

Aug 24, 2024

Version 1

Mycoplasma Testing Protocol V.1

DOI

<https://dx.doi.org/10.17504/protocols.io.81wgbxw71lpk/v1>

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Protocol Citation: Carolina Lopez 2024. Mycoplasma Testing Protocol. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.81wgbxw71lpk/v1> Version created by **Sydney Faber**

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Protocol status: Working

We use this protocol and it's working

Created: July 26, 2024

Last Modified: August 24, 2024

Protocol Integer ID: 104165

Keywords: mycoplasma testing protocol mycoplasma testing protocol, protocol, following manufacturer

Disclaimer

These protocols are adapted from the manufacturer's protocols.

Abstract

Mycoplasma Testing Protocol (Adapted following manufacturer's instructions)

Materials

Antibiotic-free media: Filter through 0.2 µm filter

	Component	Amount	Conc. Supp.	Product information
	DMEM	500 mL		Gibco Cat. # 11965092 (#11965118-cs)
	Sodium Pyruvate	5.0 mL	1mM	Corning Cat #25-000-Cl – 100mM
	L-Glutamine	5.5 mL	2 mM	Sigma Aldrich Cat #G7513 – 200mM
	FBS	50 mL	10%	

Luminescence Test Materials:

- MycoAlert PLUS Detection Kit, Lonza, cat #LT07-710.
- Opaque 96 well plate – Falcon cat #353296

PCR Test Materials

- Mycoplasma Detection Kit, Southern Biotech, Cat. No 13100-01
- Platinum Taq DNA Polymerase, Invitrogen, Cat. No 10966018
- UltraPure Agarose, Invitrogen, Cat. No 16500500

Mycoplasma Testing Protocol

1 Reagents for luminescence test:

- MycoAlert PLUS Detection Kit, Lonza, cat #LT07-710.
- Positive Control – supernatant from positive sample, 75ul aliquots stored at -80°C
- Negative Control – Water or sample from myco-free cell line, NOT unused media
- Opaque 96 well plate – Falcon cat #353296
- Antibiotic-free media (see materials)

Reagent preparation and storage conditions for MycoAlert Kit Reagent/Substrate:

- Resuspend in provided buffer, aliquot, and store at -80°C.
- Room temp – must be used within 5 hours
- 2-8°C – store for up to 5 days
- -80°C – store for up to 6 months, allow to thaw to room temp without the use of artificial heat, once thawed - aliquots should not be refrozen

2 Sample Preparation:

1. Plate cells in a 6-well plate in 2mL of Antibiotic-free media such that cells are 80-100% confluent at 48hr without being over confluent.
2. At 48hr, collect 1.5 mL of media in 1.5mL tube
3. Centrifuge at 200xg for 5 minutes
4. Transfer 1mL of supernatant to a fresh tube
5. Store at 4°C until testing (within 1 week)

3 Sample Testing:

1. Allow all reagents to warm to room temperature (~15 minutes)
2. Load 50µl of sample to a white opaque 96-well plate

Note

The company recommends using 100 µL. We scale down the reaction by half to 50 µL to save on reagents.

3. Add 50µl of MycoAlert Plus Reagent to sample and mix
4. Wait 5 minutes
5. Measure luminescence with a 1s integration time, auto gain (Read A)
6. Add 50µl of MycoAlert Plus Substrate to sample and mix
7. Wait 10 minutes
8. Measure luminescence with a 1s integration time, auto gain (Read B)

4 Results:

Divide Read B by Read A to produce a ratio

Ratio / Interpretation

< 1.2 / Negative for Mycoplasma - Okay to keep using

1.2-1.8 / Borderline, residual contamination - Test using second method (PCR)

> 1.8 / Positive for Mycoplasma - Discard cells

Note

These ratios/interpretations are adjusted from the original recommendations from the manufacturer after many rounds of testing and performing 2nd testing methods (PCR).

2nd Testing Method (PCR)

5 Reagents for PCR test:

- Mycoplasma Detection Kit, Southern Biotech, Cat. No 13100-01
- Platinum Taq DNA Polymerase, Invitrogen, Cat. No 10966018

Reagent Preparation

- Centrifuge all kit component tubes to ensure that material has completely settled to bottom
- Reconstitute primer/dNTP mix, positive, and internal control using DNase free water as follows -
 - Primer/dNTP mix - 260 µL
 - Positive control - 200 µL
 - Internal control - 200 µL
- Vortex for 5 seconds to ensure thorough mixing and equilibrate at room temperature for 5 minutes
- Vortex, then centrifuge again
- Reconstituted reagents should be stored at or below -20°C; avoid multiple freeze/thaw cycles

6 Sample Preparation

1. Use the sample collected from the 6-well plate in the above step.
2. Transfer 100 µL of the cell culture supernatant to a sterile tube; ensure the lid has been tightly sealed to prevent evaporation
3. Heat the sample at 95°C for 5 minutes
4. Quickly spin the sample supernatant at maximum speed for 5 seconds to remove cell debris; the supernatant is now ready to add to the PCR master mix

7 Sample Testing

1. Prepare PCR Master Mix.

- Total volume per reaction is 50 μL (25 μL for 1/2)

Note

The company recommends the total volume per reaction to be 50 μL . We have tried cutting the volumes in 1/2 while maintaining ratios to save reagents and have had no issues. If using the 1/2 reaction for the first time, run controls with the full reaction volume to ensure the results are as expected.

- When preparing reactions, calculations should include enough reagents for positive and negative controls
- Contents of the master mix are provided below -

	Components	1/2 reaction (μL)	Full reaction (μL)
	Water	17.8	35.6
	10X reaction buffer	2.5	5
	Primer/dNTP mix	2.5	5
	Internal control	1	2
	Taq polymerase	0.2	0.4

2. Add 48 μL (24 μL for 1/2) of master mix to each tube

3. Add 2 μL (1 μL for 1/2) of DNase-free water as a negative control into the appropriate PCR reaction tube

3. Add 2 μL (1 μL for 1/2) of heat-treated test sample into the appropriate PCR reaction tube

4. Add 2 μL (1 μL for 1/2) of the reconstituted positive control DNA supplied in the kit into the appropriate PCR reaction tube

5. Run according to the thermal cycling program

- Incubation times are dependent on the type of polymerase used; the template described below has been optimized for Hot Start Taq polymerase

	Temp ($^{\circ}\text{C}$)	Time	Cycles
	95 $^{\circ}\text{C}$	5 minutes	1
	95 $^{\circ}\text{C}$	30 seconds	35
	58 $^{\circ}\text{C}$	40 seconds	
	72 $^{\circ}\text{C}$	1 minute	



	Temp (°C)	Time	Cycles
	4°C	–	Hold

6. Prepare a 1.2% agarose gel containing 0.5µg/mL Ethidium bromide
7. Load 18 µL of each PCR reaction mixed with 3 µL of 6X loading buffer into each well
8. Run at 110 volts for ~30 minutes
9. Detect PCR product bands using ChemiDoc

8 Results (Gel Evaluation)

- Samples containing *Mycoplasma* infection will contain a band (or multiple bands) between ~448 bp to ~611 bp
- Samples containing the supplied internal control DNA will contain a distinct 270bp PCR product that is clearly distinguishable from the ~488 bp to ~611 bp band(s) found in the positive samples
 - The internal control indicates a successfully performed PCR reaction
 - PCR inhibition may have occurred if the internal control band disappears in some samples, but the band is present in the PCR reaction of the negative control (water added instead of the test sample). If the PCR of a sample is inhibited, the inhibitors can be easily removed by performing a DNA extraction.
- If the cell culture is heavily contaminated with *Mycoplasma*, amplification of the ~488 bp to ~611 bp product(s) may diminish or completely eliminate the 270-bp internal control product
- Low-intensity bands smaller than 100 bp indicate the presence of non-specific, self-annealing primers; this does not indicate a positive result and will not affect the precision of the test

Protocol references

[MycoAlert PLUS Mycoplasma Detection Kit](#)

[MycoAlert PLUS Manufacturer Protocol](#)

[SouthernBiotech Mycoplasma Detection Kit](#)

[SouthernBiotech Mycoplasma Detection Kit User Guide](#)