BioLegend®

Enabling Legendary Discovery™

LEGEND MAX™

ELISA Kit

Human CCL5(RANTES)

Cat. No. 440807

ELISA Kit for Accurate Quantitation of Human CCL5 (RANTES) from Cell Culture Supernatant, Serum, Plasma and Other Biological Fluids

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It is highly recommended that this manual be read in its entirety before using this product. Do not use this kit beyond the expiration date.

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LEGEND MAX™ Human CCL5(RANTES) ELISA Kit

Introduction:

CCL5 is a member of the CC family of chemokines, also known as RANTES. The precursor of CCL5 contains 91 amino acids, with an N-terminal signal peptide of 23 amino acids which is cleaved to generate the 68 amino acids mature protein (7.8 kD). Human mature CCL5 shares 82% - 90% amino acid seqeuence identity with equine, porcine, bovine, mouse, rat and canine CCL5.

CCL5 is expressed by T cells and monocytes/macrophages. It plays an active role in recruiting leukocytes into inflammatory sites through receptors CCR1, CCR3, CCR4, and CCR5. CCL5 induces the proliferation and activation of certain natural-killer (NK) cells. It also inhibits HIV-1 entry and infection. In addtion, CCL5 plays a crucial role in the migration and metastasis of cancer cells. Increased CCL5 levels are found in melanoma, breast, cervical, prostate, gastric, and pancreatic cancers.

The LEGEND MAX™ Human CCL5(RANTES) ELISA kit is a Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) with a 96-well strip plate that is pre-coated with a monoclonal mouse anti-human CCL5 capture antibody. The detection antibody is a biotinylated polyclonal goat anti-human CCL5. This kit is specifically designed for the accurate quantitation of human CCL5(RANTES) from cell culture supernatant, serum, plasma, and other biological fluids. This kit is analytically validated with ready-to-use reagents.

Materials Provided:

Description	Quantity	Volume (per bottle)	Part #
Anti-Human CCL5 Pre-coated 96 well Strip Microplate	1 plate		77076
Human CCL5 Detection Antibody	1 bottle	12 mL	77077
Human CCL5 Standard	1 vial	lyophilized	77087
Avidin-HRP A	1 bottle	12 mL	79131
Assay Buffer B	1 bottle	25 mL	79128
Wash Buffer (20X)	1 bottle	50 mL	78233
Substrate Solution D	1 bottle	12 mL	78115
Stop Solution	1 bottle	12 mL	79133
Plate Sealers	4 sheets		78101

Tel: 858-768-5800

Materials to be Provided by the End-User:

- Microplate reader able to measure absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 1 µL to 1,000 µL
- Deionized water
- Wash bottle or automated microplate washer
- Log-Log graph paper or software for data analysis
- Tubes to prepare standard dilutions
- Timer
- Plate Shaker
- Polypropylene vials

Storage Information:

Store unopened kit components between 2°C and 8°C. Do not use this kit beyond its expiration date.

<table>
<tr><th colspan="2">Opened or Reconstituted Components</th></tr>
<tr><td>Microplate wells</td><td>If not all microplate strips are used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal. Store between 2°C and 8°C for up to one month.</td></tr>
<tr><td>Standard</td><td>The remaining reconstituted standard stock solution can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze-thaw cycles.</td></tr>
<tr><td>Detection Antibody</td><td rowspan="6">Store opened reagents between 2°C and 8°C and use within one month.</td></tr>
<tr><td>Avidin-HRP A</td></tr>
<tr><td>Assay Buffer B</td></tr>
<tr><td>Wash Buffer (20X)</td></tr>
<tr><td>Substrate Solution D</td></tr>
<tr><td>Stop Solution</td></tr>
</table>

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online at BioLegend's website for details (www.biolegend.com/msds).
2. Substrate Solution D is harmful if inhaled or ingested. Avoid skin, eye and clothing contact.
3. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum, plasma and other biological fluids in accordance with

NCCLS regulations.

4. Stop Solution contains strong acid. *Wear eye, hand, and face protection.*
5. Before disposing of the plate, rinse it with an excess amount of tap water.

Specimen Collection and Handling:

Specimens should be clear and non-hemolyzed. If possible, unknown samples should be run at a number of dilutions to determine the optimal dilution factor that will ensure accurate quantitation.

Serum: Use a serum separator tube and allow clotting for at least 30 minutes, then centrifuge for 10 minutes at 1,000 x *g*. Remove serum layer. *Assay immediately or store serum samples at < -70°C. Avoid repeated freeze-thaw cycles.*

Plasma: Collect blood samples in citrate, heparin or EDTA-containing tubes. Centrifuge for 10 minutes at 1,000 x *g* within 30 minutes of collection. *Assay immediately or store plasma samples at < -70°C. Avoid repeated freeze-thaw cycles*.

Reagent and Sample Preparation:

Note: All reagents should be diluted immediately prior to use.

1. Dilute the 20X Wash Buffer to 1X with deionized water. For example, make 1 liter of 1X Wash Buffer by adding 50 mL of 20X Wash Buffer to 950 mL of deionized water. If crystals have formed in the 20X Wash Buffer, bring to room temperature and mix until dissolved.
2. Reconstitute the lyophilized Human CCL5 Standard by adding the volume of Assay Buffer B to make the 20 ng/mL standard stock solution (Refer to LEGEND MAX Kit Lot-Specific Certificate of Analysis/LEGEND MAX Kit Protocol). Allow the reconstituted standard to sit at room temperature for 15 minutes, then briefly vortex to mix completely.
3. For cell culture supernatants samples, the end user may need to determine the dilution factors in a preliminary experiment. Samples should be diluted in Assay Buffer B if dilutions are necessary.
4. It is recommended that serum and plasma samples be diluted 1:50 with Assay Buffer B prior to analysis. If further dilution is needed, samples should be diluted with Assay Buffer B.

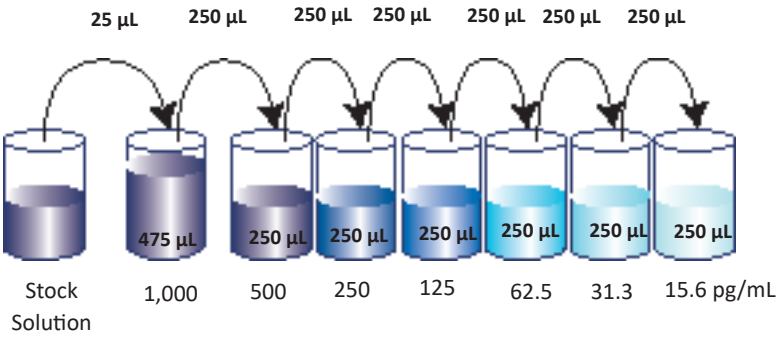
5. Assay Procedure:

Note: Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this kit.

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate. A standard curve is required for each assay.

2. If not all microplate strips will be used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal.

3. Prepare 500 µL of the 1,000 pg/mL top standard by diluting 25 µL of the standard stock solution in 475 µL of Assay Buffer B. Perform six two-fold serial dilutions of the 1,000 pg/mL top standard in separate tubes using Assay Buffer B as the diluent. Thus, the human CCL5 standard concentrations in the tubes are 1,000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL and 15.6 pg/mL, respectively. Assay Buffer B serves as the zero standard (0 pg/mL).



4. Wash the plate 4 times with at least 300 µl of 1X Wash Buffer per well and blot residual buffer by firmly tapping the plate upside down on absorbent paper. All subsequent washes should be performed similarly.

5. Add 50 µLlof Assay Buffer B to each well that will contain either standard dilutions or samples.

6. Add 50 µL of standard dilutions or samples to the appropriate wells.

7. Seal the plate with a Plate Sealer included in the kit and incubate the plate at room temperature for 2 hours while shaking at 200 rpm.

8. Discard the plate contents into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.

9. Add 100 µL of Human CCL5 Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour while shaking.

10. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.

11. Add 100 µL of Avidin-HRP A solution to each well, seal the plate and incubate at room temperature for 30 minutes while shaking.

12. Discard the contents of the plate into a sink, then wash the plate 5 times with 1X Wash Buffer as in step 4. For this final wash, soak wells in 1X Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.

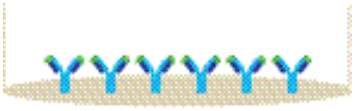
13. Add 100 µL of Substrate Solution D to each well and incubate for 10 minutes in the dark. Wells containing human CCL5 should turn blue in color with intensity proportional to concentration. It is not necessary to seal the plate during this step.

14. Stop the reaction by adding 100 µL of Stop Solution to each well. The well color should change from blue to yellow.

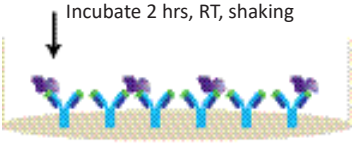
15. Read absorbance at 450 nm within 20 minutes. If the reader is capable of reading at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

Assay Procedure Summary

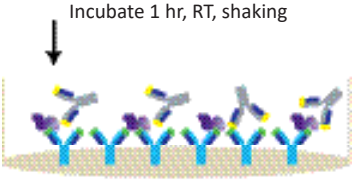
1. Wash 4 times
Add 50 µL Assay Buffer B



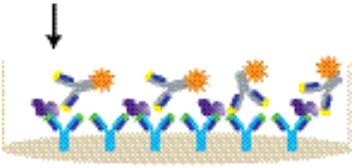
2. Add 50 µL standards or samples
Incubate 2 hrs, RT, shaking



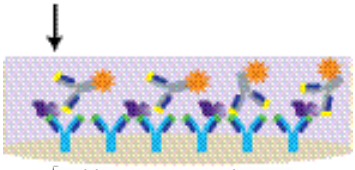
3. Wash 4 times
Add 100 µL Detection Antibody solution
Incubate 1 hr, RT, shaking



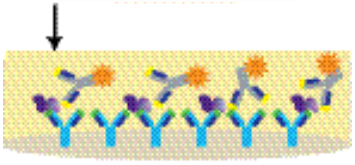
4. Wash 4 times
Add 100 µL Avidin-HRP A solution
Incubate 30 mins, RT, shaking



5. Wash 5 times
Add 100 µL Substrate Solution D
Incubate 10 mins, RT, in the dark



6. Add 100 µL Stop Solution



7. Read absorbance at 450 nm and 570 nm

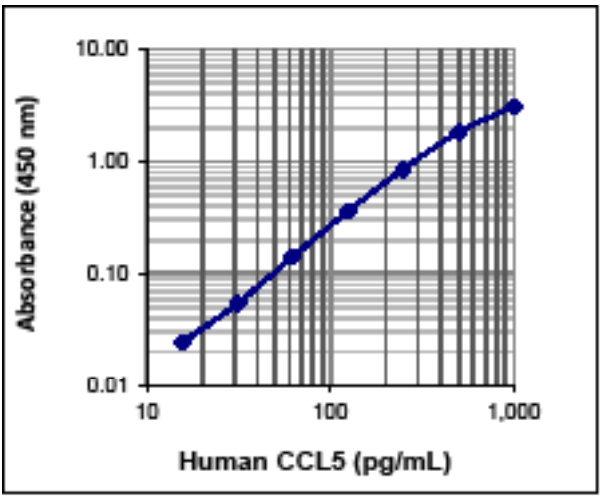
Calculation of Results:

The data can be best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm. If appropriate software is not available, use log-log graph paper to determine sample concentrations. Determine the mean absorbance for each set of duplicate or triplicate standards, controls, and samples. Plot the standard curve on log-log graph paper with cytokine concentration on the X-axis and absorbance on the Y-axis. Draw a best fit line through the standard points. To determine the unknown cytokine concentrations, find the mean absorbance value of the unknown concentration on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the cytokine concentration.

If samples were diluted, multiply the concentration by the appropriate dilution factor. If a test sample's absorbance value falls outside the linear portion of the standard curve, the test sample needs to be re-analyzed at a higher (or lower) dilution as appropriate.

Typical Data:

This standard curve was generated at BioLegend for demonstration purposes only. A standard curve must be run with each assay.



Performance Characteristics:

Specificity: No cross-reactivity was observed when this kit was used to analyze the following recombinant cytokines/chemokines, each at 50 ng/mL.

Human	CCL1, CCL2, CCL3, CCL8, CCL11, CCL17, CCL19, CCL21, CCL23, CCL26, CCL27, CXCL1, CXCL2, CXCL3, CXCL5, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12
Mouse	CCL1, CCL2, CCL5, CCL7, CCL8, CCL11, CCL14, CCL20, CCL21, CCL22, CCL28, CXCL1, CXCL2, CXCL5, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CX3CL1
Rat	CCL2

Sensitivity: The average minimum detectable concentration of human CCL5 is 3.0 ± 0.4 pg/mL.

Recovery: Three levels of recombinant Human CCL5 (250 pg/mL, 62.5 pg/mL and 15.6 pg/mL) were spiked into four human serum and plasma samples (1:50 diluted), and analyzed with the LEGEND MAX™ Human CCL5 (RANTES) ELISA Kit. On average, 101% of the cytokine was recovered from serum samples and 118% from plasma samples.

Linearity: Four human serum and plasma samples containing high concentration of CCL5 were diluted with Assay Buffer B to produce sample concentrations within the dynamic range of the assay. On average, 105% of the expected cytokine was detected from serum samples and 104% from plasma samples.

Intra-Assay Precision: Two samples with different concentrations of human CCL5 were tested with 16 replicates in one assay.

	Sample 1	Sample 2
Number of Replicates	16	16
Mean Concentration (pg/mL)	478.3	121.9
Standard Deviation	7.9	2.1
% CV	1.7	1.7

Inter-Assay Precision: Two samples with different concentrations of human CCL5 were assayed in three independent assays.

	Sample 1	Sample 2
Number of Assays	4	4
Mean Concentration (pg/mL)	516.9	124.7
Standard Deviation	23.6	5.5
% CV	4.6	4.4

Biological Samples:

Serum and Plasma

	Serum (N=20)	EDTA Plasma (N=4)	Heparin Plasma (N=4)	Citrate Plasma (N=5)
Detectable %	100	100	100	100
Mean (pg/mL)	1,883.6	5,505	13,986.3	4,946
Median (pg/mL)	1,723	5,075	13,800	4,085
Max (pg/mL)	4,350	9715	25,000	8,035
Min (pg/mL)	790	2,155	3,345	1,760

Cell Culture Supernatant

Human peripheral blood mononuclear cells at a concentration of $2x10^6$ cells/mL were stimulated with 1 μg/mL LPS, or 50 ng/mL PMA + 1 μg/mL Ionomycin at 37°C for 2 days. The cell culture supernatants were collected and assayed for the concentrations of natural human CCL5.

PBMC Cell Culture Supernatant	pg/mL
Unstimulated	0
+ LPS	1,890
+ PMA/ Iono.	540

Troubleshooting Guide:

Problem	Probable Cause	Solution
High Background	Background wells were contaminated	Avoid cross-well contamination by using the provided plate sealers. Use multichannel pipettes and change tips between pipetting samples and reagents.
	Insufficient washes	Increase number of washes. Increase soaking time between washes prior to addition of substrate solution.
	TMB Substrate Solution was contaminated	TMB Substrate Solution should be clear and colorless prior to addition to wells. Use a clean container prior to pipetting substrate solution into wells.
No or poor signal	Detection Antibody, Avidin-HRP or Substrate solution were NOT added	Rerun the assay and follow the protocol.
	Wrong reagent or reagents were added in wrong sequential order	
	Insufficient plate agitation	The plate should be agitated during all incubation steps using a plate shaker at a speed where solutions in wells are within constant motion without splashing.
	The wash buffer contains Sodium Azide (NaN3)	Avoid Sodium Azide contamination in the wash buffer as it inhibits HRP activity.
	Incubations were done at an inappropriate temperature, timing or without agitation	Rerun the assay and follow the protocol.
Low or poor standard curve signal	The standard was incorrectly reconstituted or diluted	Adjust the calculations and follow the protocol.
	Standard was inappropriately stored	Store the reconstituted standard stock solution in polypropylene vials at -70°C. Avoid repeated freeze-thaw cycles.
	Reagents added to wells with incorrect concentrations	Check for pipetting errors and the correct reagent volume.

Problem	Probable Cause	Solution
Signal is high, standard curves have saturated signal	Standard reconstituted with less volume than required	Reconstitute new lyophilized standard with the correct volume of solution recommended in the protocol.
	Standards/samples, detection antibody, Avidin-HRP or substrate solution were incubated for too long	Rerun the assay and follow the protocol.
Sample readings are out of range	Samples contain no or below detectable levels of the analyte	If samples are below detectable levels, it may be possible to use a larger sample volume. Contact technical support for appropriate protocol modifications.
	Samples contain analyte concentrations greater than highest standard point	Samples may require dilution and analysis.
High variation in samples and/or standards	Multichannel pipette errors	Confirm that pipette calibrations are accurate.
	Plate washing was not adequate or uniform	Ensure pipette tips are tightly secured. Ensure uniformity in all wash steps.
	Non-homogenous samples	Thoroughly mix samples before assaying.
	Samples may have high particulate matter	Remove particulate matter by centrifugation.
	Cross-well contamination	Do not reuse plate sealers. Always change tips for reagent additions. Ensure that pipette tips do not touch the reagents on the plate.

ELISA Plate Template

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

LEGEND MAX™ Kits are manufactured by **BioLegend Inc.**
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