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MPCR Kit for Rat TH1/TH2 Cytokines Set-2
Cat No. MP-70154: 50 reactions
Cat No. MP-70152: 100 reactions

**INSTRUCTION
MANUAL**

ID-M10039
Revised March 4, 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

The terms TH1 Cytokines (also referred to as T helper Type-1 cytokines) and TH2 cytokines (also referred to as T helper Type-2 cytokines) refer to the patterns of cytokines secreted by two different subpopulations of CD4 (+)T-cells which determine the outcome of an antigenic response toward humoral or cell-mediated immunity.

TH1 cells, which produce interferon (IFN)-gamma, interleukin (IL)-2, and tumor necrosis factor (TNF)-beta, evoke cell-mediated immunity and phagocyte-dependent inflammation. TH2 cells, which produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, evoke strong antibody responses (including those of the IgE class) and eosinophil accumulation, but inhibit several functions of phagocytic cells (phagocyte-independent inflammation). The different patterns of cytokine secretion correspond with different functions of immune effectors. TH1 cells promote cell-mediated effector responses. TH2 cells are mainly helper cells that influence B-cell development and augment humoral responses such as the secretion of antibodies, predominantly IgE, by B-cells. Both types of TH cells influence each other by the "cytokines" they secrete; IFN-gamma, for example, can downregulate TH2 clones while TH2 cytokines, such as IL-10, can suppress TH1 functions. IFN-gamma has been also shown to inhibit the proliferation of TH2 cells but not of TH1 helper T-lymphocyte clones. Thus, it appears that the TH1 and TH2 subsets are mutually antagonistic, such that the decision of which subset predominates within an infection may also determine its outcome (1,2,3,4).

The synthesis and secretion of IL-2 represents the early consequences of antigen or mitogen-induced activation of mature resting T cells. IL-2 interaction with its high-affinity receptor promotes clonal expansion of the effector T-cell population originally activated by antigen. IL-12 has multiple effects on T cells. In T-helper cells, IL-12 directly induces initial TH1 development and the production of IFN- γ secreting TH1 cells. From a humoral immunity standpoint, recombinant IL-12 has been shown to be a suppressor of IL-4 induced IgE production. Also, IL-12 has been hypothesized to play an early role in hematopoiesis.

IFN- γ is known to be both an inhibitor of viral replication and a regulator of many immunological functions. IFN- γ controls the class of antibody produced in B cells, up-regulates class I and II MHC complex antigens, and increases the efficiency of macrophage-mediated killing of intracellular parasites.

In T lymphocytes, IL-4 is an important immunomodulator for the differentiation of T helper cells to TH2 cells. TH2 cells mediate humoral immunity and assist in antibody production. IL-4 secretion by TH2 cells elicits humoral response by inducing the selective production of IgG, IgE and IgA isotypes. On B-lymphocytes, IL-4 regulates the expression of surface antigens, resulting in the enhancement of antigen-presenting capacity of B-cells.

IL-5 has a major role in the host as an eosinophil hematopoietic growth factor. IL-5 can also modify basophil function and plays a role in the pathogenic inflammatory responses of hypersensitivity and other diseases associated with eosinophil infiltration. IL-5 also induces stimulated B-cells to differentiate into Ig-secreting cells.

IL-8 is one of the inflammatory cytokines present in diseased periodontal tissues. The unrestricted production of such cytokines seems to play a role in chronic leukocyte recruitment and tissue destruction. IL-4 directly induces IL-8 release and amplifies the release of IL-8 in response to TNF- α . IL-13 is less active and IL-10 has an inhibitory effect. Epithelial cells are able to interact, therefore, with products of both TH1 and TH2 cells with respect to modulating release of IL-8.

IL-10 has a broad range of activity on a variety of cell types including both immunosuppressive and immunostimulatory cells. IL-10 produced in TH2 cells inhibits the production of cytokines, especially IFN- γ by Th1 cells responding to antigen. IL-10 is a potent down-regulator of cell-mediated immune responses, which results in potent anti-inflammatory activity.

IL-13 is a pleiotropic cytokine produced by activated TH2 cells. In humans, IL-13 brings about changes in the morphology and phenotype of monocytes by inducing expression of the IgE receptor and upregulating expression of MHC class II. IL-13 is involved in IgE switching and the induction of IL-4-independent IgG4 and IgE synthesis in the presence of T cells.

The production of IL-14 (also known as high molecular weight B-cell growth factor [BCGF]) by aggressive intermediate (diffuse large cell) lymphomas of the B-cell type non-Hodgkin's lymphoma (NHL-B) in patients with lymphomatous effusions. Autocrine or paracrine production of IL-14 may play a significant role in the rapid proliferation of aggressive NHL-B. Interrupting this pathway could be a useful goal of therapy for patients resistant to conventional chemotherapy.

TGF- β 1 is another exogenous factor which influences the development of undifferentiated CD4(+)T-cells towards either the TH1 or TH2 phenotype. To date, it has been difficult to develop a comprehensive picture of the effect of TGF- β 1 on T lymphocytes because TGF- β directly acts on T lymphocytes and indirectly by regulating the function of antigen-presenting cells. TGF- β is recognized as an antiapoptotic survival factor for T lymphocytes. The effect of TGF- β on T lymphocytes is shown to strongly depend on their stage of differentiation and on the cytokine milieu. TGF- β cannot be classified as a classical TH1 or TH2 cytokine. However, the existence of the TGF- β -producing TH3 subset was recently described which may play an important regulatory role during an immune response.

INTRODUCTION Continued

TNF-alpha is one of the TH2 Cytokines. Among the TH2 cytokines, there was a significant increase in the levels of IL-6, IL-10, and TNF alpha in both patient groups: IL-10 and TNF alpha values were significantly raised in patients with dual HIV and candida infections as compared to the other patients. There was no difference in IL-4 values across the subject groups. A positive correlation between CD4 cell counts and TH1 cytokine levels and a negative correlation with TH2 cytokines were noted. Such correlation were stronger in-patients with both HIV and candidiasis. Thus, HIV infected patients showed a TH1/TH2 cytokine imbalance with CD4 cell count reduction.

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 (9, 10, 11). 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 Rat TH1/TH2 Cytokine Set 2 (rTH12S2G) MPCR kits have been designed to detect the expression of Rat cytokines: IFN-g, IL-2, IL-4, IL-5, IL10, IL-12p40 and IL-13. The PCR primers have similar Tm and no obvious 3'-end overlap to enhance multiple amplification. The 658 bp (GAPDH), 525 bp (IL-10), 439 bp (IL-2), 381 bp (IL-4), 328 bp (IL-5), 283 bp (IFN-g), 238 bp (IL-12p40) and 201 bp (IL-13) PCR products can be generated from Rat RNA, or the positive control which is included in this kit. Therefore, the rTH12S2G MPCR kit provides a quick and simple method to analyze Rat IFN-g, IL-2, IL-4, IL-5, IL-10, IL-12P40 and IL-13 gene 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 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 (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 digoxigenin-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 (15).

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 0.1-1 μg 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:

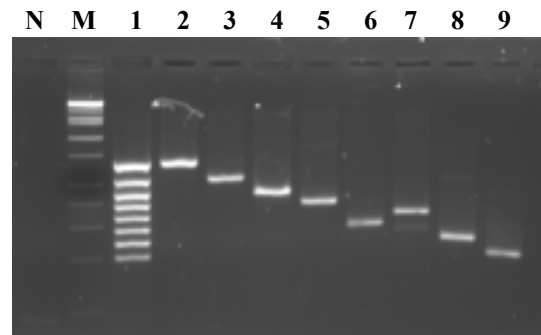
- 50X 50 μ L reaction kits
- 100X 50 μ L reaction kits

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 rTH12S2G Primers without positive (Negative)

Lane 1: PCR using rTH12S2G Primers with positive control

Lane 2: PCR using Rat GAPDH Primers with positive control

Lane 3: PCR using Rat IL-10 Primers with positive control

Lane 4: PCR using Rat IL-2 Primers with positive control

Lane 5: PCR using Rat IL-4 Primers with positive control

Lane 6: PCR using Rat IL-5 Primers with positive control

Lane 7: PCR using Rat IFN-gamma Primers with positive control

Lane 8: PCR using Rat IL-12 Primers with positive control

Lane 9: PCR using Rat IL-13 Primers with positive control

Lane M: DNA M.W. Marker

MPCR PRIMER INFORMATION

Product Code	Gene	5'/3' Tm	Amplicon Size	Accession No.	Intron Span	Genomic Size
rTH12S2G-IFG	Rat IFN- γ	66°C/70°C	283bp	AF010466	no	283bp
rTH12S2G-IL2	Rat IL-2	69°C/66°C	439bp	M22899	no	439bp
rTH12S2G-IL4	Rat IL-4	66°C/68°C	381bp	X16058	no	381bp
rTH12S2G-IL5	Rat IL-5	66°C/68°C	328bp	X54419	no	328bp
rTH12S2G-IL13	Rat IL-13	71°C/67°C	201bp	L26913	no	201bp
rTH12S2G-IL12	Rat IL-12	68°C/70°C	238bp	U16674	no	238bp
rTH12S2G-IL10	Rat IL-10	70°C/67°C	525bp	L02926	no	525bp
rTH12S2G-GAP	Rat GAPDH	68°C/68°C	658bp	M17701	yes	1252bp

KIT COMPONENTS

MP-70154

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

Product Code	Kit Component	Amount
rTH12S2G -B001	2X rTH12S2G MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 μ l
rTH12S2G -C002	10X rTH12S2G MPCR Pos. Control	50 μ l
rTH12S2G -P002	10X rTH12S2G MPCR Primers	250 μ l
MRB-0014	DNA M.W. Marker (100bp Ladder)	100 μ l
MRB-0011P	ddH ₂ O (DNase free)	2.0 ml
	Instruction Manual	

MP-70152

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

Product Code	Kit Component	Amount
rTH12S2G -B001	2X rTH12S2G MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 μ l X2
rTH12S2G -C002	10X rTH12S2G MPCR Pos. Control	50 μ l X2
rTH12S2G -P002	10X rTH12S2G MPCR Primers	250 μ l X2
MRB-0014	DNA M.W. Marker (100bp Ladder)	100 μ l X2
MRB-0011P	ddH ₂ 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 $0.5-1 \times 10^5$ 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 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	0.5 μl
5 X RT buffer	250mM Tris-HCl (pH8.3) 375mM KCl, 15mM MgCl_2 , 50mM DTT	10 μl
dNTPs	1mM each	20 μl
MMLV RT	250U/ μl	1 μl

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	<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)

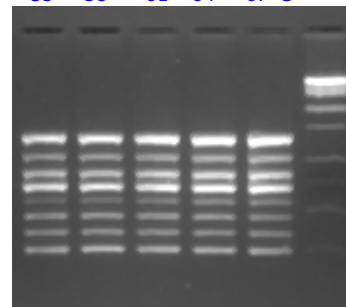
- *: ³²P dNTPs may be used here to achieve higher sensitivity and better quantitation. 5-10 µCi [³²P]dCTP (3000 Ci/mmmole) should be used here per MPCR. Keep final dNTPs concentration same as without ³²P-dNTPs.
- B. EDTA concentration in test sample must not exceed 0.5 mM because Mg⁺⁺ concentration in 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	

55 58 61 64 67°C



*The performance of MPCR kit against annealing temperatures. The above gel picture is an illustration of different annealing tepeatures on MPCR kit MP-70152.

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.	1.1a. Decrease PCR annealing temperature 3-5°C gradually.
	1.1b. Dominant primer dimers.	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.	1.2a. Increase PCR annealing temperature 3-5°C gradually.
	1.2b. Pre-PCR mispriming.	1.2b. Use any one of "Hot Start" PCR procedures.
	1.2c. cDNA is interfering with MPCR	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.	1.3a. Decrease PCR cycle number or decrease the input cDNA.
	1.3b. Variation in sample preparation, RT reaction and amounts of 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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