MANUAL

AlphaLISA® Mouse Interleukin-18 Detection Kit

Product number: AL3189HV/C/F

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed for the quantitative determination of **mouse interleukin-18 (IL-18)** using a homogeneous no wash AlphaLISA assay.

Kit contents: The kit contains 6 components: AlphaLISA Acceptor Beads coated with anti-mIL-18 Antibody, Streptavidin-coated Donor beads, Biotinylated anti-mIL-18 Antibody, Lyophilized mIL-18, 10X AlphaLISA Immunoassay Buffer and 5X AlphaLISA Lysis Buffer.

Sensitivity: Lower Detection Limit (LDL): **18 pg/mL**
Lower Limit of Quantification (LLOQ): **89 pg/mL**
EC_{50}: **28 ng/mL**

Quantitative Range: **90 – 150 000 pg/mL**

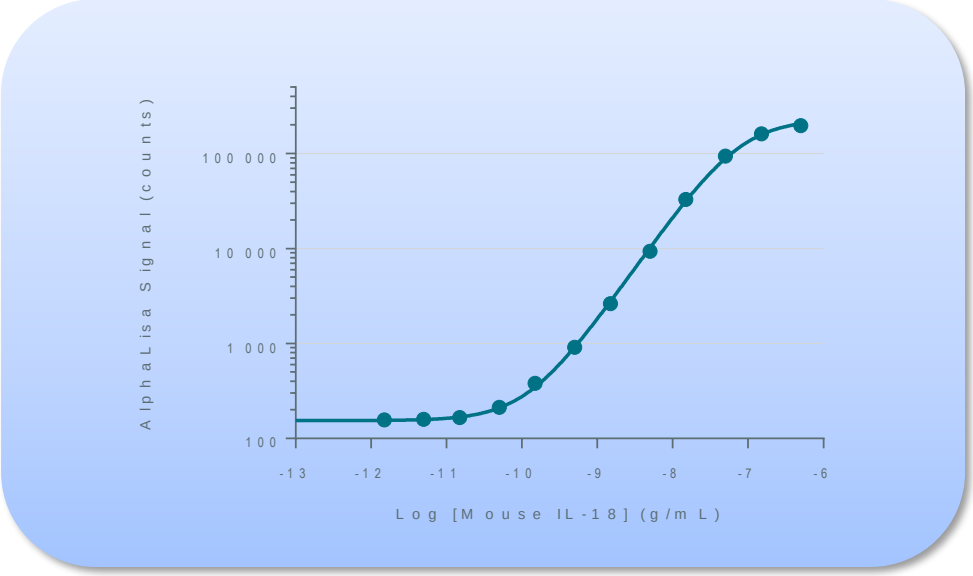


Figure 1. Typical sensitivity curve in AlphaLISA Immunoassay Buffer. The data was generated using a grey AlphaPlate™-384 microplate and the EnVision® Multilabel Plate Reader 2102 with Alpha option.

Storage: Store kit in the dark at 4 ºC. For reconstituted analyte, aliquot and store at -20 ºC for up to 1 month. **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

Interleukin-18 is a cytokine of the interleukin-1 family. It is generated as a pro-form that is transformed into the mature form by cleavage of a pro-peptide by caspase-1 (also known as ICE). Interleukin-18 is generated by many types of cells including hematopoietic (such as macrophages) and other types such as keratinocytes. Its main function is to activate immune cells after infection by generating the production of interferon gamma. Interleukin-18 is a strong activator of inflammation response and is considered a biomarker of inflammation.

The AlphaLISA Mouse Interleukin-18 Detection Kit is designed to detect mouse interleukin-18 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 interleukin-18 antibody binds to the streptavidin coated AlphaLISA Donor beads, while another anti-mouse interleukin-18 antibody is conjugated to AlphaLISA Acceptor beads. In the presence of mouse interleukin-18, 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).

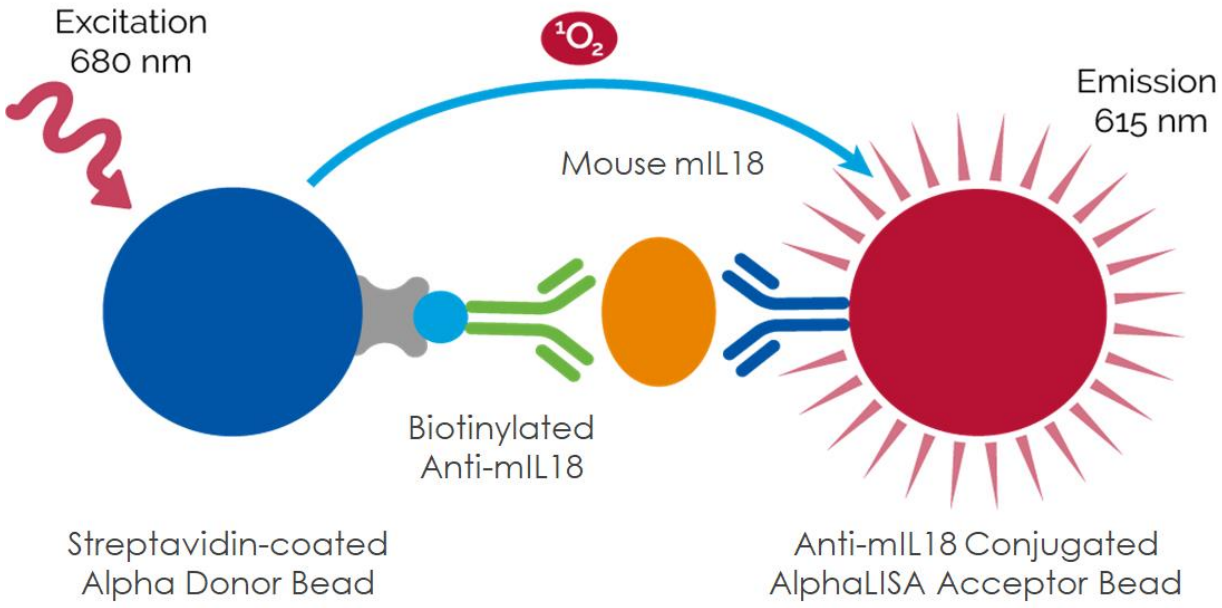


Figure 2. AlphaLISA Mouse Interleukin-18 Detection Assay Principle.

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 interleukin-18 antibody contains sodium azide. Contact with skin or inhalation should be avoided.

Kit Content: Reagents and Materials

Kit components	AL3189HV (100 assay points***)	AL3189C (500 assay points***)	AL3189F (5000 assay points***)
AlphaLISA Anti-Mouse IL-18 Acceptor beads stored in PBS, 0.05% Kathon CG/ICP II, pH 7.2	20 µL @ 5 mg/mL (1 brown tube, white cap)	50 µL @ 5 mg/mL (1 brown tube, white cap)	500 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	80 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 IL-18 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 IL-18*	1 µg (1 tube, clear cap)	1 µg (1 tube, clear cap)	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
AlphaLISA Lysis Buffer (5X)**	2 mL, 1 small bottle	5 mL, 1 small bottle	50 mL, 1 medium 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 # AL3189S).

** Extra buffer can be ordered separately (AlphaLISA Immunoassay Buffer (IAB): cat # AL000C: 10 mL, cat # AL000F: 100 mL, AlphaLISA Lysis Buffer: 10 mL cat#AL003C, 100 mL cat# AL003F).

*** 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 interleukin-18 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	
AL3189HV	100	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL3189C	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)
AL3189F	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 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*. **This manual includes a dilution of samples and analyte 1:1 in AlphaLISA Lysis Buffer.**

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 1X AlphaLISA Lysis Buffer:

   Add 2 mL of 5X AlphaLISA Immunoassay Buffer to 8 mL Milli-Q® grade H_2O.

3) Preparation of Matrix Diluent:

   Add 1 mL of matrix (culture media or FBS) to 1 mL of 1X AlphaLISA Lysis Buffer

4) Preparation of Mouse IL-18 standard dilutions:
   a. Reconstitute lyophilized Mouse IL-18 (1 µg) in 100 µL Milli-Q® grade H_2O.
   b. Dilute analyte by adding 100 uL of 1X AlphaLISA Lysis buffer
   c. Prepare standard dilutions as follows in **Matrix Diluent** (change tip between each standard dilution)

Tube	Vol. of Mouse IL-18 (µL)	Vol. of Matrix Diluent (µL) *	[Mouse IL-18] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted Mouse IL-18	90	**5.00E-07**	**500 000**
B	60 µL of tube A	140	**1.50E-07**	**150 000**
C	60 µL of tube B	120	**5.00E-08**	**50 000**
D	60 µL of tube C	140	**1.50E-08**	**15 000**
E	60 µL of tube D	120	**5.00E-09**	**5 000**
F	60 µL of tube E	140	**1.50E-09**	**1 500**
G	60 µL of tube F	120	**5.00E-10**	**500**
H	60 µL of tube G	140	**1.50E-10**	**150**
I	60 µL of tube H	120	**5.00E-11**	**50**
J	60 µL of tube I	140	**1.50E-11**	**15**
K	60 µL of tube J	120	**5.00E-12**	**5**
L	60 µL of tube K	140	**1.50E-12**	**1.5**
M ** (background)	0	100	**0**	**0**
N ** (background)	0	100	**0**	**0**
O ** (background)	0	100	**0**	**0**
P ** (background)	0	100	**0**	**0**

Important Note: Pay attention to use Matrix Diluent as diluent for standards, not the IAB.

* Dilute standards in Matrix Diluent (e.g., 1X AlphaLISA **Lysis Buffer or 1:1 dilution of 1X AlphaLISA Lysis Buffer and cell culture media or FBS, depending on sample type**).
At low concentrations of mouse IL-18, a significant amount of mouse IL-18 can bind to the vial. Therefore, load the mouse IL-18 standard dilutions in the assay microplate within 60 minutes of preparation.

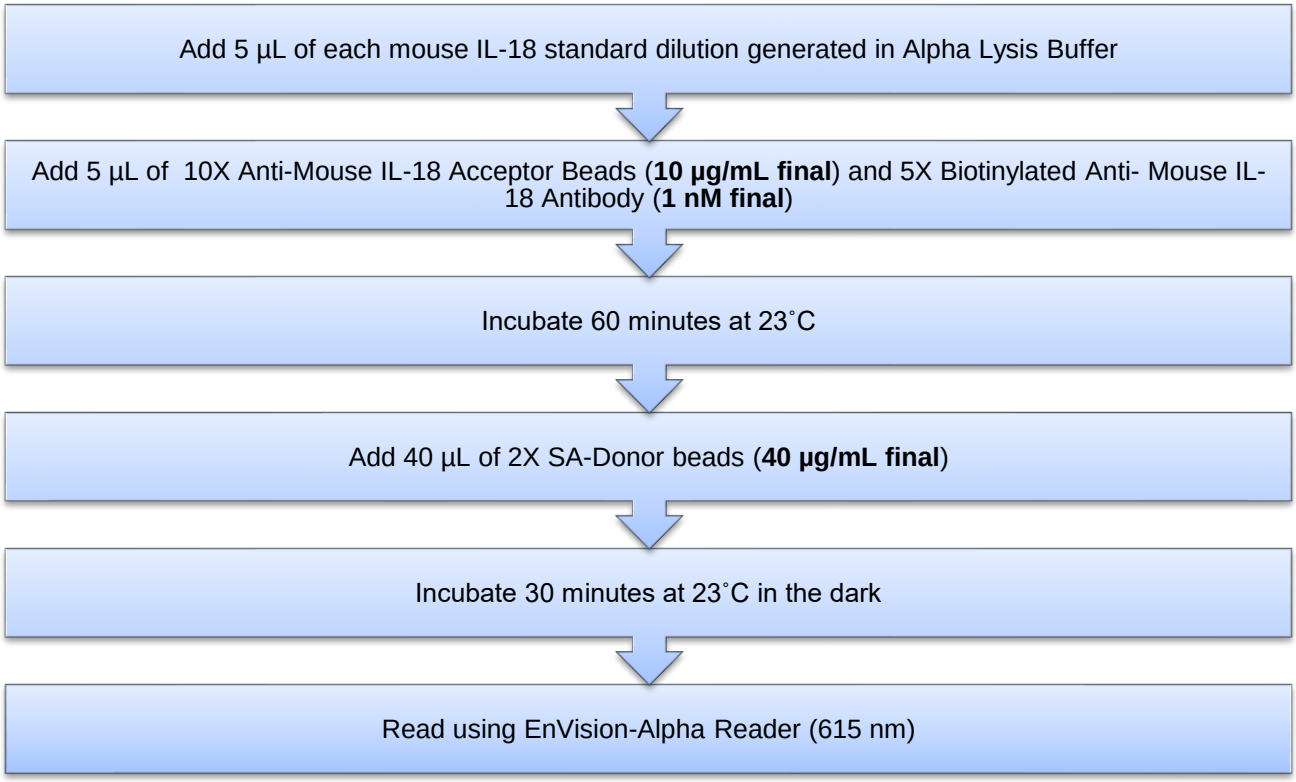
5) **Dilute all samples to be analyzed in 1:1 in 1X AlphaLISA Lysis Buffer**

6) Preparation of 10X AlphaLISA Anti- Mouse IL-18 Antibody Acceptor beads (100 µg/mL) and Biotinylated Anti- Mouse IL-18 Antibody (10 nM):

   a. Prepare just before use.
   b. Add 50 µL of 5 mg/mL AlphaLISA Anti- Mouse IL-18 Antibody Acceptor and 50 µL of 500 nM Biotinylated Anti- Mouse IL-18 Antibody to 2400 µL of 1X AlphaLISA Immunoassay Buffer.

7) 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.

8) 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, with 1X AlphaLISA Lysis Buffer. 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. Performance of each standard curve was analyzed by preparing analytes in each analyte diluent listed in the table below and then diluting 1:1 in AlphaLISA Lysis Buffer. 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
18	1X AlphaLISA Lysis Buffer
24.8	DMEM
16.4	DMEM + 10% FBS
11.7	RPMI
16.4	RPMI 1640 + 10% FBS
49.6	100% FBS
20.5	10% FBS

- Assay Precision:

The following assay precision data were calculated from three independent assays. Mouse plasma was spiked with 50 ng/mL, 5 ng/mL and 0.5ng/mL of mouse Interleukin-18. Data were analyzed on a standard curve of mouse Interleukin-18 prepared in FBS. All samples and analytes were diluted 1:1 in AlphaLISA Lysis Buffer. 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% on concentration.

- Intra-assay precision:

Mouse Interleukin-18	50 ng/mL	5 ng/mL	0.5 ng/mL
Intra-CV (%) n=24 points	6.7	4.1	10.1

- Inter-assay precision:

Mouse Interleukin-18	50 ng/mL	7 500 pg/mL (2 500 cells per well)	13 000 pg/mL (5 000 cells per well)
Inter-CV (%) n=3 experiments	3.8	4.9	4.2

- Spike Recovery:

Four known concentrations of analyte were spiked into mouse serum and plasma. Standard curves were performed in a matrix diluent of 1:1 FBS and 1X AlphaLISA Lysis Buffer.

Spiked	Recovery in Serum	Recovery in Plasma
30000 pg/mL	100%	97.8%
10000 pg/mL	89.2%	89.8%
3000 pg/mL	90.5%	97.4%
1000 pg/mL	90.4%	125%

- Cross-reactivities

Cross-reactivity of the AlphaLISA Mouse Interleukin-18 Detection Kit was tested using recombinant IL-18 from rat and human as analytes in an assay using the manual as described above. The cross reactivities were calculated using the signals of 500 ng/mL mouse interleukin-18 as 100%. Selectivity was measured by testing mouse IL-1beta and mouse IL-33.

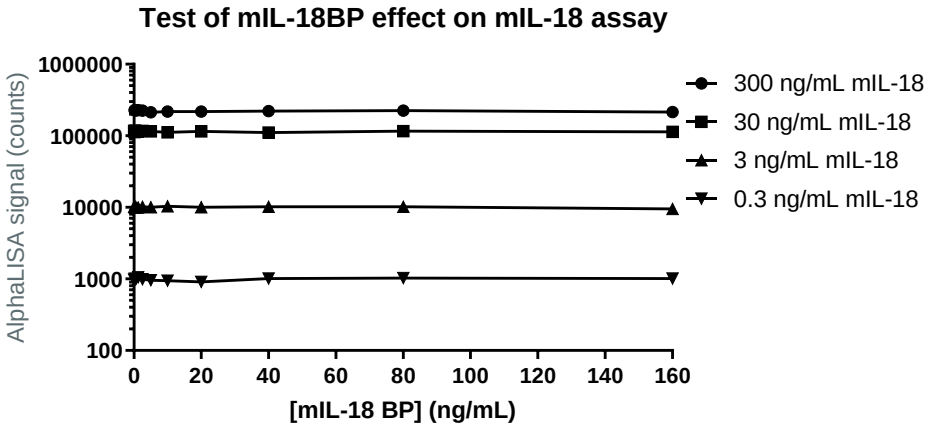
Proteins	Cross Reactivity (%)
Human Interleukin-18	None
Rat Interleukin-18	0.6%
Mouse Interleukin-1b	None
Mouse Interleukin-33	None

- IL-18BP assay

In vivo, IL-18 circulating in the blood is normally bound tightly to a regulator protein called IL-18 Binding Protein in order to control its pro-inflammatory capacity. Binding of such a protein could interfere with binding of an antibody, making results in biological fluids difficult to validate. To test this, we have incubated various amounts of IL-18 with a titration curve of IL-18BP and tested the results.

The following manual was used:

- In three wells of a 384 AlphaPlate add the following (all additions in appropriate buffer or diluent)
  - 5 µL of mouse IL-18 at 300, 30, 3 and 0.3 ng/mL, each concentration mixed with mouse IL-18BP at the following dilutions:
    - 160, 80, 40, 20, 10, 5, 2.5, 1.25, 0.63, 0.32, 0.16 and 0 ng/mL
  - 5 µL of acceptor beads at 100 ug/mL and biotin antibody at 10 nM
  - Incubate 60 minutes at 23ºC
  - 40 µL of streptavidin donor beads at 50 µg/mL
  - Incubate 30 minutes at 23ºC
  - Read on EnVision 2105.



Results show no effect of mouse IL-18BP on the detection assay regardless of the concentration of IL-18 or IL-18BP. Note the use of detergent might help to disassociate these complexes.

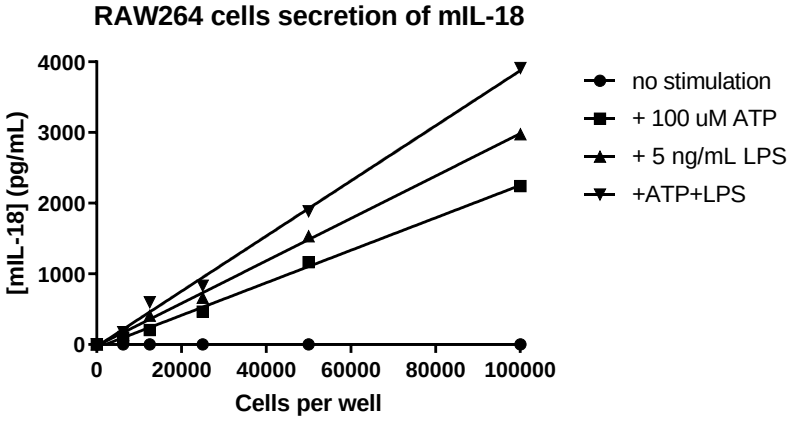
- Cell Experiments:

Mouse RAW264.7 macrophagic cell line was used for testing mouse interleukin-18 secretion. RAW264.7 cells treated with LPS and/or ATP are known to secrete significant amounts of mouse interleukin-18.

- Cell culture
  - MIN-6 cells 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 CellEx counter 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
  - Cells were incubated for 24 hours.
  - The following activators were added:
    i) 100 uM adenosine triphosphate (ATP)
    ii) 5 ng/mL lipopolysaccharide from E. coli 0H13 (LPS)
    iii) 100 uM ATP and 5 ug/mL LPS
    iv) PBS
  - Cells were incubated for 6 hours. *Note: after longer incubation, IL-18 can be degraded by proteases.*
  - Supernatant was removed and kept for testing
  - Concentrations of mouse IL-18 were calculated from interpolating the signal generated in each sample from a standard curve prepared in a matrix diluent of a 1:1 mix of 1X AlphaLISA Lysis Buffer and DMEM



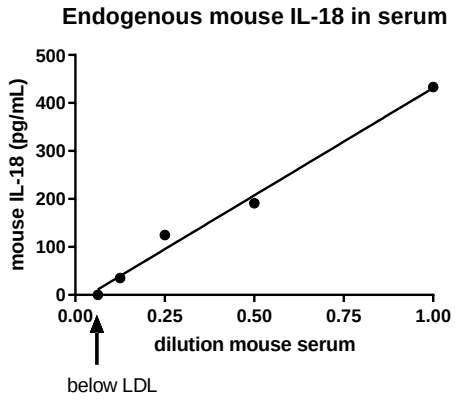
Results show that mouse interleukin-18 secretion in cells is stimulated by LPS and ATP in a synergic manner.

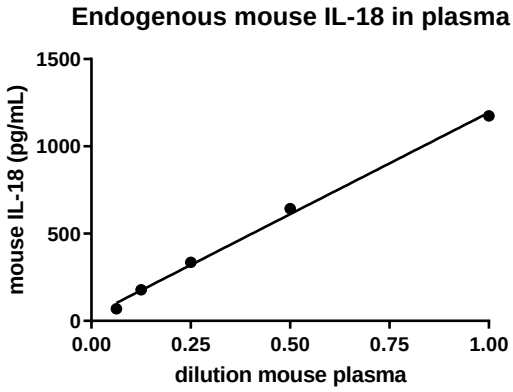
- Test of serum and plasma specimens

The following biological fluids were tested for concentration of mouse interleukin-18.

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

All samples were used as provided, then diluted at half-log dilutions in FBS. Samples were then diluted 1:1 in 1X AlphaLISA Lysis Buffer and tested on a standard curve made in a matrix diluent of FBS:Lysis Buffer 1:1. The results that were above the assay LDL were plotted on the standard curve.





- Mouse interleukin-18 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.

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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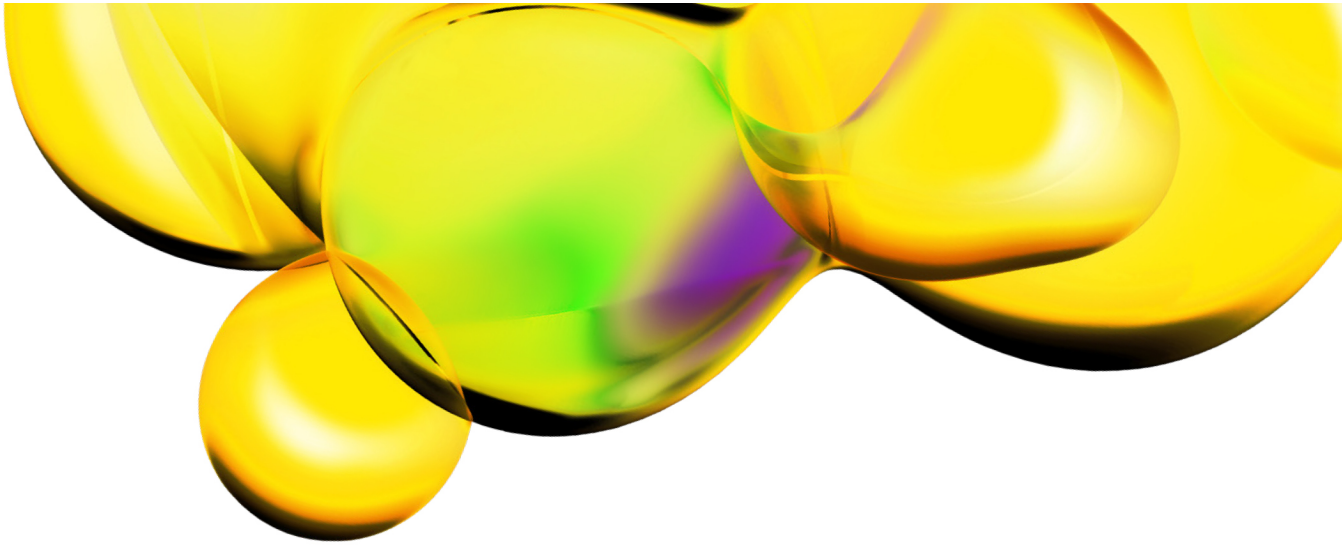

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