AnyGenes®
biomarkers & signaling pathways profiler

MycoDiag *Mycoplasma* Detection Assays

User Guide

For detection of *Mycoplasma* DNA in cell cultures by qPCR (or PCR)

(for 20, 50 or 100 reactions)

For Research Use Only

Store at -20°C & keep away from light

2021

SUMMARY

I. Product information

1) Introduction

In order to detect as soon as possible contamination of cell cultures by *Mycoplasma*, AnyGenes developed the **MycoDiag *Mycoplasma* Detection assays,** that are fast and complete solutions (Perfect Master Mix SYBR Green, MycoDiag Primer sets and Quality Controls) intended to the detection of *Mycoplasma* DNA by **qPCR (or PCR)**, directly from cell culture supernatants.

With primer sets designed with very stringent criteria and **experimentally validated** by strict quality control process, the performance of AnyGenes® MycoDiag assays has been carefully optimized to provide you a **very high sensitivity and specificity** to detect the **25 most representative *Mycoplasma* species** (that represents ~95% of cell culture contaminations). Our MycoDiag Primer Sets allows the detection and amplification of DNA from: *M. agalactiae,* ***M. arginini,*** *M. arthritidis, M. bovis, M. cloacale, M. falconis, M. faucium,* ***M. fermentans,*** *M. gallisepticum, M. genitalium,* ***M. hominis, M. hyorhinis,*** *M. hyosynoviae, M. opalescens,* ***M. orale, M. pirum,*** *M. pneumoniae M129, M. pulmonis,* ***M. salivarium,*** *M. spermatophilum, M. synoviae, M. timone,* ***A. laidlawii,*** *Spiroplasma, U. urealyticum*.

Directly from cell culture supernatants, MycoDiag assays allow to detect *Mycoplasma* contamination **in less than 2 hours**, according to your qPCR or PCR instrument, supported by quality controls (Negative Control, Positive Control with a *Mycoplasma* DNA, and Internal Control to validate the absence of PCR inhibitors in your samples).

For more details, see www.anygenes.com.

✓ **Quality Control**

As part of our routine quality assurance program, all AnyGenes® products are monitored to ensure the highest levels of performance and reliability.

2) Intended use

For molecular biology research use only. This kit is not intended for diagnosis, prevention or therapeutic applications. AnyGenes® will be not responsible of the misuse of their products.

3) Kit contents

These MycoDiag assays contain according to our catalog references:

Catalog Ref #	Contents	Number of reactions
MycoDiag-X20	1 x MycoDiag-Primers-20 Primer Sets vial (192 µl) 1 x InternalCTR-Primers-20 Primer Sets vial (144 µl) 1 x Perfect Master Mix SYBR Green (2X) vial (PMS1-X / 0.72 ml) 1 x Myco POS-20 vial (48 µl) 1 x Internal CTR-20 vial (48 µl) 1 x PCR grade H_2O vial (1 ml)	20 reactions
MycoDiag-X50	1 x MycoDiag-Primers-50 Primer Sets vial (480 µl) 1 x InternalCTR-Primers-50 Primer Sets vial (360 µl) 1 x Perfect Master Mix SYBR Green (2X) vial (PMS1-X / 1.8 ml) 1 x Myco POS-50 vial (120 µl) 1 x Internal CTR-50 vial (120 µl) 1 x PCR grade H_2O vial (1 ml)	50 reactions
MycoDiag-X100	1 x MycoDiag-Primers-50 Primer Sets vial (960 µl) 1 x InternalCTR-Primers-50 Primer Sets vial (720 µl) 1 x Perfect Master Mix SYBR Green (2X) vial (PMS1-X / 3.6 ml) 1 x Myco POS-50 vial (240 µl) 1 x Internal CTR-50 vial (240 µl) 2 x PCR grade H_2O vial (2 x 1 ml)	100 reactions

* X = W, R, LR, F type according to your qPCR instrument or P for PCR application
Please refer to our qPCR instruments & AnyGenes® Perfect Master Mix compatibility file before ordering

For more product information, please visit **www.anygenes.com** or contact us at **technical@anygenes.com**

4) Storage & stability

Upon receipt, store MycoDiag assays at -20°C until their use. These storage conditions guarantee a long-term storage of AnyGenes® products for a minimum period of 6 months after their receipt. Moreover, in order to guarantee the stability of these products, avoid repeated freezing and thawing cycles. If small volumes of reagents are frequently required, we recommend to stock alicots at -20°C.

5) Additional reagents and equipment required

A) Reagents :

- Cell culture supernatants
- Ultra-pure & sterile « nuclease, *RNAse, DNAse free »* H_2O

Caution : Do not use DEPC H_2O !!!

B) Material :

- Heat-Block
- 96- or 384-well qPCR plates or equivalent

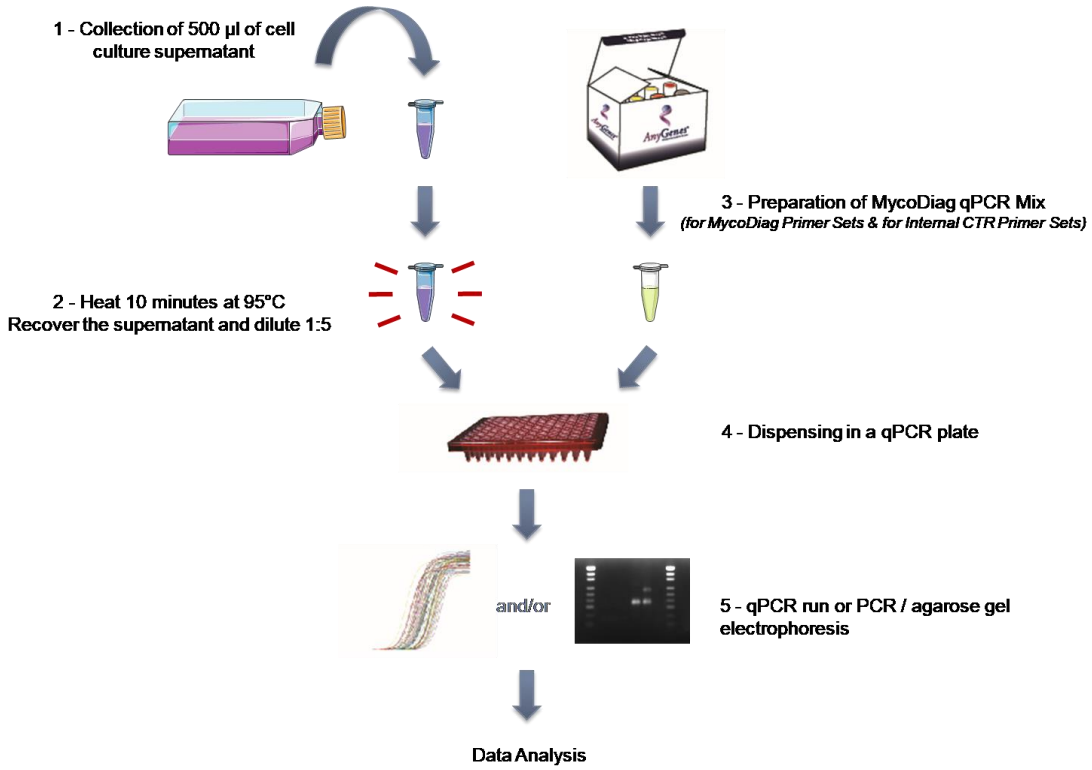
- Real-time quantitative PCR instrument (Light Cycler® 480 (Roche®), ABI 7900®, ABI 7500® (Applied Biosystems® / Life Technologies®)...) or PCR Thermocycler
- PCR plates centrifuge
- Vortex mixer and Mini-centrifuge
- Sterile “nuclease, RNase, DNase free” tips and tubes
- Pipettes

II. Protocol

1) Before you start...

To obtain reliable and reproducible results and avoid contamination and false-positive signals, it is important and necessary to follow Good Laboratory Practices.

2) Quick Procedure Scheme



3) Procedure

To optimize the detection of potential *Mycoplasma* contamination, we strongly recommend to collect your samples **after 24h of incubation of with new cell culture medium** (whether after thawing cells, or medium change), when cells are **at more than 70-80% confluence.** Because of the potential accumulation of PCR inhibiting substances, avoid to keep too long older cell cultures.

Some other few points to follow :

- **Do not test cell culture directly from a cryotube** because the presence of DMSO can strongly alter the qPCR/PCR reaction and the potential *Mycoplasmas* may be not sufficient to overpass the detection limit.

- Always collect your supernatant before adding trypsin. Trypsin may inhibit subsequent detection by qPCR/PCR.

Preparation of your cell culture samples

1) Prepare a Heat-Block at 95°C.

NB : Please avoid to use water bath that could also contaminate your samples.

2) Collect 500 µl of cell culture supernatant in a new 1.5 ml sterile tube.

3) **Tightly close the caps** with parafilm or equivalent, in order to prevent the opening of your tubes and so, potential cross contamination, during the heating step.

4) Incubate your tube at **95°C during 10 minutes.**

5) Centrifuge briefly the tubes during 30 seconds at 13 000 rpm to pellet cellular debris.

6) **Transfer 10 µl of the supernatant** in a new 0.5 ml sterile tube.

NB : We strongly recommend to perform your qPCR directly after collection or your cell culture supernatants. These samples can be kept 48h at 4°C or 1 week at -20°C before their analysis. Keep it out of the work area during the qPCR preparation mix to avoid any possible contamination.

7) **Dilute each supernatant sample with 40 µl PCR grade H_2O**.

NB : The performance of MycoDiag assays is such that it allows to detect *Mycoplasma* contamination in 1:5 diluted samples, without affecting the detection sensitivity.

Preparation of your qPCR or PCR mix

1) Thaw MycoDiag reagents 20 minutes before use, in order that slowly reaches room temperature. You can also work with your samples on ice.

2) Prepare the work area (highly recommended under workstation) by carefully cleaning all material and areas with a suitable detergent and then decontaminating the workstation through exposure to UV.

3) Meanwhile, briefly centrifuge tubes and reagents and **prepare the 2 reaction mixes** (1 with MycoDiag Primers and 1 with Internal CTR Primers) in a 1.5 ml tube according to the following table :

Reagents	**Volumes / reaction**	**Reaction mix with MycoDiag Primers**	**Reaction mix with Internal CTR Primers**
		Volumes x (n+3) reactions	**Volumes x (n+2) reactions**
2X Perfect Master Mix SYBR Green®	**10 µl**	**(n+3) x 10 µl**	**(n+2) x 10 µl**
PCR grade H_2O	**2 µl or 4 µl** *according to the qPCR mix*	**(n+3) x 4 µl**	**(n+2) x 2 µl**
Primer Sets	**4 µl**	**(n+3) x 4 µl**	**(n+2) x 4 µl**
Total Reaction Volume	**16 µl**	**(n+3) x 18 µl**	**(n+2) x 16 µl**

NB: n = number of analyzed cell culture supernatants
- For MycoDiag Primers : (n + 3) corresponds to the number of analyzed cell culture supernatants + negative control (with H_2O) + positive control with MycoPOS + 1
- For Internal CTR Primers : (n + 2) corresponds to the number of analyzed cell culture supernatants + negative control (with H_2O) + 1

4) Mix thoroughly with a pipette or briefly centrifuge the 2 qPCR mixes.

5) Prepare the qPCR plate layout, depending on the number of cell culture supernatants. Please see below an example for a qPCR test performed for 3 analyzed cell culture supernatants.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		NEG	NEG									
C		SUPERNA-TANT 1	SUPERNA-TANT 1									
D		SUPERNA-TANT 2	SUPERNA-TANT 2									
E		SUPERNA-TANT 3	SUPERNA-TANT 3									
F		MYCO POS										
G												
H												

Legend :
In blue : 18 µl per well of the qPCR reaction mix with MycoDiag Primers + 2 µl of sample or control
In green : 16 µl per well of the qPCR reaction mix with Internal CTR Primers + 2 µl of sample & 2µl of internal control (or 4µl H_2O for negative control)

6) **Heat 5 minutes at 50°C the Myco POS and Internal CTR vials.**

7) Dispense **18 µl per well of the qPCR reaction mix with MycoDiag Primers** on the qPCR plate or equivalent. Dispense **16 µl per well of the qPCR reaction mix with Internal CTR Primers** on the qPCR plate or equivalent, in another column or row.

8) **In MycoDiag qPCR mix wells, add 2 µl of your diluted cell culture supernatant** per well **for the quality control of your samples, PCR grade H_2O for negative control (NEG), or Myco POS for positive control (POS),** according to the following table :

	Negative Control	Quality Control of cell culture supernatants	Positive Control
Reagents	**Volumes / reaction**		
MycoDiag qPCR mixes	**18 µl**	**18 µl**	**18 µl**
Diluted Samples	-	**2 µl**	-
PCR grade H_20	**2 µl**	-	-
Myco POS	-	-	**2 µl**
Total Reaction Volume	**20 µl**	**20 µl**	**20 µl**

NB: Change tips to avoid cross contamination once it is necessary.

9) **In Internal CTR qPCR mix wells, add 2 µl of your diluted cell culture supernatant + 2 µl of Internal CTR** per well **for the quality control of your samples, or 4 µl of PCR grade H_2O for negative control (NEG),** according to the following table :

	Negative Controls	Quality Control of cell culture supernatants
Reagents	**Volumes / reaction**	
Internal CTR qPCR mixes	**16 µl**	**16 µl**
Diluted Samples	-	**2 µl**
PCR grade H_20	**4 µl**	-
Internal CTR	-	**2 µl**
Total Reaction Volume	**20 µl**	**20 µl**

NB: Change tips to avoid cross contamination once it is necessary.

10) Cover the plate with a suitable optical sealing foil.

Caution: Do not prepare your qPCR mix too early to ensure reliable and reproducible results. However, if your plate was prepared before the start of the qPCR run, keep the qPCR plate on ice or at 4°C in a refrigerator.

11) Centrifuge the plate 15-60 s at 1 000 g to remove any bubbles.

12) Meanwhile, prepare and check the run program under the following qPCR conditions (compatible with most qPCR instruments):

Phase	Number of cycles	Time	Temperature	Acquisition mode	Commentaries
Initial denaturation - HOT start Taq activation	**1**	**10 min**	**95°C**	**/**	**« Hot-start DNA Taq polymerase » activation**
Amplification	**40-45**	**10 s**	**95°C**	**/**	**Denaturation of cDNA brands**
		30 s	**60°C**	**quantification**	**Hybridation & elongation steps with fluorescence acquisition**
Melting curves	**1**	**10 s**	**95°C**	**/**	**Melting curves**
		30 s	**65°C**	**/**	
		0 s	**95°C**	**continuous**	

For further informations, please contact technical support AnyGenes® via technical@anygenes.com

13) Place the qPCR plate in your qPCR instrument.

14) Start the qPCR run, following the manufacturer's recommendations and protocols.

III. Interpretation of the results

This qPCR/PCR test has to include all quality controls (negative and positive controls) provided in this MycoDiag assay, to ensure the success of your performed qPCR and so, the reliability of the obtained results.

The presence of *Mycoplasma* contamination in your samples corresponds to an qPCR amplification of with MycoDiag Primer Sets (Cq < 35), if and only if, (1) the negative controls are correct (Cq > 35), (2) the internal controls are adequate for the corresponding analyzed samples (25 < Cq < 29), showing the good

performance of the qPCR/PCR reaction with your cell culture medium components, (3) all the protocol instructions have been carefully followed.

If the quality controls are not those expected, we strongly recommend to repeat the qPCR test.

1) Validation by qPCR analysis

Primer Sets	Analyzed Sample	Expected Result	Interpretation	Incorrect result & Possible solution(s)
MycoDiag Primer Sets	Negative Control (H_2O)	Cq > 35	Correct negative quality control	If Cq < 35 : possible contamination of your qPCR mix preparation, H_2O, tips or tubes. Please check the Tm (only primer dimers formation if Tm < 78)
	Cell culture supernatant	Cp > 35	No *Mycoplasma* contamination	*
		Cp < 35	Possible *Mycoplasma* contamination	*
	Myco POS	Cp < 35	*Mycoplasma* contamination	Cq > 35 : Failed positive control reaction
Internal CTR Primer Sets	Negative Control (H_2O)	Cq > 35	Correct negative quality control	If Cq < 35 : possible contamination of your qPCR mix preparation, H_2O, tips or tubes. Please check the Tm (only primer dimers formation if Tm < 78)
	Cell culture Supernatant + Internal CTR	25 < Cq < 29	No PCR inhibition	If Cq > 30 : qPCR reaction is inhibited, please repeat the test

2) Validation by Agarose Gel Electrophoresis

1) Prepare a 1,5-2% agarose gel.

2) Briefly mix your qPCR products.

3) In new tubes or a PCR plate, mix 5-10µl of PCR products with an appropriate volume of your 6X loading buffer.

4) Load each sample in an additional well, and add an appropriate size marker on both sides of your gel.

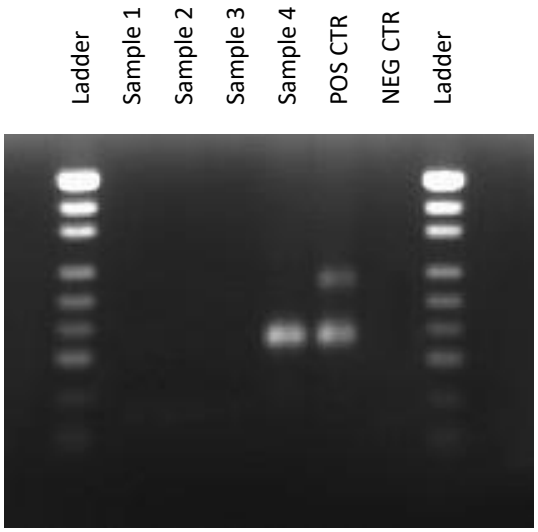
5) Run the gel at ~100-120V during 30 minutes (it can depend on your electrophoresis system).

6) Observe the results under the UV light system, and take a picture.

7) Analyze your results according the following table :

Primer Sets	Analyzed Sample	Expected Result	Interpretation	Incorrect result & Possible solution(s)
MycoDiag Primer Sets	Negative Control (H_2O)	No signal	Correct negative quality control	If PCR product : possible contamination of your qPCR mix preparation, H_2O, tips or tubes.
	Cell culture supernatant	No signal	No *Mycoplasma* contamination	*
	Cell culture supernatant	PCR product(s)	Possible *Mycoplasma* contamination	*
	Myco POS	PCR products at 129-142 bp & 230 bp	*Mycoplasma* contamination	The intensity of the bands can differ without altering the results If no PCR product : Failed positive control reaction
Internal CTR Primer Sets	Negative Control	No signal	Correct negative quality control	If PCR product : possible contamination of your qPCR mix preparation, H_2O, tips or tubes.
	Cell culture Supernatant + Internal CTR	PCR product at 92 bp	No PCR inhibition	If no PCR product : PCR reaction is inhibited, please repeat the test

Example of 2% Agarose Gel Electrophoresis :



Legend: Samples 1, 2 and 3 : negative results ; Sample 4 : positive result, so Mycoplasma contamination in this cell culture supernatant ; POS CTR : Positive Control with bands at 129-142 and 230 bp, NEG CTR : Negative Control , absence of amplification

V. Additional Information

For any further information, please contact the AnyGenes® technical support via the following email address : **technical@anygenes.com**

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