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Immunoassays for Free and Complexed PSA with fg/mL Sensitivity

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1 Abstract

Measurement of free and complexed PSA (prostate specific antigen; complexed with ACT) in certain patient groups such as prostatectomy patients and women at risk of breast and ovarian cancer has been limited by the sensitivity of current immunoassay technology (5-30 pg/mL for common clinical analyzers). Fifth generation assays for total PSA with detection limits of 0.05 to 0.1 pg/mL have been described (McDermet 2009; Thaxton 2009; Wilson 2011). We developed a next-generation assay format based on MSD's MULTI-ARRAY® electrochemiluminescence technology with detection limits of 2 fg/mL (0.002 pg/mL) for complexed PSA (cPSA) and 20 fg/mL for free PSA (fPSA), requiring only 25 µL of serum or plasma per measurement.

Assays were calibrated against the WHO standards 96/668 (free PSA) and 98/670 (90% complexed, 10% free PSA). The dynamic range of both assays was three to four orders of magnitude. Typical intra-plate coefficients of variation ranged from 5% to 15%. LLOQ's of cPSA and fPSA assays were 5 fg/mL and 55 fg/mL, respectively. Spike recovery and dilution linearity were between 80% and 120%. Approximately 100 serum samples from women were evaluated. Free PSA was detectable in 95% of samples and cPSA was detectable in all samples.

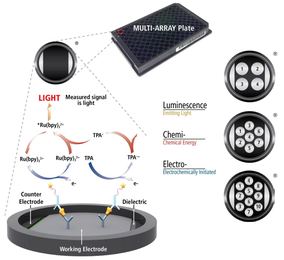
Multiple literature reports indicate that free PSA might be a marker for early detection of breast cancer; however, the concentrations of many samples were close to or below the detection limits of the assays used for the studies. We tested 48 serum samples from patients with breast cancer and 27 serum samples from apparently healthy women. As the table[1] below shows, there was no clear difference between PSA concentrations in cases and controls.

In conclusion, we developed a next-generation assay format that is 100 to 1000 times more sensitive than the currently available clinical PSA assays. This enables accurate determination of free PSA and cPSA concentrations in the serum of females.

[1] See Section 6.

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.
We developed the S-PLEX™ assay platform, a next-generation MULTI-ARRAY technology with significantly higher sensitivity.



Electrochemiluminescence Technology

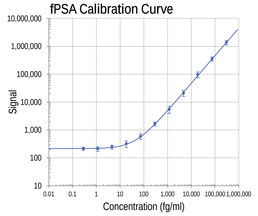
- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed 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.

3 Assay Performance Characterization

The performance of free and complexed PSA assays (fPSA and cPSA) was characterized.
Essentially all experiments had the following plate layout:
- Point-symmetrical plate layout; calibrators, QC samples, and unknowns measured in duplicates.
- 3 QC samples spanning the assay range and a female serum pool control (QC-4).

- Performance characterization included determination of limits of detection, upper and lower limits of quantitation, within plate and total reproducibility, spike recovery, and dilution linearity.
- Approximately 200 samples were tested, including approximately 100 serum samples from breast cancer patients and controls, and a set of matched serum, EDTA plasma, and heparin plasma samples from apparently healthy females and males.
- All data presented in this poster were generated in an individual assay format.

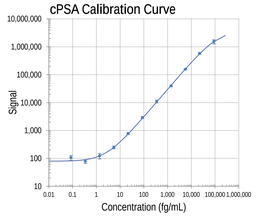
4 Calibration Curves



fPSA: Determination of LLOQ and ULOQ

	fg/mL expected	ECL counts	fg/mL measured	CV (n=8)
ULOQ	100,000	622,644	87,717	31%
	66,667	461,446	61,406	9%
	44,444	326,647	41,841	9%
	219	1,894	186	6%
	109	960	81	13%
	55	616	43	18%
LLOQ	27	404	20	54%
	14	330	11	72%

- Assay Range:
  - LOD: 18 fg/mL
  - LLOQ: 55 fg/mL
  - ULOQ: 67,000 fg/mL



cPSA: Determination of LLOQ and ULOQ

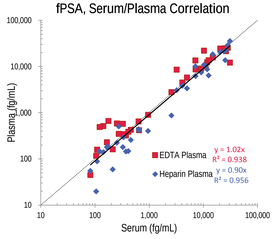
	fg/mL expected	ECL counts	fg/mL measured	CV (n=8)
ULOQ	30,000	1,235,282	28,561	26%
	20,000	1,027,645	22,829	6%
	13,333	725,182	15,425	5%
	33	2,267	42	9%
	16	1,141	21	6%
	8	632	11	9%
LLOQ	4	358	5	19%
	2.0	341	5	164%

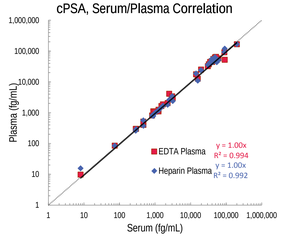
- Assay Range:
  - LOD: 1.9 fg/mL
  - LLOQ: 4.0 fg/mL
  - ULOQ: 20,000 fg/mL

A detection limit of 1.9 fg/ml for a 25 µl sample corresponds to approximately 1,000 cPSA molecules (MW PSA 28kDa).

5 Normal Serum /Plasma

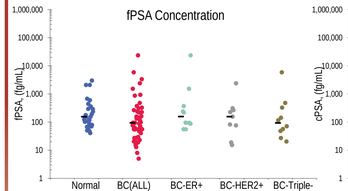
Sixty matched serum, EDTA plasma and heparin plasma samples from 20 normal female donors and 77 samples from 40 male donors including 17 normal matched serum, EDTA plasma and heparin plasma samples were run on both the fPSA and cPSA assays. Female samples were run neat and male samples run 100-fold diluted.
fPSA was detectable in all and quantifiable in 97% of normal female serum/plasma samples; cPSA was detectable and quantifiable in all normal female serum/plasma samples. Female samples are recommended to be run neat on the fPSA assay and neat or 2-fold diluted on the cPSA assay. Normal male samples are recommended to be run 100-fold diluted on the fPSA assay and 1,000 or 2,000-fold diluted on the cPSA assay.
Correlation between PSA concentration detected in serum and plasma samples is shown on the graphs below. R^2 and slopes were calculated using log-transformed concentrations.

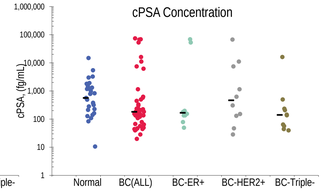


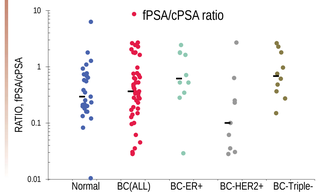


6 Breast Cancer Samples

	fPSA [fg/mL] (Median, IQR)	cPSA [fg/mL] (Median, IQR)	fPSA/cPSA (Median, IQR)
Apparently Healthy (n=27)	150 (80-260)	560 (210-1,500)	0.29 (0.18-0.73)
Breast Cancer (n=48)	90 (50-260)	180 (90-480)	0.36 (0.17-0.77)
Breast Cancer, ER positive (n=10)	160 (80-660)	170 (120-13,000)	0.62 (0.22-1.80)
Breast Cancer, HER2 positive (n=10)	160 (50-290)	460 (110-8,300)	0.10 (0.03-0.44)
Breast Cancer, triple negative (n=10)	90 (40-370)	140 (50-300)	0.68 (0.34-1.90)





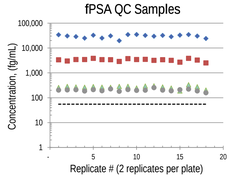


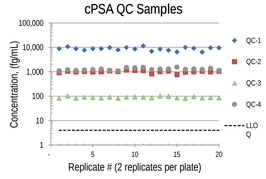
A set of 48 serum samples from patients with breast cancer was tested: 10 were ER positive, 10 were HER2 positive, 10 were triple negative, and no breast cancer subtype information was available for the remaining 18 samples. 27 serum samples from apparently healthy women were tested as controls.
Median cPSA and fPSA concentrations and ratios of fPSA to cPSA (fPSA/cPSA) for each group were calculated. The interquartile ranges are shown for both concentrations and ratios.
As the table below shows, there is no clear difference between PSA concentrations and fPSA/cPSA ratios in breast cancer cases and controls. Ratios of fPSA/cPSA have certain trends in different diseased groups; however a larger sample set should be tested to confirm the observed differences between the diseased and normal groups.

7 Spike Recovery, Dilution Linearity

Serum, EDTA plasma, and heparin plasma samples (7-8 samples total) were spiked with calibrator at two or three concentrations. The non-complexing form of PSA that does not bind to a1 Antichymotrypsin (ACT) was used in spike recovery experiments for the fPSA assay. Average spike recoveries for the fPSA and cPSA assays were 88% and 90%, respectively.
Serum, EDTA plasma, and heparin plasma samples (7-8 samples total) were diluted 2x, 4x, and 8x. Average dilution linearities for the fPSA and cPSA assays were 114% and 109%, respectively.

8 Reproducibility





		fPSA					cPSA				
		fg/mL	Mean ECL	n	CV	recovery	fg/mL	Mean ECL	n	CV	recovery
Inter-plate	QC-1	29,981	270,877	18	14%	100%	8,750	420,231	20	15%	97%
	QC-2	3,304	31,285	18	11%	110%	1,008	50,158	20	10%	112%
	QC-3	268	2,861	18	12%	89%	89	4,432	20	7%	99%
	QC-4	202	2,249	18	10%	NA	1,278	63,396	20	11%	NA
Within-plate	Mid Cal	2000	18,390	96	18%	103%	643	20,040	96	15%	108%

QC-1,2,3: fPSA or cPSA calibrator; QC-4- female serum pool

The graphs show inter-plate reproducibility data.
Nine to ten plates were run over a period of one month. Each plate included an 8-point calibration curve (duplicates) and two replicates each of 4 QC samples. The plate layout was point-symmetrical, with calibrators in columns 1 and 12, and QC samples in columns 2 and 11.
The table summarizes inter-plate and within-plate reproducibility.
Inter-plate reproducibility was estimated using QC samples. Within-plate reproducibility was estimated using 96 replicates of a mid-range concentration calibrator.

9 Conclusion

A next-generation assay for PSA was developed, based on MSD's ultrasensitive S-PLEX technology. This novel technology is 100 to 1,000 times more sensitive than the currently available clinical PSA assays. This enables accurate determination of free PSA and cPSA concentrations in the serum and plasma of females and presumably in post-prostatectomy prostate cancer patients.
This new format assays can be run on any standard MSD® instrument and can be performed within a normal workday using common lab equipment.
The table below summarizes the assay performance for the free and complexed PSA S-PLEX assays.

Summary Table		
	fPSA	cPSA
Detection Limit (LOD)	20 fg/mL	2 fg/mL
Lower Limit of Quantitation (LLOQ)	55 fg/mL	4 fg/mL
Upper Limit of Quantitation (ULOQ)	67,000 fg/mL	20,000 fg/mL
Median concentration in female normal serum	150 fg/mL	560 fg/mL
Median concentration in male normal serum/plasma	12,000 fg/mL	47,000 fg/mL
Inter-plate CV	12%	11%
Spike Recovery	88%	90%
Dilution Linearity	114%	135%
Percentage of tested female samples above the LLOQ (~150 samples)	95%	100%
Sample dilution of male samples required	100x	1000-2000x

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