Polymerase Chain Reaction

Variations of PCR in the Diagnostic Lab

The most common variations of standard PCR used in the diagnostic laboratory are:

- Reverse Transcriptase PCR (RT-PCR)
- Nested PCR (n-PCR)
- Multiplex PCR (m-PCR)
- Real-time PCR

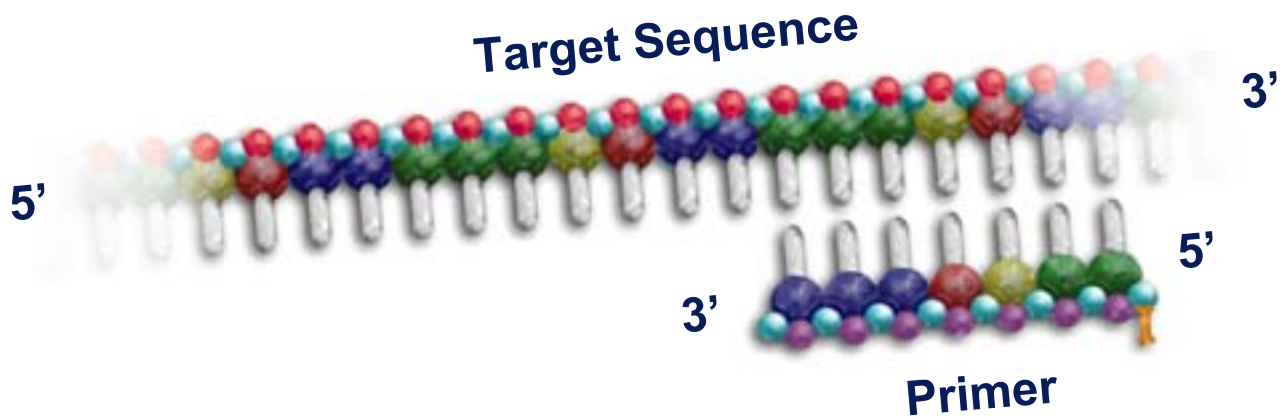
- PCR amplifies DNA targets
- Many viruses contain a RNA genome
- Amplification requires RT-PCR

Difference between RNA and DNA

Difference between RNA and DNA		
	RNA	DNA
Sugar	Ribose	Deoxyribose
Bases	Adenine (A) Cytosine (C) Uracil (U) Guanine (G)	Adenine (A) Cytosine (C) Thymine (T) Guanine (G)
No. of strands	Usually single	Double
Heat stable?	No	Yes

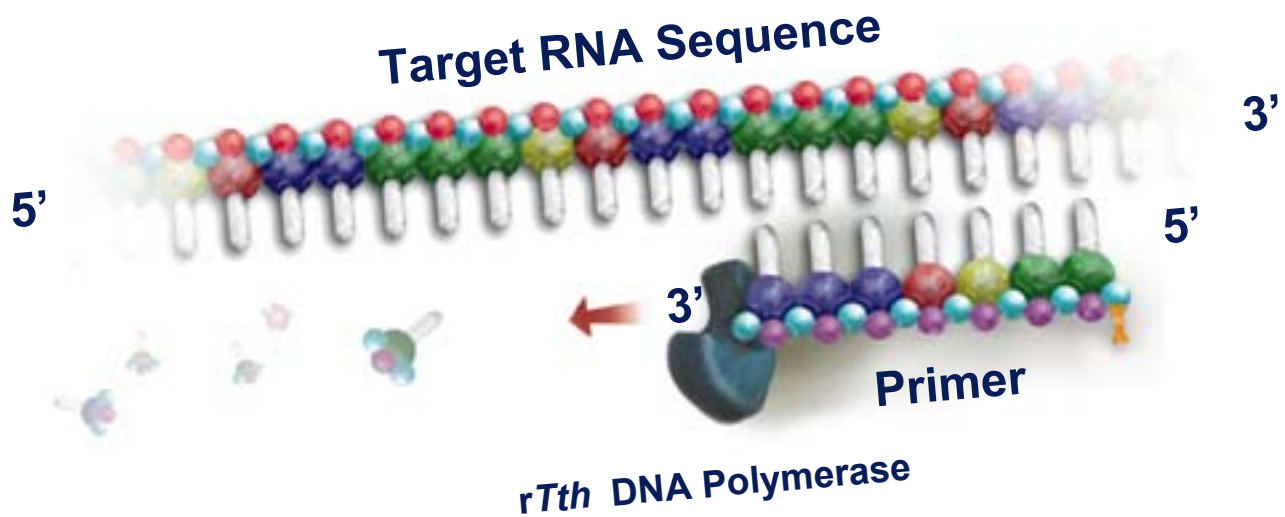
Reverse Transcription - Step 1 –

Primer Anneals to Target RNA Sequence



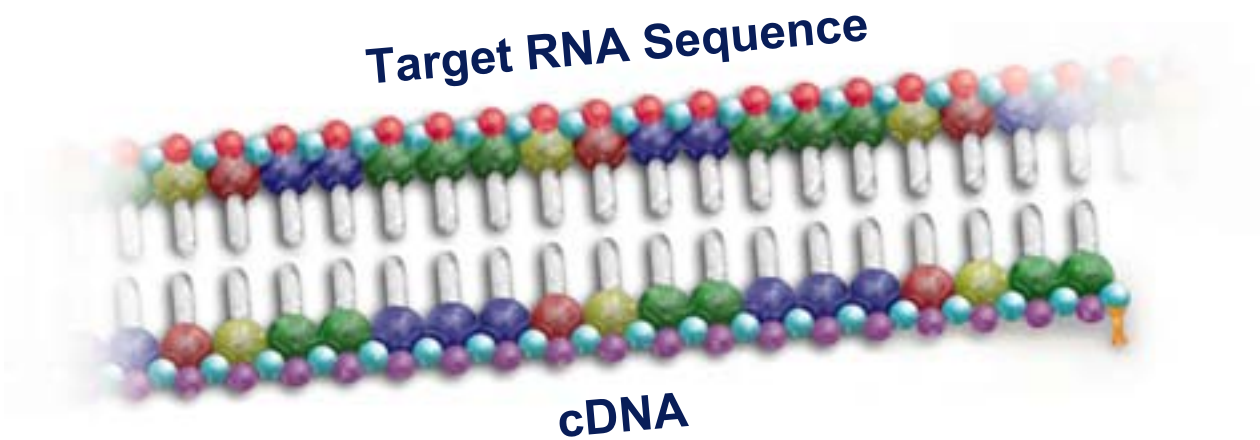
Reverse Transcription - Step 2 –

r*Tth* DNA Polymerase also has RT activity Catalyses Primer Extension by Incorporating Complementary Nucleotides

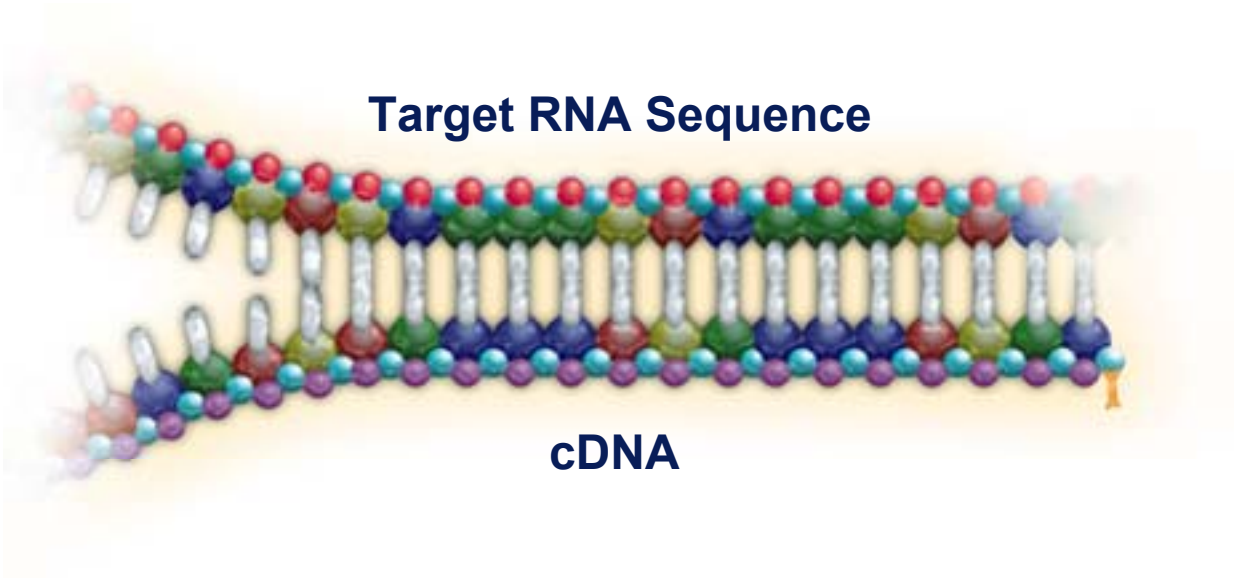


End of Reverse Transcription - Step 3 –

Results in Synthesis of Complementary DNA (cDNA) to the RNA Target Sequence



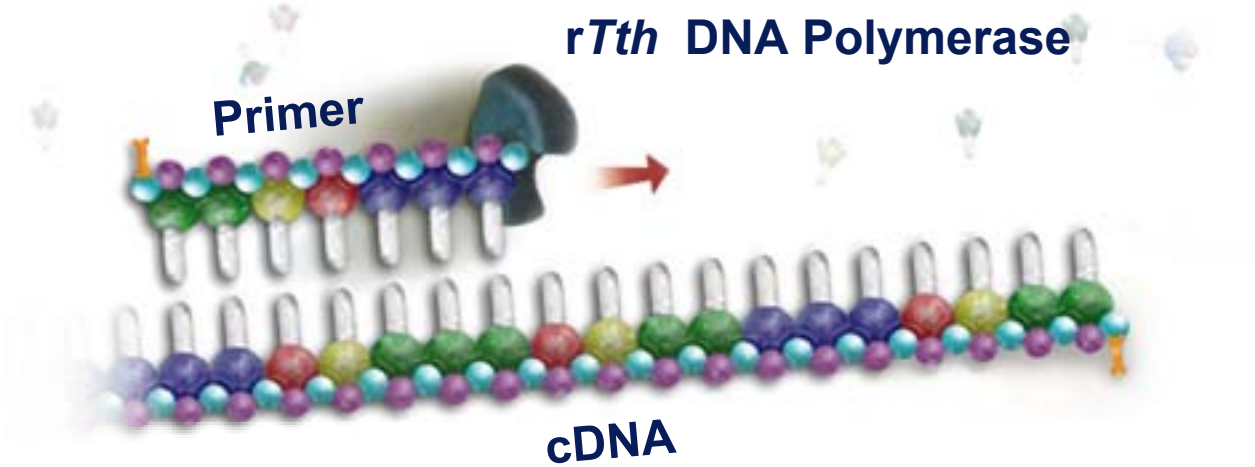
PCR Step 1 - Denaturation by Heat



PCR Step 2 - Annealing of Primer to cDNA

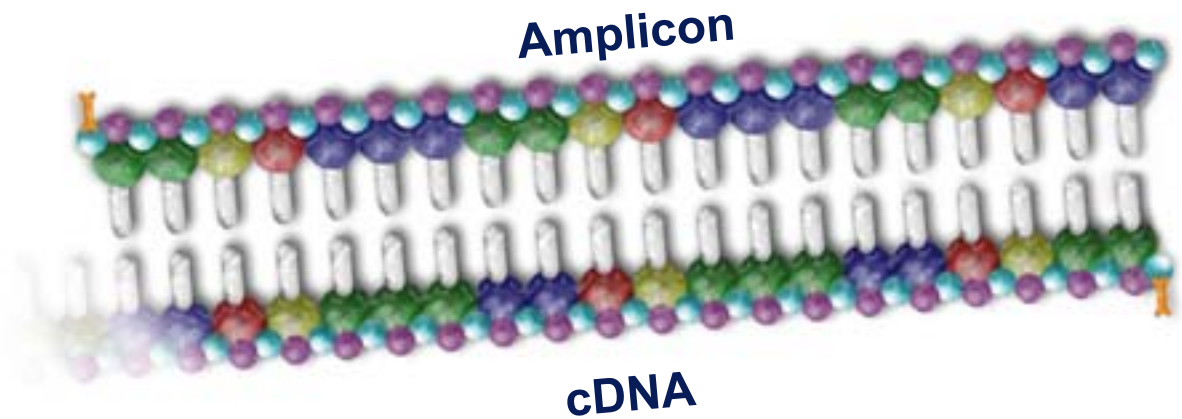
PCR Step 3 –

r*Tth* DNA Polymerase Catalyses Primer Extension



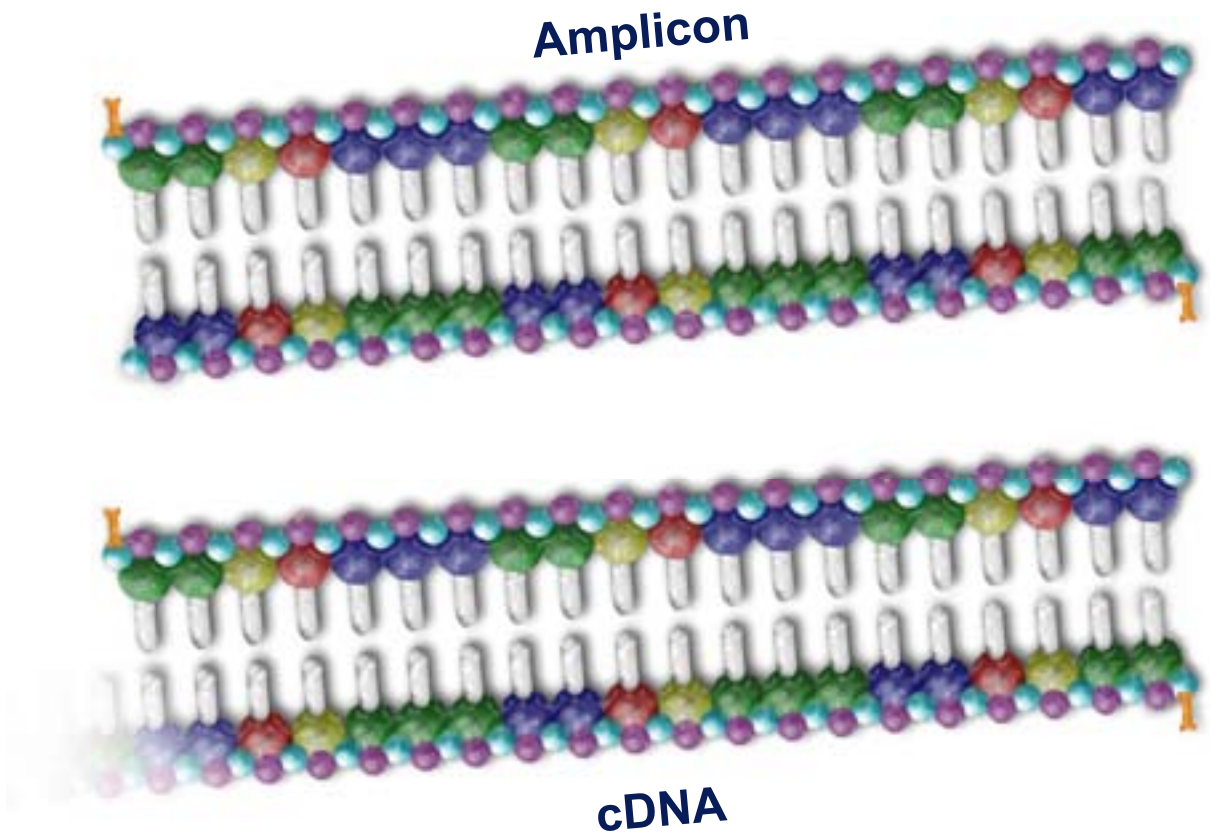
End of 1st PCR Cycle –

Yields a Double-Stranded DNA Copy (Amplicon) of the Target Sequence



PCR End of Second Cycle –

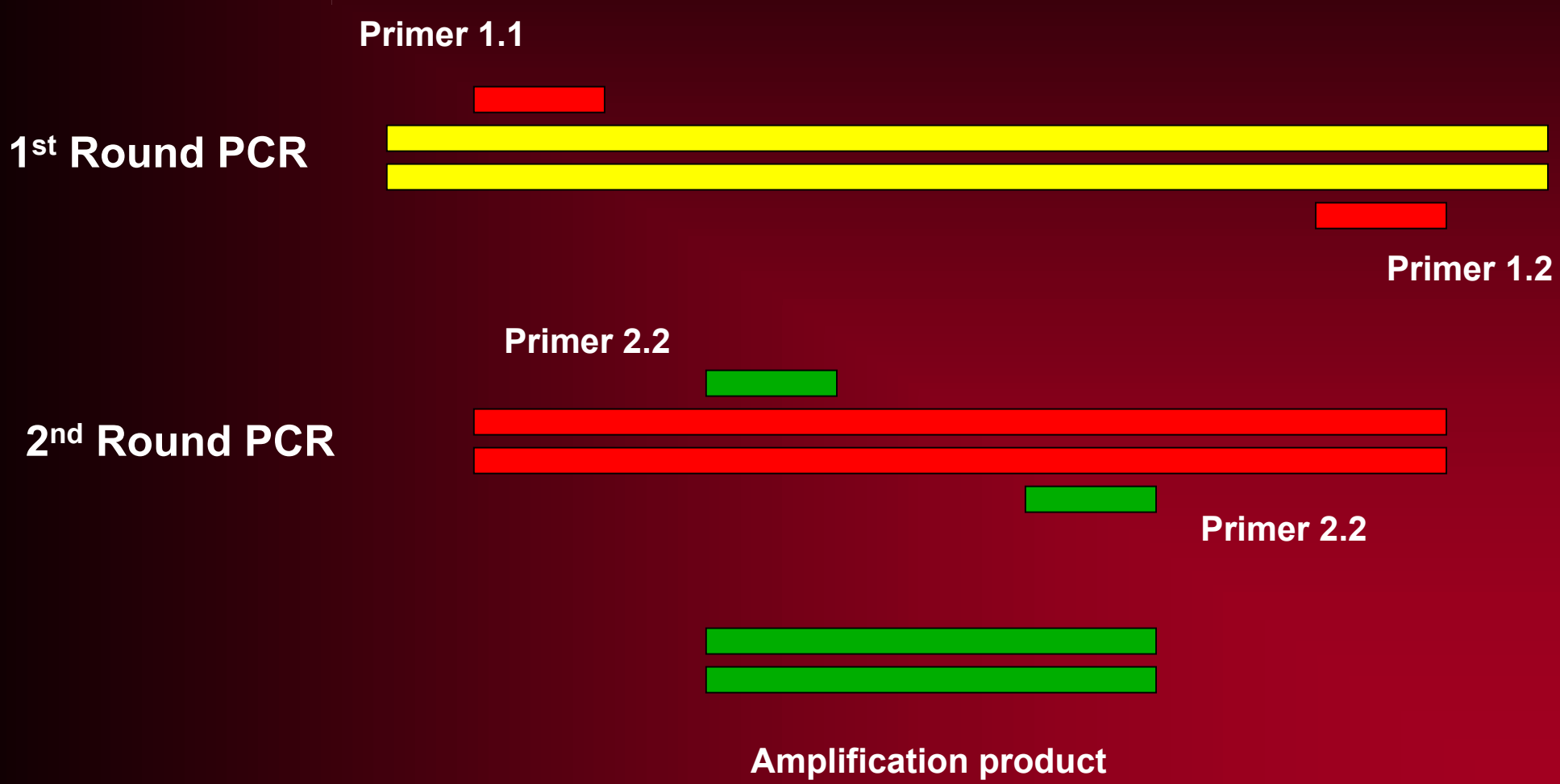
r*Tth* DNA Polymerase Catalyses Primer Extension



PCR – Variations of the Technique

- **Nested PCR**
- **Multiplex PCR**

NESTED PCR


Primer 1.1
1st Round PCR
Primer 1.2
Primer 2.2
2nd Round PCR
Primer 2.2
Amplification product

NESTED PCR

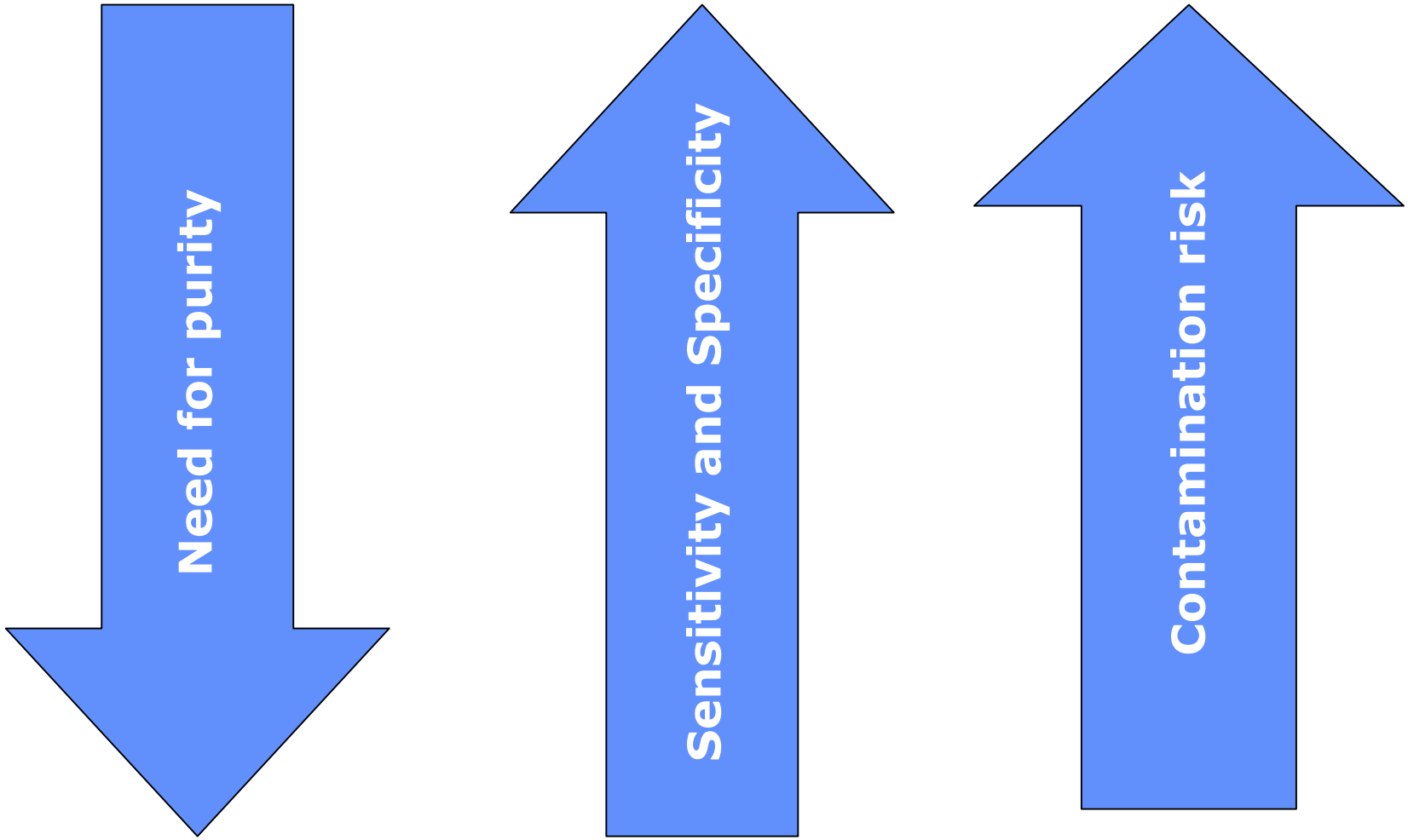
The advantages of n-PCR are:

- Its increased specificity (specific binding of 2nd primer pair).
- Increased sensitivity (2nd round of PCR amplification)

n-PCR is used to detect organisms present in low copy numbers

- Viruses in CSF (herpes simplex, JCV)
- Eye samples (adenovirus, herpes simplex)

Nested PCR


Need for purity
Sensitivity and Specificity
Contamination risk

Multiplex PCR

m-PCR is a rapid method of detecting multiple targets in a single reaction

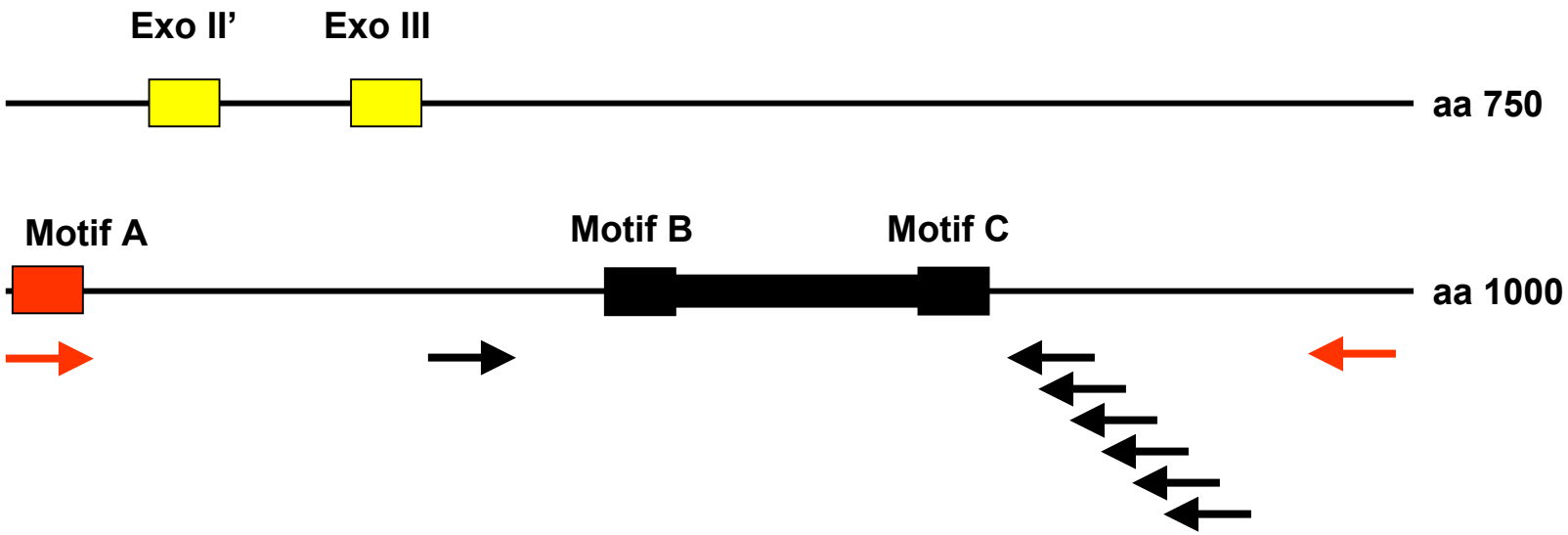
PCR reagent mix contains multiple sets of primers. Any one of these may be amplified during the PCR

- Primer sets to multiple organisms
- Primer sets to multiple target genes in the same organism

Major advantage is the reduction in test processing time

Herpes virus Multiplex Primers

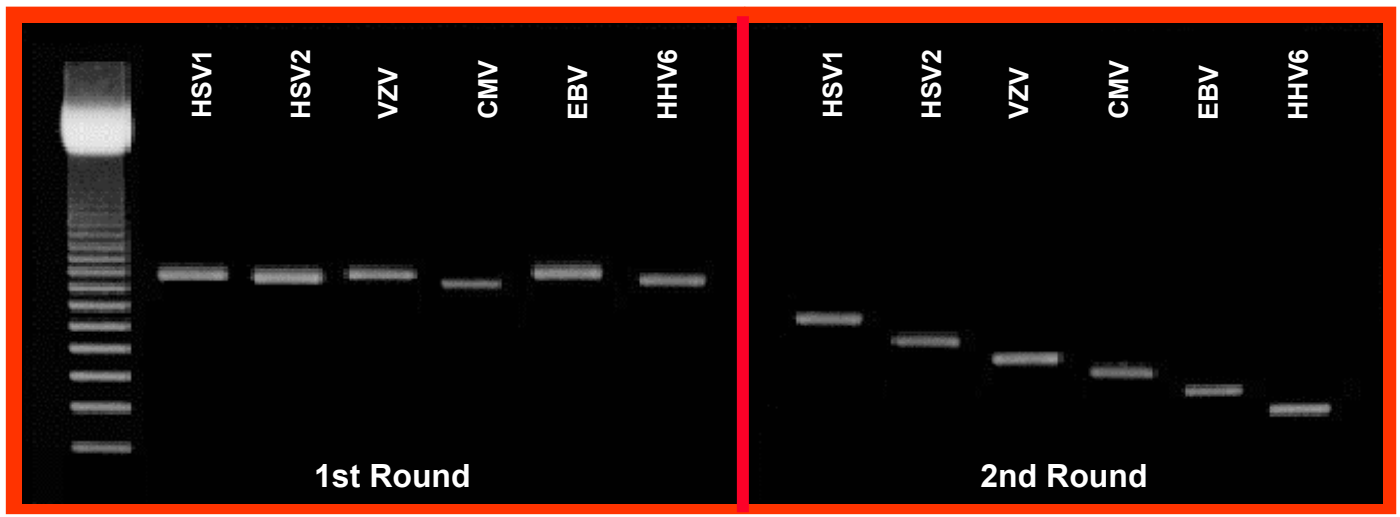
Herpesvirus DNA polymerase gene



Reference: Heringa and Argos 1994

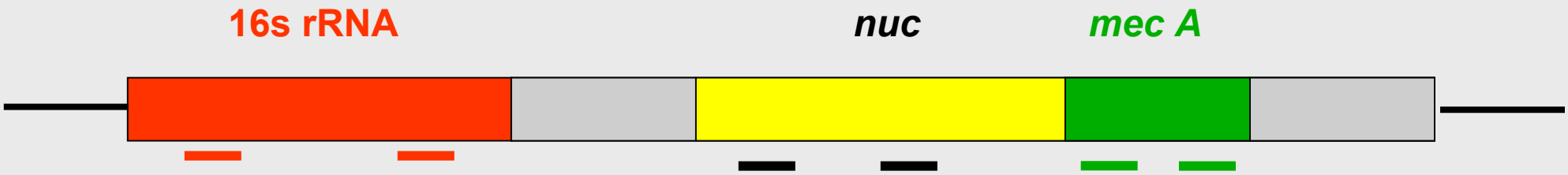
HERPES MULTIPLEX PCR

Results of PCR amplification with external and internal primers



APPLICATION OF m-PCR

Staphylococcus aureus Genome



- **Detection of 16s rRNA gene - common to ALL bacteria**

- **Detection of *genus-specific* gene sequences**
  - *nuc* gene is specific for ALL Staph aureus

- **Detection of drug resistance**
  - *mec A gene* confers methicillin resistance in MRSA

SUMMARY

- PCR is now widely used in research
- "in house" diagnostic tests are becoming accepted
- Multiplex PCR tests offer great flexibility
- Nested PCR needs stringent protocol to prevent contamination
- Instrumentation offers exciting possibilities for the future (Quantitation)

Detection of Amplification Products

- Agarose Gel Detection
- Solid Phase Hybridisation and Colour Detection
- Real-time Detection

Confirmation and Identification of PCR Products

- **Gel electrophoresis**
- **Confirmatory methods**
  - **Sequencing of the amplification product**
  - **Southern blotting**
  - **Restriction fragment length polymorphism (RFLP) analysis**
  - **high density DNA microarrays**

DNA Detection

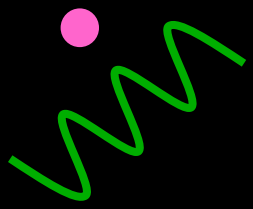
Traditional methods of amplicon detection are:

- time consuming
- limited number of specimens
- carcinogenic reagents
- relatively insensitive

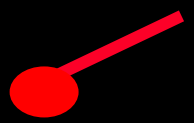
Plate hybridisation with colour detection

- process large numbers of specimens
- uses standard ELISA reagents
- increased sensitivity (100-1000 X)
- increased specificity
- allows quantitation

Colour detection of PCR Products

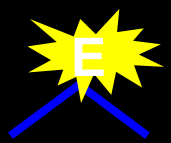


PCR incorporates digoxigenin in amplicon



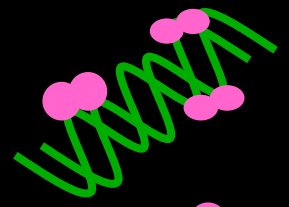
Amplicon is heat denatured and mixed with biotin labelled probe

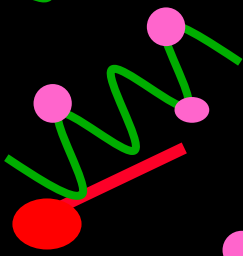
Added to plate coated with avidin

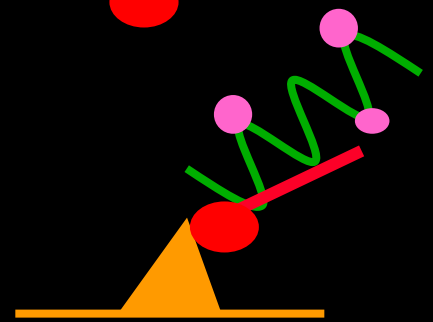


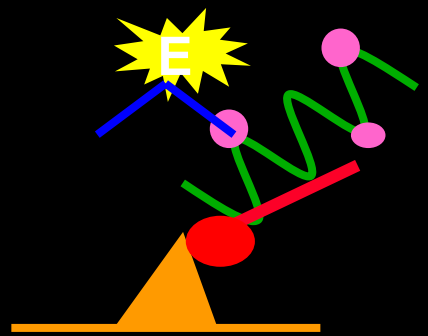
Detected with α-DIG enzyme conjugate

TMB substrate - OD_{450}

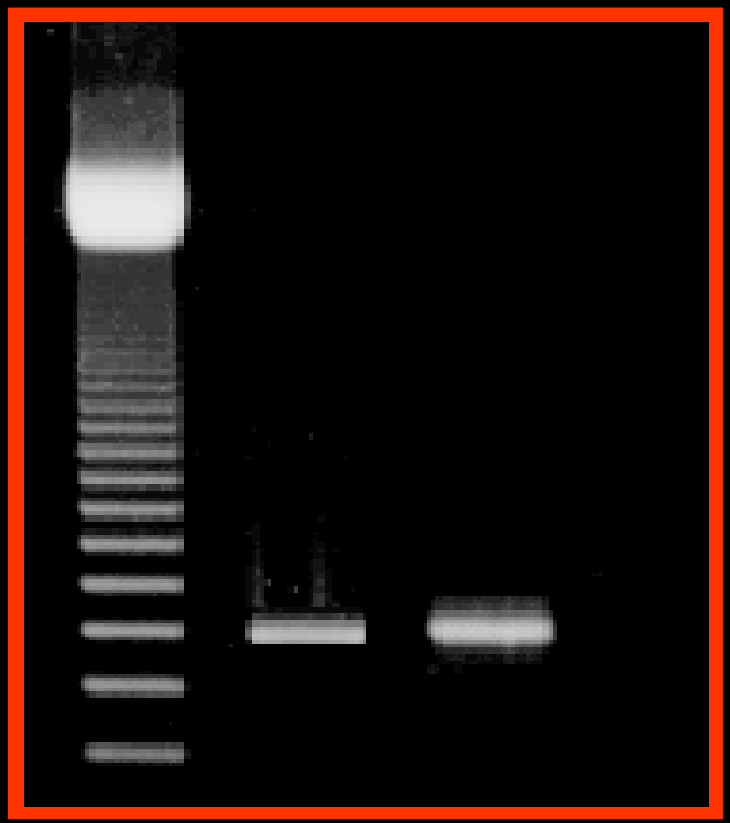








Colour Detection of PCR Products



Agarose Gel

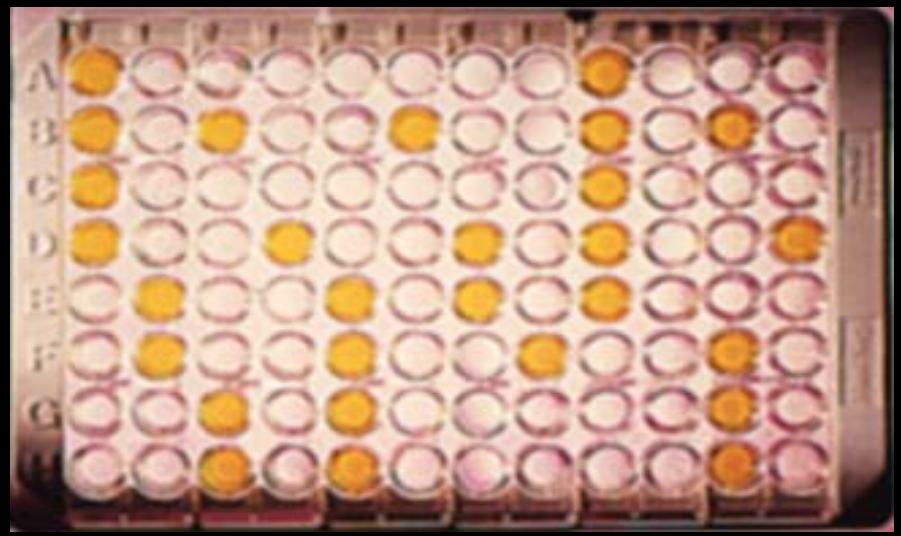
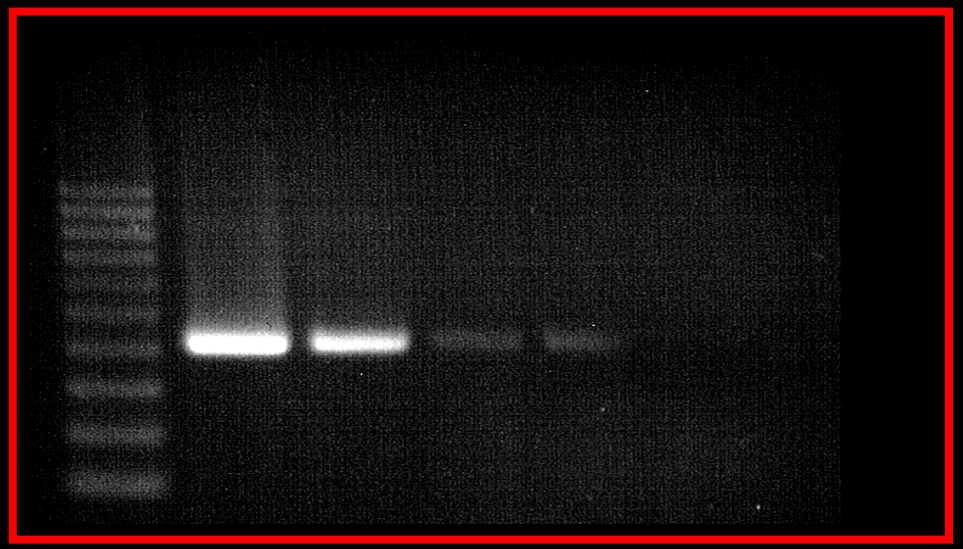
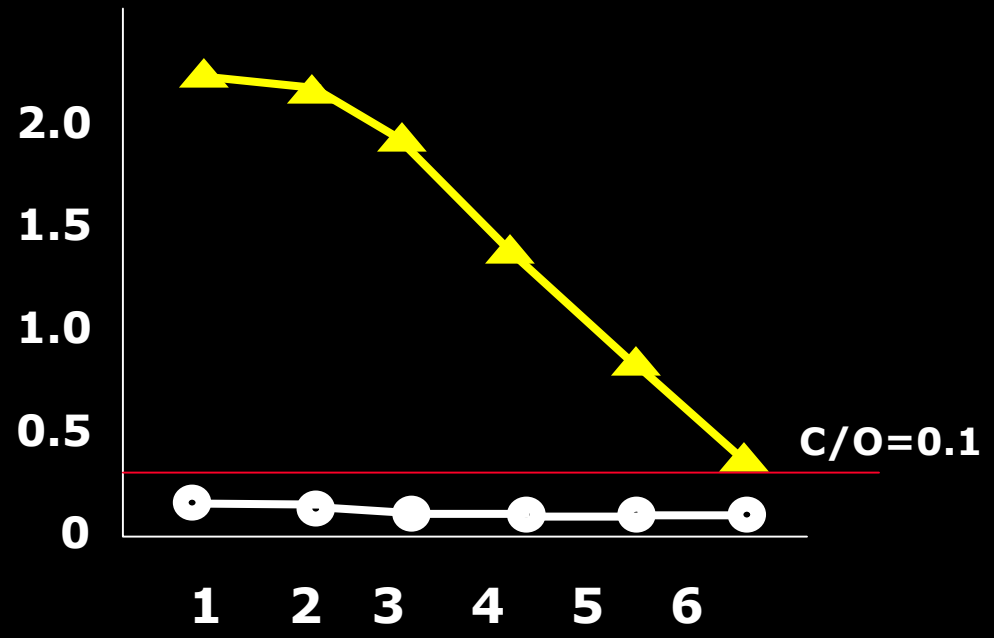


Plate Hybridisation

Gel Detection vs Colour



L – Ladder
1 - Undiluted
2 - 1 : 4
3 - 1 : 8
4 - 1 : 16
5 - 1 : 32
6 - 1 : 64



Summary

- Gel detection is fast and relatively easy, but not suitable for large numbers of specimens
- Colour detection is more labour intensive and more expensive. More sensitive and useful in quantitation
- Real-time detection is fast and relatively inexpensive. Capital outlay is considerable.