Validation Report October 2023

Evaluation of the mycoplasma detection capability of the QIAcuity® Mycoplasma Quant Kit

Introduction

Mycoplasmas are 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 published guidance on mycoplasmas safety for the manufacture of biological products as, for instance, the *European Pharmacopoeia*, chapter 2.6.7., "Mycoplasmas", the *US Pharmacopeia* chapter <63>, or the *Japanese Pharmacopoeia, 17th edition*, chapter G3.

The QIAcuity® Mycoplasma Quant Kit detects *Mollicutes* (*Mycoplasma*, *Acholeplasma*, and *Spiroplasma*) contamination in cell cultures and other cell culture derived biologicals. The kit utilizes the digital polymerase chain reaction (dPCR) after a reverse transcription (RT), established as the method of choice for high sensitivity. The kit includes a Primer/Probe/Nucleotide mix containing a FAM™-labelled probe specific for a broad range of different mycoplasma species. The internal amplification control prevents false negative results due to PCR inhibitors, improper RNA extraction, or improper RT reaction. It is possible to add the QIAcuity Internal Control RNA directly to the sample prior to RNA extraction and

analysis for verification of the complete process (RNA extraction, RT, and PCR reaction). If added to the PCR master mix directly, the Internal Control RNA acts as an RT and PCR control only. The amplification of the internal control is detectable on the QIAcuity at 560 nm (yellow channel). The mycoplasma-specific amplification is detectable at 520 nm (green channel).

Objective

We designed and completed a study to evaluate the Mycoplasma detection capability for the QIAcuity Mycoplasma Quant Kit for dPCR. Section 2.6.7 "Mycoplasmas" of the *European Pharmacopoeia* describes a protocol for validation of a kit as burden of the manufacturer. This chapter includes guidelines and specifications for relevant parameters like specificity, detection limit, and robustness in comparison to the traditional culture method.

The product modifications required a full validation of all relevant performance aspects 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. The employed method obtains qualitative result only (positive/negative). Compliance with a selection of criteria, based on the requirements of the *European Pharmacopoeia* 2.6.21 and the requirements of ICH Q2B for a qualitative/limit impurity analysis, described in ICH Q2B was demonstrated. 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.

The validation of the QIAcuity Mycoplasma Quant Kit includes the determination of sensitivity for *Mycoplasma arginine*, *Mycoplasma orale*, *Mycoplasma gallisepticum*, *Mycoplasma pneumoniae*, *Mycoplasma synoviae*, *Mycoplasma fermentans*, *Mycoplasma hyorhinis*, *Mycoplasma salivarium*, *Acholeplasma laidlawii*, and *Spiroplasma citri* in order to fullfill the requirements of the *European Pharmacopeia* (EP), chapter 2.6.7, "Mycoplasmas", the *US Pharmacopeia* (USP), chapter <63>, "Mycoplasma Tests", and the *Japanese Pharmacopoeia* (JP), chapter G3, paragraph "Mycoplasma testing for cell substrates used for the Production of Biotechnological/Biological products" [6].

Definition and Abbreviations

Abbreviation	Meaning
ATCC	American Type Culture Collection
CFU	colony-forming unit
CFU/mL	colony-forming units per milliliter
CoA	Certificate of Analysis
DMEM	Dulbecco's modified Eagles medium
DNA	deoxyribonucleic acid
ENC	Extraction negative control
EP	European Pharmacopoeia
FBS	Fetal Bovine Serum
g	*g*-force (unit for measurement of rotation speed of centrifugation)
GC	genome copy
IC	internal amplification control
ICH	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
JP	Japanese Pharmacopoeia
LOB	Limit of blank
LOD95	Limit of detection, concentration, where 95% of all samples were positive
mg/mL	milligram per milliliter
MV	mean value
N/A, n.a.	not applicable
NEC	no extraction control
N/M	not measured
nm	nanometer
N/P	not provided
NTC	no template control
OD260	optical density (at a wavelength of 260 nm)
PBS	phosphate buffered saline
PC	positive control

PCR	polymerase chain reaction
pH	potentia hydrogenii
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	reverse transcription
s	second
TE80	10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA
Tris	tris (hydroxyl methyl amino methane)

Responsibilities

The validation of the QIAcuity Mycoplasma Quant Kit was performed by a cooperation partner (Minerva Biolabs GmbH) that developed the test protocol.

Test Materials

This chapter lists the test systems, product solutions, and material used for the study.

Test system

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

Table 1. Test system information

System type	Supplied by	Cat. No.	Storage Conditions
QIAcuity Mycoplasma Quant Kit	QIAGEN GmbH	250261	–30°C to –15°C
Venor®GeM Sample Preparation Kit	Minerva Biolabs GmbH	56-3010 / 56-3100 / 56-3250	Ambient temperature (18–25°C) and 4–8°C

Sample matrix

The specificity testing used defined cell culture medium components as described in Table 2. The robustness testing used DMEM medium containing 10% (v/v) FBS as sample matrix for all spiking experiments.

Table 2. Matrix formulation

Product ingredient	Manufacturer	Cat. no.	Lot. no.
FBS Xtra	Capricorn Scientific GmbH	FBS-16A	CP22-5406
DMEM	Capricorn Scientific GmbH	DMEM-LPSTA	CP236027
RPMI 1640	Capricorn Scientific GmbH	RPMI-STA	CP22-5272
HEK293	In-house culture (Minerva Biolabs GmbH)	–	HEK293_CRL-1573_20K1

Microorganisms and eukaryotic material

Tables 3, 4, 5, 6, 7, and 8 describe the microorganisms and eukaryotic material used for spiking or specificity testing during the study as well as the required reagents used for cultivation.

Table 3. Description of Mollicutes species – quantified by traditional culture method

Species	Natural host	NCTC code	DSM code	ATCC code	GC:CFU ratio	New nomenclature
Acholeplasma laidlawii	Ubiquitous	10116	23060	23206	3.4	*Acholeplasma laidlawii*
Spiroplasma citri	–	10164	21846	27556	N/A	*Spiroplasma citri*
Mycoplasma salivarium	–	10113	–	23064	2.8	*Metamycoplasma salivarium*
Mycoplasma fermentans	Human	10117	19202	19989	4.9	*Mycoplasmopsis fermentans*
Mycoplasma hyorhinis	Mammal	10130	25591	17981	1.4	*Mesomycoplasma hyorhinis*
Mycoplasma orale	Human	10112	25590	23714	2.4	*Metamycoplasma orale*
Mycoplasma pneumoniae	Human	10119	22911	15531	N/A	*Mycoplasmoides pneumoniae*
Mycoplasma gallisepticum	Bird	10115	19817	19610	1.4	*Mycoplasmoides gallisepticum*
Mycoplasma synoviae	Mammal	10124	21430	25204	2.6	*Mycoplasmopsis synoviae*
Mycoplasma arginini	Mammal	10129	–	23838	2.1	*Mycoplasmopsis arginine*

Table 4. Description of international reference material

Species	Supplier	NCTC code	Cat. no.
WHO International Standard for Mycoplasma DNA	Paul-Ehrlich-Institut	10117	8293/13

Table 5. Description of non-Mollicutes bacterial strains – quantified photometrical based on OD260 excitation

Product ingredient	DSM code	ATCC code	NCTC code
Bacillus subtilis	347	6633	10400
Clostridium sporogenes	1664	19404	532
Kocuria rhizophila	348	9341	–
Pseudomonas aeruginosa	1128	9027	–
Staphylococcus aureus	6538	6538	10788
Staphylococcus pyogenes	19615	19615	–

Table 6. Description of eukaryotic cells – quantified by counting

Species	Family	Origin	Supplier	No.
CHO-K1	Ovary	Hamster	ATCC	CCL-61
HEK293	Kidney	Human	ATCC	CRL-1573
Vero-B4	Kidney	African green monkey	ATCC	CCL-81
HL-60	Leukemia	Human	–	–

Mycoplasma tests, according to the *European Pharmacopoeia* (EP) Chapter 2.6.7, require a sensitivity of 10 colony-forming units (CFU) per mL sample volume for NAT-based methods such as PCR in order to replace traditional culture method. To replace traditional indicator cell culture method by a NAT-based method, a sensitivity of 100 CFU/mL sample volume is required. The sensitivity must accomplish 10 CFU/mL or 100 CFU/mL, depending on the traditional method to replace, as part of the robustness testing for any particular sample matrix. Using vital mycoplasma is not acceptable for the majority of cell culture laboratories due to safety regulations. 10 CFU™ sensitivity standards contain non-vital material and allow safe and reliable validation.

The mycoplasma grew in culture medium as described in EP 2.6.7, subsequently titrated and plated on Hayflick and Frey medium for CFU determination. The mycoplasma were harvested in the early logarithmic growth phase to ensure a high ratio of vital to non-vital mycoplasma and thus a low ratio of GC to CFU. All strains were obtained from official culture collections and cultivated in low passages.

In more details, all mycoplasma listed in Table 3 had been cultivated in broth according to EP 2.6.7 until a slight color change of the phenol red indicator contained in either Frey or Hayflick medium became visible. The culture broth was divided into 2 portions: One portion was used for quantification of the mycoplasma. The broth was vortexed intensively before titration to break up mycoplasma clusters. Two tenfold dilution series were prepared in culture broth. Of each dilution step, 2 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 titer calculated as CFU/mL culture broth.

The second portion of the culture broth was aliquoted in 500 µL per tube. Aliquots were stored at –80°C until use.

Table 7. Mycoplasma cultivation media

Species	Manufacturer	Cat. no.
Frey Bouillon acc. EP	MerckMillipore	146311
Frey-Agar acc. EP	MerckMillipore	146006
Hayflick Bouillon acc. EP	MerckMillipore	146452
Hayflick-Agar acc. EP	MerckMillipore	146029

Table 8. Reagents for cell culture

Species	Manufacturer	Cat. no.	Lot no.
Trypsin-EDTA Solution	Merck KGaA (Sigma Aldrich)	T4174	SLCH8967
Ham's F-12K (Kaighn's) Medium	Thermo Fisher Scientific Inc. (Gibco)	21127022	2579500
FBS Superior	Merck KGaA (Sigma Aldrich)	50615	0001659021
FBS Xtra	Capricorn Scientific GmbH	FBS-16A	CP22-5406
Dulbecco's PBS (1x)	Capricorn Scientific GmbH	PBS-1A	CP23-6116

For the titration of the mycoplasma spike, the quality of the culture medium is of severe relevance for the subsequent spiking experiments. Each lot of the mycoplasma culture material was quality controlled with EDQM standards. Certificates of Analysis (CoA) were available for each lot.

Equipment

The following lab equipment (Table 9) and consumables (Table 10) were used for the study:

Table 9. Lab equipment

Equipment, brand	Manufacturer	Equipment ID (internal/serial no.)
Vortex	Biozym Scientific GmbH	VB181AF0000415 E124
Vortex	Carl Roth GmbH & Co. KG	VA181AB000046 E134
Centrifuge	Sarstedt AG & Co. KG	16111010 E141
Tacta® Mechanical Pipette, 8-Channel, 5–100 µL	Sartorius Lab Instruments GmbH & Co. KG	42084372 E152
Eppendorf Reference 2, 1-Channel, 100–1000 µL	Eppendorf SE	O10885D E05
Eppendorf Reference 2, 1-Channel, 10–100 µL	Eppendorf SE	L10238D E04
Picus® Electronic Pipette, 1-Channel, 10–300 µL	Sartorius Lab Instruments GmbH & Co. KG	15011121 E60
Picus Electronic Pipette, 1-Channel, 10–300 µL	Sartorius Lab Instruments GmbH & Co. KG	15011122 E57
Picus Electronic Pipette, 1-Channel, 10–300 µL	Sartorius Lab Instruments GmbH & Co. KG	42490283 E160
Eppendorf Reference 2, 1-Channel, 2–20 µL	Eppendorf SE	G26555D E15
Tacta Mechanical Pipette, Single Channel, 100–1000 µL	Sartorius Lab Instruments GmbH & Co. KG	19011091 E133
Tacta Mechanical Pipette, Single Channel, 10–100 µL	Sartorius Lab Instruments GmbH & Co. KG	19004053 E132
Tacta Mechanical Pipette, Single Channel, 100 –1000 µL	Sartorius Lab Instruments GmbH & Co. KG	18026710 E103
Tacta Mechanical Pipette, Single Channel, 10–100 µL	Sartorius Lab Instruments GmbH & Co. KG	18020324 E102

Eppendorf Reference 2, 1-Channel, 100–1000 µL	Eppendorf Vertrieb Deutschland GmbH	Q24166G E87
Sicherheitwerkbank Safe Comfort Plus	TH.Geyer GmbH & Co.KG	SCS Evo 2-4-776 E101
Sicherheitwerkbank Safe Comfort Plus	TH.Geyer GmbH & Co.KG	SCS Evo 2-4-777 E100
QIAcuity One	QIAGEN GmbH	00159 (E143) 00424 (E154)
QIAcuity Four	QIAGEN GmbH	02032 (E174)
Nanoplate Tray	QIAGEN GmbH	n.a.
QIAcuity Plate Roller	QIAGEN GmbH	n.a.
Vortex	Phoenix Instrument	VB4B016641 E27
Picus Electronic Pipette, 1-Channel, 100–5000 µL	Sartorius Lab Instruments GmbH & Co. KG	15015144 E59
Picus Electronic Pipette, 1-Channel, 50–1000 µL	Sartorius Lab Instruments GmbH & Co. KG	15017005 E67
Eppendorf Reference 2, 1-Channel, 2–20 µL	Eppendorf SE	G26420D E16
KingFisher Flex	IST Innuscreen GmbH	711-85746 E156
Thermomixer	Eppendorf Vertrieb Deutschland GmbH	5382GL720102 E81
Eppendorf Reference 2, 1-Channel, 0.5–10 µL	Eppendorf Vertrieb Deutschland GmbH	K12812G E85
CO2-Inkubator	Memmert GmbH & Co. KG	O718.0250 E99
Centrifuge	Eppendorf Vertrieb Deutschland GmbH	5427FR524310 E80
Vortex	VWR International GmbH	170925031 E82
Pipettor	VWR International GmbH	82211313 E168

Inverses Mikroskop VT Serie	Euromex Microscopen BV	800103 E170
Nanoplate Tray	Biozym Scientific GmbH	VB181AF0000415 E124
QIAcuity Plate Roller	Carl Roth GmbH & Co. KG	VA181AB000046 E134
Vortex	Sarstedt AG & Co. KG	16111010 E141
Picus Electronic Pipette, 1-Channel, 100–5000 µL	Sartorius Lab Instruments GmbH & Co. KG	42084372 E152
Picus Electronic Pipette, 1-Channel, 50–1000 µL	Eppendorf SE	O10885D E05
Eppendorf Reference 2, 1-Channel, 2–20 µL	Eppendorf SE	L10238D E04
KingFisher Flex	Sartorius Lab Instruments GmbH & Co. KG	15011121 E60
Thermomixer	Sartorius Lab Instruments GmbH & Co. KG	15011122 E57
Eppendorf Reference 2, 1-Channel, 0.5–10 µL	Sartorius Lab Instruments GmbH & Co. KG	42490283 E160
CO2-Inkubator	Eppendorf SE	G26555D E15
Centrifuge	Sartorius Lab Instruments GmbH & Co. KG	19011091 E133
Vortex	Sartorius Lab Instruments GmbH & Co. KG	19004053 E132
Pipettor	Sartorius Lab Instruments GmbH & Co. KG	18026710 E103
Inverses Mikroskop VT Serie	Sartorius Lab Instruments GmbH & Co. KG	18020324 E102

Table 10. Reagents, materials, and critical labware

Article name	Cat. no.	Lot no.	Manufacturer/supplier
1-Propanol	9169.2	261300031	QIAGEN GmbH
QIAcuity Nanoplate 26k 24-Well	250001	5724100188 5724100147 5724100059 5754100073 5754100059	QIAGEN GmbH
QIAcuity OneStep® Advanced Probe Kit (5 mL)	250132	175022670 175027119 175029708	QIAGEN GmbH
Filter tip, 100 µL, transparent, Biosphere® plus, Refill	70.3030.355	3050221 3051521	Sarstedt AG & Co.KG
Filter tip, XL, 1000 µL, transparent, Biosphere plus, Refill	70.3060.355	2054521 2054621	Sarstedt AG & Co.KG
Filter tip, 300 µL, transparent, Biosphere plus	70.3040.255	746321	Sarstedt AG & Co.KG
Filter tip, 300 µL, transparent, Biosphere plus, Refill	70.3040.355	2052321	Sarstedt AG & Co.KG
PCR SingleCap 8er-SoftStrips 0.2 mL	710970	22073 22423 23033	Biozym Scientific GmbH
InnuPREP KFFLX Plate set 960 rxn	845-KF-1296010	09052023 14062023	IST Innuscreen GmbH
Pipette tip, 5 mL	70.1183.102	16411	Sarstedt AG & Co.KG
DNA LoBind Tubes, 1.5 mL	022431021	L208302R	Eppendorf
DNA LoBind Tubes, 5 mL	0030108310	1183960K	Eppendorf
DNA LoBind Tubes, 50 mL	0030122232	I183238L	Eppendorf
Serological pipette, 10 mL	86.1254.001	2308E	Sarstedt AG & Co.KG
Serological pipette, 25 mL	86.1685.001	3150K	Sarstedt AG & Co.KG
Serological pipette, 5 mL	86.1253.001	3072E	Sarstedt AG & Co.KG
Cell culture flask, T-175	83.3912.002	3021221	Sarstedt AG & Co.KG
Cell culture flask, T-75	83.3911.002	1024121	Sarstedt AG & Co.KG
Cell culture flask, T-25	83.3910.002	902411	Sarstedt AG & Co.KG

Test Procedure

Based on the results of different proficiency tests RNA extraction prior testing is strictly required for highest confidence and sensitivity. The design and performance of pre-analytical procedures are part of this study in respect of the intended use but cannot reflect the diversity of the sample material in total. The performance of the kit within the entire analytical process has to be demonstrated by the user. The templates for the RT-PCR analysis are prepared by direct extracting the sample and subsequent RT-PCR analysis.

RNA extraction

The extraction of mycoplasma RNA was carried out according to the up to date version of the instruction manual using the Venor®GeM Sample Preparation Kit on the KingFisher Flex. In detail:

The extraction program was downloaded from the webpage from Minerva Biolabs, uploaded onto, and was chosen at the KingFisher Flex. For a schematic view on the loading of volumes of extraction reagents required for automated extraction, see Table 11.

Preparing solutions

1. Visually check for precipitant formation of the Washing Buffers. Any precipitates were dissolved by heating at 37°C until all precipitates are dissolved.
2. Add appropriate volume of 1-propanol.
    - Binding Buffer, 33 mL
    - Wash Buffer 1, 14 mL
    - Wash Buffer 2, 14 mL

Rehydration of the reagents

1. Centrifuge tubes with lyophilized components (5 s at maximum speed).
2. Add 550 µL of Rehydration Buffer to the Proteinase K.
3. Add 120 µL RNase-Free Water to Mycoplasma Internal Control (final concentration 6000 copies/µL).
4. Incubate for 5 minutes at ambient temperature.
5. Vortex and centrifuge.

Preparing Lysis Buffer – IC Mix

1. Mix 250 µL Lysis Buffer and 2.5 µL QIAcuity Mycoplasma Internal Control per extraction gently.

Preparing Magnetic Bead Suspension

1. Intensively vortex Magnetic Bead Suspension directly before usage to ensure a homogenous distribution of the beads to the samples.

Table 11. Scheme of volumes of extraction reagents for automated extraction

Step / Plate	Lysis / Plate 1			Binding / Plate 2		Wash Buffers / Plate 3-5	Elution / Plate 6
Component	Proteinase K Solution	Sample	Lysis Buffer – IC Mix	Binding Buffer	Magnetic Bead Suspension	Wash Buffers 1-2	Elution Buffer
Volumes	20 µL	250 µL	252.5 µL	400 µL	20 µL	400 µL each	80 µL
Condition	–	–	10 min, 55°C	–	5 min, ambient temperature	1 min, ambient temperature	–

The deep well plates, except the lysis plate, were loaded according to the scheme in Table 11, and loaded onto the KingFisher Flex according to the program. 20 µL Proteinase K solution was placed into the wells of the lysis plate. First, 250 µL sample were added, followed by 252.5 µL Lysis Buffer – IC Mix. The plate was placed onto the KingFisher Flex according to

the program. The program was started by pressing **Start** on the device. The program stopped for the addition of 400 µL Binding Buffer and 20 µL Magnetic Bead Suspension to each sample. The binding was enabled by incubating for 5 min at ambient temperature. The lysis plate containing the Binding Buffer and Magnetic Bead Suspension was placed onto the KingFisher Flex and the program was continued by pressing **Start** on the device. After the program was finished, the elution plate was removed from the KingFisher Flex instrument and the eluates were directly used for the analytical procedure on the QIAcuity using the QIAcuity Mycoplasma Quant Kit.

Analytical procedures

The detection of mycoplasma RNA was carried out according to the up to date version of the instruction manual. In detail:

Rehydration of Control:

1. Centrifuge tubes with lyophilized components (5 s at maximum speed).
2. Add 400 µL RNase-Free Water to the Positive Control.
3. Incubate for 5 minutes at ambient temperature.
4. Vortex and centrifuge.

PCR Master Mix Setup:

1. Total volume per reaction is 40 µL including 20 µL of sample. When setting up reactions, calculations include positive (PC) and negative template controls (NTC).

Pipetting scheme:

Table 12. Pipetting scheme

	For 1 reaction (µL)	For 25 reactions (µL)
OneStep Adv. Probe Master Mix, 4x	10.0	250.0
OneStep Adv. RT Mix, 100x	0.4	10.0
OneStep Enhancer GC	5	125.0
QIAcuity Mycoplasma Assay, 20x	2	50.0
RNase-Free Water	2.6	53.0

20 µL/sample of master mix were dispensed into the wells of a standard PCR plate. 20 µL of samples and controls were added to the respective wells according to the loading scheme. Control volume was adjusted to 20 µL with RNase-Free water. It is recommended to pipette the negative template control (20 µL of RNase-Free water or elution buffer of RNA extraction kit) first and then seal the wells before proceeding with the samples and remaining controls. The wells were sealed completely before proceeding with the positive control (20 µL) in order to avoid cross-contamination. Pre-plate was gently mixed and liquids were settled at the bottom by centrifugation. Reaction mixes were transferred to wells of 26k 24-well nanoplate while avoiding bubbles.

Programming the dPCR instrument QIAcuity:

- Program Step 1: Partitioning
  - Standard partitioning
- Program Step 2: Amplification

Table 13. Cycling

Setting	Time	Temperature (°C)	Cycles
Reverse Transcription	40 min	50	1
PCR initial heat activation	2 min	95	1
Denaturation	15 s	95	40
Annealing / Elongation	1 min	59	

Table 14. Imaging

Fluorophone	Exposure/Gain
Green (Mycoplasma)	500 – 1000/6
Yellow (Internal Control)	500/6

Result interpretation:

The presence of mycoplasma in the sample is indicated by an increasing fluorescence signal in some partitions in the mycoplasma green channel during RT-PCR. The functionality of the RT-PCR reaction is indicated by an increasing fluorescence signal in some partitions in the internal control yellow channel during RT-PCR.

Detection of Mollicutes Green channel	Detection of Internal Control Yellow channel	Interpretation
Positive (positive partitions > 3)	Irrelevant	Mollicutes positive
Negative (no positive partition)	Negative (no positive partition)	PCR inhibition
Negative (no positive partition)	Positive (no positive partitions 600)	Mollicutes negative

A successfully performed RT-PCR without inhibition is indicated by a concentration of the internal control of 150 copies/µL ±30%, provided the Internal Control was added to the RT-PCR master mix (rehydrated in 120 µL). Using Internal Control as extraction control can lead to a different concentration of the Internal Control, depending the used amount and elution volume. Processing the samples according to the protocol, results in an expected volume of 93.75 copies/µL expecting a 100% recovery in RNA extraction. Mycoplasma DNA and Internal Control DNA are competitors in RT-PCR. Because of the very low concentration of Internal Control in the RT-PCR mix, the signal strength in this channel is reduced with increasing mycoplasma DNA loads in the sample. This is an expected behavior.

Data analysis

The concentration of the target Mycoplasma in the reaction was measured in copies/µL (cp/µL) by means of the **Absolute quantification** tool of the QIAcuity Software Suite, version 2.1.7.182. The evaluation of the results for linearity and dynamic range, precision, accuracy, limit of detection, specificity, and robustness is described in detail in the following sections.

The results were accepted for wells with at least 15,000 valid partitions for the green and the yellow channels.

Linearity and dynamic range

For the determination of the working range of the QIAcuity Mycoplasma Kit, least square linear regression of the data was performed to determine the slope of the regression line. The acceptance criteria was that at least 4 consecutive dilutions had to show a R^2 value ≥0.98. The experiment was set-up using at least 3 replicates.

Precision

The precision was evaluated by means of relative repeatability and run-to-tun variation (Deprez et al., 2016). To calculate the relative repeatability (srepeat,rel) and the relative run-to-run variation (srepeat,rel) from the data collected from the working range section, the following calculations were applied: srepeat,rel = srepeat,rel = Where: MSwithin run = within run mean of squares calculated by one-way ANOVA MSwithin run = between run mean of squares calculated by one-way ANOVA $\bar{c}$sample,meas = average measured sample copy number concentration over all runs for each dilution.

Limit of blank

Limit of blank for no template control (NTC) was determined as the lowest tested sample concentration expected to be found in a maximum of 68/72 replicates when testing no template containing samples (95% confidence interval).

Limit of blank for negative extraction control (NEC) was determined as the lowest test sample concentration expected to be found in a maximum of 205/216 replicates when testing no template containing samples (95% confidence interval).

Limit of detection

Limit of detection was determined as the lowest tested sample concentration in which at least one positive partition was present in at least 23/24 replicates performed (95% confidence interval).

Specificity

The specificity was evaluated by testing the workflow using Mycoplasma unrelated bacteria and eukaryotic material as sample for Mycoplasma signals.

Robustness

Robustness was evaluated by determining the recovery rate of QIAcuity Mycoplasma Internal Control in the presence of different matrices. Tested mycoplasma concentration and species, expected Internal Control concentration, and examined matrices are listed in Table 15.

Table 15. Test conditions

Mycoplasma species	Mycoplasma concentration	IC expected concentration	Matrix
Mycoplasma orale	0 CFU/mL 10 CFU/mL 15 CFU/mL 50 CFU/mL	72 copies/µL	Cell suspension of 1 x 10^5 CHO cells/mL (DMEM + 10% FCS)
Mycoplasma orale	0 CFU/mL 10 CFU/mL 15 CFU/mL 50 CFU/mL	72 copies/µL	Cell suspension of 1 x 10^6 CHO cells/mL (DMEM + 10% FCS)
Mycoplasma orale	0 CFU/mL 10 CFU/mL 15 CFU/mL 50 CFU/mL	72 copies/µL	Cell suspension of 1 x 10^7 CHO cells/mL (DMEM + 10% FCS)
Mycoplasma orale	0 CFU/mL 10 CFU/mL 15 CFU/mL 50 CFU/mL	72 copies/µL	FBS Xtra
Mycoplasma orale	0 CFU/mL 10 CFU/mL 15 CFU/mL 50 CFU/mL	72 copies/µL	RPMI 1640
Mycoplasma orale	0 CFU/mL 10 CFU/mL 15 CFU/mL 50 CFU/mL	72 copies/µL	Cryo of 1 x 10^6 HEK293T cells/mL (DMEM + 20% FCS + 10% DMSO)

Reporting requirements

The reports generated by the QIAcuity Software Suite (version 2.1.7.182) are stored digitally. In the protocol, the Table of Results and the 1D scatterplot for both green and yellow signals are included.

Study Results

The study conditions have to provide information on all relevant validation parameters requested by ICH Q2B, EP 2.6.7, EP 2.6.21, USP 63, and JP G3-14-170. As the requirement of the method is to provide a qualitative result only, the parameter linearity, range, accuracy, and quantification limit are irrelevant.

Linearity and dynamic range

Procedure	Acceptance criterion	Results
3 biological replicates were tested at different dilutions in the range between 10,000 and 1 CFU/mL.	R^2 value above 0.98	R^2 value for all tested RT-dPCR and dPCR above 0.98

The regulations for the Mycoplasma detection do not require the assessment of the linearity. Although, we show a serial dilution of a Mycoplasma culture performed in the presence of a matrix (DMEM medium in the presence of 10% FCS). The concentration range went from 10,000 to 1 CFU/mL. The least square regression shows a high linear correlation between the sample and the measured concentration in copies/µL.

Table 16. Linearity *M. fermentans* diluted in DMEM + 10% FCS.

Conc. Sample (CFU/mL)	RT-dPCR Conc. (copies/µL)	RT-dPCR MV Conc. (copies/µL)	SD (n=3) (copies/µL)	CV (n=3)	dPCR Conc. (copies/µL)	dPCR MV Conc. (copies/µL)	SD (n=3) (copies/µL)	CV (n=3)
10,000	n.a. n.a. n.a.	n.a.	n.a.	n.a.	98.53 90.73 83.56	90.9	6.11	6.7%
1000	2878.3 2702.9 2900.7	2827.3	88.44	3.1%	9.32 8.479 9.094	9.0	0.36	4.0%
100	165.1 244.8 289.4	233.1	51.42	22.1%	0.482 0.662 0.822	0.7	0.14	21.2%
10	21.37 53.52 21.48	32.1	15.13	47.1%	0 0 0.16	0.1	0.08	141.4%
1	0 3.714 0.159	1.3	1.71	132.8%	0 0 0	0.0	0.00	n.a.
R^2 value for the linear regression (from 1000 to 1 CFU/mL)	–	0.9997	–	–	–	0.9994	–	–

Table 17. Linearity *M. gallisepticum* diluted in DMEM + 10% FCS.

Conc. Sample (CFU/mL)	RT-dPCR Conc. (copies/µL)	RT-dPCR MV Conc. (copies/µL)	SD (n=3) (copies/µL)	CV (n=3)	dPCR Conc. (copies/µL)	dPCR MV Conc. (copies/µL)	SD (n=3) (copies/µL)	CV (n=3)
10,000	13829.5 13434 14002.3	13755.3	237.87	1.7%	27.83 27.71 31.57	29.0	1.79	6.2%
1000	1143.2 1119.5 1352.5	1205.1	104.70	8.7%	2.628 2.116 2.657	2.5	0.25	10.1%
100	147.2 91.63 151.1	130.0	27.16	20.9%	0.376 0.277 0.217	0.3	0.07	22.6%
10	25.38 6.001 9.96	13.8	8.36	60.7%	0.108 0.055 0.162	0.1	0.04	40.3%
1	0.052 0.212 0.000	0.1	0.09	102.5%	0.000 0.000 0.000	0.0	0.00	n.a.
R^2 value for the linear regression	–	0.9998	–	–	–	0.9997	–	–

Table 18. Linearity *M. hyorhinis* diluted in DMEM + 10% FCS.

Conc. Sample (CFU/mL)	RT-dPCR Conc. (copies/µL)	RT-dPCR MV Conc. (copies/µL)	SD (n=3) (copies/µL)	CV (n=3)	dPCR Conc. (copies/µL)	dPCR MV Conc. (copies/µL)	SD (n=3) (copies/µL)	CV (n=3)
10,000	7608.4 7663.9 7097.8	7456.7	254.79	3.4%	32.1 27.36 25.01	28.2	6.11	21.7%
1000	827.4 829 760.9	805.8	31.73	3.9%	3.057 2.173 2.167	2.5	0.42	17.0%
100	80.22 79.34 101.1	86.9	10.06	11.6%	0.322 n.a.* 0.326	0.3	0.00	0.6%
10	5.001 13.38 12.84	10.4	3.83	36.8%	0 0.055 0.054	0.0	0.03	70.7%
1	0.000 0.000 0.107	0.0	0.05	141.4%	0 0 0	0.0	0.00	n.a.
R^2 value for the linear regression	–	0.9999	–	–	–	0.9998	–	–

* plate defect

Precision

Procedure	Acceptance criterion	Results
Data for Internal Control from limit of detection determination. 6 replicates per mycoplasma concentration on 4 different days were tested resulting in 24 datapoints per tested mycoplasma concentration.	CV ≤10%	CV range for Internal Control <10%

The precision was analyzed with the obtained results for the testing of the Mycoplasma Internal Control. This could be done because the tests performed for the determination of the working range were conducted under repeatability conditions (same analyst, same lot number of the kit and the DNA standard, same dPCR instrument, same lot number of the nanoplates). Relative repeatability and relative run-to-run variation were calculated as described in section "Precision" (page 18) by means of one-way ANOVA statistical test (Deprez et al., 2016) which results are reported in Tables 19 and 20.

Exemplary data for the WHO standard and *M. fermentans* are shown in the following tables.

Overall the CV% range for the quantification of the **Internal Control is below 10%**.

The assessment of the precision of the Mycoplasma detection in biological replicates shows a variability due to the fact that the RNA amount may vary due to gene expression and is not relevant for a qualitative assessment.

Table 19. Precision of WHO international standard

IU/mL	40				10				2.5			
	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4
IC mean value	61.71 copies/µL				61.50 copies/µL				59.85 copies/µL			
IC SD	4.37 copies/µL				4.24 copies/µL				3.90 copies/µL			
IC CV	7.08%				6.90%				6.52%			

Table 20. Precision *M. fermentans*

CFU/mL	20				10				5			
	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4
IC mean value	58.91 copies/µL				57.66 copies/µL				58.77 copies/µL			
IC SD	2.68 copies/µL				2.74 copies/µL				3.77 copies/µL			
IC CV	4.55%				4.75%				6.41%			

Limit of Blank

The employed method obtains a qualitative result. To be able to detect low levels of contamination, a low limit of blank becomes extremely important. In practice, the limit of blank is a threshold below which 95% of samples are without amplified target sequence.

Procedure	Acceptance Criterion	Results
24 NTC replicates on 3 different days were tested resulting in 72 data points. Additionally, 6 eluates of 4 different days from extracting 0 CFU/mL of 10 *Mollicutes* listed in Table 3, and WHO standard listed in Table 5 were tested each resulting in 24 NEC replicates per species and 264 data points in total.	LOB was calculated based on the mean value and standard deviation of NTC and NEC samples using Equation 1 (below).	**LOB_{NTC} = 0.057 copies/µL** **LOB_{NEC} = 0.149 copies/µL** 11 out of 72 wells NTCs contain between 1 and 3 positive partitions.

$$LOB = MV + (1.64 \times SD)$$

Equation 1: Calculation of limit of blank (LOB). LOB (limit of blank), MV (mean value), SD (standard deviation).

Limit of detection

The employed method obtains a qualitative result. Proof of linearity is not required. If however the concept of linearity extends 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).

Procedure	Acceptance criterion	Results
The Mollicutes preparations according to section "RNA extraction" (page 13) were diluted in 1:2 dilution steps (one deviating dilution step for accurate adjustment of concentration) to prepare a suspension containing 160 CFU/mL. Subsequently, dilution levels at 80, 40, 20, 10, 5, and 0 CFU/mL were prepared in DMEM medium containing 10% (v/v) FBS. WHO International Standard for Mycoplasma DNA at dilution levels 640, 160, 40, 10, 2.5, 0.625, and 0 IU/mL were prepared in DMEM medium containing 10% (v/v) FBS. Four individual dilution series were prepared at 4 different days and each dilution were analyzed in 6 replicates resulting in 24 data points per concentration. The results were confirmed testing 6 replicates for each 10 CFU standard in DMEM medium containing 10% (v/v) FBS.	23 of 24 samples containing 10 CFU/mL must be positive after subtraction of LOB_{NEC} for all species.	All samples of tested mycoplasma species containing 10 CFU/mL and the sample containing WHO International Standard at 10 IU/mL were tested mycoplasma positive. See following tables.

Table 21. LOD Mycoplasma summary

Species	Sensitivity
Acholeplasma laidlawii	10 CFU/mL
Mycoplasma arginini	5 CFU/mL
Mycoplasma fermentans	5 CFU/mL
Mycoplasma gallisepticum	10 CFU/mL
Mycoplasma hyorhinis	10 CFU/mL
Mycoplasma orale	5 CFU/mL
Mycoplasma salivarium	10 CFU/mL
Mycoplasma synoviae	10 CFU/mL
WHO International Standard	10 IU/mL

Table 22. LOD *Mycoplasma fermentans*

CFU/mL	20				10				5			
	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4
Hit rate	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
Mean value	68.65 copies/µL				32.19 copies/µL				15.60 copies/µL			
SD	17.50 copies/µL				13.07 copies/µL				9.09 copies/µL			
Out of 24	24				24				24			
IC mean value	58.91 copies/µL				57.66 copies/µL				58.77 copies/µL			
IC SD	2.68 copies/µL				2.74 copies/µL				3.77 copies/µL			
IC CV	4.55%				4.75%				6.41%			

Table 23. LOD *Mycoplasma orale*

CFU/mL	20				10				5			
	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4
Hit rate	6/6	6/6	6/6	6/6	6/6	5/6	6/6	6/6	6/6	6/6	6/6	6/6
Mean value	15.56 copies/µL				6.11 copies/µL				2.82 copies/µL			
SD	9.68 copies/µL				5.21 copies/µL				2.65 copies/µL			
Out of 24	24				23				24			
IC mean value	59.28 copies/µL				59.21 copies/µL				60.24 copies/µL			
IC SD	2.90 copies/µL				4.03 copies/µL				2.43 copies/µL			
IC CV	4.90%				6.80%				4.03%			

Table 24. LOD *Mycoplasma gallisepticum*

CFU/mL	20				10				5			
	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4
Hit rate	6/6	6/6	6/6	6/6	5/6	6/6	6/6	6/6	6/6	6/6	6/6	4/6
Mean value	34.31 copies/µL				16.56 copies/µL				8.94 copies/µL			
SD	14.55 copies/µL				9.28 copies/µL				7.22 copies/µL			
Out of 24	24				23				22			
IC mean value	64.29 copies/µL				65.44 copies/µL				63.66 copies/µL			
IC SD	3.54 copies/µL				3.65 copies/µL				3.87 copies/µL			
IC CV	5.51%				5.58%				6.07%			

Table 25. LOD *Acholeplasma laidlawii*

CFU/mL	20				10				5			
	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4
Hit rate	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	5/6	4/6	4/6	5/6
Mean value	66.42 copies/µL				41.52 copies/µL				12.88 copies/µL			
SD	47.59 copies/µL				38.87 copies/µL				15.08 copies/µL			
Out of 24	24				24				18			
IC mean value	63.33 copies/µL				63.18 copies/µL				64.11 copies/µL			
IC SD	6.04 copies/µL				5.51 copies/µL				5.24 copies/µL			
IC CV	9.54%				8.72%				8.17%			

Table 26. LOD *M. arginine*

CFU/mL	20				10				5			
	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4
Hit rate	6/6	6/6	6/6	6/6	5/6	6/6	6/6	6/6	6/6	5/6	6/6	6/6
Mean value	12.80 copies/µL				5.99 copies/µL				3.21 copies/µL			
SD	5.54 copies/µL				3.00 copies/µL				2.02 copies/µL			
Out of 24	24				24				23			
IC mean value	63.02 copies/µL				62.16 copies/µL				63.22 copies/µL			
IC SD	5.49 copies/µL				4.52 copies/µL				4.48 copies/µL			
IC CV	8.70%				7.27%				7.09%			

Table 27. LOD *M. hyorhinis*

CFU/mL	20				10				5			
	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4
Hit rate	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	5/6	5/6
Mean value	29.38 copies/µL				12.05 copies/µL				7.10 copies/µL			
SD	13.23 copies/µL				7.72 copies/µL				5.83 copies/µL			
Out of 24	24				24				22			
IC mean value	58.71 copies/µL				60.32 copies/µL				60.15 copies/µL			
IC SD	3.51 copies/µL				4.02 copies/µL				3.56 copies/µL			
IC CV	5.98%				6.67%				5.92%			

Table 28. LOD *Mycoplasma salivarium*

CFU/mL	20				10				5			
	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4
Hit rate	6/6	6/6	6/6	6/6	6/6	6/6	5/6	6/6	6/6	4/6	3/6	4/6
Mean value	27.65 copies/µL				12.52 copies/µL				9.31 copies/µL			
SD	14.30 copies/µL				10.02 copies/µL				21.29 copies/µL			
Out of 24	24				23				17			
IC mean value	63.35 copies/µL				63.93 copies/µL				65.22 copies/µL			
IC SD	3.01 copies/µL				3.00 copies/µL				2.45 copies/µL			
IC CV	4.75%				4.61%				3.76%			

Table 29. LOD *Mycoplasma synoviae*

CFU/mL	20				10				5			
	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4
Hit rate	6/6	5/6	6/6	6/6	6/6	5/6	6/6	6/6	3/6	2/6	3/6	6/6
Mean value	24.10 copies/µL				14.39 copies/µL				4.98 copies/µL			
SD	17.53 copies/µL				12.67 copies/µL				7.33 copies/µL			
Out of 24	23				23				14			
IC mean value	58.38 copies/µL				58.95 copies/µL				59.16 copies/µL			
IC SD	4.22 copies/µL				2.70 copies/µL				2.84 copies/µL			
IC CV	7.23%				4.57%				4.80%			

Table 30. LOD WHO International Standard for Mycoplasma DNA

IU/mL	40				10				2.5			
	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4
Hit rate	6 / 6	6 / 6	6 / 6	6 / 6	6 / 6	6 / 6	6 / 6	6 / 6	1 / 6	3 / 6	2 / 6	2 / 6
Mean value	6.14 copies/µL				2.37 copies/µL				0.13 copies/µL			
SD	5.93 copies/µL				3.52 copies/µL				0.12 copies/µL			
Out of 24	24				24				8			
IC mean value	61.71 copies/µL				61.50 copies/µL				59.85 copies/µL			
IC SD	4.37 copies/µL				4.24 copies/µL				3.90 copies/µL			
IC CV	7.08%				6.90%				6.52%			

Specificity

The study conditions have to provide information on all relevant validation parameters requested by ICH Q2B, EP 2.6.7, EP 2.6.21, USP <63>, and JP G3-14-170. As the requirement of the method is to provide a qualitative result only, the parameter linearity, range, accuracy, and quantification limit are irrelevant.

Sequence alignment

Procedure	Acceptance Criterion	Results
Comparison of all primer and probe sequences with genomic database. *Mollicutes* sequence alignments were performed. Even though EP 2.6.7 for specificity determination does not recommend this technique, it provides additional information for species not available for testing. Alignment was performed using TestPrime (**www.arb-silva.de/search/testprime/**) and the database SILVA ribosomal RNA database project (**www.arb-silva.de/**) in version SILVA SSU database 138.1 as of August 27, 2020, Subset SSU Ref NR 99 (**www.arb-silva.de/documentation/release-1381/**).	*Mollicutes* species showing ≤2 nucleotides mismatch of the primer sequences with the 16S rRNA genome and 0 mismatch within the second last nucleotides at the 3′ end of the primers are considered specifically detectable. Mollicutes species showing ≤2 nucleotides mismatch of the probe sequences with the 16S rRNA genome are considered specifically detectable.	At least 127 *Mollicutes* species are putatively detectable based on sequence alignment.

Table 31. Detectable *Mollicutes* species based on alignment

	Primer / Probe Mismatches		
Species (name in database)	**Forward Primer**	**Probe**	**Reverse Primer**
Acholeplasma equifetale	0	0	2
Acholeplasma granularum	0	0	0
Acholeplasma hippikon	0	0	1
Acholeplasma laidlawii	0	0	0
Acholeplasma manati	0	1	1
Acholeplasma morum	0	0	1
Acholeplasma oculi	0	0	1
Acholeplasma palmae	0	0	0
Acholeplasma pleciae	0	0	0
Acholeplasma vituli	0	1	1
Malacoplasma microti	0	1	1
Malacoplasma muris	0	1	2
Mesomycoplasma lagogenitalium	0	1	2
Mesomycoplasma molare	0	1	2
Metamycoplasma alkalescens	0	0	1
Metamycoplasma auris	0	0	1
Metamycoplasma buccale	0	0	1
Metamycoplasma canadense	0	0	1
Metamycoplasma cloacale	0	0	1
Metamycoplasma equirhinis	0	0	1
Metamycoplasma faucium	0	0	0
Metamycoplasma gateae	0	0	1
Metamycoplasma hyosynoviae	0	0	1
Metamycoplasma neophronis	0	0	2
Metamycoplasma phocicerebrale	0	0	2
Metamycoplasma salivarium	0	0	1
Metamycoplasma spumans	0	0	1
Metamycoplasma sualvi	0	0	0

Metamycoplasma subdolum	0	0	1
Mycoplasma agalactiae (*Mycoplasmopsis agalactiae*)	0	1	0
Mycoplasma alligatoris (*Mycoplasmopsis alligatoris*)	0	0	2
Mycoplasma anatis (*Mycoplasmopsis anatis*)	0	0	2
Mycoplasma anseris	0	0	1
Mycoplasma anserisalpingitis	0	0	2
Mycoplasma aquilae	0	0	0
Mycoplasma arthritidis (*Metamycoplasma arthritidis*)	0	0	1
Mycoplasma bovis (*Mycoplasmopsis bovis*)	0	0	0
Mycoplasma bovoculi (*Mesomycoplasma bovoculi*)	0	0	2
Mycoplasma buteonis	0	0	1
Mycoplasma californicum	0	0	0
Mycoplasma canis (*Mycoplasmopsis canis*)	0	0	2
Mycoplasma collis	0	1	2
Mycoplasma corogypsi	0	0	2
Mycoplasma crocodyli	0	0	2
Mycoplasma cynos (*Mycoplasmopsis cynos*)	0	0	1
Mycoplasma elephantis	0	0	1
Mycoplasma falconis	0	0	0
Mycoplasma fermentans (*Mycoplasmopsis fermentans*)	0	0	0
Mycoplasma gallisepticum	0	1	0
Mycoplasma genitalium (*Mycoplasmoides genitalium*)	0	1	2
Mycoplasma gypis	0	1	1
Mycoplasma hominis (*Metamycoplasma hominis*)	0	0	2
Mycoplasma hyorhinis (*Mesomycoplasma hyorhinis*)	0	0	0
Mycoplasma iguanae	0	1	1
Mycoplasma imitans	0	1	0
Mycoplasma indiense	0	0	1
Mycoplasma iowae	0	1	1
Mycoplasma leonicaptivi	1	0	1
Mycoplasma leopharyngis	1	0	0

Mycoplasma moatsii (*Mesomycoplasma moatsii*)	0	0	0
Mycoplasma mobile	0	0	1
Mycoplasma nasistruthionis	0	0	1
Mycoplasma orale (*Metamycoplasma orale*)	0	0	1
Mycoplasma oxoniensis	0	0	0
Mycoplasma penetrans (*Malacoplasma penetrans*)	0	1	1
Mycoplasma phocae	0	0	2
Mycoplasma pneumoniae (*Mycoplasmoides pneumoniae*)	0	1	2
Mycoplasma preputii	0	0	0
Mycoplasma simbae	0	0	1
Mycoplasma spermatophilum	0	0	0
Mycoplasma sphenisci	0	0	1
Mycoplasma synoviae (Mycoplasmopsis synoviae)	0	0	0
Mycoplasma testudineum	0	0	1
Mycoplasma zalophi	0	1	1
Mycoplasma zalophidermidis	0	0	1
Mycoplasmoides alvi	0	1	2
Mycoplasmoides gallisepticum	0	1	0
Mycoplasmoides pirum	1	1	2
Mycoplasmopsis adleri	1	0	0
Mycoplasmopsis agassizii	0	0	2
Mycoplasmopsis arginini	0	0	1
Mycoplasmopsis bovigenitalium	0	0	0
Mycoplasmopsis bovirhinis	0	0	0
Mycoplasmopsis californica	0	0	0
Mycoplasmopsis caviae	0	0	0
Mycoplasmopsis citelli	0	0	0
Mycoplasmopsis columbina	0	0	0
Mycoplasmopsis columbinasalis	0	0	0
Mycoplasmopsis columboralis	0	0	1
Mycoplasmopsis cricetuli	0	0	0

Mycoplasmopsis edwardii	0	0	2
Mycoplasmopsis equigenitalium	0	0	1
Mycoplasmopsis felifaucium	1	0	0
Mycoplasmopsis felis	1	0	1
Mycoplasmopsis gallinacea	0	0	0
Mycoplasmopsis gallinarum	0	0	0
Mycoplasmopsis gallopavonis	0	0	1
Mycoplasmopsis glycophila	0	0	1
Mycoplasmopsis hyopharyngis	0	0	1
Mycoplasmopsis iners	0	0	0
Mycoplasmopsis lipofaciens	0	0	1
Mycoplasmopsis lipophila	0	0	0
Mycoplasmopsis maculosa	0	0	0
Mycoplasmopsis meleagridis	0	0	0
Mycoplasmopsis mucosicanis	0	0	1
Mycoplasmopsis mustelae	0	0	0
Mycoplasmopsis opalescens	0	0	0
Mycoplasmopsis phocirhinis	0	0	0
Mycoplasmopsis primatum	0	0	0
Mycoplasmopsis pullorum	0	0	2
Mycoplasmopsis pulmonis	0	0	1
Mycoplasmopsis sturni	0	0	0
Mycoplasmopsis verecunda	0	0	0
Spiroplasma atrichopogonis	0	0	0
Spiroplasma chrysopicola	0	0	2
Spiroplasma citri	0	0	0
Spiroplasma endosymbiont of *Drosophila aldrichi*	0	0	0
Spiroplasma eriocheiris	0	0	1
Spiroplasma insolitum	0	0	0
Spiroplasma kunkelii	0	0	0
Spiroplasma leucomae	0	0	0

Spiroplasma melliferum	0	0	0
Spiroplasma mirum	0	0	1
Spiroplasma penaei	0	0	0
Spiroplasma phoeniceum	0	0	0
Spiroplasma poulsonii	0	0	0
Spiroplasma syrphidicola	0	0	2

Sample Matrix Cross Reactivity

Procedure	Acceptance Criterion	Results
Testing the media components according to Table 2 without mycoplasma background. The internal amplification control was added to the sample matrix as extraction control.	Positive result of all tested samples containing mycoplasma. Negative result of all tested samples without mycoplasma.	All mycoplasma containing samples were tested positive for mycoplasma. All NEC samples were considered mycoplasma negative.

Table 32. Matrix cross reactivity

Sample Matrix	Mycoplasma mean concentration (copies/µL)	Mycoplasma mean positive partitions	IC mean concentration (copies/µL)	Result
FBS Xtra	0.161	3	58.17	Negative
1 x 10^6 HEK293 cells/mL in DMEM + 20% FBS + 10% DMSO	0.00	0	34.69	Negative
RPMI	0.05	1	64.57	Negative
1 x 10^7 CHO-K1 cells/mL in DMEM + 10% FBS	0.54	424	23.02	Negative
PC	105.1	1898	0.00	Positive

Cross Reactivity

Procedure	Acceptance Criterion	Results
All non-*Mollicutes* bacteria listed in Table 6 and Table 7 were tested for mycoplasma signals at 3 different concentrations as singlet, resulting in at least 3 data points for every bacterial species. All eukaryotic cells listed in Table 26 were tested for mycoplasma signals in triplicates. 1 µL IC was spiked into RT-PCR. The amplification products of positive tested samples were analyzed by sequencing.	Negative result of all tested samples.	All samples containing no mycoplasma were tested negative.

Table 33. Microbial strains cross reactivity

Sample matrix	Spike concentration (CFU/mL)	Mycoplasma mean concentration (copies/µL)	Mycoplasma mean positive partitions	IC mean concentration (copies/µL)	Result
Bacillus subtilis	1×10^4	0.16	3	11.83	Negative
	1×10^3	0.11	2	13.11	Negative
	1×10^2	0.00	0	11.51	Negative
Bacteroides vulgatus	1×10^4	0.05	1	9.99	Negative
	1×10^3	0.00	0	12.08	Negative
	1×10^2	0.00	0	11.77	Negative
Clostridium sporogenes	1×10^4	0.05	1	12.90	Negative
	1×10^3	0.05	1	10.49	Negative
	1×10^2	0.11	2	12.45	Negative
Kocuria rhizophila	1×10^4	0.11	2	11.02	Negative
	1×10^3	0.06	1	12.59	Negative
	1×10^2	0.06	1	11.69	Negative
Pseudomonas aeruginosa	1×10^4	0.00	0	13.20	Negative
	1×10^3	0.00	0	11.43	Negative
	1×10^2	0.00	0	13.53	Negative
Staphylococcus aureus	1×10^4	0.10	2	13.15	Negative
	1×10^3	0.05	1	11.06	Negative
	1×10^2	0.05	1	11.41	Negative
Streptococcus pyogenes	1×10^4	0.05	1	10.47	Negative
	1×10^3	0.00	0	12.80	Negative
	1×10^2	0.00	0	10.62	Negative
NEC	–	0.04	1	11.43	Negative
NTC	–	0.00	0	12.08	Negative
PC	–	99.66	1806	11.53	Positive

Table 34. Cross reactivity with eukaryotic cells

Sample matrix	Spike concentration (cells/mL)	Mycoplasma mean concentration (copies/µL)	Mycoplasma mean positive partitions	IC mean concentration (copies/µL)	Result
Vero	1 x 10^6	0.02	1	11.70	Negative
HL-60 cells	1 x 10^6	0.02	1	12.70	Negative
CHO-K1*	1 x 10^7	0.05	1	23.02	Negative
	1 x 10^6	0.05	1	40.00	Negative
	1 x 10^5	0.00	0	46.86	Negative
HEK293*	1 x 10^6	0.00	0	34.52	Negative
NEC	–	0.02	1	11.30	Negative
NTC	–	0.00	0	11.81	Negative
PC	–	105.00	1893	10.66	Positive

* Data from robustness testing (without mycoplasma spike). IC was spiked as overall control during sample lysis. Hence, IC concentration is higher than for the other samples.

Robustness

The robustness testing of QIAcuity Mycoplasma Quant Kit requires the most relevant mycoplasma species from a risk-based point of view. *Mycoplasma orale* is such a relevant spike for robustness testing due to its high prevalence in cell cultures used in academic and biopharmaceutical industrial applications.

Matrix Effects

Procedure	Acceptance Criterion	Results
Testing of 3 different *Mycoplasma orale* concentrations (10 CFU/mL, 15 CFU/mL, 50 CFU/mL) in duplicates and without mycoplasma background using the media components according to Table 15. The internal amplification control was added to the sample matrix as extraction control.	Positive result of all samples.	All samples were tested positive for mycoplasma. See table below.

Table 35. Robustness using different matrices

Sample Matrix	*M. orale* spike concentration (CFU/mL)	Mycoplasma mean concentration (copies/µL)	Mycoplasma mean positive partitions	IC mean concentration (copies/µL)	Result
FBS Xtra	50	42.43	793	58.65	Positive
	15	21.00	389	61.49	Positive
	10	4.00	74	57.57	Positive
	0	0.161	3	58.17	Negative
1×10^6 HEK293 cells/mL in DMEM + 20% FBS + 10% DMSO	50	57.97	1041	41.41	Positive
	15	15.34	275	36.16	Positive
	10	5.06	90	39.90	Positive
	0	0.00	0	34.69	Negative
RPMI	50	38.27	712	64.83	Positive
	15	11.72	211	63.65	Positive
	10	3.06	56	64.08	Positive
	0	0.05	1	64.57	Negative
1×10^5 CHO-K1 cells/mL in DMEM + 10% FBS	50	41.80	774	57.55	Positive
	15	30.60	557	53.55	Positive
	10	8.69	159	53.61	Positive
	0	0	0	46.86	Negative
1×10^6 CHO-K1 cells/mL in DMEM + 10% FBS	50	42.18	769	37.50	Positive
	15	11.35	204	38.28	Positive
	10	14.66	262	42.01	Positive
	0	0.054	1	40.00	Negative
1×10^7 CHO-K1 cells/mL in DMEM + 10% FBS	50	24.00	450	25.00	Positive
	15	13.06	242	28.45	Positive
	10	5.66	104	29.64	Positive
	0	0.054	1	23.02	Negative
PC	–	105.1	1898	0.00	Positive
NTC	–	0.00	0	0.05	Negative

Conclusion

The QIAcuity Mycoplasma Quant Kit was validated in accordance to the validation protocol presented in this report ("Test Procedure", page 13). The validation report describes the test procedure itself and expected variations in sample diversity from sample types commonly tested for mycoplasma contamination such as growth media, cell cultures or pharmaceutical in-process samples and products. The QIAcuity Mycoplasma Quant Kit should be applied for mycoplasma contamination downstream of a suitable RNA extraction method. We recommend the nucleic acid extraction kit Venor®GeM Sample Preparation Kit which was used in this validation study.

The validation of the QIAcuity Mycoplasma Quant Kit has been conducted according to the *European Pharmacopeia* 11, chapter 2.6.7 (Mycoplasmas), the *United States Pharmacopeial Convention* 41, chapter 63 (Mycoplasma Tests), and the *Japanese Pharmacopeia* 17, chapter G3-14-170 (Mycoplasma Testing for Cell Substances used for the Production of Biotechnological/Biological Products), in which PCR, including digital PCR, is an alternative to the time-consuming culture method or indicator cell culture method.

In accordance to the validation criteria of the abovementioned regulations, the QIAcuity Mycoplasma Quant Kit provides:

- A Positive Control for dPCR, which cp/µL falls within an indicated range
- An Internal Control for overall control and RT-dPCR, which cp/µL falls within an indicated range
- The possibility to test negative controls for RT-dPCR with a suitable matrix proven to be free of target sequence (RNase-Free Water, Elution Buffer)

In separate kits offered are irreversibly inactivated 10 CFU/mL standards for all 10 mycoplasma species mentioned in *Ph. Eur.*, *USP*, *JP* to allow verification of the validation by the customer without dealing with infectious mycoplasma. Pharmacopeia mentioned mycoplasma species are *Mycoplasma arginini*, *Mycoplasma orale*, *Mycoplasma gallisepticum*, *Mycoplasma pneumoniae*, *Mycoplasma synoviae*, *Mycoplasma fermentans*,

Mycoplasma hyorhinis, *Acholeplasma laidlawii*, *Spiroplasma citri*, and *Mycoplasma salivarium*.

The QIAcuity Mycoplasma Quant Kit has been validated using the Venor®GeM Sample Preparation Kit upstream. Any other suitable RNA extraction kit can be used but need to be fully validated by the customer.

The study provided detailed information on the workflow performance:

- Working Range: Between 1 to 10,000 CFU/mL the lowest linear regression had a R^2 of 0.9997.
- Limit of blank: The lowest detected concentration expected to be found in 69/72 well when testing NTCs was 0.057 cp/µL, when testing NECs was 0.149 cp/µL.
- Limit of detection: The smallest concentration of mycoplasma in which at least 23/24 wells considered as positive was 10 CFU/mL at highest for all tested mycoplasma species and 10 IU/mL for the WHO International Standard.
- Precision: Relative repeatability and run-to-run variation of the internal control in the studied workflow was below 9.54%. The relative run-to-run variation of the mycoplasma RNA quantification was higher due to may differences in gene expression levels and is not required for qualitative mycoplasma testing.
- Specificity: At least 127 *Mollicutes* species are putatively detected positive. No relevant unknown cross-reactivity was found with the *in vitro* tested unrelated bacterial species, eukaryotic material, and matrices.
- Robustness: All tested matrices and sample types showed a positive mycoplasma signal at 10 CFU/mL.

The QIAcuity Mycoplasma Quant Kit can be safely used for the detection of mycoplasma contaminations in cell cultures and in the manufacturing process of pharmaceutical products using the Venor®GeM Sample Preparation Kit. In accordance to the requirements of the Ph. Eur. 10, chapter 2.6.7, the USP 41, chapter 63, and the JP 17, chapter G3-14-170, the validation described here needs to be verified by the customer using their sample matrix.

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13. TIGR CMR

Ordering Information

Product	Contents	Cat. no.
QIAcuity Mycoplasma Quant Kit	OneStep Advanced Probe Master Mix, OneStep Advanced RT Mic, OneStep Enhancer GC, QIAcuity Mycoplasma Assay, QIAcuity Mycoplasma Internal Control, QIAcuity Mycoplasma Positive Control, RNase-Free Water	250261
Mycoplasma arginini Standard CFU	QIAcuity Mycoplasma 10 CFU Standard, QIAcuity Mycoplasma Negative Control	250262
Mycoplasma orale Standard CFU	QIAcuity Mycoplasma 10 CFU Standard, QIAcuity Mycoplasma Negative Control	250263
Mycoplasma gallisepticum Standard CFU	QIAcuity Mycoplasma 10 CFU Standard, QIAcuity Mycoplasma Negative Control	250264
Mycoplasma pneumoniae Standard CFU	QIAcuity Mycoplasma 10 CFU Standard, QIAcuity Mycoplasma Negative Control	250265
Mycoplasma synoviae Standard CFU	QIAcuity Mycoplasma 10 CFU Standard, QIAcuity Mycoplasma Negative Control	250266
Mycoplasma fermentans Standard CFU	QIAcuity Mycoplasma 10 CFU Standard, QIAcuity Mycoplasma Negative Control	250267
Mycoplasma hyorhinis Standard CFU	QIAcuity Mycoplasma 10 CFU Standard, QIAcuity Mycoplasma Negative Control	250268
Acholeplasma laidlawii Standard CFU	QIAcuity Mycoplasma 10 CFU Standard, QIAcuity Mycoplasma Negative Control	250269
Spiroplasma citri Standard CFU	QIAcuity Mycoplasma 10 CFU Standard, QIAcuity Mycoplasma Negative Control	250270

Mycoplasma salivarium Standard CFU	QIAcuity Mycoplasma 10 CFU Standard, QIAcuity Mycoplasma Negative Control	250271
QIAcuity Nanoplate 26K 24-well (10)	10 QIAcuity Nanoplates 26K with 24 wells, 11 Nanoplate Seals	250001
QIAcuity One, 5plex Instrument	One-plate digital PCR instrument for detecting up to 5 fluorescent dyes, roller, USB flash memory, and QIAcuity Software Suite: includes 1 preventive maintenance visit. One year warranty on labor, travel, and parts also included.	911020
QIAcuity Four Instrument	Four-plate digital PCR instrument for detecting up to 5 fluorescent dyes, notebook computer, barcode scanner, roller, USB flash memory, and QIAcuity Software Suite: includes installation, training, and 1 preventive maintenance visit. One year warranty on labor, travel, and parts.	911040
QIAcuity Eight Instrument	Eight-plate digital PCR instrument for detecting up to 5 fluorescent dyes, notebook computer, barcode scanner, nanoplate roller, USB flash memory, and QIAcuity Software Suite: includes installation, training, and 1 preventive maintenance visit. One year warranty on labor, travel, and parts.	911050

Related Products

QIAcuity Nanoplate 8.5K 24-well (10)	10 QIAcuity Nanoplates 8.5K with 24 wells, 11 Nanoplate Seals	250011
QIAcuity Nanoplate 8.5K 96-well (10)	10 QIAcuity Nanoplates 8.5K with 96 wells, 11 Nanoplate Seals	250021

QIAcuity Nanoplate 26k 8-well (10)	10 QIAcuity Nanoplate 26k 8-well, 11 Nanoplate Seals	250031
Nanoplate Seals (11)	11 Nanoplate Seals	250099
CGT Viral Vector Lysis Kit (100)	For 100 DNase I reactions (50 µL): CGT Sample Stabilizer, CGT DNase I Buffer, DNase I, CGT Lysis Buffer, CGT Dilution Buffer and Nuclease-Free Water	250272
CGT Viral Vector Lysis Kit (1000)	For 1000 DNase I reactions (50 µL): CGT Sample Stabilizer, CGT DNase I Buffer, DNase I, CGT Lysis Buffer, CGT Dilution Buffer, and Nuclease-Free Water	250273
QIAcuity Residual DNA Quantification Kits	QIAcuity *E. coli* / CHO / HEK293 resDNA Quant Master Mix (4x) lyophilized, Positive Control, Internal Control, RNAse-Free Water, and respective Standard Kits	250220–250225
QIAcuity Cell & Gene Therapy (CGT) dPCR Assays	For 500 x 12 µL reactions (20x): QIAGEN Cell and Gene Therapy assay for GFP; ITR2/5, Sv40 promoter, AMP resistance, or others	250230–250256

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Document Revision History

Version	Description
10/2023	Initial release

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