NCPTS

# PCR

## Basic facts and how it will be used in HPV testing

Developed by Dr Melinda Paterson, PhD
NCPTS Training Team
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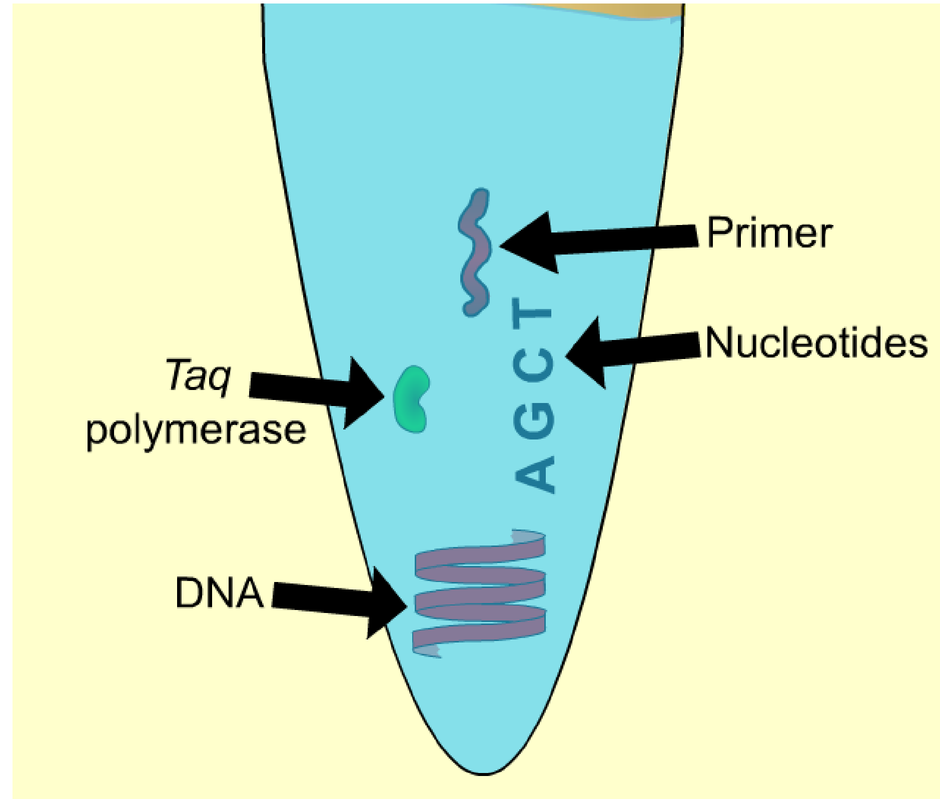
# Introduction

- Polymerase Chain Reaction (PCR) is a biochemical technique that uses thermocycling and enzymes to quickly and reliably copy DNA
- It is one of the most well-known techniques in molecular biology
- PCR is very precise and can be used to amplify or copy a specific DNA target from a mixture of DNA molecules
- Amplification is achieved by a series of 3 steps:
    - Denaturation
    - Annealing
    - Extension

# Basic PCR Ingredients

- Polymerase
- Template DNA (DNA of interest)
- Primers
- Nucleotides
- Buffer



# Polymerases

- Polymerases are enzymes which can assemble new strands of DNA from template DNA and nucleotides
- Polymerases in modern PCR come from one of two thermophilic bacteria sources, *Thermus aquaticus* (Taq) or *Pyroccus furiosus* (Pfu)
- Commercial Taq and Pfu polymerases are engineered for speed, accuracy, processing efficiency and their ability to read GC rich templates

# Template DNA

- The template DNA is the DNA that you design your primers to and is the DNA that your polymerase will read and copy
- It can be genomic (nuclear), plasmid (cytoplasmic) or cDNA (created from RNA)
- This template DNA needs to be intact and pure for successful PCR.
- The ideal amount of DNA is also important. Usually 1pg-1ng of plasmid DNA or 1ng-1µg of genomic DNA is required per reaction

# Primers

- Primers are short fragments of synthesized DNA that bind to your template DNA
- A "forward" and "reverse" primer needs to be designed
- The forward primer is the start of your PCR and its sequence is the same as your 5'-3' template DNA sequence
- The reverse primer is the end of your PCR and its sequence is the reverse complement of your template DNA
- Usually 18-22 base pairs long
- Primers should have a melting temperature of around 54-60$^{o}$C and be as similar to each other as possible

# Nucleotides

- Nucleotides are the monomers of DNA and are necessary for making DNA copies
- Deoxynucleoside triphosphates (dNTPs) are generally used either separately or as a mix
- Nucleotides are very sensitive to freeze/thaw cycles so it is best to create small aliquots

# Buffer

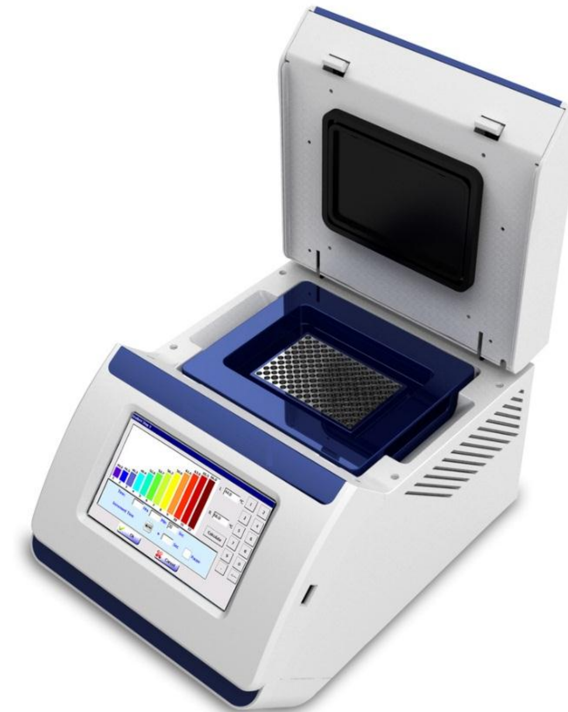
- Most commercial polymerases come supplied with their ideal buffer
- This buffer ensures the correct pH and also may have additives like magnesium, potassium or DMSO which can help optimize DNA denaturing, renaturing and polymerase activity
- Changing the concentration of $MgCl_2$ can be beneficial in optimizing conditions

# Thermocycling

- All of the above ingredients are added to a PCR tube and the tube is thermocycled
- A thermal cycler is an instrument that automates temperature cycling and incubation times for PCR
- The following is a typical PCR thermocycler profile:

i. Initialization
ii. Denaturation
iii. Annealing
iv. Extension
v. Final elongation

(ii–iv) repeated 15-40 times



# Initialization

- Reaction is heated to 94-96$^{o}$C for 30 secs to several minutes.
- Usually done once at the start of the PCR
- This step is important for activating hot-start polymerases and also to denature your template DNA
- If your template is GC rich then you may need to perform an extra-long initialization step

# Denaturation

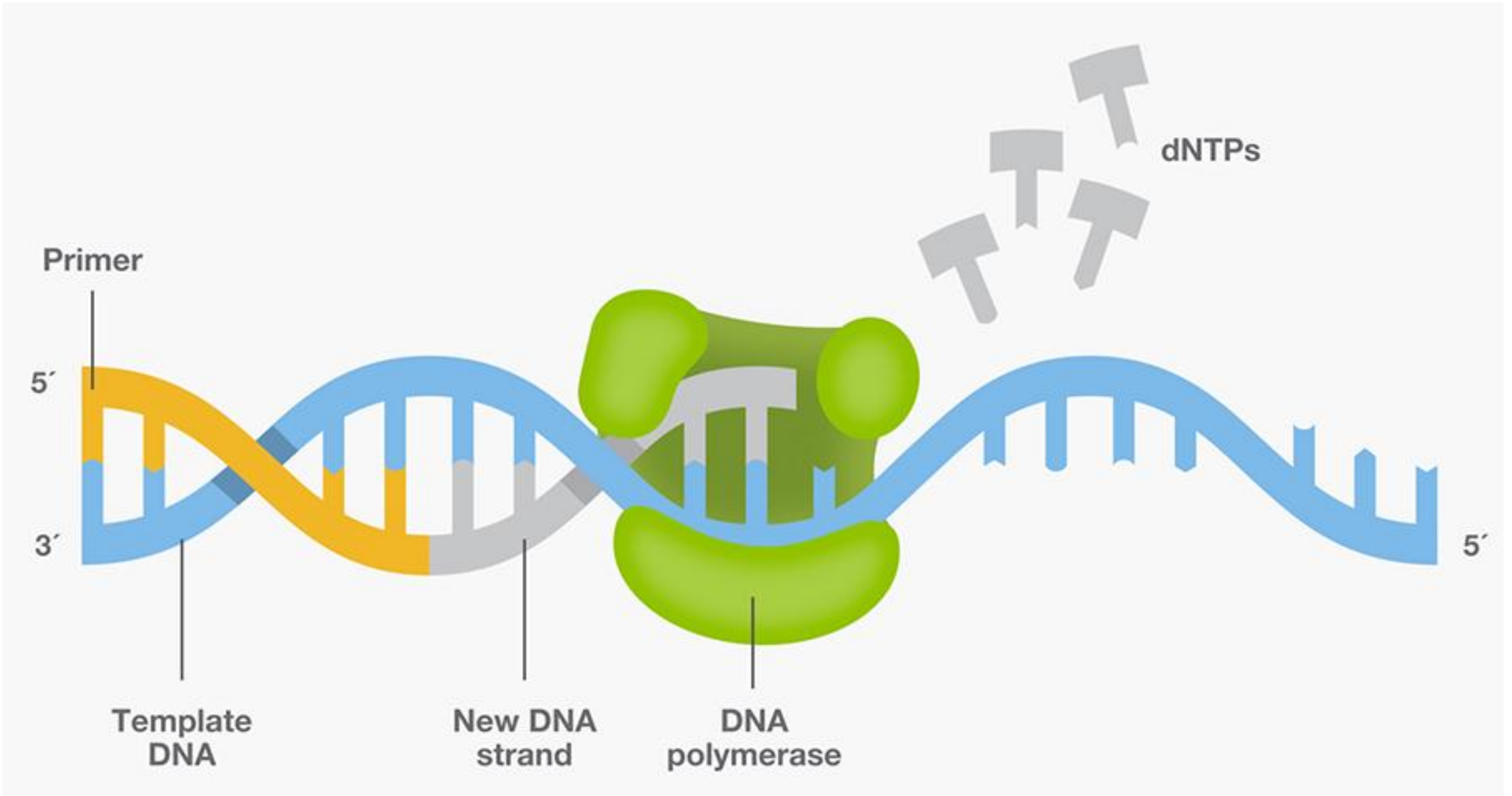
- Reaction is heated to 94-98$^{o}$C for 15-30 secs.
- This step denatures your DNA and primers which will allow them to anneal to each other in the next step

# Annealing

- Reaction's temperature is lowered to 50-64$^{o}$C for 20-40 secs.
- The temperature needs to be low enough that your denatured primers can form Watson-Crick base pairs with your template DNA, but high enough that only the most stable double-stranded DNA structures can form to prevent mis-priming
- The perfect annealing temperature is a few degrees lower than the calculated melting temperature of your primer pair
- A rough calculation of the melting temperature can be done via the 4xGC+2xAT method, but there are more accurate online tools
- The polymerase also binds to your primer/template DNA complex during this step

# Extension

- The reaction is rapidly heated to 68-74$^{o}$C.
- This is when the polymerase begins to read your DNA template in the 3’-5’ direction and synthesizes the complementary daughter strand in the 5’-3’ direction
- Exact temperature will be determined by your polymerase
- The length of the step depends on how long your DNA copy will be
- You will need to allow at least 1 minute of extension time per 1000 bases
- At the end of this incubation new double stranded pieces of DNA will have been created consisting of both template and new DNA



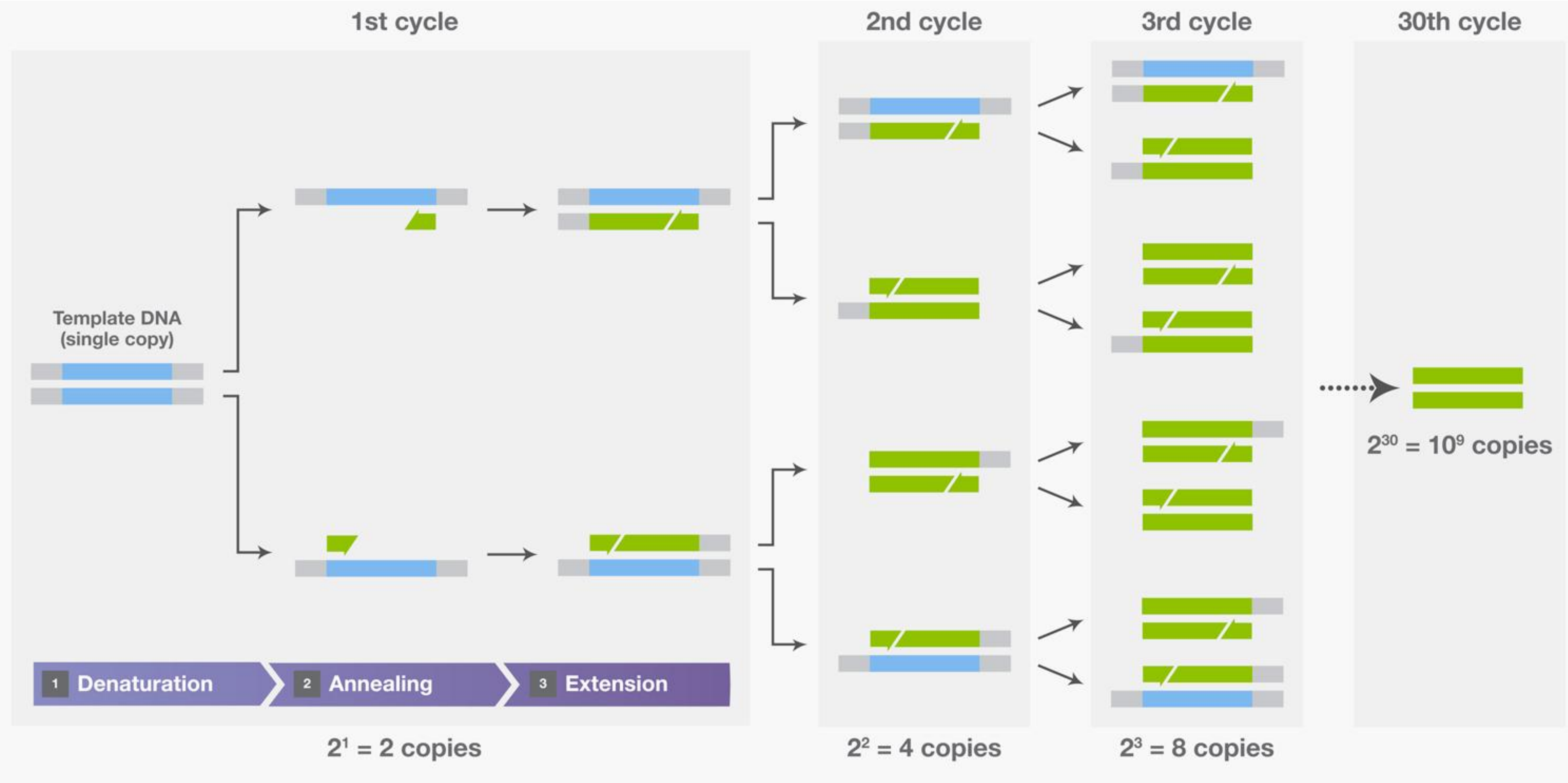

DNA Polymerase extending the 3’ end of a PCR primer in the 5’-3’ direction (Taken from the ThermoFisher Scientific website)

# Cycles

- Denaturation, Annealing and Extension steps are repeated 15-40 times
- The more cycles you program the more DNA copies you will create, but after an initial exponential amplification phase the amplification plateaus due to the depletion of primers and nucleotides
- The number of DNA copies formed after a given number of cycles is $2^n$
- There is an upper limit for the number of PCR cycles and less but good/specific clean PCR product is preferable to lots of dirty/nonspecific product
- Some DNA polymerases don‘t have a proof-reading activity leading to accumulation of errors over each cycle in synthesis

# Final Elongation and Final Hold

- This step is optional
- The reaction is held at 68-74$^{o}$C for several minutes
- This step allows the polymerases to finish reading whatever strand they are currently on and to ensure that all remaining single-stranded DNA ends are elongated
- Helps reduce the number of truncated copies in your final product
- A final 4-12$^{o}$C hold is suggested until you can analyse or use your product or transfer it to more suitable long-term storage




Three steps of PCR: denaturation, annealing, and extension (taken from ThermoFisher Scientific website)

# Controls

- Positive controls: essential so that you know the PCR has worked.
  - Typically samples proven to contain your species of interest
- Negative controls: essential to exclude contamination of your buffer, nucleotides etc.
  - Usually the normal PCR master mix but instead of adding template, you add water. This should result in no PCR products
- Inhibition control: optional
  - Can be an RNA or DNA sequence from a different organism that is amplified with sequence specific primers.
  - A positive signal within accepted range indicates that all steps carried out were successful and that no inhibitory substances were present.
- Internal Cellular control: used in HPV testing to ensure cells are present
  - Usually a cellular target like endogenous human beta globin is amplified. This evaluates cell adequacy and helps to prevent false negative results.
  - If no amplification is detected, either insufficient numbers of cells were sampled or inhibitory substances are present.

# Optimization

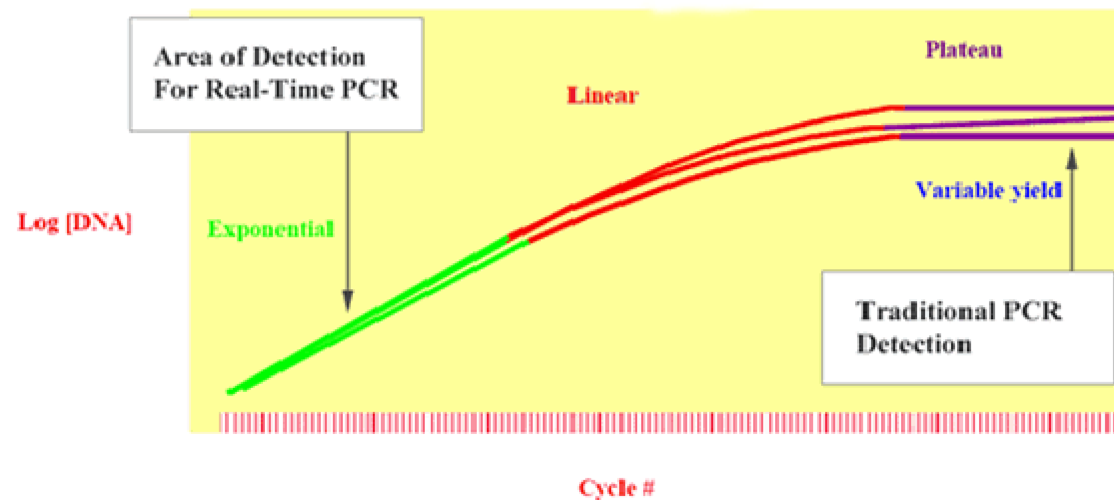
- PCR can fail for various reasons
- Contamination with extraneous DNA is the most common problem, since PCR is an extremely sensitive technique
- Primer-design techniques are important in improving PCR product yield and in avoiding the formation of spurious products
- Use of alternate buffer components or polymerase enzymes can help with amplification of long or otherwise problematic regions of DNA

# PCR Contamination

- The source of contamination could either be the laboratory environment or reagents
- The biggest source of PCR contamination is aerosolized PCR products, which are created when opening a tube or pipette amplified PCR product
- Always run negative controls because that is how you will know if you have contamination
- Setup the PCR away from where you analyse PCR results to avoid contamination
- Store PCR reagents and PCR products separately and aliquot these reagents
- Store PCR tubes/tips/racks separately
- Use a master mix and add your template last

# Real Time PCR

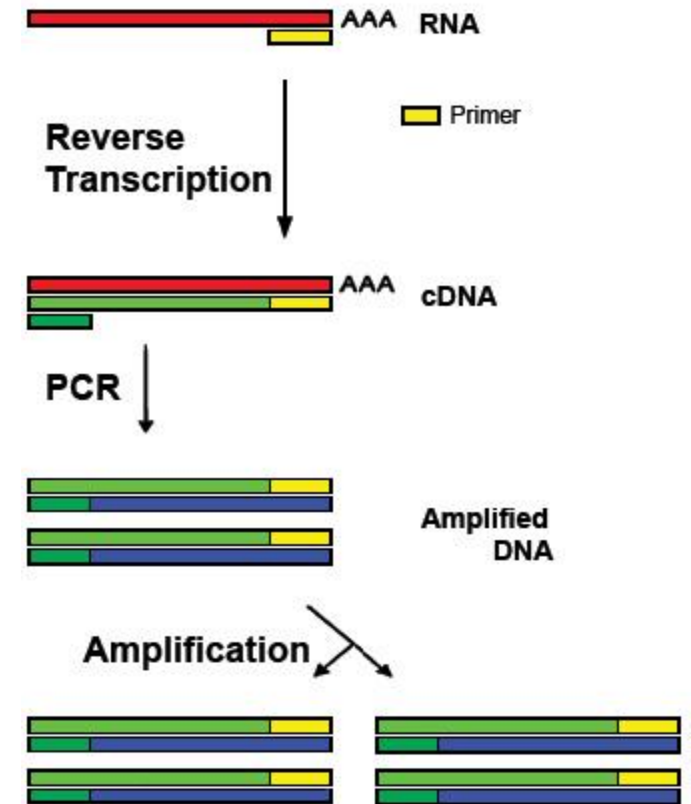
- Real-time PCR is a form of PCR where data is collected in real-time as the reaction proceeds
- In traditional PCR, results are collected after the reaction is complete (in plateau phase of the amplification reaction)
- Real-time PCR measures at the exponential phase for more accurate quantitation
- The CT value is the intersection between an amplification curve and a threshold line. It is a relative measure of the concentration of target in the PCR reaction




Taken from ThermoFisher Scientific website

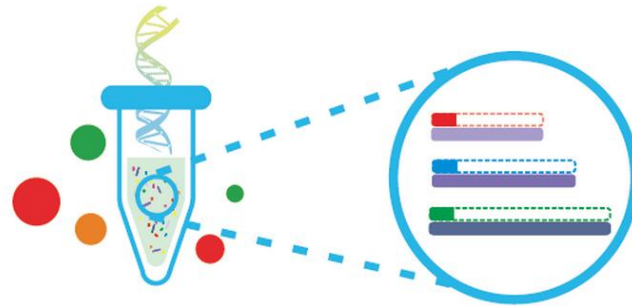
# Reverse Transcription PCR

- Reverse Transcriptase (RT-PCR) is a modification of the standard PCR technique that can be used to amplify mRNA
- The enzyme reverse transcriptase uses the mRNA template to produce a complementary single stranded DNA strand called cDNA in a process known as reverse transcription
- DNA polymerase is then used to convert the single stranded cDNA into double-stranded DNA
- The cDNA is then used as a template for exponential amplification using PCR



# Multiplex PCR

- Multiplex PCR refers to the use of PCR to amplify several different DNA sequences simultaneously
- Many separate PCR reactions are performed all together in one reaction
- The primer design for all primers pairs has to be optimized so that all primer pairs can work at the same annealing temperature during PCR



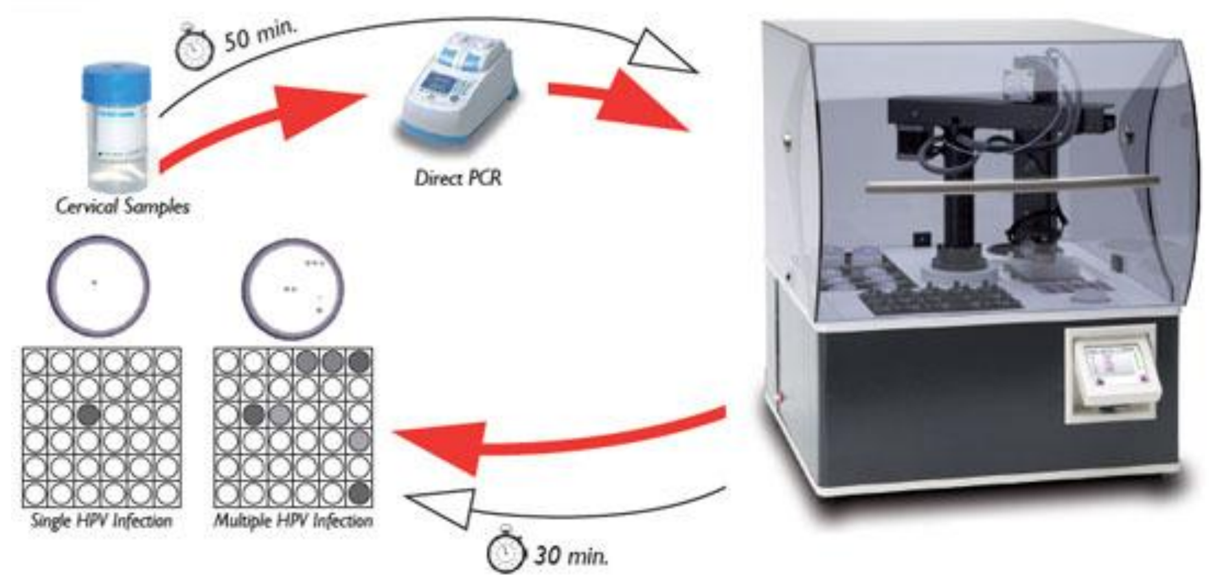
# HPV Testing

- The majority of HPV Testing methods are based on PCR
- The Roche Cobas® 4800 HPV test, Abbott RealTi*me* High-Risk HPV Assay and BD Onclarity HPV Assay all use Real-Time PCR

  Note: The other main detection method used in HPV tests like Hybrid Capture and Cervista is called signal amplification
- These HPV tests have individual genotyping of HPV-16 and 18 and pooled detection of other high-risk HPV subtypes
- These tests are usually qualitative tests (detected/not-detected) based on a platform-dependent threshold value

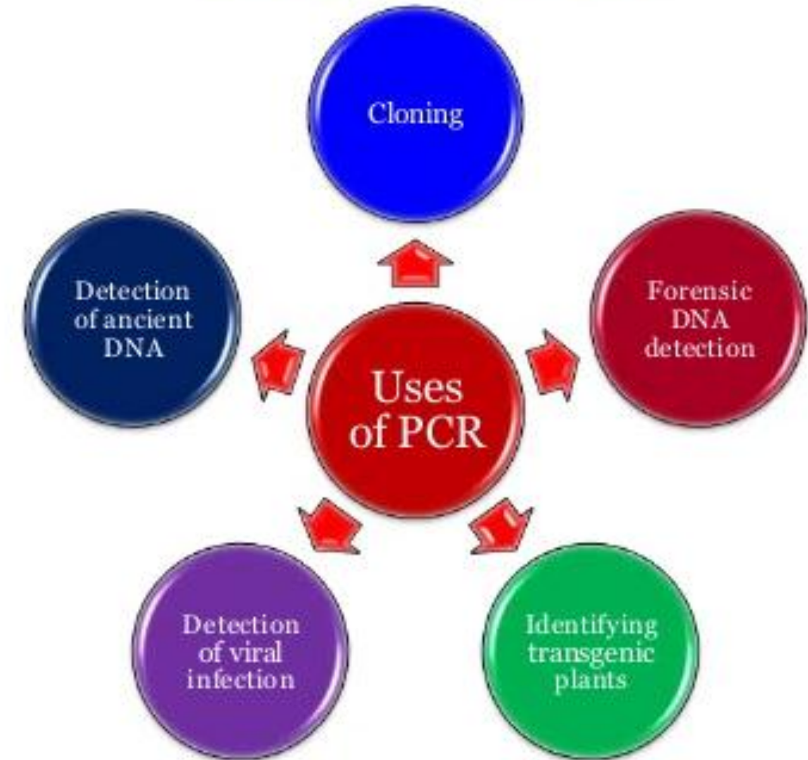
# Workflow of HPV Testing

- Sample collection
- Extraction of Nucleic Acid
- Amplification of HPV virus
- QC the results
- Analysis/LIS data transfer
- Reporting



# Applications of PCR

- Selective DNA isolation
- Amplification and quantification of DNA
- Medical applications
- Infectious disease applications
- Forensic applications
- Research applications



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