PCR: Methods and Applications

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History of PCR

- 1983 - Kary B. Mullis – Initial idea
- 1985 – Idea accepted by Cetus Corporation and rewarded as bonus for the invention
- 1989 – Taq Polymerase as molecule of the year
- 1993 – Kary Mullis awarded Nobel prize in chemistry along with Michael Smith

Polymerase Chain Reaction

- **Targeting and Amplification of a specific region of a DNA strand**
- **An *in vitro* technique to generate large quantities of a specifies DNA**
- **In contrast with normal cloning (*in vivo*), PCR technique provides multiple clones in a very short period starting with very small quantity even by a single molecule**

Purpose Of PCR

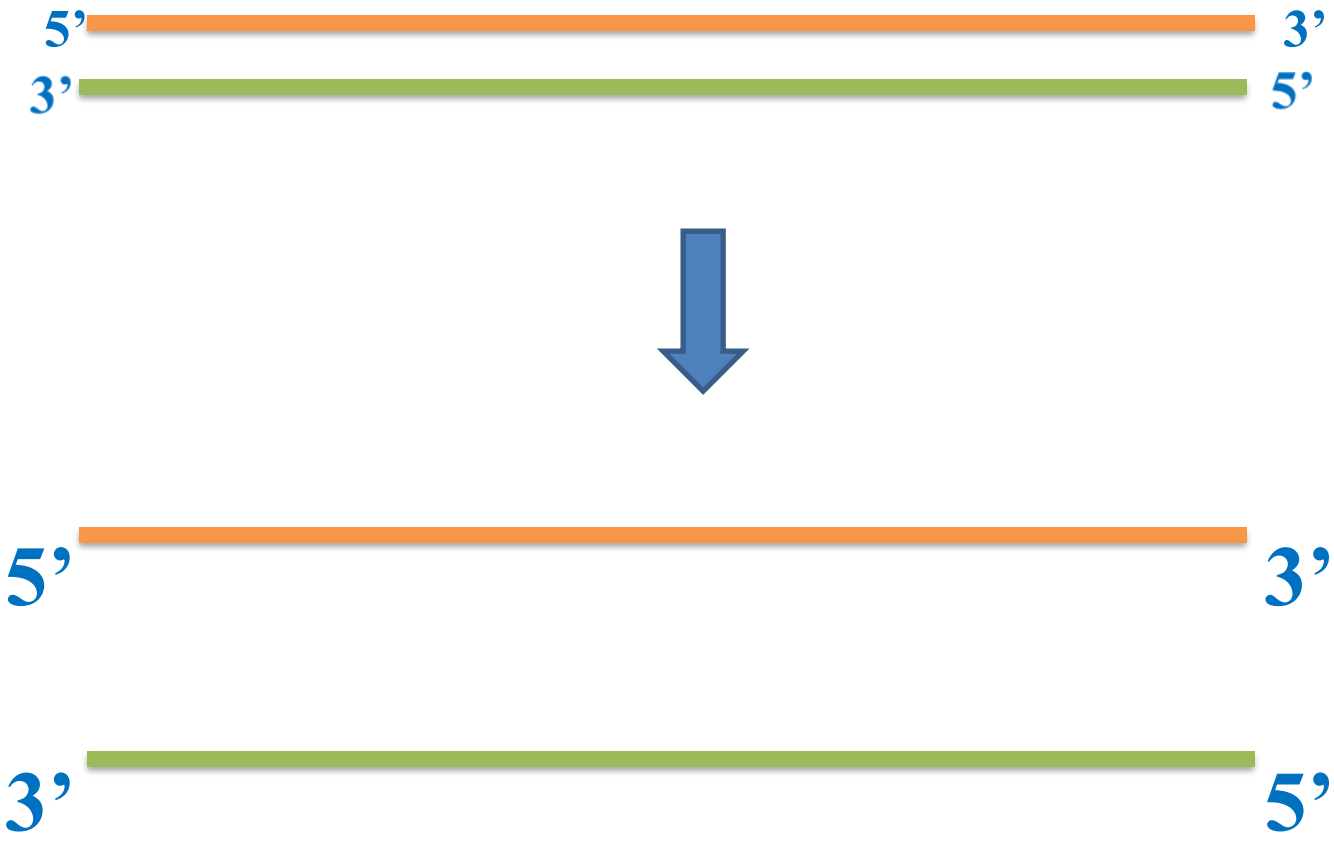
- **To amplify a lot of double stranded DNA molecules with same (identical) size, with sequence by enzymatic method and cycling condition**

Chemical Components

- DNA template (target DNA)
- Primer (two; complementary to 3’ end of target DNA)
- Polymerase enzyme: Taq Polymerase from *T. aquaticus*
- Deoxynuleosides: A,G,C, T or U
- Triphosphate (dNTPs)
- Buffer solution: 100 mM Tris-HCl pH 9.0
- Divalent cations (Mg^{2+}): Primer annealing, templet denaturation and enzyme activity
- Monovalent cations (K^{+}):

Steps Involved

1. **Denaturation** – The reaction mixture is heated to 90-98^{0}C so that the DNA get denature into single strands by disruption of hydrogen bond between complementary bases (1-2 minutes)

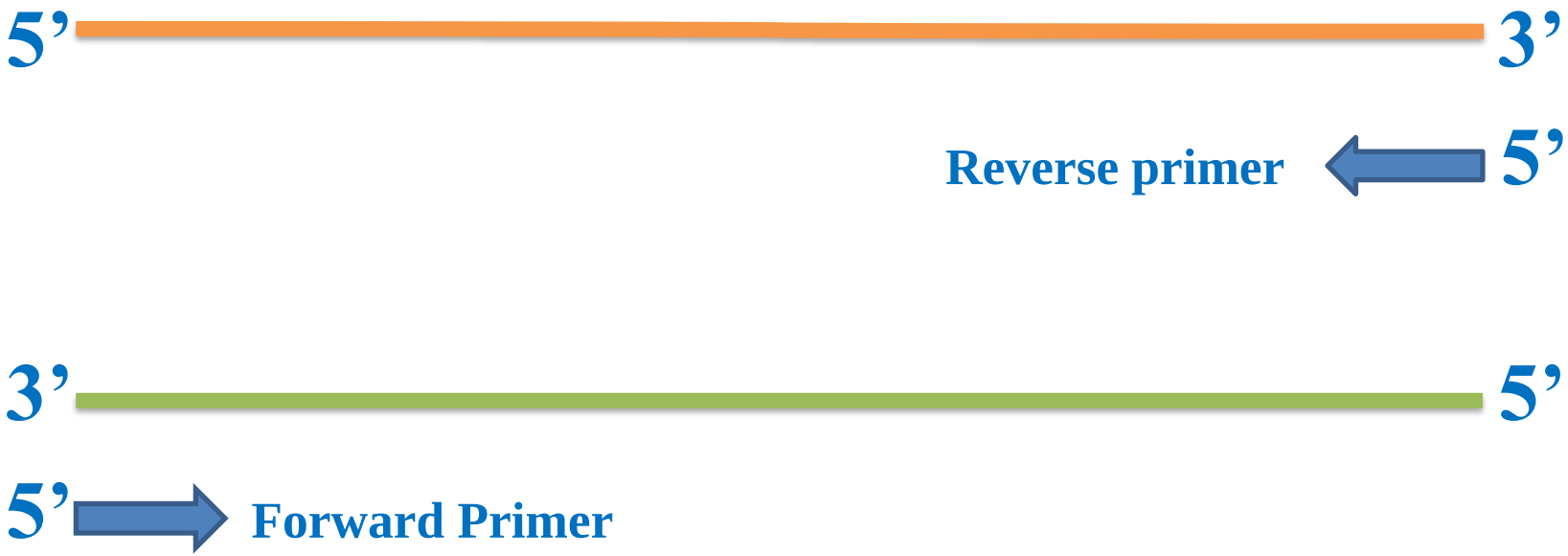


Denaturation at 94°C

2. Annealing – The temperature of reaction mixture is cooled to 45-60^{0}C

- **Primer base pair with complementary bases**
- **Hydrogen bonds reform resulted into renaturation of DNA strand**

Sense strand

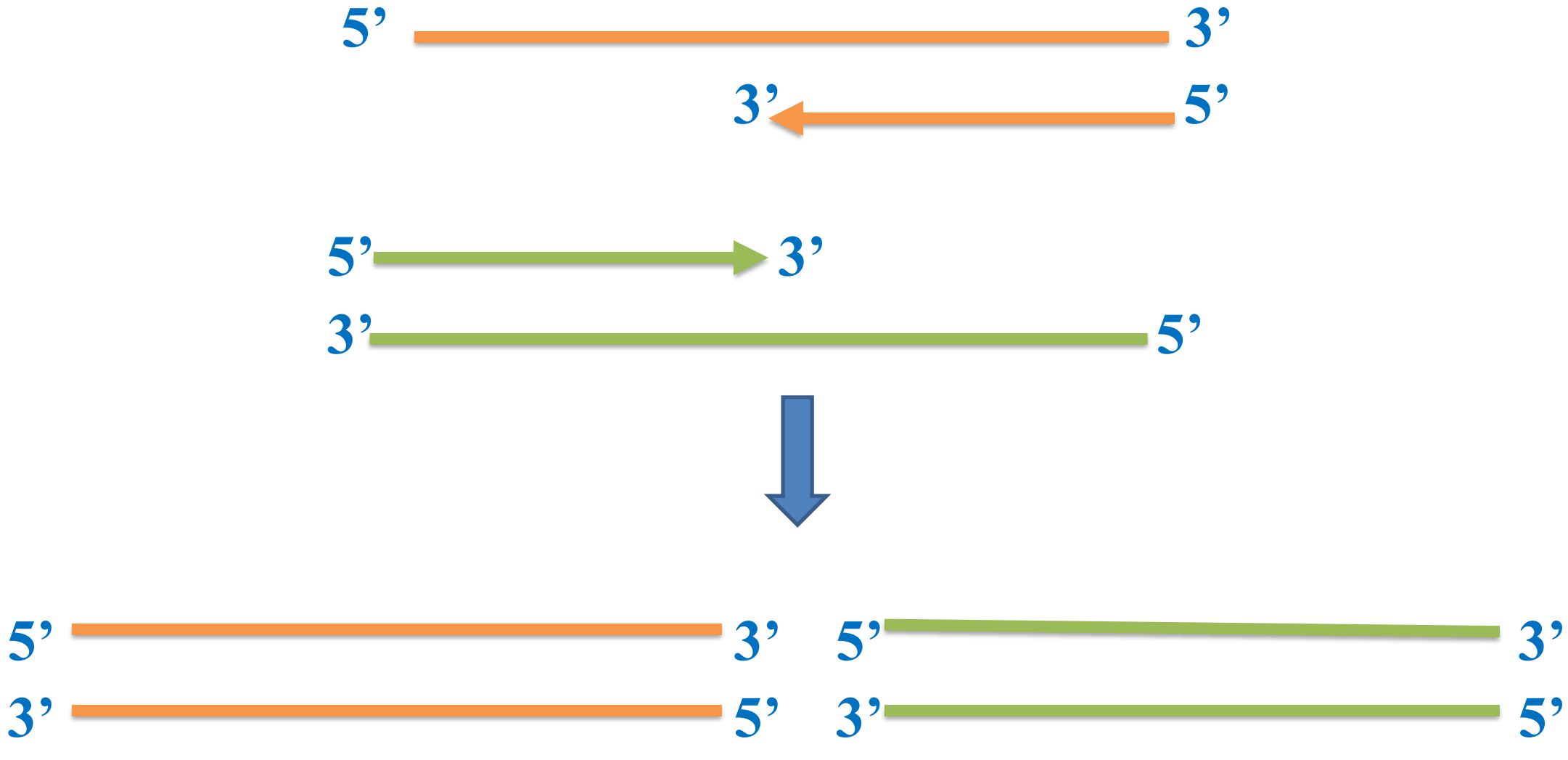


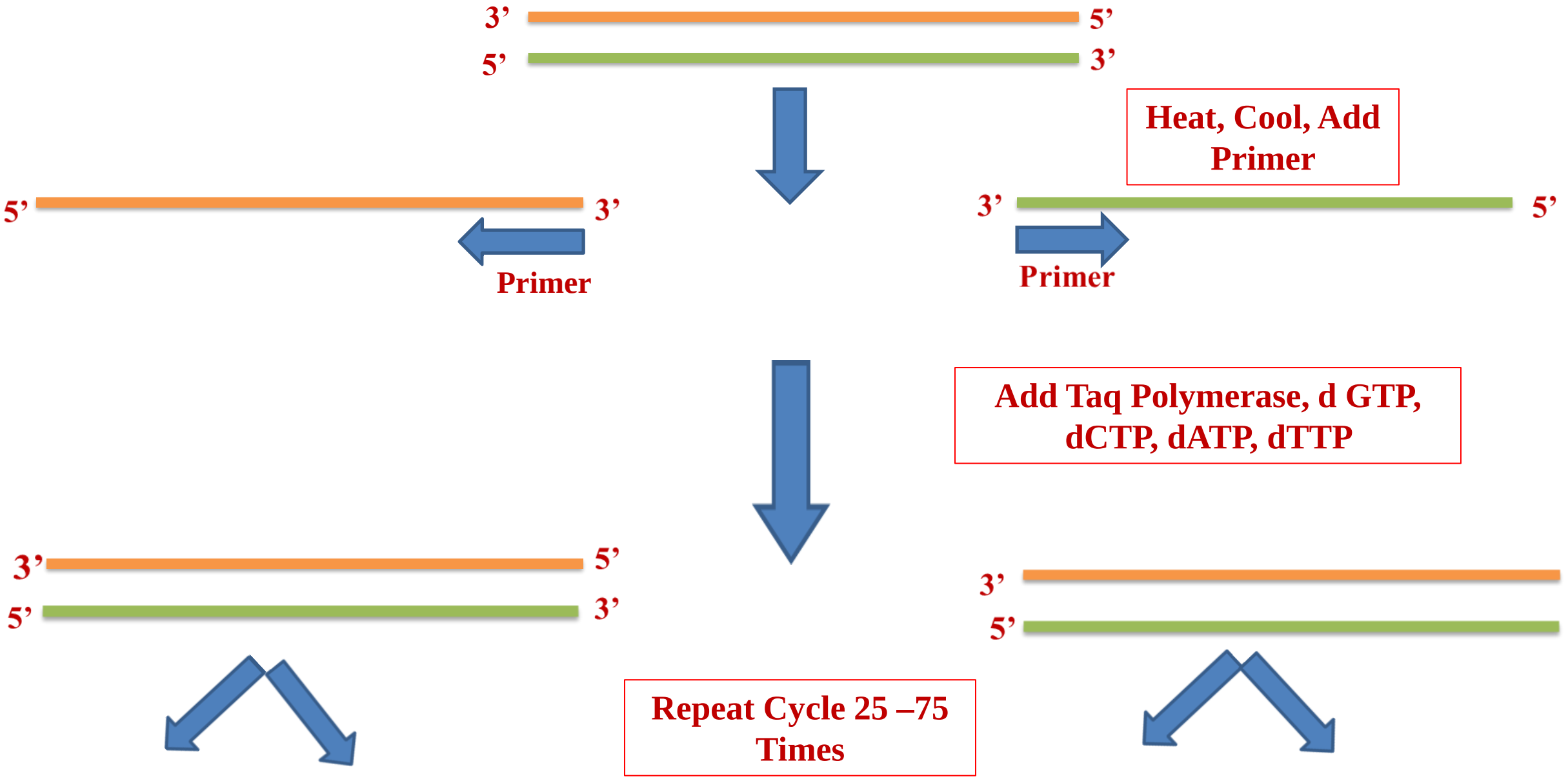
Antisense Strand

Annealing at 54^{o}C

3. Extension – The temperature is again shifted to 72^0C which is ideal for polymerase

- **Primers are extended by joining the bases complementary to DNA strand**
- **Elongation continues by addition of new bases from 5’ to 3’ end reading the template from 3’ to 5’ side**
- **The same cycle is repeated to 30-40 times**


5’
3’
3’
5’
5’
3’
3’
5’
5’
3’
3’
5’
5’
3’
3’
5’
Extension at 72°C


3’
5’
5’
3’
Heat, Cool, Add Primer
5’
3’
Primer
3’
5’
Primer
Add Taq Polymerase, d GTP, dCTP, dATP, dTTP
3’
5’
5’
3’
3’
5’
5’
3’
Repeat Cycle 25 –75 Times

Aspects of PCR

- **Specificity (Characteristics)**
- **Efficiency (Competence)**
- **Fidelity (Being Accurate or close to the original)**

Factors for Optimal PCR

1. PCR Primers –

- Correctly designed pair
- Dimer or hairpin formation should not be permitted
- Length

2. DNA Polymerases –

- Thermus aquaticus (Taq): heat resistant, no proof reading exonuclease activity, half life at 95^0C 1.6 hrs
- Thermotoga maritima (Tma): 97 kd poly I, half life at 90^0C 2hrs
- Pyrococcus furiosus (Pfu): showing lowest error rate
- Thermococcus litoralis (Vent/ Tli): half life at 95^0C 7 hrs

3. Annealing Temperature –

- Ideal annealing temperature must be low enough to enable hybridization between primer and template and high enough to prevent amplification of non-targeted sites
- It should be 2-5 ^{0}C lower then the Tm of primer DNA

4. Melting Temperature(Tm) –

- Temperature at which 2 strand of duplex get dissociated

$$Tm = (4(G+C))+ (2(A+T))$$

5. G/C Contents –

- Ideally 50% G/C
- No poly C and poly G strands should be there, which can promote non specific annealing

End Result

- **How will you make sure that the Target sequence is get amplified?**
- **Why it is very important to know the product size?**

Ans: The product is run through Agarose Gel Electrophoresis with a ladder, the with the known size showing thicker bands resulted in the success of PCR cycles

Scope of PCR

- In clinical diagnosis
- In DNA sequencing
- In forensic medicine
- In gene manipulation and expression studies
- In comparative studies of genomics
- In gene cloning

Advantages of PCR

- **Small amount of DNA is required per test**
- **Results come very quickly usually within one day**
- **Radioactive materials are not required**

Limitations of PCR

- Sequence information
- Amplification size
- Error rate during amplification
- Sensitivity to inhibitors
- Contamination

Applications

1. **Molecular Identification-**
   - Molecular Archaeology
   - Molecular Epidemiology
   - Molecular Ecology
   - DNA fingerprinting
   - Molecular taxonomy
   - Genotyping
   - Pre natal diagnosis
   - Mutation screening
   - Drug discovery
   - Genetic matching

Applications

2. Sequencing material

- Bioinformatics
- Genomic cloning
- Human genome project

3. Genetic Engineering

- Site directed mutagenesis
- Gene expression studies

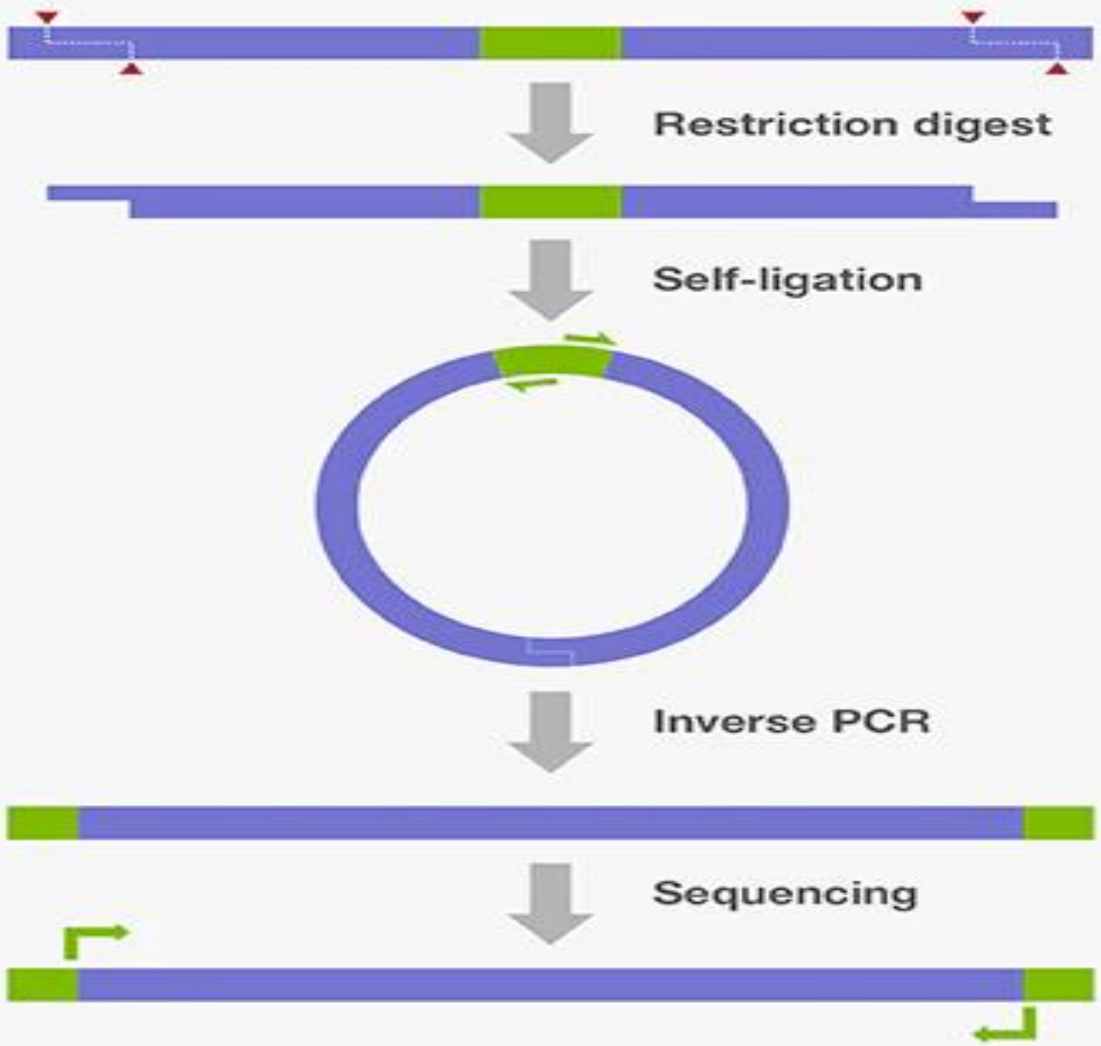
Comparison of PCR and Cloning

S.No.	Parameter	PCR	Gene Cloning
1.	Final Result	Selective amplification of specific sequence	Selective amplification of specific sequence
2.	Manipulation	In vitro	In vitro and In vivo
3.	Selectivity of specific segment from DNA complex	First Step	Last Step
4.	Quantity of starting material	Nanogram	Microgram
5.	Biological Reagents required	Taq Polymerase	Restriction enzyme, Ligase, Vector, Bacteria
6.	Automation	Yes	No
7.	Labour Intensive	No	Yes
8.	Error Probability	Less	More
9.	Applications	More	Less
10.	Cost	Less	More
11.	User Skill	Not required	Required
12.	Time for typical experiment	2-4 hrs	2-4 days

Variations of PCR methods and Their Applications

1. Inverse PCR

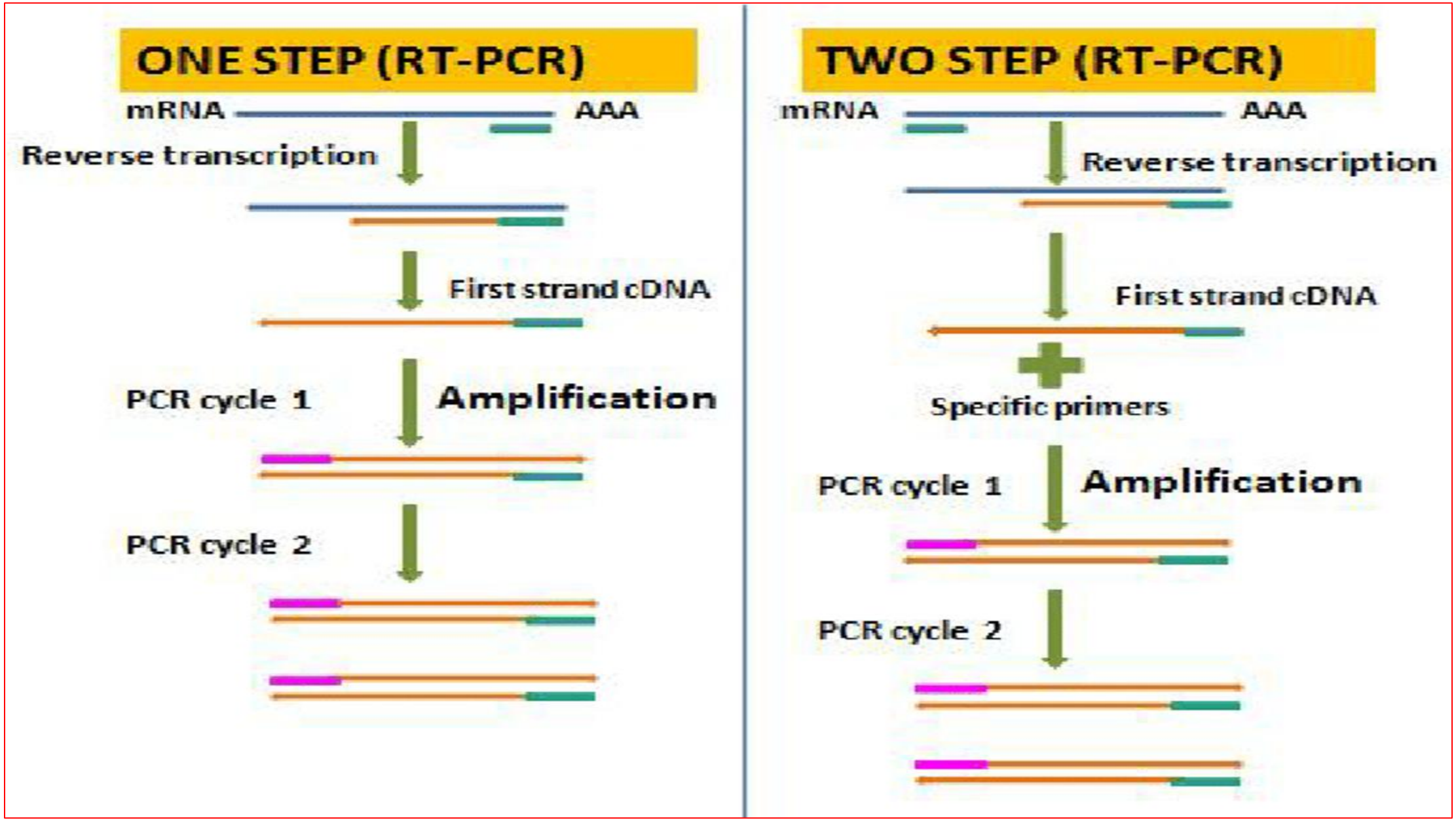
- Amplification of unknown DNA sequence from known sequence
- Includes a series of digestions and self ligation by restriction endonuclease and ligases respectively
- The primer here used are of reverse orientation
- The template is a restriction fragment ligated to form a circle
- Specially useful for identification of neighboring/adjoining sequences of DNA inserts


Restriction digest
Self-ligation
Inverse PCR
Sequencing


Known sequence
Unknown sequence
Restriction site
Inverse PCR primers
Sequencing primers

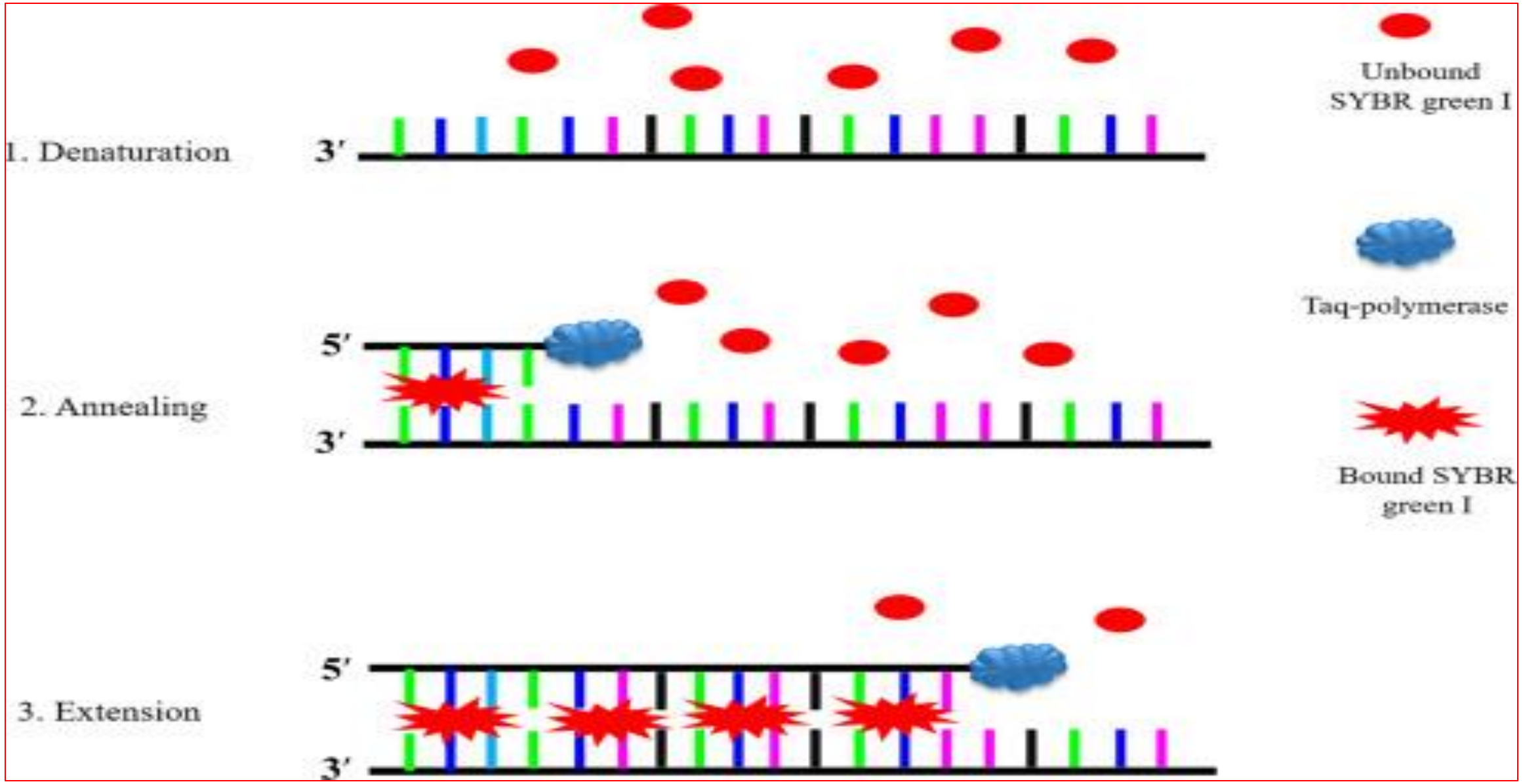
2. Reverse transcription PCR (RT PCR)

- This PCR is design for amplification of DNA from RNA
- It is commonly used for expression profiling to determine expression of a gene
- It is also used to locate the positions of exons and introns in a DNA segment


ONE STEP (RT-PCR)
mRNA
AAA
Reverse transcription
First strand cDNA
PCR cycle 1
Amplification
PCR cycle 2
TWO STEP (RT-PCR)
mRNA
AAA
Reverse transcription
First strand cDNA
Specific primers
PCR cycle 1
Amplification
PCR cycle 2

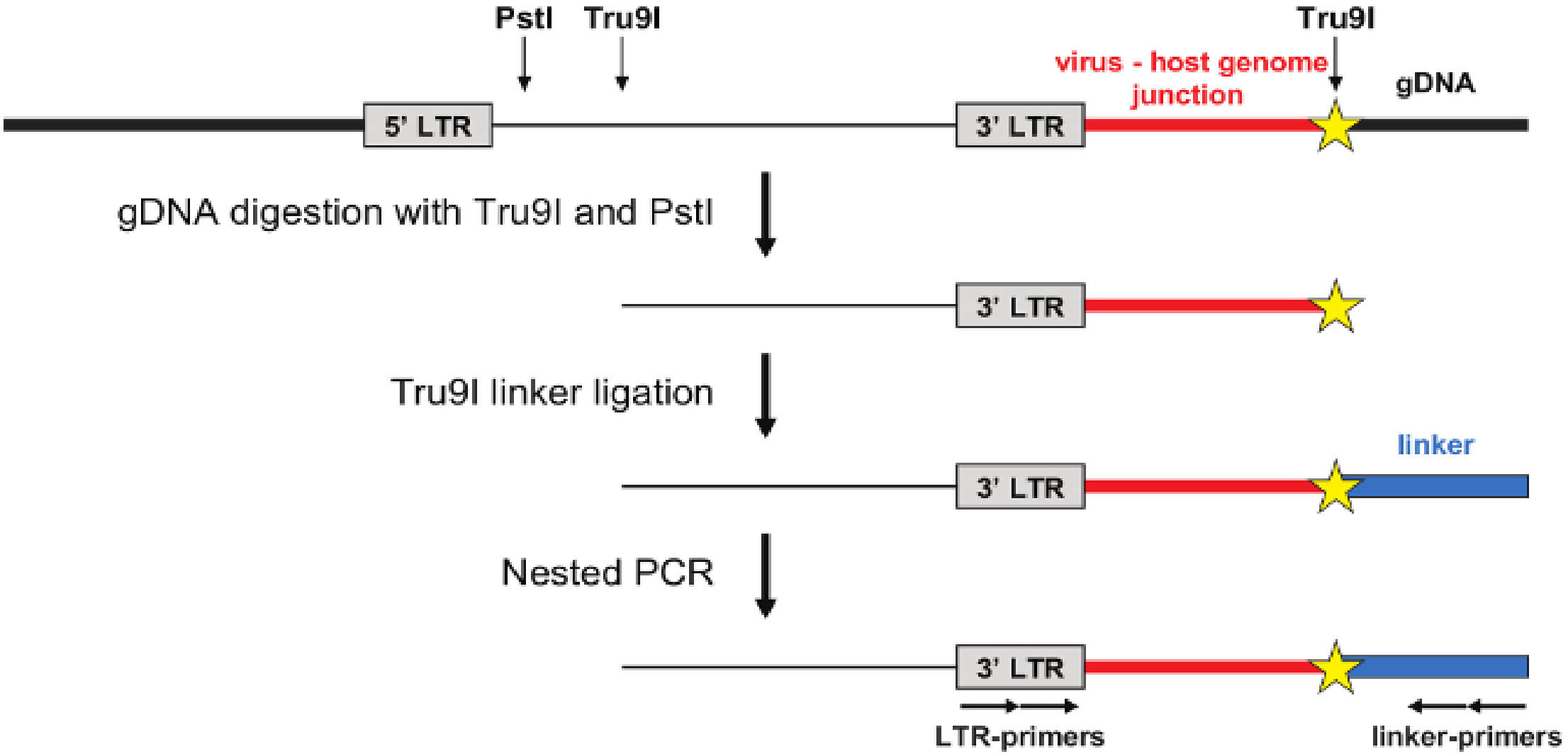
3. Quantitative Real Time PCR (QRT PCR)

- The Real Time PCR uses dyes for staining DNA such as Sybr Green, Eva Green and Fluorescent reporter probes such as Taqman
- The TaqMan probes are designed in such away that they anneal to a specific region amplified by a specific primer
- The Taq polymerase extends the primer and synthesizes nested DNA and 5'-3' exonuclease activity degrades the probe
- Degradation of probe releases the fluorophore and allowing to fluorescence
- The amount of fluorescence is directly proportional to amount of DNA template present in the PCR


1. Denaturation
3′
Unbound SYBR green I
Taq-polymerase
2. Annealing
5′
3′
Bound SYBR green I
3. Extension
5′
3′

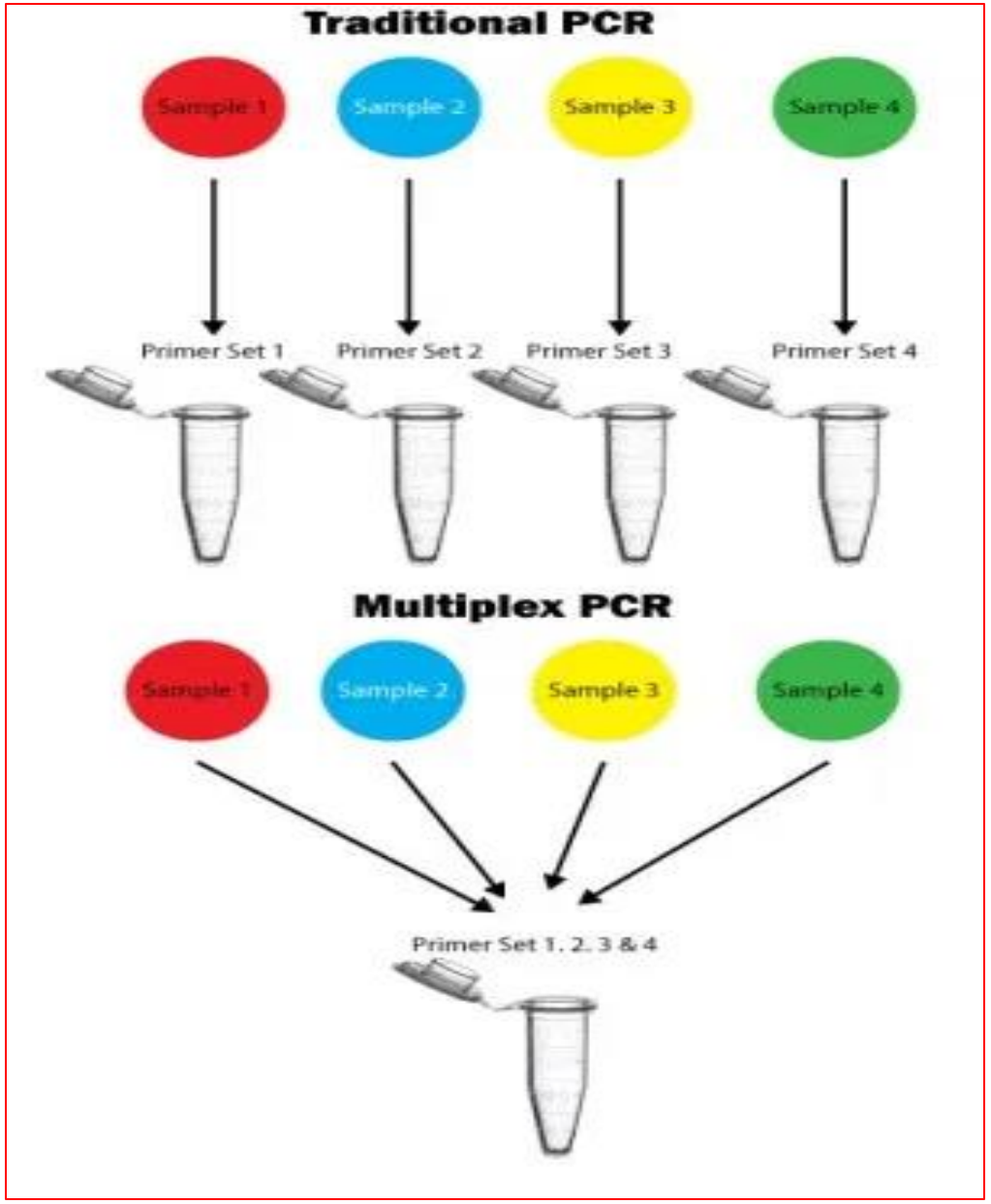
4. LM-PCR (Ligation –Mediated PCR)

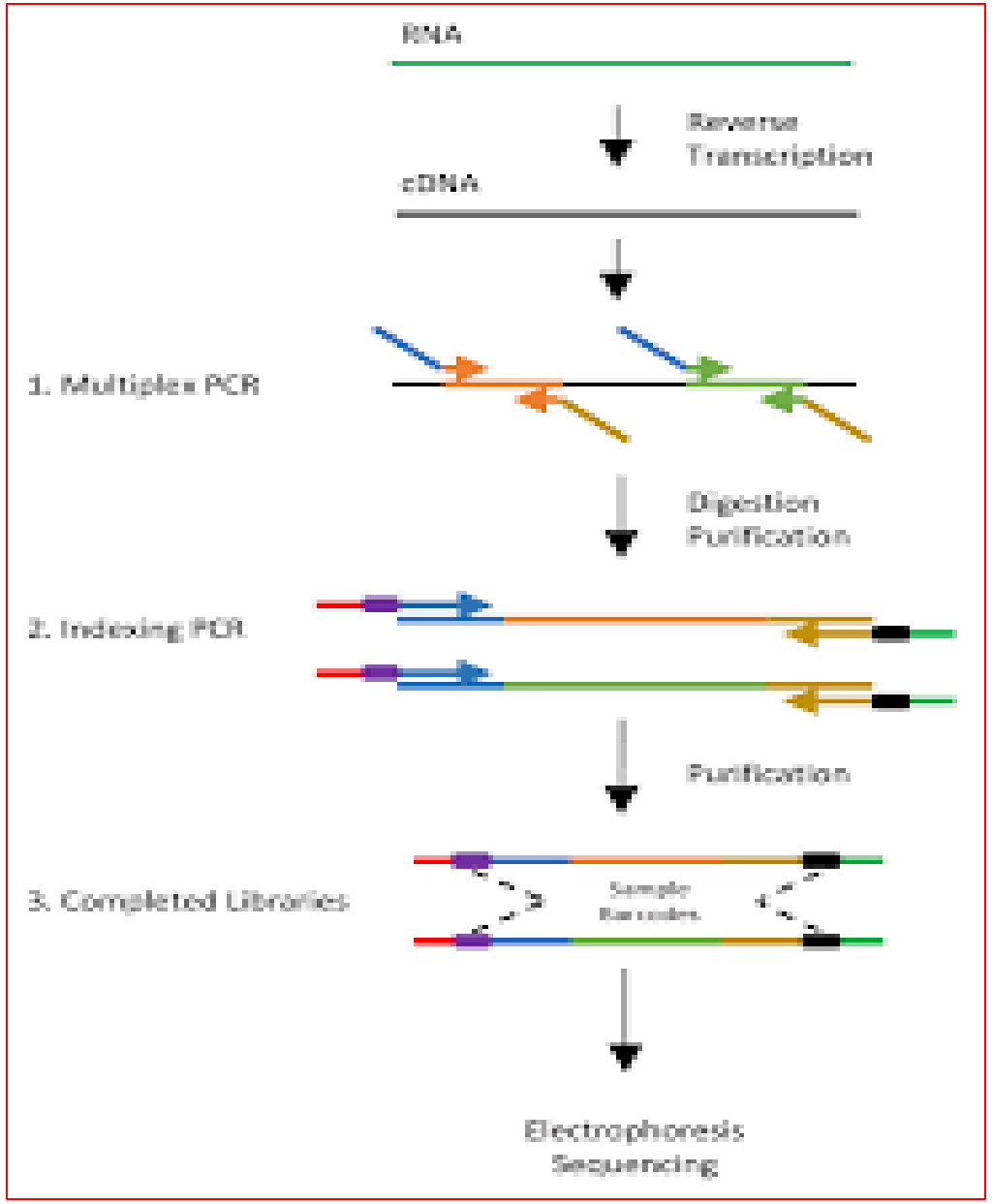
- It uses small DNA oligonucleotide linkers (adaptors) that are first ligated to fragment of target DNA
- Primer anneal to linker to be amplified
- This method is mostly used for DNA sequencing, genome walking and DNA finger printing
- Able to map single stranded DNA breaks
- Identification of DNA damages to reveal DNA -Protein interactions inside living cells


PstI
Tru9I
Tru9I
virus - host genome junction
gDNA
5' LTR
3' LTR
gDNA digestion with Tru9I and PstI
3' LTR
Tru9I linker ligation
linker
3' LTR
Nested PCR
3' LTR
LTR-primers
linker-primers

5.Multiplex PCR

- Amplification of multiple targets in a single PCR experiment
- By using multiple primer pairs in a reaction mixture, more than one targets can be amplified
- Annealing temperature must be optimized for each of the primer sets
- The amplification size (base pair length) should be different enough to visualized by gel electrophoresis
  - Single template PCR – Single genomic DNA with several pairs of forward and reverse primers to amplify specific regions within a template
  - Multiple Template PCR – Multiple templates and several primers in a same reaction tube. This may be leads to cross hybridization and possibility of mis-priming with other templates


Traditional PCR
Sample 1
Sample 2
Sample 3
Sample 4
Primer Set 1
Primer Set 2
Primer Set 3
Primer Set 4
Multiplex PCR
Sample 1
Sample 2
Sample 3
Sample 4
Primer Set 1, 2, 3 & 4

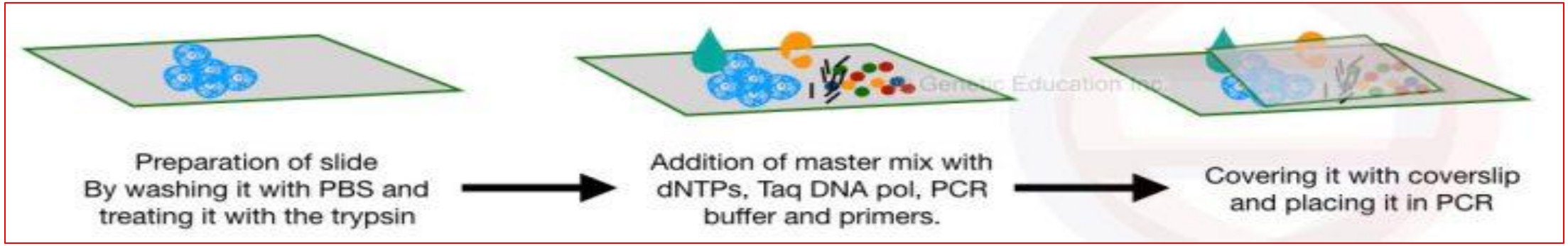

RNA
Reverse Transcription
cDNA
1. Multiplex PCR
Digestion
Purification
2. Indexing PCR
Purification
3. Completed Libraries
Sample Barcodes
Electrophoresis
Sequencing

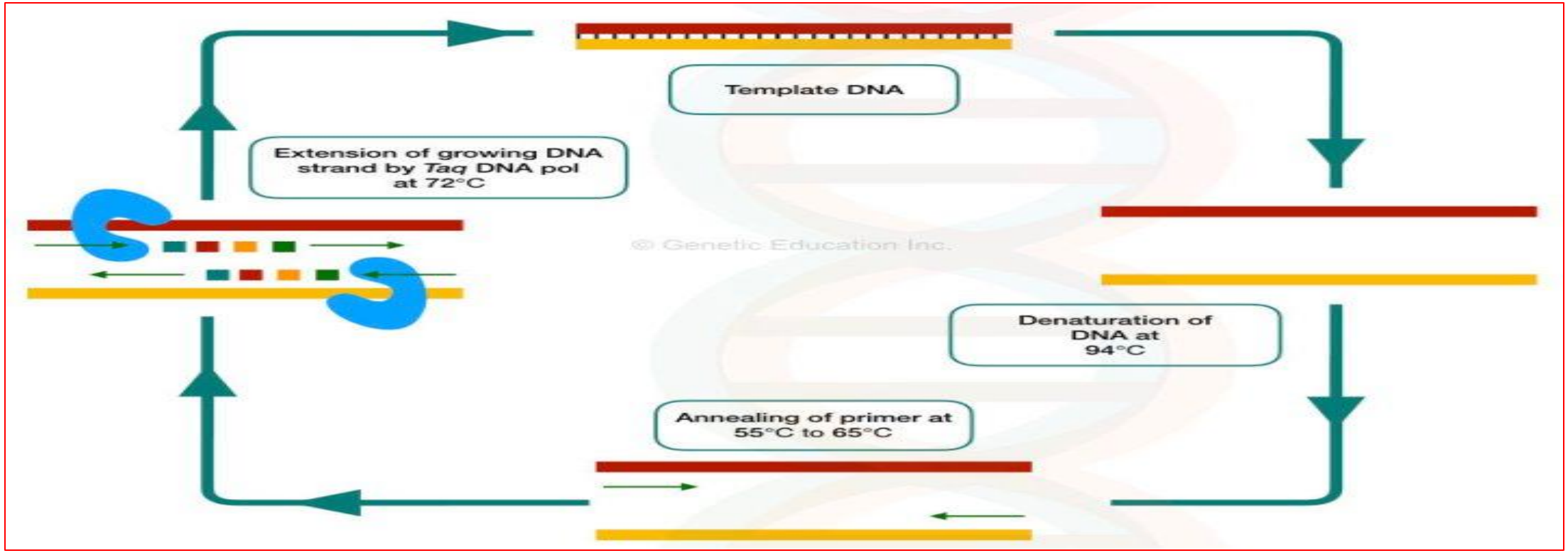
Applications of Multiplex PCR:

- Pathogen identification
- High throughput SNP genotyping
- Mutation analysis
- Gene detection analysis
- Template quantification
- Linkage analysis
- RNA detection
- Forensic studies

6. IN-Situ PCR (ISH)

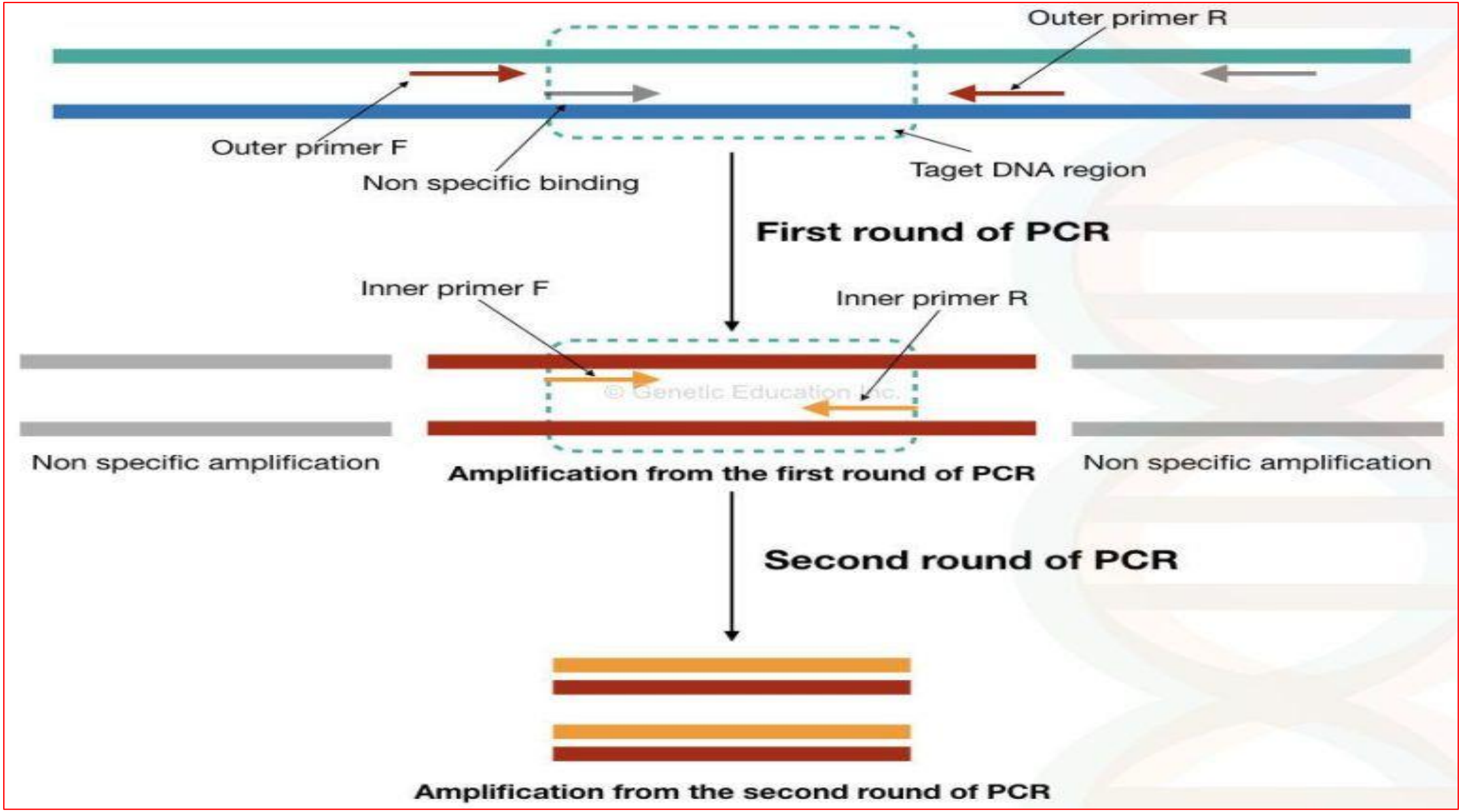
- A polymerase Chain Reaction that actually take place in side the cell on a slide
- It is performed on fixed tissue or cells
- It is used for detection and diagnosis of viruses and other infectious agents in specific cell types within tissue, detection and characterization of tumor cells within tissue, detection of gene and gene expression in a tissue, detection and diagnosis of genetic mutation in inherited diseases


Preparation of slide
By washing it with PBS and
treating it with the trypsin
Addition of master mix with
dNTPs, Taq DNA pol, PCR
buffer and primers.
Covering it with coverslip
and placing it in PCR


Template DNA
Extension of growing DNA
strand by Taq DNA pol
at 72°C
Denaturation of
DNA at
94°C
Annealing of primer at
55°C to 65°C

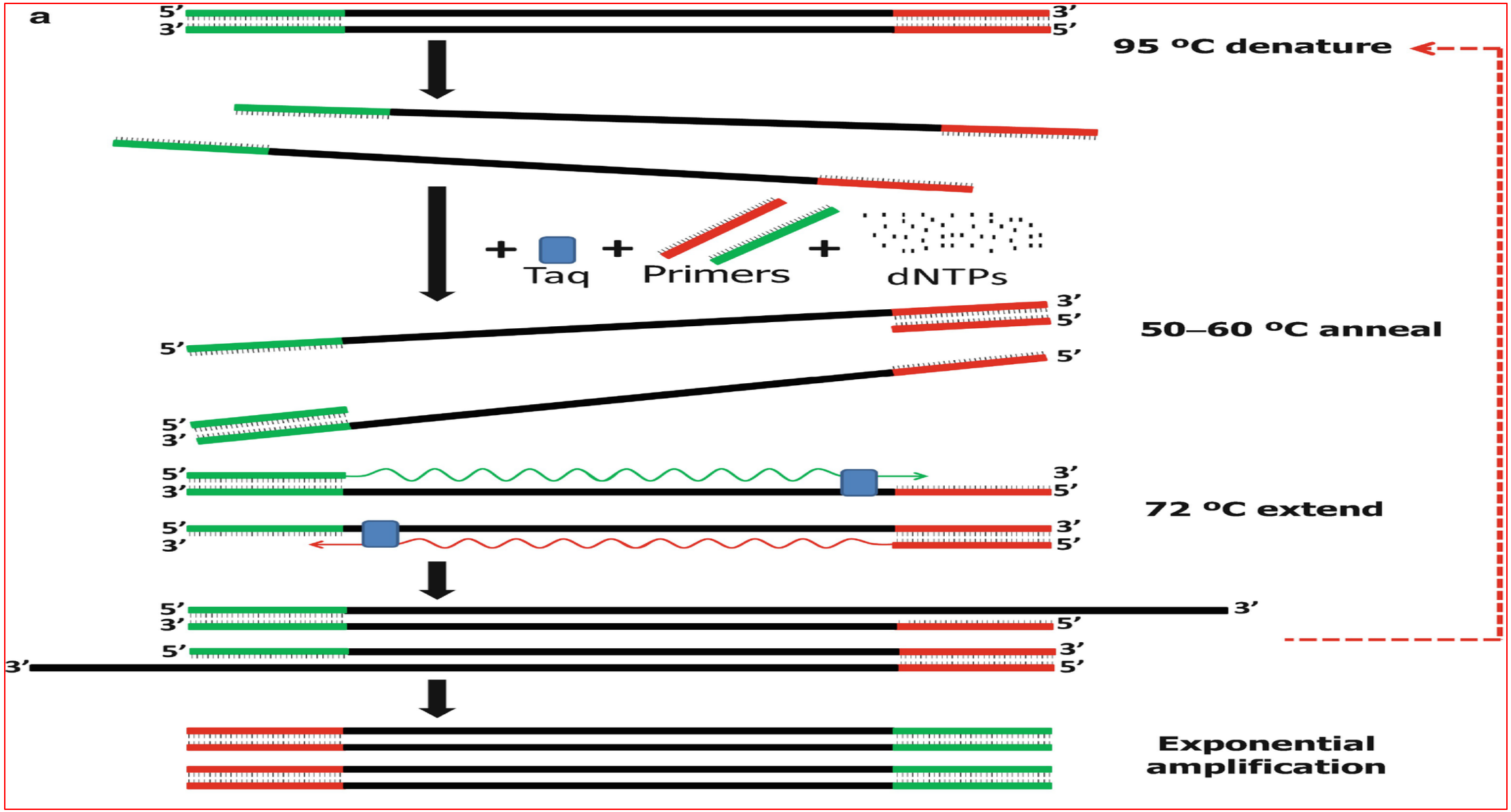
7. Nested PCR

- This method involve two PCR machines to amplify specific DNA segment
- In the first PCR the outer primer set is used to amplify the segment to generate DNA product along with some nonspecific sites
- In the second PCR the nested or internal primers are used which pair only the specific regions of interest
- The second product will be shorter than the first one


Outer primer R
Outer primer F
Non specific binding
Taget DNA region
First round of PCR
Inner primer F
Inner primer R
© Genetic Education Inc.
Non specific amplification
Amplification from the first round of PCR
Non specific amplification
Second round of PCR
Amplification from the second round of PCR

8. AFLP PCR

- It is a highly sensitive PCR based method used for detection of polymorphism in DNA
- DNA digestion with tetra cutter (Msel) and a hexa cutter (EcoRI)
- Ligation of linkers to all restriction fragments
- Amplification using restriction site specific primers
- Electrophoresis and visualization of bands
- It can also be used for genotyping individuals for a large number of loci


a
5′
3′
3′
5′
95 ºC denature
+
Taq
+
Primers
+
dNTPs
3′
5′
50–60 ºC anneal
5′
5′
3′
5′
3′
3′
5′
72 ºC extend
5′
3′
3′
5′
5′
3′
3′
5′
5′
3′
3′
5′
Exponential
amplification

9. Methylation Specific PCR (MSP PCR)

- It is used to identify the patterns of DNA methylation at cytosine- uanine (CpG) islands in genomic DNA
- Target DNA is the first treated withsodium bisulphate which converts unmethylated cytosine bases to uracil, complementary to adenine in PCR
- After two amplifications one set anneals with cytosine and other set anneals with uracil
- The changes induced by bisulphate depends upon methylation status of DNA

10. Single cell PCR

- It is used to amplify and examine minute quantities of rare genetic material as single cell
- It is commonly used in prenatal diagnostics
- It is also useful for investigation of immunological, neurological and developmental problems

11. Colony PCR

- This is used for screening of bacterial (E.coli) or yeast clones for correct ligation or plasmid products
- Selected colonies are picked with a sterile toothpick or pipette and inserted into PCR master mix or autoclaved water
- PCR is than conducted to determine if the colony contains the DNA fragment or plasmid of interest

12. Box PCR

- Box elements are repetitive sequence elements in bacterial genome such as Streptococcus genome
- Single primer targeting the repeats can be used to fingerprint bacterial species

13. Competitive PCR

- This is a method used for quantifying DNA using real time PCR
- A competitor internal sequence is co amplified with the target DNA and the target is quantified from the melting curves of the target and its competitor

14. Touchdown PCR

- This is used to reduce nonspecific background by gradually lowering the annealing temperature as PCR cycling progresses
- Initially the annealing temperature is few degree (3-5) above the Tm which in later cycles a few degree lower (3-5) the primer Tm
- Higher temperatures give greater specificity to bind the primers and the lower temperatures permit more efficient amplifications

15. **Forensic PCR** – for comparison of DNA from different sources

16. **Hairpin PCR** – DNA amplification for mutation detection

17. **PCR ELISA** – PCR products are labeled (digoxigenin) during amplification. A capture probe is used to immobilize the amplicon to immune-well plate. ELISA is done against label (antidigoxigenin) to quantitate PCR product

Thank You…..