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Not for use in diagnostic procedures.

Mycoplasma PCR ELISA

Version: 11
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Photometric enzyme immunoassay for the detection of PCR-amplified DNA of mycoplasma and acholeplasma in cell culture

Cat. No. 11 663 925 910

Store the kit at -15 to -25°C.

1. General Information

1.1. Contents

Vial / Bottle	Cap	Label	Function / Description	Content
1	red	Mycoplasma PCR ELISA, Lysis reagent	Ready-to-use solution, containing <2% sodium hydroxide.	1 vial, 1.1 ml
2	red	Mycoplasma PCR ELISA, Neutralization reagent	Ready-to-use solution, containing Tris buffer.	1 bottle, 3.6 ml
3	violet	Mycoplasma PCR ELISA, PCR ready-to-go mix	▪ Ready-to-use solution. ▪ Contains dATP, dCTP, dGTP, dUTP, DIG-dUTUP, mycoplasma-specific primers, and Taq DNA polymerase in an optimized Tris-buffered saline.	3 vials, 1.1 ml each
4	violet	Mycoplasma PCR ELISA, Denaturation reagent	Ready to-use solution, containing <0.5% sodium hydroxide.	1 bottle, 4.8 ml
5	green	Mycoplasma PCR ELISA, Hybridization buffer	Ready-to-use solution.	1 bottle, 54 ml
6	green	Mycoplasma PCR ELISA, Biotin-labeled capture probe	Solution containing a biotin-labeled oligonucleotide complementary to a mycoplasma-specific DNA sequence.	1 vial, 0.54 ml
7	black	Mycoplasma PCR ELISA, Washing buffer, 10x conc.	For hybridization and ELISA.	1 bottle, 30 ml
8	blue	Mycoplasma PCR ELISA, Anti-DIG-POD	▪ Fab fragments of a polyclonal antibody to digoxigenin (from sheep) conjugated to peroxidase. ▪ Lyophilized, stabilized	1 bottle, 240 mU
9	blue	Mycoplasma PCR ELISA, Conjugate dilution buffer	Ready-to-use solution.	1 bottle, 24 ml
10	black	Mycoplasma PCR ELISA, TMB substrate solution	Ready-to-use solution, containing the POD substrate 3,3′,5,5′-tetramethylbenzidine.	1 bottle, 15 ml
11	colorless	Mycoplasma PCR ELISA, Stop Reagent	Ready-to-use solution, containing <5% sulfuric acid.	1 bottle, 15 ml
12	blue	Mycoplasma PCR ELISA, Positive control DNA	▪ Prepared by PCR and is therefore non infectious. ▪ Solution containing a DNA fragment of *Mycoplasma orale*.	1 vial, 0.5 ml
13	foil bag	Mycoplasma PCR ELISA, Microplate	▪ Streptavidin-coated ▪ 96 wells	1 strip frame, 12 modules of 8 wells
14	–	Mycoplasma PCR ELISA, Self-adhesive Plate Cover Foil	Prevents evaporation. Cover the Microplate modules with the Cover Foils during each incubation step.	3 foils

1.2. Storage and Stability

Storage Conditions (Product)

When stored at −15 to −25°C, the kit is stable through the expiration date printed on the label.

Vial / Bottle	Cap	Label	Storage
1	red	Lysis reagent	Store at -15 to -25°C.
2	red	Neutralization reagent	
3	violet	PCR ready-to-go mix	
4	violet	Denaturation reagent	
5	green	Hybridization buffer	
6	green	Biotin-labeled capture probe	
7	black	Washing buffer, 10x conc.	
8	blue	Anti-DIG-POD	
9	blue	Conjugate dilution buffer	
10	black	TMB substrate solution	
11	colorless	Stop Reagent	
12	blue	Positive control DNA	
13	foil bag	Microplate	
14	-	Self-adhesive Plate Cover Foil	

*See section, **Working Solution** for additional information about storage and stability of kit contents.*

1.3. Additional Equipment and Reagent required

Standard Laboratory Equipment

- Microcentrifuge
- PCR thermal cycler, and mineral oil if required
- Tubes for the amplification reaction
- Microplate washer (optional)
- Microplate reader
- Microplate shaker
- Pipettes with disposable positive-displacement tips
- RNase-free, DNase-free and DNA-free aerosol-preventive pipette tips
- autoclaved cups for preparing dilutions
- Absorbant, disposable towels for washing steps

For the Preparation of Kit Working Solutions

- Autoclaved double-distilled water

Prevention of Carryover Contamination (Optional)

- Uracil-DNA Glycosylase* (UNG), 100 U (100 µl)

1.4. Application

The Mycoplasma PCR ELISA is used to detect mycoplasma and acholeplasma contamination in cell culture and other sample material.

2. How to Use this Product

2.1. Before you Begin

Sample Materials

The Mycoplasma PCR ELISA is used with cell culture supernatants.

Control Reactions

Negative Control

Pipette 10 µl autoclaved double-distilled water into a 1.5 ml reaction tube and add 10 µl of Lysis reagent (Vial 1). Proceed as described below for the other samples.

Positive Control

Pipette 10 µl Positive control DNA (Vial 12) into a 1.5 ml reaction tube and add 10 µl of Lysis reagent (Vial 1). Proceed as described below for the other samples.

General Considerations

Precautions

The Neutralization Reagent containing Tris buffer (Bottle 2) and Denaturation Reagent containing <0.5% sodium hydroxide (Bottle 4) could arrive broken or could break when thawed at high temperature. To avoid exposure:

- Wear safety glasses and protective gloves when removing Bottles 2 and 4 from the packaging.
- Thaw the contents of Bottles 2 and 4 at +15 to +25°C for 24 hours.
- After thawing, wear safety glasses and protective gloves to open Bottles 2 and 4.

General Guidelines

PCR is a sensitive method to detect low-copy number mycoplasma. To avoid contamination by PCR carryover, follow these guidelines:

- Use only autoclaved double-distilled water and autoclaved labware.
- For PCR, transfer solutions into a fresh autoclaved tube instead of directly pipetting from the original vials and bottles.
- Due to the high sensitivity of the Mycoplasma PCR ELISA (1 to 20 gene copies are detected per reaction), the presence of antibiotics in the culture medium does not interfere with the assay. However, since the number of mycoplasma can be reduced by antibiotics in the culture media, the cells may be precultured in absence of antibiotics for a minimum of two passages to ensure maximal sensitivity and reliability of the assay.
- Include at least two negative controls and one positive control in each experiment.
- The Mycoplasma PCR ELISA uses hybridization and detection in combination with a plate shaker at 300 rpm. If a suitable shaker is not available, perform the hybridization reaction in reaction tubes, see section **Hybridization and ELISA Procedure**, Step 4. The detection steps can still be carried out using the given protocol, however, signals may be lower which will influence test performance.

The experimental protocol for the Mycoplasma PCR ELISA kit consists of three stages:

1. Preparation of samples and controls.

2. Amplification of mycoplasma-specific DNA incorporating digoxigenin-labeled dUTP (DIG-dUTP) into the amplicon.

3. Hybridization and ELISA.

Safety Information

Laboratory procedures

- Handle all samples as if potentially infectious, using safe laboratory procedures. As the sensitivity and titer of potential pathogens in the sample material varies, the operator must optimize pathogen inactivation by the Lysis / Binding Buffer or take appropriate measures, according to local safety regulations.
- Do not eat, drink or smoke in the laboratory work area.
- Do not pipette by mouth.
- Wear protective disposable gloves, laboratory coats and eye protection, when handling samples and kit reagents.
- Wash hands thoroughly after handling samples and reagents.

Waste handling

- Discard unused reagents and waste in accordance with country, federal, state, and local regulations.
- Safety Data Sheets (SDS) are available online on dialog.roche.com, or upon request from the local Roche office.

Working Solution

Prepare the working solutions according to the following table.

⚠ ***To avoid confusion, label each solution with the appropriate solution number (solutions 1 to 12).***
ⓘ *Always use double-distilled water for reconstitution and dilution of reagents.*

Solution	Content	Reconstitution/Preparation of Working Solution	Storage and Stability
1	Lysis reagent (Vial 1)	Ready-to-use solution.	After opening, store at +2 to +8°C.
2	Neutralization reagent (Bottle 2)	Ready-to-use solution.	After opening, store at +2 to +8°C.
3	PCR ready-to-go mix (Vial 3)	▪ Ready-to-use solution. ▪ Thaw and dispense into suitable aliquots. ⓘ *25 µl are used for one reaction.*	Once thawed, aliquot and store at −15 to −25°C. ⚠ ***Avoid repeated freezing and thawing.***
4	Denaturation reagent (Bottle 4)	Ready-to-use solution.	After opening, store at +2 to +8°C.
5	Hybridization buffer (Bottle 5)	Ready-to-use solution.	After opening, store at +2 to +8°C.
6	Biotin-labeled capture probe (Vial 6)	Thaw and dispense into suitable aliquots. ⓘ *4.5 µl are used for one reaction.*	Once thawed, aliquot and store at −15 to −25°C. ⚠ ***Avoid repeated freezing and thawing.***
6a	Hybridization reagent	Add Biotin-labeled capture probe (Vial 6) to the Hybridization buffer at a ratio of 1:100 (v/v). ⓘ *450 µl are used for one reaction.* ⚠ ***Always prepare fresh before use; do not freeze. Warm to +20 to +25°C before use.***	Store 2 weeks at +2 to +8°C, or 2 days at +20 to +25°C, or 8 hours at +30°C.
7	Washing buffer, 10x conc. (Bottle 7)	To prepare a ready-to-use Washing buffer, mix 1 part of the Washing buffer, 10x concentrated (Bottle 7) with 9 parts of double-distilled water. ⓘ *Approximately 2 ml are used for one reaction.*	After opening, store the Washing buffer, 10x conc. at +2 to +8°C until the kit expiration date. Store the Washing buffer, 1x conc. for 1 month at +2 to +8°C.

8	Anti-DIG-POD (Bottle 8)	Reconstitute the lyophilizate in 0.24 ml double-distilled water (final antibody concentration 1 U/ml).	Store 6 months at +2 to +8°C.
8a	Anti-DIG-POD, working dilution	Add the Anti-DIG-POD to the Conjugate dilution buffer (Bottle 9) at a ratio of 1:100 (v/v) and mix. *200 µl are used for one well.* ***Always prepare fresh before use; do not freeze. Warm to +20 to +25°C before use.***	Store 2 weeks at +2 to +8°C, or 2 days at +20 to +25°C, or 8 hours at +30°C.
9	Conjugte dilution buffer (Bottle 9)	Ready-to-use solution.	After opening, store at +2 to +8°C.
10	TMB substrate solution (Bottle 10)	▪ Ready-to-use solution. ▪ Pipette the required quantity into a suitable vial just before use. ***Do not pipette directly from the bottle into the wells. Transfer an aliquot into a vial for dispensing.***	Store at +2 to +8°C. ***Keep protected from light.***
11	Stop Reagent (Bottle 11)	Ready-to-use solution.	After opening, store at +2 to +8°C.
12	Positive control DNA (Vial 12)	Thaw, mix thoroughly by vortexing, and dispense into suitable aliquots. *10 µl are used for one reaction.*	Store at −15 to −25°C.
13	Microplate	Use only the Microplate modules required for the particular experiment. Close the foil bag containing the remaining modules and the desiccant capsule tightly with adhesive tape. *The Streptavidin-coated Microplate modules are ready-to-use and do not need to be rehydrated before use.*	Once, the foil bag is opened, store Microplate modules desiccated at +2 to +8°C for approximately 12 months.

2.2. Protocols

Preparation of Samples and Controls

Mycoplasma can be detected in culture supernatant without removing cells and/or cellular debris. Centrifugation steps described below can be omitted without reducing assay sensitivity. Mix 10 µl cell culture supernatant with 10 µl Lysis reagent and process as described below. However, certain culture media may interfere with the subsequent PCR amplification. For routine applications, use the protocol below. In some cases, more quantitative methods of sample preparation may be appropriate. For best results, prepare total DNA from the sample, when a mycoplasma titer of less than 10^2 to 10^3 cfu per ml is expected. Suitable methods for quantitative DNA isolation are described in the literature and a number of kits for this purpose are available. Pure DNA preparations can be easily concentrated by ethanol precipitation and are well suited for the Mycoplasma PCR ELISA.

Use the following procedure when testing cell cultures.

Prevent sample and reagent cross-contamination by using fresh RNase-free, DNase-free and DNA-free aerosol-preventive pipette tips for all pipetting steps.

1. Transfer approximately 1 ml culture supernatant into a centrifugation tube.

2. To pellet cells and/or cellular debris, centrifuge the sample at approximately 200 × *g* for 10 minutes at +15 to +25°C.

3. Transfer the supernatant into a fresh autoclaved tube and centrifuge at 13,000 × *g* for 10 minutes at +2 to +8°C to sediment mycoplasma.
   After centrifugation, a visible pellet may not be present. For best results, label the centrifuge tubes and orient them so that the position of the pellet is known.

4. Carefully decant supernatant to avoid losing the pellet.
   – Be sure that no medium remains in the tube.

5. Add 10 µl autoclaved double-distilled water and 10 µl Lysis reagent (Vial 1), and solubilize the pellet.

6. Each experiment should be accompanied by at least two negative controls and one positive control.
   – For a positive control, pipette 10 µl of Positive control DNA (Vial 12) into a tube, and add 10 µl Lysis reagent (Vial 1).
   – For a negative control, pipette 10 µl autoclaved double-distilled water into a tube and add 10 µl Lysis reagent (Vial 1) .

7. Incubate samples and controls at +37°C for 1 hour.

8. Add 30 µl Neutralization reagent (Bottle 2) to each sample and control.

To prevent contamination by PCR carryover, decontaminate the PCR reaction mix using Uracil-DNA Glycosylase (UNG). UNG acts on single- and double-stranded dU-containing DNA by hydrolyzing uracil-glycosidic bonds at dU-containing DNA sites, efficiently digesting previously amplified DNA. To prevent PCR carryover, pipette 15 µl UNG solution (1 U) instead of water to the PCR ready-to-go mix (Vial 3). Add 10 µl sample and incubate for 10 minutes at +15 to +25°C, and start cycling. For additional information, refer to the Instructions for Use of the respective UNG.*

⚠ ***Do not treat the Positive control DNA (Vial 12) with UNG. The amplified DNA serving as the Positive control contains dU; treatment with UNG will digest the Positive control DNA, resulting in loss of signal.***

Amplification of Mycoplasma-Specific DNA

The following program was used with the GeneAmp PCR System 9600 Thermal Cycler and the DNA Thermal Cycler 480 (Perkin Elmer). Minor modifications of the program may be required for other thermal cyclers. Overlay of the reaction mixture with mineral oil may also be necessary before PCR to prevent condensation. Consult the operation manual of your thermal cycler for details.

Prevent sample and reagent cross-contamination by using fresh, RNase-free, DNase-free and DNA-free aerosol-preventive pipette tips.

1. For each sample to be tested, transfer 25 µl of the PCR ready-to-go mix (Vial 3), and 15 µl autoclaved double-distilled water into a suitable PCR tube.
   For large numbers of samples, pre-mix multiples of 25 µl of the PCR ready-to-go mix (Vial 3) and 15 µl autoclaved double-distilled water per sample in a 1.5 ml tube. Pipette 40 µl of this mixture into each PCR tube.

2. To each PCR tube, add 10 µl sample, positive control, or negative control, respectively.

3. Transfer PCR tubes to a thermal cycler, and perform PCR using the following program:

Programs		
	Temp.[°C]	Duration[hh:mm:ss]
Cycle 1	95	00:05:00
Cycles 2 – 40	94	00:00:30
	62	00:00:30
	72	00:01:00
Cycle 41	72	00:10:00
Hold	8	–

Hybridization and ELISA Procedure

1. Add 40 µl of Denaturation reagent (Bottle 4) to a suitable PCR tube using aerosol-resistant pipette tips.
   – Prepare a reaction tube for each sample and control.

2. Transfer 10 µl PCR product per tube to the tubes prepared in Step #1.
   – Incubate at +15 to +25°C for 10 minutes.

3. Add 450 µl Hybridization reagent per tube, and mix thoroughly.

4. Transfer 200 µl of the mixture per well of the Microplate.
   – Cover the wells with the Self-adhesive Cover Foil.
   – Incubate at +37°C on a shaker at 300 rpm for 3 hours.
   The amount of the Hybridization mixture allows the assay to be performed in duplicate. When a plate shaker is not available, perform the hybridization in the reaction tube at +37°C while shaking at 300 rpm for 2 hours. Transfer 200 µl of this Hybridization mixture to each well of the Microplate. Incubate the Microplate for 1 hour at +37°C.

5. Remove the Hybridization mixture by flicking, then inverting and tapping the Microplate on absorbant, disposable towels.
   – Wash three times with 250 µl 1x Washing buffer per well.
   – Remove the Washing buffer completely after each wash by flicking, inverting, and tapping the Microplate on absorbant, disposable towels.

6. Add 200 µl of Anti-DIG-POD working dilution per well.
   – Cover wells with the Self-adhesive Cover Foil.
   – Incubate at +15 to +25°C on a shaker at 300 rpm for 30 minutes.

7. Remove Anti-DIG-POD by flicking, inverting, and tapping the Microplate on absorbant, disposable towels.
   – Wash five times with 250 µl 1x Washing buffer per well.
   – Remove the Washing buffer completely after each wash by flicking, inverting, and tapping the Microplate on absorbant, disposable towels.

8. Add 100 µl TMB substrate solution (Bottle 10), prewarmed to +15 to +25°C, to each Microplate well.
   – Cover wells with the Self-adhesive Cover Foil.
   – Incubate at +15 to +25°C on a shaker at 300 rpm for approximately 20 minutes.

9. Add 100 µl Stop Reagent (Bottle 11) to each Microplate well.
   – Measure absorbance using a microplate (ELISA) reader at 450 nm, using the reference wavelength of 690 nm, within 1 hour after adding the Stop Reagent.
   Adding the Stop Reagent causes a change in the color of the reacted POD substrate TMB from blue to yellow. Stop the substrate reaction to achieve maximal sensitivity of the POD substrate TMB.

2.3. Parameters

Sensitivity

This ELISA can detect least 1 × 10^3 colony-forming units (cfu) of mycoplasma per milliliter of culture medium. To test for the sensitivity of the Mycoplasma PCR ELISA, cell cultures were infected with 1 × 10^3 and 1 × 10^6 cfu of the mycoplasma species most commonly encountered in cell cultures: *M. arginini*, *M. fermentans*, *M. hominis*, *M. hyorhinis*, *M. orale*, and *Acholeplasma laidlawii*.

Specificity

The Mycoplasma PCR ELISA detects all mycoplasma, acholeplasma, and ureaplasma species tested to date (*M. orale*, *M. arginini*, *M. fermentans*, *M. hyorhi-nis*, *M. salivarium*, *M. gallisepticum*, *M. hominis*, *M. bovis*, *M. californicum*, *M. bovoculi*, *M. Pg50 bovine group*, *M. bovigenitalium*, *M. hyopneumoniae*, *A. laidlawii*, *U. urealyticum*). DNA from other bacteria, (except for *Clostridium spec.*), yeast, and eukaryotic cells is not detected.

3. Results

Result Interpretation

Negative Control

The absorbance of the negative control should be lower than 0.25 $A_{450\ nm} - A_{690\ nm}$ units. If higher values are obtained, repeat the experiment, including the PCR.

Positive Control

The absorbance of the positive control should be higher than 1.2 $A_{450\ nm} - A_{690\ nm}$ units. If lower values are obtained, repeat the experiment, including the PCR.

Samples

Subtract the mean of the absorbance readings of the negative controls from those of the samples. Samples are mycoplasma-contaminated when the difference in absorbance (A) is higher than 0.2 $A_{450\ nm} - A_{690\ nm}$ units (see Typical Results Table).

Typical Results

The following table shows the detection of various mycoplasma species and *Acholeplasma laidlawii* using the Mycoplasma PCR ELISA or the culture method, respectively.

1. Adherent Vero cells, or NSO cells growing in suspension, were infected with mycoplasma corresponding to 10^3 and 10^6cfu (colony-forming units)/ml, respectively, and incubated for 4 hours at +37°C.

2. Detection of mycoplasma infection by the culture method was performed using porcine (Medium A) or horse serum (Medium B) supplemented media, with or without prior enrichment of mycoplasma in broth.
   ++++ = 10^4cfu/ml, +++ >$10^3$to 10^4cfu/ml, ++ = $10^2$to 10^3cfu/ml, + = 10 to 10^2cfu/ml.

3. Mycoplasma PCR ELISA was performed as described, see section **Protocols**, without prior enrichment.

4. Samples producing absorbance values (ΔA) >0.2 $A_{450nm} - A_{690nm}$ units are regarded as positive.

3. Results

Mycoplasma/ Acholeplasma Species	Cell Line	Amount of Mycoplasma Inoculated [cfu/ml]	Mycoplasma PCR ELISA ($\Delta A_{450nm} - A_{690nm}$)	Culture Method Without Prior Enrichment		Culture Method After Prior Enrichment	
				Medium A	Medium B	Medium A	Medium B
M. arginni	Vero	10^6	0.35	+++	+++	++++	++++
	Vero	10^3	0.35	++	++	++++	++++
	NSO	10^6	0.36	++++	++++	++++	++++
	NSO	10^3	0.35	++	++	++++	++++
M. fermentans	Vero	10^6	1.56	++++	++	++++	++
	Vero	10^3	1.77	+++	++	++++	+
	NSO	10^6	1.82	++++	++	++++	++
	NSO	10^3	1.70	++	+	++++	+++
M. hominis	Vero	10^6	1.54	++++	++++	+++	+++
	Vero	10^3	1.69	++	++	+++	++++
	NSO	10^6	1.76	+	+++	+	++++
	NSO	10^3	1.75	+	+	+	++++
M. hyorhinis	Vero	10^6	1.68	+++	++	+++	+++
	Vero	10^3	1.85	++	+	+++	+++
	NSO	10^6	1.83	+++	++	+++	++
	NSO	10^3	1.78	+	+	++	+
M. orale	Vero	10^6	0.49	+++	++++	+++	+++
	Vero	10^3	0.24	++	+	++	++++
	NSO	10^6	0.37	++++	+++	+	+++
	NSO	10^3	0.13	+	+	+	++++
A. Laidlawii	Vero	10^6	1.82	++++	++++	++++	++++
	Vero	10^3	1.74	+++	+++	++++	++++
	NSO	10^6	1.64	++++	++++	++++	++++
	NSO	10^3	0.64	+++	+++	++++	++++

4. Troubleshooting

Observation	Possible cause	Recommendation
Signals of positive controls are too low or even absent.	Incorrect thermal cycling program.	Optimize cycling conditions.
	Control DNA was contaminated with DNases and partially degraded.	Use clean, DNase-free reagents and tubes.
	Positive control DNA was not mixed well after thawing.	Mix control DNA thoroughly by vortexing.
	Positive control DNA was treated with uracil-DNA glycosylase (UNG).	Use fresh Positive control DNA.
	Incubation steps during the detection reaction were performed without shaking at 300 rpm.	Absolute level of absorbance values is only obtained when a microplate shaker is used.
	Stop Reagent (Bottle 11) was not added.	Absorbance measurement at 450 nm can only be performed after addition of the Stop Reagent.
	Anti-DIG-POD working dilution is inactive.	Check enzyme activity of the Anti-DIG-POD, and if necessary, prepare a fresh working dilution.
Signals are low, despite the sample having been shown to be mycoplasma infected using an alternative method.	–	Sample is free of mycoplasma (false positive in alternative assay).
	Cells were cultured in culture medium containing antibiotics.	Culture cells in antibiotic-free medium and retest.
	Mycoplasma equivalents used in the amplification reaction are below detection limit.	Culture cells in antibiotic-free medium and retest.
	Samples contain substances that inhibit the PCR.	Ensure culture supernatant is completely removed from the mycoplasma-containing pellet before adding Lysis reagent.
		To test for possible inhibitory components, mix 25 µl PCR ready-to-go mix (Vial 3) with 12 µl water and 3 µl fresh culture medium (corresponding medium volume if supernatant has been tested directly). Add 10 µl of Positive control DNA (Vial 12), and perform PCR.
		Alternatively, purify DNA by phenol/ chloroform extraction or other method.
	Hybridization was performed without shaking or at +15 to +25°C.	Perform the hybridization reaction on a shaker at 300 rpm and at +37°C.

Signals of negative controls are too high.	Negative control, Lysis reagent, Neutralization reagent, and/or PCR ready-to-go mix may be contaminated with Positive control DNA or amplification products from previous experiments or with mycoplasma DNA.	Decontaminate using Uracil-DNA Glycosylase (UNG)*, according to the instructions supplied with the product. If contaminated with mycoplasma DNA, decontamination is not possible and fresh reagents must be used.
		PCR generates millions of copies of a nucleic acid sequence, therefore, carryover of a minimal amount of DNA from a previous amplification can produce false-positive results. Minimize risk of PCR carryover by: ▪ Physical separation of pre- and post-PCR reactions. ▪ Using positive-displacement pipettes with disposable plunger and tips. ▪ Not directly pipetting stock solutions. ▪ Aliquoting reagents.
	Washing steps were not sufficient.	Increase the number of washing steps.
	Incubation with TMB Substrate Solution (Bottle10) was too long.	Stop the TMB substrate reaction after a maximum of 20 minutes after adding the TMB Substrate Solution.

5. Additional Information on this Product

5.1. Test Principle

The assay is based on the amplification of a mycoplasma-specific DNA sequence by PCR, and the subsequent detection of the amplicon by ELISA (Fig. 1). Mycoplasma contained in the sample are enriched by centrifugation of the cell-free supernatant, then lysed. A conserved and mycoplasma-specific region of the DNA is amplified by PCR in the presence of DIG-labeled dUTP. The DIG-labeled amplicon is denatured by NaOH treatment, hybridized to a biotin-labeled capture probe, and immobilized on a streptavidin-coated microplate. The immobilized amplicon is detected by means of an anti-digoxigenin, coupled to horseradish peroxidase, antibody (anti-DIG-peroxidase), and the sensitive peroxidase substrate TMB.

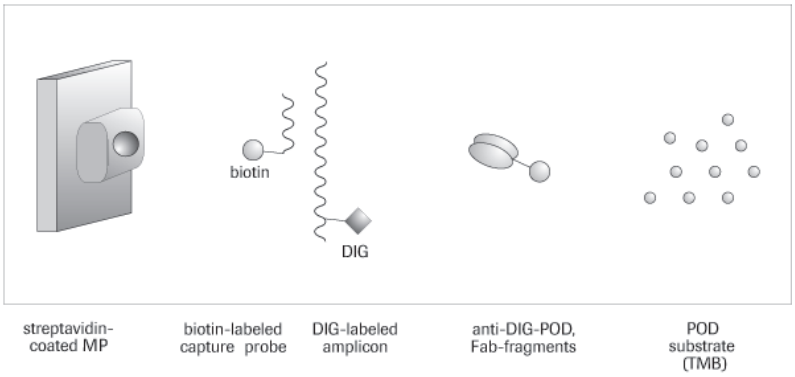

Fig. 1: Test principle

① Enrichment of mycoplasma by centrifugation of the cell-free supernatant, alkaline lysis of the mycoplasma, and neutralization of the lysate.

② Amplification of a conserved region of mycoplasma-specific DNA incorporating digoxigenin-labeled dUTP (DIG-dUTP) into the amplicon.

③ The DIG-labeled amplicon is denatured by NaOH treatment.

④ Hybridization of the single-stranded PCR product to a biotin-labeled capture probe; this complex is immobilized in the streptavidin-coated wells of the microplate.

⑤ Immobilized amplicon is detected using digoxigenin antibodies coupled to horseradish peroxidase (anti-DIG-POD), with the peroxidase-specific substrate TMB.

6. Supplementary Information

6.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
❶ ❷ ❸ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

6.2. Changes to previous version

Layout changes.
Editorial changes.
Update to include new safety Information to ensure handling according controlled conditions.

6.3. Ordering Information

Product	Pack Size	Cat. No.
Reagents, kits		
Uracil-DNA Glycosylase	100 U, 1 U/µl	11 444 646 001

6.4. Trademarks

All product names and trademarks are the property of their respective owners.

6.5. License Disclaimer

For patent license limitations for individual products please refer to:
List of biochemical reagent products.

6.6. Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

6.7. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

6.8. Contact and Support

To ask questions, solve problems, suggest enhancements or report new applications, please visit our **Online Technical Support Site**.

To call, write, fax, or email us, visit **sigma-aldrich.com**, and select your home country. Country-specific contact information will be displayed.

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