510(k) SUBSTANTIAL EQUIVALENCE DETERMINATION
DECISION SUMMARY
ASSAY ONLY

I Background Information:

A 510(k) Number

K252072

B Applicant

Center for Disease Control and Prevention

C Proprietary and Established Names

Francisella tularensis Real-time PCR Assay

D Regulatory Information

Product Code(s)	Classification	Regulation Section	Panel
SGA	Class II	21 CFR 21 CFR 866.4000 - Device To Detect And Identify Biothreat Microbial Agents In Human Clinical Specimens	

II Submission/Device Overview:

A Purpose for Submission:

The purpose of this submission is to 1) obtain a substantial equivalence determination for the *Francisella tularensis* Real-Time PCR Assay, and 2) establish a Predetermined Change Control Plan (PCCP) to validate certain future device modifications for the CDC *Francisella tularensis* Real-time PCR assay.

B Measurand:

Target chromosomal DNA sequences from *Francisella tularensis*

C Type of Test:

Nucleic Acid Amplification Test

III Intended Use/Indications for Use:

A Intended Use(s):

See Indications for Use below.

B Indication(s) for Use:

The Francisella tularensis Real-time PCR Assay is an in vitro diagnostic test for the qualitative detection of chromosomal DNA sequences from *Francisella tularensis*. The assay can be used to test whole blood EDTA, pleural fluid, and bacterial culture isolates grown on agar from individuals suspected of having tularemia.

Results generated from direct specimen testing are presumptive for the identification of *Francisella tularensis*. Results generated from culture isolate testing are used as part of the LRN *Francisella tularensis* Testing Algorithm. The diagnosis of *Francisella tularensis* infection must be made based on history, signs, symptoms, exposure likelihood, and other laboratory evidence, in addition to the identification of *Francisella tularensis* from culture isolates or from clinical specimens.

Negative results do not preclude infection with the biothreat microbial agents targeted by the device and should not be used as the sole basis for diagnosis, treatment, or other patient management decisions.

Use is limited to Centers for Disease Control and Prevention (CDC) designated laboratories.

C Special Conditions for Use Statement(s):

Rx - For Prescription Use Only

D Special Instrument Requirements:

Real-time PCR instrumentation:

- Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument
- QuantStudio Dx Real-Time PCR Instrument

IV Device/System Characteristics:

A Device Description:

The *Francisella tularensis* Real-time PCR Assay is composed of oligonucleotide primers and dual-labeled hydrolysis probes (Taqman) for use in real-time PCR. This assay is intended for the *in vitro* detection of *Francisella tularensis* chromosomal DNA targets in whole blood EDTA and pleural fluid specimens from suspect infected persons with fever and other symptoms clinically compatible with acute tularemia. Extracted DNA samples are tested using the *Francisella tularensis* Real-time PCR Assay along with an extraction control primer and probe set to demonstrate adequate DNA extraction and isolation, specimen integrity, proper function of

common reagents and equipment, and the absence of inhibitory substances. The assay can also be used to test bacterial culture isolates grown on agar from individuals suspected of having tularemia.

The *Francisella tularensis* Real-time PCR Assay includes:

- *Francisella tularensis* Real-time PCR Primer and Probe Set, consisting of 4 primers and 2 probes (Ft Primer Set 1 and Ft Primer Set 2) which target genetic regions that are unique to strains of *F. tularensis.*

- RNase P Real-time PCR Primer and Probe Set, consisting of two primers and one probe. The RNase P Real-time PCR Primer and Probe Set detects human RNase P and is used with human clinical specimens to indicate that adequate isolation of nucleic acid resulted from the extraction of the clinical specimen.

- Human Specimen Control (HSC), consisting of noninfectious, cultured human cell material suspended in Phosphate Buffered Saline (PBS). HSC is an extraction control to monitor for cross-contamination during extraction and to demonstrate successful recovery of nucleic acid by the extraction process. Purified nucleic acid from the HSC material should yield a positive result with the RNase P (RP) primer and probe set and a negative result with agent-specific primer and probe sets.

- *Francisella tularensis* Real-time PCR Positive DNA Control (FtPC), consisting of a plasmid containing targets for the *Francisella tularensis* Real-time PCR Assay primer and probe sets that serves as a positive control for the assay.

The *Francisella tularensis* Real-time PCR Assay is intended for the *in vitro* qualitative detection of *Francisella tularensis* DNA extracted from clinical specimens or culture isolates submitted to a Centers for Disease Control and Prevention (CDC) designated laboratory.

This device includes a PCCP for modifications to the *Francisella tularensis* Real-time PCR assay. See Section V.C. for the changes included in the PCCP.

B Principle of Operation:

The assay uses a fluorogenic probe, consisting of an oligonucleotide with a reporter dye attached to the 5’ end and a quencher dye attached at or near the 3’ end. The probe anneals to a specific target sequence located between the forward and reverse primers. During the extension phase of the PCR cycle, the 5’ nuclease activity of the Taq polymerase enzyme degrades the probe, causing the reporter dye to separate from the quencher dye, thereby generating a fluorescent signal. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity monitored during the PCR in real-time.

V Substantial Equivalence Information:

A Predicate Device Name(s):
FilmArray NGDS Warrior Panel

B Predicate 510(k) Number(s):
K170883

C Comparison with Predicate(s):

Device & Predicate Device(s):	K252072	K170883
Device Trade Name	*Francisella tularensis* Real-time PCR Assay	FilmArray NGDS Warrior Panel
General Device Characteristic Similarities		
Intended Use/Indications For Use	The *Francisella tularensis* Real-time PCR Assay is an *in vitro* diagnostic test for the qualitative detection of chromosomal DNA sequences from *Francisella tularensis*. The assay can be used to test whole blood EDTA, pleural fluid, and bacterial culture isolates grown on agar from individuals suspected of having tularemia. Results generated from direct specimen testing are presumptive for the identification of *Francisella tularensis*. Results generated from culture isolate testing are used as part of the LRN *Francisella tularensis* Testing Algorithm. The diagnosis of *Francisella tularensis* infection must be made based on history, signs, symptoms, exposure likelihood, and other laboratory evidence, in addition to the identification of	The FilmArray NGDS Warrior Panel is a qualitative, multiplexed, nucleic acid-based *in vitro* diagnostic test intended for use with the FilmArray 2.0 system. The FilmArray NGDS Warrior Panel detects and identifies *Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, *Coxiella burnetii*, Ebola virus, and Marburg virus nucleic acids directly from human whole blood (EDTA). The FilmArray NGDS Warrior Panel is also intended to be used to test for *Bacillus anthracis* or *Yersinia pestis* nucleic acids in blood cultures that are determined to be positive either by an automated system, by turbidity, or by daily Gram stain even without turbidity, and is indicated to be performed with concomitant Gram stain performed on positive blood culture specimens as per normal

	Francisella tularensis from cultures isolates or from clinical specimens. Negative results do not preclude infection with the biothreat microbial agents targeted by the device and should not be used as the sole basis for diagnosis, treatment, or other patient management decisions. Use is limited to Centers for Disease Control and Prevention (CDC) designated laboratories.	laboratory procedure. In addition, the FilmArray NGDS Warrior Panel may also be used to detect and identify *Yersinia pestis* and *Francisella tularensis* nucleic acids directly from sputum specimens. The FilmArray NGDS Warrior Panel is intended to test individuals with signs and symptoms of infection from biothreat agents and/or individuals who are at risk for exposure or may have been exposed to these agents. The FilmArray NGDS Warrior Panel is indicated as an aid in the diagnosis of anthrax, plague, tularemia, Q fever, and the hemorrhagic fevers caused by Ebola and Marburg viruses, in response to a suspected or confirmed bioterrorism event or outbreaks. It is for diagnostic use in conjunction with other clinical, epidemiologic, and laboratory data, in accordance with the guidelines provided by the appropriate Department of Defense and public health authorities.

		Results are for the presumptive identification of *Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, *Coxiella burnetii*, Ebola virus, and Marburg virus. The definitive identification of *Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, *Coxiella burnetii*, Ebola virus, and Marburg virus requires additional testing and confirmation procedures in consultation with the appropriate Department of Defense and public health authorities for whom reports may be necessary. Negative results do not preclude infection with these biothreat agents and should not be used as the sole basis for diagnosis, treatment, or other patient management decisions. The FilmArray NGDS Warrior Panel is solely for use by United States Department of Defense laboratories, and laboratories designated by the Department of Defense.
Analytes Detected	Nucleic acids from *Francisella tularensis* subspecies: • *F.t. tularensis,* • *F.t. holarctica,*	Nucleic acids from: • *Bacillus anthracis* (virulence plasmids pXO1 & pXO2) • *Yersinia pestis* • *Francisella*

	• *F.t. mediasiatica*	*tularensis* • *Coxiella burnetii* • Ebola Virus • Marburg
Principle of Operation	Nucleic acid amplification and fluorescent probe detection	Nested multiplex RT-PCR followed by melting analysis to confirm identity of amplified product
General Device Characteristic Differences		
Instrumentation	• Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument • QuantStudio Dx Real-Time PCR Instrument	FilmArray 2.0 system
Sample Types	Whole blood (EDTA), pleural fluid, culture isolates	Whole blood (EDTA), positive blood culture, sputum
Sample Preparation	Offboard manual or automated DNA extraction	Automated in the FilmArray NDGS Warrior pouch
Test Interpretation	A sample is considered: • positive for *F. tularensis* DNA if both agent-specific primer sets are reactive, • equivocal for *F. tularensis* DNA if only one agent-specific primer set is reactive, • negative for *F. tularensis* DNA if both agent-specific primer sets are nonreactive.	Automated test interpretation and report generation. User cannot access raw data.

In addition to the similarities and differences between the candidate and predicate devices listed in the table above, the candidate device has an authorized PCCP for modifications to the CDC

Francisella tularensis Real-time PCR assay. The PCCP specifies the validation protocols and acceptance criteria for evaluating the following modifications:

1. Addition of a real-time PCR instrument that is *in vitro* diagnostic (IVD)-labeled and used in accordance with its instructions for use.
2. Addition of PCR master mix that is IVD-labeled and used in accordance with its instructions for use or assessed and determined to be acceptable by the CDC Ancillary Reagent Qualification Program.
3. Addition of:
    a) A manual extraction method that is IVD-labeled and used in accordance with its instructions for use or assessed and determined to be acceptable by the CDC Ancillary Reagent Qualification Program.
    b) Nucleic acid extraction reagents that are IVD-labeled and used in accordance with their instructions for use or assessed and determined to be acceptable by the CDC Ancillary Reagent Qualification Program as part of a cleared manual extraction procedure.
    c) An automated extraction instrument that is IVD-labeled and used in accordance with its instructions for use.
4. Addition of oligonucleotide chemistries that are manufactured under good manufacturing practices (GMP).

VI Standards/Guidance Documents Referenced:

None referenced.

VII Validation Studies and Acceptance Criteria to Support PCCP Modifications:

Specific test methods for clinical and analytical validation are specified in the PCCP for modifications to the *Francisella tularensis* Real-time PCR assay. The following validation studies will be performed in accordance with the PCCP depending on the device modification:

1. Clinical evaluation designed using established and FDA-accepted approaches, with pre-defined acceptance criteria cleared by FDA during this review, to demonstrate substantial equivalence to the predicate device. Clinical performance studies will be conducted to evaluate the following changes according to the established PCCP: addition of a real-time PCR instrument, addition of an automated extraction instrument, addition of nucleic acid extraction reagents or manual extraction method, addition of PCR master mix, or modification of the quencher on the oligonucleotide probes.

2. Limit of Detection (LoD) determination with representative, well-characterized strains of *F. tularensis* according to established and FDA-accepted approaches. Pre-defined acceptance criteria were found to be appropriate by FDA during this review to demonstrate substantial equivalence to the predicate device. An LoD study will be conducted, according to the established PCCP, to evaluate the addition of a real-time PCR instrument, addition of an automated extraction instrument, addition of nucleic acid extraction reagents or manual extraction method, addition of PCR master mix, or modification of oligonucleotide chemistries.

3. Reproducibility study that is designed according to established and FDA-accepted approaches. Pre-defined acceptance criteria were found to be appropriate by FDA during this review to demonstrate substantial equivalence to the predicate device. A reproducibility study will be conducted, according to the established PCCP, to evaluate addition of a real-time PCR instrument or addition of an automated extraction instrument.

4. Carry-over contamination study demonstrating agreement with expected results compared to the predicate device. A carry-over study will be conducted, according to the established PCCP, to evaluate addition of a real-time PCR instrument or addition of an automated extraction instrument.

This PCCP will enable CDC to address evolving public health needs more quickly by making device changes without premarket review. This will help prevent an interruption in testing capability when there is a discontinuation or supply issue for certain instruments and reagents.

VIII Performance Characteristics (if/when applicable):

A Analytical Performance:

1. Precision/Reproducibility:

The reproducibility of the *Francisella tularensis* Real-Time PCR Assay was evaluated using the data from a multicenter study performed at seven different laboratory sites. Three laboratories performed testing using the Applied Biosystems (AB) 7500 Fast Dx instrument and four laboratories performed testing using the QuantStudio (QSDx) Real-time PCR instrument. Samples used in the study were contrived clinical whole blood EDTA and pleural fluid specimens tested collectively as a panel. The panel was composed of twelve contrived clinical specimens spiked with *F. tularensis* subspecies *holarctica* live vaccine strain (LVS) at concentrations near and above the limit of detection, and negative specimens (Table 1). The panel was tested using the *Francisella tularensis* Real-time PCR Assay. Two operators at each study site evaluated one test panel per day for five non-consecutive days resulting in each study site testing a total of ten panels. Acceptance criteria required the reproducibility of the *Francisella tularensis* Real-time PCR Assay to have less than 10% coefficient of variation in test results across sites, operators, and days of testing. Results are shown in below in Tables 2 and 3.

Table 1. *Francisella tularensis* Real-time PCR Assay Reproducibility Specimen Panel

Sample	Matrix	*F. tularensis* LVS Concentration	Volume
1	Whole blood EDTA	High positive (10x LoD)	250 µL
2	Whole blood EDTA	Negative	250 µL
3	Whole blood EDTA	Low positive (3x LoD)	250 µL
4	Whole blood EDTA	Low positive (3x LoD)	250 µL
5	Whole blood EDTA	High positive (10x LoD)	250 µL
6	Whole blood EDTA	Negative	250 µL
7	Pleural fluid	Negative	250 µL
8	Pleural fluid	Low positive (3x LoD)	250 µL

9	Pleural fluid	High positive (10x LoD)	250 µL
10	Pleural fluid	Low positive (3x LoD)	250 µL
11	Pleural fluid	Negative	250 µL
12	Pleural fluid	High positive (10x LoD)	250 µL

Table 2. Summary of Reproducibility Testing of the CDC *Francisella tularensis* Real-time RT-PCR assay using the QuantStudioDx

Panel Sample	Target	Avg. Ct	Std. Dev.	%CV	Overall Agreement	95% (CI)
Whole blood High Positive	Ft primer set 1	28.06	0.53	1.89	80/80	100% (95.42-100.0)
	Ft primer set 2	28.40	0.95	3.33		
	Control	23.45	0.69	2.93		
Whole blood Low Positive	Ft primer set 1	34.75	0.79	2.27	80/80	100% (95.42-100.0)
	Ft primer set 2	35.11	1.01	2.86		
	Control	23.73	0.72	3.03		
Whole blood Negative	Ft primer set 1	n/a	n/a	n/a	80/80	100% (95.42-100.0)
	Ft primer set 2	n/a	n/a	n/a		
	Control	23.65	0.71	3.01		
Pleural Fluid High Positive	Ft primer set 1	26.24	0.62	2.35	80/80	100% (95.42-100.0)
	Ft primer set 2	26.58	0.97	3.66		
	Control	27.85	0.71	2.55		
Pleural Fluid Low Positive	Ft primer set 1	33.14	0.93	2.81	80/80	100% (95.42-100.0)
	Ft primer set 2	33.5	1.08	3.22		
	Control	27.77	0.74	2.68		
Pleural Fluid Negative	Ft primer set 1	n/a	n/a	n/a	80/80	100% (95.42-100.0)
	Ft primer set 2	n/a	n/a	n/a		
	Control	28.14	0.75	2.68		

All panel samples tested at each of the 4 sites using the QuantStudio Dx instrument produced the expected results with each target assay in the CDC *Francisella tularensis* Real-time RT-PCR assay and with a 100% overall agreement (80 out of 80). Coefficients of variation observed across operators, sites, and days of testing were less than 5%.

Table 3. Summary of Reproducibility Testing of the CDC *Francisella tularensis* Real-time RT-PCR assay using the AB 7500 Fast Dx

Panel Sample	Target	Avg. Ct	Std. Dev.	%CV	Overall Agreement	95% CI
Whole blood High Positive	Ft primer set 1	27.59	0.61	2.21	60/60	100% (95.42-100.0)
	Ft primer set 2	27.86	0.68	2.45		
	Control	23.19	0.74	3.17		
Whole blood Low Positive	Ft primer set 1	34.20	0.79	2.32	60/60	100% (95.42-100.0)
	Ft primer set 2	34.30	1.05	3.05		
	Control	23.46	0.74	3.17		
Whole blood Negative	Ft primer set 1	n/a	n/a	n/a	59/60	98.33% (91.15-99.71)
	Ft primer set 2	n/a	n/a	n/a		
	Control	23.44	0.78	3.31		
	Ft primer set 1	25.95	0.75	2.89	60/60	100%

Pleural Fluid High Positive	Ft primer set 2	26.13	0.83	3.16		(95.42-100.0)
	Control	27.62	0.68	2.46		
Pleural Fluid Low Positive	Ft primer set 1	32.49	0.71	2.18	60/60	100% (95.42-100.0)
	Ft primer set 2	32.76	0.90	2.74		
	Control	27.54	0.68	2.45		
Pleural Fluid Negative	Ft primer set 1	n/a	n/a	n/a	60/60	100% (95.42-100.0)
	Ft primer set 2	n/a	n/a	n/a		
	Control	27.91	0.67	2.40		

All panel samples tested at two of the three sites using an AB 7500 Fast Dx instrument produced the expected results with each target assay in the CDC *Francisella tularensis* Real-time RT-PCR assay. One site produced a single inconclusive result on one of the days of testing with a negative whole blood sample. The inconclusive result was limited to one operator and was produced due to one of the two target assays showing an unexpected positive result. Therefore, the overall agreement for this sample across all three sites was 59 out of 60 (98.33%). Testing with all other samples in the panel showed 100% overall agreement (60 out 60). Coefficients of variation observed across operators, sites, and days of testing were less than 5%. The *Francisella tularensi*s Real-time PCR Assay met the acceptance criteria and the data are acceptable.

PCCP:
A reproducibility study will be conducted to evaluate addition of a real-time PCR instrument and addition of an automated nucleic extraction instrument, according to the established PCCP. Data which satisfies pre-specified acceptance criteria detailed in the PCCP will be considered acceptable to support these modifications to the device.

2. Linearity:

Not applicable.

3. Analytical Specificity/Interference:

Inclusivity

In silico Analyses

In silico analyses performed using the National Institutes of Health's National Center for Biotechnology Information's Nucleotide Basic Local Alignment Search Tool and the non-redundant nucleotide (nr/nt) and whole genome shotgun contigs (wgs) databases, indicate the amplicons from the *Francisella tularensis* Real-time PCR Assay primer sets share 100% sequence identity to amplicon sequences from 946 entries representing *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica*, the two subspecies known to cause human tularemia. Six unique sequences for *F. tularensis mediasiatica* are predicted to share 99.2 and 100% sequence identity, respectively with amplicons from the *Francisella tularensis* Real-time PCR Assay primer sets.

Testing of *F. tularensis* strains

Inclusivity testing for the *Francisella tularensis* Real-time PCR Assay was performed using genomic DNA isolated from 37 *F. tularensis* strains and analyzed using the *Francisella tularensis* Real-time PCR Assay on the AB 7500 Fast Dx instrument. Extracted DNA from the 37 *F. tularensis* strains was evaluated in PBS buffer (Table 4) and DNA from 10 of the 37 strains was evaluated in clinical matrix (Table 5).

Table 4. Inclusivity testing of *F. tularensis* subspecies in PBS buffer using the *Francisella tularensis* Real-time PCR Assay on the AB 7500 Fast Dx

		AB 7500 Fast Dx					
		Ft Primer Set 1			Ft Primer Set 2		
Species	**Strain**	**# Pos/ Total**	**Avg. Ct**	**Std. Dev**	**# Pos/ Total**	**Avg. Ct**	**Std. Dev**
F. tularensis subsp. *tularensis*	ND00-0952	3/3	28.77	0.67	3/3	28.88	0.14
	KS00-0948	3/3	28.19	0.21	3/3	28.44	0.28
	UT98-3134	3/3	30.44	0.17	3/3	30.26	0.07
	MO01-2148	3/3	26.74	0.07	3/3	26.68	0.10
	AR99-3448	3/3	26.99	0.34	3/3	27.48	0.08
	OK00-2731	3/3	29.05	0.26	3/3	29.10	0.21
	MA00-2987	3/3	28.46	0.17	3/3	28.12	0.07
	AR01-1117	3/3	26.80	0.08	3/3	27.13	0.04
	NC97-3057	3/3	28.23	0.10	3/3	28.55	0.36
	GA02-5426	3/3	27.77	0.29	3/3	28.45	0.19
	NC99-3990	3/3	27.02	0.15	3/3	27.44	0.07
	CO01-3713	3/3	27.55	0.04	3/3	28.17	0.05
	WY96-3418	3/3	26.70	0.07	3/3	27.14	0.02
	NM99-0295	3/3	27.05	0.13	3/3	27.28	0.04
	UT02-1927	3/3	27.60	0.53	3/3	27.14	0.01
	NM99-1823	3/3	27.60	0.50	3/3	27.46	0.10
	ATCC 6223	3/3	27.41	0.12	3/3	27.52	0.18
	TX00-1591	3/3	29.01	0.26	3/3	28.71	0.10
F. tularensis subsp. *holarctica*	LVS	3/3	27.18	0.10	3/3	27.58	0.16
	RC 503	3/3	27.62	0.20	3/3	27.87	0.29
	KY99-3387	3/3	27.27	0.42	3/3	27.83	0.07
	OR96-0246	3/3	27.16	0.60	3/3	27.65	0.05
	OR96-0463	3/3	28.34	0.11	3/3	28.68	0.11
	CA97-0656	3/3	26.88	0.29	3/3	27.02	0.20
	MO01-1673	3/3	27.71	0.24	3/3	27.25	0.03
	GA02-5387	3/3	27.73	0.27	3/3	27.71	0.13
	CA97-0657	3/3	27.47	0.39	3/3	27.43	0.33
	AZ00-1324	3/3	27.47	0.18	3/3	27.79	0.24
	SP98-2108	3/3	27.40	0.39	3/3	27.10	0.17
	SP03-1781	3/3	27.21	0.05	3/3	27.11	0.45
	KO97-1026	3/3	26.34	0.23	3/3	26.31	0.03
	CN98-5979	3/3	27.65	0.05	3/3	27.77	0.07
	JAP53	3/3	27.69	0.14	3/3	27.29	0.05
F. tularensis subsp.	FSC147	3/3	28.72	0.45	3/3	28.19	0.08

mediasiatica	FSC148	3/3	28.79	0.11	3/3	28.14	0.04
	FSC149	3/3	28.47	0.22	3/3	28.18	0.14
	FSC122	3/3	29.07	0.28	3/3	28.12	0.14

The data in Table 4 indicate all 37 *F. tularensis* strains tested were detected by the *Francisella tularensis* Real-Time PCR Assay.

Ten of the strains evaluated in PBS buffer were also tested in clinical matrix. The ten *F. tularensis* strains evaluated were representative of all three subspecies. Whole blood EDTA and pleural fluid specimens were spiked in triplicate with each *F. tularensis* strain at 3x limit of detection (LoD) and nucleic acid was extracted from each replicate. All strains were detected as shown in Table 5.

Table 5. Inclusivity testing of *F. tularensis* subspecies in Clinical Matrix using the *Francisella tularensis* Real-time PCR Assay on the AB 7500 Fast Dx

Subspecies	Isolate	Clinical Matrix	Avg Ct Ft Primer Set 1	Avg Ct Ft Primer Set 2	Concentration (CFU/mL)
tularensis	CO01-3713	Whole blood EDTA	33.91	32.91	2.58E+03
		Pleural fluid	33.88	33.78	8.30E+02
	NC97-3057	Whole blood EDTA	34.83	35.22	2.19E+03
		Pleural fluid	33.57	33.42	7.07E+02
	ND00-0952	Whole blood EDTA	34.77	34.69	2.10E+03
		Pleural fluid	35.04	34.81	6.41E+02
	MA00-2987	Whole blood EDTA	34.75	34.90	2.16E+03
		Pleural fluid	34.90	33.37	6.56E+02
holarctica	KY99-3387	Whole blood EDTA	35.22	33.08	2.26E+03
		Pleural fluid	34.28	33.33	7.67E+02
	CA97-0656	Whole blood EDTA	35.96	34.19	2.11E+03
		Pleural fluid	35.33	33.97	6.63E+02
	SP03-1781	Whole blood EDTA	35.05	33.84	2.18E+03
		Pleural fluid	35.01	33.61	6.96E+02
	JAP53	Whole blood EDTA	35.59	35.02	2.00E+03
		Pleural fluid	33.71	32.60	8.19E+02
mediasiatica	FSC147	Whole blood EDTA	34.26	33.97	2.27E+03
		Pleural fluid	35.50	35.05	6.15E+02
	FSC148	Whole blood EDTA	34.50	34.69	2.18E+03
		Pleural fluid	35.65	35.81	6.04E+02

These results demonstrate acceptable inclusivity of the *Francisella tularensis* Real-Time PCR Assay.

Exclusivity

Analytical exclusivity was evaluated by testing organisms that could potentially display cross reactivity with the *Francisella tularensis* Real-time PCR Assay. A panel containing high concentrations of DNA from off-panel *Francisella* species and other potential organisms causing similar symptoms, and/or flora commonly observed in blood and pleural fluid were tested using the AB 7500 Fast Dx and QSDx real-time PCR instruments. Twenty-one strains,

representing nine *Francisellaceae* members, were tested using 1.84E+06 GE/mL with the *Francisella tularensis* Real-time PCR Assay (Table 6). Nucleic acid from twenty-four non-*Francisella* species organisms was also tested (Table 7). The acceptance criteria required 100% concordance with the expected negative result with off-panel *Francisella* and near-neighbor species and other common organisms associated with clinical specimens in the selected organism panel.

Table 6. Exclusivity Testing of *F. tularensis* subspecies using the *Francisella tularensis* Real-time PCR Assay

		AB 7500 Fast Dx		QSDx*	
		Number of Positive Results / Total			
Species	**Strain**	**Ft Primer Set 1**	**Ft Primer Set 2**	**Ft Primer Set 1**	**Ft Primer Set 2**
F. novicida	GA99-3548	0/4	0/4	0/1	0/1
	GA99-3549	0/7	0/7	0/1	0/1
	GA99-3550	0/7	0/7	0/1	0/1
	TX07-6608	0/7	0/7	0/1	0/1
F. novicida-like	Fx1 (TX05-2430)	0/4	0/4	0/1	0/1
F. philomiragia	GA01-2794	0/7	0/7	0/1	0/1
	GA01-2801	0/3	0/3	NT	NT
	ATCC-25015	0/3	0/3	NT	NT
	ATCC-25016	0/3	0/3	NT	NT
	ATCC-25017	0/3	0/3	NT	NT
	ATCC-25018	0/3	0/3	NT	NT
F. salina	TX07-7308	0/7	0/7	0/1	0/1
F. uliginis	TX07-7310	0/3	0/3	NT	NT
F. noatunensis subsp. *orientalis*	FSC770	0/4	0/4	0/1	0/1
	FSC771	0/3	0/3	NT	NT
	FSC769	0/3	0/3	NT	NT
	LMG 24256	0/3	0/3	NT	NT
F. noatunensis subsp. *noatunensis*	NO12-2005	0/1	0/1	0/1	0/1
F. hispanensis	FSC454	0/4	0/4	0/1	0/1
Allofrancisella guangzhouensis	FSC996	0/3	0/3	NT	NT
F. opportunistica	PA05-1188	0/3	0/3	NT	NT

NT- Not tested
*Due to limited availability, testing was performed on the AB 7500 Fast Dx only for some strains.

None of the off-panel *Francisella* strains tested with the *Francisella tularensis* Real-time PCR Assay yielded a positive result, supporting that this assay does not cross react with off-panel species of *Francisella.*

Table 7. Exclusivity Testing of Non-*Francisella* organisms using the *Francisella tularensis* Real-time PCR Assay.

Organism		Strain	Genome GE/mL	AB 7500 Fast Dx	
				Ft Primer Set 1	Ft Primer Set 2
				# Pos/Total	# Pos/Total
Gram Negative Bacteria	*Acinetobacter baumannii*	ATCC 19606	8.16E+08	0/1	0/1
	Enterobacter cloacae	ATCC 13047	9.96E+07	0/1	0/1
	Escherichia coli	ATCC 11775	9.32E+07	0/1	0/1
	Haemophilus influenzae	b M05216	1.48E+08	0/1	0/1
	Klebsiella pneumoniae	ATCC 13883	7.20E+07	0/1	0/1
	Proteus mirabilis	ATCC 29906	5.24E+08	0/1	0/1
	Pseudomonas aeruginosa	ATCC 10145	3.85E+08	0/1	0/1
Gram Positive Bacteria	*Actinomyces israelii*	ATCC 12102	1.15E+06	0/1	0/1
	Corynebacterium diphtheriae	ATCC 11913	1.50E+06	0/1	0/1
	Enterococcus faecalis	ATCC 19433	3.17E+06	0/1	0/1
	Enterococcus faecium VRE	ATCC 700221	3.65E+07	0/1	0/1
	Streptococcus pyogenes	ATCC 12344	1.12E+07	0/1	0/1
	Streptococcus agalactiae	ATCC 13813	1.44E+07	0/1	0/1
	Methicillin resistant *Staphylococcus aureus*	ATCC BAA-1683	7.08E+06	0/1	0/1
	Micrococcus luteus	ATCC 4698	7.60E+05	0/1	0/1
	Nocardia asteroides	ATCC 19247	5.72E+06	0/1	0/1
	Propionibacterium acnes	ATCC 33179	1.17E+07	0/1	0/1
	Staphylococcus aureus	F689	8.36E+06	0/1	0/1
	Staphylococcus epidermidis	M22325	4.36E+06	0/1	0/1
	Staphylococcus haemolyticus	ATCC 700564	5.08E+06	0/1	0/1
	Streptococcus pneumoniae	19A M17850	5.24E+06	0/1	0/1
	Streptococcus mutans	ATCC 25175	8.20E+06	0/1	0/1
Virus	Hepatitis C	ATCC VR-3233SD	7.80E+07	0/1	0/1
Yeast	*Candida albicans*	ATCC 18804	2.25E+05	0/1	0/1

The *Francisella tularensis* Real-time PCR Assay did not react with any of the 24 non-

Francisella species organisms tested. Results from the exclusivity testing of the CDC *Francisella tularensis* Real-time RT-PCR assay demonstrated 100% concordance with the expected results, met the acceptance criteria, and are acceptable.

Interference

Potentially interfering substances encountered in blood specimens were evaluated for any impact on performance of the *Francisella tularensis* Real-time RT-PCR assay. Controls and contrived clinical samples were prepared in whole blood EDTA. Pleural fluid was not evaluated as 1) whole blood EDTA represents the more challenging matrix and 2) possesses higher concentrations of known potential interferents when compared to exudative pleural effusions. *Francisella tularensis* subsp. *holarctica* (LVS) was used to contrive whole blood EDTA at a concentration of 3x the LoD to create positive samples. Potentially interfering endogenous and exogenous substances and corresponding diluent material are listed in Table 8 and were tested at concentrations recommended by CLSI in document EP07-A2, Interference Testing in Clinical Chemistry.

Table 8. Substances evaluated for potential interference with the CDC *Francisella tularensis* Real-time RT-PCR assay

Substance		Test Concentration	Diluent
Endogenous	Hemoglobin	10 mg/mL	H_2O
	Bilirubin, conjugated	0.4 mg/mL	H_2O
	Bilirubin, unconjugated	0.4 mg/mL	NaOH
	Human serum albumin	120 mg/mL	H_2O
	Human genomic DNA	4 µg/mL	1x Tris EDTA
	Triglycerides	30 mg/mL	Sucrose
	Triglycerides	3 mg/mL	Sucrose
	Triglycerides	0.3 mg/mL	Sucrose
	Cholesterol	5 mg/mL	H_2O
	Hemolyzed blood	N/A	N/A
Exogenous	Acetylsalicylic acid	3620 µmol/L	EtOH
	Salicylic acid	4340 µmol/L	H_2O
	Ibuprofen	2425 µmol/L	EtOH
	Acetaminophen	1323 µmol/L	H_2O
	Gentamicin	62.8 µmol/L	H_2O
	Doxycycline	67.5 µmol/L	EtOH
	Ciprofloxacin	36.2 µmol/L	H_2O

Four samples were prepared to test each potential interferent:

1. negative control consisting of whole blood EDTA with diluent
2. negative control consisting of whole blood EDTA and potentially interfering substance in diluent
3. Positive sample consisting of *F. tularensis* LVS spiked in whole blood EDTA with diluent

4. Positive sample consisting of *F. tularensis* LVS spiked in whole blood EDTA with potentially interfering substance in diluent.

Nucleic acid from three aliquots of each sample was extracted and tested on the AB 7500 Fast Dx instrument using the CDC *Francisella tularensis* Real-time RT-PCR assay. Interference was assessed by agreement with the expected result and evaluating threshold cycle (Ct) values for statistically significant differences between samples with and without the potentially interfering substance. Acceptance criteria for interference testing required 100% agreement with the expected qualitative result from the CDC *Francisella tularensis* Real-time RT-PCR assay with samples containing potentially interfering substances.

All controls consisting of the clinical background matrix and either diluent alone or diluent with the potentially interfering substance were negative with the CDC Francisella tularensis Real-time RT-PCR assay. Samples containing thirteen potentially interfering substances yielded positive results with the *Francisella tularensis* Real-time RT-PCR assay and were without statistically significant differences when compared to their corresponding sample without the substance present. One out of three sample replicates containing cholesterol (5 mg/mL) produced a negative PCR result with the Ft Primer Set 2 assay and samples containing acetylsalicylic acid (3620 µmol/L) produced Ct results statistically significantly different (p value =0.0063) with the Ft Primer Set 2 assay than the corresponding sample without the substance present. Dilutions of cholesterol and acetylsalicylic acid were tested (in addition to the corresponding sample control) to determine the degree of interference as a function of the substance concentration. Neither substance was confirmed as interfering in the additional testing at the original level or with diluted levels of each substance (Table 9).

Table 9. Confirmatory testing and degree of interference of suspect interfering substances with CDC *Francisella tularensis* Real-time RT-PCR assay

Substance	Test Concentration	Ft Primer Set 1				Ft Primer Set 2			
		Pos/ tested	Avg. Ct	St Dev	p- value	Pos/ tested	Avg. Ct	St Dev	p-value
Cholesterol	5 mg/mL	3/3	34.76	0.09	0.6765	3/3	34.91	0.39	0.7357
	0.5 mg/mL	3/3	34.93	0.37	0.5508	3/3	34.68	0.44	0.8767
	0.05 mg/mL	3/3	35.17	0.14	0.3673	3/3	34.58	0.37	0.9329
Acetylsalicylic acid	3620 µmol/L	3/3	34.83	1.84	0.3925	3/3	34.75	1.66	0.2563
	362 µmol/L	3/3	34.26	0.17	0.8590	3/3	34.18	0.33	0.3138
	36.2 µmol/L	3/3	34.23	0.71	0.7253	3/3	34.03	0.13	0.5369

Acetylsalicylic acid (3620 µmol/L) and cholesterol (5mg/mL) initially demonstrated possible interference with the *Francisella tularensis* Real-time PCR Assay; however, neither substance demonstrated interference upon further testing. Samples containing the other thirteen substances tested showed no interference. No false-positive results were observed with any of the exogenous or endogenous substances tested alone in the background clinical matrix. Final results demonstrated 100% agreement with the expected positive qualitative results with samples containing potentially interfering substances and the data are acceptable.

4. Assay Reportable Range:

Not applicable. This is a qualitative test.

5. Traceability, Stability, Expected Values (Controls, Calibrators, or Methods):

Assay Quality Controls

The quality controls are used to monitor reagent and assay performance as follows:

- Human Specimen Control (HSC), consisting of noninfectious, cultured human cell material suspended in Phosphate Buffered Saline (PBS). HSC is an extraction control to monitor for cross-contamination during extraction and to demonstrate successful recovery of nucleic acid by the extraction process. Purified nucleic acid from the HSC material should yield a positive result with the RNase P (RP) primer and probe set and a negative result with agent-specific primer and probe sets.

- RNase P (RP) Real-time PCR Primer and Probe Set, consisting of two primers and one probe. The RNase P Real-time PCR Primer and Probe Set detects human RNase P and is used with human clinical specimens to indicate that adequate isolation of nucleic acid resulted from the extraction of the clinical specimen.

- *Francisella tularensis* Real-time PCR Positive DNA Control (FtPC), consisting of a plasmid containing targets for the Ft Primer Set 1 and Ft Primer Set 2 primers and probes that serves as a positive control for the assay.

- PCR-grade, nuclease-free water is used as a negative, no template control (NTC) for the *Francisella tularensis* Real-time PCR Assay to show that the PCR reagents are working properly and have not been contaminated with target DNA.

Specimen Stability - Room temperature (15-30°C), Refrigerated (2-8°C), and Frozen (≤-20°C)

Contrived clinical specimens were prepared in whole blood EDTA or pleural fluid using *Francisella tularensis* subsp. *holarctica* (LVS) at concentrations of 3.59E+03 CFU/mL (2.5E+02 Genome Equivalents (GE)/mL) and 1.20E+03 CFU/mL (8.33E+02 GE/mL) respectively to create positive samples at concentrations near the LoD. Five aliquots or each clinical matrix were evaluated at each predetermined timepoint for each storage temperature. The results of stability evaluations for each clinical specimen type under each storage condition and duration are summarized in Tables 10 and 11. Acceptance criteria for all stability evaluations required stored positive samples to show an average increase of no more than 3.0 Ct when compared to the original sample (Day 0) and agreement with the expected qualitative result with the *Francisella tularensis* Real-time RT-PCR assay.

Table 10. Specimen Stability for Whole Blood EDTA

Storage Temp	Assay Target	Day 0		Day 14		Day 30		Day 33		Delta Ct	Day 60		Day 66		Delta Ct
		#Pos/5	Avg. Ct	#Pos/5	Avg. Ct	#Pos/5	Avg. Ct	#Pos/5	Avg. Ct		#Pos/5	Avg. Ct	#Pos/5	Avg. Ct	
-20°C	Ft P1	5/5	34.51	5/5	34.41	5/5	34.26				5/5	34.33	5/5	33.85	-0.65
	Ft P2	5/5	34.20	5/5	34.65	5/5	34.19				5/5	34.77	5/5	33.74	-0.46
	RP	5/5	22.80	5/5	22.47	5/5	22.90				5/5	23.15	5/5	22.80	0
4°C	Ft P1	5/5	34.51	5/5	34.71	5/5	34.74	5/5	35.14	0.63					
	Ft P2	5/5	34.20	5/5	34.52	5/5	34.45	5/5	35.18	0.97					

	RP	5/5	22.80	5/5	22.31	5/5	22.07	5/5	22.00	-0.81					
30°C	Ft P1	5/5	34.51	5/5	31.43	5/5	32.21	5/5	33.01	-1.49					
	Ft P2	5/5	34.20	5/5	31.62	5/5	31.73	5/5	32.97	-1.23					
	RP	5/5	22.80	5/5	22.48	5/5	22.41	5/5	22.82	0.02					

Ft P1 – Ft Primer Set 1
Ft P2 – Ft Primer Set 2
RP – RNase P Control

Table 11. Specimen Stability for Pleural Fluid

Storage Temp	Assay Target	Day 0		Day 14		Day 30		Day 33		Delta Ct	Day 60		Day 66		Delta Ct
		#Pos/5	Avg. Ct	#Pos/5	Avg. Ct	#Pos/5	Avg. Ct	#Pos/5	Avg. Ct		#Pos/5	Avg. Ct	#Pos/5	Avg. Ct	
-20°C	Ft P1	5/5	35.70	5/5	37.11	5/5	36.05				5/5	35.88	5/5	36.68	0.97
	Ft P2	5/5	35.56	5/5	36.30	5/5	36.09				5/5	35.87	5/5	36.32	0.75
	RP	5/5	28.94	5/5	29.09	5/5	28.67				5/5	29.18	5/5	28.84	0.10
4°C	Ft P1	5/5	35.70	5/5	35.72	5/5	35.90	5/5	35.64	-0.06					
	Ft P2	5/5	35.56	5/5	36.04	5/5	35.69	5/5	35.82	0.25					
	RP	5/5	28.94	5/5	28.35	5/5	28.60	5/5	28.66	-0.28					
30°C	Ft P1	5/5	35.70	5/5	31.38	5/5	31.90	5/5	31.29	-4.41					
	Ft P2	5/5	35.56	5/5	31.44	5/5	31.98	5/5	32.00	-3.56					
	RP	5/5	28.94	5/5	28.68	5/5	29.00	5/5	29.16	0.22					

Ft P1 – Ft Primer Set 1
Ft P2 – Ft Primer Set 2
RP – RNase P Control

All replicates produced the expected qualitative result and any increases in Ct values were less than 3.0 over the storage period when tested with the *Francisella tularensis* Real-time RT-PCR Assay. The Ct values observed for the 30°C storage condition showed more change downward compared to the other storage conditions potentially indicating increased amounts of target DNA. This may be due to permissive growth conditions for the *F. tularensis* bacteria and does not negatively impact the detection of *F. tularensis* LVS by real-time PCR. The results from this specimen stability study are adequate to support the specimen stability claims in the device labeling.

Specimen Stability – Freeze-Thaw

Freeze-thaw stability was evaluated with similarly contrived specimens subjected to multiple freeze thaw cycles at -20°C. Contrived specimens were retained at -20°C for at least 23 hours before each thaw cycle. At predefined timepoints and conditions, five aliquots of each sample were individually extracted and tested on the AB 7500 Fast Dx instrument using the CDC *Francisella tularensis* Real-time RT-PCR assay. The results of stability testing of whole blood EDTA and pleural fluid contrived specimens subjected to multiple freeze-thaws are summarized in Table 12.

Table 12. Specimen Freeze-thaw Stability for Whole Blood EDTA and Pleural Fluid

Sample Type	Assay Target	Unfrozen		Freeze-Thaw 1		Freeze-Thaw 2		Freeze-Thaw 3		Delta Ct
		#Pos/5	Avg. Ct	#Pos/5	Avg. Ct	#Pos/5	Avg. Ct	#Pos/5	Avg. Ct	
	Ft P1	5/5	34.51	5/5	34.47	5/5	34.56	5/5	34.80	0.29

Whole Blood EDTA	Ft P2	5/5	34.20	5/5	34.33	5/5	34.21	5/5	34.73	0.53
	RP	5/5	22.80	5/5	22.06	5/5	23.31	5/5	22.10	-0.70
Pleural Fluid	Ft P1	5/5	35.70	5/5	36.54	5/5	35.96	5/5	35.76	0.05
	Ft P2	5/5	35.56	5/5	36.16	5/5	36.21	5/5	36.03	0.46
	RP	5/5	28.94	5/5	28.21	5/5	29.36	5/5	27.86	-1.08

Ft P1 – Ft Primer Set 1
Ft P2 – Ft Primer Set 2
RP – RNase P Control

All samples produced the expected qualitative result and minimal changes in Ct values when tested with the *Francisella tularensis* Real-time RT-PCR Assay. The results from this freeze-thaw stability study are adequate to support the specimen stability claims in the device labeling.

Specimen Stability – Agar Culture Isolate

Agar culture isolate sample stability was evaluated by streak-plating a characterized stock of *F. tularensis* LVS for positive samples and *Yersinia pestis* A1122 for negative samples. Cultures were incubated for 48 hours prior to initiating stability evaluations. Five plates of each organism were placed in each storage condition including room temperature (30°C), incubation temperature (37°C), and refrigerated temperature (4°C). Samples were prepared for testing using single, picked colonies from a plate and processing using an established CDC bacterial cell lysate preparation procedure. DNA concentrations were determined with the lysates to enable estimates of genome equivalents/mL. Five samples representing each timepoint and storage condition were individually extracted and tested on the AB 7500 Fast Dx instrument using the CDC *Francisella tularensis* Real-time RT- PCR Assay. The results of stability evaluations of bacterial culture isolates grown on agar and stored under each condition and duration are summarized in Table 13.

Table 13. Stability of *F. tularensis* Bacterial Culture Isolates Grown on Agar

Storage Temp	Assay Target	Day 0			Day 2			Day 7			Day 8			Delta Ct
		#Pos/5	Avg. GE/mL	Avg. Ct	#Pos/5	Avg. GE/mL	Avg. Ct	#Pos/5	Avg. GE/mL	Avg. Ct	#Pos/5	Avg. GE/mL	Avg. Ct	
4°C	Ft P1	5/5	1.73E+09	18.13	5/5	1.16E+09	17.39	5/5	6.08E+08	19.10	5/5	1.91E+09	16.92	-1.21
	Ft P2	5/5		17.89	5/5		17.76	5/5		18.39	5/5		17.20	-0.69
30°C	Ft P1	5/5	1.73E+09	18.13	5/5	3.37E+08	18.58	5/5	1.87E+08	29.37	5/5	2.89E+08	18.94	0.81
	Ft P2	5/5		17.89	5/5		18.92	5/5		28.85	5/5		18.73	0.84
37°C	Ft P1	5/5	1.73E+09	18.13	5/5	1.82E+09	16.85	5/5	1.01E+08	22.88	5/5	4.23E+08	17.50	-0.63
	Ft P2	5/5		17.89	5/5		17.29	5/5		22.65	5/5		17.59	-0.30

Ft P1 – Ft Primer Set 1
Ft P2 – Ft Primer Set 2

All samples from all storage conditions and durations produced the expected qualitative result. The higher Ct values on Day 7 for the 30°C storage condition exceeded the acceptance criteria of no greater than a 3.0 Ct increase relative to T = 0, however these results were acceptable due to the observed results from Day 8 which were less than 1 Ct different from the original, Day 0 results. The sponsor rationalized that Day 7 results were attributed to technician error in

processing samples collected from the agar plates. The results from this agar culture isolate stability study are adequate to support the specimen stability claims in the device labeling.

In conclusion, the following claims regarding storage and stability of specimens and samples used with the *Francisella tularensis* Real-time RT-PCR Assay are supported by the stability evaluation data:

1. Refrigerated (2-8°C) or room temperature (15-30°C) whole blood-EDTA and pleural fluid specimens tested within 30 days of collection.
2. Frozen whole blood EDTA and pleural fluid specimens (≤-20°C) tested within 60 days of collection.
3. Frozen whole blood EDTA and pleural fluid specimens (≤-20°C) that underwent no more than 3 freeze/thaw cycles.
4. Culture plates refrigerated (2-8°C), stored at room temperature (15-30°C), or incubated at 35-38°C for up to 7 days.

6. Detection Limit:

The limit of detection (LoD) of the *Francisella tularensis* Real-time RT-PCR assay was determined in Phosphate Buffered Saline (PBS), whole blood EDTA, and pleural fluid. To determine the LoD in PBS genomic DNA from *F. tularensis tularensis* SchuS4, *F. tularensis holarctica* LVS, and *F. tularensis mediasiatica* FSC147 was purified and quantified. Dilutions of genomic DNA made in PBS buffer were tested using the *Francisella tularensis* Real-time PCR Assay in 20 replicates. The LoD was determined from the lowest concentration of GE/mL that produced positive results in ≥95% of replicate samples with both the Ft Primer Set 1 and Ft Primer Set 2 primers and probes of the *Francisella tularensis* Real-time PCR Assay on both the AB 7500 Fast Dx and QSDx instruments (Table 14). The highlighted concentrations in Table 14 represent the LoD for each *F. tularensis* strain in PBS.

Table 14. *Francisella tularensis* Real-time PCR Assay LoD with Genomic DNA in PBS

		AB 7500 Fast Dx						QSDx					
		Ft Primer Set 1			Ft Primer Set 2			Ft Primer Set 1			Ft Primer Set 2		
Strain	**Conc. (GE/mL)**	**#Pos/ Total**	**Avg. Ct**	**Std. dev**	**#Pos/ Total**	**Avg. Ct**	**Std. dev**	**#Pos/ Total**	**Avg. Ct**	**Std. dev**	**#Pos/ Total**	**Avg. Ct**	**Std. dev**
SchuS4	9.62E+03	20/20	34.77	0.33	NT	NT	NT	20/20	34.18	0.29	NT	NT	NT
	4.81E+03	20/20	35.77	0.52	20/20	35.42	0.79	20/20	35.12	0.52	20/20	36.06	0.55
	2.41E+03	19/20	36.99	0.40	20/20	36.84	1.01	20/20	36.36	0.67	19/20	37.39	0.82
	9.62E+02	13/20	39.07	0.71	16/20	37.41	0.87	17/20	37.42	1.00	17/20	38.37	0.70
	9.62E+01	1/20	39.74	N/A	6/20	38.41	0.21	1/20	38.75	N/A	4/20	39.40	0.51
LVS	9.62E+03	20/20	34.08	0.84	20/20	34.46	0.30	20/20	33.91	0.39	20/20	34.53	0.29
	4.81E+03	20/20	34.91	0.39	20/20	35.70	0.38	20/20	35.21	0.44	20/20	35.78	0.32
	2.41E+03	20/20	35.98	0.58	20/20	36.64	0.44	20/20	35.90	0.76	19/20	37.06	1.74
	9.62E+02	17/20	37.65	1.24	15/20	37.71	0.68	20/20	37.19	0.85	19/20	38.03	0.77
	9.62E+01	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
FSC147	9.62E+03	20/ 20	36.55	0.71	20/ 20	35.87	0.64	20/ 20	35.95	0.75	20/20	36.73	0.53
	4.81E+03	20/ 20	37.13	0.68	19/ 20	37.38	1.58	20/ 20	37.12	0.87	19/20	37.75	0.63
	2.41E+03	16/ 20	38.21	1.22	19/ 20	37.89	0.97	16/ 20	37.75	1.34	13/20	38.86	1.24

	9.62E+02	11/ 20	38.84	0.66	12/ 20	37.97	0.69	10/ 20	38.73	0.90	9/20	39.03	0.76
	9.62E+01	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT

NT – Not Tested

Range-finding for the LoD of the *Francisella tularensis* Real-time PCR assay in clinical matrices was performed using 10-fold serial dilutions of whole blood EDTA spiked with whole cell *F. tularensis* from a frozen glycerol stock and tested in triplicate. Each dilution replicate was extracted and tested on the *Francisella tularensis* Real-time PCR assay using the AB 7500 Fast Dx and QSDx real-time PCR instruments. The preliminary LoD was determined to be the lowest concentration to generate positive results from the *Francisella tularensis* Real-time PCR assay on both instruments (5.20E+03 GE/mL). To confirm the LoD frozen glycerol stocks of *F. tularensis* LVS were used to prepare 3-fold dilution series in whole blood EDTA and pleural fluid. The concentration of *F. tularensis* LVS in the contrived samples was determined through enumeration of colony forming units (CFU/mL) by plating triplicate samples of serial dilutions in PBS. The concentration of *F. tularensis* LVS in GE/mL was determined by extracting DNA from the starting preparation of *F. tularensis LVS* and quantifying the DNA concentration. This preparation was used to generate a standard curve for both the AB 7500 Fast Dx and QuantStudio Dx instruments and to calculate the GE/mL. The confirmed LoD was determined to be the lowest concentration in CFU/mL that produced a positive result with the *Francisella tularensis* Real-time RT-PCR assay ≥95% of the time when testing 20 replicates extracted individually. Results with each instrument are summarized in Tables 15 and 16 and include dilutions 3-fold above and 3-fold below the LoD concentration, which is highlighted for each sample matrix type in each table.

Table 15. LoD confirmation of the *Francisella tularensis* Real-time PCR Assay with *F. tularensis* subsp. *holarctica* (LVS) in whole blood EDTA and pleural fluid samples on the AB 7500 Fast Dx

Sample Matrix	AB 7500 Fast Dx									
	Ft Primer Set 1					Ft Primer Set 2				
	CFU/mL	Conc. (GE/mL)	#Pos/ Total	Avg. Ct	Std. dev	CFU/mL	Conc. (GE/mL)	#Pos/ Total	Avg. Ct	Std. dev
Whole blood (EDTA)	2.53E+03	5.49E+03	19/20	34.78	1.11	2.53E+03	3.48E+03	19/20	35.11	0.98
	8.44E+02	3.11E+03	20/20	36.22	0.88	8.44E+02	3.05E+03	20/20	35.81	0.50
	2.81E+02	3.99E+02	15/20	37.63	0.93	2.81E+02	4.47E+02	8/20	37.74	0.65
Pleural fluid	5.67E+02	2.19E+03	20/20	35.68	0.62	5.67E+02	2.20E+03	20/20	34.58	0.56
	1.89E+02	7.91E+02	19/20	36.44	1.04	1.89E+02	1.03E+03	20/20	36.12	0.91
	7.57E+01	2.17E+04	11/20	36.90	1.21	7.57E+01	8.32E+03	12/20	37.03	0.81

Table 16. LoD confirmation of the *Francisella tularensis* Real-time PCR Assay with *F. tularensis* subsp. *holarctica* (LVS) in whole blood EDTA and pleural fluid samples on the QSDx

Sample Matrix	AB 7500 Fast Dx									
	Ft Primer Set 1					Ft Primer Set 2				
	CFU/mL	Conc. (GE/mL)	#Pos/ Total	Avg. Ct	Std. dev	CFU/mL	Conc. (GE/mL)	#Pos/ Total	Avg. Ct	Std. dev
	2.53E+03	5.49E+03	19/20	34.78	1.11	2.53E+03	3.48E+03	19/20	35.11	0.98
	8.44E+02	3.11E+03	20/20	36.22	0.88	8.44E+02	3.05E+03	20/20	35.81	0.50

Whole blood (EDTA)	2.81E+02	3.99E+02	15/20	37.63	0.93	2.81E+02	4.47E+02	8/20	37.74	0.65
Pleural fluid	5.67E+02	2.19E+03	20/20	35.68	0.62	5.67E+02	2.20E+03	20/20	34.58	0.56
	1.89E+02	7.91E+02	19/20	36.44	1.04	1.89E+02	1.03E+03	20/20	36.12	0.91
	7.57E+01	2.17E+04	11/20	36.90	1.21	7.57E+01	8.32E+03	12/20	37.03	0.81

The *Francisella tularensis* Real-time PCR assay LoD was approximately 4-fold lower in the pleural fluid clinical matrix in comparison to whole blood EDTA and similar assay performance was observed with the AB 7500 Fast Dx and QSDx instruments. The results from the LoD determination studies are acceptable.

PCCP:
An LoD study will be conducted to evaluate the addition of a real-time PCR instrument, addition of an automated extraction instrument, addition of nucleic acid extraction reagents or manual extraction method, addition of PCR master mix, or modification of the oligonucleotide chemistries, according to the established PCCP. Data demonstrating LoD equivalency will be considered acceptable to support these modifications to the device.

7. Assay Performance with Culture Isolates:

Pure culture stocks of *F. tularensis* LVS and SchuS4 were used to inoculate chocolate agar plates and incubated at 37°C for 48-72 hours. After confirming purity and colony morphology five individual isolated colonies were used to create cell lysates for each *F. tularensis* strain. Cell lysates were tested using the *Francisella tularensis* Real-time PCR assay primer sets on the AB 7500 Fast Dx and QSDx instruments. Acceptance criteria required the positive identification of *F. tularensis* strains prepared from isolated colonies collected from growth on chocolate agar. Table 17 shows the results for *Francisella tularensis* Real-time PCR assay testing with SchuS4 and LVS culture isolates.

Table 17. Testing of *F. tularensis* subspecies culture isolates with the *Francisella tularensis* Real-time PCR Assay

***F. tularensis* subspecies (strain)**	**AB 7500 Fast Dx**						**QSDx**					
	Ft Primer Set 1			**Ft Primer Set 2**			**Ft Primer Set 1**			**Ft Primer Set 2**		
	#Pos/ Total	**Avg. Ct**	**Std. dev**	**#Pos/ Total**	**Avg. Ct**	**Std. dev**	**#Pos/ Total**	**Avg. Ct**	**Std. dev**	**#Pos/ Total**	**Avg. Ct**	**Std. dev**
***tularensis* (SchuS4)**	5/5	15.81	0.75	5/5	15.85	0.78	5/5	15.70	1.13	5/5	15.37	1.06
***holarctica* (LVS)**	5/5	13.69	0.45	5/5	13.96	0.52	5/5	12.90	0.54	5/5	14.04	0.66

All replicates tested were positive and produced low Ct values with the assay due to the high concentration of analyte collected from culture isolates. The *Francisella tularensis* Real-time PCR assay positively identified *F. tularensis* strains prepared from isolated colonies collected from culture on chocolate agar and the results from this study are acceptable.

8. Assay Cut-Off:

A PCR is considered reactive/detected if the exponential phase of its amplification curve crosses the threshold line within 40 cycles, nonreactive/not detected if the exponential phase of its amplification curve does not cross the threshold line within 40 cycles. If both Ft Primer Set 1 and Ft Primer Set 2 are reactive the sample is considered positive. If only one Ft Primer Set is reactive the sample is considered equivocal. If both Ft Primer Set 1 and Ft Primer Set 2 are nonreactive the sample is considered negative.

9. Carry-Over:

Carry-over data for instruments cleared for use with the *Francisella tularensis* Real-time PCR assay, the Applied Biosystems 7500 Fast Dx Real-Time PCR System (K080570) and the QuantStudio Dx Real-time PCR Instrument (K123998), were reviewed and found acceptable in previous clearances.

PCCP:
A carry-over study will be conducted to evaluate the addition of a real-time PCR instrument or addition of an automated extraction instrument, according to the established PCCP. Data demonstrating pre-specified agreement with the expected results will be considered acceptable to support these modifications to the device.

B Comparison Studies:

1. Method Comparison with Predicate Device:

As a supplementary analysis, performance characteristics for the *Francisella tularensis* Real-time PCR assay were compared to the predicate device, the FilmArray NGDS Warrior Panel, by testing nine *F. tularensis* strains (inclusivity) and ten negative (exclusivity) samples (Table 18) including *Francisellaceae* strains not classified as *F. tularensis*. Samples were tested using the FilmArray NGDS Warrior Panel using a concentration of 1.0E+07 GE/mL.

Table 18. Organism Panel for Comparison of the *Francisella tularensis* Real- time PCR Assay and the FilmArray NGDS Warrior Panel

Organism	Strain ID
Francisella tularensis subsp. *tularensis*	ND00-0952
Francisella tularensis subsp. *tularensis*	NC97-3057
Francisella tularensis subsp. *tularensis*	CO01-3713
Francisella tularensis subsp. *holarctica*	SP03-1781
Francisella tularensis subsp. *holarctica*	RC 503
Francisella tularensis subsp. *holarctica*	GA02-5387
Francisella tularensis subsp. *holarctica*	CA97-0656
Francisella tularensis subsp. *holarctica*	JAP53
Francisella tularensis subsp. *mediasiatica*	FSC147
Francisella novicida	GA99-3548
Francisella novicida	TX07-6608

Francisella hispaniensis	FSC454
Francisella philomiragia	GA01-2794
Francisella noatunensis subsp. *orientalis*	FSC770
Francisella noatunensis subsp. *noatunensis*	NO12-2005
Francisella salina	TX07-7308
Francisella uliginis	TX07-7310
Allofrancisella guangzhouensis	FSC996
Francisella opportunistica	PA05-1188

The overall agreement observed between the *Francisella tularensis* Real-time PCR assay and FilmArray NGDS Warrior Panel was 100% for all *F. tularensis* strains and 70% for the non-*F. tularensis* organisms on the panel (Table 19).

Table 19. Percent agreement between the *Francisella tularensis* Real-time PCR Assay and the FilmArray NGDS Warrior Panel

		Comparator Method: FilmArray NGDS Warrior Panel		
	Assay target (result)	**Positive**	**Negative**	**% Agreement**
***Francisella tularensis* Real- time PCR Assay**	Ft Primer Set 1 (+)	9	0	100
	Ft Primer Set 2 (+)	9	0	100
	Ft Primer Set 1 (-)	3	7	70
	Ft Primer Set 2 (-)	3	7	70

The FilmArray NGDS Warrior Panel reacted with 3 non-*F. tularensis Francisella* strains. These three strains are classified as either *Francisella novicida* or *Francisella hispaniensis*. The *Francisella tularensis* Real-time PCR assay did not react with any of the non-*F. tularensis Francisella* species. The FilmArray NGDS Warrior Panel was designed to detect *Francisella novicida* and *Francisella hispaniensis* strains and discordant results were expected with these strains. Results from this study support equivalent performance between the FilmArray NGDS Warrior Panel and *Francisella tularensis* Real-time PCR assay in detecting *F. tularensis* strains.

2. Matrix Comparison:

Not applicable.

C Clinical Studies:

1. Clinical Performance

Due to the rare incidence of tularemia in humans, natural positive clinical specimens available for prospective collection and archived specimens for retrospective evaluation are difficult to obtain. Therefore, the clinical performance of the *Francisella tularensis* Real-Time PCR assay was evaluated using contrived clinical whole blood EDTA and pleural fluid specimens.

Fifty unique human whole blood-EDTA specimens and 50 unique human pleural fluid specimens were used to prepare contrived specimens for the study. The whole blood-EDTA and pleural fluid specimens were split into two aliquots. One aliquot of each unique specimen was contrived using a characterized and quantified whole cell preparation of *F. tularensis* LVS. For each sample type 25 contrived specimens were prepared at 10x LoD (high positive specimens) and 25 specimens were prepared at 3x LoD (low positive specimens). The remaining aliquot was used as a negative specimen. A total of 50 contrived positive specimens and 50 negative specimens were evaluated for each sample type. Contrived and negative specimens were labeled and assembled into a panel to allow randomization and blinding of samples prior to testing by laboratory technicians. Extractions were performed using the QIAGEN QIAamp DSP Blood Mini Kit and each sample tested with the *Francisella tularensis* Real-time PCR assay using the AB 7500 Fast Dx or QSDx instrument platforms. Specimen testing was divided among three laboratory technicians who each performed testing on three non-consecutive days using one of three separate instruments (one QSDx and two AB 7500 Fast Dx instruments).

The positive percent agreement (PPA) and negative percent agreement (NPA) with the expected result and 95% confidence intervals were calculated for each specimen type. Positive percent agreement was calculated using the equation (TP/(TP+FN)) x 100% (where TP = true positive and FN = false negative). Negative percent agreement was calculated using the equation (TN/(TN+FP)) x 100% (where TN = true negative and FP = false positive). The acceptance criteria for clinical performance of the *Francisella tularensis* Real-time PCR assay required a PPA and NPA greater than or equal to 96% for each parameter. The results of whole blood EDTA and pleural fluid clinical specimen testing with the *Francisella tularensis* Real-time PCR assay are summarized in Table 20. For equivocal results, the PPA was calculated with equivocal samples counted as either all positives or as all negatives to represent best- and worst-case scenarios.

Table 20. Clinical performance of the *Francisella tularensis* Real-time PCR assay

Specimen		Total	Positive	Equivocal	Negative	PPA (95% CI)		NPA (95% CI)
						Equivocal as Positive	Equivocal as Negative	
Whole blood-EDTA	Low Positive	25	23	2	0	100% (92.89-100%)	96% (86.29-99.51%)	
	High Positive	25	25	0	0			
	Negative	50	0	0	50			100% (92.89 - 100%)
Pleural Fluid	Low Positive	25	23	2	0	98% (89.35-99.95%)	94% (83.45-98.75%)	
	High Positive	25	24	0	1			
	Negative	50	0	0	50			100% (92.89-100%)

The results from the clinical performance evaluation met the acceptance criteria when equivocal results were treated as positives. Per the assay instructions for use, samples that yield equivocal results require further testing/analysis. These data support substantial equivalence of the *Francisella tularensis* Real-time PCR assay to the predicate device.

PCCP:
Testing of clinical samples will be conducted to evaluate addition of a real-time PCR instrument, addition of an automated extraction instrument, addition of nucleic acid extraction reagents or manual extraction method, addition of PCR master mix, or modification of the quencher on the probes, according to the established PCCP. Data demonstrating that the performance meets or exceeds pre-specified point estimates for both positive and negative percent agreement (PPA and NPA), and the associated lower bounds of the 95% confidence intervals when compared to comparator will be considered acceptable to support these modifications to the device.

D Clinical Cut-Off:

Not applicable.

E Expected Values/Reference Range:

Not applicable.

IX Proposed Labeling:

The labeling supports the finding of substantial equivalence for this device.

If the validation studies are performed according to the specified protocols in the PCCP and the validation data meet the specified acceptance criteria established in the PCCP, the modification and supporting validation study results will be included in updated device labeling. The labeling will clearly describe the added modification(s).

X Conclusion:

The submitted information in this premarket notification, which includes a PCCP for modifications to the *Francisella tularensis* Real-time PCR assay, is complete and supports a substantial equivalence decision.