Development of AccuPSA™, a novel digital immunoassay for sub-femtomolar measurement of PSA in post radical prostatectomy patients

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Introduction

The ability to detect PSA in radical prostatectomy (RP) patients 4-6 weeks after surgery (nadir PSA levels) has potential prognostic value, and monitoring of PSA at very low concentrations could help enable earlier detection of prostate cancer recurrence. With existing immunoassays, PSA is often not detectable in the serum of RP patients for several years post-surgery until levels exceed the sensitivity threshold of the assay. Much greater sensitivity is required to measure PSA at femtogram per milliliter concentrations in order to detect PSA in all of these patients shortly after surgery. The technology described here enables femtogram/mL quantification, and with unprecedented accuracy and precision.

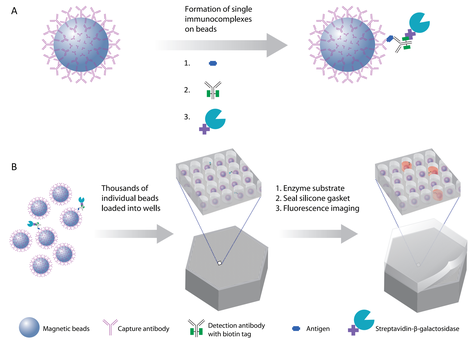
The aim of this work was to conduct an initial assessment of the readiness of an ultra high sensitivity PSA test (AccuPSA™) for clinical validation. The assay is based on Single Molecule Array (SiMoA) technology in which single molecules of PSA are detected and counted. The AccuPSA assay will represent the first fully verified and validated application of SiMoA technology for *in vitro* diagnostic purposes. The intended use of the test will be to accurately measure and monitor PSA levels in all RP patients as an aid in assessing risk of prostate cancer recurrence. The scope of this work was pre-verification testing to assess assay robustness prior to initiation of verification and clinical validation.

Methodology

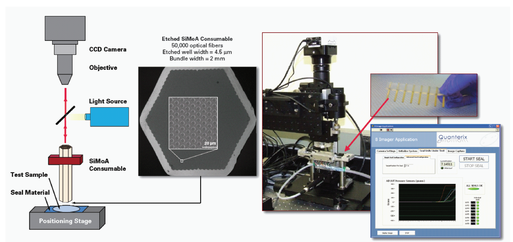
Single Molecule Array (SiMoA) Methodology

SiMoA technology is based on isolating single immunocomplexes of antibody, analyte, and a signaling enzyme in arrays of femtoliter wells. The arrays are sealed in the presence of enzyme substrate then fluorescently imaged. In contrast to typical ELISAs in which fluorescent product of the signal enzyme diffuses out into a bulk solution and is diluted, fluorescent product within each microwell is confined to a 50 femtoliter reaction volume, resulting in the buildup of a high concentration of the fluorescent product with each microwell. The concentrated fluorescent signal is strong enough that it can be easily detected using a standard fluorescence microscope (1, 2).

By using high-density arrays of femtoliter wells, hundreds to thousands of single immunocomplexes can be counted simultaneously. Isolation of single immunocomplexes with SiMoA gives rise to a dramatic increase in sensitivity over traditional bulk immunoassay detection methods. The AccuPSA assay is the world's most sensitive assay for PSA, with a limit of quantification (LOQ) of less than 0.00005 ng/mL (0.05 pg/mL or 50 fg/mL). The assay was used to measure PSA in the sera of 46 post-RP patients, all of which gave 'undetectable' results with a commercial immunoassay for PSA.

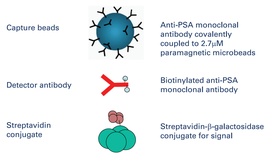


AccuPSA based on arrays of femtoliter wells. (A) Capturing and labeling single protein molecules on beads using standard ELISA reagents. (B) Loading of beads into femtoliter well arrays for isolation and detection of single molecules. (C) SEM image of a small section of a femtoliter well array after bead loading. 2.7-μm-diam. beads were loaded into an array of wells with diameters of 4.5 μm and depths of 3.25 μm. (D) Fluorescence image of a small section of the femtoliter well array after signals from single enzymes are generated.



Prototype SiMoA Instrumentation. Prototype SiMoA instrumentation was developed for assay validation and to support pharmaceutical and diagnostics collaborations. The SiMoA consumable is manufactured by etching tens of thousands of reaction vessels into the end of an optical fiber bundle. Strips of 8 fiber bundles are sized to sample one column of a microtiter plate, enabling convenient processing of up to 96-samples. The current bench-top instrument automatically seals the reaction vessels and concurrently reads the array of about 50,000 single molecule fluorescent assays. Sample preparation is done on a separate automated fluid handling workstation capable of processing hundreds of samples per shift.

Assay Components



Assay Sequence

Tecan Assay (96 well ELISA plates)

- Anti-PSA coated paramagnetic microbeads are mixed with sample and incubated.
- The microbeads are washed, and biotinylated anti-PSA detector antibody is added and incubated.
- The microbeads are again washed, and streptavidin:enzyme conjugate is added to form a labeled immunocomplex sandwich. Following incubation, the microbeads are washed a third time.

Loading and Imaging

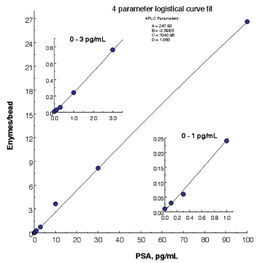
- Microbeads are loaded onto optical fiber microarrays and centrifuged at low speed for five minutes.
- Microarrays are washed and loaded onto the SiMoA imaging platform.
- Arrays are sealed against enzyme substrate (resorufin β-D-galactopyranoside, RDG).
- Arrays are interrogated for fluorescent product with five measurements of two seconds each. A rising level of fluorescence in the wells indicates the presence of labeled PSA molecules.

PSA Quantification

- Positive wells are counted as % active wells. At low PSA concentrations (up to approximately 5,000 molecules in the 50,000 well array) the wells contain either one or zero PSA molecules according to Poisson statistics. This results in a digital signal of either "positive" or "negative".
- The average intensity of the signal is converted to "average enzymes per bead", which reflects the average number of labeled PSA immunocomplexes bound to each microbead within the array. When every bead has at least one enzyme, counting is not possible and total fluorescence intensity is used to determine average enzyme per bead, thereby extending dynamic range.
- PSA concentrations in unknown samples are interpolated from a standard curve of "average enzymes per bead" vs. concentration of PSA calibrators.

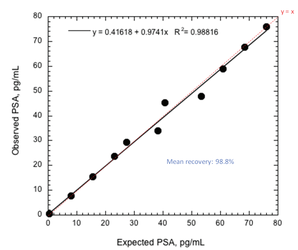
Results

Representative dose-response



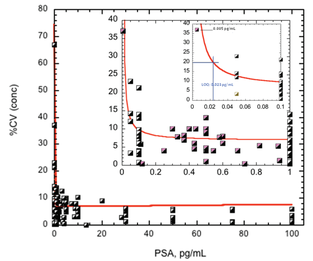
Data depict a dose-response curve (n = 3) obtained from a set of prepared WHO 90:10 standards. Y-axis units (enzymes/bead) refer to the average number of labeled immunocomplexes bound to the trapped microbeads within the array. Each labeled immunocomplex corresponds to a single molecule of PSA. The insets highlight the linear response of the test down to femtogram levels, where each microbead contains ether a single PSA molecule or no PSA molecules. Femtogram/mL sensitivity is possible due to extremely low background, which is in turn possible because of the low level of label enzyme needed to generate a measurable signal when trapped within a microwell.

Linearity



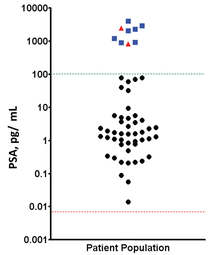
Linearity was assessed with guidance from NCCLS EP6-A. Admixtures of a 'high' spiked female serum sample and a very low female serum sample were prepared in the following ratios: 100% "high", 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, and 100% "low". Deviation from linearity at each admixture level was generally within 10%.

CV Profile and Limit of Quantification



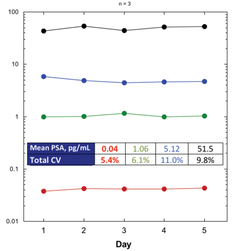
A characteristic of a SiMoA digital assay is a dose-response that results in a flat concentration CV profile extending down to fg/mL levels. Red lines depict the predicted concentration CV profile calculated from an assumed 7.1% measurement variation across the assay range (system specification) and the non-linear fit of the dose-response curve in the previous figure. The actual data represent a 6-week sampling of concentration CVs from triplicate determinations. The innermost inset shows the PSA concentration at which the CV function reaches 20%, giving an estimate of the limit of quantification (LOQ) of the assay. The predicted LOQ was 0.000023 ng/mL (0.023 pg/mL, or 23 fg/mL). Pink data points (◪) depict PSA levels measured in a set female serum samples. The lowest specimen assayed was 0.000005 ng/mL (0.005 pg/mL, or 5 fg/mL). The signal CV from triplicate replicates of this sample was 5.1%, but at this concentration, signal is approaching background, which gives rise to a relatively higher concentration CV.

Post radical prostatectomy samples



PSA was measured in 46 serum samples from patients who had undergone radical prostatectomy, RP (◆), healthy control samples (■), and Bio-Rad PSA controls (▲). All RP samples had "undetectable" PSA levels as reported by a leading clinical diagnostic assay (ADVIA Centaur). The green line represents the detection limit of the Centaur assay (0.1 ng/mL, or 100 pg/mL). All 46 patient samples were above an estimated detection limit of the AccuPSA test, shown by the red line (0.000006 ng/mL, 0.006 pg/mL, or 6 fg/mL), The lowest patient PSA concentration measured was 0.000014 ng/mL (14 fg/mL).

5-Day Reproducibility



Reproducibility was studied by repeatedly assaying a panel of "high" (50 pg/mL), medium (5 pg/mL), low (1.0 pg/mL) and ultra-low (0.05 pg/mL, 0.00005 ng/mL) spiked PSA samples over a five-day period. Each result depicted is the mean of three replicates. The excellent reproducibility of the results from the 0.05 pg/mL panel (< 6% total CV) is consistent with the LOQ estimate from the CV profile analysis (0.02 pg/mL).

Conclusion

Reproducibility robustness at extremely low PSA concentrations is essential for an ultra sensitive assay for monitoring RP patients. The data shown here indicate excellent reproducibility (between-day CV < 6%) at PSA values as low as 0.00004 ng/mL (40 fg/mL). This represents unprecedented sensitivity for reliably measuring and monitoring PSA levels in all radical prostatectomy patients, including those with very low nadir values. This could enable detection of fast recurrence of prostate cancer significantly earlier than currently available PSA tests. This level of sensitivity could also enable stratification of recurrence probability among patients with nadir PSA levels that are undetectable by current methods. Reagent lot diversity testing (not shown) indicates the test will maintain the required performance level over time for reliable monitoring. The analytical performance of the test in pre-verification studies supports its use to potentially enable earlier detection of prostate cancer recurrence and eliminate unnecessary treatment for patients unlikely to recur. The utility of the test for this intended use will be established in an upcoming clinical validation study.

References

(1) Rissin, D. M. & Walt, D. R. Digital Concentration Readout of Single Enzyme Molecules Using Femtoliter Arrays and Poisson Statistics, *Nano Letters* 2006, 6, 520-523.

(2) Rissin, D.M. et al. "Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations", *Nature Biotechnology* 28, 595 - 599 (2010)

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