Course: M.Sc. Biotechnology

Paper: BIOT4009: Genetic Engineering and Gene Therapy

UNIT – III
Polymerase Chain Reaction-2

BRIJESH PANDEY
DEPARTMENT OF BIOTECHNOLOGY
MAHATMA GANDHI CENTRAL UNIVERSITY, BIHAR

Polymerase Chain Reaction

Sensitivity, precautions and optimization

PCR has ability to amplify single template: ideally
Require precautions

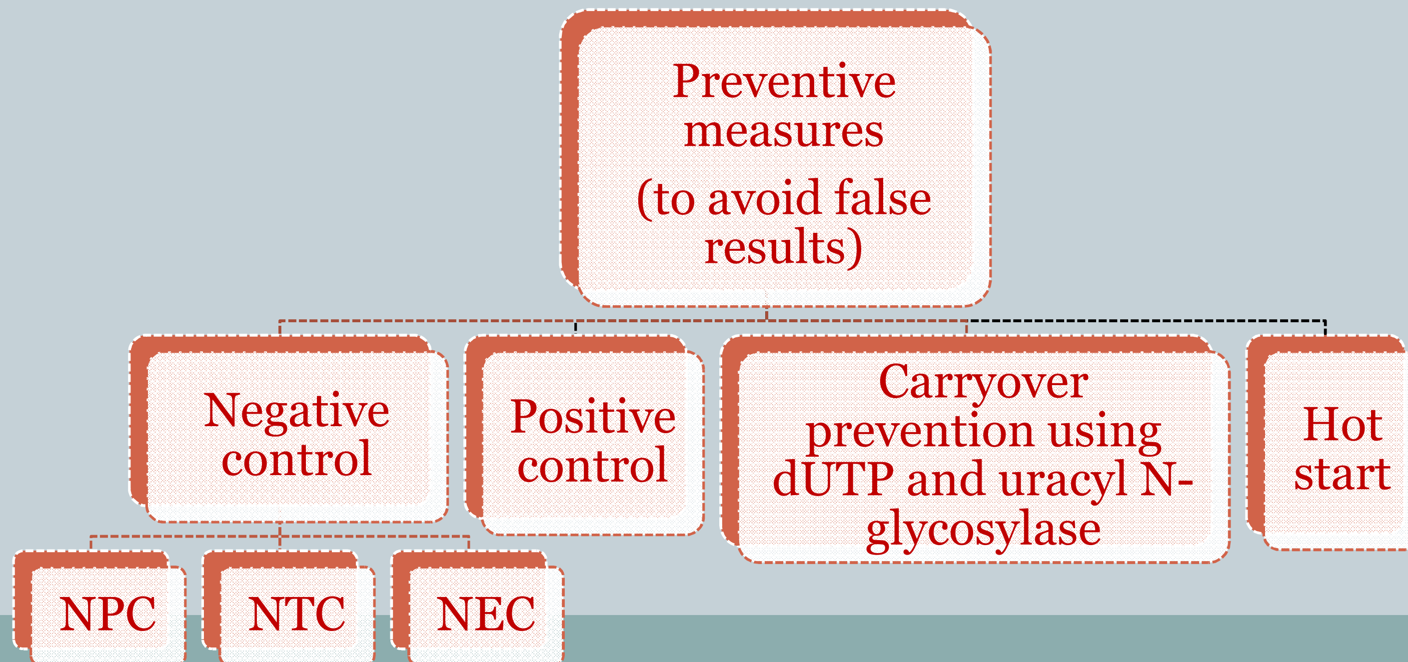
- Handling of tubes and components
- Precision in pipetting
- Avoidance of contamination, even aerosols
- Batch to batch variation in consumables
- Repetitive freeze thaw of dNTP mix

May require optimization for each template/ primer

Polymerase Chain Reaction

Sensitivity and optimization

Due to sensitivity, false results need to be avoided



Polymerase Chain Reaction

Optimization

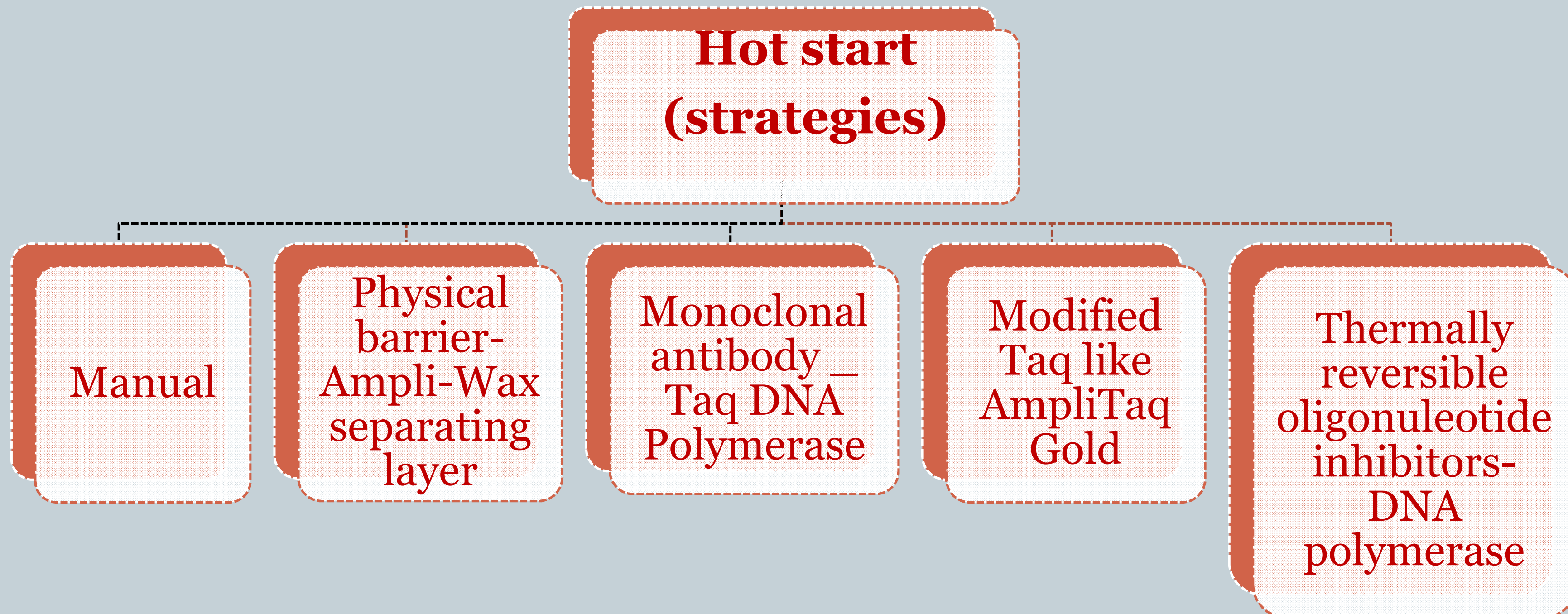
PCR reaction needs optimum conc. of ingredients and Ta

Reproducibility depends upon
Thermal profiling
Ramp rate
Reaction mixture

Optimization
- Annealing temperature
  - Touch down PCR
  - Gradient PCR
- $MgCl_2$ conc.
- dNTP con.
- Primer conc.
- Enzyme units

Polymerase Chain Reaction: PCR

Hot start: essential component is withheld until elevated temperature is reached



Polymerase Chain Reaction: PCR

PCR enzymes

Enzyme and source	Exonuclease	Fidelity	Stability (Half Life)	Remarks
Taq (Natural) *Thermus aquaticus*	5'-3'	low	40 min at 90°C/ 9 min at 97.5°C	Used in routine PCR experiment
Platinum Taq (Recombinant) *Thermus aquaticus*	3'-5'	High, 6 fold than *Taq*	-	Used in hot start
Amplitaq (Recombinant) *Thermus aquaticus*	-	low	21 min at 97.5°	Processivity is lower than full length Taq
Vent (Recombinant) *Thermococcus litoralis*	3'-5'	High, 5-15 fold than *Taq*	half-life of 23 hours at 95°C	Works with Difficult Templates: ideal for GC-rich or looped sequences

PCR enzymes contd.

Enzyme and source	Exonuclease	Fidelity	Stability (Half Life)	Remarks
Deep Vent (Recombinant) *Pyrococcus strain GB-D*	3'-5'	High 5-15 fold than Taq	~500 min at 100°C	----
Tth (Recombinant) *Thermus thermophilus*	5'-3'	low	20 min at 95°C	PCR, RT-PCR and primer extension, In the presences of Mn^{2+} RT activity enhanced,
Pfu (Natural) *Pyrococcus furiosus*	3'-5'	high	~240 min at 95°C	Used in PCR of high fidelity and primer extension
Pwo *Pyrococcus woesei*	3′→5′	high	> 2hr at 100°C	Generated blunt end product best suited for cloning

Many other enzymes are also used

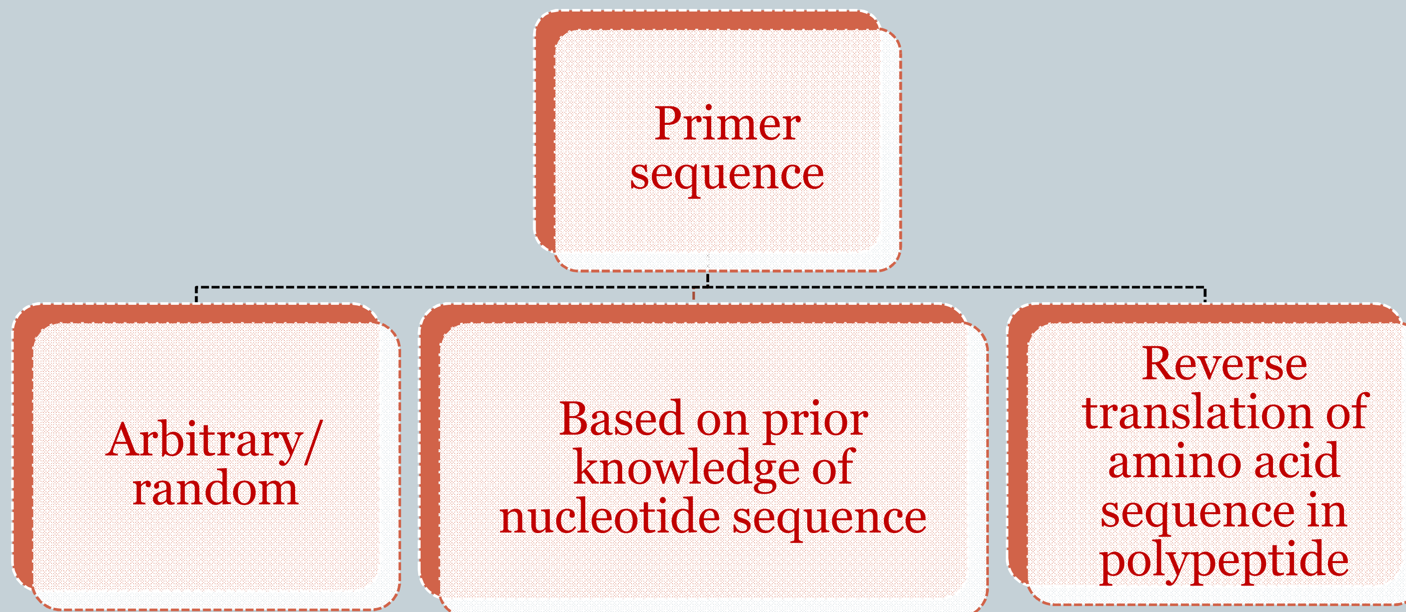
Polymerase Chain Reaction: PCR

Primers

Chemically synthesized ssDNA, complementary to template and has 3'OH

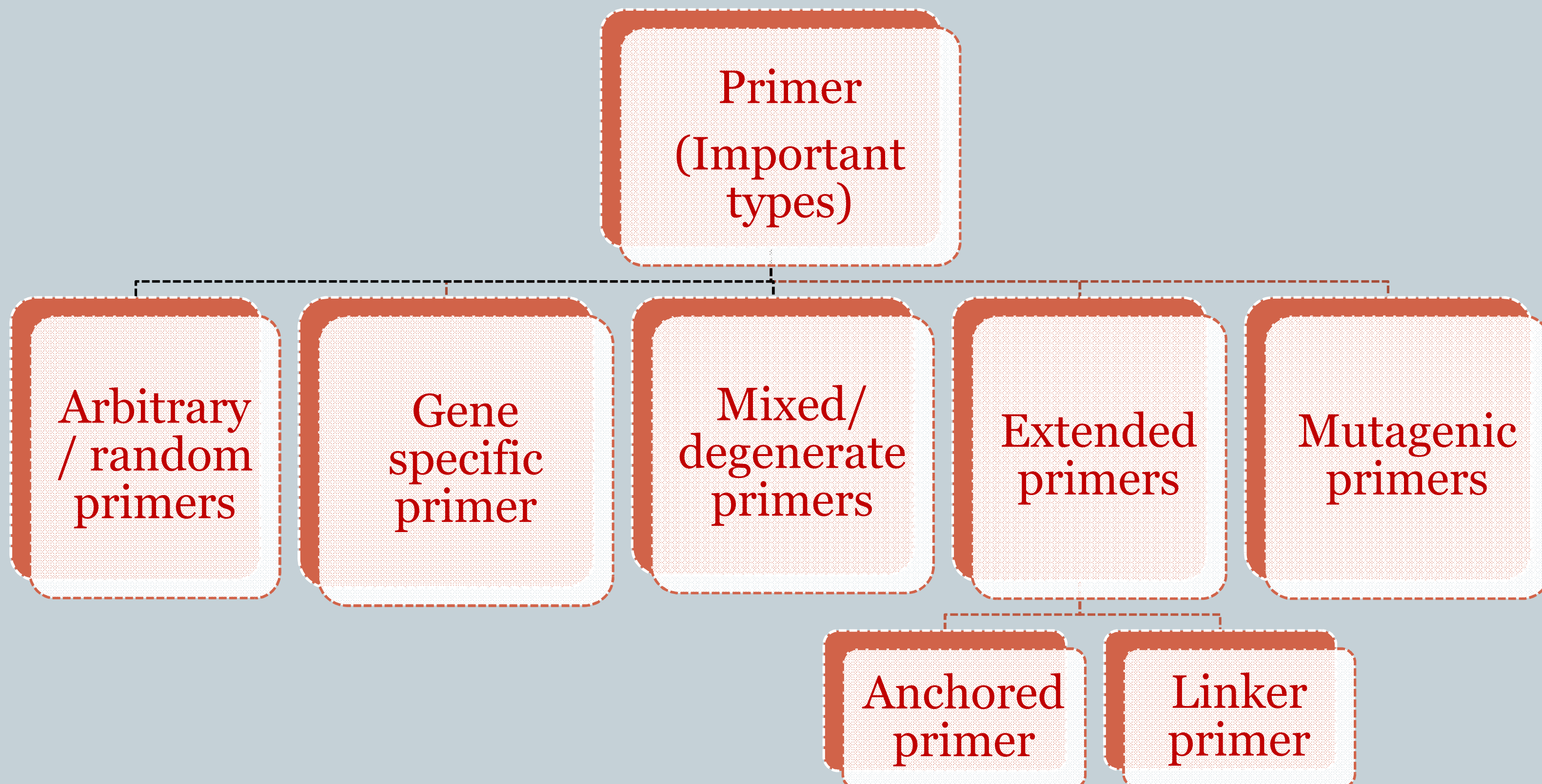
Required by DNA dependent DNA polymerase for elongation

May or may not have 5'P



Polymerase Chain Reaction: PCR

Primer types



Primer design: Optimization

Characteristics of primers:

Specificity
Specific for the intended target sequence (avoid nonspecific hybridization)

Stability
Form stable duplex with template under PCR conditions

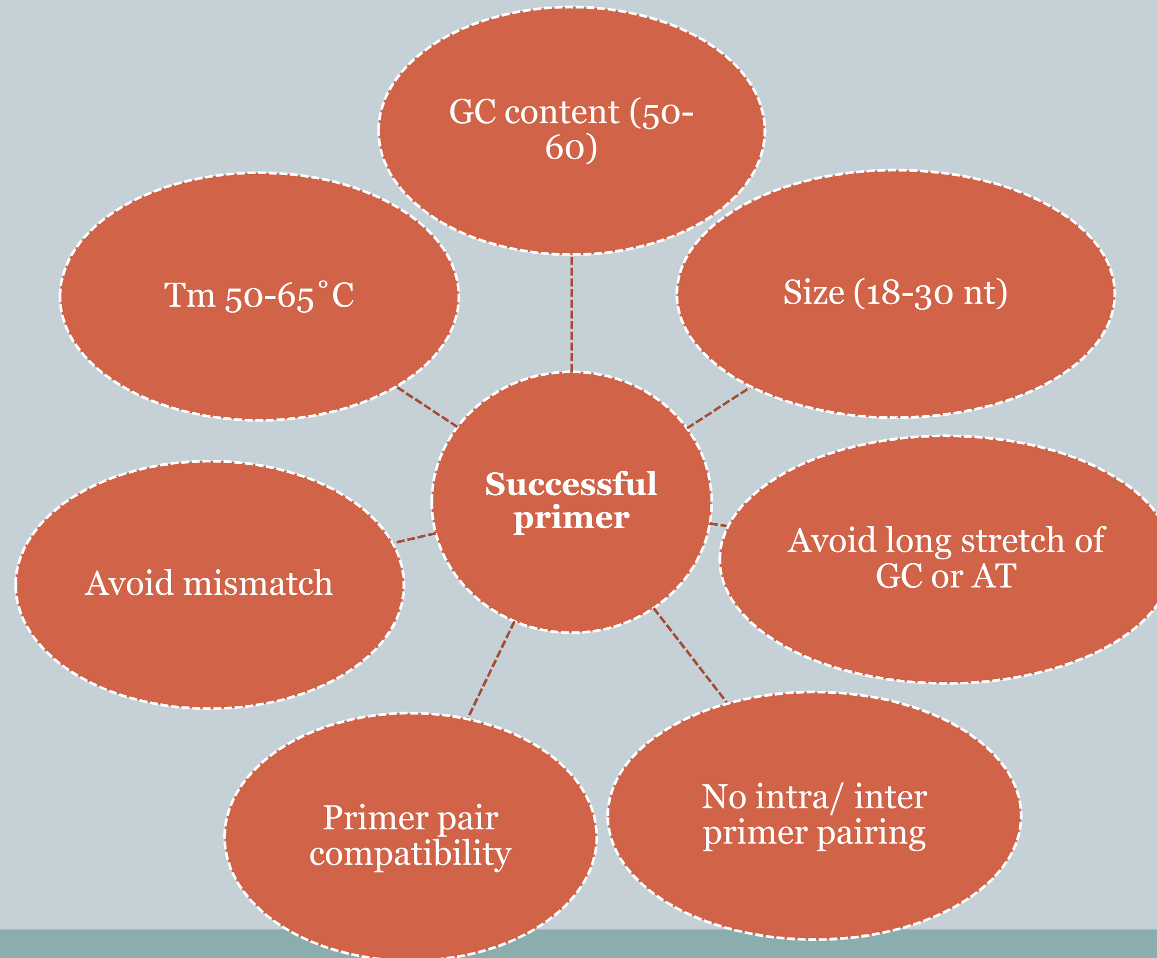
Compatibility
Primers used as a pair shall work under the same PCR condition

Thoughts on primer design:

Uniqueness
Length
Base Composition
Internal Stability
Melting Temperature
Annealing Temperature
Internal Structure
Primer Pair Matching

Polymerase Chain Reaction: PCR

Primer properties



Melting and annealing temperature

- Melting temperature (Tm): Temp at which half of the primers are single stranded
- Tm = 4(G+C) +2(A+T) °C
- Annealing temperature (Ta) is usually 3-5 °C less than Tm
- Also depends on Salt and buffer concentration other than template and primer
- It governs specificity and yield

Mismatch tolerance and advantages

❖3’ end of primer should not mismatch with template for successful PCR

❖Middle region or 5 ‘ region of primer may have mismatches

- Mutagenic primers in inverse PCR
  - For insertion of bases
  - For deletion of bases
  - For replacement of bases
  - Long primers etc.
- Linker primers for introducing restriction sites
- Anchored Primers for different purposes

Primer designing

Finding specific sequences (gene alignment/ databases) from 5 ‘ and 3’ regions of gene

Conserved/ specific sequence of 5’ as such is used as forward primer

Conserved/ specific sequence of 3’ side is converted to reverse complementary and then used as reverse primer.

Evaluation of primer properties (offline/ online tools)

- Length
- GC content
- Tm
- Ta
- Pair compatibility
- Primer- dimer possibility
- Stem loop formation within sequence

Home assignment

1. SEARCH ONLINE TOOLS FOR PRIMER DESIGNING
2. READ ITS TUTORIAL
3. DESIGN A PRIMER PAIR FOR SPECIFIC AMPLIFICATION OF ANY GENE YOU SELECT

GOOD LUCK

Thanks

To be continued