# Mycoplasma *Plus* PCR Primer Set

## Instruction Manual

**Catalog #302008**

Revision E.0

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

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# Mycoplasma *Plus* PCR Primer Set

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# Mycoplasma *Plus* PCR Primer Set

## MATERIALS PROVIDED

| Material provided | Quantity |
|---|---|
| PCR primers | 200 µl |
| Internal control template[a] | 100 µl |
| Positive control template[b] | 100 µl |
| StrataClean resin | 50 determinations |

[a] The internal control template is a cloned PCR product that contains PCR priming sites identical to the Mycoplasma PCR target.

[b] The positive control template is noninfective genomic DNA of *Mycoplasma orale*.

## STORAGE CONDITIONS

**All Components:** –20°C

## ADDITIONAL MATERIALS REQUIRED

Deoxynucleotides
*Taq* DNA polymerase or *Taq2000* DNA polymerase
10× *Taq* reaction buffer
Restriction enzyme *Sau*3A I
*Sau*3A I buffer

## NOTICE TO PURCHASER

The Stratagene Mycoplasma *Plus* PCR primer set is for research use only and is not intended for clinical diagnosis or applications involving humans. The Mycoplasma *Plus* PCR primer set must be used in accordance with NIH guidelines for recombinant DNA.

Revision E.0

## INTRODUCTION

The Mycoplasma *Plus* PCR primer set is used in the polymerase chain reaction (PCR) to detect Mycoplasma infections in cell cultures. The PCR primers can detect most Mycoplasma infections using 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. In addition to the primers, the set includes an internal control template that can be used to confirm polymerase-mediated amplification in all PCR samples and a positive control template that can be used to confirm the size of the Mycoplasma PCR amplification product.

If the cell line is infected with Mycoplasma, the PCR primers will yield a single 874-bp amplification product, regardless of which species of Mycoplasma is present in the sample. Restriction-fragment analysis of the amplification products using the restriction enzyme *Sau*3A I can corroborate the PCR results and determine which species of Mycoplasma is present in the sample. *Sau*3A I digestion of the Mycoplasma amplification products yields distinct fragmentation patterns (or fingerprints) that identify the Mycoplasma species. A species analysis is conducted by digesting a portion of the PCR sample, separating the restriction fragments using agarose gel electrophoresis, and comparing the fragmentation pattern to the fingerprints that identify the Mycoplasma species (see Figures 1 and 2).

## CRITICAL PRECAUTIONARY NOTES

- For best results, culture cells 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.

- To avoid false positives, wear gloves while preparing the template for PCR (see *Preparing the Template*), while preparing the reaction mixtures for PCR (see *Preparing the PCR Mixture*) and while performing the PCR (see *PCR Program*).

- To avoid false positives, we suggest UV-irradiating all water and water–buffer mixes used in the PCR. For example, in making the common reaction mixture for use in PCR, irradiate the *Taq* reaction buffer and water before adding the dNTPs and the *Taq* DNA polymerase. This procedure helps prevent the introduction of exogenous DNA into the PCR. Irradiate using the Stratalinker UV crosslinker set at autocrosslink mode (equivalent to 12,000 µJ/cm$^2$) or an equivalent source of UV irradiation.

- To avoid cross-contamination between samples, use aerosol-resistant pipet tips throughout the protocol and handle the samples in an area that is removed from the bench space in which the reaction mixtures for PCR are made.

- To avoid cross-contamination of the kit components, spin the component tubes in a microcentrifuge at maximum speed for 30 seconds to collect all material at the bottom of the tube. Following centrifugation, wipe the outside of each component tube with 70–100% ethanol. Repeat this procedure before every use of the components.

## SUMMARY OF THE METHOD

- Remove an aliquot of medium or harvest the cells (see *Preparing the Template*)

- Prepare the boiling extract of medium or cells (see *Preparing the Template*)

- Test the extract in a PCR (see *Preparing the PCR Mixture and PCR Program*)

- Digest the PCR product with the restriction enzyme *Sau*3A I to determine the species of Mycoplasma (see *Digesting the PCR Products with Sau3A I*).

- Analyze the results of the PCR and the digestion with *Sau*3A I using agarose gel electrophoresis (see *Electrophoresis of the PCR and Sau3A I-Digestion Products*)

# PROTOCOL

## Preparing the Template

### Boiling Extract of Cell Culture Supernatant

***Note*** *For a protocol that provides cell-equivalent standardization and detection of cell-growth-inhibiting Mycoplasma infections, see* Boiling Extract of Cell Culture 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 top of 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 (2–5 seconds) in a microcentrifuge.

4. Resuspend the StrataClean resin by vortexing the tube until no pellet remains (~30 seconds). Add 10 µl of StrataClean resin to the supernatant. Mix the resin and the supernatant by gently flicking the tube. Spin the tube in a microcentrifuge briefly (for 5–10 seconds) to pellet the resin. (No incubation is necessary.)

5. Transfer an aliquot of the treated supernatant to a fresh tube, avoiding the pelleted resin. This supernatant will be used as the template in the PCR. The template is stable for several days stored at 4°C.

### Boiling Extract of Cell Culture 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.*

1. Harvest adherent cells with trypsin using standard techniques. Pipet 1 ml of trypsin-treated adherent cells or suspension cells into a microcentrifuge tube (>50,000 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) or in a comparable isotonic solution. 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 50,000 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 in a microcentrifuge briefly (for 2–5 seconds).

5. Resuspend the StrataClean resin by vortexing the tube until no pellet remains (~30 seconds). Add 10 µl of StrataClean resin to the cell pellet extract. Gently flick the tube to mix the resin and the cell extract. Spin the tube in a microcentrifuge for 5–10 seconds to pellet the resin. (No incubation is necessary.) Assuming that 50,000 cells were resuspended in 100 µl of water, then 10 µl of straight supernatant is equivalent to 5,000 cells.

6. Transfer an aliquot of the treated supernatant to a fresh tube, avoiding the pelleted resin. This supernatant will be used as the template in the PCR. The template is stable for several days stored at 4°C.

7. Test 50 and 5,000 cell equivalents with and without the internal control template. The internal control will control for cell debris that may inhibit PCR. Prepare a 100-µl 1:100 dilution of the straight supernatant to obtain 50 cell equivalents per 10 µl. Strong Mycoplasma infections are detected in as little as 10 cell equivalents, while detection of weak or cell-growth-limiting infections requires cell equivalents in the 500–5,000 range.

## Preparing the PCR Mixture

***Notes*** *To achieve optimal accuracy, perform each detection both with and without the internal control template.*

*When calculating the number of reactions, plan to perform one negative control using either water (i.e., 5 µl of UV-irradiated $H_2O$) or an extract from a cell line known to be negative for Mycoplasma and one reaction using either the positive control or the internal control template provided in this kit. The former reaction will determine the background amplification to be seen in the absence of Mycoplasma. The latter reaction will validate that a polymerase-mediated amplification has occurred.*

*Optimal reaction conditions for Mycoplasma detection include the following:*

10 mM of Tris-HCl (pH 8.3–8.8)
50 mM KCl
1.5–2.5 mM $MgCl_2$
200 µM of each dNTP
2 U of *Taq* DNA polymerase/reaction

*For optimally efficient and accurate amplification of Mycoplasma template with the provided PCR primers, use* Taq2000 *DNA polymerase.*

## Master Mix

**Note** *Prepare the following common reaction mixture for use in the PCR. This recipe is for one reaction; it should be adjusted for the number of samples to be tested. The final reaction volume is 50 µl.*

1. Add 5 µl of 10× *Taq* reaction buffer to 35.2 µl of $H_2O$.

2. UV-irradiate this solution at 12,000 µJ/cm$^2$ (if possible). (If using the Stratalinker UV crosslinker set on the autocrosslink mode.)

3. Add the following:

   0.4 µl of dNTPs (25 mM stock, the final concentration of each dNTP in the PCR is 200 µM)
   0.4 µl of *Taq* DNA polymerase (5 U/µl stock, 2 U/PCR reaction )
   2 µl of primer mix (stock primer mix is 25 µM in each primer)

4. Add either 2 µl of the internal control template or, if the template is not used, add 2 µl of $H_2O$ instead. The total volume of the reaction mixture for PCR should be 45 µl.

   **Note** *The internal control template should not be used in samples that will be subjected to restriction analysis with* Sau*3A I.*

5. Aliquot 45 µl of the reaction mixture into each PCR tube.

6. Add 5 µl of the test template (from either step 5 of *Boiling Extract of Cell Culture Supernatant* or step 6 of *Boiling Extract of Cell Culture Cells* under *Preparing the Template*) to the appropriate reaction tube.

7. Add 5 µl of the negative control (water or negative extract) to the appropriate reaction tube.

8. Add 5 µl of the positive control template to the appropriate reaction tube.

9. If the temperature-cycler is not equipped with a heated cover, overlay each reaction with ~50 µl of DNase-, RNase-, and protease-free mineral oil (available from Sigma Chemical Company, St. Louis, Missouri).

## PCR Program

The following PCR program yields optimal amplification of the 874-bp PCR product from all species of Mycoplasma.

| Segment | Cycle(s) | Temperature | Duration |
|---|---|---|---|
| 1 | 1 | 94°C | 2 minutes |
| | | 50°C | 2 minutes |
| | | 72°C | 2 minutes |
| 2 | 40 | 94°C | 1 minute |
| | | 50°C | 1 minute |
| | | 72°C | 2 minutes |

## Digesting the PCR Product with *Sau*3A I

**Note** *Conduct the restriction analysis using a sample that does not contain the PCR product of the internal control template. The presence of the internal control template changes the restriction pattern derived from Mycoplasma amplification products.*

1. Remove a 15-µl aliquot of the PCR product and mix it with 1.5 µl of *Sau*3A I buffer.

2. Add 3–10 U of the restriction enzyme *Sau*3A I to the sample.

3. Incubate the sample for 30 minutes at 37°C.

4. Proceed with *Electrophoresis of the PCR and Sau3A I-Digestion Products*.

## Electrophoresis of the PCR and *Sau*3A I-Digestion Products

To expedite the analysis, electrophorese the undigested PCR product and the *Sau*3A I-digestion product in parallel on a high-grade 2% agarose gel.

**Note** *A high-grade 2% agarose gel allows good differentiation of the PCR products derived from the internal control and infectious Mycoplasma as well as good separation of the* Sau3*A I fragments generated during digestion of the PCR products.*

## Analyzing the Banding Patterns

1. Using the following table and Figures 1 and 2, determine if the cell culture is infected with Mycoplasma.

| PCR template | PCR product(s) | Result |
|---|---|---|
| Cell culture extract without the internal control template | None | No *Mycoplasma* infection |
| | 874 bp | *Mycoplasma* infection |
| | Triplet | *Acholeplasma* infection |
| Cell culture extract tested with the internal control template | 1kb | No *Mycoplasma* infection |
| | 874 bp and 1 kb | *Mycoplasma* infection |
| | Triplet and 1 kb | *Acholeplasma* infection |
| Positive control template | 874 bp | — |

If the cell culture is heavily infected with Mycoplasma, amplification of the 874-bp product may result in diminished amplification of the internal control template. Failure to obtain both the 1-kb and the 874-bp amplification products may indicate that the sample contains agents inhibitory to the PCR amplification.

2. To corroborate the PCR results and to determine the species of Mycoplasma, compare the fragmentation pattern of the *Sau*3A I-digested PCR product to the fingerprints that identify the five most common cell-culture-infecting species of Mycoplasma. The fragmentation patterns are displayed in Figure 1 and Figure 2 (lanes 9–13), and the sizes of the restriction fragments are listed in the following table:

| Mycoplasma species | Restriction fragments (bp) |
|---|---|
| *Mycoplasma arginini* | 680, 420, 280, 155 |
| *Mycoplasma fermentans* | 724, 150 |
| *Mycoplasma hyorhinis* | 650, 235 |
| *Mycoplasma orale* | 610, 265 |
| *Acholeplasma laidlawii* | 290, 230, 170, 145 |

***Note*** *There may be variations in the quantities of amplification products. As a result, the intensity of the amplified "Internal Control plus positive control" products may differ from the depiction in Figure 1.*

Should the fragmentation pattern of the sample differ considerably from those shown in Figures 1 and 2, it is possible that the cell culture is infected with either an uncharacterized species of Mycoplasma or a prokaryotic organism other than Mycoplasma. In this case, testing for a bacterial or a fungal infection may be the next step.

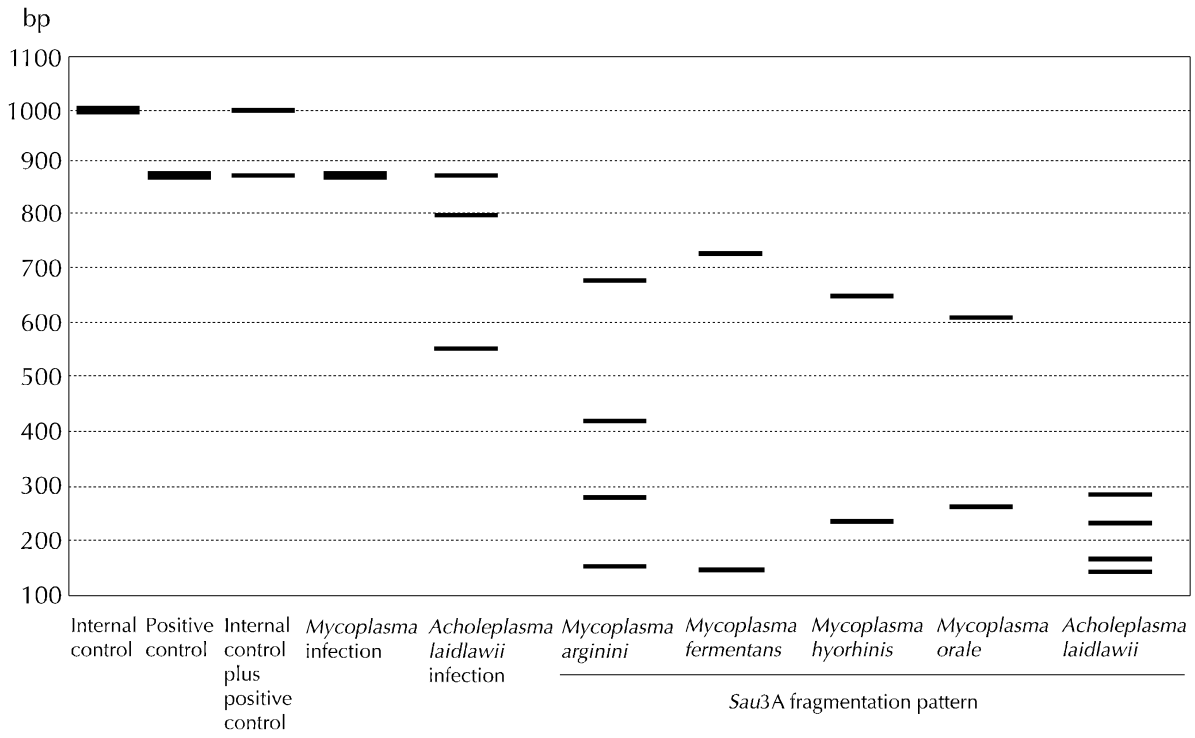


**FIGURE 1**. PCR products and fingerprints of the five most common cell-culture-infecting species of Mycoplasma.

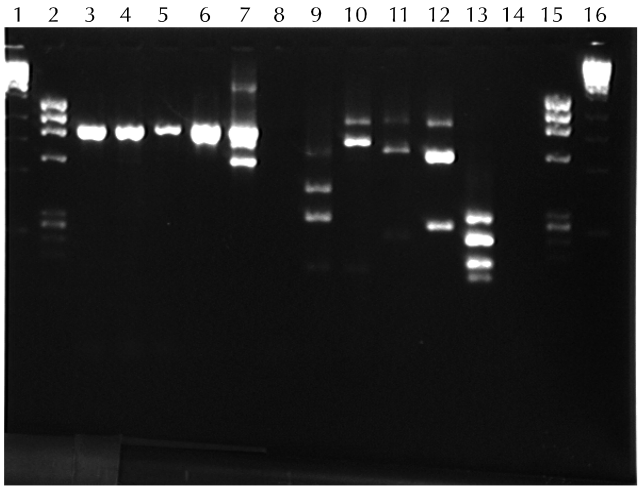


**FIGURE 2** PCR products and *Sau*3A I-digested PCR products of the five most common cell-culture-infecting species of Mycoplasma. **Lane 1**, 1-kb molecular weight marker; **Lane 2**, *Hae*III-digested ϕX174 DNA (marker); **Lane 3**, *Mycoplasma arginini* (undigested); **Lane 4**, *Mycoplasma fermentans* (undigested); **Lane 5**, *Mycoplasma hyorhinis* (undigested); **Lane 6**, *Mycoplasma orale* (undigested); **Lane 7**, *Acholeplasma laidlawii* (undigested); **Lane 8**, negative control in which supplemented RPMI medium was used in the PCR (undigested); **Lane 9**, *Mycoplasma arginini* (*Sau*3A I-digested); **Lane 10**, *Mycoplasma fermentans* (*Sau*3A I-digested); **Lane 11**, *Mycoplasma hyorhinis* (*Sau*3A I-digested); **Lane 12**, *Mycoplasma orale* (*Sau*3A I-digested); **Lane 13**, *Acholeplasma laidlawii* (*Sau*3A I-digested); **Lane 14**, negative control in which supplemented RPMI medium was used in the PCR (*Sau*3A I-digested); **Lane 15**, *Hae*III-digested ϕX174 DNA (marker); **Lane 16**, 1-kb molecular weight marker.

**Methods** Crude extracts of each Mycoplasma species (obtained from American Type Culture Collection) were diluted in RPMI medium supplemented with 10% fetal calf serum and L-glutamine and PCR amplified using the PCR primers in the Mycoplasma *Plus* PCR Primer Set, Stratagene *Taq2000* DNA polymerase, and 10× *Taq* polymerase buffer. One microliter of 10× universal buffer and 10 U of *Sau*3A I were added to a 10-µl aliquot of each PCR reaction. The digestion mixture was incubated for 30 minutes at 37°C and then electrophoresed on a 2% agarose gel using 1× TBE buffer. For comparison, 10 µl of the undigested PCR amplification products were analyzed in parallel. The results were recorded using the Eagle Eye II still video system.

## TROUBLESHOOTING

When used according to the instructions, the Mycoplasma *Plus* PCR Primer Set provides a sensitive means to detect Mycoplasma infection in cell lines. Under optimal conditions, templates derived from supernatants of an infected cell culture will yield a maximum signal in the PCR, whereas an uninfected cell line will yield no PCR product. Variations in thermal cyclers and reagents may contribute to signal differences in your experiments. Use the following guidelines for troubleshooting these variations.

| Observation | Suggestion(s) |
|---|---|
| Low signal using either the internal control or the positive control template | Suboptimal reagents (e.g., *Taq* DNA polymerase) and/or the thermal cycler used in conducting the assay may account for the results. The internal control is a good indicator of amplification efficiency, because it has been titrated to yield amounts of PCR product approaching plateau levels. Low signals with this template are indicative of poor amplification efficiency |
| | Add a 10-minute incubation at 72°C after segment 2 of the PCR program. |
| Low signal in the test samples | Mycoplasma equivalents put into the assay may not be optimal. Regrow the cells in antibiotic-free conditions and harvest the supernatants from confluent cultures. Perform the PCR using these new samples. If inhibitory substances are present in the tissue culture test sample (i.e., if the internal control is not amplified or appears fainter than when amplified alone), the inhibitory substances may be removed from the sample with StrataClean resin or by phenol–chloroform extraction |
| | Alternatively, further dilution of the test sample (e.g., 1:100) may be used in the PCR to reduce inhibitory effects. Suboptimal reagents (e.g., *Taq* DNA polymerase) and/or the thermal cycler used in conducting the assay may account for the results. To increase the specific signal, one may increase the number of cycles used in segment 2 of the PCR program. Please note that such changes may increase both the specific and nonspecific signals |
| No amplification of the internal control in the test sample | Try the guidelines above for confirming quality of PCR reagents and titrating the test sample to dilute any inhibitory components. If these attempts do not improve amplification of the internal control in the test sample, it may be necessary to repeat the experiment using both cells and supernatant samples. Amplification of the internal control should be observed with at least one of these samples. |
| *Sau*3A I fragmentation pattern does not match the expected patterns for Mycoplasma-derived PCR-amplification products | Do not include the internal control template in the PCR samples that are to be digested. The presence of the internal control template changes the restriction pattern derived from Mycoplasma amplification products |
| | The PCR-amplification products are derived from Mycoplasma templates other than those shown in Figures 1 and 2, or they are derived from another prokaryotic species |

## MSDS INFORMATION

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

# *Mycoplasma Plus* PCR Primer Set

Catalog #302008

## QUICK-REFERENCE PROTOCOL

### Preparing the PCR Mixture

- Add 5 µl of 10× Taq reaction buffer to 35.2 µl of $H_2O$ in each PCR tube and UV-irradiate at 12,000 µJ/cm$^2$
- Add the following to each tube:

  0.4 µl of dNTPs (25 mM stock, 200 µM is the final concentration of each dNTP in the PCR)

  0.4 µl of *Taq* DNA polymerase (5 U/µl stock, 2 U/PCR reaction )

  2 µl of primer mix (stock primer mix is 25 µM in each primer)

- Add either 2 µl of the internal control template to each tube or, if the template is not used, add 2 µl of $H_2O$ instead.
- Aliquot 45 µl of the reaction mixture into each PCR tube.
- Add 5 µl of the test template to the appropriate reaction tube.
- Add 5 µl of the negative control (water or negative extract) to the appropriate reaction tube.
- Overlay each reaction with mineral oil or use a temperature cycler equipped with a heated cover.

### Digesting the PCR Product with *Sau*3A I

- Mix a 15-µl aliquot of the PCR product with 1.5 µl of *Sau*3A I buffer, add 3–10 U of *Sau*3A I, and incubate the sample for 30 minutes at 37°C

### Electrophoresis of the PCR and Restriction Digestion Products

- Electrophorese the samples on a high-grade 2% agarose gel
- Analyze the banding pattern