Laboratory Diagnosis of Virus Infections MBChB III (Part 2) – 22May19

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Molecular & Cell Culture Techniques

Blotting Techniques

- Western
- Southern
- Northern

Virology Serology

Western Blotting

1. Viral proteins are separated in SDS-PAGE gel
2. Transferred to a nitrocellulose filter
3. Detected by labeled antibodies

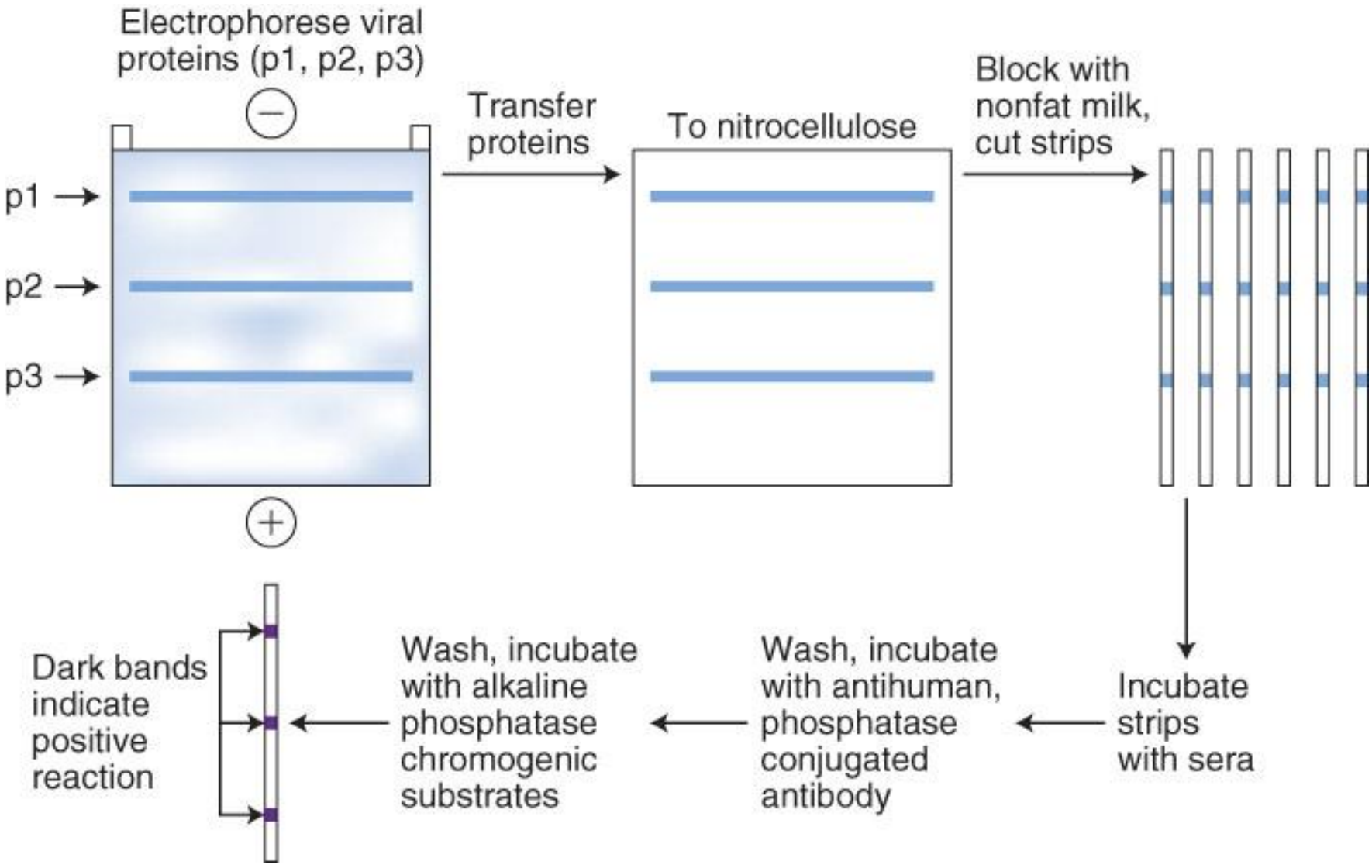
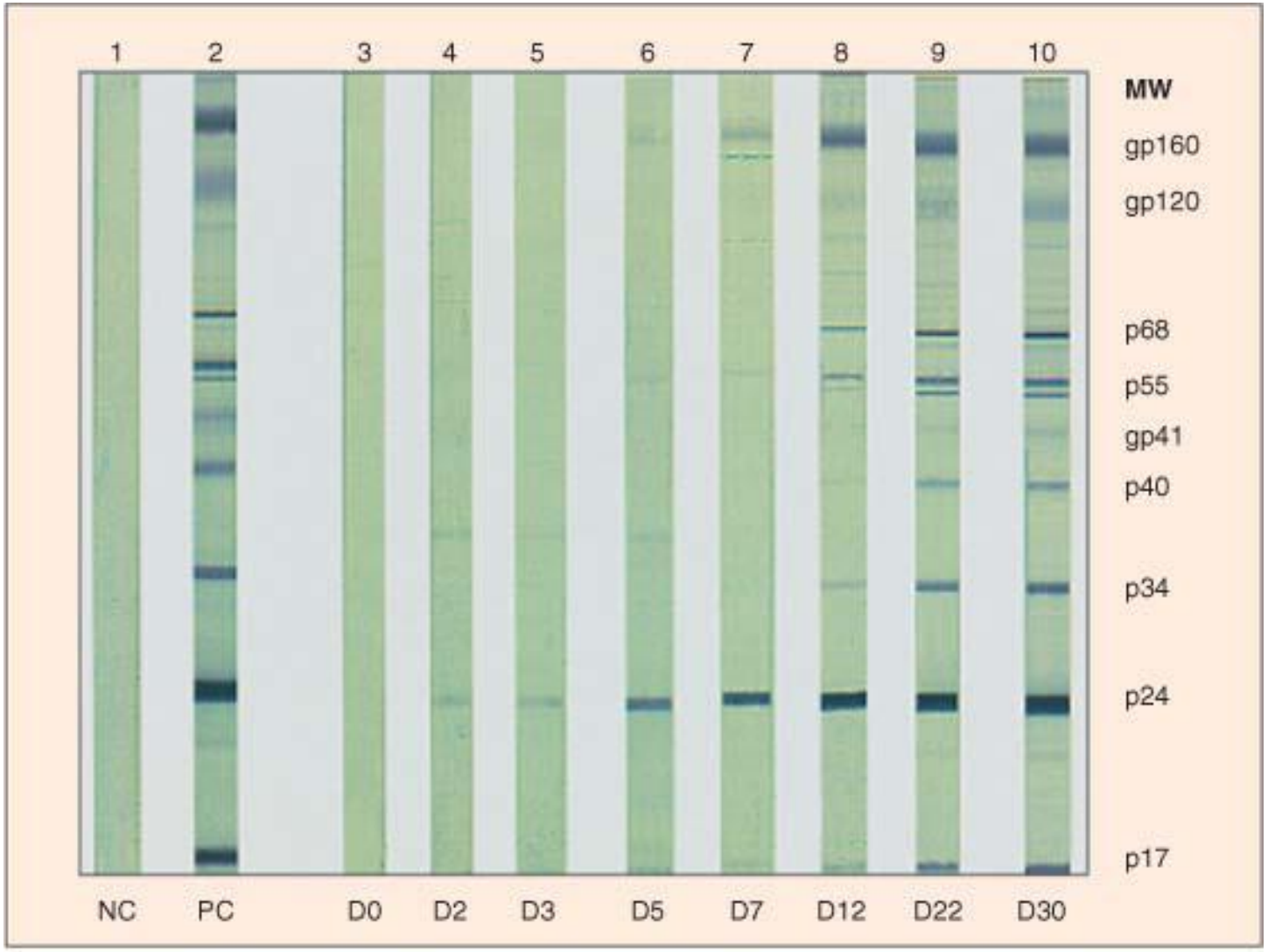


Figure 5.21a: The basic principles behind the Western blotting procedure.

Antibody detection: western blot



From Medical Microbiology, 5thed., Murray, Rosenthal & Pfaller, Mosby Inc., 2005, Fig. 51-7.

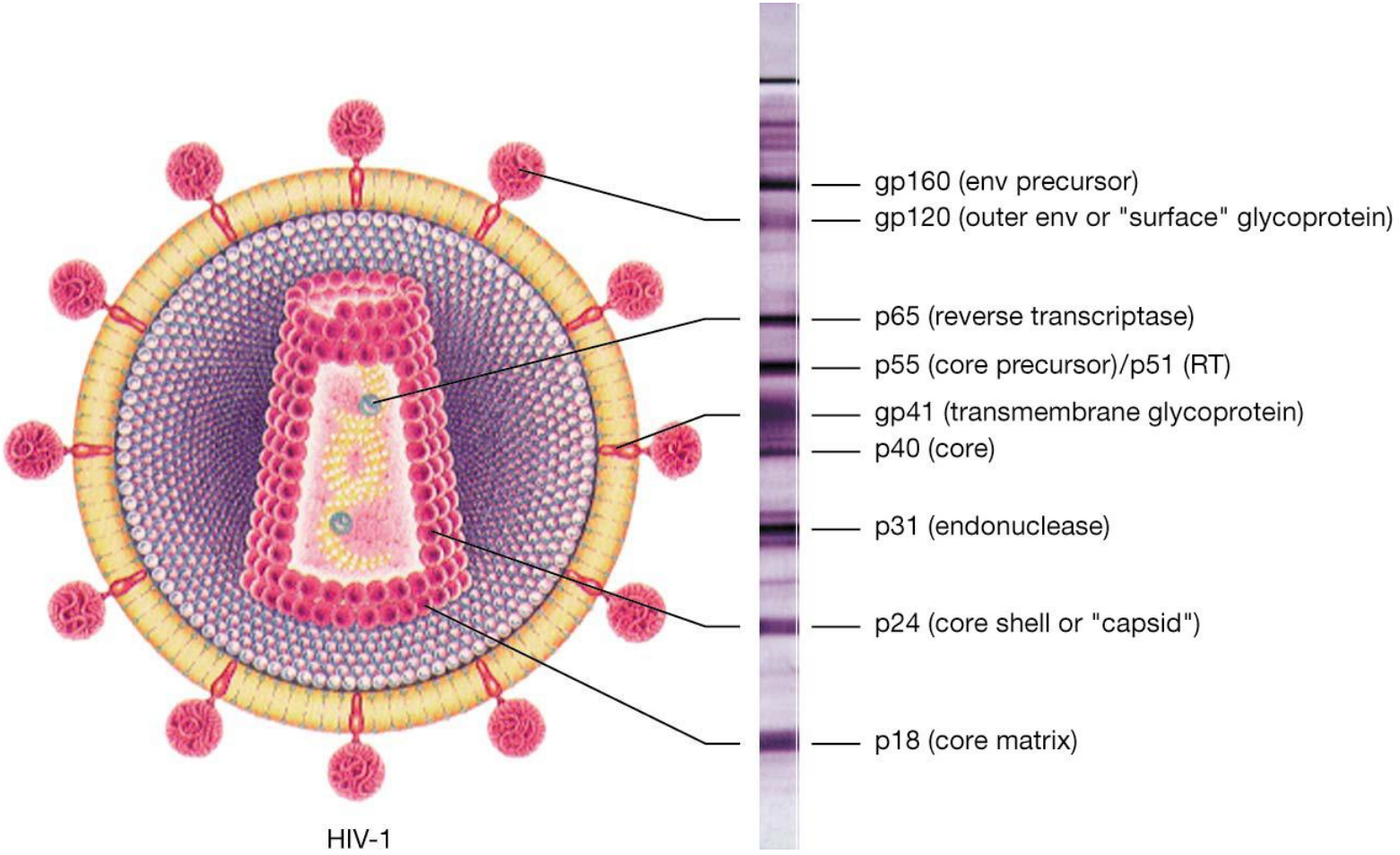
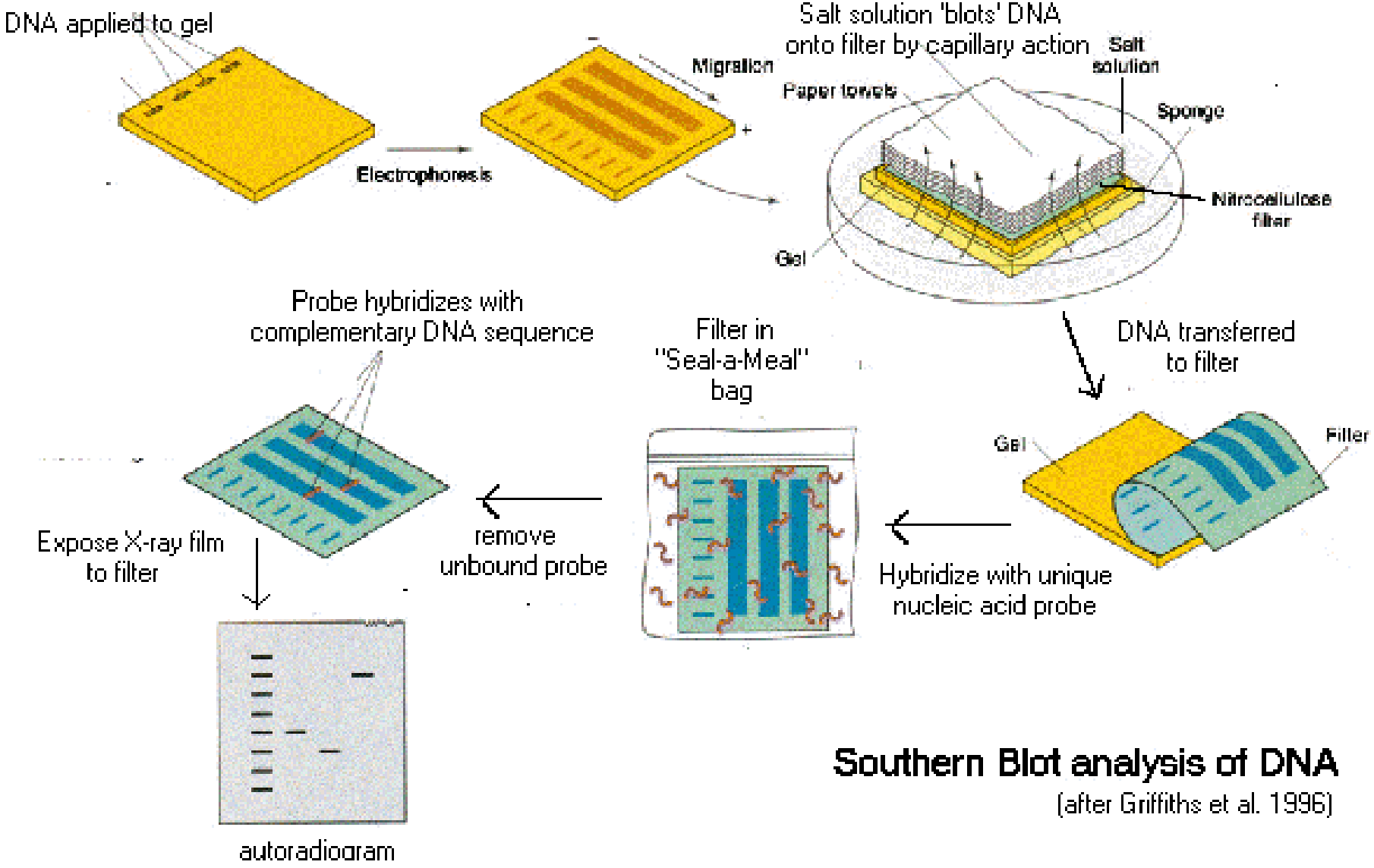


Figure 5.21b: The structure of HIV-1.

Figure 5.21c: The typical results of a Western blot testing patient serum for HIV-1 antibodies.

Southern blotting

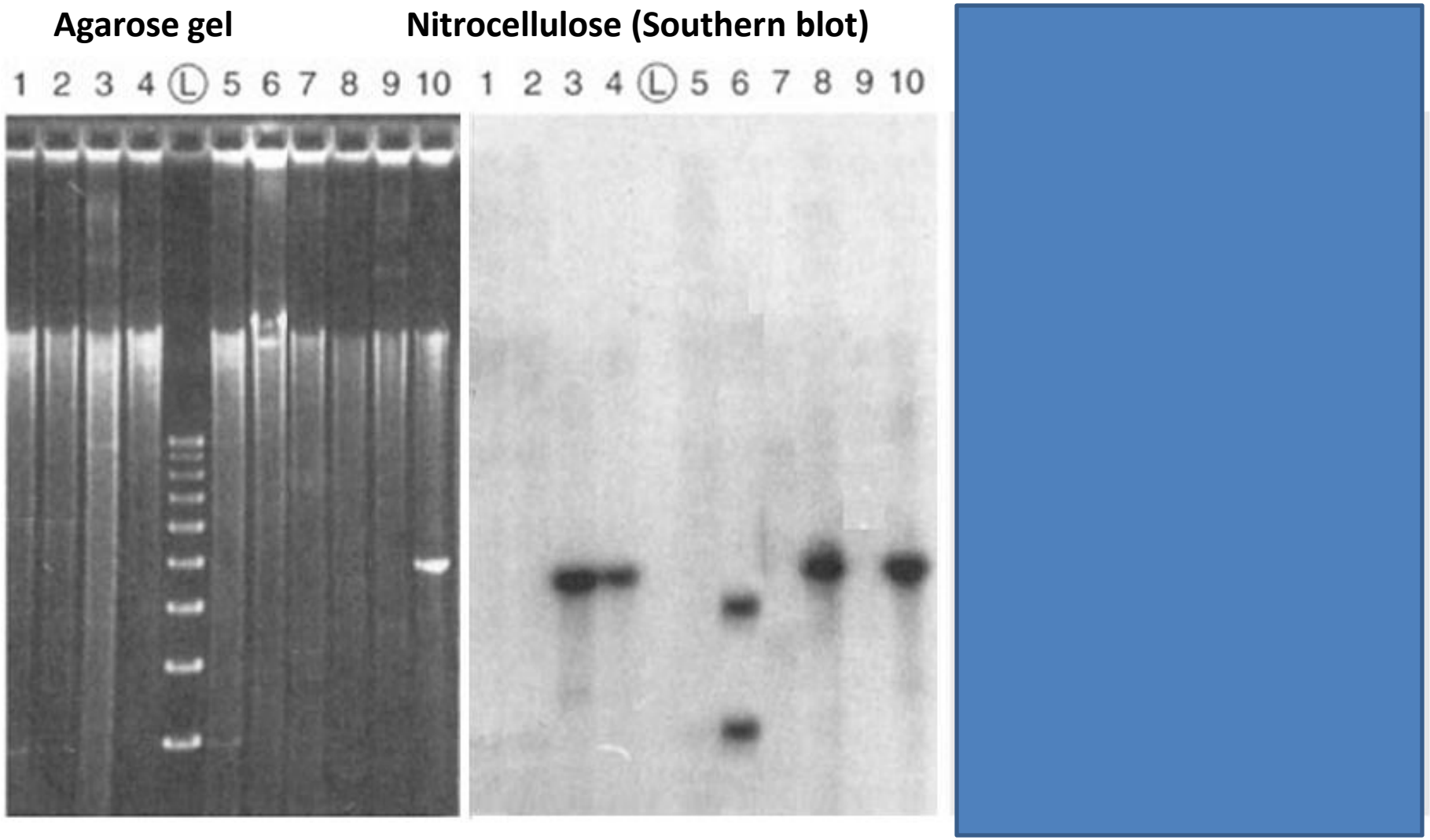
- DNA – starting material
- Restriction Enzyme digestion
- Resolve DNA fragments on agarose gel
- Transfer DNA from gel -> nitrocellulose membrane (NM)
- Probe for target DNA with radio-labelled oligonucleotide probe
- Expose NM to X-ray film
- Develop X-ray film
- Observe DNA bands



Southern Blot analysis of DNA

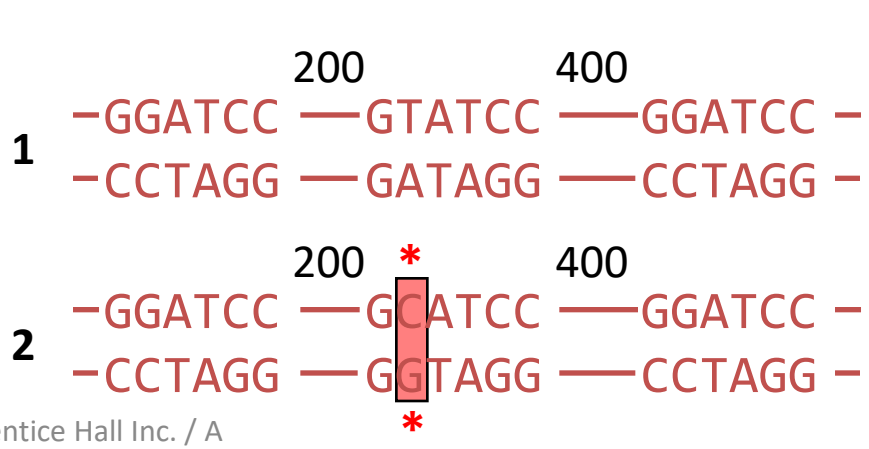
(after Griffiths et al. 1996)

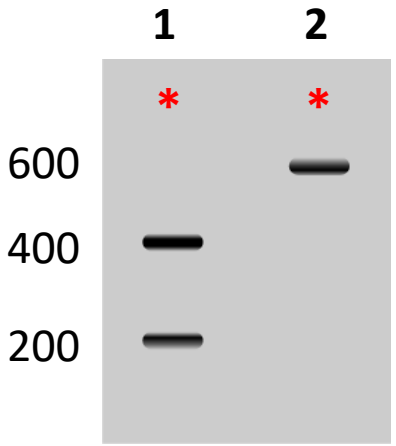
Southern blot



RFLP

- Restriction-fragment length polymorphism
  - Cut genomic DNA from two individuals with restriction enzyme
  - Run Southern blot
  - Probe with different pieces of DNA
  - Sequence difference creates different band pattern





RFLP – Restriction Fragment Length Polymorphism

- DNA cut with Restriction Enzyme
- Gel electrophoresis
- DNA hybridization
- Compare bands

- **Applications: Catching the bad guys**
- **DNA fingerprinting**

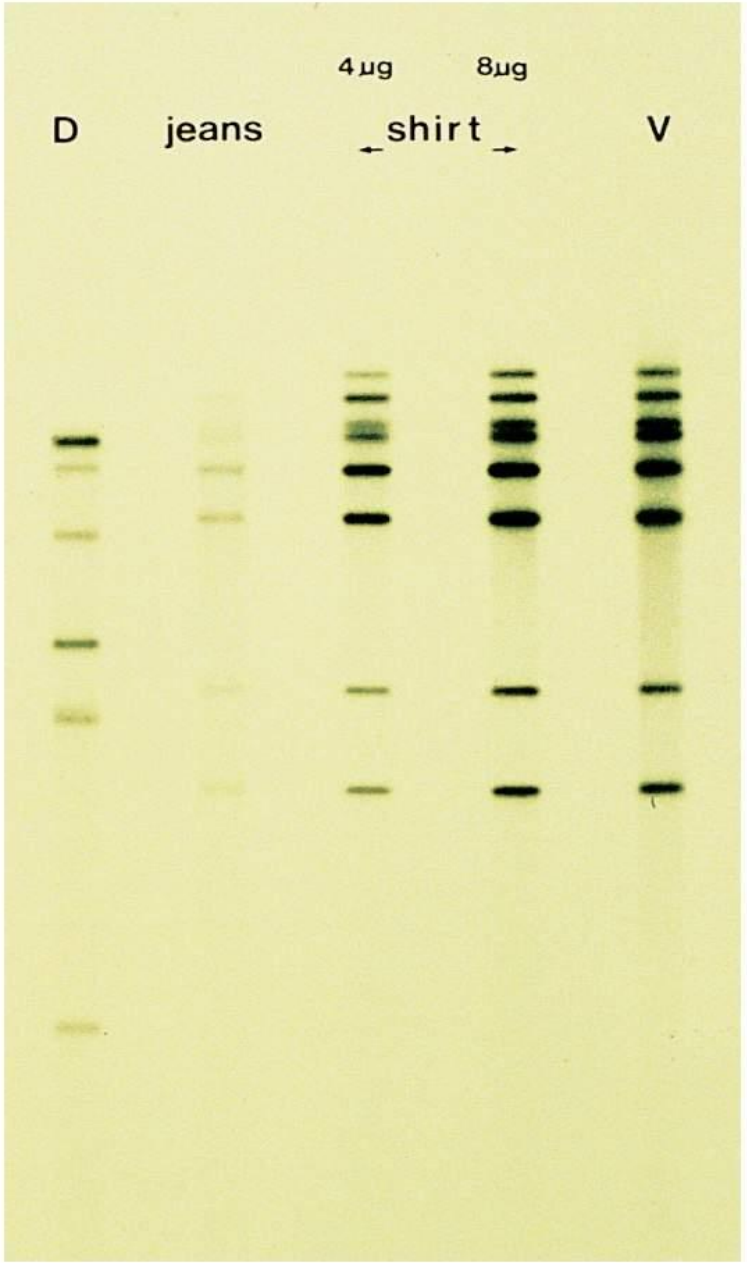
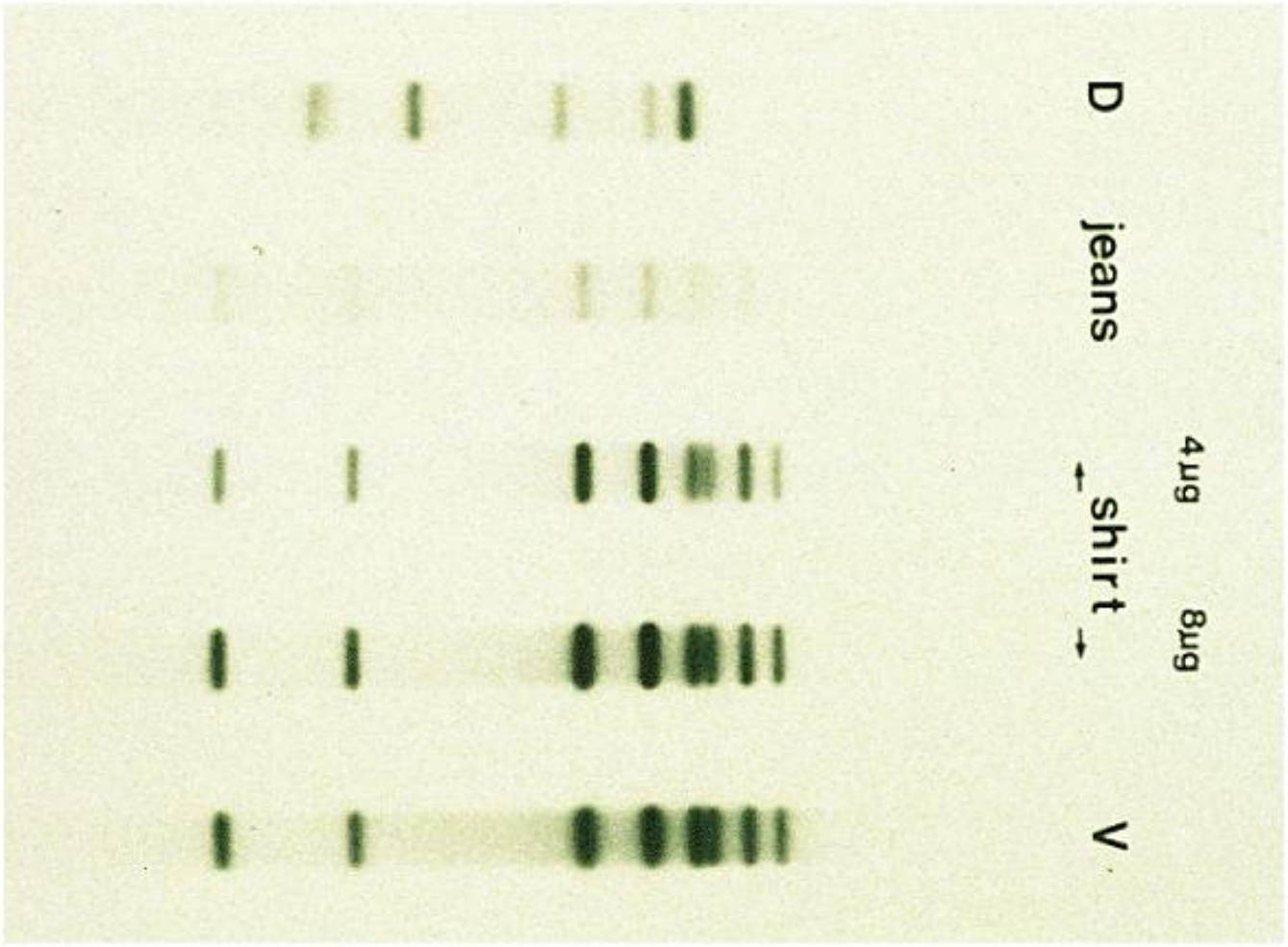


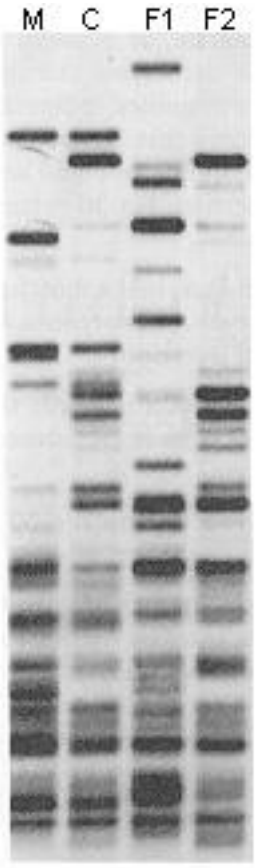
Figure 20.17 DNA fingerprints from a murder case



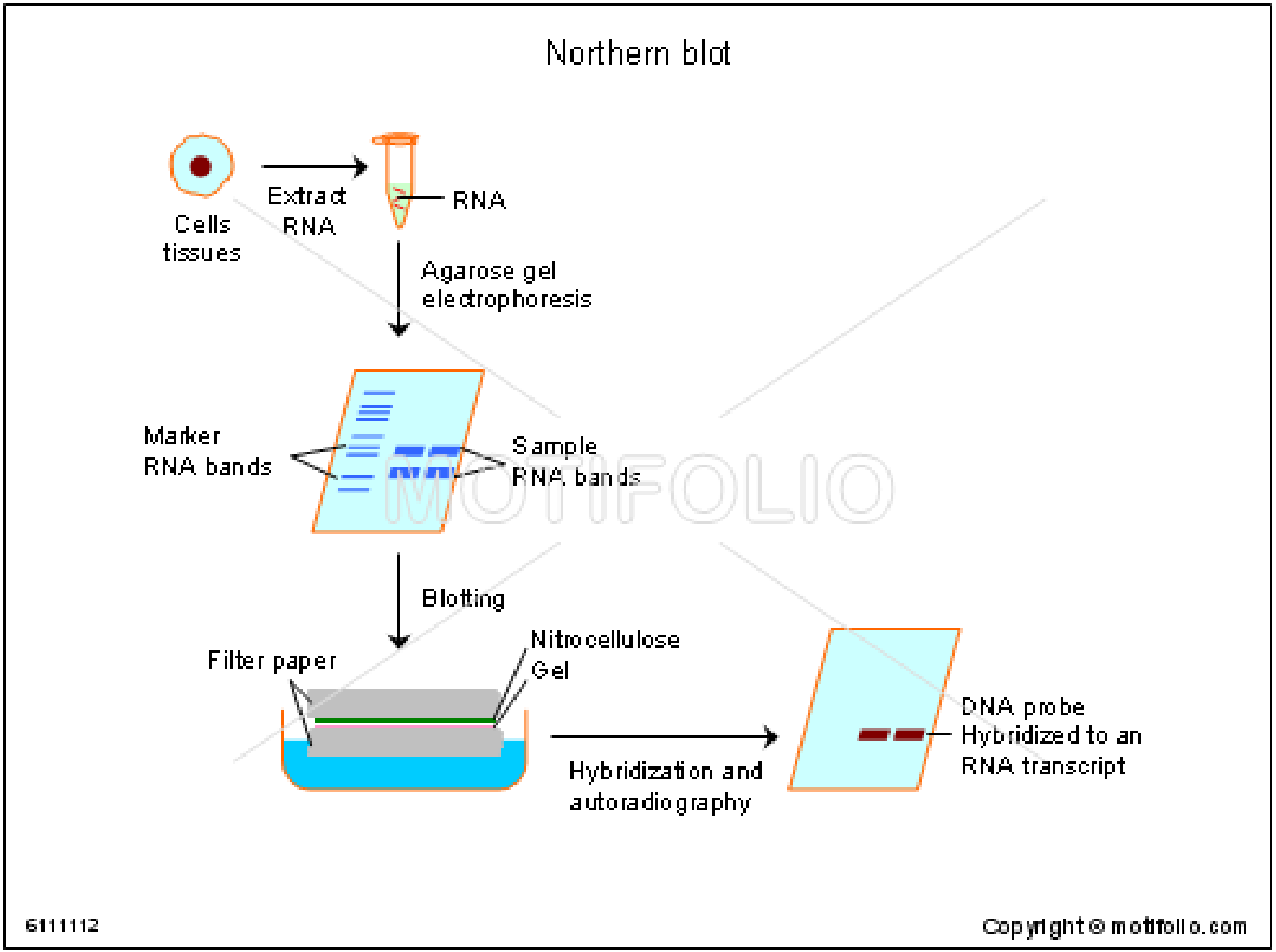
PCR amplify small amounts of DNA from crime scene

Digest DNA and compare pattern of bands – DNA fingerprint

DNA FINGERPRINTS CAN BE USED TO DETERMINE PATERNITY



Northern blot (RNA)



Polymerase-Chain Reaction (PCR)

Nucleotide sequence

```
1       GAATTCCATA TTTAAATGGA TCTTTCGTCA GGTCTGCGTA CCCTTTTAGT TATTATAATT
61      ATTGTGATTA TAATATTTGT GACAAACTCT TATTGTAATC AAAAAAAAGA AAACAAAGAA
121     GTGGATTCTC CCAAATTTGT GATGGATTCT AATTTTTTAA CACATTTGAG TATACACACG
181     TCATTAGAAA AAAAGATTAT AGAATTGGAA GATGAGTTGC AAAAATATAA AAAGTCCAAT
241     AATCTAGACA AGTCAGAAGA ACAAGATATA CCCAGTCCAA AACCAAAATC AAAATCCAAA
301     ACAAAAAAGG CATCTTCTTC TGATGATAGT GTTAAAATAA GTGACGAATC CAAATAAAAA
361     TTTTTCTATA AATGGATTTT TTATATGTAC TAATGATTTT TATTATATTT TTTGCTATTG
421     CATACAATGT GAGTATTTTG CTTAATGAGG ATAAAATTTT CAAAGCAAAA CAGTTTACCA
481     TTGATTACTT CTTGGGGGTC AAGGAGCAAG TTCCAACACG AAAAACCCAA GAAACCACTT
541     TAAATCCTGA AAAGATACGA GCACTCCTAG AAGTTTTACG TAATGACCAA ATCGCGTTTA
```

DNA is double-stranded (dsDNA)

Fig. 1. A double-stranded DNA molecule. The numbers 3' and 5' (pronounced "three prime" and "five prime") indicate the direction of the DNA strand; the lines between the two strands indicate complementary base-pairing.

PCR

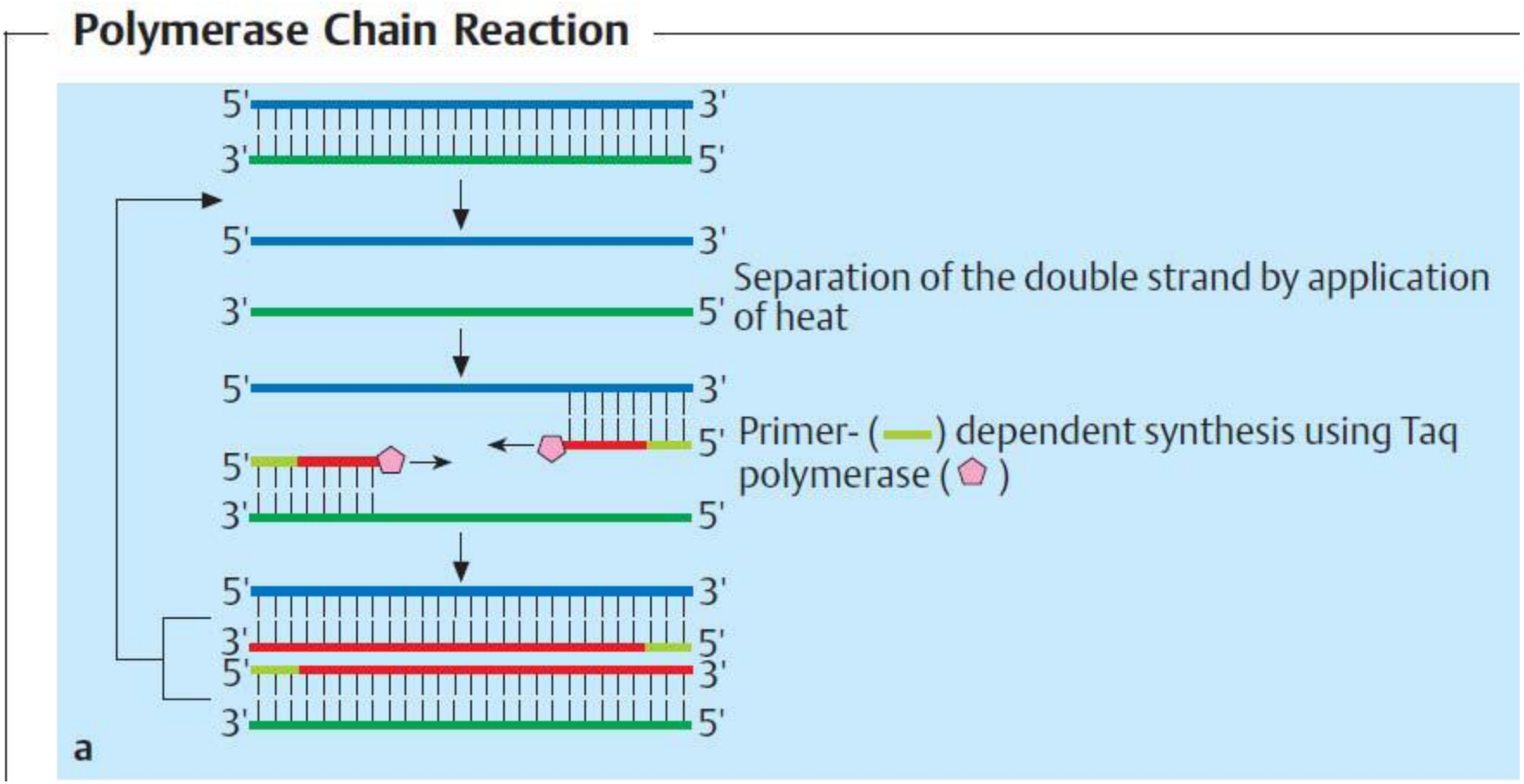
Ingredients:

- DNA (template) – e.g. from patient
- Primers
- DNA polymerase (Taq polymerase)
- Nucleotides (A, C, G, T)

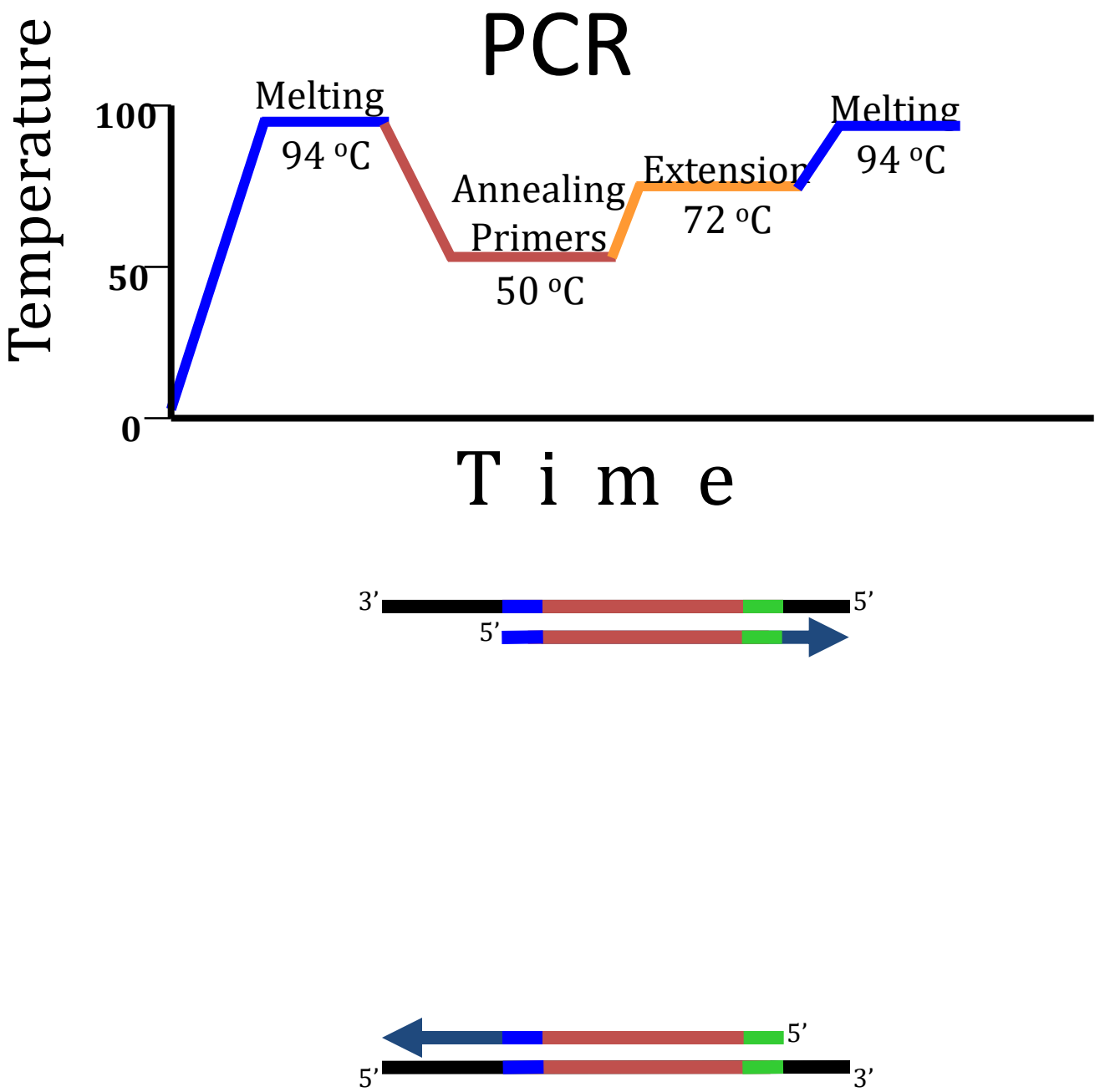
Steps

1. Denaturation (95C)
2. Annealing (55C)
3. Extension (72C)

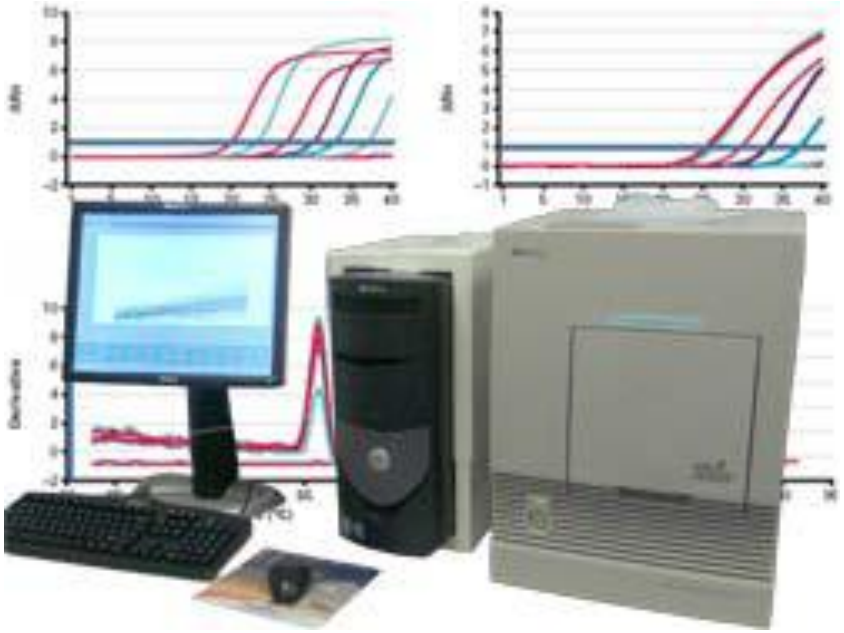
1. Polymerase Chain Reaction



Each cycle doubles the copy number of the target

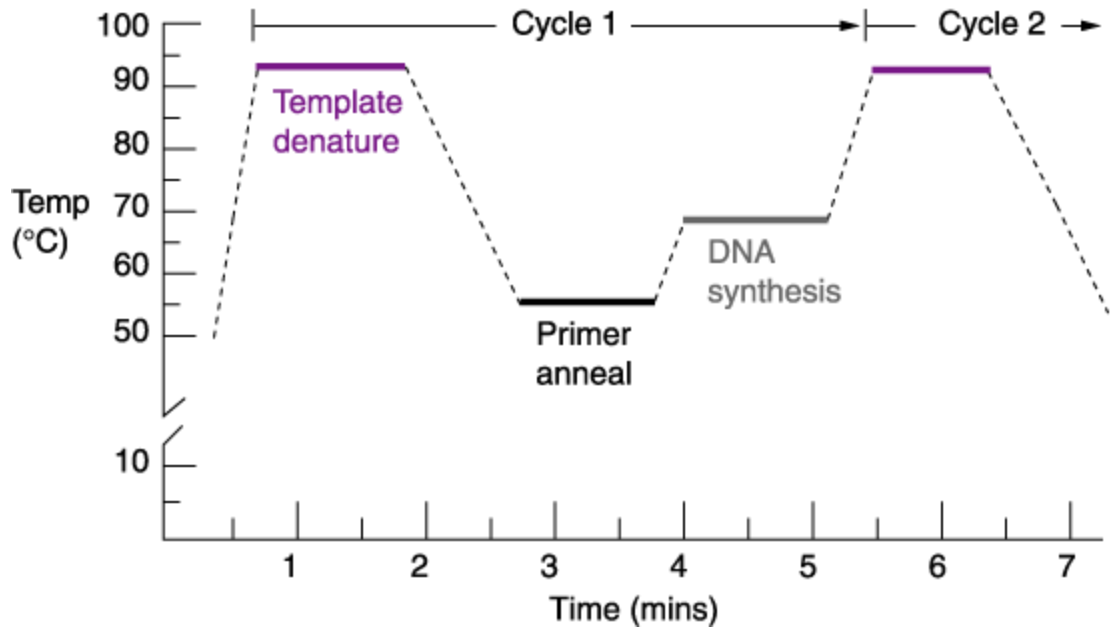

PCR
Temperature
100
50
0
Melting
94 °C
Annealing
Primers
50 °C
Extension
72 °C
Melting
94 °C
T i m e
3’
5’
5’
5’
5’
3’

PCR machine

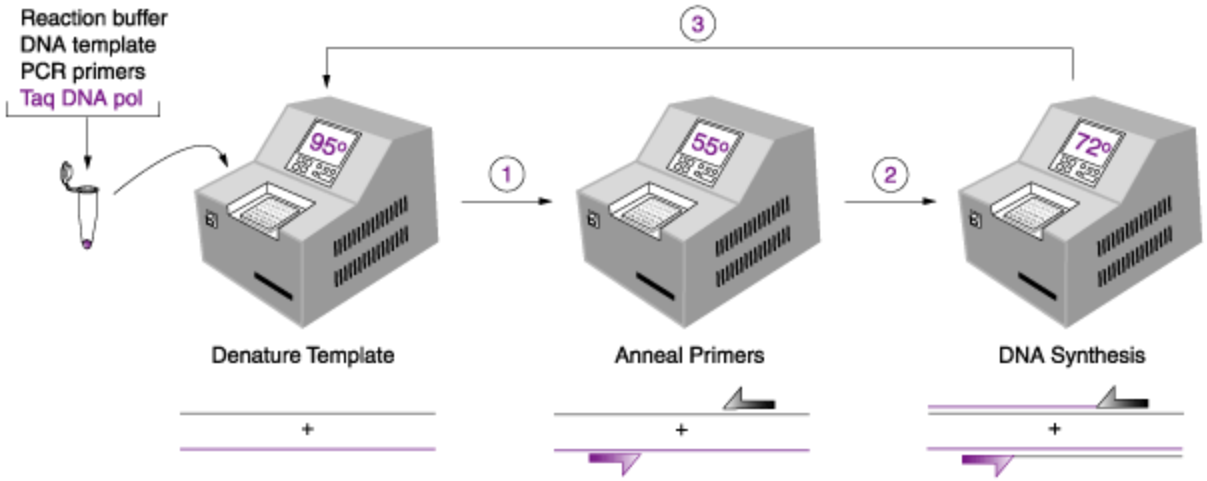


Real-time PCR machine

Temperature profile of PCR



The 3 steps in PCR



PCR amplification

Kary Mullis

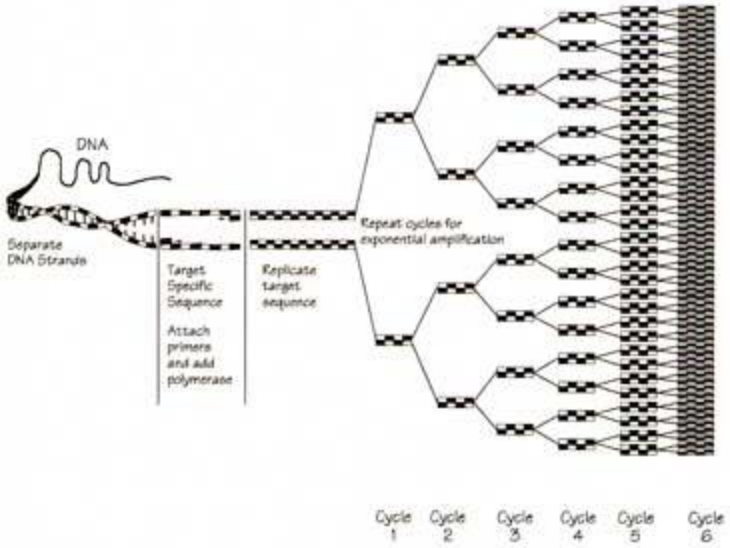


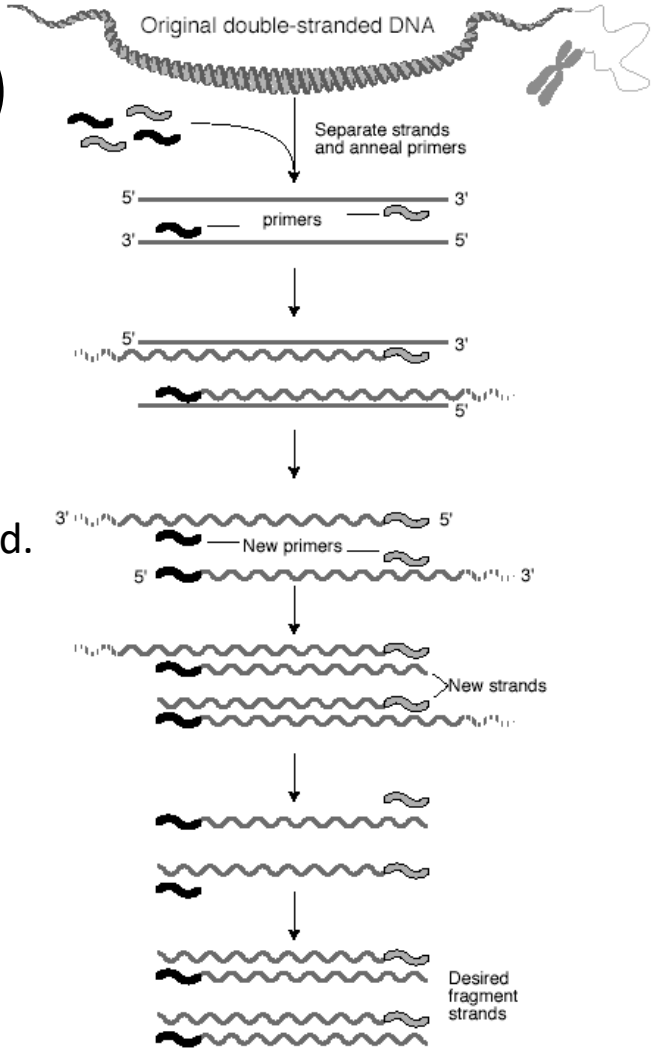
FIGURE 1. Polymerase Chain Reaction

Exponential amplification of PCR

Cycles	Copies
1	2
2	4
4	16
10	1,024
15	32,768
20	1,048,576
25	33,554,432
30	**1,073,741,824**

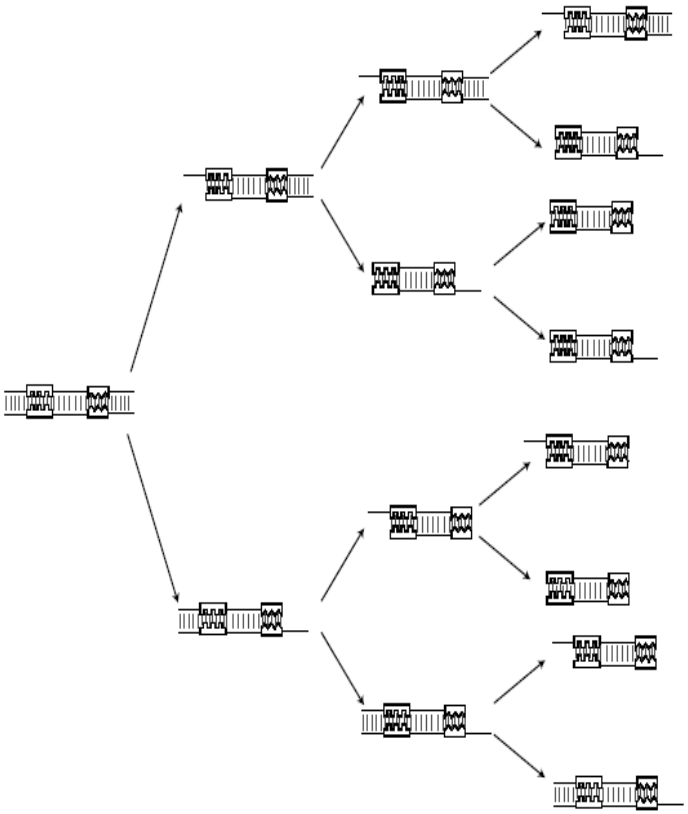
Polymerase Chain Reaction (PCR)

- Polymerase Chain Reaction (PCR)
  - Used to massively replicate DNA sequences.
- How it works:
  - Separate the two strands with low heat
  - Add some base pairs, primer sequences, and DNA Polymerase
    - Creates double stranded DNA from a single strand.
    - Primer sequences create a seed from which double stranded DNA grows.
  - Now you have two copies.
  - Repeat. Amount of DNA grows exponentially.
    - 1→2→4→8→16→32→64→128→256…

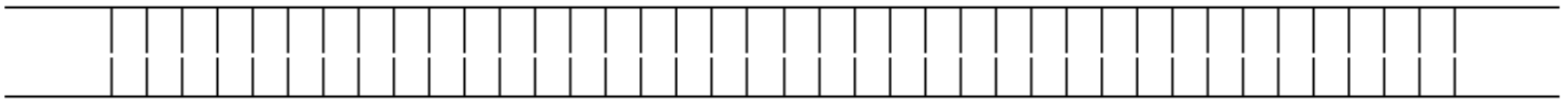


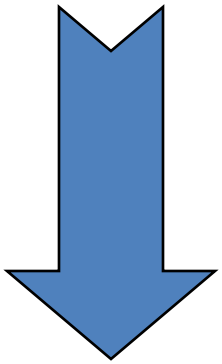
Polymerase Chain Reaction

- **Problem**: Modern instrumentation cannot easily detect single molecules of DNA, making amplification a prerequisite for further analysis
- **Solution**: PCR doubles the number of DNA fragments at every iteration

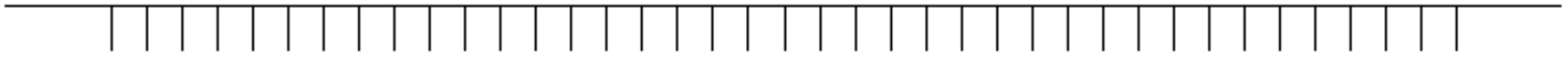


Denaturation



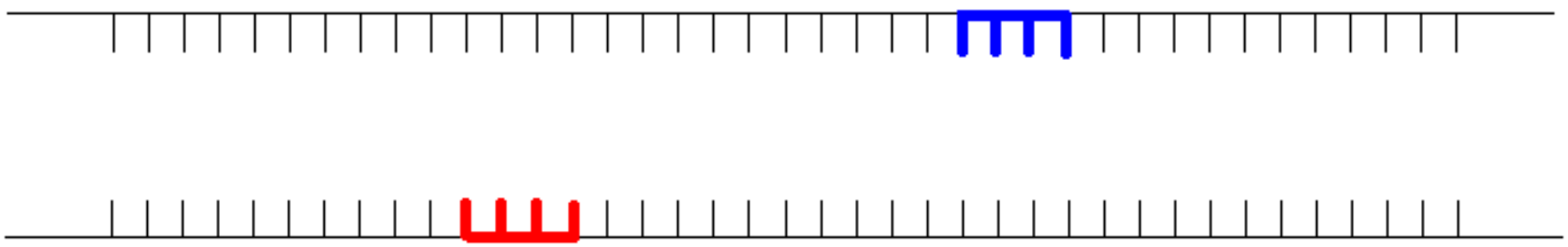


Raise temperature to 94^{o}C to separate the duplex form of DNA into single strands



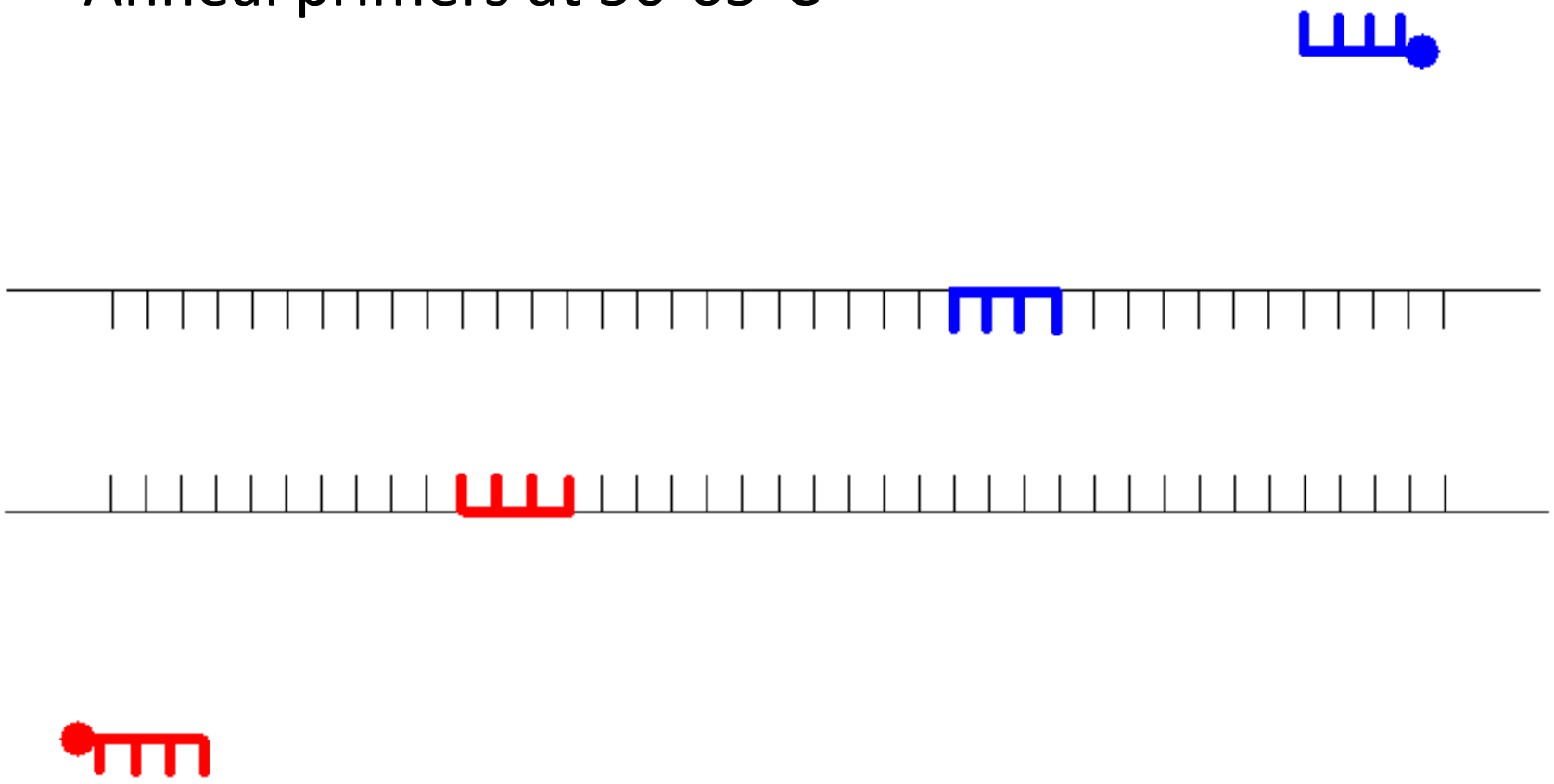
Design primers

- To perform PCR, a 10-20bp sequence on either side of the sequence to be amplified must be known because DNA pol requires a primer to synthesize a new strand of DNA



Annealing

- Anneal primers at 50-65ºC



Annealing

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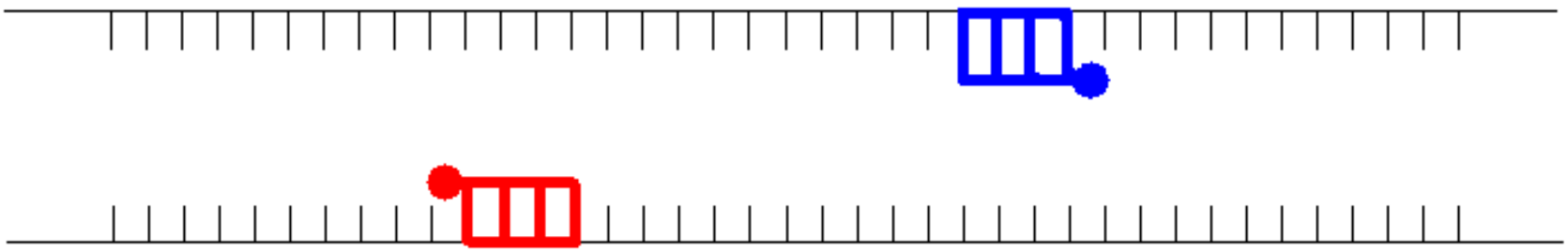
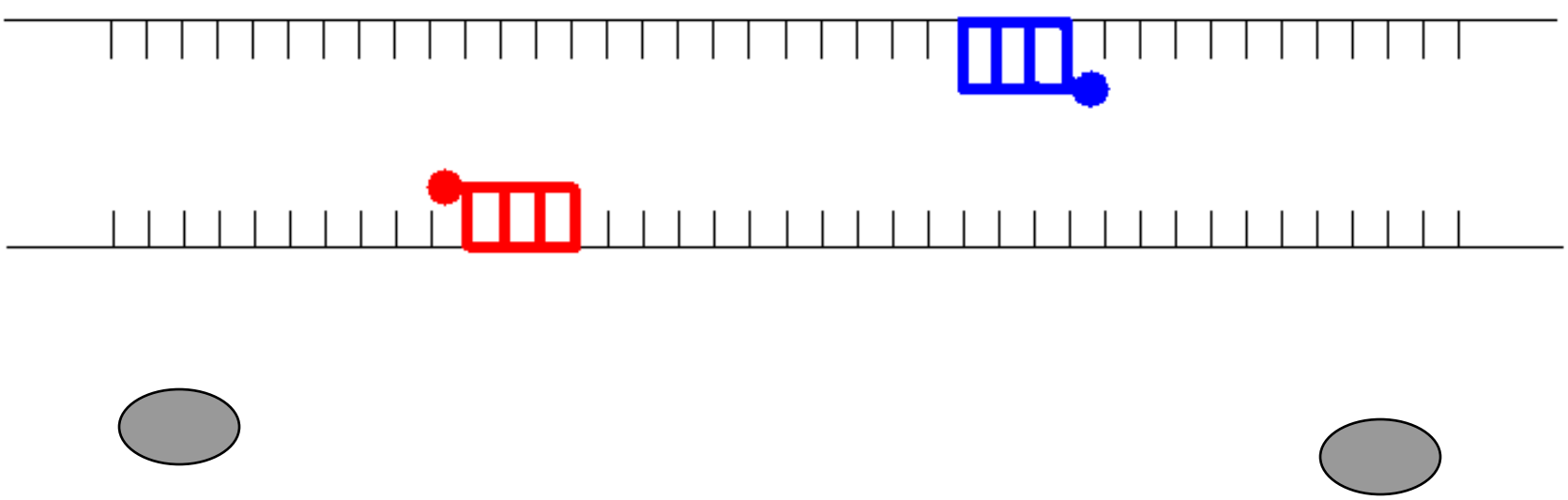


Extension

- Extend primers: raise temp to 72°C, allowing Taq pol to attach at each priming site and extend a new DNA strand



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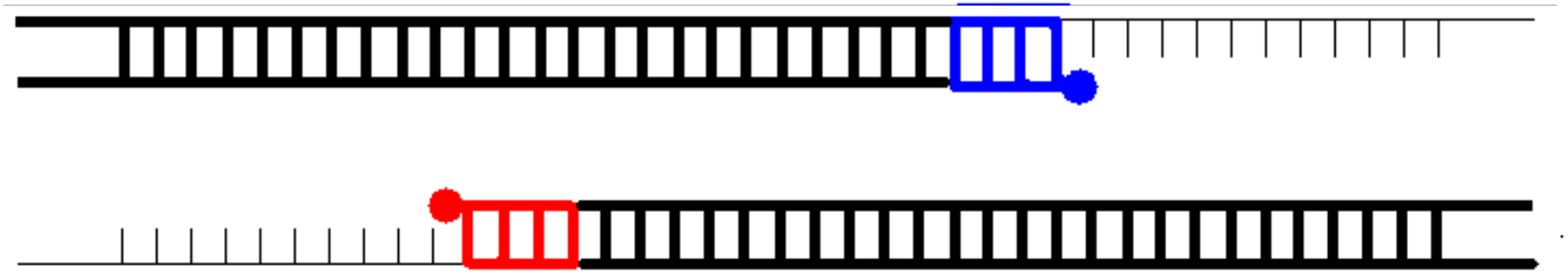
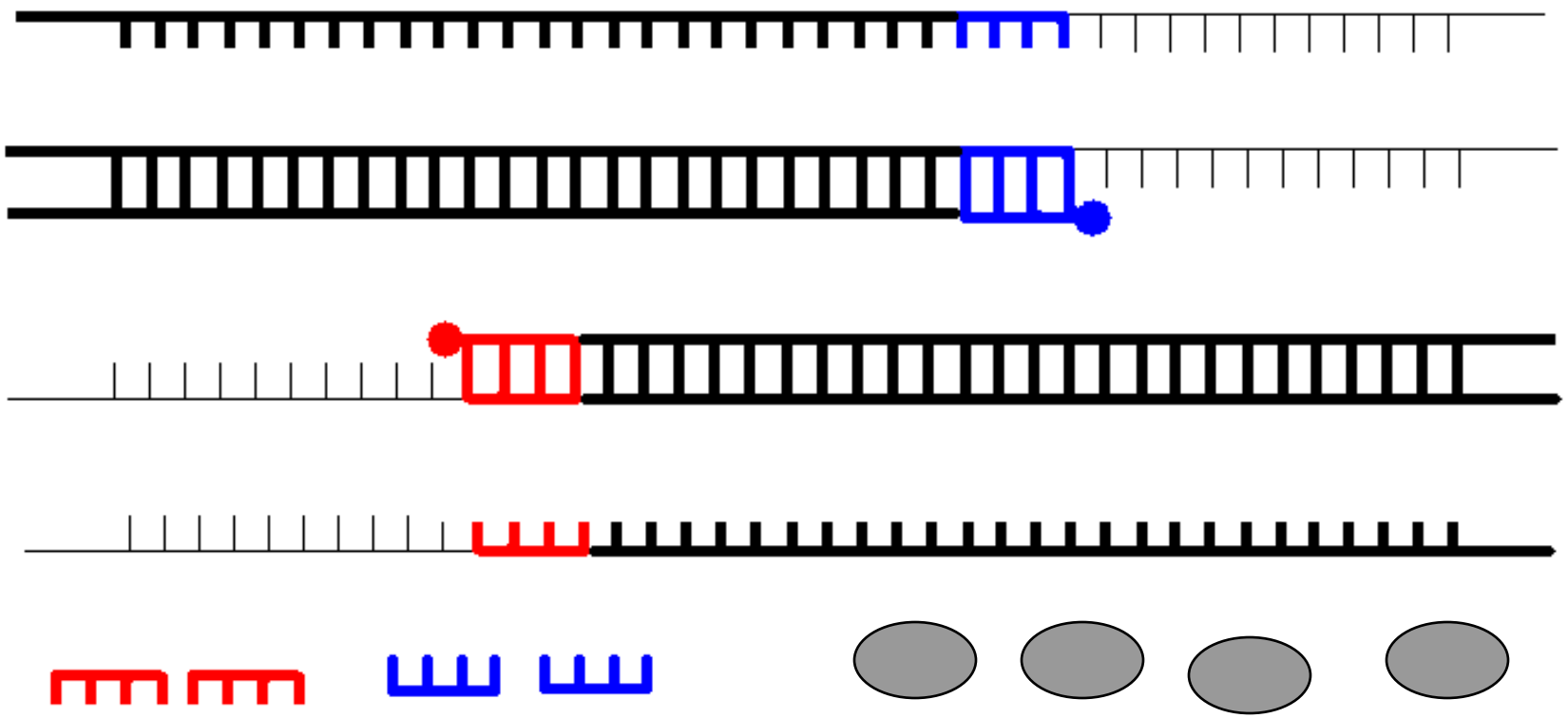
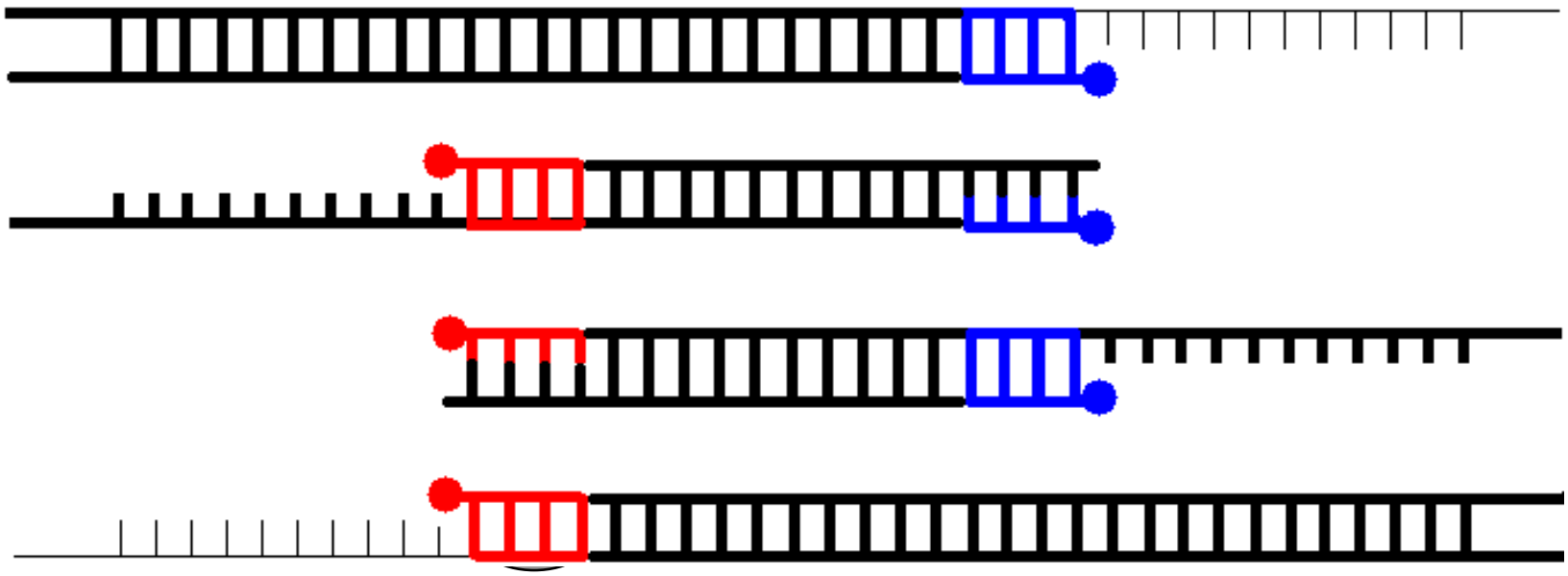


Repeat

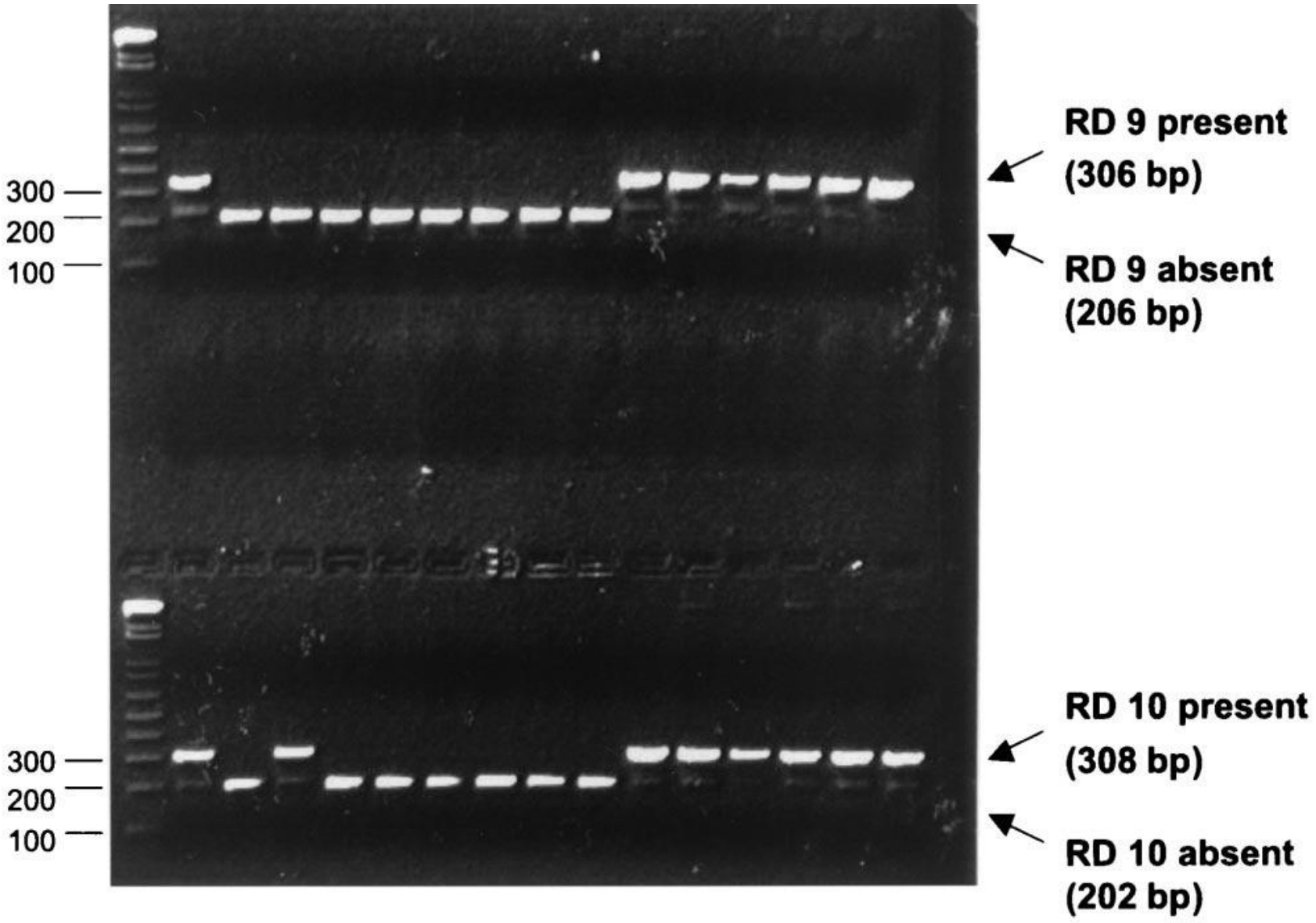
- Repeat the Denature, Anneal, Extension steps at their respective temperatures…



Polymerase Chain Reaction



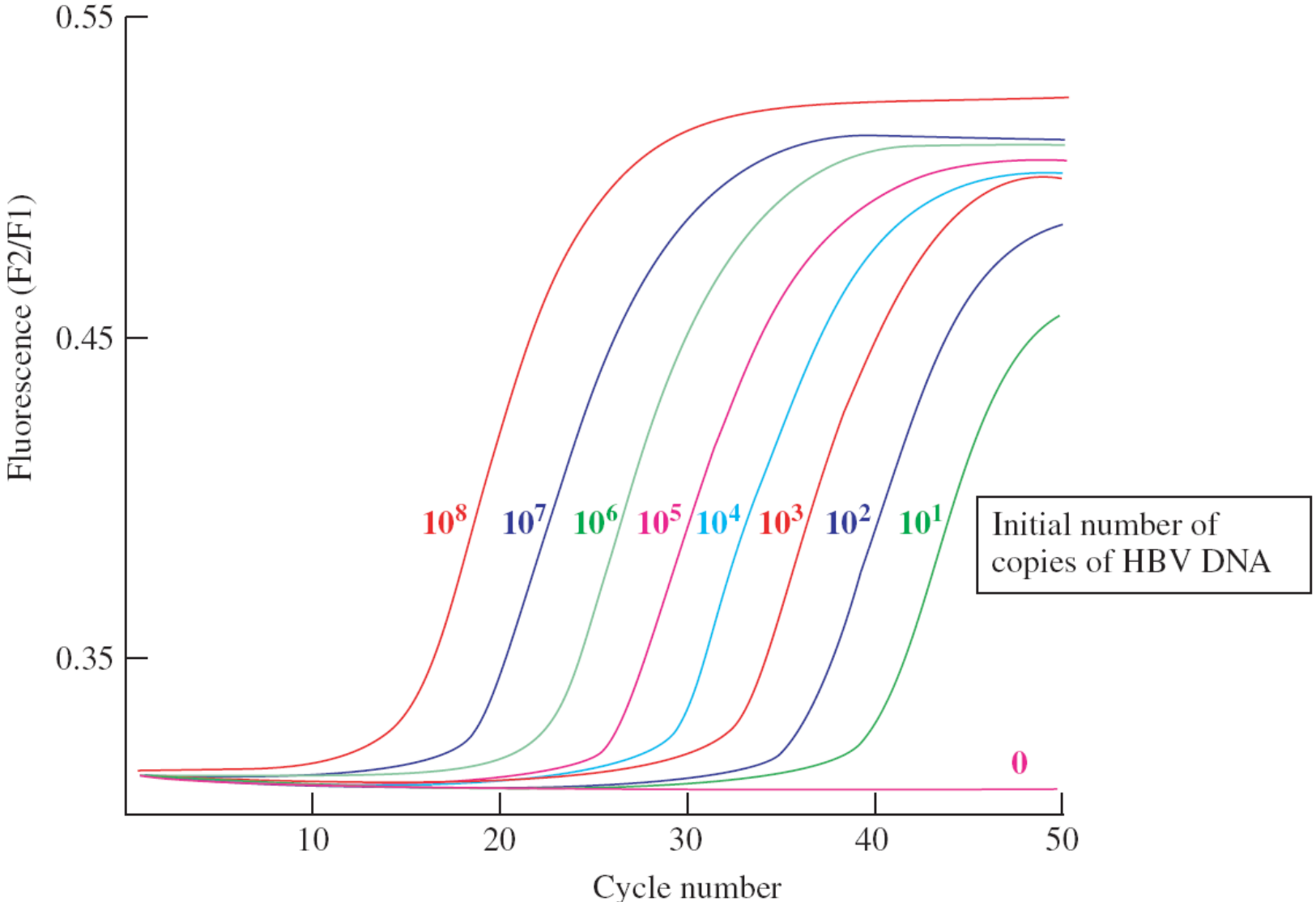
PCR product on agarose gel



PCR - Summary

- Uses dsDNA (template)
- Prior sequence of target a must
- Use sequence to make two primers
- Exponential amplification of target
  - Denaturation (95C)
  - Annealing (variable, 55C)
  - Extension (72C)
- Analyse amplicons
  - agarose gels
  - real-time

Real-time PCR



Polymerase Chain Reaction

Advantages:

- Extremely high sensitivity
- Easy to set up
- Fast turnaround time

Disadvantages

- Extremely liable to contamination
- High degree of operator skill required
- Expensive

PCR - Applications

PCR is used in the following conditions:

- Assays for the presence of infectious agents
- Prenatal diagnosis of genetic diseases
- Direct cloning from genomic DNA or cDNA
- Quantitation of rare DNA by PCR
- Amplification of RNA by PCR (RT-PCR)
- *In vitro* mutagenesis and engineering of DNA
- Genetic fingerprinting of forensic samples
- Direct nucleotide sequencing of genomic DNA or cDNA
- Direct sequencing of PCR products

Cell Culture Techniques

Virus Isolation
(Indirect Examination)

VIRUS CULTIVATION SYSTEMS

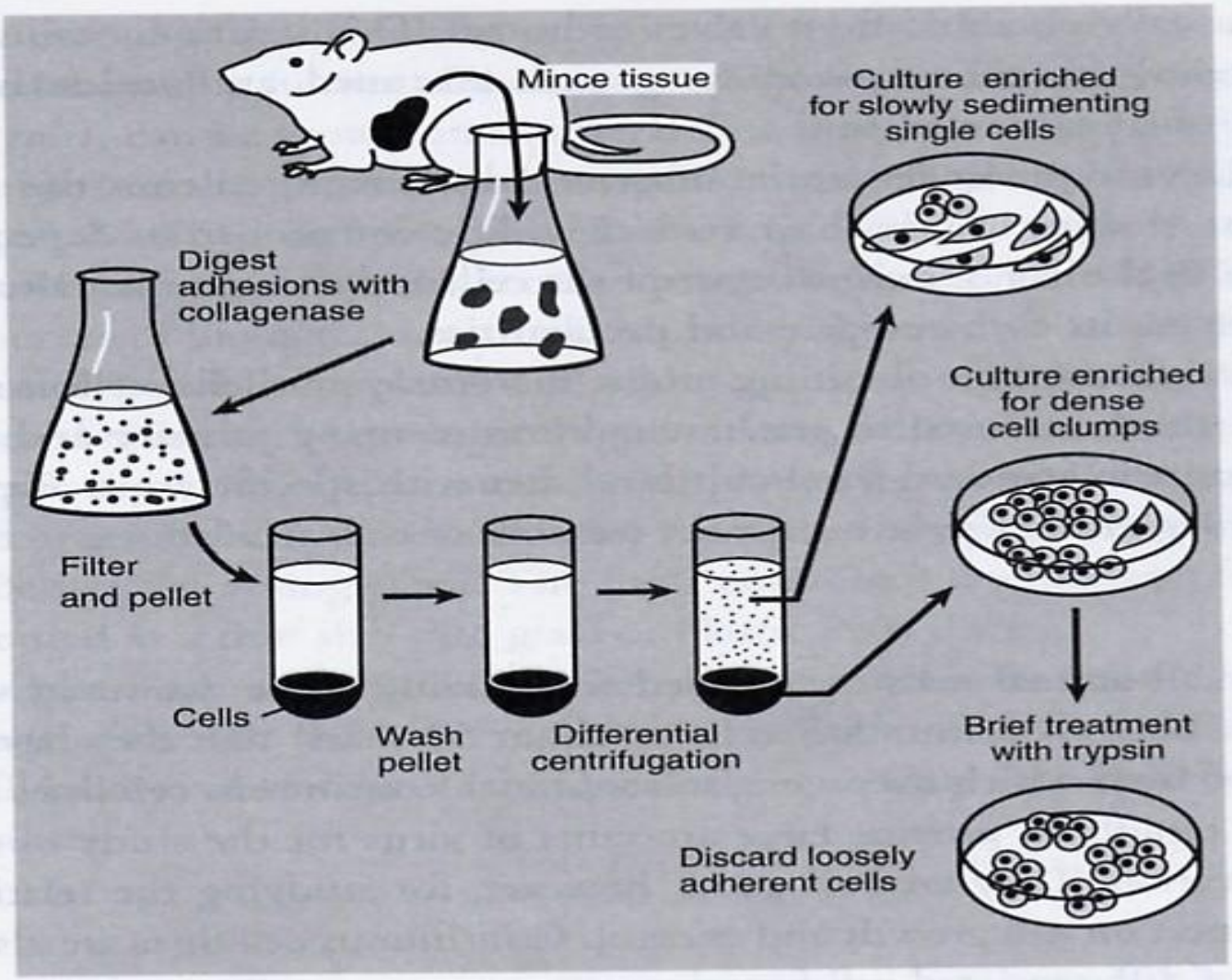
- Tissue culture system
- Embryonated eggs system
- Whole animal systems
  - a) Natural host
  - b) Experimental animals
  - c) Transgenic animals

Virus Isolation – Cell cultures

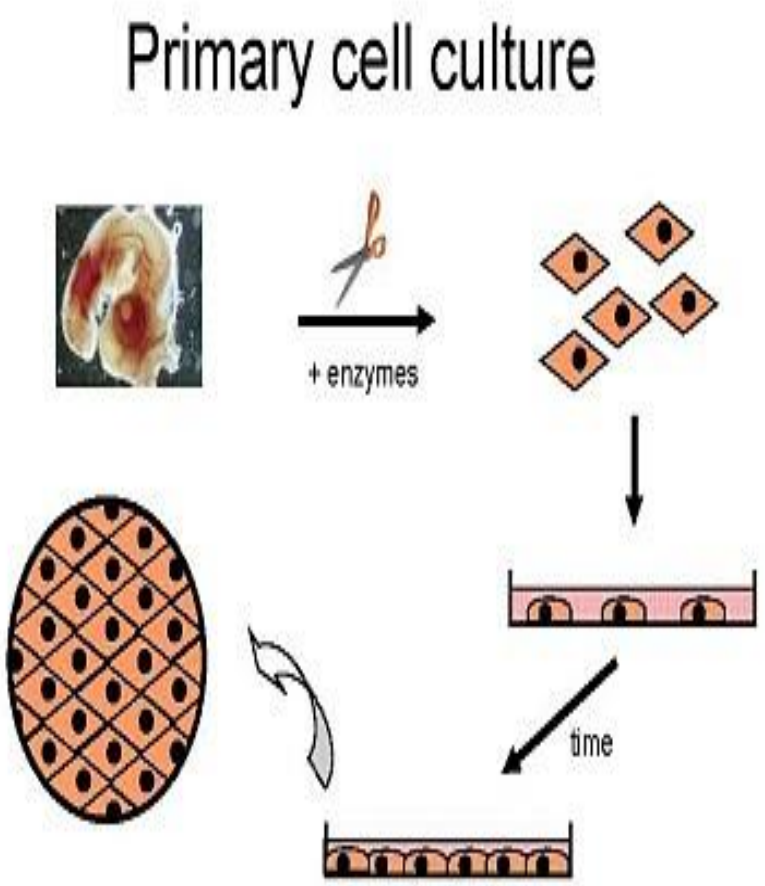
Three types

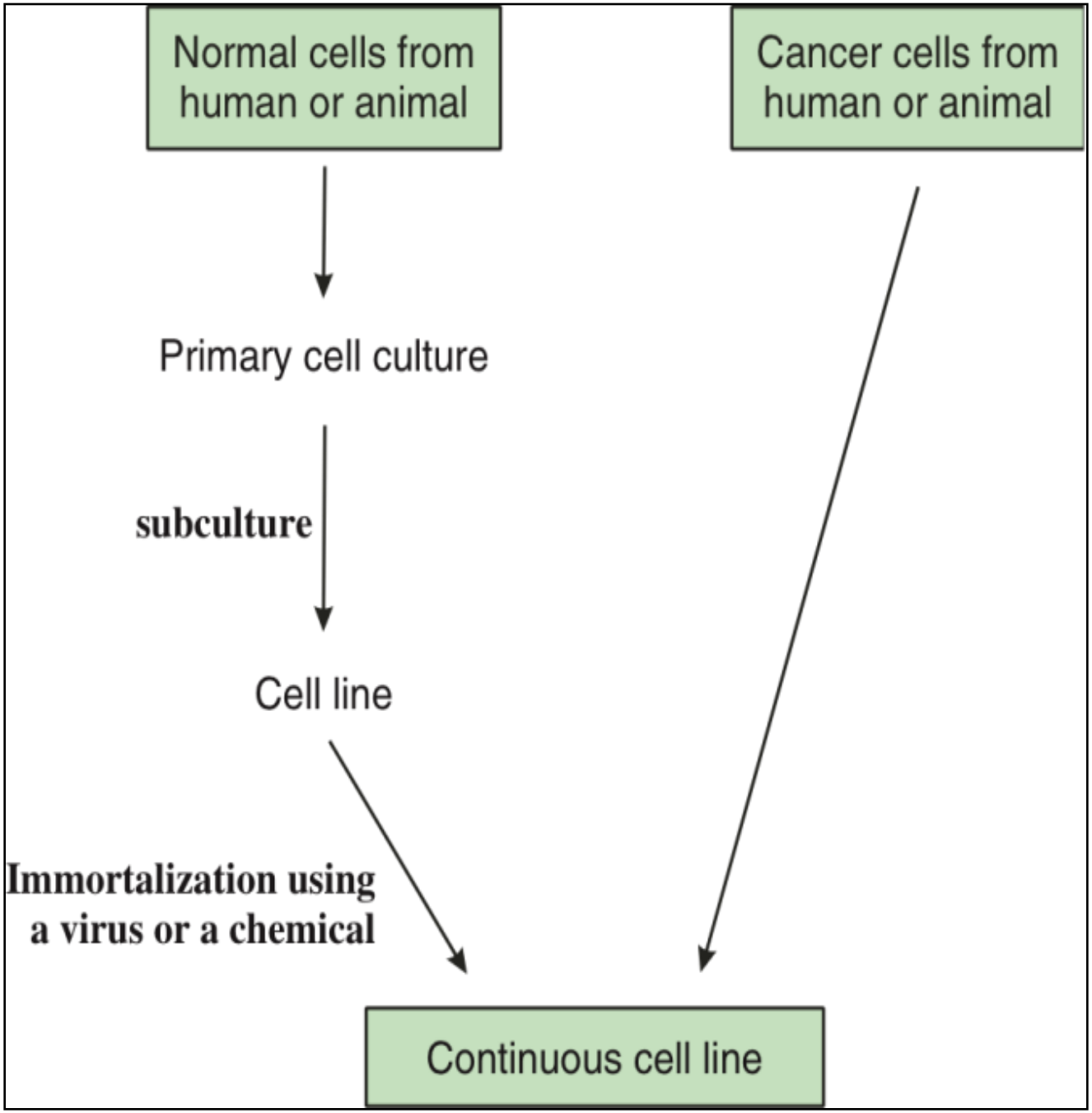
1. Primary cells – e.g. Monkey Kidney
2. Semi-continuous cells – e.g. Human embryonic kidney and skin fibroblasts
3. Continuous cells – e.g. HeLa, Vero

Making a primary cell line



PRIMARY CULTURE




Normal cells from human or animal
Cancer cells from human or animal
Primary cell culture
subculture
Cell line
Immortalization using a virus or a chemical
Continuous cell line

Cultured Cells

1. Primary
   - Heterogeneous –many cell types (several viruses can grow)
   - Closest to animal
   - Technical hassle
   - Expensive
2. Diploid cell strain
   - Relatively homogeneous –fewer cell types
   - Further from animal
   - Technically less hassle
3. Continuous cell line
   - Immortal
   - Most homogeneous
   - Genetically weird –furthest from animal
   - Hassle free
   - Suspension or monolayer
   - Limited # of viruses supported

Laminar Flow Hoods

1. Virologist's facility
2. Laminar vertical flow hoods
   - Contains HEPA filter
   - Removes particles of 0.3µM or higher

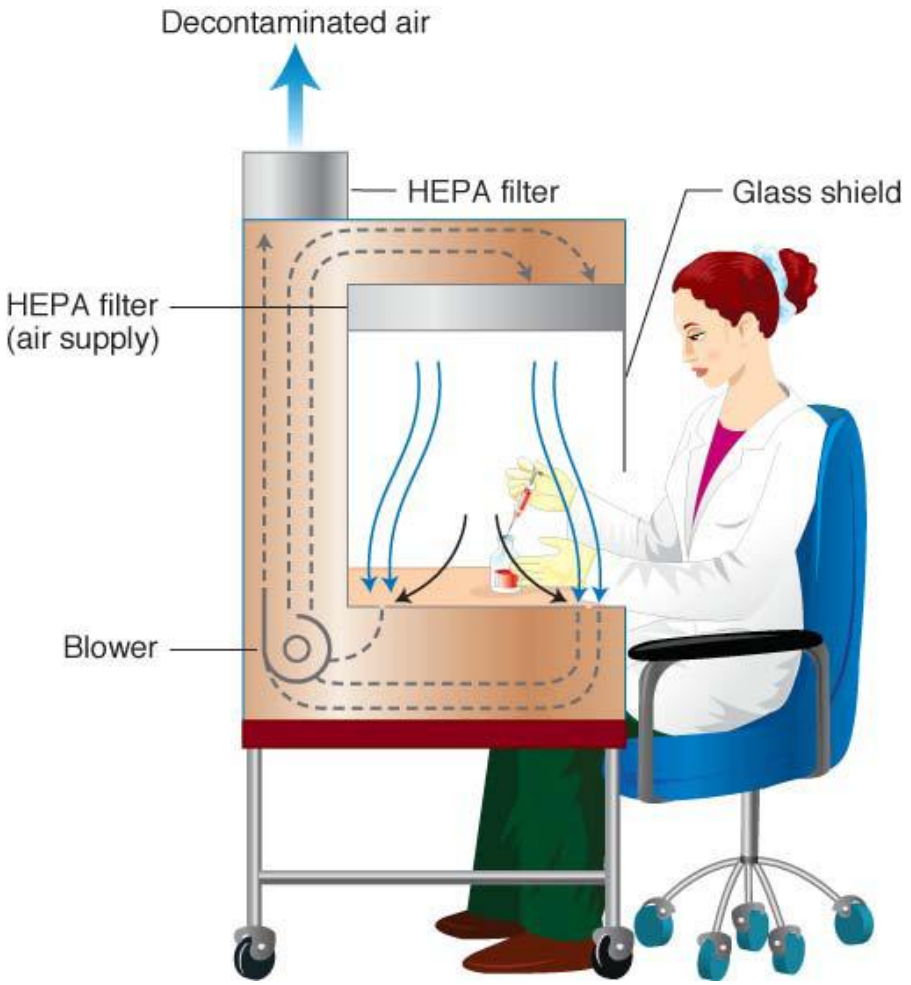


Figure 5-3: Vertical laminar flow hood.

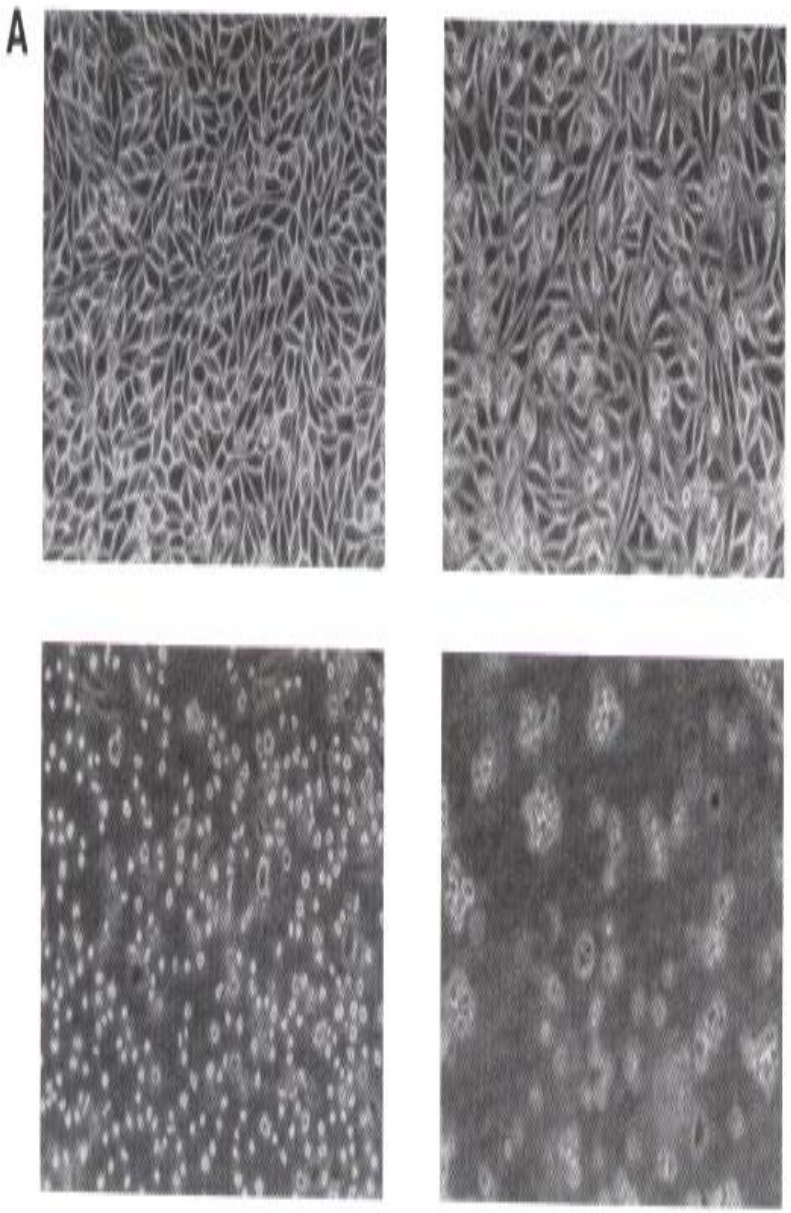
Virus Culture

- Grow a susceptible cell line
- Expose cell line to virus dilution
- Allow virus to infect cells (1 hr) – occasional gentle rocking
- Remove inoculum
- Add culture medium
- Allow infected cells to grow at 37C incubator
- Observe changes in cell morphology (daily)

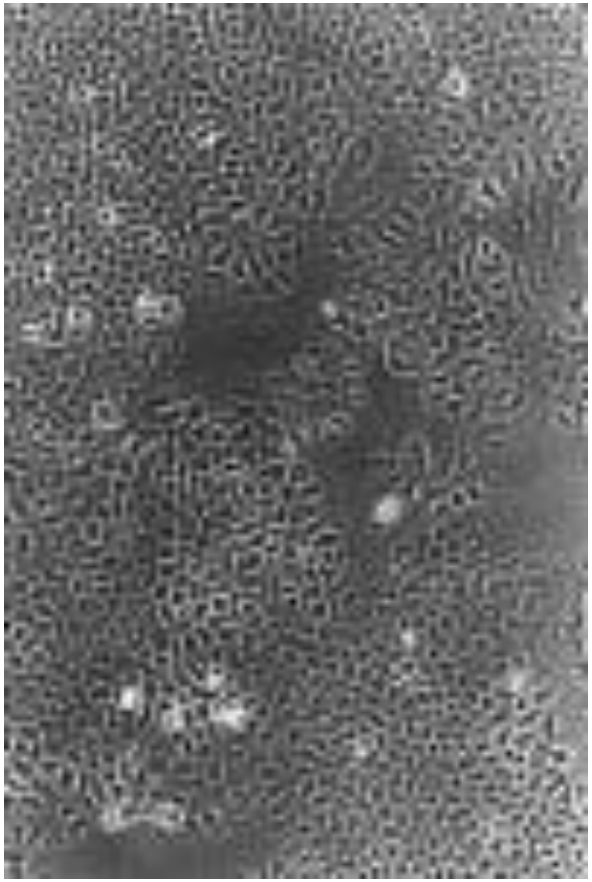
Cytopathic Effects (CPE)

- Visible results of viral infection
- Cell death by
  - Multiplying viruses
  - Inhibition of DNA, RNA or protein synthesis
  - Effects on permeability of membrane
- Observed with inverted light microscopes
  - Rounding/detachment from plastic flask
  - Syncytia/fusionFusion of cells
  - Shrinkage
  - Increased refractiability
  - Aggregation
  - Loss of adherence
  - Cell lysis/death
- Common observations of CPEs
  - Inclusion body formation (Intracellular virus parts - replication or assembly)

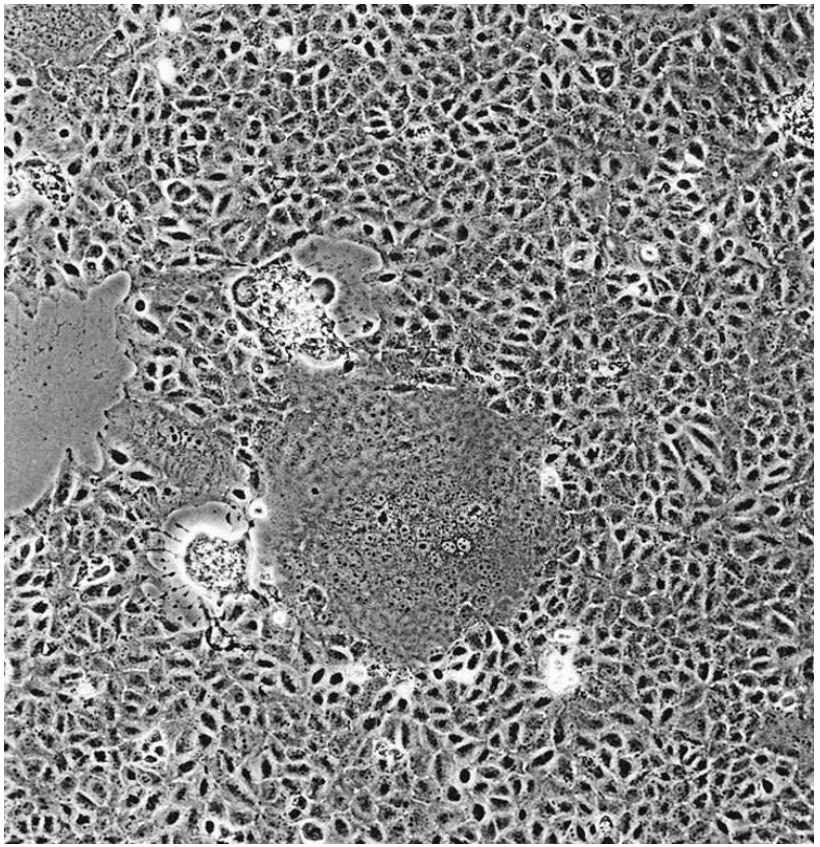
Timecourse of polio infection. Note how cells round up and die



CPE: Measles on human lung carcinoma (A549)



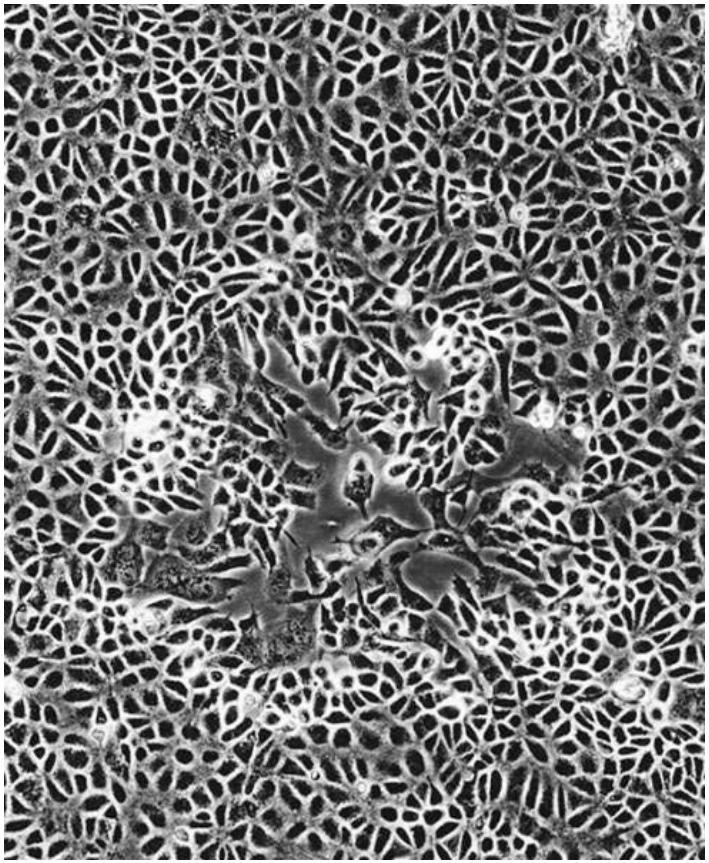
Uninfected cells



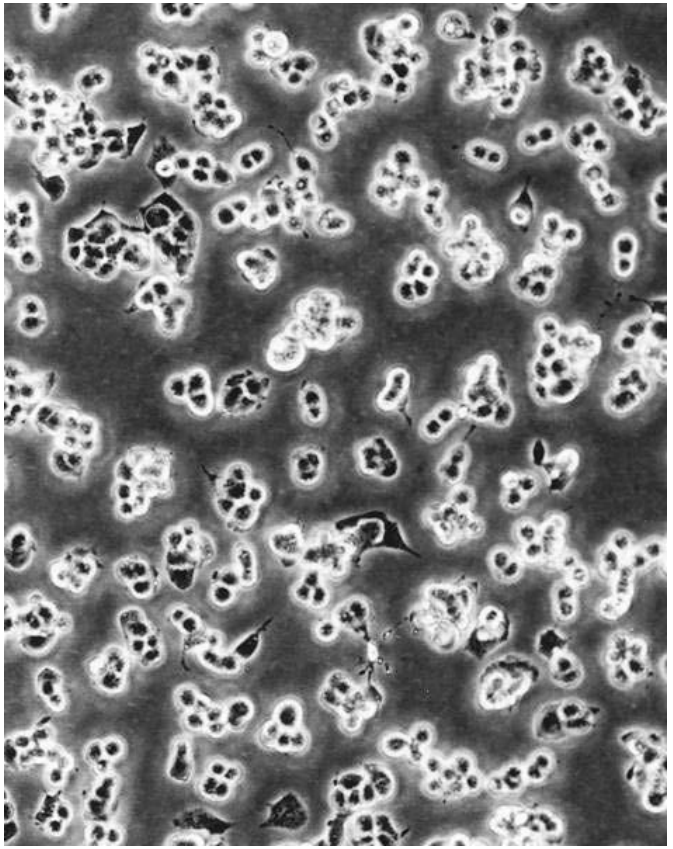
Infected cells

Fields Virology, 4th ed, Knipe & Howley, eds, Lippincott Williams & Wilkins, 2001, Fig. 2-4

CPE: vaccinia on monkey kidney (BSC40)



Low multiplicity of infection (moi)



High moi

Problems with cell culture

1. Long period (up to 4 weeks) required for result.
2. Poor sensitivity (most times)
3. Susceptible to bacterial contamination.
4. Susceptible to toxic substances which may be present in the specimen.
5. Not all viruses grow

Laminar Flow Hoods

1. Virologist's facility
2. Laminar vertical flow hoods
   - Contains HEPA filter
   - Removes particles of 0.3µM or higher

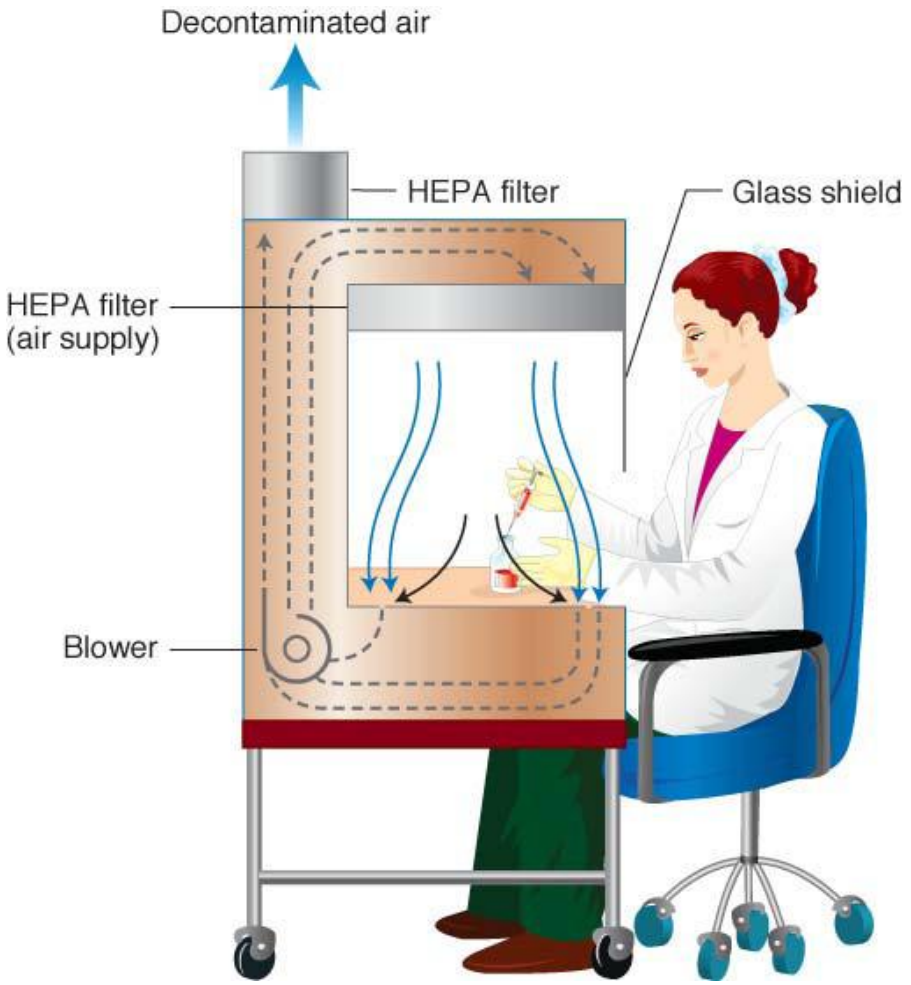


Figure 5-3: Vertical laminar flow hood.

Biosafety Levels 1, 2, 3, & 4

BSL-1 Laboratory

- BSL–1

  - Lowest
  - Low risk microbes (little or no threat of infection
  - E.g. nonopathogenic strain of E. Coli
  - Research taking place on benches (no special contaminant equipment)
  - No requirement for isolation of lab from surrounding facilities

- Only standard microbial practices required

a) Mechanical pipetting only (no mouth pipetting allowed)
b) Safe sharps handling
c) Avoidance of splashes or aerosols
d) Daily decontamination of all work surfaces when work is complete
e) Hand washing
f) Prohibition of food, drink and smoking materials in lab setting
g) Personal protective equipment, such as; eye protection, gloves and a lab coat or gown
h) Biohazard signs

BSL-2 Lab

- Agents associated with human disease (i.e. pathogenic or infectious organisms) -> pose moderate health hazard
- E.g. HIV, *Staphylococcus aureus (staph infections).*
- Maintain same std microbial practices as BSL-1 but also includes measures

- Appropriate personal protective equipment (PPE)
- Working within biological safety cabinet (BSC) – aerosol/splashes procedures
- Decontamination (autoclave, proper disposal)
- Self-closing lockable doors
- Sink & eyewash station
- Biohazard warning signs
- Restricted entry (non-lab workers or those with increased risk of contamination)

BSL-3 Lab

- Microbes that are either indigenous or exotic, and can cause serious or potentially lethal disease through inhalation.
- E.g. YFV, WNV, and the bacteria that causes tuberculosis.
- Lab personnel under medical surveillance
- In addition to BSL-2, requires;

- Std PPE must be worn (respirators might be required)
- Solid-front wraparound gowns, scrub suits or coveralls are often required
- Appropriate BSC
- Hands-free sink and eyewash are available near the exit
- Sustained directional airflow to draw air into the laboratory from clean areas towards potentially contaminated areas (Exhaust air cannot be re-circulated)
- Self closing set of locking doors with access away from general building corridors
- Access to a BSL-3 laboratory is restricted and controlled at all times.

BSL-4 Lab

- Rare
- Highest level
- Highly dangerous microbes (infections frequently fatal, often no treatment or vaccines)
- E.g. Ebola, Marburg viruses
- In addition to BSL-3:

a. Personnel are required to change clothing before entering, shower upon exiting
b. Decontamination of all materials before exiting
c. Personnel must wear appropriate PPE from prior BSL levels, as well as a full body, air-supplied, positive pressure suit
d. A Class III biological safety cabinet
e. Extremely isolated—often located in a separate building or in an isolated and restricted zone of the building.
f. Dedicated supply and exhaust air, as well as vacuum lines and decontamination systems.

Biosafety Level	BSL-1	BSL-2	BSL-3	BSL-4
Description	· No Containment · Defined organisms · Unlikely to cause disease	· Containment · Moderate Risk · Disease of varying severity	· High Containment · Aerosol Transmission · Serious/Potentially lethal disease	· Max Containment · "Exotic," High-Risk Agents · Life-threatening disease
Sample Organisms	E.Coli	Influenza, HIV, Lyme Disease	Tuberculosis	Ebola Virus
Pathogen Type	Agents that present minimal potential hazard to personnel & the environment.	Agents associated with human disease & pose moderate hazards to personnel & the environment.	Indigenous or exotic agents, agents that present a potential for aerosol trans-mission, & agents causing serious or potentially lethal disease.	Dangerous & exotic agents that pose a high risk of aerosol-transmitted lab-oratory infections & life-threatening disease.
Autoclave Requirements	None	None	Pass-thru autoclave with Bioseal required in laboratory room.	Pass-thru autoclave with Bioseal required in laboratory room.

Quantitative Assays

Plaque assays

- Cell culture-based
- Lytic viruses only
- Steps
  - -Serial dilution of virion-containing solution
  - -Create tissue culture plates
  - -Spread diluted virus
  - -Overlay with agar—prevents diffusion
  - -Count number of plaques
  - -Each plaque represents 1 PFU (Plaque Forming Unit)

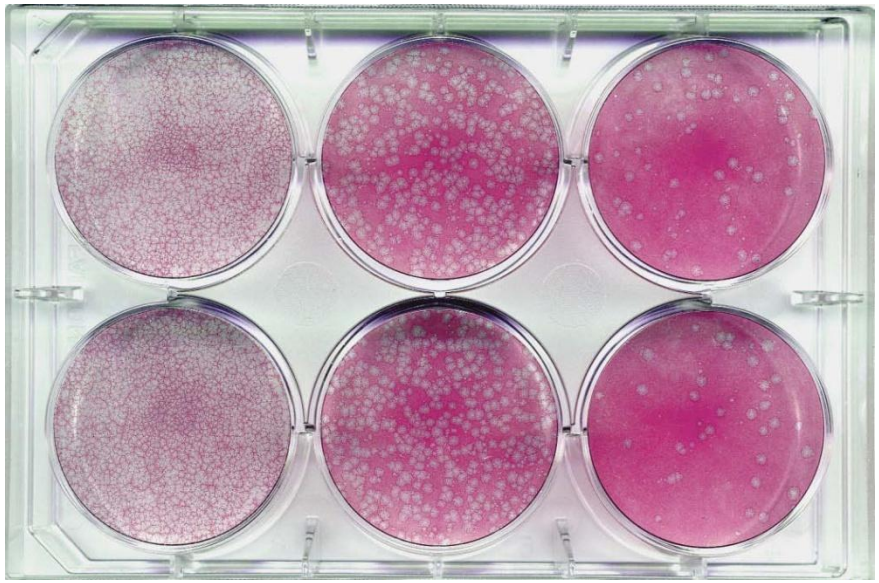
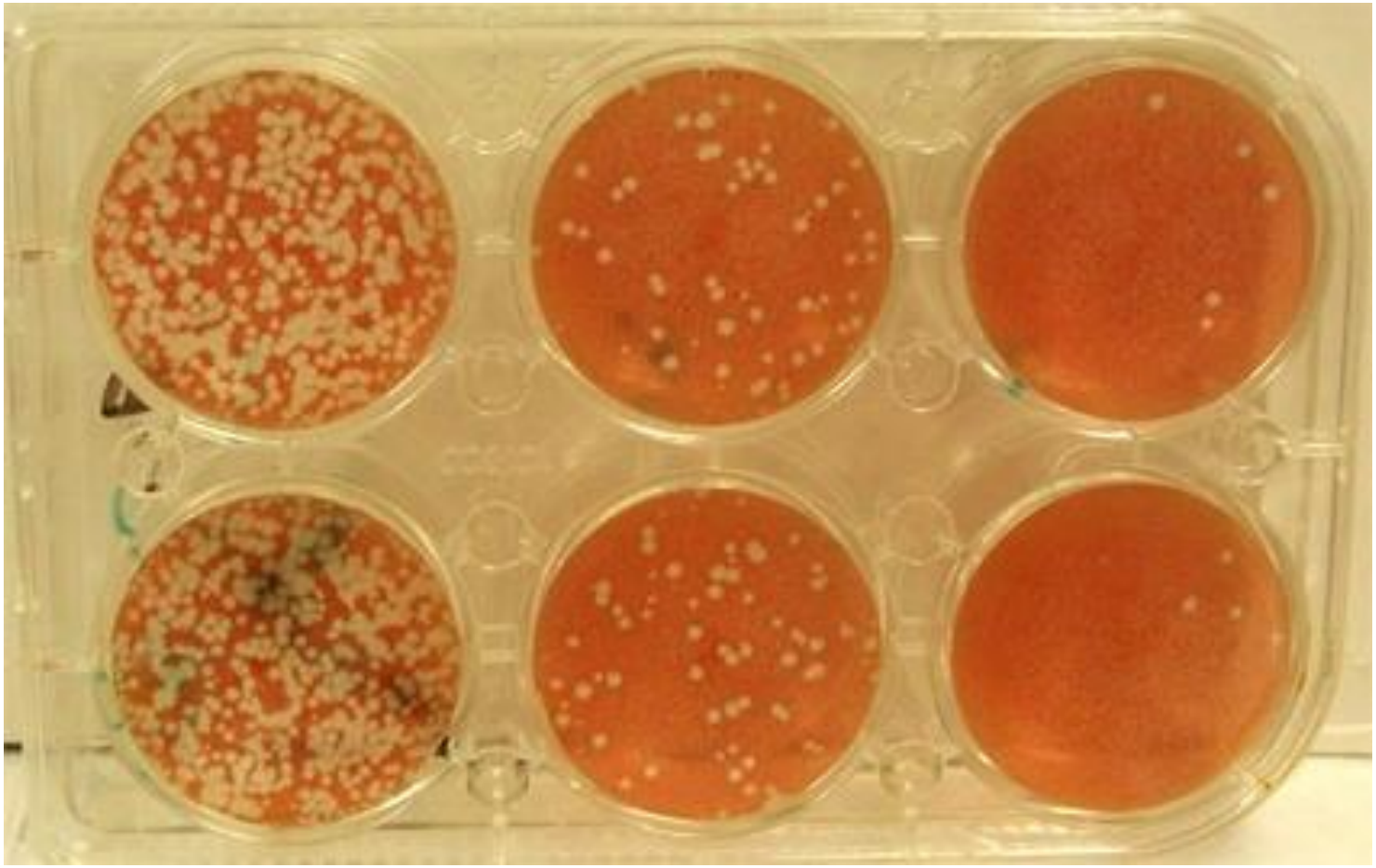
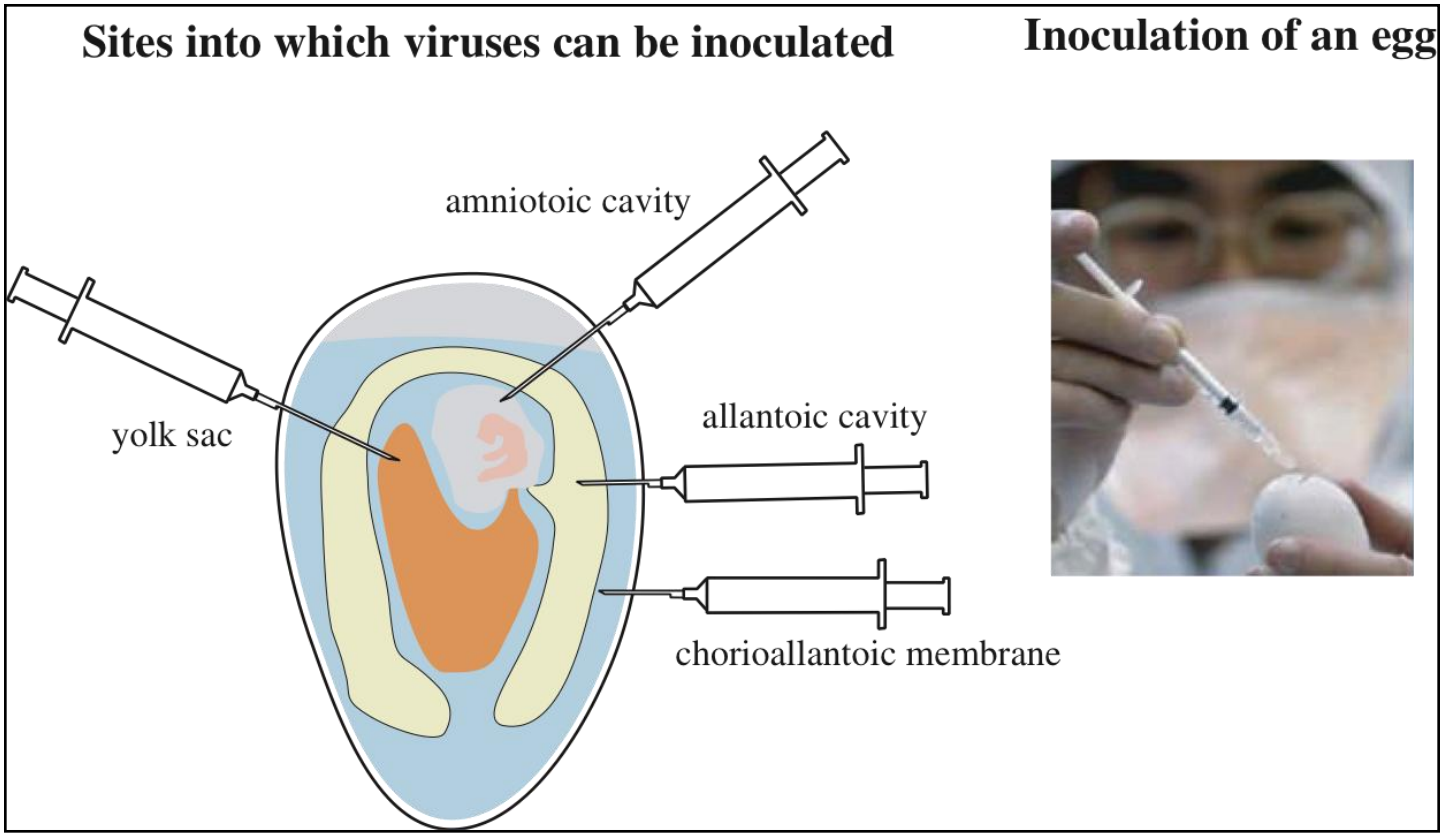


Figure 5.8: Plaque assays used to quantitate a viral stock.

Plaque Assay (e.g. vaccinia virus)
10-fold dilution (duplicate)



Embryonated Eggs

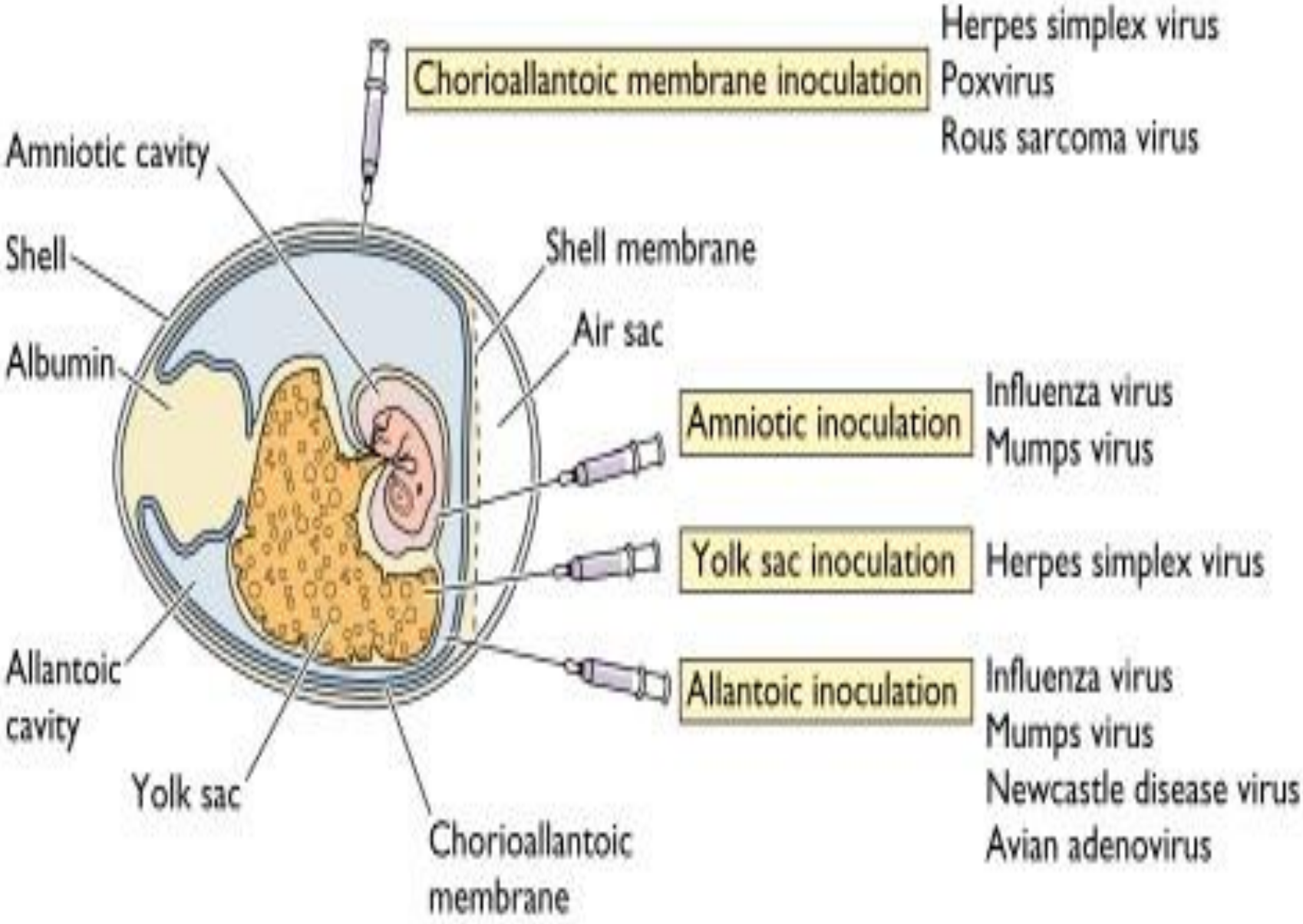


Generates large quantity of virus
Used for vaccine production

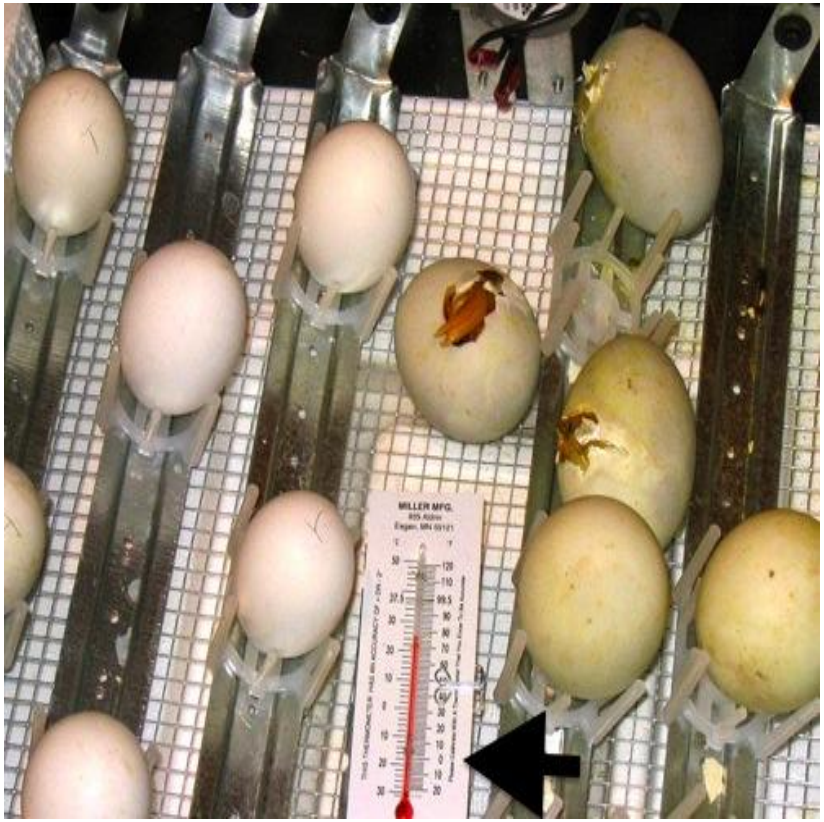
DRILL THE HOLE – Innoculate virus

– Engrotool – mode of drilling pinpoint whole – Embryonating chicken egg.

rborandum disc – Blood vessel free area
hicken eggs.


Chorioallantoic membrane inoculation
Herpes simplex virus
Poxvirus
Rous sarcoma virus
Amniotic cavity
Shell
Albumin
Shell membrane
Air sac
Amniotic inoculation
Influenza virus
Mumps virus
Yolk sac inoculation
Herpes simplex virus
Allantoic cavity
Allantoic inoculation
Influenza virus
Mumps virus
Newcastle disease virus
Avian adenovirus
Yolk sac
Chorioallantoic membrane

EGGS INCUBATE AT 36C FOR 2-3 DAYS



Embryonated eggs

Signs of viral growth include:

1. Death of the embryo,
2. Defects in embryonic development,
3. Localized areas of damage in the membranes
   – Result: discrete, opaque spots called pocks
1. Embryonic fluid & tissue - exam with EM

Virus Isolation-animal inoculation

- Choice of animal and inoculation pathways
- Mouse, rat, rabbit, monkey, etc