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MPCR Kit for Human VEGF and Its Receptors Set-3
Cat No. MP-70174: 50 reactions
Cat No. MP-70171: 100 reactions

**INSTRUCTION
MANUAL**

ID-M10077
Revised February 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

Angiogenesis process is tightly regulated and depends on a dynamic balance between stimulators and inhibitors. The vascular endothelial growth factor (VEGF) family of proteins, together with their receptors are among the most studied stimulators, and play a crucial role in embryonic development and angiogenesis. Three VEGF receptors have been identified as Flt-1, Flk-1 and Flt-4.

Analysis of the temporal and spatial distribution of RNA expression can provide researchers with important clues about the function of these cytokines within their own systems (1). 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 (2,3). 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 b-actin or 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 (4). Differences in gene expression can be determined by normalizing its expression against GAPDH or b-actin expression.

This custom MPCR kit has been designed to detect the expression of human VEGF #165, #121, #189 and VEGF receptors flt-1 and flk-1 genes, Angiopoietin-1 & 2, and TIE-2. The PCR primers have similar T_m and no obvious 3'-end overlap to enhance multiple amplification. The 750 bp (GAPDH), 554 bp (flt-1), 472 bp (Angiop-1), 408 bp (flk-1), 350 bp (TIE-2), 306 bp (VEGF #189), 234 bp (VEGF #165), 200 bp (Angiop-2) and the 104 bp (VEGF #121) PCR products can be generated from human RNA or the positive control, which is included in this kit. Therefore, the expression of human VEGFs and its related genes can be monitored and normalized against the GAPDH 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 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}P , which is incorporated into the PCR products and then assayed for radioactivity (6).

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 (7), 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)

- √ Non-isotope method with high sensitivity
0.1-1 μg total RNA per MPCR
- √ Whole process takes only a few hours
- √ 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.)
- √ Non-specific products can be eliminated by using probes and southern hybridization.
- √ Ready-to-use

RPA (RNase Protection Assay)

- √ Isotope or Non-Isotope methods
1-20 μg total RNA per RPA assay
- √ Whole process takes two days
- √ Detect Multiple Genes Simultaneously & Quantitatively
- √ Signal can be quantified directly from gel
- √ Non-specific signal can be generated by either low stringent conditions or high-secondary-structure template.
- √ 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:

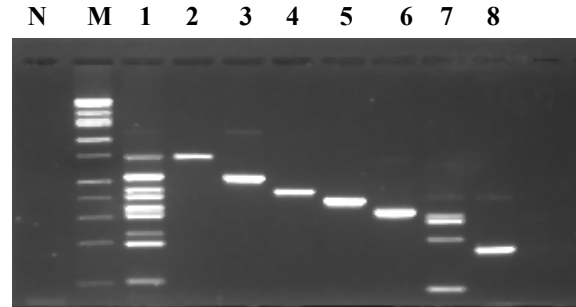
- 50X 50 μ L reaction kits
- 100X 50 μ L reaction kits

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 N: PCR using hVEGF3G Primers without positive (Negative)
 Lane 1: PCR using hVEGF3G Primers with 1X positive
 Lane 2: PCR using Human GAPDH Primers
 Lane 3: PCR using Human flt-1 Primers
 Lane 4: PCR using Human ANG1 Primers
 Lane 5: PCR using Human flk-1 Primers
 Lane 6: PCR using Human TIE-2 Primers
 Lane 7: PCR using Human VEGF Primers
 Lane 8: PCR using Human ANG2 Primers
 Lane M: DNA M.W. Marker

MPCR PRIMER INFORMATION

Product Code	Gene	5'/3' Tm	Amplicon Size	Accession No.	Intron Span	Genomic Size
hVEGF3G-FLT1	Human flt-1	72°C/69°C	554bp	AF063657	yes	8131bp
hVEGF3G-ANG1	Human Angiop-1	67°C/69°C	472bp	U83508.1	yes	42097bp
hVEGF3G-FLK1	Human flk-1	70°C/72°C	408bp	AF035121	no	408bp
hVEGF3G-TIE2	Human TIE-2	70°C/71°C	350bp	L06139.1	yes	5570bp
hVEGF3G-ANG2	Human Angiop-2	69°C/70°C	200bp	NM_001147.1	no	200bp
hVEGF3G-GAP	Human GAPDH	66°C/67°C	750bp	M33197	yes	1253bp
hVEGF3G-VEGF	Human VEGFs	69°C/72°C	306bp (type 189) 234 bp (type 165) 104 bp (type 121)	AF214570 AB021221	yes	6076bp

KIT COMPONENTS

MP-70174

50X50 μ L MPCR reaction kit
Store all reagents at -20°C

Product Code	Kit Component	Amount
hVEGF3G-B001	2X hVEGF3G-MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 μ l
hVEGF3G-C001	10X hVEGF3G-MPCR Pos. Control	50 μ l
hVEGF3G-P001	10X hVEGF3G-MPCR Primers	250 μ l
MRB-0014	DNA MW. Marker (100 bp ladder)	100 μ l
MRB-0011P	ddH ₂ O (DNase free)	2.0ml
	Instruction Manual	

MP-70171

100X50 μ L MPCR reaction kit
Store all reagents at -20°C

Product Code	Kit Component	Amount
hVEGF3G-B001	2X hVEGF3G-MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 μ l X2
hVEGF3G-C001	10X hVEGF3G-MPCR Pos. Control	50 μ l X2
hVEGF3G-P001	10X hVEGF3G-MPCR Primers	250 μ l X2
MRB-0014	DNA MW. Marker (100 bp ladder)	100 μ l X2
MRB-0011P	ddH ₂ O (DNase free)	2.0ml 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 ($1\mu\text{g}$ 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 $0.5-1 \times 10^5$ cells ($0.5-1\mu\text{g}$ cDNA derived from RNA) for each MPCR reaction.
2. Equilibrate 3 water baths: 37°C , 70°C and 95°C .
3. **On ice**, pipet $1-2 \mu\text{g}$ mRNA or $10 \mu\text{g}$ total RNA (from 10^6 cells) dissolved in pure water or $2 \mu\text{l}$ 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 $14.5 \mu\text{l}$.
5. Add $4 \mu\text{l}$ 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	$130\text{U}/\mu\text{l}$	$0.5\mu\text{l}$
5 X RT buffer	250mM Tris-HCl (pH8.3) 375mM KCl, 15mM MgCl_2 , 50mM DTT	$10\mu\text{l}$
dNTPs	1mM each	$20\mu\text{l}$
MMLV RT	$250\text{U}/\mu\text{l}$	$1\mu\text{l}$

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 **$50 \mu\text{l}$** water or 0.1X TE buffer.
11. **$2-5 \mu\text{l}$** 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	<i>Taq</i> DNA Polymerase(5U/µl)
5.0µl	Specimen cDNA or 10X Control cDNA from kit
14.5µl	H ₂ O
50.0µl	Mineral Oil (optional)

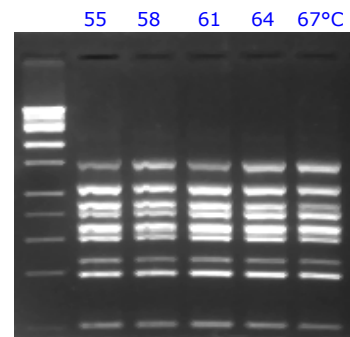
*: ³²P dNTPs may be used here to achieve higher sensitivity and better quantitation. 5-10 uCi [³²P]dCTP (3000 Ci/mmmole) should be used here per MPCR. Keep final dNTPs concentration same as without ³²P-dNTPs.

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	1 min	2X
58-60°C*	4 min	
94°C	1 min	28-35X
58-60°C*	2 min	
70°C	10 min	1X
25°C	soak	



*The performance of MPCR kit against annealing temperatures. The above gel picture is an illustration of different annealing temperatures on MPCR kit MP-70171.

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 the 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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