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Discovery of Alzheimer's Disease Biomarkers Associated with Inflammation Using a Novel, Validated, Cytokine Screening Panel

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1 Abstract

Background: Neuroinflammation plays a key role in Alzheimer's disease (AD) pathogenesis. Recent evidence suggests that multi-target therapeutic agents that modulate discrete inflammatory pathways may provide greater efficacy than single-target therapies. We have developed and validated a multiplexed panel of 30 inflammation-related assays using the MSD® platform that can be used to study candidate biomarkers of AD. This panel allows simultaneous detection of cytokines and chemokines with high precision and accuracy in human matrices.

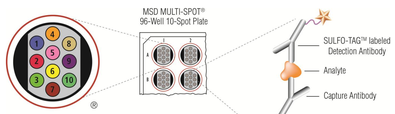
Methods: The screening panel was developed using highly characterized, qualified reagents according to industry guidelines. The panel can measure IFNγ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNFα, IL-5, IL-7, GM-CSF, IL-1α, IL-17A, IL-15, IL-16, IL-12/IL-23 p40, TNFβ, VEGF, MIP-1α, MIP-1β, Eotaxin, Eotaxin-3, IP-10, TARC, IL-8 (high abundance), MDC, MCP-1, and MCP-4 in multiple human sample types. To address problems with cytokine assays offered by other vendors, the MSD panel was analytically validated, and consistency in performance across multiple assay lots was confirmed. Assay protocol and diluents were optimized to maximize robustness. Controls were developed and used to assess accuracy and precision.

Results: The panel quantifies inflammatory biomarkers in multiple sample types with superior sensitivity and performance, quantifying many low abundance analytes at levels below 0.5 pg/mL. It demonstrates suitable spike recovery and dilution linearity performance in different biological matrices and across lots. Each assay in the panel is well characterized and shows low non-specific binding. Each assay has been evaluated for potential assay and analytical interference.

Conclusion: This MSD validated multiplexed panel is a suitable tool for accurate quantification of cytokines and chemokines. More than 80% of the panel assays are sensitive enough to detect these very low abundance biomarkers in normal biological matrices, including serum, plasma, urine, and CSF. We present a summary of the development and validation of this panel and results from measurements of AD patient samples.

2 Methods

The multiplex panel was developed as three 10-plex panels designed for optimal performance based on individual assay characteristics, native analyte levels, and suitable dilution, diluent, and assay compatibility. The Proinflammatory Panel 1 (human), Cytokine Panel 1 (human), and Chemokine Panel 1 (human) kits follow the same, simple 3-step protocol.



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.

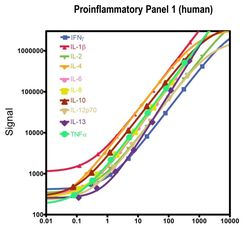
Development and Validation

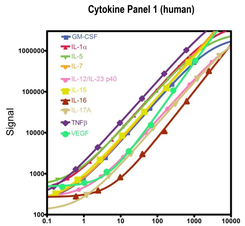
The assays presented here were developed and validated following fit-for-purpose principles and design control procedures. Accuracy, precision, and specificity were evaluated by multiple operators using independently built kit lots to test calibrators, controls, and matrix-based samples over multiple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

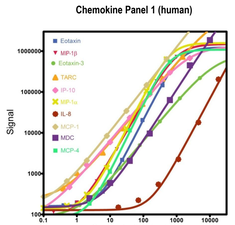
- **Sensitivity -** Limits of detection (LOD) were calculated based on assay signal and standard deviation of background signal. Limits of quantification (LOQ) were determined by spiking a known value of calibrator into diluent to assess the accuracy and precision of the samples across multiple lots.
- **Precision -** Precision is presented as the coefficient of variance (CV). Precision was determined for calibrators, controls, and LOQs on both intra- and inter-day runs.
- **Dilution Linearity and Spike Recovery -** Spike recovery and dilution linearity were assessed across multiple lots using a variety of biological matrices.
- **Specificity -** Assay specificity and interferences 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 below for each of the 30 assays. 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). Signals and sensitivity are from >30 runs collected over 2 months by 4 operators.

	LLOD Median (pg/ml)	LLOD Range (pg/ml)
IFNγ	0.19	0.05–0.62
IL-1β	0.04	0.05–0.16
IL-2	0.08	0.01-0.29
IL-4	0.01	0.01-0.05
IL-6	0.04	0.01–0.11
IL-8	0.04	0.00–0.13
IL-10	0.02	0.00–0.07
IL-12p70	0.10	0.00–0.51
IL-13	0.23	0.03–0.73
TNFα	0.04	0.01–0.13

	LLOD Median (pg/ml)	LLOD Range (pg/ml)
GM-CSF	0.13	0.10–0.47
IL-1α	0.08	0.05–2.4
IL-5	0.08	0.05–0.56
IL-7	0.15	0.11–0.57
IL-12/IL-23 p40	0.39	0.30–2.59
IL-15	0.11	0.08–0.24
IL-16	2.76	0.88–9.33
IL-17A	0.72	0.45–2.84
TNFβ	0.04	0.04–0.29
VEGF	1.09	0.55–6.06

	LLOD Median (pg/ml)	LLOD Range (pg/ml)
Eotaxin	2.73	0.21–4.80
MIP-1β	0.57	0.00–2.47
Eotaxin-3	0.69	0.03–3.27
TARC	0.14	0.03–0.79
IP-10	0.08	0.02–0.39
MIP-1α	1.83	0.41–4.51
IL-8	65.30	32.70–94.90
MCP-1	0.08	0.01–0.22
MDC	1.90	0.39–7.69
MCP-4	1.75	0.738–4.67

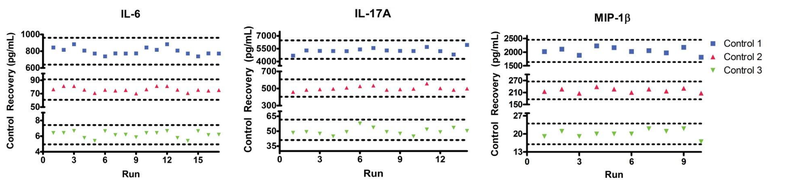
Protocol

1. Add 50 µL/well of calibrator, controls, or diluted samples; incubate 2 hours at room temperature (RT).
2. Wash; add 25 µL/well of detection antibody solution; incubate 2 hours at RT.
3. Wash; add 150 µL/well of Read Buffer T; read plate on MSD instrument.

5 Specificity and Reproducibility

Each assay in the 30-plex panel was evaluated for cross-reactivity with other analytes in the panel as well as related analytes that are not in the panel. The cross-reactivity for all assays was <1%.

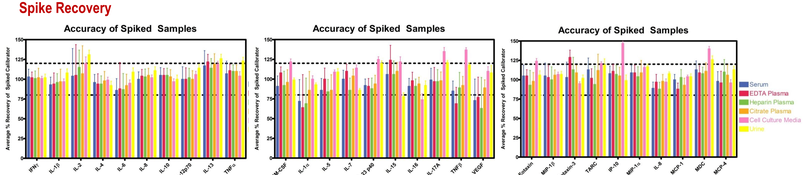
To evaluate the reproducibility of the panels, 3 levels of controls spanning the quantifiable range were prepared and measured over multiple runs for each panel. The measured concentrations for a representative assay from each of the 10-plex panels are shown below. The controls were prepared in a non-human matrix, and between 10 and 17 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 serum, heparin plasma, EDTA plasma, citrate plasma, urine, and cell culture media. Representative data are presented below showing overall good assay performance with almost all sample types for all assays accurately recovering expected concentrations of spiked analyte. (Dashed lines are at ±20% of expected value.) 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.

Spike Recovery



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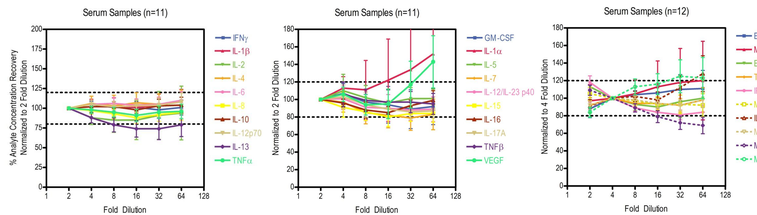
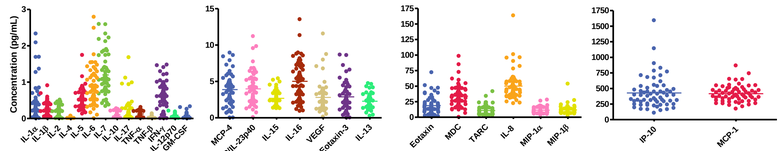
Dilution Linearity



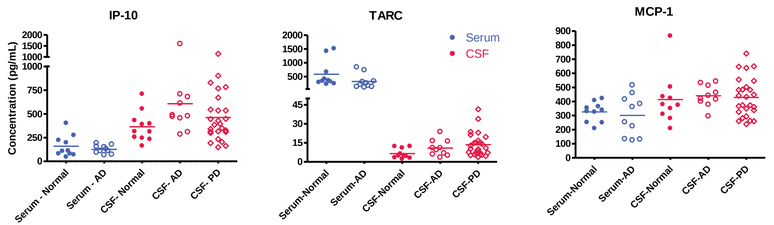
7 CSF Sample Testing

Well-curated individual human CSF samples (n=83) were evaluated for native analyte levels. The majority of samples had analyte levels above the limit of detection for 17 of the assays. Results for each assay are plotted below; analytes are grouped according to abundance.



8 Cytokines and Chemokines in Serum and CSF Samples

Cytokine and chemokine levels were measured in serum and CSF samples from normal control, Alzheimer's disease (AD), or Parkinson's disease (PD) patients. AD classification of samples was made prior to this study based on Aβ42 and tau biomarker profile. PD samples were classified based on UK Parkinson's Disease Society Brain Bank Clinical Diagnostic Criteria including standardized levodopa testing. A sampling of the results is presented below. Some analytes, such as IP-10, showed higher concentrations in CSF than serum. Other analytes were present at lower concentrations in CSF than serum; for example, TARC levels were ~50 fold lower in CSF than serum. MCP-1 and other analytes were present at similar concentrations in serum and CSF samples.



9 Conclusion

A 30-analyte panel has been developed and analytically validated for use in quantifying cytokines and chemokines in a variety of human matrices. It uses a simple 3-step protocol that requires just 75 µL of sample to measure all 30 analytes. The assays in the panel were run as 3 multiplex panels that were each optimized to achieve the best assay performance. Consistent results were demonstrated for calibration curves and control samples over multiple runs on different days by multiple operators. The excellent sensitivity, reproducibility, accuracy, and sample performance of this panel opens the door for investigation of the role of cytokines and chemokines in various disease states and in a variety of matrices, including CSF, a critical matrix in the study of neurodegenerative diseases.

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