HUMAN CYTOKINE LINCO*plex* KIT
SINGLE PLEX RANTES
96 Well Plate Assay (Cat. #HCYTO-60K-1RNTS)

*Luminex instrumentation refers to Luminex®100, Luminex 200 and other Luminex instruments from MiraiBio® (MasterPlex CT™), ACS® (STarStation™), Bio-Rad Laboratories, Inc.® (Bio-Plex™) and Qiagen® (LiquiChip™). For technical assistance on running LINCO*plex* Kits, contact our technical service department Toll Free U.S. at 866-441-8400 or 636-441-8400 or email us at info@lincoresearch.com.

HUMAN CYTOKINE LINCO*plex* KIT
SINGLE PLEX RANTES

I. INTENDED USE

This is a single plex assay kit manufactured by Linco Research to be used for the quantitative determination of the human RANTES. This kit may be used for the analysis of the above cytokine in serum, plasma, or tissue culture samples.

This kit is for research purposes only.

II. REAGENTS SUPPLIED

A. Antibody-Immobilized Beads

1 bottle containing #69-Human RANTES (20X Concentrated)

Mix beads and dilute with Bead Diluent as described in Section VIII. D.

Quantity: 0.2 ml antibody-immobilized beads/tube

B. Bead Diluent

1 vial containing diluent for bead preparation

Quantity: 3.5 ml/bottle

C. Human Cytokine/Chemokine Standard Cocktail

1 vial containing human cytokine standard cocktail, lyophilized

Quantity: 1 vial

D. Human Cytokine/Chemokine Quality Controls

Control I – 1 vial containing mixed cytokine cocktail, lyophilized
Control II – 1 vial containing mixed cytokine cocktail, lyophilized

Quantity: 1 vial/Control

E. Human RANTES Detection Antibody

1 bottle containing a cocktail of biotinylated detection antibody in Assay Buffer

Quantity: 3.2 ml/bottle

F. Streptavidin-Phycoerythrin

1 bottle containing Streptavidin-Phycoerythrin prepared in Assay Buffer

Quantity: 3.2 ml/bottle

II. REAGENTS SUPPLIED (continued)

G. Assay Buffer
50 mM PBS with 25 mM EDTA, 0.08% Sodium Azide, 0.05% Tween 20, and 1% BSA, pH 7.4.

Quantity: 2 bottles, 30 ml/bottle

H. 10X Wash Buffer

1:10 dilution required with deionized water to give 10 mM PBS with 0.05% Proclin, and 0.05% Tween 20, pH 7.4.

Quantity: 30 ml/bottle

I. Mixing Bottle

Quantity: 1 Bottle

J. Microtiter Filter Plate

Quantity: 1- 96 Well Filtration Plate

K. Plate Sealers

Quantity: 2 Plate Sealers

III. STORAGE CONDITIONS UPON RECEIPT

Recommended storage for kit components is 2 - 8°C. See individual vials for long-term storage recommendations.

Once the standards and controls have been reconstituted, immediately transfer contents into polypropylene vials. **DO NOT STORE RECONSITUTED STANDARDS OR CONTROLS IN GLASS VIALS**. For long-term storage, freeze reconstituted standards and controls at ≤ -20°C. Avoid multiple (>2) freeze thaw cycles.

DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin.

IV. REAGENT PRECAUTIONS

Sodium Azide has been added to some reagents as a preservative. Although the concentrations are low, sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.

V. MATERIALS REQUIRED BUT NOT PROVIDED

A. Reagents

Luminex Sheath Fluid (Luminex Catalogue #40-50000)

B. Instrumentation/Materials

1. Adjustable Pipettes with Tips capable of delivering 25 µl to 1000 µl
2. Multichannel Pipettes capable of delivering 5 µl to 50 µl or 25 µl to 200 µl
3. Reagent Reservoirs
4. Polypropylene Microfuge Tubes
5. Aluminum Foil
6. Absorbent Pads
7. Laboratory Vortex
8. Sonicator (Branson Ultrasonic Cleaner, Model #B200 or equivalent)
9. Titer Plate Shaker (Lab-Line Instruments, Model #4625, or equivalent)
10. Vacuum Filtration Unit (Millipore Vacuum Manifold Catalogue #MAVM0960R, or equivalent)
11. Luminex Instrument

VI. SPECIMEN COLLECTION AND STORAGE

A. A maximum of 25 µl per well of 1:50 diluted serum or plasma can be used. Cell/tissue culture supernatant or lysate may also be used.

B. Preparation of Tissue Culture Supernatant:

Centrifuge the sample to remove debris and assay immediately or aliquot and store samples at ≤ -20ºC. Avoid multiple (>2) freeze/thaw cycles. Tissue Culture Supernatent may require a dilution with the appropriate medium prior to assay.

C. Preparation of Serum Samples:

Allow the blood to clot for at least 30 minutes before centrifugation for 10 minutes at 1000 Xg. Remove serum and assay immediately or aliquot and store samples at ≤ -20ºC. Avoid multiple (>2) freeze/thaw cycles. **Serum samples need to be diluted 1:50 in the Assay Buffer prior to the assay**. (Recommended dilution: Add 5 µl sample to 245 µl Assay Buffer.)

D. Preparation of Plasma Samples:

Plasma collection using EDTA as an anticoagulant is recommended. Centrifuge for 10 minutes at 1000 Xg within 30 minutes of blood collection. Remove plasma and assay immediately or aliquot and store samples at ≤ -20ºC. Avoid multiple (>2) freeze/thaw cycles. It is recommended to centrifuge samples again prior to assay setup. **Plasma samples need to be diluted 1:50 in the Assay Buffer prior to the assay**. (Recommended dilution: Add 5 µl sample to 245 µl Assay Buffer.)

E. All samples must be stored in polypropylene tubes. **DO NOT STORE SAMPLES IN GLASS.**

F. Avoid using samples with gross hemolysis or lipemia.

G. Care must be taken when using heparin as an anticoagulant, since an excess will provide falsely high values. Use no more than 10 IU heparin per ml of blood collected.

VII. TECHNICAL GUIDELINES

To obtain reliable and reproducible results, the operator should carefully read this entire manual and fully understand all aspects of each assay step before attempting to run the assay. The following notes should be reviewed and understood before the assay is set-up.

A. The Antibody-Immobilized Beads are light sensitive and must be covered with aluminum foil at all times. Cover the assay plate containing beads with aluminum foil during all incubation steps.

B. It is critical to allow all reagents to warm to room temperature (20-25°C) before use in the assay.

C. The bottom of the Microtiter Filter Plate should not be in direct contact with any absorbent material during assay set-up or incubation times. The plate can be set on the non-flat side of the plate cover or any other plate holder to raise the plate from the surface.

D. After the wash steps, keep the bottom of the Microtiter Filter Plate clean by blotting on paper towels or absorbent pads to prevent any leakage due to capillary action.

E. Keep the vacuum suction on the plate as low as possible. It is recommended to have a vacuum setting that will remove 200 µl of buffer in $\geq$ 5 seconds (equivalent to < 100 mmHg).

F. After hydration, all standards and controls must be transferred to polypropylene tubes.

G. The standards prepared by serial dilution must be used within 1 hour of preparation. Discard any unused standards except the 10 ng/ml stock standard which may be stored at ≤ -20°C for 1 month and at ≤ -80°C for greater than one month.

H. Any unused mixed Antibody-Immobilized Beads may be stored in the bead mix bottle at 2-8°C for up to one month.

I. During the preparation of the standard curve, make certain to vortex the higher concentration well before making the next dilution. Use a fresh tip with each dilution.

J. The plate should be read immediately after the assay is finished. If, however, the plate cannot be read immediately, seal the plate, cover with aluminum foil, and store at 2-8°C for up to 24 hours. Prior to reading, agitate the plate on the plate shaker at room temperature for 10 minutes.

K. The titer plate shaker should be set at a speed to provide maximum agitation without splashing of liquid outside the wells. For the recommended plate shaker, this would be a setting of 5-7 which is approximately 500-800 rpm.

L. Ensure the needle probe is clean. This may be achieved by sonication and/or Alcohol Flushes. Adjust probe height to the Lincoplex filter plate prior to reading an assay.

M. For cell culture supernatants or tissue extraction, use the culture or extraction medium as matrix in blank, standard curve and controls. If samples are diluted in Assay Buffer, use the Assay Buffer as matrix.

VIII. PREPARATION OF REAGENTS FOR IMMUNOASSAY

A. Preparation of Antibody-Immobilized Beads

Sonicate the antibody-bead bottle for 30 seconds; vortex for 1 minute. Add 0.15 ml from the antibody bead bottle to the Mixing Bottle and bring final volume to 3.0 ml with Bead Diluent. Vortex well. Unused portions may be stored at 2-8°C for up to one month.

B. Preparation of Human Cytokine Standard Cocktail

1.) Before use, reconstitute the Human Cytokine Standard Cocktail with 250 µl Deionized Water to give a 10,000 pg/ml concentration of standard. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to set for 5-10 minutes and then transfer the standard to appropriately labeled polypropylene microfuge tube. This will be used as the 10,000 pg/ml standard; the unused portions may be stored at ≤ -20°C for up to one month.

2). Preparation of Working Standards

Label five polypropylene microfuge tubes 2000, 400, 80, 16, and 3.2 pg/ml. Add 200 µl of Assay Buffer to each of the five tubes. Prepare serial dilutions by adding 50 µl of the 10,000 pg/ml reconstituted standard to the 2000 pg/ml tube, mix well and transfer 50 µl of the 2000 standard to the 400 pg/ml tube, mix well and transfer 50 µl of the 400 standard to the 80 pg/ml tube, mix well and transfer 50 µl of the 80 standard to 16 pg/ml tube, mix well and transfer 50 µl of the 16 pg/ml standard to the 3.2 pg/ml tube and mix well. The 0 pg/ml standard (Background) will be Assay Buffer.

Standard Concentration (pg/ml)	Volume of Deionized Water to Add	Volume of Standard to Add
10,000	250 µl	0

Standard Concentration (pg/ml)	Volume of Assay Buffer to Add	Volume of Standard to Add
2000	200 µl	50 µl of 10,000 pg/ml
400	200 µl	50 µl of 2000 pg/ml
80	200 µl	50 µl of 400 pg/ml
16	200 µl	50 µl of 80 pg/ml
3.2	200 µl	50 µl of 16 pg/ml

C. Preparation of Controls

Before use, reconstitute Human Cytokine Control I and Human Cytokine Control II with 250 µl Deionized Water. Invert the vial several times to mix and vortex. Allow the vial to set for 5-10 minutes and then transfer the controls to an appropriately labeled polypropylene microfuge tube. Unused portions may be stored at ≤ -20°C for up to one month.

VIII. PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

D. Preparation of Wash Buffer

Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution. Dilute 30 ml of 10X Wash Buffer with 270 ml deionized water. Store unused portions at 2-8°C for up to one month.

IX. IMMUNOASSAY PROCEDURE

Prior to beginning this assay, it is imperative to read this protocol completely and to thoroughly understand the Technical Guidelines outlined in Section VII.

Allow all reagents to warm to room temperature (20-25°C) before use in the assay.

1. Diagram placement of Standards, 0 (Background) 3.2, 16, 80, 400, 2000, and 10,000 pg/ml, Controls I and II, and samples on Well Map Worksheet in a vertical configuration. (Note: the instrument will only read the 96-well plate vertically). It is recommended to run the assay in duplicate.
2. Block the filter plate by pipetting 200 µL of Assay Buffer into each well of the microtiter plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
3. Remove Assay Buffer by vacuum. (**NOTE: DO NOT INVERT PLATE**). Remove any excess Assay Buffer from the bottom of the plate by blotting on an absorbent pad or paper towels.
4. Add 25 µL of Assay Buffer to the 0 Standard (Background).
5. Add 25 µL of Assay Buffer to the Sample wells.
6. Add 25 µL of each Standard or Control into the appropriate wells.
7. Add 25 µL of a proper matrix solution to the Background, Standards, and Control wells. When assaying 1:50 diluted serum or plasma samples, use the Assay Buffer as the matrix solution and sample diluent. When assaying Tissue Culture or other supernatent, use the control culture medium as the matrix Solution and diluent. When assaying cell/tissue lysates or homogenate, use the extraction (lysis) buffer as the matrix solution and sample diluent.
8. Add 25 µL of Sample (tissue culture supernatent, cell lysate, or 1:50 diluted serum/plasma etc.) into the appropriate sample wells.
9. Vortex Bead Bottle and add 25 µL of Mixed Beads to each well. (Note: during addition of Mixed Beads, shake bead mix intermittently to avoid settling)
10. Seal, cover with aluminum foil, and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C).
11. Gently remove fluid by vacuum.
12. Wash plate 2 times with 200 µL/well of Wash Buffer, removing Wash Buffer by vacuum filtration between each wash. Remove any excess Wash Buffer from the bottom the plate by blotting on an absorbent pad or paper towels.
13. Add 25 µL of Detection Antibody Cocktail into each well. (Note: allow the Detection Antibody to warm to room temperature prior to addition.)

IX. IMMUNOASSAY PROCEDURE (continued)

14. Seal, cover with aluminum foil, and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C). **DO NOT VACUUM AFTER INCUBATION.**
15. Add 25 µL Streptavidin-Phycoerythrin to each well containing the 25 µL of Detection Antibody Cocktail.
16. Seal, cover with aluminum foil, and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).
17. Gently remove all contents by vacuum.
18. Wash plate 2 times with 200 µL/well Wash Buffer, removing Wash Buffer by vacuum filtration between each wash. Wipe any excess buffer on the bottom of the plate with a tissue.
19. Add 100 µL of Sheath Fluid to all wells. Cover with aluminum foil and resuspend the beads on a plate shaker for 5 minutes.
20. Run plate on Luminex Instrument.
21. Save and evaluate the median data using a 5-parameter or spline fit data reduction.

X. EQUIPMENT SETTINGS

Select the following equipment settings:

Events:	50
Sample Size:	50 µl
Bead Set:	069 for RANTES

**Gate (for IS System):	8,000 to 15,000
**Gate (for 1.7 System):	8,060 to 13,000

**These specifications are for the Luminex100 or Luminex200 with software v. 1.7 or IS. Luminex instruments with other software (e.g. Masterplex, Starstation, LiquiChip, Bioplex, LABScan100) would need to follow instrument instructions for gate settings and additional specifications

XI. QUALITY CONTROLS

The ranges for Quality Control 1 and 2 are provided on the card insert or can be located at the Linco Research website www.lincoresearch.com.

XII. ASSAY CHARACTERISTICS

A. Precision

Cytokine	Intra-assay Precision (%CV)	Interassay Precision (%CV)
RANTES	1.7	<10

Intra-assay precision is generated from the mean of the %CV's from 5 reportable results across three different concentration of cytokines in a single assay. Interassay precision is generated from the mean of the %CV's from 5 reportable results across three different concentrations of cytokine across six different assays.

C. Accuracy

Cytokine	%Recovery	Standard Deviation
RANTES	107	8.4

Accuracy, defined as Percent Recovery, is generated from calculating the %Recovery of three different levels of cytokine spiked into 6 different human serum samples with known low or no measurable cytokine levels.

D. Linearity of Dilution (% 1:50 Diluted Samples)

Cytokine	1:100 Dilution	1:200 Dilution	1:400 Dilution
RANTES	104.5	109.8	116.8

XIII. REPLACEMENT REAGENTS

REAGENTS	**Cat #**
Human Cyto/Chemo Standard Cocktail	L-8060
Human Cyto/Chemo Quality Controls	L-6060
Human RANTES Detection Antibody	L-1060-RANTES
Streptavidin-Phycoerythrin	L-SAPE2
Assay Buffer	L-AB
Set of two 96-Well Filter Plates with sealers	L-PLATE
10X Wash Buffer	L-WB
Bead Diluent	LBD
69-Human RANTES Beads	HRANTES

XIV. ORDERING INFORMATION

A. To place an order:

To assure the clarity of your custom cytokine kit order, please FAX the following information to our customer service department:

1. Your name, telephone and/or fax number
2. Customer account number
3. Shipping and billing address
4. Purchase order number
5. Catalog number and description of product
6. Quantity of kits
7. Selection of Serum Matrix Requirements

FAX: (636) 441-8050
Toll Free US: (866) 441-8400
(636) 441-8400

MAIL ORDERS: Linco Research
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

For International Customers:
To best serve our international customers, it is LINCO's policy to sell our products through a network of distributors. To place an order or to obtain additional information about LINCO products, please contact your local distributor.

B. Conditions of Sale

All products are for research use only. They are not intended for use in clinical diagnosis or for administration to humans or animals. All products are intended for *in vitro* use only.

C. Material Safety Data Sheets (MSDS)

Material Safety Data Sheets for Linco Research products may be ordered by fax or phone.

WELL MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 pg/ml Standard (Background)	400 pg/ml Standard	QC-II Control									
B	0 pg/ml Standard (Background)	400 pg/ml Standard	QC-II Control									
C	3.2 pg/ml Standard	2000 pg/ml Standard										
D	3.2 pg/ml Standard	2000 pg/ml Standard										
E	16 pg/ml Standard	10,000 pg/ml Standard										
F	16 pg/ml Standard	10,000 pg/ml Standard										
G	80 pg/ml Standard	QC-I Control										
H	80 pg/ml Standard	QC-I Control										

HUMAN CYTOKINE LINCO*plex* KIT
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(Cat. #HCYTO-60K-1RANTES)

Well #	Well Identification	Assay Buffer		Assay Buffer	Standard/ Control/Sample	Serum Matrix/ Test Media	RANTES Beads		Detection Antibody		SA-PE		Sheath Fluid
1A, 1B	0 pg/ml Standard (Background)	200 µl	**Seal, Agitate, Incubate 10 minutes at Room Temperature. Remove Assay Buffer by Vacuum**	25 µl	-	25 µl	25 µl	**Seal, Agitate, Incubate 1 hour at Room Temperature. Wash 2X with 200 µl Wash Buffer**	25 µl	**Seal, Agitate, Incubate 30 minutes at Room Temperature.**	25 µl	**Seal, Agitate, Incubate 30 minutes at Room Temperature. Wash 2X with 200 µl Wash Buffer**	100 µl
1C, 1D	3.2 pg/ml Standard			-	25 µl	25 µl							
1E, 1F	16 pg/ml Standard			-	25 µl	25 µl							
1G, 1H	80 pg/ml Standard			-	25 µl	25 µl							
2A, 2B	400 pg/ml Standard			-	25 µl	25 µl							
2C, 2D	2000 pg/ml Standard			-	25 µl	25 µl							
2E, 2F	10,000 pg/ml Standard			-	25 µl	25 µl							
2G, 2H	Control I			-	25 µl	25 µl							
3A, 3B	Control II			-	25 µl	25 µl							
3C, 3D	Sample			25 µl	25 µl	-							
3E, 3F	Sample			25 µl	25 µl	-							
3G, 3H	Sample			25 µl	25 µl	-							
4A, 4B ↓	Sample			25 µl	25 µl	-							
Final Sample	Sample			25 µl	25 µl	-							