December 2023

QIAcuity™ Mycoplasma Quant Kit Handbook

For determination of Mycoplasma using the QIAcuity dPCR system

Sample to Insight

Contents

Kit Contents

QIAcuity Mycoplasma Quant Kit Catalog number Number of reactions	250261 96*	Cap color
4x OneStep Advanced Probe Master Mix	1 x 1 mL	Red
100x OneStep Advanced RT Mix	1 x 0.045 mL	Purple
OneStep Enhancer GC	1 x 1 mL	Yellow
20x QIAcuity Mycoplasma Assay	1 x 0.2 mL	Blue
QIAcuity Mycoplasma Internal Control, lyophilized	2 vials	Orange
QIAcuity Mycoplasma Positive Control, lyophilized	1 vial	Green
RNase-Free Water	2 x 1.9 mL	Neutral

* The number of reactions is calculated based on 40 µL QIAcuity dPCR reactions.

Mycoplasma Standard CFU Kit Catalog number Volume of Standard	250262–250271* 3 mL**	Cap color
QIAcuity Mycoplasma Std. CFU, lyophilized	3 vials	Black
QIAcuity Mycoplasma Negative Control, lyophilized	1 vial	Purple

* All available Mycoplasma 10 CFU Standard Kits are listed below.
** The volume of standard is calculated based on recommended reconstitution with 1 mL matrix leading to a final concentration of 10 CFU/mL.

Mycoplasma Standard CFU Kits	Catalog number
Mycoplasma arginini Standard CFU Kit	250262
Mycoplasma orale Standard CFU Kit	250263
Mycoplasma gallisepticum Standard CFU Kit	250264
Mycoplasma pneumoniae Standard CFU Kit	250265
Mycoplasma synoviae Standard CFU Kit	250266
Mycoplasma fermentans Standard CFU Kit	250267
Mycoplasma hyorhinis Standard CFU Kit	250268
Acholeplasma laidlawii Standard CFU Kit	250269
Spiroplasma citri Standard CFU Kit	250270
Mycoplasma salivarium Standard CFU Kit	250271

Shipping and Storage

The QIAcuity Mycoplasma Quant Kit is shipped on dry ice.

The QIAcuity Mycoplasma Quant Kit should be stored protected from light at –30°C to –15°C upon receipt. Under these conditions, the components are stable until expiry date printed on their labels without showing any reduction in performance and quality, unless otherwise indicated on the labels. QIAcuity Mycoplasma Internal Control (before reconstruction), QIAcuity Mycoplasma Positive Control (before reconstruction), and RNase-Free Water can be stored both at 2–8°C (as indicated on the label) and at –30°C to –15°C. After reconstitution, QIAcuity Mycoplasma Internal Control and QIAcuity Mycoplasma Positive Control are stable at –30°C to –15°C for up to 14 days, but maximal until expiry date printed on their labels. More than 5 freeze–thaw cycles should be avoided. If QIAcuity Mycoplasma Internal Control, and QIAcuity Mycoplasma Positive Control were aliquoted, usage of DNA low binding tubes is strongly recommend to minimize loss of material due to binding to plastic.

The Mycoplasma Standard CFU Kits are shipped on gel packs.

Upon receipt, the Mycoplasma Standard CFU Kits should be stored at 2–8°C. Under these conditions, the components are stable until expiry date printed on their labels without showing any reduction in performance and quality, unless otherwise indicated on the labels.

Intended Use

The QIAcuity Mycoplasma Quant Kit is intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety**, where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of QIAcuity Mycoplasma Quant Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

The QIAcuity Mycoplasma Quant Kit detects and quantifies the presence of mycoplasma rRNA reliably in a variety of sample types. In combination with a recommended sample prep method QIAGEN offers a robust, European, US, and Japanese Pharmacopeia compliant workflow for mycoplasma testing without the need of time-costly cultivation of mycoplasma. The recommended workflow is particularly suited to a variety of sample types of different purity (e.g., from cell bank samples over in-process samples such as virus harvest to final lots/batches).

The QIAcuity Mycoplasma Quant Kit provides a consistent, accurate, precise, and highly repeatable detection of mycoplasma presence.

Principle and procedure

The QIAcuity Mycoplasma Quant Kit, together with the QIAcuity instrument, forms a unique system for detection and quantification of mycoplasma contamination that offers the best combination of performance and ease-of-use. This system is embedded into a workflow, validated according to the European Pharmacopeia (EP; chapter 2.6.7), the US Pharmacopeia (UP; chapter <63>), and the Japanese Pharmacopeia (JP; chapter G3-14-170) for all 10 Mycoplasma species mentioned in these Pharmacopeias: *Mycoplasma arginini*, *Mycoplasma orale*, *Mycoplasma gallisepticum*, *Mycoplasma pneumoniae*, *Mycoplasma synoviae*, *Mycoplasma fermentans*, *Mycoplasma hyorhinis*, *Acholeplasma laidlawii*, *Spiroplasma citri*, and *Mycoplasma salivarium*. The validation was performed by Minerva Biolabs GmbH (Berlin, Germany). The validation report is available for download on **qiagen.com**. Additionally, 127 mycoplasma species are specifically detected by the QIAcuity Mycoplasma Quant Kit.

The validated workflow consists of 2 steps in which the nucleic acids are extracted from the sample and subsequently analyzed via RT-dPCR. Using the QIAcuity OneStep Advanced Probe chemistry, the required reverse transcription and PCR are done within a single hands-on step.

Although we recommend to detect Mycoplasma using the RT-dPCR protocol as described below, the assay also detects DNA and can be used in a dPCR without the RT-step. We recommend conducting sample isolation for RNA and DNA.

Sample preparation

Elimination of PCR inhibitors and other impurities is required to have a reliable detection of any mycoplasma contamination within the sample. As overall control, the QIAcuity Mycoplasma Internal Control can optionally be given to the starting material before the sample extraction. The eluate can be used directly for mycoplasma detection using the QIAcuity Mycoplasma Quant Kit with the corresponding QIAcuity OneStep Advanced Probe Chemistry. We recommend to use a sample isolation method for RNA and DNA.

QIAcuity OneStep Advanced Probe Chemistry

The HotStart Reverse Transcription (RT) Enzyme, in combination with the HotStart QuantiNova® DNA Polymerase and proprietary chemical components, enables an optimal reverse transcription and subsequent PCR reaction for detection of any mycoplasma contamination using the QIAcuity Mycoplasma Assay without the need of a separate RT-reaction setup.

QIAcuity Mycoplasma Assay

The QIAcuity Mycoplasma Assay is provided as FAM™-labeled 20x primer-probe mix. Also included is a HEX-labeled primer-probe mix specific to the optional QIAcuity Mycoplasma Internal Control. This duplex reaction is proven for cross-talk and enables an overall control for RNA extraction, reverse transcription, amplification, and detection without setting up any extra reaction.

Description of protocols

This handbook contains one protocol for the detection of mycoplasma with the recommended spike-in of the QIAcuity Mycoplasma Internal Control as a sample extraction control during sample lysis, and a second protocol for the detection of mycoplasma contamination with the use of the QIAcuity Mycoplasma Internal Control as RT-dPCR control only. A recommendation for the extraction method is given for both protocols as the sample preparation method is crucial for the successful detection. A third protocol describes the use of Mycoplasma Standard CFU Kits. The described protocol using the QIAcuity Mycoplasma Internal Control as Spike-In control during sample preparation was used for the workflow validation according to the European, US, and Japanese Pharmacopeia at Minerva Biolabs GmbH.

Detection of Mycoplasma using the QIAcuity Mycoplasma Quant Kit with the use of the Spike-In Internal Control during sample preparation (recommended)

Description of the recommended protocol for mycoplasma detection with the use of the Spike-In of the QIAcuity Mycoplasma Internal Control during sample lysis to be used as overall control (extraction, reverse transcription, and RT-PCR control) with the recommended extraction method.

Detection of Mycoplasma using the QIAcuity Mycoplasma Quant Kit with the use of the Internal Control as RT-PCR control

Detailed description of reaction set up for the mycoplasma RNA quantification using the QIAcuity dPCR system with the use of the QIAcuity Mycoplasma Internal Control as RT-PCR control only.

Preparation of Mycoplasma Standard CFU Kits

Detailed description of use of Mycoplasma Standard CFU Kits for in-house validation or as positive control for sensitivity of mycoplasma testing without introducing vital mycoplasma.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- Mycoplasma Standard CFU kits (cat. nos. 250262–250271), optional
- QIAcuity Nanoplates (cat. no. 250001)
- Microcentrifuge tubes or PCR plates or strip tubes with appropriate sealing foil or cap.
- Single-channel or multichannel pipettor (manual or automatic) with nuclease-free, aerosol-barrier pipette tips
- Vortexer
- Centrifuge for tubes and plates
- QIAcuity One (5plex), Four, or Eight (cat. nos. 911021, 911042, or 911052)

Important Notes

Mycoplasma testing

Mycoplasma is a common contaminant of mammalian cell cultures. Contamination with the gram-negative bacteria is not detectable by microscopy due to the very small size but can have drastic impact on the vitality and gene expression of mammalian cells which affects any cell-derived products such as AAVs, proteins, or vaccines. To prevent the application of such contaminated products to patients, mycoplasma testing is mandatory during the production process and as final release criteria. Regulations such as the European, the US, or the Japanese Pharmacopeia describe such a test procedure.

Pharmacopeia compliant workflow

The European Pharmacopeia (EP), the US Pharmacopeia (USP), and Japanese Pharmacopeia (JP) describe in discrete chapters (EP 2.6.7, USP <63>, JP G3-14-170) how mycoplasma testing needs to be performed.

As alternatives to the conventional and time-consuming culture method and indicator cell culture method, faster nucleic acid amplification techniques (NAT) such as digital PCR are applicable for mycoplasma testing. The required validation requires the complete workflow which includes the sample preparation, the reverse transcription and the PCR reaction. An internal control which is spiked-in before sample preparation is strongly recommended to function as overall control.

NAT mycoplasma testing is a qualitative testing with a required minimal sensitivity depending on the conventional method which should be replaced. The required minimal sensitivity of the selected NAT system needs be at 100 CFU/mL to replace the indicator cell culture method, or at 10 CFU/mL to replace the culture method. The sensitivity needs to be shown for all application relevant mycoplasma species by the user.

Starting material

The QIAcuity Mycoplasma Quant Kit is optimized for detecting the presence of mycoplasma nucleic acids. Test material can contain various matrices such as PBS, cell culture media like DMEM (Dulbecco's modified Eagles medium) with FCS (fetal calf serum) in the presence or absence of cell background, final ATMPs (advanced therapeutic medicinal products) like gene therapy tools or vaccines in different production steps. In combination with a suitable extraction method such as the Venor®GeM SP Kit – Beads (Minerva Biolabs GmbH), the QIAcuity Mycoplasma Quant Kit is very robust against potential inhibitors such as coating agents, secreted intracellular, and extracellular material carried over by tested cell suspensions like HEK 293 cells or CHO cells.

Protocol: Detection of Mycoplasma Using the QIAcuity Mycoplasma Quant Kit with the Use of the Spike-In Internal Control During Sample Preparation

This protocol describes the use of the QIAcuity Mycoplasma Quant Kit in conjunction with the use of the QIAcuity Mycoplasma Internal Control as spike-in during nucleic acid extraction to be used as overall control.

Important points before starting

- See “Important Notes” on page 13.
- The Pharmacopeia (EP, USP, and JP) compliant mycoplasma testing requires a nucleic acid purification prior PCR which needs to be validated as a workflow. QIAGEN offers a validation report as download from the product page to demonstrate the performance of the mycoplasma testing workflow using the Venor®GeM SP Kit – Beads (Minerva Biolabs GmbH, Berlin, cat. no. 56-3100) for nucleic acid purification and QIAcuity Mycoplasma Quant Kit for RT-dPCR detection.
- As performance control of the procedure (nucleic acid purification, reverse transcription and digital PCR) an internal control spike-in is required. To avoid degradation of the internal control by RNases within the sample matrix, the internal control has to be spiked-in together with the lysis buffer or after addition of the lysis buffer.
- The protocol using the QIAcuity Mycoplasma Quant Kit with the use of the spike-in internal control during sample preparation uses a 10-fold higher concentration for the QIAcuity Mycoplasma Internal Control to lower the required Spike-In volume by factor 10.
- The 4x QIAcuity OneStep Advanced Probe Master Mix contains the HotStart QuantiNova DNA polymerase, which is inactive at ambient temperature (15–25°C). The

PCR protocol must start with a mandatory initial incubation step of 2 min at 95°C to activate the enzyme.

- The 100x QIAcuity OneStep Advanced RT Mix contains a HotStart Reverse Transcription (RT) Enzyme. This enzyme is inactive at ambient temperature, allowing users to assemble up to 4 or 8 plates and run them in parallel on the QIAcuity Four or QIAcuity Eight instrument, respectively. Nevertheless, during reaction setup, 100x QIAcuity OneStep Advanced Reverse Transcription Mix should be stored on ice (2–8°C) to ensure constant performance.
- A fluorescent dye is provided as a component of the QIAcuity Probe PCR Master Mix, for reliable detection of proper filling in the dPCR plates.
- Pipetting accuracy and precision affect the consistency of quantification results. Make sure that no air bubbles are introduced into the wells of the dPCR nanoplates during pipetting.

Things to do before starting

- Have an appropriate RNA purification method or nucleic acid purification method ready-to-use. QIAGEN recommends the Venor®GeM SP Kit – Beads (Minerva Biolabs GmbH, Berlin, cat. no. 56-3100). Alternative solutions can be used after performance assessment. Be sure to choose a solution that isolates RNA from the sample to be compatible with the QIAcuity Mycoplasma Quant Kit.
- Prepare all components of the nucleic acid purification method according to the respective protocol, including preheating of potentially required heat blocks.
- Prepare all buffers and reagents of an appropriate nucleic acid purification method according to the respective protocol before starting the procedure.
- For an overall control reconstitute the lyophilized QIAcuity Mycoplasma Internal Control: Briefly centrifuge the tubes to remove any material from the lid. To end up with a 10-fold higher concentrated Internal Control than for RT-dPCR reaction only, dissolve lyophilized QIAcuity Mycoplasma Internal Control (720,000 copies) by adding 120 µL RNase-Free

Water to reach a final concentration of 6000 copies/µL. Incubate 5 min at ambient temperature (18–25°C), then vortex and spin the reconstituted reagents.
    - QIAcuity Mycoplasma Internal Control can be stored at –15°C to –30°C for up to 14 days. Avoid more than 5 freeze–thaw cycles.
- Reconstitute lyophilized QIAcuity Mycoplasma Positive Control: Briefly centrifuge the tubes to remove any material from the lid. Dissolve lyophilized QIAcuity Mycoplasma Positive Control (80,000 copies) by adding 400 µL RNase-Free Water to reach a final concentration of 200 copies/µL. Incubate 5 min at ambient temperature, then vortex and spin the reconstituted reagents.
    - QIAcuity Mycoplasma Positive Control can be stored at –15°C to –30°C for up to 14 days. Avoid more than 5 freeze and thaw cycles.
- Thaw all kit components except the 100x QIAcuity OneStep Advanced Reverse Transcription Mix and mix all components (also components stored at ambient temperature and on ice) right before use.
- Place the 100x QIAcuity OneStep Advanced Reverse Transcription Mix on ice.

Procedure

1. **Optional:** Prepare a Lysis-Buffer-IC Mix: Add 2.5 µL of the reconstituted QIAcuity Mycoplasma Internal Control to the required volume lysis buffer per sample (according to the protocol for the nucleic acid purification method). Adjust accordingly for the amount of processed samples.

   Note: As an example, using the recommended Venor®GeM SP Kit – Beads (Minerva Biolabs GmbH, Berlin, cat. no. 56-3100), add 2.5 µL reconstituted QIAcuity Mycoplasma Internal Control to the needed 250 µL lysis buffer per sample to be processed. Mix carefully by inverting for at least 15 times to avoid foam formation. Foam formation does not affect lysis performance but make sure to pipette liquid and not foam.
2. Follow the nucleic acid purification protocol until the addition of lysis buffer. Here, the internal control needs to be spiked-in for an overall process control. Two options for spiking-in the internal control are appropriate.

2a. Add the appropriate volume of prepared Lysis–Buffer–IC Mix to the sample. Vortex for 2 s and spin down.

Important: If foam formation took place, make sure to pipette liquid and not foam.

Note: The appropriate volume lysis buffer is defined by the protocol of the nucleic acid purification method.

2b. Add the appropriate volume of lysis buffer. Vortex for 2 s and spin down. Add 2.5 µL QIAcuity Mycoplasma Internal Control to the sample. Vortex for 2 s and spin down.

Note: The appropriate volume lysis buffer is defined by the protocol of the nucleic acid purification method.

3. Proceed with the nucleic acid purification protocol. It is recommended to elute into DNA low binding tubes. Directly proceed with step 4 of this protocol.
   Long-term storage of eluates is not recommended due to low RNA content within the sample.
   Important: If a bead-based system is used for nucleic acid purification, it is important to avoid bead carry-over into the eluate. Beads within the RT-dPCR reaction can negatively affect the system performance.
4. Prepare the RT-dPCR reaction mix using the QIAcuity Mycoplasma Quant Kit according to Table 1 in a standard PCR plate.
5. Add the eluate obtained from the sample preparation. We recommend to add the maximum volume of 22.6 µL to the reaction, but this can be adjusted.

   Note: You can add 10 µL of the reconstituted QIAcuity Mycoplasma Positive Control instead of the eluate to one or more wells to check the reaction performance. Adjust the addition of water accordingly.

Table 1. PCR reaction setup

Component	Nanoplate 26K (8-well and 24-well)	Final concentration in reaction
4x OneStep Adv. Probe Master Mix	10 µL	1x
100x OneStep Adv. RT Mix	0.4 µL	1x
OneStep Enhancer GC	5 µL	–
20x QIAcuity Mycoplasma Assay	2 µL	1x
QIAcuity Mycoplasma Internal Control	–*	93.75 copies/µL†
Sample‡ or QIAcuity Mycoplasma Positive Control	Up to 22.6 µL sample or 10 µL QIAcuity Mycoplasma Positive Control	50 copies/µL§
RNase-Free Water	Fill up to 40 µL	–
Total reaction volume	**40 µL**	–

* QIAcuity Mycoplasma Internal Control is added in lysis step of nucleic acid purification; hence, no addition during RT-PCR setup is needed.

† The final concentration of QIAcuity Mycoplasma Internal Control depends on the elution volume of the nucleic acid purification and the sample volume analyzed in the RT-PCR reaction. The final concentration can be calculated using the formula:

IC concentration in RT-dPCR =

(IC spike-in vol. x IC starting conc. / elution vol.) x eluate vol. in RT-dPCR / total reaction vol.

Example: According to the recommended nucleic acid purification:

IC concentration in RT-dPCR = (2.5 µL x 6000 copies/µL / 80 µL) x 20 µL / 40 µL = 93.75 copies/µL.

‡ Sample extract should be generated using an appropriate RNA purification technique. For recommendation see "Things to do before starting".

§ Exemplary volume of the QIAcuity Mycoplasma Positive Control. If 10 µL QIAcuity Mycoplasma Positive Control is added, a final concentration of 50 copies/µL is expected. The final concentration can be calculated using the formula: PC concentration in dPCR = (PC starting conc. x PC vol. in dPCR) / total reaction vol.

6. Seal the plate and mix thoroughly by vortexing the reaction mix 5 times, 1 s each. Spin down the plate by briefly centrifuging.
7. Transfer the content of each well to a 26k nanoplate avoiding bubbles. Seal the nanoplate and load it into the QIAcuity instrument. Start the run.

Thermal cycling and imaging conditions

1. In the QIAcuity Software Suite or on the QIAcuity instrument, under the dPCR parameters, set the cycling conditions according to Table 2.

Table 2. Cycling conditions

Step	Time	Temperature
Reverse Transcription reaction		
Reverse transcription	40 min	50°C
RT Enzyme inactivation	2 min	95°C
Two-step cycling (40 cycles)		
Denaturation	15 s	95°C
Combined annealing and elongation	1 min	59°C

2. Under the dPCR parameters in the QIAcuity Software Suite or on the QIAcuity instrument, activate all needed channels in **Imaging**. Start with the default imaging settings according to Table 3.

Table 3. Imaging settings*

Channel	Exposure	Gain
Green (FAM)	500 ms	6
Yellow (HEX)	500 ms	6

* Imaging settings might need to be adjusted according to the assay. Always start with the recommended settings.

3. Place the Nanoplate into the QIAcuity instrument and start the dPCR program.

Data analysis

1. To set up a plate layout according to the experimental design, open the QIAcuity Software Suite and define the reaction mixes, samples, and controls. Plate layout can be defined before or after the Nanoplate run.

   Note: Refer to the *QIAcuity User Manual* for details on setting up the plate layout.

2. After the run is completed, the raw data are automatically sent to the QIAcuity Software Suite.
3. For data analysis, open the QIAcuity Software Suite and select the individual Nanoplate for the analysis in **Plate Overview** of the Software Suite.

   Note: See the *QIAcuity User Manual Extension: Application Guide* and *QIAcuity User Manual* for details on how to analyze absolute quantification data.

Protocol: Detection of Mycoplasma Using the QIAcuity Mycoplasma Quant Kit with the Use of the Internal Control as RT-PCR Control

This protocol describes how to setup a QIAcuity RT-PCR using the QIAcuity Mycoplasma Quant Kit for a mycoplasma RNA detection in mycoplasma test samples processed by an appropriate nucleic acid purification method without the prior addition of the QIAcuity Mycoplasma Internal Control during the sample lysis.

Important points before starting

- See "Important Notes" on page 13.
- The protocol using the QIAcuity Mycoplasma Quant Kit with the use of the Internal Control as RT-PCR control uses a 10-fold lower concentration for the QIAcuity Mycoplasma Internal Control than in the previous protocol to enable a good pipetting volume.
- The 4x QIAcuity OneStep Advanced Probe Master Mix contains the HotStart QuantiNova DNA polymerase, which is inactive at room temperature. The PCR protocol must start with a mandatory initial incubation step of 2 min at 95°C to activate the enzyme.
- The 100x QIAcuity OneStep Advanced RT Mix contains a HotStart Reverse Transcription (RT) Enzyme. This enzyme is inactive at ambient temperature, allowing users to assemble up to 4 or 8 plates and run them in parallel on the QIAcuity Four or QIAcuity Eight instrument, respectively. Nevertheless, during reaction set up, 100x QIAcuity OneStep Advanced Reverse Transcription Mix should be stored on ice (2–8°C) to ensure constant performance.
- A fluorescent dye is provided as a component of the QIAcuity Probe PCR Master Mix, for reliable detection of proper filling in the dPCR plates.

- Pipetting accuracy and precision affect the consistency of quantification results. Make sure that no air bubbles are introduced into the wells of the dPCR nanoplates during pipetting.

Things to do before starting

- Have an appropriate RNA purification method or nucleic acid purification method ready-to-use. QIAGEN recommends the Venor®GeM SP Kit – Beads (Minerva Biolabs GmbH, Berlin, cat. no. 56-3100). Alternative solutions can be used after performance assessment. Be sure to choose a solution that isolates RNA from the sample.
- Prepare all components of the nucleic acid purification method according to the respective protocol including preheating of potentially required heat blocks.
- Perform nucleic acid purification as described in the respective protocol and store the eluate for reaction set-up in step 2 of this protocol on ice.
- If not already used as overall control of the procedure (nucleic acid purification, reverse transcription, digital PCR) as described in "Protocol: Detection of Mycoplasma Using the QIAcuity Mycoplasma Quant Kit with the Use of the Spike-in Internal Control During Sample" on page 15, reconstitute lyophilized QIAcuity Mycoplasma Internal Control by briefly centrifuging the tubes to remove any material from the lid. Dissolve lyophilized QIAcuity Mycoplasma Internal Control (720,000 copies) by adding 1200 µL RNase-Free Water to reach a final concentration of 600 copies/µL. Incubate 5 min at ambient temperature, then vortex and spin the reconstituted reagents.
  - QIAcuity Mycoplasma Internal Control can be stored at –15°C to –30°C for up to 14 days. Avoid more than 5 freeze–thaw cycles.
- Reconstitute lyophilized QIAcuity Mycoplasma Positive Control: Briefly centrifuge the tubes to remove any material from the lid. Dissolve lyophilized QIAcuity Mycoplasma Positive Control (80,000 copies) by adding 400 µL RNase-Free Water to reach a final concentration of 200 copies/µL. Incubate for 5 min at ambient temperature, then vortex and spin the reconstituted reagents.

- QIAcuity Mycoplasma Positive Control can be stored at –15°C to –30°C for up to 14 days. Avoid more than 5 freeze–thaw cycles.

- Thaw all kit components except the 100x QIAcuity OneStep Advanced Reverse Transcription Mix and mix all components (also components stored at ambient temperature and on ice) right before use.
- Place the 100x QIAcuity OneStep Advanced Reverse Transcription Mix on ice.

Procedure

1. Prepare the RT-dPCR reaction mix using the QIAcuity Mycoplasma Quant Kit according to Table 4 in a standard PCR plate.

   Note: The use of the QIAcuity Mycoplasma Internal Control is highly recommended to check the presence of inhibitors in the sample.

2. Add the eluate obtained from the sample preparation. We recommend to add the maximum volume of 22.6 µL to the reaction, but this can be adjusted.

   Note: You can add 10 µL of the reconstituted QIAcuity Mycoplasma Positive Control instead of the eluate to one or more wells to check the reaction performance. Adjust the addition of water accordingly.

Table 4. PCR reaction setup

Component	Nanoplate 26K (8-well and 24-well)	Final concentration in reaction
4x OneStep Adv. Probe Master Mix	10 µL	1x
100x OneStep Adv. RT Mix	0.4 µL	1x
OneStep Enhancer GC	5 µL	–
20x QIAcuity Mycoplasma Assay	2 µL	1x
QIAcuity Mycoplasma Internal Control (if not used as Spike-in control)	1 µL*	15 copies/µL†
Sample‡ or QIAcuity Mycoplasma Positive Control	up to 22.6 µL sample or 10 µL QIAcuity Mycoplasma Positive Control	50 copies/µL§
RNase-Free Water	Fill up to 40 µL	–
Total reaction volume	**40 µL**	–

* Just add QIAcuity Mycoplasma Internal Control during RT-PCR setup if it was not added during nucleic acid purification. Otherwise, misleading results will be obtained.

† Exemplary volume of the QIAcuity Mycoplasma Internal Control. If 1 µL QIAcuity Mycoplasma Internal Control is added, a final concentration of 15 copies/µL is expected. The final concentration can be calculated using the formula: IC concentration in dPCR = (IC starting conc. x IC vol. in dPCR) / total reaction vol.

‡ Sample extract should be generated using an appropriate RNA purification technique. For recommendation see "Protocol: Detection of Mycoplasma Using the QIAcuity Mycoplasma Quant Kit with the Use of the Spike-In Internal Control During Sample Preparation" on page 15.

§ Exemplary volume of the QIAcuity Mycoplasma Positive Control. If 10 µL QIAcuity Mycoplasma Positive Control are added, a final concentration of 50 copies/µL is expected. The final concentration can be calculated using the formula: PC concentration in dPCR = (PC starting conc. x PC vol. in dPCR) / total reaction vol.

3. Seal the plate and mix thoroughly by vortexing the reaction mix 5 times, 1 s each. Spin down the plate by briefly centrifuging.
4. Transfer the content of each well to a 26k nanoplate avoiding bubbles. Seal the nanoplate and load it into the QIAcuity instrument. Start the run.

Additional information regarding RT-PCR setup, recommended cycling, and imaging conditions can be found in "Protocol: Detection of Mycoplasma Using the QIAcuity Mycoplasma Quant Kit with the Use of the Spike-in Internal Control During Sample Preparation" on page 15.

Protocol: Preparation of Mycoplasma Standard CFU Kits

This protocol describes the preparation of the Mycoplasma Standard CFU Kits to validate the mycoplasma test procedure according to the EP, USP, and JP without introducing vital mycoplasma.

Important points before starting

- See "Important Notes" on page 13.
- The Pharmacopeia (EP, USP, and JP) compliant mycoplasma testing requires a nucleic acid purification prior PCR which needs to be validated as a workflow. QIAGEN offers a validation report on request to demonstrate the performance of the mycoplasma testing workflow using the Venor®GeM SP Kit – Beads (Minerva Biolabs GmbH, Berlin, cat. no. 56-3100) for nucleic acid purification and QIAcuity Mycoplasma Quant Kit for RT-dPCR run.
- The validation of the mycoplasma test procedure needs to be performed using the sample matrix in which samples will be tested to show, the procedure is functional on the respective sample matrix.
- As performance control of the procedure (nucleic acid purification, reverse transcription and digital PCR) an internal control spike-in is required. To avoid degradation of the internal control by RNases within the sample matrix, the internal control has to be spiked-in together with the lysis buffer or after addition of the lysis buffer.

Things to do before starting

- Have an appropriate RNA purification method or nucleic acid purification method ready-to-use. QIAGEN recommends the Venor®GeM SP Kit – Beads (Minerva Biolabs GmbH,

Berlin, cat. no. 56-3100). Alternative solutions can be used after performance assessment. Be sure to choose a solution that isolates RNA from the sample.

- Prepare all components of the nucleic acid purification method according to the respective protocol. Including preheating of potentially required heat blocks.
- Prepare all buffers and reagents of an appropriate nucleic acid purification method according to the respective protocol before starting the procedure.
- For an overall control reconstitute the lyophilized QIAcuity Mycoplasma Internal Control: Briefly centrifuge the tubes to remove any material from the lid. To end up with a 10-fold higher concentrated Internal Control than for RT-dPCR reaction only, dissolve lyophilized QIAcuity Mycoplasma Internal Control (720,000 copies) by adding 120 µL RNase-Free Water to reach a final concentration of 6000 copies/µL. Incubate 5 min at ambient temperature (18–25°C), then vortex and spin the reconstituted reagents.
  - QIAcuity Mycoplasma Internal Control can be stored at –15°C to –30°C for up to 14 days. Avoid more than 5 freeze–thaw cycles.
- Reconstitute lyophilized QIAcuity Mycoplasma Positive Control: Briefly centrifuge the tubes to remove any material from the lid. Dissolve lyophilized QIAcuity Mycoplasma Positive Control (80,000 copies) by adding 400 µL RNase-Free Water to reach a final concentration of 200 copies/µL. Incubate 5 min at ambient temperature, then vortex and spin the reconstituted reagents.
  - QIAcuity Mycoplasma Positive Control can be stored at –15°C to –30°C for up to 14 days. Avoid more than 5 freeze–thaw cycles.
- Thaw all kit components except the 100x QIAcuity OneStep Advanced Reverse Transcription Mix and mix all components (components are also stored at ambient temperature and on ice) right before use.
- Place the 100x QIAcuity OneStep Advanced Reverse Transcription Mix on ice.

Procedure

1. Briefly centrifuge the tubes to remove any material from the lid. Dissolve lyophilized QIAcuity Mycoplasma Std. CFU (10 CFU) by adding 1000 µL sample matrix to reach a final concentration of 10 CFU/mL.
2. Prepare a Lysis-Buffer-IC Mix: Add 2.5 µL of the reconstituted QIAcuity Mycoplasma Internal Control to the required volume sample lysis buffer per sample (according to the protocol for the nucleic acid purification method).
   Note: As an example, using the recommended Venor®GeM SP Kit – Beads (Minerva Biolabs GmbH, Berlin, cat. no. 56-3100) add 2.5 µL reconstituted QIAcuity Mycoplasma Internal Control to the needed 250 µL lysis buffer per sample to be processed. Adjust accordingly for the amount of processed samples. Mix carefully by inverting for at least 15 times to avoid foam formation. Foam formation does not affect lysis performance but make sure to pipette liquid and not foam.
3. Follow the nucleic acid purification protocol using the reconstituted QIAcuity Mycoplasma Std. CFU as sample until the addition of lysis buffer. Add the appropriate volume of prepared Lysis–Buffer–IC Mix to the sample. Vortex 2 s and spin down.
   Important: If foam formation took place, make sure to pipette liquid and not foam.
   Note: The appropriate volume lysis buffer is defined by the protocol of the nucleic acid purification method.
4. Proceed with the nucleic acid purification protocol. It is recommended to elute into DNA low binding tubes. Directly proceed with step 5 of this protocol.
   Long-term storage of eluates is not recommended.
   Important: If a bead-base system is used for nucleic acid purification, it is important to avoid bead carry-over into the eluate. Beads within the RT-dPCR reaction can negatively affect the system performance.
5. Prepare the RT-dPCR reaction mix using the QIAcuity Mycoplasma Quant Kit according to Table 5 in a standard PCR plate.
6. Add the eluate obtained from the sample preparation. We recommend to add the maximum volume of 22.6 µL to the reaction, but this can be adjusted.

Note: You can add 10 µL of the reconstituted QIAcuity Mycoplasma Positive Control instead of the eluate to one or more wells to check the reaction performance. Adjust the addition of RNase-Free Water accordingly.

Table 5. PCR reaction setup

Component	Nanoplate 26K (8-well and 24-well)	Final concentration in reaction
4x OneStep Adv. Probe Master Mix	10 µL	1x
100x OneStep Adv. RT Mix	0.4 µL	1x
OneStep Enhancer GC	5 µL	–
20x QIAcuity Mycoplasma Assay	2 µL	1x
QIAcuity Mycoplasma Internal Control	–*	93.57 copies/µL†
Sample‡ or QIAcuity Mycoplasma Positive Control	Up to 22.6 µL sample or 10 µL QIAcuity Mycoplasma Positive Control	50 copies/µL§
RNase-Free Water	Fill up to 40 µL	–
Total reaction volume	**40 µL**	–

* QIAcuity Mycoplasma Internal Control is added in lysis step of nucleic acid purification; hence, no addition during RT-PCR setup is needed.

† The final concentration of QIAcuity Mycoplasma Internal Control depends on the elution volume of the nucleic acid purification and the sample volume analyzed in the RT-PCR reaction. The final concentration can be calculated using the formula:

IC concentration in RT-dPCR =

(IC spike-in vol. x IC starting conc. / elution vol.) x eluate vol. in RT-dPCR / total reaction vol.

Example: According to the recommended nucleic acid purification:

IC concentration in RT-dPCR = (2.5 µL x 6000 copies/µL / 80 µL) x 20 µL / 40 µL = 93.75 copies/µL.

‡ Sample extract should be generated using an appropriate RNA purification technique. For recommendation see handbook.

§ Exemplary volume of the QIAcuity Mycoplasma Positive Control. If 10 µL QIAcuity Mycoplasma Positive Control are added, a final concentration of 50 copies/µL is expected. The final concentration can be calculated using the formula: PC concentration in dPCR = (PC starting conc. x PC vol. in dPCR) / total reaction vol.

7. Transfer the content of each well to a 26k nanoplate avoiding bubbles. Seal the nanoplate and load it into the QIAcuity instrument. Start the run.

   Additional information regarding PCR setup, recommended cycling, and imaging conditions can be found in “Protocol: Detection of Mycoplasma Using the QIAcuity Mycoplasma Quant Kit with the Use of the Spike-In Internal Control During Sample” on page 19 of this handbook.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx (for contact information, visit www.qiagen.com).

	Comments and suggestions
RNA purification	
Insufficient mycoplasma lysis	**Suboptimal buffer conditions for efficient mycoplasma lysis**: Possibly high viscosity of the Lysis Buffer. Make sure to pipette indicated volume without air bubbles. **Incubation within lysis step skipped**: Any incubation step described in RNA purification protocol is mandatory. Lysis performed at different temperature or for a different amount of time can lead to a reduction in mycoplasma yield and, as a consequence, to misleading test results.
Bead carry-over	**Not all beads are bound by the magnet**: Make sure the tube is placed properly in the magnetic rack to enable optimal bead binding. It is required to place the tubes for indicated period of time in the magnetic rack to enable optimal bead binding.
Sample storage after purification	**Underquantification of the mycoplasma concentration**: Sample storage after RNA extraction is possible, even though, direct processing of eluates is strongly recommended. If direct processing is not possible, RNA containing eluates should be stored at –80°C. Storage at higher temperatures can lead to degradation of RNA.
PCR	
Lower mycoplasma concentration than expected	**Insufficient RNA extraction**: Please make sure the used extraction method is sufficient for RNA extraction and follow instruction of sample extraction as described in the respective protocol. It is recommended to use Venor®GeM SP Kit – Beads (Minerva Biolabs GmbH, Berlin, cat. no. 56-3100). **RNA degradation during processing**: Make sure to perform mycoplasma testing in a RNase-free surrounding. **Loss of RNA during storage**: Make sure to use RNase-free consumables and low binding tubes. If storage cannot be avoided, eluates need to be stored at –80°C until analyzes. Repeated thaw-freeze cycles should be avoided.

	Comments and suggestions
Lower Internal Control concentration than expected	**Insufficient rehydration**: Make sure to add the appropriate volume RNase-Free Water to QIAcuity Mycoplasma Internal Control. **Insufficient RNA extraction**: Please make sure the used extraction method is sufficient for RNA extraction and follow instruction of sample extraction as described in the respective protocol. It is recommended to use Venor®GeM SP Kit – Beads (Minerva Biolabs GmbH, Berlin, cat. no. 56-3100).
No positive partitions	**Sample input below LOD.** Increase sample input into RT-PCR. It is recommend to load 22.6 µL sample. **Cycling conditions**: Check and optimize annealing/extension temperature with custom designed assays or assays from other suppliers. Please follow recommended cycling program when using QIAGEN assays. **Imaging setup**: Check channel choice and match with probe dyes. Any positive mycoplasma signal is detected in the green channel, the QIAcuity Mycoplasma Internal Control is detected in the yellow channel. **Inhibition**: Make sure the used extraction method does not carry over any PCR inhibitor. **Shortened reverse transcription**: Ensure to run the RT-PCR with an initial RT step for 40 min at 50°C. **Prolonged initial denaturation**: Ensure to run the RT-PCR using the QIAcuity OneStep Advanced Probe mix with an initial denaturation of 2 min at 95°C which is also required to inactivate the RT enzyme. Prolonged denaturation might negatively affect PCR performance.
Infinity signal/no negative partitions	**Sample dilution**: Increase dilution to fit into the dPCR concentration range.

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

Revision	Description
10/2023	Initial release
12/2023	Added recommendation of performing sample isolation of RNA and DNA, page 10. Updated product name of Venor®GeM Sample Preparation to Venor®GeM SP Kit – Beads.

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