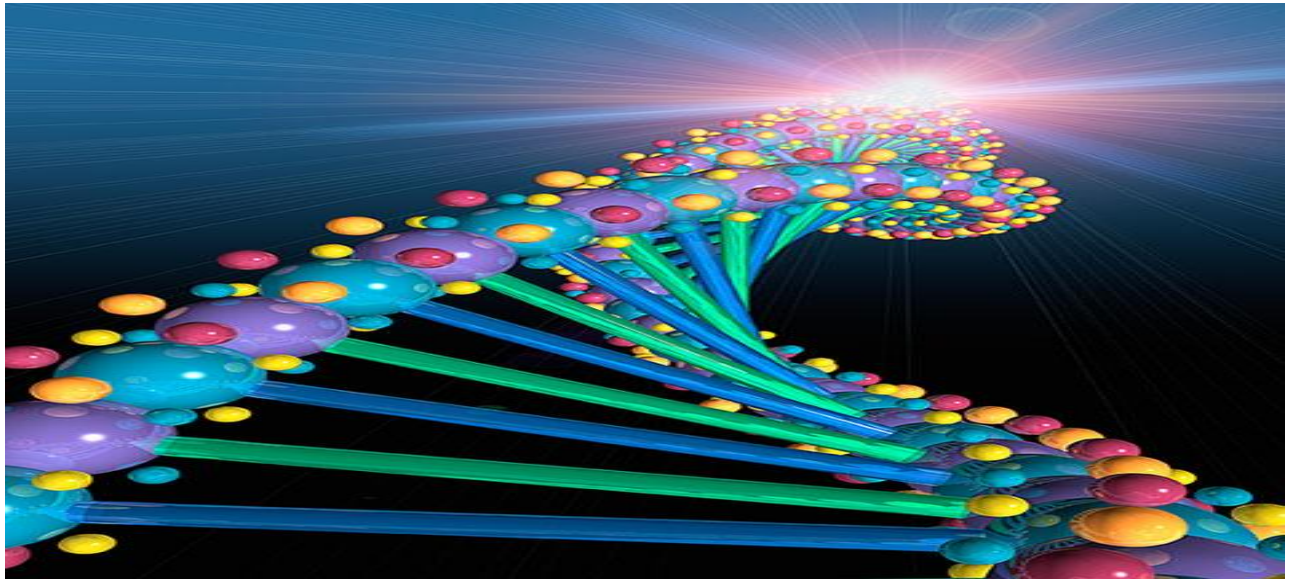
VirginiaTech
Invent the Future

RT-PCR

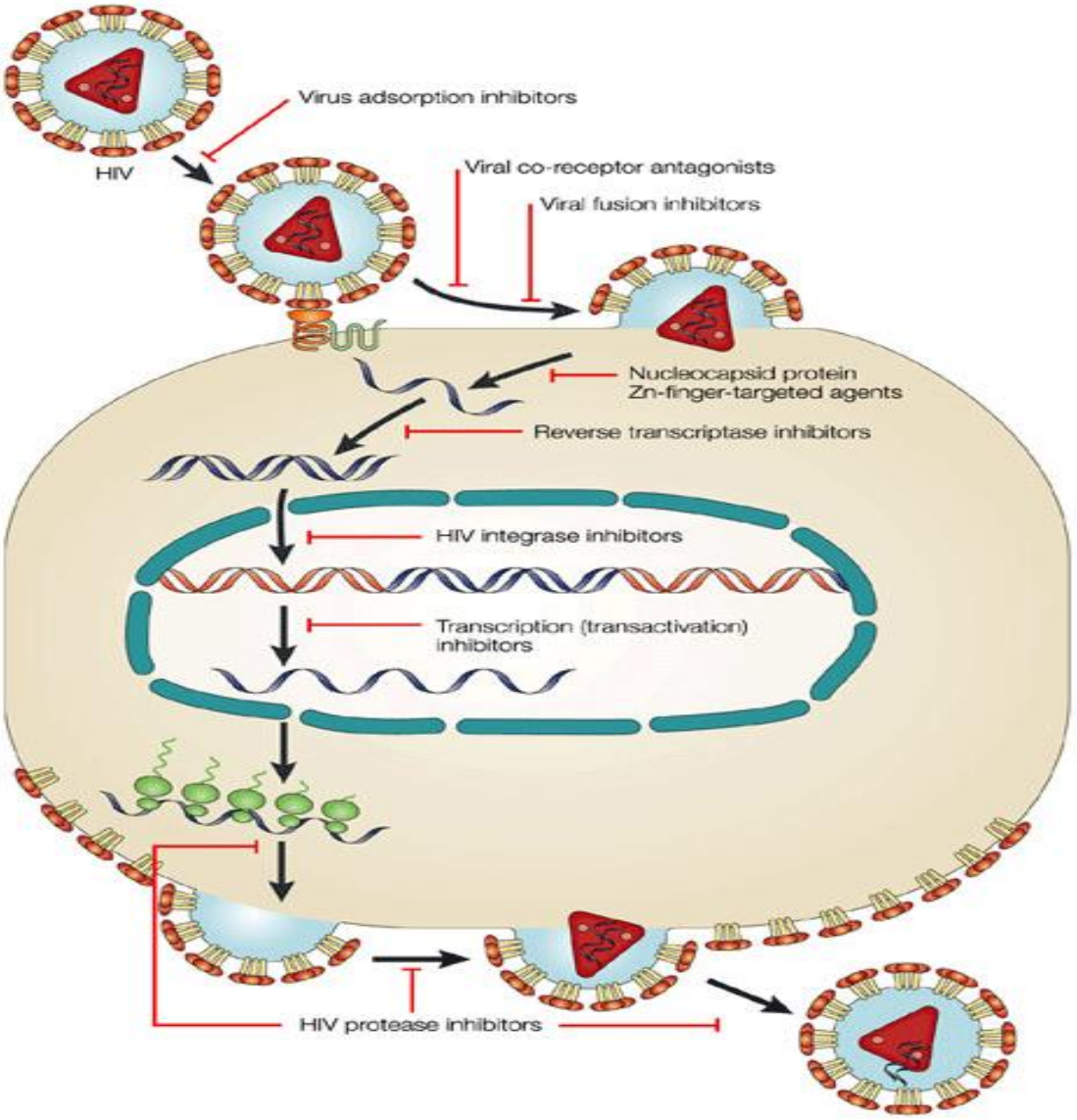


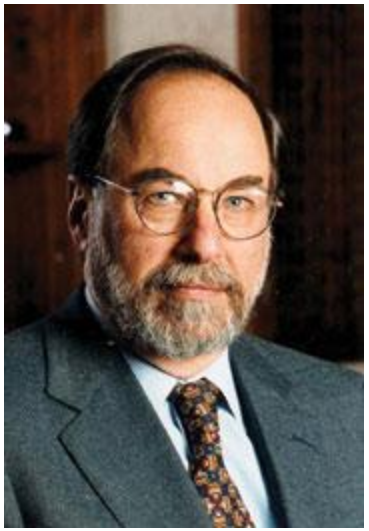
Mohamed N. Seleem

RT-PCR

- Reverse Transcriptase PCR (RT-PCR)
- Real Time PCR (*q*RT-PCR)

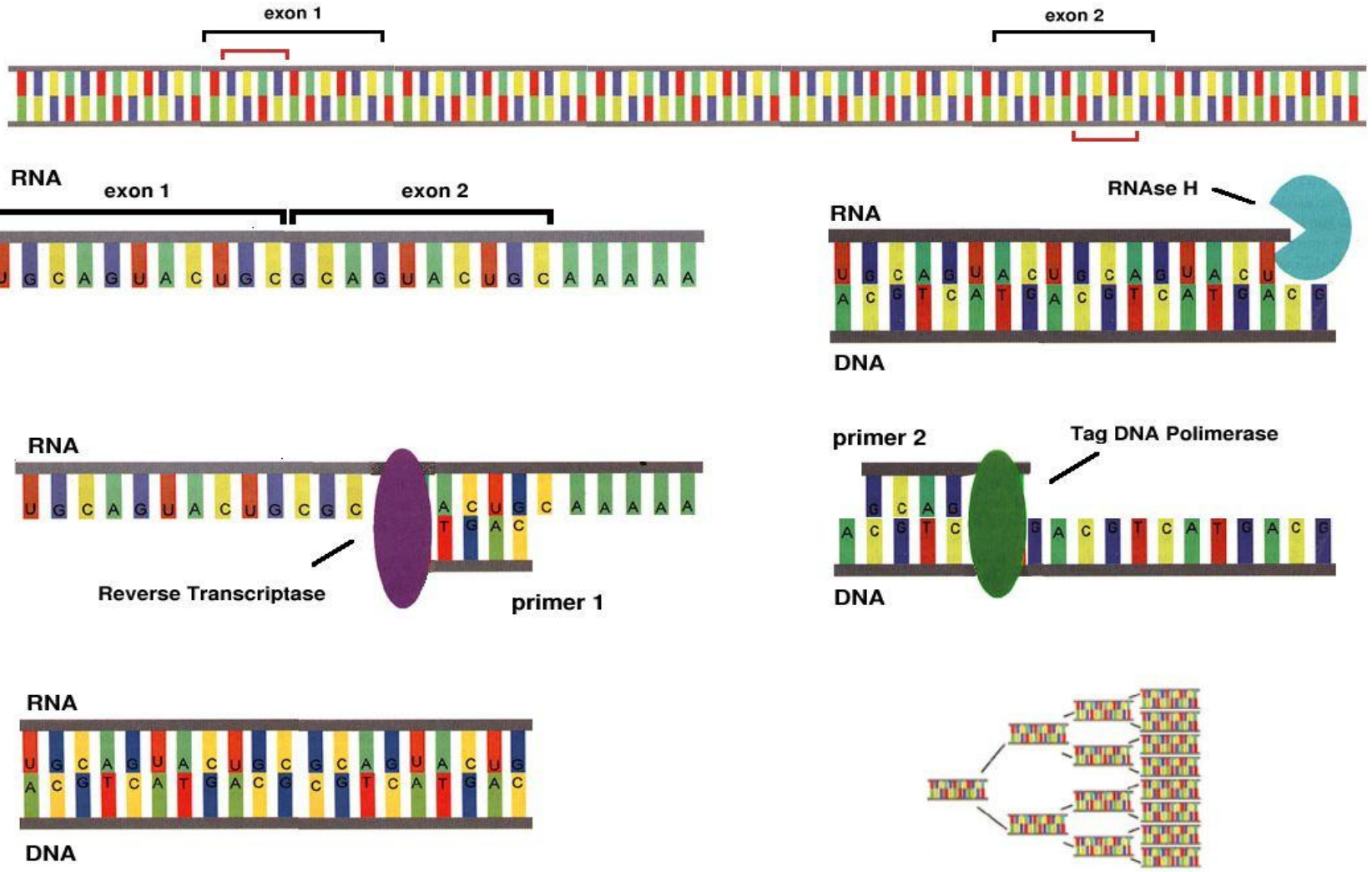
HIV– Reverse transcriptase





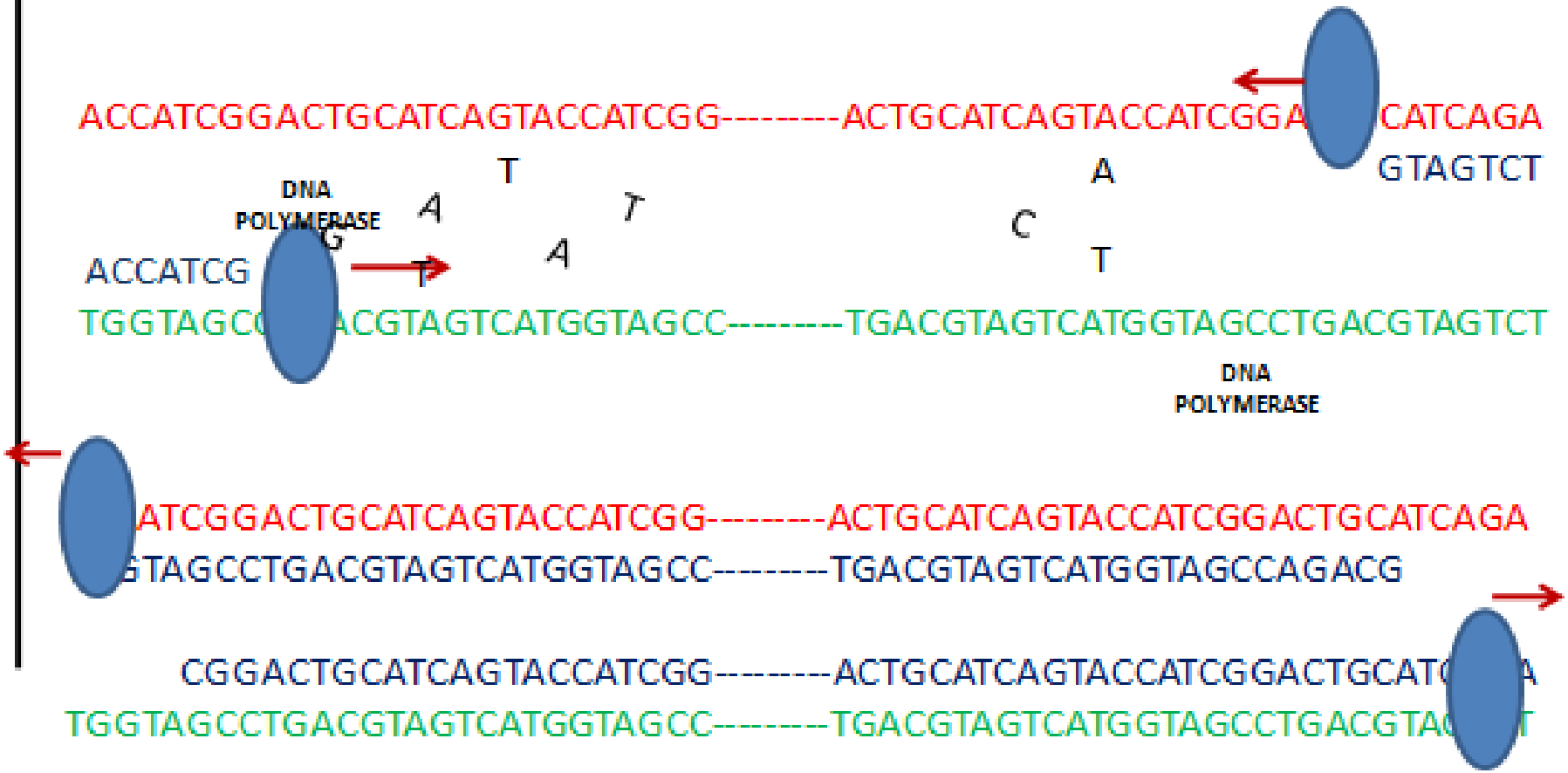
David Baltimore
1975 Nobel Prize
in Medicine

RT–PCR= reverse transcriptase PCR



PCR Cycles

- **Extension** — 72 °C for XXX sec

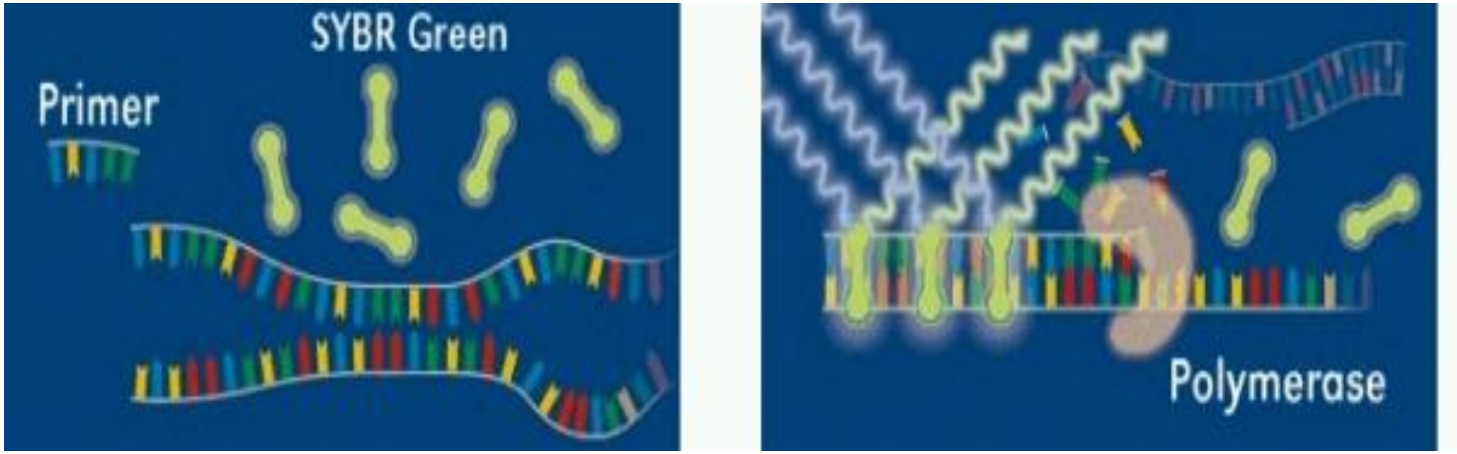


Disadvantages for PCR

- Gel electrophoreses
- Limit of detection
- Time consuming
- Not easy to quantify

SYBR Green

- The accumulation of double-stranded DNA, as indicated by a fluorescent dsDNA-binding dye (SYBR Green I), can also be used to quantitate the accumulation of a PCR product in a QPCR assay. SYBR Green I binds to the minor groove of the DNA double helix with a higher affinity for dsDNA than for single-stranded DNA (ssDNA) or RNA. In solution, the unbound dye exhibits very little fluorescence. However, fluorescence is greatly enhanced (1000-fold) upon DNA-binding making this dye a sensitive indicator for the quantity of dsDNA present in the reaction mixture at any given time.



the dye only fluoresces when bound to the dsDNA (i.e., the PCR product).

- However, specific and non-specific double-stranded PCR products generate the same fluorescence signal upon binding SYBR Green I dye. To distinguish between fluorescence derived from specific and non-specific products, SYBR Green I dye-based QPCR assays include a dissociation curve following the amplification reaction. During the dissociation curve step, dsDNA product is melted into ssDNA by a stepwise increase in temperature, with fluorescence data being collected at each step. The magnitude of the reduction in fluorescence intensity at the melting temperature of the specific PCR product of interest provides a qualitative indicator of the proportion of dsDNA attributable to the specific PCR product.

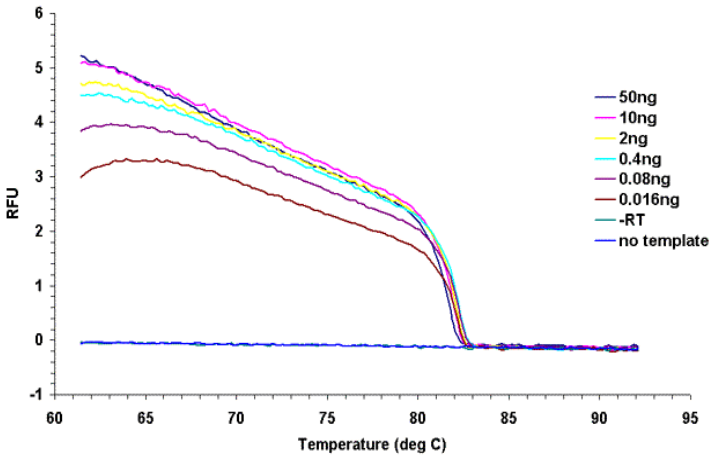
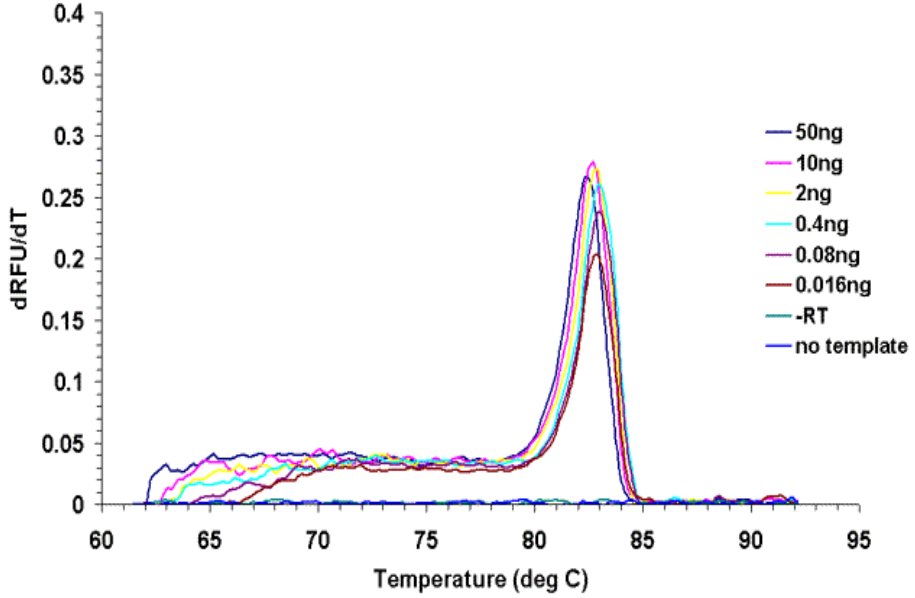
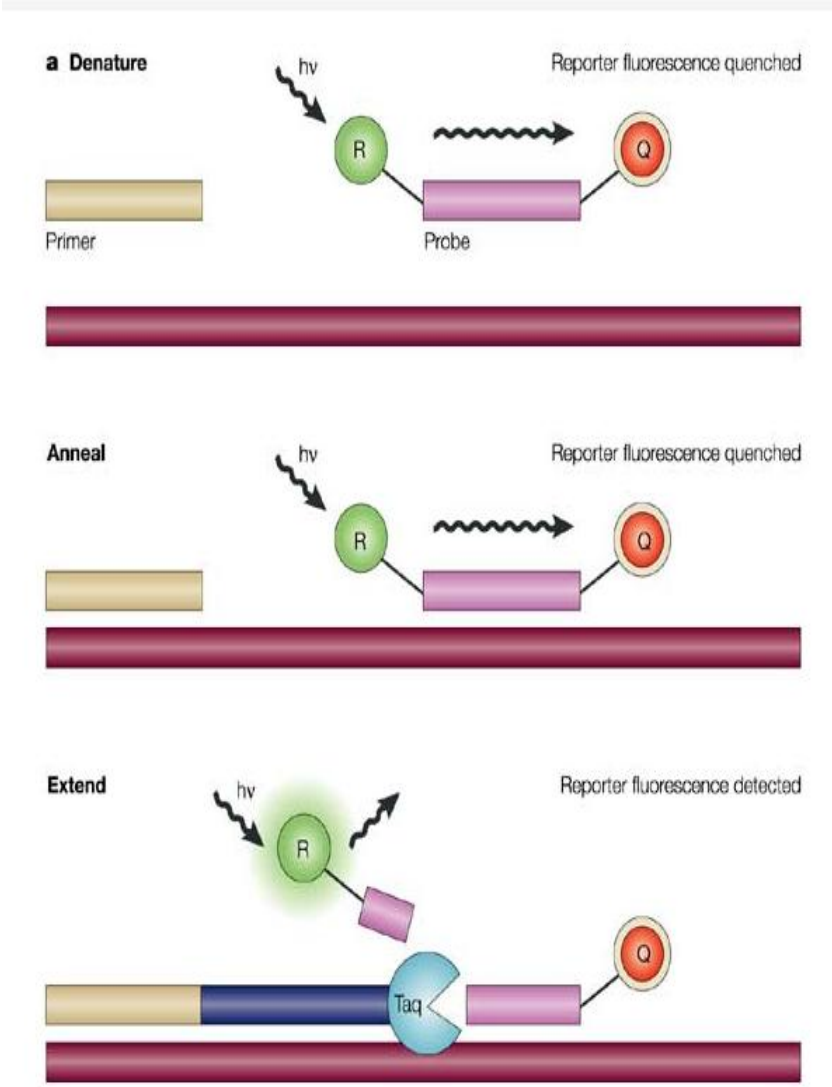


Figure 3: Melting Curve for Standard Curve Samples in Real Time for GAPDH



TaqMan Probes

- Fluorescence-labeled oligonucleotides (TaqMan® probes) are used for QPCR detection when the most accurate quantitation of PCR product accumulation is desired.
- TaqMan probes are linear oligonucleotides designed with the fluorophore and quencher in close proximity at opposite ends of the oligonucleotide. Probe molecules are cleaved during the amplification process to physically separate the fluorophore from the quencher.
- TaqMan probes are complementary to a region of the target gene located between the upstream and downstream primer binding sites.
- As the DNA polymerase extends the upstream primer, it encounters the bound probe. The 5' to 3' exonuclease activity of the polymerase cleaves the probe, releasing the fluorophore into solution, where it is allowed to fluoresce. As a result, the amount of fluorescence at any given cycle is directly proportional to the amount of specific product present at that time.
- The existence of a variety of spectrally distinct fluorophores available for labeling TaqMan probes introduces the possibility of multiplexing, or quantitating multiple targets with different probes in the same reaction well.



Method Comparison

TaqMan Probe

Advantages:

- Increased specificity due to presence of primers AND probe.
- Use when the most accurate quantitation of PCR product accumulation is desired.
- Option of detecting multiple genes in the same well (multiplexing).

Disadvantages:

- Relative high cost of labeled probe.

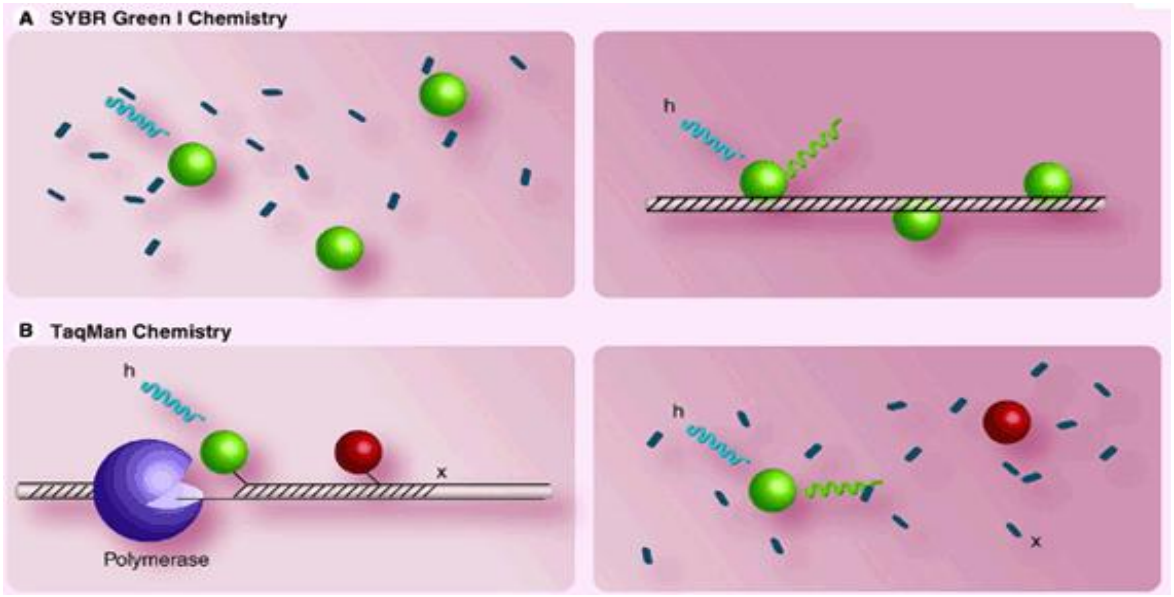
SYBR Green

Advantages:

- Relative low cost of primers.
- No fluorescent-labeled probes required.

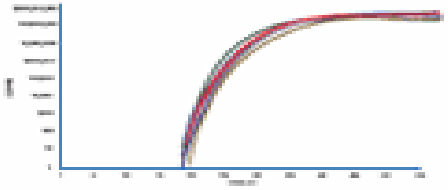
Disadvantages:

- Less specific – only primers determine specificity.
- Specific and non-specific double-stranded PCR products generate the same fluorescence signal upon binding SYBR Green I dye.
- Not possible to multiplex multiple gene targets.

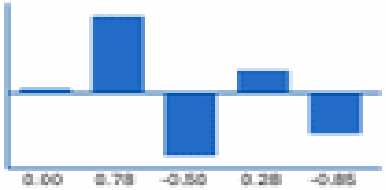


qRT–PCR vs PCR

More sensitive



More Quantitative



Faster—no gel needed

Safer—no ethidium bromide or radioactivity

Real Time PCR (*q*RT-PCR)

Rapid results

Time to result is dependent on organism load, In some cases, results may be available in <30 minutes (Mobile clinics, outreach programs, etc)

Multiplex testing

Simultaneous detection of multiple organisms from a single specimen (Detect multiple targets of the same organism)

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Questions!!!