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Application Note

Simultaneous Quantitative Profiling of 48 Immune Factors Using the MILLIPLEX® Non-Human Primate Cytokine/Chemokine/Growth Factor Panel A

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Introduction

Cytokines are a diverse group of small proteins, peptides, or glycoproteins secreted by lymphocytes, monocytes, macrophages, and other cells that regulate immune responses, hematopoiesis, and lymphocyte development. Cytokines include interleukins, interferons, chemokines, and other signaling molecules. Growth factors are extracellular polypeptides that have a positive effect on cell growth and proliferation. Growth factors and cytokines are similar in structure and the way of action. Each cytokine or growth factor acts through its own receptor on target cells to generate signaling pathways, and consequently to regulate biological processes. The expression of cytokines or growth factors and their receptors is highly regulated. Dysregulation may contribute to many diseases such as infectious disease, autoimmune and chronic inflammatory disease, cardiovascular disease, metabolic syndrome, neurological disorders, and cancer. Cytokine and growth factor research play a significant role in achieving a deeper understanding of the immune system and combating its related diseases. As the most closely related species to humans, non-human primates are critical models for those studies.

With our new MILLIPLEX® Non-Human Primate Cytokine/Chemokine/Growth Factor Panel A, researchers are now able to profile the largest selection of immune factors available for non-human primates on one 96-well plate. Save time and sample volume, with the ability to generate more than 1,800 data points in a single plate when samples are analyzed in duplicate. The MILLIPLEX® Non-Human Primate Cytokine/Chemokine/Growth Factor Panel A was built with flexibility in mind, offering the option to select only the analytes needed to meet research needs. Additionally, a customized premix can be selected, and the panel is available as a fixed 38-plex or 48-plex premixed bead kit. **Table 1** outlines the available kits and formats.

This application note will review the development and verification testing performed using MILLIPLEX® Non-Human Primate Cytokine/Chemokine/Growth Factor Panel A, with an emphasis on comparing analyte performance to assays in our non-human primate MILLIPLEX® portfolio.

Cat. No.	Number of Analytes	Included Analytes	Panel Notes
PRCYTA-40K[1]	Up to 48	BCA-1, sCD137, sCD40L, Eotaxin, sFasL, FGF-2, Fractalkine, G-CSF, GM-CSF, Granzyme A, Granzyme B, IFNα2, IFNγ, IL-1α, IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-15, IL-16, IL-17A, IL-17E, IL-18, IL-21, IL-22, IL-23, IL-28A, IL-31, IL-33, IP-10, I-TAC, MCP-1, MIG, MIP-1α, MIP-1β, MIP-3α, Perforin, RANTES, TGFα, TNFα, TNFβ, VEGF-A	[1]Customizable panel: choose your analytes and receive individual bead vials or use our convenient service for customizing your own bead premix (additional charge may be applied).
PRCYTA-40K-PX38[2], **PRCYTA-40K-BK38**[3]	38	sCD40L, sCD137, IFNγ, sFasL, G-CSF, GM-CSF, Granzyme A, IFNα2, IL-10, IL-2, IL-15, IL-17A, IL-6, Granzyme B, IL-8, IL-1RA, IL-1β, IL-23, IL-12(p70), IL-33, IL-21, IL-4, IL-18, IL-22, IL-5, IP-10, MCP-1, IL-7, I-TAC, MIG, MIP-1α, MIP-3α, MIP-1β, *RANTES, TNFα, TGFα, VEGF-A, Perforin	[2]Premix kits contain either a 37- or 47-plex bead set, plus a vial of *RANTES beads. [3]Bulk kits match the formatting of premixed kits but arrive in Space Saver packaging. *RANTES is provided as a separate bead vial due to different dilution requirements for serum/plasma samples.
PRCYTA-40K-PX48[2], **PRCYTA-40K-BK48**[3]	38	BCA-1, sCD137, sCD40L, Eotaxin, sFasL, FGF-2, Fractalkine, G-CSF, GM-CSF, Granzyme A, Granzyme B, IFNα2, IFNγ, IL-1α, IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-15, IL-16, IL-17A, IL-17E, IL-18, IL-21, IL-22, IL-23, IL-28A, IL-31, IL-33, IP-10, I-TAC, MCP-1, MIG, MIP-1α, MIP-1β, MIP-3α, Perforin, *RANTES, TGFα, TNFα, TNFβ, VEGF-A	

Table 1. Kit catalog numbers, formats, and analytes included in Non-Human Primate Cytokine/Chemokine/Growth Factor Panel A.

Materials and Methods

MILLIPLEX® Assay Development

The MILLIPLEX® Non-Human Primate Cytokine/Chemokine/Growth Factor Panel A kit was developed using magnetic microsphere beads from Luminex® Corporation[1]. Each set of beads is distinguished by different ratios of two internal dyes yielding a unique fluorescent signature for each bead set. Capture antibodies were coupled to the magnetic beads. **Figure 1** shows the Luminex® methodology and instrumentation.

(A)

(B)

xMAP® INTELLIFLEX System | **FLEXMAP 3D® System** | **Luminex® 200™ System** | **MAGPIX® System**

Figure 1. (A) Luminex® protein detection immunoassay method. (B) Instruments for use with MILLIPLEX® kits (xMAP® INTELLIFLEX, FLEXMAP 3D®, Luminex® 200™, and MAGPIX® systems).

MILLIPLEX® Quality and Assay Verification

Kit development and verification begin with testing for selectivity and specificity to ensure negligible cross-reactivity in the tested sample types and to confirm the consistent performance of an assay in single-plex vs. multiplex formats. Buffers and diluents are optimized to enhance antibody specificity, such that only those analytes of interest are detected in samples. The serum matrix was carefully selected and optimized for use in the standard curve and Quality Control (QC) wells when using serum or plasma samples to mimic the sample matrix most closely, thus normalizing assay performance. The streptavidin-phycoerythrin (SAPE) concentration was titrated in-house for optimal signal and is provided ready-to-use with no dilution required. Additionally, all MILLIPLEX® kits are rigorously tested for shipping stability, and samples are also tested for temperature and freeze/thaw tolerance.

Sample dilution testing was also performed to ensure biologically relevant samples are detectable and fall on the linear portion of the standard curves. Using MILLIPLEX® Non-Human Primate Cytokine/Chemokine/Growth Factor Panel A, serum and plasma from five non-human primate species were tested. The assay was fully verified with two of the most studied non-human primates, rhesus macaques (*Macaca mulatta*) and cynomolgus macaques (*Macaca fascicularis*), with additional verification testing performed using baboon (*Papio cynocephalus*), chimpanzee (*Pan troglodytes*), and African green monkey (*Cercopithecus aethiops*) serum and plasma samples. Additionally, cynomolgus macaque peripheral blood mononuclear cells (PBMCs) were evaluated.

QCs, which are low and high dilutions of recombinant proteins for each analyte, are manufactured such that each QC has optimal placement on each standard curve. QC range sheets are provided with each kit. Additionally, it is recommended that users include experiment-specific samples for use as controls in each assay.

Sample Preparation

Serum and Plasma

For serum samples, blood was allowed to clot for at least 30 minutes before centrifugation for 10 minutes at 1,000 x g. Serum was removed and either assayed immediately or aliquoted and stored at -20°C or lower temperature. Plasma samples, with EDTA as the recommended anticoagulant, were centrifuged at 1,000 x g within 30 minutes of blood collection. Plasma was removed and assayed immediately or aliquoted and stored at -20°C or lower temperature. Frozen samples were thawed completely, vortexed, and centrifuged prior to use, to remove particulates.

PBMCs

For PBMCs, the cells were cultured in RPMI containing 10% FBS and 1% Penicillin/Streptomycin and stimulated at 10^6 cells/mL with 1 µg/mL Lipopolysaccharide (LPS; Cat No. L6529) or Concanavalin A (Con A; Cat. No. C0412) for 48 hours at 37°C, 5% CO_2, after which unstimulated control or stimulated cell-free supernatants were collected. The PBMCs were either assayed immediately or aliquoted and stored at -20°C or lower temperature.

Results

Immunoassay Workflow

The MILLIPLEX® Non-Human Primate Cytokine/ Chemokine/Growth Factor Panel A includes a detailed, easy-to-follow protocol with a familiar workflow. Additionally, many of our kit components arrive ready-to-use, reducing assay variability and the amount of time spent preparing reagents. Detecting 48 immune makers at pg/mL levels with standard curve concentrations that remain the same from lot to lot affords a user-friendly experience. Consistent standard curves across lots allow for consistency in sample results across a project, and therefore reliable results.

Standard Curves

The 48-plex standard curves and curve ranges for the MILLIPLEX® Non-Human Primate Cytokine/ Chemokine/ Growth Factor Panel A are shown in **Figure 2** and **Table 2**. **Table 2** also shows the comparison non-human primate panel and its corresponding standard curve range for that analyte.

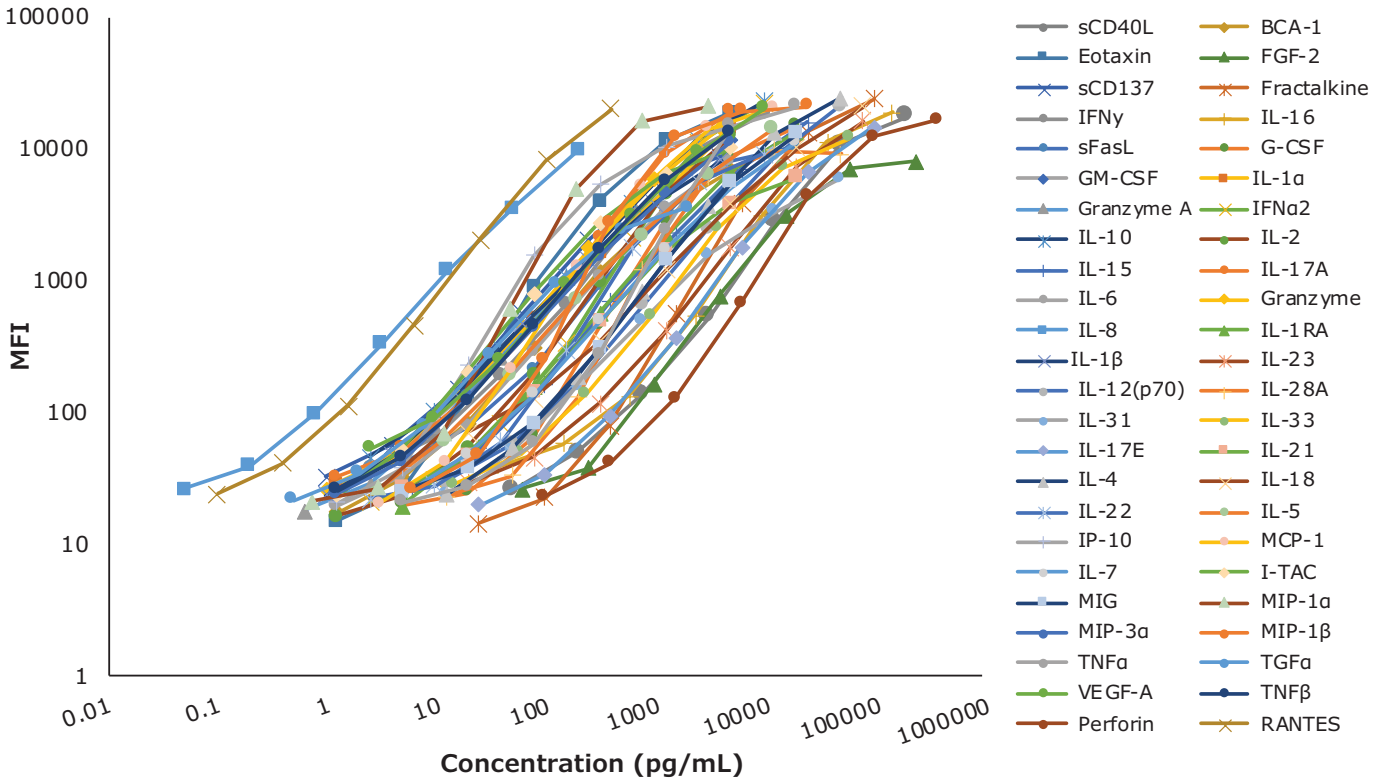

Figure 2. MILLIPLEX® Non-Human Primate Cytokine/Chemokine/Growth Factor Panel A Standard Curves. Standard curves for all analytes were assayed in MXPRSM-A serum matrix, except RANTES was in L-AB assay buffer. This assay was performed using the Cat. No. PRCYTA-40K overnight protocol.

Analyte	PRCYTA-40K Range (pg/mL)	Comparison Kit	Comparison Kit Range (pg/mL)
sCD40L	48.83-200,000	**PRCYTOMAG-40K**	Variable, see QC sheet
BCA-1	1.22-5,000	**New**	-
Eotaxin	1.22-5,000	**PRCYT2MAG40K**	3-3,000
GM-CSF	1.22-5,000	**PRCYTOMAG-40K**	2.4-10,000
IL-1α	1.22-5,000	**PRCYT2MAG40K**	29-30,000
IL-2	1.22-5,000	**PRCYTOMAG-40K** **PRCYT2MAG40K**	2.4-10,000 10-10,000
IL-17A	1.22-5,000	**PRCYTOMAG-40K** **PRCYT2MAG40K**	2.4-10,000 1-1,500
IL-6	1.22-5,000	**PRCYTOMAG-40K** **PRCYT2MAG40K**	2.4-10,000 3-3,000
IP-10	1.22-5,000	**PRCYT2MAG40K**	10-10,000
I-TAC	1.22-5,000	**New**	-
MIP-3α	1.22-5,000	**PRCYT2MAG40K**	7-7,000
TNFβ	1.22-5,000	**PRCYT2MAG40K**	98-100,000
FGF-2	61.04-250,000	**PRCYT2MAG40K**	20-20,000
sCD137	0.98-4,000	**PRCYT2MAG40K**	10-10,000
Granzyme B	0.98-4,000	**PRCYT2MAG40K**	4-4,000
IL-1β	0.98-4,000	**PRCYTOMAG-40K**	2.4-10,000
Fractalkine	24.41-100,000	**PRCYT2MAG40K**	98-100,000
IL-17E	24.41-100,000	**PRCYT2MAG40K**	293-300,000
IFNγ	2.44-10,000	**PRCYTOMAG-40K**	2.4-10,000
IFNα2	2.44-10,000	**New**	-
trIL-10	2.44-10,000	**PRCYTOMAG-40K**	12.2-50,000
IL-22	2.44-10,000	**PRCYT2MAG40K**	98-100,000
VEGF-A	2.44-10,000	**PRCYTOMAG-40K**	2.4-10,000
IL-16	36.62-150,000	**PRCYT2MAG40K**	73-75,000
sFasL	4.88-20,000	**PRCYT2MAG40K**	8-8,000
G-CSF	4.88-20,000	**PRCYTOMAG-40K**	2.4-10,000
IL-1RA	4.88-20,000	**PRCYTOMAG-40K**	2.4-10,000
IL-21	4.88-20,000	**PRCYT2MAG40K**	15-15,000
IL-7	4.88-20,000	**New**	-
MIG	4.88-20,000	**New**	-
TNFα	4.88-20,000	**PRCYTOMAG-40K**	2.4-10,000
Granzyme A	0.61-2,500	**PRCYT2MAG40K**	78-80,000
IL-15	6.10-25,000	**PRCYTOMAG-40K**	2.4-10,000
MIP-1β	6.10-25,000	**PRCYTOMAG-40K**	2.4-10,000
IL-8	0.05-200	**PRCYTOMAG-40K**	2.4-10,000
IL-23	19.53-80,000	**PRCYT2MAG40K**	24-25,000
IL-18	19.53-80,000	**PRCYTOMAG-40K**	12.2-50,000
IL-12p70*	12.21-50,000	**PRCYTOMAG-40K**	2.4-10,000
IL-28A	12.21-50,000	**PRCYT2MAG40K**	29-30,000
IL-31	12.21-50,000	**PRCYT2MAG40K**	15-15,000
IL-4	12.21-50,000	**PRCYTOMAG-40K** **PRCYT2MAG40K**	4.9-20,000 146-150,000
IL-33	14.65-60,000	**PRCYT2MAG40K**	20-20,000
IL-5	2.93-12,000	**PRCYTOMAG-40K**	2.4-10,000
MCP-1	3.05-12,500	**PRCYTOMAG-40K**	2.4-10,000
MIP-1α	0.73-3,000	**PRCYTOMAG-40K**	2.4-10,000
RANTES	0.10-400	**PRCYT2MAG40K**	1-750
TGFα	0.49-2,000	**PRCYTOMAG-40K**	2.4-10,000
Perforin	97.66-400,000	**PRCYT2MAG40K**	98-100,000

*The IL-12p70 standard curve range is compared to the IL-12/IL-23p40 standard curve range.

Table 2. Standard curve ranges for Cat. No. PRCYTA-40K compared to ranges in Cat. Nos. PRCYTOMAG-40K and PRCYT2MAG40K comparison MILLIPLEX® kits.

Sample Detectability

Improvement in sample detection was an important factor in the development of the MILLIPLEX® Non-Human Primate Cytokine/Chemokine/Growth Factor Panel A, in addition to matching sample concentrations when evaluating against comparison MILLIPLEX® kits (Cat. Nos. PRCYTOMAG-40K and PRCYT2MAG40K). We demonstrated comparable performance in analyte concentrations between Cat. No. PRCYTA-40K and comparison panels, shown in **Figure 3.** Additionally, we noted overall improved sample detectability in Cat. No. PRCYTA-40K when analyzed side-by-side with comparison MILLIPLEX® panels, outlined in **Table 3**.

Six analyte concentrations were adjusted to match literature values more closely, including IL-10[2], MIP-1β[3,4], VEGF-A[5], IL-1α[6], IP-10[7,8], and IL-22[9].

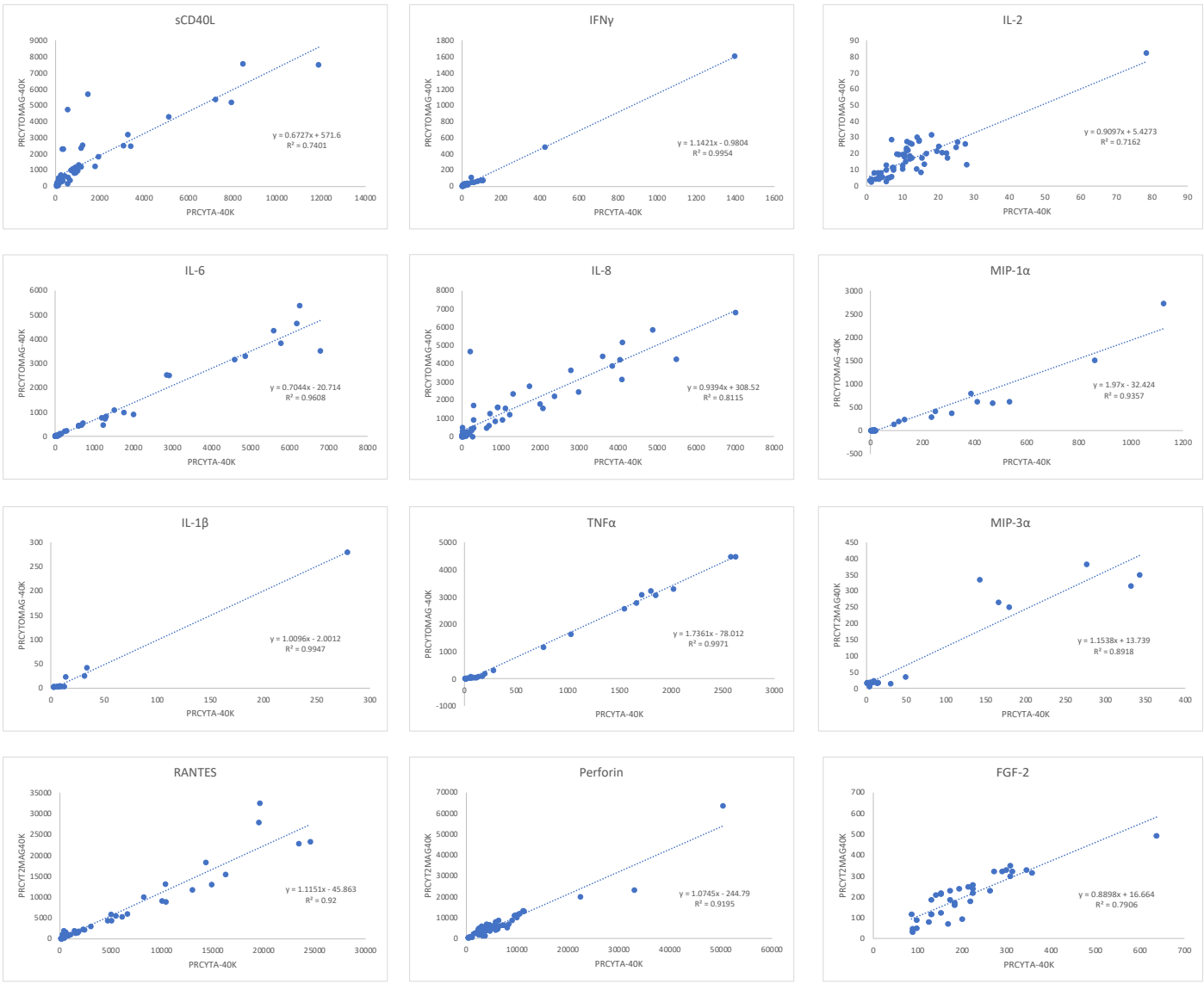

Figure 3. Sample correlation graphs for Cat. No. PRCYTA-40K compared to Cat. Nos. PRCYTOMAG-40K or PRCYT2MAG40K. Samples included serum and plasma from the five non-human primate species noted previously, and cynomolgus PBMC samples.

(A)

Analyte	sCD40L	IFNγ	G-CSF	GM-CSF	IL-10	IL-15	IL-8	IL-1Ra	IL-1β	IL-18	IL-5
PRCYTA-40K	90	67	67	93	66	93	100	100	91	40	97
PRCYTOMAG-40K	98	95	67	81	97	100	98	97	29	50	43

Analyte	MCP-1	MIP-1α	MIP-1β	TNFα	TGFα	VEGF-A
PRCYTA-40K	100	86	100	53	100	45
PRCYTOMAG-40K	100	84	98	74	74	90

(B)

Analyte	Eotaxin	FGF-2	sCD137	Fractalkine	IL-16	sFasL	IL-1α	Granzyme A	Granzyme B	IL-23	IL-28A
PRCYTA-40K	100	64	57	97	38	90	93	95	78	93	90
PRCYT2MAG40K	55	97	88	55	26	9	22	16	22	31	14

Analyte	IL-31	IL-33	IL-17E	IL-21	IL-22	IP-10	MIP-3α	RANTES	TNFβ	Perforin
PRCYTA-40K	48	38	90	90	93	100	98	100	100	100
PRCYT2MAG40K	19	16	14	22	17	41	45	100	17	98

(C)

Analyte	IL-2	IL-17A	IL-6	IL-4
PRCYTA-40K	100	59	100	55
PRCYTOMAG-40K	97	22	83	83
PRCYT2MAG40K	88	91	57	9

Table 3. Percent (%) sample detectability in Cat. No. PRCYTA-40K compared to (A) Cat. No. PRCYTOMAG-40K, (B) Cat. No. PRCYT2MAG40K, and (C) both comparison MILLIPLEX® panels. A total of 58 samples were evaluated, including rhesus serum/plasma (n=20), cynomolgus serum/plasma (n=20), cynomolgus PBMC (n=6), baboon serum/plasma (n=4), chimpanzee serum/plasma (n=4), and African green serum/plasma (n=4).

Cynomolgus macaque PBMCs demonstrated increased concentrations in several analytes when stimulated with either Con A or LPS compared to the unstimulated control. Representative data is shown in **Figure 4.**

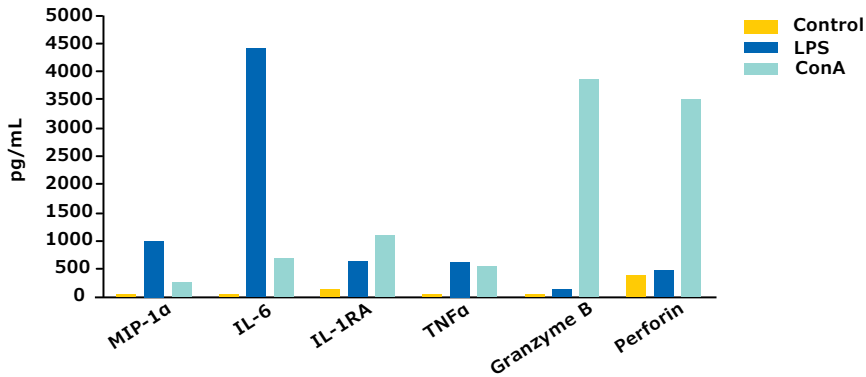

Figure 4. Average concentrations of six analytes in two cynomolgus macaque PBMC samples. PBMCs were tested as unstimulated control, stimulated with LPS, and stimulated with Con A.

New Analyte Performance

Five new analytes not previously available in MILLIPLEX® non-human primate panels are included in MILLIPLEX® Non-Human Primate Cytokine/Chemokine/Growth Factor Panel A. The analytes include BCA-1, I-TAC, IFNα2, IL-7, and MIG. **Table 4** outlines the performance of the new analytes.

Analyte	Rhesus Average Linearity (%) n=5	Cynomolgus Average Linearity (%) n=5	Average Recovery in Serum Matrix (%) n=6	Inter-assay Precision (%) n=5	Intra-assay Precision (%) n=16
BCA-1	105	126	92	12	8.6
I-TAC	129	121	87	8	9.0
IFNα2	126	124	83	11	8.1
IL-7	129	104	91	10	9.2
MIG	127	123	85	18	5.7

Table 4. Assay characteristics for five new analytes in the MILLIPLEX® Non-Human Primate Cytokine/Chemokine/Growth Factor Panel A.

Reproducibility

Consistent plate-to-plate performance is important when evaluating samples over time. To test reproducibility in the MILLIPLEX® Non-Human Primate Cytokine/Chemokine/Growth Factor Panel A, we tested duplicate wells of our QC1 and QC2 across ten 96-well plates during assay verification. Representative QC data from four analytes is illustrated in **Figure 5.**

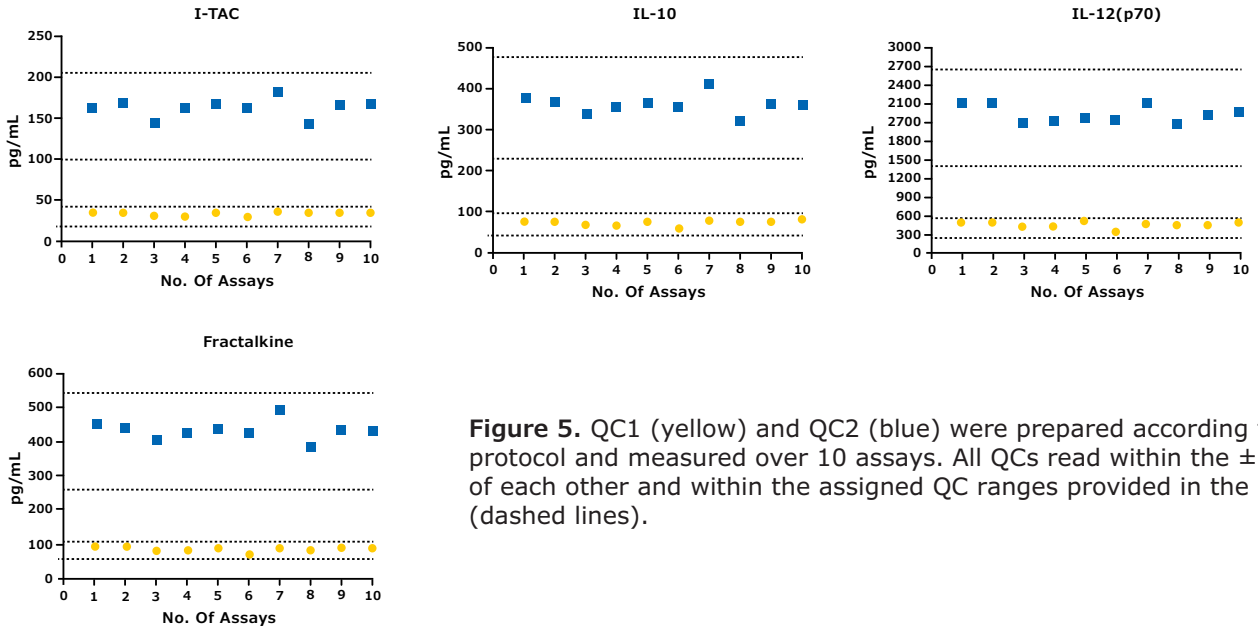

Figure 5. QC1 (yellow) and QC2 (blue) were prepared according to the protocol and measured over 10 assays. All QCs read within the ±20% of each other and within the assigned QC ranges provided in the kit (dashed lines).

Summary

The MILLIPLEX® Non-Human Primate Cytokine/Chemokine/Growth Factor Panel A offers an expanded selection of analytes in one easy-to-use panel, with the flexibility to choose any analyte combination, a customized premix, or a fixed 38- or 48-plex premix. Our new non-human primate MILLIPLEX® panel includes five new analytes, including BCA-1, IFNα2, IL-7, I-TAC, and MIG. Protocol and workflow improvements now allow for all analytes, excluding RANTES and IL-8 in certain non-human primate species, to be tested with neat serum and plasma samples. Previous MILLIPLEX® non-human primate multiplex assays required either neat samples or a 1:2 dilution. This simple sample preparation improvement provides a straightforward and time-saving workflow over previous assays.

The data presented in this application note highlights the value of MILLIPLEX® Non-Human Primate Cytokine/Chemokine/Growth Factor Panel A for the study of relevant disease biomarkers in serum and plasma, as well as PBMCs and cell culture supernatants. Standard curve ranges were expanded or shifted to allow for improved sample detectability when evaluated against comparison MILLIPLEX® non-human primate panels. The standard curve ranges are fixed to allow for lot-to-lot consistency and were specifically optimized for each analyte. Sample values were compared and correlated to comparative MILLIPLEX® assays to provide users with confidence in performance. Moreover, the MILLIPLEX® Non-Human Primate Cytokine/Chemokine/Growth Factor Panel A takes literature-reported analyte concentrations into account when calibrating standard curves to provide reliable results.

Sample correlation was an important factor in developing the MILLIPLEX® Non-Human Primate Cytokine/Chemokine/Growth Factor Panel A in comparison to other non-human primate assays in the MILLIPLEX® portfolio. This measure provides confidence in results generated, no matter the MILLIPLEX® assay that is selected. It is important to note that absolute values can change, but sample trends should remain the same. It is recommended that researchers bridge assays in their own labs by comparing kits side-by-side before switching to our larger, more versatile non-human primate panel.

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