Development of a Multiplexed Immunoassay Panel for Ten Cancer Markers: Alpha-Fetoprotein, Cancer Antigen 125, Cancer Antigen 19-9, Carcinoembryonic Antigen, cKit, E-cadherin, Epidermal Growth Factor Receptor, Erythroblastic Leukemia Viral Oncogene Homolog 2 (ErbB2), Matrix Metallopeptidase 9, and Osteopontin

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An electrochemiluminescence-based multiplexed immunoassay panel was developed for simultaneous measurement of ten classical and emerging cancer markers per well in a 96-well format. The assay requires only 25 µl of a five-fold diluted sample, i.e. 5 µl for all ten assays combined.

The assay format is simple: assay diluent and diluted sample or calibrator are added to blocked and washed plates. After a two-hour incubation with agitation, plates are washed, and detection antibody reagent is added. After a second two-hour incubation, plates are washed and read on a MSD SECTOR® Imager 6000 instrument (throughput of one plate per minute).

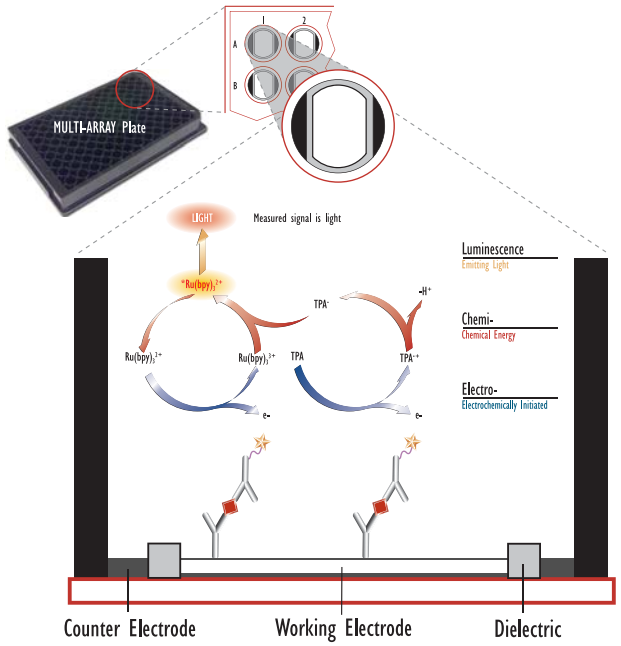
The assays are sensitive enough to measure these biomarkers in normal samples, and the dynamic range is sufficient for measurement of the elevated levels expected in disease states without additional serial sample dilution.

Analyte	Assay Range	Analyte concentration in ~20 normal serum & plasma samples	
		Median	Range
AFP	0.15 - 500 ng/mL	3 ng/ml	1 – 14 ng/ml
Ca125	3 – 5,000 U/ml	8 U/ml	<3 – 90 U/ml
Ca19-9	5 – 5,000 U/ml	26 U/ml	<5 – 170 U/ml
CEA	0.1 – 500 ng/ml	4 ng/ml	1 – 6 ng/ml
cKit	3 – 2,000 ng/ml	130 ng/ml	60 – 210 ng/ml
E-Cadherin	0.1 – 500 ng/ml	18 ng/ml	10 – 48 ng/ml
EGFR	0.6 – 1,000 ng/ml	67 ng/ml	48 – 82 ng/ml
$ErbB_2$ (Her_2/neu)	0.2 – 500 ng/ml	3 ng/ml	2 – 4 ng/ml
MMP-9	0.2 – 3,000 ng/ml	50 ng/ml	25 – 1,600 ng/ml
Osteopontin	0.05 – 100 ng/ml	3 ng/ml	0.8 – 17 ng/ml

Spike recovery and dilution linearity were in the range of 80% to 120%. Intra-plate CVs were approximately 5 – 15%. Each analyte in the multiplex panel was measured accurately even in the presence of other, high abundance analytes.

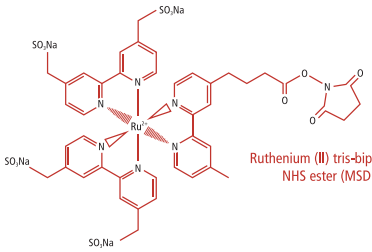
The MSD Platform

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 Features:

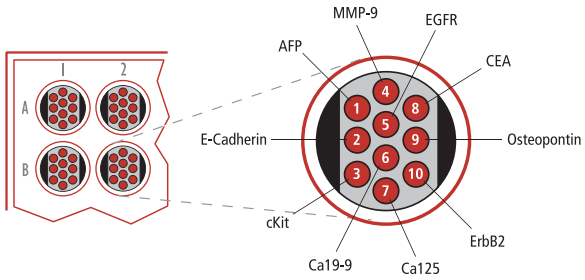
- Minimal background signals and high signal to background ratios - the stimulation mechanism (electricity) is decoupled from the signal (light)
- Proximity - only labels bound near the electrode surface are detected, enabling non-washed assays
- Flexibility - labels are stable, non-radioactive, and are conveniently conjugated to biological molecules
- Emission at ~620 nm - eliminating problems with color quenching
- Signal amplification - multiple excitation cycles of each label enhance light levels and improve sensitivity



Ruthenium (II) tris-bipyridine-(4-methylsulfonate) NHS ester (MSD SULFO-TAGTM label)

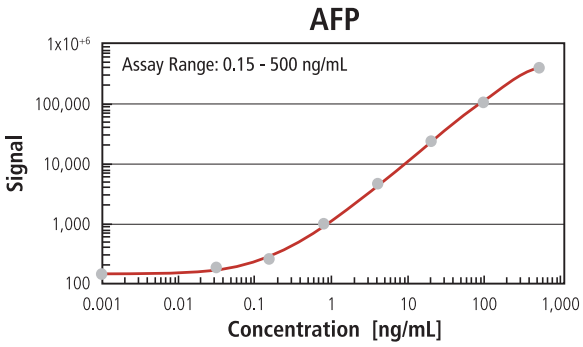
Assay Protocols

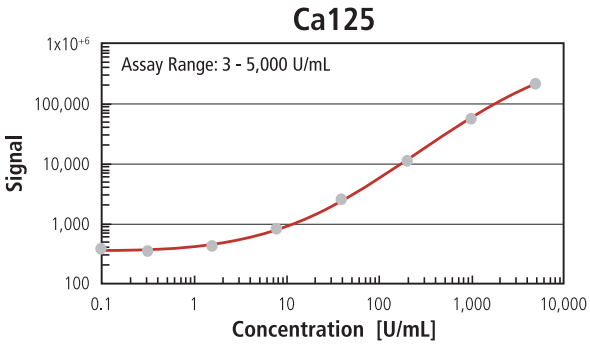
1. Block MSD MULTI-SPOT plate for 1 hour, wash.
2. Add 25 µl Assay Diluent to each well.
3. Add 25 µl calibrator or fivefold diluted sample to each well.
4. Incubate with shaking for 2 hours, wash.
5. Add 25 µl labeled antibody solution to each well.
6. Incubate with shaking for 1-2 hour, wash.
7. Add 150 µl MSD Read Buffer to each well.
8. Read plate on MSD Reader.

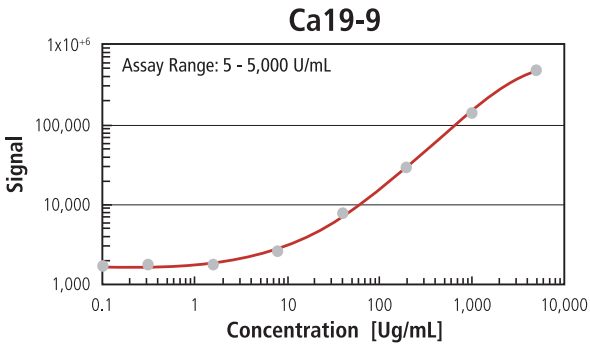


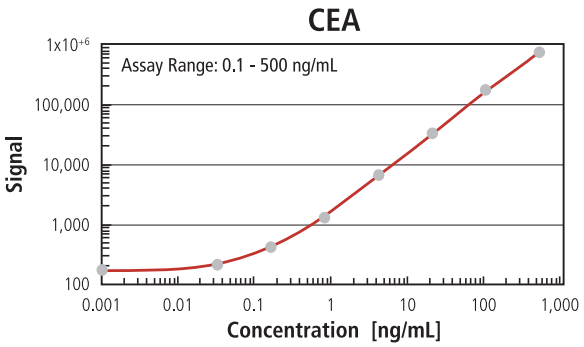
Patterned Array of Antibodies on Working Electrode

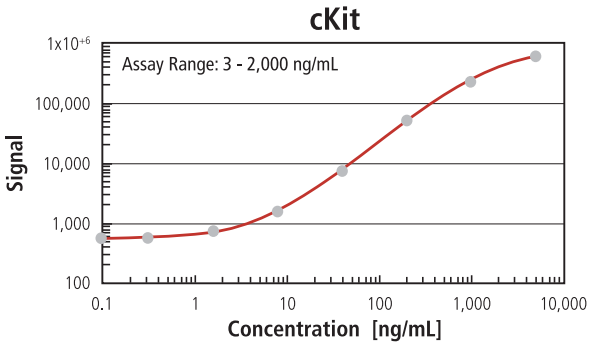
10-plex Cancer Markers Assay

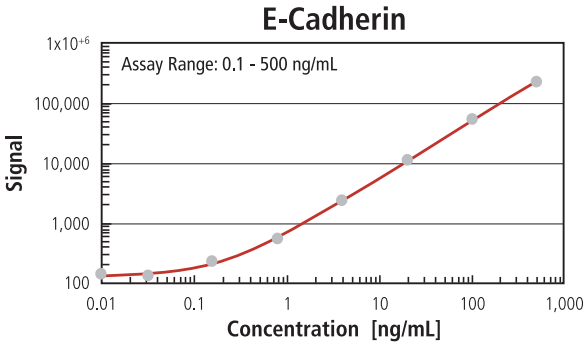












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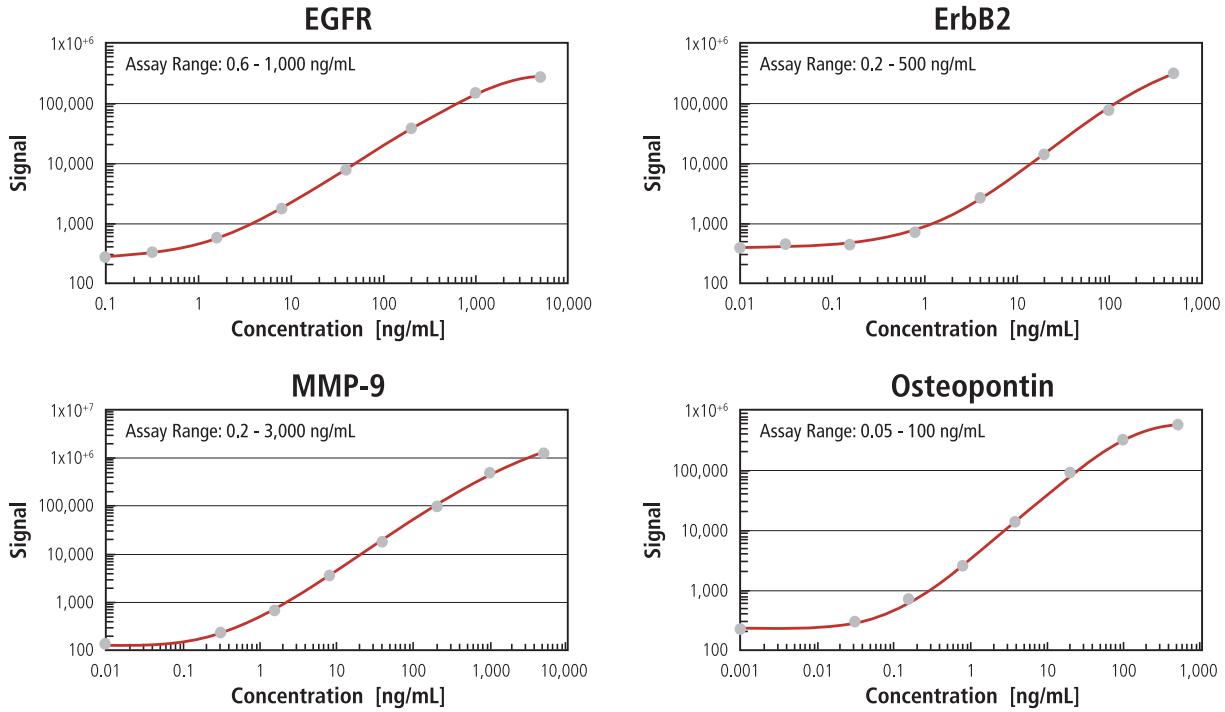

Assay Ranges and Analyte Concentrations (Healthy Donors)

The assays are sensitive enough to measure the biomarkers in normal samples. The dynamic range is sufficient for measurement of the elevated levels expected in disease states without additional serial sample dilution.

Analyte	Assay Range	Analyte concentration in ~20 normal serum & plasma samples	
		Median	Range
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Cross-Reactivity

One calibrator at a time at an elevated level was tested in a 10-spot plate.

%Cross-reactivity is defined as (signal – background on "wrong" spot)/ signal on correct spot]

% Cross-reactivity was less than 1% for most combinations. The apparent 6% cross-reactivity of Ca125 calibrator on the Ca19.9 spot is not an assay cross-reactivity, but caused by Ca19-9 contamination of the Ca125 calibrator. This calibrator is purified from an ovarian carcinoma cell line and contains (according to the manufacturer) 5 units of Ca19-9 per 1,000 units of Ca125.

Lower, but detectable cross-reactivity (~ 2%) is seen for Ca125 and Ca19-9 calibrator on the Osteopontin spot (likely also calibrator contamination) and for E-cadherin on the CEA spot.

	Single Calibrator (%)									
Assay	AFP	Ca125	Ca19-9	CEA	cKit	E-Cadherin	EGFR	ErbB2	MMP-9	Osteopontin
AFP	100	-0.1	0.0	-0.2	0.0	0.0	-0.1	-0.1	0.0	0.0
Ca125	0.0	100	0.4	-0.1	0.0	0.0	0.1	-0.1	0.0	0.0
Ca19-9	0.2	6.4	100	0.2	0.0	0.0	-0.1	0.0	0.0	-0.1
CEA	0.0	0.0	0.2	100	0.0	1.8	0.0	0.0	0.0	0.0
cKit	0.0	0.0	0.1	0.1	100	0.0	0.1	0.0	0.1	0.1
E-Cadherin	0.0	0.0	0.0	0.0	0.1	100	0.0	0.0	0.0	0.0
EGFR	0.0	-0.1	0.0	0.0	0.0	0.0	100	0.0	0.0	0.0
ErbB2	0.0	-0.1	-0.1	-0.2	0.0	0.0	-0.1	100	0.0	0.0
MMP-9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100	0.0
Osteopontin	0.0	1.6	2.2	-0.2	0.0	0.0	-0.1	0.1	0.0	100

Dilution Linearity

Eight serum or plasma samples from apparently healthy donors were diluted to 75%, 50%, and 25% in addition to the standard fivefold dilution (presented as 100%). The table shows the averages and standard deviations of the normalized concentrations per dilution level for each of the ten assays.

Most Ca125 (7/8) and several AFP (3/8) concentrations were too low in the selected healthy donors to measure diluted samples accurately. Results from these samples were excluded from the calculations.

	Dilution Linearity		
Assay	75% Dilution	50% Dilution	25% Dilution
AFP	73% ± 10%	44% ± 7%	27% ± 9%
Ca125	71%	58%	31%
Ca19-9	80% ± 9%	53% ± 12%	27% ± 3%
CEA	76% ± 7%	54% ± 9%	27% ± 4%
cKit	76% ± 2%	52% ± 3%	27% ± 1%
E-Cadherin	70% ± 7%	39% ± 6%	17% ± 6%
EGFR	77% ± 4%	54% ± 5%	28% ± 4%
ErbB2	81% ± 8%	55% ± 6%	33% ± 6%
MMP-9	78% ± 7%	53% ± 5%	29% ± 3%
Osteopontin	78% ± 5%	52% ± 5%	23% ± 3%

Spike Recovery

A single combined calibrator was spiked into seven individual serum or plasma samples (after the samples were diluted five fold according to the assay protocol) and into calibrator diluent as a control. Spike recovery was determined as the percentage of [measured difference between spiked and non-spiked sample] divided by [spiked calibrator diluent].

The summary table shows the average spike recovery and the standard deviation of the spike recovery for each assay.

Assay	Spike Recovery
AFP	92% ± 11%
Ca125	96% ± 13%
Ca19-9	103% ± 8%
CEA	102% ± 9%
cKit	106% ± 3%
E-Cadherin	86% ± 6%
EGFR	52% ± 14%
ErbB2	71% ± 9%
MMP-9	87% ± 4%
Osteopontin	81% ± 9%

Reproducibility

Reproducibility was assessed by running a midrange combined calibrator in every well of a 96-well plate. The table shows the CVs per plate for the ten assays for a representative experiment.

Assay CV (n=96)	
AFP	16%
Ca125	13%
Ca19-9	9%
CEA	7%
cKit	10%
E-Cadherin	6%
EGFR	7%
ErbB2	13%
MMP-9	10%
Osteopontin	13%

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