

Mycoplasma PCR detection kit Venor® GeM Advance: Use in detail

Biochrom AG Information

Venor® GeM Advance is a ready-to-go version of Venor® GeM. The reaction tubes included with the kit are pre-coated with appropriate dNTPs and primers to reduce the total assay time compared to the general protocol that requires individual loading of reaction tubes.

For additional convenience the gel loading buffer and dye are already included in the reaction tubes. After thermal cycling the PCR can be loaded directly on the agarose gel.

1 Test principle

Venor® GeM Advance utilizes the polymerase chain reaction, which is established as the method of choice for highest sensitivity in the detection of mycoplasma and acholeplasma contamination in cell cultures and other cell culture derived biologicals. The primer set is specific to the highly conserved 16S rRNA coding region in the mycoplasma genome. This allows for detection of all mycoplasma species usually encountered as contaminants in cell cultures. The reaction tubes also contain DNA to serve as an internal control. For the internal control DNA, a successfully performed reaction is indicated by a distinct 190 bp band on the agarose gel. A separate strip of vials contains non-infectious *Mycoplasma orale* DNA as a positive control.

We highly recommend our reliable hot-start MB *Taq* DNA polymerase (cat. no. W 53-0200) with Venor® GeM Advance. Use of other *Taq* polymerase products may require reaction optimization to achieve proper results.

2 Reagents and materials

2.1 Kit components

- | | |
|---|---|
| ➤ Test reaction tubes
tubes pre-coated with lyophilized primers, dNTPs, internal control DNA, and gel loading buffer/dye | 3, 6, 12, or 30 strips of 8 transparent tubes each, depending on package size |
| ➤ Positive control reaction tubes
Test reaction tubes containing DNA-fragments of mycoplasma orale genome | 1, 2, 3, or 5 strips of 8 red tubes each, depending on package size |
| ➤ Caps for PCR tubes | 4, 6, 15, or 35 strips of 8 tubes each, depending on package size |
| ➤ Rehydration buffer | 1.6 ml |

2.2 Stability and storage

Kit components are stable during shipping. Upon receipt, store at +2 to +8 °C. The kit is stable until the expiration date stated on the product box.

2.3 Supplemental requirements

- PCR thermal cycler
- agarose gel electrophoresis apparatus
- micro centrifuge, micropipettes and filtered tips
- polymerase

The test provides excellent results with MB *Taq* DNA polymerase (cat. no. W 53-0050/0100/0200/0250). We can neither guarantee a high level of sensitivity nor compatibility with other polymerases. If you intend to test our MB *Taq* DNA polymerase in parallel with your in-house polymerase, please feel free to contact us and get a gratis MB *Taq* sample (10 units). However, if you want to use your own polymerase, it may be necessary to use the specific buffer provided with this polymerase.

3 Application and test principle

Venor[®] *GeM* Advance utilizes the polymerase chain reaction (PCR), which was established as the method of choice for highest sensitivity in the detection of *Mycoplasma* and *Acholeplasma* contamination in cell cultures and other cell culture derived biologicals. Detection requires as little as 1 to 5 fg of mycoplasma DNA corresponding to 2-5 mycoplasma per sample volume. The primer set is specific to the highly conserved rRNA operon, or more specifically, the 16S rRNA coding region in the mycoplasma genome. This allows for detection of *M. orale*, *M. hyorhinitis*, *M. arginini*, *M. fermentans*, *M. salivarium*, *M. hominis*, usually encountered as contaminants in cell cultures, but also *M. pneumoniae*, *Acholeplasma laidlawii*, *M. synoviae* and *Ureaplasma* species. Cross-detection of bacteria with close phylogenetic relation to *Mycoplasma* is not monitored. The „European Pharmacopoeia“ recommends to check for unspecific detection of *Clostridium*, *Lactobacillus*, and *Streptococcus*. None of the following species is detected with Venor[®] *GeM* Advance: *Clostridium acetobutylicum*, *Lactobacillus acidophilus* and *Streptococcus pneumoniae*. Furthermore there is no positive signal with human DNA as template.

Because the reaction tubes included with the kit are pre-coated with appropriate dNTPs and primers, the total assay time is greatly reduced compared to general protocols that require individual loading of reaction tubes. After thermal cycling the PCR can be loaded directly on the agarose gel.

Venor[®] *GeM* Advance also contains internal control DNA in the PCR mix. When running the PCR a successfully performed reaction is indicated by a 191 bp band on the agarose gel.



Venor[®] *GeM* Advance is intended for research use only. Not for clinical diagnostics or testing of human samples.

4 Test protocol

4.1 Preparation of sample material

Samples should be derived from cultures which are at 90-100 % confluence. PCR inhibiting substances may accumulate in the medium of older cultures. For these sample materials a DNA extraction is strictly recommended prior testing. Penicillin and streptomycin in the culture media do not inhibit mycoplasma or affect test sensitivity. Only cell culture supernatant should be applied to test for mycoplasma. Cell pellets should only be tested after suitable DNA extraction, since debris will interfere with the PCR reaction. With average titers at 10^6 and a maximum titer at 10^8 you will find sufficient mycoplasma in the supernatant to guarantee a sensitive PCR. However, other materials that can be tested are Fetal Bovine Serum, vaccines, and paraffin-embedded samples following DNA extraction. If necessary, templates for PCR analysis are prepared by DNA extraction using commercially available extraction kits. Please be sure to remove any alcohol containing wash buffer from the preparation to avoid coelution of alcohol and sample material. Any remaining alcohol may inhibit the PCR. 2 μ l of the extract can be used directly as PCR template. To avoid false positive results, we recommend the use of the PCR grade water delivered with the kit, aerosol-preventive filter tips and gloves.

The preparation of sample material could be performed by one of the following methods:

Heat-inactivation of the sample material

The templates for the PCR analysis are prepared by direct heating of the cell culture supernatant or the biological sample material:

- 100 μ l liquid supernatant of the sample material is transferred into a sterile reaction tube;
- the supernatant is incubated at 95 °C for 10 minutes;
- the supernatant is centrifuged briefly (5 seconds, 1000xg) to remove cellular debris.
- the supernatant is used in the PCR. Alternatively, the DNA can be purified with a commercial extraction kit

Enrichment of mycoplasma by centrifugation

- 1 ml liquid supernatant of the sample material is transferred into a sterile reaction tube;
- the supernatant is centrifuged (15 minutes, 10000xg) to sediment mycoplasma particles. Alternatively: centrifuge the supernatant 6 min with 13000xg.
- the supernatant is rejected and the pellet is suspended into 50 μ l buffer (10 mM Tris, pH 8.4).
- the sample should be vortexed and finally heated up to 95 °C for 10 min.

The extracts can be stored at a temperature of at least -18 °C for a period of one year. Repeated freezing and defrosting, or storage in the refrigerator for longer than 12 hours should be avoided. The sample should not contain more than 100 μ g/ml DNA.

4.2 Thermal profile

The programming process of the cycler is explained in the manual of the instrument.

Program:

1 cycle	94 °C for 2 min
39 cycles	94 °C for 30 sec 55 °C for 30 sec 72 °C for 30 sec
cool down to +4 °C to +8 °C	



The incubation time depends on the polymerase used. Some hot start enzymes need to be activated at 94 °C for more than 2 minutes. Please see polymerase data sheet for duration.

4.3 PCR setup

1. Mix rehydration buffer and polymerase
For optimal comparison of all samples tested the polymerase should not be added separately to the PCR tubes but premixed with the rehydration buffer.

Determine the total number of reactions including negative and positive controls. Calculate the required amount of rehydration buffer and polymerase (1 unit/reaction) including an additional reaction (23 µl) to compensate for pipetting losses:

	Test Reaction	Negative Control	Positive Control
Rehydration buffer	22.8 µl	22.8 µl	24.8 µl
Polymerase (5 U/µl)	0.2 µl	0.2 µl	0.2 µl

For other polymerase concentrations the amount of water needs to be adjusted.

Vortex the rehydration buffer vigorously (at least 30 sec), transfer the calculated amount into a fresh minicentrifuge tube and add the required amount of polymerase. Mix the rehydration buffer/polymerase. Mix by flicking the tube.



Do not vortex diluted polymerase!

2. Rehydration of PCR reagents

Remove and cut off the required amount of test reaction tubes (transparent) and positive control tubes (red) from the bag. Replace remaining tubes in bag and seal properly. Label tubes as appropriate. Centrifuge or tip the tubes on the table to collect the lyophilized material at the bottom of the tube. Peel off protective film from tubes. Aliquot 23 µl of the prepared rehydration buffer/polymerase mix into each PCR reaction tube. Rehydrate positive control tubes with 25 µl of the mix.



Avoid cross contamination by preparing the positive controls after finishing the negative controls and test reactions!

3. Sample addition

The total volume per reaction is 25 µl. Add the corresponding sample material directly to the PCR tubes:

negative control: 2 µl of deionized water or elution buffer if extraction kit used
test reaction: 2 µl of sample
positive control: no sample required

Close tubes with caps for PCR tubes included in kit. Mix contents of each tube thoroughly by flicking tubes. Do not vortex! Collect liquid contents at the bottom of the PCR tube by brief centrifugation. Incubate at room temperature for 5 minutes. Proceed immediately to thermal cycling.

4.4 Agarose gel run

- Prepare a 1.5% standard agarose gel, approx. 5 mm thick, with a 5 mm-comb.
- Load 5 µl of each PCR reaction. No loading buffer and running dye are required.
- Stop electrophoresis after 2 cm run distance (depending on the electrophoresis chamber used e.g. run for 20 minutes at 100 V).

4.5 Gel evaluation

A distinct 191 bp band should appear in every lane indicating a successfully performed PCR. This band may fade out with increased amounts of amplicons formed, caused by mycoplasma DNA loads of $> 5 \times 10^6$ copies/ml. The initial concentration of positive control DNA exceeds 5×10^6 copies/ml in order to account for DNA loss resulting from repeated freezing and thawing.

- Relevant amplicon sizes:

internal control 191 bp
Mycoplasma spp. 265-278 bp
(see table in the appendix)

➤ Controls:

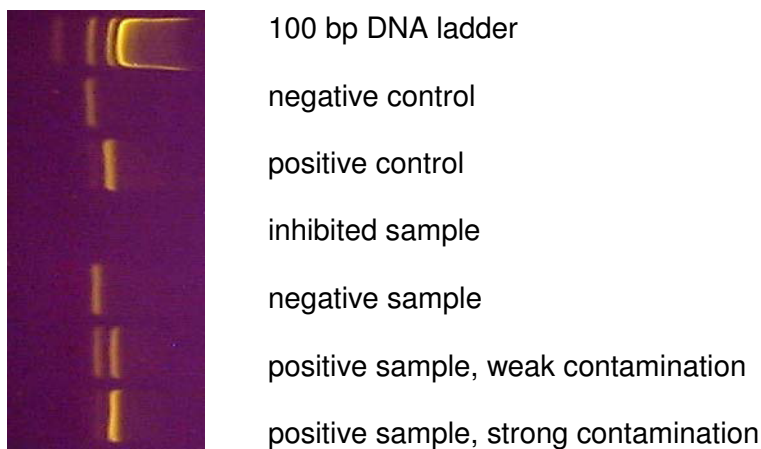
negative control band at 191 bp
 positive control band at 267 bp , possibly an additional band at 191 bp

➤ Interpretation of test reaction band patterns:

Band pattern	Interpretation
band at 191 bp	negative sample
band at 270 bp and at 191 bp	mycoplasma-positive sample with weak contamination
strong band at 270 bp	mycoplasma-positive sample, highly contaminated
no band	PCR inhibition or activity of polymerase is insufficient

With Venor[®]GeM designed for high sensitivity and therefore prone to nonspecific annealing, bands of various length that are less intensive can be produced, but do not indicate positive results. Possible primer self-annealing produces another band of 80-90 bp in length, but also does not affect the precision or results of the test. If the PCR of a sample is inhibited, PCR inhibitors can easily be removed from the sample by performing a DNA extraction with a commercially available kit. A list of recommended DNA extraction kits is provided in the appendix.

Fig. 1: Amplicon pattern



5 Trouble shooting

No amplification of control DNA may be due to the following reasons:

- activity of polymerase is insufficient
- control DNA tubes have not been spun down before rehydration
- programming mistake
- pipetting mistake

Before re-run of a negative and a positive control please check thermocycler protocol and pipetting scheme. When using polymerases other than the MB *Taq* DNA polymerase, please note the comments made under chapter 2.3. The enzyme concentration can then be raised up to 2.5 U/reaction. Please note the complete change of the pipetting scheme.

6 Details on Venor[®] GeM Advance

Parameters	Venor [®] GeM Advance			
Cat. No.	W 11-7024	W 11-7048	W 11-7096	W 11-7240
Unit	24 tests	48 tests	96 tests	240 tests
Storage	+2 - +8 °C			
Raw material	<ul style="list-style-type: none"> ➤ Primer/Nucleotide mix, internal control DNA and gel loading buffer in PCR tubes ➤ Rehydrating buffer ➤ Positive control DNA without MB <i>Taq</i> DNA polymerase 			
Use	Mycoplasma detection			
Please note	for <i>in vitro</i> use			

Products for mycoplasma detection from Biochrom AG:

<http://www.biochrom.de/en/products/mycoplasma-detection/>

Appendix

Detection Range and Sizes of Amplicons

No. species	amplicon size (bp)
1 <i>Mycoplasma orale</i> ^{1,2}	266
2 <i>Mycoplasma pneumoniae</i> ²	273
3 <i>Mycoplasma penetrans</i>	274
4 <i>Mycoplasma pirum</i>	274
5 <i>Acholeplasma laidlawii</i> ²	273
6 <i>Mycoplasma fermentans</i>	267
7 <i>Ureaplasma urealyticum</i>	273
8 <i>Mycoplasma hyorhinis</i> ²	268
9 <i>Mycoplasma pulmonis</i>	268
10 <i>Mycoplasma falconis</i>	268
11 <i>Mycoplasma arthritidis</i>	267
12 <i>Mycoplasma arginini</i>	267
13 <i>Mycoplasma spermatophilum</i>	267
14 <i>Mycoplasma opalescens</i>	266
15 <i>Mycoplasma primatum</i>	267
16 <i>Mycoplasma maculosum</i>	267
17 <i>Mycoplasma bovis</i>	267
18 <i>Mycoplasma cloacale</i>	266
19 <i>Mycoplasma hyosynoviae</i>	265
20 <i>Mycoplasma synoviae</i> ²	266
21 <i>Mycoplasma salivarium</i>	266
22 <i>Mycoplasma faucium</i>	265
23 <i>Mycoplasma hominis</i>	266
24 <i>Mycoplasma genitalium</i>	273
25 <i>Mycoplasma bovigenitalium</i>	267
26 <i>Mycoplasma sp. ovine/caprine</i>	267
27 <i>Mycoplasma agalactica</i>	267
28 <i>Mycoplasma timone</i>	266

¹ provided as positive control DNA

² Test strain according to European Pharmacopoeia 2.6.7. Mycoplasma