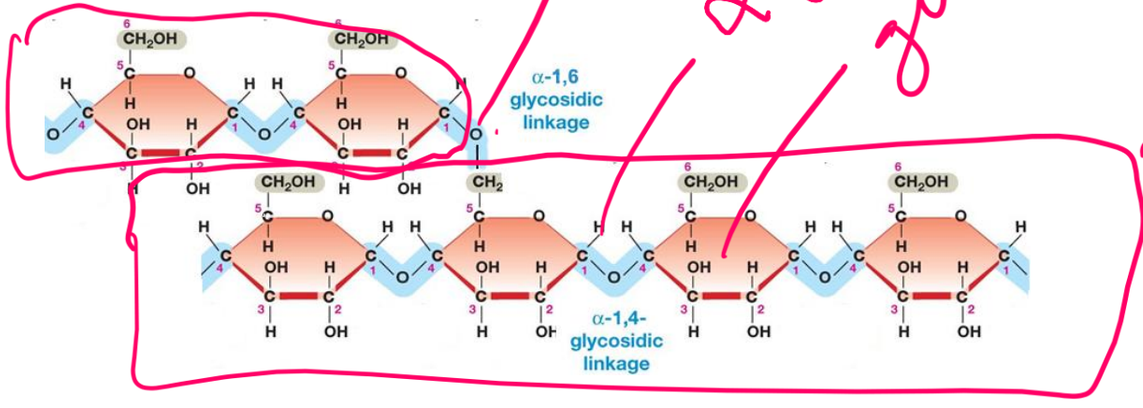
Lecture 4
Nucleic Acids & Immunology
(and a little polysaccharides)

- Nucleic Acid Technologies
- Immunotherapies

Please view the posted video on Enzyme Kinetics before our next class.

Polysaccharides as Energy Storage – Glycogen Storage Disease

Glycogen and is made entirely of glucose units and is used for glucose storage.

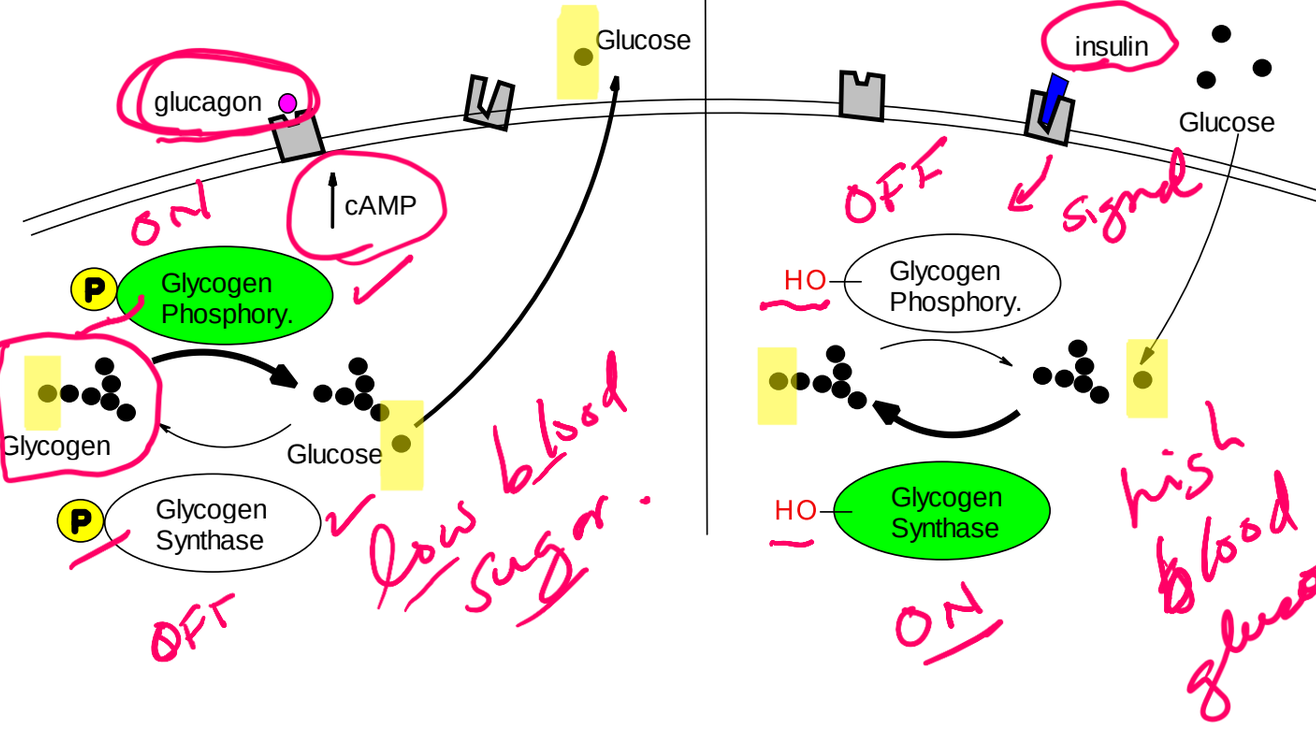


Glycogen Levels are regulated by hormones secreted due to blood glucose levels.

- Glucagon – low blood sugar
- Insulin – high blood sugar

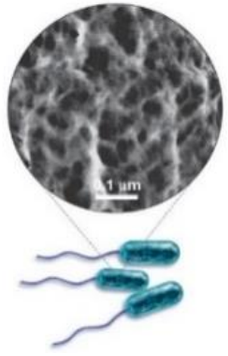
Two enzymes degrade or synthesize glycogen

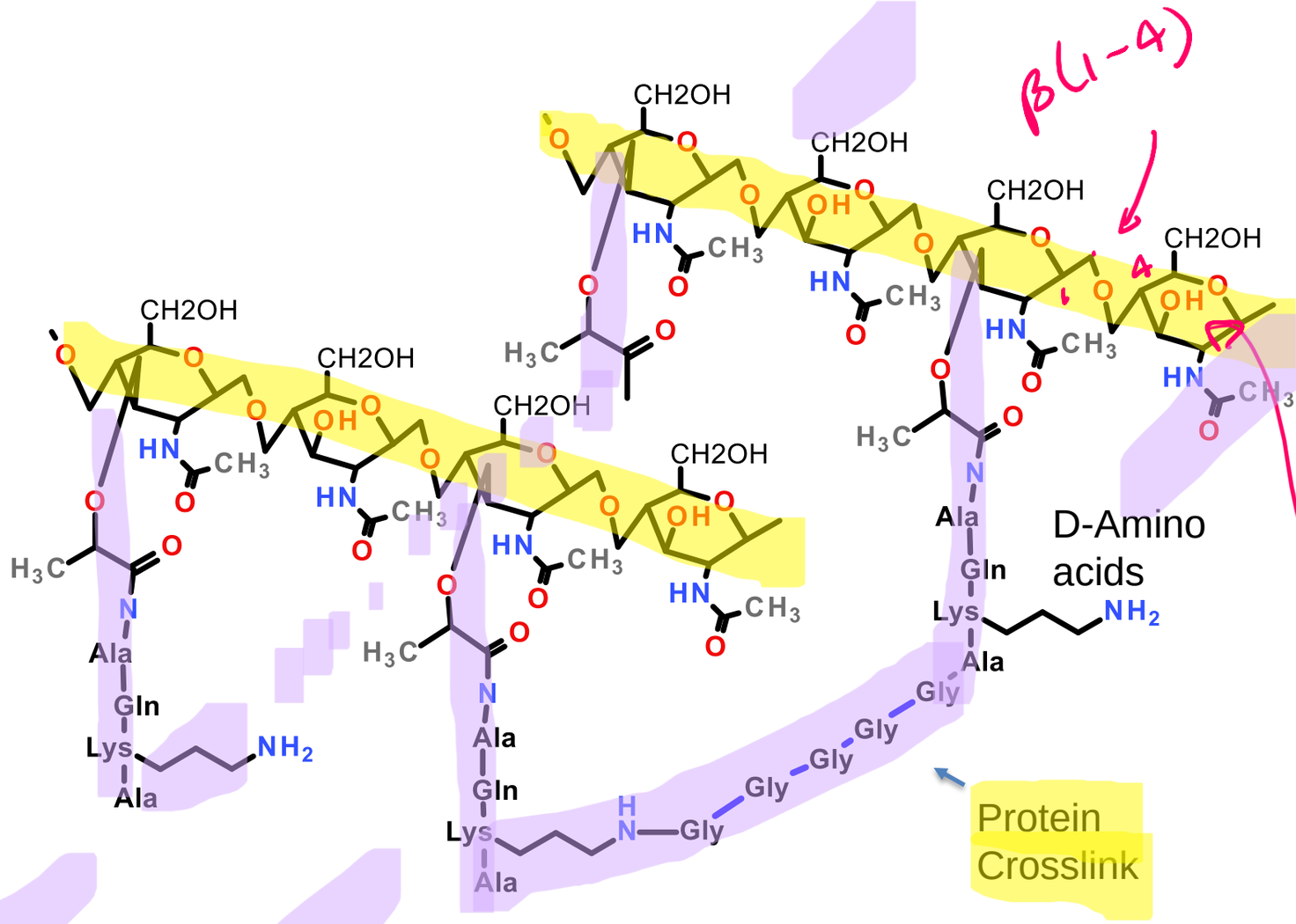
- Glycogen phosphorylase – releases glucose from glycogen
- Glycogen synthase – stores glucose in glycogen



Polysaccharides as Structural Molecules

Peptidoglycan (protein + sugar) in bacterial cell wall





β(1-4)

cell wall covalently crosslinked polymer

modified glucose

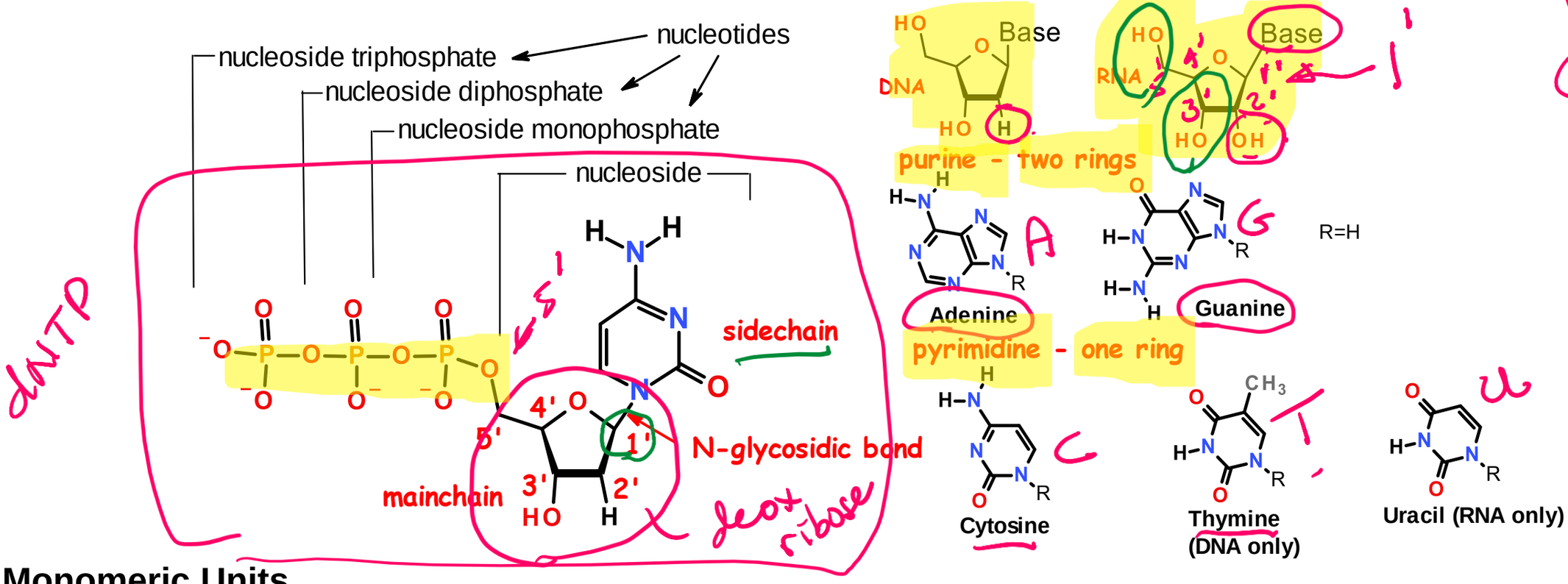
Many antibiotics interfere with cell wall synthesis (e.g. penicillin)

Peptidoglycan (Bacterial Cell Wall)

Nucleic Acid Technologies

- Review of DNA Structure
- Review of DNA Polymerase activity
- Nucleic Acid Technologies – PCR & Sequencing

Nucleic Acid Structure



Monomeric Units

a) Nucleoside triphosphates are the building blocks of nucleic acids (**dNTP = dATP, dGTP, dCTP, dTTP**)

b) The base ("sidechain") is connected to the C1' of the sugar ("mainchain") by an **N-linked glycosidic** bond.
   Base + sugar = **nucleoside**.
   Base + sugar + n-phosphates = **nucleotide**

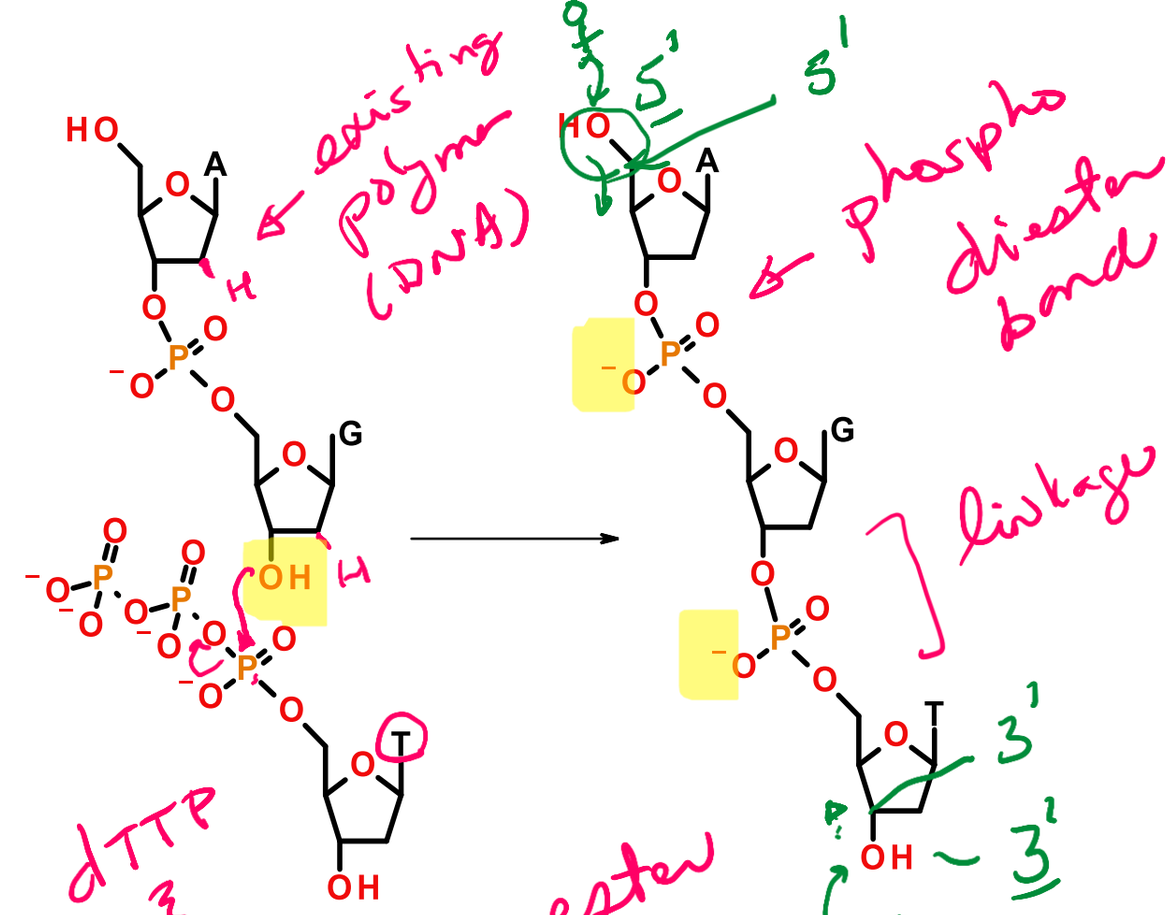
c) The carbon atoms on the sugar are numbered 1' to 5'. The primes distinguish the atoms on the sugar from those on the base.

d) DNA differs from RNA in the sugar (deoxyribose versus ribose) and one base.

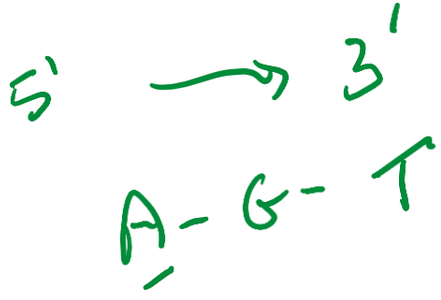
e) Four different monomers, A, G, C, T in DNA. U replaces T in RNA.

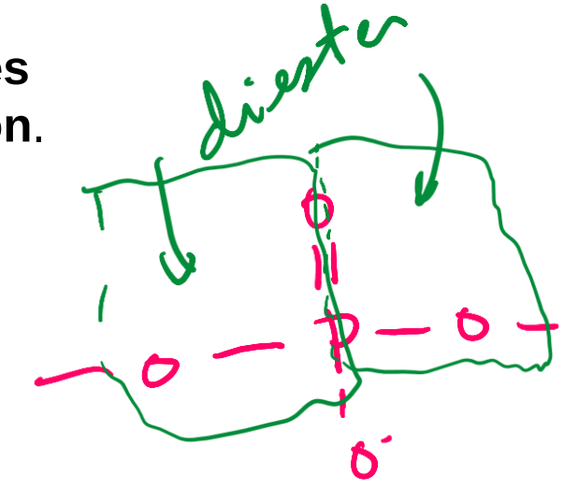
DNA and RNA are Polynucleotides:

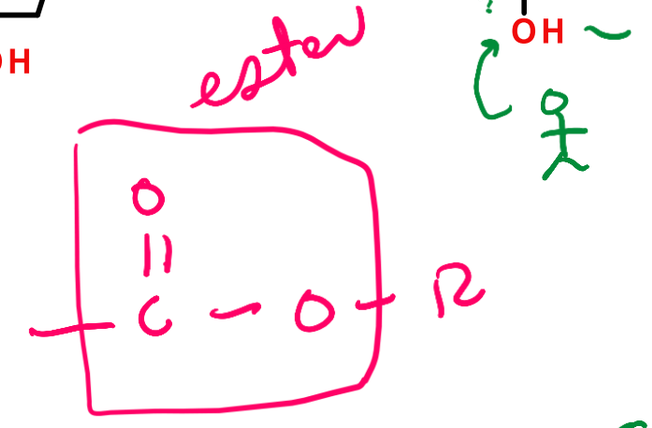
- Two phosphates are lost during polymer formation.
- The **phosphodiester** backbone is comprised of deoxyribose (DNA) or ribose (RNA) sugars bridged by one phosphate between the **3' and 5'** positions of the sugars. *Be able to draw this structure.*
- The phosphates are always ionized (pK_a~1), nucleic acids are **polyanions**. The negative charge is important for protein interactions (and electrophoresis).
- Note the polarity: 5' → 3'. *Be able to identify the 5' and 3' ends:*
  - Start at the end atom and move down the chain. The first carbon you find defines the end.

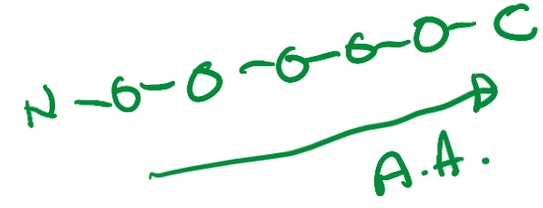


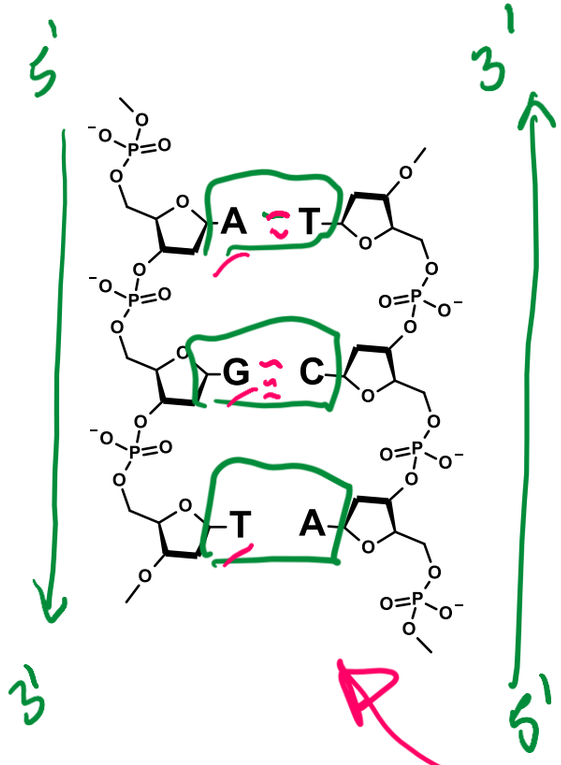
Sequence of nucleotide bases is written in the 5'-3' direction.

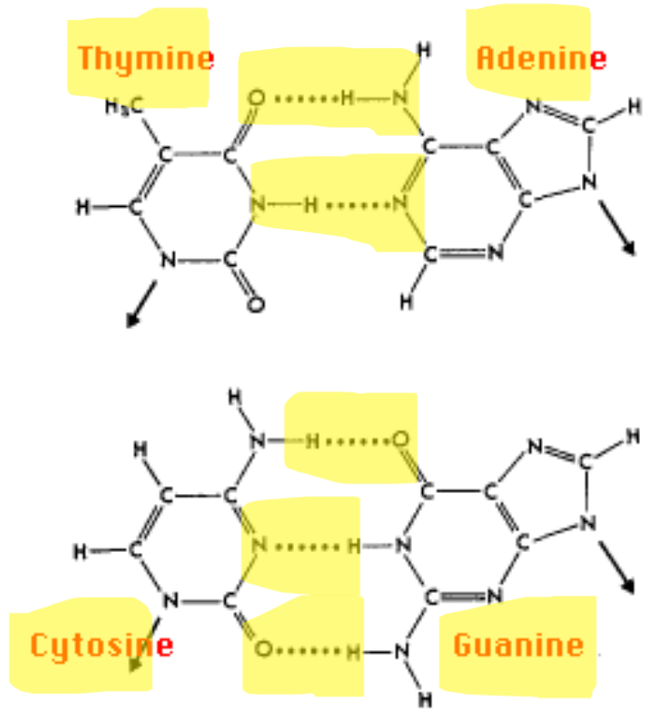












Nomenclature

5' AGT 3'

3' TCA 5'

≡ AGT
TCA

https://www.andrew.cmu.edu/user/rule/jsmol/nucleic.html

Double Helical Structures: B-DNA

a) The helix is right-handed; the chains are **antiparallel**.
b) **10 bp/turn**.
c) The helix interior is filled with stacked base, phosphates and deoxyriboses on the outside.
d) T pairs with A via two "Watson-Crick H-bonds"
e) C pairs with G via three "Watson-Crick hydrogen bonds"
f) Opposite strand termed “complimentary strand”. Top strand is always written 5’->3’, lower strand 3’ -> 5’.

Introduction to Central Dogma

Genome: Entire DNA content of an organism, contains all of the instructions for life. Single circular molecule in Proks, multiple linear molecules (chromosomes) in Euks. The genome is *replicated* when cells divide.

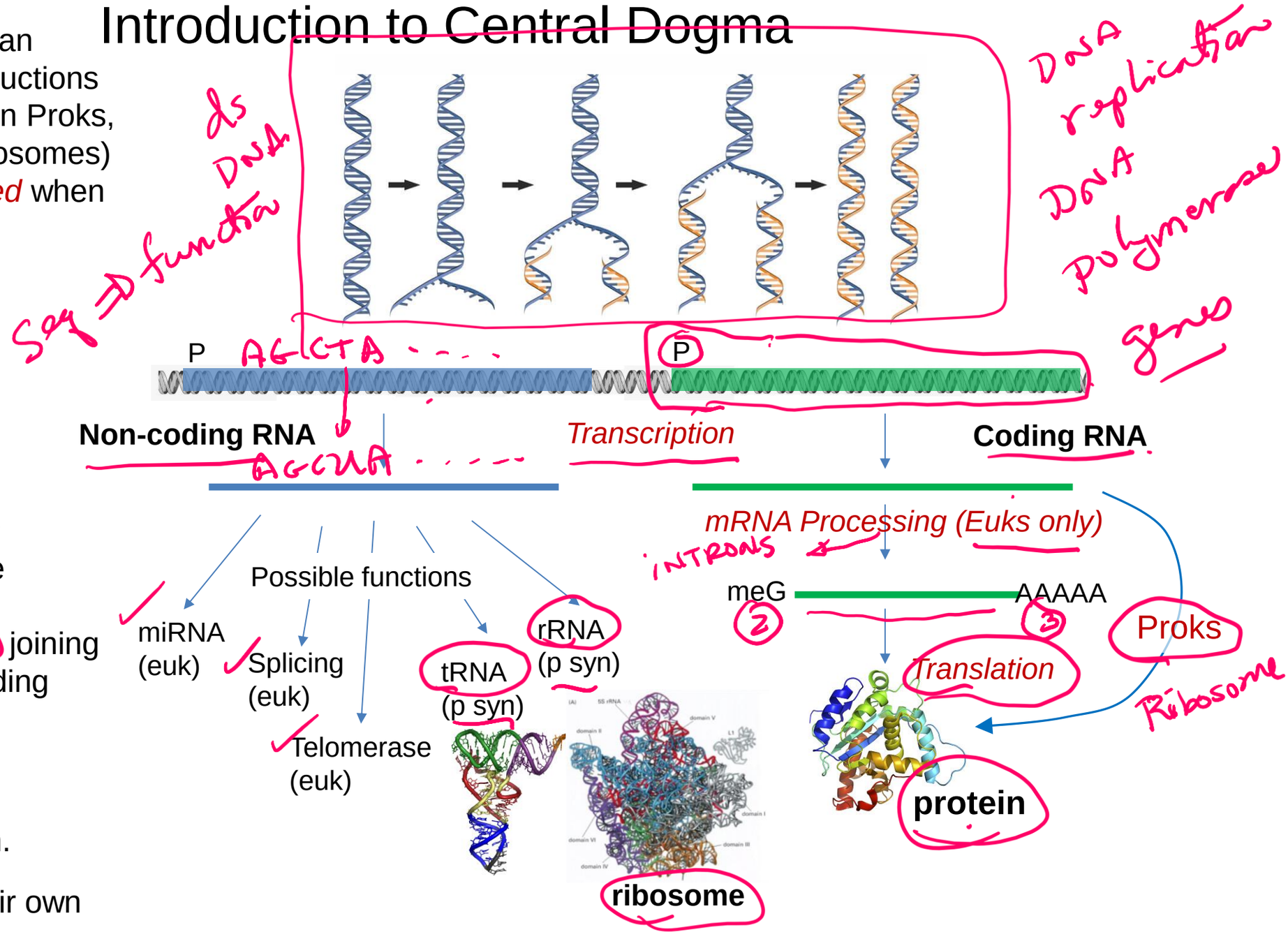
Gene – a segment of DNA that is converted (*transcribed*) to RNA. A *promoter* (P) sequence on the DNA is the minimal requirement for the production of RNA.

RNA molecules are processed in **Eukaryotic cells** before they are functional. Processing includes:

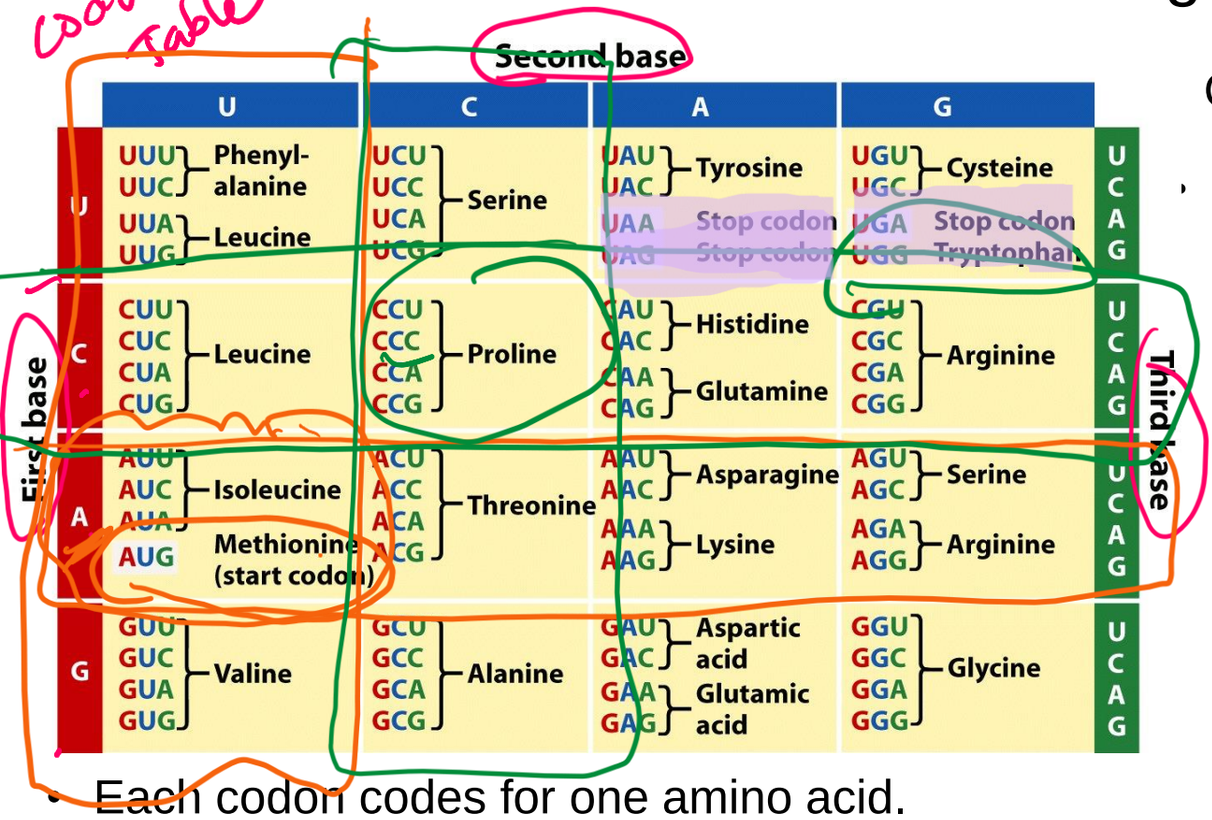
- Splicing – removal of **introns**, joining **exons** to generate protein coding region.
- 5' capping
- 3' polyA tail

mRNA are *translated* to a protein.

Many RNAs are functional on their own



The Genetic Code – Converting a DNA/RNA Sequence to a Protein



- Each codon codes for one amino acid.
- Many amino acids are coded by more than one codon.
- Most organisms use the same codon table – some codons have different meanings in some organisms.

Special Codons:

AUG = Is used to begin almost all proteins that are synthesized on the ribosome, codes for methionine when found internally.
UAA, UAG, UGA = stop codons, terminate synthesis

Codon = 3 bases that code for an amino acid

....ATATGCCCATGTGGTAA..

(DNA Sequence)

...AUAUGCCCAUGUGGUAA..

(mRNA Sequence)

..U–AUG–CCC–AUG–UGG–UAA

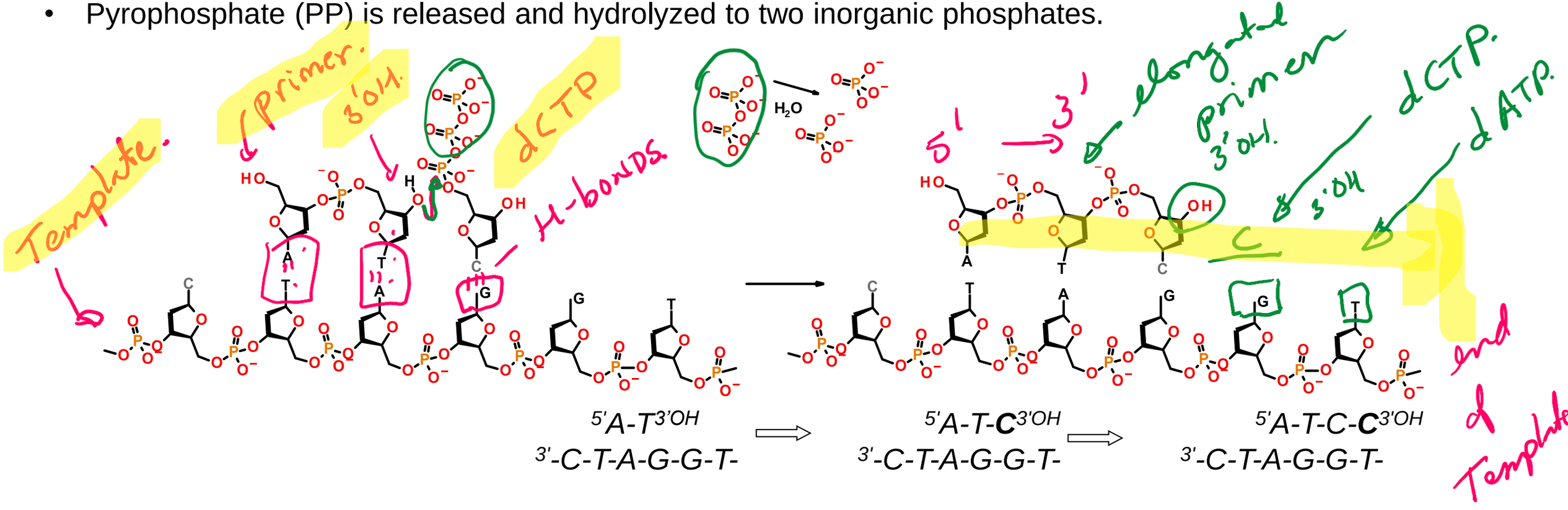
(Punctuated RNA sequence – how the ribosome interprets the sequence)

Met – Pro – Met – Trp

(Protein Sequence)

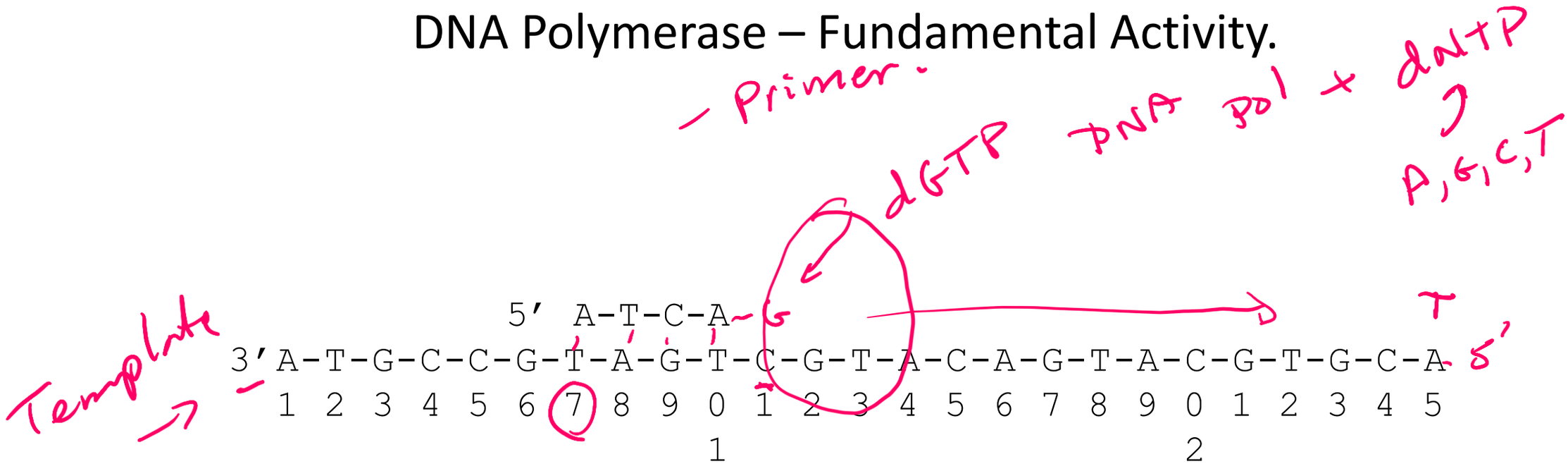
DNA Polymerases – Used in DNA Sequencing and PCR

- **DNA polymerases** utilize a **template** to direct the order of added bases,
- The enzyme will continue to the end of the template.
- Require a basepaired **primer** with a 3'OH. Primer can be DNA or RNA, DNA is used for laboratory work, RNA is used by the cell during replication
- New dNTP added to the 3' hydroxyl of the existing polymer, elongation in the **5' to 3' direction.**
- Pyrophosphate (PP) is released and hydrolyzed to two inorganic phosphates.



Expectations: Know the features of this reaction.

DNA Polymerase – Fundamental Activity.



A short 4 base primer (ATCA) is added to a template, and the temperature is lowered to allow annealing (basepairing) of the primer to the template.

1. *Where (what position) will this primer anneal?* 7
2. *What is the first base added by the polymerase?* A **G** C T
3. *What is the last base added by the polymerase?* A G C **T**

Repeat Expansion Diseases – Errors in DNA Replication

Handbook of Clinical Neurology
Volume 147, 2018, Pages 105-123

Chapter 9 - Repeat expansion diseases

Henry Paulson

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https://doi.org/10.1016/B978-0-444-63233-3.00009-9

Second base

First base	U	C	A
U	UUU Phenyl-alanine UUC Phenyl-alanine UUA Leucine UUG Leucine	UCU Serine UCC Serine UCA Serine UCG Serine	UAU Tyrosine UAC Tyrosine UAA Stop codon UAG Stop codon
C	CUU Leucine CUC Leucine CUA Leucine CUG Leucine	CCU Proline CCC Proline CCA Proline CCG Proline	CAU Histidine CAC Histidine CAA Glutamine CAG Glutamine

- CAG – at least 10 diseases (Huntington disease, spinal and bulbar muscular atrophy, dentatorubral-pallidoluysian atrophy and seven SCAs)
- CGG – fragile X, fragile X tremor ataxia syndrome, other fragile sites (GCC, CCG)
- CTG – myotonic dystrophy type 1, Huntington disease-like 2, spinocerebellar ataxia type 8, Fuchs corneal dystrophy
- GAA – Friedreich ataxia
- GCC – FRAXE mental retardation
- GCG – oculopharyngeal muscular dystrophy
- CCTG – myotonic dystrophy type 1
- ATTCT – spinocerebellar ataxia type 10
- TGGAA – spinocerebellar ataxia type 31
- GGCCTG – spinocerebellar ataxia type 36
- GGGGCC – C9ORF72 frontotemporal dementia/amyotrophic lateral sclerosis
- CCCCGCCCCGCG – EPM1 (myoclonic epilepsy)

- Repeats in coding regions of genes will generate long stretches of the same amino acid.
  - CAGCAGCAG = GlnGlnGln
- Repeats outside of coding regions can affect gene expression by changing binding of transcription factors.
- These repeats can grow due to slippage of primer during replication
- More repeats = more chance of developing disease.

- The number of repeats can be detected by:
  - DNA sequencing
  - PCR

Repeat Expansions – How Do They Grow?

Original Sequence - 3 repeats (CAG)

-TATATCCAGCAGCAGAGTATA-
-ATATAGGTCGTCGTCTCATAT-

During Replication in the cell

Primer

-TA
-ATATAGGTCGTCGTCTCATAT-

-TATATCCAGCAGCAG
-ATATAGGTCGTCGTCTCATAT-

3' end comes loose (primer slippage)

-TATATCCAGCAGCAG
-ATATAGGTCGTCGTCTCATAT-

Looped out DNA

dCTP ....

-TATATCCAGCAG
-ATATAGGTCGTCGTCTCATAT-

Replication continues

Template

-TATATCCAGCAGCAGAGTATA-
-ATATAGGTCGTCGTCTCATAT-

Next replication (upper strand as the template)

-TATATCCAGCAGCAGCAGAGTATA-
TCATAT-

Poly

4 repeats

-TATATCCAGCAGCAGCAGCAGAGTATA-
-ATATAGCTCGTCGTCGTCGTCTCATAT-

5 repeats etc.

DNA Sequencing – Sanger (dideoxy) Sequencing

DNA Sequencing - Determining the Order of Bases in the DNA.

Maxim & Gilbert: Chemical cleavage, no prior sequence information required. This method was not widely adopted because it used hydrazine (rocket fuel)

Sanger Sequencing:

- Second method to generate long (~1000 base) sequence information (Sanger was awarded his 2nd Nobel prize for this work in 1980, shared with Gilbert.
- Requires knowledge of some sequence for priming.

Determine the position of all four bases in a DNA strand = Sequence (video)

Sanger Sequencing:

Primer

Template 3′ G-A-A-G-T-C-G-A-A-G-G-T-A-T-A-C-C-A-T-T-A-G-G-C-C-A-T-G-C-A-C-G-T----

5′ C-A-T-A-T-G OH

Known Seq (plasmid) Unknown sequence (insert)

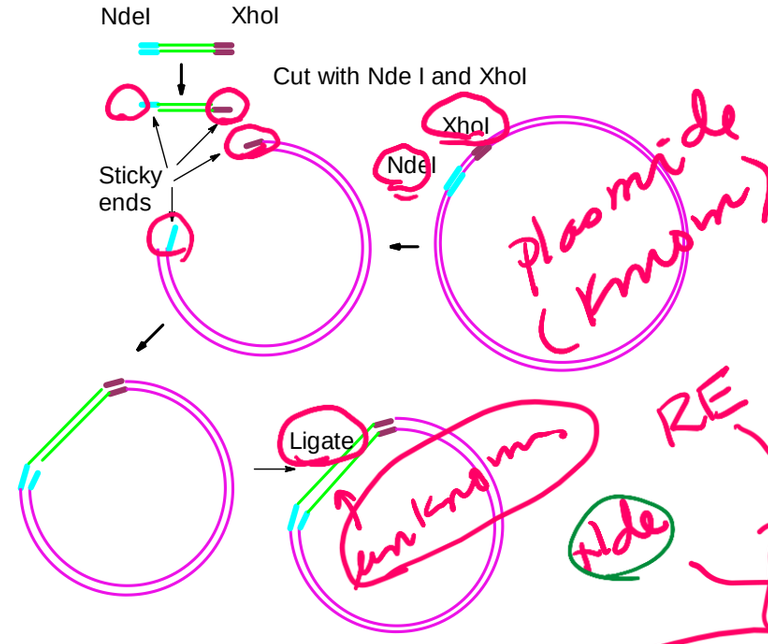
Sequenced region (~1000 bases)

5′ C-A-T-A-T-G-G-T-A-A-T-C-C-G-G-T-A-C-G-T-G-C-A----

Template 3′ G-A-A-G-T-C-G-A-A-G-G-T-A-T-A-C-C-A-T-T-A-G-G-C-C-A-T-G-C-A-C-G-T----

DNA Sequencing - Determining the Order of Bases Added by DNA Polymerase

- The DNA to be sequenced is inserted into a circular piece of double stranded DNA called a plasmid. *The DNA sequence of the plasmid is known.*
- The insertion is often accomplished using restriction enzymes that generate single stranded overhangs that allow DNA molecules to be efficiently joined.
- Restriction sites can be added to any DNA fragment using a number of techniques:
  - Addition of a short linker (same site on both ends)
  - PCR (different sites on each end)



Restriction Enzymes

- Recognize a specific sequence in the DNA
- Sequence has 2-fold symmetry – same on the top and bottom strand
- Cuts both strands, most generate single-stranded DNA (sticky ends).
- Complementary sticky ends can bind to each other.

Key Point 1. Start sequencing at known location with primer that anneals at a ***unique*** location on the plasmid, "upstream" from the region to be sequenced.

All DNA molecules begin here.

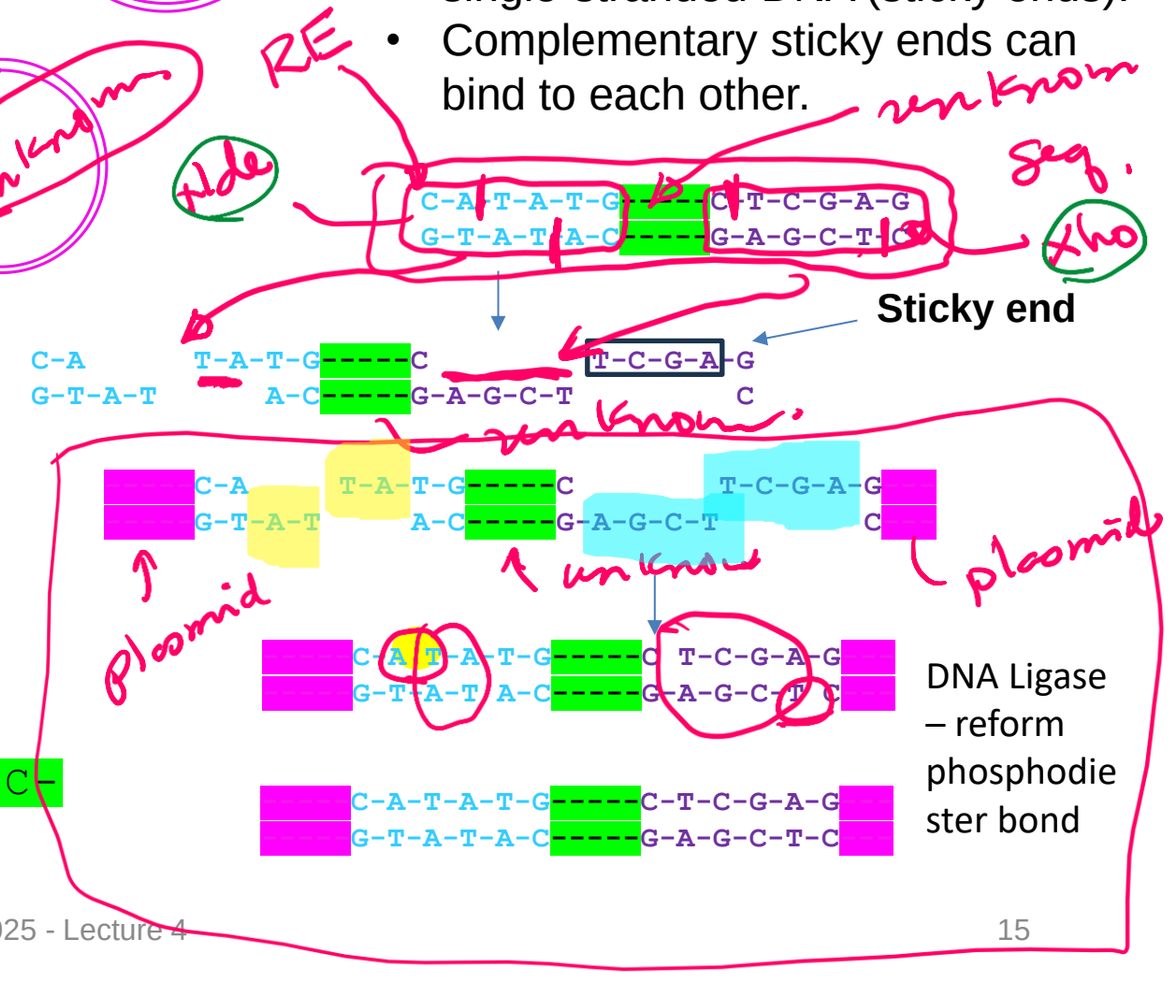
First base is added here.

Lower strand is the template

5′ C-A-T-A-T-G OH
3′ -A-G-G-T-A-T-A-C-C-A-T T A G-G-C-C-

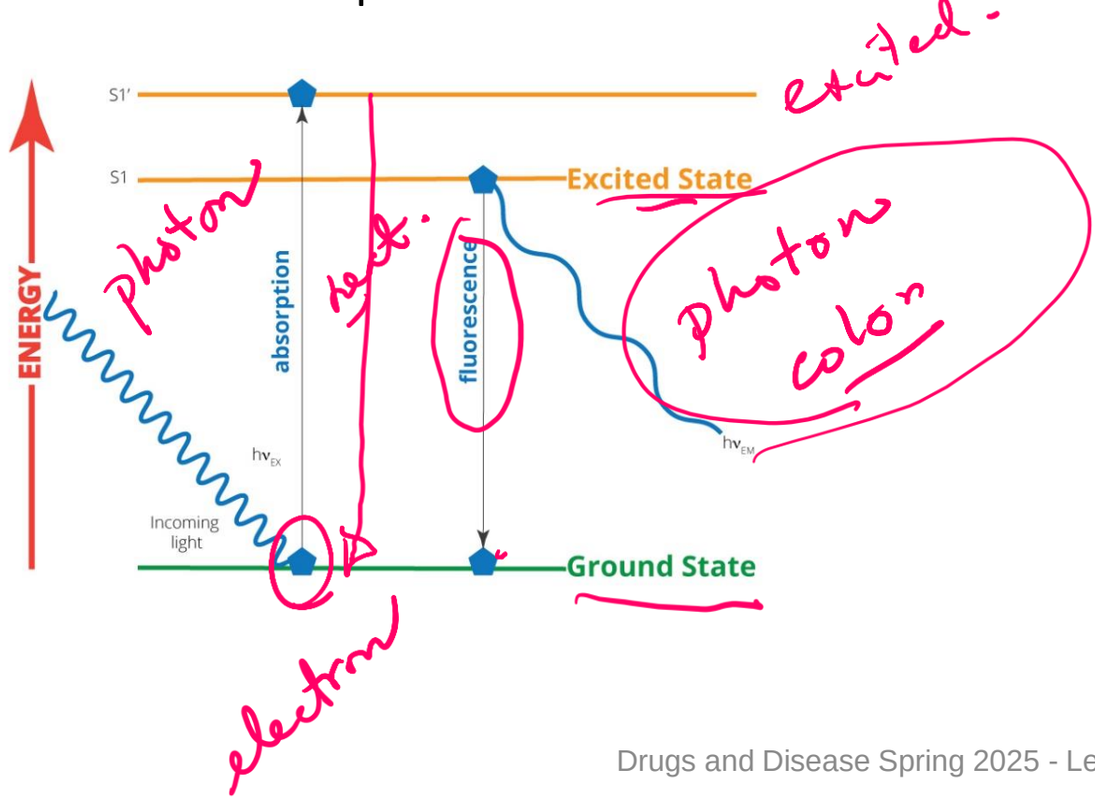
Known Seq (plasmid)

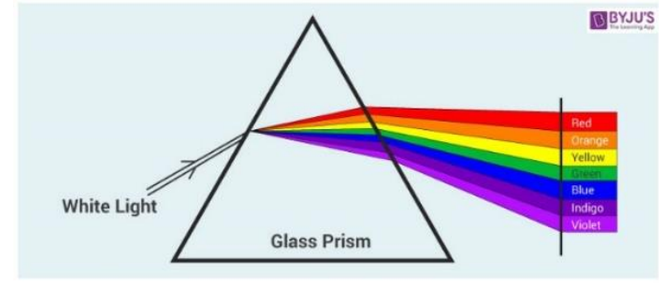
Unknown sequence (insert)



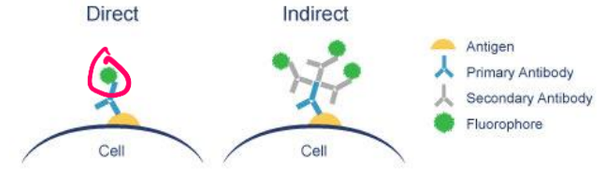
DNA Sequencing Methods Use Fluorescent Bases - What is Fluorescence?

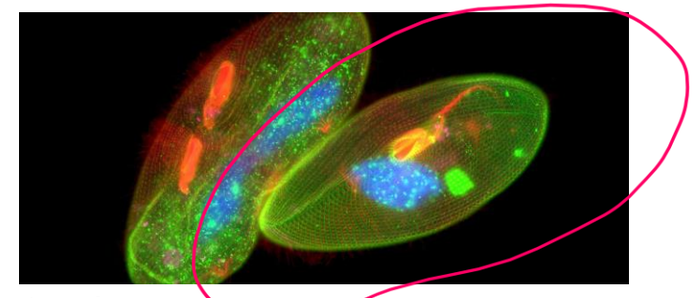
- When molecules absorb light an electron goes from a lower shell to a higher shell. This is where the energy from the light goes.
- In most molecules the electron goes back down to its original shell with the release of heat.
- Fluorescent molecules emit the energy as light of a longer wavelength (different color).
- The color that is emitted depends on the molecule.





Fluorescently tagged antibodies can be used to stain components of cell with fluorophores.

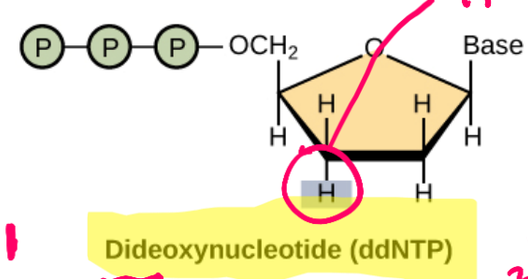


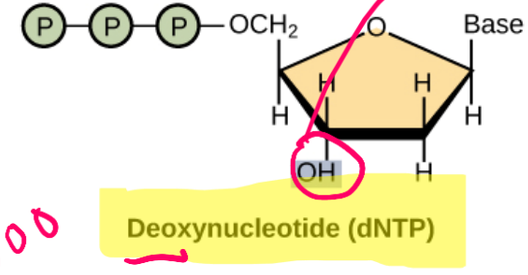


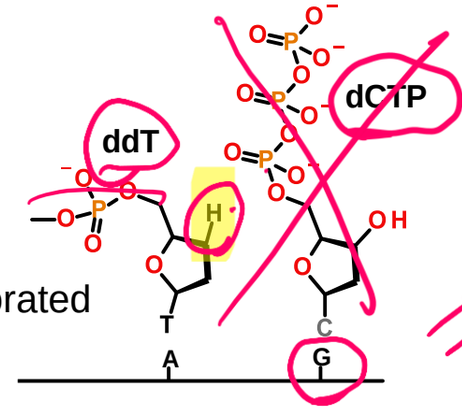
DNA Sequencing - Determining the Order of Bases Added by DNA Polymerase

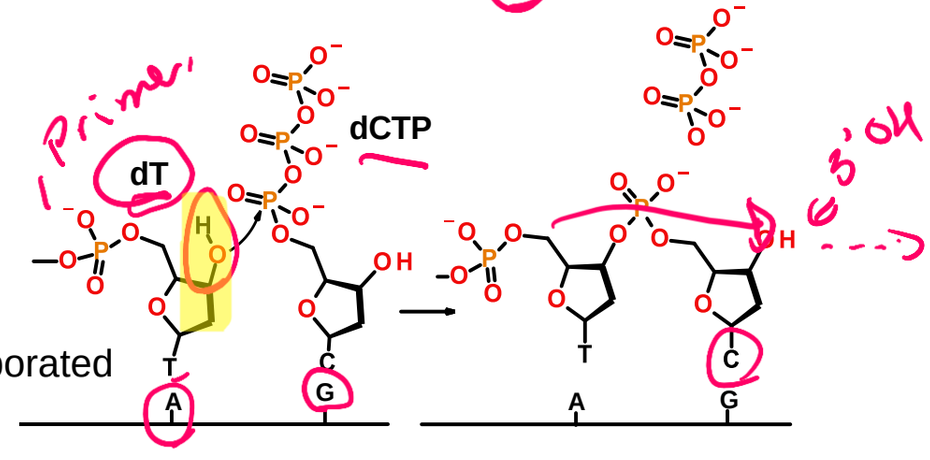
Key point 2. Use a mixture of normal bases (dNTPs) and dideoxy bases (ddNTP) for polymerization. Ratio of dNTP to ddNTP is (100:1), ***most of the time elongation occurs.***

- ddNTPs can be added to the DNA since they have a 5'-triphosphate but ***terminate*** the chain due to the lack of a 3'-OH. ~ 1 in 100 chains terminate at each base addition





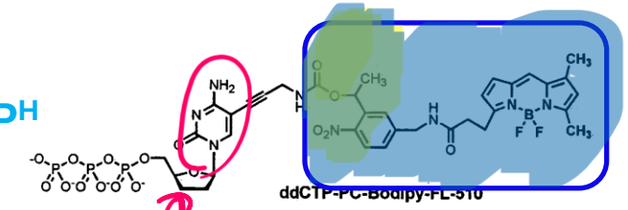




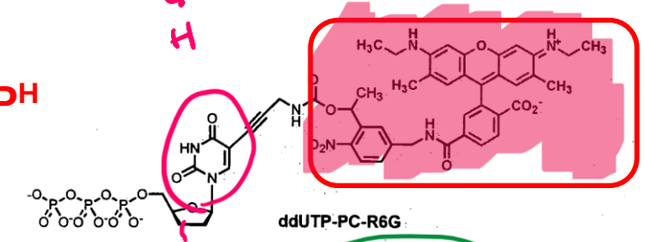
Key point 3. The ddNTPs are color coded by different fluorescent emission wavelengths.

The ddNTP that terminated the chain is known from its fluorescent color.

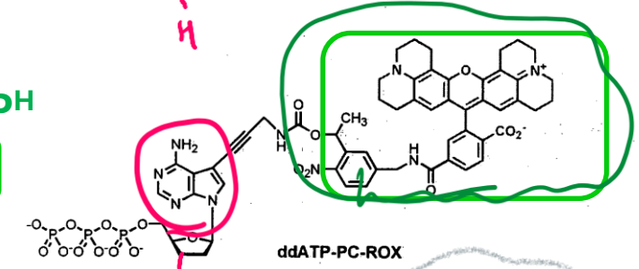
ddCTP^H



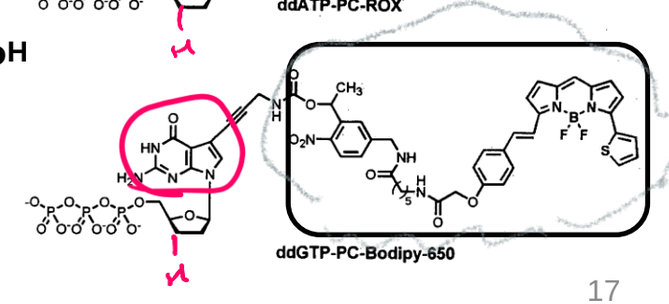
ddTTP^H



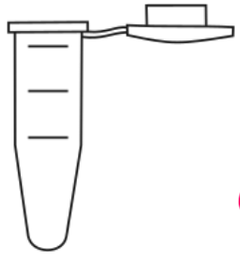
ddATP^H



ddGTP^H



DNA Sequencing – Generation of Fluorescent Fragments



Template
Primer
DNA Pol
dTNP, ddNTP

5′-C-A-T-A-T-G
3′-A-G-G-T-A-T-A-C-C-A-T-T-A-G-G-C

(1000 molecules)

dTTP
ddTTP

5′-C-A-T-A-T-G-G^{OH}
3′-A-G-G-T-A-T-A-C-C-A-T-T-A-G-G-C

(990 molecules)

5′-C-A-T-A-T-G-G-T^{OH}
3′-A-G-G-T-A-T-A-C-C-A-T-T-A-G-G-C

(980 molecules)

5′-C-A-T-A-T-G-G-T-A^{OH}
3′-A-G-G-T-A-T-A-C-C-A-T-T-A-G-G-C

ddGTP
dGTP
dGTP
ddGTP

Length=7, Black fluor.

(10) 5′-C-A-T-A-T-G-G^{H} Term
3′-A-G-G-T-A-T-A-C-C-A-T-T-A-G-G-C

(990) 5′-C-A-T-A-T-G-G^{OH} 3'OH
3′-A-G-G-T-A-T-A-C-C-A-T-T-A-G-G-C

ddTTP
dTTP

Length=8, Red fluor.

(10) 5′-C-A-T-A-T-G-G-T^{H} Term
3′-A-G-G-T-A-T-A-C-C-A-T-T-A-G-G-C

(980) 5′-C-A-T-A-T-G-G-T^{OH} 3'OH
3′-A-G-G-T-A-T-A-C-C-A-T-T-A-G-G-C

Length=9, Green fluor

(10) 5′-C-A-T-A-T-G-G-T-A^{H}
3′-A-G-G-T-A-T-A-C-C-A-T-T-A-G-G-C

(970) 5′-C-A-T-A-T-G-G-T-A^{OH}
3′-A-G-G-T-A-T-A-C-C-A-T-T-A-G-G-C

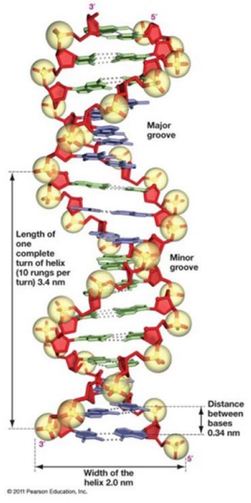
***All* Possible Fragments are Made:**

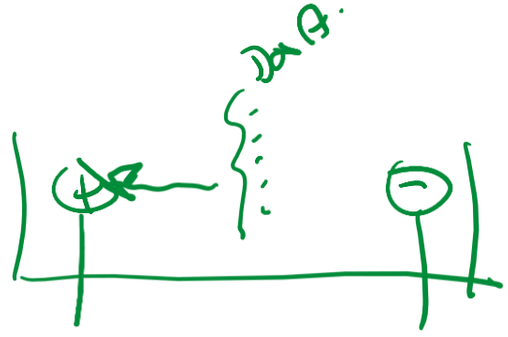
1. Each begins with the primer
2. Each ends with a *known* ddNTP, based on the color of the fluorescence.
3. Each is one longer than the previous.

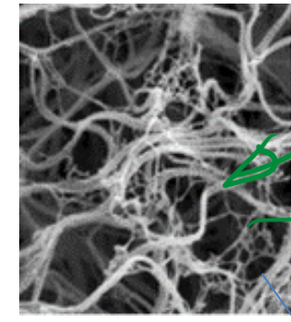
Primer
C-A-T-A-T-G-G
C-A-T-A-T-G-G-T
C-A-T-A-T-G-G-T-A
C-A-T-A-T-G-G-T-A-A
C-A-T-A-T-G-G-T-A-A-T
C-A-T-A-T-G-G-T-A-A-T-C
C-A-T-A-T-G-G-T-A-A-T-C-C
C-A-T-A-T-G-G-T-A-A-T-C-C-G

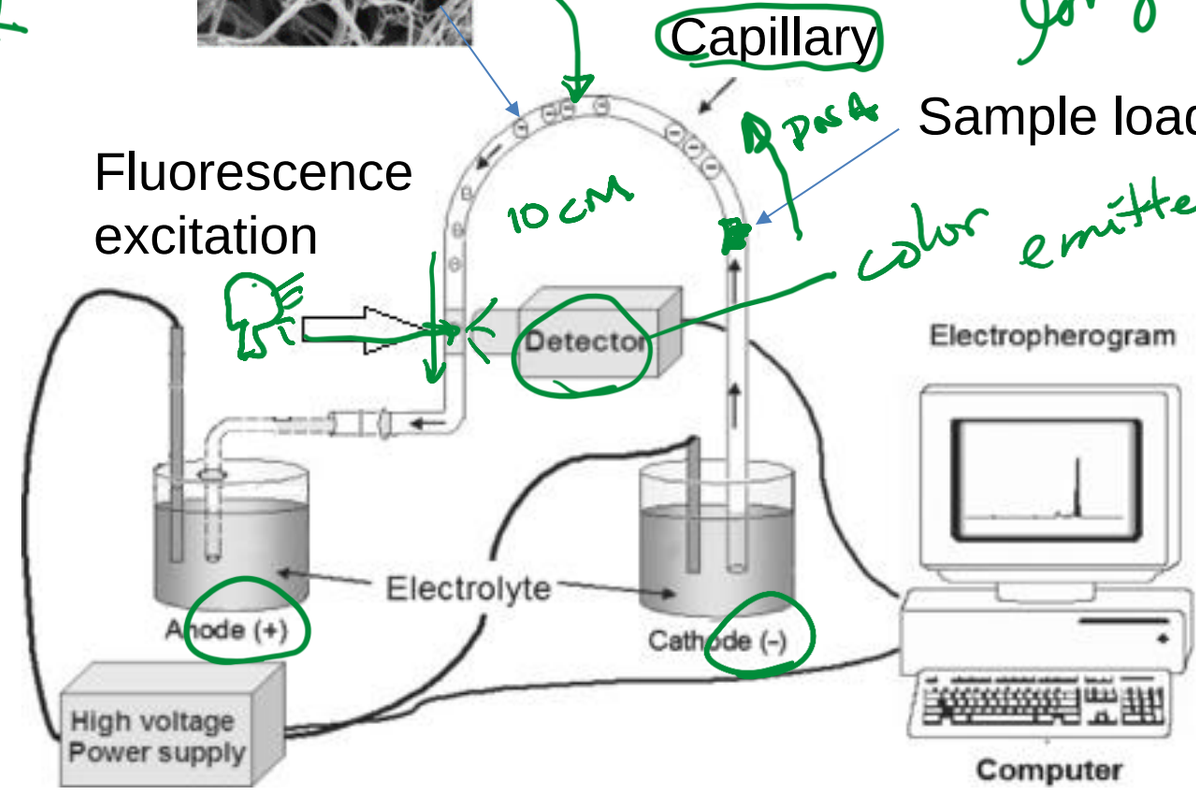
Primer | Added by Pol.

Size Determination of Fragments from DNA Sequencing Capillary Electrophoresis









DNA has a negative charge.
It will migrate towards the anode.

Capillary is filled with a gel that causes separation by size.

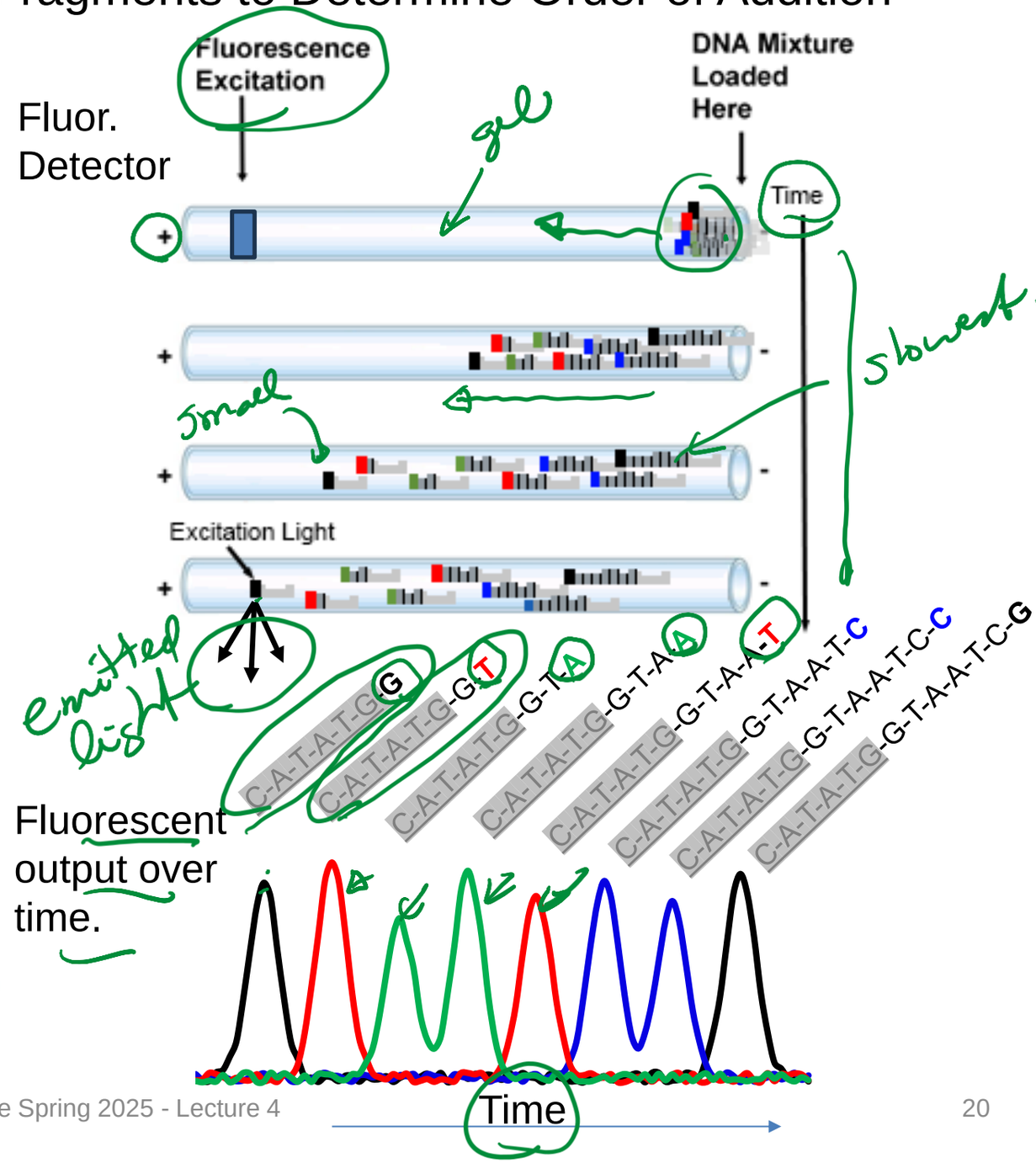
DNA molecules that are smaller migrate _____.

DNA Sequencing – Analysis of Fragments to Determine Order of Addition

4. Capillary Gel Electrophoresis

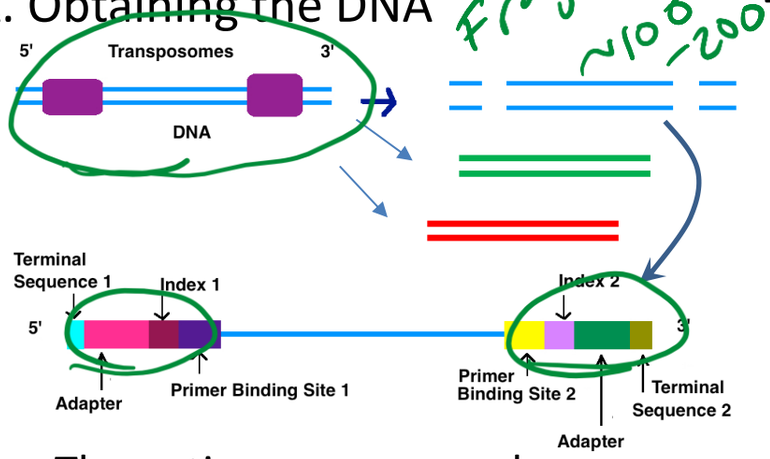
- Migration due to the voltage because of the neg. charge on DNA phosphates
- Separation of DNA molecules by size, smaller travel through gel faster.
- Fragments reach the detector in the order of their length: primer+1 first, primer+2 second, etc.
- At the detector, a laser excites the fluorescence.
- Only fluorescent DNA molecules (terminated with ddNTP) give a signal.
- The color of the emitted fluorescence gives the dideoxy base at the 3' end of the DNA fragment.
- The order of peaks gives the sequence that is complementary to the template (= strand with primer).

5'-C-A-T-A-T-GG-T-A-A-T-C-C-G
3'-A-G-G-C-T-A-T-A-C-C-A-T-T-A-G-G-C

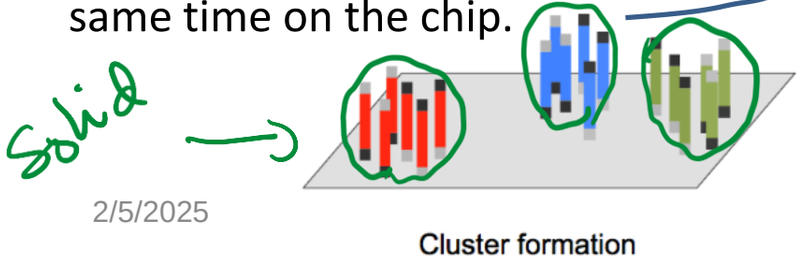


Newer Sequencing Methods-Illumina Dye Sequencing – Next Generation High Throughput

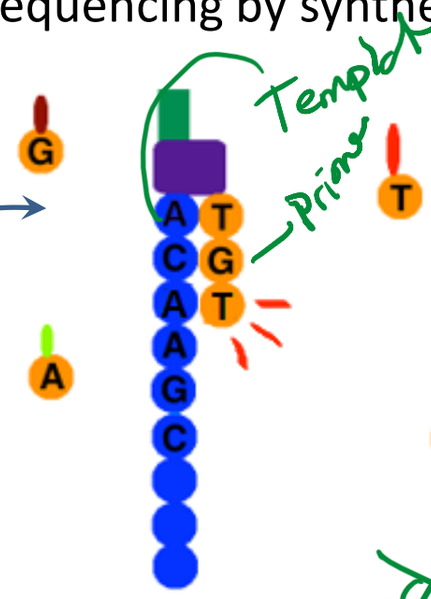
A. Obtaining the DNA

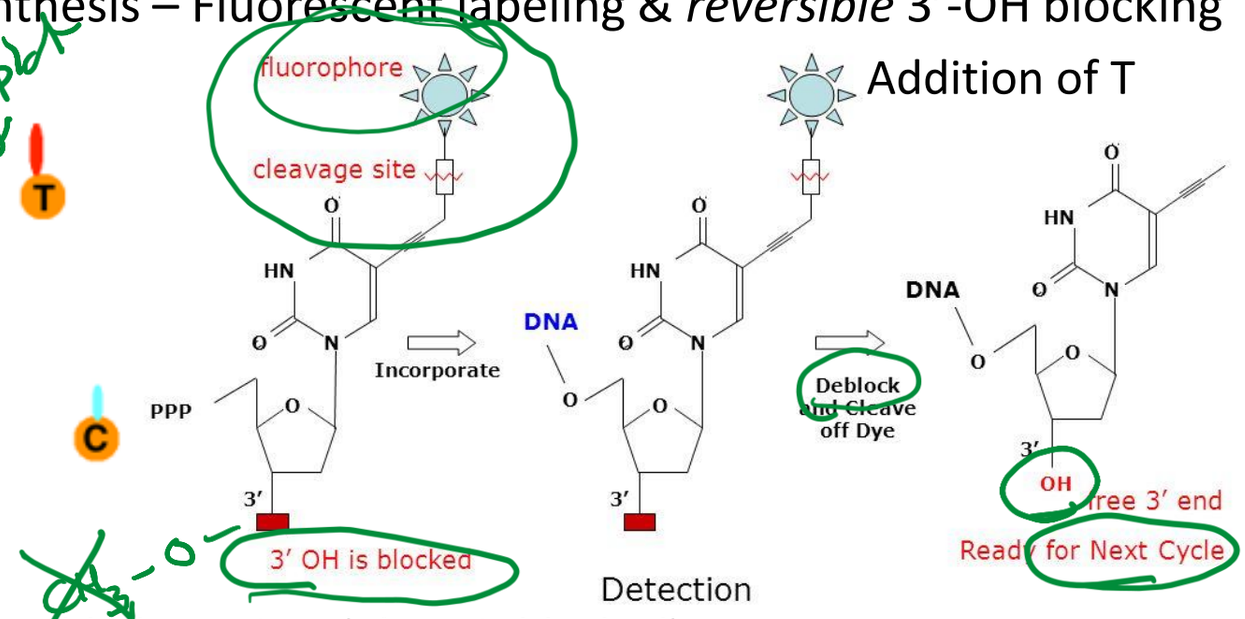


- The entire genome can be sequenced.
- The DNA is fragmented into small 100 base pieces.
- Synthetic DNA is added to the ends (sites for primers for sequencing)
- Different fragments are bound to different location on a solid surface (chip).
- All fragments are sequenced at the same time on the chip.



B. Sequencing by synthesis – Fluorescent labeling & *reversible* 3'-OH blocking



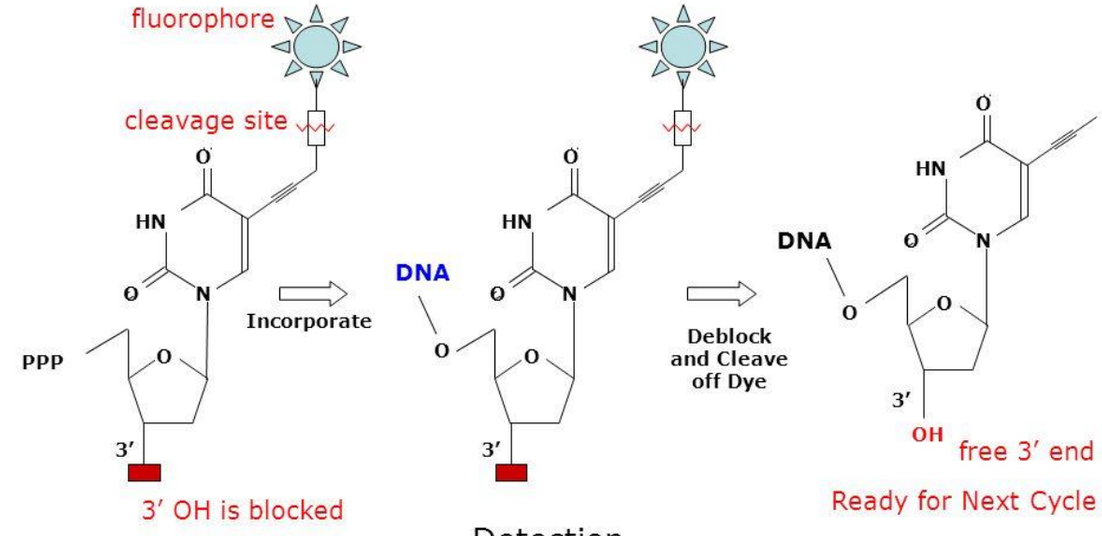


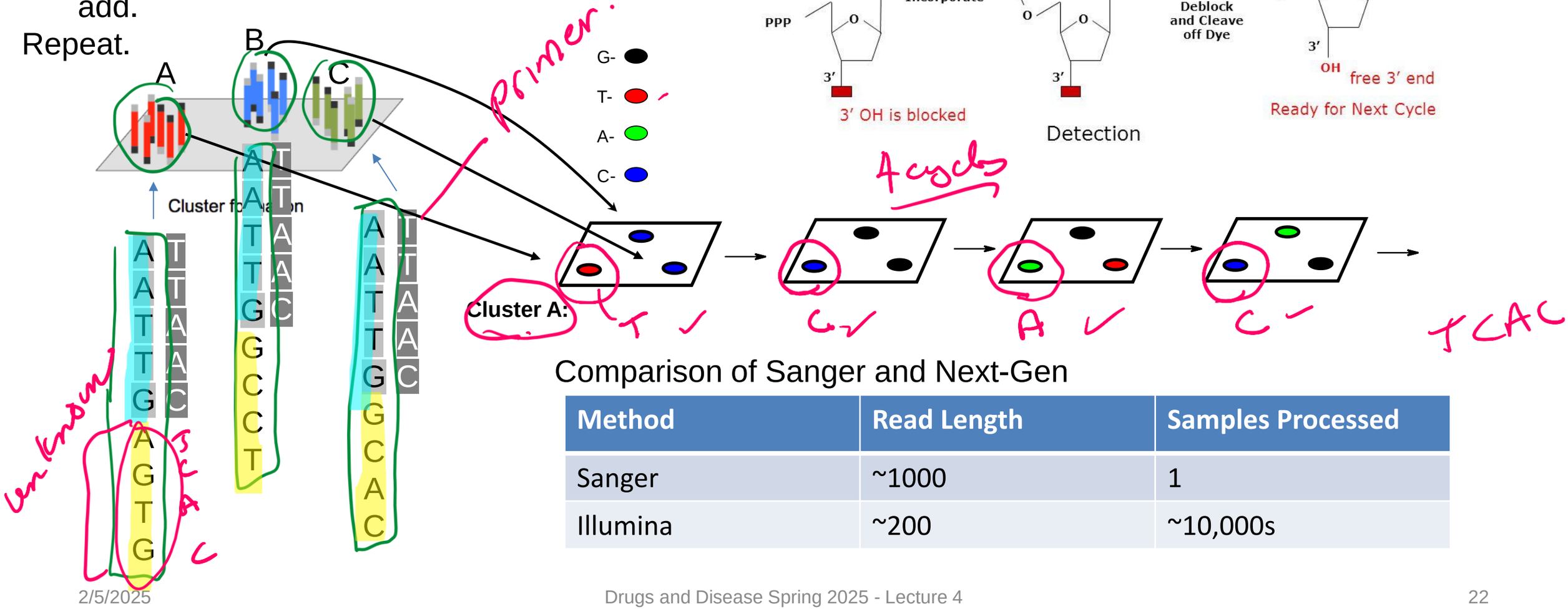
1. Only one base is added at a time (3'-OH is blocked)
2. The base that is added is determined by the color of the fluorescent base.
3. 3'-OH blocking group and the fluorescent group are removed prior to the next addition. ~100 cycles can be performed.

Next Generation - Data

1. Primer anneals
2. Add dNTPs (3'blocked) + Polymerase
3. Wash to remove unincorporated dNTPs
4. Detect which base was added to each cluster using fluorescent emission
5. Unblock 3'-OH to allow the next base to add.

Repeat.



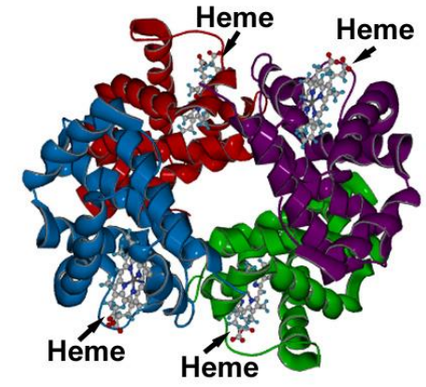


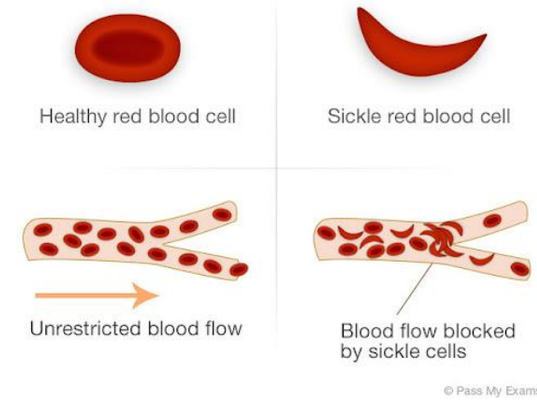
Comparison of Sanger and Next-Gen

Method	Read Length	Samples Processed
Sanger	~1000	1
Illumina	~200	~10,000s

Genotyping at the Molecular Level with DNA Sequencing.

- Sickle cell anemia is caused by a single mutation in the beta chain of hemoglobin
- This mutation causes the hemoglobin to form long polymers that distort the shape of the red blood cell.
- Determining whether someone has the mutation can be useful for treatment.



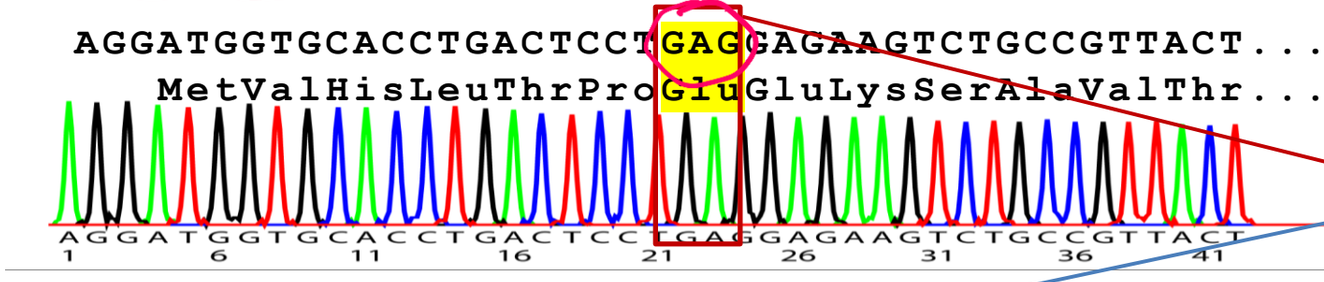


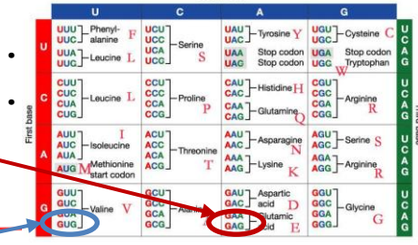
The 5' end of the Hb gene is shown on the right (ATG=start). Using GGTGCCAG as a sequencing primer gives the following sequences for the normal and mutant genes:

First dd-base added by polymerase

```
GGTGCCAGAGGATGGTGCACCTGACTCCTGAGGAGAAGTC..
CCACGGTCTCCTACCACGTGGACTGAGGACTCCTCTTCAG..
```

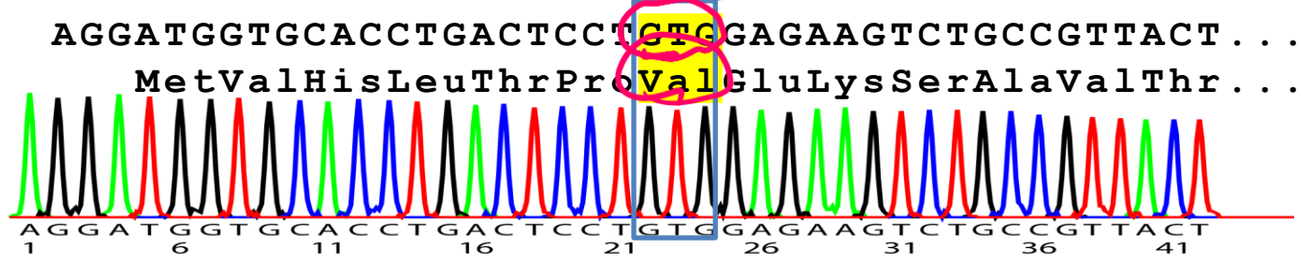
Sequencing data for the normal beta chain is:





Glu6

Sequencing data for the mutation:



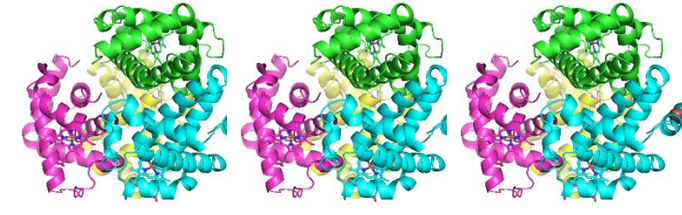
False color code:

A=Green

G=Black

T=Red

C=Blue



Val6

Sequencing Summary

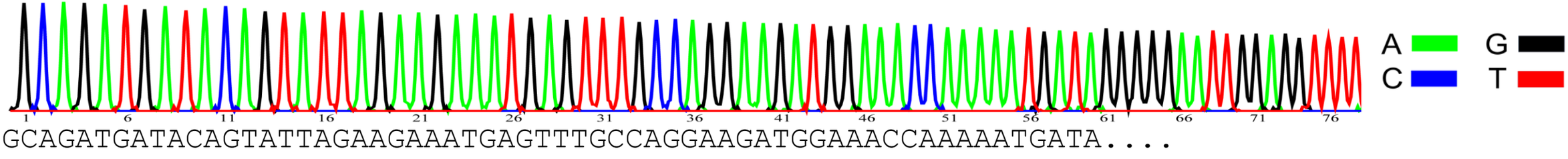
Sanger Sequencing:

- Gives the sequence that is complementary to the template strand = “top” strand, same strand at the primer.
- The start of the sequencing information is defined by a primer that anneals to the template (therefore some of the sequence has to be known, how this is done will be described later)
- Dideoxy sequencing is carried out by adding both dideoxynucleotide triphosphates (ddNTPs) and deoxyribonucleotide triphosphates (dNTPs) to the synthesis reactions, at a ratio of 1:100. Most growing chains do not terminate.
- ddNTPs are identical to dNTPs except that they lack the 3' hydroxyl group. Because of the missing 3’-OH, DNA polymerization stops once one ddNTP is added to a growing strand.
- The type of the added base is determined by “color coding” each base.
- The location of added bases is determined by measuring the size of the DNA fragment that was terminated by the ddNTP.
- It is possible to sequence approximately 1000 bases by this method.

Next Gen-Sequencing:

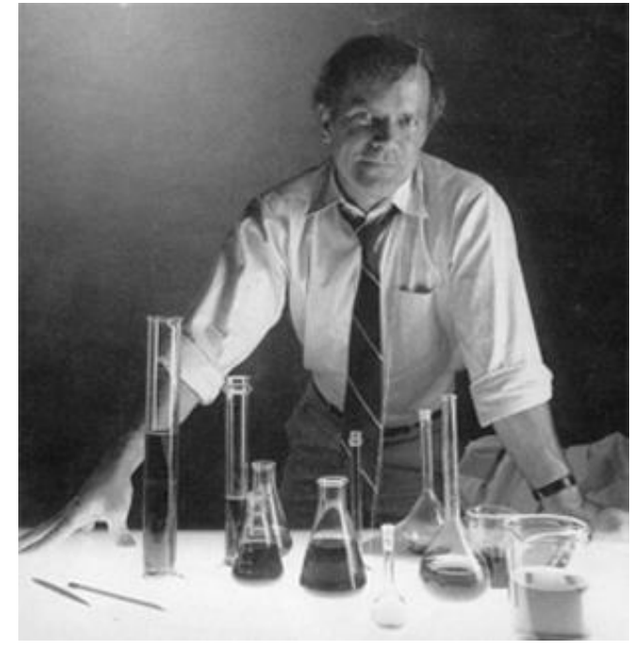
- Simultaneous sequencing of a large number of fragments
- Shorter “reads” 100 versus 1000 bases/template

8:30
8:35



Polymerase Chain Reaction - PCR

- In 1983, Kary Mullis developed the molecular biology technique that has since revolutionized genetic research, earning him the Nobel Prize in 1993.
- PCR had an impact on four main areas of biotechnology: gene mapping, cloning, DNA sequencing, and gene detection (e.g. coronavirus).
- PCR is now used as a medical diagnostic tool to detect specific mutations that may cause genetic disease, in criminal investigations and courts of law to identify suspects on a molecular level, and in the sequencing of the human genome.

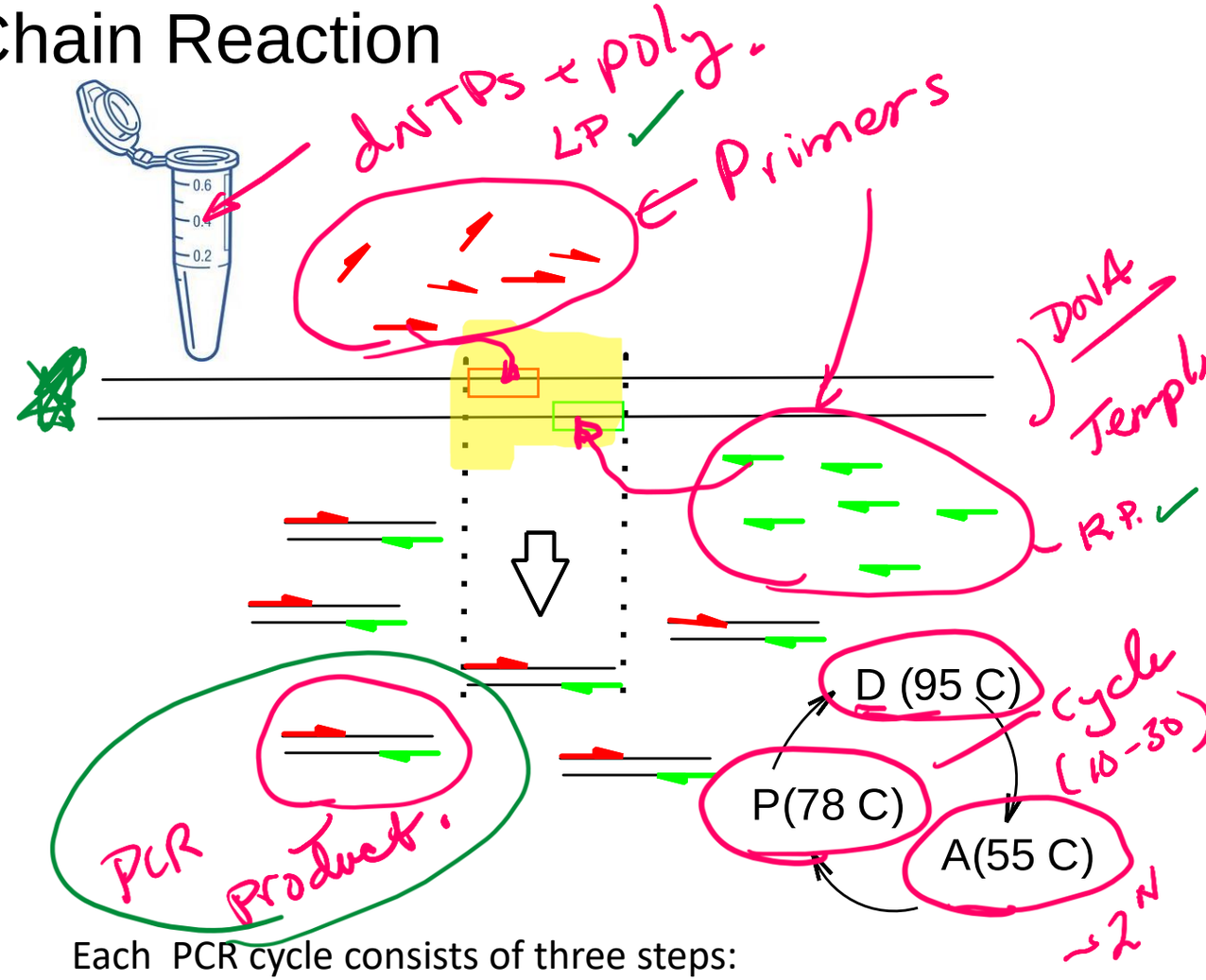


Expectations:

1. Be able to explain how PCR works to amplify a segment of DNA.
2. Be able to give the left and right primers.
3. Apply PCR approaches to determine genotype and detection of viruses.

Polymerase Chain Reaction

- PCR is an *in vitro* DNA synthesis reaction in which a specific section of DNA is replicated over and over generating exponentially large amounts of a specific piece of DNA from trace amounts of starting material (template).
- Template can be trace amounts of DNA from a drop of blood, a single hair follicle, or a cheek cell.
- The region of DNA that is copied is specified by the sequence of two primers, which are short ssDNA that initiate polymerase activity. The primers are in vast excess over the DNA.
- The location of the amplified segment is *defined* by two primers (left = upstream, right = downstream):
  - they anneal to their templates according to Watson-Crick pairing rules (A-T, G-C),
  - initiate polymerization from those sites,
  - they are incorporated into the final PCR product.
  - Left primer = sequence of top strand at left boundary
  - Right primer = sequence of bottom strand at right boundary
- The primers are DNA and are synthesized chemically, they can be any desired sequence.
- If there is no homology between the primers and the input DNA, then no PCR product will be formed.



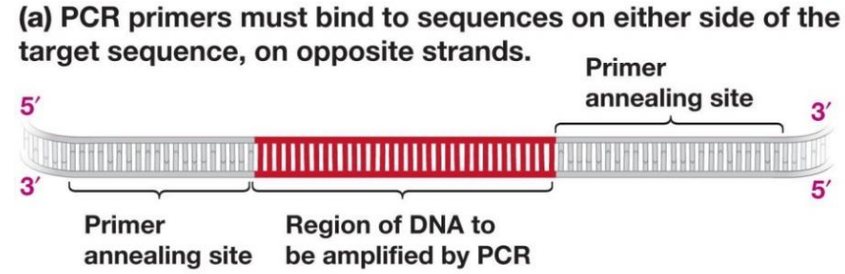
Each PCR cycle consists of three steps:

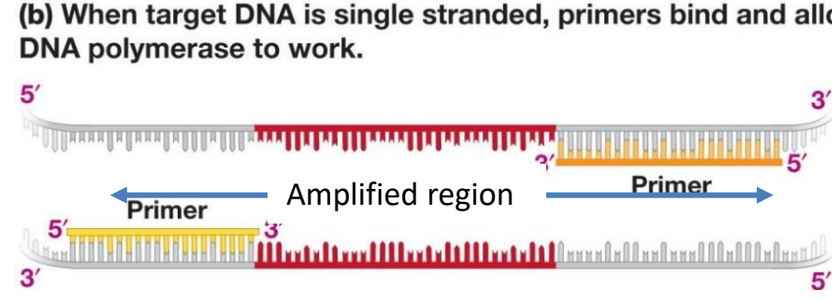
1. Denaturation of the DNA to make it single stranded (2 min at 98 C)
2. Lowering of temperature to let the primers form double-stranded DNA (1 min at 55 C)
3. Elongation by DNA polymerase (1 min/kb at 78 C)

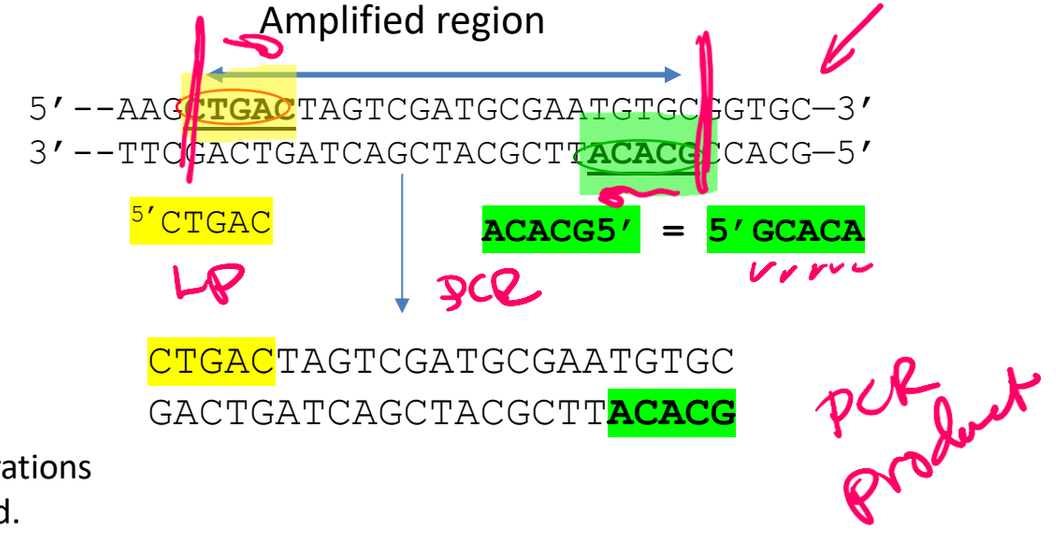
PCR – Primer Design

- Before a region of DNA can be amplified, one must identify and determine the sequence of a piece of DNA upstream and downstream of the region of interest.
- These areas are then used to determine the sequence of oligonucleotide primers that will be synthesized and used as starting points for DNA replication.
- Primers are complimentary to the up- and down-stream regions of the sequence to be amplified, so they stick, or anneal, to those regions.
  - Left primer = sequence of top strand on the left. This primer will anneal to the bottom strand.
  - Right primer = sequence of bottom strand on the right. This primer will anneal to the top strand.
- Primers are in large excess over the template DNA, they are never used up.
- The primers are incorporated into the final PCR product.

Know these rules!

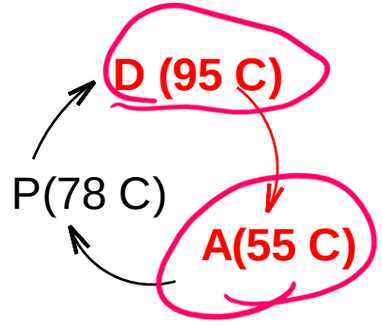






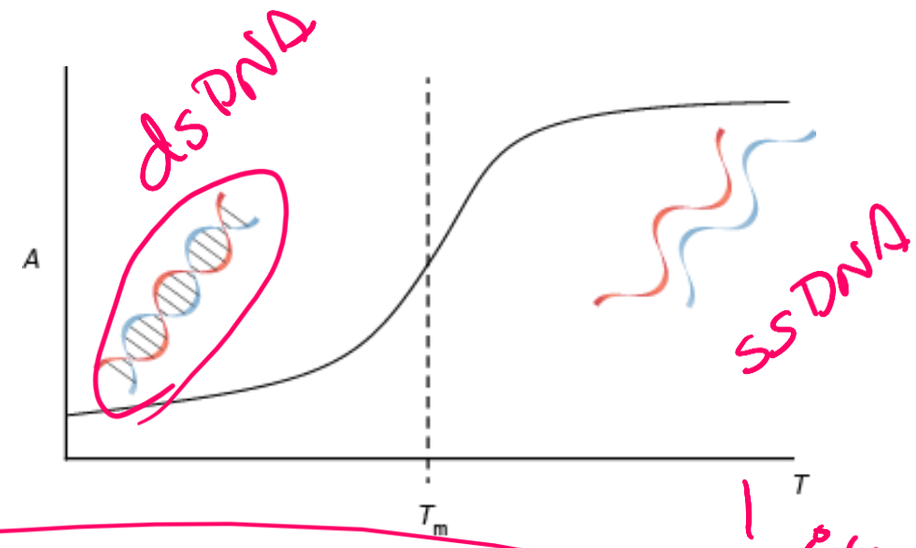
Note: Actual primer lengths are 20-30 bases, in the illustrations here and on problem sets, much shorter primers are used.

PCR Step 1 - Thermal Stability of Double Stranded DNA (dsDNA)



3′A-T-G-C-C-G-T-A-G-T-C-G-T-A-C-A-G-T-A-C-G-T-G-C-A

5′ A-T-C-A



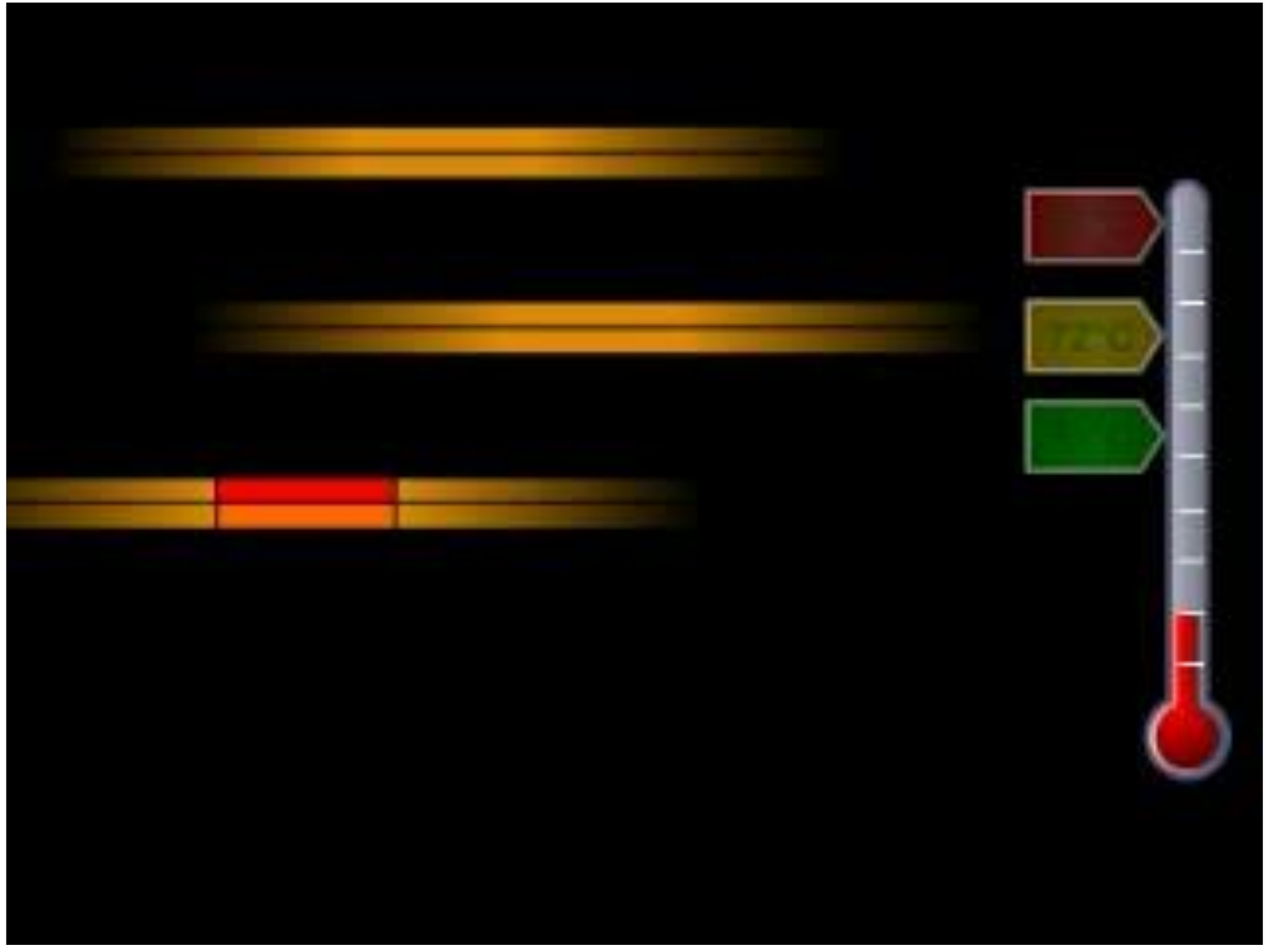
5′ A-T-C-A

3′A-T-G-C-C-G-T-A-G-T-C-G-T-A-C-A-G-T-A-C-G-T-G-C-A

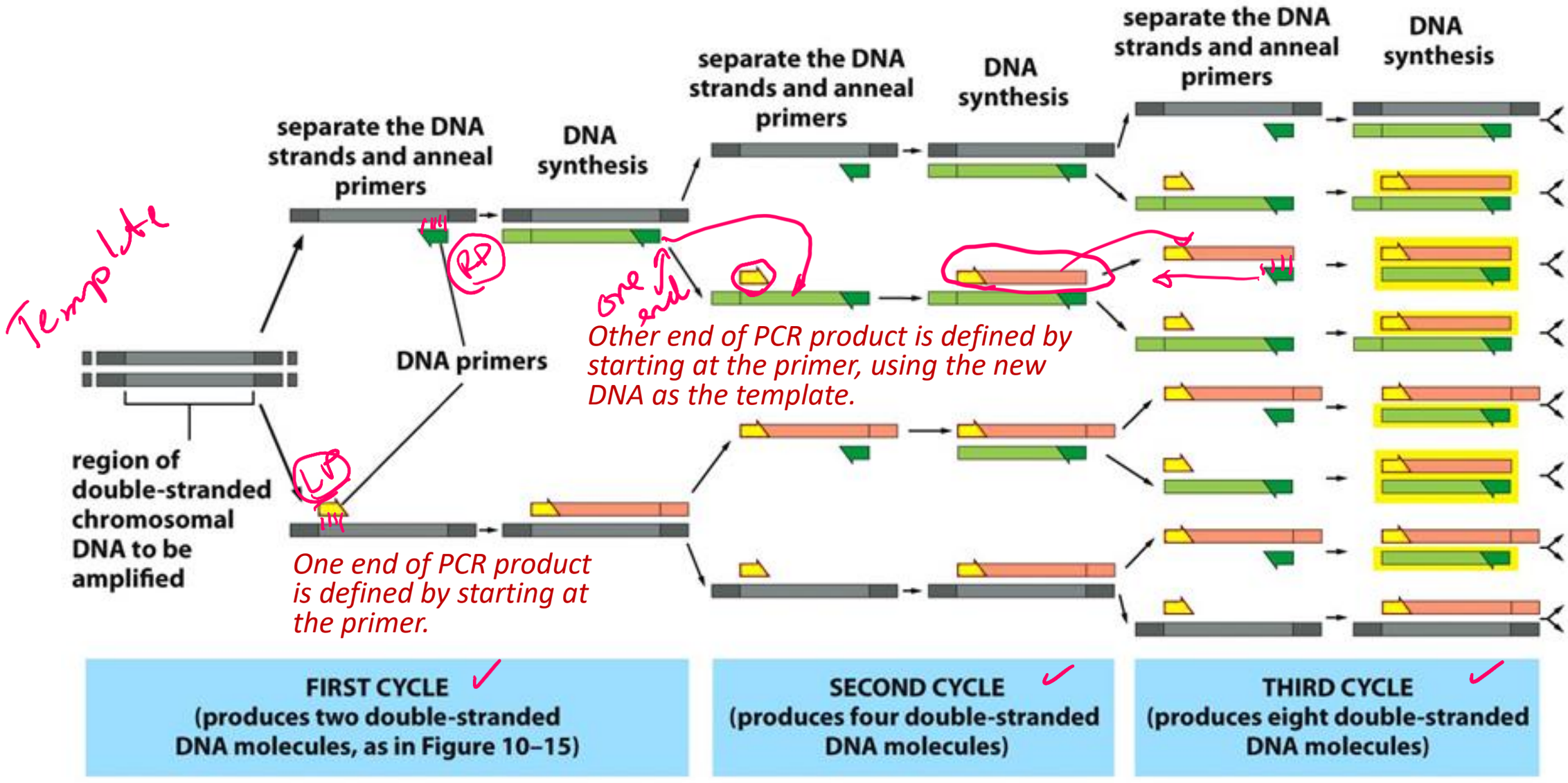
- Polymerase Characteristics
  - Since the first step of each cycle (D) requires heating to high temperature, a thermostable polymerase is required.
  - The first polymerase, Taq, was isolated from *Thermus Aquaticus,* a bacterial living in hot springs (Yellowstone National Park)
  - A number of different polymerases with improved properties have been developed.

PCR Animation

Watch Me!



Three PCR Cycles



After 30 cycles there will be 2^{30}, or over 1 billion times more copies than at the beginning!!!

Detailed Events During First Three PCR Cycles

Note:

- Polymerization starts at the primer (add to 3'-OH)
- Polymerase always goes to the end of the template.

Detailed Events during first Three PCR Cycles

Cycle 3

5′CTGACTAGTCGATGCGAATGTGC
--TTCGACTGATCAGCTACGCTTACACG5′

5′CTGACAGTCGATGCGAATGTGCGGTGC--
GACTGTCAGCTACGCTTACACG5′

Denature & Anneal

5′CTGACTAGTCGATGCGAATGTGC
ACACG5′

--TTCGACTGATCAGCTACGCTTACACG5′

5′CTGACAGTCGATGCGAATGTGCGGTGC--

5′CTGAC
GACTGTCAGCTACGCTTACACG5′

Polymerase

5′CTGACTAGTCGATGCGAATGTGC
GACTGATCAGCTACGCTTACACG5′

--TTCGACTGATCAGCTACGCTTACACG5′

5′CTGACAGTCGATGCGAATGTGCGGTGC--

5′CTGACAGTCGATGCGAATGTGC
GACTGTCAGCTACGCTTACACG5′

Now have complete PCR product.
The product will double in each of the following cycles.

Note that the primers are the first bases at the ends of each strand of the PCR product.

Example – follow the PCR cycles for the following template with primers 5′ AATT (left) and 5′ GGCC (right)

----AATT----------GGCC----
----TTAA----------CCGG----

cycle 1

cycle 2

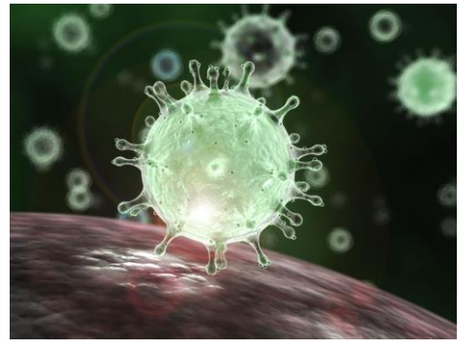
cycle 3

AATT----------GGCC
TTAA----------CCGG

PCR product

PCR Applications - Detection of Viruses

Coronavirus



Sequence of Covid-19 (top strand only)

```
1     attaaaggtt tataccttcc caggtaacaa accaaccaac tttcgatctc ttgtagatct
61    gttctctaaa cgaactttaa aatctgtgtg gctgtcactc ggctgcatgc ttagtgcact
121   cacgcagtat aattaataac taattactgt cgttgacagg acacgagtaa ctcgtctatc
181   ttctgcaggc tgcttacggt ttcgtccgtg ttgcagccga tcatcagcac atctaggttt

28261 cgaacaaact aaaatgtctg ataatggacc ccaaaatcag cgaaatgcac cccgcattac
28321 gtttggtgga ccctcagatt caactggcag taaccagaat ggagaacgca gtggggcgcg
28381 atcaaaacaa cgtcggcccc aaggtttacc caataatact gcgtcttggt tcaccgctct
28441 cactcaacat ggcaaggaag accttaaatt ccctcgagga caaggcgttc caattaacac

29701 gggaggactt gaaagagcca ccacattttc accgaggcca cgcggagtac gatcgagtgt
29761 acagtgaaca atgctaggga gagctgccta tatggaagag ccctaatgtg taaaattaat
29821 tttagtagtg ctatccccat gtgattttaa tagcttctta ggagaatgac aaaaaaaaaa
29881 aaaaaaaaaa aaaaaaaaaa aaa
```

CDC Recommended PCR Primers

2019-Novel Coronavirus (2019-nCoV) Real-time rRT-PCR Panel Primers and Probes

Name	Description	Oligonucleotide Sequence (5'>3')	Label[1]	Working Conc.
2019-nCoV_N1-F	2019-nCoV_N1 Forward Primer	5'-GAC CCC AAA ATC AGC GAA AT-3'	None	20 µM
2019-nCoV_N1-R	2019-nCoV_N1 Reverse Primer	5'-TCT GGT TAC TGC CAG TTG AAT CTG-3'	None	20 µM

dsSeq of above bold & circled region

```
28271 aaaatgtctgataatggACCCCAAAATCAGCGAAATgcaccccgcattacgtttggtggaccctcagattcaactggcagtaaccagaatggagaacgca
      ttttacagactattacctggggttttagtcgctttacgtggggcgtaatgcaaaccacctgggaGTCTAAGTTGACCGTCATTGGTCTtacctcttgcgt
```

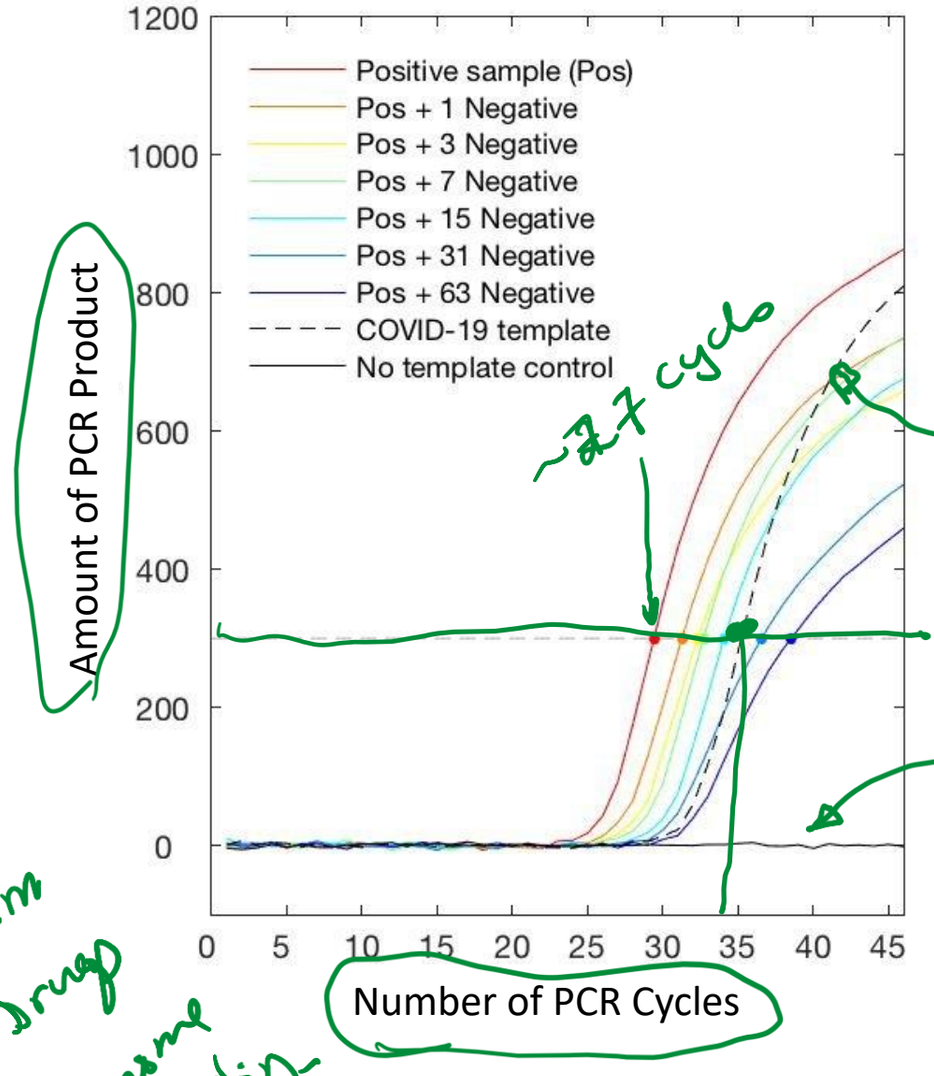
PCR Product

GACCCCAAAATCAGCGAAATGCACCCCGCATTACGTTTGGTGGACCCTCAGATTCAACTGGCAGTAACCAGA
CTGGGGTTTTAGTCGCTTTACGTGGGGCGTAATGCAAACCACCTGGGA**GTCTAAGTTGACCGTCATTGGTCT**

Will PCR generate products if the viral DNA is not present?

Covid 19 PCR Test: Detection of the PCR Product.

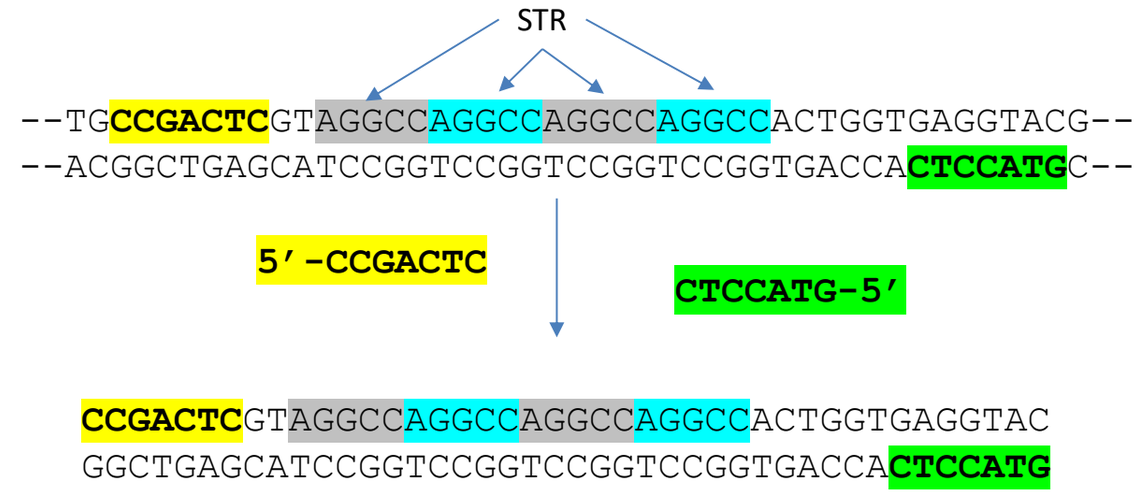
https://www.medrxiv.org/content/10.1101/2020.03.26.20039438v1



- Production of PCR products (double stranded DNA) causes an increase in signal (fluorescence)
- Signal above 300 considered to be positive (dashed gray line)
- Dots represent when a sample crosses the fluorescence threshold.
- Red curve (Positive sample) shows a threshold level of PCR product after 27 cycles.
- - - - is a ***positive control*** amount of Covid template. It shows that you can detect a PCR product if the covid genome is present.
- Solid black line is a ***negative control***, no Covid DNA. It shows that addition of covid template will lead to a signal.
- Other samples are the positive sample mixed with up to 63 negative samples, showing that it is possible to test pooled samples.

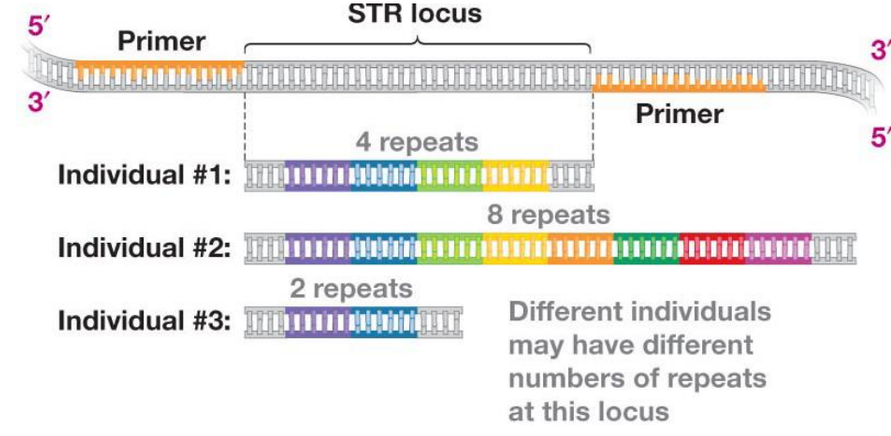
PCR Applications – Identification of Individuals

- Regions of DNA have variable numbers of repeated DNA sequences (Short tandem repeats, STR). The number of STR can differ from one person to the next.
- Individuals will inherit one copy of the repeat from each parent. The length of the inherited DNA can be the same or different.
- PCR Primers are designed to be outside the repeated region, so that they will anneal to a single location on the chromosome and then amplify the region containing the STR
- PCR Product length = primer lengths + number of tandem repeats (+ any DNA between the primers and the repeats). *Individuals can be differentiated by the length of the PCR product if they have different numbers of STR*



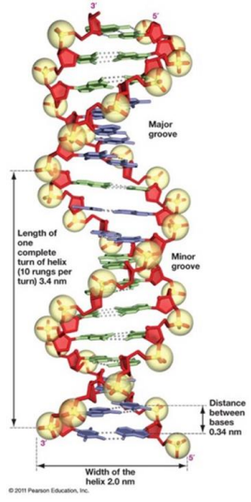
Which individual has the shortest PCR product?

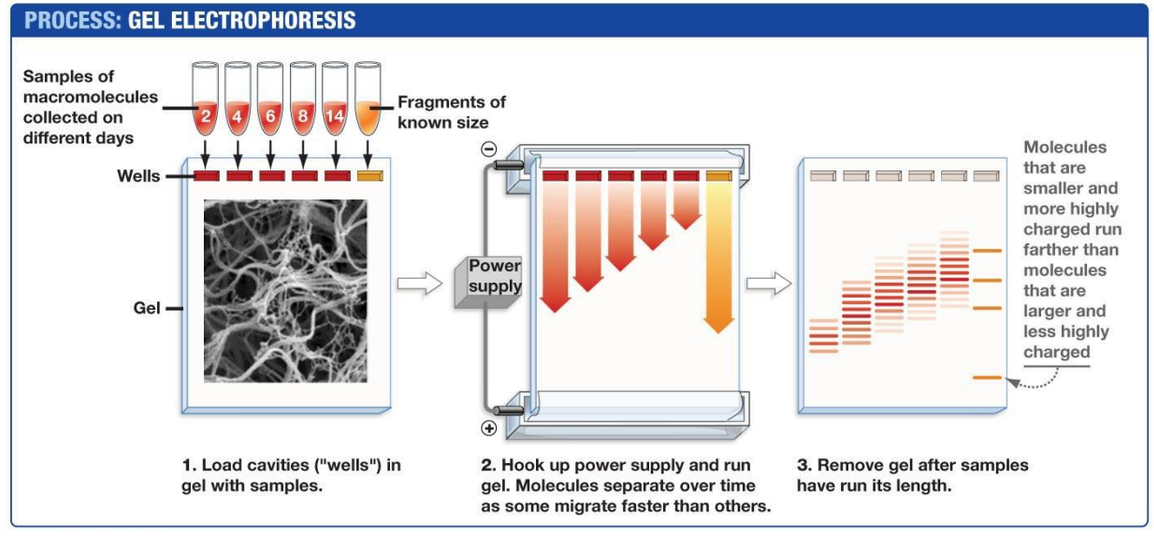
Which has the longest?

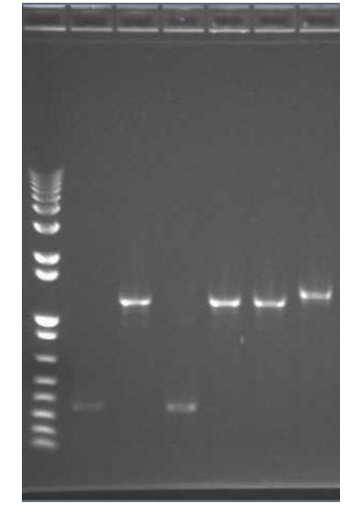


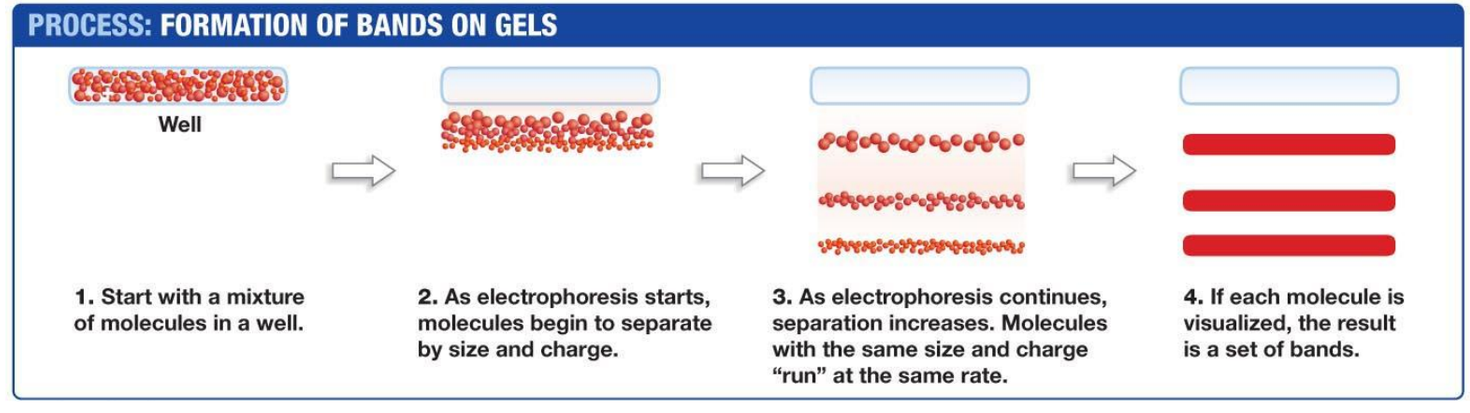
Size Determination of PCR products - Agarose Gel Electrophoresis.

https://dnalc.cshl.edu/resources/animations/gelelectrophoresis.html





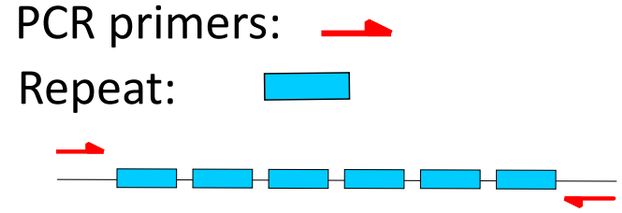




Which are the smallest PCR fragments?

Short Tandem Repeats to Test Paternity

1. DNA samples (blood, cheek cells) would be obtained from:
   - Mother
   - Child
   - Candidate fathers.
2. PCR would be preformed using primers that amplify a segment of the chromosome containing repeats.
3. Each individual would show 2 bands on the gel, corresponding to the PCR product from each chromosome (we have two copies of each chromosome).
4. The child would inherit one copy from the mother and the other from the father:
   - One of the child's PCR product would match one of the mothers.
   - The other PCR product from the child would match one of the PCR products from the father.



Lane 1: Child
Lane 2: Mother

Lanes A, B, C: Possible Fathers

1. *For the child, which PCR product is from the mother? From the father?*

2. *Who is **not** the father?*

3. *Who **may** be the father?*