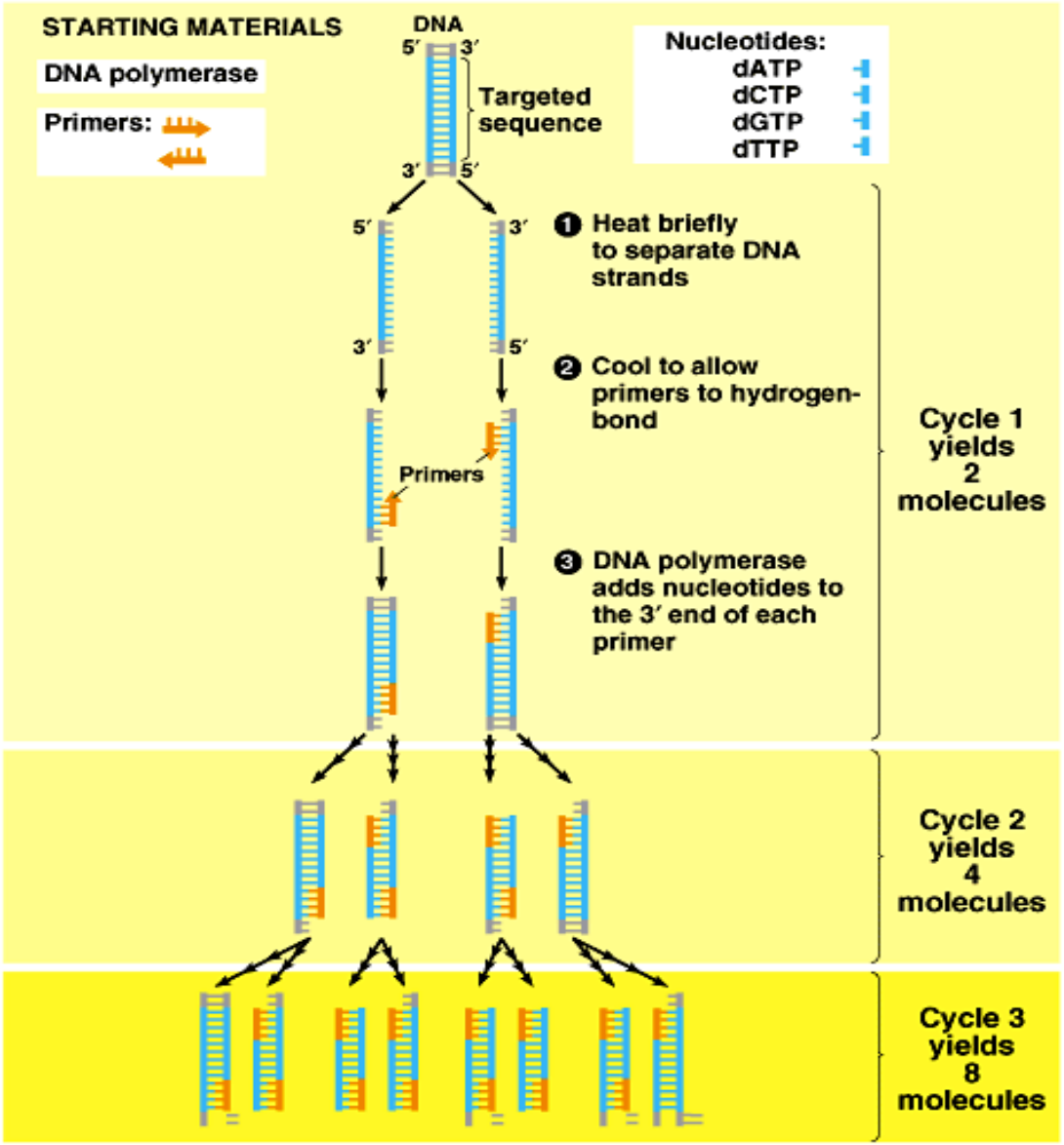
REAL TIME PCR

Source- C:\Users\admin\Documents\Ritu\Real-Time PCR\Real Time PCR Tutorial.mht

- PCR- Recap
- Real-time PCR (machine and detection)
  - Detection Ways
    - SyBr Green
    - Taqman Probe
    - Molecular Beacons
- PCR vs Real-Time PCR
- Reading the Graphs
    - X and Y axis
    - Threshold and Ct value
    - Melting Temperature
    - Controls, Normalization Controls, Efficiency and Slope
    - Quantification- Absolute and Relative Quantification
- Precautions
- Uses

Copy DNA without plasmids? PCR!

- Polymerase Chain Reaction
- Rapid procedure for in vitro enzymatic amplification of a specific segment of DNA
- It takes place in three steps:
- Denaturation at 94C
- Annealing of Primers at 50-65C
- Extension by DNA polymerase at 72C
- A total of 25-40 cycles.



Good primer (pair) properties

Primers should have

- 18-24 bases
- 40-60% G/C
- Balanced distribution of G/C and A/T bases
- Avoid runs (≥4) of an identical nucleotide (especially G's)
- Tm that allows annealing at 55-65 °C
- No internal secondary structures (hair-pins)
- The five nt at the 3'end should have no more than two G and/or C bases

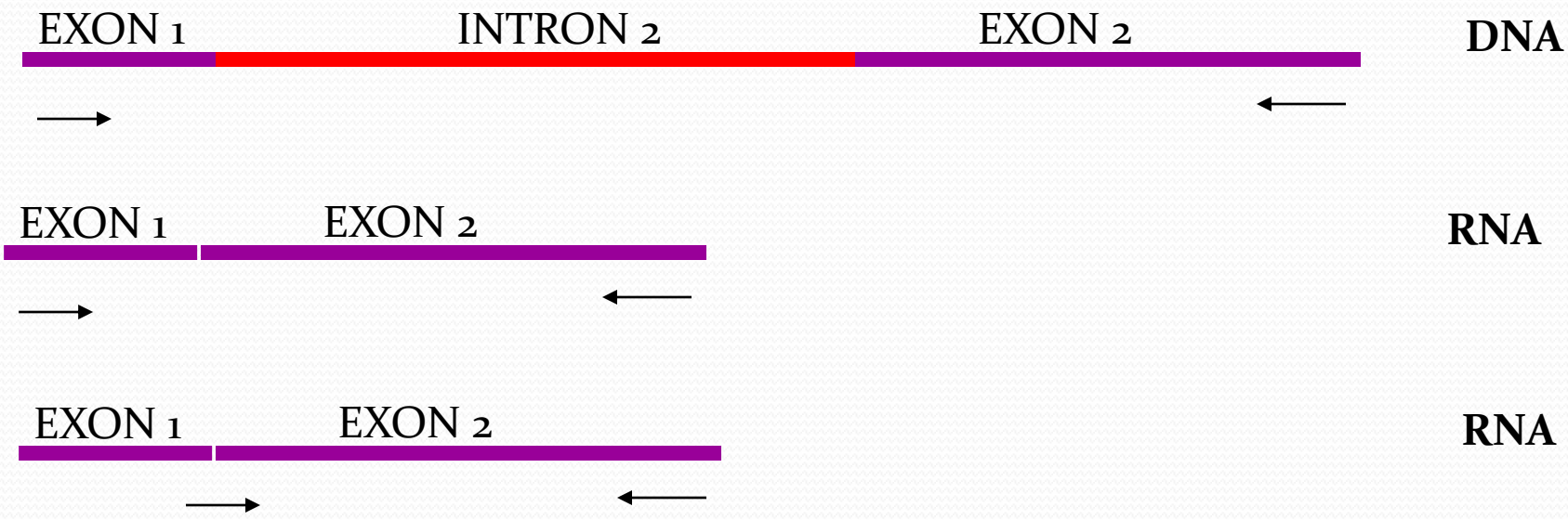
Primer pairs should have

- Similar melting temperatures, T_m , within 2-3 °C
- No significant complementarity (> 2-3 bp), particularly not in the 3'-ends

Reverse transcription polymerase chain reaction- RT-PCR

- **Reverse transcription polymerase chain reaction** (**RT-PCR**) is a variant of polymerase chain reaction (PCR), commonly used in molecular biology to generate many copies of a DNA sequence, a process termed "amplification". In RT-PCR, however, RNA strand is first reverse transcribed into its DNA complement using the enzyme reverse transcriptase, and the resulting cDNA is amplified using traditional or real-time PCR..

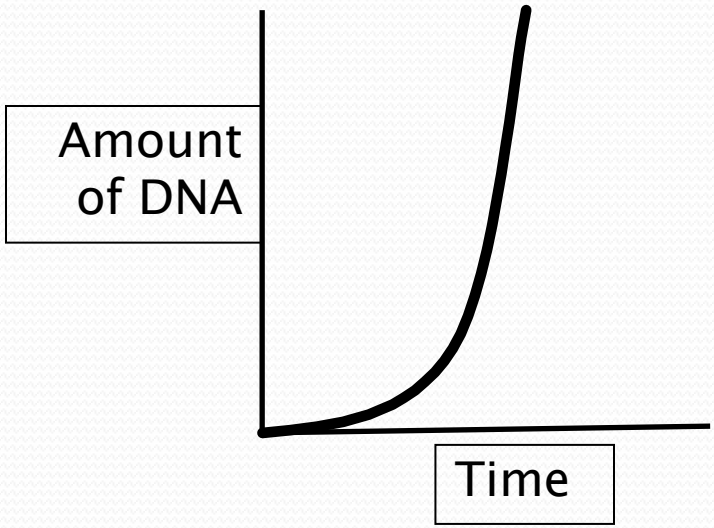
Consideration while designing primers for detection of a transcript

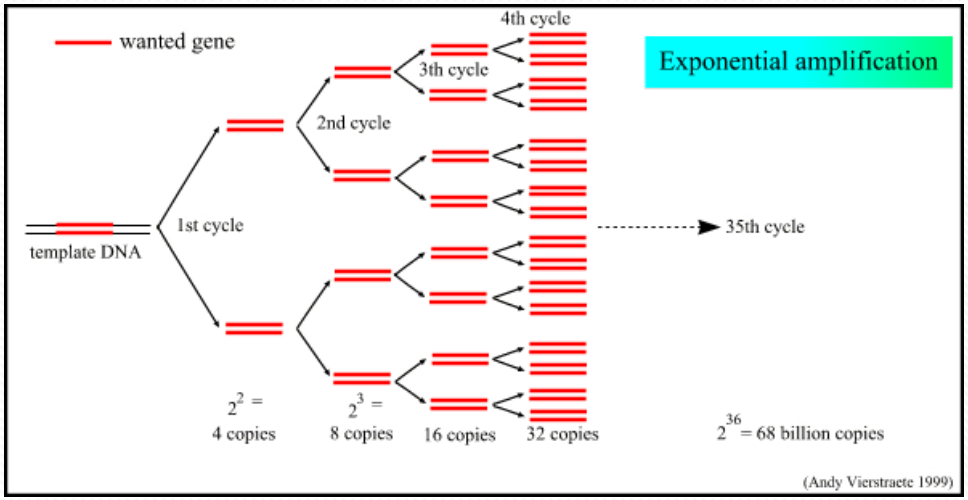


Utilize the differences in the DNA and RNA (lack of introns)
Design primers spanning the intron-exon boundary

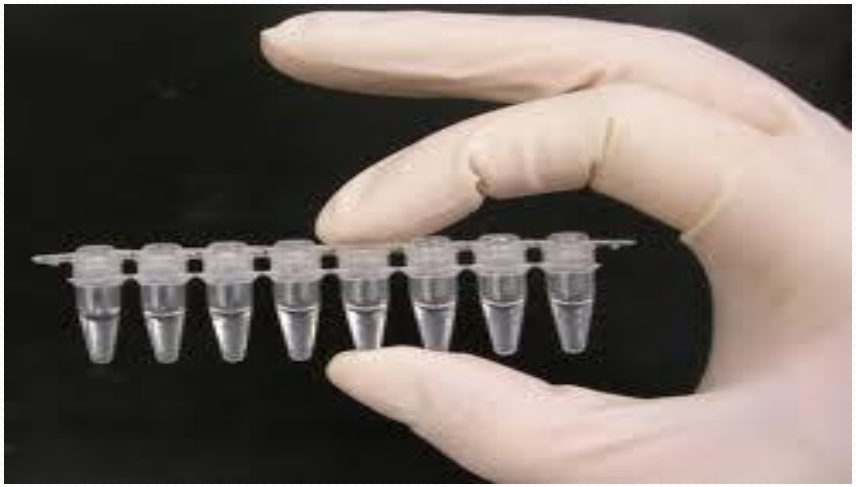
Amplification

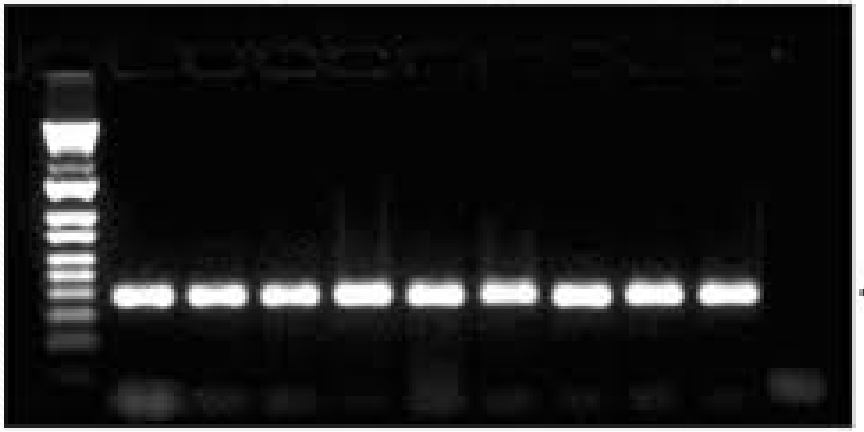
- It has an exponential increase in the number of target DNA fragments
- 30 cycles = 2^{30} copies
- $Y = A \times 2^n$
  - A= initial number of copies of target DNA
  - N= number of cycles
  - Y number of copies of DNA after the reaction



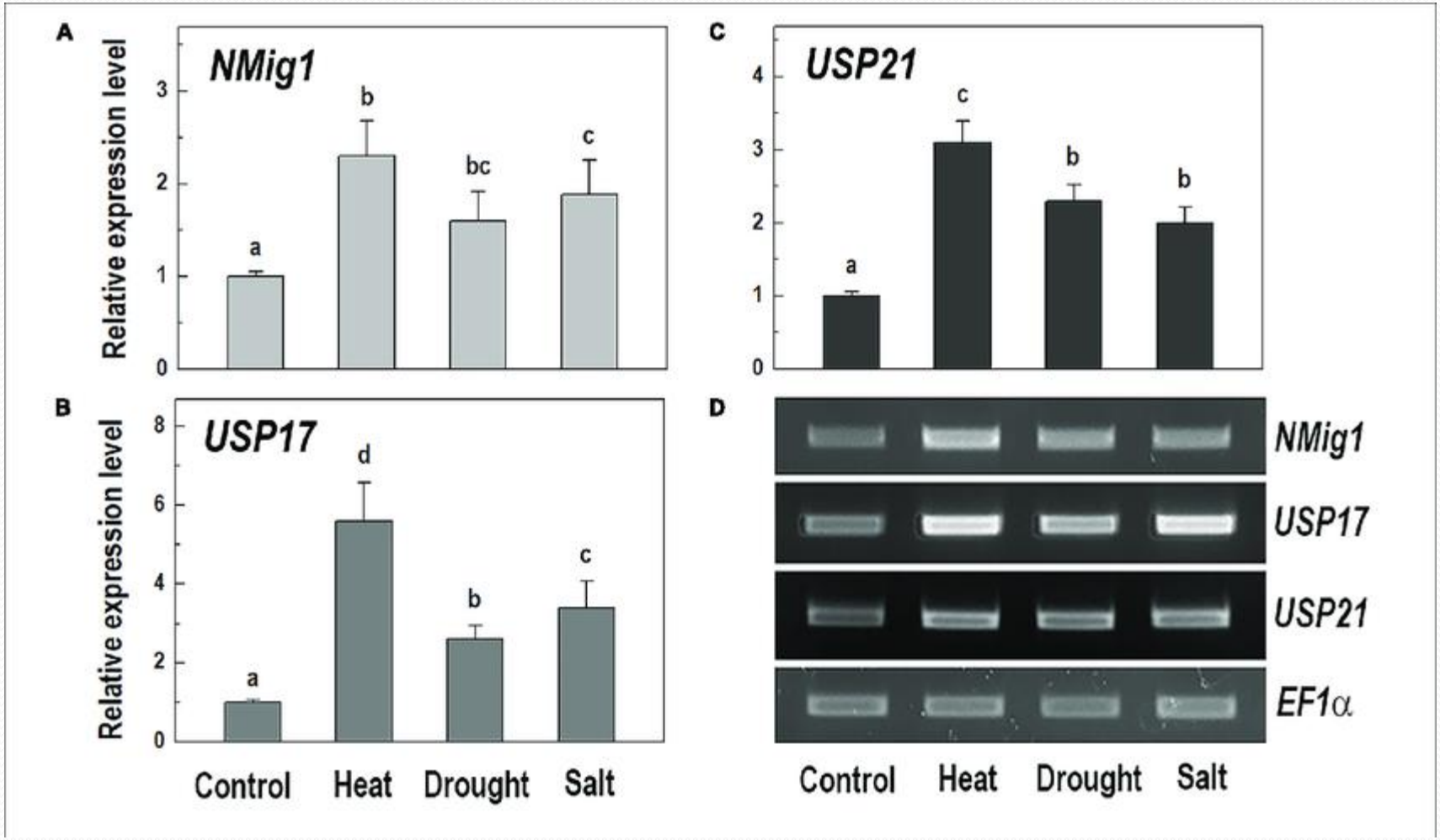


PCR AMPLIFICATION

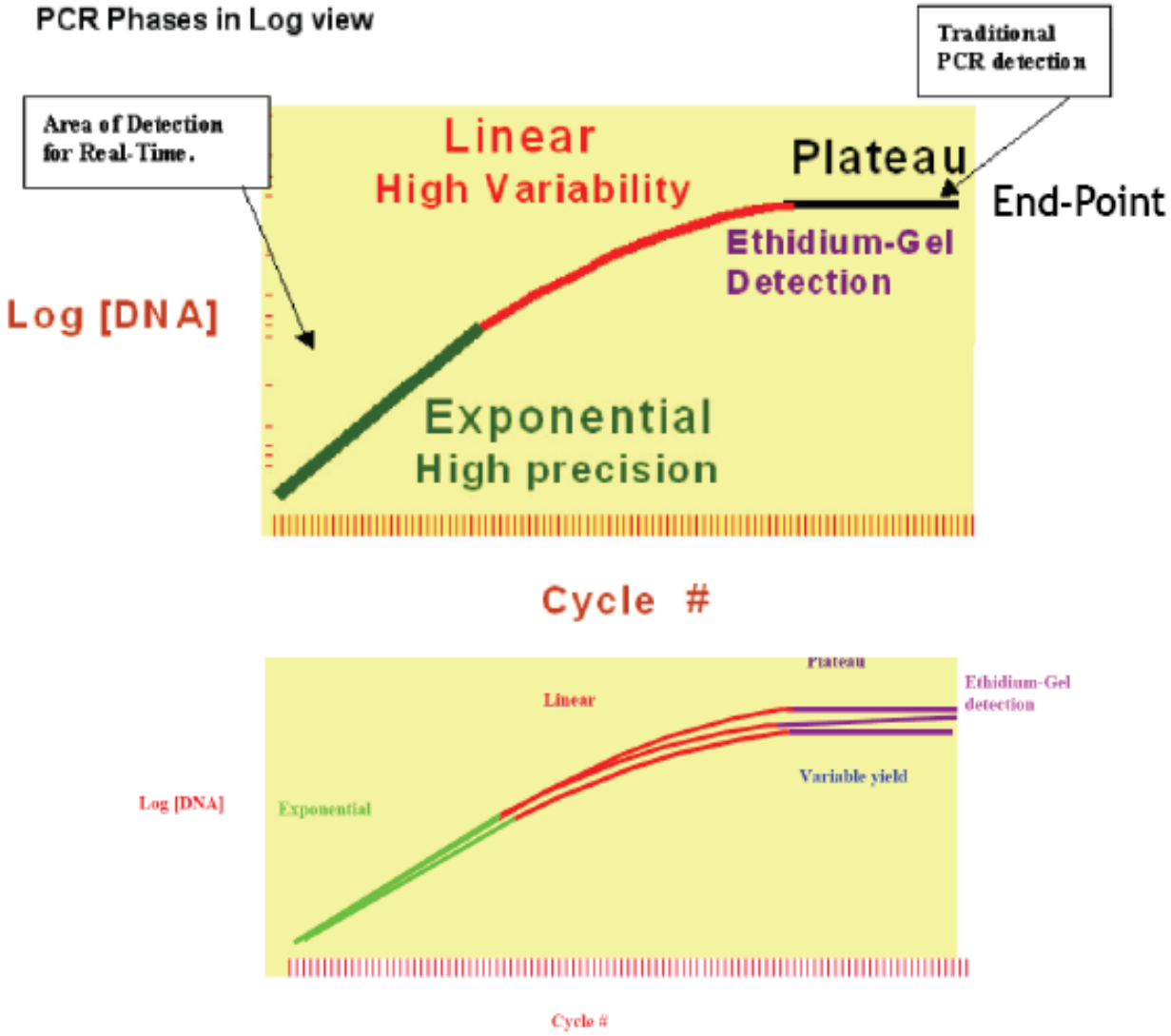




End-point Detection

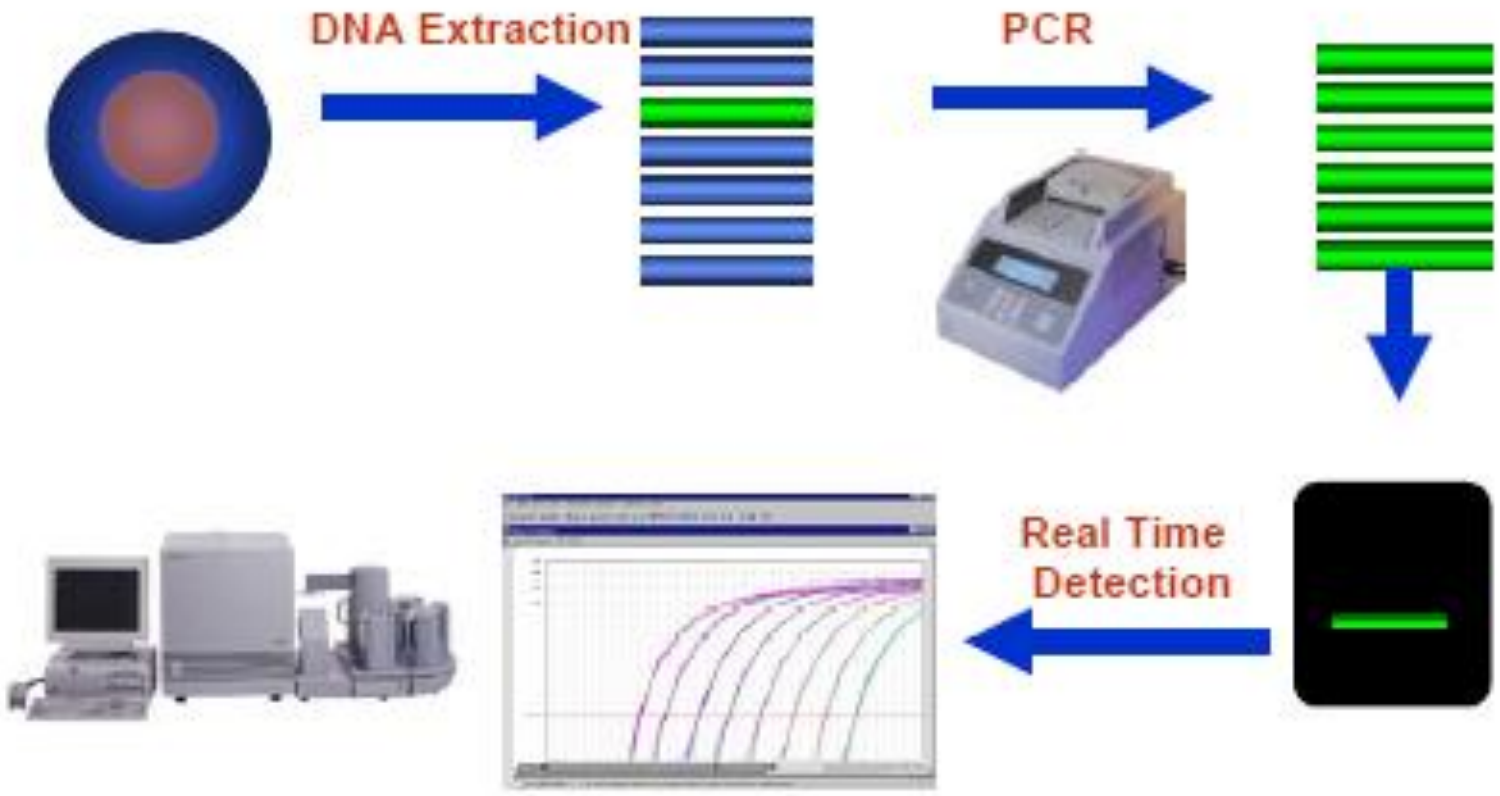

A
NMig1
Relative expression level
0
1
2
3
a
b
bc
c
B
USP17
Relative expression level
0
2
4
6
8
a
d
b
c
Control
Heat
Drought
Salt
C
USP21
0
1
2
3
4
a
c
b
b
D
NMig1
USP17
USP21
EF1α
Control
Heat
Drought
Salt

Real-time vs traditional PCR



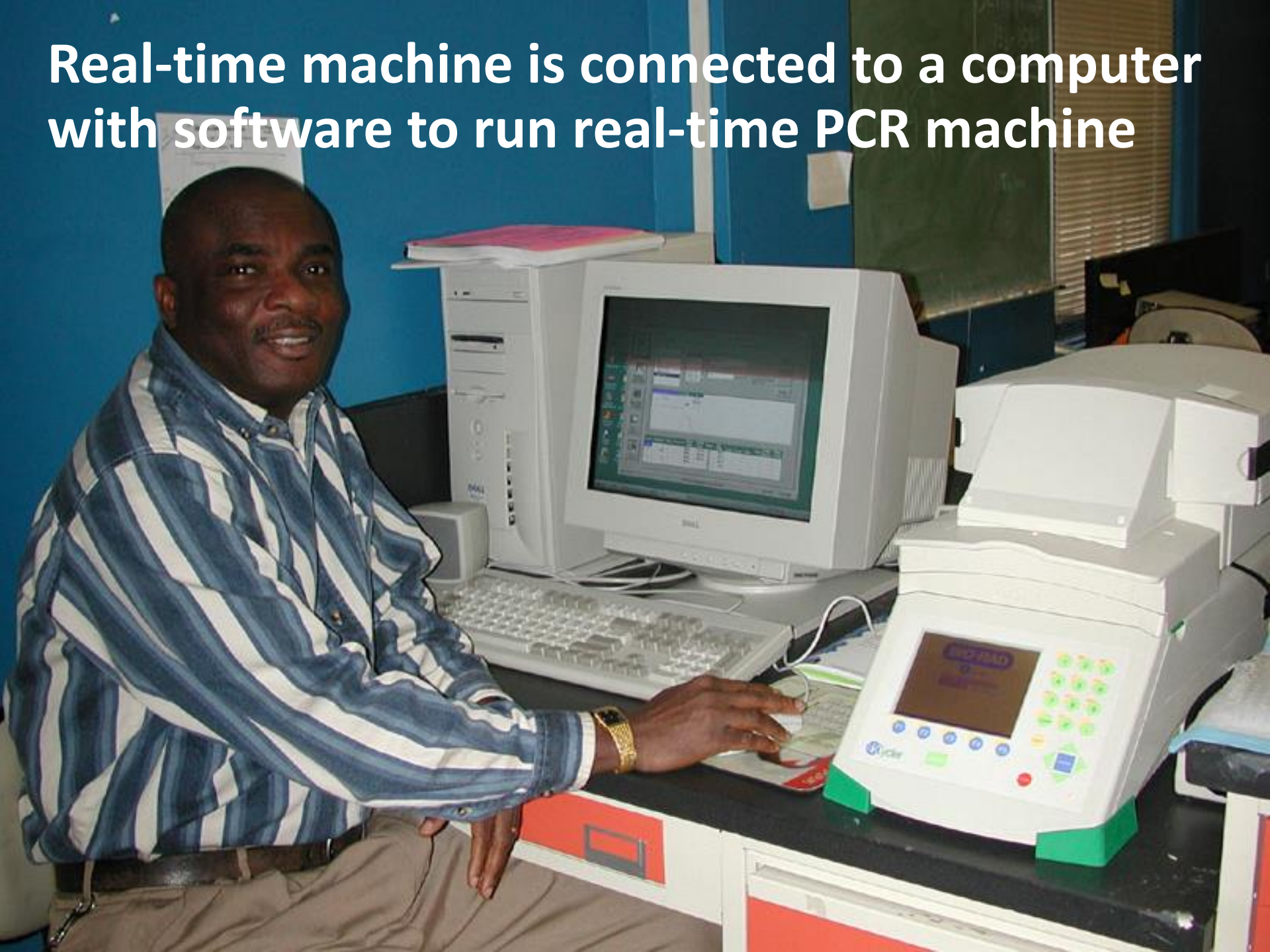
CYCLE NUMBER	AMOUNT OF DNA
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
11	2,048
12	4,096
13	8,192
14	16,384
15	32,768
16	65,536
17	131,072
18	262,144
19	524,288
20	1,048,576
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824
31	1,400,000,000
32	1,500,000,000
33	1,550,000,000
34	1,580,000,000

EVOLUTION OF REAL-TIME PCR



Real-time polymerase chain reaction

- **Real-time polymerase chain reaction**, also called *quantitative real time polymerase chain reaction* (Q-PCR/qPCR) is a laboratory technique based on the polymerase chain reaction, which is used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of a specific sequence in a DNA sample.


Real-time machine is connected to a computer with software to run real-time PCR machine

LOADING SAMPLES IN REAL-TIME PCR MACHINE

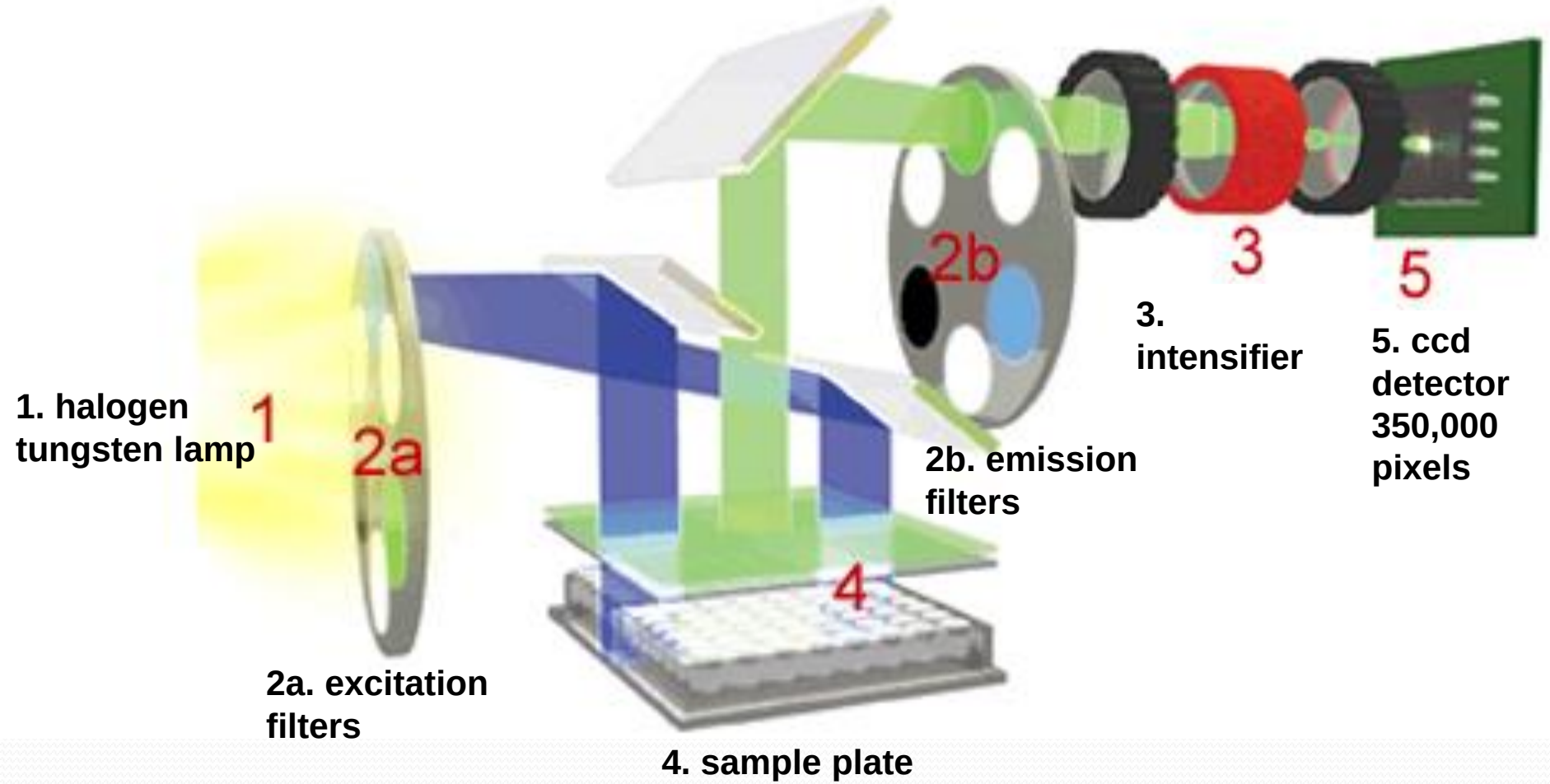


Fig. 1.2. Representation of Optical Detection System layout.

www.biorad.com

METHODS OF DETECTION

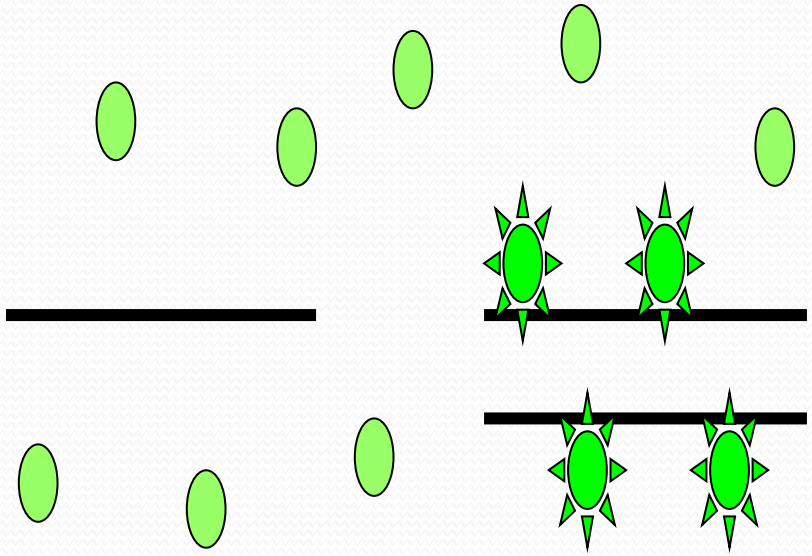
All real time PCR systems rely upon the detection and quantification of fluorescent reporter, the signal of which increases in direct proportion of the amount of PCR product in a reaction.

Two common methods of quantification

1. use of fluorescent dyes that intercalate with double-stranded DNA.
2. modified DNA oligonucleotide probes that fluoresce when hybridized with a complementary DNA.

USING FLUORESCENT DYES

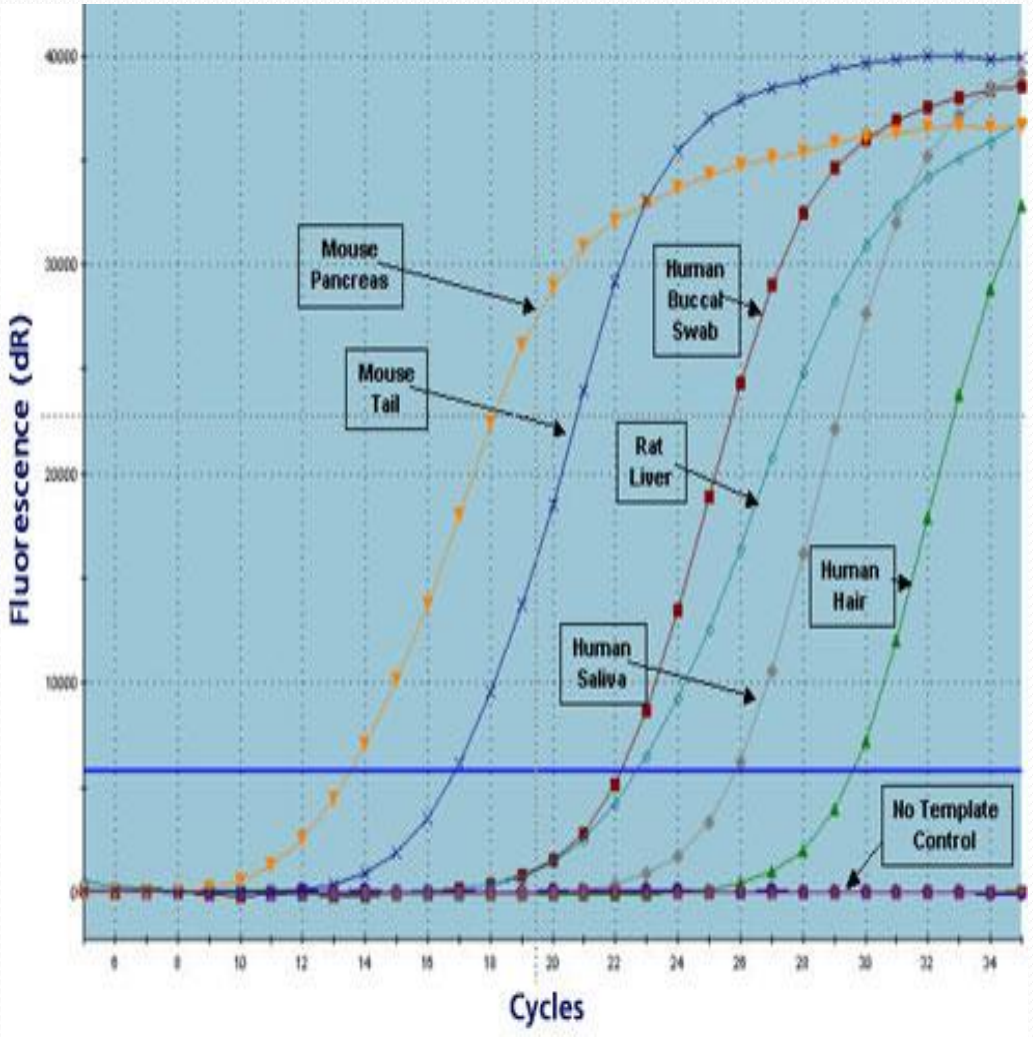
- The reaction is prepared as usual, with the addition of fluorescent dsDNA dye like SYBR GREEN.
- The reaction is run in a thermocycler, and after each cycle, the levels of fluorescence are measured with a detector; the dye only fluoresces when bound to the dsDNA.
- The resulting DNA-dye-complex best absorbs 497 nanometer blue light (λ_{max} = 497 nm) and emits green light (λ_{max} = 520 nm).



How SYBR Green dye works

Sybr green is a dye which binds to double stranded DNA but not to single-stranded DNA

- SYBR green binds to double stranded DNA and upon excitation emits light at 520nm
- Thus as PCR product accumulates the fluorescence increases



Typical Real-Time PCR cycle

1. 95 C- 10 min

2. 95- 30 sec

3. 60- 30 sec

4. 72-30 sec

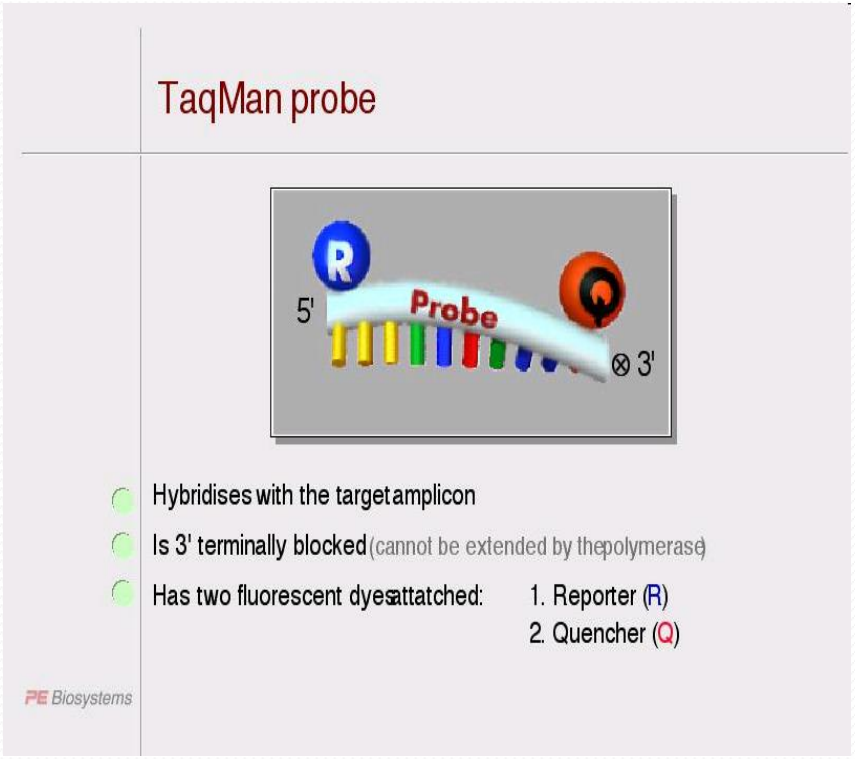
5. Read

6. Go to step 2 for 39 more cycles

7. The melt curve protocol follows with 30 seconds at 95°C and then cooling to Anneaing temp (60C) for 30 sec. and then10 seconds each at 0.2°C increments between 60°C and 95°C. Data collection is enabled at each increment of the melt curve.

Other Emerging Alternatives

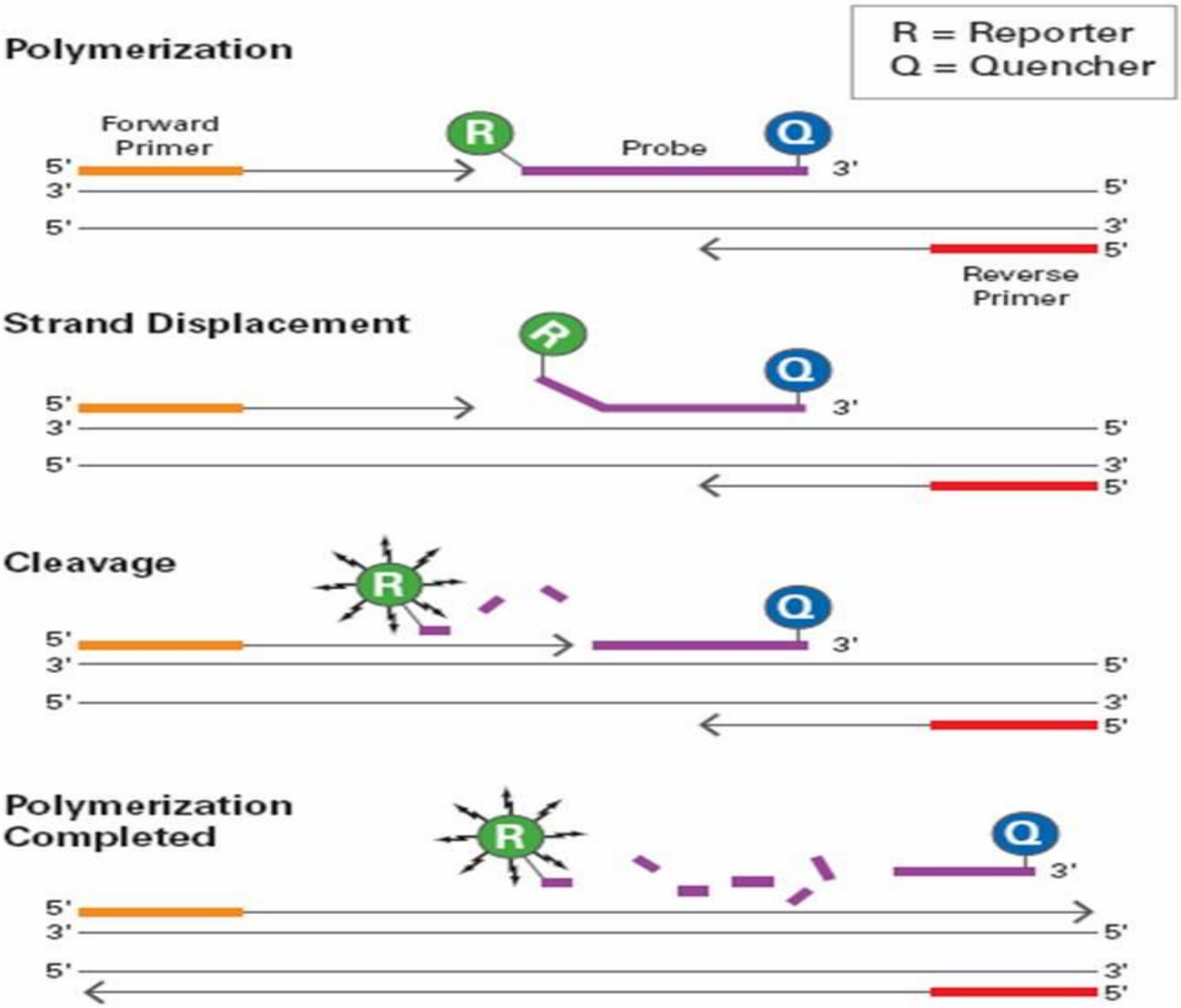
- Two most popular alternatives to SYBR green are **TaqMan®** and **Molecular Beacons**.
- Both technologies depend on hybridization probes.



USING PROBES

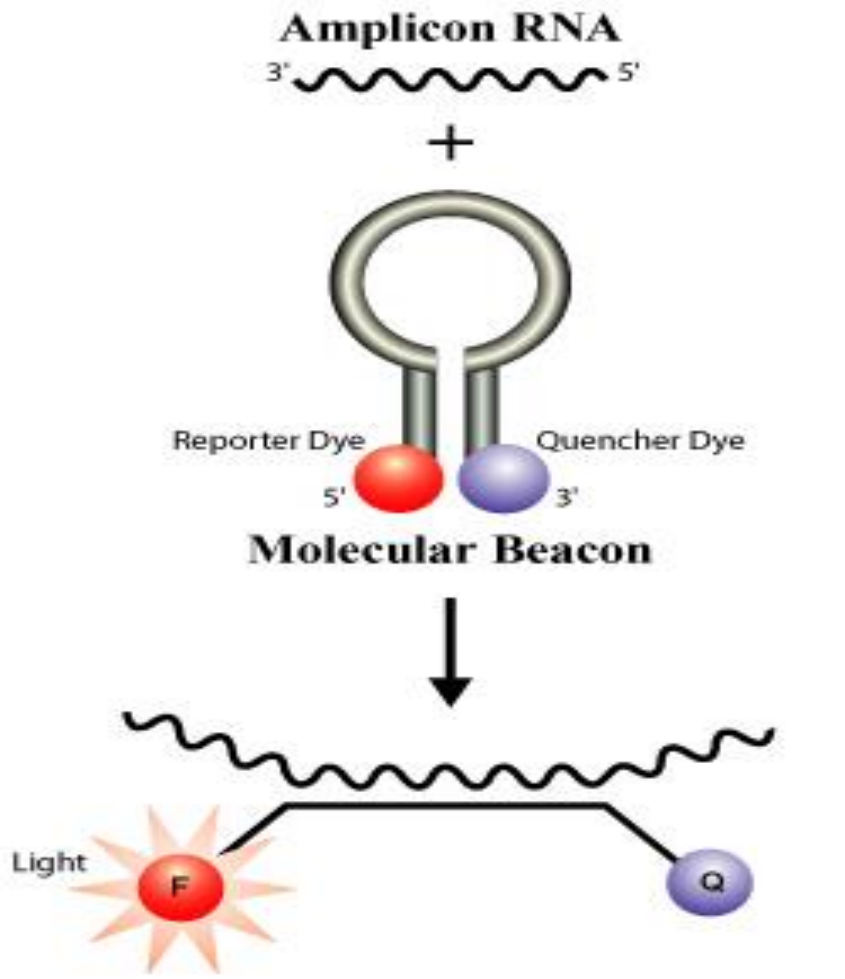
- The PCR reaction is prepared as usual and the reporter probe is added.
- both probe and primers anneal to the DNA target.
- once the polymerase reaches the probe, its 5'-3-exonuclease degrades the probe, physically separating the fluorescent reporter from the quencher, resulting in an increase in fluorescence.
- Fluorescence is detected and measured in the real time PCR thermocycler

Taqman Probes



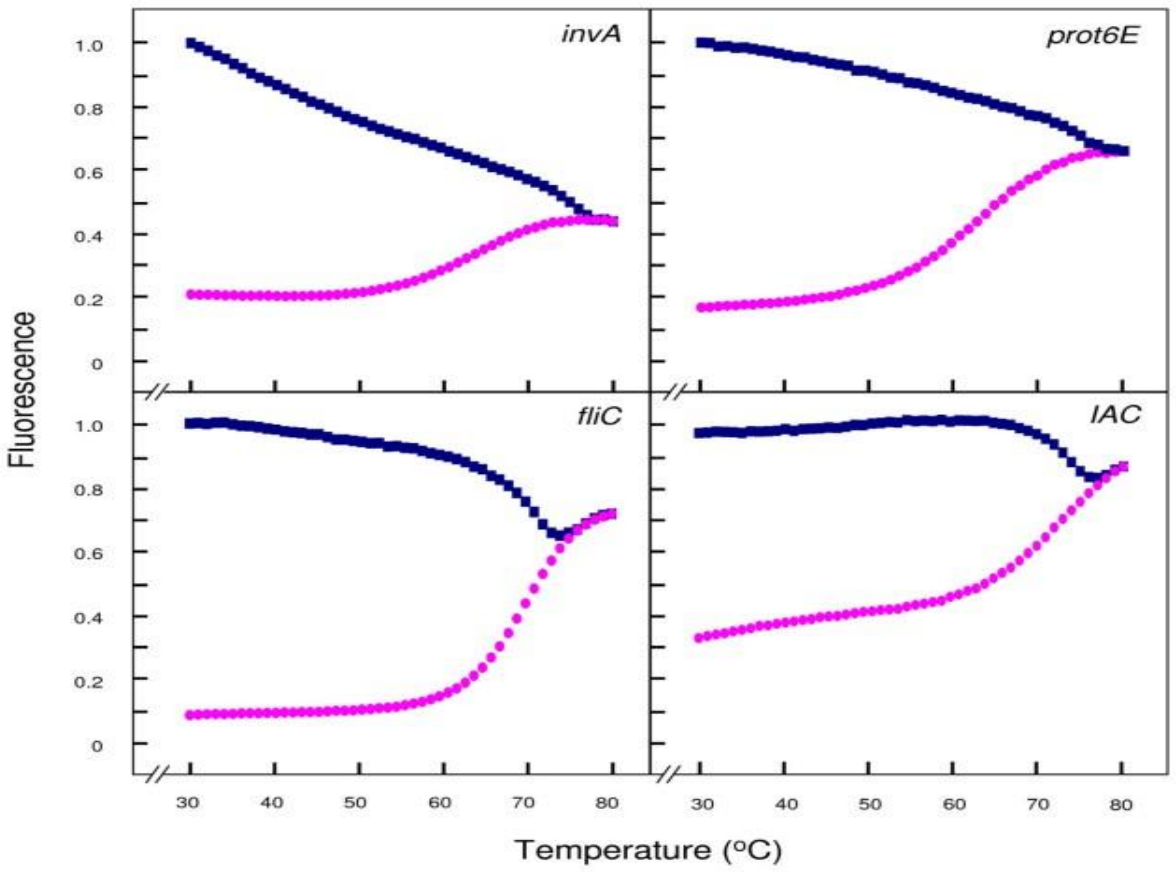
Molecular Beacons

- Molecular beacons are short segments of single-stranded DNA .
- They are designed to adopt a hairpin structure while free in solution, bringing the fluorescent dye and quencher in close proximity. When a molecular beacon hybridizes to a target the fluorescent dye emits light upon irradiation, and rebind to target in every cycle for signal measurement.



Molecular Beacon Cycle-

Activation step at 95°C for 10 min, followed by 50 cycles, each consisting of 95°C for 15 s, 50°C for 30 s (**data collection step**) and 72°C for 30 s.



Thermal denaturation profiles of the molecular beacons. Thermal denaturation profiles of the molecular beacons used in this study as established by melting curve analysis (described in Materials and Methods). The figure shows normalised fluoresence thermal transitions of molecular beacon plotted in pink circles and beacon-target complexes plotted in blue squares.

Table 2.1. Commonly used combinations of reporters and quenchers.

Reporter	Compatible Quencher
FAM	BHQ1, DABCYL,* TAMRA**
TET	BHQ1, DABCYL*
HEX	BHQ1, BHQ2, DABCYL*
TAMRA	BHQ2, DABCYL*
Texas Red	BHQ2, BHQ3, DABCYL*
ROX	BHQ2, BHQ3, DABCYL*
Cy5	BHQ2, BHQ3

* DABCYL is a mechanical quencher only appropriate for molecular beacons.

** TAMRA is not recommended as a quencher, but will work for single-color FAM assays. TAMRA will not work well as a quencher on molecular beacons.

Typical Real-Time PCR cycle

1. 95 C- 10 min

2. 95- 30 sec

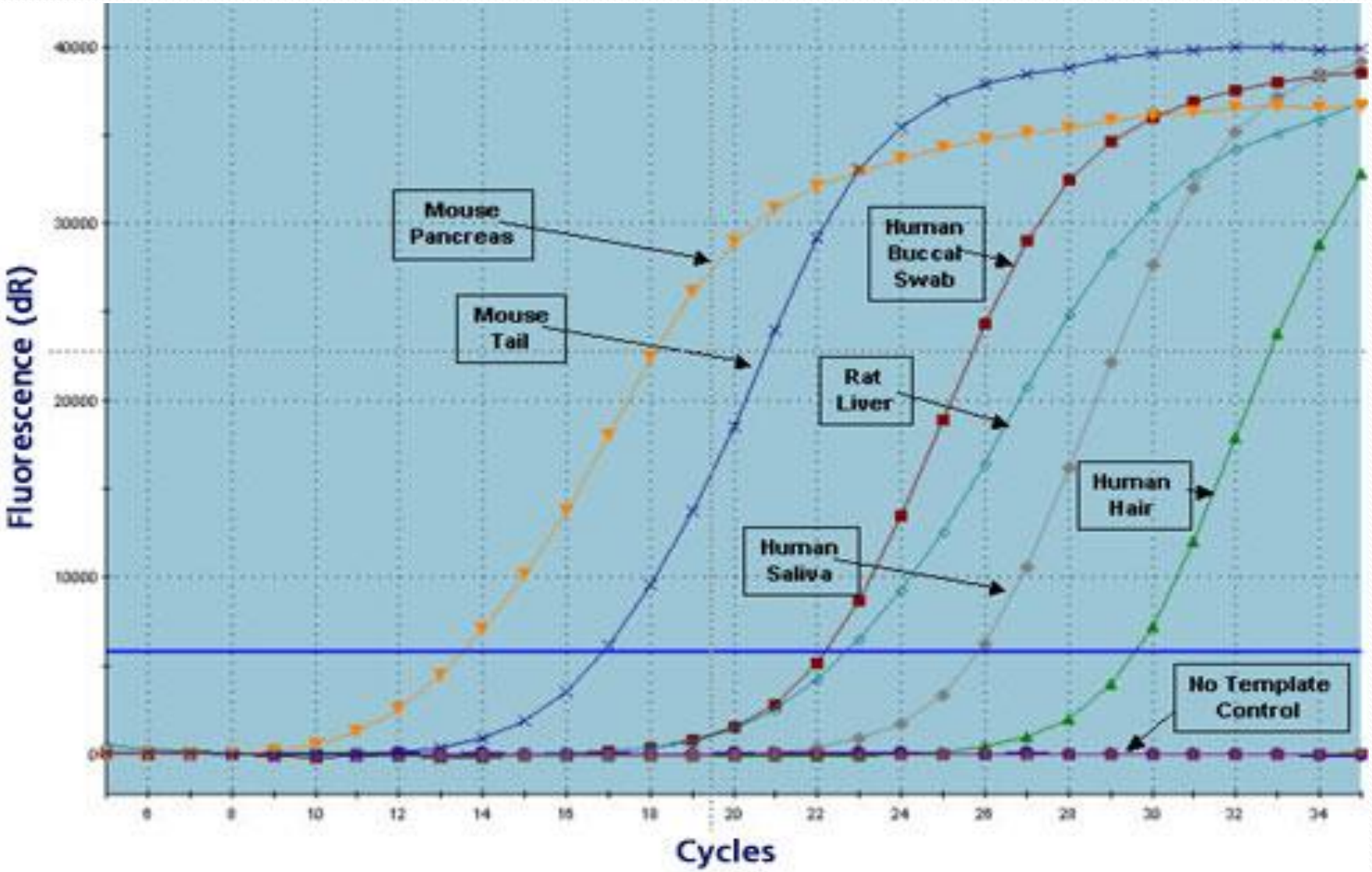
3. 60- 30 sec

4. 72-30 sec

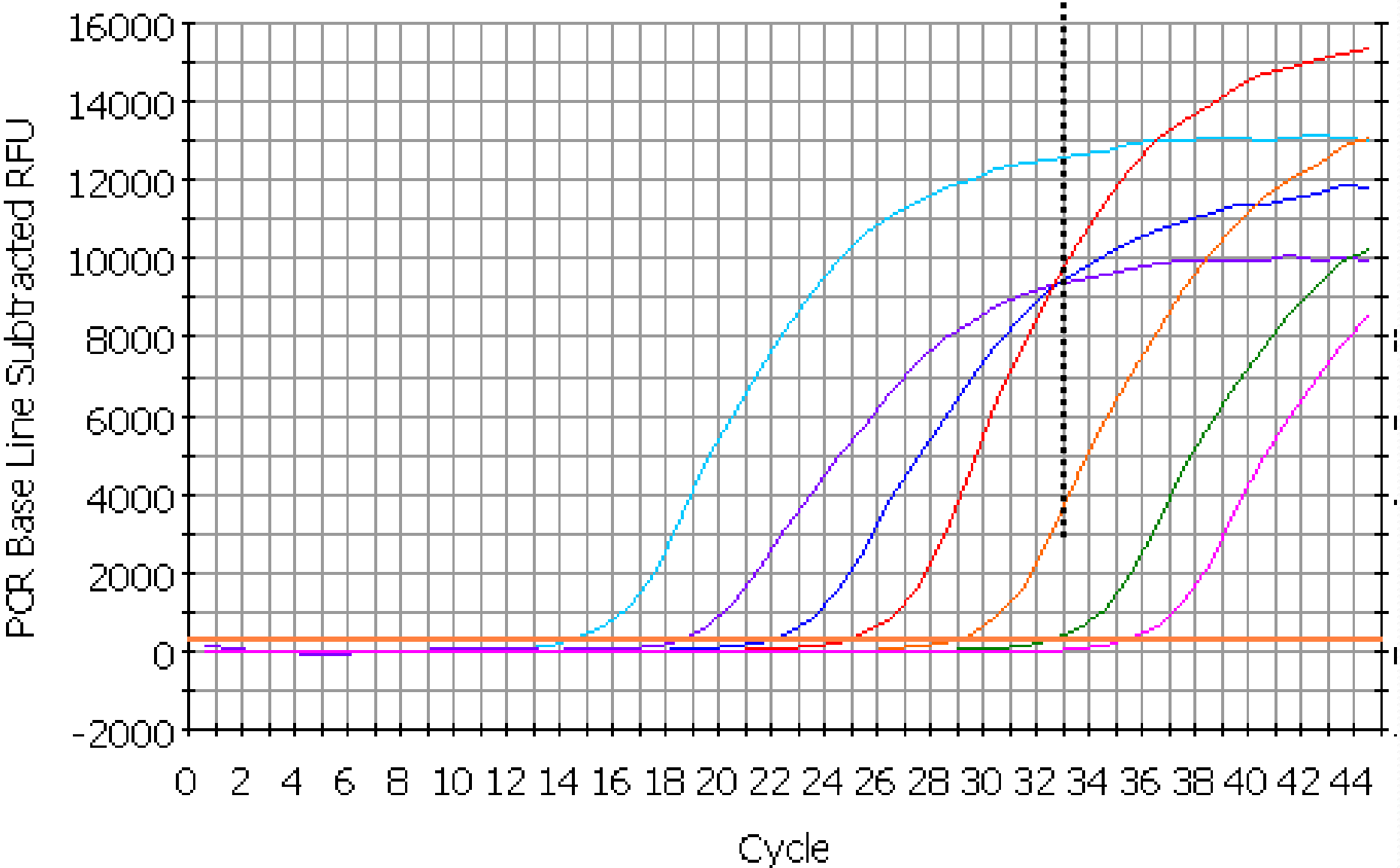
5. Read

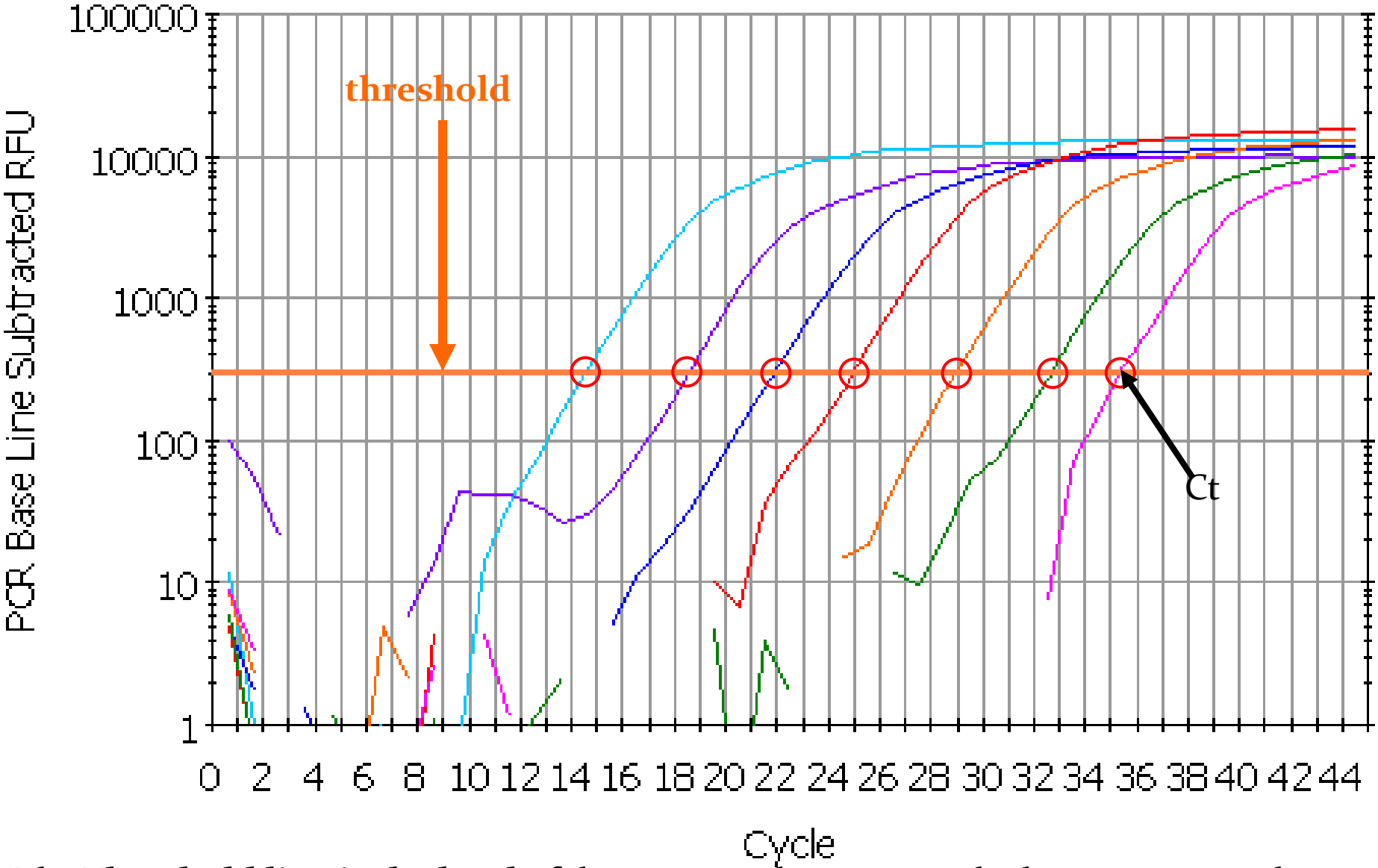
6. Go to step 2 for 39 more cycles

7. The melt curve protocol follows with 30 seconds at 95°C and then cooling to Anneaing temp (60C) for 30 sec. and then10 seconds each at 0.2°C increments between 60°C and 95°C. Data collection is enabled at each increment of the melt curve.


Fluorescence (dR)
40000
30000
20000
10000
0
Cycles
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
Mouse Pancreas
Mouse Tail
Human Buccal Swab
Rat Liver
Human Hair
Human Saliva
No Template Control

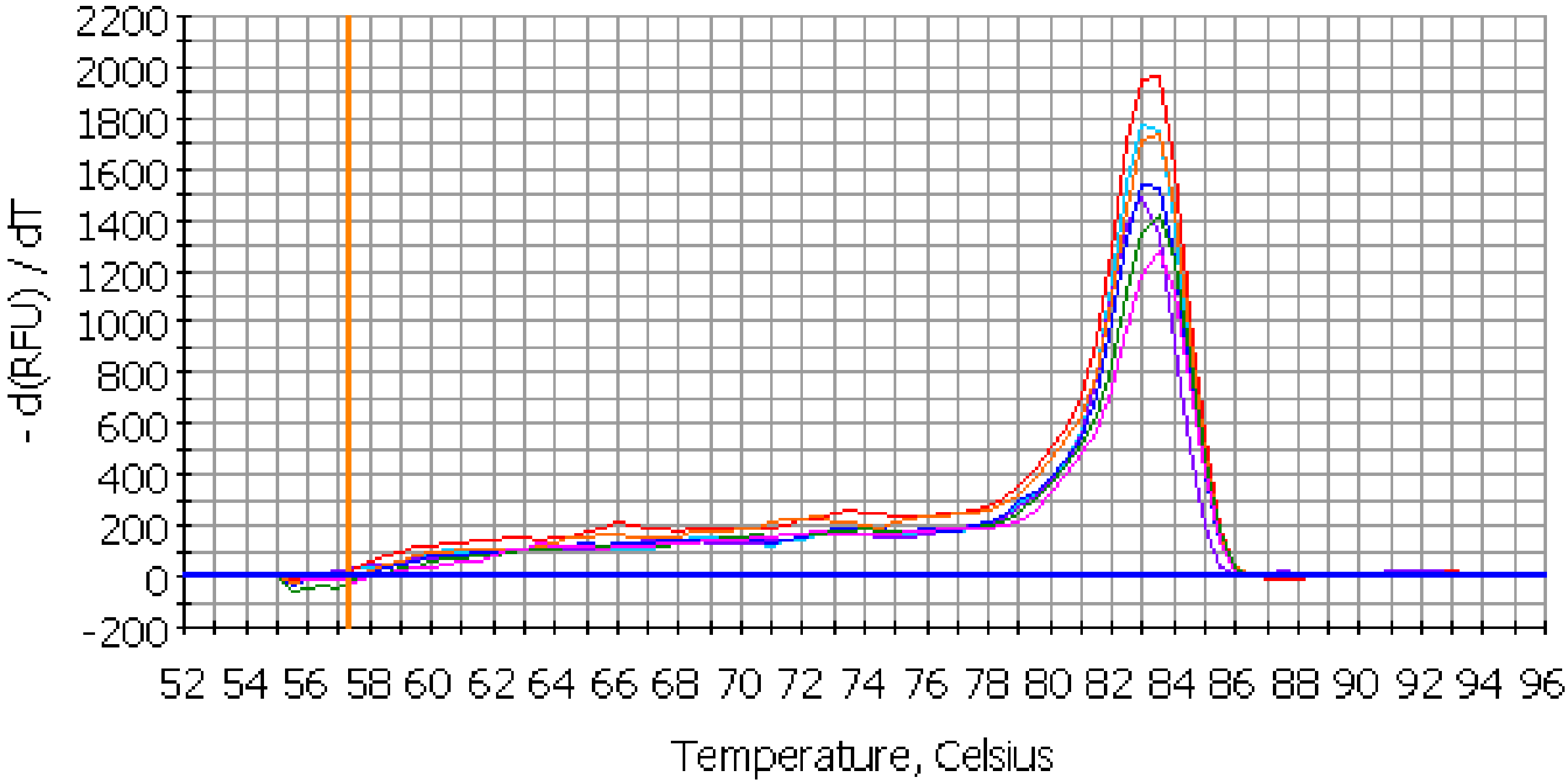
SERIES OF 10-FOLD DILUTIONS





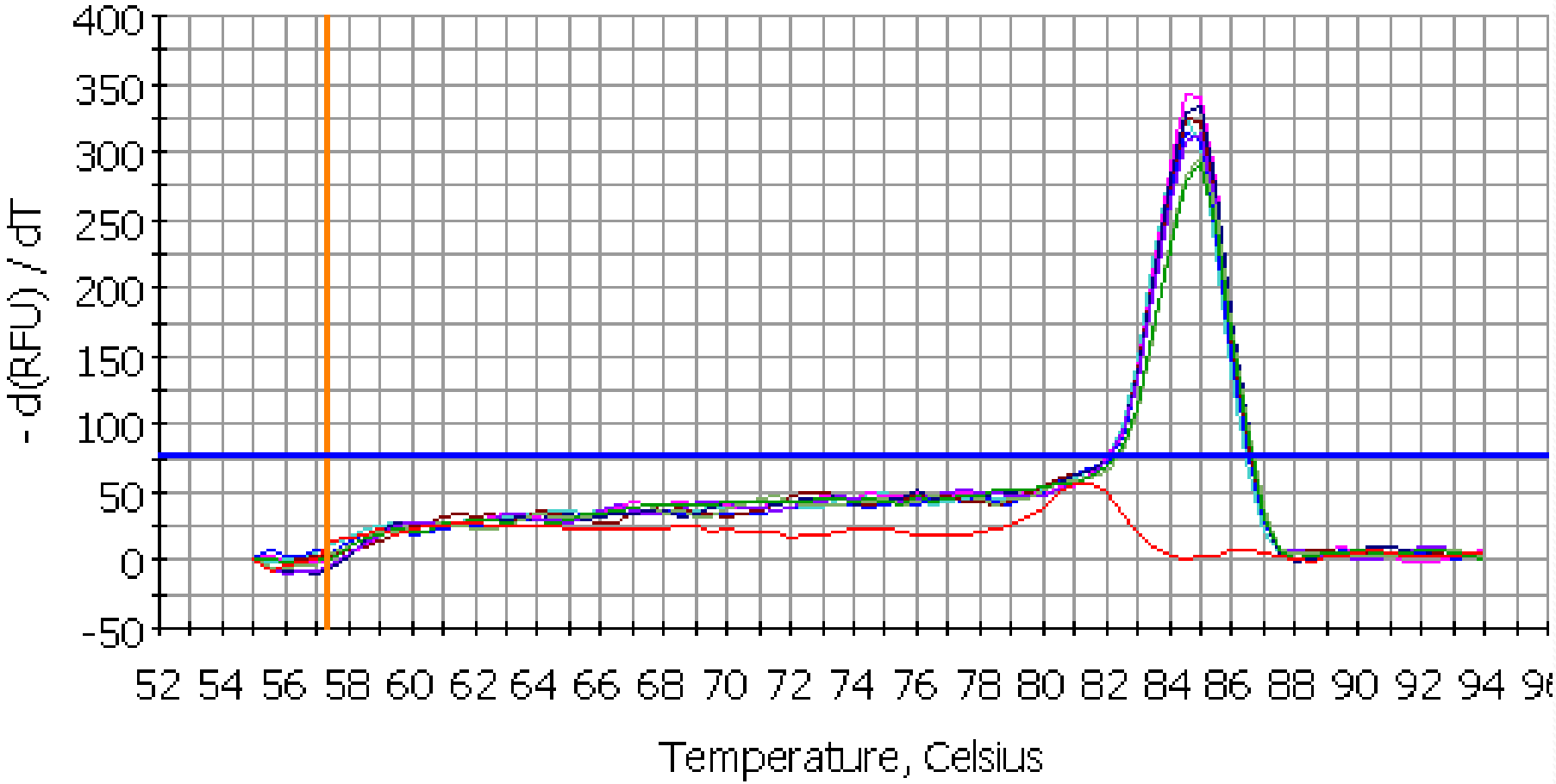
The **Threshold line is the level of** detection or the point at which a reaction reaches a fluorescent intensity above background. The threshold line is set in the exponential phase of the amplification for the most accurate reading. The cycle at which the sample reaches this level is called the **Cycle Threshold, Ct.**

MELTING CURVE



The software plots the rate of change of the relative fluorescence units (RFU) with time (T) (-d(RFU)/dT) on the Y-axis versus the temperature on the X-axis, and this will peak at the melting temperature (Tm).

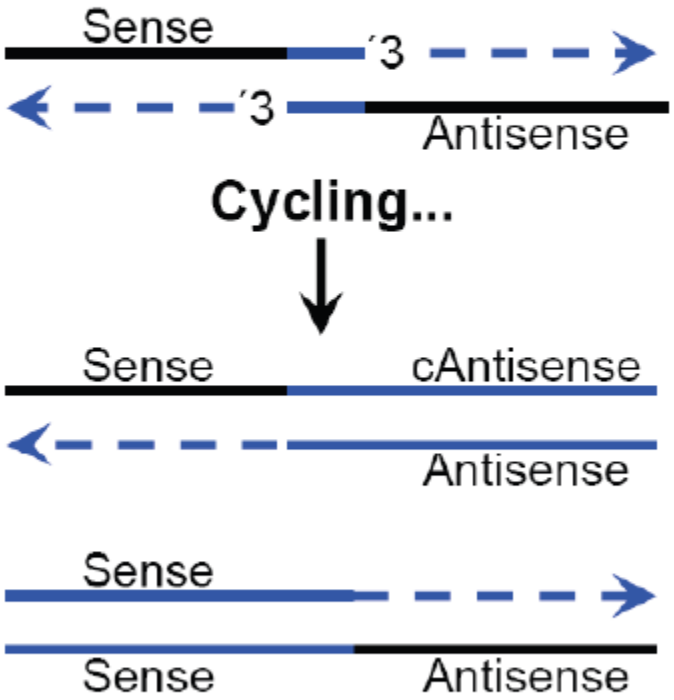
MELTING CURVE

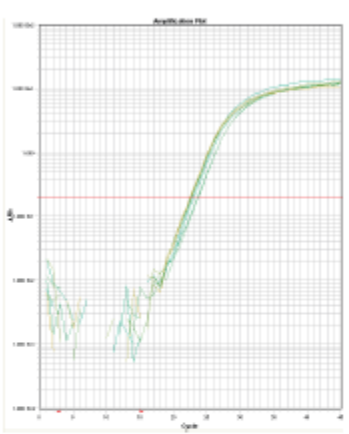


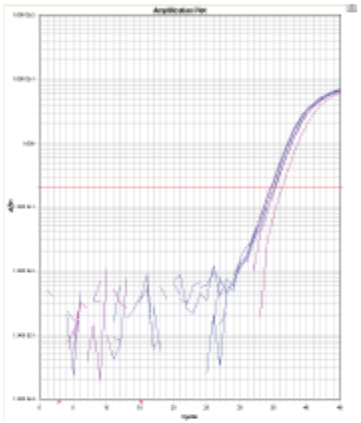
Melt Peak: Data 10-Mar-03 1259 ed.opd

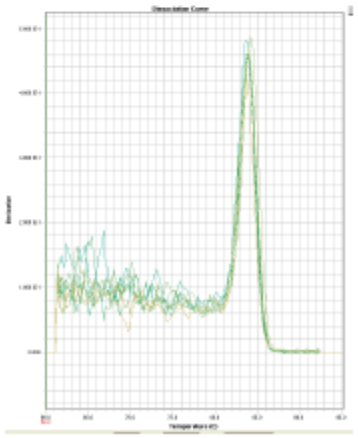
MELTING CURVE

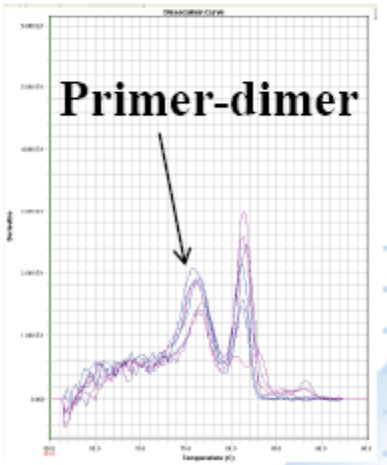
SYBR Green - dissociation curve (T_m)







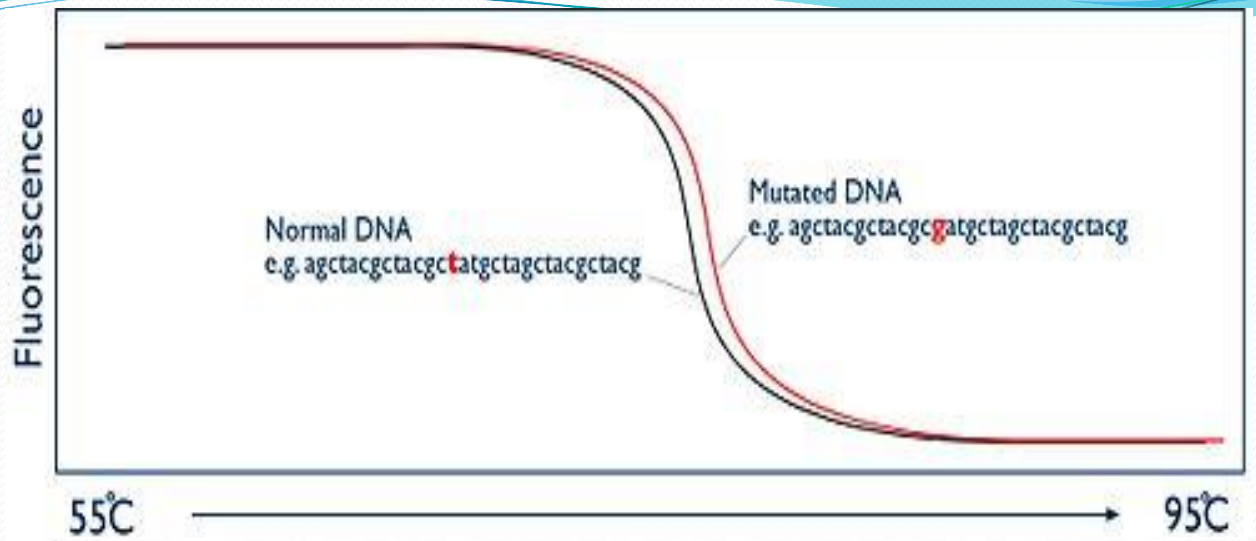


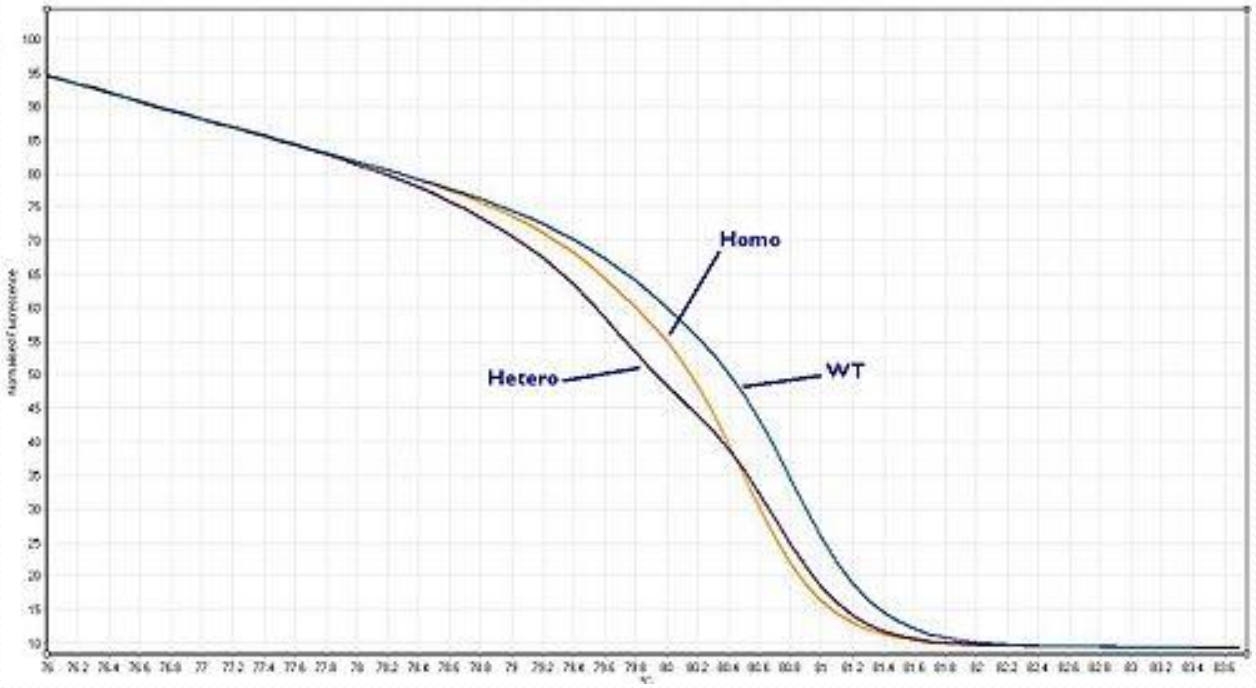


High Resolution Melt (HRM) analysis

High Resolution Melt (HRM) analysis is a powerful technique in molecular biology for the detection of mutations, polymorphisms and epigenetic differences in double-stranded DNA samples. It was discovered and developed by Idaho Technology and the University of Utah. It has advantages over other genotyping technologies, namely:

It is cost-effective vs. other genotyping technologies such as sequencing and TaqMan SNP typing. This makes it ideal for large scale genotyping projects.
It is fast and powerful thus able to accurately genotype many samples rapidly.
It is simple. With a good quality HRM assay, powerful genotyping can be performed by non-geneticists in any laboratory with access to an HRM capable real-time PCR machine.


Fluorescence
Normal DNA
e.g. agctacgctacgctatgctagctacgctacg
Mutated DNA
e.g. agctacgctacgcgatgctagctacgctacg
55°C
95°C


Homo
Hetero
WT

CONTROLS

No template control (NTC): A control reaction that contains all essential components of the amplification reaction except the template. This enables detection of contamination.

RT minus control: RNA preparations may contain residual genomic DNA, which may be detected in real-time PCR if assays are not designed to only detect and amplify RNA.

DNA contamination can be detected by performing a control reaction in which no reverse transcription takes place.

Standard: Sample of known concentration or copy number used to construct a standard curve.

Standard curve: To generate a standard curve, C_T values/crossing points of different standard dilutions are plotted against the logarithm of input amount of standard material. The standard curve is commonly generated using a dilution series of at least 5 different concentrations of the standard. Each standard curve should be checked for validity, with the value for the slope falling between –3.3 to –3.8. Standards are ideally measured in triplicate for each concentration. Standards which give a slope differing greatly from these values should be discarded.

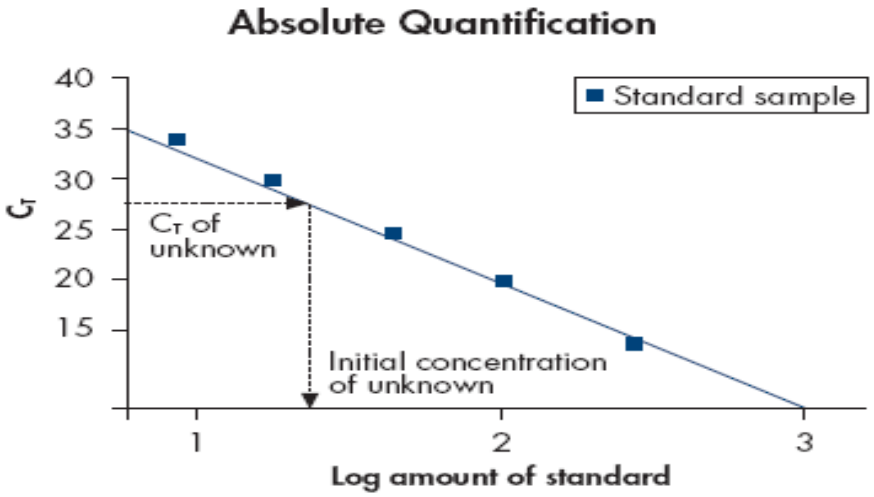


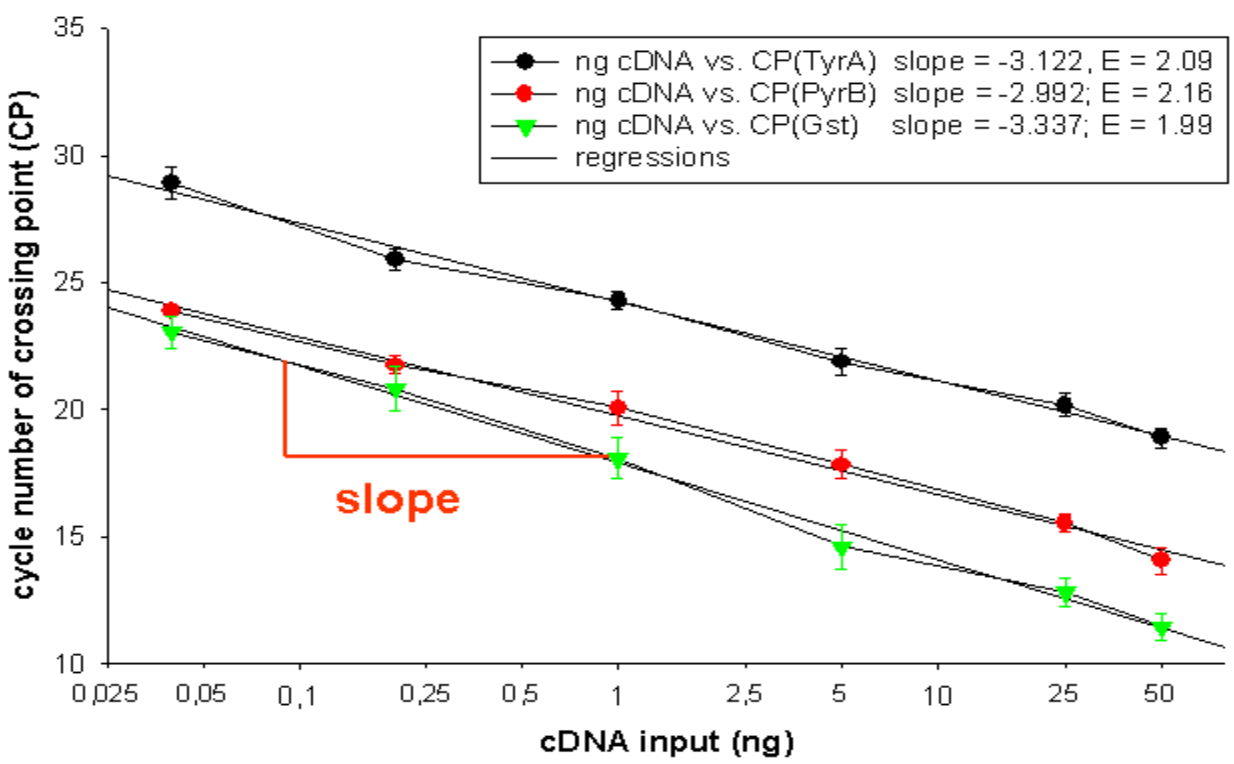
Figure 12 Typical standard curve showing determination of concentration of sample of interest.

SLOPE AND EFFICIENCY

real-time PCR. A slope of –3.322 means that the PCR has an efficiency of 1, or 100%, and the amount of PCR product doubles during each cycle. A slope of less than –3.322 (e.g., –3.8) is indicative of a reaction efficiency <1. Generally, most amplification reactions do not reach 100% efficiency due to experimental limitations. A slope of greater than –3.322 (e.g., –3.0) indicates a PCR efficiency, which appears to be greater than 100%. This can occur when values are measured in the non-linear phase of the reaction or it can indicate the presence of inhibitors in the reaction (see page 24).

amplification efficiency (E) for each target can be calculated according to the following equation:

$E = 10^{(-1/S)} - 1$, where S is the slope of the standard curve.



Quantification

Absolute and Relative

Absolute Quantification

A doctor is interested in determining the number of viral particles per ml of blood from a patient. To do so, the doctor extracts DNA from 10 ml of blood and determines the number of viral particles by running a real-time PCR assay specific to the viral DNA. By comparing the C_T value obtained for the test sample to a standard curve of known amounts of viral particles, the doctor can interpolate that there are 10,000 viral particles in the assayed DNA sample. Based on this information, she is then able to determine that there are 1,000 viral particles/ml of blood.

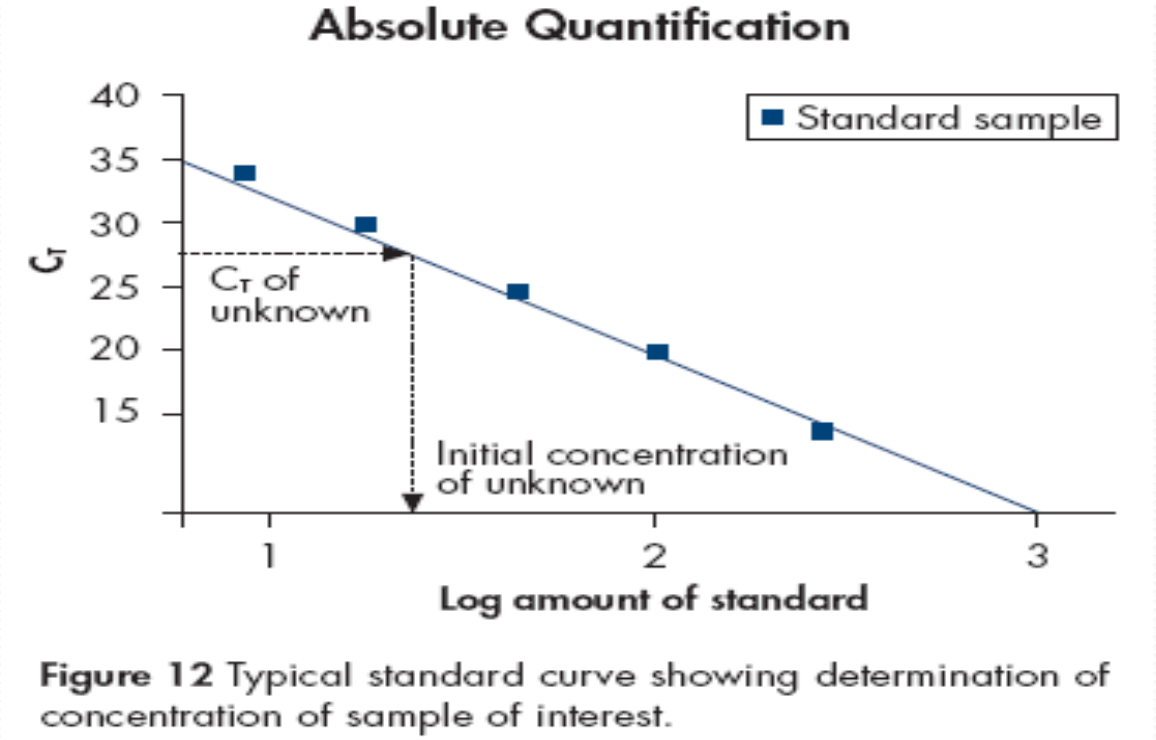


Figure 12 Typical standard curve showing determination of concentration of sample of interest.

Relative Quantification

Now let's examine a sample case that calls for relative quantification. A researcher wants to know whether the expression level of p53 differs between cancerous and normal ovarian cells, and if so, by how much. He wants to find out the relative amount, sometimes called the "fold difference", of p53 mRNA in equivalent amounts of the two tissues. The researcher can choose one of the following experimental designs depending on the type of normalizer he wishes to employ:

NORMALIZATION CONTROLS

Table 2. Housekeeping Genes Commonly Used as Endogenous References

Gene name	Relative expression level*
18S ribosomal RNA	++++
Glyceraldehyde-3-phosphate dehydrogenase	+++
β-Actin, cytoplasmic	+++
β-2-microglobulin	+++
Phosphoglycerate kinase 2	+++
Peptidylprolyl isomerase A (cyclophilin A)	+++
Ribosomal protein, large, PO	+++
Hypoxanthine phosphoribosyl transferase 1	++
β-Glucuronidase	+
TATA box binding protein	+
Transferrin receptor	+

RELATIVE QUANTITATION

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

Example: cDNAs representing 50 ng of total RNA isolated from both normal and tumor ovarian cells were assayed for p53 (target gene) and GAPDH (reference gene) message. GAPDH can serve as a reference gene for this study because previous studies showed that GAPDH expression does not vary between normal and tumor cells. The C_T values for each sample are shown below:

Sample	C_T p53 (target)	C_T GAPDH (reference)
Normal (calibrator)	15.0	16.5
Tumor (test)	12.0	15.9

To calculate relative expression using the steps described above, the C_T of the target gene is normalized to the C_T of the reference gene for both the test sample and the calibrator sample:

$$\Delta C_{T(normal)} = 15.0 - 16.5 = -1.5$$

$$\Delta C_{T(tumor)} = 12.0 - 15.9 = -3.9$$

Second, the ΔC_T of the test sample is normalized to the ΔC_T of the calibrator:

$$\Delta\Delta C_T = \Delta C_{T(tumor)} - \Delta C_{T(normal)}$$

$$= -3.9 - (-1.5) = -2.4$$

Finally, the expression ratio is calculated:

$$2^{-\Delta\Delta C_T} = 2^{-(-2.4)} = 5.3$$

Tumor cells are expressing p53 at a 5.3-fold higher level than normal cells.

The ΔC_T method using a reference gene

- The ΔC_T method using a reference gene is a variation of the Livak method that is simpler to perform and gives essentially the same results. This method uses the difference between reference and target C_T values for each sample.

$$\text{Ratio (reference/target)} = 2^{C_T(\text{reference}) - C_T(\text{target})}$$

Sample	C_T p53 (target)	C_T GAPDH (reference)
Normal (calibrator)	15.0	16.5
Tumor (test)	12.0	15.9

$2^{(C_T(\text{GAPDH}) - C_T(\text{p53}))} = \text{Expression}$

For normal cells, this yields $2^{(16.5 - 15)} = 2.8$

For tumor cells, this yields $2^{(15.9 - 12)} = 14.9$

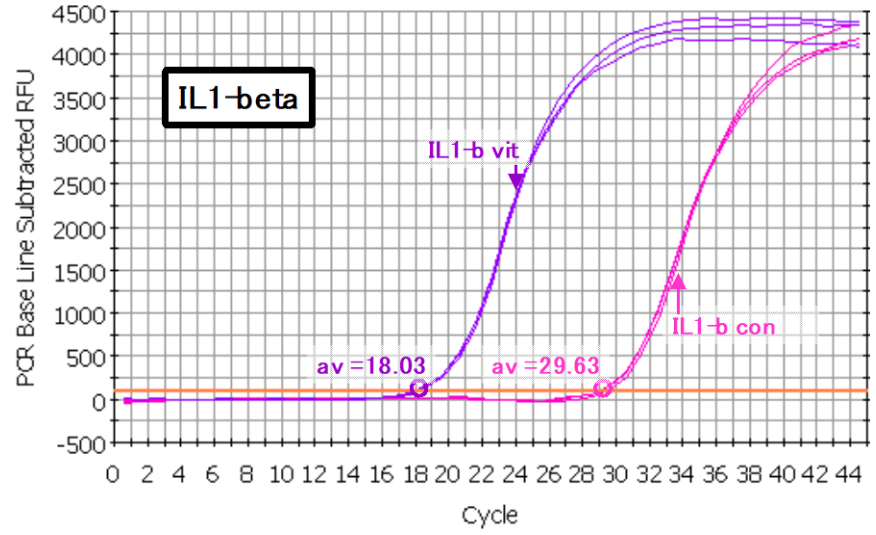
Normal/Normal = 2.8/2.8 = 1

Tumor/Normal = 14.9/2.8 = 5.3

4.2.3.3 The Pfaffl Method

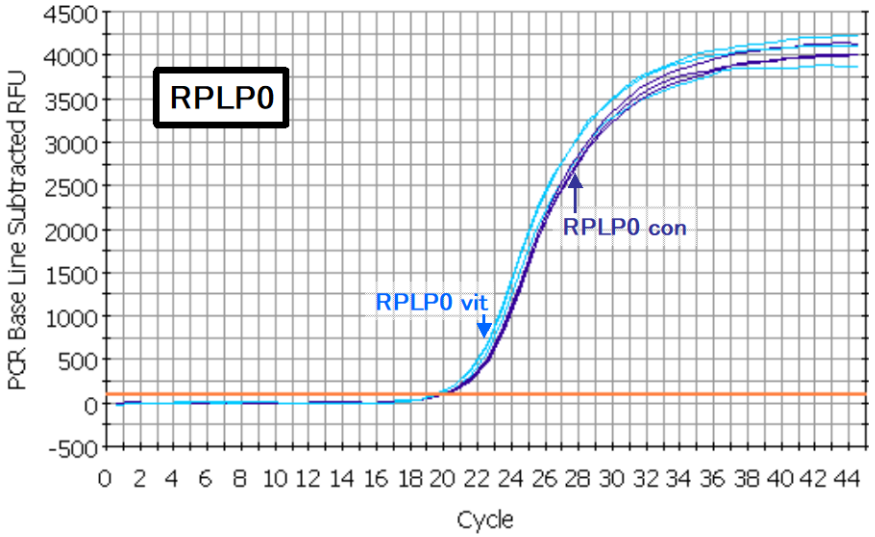
The $2^{-\Delta\Delta C_T}$ method for calculating relative gene expression is only valid when the amplification efficiencies of the target and reference genes are similar. If the amplification efficiencies of the two amplicons are not the same, an alternative formula must be used to determine the relative expression of the target gene in different samples. To determine the expression ratio between the sample and calibrator, use the following formula:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta C_T,\ \text{target (calibrator} - \text{test)}}}{(E_{\text{ref}})^{\Delta C_T,\ \text{ref (calibrator} - \text{test)}}}$$



AFTER N CYCLES: increase = (efficiency)^n

Ratio vit/con = $(1.93)^{29.63-18.03} = 1.93^{11.60} = 2053$

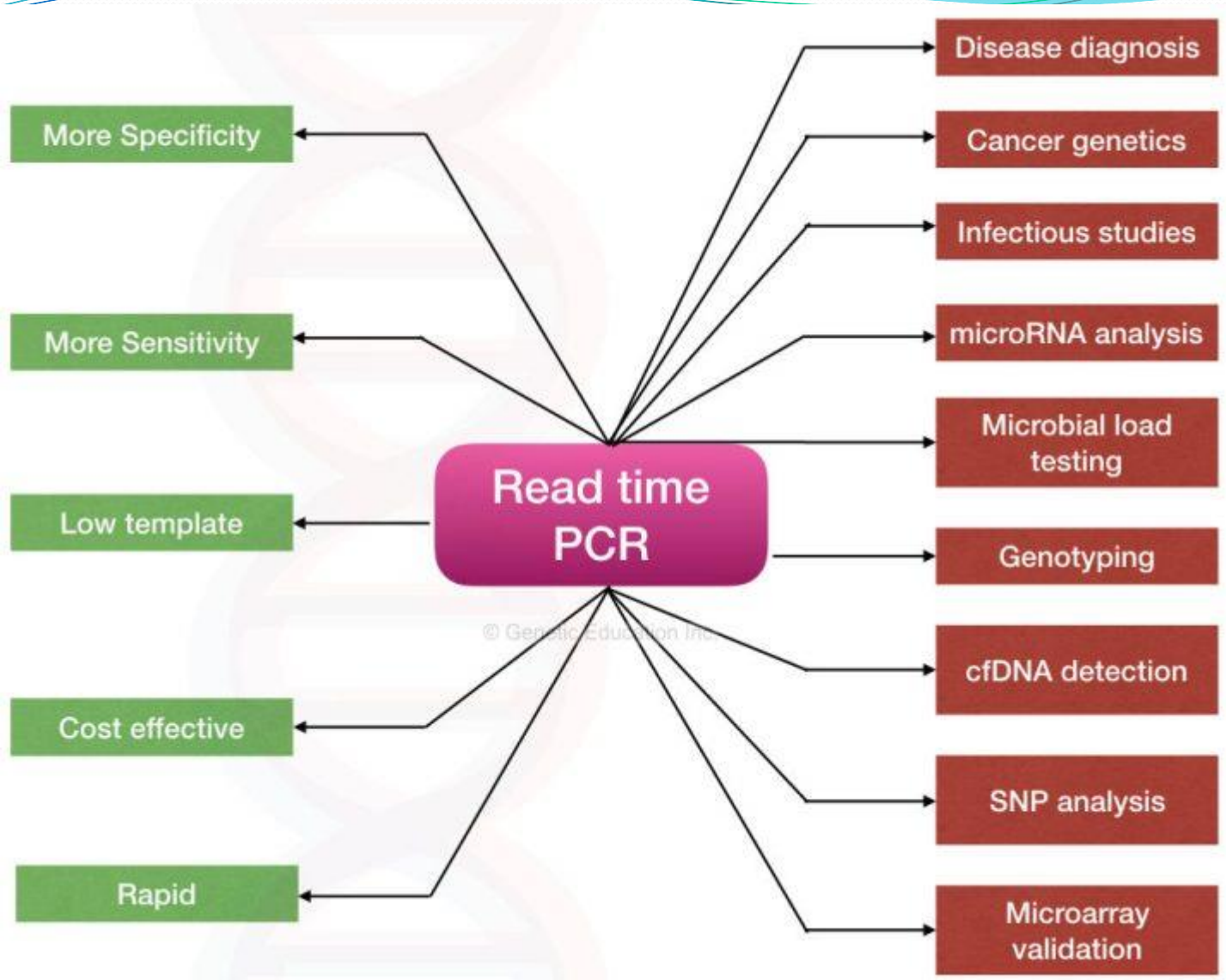


AFTER N CYCLES: increase = (efficiency)^n

Ratio vit/con = $(1.87)^{19.93-19.80} = 1.87^{0.13} = 1.08$

$$\text{ratio} = \frac{\text{change in IL1-B}}{\text{change in RPLP0}} = 2053/1.08 = 1901$$

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


Read time PCR
More Specificity
More Sensitivity
Low template
Cost effective
Rapid
Disease diagnosis
Cancer genetics
Infectious studies
microRNA analysis
Microbial load testing
Genotyping
cfDNA detection
SNP analysis
Microarray validation

Precautions while setting up RT-PCR

The following precautions can help you avoid contamination problems:

• Wipe down workstations with dilute solutions of bleach or with commercially available decontamination solutions

• Prepare samples in a designated clean room, hood, or benchtop workstation equipped with a UV lamp. Ideally, this should be located in a different area from the thermal cycler. Take care to avoid contaminating the area with plasmids or amplicons; never bring postamplification products into the designated clean area

• Change gloves frequently during sample preparation and reaction setup

• Use shaftguard pipets and aerosol-barrier pipet tips

• Clean pipets frequently with a dilute solution of bleach

• Use PCR-grade water and reagents dedicated for PCR use only

• Use screw-capped tubes for dilutions and reaction setup

Always set up a no-template control and preferably use a hot-star polymerase to prevent amplification before reaction begins.