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**MPCR Kit for Respiratory Infection Associated Viruses Set-3**  
**Cat No. MP-70203: 50 reactions**  
**Cat No. MP-70202: 100 reactions**

**INSTRUCTION  
MANUAL**

**ID-M10086**  
**Revised February 24, 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

**Adenoviruses** commonly cause respiratory illness; however, depending on the infecting serotype, they may also cause various other illnesses, such as gastroenteritis, cystitis, and rash illnesses. Symptoms of respiratory illness caused by adenovirus infection range from the common cold syndrome to pneumonia, croup, and bronchitis. Patients with compromised immune systems are especially susceptible to severe complications of adenovirus infection.

**Respiratory syncytial virus (RSV)** is the most common cause of bronchiolitis and pneumonia among infants and children under 1 year of age. During their first RSV infection, between 25% and 40% of infants and young children have signs or symptoms of bronchiolitis or pneumonia. RSV also causes repeated infections throughout life, usually associated with moderate-to-severe cold-like symptoms. However, severe lower respiratory tract disease may occur at any age, especially among the elderly or among those with compromised cardiac, pulmonary, or immune systems.

**Influenza viruses (an Orthomyxovirus)** are a common cause of lower respiratory tract disease in young children and can also cause serious lower respiratory tract disease with repeated infection (e.g. pneumonia, bronchitis, and bronchiolitis) among the elderly and those with compromised immune systems. Human influenza viruses spread from respiratory secretions through close contact with infected persons or contaminated surfaces or objects. The influenza virus is a class of virus that contains RNA as its hereditary material. It replicates by entering a host cell and using host resources to produce hundreds of copies of viral RNA. Virions are roughly spherical and approximately 200nm in diameter. Influenza viruses are divided into three types, designated A, B, and C. Type C may not be a true influenza virus and usually causes only mild or asymptomatic disease. Influenza B viruses generally cause minor illness, but have the potential to cause more severe disease, while In-

fluenza A viruses cause pandemics. Recurrent outbreaks are common because the influenza A virus undergoes periodic antigenic shifts in its two outer membrane glycoproteins hemagglutinin (H) and neuraminidase (N), thus effectively introducing a new virus into a population that has no protective serum antibody. No different subtypes of H and N have been identified for influenza B and C.

**Human parainfluenza viruses (HPIVs)** are second only to **respiratory syncytial virus (RSV)** as a common cause of lower respiratory tract disease in young children. Similar to RSV, HPIVs can cause repeated infections throughout life, usually manifested by an upper respiratory tract illness (e.g. a cold and/or sore throat). HPIVs can also cause serious lower respiratory tract disease with repeat infection (e.g. pneumonia, bronchitis, and bronchiolitis), especially among the elderly, and among patients with compromised immune systems. Each of the four HPIVs has different clinical and epidemiological features. The most distinctive clinical feature of HPIV-1 and HPIV-2 is croup (i.e. laryngotracheobronchitis); HPIV-1 is the leading cause of croup in children, whereas HPIV-2 is less frequently detected. Both HPIV-1 and HPIV-2 can cause other upper and lower respiratory tract illnesses. HPIV-3 is more often associated with bronchiolitis and pneumonia.

Maxim's Viral MPCR Kits are designed to detect the expression of mouse immune interferon gamma, influenza A virus, adenovirus type 3, human parainfluenza virus 1, human parainfluenza virus 2, human parainfluenza virus 3, human respiratory syncytial virus, and human influenza B virus. The PCR primers have similar T<sub>m</sub> and no obvious 3'-end overlap to enhance multiple amplifications. The 284 bp (IFG), 191 bp (IFZA), 463bp (AAV), 530 bp (PAR1), 368 bp (PAR2), 285 bp (PAR3), 246 bp (RSV), and 318 bp (IFBZ) PCR products can be generated from human RNA or the positive control, which is included in this kit.

# 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 (10).

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 (11), 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 (12).

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:

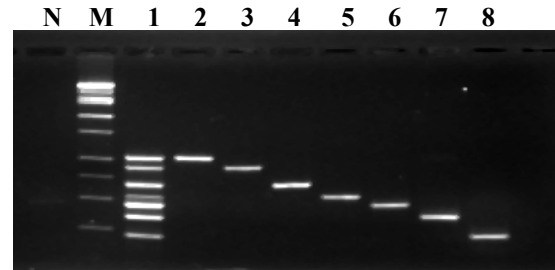
- 50X 50 $\mu$ L reaction kits
- 100X 50 $\mu$ L reaction kits

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 VIR3-P002 Primers without positive (Negative)
- Lane 1: PCR using VIR3-P002 Primers with 1X positive
- Lane 2: PCR using Parainfluenza virus 1 Primers
- Lane 3: PCR using Adenovirus Primers
- Lane 4: PCR using Parainfluenza virus 2 Primers
- Lane 5: PCR using Influenza Virus B Primers
- Lane 6: PCR using Parainfluenza virus 3 Primers
- Lane 7: PCR using RSV Primers
- Lane 8: PCR using Influenza Virus A Primers
- Lane M: DNA M.W. Marker

## MPCR PRIMER INFORMATION

Product Code	Gene	5'/3' Tm	Amplicon Size	Accession No.	Intron Span	Genomic Size
hVIR3G-IFZA	Influenza Virus	68°C/67°C	191bp	AF255370	no	191bp
hVIR3G-AAV	AAV Virus	66°C/67°C	463bp	AJ272604	no	463bp
hVIR3G-PAR1	PAR 1 Virus	67°C/68°C	530bp	X57213	no	530bp
hVIR3G-PAR2	PAR 2 Virus	66°C/66°C	368bp	X57559	no	368bp
hVIR3G-PAR3	PAR 3 Virus	66°C/67°C	285bp	NC_001796	no	285bp
hVIR3G-RSV	RSV Virus	67°C/67°C	246bp	M11486	no	246bp
hVIR3G-IFZB	Influenza Virus	66°C/67°C	318bp	NC_002204	no	318bp

## KIT COMPONENTS

### MP-70203

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

Product Code	Kit Component	Amount
VIR3-B001	2X Virus MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 $\mu$ l
VIR3-C002	10X Virus Pos. Control	50 $\mu$ l
VIR3-P002	10X Virus MPCR Primers	250 $\mu$ l
MRB-0014	DNA M.W. Marker (100 bp ladder)	100 $\mu$ l
MRB-0011P	ddH <sub>2</sub> O (DNase free)	2.0ml
	Instruction Manual	

### MP-70202

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

Product Code	Kit Component	Amount
VIR3-B001	2X Virus MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 $\mu$ l X2
VIR3-C002	10X Virus Pos. Control	50 $\mu$ l X2
VIR3-P002	10X Virus MPCR Primers	250 $\mu$ l X2
MRB-0014	DNA M.W. Marker (100 bp ladder)	100 $\mu$ l X2
MRB-0011P	ddH <sub>2</sub> 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:  $37^\circ\text{C}$ ,  $70^\circ\text{C}$  and  $95^\circ\text{C}$ .
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  $70^\circ\text{C}$  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	130U/ $\mu\text{l}$	$0.5\mu\text{l}$
5 X RT buffer	250mM Tris-HCl (pH8.3) 375mM KCl, 15mM $\text{MgCl}_2$ , 50mM DTT	$10\mu\text{l}$
dNTPs	1mM each	$20\mu\text{l}$
MMLV RT	250U/ $\mu\text{l}$	$1\mu\text{l}$

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

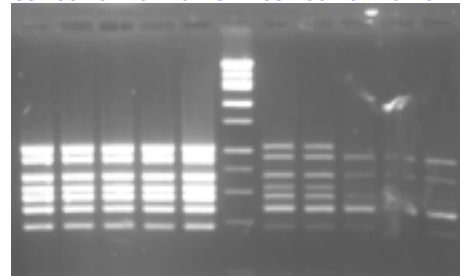
- \*: <sup>32</sup>P dNTPs may be used here to achieve higher sensitivity and better quantitation. 5-10 uCi [<sup>32</sup>P]dCTP (3000 Ci/mmmole) should be used here per MPCR. Keep final dNTPs concentration same as without <sup>32</sup>P-dNTPs.
- B. EDTA concentration in test sample must not exceed 0.5 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
<b>58-60°C*</b>	4 min	
94°C	1 min	28-35X
<b>58-60°C*</b>	2 min	
70°C	10 min	1X
25°C	soak	

55 58 61 64 67°C    55 58 61 64 67°C



Positive control from the kit                      Viruses cDNA

\*The performance of MPCR kit against annealing temperatures. The above gel picture is an illustration of different annealing temperatures on MPCR kit MP-70202.

**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

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### 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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