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MPCR Kit for Mouse TGF-b Superfamily Genes
Cat No. MP-70192: 50 reactions
Cat No. MP-70193: 100 reactions

INSTRUCTION MANUAL

ID-M10046
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INTRODUCTION

Transforming growth factor-alpha (TGF-alpha) and transforming growth factor-beta (TGF-beta) have been implicated in diverse physiologic and pathophysiologic functions including immunological, inflammatory, and neoplastic processes. TGF-beta is one of the most potent immunoregulatory molecules known. It is a 25,000-dalton homodimeric protein, with three known isoforms in man, TGF-beta1, TGF-beta2 and TGF-beta3.

TGF-beta´s are synthesized and secreted by various transformed and normal cells including lymphocytes and monocytes. TGF-beta is also a multipotent growth factor affecting development, homeostasis, angiogenesis, and tissue repair. When added to wounds, TGF-beta accelerates the repair process. In addition, increased expression of TGF-beta has been reported in different malignancies, suggesting a role for this growth factor in tumorigenesis.

Differential expression of the isoforms of TGF-beta is controlled both *in vivo* and *in vitro*. Selective regulation of expression of the TGF-beta's include such factors as oncogenes and tumor suppressor genes. In addition to transcriptional control, TGF-betas appears to be regulated posttranscriptionally. TGF-beta1 has a special importance in immunoregulation, dramatically illustrated by early death in massive inflammation affecting every organ in the body of mice, without functional TGF-beta1. Expression of TGF-beta 3, but not of TGF-beta 2 or TGF-beta 1, was correlated strongly with disease progression. These data suggest that increased expression of TGF-beta isoforms, especially TGF-beta 3, may play a role in osteosarcoma progression (1, 2, 3).

Connective tissue growth factor (CTGF) is a novel peptide that exhibits platelet-derived growth factor-like activities and is secreted by fibroblasts after activation with transforming growth factor beta (TGF-beta). In mammal, CTGF functions as a downstream mediator of TGF-beta action on connective tissue cells, where it stimulates cell proliferation and extracellular matrix synthesis. Coordinate expression of TGF-beta and CTGF during wound repair suggests a cascade process for control of tissue regeneration. While CTGF does not appear to act on epithelial cells or immune cells, CTGF may serve as a more specific target for selective intervention in processes involving connective tissue formation during wound repair or fibrotic disorders. CTGF has been implicated in skin fibrosis and atherosclerosis. Hybridization studies have demonstrated that CTGF is coordinately expressed with TGF-beta in every fibrotic disorder examined to date (4, 5, 6).

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 (7). 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 (8, 9, 10). 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 or beta-actin, 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 (8, 9). Differences in gene expression can be determined by normalizing its expression against beta-actin or GAPDH expression.

This TGF superfamily MPCR kit has been designed to detect the expression of mouse TGF-beta-1, TGF-beta-2 , TGF-beta-3, TGF-beta Receptor 1, TGF-beta Receptor 2 and GAPDH genes. The PCR primers have similar Tm and no obvious 3'-end overlap to enhance multiple amplification. The 532bp (GAPDH), 429 bp (TGF-b R2), 370 bp (TGF-b R1), 321 bp (TGF-b3), 283 bp (TGF-b2), and 247 bp (TGF-b1) and PCR products can be generated from human RNA or the positive control, which is included in this kit. Therefore, this TGF MPCR kit provides a quick and simple method to analyze human TGF-b1, TGF-b2, TGF-b3, TGF-beta Receptor 1 and Receptor 2 genes expression, and normalize their expression against 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 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 (12).

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 (13), 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 (14).

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.

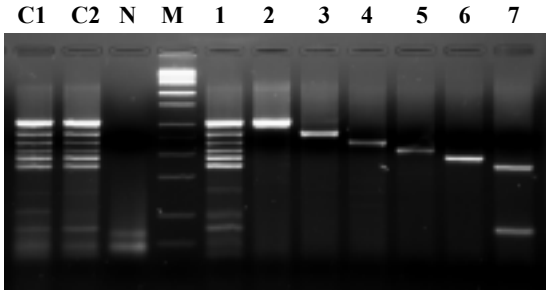
MPCR Kits come in two quantities:
- 50X 50µL reaction kits
- 100X 50µL reaction kits

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 C1:TGB1G MPCR Primers with Mouse Brain cDNA
Lane C2:TGB1G MPCR Primers with Mouse Brain cDNA with Enhancer
Lane N: TGB1G MPCR Primers without positive control (Negative)
Lane 1: TGB1G MPCR Primers with 1X positive control
Lane 2: GAPDH Primers with 1X positive control
Lane 3: TGBR2 Primers with 1X positive control
Lane 4: TGBR1 Primers with 1X positive control
Lane 5: TGB3 Primers with 1X positive control
Lane 6: TGB2 Primers with 1X positive control
Lane 7: TGB1 Primers with 1X positive control

MPCR PRIMER INFORMATION

Product Code	Gene	5'/3' Tm	Amplicon Size	Accession No.	Intron Span	Genomic Size
mTGFsG-TGB1	Mouse TGF-β1	68°C/68°C	247bp	BC013738	no	247bp
mTGFsG-TGB2	Mouse TGF-β2	67°C/67°C	283bp	NM_009367	no	283bp
mTGFsG-TGB3	Mouse TGF-β3	68°C/69°C	321bp	NM_009368	no	321bp
mTGFsG-TGBR1	Mouse TGB-R1	68°C/67°C	370bp	NM_009370	no	370bp
mTGFsG-TGBR2	Mouse TGB-R2	68°C/68°C	429bp	NM_009371	no	429bp
mTGFsG-GAP	Mouse GAPDH	66°C/65°C	532bp	M32599	no	532bp

KIT COMPONENTS

MP-70192

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

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

MP-70193

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

Product Code	Kit Component	Amount
mTGFsG-B001	2X mTGFsG MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 µl X2
mTGFsG-C001	10X mTGFsG Pos. Control	50µl X2
mTGFsG-P001	10X mTGFsG MPCR Primers	250µl X2
MRB-0014	DNA M.W. Marker (100 bp ladder)	100µl X2
MRB-0011P	ddH_2O (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 (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 μM) or Oligo(dT) (50 μM).
   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 $MgCl_2$, 50mMDTT	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.

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.

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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