

# REVERSE TRANSCRIPTION -POLYMERASE CHAIN REACTION (RT-PCR)

## General Information

Reverse transcription polymerase chain reaction (RT-PCR) is a process where DNA is first reverse transcribed from ribonucleic acid (RNA) to complementary DNA (cDNA) and is subsequently amplified by PCR. RNA and cDNA are essential in molecular biology studies because they have the same coding genes (exons) as DNA but they do not contain introns that usually consist of house-keeping and non-coding genes.

RT-PCR amplifies minute amounts of all types of RNA (mRNA, rRNA, tRNA etc.). RT-PCR has been employed in various types of applications, for example generation of high fidelity cDNA products for cloning, probe synthesis, library construction and sequencing, quantification of gene expression, detection and quantification of infectious micro-organisms, identification of cancer cells and genetic disorders in clinical diagnostics. Changes in cellular activities concerning survival, growth and differentiation are reflected in altered patterns of gene expression. Hence, the quantification of transcription levels of specific genes has always been vital to any gene function related research. Recently, the emergence of molecular medicine has resulted in the increased use of techniques able to quantify levels of RNA in clinical diagnostics.

## Reverse Transcriptase

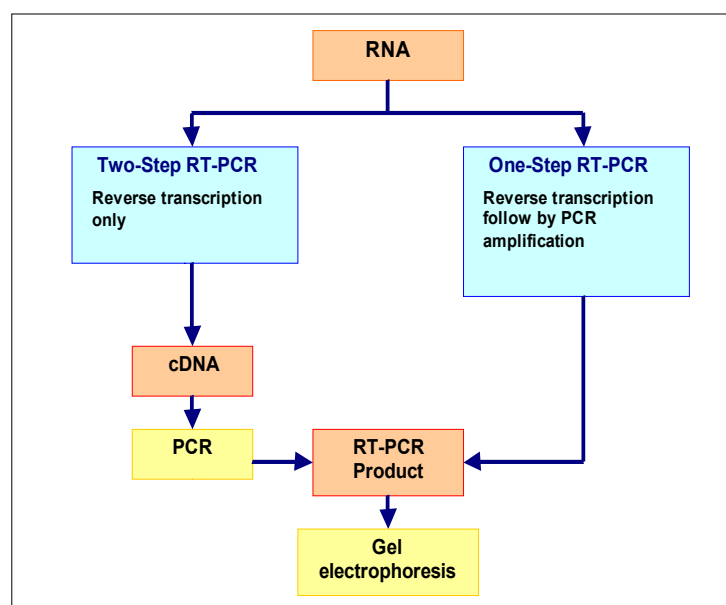
The emergence of RT-PCR is due to a shortcoming in PCR. A major limitation in conventional PCR is that it amplifies DNA templates only. It cannot amplify RNA in a similar manner. This problem can be overcome in RT-PCR by using a reverse transcriptase, which generates cDNA from RNA templates for subsequent amplification. Reverse transcriptases are enzymes synthesized naturally by retroviruses, for example the human immunodeficiency virus. The role of the reverse transcriptase is to generate DNA from viral RNA. The virus-derived DNA can then be inserted into the host's genome. In the laboratory, reverse transcriptase is used to convert RNA to cDNA that can then be used for multiple applications. There are several commonly used reverse transcriptases, including Avian Myeloblastosis Virus (AMV) reverse transcriptase, Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase or engineered enzymes that are able to enhance polymerase activity or decrease unwanted nuclease activities.

AMV reverse transcriptase is more robust than M-MuLV reverse transcriptase as it can retain significant polymerization activity up to 65°C. This is important if the template RNA has significant secondary structures. Cloned M-MuLV has been engineered to produce reverse transcriptase that is RNase H (Ribonuclease H) negative. RNase H is an enzyme that competes with the DNA polymerase for the hybrid formed between the RNA template and the growing cDNA strand and degrades the RNA strand. AMV has stronger intrinsic RNase H activity that makes it unsuitable for synthesizing long cDNAs (e.g. more than 5kb). Thus M-MuLV reverse transcriptase is suitable for generating longer cDNAs while AMV reverse transcriptase is suitable for synthesis of short cDNAs.

## Types of RT-PCR

There are three common types of RT-PCR: conventional RT-PCR, real-time RT-PCR and competitive RT-PCR. The initial step in conventional RT-PCR is the production of a single-strand cDNA copy of the RNA using reverse transcriptase followed by exponential amplification by PCR. The reaction can be performed in either one-step or two-step reactions. In the one-step reaction the reverse transcription reaction and PCR takes place sequentially in a single tube, whereas in the two-step reaction each step is performed under optimal conditions in separate tubes, where 10% of the reverse transcription reaction product is subjected to PCR cycling.

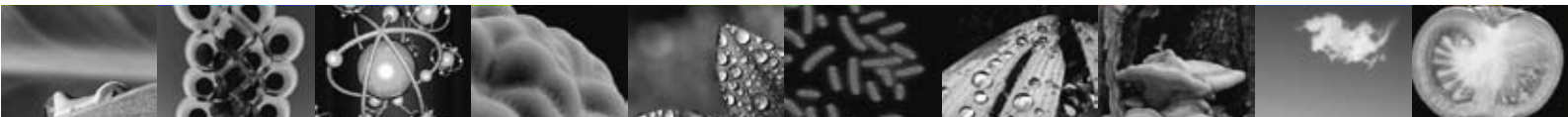
Figure 1 illustrates the flowchart of conventional RT-PCR. Two-step RT-PCR is popular and useful for detecting multiple targets from the same sample, whereas one-step RT-PCR is more advantageous for multiple samples, as it minimizes the carryover contamination. Generally, it is more efficient to use a two-step reaction rather than a one-step reaction as the two-step RT-PCR protocol may be more sensitive compared to the one-step protocol for amplifying cDNA targets. Gel electrophoresis is commonly used to confirm the identity of the PCR products, but sometimes Southern blotting and DNA sequencing or both methods may be required. The final step involves manual or automated analysis for quantifying the bands on the gel.



**Figure 1: Flow chart of conventional RT-PCR. Conventional RT-PCR can be either performed as a one step or two-step reaction. The resulting RT-PCR product is subjected to gel electrophoresis for identity confirmation.**

Real-time RT-PCR is a highly sensitive and versatile technique for amplification and quantification of RNA. The benefit of real-time PCR is that it allows the determination of the amount of starting DNA in the sample before PCR amplification starts. Present day real-time methods generally involve fluorogenic probes that emit light to indicate the amount of DNA present at each cycle of PCR. Several types of probes exist, including DNA-binding dyes like ethidium bromide (EtBr) or SYBR green I, hydrolysis probes (5'-nuclease probes) like TaqMan probe, hybridization probes, molecular beacons, sunrise and scorpion primers, and peptide nucleic acid (PNA) light-up probes. Each type of probe has its own unique characteristics, but all of them will generate the same outcome – fluorescence when DNA is amplified.

RT-PCR requires specific instrumentation to detect the fluorescent signal and record the progress, a thermocycler to carry out PCR and appropriate computer hardware and data-acquisition and analysis software. RT-PCR is able to quantify nucleic acids over a wide dynamic range, giving it its extreme sensitivity for the detection of less than five copies of a target sequence, making it possible to analyze small samples.



The relative amount of a cDNA generated by reverse transcription is proportional to the relative amount of its RNA template under the appropriate reaction conditions. The cDNA can then be used as raw material for real-time PCR, thereby determining changes in gene expression (e.g. RNA levels) based on its precision and sensitivity. RT-PCR is used frequently for two reasons: first, as a primary investigative tool to determine gene expression; second as a secondary tool to validate the results of DNA microarray. Slight changes in gene expression can be detected by real-time RT-PCR due to its precision and sensitivity. Thus real-time PCR can be used to assess both DNA and RNA levels with great sensitivity and precision.

In competitive RT-PCR, a dilution series of a DNA or RNA competitor is co-amplified with known amounts of total RNA or cDNA in the same reaction tube. The competitor has the same primer binding sites as the target sequence but is usually modified by introducing a small deletion, insertion, or mutation to distinguish it during electrophoresis. The competitor competes with the native sequence of the gene of interest for primers, deoxynucleotide triphosphates (dNTPs), enzyme, and other reagents, thus reducing the signal of the native gene when the amount of competitor is in excess. As the amount of the competitor increases, the signal of the native gene decreases. Co-amplifying a DNA competitor provides an efficient method of relating the product yield to the initial amount of transcript. Since both the competitor and target sequence of interest are presumed to be amplified with almost equal efficiency, the product will accumulate with approximately the same kinetics, even when the PCR reagents are limiting. In environmental microbiology, competitive RT-PCR is especially advantageous as some environmental samples may contain contaminants including organic solvents and humic acids that will inhibit PCR amplification.

## Two-step RT-PCR protocol

### Reverse transcription protocol using Vivantis AMV reverse transcriptase (ME2301 -04):

Components	VolumeFinal	Concentration
<b>Mixture A:</b>		
Total RNA or mRNA	variable	0.1-5.0µg
<b>Primer:</b>		
Specific primer or Random primer or Oligo(dT)	variable	15-20pmol  0.2µg  0.5µg
10mM dNTP mix	2µl	1mM of each dNTP
Nuclease free deionized water	up to 14.5µl	-
<b>Mixture B:</b>		
5X AMV-RT buffer	4µl	1X
RNase Inhibitor (50u/µl)	1µl	50 units
AMV-RT (35u/µl)	0.5µl	17.5 units
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<b>Total Volume</b>	<b>20.0µl</b>	
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1. Prepare reaction mixture A in a clean and sterile PCR tube.
2. Heat mixture A at 65°C in a water bath or thermocycler for 5 minutes and chill on ice immediately for 1-2 minutes.
3. Centrifuge briefly and add reaction mixture B.
4. Mix everything gently and incubate at 42°C for 45 minutes in a PCR machine.
5. Stop the reaction by heating at 85°C for 10 minutes.
6. Proceed with PCR reaction. The cDNA can be stored up to one week at -20°C. It is recommended to perform PCR right after the reverse transcription.
7. Use 2-4µl of cDNA to perform PCR.

### Reverse transcription protocol using Vivantis M-MuLV reverse transcriptase: (ME2305 - 06):

Components	VolumeFinal	Concentration
<b>Mixture A:</b>		
Total RNA or mRNA	variable	0.1-5.0µg
<b>Primer:</b>		
Specific primer or Random primer or Oligo(dT)	variable	15-20pmol  0.2µg  0.5µg
Nuclease free deionized water	up to 14µl	-
<b>Mixture B:</b>		
10X M-MuLV-RT buffer	2µl	1X
RNase Inhibitor (50u/µl)	1µl	50 units
10mM dNTP mix	2µl	1mM of each dNTP
<b>Add Separately:</b>		
M-MuLV-RT (250u/µl)	1µl	250 units
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<b>Total Volume</b>	<b>20.0µl</b>	
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1. Prepare reaction mixture A in a clean and sterile PCR tube.
2. Heat the mixture at 65°C for 5 minutes in a water bath or a thermocycler and chill on ice immediately for 1-2 minutes.
3. Centrifuge briefly and add reaction mixture B without adding M-MuLV-RT.
4. Incubate the reaction mixture at 37°C for 5 minutes in a PCR machine. If random primers are used, incubate at 25°C for 5 minutes. Do not remove the tube from PCR machine at the end of incubation.
5. Add 1µl of M-MuLV-RT into the tube which is in the PCR machine.
6. Incubate the mixture at 37°C for 60 minutes (for both oligo(dT) primers or specific primers). If using random primers, incubate at 25°C for 10 minutes and follow by 37°C for 60 minutes.
7. Stop the reaction by heating at 70°C for 10 minutes.
8. Proceed with PCR reaction. The cDNA can be stored up to one week at -20°C. It is recommended to perform PCR right after the reverse transcription.
9. Use 2-4µl of cDNA to perform PCR.

## Factors affecting RT-PCR

### Primers

Priming of RNA template for cDNA synthesis can be carried out using random primers, oligo(dT), a mixture of both or target-specific primers.

#### i) Random primers

This method yields the most cDNA, more than one cDNA transcript is produced per target since the cDNAs originate from multiple locations along the messenger RNA (mRNA). The major benefits of using random primers are the synthesis of shorter cDNA fragments and increased probability of the conversion of mRNA 5'-ends to cDNA. However, the reverse transcriptase is not usually able to reverse transcribe the 5'- end of long mRNAs especially when RNase H activity is present. Furthermore, the majority of cDNA synthesized from total RNA will mainly derive from ribosomal RNA (rRNA) and may compete with a target that is present at very low levels. As the annealing temperature (T<sub>m</sub>) of random primers is low, they cannot be used with thermostable reverse transcriptases.



## ii) Oligo(dT) primers

cDNA synthesis using oligo(dT) is more specific than random priming, as it will not result in the priming from rRNAs. It is the best method to use when the aim is to obtain cDNAs reverse transcribed from mRNAs, although it will not prime any RNAs that lack a polyadenylated (poly-A) tail. In addition, oligo(dT) priming requires very high-quality RNA that is in full length, and hence is not suitable for priming from fragmented RNAs. Furthermore, the reverse transcription may fail to reach the primer/probe-binding site if secondary structures exist or if the primer/probe-binding site is at the extreme 5'-end of a long mRNA. It is possible to mix random primers and oligo(dT). However, if applied in mRNA quantification, this may affect accurate quantification as the variable priming of the random oligonucleotides is likely to introduce variability.

## iii) Target-specific primers

Target-specific primers synthesize the most specific cDNAs and it is the most sensitive method available. The main disadvantage of these primers is that they require separate priming reactions for each target, which can be a disadvantage when limited amounts of RNA are available.

## iv) RNA quality and RT-PCR inhibitors

Naked RNA is extremely susceptible to degradation by endogenous RNases that are present in all living cells. Moreover, there are inhibitors present during RNA preparation that can inhibit RT-PCR. Common inhibitors include various components of body fluids and reagents present in clinical and forensic samples (e.g. haemoglobin and urea), food constituents (e.g. organic and phenolic compounds, and fats) and environmental compounds (e.g. humic acids and heavy metals). Factors such as DNA fragmentation will negatively affect PCR efficiency. Furthermore, traces of chemical from laboratory plasticware have been identified as one potential source of PCR inhibitors. Different polymerases display variable sensitivity to the presence of inhibitors such as blood, ions or biological samples. Thus the PCR-inhibiting effect of various components in biological samples can, to some extent, be eliminated by the use of the appropriate thermostable DNA polymerase.

## v) Magnesium concentration

Magnesium ion ( $Mg^{2+}$ ) is a divalent cation that is required for the enzymatic activity of AMV and M-MuLV reverse transcriptases and DNA polymerases.  $Mg^{2+}$  concentration affects product specificity, primer annealing, formation of primer-dimer artefacts, melting temperature, and DNA polymerase activity and fidelity. The sensitivity of the amplification reaction to  $Mg^{2+}$  concentration may be different depending on the nature and abundance of the target as well as the concentration of other reaction components.

## vi) dNTP concentration

dNTPs are required for reverse transcriptase and Taq DNA polymerase-mediated amplification. The final concentration of the dNTPs is important, low concentration may result in inefficient amplification whereas high concentration may result in dilution of other components in the PCR reaction mixture such as  $Mg^{2+}$ .

## RT-PCR Guidelines for beginners

### 1. Primer selection

There are two alternatives in performing RT-PCR: one-step or two-step reaction. In one-step reaction or single tube RT-PCR, PCR is performed subsequently after the reverse transcription without stopping the reaction. The primer used for reverse transcription is also the primer for PCR amplification, thus specific primers are generally used for one-step reaction. For the two-step reaction, where the reactions are being performed separately, the choice of primer for reverse transcription can be any of the three: oligo(dT) primer, random primer or specific primer.

### 2. Reverse transcriptase selection

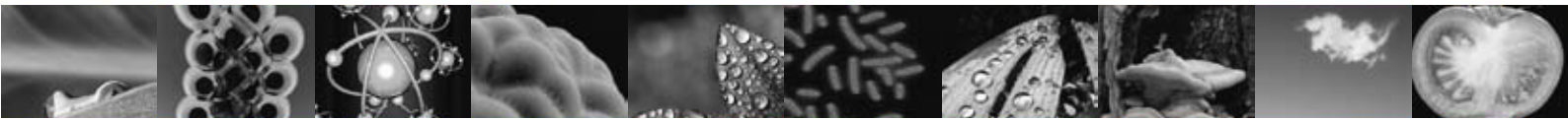
AMV is suitable for synthesis of short cDNAs as it has strong RNase H activity that degrades RNA in RNA:cDNA hybrids before the polymerization of the long cDNA strand is complete to allow primer binding and second-strand DNA synthesis. AMV is also suitable for reverse transcription of RNA templates that exhibit significant secondary structure and high GC-content as it displays optimal activity at high temperature, up to 65°C. M-MuLV is suitable for synthesis of long cDNA as it lacks of RNase H activity.

### 3. Effects of $Mg^{2+}$ and dNTP concentration

$Mg^{2+}$  and dNTPs can affect the PCR sensitivity and specificity. Studies have shown that there is a relationship between the  $Mg^{2+}$  concentration and dNTP concentration. DNA polymerase requires free  $Mg^{2+}$  as cofactor for its activity, high concentration of dNTP will result in binding of most of the  $Mg^{2+}$  to dNTPs, causing the decrease of free  $Mg^{2+}$  and this may affect the DNA polymerase activity thus inhibiting PCR reaction. RT-PCR specificity can be increased by performing a hot start PCR. The DNA polymerase for hot start PCR performs its polymerization at very high temperature that will decrease the amplification of non specific products and primer-dimer formation.

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