AKRON BIOTECH

TEL: (561) 750-6120 FAX: (561) 750-6140
1095 Broken Sound Parkway
Boca Raton, FL 33487
www.akronbiotech.com

MycoSolutions™ - Real-Time PCR Mycoplasma Detection Kit

Ready-to-use Real Time PCR Mix for the detection of Mycoplasma in cell culture

Methods of Use

Akron Catalog # **AK9752-0020** (20 tests)

Introduction:

MycoSolutions™ - Mycoplasma Real-Time PCR Detection Kit is designed to detect the presence of *Mycoplasma* contaminating biological materials, such as cultured cells.

Mycoplasma detection by the direct culture procedure is time-consuming and some *Mycoplasma* species are difficult to cultivate. With real-time PCR testing, results are obtained in less time than the traditional PCR methods, since the presence of contaminant *Mycoplasma* are detected in real-time as the DNA samples get amplified.

Only one protocol is needed for the detection of all *Mycoplasma* species. The primer set is highly specific and allows for detection of over 100 different *Mycoplasma* species, including *M. orale*, *M. hyorhinis*, *M. arginini*, *M. fermentans*, *M. salivarium*, *M. hominis*, usually encountered as contaminants in cell cultures, but also *M. pneumoniae*, *Acholeplasma laidlawii*, *M. synoviae*, *Spiroplasma citri*, and *Ureaplasma* species. Eukaryotic and bacterial DNA is not amplified by using the kit.

Kit Components:

Component	Volume	Description	Storage
Lysis Buffer	1000 µl	Lytic Agent	4°C
SYBR Green PCR Master Mix 2X	400 µl	Buffer, dNTPs mix, taq polymerase, SYBR® Green dye and ROX(a passive dye for proper well to well fluorescent normalization)	4°C
10X Primer Solution	70µl	Proprietary mix of Forward and Reverse Primers	-20°C
High Melting Positive Control (DPC)*	100 µl	1000 copies/µl of modified *Mycoplasma* DNA sequence in TE Buffer*	-20°C minimize freeze-thaw cycles
Negative Control	250 µl	Molecular Biology Water	-20°C

* The Discriminatory Positive Control (DPC) is a plasmid containing a modified *Mycoplasma* DNA sequence. When this DNA is used as a template for PCR reaction it generates an amplicon which has a melting temperature (T_m) of approximately 86°C. This melting point is outside the range of amplicons generated from real *Mycoplasma* samples (75 - 85°C).
Therefore, this property let to discriminate between a real positive test result from *Mycoplasma* and the positive control.
This particular positive control DNA enables the user to test samples for inhibitory conditions detecting false negatives and at the same time eliminates the possibility of a false positive test result due to accidental cross contamination of a test sample with the positive control DNA.

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Equipment Required (not included):

1. qPCR device with filters for the detection of the fluorescence dyes FAM and ROX
2. PCR reaction tubes for the specific qPCR device
3. 1.5 ml sterile microcentrifuge tubes, DNA and RNA free
4. Pipettes with corresponding filter tips (10, 100, 1000 µl)

Test Length:

The test takes approximately 85 minutes to complete, less than four hours total from sample to results.

Storage:

See table above for storage instructions of different components.

Principle:

Real-time PCR Mycoplasma Test utilizes real-time polymerase chain reaction (RT-PCR or qPCR). The primer set is specific for the highly conserved rRNA operon, or more specifically, the 16S-23S rRNA coding region in the *Mycoplasma* genome. rRNA gene sequences of prokaryotes, including *Mycoplasmas*, are well conserved, whereas, the lengths and sequences of the spacer region in the rRNA operon (the region between 16s and 23s gene) differ from species to species.

The detection procedure consists of:

1. Amplification of gene region using two primers in a real-time PCR device.
2. Detection of the amplified fragment by computer-generated Mycoplasma DNA concentration graph.

To determine a negative or positive reaction, see the section titled "Guidance for test samples" section G. below.

Note: This system does not allow the amplification of DNA originating from the sources, such as tissue samples or bacteria, which could affect the detection result.

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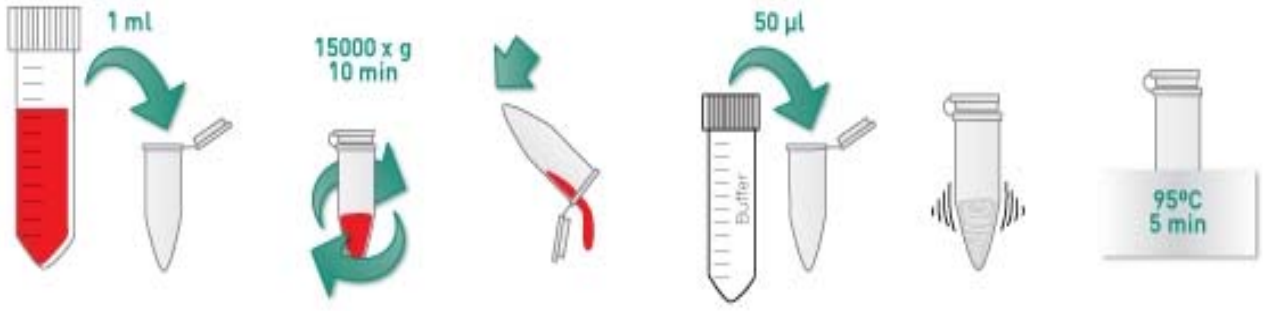
Protocol:

A. Test Sample Preparation

- Transfer 1.0 ml cell culture supernatant into a 1.5 ml sterile centrifuge tube.
- To pellet cellular debris, centrifuge the sample at 250 x g briefly.
- Transfer the supernatant into a fresh sterile 1.5 ml tube.
- Centrifuge at 15,000 x g for 10 minutes to pellet *Mycoplasma*.
- Carefully decant the supernatant and keep the pellet (the pellet will not always be visible).
- Re-suspend the pellet with 50µl of the Lysis Buffer.
- Vortex the sample.
- Heat to 95ºC for 5 minutes.
- Use up to 12µl per reaction of this solution to test for *Mycoplasma* contamination or store at -20ºC for later use.



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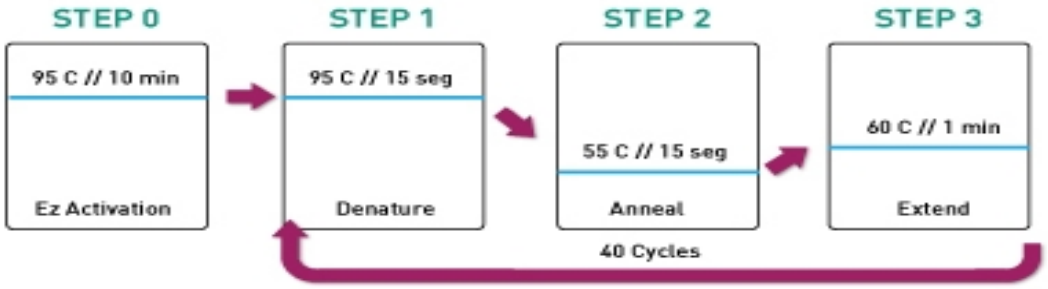
B. Set up the Experiment on a Real Time PCR Device (Prepared to SYBR Green detection)

- Prepare the plate document.
- Select Absolute Quantification.
- Select SYBR detector with: Quencher Dye set to (none) or (Non Fluorescent) and Passive Reference set to ROX.
- Set thermal-cycling conditions as indicated in the table below. Refer to your Real-Time PCR System Manual for specific details.

Steps	Hot Start Polymerase Activation	PCR			Dissociation Melt Curve
	Hold	40 Cycles			Add Dissociation Stage*
		Denature	Anneal	Extend	
Temp	95ºC	95ºC	55ºC	60ºC	
Time	10 min	15 sec	15 sec	1 min	

* Add Dissociation Stage (Refer to you Real-Time PCR System Instruction Manual for specific details).



- Set sample volume to 30 µl.

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C. Prepare the Kit Reagents and Premix Solution

- Thaw all kit reagents completely and keep at 4ºC throughout the experiment.
- Vortex and spin down reagents.
- Label a micro centrifuge tube for the Premix solution.
- Prepare the premix solution according to the following table:

Premix Solution Component	Volume for one reaction (µL)	Volume for four reactions (µL)*
SYBR Green PCR Master Mix 2x	15	66
10X Primer Solution	3.0	13.2
Final Premix Solution	**18**	**79.2**

*10% excess to compensate pipetting errors.

- Mix gently by pipetting up and down.

D. Prepare the PCR Reactions

- Pipette the following reagent into a labeled reaction strip or plate:

Reaction	Each Tube
Negative Control Reaction	18 µL of Premix Solution 12 µL of Negative Control (Water)
Unknown Sample Reaction	18 µL of Premix Solution 10 µL of unknown sample (up to 12 µL) 2µL of water (up to a final volume of 30 µL)
Inhibition Control Reaction	18 µL of Premix Solution 10 µL of unknown sample 2 µL of Discriminatory Positive Control (DPC)
Positive Control Reaction	18 µL of Premix Solution 2 µL of Discriminatory Positive Control (DPC) 10 µL of water

- For each row of wells that you use, place in sequence from left to right: negative control, unknown sample, inhibition control, positive control.

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E. Perform PCR

On a Real-Time PCR System:

- Open the plate document previously created.
- Load the reaction plate into the real-time PCR system.
- Begin the run.

F. Analyze the Results

The acceptance criteria provided in this section are based on our current knowledge of assay performance in detection of Mycoplasma recovered from a wide variety of test sample matrices. We recommend that you qualify and validate the assay internally using samples that are specific to your process and manufacturing environment.

For instructions on how to analyze your results, refer to the user guide of your real-time PCR instrument.

As general guidelines:
View the plate completely (amplification plots, melting curves and derivative values, Ct values).
For all reactions, use the default Analysis Settings:

- Select Manual CT, and then set threshold to 0.2.
- Select Manual Baseline, then enter the following settings:
  - Start (cycle): 3
  - End (cycle): 15

G. Guidance for Test Samples

The table shows criteria for positive and negative calls. A positive call indicates that at least one genome copy of Mycoplasma was present in the test reaction and the sample is positive for the presence of Mycoplasma.

Criteria for Test Samples

Result	Ct	Tm
Mycoplasma Positive Sample	≤ 36	75°C - 85°C
Mycoplasma Negative Sample	≥ 36	75°C - 85°C

Criteria for controls

Result	Ct	Tm
Discriminatory Positive Control (DPC)	25-28	> 85°C
Negative Control	≥ 36	< 75°C
Inhibition Control (Test Sample plus DPC)	Non less than 3Ct value comparing to DPC Ct value	Both pikes (DPC+Sample)

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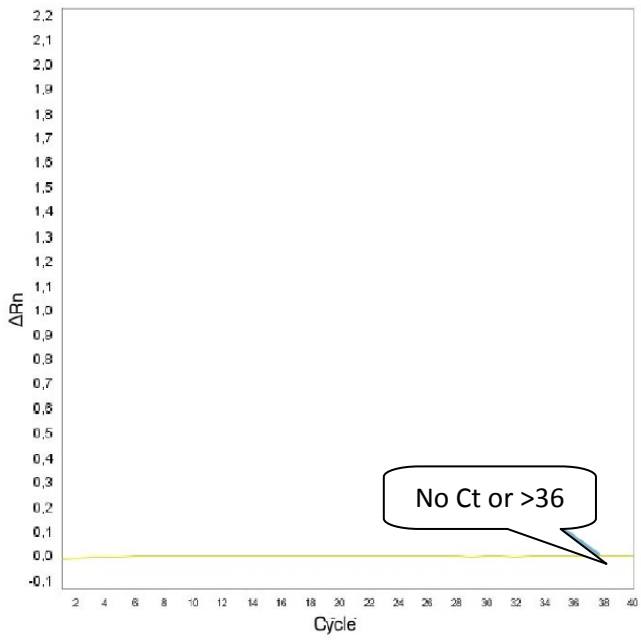
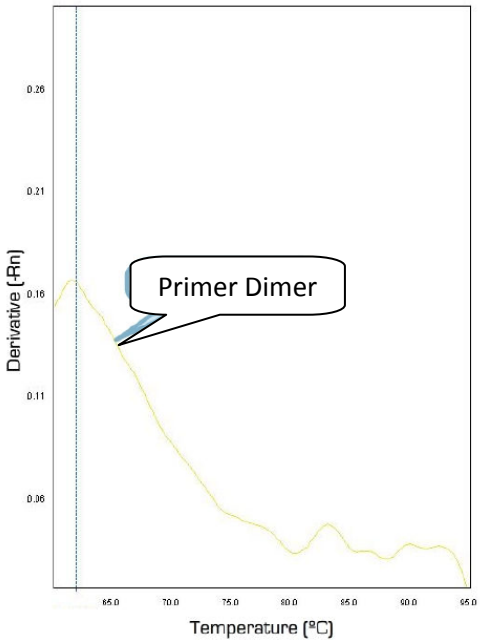
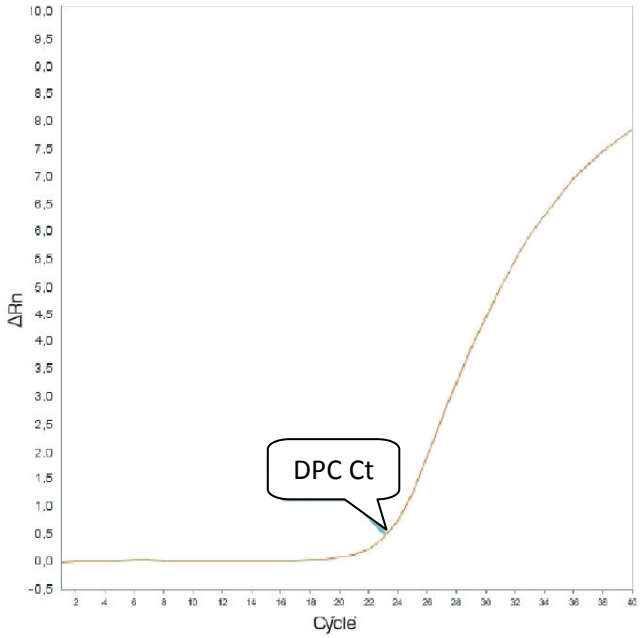
H. Example of a Positive Result (Validated by Controls)

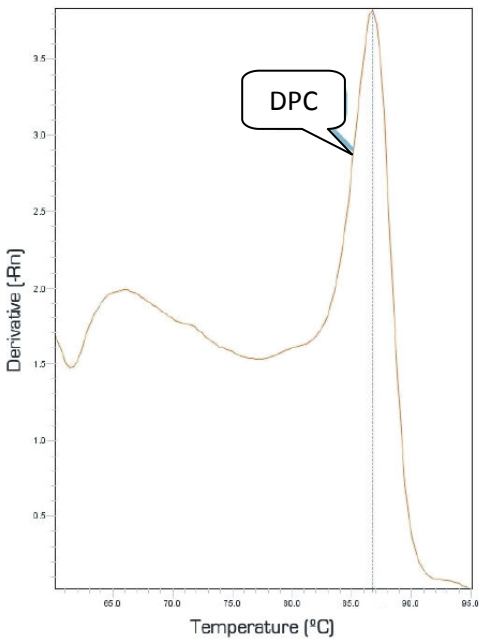
- Negative Control *Negative Result*





- Discriminatory Positive Control *DPC (2000 copies per tube)*





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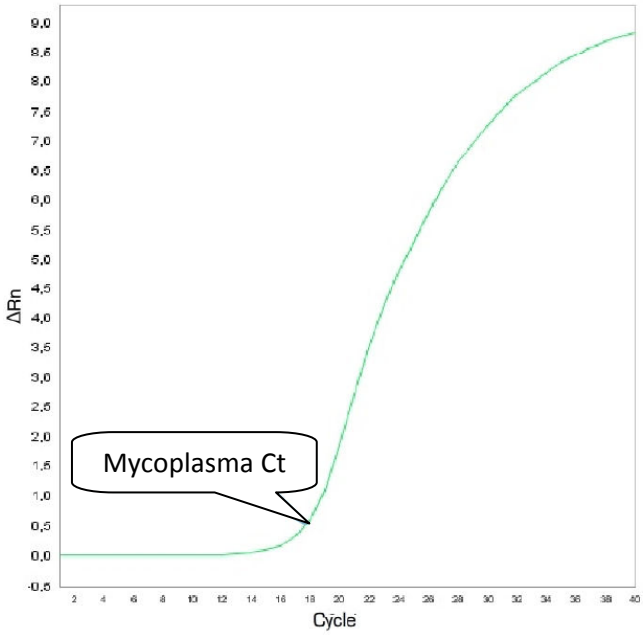
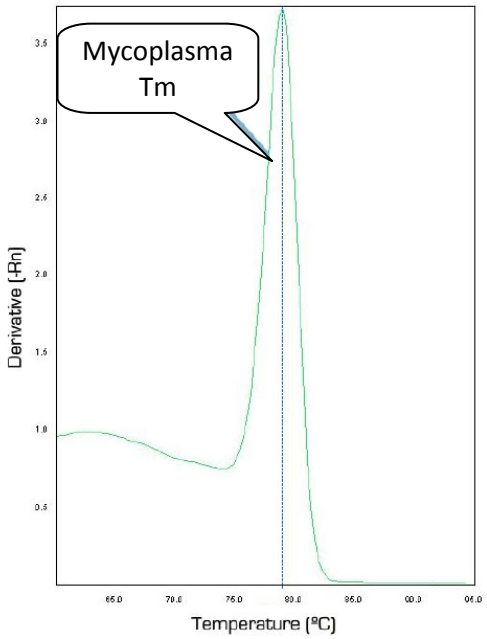
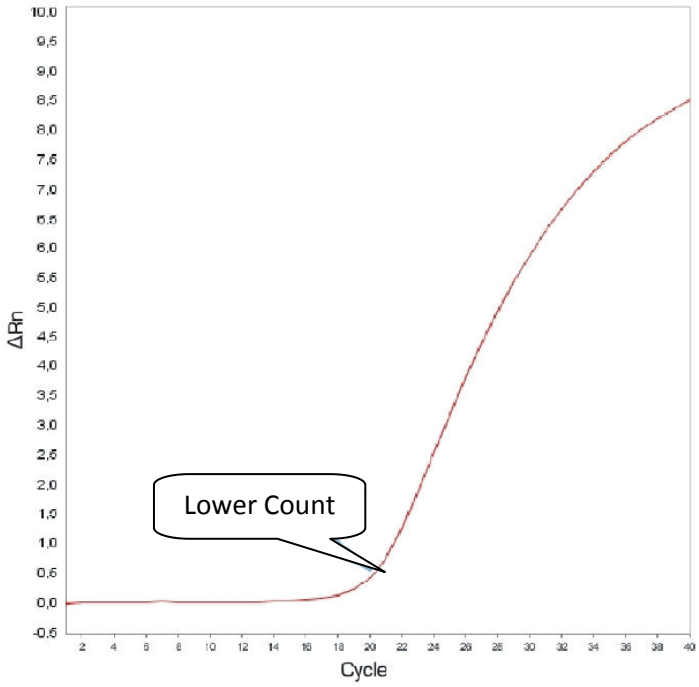
- Unknown Sample Mycoplasma *contamination (approximately 10,000 copies per RT-PCR reaction)*

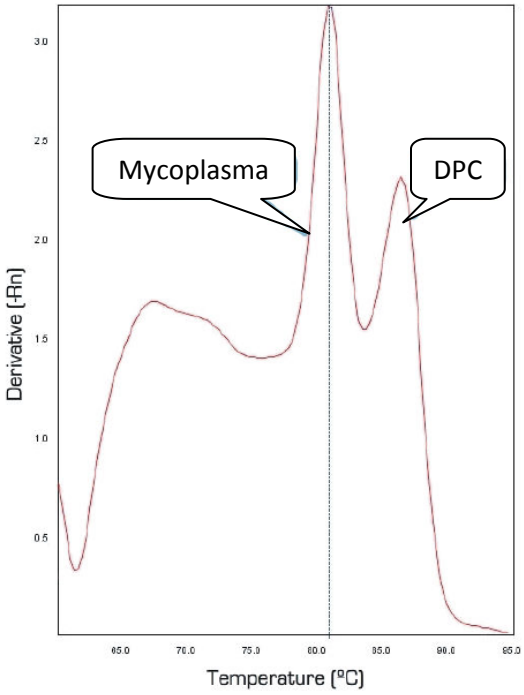




- Inhibition Control Reaction *for a heavily and a weakly contaminated sample*

Mycoplasma Positive Sample (high copies) with DPC (2000 per tube)





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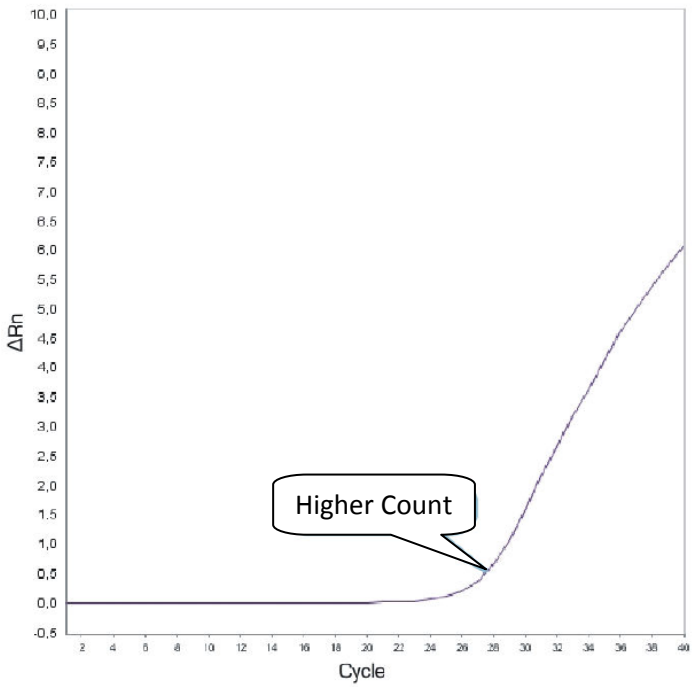
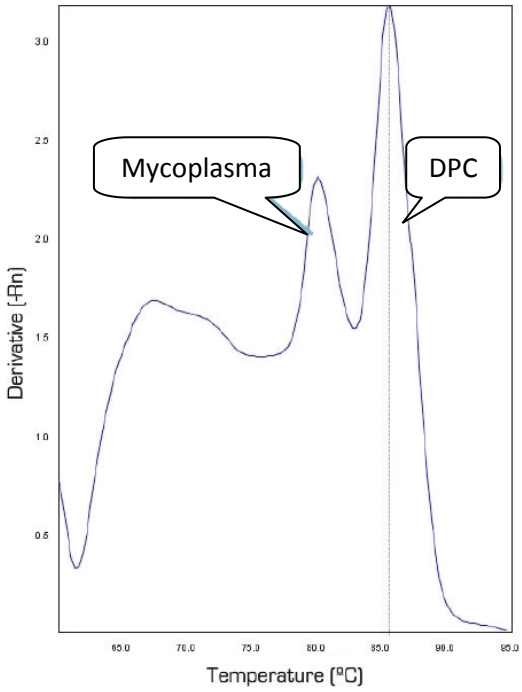
Mycoplasma Positive Sample (low copies) with DPC (2000 per tube)





PELOBIOTECH GmbH
Am Klopferspitz 19
82152 Planegg / Germany
(P) +49 89 517 286 59-0
(F) +49 89 517 286 59-88
Email:info@pelobiotech.com
www.pelobiotech.com