Course: M.Sc. Biotechnology

Paper: BIOT4009: Genetic Engineering and Gene Therapy

UNIT – III
POLYMERASE CHAIN REACTION-6

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Beyond fluorescence in SYBR Green method

- Non specific amplification (mistargetted product, primer dimers) also adds to the fluorescence due to bound SYBR Green
- Based on fluorescence, genuine/ ingenuine product formation can not be differentiated/ confirmed
- Melting curve analysis may differentiate between primer dimer and genuine product in Real-Time PCR.
- It is temperature dependent dissociation characteristic of dsDNA which is dependent upon length and GC content of product

Melting Curve analysis

- **Shape of melting curve indicates about homogeneity of product**
- ***Tm* indicates reassurance of genuine product**
- **Primer dimers (PDs) generate noise in resulting curve**
- **PDs exhibit denaturation at lower temperature**
- **Genuine PCR amplicons show denaturation at higher temperature**

Fluorescence Probe based estimation

- Phenomenon of FRET (Fluorescence Resonance Energy Transfer)
- Fluorescence donor and fluorescence acceptor are in close proximity, the fluorescence emitted by fluorophore is quenched by quencher and no net gain in fluorescence is observed.
- These two molecules are separated by defined number of nucleotides in FRET probe.

TaqMan probe method

20-24 bases long oligonucleotide specific to sequence between two primer binding sites on template

At 5’ end linked to fluorescence donor (FAM/ TET/HEX etc.) and at 3’ end linked to quencher (TAMRA etc.)

During annealing and initiation of extension, when probe is intact the FRET remains operative and no fluorescence is added to the system

With elongation of primer when complementary strand reaches 5’ of probe, 5’ -3’ exonuclease activity of Taq DNA polymerase removes 5’ labeled nucleotide having fluorophore.

It increases the distance between the fluorophore and quencher and FRET breaks leading to increased fluorescence

After each cycle DNA copy increases, bound probe increases and thus upon release of fluorophore fluorescence increases.

Properties of TaqMan probes

- ✓ 15-30 (sometimes 24-30) Oligonucleotide probe
  Labeled with a reporter dye on the 5’ end (**FAM,VIC, HEX,JOE etc.)**
  And a quencher dye on the 3’ end. (**BHQ- 1,2,3, TAMRA**)
- ✓ Tm value 5-10℃ above the Tm of the primer
- ✓ G+C content 30-80%
- ✓ More Cs than Gs and no G at the 5' end

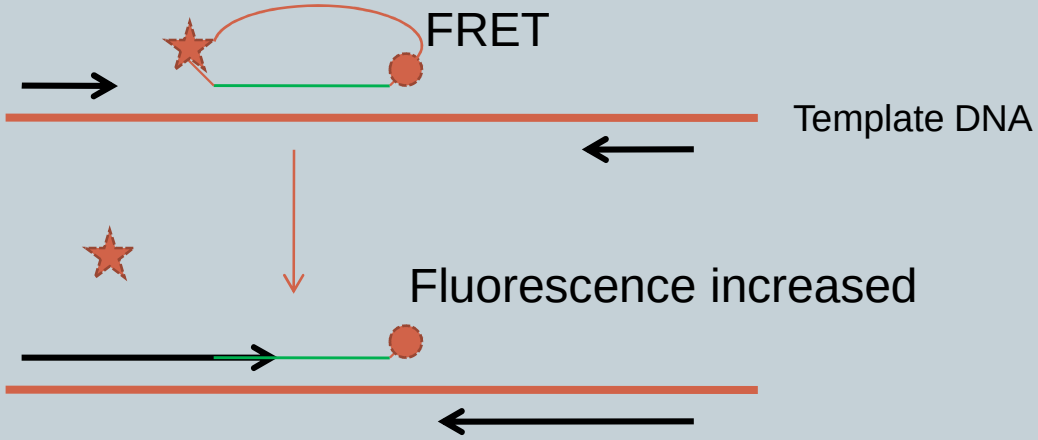
Molecular Beacons

Non degradative mechanism

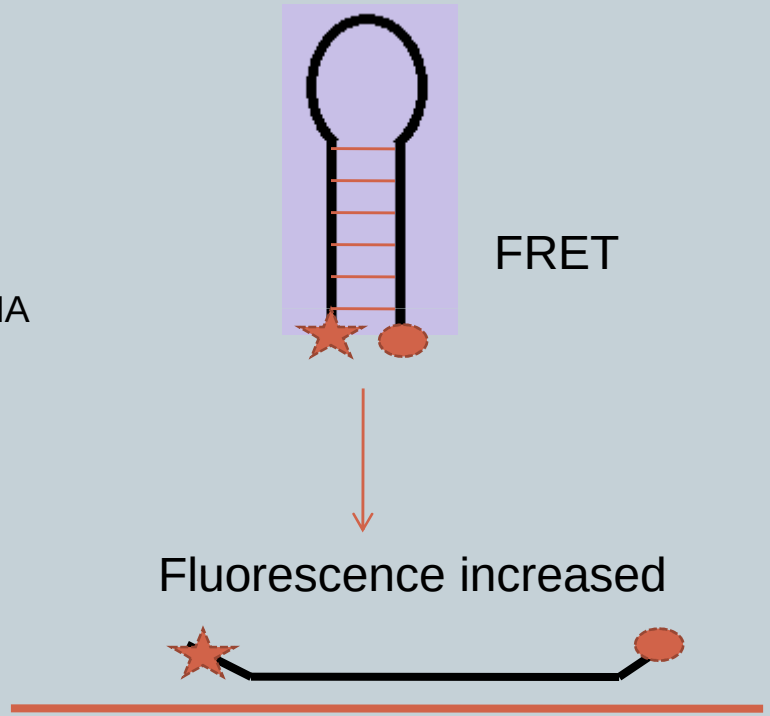
- ✓ Hairpin-shaped oligonucleotide (form stem-loop sequence) molecule that has a fluorophore and a nonfluorescent quencher dye attached to the 3' and 5' ends
- ✓ Beacons hybridizes to the target
- ✓ Loop 17-21 nt, Stem 5-8bp
- ✓ Tm is 8-10°C higher than the Tm of the primers
- ✓ >80% GC rich to form a stable complex

Fluorescence probe

TaqMan Probe



Molecular Beacons



Fluorophore
Quencher

Absolute quantification

- **Absolute standard curve for each target is constructed** (based on a serial dilution of a standard sample with known copy number)
- **Ct of each standard sample is plotted against the logarithm of the known concentration**
- **The standard curve is then used to estimate concentrations of unknown samples**

Method: Gene expression analysis

Absolute quantification/ comparative expression analysis

$$\Delta C_{T(test)} = C_{T(target,\ test)} - C_{T(ref,\ test)}$$

$$\Delta C_{T(calibrator)} = C_{T(target,\ calibrator)} - C_{T(ref,\ calibrator)}$$

$$\Delta\Delta C_T = \Delta C_{T(test)} - \Delta C_{T(calibrator)}$$

$$2^{-\Delta\Delta C_T} = \text{Normalized expression ratio}$$

The $2^{-\Delta\Delta CT}$ (Livak) method

$$\text{ratio} = \frac{(E_{target})^{\Delta Ct\ target\ (control-treated)}}{(E_{ref})^{\Delta Ct\ ref\ (control-treated)}}$$

The Pfaffl method

Thanks

TO BE CONTINUED