Polymerase Chain Reaction

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Slides prepared by Kelly M. Elkins, Ph.D., Towson University, 2025

Background

In October 2023, Towson University was awarded a cooperative agreement from NIST to develop a standardized DNA training curriculum for the United States that addresses the components in ANSI/ASB Standard 115, *Standards for Training in Forensic Short Tandem Repeat Typing Methods Using Amplification, DNA Separation, and Allele Detection*. 2020. 1st Ed.

This presentation addresses the knowledge-based portion of the training program and covers the topic outlined in 4.2.3b in ANSI/ASB Standard 115.

Learning Objectives

This material will provide trainees with an understanding of PCR, including:

1. history of development and use;
2. biochemical principles;
3. hot-start PCR;
4. multiplex PCR;
5. function of reagents;
6. specificity, fidelity, and optimization;
7. limitations of the technology;
8. PCR inhibitors;
9. stochastic effects;
10. amplification artifacts;
11. contamination and quality control

Terms & Definitions (ANSI/ASB 115)

- **Allele.** One of two or more versions of a genetic sequence at a particular location in the genome.
- **Amplification.** An increase in the number of copies of a specific DNA fragment. In forensic DNA testing laboratories, this refers to the use of the PCR technique to produce many more copies of fragments at specific genetic loci from samples of known and unknown origin for the purpose of generating DNA profiles for comparison.
- **Electrophoresis.** A technique used in laboratories to separate macromolecules based on size and charge. Negatively charged molecules (e.g., DNA and RNA) migrate towards a positively charged pole through a sieving matrix, which permits a size-dependent separation.

Terms & Definitions (ANSI/ASB 115)

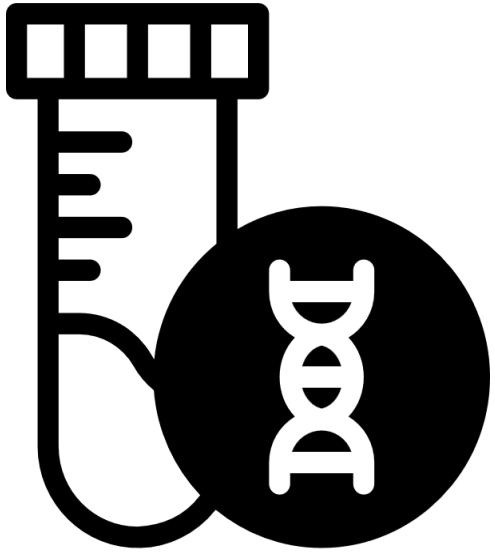
- **Inhibitor.** As related to the polymerase chain reaction (PCR), any substance that interferes with or prevents the synthesis of DNA during the amplification process.
- **Polymerase chain reaction.** An enzymatic process by which a specific region of DNA is replicated during repetitive cycles that consist of the following: denaturation of the template; annealing of primers to complementary sequences at an empirically determined temperature; and extension of the bound primers by a DNA polymerase. The goal of the PCR process is to generate many copies (termed products or amplicons) of a specific region of DNA for further analysis.

Polymerase Chain Reaction (PCR)

- Copies *or amplifies* a target region or locus of DNA
- *In vitro* (outside the cell) in the lab
- Repetitive temperature cycling (typically 28-40 cycles of 2-3 steps per cycle)
  - **Denaturation** of the template (at 90-95 °C)
    - DNA as well as proteins including most DNA polymerases are denatured at this temperature
  - **Annealing** of primers to complementary sequences (typically 55-70 °C, empirically determined)
  - **Extension** of the bound primers by a heat stable DNA polymerase (at 72 °C)(polymerase will amplify DNA at the annealing step so extension step may be combined or deleted)

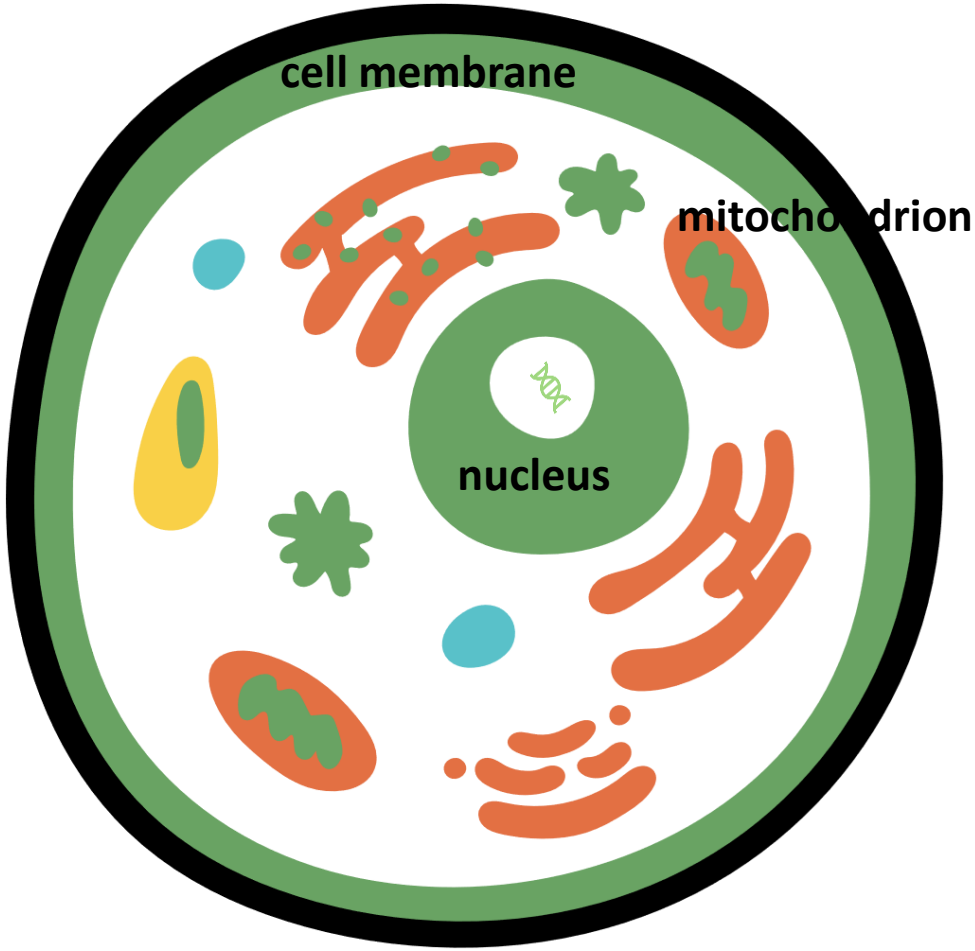
PCR Amplification

- PCR is a method used to copy DNA strands *in vitro* outside the cell in a test tube
- Forensic evidence often has low quantities of biological material and cells, and PCR can be used to amplify the extracted DNA so that it can be detected by the instrumentation.



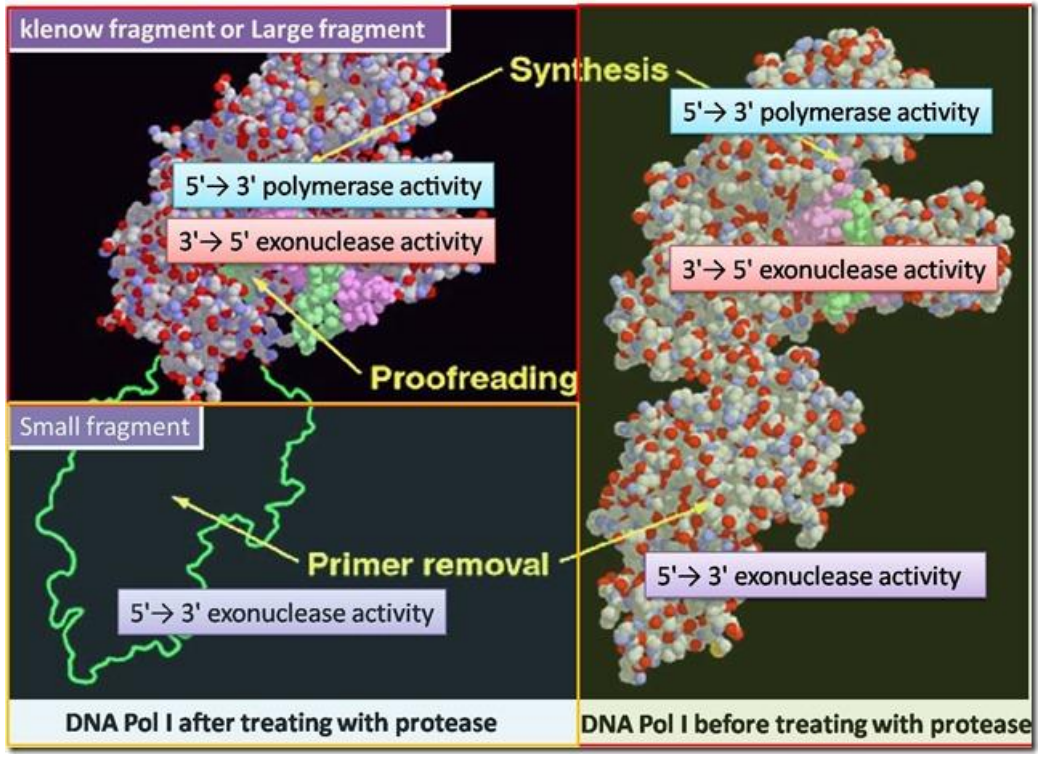
Biochemical Principles: The Cell

- The cell is surrounded by the cell membrane
- The cell contains organelles such as mitochondria and chloroplasts (not shown) as well as vesicles and small molecules
- The nucleus contains the DNA
- Products including proteins and enzymes (e.g., proteases, DNases) coded by the DNA perform the work in the cell



History of PCR

- Invented by Kary Mullis in 1985
- *E. coli* enzyme DNA polymerase I was the first used to copy DNA *in vitro*
  - DNA pol I can be cleaved by subtilisin to two fragments, Klenow fragment (the larger fragment) and a smaller fragment
    - The enzyme binds DNA and catalyzes chemical reactions
    - Klenow has 5' to 3' polymerase activity to insert bases
    - 3' to 5' exonuclease activity can remove inserted bases



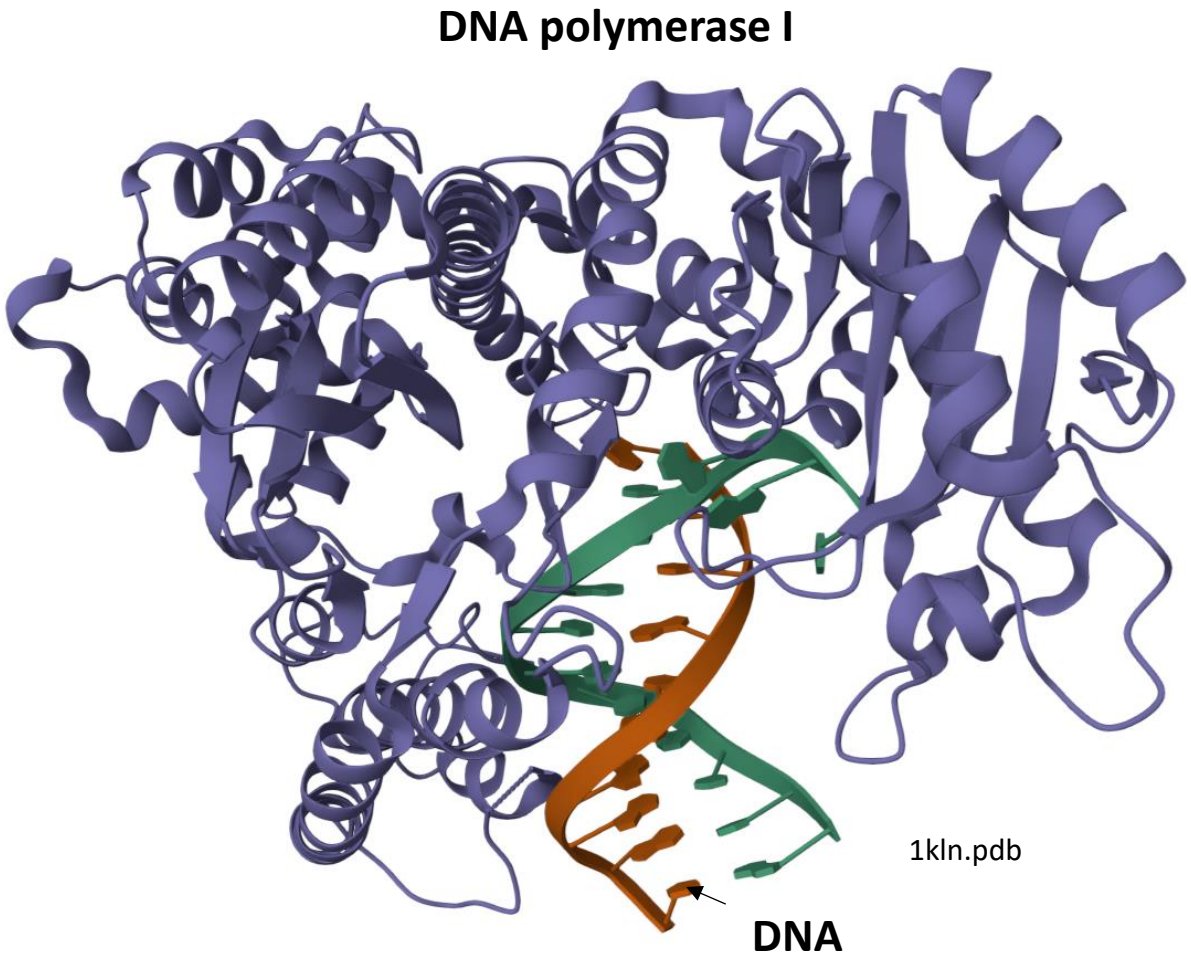
Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H. Specific Enzymatic Amplification of DNA In Vitro: The Polymerase Chain Reaction. *Cold Spring Harb Symp Quant Biol.* 1986;51: 263-273.doi:10.1101/SQB.1986.051.01.032

Mullis K, Faloona FA. Specific Synthesis of DNA *in vitro via* a Polymerase-Catalyzed Chain Reaction. *Methods Enzymol.* 1987;155: 335-350.

Ollis DL, Brick P, Hamlin R, Xuong NG, Steitz TA. Structure of large fragment of Escherichia coli DNA polymerase I complexed with dTMP. *Nature*. 1985;6;313(6005):762-6. doi: 10.1038/313762a0.

Biochemical Principles

- Thomas Steitz and colleagues solved the first X-ray crystal structure of a DNA polymerase I from *E. coli* in 1985.
- The *E. coli* Klenow fragment of the DNA polymerase I (purple) complex with DNA was published in 1993.
- As it is *not heat stable*, in early PCR DNA pol I was (re)added each cycle



Structure reference: Lorena S. Beese, Victoria Derbyshire, Thomas A. Steitz. Structure of DNA Polymerase I Klenow Fragment Bound to Duplex DNA. *Science* **260**,352-355(1993).DOI:10.1126/science.8469987

History of PCR

- Yellowstone Hot Springs' *Thermus aquaticus* (source of *Taq* DNA polymerase) was discovered in 1969
- *Taq* is *thermally stable* and most active at hot temperatures (not inactivated by hot temperatures)
- First identified enzyme suitable for *multicycle* amplification

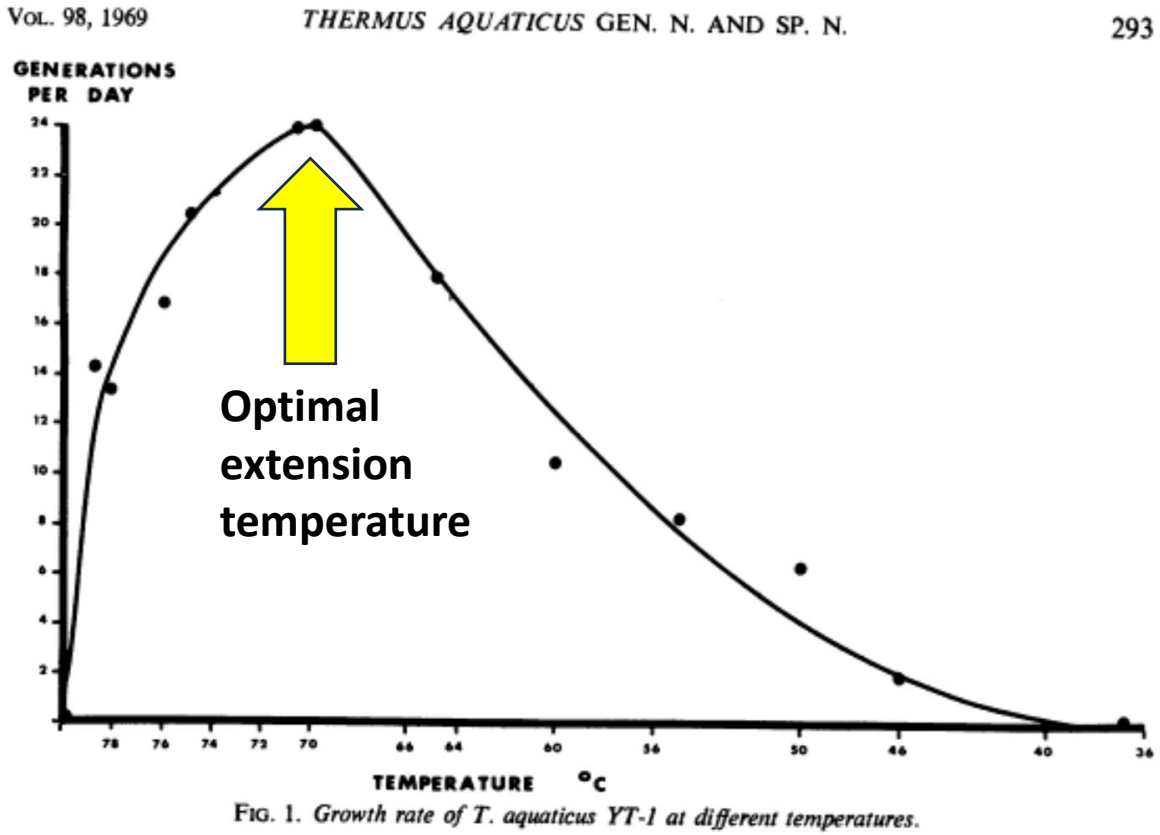
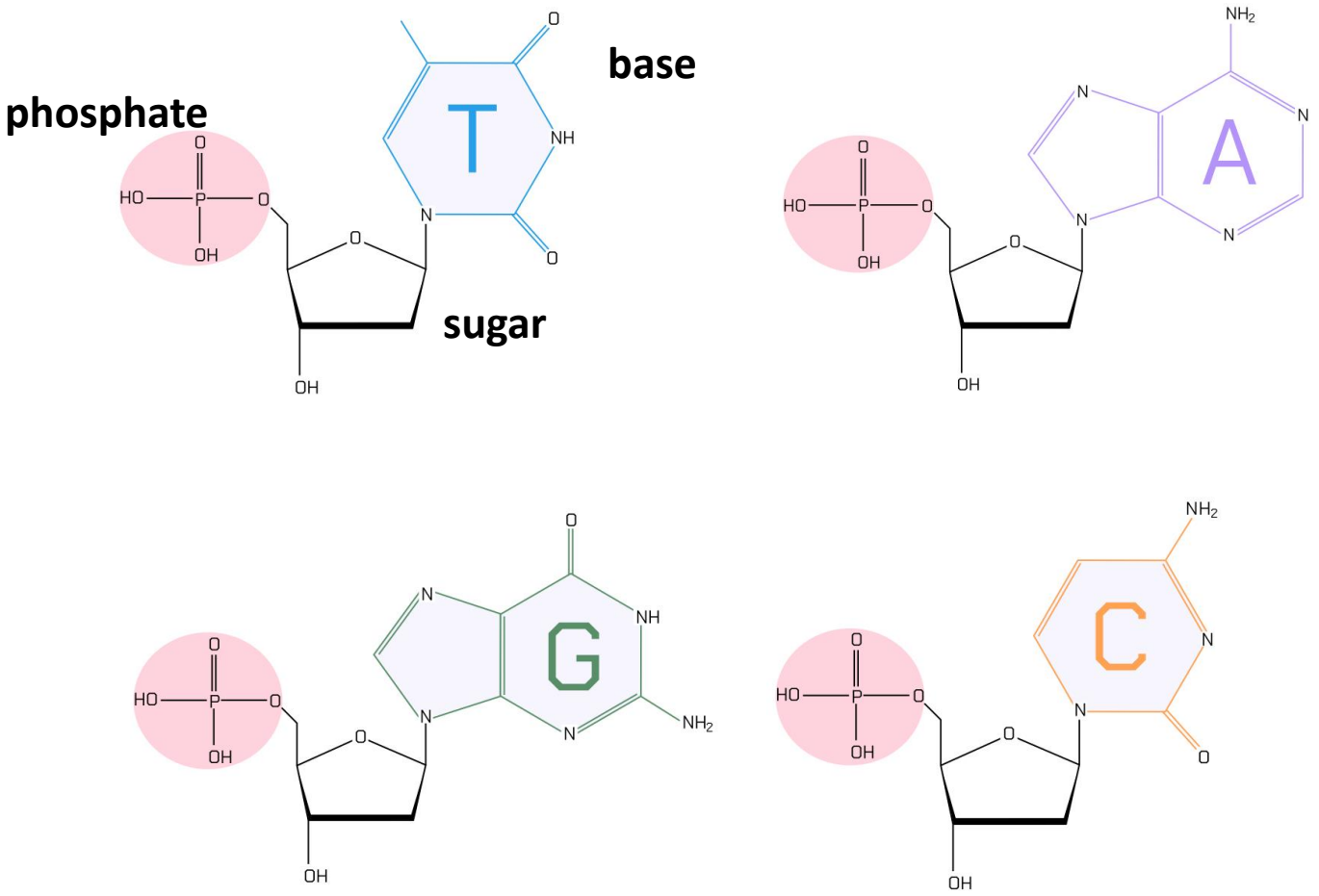


FIG. 1. *Growth rate of T. aquaticus YT-1 at different temperatures.*

Brock TD, Freeze H. Thermus aquaticus gen. n. and sp. n., a nonsporulating extreme thermophile. *J Bacteriol.* 1969 Apr;98(1):289-97. doi: 10.1128/jb.98.1.289-297.1969.

Biochemical Principles: What is DNA?

- DNA is **d**eoxyribo**n**ucleic **a**cid and is composed of
  - deoxyribose sugar (numbered with primes),
  - a phosphate (Pi) group attached at the 5'-end of the deoxy sugar,
  - and a nitrogenous base.
- A generic deoxyribonucleic acid triphosphate is called a dNTP.
- Forms a double helix through hydrogen bonding between nitrogenous bases
  - H-bonds are a special dipole-dipole interaction from a partially positively charged hydrogen and a partially negatively charged atom such as oxygen



Structures credit: Nihal K. Chana

Biochemical Principles: What is DNA?

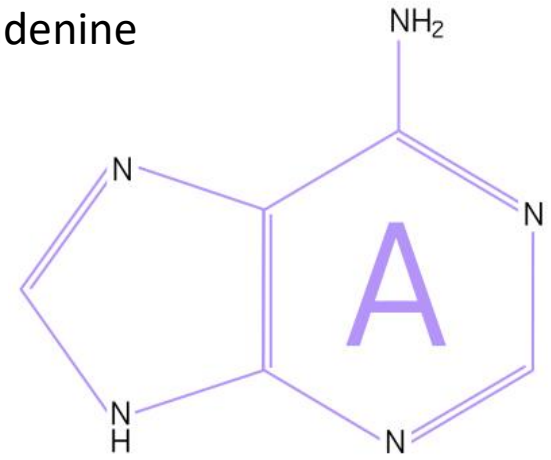
- Informational portion is nitrogenous bases
  - **purines** (A, G) and
  - **pyrimidines** (C, T)

  (T substituted by U in RNA)

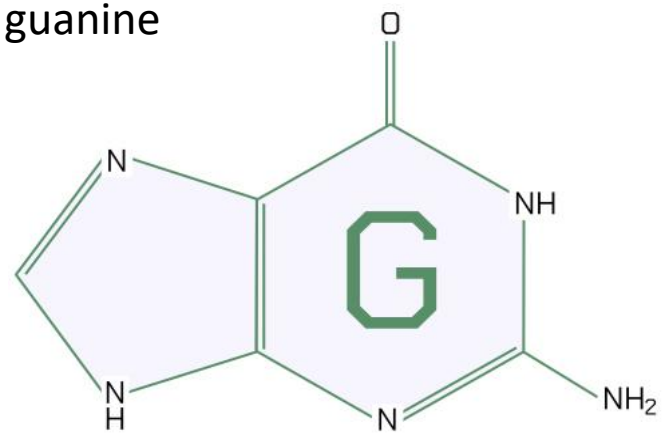
Structures credit: Nihal K. Chana

Purines

adenine

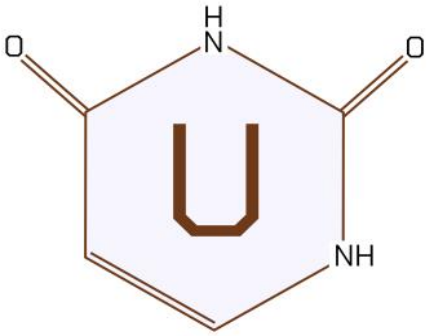


guanine

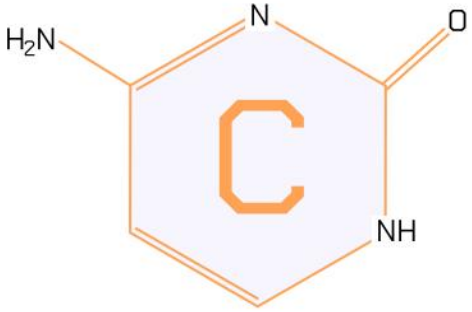


Pyrimidines

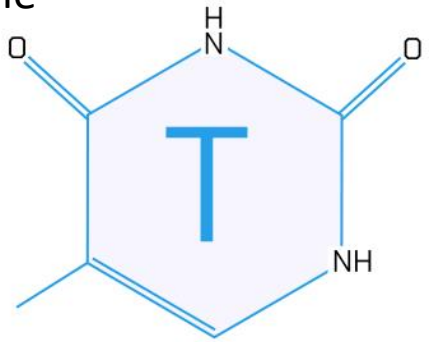
uracil



cytosine



thymine



Biochemical Principles: What is DNA?

Following addition to the 3'-O on the sugar, a new deoxynucleoside monophosphate (dNMP) is attached to grow the chain with pyrophosphate (PPi) driving the reaction through its degradation to 2 inorganic phosphate (Pi). The strands run antiparallel.

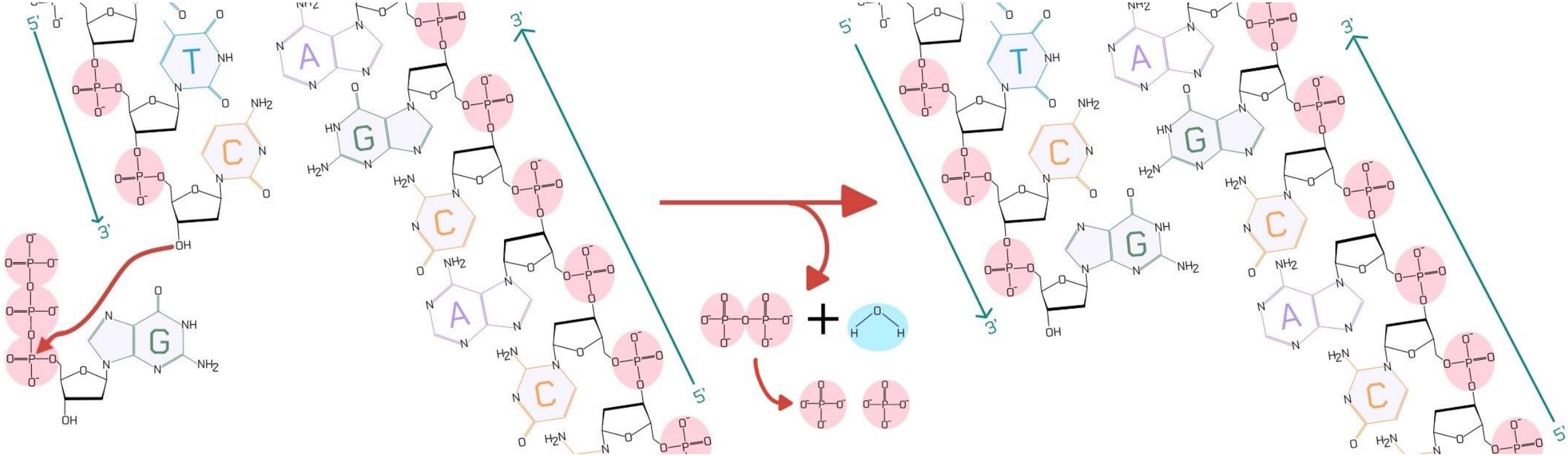


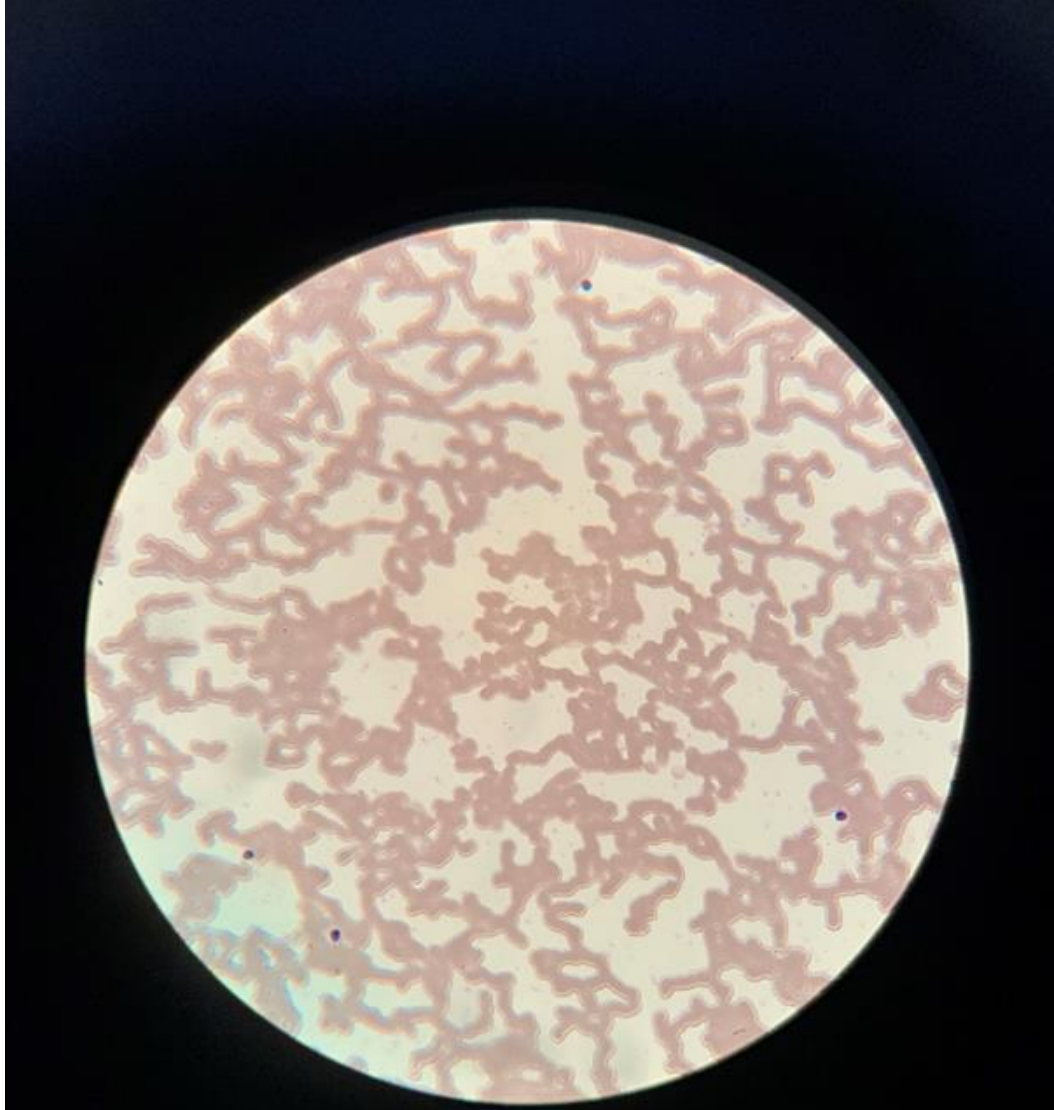
Figure credit: Nihal K. Chana

Quantity of DNA in a Human Cell

- One copy of the genome
  - 3.2 billion base pairs*
  - 3.3 pg of DNA
- Sperm or egg cell
  - 3.3 pg DNA (haploid)
- White blood cell[#], epithelial cell, etc.
  - 6.6 pg DNA (diploid)

*By definition, one haploid set of chromosomes

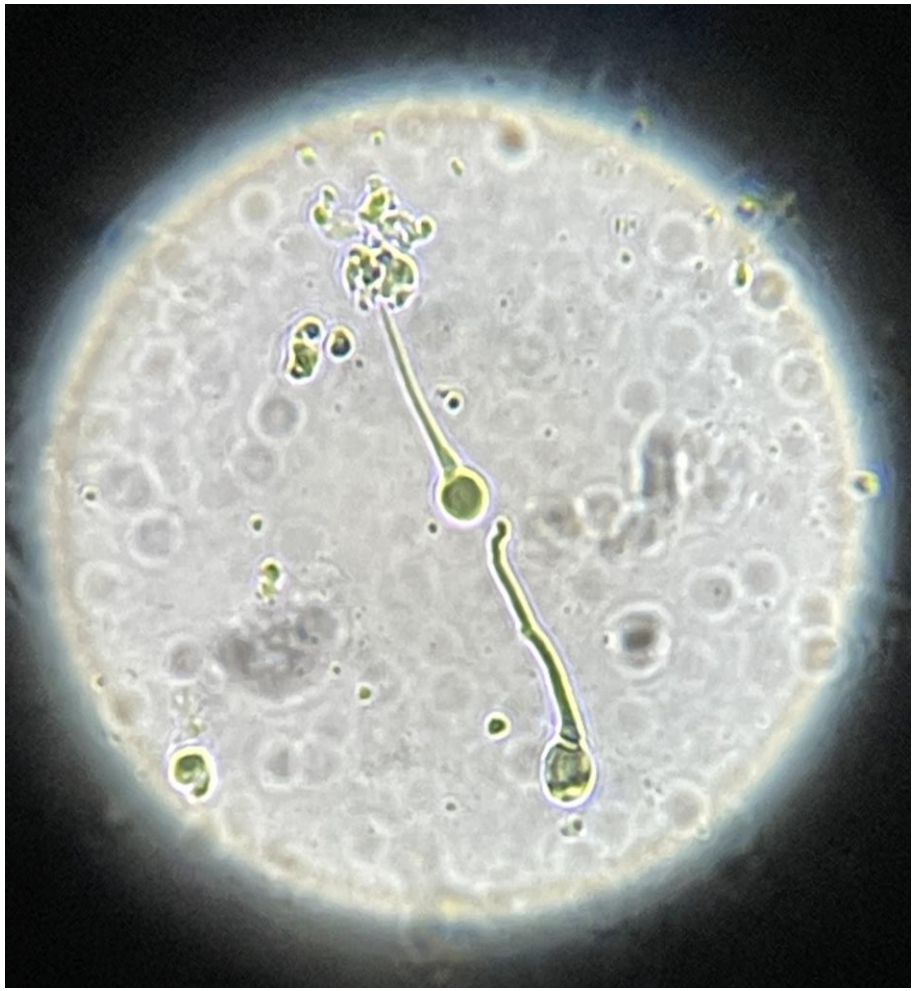
[#]Red blood cells do not contain DNA



Red Blood Cells
40x

Quantity of DNA in a Human Cell

- Sperm cell
  - 3.3 pg DNA in the head (haploid)



Calculating the Quantity of DNA in a Human Cell

1. Recall the Relative Molecular Mass of a DNA Base Pair = 618 g/mol

   A = 313 g/mol G = 329 g/mol

   T = 304 g/mol C = 289 g/mol

   A-T base pairs = 617 g/mol & G-C base pairs = 618 g/mol (2 per 152 diploid genomes)

2. There are 3.2 billion base pairs in a haploid cell so we can compute the MW

   3.2×10^9 base pairs x (618 g/mol per base pair) = 1.98×10^{12} g/mol

3. Using Avogadro's number, we can compute the number of grams of DNA

   1.98×10^{12} g/mol x 1 mole/6.02×10^{23} molecules = 3.3×10^{-12} g/molecule or 3.3 pg

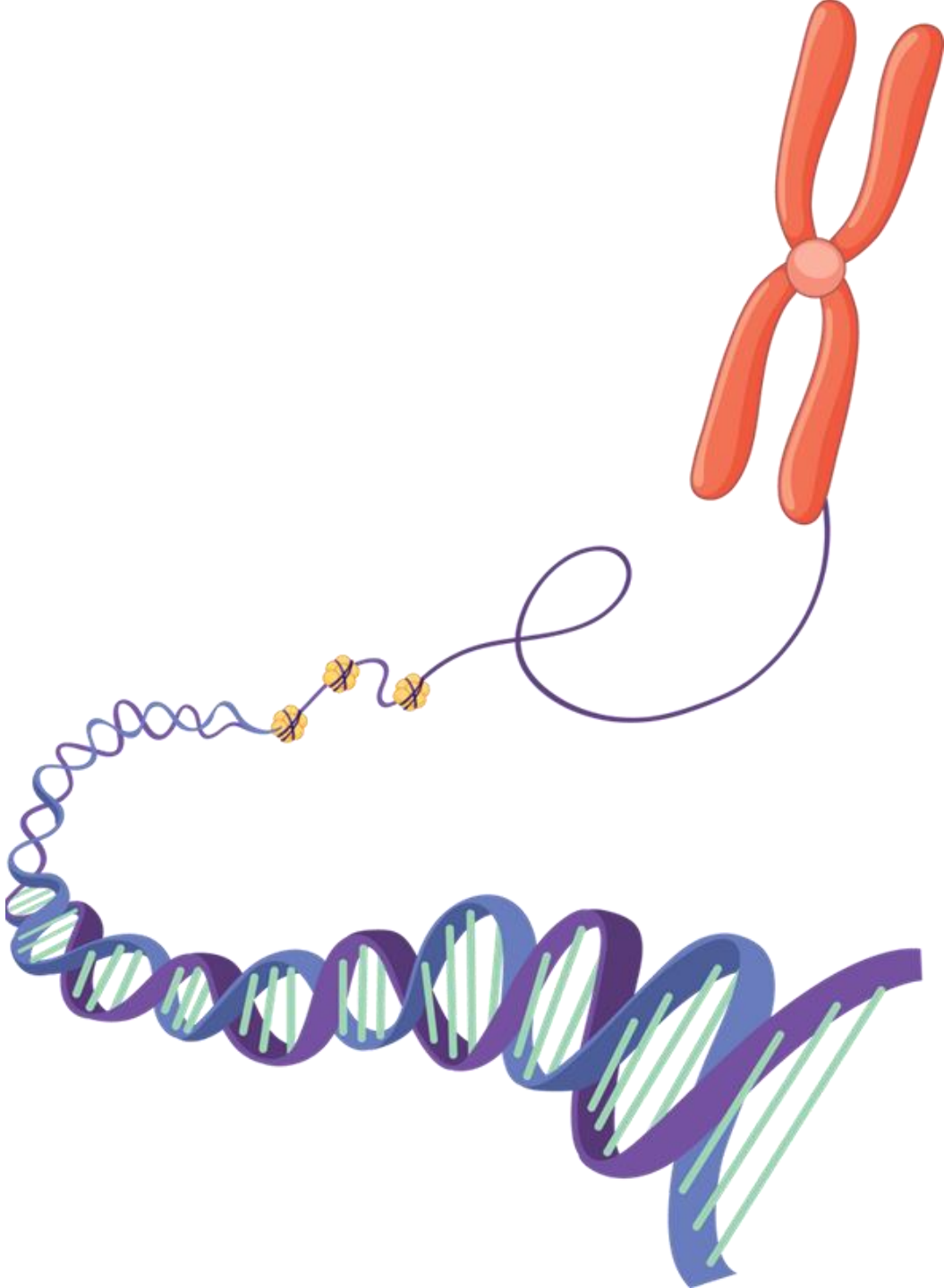
4. A diploid cell would contain twice as much so 6.6 picograms of DNA.

5. Thus, one nanogram of human DNA from a diploid cell is

   1 ng or 1×10^3 pg / 6.6 pg/cell = 152 cells or approximately 303 copies of each locus.

Biochemical Principles: DNA

- DNA is found in the nucleus (nDNA), mitochondria (mtDNA) and chloroplasts in cells.
- DNA is supercoiled and packaged in chromosomes.
- DNA wraps around histones (yellow) to pack to form the chromosomes.
- Humans have 46 chromosomes including 22 paired autosomes and two sex-determining chromosomes, X and Y.
- DNA consists of complementary hydrogen-bonded (H-bonded) strands which form helical secondary structures.



Biochemical Principles

- Topoisomerase is an enzyme that relaxes supercoiled DNA.
- Helicase unwinds DNA *in vivo* (in the cell) at the replication fork; heat is used for this purpose *in vitro* (outside the cell, in the lab)
- The unwound strands serve as templates for interpretation by their *complement.*
- Small strands of single-stranded RNA or DNA (primers) need to be complementarily bound to a DNA strand via H-bonds for the DNA polymerase I to bind.
- Replication is semi-conservative.

Figure credit: Anna-Sewell Killingstad

Biochemical Principles

- Watson-Crick base pairing
  - Adenine (A) is complementary to thymine (T)
  - Guanine (G) is complementary to cytosine (C)
- The sequence of one strand predicts the sequence of the complement

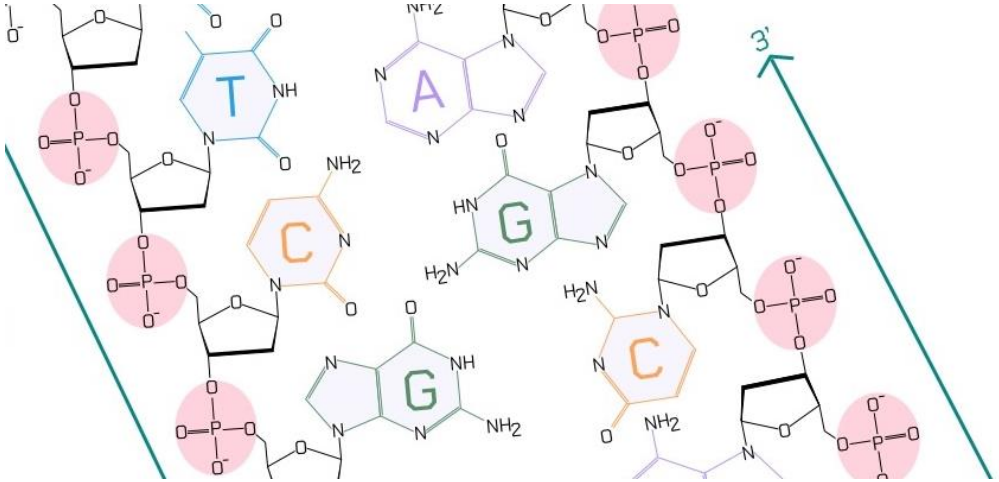
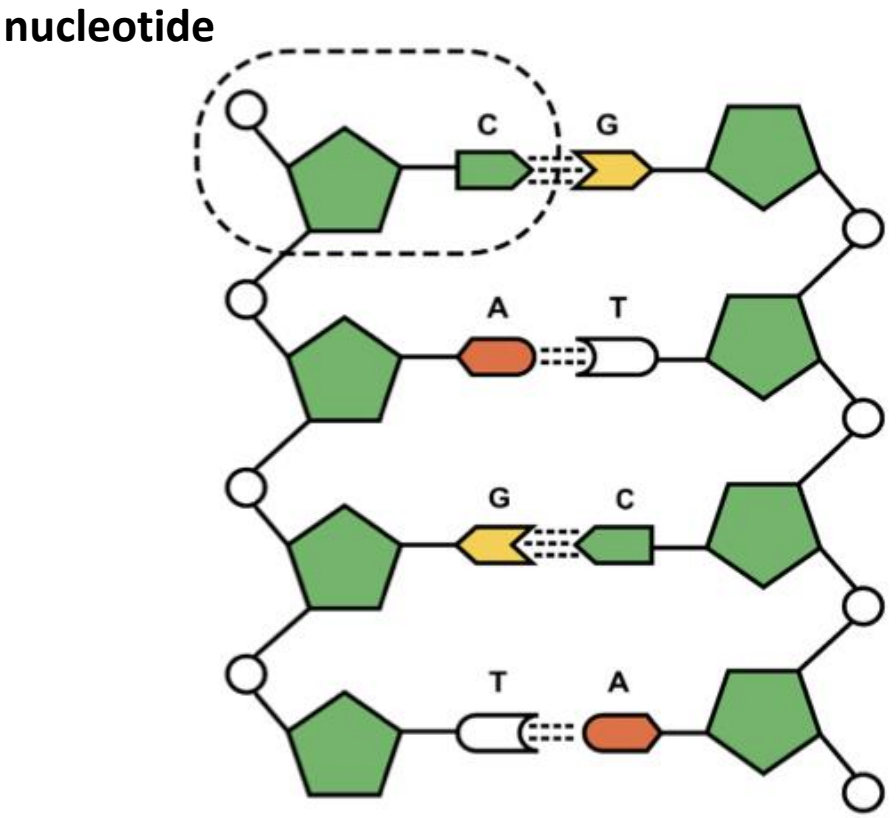


Figure credit: Nihal K. Chana

Biochemical Principles

- Watson-Crick base pairing
  - H-bonding



Biochemical Principles

- RNA primers serve *in vivo* while more stable DNA primers are used *in vitro.*
  - Primers are assembled by primase.
  - The DNA polymerase catalyzes the covalent attachment of the complementary nucleotide to the primer strand.
  - Extension occurs along the strand in the sugar carbon 5' to 3' direction.
  - Short fragments result from the replication of the lagging strand which are termed Okazaki fragments; the fragments are connected by the enzyme DNA ligase.

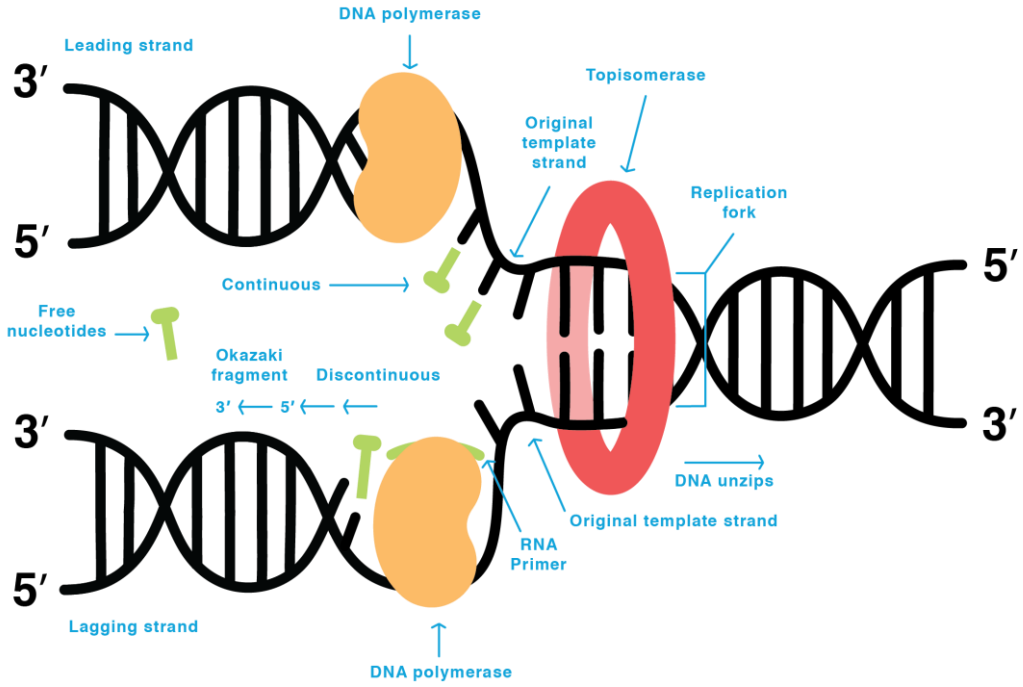
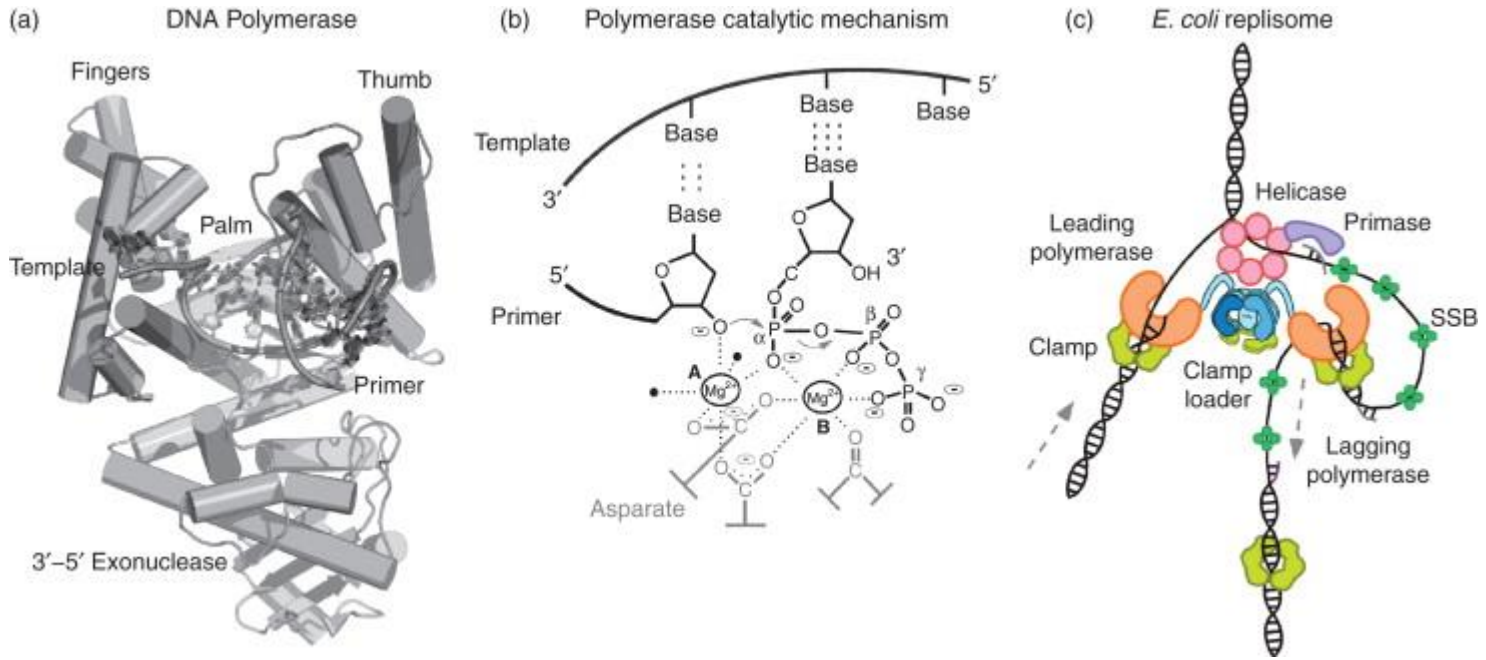


Figure credit: Anna-Sewell Killingstad

Biochemical Principles: Mechanism of DNA Polymerase I



"Figure 1. (a) Crystal structure of *Bacillus stearothermophilus* DNA polymerase showing the characteristic 'fingers', 'thumb', and 'palm' domain and the cleft containing the active site that binds the primer–template junction. (b) Diagram of a polymerase active site and catalytic mechanism. (c) Diagram of the *E. coli* replisome." Hingorani 2013 caption

M.M. Hingorani, in *Brenner's Encyclopedia of Genetics* (Second Edition), 2013

Biochemical Principles

- DNA polymerase I catalyzes condensation (or dehydration synthesis) reactions.
  - Nucleophilic addition
  - Magnesium cofactor

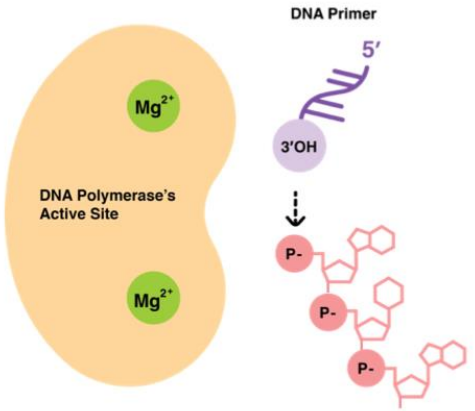


Figure credit: Anna-Sewell Killingstad

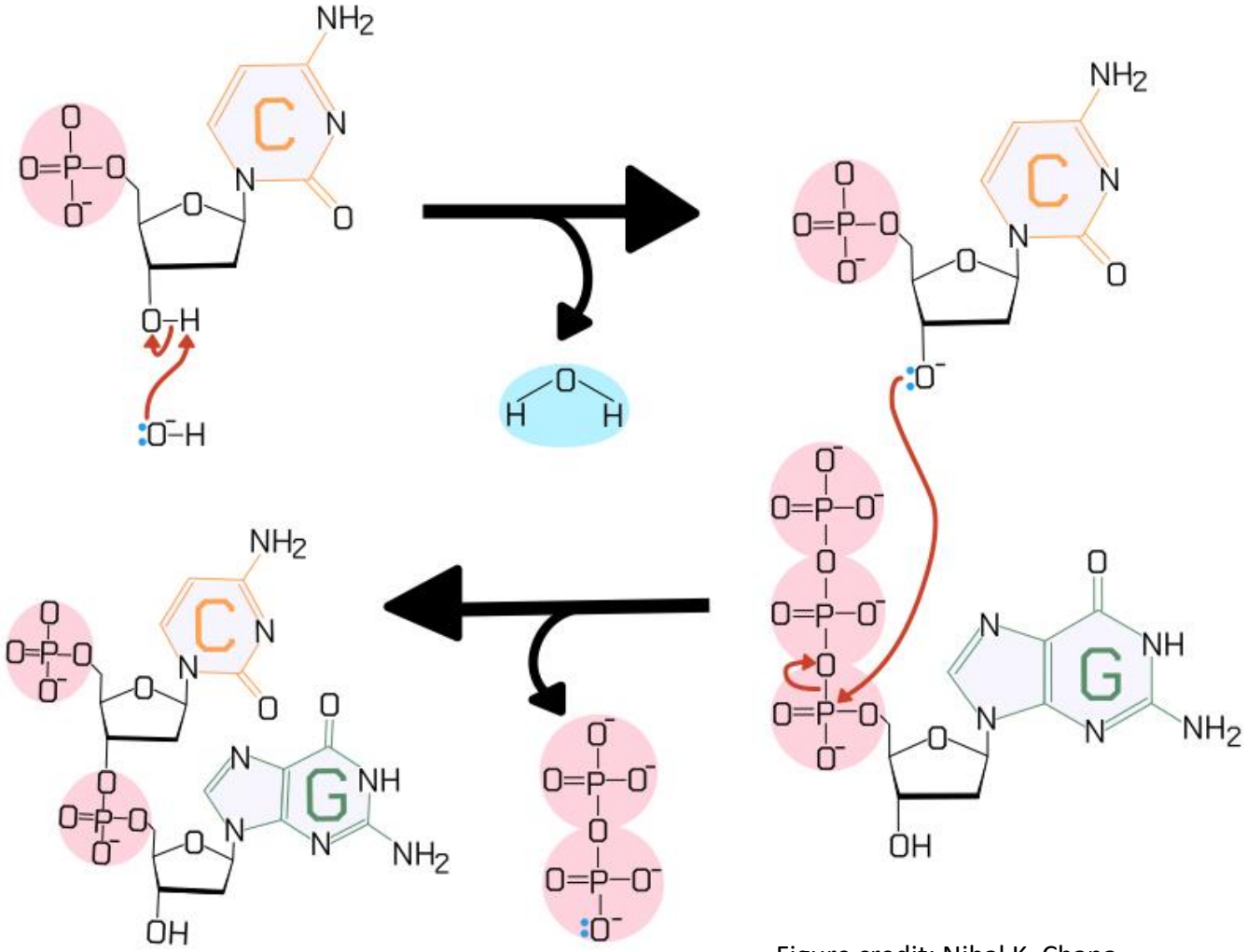
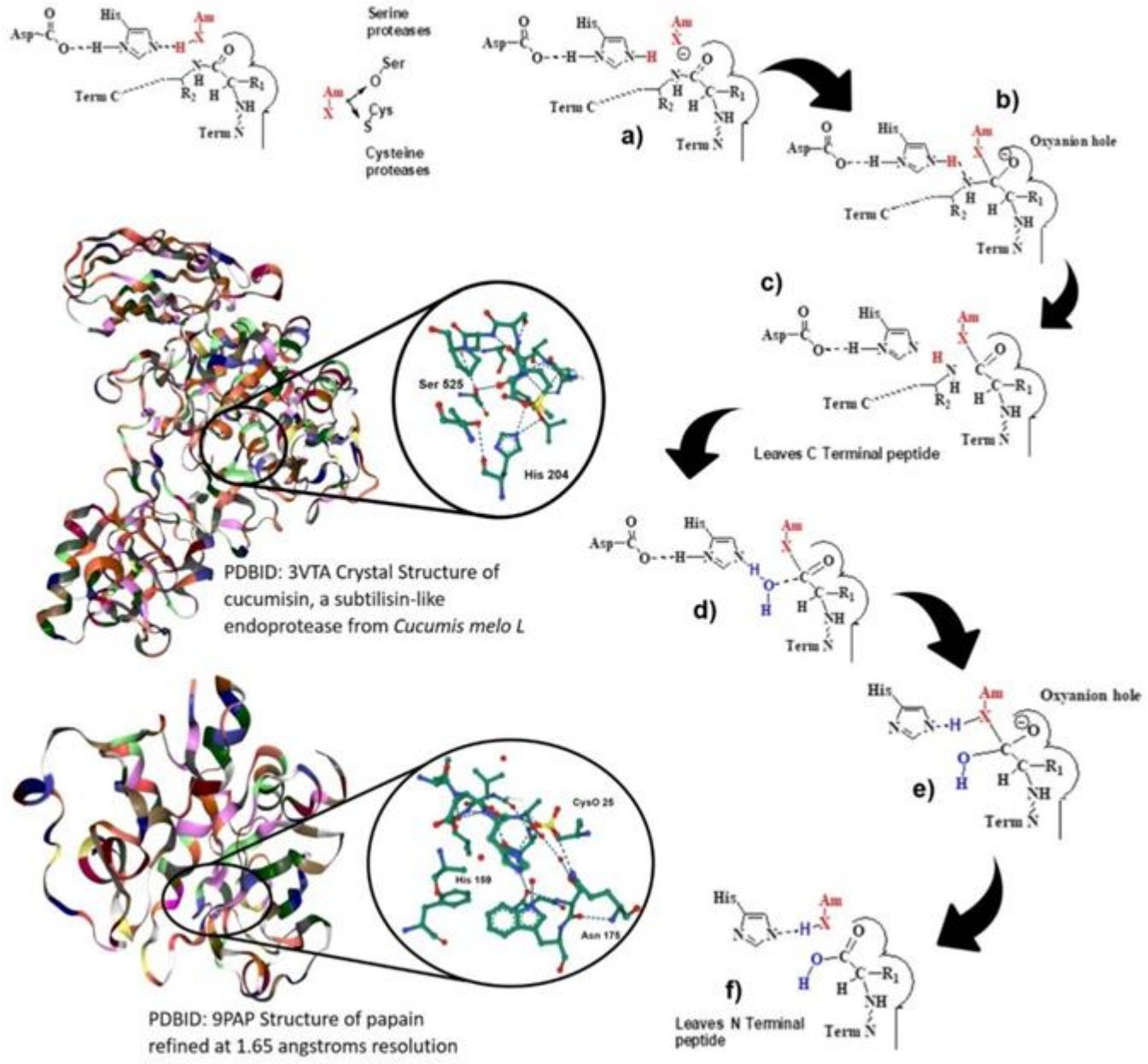


Figure credit: Nihal K. Chana

Proteases Degrade Enzymes

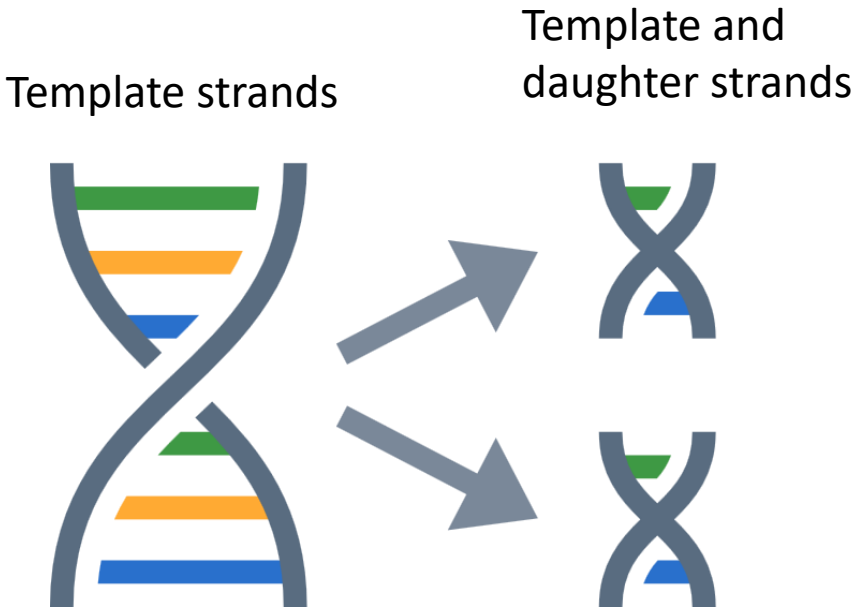
Structures and Mechanisms of Proteases



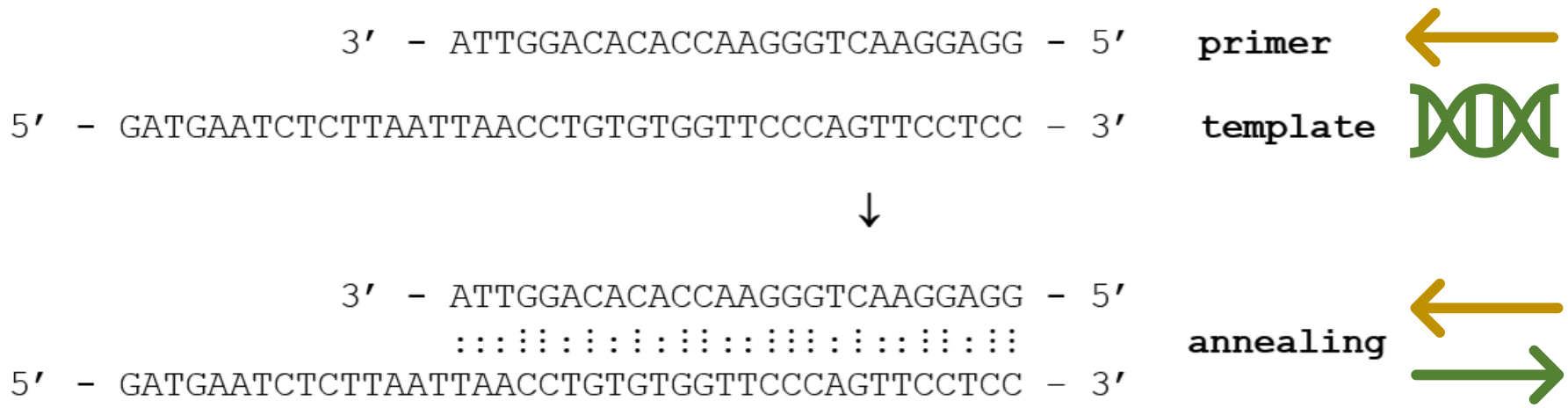
David Troncoso F, Alberto Sánchez D, Luján Ferreira M. Production of Plant Proteases and New Biotechnological Applications: An Updated Review. ChemistryOpen. 2022 Mar;11(3):e202200017. doi: 10.1002/open.202200017.

PCR

- At 100% efficiency, each cycle of PCR leads to a copy of each parent strand to yield two new daughter strands
- At less than 100% efficiency, a full set of daughter strands are not amplified in that cycle



PCR Amplification: Specific Primers are needed



Shown is Promega reverse primer for the TPOX locus

PCR Reagents and Functions

- DNA polymerase – heat stable native *Taq, Pfu, Tfl*, *Tth* enzymes, or engineered fusion or chimeric
- Mg^{2+} – cofactor for DNA polymerase and needed to stabilize DNA duplexes, too high concentrations can produce non-specific PCR products or errors from the DNA polymerase in adding the correct dNTP
- dNTPs (i.e., dCTP, dGTP, dATP, dTTP) – DNA nucleotide triphosphates for building new strands, may need to increase concentration to amplify long fragments but too high of concentrations can inhibit PCR
- Buffer, Tris-HCl pH 8.4 – control the pH for DNA polymerase activity
- BSA – bovine serum albumin, binds inhibitors
- KCl – promotes primer annealing

Optional: Intercalating dye link such as SYBR Green for detection

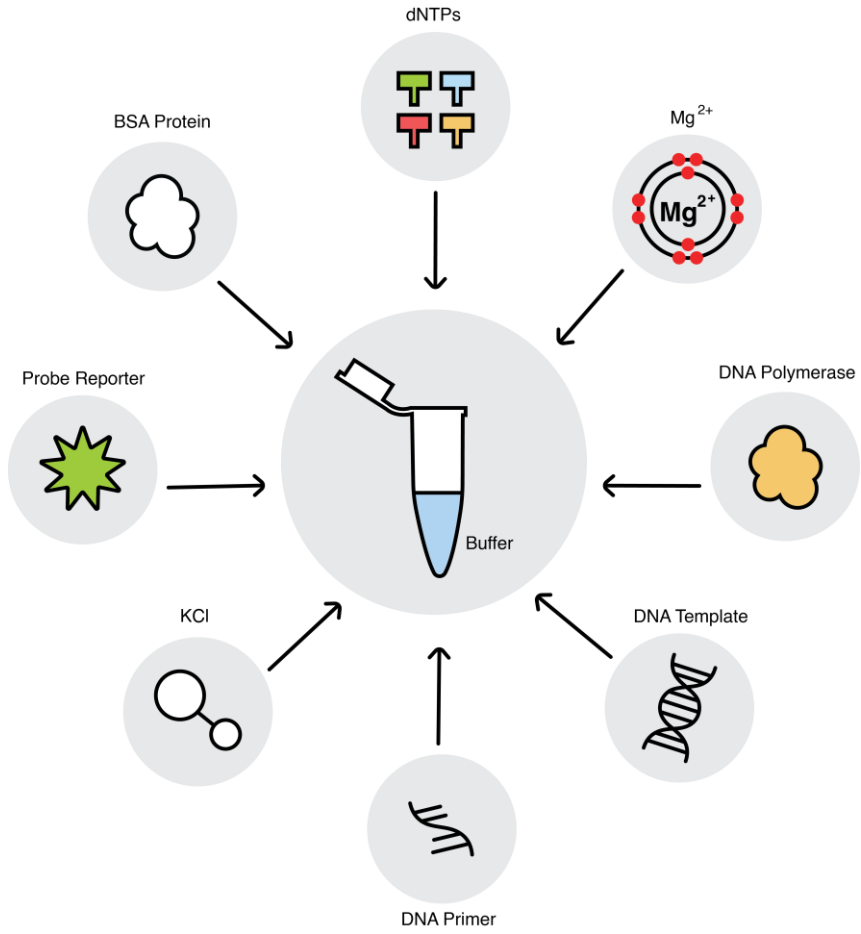


Figure credit: Anna-Sewell Killingstad

Primer Specificity and Stringency

- Impacted by the quality of the DNA template, concentration of the primers and magnesium ions, and complementarity of the primers
- High stringency is achieved when there is high base-pairing homology of the primer or probe and the template strand

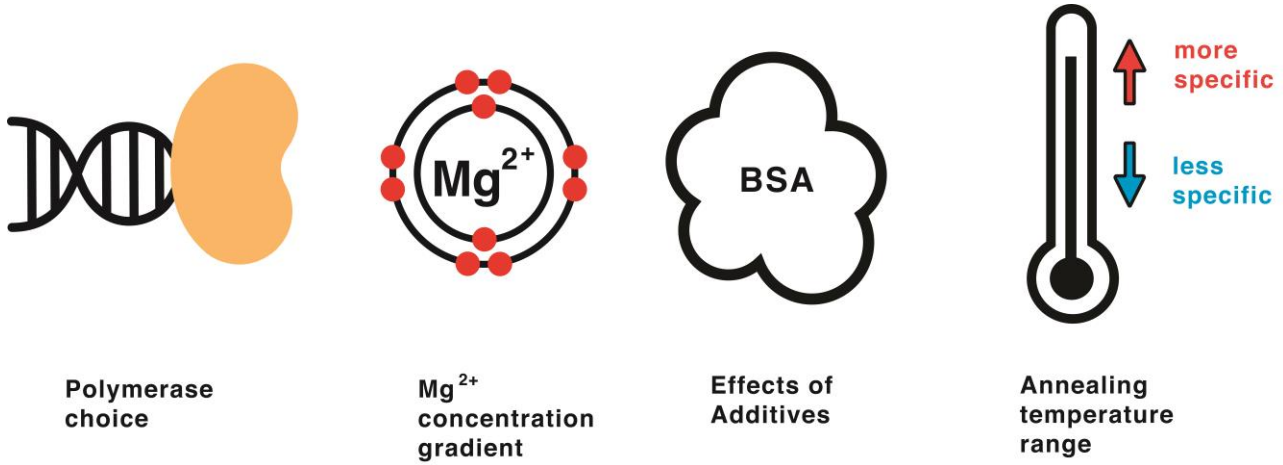


Figure credit: Anna-Sewell Killingstad

Hot-start PCR

- Modified DNA polymerase that is inactive until heat activated (e.g., AmpliTaq Gold)
  - Aptamer or antibody-bound
  - Modified lysine by heat/pH changes
  - PPi bound Mg^{2+} released by pyrophosphatase
- Reduces nonspecific amplification and mispriming
- Activated by an initial hold (10 min) long step at 95 °C
- Not used for amplification of long (>2 kb) templates sensitive to long holds at high temperatures

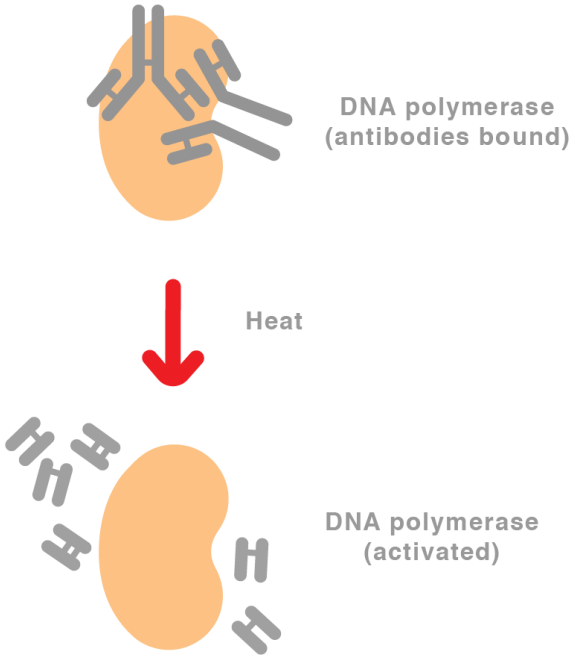
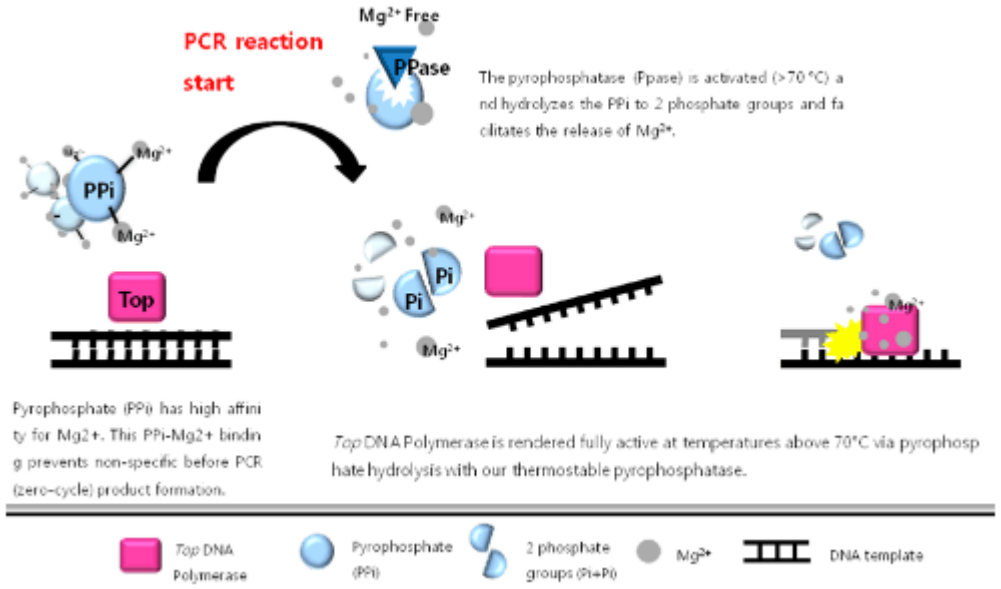


Figure credit: Anna-Sewell Killingstad

Śpibida M, Krawczyk B, Olszewski M, Kur J. Modified DNA polymerases for PCR troubleshooting. J Appl Genet. 2017 Feb;58(1):133-142. doi: 10.1007/s13353-016-0371-4.

Hot-start PCR Modifications and & Lysine and pH



https://us.bioneer.com/product/DNA_RNA_Amplification/DNA_Amplification/HotStart_PCR/AccuPower_amp_reg_PyroHotStart_TaqPCR_PreMix

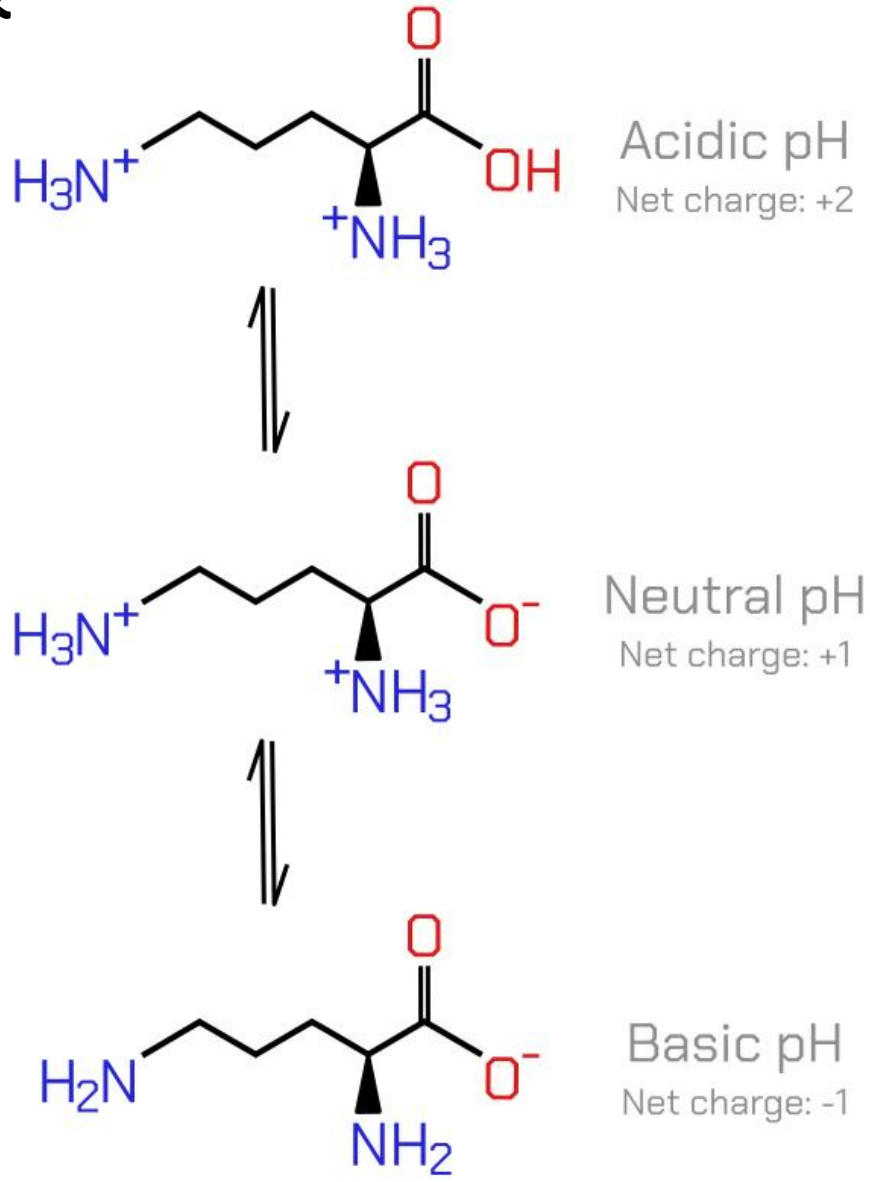
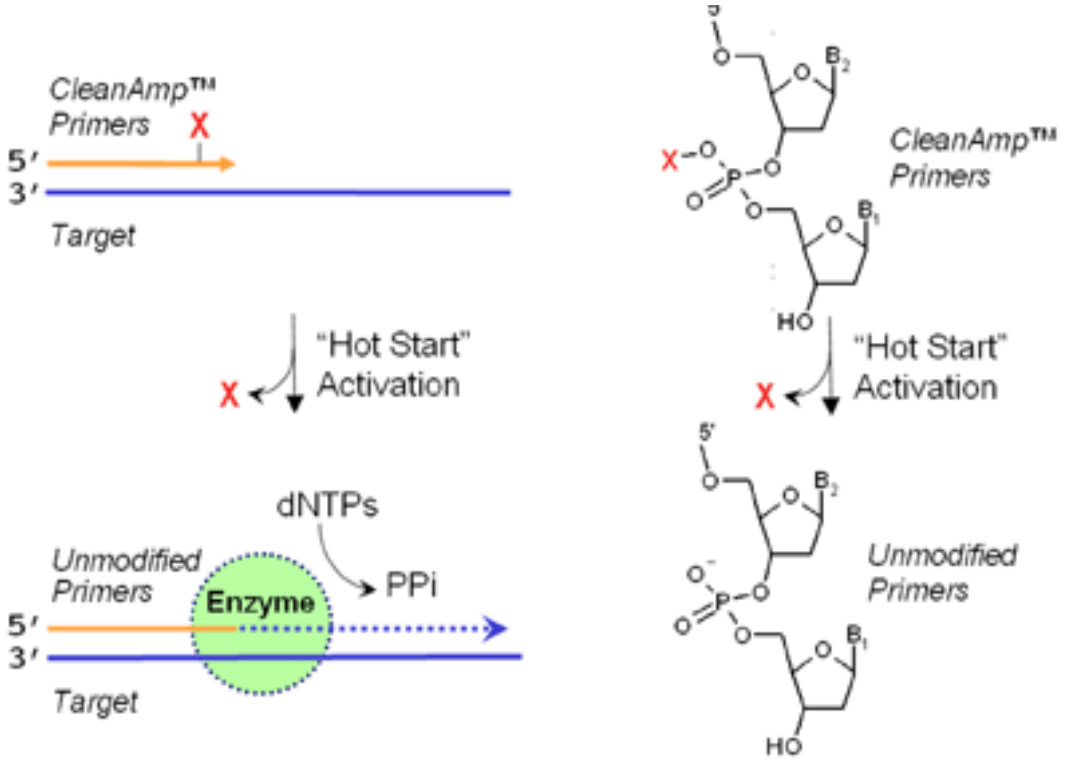


Figure credit: Nihal K. Chana

Hot-start PCR

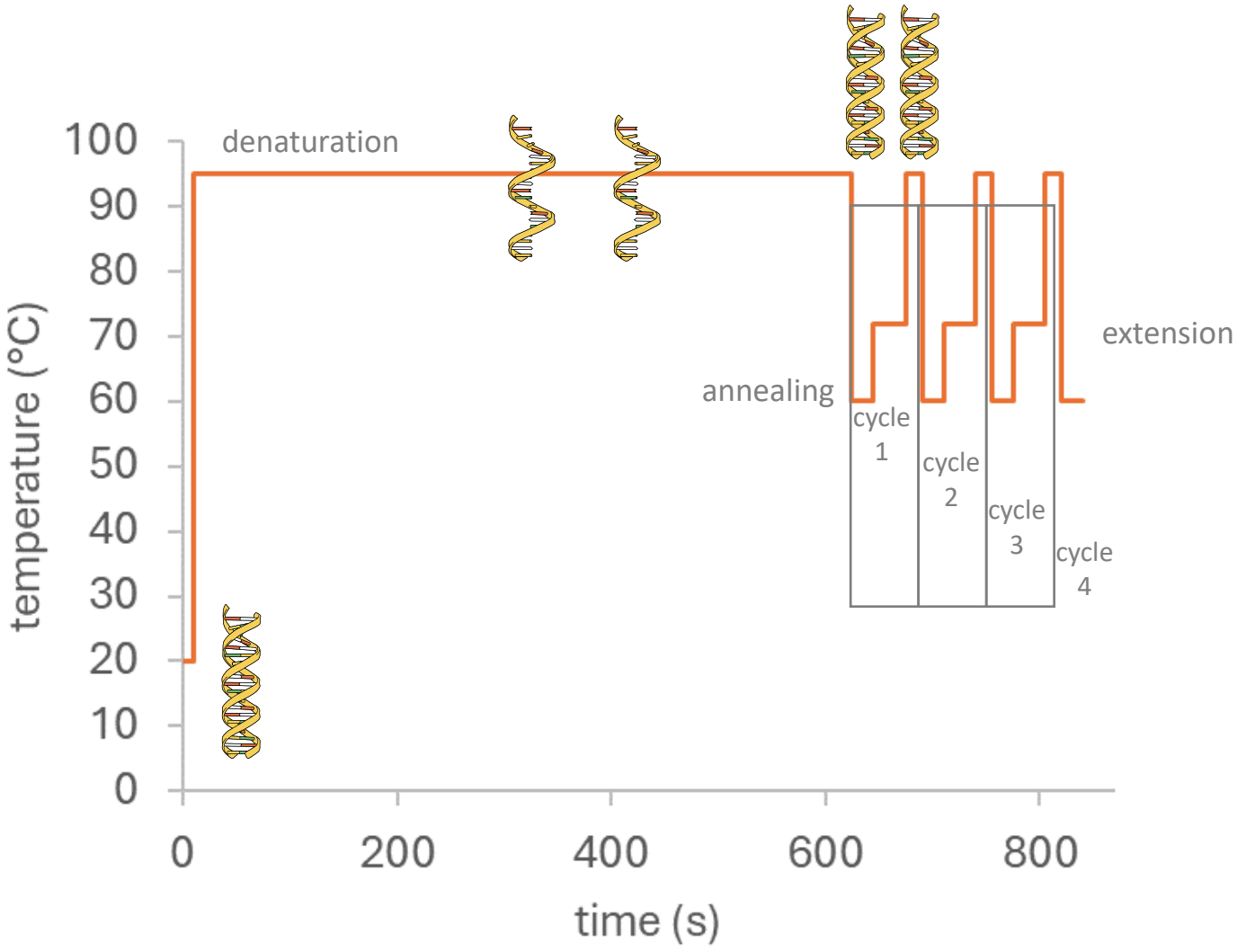
- Primers can also be modified and activated using hot-start



https://www.glenresearch.com/reports/gr21-11

Steps in Traditional PCR

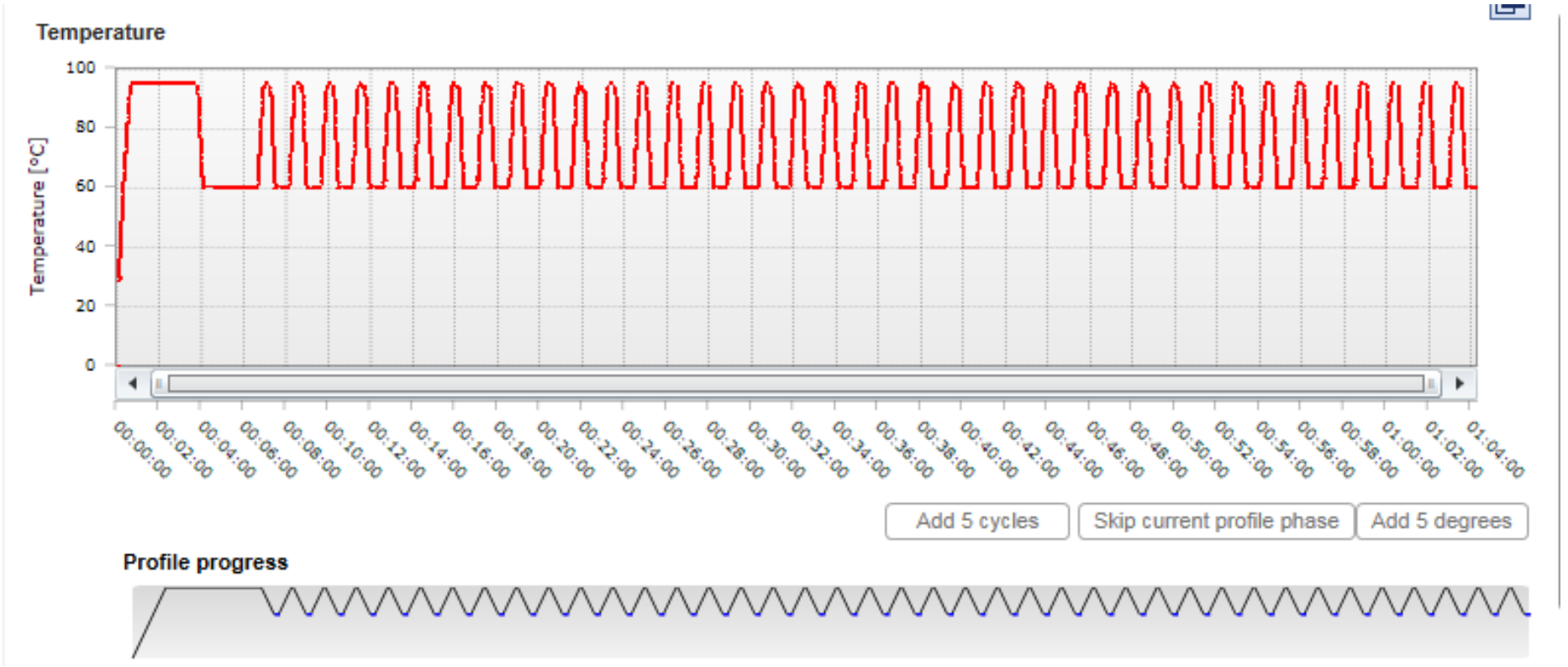
- **Denaturation** – break H-bonds connecting the strands
  - Typically 90-95 °C
- **Annealing** – binding of primer to target or template strand
  - Most frequently between 55-65 °C, can range from 45-72 °C
- **Extension** – DNA polymerase binds DNA template and primer and catalyzes new covalent bonds to the primer and dNTPs complementary to the target
  - 72 °C or annealing temperature



*In two-step PCR, the extension and annealing steps are combined. This is possible as while the DNA polymerase is most active at 72 °C, it has sufficient activity to catalyze the reaction at lower temperatures.

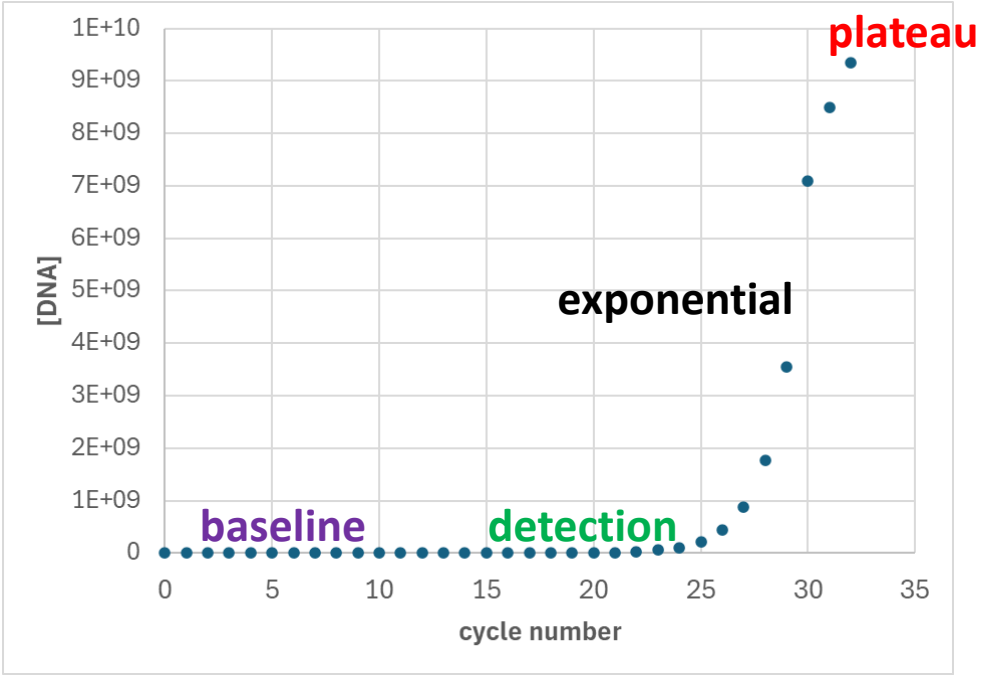
Note: In PCR, early errors will be exponentially amplified so touchdown may be employed. Touchdown assigns a higher annealing temperature (e.g., 65 °C in the first step and lowers by a value (e.g., 1 °C for a number of steps before continuing at the lower annealing temperature (e.g., 55 °C)

Quantitative PCR Cycling



How much DNA is produced in PCR?

- **Exponential amplification**
- **One double-stranded copy (6.6 pg) will be copied a *billion* times and yield $7.08x10^9$ pg or $7.08x10^6$ ng after 30 cycles, if it is copied every time.**
- **$P=(2^n)T-2n$**
- **P** = product concentration
- **T** = template concentration
- ***n*** = number of cycles
- Detection at fluorescence threshold (C_T)
  - The lower the cycle, the more DNA is present at start
- Continues until reaction components are exhausted and plateau is reached
- 10-fold increase in [DNA] each 3.32 cycles with 100% efficiency



What are some explanations for why the quantity of amplification product plateaus in PCR?

- All dNTPs are consumed or thermally degraded
- All primer molecules are consumed or thermally-degraded
- DNA polymerases are denatured or pH-inactivated (e.g., Lysine amino acids can be deactivated at low pH due to protonation of their amino groups)
- Amplified primer dimers or double stranded templates and copies bind the DNA polymerase and hinder amplification
- Not using a high-fidelity DNA polymerase that accurately amplifies the target

Jansson L, Hedman J. Challenging the proposed causes of the PCR plateau phase. Biomol Detect Quantif. 2019 Mar 2;17:100082. doi: 10.1016/j.bdq.2019.100082.

PCR Optimization

Poor amplification of target

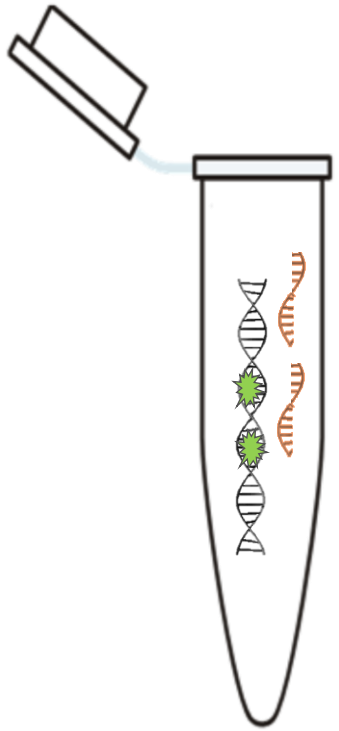
- Increase the input DNA concentration
- Increase primer concentration
- Annealing temperature touchdown protocol or decrease annealing temperature
- Increase dNTP concentration to amplify long fragments
- Add BSA to bind inhibitors or repurify
- Add KCl to promote primer annealing
- Increase final extension time

Low stringency

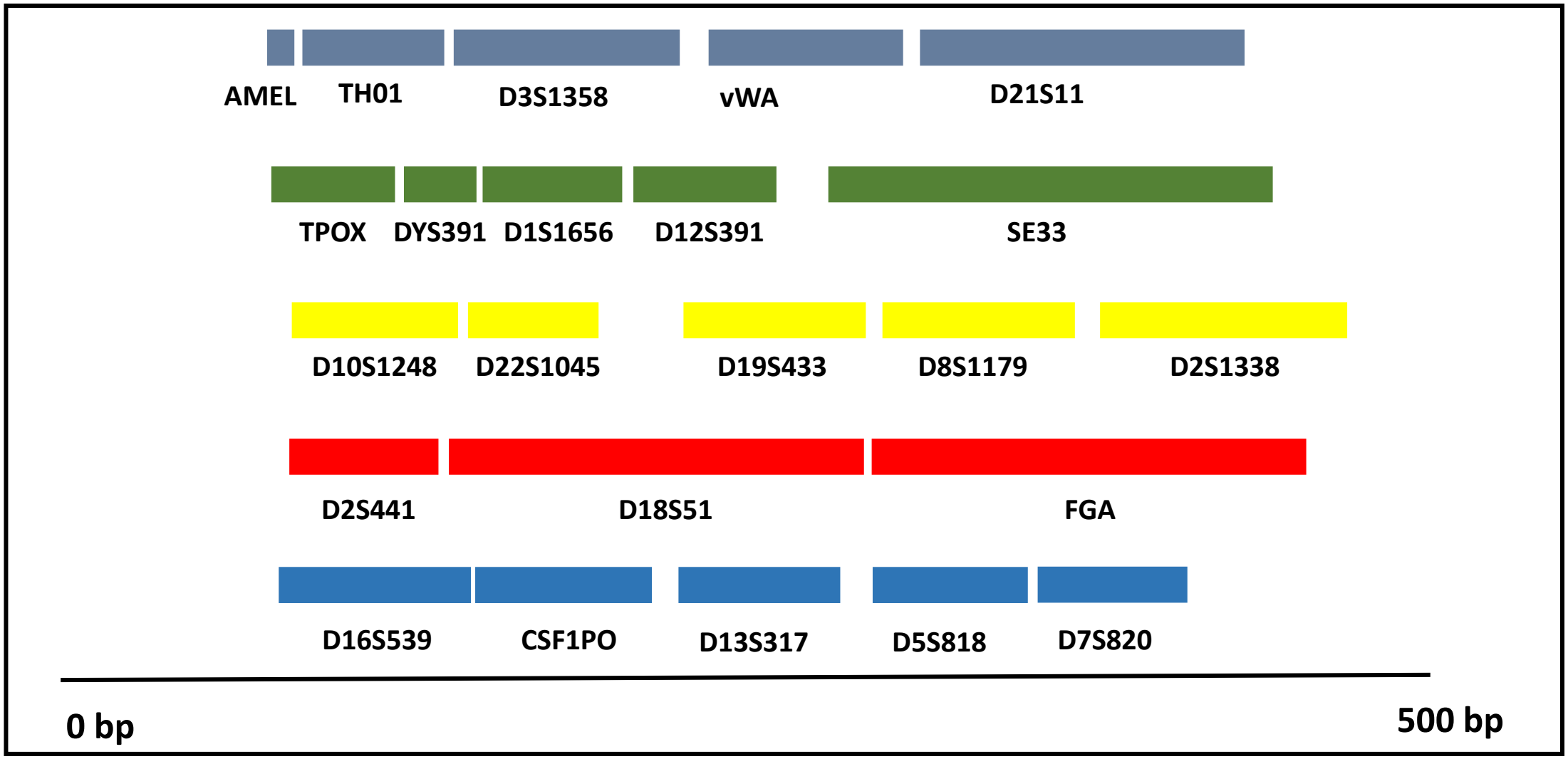
- Redesign the primers to have a longer or more tightly binding complement
- Increase the annealing temperature
- Demultiplex and test each primer set individually

DNA Typing is more efficient with multiplexes

- What is a singleplex?
  - A single set of PCR primers is used in a PCR reaction
- What is meant by multiplex?
  - 2 or more sets of PCR primers are used in PCR reaction
  - 24 loci in GlobalFiler, 24plex GO!, 24plex QS, PowerPlex® Fusion

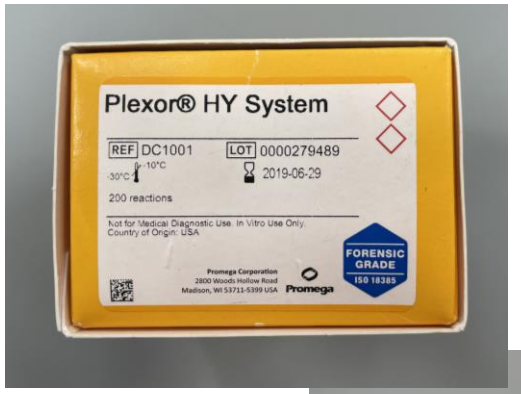


Investigator 24plex STR Loci: 5 dye labels and 24 sets of PCR primers



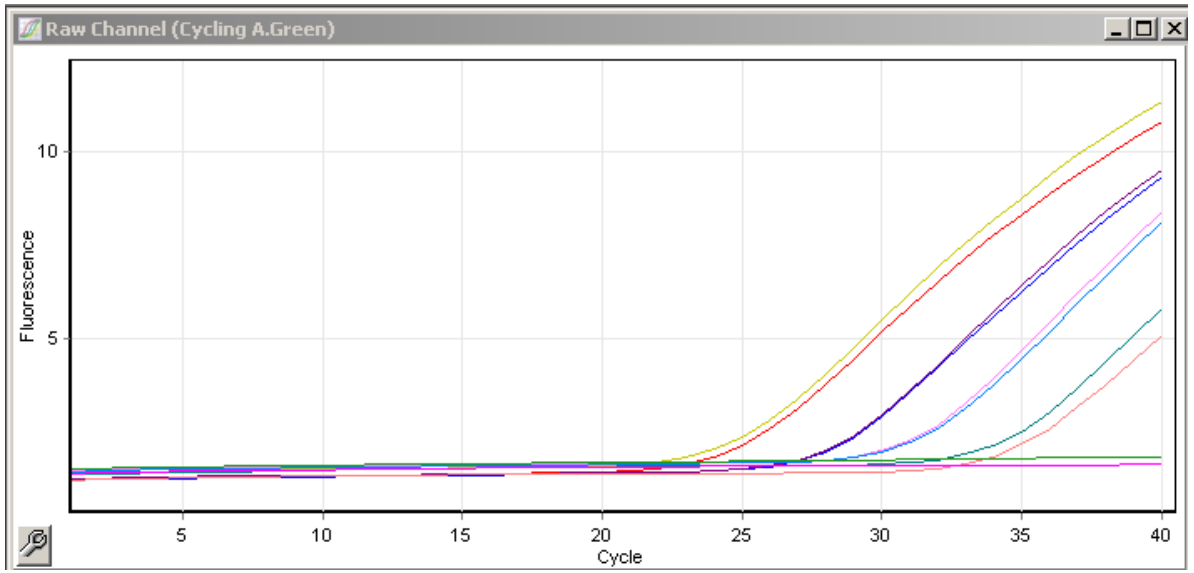
Quantitative PCR

- Perform analysis of input DNA quantity per volume *in situ* using fluorescence detection technology
- Amplify unknowns with standard DNA of known concentration
- Quantitation prior to STR typing is required by FBI Quality Assurance Standard (QAS) for evidence samples (Standard 9.4)
- Determine the quantity of evidence DNA to input quantity within kit range for STR typing
  - Kits work optimally within a narrow range of input DNA
  - Too much input DNA can lead to issues with pull up and detecting contamination
  - Too little input DNA can lead to stochastic effects
  - Often employ multiplexes



Human Quantitative PCR (qPCR) Kits

- Primers specific to human DNA
- Very sensitive method (nanogram to picogram)
- Multiplex can amplify multiple targets
  - Short and long male DNA targets
  - Short and long human autosomal DNA targets
  - IPC
- Can monitor fluorescence in real-time
- Can compute degradation index using the ratio of long to short amplicon amplification and detect inhibition



TaqMan qPCR Assays

- TaqMan minor groove binder and non-fluorescent quencher is at one end and fluorescent dye reporter at at the other end of the probe
  - Binds to complementary DNA sequence
  - Does not interfere with PCR amplification
  - Increase the melt temperature thus enabling shorter probes to be used for shorter amplicon targets
  - Fluorescence resonance energy transfer (FRET)

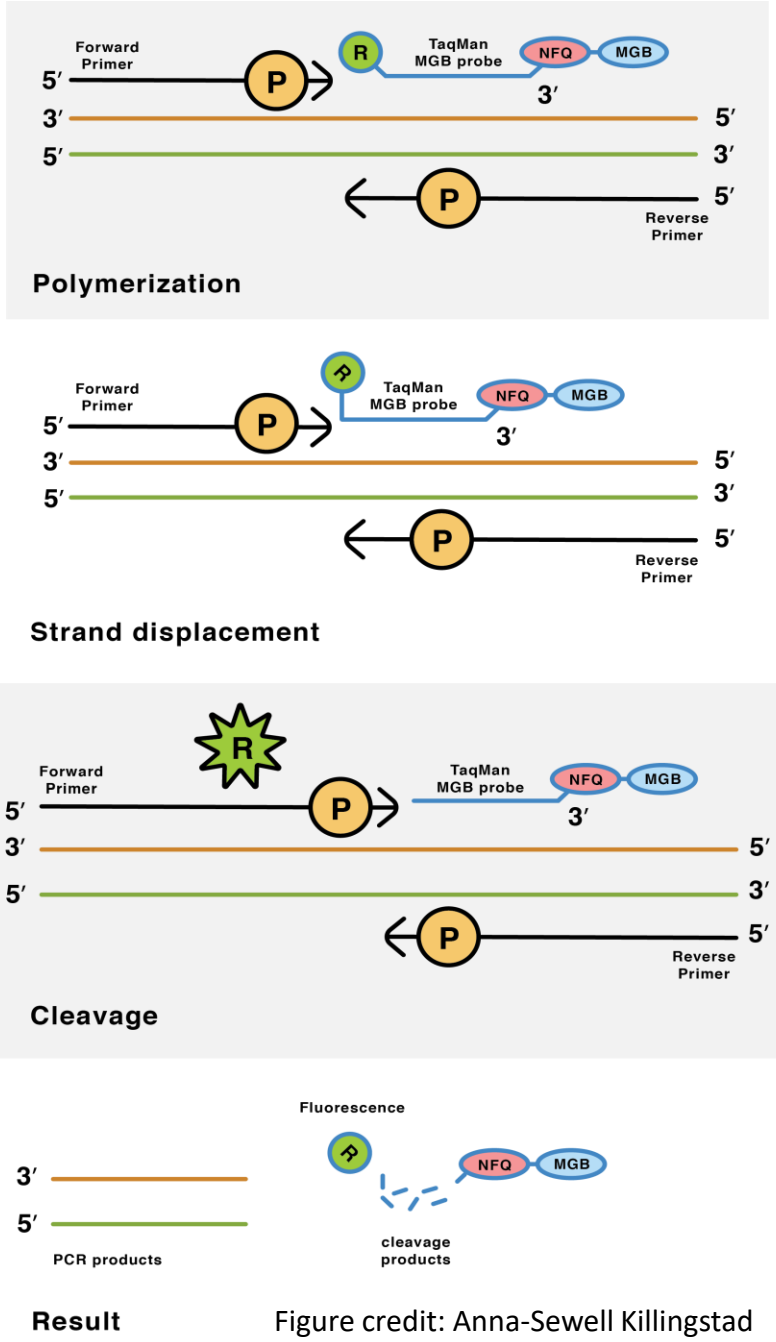


Figure credit: Anna-Sewell Killingstad

TaqMan qPCR Assays

- Principle of fluorescence resonance energy transfer (FRET)
  - Theory proposed by Thomas Förster in 1940s
    - Green fluorescent dye has a higher emission energy than red dye due to its shorter wavelength
    - If the red dye is in close proximity to the green dye, the energy emission of the green dye will transfer to the red dye due to spectral overlap
    - Energy is transferred from a higher energy level to a lower energy level "quenching" the green dye
  - The dyes must be in close proximity for the quenching, or *emission suppression* or *absorption by the emission acceptor*, to occur.
  - Taq DNA pol I will cleave the probe bound to the target in the middle of the amplicon.
  - Fluorescent signal is specific to the DNA target being copied.

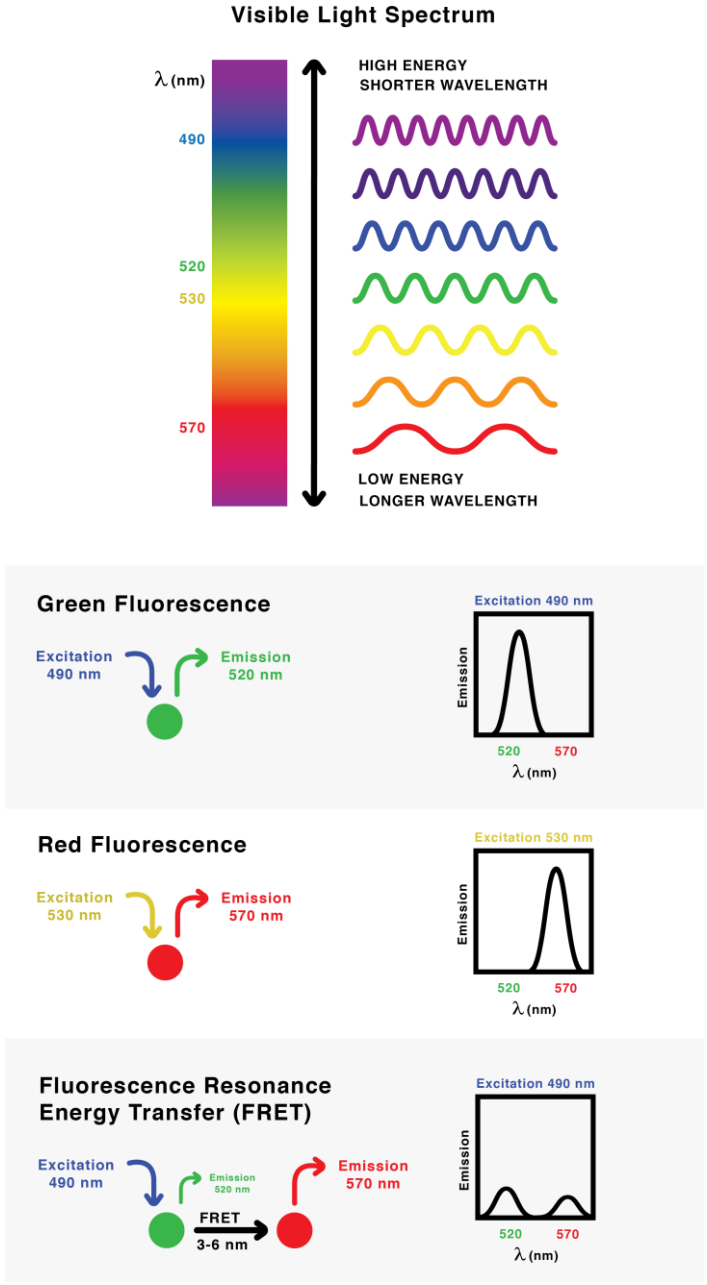
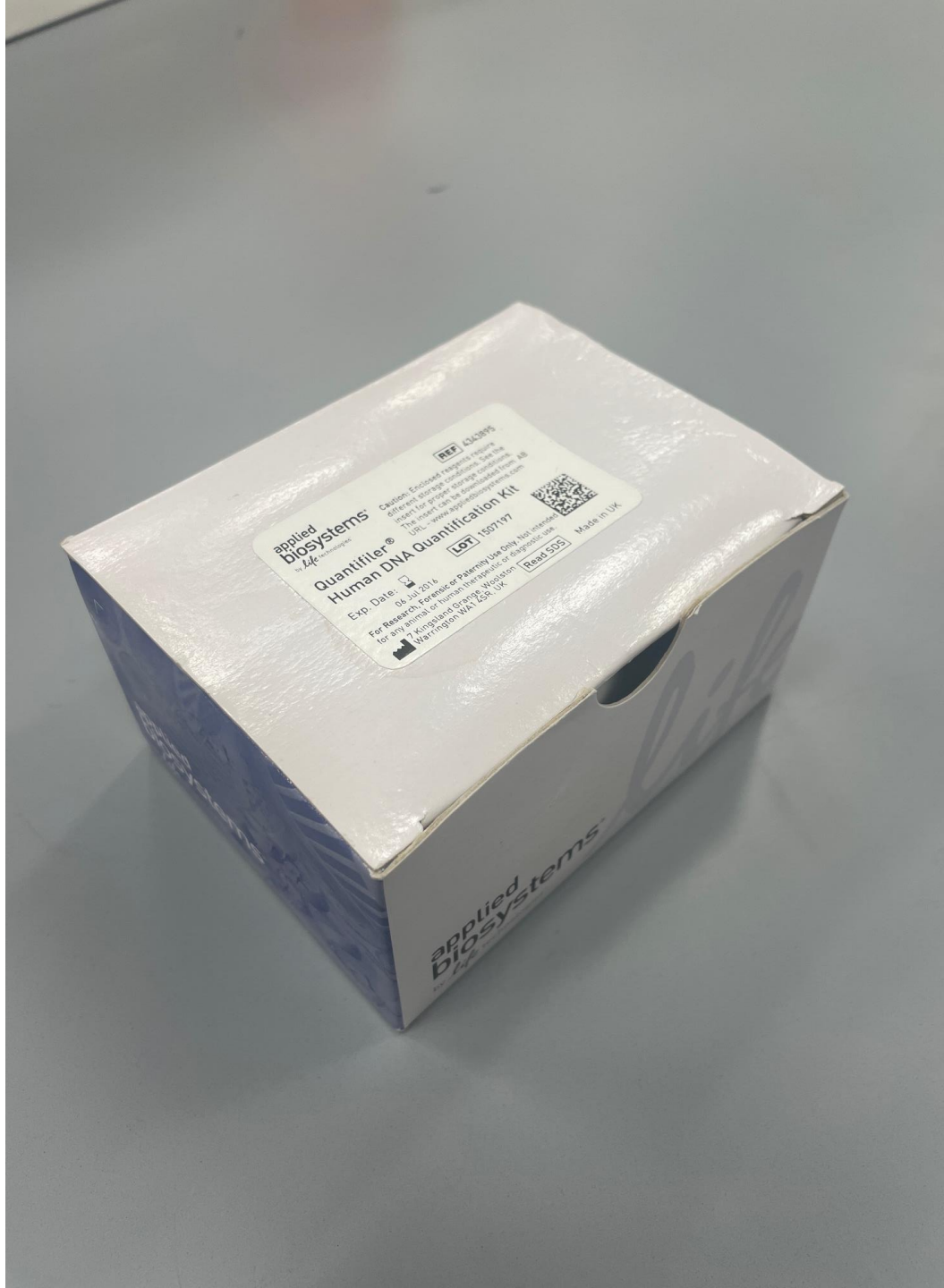


Figure credit: Anna-Sewell Killingstad

TaqMan qPCR Assays

- Quantifiler
  - 62 bp short autosomal target detected via FAM (blue) dye and MGB non-fluorescent quencher
  - Synthetic sequence used as IPC detected via VIC (green) dye and MGB quencher
  - Range: standard curve 50 ng/µL to 0.023 ng/µL
- Quantifiler Y
  - 64 bp short autosomal target detected via FAM (blue) dye and MGB non-fluorescent quencher
  - Synthetic sequence used as IPC detected via VIC (green) dye and MGB quencher
  - Range: standard curve 50 ng/µL to 0.023 ng/µL



TaqMan qPCR Assays

- Quantifiler Trio
  - 80 bp short autosomal target detected via VIC (green) dye and MGB non-fluorescent quencher
  - 214 bp long autosomal target detected via ABY (yellow) dye and QSY non-fluorescent quencher
  - 75 bp Y-chromosome target detect via FAM (blue) dye and MGB quencher
  - 130 bp synthetic sequence used as IPC detected via JUN (red) dye and QSR quencher
  - Range: standard curve 100 ng/µL to 0.005 ng/µL

TaqMan qPCR Assays

- Plexor HY
  - 99 bp short autosomal target detected via fluorescein (green) dye.
  - 133 bp Y-chromosome target detect via CAL Fluor® Orange 560 dye.
  - 150 bp IPC novel DNA sequence detected via CAL Fluor® Red 610 dye.
  - Range: standard curve 50 ng/µL to 0.0032 ng/µL

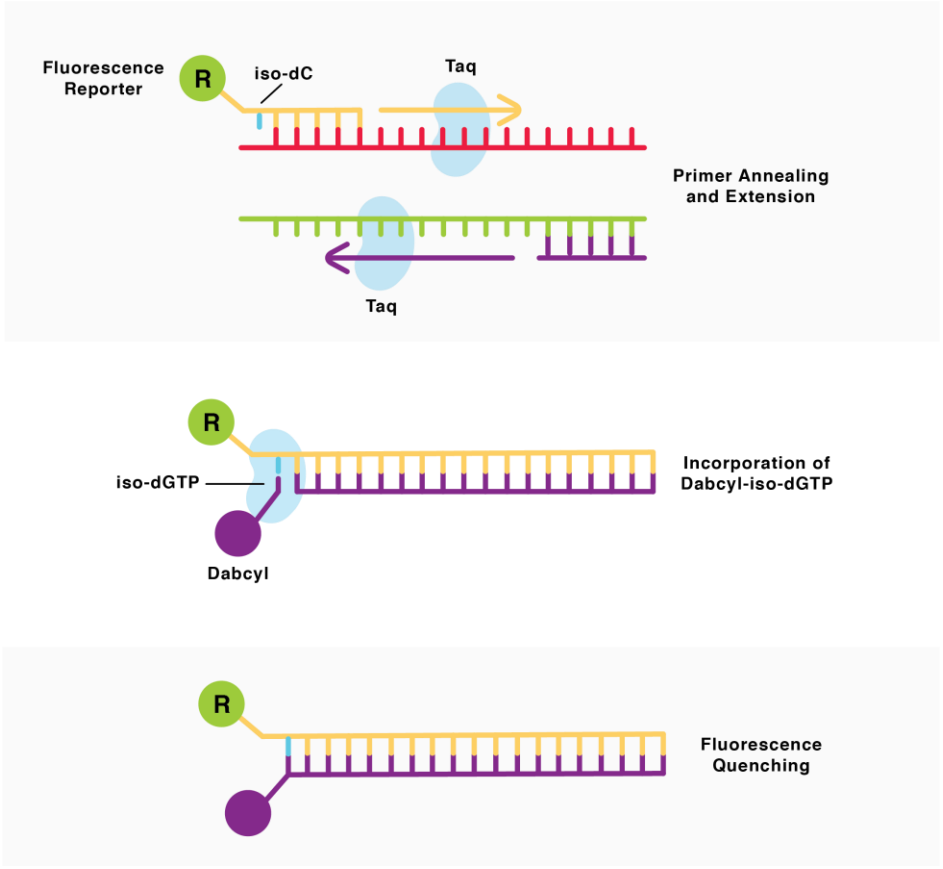


Figure credit: Anna-Sewell Killingstad

TaqMan qPCR Assays

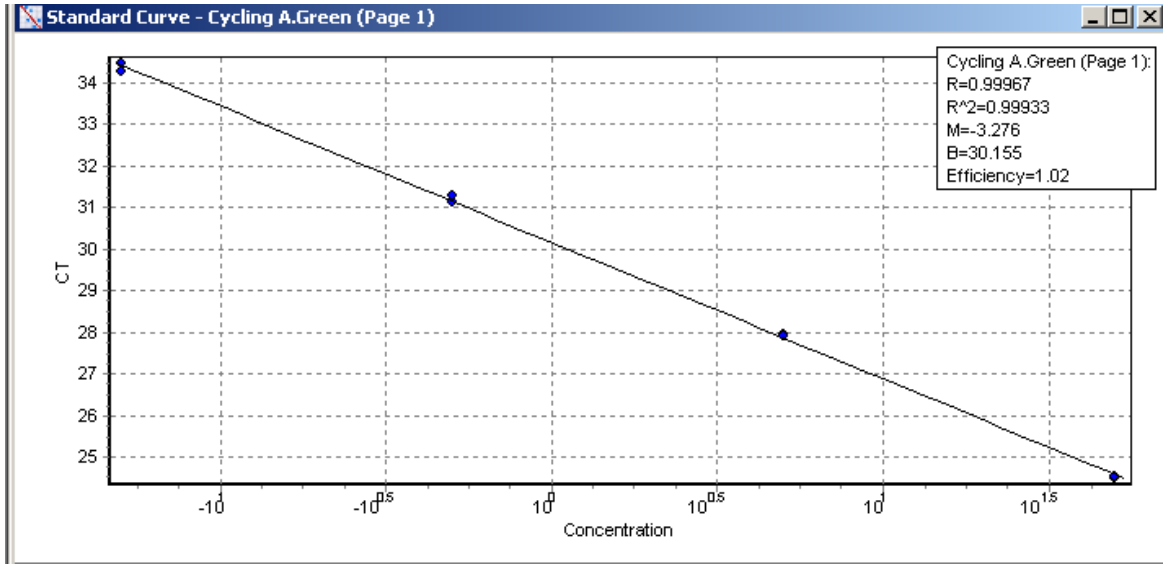
- Quantiplex Pro RGQ
  - 91 bp short autosomal target detected via **FAM™** (blue) dye.
  - 353 bp long autosomal target detected via **TAMRA™** (orange-red) dye.
  - 81 bp Y-chromosome target detect via **Cy®5** (far red) dye.
  - 434 bp IPC target detected via **JOE®** (green) dye.
  - Range: standard curve 200 ng/µL to 0.005 ng/µL

Dye Channel Selection Chart

Channel	Source	Detector	Dyes
Green	470nm	510nm	FAM, SYBR Green 1, Fluorescein, EvaGreen, Alexa Fluor 488
Yellow	530nm	555nm	JOE, VIC, HEX, TET, CAL Fluor Gold 540, Yakima Yellow
Orange	585nm	610nm	ROX, CAL Fluor Red 610, Cy3.5, Texas Red, Alexa Fluor 568
Red	625nm	660nm	Cy5, Quasar 670, Alexa Fluor 633
Crimson	680nm	710hp	Quasar705, Alexa Fluor 680
HRM	460nm	510nm	SYTO 9, EvaGreen

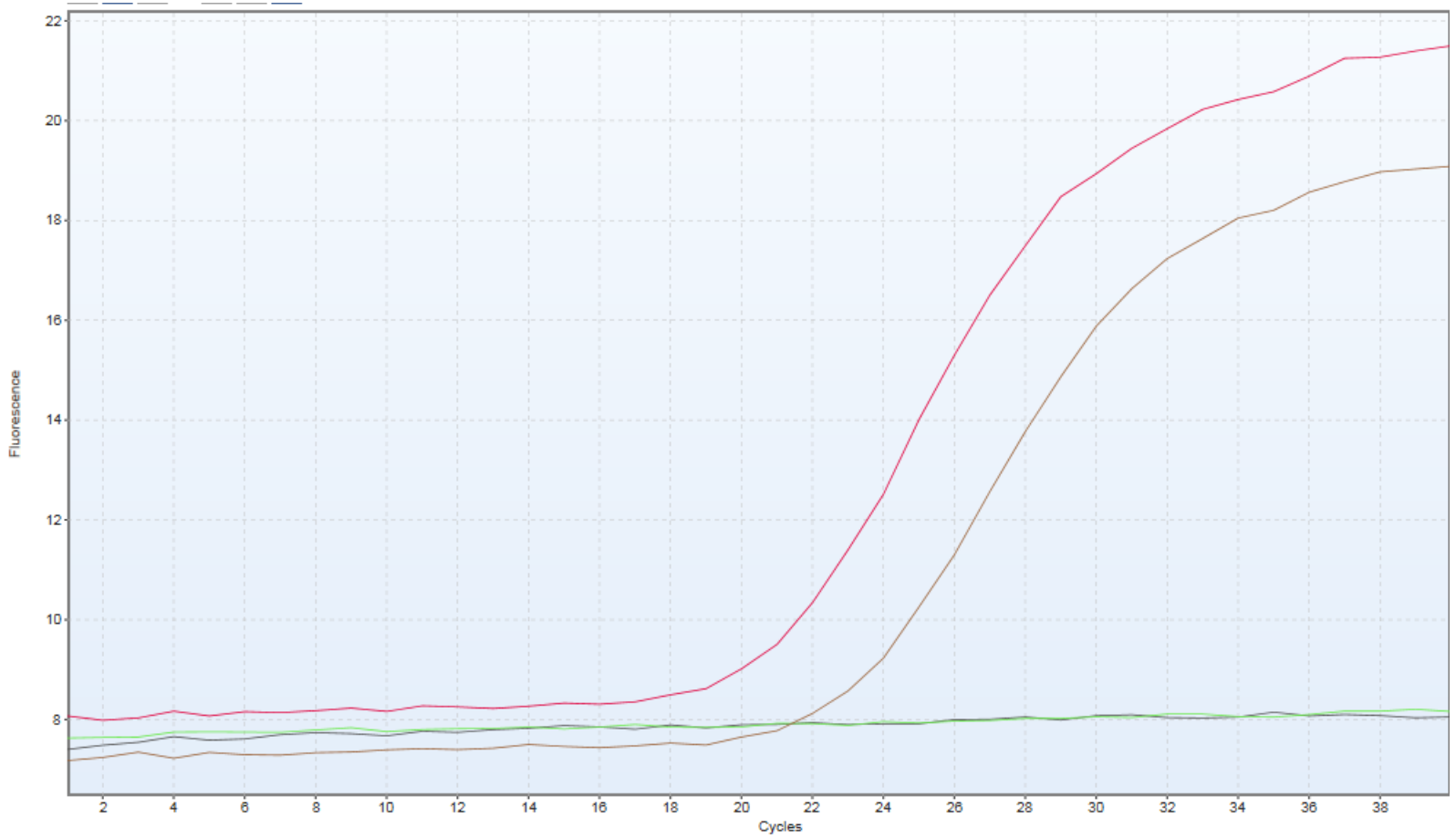
TaqMan qPCR Standard Curve

- Plot of DNA concentration vs. cycle threshold (C_T)
- R^2 is a measure of how well the data fits the standard curve
  - Perfect fit: $R^2 = 1$
- The slope measures the reaction efficiency
  - -3.32 is 100% efficient meaning that all of the DNA strands were amplified (doubled) each cycle during the PCR
- The slope and y-intercept (b) are used to compute the concentrations of the unknowns: absolute quantification
  - Passing R^2: ≥ 98%
  - Passing slope: -3.0 to –3.6
  - Long amplicon: b = 24.3 - 29.5 (Quant Trio)
  - Short amplicon and Y target: b= 24.5 - 29.5 (Quant Trio)



Quantiplex Pro RGQ qPCR

Some Samples Fail to Amplify



Did not amplify

Creating More Specific Primers

- Aim for a higher annealing temperature
- Design a longer primer
- Avoid primer dimers (primers that bind each other at a stretch of > 4 bp and are likely extended by the DNA polymerase)
- Blast the target genome and select a single copy primer binding location

Primer Feature	Optimal Values
Length	18-30 bases
Annealing Temperature	55-65 °C
GC-content	40-60%
Hairpin melt temperature	<40 °C
Primer dimer	<5 consecutive bases

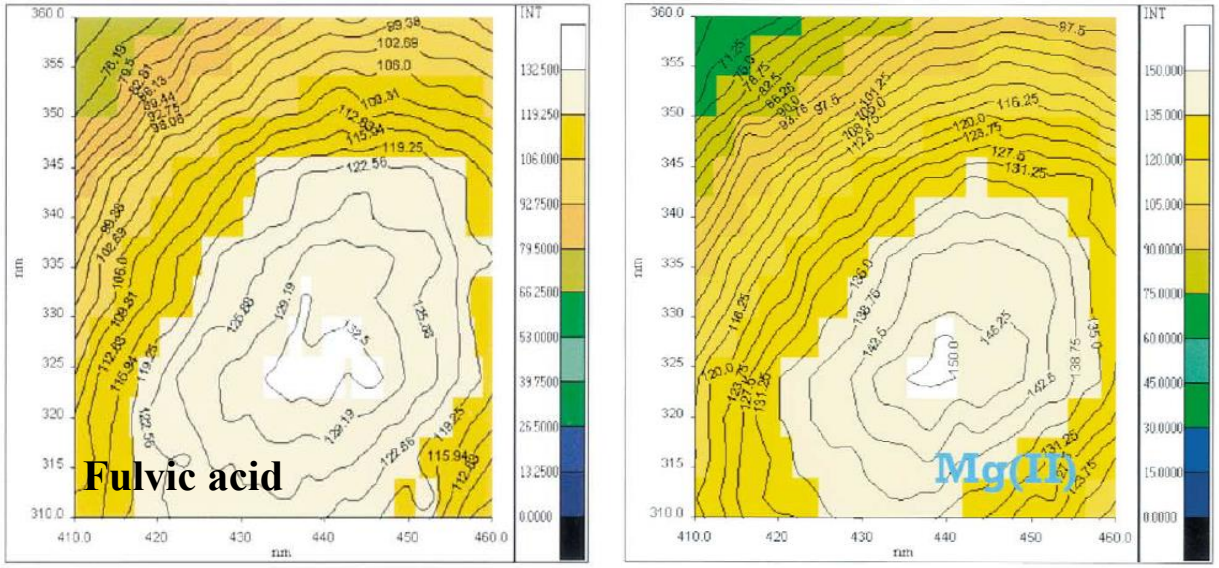
Limitations of the Technology

- Limited to short amplicons (<500 bp)
- DNA polymerase mutation errors
- Nonspecific primer annealing
- Incomplete amplification products
- Primer dimers
- Sensitive to contamination (especially with more cycles)

ATAGATAGATAGATAGATAGATAGATAGATAGATAG
ATAGATAGATAGATAGATAGATAGATAGATAGATA
ATAGATAGATAGATAGATAGATAGATAGATAGAT
ATAGATAGATAGATAGATAGATAGATAGATAGA
ATAGATAGATAGATAGATAGATAGATAGATAG
ATAGATAGATAGATAGATAGATAGATAGATA
ATAGATAGATAGATAGATAGATAGATAGAT
ATAGATAGATAGATAGATAGATAGATAGA
ATAGATAGATAGATAGATAGATAGATAG
ATAGATAGATAGATAGATAGATAGATA

PCR inhibitors are often present in evidence

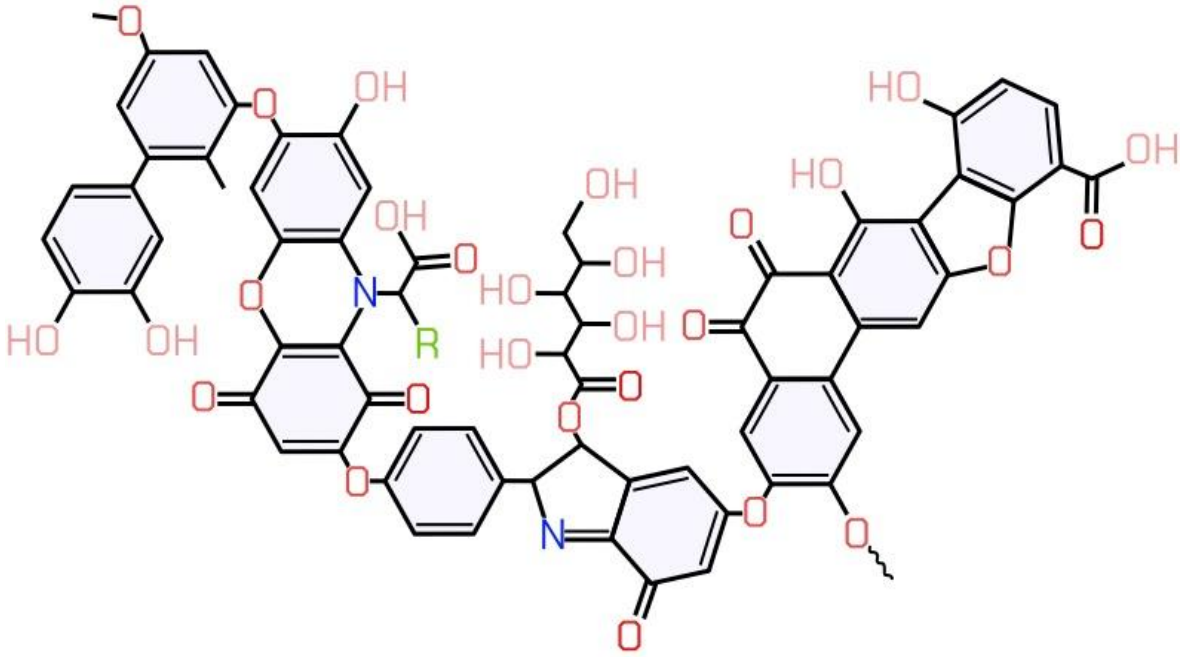
- Inhibitors prevent PCR amplification, often by binding the Mg^{2+} cofactor, but also by digesting DNA polymerase
  - Humic acids and natural organic matter (NOM) in soil
    - Fluorescence experiments demonstrate evidence of Mg^{2+} binding to acidic NOM
  - Heme in hemoglobin and myoglobin
  - Indigo dye in blue jeans
  - Proteases in soil
  - Melanin in hair and skin
  - Tannins in leather



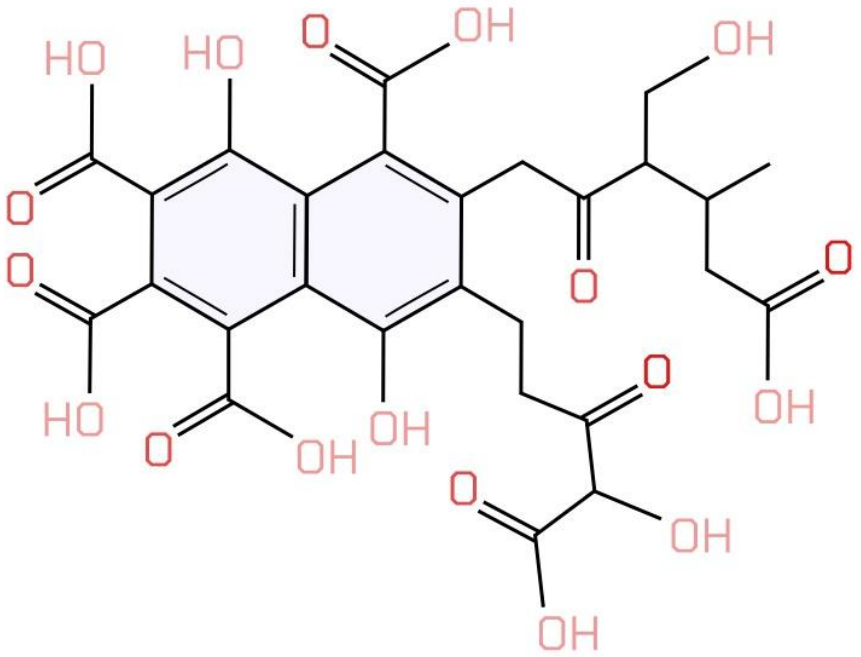
Elkins, K.M. and D.J. Nelson. 2002. Spectroscopic approaches to the study of the interaction of aluminum with humic substances, Coordination Chemistry Reviews, 228(2): 205-225

Structures of PCR Inhibitors: NOM Mg^{2+} Chelators

Humic Acid



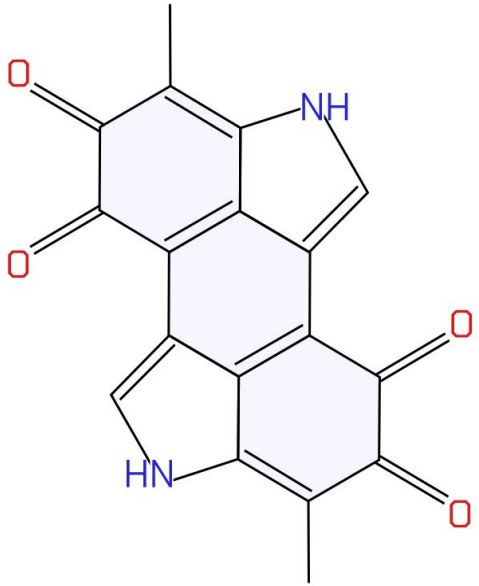
Fulvic acid



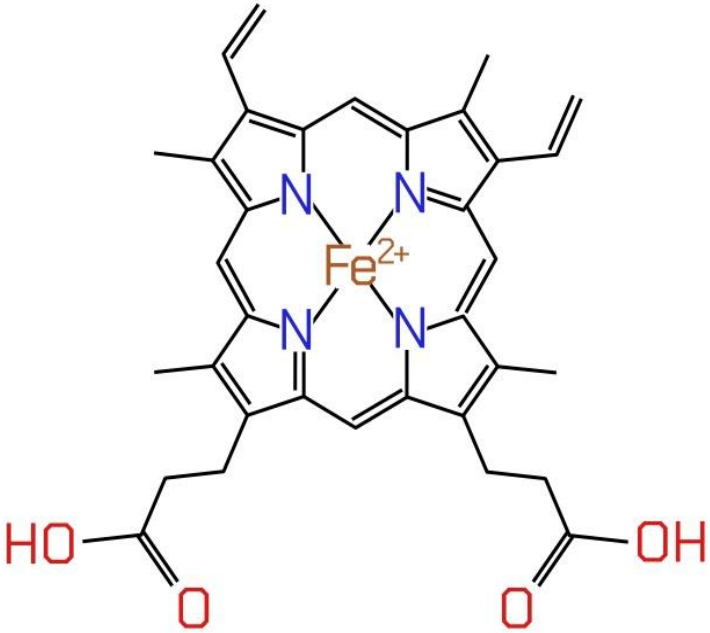
Structures credit: Nihal K. Chana

Structures of PCR Inhibitors: Mg^{2+} Chelators

Melanin



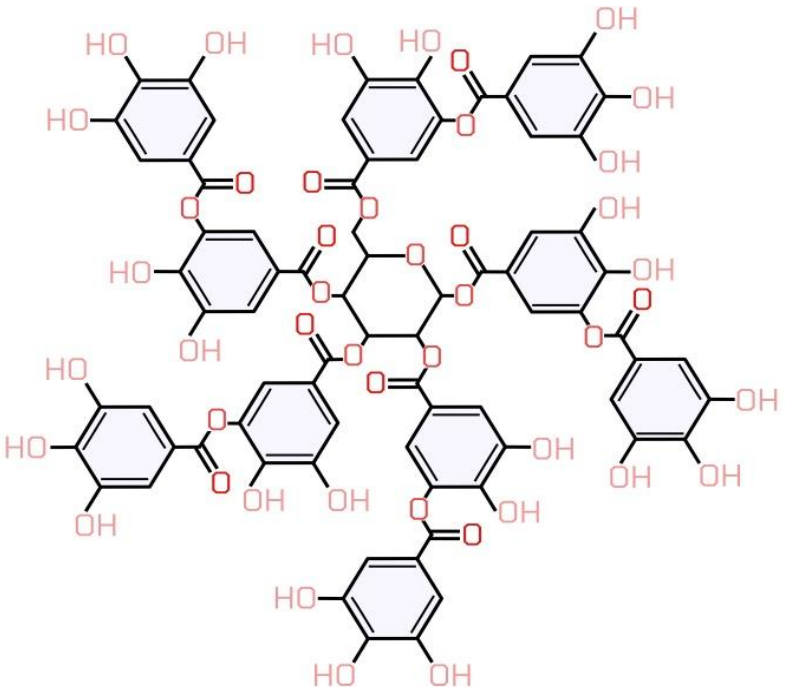
Heme bound to iron



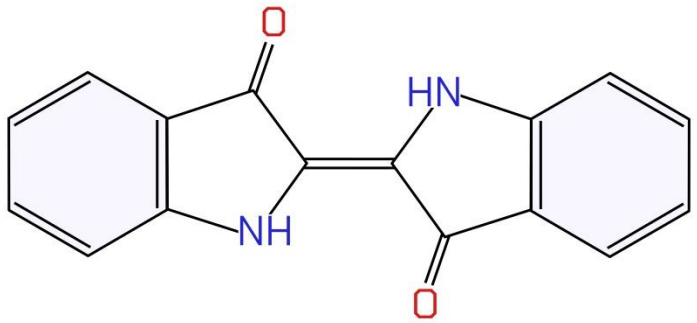
Structures credit: Nihal K. Chana

Structures of PCR Inhibitors: Mg^{2+} Chelators

Tannic Acid



Indigo

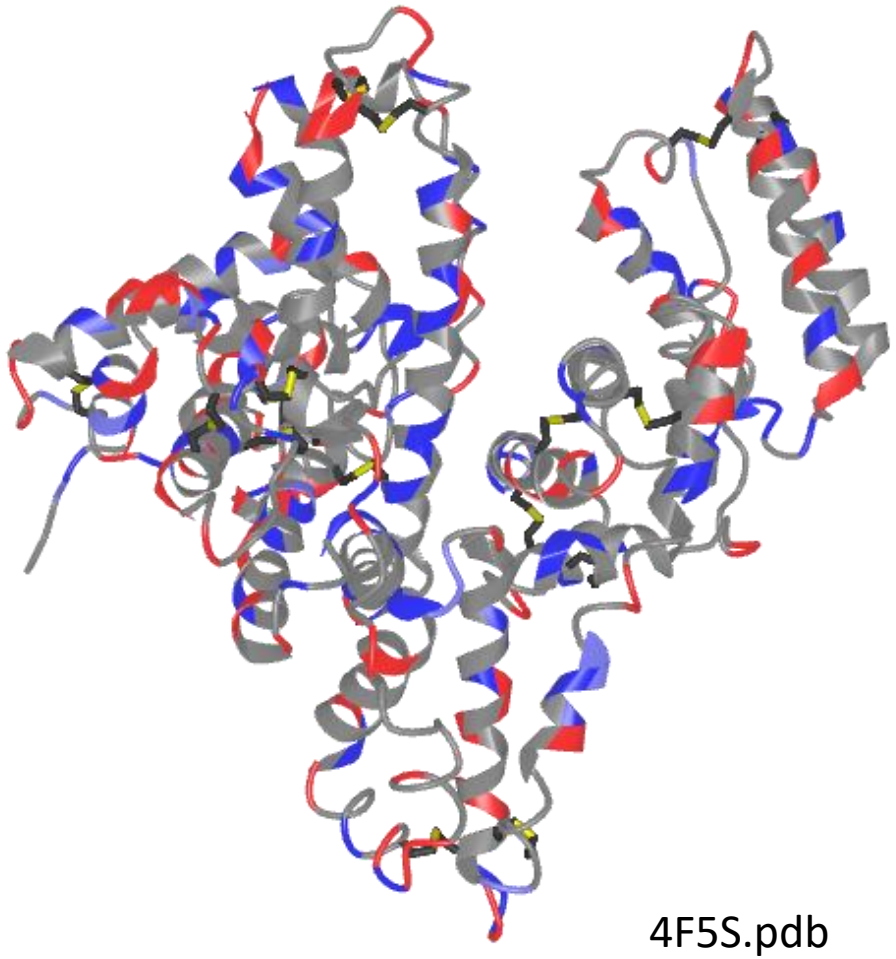


Structures credit: Nihal K. Chana

Solutions to PCR Inhibition

- Dilute genomic DNA template, which will in turn dilute inhibitors, then reamplify
- Add more DNA polymerase to compensate for PCR inhibitors present
- Add bovine serum albumin (BSA) which binds the inhibitors in the sample thus mitigating inhibition
- Treat with NaOH which will neutralize inhibitors of *Taq* DNA polymerase
- Perform additional purification/washes during DNA extraction

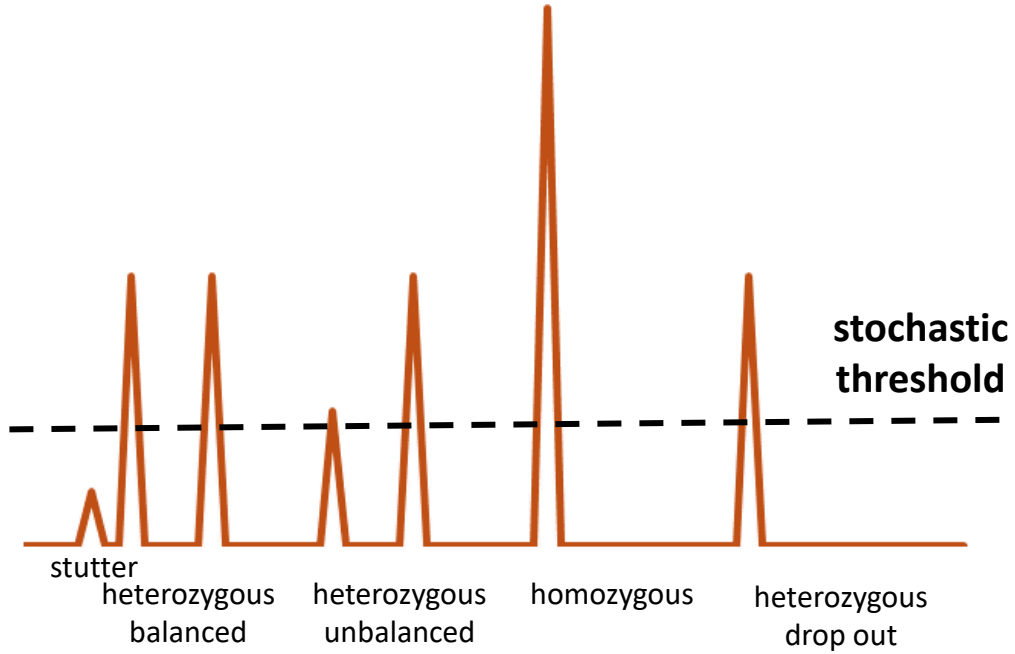
BSA



4F5S.pdb
Colored by charge

Stochastic Effects

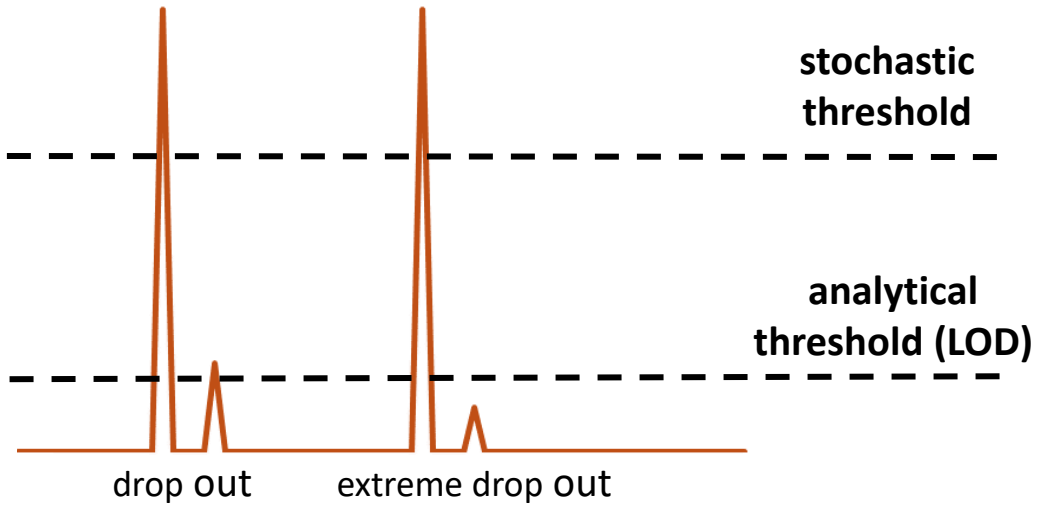
- Another term for *stochastic* is random.
- For chemical reactions to occur, the components must come together.
  - In a tube with all of the PCR reagents and many copies of the full DNA genome, there are many chances for the DNA template of interest to bond with its primer and for the complex to become bound by DNA polymerase and for it to find the dNTPs it needs to grow the chain, and so on.
  - However, in the case of only one or a few copies of the DNA genome, there will be limited chances for the primer to find the template.
  - With each PCR cycle and new copy produced, the number of incorrect binding molecules increases so the chance decreases.
- This can lead to some targets not being extended or insufficiently extended so they are not detected. This is called *allelic dropout*.
- Preferential amplification is a phenomenon when some targets are copied well while others are not. The observed result is *peak imbalance*.
- Once bound, sometimes the template strand slips in the DNA polymerase when copying repeat regions causing a repeat (or more) not to be amplified (*stutter*).



Haarkötter, C., Saiz, M., Alvarez-Cubero, M., Álvarez, J.C., Lorente, J. (2021). Challenges in the DNA Analysis of Compromised Samples. In: Dash, H.R., Shrivastava, P., Lorente, J.A. (eds) Handbook of DNA Profiling. Springer, Singapore. https://doi.org/10.1007/978-981-15-9364-2_51-1

Issues that Lead to Poor Amplification

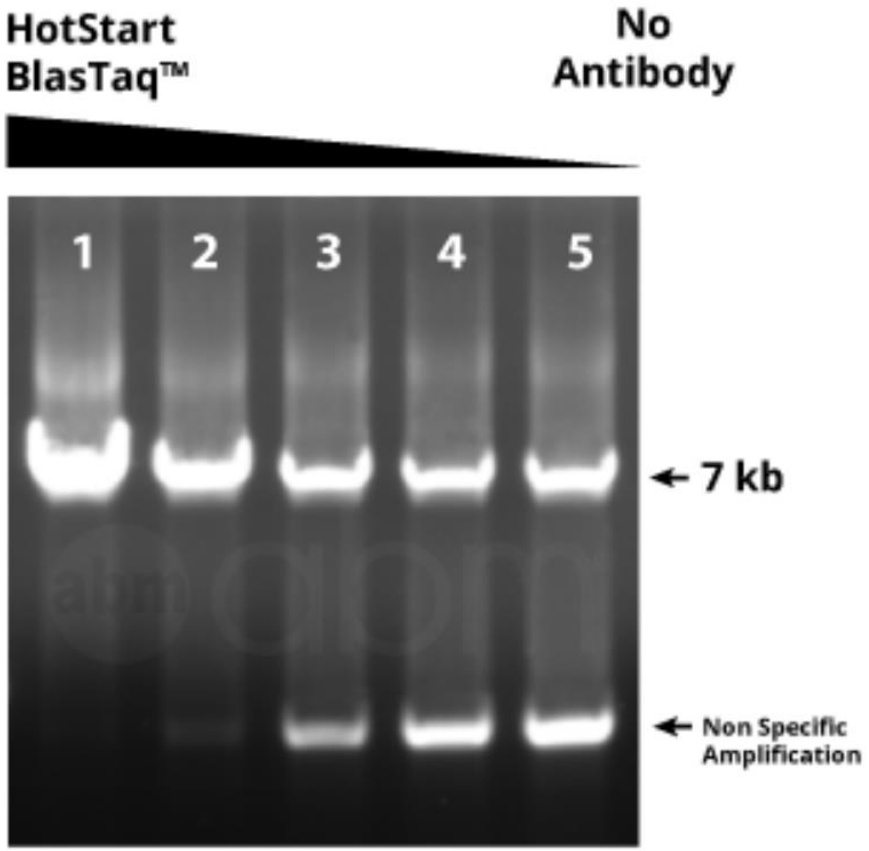
- Inputting low quantity or low template DNA may lead to stochastic effects such as allele dropout, extreme drop out or undetectable amplification



Gill P, Puch-Solis R, Curran J. The low-template-DNA (stochastic) threshold--its determination relative to risk analysis for national DNA databases. Forensic Sci Int Genet. 2009 Mar;3(2):104-11. doi: 10.1016/j.fsigen.2008.11.009.

Issues that Lead to Poor Amplification

- Preferential amplification of primer dimers, as opposed to target DNA
- Non-specific amplification – the primer binds weakly at a location other than the desired location on the template and is extended at that location by DNA polymerase



https://www.abmgood.com/assets/images/catalogPage/pcr/pcr-polymerase-hotstart-data-1.png

Issues that Lead to Poor Amplification

- DNA crosslinks including thymine dimers due to UV exposure

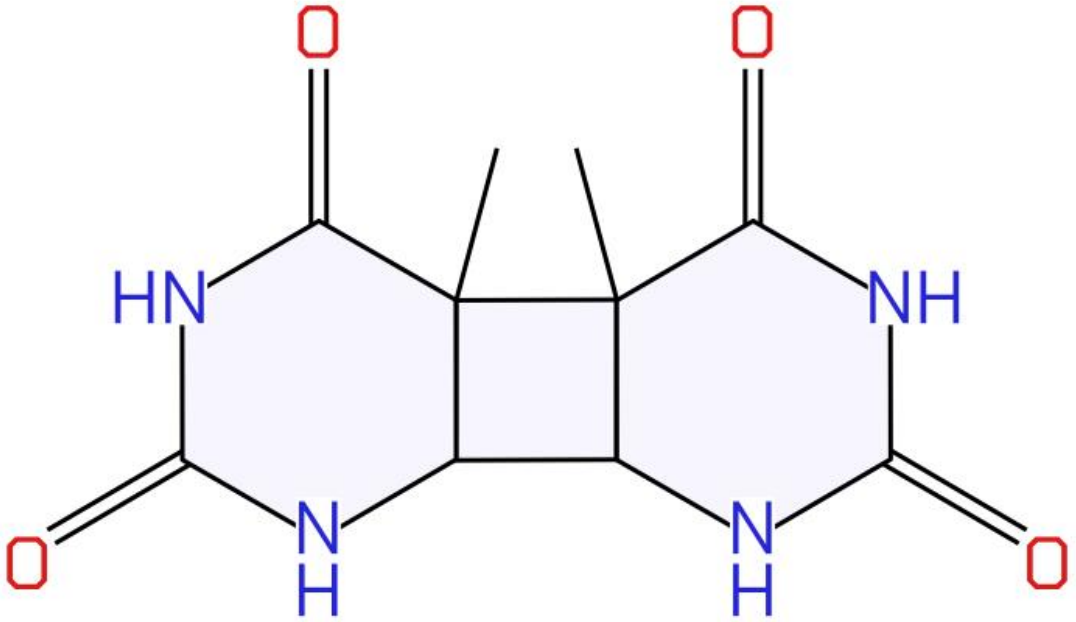


Figure credit: Nihal K. Chana

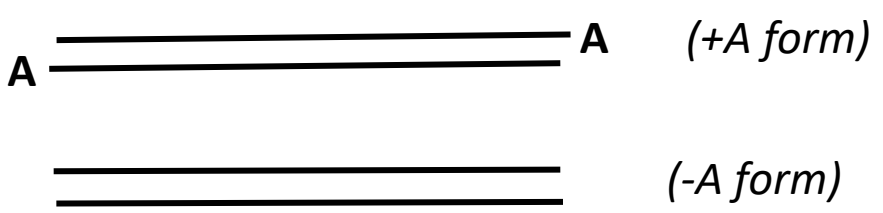
Amplification Artifacts and Solutions

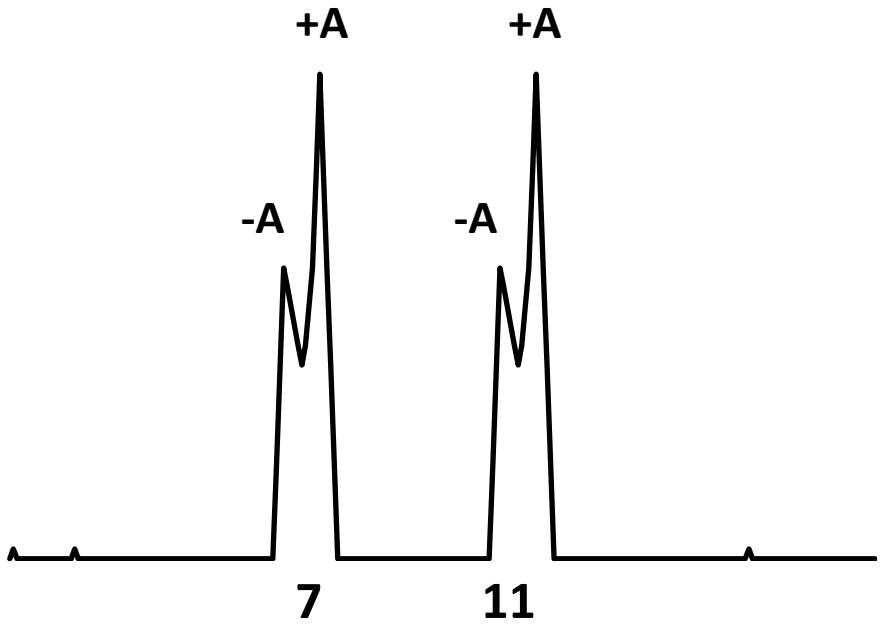
- Primer dimers – decrease length of self-complementary stretches
- Heteroduplexes – decrease number of PCR cycles
- Stochastic effects to due amplification bias – input more DNA
- Chimeras – rerun to verify
- Accuracy – check specificity in Blast
- Deletion – avoid homostretches of a base
- Insertion - avoid homostretches of a base
- Base substitution – decrease dNTP concentration
- Poly A addition – reduced if primer ends with A
- Stutter- reducing the template concentration can reduce stutter

Figure credit: Nihal K. Chana

Inducing Poly-A addition

- Allow sufficient time to extend strand
- Create primer ending in A
  - One base longer
- PIGtailing
  - Beginning reverse primers with GTTTCTT on the 5'-end

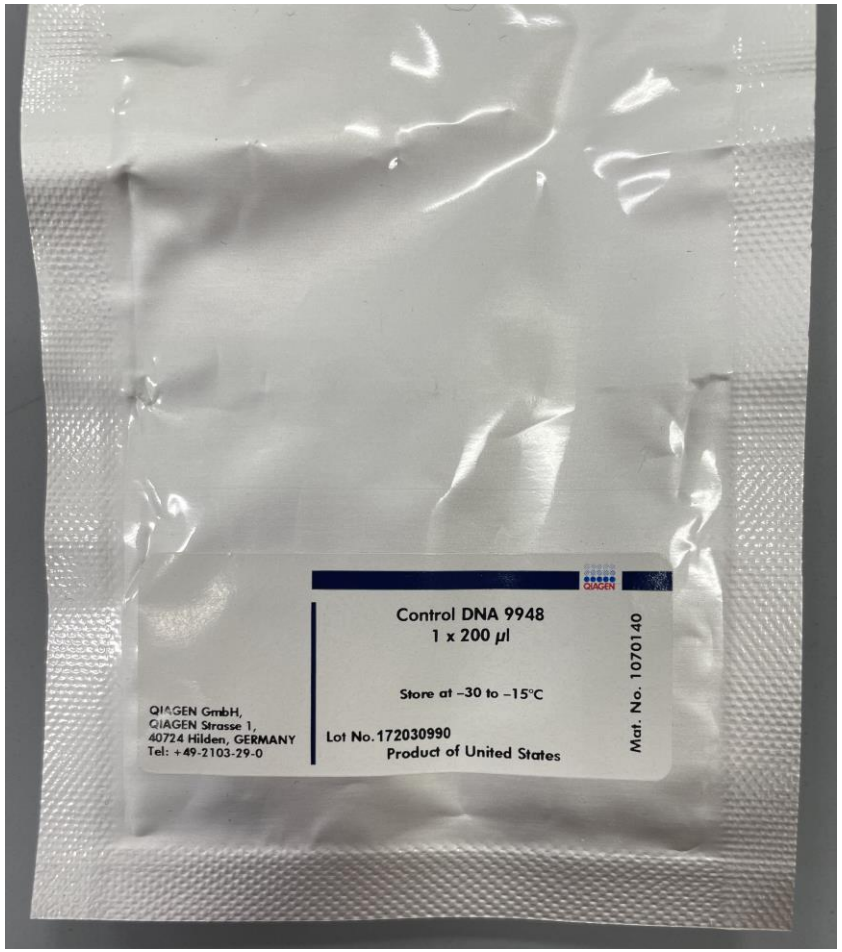




Brownstein MJ, Carpten JD, Smith JR. Modulation of non-templated nucleotide addition by Taq DNA polymerase: primer modifications that facilitate genotyping. Biotechniques. 1996 Jun;20(6):1004-6, 1008-10. doi: 10.2144/96206st01.

Quality Control

- PCR is very sensitive
  - Trace and touch DNA can serve as templates for amplification and compete with evidence target template
  - Quality control samples
    - Positive control such as a known standard (e.g., K562, 2800M, NIST SRM 2391d, etc.)
    - Negative controls such as a no template control and substrate/reagent blank control
    - Elimination samples for comparison such as analyst and CSI DNA samples



Contamination and Quality Control

- Collect elimination samples from CSI, lab personnel and visitors
- Clean surfaces regularly
- Wear PPE including lab coat, hair covering, gloves, mask
- Follow clean technique
- Positive ventilation, as indicated
- Samples should proceed in time and space from evidence to sampling, extraction, amplification and analysis but not back again
- Store post-amplification samples separately from pre-amplification

Photo credit: Cassie Skrant

Study Questions

- Define PCR. What is the purpose of PCR? What are the advantages of PCR? What are the limitations of PCR?
- Explain the functions and reactions of DNA polymerases.
- List and explain the functions of the PCR reagents.
- Explain what nucleases are and why it is important to remove them during extraction.
- List examples of PCR inhibitors.
- Explain how the probe works in a TaqMan assay.
- Explain how multiplexing is employed in qPCR assays.
- Explain the meaning of R^2, slope, y-intercept, IPC, inhibition index, and degradation index in qPCR assays. What are "passing" values?
- What is a standard curve? How is it generated? How do we use it to determine quantitation values of unknown samples?
- What does it mean when a value for IPC is not obtained for a sample?

Suggested Readings

- Afonina, I., et al. Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder. Nucleic Acids Res. 1997 July 1; 25(13): 2657-60
- Timken, M.D., et al. A Duplex Real-Time qPCR Assay for the Quantification of Human Nuclear and Mitochondrial DNA in Forensic Samples: Implications for Quantifying DNA in Degraded Samples. Journal of Forensic Sciences, Sept 2005, Vol. 50, No. 5 (1044-1060)
- Waye J.S. et al. Sensitive and specific quantitation of human genomic DNA in forensic specimen specimens: casework examples. Journal of Forensic Sciences 1991; 36(4): 1198-1203.
- Waye J.S. et al. A simple and sensitive method for quantifying human genomic DNA in forensic specimen extracts. BioTechniques Vol. 7, No. 8 (1989).
- Walsh, P.S. et al. 1992. A rapid chemiluminescent method for quantitation of Human DNA. Nucleic Acids Research. 20: 5061-5065.
- Butler, J.M. 2011. Advanced Topics in Forensic DNA Typing: Methodology. Chapter 3: Quantitation. Elsevier.