Ultrasensitive detection of *Bacillus anthracis* by real time PCR targeting a polymorphism in multi-copy 16S rRNA genes and their transcripts

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6. Supplementary Material

6.1. Supplementary Tables

Table S1: Temperature optimum.

Annealing temperature (°C)	Ct-values (Ø±SD)*	Fluorescence (arbitrary units)**
61	33.1±0.1	27.9±0.9
62	32.7±0.2	32.1±1.1
63	32.5±0.2	28.0±0.9
64	32.7±0.1	25.2±0.4

* averages (Ø) ± standard deviations (SD) of n = 3 tests; ** value at the end of the 42th PCR cycle.

Table S2: Primer titration.

Primer concentrations (µM)	Ct-values (Ø±SD)*	Fluorescence (arbitrary units)**
0.5 /0.5	26.6±0.9	13.8±0.4
0.25 / 0.5	26.5±0.5	9.7±0.4
0.5 / 0.25	27.2±0.1	11.7±0.2
0.25 / 0.25	26.6±0.1	6.0±0.4
1 / 0.5	27.6±0.2	12.8±0.6
0.5 / 1	27.3±0.2	15.0±0.4
1 / 1	26.6±0.7	13.0±0.5

* Average (Ø) ± standard deviations (SD) of n = 3 tests; ** Value at the end of the 42th PCR cycle.

Table S3: Probe titration.

Probe concentration BA* / BC** (µM)	Ct-values (Ø±SD)***	Fluorescence (arbitrary units)****
0.25 / 0.25	27.5±0.3	15.9±0.2
0.125 / 0.25	27.5±0.2	9.2±0.5
0.25 / 0.125	27.8±0.1	16.2±0.1
0.125 / 0.125	27.4±0.6	9.9±0.5
0.5 / 0.25	27.3±0.6	24.8±1.6
0.25 / 0.5	27.5±0.3	14.7±0.3
0.5 / 0.5	27.7±0.4	23.2±1.7

* 6-FAM-labeled (hybridizes against the *B. anthracis*-specific allele of the 16S rRNA gene); ** „dark" probe lacking fluorescent label (hybridizes against the *B. cereus* allele of the 16S rRNA gene); *** averages (Ø) ± standard deviations (SD) of n = 3 tests; **** value at the end of the 42th PCR cycle.

Table S4: Magnesium ion titration.

$MgCl_2$ concentration (mM)	Ct-values (Ø±SD)*	Fluorescence (arbitrary units)**
1	28.8±0.3	2.8±0.3
2	27.5±0.7	10.0±0.5
3	28.1±0.4	23.3±1.9
4	27.8±0.4	21.3±1.2

* averages (Ø) ± standard deviations (SD) of n = 3 tests; ** value at the end of the 42th PCR cycle.

Table S5: Pipetting error (5%).

Reaction volume (µl)	Ct-values (Ø±SD)*	Fluorescence (arbitrary units)**
19	28.6±0.4	47.3±6.0
20	28.9±0.1	46.4±3.8
21	29.1±0.1	44.5±5.4

* averages (Ø) ± standard deviations (SD) of n = 3 tests; ** value at the end of the 42th PCR cycle.

Table S6: Intra assay variability*.

Strain	Assay 1	Assay 2	Assay 3	Ct-values (Ø±SD)**	CV***
L3-2641	32.4	32.7	33.1	32.7±0.3	1.0
L3-2649	34.3	33.8	33.6	33.9±0.3	1.1
L3-2657	33.8	33.9	33.9	33.9±0.1	0.2
L3-2665	31.7	31.9	31.9	31.8±0.1	0.4
L3-2866	31.7	31.9	31.9	31.8±0.1	0.4
L3-3391	24.2	24.2	24.3	24.2±0.0	0.3
L3-3399	22.8	22.7	22.7	22.7±0.0	0.1
L3-3407	23.4	23.4	23.3	23.4±0.0	0.2
L3-3408	22.9	22.9	22.9	22.9±0.0	0.3
L3-3415	22.4	22.4	22.4	22.4±0.0	0.0
L3-3416	23.5	23.6	23.7	23.6±0.1	0.3
L3-3421	22.5	22.4	22.2	22.4±0.2	0.2

* replicates (n=3) of each PCR reaction with target DNA were run on the same day; ** averages (Ø) ± standard deviations (SD) of n = 3 tests; *** coefficient of variation (%).

Table S7: Inter assay variability*.

Template copies	Assays day 1	Assays day 2	Assays day 3	Ct-value (Ø±SD)**	CV***
10^4	25.9; 25.8; 26.0	26.2; 26.3; 26.3	25.6; 25.6; 25.6	25.9±0.3	1.2
10^2	32.3; 32.9; 32.3	32.9; 33.0; 32.8	32.4; 32.2; 32.0	32.5±0.3	1.1

* replicates (n=3) of each PCR reaction with target DNA were run on three consecutive days; ** averages (Ø) ± standard deviations (SD) of n = 3 x 3 tests; *** coefficient of variation (%).

Table S8: Probe titration in the presence of 10^9 copies of 16S-BC-allele (absence of 16S-BA-allele).

Probe concentration BA*/BC** (µM) [BA/BC]	Ct-values	Fluorescence (arbitrary units)****
0.25 / 0.25 [1:1]	-***	1.6±0.0
0.25 / 0.5 [1:2]	-***	1.1±0.4
0.25 / 0.75 [1:3]	-***	0.82±0.4
0.25 / 1.0 [1:4]	-***	1.4±0.3

* 6-FAM-labeled (hybridizes against the *B. anthracis*-specific 16S-BA-allele of the 16S rRNA gene); ** „dark" probe lacking fluorescent label (hybridizes against the *B. cereus* 16S-BC-allele of the 16S rRNA gene); *** no regular amplification, no CT-value; **** value at the end of the 42th PCR cycle.

Table S9: Optimized probe concentration for the detection of the 16S-BA-allele (100 target copies) in the presence of increasing copies of the alternative BC-allele.

Assay Number	16S-BC-allele concentration (copies per reaction)*	Ct value (Ø±SD)**	Fluorescence (arbitrary units)***
1	10^8	****	3.63±0.3
2	10^7	****	3.44±0.2
3	10^6	****	3.35±0.1
4	10^5	33.4±0.6	3.8±0.1
5	7.5×10^4	34.0±1.3	4.8±0.7
6	5×10^4	35.0±0.4	5.2±0.5
7	2.5×10^4	34.2±0.3	6.9±0.3
8	10^4	33.4±0.8	8.6±0.8
9	10^3	32.6±0.1	17.9±0.6
10	10^2	32.9±0.1	25.7±0.7
11	no BA. only 10^5 BC	****	2.6±0.1
12	no BA. only 0.5×10^5 BC	****	2.3±0.4
13	positive control (only BA)	33.0±0.2	31.3±4.3
14	negative control (H_2O)	-	-

* 16S-BA-allele concentration constant at 100 copies per reaction; ** averages (Ø) ± standard deviations (SD) of n = 3 tests; *** value at the end of the 42th PCR cycle; **** no regular amplification, no CT-value.

Table S10: Sensitivity panel - target organism *B. anthracis*.

# of *B. anthracis* strains	Phylogeny*	PCR result**
1	C.Br. A1055	positive
3	B.Br. CNEVA	positive
1	B.Br. Kruger B	positive
5	A.Br. 001/002	positive
1	A.Br. 011/009; A.Br.118 (STI)	positive
1	A.Br. 005/006	positive
1	A.Br. Vollum	positive
1	A.Br. Aust 94	positive
5	A.Br. Aust 94; A.Br.014	positive
1	A.Br. Aust 94; A.Br.015	positive
2	A.Br. 008/011; A.Br.127 (Pstr)	positive
1	A.Br. 008/011; A.Br.127(BUL)	positive
1	A.Br. 008/011; A.Br.161 (Heroin)	positive
1	A.Br. WNA	positive

* phylogeny according to (1) and (2); ** results of n = 3 tests.

Table S11: Specificity panel - potentially cross-reacting organisms.

Organism	Strain number	PCR result**
B. cereus	ATCC 10987	negative
B. cereus	2998	negative
B. cereus	3093	negative
B. cereus	LGL 3094	negative
B. cereus	ATCC 4342	negative
B. cereus bv. anthracis	CI-1	negative
B. cereus bv. anthracis	CA-1	negative
B. cereus	ATCC 33019	negative
B. thuringiensis	ATCC 10792	negative
B. thuringiensis	DSM 046	negative
B. paranthracis	2002	negative
B. weihenstephanensis	B-0293	negative
B. mycoides	B-298	negative
B. subtilis	ATCC 6091	negative
B. megaterium	ATCC 14581	negative
*Homo sapiens****	n.a.*	negative
*Bos taurus****	n.a.*	negative
*Capra aegagrus hircus****	n.a.*	negative
*Ovis gmelini aries****	n.a.*	negative
*Equus caballus****	n.a.*	negative

* not applicable (n.a.); ** results of n = 3 tests; ***typical host organisms.

Table S12: Specificity panel – organisms relevant for differential diagnostics and other pathogens.

Organism	Strain number	PCR result*	Organism	Strain number	PCR result*
Brucella sp.	F-070660	negative	*Moraxella catarrhalis*	B-0433	negative
Burkholderi mallei	L3-2962	negative	*Neisseria meningitidis*	B-1332	negative
Burkholderi pseudomallei	L3-0711	negative	*Propionibacterium acnes*	B-0438	negative
Burkholderia thailandensis	B-1668	negative	*Pseudomonas aeruginosa*	B-0040	negative
Campylobacter jejuni	B-1229	negative	*Serratia marcescens*	B-0014	negative
Candida albicans	B-1266	negative	*Sphingomonas zeae*	JM-791	negative
Citrobacter freundii	B-0022	negative	*Staphylococcus aureus*	B-0946	negative
Clostridium paraperfringens	B-1435	negative	*Staphylococcus epidermidis*	B-0026	negative
Clostridium sporogenes	B-1450	negative	*Stenotrophomonas maltophila*	B-0055	negative
Eikenella corrodens	B-0614	negative	*Streptococcus pneumoniae*	B-0847	negative
Escherichia coli	B-1324	negative	*Streptococcus pyogenes*	B-0846	negative
Francisella tularensis holarctica	F-0049	negative	*Vibrio cholerae*	B-1302	negative
Haemophilus influenzae	B-0850	negative	*Yersinia enterocolitica*	B-0099	negative
Klebsiella pneumoniae	B-0008	negative	*Yersinia pestis*	EV-76	negative
Legionella pneumophila	B-1341	negative	*Monkey Pox Virus*	MSF-6	negative
Listeria monocytogenes	DSM-12464	negative	*Vaccinia Virus*	VACV-0273/2004	negative
			Varicella Zoster Virus	none	negative

* results of n = 3 tests.

Table S13: Linearity of the 16S rRNA SNP-PCR*.

[Template]**	Assays day 1			Assays day 2			Assays day 3			(Ø±SD)***
A.										
10^9	9.3	9.4	9.5	9.7	9.8	9.8	9.1	9.1	9.1	9.4±0.3
10^8	12.6	12.6	12.5	13.0	13.1	13.0	12.3	12.3	12.3	12.6±0.3
10^7	16.0	16.0	16.0	16.7	16.6	16.6	15.8	15.8	15.9	16.1±0.3
10^6	19.2	19.1	19.0	19.5	19.6	19.6	18.8	18.8	18.8	19.2±0.3
10^5	22.6	22.7	22.6	23.0	23.1	23.2	22.5	22.3	22.3	22.7±0.3
10^4	25.9	25.8	26.0	26.2	26.3	26.3	25.6	25.6	25.6	25.9±0.3
10^3	29.5	29.4	29.6	30.0	29.9	29.9	29.2	29.1	29.0	29.5±0.3
10^2	32.3	32.9	32.3	32.9	33.0	32.8	32.4	32.2	32.0	32.5±0.3
10^1	35.8	35.7	36.2	36.1	35.8	35.8	35.9	36.2	35.8	35.9±0.2
10^0	36.9	37.3	38.8	39.2	38.9	38.6	-	37.2	-	38.1±1.0
B.										
10^7	17.1	17.0	17.1	16.7	16.8	16.8	16.8	16.8	16.8	16.9±0.2
10^6	20.4	20.7	20.4	20.2	20.2	20.2	20.3	20.3	20.3	20.3±0.2
10^5	24.0	24.0	24.1	23.8	23.7	23.7	23.8	23.7	23.9	23.9±0.2
10^3	27.4	27.3	27.5	26.9	27.0	26.8	27.0	27.1	27.1	27.1±0.2
10^2	30.8	29.6	30.7	30.5	30.5	30.6	30.5	30.7	30.4	30.5±0.3
10^1	34.0	34.1	33.8	33.7	33.7	33.8	33.6	34.0	33.9	33.8±0.2
10^0	38.3	36.7	37.1	36.1	36.9	36.7	37.3	37.1	36.6	37.0 ±0.6

* Replicates (n=3) of each PCR reaction with target DNA were run on three consecutive days; ** templates at indicated concentration per reaction were cloned fragment (A) or *B. anthracis* Ames DNA (B); *** averages (Ø) ± standard deviations (SD) of n = 3 x 3 tests.

Table S14: Probit-analysis of the 16S rRNA SNP-PCR.

Copies/reaction	# of tests	# of positive tests
10	12	12
8	12	12
6	12	12
4	12	12
3	12	12
2	12	7
1	12	4

Table S15: Linearity of the 16S rRNA SNP RT-PCR*.

[Template]**	Triplicates			(Ø±SD)***
10^8	10.2	9.8	10.4	10.1±0.3
10^7	13.7	13.7	13.8	13.7±0.1
10^6	16.7	16.7	16.8	16.7±0.1
10^5	20.0	19.5	21.2	20.2±0.9
10^4	24.5	24.3	23.8	24.2±0.3
10^3	26.6	26.9	26.6	26.7±0.2
10^2	29.8	31.2	30.9	30.6±0.7
10^1	34.7	35.0	34.9	34.8±0.2
10^0	40.3	37.0	40.0	39.1±1.8

* Replicates (n=3) of each PCR reaction with target DNA were run; ** templates at indicated concentration per reaction were RNAs (including genomic) DNA of *B. anthracis* Sterne; *** averages (Ø) ± standard deviations (SD) of n = 3 tests.

Table S16: Probit-analysis of the 16S rRNA SNP RT-PCR.

Copies/reaction	# of tests	# of positive tests
15	12	12
12	12	12
9	12	12
7.5	12	12
6	12	10
4.5	12	10
3	12	8
1.5	12	8
0	12	0

6.2. Supplementary Figures

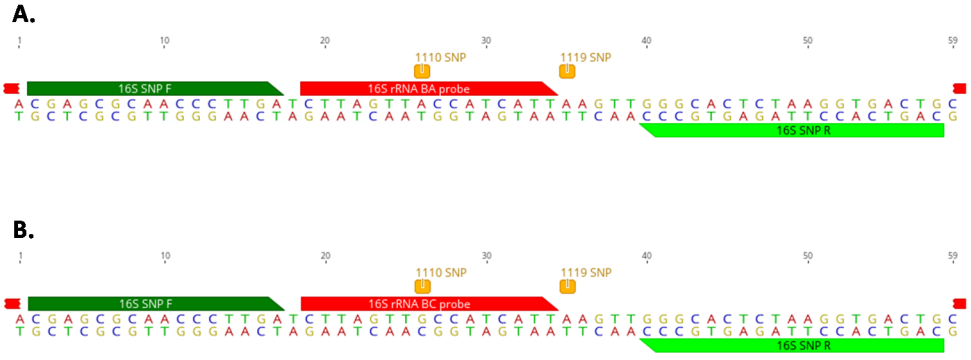


Figure S1: Partial sequence of different alleles of the 16S rRNA genes of *B. anthracis* with specific SNP ("A" at position 1110 in *B. anthracis* Ames Ancestor NC_007530; position 1139 according to (3))* and primer and probe positions. (A) *B. anthracis*-specific 16S-BA-allele. (B) alternative 16S rRNA gene allele (16S-BC-allele) in *B. anthracis* common for the *B. cereus*-group with alternative SNP state ("G" at position 1110 in *B. anthracis* strain Ames Ancestor, NC_007530). *a second SNP at position 1119 present in some of the 16S rRNA gene alleles was found not to be specific for *B. anthracis* (3).

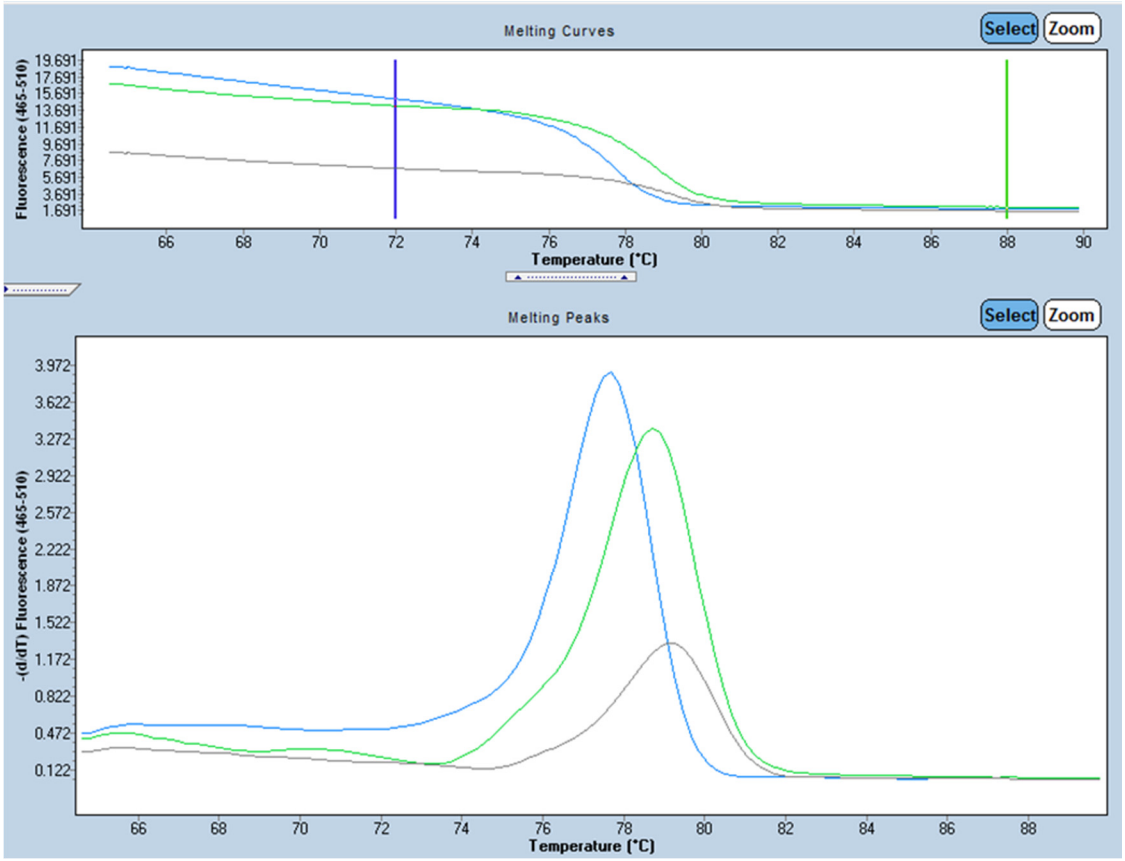


Figure S2: Melting point analysis of PCR products. Shown are melting point analyses as fluorescence change (upper panel) or as its first derivative (lower panel) over a temperature gradient. Amplificates were 16S-BA-allele (100 template copies, blue line), 16S-BC-allele (100 template copies, green line) or water-only control (grey line).

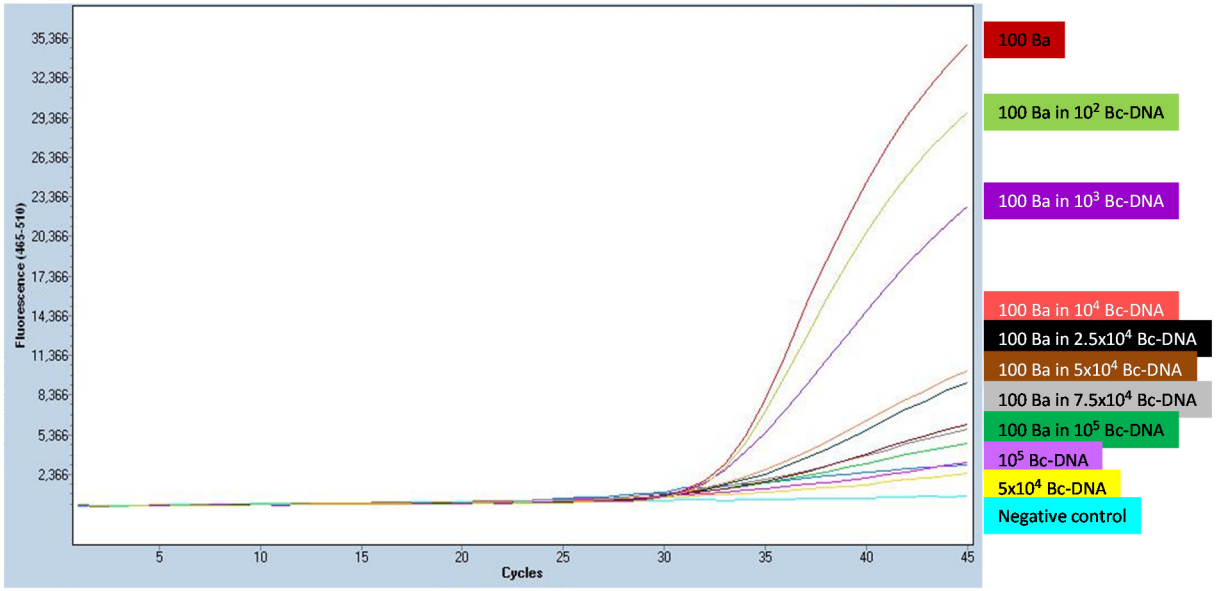


Figure S3: Competitive inhibition the 16S rRNA SNP-PCR assay by the alternative 16S-BC-allele. A constant 100 template copies of the 16S-BA-allele per reaction were titrated against increasing copy numbers of the alternative 16S–BC-allele and fluorescence recorded (shown are representative curves; see Table S9 for numerical data as replicates).

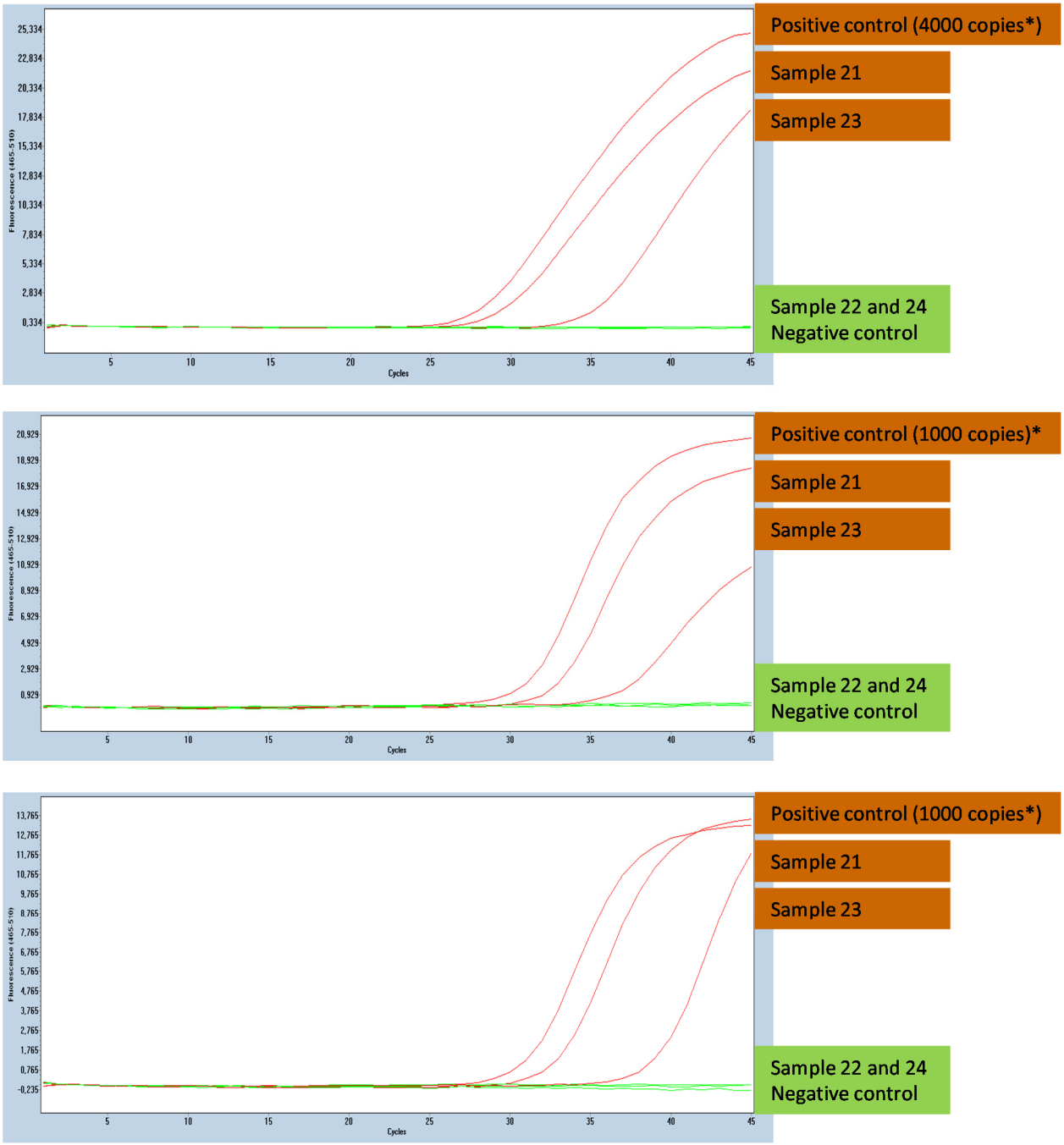


Figure S4: Challenge of the new 16S rRNA-based multi-copy assay with samples from a ring trial. Samples were subjected to real time PCR using the new 16S rRNA SNP assay (upper panel), published *dhp61* gene assay (4) (middle panel) or published *PL3* gene assay (5) (lower panel). Representative amplification curves (from n=3 with similar results) are shown. *Positive controls were 1000 genomes of *B. anthracis* Ames (harboring 4000 copies of the 16S-BA-allele but only 1000 copies of *dhp61* or *PL3*, respectively.

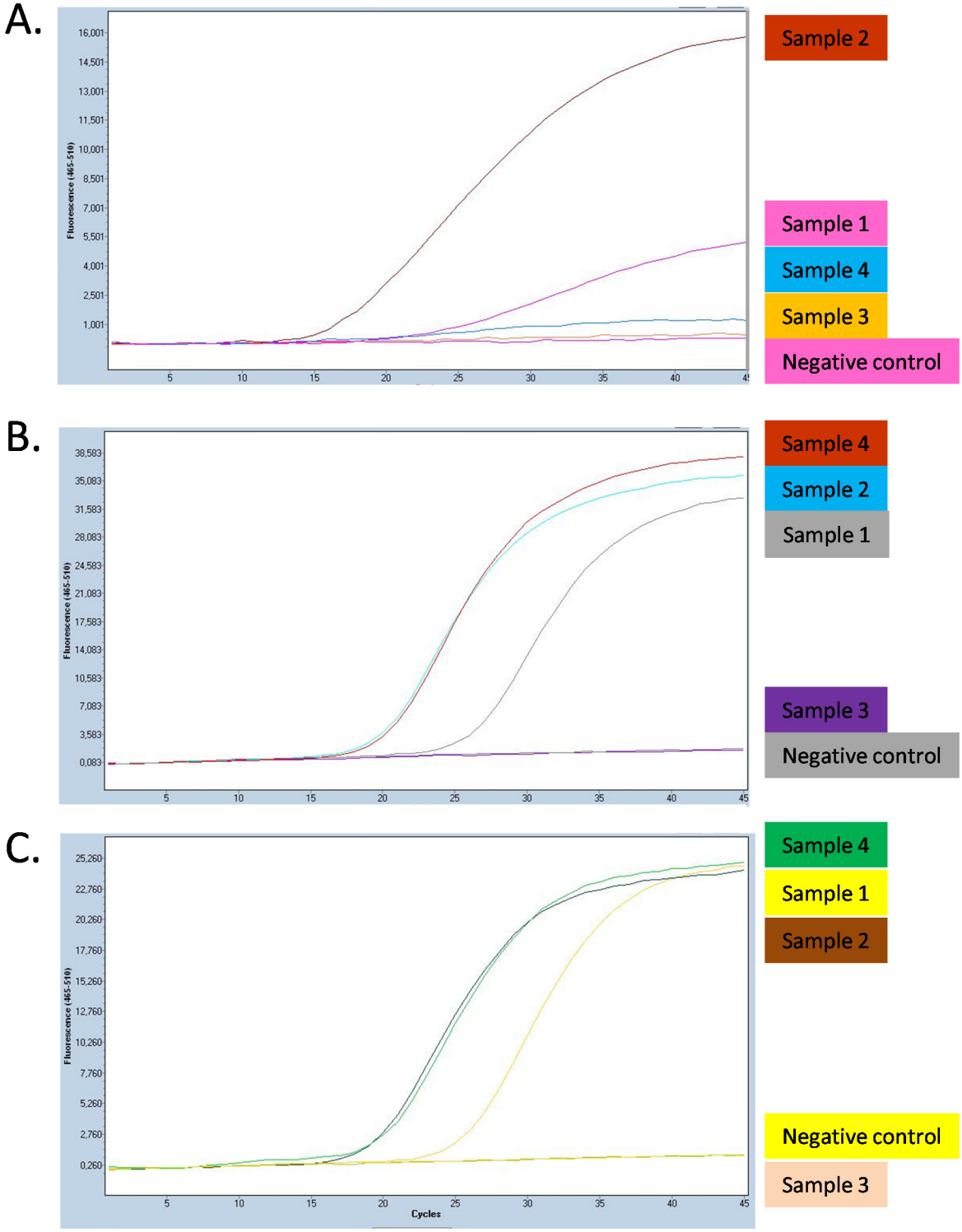


Figure S5: Challenge of the new 16S rRNA-based multi-copy assay with total DNA from spiked soil. Samples were subjected to real time PCR using the new 16S rRNA SNP assay (upper panel), published *dhp61* gene assay (4) (middle panel) or published *PL3* gene assay (5) (lower panel). Representative amplification curves (from n=3 with similar results) are shown.

References

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