# Discovering Cancer Biomarkers Using a Novel Cytokine Screening Panel

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## 1 Abstract

Cytokines and chemokines that are produced in the tumor micro-environment play major roles in tumorigenesis, tumor progression, and apoptosis. They are involved in the modulation of angiogenesis and consequently, in tumor growth and metastases. Cytokine/chemokine expression by tumor cells leads to tumor proliferation and enhances tumor cell survival. Profiling these biomarkers may be useful for cancer diagnosis. For example, biomarkers such as IFNg, TNFa, IL-1b, MCP-1, and IL-8 are overexpressed in breast carcinoma; IL-2, IL-4, IL-6, IL-8, IL-10, IL-16, and TARC are associated with prostate cancer; and increased expression levels of TNFa, IL-6, and IL-1b are implicated in colon cancer.

We have developed and validated multiplexed panels of 30 assays that allow users to simultaneously detect cytokines and chemokines with high precision and accuracy in different human matrices. These panels detect IFNg, IL-1b, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNFa, IL-5, IL-7, GM-CSF, IL-1a, IL-17A, IL-15, IL-16, IL-12/IL-23 p40, TNFb, VEGF, MIP-1a, MIP-1b, Eotaxin, Eotaxin-3, IP-10, TARC, IL-8 (high abundance), MDC, MCP-1, and MCP-4. These assays have good sensitivity and performance, quantifying many low abundance analytes at levels below 0.5 pg/mL.

A challenge with current multiplex assays is a lack of analytical validation and lot-to-lot reproducibility. These multiplex panels were developed with highly characterized and qualified reagents, and the assays were developed in accordance with industry guidelines. The panels demonstrated suitable spike recovery and dilution linearity performance in different biological matrices. Each assay in the panel was characterized and showed low non-specific binding with minimal assay and analytical interference. Quality control samples were produced and used to evaluate each panel.

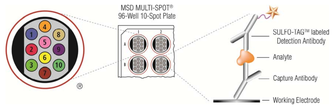
These panels are suitable tools for accurately quantitating cytokines and chemokines in various tumor samples. Data are presented for samples from normal subjects as well as samples from benign prostatic hyperplasia and prostate cancer patients (serum), cirrhosis and liver cancer patients (serum), kidney cancer patients (plasma), and colon cancer patients (serum).

## 2 Methods

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

### 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.




### Protocol

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

## 3 Sensitivity

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 over 2 months by 4 operators.

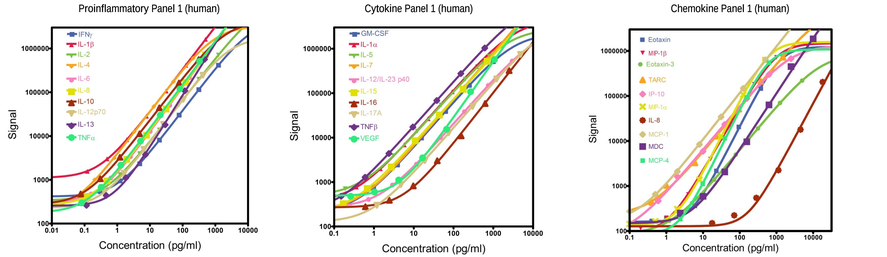
| | LLOD Median (pg/ml) | LLOD Range (pg/ml) |
|---|---|---|
| IFNγ | 0.193 | 0.045-0.615 |
| IL-1β | 0.043 | 0.053- 0.163 |
| IL-2 | 0.084 | 0.013-0.291 |
| IL-4 | 0.013 | 0.005-0.048 |
| IL-6 | 0.044 | 0.006-0.111 |
| IL-8 | 0.037 | 0.002-0.134 |
| IL-10 | 0.022 | 0.003-0.070 |
| IL-12p70 | 0.095 | 0.001-0.512 |
| IL-13 | 0.229 | 0.026-0.729 |
| TNFα | 0.039 | 0.006-0.129 |

| | LLOD Median (pg/ml) | LLOD Range (pg/ml) |
|---|---|---|
| GM-CSF | 0.134 | 0.104-0.470 |
| IL-1α | 0.076 | 0.051-2.40 |
| IL-5 | 0.084 | 0.054-0.559 |
| IL-7 | 0.147 | 0.107-0.567 |
| IL-12/IL-23 p40 | 0.394 | 0.299-2.59 |
| IL-15 | 0.107 | 0.077-0.238 |
| IL-16 | 2.76 | 0.875-9.33 |
| IL-17A | 0.723 | 0.449-2.84 |
| TNFβ | 0.043 | 0.036-0.293 |
| VEGF | 1.09 | 0.550-6.06 |

| | LLOD Median (pg/ml) | LLOD Range (pg/ml) |
|---|---|---|
| Eotaxin | 2.73 | 0.210 – 4.80 |
| MIP-1β | 0.574 | 0.089-2.47 |
| Eotaxin-3 | 0.685 | 0.027-3.27 |
| TARC | 0.139 | 0.029-0.792 |
| IP-10 | 0.084 | 0.024-0.385 |
| MIP-1α | 1.83 | 0.409-4.51 |
| IL-8 | 65.3 | 32.7-94.9 |
| MCP-1 | 0.083 | 0.006-0.216 |
| MDC | 1.90 | 0.386-7.69 |
| MCP-4 | 1.75 | 0.738-4.67 |

## 4 Calibration Curves

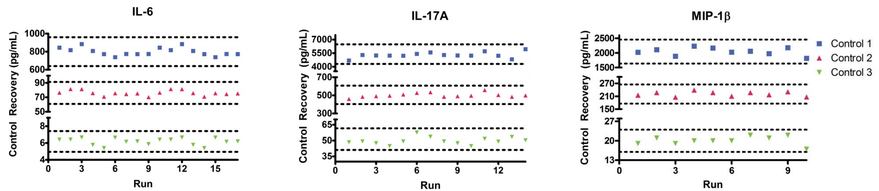
Representative calibration curves are shown below for each of the 30 assays. The curves were generated by taking the average of at least 26 runs conducted by 4 operators over a minimum of 8 days.




## 5 Specificity and Reproducibility

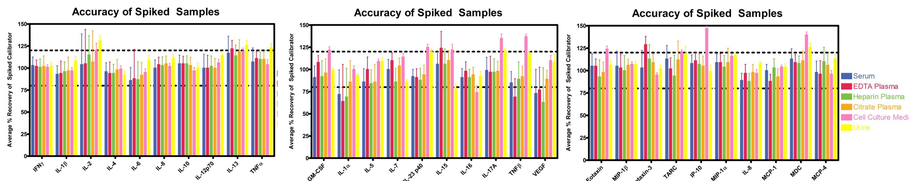
Each assay in the 30-plex panel was evaluated for cross-reactivity with other analytes on the panel as well as related analytes that are not on the panel. The cross-reactivity for all assays was <1%.

To evaluate reproducibility, 3 levels of controls spanning the quantifiable range of the panels were prepared and measured over multiple runs. The measured concentrations for a representative assay from each of the 10-plex panels are shown below. The controls were prepared in non-human matrix, and between 10 and 17 runs were conducted, depending on the panel. All controls tested were measured within the target range of +/- 20% of assigned concentration (dashed lines).



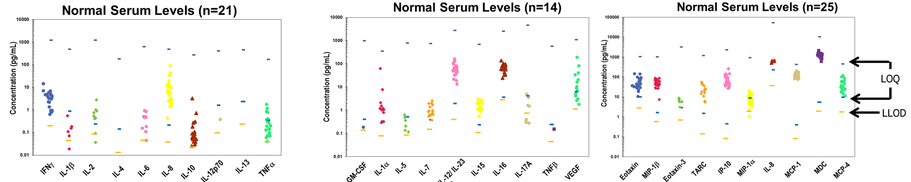

## 6 Spike Recovery and Dilution Linearity

Spike and recovery testing was conducted in serum, heparin plasma, EDTA plasma, citrate plasma, urine, and cell culture media. Representative data are presented below for all of the assays showing overall good assay performance with almost all sample types for all assays accurately recovering expected concentrations of spiked analyte (within 20% of expected, indicated by dashed lines). Dilution linearity testing was also conducted on all assays (not shown). Results were similar with almost all samples recovering within 20% of expected concentrations across all assays. Exceptions were IL-13, IL-1a, and MDC which recovered just outside of 80% to 120% of expected at the highest sample dilutions.



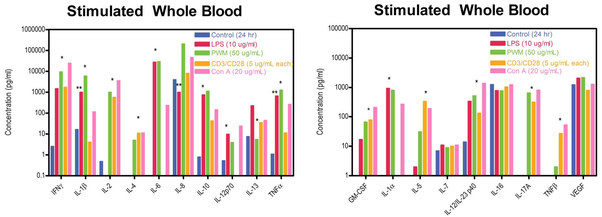

## 7 Normal Sample Testing

Normal human matrices were evaluated for native analyte levels. The representative data for serum is shown below with limits of quantitation and detection noted for each assay. Nearly all samples were detectable across the 30-plex and most samples were within the limits of quantitation.




## 8 Whole Blood Stimulation

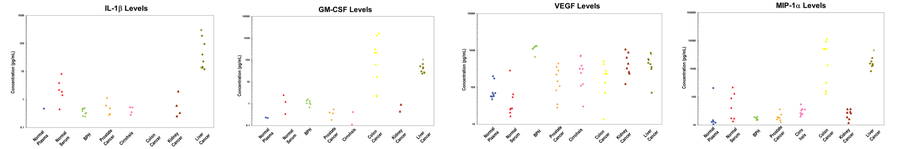
Normal human whole blood was enriched for leukocytes and platelets and then treated with various stimulants for 24 hours. The stimulated samples were tested with the Proinflammatory Panel 1 (human) and Cytokine Panel 1 (human) kits. Single asterisk denotes significant over-expression with stimulation; double asterisk denotes concentrations above saturation levels.




## 9 Disease Sample Testing

The following sample types were evaluated: 9 normal serum, 9 normal plasma, 9 benign prostatic hyperplasia (BPH) serum, 9 prostate cancer serum, 9 cirrhosis serum, 9 colon cancer serum, 9 kidney cancer serum, and 9 liver cancer serum. The 30-plex panel was run as 3 fit-for-purpose 10-plex panels. A number of biomarkers were found to be over-expressed in different conditions. A summary of the results is provided with representative data sets for assays from each of the panels shown below.

The proinflammatory cytokines IL-1b, IL-6, and TNFa were over-expressed in liver cancer serum. A number of them were also over-expressed with concentrations above the quantifiable range in colon cancer serum. GM-CSF was over-expressed in colon and liver cancers while VEGF was shown to be over-expressed in BPH, cirrhosis, and all the cancer conditions tested. Along with VEGF, IL-1a was also over-expressed in BPH serum. IL-16 was over-expressed in kidney cancer serum, and IL-7 was over-expressed in prostate, colon, and liver cancers. Eotaxin was found to be over-expressed in colon cancer serum, while eotaxin-3 was over-expressed in BPH serum. IL-8, MIP-1a, MIP-1b, and MCP-1 were over-expressed in colon and liver cancer serum.




## 10 Conclusion

A 30-plex screening panel has been developed and validated for use in quantitating cytokines and chemokines in a variety of human matrices. The assays in the panel have sufficient sensitivity, reproducibility, accuracy, and sample performance to allow testing of multiple sample types in cancer biology applications. Consistent results were demonstrated for calibration curves as well as for control samples over multiple runs on different days by different operators. All of the assays in the panel can be run using 3, fit-for-purpose-designed, 10-plex panels that use a simple 3-step protocol. Biomarker screening can be performed with as little as 75 µL of sample. Evaluation of various disease samples using this panel demonstrate that it can be an effective tool for cancer biomarker discovery and validation.

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