# QUALICHEK™ Mycoplasma qPCR Detection Kit

**Cat No: KQD-Sample / KQD-50 / KQD-100**

## Introduction:

The QUALICHEK™ Mycoplasma qPCR Detection Kit is used to detect Mycoplasma infection of cell cultures by real-time quantitative PCR (qPCR) using Probe. The QUALICHEK™Mycoplasma qPCR Detection Kit includes a Primer and Probe mixes. These mixes contain FAM labeled probe specific for mycoplasma species and Hex labeled probe for internal control DNA. The primer set is specific to the highly conserved the 16SrRNAcoding region in the mycoplasma genome. This allows the detection of *M. orale*, *M. hyorhinis*, *M. arginini*, *M. fermentans*, *Acholeplasma laidlawii*, *M. hominis*, usually encountered as contaminants in cell cultures. Furthermore, this kit can detect *M. pneumoniae*, *M. salivarium*, *M. synoviae* and Ureaplasma species. Eukaryotic and bacterial DNA is not amplified by QUALICHEK™ Mycoplasma Real-Time PCR Detection Kit. The QUALICHEK™ Mycoplasma qPCR Detection Kit is capable of detecting Mycoplasma infections in cell cultures in less than three hours, depending on the spectrofluorometric thermal cycler used for detection.

**Materials Provided:**

| Materials provided | Quantity | |
|---|---|---|
| | KQD-50 | KQD-100 |
| 2X qPCR Master Mix for Probe | 750 ul | 1.5 ml |
| Primer and Probe Mix* | 100 ul | 200 ul |
| Internal Amplification Control DNA (Orange cap) | 100 ul | 200 ul |
| Internal Amplification Control DNA for sample prep. | 1 ml | 2 ml |
| Positive Control DNA | 25 ul | 50 ul |
| 50X ROX (Reference Dye); High Rox/Low Rox | 30 ul | 60 ul |
| DNase Free Water | 600 ul | 1.2 ml |

## Storage Condition

1. Upon receipt store at -20ºC
2. Avoid repeated freeze/thaw cycles
3. Aliquot the reagents, if frequent freeze and thaw is needed

## Assay Procedure

### 1. Sample Preparation

Samples should be derived from cultures which are at 90-100% confluence. Penicillin and streptomycin in the culture media do not inhibit PCR or affect test sensitivity. To avoid false positive results, we recommend the use of the PCR grade water delivered with the kit, aerosol- preventive filter tips and gloves.

**Preparation of Sample Screening:**

**Sample Preparation from Cell Culture Media**

1. 1.2 ml liquid supernatant of the sample is transferred into a 1.5ml tube and centrifuged(5 minutes, 1,000 rpm) to sediment cell debris
2. 1ml of the supernatant is transferred into a 1.5ml tube
3. Centrifuged (10 minutes, 13,000 rpm) to sediment mycoplasma particles
4. Discard supernatant and wash the pellet once with 1ml of PBS. (Repeat step 3).
5. Discard supernatant and add 50 ul DNase free water or TE buffer to the pellet
6. Heat the samples at 98ºC for 10min, and vortex for 5~10 sec. Then, centrifuge for 5 min at 12,000 rpm with a microcentrifuge. **(Caution!! Be careful when you heat the sample at 98ºC. Heating it in PCR machine with heating lid is recommended.)**
7. Transfer the heated supernatant to a fresh tube. This supernatant will be used as the template in the PCR. Take 5µl supernatant as template for qPCR reaction

*If the template contains PCR inhibition materials, the DNA can be purified with a commercial extraction kit.

**Preparation of sample for EP 2.6.7 Guideline:**

**Genomic DNA extraction**

*DNA was isolated using a commercial kit, DNeasy ® Blood & Tissue Kit (Cat# 69504, Qiagen, Valencia, CA) following the procedure provided by the vendor

**Preparation**

All centrifugation steps are carried out at room temperature (15–25°C) in a micro centrifuge. PBS is required for use in step 2. Buffer ATL is not required in this protocol.

**Pre-Experimental Setup:**

1. Buffer AL may form a precipitate upon storage. If necessary, warm to 56°C until the precipitate has fully dissolved.
2. Buffer AW1 and Buffer AW2 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution. Preheat a thermo mixer, shaking water bath, or rocking platform to 56°C for use in step 4.

**Procedure**

1. Collect 1ml cell culture (1 x $10^6$ cell/ml) to a tube. Centrifuge for 5 min at 15,000 rpm.
   *When using a frozen cell pellet, allow cells to thaw before adding PBS until the pellet can be dislodged by gently flicking the tube.*

2. Decant the supernatant and re-suspend the pellet in 200 ul PBS.

3. Add 20 ul proteinase K and 20ul Internal Control DNA*. Continue with step 4.
   **The Internal Control DNA for sample prep. of the QUALICHEK™ kit is used to verify the DNA extraction step as well.*

4. Add 200 ul Buffer AL (without added ethanol). Mix thoroughly by vortexing, and incubate at 56°C for 10 min.
   *It is essential that the sample and Buffer AL are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution*

5. Add 200 ul ethanol (96–100%) to the sample, and mix thoroughly by vortexing.
   *It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution*

6. Pipet the mixture from step 5 into the DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at 10,000 rpm for 1 min. Discard flow-through and collection tube.

7. Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 ul Buffer AW1, and centrifuge for 1 min 10,000 rpm. Discard flow-through and collection tube.

8. Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 ul Buffer AW2, and centrifuge for 1 min at 20,000 x g (14,000 rpm). (Repeat step 8).

9. Remove the 2ml collection tube solution and centrifuge at 14,000rpmfor 3 minutes to dry the column membrane.
   *It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution. Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x g (14,000 rpm)*

10. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube, and pipet 100 ul Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 3 min at 10,000 rpm to elute.

| **Reaction Components** | **Sample Reaction** | **Control Reactions** | |
|---|---|---|---|
| | | **Positive Control** | **NTC (No Template Control)** |
| 2X qPCR Master Mix (Blue Cap) | 15 ul | 15 ul | 15 ul |
| Primer & Probe Mix including Internal DNA (Amber Tube and Cap) | 2 ul | 2 ul | 2 ul |
| Test Sample | 5 ul | - | - |
| Internal Amplification Control DNA (Orange cap) | -* | 2 ul | 2 ul |
| Positive Control DNA (Yellow Cap) | - | 1 ul | - |
| 50X ROX Reference dye** Low ROX / High ROX (Amber tube and Cap) | 0 ul (No ROX) Low ROX 0.6 ul (1X) High ROX 0.6µl (1X) | | |
| DNase Free Water (White Cap) | Up to 30 ul | | |
| Final Volume | 30 ul | 30 ul | 30 ul |

In the sample reaction, internal amplification control DNA is not added separately because the sample already include internal amplification control DNA through the sample preparation of DNA extraction procedure.

** ROX concentration for Instruments*

| Instruments | Reference Dye |
|---|---|
| **BioRad:** iCycler, MyiQ, MiQ 2, iQ 5, CFX-96, CFX-384,<br>**MJ Research:** Opticon, Option2, Chromo4, MiniOpticon<br>**Qiagen:** Roto-Gene Q, Roto-Gene3000, Roto-Gene 6000<br>**Eppendorf:** Mastercycler realplex<br>**Illumina:** Eco RealTime PCR System<br>**Roche:** LIghtCycler 480, LightCycler 2.0 | No ROX |
| **ABI:** 5700, 7000, 7300, 7700, 7900, 7900HT, 7900HT Fast, StepOne, StepOne plus | High ROX(1x) |
| **ABI:** 7500, 7500 Fast, Quantstudio (3, 5, 7)<br>**Stratagene:** MX3000, MX3005P, MX4000 | Low ROX(1x) |

Set up the qPCR instrument to run the PCR cycling (amplification) program specified below

| Steps & Cycle | | | Temp (ºC) | Time |
|---|---|---|---|---|
| Pre Heat | | | 95 | 5 min |
| **PCR** | **45 Cycles** | **Denature** | 95 | 20 sec |
| | | **Anneal*** | 60 | 30 sec |
| | | **Extend** | 72 | 30 sec |
| | | ***Acquisition Mycoplasma DNA - FAM(470~510nm), Green Channel<br>Internal DNA - HEX(535~555), Yellow Channel** | | |

## Results

A successfully performed PCR without inhibition is indicated by an increasing fluorescence signal in the internal control channel (HEX). The internal DNA can be detected with a yellow filter (535–555 nm for HEX). The presence of mycoplasma DNA in the sample is indicated by an increasing fluorescence signal at 510 nm (FAM) and is usually detected with a green filter (470–510 nm).

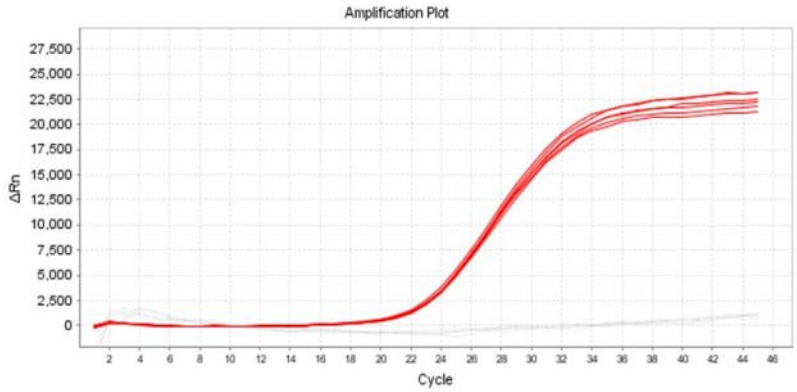
False-negative results (due to inhibition of PCR reaction by the sample matrix) can be detected individually for each sample as these reactions do not show any fluorescence signal.

Using the following table, determine whether the test cell culture is infected with Mycoplasma.

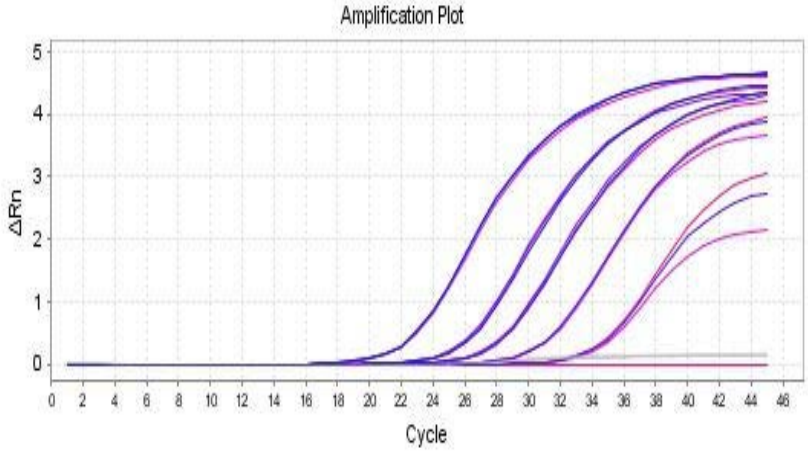
| FAM channel (Mycoplasma PCR) | HEX channel (Internal DNA) | Interpretation |
|---|---|---|
| Positive | Positive | Mycoplasma Contamination |
| Negative | Positive | Mycoplasma Non-contamination |
| Negative | Negative | PCR Inhibition or Inadequate sample preparation |

* In case of severe mycoplasma contamination, HEX can be not detected

Internal control amplification – HEX (yellow channel)




Sample and positive control amplification – FAM (green channel)




## Sensitivity

The sensitivity of the PCR using this kit is 1 to 10 copies of the target DNAper reaction. Sensitivity of the assay in real culture samples depends on the quality of the sample preparation.

## Specificity

The QUALICHEK™ Mycoplasma qPCR Detection Kit detects Mycoplasma species simply, reliably, and rapidly. To detect the presence of these microorganisms, the assay uses the polymerase chain reaction (PCR) to amplify a target unique to a wide variety of mycoplasmas. The kit can detect more than 50 different *Mycoplasma species, including Acholeplasma laidlawii and Spiroplasma citri*. The kit does not detect other genera or cell-line DNA.

### Mycoplasma species detected by QUALICHEK Mycoplasma qPCR Detection Kit

1. *Acholeplasma laidlawii*
2. **Mycoplasma arginine*
3. ** Mycoplasma fermentans*
4. **Mycoplasma gallisepticum*
27. *Mycoplasma imitans*
28. *Mycoplasma maculosum*
29. *Mycoplasma meleagridis*
30. *Mycoplasma mycoides*

5. *Mycoplasma hyorhinis*
6. *Mycoplasma orale*
7. *Mycoplasma pneumonia*
8. *Mycoplasma synoviae*
9. *Spiroplasma citiri*
10. *Mycoplasma agalactica*
11. *Mycoplasma alligatoris*
12. *Mycoplasma anatis*
13. *Mycoplasma arthritidis*
14. *Mycoplasma bovis*
15. *Mycoplasma bovigenitalium*
16. *Mycoplasma capricolum*
17. *Mycoplasma cloacale*
18. *Mycoplasma falconis*
19. *Mycoplasma faucium*
20. *Mycoplasma flocculare*
21. *Mycoplasma gallinarum*
22. *Mycoplasma genitalium*
23. *Mycoplasma hominis*
24. *Mycoplasma pneumonia*
25. *Mycoplasma hyopharyngis*
26. *Mycoplasma hyosynoviae*
31. *Mycoplasma muris*
32. *Mycoplasma neurolyticum*
33. *Mycoplasma opalescens*
34. *Mycoplasma penetrans*
35. *Mycoplasma pirum*
36. *Mycoplasma primatum*
37. *Mycoplasma pulmonis*
38. *Mycoplasma putrefaciens*
39. *Mycoplasma salivariumstrain*
40. *Mycoplasma spermatophilum*
41. *Mycoplasma sp. ovine/caprine*
42. *Mycoplasma sp. Putative*
43. *Mycoplasma suis*
44. *Mycoplasma sualvi*
45. *Mycoplasma timone*
46. *Ureaplasma diversum*
47. *Ureaplasma urealyticum*

**Mycoplasma species in EP 2.6. 7. Guidance on the Mycoplasma Test.*



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