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MPCR Kit for Human Apoptic Genes Set-7

Cat No. MP-70019: 50 reactions
Cat No. MP-70011: 100 reactions

INSTRUCTION MANUAL

ID-M10053
Revised February 5, 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

Mammalian cells can self-destruct via intrinsic programmed cell death (1). Apoptosis, a form of programmed cell death, is characterized by specific morphologic and biochemical properties. Apoptosis plays a major role in many biological processes, including embryogenesis, development of the immune system and tissue regeneration. Like growth and differentiation, apoptosis requires active and coordinated regulation of specific genes. Some molecular components of the apoptotic program have been conserved through evolution. Genetic studies of C. *elegans* have led to the identification of mutations, on 14 genes, that affect programmed cell death in this organism (1). Three of these genes, CED-3, CED4 and CED-9, are essential for apoptosis. Each of these has at least one functional equivalent in mammals: CED-9 is a member of the Bcl-2 family, CED-3 is the prototypical worm caspase, and CED-4 is homologous to Apaf-1.

Ecotopic expression of bcl-2 has been shown to block apoptosis in many experimental systems (2). Although the mechanism of bcl-2 action is unknown, biochemical studies have implicated this protein in the regulation of cell redox potential. Genetic evidence indicates that bcl-2 belongs to an emerging family. Some members of the bcl-2 family, like bcl-xL (3), suppress apoptosis, while other members, such as bax and bcl-xS (3,4), increase the susceptibility of cells to apoptotic stimuli. In addition, bcl-2 family members form hetero- and homo-dimers, suggesting that cellular susceptibility to apoptosis is partly influenced by the level of gene expression.

ICE (Caspase-1) encodes a cystein protease that cleaves peptide bonds after Asp residues (5). The ICE (Interleukin-1beta Converting Enzyme) family -recently named the caspases for cysteine aspartate-specific proteases- plays a central role in apoptosis and may well constitute part of the conserved core mechanism of the process. A family of caspase genes (Caspase #1 to #13) have been identified, which cleave key targets in the cell (6). Cell death is the outcome of a programmed intracellular cascade of genetically steps involving caspase activation and their subsequently catalytic reactions. Caspases normally exist in cells as inactive proenzymes; Proteolytic processing at a few specific sites unleashes their latent enzymatic activity and may triggers cell destruction.

Caspases can be activated by two distinct mechanisms. Because all caspases have similar cleavage specificity, the simplest way to activate a procaspase is to expose it to a previously activated caspase molecule. This "caspase cascade" is used extensively by cells for the activation of the downstream effector caspases: caspase-3, caspase-6 (6).

The second strategy, "induced proximity", was first observed in caspase-8, an initiator caspase that acts downstream of Fas/FasL(7). Upon ligand binding, Fas receptor aggregate into a membrane-bound complex. This signaling complex recruits, via the receptor-bound adapter protein FADD, several procaspase-8 molecules, resulting in a high local concentration of procaspase-8. Under these conditions, the low protease activity inherent to procaspases is sufficient to drive intermolecular proteolytic activation of the receptor-associated procaspase-8 molecules (6).

Analysis of the temporal and spatial distribution of RNA expression provides researchers with important clues about the function of apoptosis regulating genes in their own systems. Northern Blot and RNase Protection Assay are the most widely used procedures for determining the abundance of a specific mRNA in a total or poly(A) RNA sample. RT-MPCR provides an alternate and accurate method to detect multiple gene expression by amplifying all the genes under the same conditions (9, 10, 11). Variations in RNA isolation, initial quantitation errors or tube-to-tube variations in RT and PCR can be compensated by including a house-keeping gene, such as GAPDH, in MPCR. Alternatively, a parallel RT-PCR using the same cDNA, PCR conditions and primers for one of house-keeping genes may be run to offset any variations. Differences in gene expression can be determined by normalizing its expression against GAPDH expression.

Maxim's APO RT-MPCR kits have been designed to detect the expression of human bad, bcl-2, bax, bim, c-myc, bag-1, P53 and GAPDH genes. The PCR primers have similar Tm and no obvious 3'-end overlap to enhance multiple amplification. The 615 bp(GAPDH), 495 bp(P53), 421 bp(bag-1), 381 bp(c-myc), 313 bp(bim), 272 bp(bax), 235 bp(bcl-2) and 192 bp(bad) PCR products can be generated from human RNA. Therefore, the APO-M057G RT-MPCR kit provides a quick and simple method to analyze human bad, bcl-2, bax, bim, c-myc, bag-1, P53 and GAPDH genes expression during apoptosis & normalize their expression against GAPDH gene.

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 (12).

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.

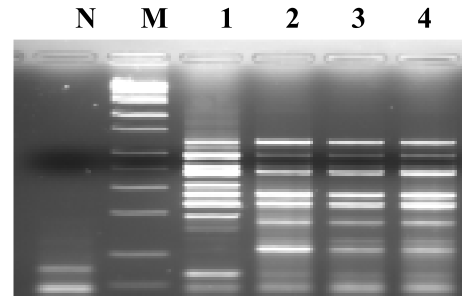
MPCR Kits come in two quantities:
- 50X 50µL reaction kits
- 100X 50µL reaction kits

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



Lane N: Negative Control
Lane 1: 1X APO1G Positive Control with 1X hAPO7G MPCR Primer
Lane 2: Human Mononucleated with 1X hAPO7G MPCR Primer
Lane 3: Human PHA-treated Mononucleated cells with 1X hAPO7G MPCR Primer
Lane 4: Human PHA & CD molecule-treated Mononucleated with 1X hAPO7G MPCR Primer

MPCR PRIMER INFORMATION

Product Code	Gene	5'/3' Tm	Amplicon Size	Accession No.	Intron Span	Genomic Size
hAPO7G-GAP	Human GAPDH	70°C/70°C	615bp	XM_033259	yes	990bp
hAPO7G-P53	Human P53	66°C/67°C	495bp	XM_113328	yes	4394bp
hAPO7G-BAG	Human BAG	66°C/67°C	421bp	XM_036229	yes	5399bp
hAPO7G-BAD	Human BAD	72°C/74°C	192bp	AF021792.1	no	192bp
hAPO7G-BCL	Human Bcl-2	70°C/71°C	235bp	M14745	no	235bp
hAPO7G-BAX	Human BAX	69.5°C/70°C	272bp	L22473	yes	370bp
hAPO7G-BIM	Human BIM	70°C/71°C	313bp	AF032458.1	yes	40383bp
hAPO7G-MYC	Human c-myc	72°C/70°C	381bp	J00120 X00364	no	381bp

KIT COMPONENTS

MP-70019

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

Product Code	Kit Component	Amount
hAPO7G-B001	2X hAPO7G MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 µl
hAPO7G-C001	10X hAPO7G MPCR Pos. Control	50µl
hAPO7G-P001	10X hAPO7G MPCR Primers	250µl
MRB-0014	DNA M.W. Marker (100bp Ladder)	100 µl
MRB-0011P	ddH_20 (DNase free)	2.0 ml
	Instruction Manual	

MP-70011

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

Product Code	Kit Component	Amount
hAPO7G-B001	2X hAPO7G MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 µl X2
hAPO7G-C001	10X hAPO7G MPCR Pos. Control	50µl X2
hAPO7G-P001	10X hAPO7G MPCR Primers	250µl X2
MRB-0014	DNA M.W. Marker (100bp Ladder)	100 µl X2
MRB-0011P	ddH_20 (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

RT Protocol:

The isolation of undegraded, intact RNA is an essential prerequisite for successful first strand synthesis and PCR amplification. Care should be taken to avoid RNase contamination of buffers and containers used for RNA work by pretreating with DEPC, autoclaving, and baking. Always wear sterile gloves when handling reagents. Use cDNA derived from 10^5 cells (1µg cDNA) and apply them to each MPCR reaction.

1. Prepare total RNA, mRNA or use the control GAPDH RNA which is provided in Maxim's MPCR kit. ***NOTE***: It is best to use cDNA derived from 0.5-1 x 10^5 cells (0.5-1µg cDNA derived from RNA) for each MPCR reaction.

2. Equilibrate 3 water baths: 37°C, 70°C and 95°C.

3. **On ice**, pipet 1-2 µg mRNA or 10 µg total RNA (from 10^6 cells) dissolved in pure water or 2 µl control GAPDH RNA into a RNAase free reaction vial. We strongly recommend including a positive control reaction when setting up an RT-PCR reaction for the first time.

4. Add sterile water to a final volume of 14.5 µl.

5. Add 4 µl random hexamer (50 mM) or Oligo(dT) (50 mM).
   NOTE: The hexamer and Oligo(dT) RT reactions may be run simultaneously.

6. Incubate tube(s) at 70°C for 5 minutes and quickly chill on ice.

7. Begin your RT reaction by adding the following reagents to your hexamer or Oligo mixture:

Reagent	Description	Volume per Reaction
RNase Inhibitor	130U/µl	0.5µl
5 X RT buffer	250mM Tris-HCl (pH8.3) 375mM KCl, 15mM $MgCl_2$, 50mM DTT	10µl
dNTPs	1mM each	20µl
MMLV RT	250U/µl	1µl

8. Incubate the RT mixture at 37°C for 60 minutes.

9. Then, heat RT mixture at 95°C for 10 minutes and quickly chill on ice. This will help to eliminate the RT enzyme interference of MPCR reaction later.

10. Add another **50 µl** water or 0.1X TE buffer.

11. **2-5 µl** of above cDNA is sufficient for most genes in a standard MPCR reaction. However, more or less DNA may be needed in PCR depending on the copy number of the specific gene.

NOTE: Please do not use excess amount of cDNA. The salt from RT reaction may interfere the performance of MPCR.

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:

PROCEDURE

Volume (Per assay)	Reagent (Add in order)
25.0 µl	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)

*: :32P 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 measure ments.

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	

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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