

780 Dubuque Avenue  
So. San Francisco, CA 94080, U.S.A.  
Tel: (800) 989-6296 / Fax:(650)871-2857  
<http://www.maximbio.com>  
E-mail: mbi@maximbio.com

**MPCR Kit for Rat VEGF and Its Receptors Set-1**  
**Cat No. MP-70175: 50 reactions**  
**Cat No. MP-70176: 100 reactions**

**INSTRUCTION  
MANUAL**

ID-M10045  
Revised November 1, 2002

\*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 is the development of new blood vessels via the migration and proliferation of endothelial cells from preexisting blood vessels. Angiogenesis is tightly regulated and depends on a dynamic balance between stimulators and inhibitors. The vesicular endothelial growth factor (VEGF), a homodimer glycoprotein of relative molecular mass 45,000, is the only mitogen that specifically acts on endothelial cells and may be a major regulator of tumor angiogenesis *in vivo*. VEGF and placental growth factor constitute a family of regulatory peptides capable of controlling blood vessel formation and permeability by interacting with two endothelial tyrosine kinase receptors, FLT1 and FLK1. A third member of family may be the ligand of the related FLT4 receptor involved in lymphatic vessel development. VEGF is known to occur as at least six differentially spliced variants, giving rise to mature isoforms containing 121, 145, 165, 183, 189 or 206 amino acids. VEGF-121 is more angiogenic and tumorigenic than are the 165 and 189 isoforms due to its ability to freely diffuse from the cells (1).

Angiogenesis requires two signals. The combination of Angiopoietin-2 and VEGF stimulate the loosening of support cells and the growth of newly exposed endothelial cells. Angiopoietin-2 blocks the Tie-2 signal, and in the presence of VEGF signal, induces angiogenesis. If Angiopoietin-2 inhibits the Tie-2 kinase in the absence of VEGF, the endothelium of the blood vessel separates from the peri-endothelial cells, the endothelial cells lose contact with each other, and the blood vessels regress. The building and remodeling of blood vessels is a critical event in the formation of every organ. The relationship between blood vessels and the tissue they serve is tightly balanced between stasis, growth and regression

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 (2a,2b & 2c). 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 rVEGF-MPCR kits have been designed to detect the expression of rat GAPDH, Tie-2, Cox-2, Angiopoietin-2, VEGF and Angiopoietin-1 genes. The PCR primers have similar  $T_m$  and no obvious 3'-end overlap to enhance multiple amplification. The 532 bp(GAPDH), 347 bp(Tie-2), 300 bp (Cox-2), 249 bp (ANG2), 199 bp (VEGF) and 168 bp (ANG1) PCR products can be generated from human RNA or the positive control, which is included in this kit. Therefore, the rVEGF-MPCR kit provides a quick and simple method to analyze rat Tie-2, Cox-2, Angiopoietin-2, VEGF and Angiopoietin-1 gene expression during angiogenesis.

# 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  $^{32}\text{P}$ , which is incorporated into the PCR products and then assayed for radioactivity (4).

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 (5), 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 (6).

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

<b>MPCR (Multiplex Polymerase Chain Reaction)</b>	<b>RPA (RNase Protection Assay)</b>
√ Non-isotope method with high sensitivity 0.1-1 $\mu\text{g}$ total RNA per MPCR	√ Isotope or Non-Isotope methods 1-20 $\mu\text{g}$ 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:

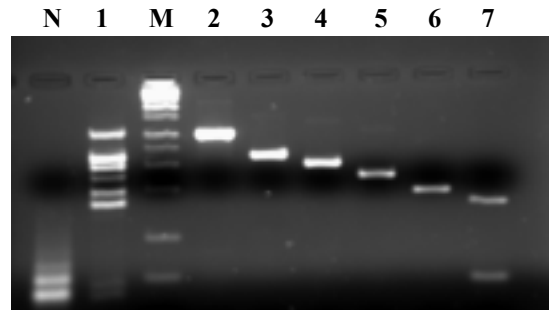
- 50X 50 $\mu$ L reaction kits
- 100X 50 $\mu$ L reaction kits

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 rVEGF1G Primers without positive (Negative)  
 Lane 1: PCR using rVEGF1G Primers  
 Lane 2: PCR using Rat GAPDH Primers  
 Lane 3: PCR using Rat Tie-2 Primers  
 Lane 4: PCR using Rat Cox-2 Primers  
 Lane 5: PCR using Rat ANG2 Primers  
 Lane 6: PCR using Rat VEGF Primers  
 Lane 7: PCR using Rat ANG1 Primers  
 Lane M: DNA M.W. Marker

## MPCR PRIMER INFORMATION

Product Code	Gene	5'/3' Tm	Amplicon Size	Accession No.	Intron Span	Genomic Size
rVEGF1G-GAP	Rat GAPDH	70°C/67°C	532bp	M17701	yes	817bp
rVEGF1G-TIE	Rat Tie-2	68°C/68°C	347bp	X71426,X67553	no	347bp
rVEGF1G-COX	Rat Cox-2	68°C/68°C	300bp	S67722	no	300bp
rVEGF1G-ANG2	Rat Angiopoietin-2	67°C/68°C	249bp	AF311728	no	249bp
rVEGF1G-VEGF	Rat VEGF	67°C/68°C	199bp	NM_031836	no	199bp
rVEGF1G-ANG1	Rat Angiopoietin-1	67°C/68°C	168bp	AY052399	no	168bp

## KIT COMPONENTS

### MP-70175

50X50 $\mu$ L MPCR reaction kit  
Store all reagents at -20°C

Product Code	Kit Component	Amount
rVEGF1G-B001	2X rVEGF1G MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 $\mu$ l
rVEGF1G-C001	10X rVEGF1G Pos. Control	50 $\mu$ l
rVEGF1G-P001	10X rVEGF1G MPCR Primers	250 $\mu$ l
MRB-0014	DNA M.W. Marker (100bp Ladder)	100 $\mu$ l
MRB-0011P	ddH <sub>2</sub> O (DNase free)	2.0 ml
	Instruction Manual	

### MP-70176

100X50 $\mu$ L MPCR reaction kit  
Store all reagents at -20°C

Product Code	Kit Component	Amount
rVEGF1G-B001	2X rVEGF1G MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 $\mu$ l X2
rVEGF1G-C001	10X rVEGF1G Pos. Control	50 $\mu$ l X2
rVEGF1G-P001	10X rVEGF1G MPCR Primers	250 $\mu$ l X2
MRB-0014	DNA M.W. Marker (100bp Ladder)	100 $\mu$ l X2
MRB-0011P	ddH <sub>2</sub> O (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 ( $1\mu\text{g}$  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  $0.5\text{-}1 \times 10^5$  cells (  $0.5\text{-}1\mu\text{g}$  cDNA derived from RNA) for each MPCR reaction.
2. Equilibrate 3 water baths:  $37^\circ\text{C}$ ,  $70^\circ\text{C}$  and  $95^\circ\text{C}$ .
3. **On ice**, pipet  $1\text{-}2\mu\text{g}$  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 ( $50\mu\text{M}$ ) or Oligo(dT) ( $50\mu\text{M}$ ).  
**NOTE:** The hexamer and Oligo(dT) RT reactions may be run simultaneously.
6. Incubate tube(s) at  $70^\circ\text{C}$  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	$250\text{mM}$ Tris-HCl (pH8.3) $375\text{mM}$ KCl, $15\text{mM}$ $\text{MgCl}_2$ , $50\text{mM}$ DTT	$10\mu\text{l}$
dNTPs	$1\text{mM}$ each	$20\mu\text{l}$
MMLV RT	$250\text{U}/\mu\text{l}$	$1\mu\text{l}$

8. Incubate the RT mixture at  $37^\circ\text{C}$  for 60 minutes.
9. Then, heat RT mixture at  $95^\circ\text{C}$  for 10 minutes and quickly chill on ice.
10. Add another  **$50\mu\text{l}$**  water or 0.1X TE buffer.
11.  **$2\text{-}5\mu\text{l}$**  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.

### 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 <sub>2</sub> O
50.0µl	Mineral Oil (optional)

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

B. EDTA concentration in test sample must not exceed 0.5 mM because Mg<sup>++</sup> concentration in MPCR Buffers is limited to certain ranges. Additional Mg<sup>++</sup> 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	

**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

---

---

1. Zhang, H.T. et al (2000) Br. J. Cancer 83: 63-68.
- 2a. Maxim Biotech Tools, 1, 2-5, 1995.
- 2b. Chamberlain, J.S. et al., In: *The polymerase chain reaction*. Mullis K, Ferre F and Gibbs R, eds. Birkhauser Boston Press, 38-46, 1994.
- 2c. Kumar, A. et al., (1997) Science 278, 1630-1632.
3. Chumakov, K.M. (1994), RT can inhibit PCR and stimulate primer-dimer formation. *PCR Methods and Applications*. 4: 62-64.
4. Hayashi, K., Orita, M., Suzuki, Y. & Sekiya, T. (1989) Nucleic Acids Res. 17:3605.
5. Landgraf, A., Reckmann, B., & Pingoud, A. (1991) Analytical Biochemistry 193:231.
6. Bloch, W. (1991) Biochemistry 30:2735.