MycoSensor PCR Assay Kit

Instruction Manual

Catalog #302108 (100 reactions) and #302109 (50 reactions)

Revision D.0

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MycoSensor PCR Assay Kit

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MycoSensor PCR Assay Kit

MATERIALS PROVIDED

Materials Provided	Quantity	
	Catalog #302108	**Catalog #302109**
Mycoplasma primer set	200 µl (100 reactions)	100 µl (50 reactions)
dNTP/dUTP mix	100 µl (100 reactions)	50 µl (50 reactions)
Internal control template[a]	400 µl (100 reactions)	200 µl (50 reactions)
Mycoplasma orale positive control template[b]	125 µl (25 reactions)	65 µl (13 reactions)
StrataClean resin	50 determinations	25 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 noninfectious genomic DNA.

STORAGE CONDITIONS

All Components: –20°C

ADDITIONAL MATERIALS REQUIRED

Taq DNA polymerase (e.g., *Taq2000* DNA polymerase or SureStart *Taq* DNA polymerase)
10× *Taq* reaction buffer
phosphate-buffered saline (PBS) or cell culture media
temperature cycler
PCR tubes
PCR-grade water
uracil-DNA g1ycosylase (UDG) or uracil-N g1ycosylase (UNG) (optional)
Brilliant QPCR Master Mix (Catalog #600549; optional)

NOTICE TO PURCHASER

The Stratagene MycoSensor PCR assay kit is for research use only and is not intended for clinical diagnosis or applications involving humans. The MycoSensor PCR assay kit must be used in accordance with NIH guidelines for recombinant DNA.

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INTRODUCTION

The MycoSensor PCR Assay Kit primer mix is capable of detecting *Mycoplasma* infections in cell cultures in less than three and a half hours. 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, from as few as 10 copies of genomic DNA.

The MycoSensor PCR assay kit detects the following eight *Mycoplasma* and *Acholeplasma* species, which make up the most commonly encountered agents of tissue culture infections: *M. arginini, M. fermentas, 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.

A positive control template is included to validate that a polymerase-mediated amplification has occurred and to confirm the size of the PCR product in the test samples. If the test cell line is infected with *Mycoplasma*, the Mycoplasma primer mix will generate a single 315-bp amplification product, regardless of which species of *Mycoplasma* is present in the sample. 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, minimizing the possibility of false positives. The internal control template generates a second 500-bp band that is easily distinguishable from the *Mycoplasma* target band. The internal control confirms the absence of PCR inhibitors in all PCR samples, reducing the occurrence of false negative results. StrataClean resin is included 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.).

The dNTP/dUTP nucleotide mix provided contains dUTP for optional dUTP/UNG decontamination treatment with uracil-N-glycosylase (UNG) or uracil-DNA glycosylase (UDG) (decontamination enzymes not included). When this strategy is put to use, carry-over PCR product contamination will be eliminated while template DNA lacking uracil will be left intact, preventing false-positives due to cross-contamination.

Using the Brilliant QPCR master mix simplifies PCR set-up. The Brilliant QPCR master mix (not included) is formulated with SureStart *Taq* DNA polymerase for hot start PCR, a nucleotide mix with dUTP for optional dUTP/UNG decontamination treatment, and buffer component concentrations optimal for PCR detection of *Mycoplasma* using the Mycoplasma primer mix provided.

Preprotocol Considerations

Critical Precautionary Notes

- 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.

- To avoid false positives, wear gloves while preparing the template for PCR, while preparing the reaction mixtures for PCR, and while performing the PCR. It is good practice to perform pre-PCR steps in an area separated from post-PCR areas (e.g., thermocycler benches, electrophoresis equipment, etc.) to minimize carry-over of amplified material into future experiments, especially if the dUTP/UNG decontamination strategy is not being used. Including a no-template (negative control) reaction will help to assess if carry-over contamination has occurred.

- To avoid false positives, UV-irradiating all water and water–buffer mixtures used in the PCR may be helpful. For example, in making the common reaction mixture for use in PCR, irradiate the *Taq* reaction buffer and water before adding the dNTP/dUTP mix 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 $\mu J/cm^2$) or an equivalent source of UV irradiation.

- To avoid cross-contamination between samples, use aerosol-resistant pipet tips throughout the protocol.

- To avoid cross-contamination of the kit components, the component tubes should be spun in a microcentrifuge at maximum speed for 30 seconds to ensure all material is 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.

PCR Guidelines

Processing Multiple PCR Control and Sample Reactions

The PCR recipes listed in Table I are for one reaction each and must be adjusted for multiple samples. 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 positive control reaction using the positive control template provided. The former reaction will determine the background amplification to be seen in the absence of *Mycoplasma*, and ensure that the PCR reagents have not been contaminated with template or carry-over product. The latter reaction will validate that a polymerase-mediated amplification has occurred and generate a 315-bp band for comparison to test samples. The internal control template should be present in each control and sample reaction to confirm the absence of PCR inhibitors, indicated by a 500-bp PCR product.

Optimal Concentrations of *Taq* DNA Polymerase and PCR Reaction Buffer Components

The optimal reaction conditions for *Mycoplasma* detection include the following:

10 mM of Tris-HCl (pH 8.3–8.8)
50 mM KCl
3.0–4.0 mM $MgCl_2$
2.5 U of *Taq* DNA polymerase/50-µl reaction

The optimal magnesium chloride concentration is typically between 3.0 and 4.0 mM in the final reaction mixture. Using less than 3.0 mM $MgCl_2$ can drastically reduce sensitivity while using greater that 4.0 mM $MgCl_2$ may result in non-specific amplification that could complicate results. Optimization testing may be required to find the exact $MgCl_2$ concentration that maximizes detection sensitivity without introducing non-specific products. Since many *Taq* DNA polymerase buffers contain only 1.5 mM $MgCl_2$, additional $MgCl_2$ may need to be added to the PCR.

Alternatively, you may use the Brilliant QPCR master mix, which provides optimal buffer conditions for *Mycoplasma* detection. In addition, the Brilliant QPCR master mix contains SureStart *Taq* DNA polymerase for hot start PCR and includes dUTP in the nucleotide mix for optional dUTP/UNG decontamination treatment.

We do not recommend the use of archaeal polymerases, such as *Pfu* DNA polymerase, with this kit due to potential poisoning by dUTP.

Preventing Template Cross-Contamination

The nucleotide mix provided contains dUTP instead of dTTP for optional dUTP/UNG treatment of contaminating PCR products from previous amplification reactions. When dUTP replaces dTTP in PCR amplification, treatment with uracil-N-glycosylase (UNG) or uracil-DNA-glycosylase (UDG) (not provided in this kit) acts on single- and double-stranded dU-containing DNA by hydrolysis of uracil-glycosidic bonds at dU-containing DNA sites, degrading carry-over PCR products so that they will not act as template in future testing assays.

PROTOCOL

Preparing the Template

Boiling Extract of Cell Culture Supernatant

Notes *For a protocol that provides cell-equivalent standardization and detection of weak or cell-growth-inhibiting* Mycoplasma *infections, see* Boiling Extract of Cell Culture 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 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 (30–60 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 for 1 minute 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 when stored at 4°C.

   Note *Carry-over StrataClean resin may inhibit PCR amplification. When removing the treated supernatant from the pelleted StrataClean resin, avoid transferring the resin. Performing a second spin to pellet the resin may further reduce carry-over.*

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*. 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. For some cell lines, washing with cell culture media rather than PBS may improve the sensitivity of detection.

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. 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 1 minute to pellet the resin. No incubation is necessary. Assuming that 100,000 cells were resuspended in 100 μl of water, then 5 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 when stored at 4°C.

   Note *Carry-over StrataClean resin may inhibit PCR amplification. When removing the treated supernatant from the pelleted StrataClean resin, avoid transferring the resin. Performing a second spin to pellet the resin may further reduce carry-over.*

7. For PCR, test 50 and 5,000 cell equivalents. The internal control template will control for cell debris that may inhibit PCR. Prepare a 100-μl of a 1:100 dilution of the straight supernatant to obtain 50 cell equivalents per 5 μl. Strong *Mycoplasma* infections are detected in as little as 10 cell equivalents, while weak or cell-growth-limiting infections require cell equivalents from the 500–5,000 range.

Preparing the PCR Mixture

Four separate PCR recipes are provided in Table I depending on the reagents used. The PCR recipes listed are intended for one reaction and must be adjusted for multiple samples. Prepare a reaction mixture for the appropriate number of samples to be tested, including the negative and positive controls. The final volume of each sample reaction is 50 µl.

TABLE I Reaction Mixture for a Single PCR Amplification

Component	Amount per reaction			
	PCR using *Taq* DNA polymerase	**PCR using *Taq* DNA polymerase with dUTP/UNG decontamination**	**PCR using the Brilliant QPCR master mix**	**PCR using the Brilliant QPCR master mix with dUTP/UNG decontamination**
Sterile water	*X* µl	*X* µl	14 µl	13.5 µl
Brilliant QPCR master mix	—	—	25 µl	25 µl
magnesium chloride[a] (3.0–4.0 mM final concentration)	*X* µl	*X* µl	—	—
10× *Taq* reaction buffer	5 µl	5 µl	—	—
dNTP/dUTP mix	1 µl	1 µl	—	—
Mycoplasma primer mix	2 µl	2 µl	2 µl	2 µl
Internal control template	4 µl	4 µl	4 µl	4 µl
Taq DNA polymerase (5 U/µl)	0.5 µl	0.5 µl	—	—
Uracil N-glycosylase (UNG) or Uracil-DNA glycosylase (UDG) (2 U/µl)	—	0.5 µl	—	0.5 µl
Positive control template or test sample	5 µl	5 µl	5 µl	5 µl
Total reaction volume	50 µl	50 µl	50 µl	50 µl

[a] The magnesium chloride concentration must be between 3.0 and 4.0 mM in the final reaction mixture. Since many *Taq* DNA polymerase buffers contain only 1.5 mM $MgCl_2$, additional $MgCl_2$ may need to be added to the PCR.

1. Determine which type of PCR will be performed (see Table I) and combine the corresponding components, *in order*, excluding the positive control or test template.

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

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

4. Add 5 µl of the positive control template or negative control (water or negative extract) to the appropriate reaction tubes.

5. 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).

Cycling the PCR Reactions

Determine which set of cycling parameters is appropriate depending on the reagents used in the PCR (see Table II). The following PCR programs yield optimal amplification of the 315-bp PCR product from the most common species of *Mycoplasma* and the 500-bp internal control product.

Table II PCR Cycling Parameters

Cycle(s)	Temperature	Duration			
		PCR using *Taq* DNA polymerase[a]	**PCR using *Taq* DNA polymerase with dUTP/UNG decontamination**	**PCR using the Brilliant QPCR master mix**	**PCR using the Brilliant QPCR master mix with dUTP/UNG decontamination**
1	37°C	—	10 minutes	—	10 minutes
	94°C	—	10 minutes	10 minutes	10 minutes
35	94°C	30 seconds	30 seconds	30 seconds	30 seconds
	55°C	1 minute	1 minute	1 minute	1 minute
	72°C	1 minute	1 minute	1 minute	1 minute

[a] If using a chemically-based hot start *Taq* DNA polymerase (e.g., SureStart *Taq* DNA polymerase), include a 10-minute preincubation step at 94°C. See the hot start polymerase manufacturer's recommendations for optimal polymerase activation.

Electrophoresis of the PCR Products

For optimal separation between the internal control and the *Mycoplasma* target bands, use a 2% gel for electrophoresis.

1. Mix 10–20 µl of each PCR reaction with gel electrophoresis loading buffer.
2. Load each sample in individual wells of a 2% agarose gel and electrophorese at the specifications recommended by the gel box manufacturer. Visualize the bands with ethidium bromide.

Expected Results

Ensure that the reaction controls are giving the expected results and determine if the test cell culture is infected with *Mycoplasma* using the following table.

PCR template	PCR product(s)	Result
Cell culture extract + internal control template	None	Inhibited PCR reaction
	315 bp and 500 bp	*Mycoplasma* infection
	315 bp only	Heavy *Mycoplasma* infection
	500 bp only	No *Mycoplasma* infection
Positive control (*M. orale*) template + internal control template	None	Failed PCR reaction
	315 bp only	Failed PCR reaction
	315 bp and 500 bp	Expected control result
Negative control (e.g., water) + internal control template	None	Failed PCR reaction
	500 bp	Expected control result
	315 bp and 500 bp	Contaminated reagents

- Failure to obtain the 500-bp internal control amplification product in the sample reaction may indicate that the sample contains agents inhibitory to the PCR amplification.
- If the culture is contaminated with *Mycoplasma* (>10-50 cell equivalents per sample), a 315-bp band will be observed on the gel. In addition, a 500-bp band may be amplified from the internal control template. If the cell culture is heavily infected with *Mycoplasma* (>10^5 *Mycoplasma* cfu per ml of culture/media), amplification of the 315-bp product may result in diminished or no amplification of the 500-bp internal control product, depending on the degree of *Mycoplasma* infection.
- If the culture is free of *Mycoplasma* and PCR inhibitors, only the 500-bp internal control product will be amplified.
- Failure to obtain the 500-bp internal control product band in the negative or positive control reactions is indicative of a failed PCR reaction due to suboptimal PCR reagents or improper set up.
- Each positive control reaction will produce a 315-bp product, as well as a 500-bp product from the internal control template. However, the internal control amplification product may appear faint or barely visible due to potential variations in PCR set-up, reagents not supplied with the kit, or equipment.
- Negative control (e.g., water) reactions should produce the 500-bp internal control product only. Additional DNA bands indicate contamination of one or more PCR mixture components.

TROUBLESHOOTING

When used according to the instructions, the MycoSensor PCR assay kit will provide a sensitive means to detect *Mycoplasma* infection in cell lines. Under optimal reaction conditions, infected cell lines will generate a strong signal in the PCR, whereas an uninfected cell line will not produce a 315-bp PCR product. There may be variations in thermal cyclers and reagents that may contribute to signal differences in your experiments. Refer to the following guidelines for troubleshooting these variations.

Observation	Suggestion(s)
Low or no signal for both the internal control and the positive control templates	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 and positive control together are indicative of poor amplification efficiency. Suboptimal reagents (e.g., *Taq* DNA polymerase), improper magnesium concentration, and/or the thermal cycler used in conducting the assay may account for the results.
Low or no signal in the test samples, but the internal control is amplified	Either the culture is not contaminated, or the *Mycoplasma* contamination is extremely low (below the detection limit of the assay). If a crude extract of cell culture supernatant was performed, the cells may need to be regrown in antibiotic-free conditions, and directly harvested from confluent cultures by scraping. Perform the PCR using these new samples. If the new PCR reaction gives results indicating that the cells are free of *Mycoplasma* infection, but the culture is still suspect for contamination, the cells can be grown for longer periods and the PCR rerun.
No amplification of the internal control in the test sample, but the amplification of the positive control is optimal	If the 315-bp *Mycoplasma* target band is observed in test samples, the internal control may not be seen due to competition with the *Mycoplasma* DNA template. In this case, the sample should be considered a strong positive.
	The test sample may contain inhibitors that are reducing the efficiency of amplification. Try treating the extract with a greater volume of StrataClean resin, extracting with phenol-chloroform, or applying the prepared extract to a DNA purification spin column (e.g., StrataPrep PCR purification kit, Catalog #400771 or #400773). Alternatively, dilution of the test sample may be performed, but detection sensitivity will be reduced.
	Cell cultures with supernatant that is consistently inhibitory to PCR amplification should be harvested and washed according to the procedures outlined in *Boiling Extract of Cell Culture Cells* in *Preparing the Template*.
Spurious bands	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, polymerase buffer, etc.), or improper magnesium concentration. If using *Taq* DNA polymerase, titrate the $MgCl_2$ concentration to find an amount the produces sufficient detection sensitivity for your system without introducing non-specific PCR products.
Low gel signal	Due to differences in PCR reagents not supplied with the kit, PCR product intensity may vary. Use a different *Taq* polymerase (e.g., hot start), use the Brilliant QPCR Master Mix which works optimally with the MycoSensor assay kit, increase magnesium concentration by 0.25–0.5 mM (a minimum of 3.0 mM is recommended), or use more sample when performing electrophoresis.

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 PCR Assay Kit

Catalog #302108, #302109

Quick-Reference Protocol

Preparing the Template from Cell Culture Supernatant

Note *For template preparation from cell culture cells, see* Boiling Extract of Cell Culture Cells *in* Preparing the Template.

- Boil (or 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.
- Add 10 µl of resuspended StrataClean resin to the supernatant and mix the contents of the tube by gentle flicking.
- Spin the tube in a microcentrifuge for 1 minute to pellet the resin.
- Transfer an aliquot of the treated supernatant to a fresh tube, avoiding the pelleted resin.

Preparing and Cycling the PCR Mixture

Note *See* Preparing the PCR Mixture *and* Cycling the PCR Reactions *when using the Brilliant QPCR Master Mix or the dUTP/UNG decontamination strategy.*

- The PCR recipe listed is for one reaction and must be adjusted for multiple samples. Prepare a reaction mixture for the appropriate number of samples to be tested.

Sterile water	*X* µl
Magnesium chloride (3.0–4.0 mM final concentration)	*X* µl
10× *Taq* reaction buffer	5 µl
dNTP/dUTP mix	1 µl
Mycoplasma primer mix	2 µl
Internal control template	4 µl
Taq DNA polymerase (5 U/µl)	0.5 µl
Positive control or test template	5 µl
Total reaction volume	50 µl

- Combine the reaction components, in order, excluding the positive control or test template.
- Aliquot 45 µl of the reaction mixture into each PCR tube.
- Add 5 µl of each test template, positive control template, or negative control (water or negative extract) to the appropriate reaction tubes for a final reaction volume of 50 µl.

- Overlay each reaction with mineral oil or use a temperature cycler equipped with a heated cover. Place the tubes in the PCR temperature cycler and run the following PCR program:

Cycle(s)	Temperature	Duration
35	94°C	30 seconds
	55°C	1 minute
	72°C	1 minute

Note *If using a hot start Taq DNA polymerase, see the hot start polymerase manufacturer's recommendations for optimal polymerase activation.*

Electrophoresis of the PCR Products

- Electrophorese the samples on a high-grade 2% agarose gel.

- Analyze the banding pattern. Reactions containing the internal control and positive control template should yield PCR products of 500 and 315 bp respectively. Expect a 315-bp band from *Mycoplasma* -infected cell cultures.