Methods in virus diagnosis PCR techniques

450 MBIO

PRACTICAL LESSON 5

Molecular Methods

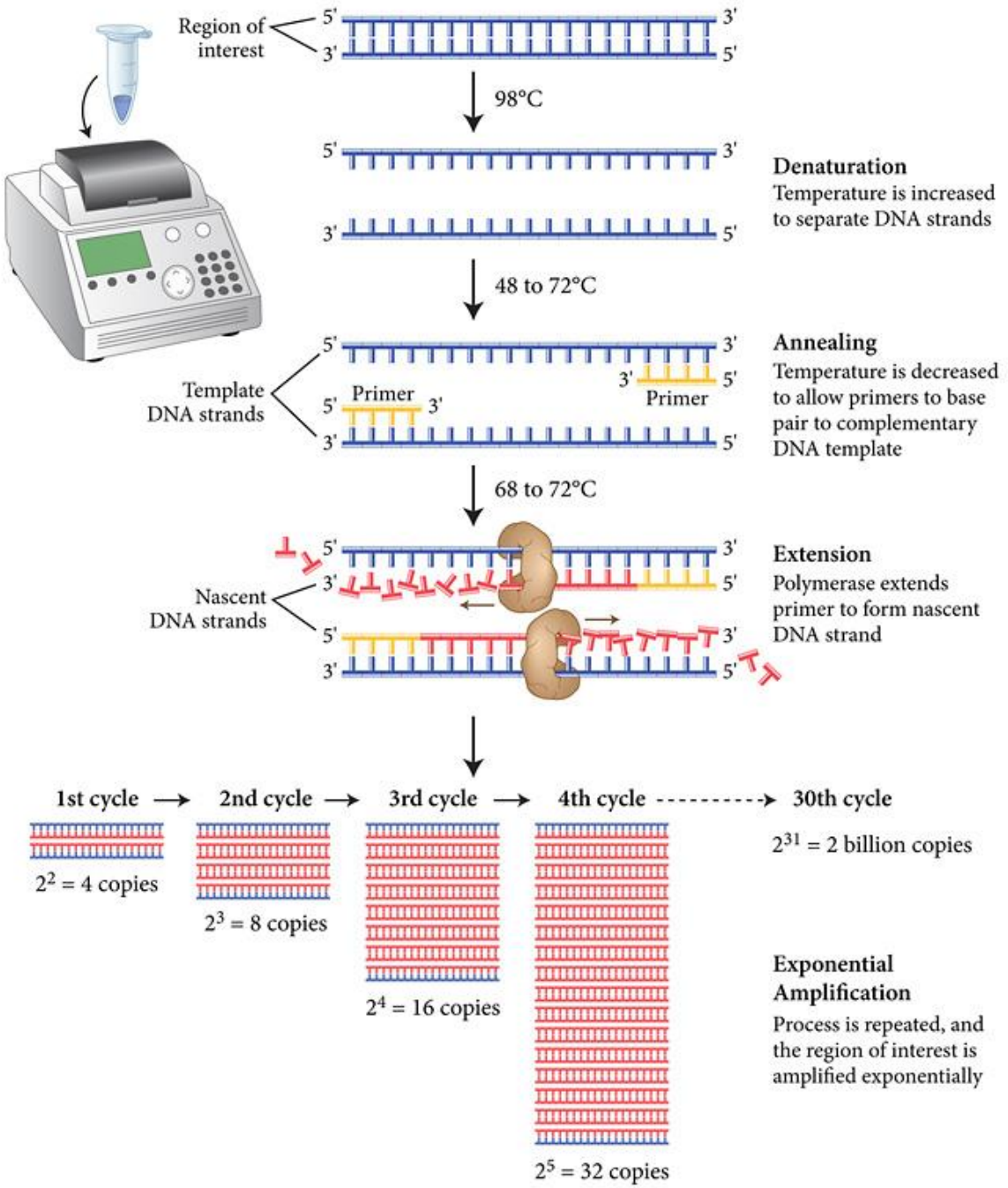
- Methods based on the detection of viral genome are also commonly known as molecular methods. It is often said that molecular methods is the future direction of viral diagnosis.
- However in practice, although the use of these methods is indeed increasing, the role played by molecular methods in a routine diagnostic virus laboratory is still small compared to conventional methods.
- It is certain though that the role of molecular methods will increase rapidly in the near future.

Classical Molecular Techniques

- Dot-blot, Southern blot, in-situ hydridization are examples of classical techniques. They depend on the use of specific DNA/RNA probes for hybridization.
- The specificity of the reaction depends on the conditions used for hybridization. However, the sensitivity of these techniques is not better than conventional viral diagnostic methods.
- However, since they are usually more tedious and expensive than conventional techniques, they never found widespread acceptance.

Polymerase Chain Reaction

- PCR allows the in vitro amplification of specific target DNA sequences by a factor of 10^6 and is thus an extremely sensitive technique.
- It is based on an enzymatic reaction involving the use of synthetic oligonucleotides flanking the target nucleic sequence of interest.
- These oligonucleotides act as primers for the thermostable Taq polymerase. Repeated cycles (usually 25 to 40) of denaturation of the template DNA (at 94^{o}C), annealing of primers to their complementary sequences (50^{o}C), and primer extension (72^{o}C) result in the exponential production of the specific target fragment.
- Further sensitivity and specificity may be obtained by the nested PCR.
- Detection and identification of the PCR product is usually carried out by agarose gel electrophoresis, hybridization with a specific oligonucleotide probe, restriction enzyme analysis, or DNA sequencing.


Region of interest
5'
3'
3'
5'
98°C
Denaturation
Temperature is increased to separate DNA strands
48 to 72°C
Annealing
Temperature is decreased to allow primers to base pair to complementary DNA template
Template DNA strands
Primer
68 to 72°C
Extension
Polymerase extends primer to form nascent DNA strand
Nascent DNA strands
1st cycle
2nd cycle
3rd cycle
4th cycle
30th cycle
2^2 = 4 copies
2^3 = 8 copies
2^4 = 16 copies
2^5 = 32 copies
2^{31} = 2 billion copies
Exponential Amplification
Process is repeated, and the region of interest is amplified exponentially

Polymerase Chain Reaction

Advantages of PCR:

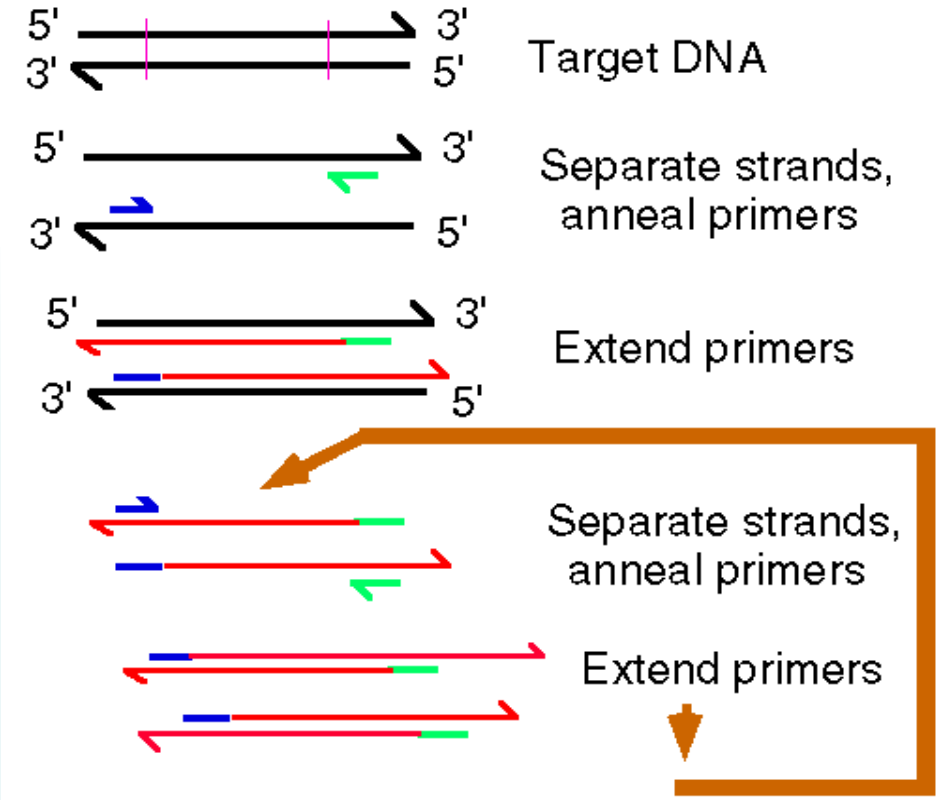
- Extremely high sensitivity, may detect down to one viral genome per sample volume
- Easy to set up
- Fast turnaround time

Disadvantages of PCR

- Extremely liable to contamination
- High degree of operator skill required
- Not easy to set up a quantitative assay.
- A positive result may be difficult to interpret, especially with latent viruses such as CMV, where any seropositive person will have virus present in their blood irrespective whether they have disease or not.

These problems are being addressed by the arrival of commercial closed systems such as the Roche Cobas Amplicor which requires minimum handling. The use of synthetic internal competitive targets in these commercial assays has facilitated the accurate quantification of results. However, these assays are very expensive.

Schematic of PCR



Each cycle doubles the copy number of the target

Other Newer Molecular Techniques

- Branched DNA is essentially a sensitive hydridization technique which involves linear amplification. Whereas exponential amplification occurs in PCR.
- Therefore, the sensitivity of bDNA lies between classical amplification techniques and PCR. Other Newer molecular techniques depend on some form of amplification.
- Commercial proprietary techniques such as LCR, NASBA, TMA depend on exponential amplification of the signal or the target.
- Therefore, these techniques are as susceptible to contamination as PCR and share the same advantages and disadvantages.
- PCR and related techniques are bound to play an increasingly important role in the diagnosis of viral infections.
- DNA chip is another promising technology where it would be possible to detect a large number of viruses, their pathogenic potential, and their drug sensitivity at the same time.

Comparison between PCR and other nucleic acid Amplification Techniques

Method	Target Amplification	Signal Amplification	Thermocycling	Sensitivity	Commercial Examples
PCR	Exponential	No	Yes	High	Roche Amplicor
LCR	No	Exponential	Yes	High	Abbot LCX
NASBA	Exponential	No	No	High	Organon Teknika
TMA	Exponential	No	No	High	Genprobe
Qß-Replicase	No	Exponential	No	High	None
Branched DNA	No	Linear	No	Medium	Chiron Quantiplex

Real-time PCR

What is Real-Time PCR?

- Real-Time PCR a specialized technique that allows a PCR reaction to be visualized “in real time” as the reaction progresses.

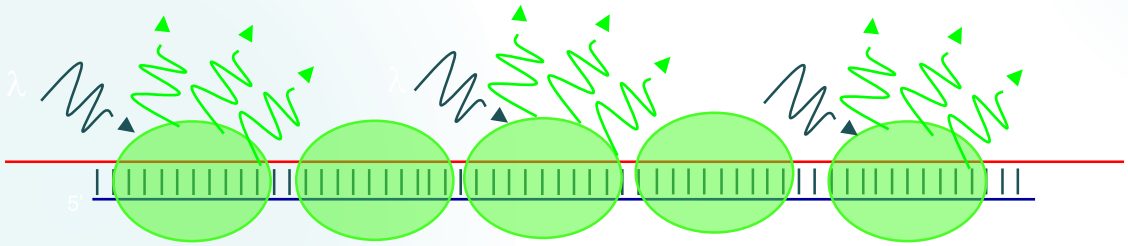
- As we will see, Real-Time PCR allows us to measure minute amounts of DNA sequences in a sample!

Real-Time PCR Applications

- quantitation of gene expression
- drug therapy efficacy / drug monitoring
- viral quantitation
- pathogen detection

How do We Measure DNA in a PCR Reaction?

We use reagents that fluoresce in the presence of amplified DNA.



Measuring DNA:

Ethidium Bromide

+ = common and well known

- = toxic, not very bright

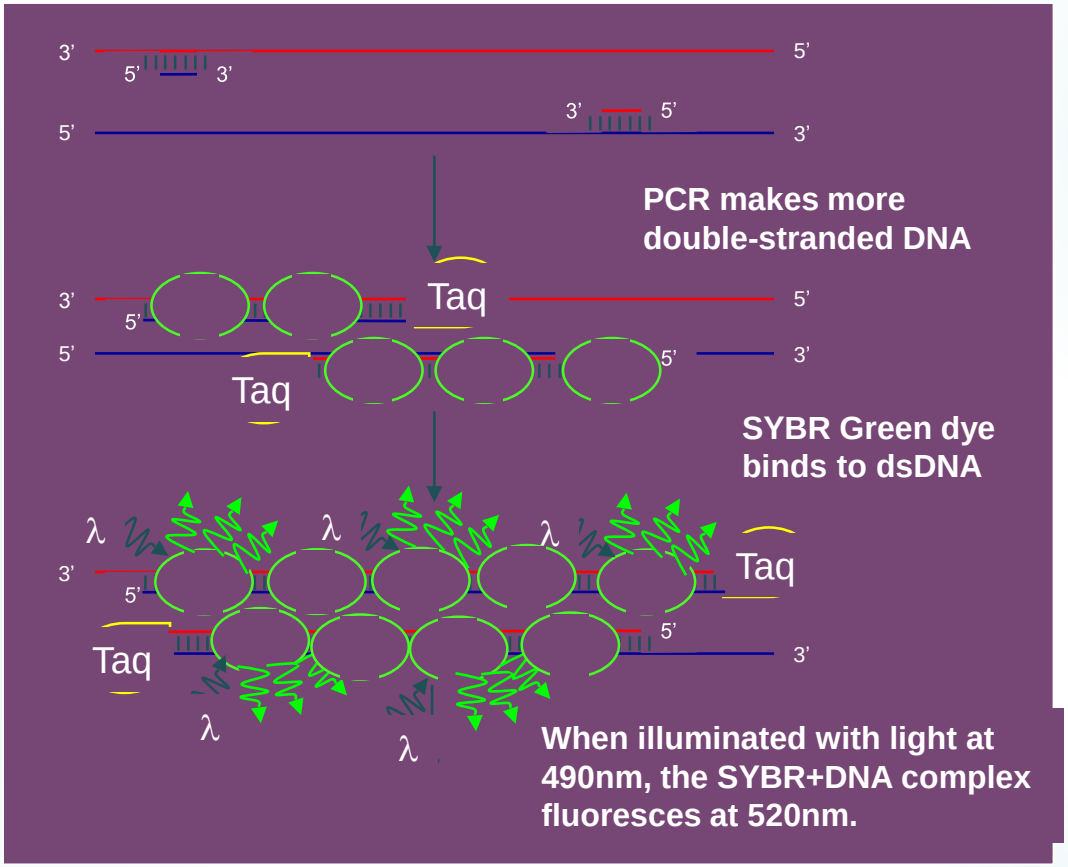
SYBR Green I

+ = Bright fluorescence!

+ = Low toxicity!

Fluorescent Dyes in PCR
Intercalating Dyes

SYBR Green in Action!



Fluorescent Dyes in PCR
Other Options

- Even more ways to detect PCR products

•Other Intercalating Dyes
  - Eva Green

•Probes
  - TaqMan Probes

•Primer/Probe Combinations
  - Scorpions
  - LUX Primers

What Type of Instruments are used with Real-Time PCR?

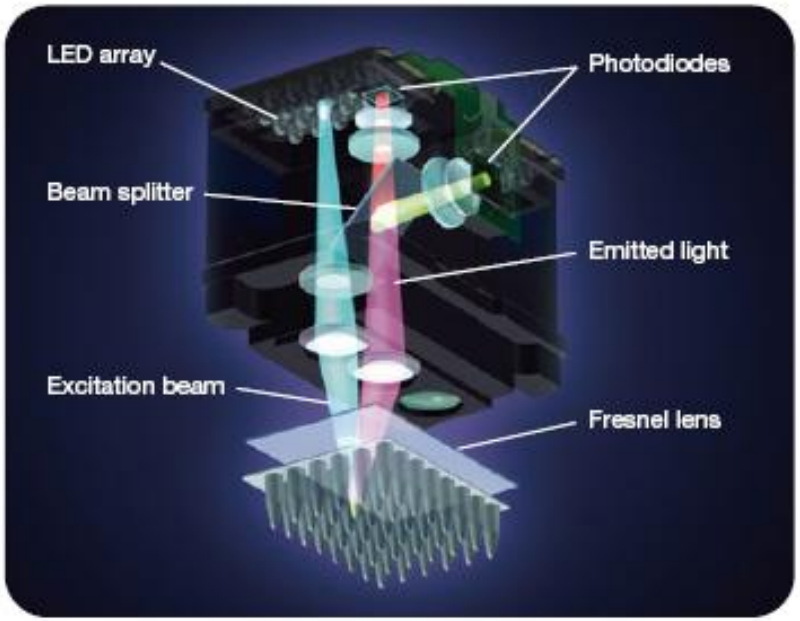
Real-time PCR systems consist of THREE main components:

1. Thermal Cycler (PCR machine), linked to a…
2. Optical Module (to detect fluorescence in the tubes during the run), linked to a…
3. Computer (to translate the fluorescence data into meaningful results).

What Type of Instruments are used with Real-Time PCR?

- A good example is the MiniOpticon real-time instrument

Optical Module
Thermal Cycler Base

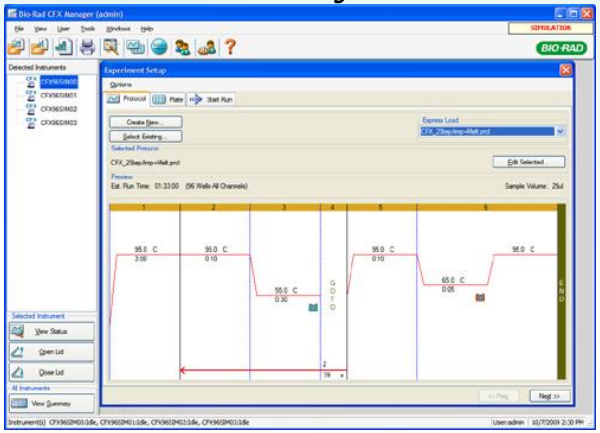


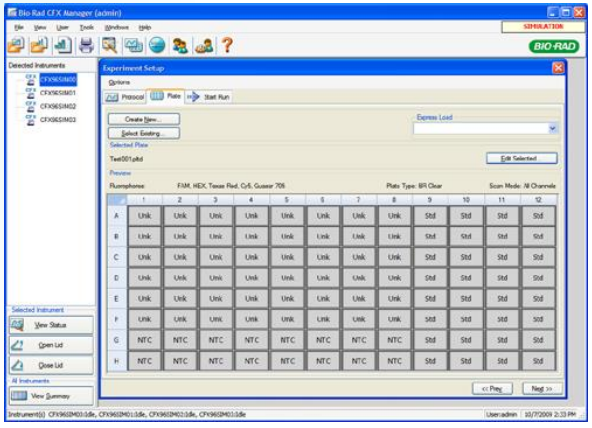
What Type of Instruments are used with Real-Time PCR?

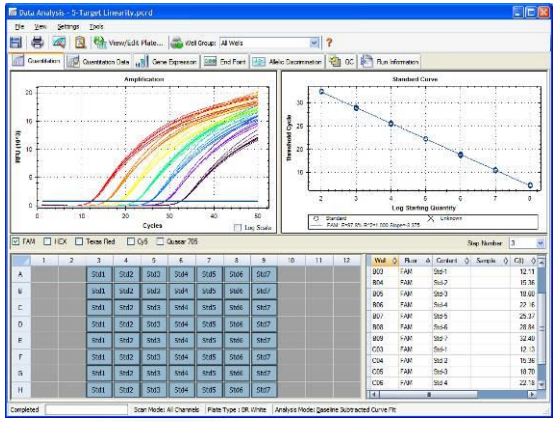
The computer, running real-time software, converts the fluorescent signals in each well to meaningful data.

Workflow:

1. Set up PCR protocol.
2. Set up plate layout.
3. Collect data.
4. Analyze data.



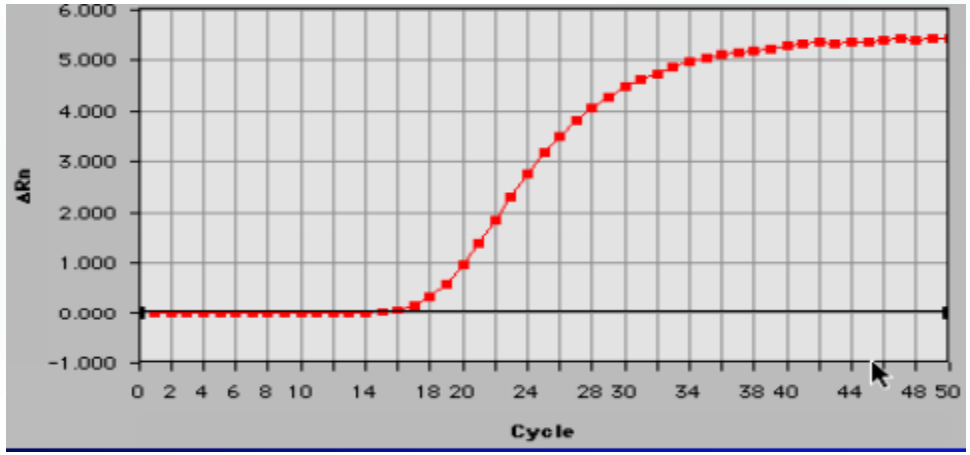




Real-time PCR is kinetic

- Detection of “amplification-associated fluorescence” at each cycle during PCR
- No gel-based analysis at the end of the PCR reaction
- Computer based analysis of the cycle-fluorescence time course

Increasing fluorescence



Linear plot

PCR cycle

Real-time PCR advantages

- not influenced by non-specific amplification
- amplification can be monitored real-time
- no post-PCR processing of products

(high throughput, low contamination risk)

- requirement of 1000-fold less RNA than conventional assays

(3 picogram = one genome equivalent)

- most specific, sensitive and reproducible

Real-time PCR disadvantages

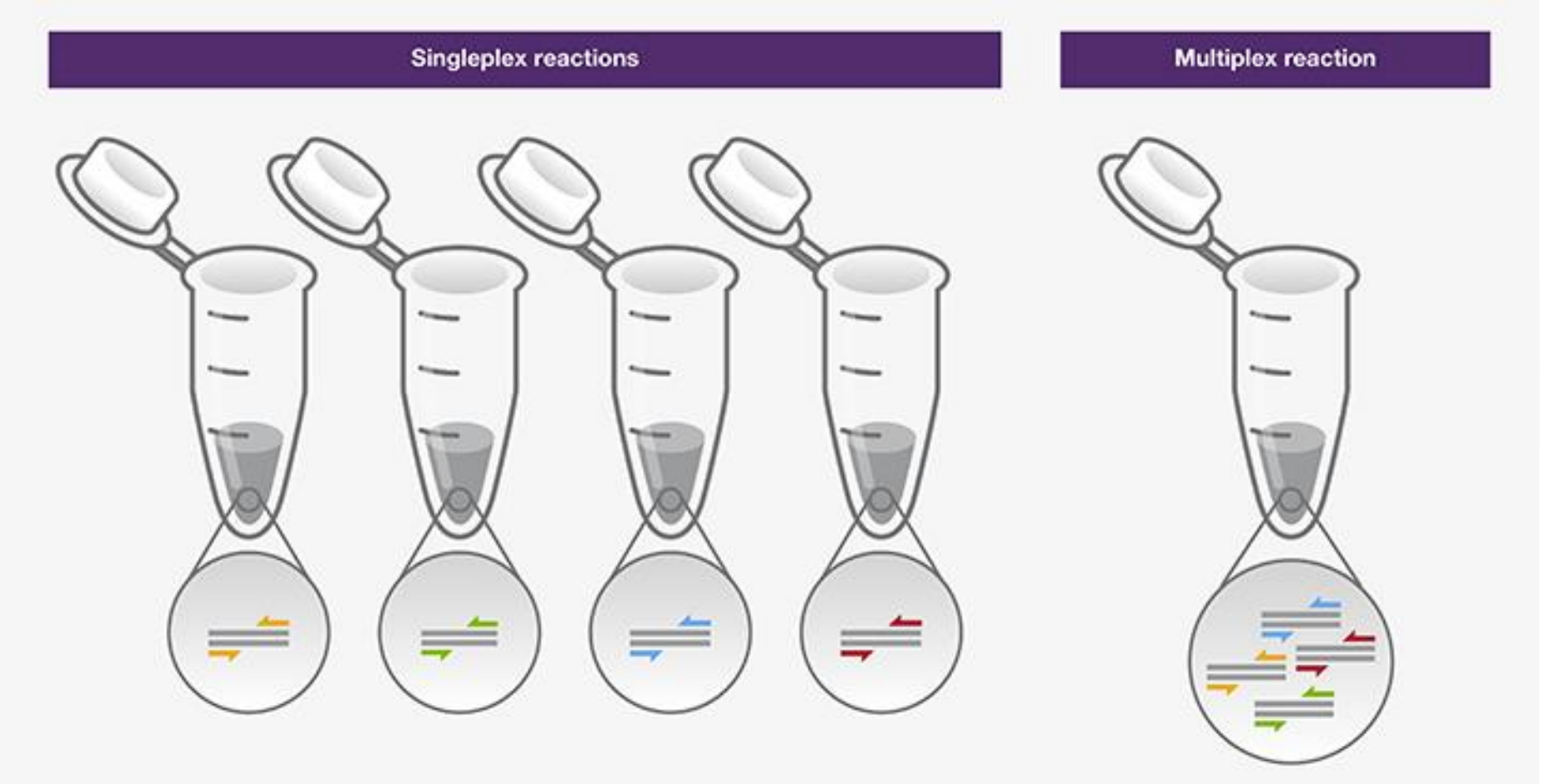
- setting up requires high technical skill and support
- high equipment cost
- Runs are more expensive than conventional PCR
- DNA contamination (in mRNA analysis)

Multiplex and Singleplex PCR

Singleplex PCR: a single target is amplified in a single reaction tube. it is ideal for those testing varied target sequences, when performing initial scouting experiments, or even for use in massively parallel technology.

Multiplex PCR: allows for simultaneous amplification of multiple target sequences in a single tube using specific primer sets in combination with probes labeled with spectrally distinct fluorophores.

multiplex PCR is commonly used for variety of research applications, including: gene panel expression, pathogen detection, SNP genotyping, gene deletion analysis and template quantification, among many others.


Singleplex reactions
Multiplex reaction

Advantages of Singleplex PCR

•It's inherently simpler in its design, implementation, and optimization because does not require specific probes.
•It can be fast and easy to perform with little optimization required.
•Reduction in the amount of starting template required, which is important when the amount of starting material is limited
• Reduction in false negatives, if a control target is amplified within each sample
• Increased laboratory throughput with a concomitant reduction in reagent costs
• Minimization of sample handling and associated opportunities for laboratory contamination

Advantages of Multiplex PCR

- Use for repeate and high-volume analysis of the same targets.
- The ability to simultaneously analyze multiple target sequences in a single tube allows for maximizing use of precious starting material
- With optimization, this can translate to increased time and cost-efficiency, yielding more data from each reaction and utilizing fewer reagents (e.g. dNTPs, enzymes, etc).
- This allows for increased throughput, with fewer pipetting steps and, thus, a decreased risk for pipetting errors.
- Additionally, multiplexing opens the possibility for an internal reference for each sample. When multiple targets are amplified, this can significantly increase the accuracy of comparative analysis with improved normalization.

Multiplex PCR