Dual Step RT PCR Kit

 # G7114 50 reactions

Store at -20^{0}C

Kit Contents	Amount
Max M-MLV Reverse Transcriptase (M-MLV RT) (200u/µl)	50 ul
10X M-MLV RT Buffer with $MgCl_2$	100 ul
10 mM dNTP mix	50 ul
Oligo dT (50uM)	50ul
Random Hexamers (50uM)	50ul
2X Hi G9 Taq PCR Master Mix (MM)	1.25 ml
Sterile, DEPC water	1.5 ml

Description

The dual step RT-PCR kit is designed for the sensitive detection of mRNAs in a two-step process. Each reaction is optimized for maximum results leading to greater sensitivity and higher yield. Multiple transcripts can be detected from a single first strand cDNA synthesis. Semi-quantitative analysis of mRNA levels can be achieved by agarose gel electrophoresis. In the 1st step, M-MLV RT is used with random primer, oligodT or gene-specific primer for generation of cDNA from RNA sample. In the second step, PCR amplification is performed in a separate tube using gene-specific primers. A ready-to-use 2X Hi G9 Taq PCR MM is provided for its convenient and consistent amplification performance.

Max M-MLV RT Storage Buffer

The enzyme is supplied in: 50mM Tris-HCl (pH 7.6), 150mM NaCl, 0.1mM EDTA, 1mM DTT, 0.1% (v/v) Nonidet P40, 50% glycerol (v/v
Max M-MLV RT 10X Reaction Buffer
500 mM Tris-HCl, pH 8.0; 750 mM KCl; 40 mM $MgCl_2$; 100 mM DTT.

Unit Definition

One unit incorporates 1 nmole of dTTP into acid-precipitable material in 10 min at 37°C using poly(A)•oligo(dT) as template-primer.
2X Hi G9 Taq PCR Master Mix contents

The Master Mix contains Hi G9 Taq DNA polymerase with reaction buffer, 5mM $MgCl_2$, and 0.4mM of each dNTP. The amount of enzyme will be 1 Unit in 50 µl reactions finally. When cloning fragments amplified with Hi G9 Taq DNA Polymerase an extra adenine overhang is incorporated at the 3' end of the amplified PCR product. The 'A' overhang is required to perform the TA cloning.

Protocol

For 1st Strand cDNA synthesis
A 20-µl reaction volume can be used for 1 ng–5 µg of total RNA or 1–500 ng of mRNA.
1. Add the following components to a nuclease-free microcentrifuge tube:

Heat mixture to 65°C for 5 min and quick chill on ice. Collect the contents of the tube by brief centrifugation and add:

Component	Amount
Oligo (dT)12-18 (500 µg/ml), or random primers, or gene-specific primer	1 µl 50–250 ng 2 pmole
Total RNA or mRNA	1ng - 5µg (total RNA) 1ng – 500ng (mRNA)
dNTP mix, 10 mM	1 µl
Sterile, DEPC water	Upto 12 µl

Heat mixture to 65°C for 5 min and quick chill on ice. Collect the contents of the tube by brief centrifugation and add:

- 2 µl 10X Max M-MLV RT Buffer with $MgCl_2$
- 4 µl nuclease-free water
- 1µl Ribonuclease Inhibitor (40 units/µl) ((Cat # G4648))

Mix contents of the tube gently and incubate at 25°C for 5 min.

2. Add 1 µl (200 units) of Max M-MLV RT and mix by pipetting gently. If using random primers, incubate tube at 25°C for 10 min.

3. Incubate 60 min at 42°C.

4. Inactivate the reaction by heating at 70°C for 15 min.

The cDNA can now be used as a template for amplification in PCR. It is recommended to use not more than 10% volume of cDNA of the final PCR reaction volume (i.e. 5ul/50 ul).

For PCR Amplification
Table 1. Pipetting instructions (add in this order).

Component	50 µl reaction	Final conc.
H_2O	To 50µl	
2X Hi G9 Taq PCR MM	25 µl	1X
Forward primer	X µl	0.5µM
Reverse Primer	X µl	0.5µM
cDNA	X µl	-

Table 2. Cycling instructions

Cycle step	3-step protocol		Cycles
	Temp.	Time	
Initial denaturation	94^{0}C	5min	1
Denaturation	94^{0}C	15-30sec	25-35
Annealing	45-63^{0}C	15-60sec	
Extension	72^{0}C	1kb/60sec	
Final extension	72^{0}C	10 min	1
Store	4^{0}C	Hold	

Troubleshooting

No product at all or low yield

- Optimize annealing temperature.
- Repeat and make sure that there are no pipetting errors.
- Make sure the cycling protocol was performed as recommended.
- Use fresh high-quality dNTPs.
- Do not use dNTP mix or primers that contain dUTP or dITP.
- Titrate template amount. Template DNA may be damaged. Use freshly prepared template.
- Increase extension time.Increase the number of cycles.
- Titrate DMSO (2−8 %) in the reaction.
- Denaturation temperature may be too low. Optimal denaturation temperature for most templates is 94-98°C.
- Optimize denaturation time.
- Check the purity and concentration of the primers.
- Check primer design.

Non-specific products - High molecular weight smears

- Make sure the extension time used was not too long.
- Reduce the total number of cycles.
- Increase annealing temperature or try 2-step PCR protocol.
- Titrate template amount.
- Optimize denaturation temperature.
- Decrease primer concentration.

Non-specific products - Low molecular weight discrete bands

- Increase annealing temperature
- Make sure the extension time used was not too long.
- Titrate template amount.
- Decrease primer concentration.
- Design new primers.