Sample-Sparing Immunoassays for Early Detection of Cancer

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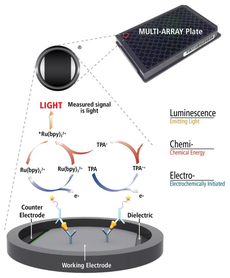
❶ Abstract

As an Early Detection Research Network (EDRN) Biomarker Characterization Center, we are developing Research Use Only (RUO) sample-sparing Meso Scale Discovery MULTI-ARRAY immunoassays covering a wide range of well-established cancer markers. Our primary focus is the development of assays to support research of lung and ovarian cancer, but many of the selected markers are also relevant to other cancer types. These immunoassays include a serology panel and three biomarker panels. The serology panel includes assays to detect autoantibodies to p53, CTAG-1, and CTAG-2. This panel requires 25 µL of 2,500-fold diluted serum or plasma. A biomarker panel requiring 25 µL of 100-fold diluted serum or plasma detects the following biomarkers: CA15-3, SCFR/Kit, ErbB2, IGFBP-2, MIF, MMP-2, MMP-9 (total), REG4, S100A6, and TNF-RI. A second biomarker panel requiring 25 µL of 10-fold diluted serum or plasma detects the following biomarkers: CA125, Ca19-9, CEACAM-5 (CEA), EGFR, VEGFR-1/Flt-1, FLT3L, HE4, MMP-3 (total), and osteopontin. A third biomarker panel requiring 25 µL of serum/plasma detects the core lung cancer biomarker cytokeratin-19 multiplexed with NSE and HGF. All assays have dynamic ranges that span 3-4 logs and are sufficiently sensitive to measure native levels in commercially sourced samples from apparently healthy individuals. We conducted a preliminary evaluation of the assays by testing more than 100 commercially sourced serum or plasma samples from apparently healthy individuals and more than 100 commercially sourced serum or plasma samples from individuals with lung, ovarian, or other cancers. Many of the biomarkers had significantly different concentrations in several cancers compared to apparently healthy individuals. These RUO assays may be useful in identifying biomarkers for a multi-marker cancer panel.

❷ Methods

MSD® 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.



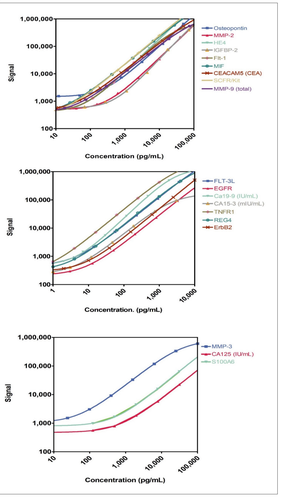
MULTI-ARRAY Electrochemiluminescence Technology

- 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.

❸ Multiplex Protein Biomarker Panels

Nineteen assays were combined into two multiplex panels (Table 1) and formulated as kits containing 96-well MULTI-SPOT 10-spot plates, blended calibrators, controls and detection antibody solutions, diluents, and read buffer. The assays were run following the 2-step protocol below using the sample dilutions listed in the table. Lower Limit of Detection (LLOD) is calculated as the concentration that gives a signal 2.5 SD above the blank.

A



B

Analyte	Fold Sample Dilution	LLOD	TOC	Units
CA125	10	219	500,000	mIU/mL
CEACAM5 (CEA)		5.1	50,000	pg/mL
HE4		11	20,000	pg/mL
Osteopontin		28.7	50,000	pg/mL
Ca19-9		0.04	200	IU/mL
EGFR		49	200,000	pg/mL
Flt-1		11.7	30,000	pg/mL
FLT-3L		0.5	3,600	pg/mL
MMP-3		3.5	100,000	pg/mL
CA15-3	100	2.38	3,000	mIU/mL
ErbB2		1.74	10,000	pg/mL
IGFBP-2		167	70,000	pg/mL
MIF		4.50	27,000	pg/mL
MMP-2		65.5	40,000	pg/mL
MMP-9 (total)		10.3	75,000	pg/mL
REG4		0.63	4,000	pg/mL
S100A6		79.7	500,000	pg/mL
SCFR/Kit		4.48	40,000	pg/mL
TNFR1		0.21	1,000	pg/mL

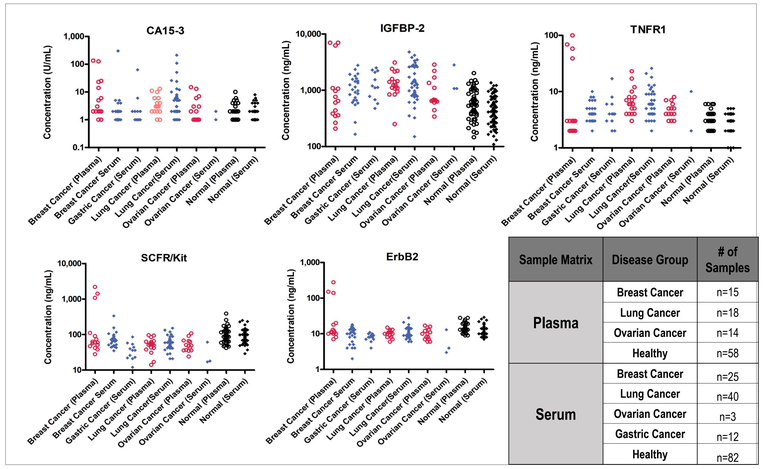
Protocol

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

Figure 1 (A) Typical calibration curves for the assays in two multiplex panels. **(B)** Table of the Lower Limits of Detection (LLOD) and Top of Curve (TOC) concentrations for each analyte in the multiplex panels. Values in this table are not corrected for sample dilution.

❹ Individual Serum and Plasma Sample Testing

Commercially sourced serum and plasma samples from apparently healthy individuals and from individuals with lung, breast, gastric or ovarian cancer were used to evaluate two multiplex protein biomarker panels. The data represented highlight five biomarkers that displayed significantly different concentrations in various cancer samples compared to samples from apparently healthy individuals.



Sample Matrix	Disease Group	# of Samples
Plasma	Breast Cancer	n=15
	Lung Cancer	n=18
	Ovarian Cancer	n=14
	Healthy	n=58
Serum	Breast Cancer	n=25
	Lung Cancer	n=40
	Ovarian Cancer	n=3
	Gastric Cancer	n=12
	Healthy	n=82

Figure 2 Sample concentrations (corrected for sample dilution) for five of the markers in the multiplex protein biomarker panels. Samples with concentrations above TOC concentrations are assigned those respective values.

❺ Serology Assay

A multiplexed serology panel was developed to detect autoantibodies against p53, CTAG-1, and CTAG-2. Plates are provided with antigens on spots in the wells of a 96-well plate. Antibodies in the sample bind to the antigens on the spots and anti-human IgG antibody conjugated with MSD SULFO-TAG is used for detection. Commercially sourced plasma or serum samples were tested at 2,500-fold dilution on the serology panel. Serology results are shown for p53 and CTAG-1. CTAG-2 results were comparable to those for CTAG-1.

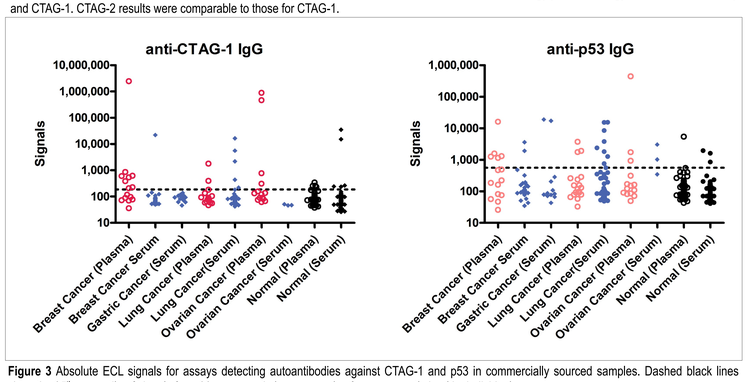


Figure 3 Absolute ECL signals for assays detecting autoantibodies against CTAG-1 and p53 in commercially sourced samples. Dashed black lines show the 95th percentile of signals from 98 serum and plasma samples from apparently healthy individuals.

❻ NSE / HGF / Cytokeratin-19 Multiplex

A multiplex panel with cytokeratin-19, HGF and NSE was developed using a one-step protocol where the detection antibody and samples are added simultaneously. The assay performance was evaluated using commercially sourced samples, including plasma or serum samples from apparently healthy individuals and plasma or serum samples from breast, lung, and ovarian cancer patients. These assays demonstrated sufficient sensitivity in measuring native cytokeratin-19, HGF and NSE levels in apparently healthy individuals. Samples can be further diluted when sample volume is limited.

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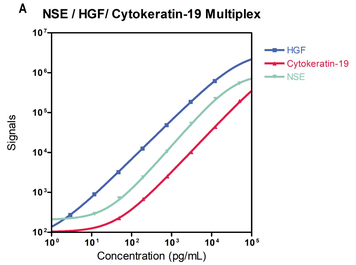

B

Analyte	LLOD	TOC	Units
Cytokeratin-19	25.5	200,000	pg/mL
HGF	1.1	12,000	pg/mL
NSE	10.3	50,000	pg/mL

Protocol

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

Figure 4 (A) Typical calibration curves for the Cytokeratin-19, HGF and NSE Triplex. **(B)** Table of the LLOD and TOC concentrations for each analyte in the multiplex panels. Values in this table are not corrected for sample dilution.

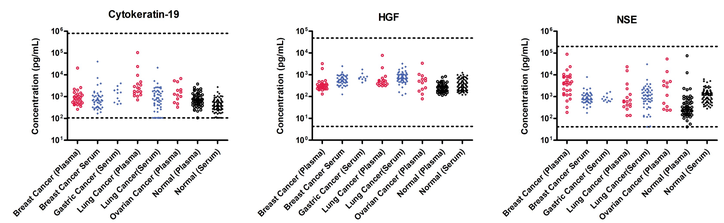

Figure 5 Approximately 300 commercially sourced serum or plasma samples from apparently healthy individuals and from individuals with lung, ovarian and breast cancer were tested. The assays have a wide dynamic range and are sufficiently sensitive to allow serum and plasma testing at 4-fold dilution. Dashed black lines show the dilution corrected TOC and LOD. Samples with concentrations below LOD concentrations are assigned those respective values. NSE measurements are susceptible to pre-analytical issues. Careful attention to pre-analytical conditions is crucial for reliable NSE interpretation.

❼ Conclusions

- We successfully developed three Research Use Only (RUO) multiplexed cancer biomarker detection panels and a multiplexed serology panel to measure biomarkers that are relevant to lung or other cancer types.
- The assays performed well in the assessment of samples (including cancer and normal samples) from commercial sources.
- The assays are sensitive enough to detect all markers in most samples.
- These RUO assays, including CA15-3, CA125, CEACAM5 (CEA), IGFBP-2, TNFR1, SCFR/Kit, ERbB2, EGFR, FLT-3L, HE4, osteopontin, HGF, NSE, and cytokeratin-19, as well as autoantibodies against p53, CTAG-1, and CTAG-2, may be useful in identifying biomarkers for a multi-marker panel to study cancer.

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❽ Acknowledgement

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