Quantikine® ELISA

Human CCL5/RANTES Immunoassay

Catalog Number DRN00B
SRN00B
PDRN00B

For the quantitative determination of human Regulated upon Activation, Normal T cell Expressed and presumably Secreted (RANTES) concentrations in cell culture supernates, serum, platelet-poor plasma, and urine.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

RANTES (Regulated upon Activation, Normal T cell Expressed and presumably Secreted), also known as CCL5, is a member of the "CC" subfamily of chemokines. It plays a primary role in the inflammatory immune response via its ability to chemoattract leukocytes and modulate their function. The cDNA for RANTES was initially discovered by subtractive hybridization as a T cell specific sequence (1, 2). Human RANTES cDNA encodes a highly basic 91 amino acid (aa) residue precursor polypeptide with a 23 aa hydrophobic signal peptide that is cleaved to generate the 68 aa mature protein (1, 2). Human RANTES exhibits approximately 85% homology with mouse RANTES at the deduced aa level (3, 4).

RANTES is a potent chemoattractant for a number of different cell types including unstimulated $CD4^+$/$CD45RO^+$ memory T cells and stimulated $CD4^+$ and $CD8^+$ T cells with naive and memory phenotypes, NK cells, basophils, eosinophils, dendritic cells, mast cells, monocytes, and microglia (5-13). In addition to its effects on migration, RANTES can activate a number of cell types including T cells (14-16), monocytes (17), neutrophils (17), NK cells (7), dendritic cells (18), and astrocytes (19). T cell activation generally requires relatively high RANTES concentrations (~ 1 μM) and is dependent upon aggregation of the molecule and association with cell surface glycosaminoglycans (GAGs) (15-17). Whether this activity occurs *in vivo* remains unclear although in mice, intraperitoneally injected RANTES mutants that are unable to aggregate and/or bind GAG, are not capable of attracting leukocytes when compared to wild-type controls (20). Other *in vivo* studies show that RANTES knockout mice exhibit deficient recruitment of leukocytes to sites of acute inflammation (21).

RANTES, is known to interact with four identified seven transmembrane G-protein coupled receptors: CCR1, CCR3, CCR4, and CCR5 (22-25). RANTES stimulation can initiate a variety of signaling cascades that are cell context dependent. For instance, in T-cells, RANTES can stimulate elevations of intracellular Ca^{2+} (26), and activation of focal adhesion kinase (FAK) (27), protein kinase A (28), PI3-kinase (14), Rho GTPase (29), and JAK/STAT signaling pathways (30). The cytomegalovirus protein US28 exhibits significant homology with CC chemokine receptors and is capable of binding RANTES (31). Membrane-spanning US28 can, depending on the context, signal in a constitutive manner (32), bind RANTES and initiate G-protein-mediated signaling cascades (33), or sequester RANTES and potentially alter inflammatory responses (34-36).

The RANTES receptor CCR5 is also the primary co-receptor for R5 (M-tropic) variants of HIV-1 (37, 38). It has been demonstrated that RANTES, as well as the other CCR5 ligands, macrophage inflammatory protein (MIP)-1α and MIP-1β, can competitively inhibit CCR5/HIV-1 interaction and suppress viral infection *in vitro* (39, 40). These effects apparently do not require fully intact signaling from the CCR5 receptor (41). Consequently, modified forms of RANTES and non-peptide compounds that block the interaction of HIV-1 with CCR5 show promise for future therapies (41-44). In contrast, several reports show that RANTES can enhance *in vitro* replication of X4 (T-tropic) variants of HIV-1 that use CXCR4 as a co-receptor rather than CCR5 (45, 46). This activity usually requires relatively high RANTES concentrations (~μM) and is dependent upon interaction with cell surface GAGs, oligomerization, and activation of tyrosine kinase and MAP kinase signaling cascades (46, 47).

The Quantikine Human CCL5/RANTES Immunoassay is a 3.5 hour solid phase ELISA designed to measure human RANTES levels in cell culture supernates, serum, platelet-poor plasma, and urine. It contains *E. coli*-expressed recombinant human RANTES and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate the recombinant factor. Results obtained using natural human RANTES showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values of natural human RANTES.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human RANTES has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any RANTES present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human RANTES is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of RANTES bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with the appropriate Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	CATALOG # DRN00B	CATALOG # SRN00B	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
RANTES Microplate	890156	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human RANTES.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
RANTES Conjugate	890157	1 vial	6 vials	21 mL/vial of a polyclonal antibody specific for human RANTES conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
RANTES Standard	892529	1 vial	6 vials	10 ng/vial of recombinant human RANTES in a buffered protein base with preservatives; lyophilized.	
Assay Diluent RD1W	895117	1 vial	6 vials	11 mL/vial of a buffered protein base with preservatives.	
Calibrator Diluent RD6-11	895489	1 vial	6 vials	21 mL/vial of a concentrated buffered protein base with preservatives. *Used diluted 1:2 for platelet-poor plasma samples. Used diluted 1:5 for cell culture supernate/serum/urine samples*	
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	1 vial	6 vials	12 mL/vial of stabilized hydrogen peroxide.	
Color Reagent B	895001	1 vial	6 vials	12 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	1 vial	6 vials	6 mL/vial of 2 N sulfuric acid.	
Plate Sealers	N/A	4 strips	24 strips	Adhesive strips.	

* Provided this is within the expiration date of the kit.

DRN00B contains sufficient materials to run an ELISA on one 96 well plate.
SRN00B (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PDRN00B). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 500 mL graduated cylinders.
- **Polypropylene** test tubes for dilution of standards and samples.
- Human RANTES Controls (optional; available from R&D Systems).

PRECAUTIONS

Calibrator Diluent RD6-11 contains sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

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SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Normal human serum added to cell culture media may contain high levels of RANTES. For best results, do not use normal human serum for growth of cell cultures if assaying for RANTES production. Because there is no species cross-reactivity of this kit, human RANTES levels in culture media containing up to 10% bovine or fetal bovine serum can be assayed without interference.*

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Platelet-poor Plasma - Collect plasma on ice using citrate, EDTA, or heparin as an anticoagulant. Centrifuge for 15 minutes at 2-8 °C at 1000 x g within 30 minutes of collection. An additional centrifugation step of the plasma at 10,000 x g for 10 minutes at 2-8 °C is recommended for complete platelet removal. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

RANTES is present in platelet granules and is released upon platelet activation. Therefore, to measure circulating levels of RANTES, platelet-free plasma should be collected for measurement. It should be noted that many protocols for plasma preparation, including procedures recommended by the Clinical and Laboratory Standards Institute (CLSI), result in incomplete removal of platelets from blood.

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Polypropylene tubes must be used. Do not use glass tubes.

Serum samples require a 100-fold dilution. A suggested 100-fold dilution is 10 μL of sample + 990 μL of Calibrator Diluent RD6-11 (diluted 1:5).*

Platelet-poor plasma samples require a 4-fold dilution. A suggested 4-fold dilution is 100 μL of sample + 300 μL of Calibrator Diluent RD6-11 (diluted 1:2).*

Urine samples require a 2-fold dilution. A suggested 2-fold dilution is 200 μL of sample + 200 μL of Calibrator Diluent RD6-11 (diluted 1:5).

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

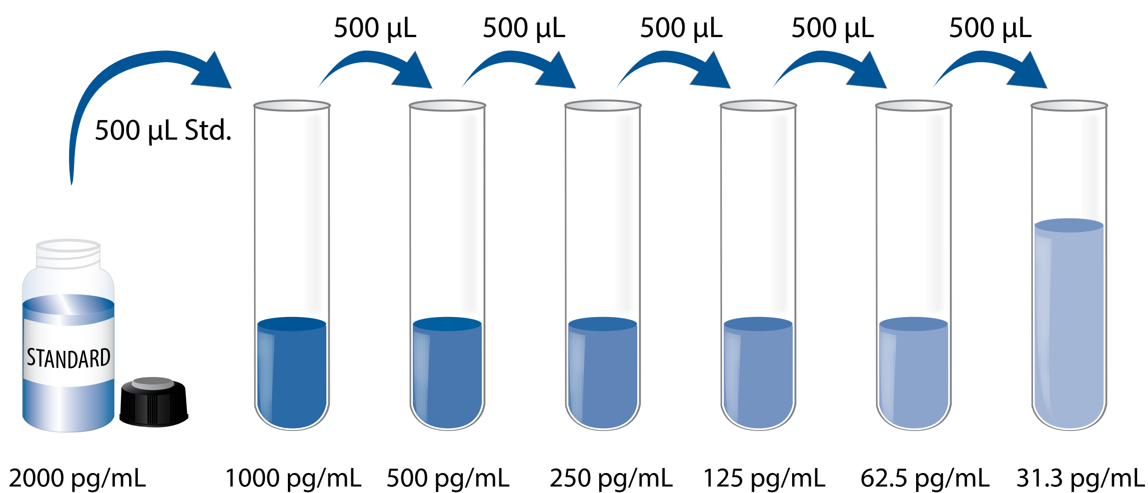
Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μL of the resultant mixture is required per well.

Calibrator Diluent RD6-11 (diluted 1:2) - **For platelet-poor plasma samples.** Add 20 mL of Calibrator Diluent RD6-11 to 20 mL of deionized or distilled water to prepare 40 mL of Calibrator Diluent RD6-11 (diluted 1:2).

Calibrator Diluent RD6-11 (diluted 1:5) - **For cell culture supernates, serum, and urine samples.** Add 20 mL of Calibrator Diluent RD6-11 to 80 mL of deionized or distilled water to prepare 100 mL of Calibrator Diluent RD6-11 (diluted 1:5).

RANTES Standard - Reconstitute the RANTES Standard with 5 mL of Calibrator Diluent RD6-11 (diluted 1:5) (*for cell culture supernate, serum, and urine samples*) or Calibrator Diluent RD6-11 (diluted 1:2) (*for platelet-poor plasma samples*). This reconstitution produces a stock solution of 2000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 500 μL of Calibrator Diluent RD6-11 (diluted 1:2) (*for platelet-poor plasma samples*) or Calibrator Diluent RD6-11 (diluted 1:5) (*for cell culture supernates, serum, and urine samples*) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted standard serves as the high standard (2000 pg/mL). The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL).



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ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

1. Prepare all reagents, working standards, controls, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Assay Diluent RD1W to each well.
4. Add 100 µL of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process twice for a total of three washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of RANTES Conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. **Protect from light.**
9. Add 50 µL of Stop Solution to each well.The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

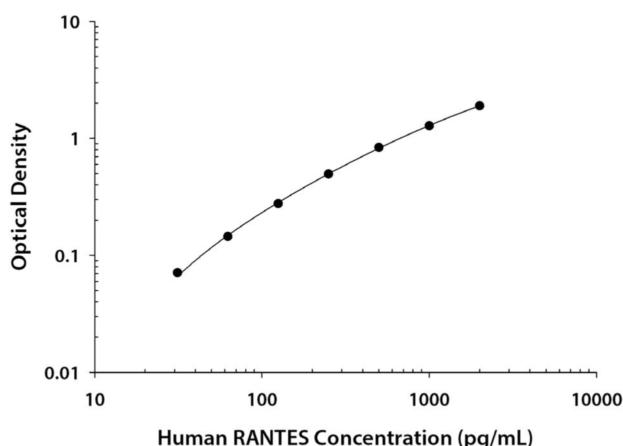
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human RANTES concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

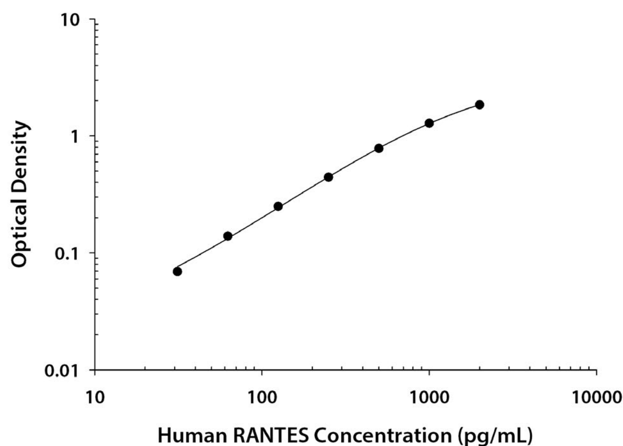
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATES/SERUM/URINE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.054 0.055	0.054	—
31.3	0.124 0.127	0.125	0.071
62.5	0.198 0.201	0.199	0.145
125	0.329 0.334	0.331	0.277
250	0.541 0.558	0.549	0.495
500	0.875 0.908	0.891	0.837
1000	1.327 1.340	1.333	1.279
2000	1.904 1.996	1.950	1.896

PLATELET-POOR PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.053 0.055	0.054	—
31.3	0.123 0.123	0.123	0.069
62.5	0.189 0.196	0.193	0.139
125	0.301 0.307	0.304	0.250
250	0.493 0.500	0.497	0.443
500	0.824 0.843	0.834	0.780
1000	1.307 1.362	1.335	1.281
2000	1.851 1.941	1.896	1.842

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

CELL CULTURE SUPERNATE/SERUM/URINE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (pg/mL)	91.9	573	1120	92.0	511	1105
Standard deviation	2.30	10.0	32.4	6.20	32.7	70.7
CV (%)	2.5	1.7	2.9	6.7	6.4	6.4

PLATELET-POOR PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (pg/mL)	108	599	1191	88.0	494	1063
Standard deviation	3.90	14.3	41.5	9.10	43.1	89.6
CV (%)	3.6	2.4	3.5	10.3	8.7	8.4

RECOVERY

The recovery of human RANTES spiked to three different levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	101	95-110%
Serum* (n=5)	101	94-107%
Platelet-poor EDTA plasma* (n=4)	98	93-103%
Platelet-poor heparin plasma* (n=4)	99	93-105%
Platelet-poor citrate plasma* (n=4)	99	93-105%
Urine* (n=10)	102	88-114%

*Samples were diluted prior to assay as directed in the Sample Preparation section.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human RANTES were serially diluted with the appropriate Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum* (n=8)	Platelet-poor			Urine* (n=4)
				EDTA plasma* (n=4)	Heparin plasma* (n=4)	Citrate plasma* (n=4)	
1:2	Average % of Expected	108	107	107	106	104	103
	Range (%)	103-114	92-118	99-114	100-112	98-108	94-111
1:4	Average % of Expected	112	106	110	112	103	103
	Range (%)	108-119	91-113	108-114	102-120	95-110	99-105
1:8	Average % of Expected	111	105	102	113	101	106
	Range (%)	105-116	100-110	91-113	102-120	99-104	98-123
1:16	Average % of Expected	112	112	102	110	99	102
	Range (%)	103-130	101-131	89-110	97-120	92-103	95-112

*Samples were diluted prior to assay as directed in the Sample Preparation section.

SENSITIVITY

Six assays were evaluated and the minimum detectable dose (MDD) of human RANTES ranged from 1.7-6.6 pg/mL. The mean MDD was 2.0 pg/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human RANTES produced at R&D Systems.

The NIBSC Non-WHO RANTES reference material 92/520 which is intended as a potency standard, was evaluated in this kit. This standard is an *E. coli*-expressed recombinant human RANTES.

The reference material parallels the Quantikine standard curve. To convert sample values obtained with the Quantikine Human RANTES kit to approximate NIBSC 92/520 units use the equation below:

NIBSC (92/520) approximate value (U/mL) = 0.0119 x Quantikine Human RANTES value (pg/mL)

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SAMPLE VALUES

Serum/Platelet-poor Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of RANTES in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	% Detectable	Range (pg/mL)
Serum (n=11)	57,270	100%	23,117-77,354
Platelet-poor EDTA plasma (n=11)	849	100%	262-1587
Platelet-poor heparin plasma (n=11)	1340	100%	308-3527
Platelet-poor citrate plasma (n=11)	467	100%	202-874
Urine (n=10)	656	80%	ND-1540

ND=Non-detectable

Cell Culture Supernates - Human peripheral blood mononuclear cells (1×10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal calf serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were cultured stimulated with 10 µg/mL PHA for 1 and 5 days. Aliquots were removed and assayed for levels of human RANTES.

Condition	Day 1 (pg/mL)	Day 5 (pg/mL)
Unstimulated cells	375	667
Stimulated cells	4660	2830

Note: *Sample collection and handling procedures have a significant impact on measured RANTES levels. Refer to the Sample Collection and Storage section for the recommended sample collection procedures.*

SPECIFICITY

This assay recognizes natural and recombinant human RANTES.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human RANTES control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

ANG
CNTF
β-ECGF
EGF
Epo
FGF acidic
FGF basic
FGF-4
G-CSF
GM-CSF
GROα
IFN-γ
IGF-I
IGF-II
IL-1α
IL-1β
IL-1ra
IL-2
IL-3
IL-5
IL-6
IL-6 R
IL-7
IL-8
IL-9
IL-10
IL-11
LIF
MCP-1
MIP-1α
MIP-1β
M-CSF
OSM
PDGF-AA
PDGF-AB
PDGF-BB
SCF
SLPI
TGF-α
TGF-β1
TGF-β2
TGF-β3
TNF-α
TNF RI
TNF RII

Recombinant mouse:

GM-CSF
IL-1a
IL-1b
IL-3
IL-4
IL-5
IL-6
IL-7
IL-9
MIP-1α
MIP-1β
SCF
TNF-α
TNF-β

Other recombinants:

mouse EGF
bovine FGF acidic
bovine FGF basic
human PDGF
porcine PDGF
human TGF-β1
porcine TGF-β1
porcine TGF-β2

Recombinant mouse RANTES cross-reacts approximately 12% in this assay.

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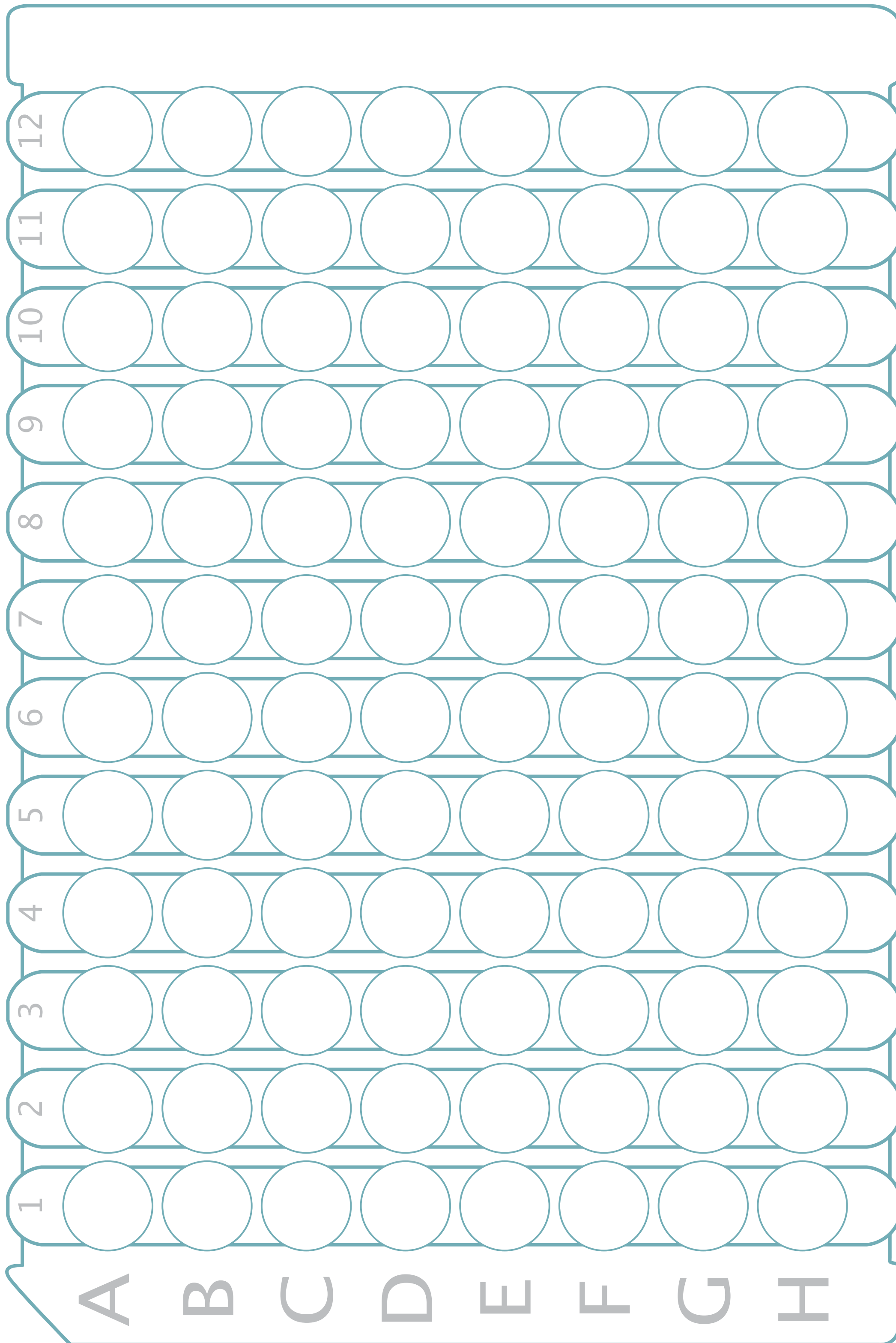
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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