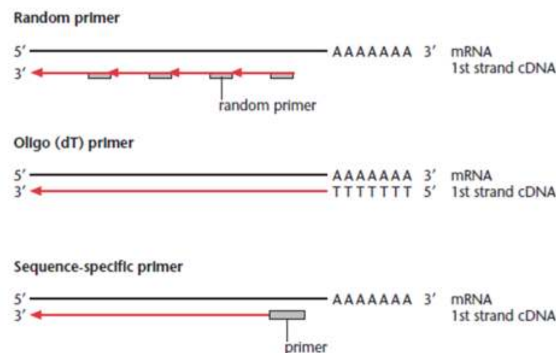
Reverse Transcription PCR

Reverse transcription followed by the PCR (RT-PCR) leads to the amplification of RNA sequences in cDNA form. No modification to the basic PCR strategy is required.
In order to apply PCR methodology to the study of RNA expression, the RNA sample must first be reverse-transcribed to cDNA to provide the necessary DNA template for the thermostable polymerase. This process is called reverse transcription (RT), hence the name RT-PCR.

1. The RNA template is converted into complementary (c)DNA by the enzyme reverse transcriptase. The cDNA serves later as a template for exponential amplification using PCR.
2. Obtaining high quality and intact RNA is the first and often the most critical step in performing RT-PCR. The isolated RNA must be DNA, RNAse and protein free.
3. Reverse transcription involves the synthesis of DNA from RNA by using an RNA-dependent DNA polymerase, the reverse of normal transcription, which is from RNA to DNA. M-MLV reverse transcriptase is the preferred reverse transcriptase in cDNA synthesis for long messenger RNA (mRNA) templates (>5 kb).
4. RT-PCR can be undertaken in one or two steps.
    a. One-step RT-PCR combines the RT reaction and PCR reaction in the same tube. Only sequence-specific primers may be used.
    b. During two-step RT-PCR, the synthesized cDNA is transferred into a second tube for PCR. Oligo (dT), random hexamer or gene-specific primers can be used. Oligo (dT) primers are generally preferred as they hybridize to the 3′ poly (A) tails in mRNAs (transcribed gene sequences), whereas random primers prime anything including ribosomal RNA .



5. There are three basic strategies for synthesis of first-strand cDNA. (a) Random primer; (b) oligo (dT) primer; (c) sequence-specific primer.

RT PCR using oligo (dT) primers.

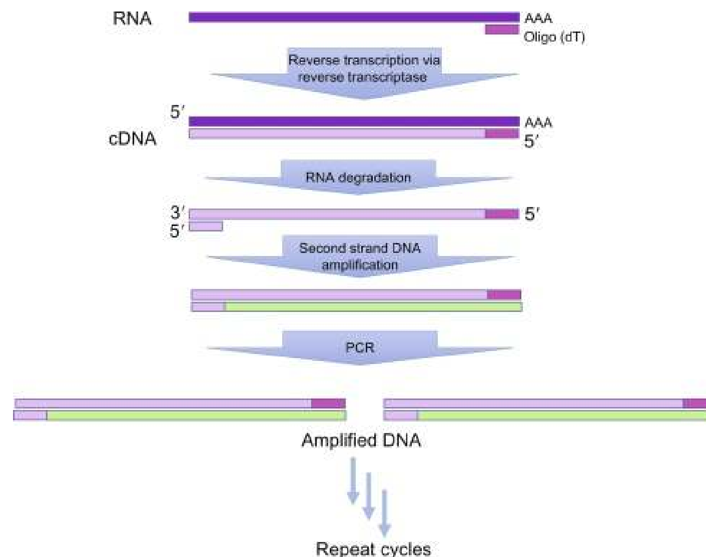
- Reverse Transcription (RT reaction) The synthesis of DNA from an RNA template, via reverse transcription, produces complementary DNA (cDNA). Reverse transcriptases (RTs) use an RNA template and a short primer complementary to the 3' end of the RNA to direct the synthesis of the first strand cDNA, which can be used directly as a template for the Polymerase Chain Reaction (PCR).
- The single-stranded RNA is reverse transcribed into complementary DNA (cDNA) by using:
    i. total cellular RNA or poly(A) RNA,
    ii. reverse transcriptase enzyme,
    iii. a primer,
    iv. dNTPs and an
    v. RNase inhibitor.

SUBHADIPA 2021

The resulting cDNA can be used in RT-PCR reaction. RT reaction is also called first strand cDNA synthesis.

- For a RT reaction, 1-2 micrograms of RNA is typically used. Steps are:
  i. RNA is first incubated with a primer at 70 degree to denature RNA secondary structure and then quickly chill on ice to let the primer anneal to the RNA.
  ii. Other components of RT are added to the reaction including dNTPs, RNase inhibitor, reverse transcriptase and RT buffer.
  iii. RT reaction is extended at 42 degree for 1 hr.
  iv. Heat the reaction at 70 degree to inactivate the enzyme. Sometimes removal of the template RNA by treating the RT reaction with RNase H is necessary before using the reaction in RT-PCR.
  v. This combination of reverse transcription and PCR (RT-PCR) allows the detection of low abundance RNAs in a sample, and production of the corresponding cDNA, thereby facilitating the cloning of low copy genes.
- Alternatively, the first-strand cDNA can be made double-stranded using DNA Polymerase I and DNA Ligase. These reaction products can be used for direct cloning without amplification.
- Many RTs are available from commercial suppliers. Avian Myeloblastosis Virus (AMV) Reverse Transcriptase and Moloney Murine Leukemia Virus (M-MuLV, MMLV) Reverse Transcriptase are RTs that are commonly used in molecular biology workflows.

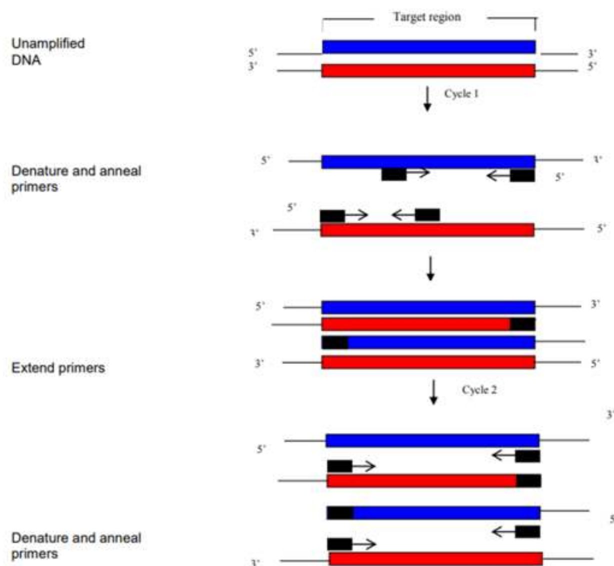
SUBHADIPA 2021



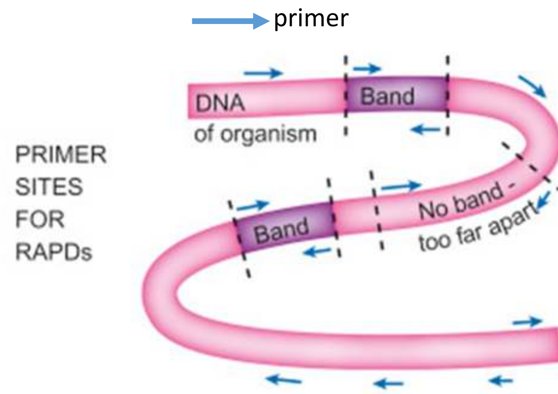
The RAPD technique

- The RAPD technique is based on the polymerase chain reaction (PCR).
- A target DNA sequence is exponentially amplified with the help of:
  - ***arbitrary primers***,
  - a thermostable DNA polymerase,
  - dideoxy nucleotide tri - phosphates,
  - magnesium and reaction buffer.
- The reaction involves repeated cycles, each consisting of:
  i. a denaturation: In the first step the DNA is made single stranded by raising the temperature to 94°C
  ii. a primer annealing: lowering of the temperature to about 40 to 65°C results in annealing of the primer to their target sequences on the template DNA
  iii. an elongation step:, temperature is chosen where the activity of the thermostable Taq DNA polymerase is optimal, i.e., usually 72° C

SUBHADIPA 2021



- The polymerase now extends the 3’ ends of the DNA-primer hybrids towards the other primer binding site. Since this happens at both primer-annealing sites on both the DNA strands, the target fragment is completely replicated. Repeating these three step cycles 40 to 50 times results in the exponential amplification of the target between the 5’ ends of the two primer binding sites.
- Amplification products are separated by gel electrophoresis and visualized by ethidium bromide staining



SUBHADIPA 2021

Principle

- The trick of RAPD analysis is to design primers(5-10bp only) that will bind to genomic DNA at random sites that are neither too rare nor too common. In this example, the primers were sufficiently long to bind the genomic DNA at a dozen places.
- For PCR to be successful, two primers must anneal at sites facing each other but on opposite strands. In addition, these paired primer sites must be close enough to allow synthesis of a PCR fragment. In this example, there are three pairs but only two of these pairs were close enough to actually make the PCR product.
- Consequently, this primer design will result in two PCR products as seen in the first lane of the gel (marked "First organism").
- The same primers are then used to amplify genomic DNA from other organisms that are suspected of being related. In this example, suspect #2 shows the same banding pattern as the first organism and is presumably related. The other two suspects do not match the first organism and are therefore not related.