applied biosystems

MycoSEQ™ Mycoplasma Real-Time PCR Detection Kit

for use with the PrepSEQ™ Mycoplasma Sample Preparation Kit

Catalog Numbers 4460623, 4460626

Publication Number 4465874

Revision C.0

For Research Use Only. Not for use in diagnostic procedures.

ThermoFisher SCIENTIFIC

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Revision history: Pub. No. 4465874

Revision	Date	Description
C.0	24 May 2018	Updated template, legal, and content information. Reorganized content. Added information about using the AccuSEQ™ Software v2.0 Mycoplasma SEQ module.
B.0	04 Dec 2013	Baseline for this revision history.

Contents

Product information

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

Product description

The MycoSEQ™ Mycoplasma Real-Time PCR 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 *Mycoplasma* species.

Contents and storage

Table 1 MycoSEQ™ Mycoplasma Real-Time PCR Detection Kit (Cat. No. 4460623)

Contents [1]	Cap color	Amount [2]	Storage
Box 1: MycoSEQ™ Mycoplasma Real-Time PCR Detection Kit			
10× *Mycoplasma* Real-Time PCR Primer Mix	blue	325 µL	–25°C to –15°C on receipt 2–8°C after first use
Negative Control	white	1,000 µL	
2× *Power* SYBR™ Green PCR Master Mix	white	2 × 1,000 µL	–25°C to –15°C on receipt, protected from light. 2–8°C after first use, protected from light
Box 2: MycoSEQ™ Discriminatory Positive/Extraction Control			
MycoSEQ™ Discriminatory Positive/ Extraction Control, 1,000 copies/µL	yellow	700 µL	–25°C to –15°C

[1] To purchase the MycoSEQ™ Mycoplasma Real-Time PCR Detection Kit that includes the PrepSEQ™ Mycoplasma Sample Preparation Kit, use Catalog Number 4460626.

[2] The kit contains reagents for 100 reactions.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
Instruments; choose one:	
7500 Fast Real-Time PCR System with AccuSEQ™ software v2.0 or later	Contact your local sales representative
(Optional) 7500 Real-Time PCR System	
Consumables	
Disposable gloves	MLS
Aerosol-resistant pipette tips	MLS
Pipettors: • Positive-displacement • Air-displacement • Multichannel	MLS
MicroAmp™ Optical 96-Well Reaction Plate with Barcode, 20 plates, 0.2-mL well; for use with 7300, 7500, and 7900HT Fast Real-Time PCR Systems	4306737[1]
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL, 20 plates; for use with 7500 Fast Real-Time PCR System	4346906
MicroAmp™ Optical 96-Well Reaction Plate with Barcode & Optical Adhesive Films, 100 plates with covers; for use with 7300 and 7500 Fast Real-Time PCR Systems	4314320
MicroAmp™ Optical 8-Cap Strips, 300 strips	4323032
MicroAmp™ Optical Adhesive Film Kit, 20 covers, 1 applicator, 1 optical cover compression pad	4313663
MicroAmp™ Optical Adhesive Film, 25 or 100 covers	4360954, 25 covers 4311971, 100 covers

[1] Not recommended for use with the 7500 Fast system. For 7500 Fast system reactions, use Cat. No. 4346906.

Workflow

Prepare the sample (page 8)

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Prepare for PCR using AccuSEQ™ Software v2.0 Mycoplasma SEQ module:

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Methods

IMPORTANT! This chapter describes how to prepare and run PCR samples using AccuSEQ™ Software v2.0. If you are using SDS software v1.4 or later, see Appendix A, "Use the kit with 7500 System SDS Software v1.4 or later".

IMPORTANT! For information on how to avoid PCR contamination, see Appendix E, "Good PCR practices".

Prepare the sample

Prepare the DNA template for the PCR reactions using the PrepSEQ™ *Mycoplasma* Nucleic Acid Extraction Kit.

For more information, see:

- The *PrepSEQ™ Sample Preparation Kits for Mycoplasma, MMV, and Vesivirus User Guide* (Pub. No. 4465957)
- The *PrepSEQ™ Express Nucleic Acid Extraction Kit for Mycoplasma, MMV, and Vesivirus Detection User Guide* (Pub. No. MAN0016799)

Prepare for PCR using AccuSEQ™ Software v2.0 Mycoplasma SEQ module

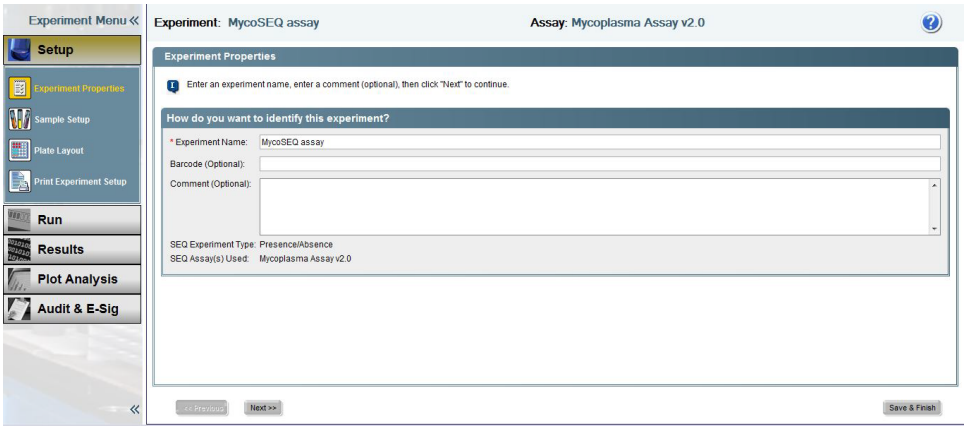
Create a new experiment

1. In the desktop, double–click the AccuSEQ™ software icon to start the software.
2. Log into the software. In the **Home** screen, click **Create MycoSEQ Experiment** to open the **Mycoplasma Assay v2.0** workflow.

Define the experiment properties

In the Experiment properties screen:

1. Enter an experiment name.

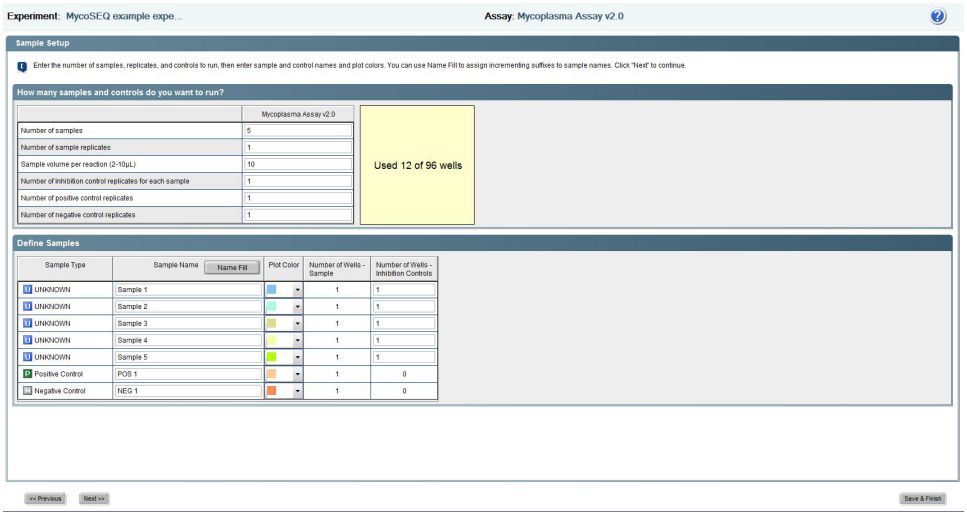


2. *(Optional)* Enter a barcode to identify the reaction plate.
3. *(Optional)* Enter comments to describe the experiment.
4. Verify the SEQ experiment type and assay to use, then click **Next**.

Setup the samples and controls

In the **Sample Setup** screen:

1. Specify the number of samples and replicates:



Field	Minimum entry[1]
Samples	1
Sample replicates	1
Inhibition control replicates for each sample	0
Positive control replicates	1
Negative control replicates	1

[1] We recommend that you use at least one negative and one positive control per run, and at least one inhibition control per sample.

2. Set the Sample volume to 10 µL per reaction.
3. Enter sample names, and *(optional)* set plot colors.
4. Click **Next**.

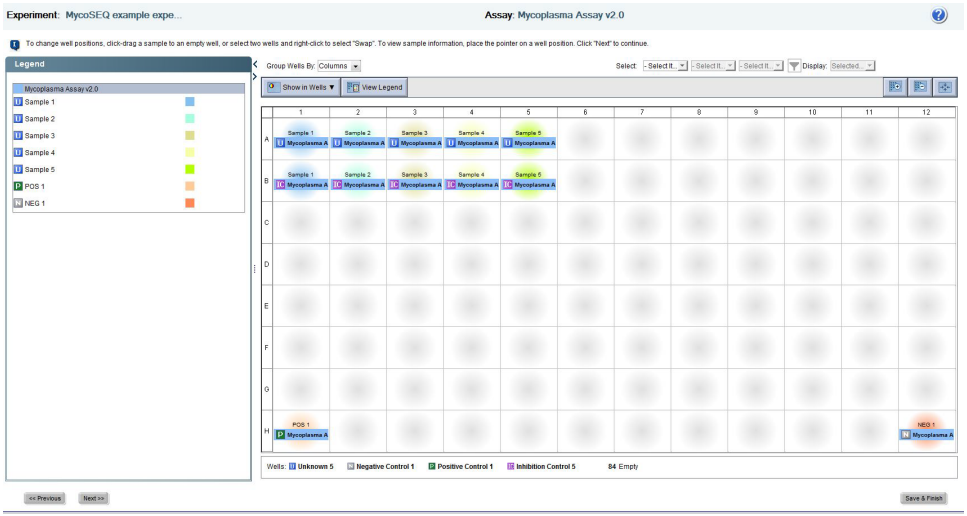
View the plate layout

The AccuSEQ™ software v2.0 uses the sample information that you enter in the **Sample Setup** screen to fill the wells in the plate layout and to calculate the required reaction component volumes for each sample type, based on the Mycoplasma Real-Time PCR Detection Kit guidelines.

To view and edit the plate layout before starting an instrument run:

1. Click **Setup** ▸ **Plate Layout** in the navigation pane.
2. Review the initial well selections in the Plate Layout screen. Drag-and-drop samples to create the layout of your choice.

   Here is an example:



3. Review the **Sample Setup** window to ensure that the number of Unknowns, Inhibition Controls, Positive Controls, and Negative Controls match your experiment sample setup. In the example, this is 5 Unknowns, 5 Inhibition Controls, 1 Positive Control, and 1 Negative Control.
4. Review the run method and click **Next**.

Save the experiment

1. At the bottom of the AccuSEQ™ software screen, click **Save & Finish**.
2. In the **Save Experiment** dialog box, verify the Mycoplasma Presence Absence Detection by MycoSEQ Example.eds file name, then click **Save**.

Prepare the kit reagents and premix solution

1. Thaw all kit reagents completely.
2. Vortex briefly, then spin down the reagents.
3. Prepare the Premix Solution according to the following table.

Component for premix solution	Volume for one 30-µL reaction	Volume for four 30-µL reactions[1]
Power SYBR™ Green PCR Master Mix, 2×	15.0 µL	66.0 µL
Mycoplasma Real-Time PCR Primer Mix, 10×	3.0 µL	13.2 µL
Total premix solution volume	**18.0 µL**	**79.2 µL**

[1] Includes 10% excess to compensate for pipetting errors.

4. Mix the Premix Solution by gently pipetting up and down, then cap the tube.

Prepare the PCR reactions

1. Dispense the following into each well to be used, gently pipetting at the bottom of the well.

To prepare...	In each tube or well...
Negative control reaction	• Add 18 µL of Premix Solution • Add 12 µL of Negative Control (water)
Your unknown sample reaction	• Add 18 µL of Premix Solution • Add 10 µL of unknown sample • Add 2 µL of Negative Control (water)
Inhibition-control reaction	• Add 18 µL of Premix Solution • Add 10 µL of unknown sample • Add 2 µL of the Discriminatory Positive Control (DPC)
Positive control reaction	• Add 18 µL of Premix Solution • Add 2 µL of the DPC • Add 10 µL of Negative Control (water)

Note: The MycoSEQ™ *Mycoplasma* Discriminatory Positive/Extraction Control can be used as a spike control that is added to the test sample or lysate before sample preparation.

For units:

- With standard 0.2-mL block – Dispense into a standard optical 96-well plate (Cat. No. 4306737).
- With Fast 0.1-mL block – Dispense into a Fast optical 96-well plate (Cat. No. 4346906).

2. If using a standard 0.2-mL plate, mix each sample by gently pipetting up and down.

3. Seal the plate with MicroAmp™ Optical Adhesive Film. See "Seal the plates" on page 28.

4. Briefly centrifuge the reaction plate.

Start the run

1. Double-click Mycoplasma SEQ Example Setup.eds to open the example experiment file you created in “Save the experiment“ on page 11.
2. Load the reaction plate into the instrument.
3. To start your 7500 Fast instrument:
    1. Click **Run** in the navigation pane.
    2. Click **START RUN** at the top of any run screen.

Analyze the results

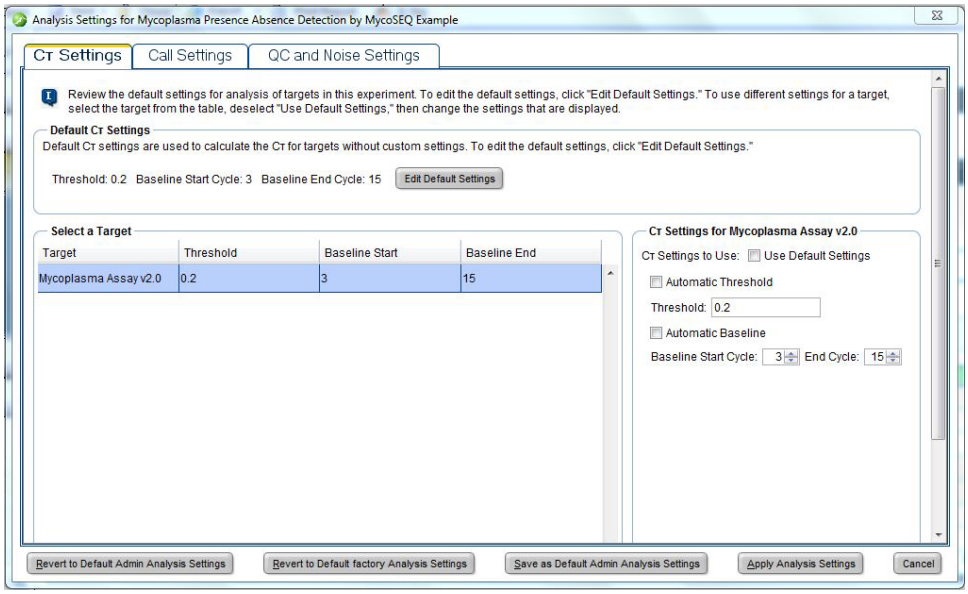
The acceptance criteria that are provided in this section are based on our current knowledge of assay performance in detection of *Mycoplasma* recovered from a wide variety of test sample matrices. We recommend that you qualify and validate the assay internally using samples that are specific to your process and manufacturing environment (raw materials, bioreactor, or cell line samples) to ensure that these criteria are appropriate.

For specific sample types, it may be necessary to make slight changes to the acceptance criteria based on specific results. We can provide you with one-on-one support during this process.

Set the baseline and threshold values

For all reactions, use the default Analysis Settings:

1. Select **Manual C_t**, then set Threshold to **0.2**.
2. Select **Manual Baseline**, then enter the following settings:
    - Start (cycle): **3**
    - End (cycle): **15**



Note: Autobaseline can also be used. To edit the baseline go to **Analysis ▸ Analysis Settings**.

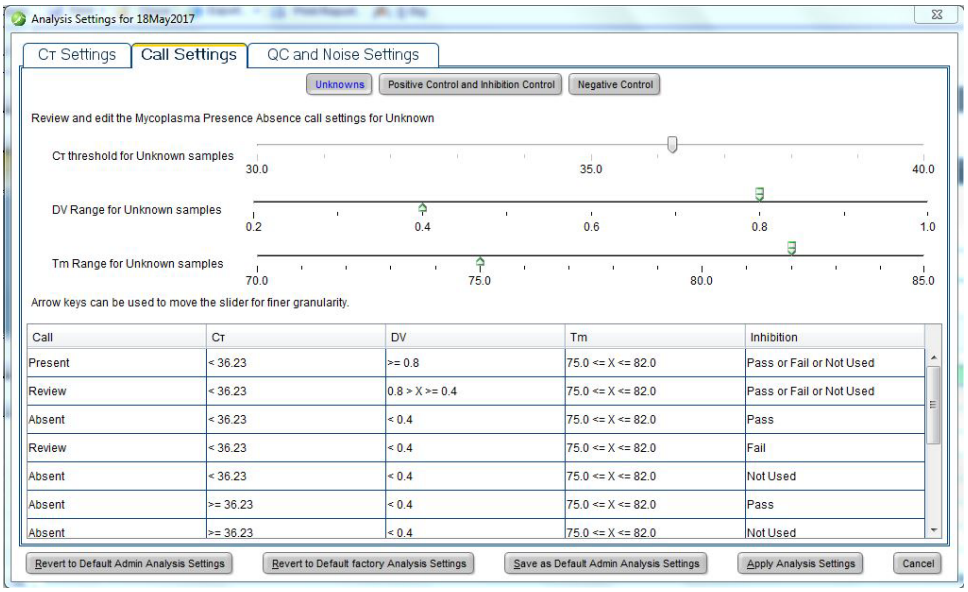
Review the Results Summary

AccuSEQ™ v2.0 software uses the acceptance criteria in Table 3 to provide an automated call summary for each reaction. Use the Call Summary, Plate Layout, and Table views in the **Results Summary** screen to review the experiment results.

1. From the navigation pane, select **Results ▸ Results Summary**.

2. Review the **Call Summary** for results.

3. *(Optional)* Adjust the *Mycoplasma* presence/absence analysis to meet your method qualifications. In the **Call Settings** tab, modify the values of the threshold cycle (C_t), derivative value (DV), and melting temperature (T_m) values. Use the slider interface at the top-half of the screen, to automatically update the comprehensive table at the bottom half of the screen.



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* DNA was present in the test reaction and the sample is positive for the presence of *Mycoplasma*. The automated threshold setting for derivative value (DV) of 0.8 for AccuSEQ™ software v2.0 (or later) is equivalent to the 0.05 setting for SDS v1.4 (or later) software.

Note: The values in the tables are subject to your own validation.

Table 2 Recommended acceptance criteria for test samples: AccuSEQ™ software v2.0 or later

Result	C_t	T_m	DV
Positive	< 36.23 C_t	75 – 82°C	≥0.8
Negative	≥ 36 C_t	< 75°C	N/A

Guidance for controls

The values in the tables are subject to your own validation.

Table 3 Recommended acceptance criteria for controls: AccuSEQ™ software v2.0 or later.

Control	C_t	T_m	DV
PCR positive control	< 36.23 C_t	82–86°C	> 0.8
Extraction spike control	< 36.23 C_t	82–86°C	> 0.8
No template control	≥ 36.23 C_t	< 75°C	N/A
Blank extraction control	≥ 36.23 C_t	< 75°C	N/A
Inhibition control	ΔC_t < 2	82–86°C	N/A

Guidance for inconclusive results with AccuSEQ™ software v2.0

If a MycoSEQ™ assay does not meet all of the criteria for a positive or negative automatic call, the well displays (inconclusive). For information about these results:

- Click Quality Summary (Quality Summary) in the Results navigation pane of the AccuSEQ™ software v2.0 screen.
- Click (Help) in the toolbar at the top of the AccuSEQ™ software v2.0 screen.
- See Appendix B, "Troubleshooting".
- Refer to the *AccuSEQ™ Real-Time PCR Detection Software Mycoplasma SEQ Experiments Getting Started Guide*.

Example results with AccuSEQ™ Software v. 2.1.1

Positive control

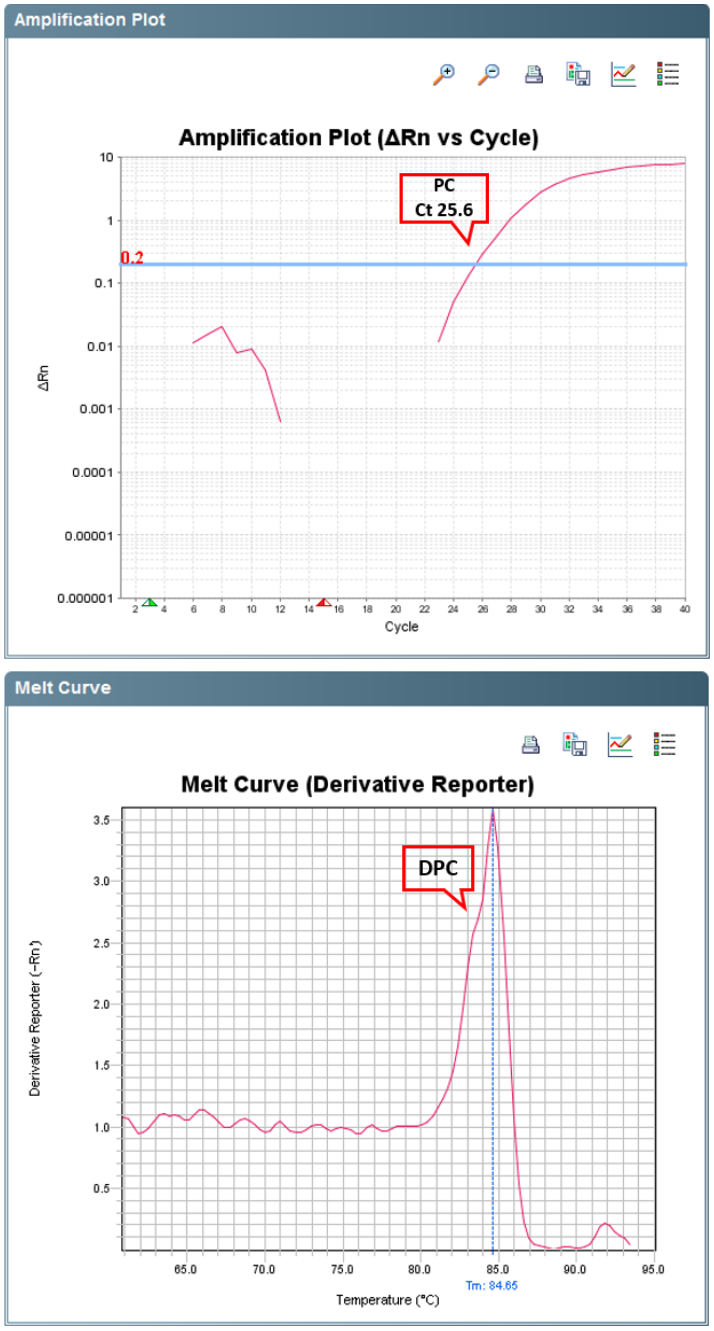


Figure 1 PCR positive control spiked with 2,000 copies of DPC.

Negative control

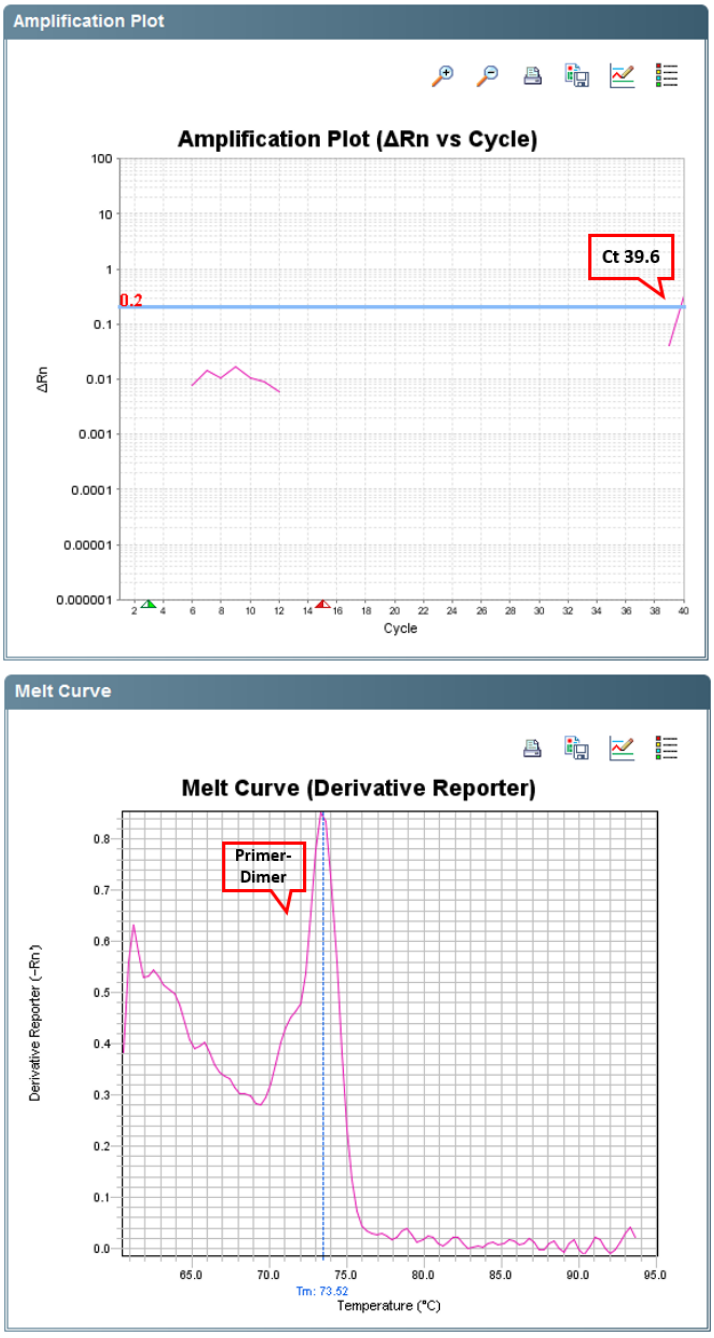


Figure 2 No template PCR control.

Blank extraction control

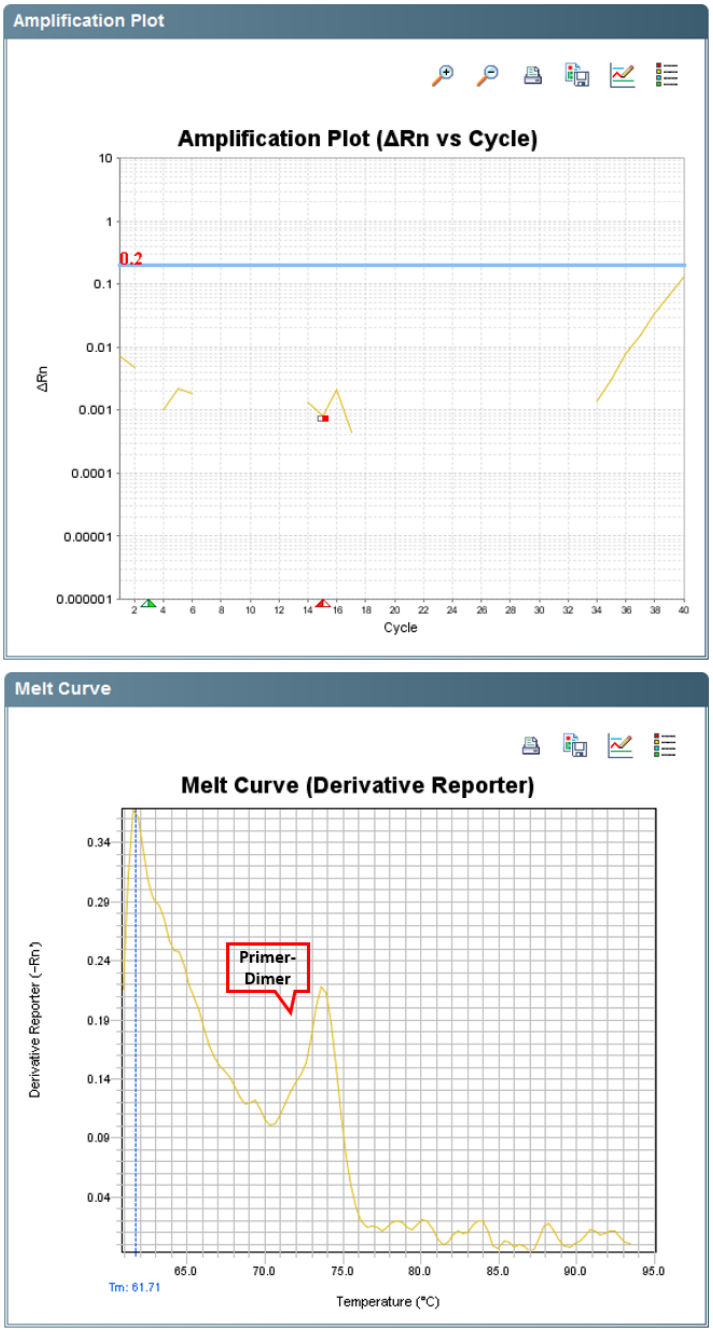


Figure 3 Blank extraction control with PBS.

Positive extraction control

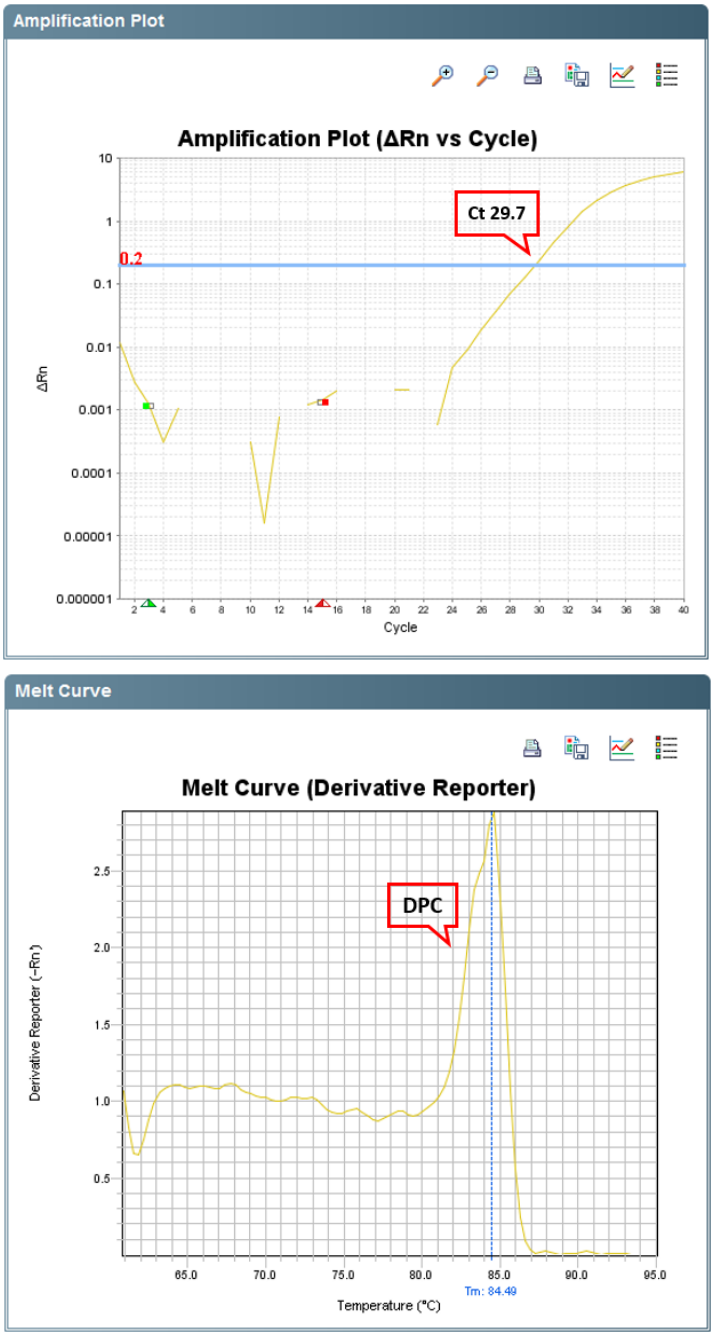


Figure 4 Sample spiked with 2,000 copies of DPC before DNA extraction.

Inhibition control and positive control

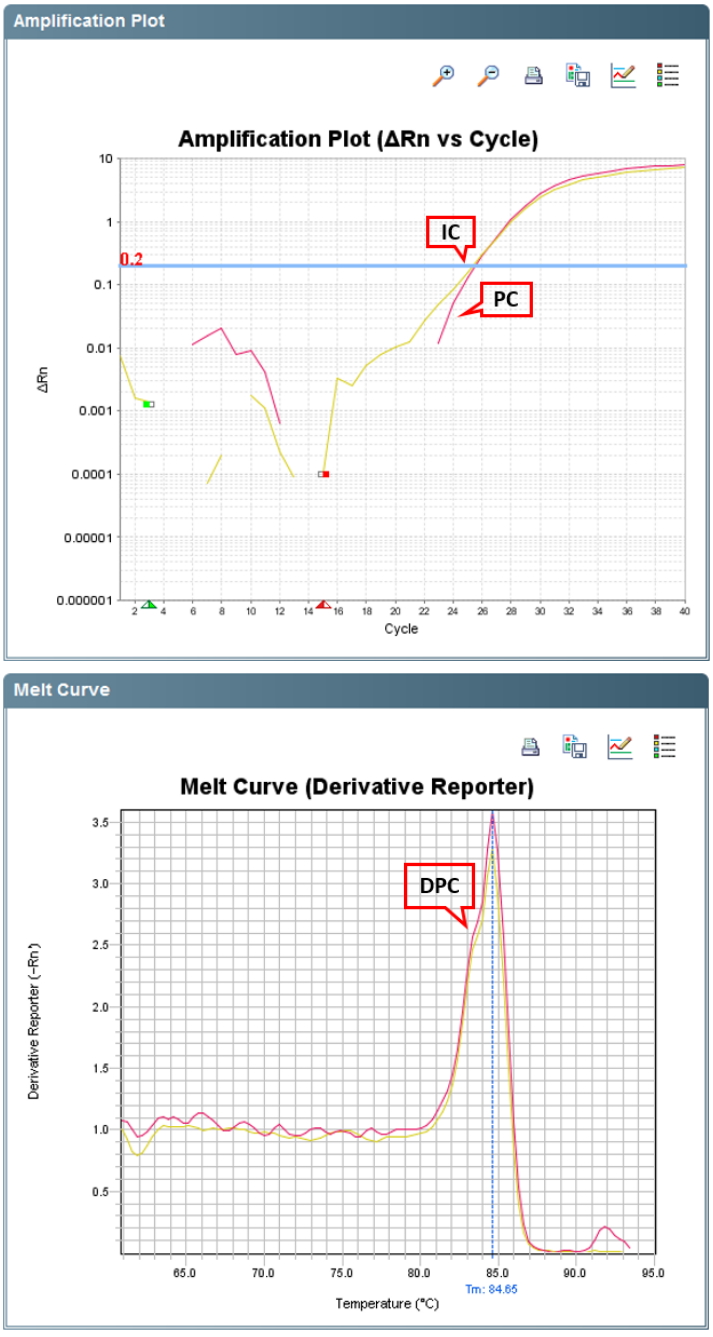


Figure 5 No PCR inhibition present; inhibition control and PCR positive control overlaid, with a $\Delta C_t < 2$.

Test sample: Negative result

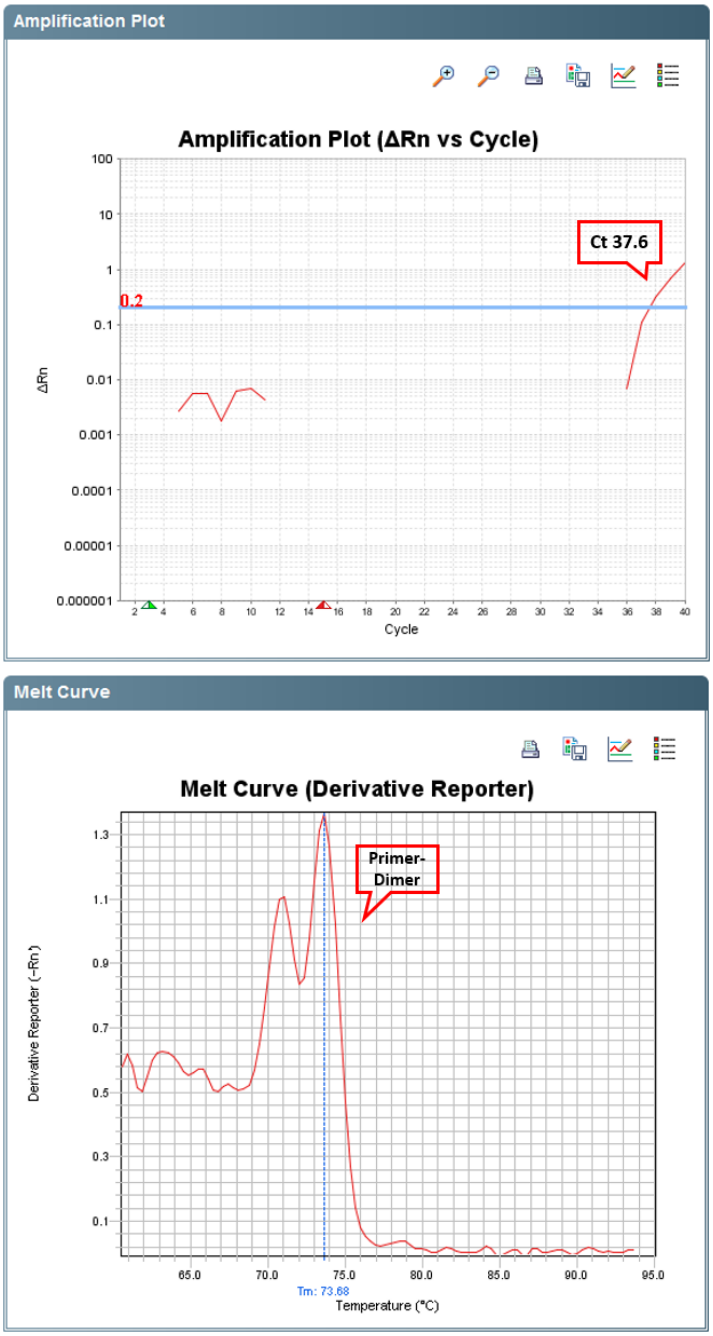


Figure 6 Negative result; C_t > 36.23 and T_m < 75°C.

Test sample: Positive result

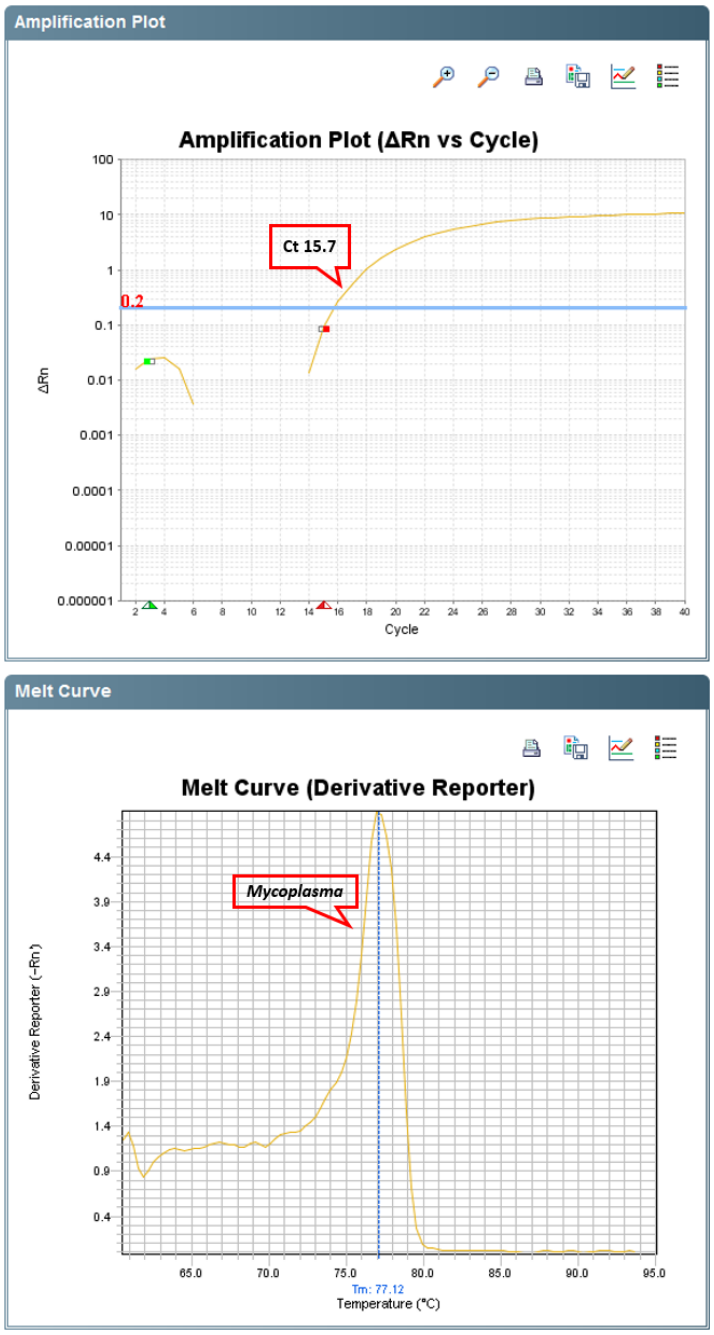


Figure 7 Positive result; C_t = 15.69, T_m > 75°C, and Derivative Reporter >0.8.

Test sample: Positive result with decreased detection of DPC

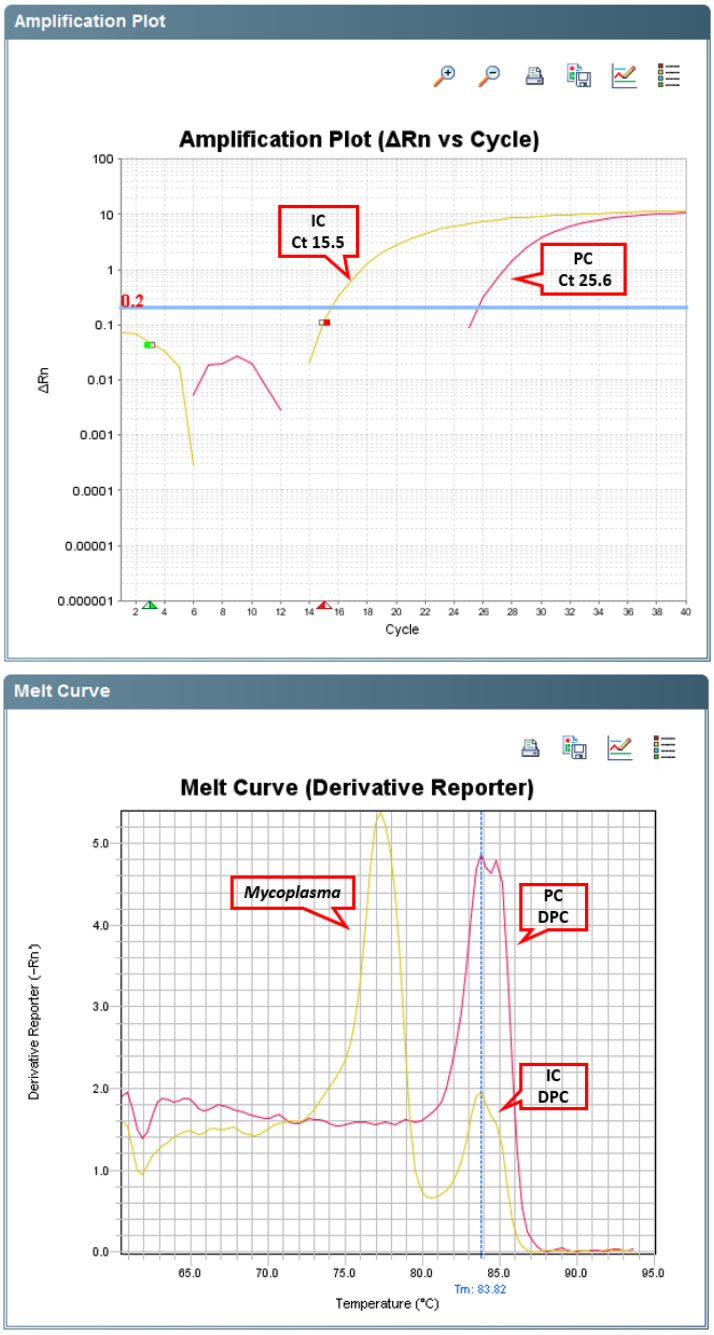


Figure 8 Decreased DPC signal can be observed in the presence of very high *mycoplasma* contamination.

PCR inhibition

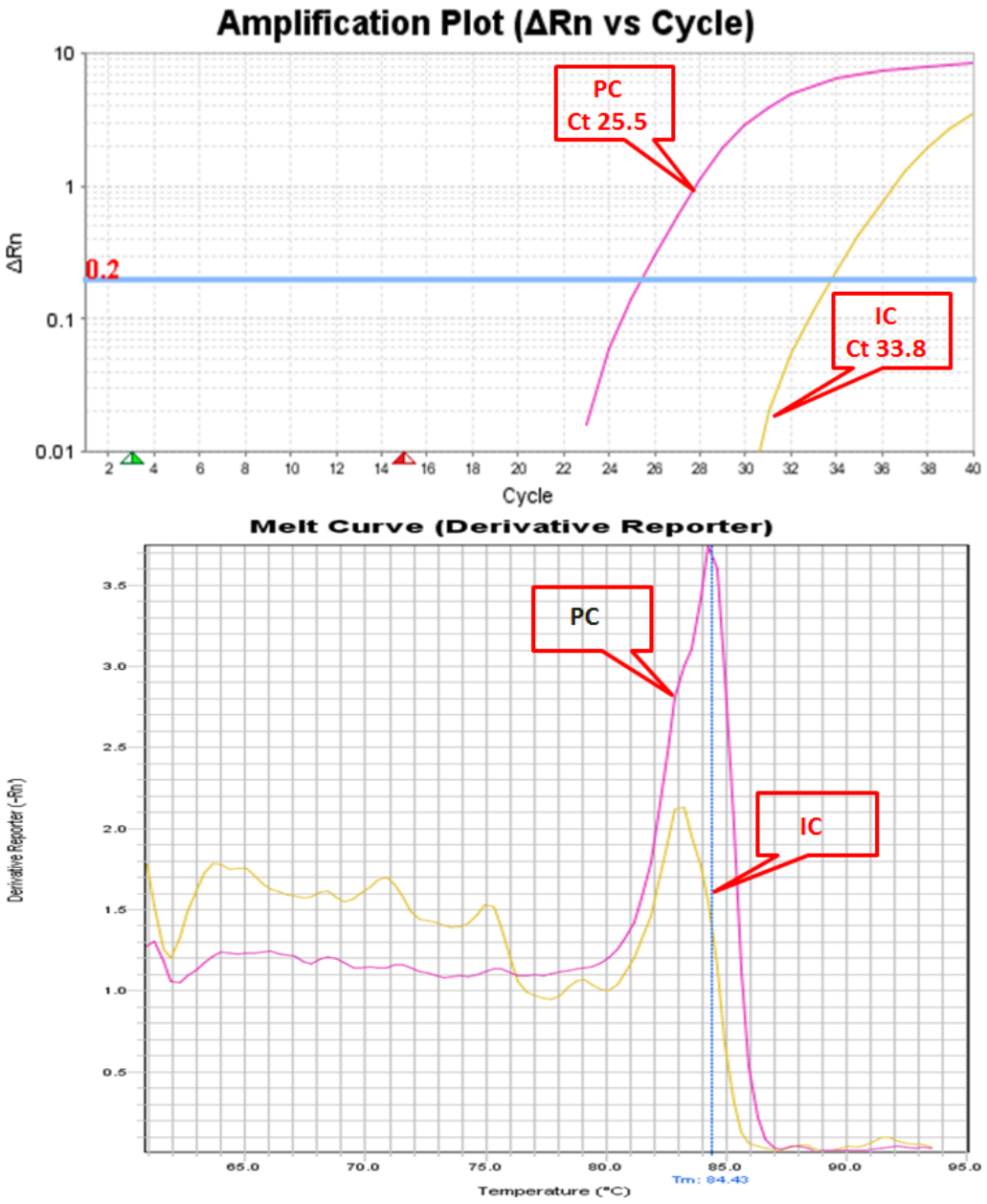


Figure 9 PCR inhibition, $\Delta C_t > 2$.

Multicomponent plots

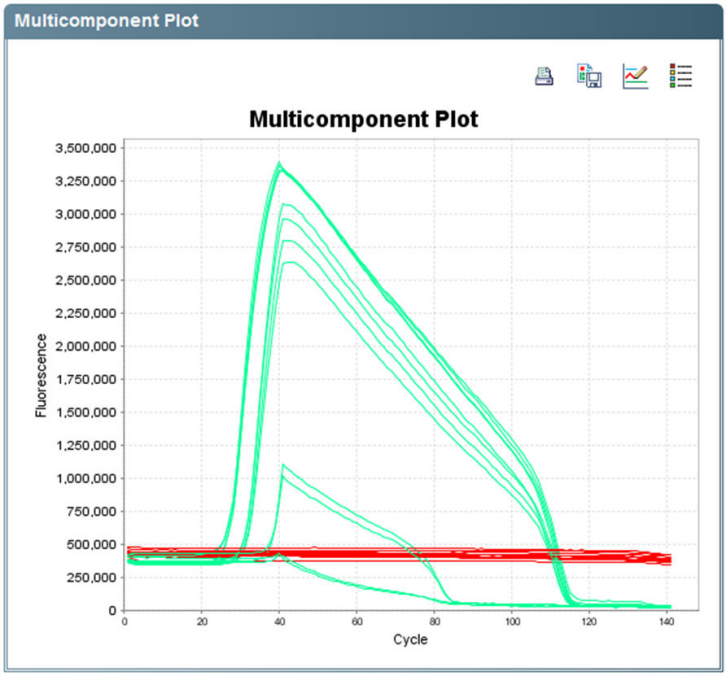


Figure 10 An example of a multicomponent plot.

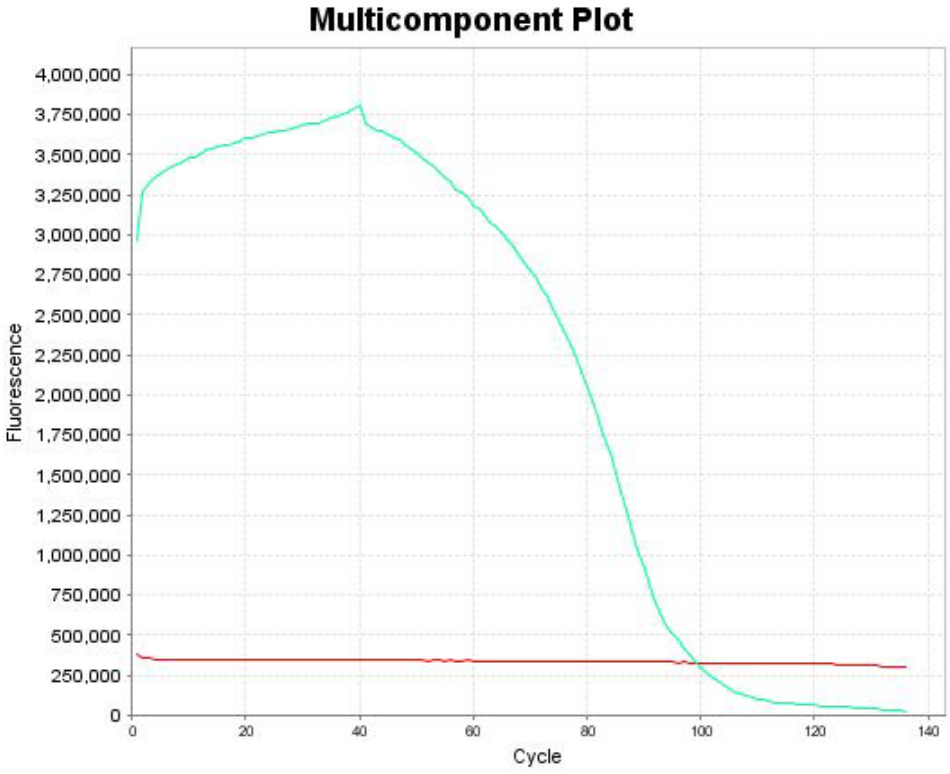


Figure 11 A multicomponent plot with high background signal which will result in PCR inhibition.

A Use the kit with 7500 System SDS Software v1.4 or later

Prepare the kit reagents and premix solution

1. Thaw all kit reagents completely.
2. Vortex briefly, then spin down the reagents.
3. Prepare the Premix Solution according to the following table.

Component for premix solution	Volume for one 30-µL reaction	Volume for four 30-µL reactions[1]
Power SYBR™ Green PCR Master Mix, 2×	15.0 µL	66.0 µL
Mycoplasma Real-Time PCR Primer Mix, 10×	3.0 µL	13.2 µL
Total premix solution volume	**18.0 µL**	**79.2 µL**

[1] Includes 10% excess to compensate for pipetting errors.

4. Mix the Premix Solution by gently pipetting up and down, then cap the tube.

Prepare the PCR reactions

1. Dispense the following into each well to be used, gently pipetting at the bottom of the well.

To prepare...	In each tube or well...
Negative control reaction	• Add 18 µL of Premix Solution • Add 12 µL of Negative Control (water)
Your unknown sample reaction	• Add 18 µL of Premix Solution • Add 10 µL of unknown sample • Add 2 µL of Negative Control (water)
Inhibition-control reaction	• Add 18 µL of Premix Solution • Add 10 µL of unknown sample • Add 2 µL of the Discriminatory Positive Control (DPC)
Positive control reaction	• Add 18 µL of Premix Solution • Add 2 µL of the DPC • Add 10 µL of Negative Control (water)

Note: The MycoSEQ™ *Mycoplasma* Discriminatory Positive/Extraction Control can be used as a spike control that is added to the test sample or lysate before sample preparation

For units:

- With standard 0.2-mL block – Dispense into a standard optical 96-well plate (Cat. No. 4306737).
- With Fast 0.1-mL block – Dispense into a Fast optical 96-well plate (Cat. No. 4346906).

2. For each row of wells that you use, place in sequence from left to right the negative control, unknown sample, inhibition control, then positive control. See "Plate layout suggestions" on page 43 for more information.

   Pipetting guidelines:

   - Use at least one negative and one positive control per run.
   - Mix each sample gently by pipetting up and down.
   - Use a new tip for each well, even when aliquoting the same solution.

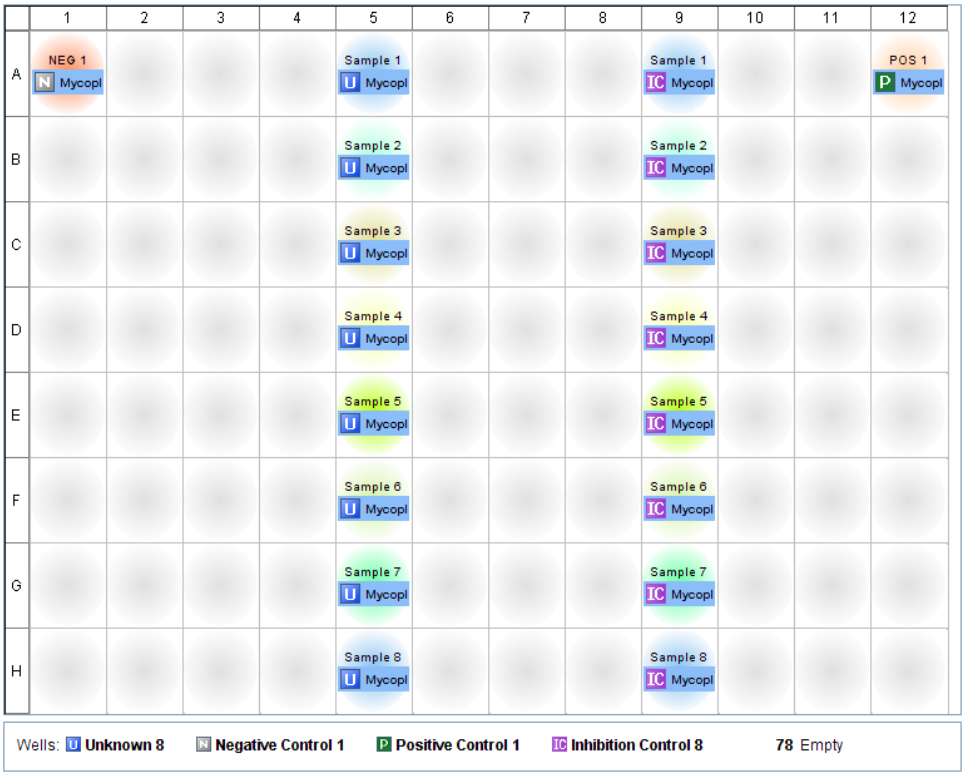
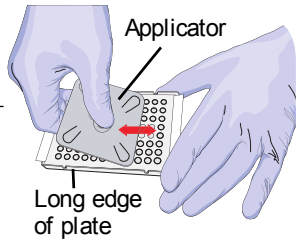


Figure 12 Example plate layout.

Seal the plates

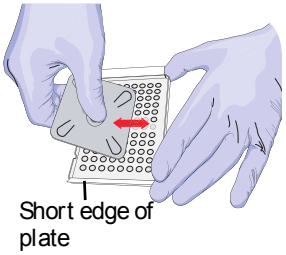
1. Place an optical adhesive cover on the plate, then rub the flat edge of the applicator back and forth along the **long** edge of the plate.



IMPORTANT! Apply significant downward pressure on the applicator to completely seal the wells. Pressure is required to activate the adhesive on the optical cover.

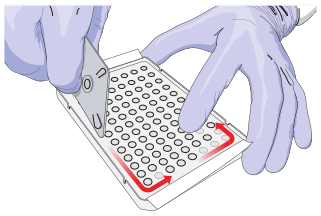
2. Rub the flat edge of the applicator back and forth along the **short** edge (width) of the plate.

3. Rub the edge of the applicator horizontally and vertically between all wells.



4. Rub the edge of the applicator around all outside edges of the plate using small back and forth motions to completely seal around the outside wells.

5. Briefly spin down the plate using a centrifuge with a plate adapter.



IMPORTANT! Make sure that the reagents (and no bubbles) are in the bottom of the wells.

Prepare the plate document

Set up the plate document in the SDS software. For more details, see the *Applied Biosystems™ 7300/7500/7500 Fast Real-Time PCR System Getting Started Guide: Absolute Quantitation using Standard Curve* (Pub. No. 4347825).

1. In the **Assay** drop-down list, select **Absolute Quantification**.

2. Select **SYBR™ detector** with:
   - Quencher Dye set to **none** or **Non Fluorescent**
   - Passive Reference set to **ROX™**

3. Set thermal-cycling conditions as indicated in the table below.

   Note: For instruments using the AccuSEQ™ 2.0 Real-Time PCR Software *Mycoplasma* Module, the cycling conditions are pre-programmed in the software.

Step	AmpliTaq Gold™ enzyme activation	PCR		Dissociation[1,2,3]			
	HOLD	Cycle (40 cycles)		Melt			
		Denature	Anneal/ extend				
Temp	95°C	95°C	60°C	95°C	60°C	95°C	60°C
Time	10 min	15 sec	1 min	15 sec	1 min	15 sec	15 sec

[1] 7500 and 7500 Fast Systems: from the Instrument tab, click **Add Dissociation Stage** (see Figure 13).

[2] Applied Biosystems™ Real-Time PCR Instruments: from the Instrument tab, click **Add Dissociation Stage** (see Figure 13). Use default settings..

[3] For other instruments, refer to their corresponding user guides for dissociation-curve setup information.

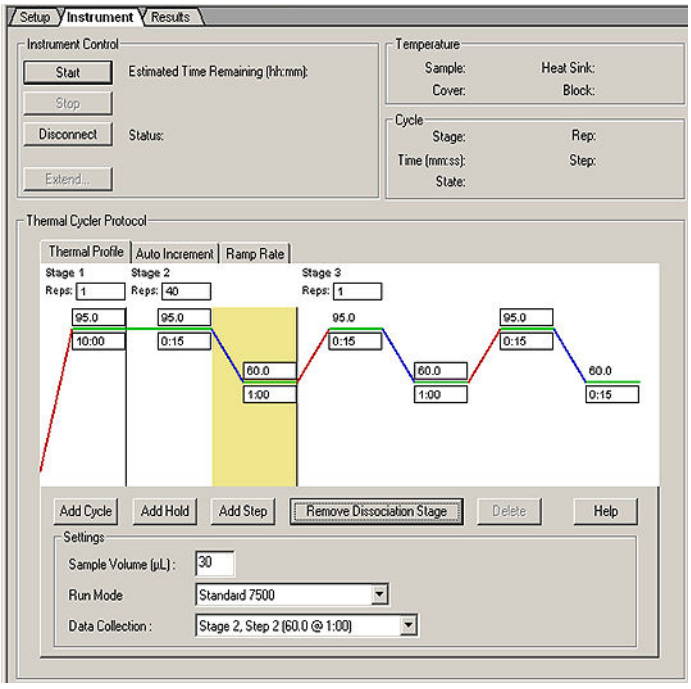


Figure 13 The instrument tab for 7500 Fast Real-Time PCR platform with SDS v1.4 software. The run mode is set to Standard 7500.

4. Set Sample Volume to **30** µL.
5. Select the Standard Run Mode for use with SYBR™ Green I dye.

Perform PCR

On an Applied Biosystems™ Real-Time PCR System:

1. Open the plate document that corresponds to the reaction plate (“Prepare the plate document“ on page 29).
2. Load the reaction plate into the real-time PCR system.
3. Start the run.

Analyze the results

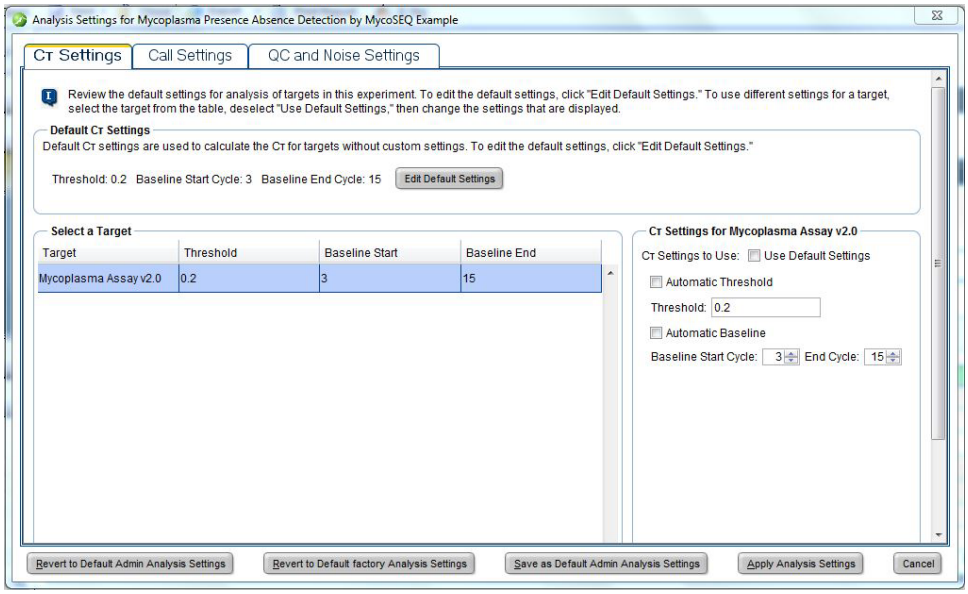
The acceptance criteria that are provided in this section are based on our current knowledge of assay performance in detection of *Mycoplasma* recovered from a wide variety of test sample matrices. We recommend that you qualify and validate the assay internally using samples that are specific to your process and manufacturing environment (raw materials, bioreactor, or cell line samples) to ensure that these criteria are appropriate.

For specific sample types, it may be necessary to make slight changes to the acceptance criteria based on specific results. We can provide you with one-on-one support during this process.

Set the baseline and threshold values

For all reactions, use the default Analysis Settings:

1. Select **Manual C_t**, then set Threshold to **0.2**.
2. Select **Manual Baseline**, then enter the following settings:
   - Start (cycle): **3**
   - End (cycle): **15**



Note: Autobaseline can also be used. To edit the baseline go to **Analysis ▸ Analysis Settings**.

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* DNA was present in the test reaction and the sample is positive for the presence of *Mycoplasma*. The automated threshold setting for derivative value (DV) of 0.8 for AccuSEQ™ 2.0 (or later) software is equivalent to the 0.05 setting for SDS v1.4 (or later) software.

Note: The values in the tables are subject to your own validation.

Table 4 Recommended acceptance criteria for test samples: SDS software v1.4 or later.

Result	C_t	T_m	DV
Positive	< 36 C_t	75°C – 81°C	≥0.05
Negative	≥ 36 C_t	< 75°C	N/A

Guidance for controls

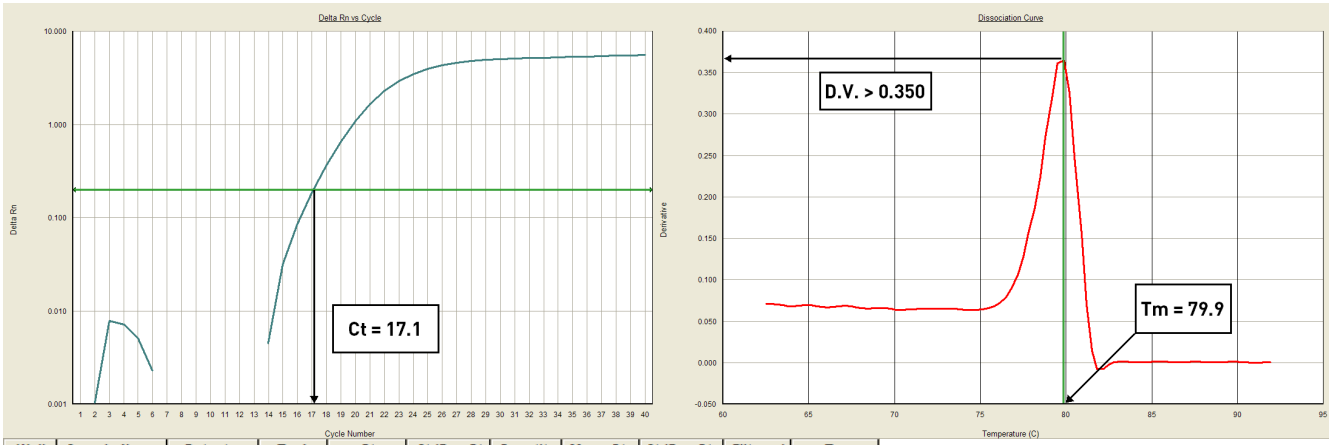
The values in the tables are subject to your own validation.

Table 5 Recommended acceptance criteria for controls: SDS software v1.4 or later.

Control	C_t	T_m	DV
PCR positive control	< 36 C_t	≈84°C	> 0.05
Extraction spike control	< 36 C_t	≈84°C	> 0.05
No template control	≥ 36 C_t	< 75°C	N/A
Blank extraction control	≥ 36 C_t	< 75°C	N/A
Inhibition control	ΔC_t < 2	≈84°C	N/A

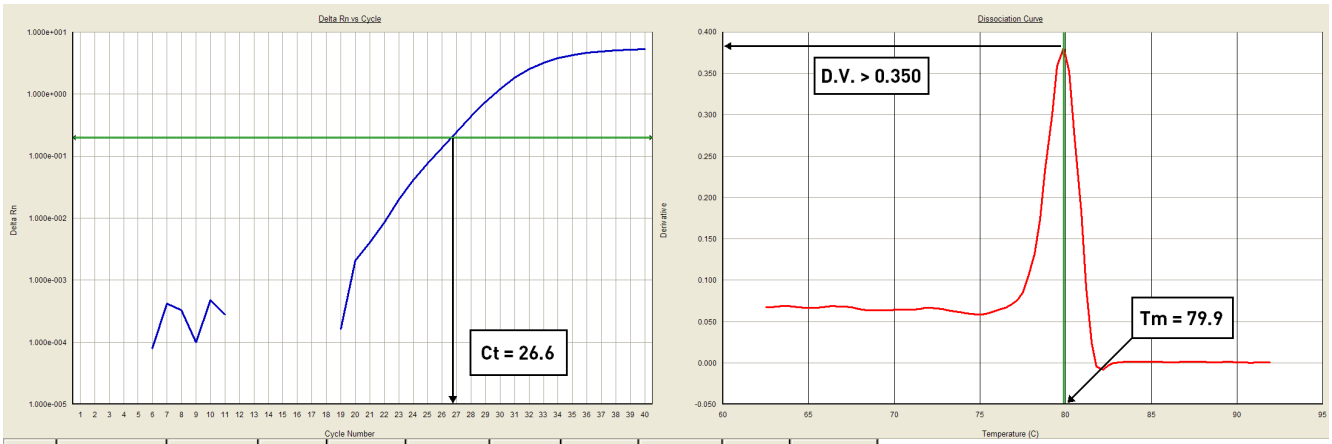
- Both the PCR positive control and the extraction spike control may present extra peaks with T_m < 75°C. These peaks represent primer dimer formation, and they do not interfere with the final results.
- The difference in C_t between the DPC and the inhibition control reaction should be less than 2 cycles. If the unknown sample is negative and the inhibition control shows a ΔC_t > 2 when compared to the positive control, then the PCR is likely inhibited. The sample should be re-purified and the assay repeated. The ΔC_t is calculated by C_t (of inhibition control reaction) – C_t (of positive control reaction).

Example positive results with SDS v1.4 software



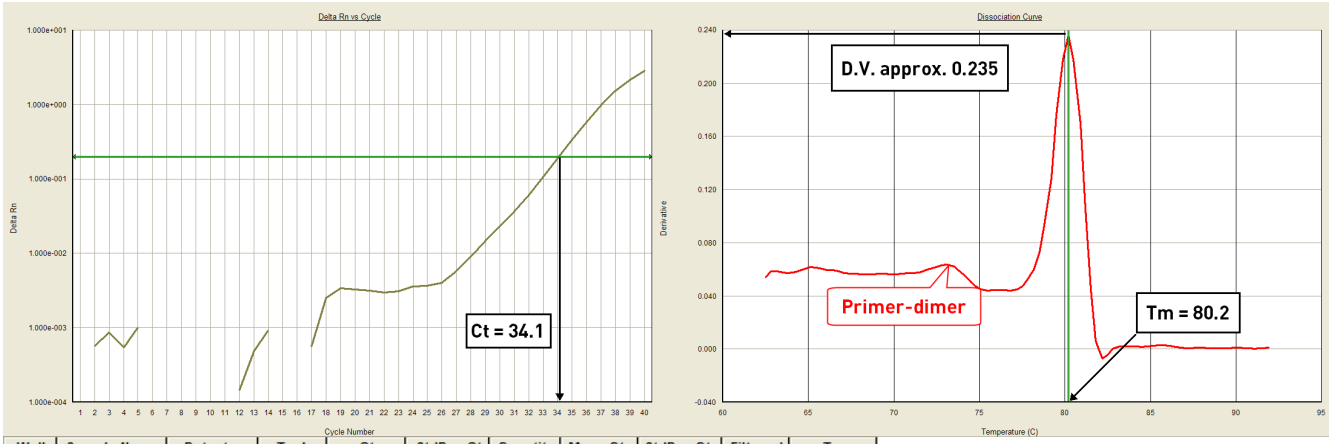
Well	Sample Name	Detector	Task	Ct	StdDev Ct	Quantity	Mean Qty	StdDev Qty	Filtered	Tm
E1	3	Mycoplasma	Unknown	17.0813						79.9 °C

Figure 14 *Mycoplasma* contamination (approximately 3 × 10^6 copies per PCR reaction).



Well	Sample Name	Detector	Task	Ct	StdDev Ct	Quantity	Mean Qty	StdDev Qty	Filtered	Tm
A3	5	Mycoplasma	Unknown	26.5762						79.9 °C

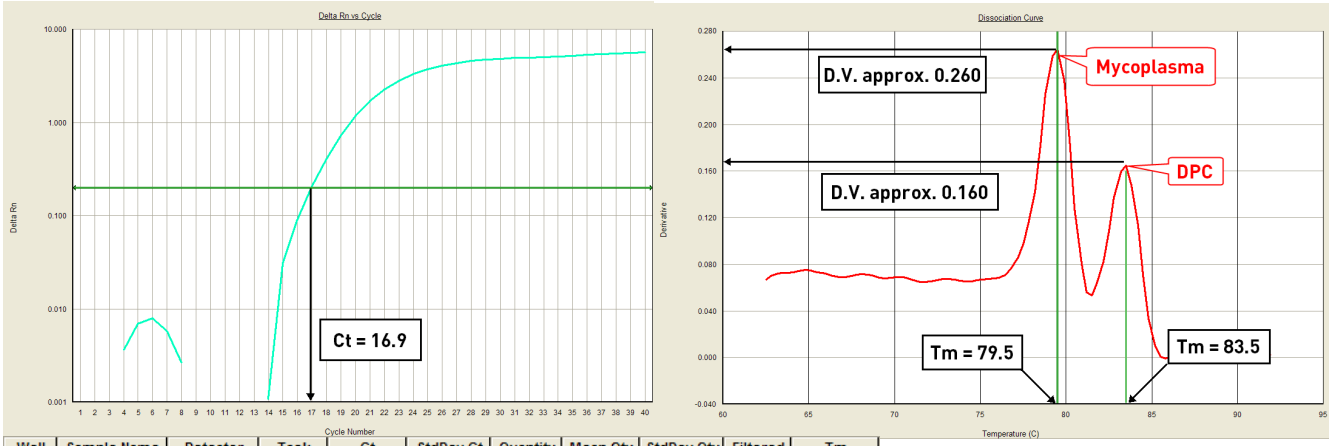
Figure 15 *Mycoplasma* contamination (approximately 2,000 copies per PCR reaction).



Well	Sample Name	Detector	Task	Ct	StdDev Ct	Quantity	Mean Qty	StdDev Qty	Filtered	Tm
E3	7	Mycoplasma	Unknown	34.0907						80.2 °C

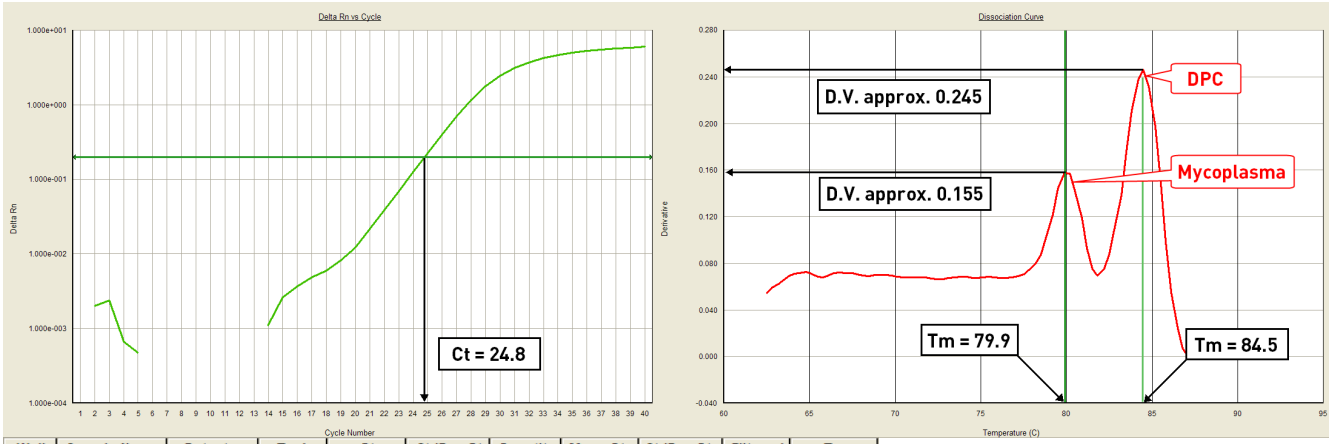
Figure 16 *Mycoplasma* contamination (less than 10 copies per PCR reaction).

Example positive control extraction results with SDS v1.4 software



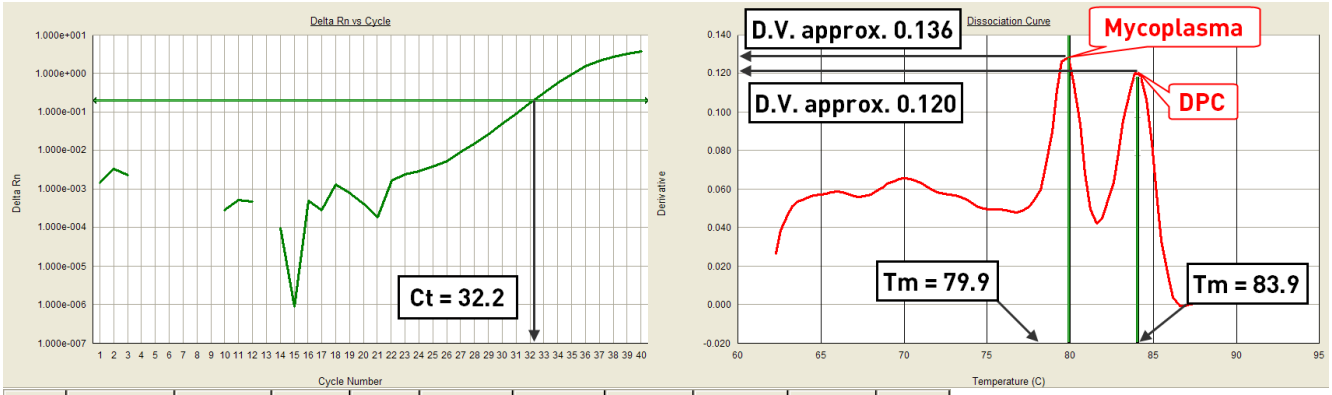
Well	Sample Name	Detector	Task	Ct	StdDev Ct	Quantity	Mean Qty	StdDev Qty	Filtered	Tm
G1	4	Mycoplasma	Unknown	16.9588						79.5 °C

Figure 17 Sample spiked with 2,000 copies of DPC and contaminated with *Mycoplasma* (3×10^6 copies).



Well	Sample Name	Detector	Task	Ct	StdDev Ct	Quantity	Mean Qty	StdDev Qty	Filtered	Tm
C3	6	Mycoplasma	Unknown	24.7689						84.5 °C

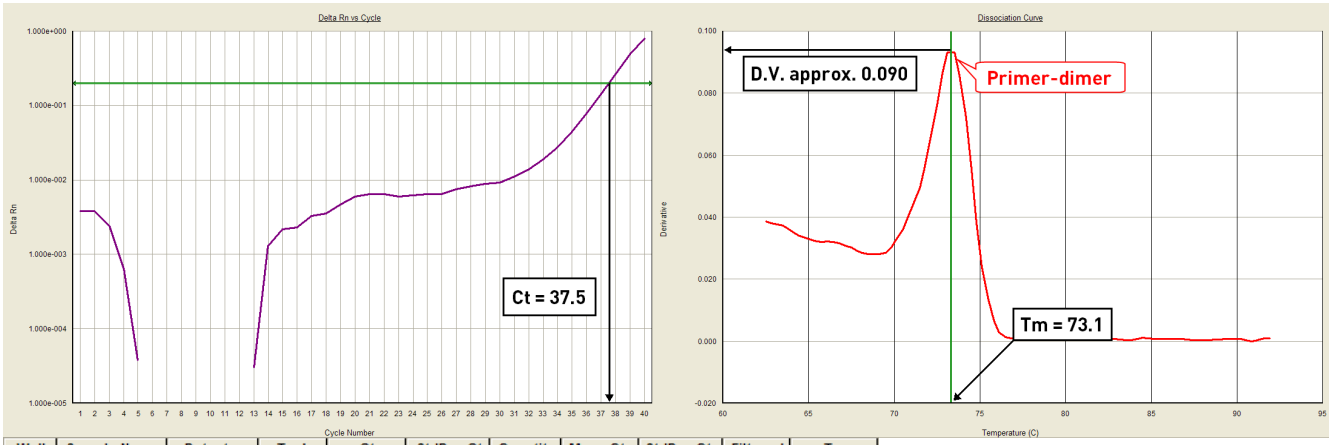
Figure 18 Sample spiked with 2,000 copies of DPC and contaminated with *Mycoplasma* (approximately 2,000 copies).



Well	Sample Name	Detector	Task	Ct	StdDev Ct	Quantity	Mean Qty	StdDev Qty	Filtered	Tm
A5	Sample D	Mycoplasma	Unknown	32.2065						79.9 °C

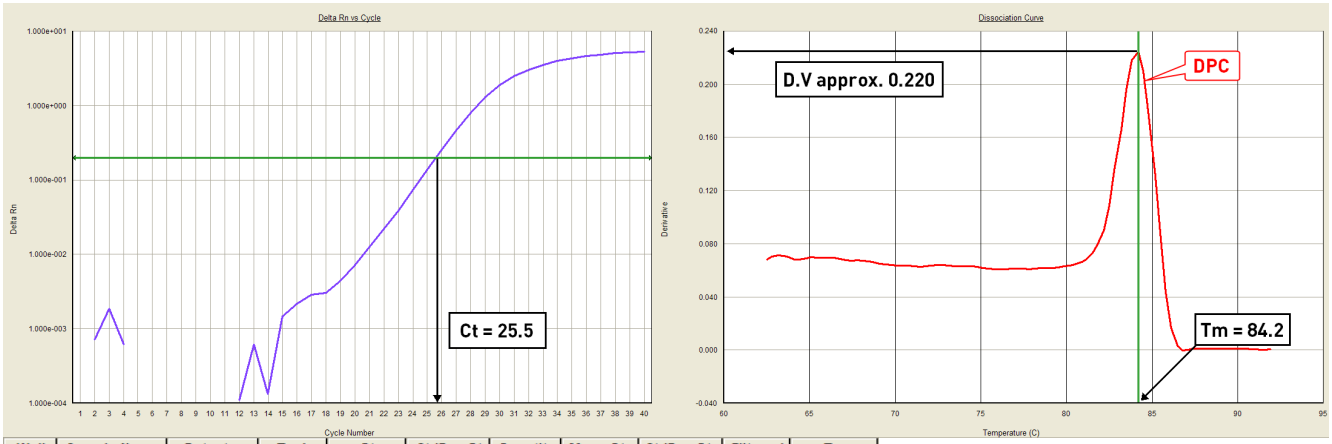
Figure 19 Sample containing 25 copies of *Mycoplasma* and 25 copies of DPC.

Example negative results with SDS v1.4 software



Well	Sample Name	Detector	Task	Ct	StdDev Ct	Quantity	Mean Qty	StdDev Qty	Filtered	Tm
E5	11	Mycoplasma	Unknown	37.4719						73.1 °C

Figure 20 Negative result.



Well	Sample Name	Detector	Task	Ct	StdDev Ct	Quantity	Mean Qty	StdDev Qty	Filtered	Tm
G5	12	Mycoplasma	Unknown	25.5402						84.2 °C

Figure 21 Negative sample spiked with 2,000 copies of DPC.

B

Troubleshooting

AccuSEQ™ 2.0 software

The following table shows some common reasons for inconclusive results with AccuSEQ™ 2.0 software. For a complete list, click (Help) in the toolbar at the top of the AccuSEQ™ 2.0 software screen. Refer to the *AccuSEQ™ software: Mycoplasma Getting Started Guide* (Part No. 4425587) for more data analysis information and example results.

Analysis result	Description	Possible cause	Recommended action
Inconclusive	Based on one or more parameter, the software did not make a positive or negative call.	Low sample concentration of *Mycoplasma*.	• Call manually according to laboratory guidelines. *or* • Allow the culture to grow for an additional 24 hours, then repurify the sample and repeat the experiment using assay components that were stored correctly.
Border-line C_t result		Incorrect baseline setting applied	Re-analyze samples using auto baseline, or change to manual start and end baseline settings.
		High SYBR signal from contaminating host cell DNA.	Apply RNase treatment during sample preparation. Contact your local FAS for more information.

Analysis result	Description	Possible cause	Recommended action
Sample inhibits amplification	PCR inhibition shown by: • Negative unknown sample *and* • Inhibition control ΔC_t>2 compared to the positive control	• Inappropriate sample preparation that results in carryover of chemicals from the lysis buffer. • Excess DNA or RNA. SYBR™ signals will have increased signal in Component View. • Components in cell culture media or additive (for example, dextran sulfate) may inhibit PCR and change T_m.	• Repurify the sample preparation and make sure of appropriate performance of wash and elution that does not carry over chemicals from reagents. • Repurify the sample using protocol with RNase and/or DNase treatment. Contact your Field Applications Specialist (FAS) for more information. • Most cell culture media inhibit PCR or change T_m without sample preparation. • Check if additives to cell culture media inhibit PCR. • To address inhibition from Dextran sulfate or Heparin, contact your FAS for more information.
High background signal	High background fluorescence signal: >500,000 fluorescent standard units (FSU).	The sample block is contaminated. Sample may have high concentration of nucleic acid carried from the cell culture during sample preparation.	Run a background calibration to identify the contaminated wells, then decontaminate the sample block. Repeat the experiment using assay components that were stored correctly.

MycoSEQ™ kit

Observation	Possible cause	Action
No positive control or target-specific SYBR™ Green dye signal is detected in inhibition control and/or positive control wells	Improper storage of Power SYBR™ Green PCR Master Mix.	Repeat the assay using properly stored assay components. Avoid freezing and thawing assay components. Protect Power SYBR™ Green PCR Master Mix from light.
	Improper storage of target-specific *Mycoplasma* Real-Time PCR Primer Mix (10×).	

Observation	Possible cause	Action
No positive control or target-specific SYBR™ Green dye signal is detected in inhibition control and/or positive control wells	Pipetting error (no premix solution added).	Repeat the assay. Make sure to pipet premix solution into all wells.
	Pipetting error (no positive control added).	Repeat the assay. Make sure to pipet positive control into all positive-control wells.
Target-specific signal is detected in negative control wells	Carryover contamination.	Repeat the assay using fresh aliquots of all reagents and clean pipetting equipment. If the negative control continues to show contamination, repeat the assay using a new kit. If the negative control continues to show contamination, contact your Application Specialist.
	High level of nonspecific product formation.	Check the dissociation curve to confirm. Repeat the assay using properly stored assay components. Avoid freezing and thawing assay components. Protect Power SYBR™ Green PCR Master Mix from light.

C

Background information

Mycoplasmas are the smallest and simplest self-replicating organisms. Their genome sizes range from about 540 kb to 1300 kb, with a G+C content of 23 mol to 41 mol%. Although mycoplasmas are derived from the gram-positive branch of walled eubacteria, their evolution from these walled bacteria resulted in a substantial reduction in genome size and loss of the functions required for synthesis and maintenance of a bacterial cell wall.

Mycoplasmas are a common bacterial contaminant of cell culture samples. Infection is persistent, difficult to detect and diagnose, and very difficult to cure. Mycoplasmas vary in size from 0.2 µm to 0.8 µm, so they can pass through some filters that are used to remove bacteria. Mycoplasma in infected cell cultures can change many cell processes, including altering cell growth rate, inducing morphological changes or cell transformation, and mimicking virus infection. Cell culture in pharmaceutical production must be *Mycoplasma*-free as required by the U.S. Pharmacopoeia and FDA regulatory requirements. Therefore, there is an absolute requirement for routine, periodic testing of possible contamination of all cell cultures used in pharmaceutical manufacturing. Because mycoplasmas grow slowly (the colonies can take up to 3 weeks to develop), traditional culture methods are unacceptable for rapid high-throughput testing. The recently introduced and validated rapid bacterial testing methods that are used in this kit provide for fast *Mycoplasma* screening.

D

Kit specificity

Sensitivity

The sensitivity of the PCR using this kit is 1 to 10 copies of the target DNA per reaction. Sensitivity of the assay in real culture samples depends on the quality of the sample preparation method that is used. The sample preparation procedure in the *PrepSEQ™ Sample Preparation Kits for Mycoplasma, MMV, and Vesivirus User Guide* (Pub. No. 4465957) allows you to detect:

- 4 to 10 CFU/mL of *Mycoplasma* from 10 mL of cell culture
  or
- 4 CFU/mL of *Mycoplasma* from 1 mL of media

Kit specificity

The MycoSEQ™ Mycoplasma Real-Time PCR Detection Kit can detect more than 90 different *Mycoplasma* species, including *Acholeplasma laidlawii* and *Spiroplasma citri*. The kit does not detect other genera or cell-line DNA.

Inclusivity – detectable species

The kit procedure in this protocol is designed to detect over 90 species, including the 14 shown below in the first table. For a complete list of species, contact Technical Support.

Species	Strain/source
Acholeplasma laidlawii	ATCC 23206D
Mycoplasma arginini	ATCC 23838D
Mycoplasma fermentans	ATCC 19989D
Mycoplasma gallisepticum	ATCC 15302
Mycoplasma genitalium	ATCC 33530D
Mycoplasma hominis	ATCC 23114D
Mycoplasma hyorhinis	ATCC 17981D
Mycoplasma hyponeumoniae	ATCC 25095
Mycoplasma orale	ATCC 23714D
Mycoplasma pirum	ATCC 25960D

Species	Strain/source
Mycoplasma pneumoniae	ATCC 15531D
Mycoplasma salivarium	ATCC 23064D
Mycoplasma sinoviae	ATCC 25204
Spiroplasma citri	ATCC 27556D

Exclusivity – undetectable organisms

Organism	Strain/source
Bacillus cereus	ATCC 10876
Bacillus subtilis	ATCC 6051
Campylobacter jejuni	ATCC 29428
Citrobacter freundii	6879
Clostridium perfringens	ATCC 12915
Enterobacter aerogenes	Q87
Enterobacter sakazaki	ATCC 51329
Enterococcus faecalis	ATCC 29212
Escherichia coli O157:H7	43888
Klebsiella oxytoca	ATCC 43165
Lactobacillus bulgaris	ATCC 11842
Listeria ivanovii	ATCC 19119
Listeria monocytogenes	ATCC 7644
Pseudomonas aeruginosa	ATCC 27853
Pseudomonas aeruginosa	ATCC 17423
Shigella	Sfla 395
Shigella	SFL 153
Shigella dysenteriae	ATCC 13313
Shigella dysenteriae	ESCL7-JHH
Staphylococcus aureus	ATCC 43300
Staphylococcus aureus aureus	PE491
Streptococcus faecalis	ATCC 9790

Organism	Strain/source
Vibrio cholerae	O36
Yersinia enterocolitica	ATCC 9610
Cat	Novagen™, Cat. No. 69235-3
Cow	Novagen™, Cat. No. 69238-3
Chicken	Novagen™, Cat. No. 69233-3
Chimpanzee	Bios, Inc.[1]
CHO	ATCC CCL-61
HeLa	ATCC CCL-2
Horse	Pel-Freez Biologicals, Cat. No. 39339-5
Orangutang	Bios, Inc.[1]
Pig	Novagen™, Cat. No. 69230-3
Rabbit	Pel-Freez Biologicals, Cat. No. 31130-1
Rat	Novagen™, Cat. No. 69238-3
Sheep	Novagen™, Cat. No. 69231-3

[1] No longer available

Appendix E Good PCR practices

PCR assays require special laboratory practices to avoid false positive amplifications. The high throughput and repetition of these assays can lead to amplification of one DNA molecule. Follow the guidelines below to prevent contamination and nonspecific amplification.

Good laboratory practices for PCR and RT-PCR

When preparing samples for PCR or RT-PCR amplification:

- Wear clean gloves and a clean lab coat.
  - Do not wear the same gloves and lab coat that you have previously used when handling amplified products or preparing samples.
- Change gloves if you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation and reaction setup.
  - Amplification and analysis of products.
- Do not bring amplified products into the reaction setup area.
- Open and close all sample tubes carefully. Avoid splashing or spraying samples.
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipettor or aerosol-resistant barrier pipette tips.
- Clean lab benches and equipment periodically with 10% bleach solution or DNA decontamination solution.

Plate layout suggestions

- For each plate row, dispense in sequence from left to right: negative controls, unknown samples, inhibition controls, and positive controls (at the end of the row or column).
- Place positive controls in one of the outer columns.
- If possible, separate all samples from each other by at least one well. If space is limited, place at least one well between unknown samples and controls.

Documentation and support

Related documentation

Document	Pub. No.	Description
MycoSEQ™ Mycoplasma Real-Time PCR Detection Kit Quick Reference	4465876	Provides brief, concise instructions on using the MycoSEQ™ Mycoplasma Detection Kit.
ViralSEQ™ Mouse Minute Virus (MMV) Real-Time PCR Detection Kit Quick Reference	4445236	Provides brief, concise instructions on using the ViralSEQ™ Mouse Minute Virus Real-Time PCR Detection Kit.
ViralSEQ™ Mouse Minute Virus (MMV) Real-Time PCR Detection Kit User Guide	4445235	Describes the ViralSEQ™ Mouse Minute Virus Real-Time PCR Detection Kit and provides information on preparing, running, and troubleshooting MMV detection.
PrepSEQ™ Sample Preparation Kits for Mycoplasma, MMV, and Vesivirus Quick Reference	4465875	Provides brief, concise instructions on using the PrepSEQ™ Sample Preparation Kits.
PrepSEQ™ Sample Preparation Kits for Mycoplasma, MMV, and Vesivirus User Guide	4465957	Describes the PrepSEQ™ Sample Preparation Kits and provides information on preparing, running, and troubleshooting sample preparation.
PrepSEQ™ Nucleic Acid Extraction Kit Quick Reference	4406303	Provides brief, concise instructions on using the PrepSEQ™ Nucleic Acid Extraction Kit.
PrepSEQ™ Nucleic Acid Extraction Kit User Guide	4400739	Describes the PrepSEQ™ Nucleic Acid Extraction Kit and provides information on preparing, running, and troubleshooting nucleic acid extractions.
Introduction to TaqMan® and SYBR™ Green Chemistries for Real-Time PCR Protocol	4407003	Describes the TaqMan® and SYBR™ Green Chemistries for Real-Time PCR and provides information on preparing, running, and troubleshooting PCR.

Document	Pub. No.	Description
AccuSEQ™ software: Mycoplasma Getting Started Guide	4425587	Provides brief, step-by-step procedures for *Mycoplasma* detection. It is designed to help you quickly learn to use the AccuSEQ™ Real-Time PCR Detection Software for Mycoplasma SEQ Experiments.
Applied Biosystems™ 7300/7500/7500 Fast Real-Time PCR System Getting Started Guide: Absolute Quantitation using Standard Curve	4347825	Provides brief, step-by-step procedures for absolute quantitation using a standard curve. It is designed to help you quickly learn to use the Applied Biosystems™ 7300/7500/7500 Fast Real-Time PCR System.

For information on new assays and updated product documentation, go to **thermofisher.com** .

Portable document format (PDF) versions of this guide and the documents listed above are available at **thermofisher.com**

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  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support
- Product documentation, including:
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

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