Munmun Chatterjee

PCR : POLYMERASE CHAIN REACTION

- MUNMUN CHATTERJEE.

- Definition : The in vitro amplification of a target DNA sequence by means of repeated cycles of replication with the help of a thermo-stable DNA polymerase, is called as polymerase chain reaction or PCR. It is a powerful technique that from single copy of DNA molecule, millions of copies can be obtained with high accuracy.

- Kary Mulis of Cetus corporation, San Francisco, USA, in 1983, introduce this technique in the biotechnological applications.

- **Basic principle : In principle, PCR involves three temperature cycles –**

  a) Temperature 90-98°C separates two strands of the target DNA,

  b) Temperature 40-60°C anneals two complementary primers to ends of the separated single strands of target DNA, &

  c) Temperature 72°C allows thermostable Taq DNA polymerase to use single strands of target as template to synthesize new strands.

These three temperature cycles are repeated 25-30 times and as a result there is a million fold increase of the copies of the target DNA. This reaction usually occurs in DNA Thermal Cycler (Perkin Elmer Cetus Instrument) using three step cycle Program set to –

- Denature at 94°C for 20 sec.
- Anneal at 55°C for 20 sec.
- Extend at 72°C for 30 sec.

- Earlier DNA polymerase enzyme from *E. coli* was used but it was very much sensitive to heat, so after each cycle as heat destroyed the enzyme, a new enzyme had to added every time. Use of thermostable DNA polymerase enzyme of *Thermophilus aquaticus* (from hot spring) was then used in 1988 and that was the major breakthrough in PCR development. Other thermostable enzymes used today are Pflu DNA polymeraes (from *Pyrococcus funosus*) and Vent polymerase (from *Thermococcus litoralis*) and they are found more efficient.

- PCR reaction components :

i. Taq polymerase – 2.5 units / µl reaction mixture;

ii. Oligonucleotide primers – two, optimum length of primer is 20-30 bases although much shorter or longer molecules may be used.

iii. Buffers – Tris buffer of pH- 8.8 at 25°C gives pH- 7.3 at 72°C.

iv. Mg^{2+} required by Taq enzyme activity.

v. Detergents (nonionic) :– Triton X –100, NP-40 or Tween 80 (final concentration of 0.01 %) added both in storage solution as well as in reaction mixture. Taq is highly hydrophobic in nature and tend to precipitate from aqueous solution. Detergents helps maintain their activity.

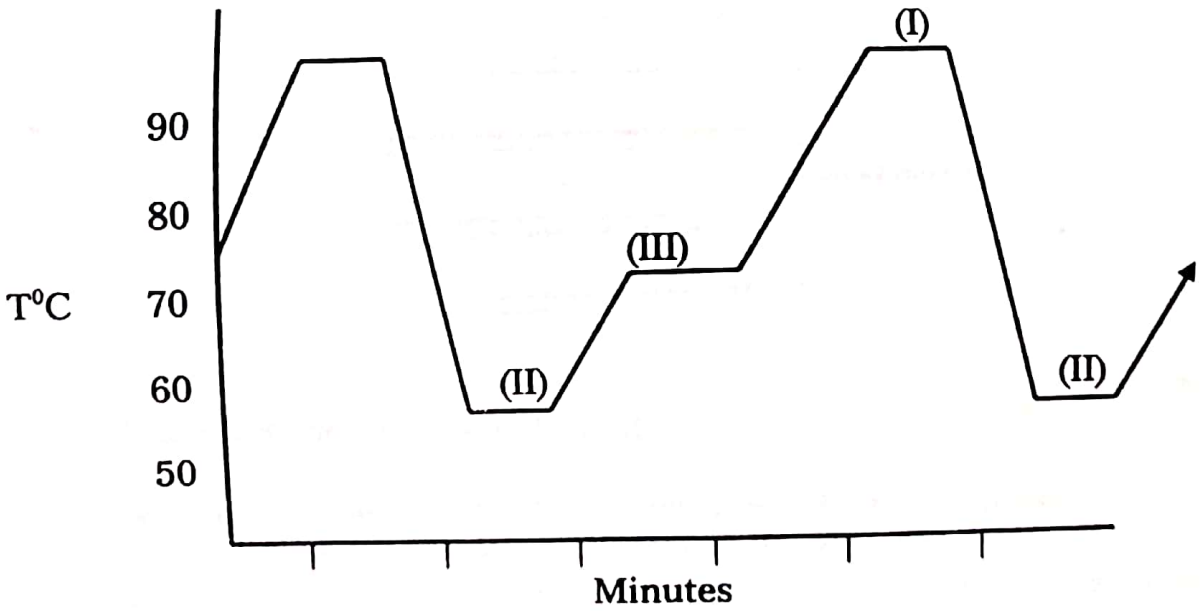
vi. Salts other than Mg^{2+} are not at all required, KCl, phosphates are inhibitory.

vii. Nucleotides – 800 µM of total dNTPs added in reaction mixture. This is sufficients to produce 13 µg of DNA in µl reaction.

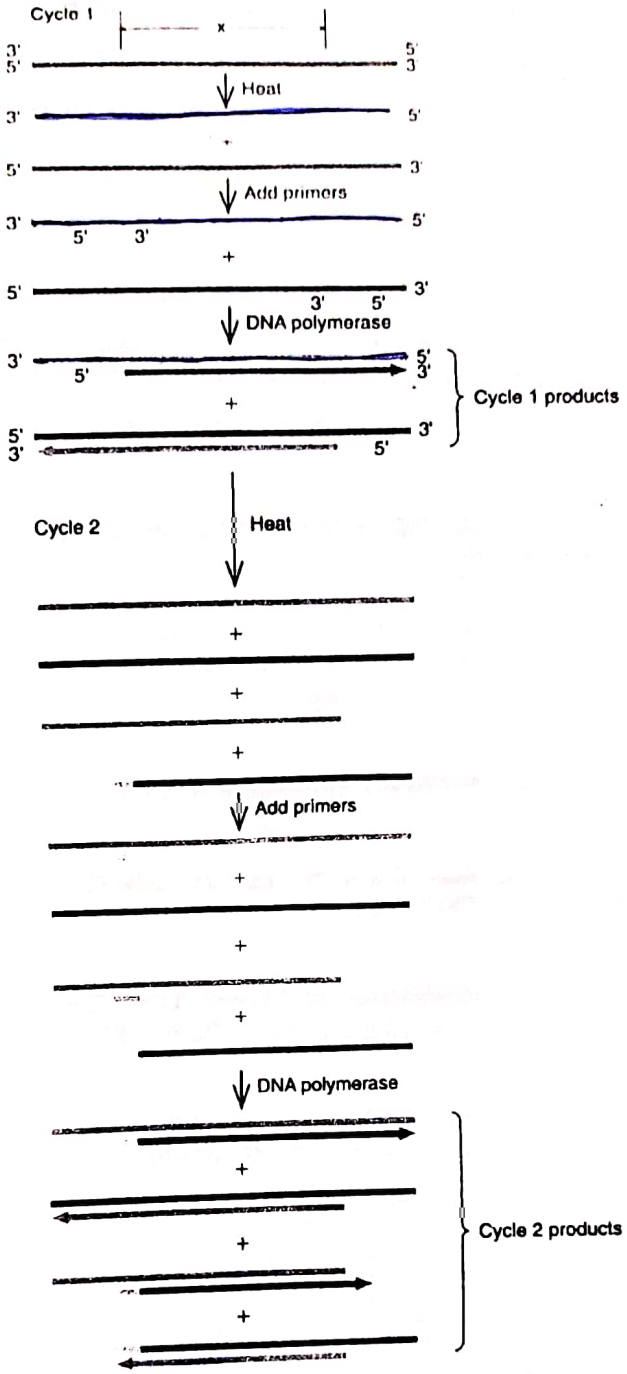
viii. Mercaptoethanol or dithiothreitol (10mM final concentration) is added to PCR mixture to stabilize proteins during thermal cycling.

ix. Template DNA – sample DNA need not be pure. Even less than 0.2 µg is sufficient.

- *Graphical representation of the basic reaction of standard PCR :*



I. Denaturation – temperature 1 – 94°C / 20 sec.
II. Annealing – temperature 2 – 55°C / 20 sec.
III. Extending – temperature 3 – 72°C / 30 sec.



- RT-PCR and its use in cDNA cloning :

i. If one wants to clone a cDNA from just one mRNA whose sequence is known, one can use a type of PCR known as RT-PCR or Reverse Transcriptase PCR.
ii. The main difference between this procedure and the PCR method is that this one starts with an mRNA instead of a double stranded DNA.
iii. This one begins by converting mRNA to DNA.
iv. As usual, this RNA → DNA step can be done with reverse transcriptase : one reverse transcribes the mRNA to make a single stranded DNA, then uses a primer to convert the single stranded DNA to double stranded.

v. Then one can use standard PCR to amplify the cDNA until enough is available for cloning.

vi. One can even add restriction sites to the ends of the cDNA by using primers that contain these sites.

vii. In the following example, a *Bam*HI site is present on one primer and a HindIII site is present on the other (placed a few nucleotide away from the ends to allow the restriction enzymes to cut efficiently).

viii. Thus the PCR product is a cDNA with these two restriction cut sites at its two ends. Cutting the PCR products with these two restriction enzymes creates sticky ends that can be ligated into the vectors of choice.

ix. Having two different sticky ends allows directional cloning, so the cDNA will have only one of the two possible orientations in the vectors.

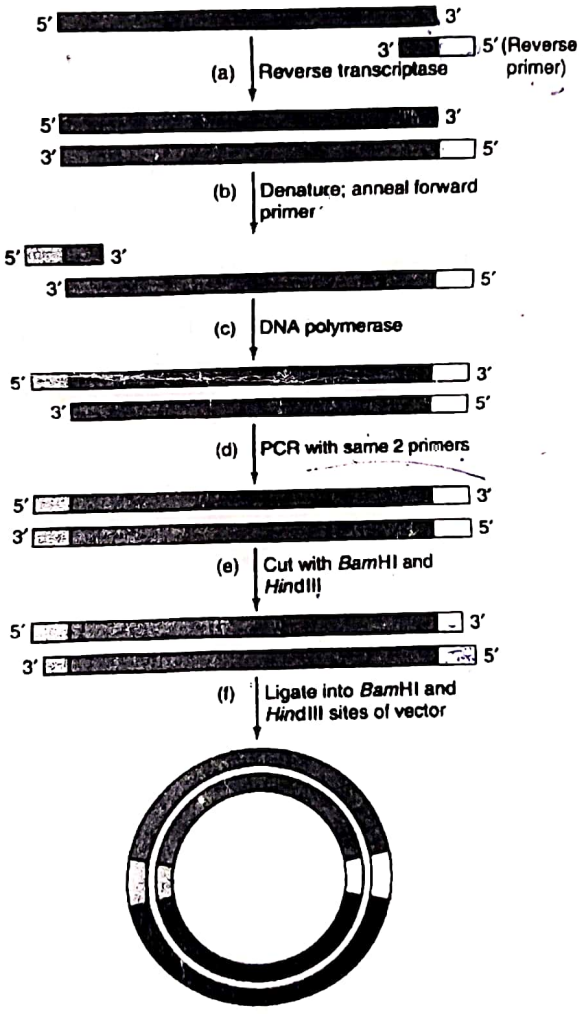


Figure 4.15 Using RT-PCR to clone a single cDNA. (a) Use a reverse primer (red) with a *Hind*III site (yellow) at its 5′-end to start first-strand cDNA synthesis, with reverse transcriptase to catalyze the reaction. (b) Denature the mRNA–cDNA hybrid and anneal a forward primer (red) with a *Bam*HI site (green) at its 5′-end. (c) This forward primer initiates second-strand cDNA synthesis, with DNA polymerase catalyzing the reaction. (d) Continue PCR with the same two primers to amplify the double-stranded cDNA. (e) Cut the cDNA with *Bam*HI and *Hind*III to generate sticky ends. (f) Ligate the cDNA to the *Bam*HI and *Hind*III sites of a suitable vector. Finally, transform cells with the recombinant cDNA to produce a clone.