MycoSensor QPCR Assay Kit

Instruction Manual

Catalog #302106 (100 reactions) and #302107 (50 reactions)

Revision C.0

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302106-12

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MycoSensor QPCR Assay Kit

Contents

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MATERIALS PROVIDED

Materials provided	Quantity	
	Catalog #302106[a]	**Catalog #302107[b]**
2× MycoSensor QPCR Master Mix	2 × 1.25 ml	1 × 1.25 ml
MycoSensor Primer Mix	100 µl	50 µl
Amplification Control[c]	100 µl	50 µl
M. orale Positive Control [d]	125 µl	75 µl
A. laidlawii Positive Control [d]	125 µl	75 µl
Reference Dye, 1 mM[e]	100 µl	100 µl
DNA Purification Kit	1 kit (40 template purification reactions)	1 kit (40 template purification reactions)

[a] Sufficient PCR reagents are provided for one hundred, 50-µl PCR reactions.

[b] Sufficient PCR reagents are provided for fifty, 50-µl PCR reactions.

[c] The amplification control template is a plasmid DNA template that does not share any homology with human, mouse or rat genomic DNA sequences.

[d] The positive control templates are noninfectious genomic DNA.

[e] The reference dye is light sensitive and should be kept away from light whenever possible.

STORAGE CONDITIONS

Master mix, primer mix, control templates and reference dye: Upon receipt, store at –20°C. Store the 2× master mix at 4°C after thawing. Once thawed, full activity is guaranteed for 6 months.
DNA purification kit: Store at room temperature.

Note *The SYBR Green I dye (present in the master mix) and the reference dye are light sensitive and should be kept away from light whenever possible.*

ADDITIONAL MATERIALS REQUIRED

Spectrofluorometric thermal cycler
Nuclease-free PCR-grade water
70% (v/v) Ethanol
Microspin cup elution buffer [5 mM Tris (pH 8), 0.1 mM EDTA]

Revision C.0

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INTRODUCTION

The MycoSensor QPCR Assay Kit is used to detect *Mycoplasma* infection of cell cultures by real-time quantitative PCR (QPCR), using SYBR® Green dye detection. The MycoSensor QPCR assay kit includes a primer mix that amplifies the eight most common species of *Mycoplasma* that infect cell cultures and a convenient master mix containing the components necessary for PCR amplification and fluorescence detection.

The MycoSensor QPCR assay kit detects the following *Mycoplasma* and *Acholeplasma* species, which make up the eight most commonly-encountered agents of tissue culture infections: *M. arginini, M. fermentans, M. hominis, M. hyorhinis, M. pirum, M. salivarium, M. orale* and *A. laidlawi*. Although detection of other, less common species has not been specifically tested, the assay may detect the presence of other related species.

The MycoSensor QPCR assay kit primer mix is capable of detecting *Mycoplasma* infections in cell cultures in less than two hours, depending on the spectrofluorometric thermal cycler used for detection. The Mycoplasma primer mix, used in the polymerase chain reaction (PCR), can detect most *Mycoplasma* infections using template from as little as 100 µl of cell culture supernatant. Cell-growth-inhibiting or weak *Mycoplasma* infections can be detected by testing extracts made directly from cells.

Control Reactions and Assay Validation

The system includes several controls and design features to validate assay results. Two positive control templates (*M. orale* and *A. laidlawii* positive controls) are included to validate that polymerase-mediated amplification of *Mycoplasma* templates may be successfully detected in the experiment.

A second type of control is performed to confirm the absence of PCR inhibitors in each culture sample, reducing the occurrence of false negative results. These control reactions are performed by adding an amplification control template to reactions containing the cell culture sample. PCR amplification of the amplification control template generates a fluorescence signal that can be distinguished from the *Mycoplasma* fluorescence signal. DNA purification reagents are included in the kit to reduce the concentration of components in the sample that are inhibitory to PCR amplification (e.g., fetal calf serum, metabolic products, cell debris, etc.).

To minimize the occurrence of false positives, the PCR primers that constitute the Mycoplasma primer mix do not extend off of *E. coli* templates so that any potential *E. coli* DNA contamination present in cloned *Taq* DNA polymerase will not be detected. Include a negative (no template) control reaction in each experiment to demonstrate that any fluorescence signal generated is dependent on template contributed by the test cell cultures or the control templates.

Detection using SYBR® Green I Dye

SYBR Green I dye[1] has a high binding affinity to the minor groove of double-stranded DNA (dsDNA). It has an excitation maximum at 497 nm and an emission maximum at 520 nm. In the unbound state the dye exhibits little fluorescence; however, when bound to dsDNA, the fluorescence greatly increases, making it useful for the detection of product accumulation during real-time PCR.

The mechanism of SYBR Green dye detection during PCR is shown in Figure 1. During denaturation, all DNA becomes single-stranded. At this stage, SYBR Green is free in solution and produces little fluorescence. During the annealing step, the primers will hybridize to the target sequence, resulting in dsDNA to which SYBR Green I can bind. As the PCR primers are extended in the elongation phase, more DNA becomes double-stranded, and a maximum amount of SYBR Green I is bound. The increase in fluorescence signal intensity depends on the initial concentration of target present in the PCR reaction.

An important consideration when using SYBR Green I, however, is that signal can also be generated from non-specific dsDNA (e.g. primer-dimers and spurious PCR products). The fluorescence resulting from amplification of the target amplicon will not be initially distinguishable from fluorescence attributable to the spurious PCR products. To distinguish between fluorescence derived from specific and non-specific products, the assay includes a dissociation curve. During the dissociation curve, dsDNA is melted into ssDNA by a stepwise increase in temperature, with fluorescence data collected at each step. The dissociation curve fluorescence data is analyzed to reveal the temperature(s) at which major populations of dsDNA are converted to ssDNA (i.e. the major Tm peaks). The *Mycoplasma* amplicons amplified using the MycoSensor primer mix have a Tm of ~82°C. In contrast, fluorescence due to primer-dimers displays a Tm of <75°C (typically, the major non-specific peak generated using the MycoSensor assay system is seen at 74°C).

A passive reference dye, used to control for any non-PCR-directed variation in fluorescence, is also provided with the kit. Providing this reagent in a separate tube makes the MycoSensor QPCR assay kit adaptable for many real-time QPCR platforms. The MycoSensor QPCR assay kit has been optimized for maximum performance on the Mx3000P, Mx3005P and Mx4000 multiplex quantitative PCR instruments.

2× MycoSensor QPCR Master Mix

The 2× MycoSensor QPCR master mix in the MycoSensor QPCR assay kit includes SureStart *Taq* DNA polymerase, a modified version of *Taq2000* DNA polymerase with hot start capability. SureStart *Taq* DNA polymerase improves PCR amplification reactions by decreasing background from non-specific amplification and increasing amplification of desired products. In addition, the master mix contains dNTPs and optimized buffer components.

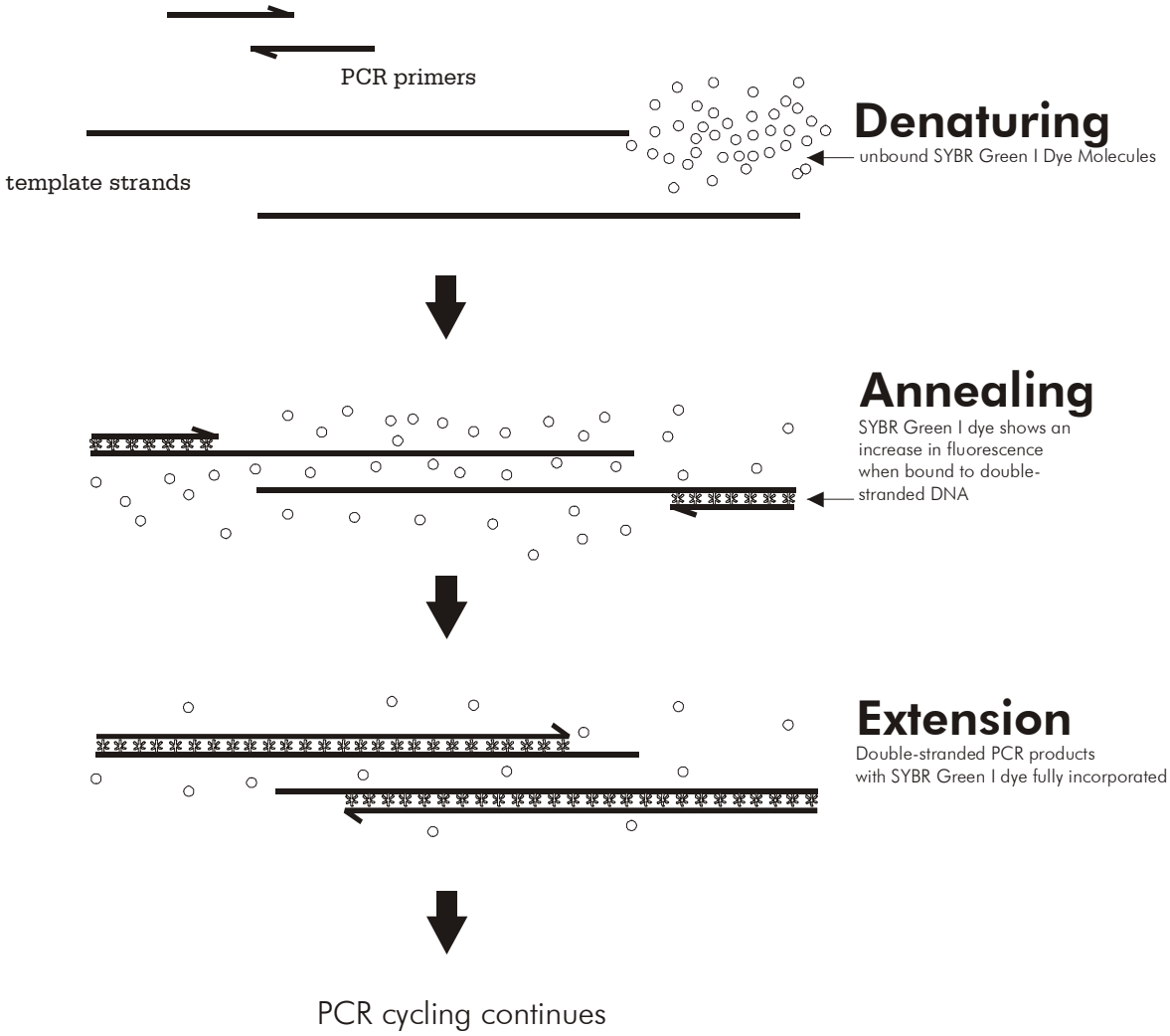

Figure 1 SYBR Green I dye has a higher affinity for double-stranded DNA (dsDNA) than for single-stranded DNA or RNA. Upon binding dsDNA, the fluorescence yield of SYBR Green I increases by approximately 1000 fold, making it ideal for detecting the accumulation of dsDNA.

Fluorescence Monitoring in Real-Time

When fluorescence signal from a PCR reaction is monitored in real-time, the results can be displayed as an amplification plot (see Figure 2), which reflects the change in fluorescence during cycling. This information can be used to derive the threshold cycle (Ct), from which the initial copy number may be quantified.[2] Ct is defined as the cycle at which fluorescence is statistically significant above background. The threshold cycle is inversely proportional to the log of the initial copy number.[2] The more template that is initially present, the fewer the number of cycles required for the fluorescence signal to become detectable above background.

Figure 2 shows amplification curve plots for a MycoSensor QPCR assay, collected on the Mx3000P instrument. Reactions containing the *Mycoplasma* positive control template (*M. orale Positive Control)* or the Amplification Control template are shown alongside a negative control reaction (*No Template Control*). The reactions containing template show a significant increase in fluorescence and have Ct values of ~29 and ~25, respectively. The *No Template Control* reaction has a Ct of $\cong 37$.

Figure 3 shows dissociation curve plots for the same set of reactions. In the dissociation curve, PCR samples are subjected to an increase in temperature from 55°C to 95°C; with fluorescence measurement taken at each temperature increment. As the temperature increases, the amplification products in each tube will melt according to their composition. If primer-dimer or non-specific products were made during the amplification step, they will generally melt at a lower temperature (T_m) than the desired products. The melting of products results in SYBR Green dissociation, which results in decreased fluorescence. After data collection is complete, fluorescence is plotted versus temperature. For an easy interpretation of the dissociation profile the first derivative of fluorescence should be displayed, i.e. $-R'(T)$ or $-Rn'(T)$.

The dissociation curve plot of these samples shows three fluorescence peaks. The peak in the *M. orale Positive Control* reaction, centered around 82°C, corresponds to *Mycoplasma* amplicon. The peak in the *Amplification Control* reaction, centered around 85°C, corresponds to the control amplicon. The peak in the *No Template Control* reaction, centered around 74°C, corresponds to primer-dimer. In this way, analysis of dissociation curve data can be a very powerful tool in the interpretation of SYBR Green dye fluorescence data.

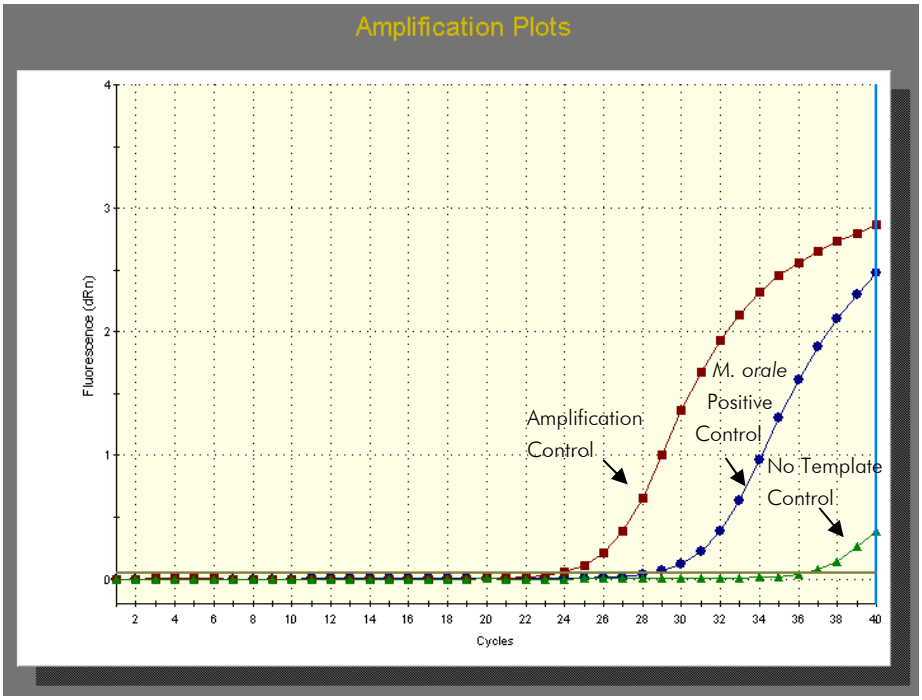

Figure 2 Amplification curve plots for reactions with and without the indicated MycoSensor kit template DNA. Data was collected and analyzed on the Mx3000P real-time PCR instrument.

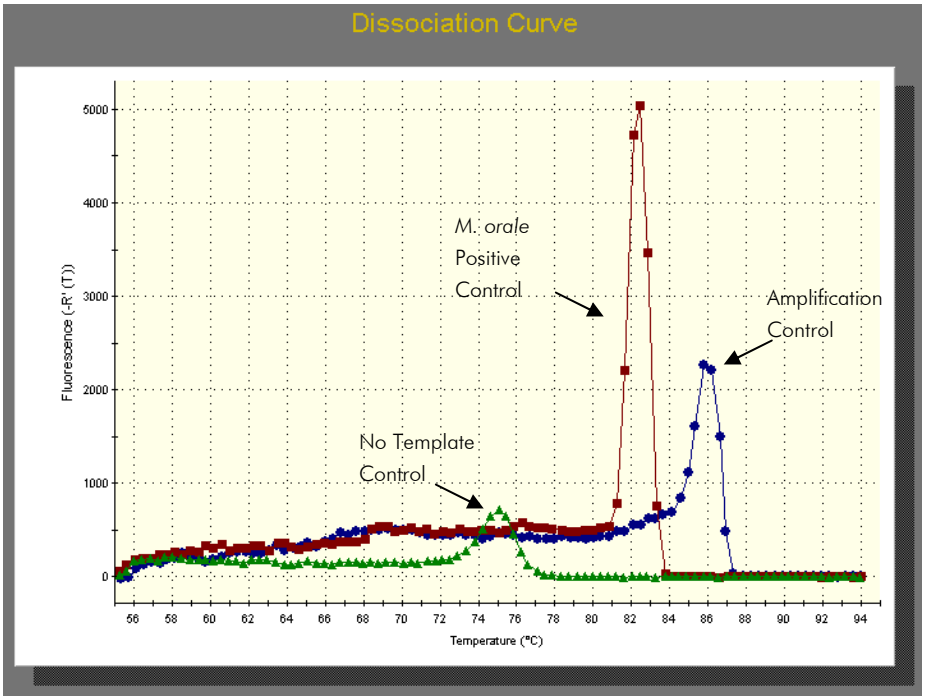

Figure 3 Dissociation curve plots for reactions with and without the indicated MycoSensor kit template DNA. When the amplified products are subjected to dissociation curve analysis, the fluorescence peak corresponding to the amplicons (centered around 82°C and 85°C) are distinguishable from each other and from the peak due to primer-dimer (centered around 74°C). Data was collected and analyzed on the Mx3000P real-time PCR instrument.

PREPROTOCOL CONSIDERATIONS

Cell Culture Conditions

Cells should be cultured in the absence of antibiotics for several days in order to maximize the strength of the signal that is observed in PCR. Test supernatants to be used in PCR should be derived from cells that are at or near confluence.

Reference Dye

A passive reference dye is included in this kit and may be added to compensate for non-PCR related variations in fluorescence. Fluorescence from the passive reference dye does not change during the course of the PCR reaction but provides a stable baseline to which samples are normalized. In this way, the reference dye compensates for changes in fluorescence between wells caused by slight volume differences in reaction tubes. The excitation and emission wavelengths of the reference dye are 584 nm and 612 nm, respectively. Although addition of the reference dye is optional when using the Mx4000, Mx3000P or Mx3005P system, with other instruments (including the ABI 7900HT and ABI PRISM® 7700) the use of the reference dye may be required for optimal results.

Reference Dye Dilution Recommendations

Prepare **fresh*** dilutions of the reference dye prior to setting up the reactions, and **keep all tubes containing the reference dye protected from light as much as possible**. Make initial dilutions of the reference dye using nuclease-free PCR-grade H_2O. If you are using a Mx3000P, Mx3005P or Mx4000 instrument, use the reference dye at a final concentration of 30 nM. If you are using the ABI PRISM 7700 or the GeneAmp® 5700 instrument, use the reference dye at a final concentration of 300 nM. For other instruments, use the following guidelines for passive reference dye optimization. For instruments that allow excitation at ~584 nm (including most tungsten/halogen lamp-based instruments and instruments equipped with a ~584 nm LED), begin optimization using the reference dye at a final concentration of 30 nM. For instruments that do not allow excitation near 584 nm, (including most laser-based instruments) begin optimization using the reference dye at a final concentration of 300 nM.

Data Acquisition with a Spectrofluorometric Thermal Cycler

The instrument should be set to collect SYBR Green I fluorescence data in real-time at both the annealing step and the extension step of each cycle during amplification. In addition, data should be collected at each temperature increment in the dissociation curve. See the *Protocols* section for specific amplification and dissociation thermal profile recommendations. Consult the instrument manufacturer's instruction manual for data acquisition instructions.

* The diluted reference dye, if stored in a light-protected tube at 4°C, can be used within the day for setting up additional assays.

Preventing Template Cross-Contamination

Take precautions to minimize the potential for carryover of nucleic acids from one experiment to the next. Use separate work areas and pipettors for template preparation and PCR setup steps. Use positive displacement pipets or aerosol-resistant pipet tips.

The 2× MycoSensor QPCR master mix contains dUTP instead of dTTP. When dUTP replaces dTTP in PCR amplification, UNG treatment (Uracil-N-glycosylase, not provided in this kit) can prevent the subsequent reamplification of dU-containing PCR products. UNG acts on single- and double-stranded dU-containing DNA by hydrolysis of uracil-glycosidic bonds at dU-containing DNA sites. When this strategy is put to use, carry-over contamination will be eliminated while template DNA (DNA containing T) will be left intact.

PROTOCOLS

Preparing the Template

Cell Extract Protocol 1: Boiling Extract from the Cell Culture Supernatant

Notes *For a protocol that provides cell-equivalent standardization and detection of weak or cell-growth-inhibiting* Mycoplasma *infections, see* Cell Extract Protocol 2: Boiling Extract from the Cultured Cells.

Cell culture supernatants may contain media components that inhibit PCR amplification (e.g., fetal calf serum, metabolic products, etc.). For an alternative protocol that avoids the introduction of cell culture medium into the PCR, see Cell Extract Protocol 2: Boiling Extract from the Cultured Cells.

1. Prepare a boiling water bath or set a thermal cycler heat block at 95°C.

2. Transfer 100 µl of supernatant from the test cell culture to a microcentrifuge tube. Tightly close the tube to prevent opening during the subsequent heating step.

3. Boil (or heat to 95°C) the supernatant for 5 minutes. Spin the tube briefly (30–60 seconds) in a microcentrifuge.

4. Transfer 100 µl of the supernatant to a fresh tube. Proceed to *Purification of Template DNA.*

Cell Extract Protocol 2: Boiling Extract from the Cultured Cells

Note *This protocol, while more involved, provides cell-equivalent standardization and a more sensitive detection limit for cell lines whose growth is inhibited by* Mycoplasma*. This protocol may also be used as an alternative to testing cell culture supernatants that may be inhibitory to PCR.*

1. Harvest adherent cells by scraping the cells from the plate. **Do not treat the cells with trypsin**, as this may inhibit subsequent detection by PCR. Pipet 1 ml of scraped adherent cells or suspension cells into a microcentrifuge tube (>100,000 cells are needed to complete this protocol). Spin the tube in a microcentrifuge for 10–15 seconds. Carefully decant the supernatant.

2. Resuspend the cells in 1 ml of sterile Dulbecco's phosphate-buffered saline (PBS). Spin the tube in a microcentrifuge for 10–15 seconds. Carefully decant the supernatant. Repeat this wash step.

3. Resuspend the cells once more as indicated in step 2 and count the cells under a microscope. Aliquot 100,000 cells in a fresh microcentrifuge tube. Spin the tube in a microcentrifuge for 10–15 seconds. Carefully aspirate the supernatant with a micropipet. Add 100 µl of sterile UV-irradiated water to the cell pellet.

4. Prepare a boiling water bath or set a thermal cycler heat block to 95°C. Boil the tube containing the cells for 10 minutes. Spin the tube briefly (30–60 seconds) in a microcentrifuge.

5. Transfer 100 µl of the supernatant to a fresh tube. Proceed to *Purification of Template DNA.*

Purification of Template DNA

1. To the 100 µl of cell culture extract prepared using either *Cell Extract Protocol 1* or *Cell Extract Protocol 2*, above, add 100 µl of the DNA-binding solution provided with the DNA purification kit. Add 200 µl of 70% (v/v) ethanol and then mix well.

2. Place a microspin cup in a 2-ml receptacle tube (both provided with the DNA purification kit). Using a pipet, transfer the mixture from step 1 into the seated microspin cup. (Exercise caution to avoid damaging the fiber matrix with the pipet tip.) Snap the cap of the 2-ml receptacle tube onto the top of the microspin cup.

3. Spin the tube in a microcentrifuge at maximum speed for 30 seconds.

   Notes *To avoid cross-contamination, it is important to cap the microspin cups prior to each spin.*

   The template DNA is retained in the fiber matrix of the microspin cup.

4. Retain the microspin cup and discard the receptacle tube. Seat the microspin cup in a fresh 2-ml receptacle tube.

5. Prepare 1× wash buffer by adding 40 ml of 100% ethanol to the bottle of 5× wash buffer (provided), and then mark the label container *[√] 1× (Ethanol Added)*. Store the 1× wash buffer at room temperature.

6. Add 750 µl of 1× wash buffer to the microspin cup. Snap the cap of the receptacle tube onto the top of the microspin cup.

7. Spin the tube in a microcentrifuge at maximum speed for 30 seconds.

8. Retain the microspin cup and discard the receptacle tube. Seat the microspin cup in a fresh 2-ml receptacle tube.

9. Spin the tube in a microcentrifuge at maximum speed for 30 seconds to remove residual wash buffer. Upon removal from the centrifuge, make sure that all of the wash buffer is removed from the microspin cup.

10. Transfer the microspin cup to a fresh 2-ml microcentrifuge tube.

11. Add 50 µl of elution buffer [5 mM Tris (pH 8), 0.1 mM EDTA] directly onto the top of the fiber matrix of the microspin cup. Avoid touching the fiber matrix with the pipet tip.

12. Snap the cap of the receptacle tube onto the microspin cup. Incubate the tube at room temperature for 5 minutes.

13. Spin the tube in a microcentrifuge at maximum speed for 30 seconds.

14. The purified template is in the bottom of the 2-ml receptacle tube. Transfer the cap from the microspin cup to the receptacle tube and discard the microspin cup.

Preparing the QPCR Reactions

Prepare the set of reactions listed in the following table in duplicate. These include three types of control reactions: 1) an amplification control for each cell culture extract, 2) positive control reaction(s) containing Mycoplasma positive control template DNA, and 3) a negative (no template) control.

	Reaction Types			
Reaction Components	**Cell Culture Tests**	**Amplification-Positive Controls (for each extract)**	**Mycoplasma-Positive Control**	**Negative Control**
PCR Template(s) added to reaction	Cell extract	Cell extract + Amplification Control template	*M. orale* or *A. laidlawii* Positive Control template	None
Reagent Mixture (from step 3 below)	44 µl	44 µl	44 µl	44 µl
Test cell extract	5 µl	5 µl	—	—
Amplification Control template	—	1 µl	—	—
M. orale or *A. laidlawii* Positive Control template	—	—	5 µl	—
H_2O	1 µl	—	1 µl	6 µl
Final reaction volume	50 µl	50 µl	50 µl	50 µl

When setting up the reactions, prepare a single reagent mixture (as specified in steps 1–3), then aliquot this mixture to the individual reaction tubes and add the specified PCR template(s).

Prepare the Reagent Mixture

1. If the reference dye will be included in the reaction, (optional), dilute the dye solution provided **1:500 (for the Mx3000P, Mx3005P, or Mx4000 instruments)** or **1:50 (for the ABI PRISM 7700 instrument)** using nuclease-free PCR-grade H_2O. See the *Reference Dye* section of *Preprotocol Considerations* if using another type of instrument. **Keep all solutions containing the reference dye protected from light.**

   Note *If using a system other than the Mx4000, Mx3000P or Mx3005P instruments, the use of the reference dye may be required for optimal results.*

2. Prepare the reagent mixture by adding the listed components *in order*. The recipe shown is for one reaction. Multiply the volumes listed according to the number of reactions included in the experiment.

    17.25 µl Nuclease-free PCR-grade H_2O
    25 µl of 2× MycoSensor QPCR master mix
    1 µl of Mycoplasma primer mix
    0.75 µl of diluted reference dye (optional) or PCR-grade H_2O

Note *Once the tube containing the MycoSensor QPCR master mix is thawed, store it on ice while setting up the reactions. Following initial thawing of the master mix, store the unused portion at 4°C. Multiple freeze-thaw cycles should be avoided.* ***SYBR Green I dye (present in the master mix) is light-sensitive, solutions containing the master mix should be protected from light whenever possible.***

3. Gently mix the solution without creating bubbles (do not vortex).

4. Aliquot 44 µl of the reagent mixture into each PCR tube.

Add the Template

5. To the cell culture test reaction tubes, add 5 µl of the corresponding purified test culture template (from step 15 of *Purification of Template DNA*) plus 1 µl of PCR-grade H_2O.

6. To the amplification positive control reaction tubes, add 5 µl of the corresponding purified test culture template (from step 15 of *Purification of Template DNA*) plus 1µl of amplification control template.

7. To the Mycoplasma positive control reaction tubes, add 5 µl of *M. orale* or *A. laidlawii* positive control template plus 1 µl of PCR-grade H_2O.

8. To the negative control reaction tubes, add 6 µl PCR-grade H_2O (or negative extract).

9. Gently mix the reactions without creating bubbles (do not vortex) and then centrifuge the reactions briefly.

    Note *Bubbles interfere with fluorescence detection.*

Running the PCR and Dissociation Curves

Set up the QPCR instrument to run the PCR cycling (amplification) program specified below, followed by the appropriate dissociation curve program.

PCR Cycling Program

Place the reactions in the QPCR instrument and run the PCR program below.

Cycles	Duration of cycle	Temperature
1	10 minutes	95°C
40	15 seconds	95°C
	1.0 minute[a]	60°C
	30 seconds	79°C

[a] Set the temperature cycler to detect and report fluorescence during the annealing (60°C) step and the extension (79°C) step.

Dissociation Curve

Mx4000 Instrument: Prior to the dissociation curve, incubate the reactions for 1 minute at 95°C to denature the PCR products. Ramp down to 55°C. For the dissociation curve, complete 81 cycles (30 seconds/cycle) of incubation where the temperature is increased by 0.5°C/cycle, beginning at 55°C and ending at 95°C. Figure 4 shows how to set the *Thermal Profile* (left) and the *Plateau Properties* (right) for the dissociation curve program on the Mx4000 instrument.

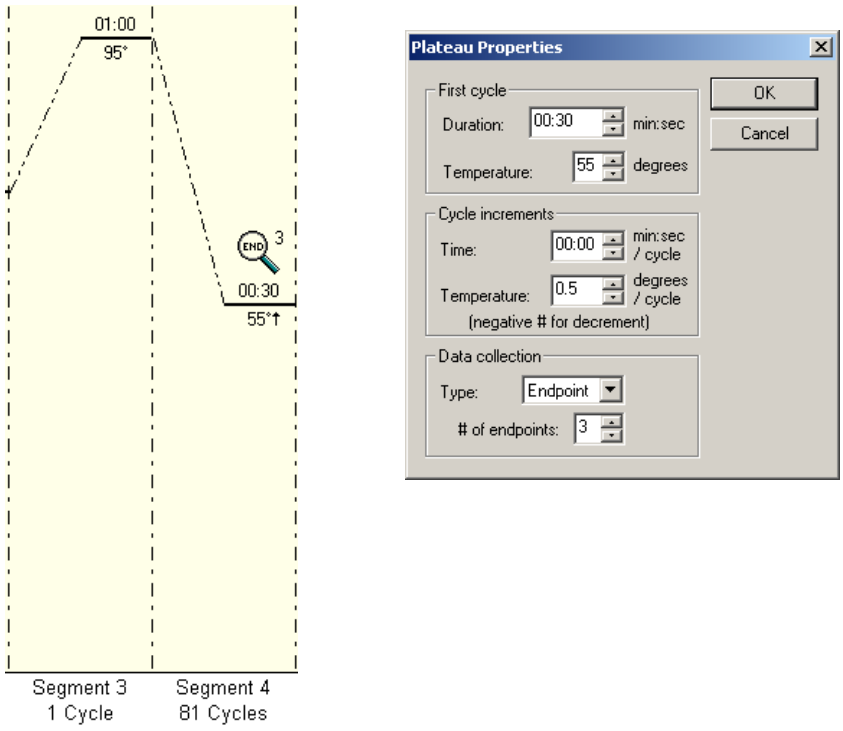

Figure 4 Dissociation program settings on the Mx4000 instrument.

Mx3000P and Mx3005P Instruments: Prior to the dissociation curve, incubate the reactions for 1 minute at 95°C to denature the PCR products. Ramp down to 55°C. For the dissociation curve, ramp up the temperature from 55°C to 95°C (at the instrument default rate of 0.2°C/sec) and collect fluorescence data continuously on the 55–95°C ramp. Figure 5 shows how to set the *Thermal Profile* for the dissociation curve program on the Mx3000P or theMx3005P instrument.

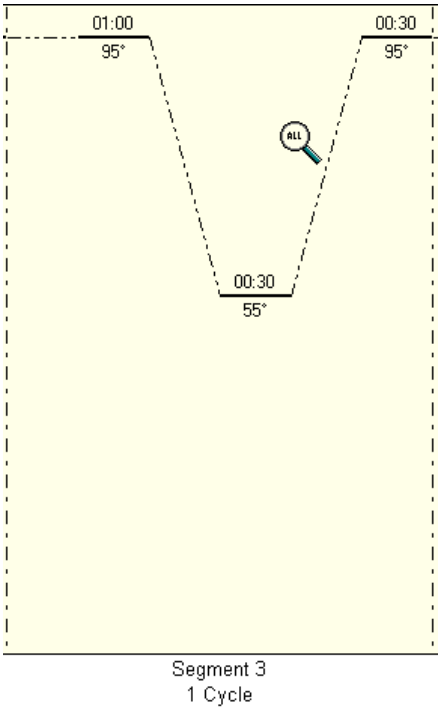

Figure 5 Dissociation program settings on the Mx3000P or Mx3005P instrument.

Other Instruments: follow the instrument manufacturer's guidelines for setting up a dissociation curve.

EXPECTED RESULTS

Using the following table, verify that the positive and negative controls give the expected results, and determine whether the test cell culture is infected with *Mycoplasma*. Refer to Figures 2 and 3 for sample control reaction amplification plot and dissociation curve data.

Note *If the cell culture is heavily infected with Mycoplasma (>10^5 Mycoplasma cfu per ml of culture/media), amplification of the Mycoplasma product may result in diminished or no amplification of the Amplification Control product (Tm ~85°C).*

PCR template	Amplification Plot: Threshold Cycle (Ct) Observations	Dissociation Profile: Melting Point (Tm) Observations	Interpretation
M. orale or *A. laidlawii* positive control template	Ct observed	Tm peak around 82°C	Expected positive control result
	No Ct	No peak	Failed positive control reaction
Negative control (i.e., water or negative extract)	No Ct	No peak	Expected negative control result (a late Ct value with Tm peak of ~74°C is also acceptable, see below)
	Ct observed	Tm peak around 74°C	Primer dimer formation (acceptable negative control result)
	Ct observed	Tm peak around 82°C	Contamination with *Mycoplasma* or positive control DNA
	Ct observed	Tm peak around 85°C	Contamination with the amplification control template
Cell culture extract (without amplification control)	Ct observed	Tm peak around 82°C	*Mycoplasma* infection
	No Ct	No peak around 82°C	Putative negative for *Mycoplasma* infection. Check the results for cell culture extract + amplification control to confirm absence of PCR inhibition.
Cell culture extract + amplification control template	No Ct	No peak	Inhibited PCR reaction, repeat cell extract preparation and repeat assay
	Ct observed	Single Tm peak around 85°C	No PCR inhibition, no *Mycoplasma* infection
	Ct observed	Two Tm peaks around 82°C and 85°C	Confirms *Mycoplasma* infection
	Ct observed	Single Tm peak around 82°C	Heavy *Mycoplasma* infection (amplification of abundant *Mycoplasma* DNA may interfere with control amplification)

TROUBLESHOOTING

Observation	Suggestion(s)
Unexpectedly late or no Ct for the positive control templates	Obtaining a late or no Ct with this template is indicative of poor amplification efficiency. Suboptimal storage or handling of the reagents, (especially the 2× MycoSensor QPCR master mix) and/or the performance of the spectrofluorometric thermal cycler may account for the results.
No amplification of the amplification control in the test culture sample (as indicated by lack of the 85°C Tm peak in the dissociation curve), but the amplification of the positive controls are optimal	The test sample may contain inhibitors that reduce the efficiency of amplification. Repeat the template DNA purification steps for the affected culture. Alternatively, dilution of the test sample may be performed, but detection sensitivity will be reduced.
	If *Mycoplasma* target amplification is observed in test culture samples (as indicated by an 82°C Tm peak in the dissociation curve), the amplification control amplicon may not be seen due to competition with the *Mycoplasma* DNA template. In this case, the sample should be considered a strong positive.
	Cell culture with supernatant that is consistently inhibitory to PCR amplification should be harvested and washed according to the procedures outlined in *Cell Extract Protocol 2: Boiling Extract from the Cultured Cells*.
Excess non-specific PCR products (as indicated by an abundance of low-Tm amplicons in the dissociation curve)	The presence of non-specific PCR product(s) may indicate PCR stringency variation due to minor differences in thermal cycling equipment, PCR reagents (e.g., water), or improper magnesium concentration. It is not necessary to supplement the PCR reaction with $MgCl_2$ when using the QPCR master mix. If excess primer-dimer is observed, the extension temperature of the PCR can be raised, thereby reducing the signal from primer-dimers.

REFERENCES

1. Molecular Probes, Inc., at http://www.probes.com/media/pis/mp07567.pdf.
2. Higuchi, R., Fockler, C., Dollinger, G. and Watson, R. (1993) *Biotechnology (N Y)* 11(9):1026-30.

ENDNOTES

ABI PRISM® is a registered trademark of The Perkin-Elmer Corporation.
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MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.

MycoSensor QPCR Assay Kit

Catalog #302106, #302107

QUICK-REFERENCE PROTOCOL

Preparing the Template from Cell Culture Supernatant

Note *For template preparation from cell culture cells, see* Cell Extract Protocol 2: Boiling Extract from the Cultured Cells *in* Preparing the Template.

- Boil (heat to 95°C) 100 µl of the cell culture supernatant in a microcentrifuge tube for 5 minutes.
- Spin the tube briefly (30–60 seconds) in a microcentrifuge.
- Transfer 100 µl of the supernatant to a fresh tube and add 100 µl of DNA binding solution. Add 200 µl of 70% (v/v) ethanol and mix well.
- Transfer the mixture into a microspin cup seated in a 2-ml microcentrifuge tube (both provided with the DNA purification kit). Cap the microspin cup and then spin the tube in a microcentrifuge at maximum speed for 30 seconds.
- Retain the microspin cup and discard the receptacle tube. Seat the microspin cup in a fresh 2-ml receptacle tube.
- Add 750 µl of 1× wash buffer to the microspin cup. Cap the microspin cup and then spin the tube in a microcentrifuge at maximum speed for 30 seconds.
- Discard the receptacle tube and transfer the microspin cup to a fresh 2-ml tube. Remove residual liquid from the spin cup by spinning the tube in a microcentrifuge at maximum speed for 30 seconds.
- Transfer the microspin cup to a fresh 2-ml microcentrifuge tube. Add 50 µl of elution buffer onto the top of the fiber matrix. Cap the microspin cup and then incubate at room temperature for 5 minutes.
- Spin the tube in a microcentrifuge at maximum speed for 30 seconds. The purified temple is in the bottom of the 2-ml microcentrifuge tube.

Preparing and Cycling the PCR Mixture

- Prepare a reagent mixture for the appropriate number of samples to be tested, using multiples of the quantities listed below.

PCR-grade H_2O	17.25 µl
2× MycoSensor QPCR master mix	25 µl
Mycoplasma primer mix	1 µl
Diluted reference dye (optional) or PCR-grade H_2O	0.75 µl
Total volume (per reaction)	44 µl

Note *If using a system other than the Mx4000, Mx3000P or Mx3005P instruments, the use of the reference dye may be required for optimal results.*

- Aliquot 44 µl of the reaction mixture into each PCR tube.
- Add 5 µl of each test template, positive control template, or water (negative control) to the appropriate reaction tubes.
- Add 1µl of amplification control template to the amplification control tubes and 1µl of water to the remaining tubes for a final reaction volume of 50 µl.
- Place the reactions in the QPCR instrument and run the PCR program below.

Cycles	Duration of cycle	Temperature
1	10 minutes	95°C
40	15 seconds	95°C
	1.0 minute[a]	60°C
	30 seconds	79°C

[a] Set the temperature cycler to detect and report fluorescence during the annealing (60°C) step and the extension (79°C) step.

- Run a dissociation curve, using settings appropriate for the QPCR instrument, and according to the following guidelines. Prior to the dissociation curve, incubate the amplified products for 1 minute at 95°C. Ramp down to 55°C and then complete a dissociation curve by increasing the temperature to 95°C, collecting fluorescence data throughout the 55–95°C temperature interval. See the *Dissociation Curve* section of the manual for more information.