Direct Detection of Bacterial DNA and viral RNA at Subfemtomolar Concentrations Using Single Molecule Arrays (Simoa)

Song L, Shan D, Zhao MW, Pink BA, Minnehan KA, York L, Gardel M, Sullivan S, & Duffy DC.
Quanterix Corp, 113 Hartwell Ave, Lexington, MA 02421 USA

Introduction

The sensitive measurement of nucleic acids (NA) is important in a number of fields such as clinical diagnostics, environmental monitoring, detection of biological threats, and food safety. The detection and quantification of NA at low concentrations from viruses and bacteria is especially important in the early diagnosis of infectious diseases and testing of blood supplies. We report the development of a method based on the direct detection of single molecules of DNA and RNA extracted from bacteria or viruses that enables sensitive measurements suitable for these applications. We have demonstrated the detection of genomic DNA purified from *S. aureus and* genomic RNA purified from Sendai virus, both with an average limit of detection (LOD) of less than 0.1 fM (equivalent to 3,000 DNA/RNA molecules per 50 µL sample). We also applied this Simoa technology to clinical and environmental samples for bacteria detection with subfemtomolar sensitivity. The capability of detecting single enzyme molecules using Simoa enables the direct detection of target DNA and RNA without requiring target amplification, and provides a highly sensitive alternative approach to polymerase chain reactions (PCR) that is much less susceptible to carryover.

Methodology

Detection of nucleic acids using Simoa is based on first generating short fragments from large genomic DNA/RNA molecules either via random sonication or digestion using specific restriction enzymes. For DNA molecules, heat-denaturation is used to generate single-stranded fragments for capture. The melted single-stranded DNA fragments or RNA targets are exposed to complementary capture probes that are covalently conjugated on paramagnetic beads and multiple biotinylated detection probes for hybridization. The resulting hybridized DNA/DNA or DNA/RNA complexes are then labeled with an enzyme (streptavidin-conjugated beta-galactosidase). Tens of thousands of Individual beads, with or without hybridized complexes, are then loaded and isolated into individual femotoliter-sized microwells in the presence of enzyme substrate, sealed, and arrays of microwells are imaged at the characteristic fluorescence of the enzymatic product. The fraction of beads associated with at least one enzyme is then determined by counting and related to target concentration via Poisson statistics. At low concentrations, where there are fewer labeled target molecules than beads, there are a significant fraction of beads associated with single molecules. At high concentrations, each bead carries multiple enzyme labels, and the average number of enzyme labels per bead (AEB) is determined from the ratio of average fluorescence intensity over all beads in an array to the average fluorescence intensity produced by a single enzyme. The concentration of DNA is quantified by determining the average number of enzyme per bead (AEB) via Poisson statistics (digital) or average bead intensity (analog).

Assay work flow

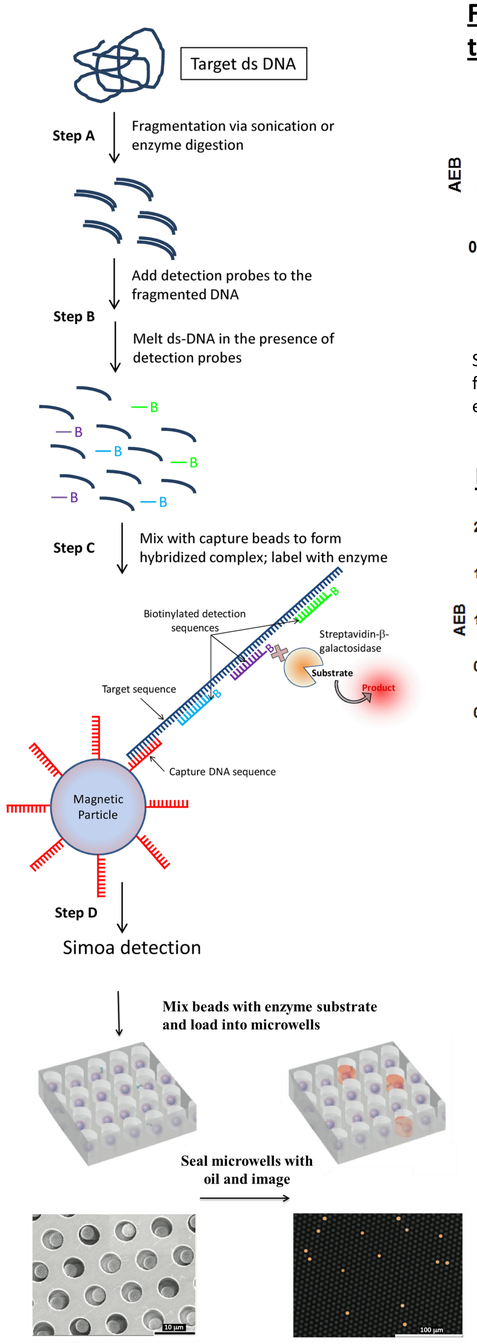
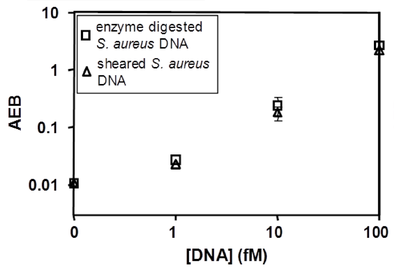


Figure 1. Schematic of the process for the direct detection of genomic DNA targets using Simoa.

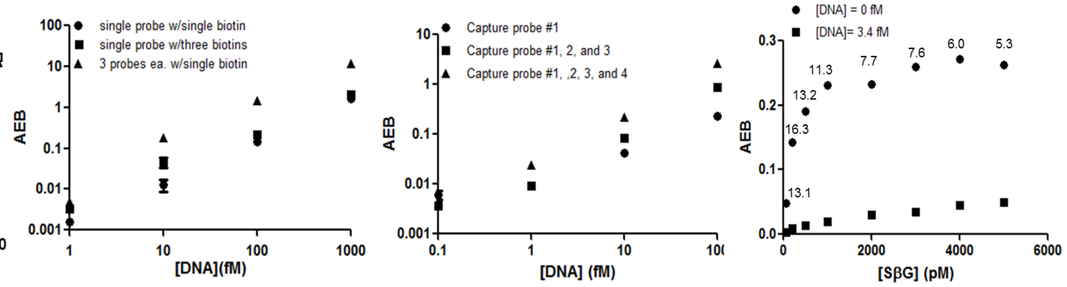
Results

Fragmentation of DNA/RNA targets (step A)



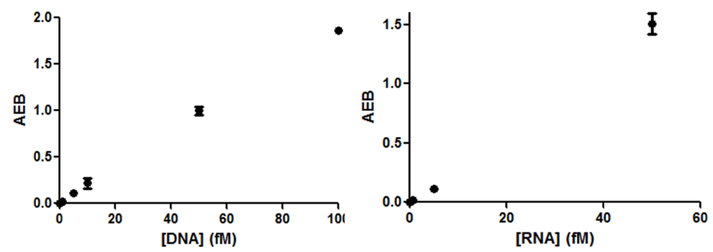
Simoa detection of genomic DNA of S. aureus fragmented using sonication and restriction enzymes.

Capture and labeling of hybridized complexes on beads (Step B and C)



Left: Measurement of plasmid DNA using three different approaches to the detection probe. **Middle:** Measurement of genomic DNA purified from S. aureus using three different combinations of capture probes. **Right:** Plots of AEB against concentration of SβG; the highest signal-to-background ratio as indicated above the symbol for the 3.4 fM data was 16.3 observed at [SβG] = 200 pM.

Dose response curves and analytical sensitivity



Dose response curves for purified genomic DNA of S. aureus (left) and RNA from Sendai virus (right) generated using Simoa.

Summarized limits of detection (LOD) for both purified DNA and RNA samples

	purified *DNA* from S. aureus		purified *RNA* from Sendai virus	
# of run	LOD*, fM	LOD, DNA molecules/ 100ul	LOD*, fM	LOD, RNA molecules/ 100ul
1	0.02	1200	0.06	3600
2	0.094	5640	0.06	3600
3	0.094	5640	0.087	5220
4	0.015	900	0.098	5880
5	0.08	4800	0.061	3660
6	0.025	1500	0.059	3540
7	0.08	4800	0.076	4560
8	0.09	5400	0.01	600
9	0.036	2160	0.008	480
10	0.086	5160	0.004	240
average	**0.062**	**3720**	**0.052**	**3138**

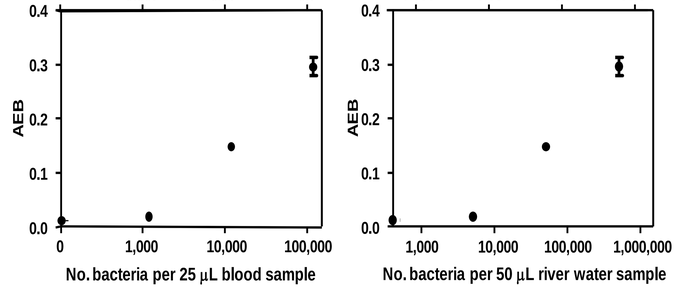
*3xSD above background

Comparable sensitivity to real-time PCR

Technology	Strain of bacteria	Target gene	LOD, fM	LOD, number of molecules	Sample volume, µl
Simoa	*S. au* 25923	*nuc*	**0.062**	**1860**	**50**
Heminested RT-PCR[a]	*S. au* 25923	*nuc*	**0.083**	**50**	**1**
RT-PCR[b]	*S. au* 25923	*nuc*	**0.5-1**	**1200-2400**	**4**

[a]: Banada, P. P., *etc*. PLoS One 2012, 7 (2), e31126. [b]: results from internal tests using real-time PCR

Detection of *S. aureus* spiked in environmental and clinical samples



Bacteria of *S. aureus* were spiked into 25 µL whole blood from a human (left) and 50 mL of water from the Charles River (right), and then detected using the Simoa DNA assay. The LODs were ***0.026 fM***, or ***396 DNA molecules*** per 25 µL of whole blood sample; and ***0.042 fM***, or ***1, ,271 DNA molecules*** per 50 µL sample of river water.

Conclusion

Simoa shows promise for the sensitive detection of genomic DNA/RNA in complex samples.. The process of DNA fragmentation, melting, capture, and labeling is highly efficient, making Simoa a highly sensitive DNA assay. The Simoa DNA assay represents a complementary approach to methods based on amplification using polymerases, such as PCR, and can be employed in situations where PCR is deficient.

References:
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D. Rissin *etc*. *Anal. Chem.* 2011, 83, 2279-2285.
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