#M1029

Development and Validation of Multiplexed Non-Human Primate Cytokine V-PLEX® Panels

Christopher Campbell, Christopher Shelburne, Joseph Manimala, Ken Wong, Prachi Gupte, Qian Ning, Laura Schaefer, David Stewart, Pankaj Oberoi, and Jacob N. Wohlstadter

Meso Scale Discovery, Rockville, Maryland, USA

1 Abstract

Purpose: Cytokine assays are widely used to monitor immune responses triggered by toxins and other trauma. Multiplexed assays provide large datasets from low-volume samples, making them ideal for monitoring toxicological responses in drug studies. Because of high homology across species, assays can often be applied to multiple non-human primate (NHP) models. We report the validation of multiple V-PLEX cytokine assays for applications in rhesus and cynomolgus monkeys. Validation included characterization of assay performance as well as intra- and inter-lot reproducibility, which is essential to support long-term studies in drug development programs.

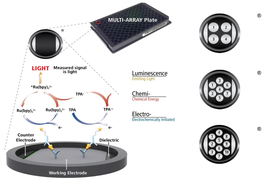
Methods: We developed and validated cytokine panels that detect up to 24 NHP cytokines in multiple matrices from rhesus and cynomolgus monkeys as well as cytokines produced by in vitro cell stimulation. The assays include IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, GM-CSF, IL-5, IL-7, IL-12/IL-23p40, IL-15, IL-16, IL-17A, TNFβ , VEGF, MIP-1β, Eotaxin-3, TARC, IP-10, MIP-1α, IL-8 (high abundance), MCP-1, MDC and MCP-4. The analytical validation process evaluated precision and accuracy, spike recovery, dilution linearity, non-specificity, and quantification of known and unknown samples.

Results: We present the validation procedure and performance data for NHP cytokine multiplex panels. Data include matrix tolerance and intra- and inter-lot sample correlations. Tests of media conditioned by rhesus and cynomolgus peripheral blood lymphocytes showed spontaneous or stimulated changes for all tested cytokines. Moreover, all analytes were detected using serum, EDTA plasma, or urine specimens.

Conclusions: We developed and validated multiplex panels for the measurement of 24 NHP cytokines using multiple assay lots. These assays are suitable for rigorous applications, such as ongoing studies of animal models of disease and therapeutic intervention.

2 Methods

The NHP cytokine multiplex panel was developed as three panels designed for optimal performance based on individual assay characteristics, native analyte levels and suitable dilution, diluent, and assay compatibility. The Proinflammatory Panel 1 (NHP), Cytokine Panel 1 (NHP) and Chemokine Panel 1 (NHP) kits are based on MSD® ECL technology and follow the same simple three-step protocol.



Protocol

1. Add calibrator, control or sample (25 µL/well). Incubate 2 hours at room temperature (RT).
2. Wash and add detection antibody solution (25 µL/well). Incubate 1 hour at RT.
3. Wash and add read buffer (150 µL/well). Analyze with MSD instrument.

Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

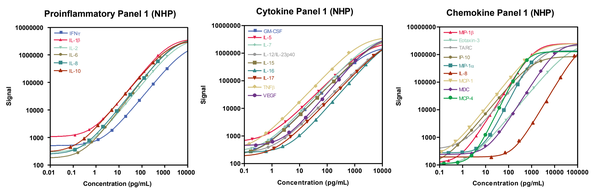
Development and Validation

The assays presented were developed and validated following fit-for-purpose principles and design control procedures. Accuracy, precision, and specificity were evaluated by multiple operators using independently built kit lots to test calibrators, controls, and matrix-based samples over multiple days and runs. Data analysis methods are as follows:

- **Sensitivity –** Limits of detection (LOD) were calculated based on assay signal and standard deviation of background signal. Limits of quantification (LOQ) were determined by spiking a known value of calibrator into diluent to assess the accuracy and precision of the samples across multiple lots.
- **Dilution Linearity and Spike Recovery –** Dilution linearity and spike recovery were assessed across multiple lots using a variety of biological matrices.
- **Specificity –** Assay specificity and interference were evaluated across multiple lots using a panel of related proteins and proteins within the panel.
- **Stability –** The robustness of the product protocol was evaluated to examine the boundaries of the selected incubation times. Assay component stability was assessed through freeze–thaw testing and accelerated stability studies for calibrators, antibodies, and controls. The validation program includes a real-time stability study with scheduled performance evaluations of complete kits for at least 30 months from date of manufacture.

3 Calibration Curves

Representative calibration curves are shown for each assay. The curves were generated by taking the average of at least 26 runs conducted by 4 operators over a minimum of 8 days.



4 Sensitivity and Assay Protocol

The lower limit of detection (LLOD) is a calculated concentration corresponding to the signal 2.5 standard deviations above the background (zero calibrator). The lower limit of quantification (LLOQ) is the highest concentration at which the CV of the calculated concentration is <20% and the recovery of the analyte is within 80% to 120% of the known value (75% to 125% for IL-8). The upper limit of quantification (ULOQ) is the highest concentration at which the CV of the calculated concentration is <20% and the recovery of the analyte is within 80% to 120% of the known value (75% to 125% for IL-8). The data are from >30 runs collected over 2 months by 4 operators.

Proinflammatory Panel 1 (NHP)			
Analyte	LLOD Median (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
IFN-γ	0.20	7.47	938
IL-1β	0.04	2.14	375
IL-2	0.09	0.89	938
IL-6	0.06	1.58	468
IL-8	0.04	1.13	375
IL-10	0.03	0.68	233

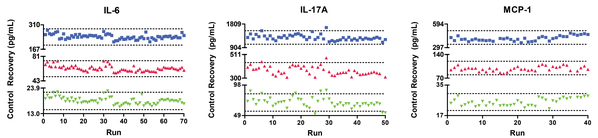
Cytokine Panel 1 (NHP)			
Analyte	LLOD Median (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
GM-CSF	0.14	1.90	750
IL-5	0.22	6.28	562
IL-7	0.16	1.37	563
IL-12/IL-23 p40	0.39	5.68	2250
IL-15	0.17	1.40	525
IL-16	2.83	19.1	1875
IL-17A	0.74	9.32	3653
TNFβ	0.05	1.15	458
VEGF	1.12	7.70	784

Chemokine Panel 1 (NHP)			
Analyte	LLOD Median (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
MIP-1β	0.37	2.27	750
Eotaxin-3	1.77	10.2	3750
TARC	0.22	3.32	1120
IP-10	0.37	1.37	500
MIP-1α	3.02	13.8	743
IL-8[1]	95.6	713	43400
MCP-1	0.09	1.09	375
MDC	1.22	88.3	7500
MCP-4	1.69	5.13	469

[1]Because of the high abundance of IL-8 in some sample types, Chemokine Panel 1 (NHP) uses a low sensitivity IL-8 assay.

5 Reproducibility

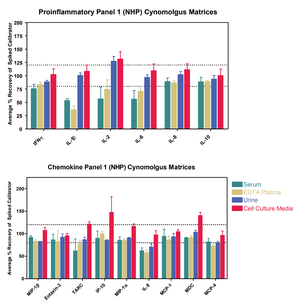
To evaluate the reproducibility of the panels, three levels of controls spanning the quantifiable range were prepared and measured over multiple runs for each panel. Representative plots of an analyte from each of the NHP panels are also shown. The controls were prepared in a non-human matrix, and between 40 and 70 runs were conducted depending on the panel. All controls tested were within the target range of ±20% of assigned concentration (dashed lines).



6 Spike Recovery and Dilution Linearity

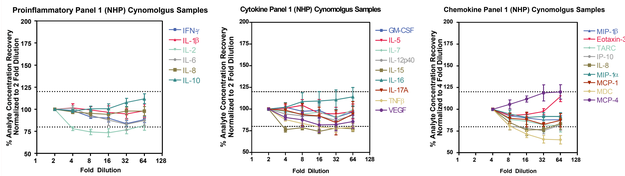
Spike recovery testing was conducted in NHP serum, heparin plasma, EDTA plasma, citrate plasma, urine, and cell culture media. Representative data are presented for assays performed in cynomolgus matrices. Almost all assays accurately recovered expected concentrations of spiked analyte (dashed lines are at ±20% of expected value). Similar data were obtained using matrices from rhesus macaques.

Spike Recovery



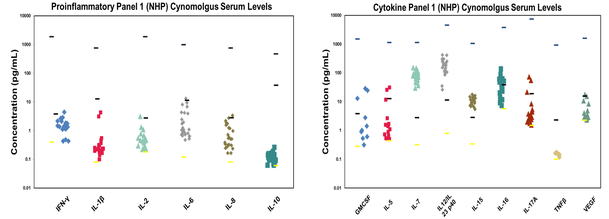
Dilution Linearity

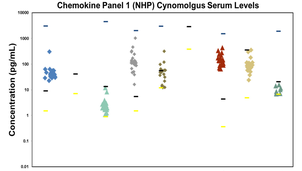
Dilution linearity testing was also conducted on all assays. Results were similar with almost all samples recovering within 20% of expected concentrations across all assays. Results from cynomolgus urine samples are displayed. Error bars are the standard deviation of 5 samples. Similar data were obtained with urine from rhesus macaques.



7 Normal Sample Testing

To evaluate the ability of our anti-NHP antibody pairs to recognize native analytes in cynomolgus and rhesus matrices, we screened normal cynomolgus and rhesus matrices (obtained from a commercial vendor) for native analyte levels using the Proinflammatory Panel 1 (NHP), Cytokine Panel 1 (NHP), and Chemokine Panel 1 (NHP) kits. Representative data for Cynomolgus serum is shown with upper limits of quantitation (ULOQ, dark blue bars), lower limits of quantitation (LLOQ, black bars) and lower limits of detection (LLOD, yellow bars) noted for each assay. Nearly all analytes were detectable (except Eotaxin-3 and IL-8) in both cynomolgus and rhesus matrices.





8 Stimulated Sample Testing

We also evaluated the ability of our antibody pairs to detect NHP analytes in the supernatants of activated cynomologus or rhesus peripheral blood lymphocytes (PBLs). PBLs were treated with nothing, ConA, LPS, PHA or PWM for 6, 24, or 48 hours. The stimulated samples were tested on Proinflammatory Panel 1 (NHP), Cytokine Panel 1 (NHP), and Chemokine Panel 1 (NHP) kits. Results are expressed as fold-change compared to unstimulated controls. When analytes were initially undetectable, calculations for fold increase were based on the LLOD of the assay.

Cynomolgus Samples

Proinflammatory Panel 1 (NHP)					
Assay	ConA	LPS	PHA	PWM	Spontaneous
IFN-γ	+++	-	+++	+++	-
IL-1β	-	+	+	+	-
IL-2	++	+	++	++	+
IL-6	+	-	+	+	-
IL-8	+	+	++	+	+
IL-10	+	+	-	+	++

Cytokine Panel 1 (NHP)					
Assay	ConA	LPS	PHA	PWM	Spontaneous
GM-CSF	+++	+	+++	+++	-
IL-5	++	-	+++	++	-
IL-7	-	-	+	+	-
IL-12p40	+	-	-	+	-
IL-15	-	-	-	+	++
IL-16	-	+	+	+	-
IL-17A	++	+	++	++	-
TNFβ	+++	+	+++	+++	-
VEGF	+	+	+	+	++

Chemokine Panel 1 (NHP)					
Assay	ConA	LPS	PHA	PWM	Spontaneous
MIP-1β	+	+	+	++	++
Eotaxin-3	-	++	-	-	-
TARC	+	-	-	-	-
IP-10	+	+	+	+	+
IL-8 (HA)	+	+	+	++	+
MIP-1α	+	+	++	++	++
MCP-1	+	+	+	+	-
MDC	+	+	+	+	+
MCP-4	-	-	+	-	+

Rhesus Samples

Proinflammatory Panel 1 (NHP)					
Assay	ConA	LPS	PHA	PWM	Spontaneous
IFN-γ	+++	+	+++	++	+
IL-1β	+	+	+	+	-
IL-2	++	+	++	+	+
IL-6	+	-	+	+	+
IL-8	+	+	++	+	+
IL-10	+	+	+	+	+

Cytokine Panel 1 (NHP)					
Assay	ConA	LPS	PHA	PWM	Spontaneous
GM-CSF	+	+	+	++	-
IL-5	-	-	+	-	-
IL-7	-	-	-	-	-
IL-12p40	-	+	-	+	-
IL-15	-	-	-	+	++
IL-16	-	+	+	-	++
IL-17A	+++	+	+++	+++	+
TNFβ	+++	+	++	+++	-
VEGF	-	+	+	+	-

Chemokine Panel 1 (NHP)					
Assay	ConA	LPS	PHA	PWM	Spontaneous
MIP-1β	-	-	+	+	+
Eotaxin-3	-	+	-	+	-
TARC	++	-	+	+	-
IP-10	++	+	+	+	+
IL-8 (HA)	+	+	+	++	++
MIP-1α	-	+	-	-	+
MCP-1	+	+	+	+	+
MDC	+	+	+	-	+
MCP-4	+	-	-	+	-

+++ >100-fold
++ >10-fold
+ >2-fold
- No significant response

9 Conclusion

NHP cytokine and chemokine multiplex panels have been developed and analytically validated for use in the quantification of NHP cytokines and chemokines in multiple NHP matrices. All assays were optimized to achieve the best assay performance and for ease of use, featuring the same simple 3-step protocol requiring just 25 µL of sample to measure all 24 NHP analytes. Consistent results were demonstrated for calibration curves and control samples over multiple runs on different days by multiple operators. Moreover, all antibody pairs were able to recognize their NHP analyte in normal samples or in culture supernatants from stimulated NHP PBL. The reproducibility, accuracy, and sensitivity of these assays should facilitate the development of NHP models of disease and therapeutic interventions.

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