Analysis of sensitivity and rapid hybridization of a multiplexed Microbial Detection Microarray

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LLNL-JRNL-594212

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This work performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

ABSTRACT

Microarrays have proved to be useful in rapid detection of many viruses and bacteria. Pathogen detection microarrays have been applied to diagnosis of viral and bacterial infections in clinical samples and to evaluate the safety of biological drug materials. We developed a multiplexed pathogen detection microarray, a modified version of the Lawrence Livermore Microbial Detection Array and evaluated the sensitivity of the array against viruses, analyzed different whole genome amplification protocols to detect a mixture of viral and bacterial agents, and also analyzed the performance of the array with rapid 1 hr hybridization. The array detected 2 ng of a DNA virus with 1 hr hybridization without any amplification, and is able to identify a mixture of viruses and bacteria. The array can detect 20 fg or 100 genome equivalents of a DNA virus when combined with random whole genome amplification. This multiplexed microarray is an efficient tool to analyze larger number of samples for rapid detection and identification of viral and bacterial pathogens.

INTRODUCTION

Rapid detection and characterization of bacterial and viral pathogens is important for public health, drug and food safety. Various detection technologies based on nucleic acid signatures have emerged in the past few years, including TaqMan and Luminex bead based systems. While these technologies are able to rapidly identify selected pathogens at the species or strain level, they do not have the capability to provide broad functional information about known or novel organisms. Characterization of known, emerging, engineered, or unknown pathogens requires a platform that has the capacity to assess whole genome sequence content from a variety of pathogens very rapidly. To meet this need, we developed the Lawrence Livermore Microbial Detection Array (LLMDA) which contains 388,000 DNA probes [1]. This array can currently detect any of the sequenced viruses or bacteria within 24 hours. In addition, the long (50-65 base-pair) oligonucleotide probes were selected to enable detection of novel, divergent species with homology to sequenced organisms. LLMDA has been used in a number of applications including biodefense, human and animal health and food and product safety, where broad detection of specific subsets of microbial organisms is desired. We recently used this array to identify a contaminating pig virus from a rotavirus vaccine [2], a vaccine used worldwide in infants to prevent rotavirus infection. We have also used this array to detect viral infections from various human clinical samples [3].

Recent development of multiplexed microarrays makes screening of larger number of samples more cost-effective and higher-throughput. To test the functionality of a multiplexed microarray, we developed a modified version of the LLMDA, using the 4x72K Roche NimbleGen format of the microarray. In this array, we have a total of 72,000 of viral and bacterial probes with an average of 30 probes per viral genome and 15 probes per bacterial genome. We performed testing to determine limit of detection of both amplified and unamplified viruses, a mixture of viral and bacterial templates and we also tested the array using known or unknown clinical samples. We analyzed various amplification techniques and also evaluated the use of the array for clinical sample analysis.

We could detect down to 500 pg (or 1.3×10^7 copies) of virus with 17 hr hybridization. Rapid 1 hr hybridization could detect targets present with less than 2 ng (or 5.2×10^7 copies) of sample without any prior whole genome amplification. When combined with whole genome

amplification, 100 genome equivalents of a DNA virus can be detected on the 72K LLMDA array. Bacterial and viral organisms could be accurately identified to the strain level if the target strain had been sequenced, and to the species level for unsequenced strains.

MATERIALS AND METHODS

Probe design for the 72k LLMDA array

Microarray probe design was described in Gardner et al. [1]. The probes were down-selected to have a total of 70,916 probes total, instead of the previously reported 388,000 probes, as indicated in Table 1. All complete viral and bacterial genomes, segments, and plasmids available as of spring 2007 were used as targets for probe design. Each viral genome or segment component was represented by 10-20 probes on the 72K array, in contrast to the 50 probes per component in the larger 388K version. The same process was used to downselect from the candidate pool of probes as was described in Gardner et al. 2010, favoring probes that were more conserved within the target family and breaking ties by picking the most distant probe in a target genome from other probes that were already selected for that target, building up the total until all viral genomes and segments were represented by the specified (10 or 20) number of probes. The same bacterial probes were used as on the 388K version, with 15 probes per genome. The arrays were custom ordered using the 4-plex 72K arrays through Roche NimbleGen.

Nucleic acid extraction from bacterial and viral samples

Adenovirus type 7 strain Gomen (Adenoviridae) and Respiratory syncycial virus (RSV) was purchased from the National Veterinary lab and grown at our laboratory. For purification of adenovirus DNA, a solution of 10mL virus culture, 1mL 5.5% Triton X-100, and 460µL 0.5M EDTA was added to a 50mL tube and vortexed vigorously. Following vortexing, 1.28mL 10% SDS and 540µL 10U/mL proteinase K was added to the mixture and incubated at 55°C for 1 hour with mixing every 10 minutes. After incubation, 420µL 5M NaCl and 13.7mL 25:24:1 phenol/chloroform/isoamyl alcohol was added to the tube and inverted until completely homogenous. Once homogenous, the solution was centrifuged at 10,000 rpm for 10 minutes and

the upper aqueous layer was transferred to a new tube. A total of 27.4mL of 100% ethanol was added to the aqueous layer and incubated at -20°C for 1 hour. Following incubation, the solution was centrifuged at maximum speed for 10 minutes and the supernatant was discarded. The pellet was washed once with a solution of 70% ethanol and 150mM NaCl. The liquid was removed and pellet was allowed to air dry before being re-suspended in nuclease free water.

For extraction of RNA from RSV virus, a solution of 2mL of virus culture and 6mL TRIzol LS Reagent from Life Technologies (#10296-010) was mixed vigorously and incubated 15 minutes at room temperature. Following incubation, 1.6mL chloroform was added to the cap of the tube, shaken vigorously for 15 seconds, and incubated at room temperature for 15 minutes. The solution was centrifuged at 4°C for 15 minutes at 3000xg and the aqueous layer was transferred to a new tube. 70% isopropyl alcohol was added to the aqueous layer, mixed by inverting several times, and incubated for 10 minutes at room temperature. The solution was centrifuged at maximum speed for 10 minutes at 4°C and the liquid was carefully poured off. The remaining pellet was washed with 70% ice cold ethanol. The ethanol was poured off and the pellet was air dried before being re-suspended in RNase free water.

Ebola Zaire 95 and Rift Valley Fever (RVF) virus RNA were obtained from National Bioforensic Analysis and Countermeasures Center (NBACC). Copy number quantitated Vaccinia Lister DNA was purchased through Advanced Biotechnologies Inc. (Columbia, MD). *B. anthracis* Ames DNA and *Salmonella enterica* DNAs were obtained from internal sources at LLNL. Influenza virus, Aichi virus, Rotavirus A, Human adenovirus type 7 vaccine strain were cultured at the Taiwan Center for Disease control and nucleic acids were extracted.

Nucleic acid amplification

The clinical samples were amplified using four different protocols: a protocol developed by Wang et al., 2003 [4], here referred to as Random PCR1, the same protocol with modified primers [5], referred to as Random PCR2, the TransPlex® Whole Transcriptome Amplification Kit (Cat. WTA1) from Sigma Aldrich (St. Louis, MO) [6] and QuantiTect Whole Transcriptome Kit (Cat. 207043) from Qiagen (Valencia, CA) [3,7]. One ng of each DNA and RNA from viruses including Ebola Zaire 95 (9.34×10^7 genome copies), Vaccinia Lister (4.76×10^6 genome

copies), *B. anthracis* Ames (1.77 x 10^5 genome copies) and RVF (1.48 x 10^8 genome copies) were mixed and amplified using the four protocols respectively.

Sample preparation for limit of detection testing

Vaccinia Lister DNA was 10-fold serially diluted from 100,000 copies to 100 copies for limit of detection testing without any whole genome amplification. The viral DNA was also 10 fold serially diluted from 10,000 copies to 10 copies and amplified using the Random PCR2 protocol, and the entire amplified product was hybridized to the array. Copy number quantitation was performed by the manufacturer by real time PCR using the Roche LightCycler.

RSV was titered at LLNL and tissue culture infectious dose 50 ($TCID_{50}$) was determined. The viral RNA was 10-fold serially diluted from 1,000,000 $TCID_{50}$ to 1000 $TCID_{50}$ and reverse transcribed into cDNA. Double-stranded cDNA was synthesized according to the Invitrogen SuperScript® Double-Stranded cDNA Synthesis Kit (Cat. 11917-010) instructions. RSV RNA was also 10 fold serially diluted from 100 $TCID_{50}$ to 0.1 $TCID_{50}$ and amplified using Random PCR2 amplification. The entire amplified product was labeled and hybridized to the 4plex arrays.

For limit of detection of adenovirus Gomen strain DNA, the concentration of fluorescently labeled DNA was measured by NanoDrop spectrophotometer. The input quantity to the labeling reaction was 250 ng (6.52 x 10^9 copies). Because the DNA concentration is not quantified by real time PCR assay, the DNA concentration measured could also include cells where the virus was grown, therefore the actual adenoviral DNA amount could be much less than the expected. Following labeling, approximately 16 µg (4.16 x 10^{11} copies) of Cy3-labeled DNA was obtained. The labeled sample was serially diluted 2 fold from 2000 ng (5.22 x 10^{10} copies) to 0.5 ng (1.3 x 10^7 copies) and hybridized to the arrays.

***S. enterica* DNA preparation**

To evaluate the specificity of the 72K LLMDA, 8 serotypes of *Salmonella enterica* Typhi, Paratyphi A, Typhimurium, Agona, Javiana, SaintPaul, Kentucky, Heidelberg were directly fluorescently labeled using 500 ng of DNA each. Genomic DNA was sonicated for three

30-second intervals prior to fluorescent labeling. One µg of each labeled DNA was hybridized to the 72K LLMDA.

Microarray hybridization

DNA microarrays were synthesized by Roche NimbleGen. This array has 388K probes total on one slide that's divided into four sub-arrays with 72K probes on each array. Four separate samples can be run on the 4-plex array simultaneously to save time and cost. Amplified or unamplified DNA or cDNA samples were fluorescently labeled using the Roche NimbleGen One-Color DNA Labeling Kit (Cat. 05223555001) according to the recommended protocols. The labeled sample was purified after labeling, and hybridized using the NimbleGen Hybridization Kit (Cat. 05583683001) to the 4plex 72K array according to manufacturers' instructions. The microarrays were allowed to hybridize for 17 hours or 1 hr, and washed using the NimbleGen Wash Buffer Kit (Cat. 05584507001) as described [1]. Microarrays were scanned on an Axon GenePix 4000B 5 µM scanner from Molecular Devices (Sunnyvale, CA). The array experiments for the four samples from the Taiwan Center for Disease Control were performed at the Taiwan CDC following a similar protocol.

Microarray data analysis

Microarray data was analyzed using the Composite Likelihood Maximization method (CLiMax) as described [1]. The target database for the 4plex 72K array was built at a later date then 388K version of the LLMDA. Probes with signal intensity at 99% above the random control were used for data analysis.

RESULTS:

Comparison of different amplification protocols to amplify a mixture of pathogens

We performed four different random amplification protocols on a mixture of bacterial and viral RNA/DNA samples including *B. anthracis* Ames, Rift Valley Fever virus, Vaccinia Lister, Ebola virus Zaire 95 to evaluate the efficiencies of the different protocols. The significant

viral and bacterial hits detected on the array from different amplification methods are shown in Table 2. The log-odds scores for each of the target are also listed.

All four amplification protocols correctly identified Ebola Zaire 95 and RSV to the species level. All three segments, S, L and M of the RSV were detected on arrays using all four methods. Random PCR 1 and 2 and Qiagen whole transcriptome kit identified *Bacillus anthracis* to the species level. Sigma transplex kit detected as *B. cereus* as top hit instead, though *B. cereus* and *B. anthracis* are closely related phylogenetically. Random PCR2 and the Sigma Transplex kit identified Vaccinia virus as top hit correctly. Random PCR1 and Qiagen WTA identified Cowpox virus as top hit, and variola virus was also detected as a second hit. Random PCR2 appeared slightly better in accuracy of detection, though the four kits performed similarly. In this paper, we used random PCR 2 to determine the detection limit of amplified pathogens on the microarray.

Analysis of *Salmonella enterica* subspecies enteric serovar on microarrays

We hybridized eight *Salmonella enterica* DNAs on the 4plex 72K MDA array including Typhi, Paratyphi A, Typhimurium, Agona, Javiana, SaintPaul, Kentucky, Heidelberg. The Salmonella enterica serovars detected on the array is listed in Table 3. The array correctly identified Typhi, Paratyphi A, Typhimurium and Agona. The top hit for the array hybridized with Javiana was Typhimurium, with Javiana detected after Typhimurium and Kentucky. The array hybridized with SaintPaul was predicted to be Kentucky. The full genomic sequence for SaintPaul has not been published, so we may not have probes that are specific to this strain. The top hit for the array hybridized with Kentucky was Choleraesuis, with Kentucky as the second highest hit. The top hit for the array hybridized with Heidelberg was Typhimurium, with Heidelberg detected a second highest hit.

Limit of detection of unamplified vaccinia virus and RSV

Vaccinia Lister DNA at 100 copies, 1000 copies, 10,000 copies and 100,000 copies were applied the 72K MDA array to analyze the limit of detection. Figure 1 shows the sensitivity of

the array at different Vaccinia concentrations. The Y axis is calculated using the percentage of probes specific to Vaccinia virus that were detected on the array. Two replicate experiments were performed. The data was able to detect Vaccinia Lister DNA at 100,000 copies of the viral DNA.

RSV cDNA at 1000, 10,000, 100,000 and 1,000, 000 $TCID_{50}$ were applied to the 72K MDA array to analyze the limit of detection. Figure 2 shows the sensitivity of the array at different RSV concentrations. Two replicate experiments were performed. The array was able to detect RSV cDNA at 100,000 $TCID_{50}$.

Limit of detection of amplified vaccinia virus and RSV

Vaccinia Lister DNA at 10 copies, 100 copies, 1,000 copies and 10,000 copies concentration were amplified using the random PCR method and the amplified samples were applied the 72K MDA array to analyze the limit of detection. Figure 3 shows the sensitivity of the array at different Vaccinia concentrations after the random amplification. Two replicate experiments were performed. The array was able to detect Vaccinia Lister DNA at 100 copies of the viral DNA.

RSV cDNA at 0.1, 1, 10 and 100 $TCID_{50}$ were applied to the 72K MDA array to analyze the limit of detection. Figure 4 shows the sensitivity of the array at different RSV concentrations. Two replicate experiments were performed. The array was able to detect RSV cDNA at 1 $TCID_{50}$.

Detection of adenovirus after 1 hr and 17 hr hybridization

After labeling the adenovirus DNA, a 2 fold serial dilution of the labeled DNA was performed from 2 µg to 0.5 ng and the diluted DNA was hybridized to the arrays for 17 hr. The signal of adenovirus is shown in Figure 5A. At 0.5 ng, adenovirus type 3 strain NHRC 1276 was detected as top hit, instead of the type 7 Gomen strain. At 1 ng and above, the top hit was the type 7 adenovirus Gomen strain. To analyze the speed of detection using this microarray, we also performed hybridization of adenovirus type 7 Gomen strain for 1 hr. Serially diluted DNA from 2 µg to 0.5 ng of DNA was hybridized to the array and data analyzed (Figure 5B). Adenovirus

type 7 Gomen strain was positively identified at 8 ng of DNA after 1 hr hybridization as compared to 1 ng of DNA with 17 hr hybridization.

DISCUSSION

Microarrays, along with PCR and DNA sequencing are the most popular methods for microbial detection and discovery using nucleic acid samples. Microarrays occupy a middle ground between low cost, narrowly focused assays such as multiplex PCR and more expensive, broad-spectrum technologies like high-throughput sequencing. Several groups have applied microarray technology to pathogen detection for clinical diagnostics, food safety testing, environmental monitoring and biodefense [8,9]. We developed a multiplexed pathogen detection microarray, a modified version the previously reported Lawrence Livermore Microbial Detection Array to further improve the efficiency, cost-effectiveness and throughout of pathogen detection microarrays.

In this study, we evaluated the detection limit of the array by using serially diluted DNA virus, Vaccinia Lister, and a RNA virus, RSV. Without whole genome amplification, the detection limit for RSV is 100,000 (or 10^5) $TCID_{50}$. This is similar in range to other molecular assays such as the real time PCR assay, multiplex ligation-dependant probe amplification (MLPA) and a dual priming oligonucleotide system (DPO) where the lowest concentration detected for RSV was 10^5 $TCID_{50}$ [10]. For Vaccinia Lister DNA, the array was able to detect 100,000 genome copies or about 20 pg of Vaccinia DNA.

We found that the limit of detection for vaccinia virus DNA is 100 copies when random whole genome amplification was used. This detection limit is similar in range to PCR based technologies, though not as sensitive as PCR based technologies. A study using real time PCR assays to identify variants of Vaccinia virus was able to detect 10 fg or around 50 genome copies of vaccinia DNA [11]. The difference in sensitivity between PCR and microarray is partially due to the random amplification, using random primers, instead of a viral specific PCR amplification. Our detection limit for the RSV virus is around 1 $TCID_{50}$ when coupled with whole genome amplification. This sensitivity is similar to multiplexed PCR based Luminex assays. A group that developed a Luminex bead assay panel to detect 20 human respiratory viruses reported a detection limit of 1 $TCID_{50}$ for RSV type A and 10 $TCID_{50}$ for RSV type B [12].

We analyzed four different amplification protocols, two of which use a random PCR based approach, the Qiagen protocol uses Phi29 DNA polymerase based approach, and the Sigma transplex kit also uses PCR based amplification. When we applied the four different protocols to a mixture of four viral and bacterial pathogens, three of the four pathogens were correctly identified. There didn't appear to be a significant difference between the four amplification protocols.

In collaboration with other institutions, we tested several viral samples and human clinical samples on the 4plex 72K MDA array, and the array was able to positively identify the virus present in the sample, confirmed the PCR or culture results.

The primary goal for the MDA approach was to identify viruses or bacteria to the family or species level. Some array experiments showed that for those bacteria or viruses that have been fully sequenced, the array can detect up to the strain level. We tested eight different *Salmonella enterica* serovar samples, and the array positively identified to the strain level, four of the eight samples. One of the strains (SaintPaul) was not sequenced before. So it is likely that the array doesn't have any specific probes to detect this strain. This analysis showed that the array can not only be applied for species level detection of food borne pathogens, but potentially used for genotyping of food borne pathogens and track food borne pathogen outbreaks. For even higher resolution of strain level detection, using a microarray with probes to detect whole genome wide single nucleotide polymorphisms is another option.

Multiplexed microarrays present a new opportunity for high-throughput and cost-effective screening of thousands of microbial species. This new format of microarray will be more advantageous over PCR or multiplex based PCR assays in cost and throughput. Further advances in array technology such as automated array sample loading and image scanning; faster hybridization times; label-free methods to detect probe-target binding will broaden the applications of microarrays even further. Ultimately, microarrays could be advanced into point-of-care devices which can deliver results in less than an hour.

ACKNOWLEDGEMENTS

This work performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

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FIGURE LEGENDS

Figure 1. Limit of detection of unamplified Vaccinia Lister viral DNA on the 72K MDA array. Figure 1 shows the sensitivity of the array at different Vaccinia Lister virus DNA concentrations. The Y axis is calculated using the percentage of probes specific to Vaccinia virus that were detected on the array. Two replicate experiments (shown in orange diamond and green cross) were performed.

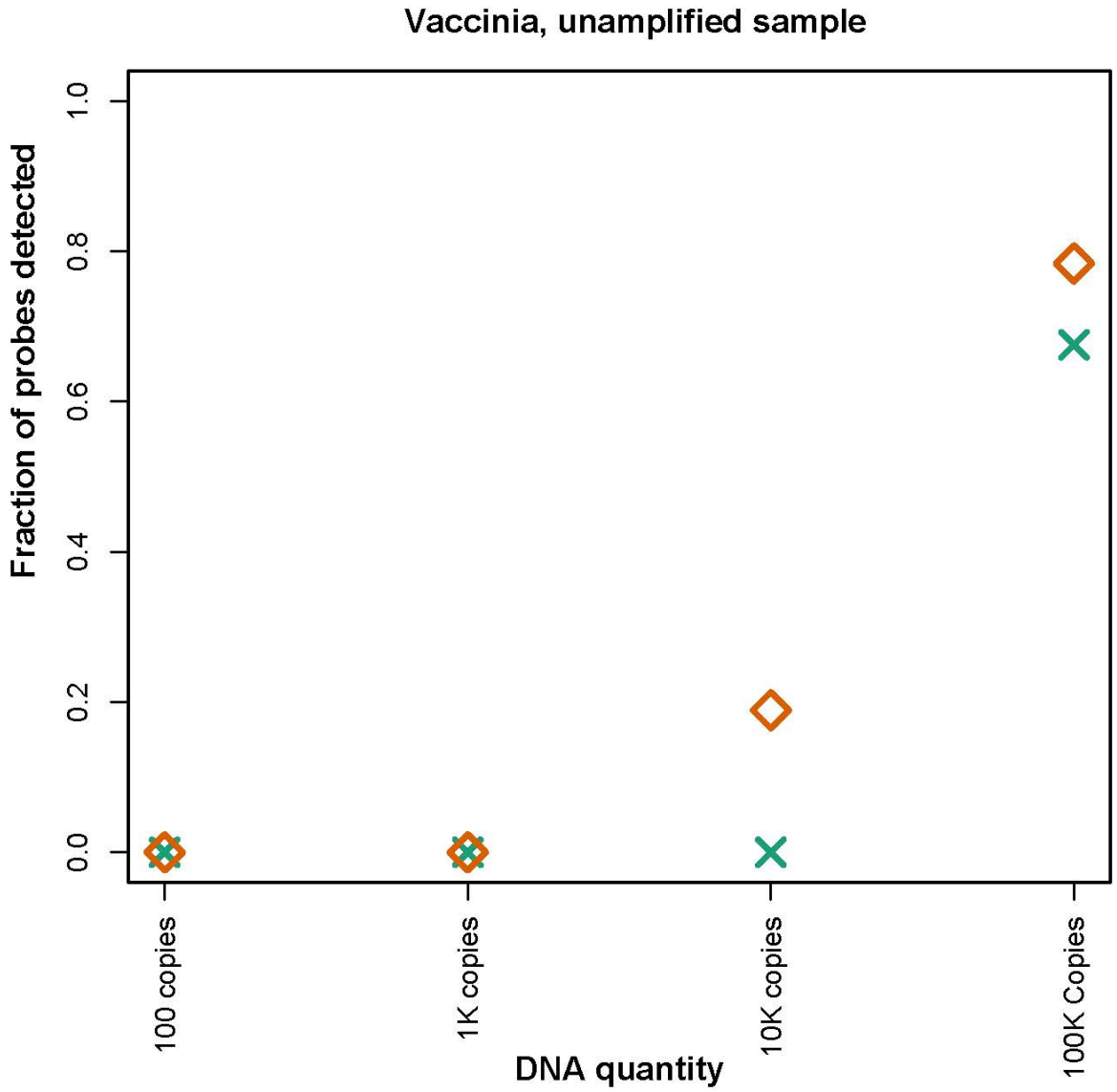

Figure 2. Limit of detection of unamplified RSV cDNA on the 72K MDA array. This figure shows the sensitivity of the array to detect RSV virus cDNA at different RSV TCID50 concentrations. The Y axis is calculated using the percentage of probes specific to RSV virus that were detected on the array. Two replicate experiments (shown in orange diamond and green cross) were performed.

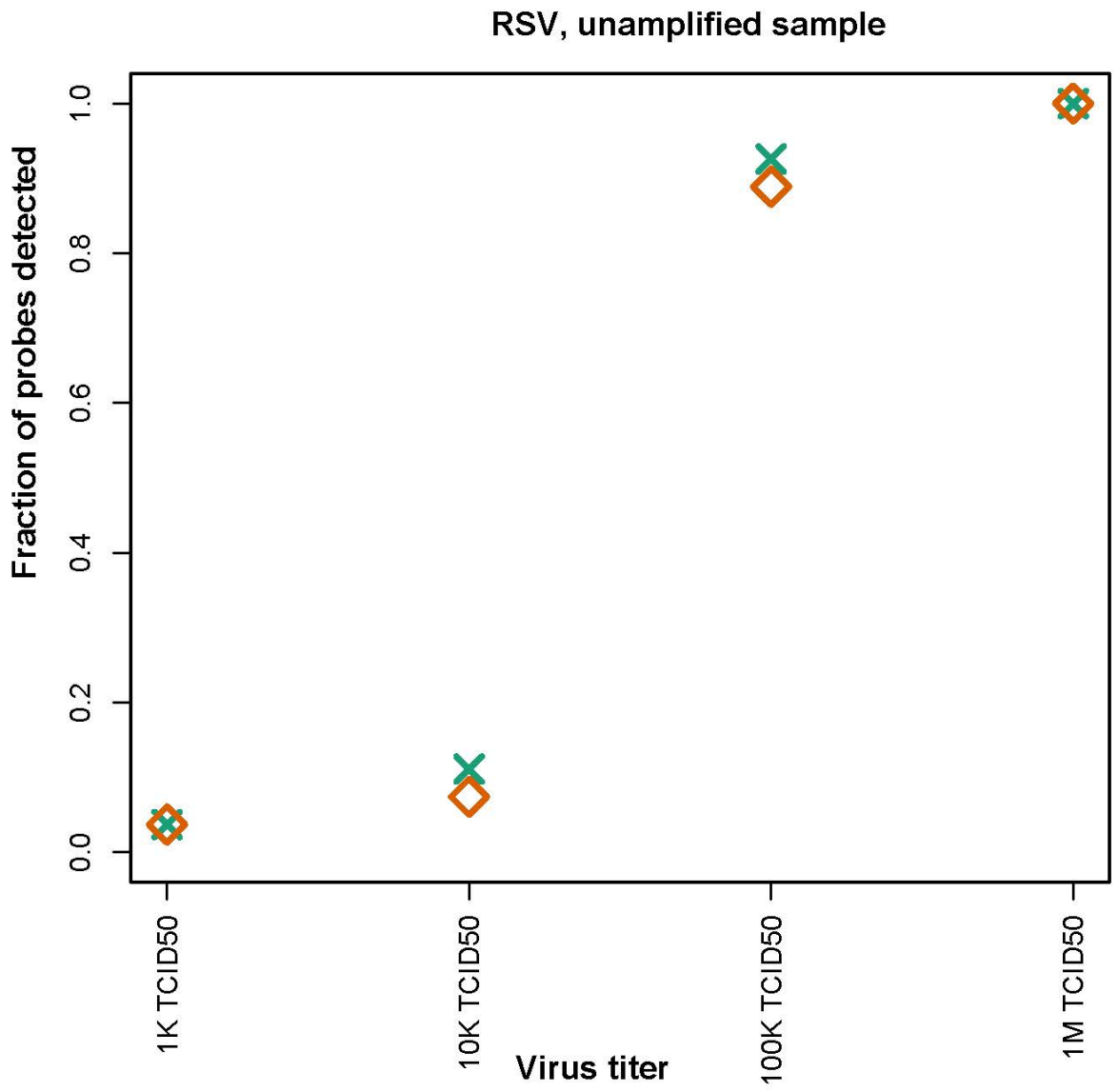

Figure 3. Limit of detection of amplified Vaccinia Lister viral DNA on the 72K MDA array. This figure shows the sensitivity of the array at different Vaccinia Lister virus DNA concentrations, after the DNA was amplified using random amplification. The Y axis is calculated using the percentage of probes specific to Vaccinia virus that were detected on the array. Two replicate experiments (shown in orange diamond and green cross) were performed.

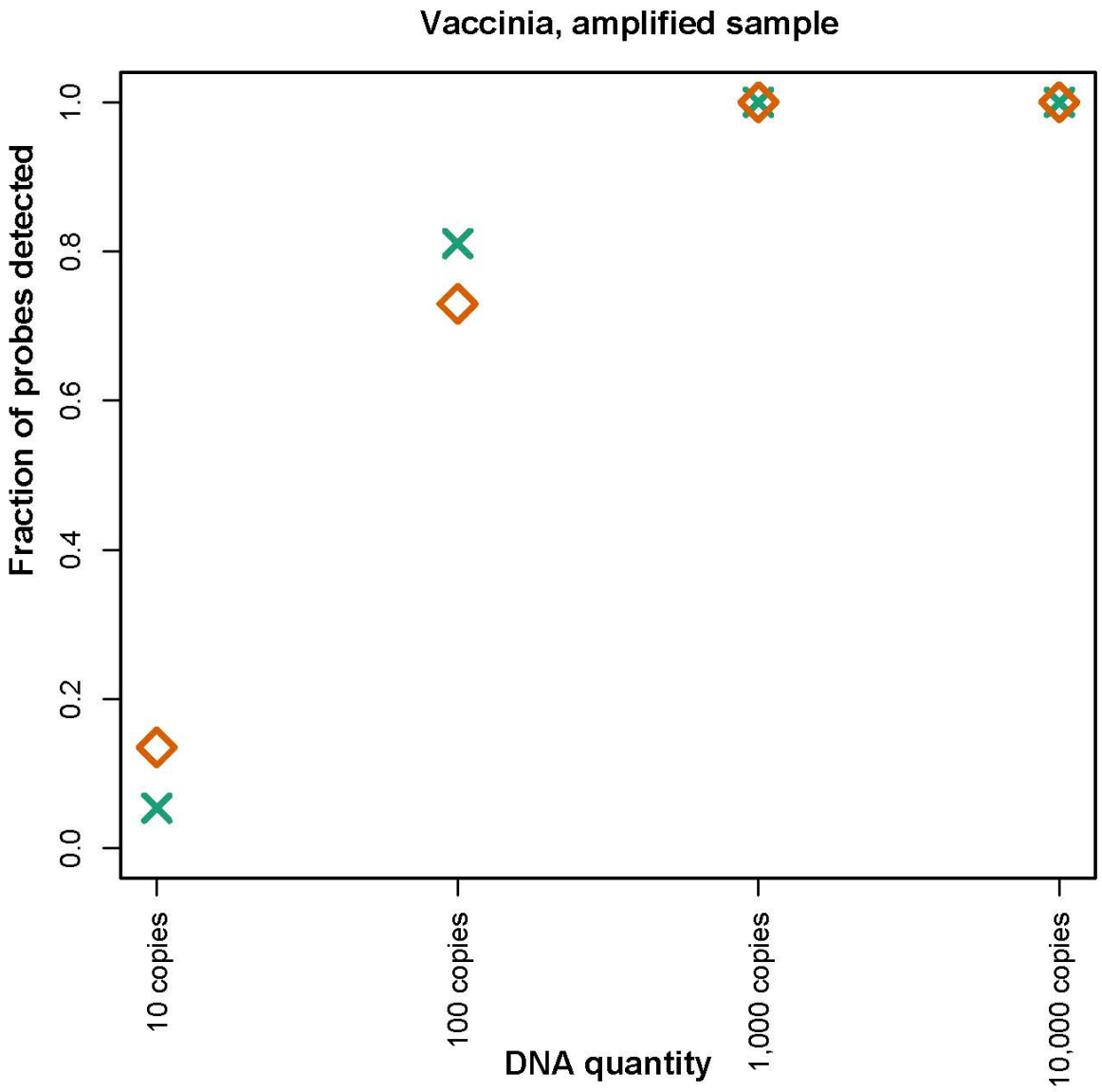

Figure 4. Limit of detection of amplified RSV cDNA on the 72K MDA array. This figure shows the sensitivity of the array to detect RSV virus cDNA at different RSV TCID50 concentrations, after the cDNA was amplified using random amplification. The Y axis is calculated using the percentage of probes specific to RSV virus that were detected on the array. Two replicate experiments (shown in orange diamond and green cross) were performed.

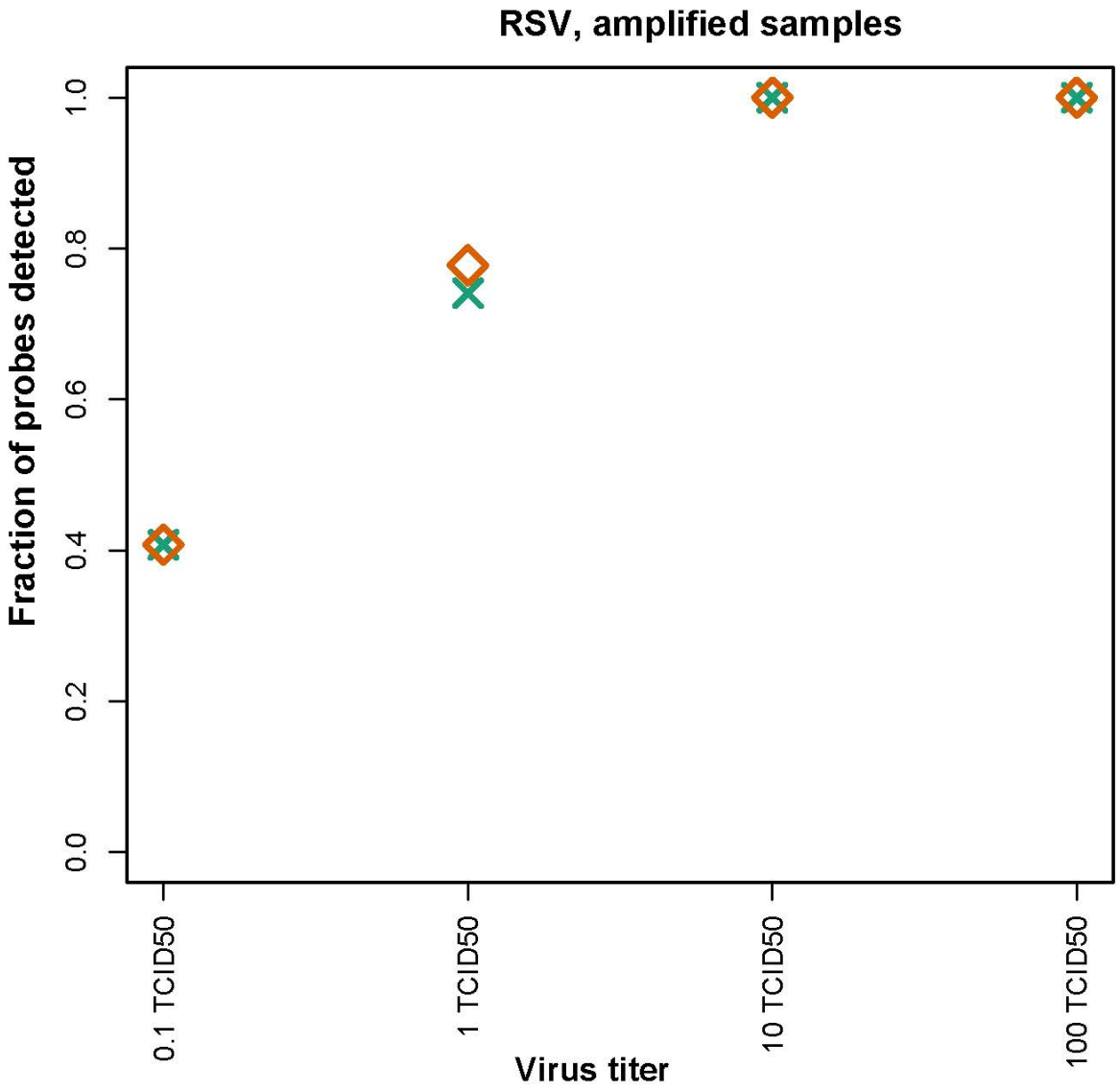

Figure 5A. Detection of Adenovirus DNA after 17 hr hybridization on the 72K MDA array. The Y axis is calculated using the percentage of probes specific to adenovirus that were detected on the array. Only one replicate experiments (shown in green cross) was performed.

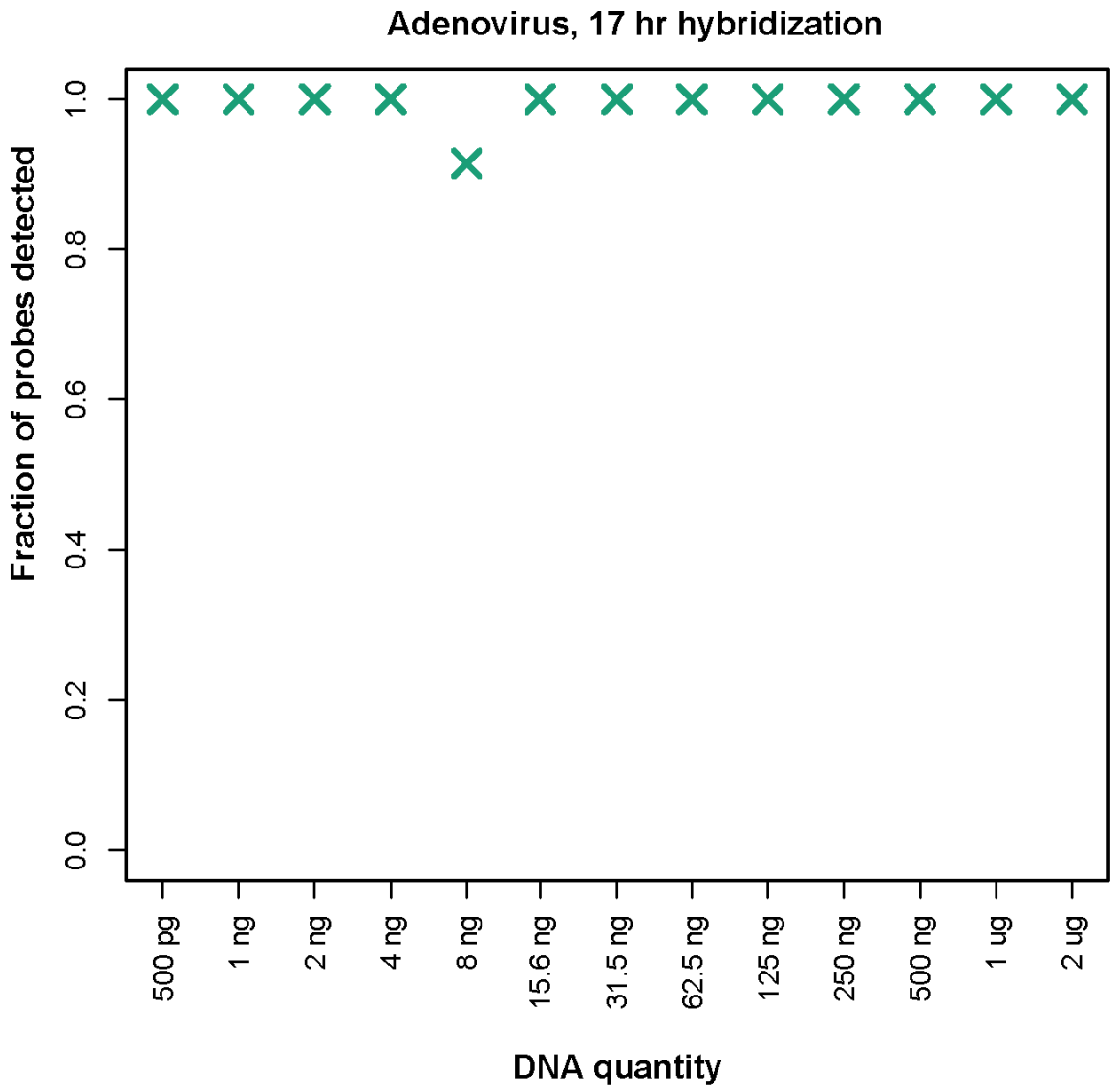

Figure 5B. Detection of Adenovirus DNA after 1 hr hybridization on the 72K MDA array. The Y axis is calculated using the percentage of probes specific to adenovirus that were detected on the array. Two replicate experiments on selected concentrations (shown in orange diamond and green cross) were performed.

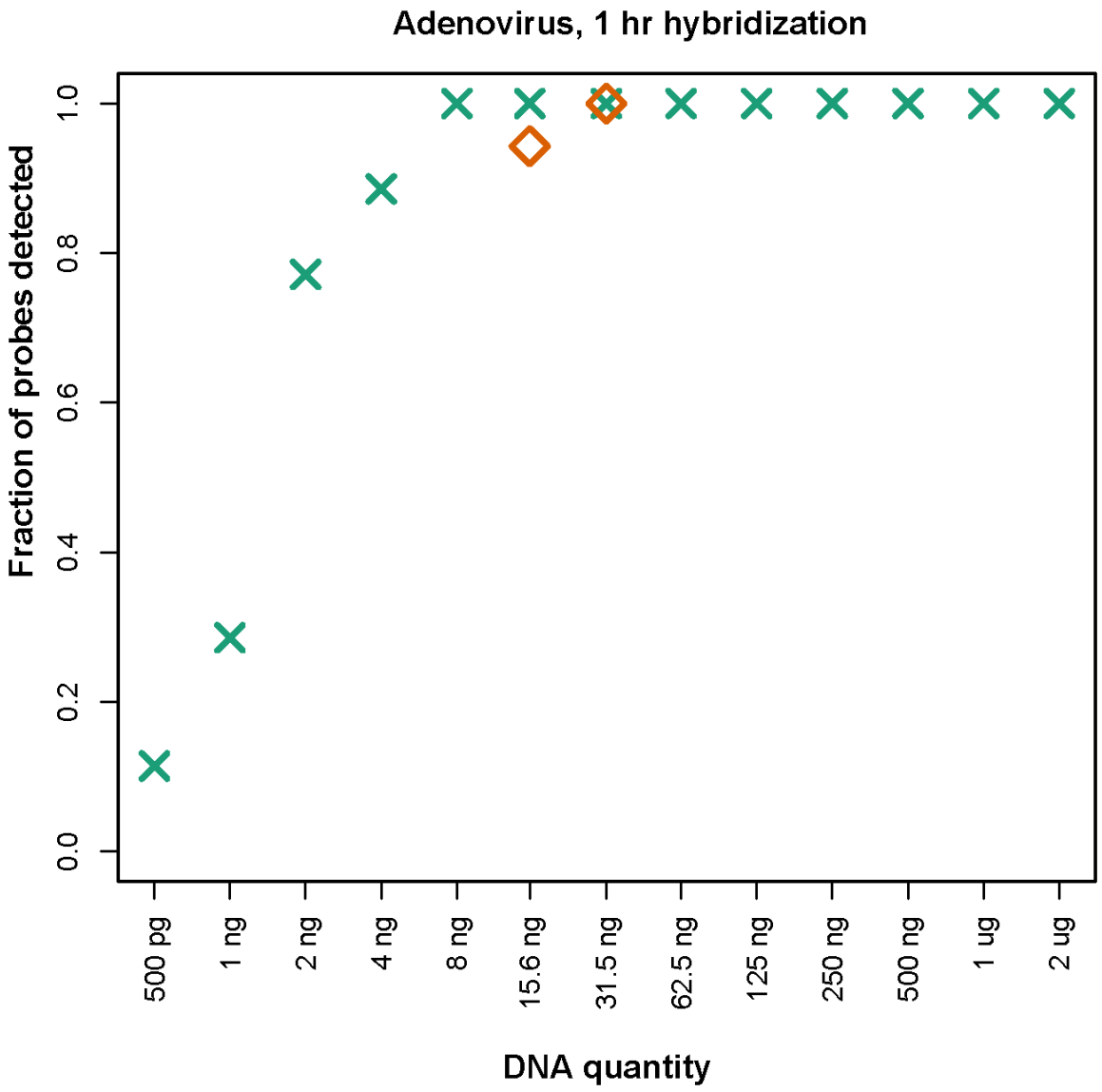

Table 1: Probe summary for 72K LLMDA array

Number of probes	Probes per sequence	Target Sequences
48893	20	All Viral families except Orthomyxoviridae and family unclassified complete viral genomes and segments
7777	10	Segments in the Orthomyxoviridae family
2972	10	Family unclassified viral genomes and complete segments
7864	15	Bacterial genomes and plasmids
3410	-	Random controls with GC% and length distribution matched to target probes
70916		Total

Table 2: Microarray analysis of a mixture of viral and bacterial templates after different random amplification protocols.

Amplification Method	Detected viruses/bacteria	Log-Odds Score
Random PCR 1	*Bacillus anthracis* str. Vollum	474.5
	Bacillus cereus ATCC 10987 plasmid pBc10987	98.6
	Bacillus anthracis str. A1055 plasmid pXO2	135.6
	Cowpox virus UK2000_K2984	222.9
	Variola virus	130.4
	RVF virus 2269/74 segment S	130.7
	RVF strain SA−51 (Van Wyck) segment L	123.3
	RVF strain Entebbe segment M	120.9
	RVF strain Kenya 57 segment M	120.8
	Ebola Zaire 95	120.1
Random PCR 2	*Bacillus anthracis* Australia 94	473.3
	Bacillus cereus ATCC 10987 plasmid pBc10987	97.2
	Vaccinia virus strain LC16m8	126.6
	Variola virus	129
	RVF virus 2269/74 segment S	129.3
	RVF strain OS-9 segment L	122.6
	RVF strain Entebbe segment M	119.4
	RVF strain OS-3 segment L	122.6
	RVF strain Kenya 57 segment M	119.4
	Ebola Zaire 95	118.7
Sigma Transplex	*Bacillus cereus* 03BB108	143
	Vaccinia virus strain LC16m8	223.1
	Variola virus	135.8
	RVF virus ZH−548 segment S	139.2
	RVF strain OS-3 segment L	129.4
	RVF strain Entebbe segment M	126.2
	RVF virus 2269/74 segment S	139
	RVF strain OS-8 segment L	129.4
	RVF strain Kenya 57 segment M	126.2
	Ebola Zaire 95	125.5
Qiagen WTA	*Bacillus anthracis* str. Vollum	504.1
	Bacillus cereus plasmid pPER272	133.3
	Bacillus cereus plasmid pCER270	121.4
	Cowpox virus strain GRI−90	236.6
	Variola virus	136.2
	RVF virus 2250/74 segment S	142.2
	RVF strain SA−51 (Van Wyck) segment L	129
	RVF strain Entebbe segment M	126.6
	RVF strain 2269/74 segment S	142.1
	Ebola Zaire 95	119.8

Table 3: Detection of Salmonella enterica strains on the 72K MDA array

***S. enterica* subsp. enterica serovar on array**	**Top hit on 72K MDA array**	**Log-Odds Score**
Typhi	*Typhi*	392.7
Paratyphi A	*Paratyphi* A	471.6
Typhimurium	*Typhimurium*	593.7
Agona	*Agona*	408.6
Javiana	*Typhimurium**	334
SaintPaul	*Typhimurium***	450.5
Kentucky	*Choleraesuis****	339.9
Heidelberg	*Typhimurium*****	353.8

*Javiana was detected at a lower hit after *Typhimurium and Kentucky*, with log-odds of 309.3

**SaintPaul is not a sequenced strain.

***Kentucky was detected as the second highest hit with log-odds score of 319.5

****Heidelberg was detected as a second highest hit, with log odds score of 326.6.