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MPCR Kit for Human DMD/BMD Set I+II

Cat No. MP-70057: 50 reactions
Cat No. MP-70056: 100 reactions

INSTRUCTION MANUAL

ID-M10060
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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

Dystrophin-associated muscular dystrophies range from the severe Duchenne to the milder Becker muscular dystrophy (DMD and BMD). Mapping and molecular genetic studies indicate that both are the result of mutations in the gene encoding dystrophin. Two-thirds of DMD/BMD patients have large deletions localized in two hot spots, and the remainingcases are presumed to be caused by point mutations. DMD deletions usually shift the frame, although there is no clear correlation found between the extent of deletion and the severity of the disorder. The most distinct feature of DMD is a progressive proximal muscular dystrophy with characterstic pseudohypertrophy of the calves. The bulbar muscles are spared, but the myocardium is affected. Effects include massive elevation of creatin kinase levels in the blood, myopathic changes charted by electromyography, and myofiber degradation with fibrosis and fatty infiltration as revealed through muscle biopsy.

DMD and the allelic disorder BMD are common X-linked recessive neuromuscular disorders that are associated with a spectrum of genetically-based developmental, cognitive, and behavioral disabilities. Seven promoters scattered throughout the DMD/BMD gene locus code for distinct isoforms of dystrophin, which exhibits nervous system developmental, regional, and cell-type specificity. Dystrophin is a complex plasmalemmal-cytoskeletal linker protein that possesses multiple functional domains, autosomal and X-linked homologs, and associated binding proteins that form multi-unit signaling complexes whose compositions are unique within its individual cellular and developmental context. Through additional interactions with extracellular matrix, plasma membrane, cytoskeleton, and distinct intracellular compartmental proteins, brain dystrophin acquires the capability to participate in the modulatory actions of a large number of cellular signaling pathways. In adult life, dystrophin normally modulates synaptic terminal integrity, distinct forms of synaptic plasticity, and regional cellular signal integration. At a system level, dystrophin may regulate essential components of an integrated sensorimotor attention network. Dystrophin deficiency in DMD/BMD patients appears to impair intracellular calcium homeostasis and disrupt multiple protein-protein interactions that normally promote information transfer and signal integration from the extracellular environment to the nucleus within regulated microdomains. In DMD/BMD, the individual profiles of cognitive and behavioral deficits, mental retardation, and other phenotypic variations appear to depend on complex profiles of transcriptional regulation. These variations are associated with individual dystrophin mutations that result in the corresponding presence or absence of individual brain dystrophin isoforms that normally exhibit developmental, regional and cell-type-specific expression and functional regulation.

Since DMD is a serious disorder for which at present there is no effective treatment, much emphasis has been given to prevention. This involves the ascertainment of women likely to have an affected son, and the provision of genetic counseling and prenatal diagnosis for such women. Accurate carrier detection and genetic counselling depend upon identifying the mutation itself in the proband. Large deletions are easily identified using multiplex polymerase chain reaction (MPCR). Maxim has designed several MPCR kits to rapidly detect the deletions in dystrophin gene. Two sets of MPCR primers, Set I [1] and Set II[2], are used to amplify different exons of dystrophin genes. Set I amplifies exons 8(360 bp), 17 (416 bp), 19 (459 bp), 44 (268 bp), 45 (547 bp), 48 (506 bp), 12 (331 bp), 51 (388 bp) and 4 (196 bp) of the dystrophin genes. Set II amplifies exon 3 (410 bp), 6 (202 bp), 13 (238 bp), 43 (357 bp), 47 (181 bp), 50 (271 bp), 52 (113 bp), 60 (139 bp), and the muscle-specific promoter (535 bp) of the dystrophin gene. About 70% of DMD/BMD gene deletions can be detected by MPCR with Set I primers and 98% of DMD/BMD gene deletions can be detected by MPCR with Set I plus Set II primers.

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 (8).

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 (9), 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

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.

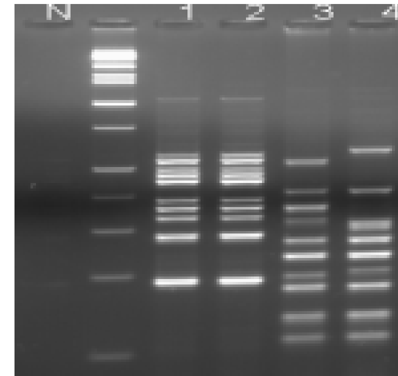
MPCR Kits come in two quantities:
- 50X 50µL reaction kits
- 100X 50µL reaction kits

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 (without Primer)
Lane 1: PCR using DMD-P001 primers with 1X Positive
Lane 2: PCR using DMD-P001 primers with 1X Positive
Lane 3: PCR using DMD-P002 Primers with 1 X Positive
Lane 4: PCR using DMD-P003 Primers with 1X Positive

MPCR PRIMER INFORMATION

Product Code	Gene	5'/3' Tm	Amplicon Size	Accession No.	Intron Span	Genomic Size
			Set I			
DMD1-Exon8	DMD exon 8	63°C/63°C	360bp	AL096699	no	360bp
DMD1-Exon17	DMD exon 17	67°C/70°C	416bp	X13045, AL031542	no	416bp
DMD1-Exon19	DMD exon 19	68°C/67°C	459bp	AL031542	no	459bp
DMD1-Exon44	DMD exon 44	67°C/70°C	268bp	X13046	no	268bp
DMD1-Exon45	DMD exon 45	72°C/66°C	547bp	X13048	no	547bp
DMD1-Exon48	DMD exon 48	68°C/64°C	506bp	X13047	no	506bp
DMD1-Exon12	DMD exon 12	64°C/67°C	331bp	AC004468	no	331bp
DMD1-Exon51	DMD exon 51	66°C/67°C	388bp	X51934	no	388bp
DMD1-Exon4	DMD exon 4	73°C/70°C	196bp	AL096699	yes	1083bp
			Set II			
DMD2-Exon3	DMD exon 3	69°C/75°C	410bp	AL096699	no	410bp
DMD2-Exon6	DMD exon 6	66°C/64°C	202bp	AL096699	no	202bp
DMD2-Exon13	DMD exon 13	65°C/69°C	238bp	AC004468	no	238bp
DMD2-Exon43	DMD exon 43	72°C/69°C	357bp	L41634	no	357bp
DMD2-Exon47	DMD exon 47	69°C/65°C	181bp	XM_051809	no	181bp
DMD2-Exon50	DMD exon 50	68°C/69°C	271bp	AJ271220	no	271bp
DMD2-Exon52	DMD exon 52	77°C/71°C	113bp	XM_051809	no	113bp
DMD2-Exon60	DMD exon 60	76°C/76°C	139bp	XM_051809	no	139bp
DMD2-PRO	DMD promoter	72°C/81°C	535bp	AL031643	no	535bp

KIT COMPONENTS

MP-70057

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

Product Code	Kit Component	Amount
DMD-B001	2X DMD Set I and II MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 µl X2
DMD-C001	10X DMD Pos. Control	50 µl
DMD-P001	10X DMD MPCR Primers Set I	250 µl
DMD-P002	10X DMD MPCR Primers Set II	250 µl
MRB-0014	DNA M.W. Marker (100bp ladder)	100 µl
MRB-0011P	ddH_2O (DNase free)	2.0 ml
	Instruction Manual	

MP-70056

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

Product Code	Kit Component	Amount
DMD-B001	2X DMD Set I and II MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 µl X4
DMD-C001	10X DMD Pos. Control	50 µl X2
DMD-P001	10X DMD MPCR Primers Set I	250 µl X2
DMD-P002	10X DMD MPCR Primers Set II	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)
25.0 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 uCi [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
94 °C	3’	1X
94 °C 53 °C 65 °C	30" 30'' 3'	30-35X
70 °C	10’	1X
25 °C	soak	

4. Agarose Gel Electrophoresis:

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

REFERENCES

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