December 2025

Validation Report

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, 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 mix containing a FAM™-labeled probe specific for a broad range of different mycoplasma species. The HEX-labeled internal amplification control prevents false negative results due to improper nucleic acid extraction, reverse transcription, or presence of PCR inhibitors. It is possible to add the QIAcuity Internal Control RNA directly to the sample prior to nucleic acid extraction and analysis for verification of the complete process (nucleic acid 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 Digital PCR System 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 of the QIAcuity Mycoplasma Quant Kit using dPCR. Section 2.6.7 "Mycoplasmas" of the *European Pharmacopoeia* describes a protocol for validation of a commercial 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.

Any modification of the presented mycoplasma detection workflow requires a full validation of all relevant performance aspects according to section 2.6.7 of the *European Pharmacopoeia* by the user, section 2.6.21 of the EP "Nucleic acid amplification techniques" (NAT, PCR), and with respect to ICH guideline Q2B. The employed method obtains a 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 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* to fulfill 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" [3, 5, 6].

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
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

Abbreviation	Meaning
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	negative 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 third-party Minerva Biolabs GmbH and by QIAGEN® GmbH.

Test Materials

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

Test system

The test systems used for the detection of mycoplasma during this study are as follows.

Table 1. Test system information

System type	Supplied by	Cat. no.	Storage temperature (°C)
QIAcuity Mycoplasma Quant Kit	QIAGEN GmbH	250261	–30 to –15
Venor® Mycoplasma Extraction (Formerly Venor Gem SP Kit Beads)	Minerva Biolabs GmbH	56-3010 / 56-3100	Ambient temperature (18–25) and 4–8
EZ1&2® Virus Mini Kit v2.0 (48)	QIAGEN GmbH	955134	Ambient temperature (15–25)

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 2a. Matrix formulation – Minerva Biolabs GmbH

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

Table 2b. Matrix formulation – QIAGEN GmbH

Product ingredient	Manufacturer	Cat. no.	Lot no.
FCS	Gibco	10437-028	2230743RP
DMEM	Gibco	11574486	2902905
FBS Extra	Capricorn Scientific GmbH	FBS-16A	CP23-6246
DMEM	Capricorn Scientific GmbH	DMEM-LPSTA	CP23-6496
HEK293	In-house culture (Minerva Biolabs GmbH)	–	–

Microorganisms and eukaryotic material

Tables 3–8 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	5.7	*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	2.8	*Mycoplasmoides pneumoniae*

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

Species	Natural host	NCTC code	DSM code	ATCC code	GC:CFU ratio	New nomenclature
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*

According to the *European Pharmacopoeia* (EP) Chapter 2.6.7, Mycoplasma tests require a sensitivity of 10 colony-forming units (CFU) per mL sample volume for NAT-based methods such as digital 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 of the NAT-based method must reach 10 CFU/mL or 100 CFU/mL, depending on the traditional method it needs to replace, as part of the robustness testing for any particular sample matrix. In the validation study presented here, vital mycoplasma was used, which was grown and quantified by culture method. Using vital mycoplasma is not acceptable for the majority of cell culture laboratories due to safety regulations. Mycoplasma Standard CFU Kits (cat. nos. 250262–250271) contain non-vital material and allow to perform reliable validation experiments without the risk of contamination.

The mycoplasma was grown in culture medium as described in EP 2.6.7, subsequently titrated and plated on Hayflick and Frey medium for CFU determination. The mycoplasma was 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 (see Table 3). All strains were obtained from official culture collections and cultivated in low passages.

In more detail, all mycoplasmas 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. After harvest, *Mycoplasma pneumoniae* was sterile filtered using a 0.45 µm filter to break strong mycoplasma clusters and all the other species were directly processed. The culture broth was divided into 2 portions. One portion was used for colony counting of the mycoplasma. The broth was vortexed intensively before titration to break up mycoplasma clusters. Two tenfold dilution series were prepared in culture broth. For 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 microscopy. Frequent counting was stopped at constant colony numbers and the titer was calculated as CFU per milliliter of 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 4. Description of international reference material

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

Table 5. Non-Mollicutes bacterial strains – quantified by colony counting using traditional culture method

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
Streptococcus pyogenes	11728	19615	–

Table 6. Eukaryotic cells – quantified by cell 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	–	–

Table 7. Mycoplasma cultivation media

Medium	Manufacturer	Cat. no.
Frey Bouillon acc. EP	Merck Millipore	146311
Frey-Agar acc. EP	Merck Millipore	146006
Hayflick Bouillon acc. EP	Merck Millipore	146452
Hayflick-Agar acc. EP	Merck Millipore	146029

Table 8. Reagents for cell culture

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

The quality of the mycoplasma culture medium is critical for the subsequent spiking experiments. Each lot of the mycoplasma culture material was quality-controlled according to EDQM standards, and a Certificate of Analysis (CoA) was available for every batch.

Equipment

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

Table 9a. Minerva Biolabs GmbH lab equipment

Equipment, brand	Manufacturer	Cat. no.	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	LH-729130	42084372 E152
Eppendorf® Reference 2, 1-Channel, 100–1000 µL	Eppendorf SE	4924000088	O10885D E05
Eppendorf Reference 2, 1-Channel, 10–100 µL	Eppendorf SE	4924000053	L10238D E04
Picus® Electronic pipette, 1-Channel, 10–300 µL	Sartorius Lab Instruments GmbH & Co. KG	LH-747061	15011121 E60
Picus Electronic pipette, 1-Channel, 10–300 µL	Sartorius Lab Instruments GmbH & Co. KG	LH-747061	15011122 E57
Picus Electronic pipette, 1-Channel, 10–300 µL	Sartorius Lab Instruments GmbH & Co. KG	LH-747061	42490283 E160
Eppendorf Reference 2, 1-Channel, 2–20 µL	Eppendorf SE	4924000037	G26555D E15
Tacta Mechanical pipette, Single Channel, 100–1000 µL	Sartorius Lab Instruments GmbH & Co. KG	LH-729070	19011091 E133

Table 9a. Minerva Biolabs GmbH lab equipment (continued)

Equipment, brand	Manufacturer	Cat. no.	Equipment ID (internal/serial no.)
Tacta Mechanical pipette, Single Channel, 10–100 µL	Sartorius Lab Instruments GmbH & Co. KG	LH-729050	19004053 E132
Tacta Mechanical pipette, Single Channel, 100 –1000 µL	Sartorius Lab Instruments GmbH & Co. KG	LH-729070	18026710 E103
Tacta Mechanical pipette, Single Channel, 10–100 µL	Sartorius Lab Instruments GmbH & Co. KG	LH-729050	18020324 E102
Eppendorf Reference 2, 1-Channel, 100–1000 µL	Eppendorf Vertrieb Deutschland GmbH	4924000088	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	911021	00159 (E143) 00424 (E154)
QIAcuity Four	QIAGEN GmbH	911042	02032 (E174)
Nanoplate Tray	QIAGEN GmbH	250098	n.a.
QIAcuity Plate Roller	QIAGEN GmbH	911105	n.a.
Vortex	Phoenix Instrument	–	VB4B016641 E27
Picus Electronic pipette, 1-Channel, 100–5000 µL	Sartorius Lab Instruments GmbH & Co. KG	LH-747101	15015144 E59
Picus Electronic pipette, 1-Channel, 50–1000 µL	Sartorius Lab Instruments GmbH & Co. KG	LH-747081	15017005 E67
Eppendorf Reference 2, 1-Channel, 2–20 µL	Eppendorf SE	4924000037	G26420D E16

Table 9a. Minerva Biolabs GmbH lab equipment (continued)

Equipment, brand	Manufacturer	Cat. no.	Equipment ID (internal/serial no.)
KingFisher Flex Cleaning System	Thermo Fisher Scientific, Inc.	5400620	711-85746 E156
Thermomixer	Eppendorf Vertrieb Deutschland GmbH	–	5382GL720102 E81
Eppendorf Reference 2, 1-Channel, 0.5–10 µL	Eppendorf Vertrieb Deutschland GmbH	4924000029	K12812G E85
CO2 incubator	Memmert GmbH & Co. KG	–	O718.0250 E99
Centrifuge	Eppendorf Vertrieb Deutschland GmbH	–	5427FR524310 E80
Vortex	VWR International GmbH	444-0004	170925031 E82
Pipettor	VWR International GmbH	–	82211313 E168
Inverted Microscope VT Series	Euromex Microscopen BV	–	800103 E170

Table 9b. QIAGEN GmbH Hilden lab equipment

Equipment, brand	Manufacturer	Cat. no.	Equipment ID (internal/serial no.)
Lamin Air Model 0.9	Holten	–	93785000 A
Finnpipette 1–5 mL	Labsystems	–	C59669
Eppendorf Reference 0.5–10 µL	Eppendorf	–	511123
PIPETMAN® 10–200 µL	Gilson	–	9856950
PIPETMAN 100–1000 µL	Gilson	–	T63109L

Table 9b. QIAGEN GmbH Hilden lab equipment (continued)

Equipment, brand	Manufacturer	Cat. no.	Equipment ID (internal/serial no.)
Vortex Gene2 Model G-560E	Scientific Industries	–	89385 2-90775 2-53935
EZ2® Connect	QIAGEN GmbH	9003210	PO 42 1044M P0322027L (Minerva Biolabs GmbH)
QIAcuity Eight	QIAGEN GmbH	911052	00095
QIAcuity Four	QIAGEN GmbH	911042	00100
Voyager Pipette 8 Channel electric 125 µL	Integra	4722	6009157
Viaflo electric Pipette 1 Channel 300 µL	Integra	4013	6004041
EVOLVE Manual Pipette 0.2–2 µL	Integra	3011	1075853
EVOLVE Manual Pipette 1–10 µL	Integra	3012	1110155
EVOLVE Manual Pipette 2–20 µL	Integra	3013	1074609
EVOLVE Manual Pipette 10–100 µL	Integra	3015	1099482
EVOLVE Manual Pipette 20–200 µL	Integra	3016	1075808 1108835
EVOLVE Manual Pipette 100–1000 µL	Integra	3018	1106097 1103136
Centrifuge Typ FVL 2400N Euro plug	Biosan	90.186.100	1075468 1109937
Minifuge (8er Strips)	Sarstedt	4722	01020224020103
Plate Fuge C2000-E	Benchmark Scientific	4013	81204104 E09180192

Table 10a. Minerva Biolabs GmbH reagents, materials, and critical labware

Article name	Manufacturer	Cat. no.	Lot no.
1-Propanol	Carl-Roth GmbH + Co. KG	9169.2	261300031
QIAcuity Nanoplate 26k 24-Well	QIAGEN GmbH	250001	5724100188 5724100147 5724100059 5754100073 5754100059 5754100185
QIAcuity OneStep® Advanced Probe Kit (5 mL)	QIAGEN GmbH	250132	175022670 175027119 175029708
Filter tip, 100 µL, transparent, Biosphere® plus, Refill	Sarstedt AG & Co.KG	70.3030.355	3050221 3051521
Filter tip, XL, 1000 µL, transparent, Biosphere plus, Refill	Sarstedt AG & Co.KG	70.3060.355	2054521 2054621
Filter tip, 300 µL, transparent, Biosphere plus	Sarstedt AG & Co.KG	70.3040.255	746321
Filter tip, 300 µL, transparent, Biosphere plus, Refill	Sarstedt AG & Co.KG	70.3040.355	2052321
PCR SingleCap 8er-SoftStrips 0.2 mL	Biozym Scientific GmbH	710970	22073 22423 23033
InnuPREP KFFLX Plate set 960 rxn	IST Innuscreen GmbH	845-KF-1296010	09052023 14062023
Pipette tip, 5 mL	Sarstedt AG & Co.KG	70.1183.102	16411
DNA LoBind Tubes, 1.5 mL	Eppendorf SE	022431021	L208302R
DNA LoBind Tubes, 5 mL	Eppendorf SE	0030108310	1183960K
DNA LoBind Tubes, 50 mL	Eppendorf SE	0030122232	I183238L

Table 10a. Minerva Biolabs GmbH reagents, materials, and critical labware (continued)

Article name	Manufacturer	Cat. no.	Lot no.
Serological pipette, 10 mL	Sarstedt AG & Co.KG	86.1254.001	2308E
Serological pipette, 25 mL	Sarstedt AG & Co.KG	86.1685.001	3150K
Serological pipette, 5 mL	Sarstedt AG & Co.KG	86.1253.001	3072E
Cell culture flask, T-175	Sarstedt AG & Co.KG	83.3912.002	3021221
Cell culture flask, T-75	Sarstedt AG & Co.KG	83.3911.002	1024121
Cell culture flask, T-25	Sarstedt AG & Co.KG	83.3910.002	902411

Table 10b. QIAGEN GmbH reagents, materials, and critical labware

Article name	Manufacturer	Cat. no.	Lot no.
50 mL Tubes	Sarstedt	62.547.254	3042521
DNA LoBind Tubes, 1.5 mL	Eppendorf SE	022431021	O 220320I
DNA LoBind Tubes, 5 mL	Eppendorf SE	0030108310	N 218294P
Griptips 12.5 µL	Integra	3415	99014390
Griptips 125 µL	Integra	3725	99031909 99030831
Griptips 300 µL	Integra	3435	99017286
Griptips 1250 µL	Integra	3445	99014646
96 er Platten	VWR	732-2387	13200
Plattenfolie	Sarstedt	95.1994	2012-04-GA
PCR 8er Strips	Sarstedt	72.991.002	4083121
5 mL Spitzen Finntip	Thermo	3725	99029651
QIAcuity Nanoplate 26k 24-Well	QIAGEN GmbH	250001	5754100185 5784100538

Table 10b. QIAGEN GmbH reagents, materials, and critical labware (continued)

Article name	Manufacturer	Cat. no.	Lot no.
QIAcuity Mycoplasma Quant Kit	QIAGEN GmbH	250261	77537 77835 78139
EZ1&2 Virus Mini Kit v2.0 (48)	QIAGEN GmbH	955134	181014237 181013231 181030202 181031690

Test Procedure

Based on the results of different proficiency tests, nucleic acid extraction prior to 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 nucleic acid extraction from the sample material. In this study, we used two nucleic acid extraction workflows: the Venor Mycoplasma Extraction Kit on the KingFisher Flex System and the EZ1&2 Virus Mini Kit v2.0 on the EZ2 Connect instrument.

Nucleic acid extraction

Mycoplasma extraction using the Venor Mycoplasma Extraction Kit

The nucleic acid extraction was carried out according to the instruction manual of the Venor Mycoplasma Extraction Kit on the KingFisher Flex System.

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

Preparing the 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, 3.3 or 33 mL, depending on kit size

- Wash Buffer 1, 1.4 or 14 mL, depending on kit size
- Wash Buffer 2, 1.4 or 14 mL, depending on kit size

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 min at ambient temperature.
5. Vortex and centrifuge.

Preparing the Lysis Buffer – IC Mix

Gently mix 250 µL Lysis Buffer and 2.5 µL QIAcuity Mycoplasma Quant Kit Internal Control per extraction.

Setup and Extraction

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

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. A volume of 20 µL Proteinase K solution was placed into the wells of the lysis plate. Subsequently, 250 µL sample was added, followed by 250 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 instrument using the QIAcuity Mycoplasma Quant Kit.

Mycoplasma extraction using the EZ2 Connect instrument

The extraction of mycoplasma nucleic acids was performed using the EZ1&2 Virus Mini Kit v2.0 on the EZ2 Connect instrument, following the *EZ1&2 Virus Mini Kit v2.0 – Mycoplasma Quick Start Protocol* (**www.qiagen.com/HB-3680**).

Preparing the solutions

- Reconstitute Carrier RNA (310 µg) in 310 µL AVE buffer to obtain a final concentration of 1 µg/µL.
- Reconstitute Mycoplasma Internal Control in 120 µL RNase-Free Water to achieve a final concentration of 6000 copies/µL.
- Prepare the Carrier RNA–Internal Control mix by combining 1 µL Carrier RNA (1 µg/µL), 2.5 µL Mycoplasma Internal Control (6000 copies/µL), and 56.5 µL AVE Buffer in a 1.5 mL tube.

Sample preparation

- Add 400 µL of sample to a 2.0 mL tube. If less than 400 µL is available, fill up to 400 µL with Buffer AVE.

Instrument setup and extraction

1. Turn on the EZ2 Connect instrument.
2. Select **Virus** on the Applications panel, then **Virus Mini Kit v2.0**.
3. Select the Mycoplasma protocol, then choose **400 µL sample** and **60 µL elution volume**.
4. Assign sample positions and IDs as required.
5. Load the EZ1&2 Virus reagent cartridges into the designated positions of the EZ2 Connect Cartridge Rack.
6. Place empty non-skirted 2 mL tubes into position 11 of the cartridges.
7. Prepare the EZ2 Connect Tip Rack:
    - Position A: 2.0 mL tube containing 400 µL sample
    - Position B: 1.5 mL tube with Carrier RNA–Internal Control mix
    - Position C: Tip holder with filter tip
    - Position D: 1.5 mL empty tube
8. Load the Cartridge Rack and Tip Rack into the instrument, remove tube caps, and start the run according to the instrument display instructions.
9. Upon completion, remove the elution tube containing purified nucleic acid from position D of the Tip Rack.
10. Discard the used cartridge and liquid waste. Optionally, follow on-screen instructions for UV decontamination of worktable surfaces.
11. Perform regular maintenance after each run.

The purified nucleic acids were directly used for downstream analytical procedures, such as quantification on the QIAcuity instrument using the QIAcuity Mycoplasma Quant Kit.

Analytical procedures

The detection of mycoplasma nucleic acids was carried out according to *QIAcuity Mycoplasma Quant Kit Handbook* (**www.qiagen.com/HB-3503**). In detail:

Rehydration of the Positive Control

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

PCR Master Mix Setup

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

Pipetting scheme

Table 12. Pipetting scheme

	For 1 reaction (µL)	For 25 reactions (µL)
OneStep Adv. Probe Master Mix, 4x	10	250
OneStep Adv. RT Mix, 100x	0.4	10
OneStep Enhancer GC	5	125
QIAcuity Mycoplasma Assay, 20x	2	50
RNase-free water	2.6	65

The master mix was dispensed into the wells of a standard PCR plate at a volume of 20 µL per well. Samples and controls were added to the respective wells according to the loading scheme. Control volume was adjusted to a volume of 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) to avoid cross-contamination. The pre-plate was gently mixed, and liquids were settled at the bottom by centrifugation. Reaction mixes were then transferred to the wells of the 26k 24-well nanoplate while avoiding bubbles.

Programming the QIAcuity dPCR instrument

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

Table 13. Cycling parameters

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

Table 14. Imaging parameters

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 (≤3 positive partition)	Negative (no positive partition)	PCR inhibition
Negative (≤3 positive partition)	Positive (>400* positive partitions)	Mollicutes negative

* The original value of 600 positive partitions was determined from a smaller dataset that excluded *Mycoplasma pneumoniae* and did not incorporate validation data from nucleic acid extraction using the EZ1&2 Virus Mini Kit v2.0.

A successful RT-PCR result without inhibition is indicated by a concentration of the internal control of 125 copies/µL ± 30% (20 µL template volume with 250 copies/µL), provided the Internal Control was added to the RT- PCR master mix (rehydrated in 120 µL at 6000 copies/µL, and diluted 1:24). Using the Internal Control as extraction control can lead to a different concentration of the Internal Control, depending on the amount used and elution volume. Processing the samples according to the protocol described here results in an expected concentration of 94 copies/µL using the Venor Mycoplasma Extraction Kit and 104 copies/µL using the EZ1&2 Virus Mini Kit v2.0 due to a difference in elution volumes and considering 100% recovery after nucleic acid extraction and a dPCR template volume of 20 µL. 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 outcome.

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, 2.5.0.1 / 3.1.1.0. 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 yellow channels.

Linearity and dynamic range

For the determination of the working range of the QIAcuity Mycoplasma Quant Kit, least squares linear regression of the data was performed to determine the slope of the regression

line. The acceptance criterion was that at least 4 consecutive dilutions had to show an 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 (15). To calculate the relative repeatability ($s_{\text{repeat, rel}}$) from the data collected from the working range section, the following calculations were applied.

$$CV\% = s_{\text{repeat, rel}} = \frac{\sqrt{MS_{\text{within run}}}}{\bar{c}_{\text{sample, meas}}}$$

Equation 1. Calculation of relative repeatability. Relative repeatability is represented by $s_{\text{repeat, rel}}$, within-run mean-of-squares calculated by one-way ANOVA is $MS_{\text{within run}}$, and the average measured sample copy number concentration over all runs for each dilution is represented by $\bar{c}_{\text{sample, meas}}$.

Limit of blank

Limit of blank was determined as the lowest tested sample concentration expected to be found in a maximum of 95% of replicates when testing no template containing samples (16). It was calculated based on the mean value and standard deviation of NTC and NEC samples using the following formula.

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

Equation 2. Calculation of the limit of blank (LOB). MV is the mean value, while SD signifies the standard deviation.

Limit of blank for the no template control (NTC) was determined as the highest tested sample concentration expected to be found in a maximum of 69/72 replicates when testing no template containing samples (95% confidence interval).

Limit of blank for the negative extraction control (NEC) was determined as the highest test sample concentration expected to be found in a maximum of 251/264 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 4 positive partitions were present in at least 23/24 replicates (95% confidence interval).

Specificity

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

Robustness

Robustness was evaluated by determining the recovery rate of the QIAcuity Mycoplasma Quant Kit 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. Robustness test conditions (Venor Mycoplasma Extraction Kit)

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

Table 16. Robustness test conditions (EZ1&2 Virus Mini Kit v2.0)

Mycoplasma species	Mycoplasma conc. (CFU/mL)	IC expected conc. (copies/µL)	Matrix
Mycoplasma orale	0 10 15 50	104	Cell suspension of 1 × 10^5 HEK293 cells/mL (DMEM + 10% FCS)
Mycoplasma orale	0 10 15 50	104	Cell suspension of 1 × 10^6 HEK293 cells/mL (DMEM +10% FCS)
Mycoplasma orale	0 10 15 50	104	Cell suspension of 1 × 10^7 HEK293 cells/mL (DMEM + 10% FCS)
Mycoplasma orale	0 10 15 50	104	FCS Xtra
Mycoplasma orale	0 10 15 50	104	RPMI 1640
Mycoplasma orale	0 10 15 50	104	Cryo of 1 × 10^6 CHO cells/mL (DMEM + 20% FCS + 10% DMSO)

Reporting requirements

The reports generated by the QIAcuity Software Suite (version 2.1.7.182, 2.5.0.1 / 3.1.1.0) are stored digitally. In the reports, 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 parameters linearity, dynamic range, accuracy, and lower quantification limit (LoQ) are not immediately relevant but offer useful information about the performance of the QIAcuity Mycoplasma Quant Kit.

Linearity and dynamic range

Procedure	3 mycoplasma culture replicates were tested at different dilutions in the range between 1 and 10,000 CFU/mL.
Acceptance criterion	R^2 value above 0.98
Results	R^2 value for all tested RT-dPCR and dPCR above 0.99

The regulations for the mycoplasma detection do not require the assessment of the linearity. Nevertheless, we show the linearity assessment of the QIAcuity Mycoplasma Quant Kit on a serial dilution ranging from 1–10,000 CFU/mL of three exemplary Mycoplasma cultures (*M. fermentans*, *M. gallisepticum*, and *M. hyorhinis*) in the presence of DMEM medium with 10% FCS. The least squares regression shows a high linear correlation between the mycoplasma concentration in the sample (CFU/mL) and the concentration measured by RT-dPCR and dPCR in copies/µL as shown in Table 17, Table 18, and Table 19. For the dPCR approach, the QIAcuity Mycoplasma Quant Kit was used but the RT enzyme was replaced with water and the RT step during cycling was skipped to detect only DNA. Linearity has only been assessed with the Venor Mycoplasma Extraction Kit.

Table 17. 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.	98.53	90.9	6.11	6.7%
	n.a.*				90.73			
	n.a.*				83.56			
1000	2878.3	2827.3	88.44	3.1%	9.32	9.0	0.36	4.0%
	2702.9				8.479			
	2900.7				9.094			
100	165.1	233.1	51.42	22.1%	0.482	0.7	0.14	21.2%
	244.8				0.662			
	289.4				0.822			
10	21.37	32.1	15.13	47.1%	0	0.1	0.08	141.4%
	53.52				0			
	21.48				0.16			
1	0	1.3	1.71	132.8%	0	0.0	0.00	n.a.
	3.714				0			
	0.159				0			
R^2 value (from 1000 to 1 CFU/mL)	–	0.9997	–	–	–	0.9994	–	–

* Well oversaturated.

Table 18. 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	13755.3	237.87	1.7%	27.83	29.0	1.79	6.2%
	13434				27.71			
	14002.3				31.57			
1000	1143.2	1205.1	104.70	8.7%	2.628	2.5	0.25	10.1%
	1119.5				2.116			
	1352.5				2.657			
100	147.2	130.0	27.16	20.9%	0.376	0.3	0.07	22.6%
	91.63				0.277			
	151.1				0.217			
10	25.38	13.8	8.36	60.7%	0.108	0.1	0.04	40.3%
	6.001				0.055			
	9.96				0.162			
1	0.052	0.1	0.09	102.5%	0.000	0.0	0.00	n.a.
	0.212				0.000			
	0.000				0.000			
R^2 value	–	0.9998	–	–	–	0.9997	–	–

Table 19. Linearity – *M. hyorhinis* diluted in DMEM + 10% FCS

Sample conc. (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	7456.7	254.79	3.4%	32.1	28.2	6.11	21.7%
	7663.9				27.36			
	7097.8				25.01			
1000	827.4	805.8	31.73	3.9%	3.057	2.5	0.42	17.0%
	829				2.173			
	760.9				2.167			
100	80.22	86.9	10.06	11.6%	0.322	0.3	0.00	0.6%
	79.34				n.a.*			
	101.1				0.326			
10	5.001	10.4	3.83	36.8%	0	0.0	0.03	70.7%
	13.38				0.055			
	12.84				0.054			
1	0.000	0.0	0.05	141.4%	0	0.0	0.00	n.a.
	0.000				0			
	0.107				0			
R^2 value	–	0.9999	–	–	–	0.9998	–	–

* Plate defect.

Precision

Procedure	Internal Control data obtained by limit of detection determination. 6 biological replicates per mycoplasma concentration were tested on 4 different days resulting in 24 data points per tested mycoplasma concentration.
Acceptance criterion	CV ≤20%
Results	CV range for Internal Control. The original acceptance criterion (≤10%) was established using a smaller dataset limited to the Venor Mycoplasma Extraction Kit and did not include *Mycoplasma pneumoniae*. Expanded validation now covers multiple operators, sample prep kits, instruments, reagent lots, reflecting more realistic operational variability. RNA targets analyzed by digital PCR inherently show higher variability due to reverse transcription and RNA integrity factors. Therefore, updating the acceptance criterion to CV ≤20% aligns with best practices, reflects true operational performance, and maintains assay integrity for its intended purpose.

The precision was calculated using the Mycoplasma Internal Control data obtained in the limit of detection determination. Relative repeatability expressed as CV% was calculated as described in the "Precision" section, using one-way ANOVA (15). Exemplary data for the WHO standard and *M. fermentans* are shown in Tables 20–23. Data for all species are provided in section "Limit of detection", Tables 26–47.

Overall, the CV% range for the quantification of the Internal Control is below 12.5% the Venor Mycoplasma Extraction Kit. The CV% range for the quantification of the Internal Control is below 17% for the EZ1&2 Virus Mini Kit v2.0.

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 differences in gene expression and which are not relevant for a qualitative assessment of Mycoplasma presence.

Table 20. WHO international standard (Venor Mycoplasma Extraction Kit)

IU/mL	40 IU/mL	10 IU/mL	2.5 IU/mL
IC mean value (copies/µL)	61.71	61.50	59.85
IC SD (copies/µL)	4.37	4.24	3.90
IC CV (%)	7.08	6.90	6.52

Table 21. *M. fermentans* (Venor Mycoplasma Extraction Kit)

CFU/mL	20 CFU/mL	10 CFU/mL	5 CFU/mL
IC mean value (copies/µL)	58.91	57.66	58.77
IC SD (copies/µL)	2.68	2.74	3.77
IC CV (%)	4.55	4.75	6.41

Table 22. WHO international standard (EZ1&2 Virus Mini Kit v2.0)

IU/mL	40 IU/mL	10 IU/mL	2.5 IU/mL
IC mean value (copies/µL)	46.84	47.24	44.65
IC SD (copies/µL)	3.79	3.04	5.03
IC CV (%)	8.08	6.43	11.27

Table 23. *M. fermentans* (EZ1&2 Virus Mini Kit v2.0)

CFU/mL	20 CFU/mL	10 CFU/mL	5 CFU/mL
IC mean value (copies/µL)	43.73	43.08	42.41
IC SD (copies/µL)	4.52	5.06	4.91
IC CV (%)	10.33	11.74	11.57

Limit of Blank

The employed method is used to obtain a qualitative result interpretation. To be able to detect low levels of mycoplasma 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	24 NTC replicates were analyzed on 3 different days resulting in 72 data points. Additionally, 6 eluates from 4 different days resulting from the extraction of 0 CFU/mL from 10 Mollicutes listed in Table 3, and WHO standard listed in Table 4 were tested each resulting in 24 NEC replicates per species and 264 data points in total. The LOB was calculated based on the mean value and standard deviation of NTC and NEC samples using Equation 2.
Acceptance criterion	For No Template Control (NTC) samples, the acceptance criterion requires that no more than 3 out of 72 replicates exhibit a concentration above the calculated Limit of Blank for NTC (LOB_{NTC}). This ensures that at least 69 out of 72 replicates, corresponding to approximately 95% confidence, are classified as mycoplasma negative when testing samples without template material. For No Extraction Control (NEC) samples, the acceptance criterion stipulates that no more than 13 out of 264 replicates show a concentration above the calculated Limit of Blank for NEC (LOB_{NEC}). Consequently, at least 251 out of 264 replicates, also representing a 95% confidence level, must be considered mycoplasma negative when evaluating samples without template content.
Results **NTC**	LOB_{NTC} = 0.057 copies/µL (1 positive partition) 2 out of 72 NTC wells contained 2 or more positive partitions.
Results **Venor Mycoplasma Extraction**	LOB_{NEC} = 0.170 copies/µL (3 positive partitions) 9 out of 264 NEC wells contained 4 or more positive partitions.
Results **EZ1&2 Virus Mini Kit v2.0**	LOB_{NEC} =0.165 copies/µL (3 positive partitions) 10 out of 264 NEC wells contained 4 or more positive partitions.

Limit of detection

The employed method is used to obtain a qualitative result interpretation. 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	The Mollicutes broth aliquots stored at -80°C were adjusted to a suspension with 160 CFU/mL by diluting accordingly in DMEM medium containing 10% (v/v) FBS. Quantification and aliquot preparation as described in section Microorganisms and eukaryotic material, below Table 3. Subsequently, dilutions of 80, 40, 20, 10, 5, and 0 CFU/mL were prepared as twofold dilution series in DMEM medium containing 10% (v/v) FBS. The WHO International Standard for Mycoplasma DNA was prepared as a dilution series of 640, 160, 40, 10, 2.5, 0.625, and 0 IU/mL in DMEM medium containing 10% (v/v) FBS. In total, four individual dilution series were prepared on 4 different days and each dilution was analyzed in 6 replicates resulting in 24 data points per concentration and Mollicutes species.
Acceptance criterion	23 of 24 samples containing 10 CFU/mL must be positive after subtraction of LOB_{NEC} (considered as hit) for all species and the WHO standard (WHO standard expressed as IU/mL, so 10 IU/mL have to be detected).
Results	All mycoplasma samples containing 10 CFU/mL and the sample containing the WHO International Standard at 10 IU/mL were tested mycoplasma positive.

Table 24. LOD of the QIAcuity Mycoplasma Quant Kit for different mollicutes (Venor Mycoplasma Extraction Kit)

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 pneumoniae	10 CFU/mL
Mycoplasma salivarium	10 CFU/mL
Mycoplasma synoviae	10 CFU/mL
Spiroplasma citri	10 CFU/mL
WHO International Standard	10 IU/mL

Table 25. LOD of the QIAcuity Mycoplasma Quant Kit for different mollicutes (EZ1&2 Virus Mini Kit v2.0)

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

Table 26. *Mycoplasma fermentans* (Venor Mycoplasma Extraction Kit)

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 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
Mycoplasma mean value (copies/µL)	68.65				32.19				15.60			
Mycoplasma SD (copies/µL)	17.50				13.07				9.09			
Overall hit rate	24/24				24/24				24/24			
IC mean value (copies/µL)	58.91				57.66				58.77			
IC SD (copies/µL)	2.68				2.74				3.77			
IC CV (%)	4.55				4.75				6.41			

Table 27. *Mycoplasma orale* (Venor Mycoplasma Extraction Kit)

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 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
Mycoplasma mean value (copies/µL)	15.56				6.11				2.82			
Mycoplasma SD (copies/µL)	9.68				5.21				2.65			
Overall hit rate	24/24				23/24				24/24			
IC mean value (copies/µL)	59.28				59.21				60.24			
IC SD (copies/µL)	2.90				4.03				2.43			
IC CV (%)	4.90				6.80				4.03			

Table 28. *Mycoplasma gallisepticum* (Venor Mycoplasma Extraction Kit)

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
Hit rate	6/6	6/6	6/6	6/6	5/6	6/6	6/6	6/6	6/6	6/6	5/6	4/6
Mycoplasma mean value (copies/µL)	34.31				16.56				8.94			
Mycoplasma SD (copies/µL)	14.55				9.28				7.22			
Overall hit rate	24/24				23/24				21/24			
IC mean value (copies/µL)	64.29				65.44				63.66			
IC SD (copies/µL)	3.54				3.65				3.87			
IC CV (%)	5.51				5.58				6.07			

Table 29. *Acholeplasma laidlawii* (Venor Mycoplasma Extraction Kit)

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
Hit rate	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	5/6	3/6	4/6	5/6
Mycoplasma mean value (copies/µL)	66.42				41.52				12.88			
Mycoplasma SD (copies/µL)	47.59				38.87				15.08			
Overall hit rate	24/24				24/24				17/24			
IC mean value (copies/µL)	63.33				63.18				64.11			
IC SD (copies/µL)	6.04				5.51				5.24			
IC CV (%)	9.54				8.72				8.17			

Table 30. *Mycoplasma arginini* (Venor Mycoplasma Extraction Kit)

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
Hit rate	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	5/6	6/6	6/6
Mycoplasma mean value (copies/µL)	12.80				5.99				3.21			
Mycoplasma SD (copies/µL)	5.54				3.00				2.02			
Overall hit rate	24/24				24/24				23/24			
IC mean value (copies/µL)	63.02				62.16				63.22			
IC SD (copies/µL)	5.49				4.52				4.48			
IC CV (%)	8.70				7.27				7.09			

Table 31. *Mycoplasma hyorhinis* (Venor Mycoplasma Extraction Kit)

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
Hit rate	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	5/6	6/6	5/6	5/6
Mycoplasma mean value (copies/µL)	29.38				12.05				7.10			
Mycoplasma SD (copies/µL)	13.23				7.72				5.83			
Overall hit rate	24/24				24/24				21/24			
IC mean value (copies/µL)	58.71				60.32				60.15			
IC SD (copies/µL)	3.51				4.02				3.56			
IC CV (%)	5.98				6.67				5.92			

Table 32. *Mycoplasma pneumoniae* (Venor Mycoplasma Extraction Kit)

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
Hit rate	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	4/6	4/6
Mycoplasma mean value (copies/µL)	18.77				8.26				4.31			
Mycoplasma SD (copies/µL)	10.83				5.12				3.36			
Overall hit rate	24/24				24/24				20/24			
IC mean value (copies/µL)	62.49				61.70				62.27			
IC SD (copies/µL)	6.81				7.28				7.76			
IC CV (%)	10.89				11.80				12.47			

Table 33. *Mycoplasma salivarium* (Venor Mycoplasma Extraction Kit)

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 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	3/6
Mycoplasma mean value (copies/µL)	27.65				12.52				9.31			
Mycoplasma SD (copies/µL)	14.30				10.02				21.29			
Overall hit rate	24/24				23/24				16/24			
IC mean value (copies/µL)	63.35				63.93				65.22			
IC SD (copies/µL)	3.01				3.00				2.45			
IC CV (%)	4.75				4.61				3.76			

Table 34. *Mycoplasma synoviae* (Venor Mycoplasma Extraction Kit)

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 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	5/6
Mycoplasma mean value (copies/µL)	24.10				14.39				4.98			
Mycoplasma SD (copies/µL)	17.53				12.67				7.33			
Overall hit rate	23/24				23/24				13/24			
IC mean value (copies/µL)	58.38				58.95				59.16			
IC SD (copies/µL)	4.22				2.70				2.84			
IC CV (%)	7.23				4.57				4.80			

Table 35. *Spiroplasma citri* (Venor Mycoplasma Extraction Kit)

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
Hit rate	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	5/6	6/6	6/6	5/6
Mycoplasma mean value (copies/µL)	14.86				5.45				3.70			
Mycoplasma SD (copies/µL)	8.00				3.25				2.50			
Overall hit rate	24/24				24/24				22/24			
IC mean value (copies/µL)	59.24				59.94				59.91			
IC SD (copies/µL)	4.51				4.24				4.12			
IC CV (%)	7.62				7.07				6.87			

Table 36. WHO International Standard for Mycoplasma DNA (Venor Mycoplasma Extraction Kit)

	40 IU/mL				10 IU/mL				2.5 IU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
Hit rate	6/6	6/6	6/6	6/6	6/6	6/6	6/6	5/6	1/6	2/6	2/6	1/6
Mycoplasma mean value (copies/µL)	6.14				2.37				0.13			
Mycoplasma SD (copies/µL)	5.93				3.52				0.12			
Overall hit rate	24/24				23/24				6/24			
IC mean value (copies/µL)	61.71				61.50				59.85			
IC SD (copies/µL)	4.37				4.24				3.90			
IC CV (%)	7.08				6.90				6.52			

Table 37. *Mycoplasma fermentans* (EZ1&2 Virus Mini Kit v2.0)

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 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
Mycoplasma mean value (copies/µL)	55.43				26.42				14.74			
Mycoplasma SD (copies/µL)	23.94				12.24				8.14			
Overall hit rate	24/24				24/24				24/24			
IC mean value (copies/µL)	43.73				43.08				42.41			
IC SD (copies/µL)	4.52				5.06				4.91			
IC CV (%)	10.3				11.7				11.6			

Table 38. *Mycoplasma orale* (EZ1&2 Virus Mini Kit v2.0)

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 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
Mycoplasma mean value (copies/µL)	57.98				28.05				16.81			
Mycoplasma SD (copies/µL)	25.35				22.51				12.67			
Overall hit rate	24/24				24/24				24/24			
IC mean value (copies/µL)	45.86				46.12				42.83			
IC SD (copies/µL)	6.36				3.95				4.66			
IC CV (%)	13.9				8.6				10.9			

Table 39. *Mycoplasma gallisepticum* (EZ1&2 Virus Mini Kit v2.0)

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 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
Mycoplasma mean value (copies/µL)	43.13				18.30				8.83			
Mycoplasma SD (copies/µL)	16.83				9.58				6.63			
Overall hit rate	24/24				24/24				24/24			
IC mean value (copies/µL)	44.83				43.38				42.81			
IC SD (copies/µL)	2.82				3.51				5.28			
IC CV (%)	6.3				8.1				12.3			

Table 40. *Acholeplasma laidlawii* (EZ1&2 Virus Mini Kit v2.0)

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
Hit rate	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	5/6	6/6	6/6	6/6
Mycoplasma mean value (copies/µL)	72.59				30.98				13.48			
Mycoplasma SD (copies/µL)	51.17				17.45				12.17			
Overall hit rate	24/24				24/24				23/24			
IC mean value (copies/µL)	31.45				30.16				29.72			
IC SD (copies/µL)	4.01				4.38				3.03			
IC CV (%)	12.8				14.5				10.2			

Table 41. *Mycoplasma arginini* (EZ1&2 Virus Mini Kit v2.0)

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 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
Mycoplasma mean value (copies/µL)	53.17				24.09				11.34			
Mycoplasma SD (copies/µL)	22.32				11.20				6.85			
Overall hit rate	24/24				24/24				24/24			
IC mean value (copies/µL)	45.80				44.16				41.80			
IC SD (copies/µL)	5.46				4.32				4.26			
IC CV (%)	11.9				9.8				10.2			

Table 42. *Mycoplasma hyorhinis* (EZ1&2 Virus Mini Kit v2.0)

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
Hit rate	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	5/6	6/6	6/6	6/6
Mycoplasma mean value (copies/µL)	36.66				29.15				9.58			
Mycoplasma SD (copies/µL)	20.34				17.45				8.34			
Overall hit rate	24/24				24/24				23/24			
IC mean value (copies/µL)	40.39				39.49				40.57			
IC SD (copies/µL)	3.98				3.89				3.44			
IC CV (%)	9.8				9.8				8.5			

Table 43. *Mycoplasma pneumoniae* (EZ1&2 Virus Mini Kit v2.0)

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
Hit rate	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	5/6	6/6	6/6
Mycoplasma mean value (copies/µL)	12.86				6.93				3.03			
Mycoplasma SD (copies/µL)	6.08				4.17				1.96			
Overall hit rate	24/24				24/24				23/24			
IC mean value (copies/µL)	37.73				36.76				37.06			
IC SD (copies/µL)	2.73				3.21				2.76			
IC CV (%)	7.2				8.7				7.5			

Table 44. *Mycoplasma salivarium* (EZ1&2 Virus Mini Kit v2.0)

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
Hit rate	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	4/6	5/6	6/6	6/6
Mycoplasma mean value (copies/µL)	44.34				18.59				15.64			
Mycoplasma SD (copies/µL)	25.13				11.50				20.64			
Overall hit rate	24/24				24/24				21/24			
IC mean value (copies/µL)	38.62				39.63				39.84			
IC SD (copies/µL)	6.35				7.00				6.10			
IC CV (%)	16.4				17.7				15.3			

Table 45. *Mycoplasma synoviae* (EZ1&2 Virus Mini Kit v2.0)

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
Hit rate	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	5/6	4/6	6/6	6/6
Mycoplasma mean value (copies/µL)	37.34				21.03				10.14			
Mycoplasma SD (copies/µL)	28.10				21.37				14.52			
Overall hit rate	24/24				24/24				21/24			
IC mean value (copies/µL)	40.37				39.54				40.56			
IC SD (copies/µL)	4.33				3.79				4.59			
IC CV (%)	10.7				9.6				11.3			

Table 46. *Spiroplasma citri* (EZ1&2 Virus Mini Kit v2.0)

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 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
Mycoplasma mean value (copies/µL)	15.80				10.03				4.71			
Mycoplasma SD (copies/µL)	4.51				3.03				1.60			
Overall hit rate	24/24				24/24				24/24			
IC mean value (copies/µL)	39.90				37.20				39.65			
IC SD (copies/µL)	3.80				4.57				3.28			
IC CV (%)	9.5				12.3				8.3			

Table 47. WHO International Standard for Mycoplasma DNA (EZ1&2 Virus Mini Kit v2.0)

	40 IU/mL				10 IU/mL				2.5 IU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
Hit rate	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	4/6	1/6	4/6	2/6
Mycoplasma mean value (copies/µL)	10.47				1.54				1.78			
Mycoplasma SD (copies/µL)	6.90				1.64				6.12			
Overall hit rate	24/24				24/24				11/24			
IC mean value (copies/µL)	46.84				47.24				44.65			
IC SD (copies/µL)	3.79				3.04				5.03			
IC CV (%)	8.1				6.4				11.3			

Specificity

Specificity, as requested by *ICH* Q2B, *EP* 2.6.7, *EP* 2.6.21, *USP* <63>, and *JP* G3-14-170, is shown by in silico sequence alignment of primers and probe as well as by wet lab experiments on cross-reactivity with sample matrices, unrelated bacteria, and standard eukaryotic cell lines.

Sequence alignment

Procedure	Mollicutes primer and probe sequences were aligned with a genomic database. This provides additional information for species not available for testing. The alignment was performed using TestPrime (**arb-silva.de/search/testprime/**) and the SILVA ribosomal RNA database project (**arb-silva.de/**) in version SILVA SSU database 138.1 as of August 27, 2020. Subset SSU Ref NR 99 (**arb-silva.de/documentation/release-1381/**).
Acceptance criterion	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.
Results	At least 127 Mollicutes species are putatively detectable based on sequence alignment.

Table 48. Detectable Mollicutes species based on primers and probe sequence 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

Table 48. Detectable Mollicutes species based on primers and probe sequence alignment (continued)

	Primer/Probe mismatches		
Species (name in database)	**Forward Primer**	**Probe**	**Reverse Primer**
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

Table 48. Detectable Mollicutes species based on primers and probe sequence alignment (continued)

	Primer/Probe mismatches		
Species (name in database)	**Forward Primer**	**Probe**	**Reverse Primer**
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

Table 48. Detectable Mollicutes species based on primers and probe sequence alignment (continued)

	Primer/Probe mismatches		
Species (name in database)	**Forward Primer**	**Probe**	**Reverse Primer**
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

Table 48. Detectable Mollicutes species based on primers and probe sequence alignment (continued)

	Primer/Probe mismatches		
Species (name in database)	**Forward Primer**	**Probe**	**Reverse Primer**
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

Table 48. Detectable Mollicutes species based on primers and probe sequence alignment (continued)

	Primer/Probe mismatches		
Species (name in database)	**Forward Primer**	**Probe**	**Reverse Primer**
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

Table 48. Detectable Mollicutes species based on primers and probe sequence alignment (continued)

	Primer/Probe mismatches		
Species (name in database)	**Forward Primer**	**Probe**	**Reverse Primer**
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	Testing the media components according to Table 2 without mycoplasma background. The internal amplification control was added to the sample matrix as extraction control.
Acceptance criterion	Negative result of all tested samples without mycoplasma. Positive result of all tested samples containing the positive control (PC).
Results	All NEC samples were considered mycoplasma negative. All PC samples were tested positive for mycoplasma.

Table 49. Matrix cross reactivity (Venor Mycoplasma Extraction Kit)

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	0.05	1	23.02	Negative
PC	105.1	1898	0.00	Positive

Table 50. Matrix cross reactivity (EZ1&2 Virus Mini Kit v2.0)

Sample Matrix	Mycoplasma mean concentration (copies/µL)	Mycoplasma mean positive partitions	IC mean concentration (copies/µL)	Result
FBS Xtra	0.00	0	35.17	Negative
1 x 10^6 CHO cells/mL in DMEM + 20% FBS + 10% DMSO	0.11	2	46.81	Negative
RPMI	0.00	0	36.74	Negative
1 x 10^7 HEK293 cells/mL in DMEM + 10% FBS	0.00	0	50.87	Negative
PC	60.31	1086	0.00	Positive

Cross-reactivity

Procedure	All non-mollicutes bacteria listed in Table 5 were tested for mycoplasma presence at 3 different concentrations as singlet, resulting in at least 3 data points for every bacterial species. All eukaryotic cells listed in Table 6 were tested for mycoplasma presence in triplicates. The RT-PCR reaction was spiked with 1 µL of IC. The amplification products of samples tested positive were subsequently analyzed by sequencing.
Acceptance criterion	Negative result of all tested samples. Positive result of all tested samples containing the positive control (PC).
Results	All samples were tested mycoplasma negative. All PC samples were tested positive for mycoplasma.

Table 51. Microbial strains cross reactivity (Venor Mycoplasma Extraction Kit)

Sample matrix	Spike conc. (CFU/mL)	Mycoplasma mean conc. (copies/µL)	Mycoplasma mean positive partitions	IC mean conc. (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

Table 51. Microbial strains cross reactivity (Venor Mycoplasma Extraction Kit) (continued)

Sample matrix	Spike conc. (CFU/mL)	Mycoplasma mean conc. (copies/µL)	Mycoplasma mean positive partitions	IC mean conc. (copies/µL)	Result
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 52. Cross-reactivity with eukaryotic cells (Venor Mycoplasma Extraction Kit)

Sample matrix	Spike conc. (cells/mL)	Mycoplasma mean conc. (copies/µL)	Mycoplasma mean positive partitions	IC mean conc. (copies/µL)	Result
Vero	1×10^6	0.02	1	11.70	Negative
HL-60 cells	1×10^6	0.02	1	12.70	Negative
CHO-K1*	1×10^7	0.05	1	23.02	Negative
	1×10^6	0.05	1	40.00	Negative
	1×10^5	0.00	0	46.86	Negative
HEK293*	1×10^6	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.

Table 53. Cross-reactivity with eukaryotic cells (EZ1&2 Virus Mini Kit v2.0)

Sample matrix	Spike concentration (cells/mL)	Mycoplasma mean concentration (copies/µL)	Mycoplasma mean positive partitions	IC mean concentration (copies/µL)	Result
CHO	1×10^6	0.11	2	46.81	Negative
HEK293*	1×10^7	0.00	0	50.87	Negative
	1×10^6	0.00	0	49.38	Negative
	1×10^5	0.00	0	40.30	Negative
NTC	–	0.00	0	0.00	Negative
PC	–	60.31	1086	0.00	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	Testing of 3 different *Mycoplasma orale* concentrations (10, 15, 50 CFU/mL) in duplicates and a sample without mycoplasma background (0 CFU/mL) using the media components according to Table 15. The internal amplification control was added to the sample matrix to serve as an extraction control.
Acceptance criterion	Positive result for all tested samples containing mycoplasma. Negative result of all tested samples without mycoplasma.
Results	All mycoplasma containing samples were tested positive for mycoplasma and mycoplasma-free samples were tested mycoplasma negative.

Table 54. Robustness using different matrices (Venor Mycoplasma Extraction Kit)

Sample Matrix	*M. orale* spike conc. (CFU/mL)	Mycoplasma mean conc. (copies/µL)	Mycoplasma mean positive partitions	IC mean conc. (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

Table 54. Robustness using different matrices (Venor Mycoplasma Extraction Kit) (continued)

Sample Matrix	*M. orale* spike conc. (CFU/mL)	Mycoplasma mean conc. (copies/µL)	Mycoplasma mean positive partitions	IC mean conc. (copies/µL)	Result
1 x 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 x 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	0.00	0	46.86	Negative
1 x 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 x 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

Table 55. Robustness using different matrices (EZ1&2 Virus Mini Kit v2.0)

Sample Matrix	*M. orale* spike conc. (CFU/mL)	Mycoplasma mean conc. (copies/µL)	Mycoplasma mean positive partitions	IC mean conc. (copies/µL)	Result
FBS Xtra	50	38.86	733	33.26	Positive
	15	29.73	547	35.16	Positive
	10	27.76	509	37.56	Positive
	0	0.00	0	35.17	Negative
1×10^6 CHO cells/mL in DMEM + 20% FBS + 10% DMSO	50	160.09	2866	49.13	Positive
	15	55.03	1012	52.61	Positive
	10	17.94	334	49.55	Positive
	0	0.11	2	46.81	Negative
RPMI	50	40.04	742	43.61	Positive
	15	5.43	100	35.52	Positive
	10	5.46	102	40.45	Positive
	0	0.00	0	36.74	Negative
1×10^5 HEK293 cells/mL in DMEM + 10% FBS	50	69.41	1260	53.58	Positive
	15	10.53	195	47.28	Positive
	10	9.12	169	54.44	Positive
	0	0.00	0	50.87	Negative
1×10^6 HEK293 cells/mL in DMEM + 10% FBS	50	42.75	790	49.11	Positive
	15	22.23	399	48.91	Positive
	10	17.69	324	45.49	Positive
	0	0.00	0	49.38	Negative

Table 55. Robustness using different matrices (EZ1&2 Virus Mini Kit v2.0) (continued)

Sample Matrix	*M. orale* spike conc. (CFU/mL)	Mycoplasma mean conc. (copies/µL)	Mycoplasma mean positive partitions	IC mean conc. (copies/µL)	Result
1×10^7 CHO-K1 cells/mL in DMEM + 10% FBS	50	15.16	289	15.35	Positive
	15	1.58	29	30.61	Positive
	10	0.81	16	14.00	Positive
	0	0.00	0	40.30	Negative
PC	–	60.31	1086	0.00	Positive
NTC	–	0.00	0	0.00	Negative

Conclusion

The QIAcuity Mycoplasma Quant Kit was validated in accordance with the validation protocol presented in this report ("Test Procedure" on page 17). The validation report describes the test procedure itself and expected variations resulting 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 testing downstream of a suitable total nucleic acid extraction method. Two nucleic acid extraction kits were evaluated in the validation study: the EZ1&2 Virus Mini Kit v2.0, which can be used for automated sample processing on the EZ2 Connect instrument, and the Venor Mycoplasma Extraction Kit, which supports both manual and automated workflows. The QIAcuity Mycoplasma Quant Kit showed higher sensitivity for mycoplasma detection when paired with the EZ1&2 Virus Mini Kit v2.0, achieving a lower limit of detection (LOD). It was able to detect a broader range of mycoplasma species at concentrations as low as 5 CFU/mL. Any other suitable total nucleic acid extraction kit can be used instead but needs to be fully validated by the user.

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* 18, 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 criterion 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 (extraction and RT-dPCR control), with cp/µL 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)

Irreversibly inactivated 10 CFU/mL standards for all 10 mycoplasma species mentioned in *Ph. Eur.*, *USP*, and *JP* are offered as separate kits by the manufacturer. These allow in-house validation of the QIAcutiy Mycoplasma Quant Kit by the user without the risk of mycoplasma contamination. The mycoplasma species described by the *Ph. Eur.*, *USP*, and *JP* are *Mycoplasma arginini*, *Mycoplasma orale*, *Mycoplasma gallisepticum*, *Mycoplasma pneumoniae*, *Mycoplasma synoviae*, *Mycoplasma fermentans*, *Mycoplasma hyorhinis*, *Acholeplasma laidlawii*, *Spiroplasma citri*, and *Mycoplasma salivarium*.

The study provided detailed information on the workflow performance:

- Working Range: Between 1 CFU/mL to 10,000 CFU/mL the lowest linear regression had an R^2 of 0.9997.
- Limit of blank: The lowest detected concentration of mycoplasma expected to be found when testing NTCs was 0.057 cp/µL (1 positive partition). When testing NECs it was 0.17 (Venor) and 0.165 (EZ2) cp/µL, both with 3 positive partitions.
- Limit of detection: The lowest detectable concentration of mycoplasma was 5 or 10 CFU/mL depending on the species, and 10 IU/mL for the WHO International Standard.
- Precision: Relative repeatability of the internal control in the studied workflow was ≤20% for all species tested. The relative repeatability of the mycoplasma nucleic acid quantification was higher due to differences in gene expression levels and is not required for qualitative mycoplasma testing.

- Specificity: At least 127 Mollicutes species are putatively detected positive based on an in silico analysis. No cross-reactivity was found with the wet-lab 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 in combination with the Venor Mycoplasma Extraction Kit or EZ1&2 Virus Mini Kit v2.0 Kit. In accordance to the requirements of the *Ph. Eur.* 11, chapter 2.6.*7*, the *USP* 41, chapter 63, and the *JP 18*, chapter G3-14-170, the validation described here needs to be verified by the user using their sample matrix.

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Ordering Information

Product	Contents	Cat. no.
QIAcuity Mycoplasma Quant Kit	OneStep Advanced Probe Master Mix, OneStep Advanced RT Mix, 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
EZ1&2 Virus Mini Kit v2.0 (48)	For 48 preps: Reagent Cartridges (Virus Mini v2.0), Disposable Filter-Tips, Disposable Tip-Holders, Sample Tubes (2 ml), Elution Tubes (1.5 ml), Buffer AVE, carrier RNA	955134
QIAcuity Nanoplate 26K 24-well (10)	10 QIAcuity Nanoplates 26K with 24 wells, 11 Nanoplate Seals	250001

Product	Contents	Cat. no.
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 included.	911020
QIAcuity Four, 5plex 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, 5plex 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
EZ2 Connect	Benchtop instrument for automated isolation of nucleic acids from up to 24 samples in parallel, using sealed prefilled cartridges; includes 1-year warranty on parts and labor	9003210
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

Product	Contents	Cat. no.
Cell and Gene Therapy Lentivirus Lysis Kit (100)	For the lysis of lentivirus (LV). The kit includes: DNase I, RDD buffer, Proteinase K, CGT Dilution Buffer, Nuclease-free Water, CGT PBS buffer, enough to process 100 LV samples	250323
Cell and Gene Therapy Lentivirus Lysis Kit (1000)	For the lysis of lentivirus (LV). The kit includes: DNase I, RDD buffer, Proteinase K, CGT Dilution Buffer, Nuclease-free Water, CGT PBS buffer, enough to process 100 LV samples	250324
QIAcuity Residual DNA Quantification Kits and Standards	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 × 12 µL reactions (20x): GFP, ITR2/5, Sv40 promoter, AMP resistance, and other targets	250230–250256, 250300–250321
QIAcuity RCL Quant Kit	This kit includes: QIAcuity MasterMix, QN Internal Control DNA dPCR, QN IC Probe Assay, VSV-G Assay, Positive Control, and RNase-free water	250322

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Document Revision History

Date	Description
10/2023	Initial release
12/2025	EZ1&2 Virus Mini Kit v2.0 (48) materials and protocol on the EZ2 Connect system included accompanied by all relevant validation data. Additionally, minor adaptations to the text were done.