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Mycoplasma Detection PCR Kit 16S rRNA Gene
Cat No. MP-70114: 50 reactions

**INSTRUCTION
MANUAL**

ID-M10064
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*These products are designed and sold for use in the Multiplex PCR (MPCR) covered by patent # 5,582,989. Use of the MPCR process requires a license. A limited, non-automated research field license under the patent to use only this amount of the product to practice the MPCR process is conveyed to the purchaser by the purchase of this product.

The Polymerase Chain Reaction (PCR) process is covered by patents owned by Hoffman-LaRoche. Use of the PCR process requires a license. A license for diagnostic purposes may be obtained from Roche Molecular System. A license for research may be obtained by the purchase and the use of authorized reagents and DNA thermocyclers from the Perkin-Elmer Corporation or by negotiating a license with Perkin-Elmer.

This product is intended for research use only and not for diagnostic purposes.

INTRODUCTION

Laboratory diagnosis of mycoplasma infections is hindered by the difficulty or total failure to cultivate the organisms *in vitro*, and by the frequently weak and poorly specific serological response of the host. DNA probes consisting of cloned ribosomal RNA genes, DNA to mycoplasmal rRNA, synthetic 16S rRNA oligonucleotide sequences, or cloned mycoplasmal protein genes, have been developed and applied as diagnostic tools in a variety of human and animal mycoplasma infections. These included primary atypical pneumonia caused by *Mycoplasma pneumoniae*, urogenital infections associated with *M. genitalium* and *Ureaplasma urealyticum*, and infections with *M. fermentans*,

M. penetrans or *M. pirum*--mycoplasmas recently incriminated as cofactors in AIDS. DNA probes were also designed to aid in diagnosis of mycoplasma diseases of farm and laboratory animals, and the hard-to-diagnose mycoplasma infections of cell cultures.

This PCR primer sets were designed to detect a segment of 16S rRNA gene of *Mycoplasma*. The Primers provided are sufficient enough for 100 X 50 ul nested PCR. For optimal results, please read and follow the instructions in this manual carefully. If you have any questions, contact Maxim Biotech Customer Service at (650) 871-1919.

PCR PRODUCT QUANTITATION

I: Radioactive Quantitation

In our experience, visual inspection of an EtBr-stained agarose gel is sensitive and precise enough to detect changes as low as two-fold. If greater discrimination is necessary, several methods are available. The simplest procedure is to add a radioactively labeled dNTP to the PCR reaction. After gel analysis, the band may be excised and counted in a scintillation counter. Alternatively the gel may be dried and an autoradiogram may be generated which can be scanned in a densitometer. Another method is to label the 5' end of one or both of the primers with ^{32}P , which is incorporated into the PCR products and then assayed for radioactivity (5).

Southern blot hybridization with synthetic DNA probes may also be performed to verify and quantitate PCR generated products, either by densitometry of an autoradiogram or by excising and counting the signal from a hybridization membrane. This method also quantitates only the target product without interference from nontarget products or primer-generated artifacts.

II: Non-Radioactive Quantitation

Nonradioactive quantitation methods include the use of biotinylated or digoxigenin-labeled primers in conjunction with the appropriate detection methods (6), use of a bioanalyzer or WAVE. For an in-depth discussion of the various methods of PCR product quantitation, refer to the review article by Bloch (7).

In addition to the above methods, several companies now offer gel video systems which can scan and quantitate EtBr-stained gel bands in much the same way a densitometer does. Lab-on-a-chip (BioAnalyzer), CE, HPLC, and WAVE may also be used to analyze MPCR products and quantitate simultaneously.

COMPARISON OF MPCR WITH RPA

MPCR (Multiplex Polymerase Chain Reaction)	RPA (RNase Protection Assay)
√ Non-isotope method with high sensitivity 0.1-1 μg total RNA per MPCR	√ Isotope or Non-Isotope methods 1-20 μg total RNA per RPA assay
√ Whole process takes only a few hours	√ Whole process takes two days
√ Detect Multiple Genes Simultaneously & Quantitatively	√ Detect Multiple Genes Simultaneously & Quantitatively
√ Signal can be quantified directly from gel if isotope is included in MPCR. Additional techniques can be used to quantify MPCR product (using Bioanalyzer, HPLC, and WAVE.)	√ Signal can be quantified directly from gel
√ Non-specific products can be eliminated by using probes and southern hybridization.	√ Non-specific signal can be generated by either low stringent conditions or high-secondary-structure template.
√ Ready-to-use	√ Make own "hot" RNA probes

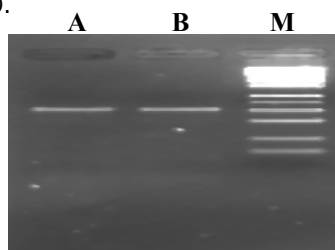
MPCR KIT DESCRIPTION

MPCR Amplification Kits include all necessary MPCR amplification reagents with the exception of *Taq* Polymerase. These kits have been designed to direct the simultaneous amplification of specific regions of human DNA.

Each kit offers Maxim's optimal primer/buffer system which will enhance amplification specificity.

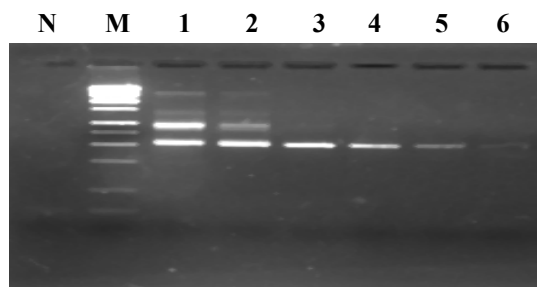
Figure 1 shows quality control MPCR results obtained by following MPCR kit manual using different concentrations of positive control.

For optimal results, please read and follow the instructions in this manual carefully. If you have any questions, please contact Maxim Biotech Customer Service at (650) 871-1919.



A: MPL-P001 primers with genomic DNA of *Mycoplasma hominis*
 B: MPL-P001 primers with genomic DNA of *Mycoplasma fermentans*
 M: GelMarker™.

Figure 1



1: MPL-P001 primers with 1,000,000 copies of *M. hominis* DNA
 2: MPL-P001 primers with 100,000 copies of *M. hominis* DNA
 3: MPL-P001 primers with 10,000 copies of *M. hominis* DNA
 4: MPL-P001 primers with 1,000 copies of *M. hominis* DNA
 5: MPL-P001 primers with 100 copies of *M. hominis* DNA
 6: MPL-P001 primers with 10 copies of *M. hominis* DNA
 N: MPL-P001 primers without DNA (Negative Control)

{* If the sample contains a high copy number, there may be two amplicon sizes (520 bp & 320 bp)}

MPCR PRIMER INFORMATION

Product Code	Gene	5'/3' Tm	Amplicon Size	Accession No.	Intron Span	Genomic Size
MPL-1001/1002*	bacteria 16S rRNA	67°C/69°C	520 bp	M24289	no	520bp
MPL-1003/1004**	bacteria 16S rRNA	64°C/60°C	320 bp	M24289	no	320bp

*: Mixtures of 9 oligos.
 **: Mixtures of 8 oligos.
 See Table Below

Sequences: (Alignment on database: Genbank accession number)

M. arginini (U15794), *M. fermentans* (M23940), *M. hominis* (M24658), *M. Hyorhinis* (M24473), *M. Orale* (M24289), *M. pirum* (M24659), *A. Laidlawii* (M23932), and others.

Table I. Restriction Fragment Length of final PCR products of common Mycoplasma Species

Mycoplasma Species	Size of Final PCR product	Size of Restriction Fragments (bp)			
		Afl III	Aat II	Bgl II	Vsp I
Arginini	329 bp	176/153	186/143		
Fermentans	327 bp	174/153	186/141	302/25	
Hominis	327 bp	174/153	186/141		257/70
Hyorhinis	327 bp	189/138	186/141		
Orale	328 bp	176/152	185/143		
Pirum	312 bp		185/127		
Laidlawii	327 bp		185/142		

KIT COMPONENTS

MP-70114

50X50 μ L MPCR reaction kit
Store all reagents at -20°C

Product Code	Kit Component	Amount
MPL-B001	2X Optimal PCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 μ l x 2
MPL-C001	10X Positive control	100 μ l
MPL-P001	10X PCR Primer Set	500 μ l
MRB-0014	DNA M.W. Marker (100 bp ladder)	100 μ l
MRP-0011P	ddH ₂ O (DNase free)	2.0 ml
	Instruction Manual	

NOTE: SPIN ALL TUBES BEFORE USING AND VORTEX ALL REAGENTS FOR AT LEAST 15 SECONDS BEFORE USING!!

PROCEDURE

PCR Protocol:

1. *Taq* DNA polymerase from Perkin-Elmer or its derivatives are highly recommended for MPCR. *Ampli-Taq Gold*, however, is not recommended because its own optimal buffer system is required.

2. Reaction Mixture Preparation:

- A. Set up MPCR reactions with the test samples and MPCR buffers provided in the MPCR kit according to the table on the next page:

Volume (Per assay)	Reagent (Add in order)
25.0 μ l	2X MPCR BufferMixture
5.0 μ l	10X MPCR Primers
0.5 μ l	<i>Taq</i> DNA Polymerase(5U/ μ l)
5.0 μ l	Specimen DNA or
	10X Control DNA from kit
14.5 μ l	H ₂ O
50.0 μ l	Mineral Oil (optional)

- *: ³²P dNTPs may be used here to achieve higher sensitivity and better quantitation. 5-10 uCi [α -³²P]dCTP (3000 Ci/mmmole) should be used here per MPCR. Keep final dNTPs concentration same as without ³²P-dNTPs.

- B. EDTA concentration in test sample must not exceed 0.5 mM because Mg⁺⁺ concentration in MPCR Buffers is limited to certain ranges. Additional Mg⁺⁺ may be added to the PCR mixture to compensate for EDTA. We strongly recommend running an MPCR reaction with the positive control provided in the kit. Since the MPCR DNA polymerase needed in each reaction is in a very small volume, it is recommended that all of the PCR components be premixed in a sufficient quantity for daily needs and then dispensed into individual reaction vials. This will help you to achieve more accurate measurements.

3. PCR thermocycle profile:

Reaction profiles will need to be optimized according to the machine type and needs of user. Please take note that temperature variations occur between different thermocyclers, therefore, the annealing temperature in the sample profile below is given as a range. It will be necessary to determine the optimal temperature for your individual thermocycler. An example of a time-temperature profile for the positive control PCR reaction optimized for Perkin Elmer machine types 480, 2400, and 9600 is provided below:

Temperature	Time	Cycles
96°C	5 min	1X
94°C	1 min	5X
62°C	1 min	
72°C	1 min	
94°C	0.5 min	25-30X
58°C	0.5 min	
72°C	0.5 min	
70°C	10 min	1X
25°C	soak	

4. Agarose Gel Electrophoresis:

To fractionate the MPCR DNA product electrophoretically, mix 10 μ l of the MPCR product with 2 μ l 6X loading buffer. Run the total 12 μ l alongside 10 μ l of DNA marker* from the MPCR kit on a 2 % agarose gel containing 0.5 mg/ml ethidium bromide. Electrophorese and photograph. (Hint: Best results are obtained when the gels are run slowly at less than 100 volts).

* DAN Marker contains linear double stranded DNA bands of 1,000; 900, 800, 700; 600; 500; 400; 300; 200; and 100 base pairs (bp).

TROUBLESHOOTING

1. MPCR AMPLIFICATION

Observation	Possible Cause	Recommended Action
1.1. No signal or missing some bands during amplification even using positive control provided in kit.	1.1a. The annealing temperature in the thermocycler is too high. 1.1b. Dominant primer dimers.	1.1a. Decrease PCR annealing temperature 3-5°C gradually. 1.1b. Use any one of "Hot Start" PCR procedures.
1.2. Too many nonspecific bands.	1.2a. The annealing temperature in the thermocycler is too low. 1.2b. Pre-PCR mispriming. 1.2c. cDNA is interfering with MPCR	1.2a. Increase PCR annealing temperature 3-5°C gradually. 1.2b. Use any one of "Hot Start" PCR procedures. 1.2c. Clean cDNA with Phenol/ Chloroform. 1.2d. Use Maxim's 3M™-MPCR Kit.
1.3. No difference in gene expression among treatments	1.3a. PCR amplification of this specific gene has passed the exponential phase. 1.3b. Variation in sample preparation, RT reaction and amounts of input cDNA.	1.3a. Decrease PCR cycle number or decrease the input cDNA. 1.3b. Run a parallel PCR with a house-keeping gene to eliminate variables.

PRECAUTIONS AND STORAGE

Storage

1. Store all MPCR Kit components at -20°C. Under these conditions components of the kit are stable for 1 year.
2. Isolate the kits from any sources of contaminating DNA, especially amplified PCR product.
3. Do not mix MPCR kit components that are from different lots. Each lot is optimized individually.

REFERENCES

1. Kobayashi H, et al. (1998) J Vet Med Sci; 60(12):1299-303
2. Razin S. (1994) Mol Cell Probes; 8(6): 497-511.
3. Stemke GW, et al. (1994) Am J Vet Res. 55(1):81-4.
4. van Kuppeveld FJ. et al. (1994) Appl Environ Microbiol. 60(1): 149-52