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MPCR Kit for H. Pylori Detection

Cat No. MP-70081: 50 reactions
Cat No. MP-70080: 100 reactions

INSTRUCTION MANUAL

ID-M10061
Revised April 1, 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

The bacteria *Helicobacter pylori* is a fastidious microaerophilic spiral gram-negative microorganism which plays a significant role in the pathogenesis of chronic gastritis, peptic ulcer, and gastric cancer. Methods that accurately detect *H.pylori* infection are therefore of major importance. Direct demonstration of *H.pylori* is possible through the use of culture, histological examination with several strains, and assays for rapid urease activity. Culture is very sensitive and specific detection method although it is very labor-intensive because of the fastidious nature of the organism.

This Kit is designed to rapidly detect Helicobacter pylori using multiplex PCR. A segment of following genes in H. pylori genome: CagA, UreaC, Flagellin and 16S rRNA (1, 2, 3, 4) will be co-amplified in a single tube. The PCR primers have similar Tm and no obvious 3'-end overlap to enhance multiple genes amplification (5, 6, 7). The kit will yield the 348 bp (Cag A), 315 bp (UreaC), 152 bp (Flagellin), and the 110 bp (16S) PCR products when using positive control DNA provided in the Kit. PCR products can be distinguished on a regular agarose gel.

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 autoradio-gram 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 (10).

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 digoxygenin-labeled primers in conjunction with the appropriate detection methods (11), 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 (9).

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 0.1-1μg 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.

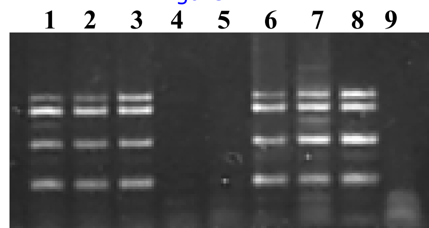
MPCR Kits come in two quantities:
- 50X 50μL reaction kits
- 100X 50μL reaction kits

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

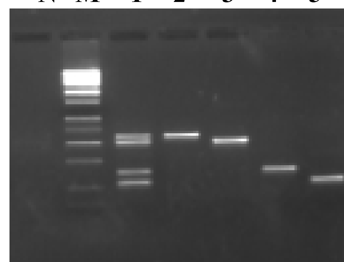
Figure 1 shows quality control MPCR results obtained by following MPCR kit manual using different concentrations of positive control.

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



Lanes 1-8: *H.Pylori* MPCR results using DNAs from 8 different patients.
Lane 9: negative control.



Lane N: PCR using HPY MPCR Primers without positive (Negative)
Lane 1: PCR using HPY MPCR Primers with positive control
Lane 2: PCR using Cag A Primers with positive control
Lane 3: PCR using Urea C Primers with positive control
Lane 4: PCR using Flagellin Primers with positive control
Lane 5: PCR using 16S Primers with positive control
Lane M: DNA M.W. Marker

MPCR PRIMER INFORMATION

Product Code	Gene	5'/3' Tm	Amplicon Size	Accession No.	Intron Span	Genomic Size
HPY-CAG	H pylori Cag A	66°C/64°C	349bp	L11714	no	349bp
HPY-URC	H pylori Urea C	67°C/66°C	315bp	M60398	no	315bp
HPY-FGN	H pylori Flagellin	64°C/64°C	152bp	X60746	no	152bp
HPY-16S	H pylori 16S	63°C/62°C	110bp	U00679	no	110bp

KIT COMPONENTS

MP-70081

50X50µL MPCR reaction kit
Store all reagents at -20°C

Product Code	Kit Component	Amount
HPY-B001	2X HPY MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 µl
HPY-C001	10X HPY Pos. Control	50 µl
HPY-P001	10X HPY MPCR Primers	250 µl
MRB-0014	DNA M.W. Marker (100bp Ladder)	100 µl
MRB-0011P	ddH_2O (DNase free)	2.0 ml
	Instruction Manual	

MP-70080

100X50µL MPCR reaction kit
Store all reagents at -20°C

Product Code	Kit Component	Amount
HPY-B001	2X HPY MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 µl X2
HPY-C001	10X HPY Pos. Control	50 µl X2
HPY-P001	10X HPY MPCR Primers	250 µl X2
MRB-0014	DNA M.W. Marker (100bp Ladder)	100 µl X2
MRB-0011P	ddH_2O (DNase free)	2.0 ml X2
	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)
25.0µl	2X MPCR BufferMixture
5.0µl	10X MPCR Primers
0.5µl	*Taq* DNA Polymerase(5U/µl)
5.0µl	Specimen cDNA or 10X Control cDNA from kit
14.5µl	H_2O
50.0µl	Mineral Oil (optional)

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

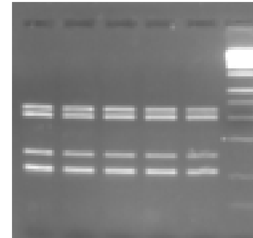
B. EDTA concentration in test sample must not exceed 0.5 mM because Mg^{++} concentration in MPCR Buffers is limited to certain ranges. Additional Mg^{++} may be added to the PCR mixture to compensate for EDTA. We strongly recommend running an MPCR reaction with the positive control provided in the kit. 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 **58-60°C***	1 min 4 min	2X
94°C **58-60°C***	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 tempeatures on MPCR kit MP-70080.

Note: A 2-step PCR thermocycle profile was found to be more effective than a 3-step PCR thermocycle profile for MPCR amplification. For 2-step PCR, use 94-95°C for denaturation and 58-60°C for annealing and extension. The 72°C step is omitted.

4. Agarose Gel Electrophoresis:

To fractionate the MPCR DNA product electrophoretically, mix 10µl of the MPCR product with 2µl 6X loading buffer. Run the total 12µl alongside 10 µl 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. 1.1b. 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.

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