Comprehensive Validation Report

Microsart® AMP Mycoplasma

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1 Introduction

Mycoplasmas are known as important contaminants of biological products derived from cell lines in the Biopharmaceutical Industry affecting every parameter of a cell culture system. Contaminated cultures can result in production loss and unsafe products. Mycoplasmas are the smallest of the free-living organisms. Unlike viruses, mycoplasmas can reproduce outside of living cells. Many species within the genera *Mycoplasma*, *Acholeplasma* and *Spiroplasma* thrive as parasites in human, bird, plants and animal hosts. Some species can cause disease in humans. Such contaminations can arise from the contamination of the source cell lines themselves (cell substrates) or from adventitious introduction of mycoplasmas during production. Based on this, contamination risk guidelines and technical papers are published to give guidance on mycoplasmas safety for the manufacture of biological products as for instance the European Pharmacopoeia, chapter 2.6.7., “Mycoplasmas”.

The diagnostic kit Microsart® AMP Mycoplasma is used for the detection of *Mollicutes* (*Mycoplasma*, *Acholeplasma*, and *Spiroplasma*) contamination in cell cultures and other cell culture derived biologicals. The kit utilizes the polymerase chain reaction (PCR), which was established as the method of choice for high sensitivity. The kit includes a Primer/Probe/Nucleotide mix containing a FAM labeled probe specific for a broad range of different mycoplasma species. False negative results due to PCR inhibitors or improper DNA extraction are detected by the internal amplification control. The Internal Control DNA can be added to the sample prior to DNA extraction and analysis for verification of the complete process (DNA extraction and PCR reaction). The Internal Control DNA can also be added directly to the PCR master mix to act as a PCR control only. The amplification of the control reaction is detected at 610 nm (ROX channel) and the mycoplasma-specific sequence at 520 nm (FAM channel). The kit contains dUTP instead of dTTP, so the option is available to degrade amplicons from previous analysis by use of uracil-N-glycosylase (UNG). Thus the occurrence of false-positive results can be minimized.

2 Objective

A study was designed to evaluate the *Mycoplasma* detection capability for the Mycoplasma Detection Kit Microsart® AMP Mycoplasma for qPCR. Mycoplasmas and the protocol for validation are described in section 2.6.7 of the *European Pharmacopoeia*. This chapter includes guidelines and specifications for relevant parameters like specificity, detection limit and robustness in comparison to the traditional culture method which had been evaluated in detail.

The general validation for the Mycoplasma Detection Kit Microsart® AMP Mycoplasma for qPCR, was done for product version 1 according to section 2.6.7 of the European Pharmacopoeia, section 2.6.21 of the EP “Nucleic acid amplification techniques” (NAT, PCR), and with respect to ICH guideline Q2B. As the method employed is used for the purpose of obtaining a qualitative result only (positive/negative), it was not necessary to demonstrate compliance with all individual requirements of ICH Q2B. This opinion is based on the requirements of the European Pharmacopoeia 2.6.21. The validation plan considered the core requirements of validation in accordance with ICH Q2B in the context of their applicability to the qualitative nature of the test employed.

With finalizing the validation of the product version 1 new results of our permanent product optimization efforts became available. As these findings were considered as relevant for the performance of the kit and for the users in general, these modifications were implemented into the user’s protocol and the product design instantly resulting in the Microsart® AMP Mycoplasma, version 2. These product modifications required a partial validation of relevant performance aspects. In detail the following improvements were evaluated:

1. Replacement of an antibody-inhibited hot-start polymerase against an aptamer-protected hot-start polymerase.
2. Polymerase-optimized buffer.
3. Sample concentration using Vivaspin 6 and 20 columns.
4. Use of the Internal Control DNA as an extraction control.
5. Updated protocol for qPCR cycler.
6. Updated protocol for DNA extraction comprising a protease incubation step which might be required for highly proteinogenic samples.
7. The robustness testing shall reflect a test method independent from mycoplasma spike material prepared during this study and commercially available to allow users a direct comparison of the test performance.

Based on a risk assessment the product modifications may affect the relevant parameters specificity, detection limit, and robustness. This report includes all data derived with product version 1 and the repeated validation of these specifications with product version 2 in comparison to the traditional culture method. All parameters were validated with characteristic and most challenging test setups.

3 Definitions and Abbreviations

Av	average
CCS	cell culture supernatant
Dev	Standard Deviation
DMEM	Dulbecco´s modified Eagles medium
DNA	deoxyribonucleic acid
EP	European Pharmacopoeia
FBS	Fetal Bovine Serum
g	g-force (unit for measurement of rotation speed of centrifugation)
g/l	gram per liter
GLP	good laboratory practice
GMP	good manufacturing practice
ICH	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
LOD_{95}	concentration, where 95 % of all samples were positive
m	meter
mg/ml	milligram per milliliter
N/A	not applicable
N/D	not defined
N/M	not measured
nm	nanometer
N/P	not provided
NTC	no template control
OD_{260}	optical density (at a wavelength of 260 nm)
PBS	phosphate buffered saline
PC	positive control
PCR	polymerase chain reaction
PDA	Parenteral Drug Association
CFU/ml	colony-forming units per milliliter
pH	*potentia hydrogenii*
RPMI	Roswell Park Memorial Institute
s	second
SSB	Sartorius Stedim Biotech
TE80	10 mM Tris.HCl (pH 8.0), 0.1 mM EDTA
Tris	tris (hydroxylmethyl amino methane)

4 Responsibilities

Minerva Biolabs GmbH was responsible for developing the test protocol in association with Sartorius Stedim Biotech GmbH (Sartorius Stedim). Sartorius Stedim was responsible for reviewing the test protocol to ensure its accuracy, completeness and validity.

Test initiation was scheduled by Minerva Biolabs and Sartorius Stedim after approval of the validation plan by signing and exchanging a copy of the plan cover page and the necessary material for testing have been received at Sartorius Stedim.

Sartorius Stedim and Minerva Biolabs technicians executed the test protocol. Minerva Biolabs and Sartorius Stedim were responsible for the execution of dedicated parts of the protocol.

Minerva Biolabs drafted the validation report and Sartorius Stedim Biotech reviewed and approved the document to ensure its validity. The report was closed by exchanging a signed copy of the report cover page.

Deviations, including test failures and protocol modifications which occurred during the execution of the test protocol had been discussed between Minerva Biolabs and Sartorius Stedim.

5 Validation Plan

5.1 Test Materials

The tests were conducted using the following test systems, product solutions and materials.

5.1.1 Test System

The test system used for the detection of mycoplasma during this study is as follows:

Table 1. Test System Information

System type	Lot	Amount	Supplied by	Storage Conditions
Microsart® AMP Mycoplasma	951S7091 951S1101 951S2101 951S1111 951S1032 9511S2052 9511S1082	70x 25 reactions 40x 25 reactions 80x 25 reactions 60x 25 reactions 50x 25 reactions 33x 25 reactions 40x 25 reactions	Minerva	2 – 8 °C
Microsart® AMP Coating Buffer	9523S1052	10 packs (20 x 2 ml each)	Minerva	2 - 8 °C
Microsart® AMP Extraction	9522S1052	4x 200 extractions	Minerva	ambient temperatures
Vivaspin 6, VS0642, 100,000	11VS0635	20 units	Sartorius	ambient temperatures
Vivaspin 20, VS2042, 100,000	11VS2029	20 units	Sartorius	ambient temperatures

5.1.2 Matrix Solution

The matrix solutions used for the study have the formulation and characteristics as follows:

Table 2. Matrix Formulation

Product Ingredient	Manufacturer	Catalog No.	Lot No.	Shelf Life
PBS Dulbecco	Biochrom AG	L1815	0448W	04.2015
RPMI medium	Biochrom AG	FG 1215	1608W	05.2012
DMEM medium	Biochrom AG	FG 0415	1499W 1004X	04.2012 10.2013
Tris buffer, pH 8.5	Minerva Biolabs GmbH	N/A	520T2031	03.2013
TE80	Minerva Biolabs GmbH	N/A	N/A	N/A
Fetal bovine serum	Biochrom AG	S0615	0248W	02.2015
Horse serum	Biochrom AG	S9133	0533W	05.2014
Goat serum, origin France	Antibody Production Services Ltd.	S-707-S	A8070711	N/D

5.1.3 Microorganisms and Eukaryotic Material

Microorganisms and eukaryotic material used for spiking or specificity testing during the study are described in the following tables:

Table 3. *Mollicutes* Description

Species	Family	Natural Host	Origin	No.	Quantification
EP 2.6.7. listed mycoplasma species used for spiking					
Acholeplasma laidlawii	*Acholeplasmataceae*	ubiquitous	NCTC	10116	See chapter 5.5
Mycoplasma fermentans	*Mycoplasmataceae*	human		10117	
Mycoplasma hyorhinis		mammal		10130	
Mycoplasma orale		human		10112	
Mycoplasma pneumoniae		human		10119	
Mycoplasma gallisepticum		bird		10115	
Mycoplasma synoviae		mammal		10124	
Mycoplasma arginini		mammal		10129	
Spiroplasma citri		plant		10164	
DNA from *Mycoplasma* species used for specificity testing					
Mycoplasma arthritidis	*Mycoplasmataceae*	mammal	Minerva Biolabs	51-0162	OD_{260}
Mycoplasma genitalium		human		51-0195	
Mycoplasma hominis		human		51-0111	
Mycoplasma penetrans		mammal		51-1746	
Mycoplasma salivarium		human		51-0113	
Ureaplasma urealyticum		human		51-0177	

Table 4. Description of non-*Mollicutes* bacterial strains and eukaryotic materials

Species	Family	Natural Host	Origin	No.	Quantification
DNA from bacteria species used for specificity testing					
Chlamydia trachomatis	*Chlamydiaceae*	human	Minerva Biolabs	51-0440	OD_{260}
Clostridium acetobutylicum	*Clostridiaceae*	ubiquitous		51-0792	
Lactobacillus acidophilus	*Lactobacillaceae*	human		51-1723	
Legionella pneumophila	*Legionellaceae*	amoeba		51-0101	
Staphylococcus aureus	*Stapylococcaceae*	ubiquitous		51-3361	
Pseudomonas aeruginosa	*Pseudomonaceae*	ubiquitous		51-0071	
Streptococcus pneumoniae	*Streptococcaceae*	human		51-0566	

Species	Family	Natural Host	Origin	No.	Quantification
DNA from bacteria species used for specificity testing (continued)					
Micrococcus luteus	*Micrococcineae*	ubiquitous	DSMZ	20030	OD_{260}
Candida albicans	*Sacharomycetales*	ubiquitous		1386	
Enterococcus faecalis	*Enterococcaceae*	Human, mammal		20478	
Enterobacter aerogenes	*Enterobacteriaceae*	ubiquitous		30053	
Escherichia coli	*Enterobacteriaceae*	ubiquitous		30083	
Proteus mirabilis	*Enterobacteriaceae*	ubiquitous		4479	
Salmonella enterica	*Enterobacteriaceae*	Human, mammal		17058	
Bacillus subtilis subsp. spizizenii	*Bacillaceae*	ubiquitous		347	
Staphylococcus epidermidis	*Stapylococcaceae*	skin		20044	
Bacillus cereus	*Bacillaceae*	ubiquitous		31	
DNA from cell cultures and tissues used for specificity testing					
Species	Family	Natural Host	Origin	No.	Quantification
Vero-B4	kidney	african green monkey	DSMZ	ACC 33	OD_{260}
Per.C6	human embryonic retinoplasts	human	Crucell	B127-006	
RK13	kidney	rabbit	ECACC	21715	
CHO-K1	ovary	hamster	DSMZ	ACC 110	
Murine Genomic DNA, 200 ng/µl	blood	mouse	Bioline	LotNo: MS-107G; CatNo: Bio-35027	
Calf Thymus DNA	thymus	bovine	Invitrogen	15633019	

5.2 Mycoplasma Harvest

All mycoplasma listed in Table 3 as used for spiking had been cultivated in broth according to EP 2.6.7 until a slight colour change of the phenol red indicator contained in either Frey or Hayflick medium became barely visible.

The culture broth was divided into two portions: One portion was used for quantification of the mycoplasma. The broth was vortexed intensively prior titration to break up mycoplasma clusters. Two tenfold dilution series were prepared in culture broth. Of each dilution step two hayflick/frey agar plates were inoculated with 20 µl each, incubated at 37 °C (30 °C for *Spiroplasma citri*) and checked frequently for colony formation by microscope. Frequent counting was stopped at constant colony numbers and titre calculated as CFU/ml culture broth. The second portion of the culture broth was filled in 1.5 ml reaction tubes at a volume of 500 µl/tube and heat inactivated (10 min, 95 °C, heat block). All tubes were stored at -80 °C until use.

Table 5. Mycoplasma Cultivation Media

Medium	Manufacturer	Catalog No.	Lot No.	Shelf Life
Frey liquid medium	Heipha Dr. Müller GmbH	397100	107717	2012-04-11
Frey agar	Heipha Dr. Müller GmbH	097e	107705	2012-01-11
Hayflick liquid medium	Heipha Dr. Müller GmbH	393100	107311	2012-03-13
Hayflick agar	Heipha Dr. Müller GmbH	093e	109515 107704 109091	2012-02-26 2012-01-01 2012-01-29

As the titration of the mycoplasma spike is of severe relevance for the subsequent spiking experiments the following materials will be tested in parallel as orthogonal materials to confirm the determined titres of the mycoplasma harvest:

Table 6. EDQM Reference Standards

Article no.	Article name	Estimated Titre
Y0000692	*Mycoplasma fermentans* Ph Eur BRP, batch 1	$9.55x10^7$ CFU/ml with a range from $1.58x10^7$ to $5.75x10^8$ CFU/ml
Y0000691	*Mycoplasma orale* Ph Eur BRP, batch 1	$4.90x10^5$ CFU/ml with a range from $9.33x10^4$ to $2.57x10^6$ CFU/ml
Y0000689	*Mycoplasma synoviae* BRP, batch 1	$1.86x10^7$ CFU/ml with a range from $5.89x10^6$ to $5.89x10^7$ CFU/ml
Y0000690	*Mycoplasma hyorhinis* BRP, batch 1	$1.17x10^8$ CFU/ml with a range from $6.76x10^7$ to $2.34x10^8$ CFU/ml

Due to a logistic mistake reference standard Y0000693 *Acholeplasma laidlawii* was not ordered from EDQM and not considered in the study.

5.3 Targeted Mycoplasma Spike Level

The targeted spike level for the study was $\geq 1 \times 10^6$ CFU per ml sample matrix as a starting material for dilution series.

5.4 Equipment

The following lab equipment were used for the study:

Table 7. Lab Equipment at Minerva Biolabs

Equipment	Equipment-ID	Manufacturer	Brand
qPCR cycler	R 04 0843, ES72	Corbett Research	RotorGene 6000
qPCR cycler	275001289, ES12	Applied BioSystems	ABI Prism 7500
qPCR cycler	Model 401513; Serial No DE00700786	Agilent Technologies	Mx3005P
Pipettes for Master Mix setup 0.5-10 µl 10–100 µl 100–1000 µl	 K0404590A, ES51 210267, ES60 315688, ES61	 Rainin Eppendorf Eppendorf	 L-10 Reference Reference
Pipettes for DNA/sample handling 10-100µl 100-1000µl	 207047,ES52 272304, ES53	 Eppendorf Eppendorf	 Reference Reference
PCR Hood	H02PC1N9861, ES29	Bioair	Aura PCR
Vortex	020314607, ES43	VWR	n/a
Centrifuge	5452YI748008, ES79	Eppendorf	MiniSpin

Table 8. Lab Equipment at Sartorius

Equipment	Equipment-ID	Manufacturer	Brand
qPCR cycler	Model 401513; Serial No DE192800511	Agilent Technologies	Stratagene Mx3005P
PCR hood	/	self made by Sartorius	/
Vortex	No 541-10000-00-0; Ser. No 020314813	Heidolph	REAX top
Micro centrifuge	Model AL220VAC; SN 053164	Roth	Rotilabo-Zentrifuge
Centrifuge	Order No. 75003280; Ser. No. 237292	Heraeus Instruments	Biofuge pico
Pipettes DNA-free 0.5 – 10 µl 2 – 20 µl 10 – 100 µl	 Test Eq. Vol/ 1071 Test Eq. Vol/ 1072 Test Eq. Vol/ 1073	Eppendorf	Eppendorf Reference
Pipettes 0.5 – 10 µl 10 – 100 µl 50 – 200 µl 100 – 1000 µl	 Test Eq. Vol/ 1067 Test Eq. Vol/ 1065 Test Eq. Vol/ 1066 Test Eq.t Vol/ 1068	Eppendorf	Eppendorf Reference

The following consumables were used:

Table 9. Reagents, materials and critical lab ware used at Minerva Biolabs

Article no.	Article name	Manufacturer / Supplier
72.690.001/ 72.699.001	Micro tubes, 1.5 ml/0.5 ml	Sarstedt
710970	PCR tubes	Biozym
56-1100	MB DNA Extraction Kit	Minerva
102-4003	10CFU™ Sensitivity Standard *Mycoplasma pneumoniae*	Minerva
S1120-3810 S1120-1840 S1126-7810	0.1-10 µL filter tips ep type 10-100 µl bevelled filter tips 101-1000 µL filter tips	Starlab

Table 10. Reagents, materials and critical lab ware used at Sartorius

Article no.	Article name	Manufacturer / Supplier
56-1100	MB DNA Extraction Kit	Minerva Biolabs GmbH
0030123.328	Tubes 1,5 ml PCR clean	Eppendorf
401428	8x Strip	Agilent Technologies
401425	Cap 8x Strip	Agilent Technologies
9065.1	Ethanol Rotipuran (for DNA Extraction)	Carl Roth
 0030077.610 0030077.547 0030077.571	ep Dualfilter T.I.P.S. 0.1 – 10 µl 2 – 100 µl 50 – 1000 µl	Eppendorf

5.5 Test Procedure

The design and performance of the pre-analytical concentration procedure is an essential part of this study in respect of the intended use and the diversity of the sample material. The performance of the kit within the entire analytical process has to be demonstrated by the user for the specific sample material.

The templates for the PCR analysis are prepared by direct DNA extraction using the Microsart® AMP Extraction Kit. The extracts can be used directly for subsequent PCR analysis.

5.5.1 Mycoplasma Concentration

Test Procedure for 1 to 5 ml samples

1 ml Microsart® AMP Coating Buffer are added to the sample and the mixture briefly vortexed. The mixture is transferred into the Vivaspin 6 units. Centrifuge for up to 30 min depending on the maximum speed of the available centrifuge and the viscosity of the sample matrix.

The dead volume of the spin column is 30 µl. The sample is concentrated to a volume < 200 µl. The volume of the retentate is estimated during transfer into a fresh reaction tube. The Vivaspin unit is rinsed with Buffer A (rinse volume = 400 µl – volume retentate) and the wash added to the retentate.

Test Procedure for 5 to 18 ml samples

2 ml Microsart® AMP Coating Buffer are added to the sample and the mixture briefly vortexed. The mixture is transferred into the Vivaspin 20. Centrifuge for up to 40 min depending on the maximum speed of the available centrifuge and the viscosity of the sample matrix.

The dead volume of the spin column is 50 µl. The sample is concentrated to a volume < 200 µl. The volume of the retentate is estimated during transfer into a fresh reaction tube. The Vivaspin unit is rinsed with Buffer A (rinse volume = 400 µl – volume retentate) and the wash added to the retentate.

5.5.2 DNA Extraction

The Microsart® AMP Extraction Kit purifies genomic DNA from whole blood, buffy coat, other body fluids and cell culture samples. Mycoplasma are lysed by a combination of a detergent and chaotropic salt. The lysate is directly applied onto the spin columns. The DNA is selectively bound to the highly specified silica membrane. Two subsequent washes remove residual contaminants, like proteins, metabolites, dyes, detergent etc. The purified DNA is eluted in Tris buffer. The DNA is ready-to-use.

The Internal Control DNA of Microsart® AMP Mycoplasma can be used to monitor the extraction process. 2 µl of the Internal Control DNA are added directly to 200 µl sample volume. The sample is vortexed briefly prior extraction. No additional Internal Control DNA is used in the reaction mix for these samples.

The isolation of DNA will be carried out according to the update version of the instruction manual. In detail:

Transfer 200 µl of sample material or the retentate from the concentration step into a fresh 1.5 ml reaction tube. For Vivaspin samples the total 400 µl sample volume are used.

Add 200 µl of Buffer A (not applicable for Vivaspin samples), vortex for at least 10 sec. For samples with protein concentrations above 10 mg/ml, add 10 µl Proteinase K solution and incubate for 15 min at 56 °C, spin briefly to remove liquid from the lid.

Add 200 µl of absolute ethanol to the mixture. Vortex immediately and very thoroughly in order to prevent any precipitation of nucleic acids.

Take one spin column per sample from the kit and insert it into a collection tube. Mark the sample identification on the lid of the spin column. Fill the sample lysate into the spin column without moistening the rim of the spin column.

Centrifuge the system for 1 min at 10,600 x g (approx. 10,000 rpm with a benchtop centrifuge). Discard the flow through from the collection tube and reassemble the spin column and the collection tube.

Add 500 µl of Buffer B. Centrifuge the system for 1 min at 10,600 x g (approx. 10,000 rpm with a benchtop centrifuge), discard the flow through and re-assemble the spin column.

Fill the spin column with 500 µl Buffer C. Centrifuge the system for 1 min at 10,600 x g (10,000 rpm), take the spin column out of the collection tube, dump the containing Buffer C, discard the flow through and re-assemble the spin column.

Centrifuge for 1 min at full speed (approx. 13,200 rpm) in order to remove the remaining Buffer C.

Discard the collection tube containing the Buffer C and place the spin column into a sample storage tube.

Pipette 60 µl of pre-heated Buffer E (70 °C) into the spin column directly onto the center of the silica membrane. The complete membrane should get in touch with the Buffer E. Secure the sample storage tube and incubate for 2 min at room temperature.

Following the incubation, centrifuge the system for 2 min at 10,600 x g (10,000 rpm).

Remove the spin column and use the eluate directly for the PCR procedure.

5.5.3 Analytical procedures

The detection of mycoplasma DNA will be carried out according to the update version of the instruction manual. In detail:

Rehydration of the Reagents:

1. Centrifuge tubes with lyophilized components (5 sec at maximum speed)
2. Add 1275 µl of Rehydration Buffer to the Primer/Probe/Nucleotide Mixes (each)
3. Add appropriate amount of deionized, DNA-free water
   Positive Control DNA 300 µl
   Internal Control DNA 300 µl
3. Incubate for 10 minutes at room temperature
4. Vortex and centrifuge again

PCR Master Mix Setup:

Total volume per reaction is 100 µl including 50 µl of sample. When setting up reactions, calculations include positive (PC) and negative controls (NC). Pipet master mix on ice into a 1.5 ml reaction tube and mix gently.

Pipetting scheme:

	for 1 reaction	for 25 reactions
primer/probe/nucleotide mix	49 µl	1225.0 µl
Internal Control DNA	1.0 µl	25.0 µl

For all samples already containing the Internal Control DNA as an extraction control 1 µl of DNA-free water has to be added per reaction instead of the Internal Control DNA.

Aliquot 50 µl of master mix into each PCR reaction tube. After pipetting the negative control (50 µl of water or elution buffer of DNA extraction kit), the tube must be sealed before proceeding with the samples. Add 50 µl of sample to each PCR reaction tube. Seal the tubes completely before proceeding with the positive control (2 µl + 48 µl of water/reaction) in order to avoid cross contamination.

Programming the qPCR cycler Rotorgene 6000 (5-plex):

Program Step 1: Pre-incubation

Setting	Hold
Hold Temperature	95°C
Hold Time	3 min 0 sec

Program Step 2: Amplification

Setting	Cycling
Cycles	45
Denaturation	95 °C for 30 sec
Annealing	55 °C for 30 sec
Detection/ Elongation	60 °C for 45 sec
Gain setting	automatic (auto gain)
Slope Correct	activated
Ignore First	deactivated

Programming the qPCR cycler ABI Prism® 7500:

Program Step 1: Pre-incubation

Setting	Hold
Hold Temperature	95°C
Hold Time	3 min 0 sec

Program Step 2: Amplification

Setting	Cycling
Cycles	45
Denaturation	95 °C for 30 sec
Annealing	55 °C for 30 sec
Detection/ Elongation	60 °C for 45 sec

Programming the qPCR cycler Mx3005p®:

Segment 1 (Pre-Melt)	95°C, 3 min 0 sec
Segment 2	95 °C for 30 sec
	55 °C for 30 sec
	60 °C for 45 sec, data collection
Cycles	45
Analysis mode:	adaptive baseline (baseline correction)

Result Interpretation:
The presence of mycoplasma in the sample is indicated by an increasing fluorescence signal in the mycoplasma FAM channel during PCR.

Detection of *Mollicutes* FAM™ channel	Internal control ROX™ channel	Interpretation
positive (C_t < 40)	irrelevant	*Mollicutes positive*
negative (no C_t)	negative (no C_t)	PCR inhibition
negative (no C_t)	positive (C_t < 40)	Mollicutes negative
borderline (C_t > 40)	positive (C_t < 40)	result not valid, repeat process including DNA extraction
borderline (C_t > 40)	negative (no C_t)	PCR inhibition

A successfully performed PCR without inhibition is indicated by an increasing fluorescence signal in the internal control channel, provided the Internal Control was added to the master mix or as extraction control to the master mix. Mycoplasma DNA and Internal Control DNA are competitors in PCR. Because of the very low concentration of Internal Control in the PCR mix, the signal strength in this channel is reduced with increasing mycoplasma DNA loads in the sample.

5.5.4 Calculations
None.

5.5.5 Reporting requirements
The reports generated by the qPCR machine will be printed in color. All run information will be printed, including protocol, sample identification, internal amplification control curves (ROX curves) and target curves (FAM filter) and filed according to the chapter structure of this validation plan. Sample identification should contain information on the species, the contained concentration in CFU/ml or alternatively the type of control (PC for positive control and NTC for No Template Control).

6 Validation Data

The study conditions have to provide information on all relevant validation parameters requested by ICH Q2B, EP 2.6.7 and EP 2.6.21. As the requirement of the method is to provide a qualitative result only, the parameter linearity, range, accuracy and quantification limit are irrelevant.

6.1 Specificity

6.1.1 Sequence Alignment

Procedure	Acceptance Criterion	Results
Comparison of all primer sequences with the genomic database. *Mollicutes* sequence alignments will be performed. Even though this technique is not recommended by EP 2.6.7 for specificity determination it provides additional information for species not available for testing.	*Mollicutes* species showing ≤ 3 nucleotides mismatch in the alignment of the primer and probe sequence with the 16S rRNA genome are considered specifically detectable.	At least 141 species are putatively detectable based on sequence alignment.

Species; Type Strain	**Primer Mismatches**		
	Forward Primer	**Probe**	**Reverse Primer**
Acholeplasma equifetale (T); C112.	0	1	2
Acholeplasma granularum (T); BTS-39.	0	1	0
Acholeplasma hippikon (T); C1.	0	1	1
Acholeplasma laidlawii (T); PG8 ATCC 23206.	0	2	0
Acholeplasma oculi (T); 19L ATCC 27350.	0	1	1
Acholeplasma pleciae (T); ATCC 49582; PS-1.	0	1	0
Mycoplasma adleri (T); G145.	1	0	0
Mycoplasma agalactiae (T).	0	0	0
Mycoplasma agassizii (T).	0	0	2
Mycoplasma alkalescens (T); PG51.	0	0	1
Mycoplasma alligatoris (T); A21JP2(T).	0	0	2
Mycoplasma alvi (T); Isley.	0	0	2
Mycoplasma amphoriforme (T); A39.	0	0	2
Mycoplasma anatis (T); 1340(T).	0	0	2
Mycoplasma anseris (T); 1219(T).	0	0	1
Mycoplasma arginini (T); G230(T).	0	0	1
Mycoplasma arthritidis (T).	0	0	1
Mycoplasma auris (T); UIA.	0	0	1
Mycoplasma bovigenitalium (T).	0	0	0
Mycoplasma bovirhinis (T); PG43.	0	0	0
Mycoplasma bovis (T); Donetta (type strain); pMb16S.	0	0	0
Mycoplasma bovoculi (T); M165/69.	0	0	2
Mycoplasma buccale (T); CH20247(T).	0	0	1
Mycoplasma buteonis (T); BbT2g(T).	0	0	1
Mycoplasma californicum (T).	0	0	0
Mycoplasma canadense (T); 275c.	0	0	1
Mycoplasma canis (T); PG14.	0	0	1
Mycoplasma capricolum.	0	0	1
Mycoplasma caviae (T); G122(T).	0	0	0
Mycoplasma citelli (T); RG-2C(T).	0	0	0
Mycoplasma cloacale (T); 383(T).	0	0	1
Mycoplasma columbinasale (T); 694(T).	0	0	0
Mycoplasma columbinum (T); MMP-1(T).	0	0	0

Species; Type Strain	Primer Mismatches		
	Forward Primer	Probe	Reverse Primer
Mycoplasma columborale (T); MMP-4(T).	0	0	1
Mycoplasma cricetuli (T); CH(T).	0	1	2
Mycoplasma crocodyli (T); MP145(T).	0	0	2
Mycoplasma cynos (T); H831(T).	0	0	1
Mycoplasma edwardii; PG24.	0	0	2
Mycoplasma elephantis (T); E42(T).	0	0	1
Mycoplasma equigenitalium (T); T37(T).	0	0	1
Mycoplasma equirhinis (T); M432/72(T).	0	0	1
Mycoplasma falconis (T); H/T1(T).	0	0	0
Mycoplasma faucium (T); DC333(T).	0	0	0
Mycoplasma felifaucium (T); ATCC 43428.	1	0	0
Mycoplasma felis (T); ATCC 23391.	1	0	1
Mycoplasma fermentans (T).	0	0	0
Mycoplasma gallinaceum (T); DD.	0	0	0
Mycoplasma gallinarum (T); PG16.	0	0	0
Mycoplasma gallisepticum str. F; 1.	0	0	0
Mycoplasma gallopavonis (T); WR1(T).	0	0	1
Mycoplasma gateae (T); ATCC 23392.	0	0	1
Mycoplasma genitalium (T); G37.	0	0	2
Mycoplasma glycophilum (T); 486(T).	0	0	1
Mycoplasma gypis (T); B1/T1(T).	0	1	1
Mycoplasma hominis (T); PG21; ATCC 23114.	0	0	2
Mycoplasma hyopharyngis (T).	0	0	1
Mycoplasma hyorhinis (T); BTS7(T).	0	0	0
Mycoplasma hyosynoviae (T); S-16.	0	0	1
Mycoplasma iguanae (T); 2327.	0	1	1
Mycoplasma imitans (T); 4229.	0	0	0
Mycoplasma indiense (T); 3T(T).	0	0	1
Mycoplasma iners (T); PG30(T).	0	0	0
Mycoplasma iowae (T).	0	0	1
Mycoplasma lagogenitalium (T); 12MS(T).	0	1	2
Mycoplasma leonicaptivi (T); ATCC 49890.	1	0	1
Mycoplasma leopharyngis (T); ATCC 49889.	1	0	0
Mycoplasma lipofaciens (T); R171(T).	0	0	1
Mycoplasma lipophilum (T).	0	0	0
Mycoplasma maculosum (T); PG15(T).	0	0	0
Mycoplasma meleagridis (T); 17529.	0	0	0
Mycoplasma microti (T); IL371.	0	0	1
Mycoplasma moatsii (T); MK405(T).	0	0	0
Mycoplasma mobile (T).	0	0	1
Mycoplasma molare (T); H542.	0	1	2
Mycoplasma mucosicanis (T); type strain: 1642.	0	0	1
Mycoplasma muris (T).	0	0	2
Mycoplasma mustelae (T); MX9(T).	0	0	0
Mycoplasma opalescens (T); MH5408(T).	0	0	0
Mycoplasma orale (T); NC10112; CH 19299; ATCC 23714.	0	0	1
Mycoplasma oxoniensis (T); 128(T).	0	0	0
Mycoplasma penetrans HF-2.	0	0	1
Mycoplasma phocae; CSL 4693.	0	0	2
Mycoplasma phocicerebrale (T); 1049; ATCC 49640.	0	0	2
Mycoplasma phocidae (T); 105; ATCC 33657.	0	0	2
Mycoplasma phocirhinis (T); 852; ATCC 49639.	0	0	0
Mycoplasma pirum (T).	0	0	2
Mycoplasma pneumoniae (T); ATCC 15531.	0	0	2
Mycoplasma primatum (T); HRC292(T).	0	0	0

Species; Type Strain	Primer Mismatches		
	Forward Primer	Probe	Reverse Primer
Mycoplasma pullorum (T); CKK.	0	0	2
Mycoplasma pulmonis (T); PG34(T).	0	0	1
Mycoplasma salivarium (T); PG20(T).	0	0	1
Mycoplasma simbae (T); ATCC 49888.	0	0	1
Mycoplasma spermatophilum (T); AH159(T).	0	0	0
Mycoplasma sphenisci; UCMJ.	0	0	1
Mycoplasma spumans (T); PG13(T).	0	0	1
Mycoplasma sturni (T); UC/MF; p170/171.	0	0	0
Mycoplasma sualvi (T); Mayfield B(T).	0	0	0
Mycoplasma subdolum (T); TB(T).	0	0	1
Mycoplasma synoviae (T); WVU 1853; pMSk3-4 pMSF16S.	0	0	0
Mycoplasma testudineum (T); H3110.	1	0	1
Mycoplasma testudinis (T); ATCC 43263.	0	0	3
Mycoplasma timone.	0	0	1
Mycoplasma verecundum (T); GIH(T).	0	0	0
Mycoplasma vulturii; Gb-V33.	0	0	3
Mycoplasma zalophidermidis; CSL 4779.	0	0	1
Ureaplasma canigenitalium (T); D6P-C.	0	0	3
Ureaplasma diversum (T); A417.	0	0	3
Ureaplasma felinum (T); FT2-B.	0	0	3
Ureaplasma parvum (T); ATCC27815.	0	0	3
Ureaplasma urealyticum (T); ATCC27618.	0	0	3

6.2 Sample Matrix Effects

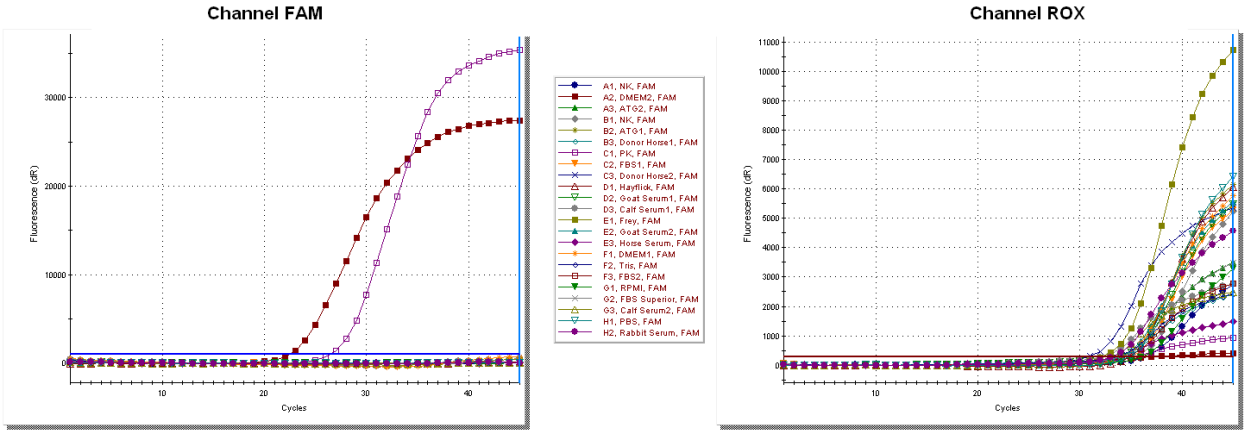
6.2.1 Direct Testing

Procedure	Acceptance Criterion	Results
Testing of at least 20 different samples using the media components according to Table 2 and 5 to exclude the possibility of false-positive results.	All tested samples shall show a negative result.	passed 19 out of 20 samples were tested negative. One sample was found true positive. Re-examination of the sample medium was found negative.

No.	Sample Matrix	Lot	Ct (FAM)	Ct (ROX)	Result
1	Hayflick Liquid Medium	107311	No Ct	35.01	negative
2	Frey Liquid Medium	107717	No Ct	32.39	negative
3	Dulbecco's MEM, bottle 1	1499W	No Ct	34.10	negative
4	Dulbecco's MEM, bottle 2	0407X	22.52	37.73	positive
5	RPMI Medium	0512X	No Ct	36.30	negative
6	PBS Dulbecco	0448W	No Ct	34.94	negative
7	anti-human-T-lymphocyte immune globulin, rabbit*	B 14 L-2	No Ct	34.24	negative
8	anti-human-T-lymphocyte immune globulin, rabbit*	B 13 L-1	No Ct	33.99	negative
9	FBS*	1231T	No Ct	35.13	negative
10	FBS*	11-3356	No Ct	34.65	negative

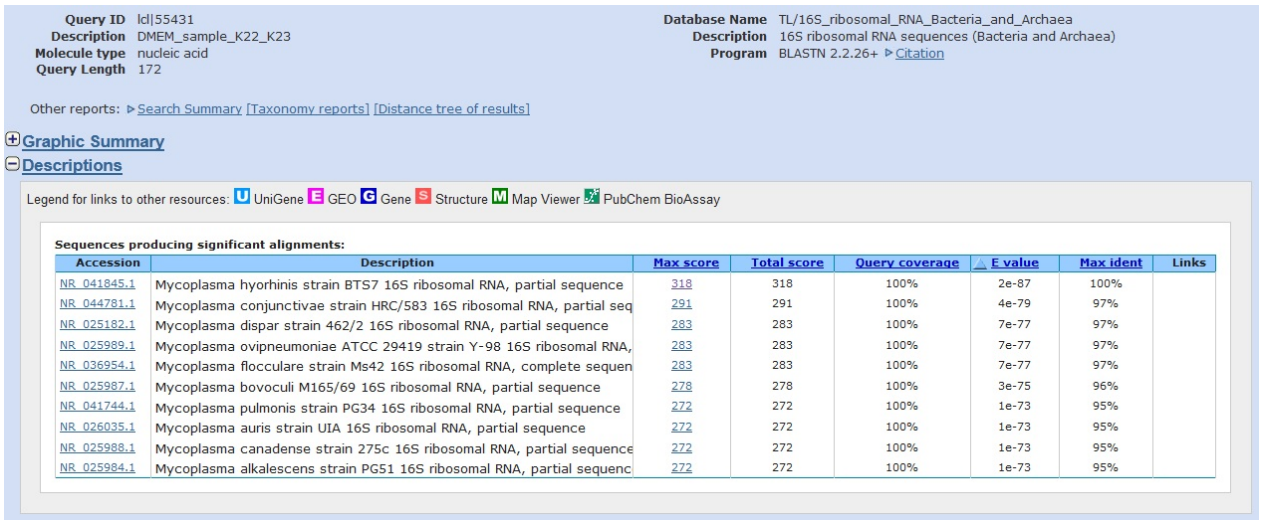
No.	Sample Matrix	Lot	Ct (FAM)	Ct (ROX)	Result
11	FBS Superior*	1201T	No Ct	34.61	negative
12	goat serum	B1060711	No Ct	33.96	negative
13	goat serum	A8070711	No Ct	33.78	negative
14	Tris buffer	118B1101	No Ct	33.67	negative
15	rabbit serum*	B03411-1243	No Ct	33.36	negative
16	donor horse serum	F11-2542	No Ct	34.11	negative
17	donor horse serum	0533W	No Ct	30.99	negative
18	calf serum*	11-3497	No Ct	32.46	negative
19	calf serum*	11-2875	No Ct	33.26	negative
20	horse serum*	10-2658	No Ct	34.32	negative
21	positive control	---	26.26	35.66	positive
22	NTC	---	No Ct	36.60	negative

* Due to confidentiality reasons no specifications can be provided for these matrices.



Sequencing Dulbecco's MEM bottle 2 (No. 4):

Sequence analysis of the amplified PCR products confirmed the presence of *Mycoplasma hyorhinis* in sample No. 4. The obtained positive PCR result is not to be interpreted as false-positive.



Query ID lcl|55431
Description DMEM_sample_K22_K23
Molecule type nucleic acid
Query Length 172

Database Name TL/16S_ribosomal_RNA_Bacteria_and_Archaea
Description 16S ribosomal RNA sequences (Bacteria and Archaea)
Program BLASTN 2.2.26+ ▹Citation

Other reports: ▹Search Summary [Taxonomy reports] [Distance tree of results]

⊕Graphic Summary
⊖Descriptions

Legend for links to other resources: U UniGene E GEO G Gene S Structure M Map Viewer PubChem BioAssay

Sequences producing significant alignments:

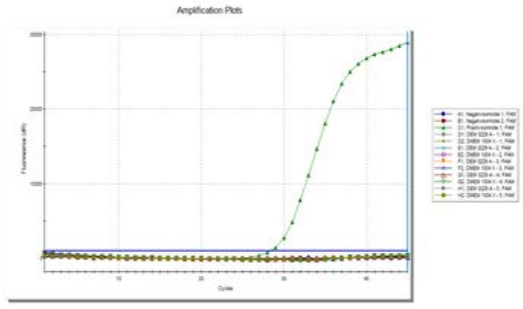
Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
NR_041845.1	Mycoplasma hyorhinis strain BTS7 16S ribosomal RNA, partial sequence	318	318	100%	2e-87	100%	
NR_044781.1	Mycoplasma conjunctivae strain HRC/583 16S ribosomal RNA, partial seq	291	291	100%	4e-79	97%	
NR_025182.1	Mycoplasma dispar strain 462/2 16S ribosomal RNA, partial sequence	283	283	100%	7e-77	97%	
NR_025989.1	Mycoplasma ovipneumoniae ATCC 29419 strain Y-98 16S ribosomal RNA,	283	283	100%	7e-77	97%	
NR_036954.1	Mycoplasma flocculare strain Ms42 16S ribosomal RNA, complete sequen	283	283	100%	7e-77	97%	
NR_025987.1	Mycoplasma bovoculi M165/69 16S ribosomal RNA, partial sequence	278	278	100%	3e-75	96%	
NR_041744.1	Mycoplasma pulmonis strain PG34 16S ribosomal RNA, partial sequence	272	272	100%	1e-73	95%	
NR_026035.1	Mycoplasma auris strain UIA 16S ribosomal RNA, partial sequence	272	272	100%	1e-73	95%	
NR_025988.1	Mycoplasma canadense strain 275c 16S ribosomal RNA, partial sequence	272	272	100%	1e-73	95%	
NR_025984.1	Mycoplasma alkalescens strain PG51 16S ribosomal RNA, partial sequenc	272	272	100%	1e-73	95%	

Repeating DMEM:

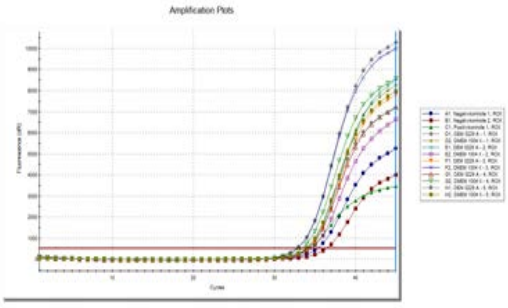
Due to the positive result found with sample 4 a new lot was purchased and 2 bottles of the medium tested with 5 repeat each. All samples had been identified as negative for *Mycoplasma* DNA.

No.	Sample Matrix	Ct (FAM)	Ct (ROX)	Result
1	Dulbecco's MEM, bottle 1, repeat 1	No Ct	32.76	negative
2	Dulbecco's MEM, bottle 1, repeat 2	No Ct	34.13	negative
3	Dulbecco's MEM, bottle 1, repeat 3	No Ct	34.28	negative
4	Dulbecco's MEM, bottle 1, repeat 4	No Ct	34.24	negative
5	Dulbecco's MEM, bottle 1, repeat 5	No Ct	33.90	negative
6	Dulbecco's MEM, bottle 2, repeat 1	No Ct	34.00	negative
7	Dulbecco's MEM, bottle 2, repeat 2	No Ct	34.70	negative
8	Dulbecco's MEM, bottle 2, repeat 3	No Ct	32.88	negative
9	Dulbecco's MEM, bottle 2, repeat 4	No Ct	33.43	negative
10	Dulbecco's MEM, bottle 2, repeat 5	No Ct	33.93	negative
11	positive control	28.47	34.37	positive
12	NTC	No Ct	35.34	negative
13	DNA extraction control	No Ct	36.40	negative

FAM signal:



ROX signal:

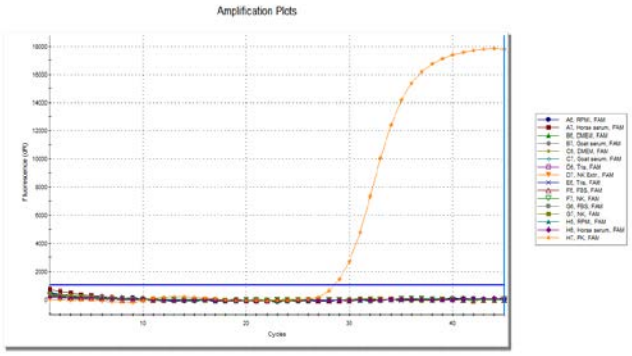


6.2.2 Using the Internal Amplification Control as Process Control in Media

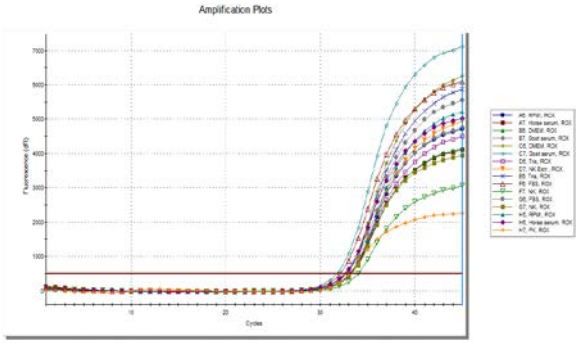
Procedure	Acceptance Criterion	Results
Testing of culture media and media components in duplicate to verify the possibility of false-positive results. The internal amplification control will be added to the sample matrix as extraction control.	All tested samples shall show a negative result.	passed

No.	Sample Matrix	Cat. No.	Lot	Ct (FAM)	Ct (ROX)	Result
1	RPMI Medium	FG 1215	1608W	No Ct	33.20	negative
2	RPMI Medium	FG 1215	1608W	No Ct	33.13	negative
3	Dulbecco's MEM	FG0415	1004X	No Ct	33.26	negative
4	Dulbecco's MEM	FG0415	1004X	No Ct	32.66	negative
5	Tris buffer, pH 8.5	N/A	520T2031	No Ct	33.21	negative
6	Tris buffer, pH 8.5	N/A	520T2031	No Ct	32.75	negative
7	Fetal bovine serum	S0615	0248W	No Ct	32.11	negative
8	Fetal bovine serum	S0615	0248W	No Ct	32.78	negative
9	Donor Horse Serum	S9133	0533W	No Ct	33.27	negative
10	Donor Horse Serum	S9133	0533W	No Ct	32.59	negative
11	Goat Serum	S-707-S	A8070711	No Ct	32.82	negative
12	Goat Serum	S-707-S	A8070711	No Ct	31.74	negative
13	Positive Control	---	---	28.55	33.16	positive
14	NTC	---	---	No Ct	33.38	negative

FAM signal:



ROX signal:

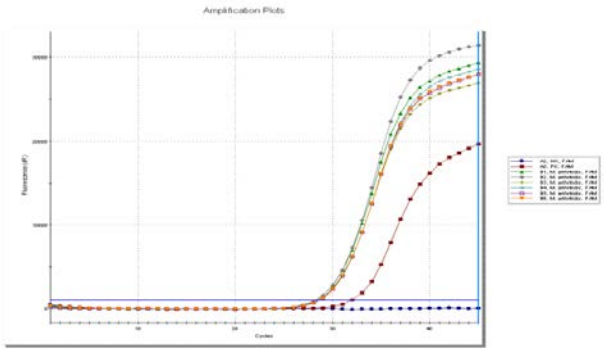


6.2.3 *Mollicutes* Detection Range

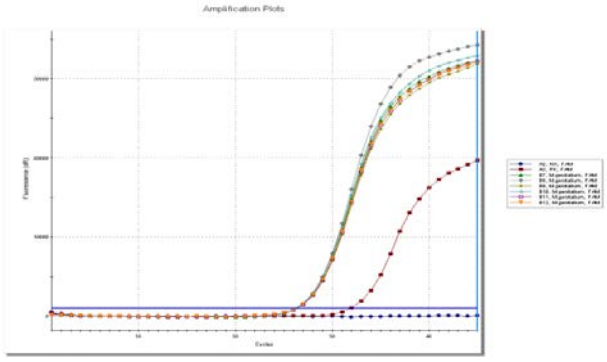
Procedure	Acceptance Criterion	Results
All DNA extracts listed in Table 3 derived from *Mollicutes* will be tested at a load of ≥ 0.1 ng/test. At least 6 repeats shall be tested for each sample.	All tested samples shall show a positive result.	passed

Species	**Results**
Acholeplasma laidlawii	data shown in chapter 6.3.2
Mycoplasma fermentans	
Mycoplasma hyorhinis	
Mycoplasma orale	
Mycoplasma pneumoniae	
Mycoplasma gallisepticum	
Mycoplasma synoviae	
Mycoplasma arginini	
Spiroplasma citri	
Mycoplasma arthritidis	passed
Mycoplasma genitalium	passed
Mycoplasma hominis	passed
Mycoplasma penetrans	passed
Mycoplasma salivarium	passed
Ureaplasma urealyticum	passed

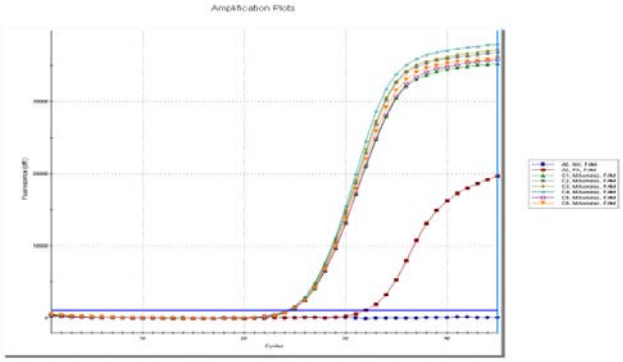
No.	**Species**	**Ct**	**Result**
1	*Mycoplasma arthritidis*	28.14	positive
2		28.33	positive
3		28.48	positive
4		28.65	positive
5		28.59	positive
6		28.72	positive



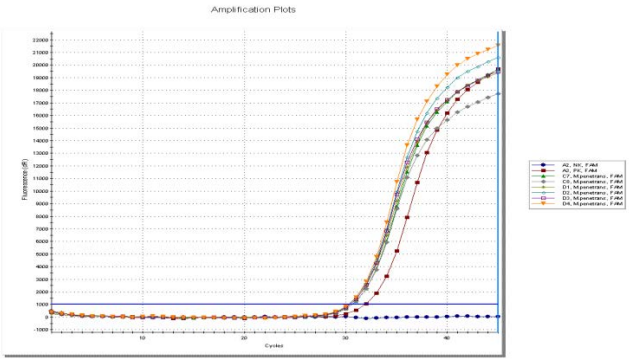
No.	Species	Ct	Result
1	*Mycoplasma genitalium*	26.45	positive
2		26.29	positive
3		26.42	positive
4		26.25	positive
5		26.43	positive
6		26.40	positive



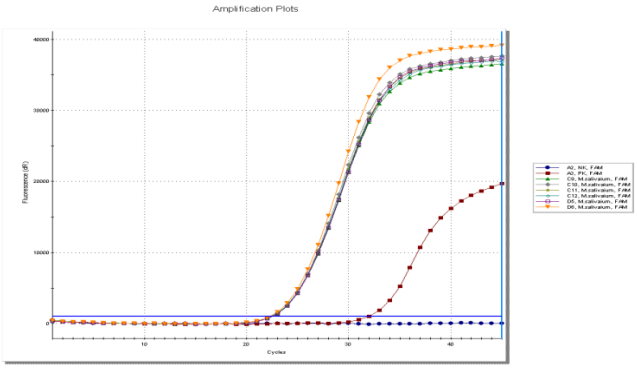
No.	Species	Ct	Result
1	*Mycoplasma hominis*	24.57	positive
2		24.54	positive
3		24.26	positive
4		24.43	positive
5		24.44	positive
6		24.64	positive



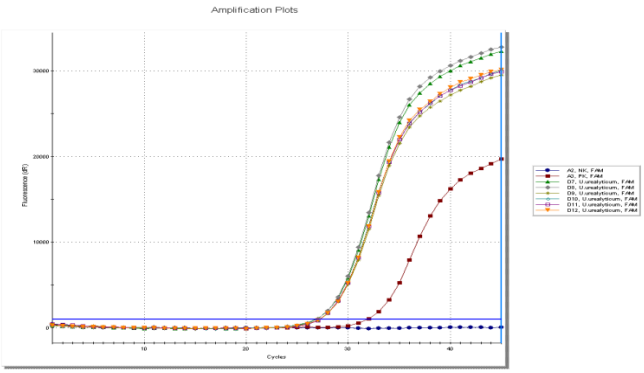
No.	Species	Ct	Result
1	*Mycoplasma penetrans*	30.67	positive
2		30.63	positive
3		30.46	positive
4		30.35	positive
5		30.48	positive
6		30.28	positive



No.	Species	Ct	Result
1	*Mycoplasma salivarium*	22.54	positive
2		22.36	positive
3		22.51	positive
4		22.48	positive
5		22.55	positive
6		22.30	positive

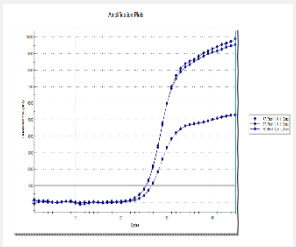
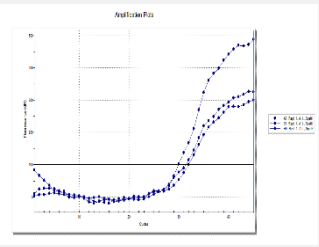
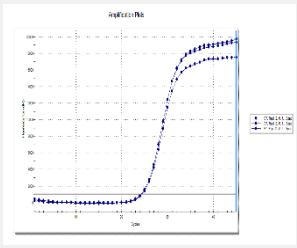
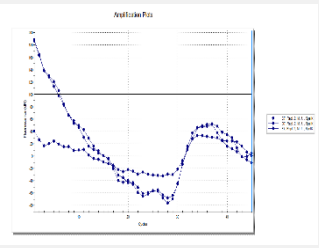
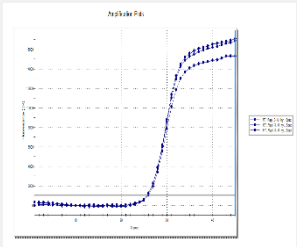
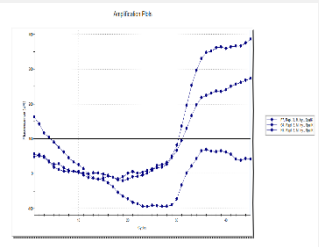
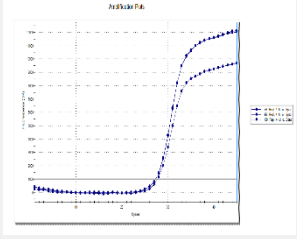
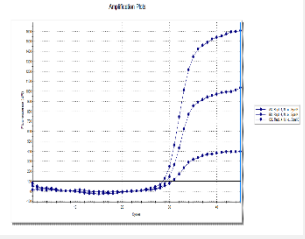


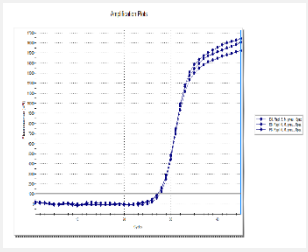
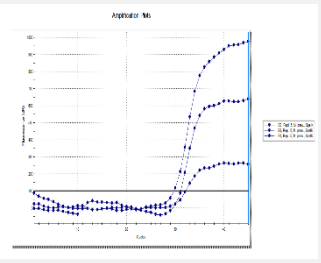
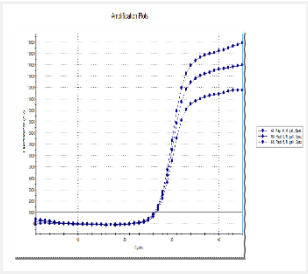
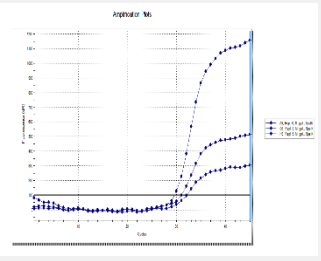
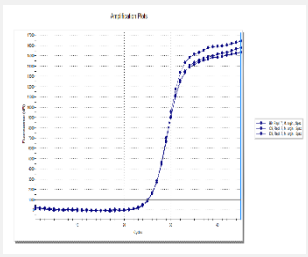
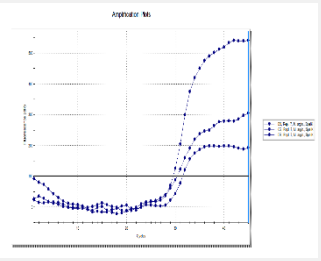
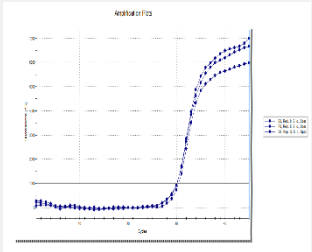
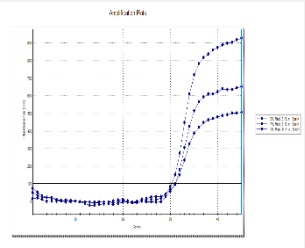
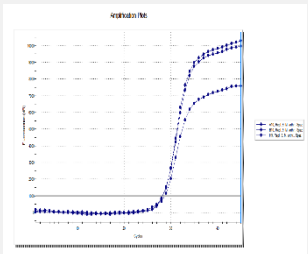
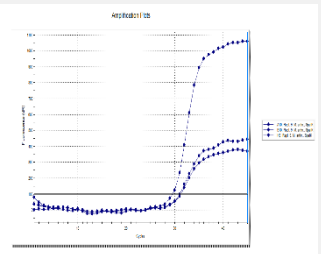
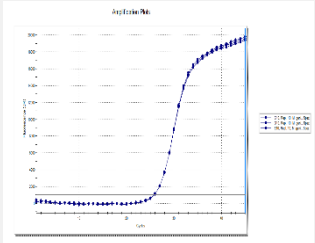
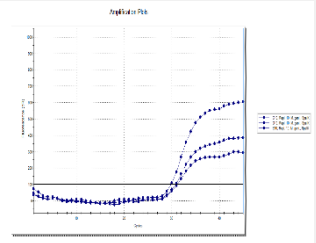
No.	Species	Ct	Result
1	*Ureaplasma urealyticum*	26.83	positive
2		26.91	positive
3		27.26	positive
4		27.19	positive
5		27.28	positive
6		27.21	positive

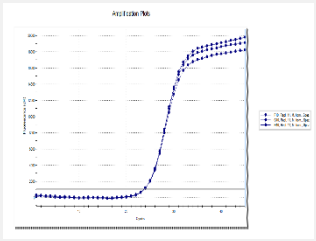
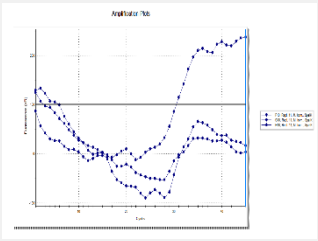
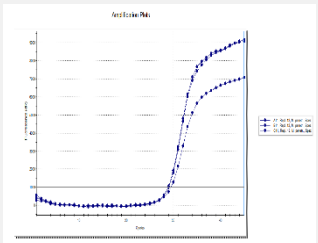
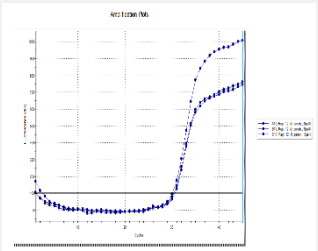
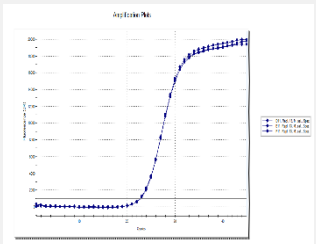
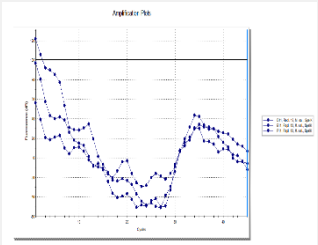
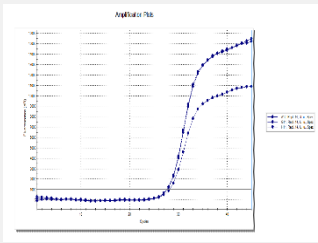
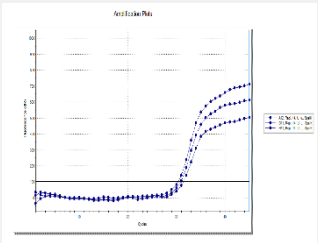
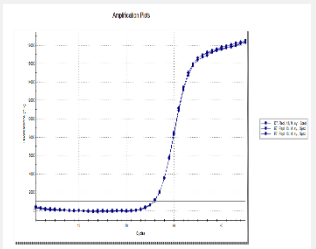
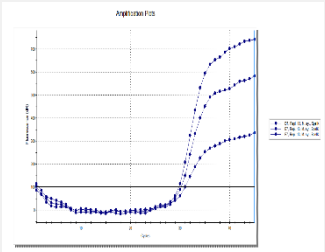


The impact of the product design change on the specificity of the kit was evaluated in part repeating the test with a reduced number of replicates:

Procedure	Acceptance Criterion	Results
All available *Mollicutes* DNA extracts will be tested at a load of ≥ 0.1 ng/test. 3 repeats shall be tested for each sample with Product Version 2.	All tested samples shall show a positive result.	passed

No	Species	Ct FAM	Ct ROX	Result	FAM	ROX
1	*A. laidlawii*	26.69	32.00	positiv		
2		25.44	29.94	positiv		
3		25.54	31.41	positiv		
1	*Mycoplasma fermentans*	24.47	No Ct	positiv		
2		24.29	No Ct	positiv		
3		24.30	No Ct	positiv		
1	*Mycoplasma hyorhinis*	25.94	31.16	positiv		
2		25.71	No Ct	positiv		
3		25.72	30.40	positiv		
1	*Mycoplasma orale*	27.77	30.60	positiv		
2		27.38	28.63	positiv		
3		27.37	29.47	positiv		

No	Species	Ct FAM	Ct ROX	Result	FAM	ROX
1	*Mycoplasma pneumoniae*	27.73	31.16	positiv		
2		27.21	29.77	positiv		
3		27.30	32.19	positiv		
1	*Mycoplasma gallisepti-cum*	26.66	32.12	positiv		
2		26.47	29.67	positiv		
3		26.27	31.02	positiv		
1	*Mycoplasma arginini*	25.21	29.62	positiv		
2		25.23	31.57	positiv		
3		25.06	30.39	positiv		
1	*Spiroplasma citri*	30.10	31.02	positiv		
2		30.49	31.06	positiv		
3		30.16	30.29	positiv		
1	*Mycoplasma arthritidis*	28.74	31.32	positiv		
2		28.27	31.00	positiv		
3		28.39	29.59	positiv		
1	*Mycoplasma genitalium*	25.88	29.88	positiv		
2		25.84	30.85	positiv		
3		25.89	31.20	positiv		

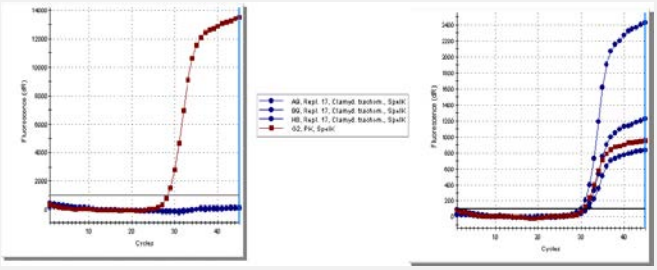
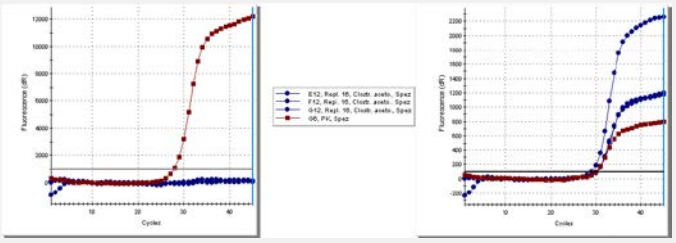
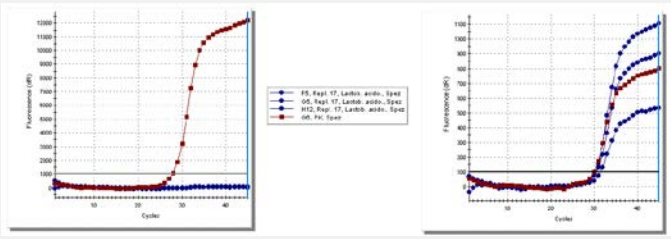
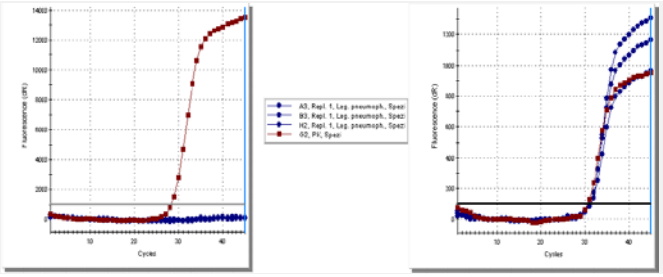
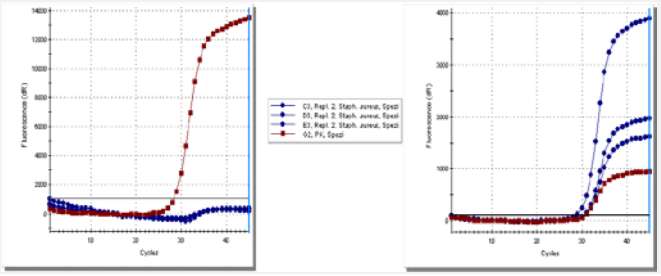
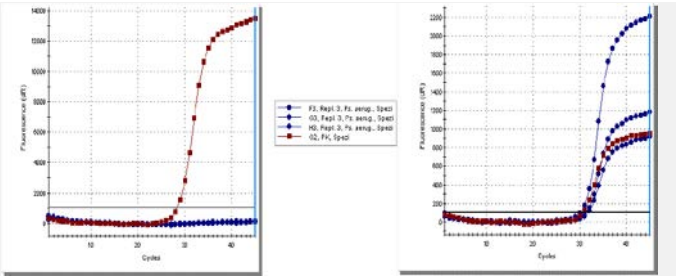
No	Species	Ct FAM	Ct ROX	Result	FAM	ROX
1	*Mycoplasma hominis*	23.79	No Ct	positiv		
2		23.78	30.55	positiv		
3		23.71	No Ct	positiv		
1	*Mycoplasma penetrans*	29.54	30.09	positiv		
2		28.92	30.42	positiv		
3		29.01	30.63	positiv		
1	*Mycoplasma salivarium*	22.68	No Ct	positiv		
2		22.74	No Ct	positiv		
3		22.84	No Ct	positiv		
1	*Ureaplasma urealyticum*	28.18	30.89	positiv		
2		27.67	30.35	positiv		
3		27.71	31.43	positiv		
1	*Mycoplasma synoviae*	25.78	30.24	positiv		
2		25.77	29.77	positiv		
3		25.88	30.97	positiv		

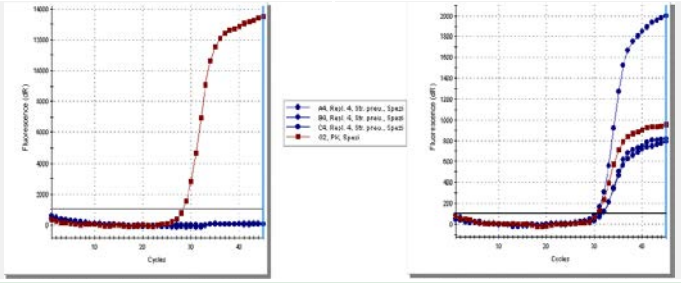
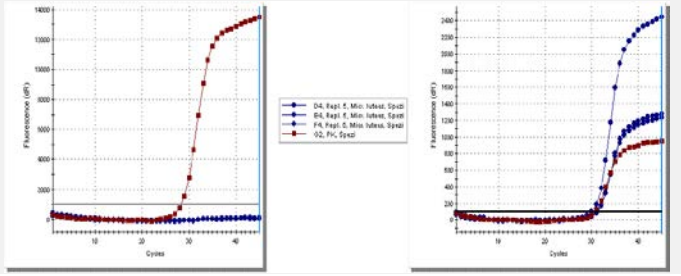
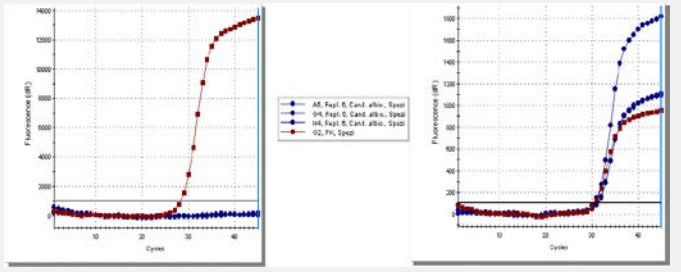
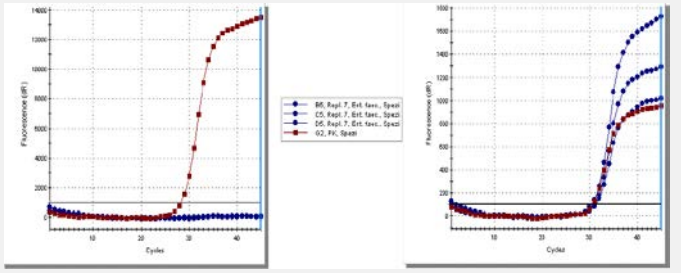
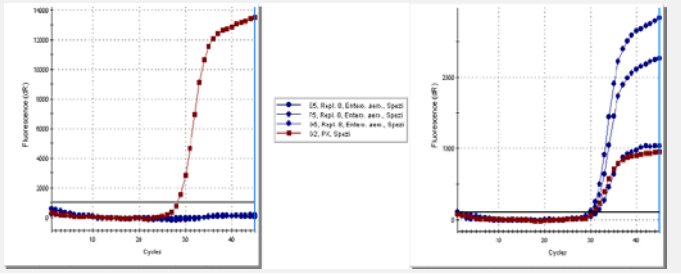
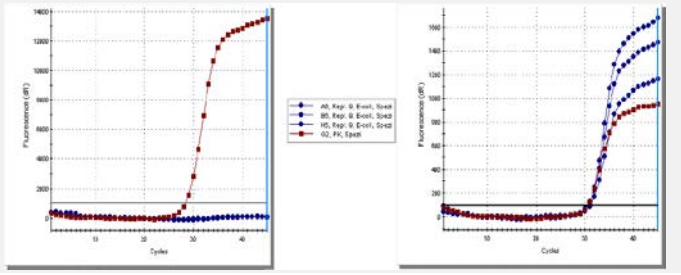
6.2.4 Cross Reactivity

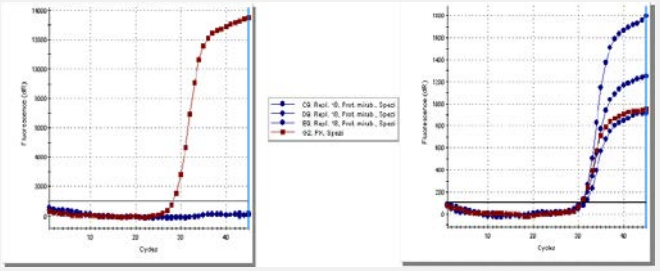
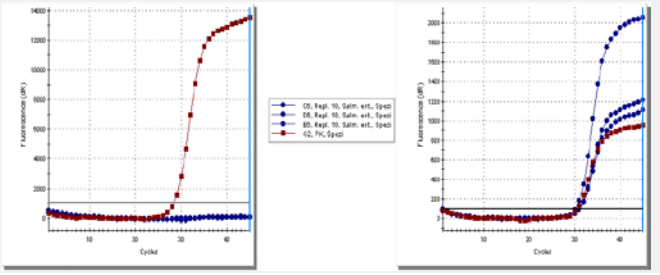
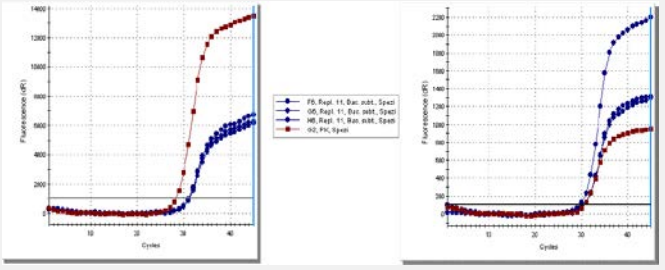
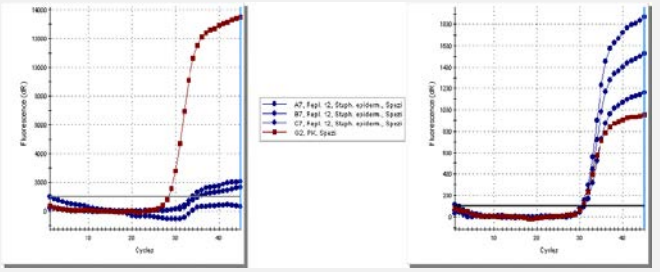
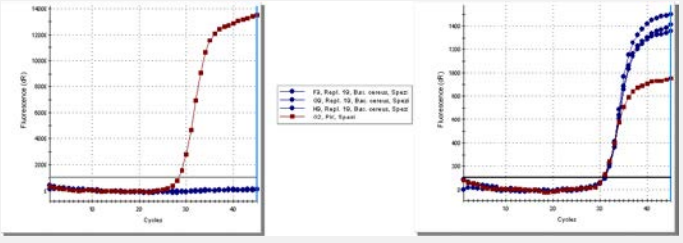
Procedure	Acceptance Criterion	Results
All DNA extracts listed in Table 4 of the validation plan derived from microorganisms and cells will be tested at a load of ≥ 0.1 ng/test for microorganisms and ≥ 30 ng for mammalian cells. At least 2 repeats shall be tested for each sample. For species showing positive results the detection limit in means of GU/µl will be determined.	All tested samples shall show a negative result.	failed Cross reactivity was found for 2 out of 23 tested microorganisms / tissues (DNA): *Bacillus subtilis, Staphylococcus epidermidis*

Species	**Results**
Chlamydia trachomatis	negative
Clostridium acetobutylicum	negative
Lactobacillus acidophilus	negative
Legionella pneumophila	negative
Staphylococcus aureus	negative
Pseudomonas aeruginosa	negative
Streptococcus pneumoniae	negative
Micrococcus luteus	negative
Candida albicans	negative
Enterococcus faecalis	negative
Enterobacter aerogenes	negative
Escherichia coli	negative
Proteus mirabilis	negative
Salmonella enterica	negative
Bacillus subtilis subsp. spizizenii	positive
Staphylococcus epidermidis	positive
Bacillus cereus	negative
Vero-B4	negative
Per.C6	negative
RK13	negative
CHO-K1	negative
Murine Genomic DNA	negative
Calf Thymus DNA	negative

Microbial DNA in Detail

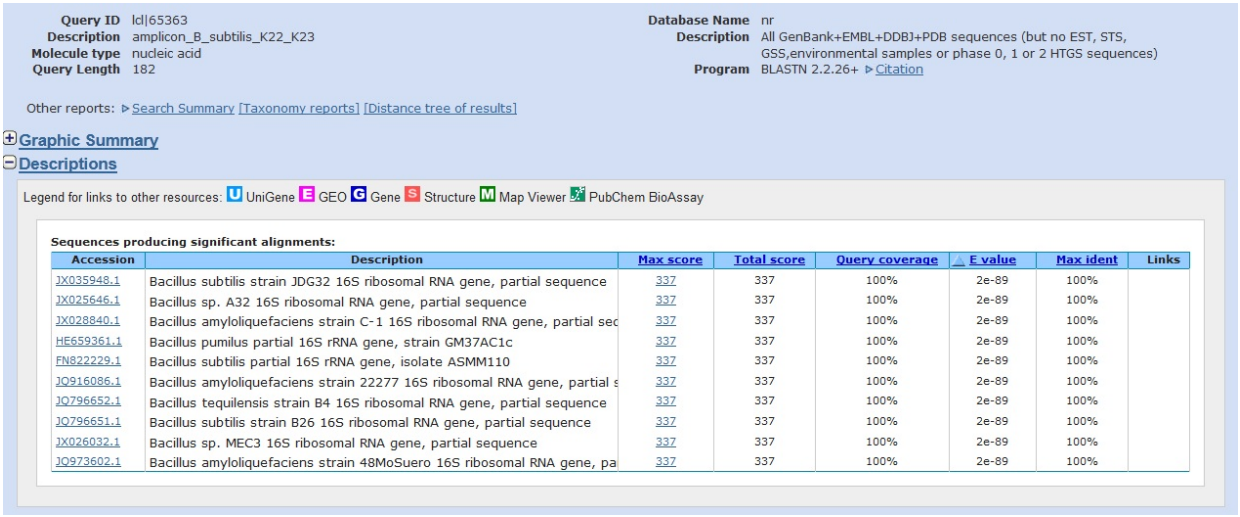
No	Species	Ct FAM	Ct ROX	Result	FAM	ROX
1	*Chlamydia trachomatis*	No Ct	31.70	negative		
2		No Ct	29.93	negative		
3		No Ct	31.30	negative		
1	*Clostridium acetobutylicum*	No Ct	30.16	negative		
2		No Ct	30.29	negative		
3		No Ct	28.98	negative		
1	*Lactobacillus acidophilus*	No Ct	31.53	negative		
2		No Ct	30.40	negative		
3		No Ct	30.54	negative		
1	*Legionella pneumophila*	No Ct	31.22	negative		
2		No Ct	31.41	negative		
3		No Ct	31.15	negative		
1	*Staphylococcus aureus*	No Ct	30.36	negative		
2		No Ct	28.50	negative		
3		No Ct	30.38	negative		
1	*Pseudomonas aeruginosa*	No Ct	30.11	negative		
2		No Ct	31.78	negative		
3		No Ct	31.42	negative		

No	Species	Ct FAM	Ct ROX	Result	FAM	ROX
1	*Streptococcus pneumoniae*	No Ct	31.79	negative		
2		No Ct	31.57	negative		
3		No Ct	30.22	negative		
1	*Micrococcus luteus*	No Ct	30.02	negative		
2		No Ct	31.29	negative		
3		No Ct	31.05	negative		
1	*Candida albicans*	No Ct	31.31	negative		
2		No Ct	30.42	negative		
3		No Ct	31.34	negative		
1	*Enterococcus faecalis*	No Ct	31.11	negative		
2		No Ct	30.46	negative		
3		No Ct	31.30	negative		
1	*Enterobacter aerogenes*	No Ct	31.42	negative		
2		No Ct	29.77	negative		
3		No Ct	30.30	negative		
1	*Escherichia coli*	No Ct	31.24	negative		
2		No Ct	30.61	negative		
3		No Ct	30.72	negative		

No	Species	Ct FAM	Ct ROX	Result	FAM	ROX
1	*Proteus mirabilis*	No Ct	31.47	negative		
2		No Ct	30.54	negative		
3		No Ct	30.61	negative		
1	*Salmonella enterica*	No Ct	31.17	negative		
2		No Ct	31.19	negative		
3		No Ct	30.17	negative		
1	*Bacillus subtilis*	31.26	30.58	positive		
2		31.04	30.65	positive		
3		31.12	29.64	positive		
1	*Staphylococcus epidermidis*	35.49	30.36	positive		
2		33.95	30.58	positive		
3		No Ct	31.23	negative		
1	*Bacillus cereus*	No Ct	30.75	negative		
2		No Ct	31.09	negative		
3		No Ct	30.85	negative		

Evaluating of the *Bacillus subtilis* PCR products:

Significant homology of the amplified PCR products to the 16S rRNA gene of *Bacillus subtilis* were confirmed by sequence analysis. Microsart® AMP Mycoplasma cross-reacts with *Bacillus subtilis* DNA



Query ID lcl|65363
Description amplicon_B_subtilis_K22_K23
Molecule type nucleic acid
Query Length 182

Database Name nr
Description All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences)
Program BLASTN 2.2.26+ ▷Citation

Other reports: ▷Search Summary [Taxonomy reports] [Distance tree of results]

⊞Graphic Summary
⊟Descriptions

Legend for links to other resources: U UniGene E GEO G Gene S Structure M Map Viewer PubChem BioAssay

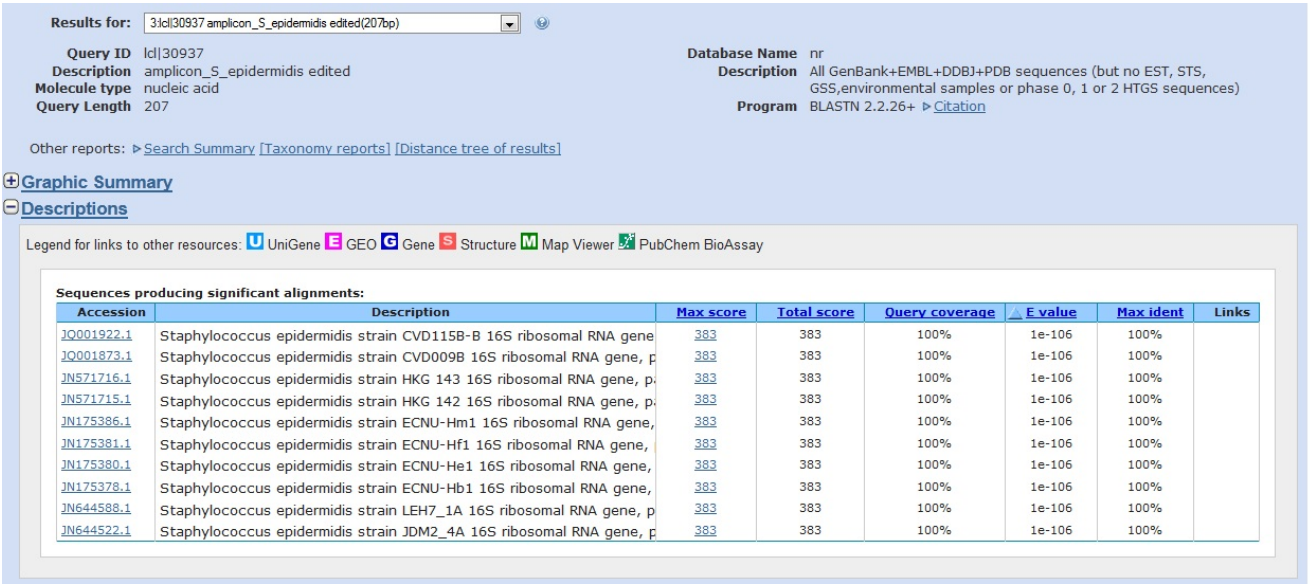
Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
JX035948.1	Bacillus subtilis strain JDG32 16S ribosomal RNA gene, partial sequence	337	337	100%	2e-89	100%	
JX025646.1	Bacillus sp. A32 16S ribosomal RNA gene, partial sequence	337	337	100%	2e-89	100%	
JX028840.1	Bacillus amyloliquefaciens strain C-1 16S ribosomal RNA gene, partial sec	337	337	100%	2e-89	100%	
HE659361.1	Bacillus pumilus partial 16S rRNA gene, strain GM37AC1c	337	337	100%	2e-89	100%	
FN822229.1	Bacillus subtilis partial 16S rRNA gene, isolate ASMM110	337	337	100%	2e-89	100%	
JQ916086.1	Bacillus amyloliquefaciens strain 22277 16S ribosomal RNA gene, partial s	337	337	100%	2e-89	100%	
JQ796652.1	Bacillus tequilensis strain B4 16S ribosomal RNA gene, partial sequence	337	337	100%	2e-89	100%	
JQ796651.1	Bacillus subtilis strain B26 16S ribosomal RNA gene, partial sequence	337	337	100%	2e-89	100%	
JX026032.1	Bacillus sp. MEC3 16S ribosomal RNA gene, partial sequence	337	337	100%	2e-89	100%	
JQ973602.1	Bacillus amyloliquefaciens strain 48MoSuero 16S ribosomal RNA gene, pa	337	337	100%	2e-89	100%	

A detection limit of 0.2 ng DNA/50 µl of sample volume per PCR was determined. Assuming a genome size of 4,214,814 bp [13] and 10 rRNA operon copies [10], the detection limit corresponds to $8.7*10^5$ cells per ml of sample.

Evaluating the *Staphylococcus epidermidis* PCR products:

Significant homology of the amplified PCR products to the 16S rRNA gene of *Staphylococcus epidermidis* were confirmed by sequence analysis. Microsart® AMP Mycoplasma cross-reacts with *Staphylococcus epidermidis* DNA.



Results for: 3|lcl|30937 amplicon_S_epidermidis edited(207bp)

Query ID lcl|30937
Description amplicon_S_epidermidis edited
Molecule type nucleic acid
Query Length 207

Database Name nr
Description All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences)
Program BLASTN 2.2.26+ ▷Citation

Other reports: ▷Search Summary [Taxonomy reports] [Distance tree of results]

⊞Graphic Summary
⊟Descriptions

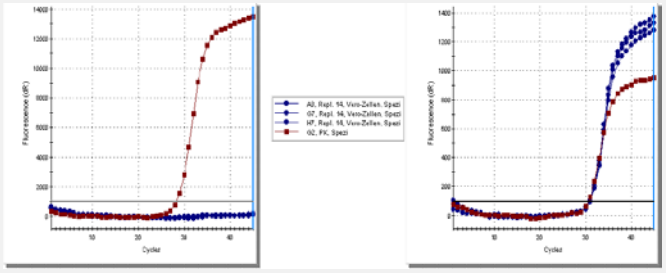
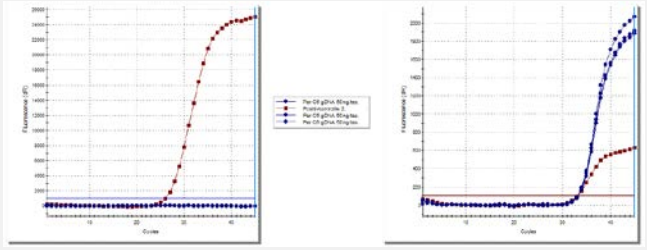
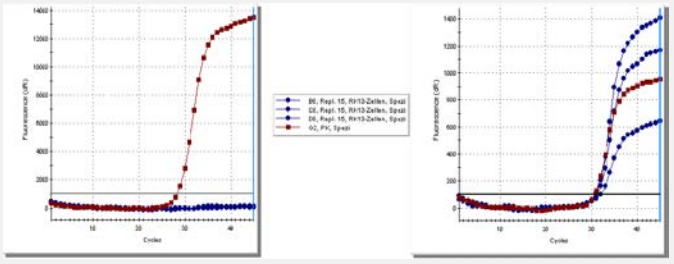
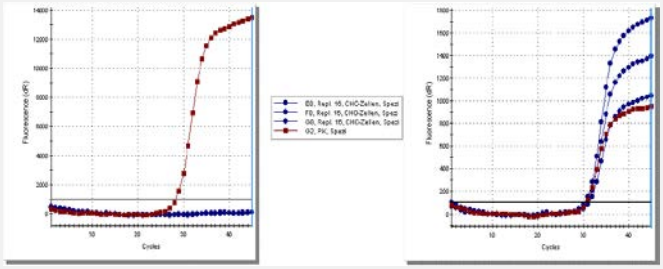
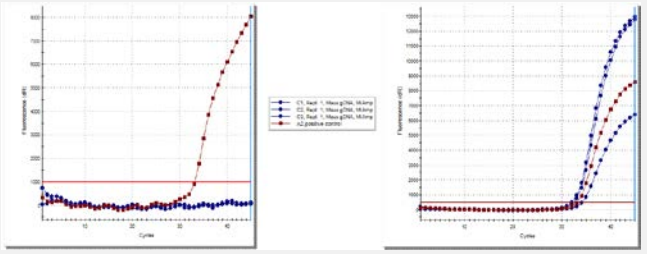
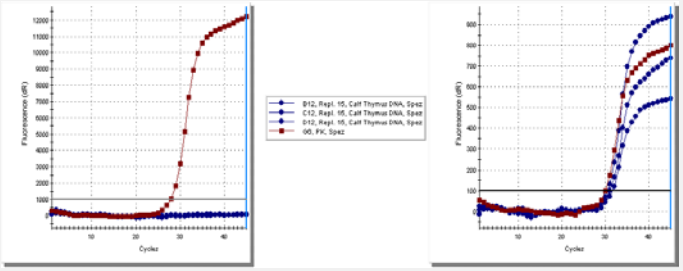
Legend for links to other resources: U UniGene E GEO G Gene S Structure M Map Viewer PubChem BioAssay

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
JQ001922.1	Staphylococcus epidermidis strain CVD115B-B 16S ribosomal RNA gene	383	383	100%	1e-106	100%	
JQ001873.1	Staphylococcus epidermidis strain CVD009B 16S ribosomal RNA gene, p	383	383	100%	1e-106	100%	
JN571716.1	Staphylococcus epidermidis strain HKG 143 16S ribosomal RNA gene, p	383	383	100%	1e-106	100%	
JN571715.1	Staphylococcus epidermidis strain HKG 142 16S ribosomal RNA gene, p	383	383	100%	1e-106	100%	
JN175386.1	Staphylococcus epidermidis strain ECNU-Hm1 16S ribosomal RNA gene,	383	383	100%	1e-106	100%	
JN175381.1	Staphylococcus epidermidis strain ECNU-Hf1 16S ribosomal RNA gene,	383	383	100%	1e-106	100%	
JN175380.1	Staphylococcus epidermidis strain ECNU-He1 16S ribosomal RNA gene,	383	383	100%	1e-106	100%	
JN175378.1	Staphylococcus epidermidis strain ECNU-Hb1 16S ribosomal RNA gene,	383	383	100%	1e-106	100%	
JN644588.1	Staphylococcus epidermidis strain LEH7_1A 16S ribosomal RNA gene, p	383	383	100%	1e-106	100%	
JN644522.1	Staphylococcus epidermidis strain JDM2_4A 16S ribosomal RNA gene, p	383	383	100%	1e-106	100%	

A detection limit of 0.1 ng DNA/50 µl of sample volume per PCR was determined. Assuming a genome size of 2,499,279 bp [12] and 5 rRNA operon copies [10], the detection limit corresponds to $7.3x10^5$ cells per ml of sample.

Mammalian DNA

No	Species	Ct FAM	Ct ROX	Result	FAM	ROX
1	Vero-B4	No Ct	31.11	negative		
2		No Ct	30.92	negative		
3		No Ct	30.85	negative		
1	PER.C6	No Ct	33.34	negative		
2		No Ct	33.12	negative		
3		No Ct	33.37	negative		
1	RK13	No Ct	32.02	negative		
2		No Ct	31.26	negative		
3		No Ct	30.87	negative		
1	CHO-K1	No Ct	30.35	negative		
2		No Ct	31.32	negative		
3		No Ct	30.98	negative		
1	Murine Genomic DNA	No Ct	34.19	negative		
2		No Ct	32.37	negative		
3		No Ct	32.02	negative		
1	Calf Thymus DNA	No Ct	31.02	negative		
2		No Ct	31.64	negative		
3		No Ct	30.62	negative		

6.3 Detection Limit

As the method employed is used only to obtain a qualitative result, proof of linearity is not required. If however the concept of linearity is extended to cover the working range, the detection limit becomes extremely important. In practice, the detection limit is determined in the form of the positive threshold (i.e. the cut-off point in the form of the minimum number of amplified target sequences by volume positively detected in 95% of the sample series).

6.3.1 Culture Media Comparison

Procedure	Acceptance Criterion	Results
For spiking, all *Mollicutes* species listed in EP 2.6.7 (see Table 3) are used. The spike will be prepared according to chapter 5.3 from fresh cultures and will be quantified immediately on culture plates. The ability of the culture method used to sustain the growth of mycoplasma in acceptable performance is confirmed by parallel testing with the EDQM Reference Standard listed in Table 6. The EDQM Reference Standards and the spikes of the same species will be diluted in culture broth with 3 replicates per dilution.	The prepared spikes shall show a calculated titer within the estimated titer range of the EDQM Reference Standard.	passed

Article No.	Article name	Estimated Titer	Result
Y0000692	*Mycoplasma fermentans* Ph Eur BRP, batch 1	$9.55x10^7$ CFU/ml with a range from $1.58x10^7$ to $5.75x10^8$ CFU/ml	$4.78x10^7$ CFU/ml
Y0000691	*Mycoplasma orale* Ph Eur BRP, batch 1	$4.90x10^5$ CFU/ml with a range from $9.33x10^4$ to $2.57x10^6$ CFU/ml	$5.88x10^5$ CFU/ml
Y0000689	*Mycoplasma synoviae* BRP, batch 1	$1.86x10^7$ CFU/ml with a range from $5.89x10^6$ to $5.89x10^7$ CFU/ml	$2.79x10^7$ CFU/ml
Y0000690	*Mycoplasma hyorhinis* BRP, batch 1	$1.17x10^8$ CFU/ml with a range from $6.76x10^7$ to $2.34x10^8$ CFU/ml	$1.99x10^8$ CFU/ml

6.3.2 LOD_{95} Determination

Procedure	Acceptance Criterion	Results
The prepared *Mollicutes* spikes according to chapter 5.3 will be diluted in 1:10 dilution steps (one deviating dilution step for accurate adjustment of concentration) in TE80 buffer to prepare a suspension with a concentration of 80 CFU/ml. Subsequently a twofold dilution series for 5 more steps will be prepared (40, 20, 10, 5, 2.5 CFU/ml). 8 individually dilution series will be prepared and dilutions from 80 to 2.5 CFU/ml tested according to chapter 8 with 3 repeats each so that at least 24 results per dilution are obtained: Analysis will be performed by four different operators (2 dilution series per person). Each sample of a dilution series will be analyzed three times (n = 8x3 = 24).	23 of 24 samples containing at least 10 CFU/ml must be tested positive for all species.	passed A Detection Limit of at least 10 cfu/ml was found for all species listed in the EP 2.6.7.

Acholeplasma laidlawii

CFU/ml	80	40	20	10	5	2.5	NTC
	29.37	29.89	27.95	32.50	33.80	34.25	No Ct
	28.59	30.35	31.44	32.20	32.59	35.34	
	29.32	29.28	30.46	31.99	34.41	33.75	
	28.44	28.52	29.23	31.75	32.51	33.95	No Ct
	27.80	29.79	30.43	31.08	32.70	31.68	
	28.53	28.70	30.27	31.27	32.65	27.23	
	27.84	28.40	29.80	30.75	31.34	32.43	No Ct
	27.60	28.09	29.52	29.51	30.41	32.21	
	27.34	28.20	28.75	30.66	30.75	31.37	
	29.49	29.94	29.86	32.09	32.35	34.21	41.05
	29.47	29.66	31.39	31.42	32.46	33.86	
	29.06	29.91	33.64	32.35	33.30	34.42	
	28.74	27.19	31.87	32.24	33.90	34.51	No Ct
	29.21	30.94	31.97	32.51	33.54	33.08	
	28.75	30.69	31.72	32.34	32.60	34.54	
	27.62	28.71	28.84	30.03	30.93	32.10	No Ct
	27.71	28.14	29.26	30.03	31.63	32.15	
	27.26	28.82	28.44	29.60	31.21	29.20	
	27.20	28.54	29.03	28.58	31.60	32.21	No Ct
	27.54	29.08	29.54	28.79	30.62	32.73	
	26.96	28.96	29.73	30.61	31.13	32.76	
	28.60	29.81	29.91	29.27	31.78	31.66	No Ct
	26.96	28.64	29.66	30.32	32.27	31.29	
	26.79	29.39	29.38	30.19	30.89	31.65	
Mw	**28.17**	**29.15**	**30.09**	**30.92**	**32.14**	**32.61**	
STABWN	0.87	0.89	1.30	1.21	1.10	1.77	
positive	24	24	24	24	24	24	
total	24	24	24	24	24	24	

Detection Limit: ≤ 2.5 cfu/ml

Mycoplasma arginini

CFU/ml	80	40	20	10	5	2.5	NTC
	34.63	34.16	34.89	35.77	35.38	No Ct	No Ct
	34.81	35.17	37.64	37.06	No Ct	No Ct	
	35.22	36.04	34.83	38.15	34.80	No Ct	
	32.25	34.55	36.05	33.04	35.26	No Ct	No Ct
	31.57	34.09	34.34	36.01	34.86	No Ct	
	32.00	33.58	33.76	40.69	35.71	36.12	
	32.48	33.03	33.78	35.80	35.58	35.40	No Ct
	32.67	33.92	No Ct	35.42	31.97	39.30	
	32.20	32.07	34.66	31.35	39.47	No Ct	
	32.16	33.83	32.28	34.33	35.53	38.71	No Ct
	31.94	33.17	32.39	36.51	No Ct	38.01	
	32.99	33.88	34.60	35.63	33.99	No Ct	
	30.45	31.01	32.46	32.38	34.48	34.06	42.24
	29.45	30.74	31.16	32.28	33.52	33.38	
	29.46	30.40	31.07	31.48	32.89	34.01	
	29.64	32.07	28.95	31.66	33.41	35.16	No Ct
	28.74	30.27	31.35	31.98	30.92	32.80	
	29.20	30.32	31.05	31.68	33.61	34.45	
	34.74	34.39	34.77	31.01	No Ct	31.36	No Ct
	33.56	38.53	36.38	34.43	36.73	30.15	
	33.68	35.87	36.04	38.15	35.63	35.78	
	32.79	33.44	34.46	34.47	35.61	36.86	No Ct
	32.76	32.65	34.14	34.92	35.86	38.42	
	33.22	33.35	34.63	35.79	35.95	35.91	
Mw	**32.19**	**33.36**	**33.73**	**34.58**	**34.82**	**35.29**	
STABWN	1.84	1.96	2.03	2.50	1.75	2.48	
positive	24	24	23	23	21	17	
total	24	24	24	24	24	24	

Detection Limit: 10 cfu/ml

Mycoplasma fermentans

CFU/ml	80	40	20	10	5	2.5	NTC
	29.54	31.12	30.92	32.42	33.45	30.44	No Ct
	29.24	31.51	29.43	33.07	32.50	34.21	
	29.95	32.01	32.85	31.56	33.00	34.31	
	30.15	31.39	31.61	32.17	31.29	36.38	No Ct
	30.24	29.52	28.85	32.98	33.29	29.98	
	30.14	29.98	31.49	33.27	31.64	34.62	
	29.95	31.48	31.23	32.51	34.23	33.77	No Ct
	29.19	30.06	30.92	31.27	32.92	34.09	
	29.82	30.94	30.48	33.29	33.64	36.97	
	30.27	31.96	31.18	32.65	35.85	37.51	No Ct
	31.10	32.74	31.66	32.11	34.05	35.23	
	31.26	30.58	32.96	32.66	33.28	37.26	
	26.75	28.42	29.60	26.49	28.02	27.76	No Ct
	27.38	27.48	29.00	29.74	28.36	29.07	
	28.24	28.00	28.42	30.52	29.15	29.55	
	30.24	24.00	32.33	32.56	31.60	34.38	40.30
	29.62	30.64	31.16	30.69	33.85	31.23	
	29.83	29.88	31.86	32.17	33.46	33.81	
	25.86	27.58	27.35	30.35	30.83	31.90	No Ct
	26.41	28.04	28.83	29.04	29.79	31.42	
	26.19	27.41	27.95	29.85	29.15	31.27	
	29.28	33.58	29.77	32.30	32.86	35.01	No Ct
	30.94	32.76	32.38	33.64	33.50	36.31	
	30.21	31.14	31.60	34.26	32.52	35.21	
Mw	**29.24**	**30.09**	**30.58**	**31.73**	**32.18**	**33.40**	
STABWN	1.55	2.16	1.55	1.70	1.99	2.72	
positive	24	24	24	24	24	24	
total	24	24	24	24	24	24	

Detection Limit: ≤ 2.5 cfu/ml

Mycoplasma gallisepticum

CFU/ml	80	40	20	10	5	2.5	NTC
	30.79	32.66	32.22	35.49	40.09	No Ct	No Ct
	31.76	32.48	33.34	35.26	41.61	34.63	
	32.21	32.88	32.02	33.73	34.55	34.54	
	28.35	31.69	33.40	34.00	35.53	36.01	No Ct
	29.51	31.46	33.73	32.08	34.17	38.31	
	30.22	31.32	31.57	34.03	35.59	35.44	
	30.86	32.53	34.89	35.12	No Ct	40.37	No Ct
	31.61	32.32	34.95	33.51	36.69	38.3	
	31.61	32.48	33.69	36.88	36.96	39.18	
	29.58	31.66	33.00	35.01	36.37	35.14	No Ct
	30.08	30.64	32.48	35.24	34.75	38.23	
	29.27	30.52	32.85	37.27	34.46	36.93	
	32.12	34.23	33.25	33.78	39.27	No Ct	No Ct
	31.56	32.52	33.51	37.87	35.35	No Ct	
	32.35	32.65	33.71	35.21	38.77	43.84	
	32.49	32.85	33.65	35.08	36.85	No Ct	No Ct
	32.23	34.04	34.73	37.00	37.83	41.14	
	32.43	32.96	33.23	36.31	39.95	35.13	
	33.61	35.12	39.35	37.20	37.79	No Ct	No Ct
	33.13	34.09	34.30	36.34	No Ct	34.44	
	33.04	33.56	36.03	36.82	40.10	No Ct	
	33.61	35.70	35.68	36.56	36.73	40.99	No Ct
	32.89	34.98	36.26	37.80	38.62	38.09	
	32.98	34.48	34.08	35.85	36.27	37.18	
Mw	**31.60**	**32.91**	**34.00**	**35.56**	**37.20**	**37.66**	
STABWN	1.43	1.35	1.62	1.48	2.07	2.61	
positive	24	24	24	24	19	14	
total	24	24	24	24	24	24	

Detection Limit: 10 cfu/ml

Mycoplasma hyorhinis

CFU/ml	80	40	20	10	5	2.5	NTC
	30.91	31.24	32.08	32.27	34.24	40.99	No Ct
	31.11	30.71	31.79	32.81	34.01	34.77	
	30.72	30.84	31.49	32.67	33.92	34.34	
	30.56	30.88	32.86	31.15	33.48	35.87	No Ct
	30.01	30.92	32.04	33.25	33.92	35.18	
	30.27	31.09	33.07	31.78	33.92	36.34	
	29.59	31.07	31.71	33.11	34.72	35.00	No Ct
	29.60	31.14	32.39	32.71	34.50	34.59	
	29.68	30.48	31.33	32.93	33.83	33.92	
	28.51	29.53	30.35	31.31	31.96	33.73	No Ct
	28.53	29.18	30.27	31.26	31.73	33.92	
	28.52	29.54	30.18	31.40	32.18	33.17	
	30.27	31.54	31.61	32.94	37.17	35.63	No Ct
	29.58	31.26	32.25	33.04	35.14	35.12	
	30.01	30.42	31.77	33.42	33.90	36.70	
	29.36	30.60	31.60	31.98	32.96	35.03	No Ct
	30.23	31.10	31.32	32.04	33.55	34.50	
	29.52	31.47	31.02	32.10	33.11	33.20	
	29.91	31.50	32.23	34.32	33.35	36.86	No Ct
	30.16	31.69	32.79	33.70	34.13	37.62	
	30.49	31.08	32.29	33.51	34.91	37.66	
	30.00	31.33	32.24	33.27	33.41	36.89	No Ct
	30.18	30.81	32.34	33.39	No Ct	35.62	
	30.20	30.90	32.09	33.35	34.60	36.21	
Mw	29.91	30.85	31.80	32.65	33.85	35.54	
STABWN	0.679	0.629	0.756	0.845	1.117	1.697	
positive	24	24	24	24	23	23	
total	24	24	24	24	24	24	

Detection Limit: ≤ 10 cfu/ml

Mycoplasma orale

CFU/ml	80	40	20	10	5	2.5	NTC
	31.23	33.48	32.37	35.53	No Ct	38.59	No Ct
	31.63	32.03	34.43	33.74	35.22	No Ct	
	32.28	32.84	34.53	34.96	31.41	No Ct	
	30.28	31.27	32.63	33.46	35.36	No Ct	No Ct
	30.76	31.10	32.72	34.47	35.65	35.75	
	30.63	31.17	33.97	34.21	37.02	39.25	
	31.16	32.14	32.73	35.20	35.20	35.51	No Ct
	31.39	31.71	32.15	35.91	35.41	39.07	
	31.13	31.43	32.63	40.89	37.15	No Ct	
	29.47	31.15	31.68	33.17	34.32	32.50	No Ct
	29.19	30.48	32.11	32.20	34.64	31.72	
	29.55	31.43	30.76	32.70	34.79	34.87	
	29.13	29.61	30.57	32.35	32.87	34.16	No Ct
	28.39	29.95	31.15	32.51	32.99	34.13	
	29.36	29.96	30.39	32.45	33.38	41.06	
	32.30	34.02	35.95	36.45	36.70	38.97	No Ct
	31.88	33.06	34.30	34.82	No Ct	No Ct	
	31.92	33.37	34.35	37.09	41.00	42.91	
	29.45	30.04	31.40	31.89	33.17	34.28	No Ct
	29.28	29.80	31.61	32.81	33.33	34.42	
	29.49	29.91	31.14	32.90	33.32	34.19	
	29.35	30.48	31.19	32.23	33.28	34.14	No Ct
	29.92	30.43	31.55	30.91	32.75	33.58	
	30.42	30.83	30.38	32.48	33.51	34.76	
Mw	**30.40**	**31.32**	**32.36**	**33.97**	**34.66**	**35.99**	
STABWN	1.122	1.258	1.489	2.118	2.020	2.969	
positive	24	24	24	23	20	17	
total	24	24	24	24	21	24	

Detection Limit: 10 cfu/ml

Mycoplasma pneumoniae

CFU/ml	80	40	20	10	5	2.5	NTC
	32.57	32.70	36.31	32.79	38.15	33.68	No Ct
	31.68	33.41	35.16	35.92	35.34	No Ct	
	30.81	32.24	33.61	40.37	No Ct	No Ct	
	32.92	32.46	32.56	35.44	32.18	37.22	No Ct
	31.92	32.69	34.11	39.87	33.41	No Ct	
	32.62	35.75	36.70	35.48	33.48	33.54	
	31.97	32.17	33.12	35.46	36.51	40.82	No Ct
	30.80	32.25	34.46	33.99	35.33	31.62	
	28.31	32.13	34.37	37.34	36.26	No Ct	
	28.40	29.02	29.32	32.84	31.83	32.21	No Ct
	28.12	29.49	30.16	31.58	32.45	31.95	
	28.78	29.46	31.09	31.32	31.80	33.31	
	29.47	33.60	31.87	33.98	34.43	No Ct	42.25
	32.08	32.80	33.98	34.03	35.95	35.64	
	30.63	30.49	33.99	35.26	34.39	32.42	
	32.90	35.85	34.78	35.74	37.13	No Ct	42.61
	30.97	32.44	33.97	34.95	No Ct	36.95	
	31.79	33.22	33.77	36.94	41.26	33.06	
	31.23	31.77	32.47	32.28	34.74	37.16	43.59
	30.39	32.38	33.42	34.28	36.48	34.61	
	32.00	32.02	33.20	33.90	35.83	35.61	
	31.54	32.68	33.75	32.68	35.66	38.09	No Ct
	29.78	32.97	33.35	35.30	34.84	37.36	
	32.16	32.51	32.24	35.29	34.75	34.93	
Mw	**30.99**	**32.35**	**33.41**	**34.88**	**35.10**	**35.01**	
STABWN	1.45	1.58	1.66	2.20	2.16	2.46	
positive	24	24	24	23	22	18	
total	24	24	24	24	24	24	

Detection Limit: 10 cfu/ml

Mycoplasma synoviae

CFU/ml	80	40	20	10	5	2.5	NTC
	31.29	31.44	31.12	33.99	36.62	39.69	No Ct
	32.03	32.57	34.51	44.25	No Ct	33.78	
	31.94	32.02	33.28	39.00	33.39	35.09	
	30.14	33.07	30.80	33.77	35.26	39.36	No Ct
	30.69	31.24	30.85	33.72	No Ct	36.35	
	32.52	30.73	32.84	34.46	34.66	39.75	
	31.44	33.24	32.94	33.95	No Ct	36.92	No Ct
	31.27	32.38	36.52	33.12	38.97	36.72	
	29.34	33.88	34.26	No Ct	33.90	36.86	
	32.00	33.32	32.11	39.01	33.64	32.85	No Ct
	31.43	31.98	34.76	30.82	34.94	37.47	
	33.18	31.48	35.30	37.10	30.73	31.42	
	32.20	33.53	33.45	36.00	34.03	39.85	No Ct
	32.07	33.17	34.68	37.45	34.26	37.35	
	32.26	31.91	34.49	36.09	41.95	No Ct	
	29.82	32.51	31.45	33.07	39.94	33.94	No Ct
	32.54	33.48	31.96	33.44	31.55	32.08	
	30.38	33.88	32.56	32.98	34.10	33.19	
	32.92	34.47	34.45	36.28	32.61	36.36	No Ct
	33.86	36.67	37.23	36.72	35.16	38.16	
	33.90	33.95	35.18	35.89	34.98	39.63	
	31.63	32.52	32.16	31.06	33.58	29.66	No Ct
	30.75	31.36	32.00	29.83	33.52	33.00	
	33.32	28.08	32.42	28.90	34.03	32.52	
Mw	31.79	32.62	33.39	34.82	34.85	35.74	
STABWN	1.184	1.574	1.716	3.305	2.580	2.963	
positive	24	24	24	23	20	23	
total	24	24	24	24	24	24	

Detection Limit: 10 cfu/ml

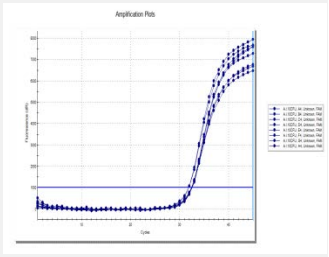
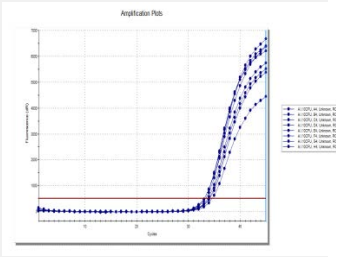
Spiroplasma citri

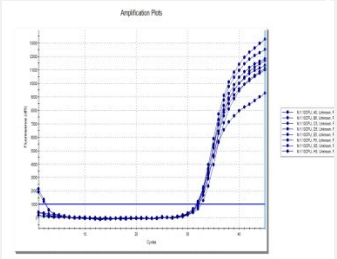
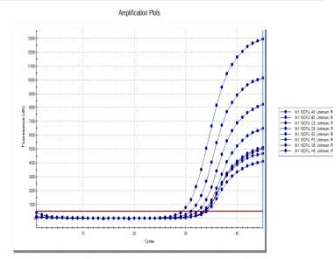
CFU/ml	80	40	20	10	5	2.5	NTC
	31.08	32.91	32.34	33.49	34.62	41.01	No Ct
	30.99	31.29	33.67	33.49	34.63	35.46	
	31.92	32.46	33.08	35.21	34.64	37.53	
	32.53	34.14	33.53	39.32	33.35	32.62	No Ct
	35.86	33.63	38.21	35.40	39.73	No Ct	
	33.49	35.82	35.18	31.98	37.92	27.65	
	32.84	34.32	33.85	34.94	35.80	No Ct	No Ct
	32.39	31.76	35.80	36.76	37.26	33.08	
	32.55	33.93	32.29	33.43	34.72	No Ct	
	34.84	34.54	33.70	No Ct	42.69	41.24	No Ct
	33.48	32.50	37.38	37.90	No Ct	No Ct	
	35.05	34.26	38.00	35.17	37.50	34.64	
	32.65	35.94	33.06	38.58	28.90	35.38	No Ct
	31.08	32.83	35.19	37.03	35.47	No Ct	
	32.53	34.91	34.77	36.18	28.08	40.67	
	32.27	34.21	35.42	35.39	33.00	44.73	No Ct
	32.45	33.04	35.12	34.72	37.98	31.44	
	33.33	33.33	34.94	34.36	34.58	38.50	
	33.97	36.05	35.39	35.34	37.14	36.81	No Ct
	32.83	37.45	35.23	35.37	36.57	37.49	
	33.44	33.37	37.25	34.04	36.71	38.83	
	32.14	33.53	34.12	34.08	36.63	35.89	No Ct
	32.48	33.62	34.41	34.46	34.85	35.86	
	33.53	33.45	33.82	34.43	35.13	36.01	
Mw	**32.91**	**33.89**	**34.82**	**35.26**	**35.56**	**36.57**	
STABWN	1.18	1.39	1.60	1.70	3.01	3.82	
positive	24	24	24	23	22	15	
total	24	24	24	24	24	24	

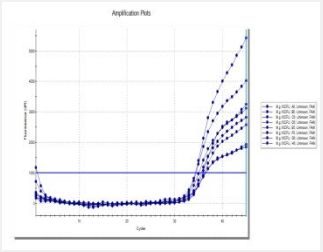
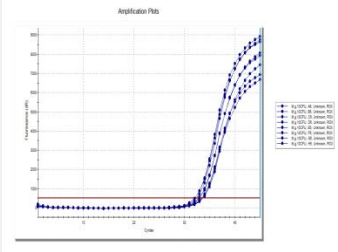
Detection Limit: 10 cfu/ml

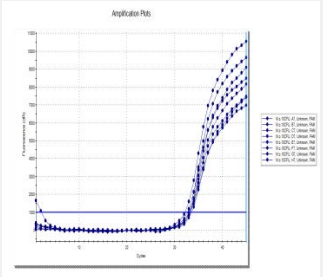
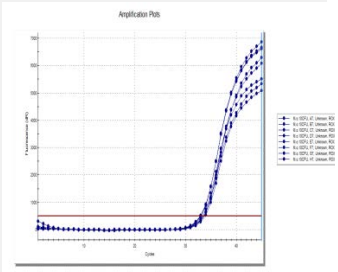
6.3.3 Cross Check Detection of 10 CFU/ml

Procedure	Acceptance Criterion	Results
All *Mollicutes* species listed in EP 2.6.7 with the exception of *Mycoplasma pneumonia* (see chapter 6.3.4) are used. The samples will be prepared from titrated mycoplasma broth. 8 samples from the dilution containing 10 CFU/ml from each species will be tested according to chapter 5.5.	All 8 reactions must be positive for all species.	passed

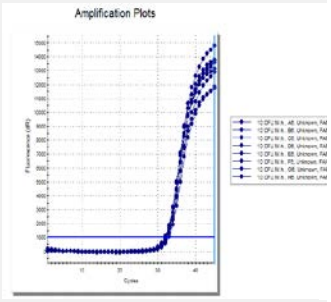
No	Species	Ct FAM	Ct ROX	Result	FAM	ROX
1	*Acholeplasma laidlawii*	32.50	34.02	positive		
2	*Acholeplasma laidlawii*	31.89	33.12	positive		
3	*Acholeplasma laidlawii*	32.46	34.19	positive		
4	*Acholeplasma laidlawii*	31.89	33.38	positive		
5	*Acholeplasma laidlawii*	32.57	33.39	positive		
6	*Acholeplasma laidlawii*	32.51	34.65	positive		
7	*Acholeplasma laidlawii*	32.62	33.81	positive		
8	*Acholeplasma laidlawii*	32.59	33.10	positive		

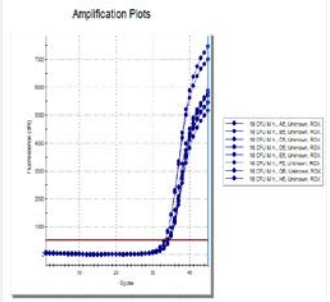
No	Species	Ct FAM	Ct ROX	Result	FAM	ROX
1	*Mycoplasma fermentans*	32.12	34.15	positive		
2	*Mycoplasma fermentans*	31.93	33.84	positive		
3	*Mycoplasma fermentans*	31.68	33.72	positive		
4	*Mycoplasma fermentans*	31.85	29.34	positive		
5	*Mycoplasma fermentans*	32.22	32.35	positive		
6	*Mycoplasma fermentans*	31.65	33.65	positive		
7	*Mycoplasma fermentans*	32.59	33.46	positive		
8	*Mycoplasma fermentans*	31.65	30.99	positive		

No	Species	Ct FAM	Ct ROX	Result	FAM	ROX
1	*Mycoplasma gallisepticum*	36.24	33.40	positive		
2	*Mycoplasma gallisepticum*	35.08	33.59	positive		
3	*Mycoplasma gallisepticum*	34.38	32.42	positive		
4	*Mycoplasma gallisepticum*	35.87	33.71	positive		
5	*Mycoplasma gallisepticum*	35.64	32.84	positive		
6	*Mycoplasma gallisepticum*	36.54	32.06	positive		
7	*Mycoplasma gallisepticum*	36.50	32.99	positive		
8	*Mycoplasma gallisepticum*	34.54	32.03	positive		

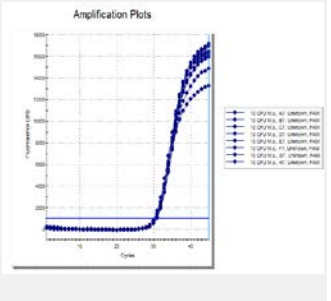
No	Species	Ct FAM	Ct ROX	Result	FAM	ROX
1	*Mycoplasma orale*	33.39	33.01	positive		
2	*Mycoplasma orale*	33.05	33.57	positive		
3	*Mycoplasma orale*	32.73	33.34	positive		
4	*Mycoplasma orale*	33.11	32.96	positive		
5	*Mycoplasma orale*	32.73	33.89	positive		
6	*Mycoplasma orale*	33.24	33.07	positive		
7	*Mycoplasma orale*	33.60	33.68	positive		
8	*Mycoplasma orale*	32.20	33.65	positive		

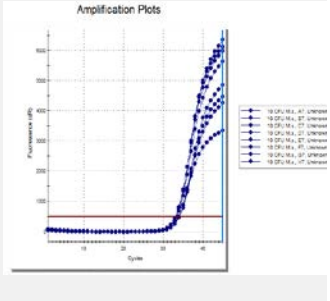
No	Species	Ct FAM	Ct ROX	Result	FAM	ROX
1	*Mycoplasma hyorhinis*	32.47	33.89	positive		
2	*Mycoplasma hyorhinis*	32.14	34.59	positive		
3	*Mycoplasma hyorhinis*	31.84	34.45	positive		
4	*Mycoplasma hyorhinis*	32.04	33.22	positive		
5	*Mycoplasma hyorhinis*	32.00	33.18	positive		
6	*Mycoplasma hyorhinis*	32.66	34.48	positive		
7	*Mycoplasma hyorhinis*	32.22	34.38	positive		
8	*Mycoplasma hyorhinis*	32.93	34.55	positive		



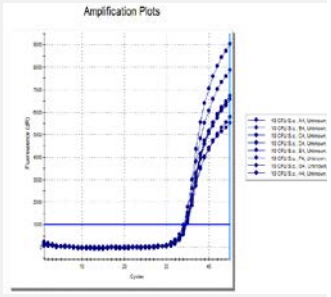


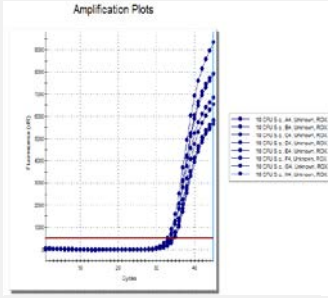
No	Species	Ct FAM	Ct ROX	Result	FAM	ROX
1	*Mycoplasma synoviae*	30.90	33.20	positive		
2	*Mycoplasma synoviae*	30.44	33.51	positive		
3	*Mycoplasma synoviae*	30.88	33.27	positive		
4	*Mycoplasma synoviae*	30.91	33.31	positive		
5	*Mycoplasma synoviae*	30.83	34.04	positive		
6	*Mycoplasma synoviae*	30.57	34.06	positive		
7	*Mycoplasma synoviae*	30.23	34.05	positive		
8	*Mycoplasma synoviae*	30.54	34.19	positive		



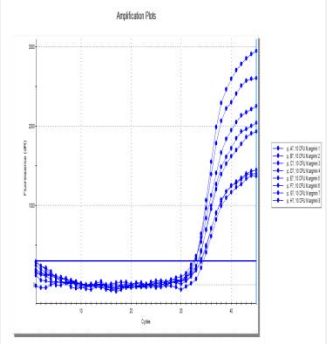


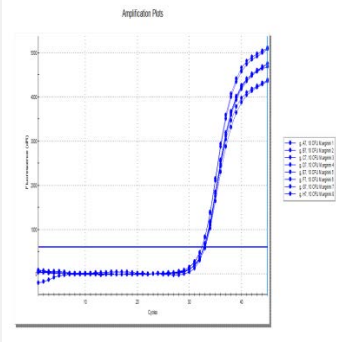
No	Species	Ct FAM	Ct ROX	Result	FAM	ROX
1	*Spiroplasma citri*	34.48	33.60	positive		
2	*Spiroplasma citri*	34.51	34.36	positive		
3	*Spiroplasma citri*	34.75	32.94	positive		
4	*Spiroplasma citri*	34.70	34.26	positive		
5	*Spiroplasma citri*	34.04	33.39	positive		
6	*Spiroplasma citri*	34.36	33.38	positive		
7	*Spiroplasma citri*	34.90	34.83	positive		
8	*Spiroplasma citri*	34.66	34.64	positive		





No	Species	Ct FAM	Ct ROX	Result	FAM	ROX
1	*Mycoplasma arginini*	34.88	32.18	positive		
2	*Mycoplasma arginini*	35.11	32.63	positive		
3	*Mycoplasma arginini*	37.64	32.55	positive		
4	*Mycoplasma arginini*	37.71	32.76	positive		
5	*Mycoplasma arginini*	36.33	32.52	positive		
6	*Mycoplasma arginini*	38.12	32.75	positive		
7	*Mycoplasma arginini*	36.06	32.54	positive		
8	*Mycoplasma arginini*	35.73	32.12	positive		





6.3.4 Cross Check LOD_{95} for *Mycoplasma pneumoniae*

Procedure	Acceptance Criterion	Results
Mycoplasma pneumoniae was found as the species showing the lowest sensitivity with Microsart® AMP Mycoplasma, version 1. The prepared *Mycoplasma pneumoniae* spike prepared during the previous study will be diluted again in 1:10 dilution steps (one deviating dilution step for accurate adjustment of concentration) in TE80 buffer to prepare a suspension with a concentration of 80 CFU/ml. Subsequently a twofold dilution series for 3 more steps will be prepared (40, 20, 10 CFU/ml). 8 individually dilution series will be prepared and the dilution containing 10 CFU/ml tested according to chapter 8 with 3 repeats each so that at least 24 results for this dilution are obtained: Analysis will be performed by four different operators (2 dilution series per person). Each sample of a dilution series will be analyzed three times (n = 8x3 = 24)	A detection limit of at least 10 CFU/ml must be reached with 23 positive reactions out of 24 reactions.	passed A Detection Limit of 10 cfu/ml was confirmed for *Mycoplasma pneumonia*e.

CFU/ml	**10**	**10**	**10**	
	33.78	35.78	35.59	
	34.67	35.12	33.68	
	34.02	35.29	36.22	
	33.11	34.92	34.94	
	33.85	34.45	33.30	
	33.08	No Ct	34.27	
	33.09	33.77	35.71	
	33.68	36.07	35.41	
NTC	No Ct	No Ct	No Ct	
Av	33.66	35.06	34.89	
Dev	0.520	0.722	0.973	
∑ positive	8	7	8	23
∑	8	8	8	24

6.4 Robustness

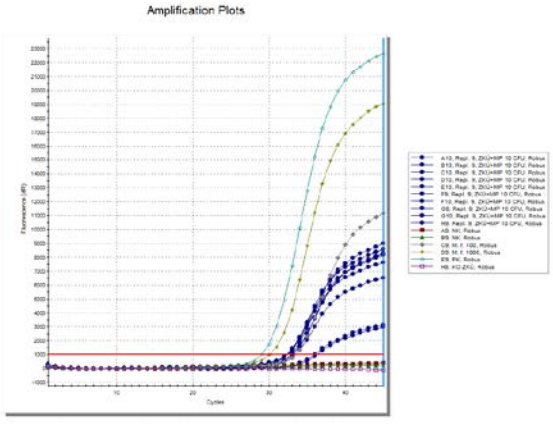
The robustness of Microsart® AMP Mycoplasma shall be shown for the mycoplasma species showing the lowest sensitivity. The results of the sensitivity study demonstrated identical sensitivities for the mycoplasma species *Mycoplasma synoviae, Spiroplasma citri, Mycoplasma arginini, Mycoplasma gallisepticum, Mycoplasma orale, and Mycoplasma pneumoniae*. Due to the intended use of the kit for testing biopharmaceuticals with an application ratio of estimated focus of 90 % on human associated species, *Mycoplasma pneumoniae* has been selected by clinical relevance for robustness testing.

6.4.1 Matrix Effects – Cell Culture Media

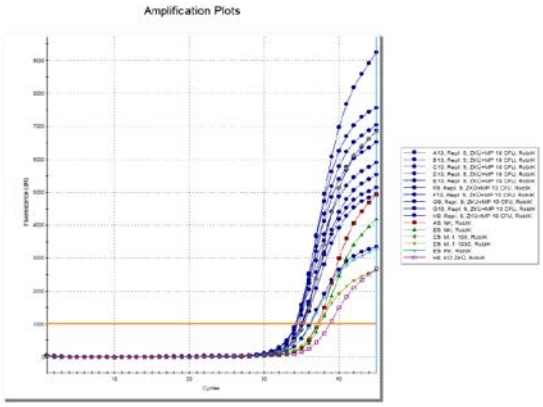
Procedure	Acceptance Criterion	Results
The robustness of the method will be demonstrated by the determination of 10 CFU/ml of the *Mycoplasma* species showing the lowest sensitivity according to chapter 6.2 in different matrices (Vero cell culture supernatant, DMEM with 5 % FCS, RPMI, RPMI with 10 % v/v FCS). At least 10 repeats shall be tested for each sample matrix.	All samples show a positive result.	passed

No	Sample	Ct - FAM	Ct - ROX	Result
1	**Cell culture supernatant** + 10 CFU/ml *M. pneumoniae*	32.41	36.15	positive
2		32.37	35.03	positive
3		32.40	34.62	positive
4		32.29	35.40	positive
5		33.47	34.38	positive
6		33.11	34.19	positive
7		36.33	34.20	positive
8		33.00	34.33	positive
9		35.94	35.02	positive
10		32.89	35.15	positive
11	NTC	No Ct	37.29	passed
12	NTC	No Ct	37.65	passed
13	Negative control (extraction)	No Ct	38.90	passed

FAM signal:

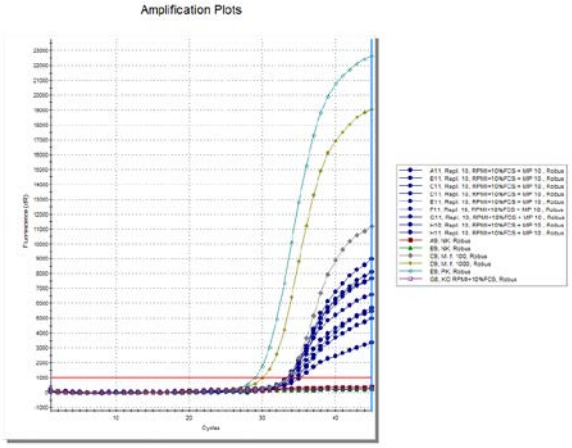


ROX signal:

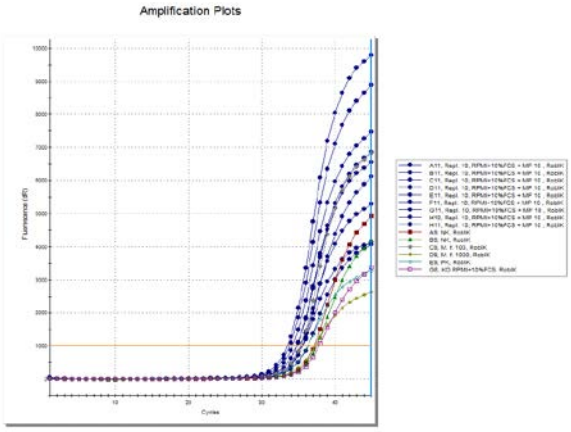


No	Sample	Ct - FAM	Ct - ROX	Result
1	**RPMI + 10 % FCS** + 10 CFU/ml *M. pneumoniae*	35.17	35.63	positive
2		33.78	34.85	positive
3		33.31	34.82	positive
4		34.56	33.85	positive
5		33.55	34.27	positive
6		33.47	35.63	positive
7		33.54	35.61	positive
8		34.04	35.38	positive
9		34.67	35.56	positive
10		33.93	36.33	positive
11	NTC	No Ct	37.29	passed
12	NTC	No Ct	37.65	passed
13	Negative control (extraction)	No Ct	37.97	passed

FAM signal:

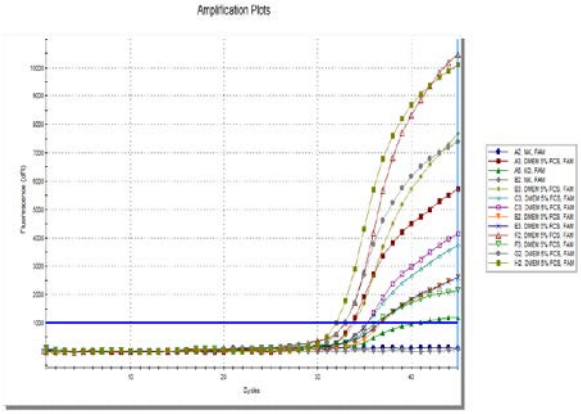


ROX signal:

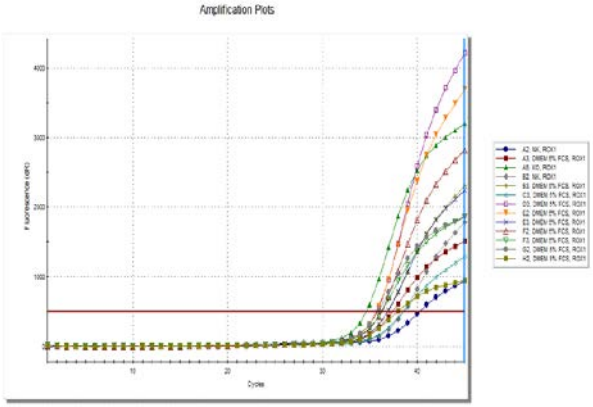


No	Sample	Ct - FAM	Ct - ROX	Result
1	**DMEM + 5 % FCS** + 10 CFU/ml *M. pneumoniae*	33,72	37,45	positive
2		33,97	36,96	positive
3		35,38	38,56	positive
4		35,37	35,81	positive
5		36,89	35,78	positive
6		36,65	36,93	positive
7		32,97	36,29	positive
8		36,26	36,33	positive
9		32,96	35,90	positive
10		31,98	37,94	positive
11	NTC	No Ct	38.25	passed
12	NTC	No Ct	37.08	passed
13	Negative control (extraction)	40,49	33.48	passed

FAM signal:

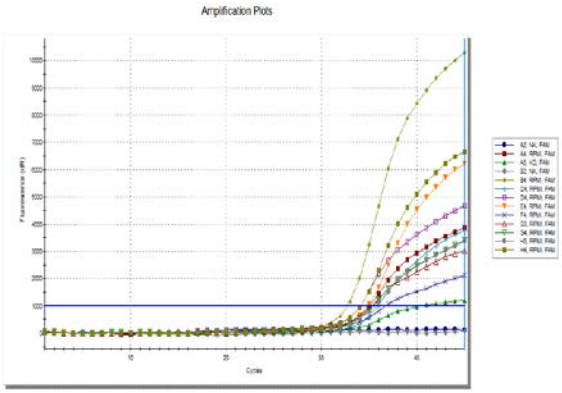


ROX signal:

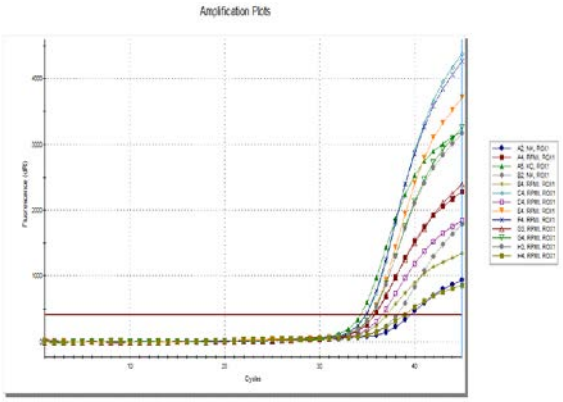


No	Sample	Ct - FAM	Ct - ROX	Result
1	**RPMI** + 10 CFU/ml *M. pneumoniae*	35,08	36,29	positive
2		32,78	37,64	positive
3		35,92	35,29	positive
4		34,13	37,03	positive
5		34,93	35,80	positive
6		36,78	35,21	positive
7		35,35	36,23	positive
8		35,47	35,87	positive
9		35,66	35,89	positive
10		34,14	39,77	positive
11	NTC	No Ct	38.25	passed
12	NTC	No Ct	37.08	passed
13	Negative control (extraction)	40,49	33.48	passed

FAM signal:



ROX signal:



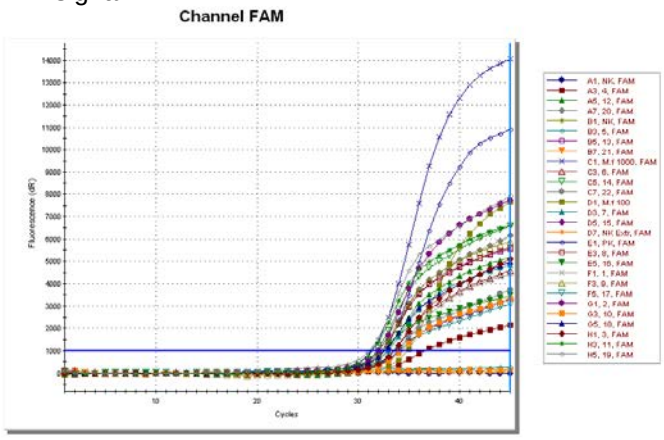
6.4.2 Matrix Effects – Cell Suspensions

Procedure	Acceptance Criterion	Results
To demonstrate robustness, at least 20 *Mycoplasma* negative samples (selected at random from samples submitted by customers for *Mycoplasma* detection) spiked with 10 CFU/ml of *Mycoplasma pneumoniae* validated with the lowest sensitivity should be tested. At least 2 repeats shall be tested for each sample.	All samples should be found positive.	passed

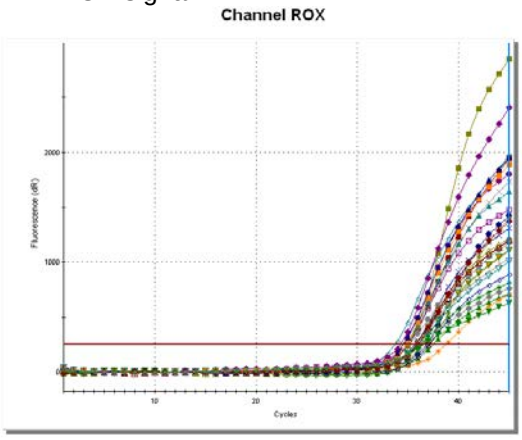
Nº	**Sample Code / Characteristics**				**Ct.** (FAM)	**Ct.** (ROX)	**Ct.** (FAM)	**Ct.** (ROX)	**Result**
					Repeat 1		Repeat 2		
1	MT 1206	cell culture supernatant	colorless	clear	33,97	35,14	31,90	32,07	positive
2	MT 1207	cell culture supernatant	colorless	clear	31,99	34,63	32,06	32,36	positive
3	P1661	cell culture supernatant	pink	clear	33,59	35,96	33,44	31,70	positive
4	P1680	cell culture supernatant	pink	clear	36,64	35,08	33,60	30,95	positive
5	B04-32	cell suspension	yellow	turbid	33,51	36,09	33,01	32,31	positive
6	B04-32	cell suspension	yellow	turbid	32,99	34,87	32,04	31,56	positive
7	36.26.10	cell suspension	pink	turbid	32,45	35,86	31,45	32,01	positive
8	47.28.15	cell suspension	pink	turbid	32,45	35,84	31,30	31,80	positive
9	NaTH119	cell culture supernatant	pink	clear	34,25	34,89	32,09	31,64	positive
10	7G5	cell culture supernatant	pink	clear	31,47	36,26	31,32	31,63	positive
11	Myco1	cell culture supernatant	pink	clear	32,82	37,55	29,51	29,66	positive
12	Panc02	cell culture supernatant	pink	clear	32,35	34,91	31,03	31,41	positive
13	HCT-8, Fl.Q	cell culture supernatant	pink	clear	31,88	36,47	32,02	31,14	positive
14	B04-35	cell suspension	colorless	turbid	34,08	34,31	32,78	31,72	positive
15	B04-35	cell suspension	pink	turbid	32,26	36,59	32,14	31,91	positive
16	MW5B-CO1	cell suspension	colorless	turbid	34,03	36,04	30,18	30,61	positive
17	MW5B-Test	cell suspension	colorless	turbid	32,70	34,62	31,56	31,08	positive
18	1-WCB10	cell culture supernatant	pink	clear	31,12	35,88	30,78	30,91	positive
19	2-WCB10	cell culture supernatant	pink	clear	32,16	36,55	30,46	30,31	positive
20	10112	plasma	yellow	turbid	33,75	35,19	33,22	32,20	positive
21	extraction control				No Ct	38,78	No Ct	35,52	negative
22	NTC				No Ct	36,91	No Ct	35,91	negative

Repeat 1:

FAM signal:

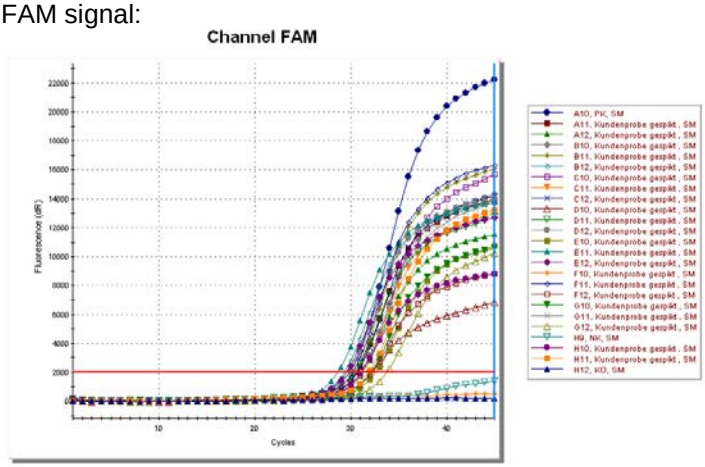


ROX signal:

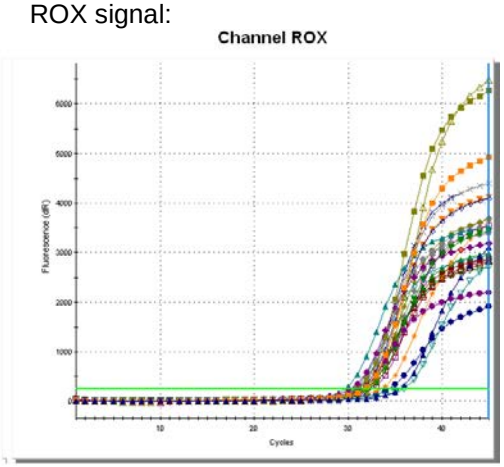


Repeat 2:

FAM signal:



ROX signal:



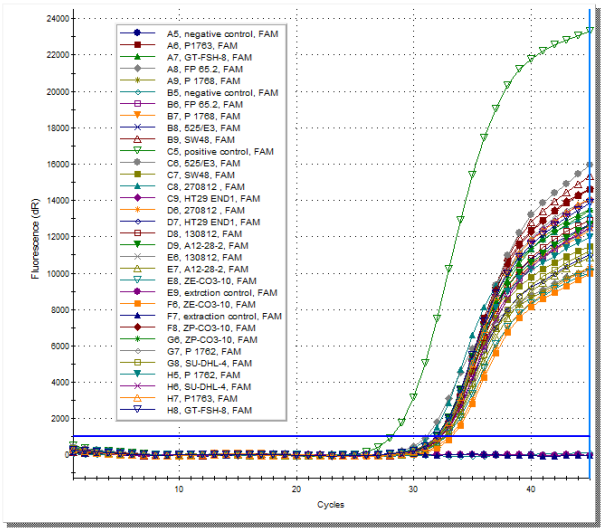
6.4.3 Internal Amplification Control Used for Monitoring the Process with Native Samples

Procedure	Acceptance Criterion	Results
12 Mycoplasma negative samples (selected at random from samples submitted by customers for mycoplasma detection) will be spiked with 10 CFU/ml of *Mycoplasma pneumoniae*. 2 repeats shall be tested for each sample.	All samples should be found positive.	passed

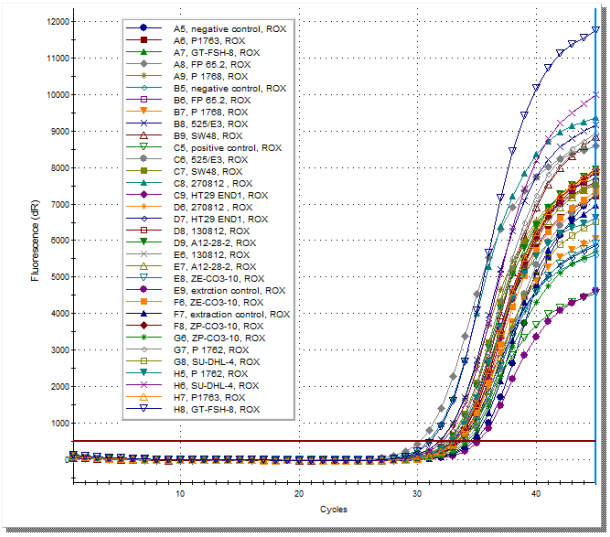
Sample				Order number	Spike volume	Ct.-value (FAM)	Ct.-value (ROX)	result
Code	**Type**	**characteristics**						
P 1762	cell culture medium	colorless	clear	D210812-1	22.7 µl	32.08 32.00	33.00 32.64	positive
P1763	cell culture medium	colorless	clear	D210812-1	22.7 µl	31.97 32. 06	33.24 33.13	positive
FP 65.2	cell suspension	pink	clear	D040912-2	22.7 µl	32.43 31.10	33.06 30.20	positive
525/E3	cell suspension	pink	clear	D040912-2	22.7 µl	32.22 32.12	34.04 31.87	positive
270812	cell suspension	colorless	turbid	D040912-3	22.7 µl	33.06 31.37	33.12 30.93	positive
130812	cell suspension	colorless	turbid	D040912-3	22.7 µl	32.64 32.39	32.76 33.16	positive

Sample				Order number	Spike volume	Ct.-value (FAM)	Ct.-value (ROX)	result
Code	Type	characteristics						
ZE-CO3-10	cell suspension	pink	turbid	D040912-4	22.7 µl	33.31 32.80	33.46 33.76	positive
ZP-CO3-10	cell suspension	yellow	turbid	D040912-4	22.7 µl	32.15 32.10	34.23 33.14	positive
SU-DHL-4	cell suspension	yellow	turbid	D300812-2	22.7 µl	32.36 32.62	32.16 33.74	positive
GT-FSH-8	cell suspension	pink	turbid	D240812-1	22.7 µl	32.18 31.92	33.03 31.12	positive
P 1768	cell culture medium	pink	clear	D310812-1	22.7 µl	32.26 31.96	33.54 31.91	positive
SW48	cell culture medium	pink	clear	D220812-2	22.7 µl	32.28 32.00	32.51 32.91	positive
HT29	cell culture medium	pink	clear	D220812-2	22.7 µl	32.39 32.11	33.60 33.45	positive
A12-28-2	cell culture medium	pink	clear	D160812-2	22.7 µl	32.70 31.95	33.20 32.63	positive
extraction control 1				---	---	No Ct	34.50	negative
extraction control 2				---	---	No Ct	35.12	negative
negative control 1				---	---	No Ct	34.78	negative
negative control 2				---	---	No Ct	33.43	negative
positive control				---	---	28.15	33.48	positive

Amplification plots of target amplification (channel FAM)



Amplification plots of internal control (channel ROX)



6.4.4 Lab-to-Lab Precision

Procedure	Acceptance Criterion	Results
From the four EDQM Reference Standards (listed in table 6) an equal dilution series is prepared in culture broth and tested by PCR by Sartorius and by Minerva.	Average Ct values for each concentration with Ct values < 30 shall not differ by more than 2 Ct.	Failed, for 2 of 4 preparations in the low copy, none-linear range of the method.

EDQM *M fermentans*

cfu/ml	SSB			Minerva			delta Ct
	Set 1	Set 2	**Av Ct**	Set 1	Set 2	**Av Ct**	
9.55E+06	16.12	16.03	**16.08**	16.63	16.93	**16.78**	-0.70
9.55E+05	18.94	19.99	**19.47**	19.66	19.43	**19.55**	-0.08
9.55E+04	22.39	22.71	**22.55**	23.24	22.49	**22.87**	-0.31
9.55E+03	25.57	26.4	**25.99**	26.51	26.64	**26.58**	-0.59
9.55E+02	28.32	28.5	**28.41**	30.22	29.47	**29.85**	-1.44
9.55E+01	28.54	30.32	**29.43**	33.95	32.51	**33.23**	-3.80

EDQM *M. hyorhinis*

cfu/ml	SSB			Minerva			delta Ct
	Set 1	Set 2	**Av Ct**	Set 1	Set 2	**Av Ct**	
1.17E+07	17.58	18.48	**18.03**	17.61	17.86	**17.74**	0.30
1.17E+06	20.19	20.88	**20.54**	21.29	20.58	**20.94**	-0.40
1.17E+05	24.02	24.98	**24.50**	24.42	23.9	**24.16**	0.34
1.17E+04	27.51	28.57	**28.04**	27.71	27.36	**27.54**	0.50
1.17E+03	29.74	30.99	**30.37**	31.59	30.51	**31.05**	-0.69
1.17E+02	31.84	30.1	**30.97**	34.35	34.97	**34.66**	-3.69
1.17E+01	32.78	31.88	**32.33**	No Ct	No Ct	N/D	N/D
1.17E+00	31.27	31.11	**31.19**	No Ct	No Ct	N/D	N/D
1.17E-01	32.51	31.16	**31.84**	No Ct	No Ct	N/D	N/D

EDQM *M. orale*

cfu/ml	SSB			Minerva					delta Ct
	Set 1	Set 2	**Av Ct**	Set 1	Set 2	Set 3	Set 4	**Av Ct**	
4.90E+04	No Ct.	22.57	**22.57**	24.39	n.d.	23.54	23.49	**23.81**	-1.24
4.90E+03	26.02	26.03	**26.03**	27.59	n.d.	26.81	26.66	**27.02**	-1.00
4.90E+02	29.27	29.19	**29.23**	30.48	n.d.	29.85	29.54	**29.96**	-0.73
4.90E+01	32.68	33.38	**33.03**	34.27	33.3	34.04	33.3	**33.73**	-0.70
2.45E+01	34.47	34.32	**34.40**	34.58	34.77	N/D	N/D	**34.68**	-0.27

EDQM *M. synoviae*

cfu/ml	SSB					Minerva				delta Ct
	Set 1	Set 2	Set 3	Set 4	**Av Ct**	Set 1	Set 2	Set 3	**Av Ct**	
1.86E+06	18.32	18.79	18.47	17.50	**18.27**	17	17.55	20.7	**18.42**	-0.15
1.86E+05	21.32	21.89	20.96	20.39	**21.14**	20.16	20.23	N/D	**20.20**	0.95
1.86E+04	25.41	25.55	24.14	23.87	**24.74**	22.97	23.01	N/D	**22.99**	1.75
1.86E+03	27.79	28.50	27.78	27.78	**27.96**	26.78	26.51	28.12	**27.14**	0.83
1.86E+02	30.29	31.56	31.27	30.56	**30.92**	29.93	28.36	31.76	**30.02**	0.90
1.86E+01	29.85	32.50	33.11	32.53	**31.99**	36.74	30.26	36.48	**33.50**	-1.51

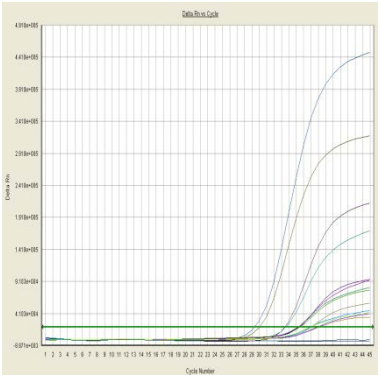
6.4.5 Device compatibility

Procedure	Acceptance Criterion	Results
As the test can basically be performed with any qPCR cycler capable of interpreting FAM and ROX signals performance of the test on these machines needs to be validated. As not all qPCR cycler commercially available are accessible for validation the following three devices representing block and air heating systems are tested: Rotor-Gene 6000, Mx3005p and ABI Prism 7500. The robustness of the method will be demonstrated by the determination of 10 CFU/ml of *Mycoplasma pneumoniae* in Tris buffer. At least 10 replicates shall be tested on each machine.	All samples show a positive result.	Mx3005p passed Rotorgene 6000 failed ABI Prism 7500 passed

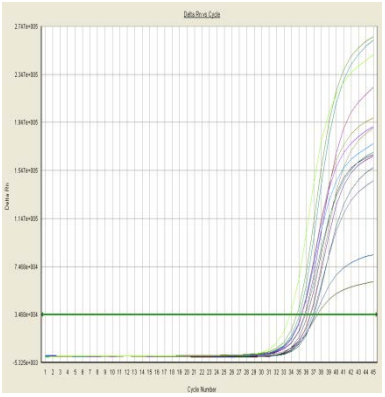
ABI Prism 7500

No	Species	Concentration	Ct (FAM)	Ct (ROX)	Result
1	*Mycoplasma pneumoniae*	10 CFU / ml	35.438	36.589	positive
2	*Mycoplasma pneumoniae*	10 CFU / ml	35.279	35.902	positive
3	*Mycoplasma pneumoniae*	10 CFU / ml	35.504	34.745	positive
4	*Mycoplasma pneumoniae*	10 CFU / ml	37.071	36.513	positive
5	*Mycoplasma pneumoniae*	10 CFU / ml	33.615	35.094	positive
6	*Mycoplasma pneumoniae*	10 CFU / ml	35.291	35.780	positive
7	*Mycoplasma pneumoniae*	10 CFU / ml	38.073	35.309	positive
8	*Mycoplasma pneumoniae*	10 CFU / ml	37.012	35.405	positive
9	*Mycoplasma pneumoniae*	10 CFU / ml	37.932	35.393	positive
10	*Mycoplasma pneumoniae*	10 CFU / ml	36.887	33.887	positive
11	NTC	---	No Ct	37.079	negative
12	PC	---	29.377	37.282	positive

FAM signal:



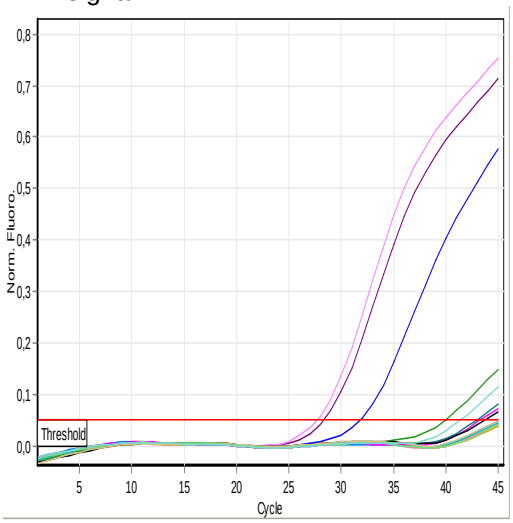
ROX signal:



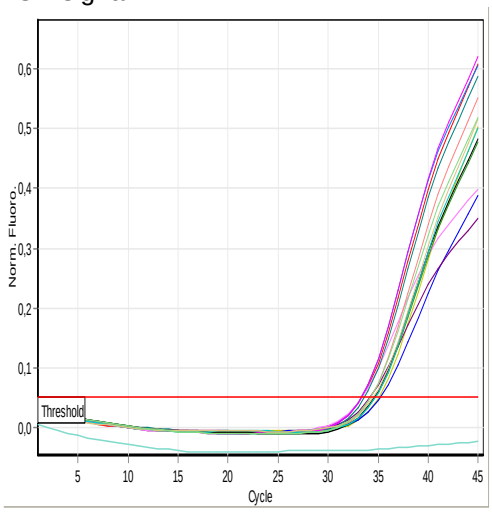
Rotorgene 6000

No	Species	Concentration	Ct (FAM)	Ct (ROX)	Result
1	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	33.35	negative
2	*Mycoplasma pneumoniae*	10 CFU / ml	42.90	33.63	positive
3	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	34.23	negative
4	*Mycoplasma pneumoniae*	10 CFU / ml	39.92	34.71	positive
5	*Mycoplasma pneumoniae*	10 CFU / ml	43.27	33.35	positive
6	*Mycoplasma pneumoniae*	10 CFU / ml	43.61	34.61	positive
7	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	34.70	negative
8	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	34.52	negative
9	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	34.23	negative
10	*Mycoplasma pneumoniae*	10 CFU / ml	41.39	No Ct	positive
11	NTC	---	No Ct	35.01	negative
12	PC	---	27.71	33.35	positive

FAM signal:



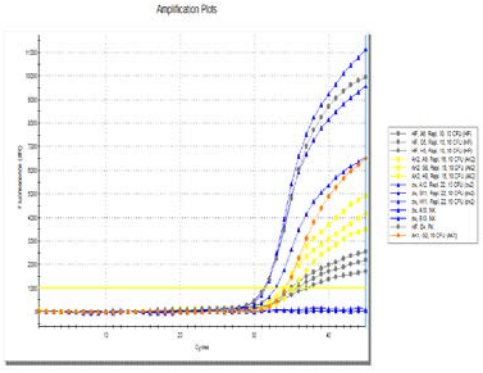
ROX signal:



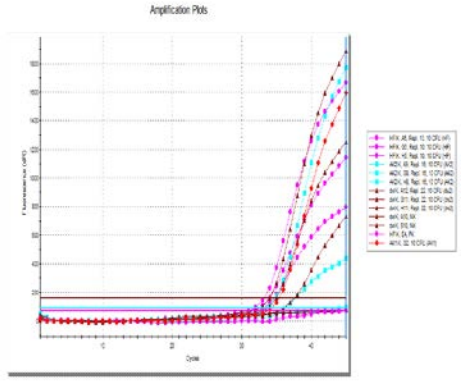
Mx3005p

No	Species	Concentration	Ct (FAM)	Ct (ROX)	Result
1	*Mycoplasma pneumoniae*	10 CFU / ml	32.84	35.00	positive
2	*Mycoplasma pneumoniae*	10 CFU / ml	31.58	34.00	positive
3	*Mycoplasma pneumoniae*	10 CFU / ml	31.32	No Ct	positive
4	*Mycoplasma pneumoniae*	10 CFU / ml	33.98	33.58	positive
5	*Mycoplasma pneumoniae*	10 CFU / ml	34.03	36.21	positive
6	*Mycoplasma pneumoniae*	10 CFU / ml	35.26	No Ct	positive
7	*Mycoplasma pneumoniae*	10 CFU / ml	35.74	32.34	positive
8	*Mycoplasma pneumoniae*	10 CFU / ml	34.95	31.57	positive
9	*Mycoplasma pneumoniae*	10 CFU / ml	36.94	31.20	positive
10	*Mycoplasma pneumoniae*	10 CFU / ml	32.28	36.40	positive
11	NTC	---	No Ct	35.94	negative
12	PC	---	31.38	44.97	positive

FAM signal:



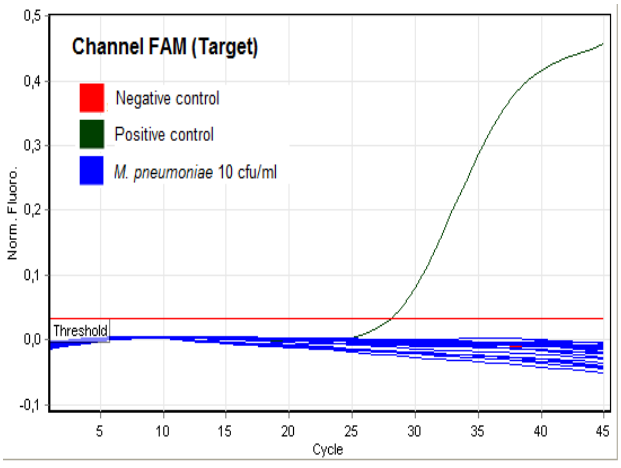
ROX signal:

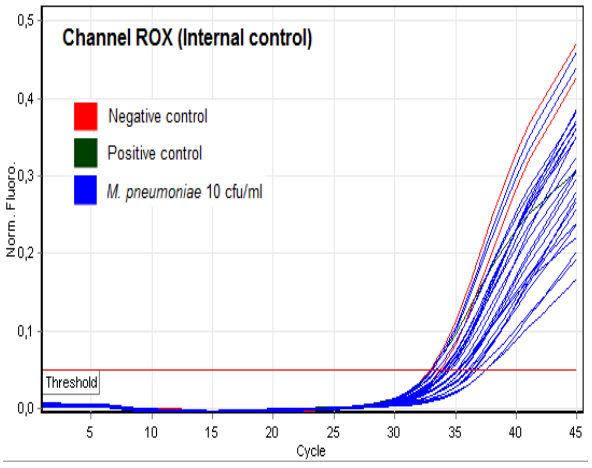


6.4.6 Updated RotorGene 6000 Protocol

Procedure	Acceptance Criterion	Results
As the test can basically be performed with any qPCR cycler capable of interpreting FAM and ROX signals performance of the test on these machines needs to be validated. As Microsart® AMP Mycoplasma failed on the RotorGene 6000 the tests will be repeated with the revised protocol with 10 CFU/ml of *Mycoplasma pneumoniae* in Tris buffer. At least 24 replicates shall be tested.	23 of 24 samples should be found positive.	failed

No	Species	Concentration	Ct FAM	Ct ROX
1	Negative control	---	No Ct	32.94
2	Negative control	---	No Ct	33.79
3	Positive control	---	28,13	33.08
4	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	33.46
5	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	35.29
6	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	33.93
7	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	34.60
8	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	37.52
9	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	35.63
10	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	36.46
11	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	35.17
12	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	35.58
13	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	34.10
14	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	36.54
15	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	36.04
16	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	36.84
17	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	35.10
18	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	34.42
19	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	33.50
20	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	34.47
21	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	33.16
22	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	35.15
23	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	37.60
24	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	36.16
25	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	33.04
26	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	34.30
27	*Mycoplasma pneumoniae*	10 CFU / ml	No Ct	34.06

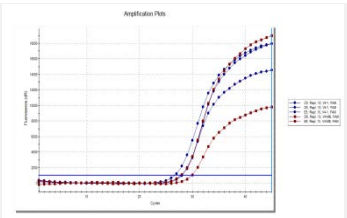
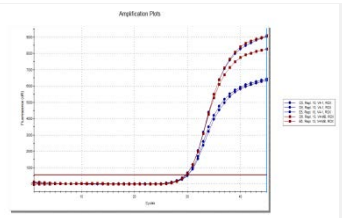




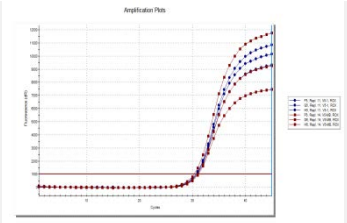
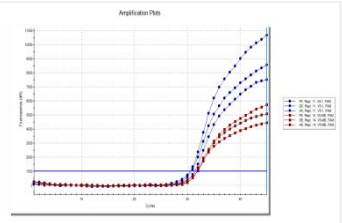
6.4.7 Concentration of 1 ml samples with Vivaspin 6

Procedure	Acceptance Criterion	Results
The performance of the concentration method using Vivaspin 6 units will be demonstrated by the determination of 10 CFU/ml *Mycoplasma pneumoniae* prepared according to chapter 5.2 in 1 ml of DMEM. At least 3 repeats shall be tested for each concentration step.	All samples should show a detection level better as with the direct test method.	passed

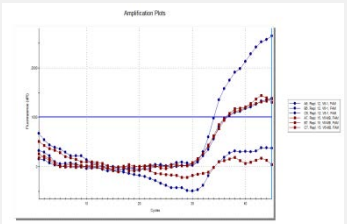
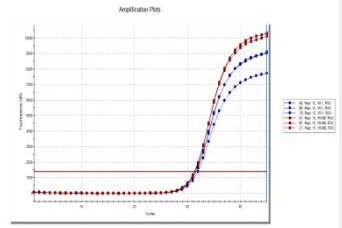
9.9 x 10^2 CFU/ml

Setup	Volume	Ct FAM	Ct ROX	FAM	ROX
Vivaspin 6	1 ml	27.92	29.97		
Vivaspin 6	1 ml	28.07	29.62		
Vivaspin 6	1 ml	26.71	30.08		

99 CFU/ml

Setup	Volume	Ct FAM	Ct ROX	FAM	ROX
Vivaspin 6	1 ml	30.55	29.59		
Vivaspin 6	1 ml	30.80	29.79		
Vivaspin 6	1 ml	31.41	29.75		

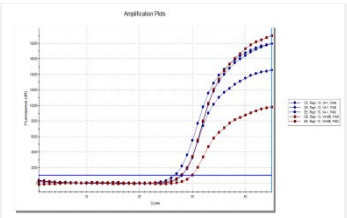
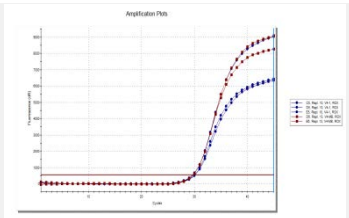
9.9 CFU/ml

Setup	Volume	Ct FAM	Ct ROX	FAM	ROX
Vivaspin 6	1 ml	34.06	29.97		
Vivaspin 6	1 ml	No Ct	30.26		
Vivaspin 6	1 ml	36.55	29.83		

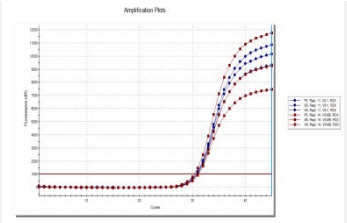
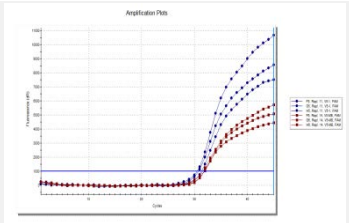
6.4.8 Concentration of 4 ml samples with Vivaspin 6

Procedure	Acceptance Criterion	Results
The performance of the concentration method using Vivaspin 6 units will be demonstrated by the determination of 3 different concentrations of *Mycoplasma pneumoniae* prepared according to chapter 5.2 in 4 ml of DMEM. At least 3 repeats shall be tested for each concentration step.	All samples should show a detection level better as with the direct test method.	passed

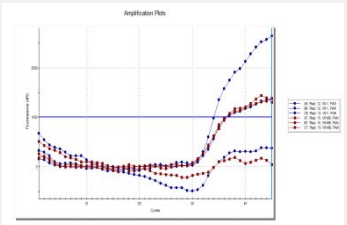
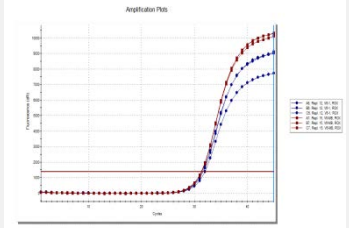
9.9 x 10^2 CFU/ml

Setup	Volume	Ct FAM	Ct ROX	FAM	ROX
Vivaspin 6	4 ml	27.92	29.97		
Vivaspin 6	4 ml	28.07	29.62		
Vivaspin 6	4 ml	26.71	30.08		

99 CFU/ml

Setup	Volume	Ct FAM	Ct ROX	FAM	ROX
Vivaspin 6	4 ml	30.55	29.59		
Vivaspin 6	4 ml	30.80	29.79		
Vivaspin 6	4 ml	31.41	29.75		

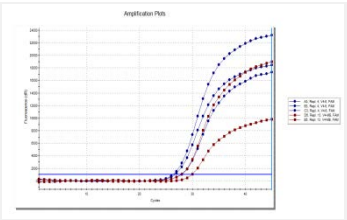
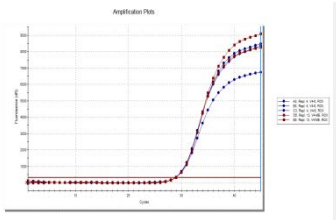
9.9 CFU/ml

Setup	Volume	Ct FAM	Ct ROX	FAM	ROX
Vivaspin 6	4 ml	34.06	29.97		
Vivaspin 6	4 ml	No Ct	30.26		
Vivaspin 6	4 ml	36.55	29.83		

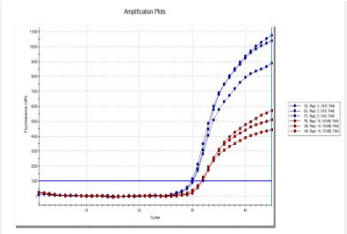
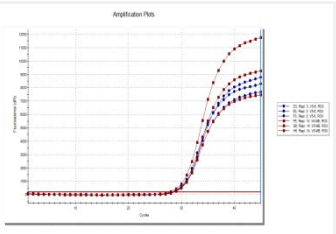
6.4.9 Concentration of 6 ml samples with Vivaspin 20

Procedure	Acceptance Criterion	Results
The performance of the concentration method using Vivaspin 20 units will be demonstrated by the determination of 3 different concentrations of *Mycoplasma pneumoniae* prepared according to chapter 5.2 in 6 ml of DMEM. At least 3 repeats shall be tested for each concentration step.	All samples should show a detection level better as with the direct test method.	passed

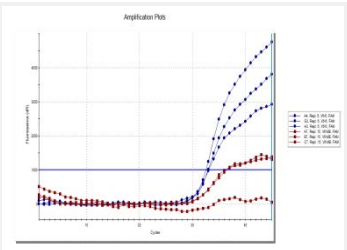
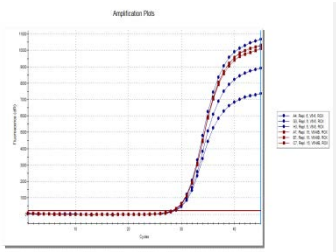
9.9 x 10^2 CFU/ml

Setup	Volume	Ct FAM	Ct ROX	FAM	ROX
Vivaspin20	6 ml	27.93	29.64		
Vivaspin20	6 ml	26.71	29.73		
Vivaspin20	6 ml	26.42	29.61		

99 CFU/ml

Setup	Volume	Ct - FAM	Ct ROX	FAM	ROX
Vivaspin20	6 ml	29.80	29.94		
Vivaspin20	6 ml	30.17	29.73		
Vivaspin20	6 ml	30.08	29.83		

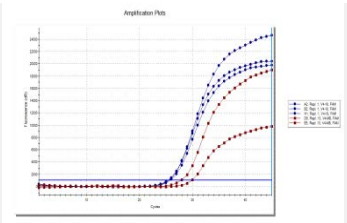
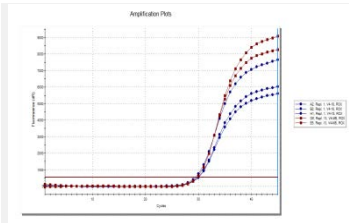
9.9 CFU/ml

Setup	Volume	Ct FAM	Ct ROX	FAM	ROX
Vivaspin20	6 ml	33.11	30.17		
Vivaspin20	6 ml	32.99	30.02		
Vivaspin20	6 ml	32.62	29.66		

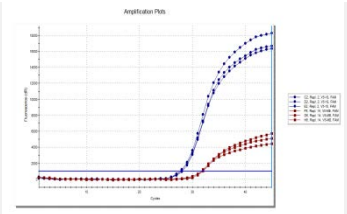
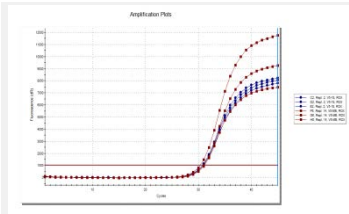
6.4.10 Concentration of 18 ml samples with Vivaspin 20

Procedure	Acceptance Criterion	Results
The performance of the concentration method using Vivaspin 20 units will be demonstrated by the determination of 3 different concentrations of *Mycoplasma pneumoniae* prepared according to chapter 5.2 in 18 ml of DMEM. At least 3 repeats shall be tested for each concentration step.	All samples should show a detection level better as with the direct test method.	passed

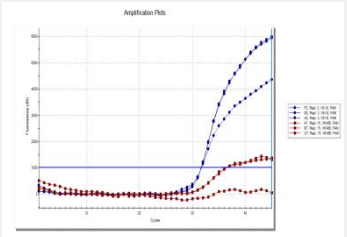
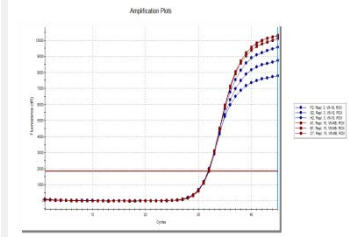
9.9 x 10^2 CFU/ml

Setup	Volume	Ct FAM	Ct ROX	FAM	ROX
Vivaspin20	18 ml	25.46	29.98		
Vivaspin20	18 ml	25.83	29.90		
Vivaspin20	18 ml	25.56	29.38		

99 CFU/ml

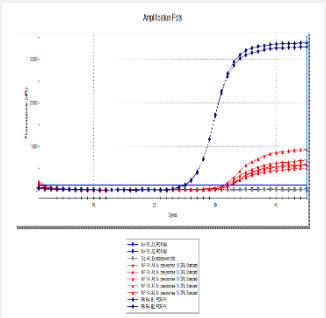
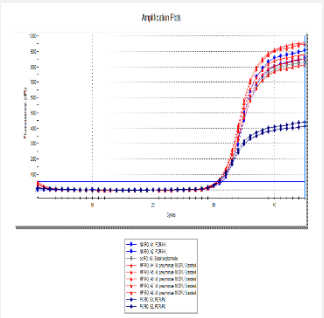
Setup	Volume	Ct FAM	Ct ROX	FAM	ROX
Vivaspin20	18 ml	27.88	29.84		
Vivaspin20	18 ml	28.11	29.88		
Vivaspin20	18 ml	27.70	29.67		

9.9 CFU/ml

Setup	Volume	Ct FAM	Ct ROX	FAM	ROX
Vivaspin20	18 ml	31.70	29.69		
Vivaspin20	18 ml	31.75	29.57		
Vivaspin20	18 ml	31.74	29.75		

6.4.11 Interlab Precision

Procedure	Acceptance Criterion	Results
At SSB DMEM cell culture medium supplemented with 5 % FCS (v/v) will be spiked with *Mycoplasma pneumoniae* using the 10CFU™ Sensitivity Standards and tested in duplicate.	All samples containing at least 10 CFU/ml of *M. pneumoniae* must be tested positive.	passed

Setup	Ct FAM	Ct ROX	FAM	ROX
Replicate 1	31.64	30.69		
Replicate 2	31.37	30.99		
Replicate 3	32.29	30.93		
Replicate 4	32.32	30.55		
Replicate 5	32.43	30.46		
PCR Negative Control	No Ct	31.27		
PCR Negative Control	No Ct	30.95		
PCR Positive Control	24.97	30.66		
PCR Positive Control	24.93	30.85		
Extraction Control	No Ct	30.98		

7 Conclusion

Microsart® AMP Mycoplasma was validated intensively in compliance with the designed validation protocol. The validation protocol reflects the method itself and variations expected by the diversity of samples from different customers during QC testing in the manufacturing process of biologicals.

Microsart® AMP Mycoplasma was found as state-of-the-art product for mycoplasma detection. This product should be applied for mycoplasma testing according to *European Pharmacopoeia* 2.6.7 for any kind of sample material occurring in the manufacturing process of biologicals, like cell culture media und supplements, cryo stocks, cell culture supernatants, cell suspensions, antibody formulations, for bulk harvest testing or final lot release. Even more, the new sample concentration protocol allows for an enhancement of mycoplasma by a factor of approx. 20 fold, increasing the sensitivity of the test system even more (appendix 1).

Additional *Mollicutes* species have been tested positive as well. As the sequences of the primer and probes remain unchanged the kit still detects at least 141 different *Acholeplasma*, *Mycoplasma*, and *Ureaplasma* species based on the sequence alignment. This feature increases the chance to detect mycoplasmas which contaminate cell culture rarely, or have not been described as contaminants so far or are not cultivable by using the traditional mycoplasma testing method.

Specificity testing showed that the kit detects *Staphylococcus epidermidis* with a detection limit of approximately 7.3 x 10^5 cells per ml of sample which corresponds to 0.1 ng DNA/PCR. The detection limit for *Bacillus subtilis,* a species genetically very closely related to *Mycoplasma* but not addressed by the EP 2.6.7 to be tested for specificity, is approximately 8.7 x 10^5 cells per ml (0.2 ng DNA/50 µl of sample volume per PCR). The detection limits for both species correspond to cell densities with significant impact on biopharmaceutical processes leading instantly to the correct interpretation of the test results. DNA of eukaryotic origin is not detected by Microsart® AMP Mycoplasma even at concentrations higher than 30 ng/PCR.

As robustness is a key issue in evaluating the characteristics of a release test, the product has been validated with different pharmaceutical relevant sample materials and qPCR cyclers. The species *Mycoplasma pneumonia*e, the most prominent mycoplasma species in means of human health risk, was used for spiking. It showed the same sensitivity as *Mycoplasma synoviae, Spiroplasma citri, Mycoplasma arginini, Mycoplasma gallisepticum,* and *Mycoplasma orale. Mycoplasma pneumoniae* was easily detectable at the required concentration of 10 CFU/ml even in complex sample matrices and multiple replicates.

The application of the kit in two different laboratories using the same sample material showed comparable results even with different qPCR cyclers down to the fifth 1:10 dilution (lab-to-lab precision, chapter 6.4.4). With continuous dilution of the sample material discrepancies became obvious most likely caused by different pipetting precisions rather than kit characteristics.

The validation of the improved protocol for the air-heated qPCR cycler RotorGene 6000 showed sensitivity close to but still failing the required detection limit of 10 CFU/ml as LOD_{95}. Currently, no validated protocol can be provided for this qPCR cycler.

Additionally, a protocol is provided to use the Internal Control to monitor the entire process including DNA extraction by spiking the sample directly. The Internal Control DNA was found in the PCR reaction with highest accuracy monitoring the DNA extraction process. Additionally these results show the robustness of the Microsart® AMP Extraction kit for the preparation of different kind of sample material.

It was shown that the product Microsart® AMP Mycoplasma fully complies with the requirements of EP 2.6.7 providing additional protocols which fulfill even higher limits in respect of process control and sensitivity for the detection of mycoplasma in any kind of sample material occurring in the manufacturing process of biologicals, like cell culture media und supplements, cryo stocks, cell culture supernatants, cell suspensions, antibody formulations, for bulk harvest testing or final lot release.

8 Reference Documents

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13. TIGR CMR

9 Appendix

Volume Dependent Enrichment Factors with the Vivaspin Protocol

M. pneumoniae dilution 9.9 x 10^2 CFU/ml

Concentration unit / sample volume	Average Ct Value / (detected/analyzed)	delta Ct	Enrichment factor
Vivaspin 20 / 18 ml	25.62	3.28	9.67
Vivaspin 20 / 6 ml	27.02	1.88	3.62
Vivaspin 6 / 4 ml	26.16	2.74	6.56
Vivaspin 6 / 1 ml	27.57	1.33	2.38
direct / 200 µl	28.90	0.00	1.00

M. pneumoniae - dilution 9.9 x 10^1 CFU/ml

Concentration unit / sample volume	Average Ct Value / (detected/analyzed)	delta Ct	Enrichment factor
Vivaspin 20 / 18 ml	27.90	3.93	21.10
Vivaspin 20 / 6 ml	30.02	1.81	4.08
Vivaspin 6 / 4 ml	29.07	2.76	8.56
Vivaspin 6 / 1 ml	30.92	0.90	2.08
direct / 200 µl	31.82	0.00	1.00

M. pneumoniae - dilution 9.9 CFU/ml

Concentration unit / sample volume	Average Ct Value / (detected/analyzed)	delta Ct	Enrichment factor
Vivaspin 20 / 18 ml	31.73	4.67	37.10
Vivaspin 20 / 6 ml	32.91	3.49	15.11
Vivaspin 6 / 4 ml	31.71	4.69	37.82
Vivaspin 6 / 1 ml	35.31	1.09	3.49
direct / 200 µl	36.40	0.00	1.00

Summary of the Enrichment Factors Achievable with the Vivaspin Protocol

Sample Volume	Enrichment Factor		Efficiency
	theoretical	practical	
18 ml	90	22.62	25.13%
6 ml	30	7.60	25.34%
4 ml	20	11.69	58.46%
1 ml	5	2.65	53.02%
200 µl	0	0	N/A