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Product Information

# HiPer® RT-PCR Teaching Kit

**Product Code: HTBM024**

**Number of experiments that can be performed: 5**

**Duration of Experiment:**
**Protocol: 4 hours**
**Agarose Gel Electrophoresis: 45 minutes**

**Storage Instructions:**

- The kit is stable for 6 months from the date of manufacture
- Store DNA Ladder, all the RT-PCR and PCR reagents at -20°C
- Store 6X Dye at 2-8°C
- Other reagents can be stored at room temperature (15-25°C)

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## Aim:

To amplify a specific RNA fragment by Reverse Transcription Polymerase Chain Reaction or RT- PCR.

## Introduction:

Reverse transcription polymerase chain reaction or RT-PCR is a modified version of PCR. This is a two step process where RNA strand is first reverse transcribed to complementary DNA (cDNA) by the enzyme reverse transcriptase and the resulting cDNA is amplified by the traditional PCR. In RT-PCR a pair of primers are used which are complementary to a defined sequence on each of the two strands of the cDNA. These primers (annealed to the cDNA) are then extended by a DNA polymerase and a copy of the strand is made after each cycle which leads to the exponential amplification. It is a very sensitive method and is used to generate large cDNA libraries from very small amount of mRNA and in gene expression studies.

## Principle:

The purpose of converting mRNA to cDNA is mainly for the analysis of the template mRNA because DNA is much stable than RNA. Once mRNA is converted to cDNA, the cDNA can be used for RT-PCR, as probe for expression analysis and for cloning of the mRNA sequence. In RT-PCR three major steps are included. The first step is reverse transcription where cDNA (complementary DNA) is synthesized *in vitro* from an mRNA template by an enzyme that has reverse transcriptase activity. Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) is an RNA-dependent DNA polymerase that can be used in cDNA synthesis. This step is very important in order to perform PCR since DNA polymerase can act only on DNA templates. The resulted cDNA is single-stranded and this process is called reverse transcription (RT) or first strand cDNA synthesis. During this step mRNA is copied to cDNA by reverse transcriptase using an oligo dT primer or random oligomers or both.

During the second step of RT-PCR, the cDNA is amplified through a standard PCR by using a gene specific primer and a thermostable DNA polymerase. In this step cDNA is denatured at more than 90$^{o}$ C so that the two strands separate and the gene specific primers can bind. The temperature is decreased until it reaches the annealing temperature and gene specific primers are annealed that are complementary to a site on each strand. The annealing temperature can vary upon the primers used. The temperature of the reaction is raised to 72$^{o}$ C at which the thermostable Taq DNA polymerase works optimally. Finally, the PCR product is detected using agarose gel electrophoresis and ethidium bromide.

The exponential amplification through RT-PCR is a very sensitive method in which a very low copy number of RNA molecules can be detected. It is widely used in the diagnosis of genetic diseases and in the determination of the abundance of specific different RNA molecules within a cell or tissue as a measure of gene expression. Due to the fact that most eukaryotic genes contain introns which are present in the genome but not in the mature mRNA, the cDNA generated from a RT-PCR reaction is the exact DNA sequence which would be directly translated into a protein after transcription. Prokaryotic cells lack the mRNA splicing mechanism and when these genes are expressed in prokaryotic cells for protein purification, the RNA produced directly from transcription need not undergo splicing as the transcript contains only exons.

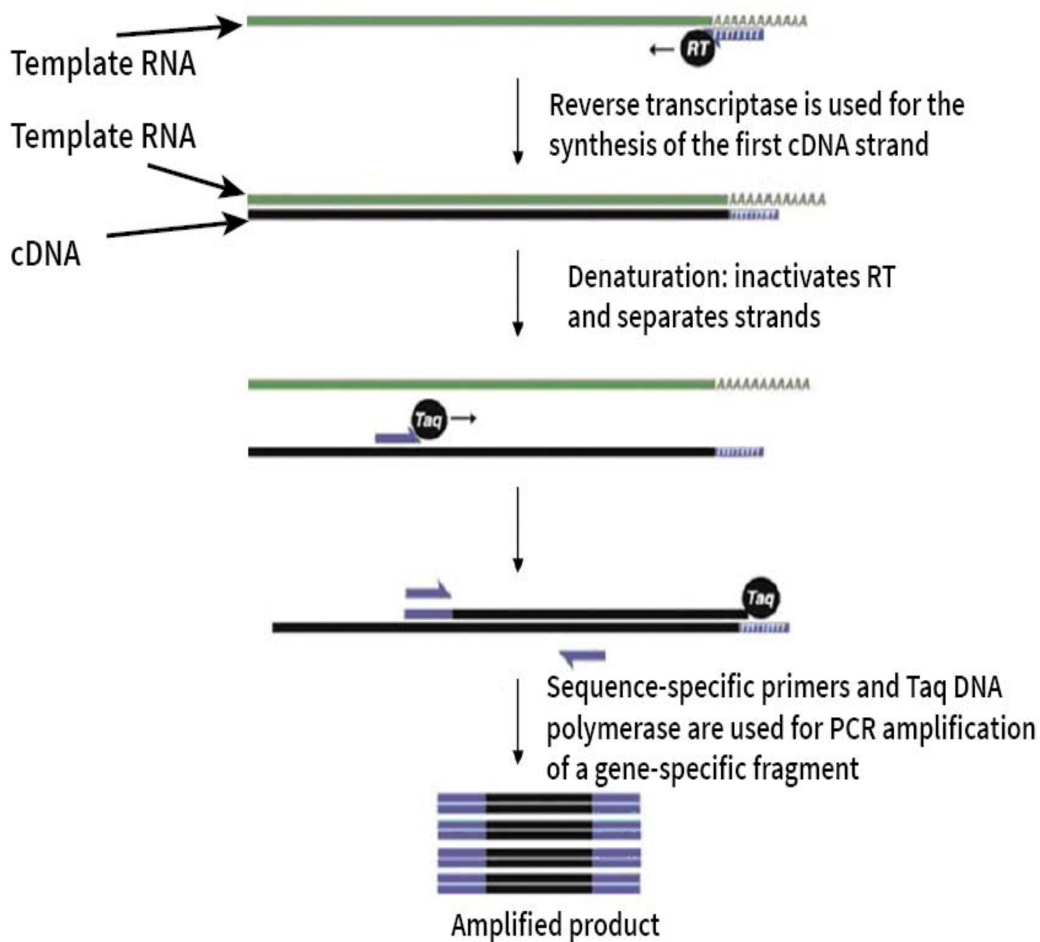



**Fig 1: Schematic representation of the amplification of RNA by RT-PCR**

## Kit Contents:

The kit can be used to reverse transcribe a RNA into its cDNA by using a reverse transcriptase and subsequent amplification of the cDNA using conventional PCR.

**Table 1: Enlists the materials provided in this kit with their quantity and recommended storage**

| Sr. No. | Product Code | Materials Provided | Quantity | Storage |
|---|---|---|---|---|
| | | | 5 expts | |
| 1 | *TKC306 | Total RNA | 0.030 ml | -20$^0$C |
| 2 | *DS0279 | RT Buffer for MMuLV | 0.025 ml | -20$^0$C |
| 3 | *DS0280 | 10X Solution for MMuLV | 0.012 ml | -20$^0$C |
| 4 | *TKC117 | 10X Assay Buffer | 0.060ml | -20$^0$C |
| 5 | *TKC118 | 2.5 mM dNTP mix | 0.060ml | -20$^0$C |
| 6 | *TKC119 | 25 mM $MgCl_2$ | 0.060ml | -20$^0$C |
| 7 | *TKC120 | Taq DNA Polymerase | 0.007ml | -20$^0$C |
| 8 | ML065 | Molecular Biology Grade Water | 0.5 ml | -20$^0$C |

|  |  | for PCR |  |  |
|---|---|---|---|---|
| 9 | *MBT059 | 100mN dNTP Mix | 0.012 ml | -20⁰C |
| 10 | *DS0146 | Random Hexamer | 0.006 ml | -20⁰C |
| 11 | *MBT072 | M-MuLV Reverse Transcriptase | 0.006 ml | -20⁰C |
| 12 | MB002 | Agarose | 3 g | RT |
| 13 | ML016 | 50X TAE | 60 ml | RT |
| 14 | ML015 | 6X Gel Loading buffer | 0.04 ml | 2-8 ⁰C |
| 15 | CG282 | Polypropylene Tubes, 0.2ml (PCR tubes) | 15 Nos. | R T |
| 16 | *TKC312 | Forward Primer (10 nM) | 0.015 ml | -20⁰C |
| 17 | *TKC313 | Reverse Primer (10 nM) | 0.015 ml | -20⁰C |
| 18 | *MBT049 | I00 bp DNA Ladder | 0.02 ml | -20⁰C |

*** Always give a quick spin before opening the vial as the liquid material may stick to the wall or to the cap of the vial.**

## Materials Required But Not Provided:

**Glasswares:** Measuring cylinder, Beaker
**Reagents:** Ethidium bromide (10 mg/ml), Molecular Biology Grade Water
**Other requirements:** Thermocycler, Electrophoresis apparatus, UV Transilluminator, Vortex Mixer, Micropipettes, Tips, Adhesive tape, Microwave/ Hotplate/ Burner, Crushed ice, water bath

## Storage:

HiPer® RT-PCR Teaching Kit is stable for 6 months from the date of manufacture without showing any reduction in performance. Store 100 bp DNA Ladder, all RT-PCR and PCR reagents at -20ºC. On receipt, store 6X Dye at 2-8ºC. Other reagents can be stored at room temperature (15-25ºC).

## Important Instructions:

- Read the entire procedure carefully before starting the experiment.
- Keep all the solutions in the icebox during use.
- The 1 Kb DNA ladder supplied in the kit is ready to use and can be directly loaded onto the agarose gel.

## Procedure:

### I. Preparation of cDNA

**Step 1**

1. Take one PCR tube and add the following ingredients:

| Sr. No. | Ingredients | Volume in µl |
|---|---|---|
| 1 | Random Hexamer | 1 µl |
| 2 | 100mM dNTP mix | 2 µl |
| 3 | Total RNA | 5 µl |
| 4 | Molecular Biology Grade Water | 2 µl |

2. Incubate the tubes at 65ºC for 5 minutes.
3. After incubation chill the tubes on ice for 1 minute.

**Step 2**

4. Prepare the reaction mixture in a total volume of 20 µl

| Sr. No. | Ingredients | Volume in µl |
|---|---|---|
| 1 | Template RNA Primer mixture (from Step 1) | 10 µl |
| 2 | RT Buffer for MMuLV | 4 µl |
| 3 | 10X solution for MMuLV | 2 µl |
| 4 | M-MULV Reverse Transcriptase | 1 µl |
| 5 | 100mM dNTP mix | 2 µl |
| 6 | Molecular Biology Grade Water | 1 µl |

5. Gently mix and ensure that all the components are at the bottom of the tube. Centrifuge briefly if needed.
6. Incubate the tube at following condition in a thermo cycler.

- 25°C for 5 minutes – 1 cycle
  42°C for 60 minutes – 1 cycle
  70°C for 5 minutes – 1 cycle
  Hold at 4°C - optional

7. The cDNA can be further used to perform PCR assay

**II) Preparation of master mix for PCR**

1) Take two PCR tubes and label them as **Negative PCR** and **Positive PCR**. To the respective tubes add the following ingredients:

**Negative PCR**

| Sr. No. | Ingredients | Volume in µl |
|---|---|---|
| 1 | 10X Assay Buffer | 5 µl |
| 2 | 25 mM $MgCl_2$ | 5 µl |
| 3 | 10mM dNTP Mix | 5 µl |
| 4 | Taq DNA Polymerase | 0.5 µl |
| 5 | Forward Primer (10 nM) | 1 µl |
| 6 | Reverse Primer (10 nM) | 1 µl |
| 7 | Molecular Biology Grade Water | 32.5 µl |
| | **Total volume** | **50 µl** |

**Positive PCR**

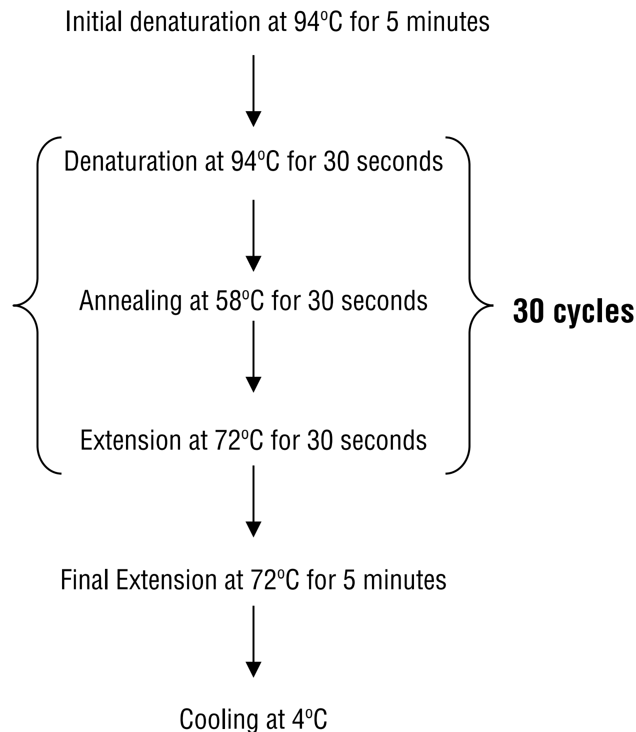
| Sr. No. | Ingredients | Volume in µl |
|---|---|---|
| 1 | 10X Assay Buffer | 5 µl |
| 2 | 25 mM $MgCl_2$ | 5 µl |
| 3 | 10mM dNTP Mix | 5 µl |
| 4 | Taq DNA Polymerase | 0.5 µl |
| 5 | Forward Primer (10 nM) | 1 µl |
| 6 | Reverse Primer (10 nM) | 1 µl |
| 7 | Molecular Biology Grade Water | 30.5 µl |
| 8 | cDNA | 2 µl |
| | **Total volume** | **50 µl** |

2) Tap the tubes for 1– 2 seconds to mix the contents thoroughly.

3) Place the tube in the thermocycler block and set the program to get DNA amplification.

**PCR Amplification Cycle:**

Carry out the amplification in a thermocycler for 30 cycles using the following reaction conditions.



## Agarose Gel Electrophoresis:

**Preparation of 1X TAE:** To prepare 500 ml of 1X TAE buffer, add 10 ml of 50X TAE Buffer to 490 ml of sterile distilled water*. Mix well before use.

**Preparation of agarose gel:** To prepare 50 ml of 1% agarose gel, add 0.5 g agarose to 50ml of 1X TAE buffer in a glass beaker or flask. Heat the mixture on a microwave or hot plate, swirling the glass beaker/flask occasionally, until agarose dissolves completely (Ensure that the lid of the flask is loose to avoid buildup of pressure). Allow the solution to cool down to about 55-60ºC. Add 0.5µl Ethidium bromide, mix well and pour the gel solution into the gel tray. Allow the gel to solidify for about 30 minutes at room temperature.

**Loading of the DNA samples:** Load 3 µl of ready to use DNA ladder into the first well. Add 2 µl of 6X gel loading buffer to 10 µl of RT-PCR product. Load the RT-PCR samples into the following wells.

**Electrophoresis:** Connect the power cord to the electrophoretic power supply according to the conventions: Red-Anode and Black-Cathode. Electrophorese at 100-120 volts and 90 mA until dye markers have migrated an appropriate distance, depending on the size of DNA to be visualized.

* Molecular biology grade water is recommended (Product code: ML024).

## Observation and Result:

After completion of the PCR, perform agarose gel electrophoresis. Compare the amplified product with the ladder and determine its size.

Lane 1: 100bp DNA Ladder

Lane 2: RT-PCR Product (Negative)

Lane 3: RT-PCR Product (Positive)

## Interpretation:

After performing agarose gel electrophoresis, one can check the amplification of a specific RT-PCR product in the positive tube and no amplified product in the negative tube. The optimized conditions result in the amplified RT-PCR product of desired size.

## Troubleshooting Guide:

| Sr. No. | Problem | Possible Cause | Solution |
|---|---|---|---|
| 1 | No amplification observed | Incorrect setup of RT reaction | Be sure to set up the reaction on ice |
| | | Missing reagent when setting up reverse-transcription reaction | Mix all reagents well after thawing, and repeat the reverse-transcription reaction |
| 2 | Poor amplification yield | Template or dNTPs may be degraded, enzymes may have been inactive | Store the kit at -20ºC and avoid repeated freeze thaw. Also keep all the materials in ice while performing the experiment |
| | | Thermocycler operation or program is improper | Ensure proper functioning of Thermocycler. Run positive control with every reaction |
| | | Inadequate mixing of the reaction tube | Mix the reaction mixture using a micropipette, avoid air bubble |

## Technical Assistance:

At HiMedia we pride ourselves on the quality and availability of our technical support. For any kind of technical assistance, mail at mb@himedialabs.com

Storage temperature

Do not use if package is damaged

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