Molecular Biology Techniques

By Matthew Hryniewicki

What will we cover?

- How we obtain fragments of DNA and gene copies
  - Restriction enzymes
  - Reverse transcriptase
- How we identify DNA sequences
  - Probes
  - Gel electrophoresis
  - Southern Blots
  - DNA sequencing
- How we amplify DNA sequences
  - Cloning
  - PCR
- Studying gene expression
  - Polymorphisms
  - Gene microarray
- HIV Diagnostics
  - ELISA
  - Western blot
  - Quantitative RT-PCR

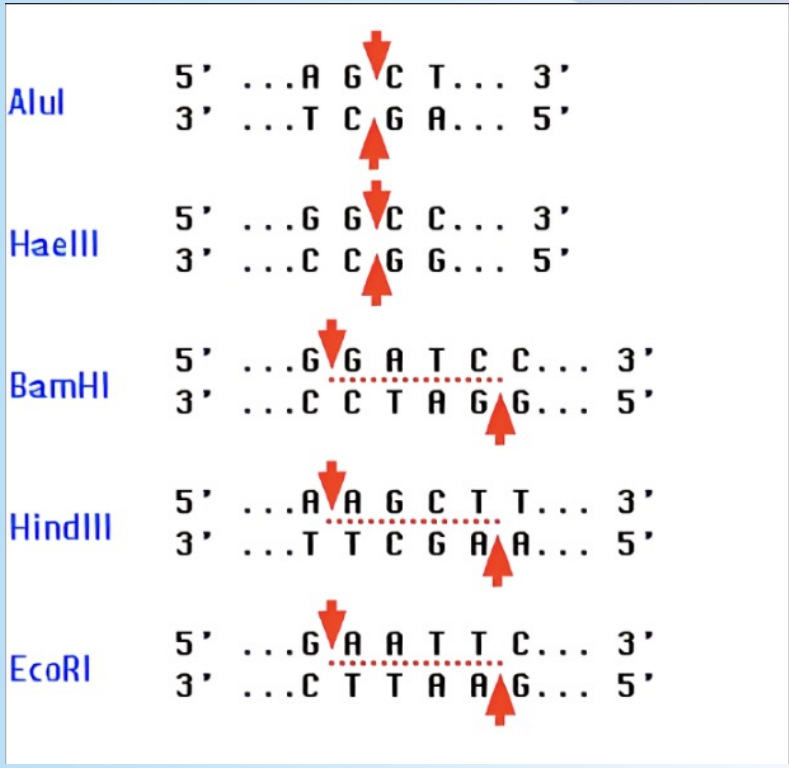
Restriction Endonucleases

Special group of bacterial enzymes that cleave dsDNA into smaller, defined fragments

Each enzyme has a restriction site (a specific nucleotide sequence)

Palindromic sequences: sequence is identical if each strand is read in 5' → 3' direction

Product: Restriction fragments (precisely defined DNA segments)



Restriction Endonucleases

Sticky or blunt ends

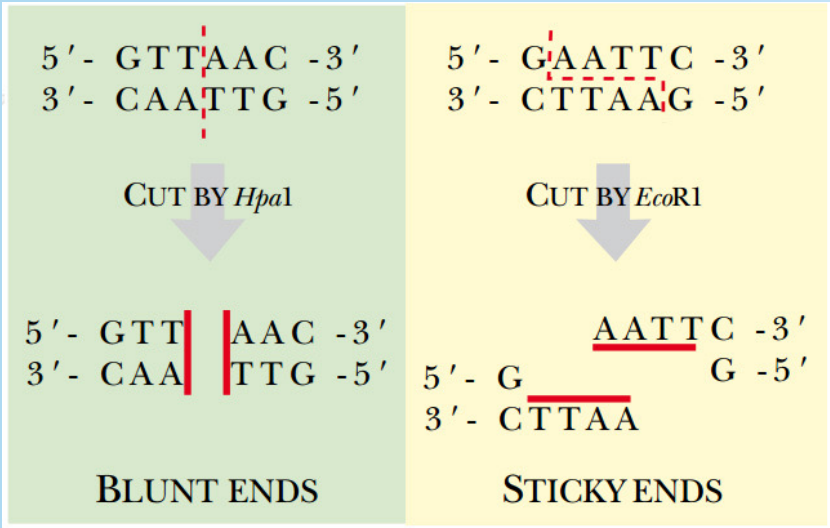
Cleavage produces a 3'-hydroxyl end and a 5'-phosphate end

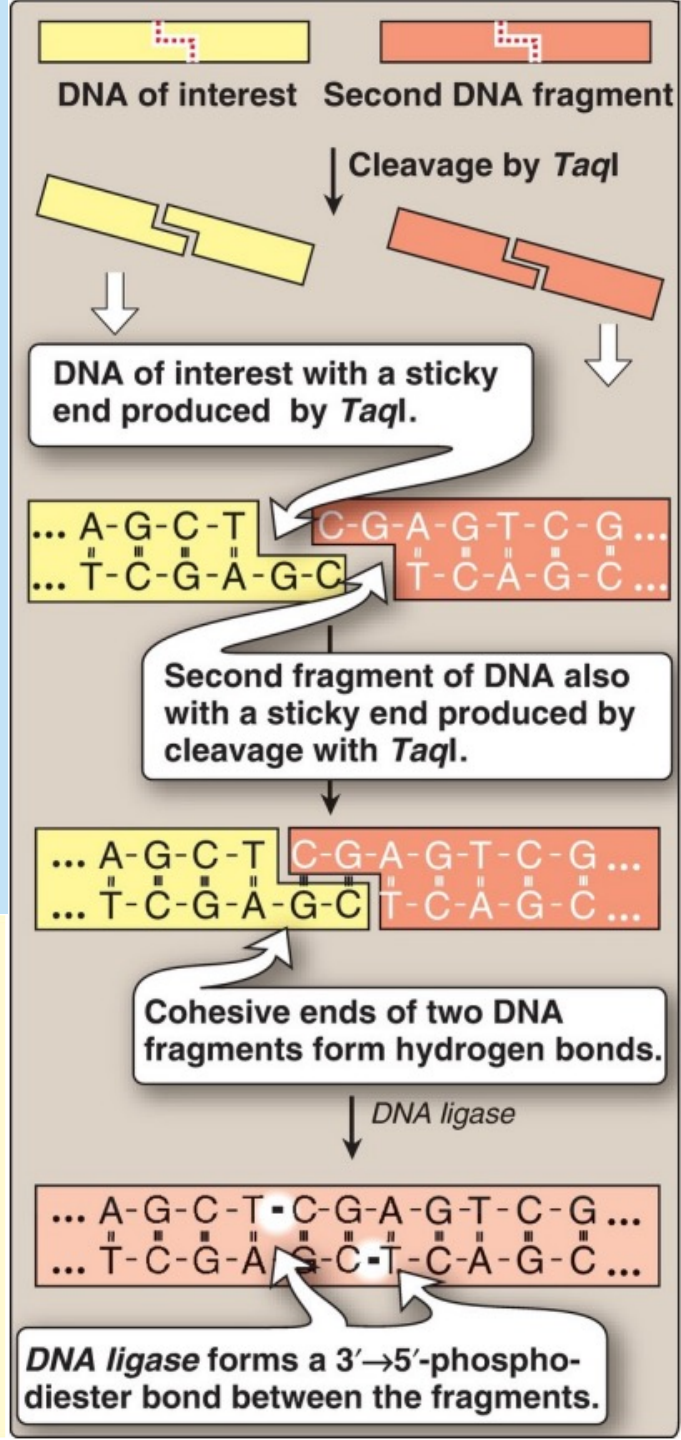
Ligase can join sticky and blunt ends of two DNA fragments together

Blunt end ligation:

-no hydrogen bonding between complementary nucleotide overhangs

- Does not require complementary ends



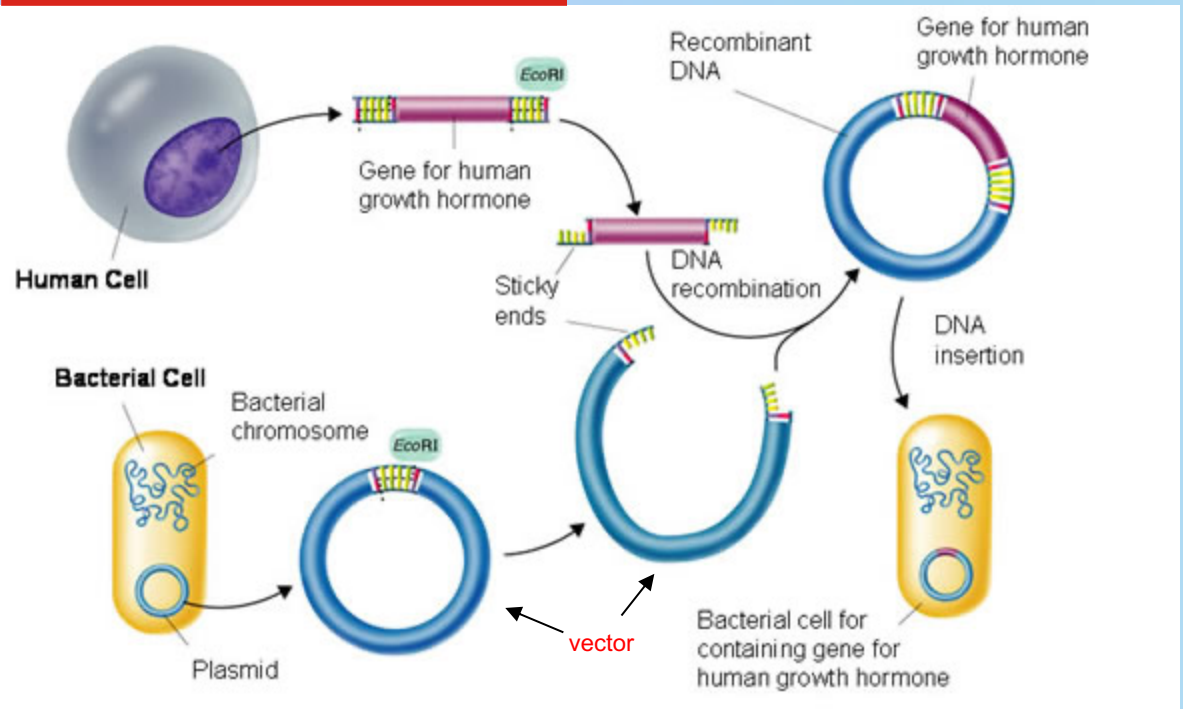


Recombinant DNA (rDNA)

Technology using enzymes to cut and paste together DNA sequences of interest

Recombined DNA sequences are placed into vectors

Suitable host cell allows for copying and expression



studyaid

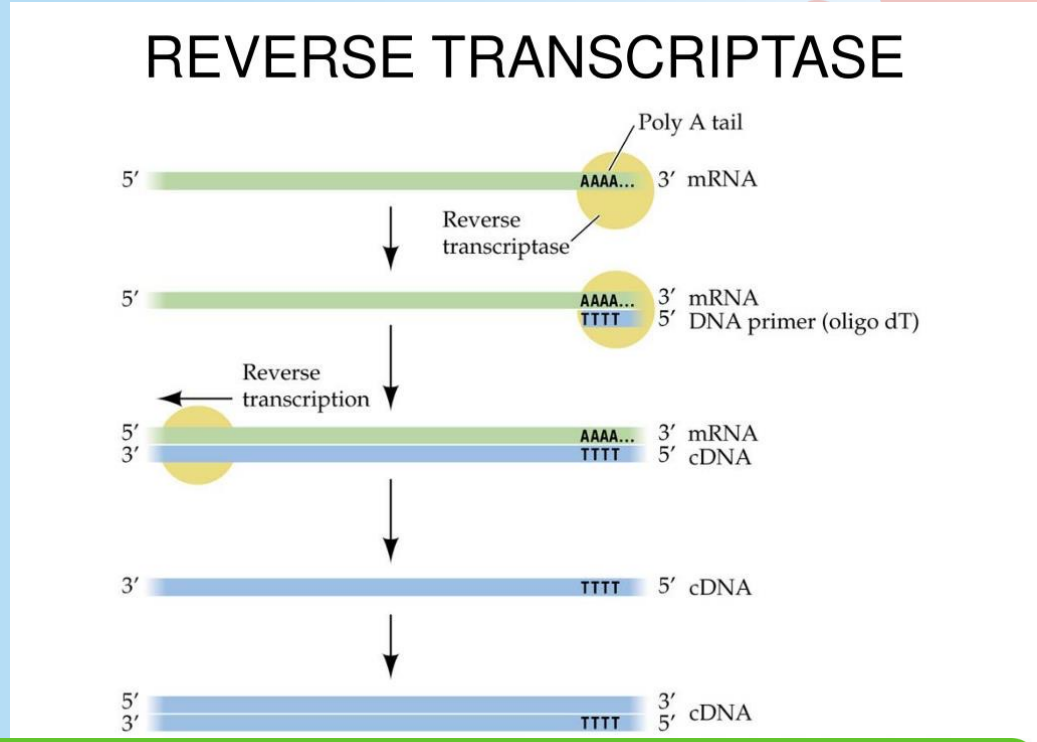
Reverse Transcriptase

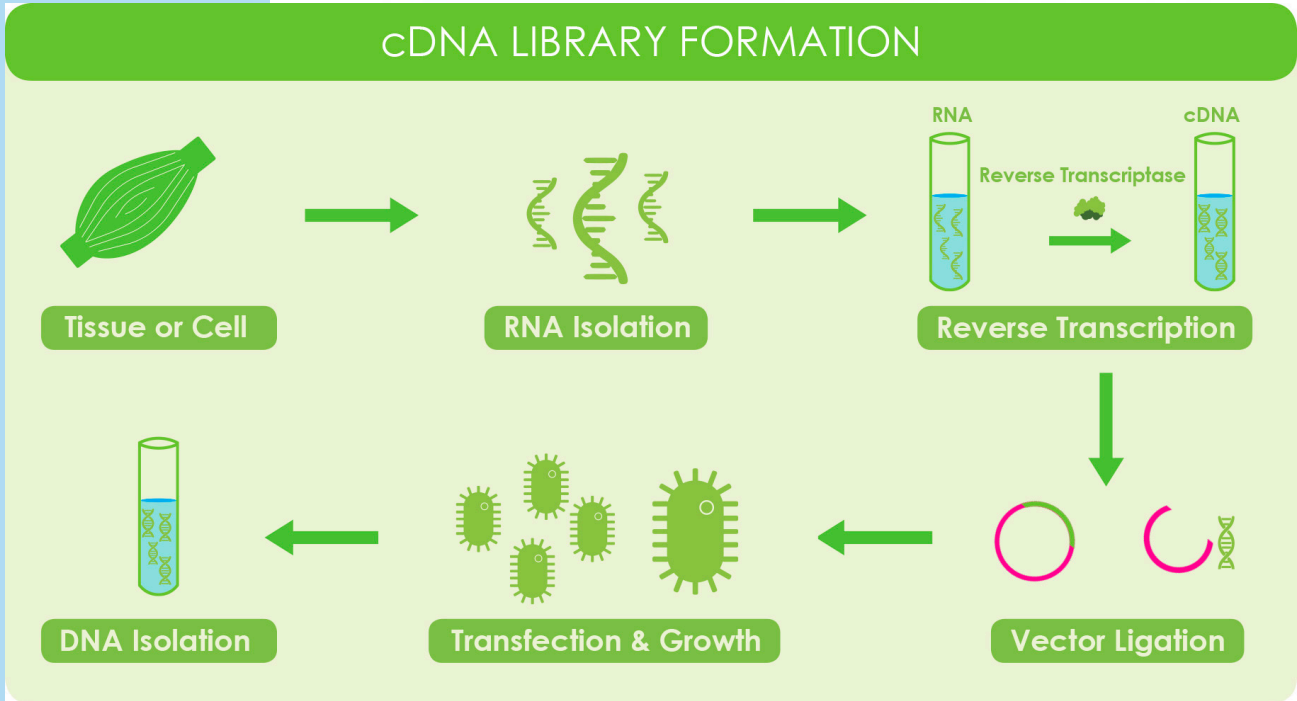
Template: mRNA

Product: cDNA

When compared to cleaved DNA fragments by restriction enzymes:

- No introns
- No regulatory regions of gene





Progress

- ✓ How we obtain fragments of DNA and gene copies
  - ✓ Restriction enzymes
  - ✓ Reverse transcriptase
- ❑ How we identify DNA sequences
  - Probes
  - Gel electrophoresis
  - Southern Blots
  - DNA sequencing
- ❑ How we amplify DNA sequences
  - Cloning
  - PCR
- ❑ Studying gene expression
  - Polymorphisms
  - Gene microarray
- ❑ HIV Diagnostics
  - ELISA
  - Western blot
  - Quantitative RT-PCR

Cloning

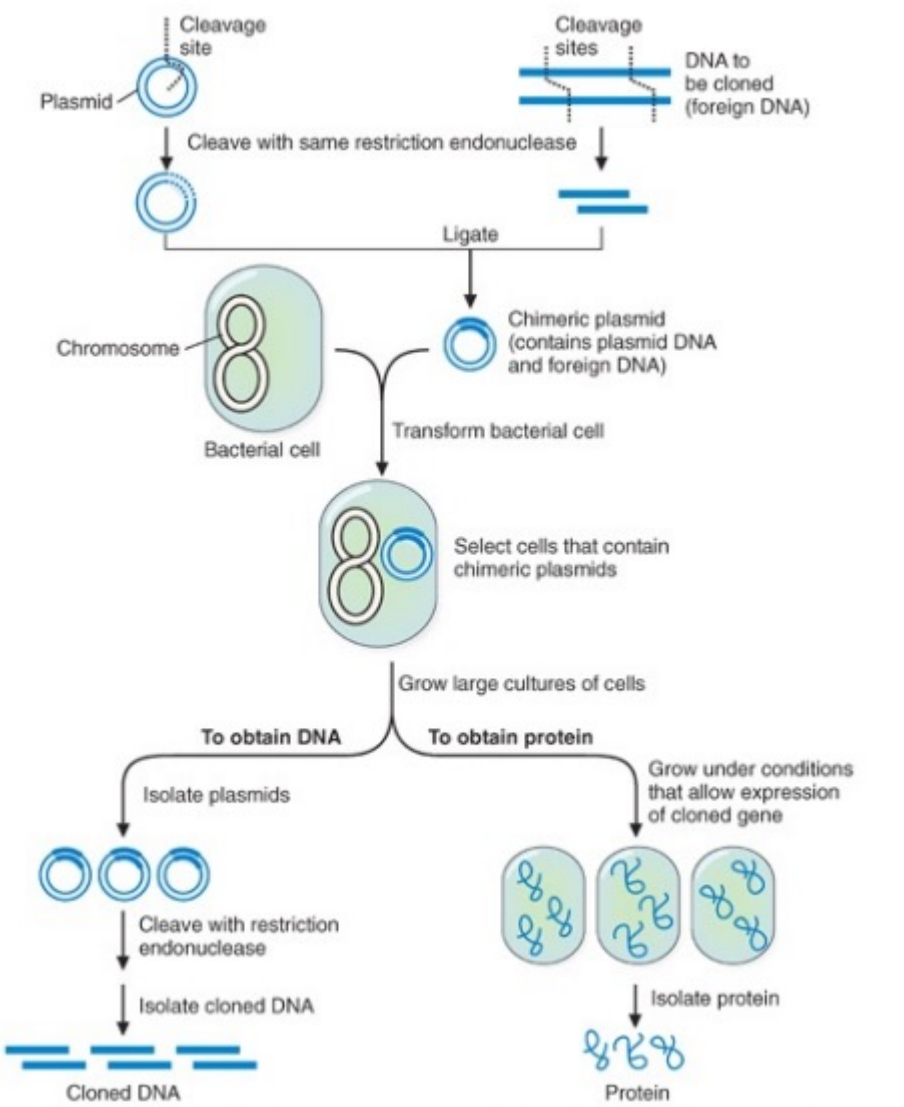
Foreign DNA (what you want amplified) is attached to a vector (carrier DNA)

This is introduced into a host cell that makes multiple copies of DNA

When host cell divides, it replicates their own DNA as well as the vector DNA <u>which includes</u> the DNA you want amplified

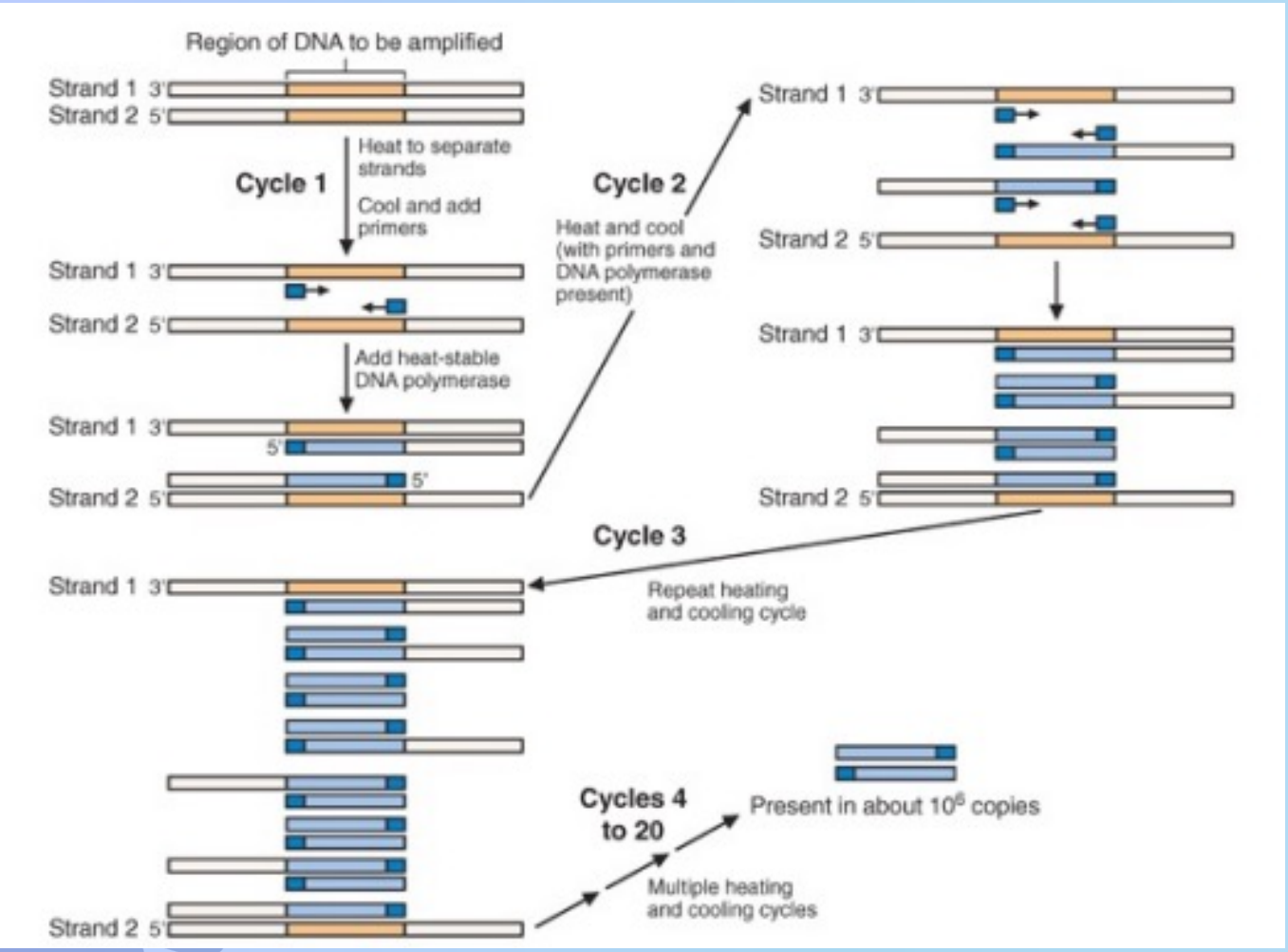
Foreign and vector DNA cut with the same restriction enzyme

<u>Extra terms to know</u>:
Transformed – host bacterial cells with recombinant DNA
Transfected – host eukaryotic cells with recombinant DNA
Transduced – when the vector is a virus



Host	Vector
Bacteria	Bacteriophage, plasmid, cosmids
Eukaryotic cells	Retroviruses, adenoviruses, free DNA, liposomes

PCR = Polymerase Chain Reaction



IN VITRO method that can be used for rapid production of very large amounts of specific segments of DNA

Needed: Primers

- 1 oligonucleotide complementary to short sequence (and 1 to other strand)

4x dNTPs

DNA polymerase

Target DNA

Progress

- ✓ How we obtain fragments of DNA and gene copies
  - ✓ Restriction enzymes
  - ✓ Reverse transcriptase
- ❑ How we identify DNA sequences
  - ❑ Probes
  - ❑ Gel electrophoresis
  - ❑ Southern Blots
  - ❑ DNA sequencing
- ✓ How we amplify DNA sequences
  - ✓ Cloning
  - ✓ PCR
- ❑ Studying gene expression
  - Polymorphisms
  - Gene microarray
- ❑ HIV Diagnostics
  - ELISA
  - Western blot
  - Quantitative RT-PCR

Probes

A single stranded polynucleotide (DNA or RNA) used to identify a complementary sequence on a larger-stranded DNA or RNA molecule

Annealing or *hybridization*: formation of these base pairs with a complementary strand

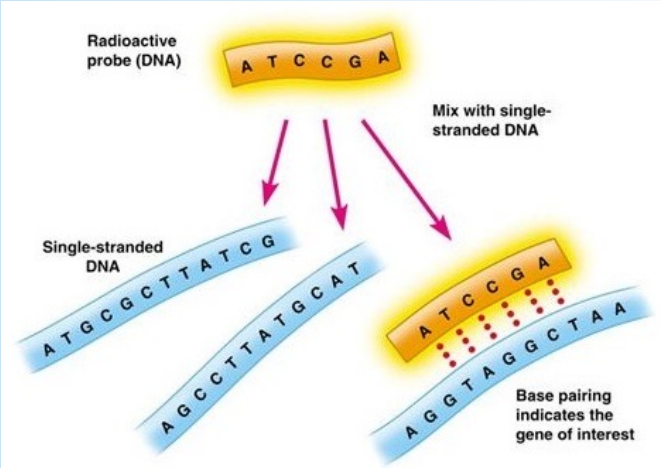
Can be:
- cDNA (produced via reverse transcriptase)
- DNA fragments (cleaved via restriction enzymes from a genome)
- Chemically synthesized oligonucleotides
- RNA (occasionally)

Stringency: how exact of a match the probe must have to the DNA it is hybridizing in order for significant hybridization to occur

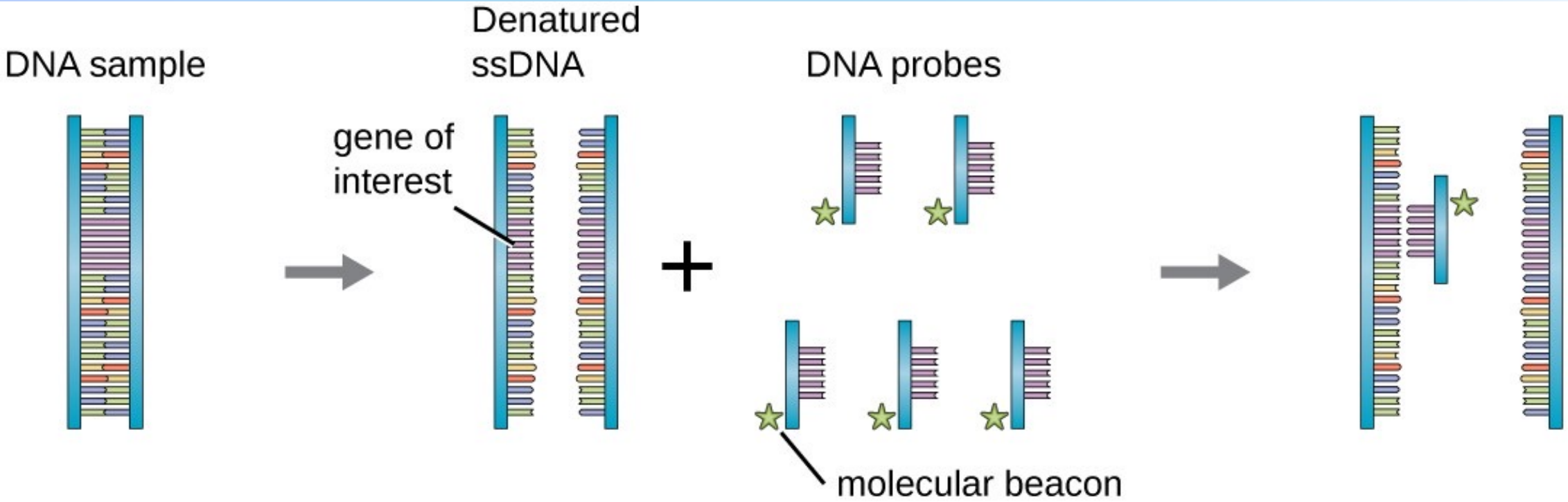
- Temperature (higher = more stringent)
- Salt concentration (higher = less stringent)

Label: needed in order identify the target sequence

Can be: radioactive: detected by autoradiography
chemical: detected by fluorescence microscopy



Probes



1. Isolate DNA from body fluid sample.

2. Denature DNA sample and combine with DNA probes. Probes are complementary to the gene of interest and labeled with a molecular beacon.

3. DNA probes will bind to the gene of interest if it is present in the DNA sample.

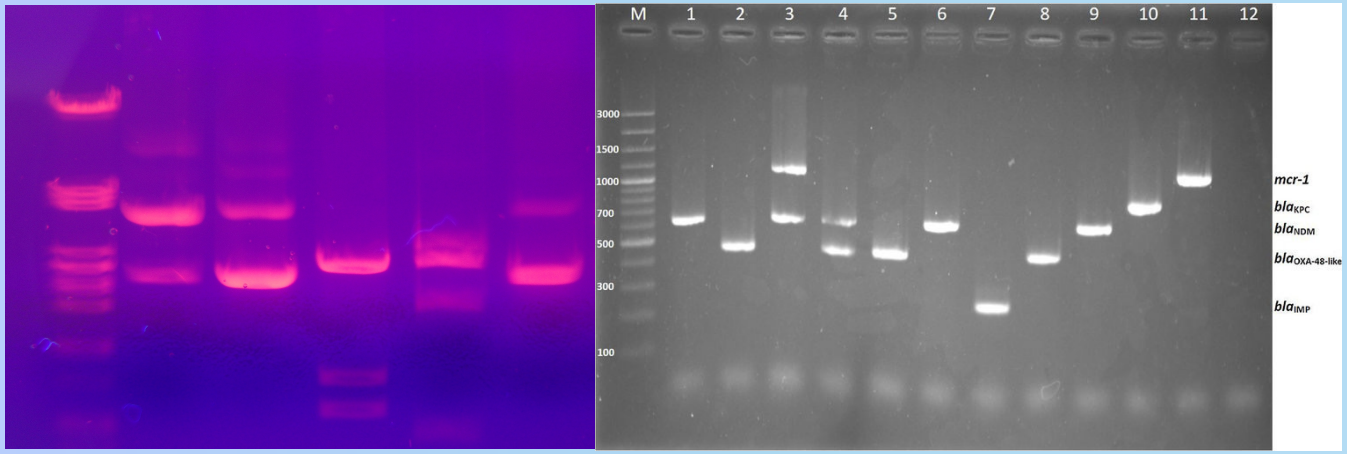
Gel electrophoresis

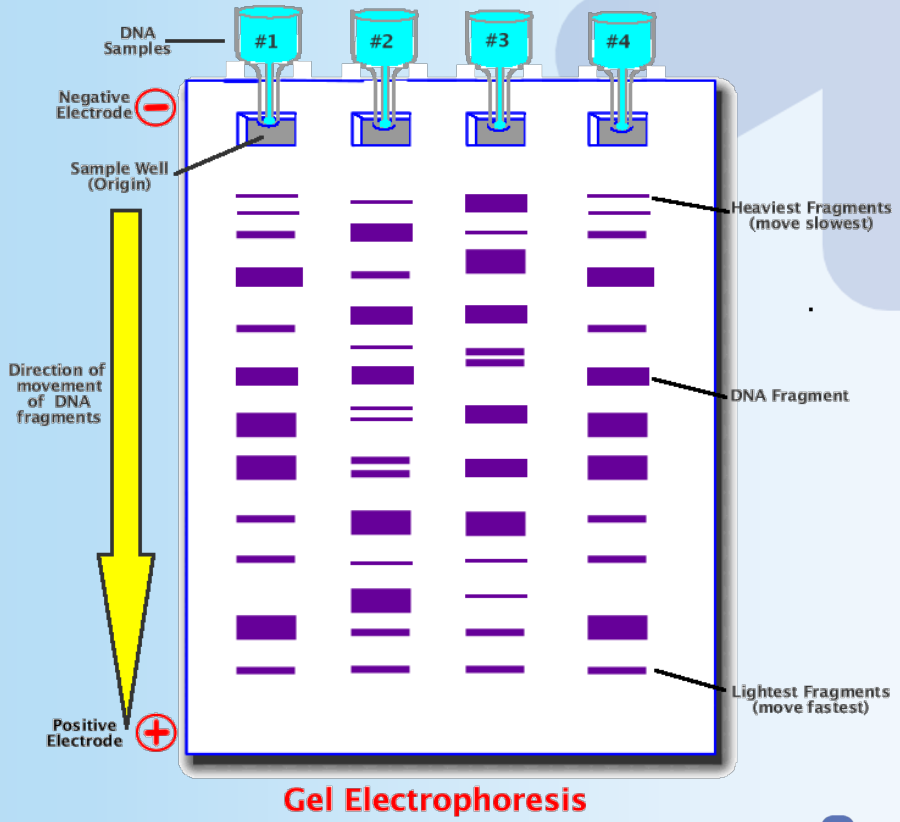
Uses an electrical field to separate molecules based on size

REMEMBER: DNA has a NEGATIVE phosphate group

Shorter molecules travel faster than longer molecules

Bands are then visualized (by dyes and other techniques)





studyaid

Blotting

Used in the detection of specific sequences

Involves:

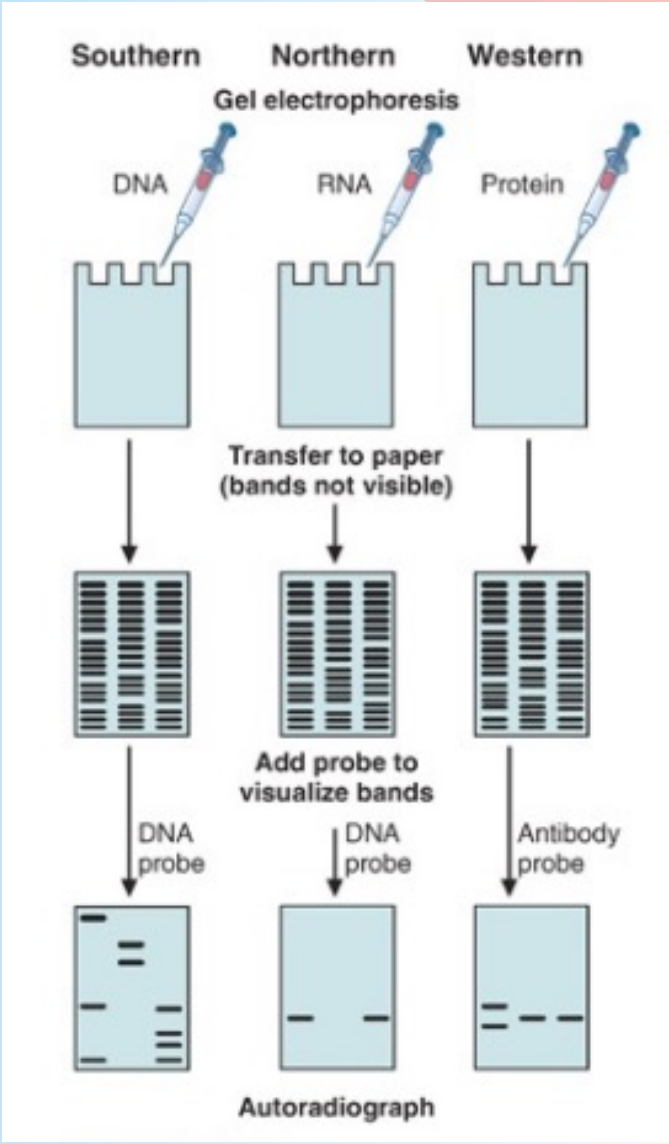
1. Separate molecules by electrophoresis
2. Transfer of molecule (from electrophoresis gel) to a solid support (nitrocellulose paper)
3. Alkaline solution (denatures DNA)+ heat (fixes to paper)
4. Hybridization with a probe
5. Identification

Western cowboy eating a steak (**protein**)

RUN DOWN UNDER

= DNA

=South



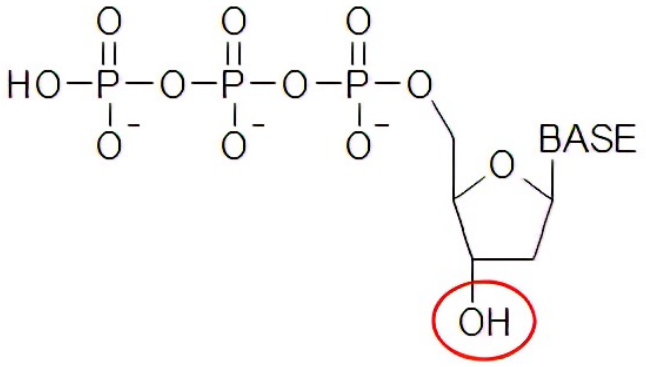
studyaid

Sanger Sequencing

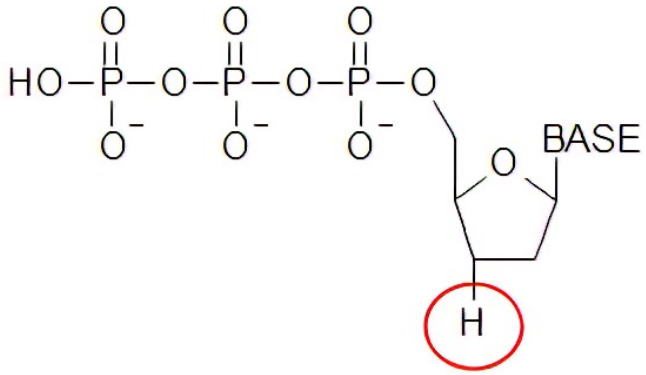
Purpose: Determining the sequence of nucleotides in a DNA strand

Materials:
- Deoxynucleotides
- DNA polymerase
- Primer
- Template strand
- ★Dideoxynucleotides★ Only 1 per reaction (either ddATP, ddTTP, ddCTP, ddGTP)

<u>Lack of 3'-hydroxyl group</u>
-Terminates polymerization

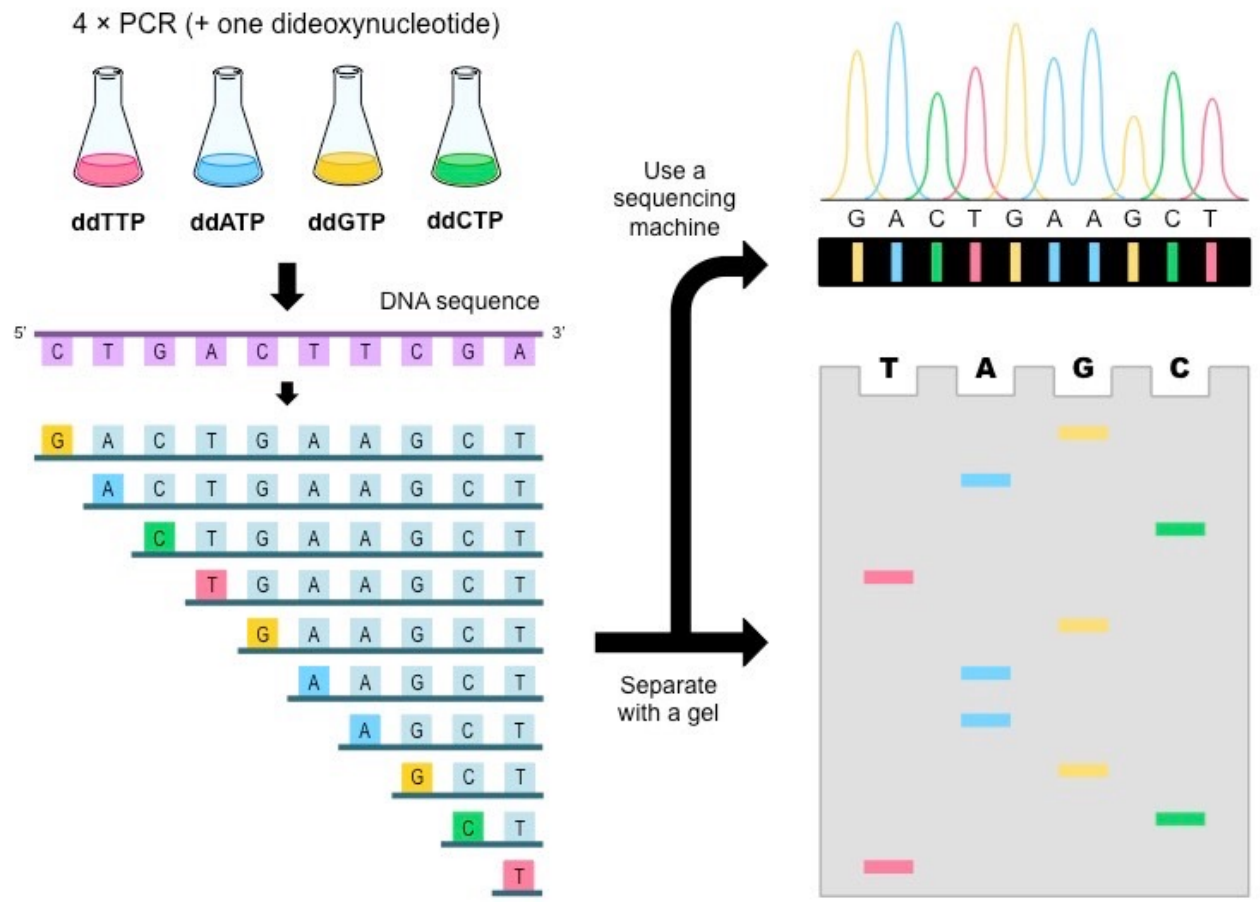


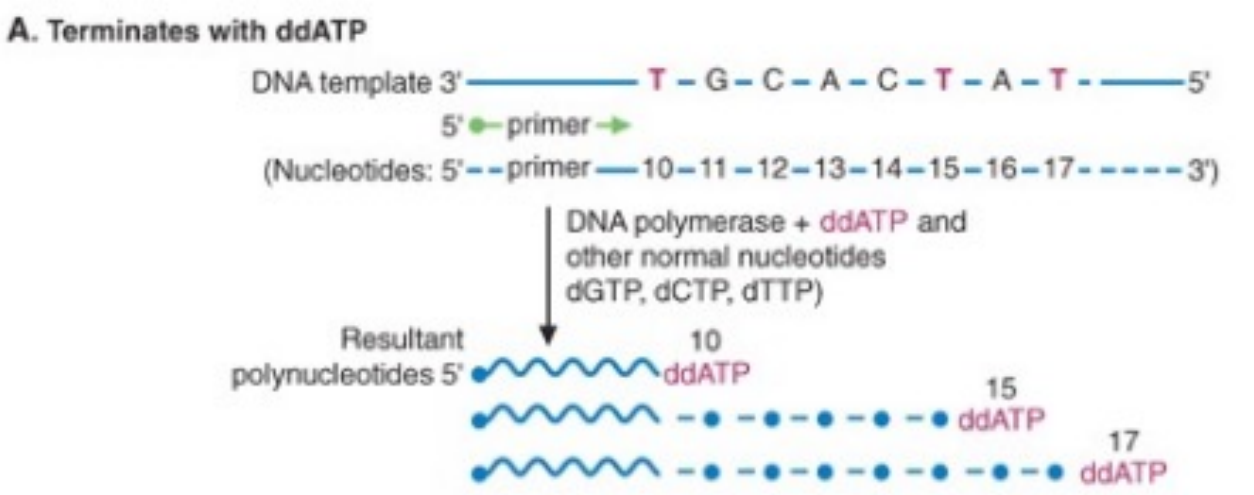
deoxynucleotide triphosphate

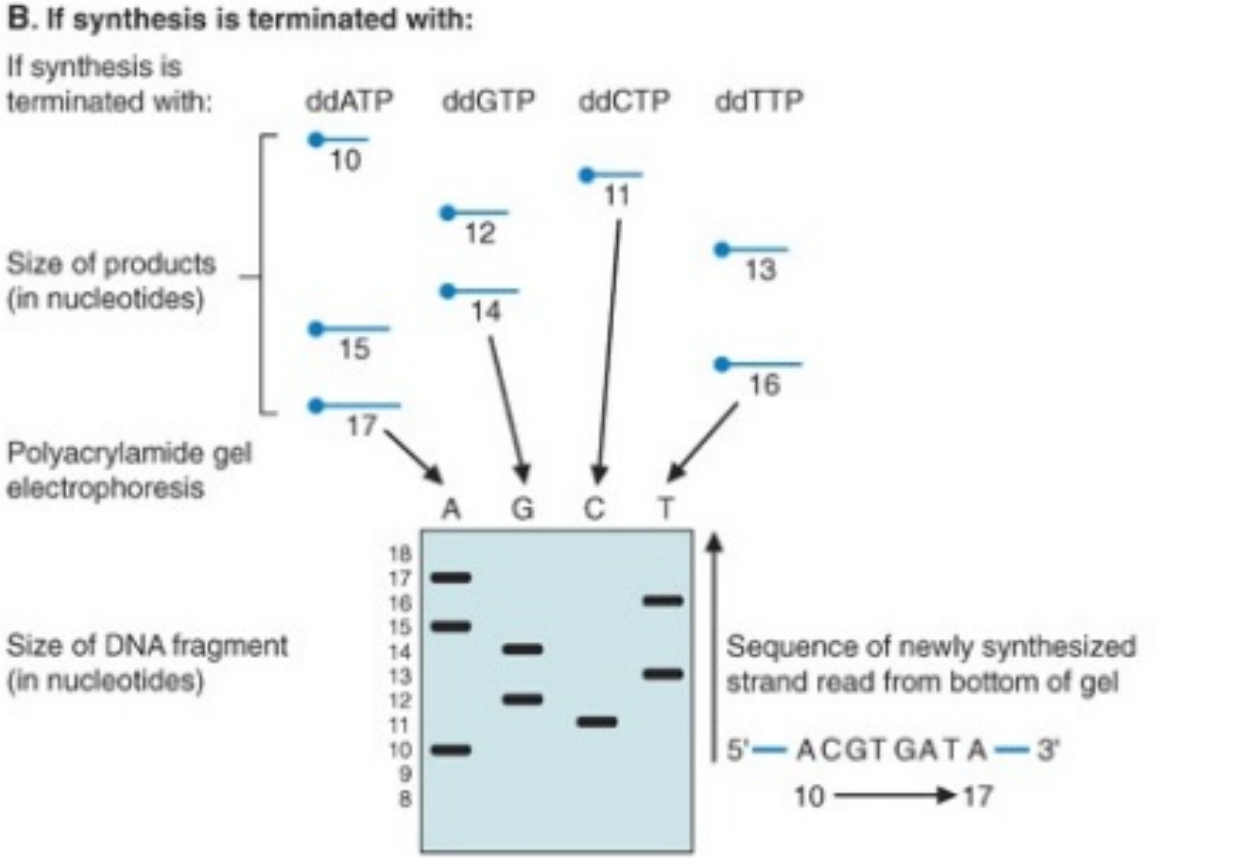


dideoxynucleotide triphosphate

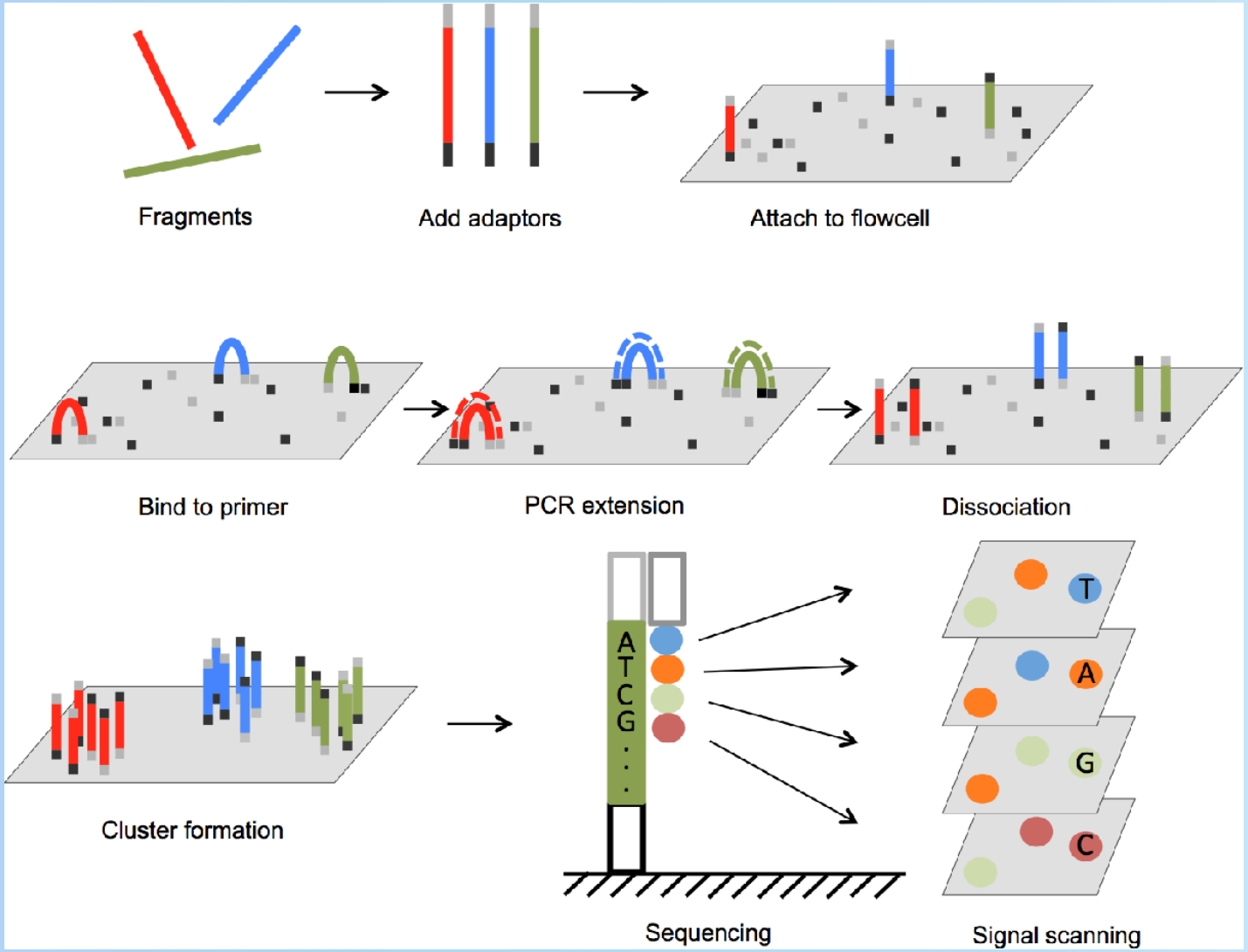
Sanger sequencing







Next-generation sequencing



Progress

- ✓ How we obtain fragments of DNA and gene copies
  - ✓ Restriction enzymes
  - ✓ Reverse transcriptase
- ✓ How we identify DNA sequences
  - ✓ Probes
  - ✓ Gel electrophoresis
  - ✓ Southern Blots
  - ✓ DNA sequencing
- ✓ How we amplify DNA sequences
  - ✓ Cloning
  - ✓ PCR
- ❑ Studying gene expression
  - Polymorphisms
  - Gene microarray
- ❑ HIV Diagnostics
  - ELISA
  - Western blot
  - Quantitative RT-PCR

Restriction Fragment Length Polymorphisms

Polymorphisms – variations among individuals of a species in DNA sequences of the genome
-point mutations
-insertions/deletions

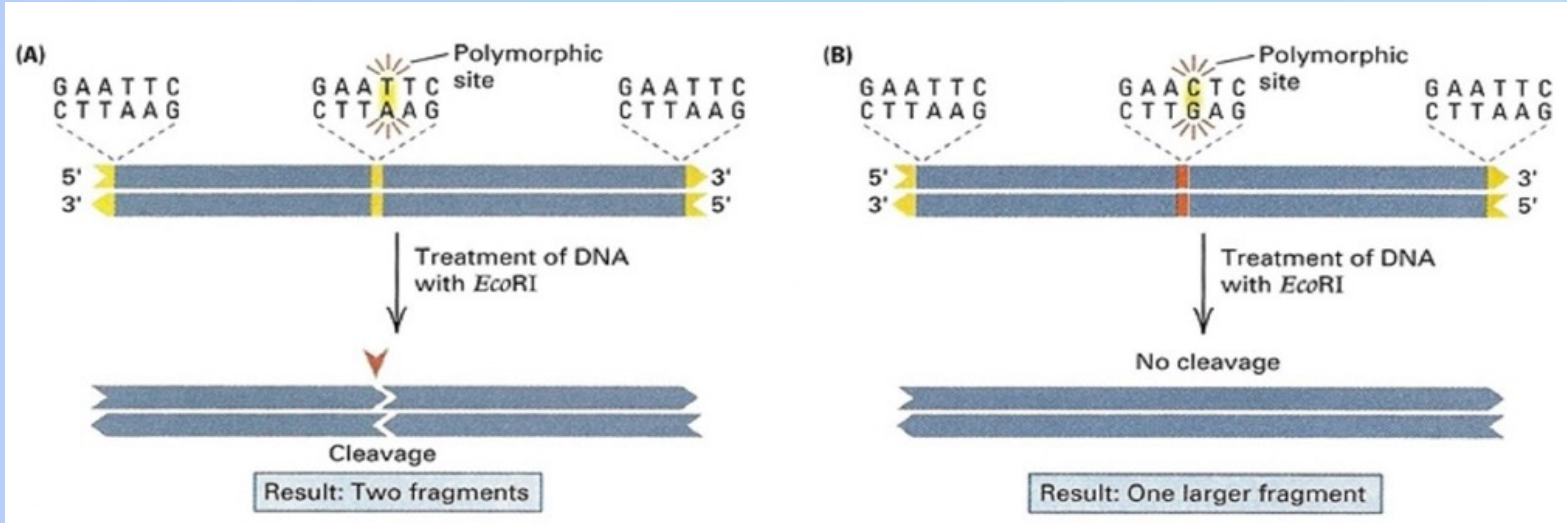
Sometimes a point mutation can occur at a recognition site for one of the restriction enzymes

Restriction enzyme CANNOT cut → larger restriction fragment

Sometimes mutations can make restriction sites not normally present

Restriction enzyme performs extra cut → smaller restriction fragment

These variations in the length of restriction fragments = RFLPs



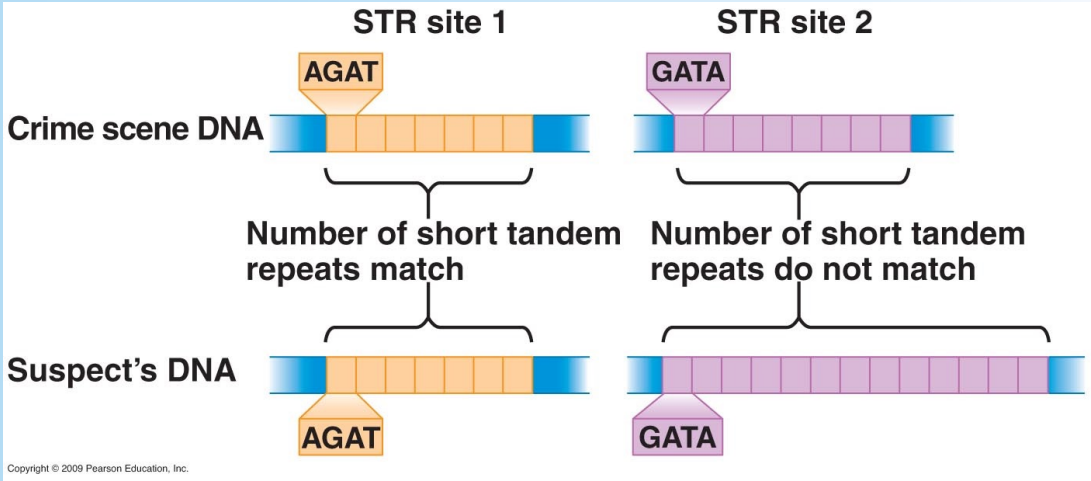
Molecular fingerprint

Variable number of tandem repeats (VNTR)

- Short sequences of DNA at scattered locations of genome, repeated in tandem
- Number of repeats differs from person to person and is unique to any given person

Short tandem repeats (STR)

- Very similar to VNTR but smaller in size
- Used in forensic science with DNA amplification by PCR



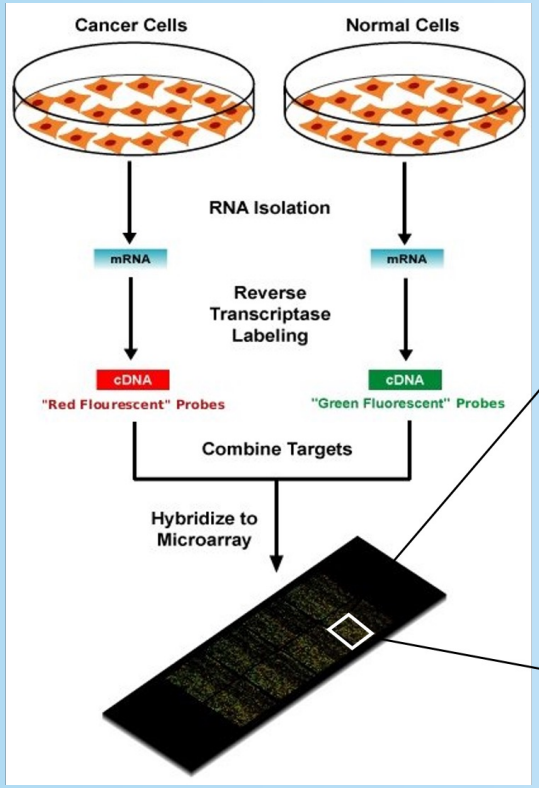
Microarrays (DNA Chips)

Inside the wells of the DNA chip there are oligonucleotides or probes corresponding to a DNA sequence we are interested in

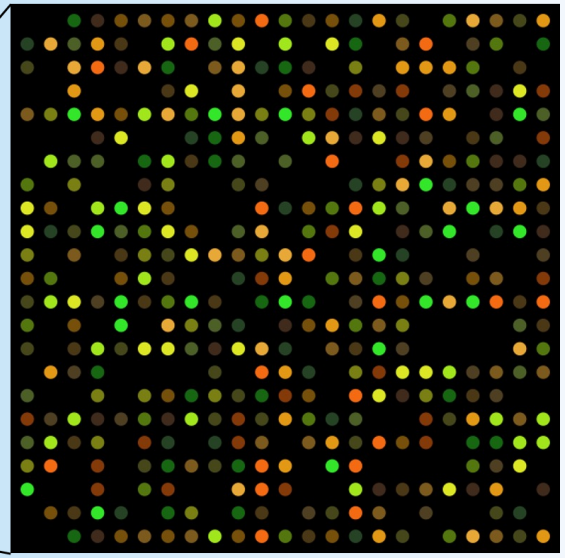
Pattern of hybridization is determined via computer analysis

Useful for:
- determining mutations for a particular genetic disease
-determining which alleles of drug-metabolizing enzymes are present
-determine which genes are being expressed in cells

A



B



Progress

- ✓ How we obtain fragments of DNA and gene copies
  - ✓ Restriction enzymes
  - ✓ Reverse transcriptase
- ✓ How we identify DNA sequences
  - ✓ Probes
  - ✓ Gel electrophoresis
  - ✓ Southern Blots
  - ✓ DNA sequencing
- ✓ How we amplify DNA sequences
  - ✓ Cloning
  - ✓ PCR
- ✓ Studying gene expression
  - ✓ Polymorphisms
  - ✓ Gene microarray
- ❑ HIV Diagnostics
  - ELISA
  - Western blot
  - Quantitative RT-PCR

HIV

Retrovirus

2 types: HIV-1 and HIV-2

Structure: icosahedral

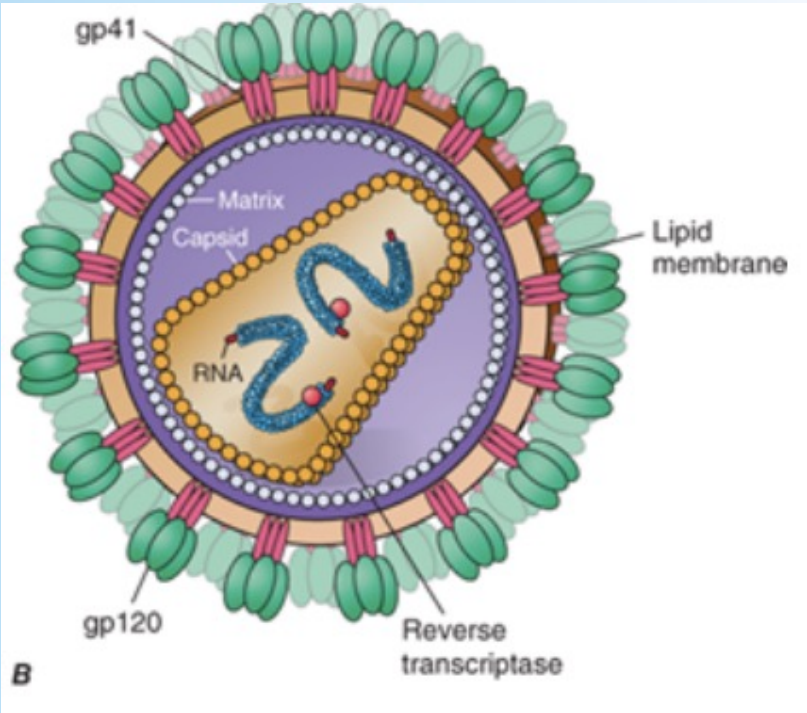
2 major envelope proteins: **gp120** (external) and **gp41** (transmembrane)

Genomic RNA

Reverse transcriptase enzyme

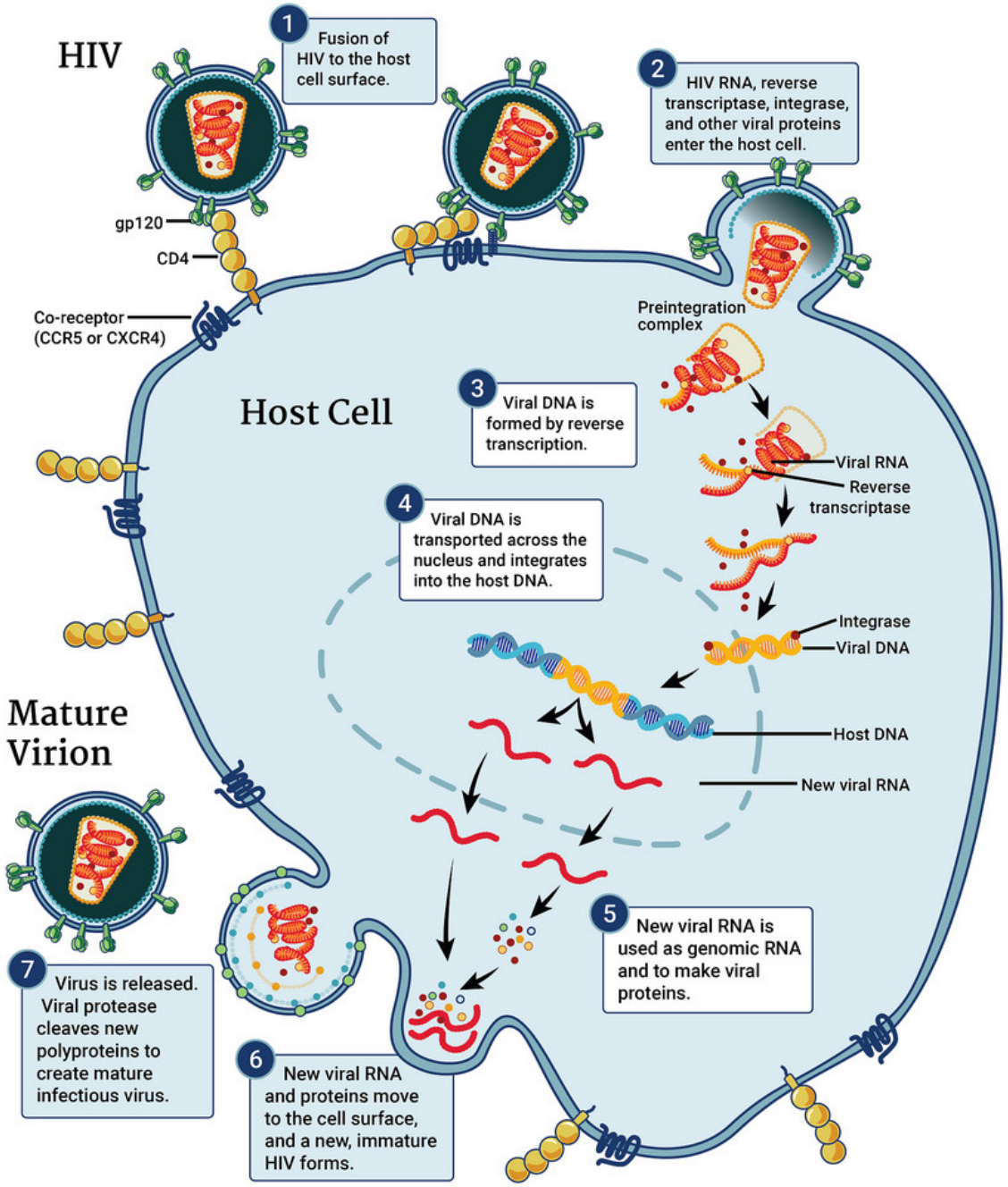
Matrix (inner membrane)

Capsid (p24 core protein)



HIV Replication Cycle

1. Binding of HIV to CD4 molecule via **gp120**
2. Conformational change of **gp120**
3. Binding of co-receptors **CCR5** and/or **CXCR4**
4. Fusion of virus with host cell membrane via **gp41**
5. Uncoating of the capsid shell → facilitates **reverse transcriptase** and forms pre-integration complex (viral RNA, enzymes, accessory proteins) surrounded by capsid and matrix
6. Preintegration complex searches for nucleus
   Reverse transcription of RNA to dsDNA
7. Viral DNA enters the nucleus through the nuclear pore
8. Integration of proviral DNA to host DNA via **integrase**
9. The provirus can be latent or active
10. If active, transcription occurs where HIV mRNA is translated to proteins that undergo modifications
11. Assembly of viral particle occurs at the plasma membrane; contains HIV proteins, enzymes, and genomic RNA
12. Budding of the progeny virion gives it an external envelope
13. During or soon after budding, **protease** cleaves a gag-pol precursor = mature virion
    1. Gag – p24, p17
    2. Pol – protease processing, reverse transcription, integration



Detection of HIV

Eclipse period – variable amount of time in which no existing diagnostic test is capable of detecting HIV

Window period – time from infection to first reactive result

HIV RNA – within 12 days (50%) with peaks at 20-30 days

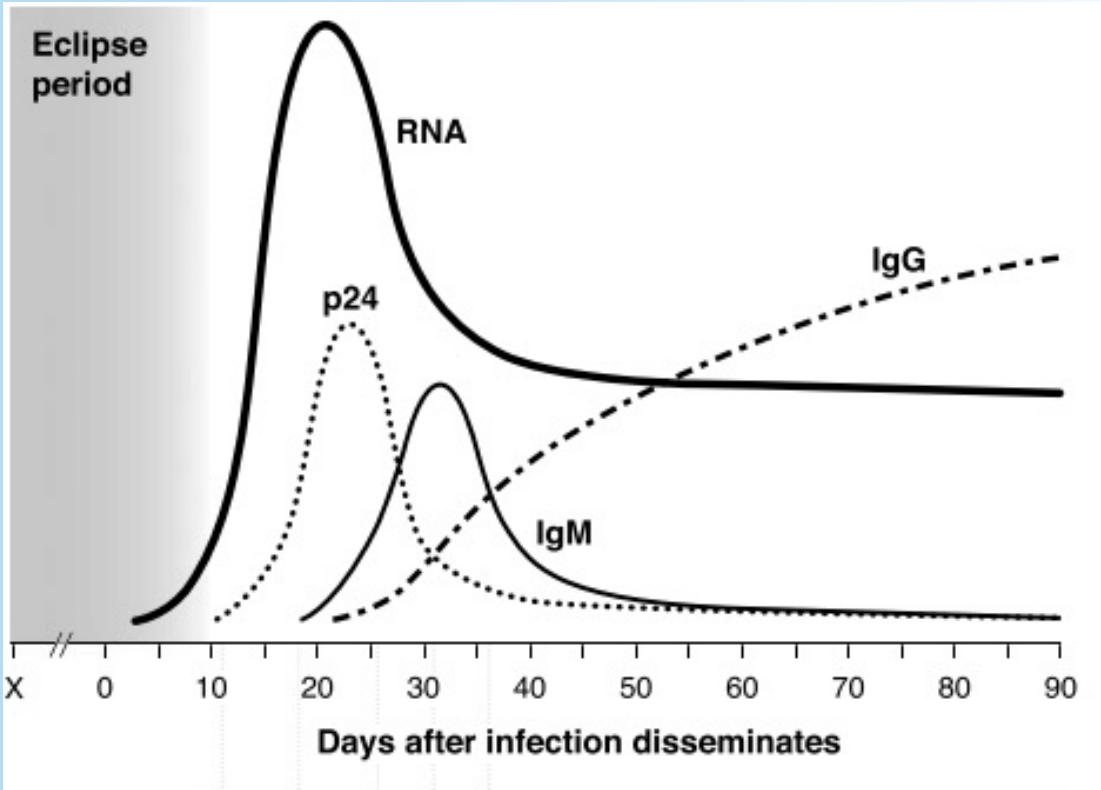
p24 – by day 15 and rises through days 25-30; by day 50, antigen is cleared from bloodstream

IgM – day 20, peaking days 30-35

IgG – days 30-35

Point of Care (POC) Testing	Window Time
IgG (1st and 2nd gen)	~31 days
IgM/IgG (3rd gen)	~23 days
Antigen/antibody (IgM+IgG+p24) (4th gen)	~18 days

Serum/plasma >> oral transudate
One key disadvantage: lower sensitivity, especially early after infection



Enzyme-linked immunosorbent assay (ELISA)

Detection of antibodies to HIV

Immovable HIV-specific proteins are used as 'bait' (=antigens) for HIV antibodies in the human blood sample

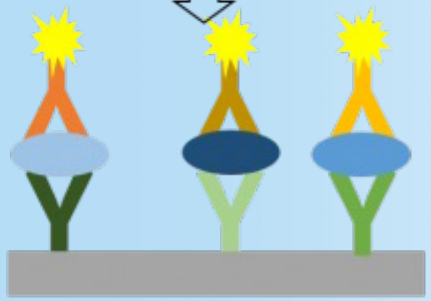
HIV antibodies bind to the antigens

Bound HIV antibodies are made visible by labelled antigens that bind to the HIV antibodies

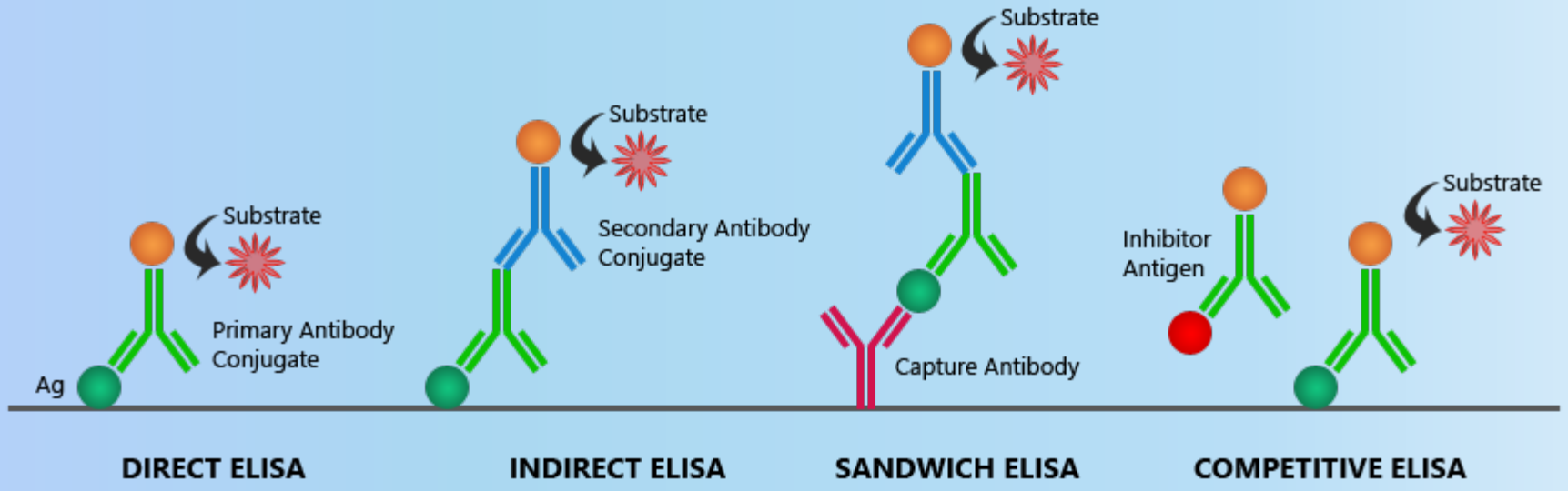
Detection of HIV-specific proteins

Immovable HIV-specific antibodies are used as 'bait' for HIV-specific proteins (i.e. antigens) in the human blood sample

HIV antigens bind to the antibodies



Bound HIV antigens are made visible by labelled antibodies that bind to the HIV antigens



Improvements in ELISA testing for HIV

1st gen	2nd gen	3rd gen	4th gen
Detects IgG Abs to HIV-1 whole viral lysate	Detects IgG Abs to HIV-1 and HIV-2 using synthetic peptides	Detects both IgG and IgM Abs to HIV-1 and HIV-2	Same as 3rd PLUS HIV-1 p24 Ag

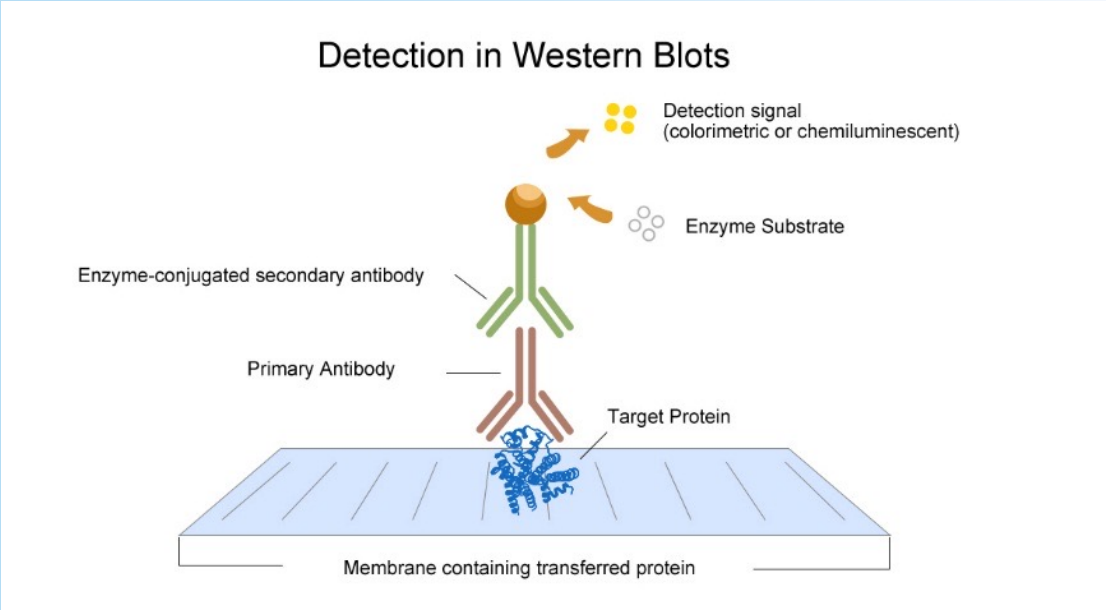
Western Blot

In a nutshell: you work with prepared samples of HIV proteins and look to see if the patient has any antibodies that stick to them

HIV envelope: gp41, gp120/go160
Core: p17, p24, p55
Enzymes: p31, p51, p66

+ = antibody against **envelope** protein AND: either one **core** or **enzyme**

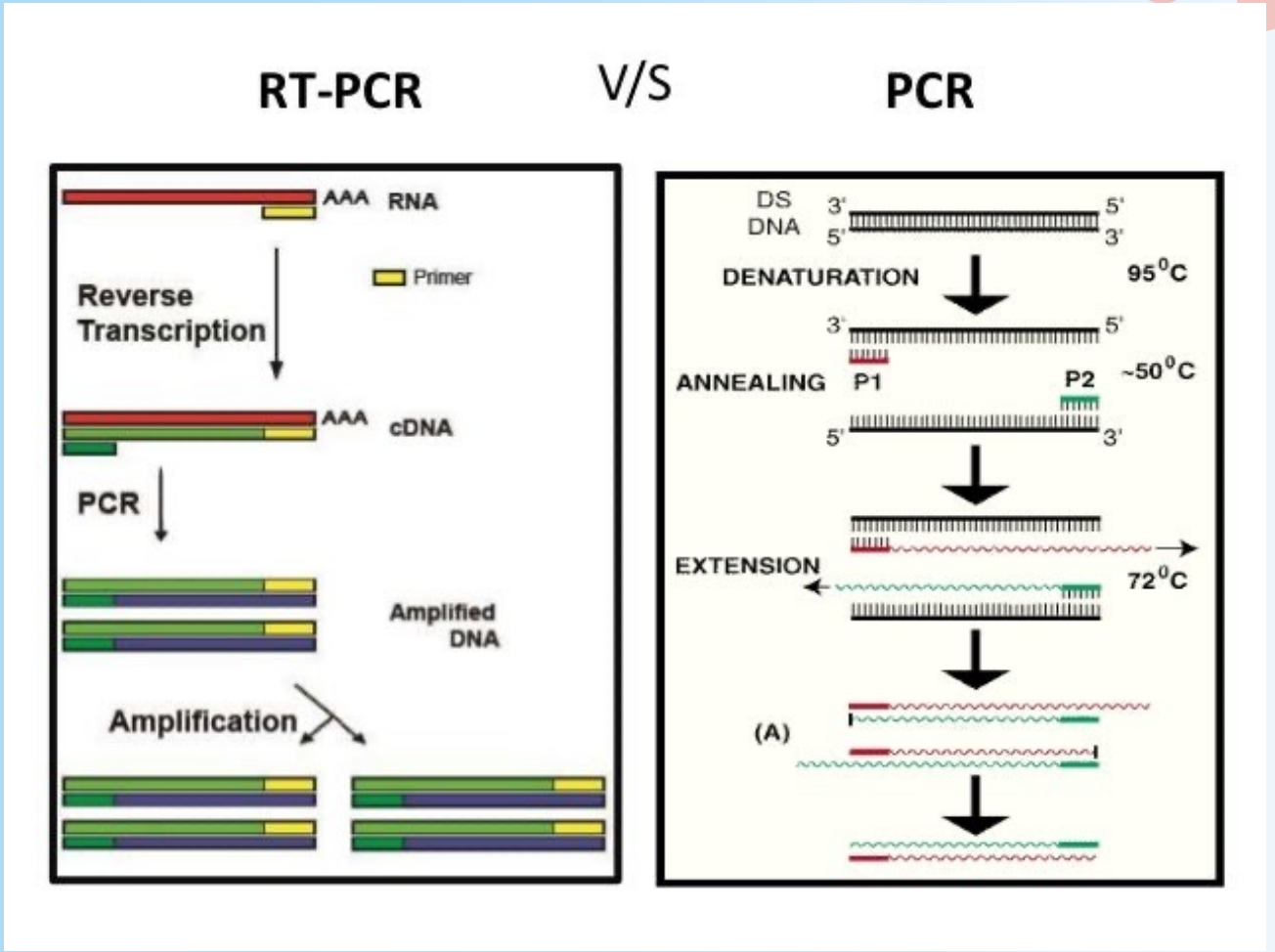
Used as confirmatory testing



RT-PCR

Materials: Viral RNA
dNTPs
RNAse
Primer
DNA polymerase
Reverse transcriptase

Product: cDNA



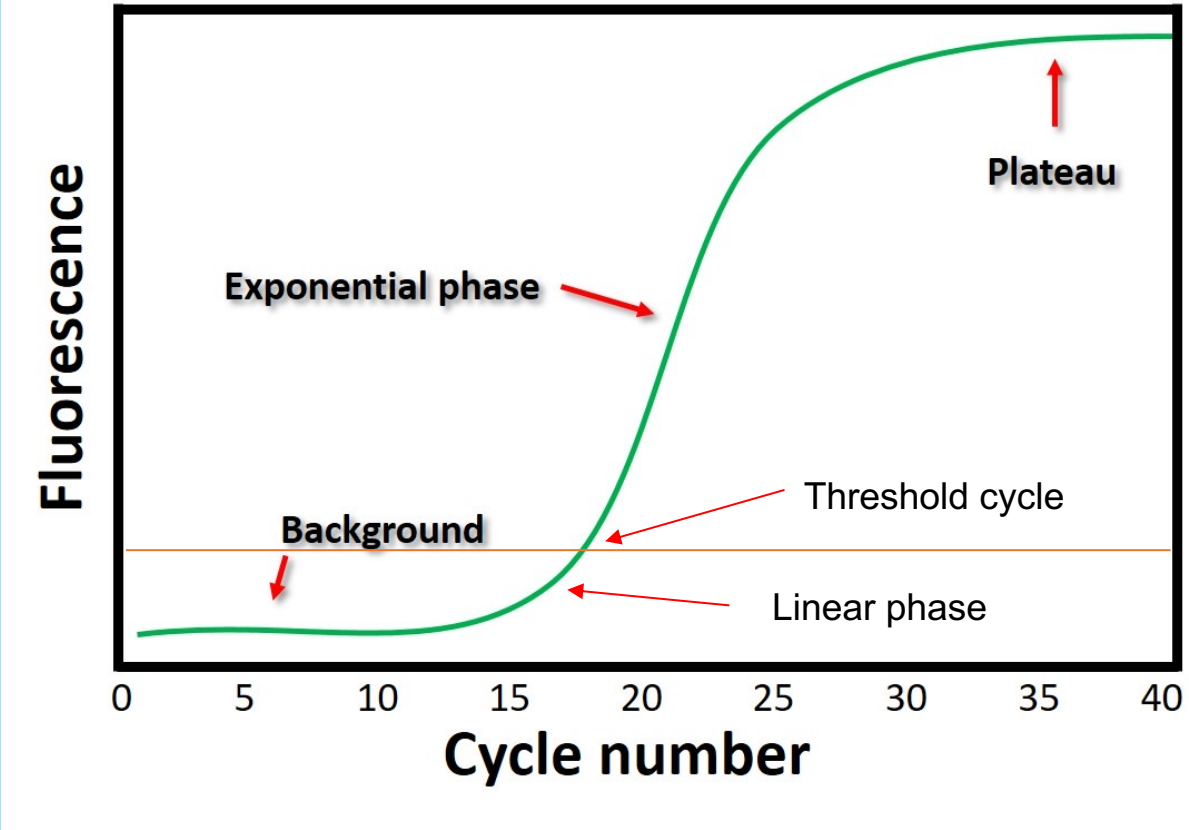
qRT-PCR

What: Amplification and product detection in one go

Fluorescent dyes specifically label DNA of interest (SYBR Green or TaqMan)

Many uses:

Gene expression analysis, cancer biomarker identification, SNP genotyping, protein analysis



Practice Questions:
https://www.wooclap.com/DZZMXB

Thank you and happy studying

Lippincott Chapter 34: Biotechnology and Human Disease