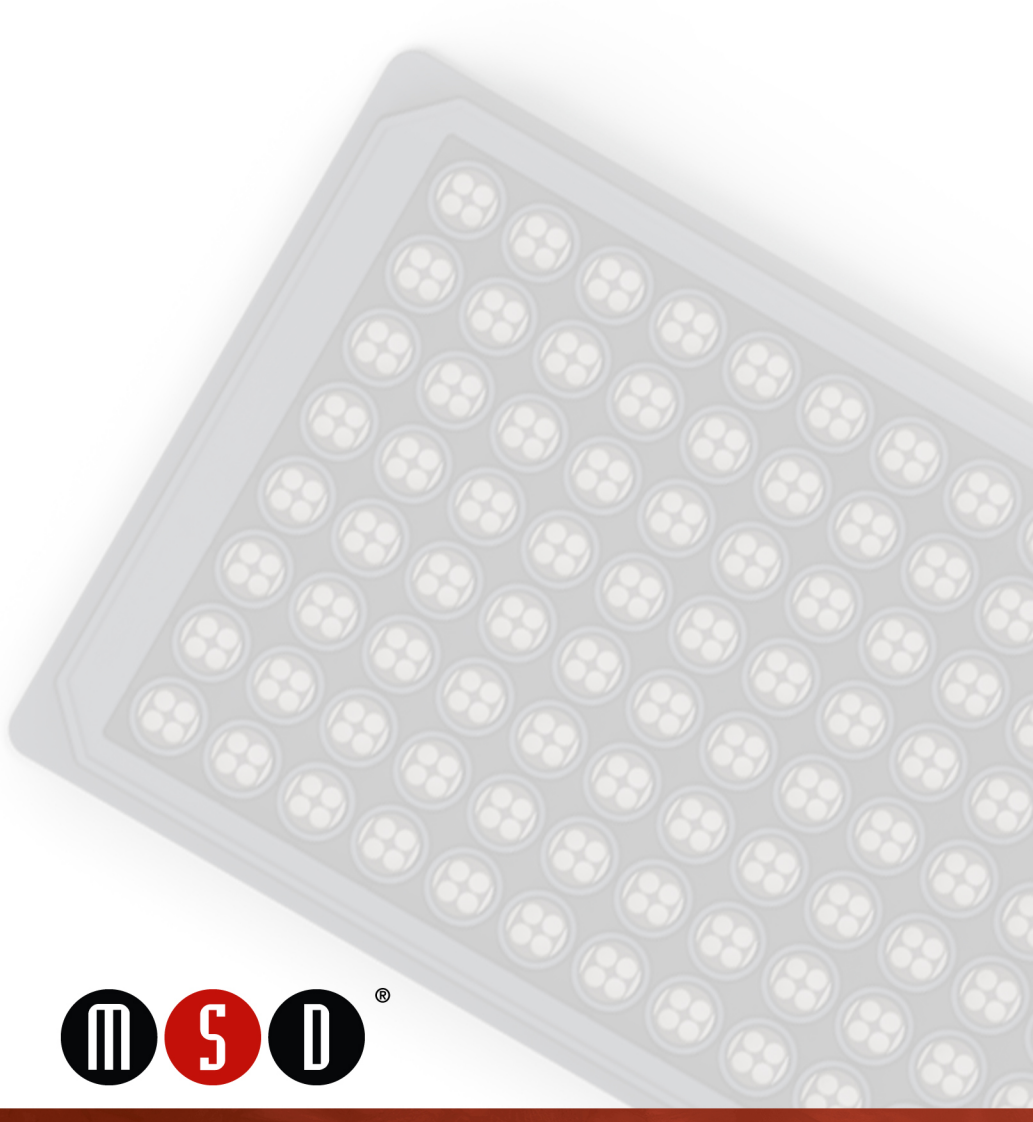
MSD® MULTI-SPOT Assay System

Acute Phase Protein Panel 1 (rat) Kit

1-Plate Kit	K15175C-1
5-Plate Kit	K15175C-2
25-Plate Kit	K15175C-4



MSD Toxicology Assays

Acute Phase Protein Panel 1 (rat) Kit

AGP, A2M

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

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NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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Introduction

Rat α2-macroglobulin (A2M) is a serum glycoprotein produced by hepatocytes that is induced in acute and chronic inflammatory injury. A2M is a major protease inhibitor and an acute phase protein comprising the C3 and C4 complement components. Rat α1-acid glycoprotein (AGP) is involved in the acute phase response but its function is largely unknown. AGP is also associated with inflammatory responses. Both AGP and A2M are abundant in serum and their concentrations can increase by several hundred fold following an inflammatory response. These markers also show a rapid response with sustained high level for several days after an inflammatory response. AGP may be indicative of liver hypertrophy as well as local and general inflammation.

Principle of the Assay

MSD assays provide a rapid and convenient method for measuring the levels of protein targets within a single, small-volume sample. The Acute Phase Protein Panel 1 (rat) is a multiplex sandwich immunoassay (Figure 1). This panel has 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] MSD provides a plate pre-coated with capture antibodies. The user adds the sample and a solution containing detection antibodies conjugated with electrochemiluminescent labels (MSD SULFO-TAG™) 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 detection antibodies by the bound analytes completes the sandwich. The user adds an MSD buffer that provides the appropriate chemical environment for electrochemiluminescence and loads the plate into an MSD® instrument where a voltage applied to the plate electrodes causes the captured labels to emit light. The instrument measures intensity of emitted light to provide a quantitative measure of analytes in the sample.

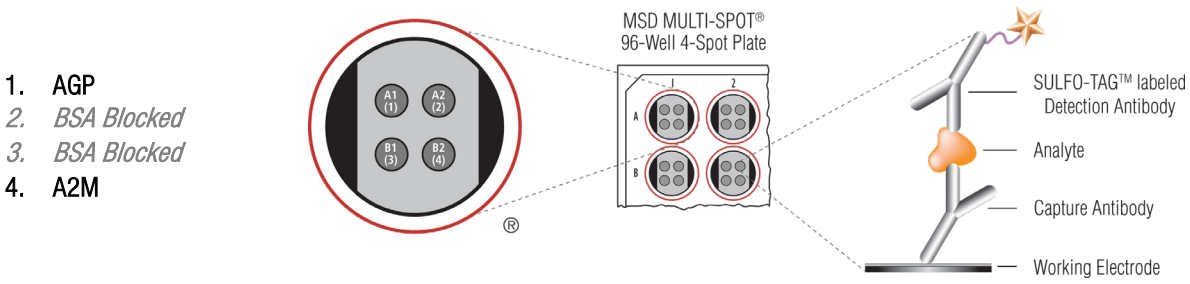


Figure 1. *Spot diagram showing placement of analyte capture antibody. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files.*

Reagents Supplied

Product Description	Storage	Quantity per Kit		
		K15175C-1	K15175C-2	K15175C-4
MULTI-SPOT® 96-Well 4-Spot Acute Phase Protein Panel 1 (rat) Plate N45175B-1	2–8 °C	1 plate	5 plates	25 plates
SULFO-TAG Anti-rat AGP Antibody[1] (50X)	2–8 °C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
SULFO-TAG Anti-rat A2M Antibody[1] (50X)	2–8 °C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
Acute Phase Protein Panel 1 (rat) Calibrator Blend (20X)	≤-70 °C	1 vial (15 µL)	5 vials (15 µL ea)	25 vials (15 µL ea)
Diluent 100 R50AA-2 (200 mL), R50AA-3 (1000 mL)	2–8 °C	1 bottle (200 mL)	1 bottle (1000 mL)	5 bottles (1000 mL ea)
Read Buffer T (4X) R92TC-3 (50 mL)	RT	1 bottle (50 mL)	1 bottle (50 mL)	5 bottles (50 mL ea)

Additional Materials and Equipment

- ❑ Appropriately sized tubes for reagent preparation
- ❑ Polypropylene microcentrifuge tubes for preparing serial dilutions
- ❑ Phosphate-buffered saline plus 0.05% Tween-20 (PBS-T) for plate washing or MSD Wash Buffer (Catalog No. R61AA-1)
- ❑ Liquid handling equipment for desired throughput, capable of dispensing 10 to 150 µL/well into a 96-well microtiter plate
- ❑ Plate washing equipment: automated plate washer or multichannel pipette
- ❑ Adhesive plate seals
- ❑ Microtiter plate shaker (rotary) capable of shaking at 500-1,000 rpm
- ❑ Deionized water
- ❑ Vortex mixer

Safety

Use safe laboratory practices and wear gloves, safety glasses, and lab coats when handling kit components. Handle and dispose of all hazardous samples properly in accordance with local, state, and federal guidelines.

Additional product-specific safety information is available in the safety data sheet (SDS), which can be obtained from MSD Customer Service or at www.mesoscale.com.

[1] SULFO-TAG conjugated detection antibodies should be stored in the dark.

Best Practices and Technical Hints

- Mixing or substituting reagents from different sources or different kit lots is not recommended. Lot information is provided in the lot-specific certificate of analysis (COA).
- Assay incubation steps should be performed between 20-26 °C to achieve the most consistent signals between runs.
- Bring frozen diluent (if applicable) to room temperature in a 24 °C water bath. Thaw other reagents on wet ice and use as directed without delay.
- Prepare calibrators, samples, and controls in polypropylene microcentrifuge tubes; use a fresh pipette tip for each dilution; vortex after each dilution before proceeding.
- Avoid prolonged exposure of detection antibody (stock or diluted) to light. During the antibody incubation step, plates do not need to be shielded from light except for direct sunlight.
- Avoid bubbles in wells at all pipetting steps. Bubbles may lead to variable results; bubbles introduced when adding Read Buffer T may interfere with signal detection.
- Do not touch the pipette tip on the bottom of the wells when pipetting into the MSD plate.
- Use reverse pipetting when necessary to avoid introduction of bubbles. For empty wells, pipette to the bottom corner.
- Plate shaking should be vigorous, with a rotary motion between 500 and 1,000 rpm. Binding reactions may reach equilibrium sooner if you use shaking at the middle of this range (~700 rpm) or above.
- When using an automated plate washer, rotate the plate 180 degrees between wash steps to improve assay precision.
- Gently tap the plate on a paper towel to remove residual fluid after washing.
- Read buffer should be at room temperature when added to the plate.
- Keep time intervals consistent between adding read buffer and reading the plate to improve inter-plate precision. Unless otherwise directed, read plate as soon as practical after adding read buffer.
- No shaking is necessary after adding read buffer.
- If an incubation step needs to be extended, leave the sample or detection antibody solution in the plate to keep the plate from drying out.
- Remove the plate seals prior to reading the plate.
- If assay results are above the top of the calibration curve, dilute the samples and repeat the assay.
- When running a partial plate, seal the unused sectors (see sector map in instrument and software manuals) to avoid contaminating unused wells. Remove all seals before reading. Partially used plates may be sealed and stored up to 30 days at 2–8 °C in the original foil pouch with desiccant. You may adjust volumes proportionally when preparing reagents.

Reagent Preparation

Bring all reagents to room temperature. Thaw the stock calibrator on ice.

Prepare Calibrator Dilutions

MSD supplies a blended calibrator for the Acute Phase Protein Panel 1 (rat) Kit at 20-fold higher concentration than the recommended highest standard. We recommend an 8-point standard curve with 4-fold serial dilution steps and a zero calibrator.

Thaw the stock calibrator and keep on ice, then add to diluent at room temperature to make the standard curve solutions. For the lot-specific concentration of the calibrator, refer to the COA supplied with the kit or available at www.mesoscale.com.

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

1) Prepare the highest standard by adding 10 µL of calibrator stock to 190 µL of Diluent 100. Mix well.
2) Prepare the next standard by transferring 60 µL of the highest standard to 180 µL of Diluent 100. Mix well. Repeat 4-fold serial dilutions 5 additional times to generate 7 standards.
3) The recommended 8th standard is Diluent 100 (i.e. zero calibrator).

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

Dilute Samples

For rat serum and plasma samples, MSD recommends a 20,000-fold dilution in Diluent 100; however, you may adjust dilution factors for the sample set under investigation. We recommend running at least two replicates per sample. The kit includes diluent sufficient for running samples in duplicate. Additional diluent can be purchased at www.mesoscale.com.

We recommend a 2-step dilution of the samples, a 1:100 dilution followed by a 1:200 dilution.

To dilute sample 20,000-fold, add 10 µL sample to 990 µL of Diluent 100 followed by 10 µL diluted sample to 1,990 µL of Diluent 100.

Prepare Detection Antibody Solution

MSD provides each detection antibody in a 50X stock solution. The working detection antibody solution is 1X.

For one plate, combine:

- ❑ 60 µL of 50X SULFO-TAG Anti-rat AGP Antibody
- ❑ 60 µL of 50X SULFO-TAG Anti-rat A2M Antibody
- ❑ 2.88 mL of Diluent 100

Note: If you omit detection antibody for an analyte not being measured, add 60 µL of Diluent 100 for each omitted antibody.

Prepare Wash Buffer

MSD provides Wash Buffer as a 20X stock solution. The working solution is 1X. PBS + 0.05% Tween-20 can be used instead.

For one plate, combine:

- ❑ 15 mL of MSD Wash Buffer (20X)
- ❑ 285 mL of deionized water

Prepare Read Buffer

MSD provides Read Buffer T as a 4X stock solution. The working solution is 2X.

For one plate, combine:

- ❑ 10 mL Read Buffer T (4X)
- ❑ 10 mL deionized water

You may prepare diluted read buffer in advance and store it at room temperature in a tightly sealed container.

Prepare MSD Plate

MSD plates are pre-coated with capture antibodies (Figure 1) and exposed to a proprietary stabilizing treatment to ensure the integrity and stability of the immobilized antibodies. Plates can be used as delivered; no additional preparation (e.g., pre-wetting) is required.

Protocol

Note: Follow **Reagent Preparation** before beginning this assay protocol.

STEP 1: Add Diluent 100

- ❑ Add 25 µL of Diluent 100 to each well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking for 30 minutes.

STEP 2: Add Sample or Calibrator

- ❑ Add 25 µL of Calibrator or diluted sample to each well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking (500-1,000 rpm) for 2 hours.

STEP 3: Wash and Add Detection Antibody Solution

- ❑ Wash the plate three times with at least 150 µL/well of PBS-T or 1X MSD Wash Buffer.
- ❑ Add 25 µL of detection antibody solution to each well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking (500-1,000 rpm) for 2 hours.

STEP 4: Wash and Read

- ❑ Wash the plate three times with at least 150 µL/well of PBS-T or 1X MSD Wash Buffer.
- ❑ Add 150 µL of 2X Read Buffer T to each well. Analyze the plate on an MSD instrument. Incubation in Read Buffer T is not required before reading the plate.

Analysis of Results

Run at least one set of calibrators in duplicate to generate the standard curve. The standard curve is modeled using least squares fitting algorithms so that signals from the calibrators can be used to calculate the concentration of analyte in the samples. The assays have a wide dynamic range (3–4 logs) which allows accurate quantification without the need for dilution in many cases. The MSD DISCOVERY WORKBENCH® analysis software uses 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 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-parameter logistic 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 controls for intra- and inter-day coefficients of variance (CVs) of less than 25%. The typical specification includes a calculated concentration CV of less than 25%, and accuracy within 25% of expected concentration. The kit specifications for this lot are provided in the enclosed COA.
  - 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 COA.

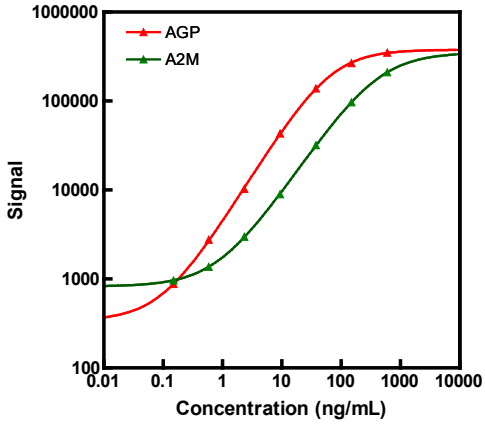
- **Robustness and Stability**

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 COA 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 COA enclosed with the kit.

Typical Data

The following standard curves illustrate the dynamic range of the assay. The actual signals may vary. Run a standard curve on each plate for the best quantification of unknown samples.



AGP		
Conc. (ng/mL)	Average Signal	%CV
0	263	3.9
0.146	876	1.6
0.586	2,757	3.9
2.34	10,279	2.0
9.38	43,117	3.4
37.5	138,099	2.5
150	266,110	3.2
600	349,199	1.5

A2M		
Conc. (ng/mL)	Average Signal	%CV
0	815	1.8
0.146	964	5.3
0.586	1,368	3.4
2.34	2,989	2.4
9.38	8,946	3.0
37.5	31,833	3.0
150	96,516	4.0
600	210,212	1.5

Sensitivity

The lower limit of detection (LLOD) is a calculated concentration based on a signal 2.5 standard deviations above 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 25% and the percent recovery of the standard is between 75% and 125%.

The ULOQ of AGP 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 75% and 125%.

The ULOQ of A2M 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 75% and 125%.

	AGP (ng/mL)	A2M (ng/mL)
LLOD	0.012	0.036
LLOQ	0.146	1.00
ULOQ	150	400

Precision

High, mid, and low controls were defined that are rat serum samples diluted 10,000-fold into assay diluent. Samples were chosen that span a large proportion of the dynamic range for each assay. The concentration values shown below are corrected for the 10,000-fold dilution.

The controls were run in triplicate on each of 9 plates run across multiple days (n>3).

Average intra-plate %CV is the average %CV of the control replicates within an individual run.

Inter-plate %CV is the variability of controls across 9 runs over 3 days.

Inter-lot %CV is the variability of controls across 2 kit lots.

	Control	Runs	Average Conc. (ng/mL)	Average Intra-plate %CV	Inter-plate %CV	Inter-lot %CV
AGP	High	9	42,706	5.3	9.9	7.1
	Mid	9	8,427	4.2	10.1	5.6
	Low	9	408	3.4	7.1	7.0
A2M	High	9	215,203	6.2	8.6	7.0
	Mid	9	20,468	2.4	6.3	5.8
	Low	9	1,870	3.1	4.0	6.8

Spike Recovery

Normal rat serum, EDTA plasma, and heparin plasma were diluted 20,000-fold then spiked with calibrators at multiple levels throughout the range of the assay.

% Recovery=measured/expected∗100

	AGP				A2M			
Sample	Spike Conc. (ng/mL)	Measured Conc. (ng/mL)	Measured Conc. %CV	% Recovery	Spike Conc. (ng/mL)	Measured Conc. (ng/mL)	Measured Conc. %CV	% Recovery
Serum	0	1.84	1.5		0	3.31	1.8	
	4.69	6.80	1.8	104	4.69	7.30	3.3	91
	18.8	23.3	1.4	113	18.8	20.9	2.0	95
	75.0	76.4	2.1	99	75	77.2	1.1	99
	300	>ULOQ	-	-	300	295	1.2	97
EDTA Plasma	0	2.78	2.6		0	6.81	0.2	
	4.69	7.76	2.5	104	4.69	10.7	0.5	93
	18.8	23.4	3.4	109	18.8	24.0	1.0	94
	75.0	64.9	3.1	83	75	76.2	2.8	93
	300	>ULOQ	-	-	300	298	5.4	97
Heparin Plasma	0	1.91	1.6		0	2.36	2.2	
	4.69	6.39	2.6	97	4.69	5.90	6.0	84
	18.8	21.8	1.5	106	18.8	17.2	3.6	81
	75.0	67.9	2.4	88	75	69.0	4.7	89
	300	>ULOQ	-	-	300	275	5.2	91

Dilution Linearity

To assess linearity, normal rat serum, EDTA plasma, and heparin plasma samples were diluted 50-fold, 100-fold, 200-fold, and 400-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).

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

		AGP			A2M		
Sample	Fold Dilution	Conc. (ng/mL)	Conc. %CV	% Recovery	Conc. (ng/mL)	Conc. %CV	% Recovery
Serum 1	10,000	40,723	3.3		68,122	0.7	
	20,000	39,630	2.3	97	69,513	1.7	102
	40,000	39,010	3.1	98	69,726	5.6	100
	80,000	40,691	0.7	104	<LLOQ	-	-
Serum 2	10,000	38,328	1.9		15,105	1.4	
	20,000	38,607	2.5	101	<LLOQ	-	-
	40,000	38,175	1.7	99	<LLOQ	-	-
	80,000	40,624	0.9	106	<LLOQ	-	-
EDTA Plasma 1	10,000	30,208	2.3		15,786	1.1	
	20,000	29,185	1.0	97	<LLOQ	-	-
	40,000	30,795	1.4	106	<LLOQ	-	-
	80,000	32,109	1.8	104	<LLOQ	-	-
EDTA Plasma 2	10,000	73,760	2.4		151,997	3.6	
	20,000	67,844	3.6	92	156,337	3.1	103
	40,000	66,470	3.6	98	159,606	2.7	102
	80,000	66,440	1.3	100	161,312	3.9	101
Heparin Plasma 1	10,000	42,268	1.8		122,512	1.1	
	20,000	38,648	0.3	91	112,659	0.8	92
	40,000	41,168	2.5	107	124,168	2.3	110
	80,000	41,636	1.5	101	115,258	0.9	93
Heparin Plasma 2	10,000	43,130	2.9		51,124	3.7	
	20,000	41,316	1.4	96	49,838	0.8	97
	40,000	42,182	2.9	102	53,127	3.7	107
	80,000	45,056	1.3	107	<LLOQ	-	-

Specificity

To assess specificity of the detection antibodies, the Acute Phase Protein Panel 1 (rat) was run using blended calibrators diluted to STD-02, with individual detection antibodies. The % cross-reactivity for each individual detection antibody was shown to be <0.5 % for all assays.

Samples

EDTA plasma samples were collected from 15 control animals and 9 animals treated with agents to induce an inflammatory response, diluted 20,000-fold, and tested with the Acute Phase Protein Panel 1 (rat). Median and range of concentrations for each sample set are displayed below. Detected level was above LLOQ for all analytes in all samples. Average CVs for measured samples was less than 10%.

Sample	Statistic	AGP	A2M
Control EDTA Plasma	Median (µg/mL)	35.8	38.5
	Range (µg/mL)	21.6–79.7	23.1–59.7
	N	15	15
Treated EDTA Plasma	Median (µg/mL)	329	1,404
	Range (µg/mL)	86.9–502	84.0–4,142
	N	9	9

Assay Components

Calibrators

Normal rat EDTA plasma samples were pooled, calibrated against internal controls, and diluted to make the Acute Phase Protein Panel 1 (rat) Calibrator Blend.

Antibodies

	Source Species	
Analyte	MSD Capture Antibody	MSD Detection Antibody
AGP	Rabbit Polyclonal	Rabbit Polyclonal
A2M	Goat Polyclonal	Goat Polyclonal

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. Ruminy P, Gangneux C, Claeyssens S, Scotte M, Daveau M, Salier JP. Gene transcription in hepatocytes during acute phase of a systemic inflammation: from transcription factors to target genes. Inflamm Res. 2001 Aug;50(8): 383-390

Summary Protocol

MSD 96-well MULTI-SPOT Acute Phase Protein Panel 1 (rat) Kit

MSD provides this summary protocol for your convenience.
Please read the entire detailed protocol prior to performing the Acute Phase Protein Panel 1 (rat) assays.

Sample and Reagent Preparation

- ❑ Bring all reagents to room temperature, and thaw the calibrator on ice.
- ❑ Prepare an 8-point standard curve using the supplied calibrator:
  - Dilute the stock calibrator blend 20-fold in Diluent 100.
  - Perform a series of 4-fold dilution steps and prepare a zero calibrator blank.
- ❑ Dilute samples 20,000-fold in Diluent 100 before adding to the plate.
- ❑ Prepare combined detection antibody solution by diluting each stock detection antibody 50-fold in Diluent 100.
- ❑ Prepare 2X Read Buffer T by diluting stock 4X Read Buffer T 2-fold with deionized water.
- ❑ If using MSD Wash Buffer, dilute stock 20X MSD Wash Buffer 20-fold with deionized water to prepare a 1X working solution.

STEP 1: Add Diluent 100

- ❑ Add 25 µL/well of Diluent 100.
- ❑ Incubate at room temperature with shaking (500–1,000 rpm) for 30 minutes.

STEP 2: Add Sample or Calibrator

- ❑ Add 25 µL/well of Calibrator or diluted sample.
- ❑ Incubate at room temperature with shaking (500–1,000 rpm) for 2 hours.

STEP 3: Wash and Add Detection Antibody Solution

- ❑ Wash the plate three times with at least 150 µL/well of PBS-T or 1X MSD Wash Buffer.
- ❑ Add 25 µL/well of 1X detection antibody solution.
- ❑ Incubate at room temperature with shaking (500–1,000 rpm) for 2 hours.

STEP 4: Wash and Read Plate

- ❑ Wash the plate three times with at least 150 µL/well of PBS-T or 1X MSD Wash Buffer.
- ❑ Add 150 µL/well of 2X Read Buffer T.
- ❑ Analyze the plate on an MSD instrument.

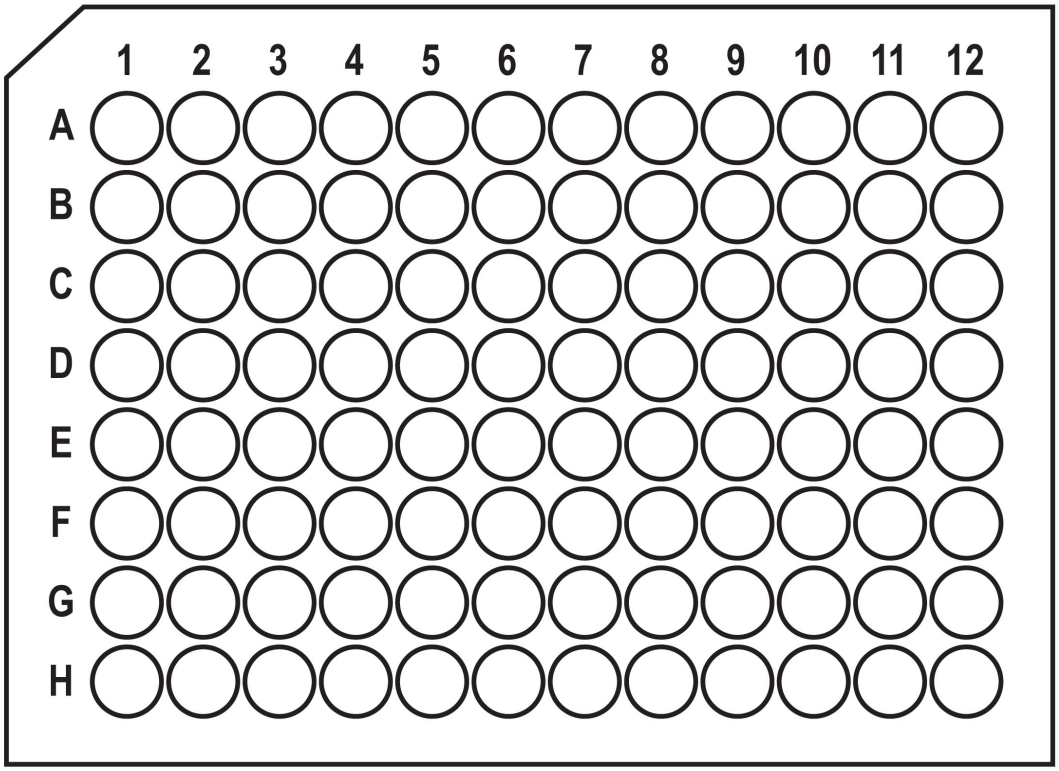
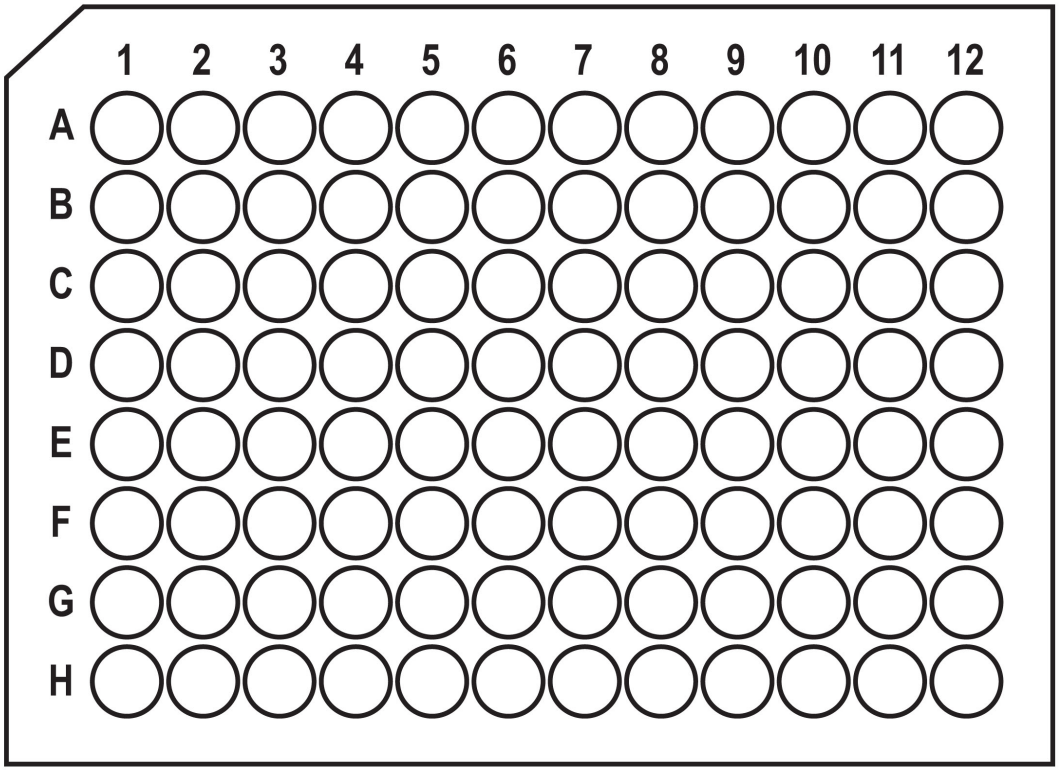



Plate Diagrams