	54250	13.9	70

Specificity

Specificity of skeletal Troponin I was demonstrated by testing rat muscle homogenates on the MSD Muscle Injury Panel 1 by running the assay with single calibrators and single detection antibodies. Rat Skeletal Troponin I Assay is the same assay used for the MSD Muscle Injury Panel 1.

Tissue homogenates from heart, fast twitch, and slow twitch muscle were tested at 100X, 1000X, and 10000X sample dilution. The assay for skeletal Troponin I was specific for fast and slow twitch skeletal muscle.

Sample Group	Cardiac TnI		Cardiac TnT		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



Samples

Serum, EDTA plasma, and heparin plasma samples collected from normal Sprague-Dawley rats were measured neat on the Rat Skeletal Troponin I 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	Skeletal Troponin I
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

Assay Components

Calibrators

Rat Skeletal Troponin I was purified from rat skeletal muscle. This analyte was calibrated against internal controls, diluted, and pooled to a final concentration of 4 µg/mL.

Antibodies

Analyte	Source Species	
	MSD Capture Antibody	MSD Detection Antibody
Skeletal Troponin I	Mouse Monoclonal	Mouse Monoclonal

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Summary Protocol

MSD 96-well MULTI-ARRAY Rat Skeletal Troponin I Assay Kit

MSD provides this summary protocol for your convenience.
Please read the entire detailed protocol prior to performing
the Rat Skeletal Troponin I 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.

If necessary, samples should be diluted in Diluent 7 + additives.

Prepare an 8-point standard curve using the supplied calibrators:

- The calibrator blend should be diluted in Diluent 7 + additives.
- Dilute the stock calibrator blend 20-fold in Diluent 7 + additives. Then perform a series of 3-fold dilution steps and prepare a zero calibrator blank.
- Incubate calibrator and samples in Diluent 7 + additives for 30 minutes before addition to plate.

Prepare detection antibody solution by diluting the 50X detection antibody to 1X in a final volume of 3.0 mL Diluent 30 per plate.

Prepare 20 mL of 1X Read Buffer T by diluting 4X Read Buffer T with deionized water.

Step 2 : Add Diluent 7 + Additives

Dispense 25 μ L/well of Diluent 7 + additives.

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

Step 3 : Add Sample or Calibrator

Dispense 25 μ L/well of 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 3 times with 300 μ L of PBS-T.

Dispense 25 μ L/well of 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 3 times with 300 μ L of PBS-T.

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

Analyze plate on SECTOR Imager.

	1	2	3	4	5	6	7	8	9	10	11	12
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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"/>

	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"/>
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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"/>
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