Lecture 4
DNA Technologies, Immunology

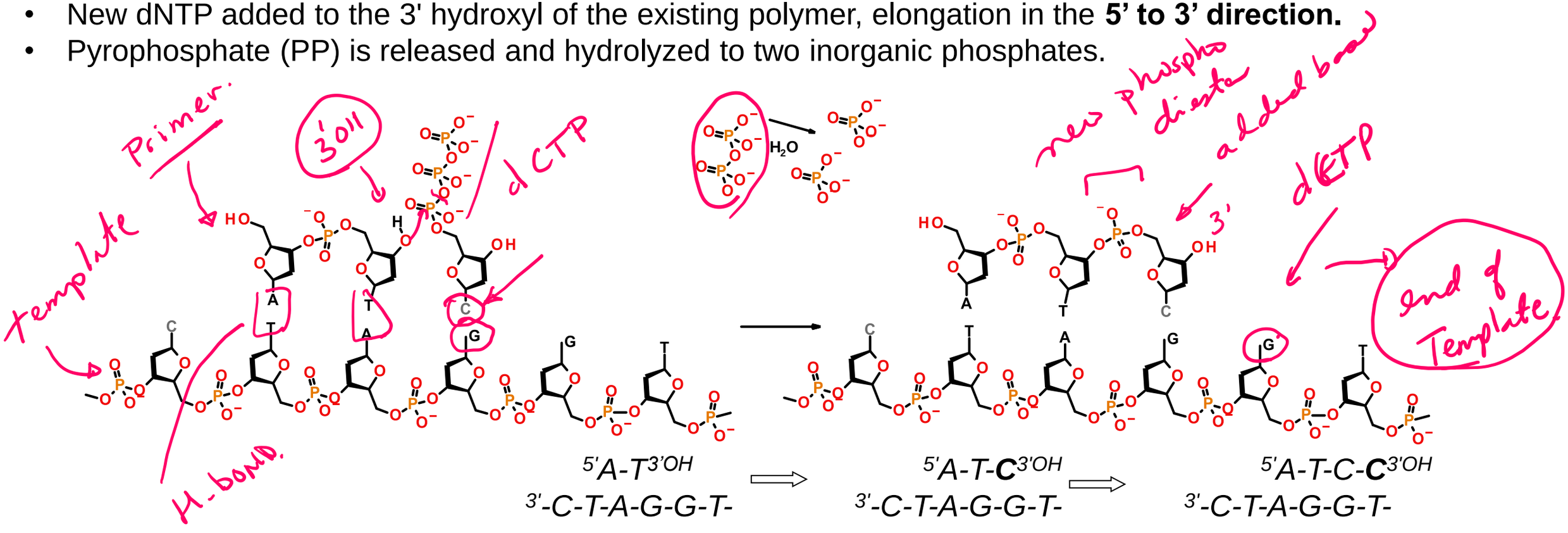
- Review of DNA polymerases
- DNA Sequencing
- Polymerase chain reaction (PCR) & Applications

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

Please send me your presentation topics as soon as you can.

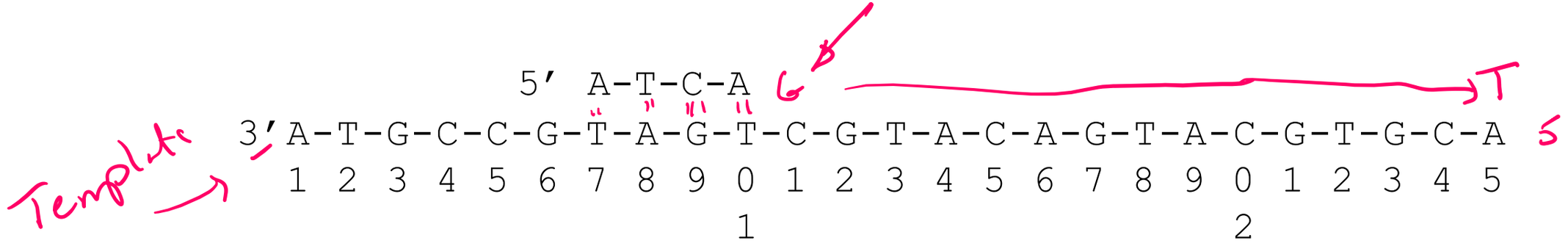
DNA Polymerases – Used in DNA Sequencing and PCR

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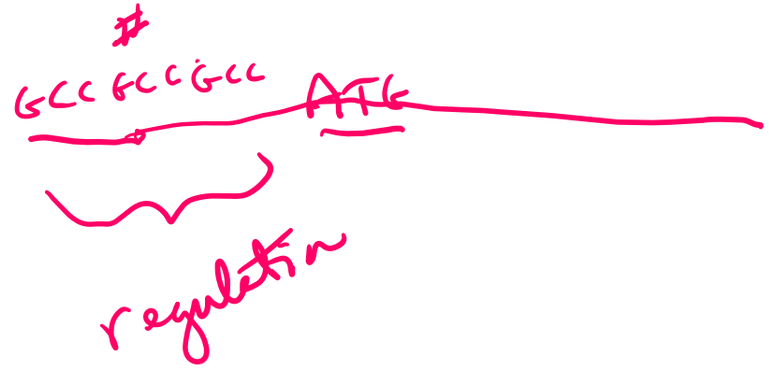
https://doi.org/10.1016/B978-0-444-63233-3.00009-9

Second base

t base	U	C	A
U	UUU, UUC – Phenylalanine UUA, UUG – Leucine	UCU, UCC, UCA, UCG – Serine	UAU, UAC – Tyrosine UAA, UAG – Stop codon
C	CUU, CUC, CUA, CUG – Leucine	CCU, CCC, CCA, CCG – Proline	CAU, CAC – Histidine CAA, 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 = GluGluGlu
- 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

-TATATCCAGCAGCAGAGTATA-
-ATATAGGTCGTCGTCTCATAT-

- During Replication in the cell

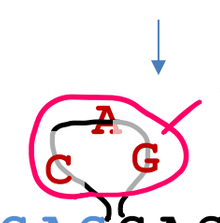
-TA 3' → Pol
-ATATAGGTCGTCGTCTCATAT-

-TATATCCAGCAGCAG 3'
-ATATAGGTCGTCGTCTCATAT-

Primer slippage 3' end comes loose

-TATATCCAGCAGCAG
-ATATAGGTCGTCGTCTCATAT-

Looped out DNA



-TATATCCAGCAG
-ATATAGGTCGTCGTCTCATAT-

Replication continues

-TATATCCAGCAGCAGCAGAGTATA-
-ATATAGGTCGTCGTCTCATAT-

Next replication (upper strand as the template)

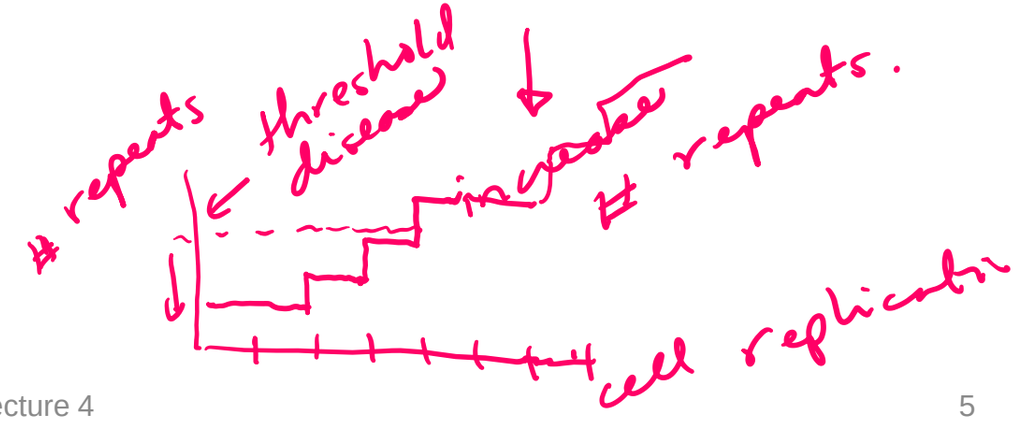
template

1 2 3 4

-TATATCCAGCAGCAGCAGCAGAGTATA-
TCATAT-
Pol

4 repeats

-TATATCCAGCAGCAGCAGCAGAGTATA-
-ATATAGCTCGTCGTCGTCGTCTCATAT-

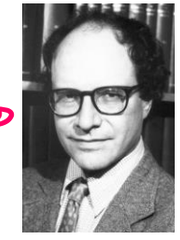


DNA Sequencing – Sanger (dideoxy) Sequencing

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

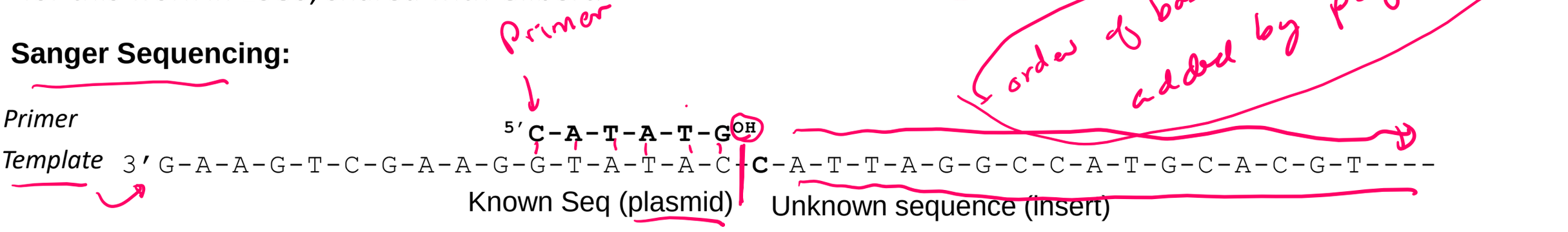
Sanger Sequencing:

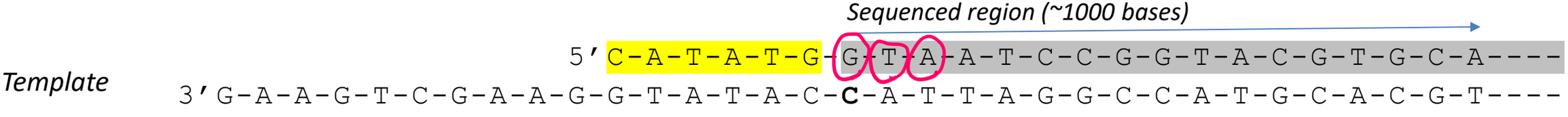
- Second method to generate long (~1000 base) sequence information (an earlier chemical method developed by Gilbert proved to be impractical for most laboratories (hydrazine = rocket fuel was required)
- Sanger was awarded his 2nd Nobel prize for this work in 1980, shared with Gilbert.



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

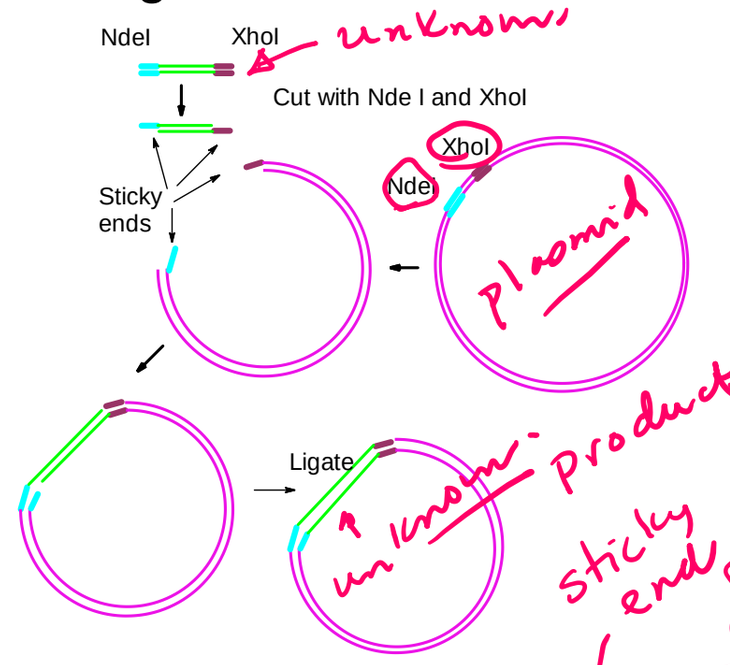
Sanger Sequencing:





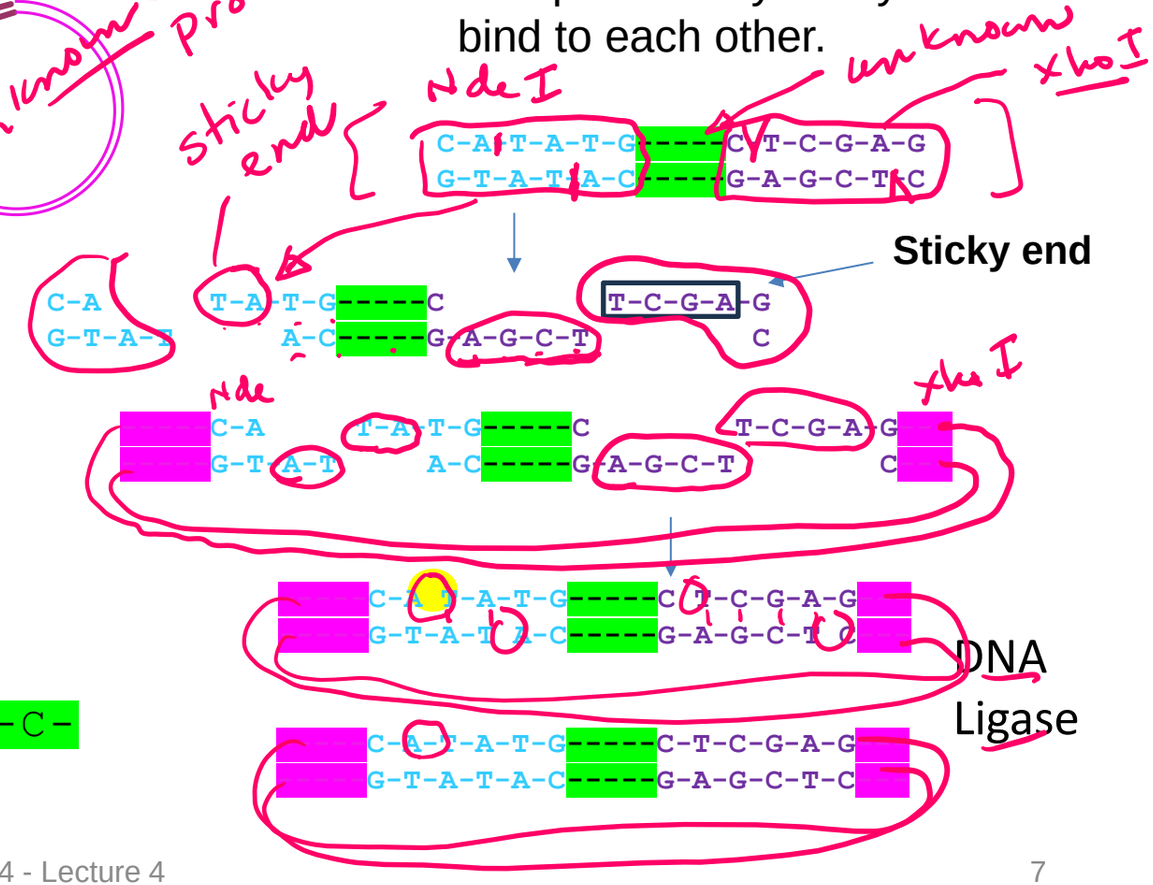
DNA Sequencing - Determining the Order of Bases Added by DNA Polymerase

- 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)

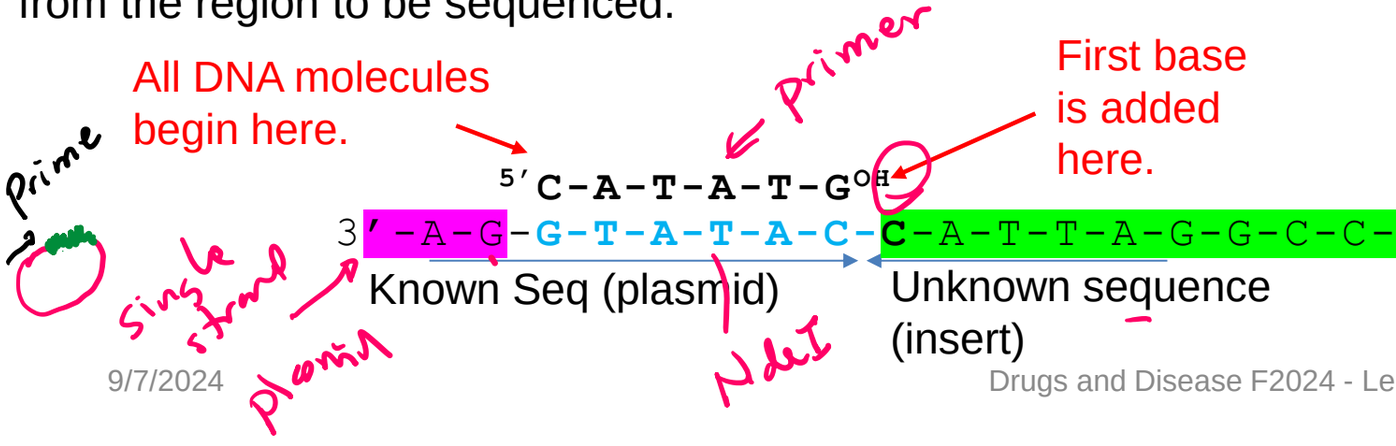


Restriction Enzymes

- Recognize a specific sequence in the DNA res. site.
- 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.

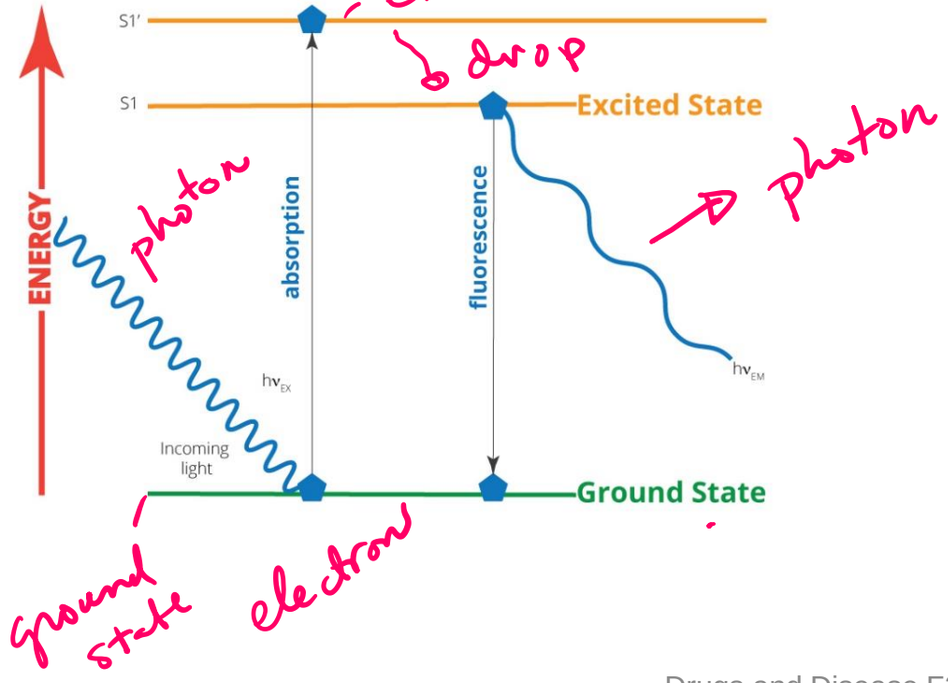


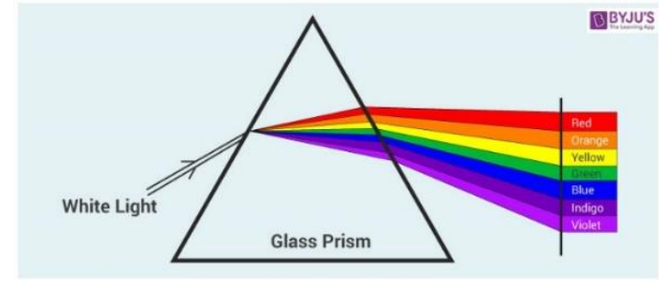
1. Start sequencing at known location with primer that anneals at a ***unique*** location on the plasmid, "upstream" from the region to be sequenced.



DNA Sequencing Methods Use Fluorescent Bases - What is Fluorescence?

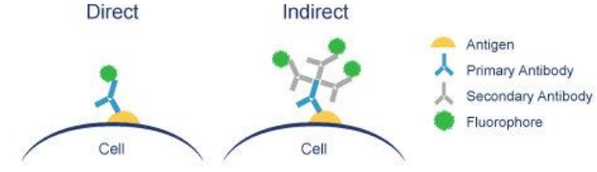
- When molecules absorb light an electron goes from a lower shell to a higher shell. This is where the energy from the light goes.
- In most molecules the electron goes back down to its original shell with the release of heat.
- Fluorescent molecules emit the energy as light of a longer wavelength (different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

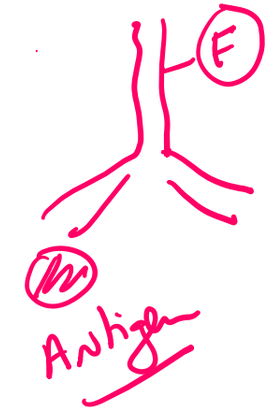




← diff colors emitted light.

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

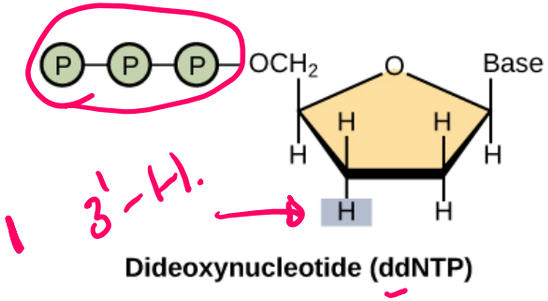


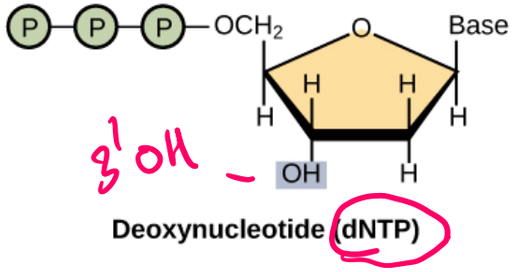


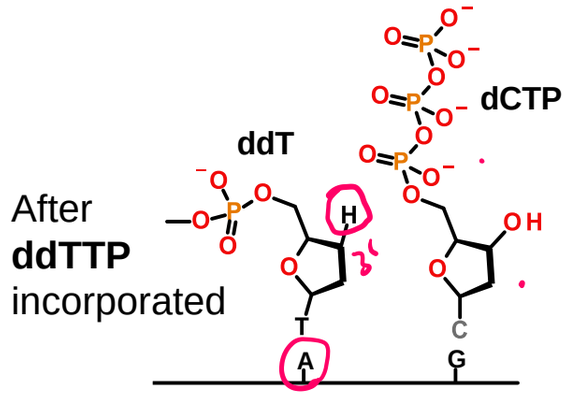
DNA Sequencing - Determining the Order of Bases Added by DNA Polymerase

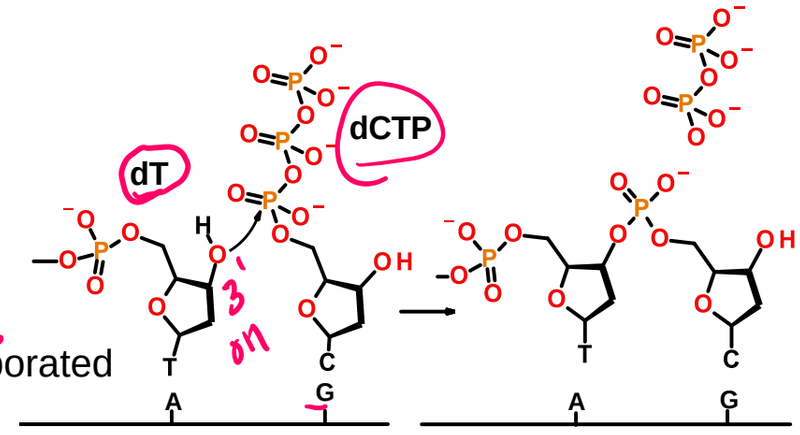
2. Use a mixture of normal bases (dNTPs) and dideoxy bases (ddNTP) for polymerization. Ratio of dNTP to ddNTP is (100:1), ***most of the time elongation occurs.***

- 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



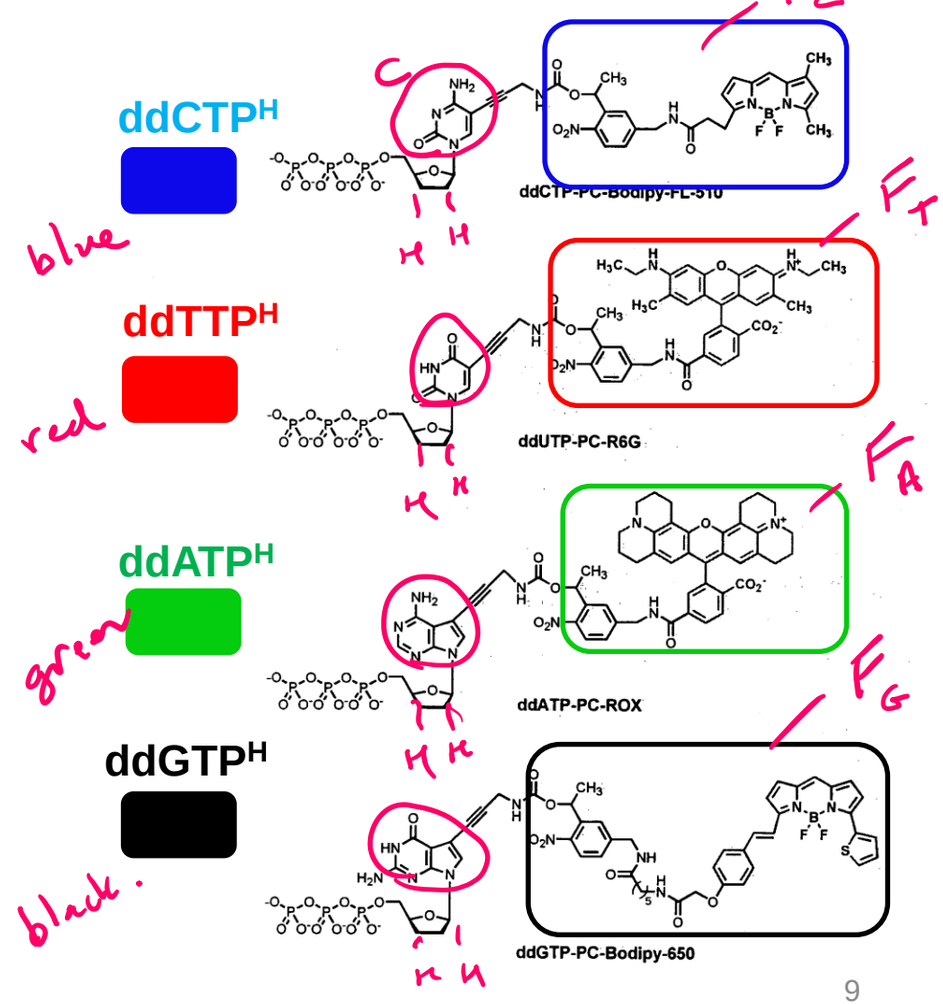




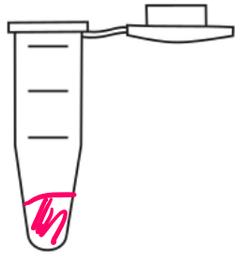


3. The ddNTPs are color coded by different fluorescent emission wavelengths.

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



DNA Sequencing – Generation of Fluorescent Fragments



Template
Primer
DNA Pol
dNTP, 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)

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

dGTP (990) 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

Length=7, Black fluor

2nd

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)

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

dTTP (980) 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

Length=8, Red fluor.

3rd

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)

ddATP (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

dATP (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

Length=9, Green fluor

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

Sort by length

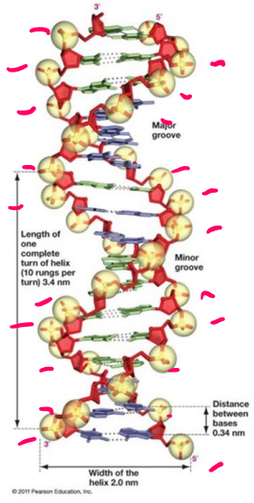
C-A-T-A-T-G-G
C-A-T-A-T-G-G-T
C-A-T-A-T-G-G-T-A
C-A-T-A-T-G-G-T-A-A
C-A-T-A-T-G-G-T-A-A-T
C-A-T-A-T-G-G-T-A-A-T-C
C-A-T-A-T-G-G-T-A-A-T-C-C
C-A-T-A-T-G-G-T-A-A-T-C-C-G

Primer | Added by Pol.

All Possible Fragments are Made:

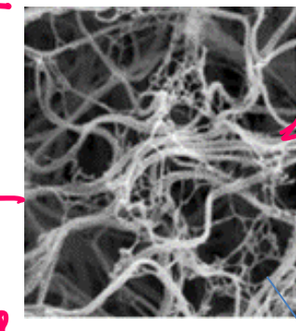
1. Each begins with the primer
2. Each ends with a *known* ddNTP, based on the color of the fluorescence.
3. Each is one longer than the previous.

Size Determination of Fragments from DNA Sequencing Capillary Electrophoresis



dsDNA

neg charge



mesh like structure

DNA

sorting by size

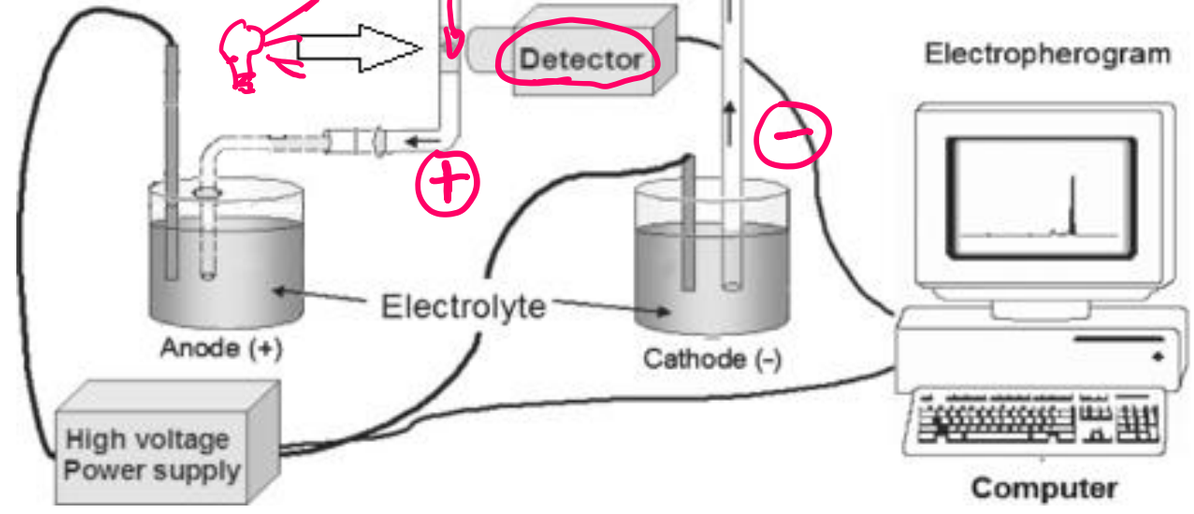
shorter DNA ⇒ faster migration

Capillary

Sample loaded

DNA

Fluorescence excitation



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 _____.

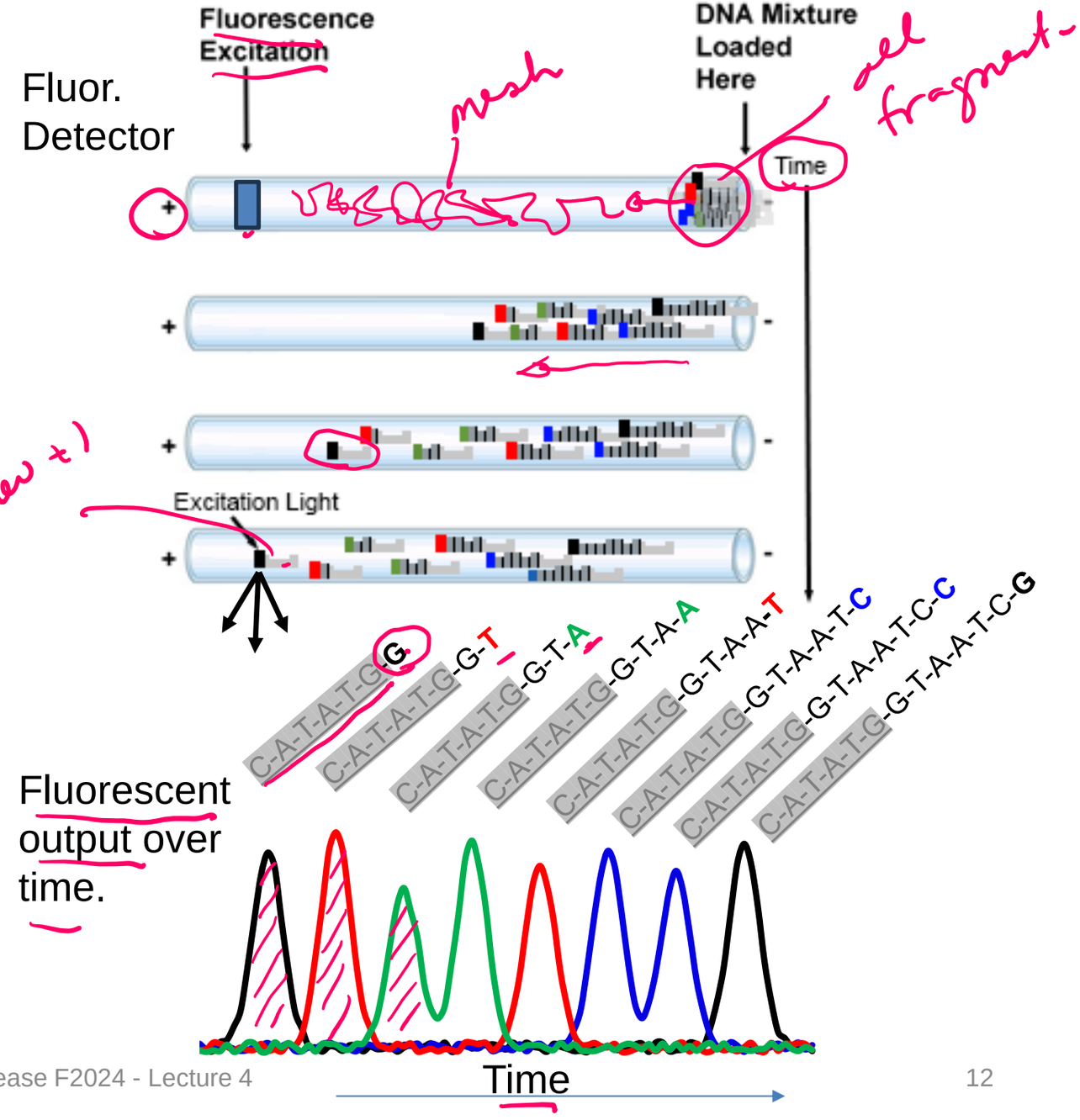
DNA Sequencing – Analysis of Fragments to Determine Order of Addition

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).

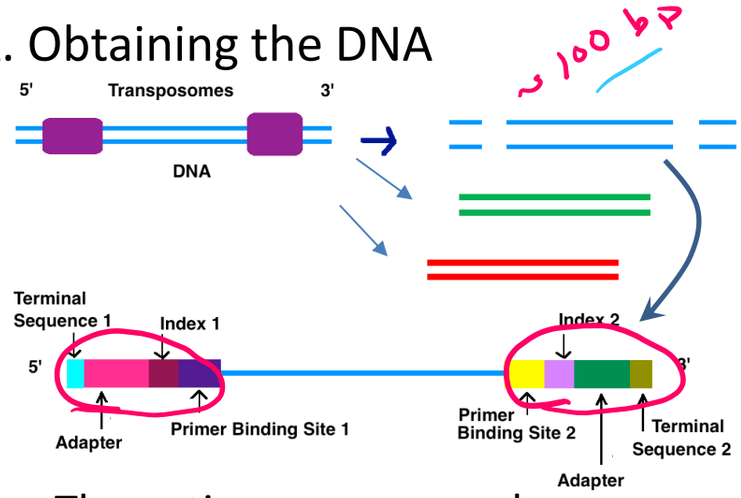
5'-C-A-T-A-T-G G-T-A-A-T-C-C-G

3'-A-G-G-C-T-A-T-A-C-C-A-T-T-A-G-G-C

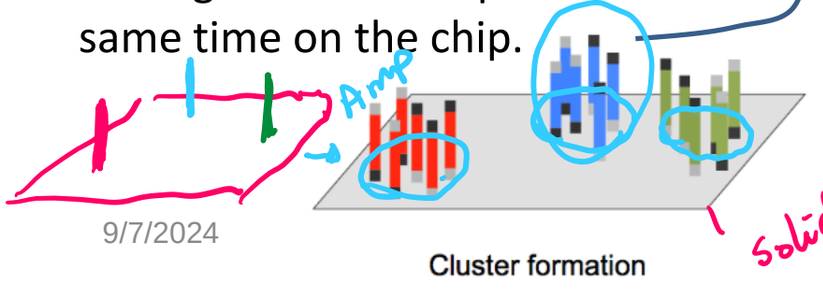


Newer Sequencing Methods-Illumina Dye Sequencing – Next Generation High Throughput

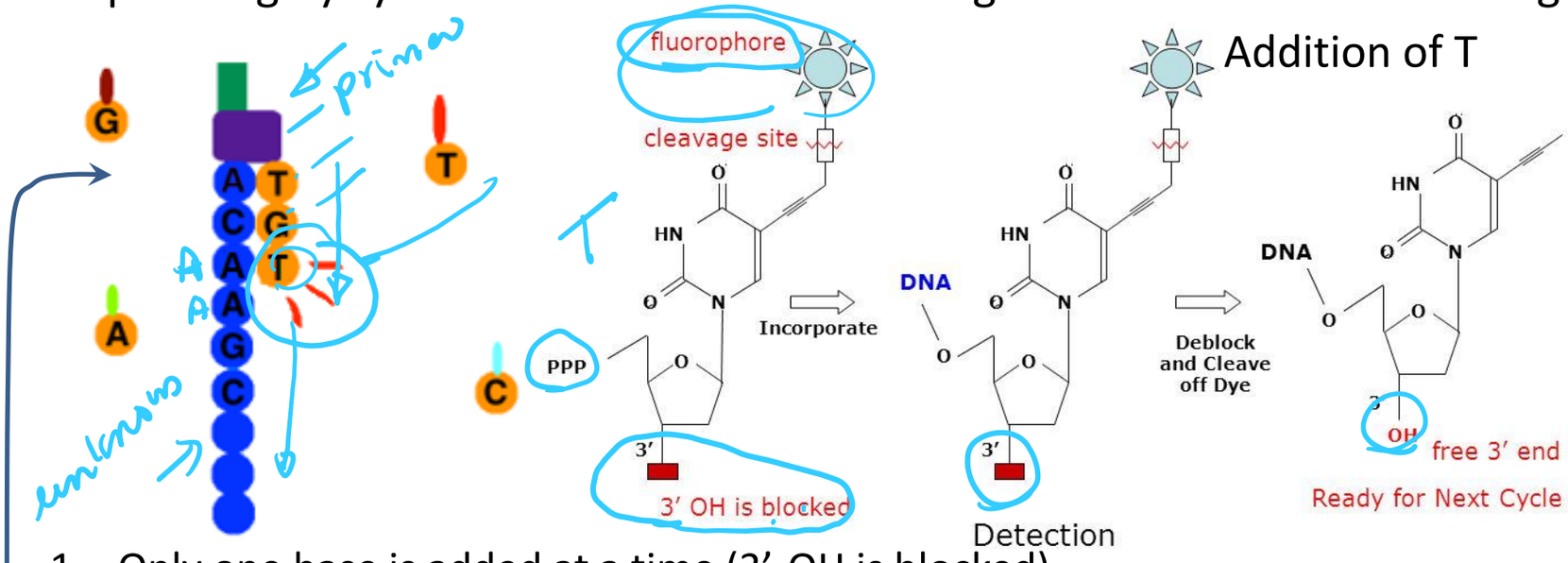
A. Obtaining the DNA



- 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



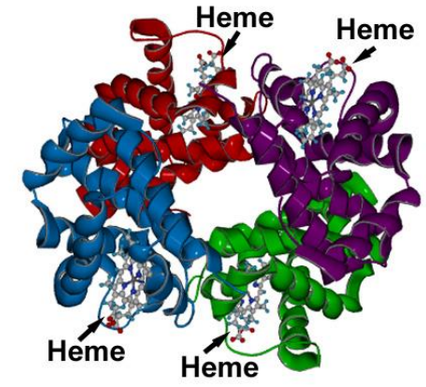
1. Only one base is added at a time (3'-OH is blocked)
2. The base that is added is determined by the color of the fluorescent base.
3. 3'-OH blocking group and the fluorescent group are removed prior to the next addition. ~100 cycles can be performed.

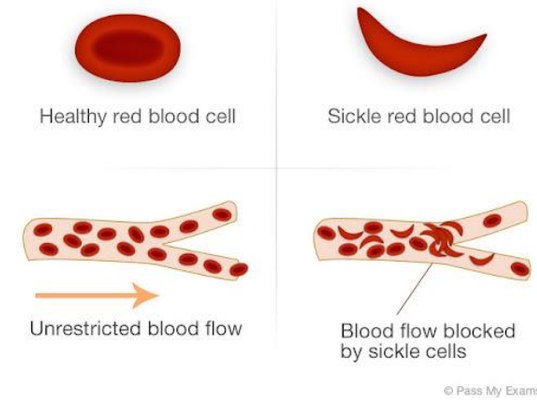
By DMLapato - Own work, CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php?curid=43777596

Method	Read Length	Samples Processed
Sanger	~1000	1
Illumina	~100	~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.





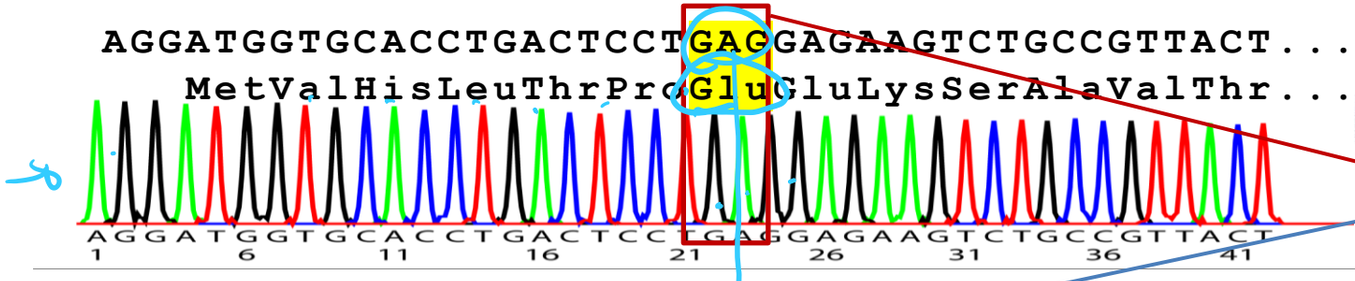
The 5' end of the Hb gene is shown on the right (ATG=start). Using GGTGCCAG as a sequencing primer gives the following sequences for the normal and mutant genes:

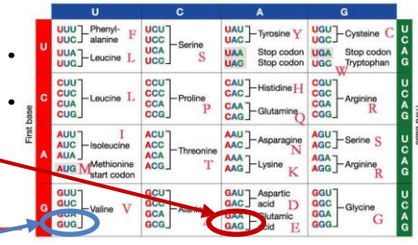
First dd-base added by polymerase

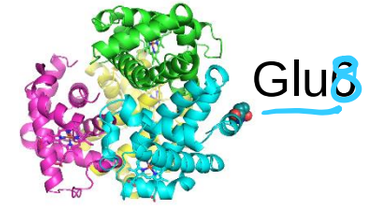
```
GGTGCCAGAGGATGGTGCACCTGACTCCTGAGGAGAAGTC..
CCACGGTCTCCTACCACGTGGACTGAGGACTCCTCTTCAG..
```

Template

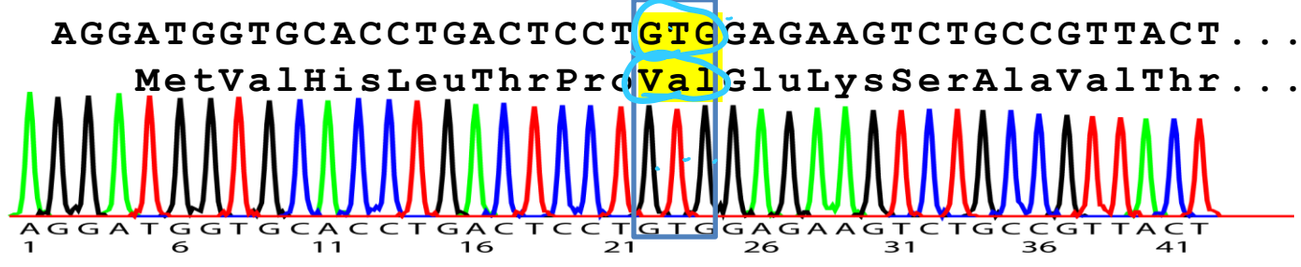
Sequencing data for the normal beta chain is:







Sequencing data for the mutation:



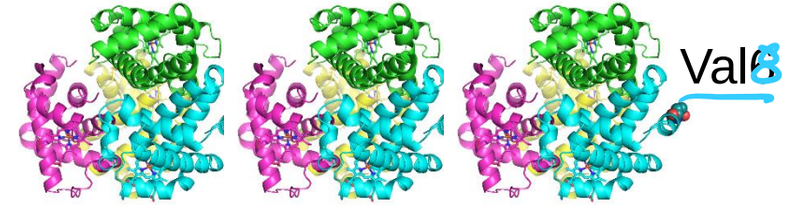
False color code:

A=Green

G=Black

T=Red

C=Blue



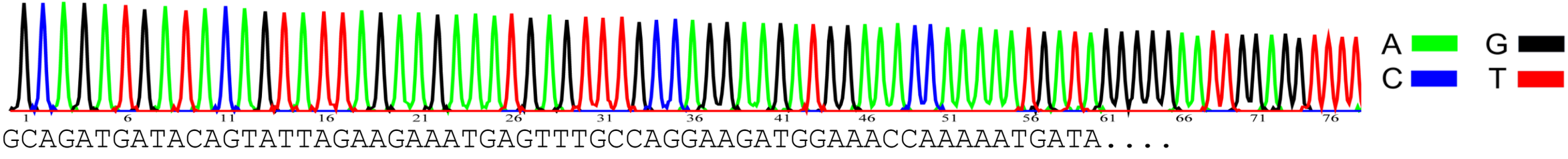
Sequencing Summary & Expectations

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.

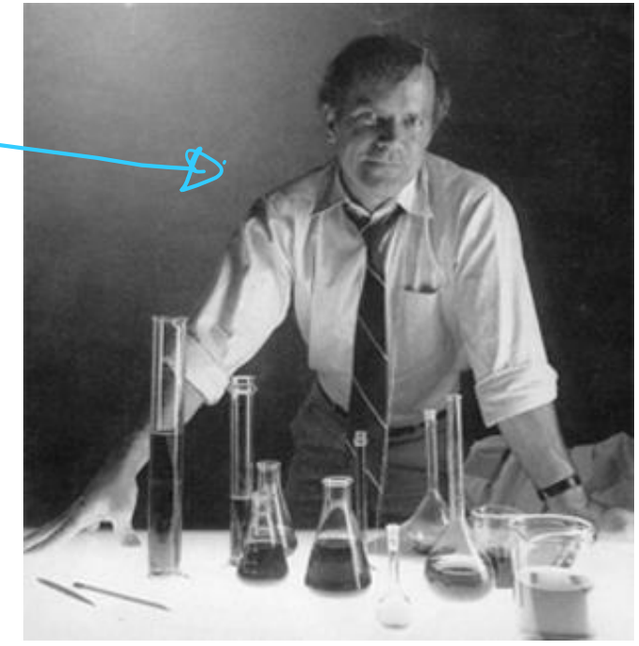
Next Gen-Sequencing:

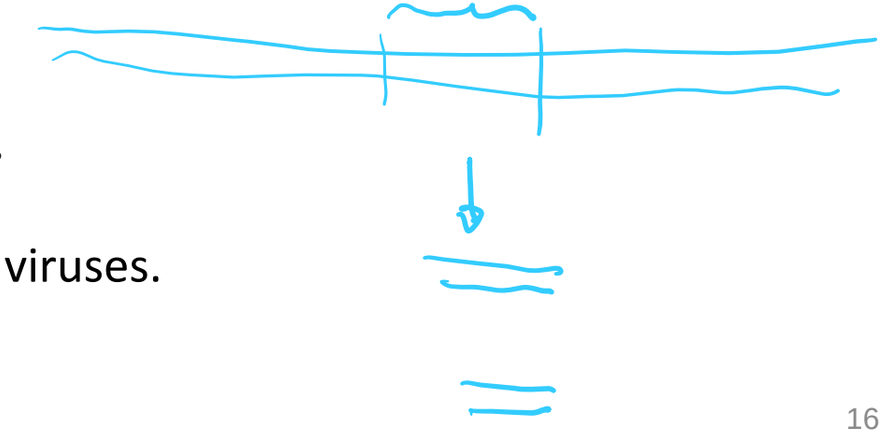
- Simultaneous sequencing of a large number of fragments
- Shorter “reads” 100 versus 1000 bases/template



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.



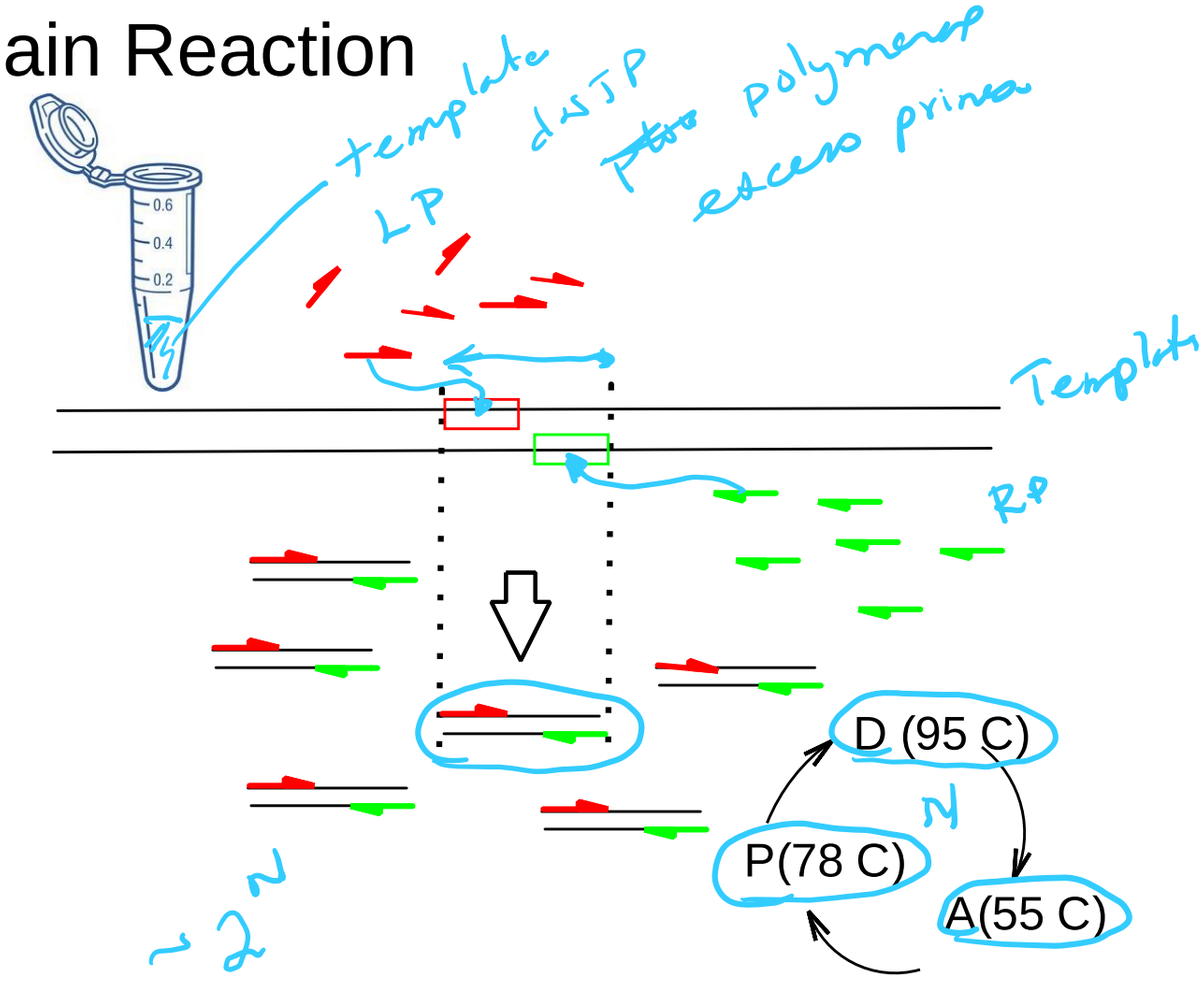


Expectations:

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.

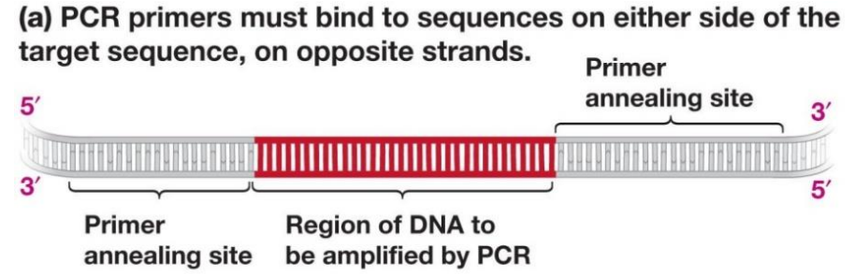


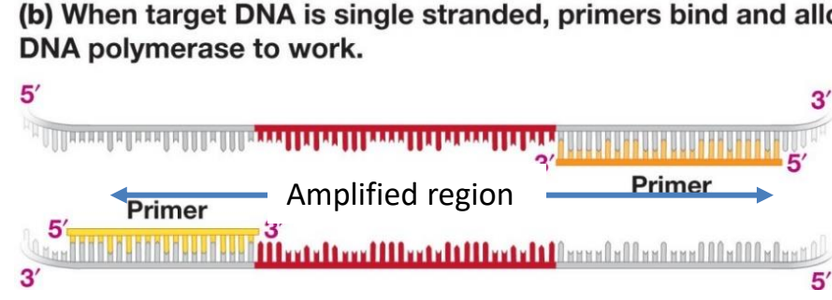
Each PCR cycle consists of three steps:

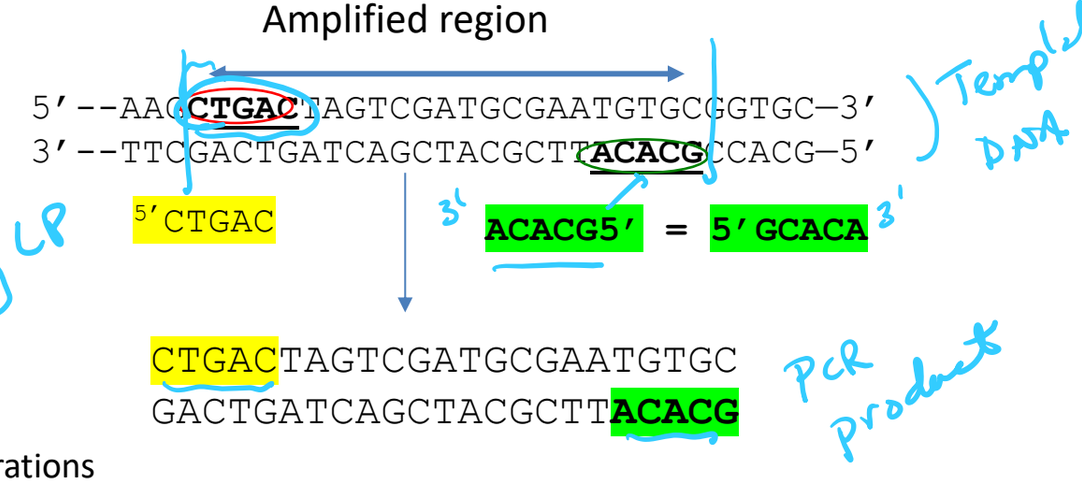
1. Denaturation of the DNA to make it single stranded (2 min at 98 C)
2. Lowering of temperature to let the primers form double-stranded DNA (1 min at 55 C)
3. Elongation by DNA polymerase (1 min/kb at 78 C)

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.



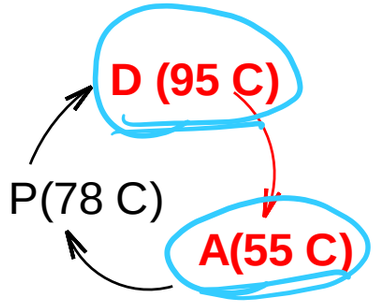




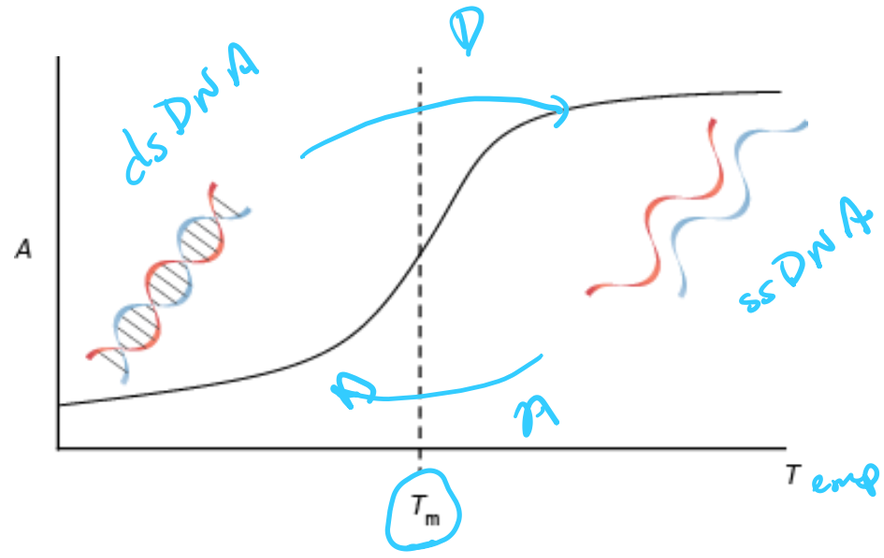
Know these rules!

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



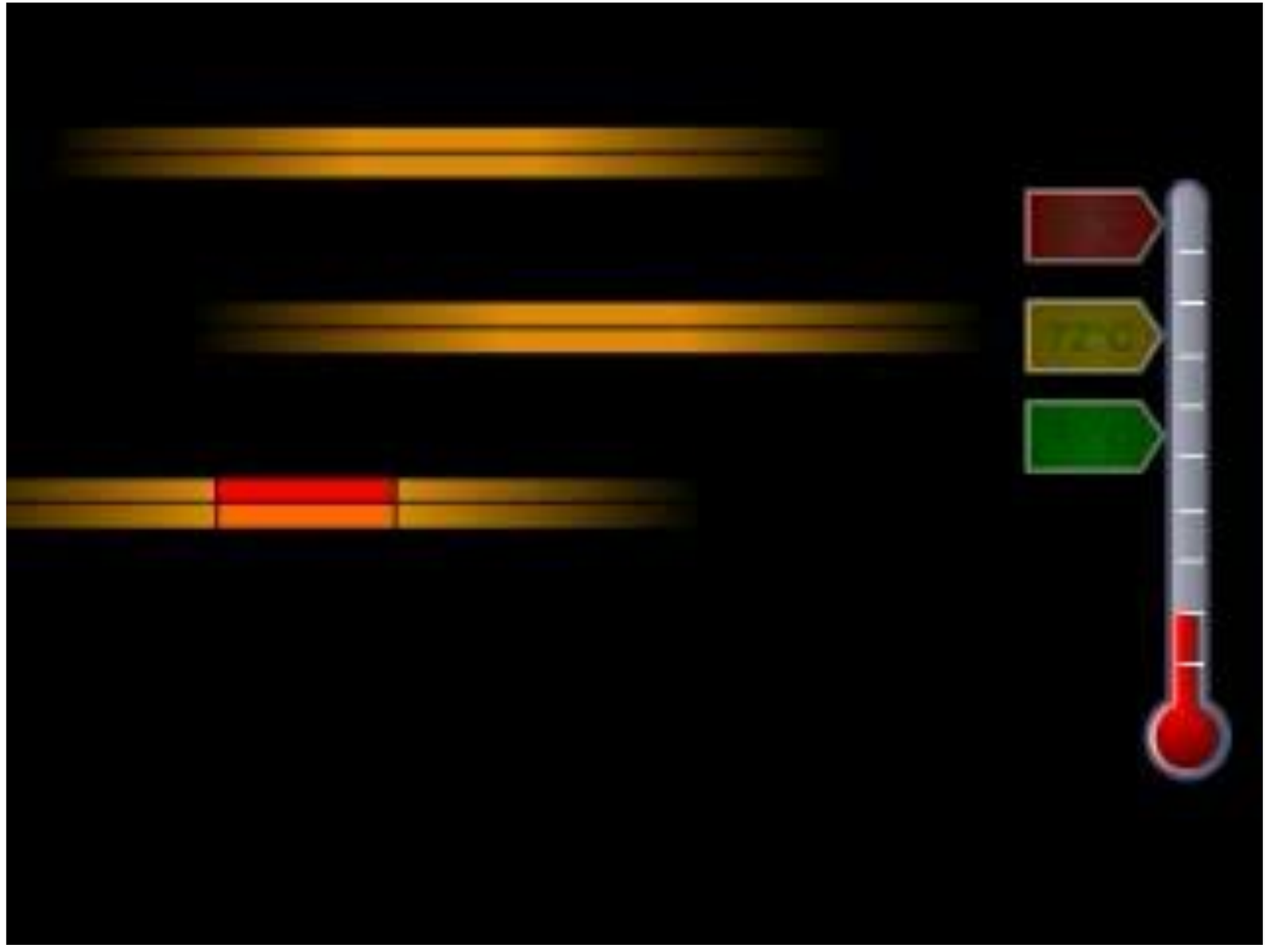
5′ A-T-C-A
3′A-T-G-C-C-G-T-A-G-T-C-G-T-A-C-A-G-T-A-C-G-T-G-C-A

Polymerase Characteristics

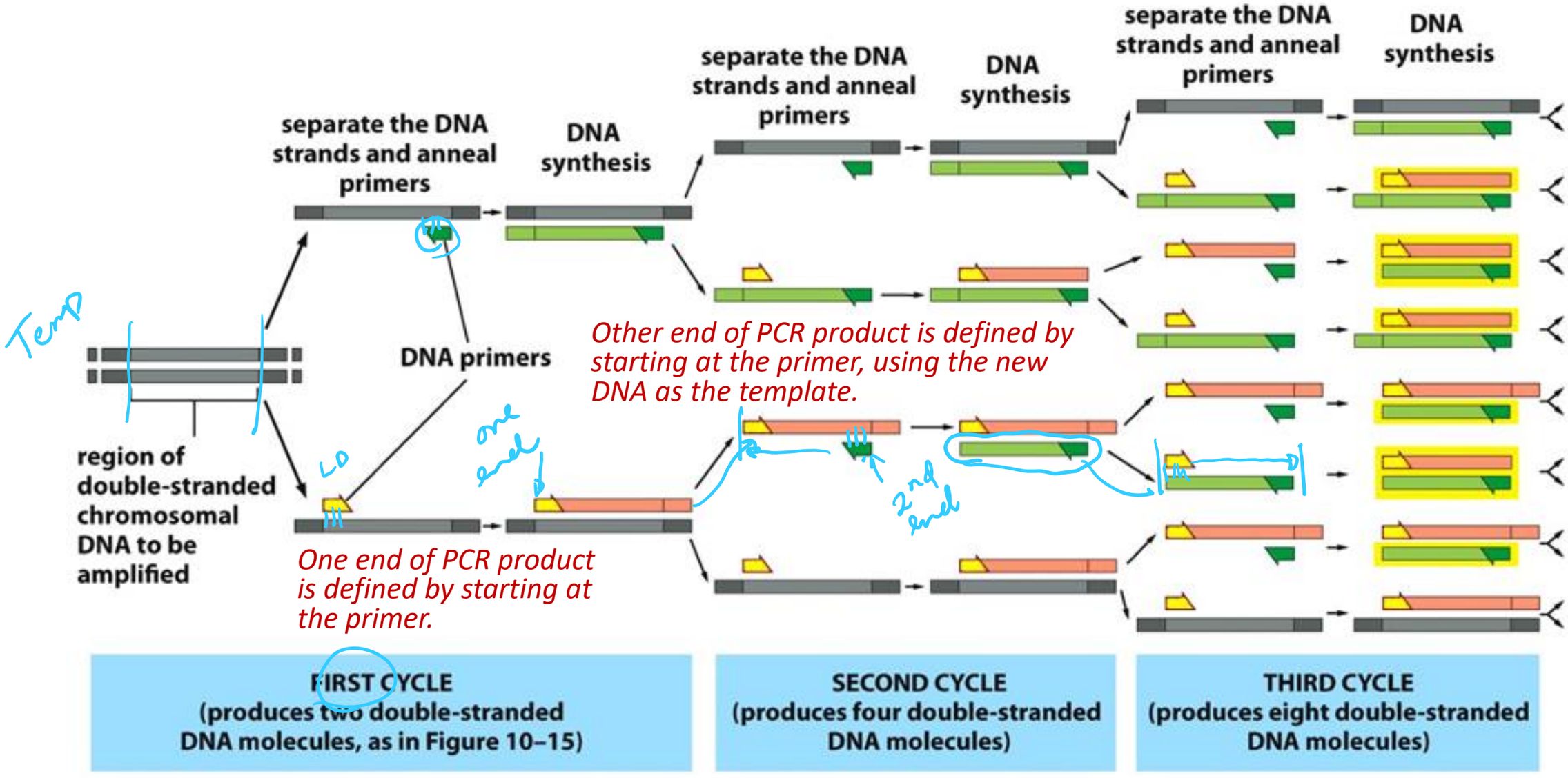
- Since the first step of each cycle (D) requires heating to high temperature, a thermostable polymerase is required.
- The first polymerase, Taq, was isolated from *Thermus Aquaticus,* a bacterial living in hot springs (Yellowstone National Park)
- A number of different polymerases with improved properties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

PCR Animation

Watch Me!



Three PCR Cycles

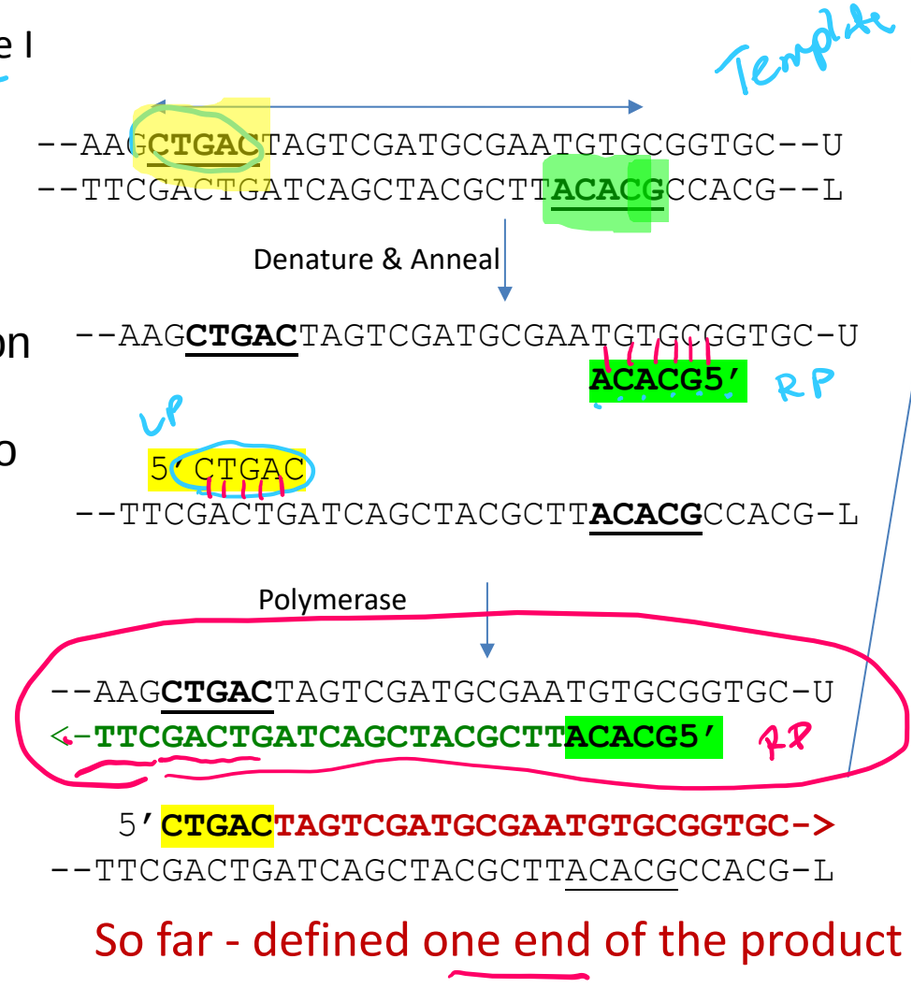


After 30 cycles there will be 2^{30}, or over 1 billion times more copies than at the beginning!!!

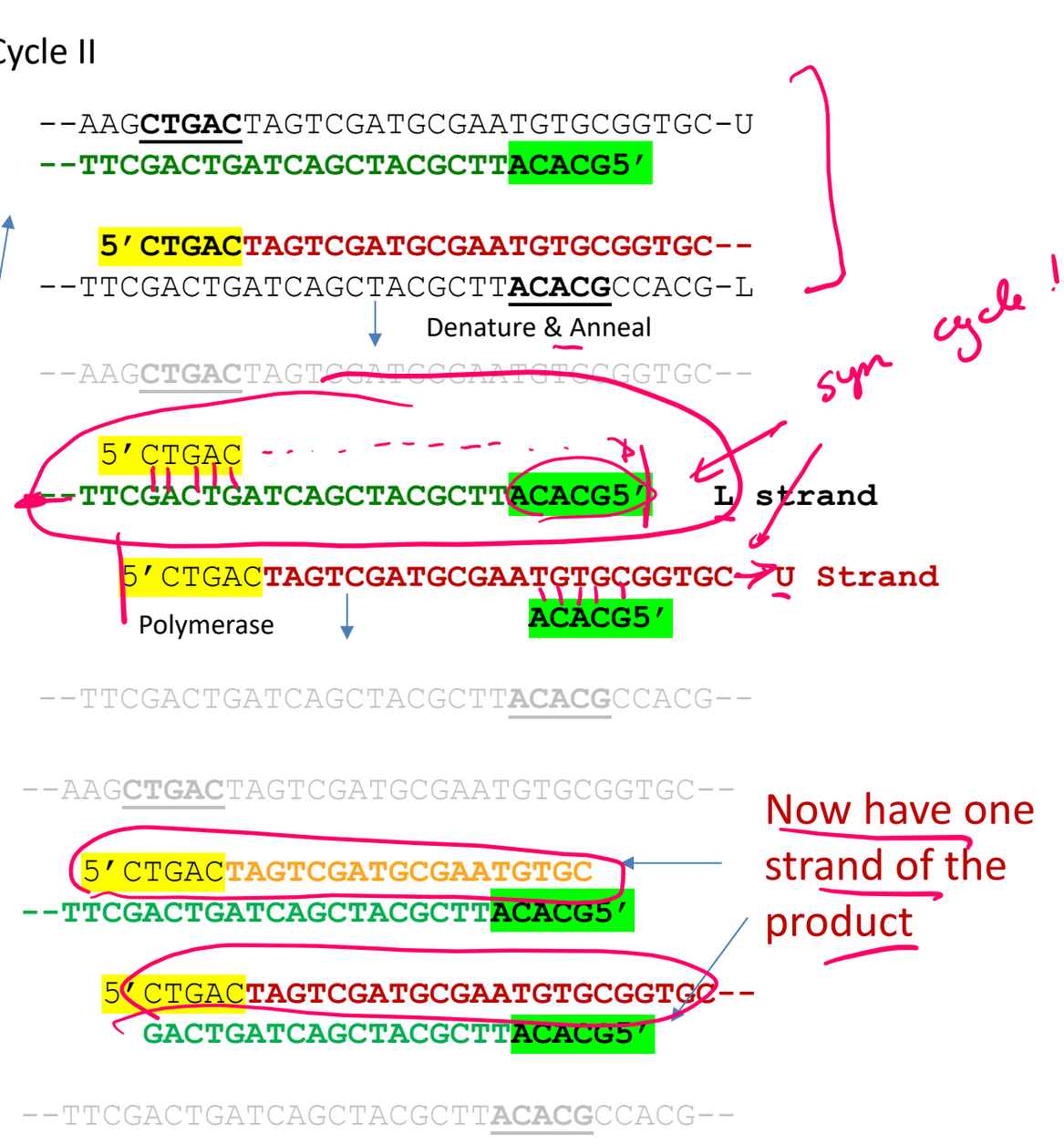
Detailed Events During First Three PCR Cycles

Note:

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



So far - defined one end of the product

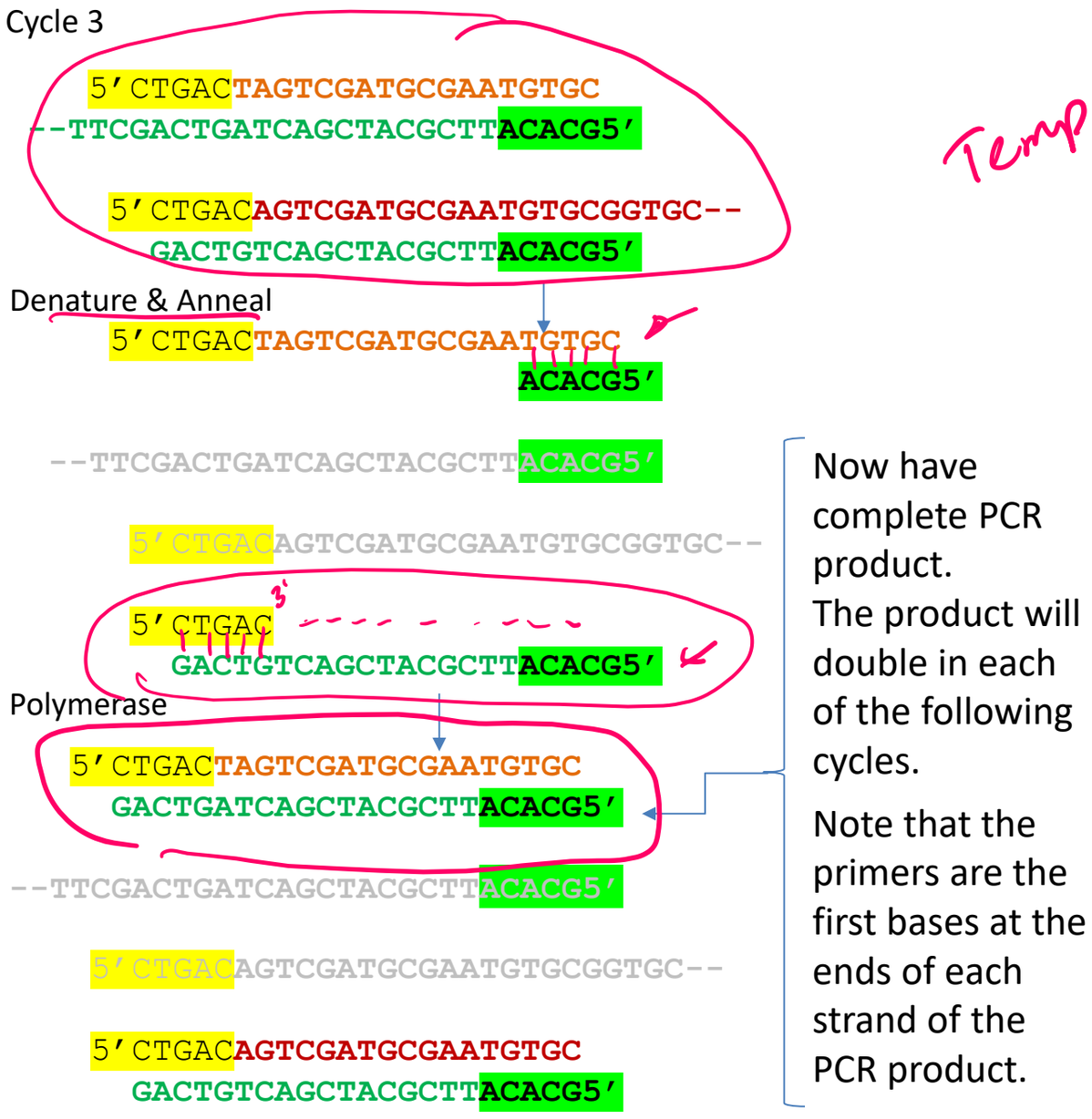


Now have one strand of the product

Final Product

CTGACTAGTCGATGCGAATGTGC
GACTGATCAGCTACGCTTACACG

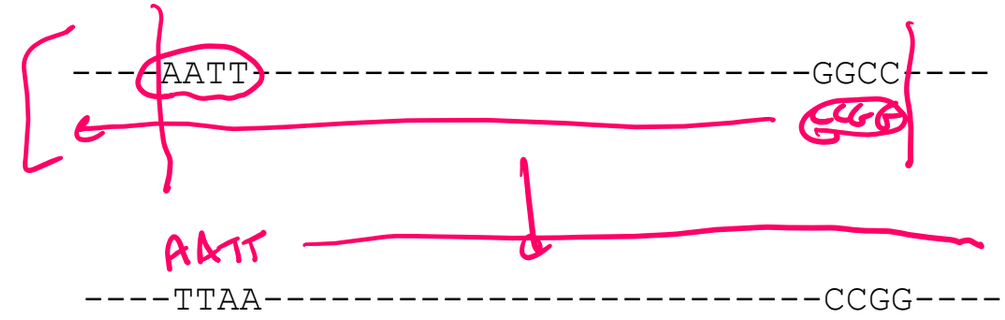
Detailed Events during first Three PCR Cycles

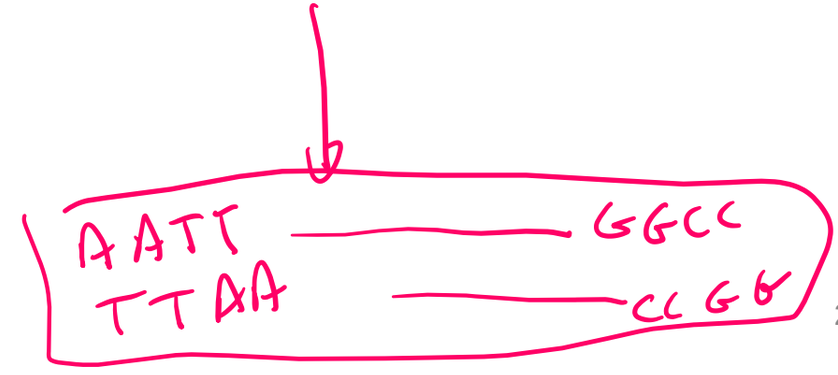


Now have complete PCR product.
The product will double in each of the following cycles.

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

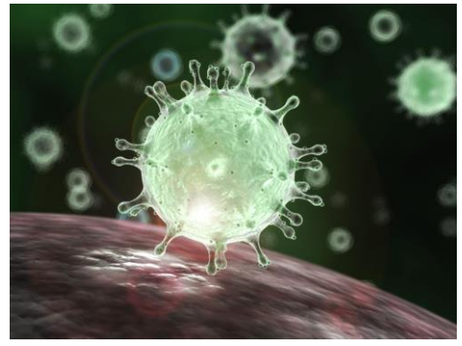
Example – follow the PCR cycles for the following template with primers 5′ AATT (left) and 5′ GGCC (right)





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
```

LP

RP

PCR Product

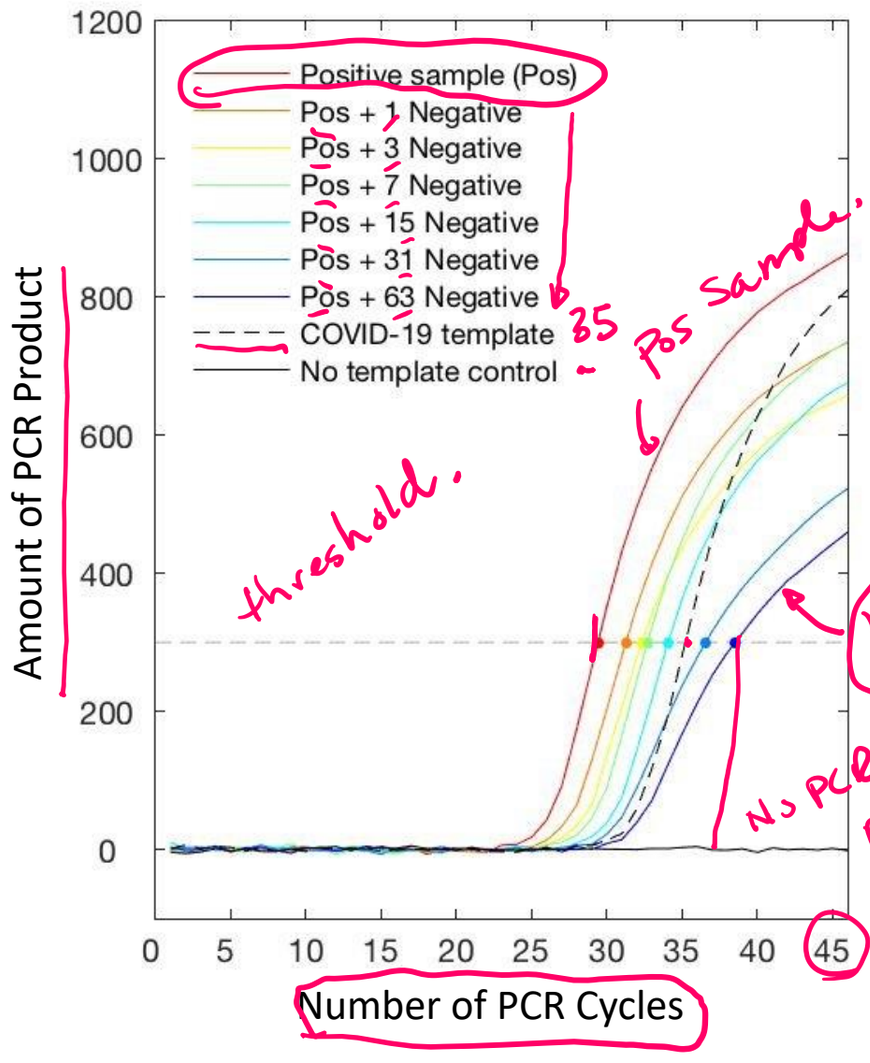
GACCCCAAAATCAGCGAAATGCACCCCGCATTACGTTTGGTGGACCCTCAGATTCAACTGGCAGTAACCAGA
CTGGGGTTTTAGTCGCTTTACGTGGGGCGTAATGCAAACCACCTGGGAGTCTAAGTTGACCGTCATTGGTCT

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

No PCR product.

Covid 19 PCR Test: Detection of the PCR Product.

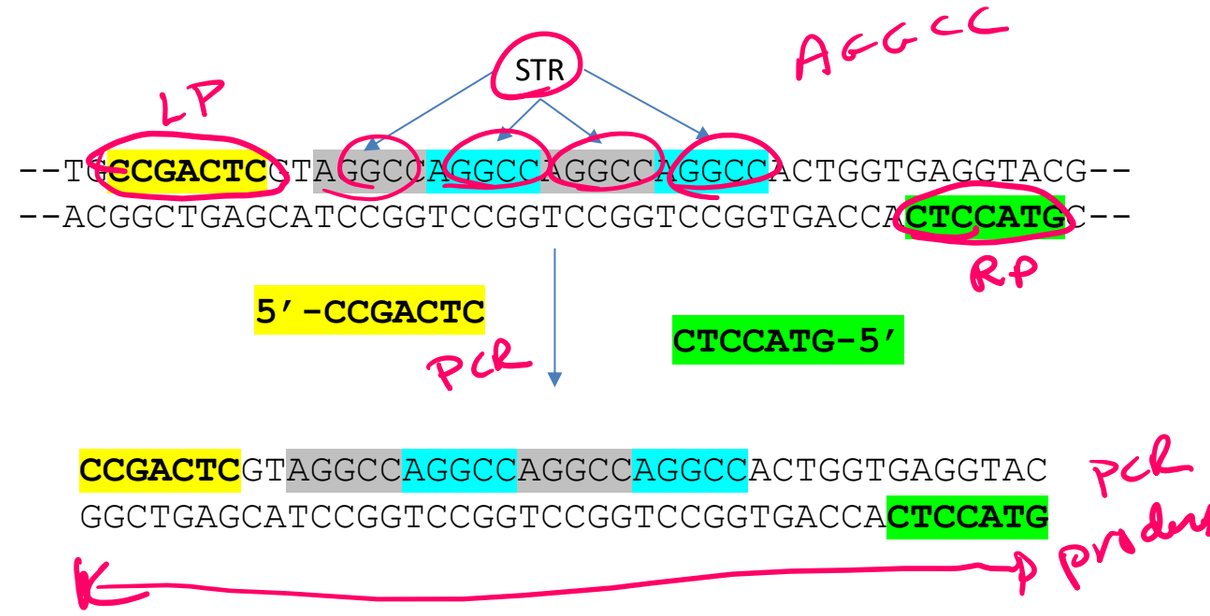
https://www.medrxiv.org/content/10.1101/2020.03.26.20039438v1



- 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.
- Next 6 samples are the positive sample mixed with up to 63 negative samples, showing that it is possible to test pooled samples.
- - - - 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.

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*

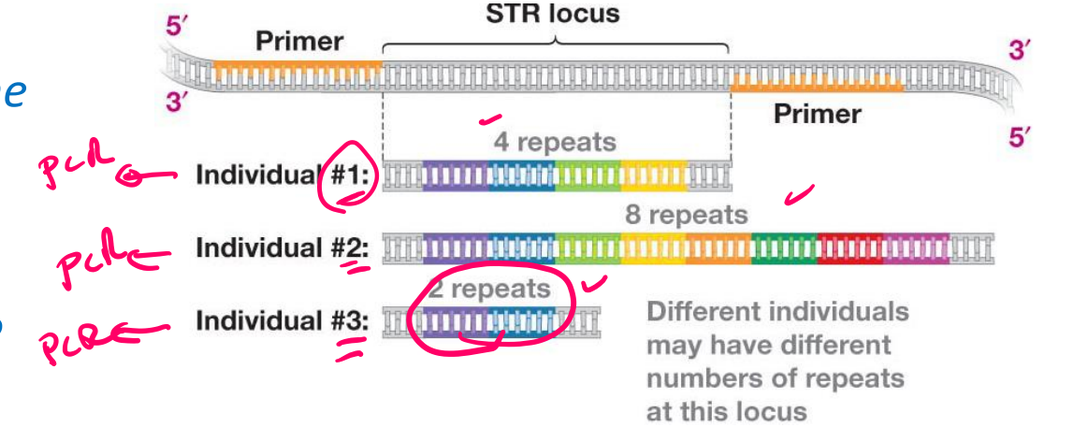


Which individual has the shortest PCR product?

3

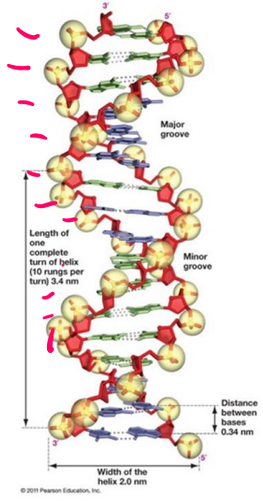
Which has the longest?

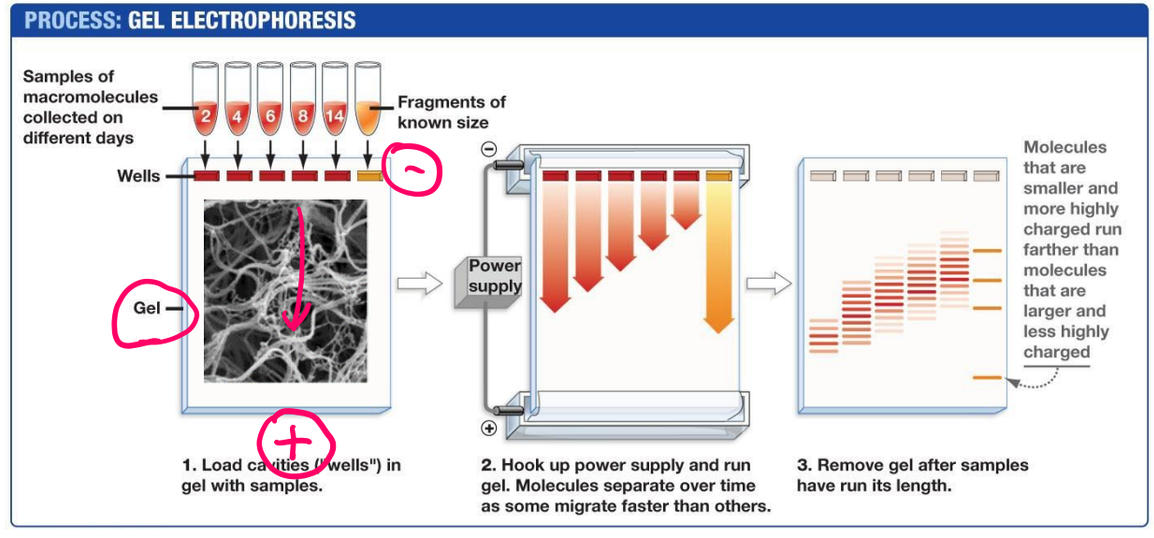
2



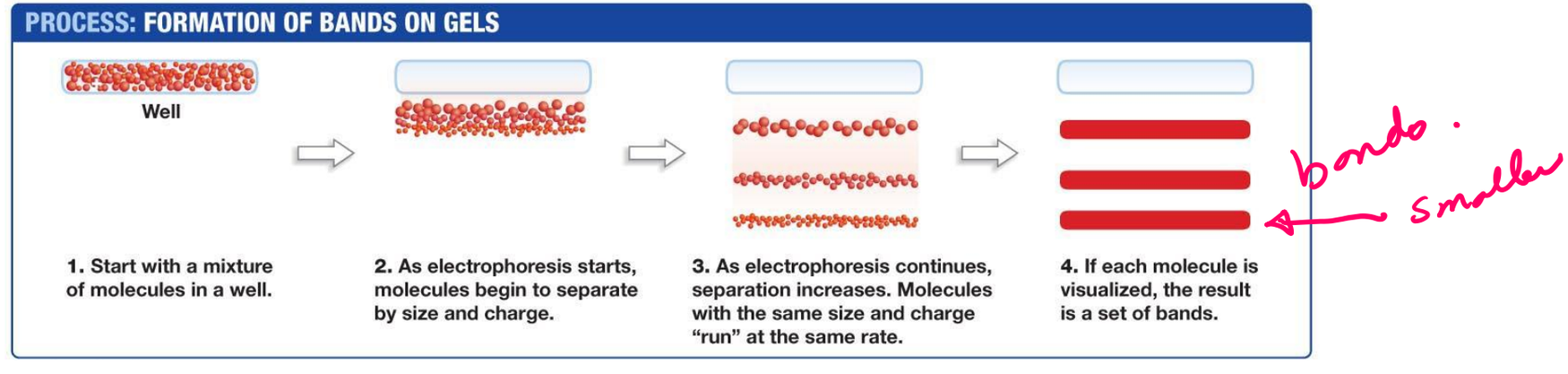
Size Determination of PCR products - Agarose Gel Electrophoresis.

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



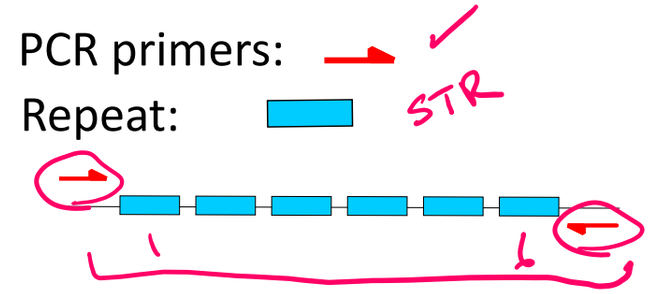


Which are the smallest PCR fragments?



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

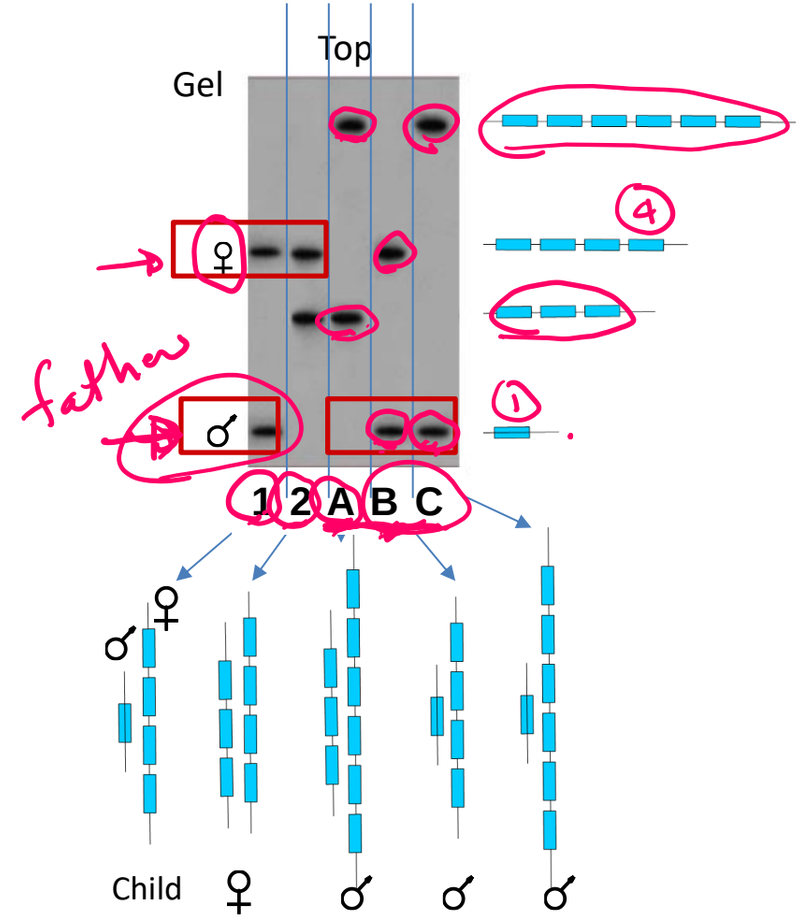
Lanes A, B, C: Possible Fathers

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

A

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

B, C



Introduction to Immunology

1. Branches of the immune system (Innate and acquired)
2. Properties of antibodies (Quaternary structure, antigen recognition)
3. How antibodies are produced:
   - Genome DNA changes
   - mRNA splicing

4. How antibodies eliminate pathogens

Key Questions:

1. Why is the innate system important?
2. What is the origin of diversity in acquired immunity?
3. How are antibodies made.

The Nobel Prize in Physiology or Medicine 2018

Ill. Niklas Elmehed. © Nobel Media

James P. Allison

Prize share: 1/2

Ill. Niklas Elmehed. © Nobel Media

Tasuku Honjo

Prize share: 1/2

The Nobel Prize in Physiology or Medicine 2018 was awarded jointly to James P. Allison and Tasuku Honjo "for their discovery of cancer therapy by inhibition of negative immune regulation."

Some Important Definitions:

Antigen = something that is recognized by the immune system, e.g. bacteria, virus, pollen.

Epitope = the part of the antigen that is contacted by the antibody.

Antibody (Ab) = Y-shaped protein that recognizes antigens, found on the surface of B-cells or secreted by plasma cells. When bound to antigen, it can initiate a process that results in the destruction of the antigen. *Specificity is high due to AA sequence in the variable segments.*

Immunoglobulin (Ig) = antibody.

B-cell = involved in antibody production and recognition of pathogen. Has antibody molecule on its surface (as part of the B-cell receptor). Develops into plasma cells after activation by T_H cells. Called B-cells because they are generated in the organ called the Bursa in birds.

Plasma cell = derived from B-cell after activation of the B-cell, produces secreted antibodies with the *same specificity as the original B-cell.*

T_H cell = T-helper: *Required* to activate both B and T_C cells, as well as other cells in the immune system. Called T-cells because they mature in the thymus.

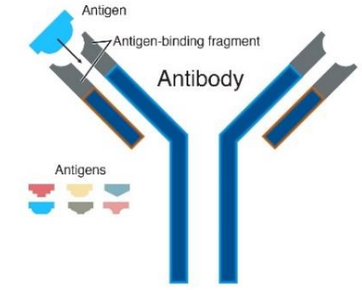
T_c cell = T-cellular: Involved in defense against viruses and cancer.

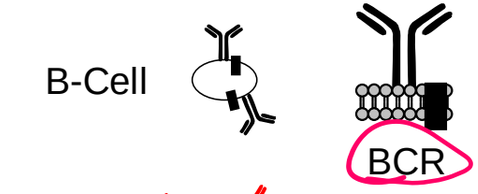
TCR = T-cell receptor – found on the surface of T-cells, recognizes MHC proteins + bound peptide, RTK.

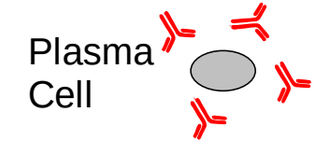
- **T_c cell** = recognizes MHC I + peptide
- **T_H cell** = recognizes MHC II + peptide

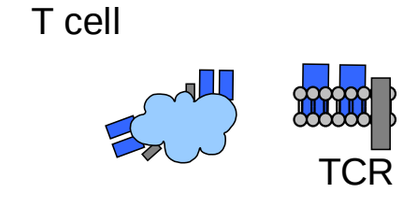
MHC = major histocompatibility complex – required for acquired immunity (basis of transplant rejection)

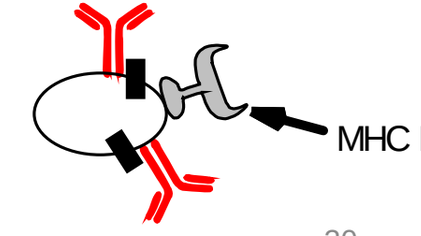
- MHC I = protein found on the surface of ***all*** cells, "presents" peptides derived from the proteins that were made by the cell. The MHC-peptide complex is recognized by T_c cells. ***Only foreign peptides produce a response***.
- MHC II = on the surface of B-cells, macrophages, and dendritic cells. Presents external peptides to T_H cells, leading to activation of the cell by T_H cells. ***Only foreign peptides produce a response***.





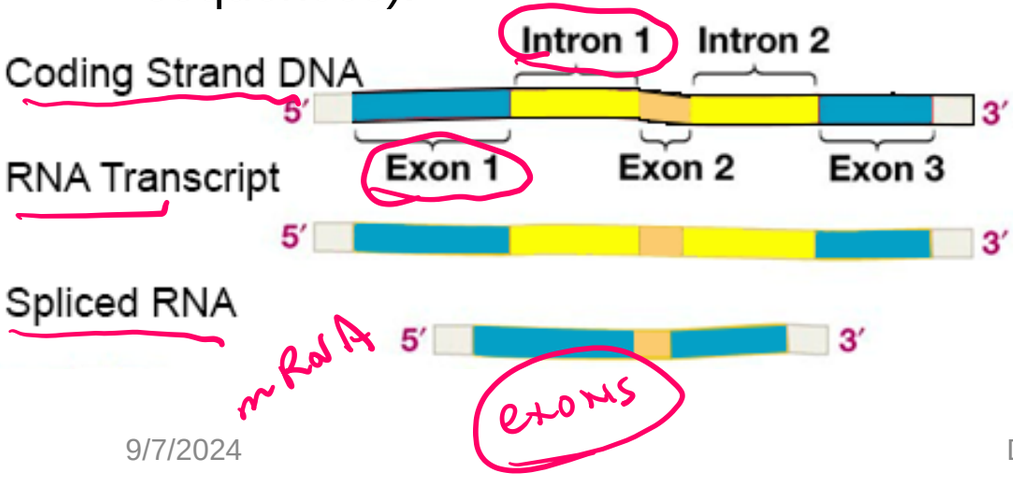




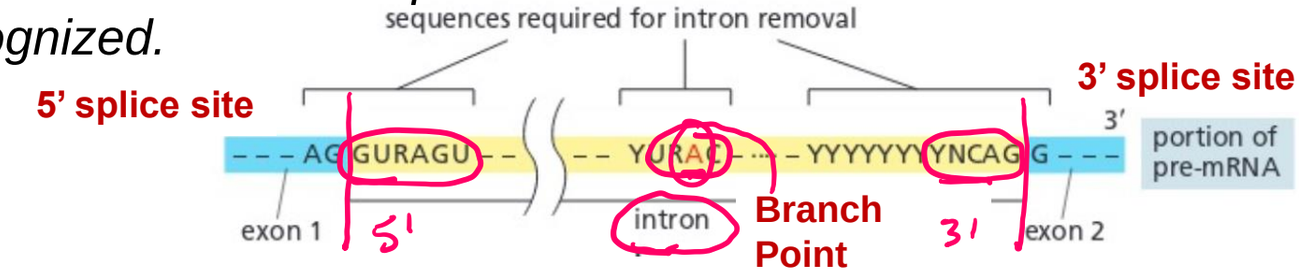


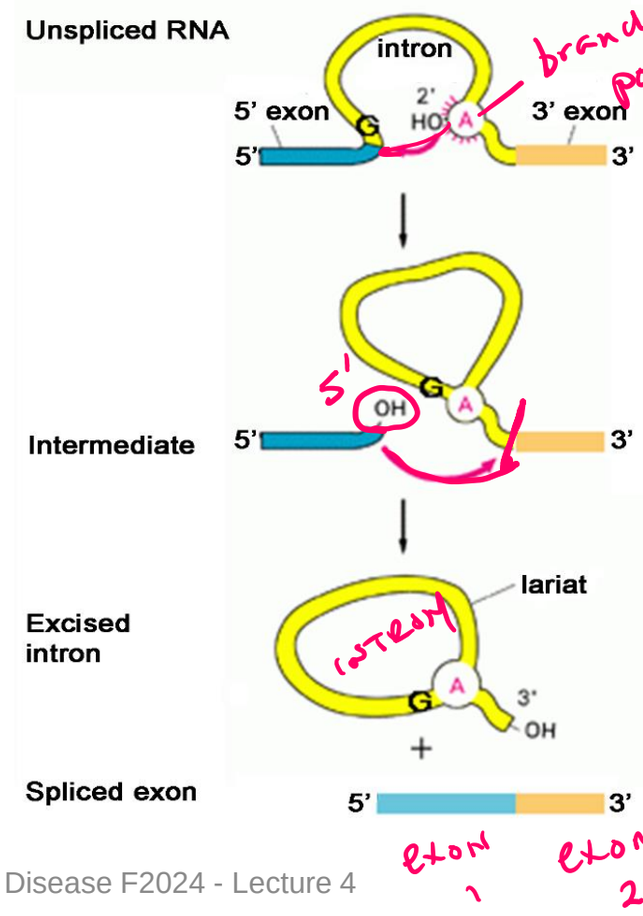
mRNA Splicing Required to Produce Functional mRNA

- When DNA sequences were aligned to RNA sequences, it was found that segments were deleted in the final RNA.
- This suggested that the gene encoding a protein was coded by segments of the DNA:
  - Those to be in the final mRNA were called **exons**.
  - Those sections not in the mRNA were called introns (intervening sequences).



Splice sites are recognized due to specific sequences at the exon-intron boundaries. *Sequences in both the exon and intron are recognized.*





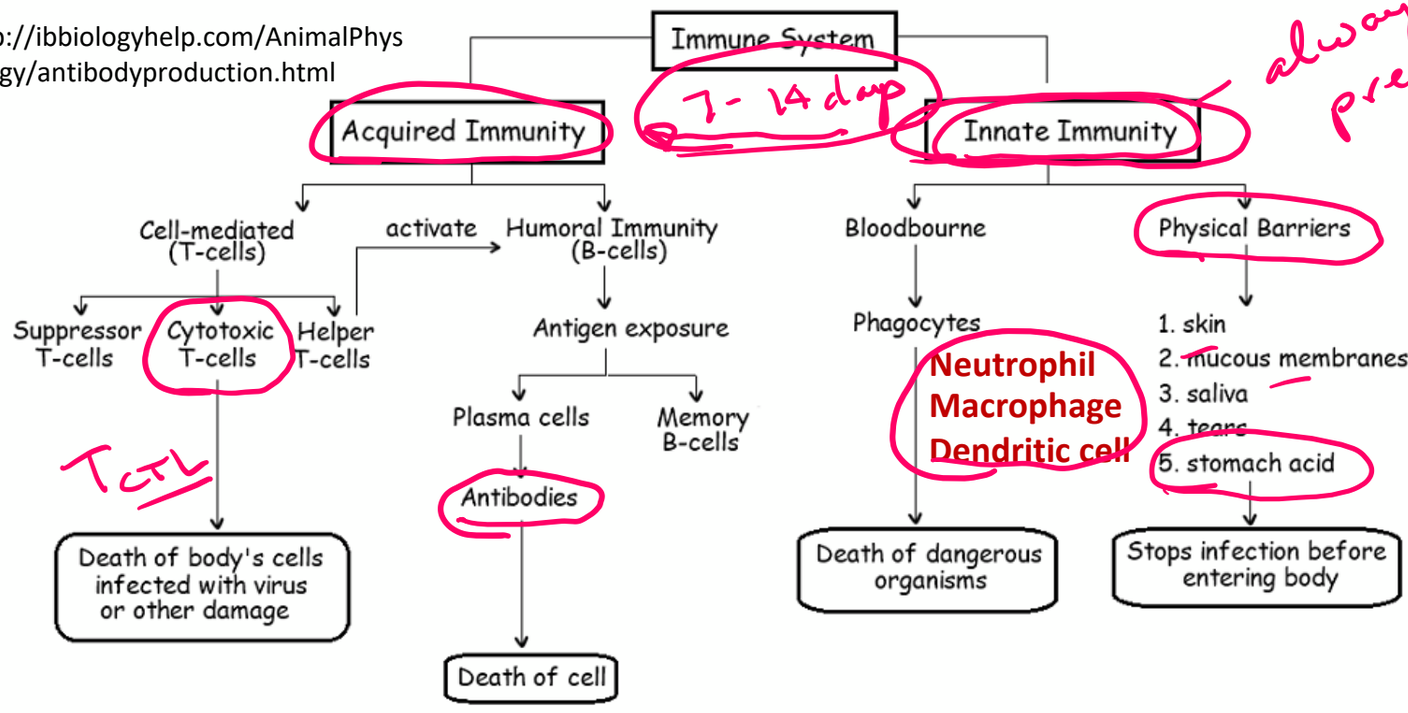
1. There is a 5' splice site with a conserved sequence: (A/C)AG|GU(A/G)AGU
2. There is a 3' splice site with a conserved sequence: CAG|G
3. There is an A in the intron (branch point) required for splicing.

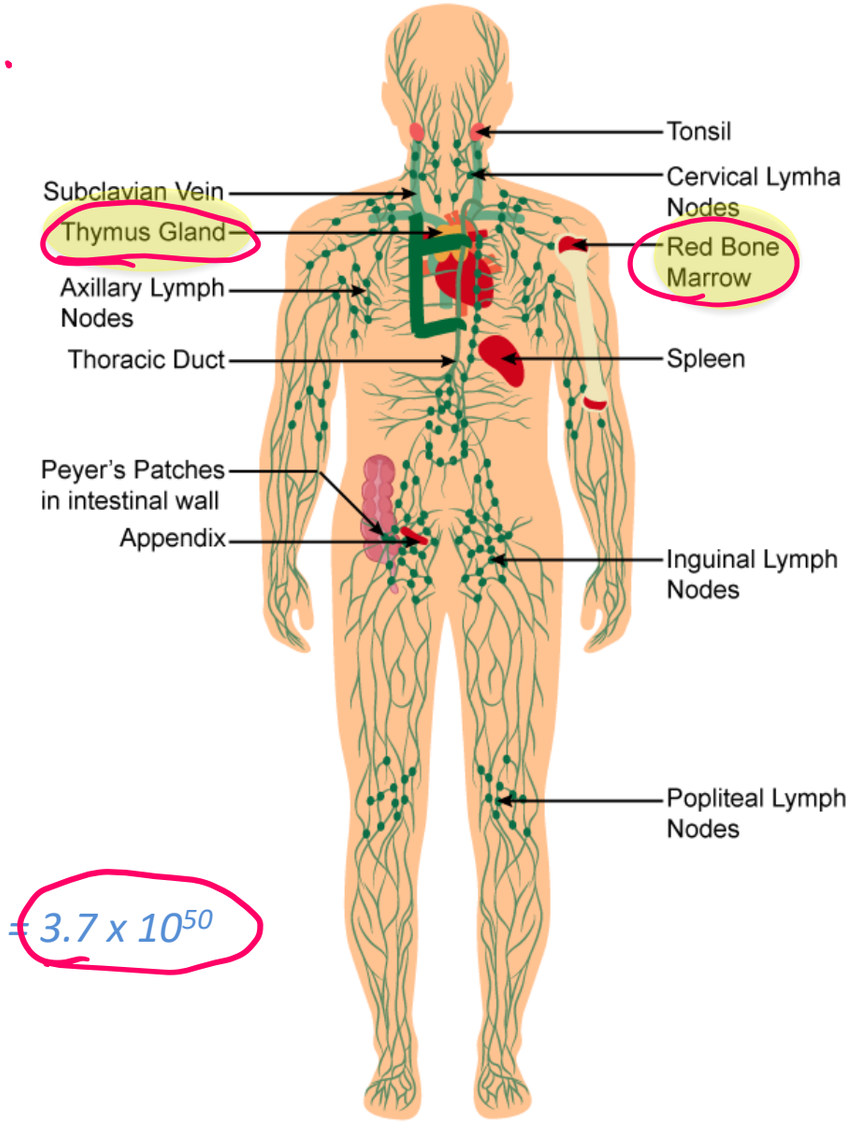
Steps:

1. The branch A breaks at the 5' splice site, forming a lariat.
2. The 5'-OH is joined to the 5' end of the downstream 3' exon.

Branches of the Immune System:

http://ibbiologyhelp.com/AnimalPhysiology/antibodyproduction.html





https://www.topperlearning.com/

Why is the innate system essential?

- *A pathogen doubles every hour.*
- *It takes 7 days to produce antibody (after 1st exposure)*
- *How many bacteria would be present if they grew uncontrolled for 7 days: $2^{24 \times 7} = 3.7 \times 10^{50}$*

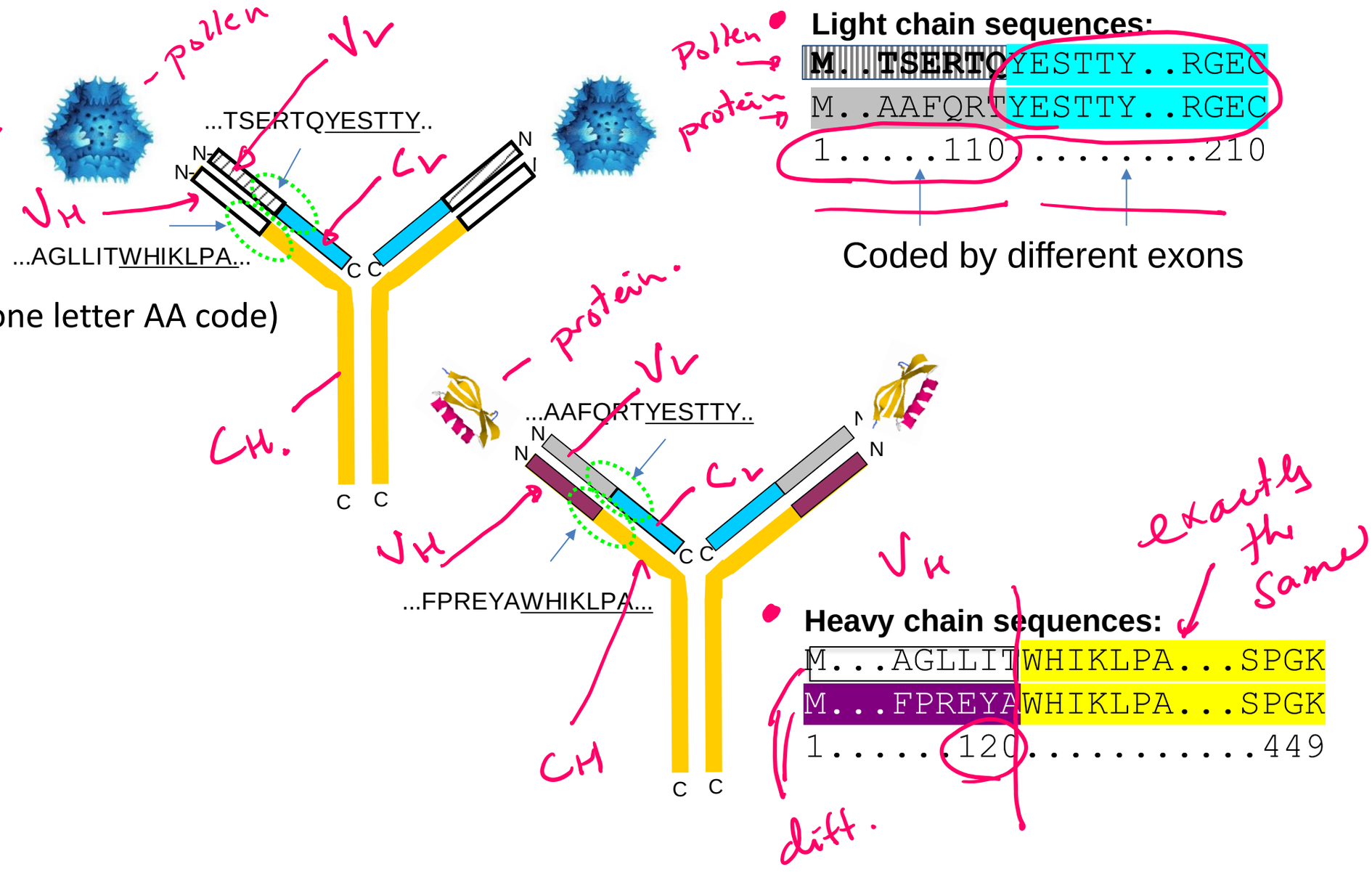
(there are approximately 10^{13} cells in the human body)

1. Important **primary** lymphatic organs: bone marrow (B), thymus (T)- Generate all immune cell.
2. Important **secondary** lymphatic organs: lymph nodes, spleen, Peyer's patches – Activation of immune cells.

Antibody Structure and Diversity

Each Antibody:

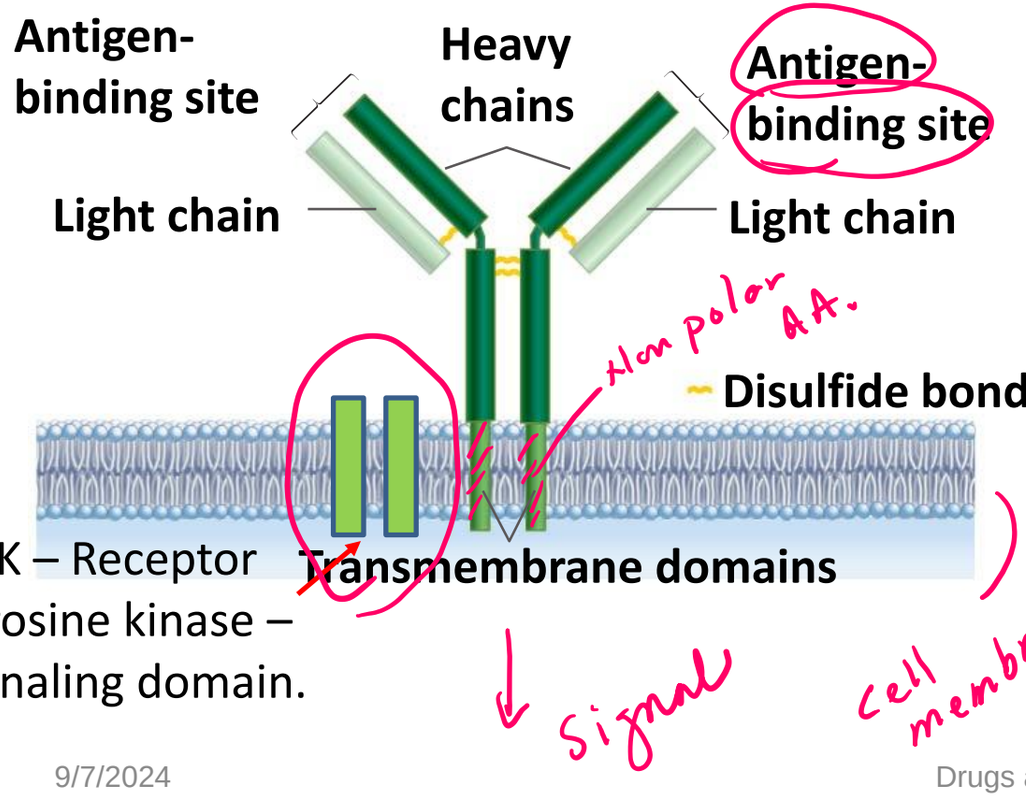
- Two identical light chains
- Two identical heavy chains
- First ~100 Amino acids on each chain are called the variable region and differ from antibody to antibody.
- Unique sequence for variable region of both heavy and light chains ***– defines specificity – different antibodies bind different antigens.***
- Constant regions - same protein sequence for all.



Production of Antibodies by B-cells & Plasma Cells

B- Cells & B-cell Receptor (BCR)

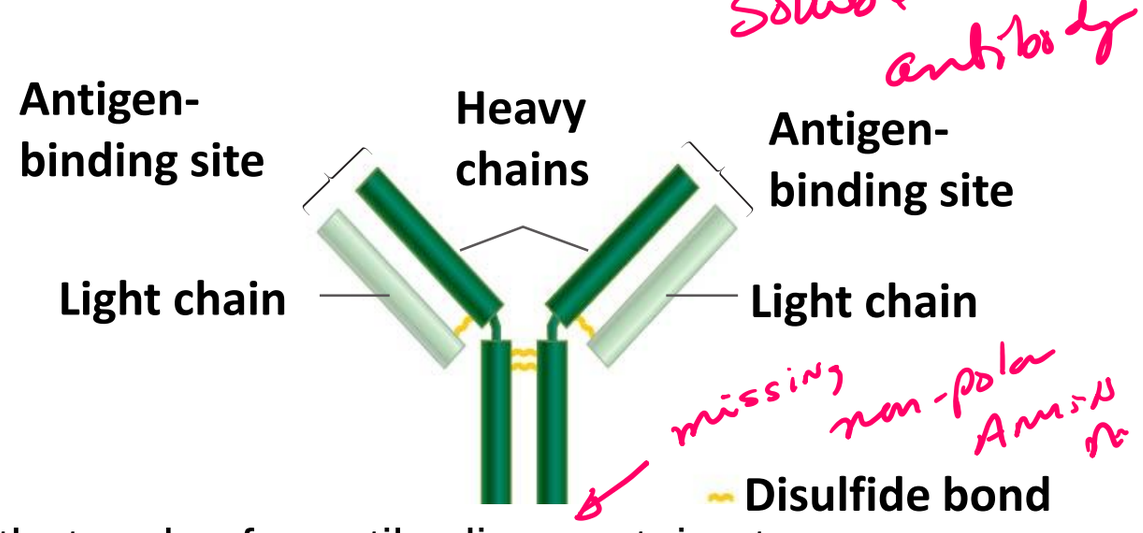
- Each B-cell has only one type of antibody as part of its BCR (B-cell receptor), i.e. the 10^5 BCRs are *homogeneous* on the same cell.
- Approximately 10^8 different specificities at any one time. i.e. *10^8 different B-cells!*



RTK – Receptor tyrosine kinase – signaling domain.

Plasma Cells:

- After activation, a B-cell develops into a plasma cell.
- The antibody is secreted.
- The same light chains are produced.
- The heavy chains differ only in the absence of the transmembrane domains.



mRNA that codes for antibodies contains two types of sequences:

- Exons – contain codons for the amino acids
- Introns – removed before translation

Different exons are used to produce membrane bound or soluble antibodies.

B-Cell Biology - From Stem Cells to Pathogen Killing.

1. Generation of high diversity of chains during development of stem cells to B-cells in bone marrow.
- DNA rearrangements to generate functional exons for variable segments of both light and heavy chain.

2. Molecular & cellular biology of membrane bound antibodies on cell surface = B-cell receptor (BCR)
- Transcriptional enhancers, mRNA splicing
- Light chain and heavy chain exported to surface of B-cells.

3. Self tolerance test to prevent autoimmune diseases, autoreactive B-cells eliminated.

4. Encounter and capture of antigen in lymph nodes

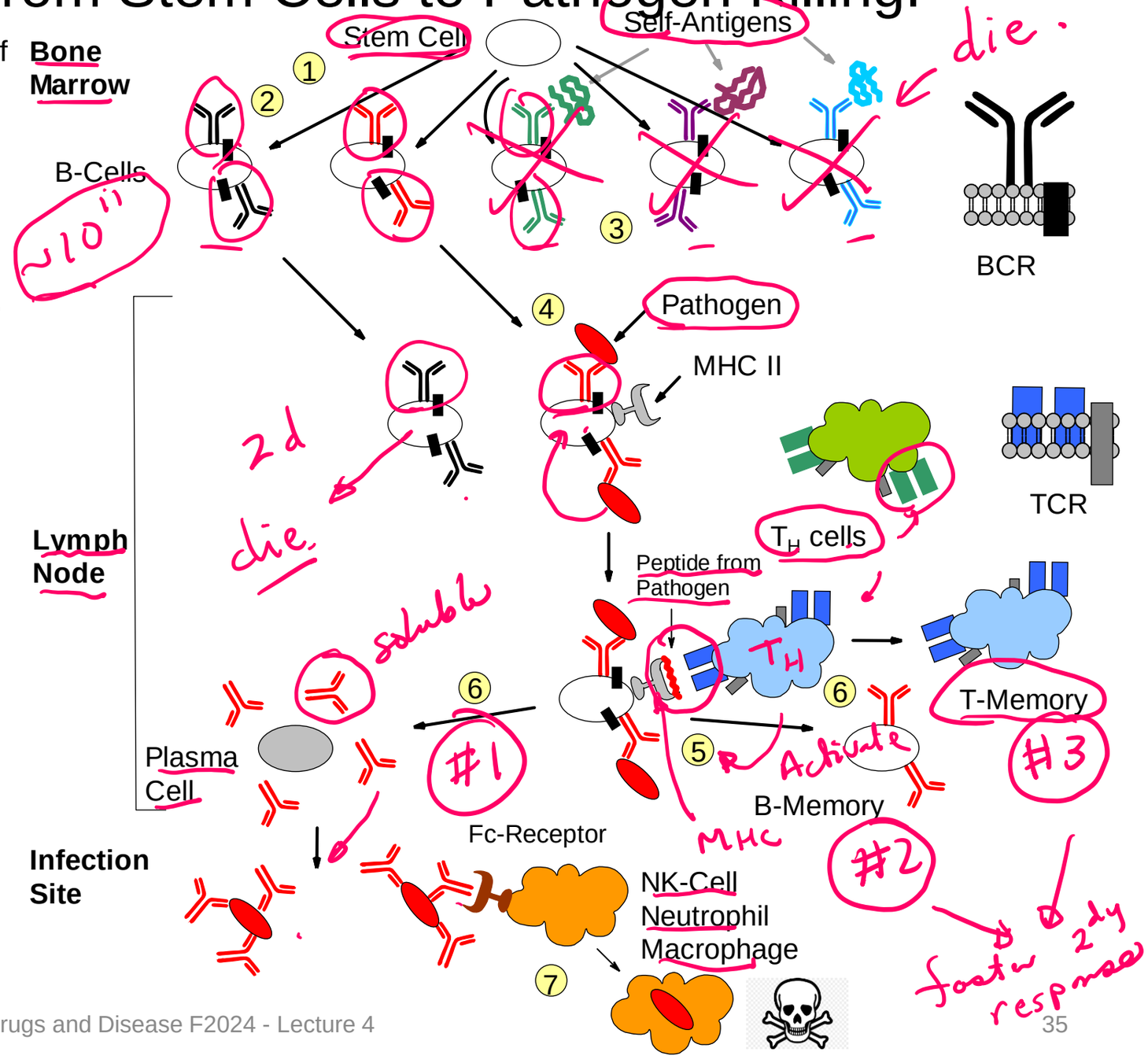
5. Activation of B-cells by T_H cells
- Peptides from pathogen presented on major histocompatibility proteins (MHC II).
- T-cell activation by tyrosine kinase receptors (T-cell Receptor, TCR), secretion of signaling molecules.

6. Development of
- Plasma cells - Production of soluble antibodies of the same specificity as the parent B-cell.
- B-memory cells (basis of immunity)
- T-memory cells (basis of immunity)

7. Destruction of Pathogens
- Fc region of antibody binds to Fc Receptor on NK cells, neutrophiles, macrophages
- Pathogen internalized and destroyed.

BCR – B-cell receptor = antibody + signaling chains.

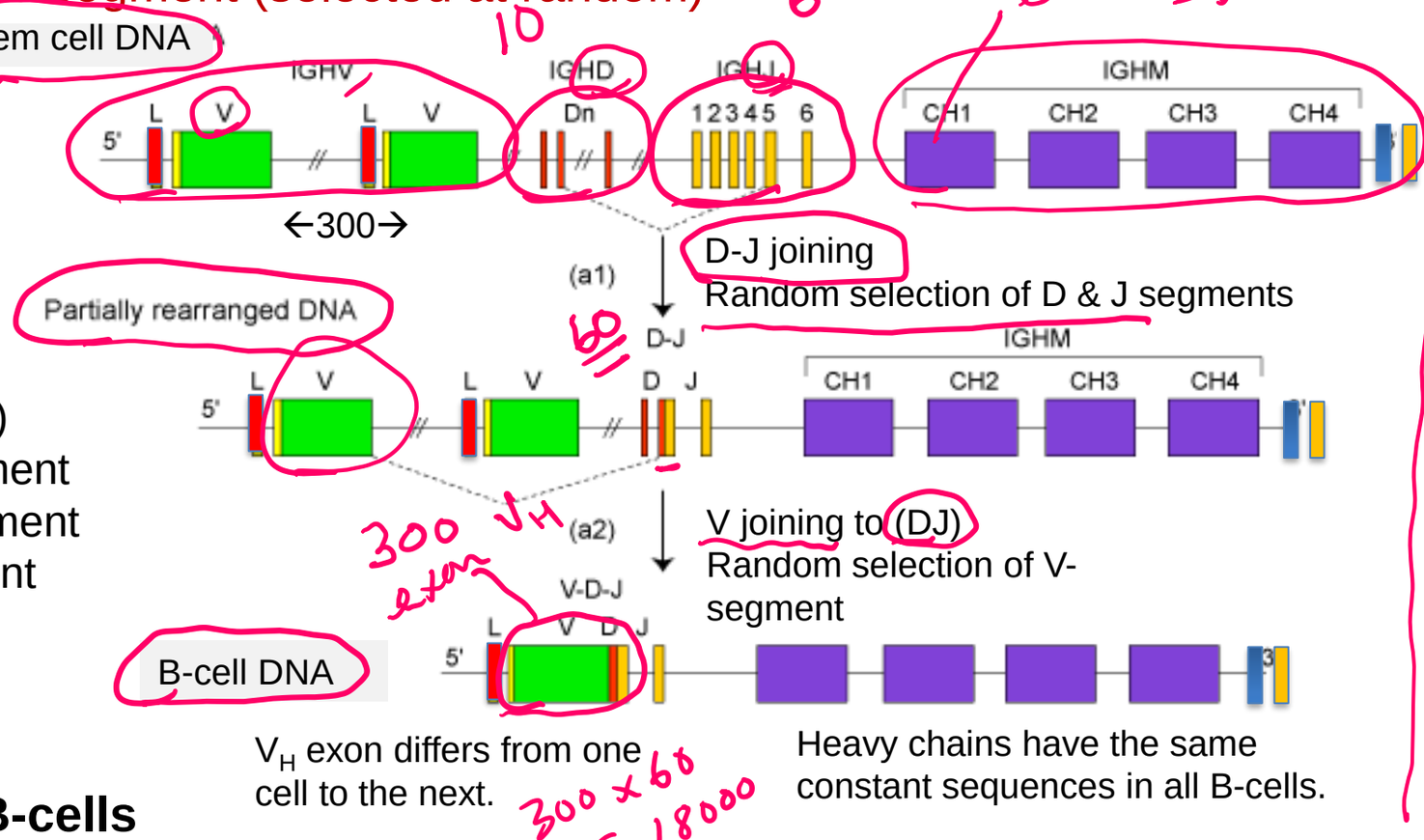
TCR – T cell receptor = MHC-peptide recognition + signaling.



Antibody Genes are Assembled From DNA Segments: Giving many different sequences.

Production of Heavy Chain Gene:

V_H exon = V+D+J segment (selected at random)

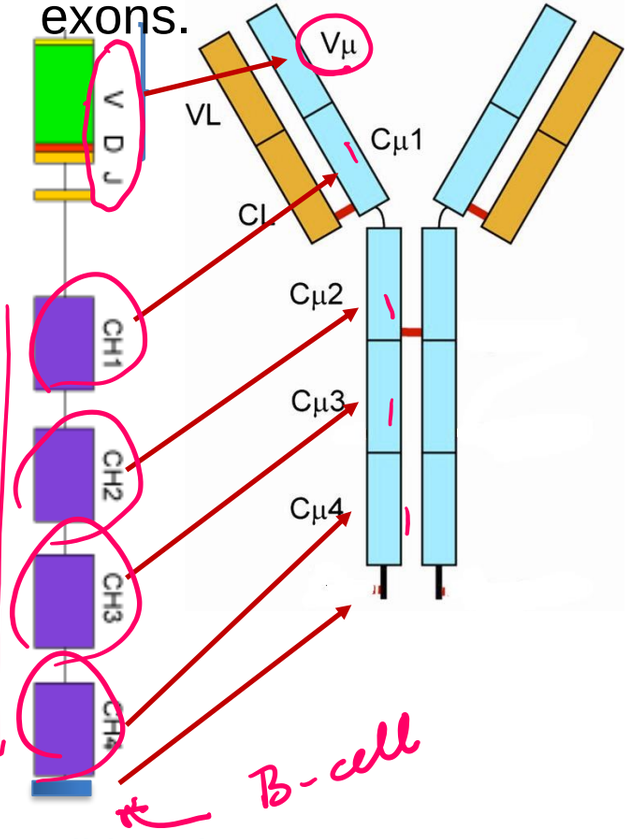


L = leader (signal)
V = variable segment
D = diversity segment
J = joining segment

V_H exon differs from one cell to the next.

Heavy chains have the same constant sequences in all B-cells.

The mRNA coding for antibodies contains 5 exons.



Stem cells -> B-cells

- The exon that codes for the variable region of the heavy chain is generated by the random joining of a V, D, and J DNA segments.
- Each B-cell will generate a unique sequence for its heavy and light chain DNA.
- This is a permanent change to the DNA (***genome)*** of the B-cell.

1. If there are 300 possible V-heavy segments, 10 possible D segments, and 6 possible J segments, how many different heavy chains can be made?