Course: M.Sc. Biotechnology

Paper: BIOT4009: Genetic Engineering and Gene Therapy

1

UNIT – III
POLYMERASE CHAIN REACTION-5

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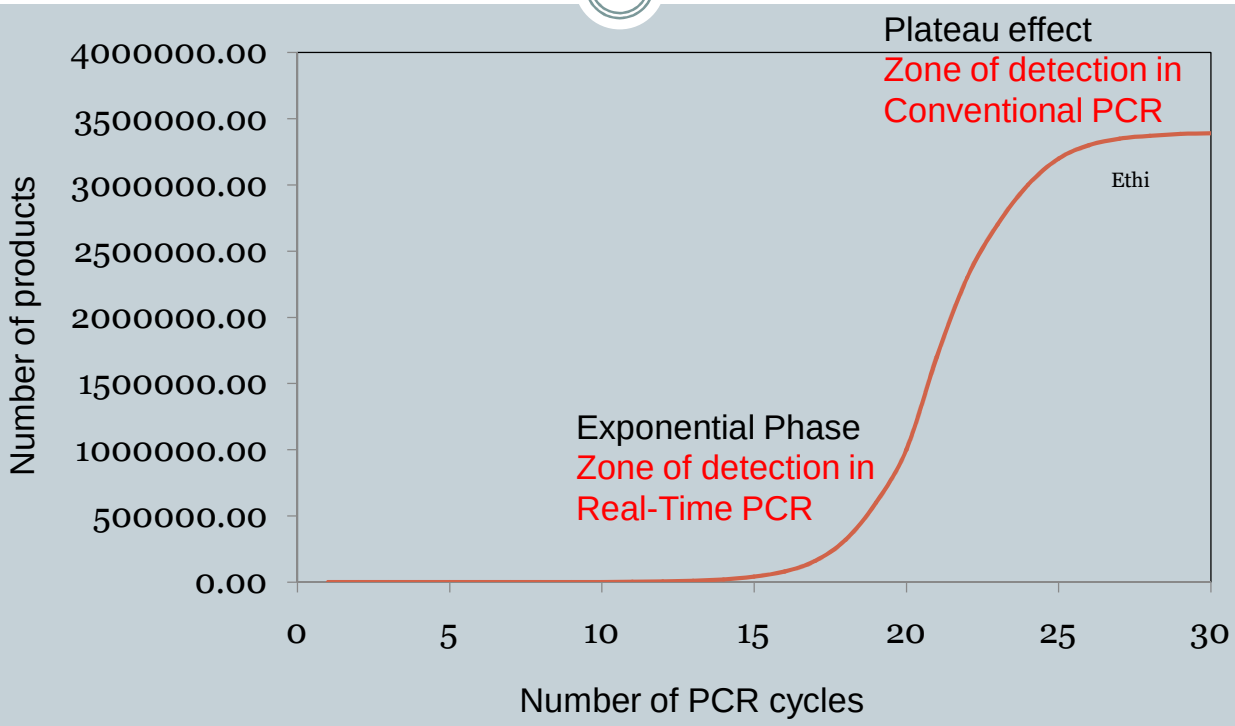
PCR kinetics

2



Some of the problems with End-Point Detection

3

- Poor Precision due to onset of plateau effect
- Onset of plateau effect may differ from sample to sample
- Low sensitivity due to ethidium bromide based detection
- Short dynamic range < 2 logs
- Low resolution
- Non - Automated
- Size-based clear discrimination only
- Results are not truly quantitative
- Post PCR processing is required

Real –Time PCR

4

- **Increase in product is monitored in Real Time of amplification, i.e. in exponential phase**
- **Real-time PCR has a thermal cycler coupled with fluorimeter**
- **It monitors the fluorescence emitted during the reaction as an indicator of amplicon production at each PCR cycle (in real time) as opposed to the endpoint detection**
- **Increase in fluorescence intensity is monitored by fluorescence emitting molecule**

Detection methods in Real-Time PCR

5

Detection Chemistry

Nucleic acid intercalating/binding dyes (Non-specific)

e.g. **SYBR Green**

Labeled probes/labeled primer-probes (Highly specific)

e.g. **TaqMan, Molecular Beacons, Scorpions etc.**

Features of SYBR Green

6

SYBR Green binds to the surface of DNA (MINOR GROOVE)

Absorption maxima ~312 nm and emission maxima at ~524 nm

(Fluorescence Of ssDNA : dsDNA :: 1:11);[doi: 10.1093/nar/gnh101]

Free Dye: dsDNA Bound dye fluorescence:: 1:>1000 [doi: 10.1007/s10895-012-1059-8]

Fluorescence is also influenced by salt and viscosity.

Fluorescence is related to amount of bound dye which is dependent on amount of dsDNA

With increase in DNA in progressing cycles, bound dye and fluorescence increases

SYBR Green chemistry: Advantages and disadvantages

7

- **Advantages:**

✓ **Relatively low cost of primers**

✓ **No fluorescent-labeled probes are required**

- **Disadvantages:**

✓ **Non- specific and can bind and fluoresce to any dsDNA.**

✓ **Only primers determine specificity**

✓ **Requires extensive optimisation**

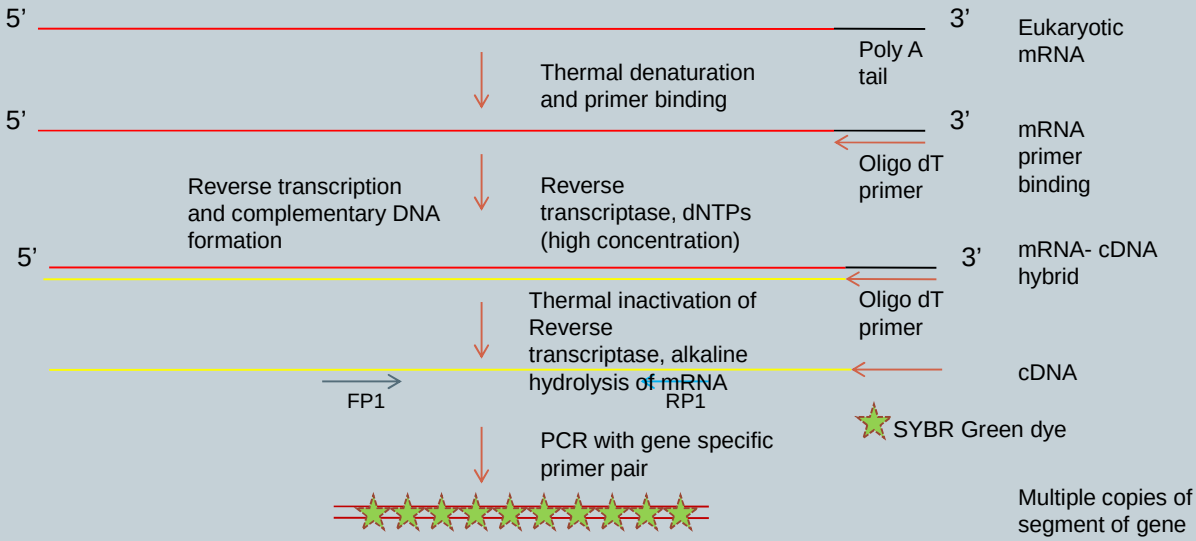
✓ **Multiplexing is not possible**

Gene expression quantitative analysis

8

Basics of Reverse Transcription PCR: Recap

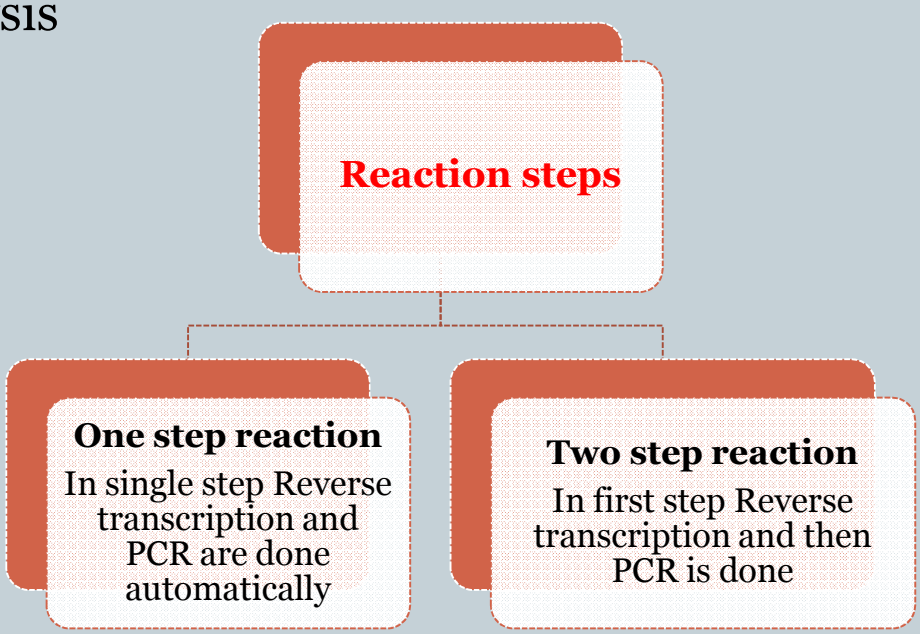
- mRNA is reverse transcribed by reverse transcriptase
- Complementary DNA thus formed in subjected to PCR with gene specific primer pairs (FP1 and RP1 may be homo or heterologous forward and reverse primers)



Types of Reaction setup

9

Strategies of SYBR Green mediated Gene expression analysis



Few important aspects of Real-Time PCR

10

Over a broad range, the amount of fluorescence is directly proportional to DNA concentration

Amount of initial template is calculated by Ct (Threshold cycle: The cycle at which statistically significant fluorescence is first detected above the baseline or background)

The linear correlation between PCR product and fluorescence intensity is used to find initial template

Thanks

11

TO BE CONTINUED