The Proinflammatory Panel 1 (mouse) V-PLEX™ Kit: A Summary Report of Development and Validation Studies Demonstrating High Performance and Lot-to-Lot Reproducibility

Meso Scale Discovery (MSD®)

A division of Meso Scale Diagnostics, LLC., 1601 Research Boulevard, Rockville, Maryland 20850 U.S.A.

ABSTRACT

A significant challenge facing the field of multiplexed immunoassays is lot-to-lot reproducibility. With the use of well-characterized and purified reagents and highly optimized assays, MSD's V-PLEX product portfolio provides consistent results from multiplexed assays. The Proinflammatory Panel 1 (mouse) comprises 10 assays for inflammation-related biomarkers: IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, and TNF-α. This panel is analytically validated for use in serum, EDTA plasma, heparin plasma, citrate plasma, urine, and cell culture supernatants. It shows excellent lot-to-lot reproducibility and superior sensitivity, precision, and accuracy when compared to other assays on the market. This report summarizes the V-PLEX development process and presents data from the validation studies for the Proinflammatory Panel 1 (mouse).

INTRODUCTION

Many commercial immunoassay kits are being used for validated studies, clinical trials, and long-term research programs. Researchers depend on these kits for measuring both novel and established biomarkers. Performance changes due to new lots of reagent, poorly developed assays, or lack of robust manufacturing can lead to variability that is especially troublesome for long-term or meta-analytical studies. It has been shown that inconsistencies between biomarker discovery studies can be attributed to variability between assay kit lots.[1-2] A lack of standard guidelines for commercial, research use only (RUO) kits contributes to inconsistencies in performance for kits from different lots and manufacturers. Even CE-marked kits may lack reproducibility because the CE mark is self-regulated, and most vendors do not publish specifications on lot-to-lot reproducibility. Researchers are required to perform their own validations and show that the kits are fit for purpose to determine whether an off-the-shelf kit will be suitable for their study. Working groups from several consortia and research societies have expressed a need for standardization in the validation of these assays. A working group of the American Association of Pharmaceutical Scientists has published fit-for-purpose guidelines for biomarker studies, highlighting the requirement for higher-grade research kits, and presented white papers on the need for critical reagent monitoring.[3-5]

MSD has addressed this need with the V-PLEX product line, a catalog of validated kits designed and developed to provide both high performance and consistency within and between kit lots. These kits are developed under rigorous design control and are fully validated according to fit-for-purpose principles in accordance with MSD's Quality Management System (MSD's Quality Management System has received ISO 9001: 2008 certification for the design, development, manufacture, distribution and service of instruments, plates, reagents, assays and kits for

research purposes). A minimum of 3 kit lots are manufactured (from independent raw material lots when possible) for the validation process. Analytical validation is performed on kits from each lot, measuring sensitivity, accuracy, precision, specificity, dilution linearity, spike recovery, and sample values. In addition to the analytical validation, robustness of the assay protocol is evaluated during development and the stability of the assay components and kits is characterized.

In this report, we present data from the development and validation of the Proinflammatory Panel 1 (mouse) V-PLEX kit (K15048D).

Development

Proinflammatory Panel 1 (mouse) development included optimization of various assay parameters such as antibody concentration, diluent composition, and protocol timing. Calibration curve concentrations for each assay were optimized for maximum dynamic range, while maintaining sufficient calibration points near the low end of the curve to ensure accurate quantification of samples that require high sensitivity. The specificity of both capture and detection antibodies was measured during assay development. Antibody specificity was assessed by first running each assay individually on a multiplex plate with assay-specific detection antibody and assay-specific calibrator. These results were then compared to the assay's performance when the plate was run with: 1) the multi-analyte calibrator and assay-specific detection antibodies and 2) assay-specific calibrator and all detection antibodies. In addition to measuring the specificity of antibodies in the multiplex kit, interference from other related biomarkers was evaluated, including selected proteins and receptors or binding partners. Stimulated whole blood, PBMCs, and/or cell line samples were used to measure samples with elevated endogenous analyte levels; this confirmed the kit's ability to quantify analytes at concentrations that are often higher than those found in normal samples. As part of development, real-time and accelerated stability testing was conducted for components and kits. Freeze–thaw and storage (2–8°C) stability were evaluated for both reconstituted calibrators and controls.

Validation

V-PLEX products are validated following fit-for-purpose principles[6] under MSD's design control system. Prior to the release of Proinflammatory Panel 1 (mouse), 3 independent kit lots were produced and analytically validated in the final product configuration. Results from multiple runs (typically greater than 50 runs performed by multiple operators) were used to establish production specifications for sensitivity, specificity, accuracy, and precision. During validation of the multiplex panel, the specificity and independence of each individual assay was demonstrated by comparing results for each assay when run in two modes: 1) individual assay mode, in which the assay is run using a multi-analyte calibrator and the full 10-spot plate with only the detection antibody for that specific assay; or 2) multiplex mode, in which the assay is run using a multi-analyte calibrator and the 10-spot plate with all 10 detection antibodies. The results from the two modes were compared, confirming that multiplexing does not compromise the results. The specificity and independence of each assay are important because they allow them to be multiplexed in any combination. In addition, each assay in the multiplex panel was analytically validated on a single-spot plate.

The Proinflammatory Panel 1 (mouse) was validated by evaluating sensitivity, quantifiable range, accuracy, precision, antibody specificity, spike recovery, and dilution linearity using 6 sample types (serum, EDTA plasma, heparin plasma, citrate plasma, urine, and cell culture supernatants) across 3 lots. Precision is reported as the coefficient of variation (CV). Accuracy and precision were evaluated by measuring calibrators and matrix-based validation samples or controls across multiple runs and multiple lots.

Matrix effects from serum, plasma, urine, and cell culture media were also assessed as part of the validation process. Dilution linearity and spike recovery studies were performed on individual samples rather than pooled samples to assess variability of results due to matrix effects across different sample types. The validation program includes a real-time stability study of the Proinflammatory Panel 1 (mouse) kit with scheduled performance evaluations of complete kits for up to 54 months from the date of manufacture.

For each production lot, every kit component is manufactured and functionally tested using specifications based on the results of the analytical validation. All components are evaluated against a reference component. Calibrators are anchored to an internal reference standard that will be maintained for the life of the product. Assembled kits are functionally evaluated to ensure consistent performance of each kit lot. Functional testing involves sensitivity, quantifiable range, accuracy, precision, and antibody specificity. Lot-specific performance is included in the certificate of analysis (C of A) that is shipped with each order.

MATERIALS AND METHODS

MSD Technology

The V-PLEX product line applies MSD's MULTI-ARRAY® electrochemiluminescence (ECL) detection technology (**Figure 1**) to sensitive, multiplexed sandwich immunoassays.

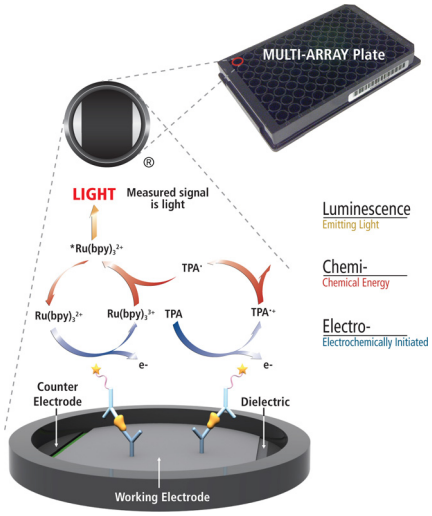


*Figure 1. 1) **Electricity** initiates the electrochemiluminescence cascade; 2) A **Chemical** reaction excites SULFO-TAG; 3) SULFO-TAG **Luminesces** as it returns to the base state. The cycle repeats, strengthening the signal.*

The bottom of each well of an MSD MULTI-SPOT® plate is divided into 10 independent, electrically conductive, well-defined regions (spots). The spots are coated with specific capture antibodies as shown in **Figure 2**. The user adds the sample and a solution containing detection antibodies conjugated with electrochemiluminescent labels (MSD SULFO-TAG™) over the course of one or more incubation periods.

Analytes in the sample bind to capture antibodies immobilized on the working electrode surface; recruitment of the detection antibodies by the bound analytes completes the sandwich. In the final step, the user adds an MSD buffer that provides the appropriate chemical environment for ECL and loads the plate into an MSD instrument where a voltage applied to the plate electrodes causes the captured labels to emit light. The instrument measures the intensity of emitted light to provide a quantitative measure of analytes in the sample.

1. **IFN-γ**
2. **IL-1β**
3. **IL-2**
4. **IL-4**
5. **IL-5**
6. **IL-6**
7. **KC/GRO**
8. **IL-10**
9. **IL-12p70**
10. **TNF-α**

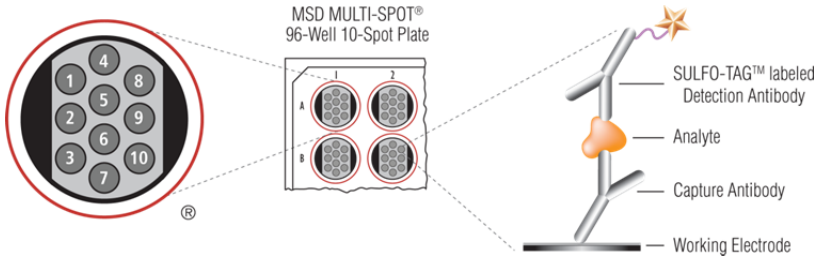


Figure 2. Multiplex plate spot diagram showing placement of analyte capture antibodies. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files.

MSD technology offers the following advantages:

High sensitivity and broad dynamic range. Multiple excitation cycles of each label amplify the signal and improve sensitivity; the wide dynamic range of the detection systems mean high and low expression levels can be measured without multiple dilutions.

Flexibility. Carbon electrode plates support the immobilization of a wide range of different biological materials, with 10X greater binding capacity than polystyrene.

Clean signals. The stimulation mechanism (electricity) affects only analytes bound near the surface of the plate, eliminating problems with background noise. The ~620 nm photons from SULFO-TAG labels are not absorbed by most components of biological samples, dramatically reducing matrix interference and color quenching.

Time- and cost-effective processing. Multiplex panels are very cost-effective and offer high processing efficiency; multiple analytes can be measured in one well using typical sample volumes of 25 µL or less without compromising speed or performance.

Fast, convenient protocols. Since only labels bound near the electrode surface are excited, protocols are simpler with fewer wash steps, so assays typically take around 4 hours to complete. MSD instruments read plates in 1–3 minutes and require no cleaning or calibration between reads.

Consistent results. Independent reports in respected journals show that MSD's assays provide highly reproducible intra- and inter-lab results. Strict manufacturing procedures keep critical reagents consistent from lot-to-lot, and simple protocols and standardized templates reduce the potential effects of inter-operator variability.

Kits and Reagents

Three independent Proinflammatory Panel 1 (mouse) kit lots (K00D0072, K0080001, and K0080002) were built and used for product validation. Controls built in mouse serum (lots A00C0160, A00C0161, and A00C0162) were used to measure precision and accuracy. In addition, individual assay kits using single spot plates—lots K0080005 (IFN-γ), K0080006 (IL-1β), K0080007 (IL-2), K0080008 (IL-4), K0080009 (IL-5), K0080010 (IL-6), K0080011 (KC/GRO), K0080012 (IL-10), K0080013 (IL-12p70), and K0080014 (TNF-α)—were validated. Normal samples, stimulated whole blood, and crude buffy coat PBMCs were purchased from Bioreclamation (Liverpool, NY), and a mouse monocyte macrophage cell line (J774A.1) was obtained from American Type Culture Collection.

Kit calibrators were built from bulk calibrators that are maintained at higher concentrations and are characterized relative to internal reference calibrators. The kit calibrators were then lyophilized and intra-lot reproducibility was evaluated by running 22 vials and calculating intra- and inter-vial precision. The final concentration for the kit calibrators was determined using an internal reference calibrator over three runs performed over multiple days. Where NIBSC/WHO reference standards are available, MSD's internal reference calibrators are anchored to them. Kit calibrator performance specifications are summarized in the calibrator C of A.

Controls were made by spiking calibrators into mouse serum at 3 levels within the quantitative range of the assay. The native expression levels of analytes in serum were initially assayed, and appropriate levels of calibrators were spiked into the serum. The controls were lyophilized, and the concentrations of each analyte were assigned as the mean concentration measured using three kit lots. Control performance specifications are summarized in the control C of A.

Kit Protocol

Unless specified otherwise, the following protocol was used for development and validation of the Proinflammatory Panel 1 (mouse) kit.

1) **Add sample:** Add 50 µL of diluted sample, calibrator, or control per well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking for 2 hours.
2) **Wash and Add Detection Antibody Solution:** Wash the plate 3 times with at least 150 µL/well of wash buffer. Add 25 µL of detection antibody solution to each well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking for 2 hours.
3) **Wash and Read:** Wash the plate 3 times with at least 150 µL/well of wash buffer. Add 150 µL of 2X Read Buffer T to each well. Read the plate on the MSD instrument. No incubation in read buffer is required before reading the plate.

Validation

Sensitivity. The following methods were used to establish the lower limit of detection (LLOD) and the lower and upper limits of quantitation (LLOQ and ULOQ, respectively). Samples for determining LLOQ and ULOQ were prepared by spiking calibrators into assay diluent.

The LLOD is a calculated concentration corresponding to the signal 2.5 standard deviations above the background (zero calibrator).

The ULOQ is the highest concentration at which the CVs of the calculated concentrations are <25% and recovery of calibrators is within 75%–125% of the known value.

The LLOQ is the lowest concentration at which the CVs of the calculated concentrations are <25% and recovery of calibrators is within 75%–125% of the known value.

The LLOD was calculated using results from multiple plates for each lot, and the median and range of calculated LLODs for a representative kit lot are reported in the product insert. The ULOQ and LLOQ were established for each lot by measuring multiple levels near the expected limits. The final LLOQ and ULOQ specifications for the product were established after assessment of all validation lots. When LLOQ and ULOQ values were assigned, we ensured that there was a point on the calibration curve below and above each value, respectively.

Accuracy and Precision. The accuracy and precision of the Proinflammatory Panel 1 (mouse) were evaluated using 3 kits from each of 3 lots tested over 3 days.

Inter- and intra-plate CVs and analyte % recovery range were calculated for:

- Calibrator concentrations within the quantifiable range of the assay
- Control recovery
- LLOQ and ULOQ sample recovery

Three lots of multiplex kits were tested by running at least 3 kits from each lot per day on 3 separate days. Single-spot kits were tested using at least 3 kits.

Dilution Linearity. To assess linearity, normal mouse serum, EDTA plasma, heparin plasma, citrate plasma, and urine samples, as well as cell culture supernatants, were spiked with recombinant calibrators and diluted 2-fold, 4-fold, 8-fold, 16-fold, 32-fold, and 64-fold. These diluted samples were tested using kit lots K00D0072, K0080001, and K0080002. Percent recovery at each dilution was calculated by dividing the dilution-adjusted concentration by the expected concentration, i.e., the dilution-adjusted concentration measured at a 2-fold dilution.

$$\%\ Recovery = \frac{measured\ concentration}{expected\ concentration} * 100$$

Spike Recovery. Spike recovery measurements using different sample types across the quantitative range of each assay were evaluated. Multiple individual mouse serum, EDTA plasma, heparin plasma, citrate plasma, and urine samples, as well as cell culture supernatants, were spiked with calibrators at 3 levels (high, mid, and low) then diluted 2-fold. They were tested using kit lots K00D0072, K0080001, and K0080002. The average % recovery for each sample type is reported along with % CV and % recovery range.

$$\%\ Recovery = \frac{measured\ concentration}{expected\ concentration} * 100$$

Specificity. To assess specificity, each assay in the panel was tested individually using single detection antibodies (lots K00D0072, K0080001, and K0080002). Nonspecific binding was also evaluated with additional recombinant mouse analytes (IL-13, IL-17, GM-CSF, MCP-1, MIP-3α, RANTES, TNF-RI, TNF-RII, and VEGF).

$$\%\ Nonspecificity = \frac{nonspecific\ signal}{specific\ signal} * 100$$

Stability. Reconstituted calibrator, reconstituted controls, and diluents were tested for freeze–thaw stability. Storage stability of reconstituted calibrators and controls was also tested at 2–8°C. Based on results of stability testing, partially used MSD plates may be sealed and stored up to 30 days at 2–8°C in the original foil pouch with desiccant. Results from control measurements changed by ≤30% after plates were stored for 30 days in the recommended manner. The validation study includes a real-time stability study with scheduled performance evaluations of complete kits for up to 54 months from date of manufacture.

Calibration. All of the calibrators in the panel are calibrated against a reference calibrator generated at MSD. Where NIBSC/WHO standards have been established for an analyte, MSD reference calibrators are evaluated against it. **Table 1** lists the NIBSC/WHO catalog numbers for analytes that have been evaluated against the MSD reference calibrator.

Table 1. Calibrator standards

Analyte	NIBSC/WHO Cat. #

IL-1β	96/668
IL-2	93/566
IL-4	91/656
IL-6	93/730
TNF-α	88/532

Samples. Normal mouse serum, EDTA plasma, heparin plasma, citrate plasma, and urine samples were diluted 2-fold and tested using lots K00D0075, K0080001, and K0080002. To prepare stimulated whole blood, freshly collected, normal, pooled, mouse whole blood was incubated at 37°C for different time periods either with lipopolysaccharide (LPS) or with peptidoglycan (PG) and zymosan (ZY); plasma was isolated at the end of incubations. In addition, a mouse monocyte macrophage cell line (J774A.1) was stimulated for 4 hours with LPS or pokeweed mitogen (PWM). The lysate was collected and tested. The concentrations were normalized for 50 µg of lysate per well.

Comparison with Current Kits

The Proinflammatory Panel 1 (mouse) Kit was evaluated against current MSD mouse cytokine kits. Mouse TH1/TH2 9-plex kit (lot K0033705) and Pro-Inflammatory 7-plex (lot K0033232) were used for this study.

Calculation of Analyte Concentrations

The calibration curves used to calculate analyte concentrations were established by fitting the signals from the calibrators to a 4-parameter logistic (or sigmoidal dose-response) model with a $1/Y^2$ weighting. Analyte concentrations were determined from the ECL signals by back-fitting to the calibration curve. The assays have a wide dynamic range (4 logs), which allows accurate quantification of samples without the need for multiple dilutions or repeated testing. The calculations to establish calibration curves and determine concentrations were carried out using the MSD DISCOVERY WORKBENCH® analysis software.

RESULTS AND DISCUSSION

Preliminary Assay Development

Antibodies were screened in pairwise combinations using recombinant proteins, stimulated cells, and serum samples to confirm recognition of native protein. Once optimal antibody pairs were selected, all raw material antibodies were purified and characterized. Dynamic light scattering was used to assess the reagents aggregation; (polyacrylamide gel electrophoresis was used to assess purity and absence of degradation products. The isoelectric point (pI) was determined for each antibody by isoelectric focusing. Together, these characterizations are used to confirm consistency in new lots of raw materials used in future kits.

Assays for relevant inflammation-related biomarkers were assembled into a panel and both the kit components and the kit as a unit were optimized. Kit components were individually optimized to ensure consistent performance. This involved optimization of antibody concentration, diluent formulation and component concentrations, buffer formulation, protocol, reagent/sample preparation and timing, and manufacturing scale up. Once the kit components were optimized, they were assembled into the final kit configuration. The final kit was used to collect assay performance data. Kit performance specifications were set, and the kit was validated against these specifications. Summaries of development and validation data are presented below. Based on development and validation results, QC specifications were established for release testing of all future kit lots.

Calibration Curves

Calibration standards were selected to provide a calibration curve with a broad dynamic range, but with sufficient points at low analyte concentration to ensure optimal assay performance near the low end of the curve. The wide dynamic range achievable with ECL technology allows simultaneous quantitation of normal and disease/stimulated samples at the same sample dilution. To maximize sensitivity, we use a minimal sample dilution. The calibrators in each kit calibrator lot were anchored to an MSD internal reference standard (See **Calibration** section below). Representative average calibration curves from kit lot K00D0072 are presented in **Figure 3**. Data were collected over 6 months of testing by 5 operators (63 runs in total). Most assays displayed a minimum 4-log dynamic range. At least 2 replicates of calibrators were run on each plate.

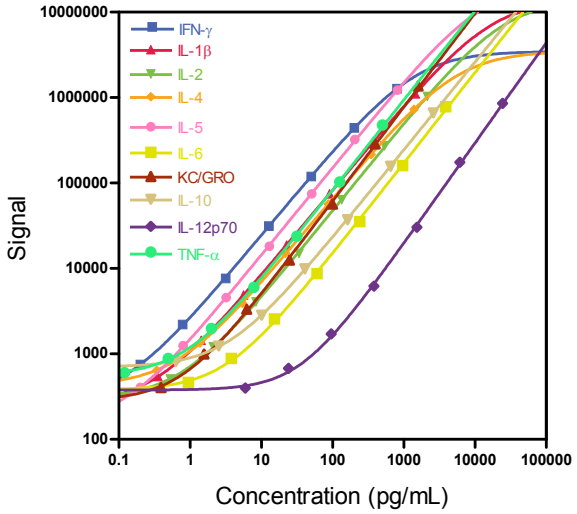


Figure 3. Representative calibration curves demonstrating broad dynamic range.

Assay and Protocol Optimization

During product development, antibody concentrations and formulations of diluents and buffers were designed to optimize performance of all 10 assays in the panel. Diluents were selected to 1) reduce assay background, 2) maximize assay-specific signals, 3) reduce assay interference, matrix effects, and non-specificity, 4) ensure reproducibility of sample quantification, and 5) provide good assay performance for all 10 assays in all of the target sample types. A minimum 2-fold dilution is recommended for optimum performance and quantitation. If analyte levels in the samples of interest are elevated, a higher sample dilution may be used to help minimize any possible sample-specific matrix effects.

During protocol optimization, assay and antibody incubation times were optimized. We tested the boundaries of selected incubation times to ensure that assay signal fluctuations stay within 20% at ± 30 minutes. A 2-hour incubation was optimal for both samples and detection antibodies. In addition, we optimized the timing and handling of reconstituted lyophilized multi-analyte calibrator and controls. We found that a 5-minute incubation with periodic vortexing was the optimal condition for reconstituting both calibrator and controls.

A number of alternate protocols were evaluated during development to accommodate various customer needs. These protocols were not used in the validation process.

Alternate Protocol 1, Extended Incubation: Incubating samples overnight at 2–8°C may improve sensitivity for some assays. All but IL-6 and KC/GRO yielded same or better sensitivity with overnight incubation. See **Appendix A** for specific assays that are affected.

Alternate Protocol 2, Reduced Wash: A simplified protocol may be used with tissue culture samples by eliminating the first wash step. Sensitivity for all assays except for IL-12p70 decreased slightly with this protocol. See **Appendix A** for assay performance using the reduced wash protocol.

Sensitivity

The calculated LLOD, LLOQ, and ULOQ for each lot are shown in **Table 2**. The quantitative range of the assay lies between the LLOQ and ULOQ. LLOQ and ULOQ samples were only tested with lots K008001 and K008002. The LLOQ and ULOQ are verified for each production lot, and the results are provided in the lot-specific C of A that is included with each kit and available at www.mesoscale.com.

Table 2: Assay Sensitivity

Lot	K00D0072	K0080001			K0080002		
Limits (pg/mL)	Median LLOD (N=63)	Median LLOD (N=31)	LLOQ	ULOQ	Median LLOD (N=25)	LLOQ	ULOQ
IFN-γ	0.04	0.04	0.39	724	0.02	0.40	734
IL-1β	0.11	0.09	0.72	1320	0.07	0.72	1320
IL-2	0.22	0.24	1.09	2020	0.19	1.03	1900
IL-4	0.14	0.16	0.71	1320	0.11	2.58	1190
IL-5	0.07	0.05	0.41	753	0.04	1.60	740
IL-6	0.63	0.69	7.05	3250	0.60	6.82	3140
KC/GRO	0.24	0.26	3.18	1470	0.17	3.01	1390
IL-10	1.06	0.86	5.2	2400	0.69	4.96	2290
IL-12p70	9.52	11.7	47.3	21800	7.27	44.7	20600
TNF-α	0.13	0.21	0.98	454	0.18	0.99	457

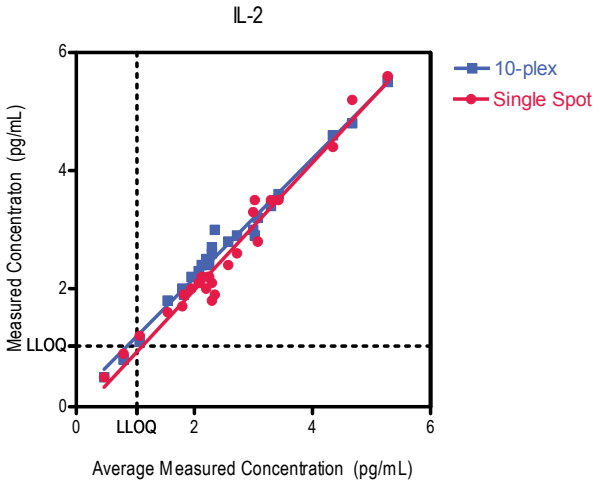
Impact of Multiplexing

To ensure that assays run equally well individually and as part of a multiplex, each assay was tested on single-spot plates (results in **Appendix B**) and on 10-spot plates with a single, assay-specific detection antibody (results in **Appendix C**). The results were compared to results from fully multiplexed assays.

In general, assays provided similar LLODs in singleplex and multiplex formats. Multiplexed assays generally displayed higher background signals because the use of multiple detection antibodies resulted in a higher concentration of labeled molecules; however, the assay-specific signals were not affected.

The same assay run on a single-spot plate yielded lower specific signals than when run on the 10-spot plate (individually or multiplexed). This is because the reported signal is actually a measure of signal per area (density). The same amount of materials captured on a smaller area results in a higher reported signal. The spot area is larger on single-spot and 4-spot plates than on 7- and 10-spot plates; therefore, the signals are higher on a 10-spot plate than on a single-spot or 4-spot plate.

To further evaluate the impact of multiplexing, correlation for recovery of IL-2 in samples was evaluated between 10-plex (lot K0080001) and single-spot (lot K0080007) assays. Recovery correlation was high (**Figure 4**). This demonstrates that the assays used in the Proinflammatory Panel 1 (mouse) provide very similar performance as single-spot assays or as part of a multiplex.



	10-Plex	Single Spot
r^2 Value	0.98	0.98
Slope	0.96	1.04

Figure 4. Comparison of recovery of IL-2 samples using single-spot and 10-plex assays.

Accuracy and Precision

The accuracy and precision of each assay was assessed using calibrators and control samples. The back-fitted concentrations of all calibrator points within the quantifiable range of each assay yielded CVs below 20% and recoveries within 80% to 120% of expected concentration (data not shown). A minimum of 5 calibrator points are within the quantifiable range of each assay. The precision and accuracy statistics for controls are listed in

Table 3. Even though the specification for precision is a concentration CV of less than 25% on both intra- and inter-day runs, for this panel, the data shows that the CVs for most assays are below 10%. The reported concentration was dilution adjusted to the concentration of the controls upon initial reconstitution in 250 µL of Diluent 41.

Table 3. Precision and accuracy statistics for controls with dilution-adjusted concentrations reported in (pg/mL).

		K00D0072 (N=37)			K0080001 (N=9)			K0080002 (N=9)			Inter-lot	
Analyte	Control	Av. Conc.	Av. Intra-run %CV	Inter-run %CV	Av. Conc.	Av. Intra-run %CV	Inter-run %CV	Av. Conc.	Av. Intra-run %CV	Inter-run %CV	Av. Inter-lot Conc.	Inter-lot %CV
IFN-γ	A00C0160	737	6.0	13.3	776	4.3	8.2	707	2.8	5.7	740	4.7
	A00C0161	58.8	2.0	8.1	57.5	2.7	7.7	51.9	2.0	7.9	56.1	6.5
	A00C0162	5.37	2.5	12.3	5.10	2.9	8.5	4.63	1.7	7.1	5.03	7.4
IL-1β	A00C0160	1417	2.3	8.2	1450	3.6	8.0	1368	2.9	5.9	1412	2.9
	A00C0161	110	1.1	7.0	106	2.4	8.2	98.6	2.5	6.9	105	5.5
	A00C0162	10.0	1.6	11.2	9.31	2.7	9.2	8.62	2.6	11.1	9.31	7.4
IL-2	A00C0160	2519	1.6	8.6	2647	2.9	8.3	2346	3.0	4.5	2504	6.0
	A00C0161	200	1.6	7.9	193	2.9	8.7	168	3.3	9.1	187	9.0
	A00C0162	15.6	1.9	12.2	15.0	3.0	8.3	13.8	3.9	10.9	14.8	6.2
IL-4	A00C0160	737	2.5	7.3	697	2.8	7.8	669	2.3	4.2	701	4.9
	A00C0161	78.3	2.0	7.7	74.3	2.5	6.4	69.3	2.7	8.1	74.0	6.1
	A00C0162	11.7	2.5	11.1	10.7	3.1	10.9	9.6	3.6	8.4	10.7	9.8
IL-5	A00C0160	858	2.5	9.3	867	2.8	8.6	772	3.0	7.3	832	6.3
	A00C0161	57.2	1.9	9.4	54.0	2.6	8.3	48.5	2.3	10.0	53.2	8.3
	A00C0162	3.04	2.8	16.3	2.81	2.8	9.2	2.78	3.7	11.5	2.88	4.9
IL-6	A00C0160	4751	2.2	11.2	5165	2.3	9.5	5179	2.4	6.1	5032	4.8
	A00C0161	538	1.7	7.4	539	2.5	8.3	549	3.5	9.3	542	1.1
	A00C0162	65.2	1.6	11.3	59.4	3.0	7.9	59.8	3.1	7.8	61.5	5.3
KC/GRO	A00C0160	1961	1.6	10.6	1909	2.5	10.9	1896	2.5	4.5	1922	1.8
	A00C0161	250	1.6	7.8	241	2.0	8.1	219	2.7	6.2	237	6.7
	A00C0162	28	3.0	10.5	25.5	2.5	9.7	23.1	2.6	5.8	25.5	9.6
IL-10	A00C0160	2897	3.4	8.9	2553	5.3	8.1	2741	3.5	4.5	2730	6.3
	A00C0161	661	2.4	8.6	573	6.4	7.6	624	2.9	9.7	619	7.1
	A00C0162	146	2.5	9.7	131	6.4	10.9	133	3.8	8.8	137	6.0
IL-12p70	A00C0160	31 936	2.2	12.6	33 910	2.1	10.4	32 536	2.4	6.8	32 794	3.1
	A00C0161	4282	1.6	12.1	4762	2.1	10.9	4439	1.8	10.7	4494	5.4
	A00C0162	683	1.7	10.4	641	2.3	9.6	604	2.8	10.9	643	6.2
TNF-α	A00C0160	364	1.8	9.6	369	3.1	6.8	406	3.5	5.0	380	6.0
	A00C0161	107	1.4	6.3	103	2.9	6.6	100	2.1	7.1	103	3.4
	A00C0162	30.4	1.8	9.4	29.3	2.8	8.6	25.7	3.0	10.3	28.5	8.6

Control Recovery

Dilution-adjusted control recoveries for each run (control lots A00C0160, A00C0161, and A00C0162) are plotted below (**Figure 5**) for 3 kit lots (kit lots K00D0072, K0080001, and K0080002). The specification for control recovery is a concentration within 75% to 125% of the average multi-lot concentration (dashed lines). We recommend that each laboratory establish its own mean values.

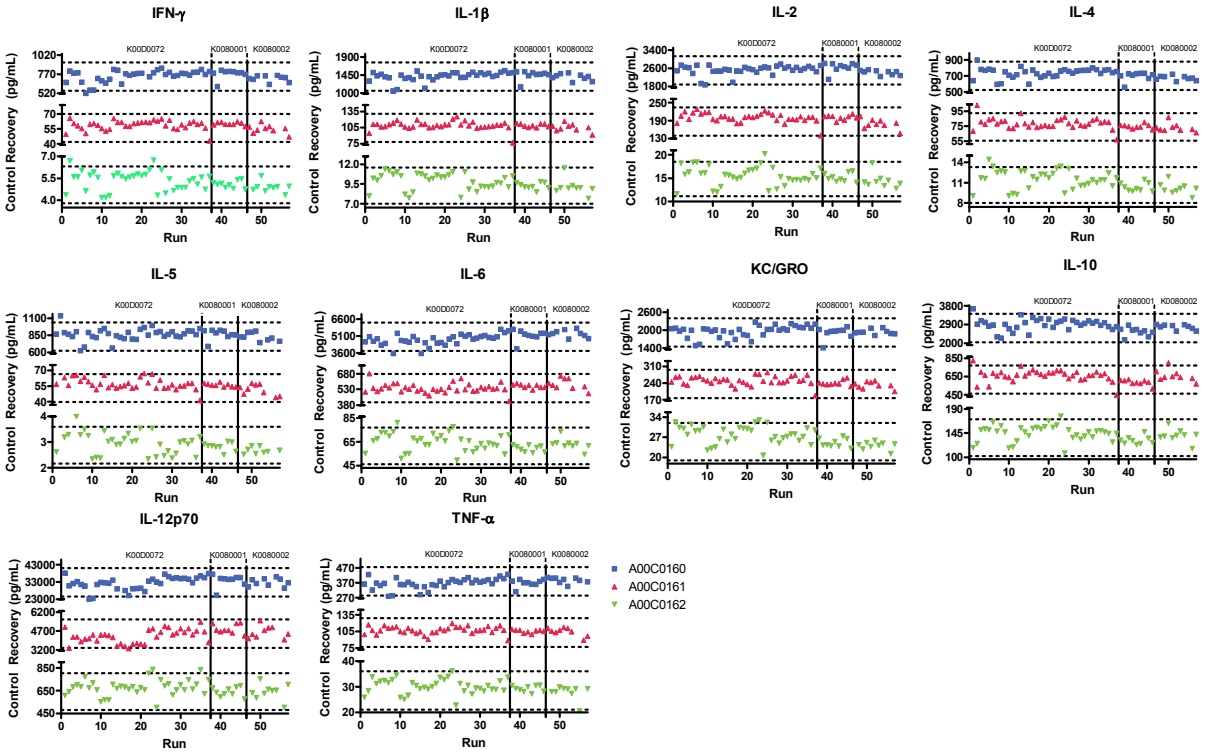


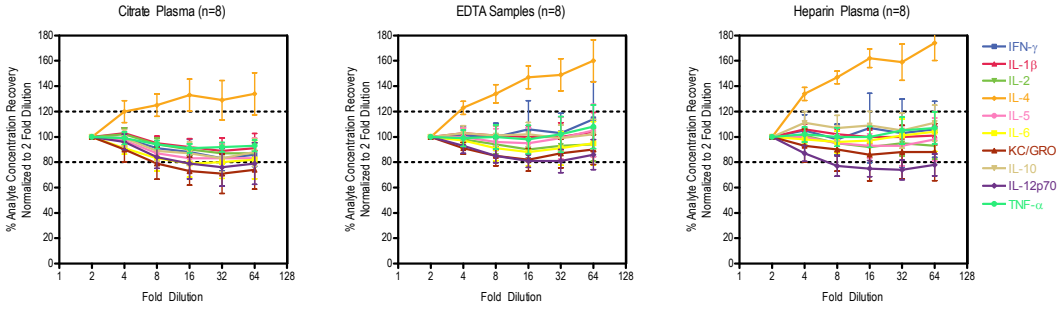
Figure 5. Control recovery graphs for low (green), mid (red), and high (blue) controls.

Dilution Linearity

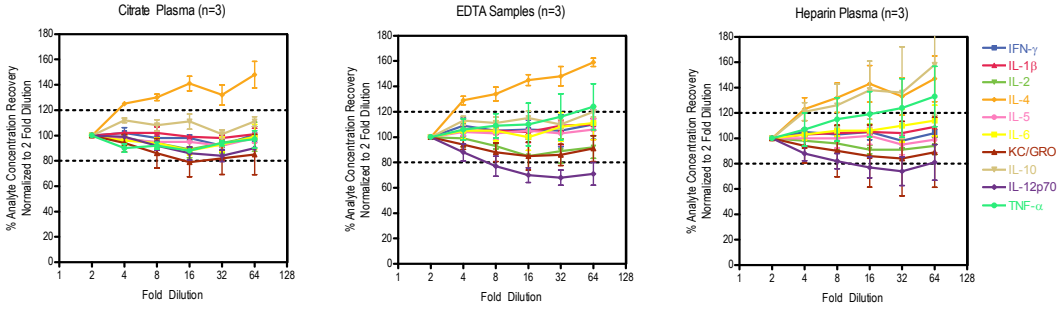
To evaluate matrix tolerance, dilution linearity was assessed by spiking recombinant protein into normal mouse serum, EDTA plasma, heparin plasma, citrate plasma, and urine and serially diluting the spiked samples. Different sets of samples were used for each of the 3 kit lots. Average recovery and standard deviations (error bars) for each assay and sample type for all 3 lots are depicted in **Figure 6**. The targeted recovery for all assays was between 80% and 120%; however, it is challenging to meet this specification for all assays since certain analytes may inherently display some matrix effects. Most assays show excellent dilution linearity in different sample types and overall high reproducibility across kit lots. IL-4 may benefit from further sample dilution, such as 8-fold or 16 fold, in serum, plasma, and urine. KC/GRO will improve with 8-fold sample dilution in urine and cell culture supernatants. The greater dilution factor may reduce the ability to detect normal levels in serum, plasma, and urine samples, but it could be viable for stimulated samples.

The error bars shown in the graphs below (**Figure 6**) represent the variability between the individual samples. We see that matrix effects are dependent on the individual samples. Some samples showed no interference. Urine samples in particular were more variable for some assays.

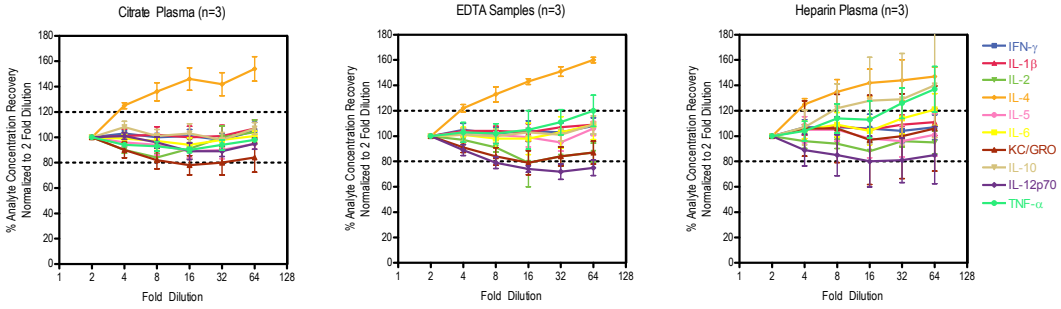
K00D0072



K0080001



K0080002



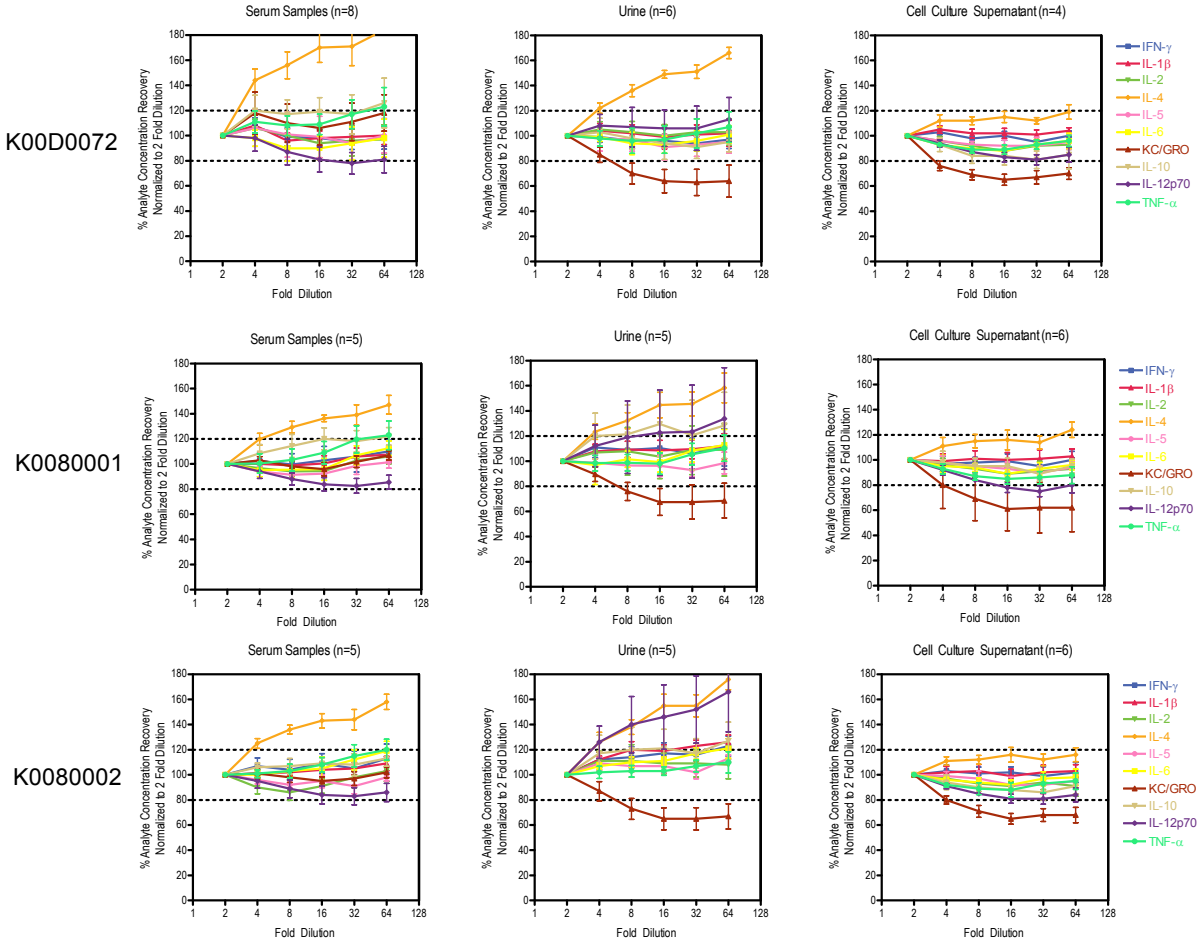


Figure 6. Dilution linearity studies in 6 matrices over 3 kit lots.

Spike Recovery

Samples were spiked with calibrators at 3 different levels spanning the quantifiable range of the assay. The average recovery and the standard deviations are plotted below for multiple sample types. **Figure 7** depicts the accuracy of spiked samples in 6 validated matrices from kit lots K00D0072, K0080001, and K0080002. Independent sets of samples were used for each lot. Overall, the assays show excellent spike recovery. As expected based on dilution linearity, IL-4 tended to under-recover in serum, plasma, and urine. KC/GRO shows over-recovery in urine and cell culture media.

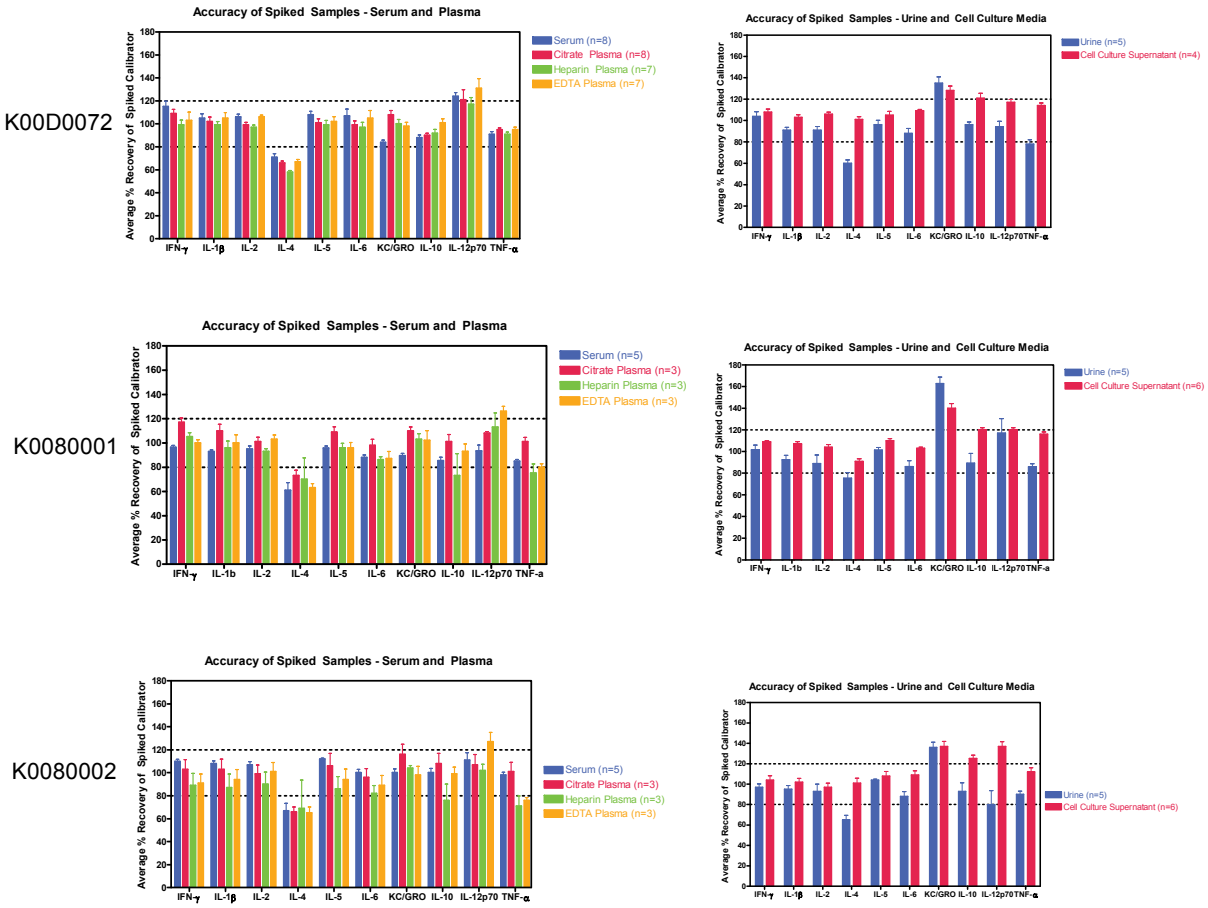


Figure 7. Recovery of spiked samples in 6 matrices over 3 kit lots.

Validation Samples

To ensure reproducibility of sample recovery across lots, 5 serum, 5 EDTA plasma, 5 heparin plasma, 5 citrate plasma, and 6 stimulated plasma samples plus an unstimulated plasma control were tested across 3 lots. Correlation is reported in **Figure 8** along with summaries of fit and slope in **Table 4**. IL-4 and IL-12p70 did not yield enough samples in the quantifiable range to evaluate the correlation for these assays. All other assays yielded excellent correlation.

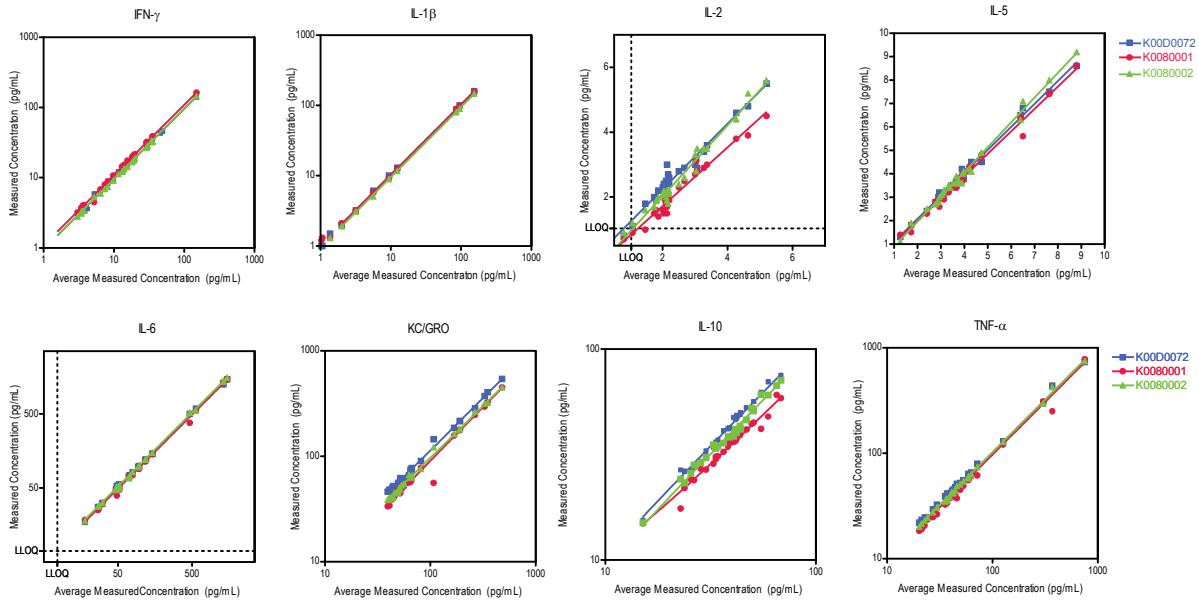


Figure 8. Recovery of spiked samples in 6 matrices over 3 kit lots.

Table 4: Slope and r-squared values for correlation graphs

Lot	Statistic	IFN-γ	IL-1β	IL-2	IL-4	IL-5	IL-6	KC/GRO	IL-10	IL-12p70	TNF-α
K00D0072	r^2 Value	1.00	1.00	0.97	N/A	0.99	1.00	1.00	0.99	N/A	0.99
	Slope	0.95	1.03	1.01	N/A	0.98	0.97	1.13	1.13	N/A	1.00
K0080001	r^2 Value	1.00	1.00	0.97	N/A	0.98	1.00	1.00	0.97	N/A	0.98
	Slope	1.09	1.02	0.9	N/A	0.96	0.98	0.93	0.83	N/A	0.98
K0080002	r^2 Value	1.00	1.00	0.98	N/A	0.99	1.00	1.00	0.99	N/A	1.00
	Slope	0.96	0.95	1.09	N/A	1.06	1.04	0.94	1.04	N/A	1.02

Normal Samples

Normal mouse serum, EDTA plasma, heparin plasma, citrate plasma, and urine samples from a commercial source were diluted 2-fold and tested. Representative data from lot K00D0072 are displayed in **Table 5**. Concentrations are corrected for sample dilution.

Table 5: Results from measurements of normal samples

Sample	Statistic	IFN-γ	IL-1β	IL-2	IL-4	IL-5	IL-6	KC/GRO	IL-10	IL-12p70	TNF-α
Serum (N=16)	Median (pg/mL)	0.95	2.27	1.02	0.43	2.72	21.6	48.3	11.0	81.0	12.0
	Range (pg/mL)	0.34–28.7	1.13–3.95	0.55–3.98	0.23–1.10	0.58–6.52	5.28–111	28.7–102	5.71–45.4	64.8–97.1	8.23–34.4
	% Detected	100	100	100	94	100	100	100	100	13	100
EDTA Plasma (N=15)	Median (pg/mL)	41.2	0.86	3.86	0.63	2.59	117	70.5	56.5	69.3	38.5
	Range (pg/mL)	18.6–262	0.46–2.40	2.60–5.89	0.48–0.70	1.50–2.88	11.0–185	54.2–96.9	31.5–74.7	50.2–171	21.3–47.0
	% Detected	100	87	100	60	100	100	100	100	73	100
Heparin Plasma (N=15)	Median (pg/mL)	262	1.62	4.63	0.75	4.01	175	269	76.4	85.6	65.3
	Range (pg/mL)	156–352	0.61–2.25	3.35–7.36	0.42–1.49	2.26–5.72	28.8–355	220–369	63.7–105	38.0–152	35.0–76.7
	% Detected	100	87	100	60	100	100	100	100	53	100
Citrate Plasma (N=16)	Median (pg/mL)	7.04	1.01	3.09	0.73	3.37	41.9	65.3	30.7	71.2	42.8
	Range (pg/mL)	0.31–122	0.45–2.02	0.65–5.03	0.39–1.47	1.72–8.24	6.84–74.2	34.9–172	5.30–68.2	50.4–107	5.45–58.8
	% Detected	100	100	94	100	100	100	100	100	94	100
Urine (N=10)	Median (pg/mL)	0.32	0.57	0.50	0.95	ND	ND	2.31	1.36	102	0.63
	Range (pg/mL)	0.09–0.66	0.35–1.34	0.49–0.65	0.33–1.31	ND	ND	1.91–2.84	0.98–1.53	67.3–125	0.48–3.90
	% Detected	70	60	30	90	0	0	100	40	90	80

ND = Non-detectable

The samples above, along with other normal samples used in the validation studies, are plotted in **Figure 9** to illustrate the measured sample concentrations in relation to the LLOD and quantifiable range (LLOQ to ULOQ). Concentration, LLOD, LLOQ, and ULOQ values are dilution adjusted. Only detectable analytes are depicted. Most assays detected analytes from a significant number of samples in all sample types. None of the samples yielded IL-4 or IL-12p70 in the quantifiable range.

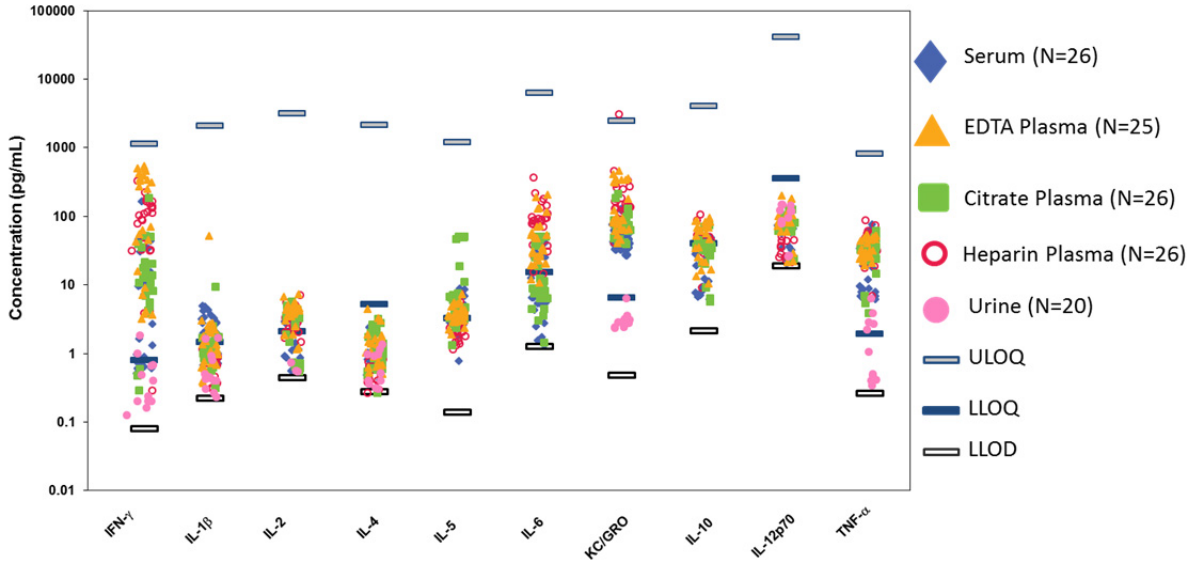


Figure 9. Sample recoveries and their relation to the LLOD and quantifiable range.

Stimulated Samples

Freshly collected, normal, pooled, mouse whole blood was incubated at 37°C for different time periods, either with LPS or with PG and ZY; plasma was isolated at the end of incubations. The dilution-adjusted concentrations (pg/mL) for each stimulation model are displayed in **Figure 10**.

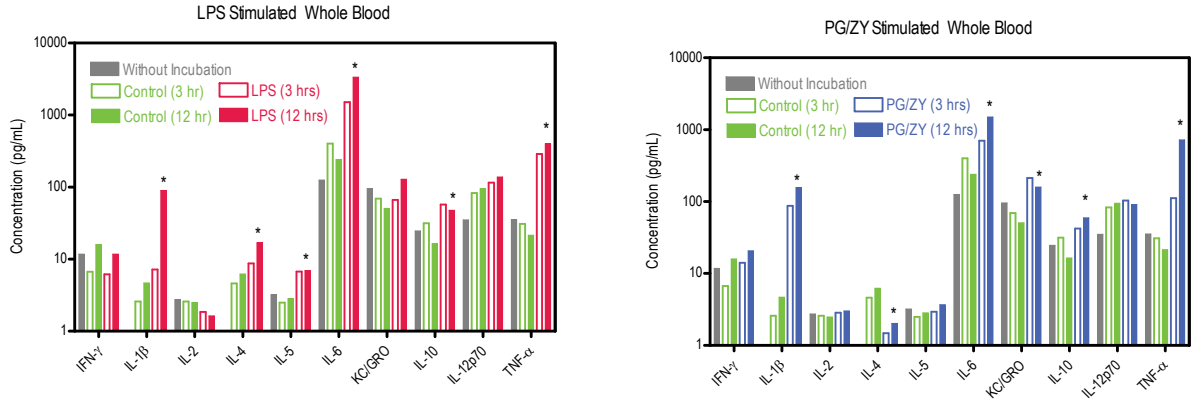


Figure 10. Assay results showing dilution-adjusted concentrations (pg/mL) for blood samples stimulated with LPS or with PG and ZY. Assays that showed a significant difference in analyte level with prolonged stimulation are identified with an asterisk.

A mouse monocyte macrophage cell line (J774A.1) was stimulated for 4 hours with LPS or pokeweed mitogen (PWM). The lysate was collected and tested and concentrations were normalized for 50 µg of lysate per well. Analyte levels for IFN-γ, IL-4, IL-5, and IL-12p70 were non-detectable (**Figure 11**).

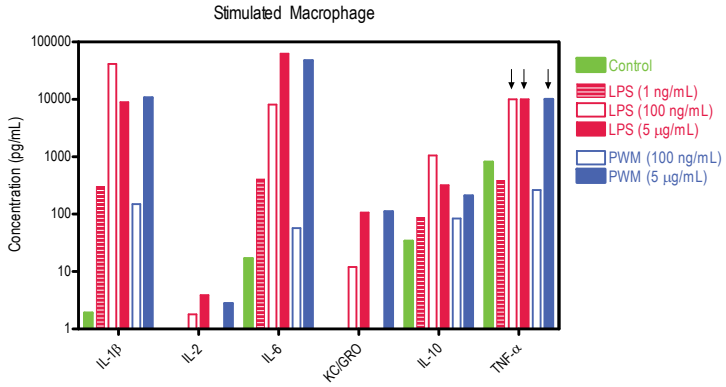


Figure 11. Assay results showing dilution-adjusted concentrations (pg/mL) for a macrophage cell line stimulated with LPS or PWM. Measurements above saturation levels are identified with an arrow.

Specificity

To assess specificity, each assay in the panel was tested individually for each kit lot. Non-specific binding measured using individual detection antibodies was less than 0.1% for all assays in the kit. Nonspecific binding was also evaluated with additional recombinant mouse analytes (IL-13, IL-17, GM-CSF, MCP-1, MIP-3α, RANTES, TNF-RI, TNF-RII, and VEGF). Nonspecific binding from the additional mouse analytes was less than 0.2% for all assays in the kit. This level of non-specificity was verified by comparing with results from 1) running each assay with individual calibrator and individual detection antibody on the 10-spot plate and 2) running the assays with individual calibrators and blended detection antibodies. All non-specific binding was below 0.8 %.

Interferences

To evaluate the TNF-α assay for interference by its receptors, 18 pg/mL of TNF-α was spiked into assay diluent and normal mouse serum with 3 different TNF-RI or TNF-RII concentrations. TNF-α concentration was measured and compared to the concentration measured in the absence of TNF-RI or TNF-RII (represented as % recovered). Results in **Table 6** indicate minimal interference.

Table 6. Percent recovery of TNF-α in the presence of its receptors

	pg/mL	Diluent (% recovered)	Serum (% recovered)
TNF-RI	0	100	100
	3000	102	108
	5000	109	111
	10 000	108	111
TNF-RII	0	100	100
	5000	110	107
	8000	113	107
	15 000	113	113

Stability

As part of V-PLEX development and validation, stability of kit components and assembled kits are evaluated. Initially, accelerated stability of components is assessed to establish shelf life. The accelerated stability of components is then supplemented with the ongoing real-time kit stability that is part of the MSD product development process. In addition to accelerated stability studies, freeze-thaw stability of frozen components is evaluated. For lyophilized components, stability after reconstitution at 2–8ºC as well as freeze-thaw tolerance of the reconstituted solution is evaluated.

Freeze–Thaw Stability

Diluent, reconstituted calibrator and reconstituted controls were tested for freeze–thaw stability. During freeze–thaw cycles, components were held at ≤-70ºC for 24 hours. The frozen components were thawed on ice. Results shown in **Figure 12** demonstrate that Diluent 41, Diluent 45, reconstituted calibrator, and reconstituted controls, can go through 5 freeze–thaw cycles without significantly affecting the performance of the assay. Data were normalized to signal or concentration from a no freeze–thaw condition. Calibrator and control performance was evaluated as part of reconstituted diluent stability studies. Frozen diluents were put through up to six freeze–thaw cycles with no discernible impact on calibrator or control performance. Reported control recoveries are the average of 3 levels (high, medium and low concentrations). Control values were evaluated against known control concentrations, while calibrators were evaluated against signal.

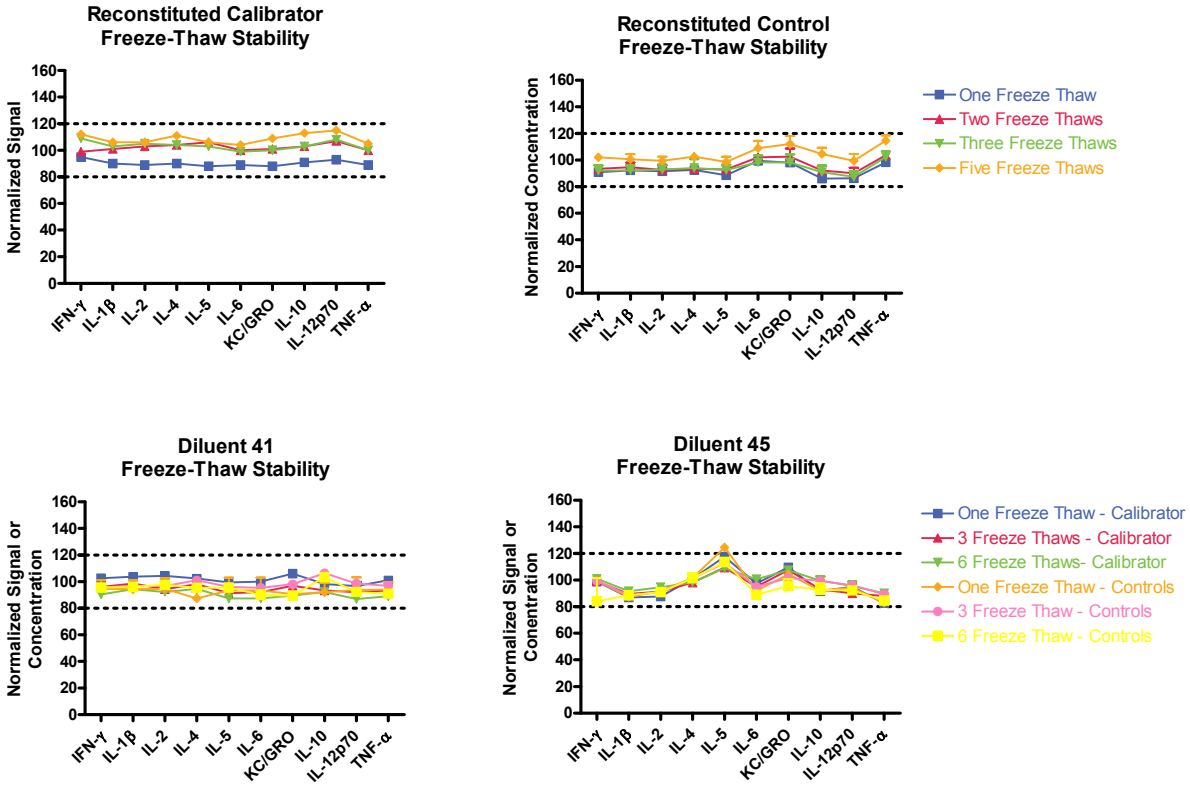


Figure 12. Results of freeze-thaw stability testing on calibrators, controls, and diluents.

Normal and stimulated plasma samples were also evaluated for freeze–thaw stability (data not shown). Samples were stable through 3 freeze–thaw cycles.

Stability of Reconstituted Lyophilized Components

Both lyophilized calibrators and controls were reconstituted in assay buffer and stored at 2–8ºC for stability evaluation. Once reconstituted, calibrator and controls are stable at 2–8°C for 33 days and 11 days respectively (**Figure 13**). Reconstituted calibrators and controls may be stored at ≤-70ºC for greater stability.

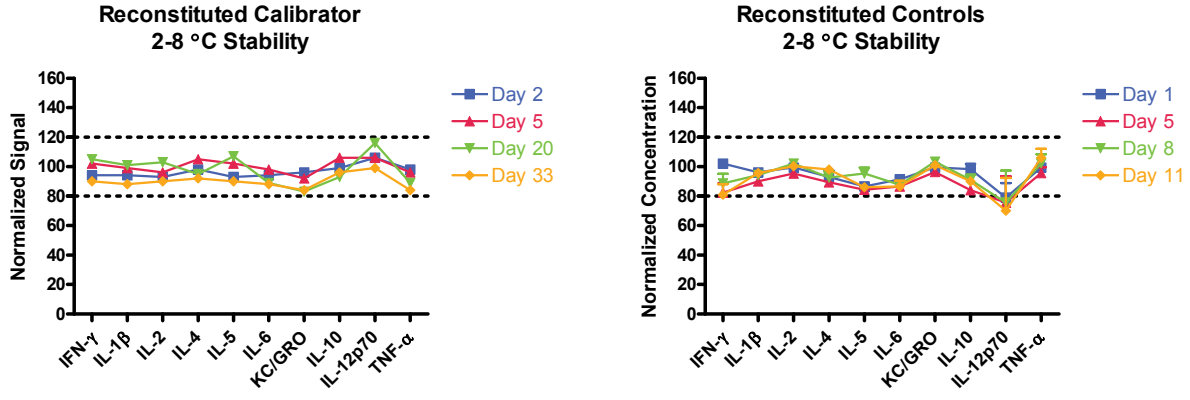


Figure 13. Stability of reconstituted calibrators and controls.

Accelerated and Real-Time Stability of Lyophilized Components

Lyophilized calibrator and controls were subjected to long-term isochronous studies to evaluate stability under 2 storage conditions, room temperature and 2–8°C. Representative data for lyophilized calibrator is shown in **Figure 14**. For the control condition, lyophilized calibrators were stored at ≤-10ºC; selected vials were moved to different storage conditions (room temperature or 2–8°C) at different time points. Stability for all vials was evaluated simultaneously after 6 months. All calibrators were reconstituted on the day of testing. The data shown in **Figure 14** was normalized to lyophilized calibrators stored at ≤-10ºC. Lyophilized calibrators are stable at room temperature for more than 6 months. Using the Q_{10} method for shelf life estimation, six months at 25 ºC equates to 5 years at 4 ºC.

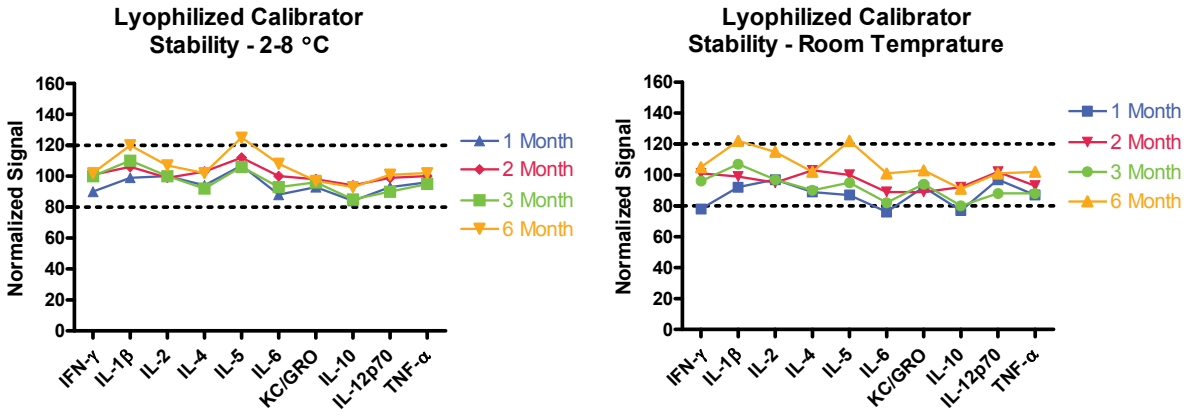


Figure 14. Representative data for stability studies on lyophilized calibrator.

A similar study was performed with lyophilized controls (data not shown). As with the calibrators, controls were stable at room temperature for 6 months.

Calibration

All the assays in the panel are calibrated against a reference calibrator generated at MSD.

During product development, the NIBSC/WHO standards for the following analytes were evaluated against the MSD reference calibrators. Upon evaluation, IL-6 and TNF-α reference calibrators differed from the NIBSC/WHO standards. As a result, these internal references were anchored to NIBSC/WHO standards. To convert sample values obtained with the Proinflammatory Panel 1 (mouse) to an approximate NIBSC/WHO concentration, multiply the calculated concentration results by the concentration ratio in **Table 7**.

Table 7. Ratio to convert MSD concentrations to an equivalent NIBSC/WHO concentration

Analyte	NIBSC/WHO Catalog Number	Concentration Ratio (MSD Reference: NIBSC)
IL-1β	96/668	1.18
IL-2	93/566	0.98
IL-4	91/656	0.89
IL-6	93/730	1.0
TNF-α	88/532	1.0

Instrument Comparison

Proinflammatory Panel 1 (mouse) kits were tested with different models of MSD instruments. All 3 instruments showed identical performance. Calibration curves are compared in **Appendix D**.

Comparison to Current MSD Assays

Proinflammatory Panel 1 (mouse) kits were evaluated against Mouse ProInflammatory 7-plex and Mouse TH1/TH2 9-plex kits. See results in **Table 8** and **Figure 15**. Overall, the Proinflammatory Panel 1 (mouse) kits yield better sensitivity and lower background compared to the other panels.

Table 8. Comparison of LLOD (pg/mL) between other MSD cytokine panels and the **Proinflammatory Panel 1 (mouse)**

MSD Cytokine Panels	IFN-γ	IL-1β	IL-2	IL-4	IL-5	IL-6	KC/GRO	IL-10	IL-12p70	TNF-α
Proinflammatory Panel 1 (mouse)	0.04	0.11	0.22	0.14	0.07	0.61	0.24	0.95	9.95	0.13
Mouse ProInflammatory 7-Plex	0.38	0.75	-	-	-	4.50	3.30	11.0	35.0	0.85
Mouse TH1/TH2 9-Plex	0.47	2.10	3.00	0.87	0.70	-	2.90	11.0	-	1.00

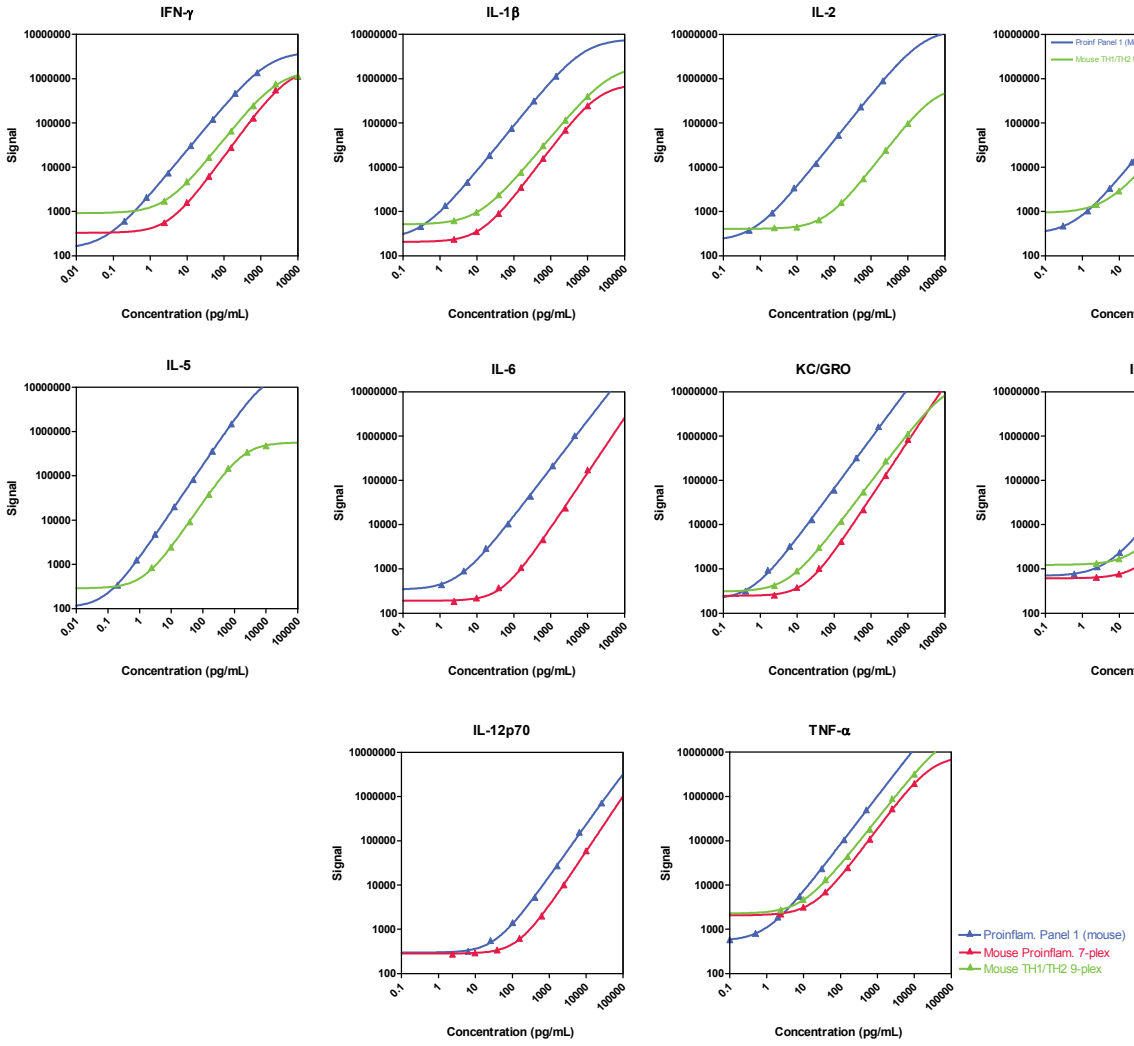


Figure 15. Performance comparison of assays in Proinflammatory Panel 1 with corresponding assays from the Mouse ProInflammatory 7-Plex and the Mouse TH1/T2 9-Plex.

To evaluate correlation between kits, 6 stimulated plasma samples, a pooled plasma sample, and 3 levels of Proinflammatory Panel 1 (mouse) controls were run on all 3 kits.

Correlations between the V-PLEX assays and corresponding assays in the other 2 panels were high (r^2 >0.8) (**Table 9**). The differences in the slope between the panels may be attributable to different diluents, antibody pairs, and/or anchoring to NIBSC/WHO standard.

Table 9. Correlation with Proinflammatory Panel 1 (mouse) V-PLEX Panel

Panel	Statistic	IFN-γ	IL-1β	IL-2	IL-4	IL-5	IL-6	KC/GRO	IL-10	IL-12p70	TNF-α
ProInflammatory 7-Plex	r^2	0.97	0.99	-	-	-	0.99	0.99	0.99	0.99	0.89
	slope	2.27	1.22	-	-	-	20.7	2.55	0.99	1.61	1.42
TH1/TH2 9-Plex	r^2	0.98	1.00	1.00	1.00	1.00	-	0.94	1.00	-	0.78
	slope	2.03	1.13	1.06	1.58	1.18	-	2.35	1.05	-	3.17

CONCLUSION

Performance consistency is the major challenge associated with commercial RUO immunoassay kits—especially multiplexed kits. MSD is addressing the need for higher quality kits through a new V-PLEX product portfolio that provides highly reproducible results. V-PLEX kits were developed using well-characterized and purified reagents and highly optimized assays. They were validated according to fit-for-purpose guidelines[6] and FDA guidance documents.[7] The Proinflammatory Panel 1 (mouse) Kit, consisting of assays for IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, and TNF-α, was validated for use with serum, EDTA plasma, heparin plasma, citrate plasma, urine, and cell culture supernatants. Three validation lots were generated (from independently produced raw material lots when possible) using established manufacturing processes. Analytical validation was performed for each kit lot, measuring sensitivity, accuracy, precision, specificity, dilution linearity, spike recovery, and recovery of samples. The development and validation data presented in this report demonstrate that the Proinflammatory Panel 1 (mouse) kit is a well-characterized product capable of generating highly reproducible results over multiple lots. The assays exhibit high sensitivity, specificity, reproducibility, and robustness. Multiplexing has minimal to no impact on performance. The kit components are stable, and the product correlates with current MSD kits. These high quality, reliable kits are excellent tools for validated studies, clinical trials, and long-term research programs.

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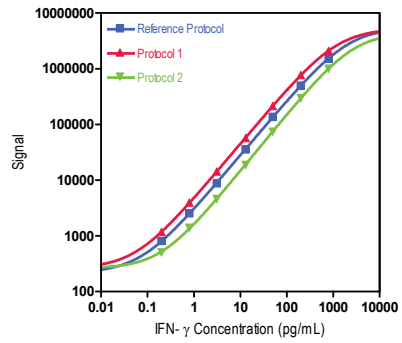
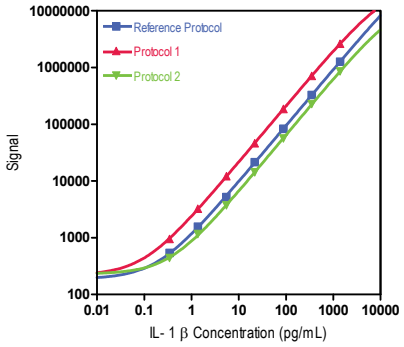
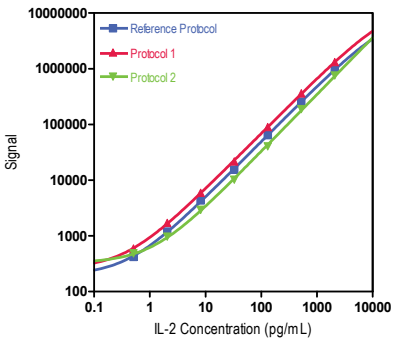
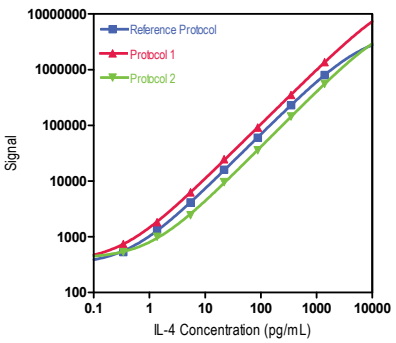
Appendix A

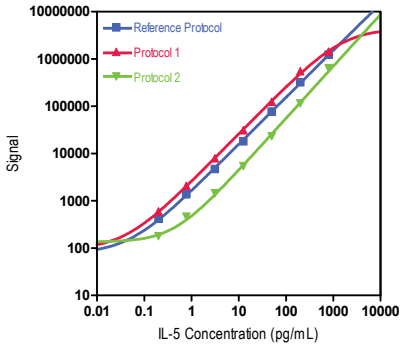
The calibration curves and LLOD comparison table below illustrate the relative sensitivity of each assay under the Reference Protocol (2-hour sample incubation/2 wash steps, blue curve), Alternate Protocol 1 (overnight sample incubation, red curve), and Alternate Protocol 2 (tissue culture: single wash, green curve).

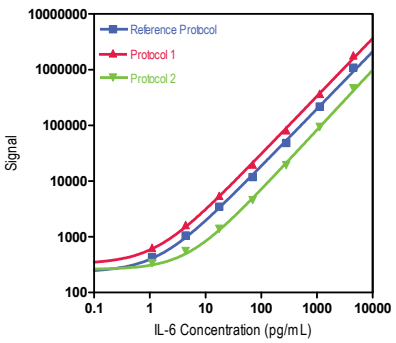


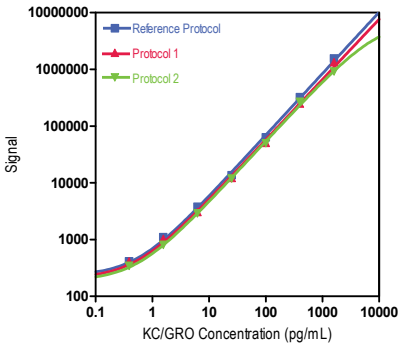


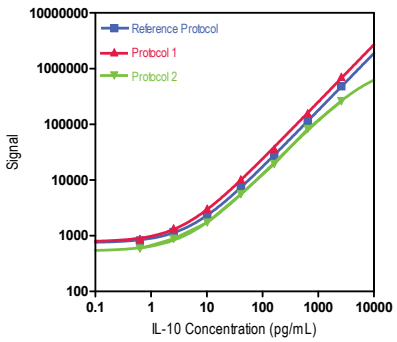


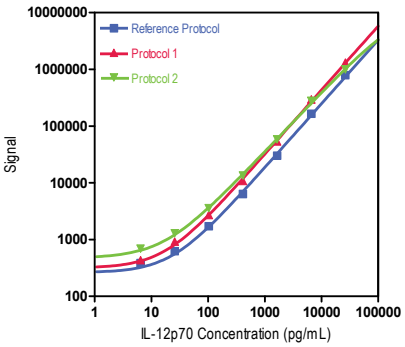


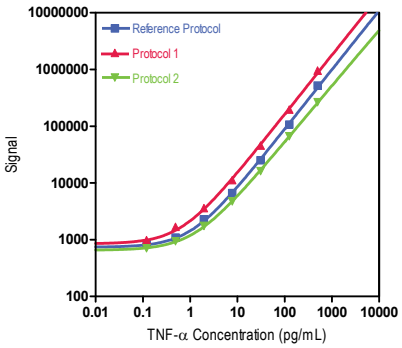








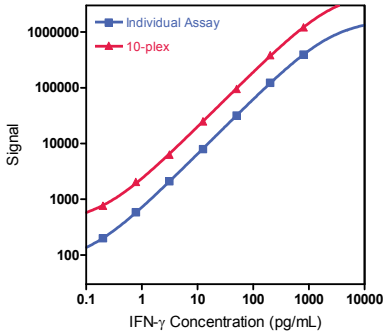


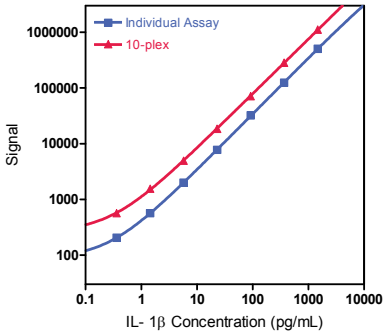


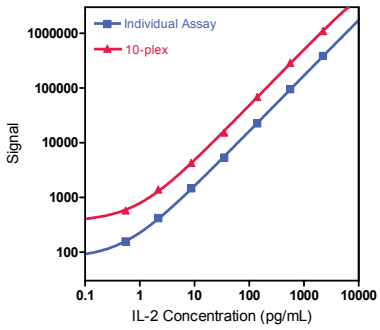
	LLOD (pg/mL)		
	Reference Protocol	Protocol 1	Protocol 2
IFN-γ	0.04	0.02	0.07
IL-1β	0.11	0.05	0.12
IL-2	0.22	0.11	0.35
IL-4	0.14	0.08	0.26
IL-5	0.07	0.03	0.25
IL-6	0.61	0.62	2.88
KC/GRO	0.24	0.29	0.51
IL-10	0.95	0.39	0.64
IL-12p70	9.95	4.83	2.92
TNF-α	0.13	0.13	0.40

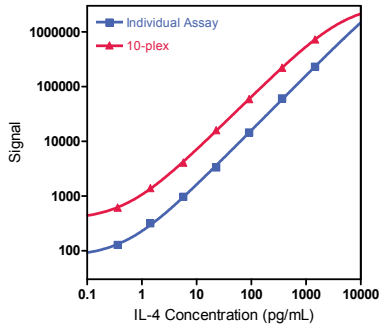
Appendix B

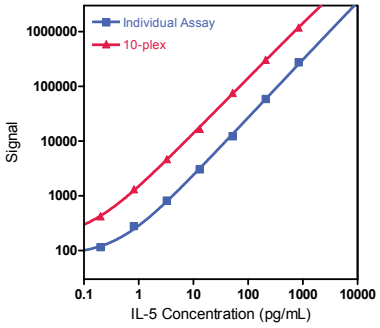
The calibration curves and LLOD table below compare results from assays run on a single-spot plate (blue) vs. the 10 spot plate (red). In both cases, only the single, assay-specific detection antibody is used. Results are highly correlated, with 10-plex plates generating slightly higher signals due to smaller spot size. (The reported signal is a measure of signal per area [density]; therefore, smaller spots generate a higher signal.)

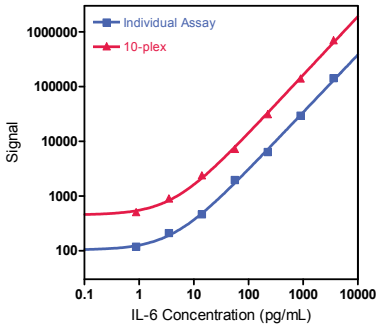


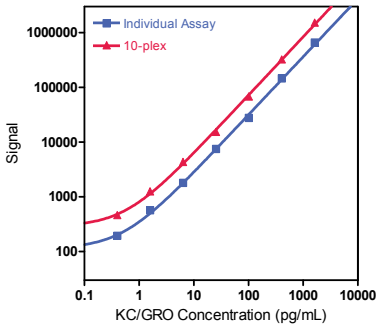


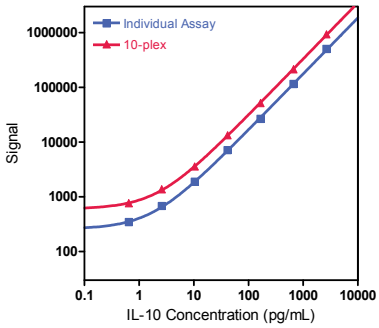


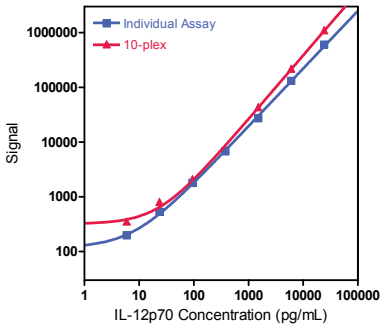


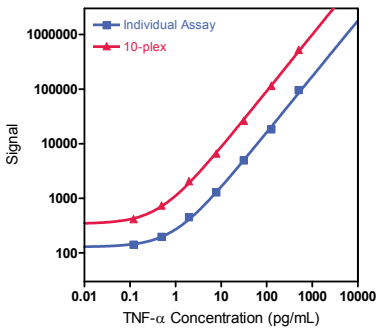








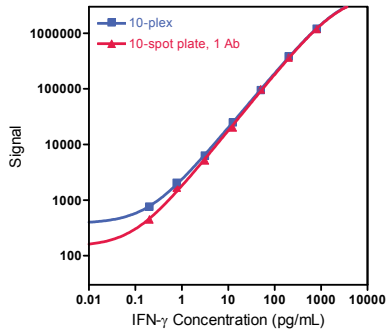


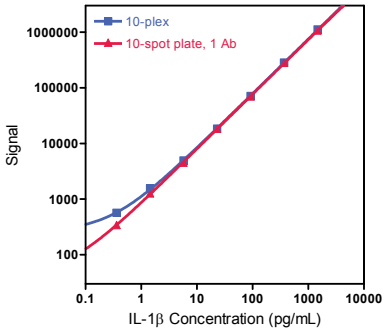


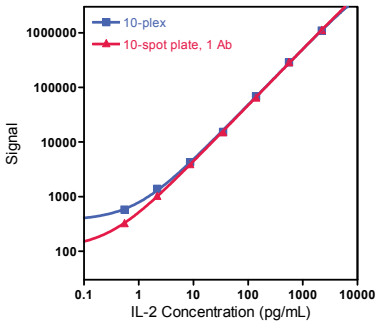
	LLOD (pg/mL)	
Assay	**Individual**	**10-plex**
IFN-γ	0.05	0.04
IL-1β	0.09	0.11
IL-2	0.29	0.22
IL-4	0.21	0.14
IL-5	0.15	0.07
IL-6	1.24	0.61
KC/GRO	0.22	0.24
IL-10	0.26	0.95
IL-12p70	3.10	9.95
TNF-α	0.18	0.13

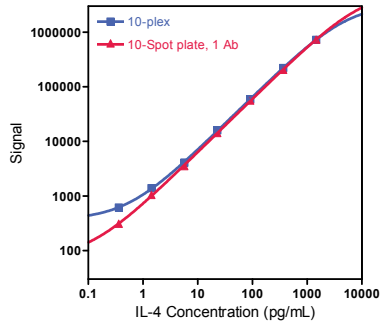
Appendix C

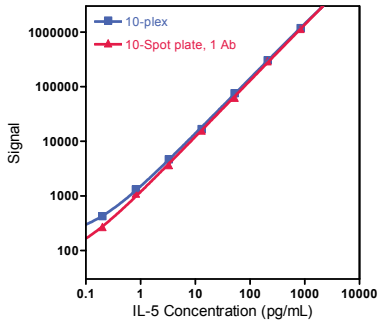
The calibration curves and LLOD table below compare results for each assay in the 10-plex panel when the plate is run as a multiplex using all 10 detection antibodies (blue) vs. running each assay using a single, assay-specific detection antibody (red). As expected, both multiplex formats yielded the same specific signal, but lower background signals were seen when using the single detection antibody.

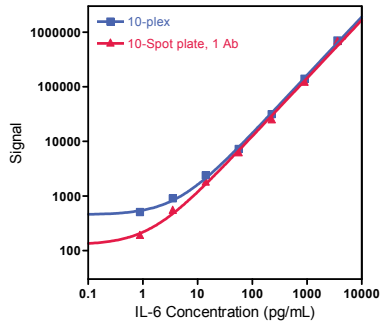


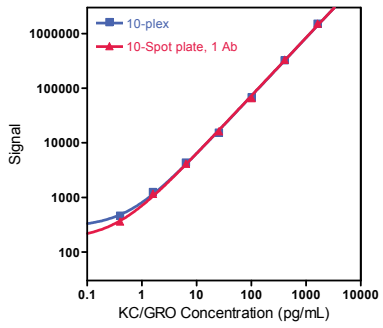


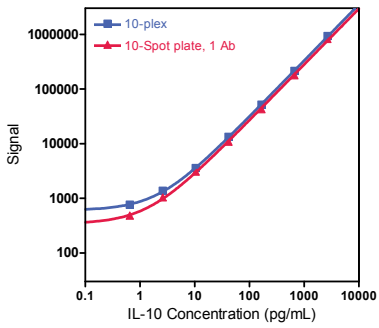


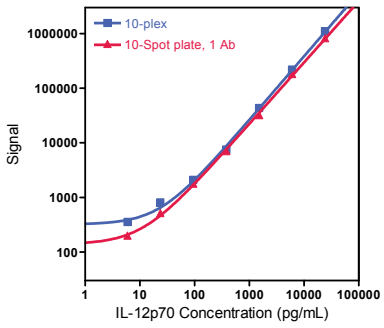


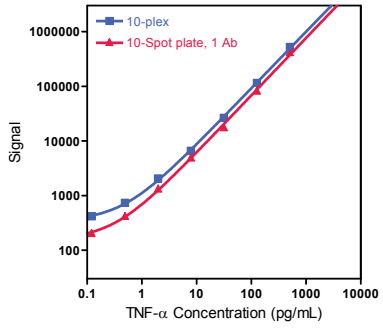












	LLOD (pg/mL)	
Assay	**10-spot plate, 1 Ab**	**10-plex**
IFN-γ	0.04	0.04
IL-1β	0.10	0.11
IL-2	0.28	0.22
IL-4	0.11	0.14
IL-5	0.05	0.07
IL-6	0.73	0.61
KC/GRO	0.13	0.24
IL-10	0.30	0.95
IL-12p70	5.23	9.95
TNF-α	0.14	0.13

Appendix D

The calibration curves below demonstrate highly correlated assay performance between the different MSD instruments. First group: SECTOR® Imager 6000 (blue) vs. SECTOR Imager 2400 (red). Second group: SECTOR Imager 6000 (blue) vs. QuickPlex™ SQ 120 (green).