Validation of a Multiplexed Luminex Assay for Immunogenicity Assessment of Cytokine Responses in Nonhuman Primates: Implications for Drug Safety and Tolerability

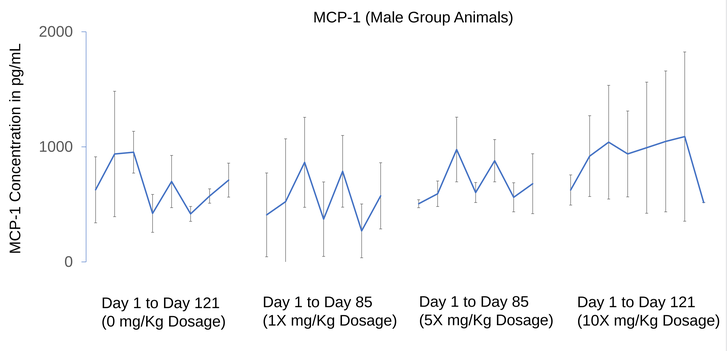
Debargh Dutta, Skylar Guthrie, Nicholas Kendrick, Arlene Leon and Kenneth Swart
Inotiv Biotherapeutics Sciences Group, Rockville, MD.

Table 1. Highlights from Validation assays

Analytes	IFN-γ	TNF-α	IL-1β	IL-2	IL-4	Il-5	Il-6	Il-8	IL-10	IL-17α	IFN-α	GM-CSF	IP-10	MCP-1	VEGF- α
ULOQ (pg/mL)	1663	4500	1450	1938	6325	3350	11025	2950	1925	6225	1125	13275	2125	3950	5400
• ULOQ CV	7.7	22.8	11.45	26.7	15.36	12.07	19	14.3	8.87	26.4	9.6	22.3	12.3	13	8.78
• ULOQ BIAS%	-3.2	-7.3	-2.13	-9.93	1.23	0.12	-7.2	-2	6.08	-5.9	-3.04	-1.1	4.39	2.86	-13.2
• ULOQ Total Error	10.9	30.1	13.6	36.7	16.6	12.2	26.2	16.3	14.9	32.2	12.6	23.4	16.7	15.8	22
LLOQ (pg/mL)	11.5	31.3	10.1	13.5	43.9	23.3	76.6	20.5	13.4	43.2	7.8	92.2	14.8	27.4	37.5
• LLOQ CV%	10.1	**38.9**	11.4	15.44	14.14	14.18	14.8	15.2	13.3	12.6	9.73	11.4	10.3	20.3	9.46
• LLOQ BIAS %	-13.4	-27.5	-12.1	-12	-7.02	-8.89	-5.7	-14.3	-15.3	-13.8	-18.2	-13.9	-4.34	-15.3	-23.8
• LLOQ Total Error	23.5	66.4	23.5	27.5	21.2	23.1	20.4	29.5	28.6	26.4	27.9	25.3	14.6	35.6	33.2
Dilution Linearity	1:8 to 1:12	1:8 to 1:12	1:8 to 1:12	1:8 to 1:12	1:8 to 1:12 (Showed under-recovery of the spike conc.)	1:8 to 1:12	1:8 to 1:12	1:8 to 1:12	1:8 to 1:12	1:8 to 1:12 (Showed under-recovery of the spike conc.)	1:8 to 1:12 (Showed under-recovery of the spike conc.)	1:8 to 1:12	1:8 to 1:12	1:8 to 1:12	1:8 to 1:12 (Showed under-recovery of the spike conc.)
Freeze Thaw Stability	Stable up to 4 freeze thaw cycles	Stable up to 4 freeze thaw cycles	Stable up to 4 freeze thaw cycles	Stable up to 4 freeze thaw cycles	Stable up to 4 freeze thaw cycles	Stable up to 4 freeze thaw cycles	Stable up to 4 freeze thaw cycles	Stable up to 4 freeze thaw cycles	Stable up to 4 freeze thaw cycles	Stable up to 4 freeze thaw cycles	Stable up to 4 freeze thaw cycles	Stable up to 4 freeze thaw cycles	Stable up to 4 freeze thaw cycles	Stable up to 4 freeze thaw cycles	Stable up to 4 freeze thaw cycles
Bench Top Stability	Stable up to 25h at RT	Stable up to 25h at RT	Stable up to 25h at RT	Stable up to 25h at RT	Stable up to 25h at RT	Stable up to 25h at RT	Stable up to 25h at RT	Stable up to 25h at RT	Stable up to 25h at RT	Not Stable at RT	Stable up to 25h at RT	Stable up to 25h at RT	Stable up to 25h at RT	Not Stable at RT	Stable up to 25h at RT
Refrigerator Stability	Stable up to 25h at RT	Stable up to 25h at RT	Stable up to 25h at RT	Stable up to 25h at RT	Stable up to 25h at RT	Stable up to 25h at RT	Stable up to 25h at RT	Stable up to 25h at RT	Stable up to 25h at RT	Stable up to 25h at RT	Stable up to 25h at RT for eHQC only	Stable up to 25h at RT for eHQC only	Stable up to 25 hours	Stable up to 25 hours	Stable up to 25h at RT for eHQC only

Carry-over test did not show an alteration of a measured concentration due to residual analyte from a preceding sample that remains in the analytical instrument. Long-term stability studies are in progress, and according to the literature, most cytokines in the matrix remain stable for up to five years when stored frozen at -75ºC from the day of collection.

Figure 1. A result from a Study conducted in NHP.



Results

The results demonstrated that our method was able to quantify several cytokines namely, IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-17α, IFN-α, GM-CSF, IP-10, MCP-1and VEGF-α with acceptable precision and accuracy with a dilution up to 1.5 times after MRD. No hook effect was observed up to the ULOQ concentrations for all analytes. Analytes are stable in Cynomolgus Monkey plasma at refrigerator and room temperatures for up to at least 24 hours and can undergo up to four freeze-thaw cycles. No carryover was observed for all cytokines.

Our assay demonstrated high sensitivity, specificity, precision and accuracy with robust standard curves for each cytokine. Three serum cytokines (IL-10, IL-17α, and IP-10) were unstable at room temperature, and all serum cytokines except MCP-1, IL-10, IL-17α, and IP-10 were stable up to four freeze and thaw cycles.

Conclusions

We were able to validate a multiplex assay for these cytokines with high sensitivity and acceptable accuracy and precision, which allows for rapid profiling of cytokine levels in NHP samples during a preclinical trial, using a small volume of limited samples for K2EDTA plasma and serum. This method provided significant advantages for investigating complex immune responses to the drug under investigation.

It allows for quantitation of cytokine levels in NHP serum or plasma to a much lower level of quantification (10 - 100 pg/mL) than methods previously reported (100 - 300 pg/mL), thereby providing better insight into the effects of drug treatment.

Introduction

To thoroughly assess the immunogenicity of a large molecular drug in non-human primates, it is crucial to establish a reliable and precise analytical method for quantifying cytokines. By utilizing a robust analytical technique, investigator can monitor cytokine profiles and effectively evaluate the drug's safety and tolerability, thus enhancing the understanding of its immunological impact in non-human primates before advancing to human trials. Most current Luminex assays used for NHP sample analysis do not offer high sensitivity for quantifying cytokines in serum or plasma. The sensitivity for major cytokines typically ranges from 100 pg/mL to 300 pg/mL. A highly sensitive method that can quantify cytokines in the range of 10 pg/mL to 100 pg/mL would be significantly more beneficial.

To offer a highly sensitive assay for preclinical sample analysis, we have developed a validated multiplex bead-based assay on a Luminex platform that simultaneously quantifies a 15 analyte panel of key pro-inflammatory and anti-inflammatory cytokines (including IFN-γ, TNF- α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-17α, IFN-α, GM-CSF, IP-10, MCP-1, and VEGF-α) in NHP serum or K2EDTA plasma samples. In developing this assay, we followed the FDA's M10 as well as other relevant guidelines.

Accuracy and precision as well additional validation criteria like assessing parallelism, dilution linearity, short-term stability at room temperature and refrigeration, freeze-thaw stability, and long-term stability for up to one year was performed. Normal serum and plasma contain detectable levels of IL-8 and MCP-1, while other critical cytokines are below the quantifiable range, which poses a challenge for assessing minimal residual dilution in the parallelism test and stability testing. This was overcome by spiking both matrices with surrogate recombinant proteins to simulate endogenous high-level samples. Stability testing was performed at both high-level and low-level endogenous samples.

Luminex Method

A quantitative Luminex-based assay designed to quantify IFN ɣ, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12/IL-23p40, IL-17A, IFN-α, GM-CSF, IP-10, MCP-1, MIP-1α and VEGF-A in Cynomolgus Monkey Plasma (K2EDTA) was validated on a Luminex instrument FLEXMAP 3D platform. It involves essential buffers, magnetic MagPlex microsphere beads, a blend of cytokines as standard stock vial, a detection antibody cocktail, and Streptavidin-PE to thus mimicking a sandwich ELISA on the beads. Each magnetic MagPlex microsphere bead is fluorescently coded with one of 500 specific ratios of two fluorophores (each spectrally distinct set is known as a bead region, fluorescent at an Excitation Wavelength (Ex) of 635 nm). IFN ɣ, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12/IL-23p40, IL-17α, IFN-α, GM-CSF, IP-10, MCP-1, MIP-1α and VEGF-α capture antibodies are bound to bead region number 43, 45, 18, 19, 20, 21, 25, 27, 28, 64, 36, 48, 44, 22, 51, 12 and 78 respectively.

To prevent non-specific binding, unbound sites of the bead are blocked with an universal assay buffer to inhibit non-specific interaction. Beads are incubated with a sample, washed, and then incubated with a biotinylated secondary antibody. The unbound secondary antibody is washed away and the added streptavidin-phycoerythrin binds to the biotin of the "sandwich" immunoassay.

The Luminex instrument FLEXMAP 3D detects individual beads by region plus the streptavidin-conjugated R-Phycoerythrin (SAPE) signal, Ex 525 nm, indicating the analyte is present and signal intensity quantifies them.

The Median Fluorescent Intensity (MFI) raw data in the CSV file is analyzed using a 4-parameter logistic for calculating cytokine concentrations. Data was collected on the Luminex platform and analysis performed using Watson LIMS 7.7.1™ software.

Data was directly imported from batch results obtained from Xponent (Luminex sample analysis tool) into Watson LIMS 7.7.1™ without a possibility of raw data alteration.