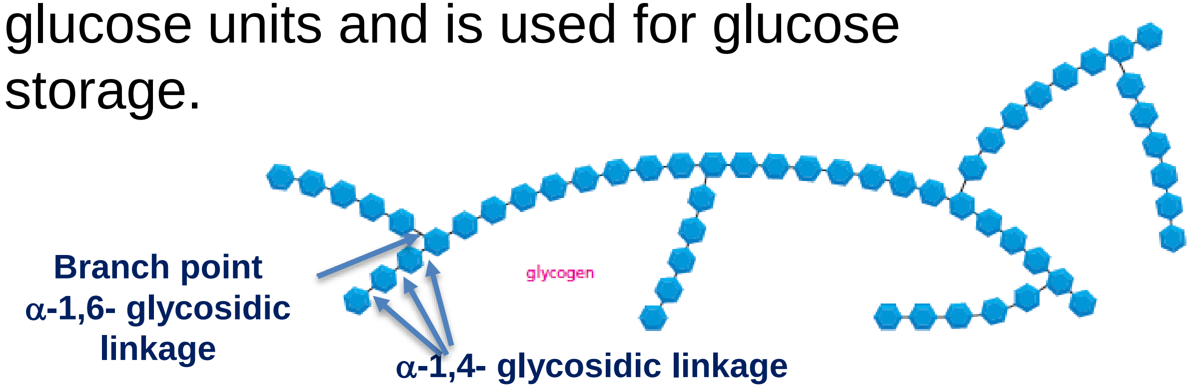
Lecture 4
Nucleic Acids & Immunology
(and a little polysaccharides)

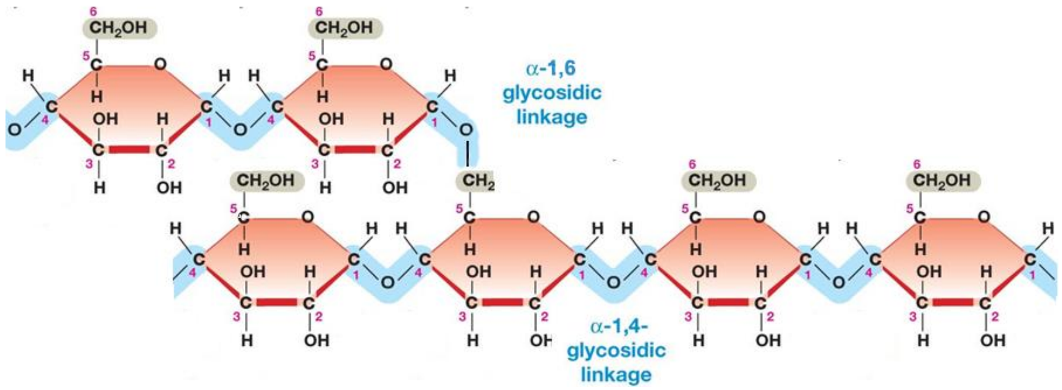
- Nucleic Acid Technologies
- Immunotherapies

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.



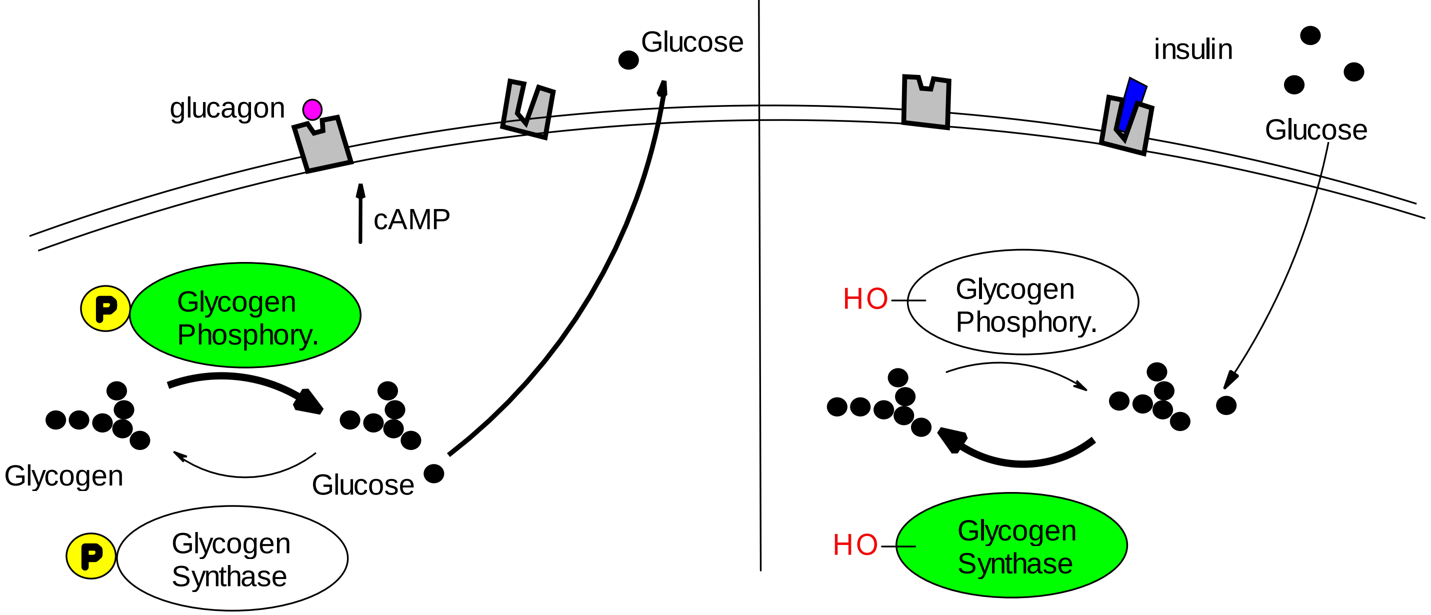


Glycogen Levels are regulated by hormones secreted due to blood glucose levels.

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

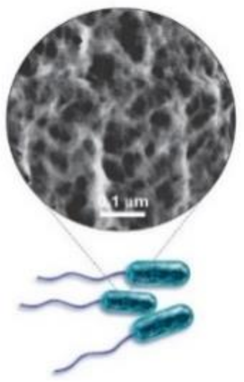
Two enzymes degrade or synthesize glycogen

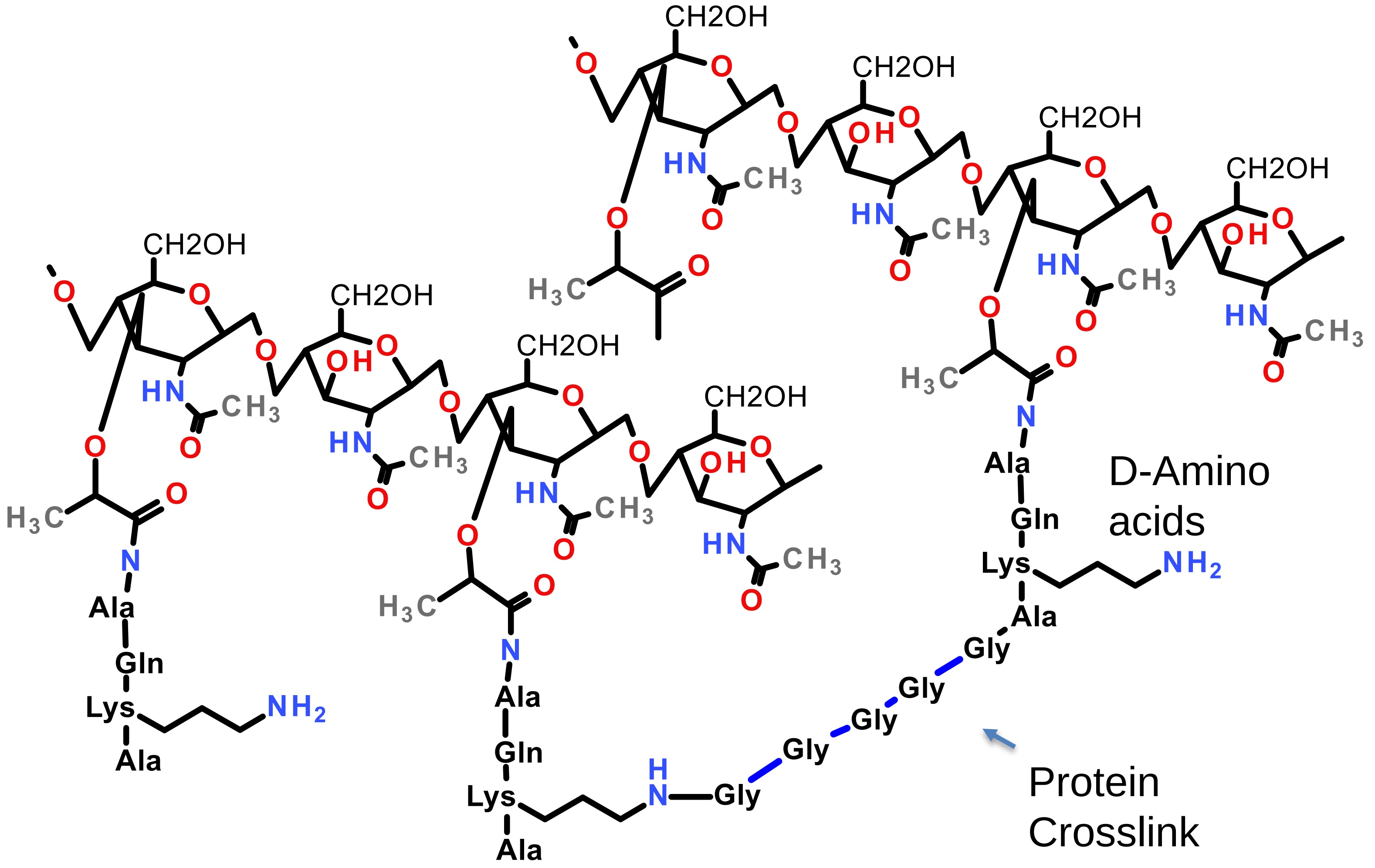
- Glycogen phosphorylase – releases glucose from glycogen
- Glycogen synthase – stores glucose in glycogen



Polysaccharides as Structural Molecules

Peptidoglycan (protein + sugar) in bacterial cell wall





D-Amino acids

Protein Crosslink

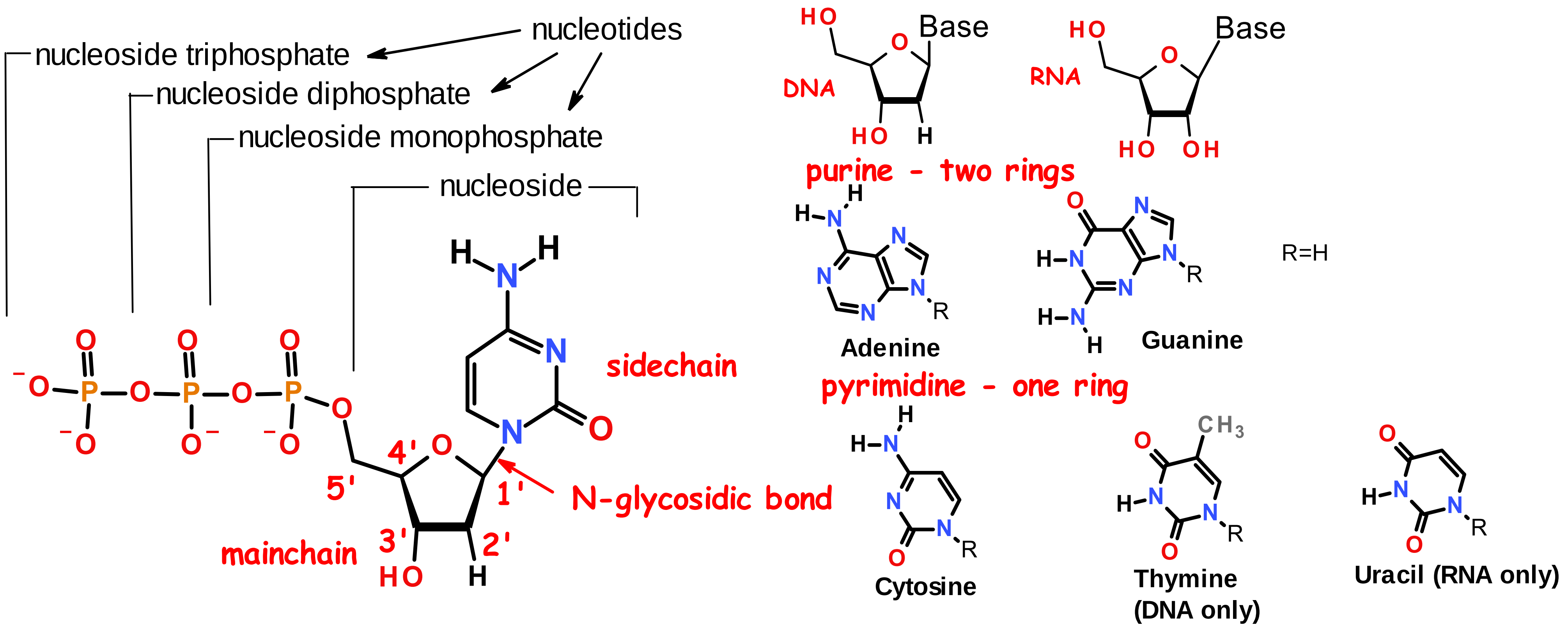
Peptidoglycan (Bacterial Cell Wall)

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

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**

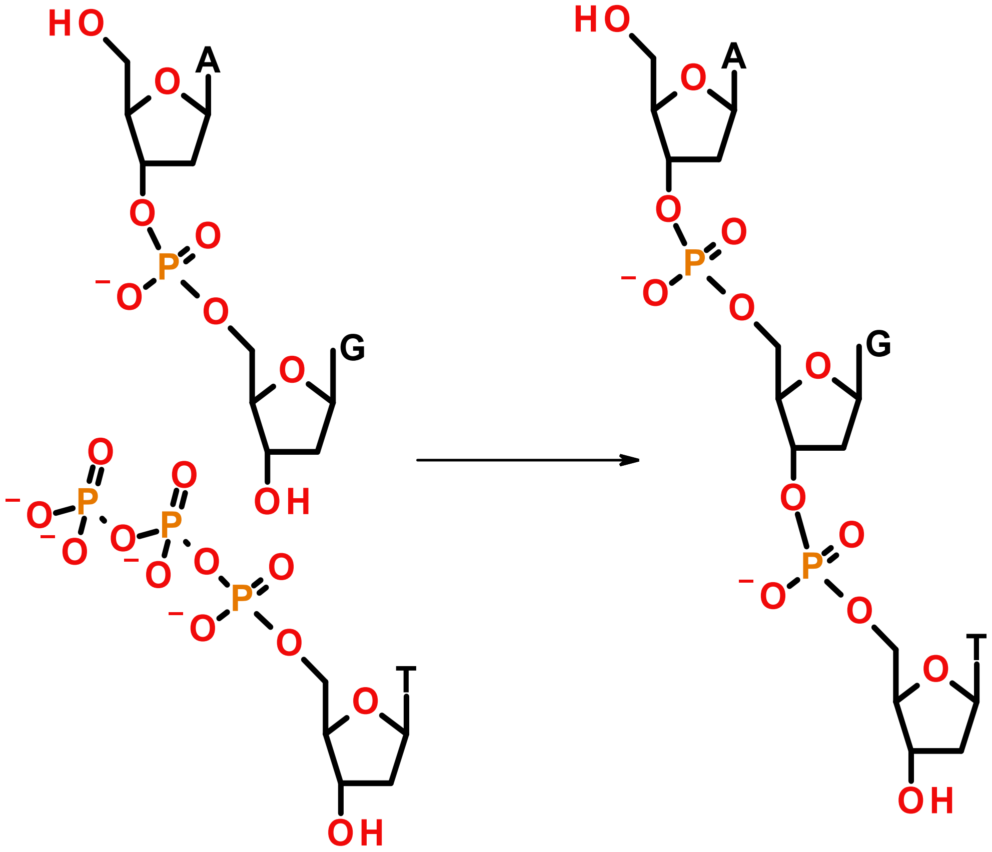
c) The carbon atoms on the sugar are numbered 1' to 5'. The primes distinguish the atoms on the sugar from those on the base.

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

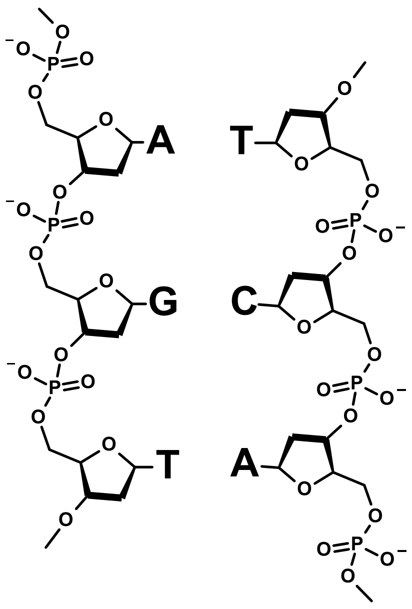
e) Four different monomers, A, G, C, T in DNA. U replaces T in RNA.

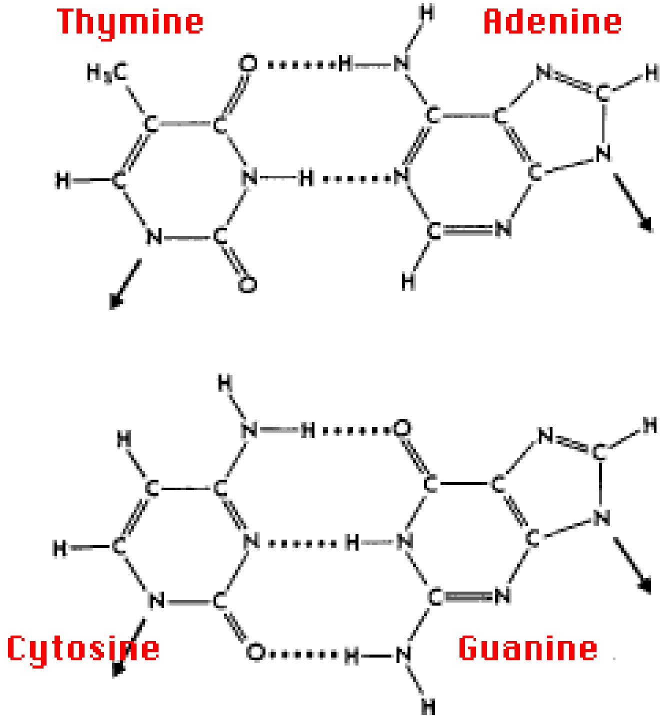
DNA and RNA are Polynucleotides:

- 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.

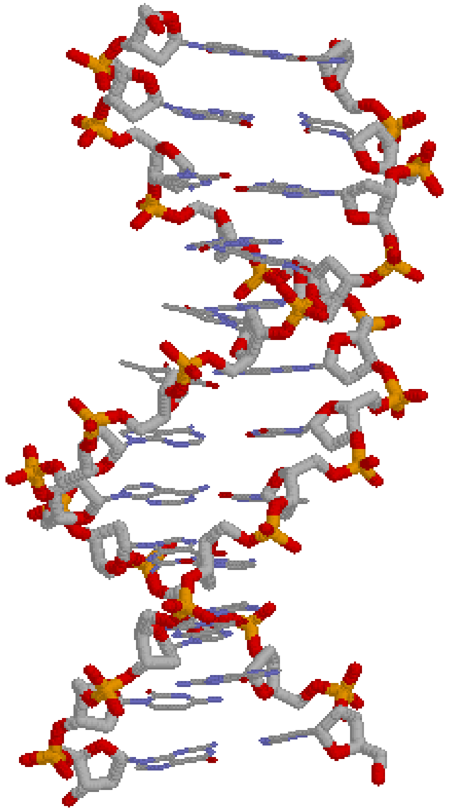




Nomenclature

5’ AGT 3’
3’ TCA 5’

= AGT
TCA



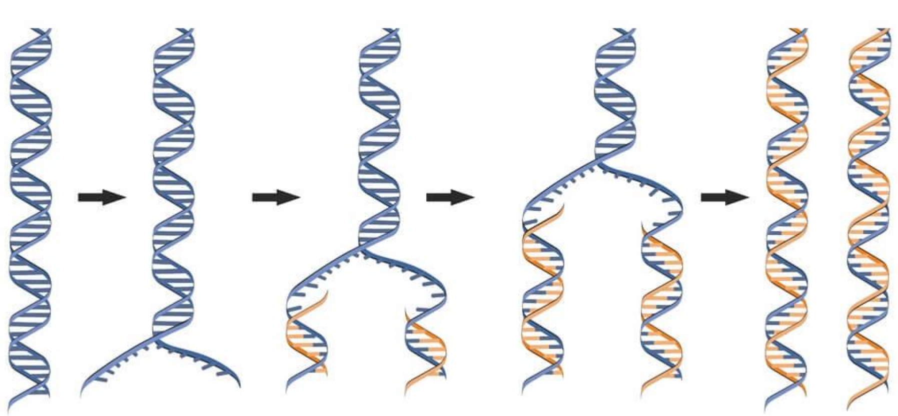
https://www.andrew.cmu.edu/user/rule/jsmol/nucleic.html

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.



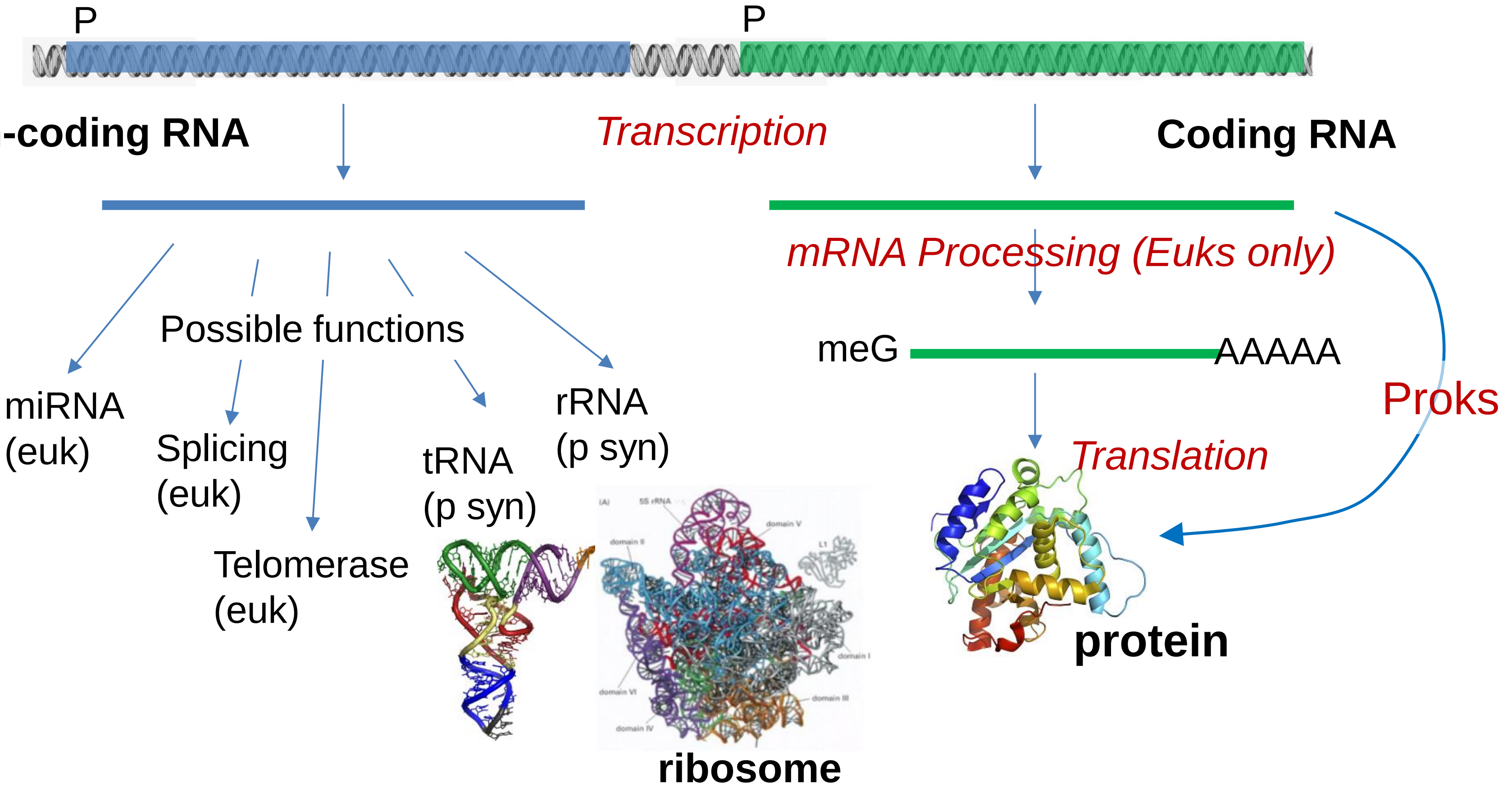
Gene – a segment of DNA that is converted (*transcribed*) to RNA. A *promoter* (P) sequence on the DNA is the minimal requirement for the production of RNA.

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



P

P

Non-coding RNA

Transcription

Coding RNA

mRNA Processing (Euks only)

meG

AAAAA

Proks

Translation

Possible functions

miRNA
(euk)

Splicing
(euk)

Telomerase
(euk)

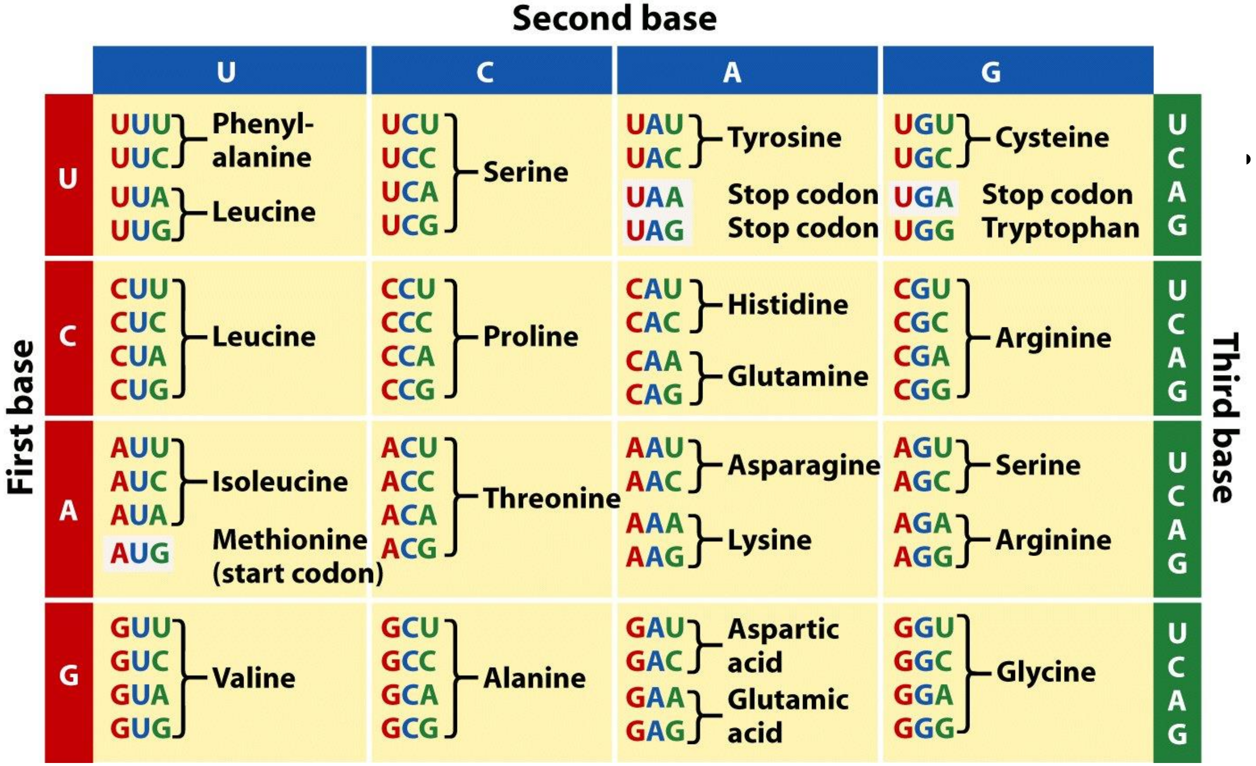
tRNA
(p syn)

rRNA
(p syn)

ribosome

protein

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



Second base

First base	U	C	A	G	Third base
U	UUU Phenyl-alanine	UCU Serine	UAU Tyrosine	UGU Cysteine	U
	UUC	UCC	UAC	UGC	C
	UUA Leucine	UCA	UAA Stop codon	UGA Stop codon	A
	UUG	UCG	UAG Stop codon	UGG Tryptophan	G
C	CUU Leucine	CCU Proline	CAU Histidine	CGU Arginine	U
	CUC	CCC	CAC	CGC	C
	CUA	CCA	CAA Glutamine	CGA	A
	CUG	CCG	CAG	CGG	G
A	AUU Isoleucine	ACU Threonine	AAU Asparagine	AGU Serine	U
	AUC	ACC	AAC	AGC	C
	AUA	ACA	AAA Lysine	AGA Arginine	A
	AUG Methionine (start codon)	ACG	AAG	AGG	G
G	GUU Valine	GCU Alanine	GAU Aspartic acid	GGU Glycine	U
	GUC	GCC	GAC	GGC	C
	GUA	GCA	GAA Glutamic acid	GGA	A
	GUG	GCG	GAG	GGG	G

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)

_______-_______-_______-______

(Protein Sequence)

- 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.

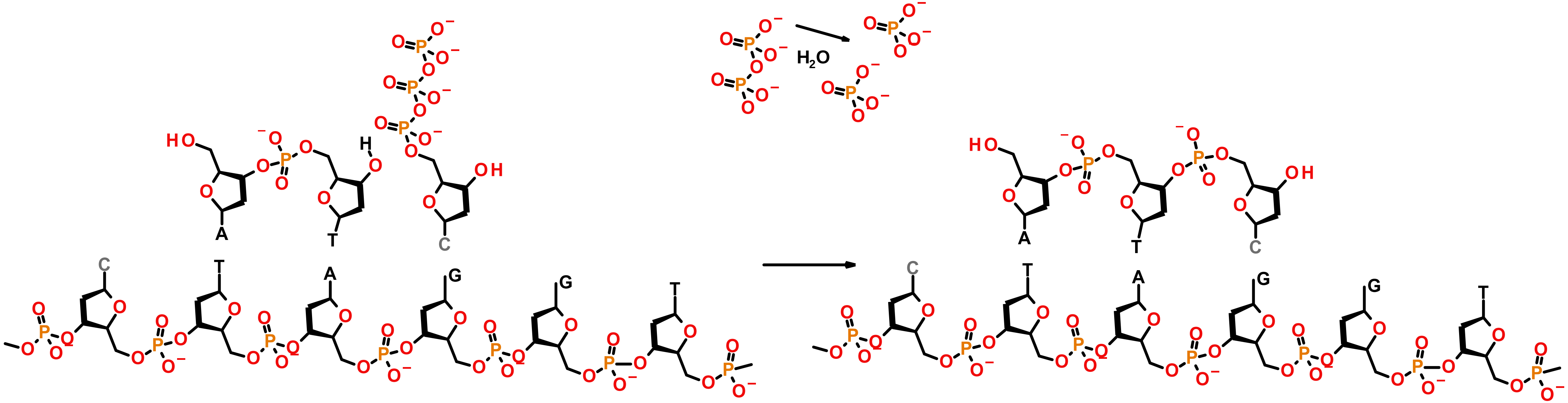
Special Codons:

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

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.



$^{5'}$A-T$^{3'OH}$
$^{3'}$-C-T-A-G-G-T-

⇒

*$^{5'}$A-T-**C**$^{3'OH}$*
$^{3'}$-C-T-A-G-G-T-

⇒

*$^{5'}$A-T-C-**C**$^{3'OH}$*
$^{3'}$-C-T-A-G-G-T-

Expectations: Know the features of this reaction.

DNA Polymerase – Fundamental Activity.

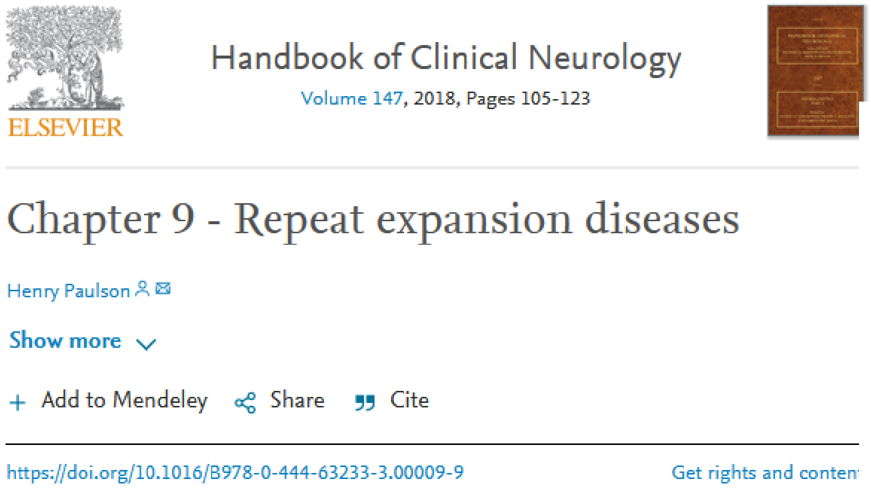
```
            5’  A-T-C-A


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
  1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5
                    1                   2
```

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

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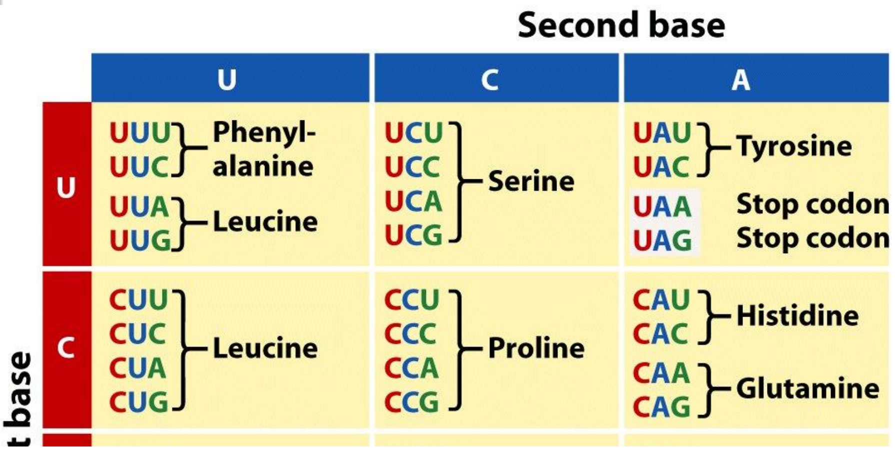


Second base

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

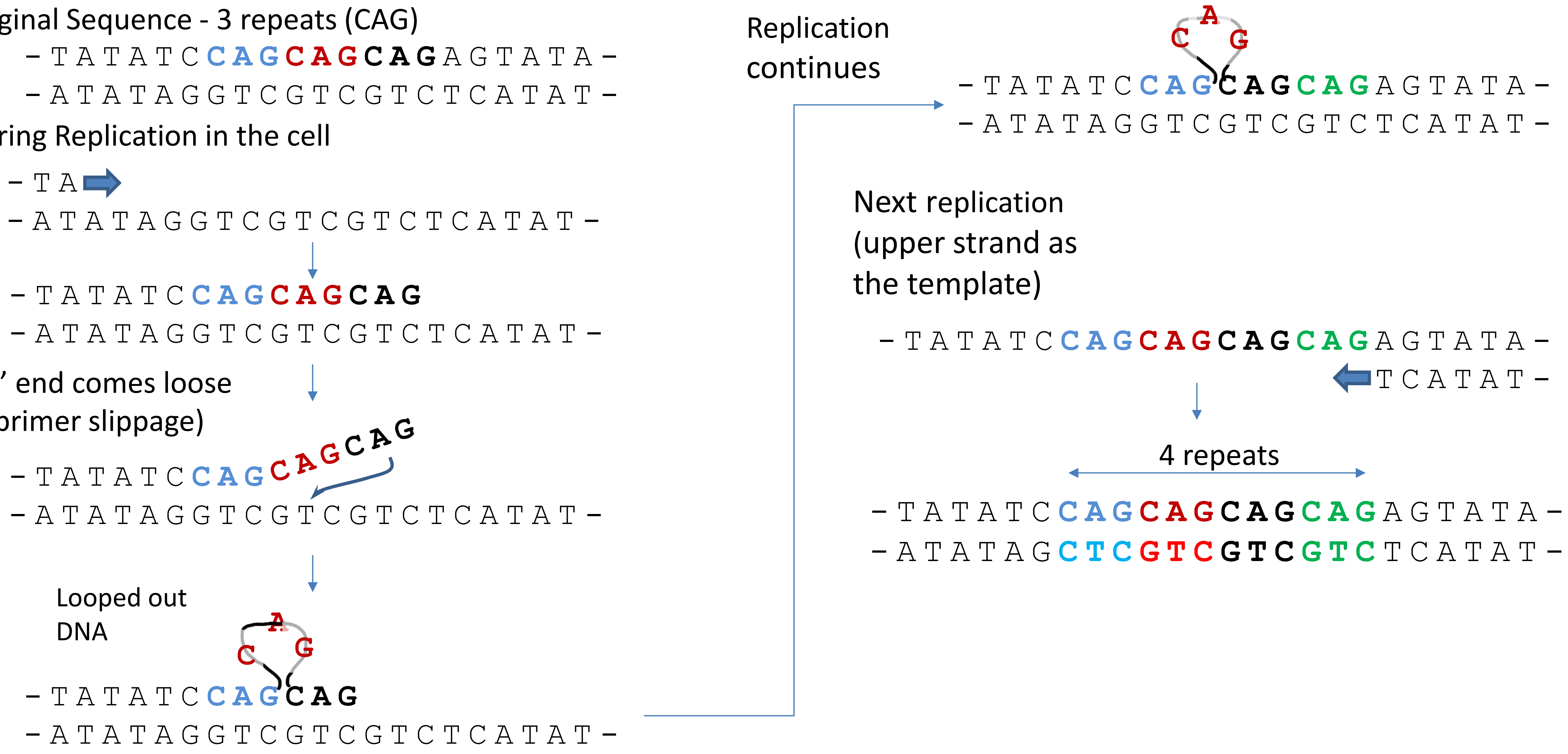
- CAG – at least 10 diseases (Huntington disease, spinal and bulbar muscular atrophy, dentatorubral-pallidoluysian atrophy and seven SCAs)
- CGG – fragile X, fragile X tremor ataxia syndrome, other fragile sites (GCC, CCG)
- CTG – myotonic dystrophy type 1, Huntington disease-like 2, spinocerebellar 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 (CAG)

-TATATCCAGCAGCAGAGTATA-
-ATATAGGTCGTCGTCTCATAT-

During Replication in the cell

-TA
-ATATAGGTCGTCGTCTCATAT-

-TATATCCAGCAGCAG
-ATATAGGTCGTCGTCTCATAT-

3' end comes loose
(primer slippage)

-TATATCCAGCAGCAG
-ATATAGGTCGTCGTCTCATAT-

Looped out
DNA

-TATATCCAGCAG
-ATATAGGTCGTCGTCTCATAT-

Replication
continues

-TATATCCAGCAGCAGAGTATA-
-ATATAGGTCGTCGTCTCATAT-

Next replication
(upper strand as
the template)

-TATATCCAGCAGCAGCAGAGTATA-
TCATAT-

4 repeats

-TATATCCAGCAGCAGCAGAGTATA-
-ATATAGCTCGTCGTCGTCTCATAT-

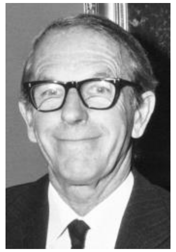
DNA Sequencing – Sanger (dideoxy) Sequencing

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 $^{5'}$**C-A-T-A-T-G**^{OH}

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----

Known Seq (plasmid) Unknown sequence (insert)

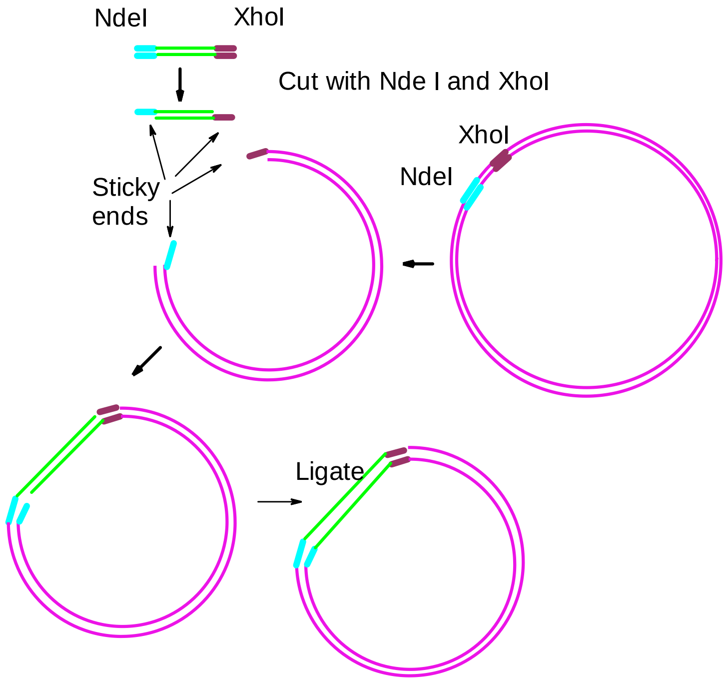
Sequenced region (~1000 bases)

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----

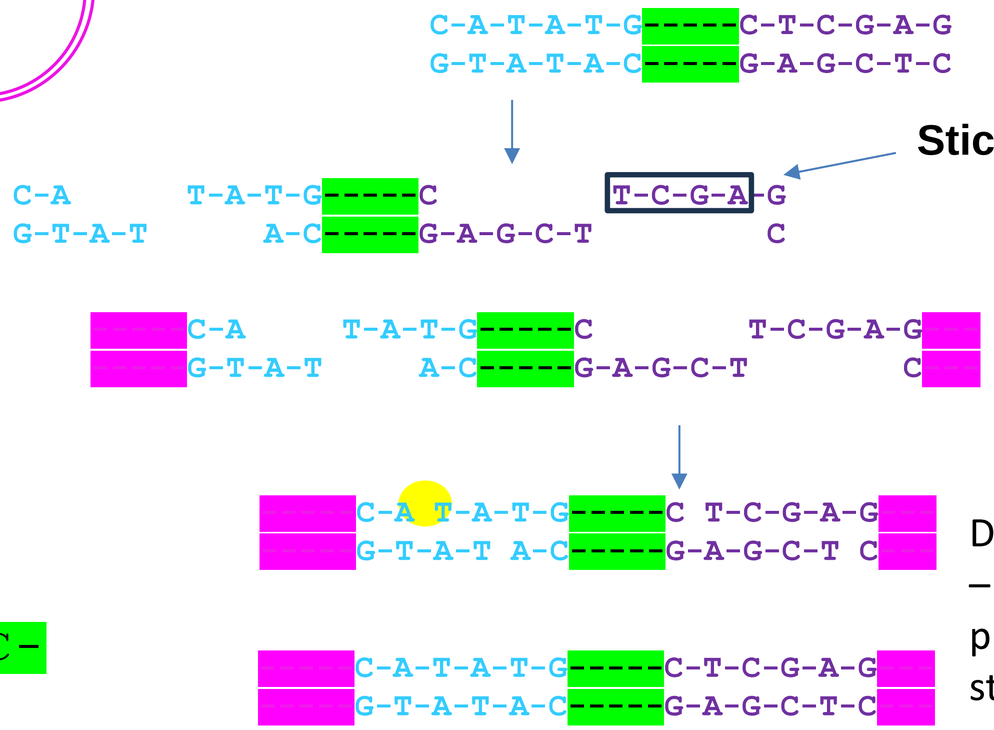
DNA Sequencing - Determining the Order of Bases Added by DNA Polymerase

- 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.



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

Sticky end

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

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

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

DNA Ligase – reform phosphodiester bond

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

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.

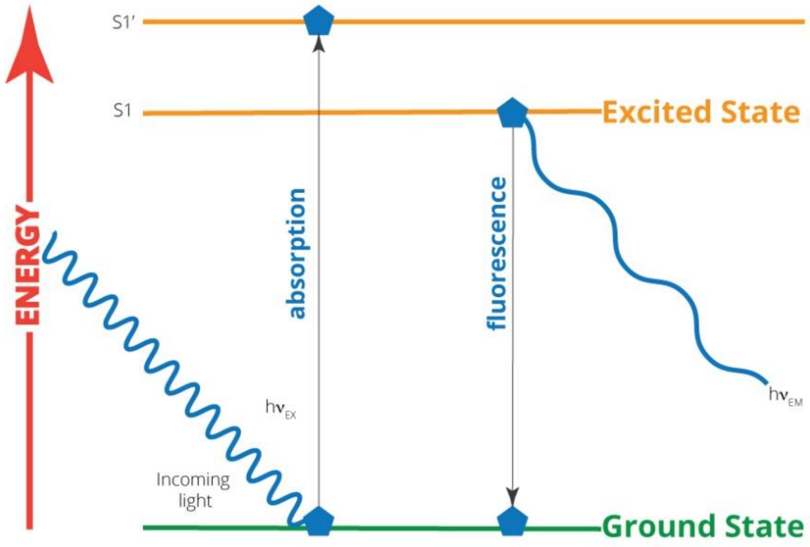
First base is added here.

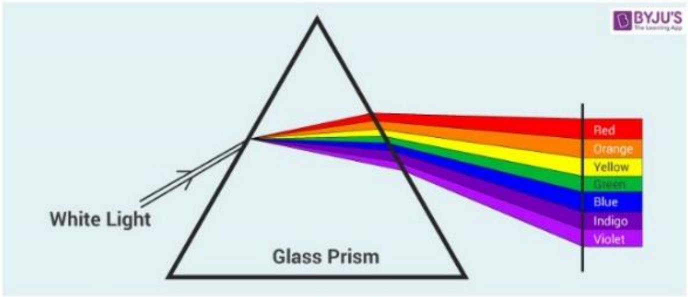
```
     5′C-A-T-A-T-G OH
3′-A-G-G-T-A-T-A-C-C-A-T-T-A-G-G-C-C-
```

Known Seq (plasmid) Unknown sequence (insert)

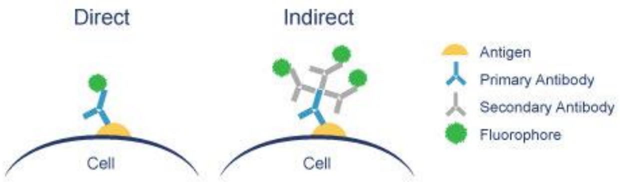
DNA Sequencing Methods Use Fluorescent Bases - What is Fluorescence?

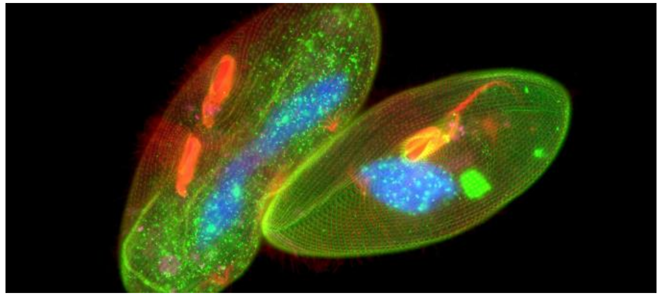
- When molecules absorb light an electron goes from a lower shell to a higher shell. This is where the energy from the light goes.
- In most molecules the electron goes back down to its original shell with the release of heat.
- Fluorescent molecules emit the energy as light of a longer wavelength (different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





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

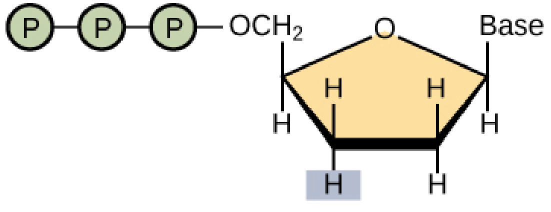




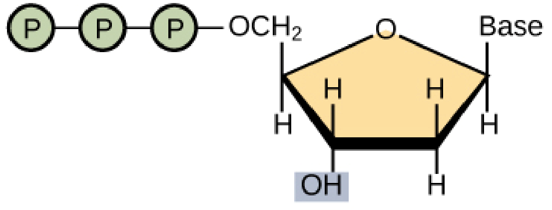
DNA Sequencing - Determining the Order of Bases Added by DNA Polymerase

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

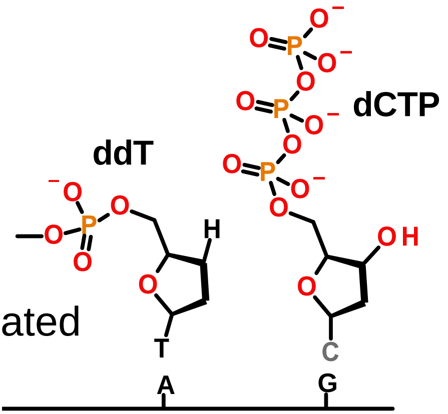


Dideoxynucleotide (ddNTP)

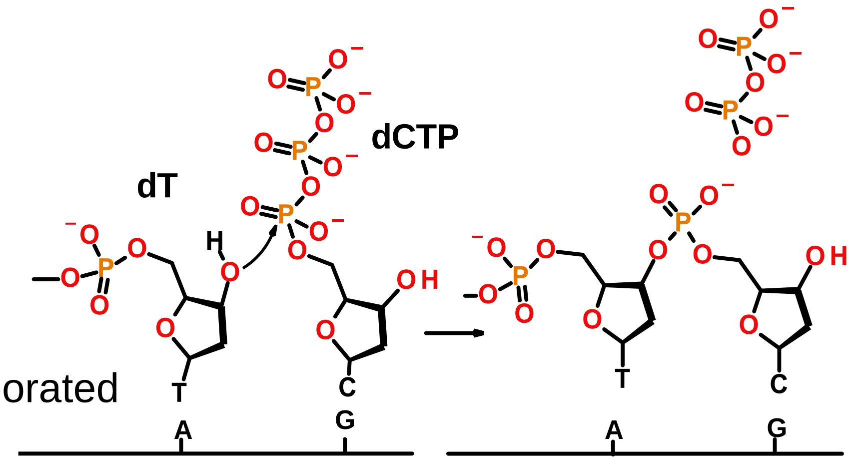


Deoxynucleotide (dNTP)

After **ddTTP** incorporated

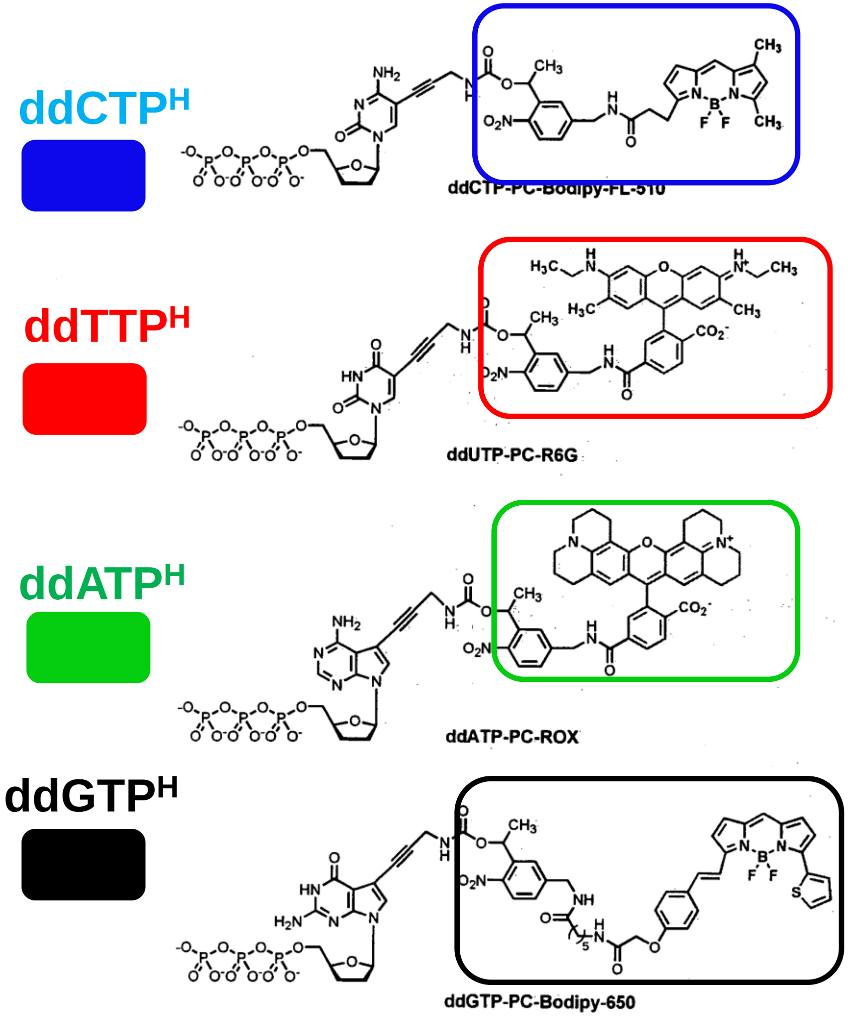


After **dTTP** incorporated



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.

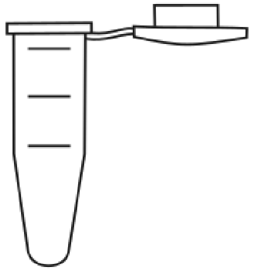


ddCTP^{H} 🟦

ddTTP^{H} 🟥

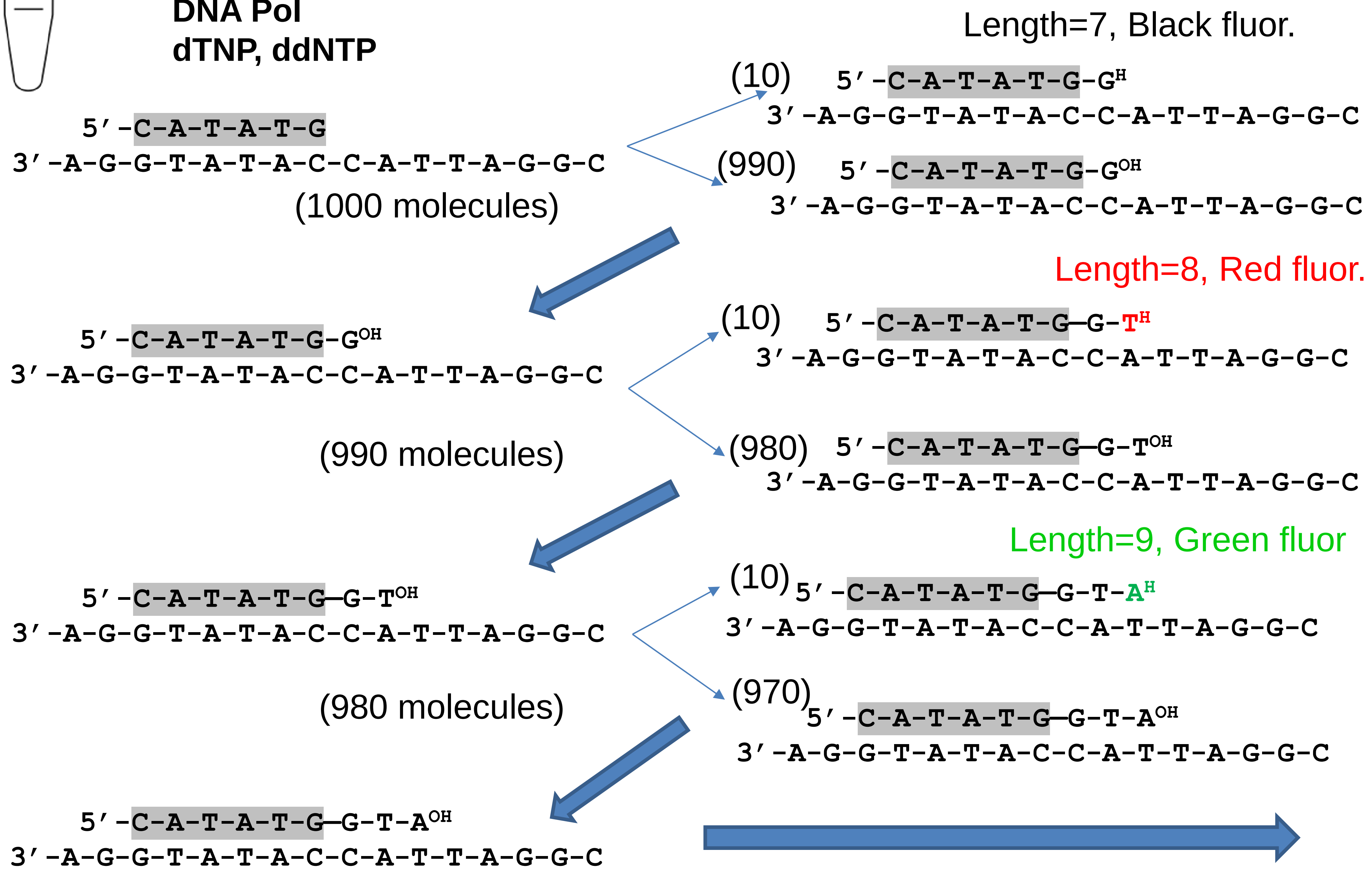
ddATP^{H} 🟩

ddGTP^{H} ⬛



Template
Primer
DNA Pol
dTNP, ddNTP

DNA Sequencing – Generation of Fluorescent Fragments



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)

Length=7, Black fluor.

(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

(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

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)

Length=8, Red fluor.

(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

(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

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)

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

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

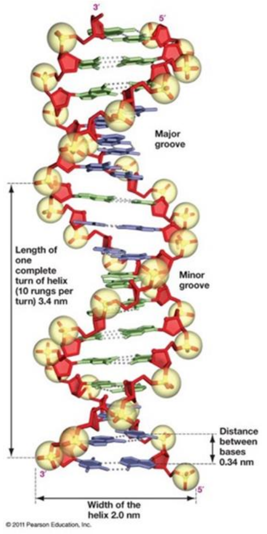
All Possible Fragments are Made:

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.

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

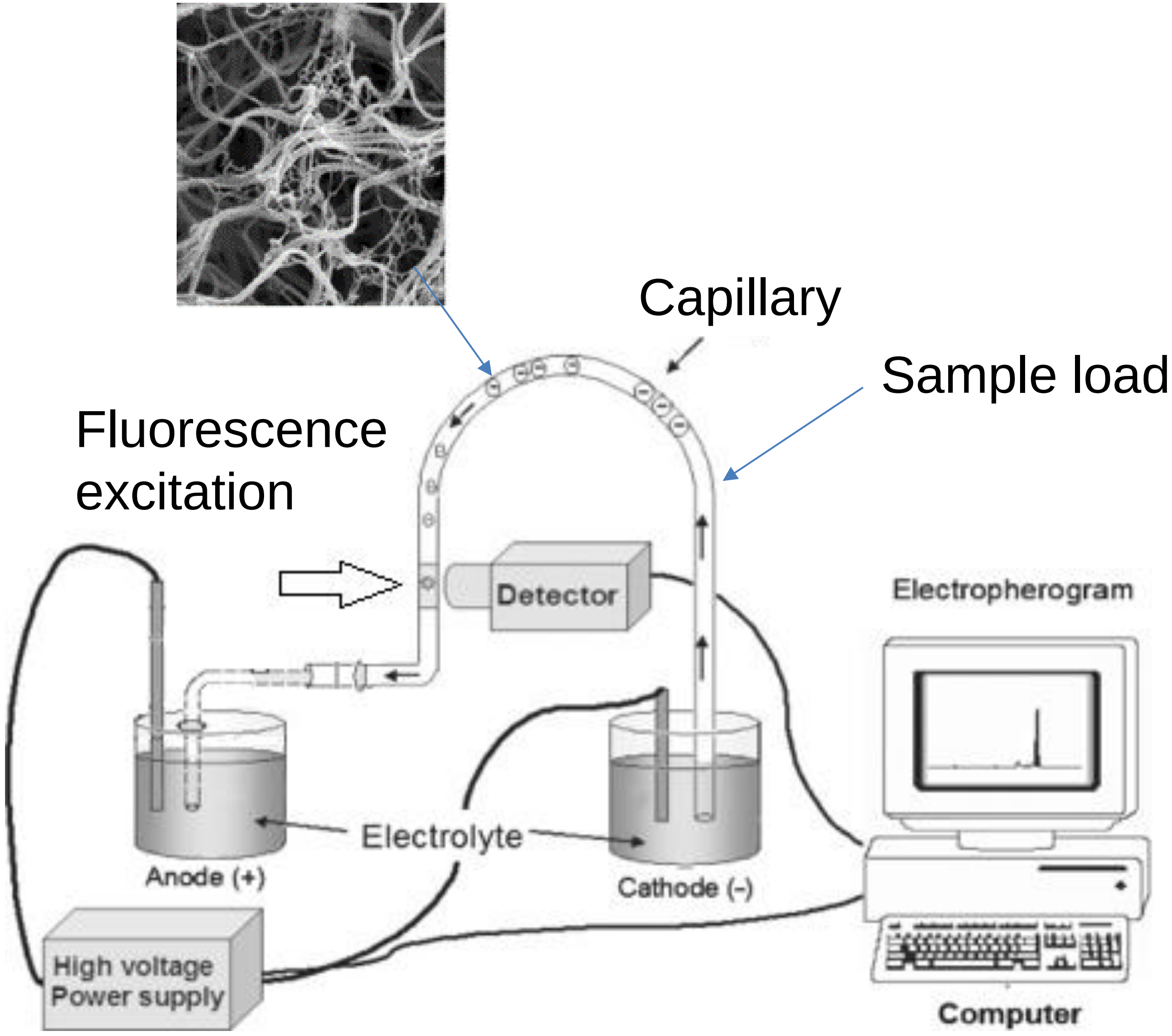


DNA has a negative charge.
It will migrate towards the anode.

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

DNA molecules that are smaller

migrate _______________.



Capillary

Sample loaded

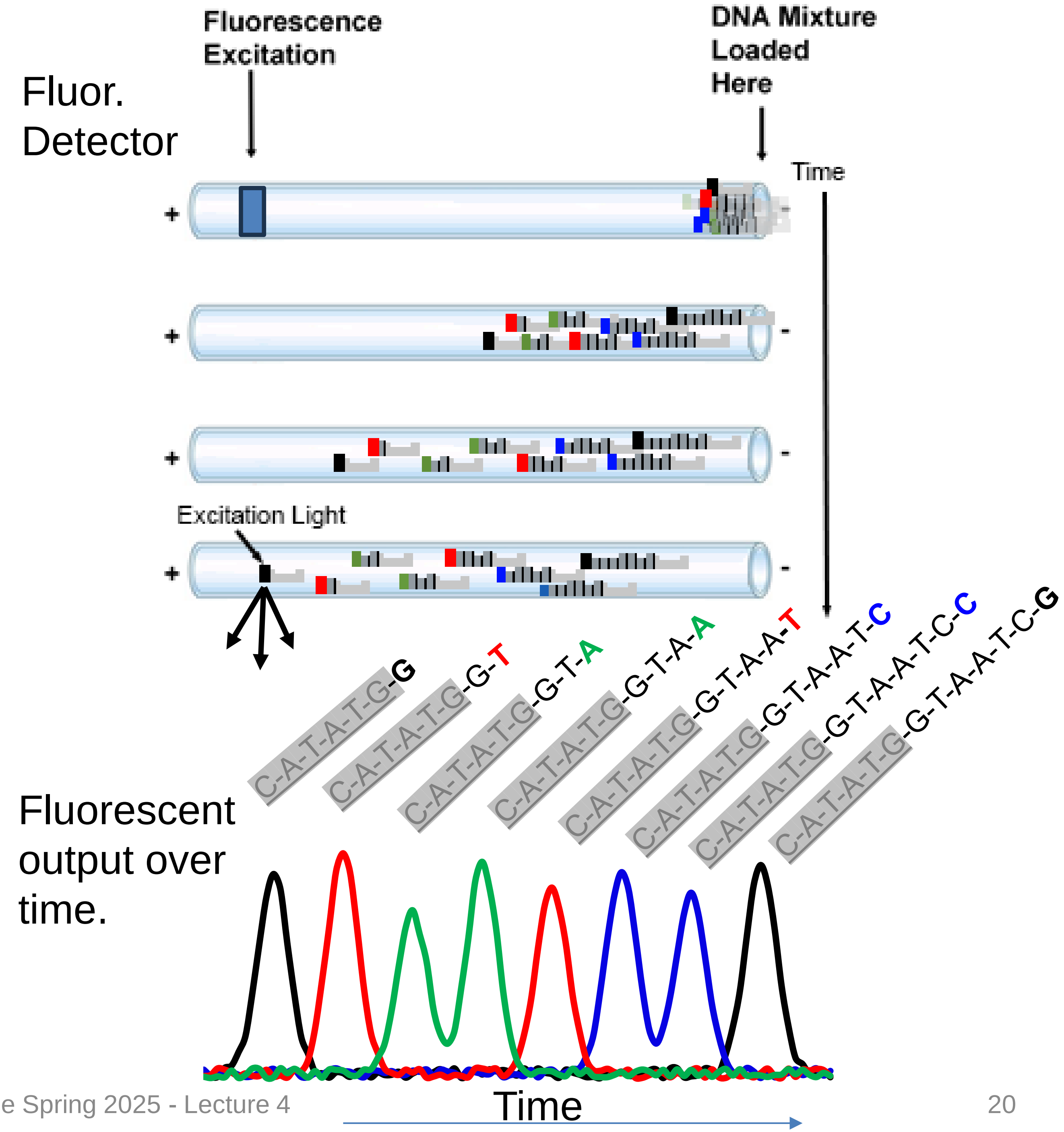
Fluorescence excitation

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

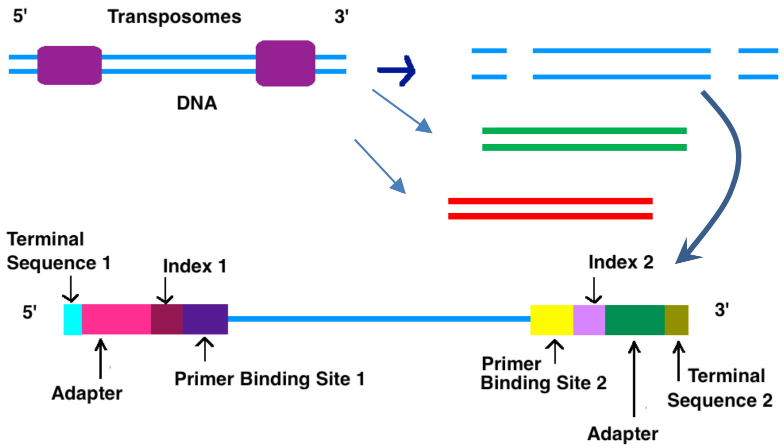


Fluor. Detector

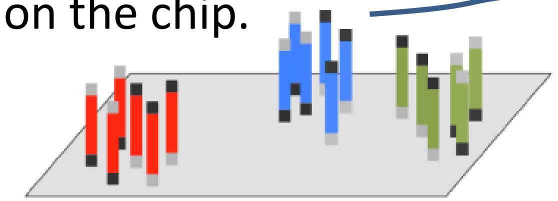
Fluorescent output over time.

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

A. Obtaining the DNA

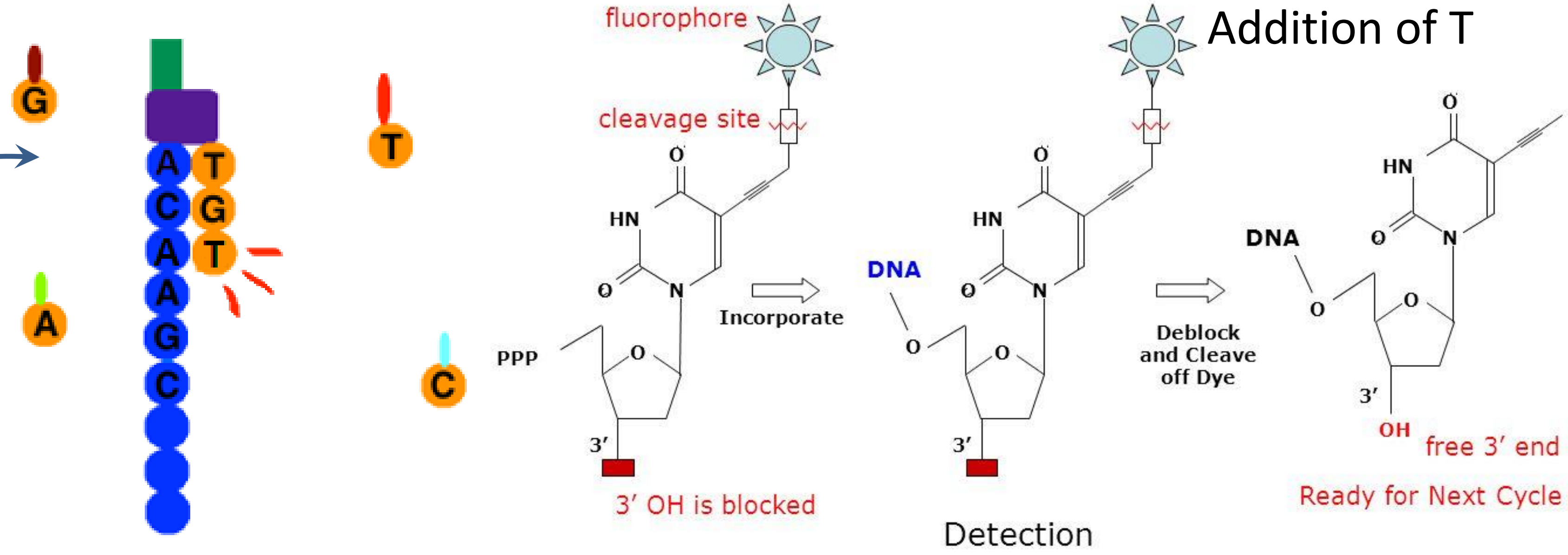


- The entire genome can be sequenced.
- The DNA is fragmented into small 100 base pieces.
- Synthetic DNA is added to the ends (sites for primers for sequencing)
- Different fragments are bound to different location on a solid surface (chip).
- All fragments are sequenced at the same time on the chip.



Cluster formation

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

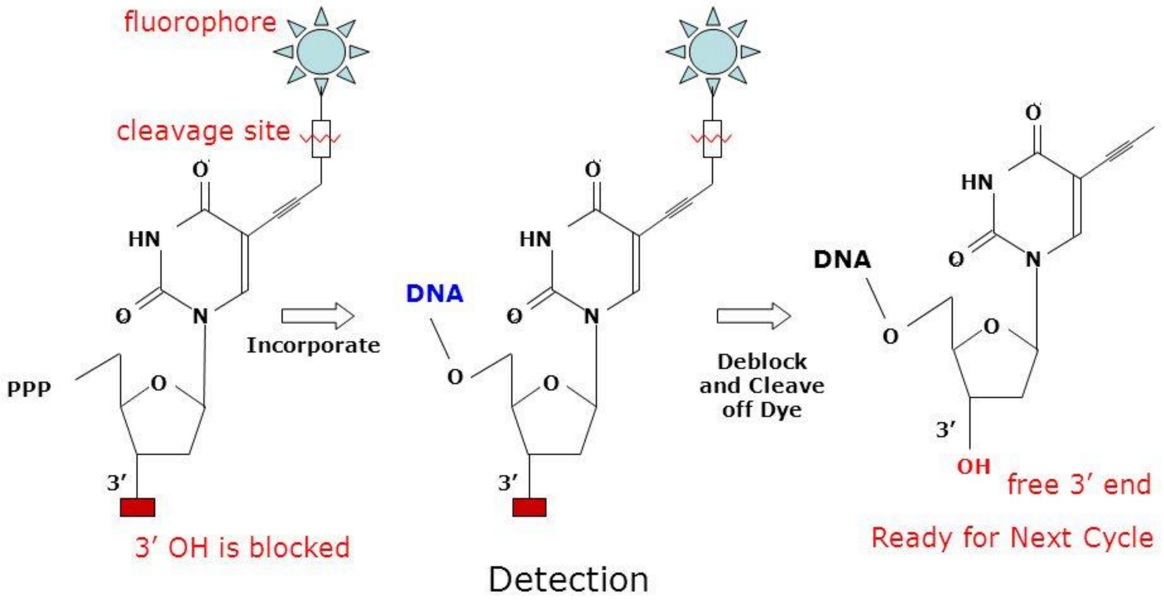


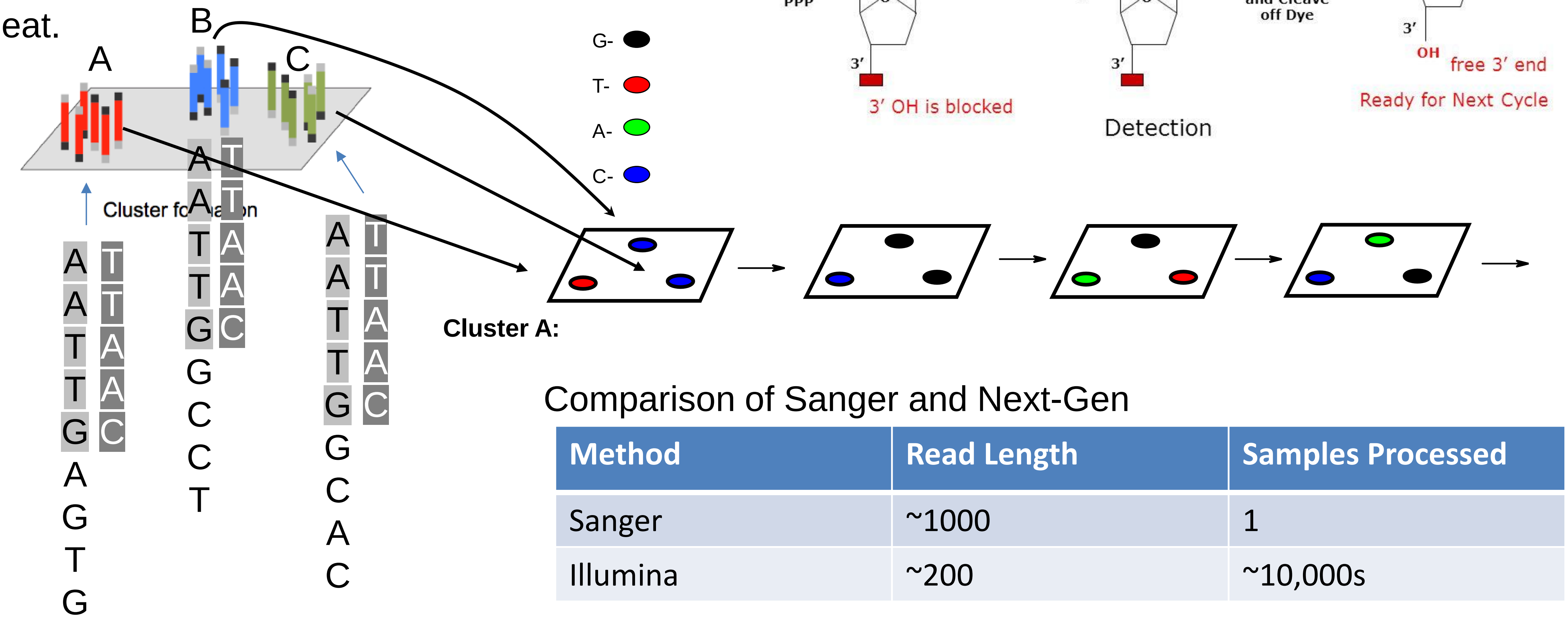
1. Only one base is added at a time (3’-OH is blocked)
2. The base that is added is determined by the color of the fluorescent base.
3. 3’-OH blocking group and the fluorescent group are removed prior to the next addition. ~100 cycles can be performed.

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.



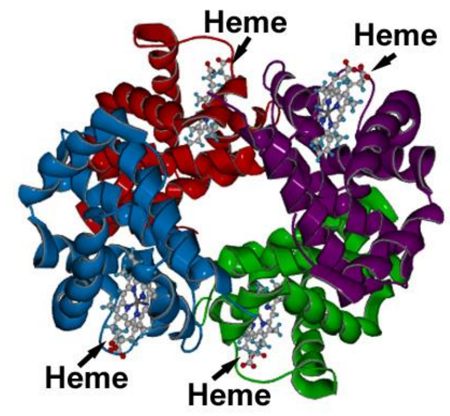


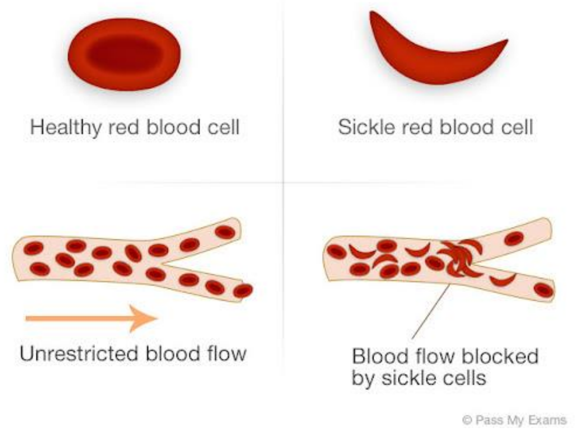
Comparison of Sanger and Next-Gen

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.





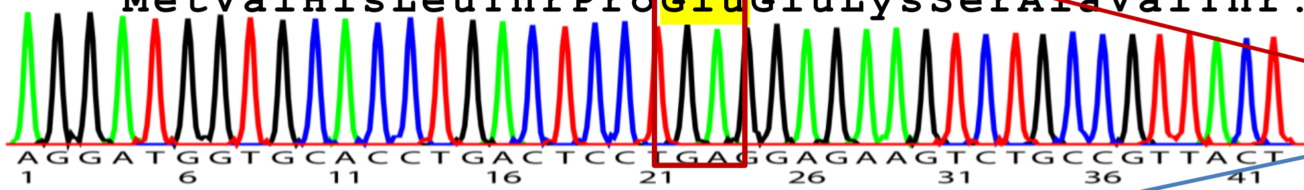
The 5’ end of the Hb gene is shown on the right (ATG=start). Using GGTGCCAG as a sequencing primer gives the following sequences for the normal and mutant genes:

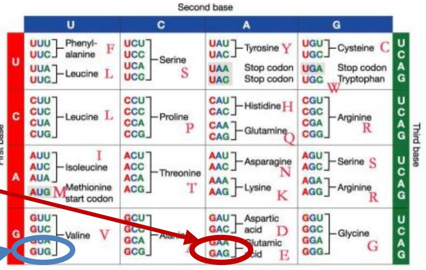
First dd-base added by polymerase

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

Sequencing data for the normal beta chain is:

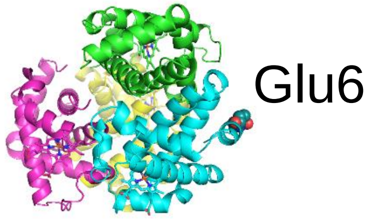
```
AGGATGGTGCACCTGACTCCTGAGGAGAAGTCTGCCGTTACT...
   MetValHisLeuThrProGluGluLysSerAlaValThr...
```





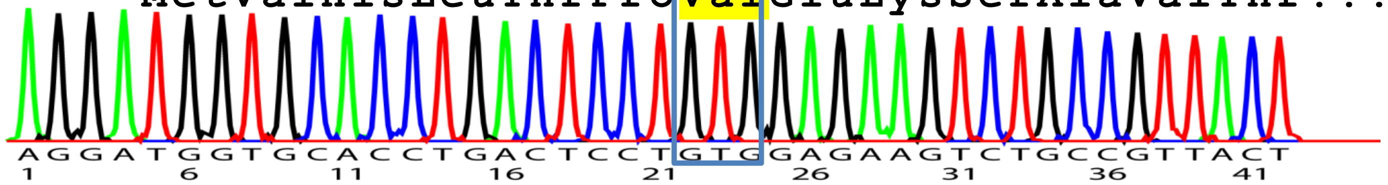
Second base

First base	U	C	A	G	Third base
U	UUU UUC Phenyl-alanine F UUA UUC Leucine L	UCU UCC UCA UCG Serine S	UAU UAC Tyrosine Y UAA Stop codon UAG Stop codon	UGU UGC Cysteine C UGA Stop codon UGC Tryptophan W	U C A G
C	CUU CUC CUA CUG Leucine L	CCU CCC CCA CCG Proline P	CAU CAC Histidine H CAA CAG Glutamine Q	CGU CGC CGA CGG Arginine R	U C A G
A	AUU AUC AUA Isoleucine I AUG Methionine start codon M	ACU ACC ACA ACG Threonine T	AAU AAC Asparagine N AAA AAG Lysine K	AGU AGC Serine S AGA AGG Arginine R	U C A G
G	GUU GUC GUA GUG Valine V	GCU GCC GCA GCG Ala	GAU GAC Aspartic acid D GAA GAG Glutamic acid E	GGU GGC GGA GGG Glycine G	U C A G



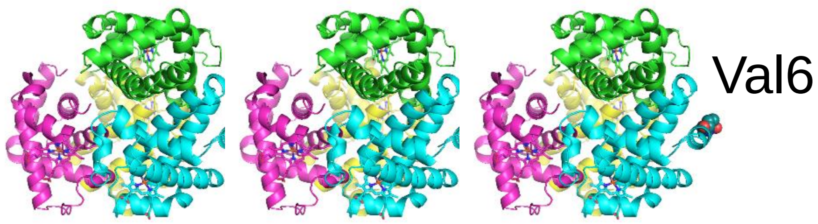
Sequencing data for the mutation:

```
AGGATGGTGCACCTGACTCCTGTGGAGAAGTCTGCCGTTACT...
   MetValHisLeuThrProValGluLysSerAlaValThr...
```



False color code:

A=Green
G=Black
T=Red
C=Blue



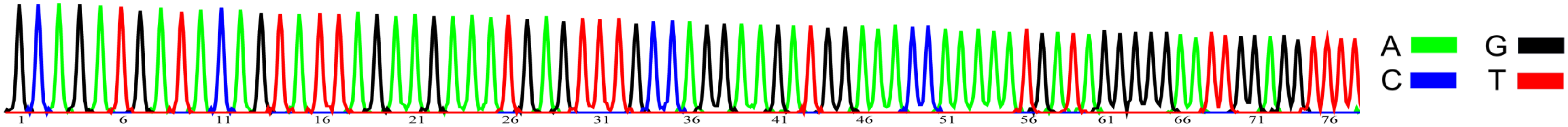
Sequencing Summary

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:

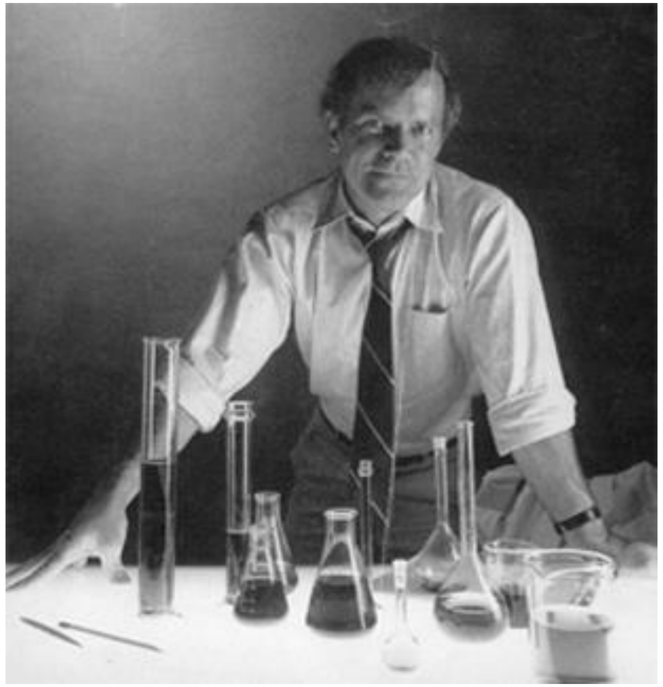
- Simultaneous sequencing of a large number of fragments
- Shorter “reads” 100 versus 1000 bases/template



GCAGATGATACAGTATTAGAAGAAATGAGTTTGCCAGGAAGATGGAAACCAAAAATGATA....

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.

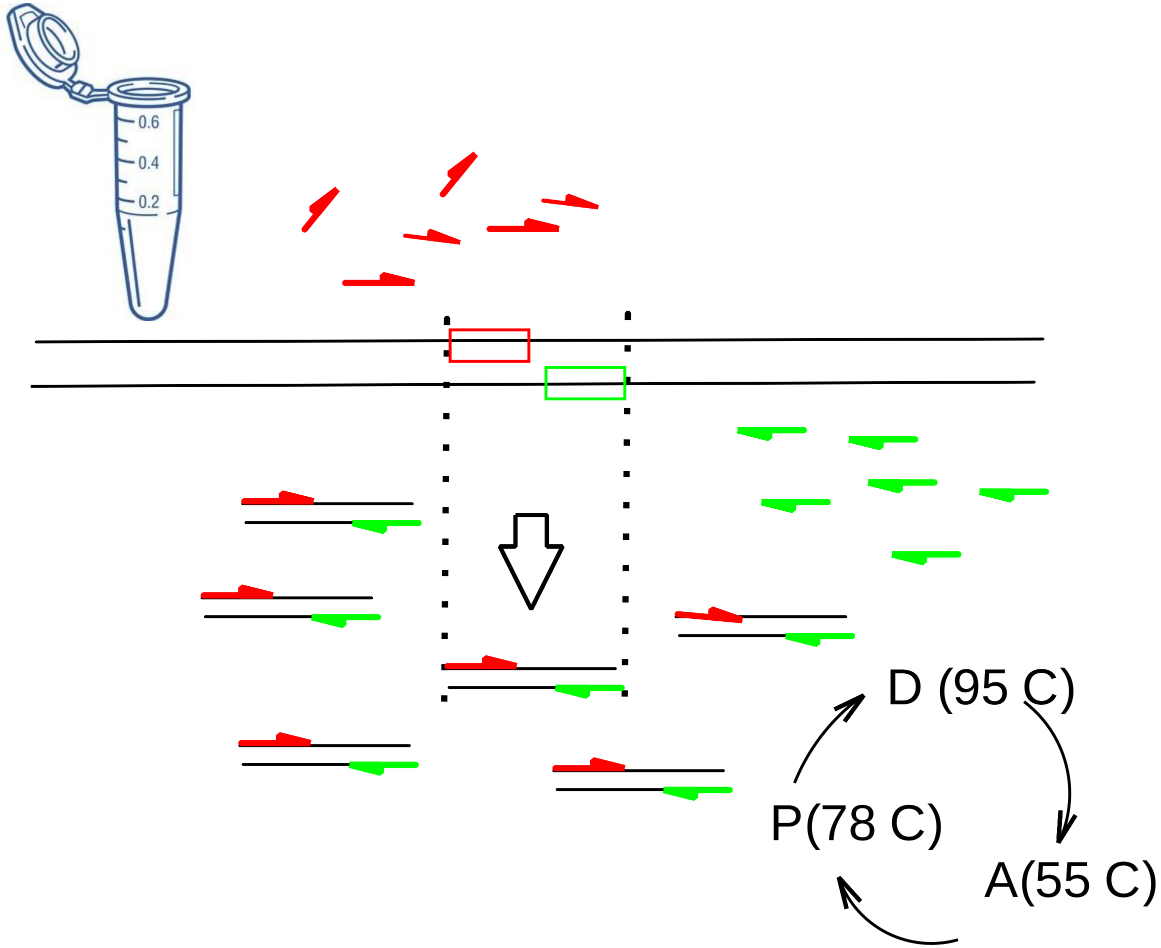


Expectations:

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.



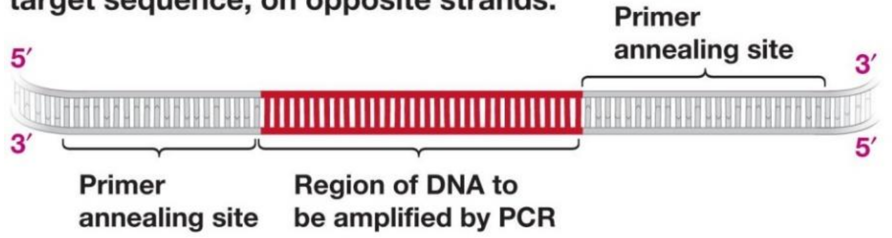
Each PCR cycle consists of three steps:

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)

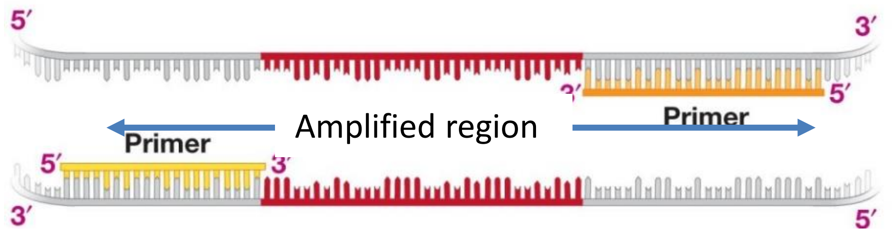
PCR – Primer Design

- 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!



(a) PCR primers must bind to sequences on either side of the target sequence, on opposite strands.



(b) When target DNA is single stranded, primers bind and allow DNA polymerase to work.

Amplified region

5′--AAG**CTGAC**TAGTCGATGCGAATGTGCGGTGC—3′

3′--TTCGACTGATCAGCTACGCTT**ACACG**CCACG—5′

5′CTGAC

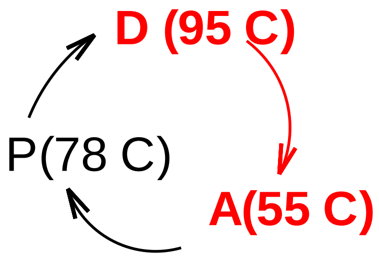
ACACG5′ = 5′GCACA

CTGACTAGTCGATGCGAATGTGC

GACTGATCAGCTACGCTT**ACACG**

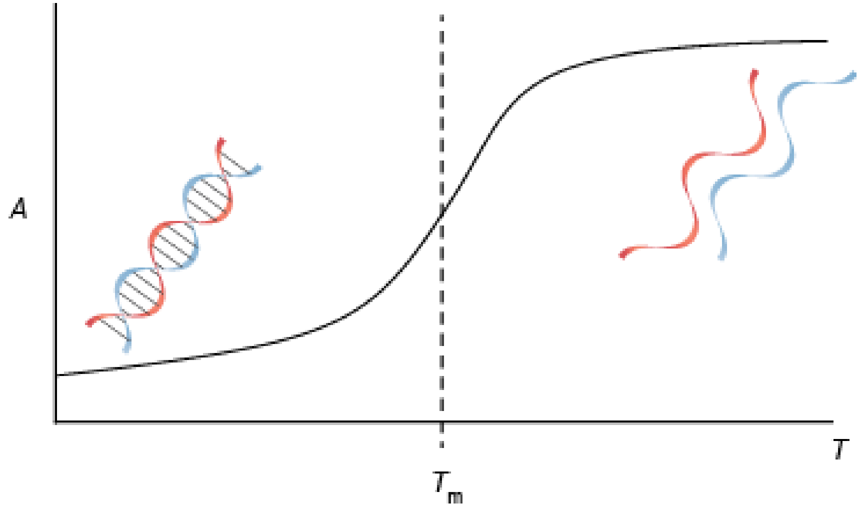
Note: Actual primer lengths are 20-30 bases, in the illustrations here and on problem sets, much shorter primers are used.

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



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



5’ A-T-C-A

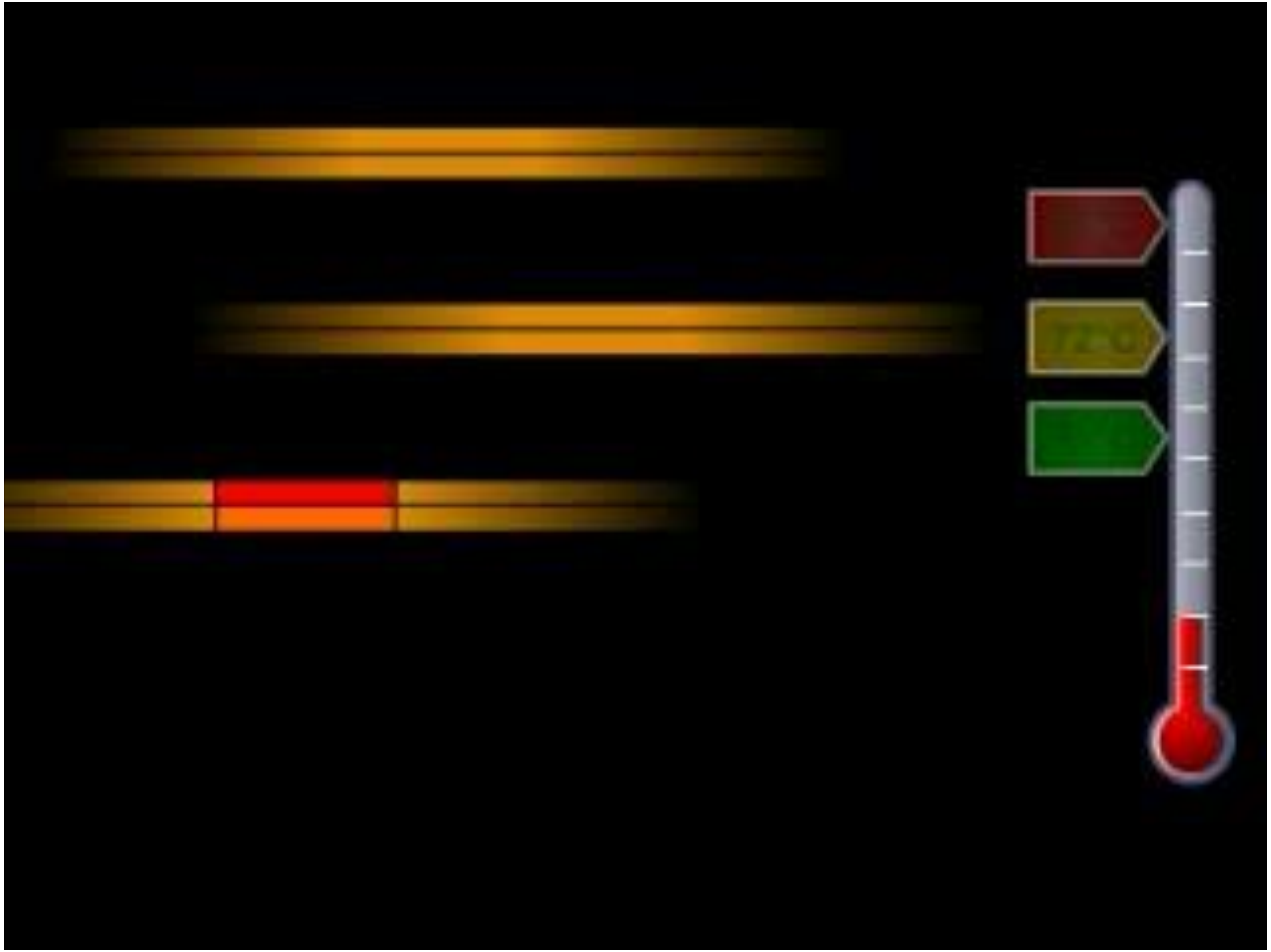
3’A-T-G-C-C-G-T-A-G-T-C-G-T-A-C-A-G-T-A-C-G-T-G-C-A

Polymerase Characteristics

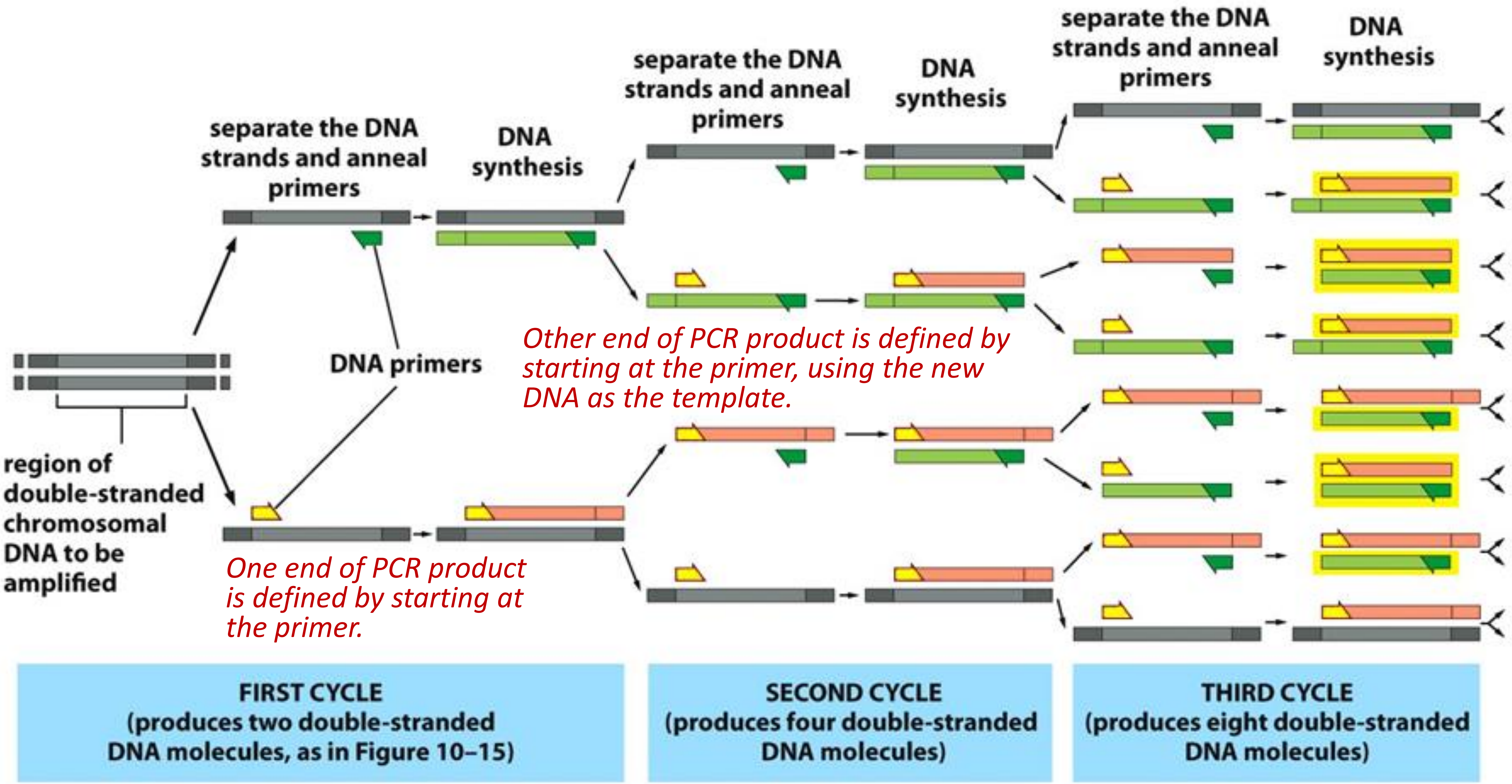
- Since the first step of each cycle (D) requires heating to high temperature, a thermostable polymerase is required.
- The first polymerase, Taq, was isolated from *Thermus Aquaticus,* a bacterial living in hot springs (Yellowstone National Park)
- A number of different polymerases with improved properties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

PCR Animation

Watch Me!



Three PCR Cycles



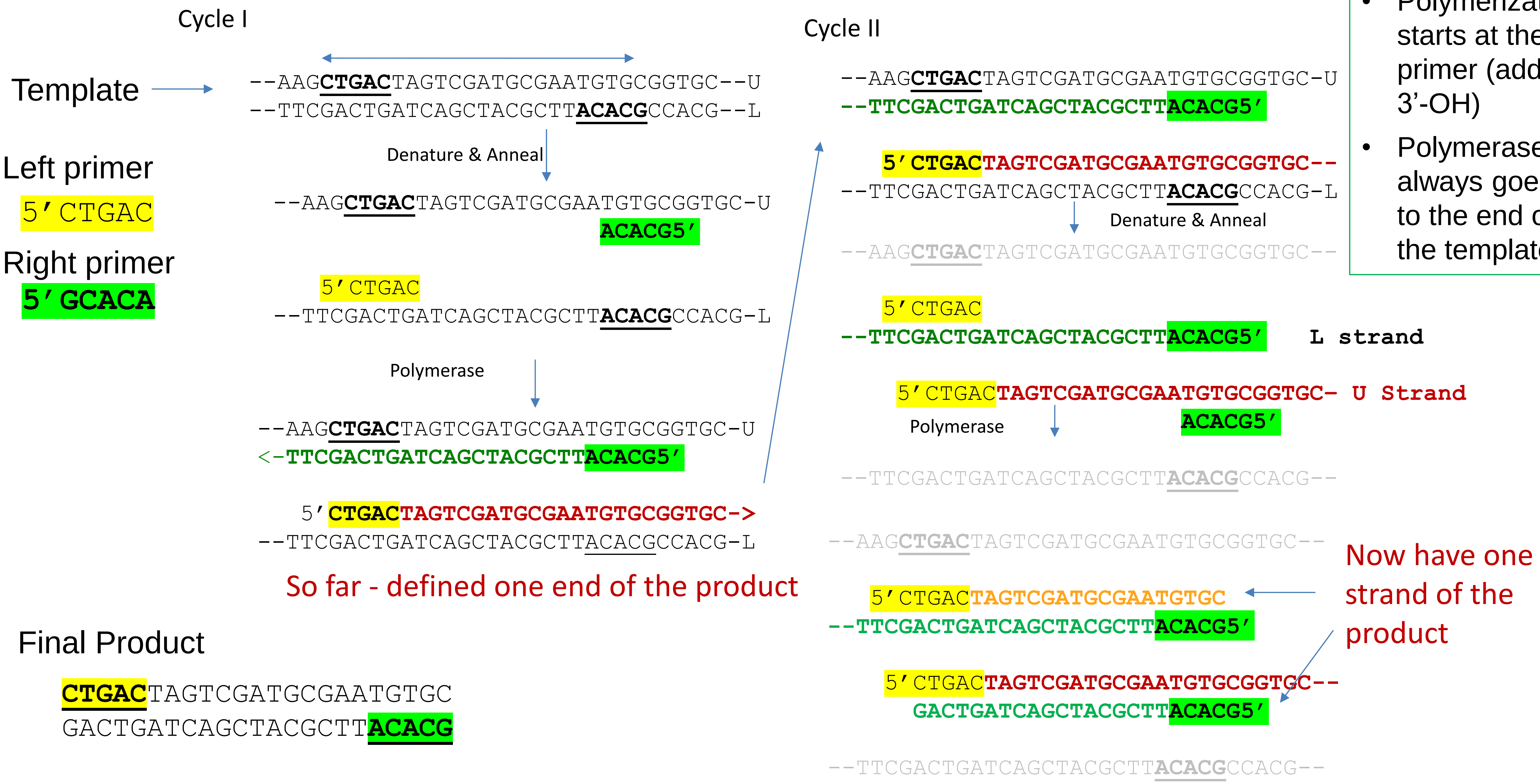
Other end of PCR product is defined by starting at the primer, using the new DNA as the template.

One end of PCR product is defined by starting at the primer.

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.



Cycle I

Template

```
--AAGCTGACTAGTCGATGCGAATGTGCGGTGC--U
--TTCGACTGATCAGCTACGCTTACACGCCACG--L
```

Left primer

5’CTGAC

Right primer

5’GCACA

Denature & Anneal

```
--AAGCTGACTAGTCGATGCGAATGTGCGGTGC-U
                         ACACG5’
```

```
  5’CTGAC
--TTCGACTGATCAGCTACGCTTACACGCCACG-L
```

Polymerase

```
--AAGCTGACTAGTCGATGCGAATGTGCGGTGC-U
<-TTCGACTGATCAGCTACGCTTACACG5’
```

```
  5’CTGACTAGTCGATGCGAATGTGCGGTGC->
--TTCGACTGATCAGCTACGCTTACACGCCACG-L
```

So far - defined one end of the product

Final Product

```
CTGACTAGTCGATGCGAATGTGC
GACTGATCAGCTACGCTTACACG
```

Cycle II

```
--AAGCTGACTAGTCGATGCGAATGTGCGGTGC-U
--TTCGACTGATCAGCTACGCTTACACG5’
```

```
   5’CTGACTAGTCGATGCGAATGTGCGGTGC--
--TTCGACTGATCAGCTACGCTTACACGCCACG-L
```

Denature & Anneal

```
--AAGCTGACTAGTCGATGCGAATGTGCGGTGC--
```

```
   5’CTGAC
--TTCGACTGATCAGCTACGCTTACACG5’      L strand
```

```
    5’CTGACTAGTCGATGCGAATGTGCGGTGC- U Strand
                         ACACG5’
```

Polymerase

```
--TTCGACTGATCAGCTACGCTTACACGCCACG--
```

```
--AAGCTGACTAGTCGATGCGAATGTGCGGTGC--
```

```
   5’CTGACTAGTCGATGCGAATGTGC
--TTCGACTGATCAGCTACGCTTACACG5’
```

Now have one strand of the product

```
   5’CTGACTAGTCGATGCGAATGTGCGGTGC--
       GACTGATCAGCTACGCTTACACG5’
```

```
--TTCGACTGATCAGCTACGCTTACACGCCACG--
```

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.

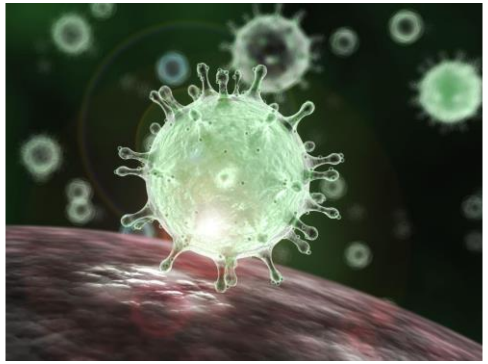
Example – follow the PCR cycles for the following template with primers 5’ AATT (left) and 5’ GGCC (right)

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

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

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 aaaatgtctgataatg**GACCCCAAAATCAGCGAAAT**gcaccccgcattacgtttggtggaccctcagattcaactggcagtaaccagaatggagaacgca
      ttttacagactattacctggggttttagtcgctttacgtggggcgtaatgcaaaccacctggga**GTCTAAGTTGACCGTCATTGGTCT**tacctcttgcgt

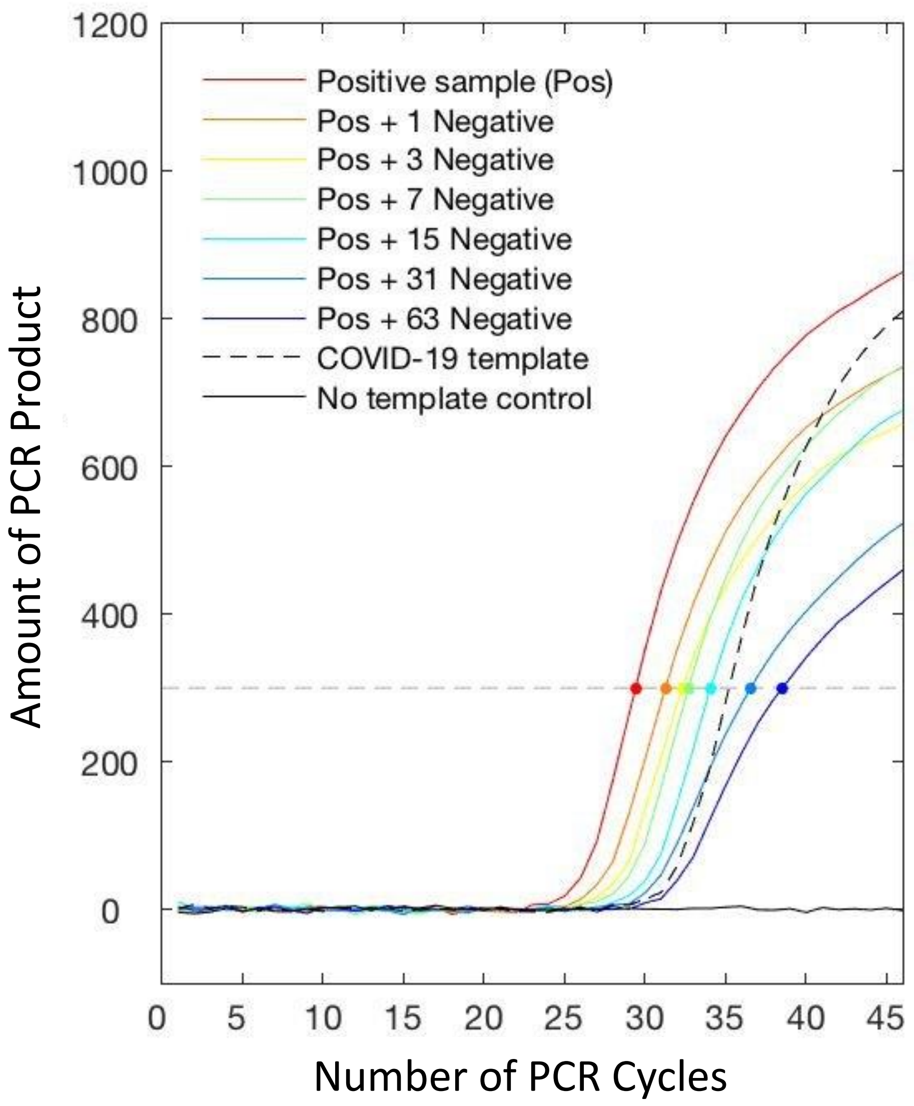
PCR Product

GACCCCAAAATCAGCGAAATGCACCCCGCATTACGTTTGGTGGACCCTCAGATTCAACTGGCAGTAACCAGA
CTGGGGTTTTAGTCGCTTTACGTGGGGCGTAATGCAAACCACCTGGGA**GTCTAAGTTGACCGTCATTGGTCT**

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

Covid 19 PCR Test: Detection of the PCR Product.

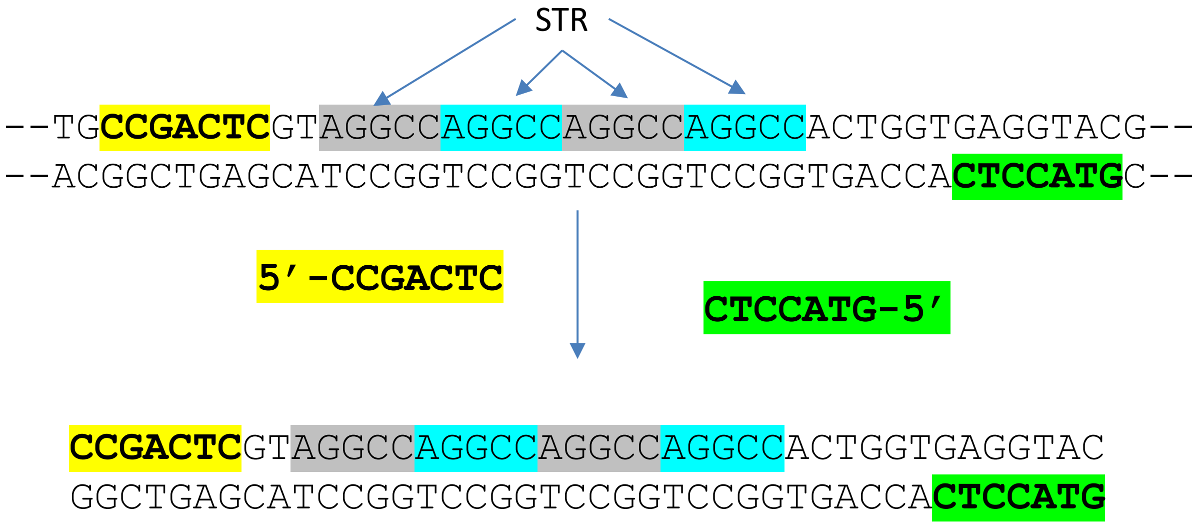
https://www.medrxiv.org/content/10.1101/2020.03.26.20039438v1



- 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*



STR

--TG**CCGACTC**GTAGGCCAGGCCAGGCCAGGCCACTGGTGAGGTACG--
--ACGGCTGAGCATCCGGTCCGGTCCGGTCCGGTGACCA**CTCCATG**C--

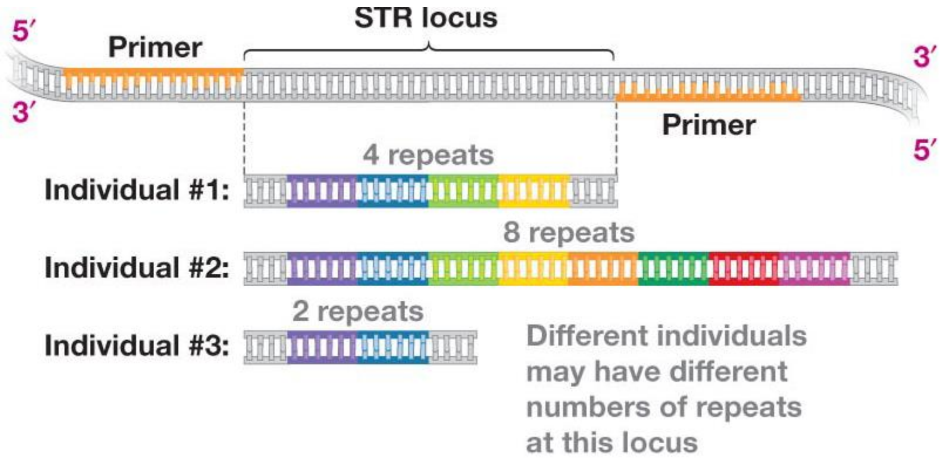
5′-CCGACTC

CTCCATG-5′

CCGACTCGTAGGCCAGGCCAGGCCAGGCCACTGGTGAGGTAC
GGCTGAGCATCCGGTCCGGTCCGGTCCGGTGACCA**CTCCATG**

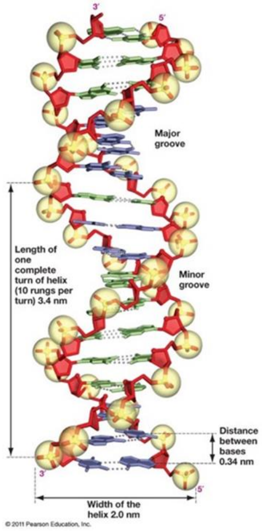
Which individual has the shortest PCR product?

Which has the longest?

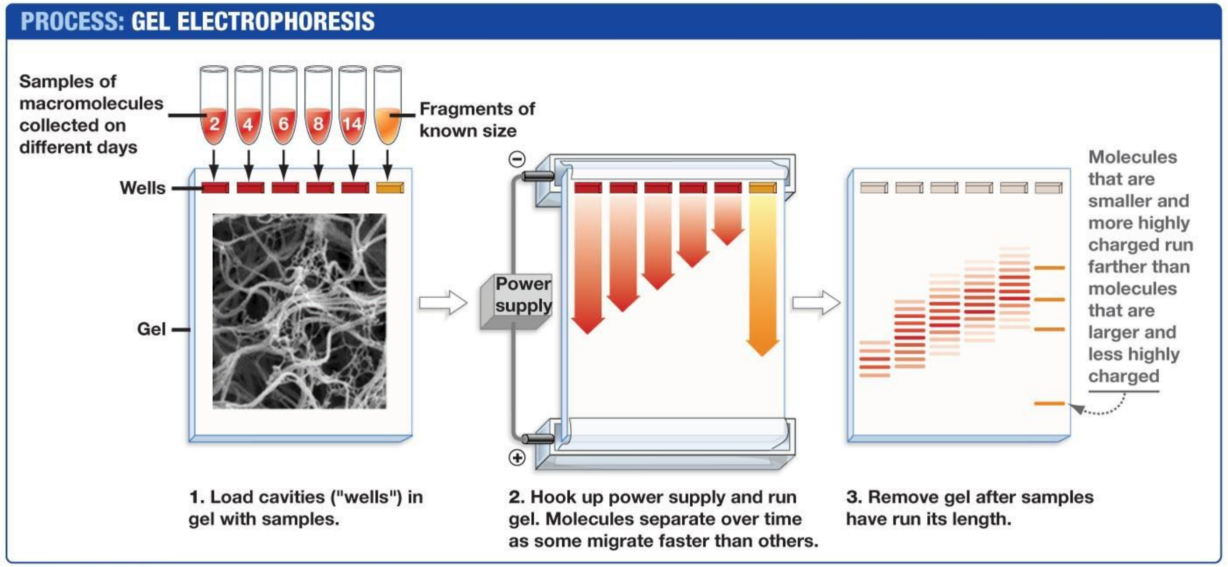


Different individuals may have different numbers of repeats at this locus

Size Determination of PCR products - Agarose Gel Electrophoresis.

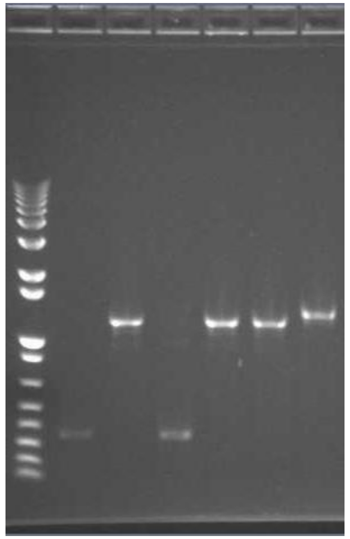


https://dnalc.cshl.edu/resources/animations/gelelectrophoresis.html

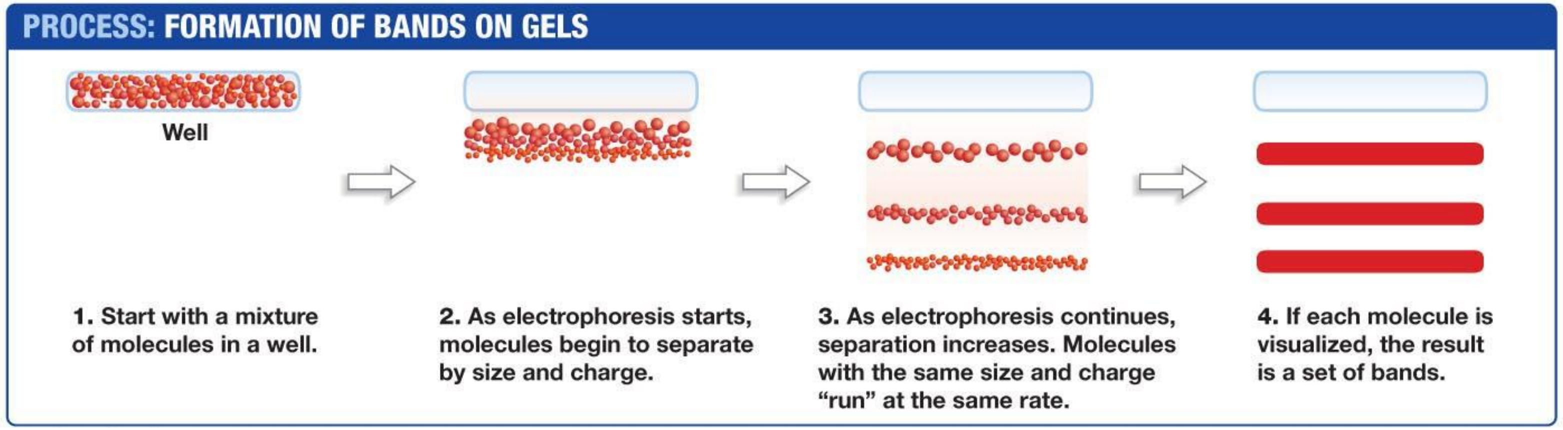


PROCESS: GEL ELECTROPHORESIS

1. Load cavities ("wells") in gel with samples.
2. Hook up power supply and run gel. Molecules separate over time as some migrate faster than others.
3. Remove gel after samples have run its length.



Which are the smallest PCR fragments?



PROCESS: FORMATION OF BANDS ON GELS

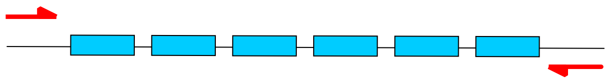
1. Start with a mixture of molecules in a well.
2. As electrophoresis starts, molecules begin to separate by size and charge.
3. As electrophoresis continues, separation increases. Molecules with the same size and charge “run” at the same rate.
4. If each molecule is visualized, the result is a set of bands.

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.

PCR primers: →

Repeat: ▭



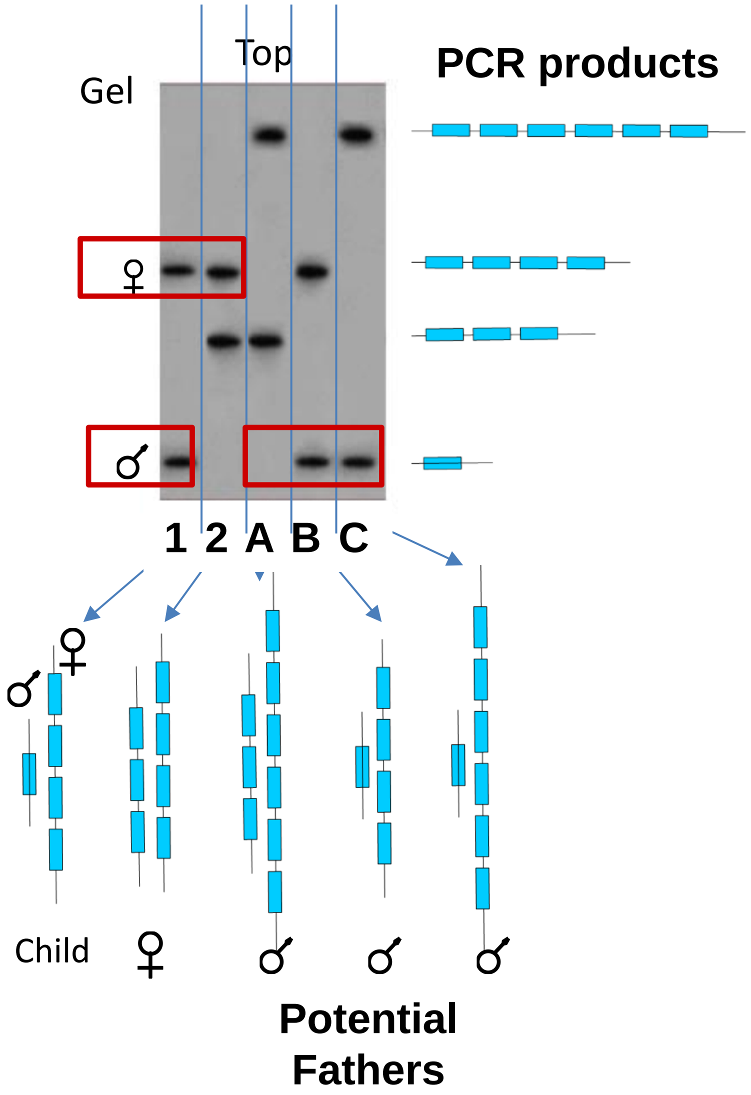
Lane 1: Child
Lane 2: Mother

Lanes A, B, C: Possible Fathers

1. Which PCR product is from the mother? From the father?

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

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



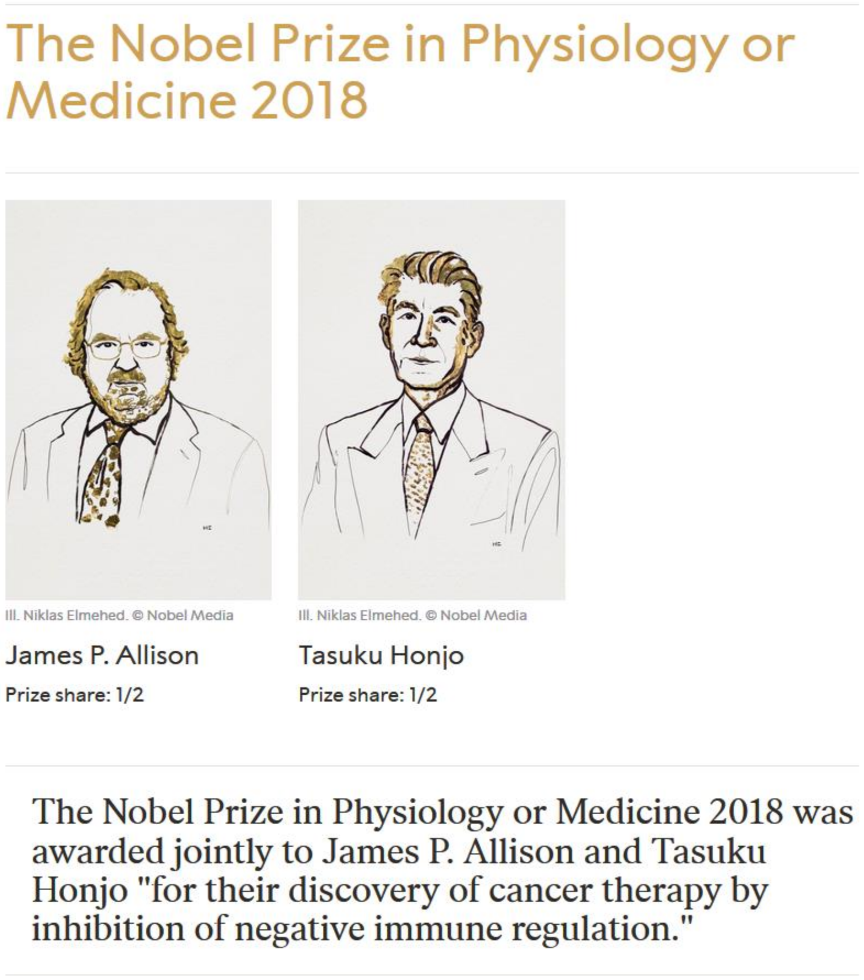
Introduction to Immunology

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.

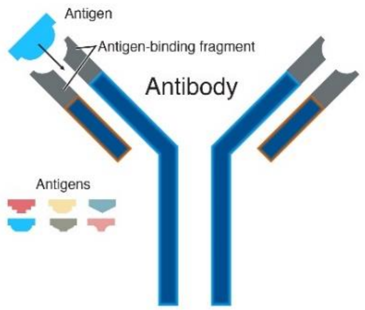
T_c cell = T-cellular: Involved in defense against viruses and cancer.

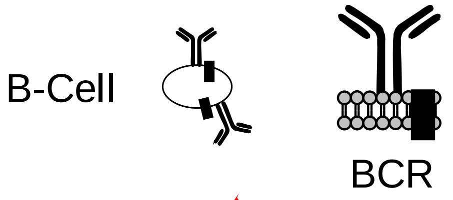
TCR = T-cell receptor – found on the surface of T-cells, recognizes MHC proteins + bound peptide, RTK.

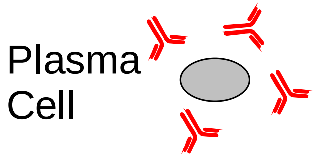
- **T_c cell** = recognizes MHC I + peptide
- **T_H cell** = recognizes MHC II + peptide

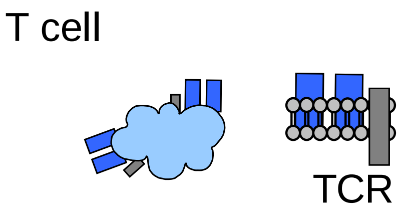
MHC = major histocompatibility complex – required for acquired immunity (basis of transplant rejection)

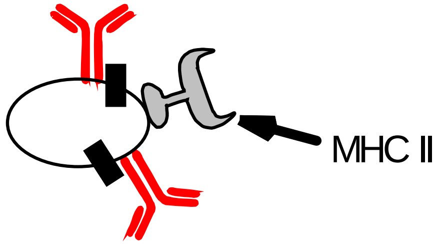
- MHC I = protein found on the surface of ***all*** cells, “presents” peptides derived from the proteins that were made by the cell. The MHC-peptide complex is recognized by T_c cells. ***Only foreign peptides produce a response***.
- MHC II = on the surface of B-cells, macrophages, and dendritic cells.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





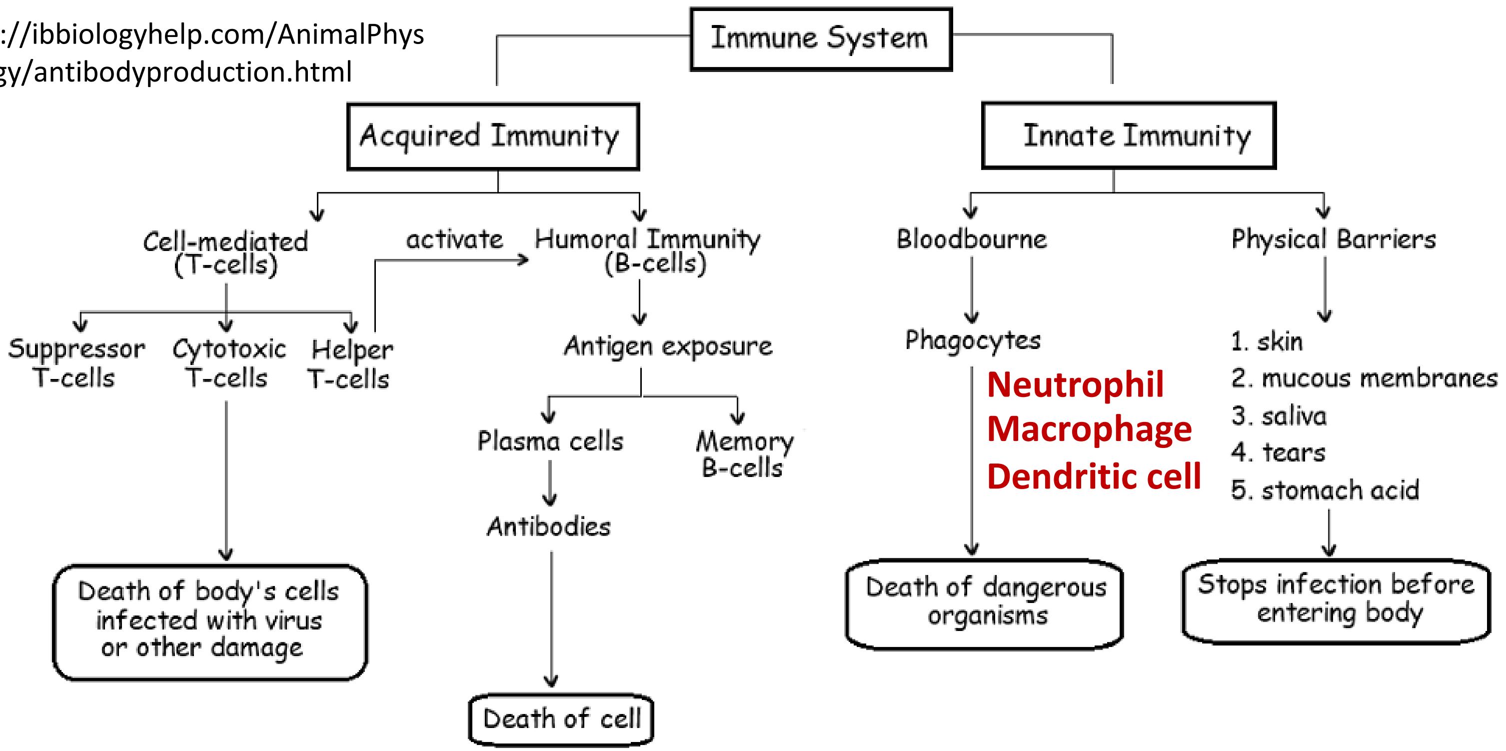




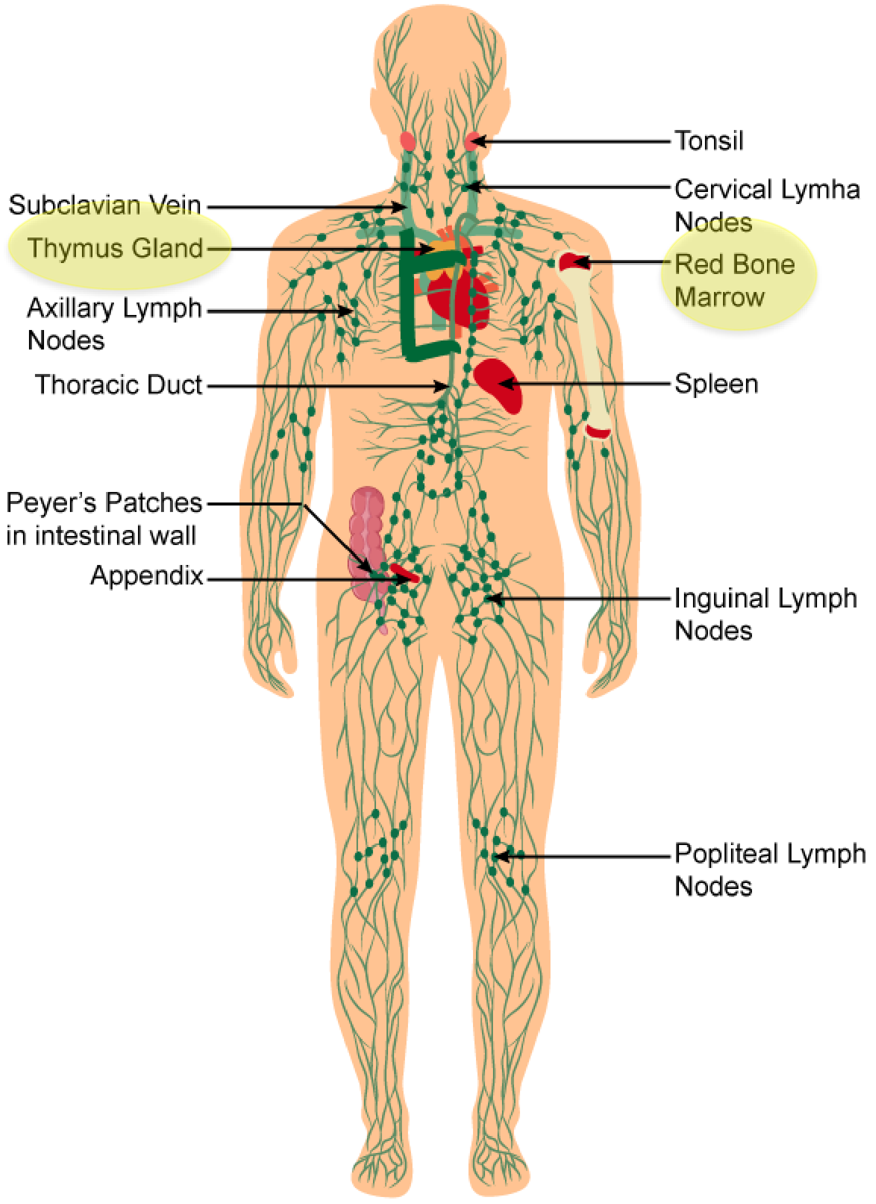


Branches of the Immune System:

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



Neutrophil
Macrophage
Dendritic cell



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)*
- *Uncontrolled growth would produce many bacteria: $2^{24 \times 7} = 3.7 \times 10^{50}$ (~10^{30} kg)*

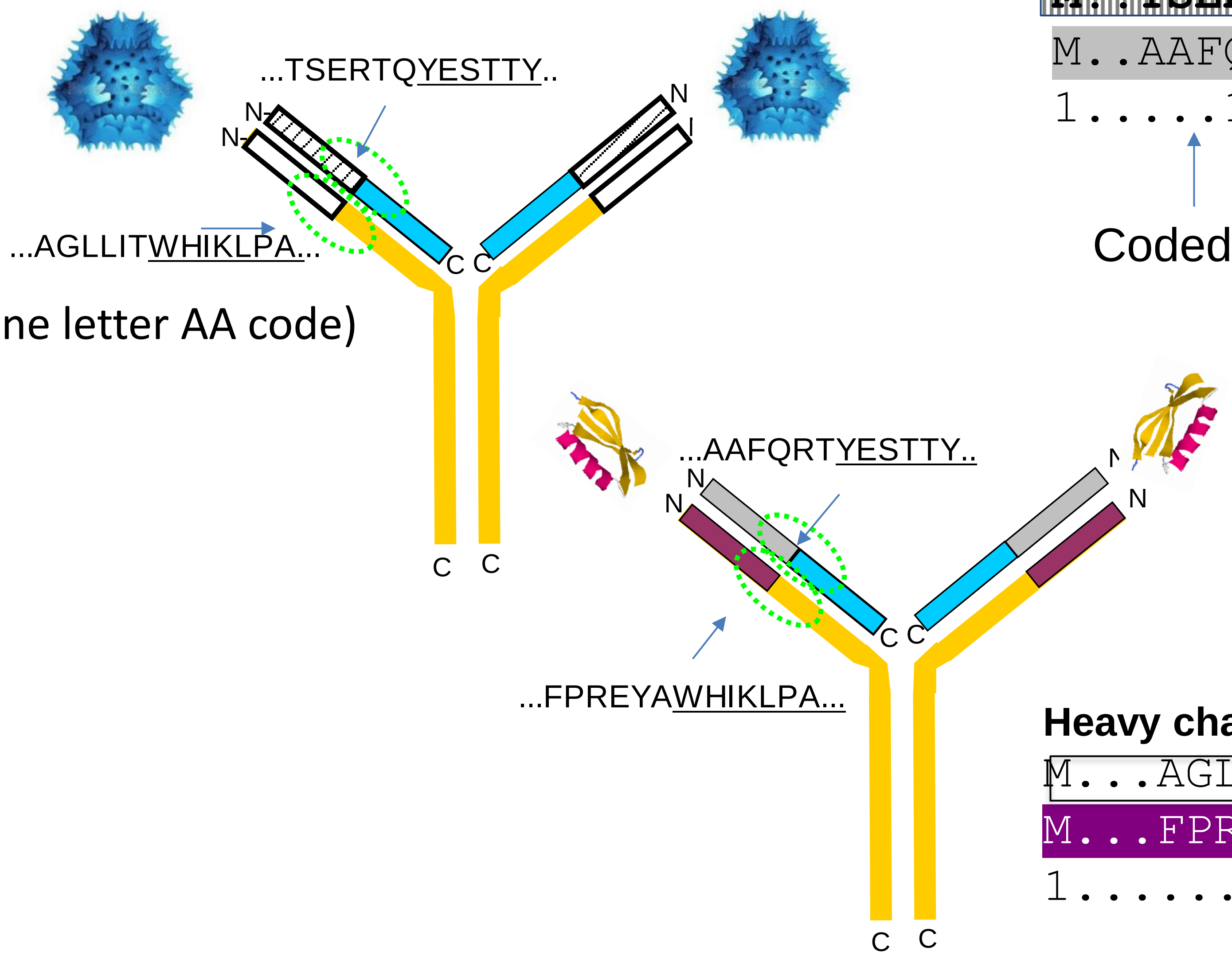
Important **primary** lymphatic organs: **bone marrow** (B), **thymus** (T)- Generate all immune cell.

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.



(one letter AA code)

Light chain sequences:

M..TSERTQYESTTY..RGEC

M..AAFQRTYESTTY..RGEC

1.....110.........210

Coded by different exons

Heavy chain sequences:

M...AGLLITWHIKLPA...SPGK

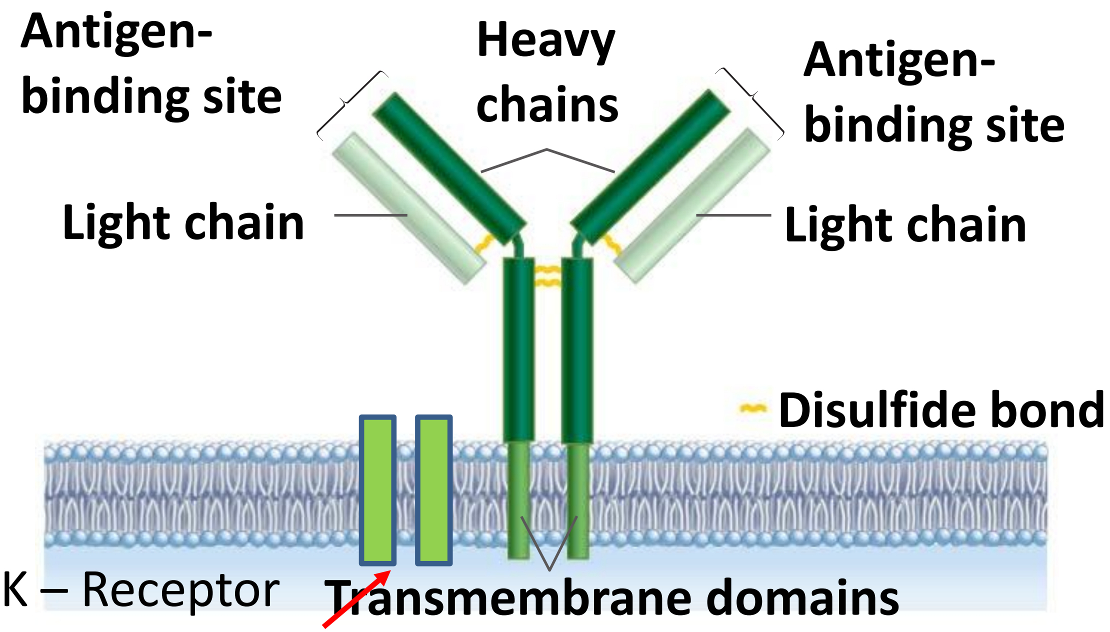
M...FPREYAWHIKLPA...SPGK

1......120...........449

Production of Antibodies by B-cells & Plasma Cells

B- Cells & B-cell Receptor (BCR)

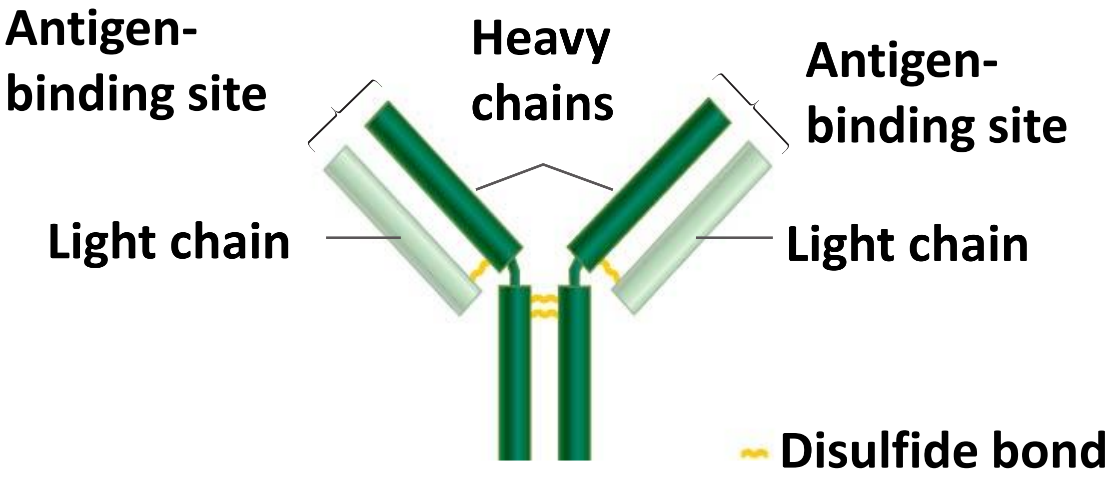
- Each B-cell has only one type of antibody as part of its BCR (B-cell receptor), i.e. the 10^5 BCRs are *homogeneous* on the same cell.
- Approximately 10^8 different specificities at any one time. i.e. *10^8 different B-cells!*



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

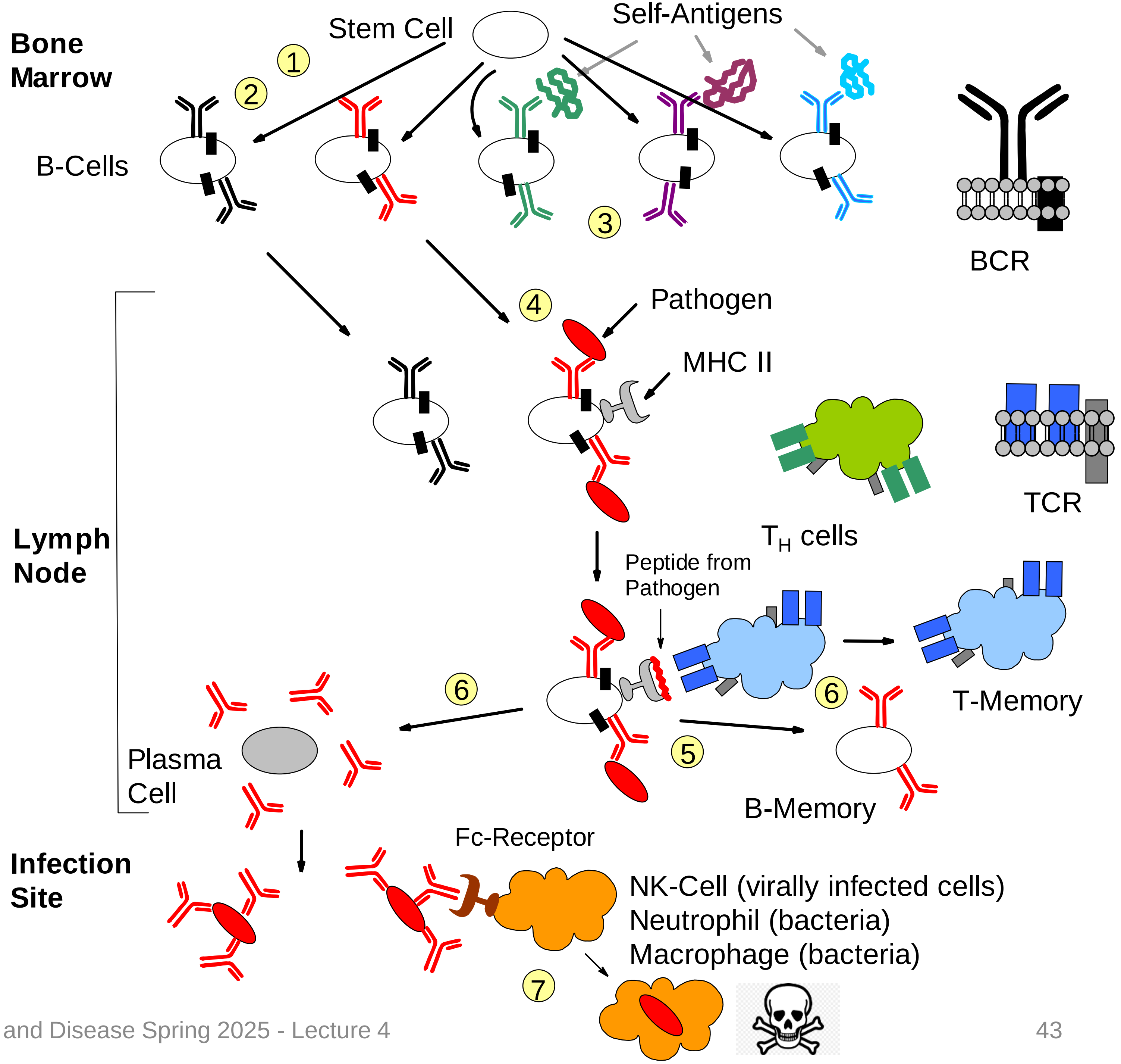
5. Activation of B-cells by T_H cells
- Peptides from pathogen presented on major histocompatibility proteins (MHC II).
- T-cell activation by tyrosine kinase receptors (T-cell Receptor, TCR), secretion of signaling molecules.

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.

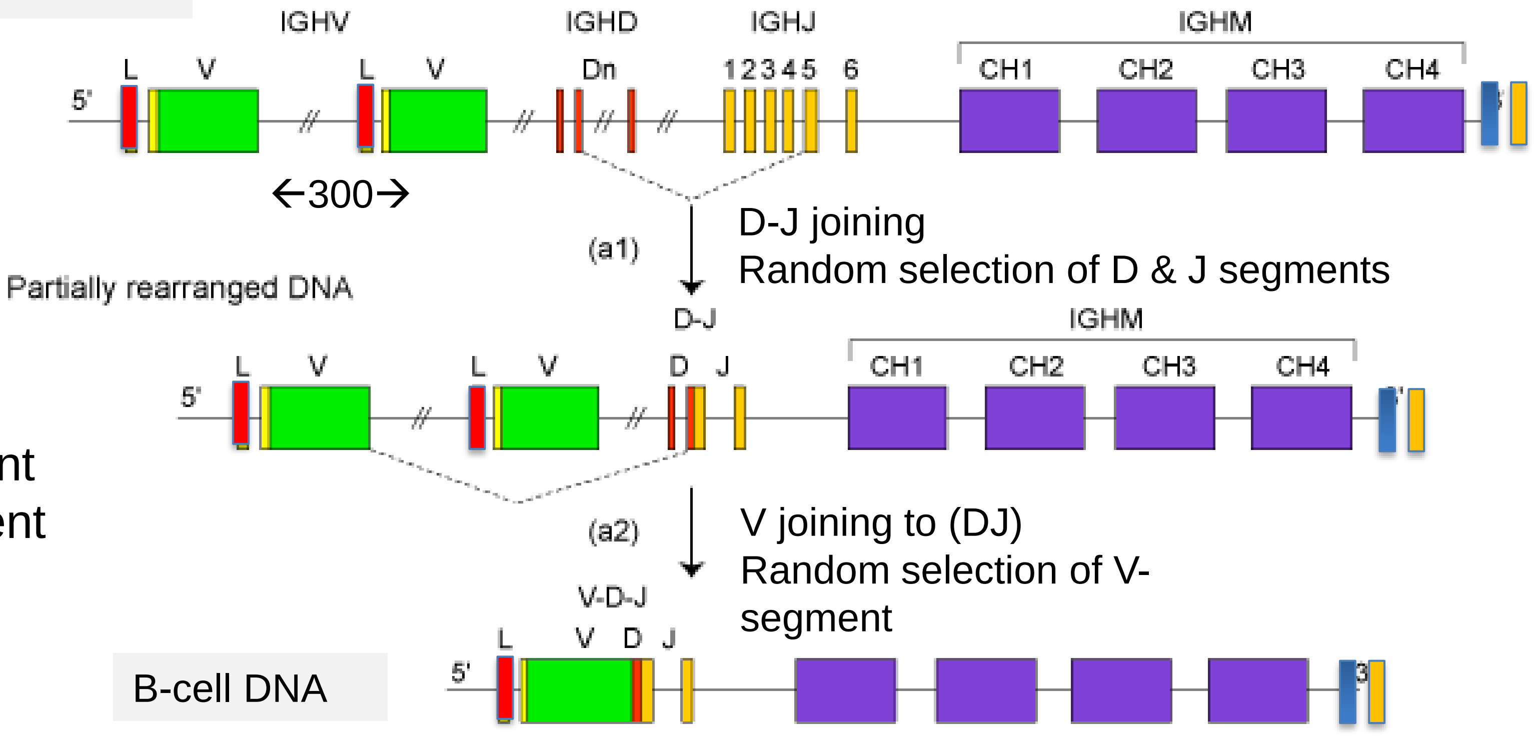


Antibody Genes are Assembled From DNA Segments: Giving many different sequences.

Production of Heavy Chain Gene:
V_H exon = V+D+J segment (selected at random)

The mRNA coding for antibodies contains 5 exons.

Germline/Stem cell DNA



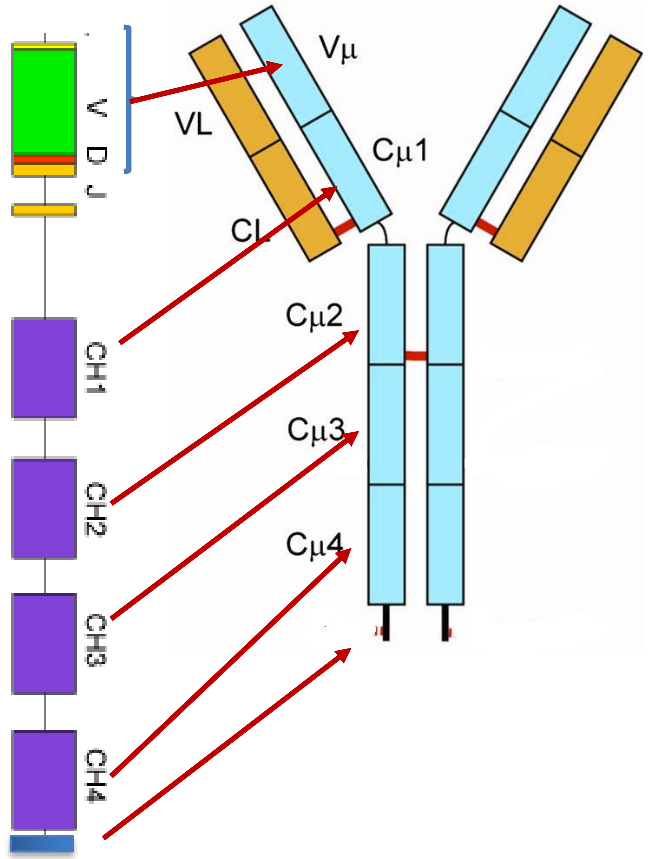
D-J joining
Random selection of D & J segments

V joining to (DJ)
Random selection of V-segment

B-cell DNA

V_H exon differs from one cell to the next.

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



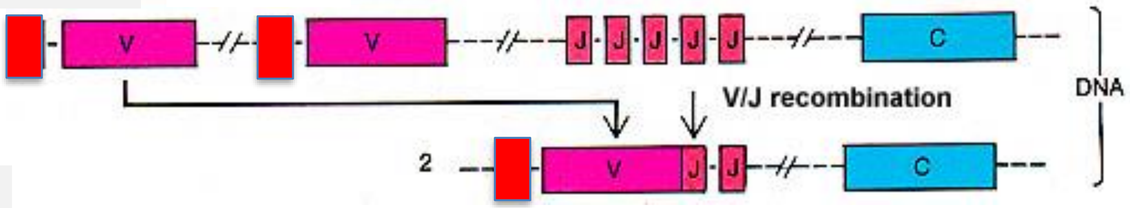
Stem cells -> B-cells

- 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?

Light-chain Genes are Assembled From DNA Segments: Giving many different sequences.

Production of Light Chain Gene



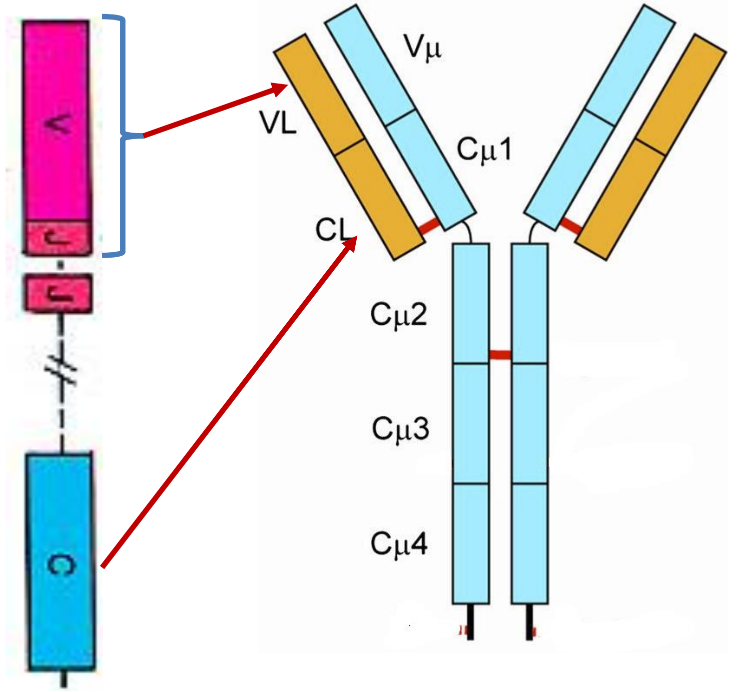
Germline/Stem cell DNA

←100→

B-cell DNA

Light chains have different V-sequences in different B-cells.

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



Antibody Diversity

1. If there are 100 possible V-heavy segments and 5 possible J segments, how many different light chains can be made?

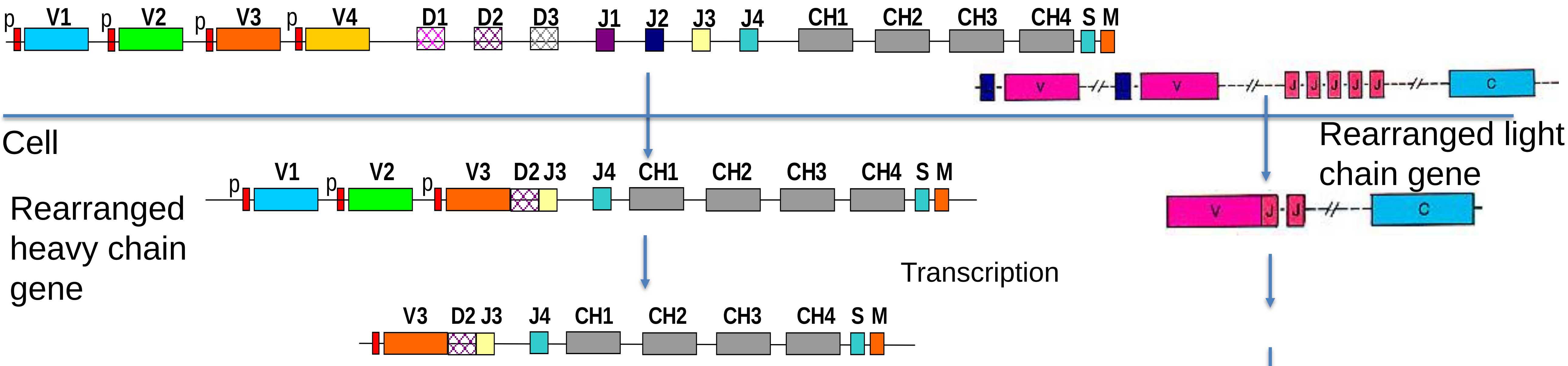
2. If any possible heavy chain can pair with any possible light chain, how many different antibodies can be generated, assuming there are 10,000 possible heavy chains and 500 different light chains?

Stem cells -> B-cells

- In the case of the light chain, the variable region is generated by VJ joining.
- 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.

Antibody Production – From Stem Cells to B-Cells

Stem Cell

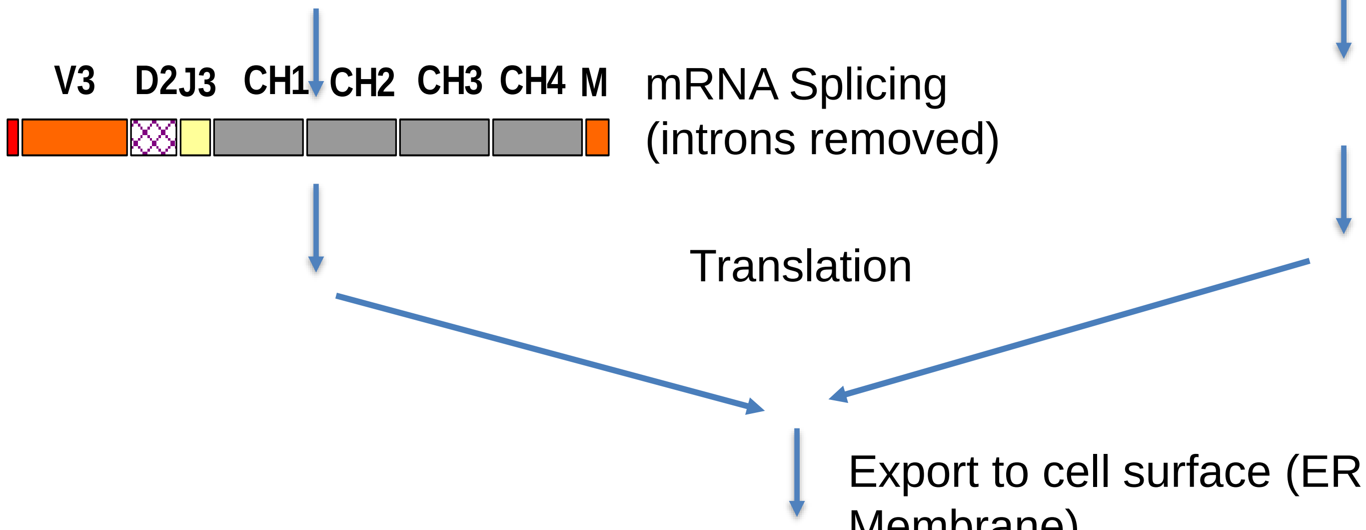


B-Cell

Rearranged heavy chain gene

Rearranged light chain gene

Transcription

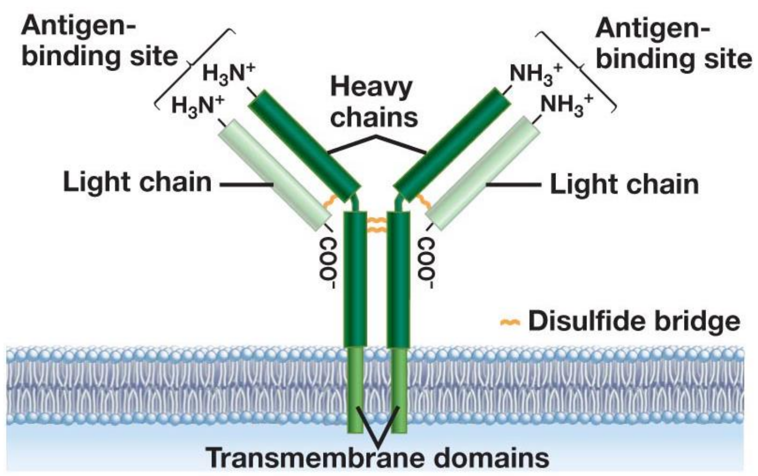


mRNA Splicing (introns removed)

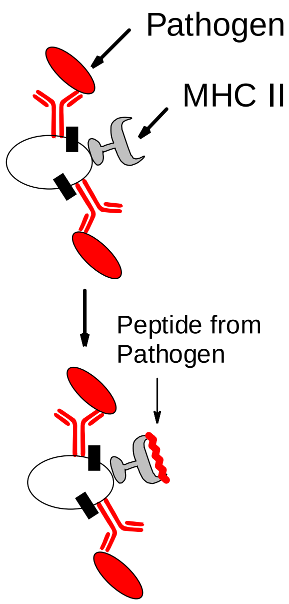
Translation

Export to cell surface (ER -> golgi -> Membrane)

(a) B-cell receptor

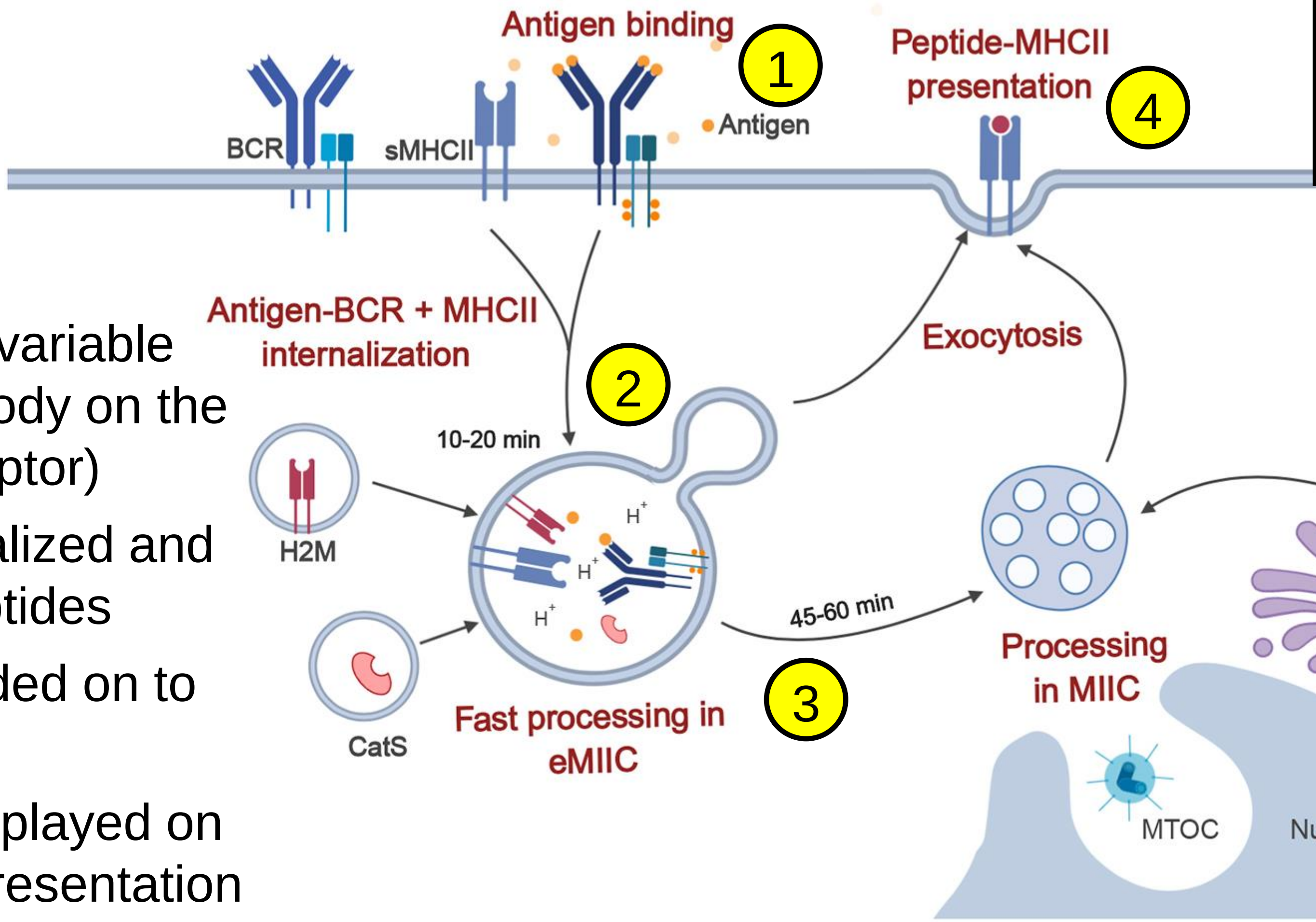


Antigen Capture by B-Cells - Endocytic Pathways

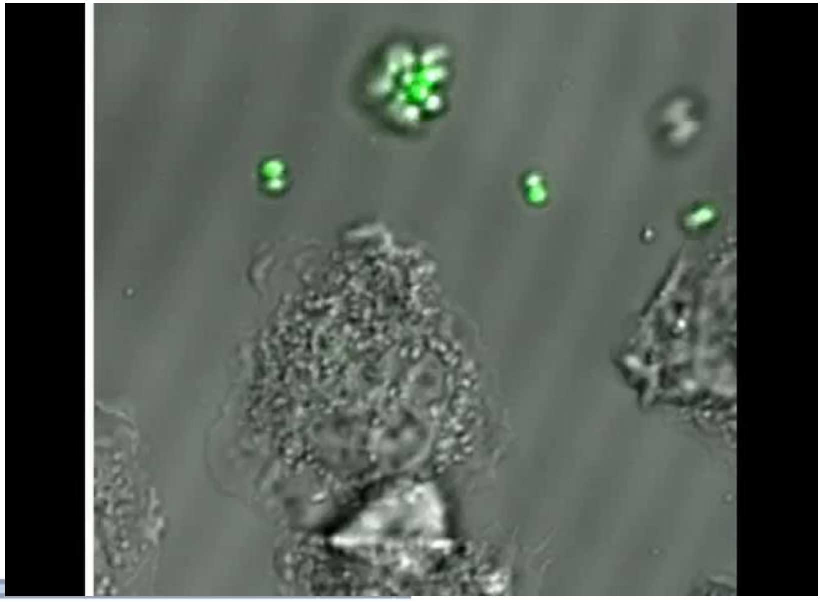


Endocytosis of bacteria by a B-cell

1. Antigen binds to variable domains of antibody on the BCR (B-cell receptor)
2. Antigen is internalized and digested into peptides
3. Peptides are loaded on to class II MHC
4. Peptide-MHC displayed on membrane for presentation to T-cells



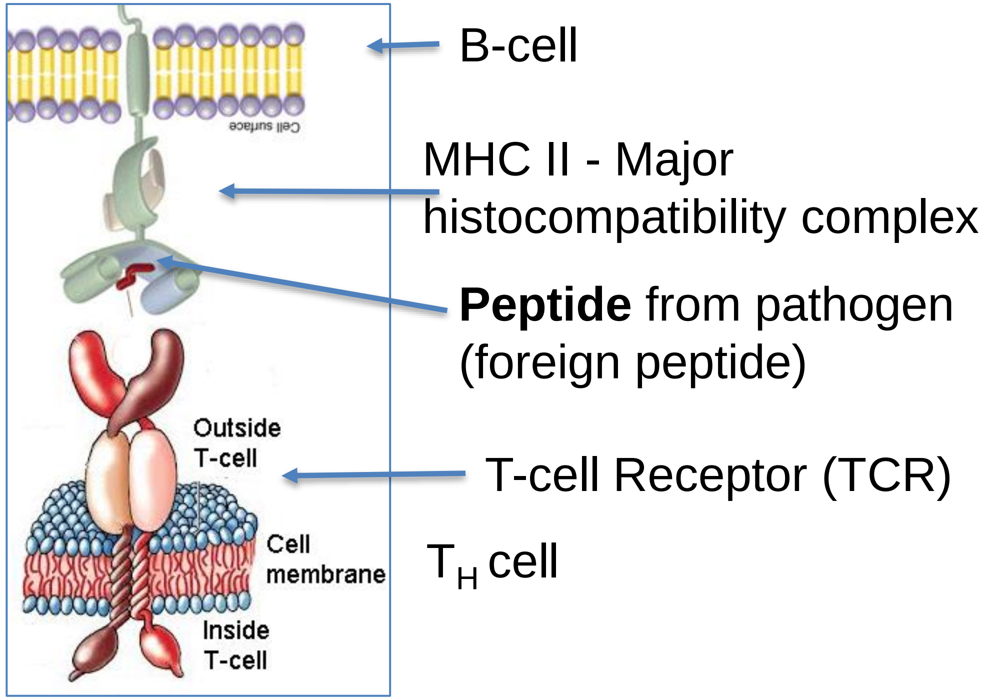
Journal of Cell Science doi: [10.1242/jcs.235192](https://doi.org/10.1242/jcs.235192)



Bacteria labeled with Green fluorescent protein.

I. Capture of the bacteria
II. Internalization (endocytosis)
III. Degradation of the bacterial proteins, producing peptides.

Activation of B cells by Antigen - Lymph Node



B-cell

MHC II - Major histocompatibility complex

Peptide from pathogen (foreign peptide)

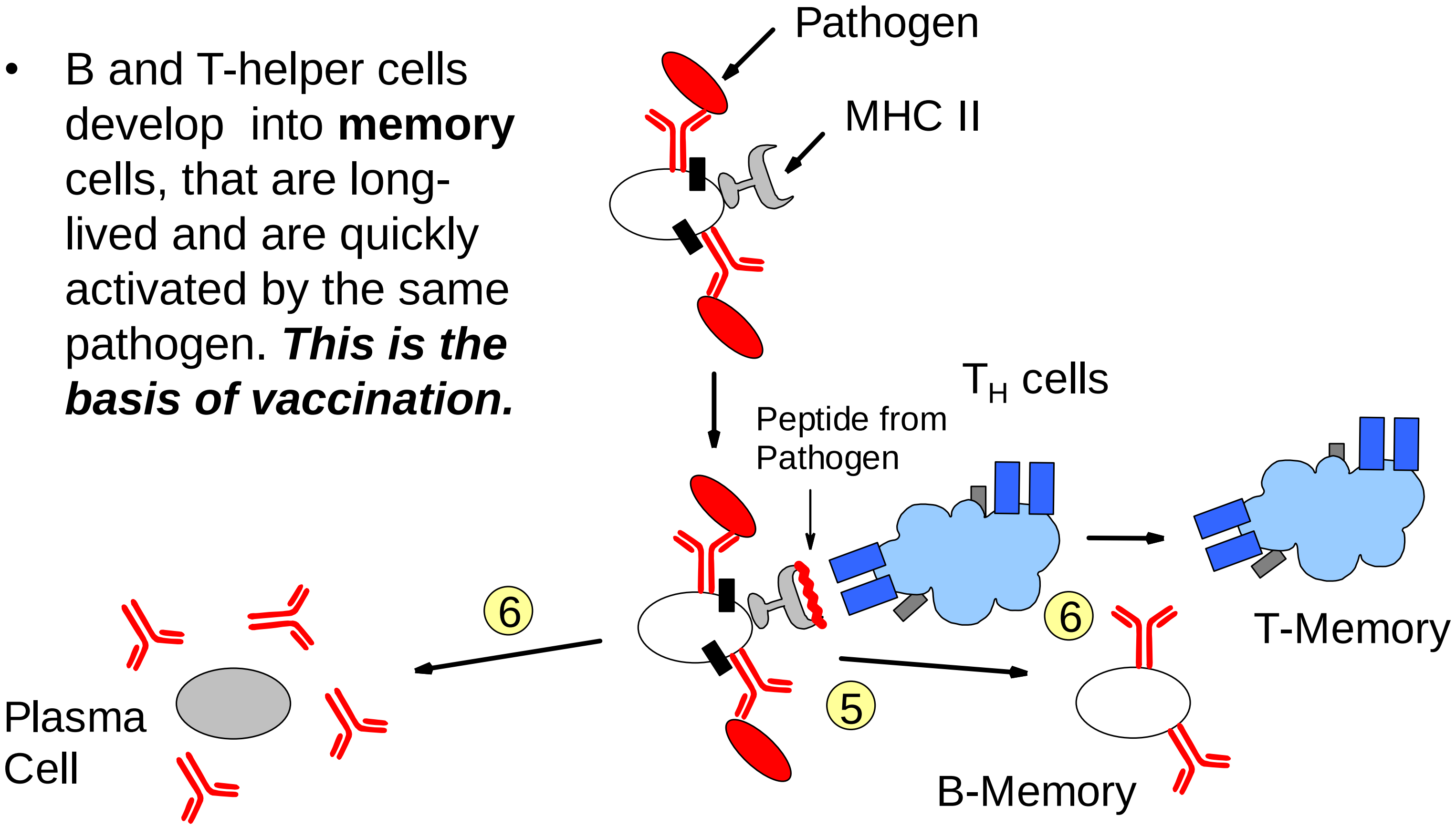
T-cell Receptor (TCR)

T_H cell

Events:

1. Recognition of MHC II-peptide by TCR
2. Tyrosine kinase signaling in T_H cell
3. Cytokines (protein messengers) produced.
4. Cytokines activate B-cells.

- B-cells develop into antibody secreting *plasma cells*.
- B and T-helper cells develop into **memory** cells, that are long-lived and are quickly activated by the same pathogen. ***This is the basis of vaccination.***


Pathogen

MHC II

T_H cells

Peptide from Pathogen

T-Memory

Plasma Cell

B-Memory

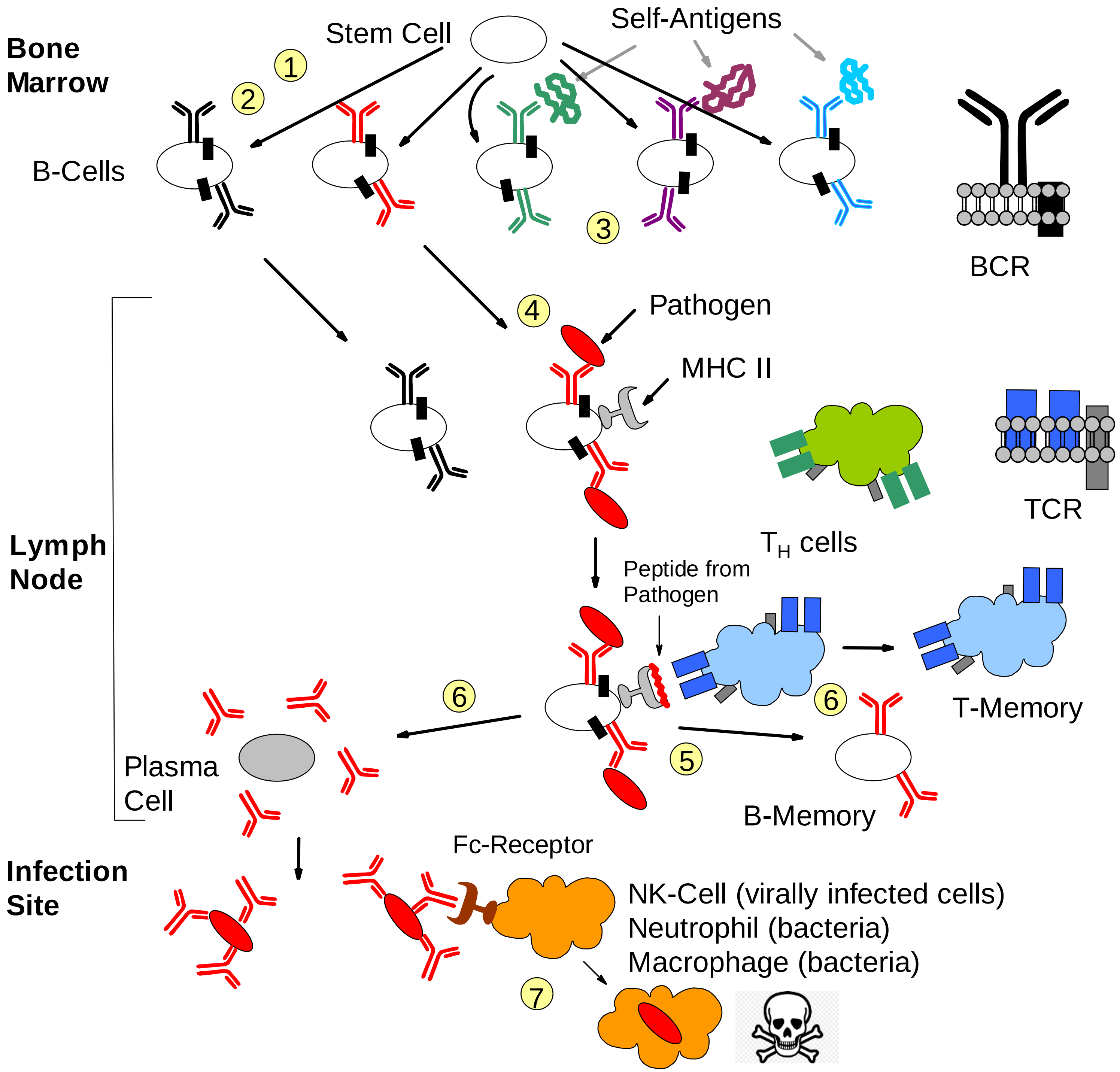
- Soluble antibody from plasma cells has the same light and heavy chains as the original B-cell.
- Membrane anchors are missing, so antibody is secreted outside the cell.

Can you:

- Describe how the genes for the heavy and light chain are generated, and how this give rise to many different antibodies?

- Do you understand the process of B-cell activation, including presentation of foreign peptides on MHC II and the role of the T-helper cell.

- Describe how antibodies inactivate pathogens?



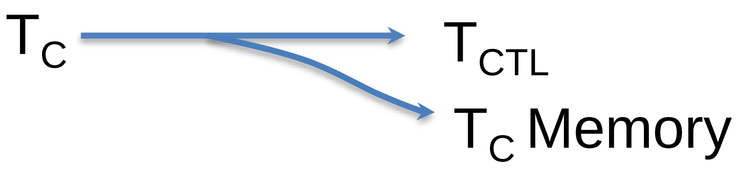
Cell Based Acquired Immunology

Key Questions:

1. How does your immune system fight viruses?
2. How does your immune system detect and destroy cancer cells?
3. How can the immune response be engineered to fight cancer?

Cell Types:

- T_H
- T_C, T_{CTL}



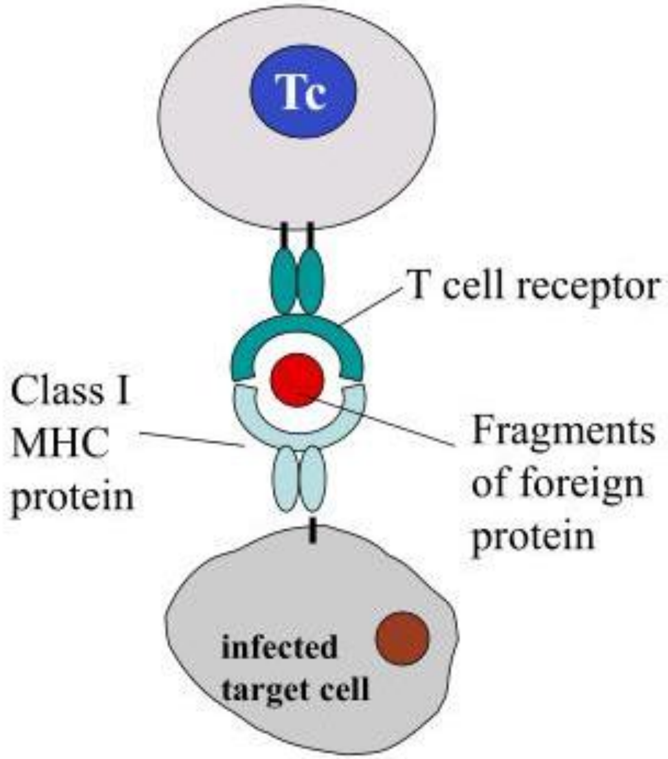
T_C

T_{CTL}

T_C Memory

Activation of Tc cells requires:

1. Recognition of ***foreign*** peptide on MHC I.
2. Assistance from T-helper cells.



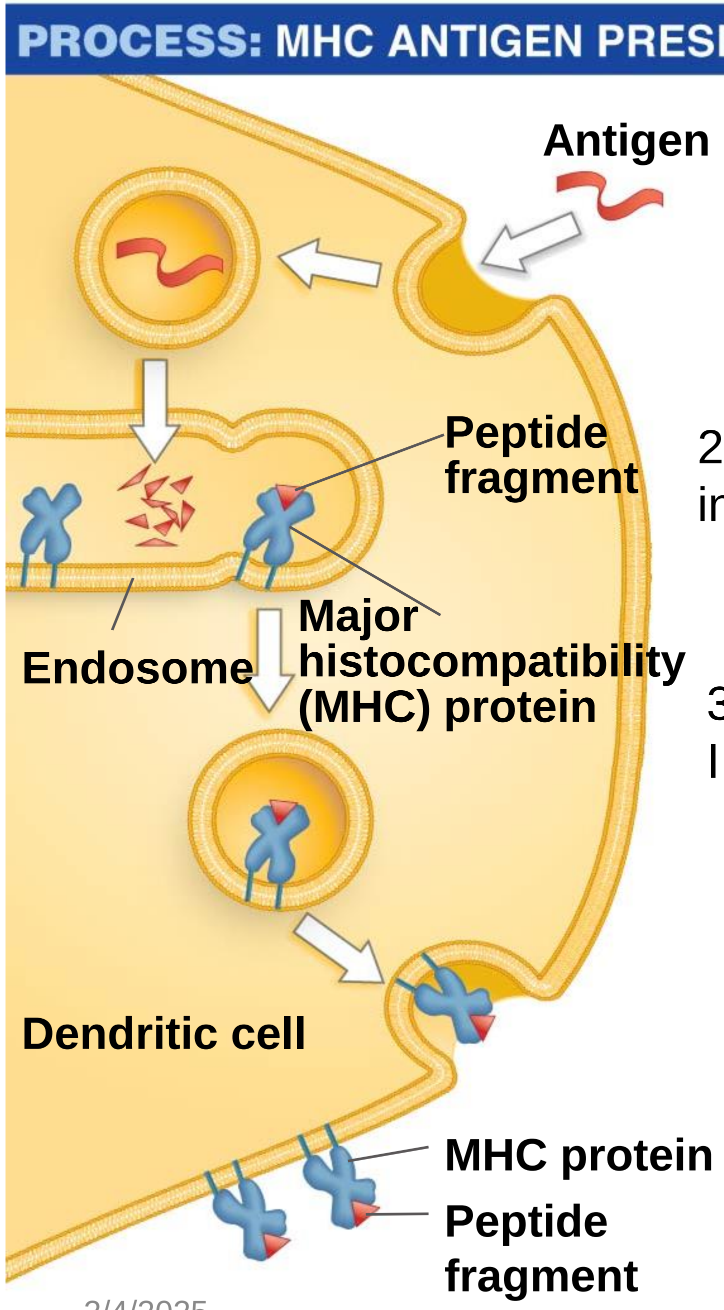
Activated Tc cell becomes a cytotoxic T-lymphocyte T_{CTL}

T_{CTL}

- Kill virally infected cells
- Kill cancer cells

Activation of Tc-Cells

A. Dendritic Cells Acquire Antigen from Viruses and Cancerous Cells



1. Dendritic cell ingests antigen via ***phagocytosis*** (intact virus, cell debris from cancer cell).

2. Enzymes break antigen proteins into peptide fragments.

3. Peptide fragments are loaded onto ***both*** class I and class II MHC proteins in endosomes.

4. MHC I & II –peptide complex is transported to cell surface.

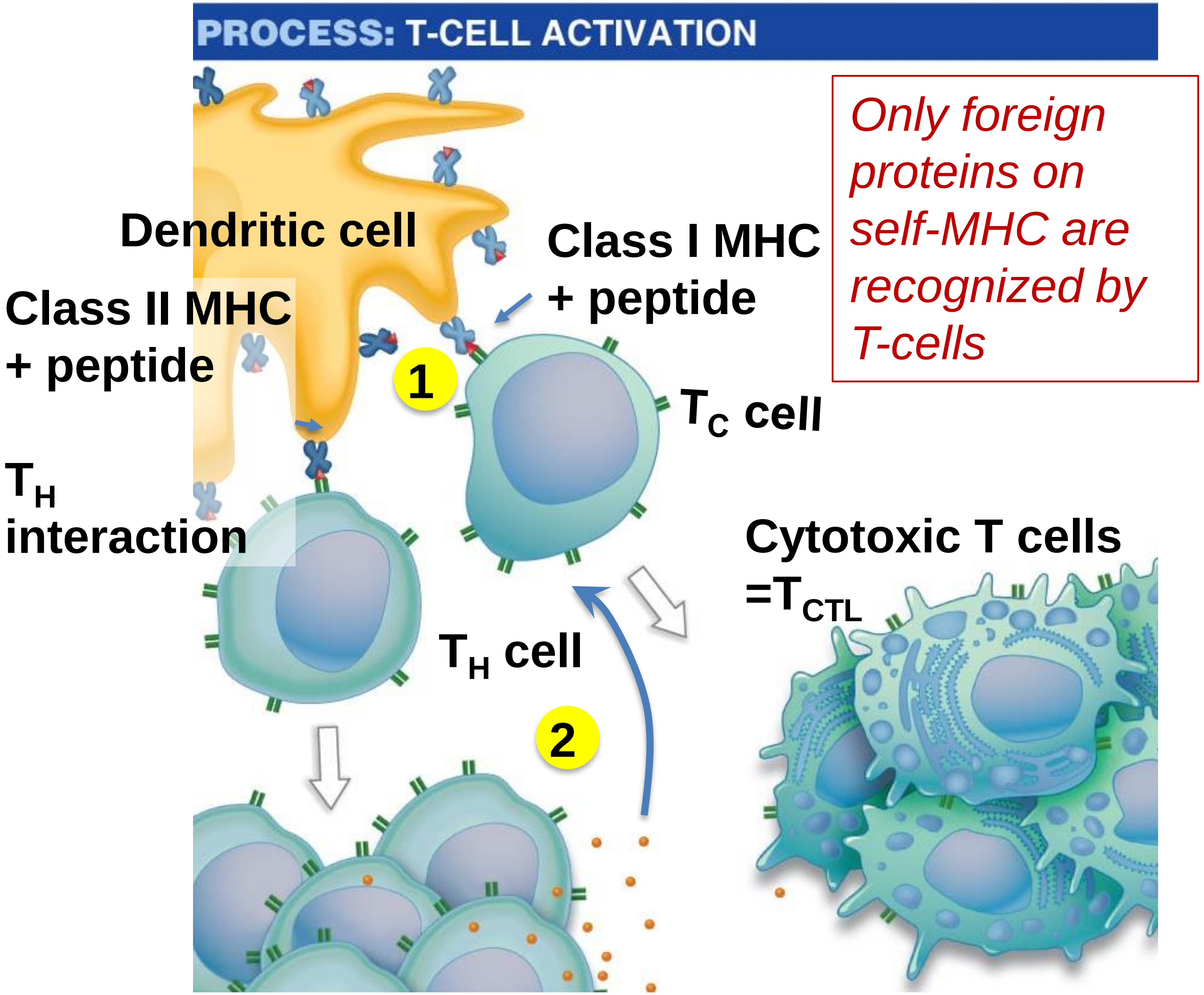
5. MHC protein presents peptide fragment on cell Surface to T-H and T-C cells.

Activation of Tc-Cells
B. Dendritic Cells Activate T_H and T_C cells.

Activation of Tc cells requires:

1. Recognition of foreign peptide on MHC I by TCR on Tc cell

2. Assistance from T-helper cells via secreted messengers (small proteins called cytokines)

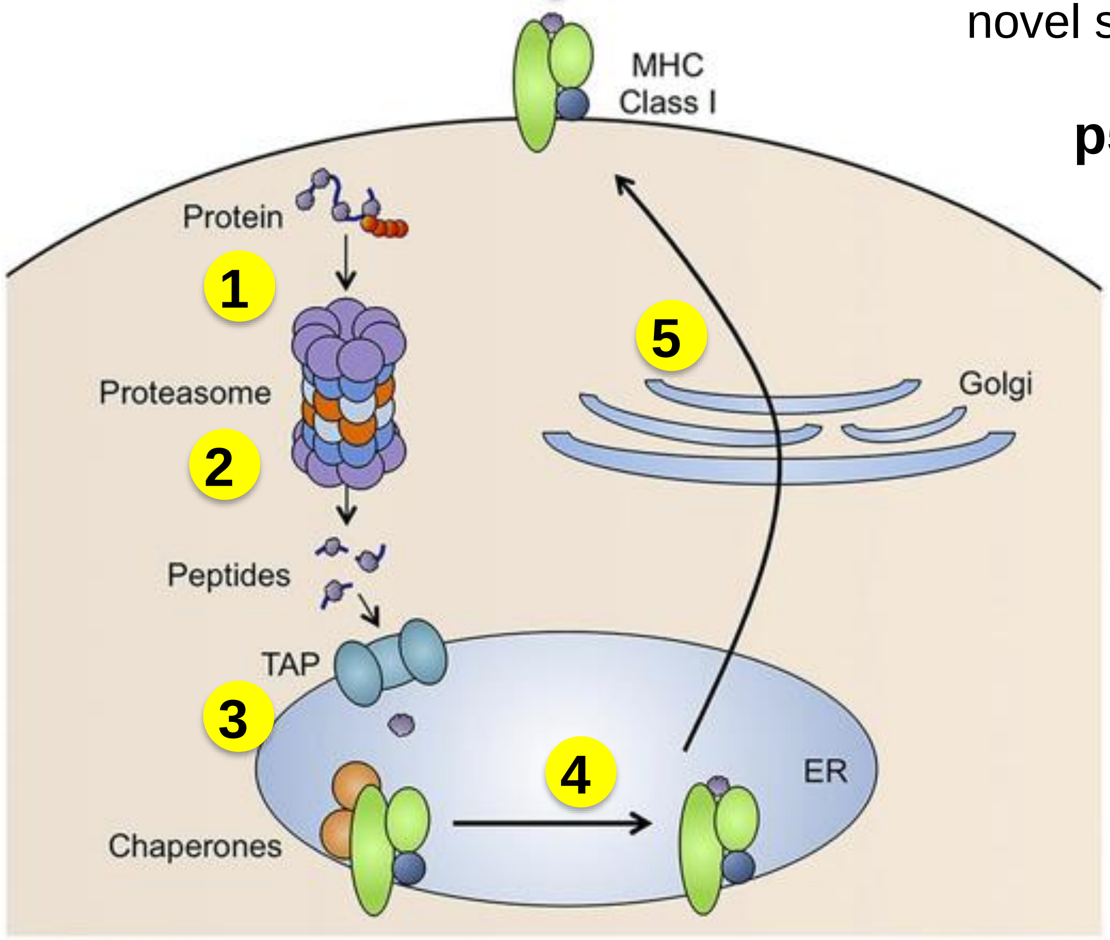


Only foreign proteins on self-MHC are recognized by T-cells

T_c Detection of Diseased/Cancer Cells - Role of MHC I

- MHC I present peptides
- Peptides are generated from of ***all*** of the proteins that are made in the cell.
- Steps:
  1. protein targeted for degradation by ubiquitin
  2. Protein digested by proteasome
  3. Peptides transported into ER
  4. Peptides loaded on to MHC I
  5. Peptide/MHC complex transported to cell membrane.

Only foreign peptides activate T-cells



Foreign Peptide Source:

1. From replication of viruses in the cell
2. From replication of intracellular bacteria (e.g. TB)
3. New coding sequences in cancer cells due to genetic changes (e.g. mutations in p53 lead to novel sequences).

p53 Protein Sequence

Zn Fingers (DNA binding)

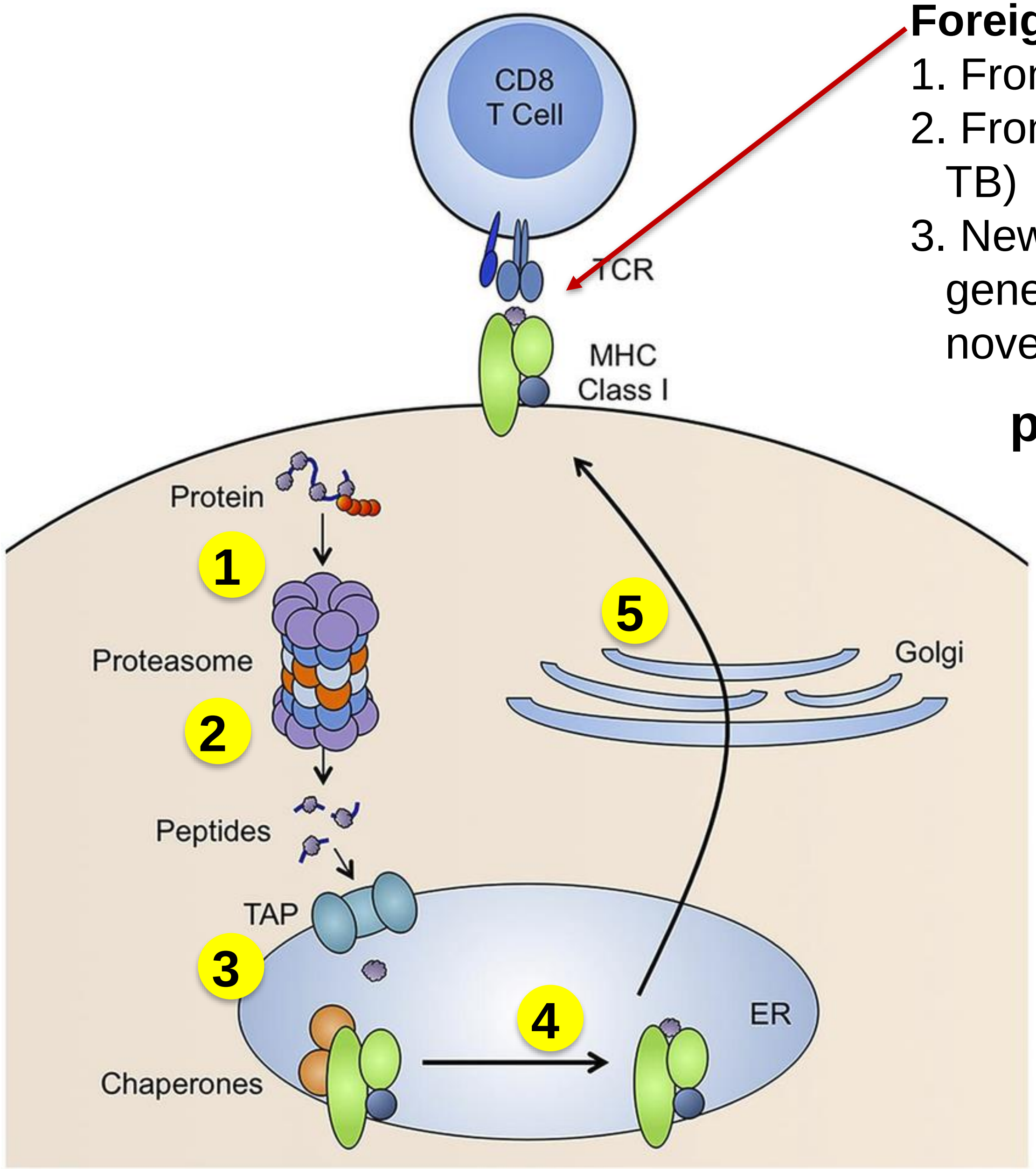
```
        10         20         30         40         50
MEEPQSDPSV EPPLSQETFS DLWKLLPENN VLSPLPSQAM DDLMLSPDDI
        60         70         80         90        100
EQWFTEDPGP DEAPRMPEAA PPVAPAPAAP TPAAPAPAPS WPLSSSVPSQ
       110        120        130        140        150
KTYQGSYGFR LGFLHSGTAK SVTCTYSPAL NKMFCQLAKT CPVQLWVDST
       160        170        180        190        200
PPPGTRVRAM AIYKQSQHMT EVVRRCPHHE RCSDSDGLAP PQHLIRVEGN
       210        220        230        240        250
LRVEYLDDRN TFRHSVVVPY EPPEVGSDCT TIHYNYMCNS SCMGGMNRRP
       260        270        280        290        300
ILTIITLEDS SGNLLGRNSF EVRVCACPGR DRRTEEENLR KKGEPHHELP
       310        320        330        340        350
PGSTKRALPN NTSSSPQPKK KPLDGEYFTL QIRGRERFEM FRELNEALEL
       360        370        380        390
KDAQAGKEPG GSRAHSSHLK SKKGQSTSRH KKLMFKTEGP DSD
```

EVVRRCPHHE Normal seq., ***ignored*** by TCR

EV**GG**CPHHE Mutant seq. in cancer, ***detected*** by TCR

T_c Detection of Diseased/Cancer Cells - Role of MHC I

- MHC I present peptides
- Peptides are generated from of ***all*** of the proteins that are made in the cell.
- Steps:
  1. protein targeted for degradation by ubiquitin
  2. Protein digested by proteasome
  3. Peptides transported into ER
  4. Peptides loaded on to MHC I
  5. Peptide/MHC complex transported to cell membrane.



Foreign Peptide Source:

1. From replication of viruses in the cell
2. From replication of intracellular bacteria (e.g. TB)
3. New coding sequences in cancer cells due to genetic changes (e.g. mutations in p53 lead to novel sequences).

p53 Protein Sequence

Zn Fingers (DNA binding)

```
        10         20         30         40         50
MEEPQSDPSV EPPLSQETFS DLWKLLPENN VLSPLPSQAM DDLMLSPDDI
        60         70         80         90        100
EQWFTEDPGP DEAPRMPEAA PPVAPAPAAP TPAAPAPAPS WPLSSSVPSQ
       110        120        130        140        150
KTYQGSYGFR LGFLHSGTAK SVTCTYSPAL NKMFCQLAKT CPVQLWVDST
       160        170        180        190        200
PPPGTRVRAM AIYKQSQHMT EVVRRCPHHE RCSDSDGLAP PQHLIRVEGN
       210        220        230        240        250
LRVEYLDDRN TFRHSVVVPY EPPEVGSDCT TIHYNYMCNS SCMGGMNRRP
       260        270        280        290        300
ILTIITLEDS SGNLLGRNSF EVRVCACPGR DRRTEEENLR KKGEPHHELP
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       360        370        380        390
KDAQAGKEPG GSRAHSSHLK SKKGQSTSRH KKLMFKTEGP DSD
```
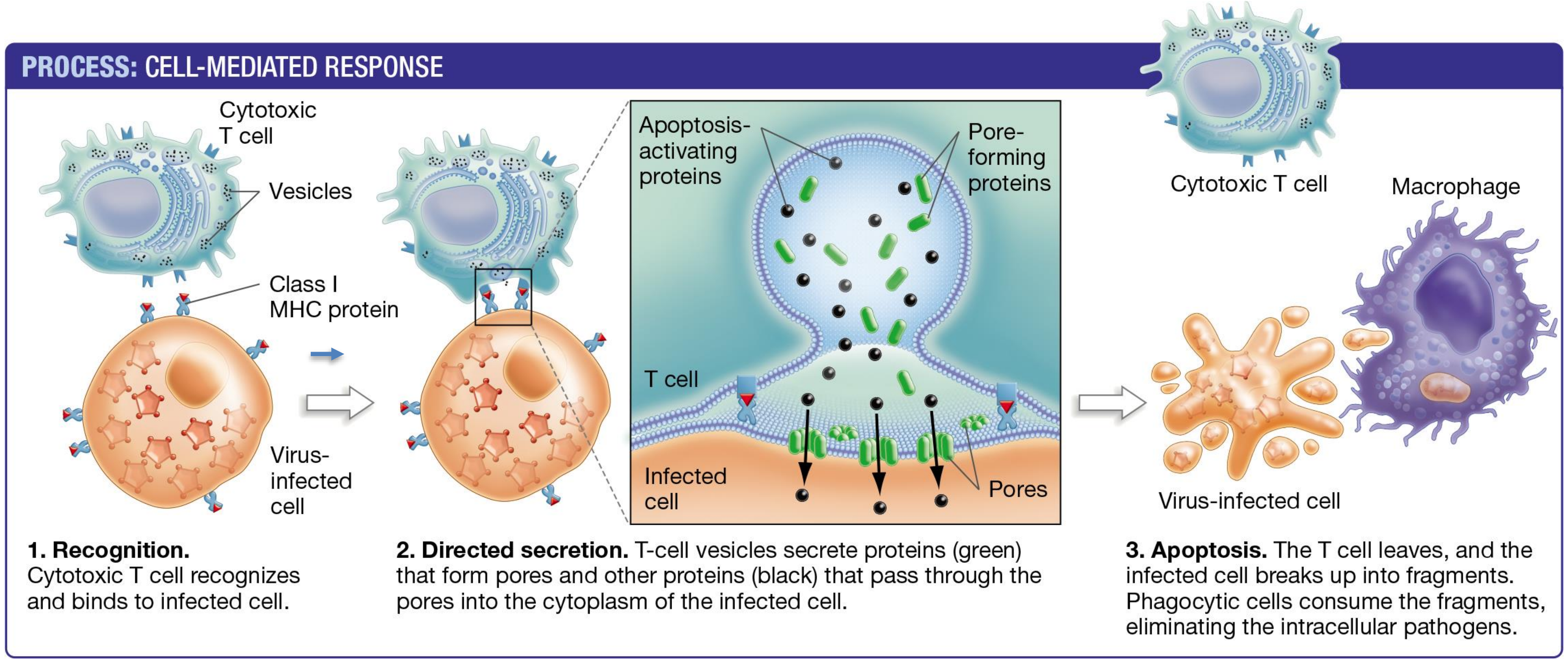

EVVRRCPHHE Normal seq., ***ignored*** by TCR

EVV**GG**CPHHE Mutant seq. in cancer, ***detected*** by TCR

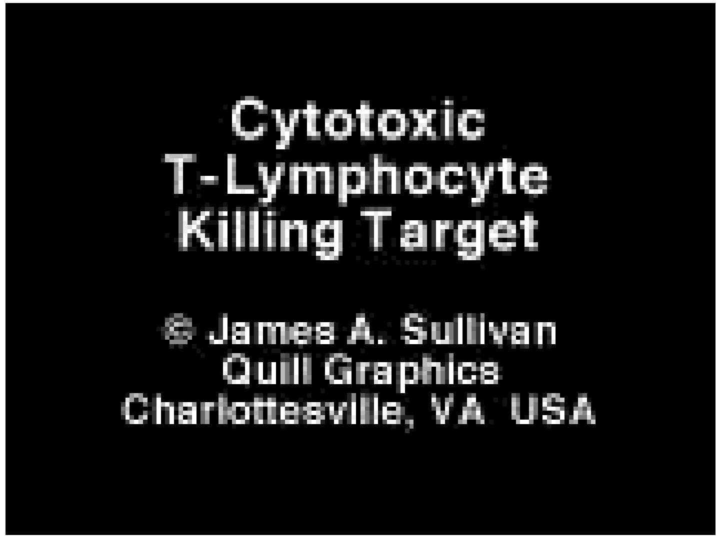
T_C Cells: Detection and Killing of Virally Infected or Cancer Cells



1. Recognition. Cytotoxic T cell recognizes and binds to infected cell.

2. Directed secretion. T-cell vesicles secrete proteins (green) that form pores and other proteins (black) that pass through the pores into the cytoplasm of the infected cell.

3. Apoptosis. The T cell leaves, and the infected cell breaks up into fragments. Phagocytic cells consume the fragments, eliminating the intracellular pathogens.


Cytotoxic
T-Lymphocyte
Killing Target

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Quill Graphics
Charlottesville, VA USA

Cancer cell or Infected cell

- Granzymes enter through perforin pore and cause cell undergo programmed cell death (apoptosis)

Summary Questions for Immunology:

1. What are the two major branches of the immune system? Why are both important?
2. What are the roles of different cell types in each system, e.g. what would happen if T_H-cells disappeared?
3. What is the quaternary structure of an antibody? Can you sketch an antibody and indicate where the antigen binds?
4. What defines the specificity of antibodies?
5. What are the steps in the production of antibody genes, at the molecular level:
   a) How do DNA rearrangements produce functional heavy and light chain genes
   b) How are is the mature mRNA generated in B-cells and Plasma cells.
   c) What is the difference between the heavy chain export process for B-cells and plasma cells.
6. Can you describe how antibodies kill/inactivate pathogens
7. How are virally infected cells and tumor cells recognized by Tc cells?
8. How does the Tc cell kill those cells?