Message

From: Pranav Patel [/O=THERANOS ORGANIZATION/OU=EXCHANGE ADMINISTRATIVE GROUP (FYDIBOHF23SPDLT)/CN=RECIPIENTS/CN=PRANAV PATEL268]
Sent: 12/11/2013 1:25:14 AM
To: Mark Pandori [mpandori@theranos.com]
Subject: TNAA LDT validation reports

Hi Mark,

As promised yesterday, attached are the TNAA reports.

Please let me have your feedback.

Thanks

Pranav

BORDETELLA PARAPERTUSSIS

TNAA LDT Validation Report

Limit of Detection = 30 cp/uL

Rate of Detection = 100 cp/uL in 30 minutes

Katie Sullivan-Bibee

THERANOS, INC.

Bordetella parapertussis TNAA Validation Report

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Bordetella parapertussis

1) PURPOSE

This report includes relevant information about the target organism, a detailed description of the primers and selected targets designed for the detection of this organism, a summary of the validation assay performance, and recommendations for future assay execution.

2) BACKGROUND

Bordetella parapertussis, like its closely related congener *B. pertussis*, is a betabacterium (order Burkholderiales) that causes whooping cough. *B. parapertussis* can infect humans and sheep, and is derived from a free living ancestor similar to *B. bronchiseptica*. *B. parapertussis* has less severe symptoms and different reporting requirements than *B. pertussis*, so an independent test is justified.

At many genetic loci there is little genetic divergence among *Bordetella* species, but they differ in transposable element content. *IS1001* is present in ~20 copies in *B. parapertussis*, but has not been found in *B. pertussis*. It is sporadically present at low copy number in *B. bronchiseptica*, but this species is very rarely observed in humans and primarily infects the respiratory tracts of small mammals.

This report describes the nucleic acid detection test developed to detect *Bordetella parapertussis*. The target region is the transposase gene of IS1001. There are two complete genome sequences for *B. parapertussis*, one isolated from a human (12822), and one from sheep (Bpp5), and they contain 21 and 24 full length transposase genes, respectively.

3) SUMMARY OF PERFORMANCE DATA

Theranos developed a Theranos Nucleic Acid Amplification (TNAA) assay specific for *Bordetella parapertussis*. The Nucleic Acid Amplification reactions contained 1x Nucleic Acid Amplification buffer (20 mM Tris Acetate, pH 7.9, 50 mM Potassium Acetate, 10 mM Magnesium Acetate and 1mM DTT), 0.08% Tween, 0.8 M betaine, 1.4 mM dNTPs, 2 uM Syto59, 0.8 uM RLX2263 primer and 0.8 uM RLX2264 primer, 20 units Bst polymerase, and template at the noted concentration. The reactions were run at 56ºC for 60 minutes. Summarized data will follow below while detailed experimental data can be found in the appendix.

Primer sequences are:

Bordetella parapertussis	RLX2263	CGCGCTTAAAGTATTCCTGGTCG
	RLX2264	TAAGCGCGCGAGGGCATCAACAA

4) LIMIT OF DETECTION

The purpose of this study is to determine the limit of detection (LOD) for the Theranos TNAA assay. The LOD_{95} is the bacterial titer at which >95% of known positive samples test positive using the TNAA assay. Statistically justified cut-off times for making positive/negative calls were determined for each target empirically. A set of experiments, repeated over four days, were conducted that included eight replicates each of three target dilutions (LoD, 10X LoD, and 100X LoD), as well as 8 NTCs, using the target primers for amplification. These data were then processed using a receiver-operator character (ROC) analysis, and the best threshold detection time for distinguishing positives and negatives determined using the Youden test statistic as implemented by the R package, pROC.

The assay reliably detected 1,802 CFU/ml of *Bordetella parapertussis* in about 37.2 minutes, as shown below. The 37.2 minute assay cut-off time was determined by ROC analysis. The assay was performed six times. Reactions with and without template (NTCs or Non-Templated Controls) were run in eight replicates each.

LOD	Samples	NumPositive	Total	Percent
100X LOD	180,155 CFU/ml	48	48	100
10X LOD	18,015 CFU/ml	48	48	100
1X LOD	1,802 CFU/ml	45	48	94
	NTC	0	48	0

5) REPRODUCIBILITY/PRECISION

The purpose of this experiment is to determine the precision of the assay, percent positive and negative at three detection limits: high-negative (0.1X LOD=180 CFU/ml), low-positive (LOD=1,802 CFU/ml), and high-positive (3X LOD=5,405 CFU/ml). The assay was performed six times. Reactions with and without template (NTCs or Non-templated Controls) were run in eight replicates each.

Precision LOD	Samples	NumPositive	Total	Percent
3X LOD	5,405 CFU/ml	47	48	98
1X LOD	1,802 CFU/ml	42	48	88
0.1X LOD	180 CFU/ml	23	48	48
	NTC	0	48	0

6) CARRYOVER

The purpose of this experiment is to determine the potential for carryover of positive samples adjacent to negative reactions. The nucleic acid template is prepared from high-positive (100X LOD = 180,155 CFU/ml), low-positive (1X LOD=1,802 CFU/ml), and non-templated controls (NTCs) which are arrayed in alternating rows of eight replicates each. There are two rows of high-positive reactions, two rows of low-positive reactions, and six rows of NTCs. The assay was performed once, with no carryover of positive samples to negative reactions.

		100X LOD	NTC	100X LOD	NTC	LOD	NTC	LOD	NTC	NTC	NTC	
	1	2	3	4	5	6	7	8	9	10	11	12
A	empty	+	-	+	-	+	-	+	-	-	-	empty
B		+	-	+	-	+	-	+	-	-	-	
C		+	-	+	-	+	-	+	-	-	-	
D		+	-	+	-	+	-	+	-	-	-	
E		+	-	+	-	+	-	+	-	-	-	
F		+	-	+	-	+	-	+	-	-	-	
G		+	-	+	-	+	-	+	-	-	-	
H		+	-	+	-	+	-	+	-	-	-	

Carryover samples	NumPositive	Total	Percent
1,802 CFU/ml	15	16	94
180,155 CFU/ml	16	16	100
NTC	0	48	0

7) INCLUSIVITY/EXCLUSIVITY

The assay for *Bordetella parapertussis* was tested to validate inclusivity and exclusivity. Various strains of *Bordetella parapertussis* were tested to verify inclusive assay performance. The assay was also tested against different species of *Bordetella* to verify exclusivity between close relatives.

All inclusive strains of *B. parapertussis* were tested in six replicates each, while there were twelve total replicates for NTC reactions. The TNAA method successfully detected all inclusive *B. parapertussis* strains.

All exclusive *Bordetella* strains were tested in eight replicates each, with eight positive control reactions and eight negative NTC replicates. The TNAA method excluded all closely related *Bordetella* strains, although one out of eight *B. holmesii* reactions was detected.

The following tables summarize the inclusivity and exclusivity pathogens to be evaluated for the *Bordetella parapertussis* assay.

Inclusivity Samples	NumPositive	Total	Percent
B. parapertussis 12822 [ATCC BAA-587] (10^6 cp/ml)	6	6	100
B. parapertussis 508 and 344 [NCTC 10853] (10^6 cp/ml)	6	6	100
B. parapertussis 509 and 609 (10^6 cp/ml)	6	6	100
B. parapertussis 517 (10^6 cp/ml)	6	6	100
B. parapertussis NCTC 5952 (10^6 cp/ml)	6	6	100
B. parapertussis PT28G (10^6 cp/ml)	6	6	100
NTC	0	12	0

Exclusivity Samples	NumPositive	Total	Percent
B. avium (10^6 cp/ml)	0	8	0
B. bronchiseptica (10^6 cp/ml)	0	8	0
B. holmesii (10^6 cp/ml)	1	8	12
B. parapertussis (10^6 cp/ml)	8	8	100
B. pertussis (10^6 cp/ml)	0	8	0
NTC	0	8	0

8) CROSS-REACTIVITY

The cross-reactivity of the assay was tested against a panel of organisms which may also be present in collected *Bordetella parapertussis* clinical samples. These organisms must be tested to ascertain that no false positives will be due to contamination from the off-target genomic material at clinically relevant viral or bacterial loads. The table below summarizes the genomic material tested and the results obtained. All potentially cross-reactive organisms were tested in replicates of eight and NTCs and the positive control were tested replicates of four. The TNAA assay was verified to not cross-react with any non-target organisms, although one out of eight *E. coli* reactions was detected.

Cross-reactivity Samples	NumPositive	Total	Percent
Adenovirus 4 (10^6 cp/ml)	0	8	0
B. parapertussis (10^5 cp/ml)	4	4	100
B. pertussis (10^8 cp/ml)	0	8	0
C. albicans (10^6 cp/ml)	0	8	0
E. coli (10^8 cp/ml)	1	8	12
Flu A/H1N1 (10^8 cp/ml)	0	8	0
Flu B/Russia/69 (10^8 cp/ml)	0	8	0
hgDNA (200ng/ml)	0	8	0
K. pneumoniae (10^6 cp/ml)	0	8	0
NTC	0	4	0
P. aeruginosa (10^7 cp/ml)	0	8	0
S. aureus MSSA (10^7 cp/ml)	0	8	0
S. pyogenes (10^7 cp/ml)	0	8	0

9) SPECIFICITY

The specificity of the assay was tested against a panel of organisms which may be present as potential contaminants in *Bordetella parapertussis* samples and whose genomic material may be carried though the sample preparation protocol. These organisms must be tested to verify that assay performance is not significantly impacted by the presence of off-target genomic material combined with *Bordetella parapertussis* at clinically relevant loads. The table below summarizes the genomic material tested and the results obtained. All organisms combined with *B. parapertussis* were tested in replicates of two. The positive control and NTCs were also tested in two replicates.

The results below show that the assay is specific to *Bordetella parapertussis* and spiking in other organisms that may be found in the same sample type does not affect assay performance. The assay tested B. parapertussis target at 10X LOD (18,015 CFU/ml) combined with the off-target organism. The off-target nucleic acid concentration reflects expected median viral/bacterial loads in clinical specimens.

Specificity Samples	NumPositive	Total	Percent
B. parapertussis + Adenovirus 4 (10^6 cp/ml)	2	2	100
B. parapertussis + B. pertussis (10^8 cp/ml)	2	2	100
B. parapertussis + C. albicans (10^6 cp/ml)	2	2	100
B. parapertussis + E. coli (10^8 cp/ml)	2	2	100
B. parapertussis + Flu A/H1N1 novel (10^8 cp/ml)	2	2	100
B. parapertussis + Flu B/Mass/3/66 (10^8 cp/ml)	2	2	100
B. parapertussis + hgDNA (200ng/ml)	2	2	100
B. parapertussis + IDTE	2	2	100
B. parapertussis + K. pneumoniae (10^6 cp/ml)	2	2	100
B. parapertussis + P. aeruginosa (10^7 cp/ml)	2	2	100
B. parapertussis + S. aureus MSSA (10^7 cp/ml)	2	2	100
NTC	0	2	0

10) INTERFERING SUBSTANCES

The following interfering substances have been evaluated to have no significant effect on the performance of the TNAA assay. The interfering substances were added to *Bordetella parapertussis* sample prep at both 10% and 0.1% of the total reaction by volume.

Interfering Substances: Endogenous and Exogenous.

Endogenous	Exogenous
Human blood	Bactroban nasal
Mucin	Flonase
Human genomic DNA	Nasonex
	Astelin
	Anefrin Nasal Spray
	Neosynphrine
	VapoRub cough suppressant
	ZiCam Allergy Relief nasal gel
	Mucin
	UTM

11) METHOD COMPARISON ON CLINICAL SAMPLES

The purpose of this study is to estimate the sensitivity and specificity of the TNAA assay using qPCR as the comparator (predicate method).

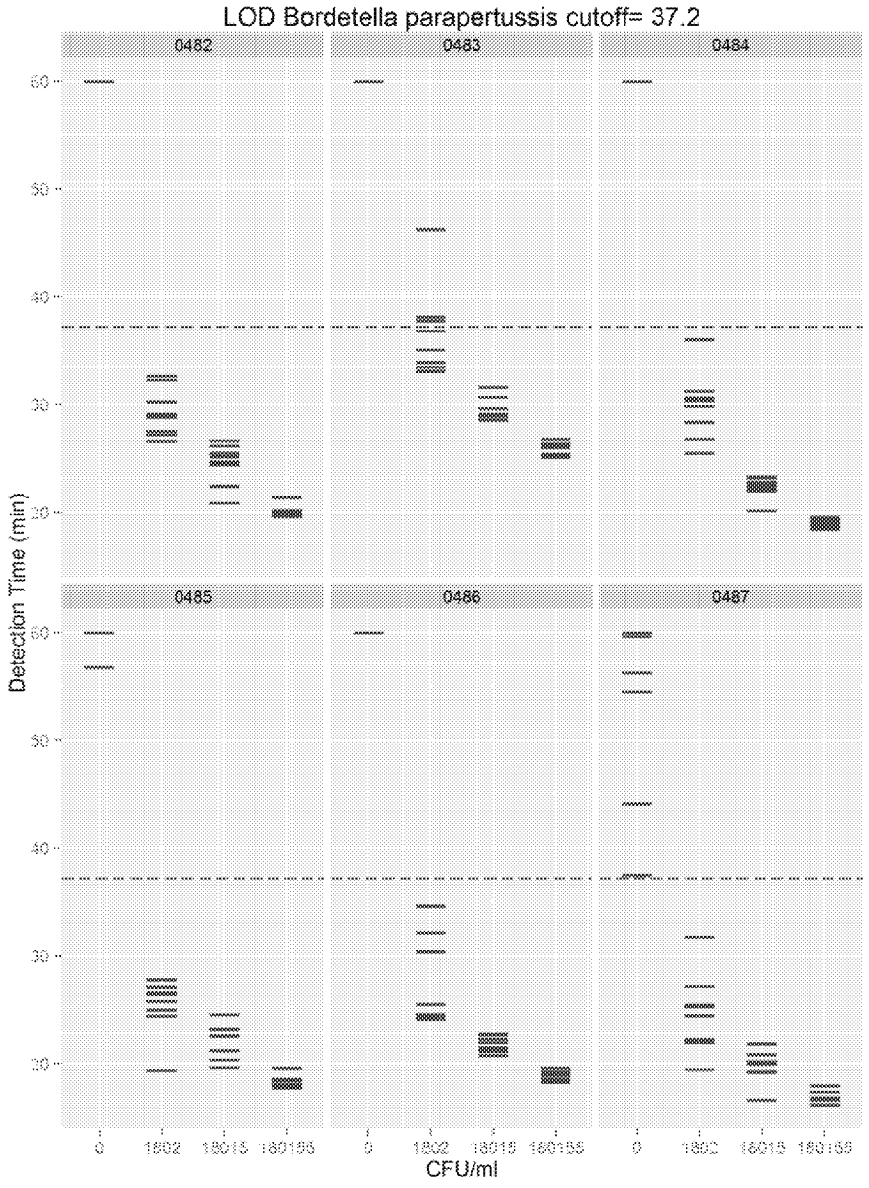
The following clinical samples were tested: 59 positive samples and 100 negative samples obtained from Fostering Tech Medical. Both pharyngeal exudate and nasal swab samples were taken from a range of individuals of both sexes and various ages. Nine of the 100 negative clinical samples were found to be positive when tested by the predicate method (qPCR). However, all of these samples were quantified to be below the *B. parapertussis* assay's Limit of Detection of 1,802 CFU/ml.

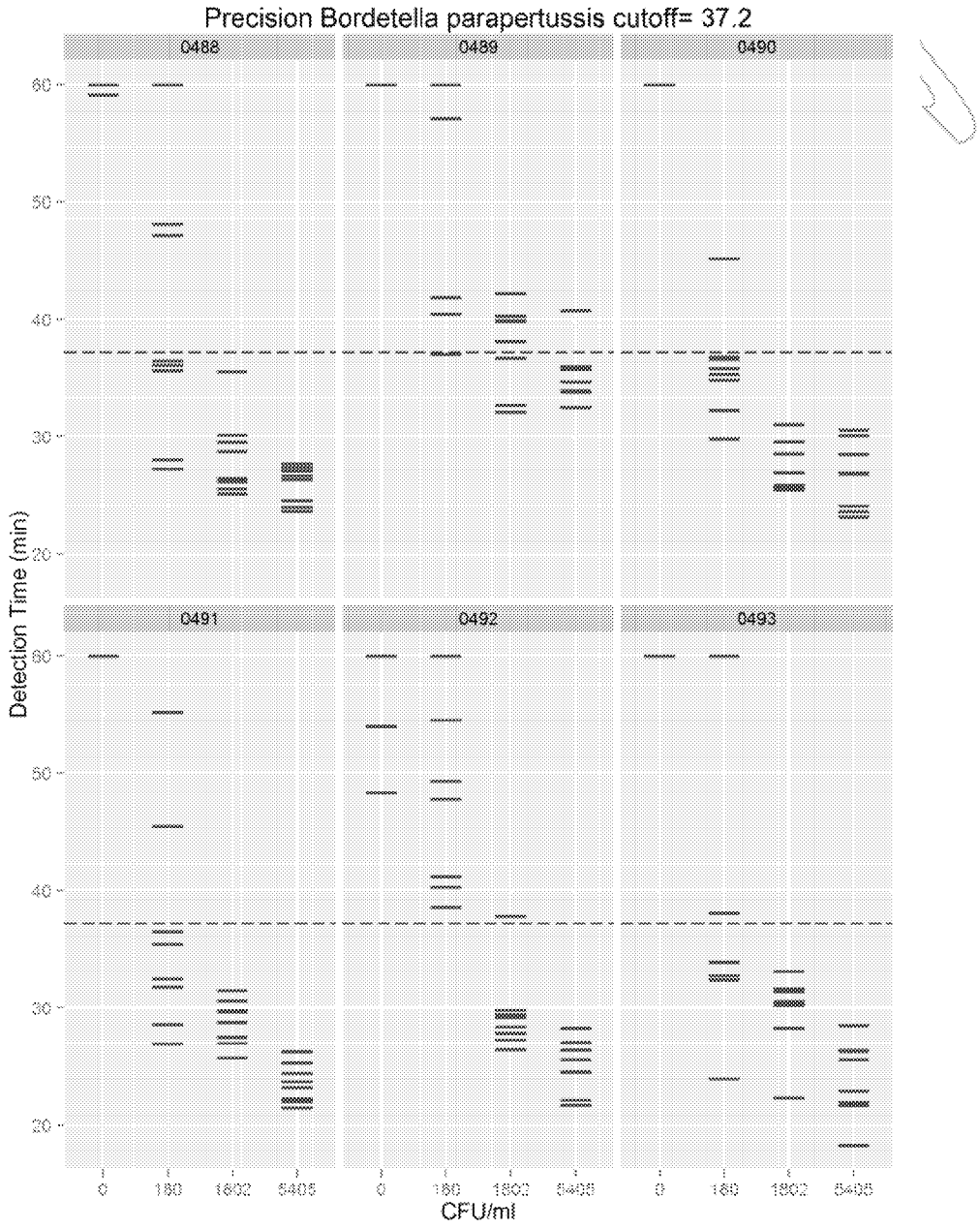
	Clinical Positive (qPCR)	Clinical Positive (TNAA)	Clinical Negative (qPCR)	Clinical Negative (TNAA)
NumPositive	59	59	9	0
Total	59	59	100	100
Percent	100	100	9	0

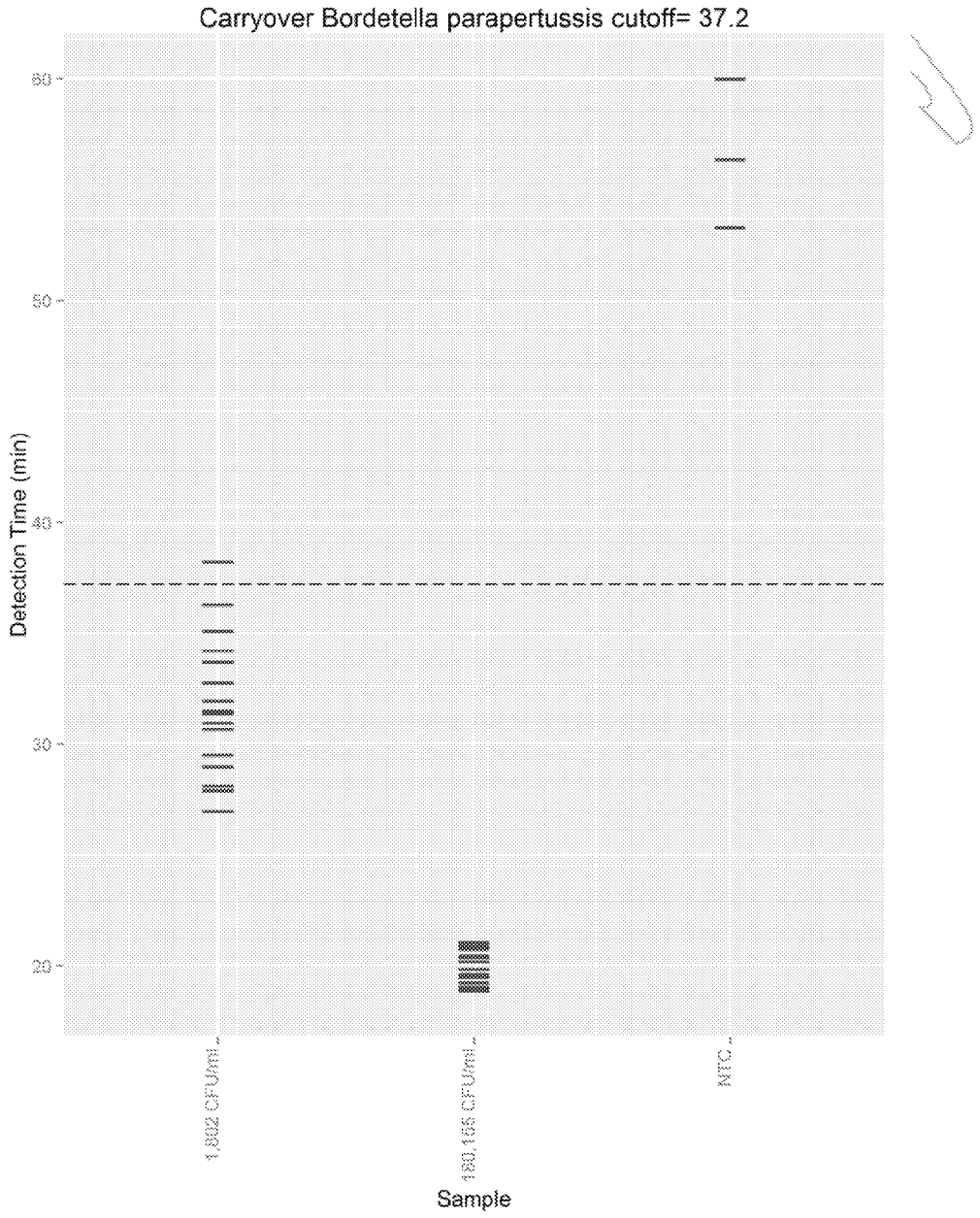
12) FINAL RECOMMENDATIONS

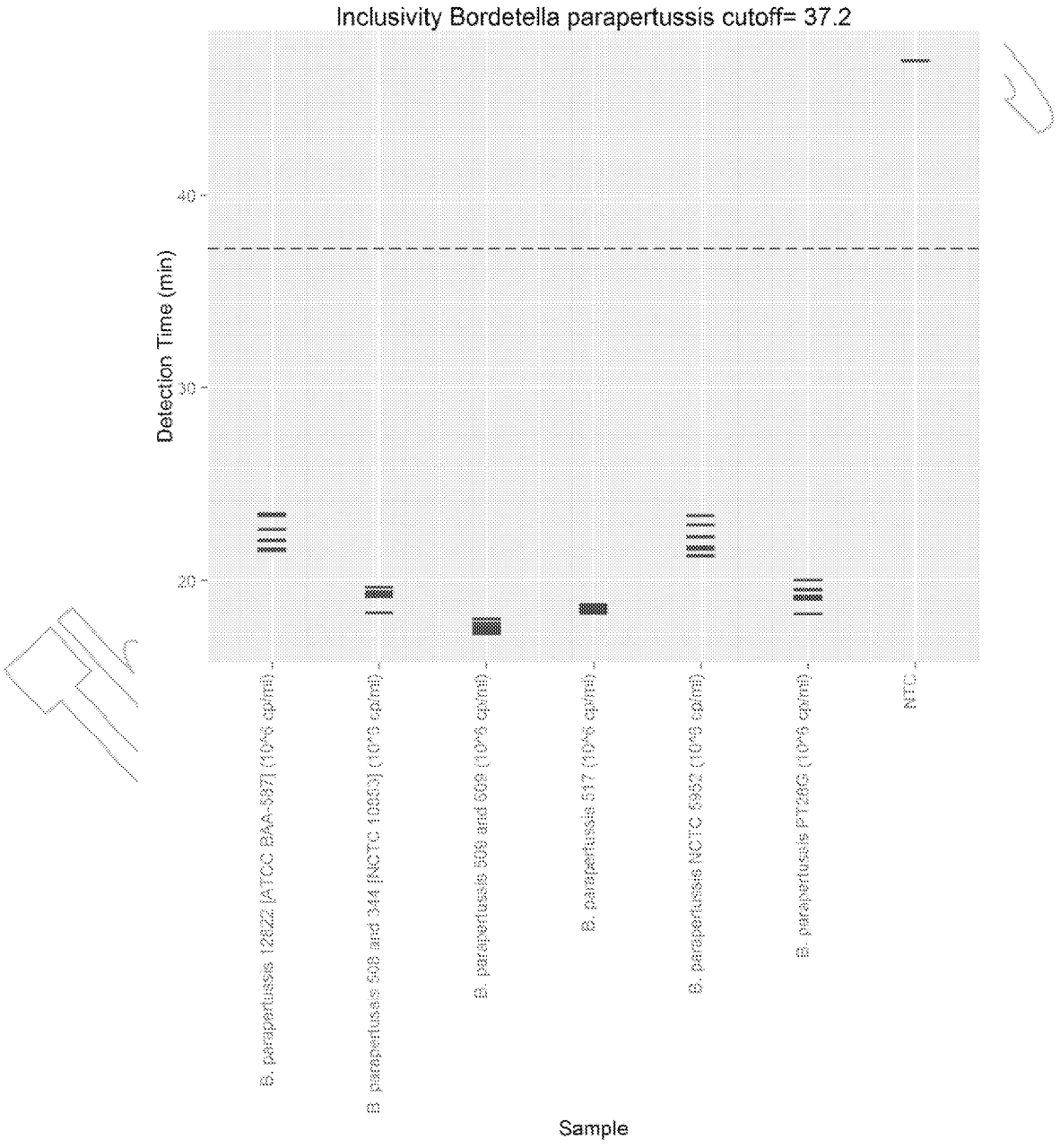
The assay for *Bordetella parapertussis* was found to meet all criteria for precision, carryover, inclusivity, exclusivity, cross-reactivity, specificity, and resistance to interfering substances. Positive and negative clinical samples were tested and compared to a predicate method. The *Bordetella parapertussis* assay specifically and reliably detects *Bordetella parapertussis*. The assay limit of detection is 1,802 CFU/ml with a recommended assay duration of 38 minutes as determined by ROC analysis.

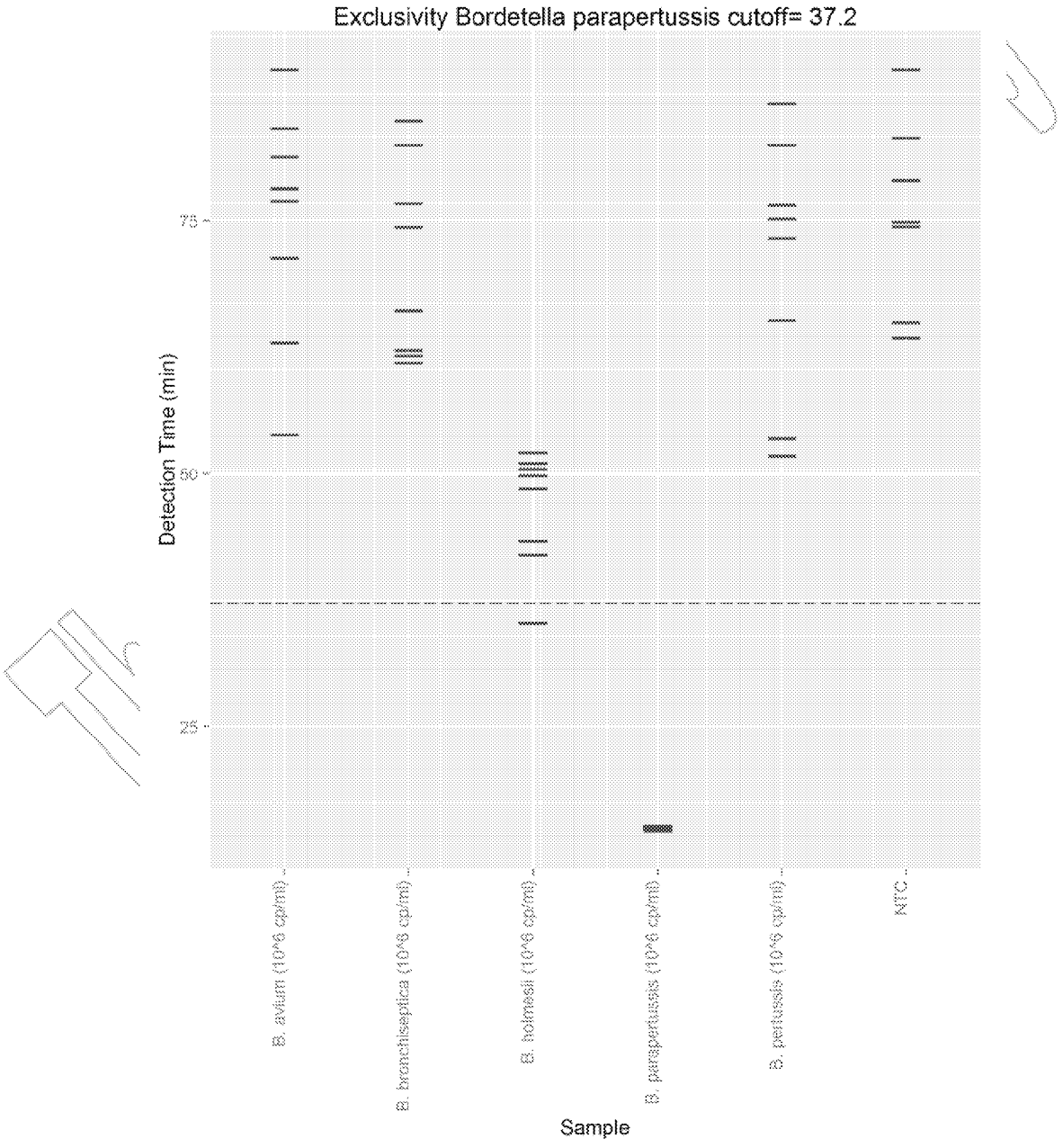
13) APPENDIX

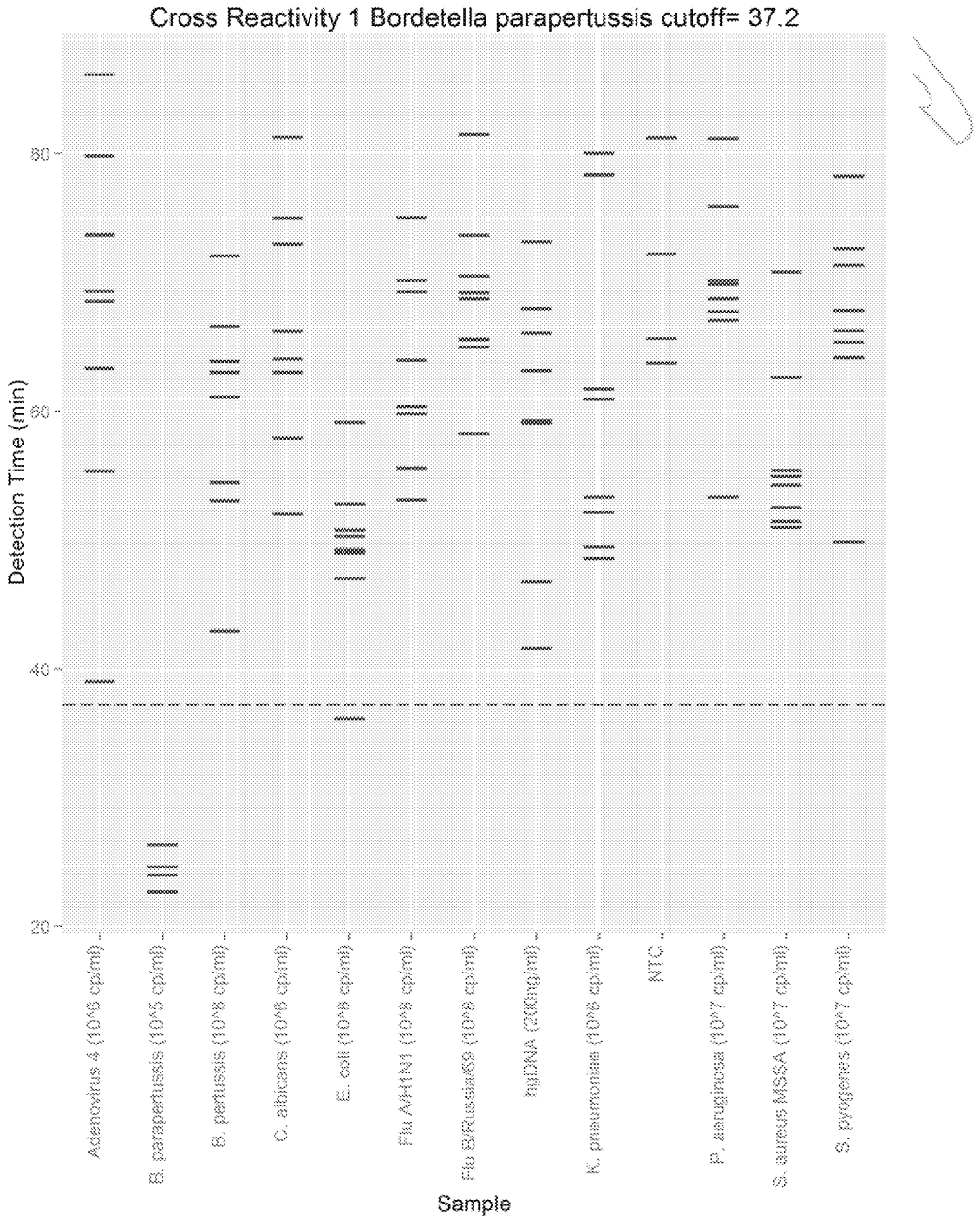


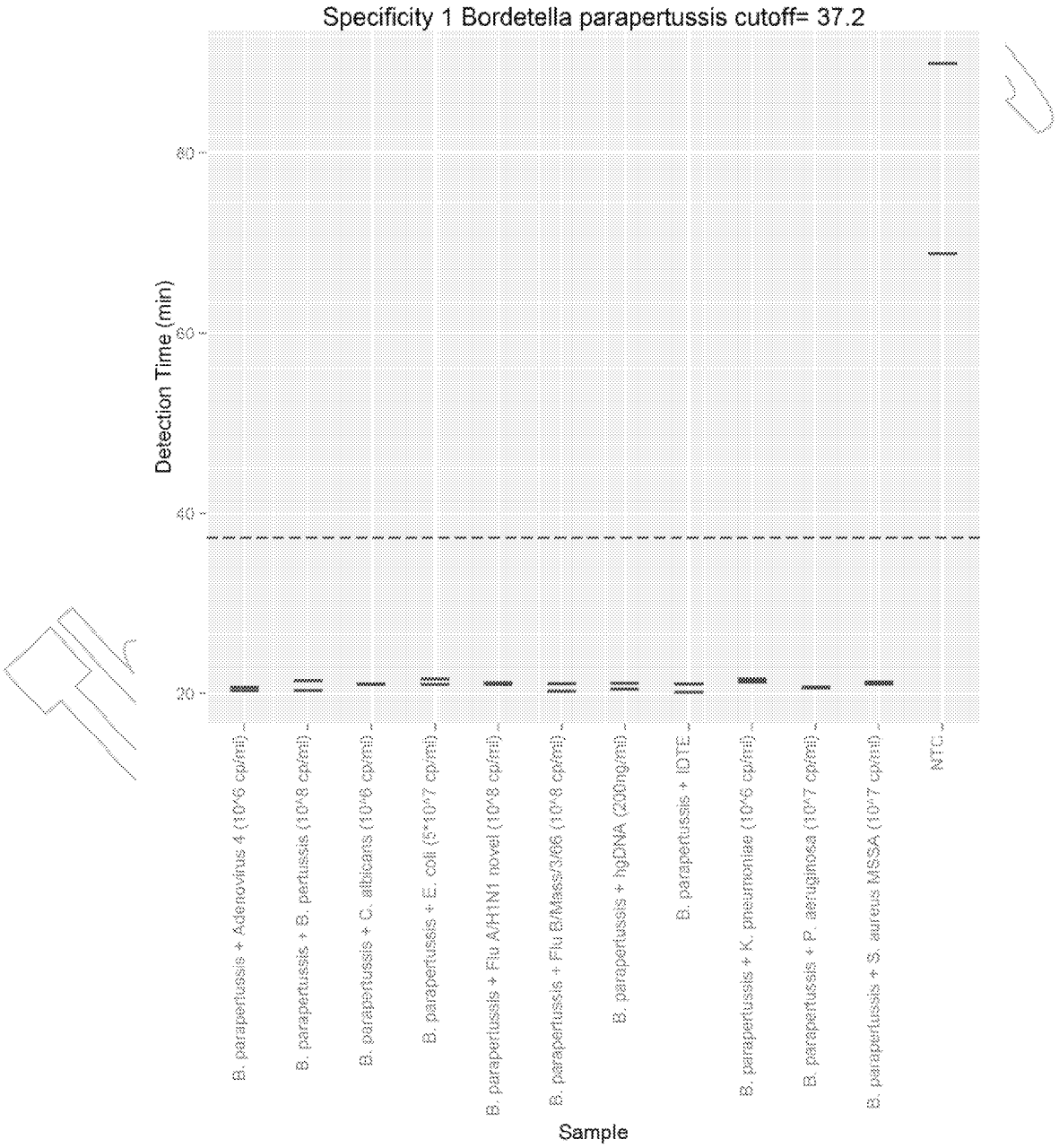


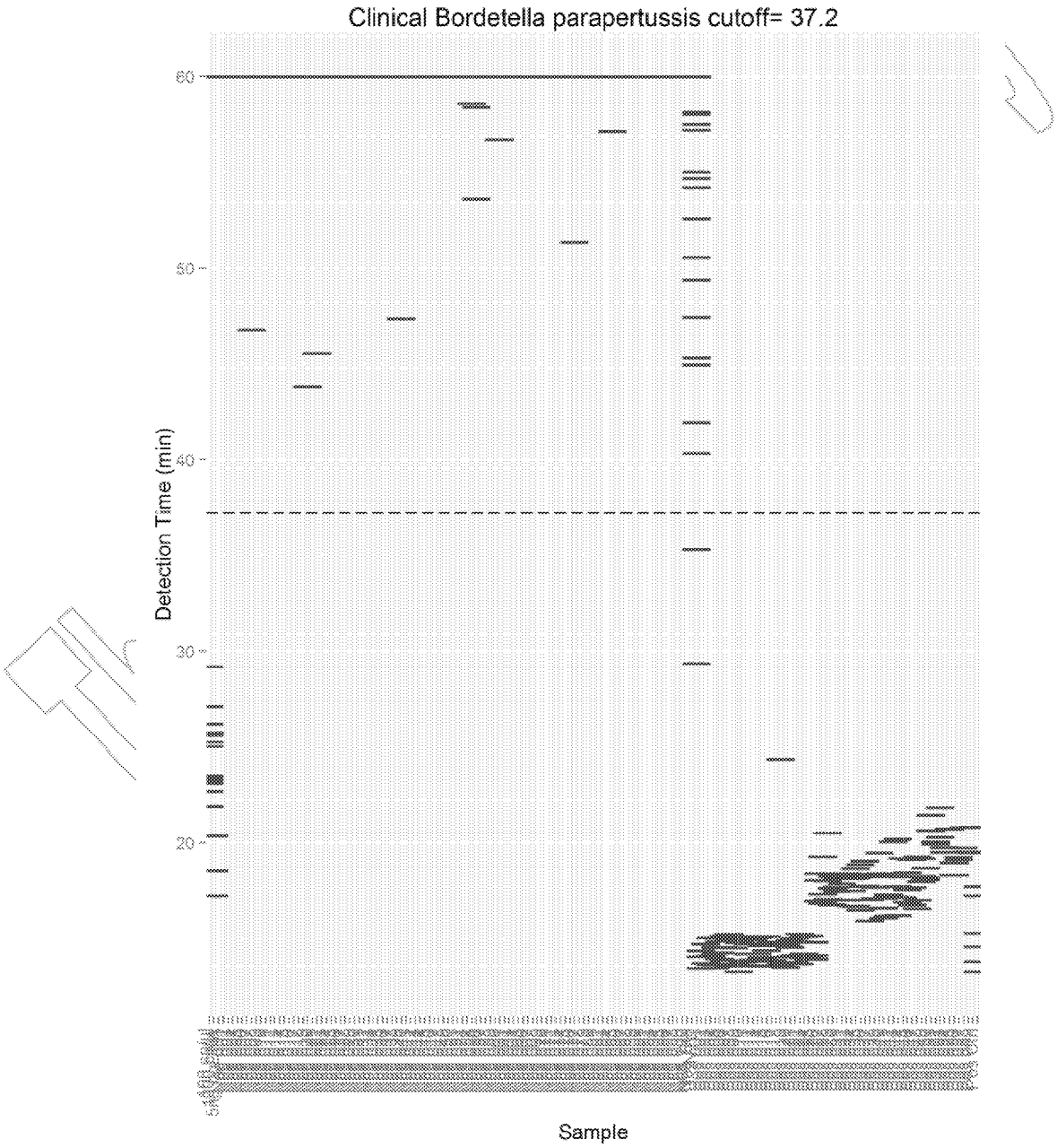












Clinical Samples TNAA Treatment	NumPositive	Total	Percent
100 cp/ul	15	16	94
1000 cp/ul	3	3	100
5ng hgDNA	0	16	0
Neg 001	0	2	0
Neg 002	0	2	0
Neg 003	0	2	0
Neg 004	0	2	0
Neg 005	0	2	0
Neg 006	0	2	0
Neg 007	0	2	0
Neg 008	0	2	0
Neg 009	0	2	0
Neg 010	0	2	0
Neg 011	0	2	0
Neg 012	0	2	0
Neg 013	0	2	0
Neg 014	0	2	0
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Neg 017	0	2	0
Neg 018	0	2	0
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Neg 020	0	2	0
Neg 021	0	2	0
Neg 022	0	2	0
Neg 023	0	2	0
Neg 024	0	2	0
Neg 025	0	2	0
Neg 026	0	2	0
Neg 027	0	2	0

Neg 028	0	2	0
Neg 029	0	2	0
Neg 030	0	2	0
Neg 031	0	2	0
Neg 032	0	2	0
Neg 033	0	2	0
Neg 034	0	2	0
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Neg 037	0	2	0
Neg 038	0	2	0
Neg 039	0	2	0
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Neg 087	0	2	0
Neg 088	0	2	0
Neg 089	0	2	0

Neg 090	0	2	0
Neg 091	0	2	0
Neg 092	0	2	0
Neg 093	0	2	0
Neg 094	0	2	0
Neg 095	0	2	0
Neg 096	0	2	0
Neg 097	0	2	0
Neg 098	0	2	0
Neg 099	0	2	0
Neg 100	0	2	0
Neg Ctrl	0	4	0
NTC	2	136	1
Pos 001	3	3	100
Pos 002	3	3	100
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Pos 050	2	2	100
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Pos 056	2	2	100
Pos 057	2	2	100
Pos 058	2	2	100
Pos 059	2	2	100
Pos Ctrl	7	7	100

BORDETELLA PERTUSSIS

TNAA LDT Validation Report

Limit of Detection = 0.4 cp/uL

Rate of Detection = 10 cp/uL in 20 minutes

Katie Sullivan-Bibee

Theranos, Inc. 1601 S. California Avenue Palo Alto, CA 94304

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Name: Daniel Young, Ph.D.	Title: Vice President

Approver(s):

Signature:	Date:
Name: Adam Rosendorff, M.D	Title: Laboratory Director

Table of Contents

Bordetella pertussis

1) PURPOSE

This report includes relevant information about the target organism, a detailed description of the primers and selected targets designed for the detection of this organism, a summary of the validation assay performance, and recommendations for future assay execution.

2) BACKGROUND

Bordetella pertussis is a Gram-negative, aerobic coccobacillus of the genus *Bordetella*, and the causative agent of pertussis or whooping cough. It is an obligate human pathogen and infects the host by colonizing lung epithelial cells. The bacterium contains a surface protein, filamentous hemagglutinin, which binds to the lactose-containing moities found on cilia of epithelial cells. Once anchored, the bacterium produces tracheal cytotoxin which stops the cilia's ability to beat and thus prevents debris clearance from the lungs. The body responds by sending the host into a coughing fit, expelling bacteria into the air, which are free to infect other hosts.

Pertussis (or whooping cough) is an infection of the respiratory system characterized by a "whooping" sound when the person breathes in, although only 50% of patients display the classic sound as they attempt to draw breath over a partially closed glottis. In the US, whooping cough killed between 10,000 and 20,000 people per year before a vaccine was available. Vaccination has transformed this; between 1985 and 1988, fewer than 100 children died from pertussis. The dropping rates of vaccination are reversing these trends.

The target gene is the transposon *IS481*. According to Parkhill et al. (Nature Genetics, 2003), this element is present in 238 copies in the reference *B. pertussis* strain Tohama I, but not present in *B. parapertussis* or *B. bronchiseptica*. However, some *B. bronchiseptica* isolates appear to contain the element (e.g. EF043395) – see Register and Sanden 2006 (Journal of Clinical Microbiology). Since *B. bronchiseptica* is almost never observed in humans and *IS481* has yet to be found in *B. parapertussis*, the target still seems like a sound choice. It is important that the assay not cross amplify *B. parapertussis*, which has less severe symptoms and different reporting requirements. *IS481* has a GC content of 65%.

3) SUMMARY OF PERFORMANCE DATA

Theranos developed a Theranos Nucleic Acid Amplification (TNAA) assay specific for *Bordetella pertussis*. The Nucleic Acid Amplification reactions contained 1x Nucleic Acid Amplification buffer (20 mM Tris Acetate, pH 7.9, 50 mM Potassium Acetate, 10 mM Magnesium Acetate and 1mM DTT), 0.08% Tween, 0.8 M betaine, 1.4 mM dNTPs, 2 uM Syto59, 0.8 uM RLX1512 primer and 0.8 uM RLX1513 primer, 20 units Bst polymerase, and template at the noted concentration. The reactions were run at 56ºC for 60 minutes. Summarized data will follow below while detailed experimental data can be found in the appendix. Primer sequences are:

Bordetella pertussis	RLX 1512	TTCATGGCCTACCAGAACTCCCA
	RLX 1513	GCCATGAACAGTTGTAGTGGTGTA

4) LIMIT OF DETECTION

The purpose of this study is to determine the limit of detection (LOD) for the Theranos TNAA assay. The LOD_{95} is the bacterial titer at which >95% of known positive samples test positive using the TNAA assay. Statistically justified cut-off times for making positive/negative calls were determined for each target empirically. A set of experiments, repeated over four days, were conducted that included eight replicates each of three target dilutions (LoD, 10X LoD, and 100X LoD), as well as eight NTCs, using the target primers for amplification. These data were then processed using a receiver-operator character (ROC) analysis, and the best threshold detection time for distinguishing positives and negatives determined using the Youden test statistic as implemented by the R package, pROC.

The assay reliably detected 228 CFU/ml of *Bordetella pertussis* in about 29.5 minutes, as shown below. The 29.5 minute assay cut-off time was determined by ROC analysis. The assay was performed eight times. Reactions with and without template (NTCs or Non-Templated Controls) were run in eight replicates each.

LOD	Sample	NumPositive	Total	Percent
100X LOD	22,829 CFU/ml	64	64	100
10X LOD	2,283 CFU/ml	64	64	100
1X LOD	228 CFU/ml	64	64	100
	NTC	0	64	0

5) REPRODUCIBILITY/PRECISION

The purpose of this experiment is to determine the precision of the assay, percent positive and negative at three detection limits: high-negative (0.1X LOD=23 CFU/ml), low-positive (LOD=228 CFU/ml), and high-positive (3X LOD=685 CFU/ml). The assay was performed eight times. Reactions with and without template (NTCs or Non-templated Controls) were run in eight replicates each.

Precision LOD	Sample	NumPositive	Total	Percent
3X LOD	685 CFU/ml	64	64	100
1X LOD	228 CFU/ml	64	64	100
0.1X LOD	23 CFU/ml	61	64	95
	NTC	1	64	2

6) CARRYOVER

The purpose of this experiment is to determine the potential for carryover of positive samples adjacent to negative reactions. The nucleic acid template is prepared from high-positive (100X LOD = 22,829 CFU/ml), low-positive (1X LOD=228 CFU/ml), and non-templated controls (NTCs) which are arrayed in alternating rows of eight replicates each. There are two rows of high-positive reactions, two rows of low-positive reactions, and six rows of NTCs. The assay was performed once, with no carryover of positive samples to negative reactions.

		100X LOD	NTC	100X LOD	NTC	LOD	NTC	LOD	NTC	NTC	NTC	
	1	2	3	4	5	6	7	8	9	10	11	12
A	empty	+	-	+	-	+	-	+	-	-	-	empty
B		+	-	+	-	+	-	+	-	-	-	
C		+	-	+	-	+	-	+	-	-	-	
D		+	-	+	-	+	-	+	-	-	-	
E		+	-	+	-	+	-	+	-	-	-	
F		+	-	+	-	+	-	+	-	-	-	
G		+	-	+	-	+	-	+	-	-	-	
H		+	-	+	-	+	-	+	-	-	-	

Carryover Samples	NumPositive	Total	Percent
22,829 CFU/ml	16	16	100
228 CFU/ml	16	16	100
NTC	0	48	0

7) INCLUSIVITY/EXCLUSIVITY

The assay for *Bordetella pertussis* was tested to validate inclusivity and exclusivity. Various strains of *Bordetella pertussis* were tested to verify inclusive assay performance. The assay was also tested against different species of *Bordetella* to verify exclusivity between close relatives.

This assay does detect *Bordetella holmesii*. A separate assay was developed for *B. holmesii* and was verified not to cross-react with *B. pertussis.*

All inclusive strains of *B. pertussis* were tested in eight replicates each, while NTCs were tested in eight replicates. The TNAA method successfully detected all inclusive *B. pertussis* strains as well as *B. holmesii.*

All exclusive *Bordetella* strains were tested in eight replicates each, with eight positive replicate reactions for *B. pertussis* and eight negative NTC replicates. The TNAA method excluded all closely related *Bordetella* strains with the exception of *B. holmesii*.

The following tables summarize the inclusivity and exclusivity pathogens to be evaluated for the *Bordetella pertussis* assay.

Inclusivity Samples	NumPositive	Total	Percent
Bordetella holmesii (10^7 cp/ml)	8	8	100
Bordetella pertussis 10-536 (10^6 cp/ml)	8	8	100
Bordetella pertussis 40103 (10^6 cp/ml)	8	8	100
Bordetella pertussis 5 [17921] (10^6 cp/ml)	8	8	100
Bordetella pertussis 5374 [3747] (10^6 cp/ml)	8	8	100
Bordetella pertussis 589 (10^6 cp/ml)	8	8	100
Bordetella pertussis CNCTC Hp 12/63 [623] (10^6 cp/ml)	8	8	100
Bordetella pertussis F (10^6 cp/ml)	8	8	100
Bordetella pertussis MN2531 (10^6 cp/ml)	8	8	100
Bordetella pertussis PT9/28G [W28] (10^6 cp/ml)	8	8	100
Bordetella pertussis Tohama 1 (10^6 cp/ml)	8	8	100
NTC	0	8	0

Exclusivity Samples	NumPositive	Total	Percent
Bordetella avium (10^7 cp/ml)	0	8	0
Bordetella bronchiseptica (10^7 cp/ml)	0	8	0
Bordetella holmesii (10^7 cp/ml)	8	8	100
Bordetella parapertussis (10^7 cp/ml)	0	8	0
Bordetella pertussis (10^7 cp/ml)	8	8	100
NTC	0	8	0

8) CROSS-REACTIVITY

The cross-reactivity of the assay was tested against a panel of organisms which may also be present in collected *Bordetella pertussis* clinical samples. These organisms must be tested to ascertain that no false positives will be due to contamination from the off-target genomic material at clinically relevant viral or bacterial loads. The table below summarizes the genomic material tested and the results obtained. All potentially cross-reactive organisms and the positive control were tested in replicates of eight, while NTCs were tested in replicates of four. The TNAA assay was verified to not cross-react with any non-target organisms, with the exception of *C. albicans* and hgDNA. However, this cross-reactivity was not found to be significant.

Cross-reactivity Samples	NumPositive	Total	Percent
Adenovirus 4 (10^7 cp/ml)	0	8	0
Bordetella pertussis (10^7 cp/ml)	8	8	100
Candida albicans (10^8 cp/ml)	1	8	12
Escherichia coli (10^8 cp/ml)	0	8	0
hgDNA (200ng/ml)	1	8	12
Influenza A/WS/33 (H1N1) (10^8 cp/ml)	0	8	0
Influenza B/Hubei-Wujiagang/158/2009 (10^8 cp/ml)	0	8	0
Klebsiella pneumoniae (10^8 cp/ml)	0	8	0
NTC	0	4	0
Pseudomonas aeruginosa (10^8 cp/ml)	0	8	0
Staphylococcus aureus MSSA (DmecA) (10^8 cp/ml)	0	8	0
Streptococcus pyogenes (10^8 cp/ml)	0	8	0

9) SPECIFICITY

The specificity of the assay was tested against a panel of organisms which may be present as potential contaminants in *Bordetella pertussis* samples and whose genomic material may be carried though the sample preparation protocol. These organisms must be tested to verify that assay performance is not significantly impacted by the presence of off-target genomic material combined

with *Bordetella pertussis* at clinically relevant loads. The table below summarizes the genomic material tested and the results obtained. All organisms combined with *B. pertussis* were tested in replicates of two. The positive control and NTCs were also tested in two replicates.

The results below show that the assay is specific to *Bordetella pertussis* and spiking in other organism species that may be found in the same sample type does not affect assay performance. The assay tested *B. pertussis* target at 10X LOD (2,283 CFU/ml) combined with the off-target organism. The off-target nucleic acid concentration reflects expected median viral/bacterial loads in clinical specimens.

Specificity Samples	NumPositive	Total	Percent
B. pertussis + Adenovirus 4 (10^6 cp/ml)	2	2	100
B. pertussis + A/Solomon Islands/3/2006 (H1N1) (10^8 cp/ml)	2	2	100
B. pertussis + Bordetella pertussis (10^8 cp/ml)	2	2	100
B. pertussis + B/Russia/69 (10^8 cp/ml)	2	2	100
B. pertussis + Candida albicans (10^6 cp/ml)	2	2	100
B. pertussis + Escherichia coli (10^8 cp/ml)	2	2	100
B. pertussis + hgDNA (200ng/ml)	2	2	100
B. pertussis + IDTE	2	2	100
B. pertussis + Klebsiella pneumoniae (10^6 cp/ml)	2	2	100
B. pertussis + Pseudomonas aeruginosa (10^7 cp/ml)	2	2	100
B. pertussis + Staphylococcus aureus MSSA (DmecA) (10^7 cp/ml)	2	2	100
NTC	0	2	0

10) INTERFERING SUBSTANCES

The following interfering substances have been evaluated to have no significant effect on the performance of the TNAA assay. The interfering substances were added to *Bordetella pertussis* sample prep at both 10% and 0.1% of the total reaction by volume.

Interfering Substances: Endogenous and Exogenous.

Endogenous	Exogenous
Human blood	Bactroban nasal
Mucin	Flonase
Human genomic DNA	Nasonex
	Astelin
	Anefrin Nasal Spray
	Neosynphrine
	VapoRub cough suppressant
	ZiCam Allergy Relief nasal gel
	Mucin
	UTM

11) METHOD COMPARISON ON CLINICAL SAMPLES

The purpose of this study is to estimate the sensitivity and specificity of the TNAA assay using qPCR as the comparator (predicate method).

The following clinical samples were tested: 54 positive samples and 100 negative samples obtained from Fostering Tech Medical. Sample types obtained were pharyngeal exudate and nasal swabs which were taken from a range of individuals of both sexes and various ages.

TNAA vs qPCR Contingency Table		qPCR		
		Positive	Negative	Total
TNAA	Positive	54	0	54
	Negative	0	100	100
	Total	54	100	154

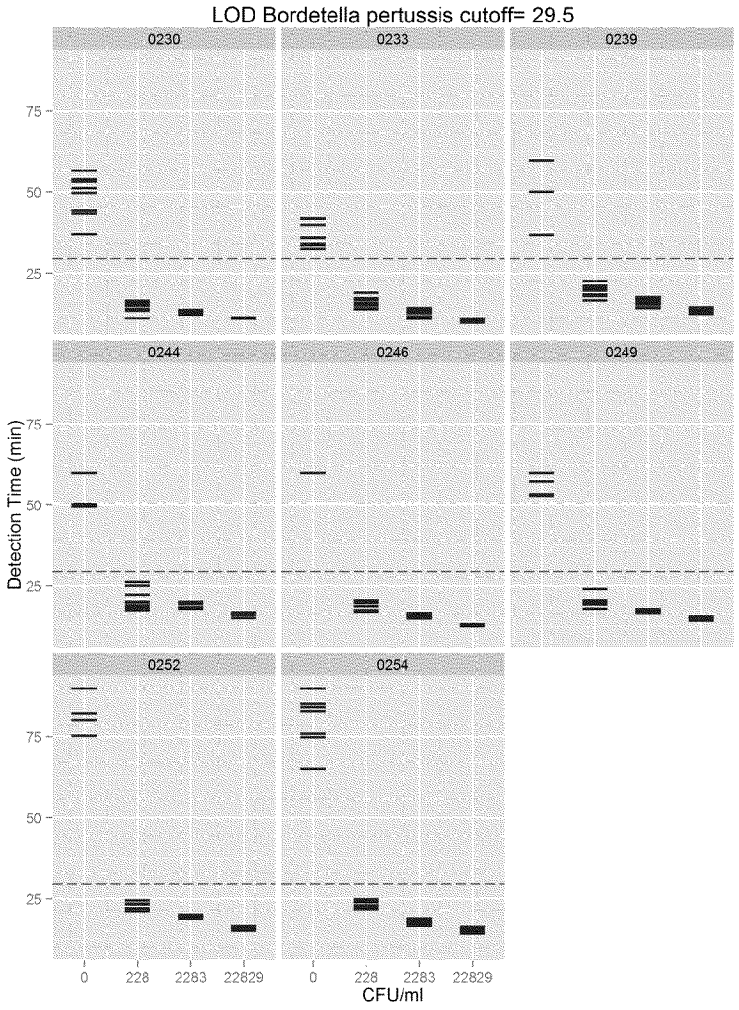
	Percent	95% Confidence Interval	
Estimated Sensitivity	100%	93%	100%
Estimated Specificity	100%	96%	100%

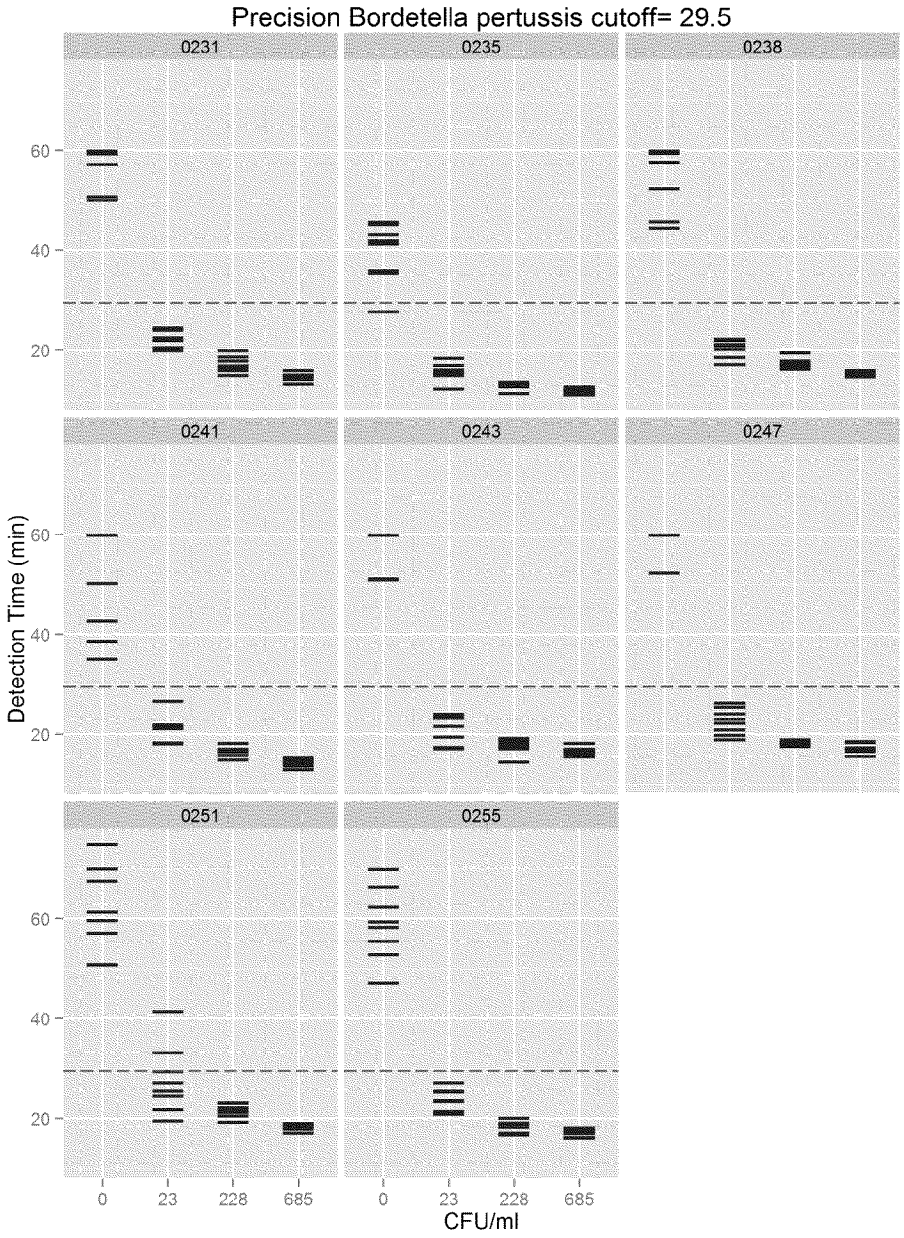
Based on a Prevalence of	**35%**
Positive Predictive Value	100%
Negative Predictive Value	100%

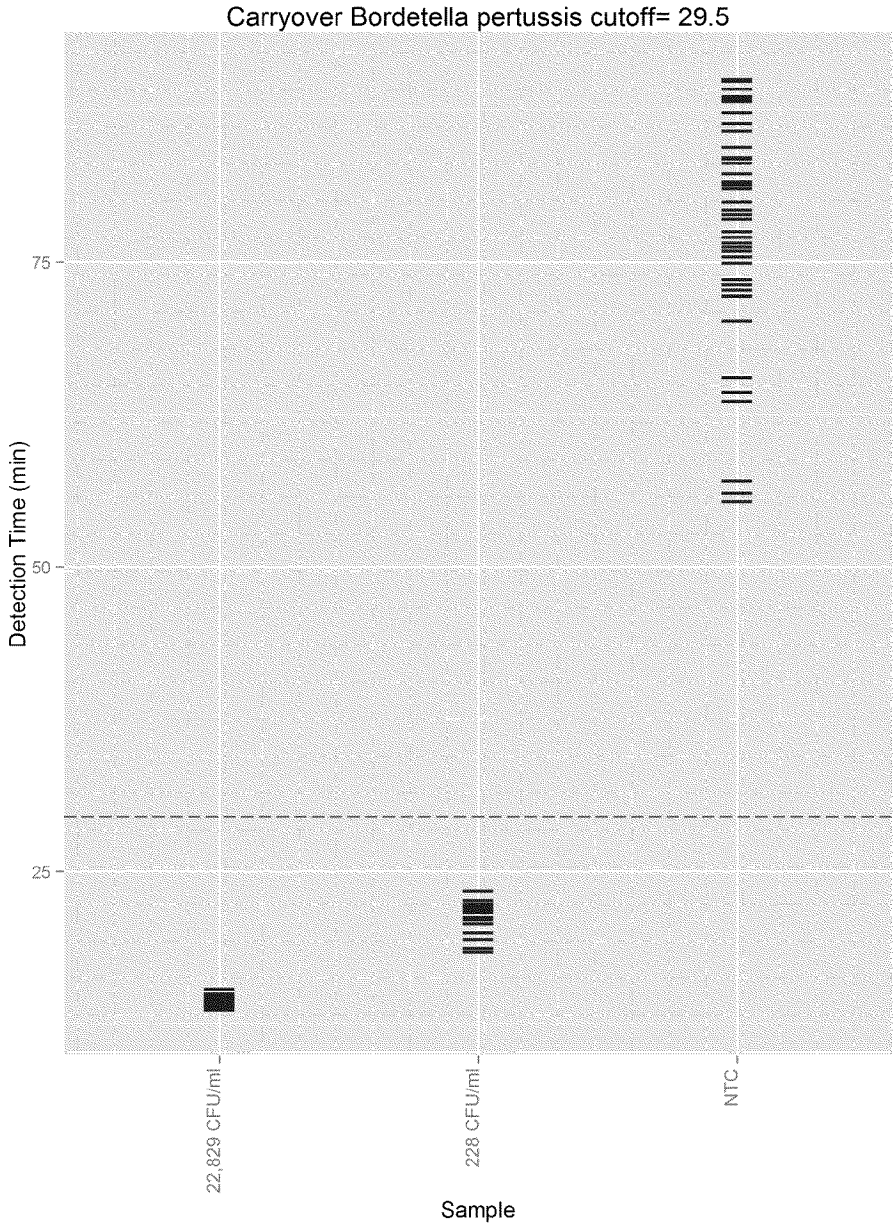
12) FINAL RECOMMENDATIONS

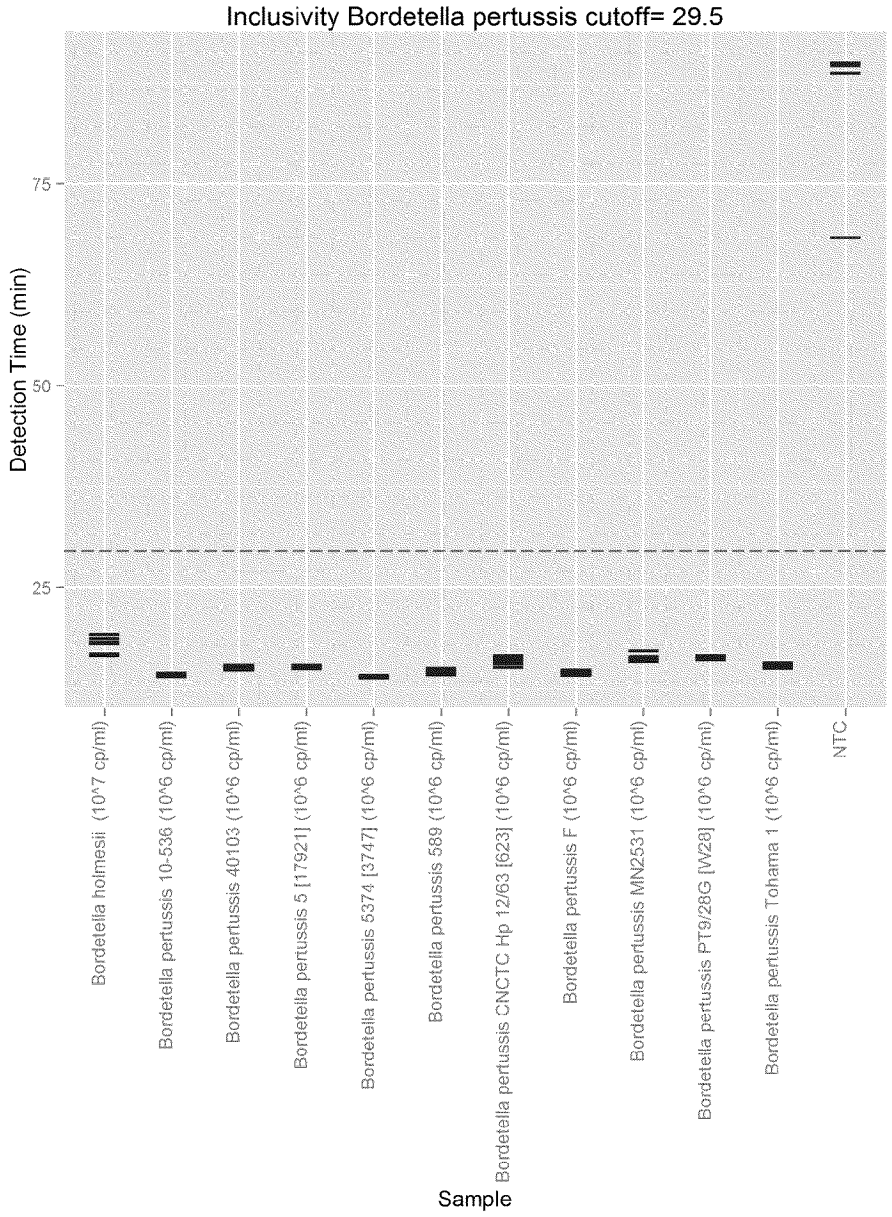
The assay for *Bordetella pertussis* was found to meet all criteria for precision, carryover, inclusivity, exclusivity, cross-reactivity, specificity, and resistance to interfering substances. Positive and negative clinical samples were tested and compared to a predicate method. The *Bordetella pertussis* assay specifically and reliably detects *Bordetella pertussis*. The assay limit of detection is 228 CFU/ml with a recommended assay duration of 30 minutes as determined by ROC analysis.

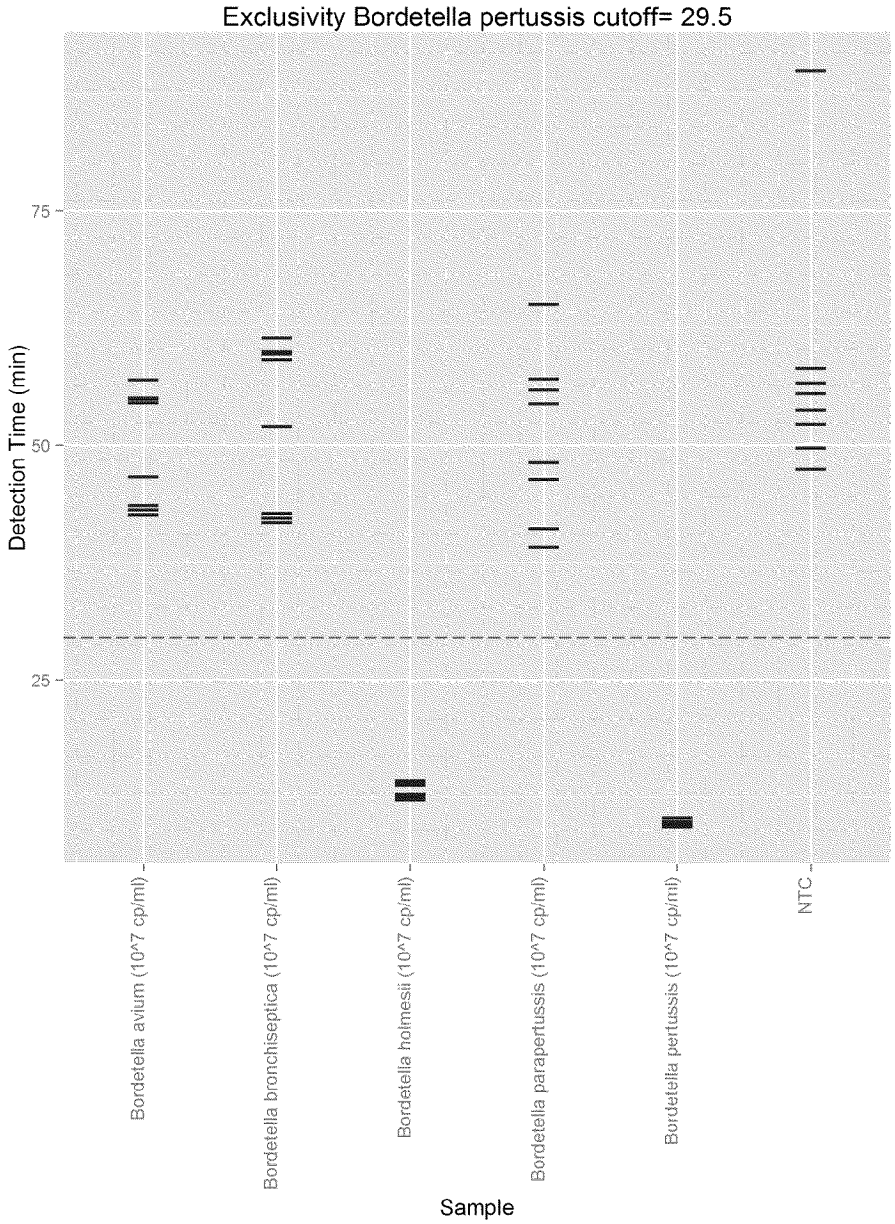
13) APPENDIX

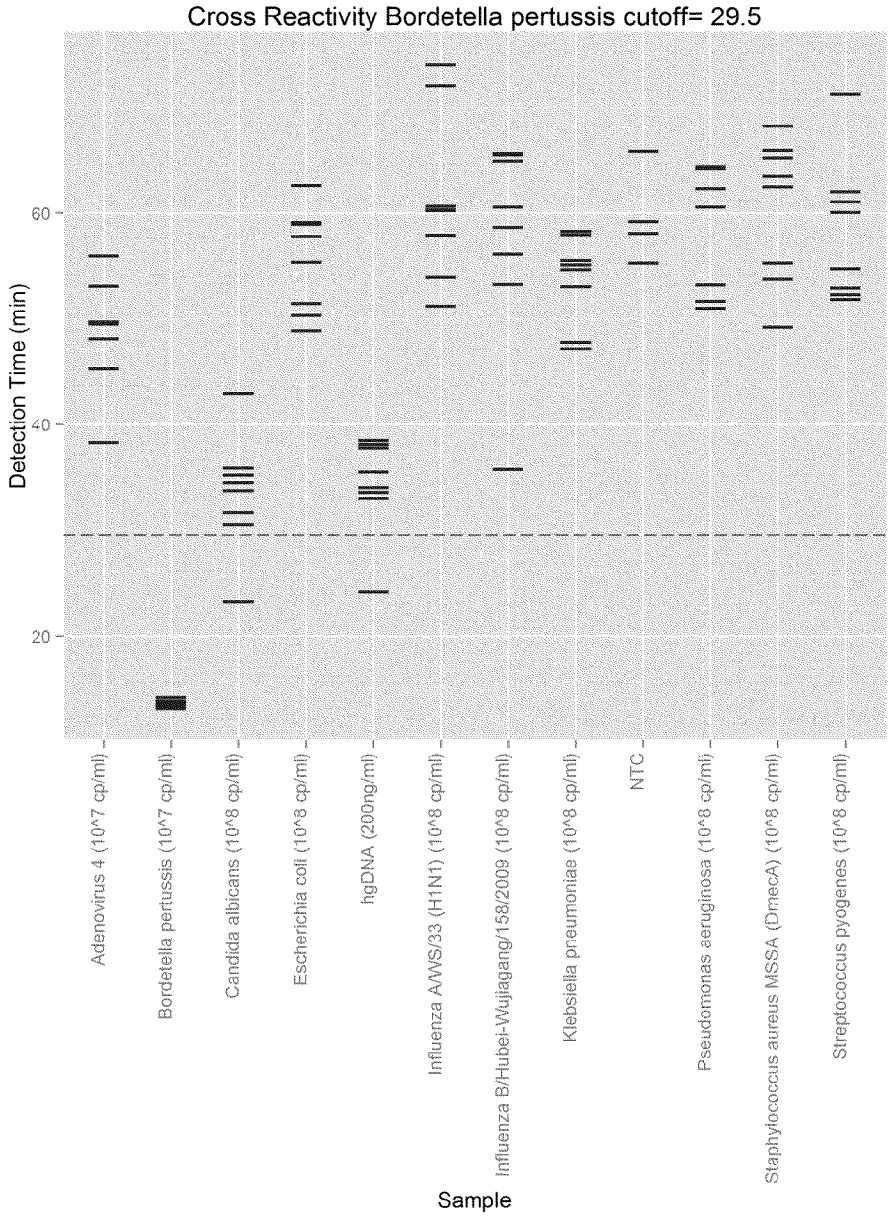


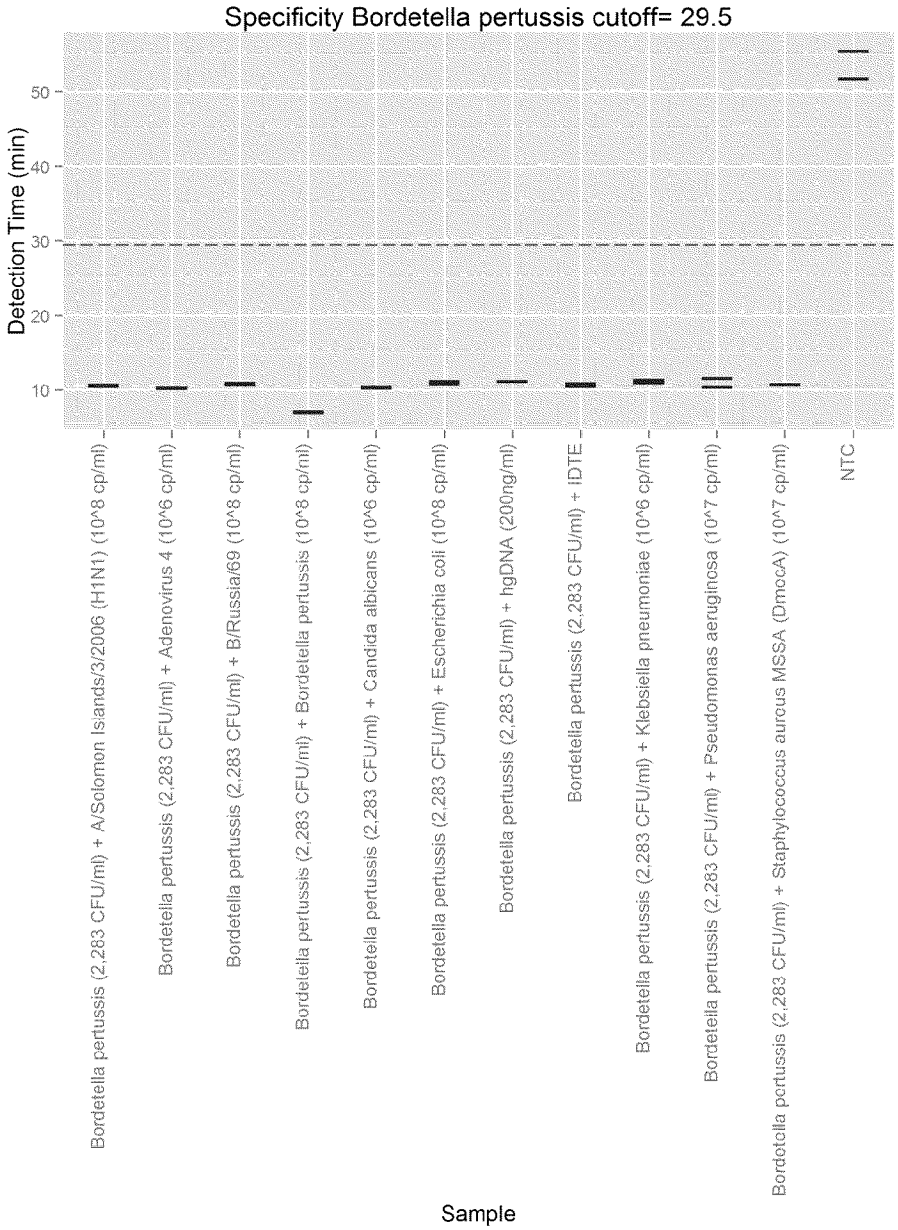


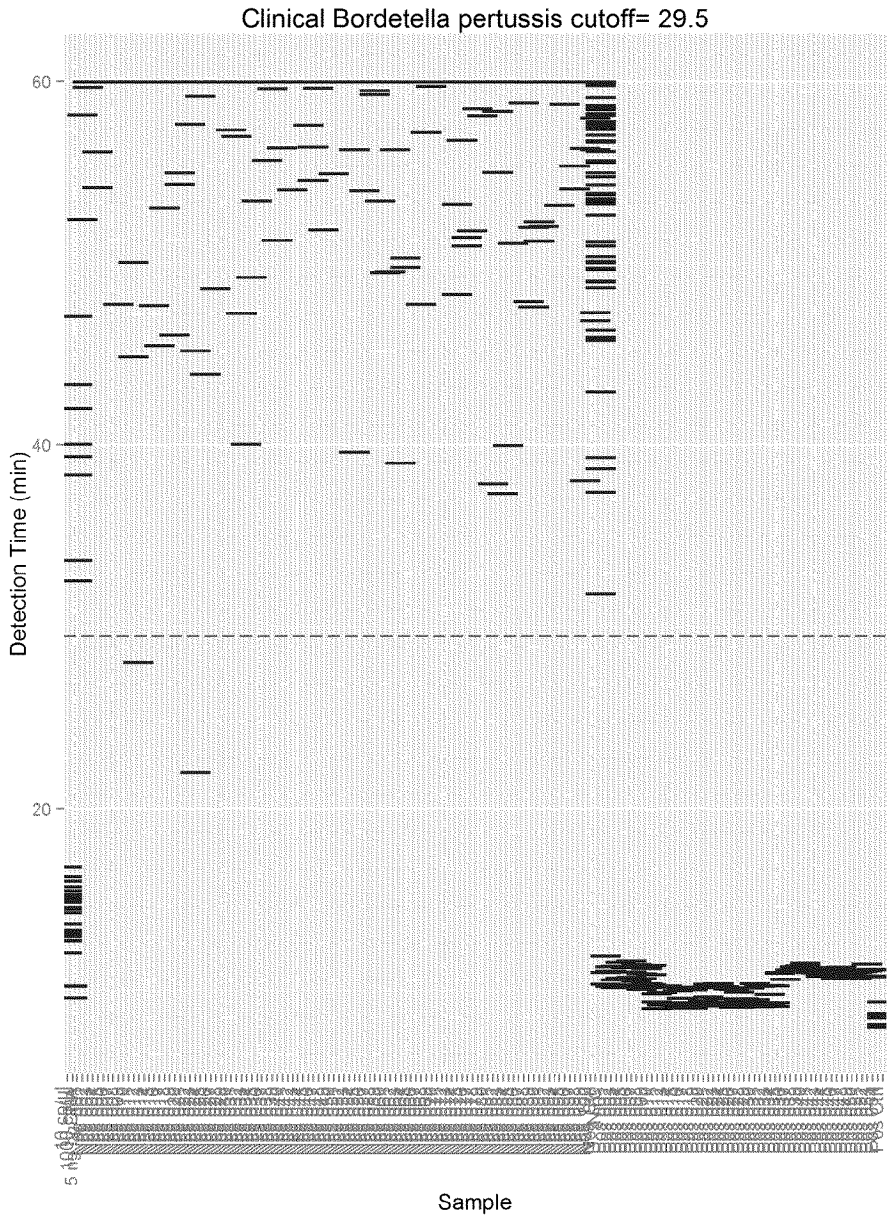












Clinical Samples TNAA: Treatment	NumPositive	Total	Percent
10 cp/ul	24	24	100
1000 cp/ul	2	3	67
5 ng hgDNA	0	8	0
Neg 001	0	2	0
Neg 002	0	2	0
Neg 003	0	2	0
Neg 004	0	2	0
Neg 005	0	2	0
Neg 006	0	2	0
Neg 007	0	2	0
Neg 008	0	2	0
Neg 009	0	2	0
Neg 010	0	2	0
Neg 011	0	2	0
Neg 012	1	2	50
Neg 013	0	2	0
Neg 014	0	2	0
Neg 015	0	2	0
Neg 016	0	2	0
Neg 017	0	2	0
Neg 018	0	2	0
Neg 019	0	2	0
Neg 020	0	2	0
Neg 021	0	2	0
Neg 022	0	2	0
Neg 023	1	2	50
Neg 024	0	2	0
Neg 025	0	2	0
Neg 026	0	2	0

Neg 027	0	2	0
Neg 028	0	2	0
Neg 029	0	2	0
Neg 030	0	2	0
Neg 031	0	2	0
Neg 032	0	2	0
Neg 033	0	2	0
Neg 034	0	2	0
Neg 035	0	2	0
Neg 036	0	2	0
Neg 037	0	2	0
Neg 038	0	2	0
Neg 039	0	2	0
Neg 040	0	2	0
Neg 041	0	2	0
Neg 042	0	2	0
Neg 043	0	2	0
Neg 044	0	2	0
Neg 045	0	2	0
Neg 046	0	2	0
Neg 047	0	2	0
Neg 048	0	2	0
Neg 049	0	2	0
Neg 050	0	2	0
Neg 051	0	2	0
Neg 052	0	2	0
Neg 053	0	2	0
Neg 054	0	2	0
Neg 055	0	2	0

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Neg 058	0	2	0
Neg 059	0	2	0
Neg 060	0	2	0
Neg 061	0	2	0
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Neg 081	0	2	0
Neg 082	0	2	0
Neg 083	0	2	0
Neg 084	0	2	0

Neg 085	0	2	0
Neg 086	0	2	0
Neg 087	0	2	0
Neg 088	0	2	0
Neg 089	0	2	0
Neg 090	0	2	0
Neg 091	0	2	0
Neg 092	0	2	0
Neg 093	0	2	0
Neg 094	0	2	0
Neg 095	0	2	0
Neg 096	0	2	0
Neg 097	0	2	0
Neg 098	0	2	0
Neg 099	0	2	0
Neg 100	0	2	0
Neg Ctrl	0	4	0
NTC	0	116	0
Pos 001	3	3	100
Pos 002	3	3	100
Pos 003	3	3	100
Pos 004	3	3	100
Pos 005	3	3	100
Pos 006	3	3	100
Pos 007	3	3	100
Pos 008	3	3	100
Pos 009	3	3	100
Pos 010	3	3	100
Pos 011	0	3	0

Pos 012	1	3	33
Pos 013	1	3	33
Pos 014	1	3	33
Pos 015	1	3	33
Pos 016	1	3	33
Pos 017	1	3	33
Pos 018	1	3	33
Pos 019	1	3	33
Pos 020	1	3	33
Pos 021	1	3	33
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Pos 042	2	2	100
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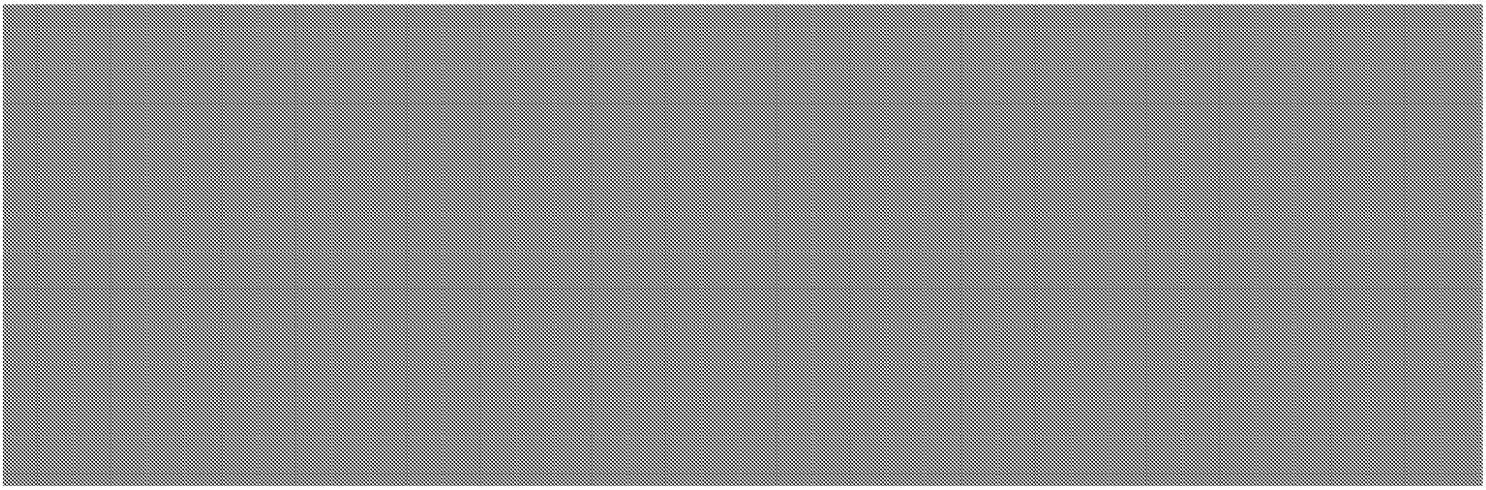

H5N1 INFLUENZA A

TNAA LDT Validation Report

Limit of Detection = 100 cp/uL

Rate of Detection = 100 cp/uL in 35 minutes

Katie Sullivan-Bibee

THERANOS, INC.

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Table of Contents

H5N1 Influenza A

1) PURPOSE

This report includes relevant information about the target organism, a detailed description of the primers and selected targets designed for the detection of this organism, a summary of the validation assay performance, and recommendations for future assay execution.

2) BACKGROUND

Influenza viruses contain a single-stranded, negative-sense, segmented RNA genome, with each segment of RNA containing one or two genes that encode for a viral protein. The influenza A genome contains 11 genes on eight segments of RNA, encoding for 11 proteins: hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), M1, M2, NS1, NS2 (NEP: nuclear export protein), PA, PB1 (polymerase basic 1), PB1-F2 and PB2. Influenza A viruses are classified into subtypes based on antibody responses to hemagglutinin (HA) and neuraminidase (NA), two large glycoproteins found on the surface of the virus. HA is a lectin that mediates binding of the virus to target cells and entry of the viral genome into the target cell, while NA is involved in the release of progeny virus from infected cells, and works by cleaving sugars that bind the mature viral particles. These different types of HA and NA form the basis of the *H* and *N* distinctions. There are 17 H and 9 N subtypes known, but only H1, H2 and H3, and N1 and N2 are commonly found in humans.

All subtypes of Influenza A naturally occur in wild birds, but most are not known to infect humans. A small subset, the Highly Pathogenic Avian Influenzas (HPAI), is the greatest risk for causing pandemics if transmitted to humans. Periodic avian flu infections are observed in humans, and although this is typically a dead-end zoonotic infection (i.e. limited to no person-to-person transmission), the symptoms are quite severe and infected patients in hospitals have a high mortality rate. Most infected patients have had contact with dead or diseased birds, often from poultry markets. The HPAIs observed in humans are H5N1, H7N3, H7N9, and H9N2.

Although rare, H5N1 Avian Influenza A periodically infects humans and can be quite deadly. Of the more than 600 cases reported to the WHO since 2003, about 60% were fatal. The virus has a large reservoir of diversity in birds and is not transmitted person-to-person. Therefore, unlike human influenza viruses, the predominant H5N1 infecting people each year is different and not derived from one seen the previous year. According to the CDC, Indonesia, Vietnam and Egypt have reported the

highest number of human HPAI H5N1 cases to date. If human-to-human spread were to evolve in these viruses, there is a possibility of a deadly pandemic. There is also concern about H5N1 being used in bioterrorism.

Theranos developed a panel of nucleic acid amplification-based assays to detect and distinguish influenza A and B, to further subtype Influenza A as H1N1, H3N2, H5N1 or H7N9, and to distinguish between H1N1 seasonal strains and novel H1N1 (swine flu) which appeared during the human outbreak in 2009.

The assay described in this report focuses on the identification of the H5N1 avian influenza. Since the H5 type of HA is only observed in humans in the context of H5N1 avian flu, the HA gene was chosen as the nucleotide target for detection. Based on the available data, about 10% of nucleotide sites are variable across the 600 bp target in HPAI H5N1 isolated in humans since 2003. The target GC content is 41%.

3) SUMMARY OF PERFORMANCE DATA

Theranos developed a Theranos Nucleic Acid Amplification (TNAA) assay specific for *H5N1 Influenza A*. The Nucleic Acid Amplification reactions contained 1x Nucleic Acid Amplification buffer (20 mM Tris Acetate, pH 7.9, 50 mM Potassium Acetate, 10 mM Magnesium Acetate and 1mM DTT), 0.08% Tween, 0.8 M betaine, 1.4 mM dNTPs, 2 uM Syto59, 0.8 uM RLX1530 primer and 0.8 uM RLX1531 primer, 20 units Bst polymerase, and template at the noted concentration. The reactions were run at 56ºC for 60 minutes. Summarized data will follow below while detailed experimental data can be found in the appendix. Primer sequences are:

H5N1 Influenza A	RLX1530	AAGGTCAATCAAACTGAGTGTTCAT
	RLX1531	TTGACCTTCAGACAAAGAATCCAC

4) LIMIT OF DETECTION

The purpose of this study is to determine the limit of detection (LOD) for the Theranos TNAA assay. The LOD_{95} is the viral titer at which >95% of known positive samples test positive using the TNAA assay. Statistically justified cut-off times for making positive/negative calls were determined for each target empirically. A set of experiments, repeated over four days, were conducted that included eight replicates each of three target dilutions (LoD, 10X LoD, and 100X LoD), as well as 8 NTCs, using the target

primers for amplification. These data were then processed using a receiver-operator character (ROC) analysis, and the best threshold detection time for distinguishing positives and negatives determined using the Youden test statistic as implemented by the R package, pROC.

The assay reliably detected 1E+5 cp/ml of H5N1 Influenza A in about 47 minutes, as shown below. The 47 minute assay cut-off time was determined by ROC analysis. The assay was performed six times. Reactions with and without template (NTCs or Non-Templated Controls) were run in eight replicates each.

LOD	Samples	NumPositive	Total	Percent
100X LOD	10,000,000 cp/ml	48	48	100
10X LOD	1,000,000 cp/ml	48	48	100
1X LOD	100,000 cp/ml	48	48	100
	NTC	0	48	0

5) REPRODUCIBILITY/PRECISION

The purpose of this experiment is to determine the precision of the assay, percent positive and negative at three detection limits: high-negative (0.1X LOD=1E+4 cp/ml), low-positive (LOD=1E+5 cp/ml), and high-positive (3X LOD=3E+5 cp/ml). The assay was performed six times. Reactions with and without template (NTCs or Non-templated Controls) were run in eight replicates each.

Precision LOD	Samples	NumPositive	Total	Percent
3X LOD	300,000 cp/ml	48	48	100
1X LOD	100,000 cp/ml	47	48	98
0.1X LOD	10,000 cp/ml	27	48	56
	NTC	0	48	0

6) CARRYOVER

The purpose of this experiment is to determine the potential for carryover of positive samples adjacent to negative reactions. The nucleic acid template is prepared from high-positive (100X LOD = 1E+7 cp/ml), low-positive (1X LOD=1E+5 cp/ml), and non-templated controls (NTCs) which are arrayed in alternating rows of eight replicates each. There are two rows of high-positive reactions, two rows of low-positive reactions, and six rows of NTCs. The assay was performed once, with no carryover of positive samples to negative reactions.

		100X LOD	NTC	100X LOD	NTC	LOD	NTC	LOD	NTC	NTC	NTC	
	1	2	3	4	5	6	7	8	9	10	11	12
A	empty	+	-	+	-	+	-	+	-	-	-	empty
B		+	-	+	-	+	-	+	-	-	-	
C		+	-	+	-	+	-	+	-	-	-	
D		+	-	+	-	+	-	+	-	-	-	
E		+	-	+	-	+	-	+	-	-	-	
F		+	-	+	-	+	-	+	-	-	-	
G		+	-	+	-	+	-	+	-	-	-	
H		+	-	+	-	+	-	+	-	-	-	

Carryover Samples	NumPositive	Total	Percent
10,000,000 cp/ml	16	16	100
100,000 cp/ml	16	16	100
NTC	0	48	0

7) INCLUSIVITY/EXCLUSIVITY

The assay for H5N1 Influenza A was tested to validate inclusivity and exclusivity. Various strains of H5N1 Influenza A were tested to verify inclusive assay performance. The assay was also tested against different species of Influenza A and B to verify exclusivity between close relatives.

All inclusive strains of H5N1 were tested in eight replicates each, while there were four total replicates for NTC reactions. The TNAA method successfully detected all inclusive *H5N1* strains.

All exclusive Influenza A and B strains were tested in four or eight replicates each and four negative NTC replicates. The TNAA method excluded all closely related Influenza A and B strains.

The following tables summarize the inclusivity and exclusivity pathogens to be evaluated for the H5N1 Influenza A assay.

Inclusivity Samples	NumPositive	Total	Percent
Flu A/Egypt/H5N1 (10^5 cp/ml)	8	8	100
Flu A/India/H5N1 (10^5 cp/ml)	8	8	100
Flu A/Turkey/H5N1 (10^5 cp/ml)	8	8	100
NTC	0	4	0

Exclusivity Samples	NumPositive	Total	Percent
Flu A/Aichi2/H3N2 (5*10^7 cp/ml)	0	8	0
Flu A/Denver/H1N1 (10^8 cp/ml)	0	8	0
Flu A/FM/H1N1 (10^8 cp/ml)	0	4	0
Flu A/H1N1 novel (10^8 cp/ml)	0	8	0
Flu A/Hong Kong/H9N2 (10^8 cp/ml)	0	8	0
Flu A/Victoria/H3N2 (10^8 cp/ml)	0	8	0
Flu B/Malaysia (10^8 cp/ml)	0	8	0
Flu B/Russia/69 (10^8 cp/ml)	0	8	0
NTC	0	4	0

8) CROSS-REACTIVITY

The cross-reactivity of the assay was tested against a panel of organisms which may also be present in collected H5N1 Influenza A clinical samples. These organisms must be tested to ascertain that no false positives will be due to contamination from the off-target genomic material at clinically relevant viral or bacterial loads. The table below summarizes the genomic material tested and the results obtained. All potentially cross-reactive organisms were tested in replicates of eight and NTCs and the positive control were tested replicates of four. The TNAA assay was verified to not cross-react with any non-target organisms.

Cross-reactivity Samples	NumPositive	Total	Percent
Adenovirus (10^6 cp/ml)	0	8	0
B. pertussis (10^8 cp/ml)	0	8	0
C. albicans (10^6 cp/ml)	0	8	0
E. coli (10^8 cp/ml)	0	8	0
Flu A/WS/33 (H1N1) (10^8 cp/ml)	0	8	0
Flu B/Hubei-Wujiagang (10^8 cp/ml)	0	8	0
H5N1 (10^5 cp/ml)	4	4	100
hgDNA (200ng/ml)	0	8	0
K. pneumoniae (10^6 cp/ml)	0	8	0
NTC	0	4	0
P. aeruginosa (10^7 cp/ml)	0	8	0
S. aureus (10^7 cp/ml)	0	8	0
S. pyogenes (10^7 cp/ml)	0	8	0

9) SPECIFICITY

The specificity of the assay was tested against a panel of organisms which may be present as potential contaminants in H5N1 Influenza A samples and whose genomic material may be carried though the sample preparation protocol. These organisms must be tested to verify that assay performance is not significantly impacted by the presence of off-target genomic material combined with *H5N1 Influenza A* at clinically relevant loads. The table below summarizes the genomic material tested and the results obtained. All organisms combined with *H5N1* were tested in replicates of four. The positive and negative controls were tested in sixteen replicates.

The results below show that the assay is specific to H5N1 Influenza A and spiking in other organisms that may be found in the same sample type does not affect assay performance. The assay tested H5N1 target at 100X, 10X, and 1X LOD combined with the off-target organism. The off-target nucleic acid concentration reflects expected median viral/bacterial loads in clinical specimens.

Specificity Samples	NumPositive	Total	Percent
H5N1 (10^5 cp/ml) + HMPV (10^6 cp/ml)	4	4	100
H5N1 (10^5 cp/ml) + IDTE	15	16	94
H5N1 (10^5 cp/ml) + PIV1 (10^6 cp/ml)	4	4	100
H5N1 (10^5 cp/ml) + PIV3 (10^6 cp/ml)	4	4	100
H5N1 (10^5 cp/ml) + RSV (10^6 cp/ml)	4	4	100
H5N1 (10^6 cp/ml) + HMPV (10^6 cp/ml)	4	4	100
H5N1 (10^6 cp/ml) + PIV1 (10^6 cp/ml)	4	4	100
H5N1 (10^6 cp/ml) + PIV3 (10^6 cp/ml)	4	4	100
H5N1 (10^6 cp/ml) + RSV (10^6 cp/ml)	4	4	100
H5N1 (10^7 cp/ml) + HMPV (10^6 cp/ml)	4	4	100
H5N1 (10^7 cp/ml) + PIV1 (10^6 cp/ml)	4	4	100
H5N1 (10^7 cp/ml) + PIV3 (10^6 cp/ml)	4	4	100
H5N1 (10^7 cp/ml) + RSV (10^6 cp/ml)	4	4	100
IDTE + HMPV (10^6 cp/ml)	0	4	0
IDTE + PIV1 (10^6 cp/ml)	0	4	0
IDTE + PIV3 (10^6 cp/ml)	0	4	0

IDTE + RSV (10^6 cp/ml)	0	4	0
NTC	0	16	0

10) INTERFERING SUBSTANCES

The following interfering substances have been evaluated to have no significant effect on the performance of the TNAA assay. The interfering substances were added to H5N1 Influenza A sample prep at both 10% and 0.1% of the total reaction by volume.

Interfering Substances: Endogenous and Exogenous.

Endogenous	Exogenous
Human blood	Bactroban nasal
Mucin	Flonase
Human genomic DNA	Nasonex
	Astelin
	Anefrin Nasal Spray
	Neosynphrine
	VapoRub cough suppressant
	ZiCam Allergy Relief nasal gel
	Mucin
	UTM

11) METHOD COMPARISON ON CLINICAL SAMPLES

The purpose of this study is to estimate the sensitivity and specificity of the TNAA assay using qPCR as the comparator (predicate method).

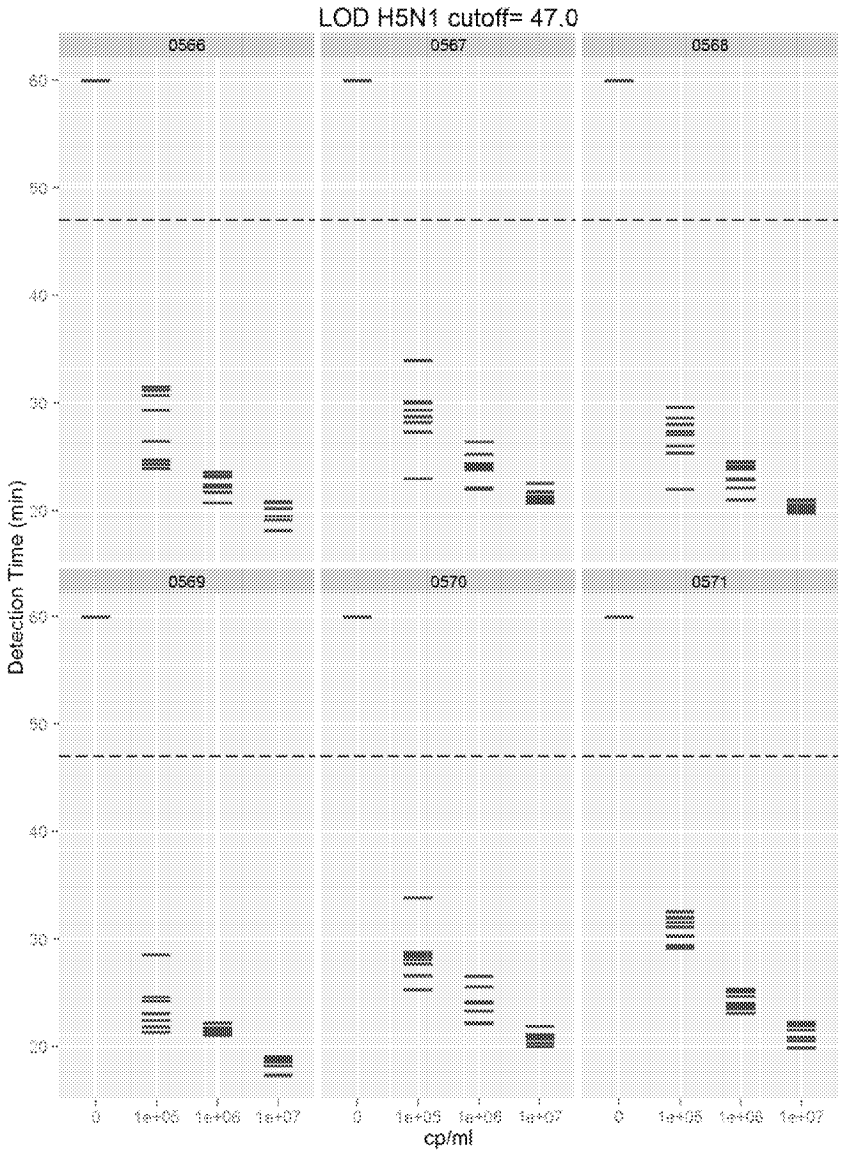
Positive clinical samples were unavailable for H5N1 Influenza A. However, 100 negative samples obtained from Fostering Tech Medical. Nasal swab samples were taken from a range of individuals of both sexes and various ages.

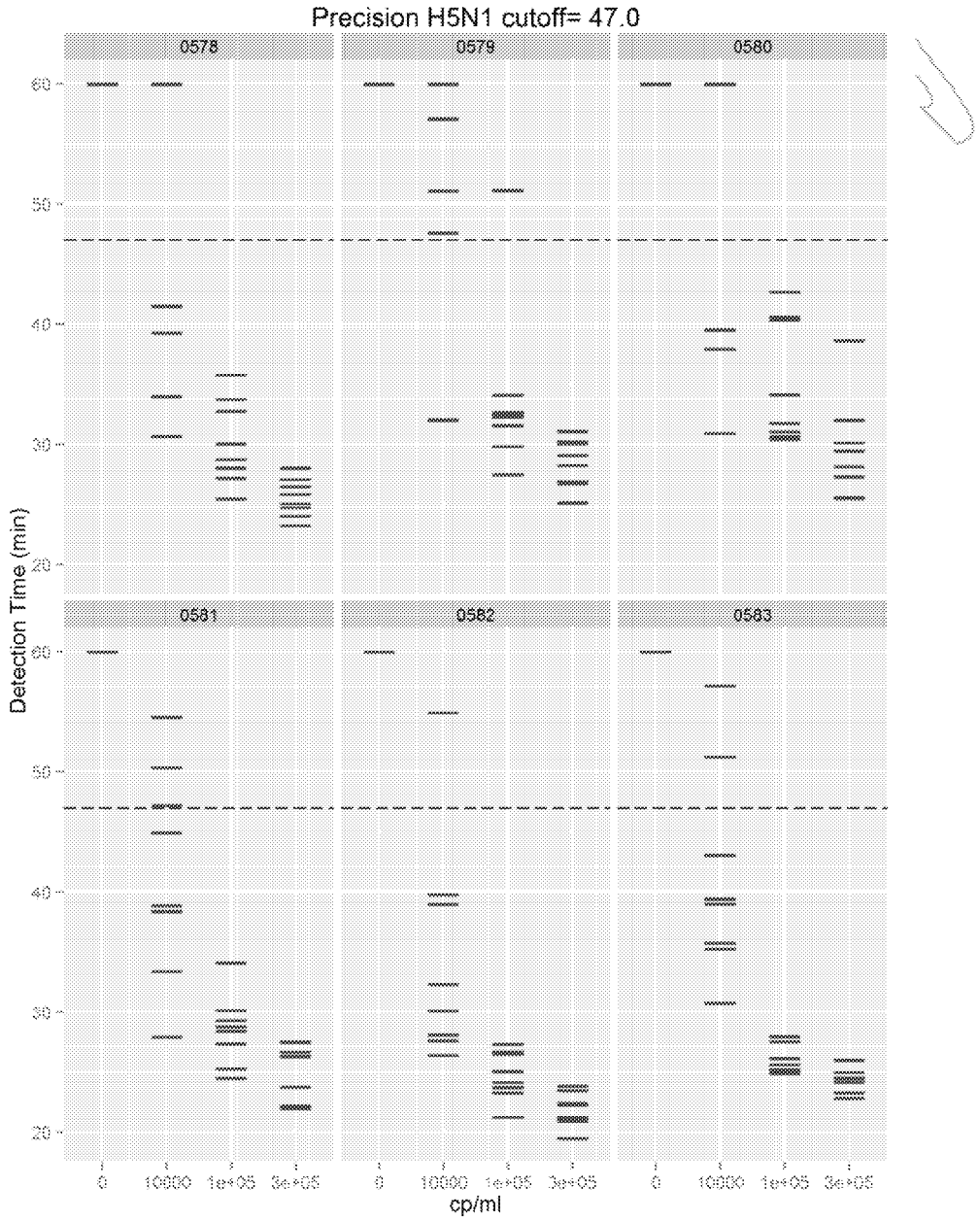
	Clinical Positive (qPCR)	Clinical Positive (TNAA)	Clinical Negative (qPCR)	Clinical Negative (TNAA)
NumPositive	N/A	N/A	0	0
Total	N/A	N/A	100	100
Percent	N/A	N/A	0	0

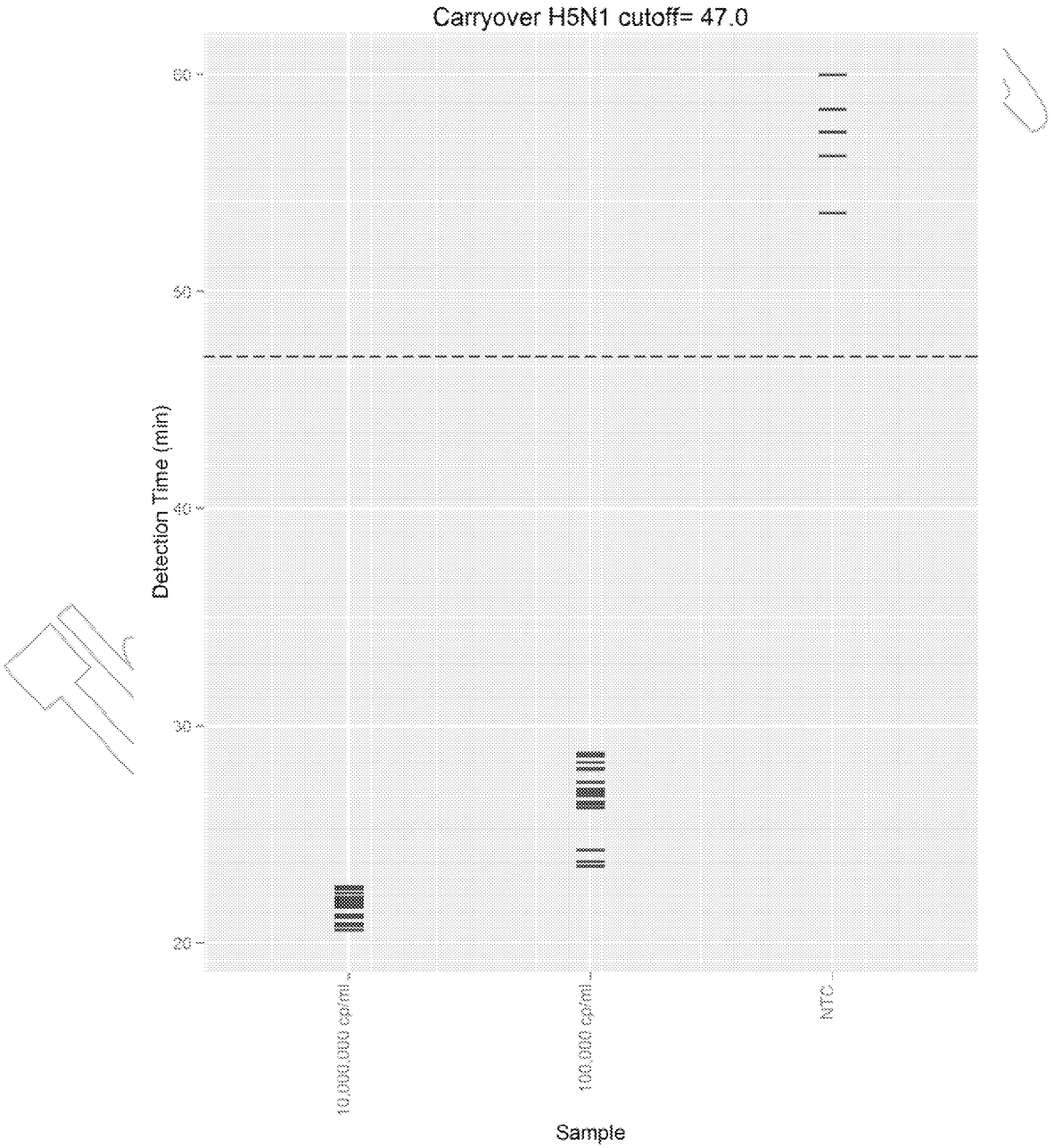
12) FINAL RECOMMENDATIONS

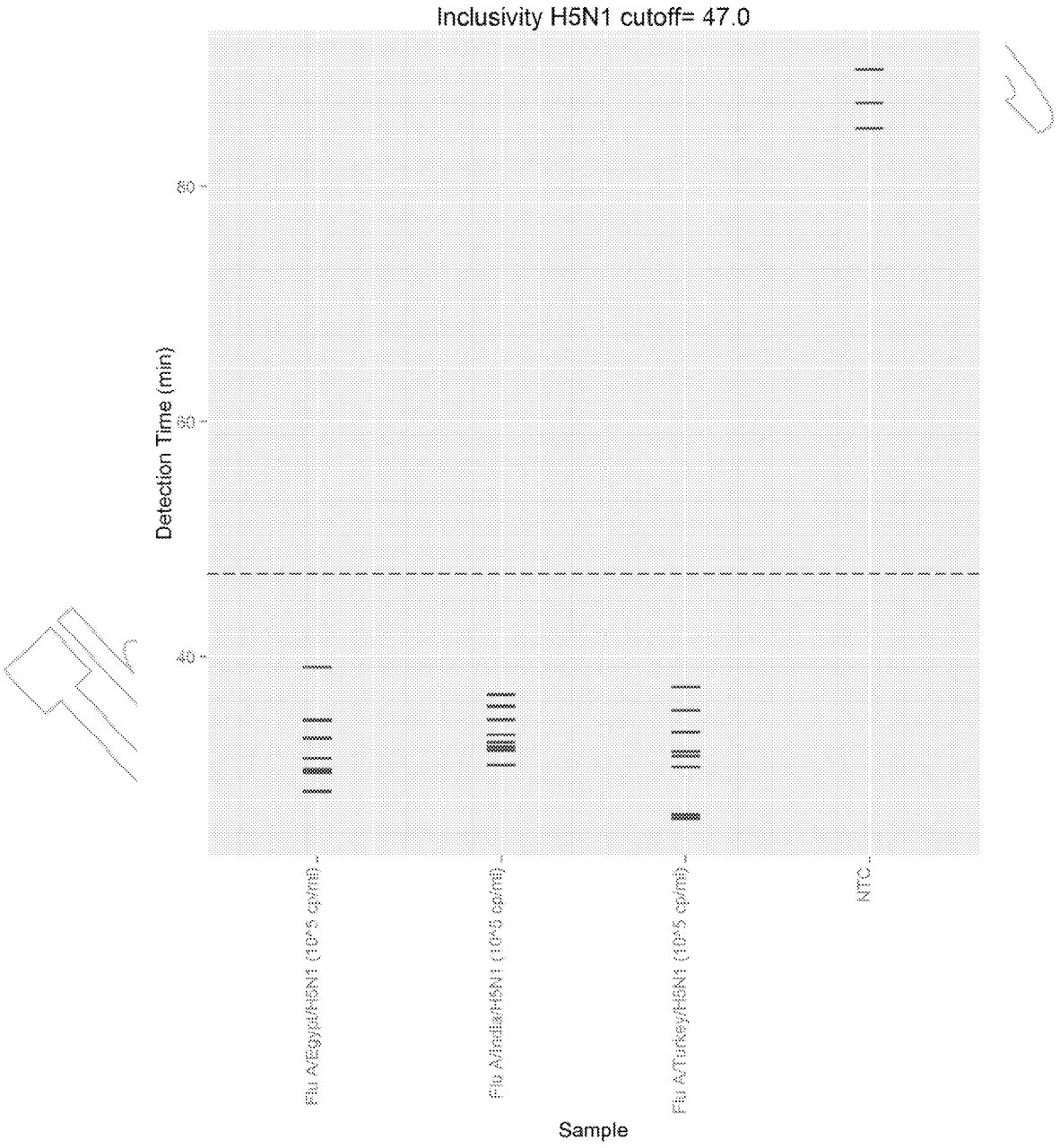
The assay for H5N1 Influenza A was found to meet all criteria for precision, carryover, inclusivity, exclusivity, cross-reactivity, specificity, and resistance to interfering substances. Negative clinical samples were tested and compared to a predicate method. The H5N1 Influenza A assay specifically and reliably detects H5N1 Influenza A. The assay limit of detection is 1E+5 cp/ml with a recommended assay duration of 47 minutes as determined by ROC analysis.

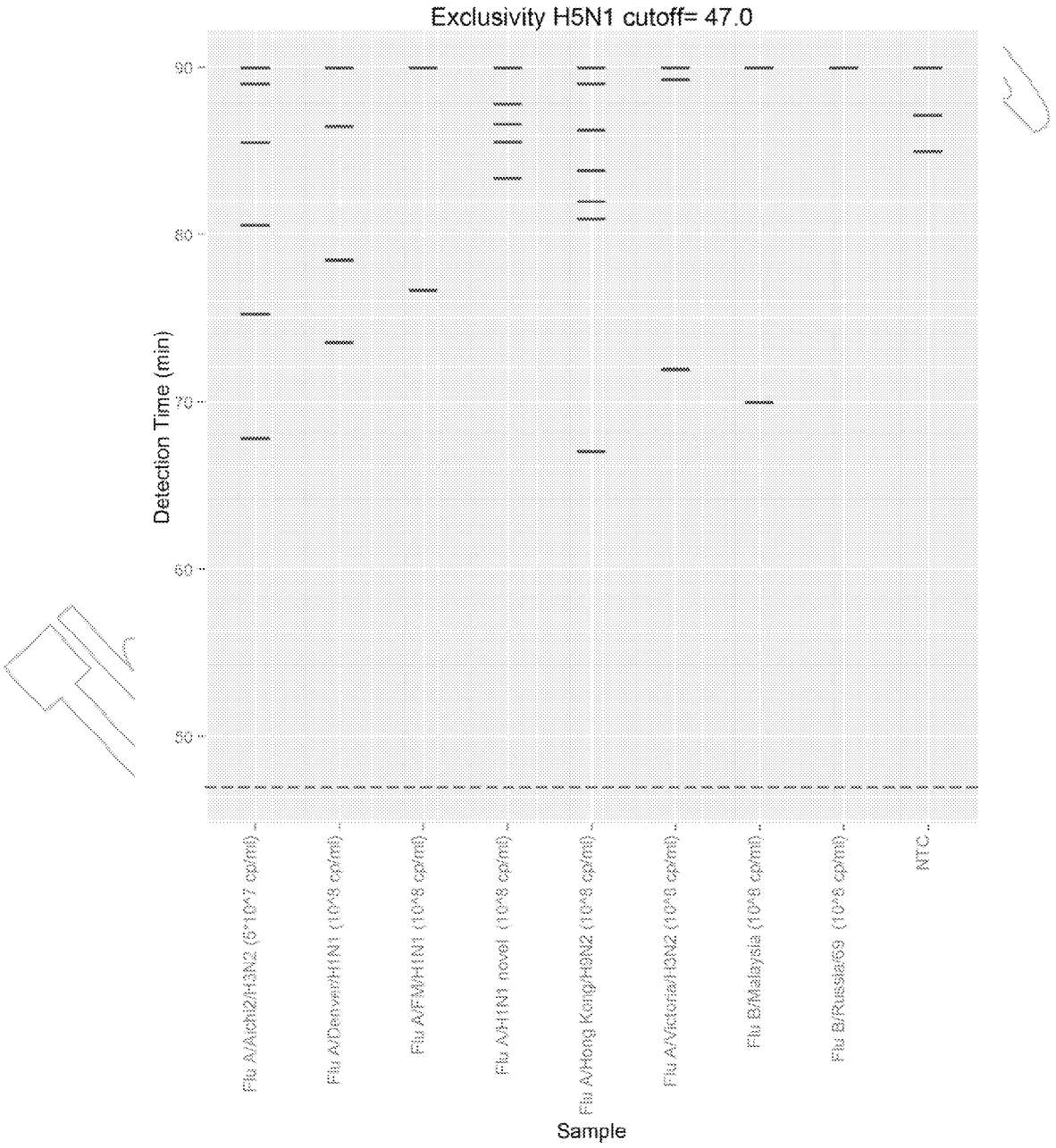
13) APPENDIX

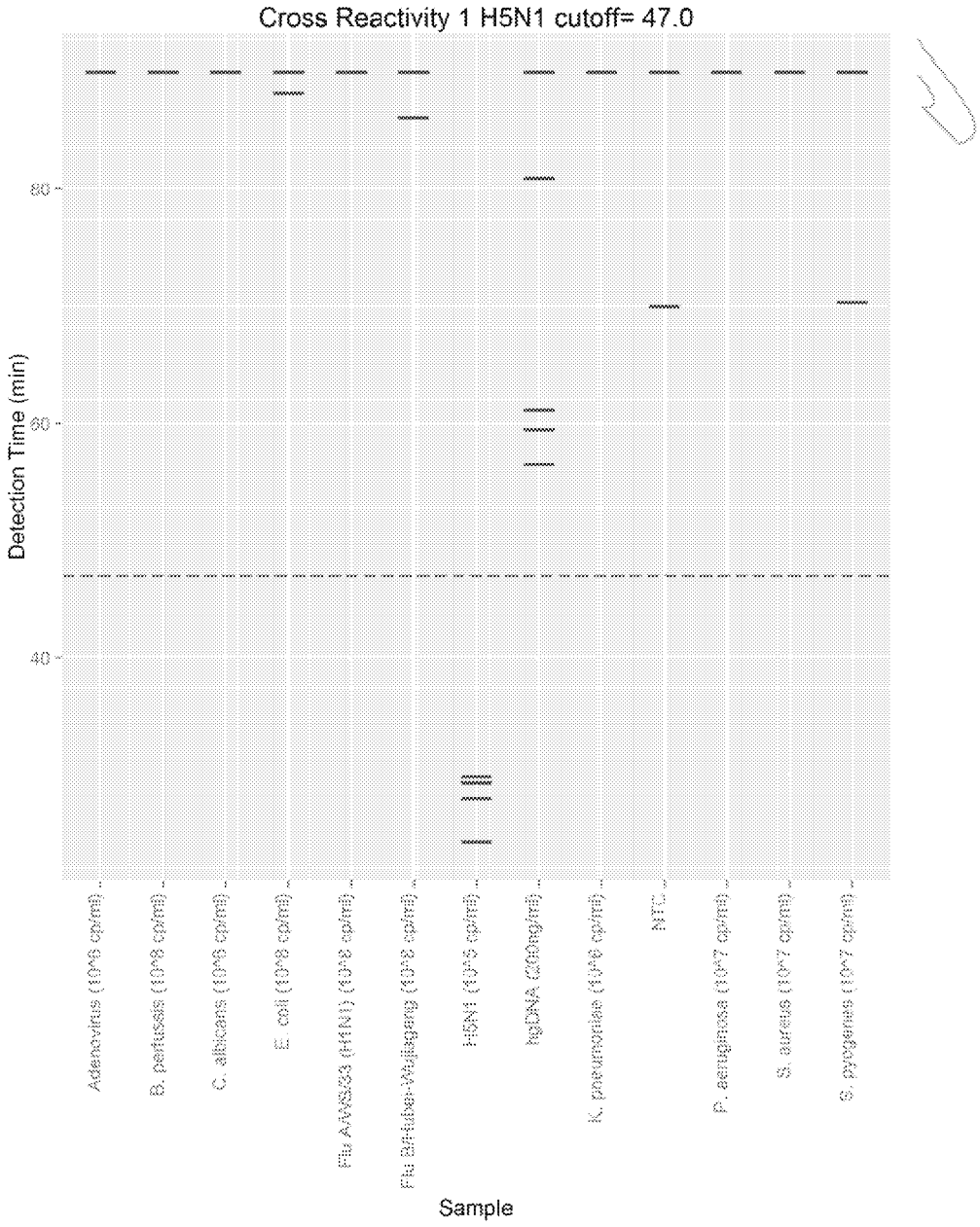


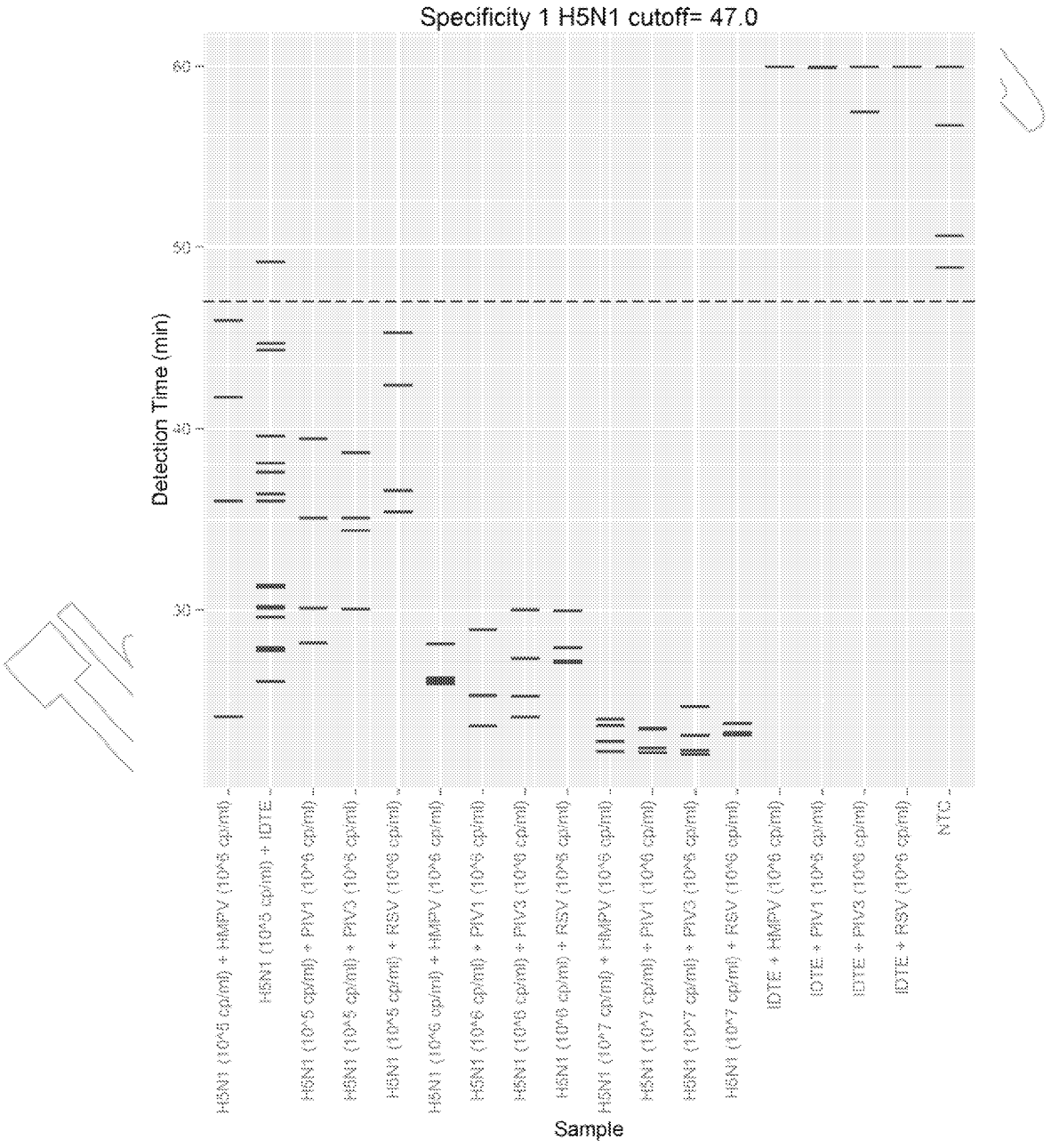


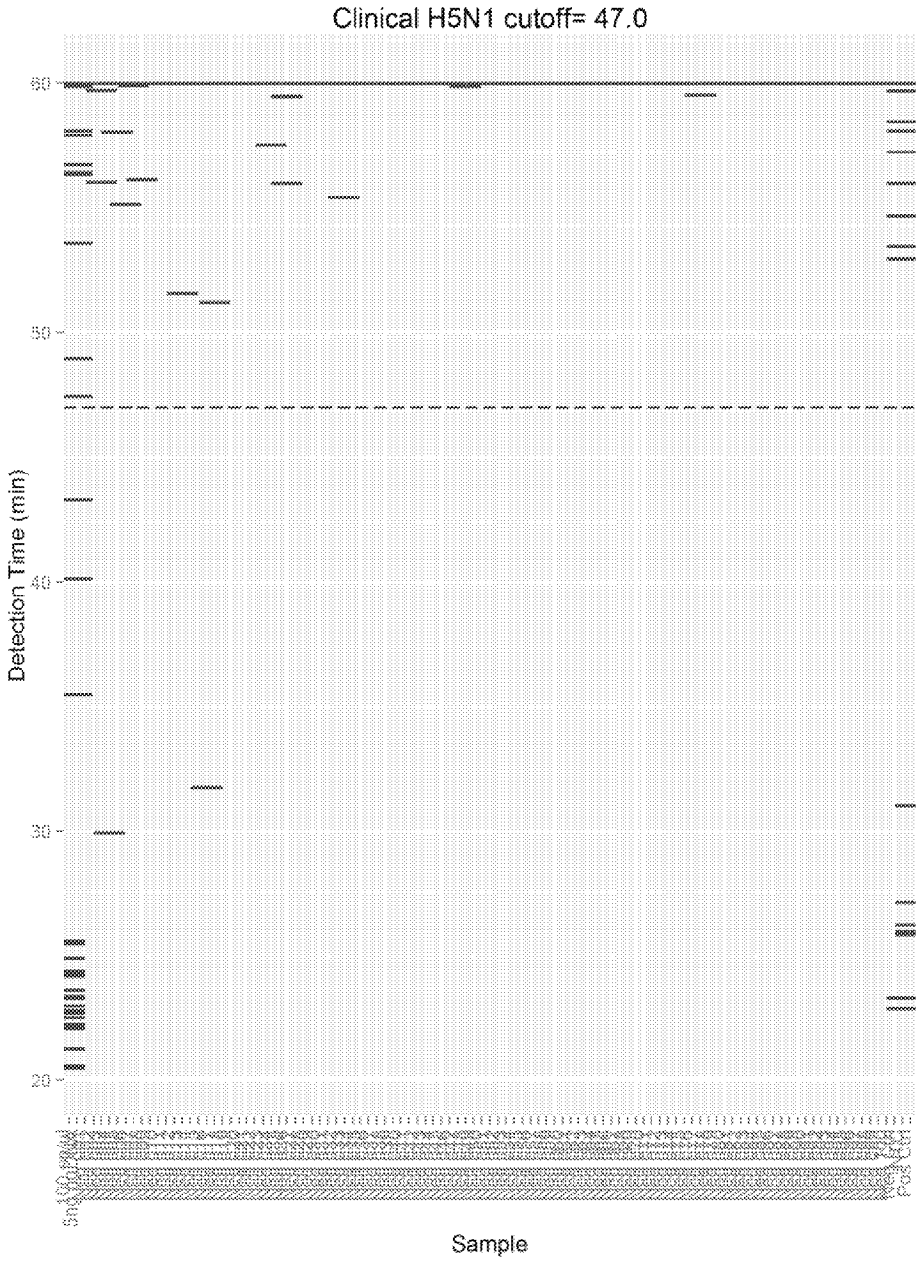












Clinical Samples TNAA Treatment	NumPositive	Total	Percent
100 cp/ul	20	20	100
5ng hgDNA	3	20	15
Neg 001	0	3	0
Neg 002	0	3	0
Neg 003	0	3	0
Neg 004	1	3	33
Neg 005	0	3	0
Neg 006	0	3	0
Neg 007	0	3	0
Neg 008	0	3	0
Neg 009	0	3	0
Neg 010	0	3	0
Neg 011	0	3	0
Neg 012	0	3	0
Neg 013	0	3	0
Neg 014	0	3	0
Neg 015	0	3	0
Neg 016	1	3	33
Neg 017	0	3	0
Neg 018	0	3	0
Neg 019	0	3	0
Neg 020	0	3	0
Neg 021	0	3	0
Neg 022	0	3	0
Neg 023	0	3	0
Neg 024	0	3	0
Neg 025	0	3	0
Neg 026	0	3	0
Neg 027	0	3	0

Neg 028	0	3	0
Neg 029	0	3	0
Neg 030	0	3	0
Neg 031	0	3	0
Neg 032	0	3	0
Neg 033	0	3	0
Neg 034	0	3	0
Neg 035	0	3	0
Neg 036	0	3	0
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Neg 091	0	2	0
Neg 092	0	2	0
Neg 093	0	2	0
Neg 094	0	2	0
Neg 095	0	2	0
Neg 096	0	2	0
Neg 097	0	2	0
Neg 098	0	2	0
Neg 099	0	2	0
Neg 100	0	2	0
Neg Ctrl	0	5	0
NTC	2	180	1
Pos Ctrl	5	5	100

[redacted]

ENTEROBACTER AEROGENES

TNAA LDT Validation Report

Limit of Detection = 10 cp/uL

Rate of Detection = 100 cp/uL in 21 minutes

Katie Sullivan-Bibee

THERANOS, INC.

Author(s):

Signature:	Date:
Name: Katie Sullivan-Bibee	Title: Research Associate

Reviewer(s)

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Signature:	Date:
Name: Adam Rosendorff, M.D	Title: Laboratory Director

Table of Contents

Enterobacter aerogenes

1) PURPOSE

This report includes relevant information about the target organism, a detailed description of the primers and selected targets designed for the detection of this organism, a summary of the validation assay performance, and recommendations for future assay execution.

2) BACKGROUND

The gram negative gammaproteobacterial family Enterobacteriaceae contains numerous species found in hospital acquired infections, including *Enterobacter aerogenes*, *E. cloacae*, *Escherichia coli*, as well as members of the genera *Salmonella*, *Klebsiella*, *Shigella*, *Proteus*, *Serratia*, and *Citrobacter*. Many of these facultative anaerobes are natural components of the human gut flora, but can infect immunocompromised patients more systematically (including bloodstream, catheter, and intubation infections). Distressingly, members of the Enterobacteriaceae frequently exchange antibiotic resistance plasmids (e.g. KPC), making treatment difficult.

Enterobacter aerogenes is a nosocomial and pathogenic bacterium that causes opportunistic infections including most types of infections. The majority are sensitive to most antibiotics designed for this bacteria class, but this is complicated by their inducible resistance mechanisms, particularly lactamase which means that they quickly become resistant to standard antibiotics during treatment, requiring change in antibiotic to avoid worsening of the sepsis. Some of the infections caused by *E. aerogenes* result from specific antibiotic treatments, venous catheter insertions, and/or surgical procedures. *E. aerogenes* is generally found in the human gastrointestinal tract and does not generally cause disease in healthy individuals. It has been found to live in various wastes, hygienic chemicals, and soil.

This report describes the nucleic acid amplification test developed to detect *Enterobacter aerogenes*. A conserved region of the *bdh* metabolic gene was chosen as a target as it shows 99% conservation within *E. aerogenes* and only 84% with closely related *Klebsiella pneumoniae*.

3) SUMMARY OF PERFORMANCE DATA

Theranos developed a Theranos Nucleic Acid Amplification (TNAA) assay specific for *Enterobacter aerogenes*. The Nucleic Acid Amplification reactions contained 1x Nucleic Acid Amplification buffer (20 mM Tris Acetate, pH 7.9, 50 mM Potassium Acetate, 10 mM Magnesium Acetate, 1mM DTT and 0.08% Tween), 0.8 M betaine, 1.4 mM dNTPs, 2 uM Syto59, 1.6 uM primer RLX2285 and 1.6 uM primer RLX2286, 20 units Bst polymerase and template at the noted concentration. The reactions were run at 56ºC for 60 minutes. Summarized data will follow below while detailed experimental data can be found in the appendix.

Primer sequences are:

Enterobacter aerogenes	RLX2285	CTCCGTGCTCTTTCGAGGCGA
	RLX2286	GCACGGAGTGGGGGCGGATTA

4) LIMIT OF DETECTION

The purpose of this study is to determine the limit of detection (LOD) for the Theranos TNAA assay. The LOD_{95} is the bacterial titer at which >95% of known positive samples test positive using the TNAA assay. Statistically justified cut-off times for making positive/negative calls were determined for each target empirically. A set of experiments, repeated over four days, were conducted that included eight replicates each of three target dilutions (LoD, 10X LoD, and 100X LoD), as well as 8 NTCs, using the target primers for amplification. These data were then processed using a receiver-operator character (ROC) analysis, and the best threshold detection time for distinguishing positives and negatives determined using the Youden test statistic as implemented by the R package, pROC.

The assay reliably detected 19,020 CFU/ml of *Enterobacter aerogenes* in about 33.8 minutes, as shown below. The 33.8 minute assay cut-off time was determined by ROC analysis. The assay was performed seven times. Reactions with and without template (NTCs or Non-Templated Controls) were run in eight replicates each.

LOD	Sample CFU/ml	NumPositive	Total	Percent
100X LOD	1,902,027 CFU/ml	56	56	100
10X LOD	190,203 CFU/ml	56	56	100
1X LOD	19,020 CFU/ml	55	56	98
	NTC	0	56	0

5) REPRODUCIBILITY/PRECISION

The purpose of this experiment is to determine the precision of the assay, percent positive and negative at three detection limits: high-negative (0.1X LOD=1,902 CFU/ml), low-positive (LOD=19,020 CFU/ml), and high-positive (3X LOD=57,061 CFU/ml). The assay was performed six times. Reactions with and without template (NTCs or Non-templated Controls) were run in eight replicates each.

Precision LOD	Sample CFU/ml	NumPositive	Total	Percent
3X LOD	57,061 CFU/ml	48	48	100
1X LOD	19,020 CFU/ml	48	48	100
0.1X LOD	1,902 CFU/ml	16	48	33
	NTC	1	48	2

6) CARRYOVER

The purpose of this experiment is to determine the potential for carryover of positive samples adjacent to negative reactions. The nucleic acid template is prepared from high-positive (100X LOD = 1,902,027 CFU/ml), low-positive (1X LOD=19,020 CFU/ml), and non-templated controls (NTCs) which are arrayed in alternating rows of eight replicates each. There are two rows of high-positive reactions, two rows of low-positive reactions, and six rows of NTCs. The assay was performed once, with no carryover of positive samples to negative reactions.

		100X LOD	NTC	100X LOD	NTC	LOD	NTC	LOD	NTC	NTC	NTC	
	1	2	3	4	5	6	7	8	9	10	11	12
A	empty	+	-	+	-	+	-	+	-	-	-	empty
B		+	-	+	-	+	-	+	-	-	-	
C		+	-	+	-	+	-	+	-	-	-	
D		+	-	+	-	+	-	+	-	-	-	
E		+	-	+	-	+	-	+	-	-	-	
F		+	-	+	-	+	-	+	-	-	-	
G		+	-	+	-	+	-	+	-	-	-	
H		+	-	+	-	+	-	+	-	-	-	

Carryover Samples	NumPositive	Total	Percent
1,902,027 CFU/ml	16	16	100
19,020 CFU/ml	16	16	100
NTC	0	48	0

7) INCLUSIVITY/EXCLUSIVITY

The assay for *Enterobacter aerogenes* was tested to validate inclusivity and exclusivity. Various strains of *Enterobacter aerogenes* were tested to verify inclusive assay performance. The assay was also tested against different species of *Enterobacter* to verify exclusivity between close relatives.

All inclusive strains of *E. aerogenes* were tested in four replicates each, while human genomic DNA was tested in eight replicates. There were twelve total replicates for NTC reactions. The TNAA method successfully detected all inclusive *E. aerogenes* strains.

All exclusive *Enterobacter* strains were tested in eight replicates each, with three positive replicate reactions for *E. aerogenes* and eight negative NTC replicates. The TNAA method excluded all closely related *Enterobacter* strains. The following tables summarize the inclusivity and exclusivity pathogens to be evaluated for the *Enterobacter aerogenes* assay.

Inclusivity Samples	NumPositive	Total	Percent
Enterobacter aerogenes 1101206 (10^6 cp/ml)	4	4	100
Enterobacter aerogenes 1101481 (10^6 cp/ml)	4	4	100
Enterobacter aerogenes AmMS 264 (10^6 cp/ml)	4	4	100
Enterobacter aerogenes CDC 120-75 (10^6 cp/ml)	4	4	100
Enterobacter aerogenes IFO 12010 (10^6 cp/ml)	4	4	100
Enterobacter aerogenes MULB-250 (10^6 cp/ml)	4	4	100
Enterobacter aerogenes NCDC 819-56 (10^6 cp/ml)	4	4	100
hgDNA (200ng/ml)	0	8	0
NTC	0	12	0

Exclusivity Samples	NumPositive	Total	Percent
Enterobacter aerogenes NCDC 819-56 (10^7 cp/ml)	3	3	100
Enterobacter cloacae 7256 (10^6 cp/ml)	0	8	0
Enterobacter hormaechei O'Hara (10^6 cp/ml)	0	8	0
NTC	0	8	0

8) CROSS-REACTIVITY

The cross-reactivity of the assay was tested against a panel of organisms which may also be present in collected *Enterobacter aerogenes* clinical samples. These organisms must be tested to ascertain that no false positives will be due to contamination from the off-target genomic material at clinically relevant viral or bacterial loads. The table below summarizes the genomic material tested and the results obtained. All potentially cross-reactive organisms were tested in replicates of three or six and NTCs were tested in nine replicates. The TNAA assay was verified to not cross-react with any non-target organisms.

Cross-reactivity Samples	NumPositive	Total	Percent
A/California/7/2009 H1N1 novel (10^8 cp/ml)	0	3	0
Acinetobacter baumannii (10^7 cp/ml)	0	3	0
Adenovirus (10^6 cp/ml)	0	3	0
Bordetella pertussis (10^8 cp/ml)	0	3	0
B/Russia/69 (10^6 cp/ml)	0	3	0
Candida albicans (10^6 cp/ml)	0	3	0
Enterobacter aerogenes NCDC 819-56 (10^7 cp/ml)	6	6	100
Enterobacter cloacae (10^7 cp/ml)	0	3	0
Escherichia coli (10^7 cp/ml)	0	6	0
Klebsiella oxytoca (10^7 cp/ml)	0	3	0
Klebsiella pneumoniae (10^6 cp/ml)	0	3	0
Klebsiella pneumoniae (10^7 cp/ml)	0	3	0
Neisseria meningitidis (10^7 cp/ml)	0	3	0
NTC	0	9	0
Pseudomonas aeruginosa (10^7 cp/ml)	0	6	0
Serratia marcescens (10^7 cp/ml)	0	3	0
Staphylococcus aureus MSSA [DmecA] (10^7 cp/ml)	0	3	0
Streptococcus agalactiae (10^7 cp/ml)	0	3	0
Streptococcus pneumoniae (10^7 cp/ml)	0	3	0
Streptococcus pyogenes (10^7 cp/ml)	0	3	0

9) SPECIFICITY

The specificity of the assay was tested against a panel of organisms which may be present as potential contaminants in *Enterobacter aerogenes* samples and whose genomic material may be carried though the sample preparation protocol. These organisms must be tested to verify that assay performance is not significantly impacted by the presence of off-target genomic material combined with *Enterobacter aerogenes* at clinically relevant loads. The table below summarizes the genomic material tested and the results obtained. All organisms combined with *E. aerogenes* were tested in replicates of two or four. The positive control and NTCs were tested in replicates of eight.

The results below show that the assay is specific to *Enterobacter aerogenes* and spiking in other organisms that may be found in the same sample type does not affect assay performance. The assay tested E. aerogenes target at 10X LOD (1,902,027 CFU/ml) combined with the off-target organism. The off-target nucleic acid concentration reflects expected median viral/bacterial loads in clinical specimens.

Specificity Samples	NumPositive	Total	Percent
E. aero + A. baumannii (10^7 cp/ml)	2	2	100
E. aero + Adenovirus 4 (10^6 cp/ml)	2	2	100
E. aero + B. pertussis (10^8 cp/ml)	2	2	100
E. aero + C. albicans (10^6 cp/ml)	2	2	100
E. aero + E. cloacae (10^7 cp/ml)	2	2	100
E. aero + E. coli (10^7 cp/ml)	4	4	100
E. aero + Flu A/H1N1 novel (10^8 cp/ml)	2	2	100
E. aero + Flu B/Russia/69 (10^6 cp/ml)	2	2	100
E. aero + IDTE	8	8	100
E. aero + K. oxytoca (10^7 cp/ml)	2	2	100
E. aero + K. pneumoniae (10^6 cp/ml)	2	2	100
E. aero + K. pneumoniae (10^7 cp/ml)	2	2	100
E. aero + N. meningitidis (10^7 cp/ml)	2	2	100
E. aero + P. aeruginosa (10^7 cp/ml)	4	4	100
E. aero + S. agalactiae (10^7 cp/ml)	2	2	100
E. aero + S. aureus (10^7 cp/ml)	2	2	100
E. aero + S. marcescens (10^7 cp/ml)	2	2	100
E. aero + S. pneumoniae (10^7 cp/ml)	2	2	100
E. aero + S. pyogenes (10^7 cp/ml)	2	2	100
NTC	0	8	0

10) INTERFERING SUBSTANCES

The following interfering substances have been evaluated to have no significant effect on the performance of the TNAA assay. The interfering substances were added to *Enterobacter aerogenes* sample prep at both 10% and 0.1% of the total reaction by volume.

Interfering Substances: Endogenous and Exogenous.

Endogenous	Exogenous
Human blood	Bactroban nasal
Mucin	Flonase
Human genomic DNA	Nasonex
	Astelin
	Anefrin Nasal Spray
	Neosynphrine
	VapoRub cough suppressant
	ZiCam Allergy Relief nasal gel
	Mucin
	UTM

11) METHOD COMPARISON ON CLINICAL SAMPLES

The purpose of this study is to estimate the sensitivity and specificity of the TNAA assay using qPCR as the comparator (predicate method).

The following clinical samples were tested: 50 positive samples and 100 negative samples obtained from Fostering Tech Medical. Both coproculture and serum samples were taken from a range of individuals of both sexes and various ages.

TNAA vs qPCR Contingency Table		qPCR		
		Positive	**Negative**	**Total**
TNAA	**Positive**	50	0	50
	Negative	0	100	100
	Total	50	100	150

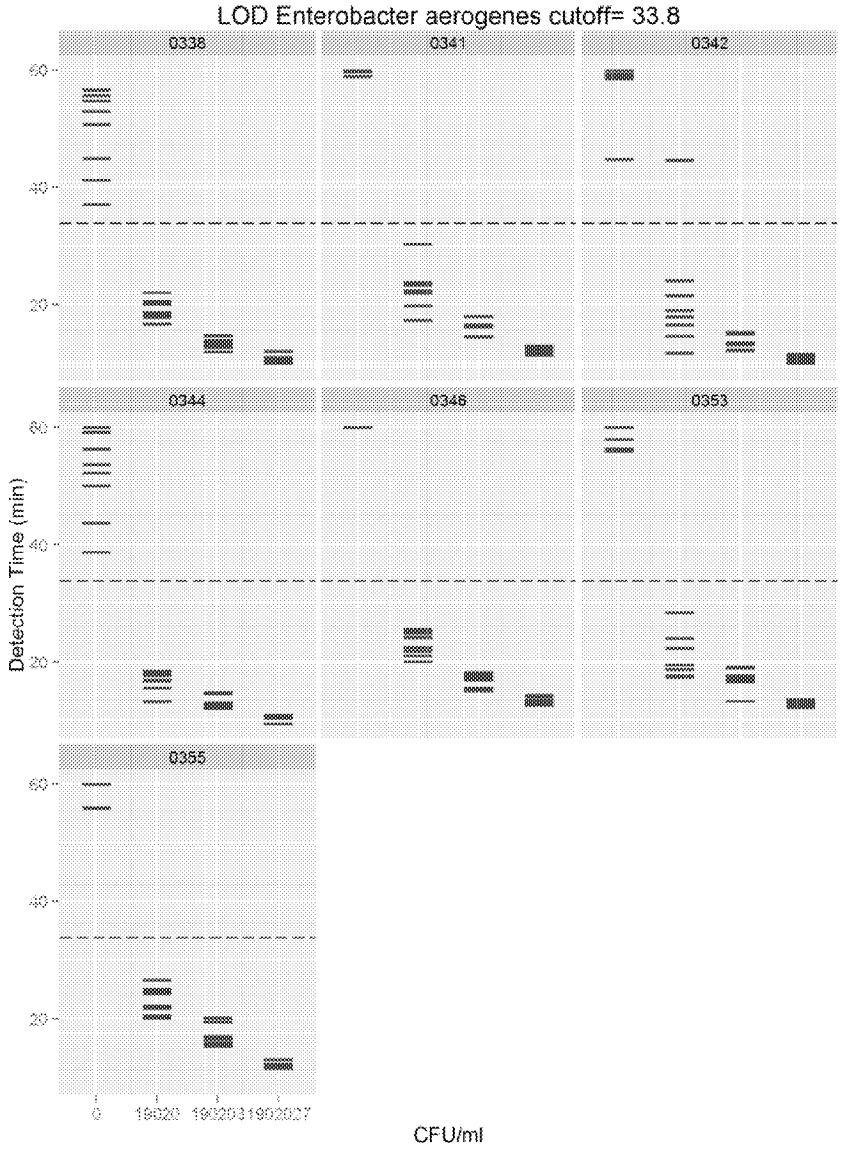
	Percent	95% Confidence Interval	
Estimated Sensitivity	100%	93%	100%
Estimated Specificity	100%	96%	100%

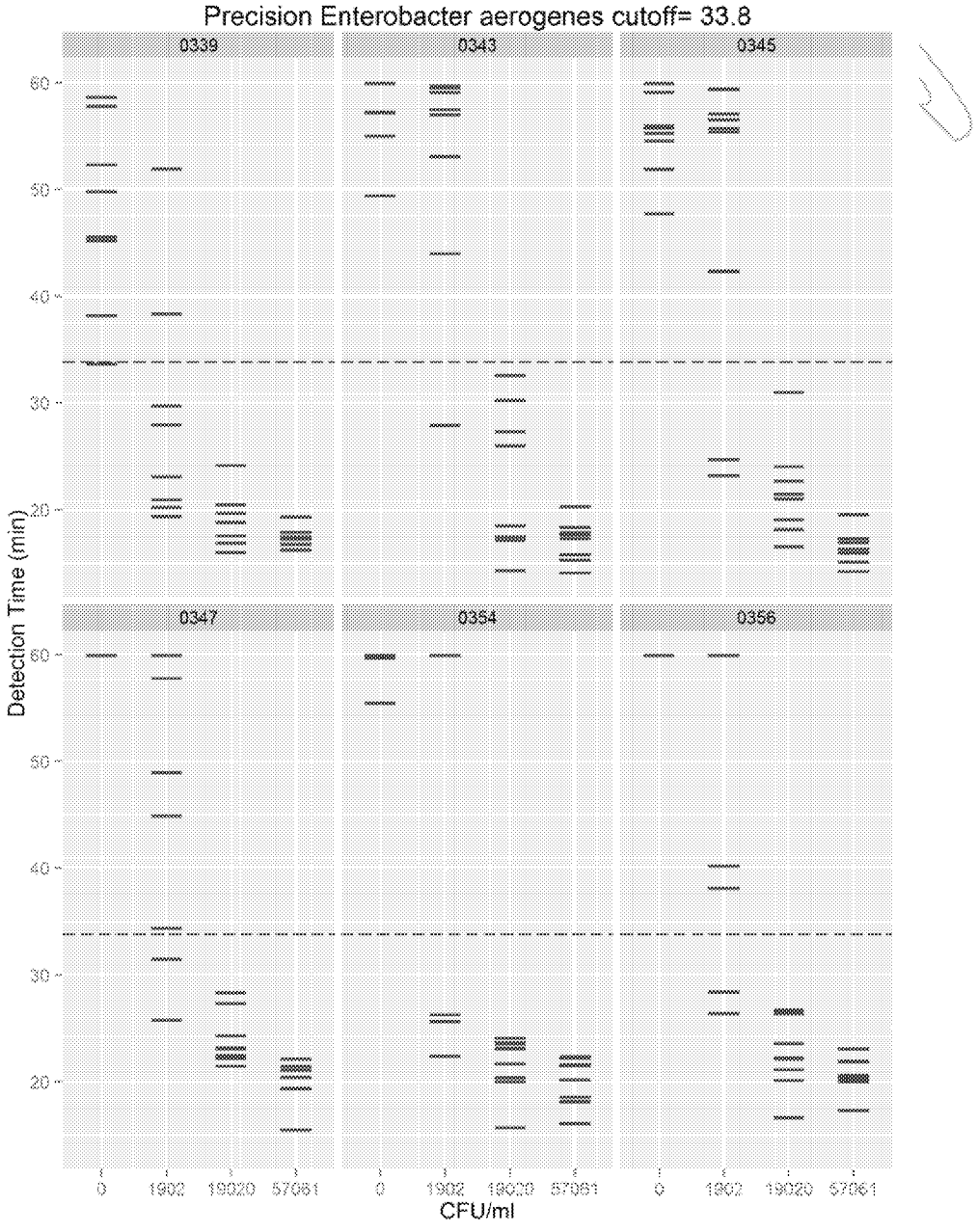
Based on a Prevalence of	**33%**
Positive Predictive Value	100%
Negative Predictive Value	100%

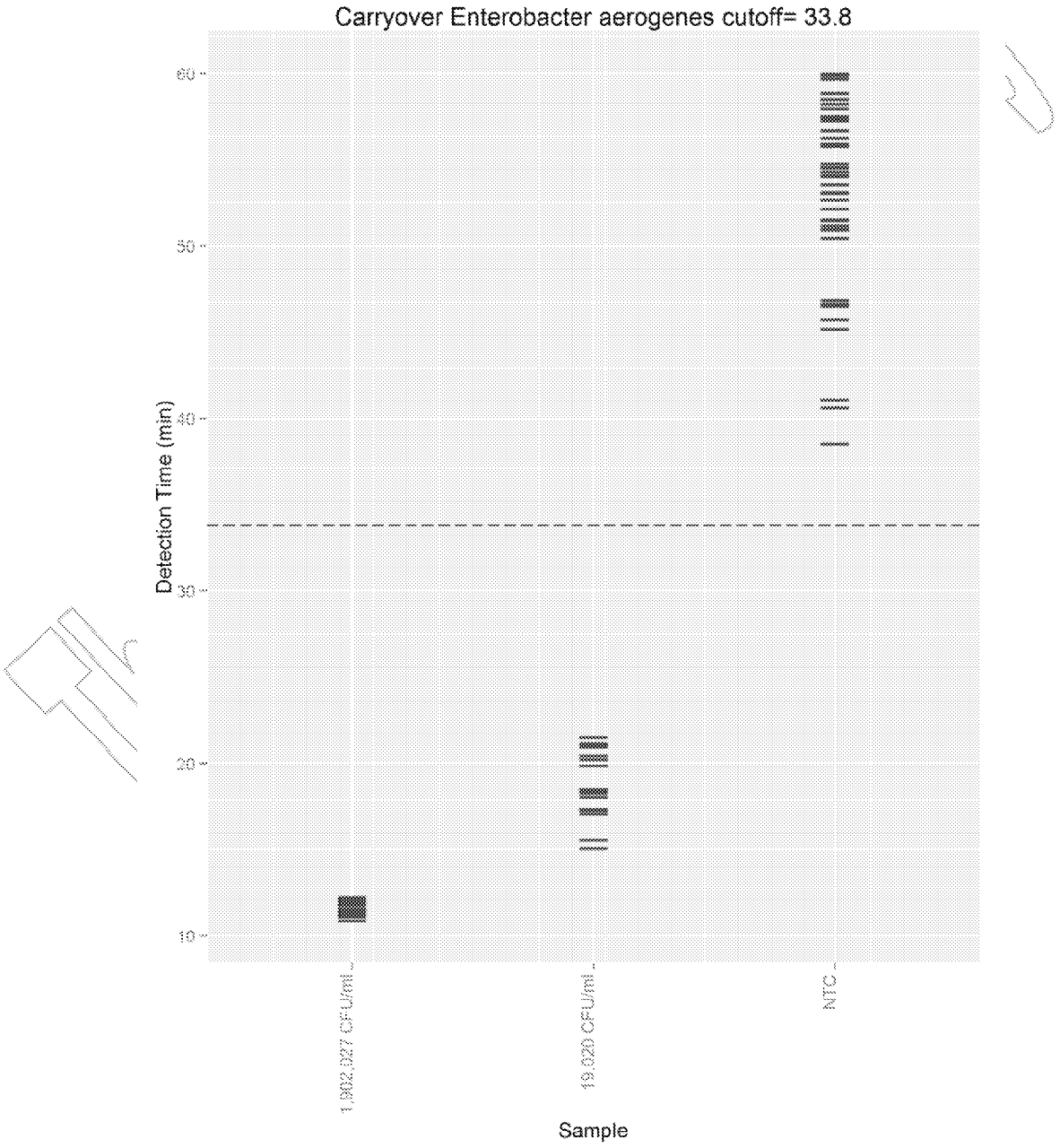
12) FINAL RECOMMENDATIONS

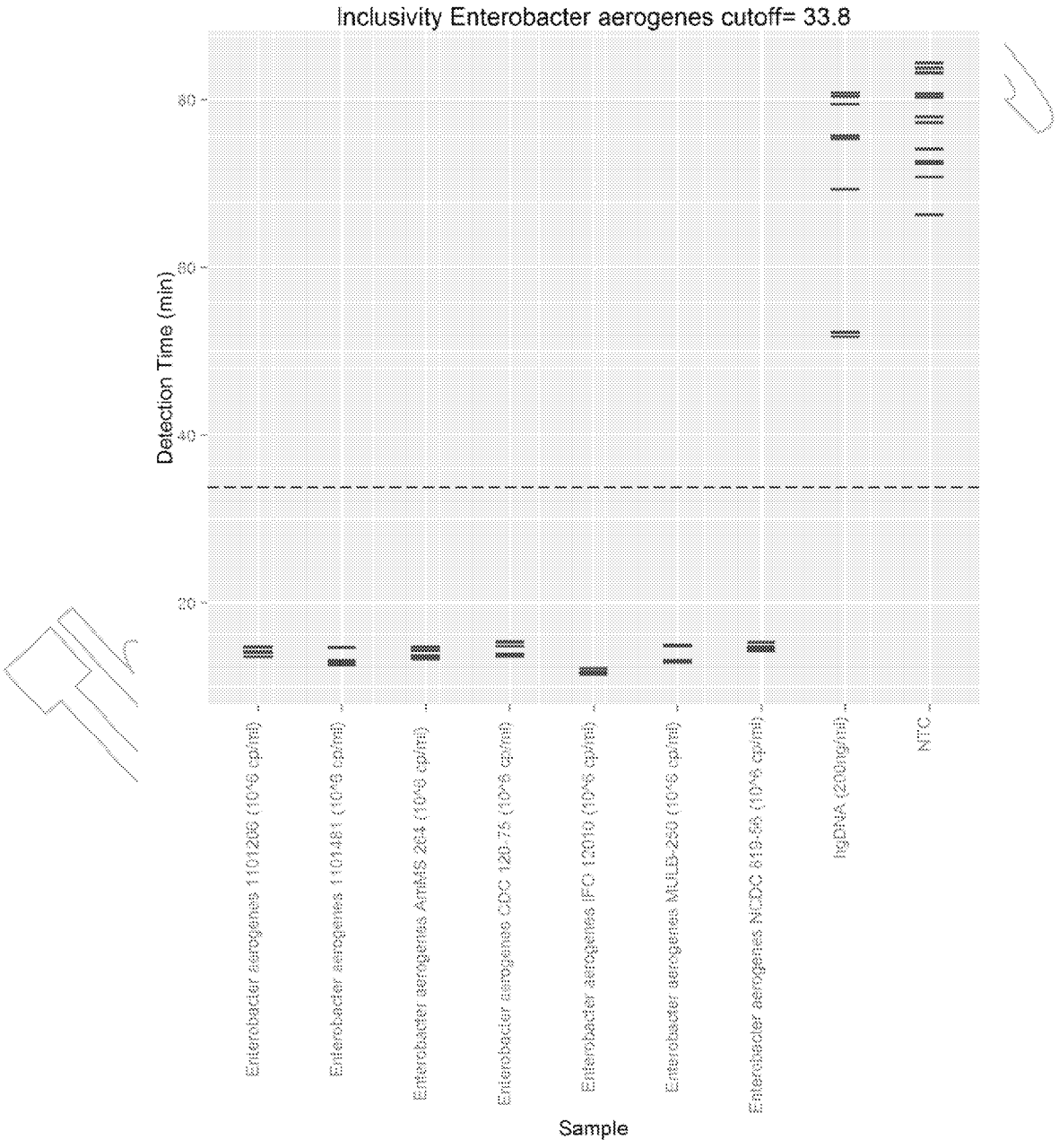
The assay for *Enterobacter aerogenes* was found to meet all criteria for precision, carryover, inclusivity, exclusivity, cross-reactivity, specificity, and resistance to interfering substances. Positive and negative clinical samples were tested and compared to a predicate method. The *Enterobacter aerogenes* assay specifically and reliably detects *Enterobacter aerogenes*. The assay limit of detection is 19,020 CFU/ml with a recommended assay duration of 34 minutes as determined by ROC analysis.

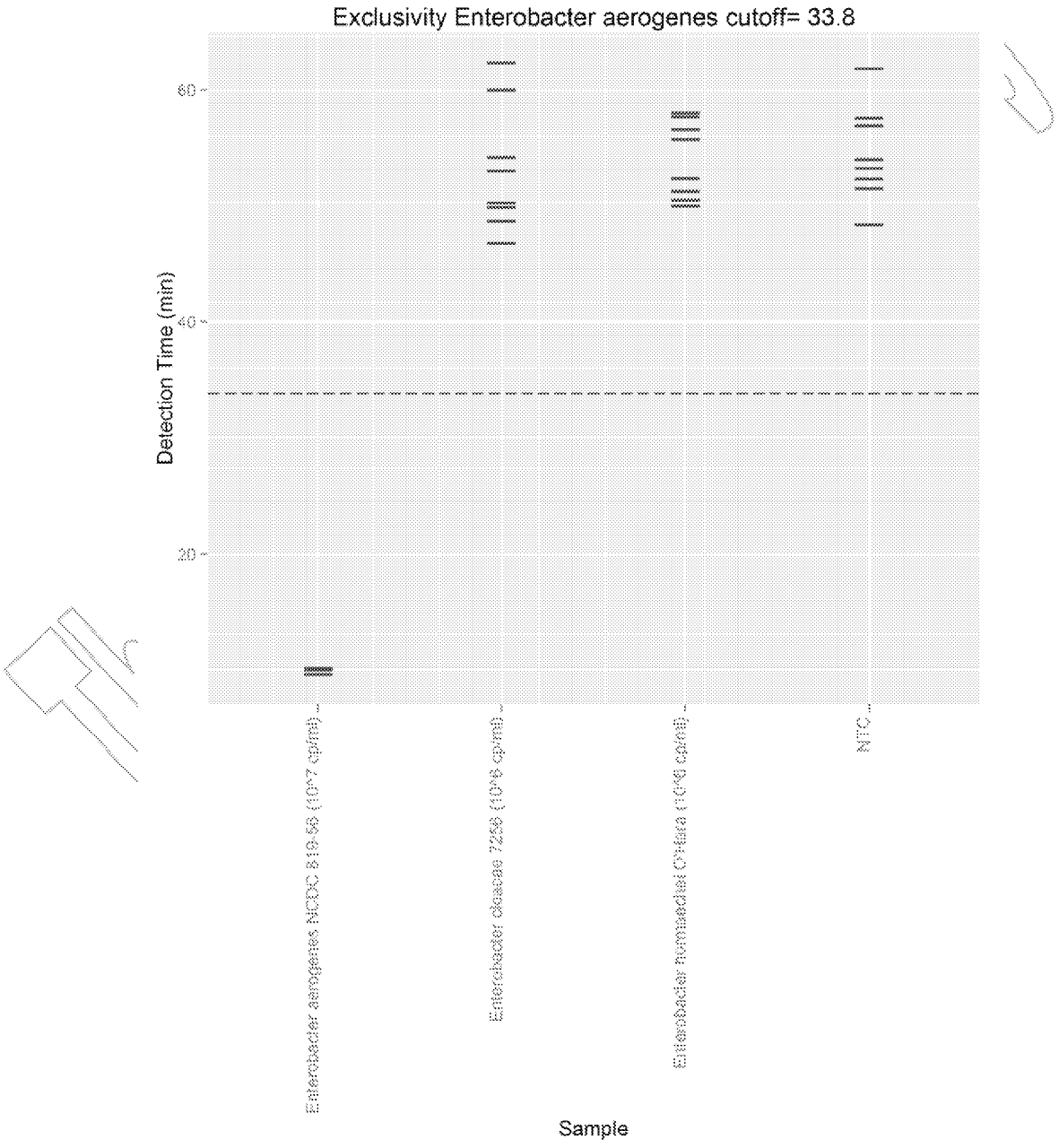
13) APPENDIX

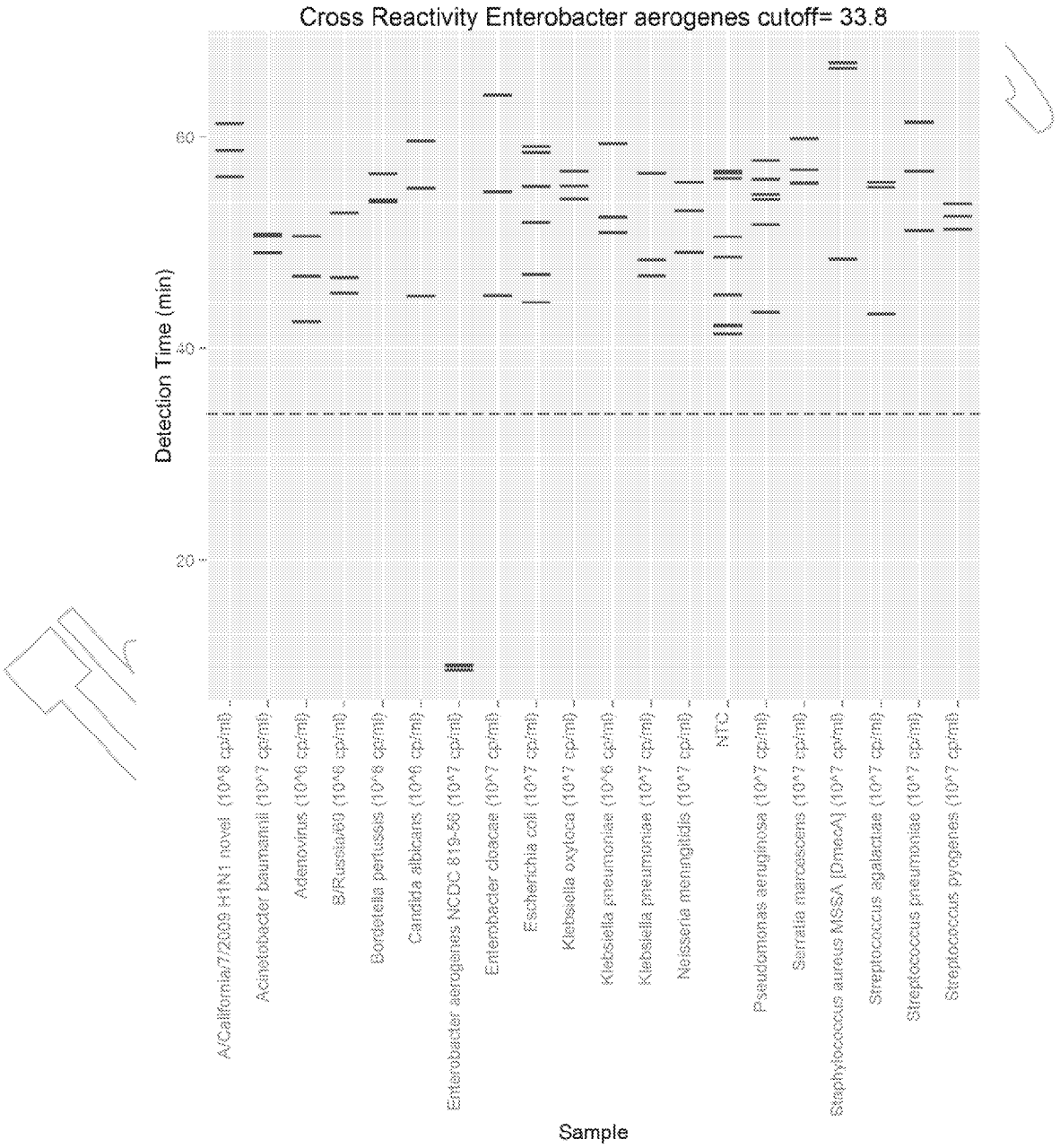


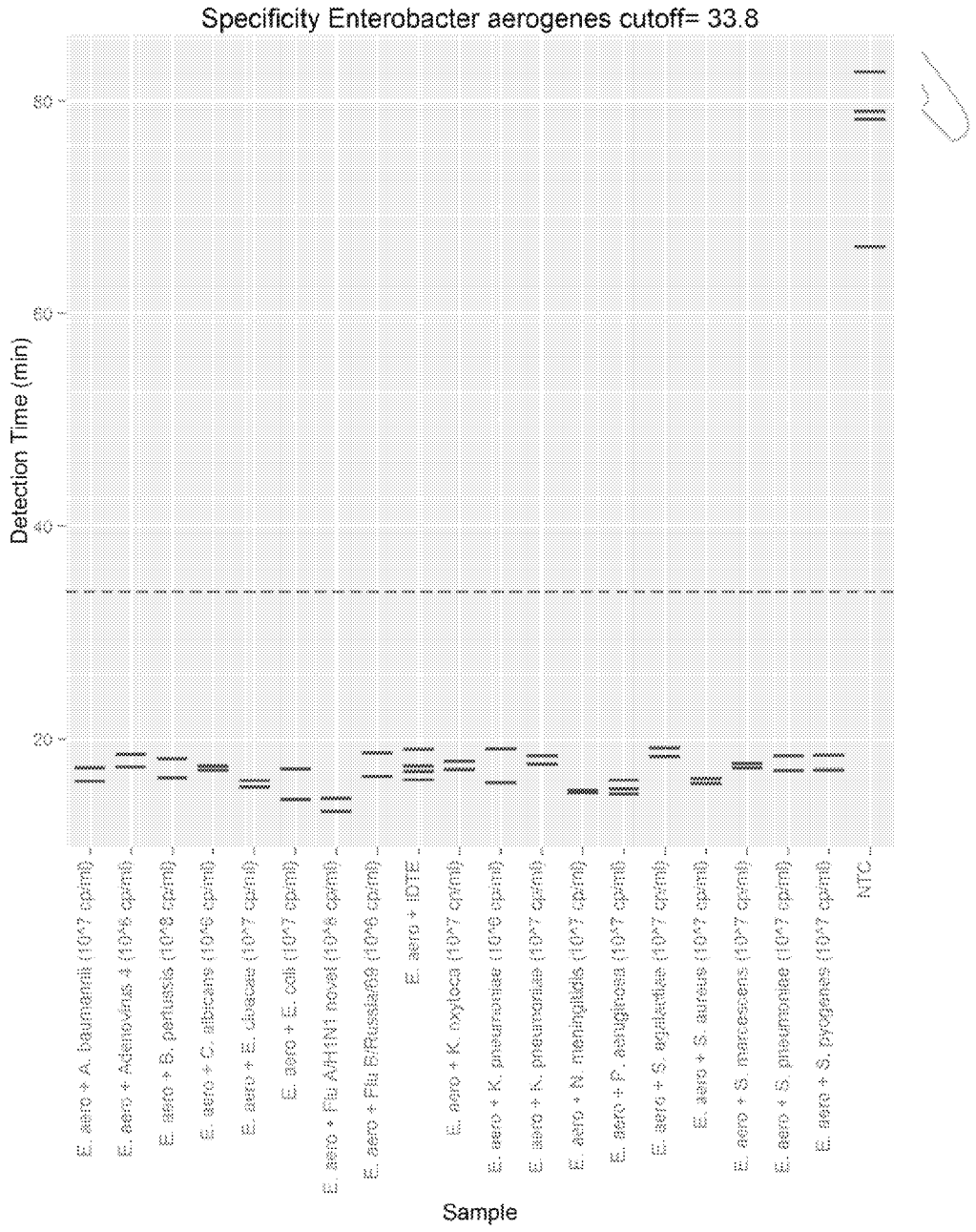


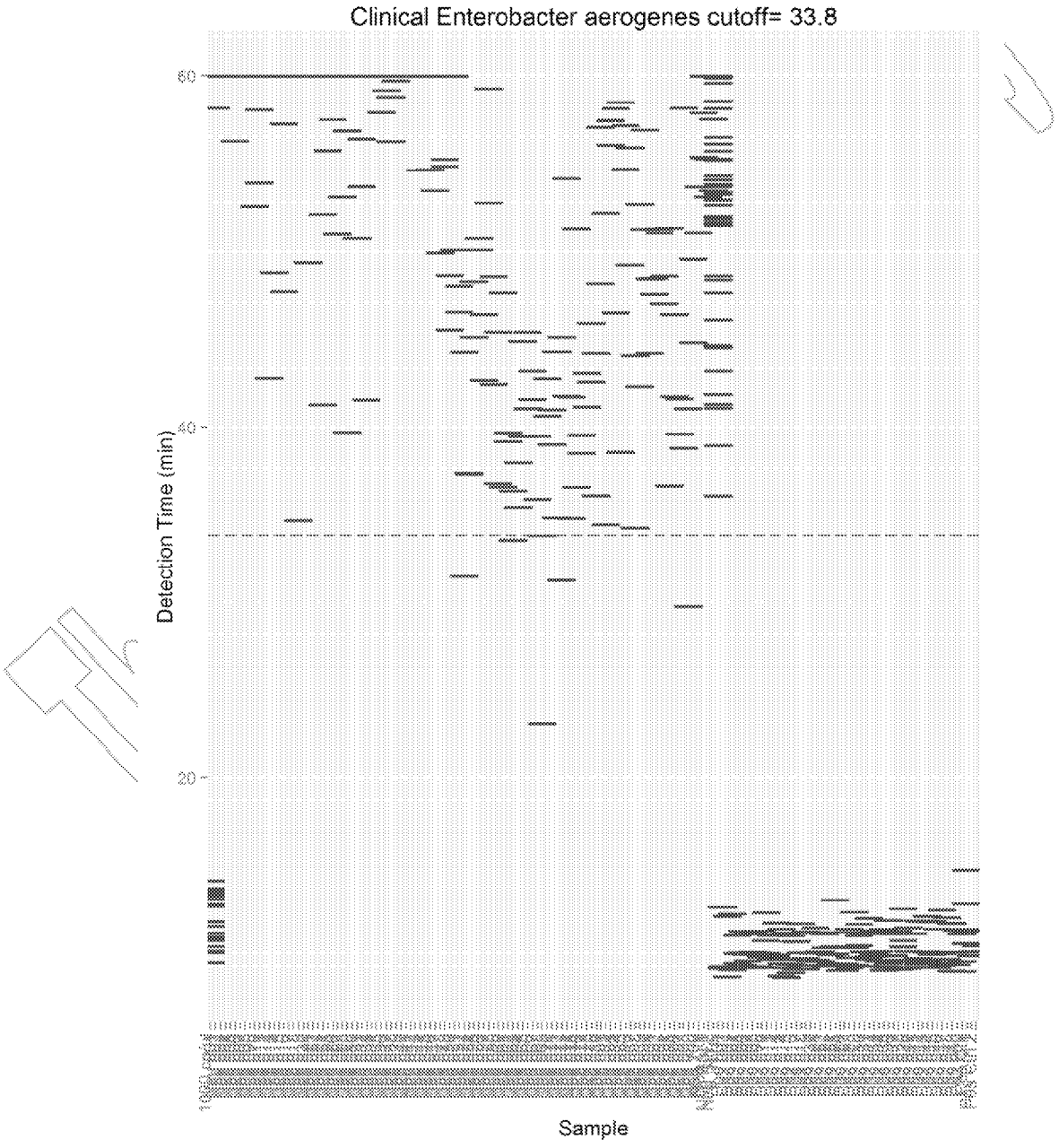












Clinical Samples TNAA: Treatment	NumPositive	Total	Percent
1000 cp/ul	22	22	100
Neg 001	0	2	0
Neg 002	0	2	0
Neg 003	0	2	0
Neg 004	0	2	0
Neg 005	0	2	0
Neg 006	0	2	0
Neg 007	0	2	0
Neg 008	0	2	0
Neg 009	0	2	0
Neg 010	0	2	0
Neg 011	0	2	0
Neg 012	0	2	0
Neg 013	0	2	0
Neg 014	0	2	0
Neg 015	0	2	0
Neg 016	0	2	0
Neg 017	0	2	0
Neg 018	0	2	0
Neg 019	0	2	0
Neg 020	0	2	0
Neg 021	0	2	0
Neg 022	0	2	0
Neg 023	0	2	0
Neg 024	0	2	0
Neg 025	0	2	0
Neg 026	0	2	0
Neg 027	0	2	0
Neg 028	0	2	0
Neg 029	0	2	0
Neg 030	0	2	0
Neg 031	0	2	0
Neg 032	0	2	0
Neg 033	0	2	0

Neg 034	0	2	0
Neg 035	0	2	0
Neg 036	0	2	0
Neg 037	0	2	0
Neg 038	0	2	0
Neg 039	0	2	0
Neg 040	0	2	0
Neg 041	0	2	0
Neg 042	0	2	0
Neg 043	0	2	0
Neg 044	0	2	0
Neg 045	0	2	0
Neg 046	0	2	0
Neg 047	0	2	0
Neg 048	0	2	0
Neg 049	0	2	0
Neg 050	0	2	0
Neg 051	0	2	0
Neg 052	1	2	50
Neg 053	0	2	0
Neg 054	0	2	0
Neg 055	0	2	0
Neg 056	0	2	0
Neg 057	0	2	0
Neg 058	0	2	0
Neg 059	0	2	0
Neg 060	0	2	0
Neg 061	0	2	0
Neg 062	1	2	50
Neg 063	0	2	0
Neg 064	0	2	0
Neg 065	0	2	0
Neg 066	0	2	0
Neg 067	0	2	0
Neg 068	1	2	50
Neg 069	0	2	0

Neg 070	0	2	0
Neg 071	0	2	0
Neg 072	1	2	50
Neg 073	0	2	0
Neg 074	0	2	0
Neg 075	0	2	0
Neg 076	0	2	0
Neg 077	0	2	0
Neg 078	0	2	0
Neg 079	0	2	0
Neg 080	0	2	0
Neg 081	0	2	0
Neg 082	0	2	0
Neg 083	0	2	0
Neg 084	0	2	0
Neg 085	0	2	0
Neg 086	0	2	0
Neg 087	0	2	0
Neg 088	0	2	0
Neg 089	0	2	0
Neg 090	0	2	0
Neg 091	0	2	0
Neg 092	0	2	0
Neg 093	0	2	0
Neg 094	0	2	0
Neg 095	0	2	0
Neg 096	0	2	0
Neg 097	0	2	0
Neg 098	1	2	50
Neg 099	0	2	0
Neg 100	0	2	0
Neg Ctrl	0	5	0
Neg Ctrl 1	0	2	0
Neg Ctrl 2	0	2	0
NTC	0	114	0
Pos 001	3	3	100

Pos 002	3	3	100
Pos 003	3	3	100
Pos 004	3	3	100
Pos 005	3	3	100
Pos 006	3	3	100
Pos 007	3	3	100
Pos 008	3	3	100
Pos 009	3	3	100
Pos 010	3	3	100
Pos 011	3	3	100
Pos 012	3	3	100
Pos 013	3	3	100
Pos 014	3	3	100
Pos 015	3	3	100
Pos 016	3	3	100
Pos 017	3	3	100
Pos 018	3	3	100
Pos 019	3	3	100
Pos 020	3	3	100
Pos 021	3	3	100
Pos 022	3	3	100
Pos 023	3	3	100
Pos 024	3	3	100
Pos 025	3	3	100
Pos 026	3	3	100
Pos 027	3	3	100
Pos 028	3	3	100
Pos 029	3	3	100
Pos 030	3	3	100
Pos 031	3	3	100
Pos 032	3	3	100
Pos 033	3	3	100
Pos 034	3	3	100
Pos 035	3	3	100
Pos 036	3	3	100
Pos 037	3	3	100

Pos 038	3	3	100
Pos 039	3	3	100
Pos 040	3	3	100
Pos 041	3	3	100
Pos 042	3	3	100
Pos 043	3	3	100
Pos 044	3	3	100
Pos 045	3	3	100
Pos 046	3	3	100
Pos 047	3	3	100
Pos 048	3	3	100
Pos 049	3	3	100
Pos 050	3	3	100
Pos Ctrl	5	5	100
Pos Ctrl 1	2	2	100
Pos Ctrl 2	2	2	100

MYCOBACTERIUM TUBERCULOSIS

TNAA LDT Validation Report

Limit of Detection = 10 cp/uL

Rate of Detection = 100 cp/uL in 24 minutes

Katie Sullivan-Bibee

THERANOS, INC.

Mycobacterium tuberculosis TNAA Validation Report

Author(s):

Signature:	Date:
Name: Katie Sullivan-Bibee	Title: Research Associate

Reviewer(s)

Signature:	Date:
Name: Pranav Patel, PhD.	Title: Team Lead

Signature:	Date:
Name: Daniel Young, Ph.D.	Title: Vice President

Approver(s):

Signature:	Date:
Name: Adam Rosendorff, M.D	Title: Laboratory Director

Table of Contents

Mycobacterium tuberculosis

1) PURPOSE

This report includes relevant information about the target organism, a detailed description of the primers and selected targets designed for the detection of this organism, a summary of the validation assay performance, and recommendations for future assay execution.

2) BACKGROUND

Mycobacterium tuberculosis (MTb), the causative agent of tuberculosis, causes 8-9 million cases of infection and 1.5 million deaths every year. Current Tb diagnostic methods often rely on culturing, which can be lengthy (2-3 weeks for diagnostic) and inaccurate. MTb has a genome of 4.5 Mb, with 4000 genes. Species wide sequence polymorphism is ~1/10,000 sites. The *M. tuberculosis* complex includes the following species: *M. tuberculosis* (primary human pathogen), *M. bovis* (cattle pathogen, close relative and potential donor lineage for human strain), *M. microtii, M. canetti, M. africanum, M. caprae* and *M. piniipedii*

Insertion sequence IS6110 is present in *M. tuberculosis* complex but not in other related species. It exists as about ~10 copies per genome. A nucleic acid amplification test was developed to detect MTb based on the presence of this insertion sequence.

3) SUMMARY OF PERFORMANCE DATA

Theranos developed a Theranos Nucleic Acid Amplification (TNAA) assay specific for *Mycobacterium tuberculosis*. The Nucleic Acid Amplification reactions contained 1x Nucleic Acid Amplification buffer (20 mM Tris Acetate, pH 7.9, 50 mM Potassium Acetate, 10 mM Magnesium Acetate and 1mM DTT), 0.08% Tween, 0.8 M betaine, 1.4 mM dNTPs, 2 uM Syto59, 0.8 uM RLX1445 primer and 0.8 uM RLX1446 primer, 20 units Bst polymerase, and template at the noted concentration. The reactions were run at 56ºC for 60 minutes. Summarized data will follow below while detailed experimental data can be found in the appendix. Primer sequences are:

Mycobacterium tuberculosis	RLX1445	TGAAAGACGATGTGTACTGAGATC
	RLX1446	CGTCTTTCACAACAAGAAGGCGTA

4) LIMIT OF DETECTION

The purpose of this study is to determine the limit of detection (LOD) for the Theranos TNAA assay. The LOD_{95} is the bacterial titer at which >95% of known positive samples test positive using the TNAA assay. Statistically justified cut-off times for making positive/negative calls were determined for each target empirically. A set of experiments, repeated over four days, were conducted that included eight replicates each of three target dilutions (LoD, 10X LoD, and 100X LoD), as well as 8 NTCs, using the target primers for amplification. These data were then processed using a receiver-operator character (ROC) analysis, and the best threshold detection time for distinguishing positives and negatives determined using the Youden test statistic as implemented by the R package, pROC.

The assay reliably detected 301 CFU/ml of *Mycobacterium tuberculosis* in about 34.8 minutes, as shown below. The 34.8 minute assay cut-off time was determined by ROC analysis. The assay was performed eight times. Reactions with and without template (NTCs or Non-Templated Controls) were run in eight replicates each.

LOD	Sample	NumPositive	Total	Percent
100X LOD	30,097 CFU/ml	64	64	100
10X LOD	3,010 CFU/ml	64	64	100
1X LOD	301 CFU/ml	64	64	100
	NTC	0	64	0

5) REPRODUCIBILITY/PRECISION

The purpose of this experiment is to determine the precision of the assay, percent positive and negative at three detection limits: high-negative (0.1X LOD=30 CFU/ml), low-positive (LOD=301 CFU/ml), and high-positive (3X LOD=903 CFU/ml). The assay was performed six times. Reactions with and without template (NTCs or Non-templated Controls) were run in eight replicates each.

Precision LOD	Sample	NumPositive	Total	Percent
3X LOD	903 CFU/ml	48	48	100
1X LOD	301 CFU/ml	48	48	100
0.1X LOD	30 CFU/ml	39	48	81
	NTC	0	48	0

6) CARRYOVER

The purpose of this experiment is to determine the potential for carryover of positive samples adjacent to negative reactions. The nucleic acid template is prepared from high-positive (100X LOD = 30,097 CFU/ml), low-positive (1X LOD=301 CFU/ml), and non-templated controls (NTCs) which are arrayed in alternating rows of eight replicates each. There are two rows of high-positive reactions, two rows of low-positive reactions, and six rows of NTCs. The assay was performed once, with no carryover of positive samples to negative reactions.

	100X LOD	NTC	100X LOD	NTC	LOD	NTC	LOD	NTC	NTC	NTC	
	1	2	3	4	5	6	7	8	9	10	11	12
A	empty	+	-	+	-	+	-	+	-	-	-	empty
B		+	-	+	-	+	-	+	-	-	-	
C		+	-	+	-	+	-	+	-	-	-	
D		+	-	+	-	+	-	+	-	-	-	
E		+	-	+	-	+	-	+	-	-	-	
F		+	-	+	-	+	-	+	-	-	-	
G		+	-	+	-	+	-	+	-	-	-	
H		+	-	+	-	+	-	+	-	-	-	

Carryover Samples	NumPositive	Total	Percent
30,097 CFU/ml	16	16	100
301 CFU/ml	16	16	100
NTC	0	48	0

7) INCLUSIVITY/EXCLUSIVITY

The assay for *Mycobacterium tuberculosis* was tested to validate inclusivity and exclusivity. Various strains of *Mycobacterium tuberculosis* were tested to verify inclusive assay performance. The assay was also tested against different species of *Mycobacterium* to verify exclusivity between close relatives.

All inclusive strains of *M. tuberculosis* were tested in eight replicates each, while there were sixteen total replicates for NTC reactions. The TNAA method successfully detected all inclusive *M. tuberculosis* strains including *M. bovis* which is part of the tuberculosis complex.

All exclusive *Mycobacterium* strains were tested in eight replicates each, with eight positive control reactions and sixteen negative NTC replicates. The TNAA method excluded all closely related *Mycobacterium* strains.

The following tables summarize the inclusivity and exclusivity pathogens to be evaluated for the *Mycobacterium tuberculosis* assay.

Inclusivity Samples	NumPositive	Total	Percent
Mycobacterium tuberculosis H37Ra (10^6 cp/ml)	8	8	100
Mycobacterium tuberculosis TMC 102 [H37Rv] (10^6 cp/ml)	8	8	100
Mycobacterium tuberculosis X003899 (10^6 cp/ml)	8	8	100
Mycobacterium tuberculosis X004439 (10^6 cp/ml)	8	8	100
Mycobacterium bovis TMC 1011 (10^6 cp/ml)	8	8	100
NTC	0	16	0

Exclusivity Samples	NumPositive	Total	Percent
Mycobacterium abscessus TMC 1543 (10^6 cp/ml)	0	8	0
Mycobacterium abscessus K.K. (10^6 cp/ml)	0	8	0
Mycobacterium abscessus SSC 210 (10^6 cp/ml)	0	8	0
Mycobacterium avium K-10 (10^6 cp/ml)	0	8	0
Mycobacterium gastri W-417 (10^6 cp/ml)	0	8	0
Mycobacterium tuberculosis (10^6 cp/ml)	8	8	100
NTC	0	16	0

8) CROSS-REACTIVITY

The cross-reactivity of the assay was tested against a panel of organisms which may also be present in collected *Mycobacterium tuberculosis* clinical samples. These organisms must be tested to ascertain that no false positives will be due to contamination from the off-target genomic material at clinically relevant viral or bacterial loads. The table below summarizes the genomic material tested and the results obtained. All potentially cross-reactive organisms were tested in replicates of eight and NTCs and the positive control were tested replicates of four. The TNAA assay was verified to not cross-react with any non-target organisms.

Cross-Reactivity Samples	NumPositive	Total	Percent
Adenovirus (10^8 cp/ml)	0	8	0
Candida albicans (10^8 cp/ml)	0	8	0
E. coli (10^8 cp/ml)	0	8	0
hgDNA (200ng/ml)	0	8	0
Influenza A (10^8 cp/ml)	0	8	0
Influenza B (10^8 cp/ml)	0	8	0
Mycobacterium tuberculosis (MTB) (10^6 cp/ml)	4	4	100
NTC	0	4	0
Pseudomonas aeruginosa (10^8 cp/ml)	0	8	0
Staphylococcus aureus (10^8 cp/ml)	0	8	0
Streptococcus pyogenes (10^8 cp/ml)	0	8	0

9) SPECIFICITY

The specificity of the assay was tested against a panel of organisms which may be present as potential contaminants in *Mycobacterium tuberculosis* samples and whose genomic material may be carried though the sample preparation protocol. These organisms must be tested to verify that assay performance is not significantly impacted by the presence of off-target genomic material combined with *Mycobacterium tuberculosis* at clinically relevant loads. The table below summarizes the genomic material tested and the results obtained. All organisms combined with *M. tuberculosis* were tested in replicates of two. The positive control and NTCs were also tested in two replicates.

The results below show that the assay is specific to *Mycobacterium tuberculosis* and spiking in other organisms that may be found in the same sample type does not affect assay performance. The assay tested M. tuberculosis target at 10X LOD (3,010 CFU/ml) combined with the off-target organism. The off-target nucleic acid concentration reflects expected median viral/bacterial loads in clinical specimens.

Specificity Samples	NumPositive	Total	Percent
M. tb + Adenovirus 4 (10^6 cp/ml)	2	2	100
M. tb + Bordetella pertussis (10^8 cp/ml)	2	2	100
M. tb + Candida albicans (10^6 cp/ml)	2	2	100
M. tb + Escherichia coli (10^7 cp/ml)	2	2	100
M. tb + IDTE	2	2	100
M. tb + Influenza A/California/7/2009 (H1N1 novel) (10^8 cp/ml)	2	2	100
M. tb + Influenza B/Russia/69 (10^6 cp/ml)	2	2	100
M. tb + Klebsiella pneumoniae (10^6 cp/ml)	2	2	100
M. tb + Pseudomonas aeruginosa (10^7 cp/ml)	2	2	100
M. tb + Staphylococcus aureus MSSA (DmecA) (10^7 cp/ml)	2	2	100
M. tb + Streptococcus pyogenes (10^7 cp/ml)	2	2	100
NTC	0	2	0

10) INTERFERING SUBSTANCES

The following interfering substances have been evaluated to have no significant effect on the performance of the TNAA assay. The interfering substances were added to *Mycobacterium tuberculosis* sample prep at both 10% and 0.1% of the total reaction by volume.

Interfering Substances: Endogenous and Exogenous.

Endogenous	Exogenous
Human blood	Bactroban nasal
Mucin	Flonase
Human genomic DNA	Nasonex
	Astelin
	Anefrin Nasal Spray
	Neosynphrine
	VapoRub cough suppressant
	ZiCam Allergy Relief nasal gel
	Mucin
	UTM

11) METHOD COMPARISON ON CLINICAL SAMPLES

The purpose of this study is to estimate the sensitivity and specificity of the TNAA assay using qPCR as the comparator (predicate method).

The following clinical samples were tested: 50 positive samples and 100 negative samples obtained from Fostering Tech Medical. Both nasal swab and sputum samples were taken from a range of individuals of both sexes and various ages.

TNAA vs qPCR Contingency Table		qPCR		
		Positive	Negative	Total
TNAA	Positive	50	0	50
	Negative	0	100	100
	Total	50	100	150

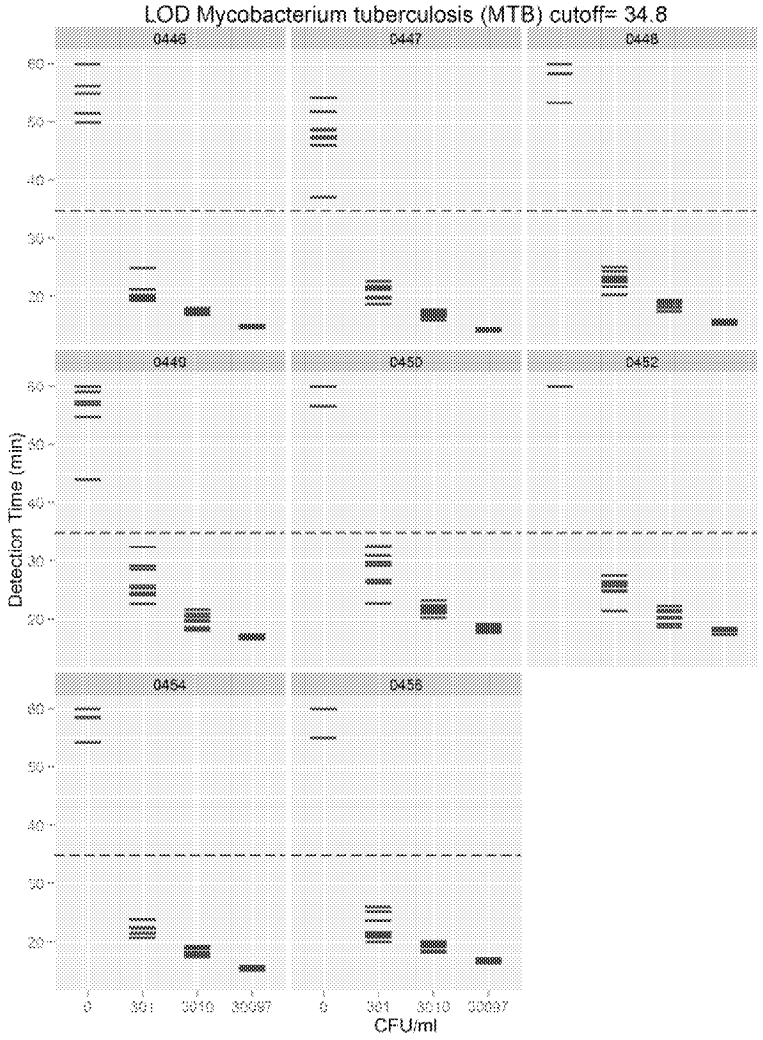
	Percent	95% Confidence Interval	
Estimated Sensitivity	100%	93%	100%
Estimated Specificity	100%	96%	100%

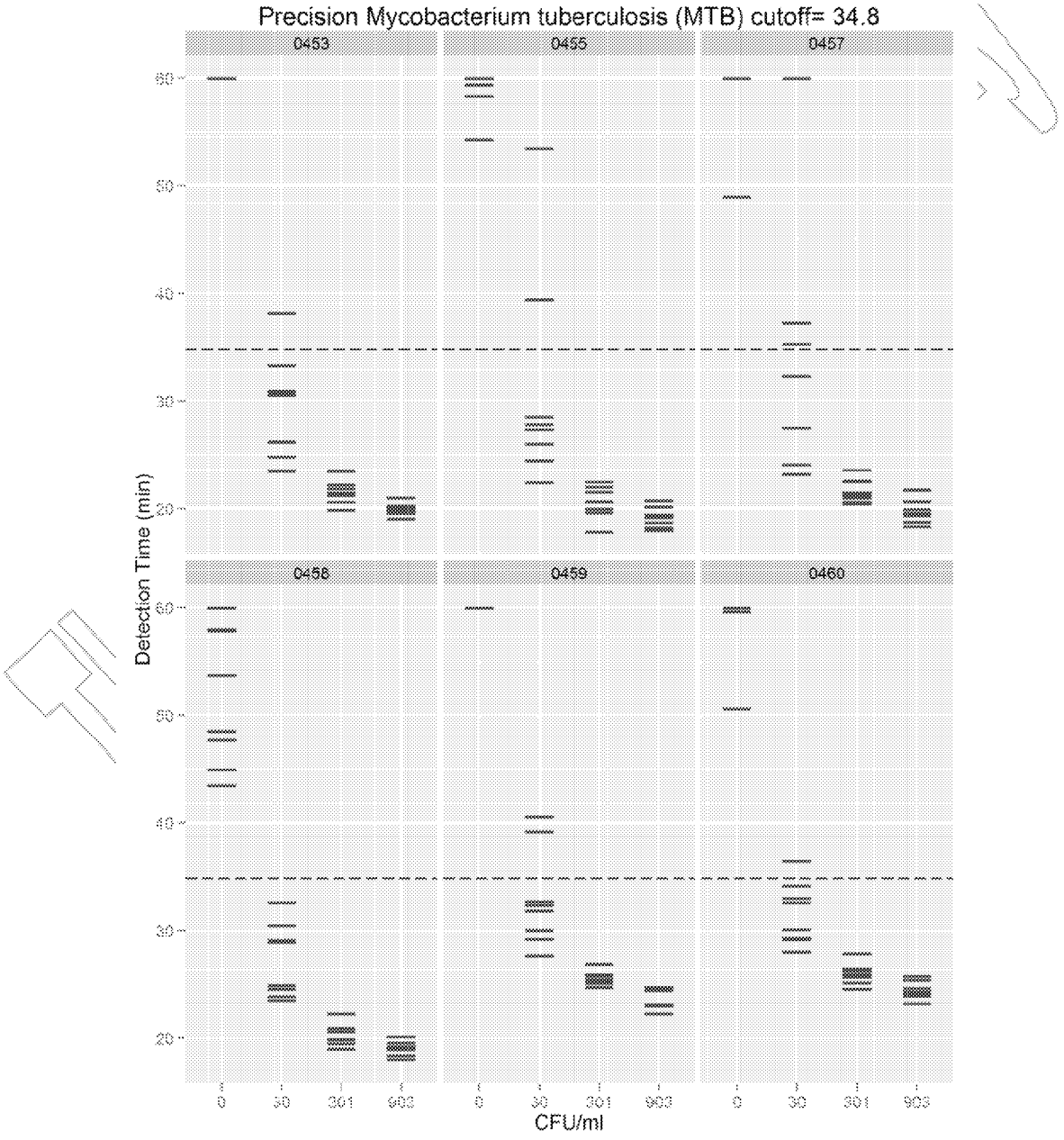
Based on a Prevalence of	**33%**
Positive Predictive Value	100%
Negative Predictive Value	100%

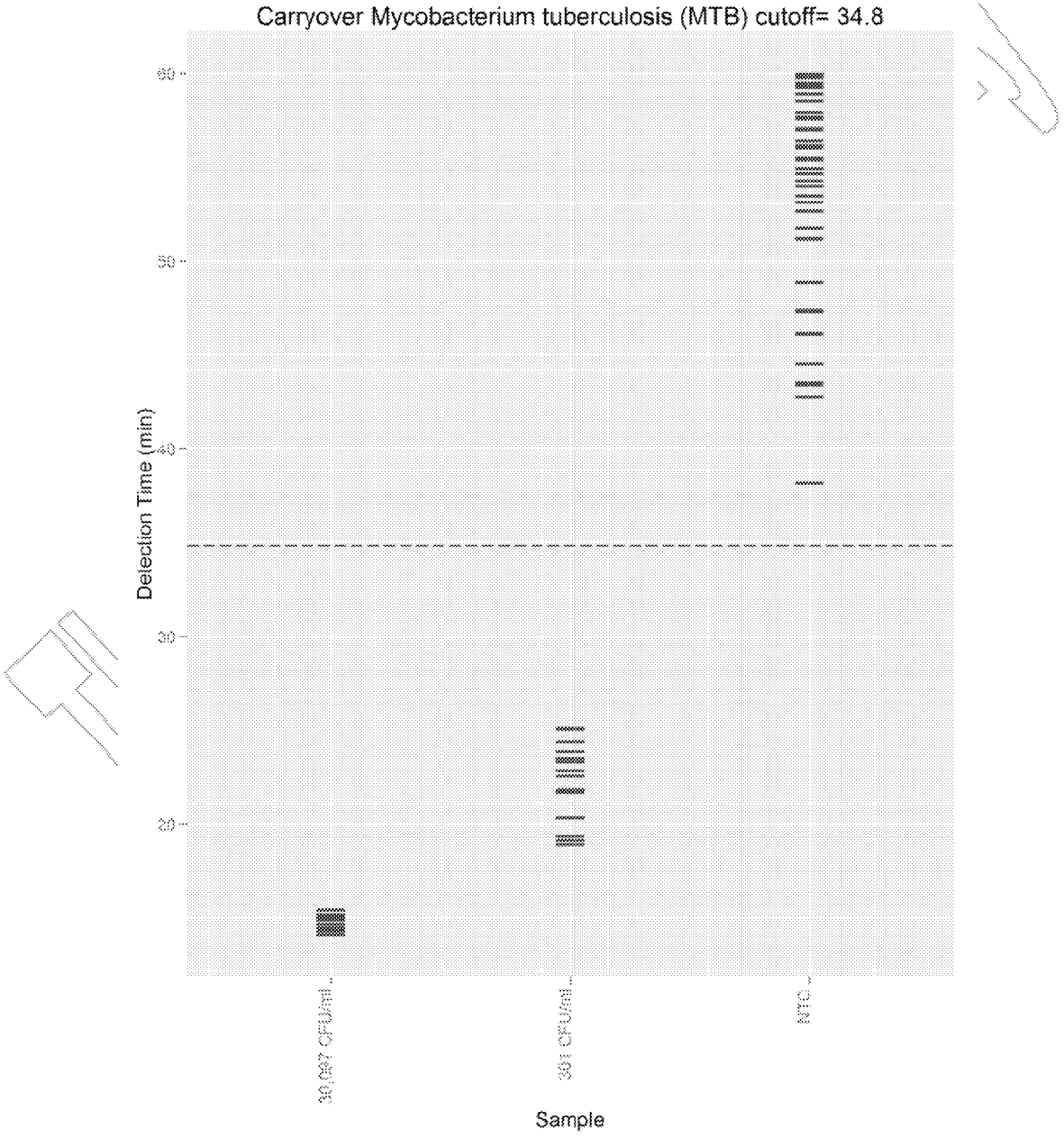
12) FINAL RECOMMENDATIONS

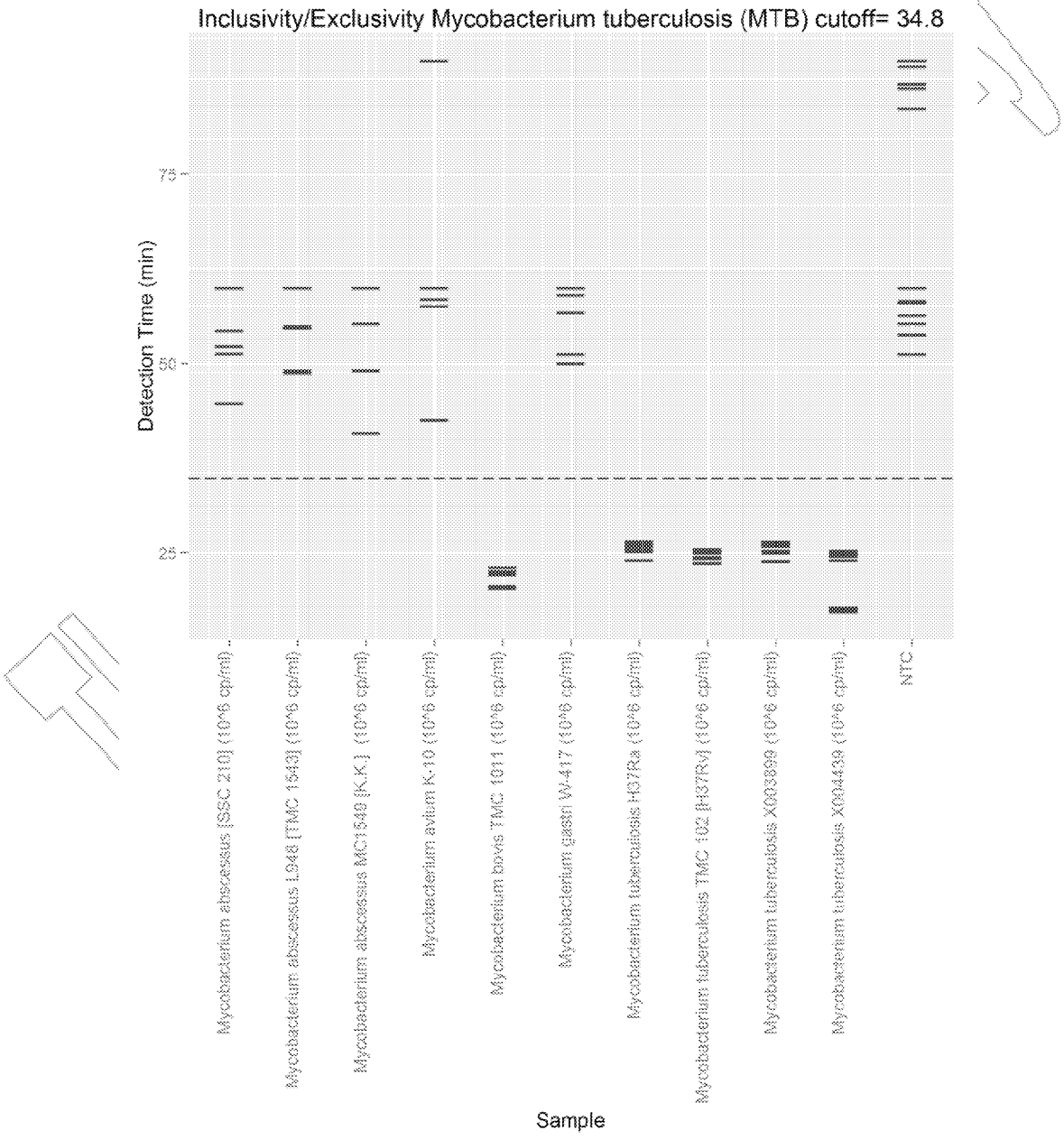
The assay for *Mycobacterium tuberculosis* was found to meet all criteria for precision, carryover, inclusivity, exclusivity, cross-reactivity, specificity, and resistance to interfering substances. Positive and negative clinical samples were tested and compared to a predicate method. The *Mycobacterium tuberculosis* assay specifically and reliably detects *Mycobacterium tuberculosis*. The assay limit of detection is 301 CFU/ml with a recommended assay duration of 35 minutes as determined by ROC analysis.

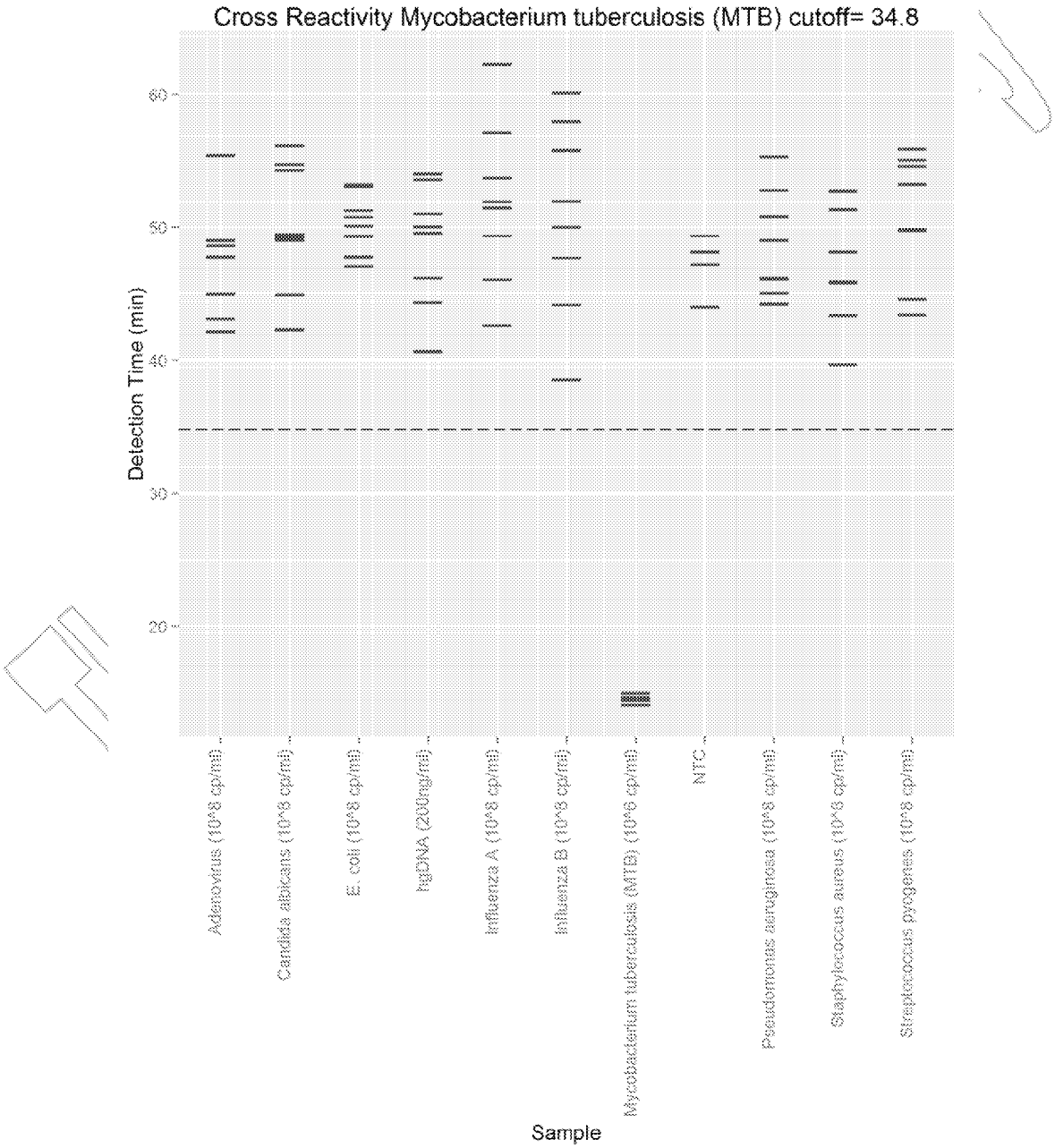
13) APPENDIX

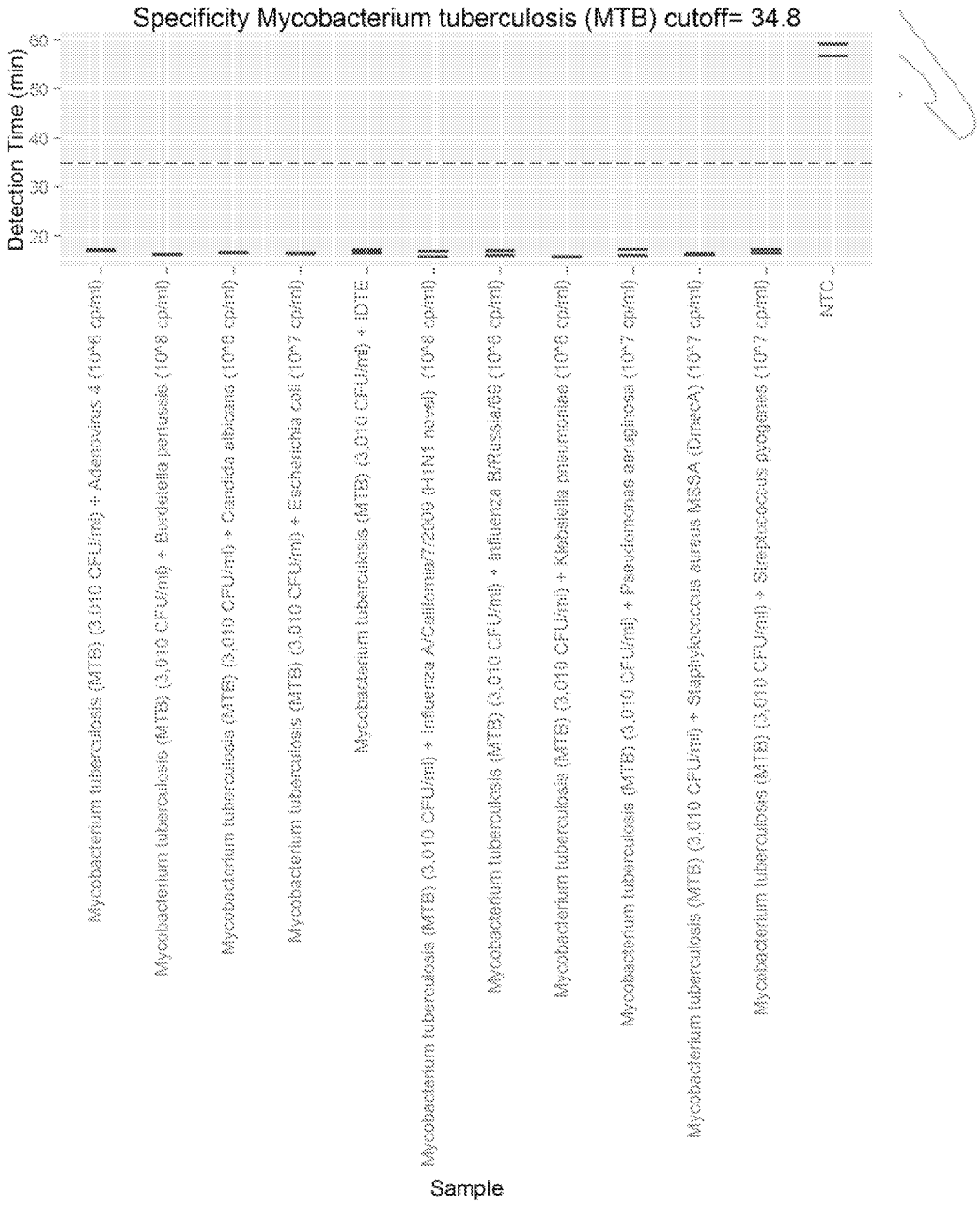


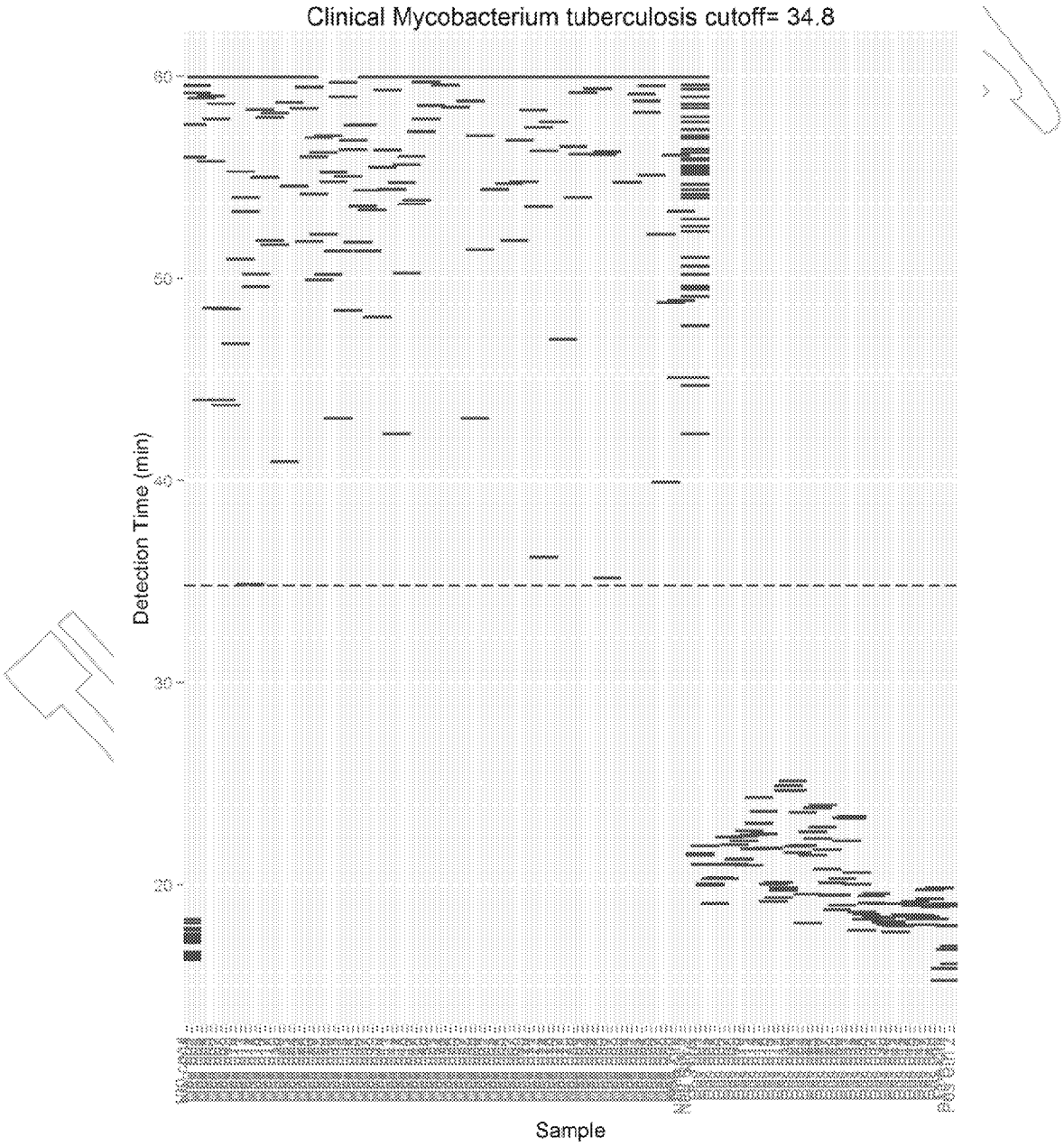












Clinical Samples TNAA: Treatment	NumPositive	Total	Percent
100 cp/ul	24	24	100
Neg 001	0	2	0
Neg 002	0	2	0
Neg 003	0	2	0
Neg 004	0	2	0
Neg 005	0	2	0
Neg 006	0	2	0
Neg 007	0	2	0
Neg 008	0	2	0
Neg 009	0	2	0
Neg 010	0	2	0
Neg 011	0	2	0
Neg 012	0	2	0
Neg 013	0	2	0
Neg 014	0	2	0
Neg 015	0	2	0
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Neg 028	0	2	0
Neg 029	0	2	0

Neg 030	0	2	0
Neg 031	0	2	0
Neg 032	0	2	0
Neg 033	0	2	0
Neg 034	0	2	0
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Neg 036	0	2	0
Neg 037	0	2	0
Neg 038	0	2	0
Neg 039	0	2	0
Neg 040	0	2	0
Neg 041	0	2	0
Neg 042	0	2	0
Neg 043	0	2	0
Neg 044	0	2	0
Neg 045	0	2	0
Neg 046	0	2	0
Neg 047	0	2	0
Neg 048	0	2	0
Neg 049	0	2	0
Neg 050	0	2	0
Neg 051	0	2	0
Neg 052	0	2	0
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Pos 046	2	2	100
Pos 047	2	2	100
Pos 048	2	2	100
Pos 049	2	2	100

Pos 050	2	2	100
Pos Ctrl	4	4	100
Pos Ctrl 1	2	2	100
Pos Ctrl 2	2	2	100

STAPHYLOCOCCUS AUREUS

TNAA LDT Validation Report

Limit of Detection = 10 cp/uL

Rate of Detection = 100 cp/uL in 19 minutes

Katie Sullivan-Bibee

THERANOS, INC.

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Table of Contents

Staphylococcus aureus

1) PURPOSE

This report includes relevant information about the target organism, a detailed description of the primers and selected targets designed for the detection of this organism, a summary of the validation assay performance, and recommendations for future assay execution.

2) BACKGROUND

Staphylococcus aureus is a facultative anaerobic gram-positive coccal bacterium that is frequently found as part of the normal skin flora on the skin and nasal passages. It is estimated that 20% of the human population are long-term carriers of *S. aureus*. It most commonly colonizes the nose and sometimes the throat and is the most common species of *Staphylococcus* to cause Staph infections. It is still one of the five most common causes of nosocomial infections and is often the cause of postsurgical wound infections. *S. aureus* is a common cause of food poisoning, due to the production of enterotoxin B, and is particularly prevalent in uncooked foods prepared by hand (such as sandwiches). Some *S. aureus* strains are resistant to antibiotics, such as methicillin (MRSA) and vancomycin (VISA/VRSA). *S. aureus* appears as grape-like clusters when viewed through a microscope, and has large, round, golden-yellow colonies. In a study of 106 *S. aureus* carriers in a hospital setting, the median bacterial load was 10^5 CFUs/nasal swab, and 95% exceed 100 CFUs/swab (White 1961).

The target is the immunoglobulin G binding protein A (spa) gene, highly conserved among *S. aureus* isolates, with fewer than 5% of nucleotide sites varying across sequenced strains in the 334 bp target region.

White, A. (1961). Quantitative studies of nasal carriers of staphylococci among hospitalized patients. Journal of Clinical Investigation, 40(1), 23.

3) SUMMARY OF PERFORMANCE DATA

Theranos developed a Theranos Nucleic Acid Amplification (TNAA) assay specific for *Staphylococcus aureus*. The Nucleic Acid Amplification reactions contained 1x Nucleic Acid Amplification buffer (20 mM Tris Acetate, pH 7.9, 50 mM Potassium Acetate, 10 mM Magnesium Acetate and 1mM DTT), 0.08% Tween, 0.8 M betaine, 1.4 mM dNTPs, 2 uM Syto59, 0.8 uM RLX1449 primer and 0.8 uM RLX1450 primer, 20 units Bst polymerase, and template at the noted concentration. The reactions were run at 56ºC for

60 minutes. Summarized data will follow below while detailed experimental data can be found in the appendix.

Primer sequences are:

Staphylococcus aureus	RLX1449	TGTACCGACAGAAACTGGTGAAG
	RLX1450	TCGGTACATAATGATAATCCACCAAA

4) LIMIT OF DETECTION

The purpose of this study is to determine the limit of detection (LOD) for the Theranos TNAA assay. The LOD_{95} is the bacterial titer at which >95% of known positive samples test positive using the TNAA assay. Statistically justified cut-off times for making positive/negative calls were determined for each target empirically. A set of experiments, repeated over four days, were conducted that included eight replicates each of three target dilutions (LoD, 10X LoD, and 100X LoD), as well as 8 NTCs, using the target primers for amplification. These data were then processed using a receiver-operator character (ROC) analysis, and the best threshold detection time for distinguishing positives and negatives determined using the Youden test statistic as implemented by the R package, pROC.

The assay reliably detected 2,142 CFU/ml of *Staphylococcus aureus* in about 49.8 minutes, as shown below. The 49.8 minute assay cut-off time was determined by ROC analysis. The assay was performed eight times. Reactions with and without template (NTCs or Non-Templated Controls) were run in eight replicates each.

LOD	Samples	NumPositive	Total	Percent
100X LOD	214,200 CFU/ml	64	64	100
10X LOD	21,420 CFU/ml	64	64	100
1x LOD	2,142 CFU/ml	64	64	100
	NTC	0	64	0

5) REPRODUCIBILITY/PRECISION

The purpose of this experiment is to determine the precision of the assay, percent positive and negative at three detection limits: high-negative (0.1X LOD=214 CFU/ml), low-positive (LOD=2,142 CFU/ml), and high-positive (3X LOD=6,426 CFU/ml). The assay was performed nine times. Reactions with and without template (NTCs or Non-templated Controls) were run in eight replicates each.

Precision LOD	Sample	NumPositive	Total	Percent
3X LOD	6,426 CFU/ml	72	72	100
1x LOD	2,142 CFU/ml	71	72	99
0.1X LOD	214 CFU/ml	37	72	51
	NTC	0	72	0

6) CARRYOVER

The purpose of this experiment is to determine the potential for carryover of positive samples adjacent to negative reactions. The nucleic acid template is prepared from high-positive (100X LOD = 21,420 CFU/ml), low-positive (1X LOD=2,142 CFU/ml), and non-templated controls (NTCs) which are arrayed in alternating rows of eight replicates each. There are two rows of high-positive reactions, two rows of low-positive reactions, and six rows of NTCs. The assay was performed once, with no carryover of positive samples to negative reactions.

		100X LOD	NTC	100X LOD	NTC	LOD	NTC	LOD	NTC	NTC	NTC	
	1	2	3	4	5	6	7	8	9	10	11	12
A	empty	+	-	+	-	+	-	+	-	-	-	empty
B		+	-	+	-	+	-	+	-	-	-	
C		+	-	+	-	+	-	+	-	-	-	
D		+	-	+	-	+	-	+	-	-	-	
E		+	-	+	-	+	-	+	-	-	-	
F		+	-	+	-	+	-	+	-	-	-	
G		+	-	+	-	+	-	+	-	-	-	
H		+	-	+	-	+	-	+	-	-	-	

Carryover Samples	NumPositive	Total	Percent
214,200 CFU/ml	16	16	100
2,142 CFU/ml	16	16	100
NTC	0	48	0

7) INCLUSIVITY/EXCLUSIVITY

The assay for *Staphylococcus aureus* was tested to validate inclusivity. Various strains of *Staphylococcus aureus* including methicillin-resistant *Staphylococcus aureus* (MRSA) were tested to verify inclusive assay performance. There was no exclusivity experiment needed for this assay.

All inclusive strains of *S. aureus or* MRSA were tested in one, seven, or eight replicates each, while there were eight total replicates for NTC reactions. The TNAA method successfully detected all inclusive *S. aureus* strains including MRSA

The following tables summarize the inclusivity pathogens to be evaluated for the *Staphylococcus aureus* assay.

Inclusivity Samples	NumPositive	Total	Percent
MRSA HDE288 (10^6 cp/ml)	8	8	100
MRSA HFH-30364 (10^6 cp/ml)	8	8	100
MRSA M10/0148 (10^6 cp/ml)	7	7	100
MRSA M10/0148 (10^6 cp/ml)	1	1	100
MRSA Mu50 [NRS1] (10^6 cp/ml)	8	8	100
MRSA NYBK2464 (10^6 cp/ml)	8	8	100
NTC	0	8	0
S. aureus FDA 209 (10^6 cp/ml)	8	8	100
S. aureus NCTC 8530 [S11] (10^6 cp/ml)	8	8	100
S. aureus PCI 1158 (8.57 * 10^5 cp/ml)	8	8	100
S. aureus Rose (10^6 cp/ml)	8	8	100
S. aureus TCH959 (10^6 cp/ml)	8	8	100
S. aureus Wood 46 (10^6 cp/ml)	8	8	100

8) CROSS-REACTIVITY

The cross-reactivity of the assay was tested against a panel of organisms which may also be present in collected *Staphylococcus aureus* clinical samples. These organisms must be tested to ascertain that no false positives will be due to contamination from the off-target genomic material at clinically relevant viral or bacterial loads. The table below summarizes the genomic material tested and the results obtained. All potentially cross-reactive organisms were tested in replicates of eight and NTCs and the positive control were tested replicates of four. The *S. aureus* assay did initially show cross-reactivity with eight out of eight Flu B reactions. A second cross-reactivity experiment was performed to test three additional Influenza B strains. This follow up experiment verified that there is no cross-reactivity between the *S. aureus* assay and Influenza B. It is likely that there was a low level of *S. aureus* contamination in the original Flu B lysate. Therefore, the *S. aureus* TNAA assay was verified to not cross-react with any non-target organisms.

Experiment 1: Cross-reactivity Samples	NumPositive	Total	Percent
Adenovirus (2*10^8 cp/ml)	0	8	0
C. albicans (10^8 cp/ml)	0	8	0
E. coli (10^8 cp/ml)	0	8	0
Flu A (10^8 cp/ml)	0	8	0
Flu B (10^8 cp/ml)	8	8	100
hgDNA (200ng/ml)	0	8	0
NTC	0	4	0
P. aeruginosa (10^8 cp/ml)	0	8	0
S. aureus (10^5 cp/ml)	4	4	100
S. aureus (10^8 cp/ml)	8	8	100
S. pyogenes (10^8 cp/ml)	0	8	0

Experiment 2: Cross-Reactivity Samples	NumPositive	Total	Percent
Influenza B/Lee/40 (10^8 cp/ml)	0	8	0
Influenza B/Malaysia/2506/2004 (10^8 cp/ml)	0	8	0
Influenza B/Russia/69 (10^7 cp/ml)	0	8	0
NTC	1	16	6
Staphylococcus aureus (MRSA) (10^5 cp/ml)	8	8	100

9) SPECIFICITY

The specificity of the assay was tested against a panel of organisms which may be present as potential contaminants in *Staphylococcus aureus* samples and whose genomic material may be carried though the sample preparation protocol. These organisms must be tested to verify that assay performance is not significantly impacted by the presence of off-target genomic material combined with *Staphylococcus aureus* at clinically relevant loads. The table below summarizes the genomic material tested and the results obtained. All organisms combined with *S. aureus* were tested in replicates of two. The positive control and NTCs were also tested in two replicates.

The results below show that the assay is specific to *Staphylococcus aureus* and spiking in other organisms that may be found in the same sample type does not affect assay performance. The assay tested S. aureus target at 10X LOD (214,200 CFU/ml) combined with the off-target organism. The off-target nucleic acid concentration reflects expected median viral/bacterial loads in clinical specimens.

Specificity Samples	NumPositive	Total	Percent
NTC	0	2	0
S. aureus + Adenovirus (10^6 cp/ml)	2	2	100
S. aureus + B. pertussis (10^8 cp/ml)	2	2	100
S. aureus + C. albicans (10^6 cp/ml)	2	2	100
S. aureus + E. coli (10^7 cp/ml)	2	2	100
S. aureus + Flu A/H1N1 novel (10^8 cp/ml)	2	2	100
S. aureus + Flu B (10^6 cp/ml)	2	2	100
S. aureus + hgDNA (200ng/ml)	2	2	100
S. aureus + IDTE	2	2	100
S. aureus + K. pneumoniae (10^6 cp/ml)	2	2	100
S. aureus + P. aeruginosa (10^7 cp/ml)	2	2	100
S. aureus + S. aureus (10^7 cp/ml)	2	2	100

10) INTERFERING SUBSTANCES

The following interfering substances have been evaluated to have no significant effect on the performance of the TNAA assay. The interfering substances were added to *Staphylococcus aureus* sample prep at both 10% and 0.1% of the total reaction by volume.

Interfering Substances: Endogenous and Exogenous.

Endogenous	Exogenous
Human blood	Bactroban nasal
Mucin	Flonase
Human genomic DNA	Nasonex
	Astelin
	Anefrin Nasal Spray
	Neosynphrine
	VapoRub cough suppressant
	ZiCam Allergy Relief nasal gel
	Mucin
	UTM

11) METHOD COMPARISON ON CLINICAL SAMPLES

The purpose of this study is to estimate the sensitivity and specificity of the TNAA assay using qPCR as the comparator (predicate method).

The following clinical samples were tested: 50 positive samples and 100 negative samples obtained from Fostering Tech Medical. Both nasal swab and pharyngeal exudate samples were taken from a range of individuals of both sexes and various ages.

TNAA vs qPCR Contingency Table		qPCR		
		Positive	Negative	Total
TNAA	Positive	50	0	50
	Negative	0	100	100
	Total	50	100	150

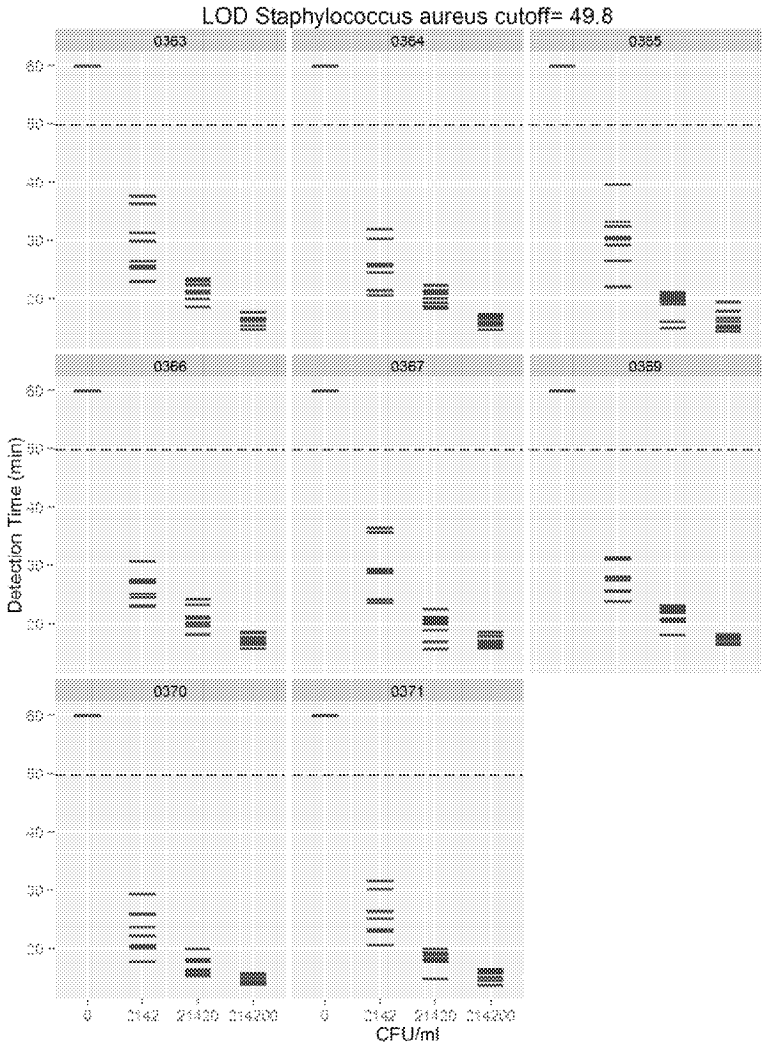
	Percent	95% Confidence Interval	
Estimated Sensitivity	100%	93%	100%
Estimated Specificity	100%	96%	100%

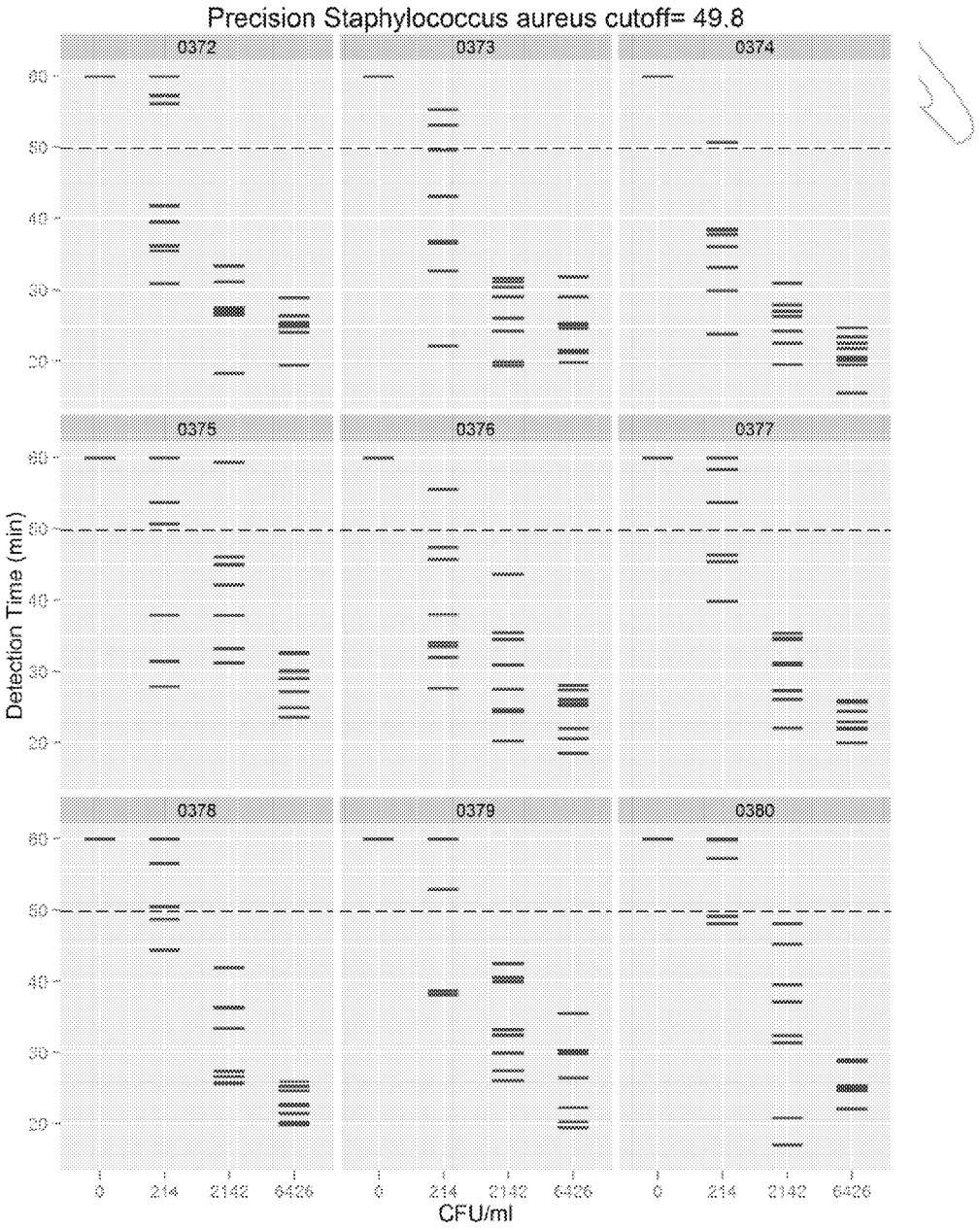
Based on a Prevalence of	33%
Positive Predictive Value	100%
Negative Predictive Value	100%

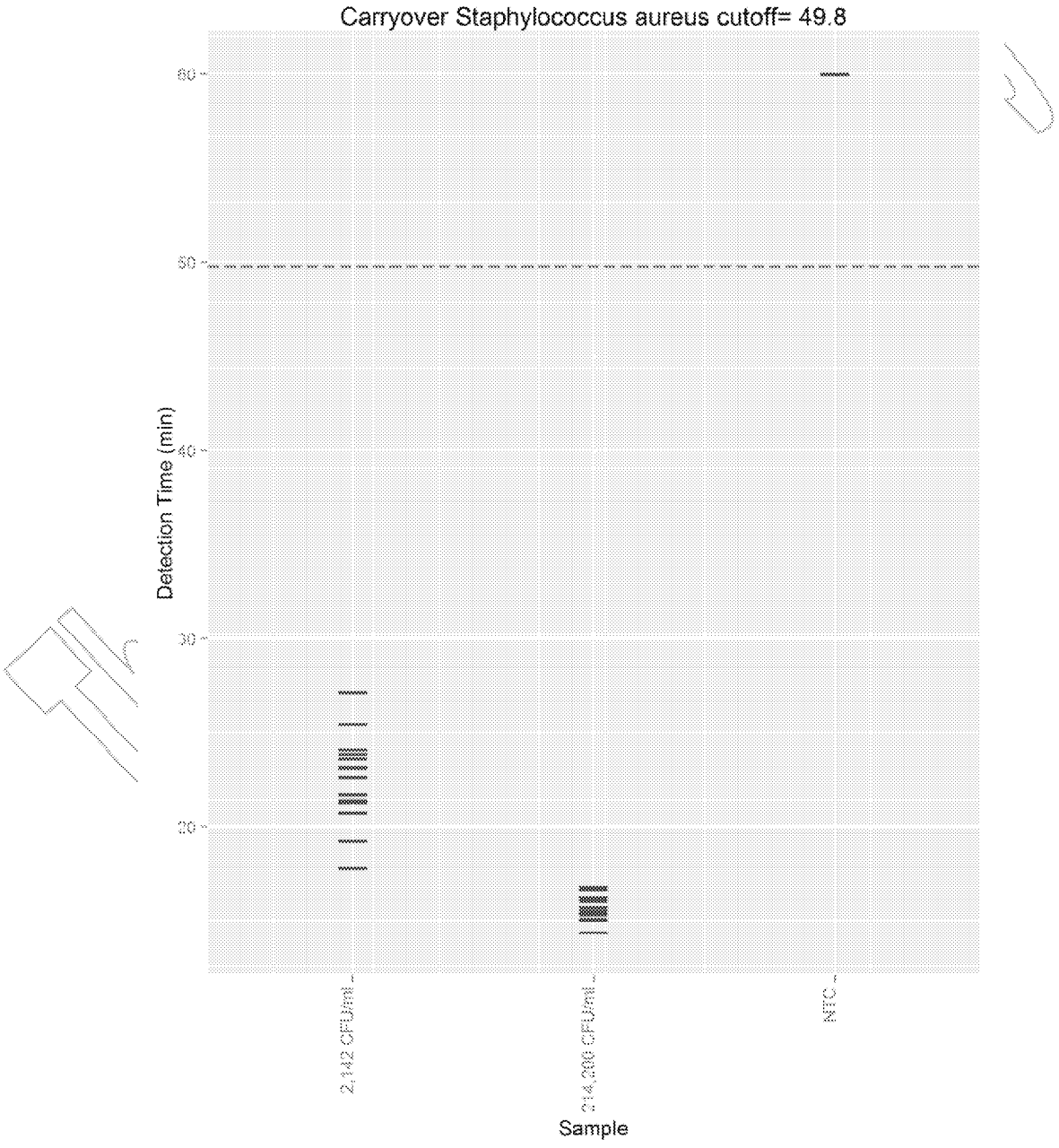
12) FINAL RECOMMENDATIONS

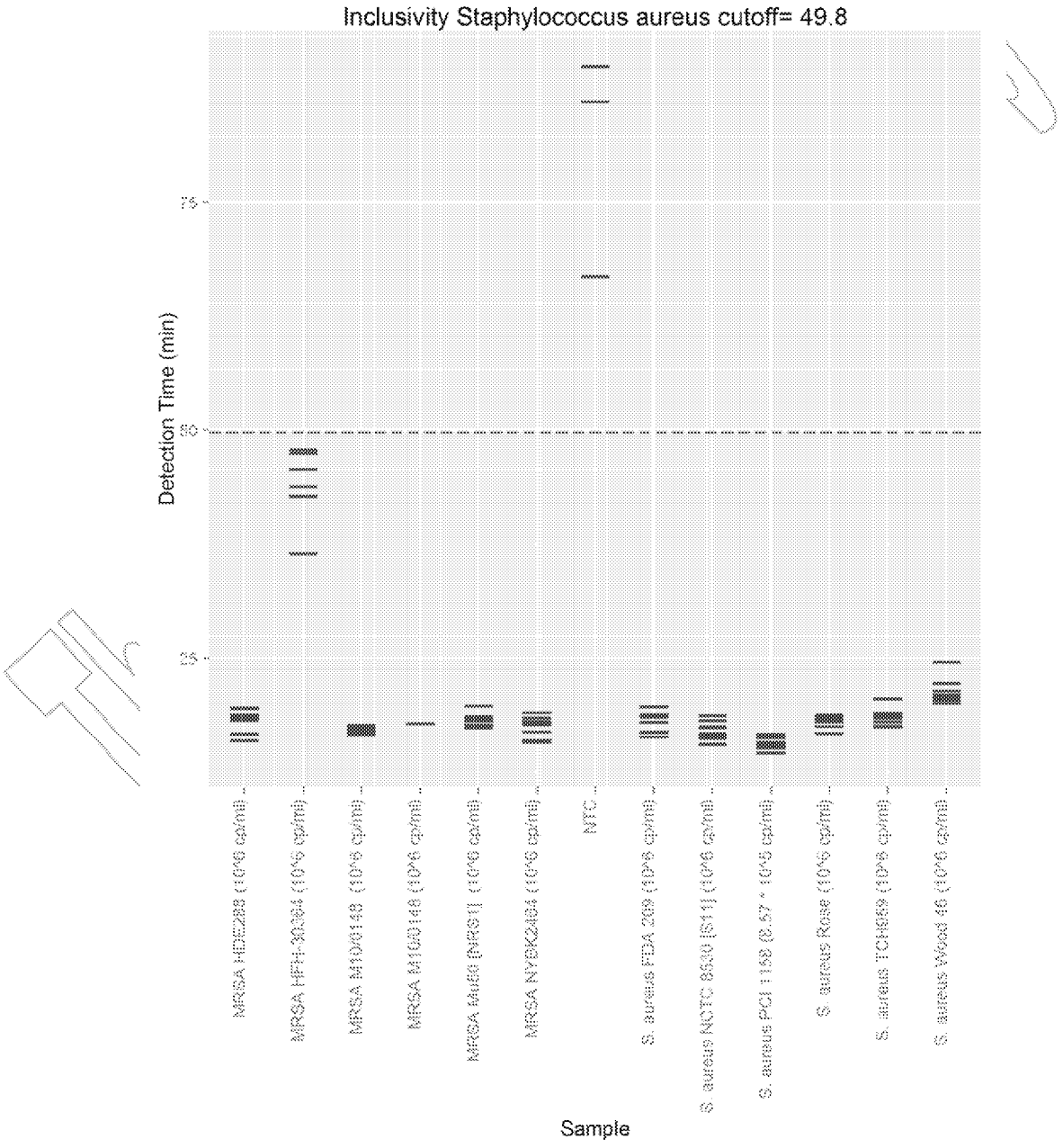
The assay for *Staphylococcus aureus* was found to meet all criteria for precision, carryover, inclusivity, cross-reactivity, specificity, and resistance to interfering substances. Positive and negative clinical samples were tested and compared to a predicate method. The *Staphylococcus aureus* assay specifically and reliably detects *Staphylococcus aureus*. The assay limit of detection is 2,142 CFU/ml with a recommended assay duration of 50 minutes as determined by ROC analysis.

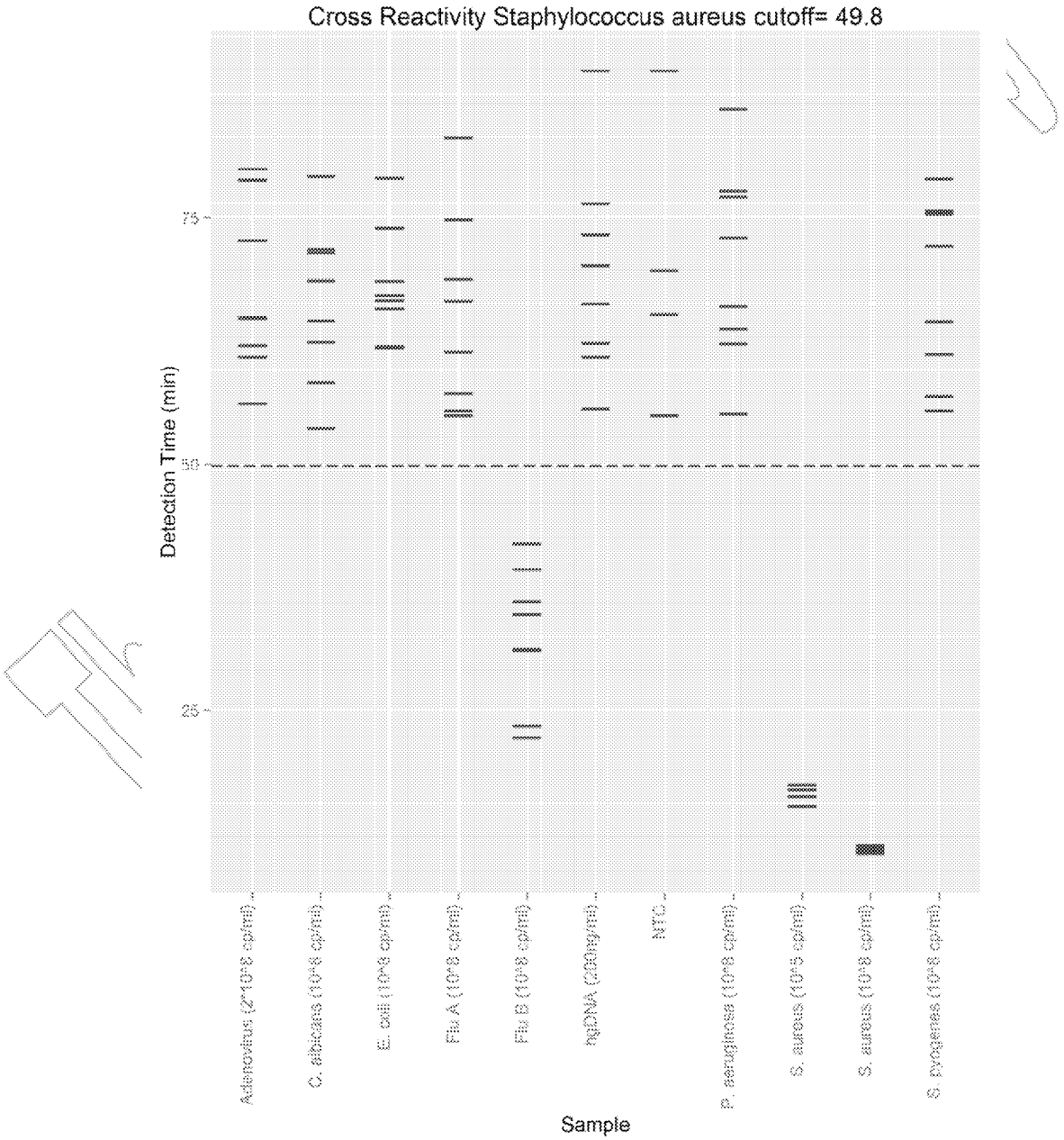
13) APPENDIX

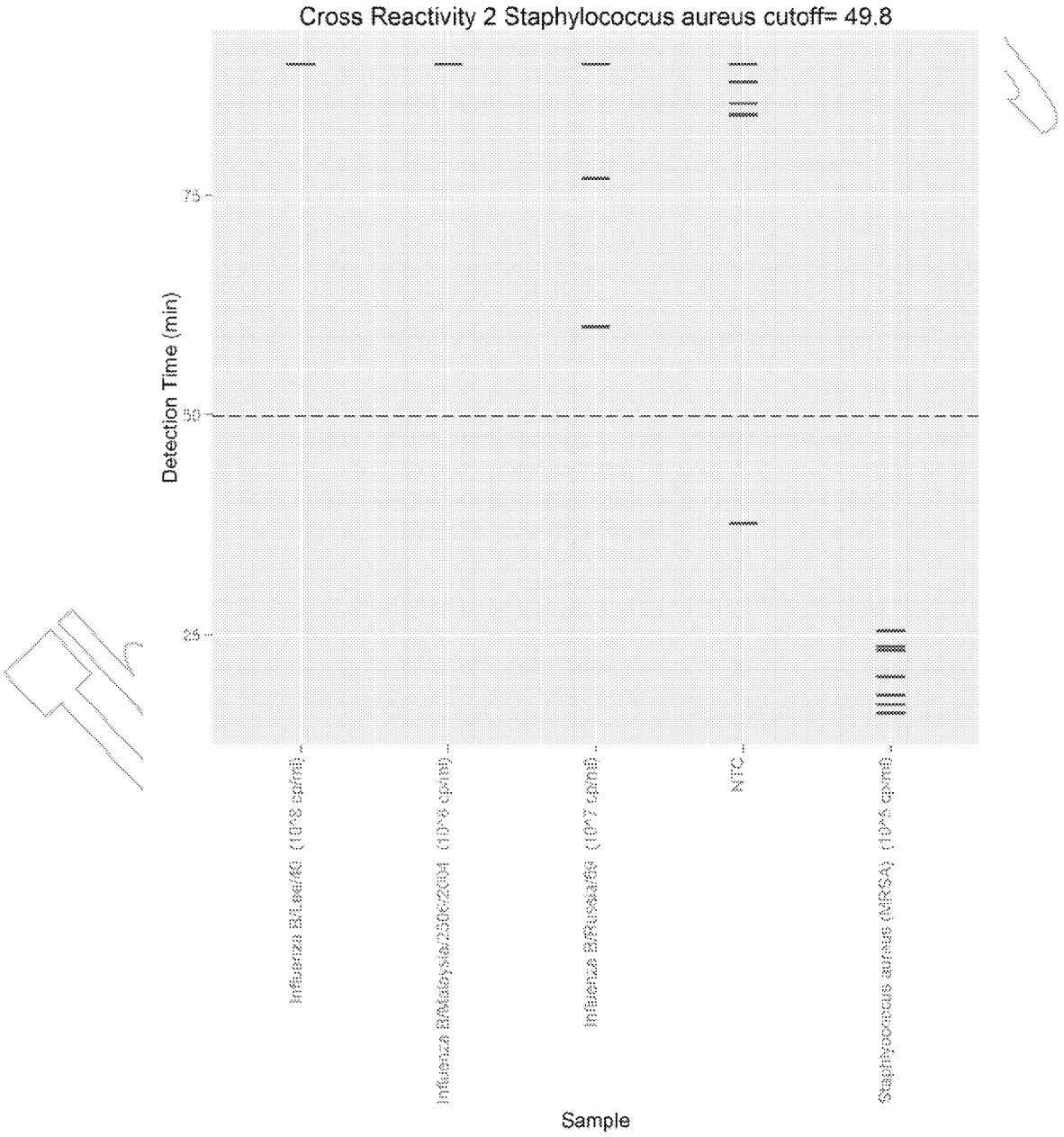


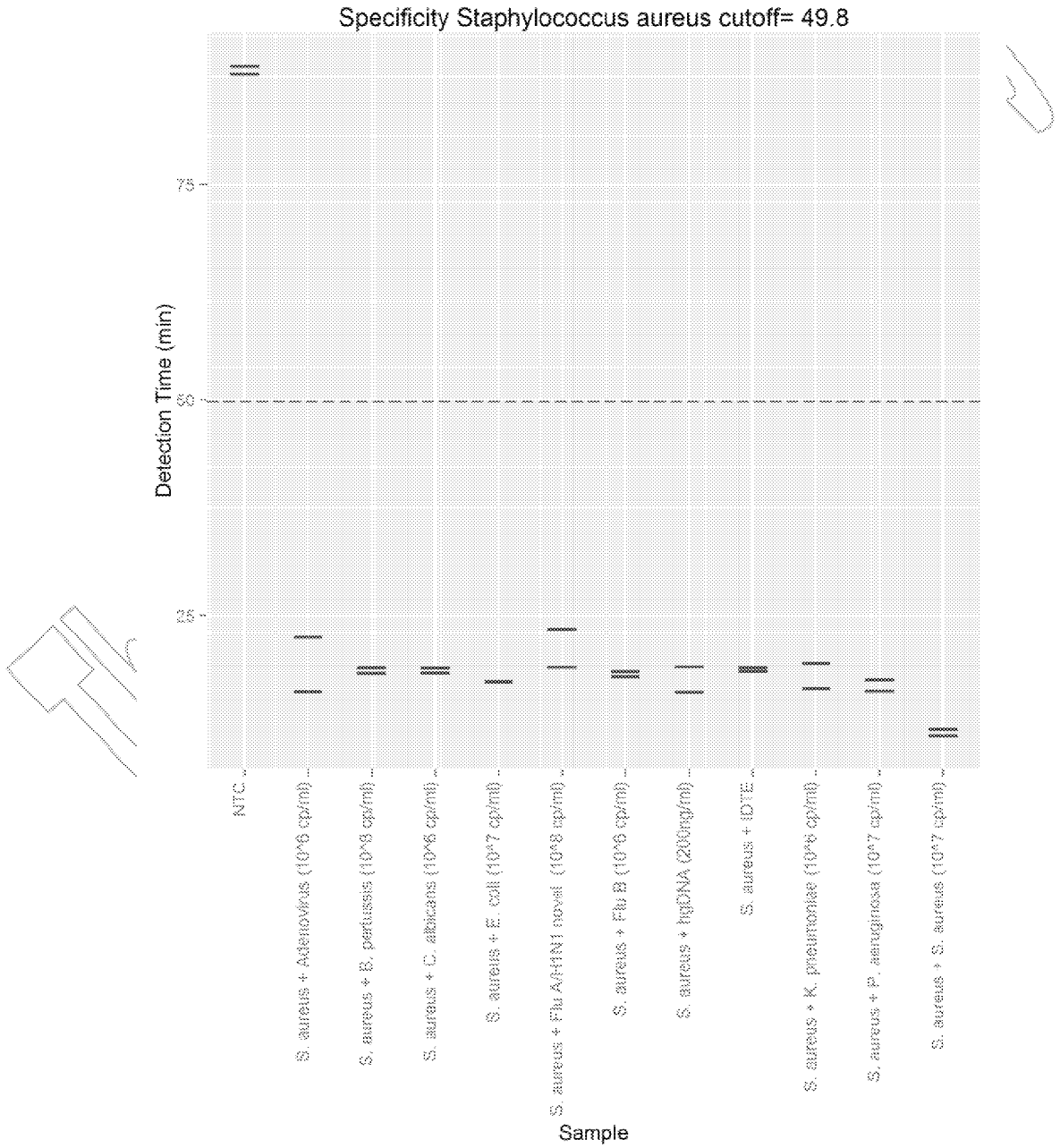


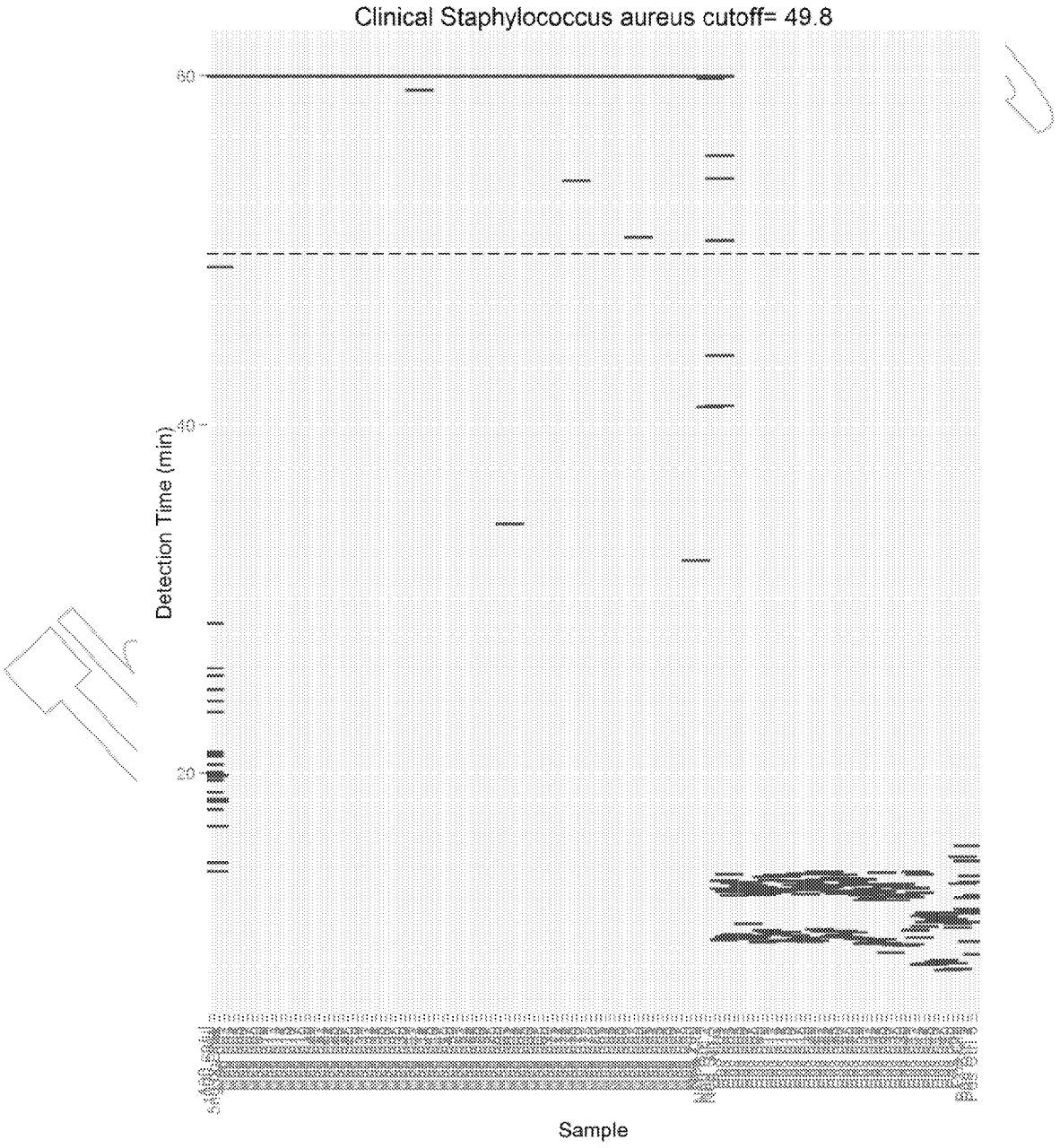












Clinical Samples TNAA: Treatment	NumPositive	Total	Percent
100 cp/ul	16	16	100
1000 cp/ul	6	6	100
5ng hgDNA	1	16	6
Neg 001	0	2	0
Neg 002	0	2	0
Neg 003	0	2	0
Neg 004	0	2	0
Neg 005	0	2	0
Neg 006	0	2	0
Neg 007	0	2	0
Neg 008	0	2	0
Neg 009	0	2	0
Neg 010	0	2	0
Neg 011	0	2	0
Neg 012	0	2	0
Neg 013	0	2	0
Neg 014	0	2	0
Neg 015	0	2	0
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Neg 027	0	2	0

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Neg 089	0	2	0

Neg 090	0	2	0
Neg 091	0	2	0
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Neg 094	0	2	0
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Neg 096	0	2	0
Neg 097	0	2	0
Neg 098	0	2	0
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STREPTOCOCCUS PNEUMONIAE

TNAA LDT Validation Report

Limit of Detection = 10 cp/uL

Rate of Detection = 100 cp/uL in 19 minutes

Katie Sullivan-Bibee

THERANOS, INC.

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Table of Contents

Streptococcus pneumoniae

1) PURPOSE

This report includes relevant information about the target organism, a detailed description of the primers and selected targets designed for the detection of this organism, a summary of the validation assay performance, and recommendations for future assay execution.

2) BACKGROUND

Streptococcus pneumoniae, a Gram-positive, alpha-hemolytic, aerotolerant anaerobic member of the genus *Streptococcus*, is a significant human pathogenic bacterium. It resides asymptomatically in the nasopharynx of healthy carriers. However, in susceptible individuals, such as the elderly, the immunocompromised, and children, the pathogen can spread to other locations and cause disease. In children and the elderly, *S. pneumoniae* is the main cause of community acquired pneumonia and meningitis, as well as the main cause of septicemia in HIV-infected individuals.

Despite its name, *S. pneumoniae* causes many types of pneumococcal infections other than pneumonia. These invasive pneumococcal diseases include acute sinusitis, otitis media, conjunctivitis, meningitis, bacteremia, sepsis, osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, cellulitis, and brain abscess. *S. pneumoniae* is one of the most common causes of bacterial meningitis in adults and young adults, along with *Neisseria meningitidis*, and *S. pneumoniae* is the leading cause of bacterial meningitis in adults in the USA. It is also one of the top two isolates found in ear infections, otitis media. Pneumococcal pneumonia is more common in the very young and the very old.

This report describes the nucleic acid amplification test developed to *detect Streptococcus pneumoniae*. The target gene, lytA, was chosen because alignment studies predict that cross-target amplification with *S. pseudopneumoniae* or *S. mitis* is very unlikely.

3) SUMMARY OF PERFORMANCE DATA

Theranos developed a Theranos Nucleic Acid Amplification (TNAA) assay specific for *Streptococcus pneumoniae*. The Nucleic Acid Amplification reactions contained 1x Nucleic Acid Amplification buffer (20 mM Tris Acetate, pH 7.9, 50 mM Potassium Acetate, 10 mM Magnesium Acetate and 1mM DTT), 0.08% Tween, 0.8 M betaine, 1.4 mM dNTPs, 2 uM Syto59, 0.8 uM RLX2255 primer and 0.8 uM RLX2256 primer, 20 units Bst polymerase, and template at the noted concentration. The reactions were run at

56ºC for 60 minutes. Summarized data will follow below while detailed experimental data can be found in the appendix. Primer sequences are:

Streptococcus pneumoniae	RLX2255	CAAGTACATCTTTAGCGTCTA
	RLX2256	TGTACTTGCATGAAGACAGGCTG

4) LIMIT OF DETECTION

The purpose of this study is to determine the limit of detection (LOD) for the Theranos TNAA assay. The LOD_{95} is the bacterial titer at which >95% of known positive samples test positive using the TNAA assay. Statistically justified cut-off times for making positive/negative calls were determined for each target empirically. A set of experiments, repeated over four days, were conducted that included eight replicates each of three target dilutions (LoD, 10X LoD, and 100X LoD), as well as 8 NTCs, using the target primers for amplification. These data were then processed using a receiver-operator character (ROC) analysis, and the best threshold detection time for distinguishing positives and negatives determined using the Youden test statistic as implemented by the R package, pROC.

The assay reliably detected 479 CFU/ml of *Streptococcus pneumoniae* in about 29.8 minutes, as shown below. The 29.8 minute assay cut-off time was determined by ROC analysis. The assay was performed six times. Reactions with and without template (NTCs or Non-Templated Controls) were run in eight replicates each.

LOD	Samples	NumPositive	Total	Percent
100X LOD	47,930 CFU/ml	48	48	100
10X LOD	4,793 CFU/ml	48	48	100
1X LOD	479 CFU/ml	48	48	100
	NTC	0	48	0

5) REPRODUCIBILITY/PRECISION

The purpose of this experiment is to determine the precision of the assay, percent positive and negative at three detection limits: high-negative (0.1X LOD=48 CFU/ml), low-positive (LOD=479 CFU/ml), and high-positive (3X LOD=1,438 CFU/ml). The assay was performed six times. Reactions with and without template (NTCs or Non-templated Controls) were run in eight replicates each.

Precision LOD	Samples	NumPositive	Total	Percent
3X LOD	1,438 CFU/ml	48	48	100
1X LOD	479 CFU/ml	48	48	100
0.1X LOD	48 CFU/ml	35	48	73
	NTC	0	48	0

6) CARRYOVER

The purpose of this experiment is to determine the potential for carryover of positive samples adjacent to negative reactions. The nucleic acid template is prepared from high-positive (100X LOD = 47,930 CFU/ml), low-positive (1X LOD=479 CFU/ml), and non-templated controls (NTCs) which are arrayed in alternating rows of eight replicates each. There are two rows of high-positive reactions, two rows of low-positive reactions, and six rows of NTCs. The assay was performed once, with no carryover of positive samples to negative reactions.

	1	2	3	4	5	6	7	8	9	10	11	12
		100X LOD	NTC	100X LOD	NTC	LOD	NTC	LOD	NTC	NTC	NTC	
A	empty	+	-	+	-	+	-	+	-	-	-	empty
B		+	-	+	-	+	-	+	-	-	-	
C		+	-	+	-	+	-	+	-	-	-	
D		+	-	+	-	+	-	+	-	-	-	
E		+	-	+	-	+	-	+	-	-	-	
F		+	-	+	-	+	-	+	-	-	-	
G		+	-	+	-	+	-	+	-	-	-	
H		+	-	+	-	+	-	+	-	-	-	

Carryover Samples	NumPositive	Total	Percent
47,930 CFU/ml	16	16	100
479 CFU/ml	16	16	100
NTC	0	48	0

7) INCLUSIVITY/EXCLUSIVITY

The assay for *Streptococcus pneumoniae* was tested to validate inclusivity and exclusivity. Various strains of *Streptococcus pneumoniae* were tested to verify inclusive assay performance. The assay was also tested against different species of *Streptococcus* to verify exclusivity between close relatives.

All inclusive strains of *S. pneumoniae* were tested in seven replicates each, while there were six total replicates for NTC reactions and six human genomic DNA reactions. The TNAA method successfully detected all inclusive *S. pneumoniae* strains.

All exclusive *Streptococcus* strains were tested in eight replicates each, with eight positive control reactions and eight negative NTC replicates. The TNAA method excluded all closely related *Streptococcus* strains.

The following tables summarize the inclusivity and exclusivity pathogens to be evaluated for the *Streptococcus pneumoniae* assay.

Inclusivity Samples	NumPositive	Total	Percent
hgDNA (200ng/ml)	0	6	0
NTC	0	6	0
S. pneumoniae Colombia 5-19 (penS) (10^5 cp/ml)	7	7	100
S. pneumoniae England 14-9 (penS) (10^5 cp/ml)	7	7	100
S. pneumoniae Greece 6B-22 (penS) (10^5 cp/ml)	7	7	100
S. pneumoniae Hungary 19A-6 (penR) (10^5 cp/ml)	7	7	100
S. pneumoniae North Carolina 6A-23 (penR) (10^5 cp/ml)	7	7	100
S. pneumoniae Poland 23F-16 (penR) (10^5 cp/ml)	7	7	100
S. pneumoniae Portugal 19F-21 (penS) (10^5 cp/ml)	7	7	100
S. pneumoniae S. Africa 6B-8 (penS) (10^5 cp/ml)	7	7	100
S. pneumoniae Spain 23F-1 (penR) (10^5 cp/ml)	7	7	100
S. pneumoniae Sweden 15A-25 (penS) (10^5 cp/ml)	7	7	100
S. pneumoniae Taiwan 19F-14 (penR) (10^5 cp/ml)	7	7	100
S. pneumoniae Taiwan 23F-15 (penS) (10^5 cp/ml)	7	7	100

Exclusivity Samples	NumPositive	Total	Percent
NTC	0	8	0
S. agalactiae (10^7 cp/ml)	0	8	0
S. mutans (10^7 cp/ml)	0	8	0
S. pneumoniae (10^7 cp/ml)	8	8	100
S. pyogenes (10^7 cp/ml)	0	8	0
S. salivarus (2.08*10^6 cp/ml)	0	8	0

8) CROSS-REACTIVITY

The cross-reactivity of the assay was tested against a panel of organisms which may also be present in collected *Streptococcus pneumoniae* clinical samples. These organisms must be tested to ascertain that no false positives will be due to contamination from the off-target genomic material at clinically relevant viral or bacterial loads. The table below summarizes the genomic material tested and the results obtained. All potentially cross-reactive organisms were tested in replicates of eight and NTCs and the positive control were tested replicates of four or eight. The TNAA assay was verified to not cross-react with any non-target organisms.

Cross Reactivity 1 Samples	NumPositive	Total	Percent
Adenovirus 4 (10^6 cp/ml)	0	8	0
B. pertussis (10^8 cp/ml)	0	8	0
C. albicans (10^6 cp/ml)	0	8	0
E. coli (10^8 cp/ml)	0	8	0
Flu A/H1N1 (10^8 cp/ml)	0	8	0
Flu B/Russia/69 (10^8 cp/ml)	0	8	0
hgDNA (200ng/ml)	0	8	0
K. pneumoniae (10^6 cp/ml)	0	8	0
NTC	0	4	0
P. aeruginosa (10^7 cp/ml)	0	8	0
S. aureus MSSA (10^7 cp/ml)	0	8	0
S. pneumoniae (10^5 cp/ml)	4	4	100
S. pyogenes (10^7 cp/ml)	0	8	0

Cross-Reactivity 2 Samples	NumPositive	Total	Percent
A. baumannii (10^7 cp/ml)	0	8	0
E. aerogenes (10^7 cp/ml)	0	8	0
E. cloacae (10^7 cp/ml)	0	8	0
E. coli (10^7 cp/ml)	0	8	0
K. oxytoca (10^7 cp/ml)	0	8	0
K. pneumoniae (10^7 cp/ml)	0	8	0
N. meningitidis (10^7 cp/ml)	0	8	0
NTC	0	4	0
P. aeruginosa (10^7 cp/ml)	0	8	0
S. agalactiae (10^7 cp/ml)	0	8	0
S. marcescens (10^7 cp/ml)	0	8	0
S. pneumoniae (10^5 cp/ml)	3	4	75
S. pneumoniae (10^7 cp/ml)	8	8	100

9) SPECIFICITY

The specificity of the assay was tested against a panel of organisms which may be present as potential contaminants in *Streptococcus pneumoniae* samples and whose genomic material may be carried though the sample preparation protocol. These organisms must be tested to verify that assay performance is not significantly impacted by the presence of off-target genomic material combined with *Streptococcus pneumoniae* at clinically relevant loads. The table below summarizes the genomic material tested and the results obtained. All organisms combined with *S. pneumoniae* were tested in replicates of two. The positive control and NTCs were also tested in two replicates.

The results below show that the assay is specific to *Streptococcus pneumoniae* and spiking in other organisms that may be found in the same sample type does not affect assay performance. The assay tested S. pneumoniae target at 10X LOD (4,793 CFU/ml) combined with the off-target organism. The off-target nucleic acid concentration reflects expected median viral/bacterial loads in clinical specimens.

Specificity 1 Samples	NumPositive	Total	Percent
NTC	0	2	0
S. pneumoniae + Adenovirus 4 (10^6 cp/ml)	2	2	100
S. pneumoniae + B. pertussis (10^8 cp/ml)	2	2	100
S. pneumoniae + C. albicans (10^6 cp/ml)	2	2	100
S. pneumoniae + E. coli (5*10^7 cp/ml)	2	2	100
S. pneumoniae + Flu A/H1N1 novel (10^8 cp/ml)	2	2	100
S. pneumoniae + Flu B/Mass/3/66 (10^8 cp/ml)	2	2	100
S. pneumoniae + hgDNA (200ng/ml)	2	2	100
S. pneumoniae + IDTE	2	2	100
S. pneumoniae + K. pneumoniae (10^6 cp/ml)	2	2	100
S. pneumoniae + P. aeruginosa (10^7 cp/ml)	2	2	100
S. pneumoniae + S. aureus MSSA (10^7 cp/ml)	2	2	100

Specificity 2 Samples	NumPositive	Total	Percent
NTC	0	2	0
S. pneumoniae + A. baumannii (10^7 cp/ml)	2	2	100
S. pneumoniae + E. cloacae (10^7 cp/ml)	2	2	100
S. pneumoniae + E. coli (10^7 cp/ml)	2	2	100
S. pneumoniae + IDTE	2	2	100
S. pneumoniae + K. oxytoca (10^7 cp/ml)	2	2	100
S. pneumoniae + K. pneumoniae (10^7 cp/ml)	2	2	100
S. pneumoniae + N. meningitidis (10^7 cp/ml)	2	2	100
S. pneumoniae + P. aeruginosa (10^7 cp/ml)	2	2	100
S. pneumoniae + S. agalactiae (10^7 cp/ml)	2	2	100
S. pneumoniae + S. marcescens (10^7 cp/ml)	2	2	100
S. pneumoniae + S. pneumoniae (10^7 cp/ml)	2	2	100

10) INTERFERING SUBSTANCES

The following interfering substances have been evaluated to have no significant effect on the performance of the TNAA assay. The interfering substances were added to *Streptococcus pneumoniae* sample prep at both 10% and 0.1% of the total reaction by volume.

Interfering Substances: Endogenous and Exogenous.

Endogenous	Exogenous
Human blood	Bactroban nasal
Mucin	Flonase
Human genomic DNA	Nasonex
	Astelin
	Anefrin Nasal Spray
	Neosynphrine
	VapoRub cough suppressant
	ZiCam Allergy Relief nasal gel
	Mucin
	UTM

11) METHOD COMPARISON ON CLINICAL SAMPLES

The purpose of this study is to estimate the sensitivity and specificity of the TNAA assay using qPCR as the comparator (predicate method).

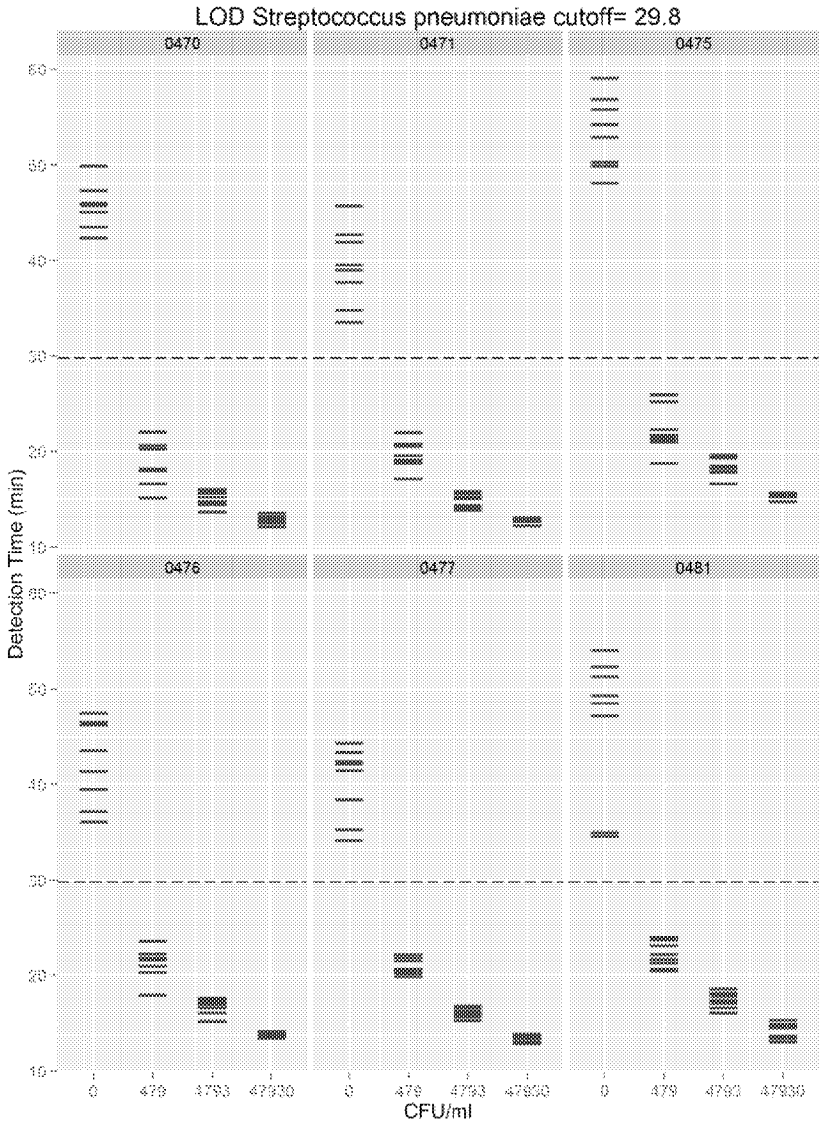
The following clinical samples were tested: 100 positive samples and 100 negative samples obtained from Fostering Tech Medical. Both nasal swab and pharyngeal exudate samples were taken from a range of individuals of both sexes and various ages.

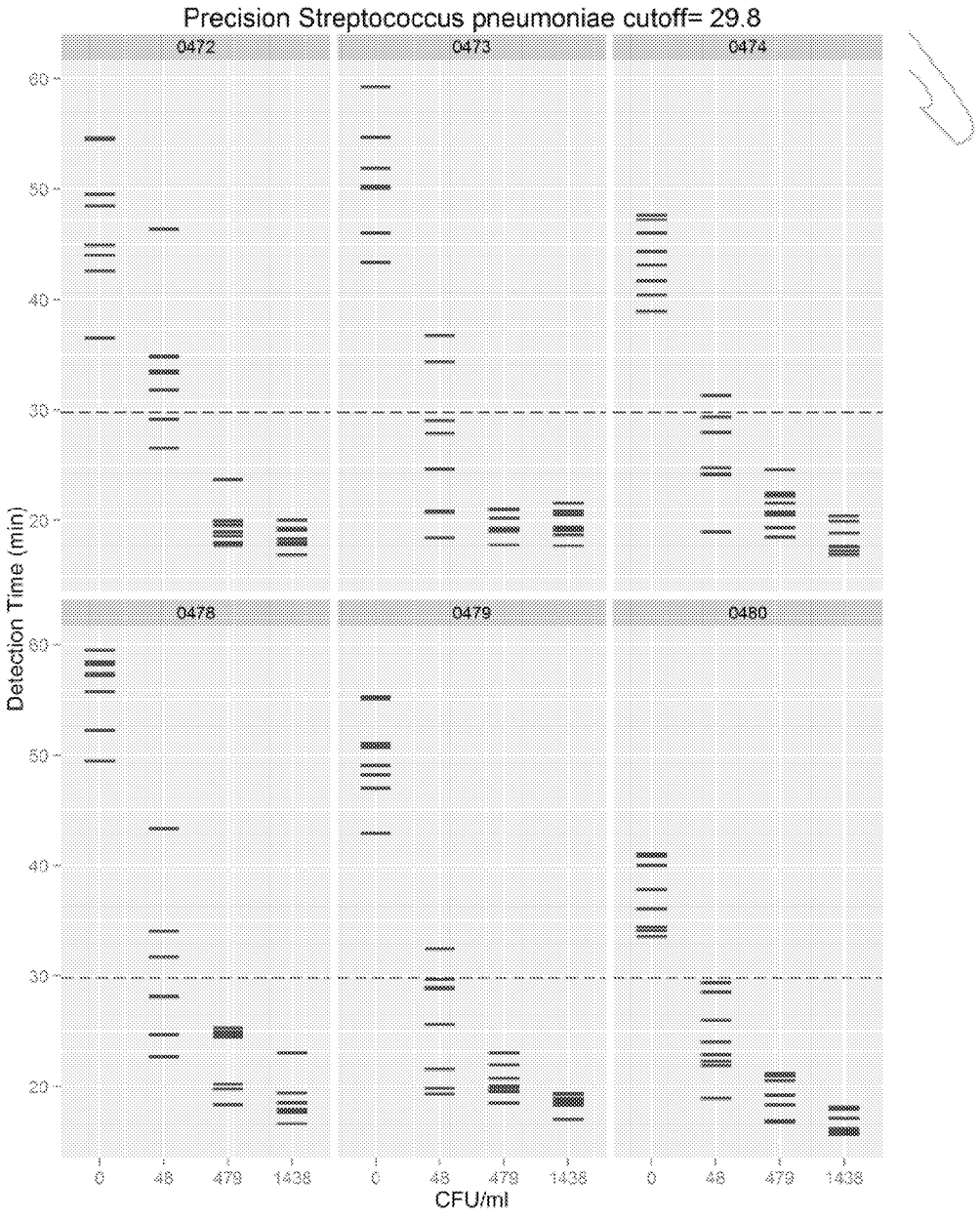
	Clinical Positive (qPCR)	Clinical Positive (TNAA)	Clinical Negative (qPCR)	Clinical Negative (TNAA)
NumPositive	100	100	0	0
Total	100	100	100	100
Percent	100	100	0	0

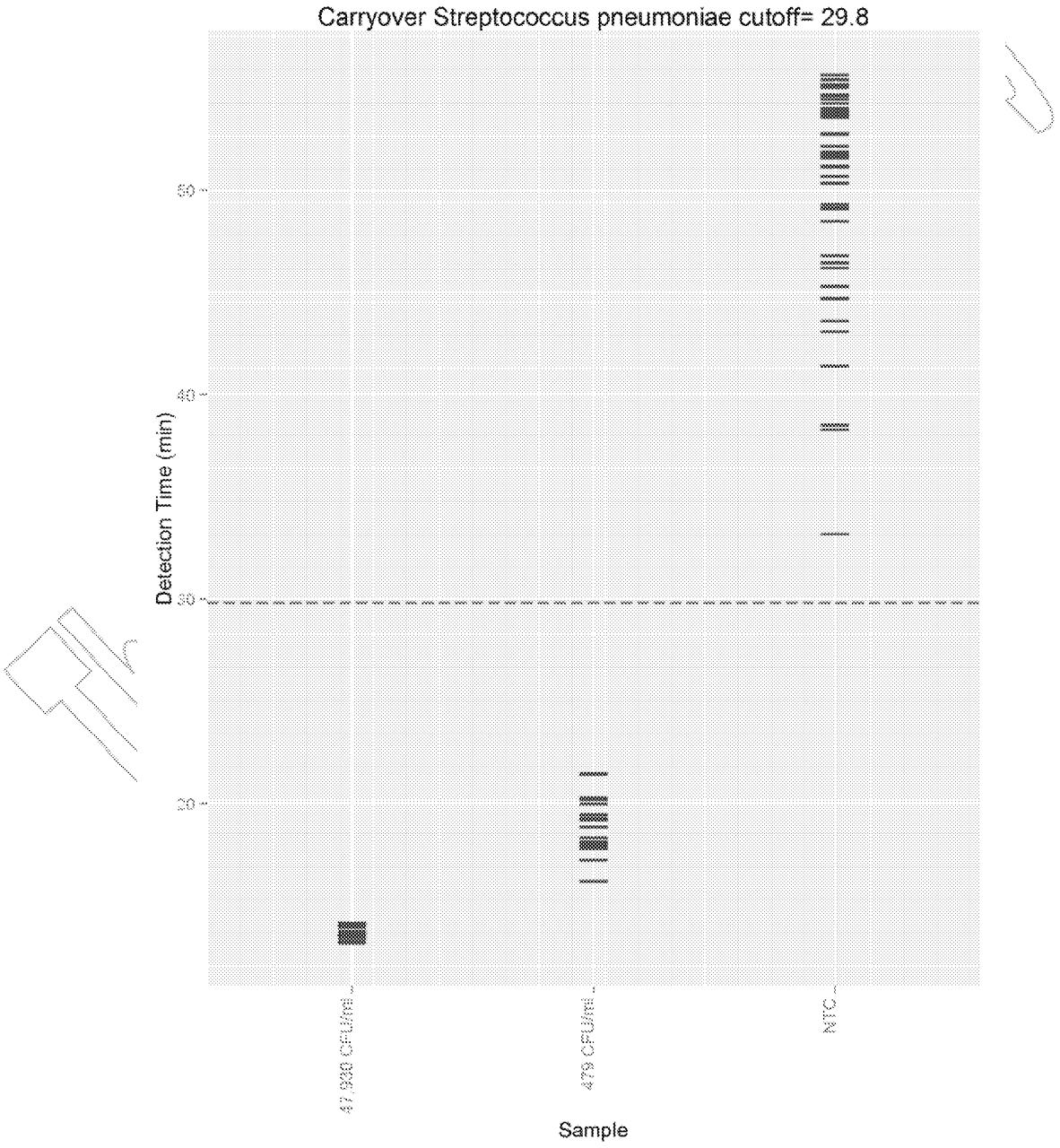
12) FINAL RECOMMENDATIONS

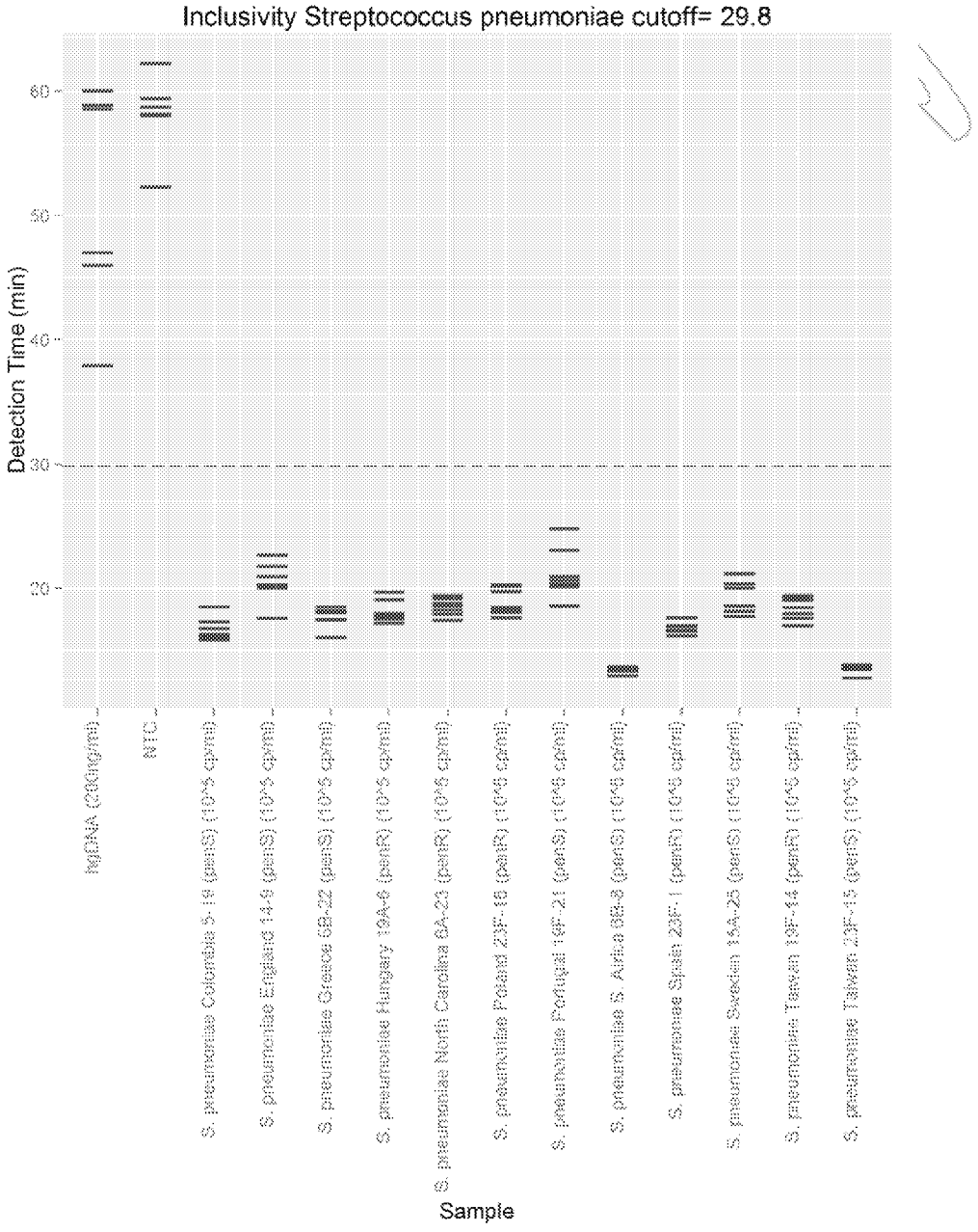
The assay for *Streptococcus pneumoniae* was found to meet all criteria for precision, carryover, inclusivity, exclusivity, cross-reactivity, specificity, and resistance to interfering substances. Positive and negative clinical samples were tested and compared to a predicate method. The *Streptococcus pneumoniae* assay specifically and reliably detects *Streptococcus pneumoniae*. The assay limit of detection is 479 CFU/ml with a recommended assay duration of 30 minutes as determined by ROC analysis.

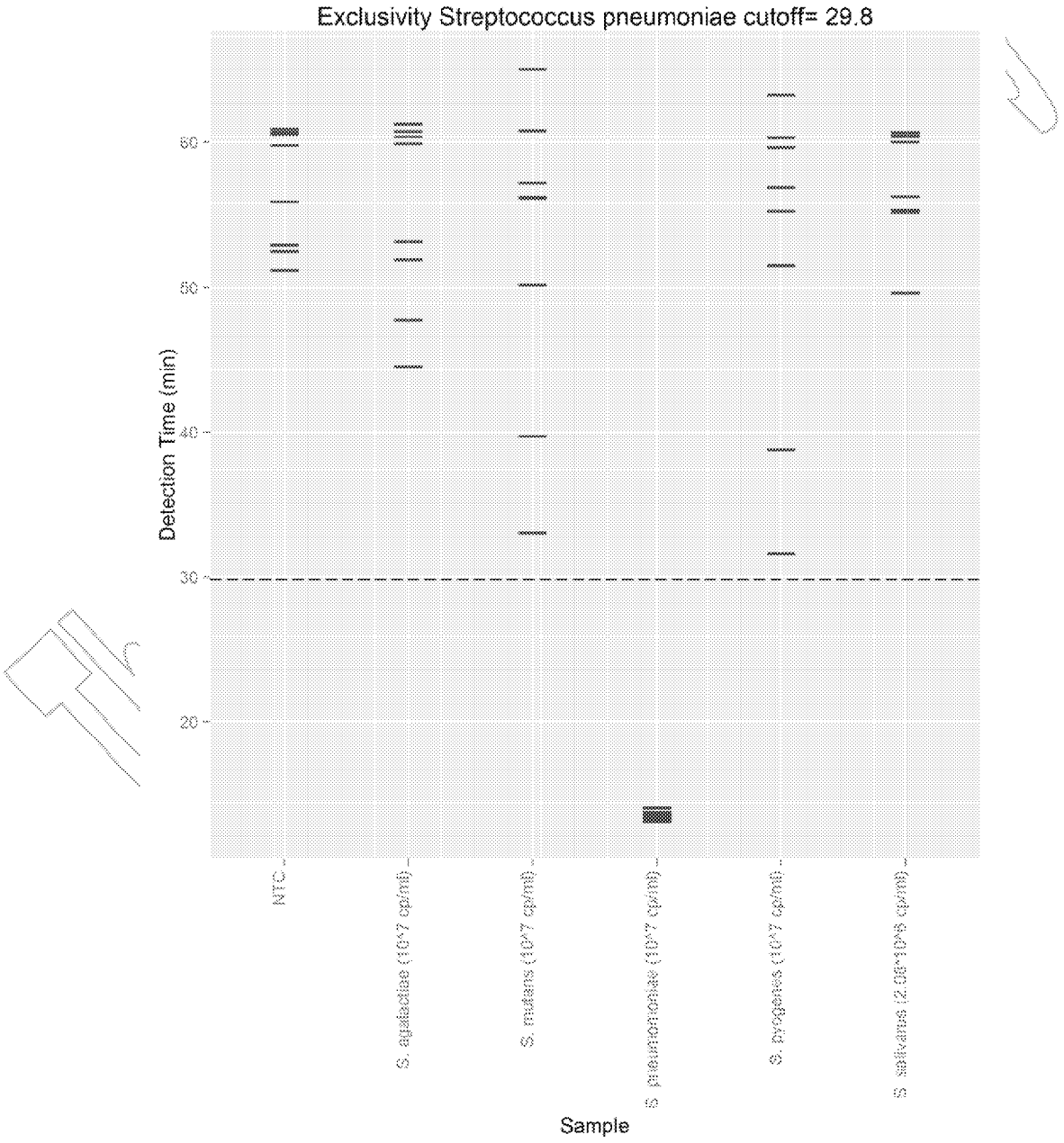
13) APPENDIX

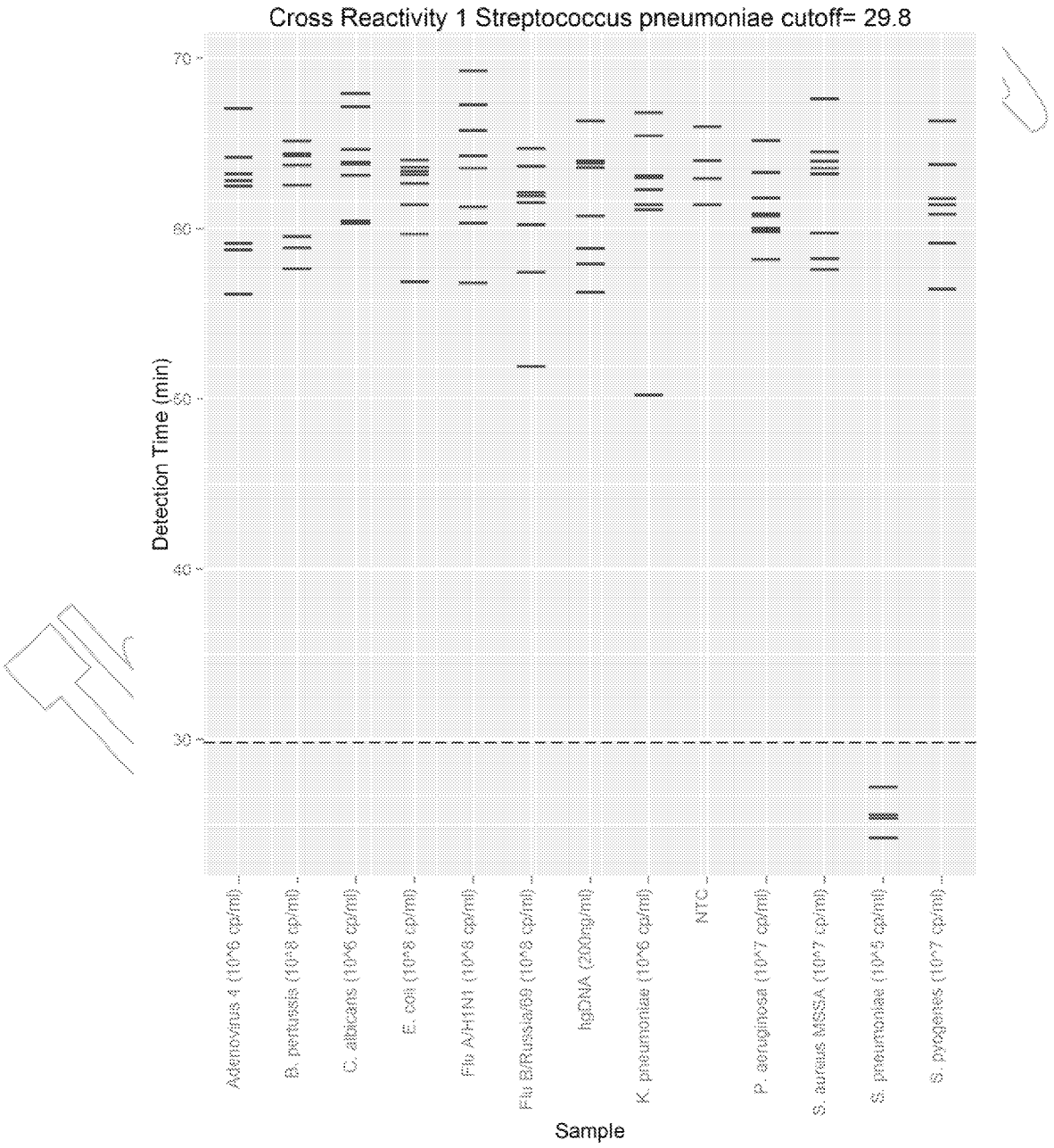


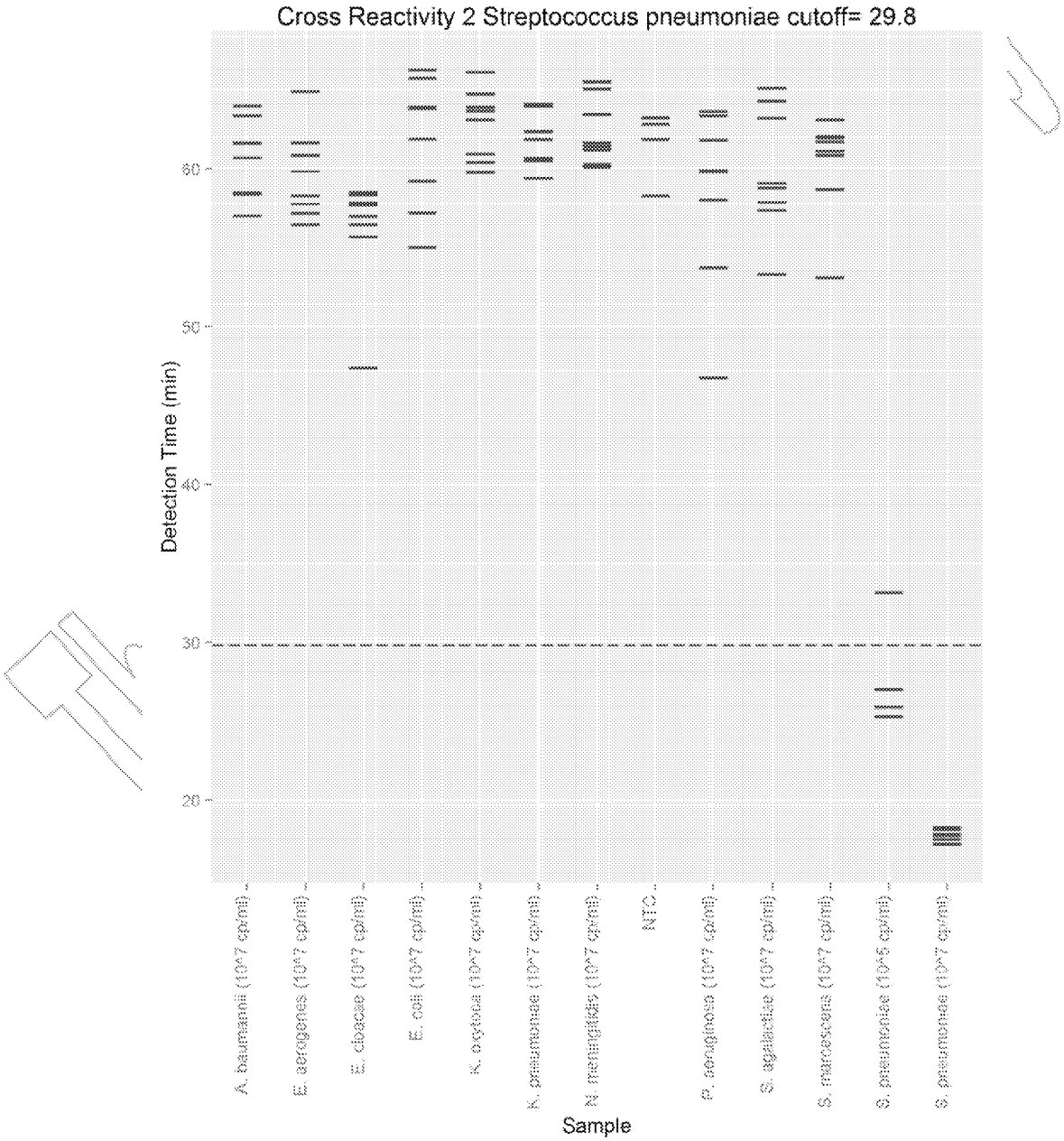


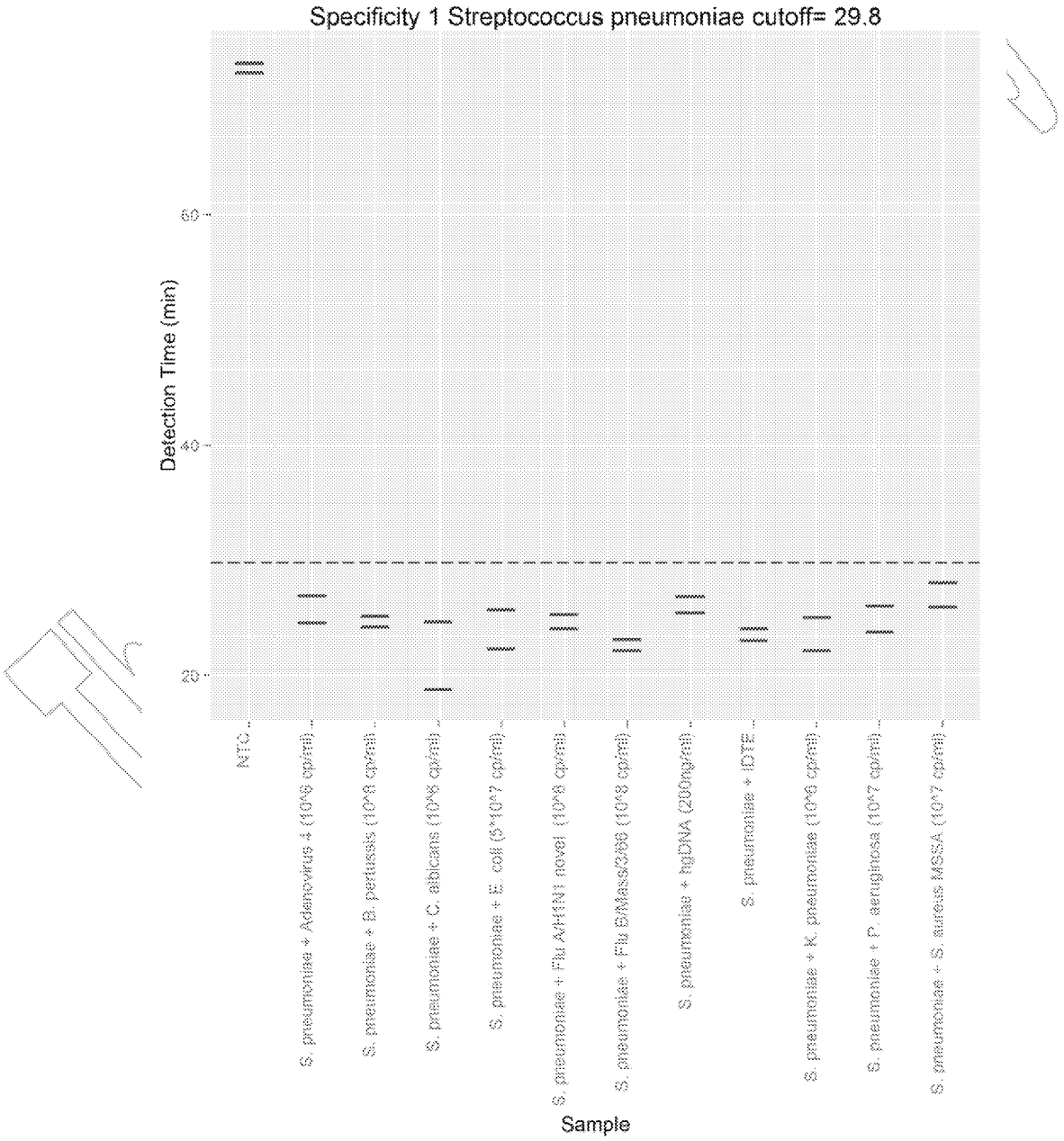


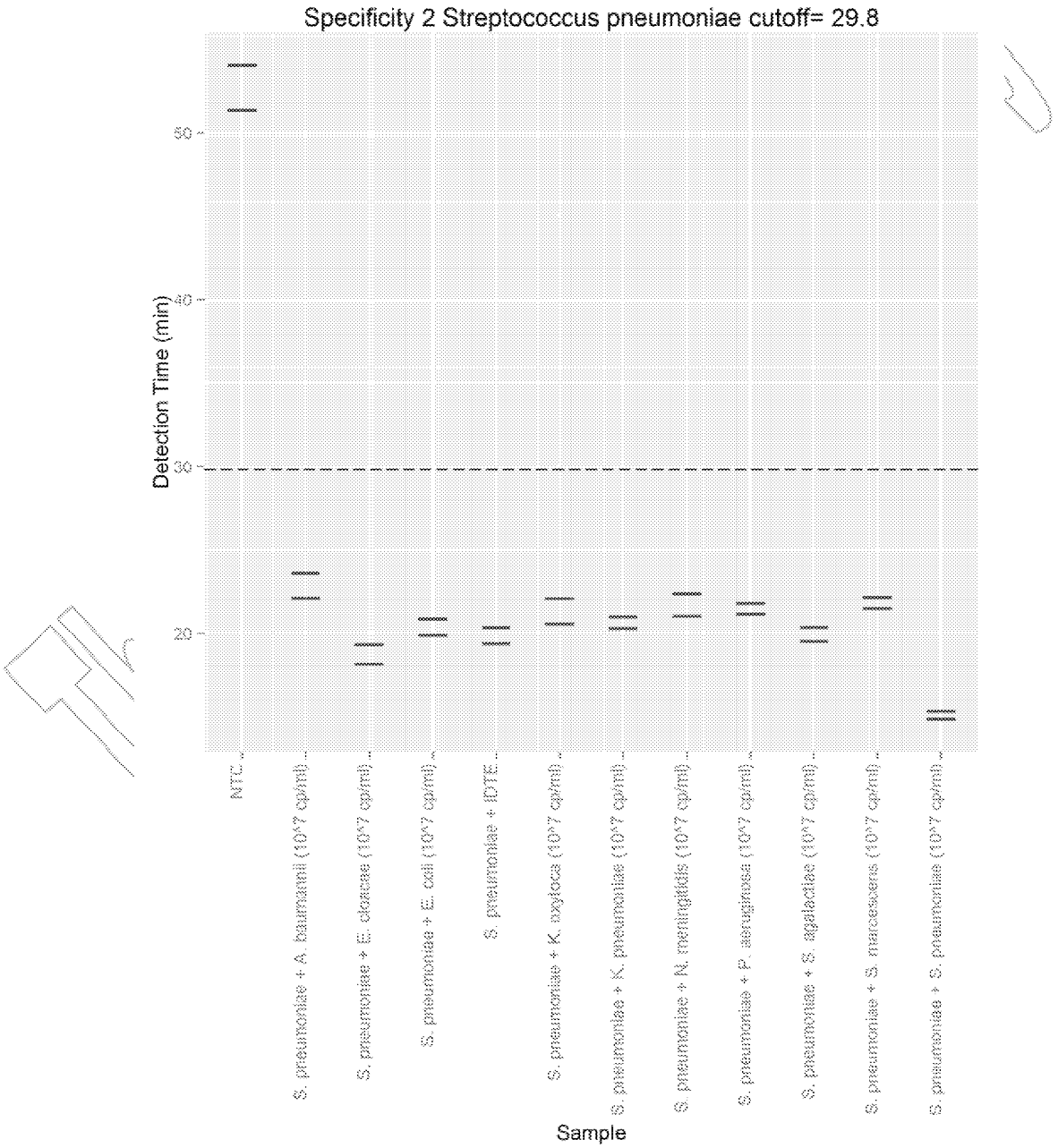


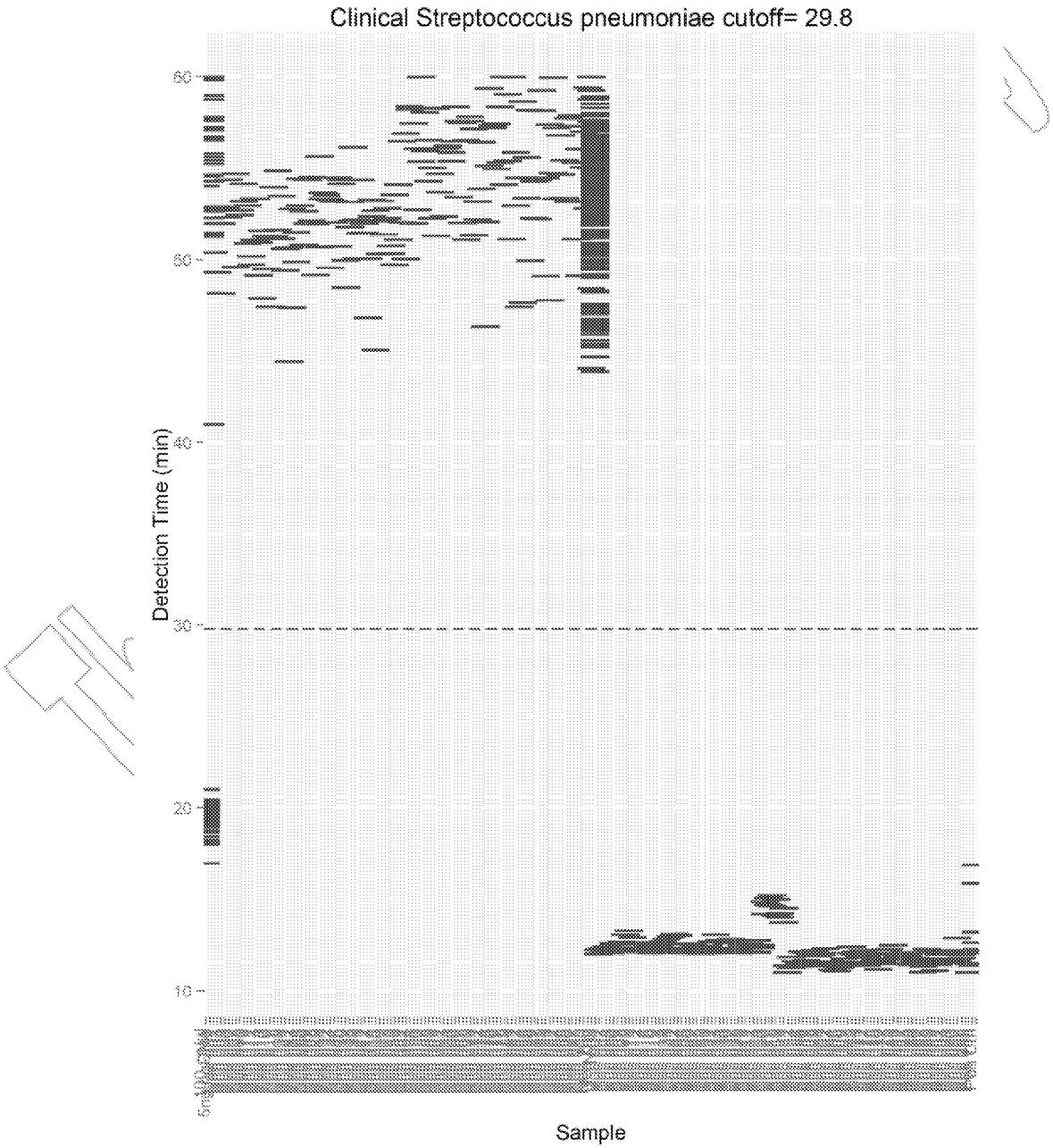












Clinical Samples TNAA Treatment	NumPositive	Total	Percent
100 cp/ul	30	34	88
5ng hgDNA	0	30	0
Neg 001	0	2	0
Neg 002	0	2	0
Neg 003	0	2	0
Neg 004	0	2	0
Neg 005	0	2	0
Neg 006	0	2	0
Neg 007	0	2	0
Neg 008	0	2	0
Neg 009	0	2	0
Neg 010	0	2	0
Neg 011	0	2	0
Neg 012	0	2	0
Neg 013	0	2	0
Neg 014	0	2	0
Neg 015	0	2	0
Neg 016	0	2	0
Neg 017	0	2	0
Neg 018	0	2	0
Neg 019	0	2	0
Neg 020	0	2	0
Neg 021	0	2	0
Neg 022	0	2	0
Neg 023	0	2	0
Neg 024	0	2	0
Neg 025	0	2	0
Neg 026	0	2	0
Neg 027	0	2	0
Neg 028	0	2	0

Neg 029	0	2	0
Neg 030	0	2	0
Neg 031	0	2	0
Neg 032	0	2	0
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Neg 035	0	2	0
Neg 036	0	2	0
Neg 037	0	2	0
Neg 038	0	2	0
Neg 039	0	2	0
Neg 040	0	2	0
Neg 041	0	2	0
Neg 042	0	2	0
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Neg 090	0	2	0

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Neg 092	0	2	0
Neg 093	0	2	0
Neg 094	0	2	0
Neg 095	0	2	0
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Neg 097	0	2	0
Neg 098	0	2	0
Neg 099	0	2	0
Neg 100	0	2	0
Neg Ctrl	0	8	0
NTC	0	232	0
Pos 001	2	2	100
Pos 002	2	2	100
Pos 003	2	2	100
Pos 004	2	2	100
Pos 005	2	2	100
Pos 006	2	2	100
Pos 007	2	2	100
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Pos 009	2	2	100
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Pos 011	2	2	100
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Pos 094	2	2	100
Pos 095	2	2	100
Pos 096	2	2	100
Pos 097	2	2	100
Pos 098	2	2	100
Pos 099	2	2	100
Pos 100	2	2	100
Pos Ctrl	8	8	100

STREPTOCOCCUS AGALACTIAE

TNAA LDT Validation Report

Limit of Detection = 10 cp/uL

Rate of Detection = 100 cp/uL in 24 minutes

Katie Sullivan-Bibee

THERANOS, INC.

Author(s):

Signature:	Date:
Name: Katie Sullivan-Bibee	Title: Research Associate

Reviewer(s)

Signature:	Date:
Name: Pranav Patel, PhD.	Title: Team Lead

Signature:	Date:
Name: Daniel Young, Ph.D.	Title: Vice President

Approver(s):

Signature:	Date:
Name: Adam Rosendorff, M.D	Title: Laboratory Director

Table of Contents

Streptococcus agalactiae

1) PURPOSE

This report includes relevant information about the target organism, a detailed description of the primers and selected targets designed for the detection of this organism, a summary of the validation assay performance, and recommendations for future assay execution.

2) BACKGROUND

Streptococcus is a genus of spherical Gram-positive bacteria that is responsible for a wide range of clinical diseases such as pharyngitis, soft tissue infections, meningitis and bacteremia. Streptococci are classified based on their hemolytic properties when grown on blood agar. Alpha-hemolytic species partially lyse surrounding blood cells and oxidize the iron in the hemoglobin, leaving a green-tinged ring around the bacterial colonies. Beta-hemolytic species lyse surrounding red blood cells, leaving a clear zone around the bacterial colonies. Beta-hemolytic streptococci are further serotyped based on the type of carbohydrates expressed on their cell wall and denoted by the Lancefield groups A through V. Alpha-hemolytic species such as *Streptococcus pneumonia* and the *Streptococcus viridans* group, as well as beta-hemolytic species such as Group A and Group B *Streptococcus*, are the most medically relevant due to their roles in human disease.

Streptococcus agalactiae (group B strep, aka GBS) is of concern for pregnant women as it can be passed to the baby during delivery, potentially leading to neonatal sepsis. Typically, a few weeks before delivery (>35 weeks gestation), a pregnant woman is tested for GBS and antibiotics administered in cases with a positive result. Approximately 15% of women are GBS carriers. The standard GBS screening involves a vaginal/rectal swab, followed by 18-24 hours of growth in enrichment broth prior to plate, hemagglutinization, or NAA test. Intrapartum testing (e.g. during preterm delivery) requires quicker turnaround than possible with a culture step, which calls for an NAA test.

The target gene for the assay was *cfb* (T132B1, AE009948.1|:2016657-2016956), found in *S. agalactiae* and not in other *Streptococcus* species. There is less than 2% sequence divergence across sequenced GBS isolates in the 300 bp of the target gene used for assay development. The GC content is only 31.7%.

3) SUMMARY OF PERFORMANCE DATA

Theranos developed a Theranos Nucleic Acid Amplification (TNAA) assay specific for *Streptococcus agalactiae*. The Nucleic Acid Amplification reactions contained 1x Nucleic Acid Amplification buffer (20 mM Tris Acetate, pH 7.9, 50 mM Potassium Acetate, 10 mM Magnesium Acetate and 1mM DTT), 0.08% Tween, 0.8 M betaine, 1.4 mM dNTPs, 2 uM Syto59, 0.8 uM RLX1453 primer and 0.8 uM RLX1454 primer, 20 units Bst polymerase, and template at the noted concentration. The reactions were run at 56ºC for 60 minutes. Summarized data will follow below while detailed experimental data can be found in the appendix. Primer sequences are:

Streptococcus agalactiae	RLX1453	AGCTTAGTTTGATATGGGATTTGGG
	RLX1454	AACTAAGCTTGAATCAACTGAAGCA

4) LIMIT OF DETECTION

The purpose of this study is to determine the limit of detection (LOD) for the Theranos TNAA assay. The LOD_{95} is the bacterial titer at which >95% of known positive samples test positive using the TNAA assay. Statistically justified cut-off times for making positive/negative calls were determined for each target empirically. A set of experiments, repeated over four days, were conducted that included eight replicates each of three target dilutions (LoD, 10X LoD, and 100X LoD), as well as 8 NTCs, using the target primers for amplification. These data were then processed using a receiver-operator character (ROC) analysis, and the best threshold detection time for distinguishing positives and negatives determined using the Youden test statistic as implemented by the R package, pROC.

The assay reliably detected 875 CFU/ml of *Streptococcus agalactiae* in about 50.2 minutes, as shown below. The 50.2 minute assay cut-off time was determined by ROC analysis. The assay was performed six times. Reactions with and without template (NTCs or Non-Templated Controls) were run in eight replicates each.

LOD	Samples	NumPositive	Total	Percent
100X LOD	87,500 CFU/ml	48	48	100
10X LOD	8,750 CFU/ml	48	48	100
1X LOD	875 CFU/ml	48	48	100
	NTC	0	48	0

5) REPRODUCIBILITY/PRECISION

The purpose of this experiment is to determine the precision of the assay, percent positive and negative at three detection limits: high-negative (0.1X LOD=88 CFU/ml), low-positive (LOD=875 CFU/ml), and high-positive (3X LOD=2,625 CFU/ml). The assay was performed six times. Reactions with and without template (NTCs or Non-templated Controls) were run in eight replicates each.

Precision LOD	Samples	NumPositive	Total	Percent
3X LOD	2,625 CFU/ml	48	48	100
1X LOD	875 CFU/ml	46	48	96
0.1X LOD	88 CFU/ml	29	48	60
	NTC	0	48	0

6) CARRYOVER

The purpose of this experiment is to determine the potential for carryover of positive samples adjacent to negative reactions. The nucleic acid template is prepared from high-positive (100X LOD = 87,500 CFU/ml), low-positive (1X LOD=875 CFU/ml), and non-templated controls (NTCs) which are arrayed in alternating rows of eight replicates each. There are two rows of high-positive reactions, two rows of low-positive reactions, and six rows of NTCs. The assay was performed once, with no carryover of positive samples to negative reactions.

		100X LOD	NTC	100X LOD	NTC	LOD	NTC	LOD	NTC	NTC	NTC	
	1	2	3	4	5	6	7	8	9	10	11	12
A	empty	+	-	+	-	+	-	+	-	-	-	empty
B		+	-	+	-	+	-	+	-	-	-	
C		+	-	+	-	+	-	+	-	-	-	
D		+	-	+	-	+	-	+	-	-	-	
E		+	-	+	-	+	-	+	-	-	-	
F		+	-	+	-	+	-	+	-	-	-	
G		+	-	+	-	+	-	+	-	-	-	
H		+	-	+	-	+	-	+	-	-	-	

Carryover Samples	NumPositive	Total	Percent
87,500 CFU/ml	16	16	100
875 CFU/ml	15	16	94
NTC	0	48	0

7) INCLUSIVITY/EXCLUSIVITY

The assay for *Streptococcus agalactiae* was tested to validate inclusivity and exclusivity. Various strains of *Streptococcus agalactiae* were tested to verify inclusive assay performance. The assay was also tested against different species of *Streptococcus* to verify exclusivity between close relatives.

All inclusive strains of *S. agalactiae* were tested in eight or 24 replicates each, while there were sixteen total replicates for NTC reactions. The TNAA method successfully detected all inclusive *S. agalactiae* strains.

All exclusive *Streptococcus* strains were tested in eight replicates each, with seven positive control reactions and nine negative NTC replicates. While the assay did detect 1 out of eight replicates of *S. mutans*, the TNAA method excluded all closely related *Streptococcus* strains.

The following tables summarize the inclusivity and exclusivity pathogens to be evaluated for the *Streptococcus agalactiae* assay.

Inclusivity Samples	NumPositive	Total	Percent
Enterobacter cloacae NCDC 279-56 (10^6 cp/ml)	0	8	0
NTC	0	16	0
S. agalactiae	0	24	0
S. agalactiae 18RS21 (10^6 cp/ml)	8	8	100
S. agalactiae 514 (10^6 cp/ml)	8	8	100
S. agalactiae 515, Type Ia (10^6 cp/ml)	8	8	100
S. agalactiae A909 (10^6 cp/ml)	8	8	100
S. agalactiae H36b (10^6 cp/ml)	8	8	100
S. agalactiae N/A (10^6 cp/ml)	8	8	100

Exclusivity Samples	NumPositive	Total	Percent
NTC (10^6 cp/ml)	0	9	0
S. agalactiae (10^6 cp/ml)	7	7	100
S. mutans (10^6 cp/ml)	1	8	12
S. pneumomoniae (10^6 cp/ml)	0	8	0
S. pyogenes (10^6 cp/ml)	0	8	0
S. salivarus (10^6 cp/ml)	0	8	0

8) CROSS-REACTIVITY

The cross-reactivity of the assay was tested against a panel of organisms which may also be present in collected *Streptococcus agalactiae* clinical samples. These organisms must be tested to ascertain that no false positives will be due to contamination from the off-target genomic material at clinically relevant viral or bacterial loads. The table below summarizes the genomic material tested and the results obtained. All potentially cross-reactive organisms were tested in replicates of eight with NTCs tested in replicates of four and the positive control were tested replicates of four and eight. While the assay did detect 1 out of eight *C. albicans*, the TNAA assay was verified to not cross-react with any non-target organisms.

Cross-reactivity Samples	NumPositive	Total	Percent
5ng human genomic DNA	0	8	0
C. albicans (10^8 cp/ml)	1	8	12
E. aerogenes (10^8 cp/ml)	0	8	0
E. coli (10^8 cp/ml)	0	8	0
HPV (10^12 cp/ml)	0	8	0
HSV2 (10^7 cp/ml)	0	8	0
L. casei (10^8 cp/ml)	0	8	0
N. gonorrhoeae (10^8 cp/ml)	0	8	0
NTC	0	4	0
P. aeruginosa (10^8 cp/ml)	0	8	0
S. agalactiae (10^8 cp/ml)	8	8	100
S. aureus MSSA (10^8 cp/ml)	0	8	0
Strep agalactiae (10^5 cp/ml)	4	4	100

9) SPECIFICITY

The specificity of the assay was tested against a panel of organisms which may be present as potential contaminants in *Streptococcus agalactiae* samples and whose genomic material may be carried though the sample preparation protocol. These organisms must be tested to verify that assay performance is not significantly impacted by the presence of off-target genomic material combined with *Streptococcus agalactiae* at clinically relevant loads. The table below summarizes the genomic material tested and the results obtained. All organisms combined with *S. agalactiae* were tested in replicates of eight. The positive control and NTCs were also tested in eight replicates.

The results below show that the assay is specific to *Streptococcus agalactiae* and spiking in other organisms that may be found in the same sample type does not affect assay performance. The assay tested S. agalactiae target at 10X LOD (3,010 CFU/ml) combined with the off-target organism. The off-target nucleic acid concentration reflects expected median viral/bacterial loads in clinical specimens.

Specificity Samples	NumPositive	Total	Percent
NTC	0	8	0
S. agalactiae (10^5 cp/mL)	8	8	100
S. agalactiae + C. albicans (10^6 cp/ml)	8	8	100
S. agalactiae + HPV (10^6 cp/ml)	8	8	100
S. agalactiae + HSV2 (10^6 cp/ml)	8	8	100
S. agalactiae + N. gonorrhoeae (10^6 cp/ml)	8	8	100

10) INTERFERING SUBSTANCES

The following interfering substances have been evaluated to have no significant effect on the performance of the TNAA assay. The interfering substances were added to *Streptococcus agalactiae* sample prep at both 10% and 0.1% of the total reaction by volume.

Interfering Substances: Endogenous and Exogenous.

Endogenous	Exogenous
Human blood	Bactroban nasal
Mucin	Flonase
Human genomic DNA	Nasonex
	Astelin
	Anefrin Nasal Spray
	Neosynphrine
	VapoRub cough suppressant
	ZiCam Allergy Relief nasal gel
	Mucin
	UTM

11) METHOD COMPARISON ON CLINICAL SAMPLES

The purpose of this study is to estimate the sensitivity and specificity of the TNAA assay using qPCR as the comparator (predicate method).

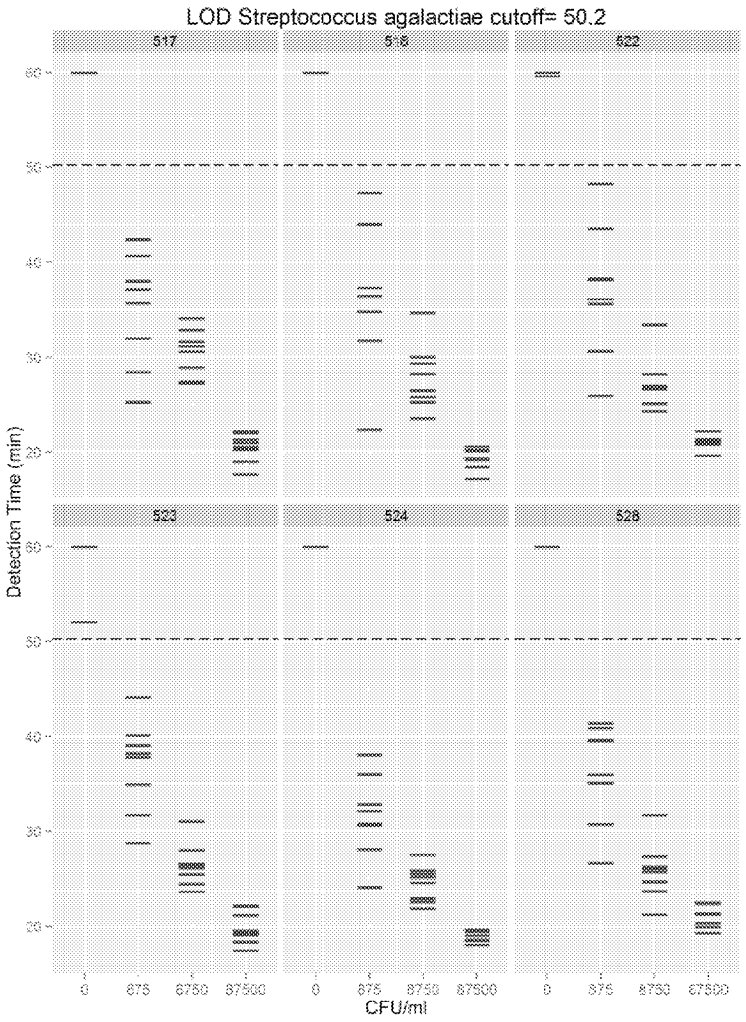
The following clinical samples were tested: 50 positive samples and 100 negative samples obtained from Fostering Tech Medical. Nasal swab samples were taken from a range of individuals of both sexes and various ages.

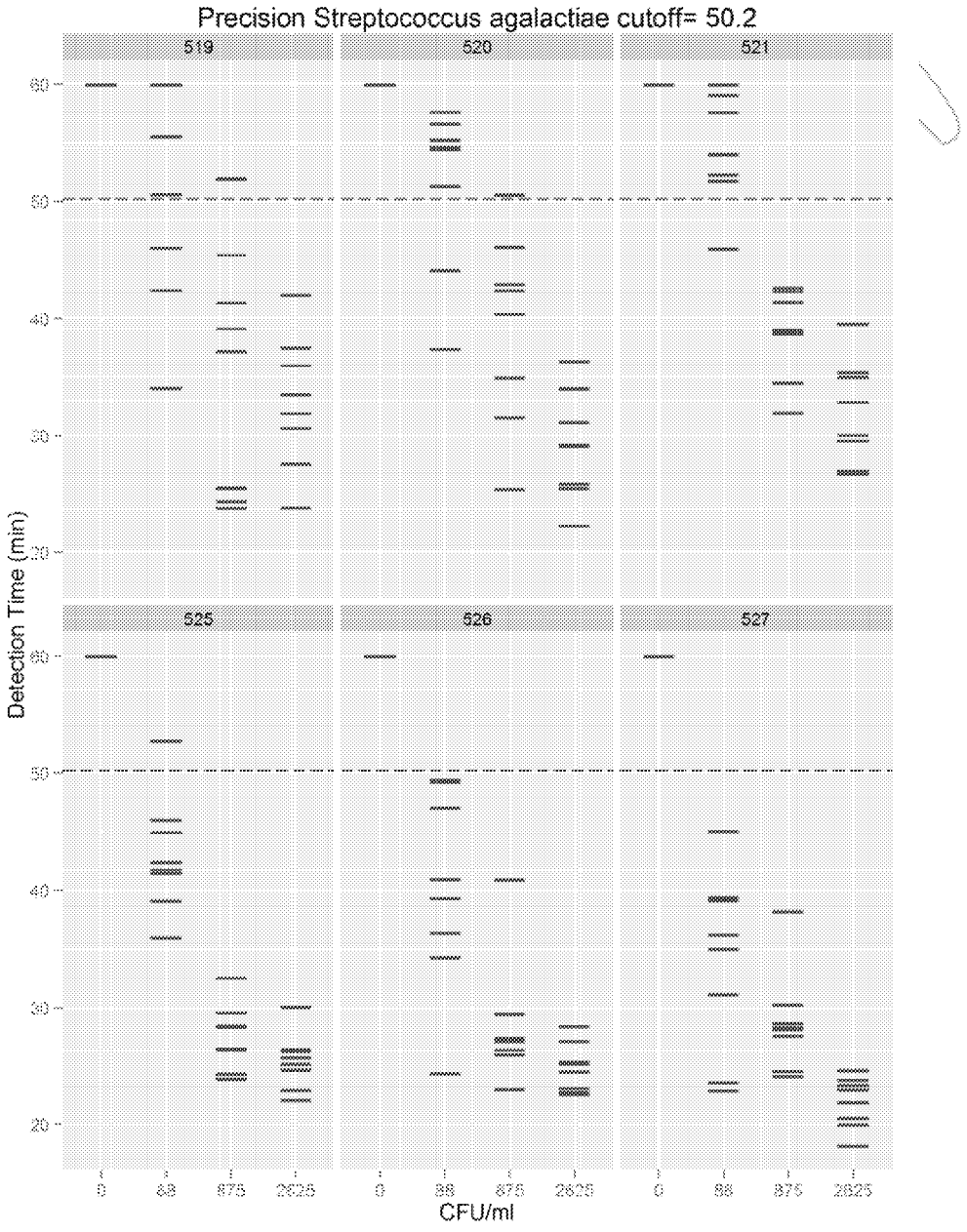
	Clinical Positive (qPCR)	Clinical Positive (TNAA)	Clinical Negative (qPCR)	Clinical Negative (TNAA)
NumPositive	50	49	0	0
Total	50	50	100	100
Percent	100	98	0	0

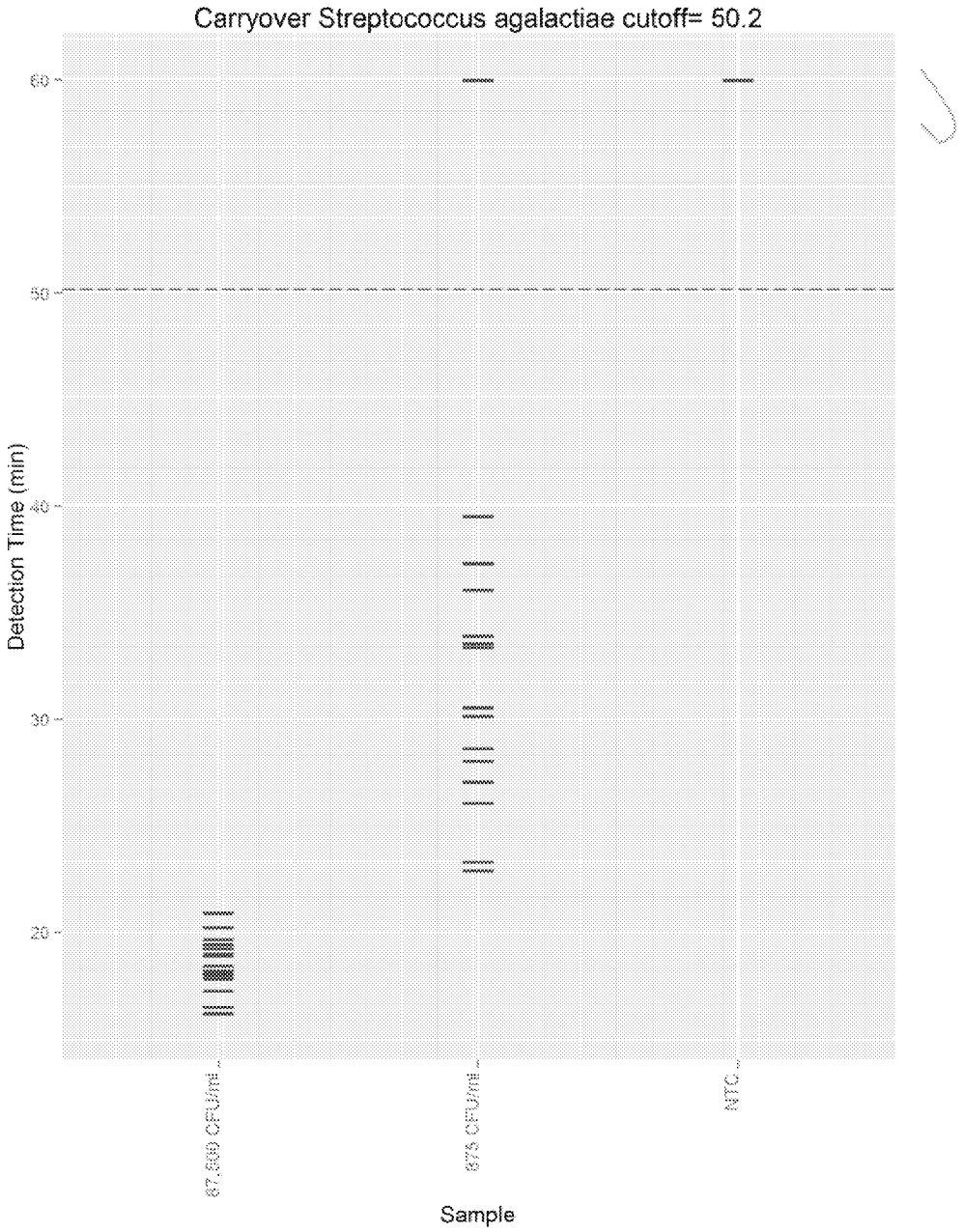
12) FINAL RECOMMENDATIONS

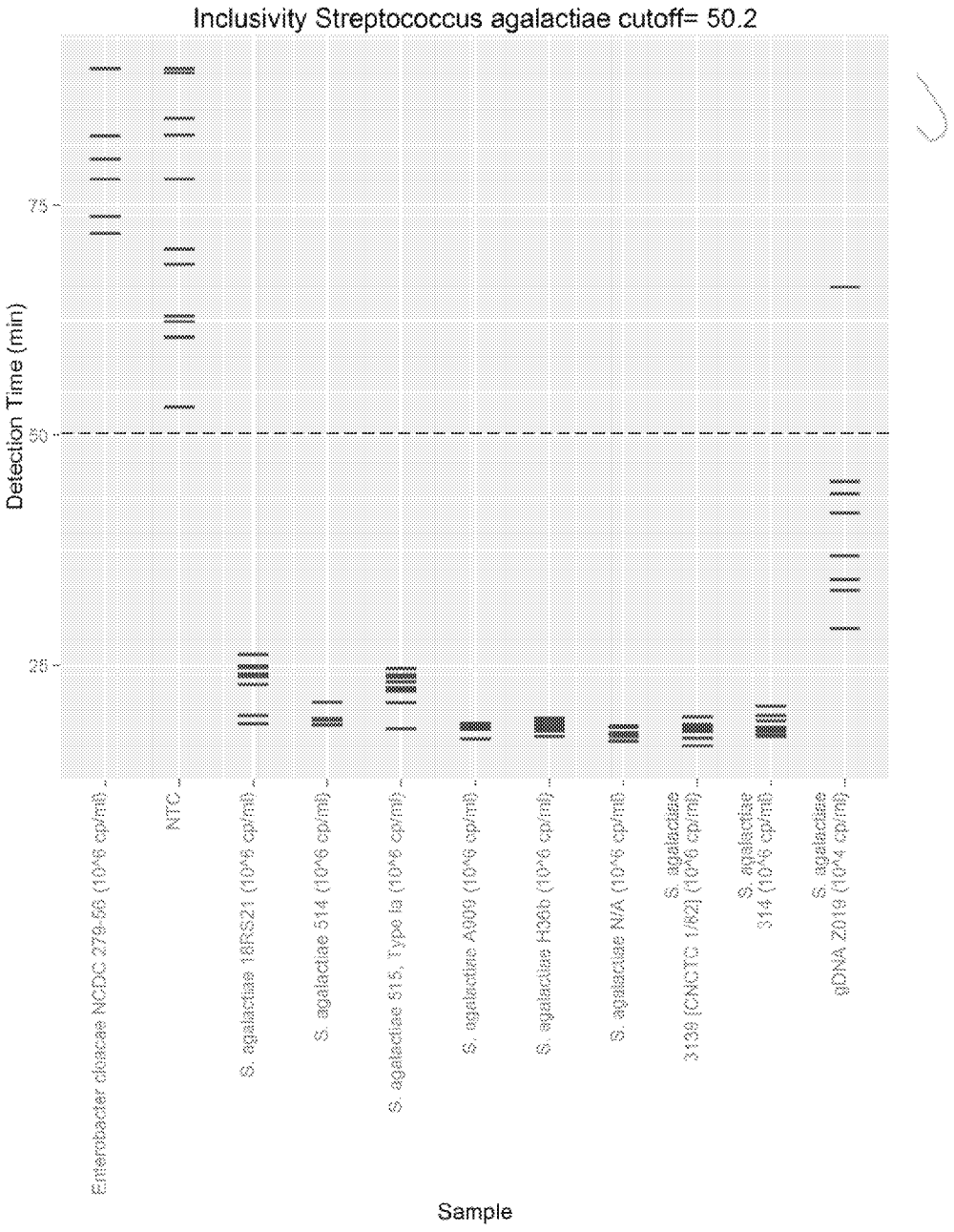
The assay for *Streptococcus agalactiae* was found to meet all criteria for precision, carryover, inclusivity, exclusivity, cross-reactivity, specificity, and resistance to interfering substances. Positive and negative clinical samples were tested and compared to a predicate method. The *Streptococcus agalactiae* assay specifically and reliably detects *Streptococcus agalactiae*. The assay limit of detection is 875 CFU/ml with a recommended assay duration of 51 minutes as determined by ROC analysis.

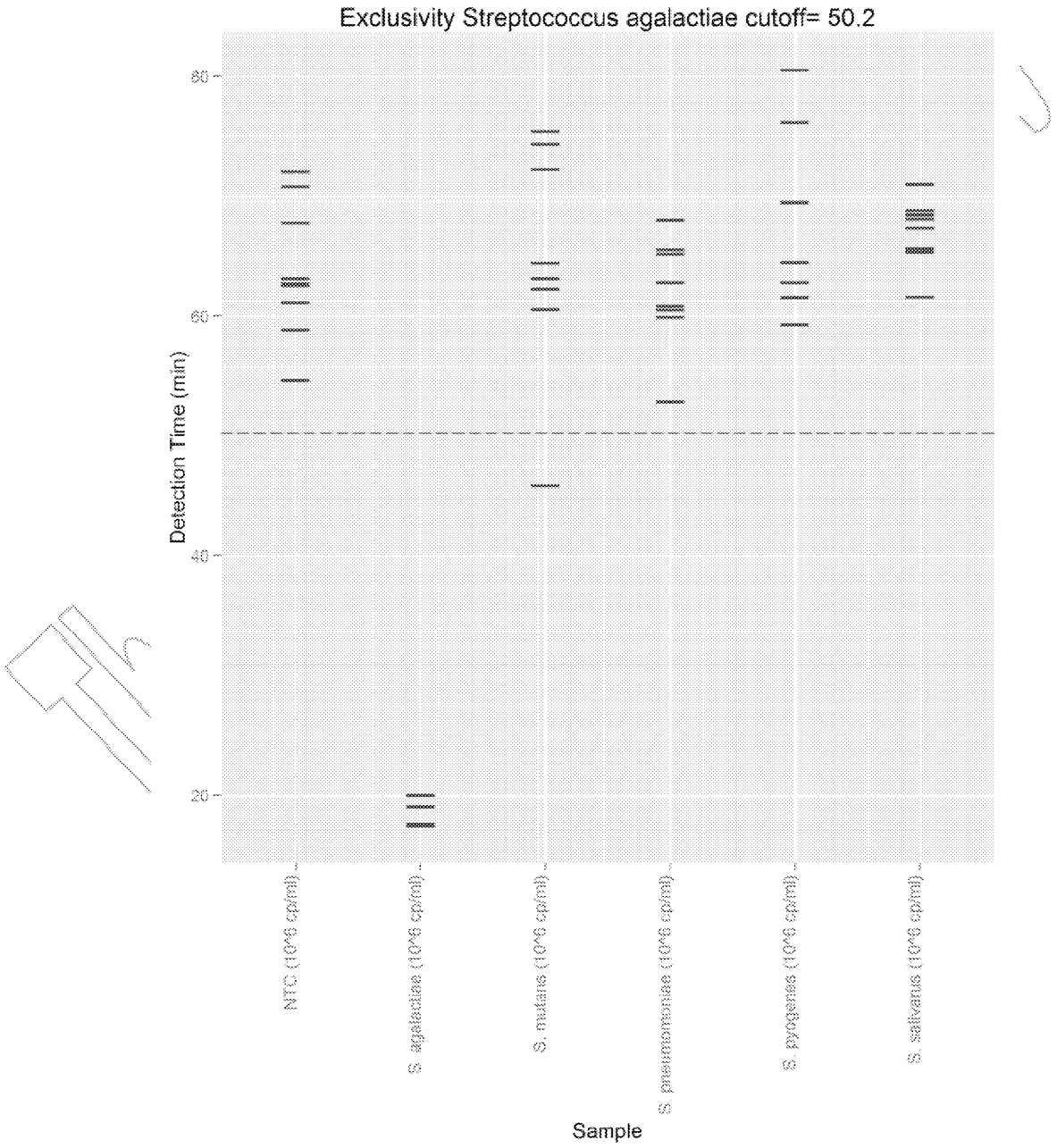
13) APPENDIX

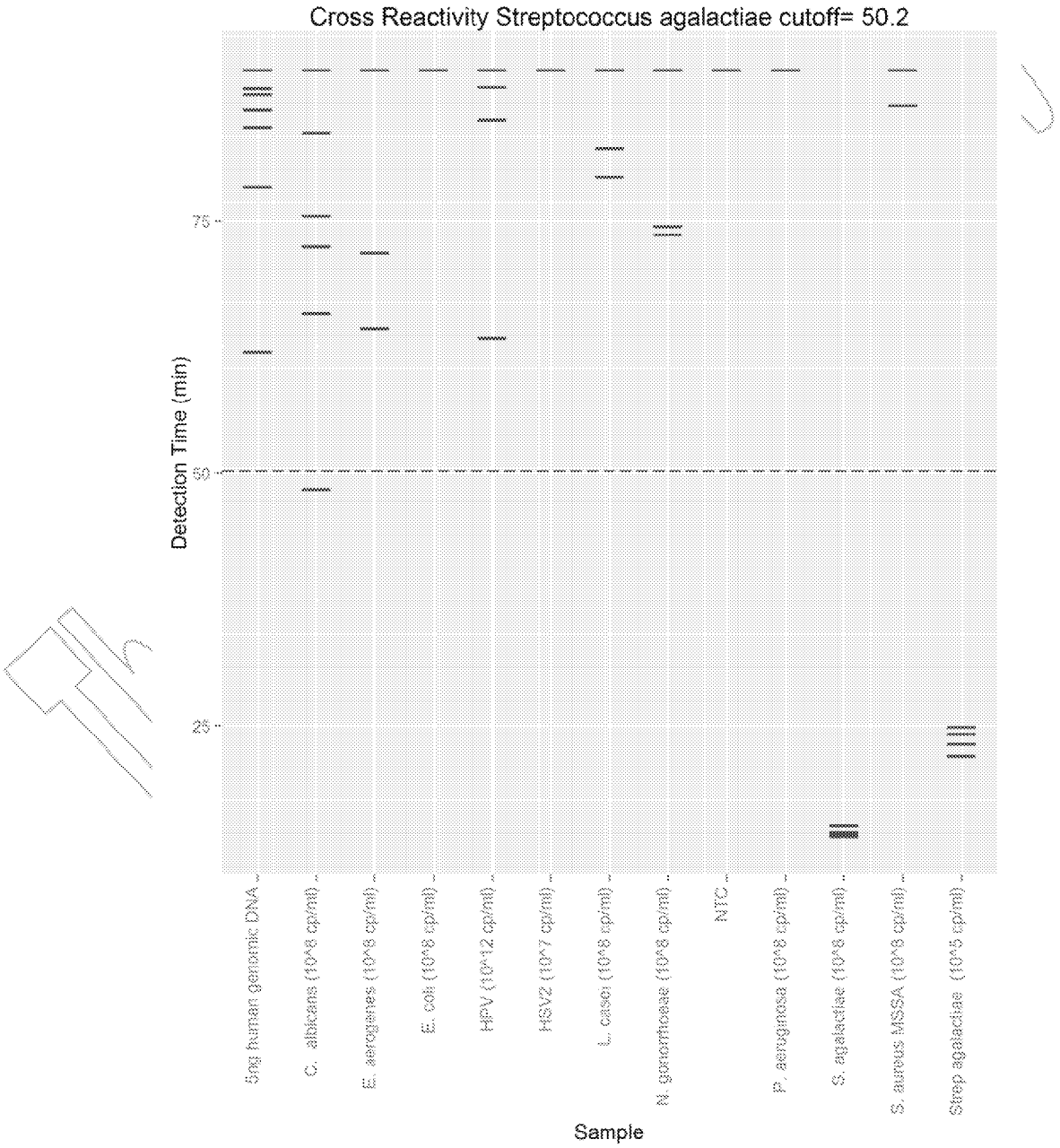


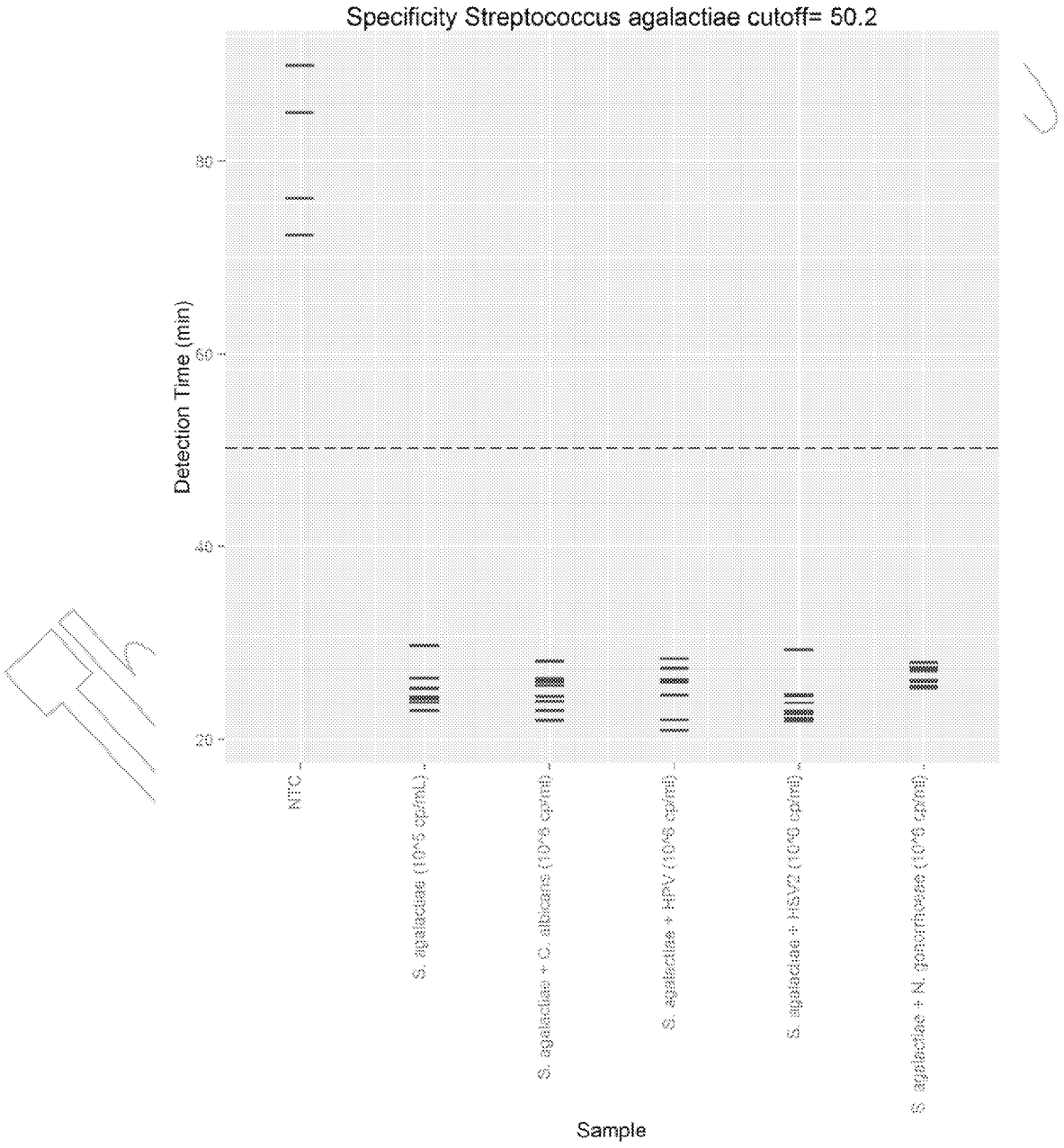


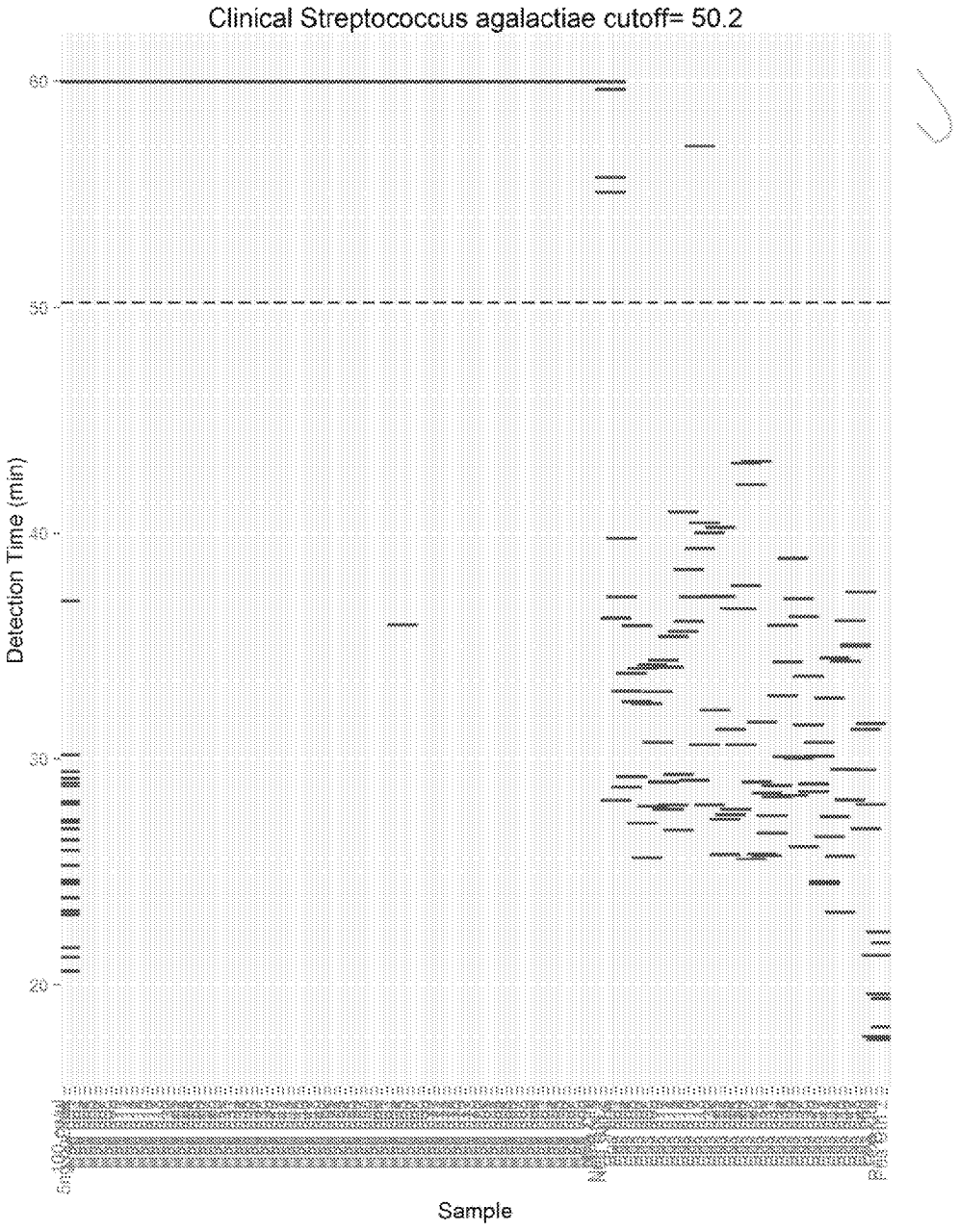












Clinical Samples TNAA: Treatment	NumPositive	Total	Percent
100 cp/ul	24	24	100
5ng hgDNA	0	24	0
Neg 001	0	2	0
Neg 002	0	2	0
Neg 003	0	2	0
Neg 004	0	2	0
Neg 005	0	2	0
Neg 006	0	2	0
Neg 007	0	2	0
Neg 008	0	2	0
Neg 009	0	2	0
Neg 010	0	2	0
Neg 011	0	2	0
Neg 012	0	2	0
Neg 013	0	2	0
Neg 014	0	2	0
Neg 015	0	2	0
Neg 016	0	2	0
Neg 017	0	2	0
Neg 018	0	2	0
Neg 019	0	2	0
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Neg 024	0	2	0
Neg 025	0	2	0
Neg 026	0	2	0
Neg 027	0	2	0
Neg 028	0	2	0

Neg 029	0	2	0
Neg 030	0	2	0
Neg 031	0	2	0
Neg 032	0	2	0
Neg 033	0	2	0
Neg 034	0	2	0
Neg 035	0	2	0
Neg 036	0	2	0
Neg 037	0	2	0
Neg 038	0	2	0
Neg 039	0	2	0
Neg 040	0	2	0
Neg 041	0	2	0
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Neg 043	0	2	0
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Neg 045	0	2	0
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Neg 059	0	2	0

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Neg 090	0	2	0

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Neg 092	0	2	0
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Neg 095	0	2	0
Neg 096	0	2	0
Neg 097	0	2	0
Neg 098	0	2	0
Neg 099	0	2	0
Neg 100	0	2	0
Neg Ctrl	0	2	0
Neg Ctrl 1	0	4	0
Neg Ctrl 2	0	4	0
NTC	0	208	0
Pos 001	2	2	100
Pos 002	2	2	100
Pos 003	2	2	100
Pos 004	2	2	100
Pos 005	2	2	100
Pos 006	2	2	100
Pos 007	2	2	100
Pos 008	2	2	100
Pos 009	2	2	100
Pos 010	2	2	100
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Pos Ctrl 2	4	4	100

[redacted]

ENTEROBACTER CLOACAE

TNAA LDT Validation Report

Limit of Detection = 10 cp/uL

Rate of Detection = 100 cp/uL in 20 minutes

Katie Sullivan-Bibee

THERANOS, INC.

Author(s):

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Reviewer(s)

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Name: Pranav Patel, PhD.	Title: Team Lead

Signature:	Date:
Name: Daniel Young, Ph.D.	Title: Vice President

Approver(s):

Signature:	Date:
Name: Adam Rosendorff, M.D	Title: Laboratory Director

Table of Contents

Enterobacter cloacae

1) PURPOSE

This report includes relevant information about the target organism, a detailed description of the primers and selected targets designed for the detection of this organism, a summary of the validation assay performance, and recommendations for future assay execution.

2) BACKGROUND

The gram negative gammaproteobacterial family Enterobacteriaceae contains numerous species found in hospital acquired infections, including *Enterobacter aerogenes*, *E. cloacae*, *Escherichia coli*, as well as members of the genera *Salmonella*, *Klebsiella*, *Shigella*, *Proteus*, *Serratia*, and *Citrobacter*. Many of these facultative anaerobes are natural components of the human gut flora, but can infect immunocompromised patients more systematically (including bloodstream, catheter, and intubation infections). Distressingly, members of the Enterobacteriaceae frequently exchange antibiotic resistance plasmids (e.g. KPC), making treatment difficult.

Enterobacter cloacae is an important nosocomial pathogen responsible for various infections, including sepsis, infections of the respiratory tract and urinary tract, wound infections, and meningitis. Multiple antibiotic-resistant strains have caused outbreaks of infections in hospitals, usually in settings where seriously ill patients are housed, such as intensive care units (ICUs). In an intensive care setting (ICU), these pathogens may cause significant morbidity and mortality, as infection management is complicated by resistance to multiple antibiotics.

This report describes the nucleic acid amplification test developed to detect *Enterobacter cloacae*. A conserved region of the *gyrA* gene was chosen as a target.

3) SUMMARY OF PERFORMANCE DATA

Theranos developed a Theranos Nucleic Acid Amplification (TNAA) assay specific for *Enterobacter cloacae*. The Nucleic Acid Amplification reactions contained 1x Nucleic Acid Amplification buffer (20 mM Tris Acetate, pH 7.9, 50 mM Potassium Acetate, 10 mM Magnesium Acetate and 1mM DTT), 0.08% Tween, 0.8 M betaine, 1.4 mM dNTPs, 2 uM Syto59, 0.8 uM RLX2364 primer and 0.8 uM RLX2365 primer, 20 units Bst polymerase, and template at the noted concentration. The reactions were run at 56ºC for

60 minutes. Summarized data will follow below while detailed experimental data can be found in the appendix.

Primer sequences are:

Enterobacter cloacae	RLX2364	ATTTACCGAAATCTGCCCGTG
	RLX2365	CGGTAAATTCGTACACCGCG

4) LIMIT OF DETECTION

The purpose of this study is to determine the limit of detection (LOD) for the Theranos TNAA assay. The LOD_{95} is the bacterial titer at which >95% of known positive samples test positive using the TNAA assay. Statistically justified cut-off times for making positive/negative calls were determined for each target empirically. A set of experiments, repeated over four days, were conducted that included eight replicates each of three target dilutions (LoD, 10X LoD, and 100X LoD), as well as 8 NTCs, using the target primers for amplification. These data were then processed using a receiver-operator character (ROC) analysis, and the best threshold detection time for distinguishing positives and negatives determined using the Youden test statistic as implemented by the R package, pROC.

The assay reliably detected 5,869 CFU/ml of *Enterobacter cloacae* in about 33.6 minutes, as shown below. The 33.6 minute assay cut-off time was determined by ROC analysis. The assay was performed nine times. Reactions with and without template (NTCs or Non-Templated Controls) were run in eight replicates each.

LOD	Samples	NumPositive	Total	Percent
100X LOD	586,854 CFU/ml	48	48	100
10X LOD	58,685 CFU/ml	48	48	100
1X LOD	5,869 CFU/ml	48	48	100
	NTC	0	48	0

5) REPRODUCIBILITY/PRECISION

The purpose of this experiment is to determine the precision of the assay, percent positive and negative at three detection limits: high-negative (0.1X LOD=587 CFU/ml), low-positive (LOD=5,869

CFU/ml), and high-positive (3X LOD=17,606 CFU/ml). The assay was performed seven times. Reactions with and without template (NTCs or Non-templated Controls) were run in eight replicates each.

Precision LOD	Samples	NumPositive	Total	Percent
3X LOD	17,606 CFU/ml	56	56	100
1X LOD	5,869 CFU/ml	56	56	100
0.1X LOD	587 CFU/ml	37	56	66
	NTC	0	56	0

6) CARRYOVER

The purpose of this experiment is to determine the potential for carryover of positive samples adjacent to negative reactions. The nucleic acid template is prepared from high-positive (100X LOD = 58,690 CFU/ml), low-positive (1X LOD=5,869 CFU/ml), and non-templated controls (NTCs) which are arrayed in alternating rows of eight replicates each. There are two rows of high-positive reactions, two rows of low-positive reactions, and six rows of NTCs. High-positive template was accidentally pipetted into well A3, which was noted at the time of experiment set up. This was procedural user error, not contamination due to true carryover. This increases the number of high-positive samples to 17 and reduces the number of non-templated controls to 47. The assay was performed once, with no carryover of positive samples to negative reactions.

		100X LOD	NTC	100X LOD	NTC	LOD	NTC	LOD	NTC	NTC	NTC	
	1	2	3	4	5	6	7	8	9	10	11	12
A	empty	+	+	+	-	+	-	+	-	-	-	empty
B		+	-	+	-	+	-	+	-	-	-	
C		+	-	+	-	+	-	+	-	-	-	
D		+	-	+	-	+	-	+	-	-	-	
E		+	-	+	-	+	-	+	-	-	-	
F		+	-	+	-	+	-	+	-	-	-	
G		+	-	+	-	+	-	+	-	-	-	
H		+	-	+	-	+	-	+	-	-	-	

Carryover Samples	NumPositive	Total	Percent
586,854 CFU/ml	17	17	100
5,869 CFU/ml	16	16	100
NTC	0	47	0

7) INCLUSIVITY/EXCLUSIVITY

The assay for *Enterobacter cloacae* was tested to validate inclusivity and exclusivity. Various strains of *Enterobacter cloacae* were tested to verify inclusive assay performance. The assay was also tested against different species of *Enterobacter* to verify exclusivity between close relatives.

All inclusive and exclusive strains of *E. cloacae* were tested in four replicates each, while there were also four total replicates for NTC reactions. The TNAA method successfully detected all inclusive *E. cloacae* strains including *E. hormaechei* which is part of the *cloacae* complex as determined by sequencing. The TNAA method excluded all closely related *Enterobacter* strains including *E. cloacae* negative (ATCC 7256) which was sequenced and found to be another *Enterobacter* species, but not in the *cloacae* complex.

The following tables summarize the inclusivity and exclusivity pathogens to be evaluated for the *Enterobacter cloacae* assay.

Inclusivity and Exclusivity Samples	NumPositive	Total	Percent
E. aerogenes (10^6 cp/ml)	0	4	0
E. cloacae (1101177) (10^6 cp/ml)	4	4	100
E. cloacae (ATCC BAA-2080) (10^6 cp/ml)	4	4	100
E. cloacae KPC+ (1101152) (10^6 cp/ml)	4	4	100
E. cloacae (NCDC 279-56) (10^6 cp/ml)	4	4	100
E. cloacae (NDM-1+) (10^6 cp/ml)	4	4	100
E. cloacae negative (ATCC 7256) (10^6 cp/ml)	0	4	0
E. hormaechei (10^6 cp/ml)	4	4	100
NTC	0	4	0

8) CROSS-REACTIVITY

The cross-reactivity of the assay was tested against a panel of organisms which may also be present in collected *Enterobacter cloacae* clinical samples. These organisms must be tested to ascertain that no false positives will be due to contamination from the off-target genomic material at clinically relevant viral or bacterial loads. The table below summarizes the genomic material tested and the results obtained. All potentially cross-reactive organisms were tested in replicates of eight, with the exception of *S. pneumoniae* which was tested in replicates of sixteen. NTCs were tested in replicates of four and the positive control was tested in replicates of both four and eight. The TNAA assay was verified to not cross-react with any non-target organisms.

Cross-reactivity Samples	NumPositive	Total	Percent
A. baumannii (10^7 cp/ml)	0	8	0
Adenovirus 4 (10^6 cp/ml)	0	8	0
B. pertussis (10^8 cp/ml)	0	8	0
C. albicans (10^6 cp/ml)	0	8	0
E. aerogenes (10^7 cp/ml)	0	8	0
E. cloacae (10^5 cp/ml)	8	8	100
E. cloacae (L601) (10^5 cp/ml)	4	4	100
E. cloacae negative (L499) (10^7 cp/ml)	0	8	0
E. coli (10^7 cp/ml)	0	8	0
E. coli (5X10^7 cp/mL)	0	8	0
Flu A (H1N1 novel) (10^8 cp/ml)	0	8	0
Flu B/mass. (10^8 cp/ml)	0	8	0
hgDNA (200ng/ml)	0	8	0
K. oxytoca (10^7 cp/ml)	0	8	0
K. pneumoniae (10^6 cp/ml)	0	8	0
K. pneumoniae (10^7 cp/ml)	0	8	0
N. meningitidis (10^7 cp/ml)	0	8	0
NTC	0	4	0
P. aeruginosa (10^7 cp/ml)	0	16	0
S. agalactiae (10^7 cp/ml)	0	8	0
S. aureus MSSA (10^7 cp/ml)	0	8	0
S. marcescens (10^7 cp/ml)	0	8	0
S. pneumoniae (10^7 cp/ml)	0	8	0
S. pyogenes (10^7 cp/ml)	0	8	0

9) SPECIFICITY

The specificity of the assay was tested against a panel of organisms which may be present as potential contaminants in *Enterobacter cloacae* samples and whose genomic material may be carried though the sample preparation protocol. These organisms must be tested to verify that assay performance is not significantly impacted by the presence of off-target genomic material combined with *Enterobacter cloacae* at clinically relevant loads. The table below summarizes the genomic material tested and the results obtained. All organisms combined with *E. cloacae* were tested in replicates of two with the exception of *P. aeruginosa, E. coli, K. pneumoniae,* and IDTE which were tested in replicates of four. The positive control and NTCs were also tested in two replicates.

The results below show that the assay is specific to *Enterobacter cloacae* and spiking in other organisms that may be found in the same sample type does not affect assay performance. The assay tested E. cloacae target at 10X LOD (58,690 CFU/ml) combined with the off-target organism. The off-target nucleic acid concentration reflects expected median viral/bacterial loads in clinical specimens.

Specificity Samples	NumPositive	Total	Percent
E. cloacae + A. baumannii (10^7 cp/ml)	2	2	100
E. cloacae + Adenovirus 4 (10^6 cp/ml)	2	2	100
E. cloacae + B. pertussis (10^8 cp/ml)	2	2	100
E. cloacae + C. albicans (10^6 cp/ml)	2	2	100
E. cloacae + E. cloacae (10^7 cp/ml)	2	2	100
E. cloacae + E. coli (10^7 cp/ml)	2	2	100
E. cloacae + E. coli (5X10^7 cp/ml)	2	2	100
E. cloacae + Flu A (H1N1 novel) (10^8 cp/ml)	2	2	100
E. cloacae + Flu B/Mass (10^8 cp/ml)	2	2	100
E. cloacae + hgDNA (200ng/ml)	2	2	100
E. cloacae + IDTE	4	4	100
E. cloacae + Klebsiella oxytoca (10^7 cp/ml)	2	2	100
E. cloacae + K. pneumoniae (10^6 cp/ml)	2	2	100
E. cloacae + K. pneumoniae (10^7 cp/ml)	2	2	100
E. cloacae + N. meningitidis (10^7 cp/ml)	2	2	100
E. cloacae + P. aeruginosa (10^7 cp/ml)	4	4	100
E. cloacae + S. agalactiae (10^7 cp/ml)	2	2	100
E. cloacae + S. aureus MSSA (10^7 cp/ml)	2	2	100

E. cloacae + S. marcescens (10^7 cp/ml)	2	2	100
E. cloacae + S. pneumoniae (10^7 cp/ml)	2	2	100
NTC	0	4	0

10) INTERFERING SUBSTANCES

The following interfering substances have been evaluated to have no significant effect on the performance of the TNAA assay. The interfering substances were added to *Enterobacter cloacae* sample prep at both 10% and 0.1% of the total reaction by volume.

Interfering Substances: Endogenous and Exogenous.

Endogenous	Exogenous
Human blood	Bactroban nasal
Mucin	Flonase
Human genomic DNA	Nasonex
	Astelin
	Anefrin Nasal Spray
	Neosynphrine
	VapoRub cough suppressant
	ZiCam Allergy Relief nasal gel
	Mucin
	UTM

11) METHOD COMPARISON ON CLINICAL SAMPLES

The purpose of this study is to estimate the sensitivity and specificity of the TNAA assay using qPCR as the comparator (predicate method).

The following clinical samples were tested: 50 positive samples and 100 negative samples obtained from Fostering Tech Medical. Both nosocomial, coproculture, and nasal swab samples were taken from a range of individuals of both sexes and various ages.

TNAA vs qPCR Contingency Table		qPCR		
		Positive	**Negative**	**Total**
TNAA	**Positive**	50	0	50
	Negative	0	100	100
	Total	50	100	150

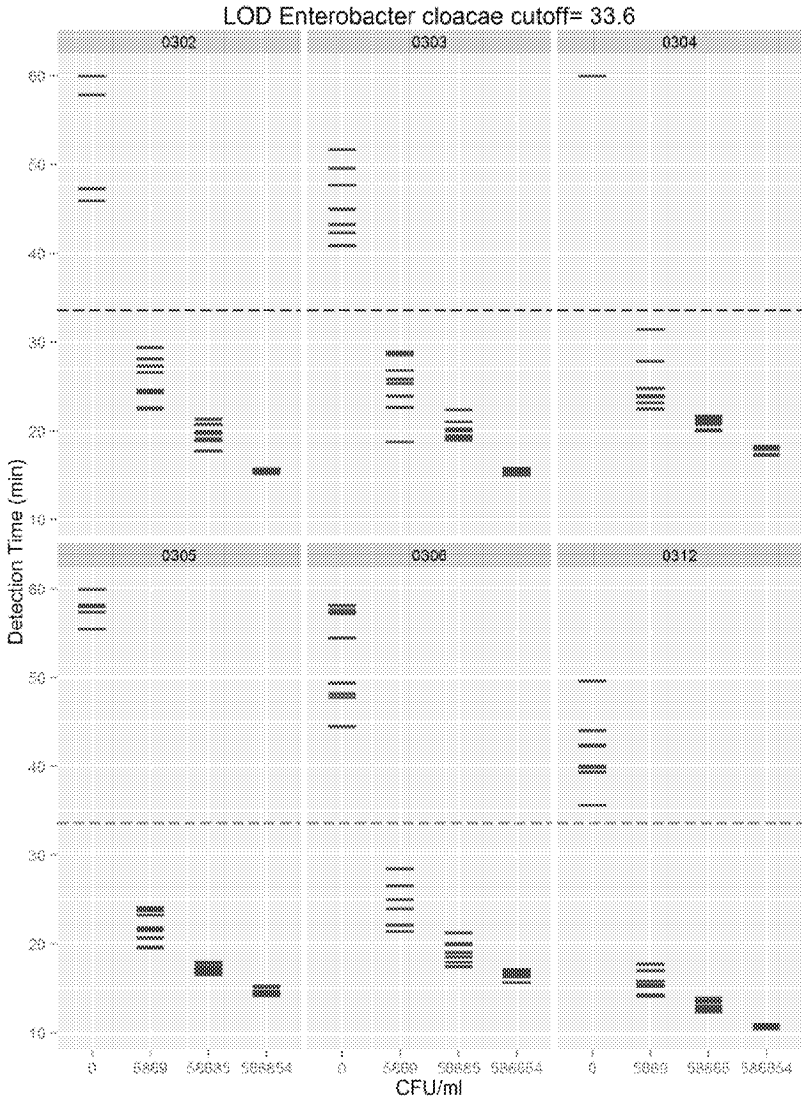
	Percent	95% Confidence Interval	
Estimated Sensitivity	100%	93%	100%
Estimated Specificity	100%	96%	100%

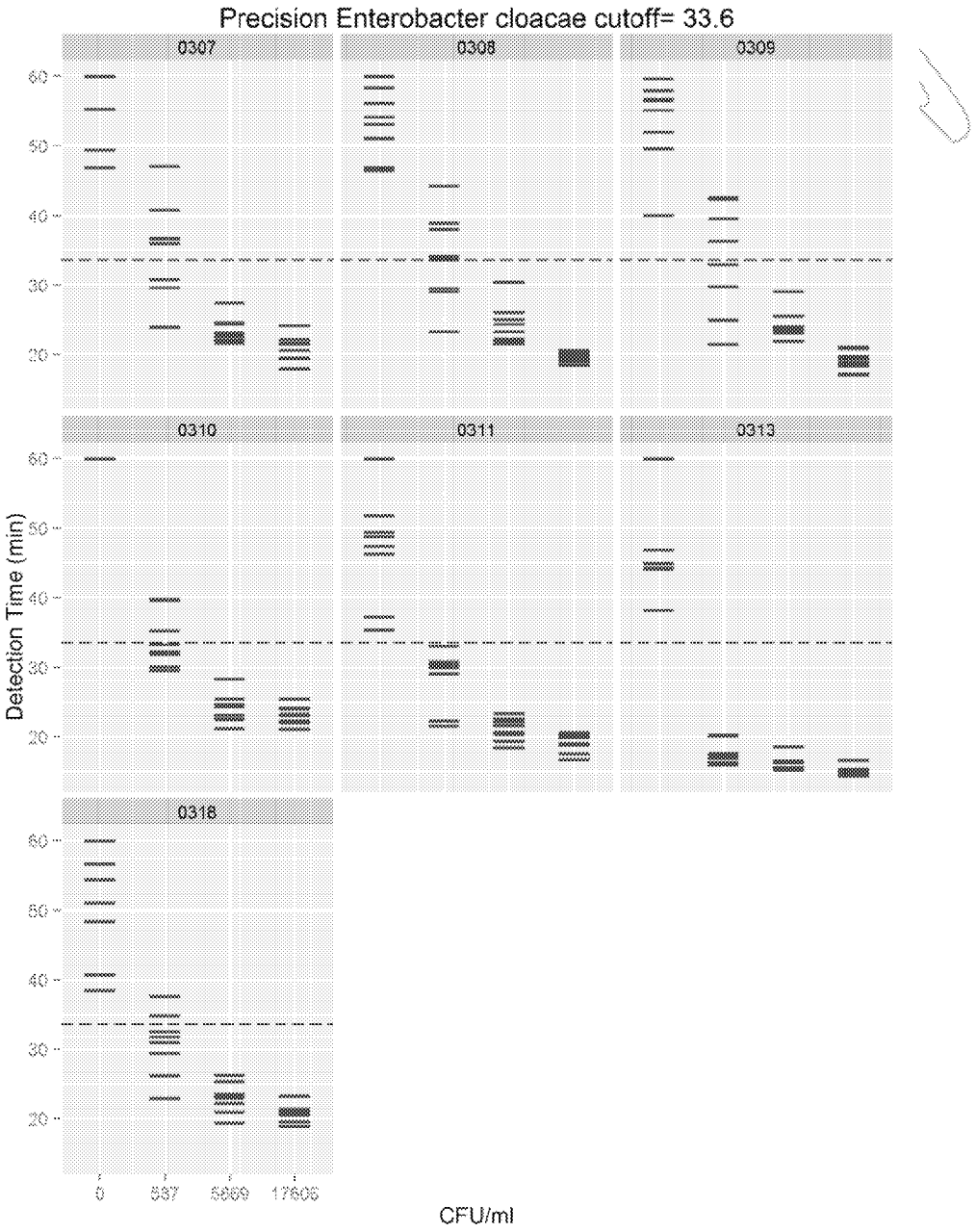
Based on a Prevalence of	**33%**
Positive Predictive Value	100%
Negative Predictive Value	100%

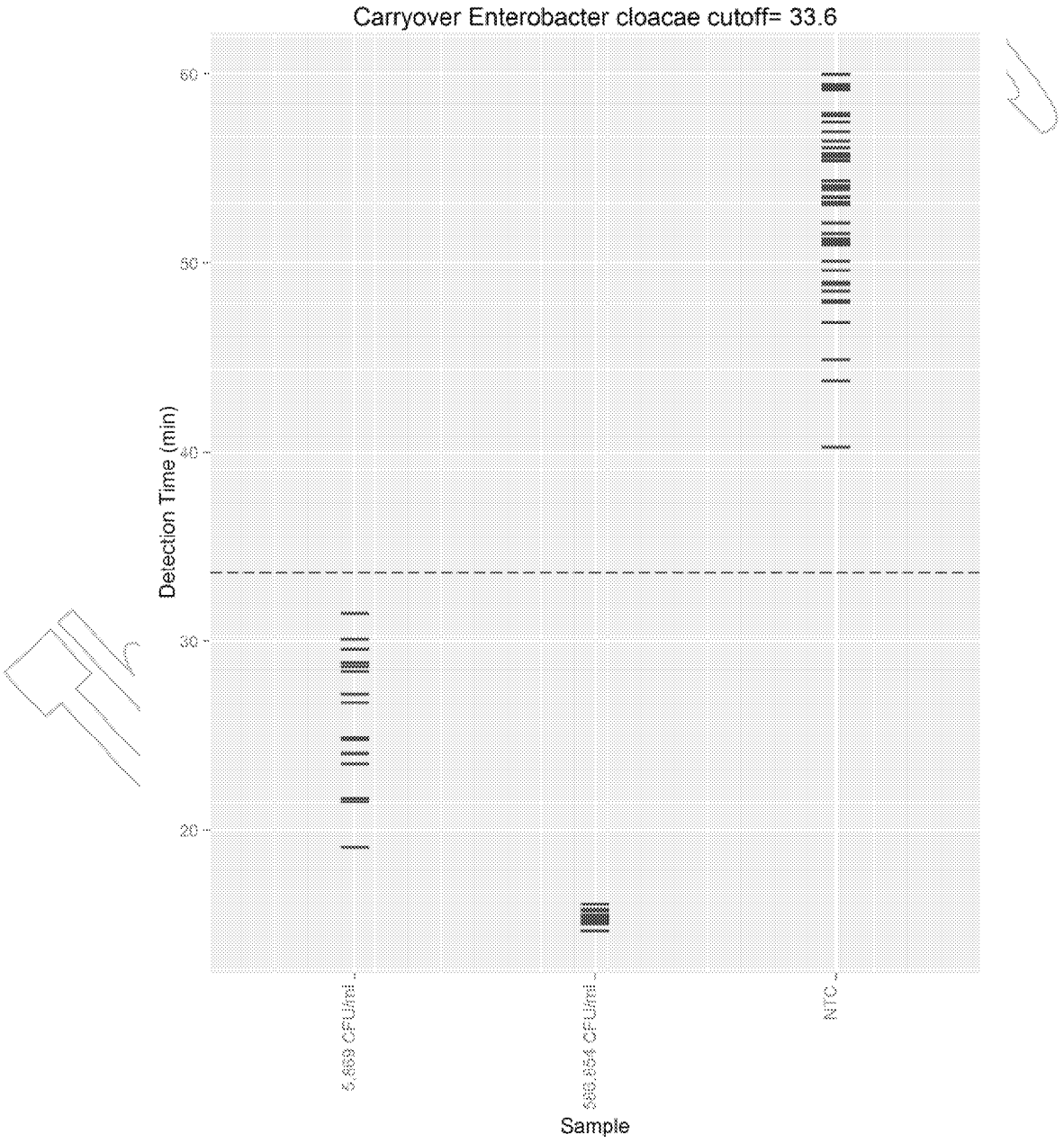
12) FINAL RECOMMENDATIONS

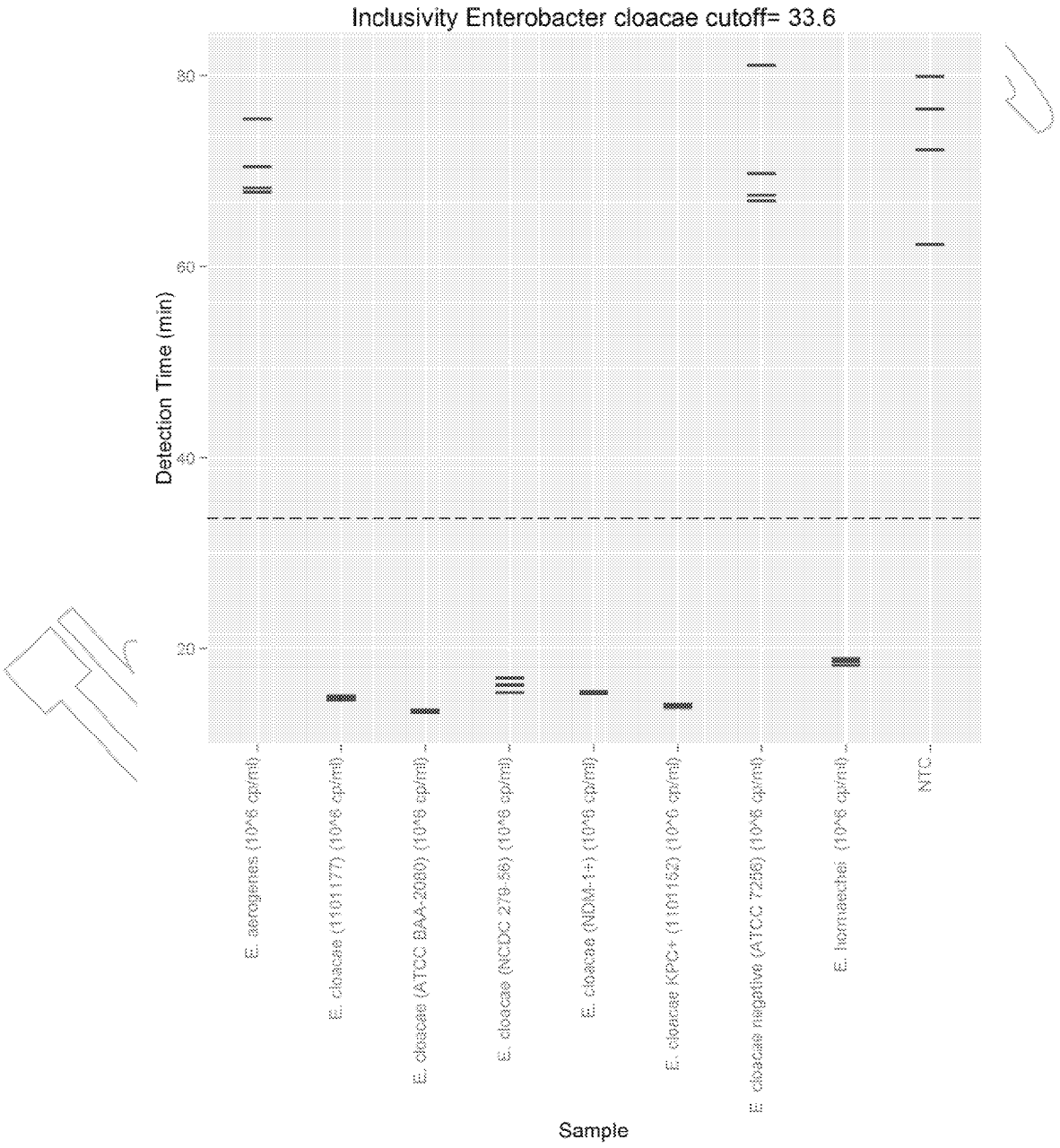
The assay for *Enterobacter cloacae* was found to meet all criteria for precision, carryover, inclusivity, exclusivity, cross-reactivity, specificity, and resistance to interfering substances. Positive and negative clinical samples were tested and compared to a predicate method. The *Enterobacter cloacae* assay specifically and reliably detects *Enterobacter cloacae*. The assay limit of detection is 5,869 CFU/ml with a recommended assay duration of 34 minutes as determined by ROC analysis.

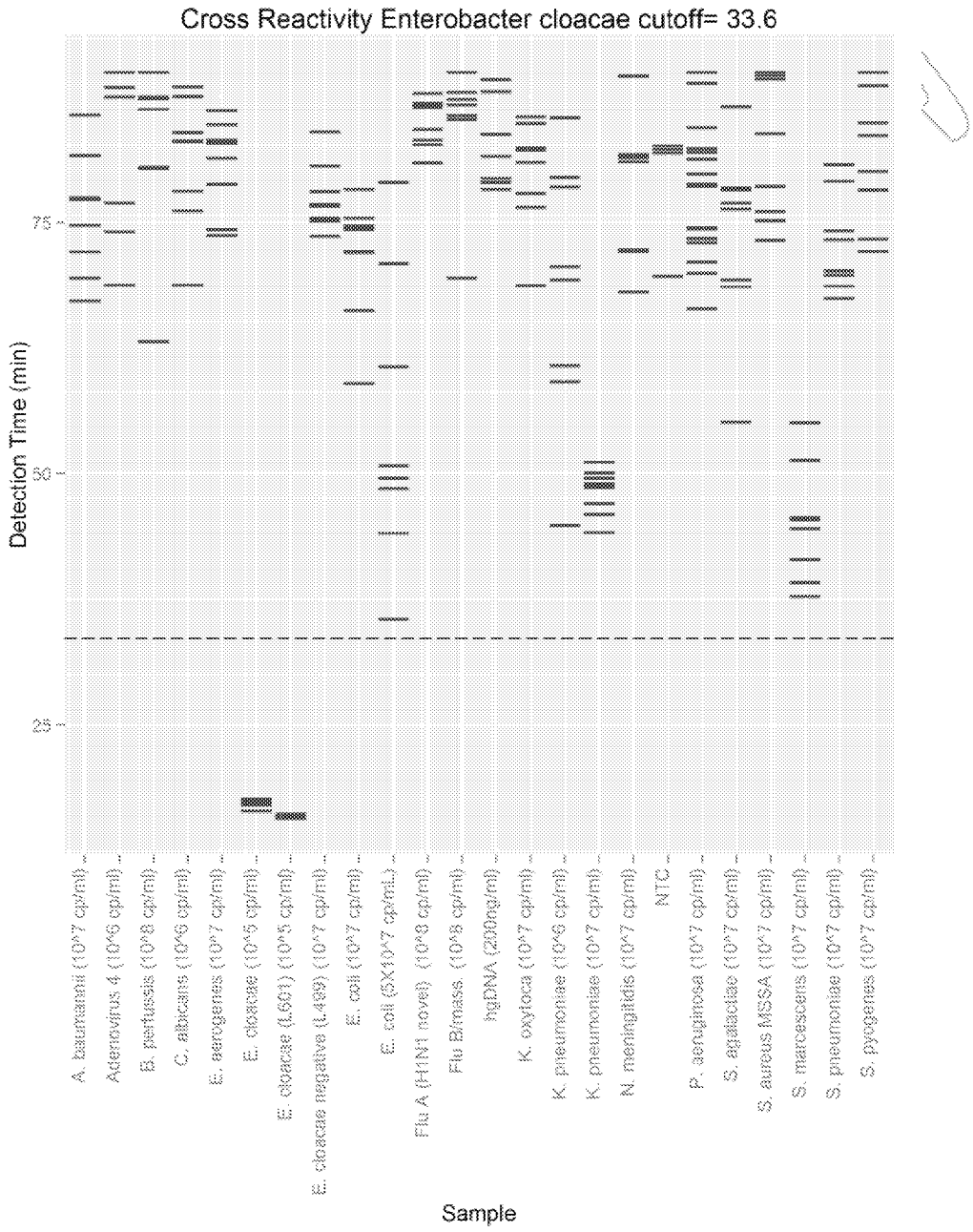
13) APPENDIX

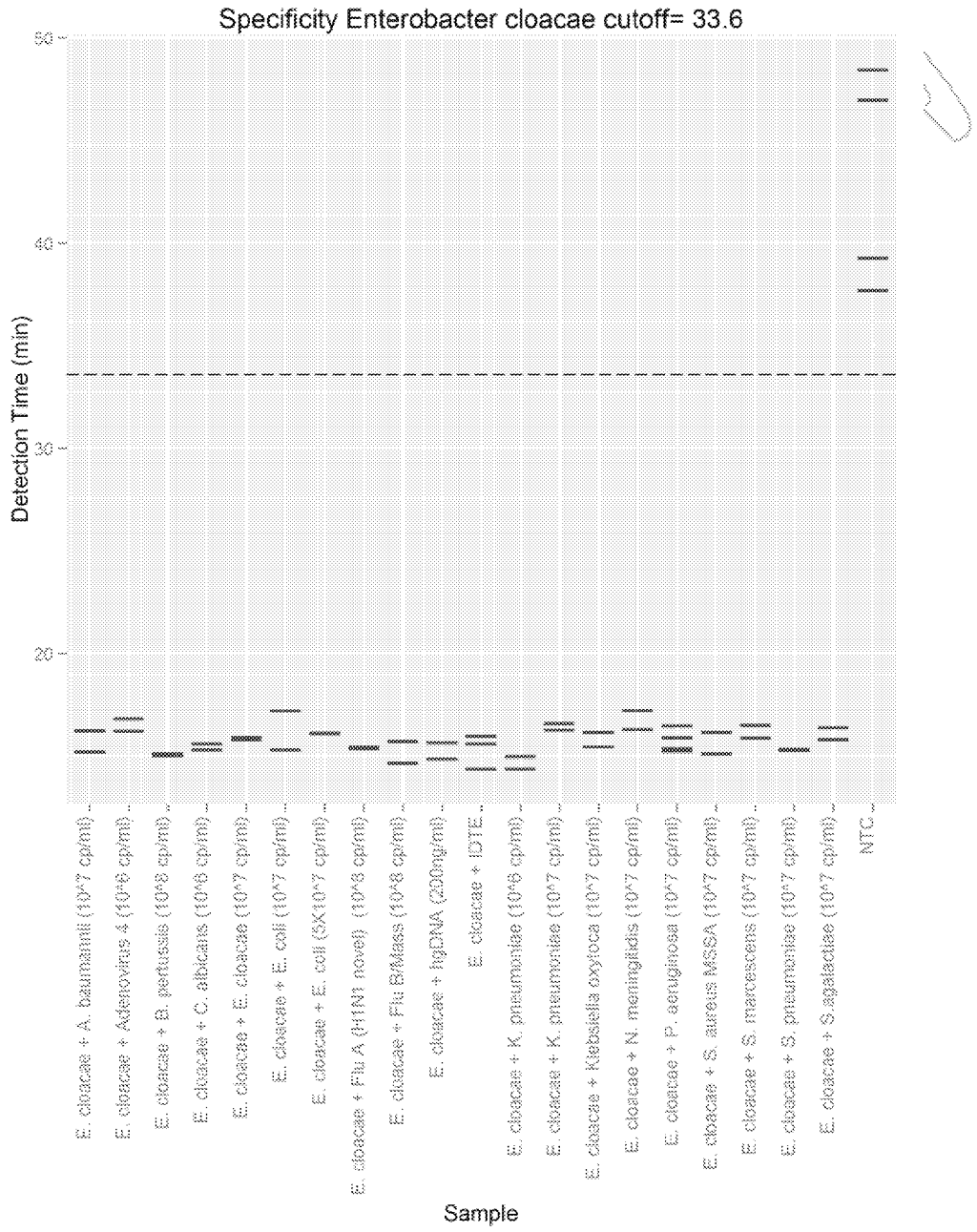


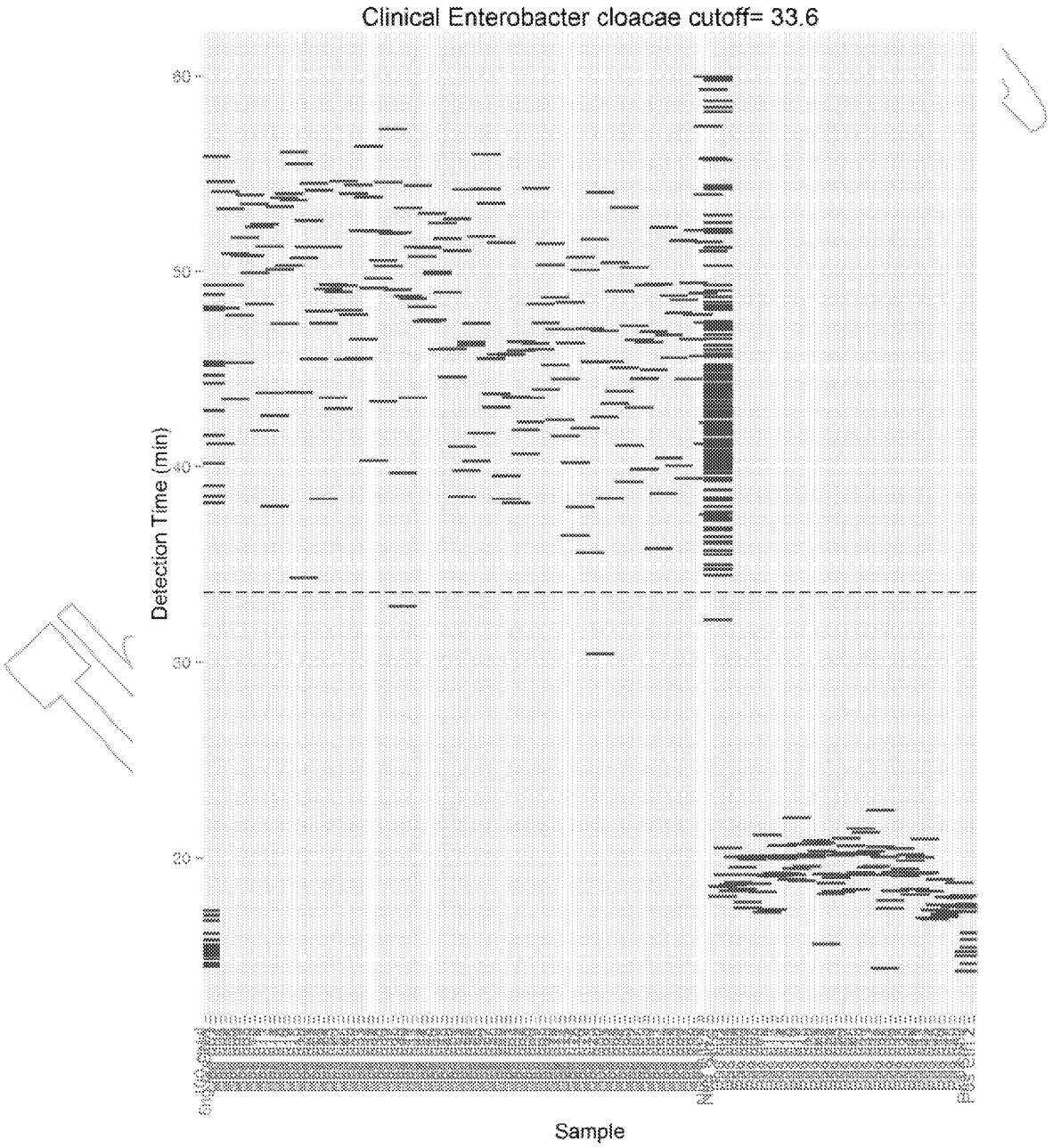












Clinical Samples TNAA: Treatment	NumPositive	Total	Percent
100 cp/ul	20	20	100
5ng hgDNA	0	16	0
Neg 001	0	2	0
Neg 002	0	2	0
Neg 003	0	2	0
Neg 004	0	2	0
Neg 005	0	2	0
Neg 006	0	2	0
Neg 007	0	2	0
Neg 008	0	2	0
Neg 009	0	2	0
Neg 010	0	2	0
Neg 011	0	2	0
Neg 012	0	2	0
Neg 013	0	2	0
Neg 014	0	2	0
Neg 015	0	2	0
Neg 016	0	2	0
Neg 017	0	2	0
Neg 018	0	2	0
Neg 019	0	2	0
Neg 020	0	2	0
Neg 021	0	2	0
Neg 022	0	2	0
Neg 023	0	2	0
Neg 024	0	2	0
Neg 025	0	2	0
Neg 026	0	2	0
Neg 027	0	2	0
Neg 028	0	2	0

Neg 029	0	2	0
Neg 030	0	2	0
Neg 031	0	2	0
Neg 032	0	2	0
Neg 033	0	2	0
Neg 034	0	2	0
Neg 035	0	2	0
Neg 036	0	2	0
Neg 037	0	2	0
Neg 038	0	2	0
Neg 039	1	2	50
Neg 040	0	2	0
Neg 041	0	2	0
Neg 042	0	2	0
Neg 043	0	2	0
Neg 044	0	2	0
Neg 045	0	2	0
Neg 046	0	2	0
Neg 047	0	2	0
Neg 048	0	2	0
Neg 049	0	2	0
Neg 050	0	2	0
Neg 051	0	2	0
Neg 052	0	2	0
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Neg 054	0	2	0
Neg 055	0	2	0
Neg 056	0	2	0
Neg 057	0	2	0
Neg 058	0	2	0
Neg 059	0	2	0

Neg 060	0	2	0
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Neg 074	0	2	0
Neg 075	0	2	0
Neg 076	0	2	0
Neg 077	0	2	0
Neg 078	0	2	0
Neg 079	1	2	50
Neg 080	0	2	0
Neg 081	0	2	0
Neg 082	0	2	0
Neg 083	0	2	0
Neg 084	0	2	0
Neg 085	0	2	0
Neg 086	0	2	0
Neg 087	0	2	0
Neg 088	0	2	0
Neg 089	0	2	0
Neg 090	0	2	0

Neg 091	0	2	0
Neg 092	0	2	0
Neg 093	0	2	0
Neg 094	0	2	0
Neg 095	0	2	0
Neg 096	0	2	0
Neg 097	0	2	0
Neg 098	0	2	0
Neg 099	0	2	0
Neg 100	0	2	0
Neg Ctrl 1	0	6	0
Neg Ctrl 2	0	6	0
NTC	1	168	1
Pos 001	2	2	100
Pos 002	2	2	100
Pos 003	2	2	100
Pos 004	2	2	100
Pos 005	2	2	100
Pos 006	2	2	100
Pos 007	2	2	100
Pos 008	2	2	100
Pos 009	2	2	100
Pos 010	2	2	100
Pos 011	2	2	100
Pos 012	2	2	100
Pos 013	2	2	100
Pos 014	2	2	100
Pos 015	2	2	100
Pos 016	2	2	100
Pos 017	2	2	100
Pos 018	2	2	100

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Pos 050	2	2	100
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