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**MPCR Kit for Chlamydia pneumoniae, Legionella pneumophila,  
Mycoplasma pneumoniae & Pneumocistis carini**

**Cat No. MP-70214: 50 reactions  
Cat No. MP-700213: 100 reactions**

**INSTRUCTION  
MANUAL**

ID-M10058  
Revised April 14, 2003

\*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

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Diagnosis of bacteria pathogens used to depend on culture and Gram's smear, enzymatic immunoassays. Recent studies show that PCR assay provides a satisfactory diagnostic tool for the detection of bacteria pathogens in clinical genital swab samples (1,2,3). PCR method is more sensitive, accurate and faster than the culturing method (5,6,7).

This PCP-MPCR kits have been designed to detect simultaneously *Chlamydia pneumoniae* (NUM), *Legionella pneumophila* (LPN), *Mycoplasma pneumoniae* (MPN), and *Pneumocystis carini* (PCP).

The PCR primers have similar  $T_m$  and no obvious 3'-end overlap to enhance multiple and equal amplification. The kit will yield the 871 bp (NUM), 375 bp (MPN), 300 bp (PCP), and 232 bp (LPN) PCR products from patient samples, or from the positive control which is included in this kit. Therefore, the PCP-MPCR kit provides a quick and simple method to detect *Chlamydia pneumoniae* (NUM), *Legionella pneumophila* (LPN), *Mycoplasma pneumoniae* (MPN), and *Pneumocystis carini* (PCP) in a single step.

## 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  $^{32}\text{P}$ , which is incorporated into the PCR products and then assayed for radioactivity (11).

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 (12), 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 (10).

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

<b>MPCR (Multiplex Polymerase Chain Reaction)</b>	<b>RPA (RNase Protection Assay)</b>
√ Non-isotope method with high sensitivity 0.1-1 $\mu\text{g}$ total RNA per MPCR	√ Isotope or Non-Isotope methods 1-20 $\mu\text{g}$ 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.

MPCR Kits come in two quantities:

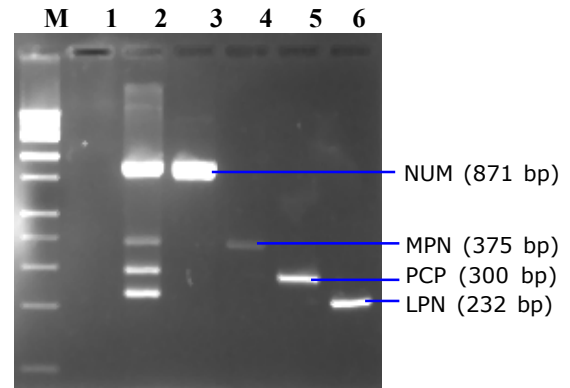
- 50X 50 $\mu$ L reaction kits
- 100X 50 $\mu$ L reaction kits

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.

Figure 1



- Lane 1: PCR using PCP MPCR Primers without positive control (Negative)
- Lane 2: PCR using PCP MPCR Primers with positive control
- Lane 3: PCR using NUM MPCR Primers
- Lane 4: PCR using MPN MPCR Primers
- Lane 5: PCR using PCP MPCR Primers
- Lane 6: PCR using LPN Primers
- Lane M: DNA M.W. Marker

## MPCR PRIMER INFORMATION

Product Code	Gene	5'/3' Tm	Amplicon Size	Accession No.
PCP-NUM	<i>C. pneumoniae</i>	64°C/65°C	871 bp	Z49873
PCP-LPN	<i>L. pneumophila</i>	68°C/68°C	232 bp	AF095228
PCP-MPN	<i>M. pneumoniae</i>	72°C/70°C	375 bp	MYCP1A,M18639
PCP-PCP	<i>P. carini</i>	69°C/68°C	300 bp	Z19053

## KIT COMPONENTS

### MP-70214

50X50 $\mu$ L MPCR reaction kit  
Store all reagents at -20°C

Product Code	Kit Component	Amount
PCP-B001	2X PCP MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 $\mu$ l
PCP-C001	10X PCP Pos. Control	50 $\mu$ l
PCP-P001	10X PCP MPCR Primers	250 $\mu$ l
MRB-0014	DNA M.W. Marker (100bp ladder)	100 $\mu$ l
MRB-0011P	Sterile ddH <sub>2</sub> O	2.0 ml
	Instruction Manual	

### MP-70213

100X50 $\mu$ L MPCR reaction kit  
Store all reagents at -20°C

Product Code	Kit Component	Amount
PCP-B001	2X PCP MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 $\mu$ l X2
PCP-C001	10X PCP Pos. Control	50 $\mu$ l X2
PCP-P001	10X PCP MPCR Primers	250 $\mu$ l X2
MRB-0014	DNA M.W. Marker (100bp ladder)	100 $\mu$ l X2
MRB-0011P	Sterile ddH <sub>2</sub> O	2.0 ml X2
	Instruction Manual	

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

## PROCEDURE

### DNA Isolation Protocol

The molecular cloning manual by Sambrook, et al. (9) contains useful information on how to isolate and handle DNA properly. Additionally, several companies offer kits for DNA isolation, including Maxim's genomic DNA Isolation Kit (EXT-0001). Following is a procedure for blood DNA isolation from Maxim's EXT-0001.

#### PROCEDURE

##### Section A:

1. Collect 5 ml of whole blood with anti-coagulation agent (i.e. EDTA at concentration: 1mg/ml blood; *Also See Note*) and transfer it to a 15 ml polypropylene centrifuge tube.
2. Add 5 ml of **BD-1 solution** and invert several times until completely mixed.
3. Centrifuge for 10 minutes at 2,200xg using a bench top centrifuge.
4. Slowly pour off the supernatant and save the nuclear pellet.
5. Add 5 ml of **BD-2 solution** to wash the pellet. Spin at 2,200xg for 10 minutes as before.
6. Discard the supernatant and gently resuspend the pellet with 0.8 ml of **BD-3 solution** by pipetting back and forth several times. Incubate at 65 °C for 15 minutes.  
(**Note:** *If a precipitate forms in **BD-3 solution**, warm up at 37°C before use*)
7. Add 0.3 ml of **BD-4 solution** into the tube and mix well.

##### Section B:

1. Spin for 10 minutes at maximum speed in a microcentrifuge. Carefully collect the supernatant and avoid the pellet. (*If a precipitate remains in the supernatant, spin again until it is clear*)
2. Add 2.0 ml of 100% ethanol the supernatant at room temperature and invert the tube several times until the DNA precipitates.
3. Spool the DNA with a one end closed pasture pipet and transfer the DNA into a microcentrifuge tube containing 1 ml of **ice-cold** 70% ethanol.
4. Spin for 5 minutes at maximum speed in a micro-centrifuge at **4 °C**.
5. Briefly dry the pellet and add 0.5 ml sterile H<sub>2</sub>O or TE buffer to the DNA.
6. Heat the tube at 65 °C for 15-30 minutes and invert several times during the incubation.  
(It is important to invert several times assisting the high molecular weight genomic DNA to dissolve)
7. Briefly spin at 4 °C and use a aliquot to determine the DNA concentration and purity.
8. It is ready to use.
9. Isolated DNA can be stored conveniently at -20°C or at -70°C for several years without appreciable deterioration. Repeated freeze and thaw cycles should be avoided.

*Note: DNA extracted from the blood with heparin can influence treatment with restriction enzymes or PCR reactions.*

##### Sample Preparation from Smear or Gastric Acid

1. Mix 250 ul of smear specimens with 20 ul Neutralize Buffer (1N NaOH).
2. Keep at 55C for 2 hours.
3. Exact once with Phenol/Chloroform.
4. Transfer the aqueous phase to new tube.
5. Add 25 ul of 3M Na(OAc) and 250 ul of Isopropanol, mix well.
6. Keep at -20C for 2 hours.
7. Microcentrifuge the DNA for 15 minutes at room temperature.
8. Discard the supernatant and dry the DNA pellet.
9. Dissolve the DNA into 100 ul ddH<sub>2</sub>O.
10. Use around 5 ul for 50ul PCR reaction.

# 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 below:

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

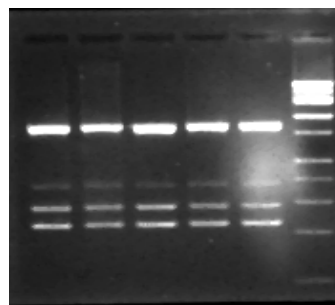
- \*: <sup>32</sup>P dNTPs may be used here to achieve higher sensitivity and better quantitation. 5-10 uCi [<sup>32</sup>P]dCTP (3000 Ci/mmole) should be used here per MPCR. Keep final dNTPs concentration same as without <sup>32</sup>P-dNTPs.
- B. EDTA concentration in test sample must not exceed 0.5 mM because Mg<sup>++</sup> concentration in MPCR Buffers is limited to certain ranges. Additional Mg<sup>++</sup> 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 <b>60-62°C*</b>	1 min 4 min	2X
94°C <b>60-62°C*</b>	1 min 2 min	28-35X
70°C	10 min	1X
25°C	soak	

55 58 61 64 67°C



\*The performance of MPCR kit against annealing temperatures. The above gel picture is an illustration of different annealing temperatures on MPCR kit MP-70214.

## PROCEDURE

### 4. Agarose Gel Electrophoresis:

To fractionate the MPCR DNA product electrophoretically, mix 10 $\mu$ l of the MPCR product with 2  $\mu$ l 6X loading buffer. Run the total 12  $\mu$ l alongside 10  $\mu$ 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 thermocycler is too high.	1.1a. Decrease PCR annealing temperature 3-5°C gradually.
	1.1b. Dominant primer dimers.	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.	1.2a. Increase PCR annealing temperature 3-5°C gradually.
	1.2b. Pre-PCR mispriming.	1.2b. Use any one of "Hot Start" PCR procedures.
	1.2c. cDNA is interfering with MPCR	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.	1.3a. Decrease PCR cycle number or decrease the input cDNA.
	1.3b. Variation in sample preparation, RT reaction and amounts of input cDNA.	1.3b. Run a parallel PCR with a house-keeping gene to eliminate variables.

## PRECAUTIONS AND STORAGE

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### 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

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1. Lee, C.H. et al. *J. Clin. Microbiol.* 31 (3), 754-757 (1993).
2. Su, C.J. et al. *Infect. Immun.* 55, 3023-3029 (1987).
3. Willoughby, J.J. et al. *Infect Immun* 59: 2463-2469, 1991
4. Grimprel, E et al. *J Clin Microbiol* 29: 1711-1718, 1991.
5. Maxim Biotech Tools, 1, 2-5, 1995.
6. Chamberlain, J.S. et al., In: *The polymerase chain reaction*. Mullis K, Ferre F and Gibbs R, eds. Birkhauser Boston Press, 38-46, 1994.
7. Kumar, A. et al., (1997) *Science* 278, 1630-1632.
8. Chou, Q et al., 1992 *Nucl. Acids Res.* 20, 1717.
9. Sambrook, J. & Maniatis, T. (1989) *Molecular Cloning Manual* Cold Spring Harbor Laboratory Press.
10. Bloch, W. (1991) *Biochemistry* 30:2735.
11. Hayashi, K., Orita, M., Suzuki, Y. & Sekiya, T. (1989) *Nucleic Acids Res.* 17:3605.
12. Landgraf, A., Reckmann, B., & Pingoud, A. (1991) *Analytical Biochemistry* 193:231.