Multiplex Panels for Cancer-Associated Biomarkers: Quantify 40 Biomarkers from 40 µL of Serum or Plasma

Martin K. Stengelin,[1] Anahit Aghvanyan,[1] Gelu Dobrescu,[2] Mingyue Wang,[1] Anu Mathew,[1] Eli N. Glezer,[1] Jacob N. Wohlstadter[1]

[1]Meso Scale Diagnostics, LLC, Rockville, MD; [2]Medimmune, Gaithersburg, MD

1 Abstract

There is currently a gap in the translational research between identification of new biomarkers and development and commercialization of FDA-approved diagnostic tests. A large number of potential cancer serum/plasma biomarkers have emerged from academic research projects. Although none of them has sufficient sensitivity and specificity to be used alone as a screening assay, using a combination of markers might allow identification of individuals who are at the earliest and most treatable stage of cancer.

Discovery and validation of biomarker panels for early detection requires samples, ideally collected before cancer diagnosis, with cases and controls collected using the same protocol. To make maximum use of these precious samples, assays must be developed to minimize sample volume requirements. Stand-alone ELISAs require a significant amount of sample, e.g. 100 µL or more per replicate and per assay.

We developed electrochemiluminescence (ECL)-based, multiplexed, serum/plasma immunoassay panels to measure more than 40 cancer-related biomarkers using a 96-well, 7-spot format. Due to the high sensitivity of ECL technology, these assays can be used with diluted serum or plasma, bringing the total sample volume required to run 45 assays down to approximately 40 µL per replicate.

These MSD® MULTI-ARRAY assay panels contain most of the classical cancer markers (AFP, CA 125, CA 15.3, CA 19.9, CEA, Cyfra 21.1, Her-2, NSE) as well as a number of cancer-associated markers such as growth factors and their receptors (e.g., VEGF, sFlt-1, cMet, SCF, cKit, EGFR), cytokines and chemokines (e.g., IL6, IL-2R), MMPs, and inflammation markers.

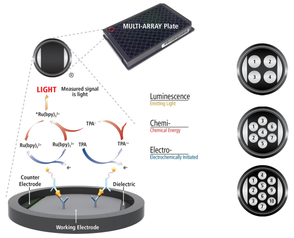
The assay format is simple: diluted sample or calibrator is added to blocked and washed incubation with agitation, plates are washed and detection antibody reagent is added. After a 1-hour incubation, plates are washed and read on an MSD imager (read time, 1–3 minutes per plate).

The assays were sensitive enough to measure these biomarkers in normal samples, and the dynamic ranges extended beyond the elevated levels expected in disease states. Multiplexing does not affect accuracy; each analyte was measured accurately even when other analytes were present in high abundance. Spike recovery and dilution linearity for most assays were in the range of 80% to 120%. Detection limits for a majority of the assays were between 1 and 10 pg/mL, and dynamic ranges were between 3 and 4 logs.

In conclusion, MULTI-ARRAY® assay panels have been developed and validated for measuring serum biomarkers relevant to the early detection, diagnosis, prognosis, and/or monitoring of various cancers. The versatility, ease of use, and high-throughput features of the technology make it ideally suited for large-scale clinical studies.

2 Methods

MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY and MULTI-SPOT® microplates.



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 200 µL MSD Blocker A solution to each well. Incubate for 30 minutes at room temperature (RT).
2. Wash with PBS-T. Add 25 µL of assay diluent and 25 µL of diluted sample or calibrator. Incubate for 2 hours at RT with shaking. (Note: For Panels F, G, & H, add 40 µL of assay diluent and 10 µL of non-diluted sample.)
3. Wash with PBS-T. Add 25 µL of detection antibody. Incubate for 1 hour at RT.
4. Wash with PBS-T. Add 150 µL of Read Buffer T. Read on MSD imager. (Note: For Panel C, use Read Buffer P.)

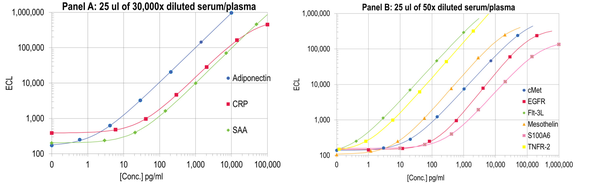
3 Multiplex Panels of Cancer-Associated Serum Biomarkers

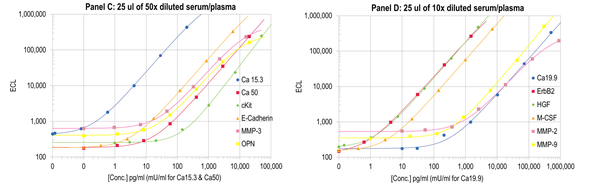
The table below shows how 45 cancer-associated markers were multiplexed into 8 panels.
The lower limit of detection (LLOD) for most assays was between 1 and 10 pg/mL. Spot coating uniformity tests resulted in CVs (coefficients of variation) generally under 10% for each assay. Total CVs for most assays were below 20%.
The LLOD is a calculated concentration corresponding to a signal 2.5 standard deviations above the background. ECL signals from 9 plates were averaged to calculate the LLODs shown below. Spot coating uniformity CVs were determined by testing 3 full plates (96 replicates per plates; plates were selected from the beginning, middle, and end of the plate manufacturing run). Total CVs were determined by running 2 quality control (QC) samples per plate (placed in opposite corners) on ~10 plates (1–3 plates per day; over a period of approximately 1 month).

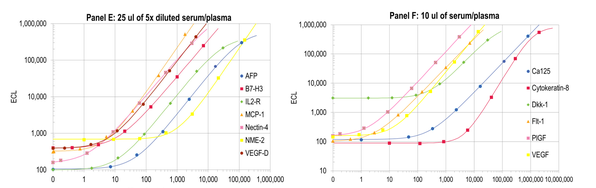
Assays	Sample Volume / Sample Dilution	Median LLOD	Spot Coating Uniformity CV%	Total CV%
Adiponectin	25 µL 30,000x	0.4 pg/mL	10	9
CRP (C-reactive Protein)		6 pg/mL	11	20
SAA (Serum Amyloid A)		6 pg/mL	7	11
cMet (HGFR)	25 µL 50x	8 pg/mL	16	18
EGFR (Epidermal Growth Factor Receptor)		70 pg/mL	5	12
Flt-3L (Flt-3 Ligand)		0.2 pg/mL	5	13
Mesothelin		3 pg/mL	5	9
S100A6		80 pg/mL	5	16
TNFR-2 (Soluble Tumor Necrosis Factor Receptor 2)		0.5 pg/mL	6	16
CA 15.3 (Cancer Antigen 15.3)	25 µL 50x	0.04 mU/mL	5	18
CA 50 (Cancer Antigen 50)		4 mU/mL	8	20
cKit (SCF Soluble Receptor)		40 pg/mL	16	22
E-Cadherin		1 pg/mL	4	17
MMP-3 (Matrix Metalloproteinase 3)		3pg/mL	4	19
OPN (Osteopontin)		10 pg/mL	3	20
CA 19-9 (Cancer Antigen 19-9)	25 µL 10x	0.1 U/mL	6	8
ErbB2 (Her2, Neu)		0.3 pg/mL	4	5
HGF (Hepatocyte Growth Factor)		0.6 pg/mL	10	13
M-CSF (Macrophage Colony-Stimulating Factor)		1 pg/mL	19	9
MMP-2 (Matrix Metalloproteinase 2)		200 pg/mL	5	8
MMP-9 (Matrix Metalloproteinase 9)		100 pg/mL	17	26
AFP (Alpha Feto-Protein)	25 µL 5x	20 pg/mL	6	10
B7-H3 (B7 Homolog 3)		2 pg/mL	3	12
IL-2R (Soluble IL-2 Receptor 2)		6 pg/mL	4	13
MCP-1 (CCL2)		1 pg/mL	5	12
Nectin-4		0.5 pg/mL	3	10
NME-2 (NME/NM23 Nucleoside Diphosphate Kinase 2)		200 pg/mL	6	11
VEGF-D (Vascular Endothelial Growth Factor D)		2 pg/mL	5	11
CA 125 (Cancer Antigen 125)	10 µL neat	0.1 U/mL	5	12
Cytokeratin-8		3 ng/mL	14	15
Dkk-1 (Dickkopf-related 1)		50 pg/mL	6	15
Flt-1 (VEGF Receptor 1)		1 pg/mL	5	12
PlGF (Placental Growth Factor)		1 pg/mL	6	24
VEGF (Vascular Endothelial Growth Factor A)		6 pg/mL	7	15
Cytokeratin-19 (Cyfra 21.1)	10 µL neat	80 pg/mL	5	11
IL-6 (Interleukin 6)		0.5 pg/mL	5	13
ITAC (CXCL11)		7 pg/mL	9	23
KGF (Keratinocyte Growth Factor; FGF-7)		0.3 pg/mL	4	11
NSE (Neuron-Specific Enolase)		30 pg/mL	5	11
OPG (Osteoprotegerin)		8 pg/mL	3	11
SCF (Stem Cell Factor)		1 pg/mL	7	15
CEA (Carcinoembryonic Antigen)	10 µL neat	20 pg/mL	4	18
GPI (Glucose Phosphate Isomerase)		10 ng/mL	6	15
MDC (CCL22)		20 pg/mL	14	24
uPA (Urokinase Plasminogen Activator)		30 mU/mL	5	16

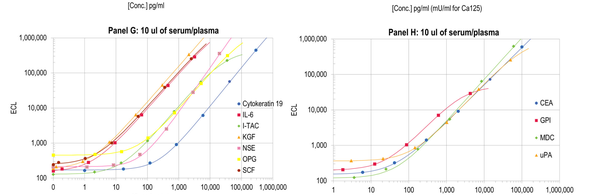
4 Calibration Curves

The eight graphs below show the calibration curves (log-log scale) for each of the eight panels. Concentrations on the x-axis refer to the concentration of the calibrator added to the well and do not include any correction for sample dilution. The ECL signal data shown below were averaged over 9 plates per panel, with 2 replicates at each calibrator level per plate. Analytical performance of the panels was verified by cross-reactivity, spike recovery, and dilution linearity experiments (not shown).



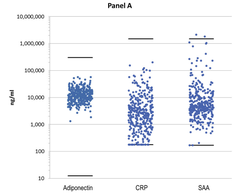


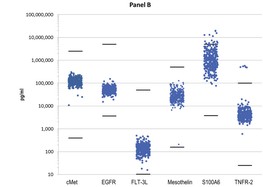


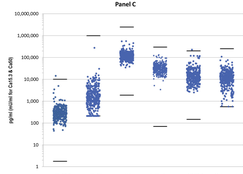


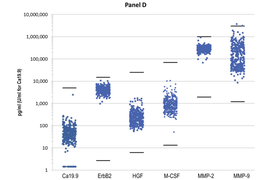
5 Clinical Serum and Plasma Samples

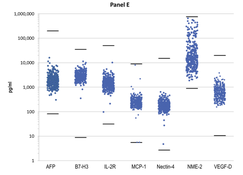
Approximately 350 serum and plasma samples from lung cancer patients, heavy smokers, and healthy controls were tested on the eight panels. Even though only 150 µL of sample volume was available, we were able to measure each of the 45 markers in duplicate.
The eight graphs below show the sample concentrations (blue dots, corrected for sample dilution) for each of the markers. The horizontal black bars for each assay show the detection limit and the upper end of the assay range.
The graphs demonstrate the wide dynamic range of the MULTI-ARRAY technology. For most assays, concentrations of all samples were within the assay range.

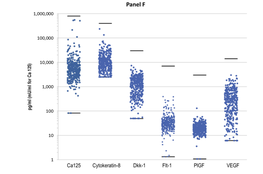


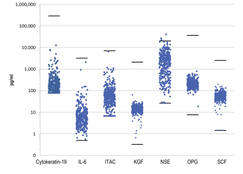


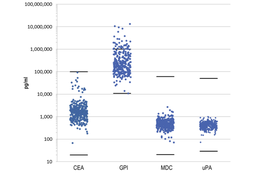












6 Conclusion

The data presented above, covering 45 cancer-related biomarkers, show that MSD MULTI-ARRAY assays provide highly sensitive and precise measurements over a wide dynamic range using a simple, fast protocol with low sample volume requirements. MSD technology is ideal for situations where a maximum amount of information needs to be extracted from a minimal amount of sample.

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