

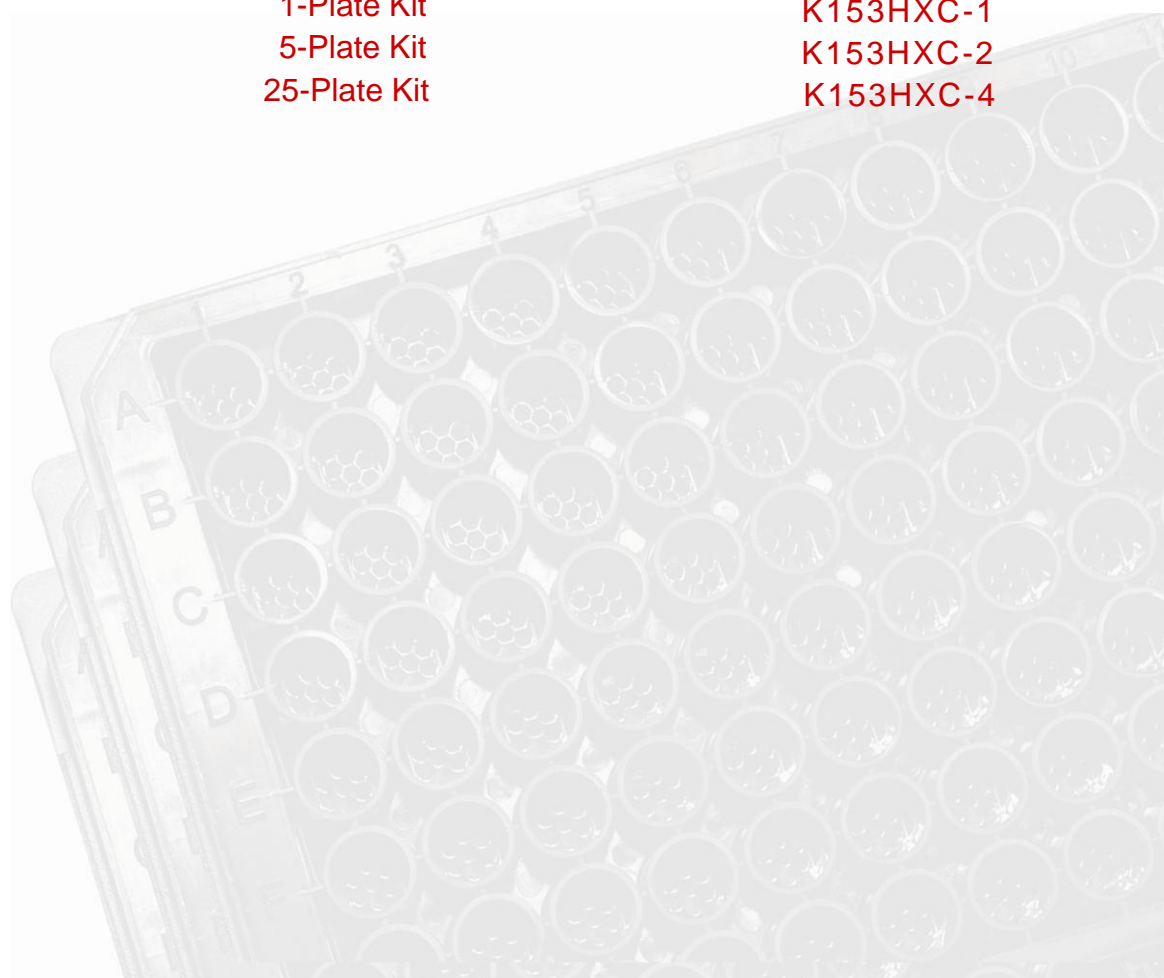
# MESO SCALE DISCOVERY

## MULTI-ARRAY Assay System

### Rat Clusterin Assay Kit

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

K153HXC-1  
K153HXC-2  
K153HXC-4



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# MSD Toxicology Assays

## Rat Clusterin Assay

*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®**

A division of Meso Scale Diagnostics, LLC.

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## Ordering Information

ordering information

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# MSD Advantage

MSD advantage

MESO SCALE DISCOVERY'S unique spot patterns are a hallmark of our MULTI-ARRAY® technology 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, 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, ten different biomarkers can be analyzed simultaneously using as little as 10-25 µL 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

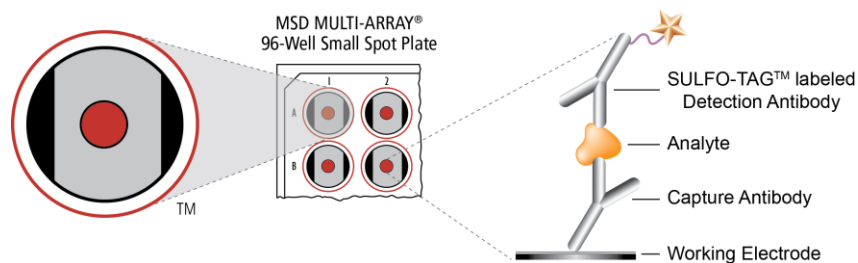
introduction

**Clusterin** is a glycoprotein that is found in most mammalian tissues. The localized over-expression of clusterin at sites of tissue damage or stress implicates that clusterin acts as a molecular chaperone displaying cytoprotective characteristics. In addition, the marked induction of clusterin in several renal disease states suggest that clusterin may be a putative biochemical marker of kidney damage and disease.

# 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. 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.<sup>1</sup> 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 Rat Clusterin Assay is a sandwich immunoassay (Figure 1). MSD provides a plate that has been pre-coated with capture antibody for clusterin. The user adds the sample and a solution containing the conjugated detection antibody—anti-clusterin 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® 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 provide a quantitative measure of clusterin 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.

# IV Reagents Supplied

reagents supplied

| Product Description   | Storage | Quantity per Kit     |                         |                          |
|---|---------|----------------------|-------------------------|--------------------------|
|   |         | K153HXC-1            | K153HXC-2               | K153HXC-4                |
| MULTI-ARRAY 96-well Rat Clusterin Plate(s)<br>L453HXA-1     | 2-8°C   | 1 plate              | 5 plates                | 25 plates                |
| SULFO-TAG Anti-rat Clusterin Antibody <sup>1</sup><br>(50X) | 2-8°C   | 1 vial<br>(75 µL)    | 1 vial<br>(375 µL)      | 5 vials<br>(375 µL ea)   |
| Rat Clusterin Calibrator<br>(20X)                           | ≤ -70°C | 1 vial<br>(15 µL)    | 5 vials<br>(15 µL ea)   | 25 vials<br>(15 µL ea)   |
| Blocker A Kit<br>R93AA-2 (250 mL)                           | RT      | 1 bottle<br>(250 mL) | 1 bottle<br>(250 mL)    | 5 bottles<br>(250 mL ea) |
| Triton X-100<br>(10%)                                       | 2-8°C   | 1 vial<br>(1.0 mL)   | 1 vial<br>(1.0 mL)      | 5 vials<br>(1.0 mL ea)   |
| Diluent 5<br>R52BA-5 (25 mL)                                | ≤ -10°C | 1 bottle<br>(25 mL)  | 3 bottles<br>(25 mL ea) | 15 bottles<br>(25 mL ea) |
| Read Buffer T (4X)<br>R92TC-3 (50 mL)                       | RT      | 1 bottle<br>(50 mL)  | 1 bottle<br>(50 mL)     | 5 bottles<br>(50 mL ea)  |

# 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 µL 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.

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

# VII Reagent Preparation

## reagent preparation

**Important:** The Rat Clusterin Calibrator stock solution should be thawed and kept on ice during all pipetting steps. The diluted Calibrators should be kept at room temperature prior to addition to the plate. Bring all other reagents to room temperature.

**Important:** Upon first thaw, separate Diluent 5 into aliquots appropriate to the size of your assay needs. This diluent can go through up to three freeze-thaw cycles without significantly affecting the performance of the assay.

### Prepare Blocker A Solution

Follow instructions included with the Blocker A Kit.

### Prepare Assay Diluent

Prepare a solution of 0.4% Triton X-100 in Diluent 5. For one plate, mix 2.88 mL of Diluent 5 with 0.12 mL of 10% Triton X-100. For each plate, 25  $\mu$ L per well of this solution will be required.

### Prepare Calibrator and Control Solutions

Calibrator for the Rat Clusterin 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, but 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](http://www.mesoscale.com)

To prepare this 8-point standard curve for up to 3 replicates:

- 1) Prepare the highest Calibrator by adding 12  $\mu$ L of the Calibrator stock vial to 228  $\mu$ L of Diluent 5 (**NOT** containing Triton X-100) (20-fold dilution).
- 2) Prepare the next Calibrator by transferring 80  $\mu$ L of the diluted Calibrator to 160  $\mu$ L of Diluent 5. Repeat 3-fold serial dilutions 5 additional times to generate 7 Calibrators.
- 3) The recommended 8<sup>th</sup> Standard is Diluent 5 (i.e. zero Calibrator).

Calibrators should be prepared at room temperature no more than 20 minutes before use.

### Dilution of Samples

A 5-fold dilution of urine samples into Diluent 5 is recommended for the Rat Clusterin Assay in order to get the analyte levels into the detection range. Depending on the sample set under investigation, higher or lower dilution factors may be necessary. For kidney tissue lysates or homogenates, a 10 to 500-fold dilution may be required.

### 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  $\mu$ L aliquot of the stock Detection Antibody into 2940  $\mu$ L of Diluent 5.

### 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 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.

# VIII Assay Protocol

assay protocol

## Notes

- 1. Addition of Blocker A Solution:** Dispense 150  $\mu\text{L}$  of Blocker A Solution into each well. Seal the plate with an adhesive plate seal and incubate for 1 hour with vigorous shaking (300–1000 rpm) at room temperature.
- 2. Wash and Addition of the Sample or Calibrator:** Wash the plate 3 times with PBS-T. Dispense 25  $\mu\text{L}$  of Assay Diluent (Diluent 5 + 0.4% Triton X-100) into each well of the MSD plate. Then, dispense 25  $\mu\text{L}$  of sample or Calibrator 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  $\mu\text{L}$  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  $\mu\text{L}$  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 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 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.

# X Assay Qualification and Verification

## assay qualification 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.<sup>1</sup>

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 qualification 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 (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 inter-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 within 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.

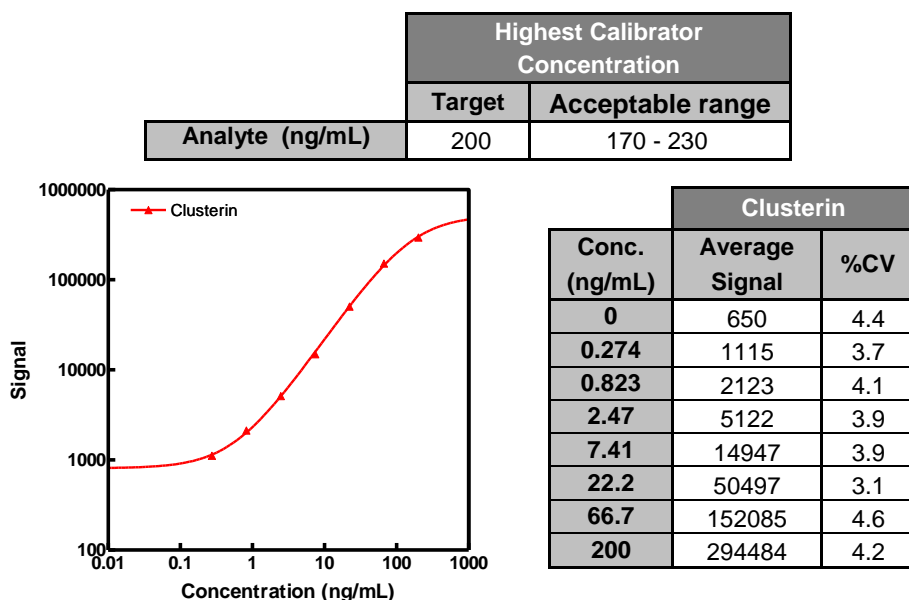
All acceptance criteria and verification conformance are defined in the C of A for all kit lots. Presented below are representative data from the assay qualification for this assay that meets the criteria described above. The actual kit-specific standard curve and measured limits of quantification can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

Some variation in the concentration of the highest Calibrator is permissible between kit lots. Below is a table that details the acceptable range of the highest Calibrator concentration. For each individual kit lot, the Calibrator concentrations are shown in the C of A.



# XII Sensitivity

## sensitivity

The lower limit of detection (LLOD) is the calculated concentration of the signal that is 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 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 20% and the percent recovery of the standard is between 80% and 120%.

|      | Clusterin (ng/mL) |
|------|-------------------|
| LLOD | 0.0571            |
| LLOQ | 0.823             |
| ULOQ | 150               |

# XIII Precision

precision

Control samples of high, mid, and low levels were measured on each plate. Controls were made by spiking Calibrator into Diluent 5.

The controls were run in quadruplicate on each of 10 plates run across multiple days (n>3).

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 14 plates over 4 days.

Inter-lot %CV is the variability of controls across 3 kit lots.

|           | Control | Plates | Average Conc. (ng/mL) | Average Intra-plate %CV | Inter-plate %CV | Inter-lot %CV |
|-----------|---------|--------|-----------------------|-------------------------|-----------------|---------------|
| Clusterin | High    | 14     | 135                   | 7.3                     | 10.2            | 12.3          |
|           | Mid     | 14     | 17.6                  | 4.9                     | 6.0             | 6.6           |
|           | Low     | 14     | 1.90                  | 6.1                     | 8.2             | 10.1          |

# XIV Spike Recovery

spike recovery

Rat urine samples were spiked with the Calibrator at multiple values throughout the range of the assay. The samples were diluted 5-fold and then spiked with Calibrator at the levels indicated in the table below. Control sample 1 is a urine sample from an individual rat and control sample 2 is a pooled urine sample.

% Recovery = measured / expected x 100

| Sample   | Clusterin           |                        |                    |            |
|----------|---------------------|------------------------|--------------------|------------|
|          | Spike Conc. (ng/mL) | Measured Conc. (ng/mL) | Measured Conc. %CV | % Recovery |
| Sample 1 | 0                   | 0.817                  | 5.6                |            |
|          | 0.565               | 1.54                   | 5.3                | 112        |
|          | 14.1                | 17.1                   | 2.3                | 115        |
|          | 124                 | 161                    | 2.5                | 129        |
| Sample 2 | 0                   | 0.818                  | 8.7                |            |
|          | 0.666               | 1.44                   | 5.9                | 97         |
|          | 14.2                | 13.9                   | 6.1                | 93         |
|          | 124                 | 118                    | 4.5                | 94         |

# XV Linearity

linearity

To assess linearity, rat urine samples were diluted 5-fold, 10-fold, 20-fold, 40-fold and 80-fold prior to testing. 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). Measurements below the assay LLOQ are shown in italics.

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

| Sample   | Fold Dilution | Clusterin     |             |            |
|----------|---------------|---------------|-------------|------------|
|          |               | Conc. (ng/mL) | Conc. %CV   | % Recovery |
| Sample 1 | 5             | 43.6          | 3.2         |            |
|          | 10            | 55.9          | 2.6         | 128        |
|          | 20            | 64.4          | 0.5         | 115        |
|          | 40            | 67.0          | 2.0         | 104        |
|          | 80            | <i>64.5</i>   | 3.3         | <i>96</i>  |
| Sample 2 | 5             | 31.9          | 1.5         |            |
|          | 10            | 36.6          | 3.3         | 115        |
|          | 20            | 41.2          | 1.4         | 112        |
|          | 40            | 42.8          | 3.9         | 104        |
|          | 80            | <i>42.8</i>   | <i>13.6</i> | <i>100</i> |
| Sample 3 | 5             | 12.9          | 4.7         |            |
|          | 10            | 14.2          | 2.5         | 111        |
|          | 20            | <i>14.3</i>   | 2.2         | <i>101</i> |
|          | 40            | <i>14.6</i>   | 5.4         | <i>102</i> |
|          | 80            | <i>15.8</i>   | 6.5         | <i>108</i> |
| Sample 4 | 5             | 103           | 3.0         |            |
|          | 10            | 141           | 2.3         | 138        |
|          | 20            | 187           | 3.5         | 132        |
|          | 40            | 216           | 1.7         | 116        |
|          | 80            | 240           | 1.2         | 111        |
| Sample 5 | 5             | 70.5          | 2.5         |            |
|          | 10            | 94.4          | 1.8         | 134        |
|          | 20            | 126           | 2.0         | 133        |
|          | 40            | 139           | 0.6         | 111        |
|          | 80            | 158           | 2.2         | 113        |

# XVI Samples

s a m p l e s

Urine samples collected from normal and treated rats were tested at 5-fold dilution, and some were re-run at higher dilution ratio to get them in range. Treated animals were dosed with nephrotoxicants prior to sample collection. Concentrations have been corrected for sample dilution. Concentrations in gray were below the LLOQ.

| Sample # | Type    | Dilution Corrected Conc. (ng/mL) | Sample # | Type    | Dilution Corrected Conc. (ng/mL) |
|----------|---------|----------------------------------|----------|---------|----------------------------------|
| 1        | Control | 8.44                             | 18       | Control | 6.52                             |
| 2        | Control | 1.81                             | 19       | Treated | 9.02                             |
| 3        | Control | 4.24                             | 20       | Treated | 469                              |
| 4        | Control | 8.33                             | 21       | Treated | 270                              |
| 5        | Control | 9.33                             | 22       | Treated | 591                              |
| 6        | Control | 3.97                             | 23       | Treated | 464                              |
| 7        | Control | 6.59                             | 24       | Treated | 1428                             |
| 8        | Control | 7.28                             | 27       | Treated | 111                              |
| 9        | Control | 7.29                             | 28       | Treated | 31.0                             |
| 10       | Control | 3.93                             | 29       | Treated | 83.0                             |
| 11       | Control | 2.52                             | 30       | Treated | 84.9                             |
| 12       | Control | 12.3                             | 34       | Treated | 356                              |
| 13       | Control | 0.789                            | 35       | Treated | 143                              |
| 14       | Control | 0.951                            | 36       | Treated | 213                              |
| 15       | Control | 1.58                             | 37       | Treated | 230                              |
| 16       | Control | 9.78                             | 38       | Treated | 231                              |
| 17       | Control | 11.0                             |          |         |                                  |

# XVII Assay Components

a s s a y c o m p o n e n t s

## Calibrator

Recombinant rat clusterin was calibrated against an internal control. The final assigned concentration of the Rat Clusterin Calibrator is defined in the C of A for each kit lot.

## Antibodies

| Analyte   | Source Species       |                        |
|-----------|----------------------|------------------------|
|           | MSD Capture Antibody | MSD Detection Antibody |
| Clusterin | Rabbit Polyclonal    | Rabbit Polyclonal      |

# XVIII References

## references

1. Lee JW, Devanarayan V, Barrett YC, Weiner R, Allinson J, Fountain S, Keller S, Weinryb I, Green M, Duan L, Rogers JA, Millham R, O'Brien PJ, Sailstad J, Khan M, Ray C, Wagner JA. Fit-for-purpose method development and validation for successful biomarker measurement. *Pharm Res.* 2006 Feb;23(2):312-28.
2. Rached, E., Hoffman, D., Blumbach, K., Weber, K., Dekant, W., Mally, A. *Evaluation of Putative Biomarkers of Nephrotoxicity after Exposure to Ochratoxin A in vivo and in vitro.* *Toxicol Sci.* 2008 Jun; 103(2):371-81

## Summary Protocol

### MSD 96-well MULTI-ARRAY Rat Clusterin Assay Kit

MSD provides this summary protocol for your convenience.  
Please read the entire detailed protocol prior to performing the Rat Clusterin Assay.

#### Step 1 : Sample and Reagent Preparation

The Rat Clusterin Calibrator solution should be thawed and kept on ice during all pipetting steps. The diluted Calibrators should be kept at room temperature prior to addition to the plate. Bring appropriate diluents and plates to room temperature.

- Prepare Blocker A Solution.
- Prepare Assay Diluent by diluting 10% Triton X-100 in Diluent 5 to a final concentration of 0.4% Triton X-100.
- Prepare an 8-point standard curve using supplied Calibrator:
  - The Calibrator should be diluted in Diluent 5 without Triton X-100.
  - Dilute the stock Calibrator 20-fold in Diluent 5 then perform a series of 3-fold dilution steps and a no Calibrator blank.
- Dilute samples 5-fold into Diluent 5 prior to addition to the plate.
- Prepare Detection Antibody Solution by diluting the 50X Detection Antibody to 1X in a final volume of 3.0 mL Diluent 5 per plate.
- Prepare 20 mL of 1X Read Buffer T by diluting 4X Read Buffer T with deionized water.

#### Step 2 : Add Blocker A Solution

Dispense 150  $\mu$ L/well Blocker A Solution.

Incubate at room temperature with vigorous shaking (300–1000 rpm) for 1 hour.

#### Step 3 : Wash and Add Sample or Calibrator

Wash plate 3 times with PBS-T.

Dispense 25  $\mu$ L/well Assay Diluent (Diluent 5 + 0.4% Triton).

Dispense 25  $\mu$ 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 3 times with PBS-T.

Dispense 25  $\mu$ 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 3 times with PBS-T.

Dispense 150  $\mu$ L/well 1X Read Buffer T.

Analyze plate on SECTOR instrument.



|   | 1                        | 2                        | 3                        | 4                        | 5                        | 6                        | 7                        | 8                        | 9                        | 10                       | 11                       | 12                       |
|---|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
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