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LLNL-TR-482763

Report for Evaluation of Canonical SNP Taqman Assays to Detect Biothreat Agents and Environmental Samples for DHS

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May 4, 2011

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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.

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INTRODUCTION

The objective of this project is to provide DHS a comprehensive evaluation of the current genomic technologies including genotyping, Taqman PCR, multiple locus variable tandem repeat analysis (MLVA), microarray and high-throughput DNA sequencing in the analysis of biothreat agents from complex environmental samples. Taqman assays were designed to detect the canonical SNPs to discriminate different strains of *Bacillus anthracis.* The assays were run to test purified DNA from several strains of *B. anthracis* and BioWatch aerosol filter extracts and soil samples that were spiked with *B. anthracis*. The assays were very sensitive. We were able to detect signatures specific to *B. anthracis* Ames at the 10-100 genome copy level after spiking *B. anthracis* Ames DNA into BioWatch aerosol filter extracts 100-1000 when spiked into soil samples.

METHODS

1. Canonical SNP Taqman assay design

We designed 13 *B. anthracis* canonical SNP Taqman assays to detect various strains of *B. anthracis*. The canonical SNPs were designed based on the phylogenetic analysis conducted by Van Ert *et al*. (1) (Figure 1). A total of 13 branch points were identified for strain level detection of *B. anthracis* strains. Two probes were designed for each SNP, one allele labeled with FAM dye and the other allele was labeled with VIC dye. The Taqman primer and probes were ordered through Applied Biosystems Inc (ABI). The canonical SNP assay ID, their corresponding branch points and the Ame and Sterne genome locations are listed in Table 1. The primer and probe sequences are listed in Table 2.

Table 1. Canonical SNP assay ID, corresponding branch points and the genomics locations on B. *anthracis* Ames and *B. anthracis* Sterne

Assay ID	**Branch point**	**Ames genome position**	**Sterne genome position**
canSNP1	A.Br.001	182106	182107
canSNP2	A.Br.002	947759	947654
canSNP3	A.Br.003	1493157	1493231
canSNP4	A.Br.004	3600659	3601360
canSNP5	A.Br.006	162509	162510
canSNP6	A.Br.007	266439	266452
canSNP7	A.Br.008	3947248	3947747
canSNP8	A.Br.009	2589823	2590283
canSNP9	B.Br.001	1455279	1455347
canSNP10	B.Br.002	1056740	1056633
canSNP11	B.Br.003	1494269	1494343
canSNP12	B.Br.004	69952	69953
canSNP13	A/B.Br.001	3697886	3698581

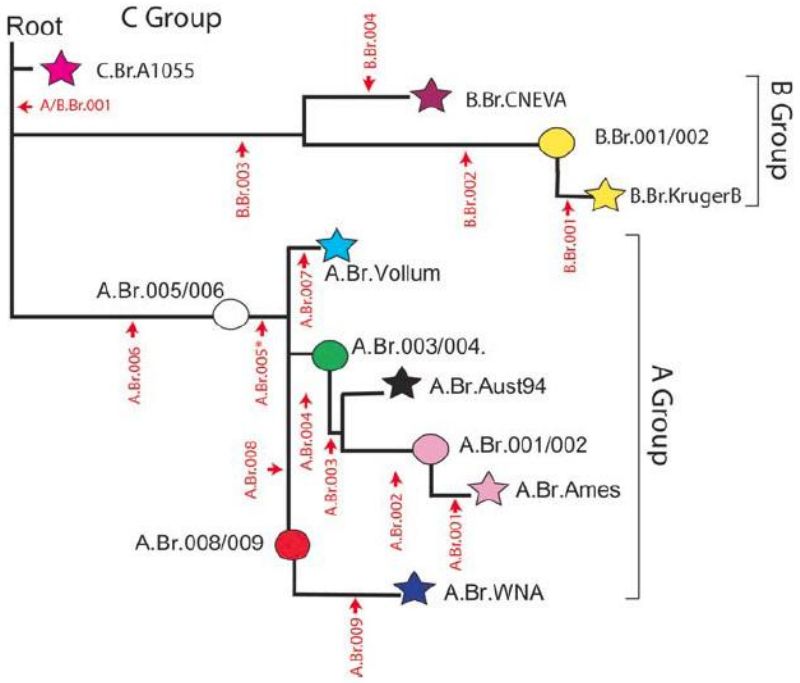

Figure 1. The relationship between canSNPs, sub-lineages and/or sub-groups: The stars in this dendrogram represent specific lineages that are defined by one of the seven sequenced genomes of *B. anthracis*. The circles represent branch points along the lineages that contain specific subgroups of isolates. These sub-groups are named after the canSNPs that flank these positions. Indicated in red are the positions and names for each of the canSNPs. (From Van Ert *et al*. 2007, PLoS ONE).

Table 2. Canonical SNP primer and probe sequences

Assay#	Forward Primer	Reverse Primer	Reporter 1 (VIC)	Reporter 2 (FAM)
canSNP1	GGCAAGCGGAACCAAATTTAATCTT	TACGTCATTGTATAATACGGTTTCCCTTT	ATCGACTTCAAGTTTCGGT	TCGACTTCAAATTTCGGT
canSNP2	GAGGCAGAAGGAGCAAGTAATGTTA	ACCATAACTGATCCAACGATACCTAAAATC	CCGCCCAACCTAAA	CGCCCAGCCTAAA
canSNP3	GCTTGCCAAGCTTTTTTTCTATTATATATAAAAGGAA	GTAGCTACTGTCATTGTATAAAAACCTCCTT	TTTCTACCTCAAGCTTAATT	TTTCTACCTCAAACTTAATT
canSNP4	CCGATACCAGTAAACGACGACATC	CTGGAATTGGTGGAGCTATGGAA	TTGGAATGCCCCTAATC	TTTGGAATGTCCCTAATC
canSNP5	TTCAAAAAATTCTTTGATCAATATGTTGTTGATCATTC	CTTCCTCATCCCAATCTAGCGTTTT	CATCGCCTAGTGCATG	ATCGCCTCGTGCATG
canSNP6	GGCGATTGCGAAAAGTATTGTTGAA	TTGGTAACGAGACGATAAACTGAATAATACC	CGAGCTGAATGTAAGGAT	TCGAGCTGAATATAAGGAT
canSNP7	GGATGCAAATAAACCAAACGGTGAAAA	CATTCGCAACTACGCTATACGTTTT	AATTCTTCGCCGCTTGT	AGATAATTCTTCTCCGCTTGT
canSNP8	GGCAATCGGCCACTGTTTT	CTACTGTGTATGTTGTTAATAAAAAGTATGAATTTTTAGGT	ACGGCTTTGCTTGCAT	AACGGCTTTACTTGCAT
canSNP9	GGGAGAAGTTATTTGCACGGTCATA	TTCAAAAGGTTCGGATATGATACCGATAC	CGGTACAATAGAAGAGGATAA	CGGTACAATAGAAGAAGATAA
canSNP10	CCGAATGGAGGAGAAGTTGCA	TGCACCTTCTGTGTTCGTTGTTAA	AAAGGAACAGAAGTAACG	AGGAACAGCAGTAACG
canSNP11	TCGCATAGAAGCAGATGAGCTTAC	TGTGCCATCAAATAACTCTTTCTCAAGT	CATAACGTGAAGTGGATAT	AACGTGAAGCGGATAT
canSNP12	ACAAGTGCTTGGGTAACCTTCTTT	GCCTTGAGCTTGGTTTAATAAGAAGAAGAA	AACGGGATGGTAGAAGT	ACGGGATGATAGAAGT
canSNP13	ACCAGTTATTCCAATCGCTGCA	ACCTTTCGGTAAATAGTCCCCGATA	CTCTTTTATTTAGGAGATAGC	CTCTTTTATTTAGAAGATAGC

2. DNA Extraction from pure bacteria and environmental samples

2.1 DNA extraction from BioWatch Filters

PSU filters from the NCR Laboratory were received from the BioWatch group at LLNL. One quarter of each filter had previously been excised at NCR, so only ¾ of each filter was available. One week of filters was collected from each season: Spring (4/20-4/26/09), Summer (7/19-7/25/09), Fall (10/25-10/31/09), and Winter (1/22/09-1/28/09). For each day, 7-11 "clean" filters were extracted (49-77 per week). Filters were determined to be "dirty" if they had an abundance of soot and dirt captured on their surface.

The ¾ PSU filters were cut into 5 roughly equal pieces using sterile equipment. Up to 24 filters were placed into a 50mL conical tube. 30mL of 100mM phosphate buffer (pH 7.4), 0.05% (v/v) Tween 80 was added to each 50mL tube. The conical tubes were vortexed for 30 seconds and placed on a rocking shaker for 15 minutes. The 30 second vortexing and 15 min shaking was repeated an additional 3 times for a total of 1 hour of washing. The filters were removed from the tube and remaining solution was centrifuged at 3200 *x g* for 30 minutes at 5^{o}C. Following centrifugation, the supernatant was removed and discarded.

To complete the DNA purification, components of the UltraClean Soil DNA Isolation Kit #12800 from MoBio (Carlsbad, CA) were utilized. The remaining pellet was resuspended with the following solutions added in this order: 100µL TE buffer, 350µL MoBio Bead Solution, 60µL MoBio Solution S1, and 200µL MoBio Inhibitor Removal Solution. A 2mL screw cap tube was loaded with 500mg each of 106 and 500mm zirconia/silica beads. The entire 700µL of resuspended pellet was added to the 2mL bead tube. The samples were bead-beated at max speed for 2 minutes. Following bead-beating, the tubes were centrifuged at 10,000 *x g* for 30 seconds. The entire supernatant (~450µL) was transferred to a sterile 2mL tube for further extraction.

To the supernatant, 250µL of MoBio Solution S2 was added, vortexed for 5 seconds, and incubated at 4^{o}C for 5 minutes. Following incubation the samples were centrifuged for 1 minute at 10,000 *x g* and the supernatant transferred to a clean 2mL tube. 2 volumes (~1.3mL) of MoBio Solution S3 was added to the supernatant and vortexed for 5 seconds. The vortexed solution was added in 700µL aliquots, until the entire sample is processed, to a MoBio spin filter and centrifuged for 1 minute at 10,000 *x g* and the flow-through discarded. The spin filter was washed 3 times by adding 300µL MoBio Solution S4, centrifuged for 30 seconds at 10,000 *x g*, and the flow-through discarded.

The spin filter was centrifuged an additional 1 minute at 10,000 *x g* to dry the filter. The filter was placed in a new 2mL collection tube and 50µL of MoBio Solution S5 was added to the membrane. The sample was centrifuged at 10,000 *x g* for 30 seconds and the eluted DNA was retained. The multiple elutions for each season were combined into one large volume. Samples were speed-vacced to ~50% of the starting volume in order to increase the DNA concentration. DNA concentration was determined by the Invitrogen Qubit fluorometer (Carlsbad, CA).

2.2 DNA Extraction from Soil

Soil was collected in the downtown areas of both Oakland, CA and San Francisco, CA. Four samples were collected in each city at various sites. Samples were extracted using the MoBio

UltraClean Soil DNA Isolation Kit #12800. The manufacturer's Alternative Protocol (For Maximum Yields) was followed for this work. The only deviation from the protocol was to wash twice (Step 15) with Solution S4 instead of just once as the protocol stated.

Following extraction, 1ng of each extracted DNA was used in a Real-Time PCR assay to test for inhibition. All samples showed a high level of inhibition of PCR. Based on this each extracted DNA was re-extracted starting from Step 12 of the MoBio Alternative Protocol. This additional extraction is intended to remove additional humic acid. DNA concentration was determined by the Invitrogen Qubit fluorometer.

2.3 *Bacillus anthracis* Ames DNA spiked in environmental samples
B. anthracis Ames DNA was acquired from the select agent laboratory within LLNL. Sterility test was performed to ensure the DNA is sterile before the DNA was transferred to our laboratory. DNA was quantified using the Invitrogen Qubit fluorometer and copy number was determined. Six concentrations of *B. anthracis* Ames DNA were made in 10 fold serial dilutions from 1-100,000 copies. Each concentration was mixed with 100 pg of extracted DNA from the Spring NCR filters for the aerosol spike experiments or 1 ng extracted DNA from the combination of soil from Oakland and San Francisco for the soil spike experiments.

3. SNP assay protocol
The SNP Taqman assays were carried out in 10 µL reactions. Each reaction consisted of 2X Taqman ABI Universal PCR Master Mix (#4304437), 40X Assay (ABI Custom Taqman SNP Genotyping Assay), and PCR grade water. All reactions were run on an ABI 7900 HT Fast Real-Time PCR System with the following parameters: 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C* for 1 min (*Assay #1 only has an annealing temperature of 63°C).

RESULTS

1. Canonical SNP assays with pure *Bacillus anthracis* DNA

We isolated genomic DNAs from *B. anthracis* Ames, Sterne and A0382. The DNAs were tested in 2 replicates with the canonical SNP assays. The data is shown in Table 3. Ames is mostly reactive to CanSNPs 1-5 (with signal from the VIC dye) as expected because these five canonical SNPs correspond to the branches with phylogenetic lineages closely related to Ames. Canonical SNP1 is specific to Ames strain. The Stern strain is mostly reactive to CanSNPs 2-5, also as expected since CanSNP1 is specific to Ames, while CanSNPs 2-5 correspond to the phylogenetic lineages of the Sterne strain. The A0382 strain has not been sequenced. It is reactive to CanSNP 10 and 11, suggesting that it is closely related to the Kruger strain.

Table 3. Canonical SNP results from *B. anthracis* DNAs

	Ames		Sterne		A0382	
	Ave Ct	**Stdev**	**Ave Ct**	**Stdev**	**Ave Ct**	**Stdev**
canSNP1	20.11	0.36	18.59	0.1	19.08	0.24
canSNP2	22.16	0.15	20.1	0.2	–	–
canSNP3	23.07	0.19	20.51	0.15	22.16	0.09
canSNP4	22.01	0.13	19.94	0.28	21.8	0.23
canSNP5	21.54	0.25	20.07	0.24	21.72	0.12
canSNP6	20.3	0.14	18.72	0.17	19.22	0.13
canSNP7	22.86	0.16	20.54	0.17	21.26	0.16
canSNP8	22.84	0.17	19.58	0.14	20.1	0.13
canSNP9	21.82	0.1	19.58	0.1	20.02	0.09
canSNP10	23.59	0.35	21.48	0.63	20.07	0.24
canSNP11	21.28	0.18	19.01	0.14	19.73	0.13
canSNP12	20.72	0.21	19.09	0.11	19.76	0.13
canSNP13	23.67	0.07	21.4	0.09	22.66	0.11

FAM

VIC

– = Undertimined

2. Determination of the limit of detection of the Canonical SNP assays using *B. anthracis* Ames spiked into BioWatch aerosol samples.

We performed limit of detection testing of the canonical SNP assays using serially diluted *B. anthracis* Ames spiked into 100 pg of DNA from BioWatch aerosol filter extracts. Duplicate experiments were run to ensure repeatability and data consistency. 1, 10, 100, 1,000, 10,000, and 100,000 copies of *B. anthracis* Ames were tested. Table 4 below shows results of the Taqman SNP assays at each of the *B. anthracis* DNA concentrations. When 10 copies of *B. anthracis* DNA were spiked into the aerosol sample, numerous canonical SNP assays were undetermined. This experiment suggested that our detection limit for *B. anthracis* Ames could be in the range between 10-100 copies when the DNA was spiked into 100 pg of the aerosol DNA sample.

Table 4. Limit of detection of *B. anthracis* Ames DNA spiked in Biowatch aerosol samples

Amount *B. anthracis* DNA	560 pg		56 pg		5.6 pg		560 fg		56 fg		5.6 fg	
***B. anthracis* DNA Copy #**	100,000 copies		10,000 copies		1,000 copies		100 copies		10 copies		1 copy	
% BA DNA in aerosol DNA	98.20%		35.90%		5.30%		0.56%		0.06%		0.01%	
	Avg	**Stdev**	**Avg**	**Stdev**	**Avg**	**Stdev**	**Avg**	**Stdev**	**Avg**	**Stdev**	**Ct-Rep1**	**Ct-Rep2**
canSNP1	21.26	0.33	25.04	0.15	28.38	0.10	31.87	0.28	36.26	1.07	–	–
canSNP2	23.99	1.51	28.71	2.38	31.66	1.03	34.83	1.31	–	–	–	–
canSNP3	22.66	1.02	27.09	0.33	30.81	0.54	34.55	0.37	37.07	N/A	–	–
canSNP4	21.97	0.84	26.11	0.38	29.60	0.54	32.93	0.30	35.81	0.14	–	–
canSNP5	22.21	0.78	26.39	0.75	30.18	0.70	33.63	0.85	36.13	N/A	–	–
canSNP6	21.34	0.54	25.50	0.32	29.12	0.45	32.24	0.43	36.17	0.10	–	–
canSNP7	23.40	0.75	27.48	0.47	30.67	0.43	33.79	0.94	36.51	N/A	–	–
canSNP8	22.55	0.54	27.18	0.62	30.58	0.45	33.86	0.59	37.05	N/A	–	–
canSNP9	22.05	0.69	26.32	0.57	29.73	0.49	33.01	0.62	36.77	0.23	–	–
canSNP10	22.76	0.19	27.08	0.13	30.65	0.40	34.11	0.07	36.87	N/A	–	–
canSNP11	21.15	0.01	25.44	0.11	28.65	0.07	32.02	0.49	36.32	0.90	–	–
canSNP12	21.85	0.13	25.77	0.01	29.33	0.02	32.83	0.15	35.97	0.00	–	–
canSNP13	21.92	0.28	26.01	0.10	29.27	0.17	32.76	0.40	36.75	N/A	–	–

FAM

VIC

– = undetermined

3. Determination of the limit of detection of the Canonical SNP assays using *B. anthracis* Ames spiked into soil samples.

We performed a similar limit of detection test of the SNP assays using serially diluted *B. anthracis* Ames spiked into 1 ng of DNA from soil extracts. The soils were collected locally in San Francisco and Oakland. Six different DNA concentration levels of *B. anthracis* Ames were tested, from 1 copy to 100,000 copies. Table 5 below shows results of the SNP assays at each of the *B. anthracis* DNA concentration. When 100 copies of *B. anthracis* DNA were spiked into the soil sample numerous SNP assays were undetermined. This experiment suggested that our detection limit for *B. anthracis* Ames could be in the range between 100-1000 copies when the DNA was spiked into 1 ng of soil DNA sample.

Table 5. Limit of detection of *B. anthracis* Ames DNA spiked in soil samples.

Amount *B. anthracis* DNA	560 pg		56 pg		5.6 pg		560 fg		56 fg		5.6 fg	
***B. anthracis* DNA Copy #**	100,000 copies		10,000 copies		1,000 copies		100 copies		10 copies		1 copy	
% BA DNA in soil DNA	35.90%		5.30%		0.56%		0.06%		0.01%		0.00%	
	Avg	**Stdev**	**Avg**	**Stdev**	**Avg**	**Stdev**	**Avg**	**Stdev**	**Avg**	**Stdev**	**Avg**	**Stdev**
canSNP1	27.18	1.19	30.25	0.28	33.33	0.32	36.62	0.51	–	–	–	–
canSNP2	30.96	2.53	32.34	N/A	–	–	–	–	–	–	–	–
canSNP3	27.54	0.23	30.50	0.53	34.46	0.15	–	–	–	–	–	–
canSNP4	24.89	0.15	27.43	0.40	31.09	0.01	35.10	0.17	–	–	–	–
canSNP5	26.74	0.60	29.18	1.35	33.54	0.18	–	–	–	–	–	–
canSNP6	24.29	0.69	27.02	0.79	31.12	0.56	35.11	0.04	–	–	–	–
canSNP7	26.66	1.40	29.01	1.07	33.01	0.28	–	–	–	–	–	–
canSNP8	24.51	0.59	27.98	0.68	31.91	0.10	36.18	0.27	–	–	–	–
canSNP9	23.73	0.98	26.71	0.64	31.10	0.30	35.05	0.17	–	–	–	–
canSNP10	27.04	N/A	29.78	0.29	34.63	N/A	–	–	–	–	–	–
canSNP11	24.78	0.22	27.21	1.02	31.34	0.38	–	–	–	–	–	–
canSNP12	26.93	0.17	29.65	0.20	34.12	0.20	–	–	–	–	–	–
canSNP13	27.12	0.33	28.26	0.77	32.30	0.02	36.44	0.20	–	–	–	–

FAM

VIC

– = undetermined

References:

1. Van Ert, M.N., Easterday, W.R., Simonson, T.S., U'Ren, J.M., Pearson, T., Kenefic, L.J., Busch, J.D., Huynh, L.Y., Dukerich, M., Trim, C.B. *et al.* (2007) Strain-Specific Single-Nucleotide Polymorphism Assays for the Bacillus anthracis Ames Strain. *J Clin Microbiol.*, **45**, 47–53.