MANUAL

AlphaLISA® Mouse CCL4/MIP-1β Detection Kit

Product number: AL3190

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed for the quantitative determination of **mouse CCL4/MIP-1b** using a homogeneous no wash AlphaLISA assay.

Kit contents: The kit contains 5 components: AlphaLISA Acceptor Beads coated with anti-mCCL4 Antibody, Streptavidin-coated Donor beads, Biotinylated anti-mCCL4 Antibody, Lyophilized mCCL4 and 10X AlphaLISA Immunoassay Buffer.

Sensitivity: Lower Detection Limit (LDL): **5.8 pg/mL**
Lower Limit of Quantification (LLOQ): **17.2 pg/mL**
EC_{50}: **7.2 ng/mL**

Quantitative Range: **17 – 30 000 pg/mL**

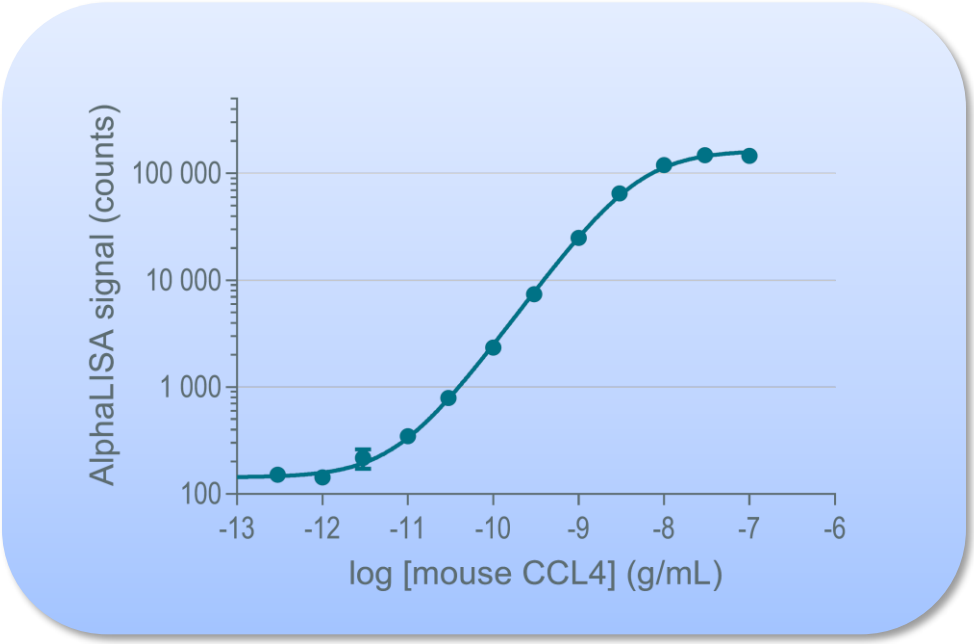


Figure 1. Typical sensitivity curve in AlphaLISA Immunoassay Buffer. The data was generated using a grey AlphaPlate™-384 microplate and the EnVision® Multilabel Plate Reader 2102 with Alpha option.

Storage: Store kit in the dark at 4 ºC. For reconstituted analyte, aliquot and store at -20 ºC for up to 6 weeks. **Avoid freeze-thaw cycles**

Stability: This kit is stable for at least 6 months from the date of manufacture when stored in its original packaging and the recommended storage conditions.

Analyte of Interest

CCL4 (also known as Macrophage Inflammatory protein 1 beta (MIP-1 beta)) is a cytokine produced by cells of the immune system, particularly monocytes and has pro-inflammatory functions upon binding to receptors, mainly by attracting inflammatory cells to a specific site. It is a member of the CC chemokine family and is known to play roles in pathological inflammation such as toxic shock or chronical diseases such as arthritis.

The AlphaLISA Mouse CCL4/MIP-1b Detection Kit is designed to detect mouse CCL4/MIP-1b in serum, plasma and cell culture media.

AlphaLISA technology allows the detection of molecules of interest in cell culture and secretion media (DMEM, RPMI 1640), in serum and plasma in a highly sensitive, quantitative, reproducible, and user-friendly mode. In this AlphaLISA assay, a biotinylated anti-mouse CCL4/MIP-1b antibody binds to the streptavidin coated AlphaLISA Donor beads, while another anti-mouse CCL4/MIP-1b antibody is conjugated to AlphaLISA Acceptor beads. In the presence of mouse CCL4/MIP-1b, the beads come into proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer within the Acceptor beads, resulting in emission with λ_{max} at 615 nm (Figure 2).

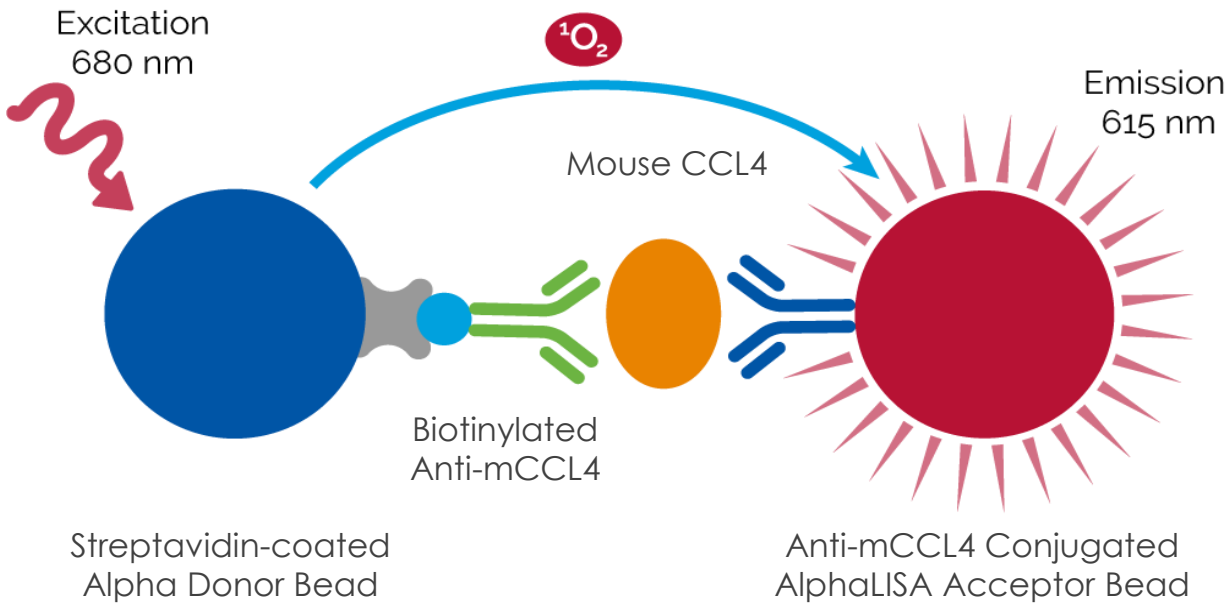


Figure 2. AlphaLISA Mouse CCL4/MIP-1b Detection Assay Principle.

Precautions

- The Alpha Donor beads are light sensitive. All the other assay reagents can be used under normal light conditions. All Alpha assays using the Donor beads should be performed under subdued laboratory lighting (< 100 lux). Green filters (LEE 090 filters (preferred) or Roscolux filters #389 from Rosco) can be applied to light fixtures.
- Take precautionary measures to avoid contamination of the reagent solutions.
- The biotinylated anti-Mouse CCL4/MIP-1b antibody contains sodium azide. Contact with skin or inhalation should be avoided.

Kit Content: Reagents and Materials

Kit components	AL3190HV (100 assay points)	AL3190C (500 assay points)	AL3190F (5000 assay points)
AlphaLISA Anti- Mouse CCL4 Acceptor beads stored in PBS, 0.05% Kathon CG/ICP II, pH 7.2	10 µL @ 5 mg/mL (1 brown tube, white cap)	25 µL @ 5 mg/mL (1 brown tube, white cap)	250 µL @ 5 mg/mL (1 brown tube, white cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Kathon CG/ICP II, pH 7.4	40 µL @ 5 mg/mL (1 brown tube, black cap)	200 µL @ 5 mg/mL (1 brown tube, black cap)	2 X 1 mL @ 5 mg/mL (2 brown tubes, black caps)
Biotinylated Anti- Mouse CCL4 Antibody stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	20 µL @ 500 nM (1 tube, black cap)	50 µL @ 500 nM (1 tube, black cap)	500 µL @ 500 nM (1 tube, black cap)
Lyophilized Recombinant Mouse CCL4	0.1 µg (1 tube, clear cap)	0.1 µg (1 tube, clear cap)	0.1 µg (1 tube, clear cap)
AlphaLISA Immunoassay Buffer (10X)	2 mL, 1 small bottle	10 mL, 1 medium bottle	100 mL, 1 large bottle

* Reconstitute lyophilized analyte in 100 µL Milli-Q® grade H_2O. IMPORTANT: **do not vortex.** The reconstituted analyte should be used within 60 minutes or aliquoted into screw-capped 0.5 mL polypropylene vials and stored at -20°C for future experiments. **The aliquoted analyte at -20°C is stable up to 6 weeks. Avoid freeze-thaw cycles.** One vial contains an amount of analyte sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL3190S).

** Extra buffer can be ordered separately (AlphaLISA Immunoassay Buffer (IAB): cat # AL000C: 10 mL, cat # AL000F: 100 mL.

*** The number of assay points is based on an assay volume of 100 µL in 96-well plates or 50 µL in 384-well assay plates using the kit components at the recommended concentrations.

Sodium azide should **not** be added to the stock reagents. High concentrations of sodium azide (> 0.001% final in the assay) might decrease the AlphaLISA signal. Note that sodium azide from the biotinylated anti-mouse CCL4/MIP-1b antibody stock solution will not interfere with the AlphaLISA signal (0.0001% final in the assay).

Specific additional required reagents and materials:

The following materials are recommended:

Item	Suggested source	Catalog #
TopSeal™-A Plus Adhesive Sealing Film	Revvity Inc.	6050185
EnVision®-Alpha Reader	Revvity Inc.	-

Recommendations

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- The volume indicated on each tube is guaranteed for single pipetting. Multiple pipetting of the reagents may reduce the theoretical amount left in the tube. To minimize loss when pipetting beads, it is preferable not to pre-wet the tip.
- Centrifuge all tubes (including lyophilized analyte) before use to improve recovery of content (2000*g*, 10-15 sec). Re-suspend all reagents by vortexing before use.
- Use Milli-Q® grade H_2O to dilute 10X AlphaLISA Immunoassay Buffer and to reconstitute the lyophilized analyte. Do not vortex the analyte.
- When diluting the standard or samples, change tips between each standard or sample dilution. When loading reagents in the assay microplate, change tips between each standard or sample addition and after each set of reagents.
- When reagents are added to the microplate, make sure the liquids are at the bottom of the well.
- Small volumes may be prone to evaporation. It is recommended to cover microplates with TopSeal-A Adhesive Sealing Films to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film in place.
- The AlphaLISA signal is detected with an EnVision Multilabel Plate Reader equipped with the Alpha option using the AlphaScreen standard settings (e.g. Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as, Emission Filter: M570w, Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75%).
- AlphaLISA signal will vary with temperature and incubation time. For consistent results, identical incubation times and temperature should be used for each plate.
- The standard curves shown in this technical data sheet are provided for information only. A standard curve must be generated for each experiment.

Assay Procedure

- The manual described below is an example for generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations). The manuals also include testing samples in 452 wells. If different amounts of samples are tested, the volumes of all reagents have to be adjusted accordingly, as shown in the table below. These calculations do not include excess reagent to account for losses during transfer of solutions or dead volumes.
- The standard dilution manual is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.
- Use of four background points in triplicate (12 wells) is recommended when LDL/LLOQ is calculated. One background point in triplicate (3 wells) can be used when LDL/LLOQ is not calculated.

Format	# of data points	Volume				Plate recommendation
		Final	Sample	AlphaLISA Acceptor Beads/biotin antibody mix	SA-Donor beads	
AL3190HV	100	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL3190C	250	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 (cat # 6005290)
	500	50 µL	5 µL	5 µL	40 µL	½ Area AlphaPlate-96 (cat # 6002350) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate™-384 (cat # 6005350)
	1 250	20 µL	2 µL	2 µL	16 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate™-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	2 500	10 µL	1 µL	1 µL	8 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL3190F	5 000	50 µL	5 µL	5 µL	40 µL	½ Area AlphaPlate-96 (cat # 6002350) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate-384 (cat # 6005350)
	12 500	20 µL	2 µL	2 µL	16 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	25 000	10 µL	1 µL	1 µL	8 µL	Light gray AlphaPlate-1536 (cat # 6004350)

The 2-Step high concentration manual described below is for 500 assay points including one standard curve (48 wells) and samples (452 wells). *If different amounts of samples are tested, the volumes of all reagents have to be adjusted accordingly.*

1) Preparation of 1X AlphaLISA Immunoassay Buffer:

   Add 1 mL of 10X AlphaLISA Immunoassay Buffer to 9 mL Milli-Q® grade H_2O.

2) Preparation of Mouse CCL4 standard dilutions:
   a. Reconstitute lyophilized Mouse CCL4 (0.1 µg) in 100 µL Milli-Q® grade H_2O.
   b. Prepare standard dilutions as follows in 1X AlphaLISA ImmunoAssay Buffer (change tip between each standard dilution):
   c. A standard curve must be generated for each experiment. The standard curve should be performed in a similar matrix as the samples (e.g. cell culture media for cell supernatant samples, FBS for serum samples).

Tube	Vol. of Mouse CCL4 (µL)	Vol. of Matrix Diluent (µL) *	[Mouse CCL4] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted Mouse CCL4	90	1E-07	100 000
B	60 µL of tube A	140	3.00E-08	30 000
C	60 µL of tube B	120	1.00E-08	10 000
D	60 µL of tube C	140	3.00E-09	3 000
E	60 µL of tube D	120	1.00E-09	1 000
F	60 µL of tube E	140	3.00E-10	300
G	60 µL of tube F	120	1.00E-10	100
H	60 µL of tube G	140	3.00E-11	30
I	60 µL of tube H	120	1.00E-11	10
J	60 µL of tube I	140	3.00E-12	3
K	60 µL of tube J	120	1.00E-12	1
L	60 µL of tube K	140	3.00E-13	0.3
M ** (background)	0	100	0	0
N ** (background)	0	100	0	0
O ** (background)	0	100	0	0
P ** (background)	0	100	0	0

* At low concentrations of mouse CCL4, a significant amount of mouse CCL4 can bind to the vial. Therefore, load the mouse CCL4 standard dilutions in the assay microplate within 60 minutes of preparation.

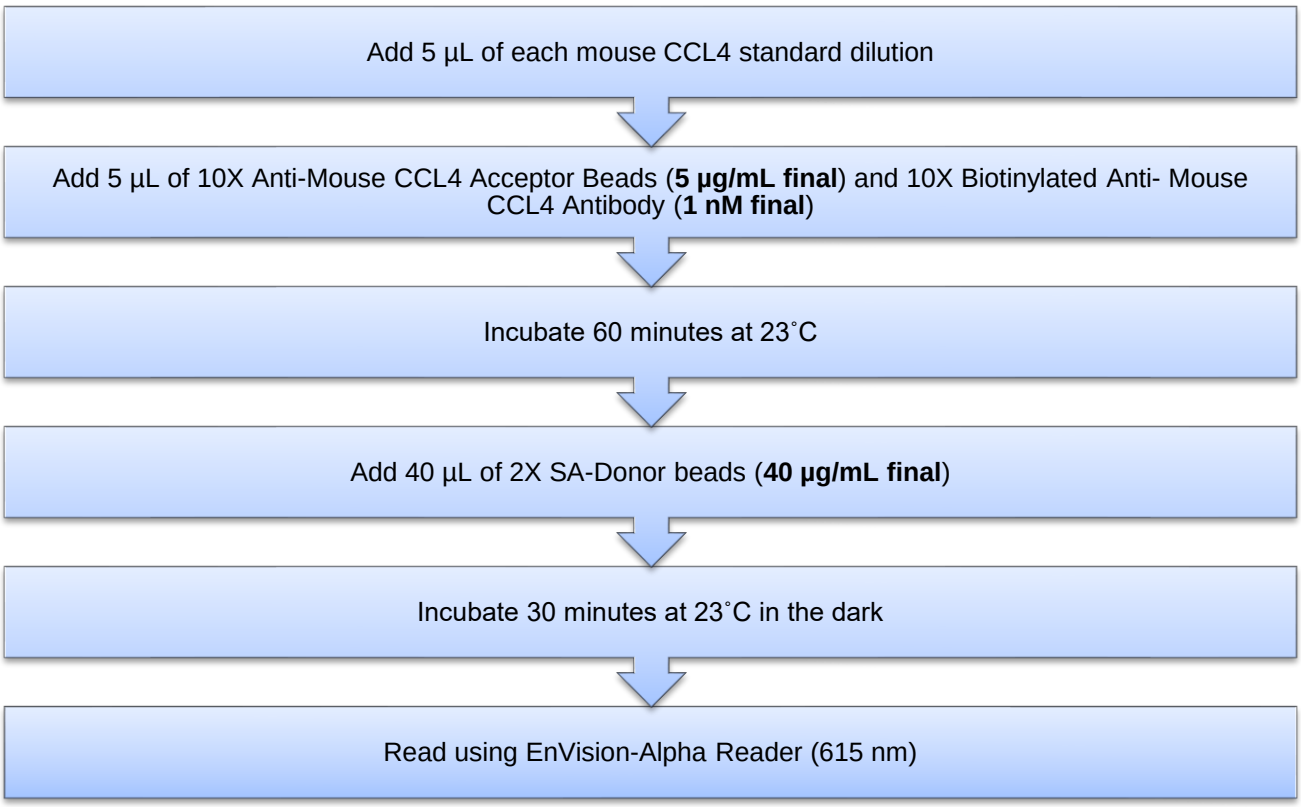
** These points are used to calculate parameters such as LDL. If not necessary, a single background point (M) can be used.

3) Preparation of 10X AlphaLISA Anti- Mouse CCL4 Antibody Acceptor beads (50 µg/mL) and Biotinylated Anti- Mouse CCL4 Antibody (10 nM):

   a. Prepare just before use.
   b. Add 25 µL of 5 mg/mL AlphaLISA Anti- Mouse CCL4 Antibody Acceptor and 50 µL of 500 nM Biotinylated Anti- Mouse CCL4 Antibody to 2425 µL of 1X AlphaLISA Immunoassay Buffer.

4) Preparation of 1.25X Streptavidin (SA) Donor beads (50 µg/mL):
   a. Prepare just before use.
   b. Keep the beads under subdued laboratory lighting.
   c. Add 200 µL of 5 mg/mL SA-Donor beads to 19800 µL of 1X AlphaLISA Immunoassay Buffer.

5) In an AlphaPlate (384 wells):



Plot analytes on the standard curve.

Data Analysis

- Calculate the average count value for the background wells.
- Generate a standard curve by plotting the AlphaLISA counts versus the concentration of analyte. A log scale can be used for either or both axes. No additional data transformation is required.
- Analyze data according to a nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) and a $1/Y^2$ data weighting (the values at maximal concentrations of analyte after the hook point should be removed for correct analysis).
- The LDL is calculated by interpolating the average background counts (12 wells without analyte) + 3 x standard deviation value (average background counts + (3xSD)) on the standard curve.
- The LLOQ as measured here is calculated by interpolating the average background counts (12 wells without analyte) + 10 x standard deviation value (average background counts + (10xSD)) on the standard curve. Alternatively, the true LLOQ can be determined by spiking known concentrations of analyte in the matrix and measuring the percent recovery, and then determining the minimal amount of spiked analyte that can be quantified within a given limit (usually +/- 20% or 30% of the real concentration).
- Read from the standard curve the concentration of analyte contained in the samples.
- If samples have been further diluted, the concentration read must be multiplied by the dilution factor.

Assay Performance Characteristics

AlphaLISA assay performance described below was determined using the 2-step high concentration manual and preparing the proper matrix diluent, depending on sample type. 1X AlphaLISA Immunoassay buffer was used for dilution of all detection reagents.

- Assay Sensitivity:

AlphaLISA sensitivity was determined using the 2-step high concentration manual described above. Performance of each standard curve was analyzed by preparing analytes in each analyte diluent listed in the table below. AlphaLISA detection reagents were prepared in 1X AlphaLISA Immunoassay Buffer. The LDL was calculated as described above. The values correspond to the lowest concentration of analyte that can be detected in a volume of 5 µL sample using the recommended assay conditions.

LDL (pg/mL)	Analyte diluent
5.8	1X AlphaLISA Lysis Buffer
8.6	DMEM
1.5	DMEM + 10% FBS
10	RPMI
7.2	RPMI 1640 + 10% FBS
7.1	100% FBS
10.1	10% FBS

- Assay Precision:

The following assay precision data were calculated from three independent assays. Mouse plasma was used at the following dilutions: raw plasma, 50% plasma in FBS and 25% plasma in FBS. Measured concentration of CCL4 in raw mouse plasma is 200 pg/mL. Data were analyzed on a standard curve of mouse CCL4/MIP-1b prepared in FBS. 24 assay points were tested at each concentration. The experiment was repeated 3 times with 3 assay points at each concentration to determine inter-assay variation. Data of variability are shown in CV% of measured concentration.

  - Intra-assay precision:

Mouse CCL4/MIP-1b	Raw plasma 200 pg/mL of CCL4	50% plasma in FBS 100 pg/mL of CCL4	25% plasma in FBS 50 pg/mL of CCL4
Intra-CV (%) n=24 points	4.7	8.9	13.0

  - Inter-assay precision:

Mouse CCL4/MIP-1b	Raw plasma 200 pg/mL of CCL4	50% plasma in FBS 100 pg/mL of CCL4	25% plasma in FBS 50 pg/mL of CCL4
Inter-CV (%) n=3 experiments	5	5.3	2.8

- Spike Recovery:

Three known concentrations of analyte (10, 1 and 0.1 ng/mL) were spiked into various matrices, including IAB, DMEM, DMEM + 10% FBS, RPMI, RPMI + 10% FBS and FBS. Standard curves were performed in the corresponding matrices and results plotted. Obtained concentrations were divided by theoretical concentration and converted into percentage.

Spiked	IAB	DMEM	DMEM + 10% FBS	RPMI	RPMI + 10% FBS	FBS
10 ng/mL	116,3%	92%	114,8%	113,8%	110,4%	110,4%
1 ng/mL	94,3%	85,1%	90,8%	87,8%	94,5%	101,1%
0.1 ng/mL	113,9%	103,5%	102,2%	103,8%	103,5%	115,1%

- Cross-reactivities

Cross-reactivity of the AlphaLISA Mouse CCL4/MIP-1b Detection Kit was tested using recombinant CCL4 from rat and human as analytes in an assay using the manual as described above. The cross reactivities were calculated using the highest signal obtained and plotting it on a standard curve of mouse CCL4 and dividing concentration obtained on the curve by the concentration of analog used then conversion into percentage. Selectivity was measured by testing mouse MIP-1a, MIP-1c and MIP-1d.

Proteins	Cross Reactivity (%)*
Human CCL4/MIP-1b	None
Rat CCL4/MIP-1b	None
Mouse MIP-1a/CCL3	None
Mouse MIP-1c/CCL9	None
Mouse MIP-1d/CCL15	None

* Results of None indicate that no measurable signal was obtained at any concentration of tested analyte.

- Cell Experiments:

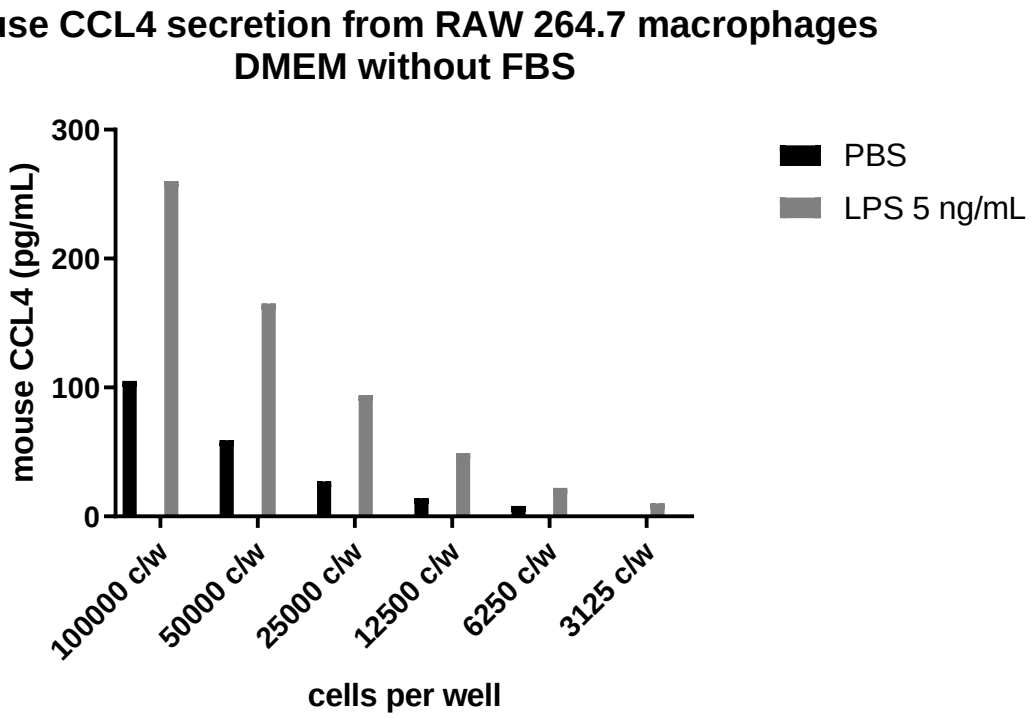
Mouse RAW264.7 macrophagic cell line was used for testing mouse CCL4/MIP-1b secretion. RAW264.7 cells treated with LPS are known to secrete significant amounts of mouse CCL4/MIP-1b.

- Cell culture
  - RAW264.7cells were thawed at 37°C for 2 minutes, spun down and resuspended in 1 mL of DMEM + 10% FBS.
  - The cells were added to a T-75 flask and left to grow until confluence in a 5% CO_2 37C incubator.
  - Cells were harvested using 0.25% trypsin solution. *Note: Macrophage cell line are known to be difficult to harvest. A trypsinization time of 15 minutes is recommended. Alternatively, a cell scrapper can be used with GENTLE strokes to detach the cells.*
  - Cells were washed twice with cold sterile PBS.
  - Cells were counted on a cell counter (Nexcellom auto T4) and were plated in 8-times replicas in a 96-well CulturPlate at the following amounts: 100000, 50000, 25000, 12500, 6250, 3125, 1560, 780, 390, 195, 98 and 0 cell per well (multiply by 10 to obtain concentration in cells per mL) in 100 µL additions in DMEM+ 10% FBS.
  - Cells were incubated for 24 hours.

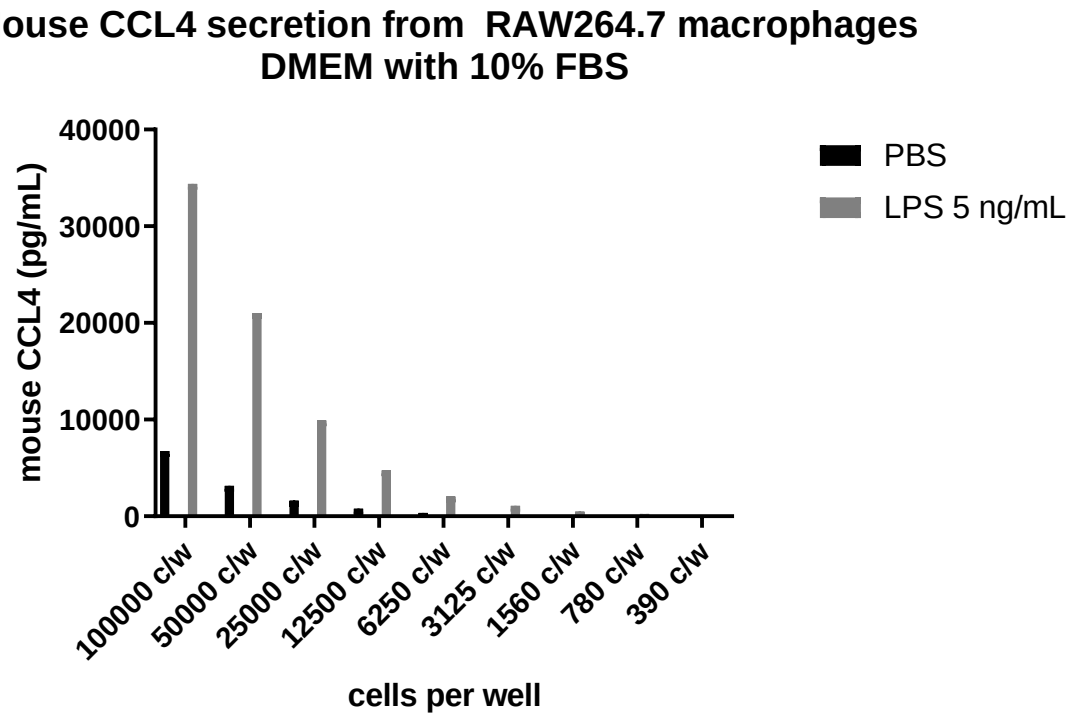
- Pharmacological treatment

    - Supernatant was removed and discarded.
    - Culture media was replaced with DMEM without serum for one assay group and with DMEM + 10% FBS for the other assay group.
    - Cells were incubated for 24 hours.
    - The following activators were added to each assay group from 100X stock solutions:
        i) 5 ng/mL lipopolysaccharide from E. coli 0H13 (LPS)
        ii) Sterile PBS 1X was used as negative control.
    - Cells were incubated for 18 hours.
    - Supernatant was removed and kept for testing.
    - Concentrations of mouse CCL4 were calculated from interpolating the signal generated in each sample from a standard curve prepared in DMEM + 10% FBS.

Results from group without FBS in culture media.



Results show that LPS does stimulate the cells to secrete mCCL4. There is also a basal signal of secretion present.



Results show that FBS has a significant impact on both basal and activated secretion levels of mouse CCL4 by RAW264.7 cells.

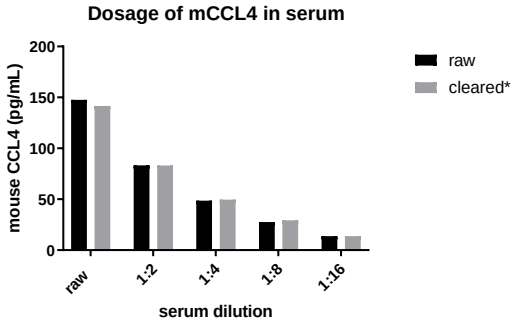
- Test of serum and plasma specimens

The following biological fluids were tested for concentration of mouse CCL4/MIP-1b.

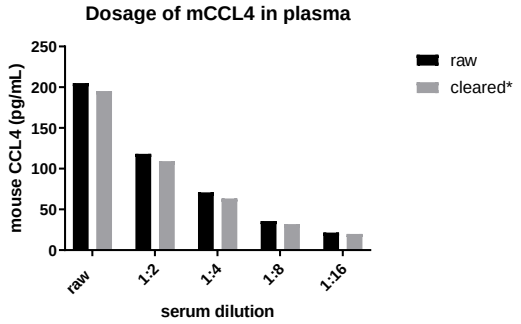
- Mouse serum (normal from pooled donors) (Innovative Research custom lot)
- Mouse plasma (Innovative Research custom lot)

As serum and plasma looked cloudy due to the presence of lipids, a comparison was made between raw samples and sampled cleared of lipids by centrifugation (15 minutes at 13.2k x *g*).

All samples were used as provided, then diluted at one in two dilutions in FBS. Samples were tested on a standard curve made in a matrix diluent of FBS. The results that were above the assay LDL were plotted on the standard curve.



* lipids removed by 15 min centrifugation at 16 000g



* lipids removed by 15 min centrifugation at 16 000g

- Mouse CCL4/MIP-1b was detected in the assay at concentrations that are within accepted ranges from the literature.
- Note that all samples are from pooled donors, with no records of sex or any treatment.

- The delipidation step appears to be unnecessary, as no difference were observed between raw and treated samples.

Troubleshooting Guide

You will find detailed recommendations for common situations you might encounter with your AlphaLISA Assay kit at: www.revvity.com

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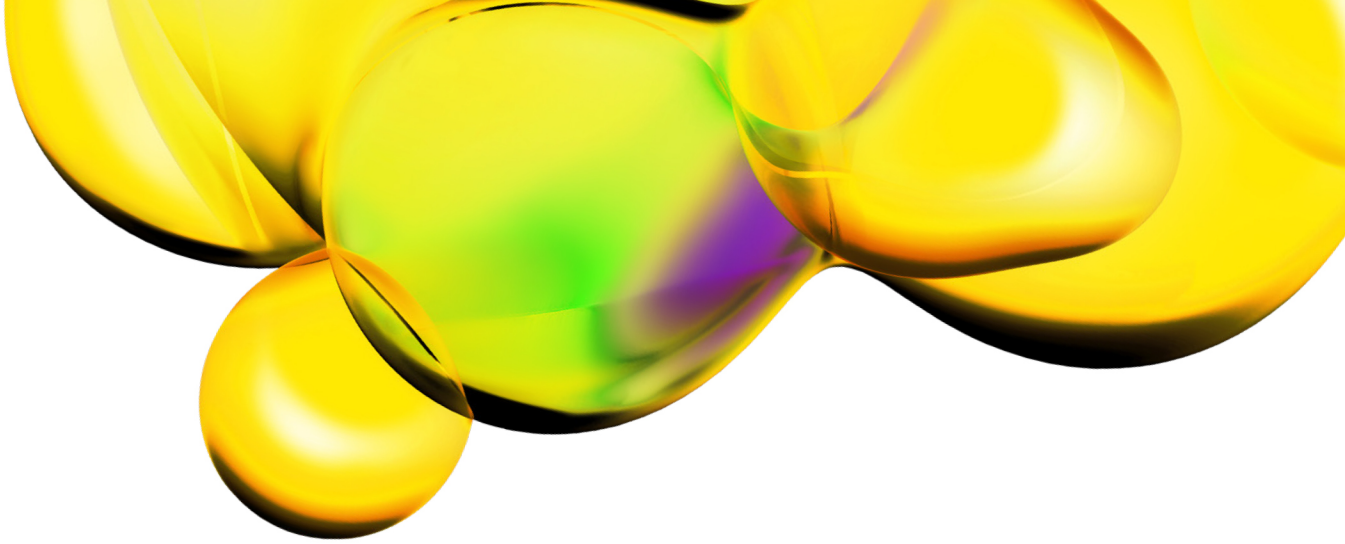

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