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MPCR Kit for Rat Alipoproteins Family

Cat No. MP-70004: 50 reactions

Cat No. MP-70003: 100 reactions

INSTRUCTION MANUAL

ID-M10012
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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

The central nervous system accounts for only 2% of the whole body mass but contains almost a quarter of the unesterified cholesterol present in the whole individual. This sterol is largely present in two pools comprised of the cholesterol in the plasma membranes of glial cells and neurons and the cholesterol present in the specialized membranes of myelin. The input of cholesterol into the central nervous system comes almost entirely from in situ synthesis, and there is currently little evidence for the net transfer of sterol from the plasma into the brain of the fetus, newborn or adult. In the steady state in the adult, an equivalent amount of cholesterol must move out of the brain and this output is partly accounted for by the formation and excretion of 24S-hydroxycholesterol. This cholesterol turnover across the brain is increased in neurodegenerative disorders such as Alzheimer's disease. Indirect evidence suggests that large amounts of cholesterol also turn over among the glial cells and neurons within the central nervous system during brain growth and neuron repair and remodeling. This internal recycling of sterol may involve ligands such as apolipoproteins E and AI, and one or more membrane transport proteins such as members of the low density lipoprotein receptor family.

Apolipoprotein A (ApoA) is a very large molecule, larger than plasminogen; it contains duplications of many kringles present in small numbers in plasminogen. ApoA-I is identical to serum PGI(2) stabilizing factor (PSF). PGI (2), or prostacyclin, is synthesized by the vascular endothelium and smooth muscle, and functions as a potent vasodilator and inhibitor of platelet aggregation.

Apolipoprotein B (ApoB), a major protein component of circulating plasma lipoproteins, exists in 2 forms: apoB-100 and apoB-48. In humans, apoB-100 is synthesized in the liver and is present in very low density lipoproteins and their metabolic products. On the other hand, apoB-48 is synthesized in the small intestine and is the form of apoB present in chylomicrons and chylomicron remnants. Apolipoprotein C-II (apoC-II) is a necessary cofactor for the activation of lipoprotein lipase, the enzyme that hydrolyzes triglycerides in plasma and transfers the fatty acids to tissues.

Apolipoprotein D (ApoD) is a 29-kDa glycoprotein that is primarily associated with high density lipoproteins in human plasma. It is an atypical apolipoprotein and, based on its primary structure, ApoD is predicted to be a member of the lipocalin family. Lipocalins adopt a beta-barrel tertiary structure and transport small hydrophobic ligands. The ApoD gene is expressed in many tissues, with high levels of expression inspleen, testes and brain. ApoD is present at high concentrations in the cyst fluid of women with gross cystic disease of the breast, a condition associated with increased risk of breast cancer. It also accumulates at sites of regenerating peripheral nerves and in the cerebrospinal fluid of patients with neurodegenerative conditions, such as Alzheimer's disease. ApoD may, therefore, participate in maintenance and repair within the central and peripheral nervous systems. While its role in metabolism has yet to be defined, ApoD is likely to be a multi-ligand, multi-functional transporter. It could transport a ligand from one cell to another within an organ, scavenge a ligand within an organ for transport to the blood or could transport a ligand from the circulation to specific cells within a tissue.

Apolipoprotein E (ApoE) and apoE-derived proteolytic fragments are present in amyloid deposits in Alzheimer disease (AD) and cerebral amyloid angiopathy (CAA). An isoform of apolipoprotein E, a protein that mediates the transport of lipids and cholesterol in the circulatory system, predisposes carriers of this allele to the common late-onset form of the disease. How this protein is related to a neurodegenerative disorder is an enigma. Evidence indicates that apolipoprotein E receptors, which are abundantly expressed in most neurons in the central nervous system, also fulfill critical functions during brain development and may profoundly influence the pathogenesis of Alzheimer's disease.

Apolipoprotein J (ApoJ, clusterin) is a ubiquitous multifunctional glycoprotein capable of interacting with a broad spectrum of molecules. In pathological conditions, it is an amyloid associated protein, co-localizing with fibrillar deposits in systemic and localized amyloid disorders. In Alzheimer's disease, the most frequent form of amyloidosis in humans and the major cause of dementia in the elderly, ApoJ is present in amyloid plaques and cerebrovascular deposits but is rarely seen in NFT-containing neurons. ApoJ expression is up-regulated in a wide variety of insults and may represent a defense response against local damage to neurons.

Maxim's Rat ALPG MPCR kits have been designed to detect the expression of Rat Genes: ApoA, ApoB, ApoC, ApoD, ApoE, ApoJ and GAPDH. The PCR primers have similar Tm and no obvious 3'-end overlap to enhance multiple amplification. The 555 bp (H18S), 387 bp (ApoJ), 336 bp (ApoB), 294 bp (ApoE), 254 bp (ApoA), 224 bp (ApoD) and 203 bp (ApoC) PCR products can be generated from Rat RNA, or the positive control which is included in this kit. Therefore, the rALPG MPCR kit provides a quick and simple method to analyze Rat ApoA, ApoB, ApoC, ApoD, ApoE and ApoJ gene expression and normalize their expression against 18S expression.

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 (13).

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 (14), 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 (8).

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.

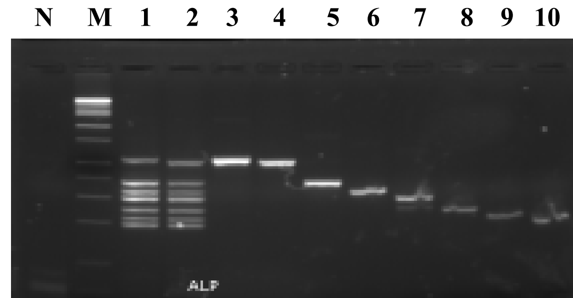
MPCR Kits come in two quantities:
- 50X 50µL reaction kits
- 100X 50µL reaction kits

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: PCR using rALPG Primers without positive (Negative)
Lane M: DNA M.W. Marker
Lane 1: PCR using rALPS Primers with 1X positive
Lane 2: PCR using rALPG Primers with 1X positive
Lane 3: PCR using Rat 18S Primers with 1X positive
Lane 4: PCR using Rat GAPDH Primers with 1X positive
Lane 5: PCR using Rat ApoJ Primers with 1X positive
Lane 6: PCR using Rat ApoB Primers with 1X positive
Lane 7: PCR using Rat ApoE Primers with 1X positive
Lane 8: PCR using Rat ApoA Primers with 1X positive
Lane 9: PCR using Rat ApoD Primers with 1X positive
Lane 10: PCR using Rat ApoC Primers with 1X positive

MPCR PRIMER INFORMATION

Product Code	Gene	5'/3' Tm	Amplicon Size	Accession No.	Intron Span	Genomic Size
rALPS-ApoJ	Rat ApoJ	66°C/70°C	387 bp	NM_012679.2	yes	3948bp
rALPS-ApoB	Rat ApoB	68°C/68°C	336 bp	U53873.1	N/A	336bp
rALPS-ApoE	Rat ApoE	68°C/68°C	298bp	J02582	N/A	302bp
rALPS-ApoA	Rat ApoA	68°C/68°C	254 bp	NM_012738.1	N/A	254bp
rALPS-ApoD	Rat ApoD	68°C/68°C	224 bp	NM_012777	N/A	224bp
rALPS-ApoC	Rat ApoC	67°C/68°C	203 bp	NM_012824.1	N/A	203bp
rALPS-18S	Rat 18S	64°C/65°C	554 bp	V01270	N/A	554bp

KIT COMPONENTS

MP-70004

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

Product Code	Kit Component	Amount
rALPS -B001	2X rALPS MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 μl
rALPS -C001	10X rALPS MPCR Pos. Control	50μl
rALPS -P001	10X rALPS 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-70003

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

Product Code	Kit Component	Amount
rALPS -B001	2X rALPS MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 μl X2
rALPS -C001	10X rALPS MPCR Pos. Control	50μl X2
rALPS -P001	10X rALPS 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)

*: $^{:32}$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 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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