Amplification of Nucleic Acids

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Objectives

- Differentiate target, probe, signal amplification
- Extensively detail Polymerase Chain Reaction (PCR)
  - Overview of the reaction
  - Reaction optimization
  - Innovations and modifications
- Explain non-PCR target, probe, and signal amplification techniques using examples:
  - Transcription-Mediated Amplification
  - Ligase Chain Reaction
  - Branched DNA Amplification
- Discuss operational considerations for laboratories performing amplification-based testing

References and Additional Resources

- Buckingham, Lela. *Molecular Diagnostics: Fundamentals, Methods, and Clinical Applications.* 3rd ed., F.A. Davis, 2019.
  - Chapter 6: Nucleic Acid Amplification
  - Chapter 15: Quality Assurance and Quality Control in the Molecular Laboratory

Nucleic Acid Amplification Techniques

Extracted DNA and RNA is generally in too low of concentrations for direct analysis. Molecular laboratories get around this through **amplification**, a process of replication that exponentially increases the sensitivity of a test system.

Amplification techniques can be sorted into three broad categories:

- **Target Amplification**: replication of a specific target region of DNA/RNA
- **Probe Amplification**: probe, complementary to the target sequence, is replicated while the number of target sequences remains unchanged
- **Signal Amplification**: probe-generated signal is amplified/accumulated without increasing the number of target sequences or probes

Polymerase Chain Reaction

An overview of the reaction.

Nucleic acid analysis was limited by the availability of DNA/RNA.

Previous methods of generating enough genomic material were labor intensive, time consuming, and ill-suited for many sequences.

- Cell culturing
- Gene cloning using bacterial plasmids

1983 - Kary Mullis invents a new method of DNA amplification known as Polymerase Chain Reaction (PCR).

- Mimics the in vivo replication of DNA, but at a much faster rate.

Mullis winning the Nobel Prize for Chemistry, 1993

Polymerase Chain Reaction

PCR is *in vitro*, primer-directed DNA polymerization resulting in amplification of specific DNA sequences.

Components:

- Nucleic Acid Template
- Molecular Biology Grade Water
- Buffer
- Mg2+
- Nucleotides
- Primers
- DNA Polymerase

PCR Components

Nucleic Acid Template

Isolated nucleic acid from human tissue, bacteria, virus, etc.

PCR requires DNA as a starting template

- For RNA samples, RNA must be converted first to its complementary DNA (cDNA) through a process called reverse transcription (more on this later).

PCR setups will require template to be diluted to a specific concentration ($C_1V_1 = C_2V_2$) using molecular biology grade water (more on this later).

- Too low of a concentration can result in amplification failure
- Too high of a concentration can result in overshooting the linearity of an assay

PCR Components

The remaining components constitute the PCR **master mix**.

- Molecular Biology Grade Water
- Buffer
- Mg^{2+}
- Nucleotides
- Primers
- DNA Polymerase

Master mix components are generally stored separately from sample nucleic acids, controls, standards, sizing ladders, etc.

- Prevents process contamination

These components are usually purchased commercially as complete kits.

PCR Components

Molecular Biology Grade Water

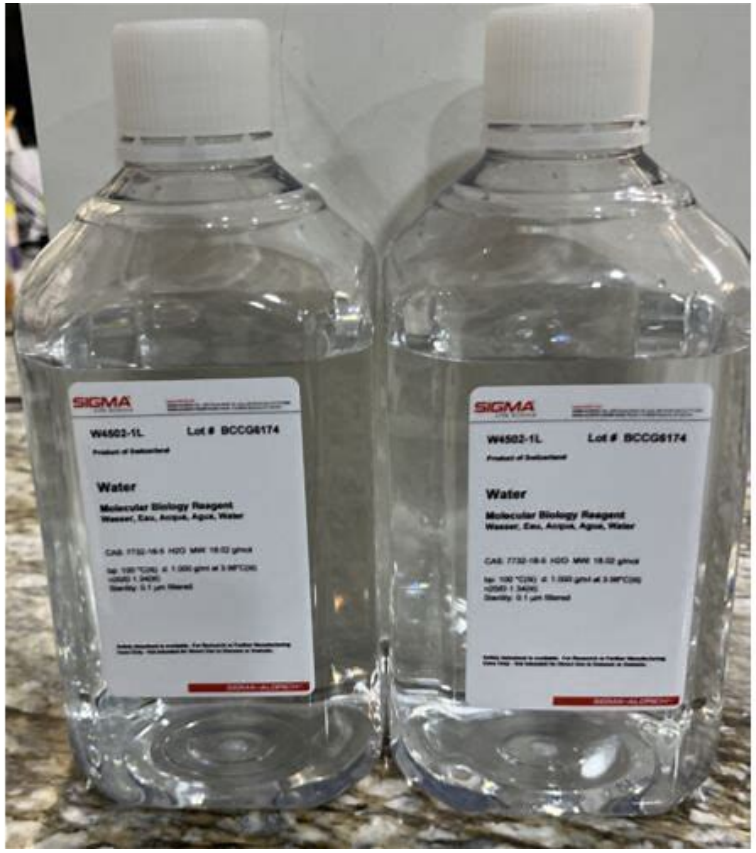
- Neutral solvent used to bring master mix to appropriate concentration
- Nuclease (DNAse/RNAse) free

Buffer

- Maintains the pH of the reaction

Mg^{2+}

- Magnesium ions are an essential cofactor for polymerase enzyme and help stabilize the DNA double helix



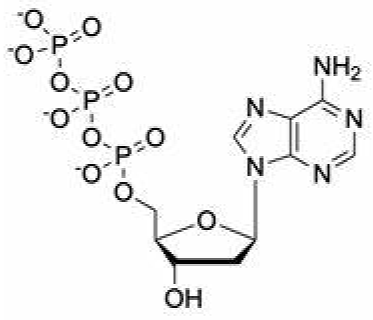
PCR Components

Nucleotides

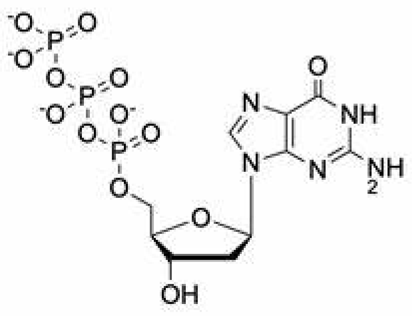
- An equal mixture of the four deoxynucleoside triphosphates (**dNTPs**)
  - dATP
  - dTTP
  - dCTP
  - dGTP

DNA Polymerase

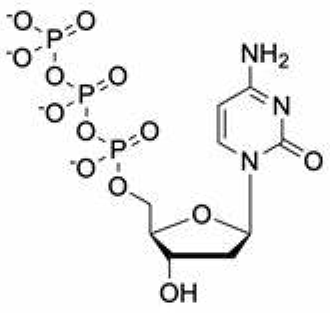
- Reads template strand and incorporates complementary dNTPs to synthesize a new strand of DNA



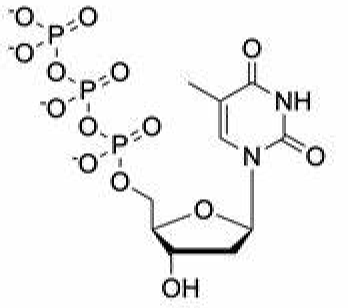
dATP



dGTP



dCTP

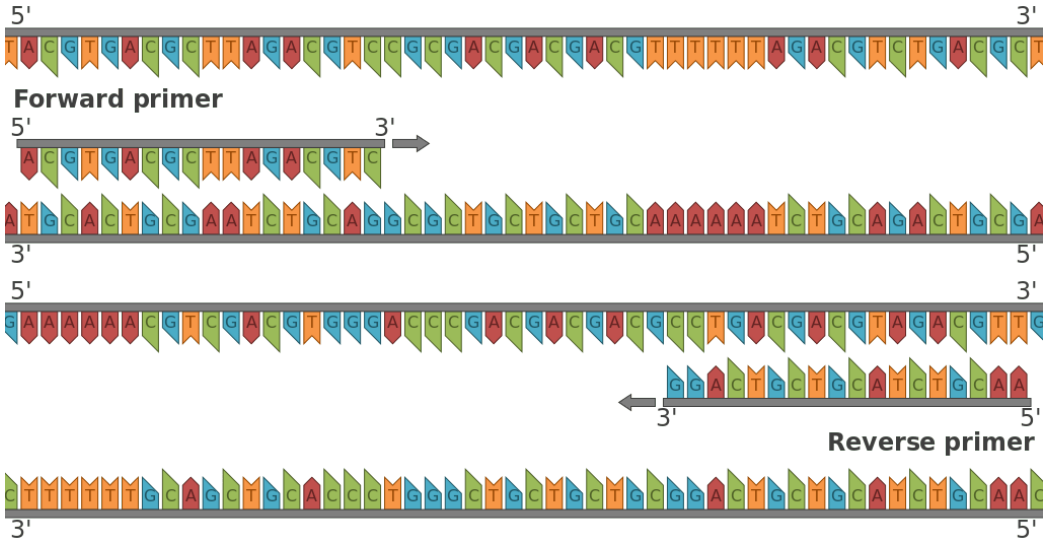


dTTP

PCR Components

Primers

- Short oligonucleotides (20-30bp) that are complementary to template DNA.
- Forward and reverse primers are designed to flank the sequence of interest, or **target**.
  - Forward = attaches 5’ to the target sequence, binds the antisense strand and primes the sense strand
  - Reverse = attaches 3’ to the target sequence, binds the sense strand and primes the antisense strand
- Primer design (more on this later) determines the final size of the PCR product.



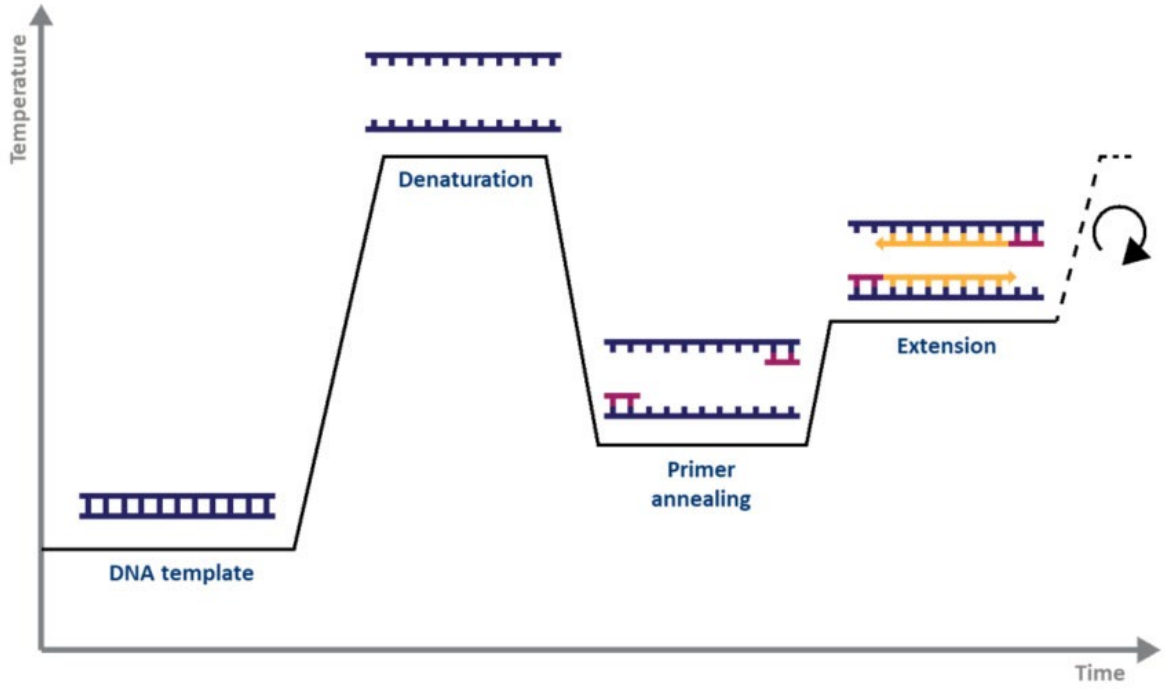
PCR Steps

PCR consists of three steps

1. Denaturation
2. Annealing (aka hybridization)
3. Extension (aka elongation)

Each step occurs when reaction mixture is held at a specific, optimal temperature.

These steps, performed in order, constitute one cycle of PCR.



Step 1 – Denaturation

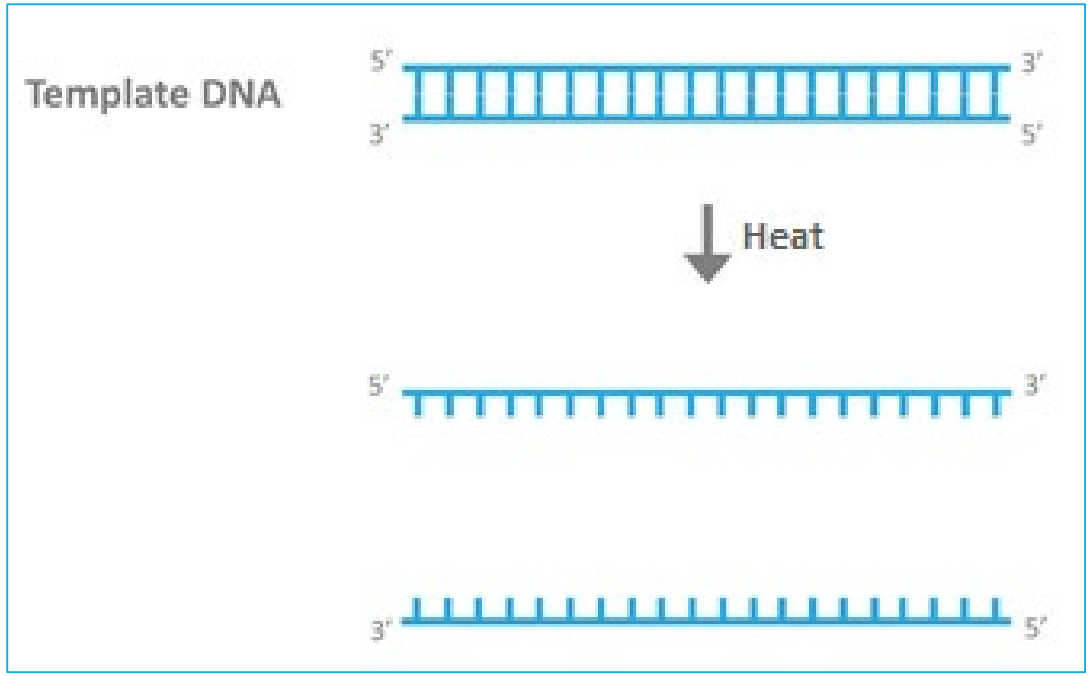
Temperature: 90°C-96°C

- The **HIGHEST** temperature

Time: 20-60 seconds

High temperature causes double-stranded DNA to separate.

Single-stranded DNA is now available for primers to attach.



Step 2 – Annealing/Hybridization

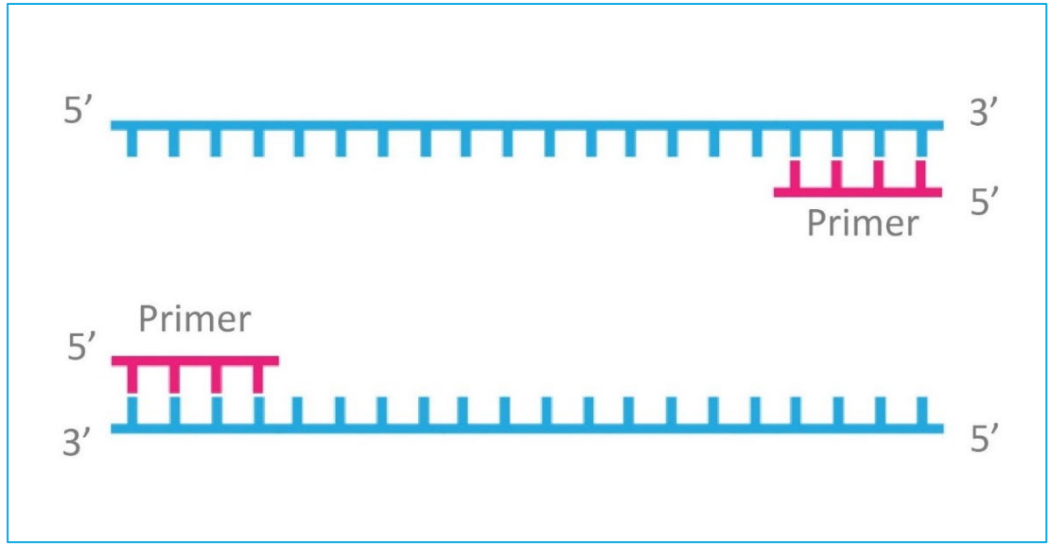
Temperature: 50°C-70°C

- The **LOWEST** temperature

Time: 20-90 seconds

Forward and reverse primers bind to complementary sequences on the template.

Optimal hybridization temperature is determined by primer sequence and reaction conditions (more on this later).



Step 3 – Extension/Elongation

Temperature: 68°C-75°C

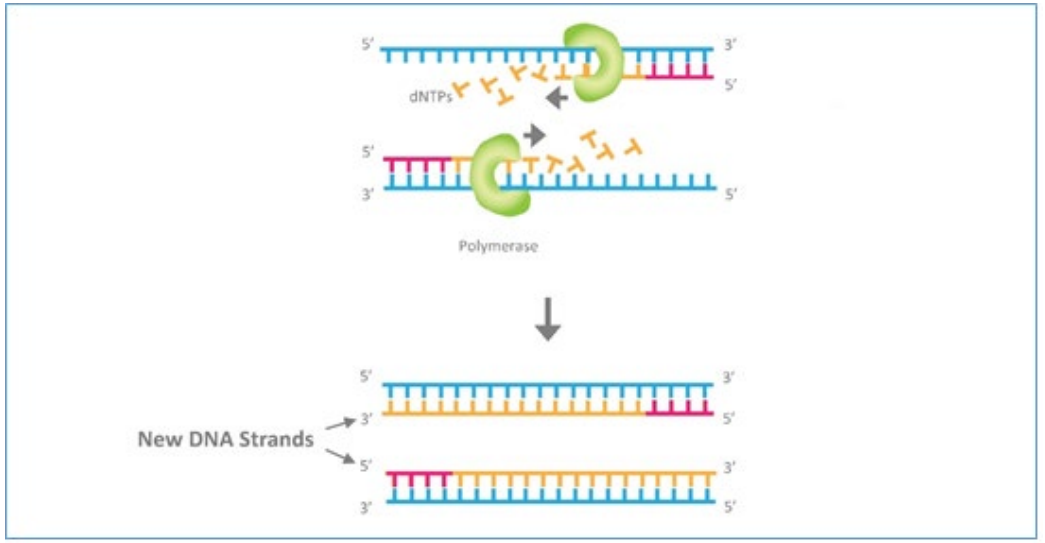
- The **MIDDLE** temperature

Time: 10-60 seconds

DNA polymerase catalyzes the addition of nucleotides to the primers, using the sample DNA as the template.

DNA polymerase simultaneously extends primers on both strands of the template, creating two **amplicons.**

Extension temperature determined by optimum temperature for activity of polymerase.



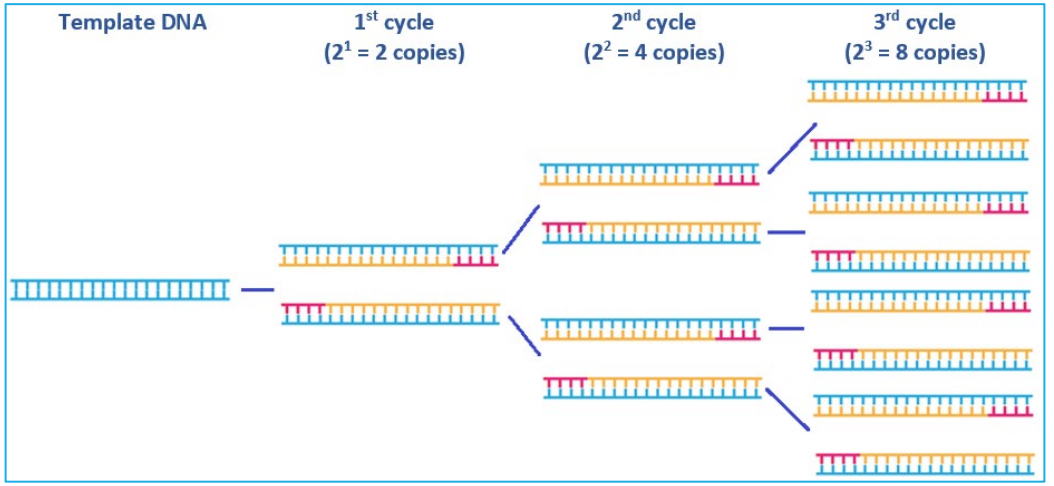
And… Repeat!

By cycling these three steps over and over, the target is amplified **exponentially.**

Each cycle of PCR creates 2^n copies, where N is the number of times the region is replicated

- *e.g.* A full PCR cycle takes 3-4 minutes, and 30 cycles yields 2^{30} copies. This means over 34 billion copies of the target can be produced in fewer than 2 hours.

Amplified products can then be further analyzed by gel or capillary electrophoresis, blotting procedures, MALDI-TOF, sequencing methods, etc.



PCR Reaction Optimization

- PCR Inhibitors
- Sample Dilution
- Primer Design
- T_m and T_a
- Stringency
- PCR Product Cleanup

Validation/verification of any PCR-based test system requires strict optimization of the reaction conditions.

Failure to account for any of the conditions listed to the left can affect the accuracy, precision, sensitivity, specificity, and reproducibility of the test.

Common PCR Inhibitors

PRESENT IN ORIGINAL SPECIMEN

Anticoagulants
- Heparin (green top tube) is especially inhibitory
- EDTA is preferred anticoagulant for most molecular testing, ACD also acceptable

Hemoglobin

Polysaccharides

Proteins

DNAse, RNAse, Protease

INTRODUCED BY PROCESSING/EXTRACTION

Phenol

Isopropanol

Ethanol

Glacial acetic acid

EDTA

Salts other than Mg+

Heavy metals

Sample Dilution

To ensure consistency across all samples, samples are often diluted with nuclease-free water prior to PCR.

The formula used to calculate the dilution is as follows:

$$C_1V_1 = C_2V_2$$

Where…

- C_1 = initial concentration of undiluted nucleic acid
- V_1 = volume of undiluted nucleic acid added to solution
- C_2 = final concentration of diluted nucleic acid
- V_2 = final volume of diluted nucleic acid

Provided you know three out of four values, you can solve for the unknown.

Sample Dilution

V_1 stays constant

A DNA sample has a quant of 140 µg/mL, but the loading concentration for PCR is 20 µg/mL.

Using an initial volume of 0.1 mL, the dilution can be calculated as follows:

$(140\ \mu g/mL)(0.1\ mL) = (20\ \mu g/mL)(V_2)$

$$V_2 = \frac{(140\ \mu g/mL)(0.1\ mL)}{20\ \mu g/mL} = 0.7\ mL$$

0.1 mL of undiluted DNA is added to 0.6 mL of water for a total final diluted volume of 0.7 mL.

V_2 stays constant

A DNA sample has a quant of 100 µg/mL, but the loading concentration for PCR is 10 µg/mL.

If the desired final volume of diluted DNA is 0.5 mL, the dilution can be calculated as follows:

$(100\ \mu g/mL)(V_1) = (10\ \mu g/mL)(0.5\ mL)$

$$V_1 = \frac{(10\ \mu g/mL)(0.5\ mL)}{100\ \mu g/mL} = 0.05\ mL$$

0.05 mL of undiluted DNA is added to 0.45 mL of water for a total final diluted volume of 0.5 mL.

Primer Design – Sequence Selection

Selecting the right primer sequence helps avoid hybridization issues, such as mispriming and primer dimers.

- Mispriming = hybridization of primer to a sequence other than the intended target
- Primer dimer = primer-primer hybridization (see next slide)

Table to the right lists some general dos and don’ts when it comes to primer design.

DO	DON’T
20 – 30 base pair length	Runs of 4+ same or alternating bp (*e.g.*, AAAA, ACAC)
40 – 60% GC content	
T_m between 50 – 75°C	Self- or cross-complementarity of primers (primer dimers)
Forward and reverse primer T_m within 5°C	

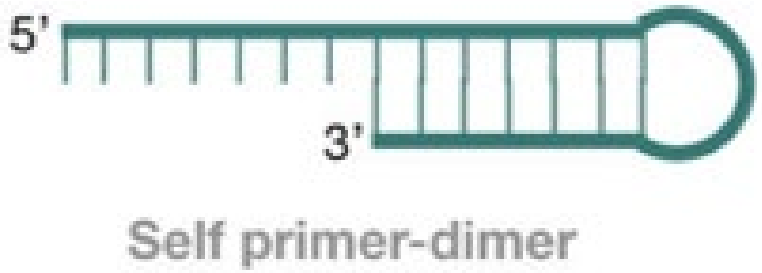
Primer Design – Primer Dimer

Primer design must take into consideration the risk of **primer dimer** interactions.

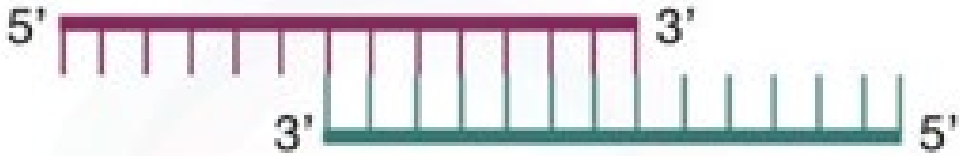
Short, complementary segments on the forward and reverse primers can hybridize to themselves or one another.

Polymerase is then able to synthesize a segment of non-template DNA about twice the size of the individual primers.

This will appear as an artifact that can be mistaken for true amplification of template.



Self primer-dimer



Cross primer-dimer

Primer Design – T_m and T_a

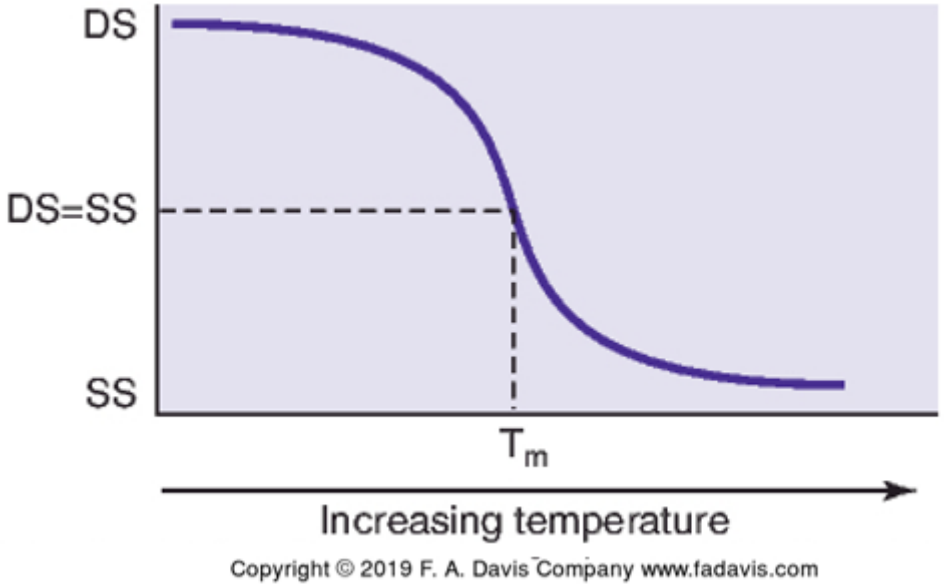
Primer sequence is a major contributor in determining the optimum annealing temperature of PCR.

Melting temperature, or **T_m**, is an expression of the amount of energy required to separate hybridized strands.

- At T_m, half ssDNA and half dsDNA

A simple calculation for short oligonucleotides (*i.e.*, primers) is based on the number of A/T and G/C pairs in sequence.

T_m = (4°C x # of GC pairs) + (2°C x # of AT pairs)



Primer Design – T_m and T_a

Ideally, forward and reverse primers should have the same or similar (within 5°C) T_m to enable co-hybridization at the same annealing temperature.

T_m is **NOT** the same thing as the optimal annealing temperature (**T_a**).

In general, optimal annealing temperature is about 5°C below the T_m.*

$$\mathbf{T_a = T_m - 5°C}$$

*This is an oversimplification but will get you through the MB(ASCP).

Primer Design - Practice

Consider a theoretical primer pair designed to amplify a 65bp target sequence:

- Forward = 5’-CAGGTTACGACATGCACCCC-3’
- Reverse = 5’-CTATCATTGCGCCATGGGGT-3’

Primer Design - Practice

What's *good* about this primer pair:

- Base pair length is between 20-30 for each
- GC content within 40-60% for both primers
  - Forward = 12 GCs/20bp = 60%
  - Reverse = 11 GCs/20bp = 55%
- Melt temperatures are between 50-75°C and within 5°C of each other:
  - T_m calculation:
  - Forward = $(12_{CG} \times 4°C) + (8_{AT} \times 2°C) = 64°C$
  - Reverse = $(11_{CG} \times 4°C) + (9_{AT} \times 2° C) = 62°C$
  - T_a calculation
  - Forward = 64°C - 5°C = 59°C
  - Reverse = 62°C - 5°C = 57°C

Primer Design - Practice

What's *bad* about this primer pair:

- Run of four cytosines at the 3'-end of the forward primer
- Run of four guanines near the 3'-end of the reverse primer

- Cross complementarity of 3' ends of forward and reverse primers which will produce a 34bp cross primer-dimer artifact.

Stringency

Stringency is the combination of conditions under which hybridization occurs.

- High stringency = more difficult to hybridize
- Low stringency = easier to hybridize

A balancing act: too high and hybridization is impossible, too low and misprimes occur.

These conditions affect any oligonucleotide-based hybridization system.

- PCR primers and probes, blotting probes, FISH probes, etc.

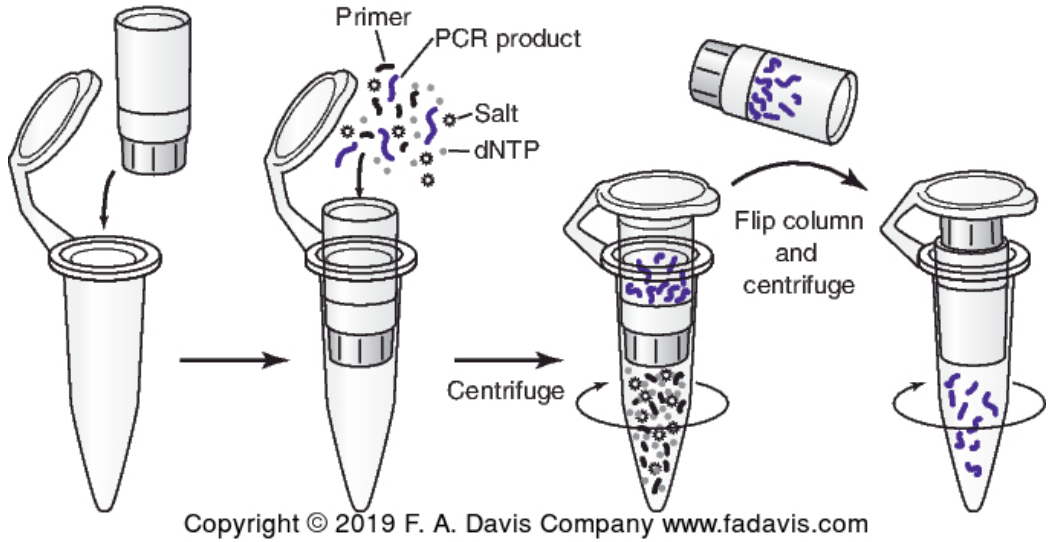
Condition	Increases Stringency	Decreases Stringency
Primer/probe length	↑	↓
G/C pairings	↑	↓
A/T pairings	↓	↑
Temperature	↑	↓
Salt concentration of PCR buffer/SSC	↓	↑
Denaturing agents (formamide, etc.)	↑	↓
Surfactants (detergents, etc.)	↑	↓

PCR Product Cleanup

Unincorporated dNTPs, PCR reaction buffer, salts, and unextended primer can all interfere with post-PCR analytic activities.

Spin-column cleanups are a common post-PCR process that use the same silica filters as in solid-phase extraction methods.

- PCR product is added to spin column.
- Amplicon binds to silica filter while other components are removed by centrifugation.
- Clean PCR product can then be eluted into new collection tube using elution buffer.

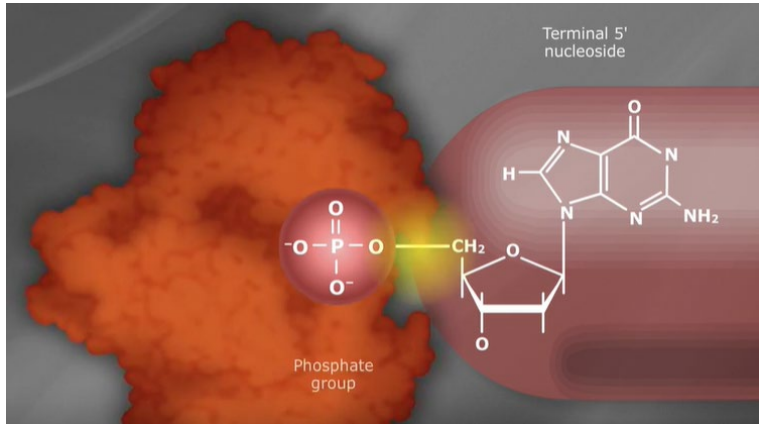


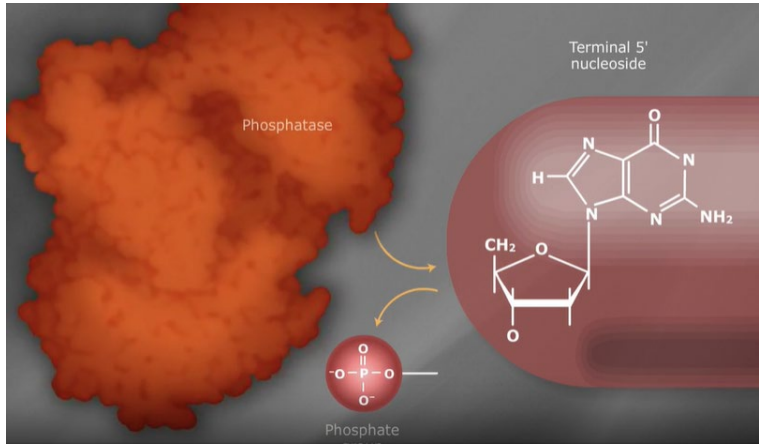
PCR Product Cleanup

Alkaline phosphatase (**AP**) incubation is an enzymatic method of cleaning up PCR product.

AP enzyme, most active at 37 °C, dephosphorylates unincorporated nucleotides and unextended primers without affecting the desired dsDNA amplicon.

Following incubation, enzyme is then denatured and inactivated at 95°C to prevent it from interfering with downstream procedures.





PCR Innovations and Modifications

- Thermal Cyclers
- Taq Polymerase
- Hot-Start PCR
- Touchdown PCR
- Multiplex PCR
- Reverse Transcriptase PCR
- Nested and Semi-Nested PCR

The original PCR process was time consuming and labor-intensive.

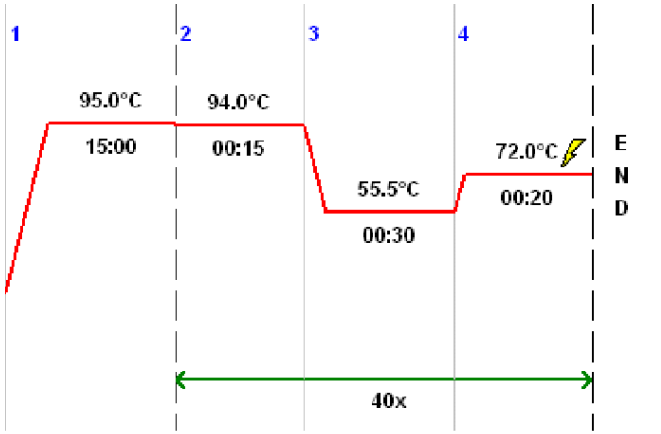
Innovations and modifications of the original PCR process have significantly improved its efficiency, sensitivity, and specificity while simultaneously reducing tech time required.

Thermal Cyclers

Thermal cyclers (or thermocyclers) can be programmed to automatically raise and lower reaction temperature with each step of PCR.

This automation significantly reduces tech time required to perform testing.

Eppendorf Mastercycler Pro S



Taq Polymerase

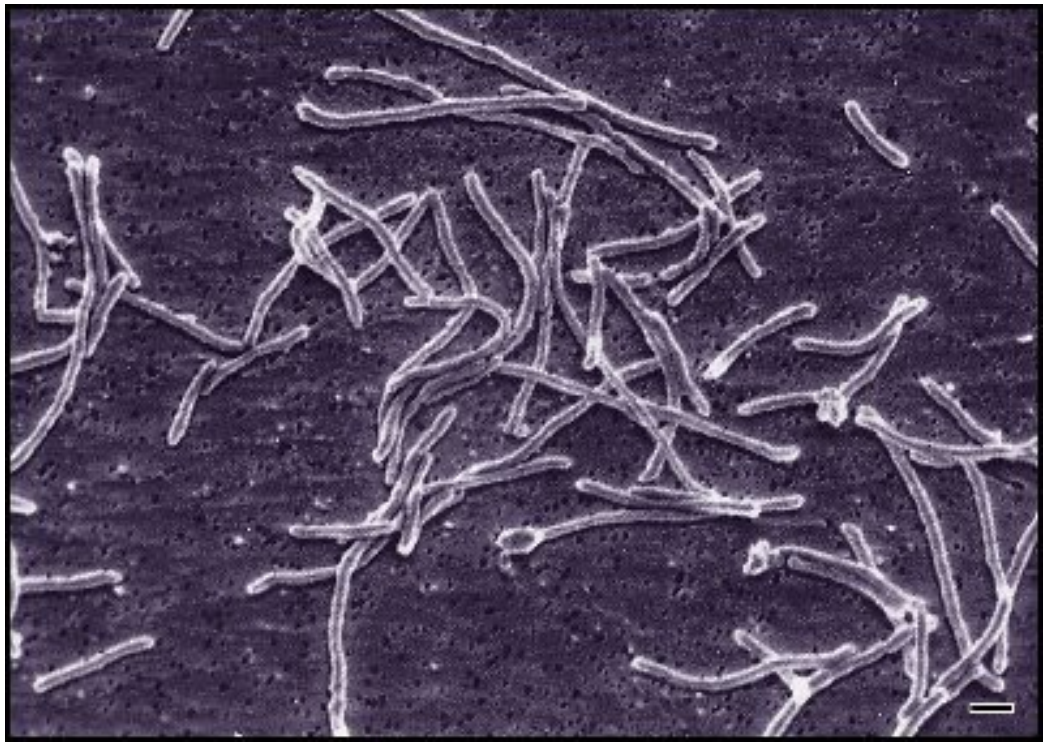
Original polymerases used in PCR were thermolabile, meaning they denatured and lost efficacy at high temperatures.

- Additional polymerase had to be added after each denaturation step of PCR.

Taq Polymerase, derived from the thermophilic bacterium *Thermus aquaticus* native to hot springs, is stable at high temps.

- Eliminates the need to re-add polymerase.

Taq, like all enzymes, is still subject to mechanical degradation from vigorous mixing or repeated freeze-thaws.



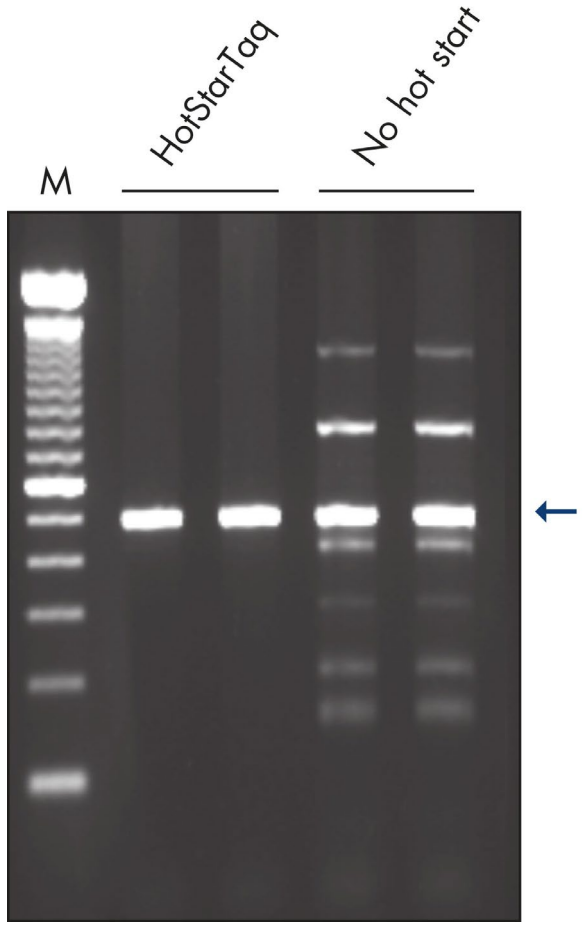
Hot-Start and Touchdown PCR

Even with well designed primers, misprimes can still occur.

- Taq polymerase has some activity at room temperature
- Ambient temperatures (~20°C) have low stringency

Two modifications to PCR help correct for this issue, often used simultaneously:

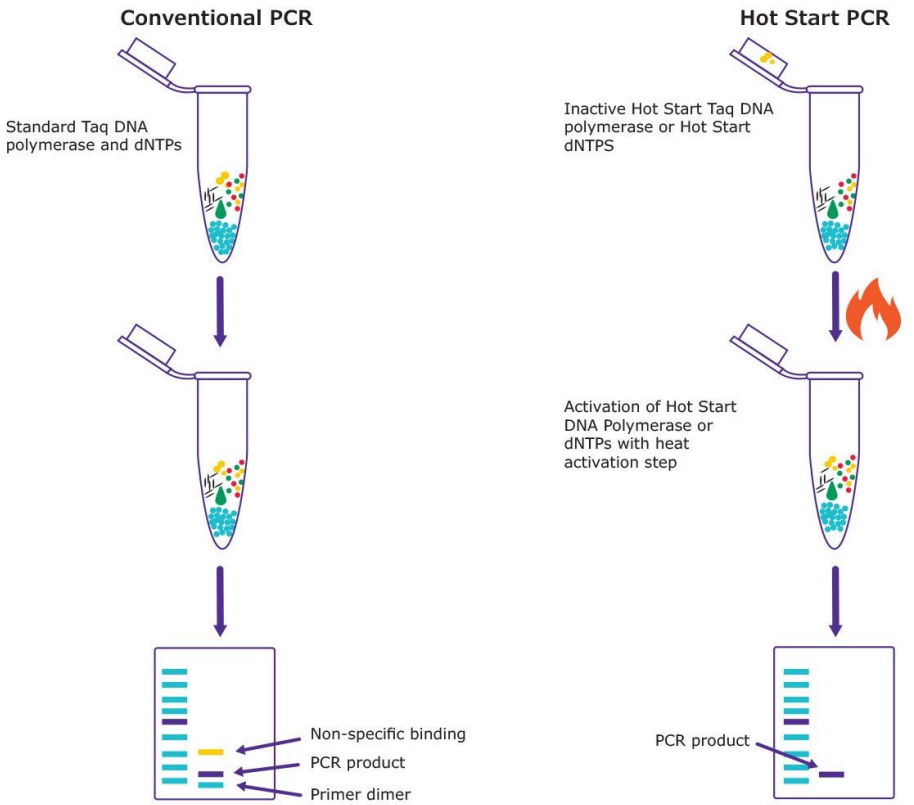
- Hot-Start PCR
- Touchdown PCR



Hot-Start and Touchdown PCR

In **hot-start PCR**, pre-denaturation polymerase activity is prevented in one of three ways:

- Mastermix is prepared over ice and placed on the thermalcycler only after denaturation temperature is reached.
- Polymerase is sequestered using wax barrier/wax beads that melt during the first denaturation step.
- **Chemical or antibody-mediated inactivation of polymerase/dNTPs, which are then activated by the heat of the first denaturation step.**

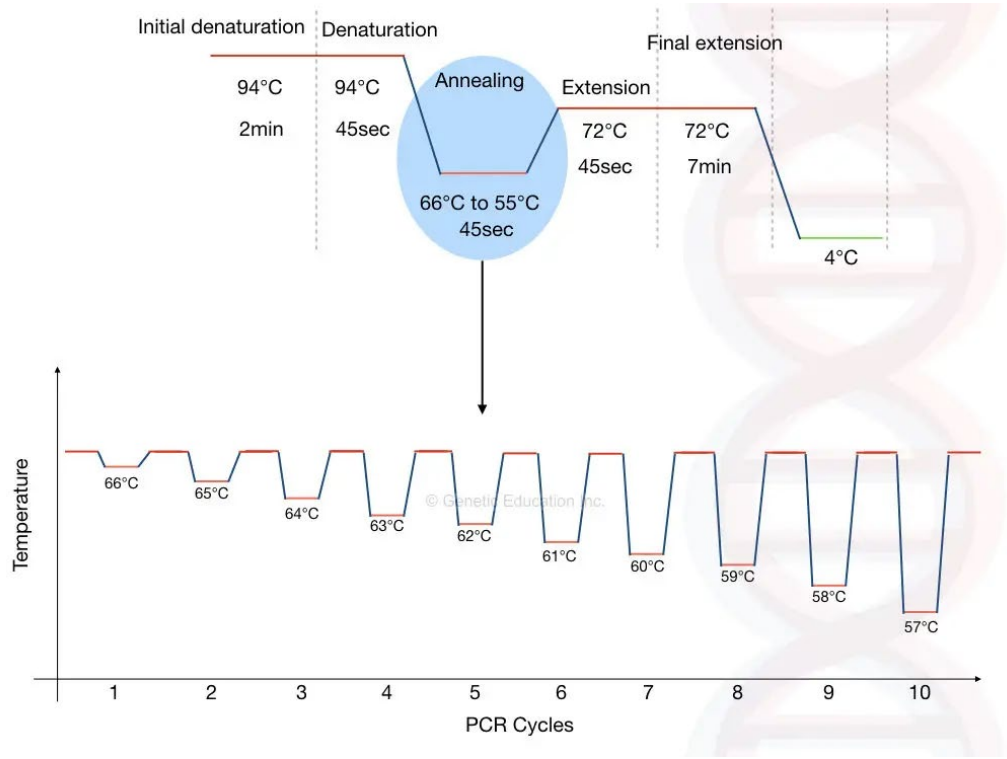


Hot-Start and Touchdown PCR

Touchdown PCR prevents mispriming with an initial increase in the temperature of the hybridization step.

Temperature of hybridization step is then lowered gradually (*i.e.*, 1°C every other cycle) until optimal annealing temperature is reached.

This increases stringency at the start of thermocycling, ensuring that subsequent PCR cycles amplify only correctly primed target sequences.



Multiplex PCR

Multiplex PCR refers to the inclusion of more than one primer pair into a single reaction well.

Useful for both genomic and infectious disease testing:

- CCF's Cystic Fibrosis Carrier Testing panel identifies 142 variants across 8 reaction wells.
- CCF Respiratory Pathogen Panel identifies 22 different pathogens in a single reaction tube.

Requires more complex reaction optimization due to differing hybridization specifications of each primer pair.

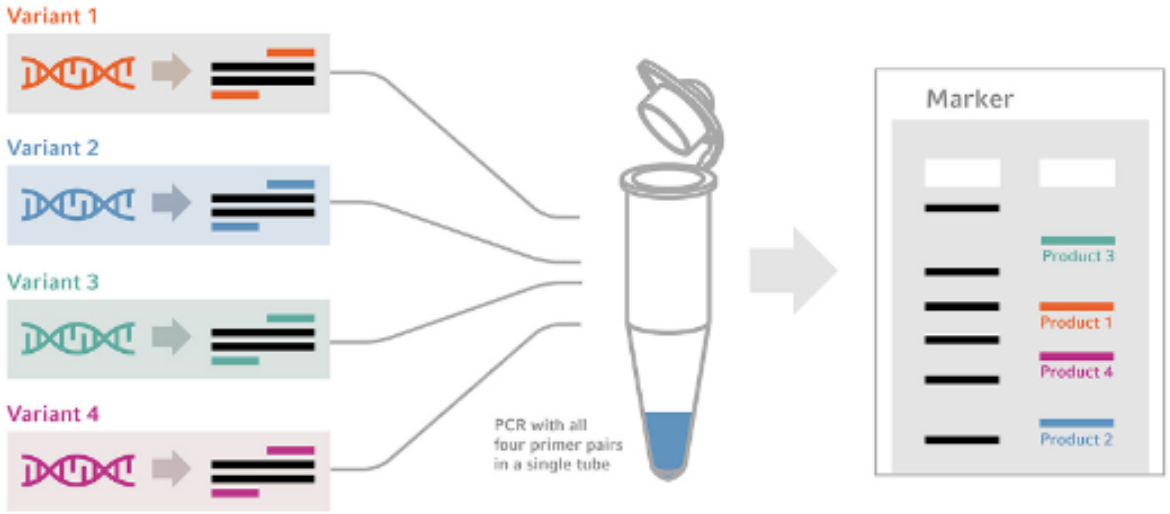


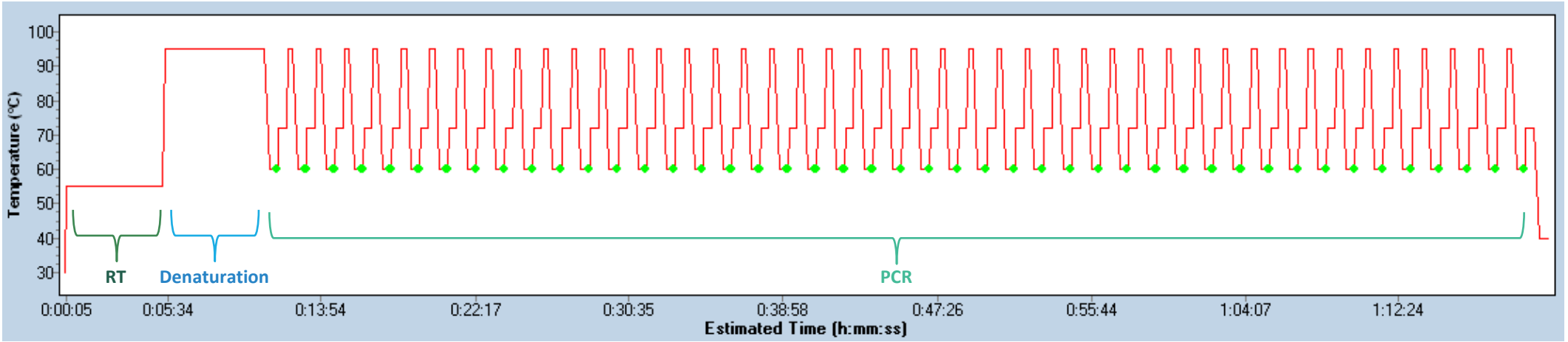
Diagram showing the multiplex PCR approach, whereby all of the primers are added to a single tube. Image credit: S. Rosel, 2021

Reverse-Transcriptase PCR

PCR requires DNA as a template, but RNA samples can still be amplified through **Reverse-Transcriptase PCR**, or **RT-PCR**.

A reverse transcriptase enzyme, most functional between 50-55°C, uses RNA as a template to synthesize its **complementary DNA** (**cDNA**)

Reverse transcription is usually the first step of a longer thermalcycling program, with subsequent PCR of RT-generated cDNA.



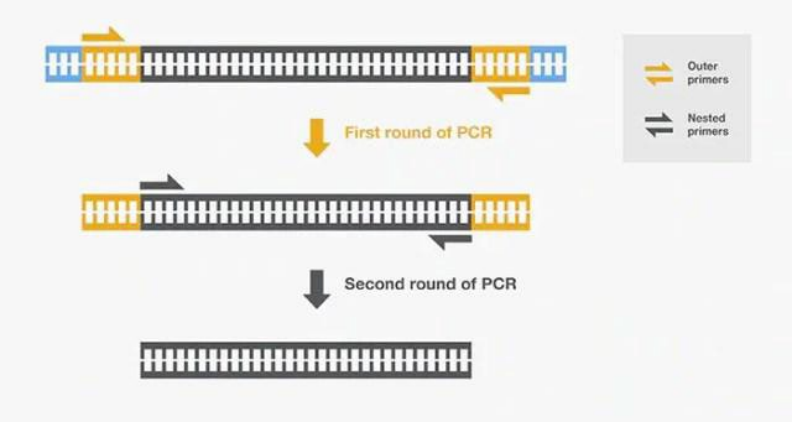
Nested and Semi-Nested PCR

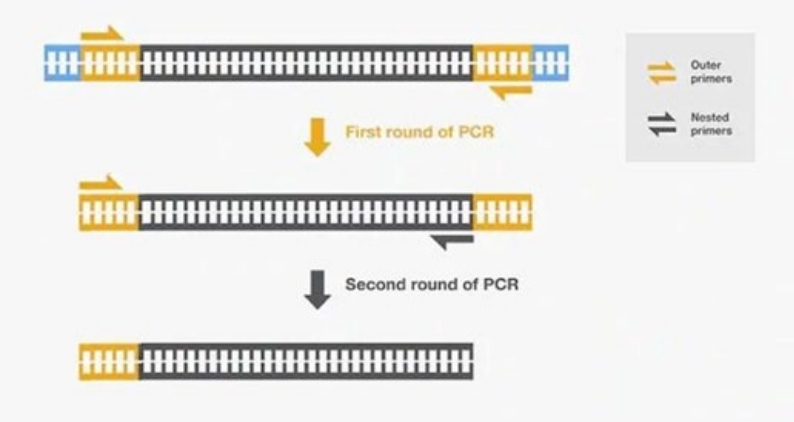
Sensitivity and specificity of PCR can be increased by nesting primers.

In **nested PCR** (top image), two pairs of primers are used to amplify a single target in two separate polymerase chain reactions.

- First-round PCR amplifies a larger product
- Second-round PCR amplifies a smaller product within the sequence amplified by round one

In **semi-nested** PCR (bottom image), the second-round PCR reuses one of the first-round primers.





Other Target Amplification

- **Transcription Mediated Amplification (TMA)**
- Nucleic Acid Sequence Based Amplification (NASBA)
- Self-Sustaining Sequence Replication (3SR)
- Loop-Mediated Amplification (LAMP)

PCR isn't the only target amplification technique used in molecular laboratories.

Several other techniques are mentioned in the textbook and all are commonly used, especially for infectious disease testing:

- Transcription-Based Amplification Systems (TAS)
  - Transcription Mediated Amplification (TMA)
  - Nucleic Acid Sequence Based Amplification (NASBA)
  - Self-Sustaining Sequence Replication (3SR)
- Loop-Mediated Amplification (LAMP)

All these methods are **isothermal**, meaning that the entire reaction occurs at a single temperature.

We'll focus on **Transcription Mediated Amplification** (**TMA**) as an example.

Transcription-Mediated Amplification (TMA)

Transcription-Mediated Amplification (TMA) is modeled after the replication of retroviruses.

Incorporates additional enzymes to generate isothermal amplification reaction:

- Reverse Transcriptase
- RNAse H
- RNA Polymerase

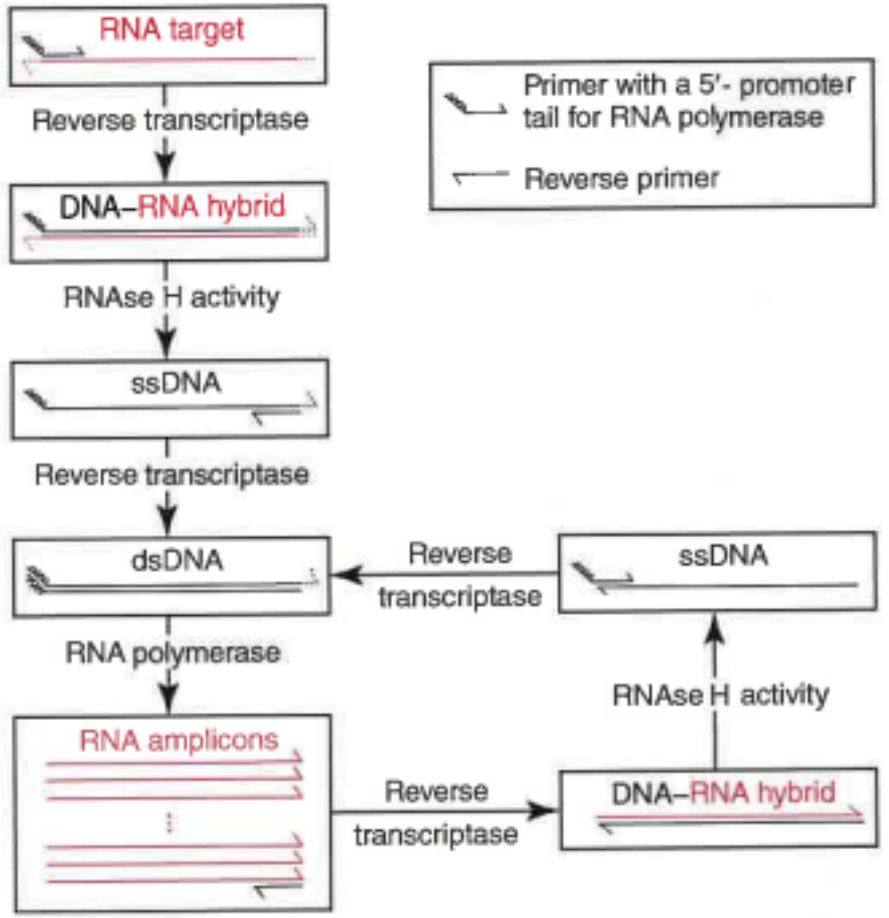
Particularly useful for the quick amplification of RNA targets from microorganisms with RNA-based genomes.

Hologic® Panther Fusion® System

Transcription-Mediated Amplification (TMA)

The process:

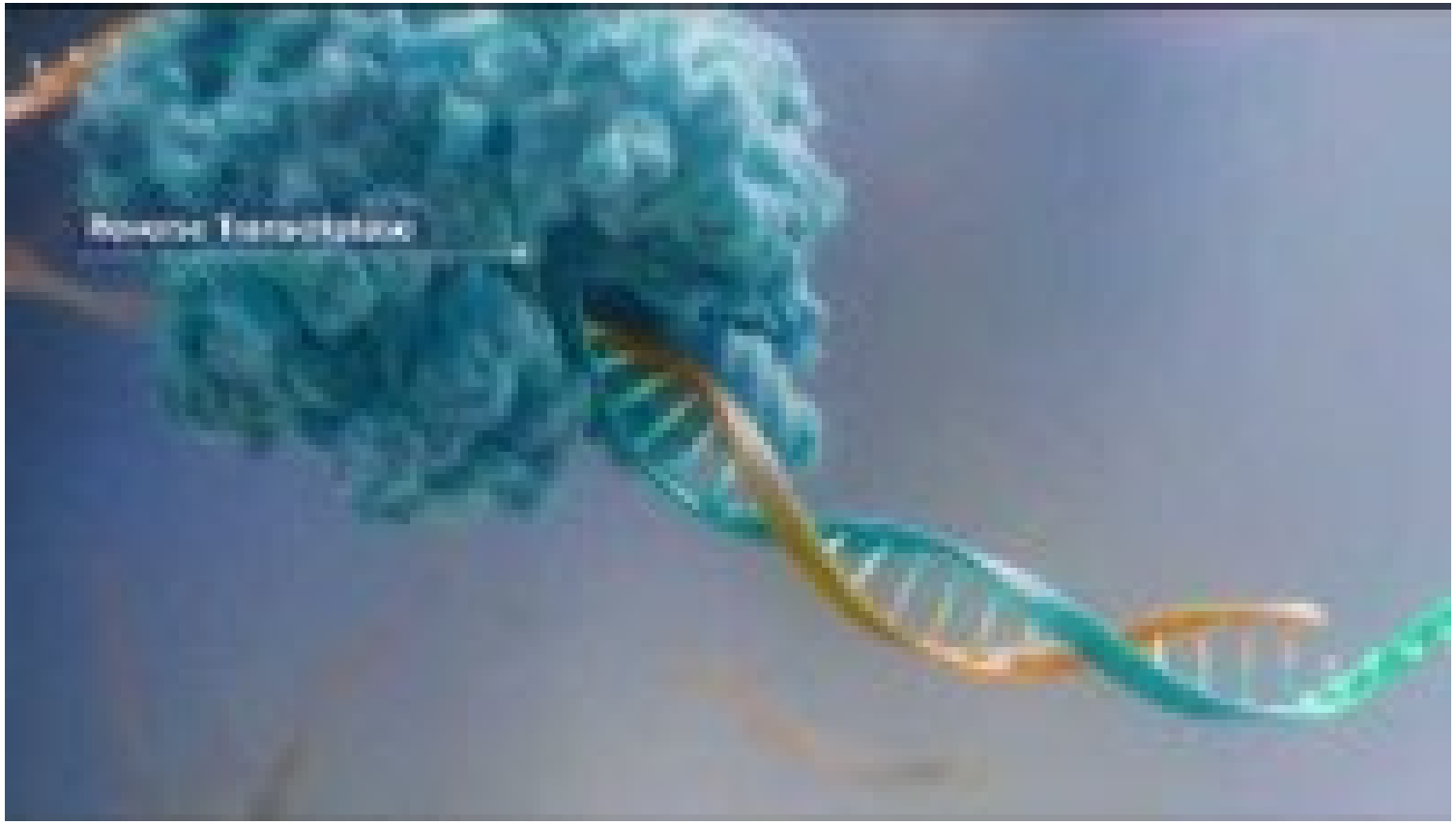
- Primer with 5’- promoter tail for RNA polymerase binds to RNA target template
- Primed RNA template is used by RT for creation of DNA:RNA duplex
- RNAse H degrades RNA template strand from the duplex, leaving ssDNA behind
- Second primer binds to ssDNA
- RT then uses primed ssDNA as template for dsDNA extension
- RNA polymerase recognizes 5’-promoter in dsDNA, uses dsDNA as template to synthesize RNA amplicons
- And repeat!



Hologic® Panther® Systems- TMA Testing

YouTube Link:

https://www.youtube.com/watch?v=RPuGrg3cBOE&t=105s



Probe Amplification

Ligase Chain Reaction (LCR)

Strand Displacement Amplification (SDA)

Qβ Replicase (Qβ)

Probe, complementary to the target sequence, is replicated while the number of target sequences remains unchanged.

Three methods described in the book:

- Ligase Chain Reaction (LCR)
- Strand Displacement Amplification (SDA)
- Qβ Replicase (Qβ)

We'll focus on **Ligase Chain Reaction** as an example.

Ligase Chain Reaction

Primer/probes are designed to be immediately adjacent to one another on the target sequence.

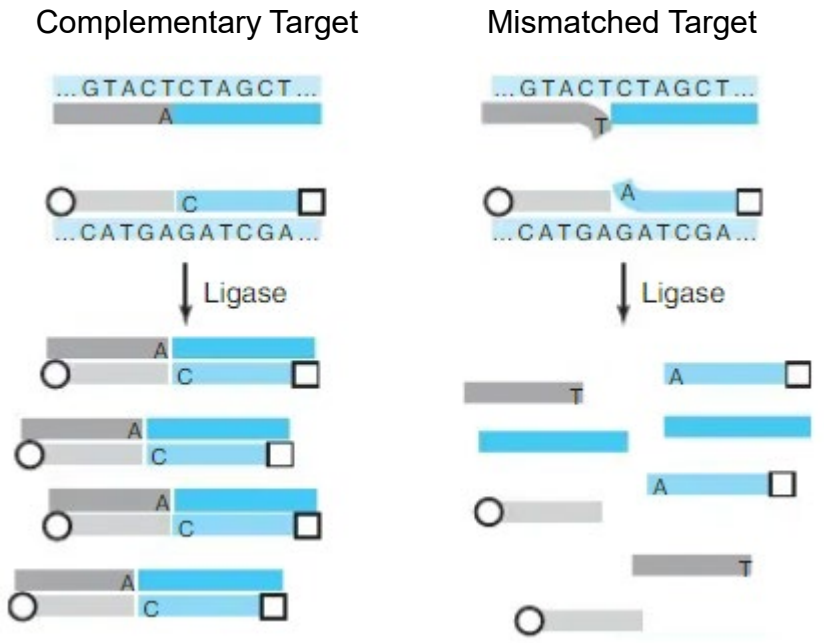
- SNP being interrogated located where both primer/probes meet.

A **ligase** enzyme then joins the two primers together if sequences are *perfectly* complementary.

- If SNP is present, ligation won't be able to occur.

To enhance sensitivity, the ligated product is further amplified using traditional PCR.

- Primer/probe is what is amplified, NOT the original target sequence.



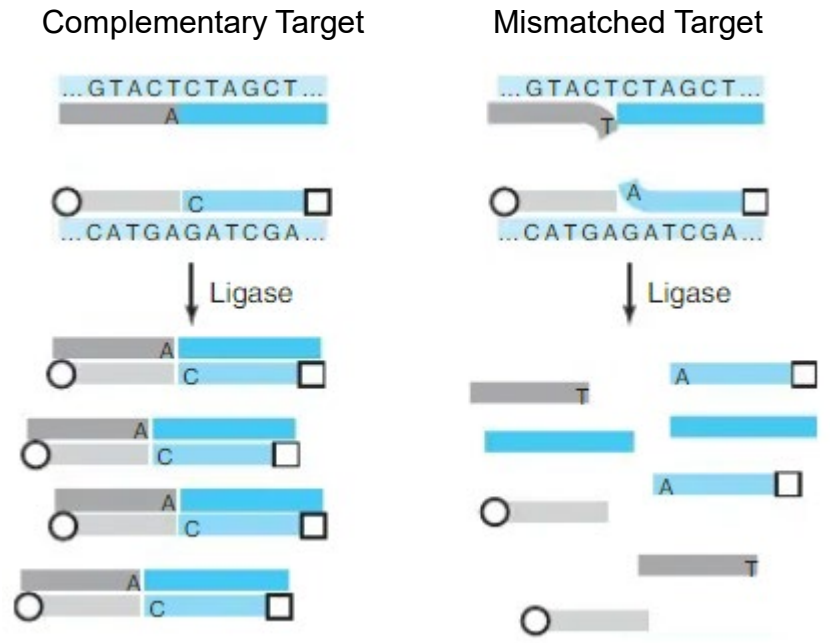
Ligase Chain Reaction

Advantages

- Highly specific, with low likelihood of cross-hybridization with other sequences

Disadvantages

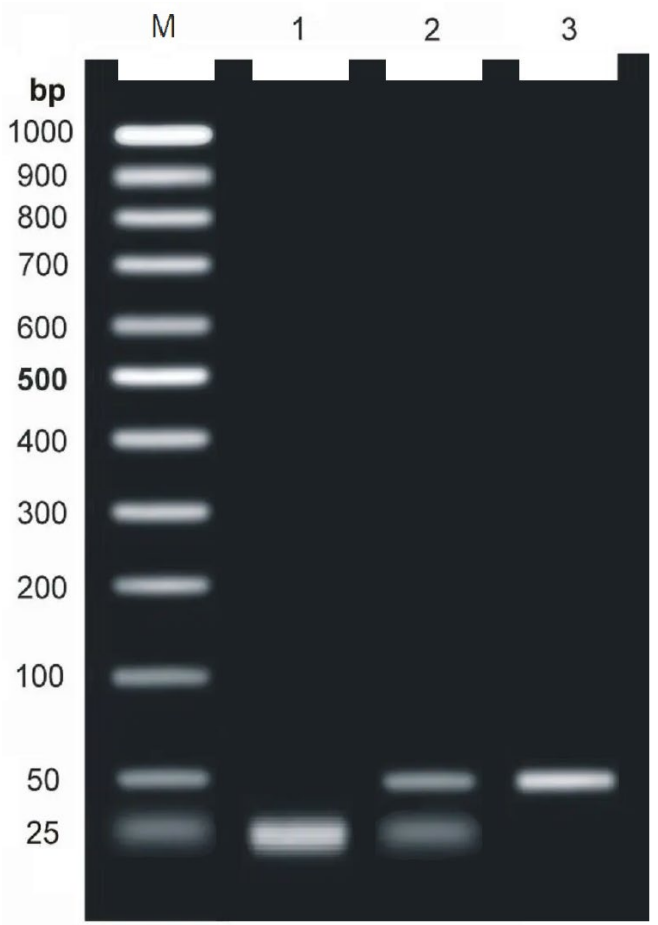
- Entire sequence must be known prior to primer design
- Longer thermalcycling time due to additional ligation step



Ligase Chain Reaction

Primer/probes are of known size, so their ligated product can be detected easily via gel or capillary electrophoresis.

- To the right: Gel image showing size marker and three patients. Assume each primer/probe is 25bp in length.
  - Patient 1 = One band (25bp). Mismatch present for all DNA, no ligation occurred. Patient is homozygous mutant.
  - Patient 2 = Two bands (25bp and 50bp). Mismatch present for half of DNA, some ligation occurred. Patient is heterozygous mutant.
  - Patient 3 = One band (50bp). No mismatch, ligation occurred completely. Patient is homozygous wildtype.



Signal Amplification

- Branched DNA Amplification (bDNA)
- Hybrid Capture Assays
- Cleavage-Based Amplification
- Cycling Probe

Probe-generated signal is amplified/accumulated without increasing the number of target sequences or probes.

Four methods described in the book:

- Branched DNA Amplification (bDNA)
- Hybrid Capture Assays
- Cleavage-Based Amplification
- Cycling Probe

We'll focus on **Branched DNA Amplification** as an example.

Branched DNA Amplification

DNA or RNA target, *if present in the sample*, is immobilized by solid-phase **capture probes**

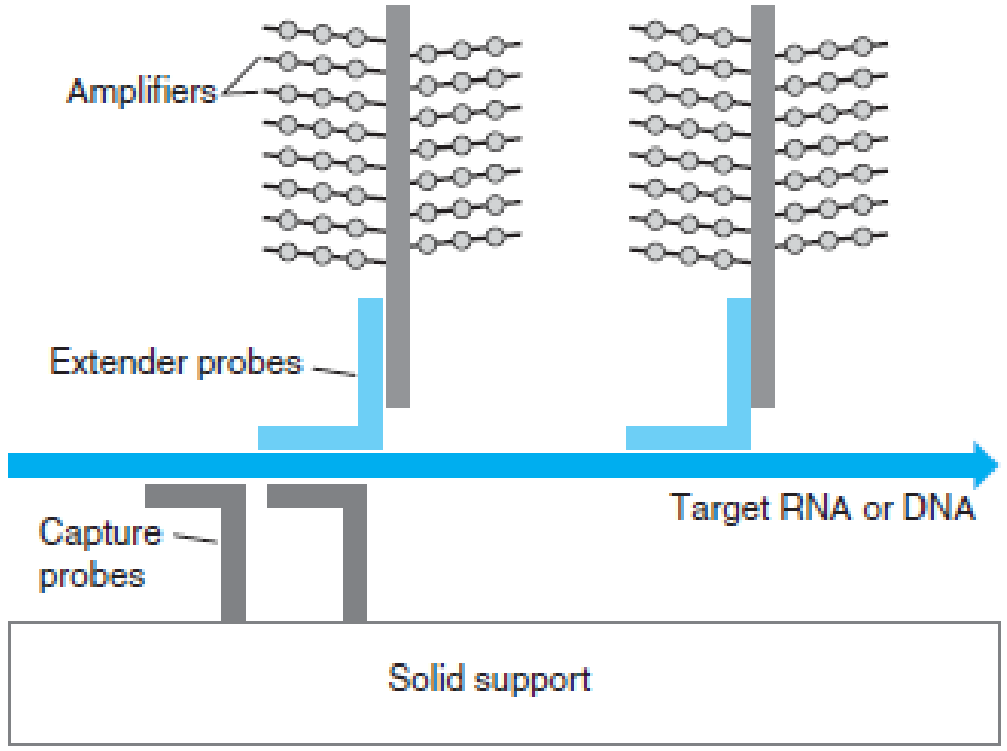
Extender probes bind to the captured target

- Contain sequences complementary to both target DNA/RNA and amplifiers.

Tree-like **amplifiers** bind to extender probe.

- Contain branches that serve as sites for alkaline phosphatase-driven chemiluminescent reaction.

If the entire series of binding occurs, reaction will produce detectable luminescence that indicates sample contained the target sequence.



First-generation bDNA

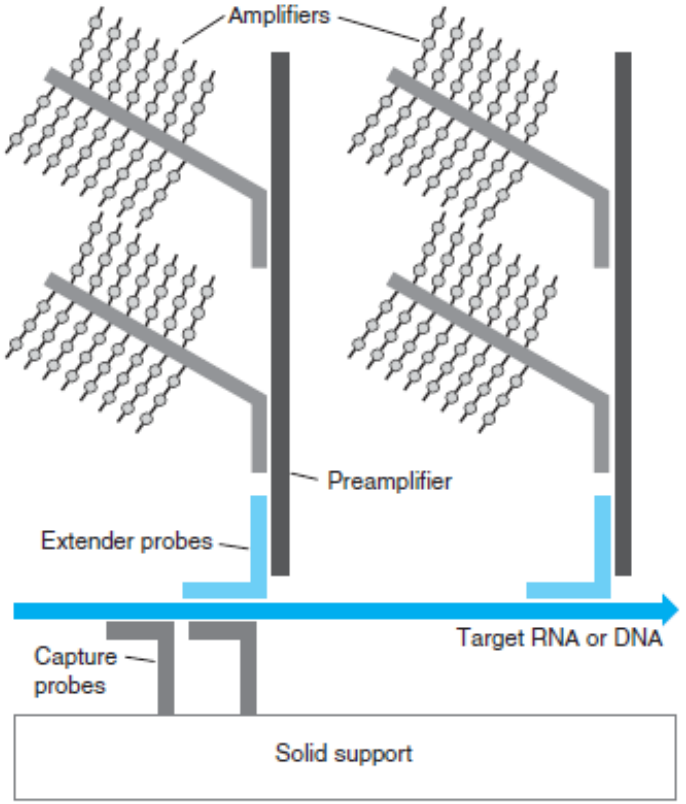
Branched DNA Amplification

Second-generation bDNA incorporates **preamplifier** that allows for binding of multiple amplifiers per target sequence

- Increases sensitivity by increasing luminescence

Since bDNA does not amplify the target sequence, there is reduced likelihood of carryover contamination to subsequent reactions.

No polymerase or RT involved, so common inhibitors of PCR (*e.g.,* EDTA) are less of a concern.



Second-generation bDNA

Operational Considerations

- Contamination Prevention
- Quality Control

Contamination is a major concern for amplification-based assays.

A PCR reaction creates billions of amplicons. The amplicons of a previous reaction are the most likely source of PCR contamination.

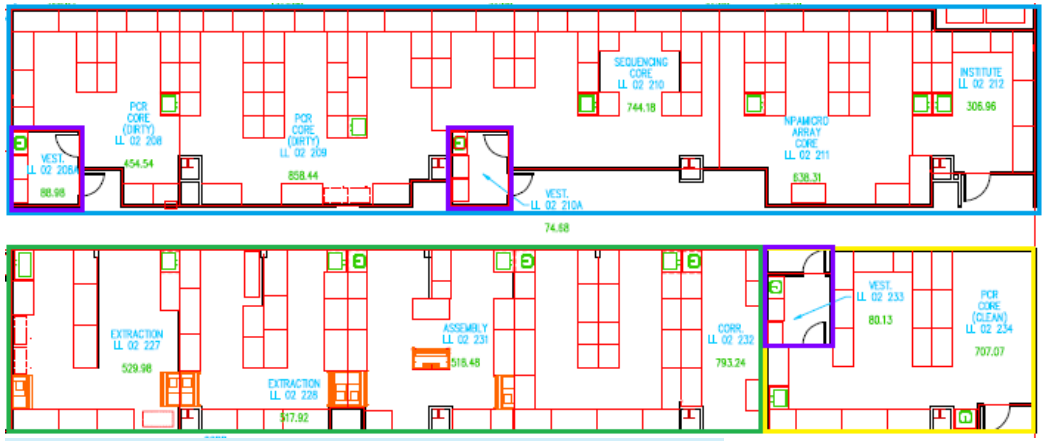
Laboratories prevent and monitor contamination in many ways:

- Unidirectional workflow design
- Topical inactivation of nucleic acids
- dUTP-UNG systems
- Wipe tests
- Addition of No Template Controls alongside other control types commonly used in the medical laboratory

Unidirectional Workflow

MDx labs maintain a **unidirectional workflow**

- Separate areas of the lab are designated for specific activities
- "**Clean Room**" designated for master mix preparation
  - ↑pressure to ensure air only flows out
- "**Main Lab**" designated for nucleic acid extraction and dilution
  - Ambient pressure
- "**Dirty Room**" designated for amplification procedures
  - ↓pressure to ensure air only flows in



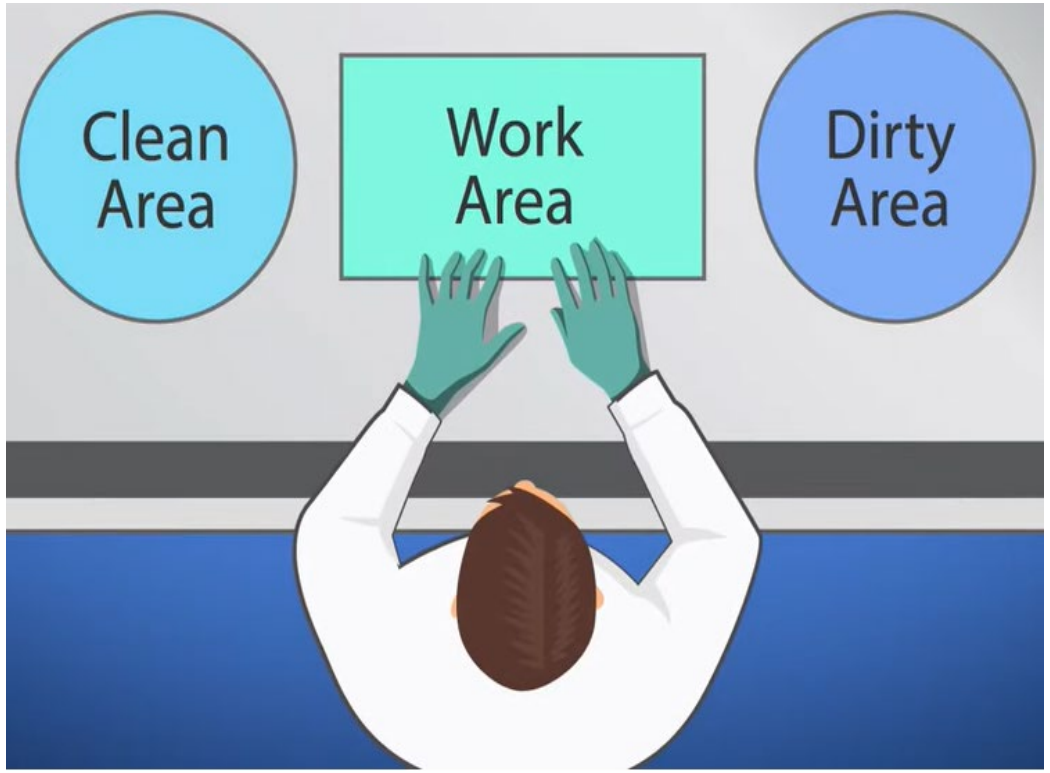
Yellow = Clean Room"
Green = Main Lab
Blue = Dirty Room
Purple = pressure controlled entrances/exits
Red = benches

Unidirectional Workflow

Bench techs also incorporate the concept of unidirectional workflow into their personal workspace.

- Designated **clean area** has reagents not in use, clean tips, and other unused consumables
- Designated **work area** has samples, reagents, and consumables that are actively being used
- Designated **dirty area** has waste containers

Allows techs to move unidirectionally:
clean tip/reagent → destination well → waste



Setup for right-handed technologist

Workspace Decontamination

Workspaces should be decontaminated before **AND** after use.

Chemical decontaminants can be used to degrade residual nucleic acid and inactivate exogenous nucleases.

- Sodium hypochlorite solution (bleach)
- Phosphoric acid (PCR Clean Wipes)
- Mixed alcohol wipes (purple wipes)

Ultraviolet light can also be used in addition to chemical decontaminants.

- Biological safety cabinets, assembly tamers, and other hooded workspaces affixed with UV bulbs

dUTP-UNG

Enzymatic method of decontamination with two components:

- **dUTP**, which substitutes dTTP during amplification reaction
- Uracil-N-glycosylase (**UNG**) enzyme that degrades nucleic acids containing uracil

Any amplicon from a previous reaction will contain uracil instead of thymine.

Adding a short UNG digestion step prior to new amplification reaction will remove contaminating amplicons.

Cobas 8800 automated extraction/real-time PCR instrument uses dUTP-UNG system, allowing it to be located in the main lab area instead of the "dirty room."

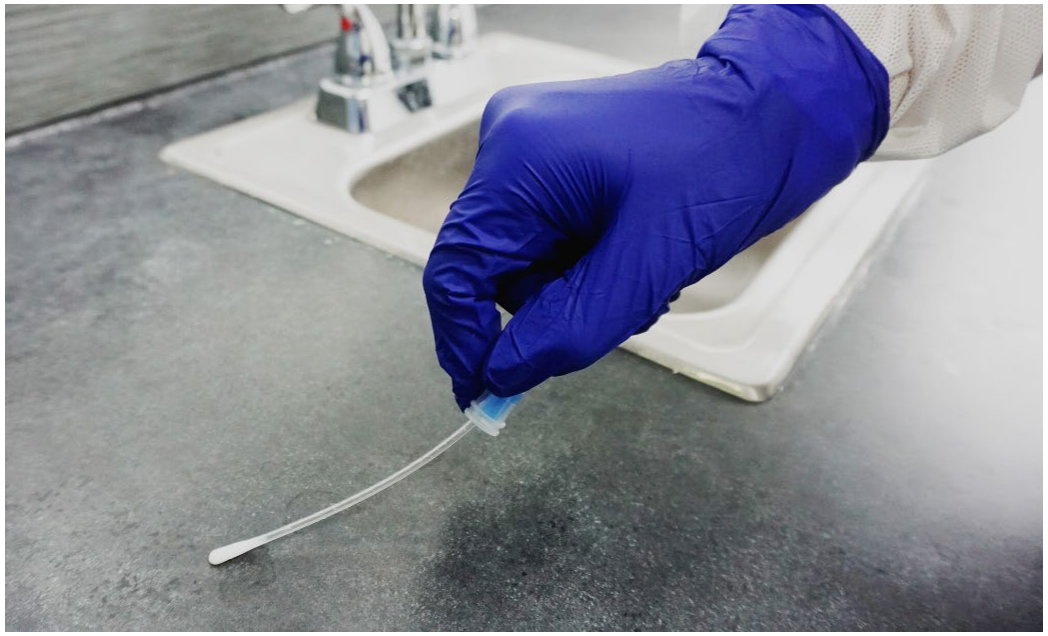
Wipe Tests

Molecular laboratories, particularly those interrogating respiratory pathogens that can transmit through the air, employ **wipe tests** to identify contaminating nucleic acids.

Workspace surfaces are routinely wiped with a sterile swab or pad (*e.g.*, once a month).

Amplification-based procedure is performed on swab.

If amplification occurs, workspace is contaminated and requires more extensive sanitization.



Quality Control

As with all medical laboratory tests, daily performance of quality control is an essential component of quality assurance.

Some high-volume molecular test systems mimic what you're used to in chemistry and heme: two levels of controls tested every 24 hours.

In practice, most molecular tests have substantially more controls and are measured with every performance of the test.

Most molecular controls fall into one of two categories:

- External
- Internal

External Controls

External controls: samples with known results that are included on the run to assess the overall performance of the test system

Separate samples with their own designated reaction wells

Several kinds:

- **Positive** control: contains the target sequence
- **Sensitivity** control: a positive control containing the target sequence at a very low level (*e.g.*, 1%), useful for quantitative assays
- **Negative** control: does NOT contain the target sequence
- No template control (**NTC**): has no DNA/RNA template present and should not amplify, water control

Failure of an external control to perform as expected indicates issues with the entire run (mis-pipetting, incorrect reagent prep, primer-dimer, contamination, etc.)

Internal Controls

Internal control: an analytical target distinguishable from the test target but detectable with reagents included in the reaction mix

Located within the same reaction well as the patient sample

Two types: **intrinsic** and **extrinsic**

- *Intrinsic internal control*: already present within the sample, usually a target found ubiquitously in all human cells
- *Extrinsic internal control*: control is "spiked in" to sample at the beginning of the analytic process

Failure of an internal control to perform as expected indicates sample-specific issues (poor sample collection, poor extraction, interfering substances, etc.)

Quality Control Example

Example:

In SARS-CoV-2 testing, nasopharyngeal swab specimens undergo a PCR-based procedure that attempts to amplify a target sequence unique to the SARS-CoV-2 virus (*e.g.*, RdRP gene).

Specimens are *also* analyzed for target that should be present in all human cells (*e.g.*, RNAse P1).

- Human target is an intrinsic internal control in this scenario

Performance of the internal control changes the interpretation of SARS-CoV-2 target amplification:

- RdRP negative, RNAse P1 positive = true negative
- RdRP negative, RNAse P1 target negative = false negative due to collection/extraction/amplification issue

Questions?

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This concludes the presentation.

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