Protocol

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Mycoplasma Detection Protocol

utilizing certus QC – mycoplasma Detection Kit

Introduction

The certus QC – mycoplasma detection kit is a ready-to-use isothermal master mix for simple, rapid and reliable detection of *Mycoplasma* and *Acholeplasma* species in culture. These bacteria are frequent contaminants of cell cultures in research laboratories and a major concern in industrial facilities working with primary cells or continuous cell lines. Contamination with these microorganisms may affect virtually every aspect of the quality and safety of biopharmaceuticals.

This assay is capable of detecting the six most common species, which account for >95% of contamination: *M. orale, M. hyorhinis, M. arginine, M. fermentans, M. hominis and A. ladilawii* (see complete species list on page 8). Due to sequence homology, many other *Mycoplasma* species will be detected as well.

The certus QC- mycoplasma detection kit has many benefits including generating **reproducible and reliable results in less than an hour, high sensitivity, minimal hands-on time, wide instrument compatibility and cost effectiveness.**

Test principle: The certus QC – mycoplasma detection kit is based on isothermal amplification of *Mycoplasma*-specific nucleic acids and real-time detection using a DNA-intercalating dye. The sample to be tested is simply added to a proprietary Lysis Buffer and the mixture is directly added to the primer/enzyme mix. Nucleic acid extraction is not necessary. Following isothermal amplification at 65°C, melting curve analysis is performed to distinguish a contaminated sample from a *Mycoplasma*-free specimen. An in-well internal control with a distinct melting temperature indicates correct performance of each amplification reaction (Figure 1). Here, we present a protocol for the use of certus QC – mycoplasma detection kit for rapid and effective detection of *Mycoplasma* and *Acholeplasma* species in cell culture.

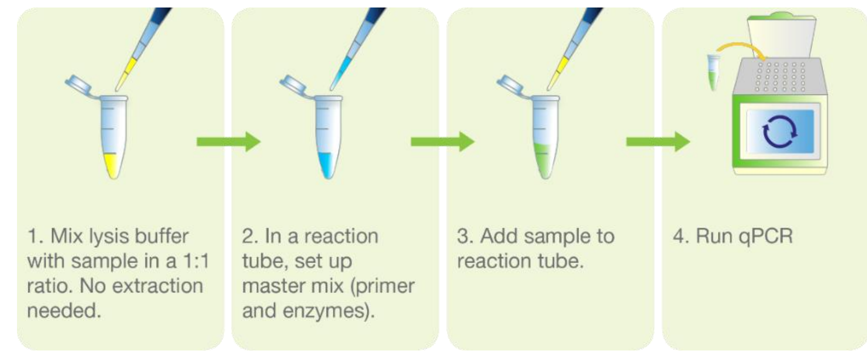

Figure 1. certus QC – mycoplasma Detection Kit protocol overview.

Materials

Included:

- certus QC – mycoplasma Detection Kit (GoldBio Catalog # MD-250)

Vial	Color Code	Label	Content (µl) 25 rxn	50 rxn	100 rxn
#1	blue	Primer Mix	55	105	210
#2	green	Enzyme Mix	400	800	1580
lys	yellow	Lysis Buffer	120	240	480
pos	red	Positive Control	60	120	240

The certus QC – mycoADVANCED detection kit includes all required controls (components are color-coded for ease of use):

- Primer Mix (blue label). Includes primers for an internal control (IC), which is only amplified in *Mycoplasma*-negative or low positive samples.
- Enzyme Mix (green label)
- A positive assay control (red label), which consists of an artificial DNA template containing the *Mycoplasma* target sequence. **It is not infectious and cannot cause contamination in cell lines**.
- Lysis Buffer (yellow label). A negative assay control can be made from diluting fresh medium in lysis buffer. Also includes an internal control (IC), which is only amplified in *Mycoplasma*-negative or low positive samples.

Not included:

- Real-time PCR instrument with appropriate PCR tubes/plates
- Micropipettes and pipette tips
- Microcentrifuge tubes

- Microcentrifuge

Storage and Handling

- The components of this kit may be shipped on blue ice and remain stable during transit.
- Upon arrival, store vials at -20°C. See vial label for expiration date.
- Repeated freeze-thaw cycles may impact the quality of the primers. Aliquot the Primer Mix (vial #1) prior to storage.

Method

Perform all steps on ice or in a cooling block and set up the reactions as quickly as possible.

1. Sample preparation
   a. Mix the sample (e.g. cell culture supernatant) with Lysis Buffer in a ratio of 1:1 in a microcentrifuge tube. A single reaction requires total volume (sample and supernatant) of 8 µl.

   Note: For increased sensitivity we recommend testing each sample in duplicates or more. For example, if a sample is tested in duplicates, mix 9 µl with 9 µl Lysis Buffer to compensate for sample loss (a single reaction requires 8 µl).

   b. For the Negative Assay Control, mix 5 µl fresh medium with 5 µl Lysis buffer in a microcentrifuge tube.
   c. For the Positive Assay Control, mix 5 µl of the Kit Positive Control with 5 µl fresh medium in a microcentrifuge tube.

2. Amplification and Detection
   a. Set up a master mix for the required amount of reactions plus an extra 2 reactions (to compensate for sample loss) in a microcentrifuge tube as shown below. All reagents must be thawed, gently mixed by inversion and centrifuged briefly before use.

Vial	Label	Volume
#1	Primer Mix	2.0 µl
#2	Enzyme Mix	15.0 µl

   b. Briefly mix and spin down the master mix, then pipette 17 µl into individual wells/tubes.

c. Add 8 µl of the prepared sample, Negative Assay Control (fresh medium diluted in Lysis Buffer) or Positive Assay Control (diluted in fresh medium) to the individual wells/tubes containing the master mix.

Note: Each run should include at least one Positive and one Negative Assay Control.

d. Seal the PCR plate or cap tubes and centrifuge briefly to remove air bubbles.

Note: Make sure that all wells are sealed properly to avoid carry-over contamination!

e. Use the following isothermal amplification and dye acquisition profile for **real-time PCR instruments**:

Component	Cycles	Temperature	Acquisition	Time	Ramp rate Roche	Ramp rate ABI
Amplification	26	65°C	None	27 s	4.4°C	100%
			Single	30 s	4.4°C	100%
Melting curve	1.0	84°C	None	5 s	4.4°C	100%
		97°C	Continuous (10 readings/°C)	1 s	0.1°C	0.3%

Note: as this is an isothermal amplification method, both steps during amplification are performed at 65°C.

f. Measure fluorescence in the SYBR Green channel.

3. Interpretation of Results
To determine if your sample is contaminated with *Mycoplasma*, compare the melting temperature (Tm) to the Positive and Negative Assay Controls (Table 1). C_t values are not relevant for result interpretation.

Table 1. Result interpretation based on melting temperatures of the reaction product.

Melting temperature [°C] comparable to	Result
Negative Assay Control ± 0.5°C	Amplification of the Internal Control (IC) **-> *Mycoplasma* - negative**
Between -1°C and +2°C of the Positive Assay Control	Amplification of *Mycoplasma* DNA **-> *Mycoplasma* - positive**
Negative Assay Control ± 0.5°C AND Between -1°C and +2°C of the Positive Assay Control	Amplification of both the IC and *Mycoplasma* DNA **->** Repeat testing of the sample if the peak for *Mycoplasma* is only very low. If the sample is repeatedly positive, it is **contaminated with a low amount of *Mycoplasma***. If it is negative, an artifact occurred during initial testing.
> +2°C of the Positive Assay Control OR < -0.5°C of the Negative Assay Control	**Contamination with other bacteria or unspecific amplification** (almost exclusively occurs in combination with a melting peak corresponding to the IC). This peak can be ignored.

Note: Even in *Mycoplasma*-negative samples and in the Negative Assay Control, an amplification curve is observed. This is due to an Internal Control (IC), which is included to detect false-negative results due to inhibition of the polymerase. In highly *Mycoplasma*-contaminated samples, amplification of the IC is suppressed.

Note: Melting temperatures can vary depending on the instrument and matrix (e. g. cell culture medium or cell density).

If the Tm of the sample corresponds to the Tm of the Negative Assay Control ±0.5°C, it is considered *Mycoplasma* negative. In this case, the Internal Control was amplified (Figure 2A). On the Roche LightCycler® 96, the Tm of the IC is around 94.5°C, the Tm may vary slightly depending on the specific instrument.

If it is between -1°C and +2°C of the Tm of the Positive Assay Control, the sample is considered *Mycoplasma*-positive (Figure 2B). On the Roche LightCycler® 96, the Tm of *Mycoplasma* is between 86 and 89°C. A higher the variance is due to differences between the *Mycoplasma* species detected.

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Samples contaminated with a low amount of *Mycoplasma* may show two melting peaks, one for the *Mycoplasma* target and one for the Internal Control (Figure 2C).

Amplification Curve | **Melting temperature (Tm)**

A) Negative sample — IC

B) Positive sample — Mycoplasma

C) Low positive sample — Mycoplasma, IC

Figure 2. Example of amplification curves and melting temperatures. If the sample is *Mycoplasma*-negative, the Internal Control (IC) is amplified around C_t 20 with a melting peak similar to that of the Negative Assay Control (A). If a sample is highly contaminated with *Mycoplasma*, a single peak shows up near the Tm of the Positive Assay Control (B). In samples with a low amount of *Mycoplasma*, the C_t is similar to a negative sample, but melting curve analysis reveals two peaks: one for *Mycoplasma* and one for the IC (C).

The following *Acholeplasma* and *Mycoplasma* species have been tested and detected:

- *A. laidlawii*
- *M. arginini*
- *M. fermentans*
- *M. hominis*
- *M. hyorhinis*
- *M. orale*
- *M. synoviae*

Additional *Mycoplasma* species that have been tested and detected include:

- *M. adleri*
- *M. arthritidis*
- *M. bovirhinis*
- *M. bovis*
- *M. bovoculi*
- *M. californicum*
- *M. ciconiae*
- *M. cottewii*
- *M. dispar*
- *M. fastidiosum*
- *M. feriruminatoris*
- *M. gallinarum*
- *M. genitalium*
- *M. lagogenitalium*
- *M. leachii*
- *M. meleagridis*
- *M. microti*
- *M. mucosicanis*
- *M. neophronis*
- *M. penetrans*
- *M. pulmonis*
- *M. salivarium*
- *M. sturnidae*
- *M. sualvi*

For research only. Not intended for diagnostic or therapeutic use.

Associated Products

- certus QC – mycoADVANCED Detection Kit (GoldBio Catalog # MD-500)

- Sterile Filtered DMSO (GoldBio Catalog # D-361)
- Sodium Pyruvate (GoldBio Catalog # S-117)
- PBS (Phosphate Buffered Saline) Tablets (GoldBio Catalog # P-271)
- HEPES, Free Acid (GoldBio Catalog # H-400)
- Ciprofloxacin (GoldBio Catalog # C-860)
- Ciprofloxacin HCl (GoldBio Catalog # C-861)
- Gentamicin Sulfate, USP Grade (GoldBio Catalog # G-400)
- Gentamicin Sulfate 50 mg/ml Solution (GoldBio Catalog # G-400-SL)
- Gentamicin Sulfate EZ Pak™ for 50 mg/ml Solution (GoldBio Catalog # G-400-EZ10)
- Kanamycin Monosulfate, USP Grade (GoldBio Catalog # K-120)
- Kanamycin Monosulfate 50 mg/ml Solution (GoldBio Catalog # K-120-SL)
- Kanamycin Monosulfate EZ-Pak™ for 50 mg/ml Solution (GoldBio Catalog # K-120-EZ)

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