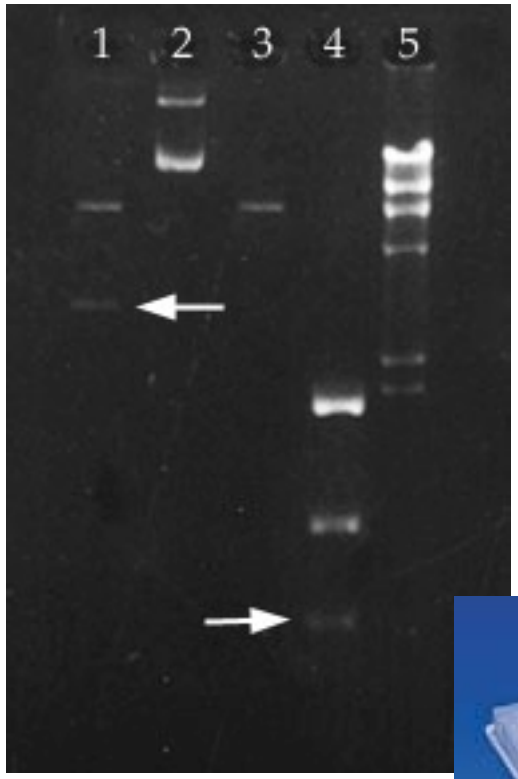
Basic lab techniques

Sandrine Dudoit

Bioconductor short course

Summer 2002

Lab techniques



Basic lab techniques for nucleic acids

- Hybridization.
- Cut: restriction enzymes.
- Amplify: PCR.
- Sort: gel electrophoresis.
- Probe: blots and microarrays.

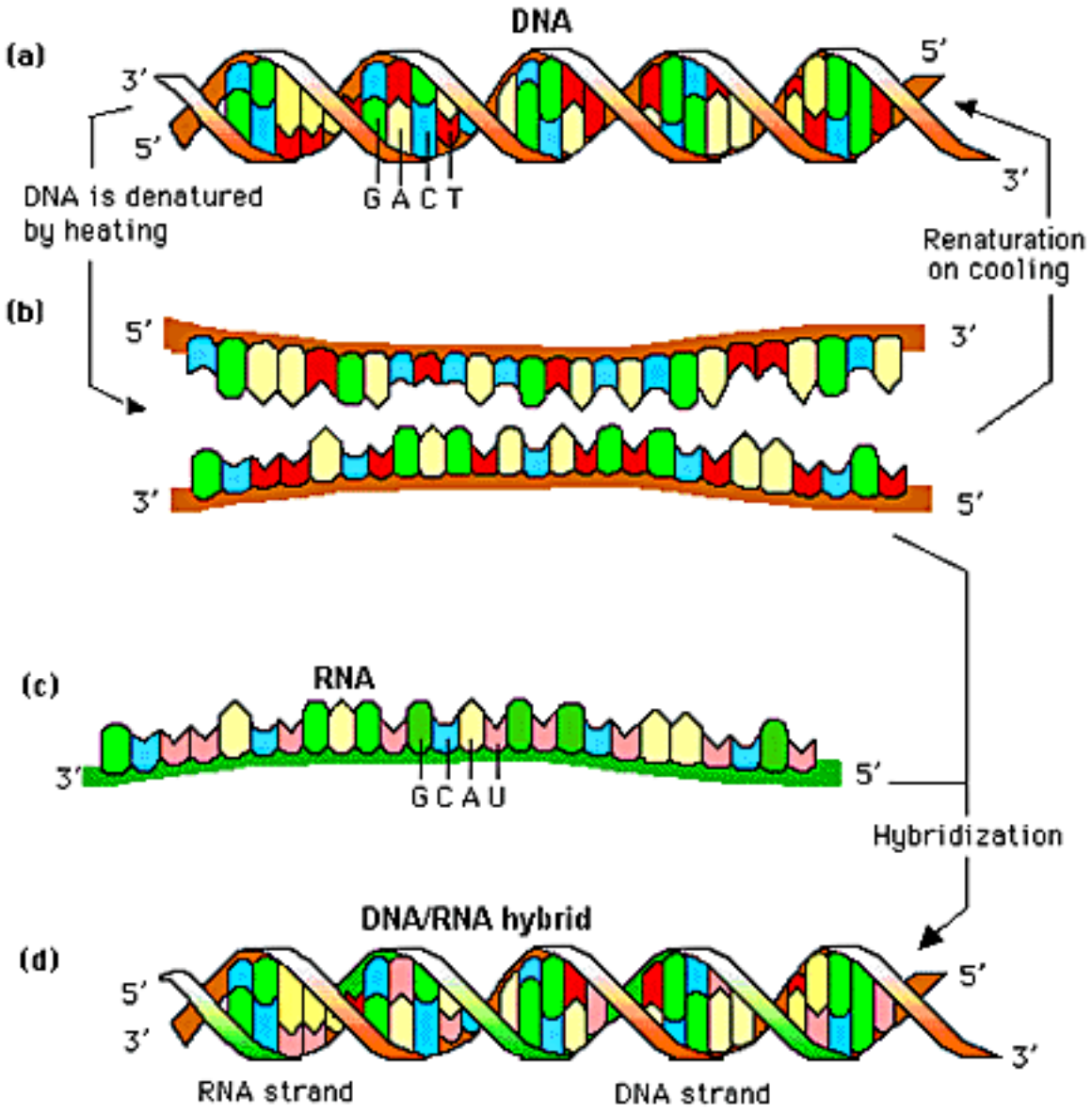
Why?

- Why cut, amplify, sort, probe?
  - Sequencing;
  - Genotyping (cf. genetic mapping, forensics);
  - Measuring gene expression;
  - Etc.

Hybridization

- **Hybridization** refers to the **annealing** of two nucleic acid strands following the base-pairing rules.

- Nucleic acid strands in a duplex can be separated, or **denatured**, by heating to destroy the hydrogen bonds.



Nucleic Acid Hybridization

Restriction enzymes

- DNA **restriction enzymes** or **restriction endonucleases** recognize short, specific sequences of DNA bases and make breaks in the sugar-phosphate backbone of the DNA.
- The recognition sites are usually **palindromes**, .i.e, the sequence in one strand is the same as that in the other strand, read in the reverse direction.
- Some restriction enzymes make staggered cuts in the opposite strand, creating complementary, single-stranded ends or **sticky ends**; others cut across both strands creating DNA fragments with **blunt ends**.

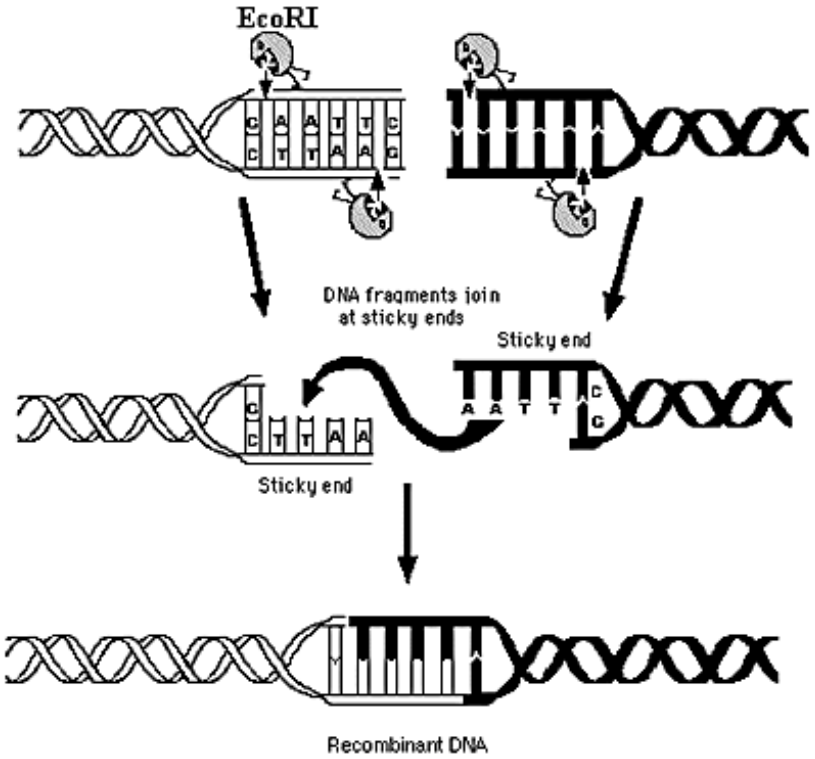
EcoRI

- Restriction enzymes allow bacteria to self-defend against invading DNA-containing organisms (e.g. virus).
- EcoRI, from *Escherichia coli* or *E. coli.*

5' G|AATTC

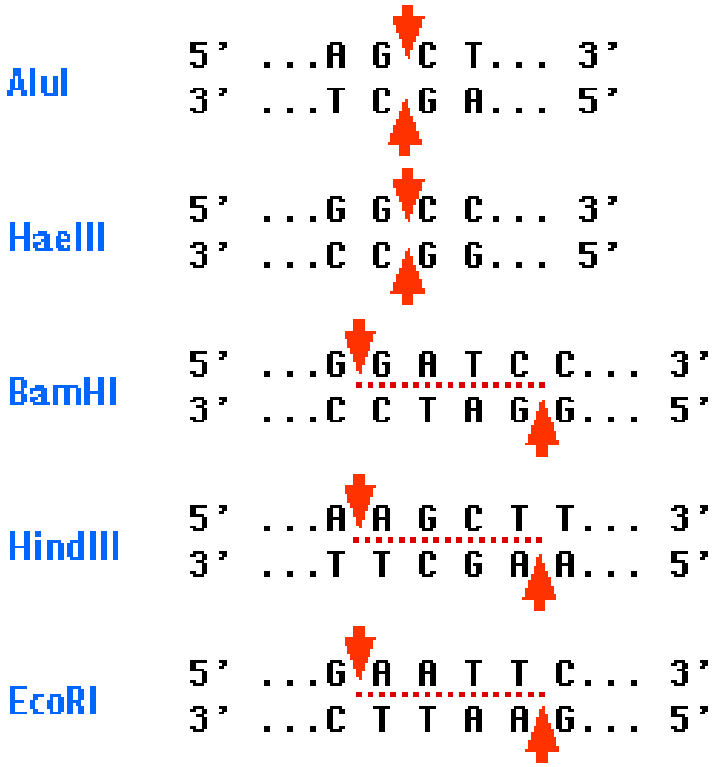
3' CTTAA|G

Restriction enzymes



Restriction Enzyme Action of EcoRI

Restriction enzymes



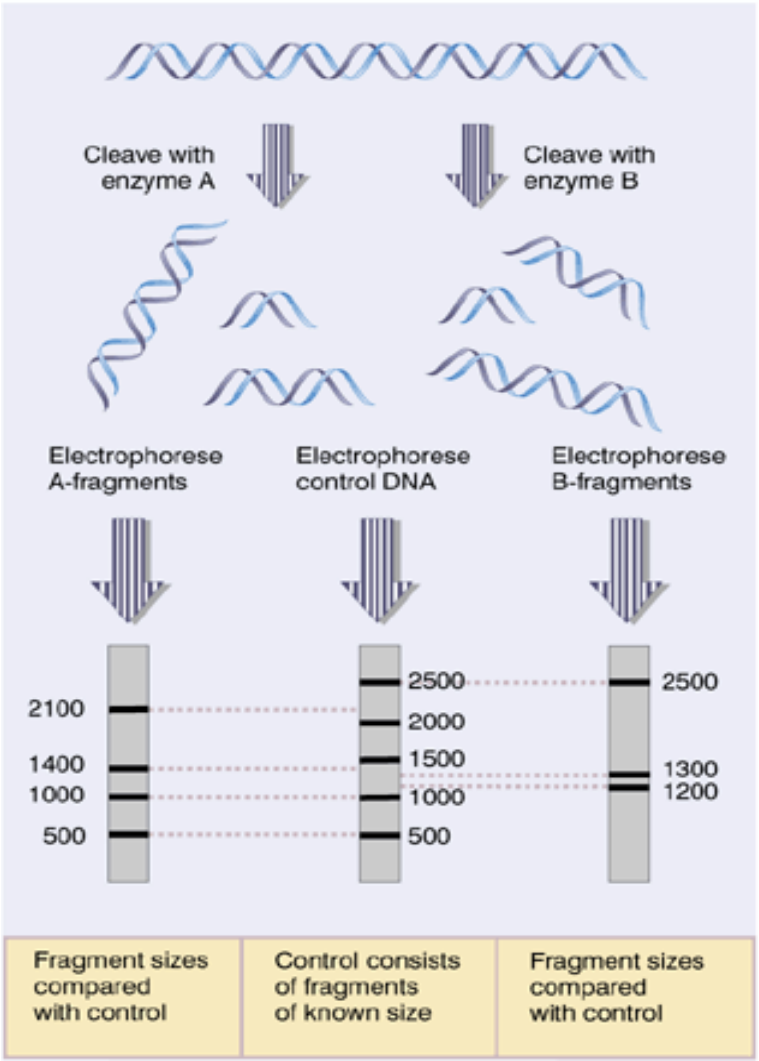
AluI and **HaeIII** produce blunt ends

BamHI **HindIII** and **EcoRI** produce "sticky" ends

http://www.ultranet.com/~jkimball/BiologyPages/

Restriction enzymes

Figure 2.1 DNA can be cleaved by restriction enzymes into fragments that can be separated by gel electrophoresis.



PCR

- **Polymerase chain reaction** or **PCR** is a widely used technique for creating billions of copies, i.e., **amplifying**, a single DNA fragment.

- It is based on nucleic acid hybridization.

PCR

- PCR relies on
  - Known sequence for the 3' end of the **template**, i.e., segment to be amplified.
  - Availability of **primers**, i.e., synthetic oligonucleotides complementary to the 3' ends of the template.
  - Use of **temperature** to control DNA **annealing** and **denaturation**.
  - Existence of a temperature resistant enzyme for DNA synthesis by primer extension: **Taq polymerase** (*Thermus aquaticus*, bacterium found in Yellowstone hot springs).

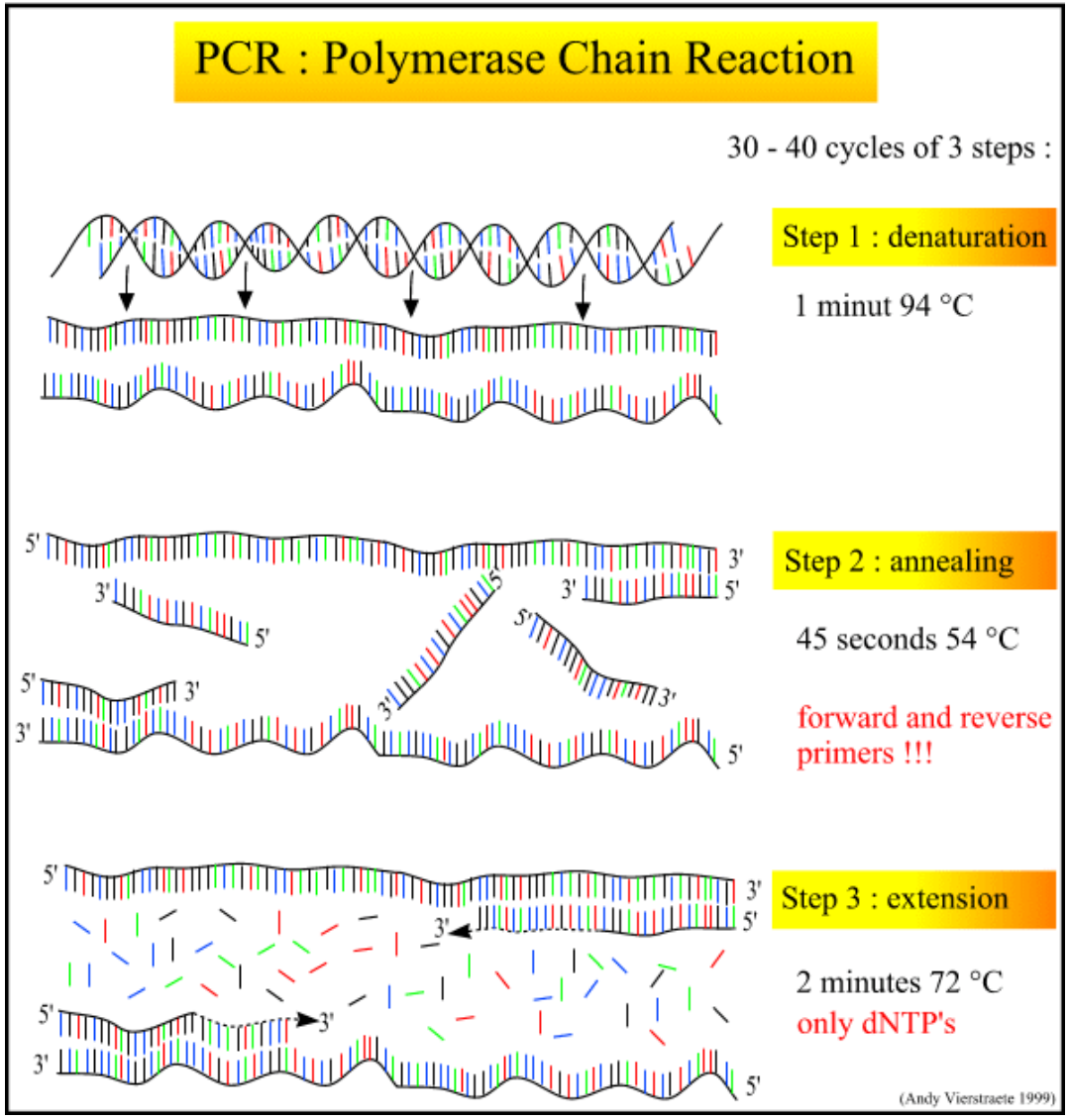
PCR

- Main ingredients:
  - DNA template,
  - primers in great excess of template,
  - dNTPs: deoxynucleotide triphosphates,
  - Taq polymerase.
- Repeated **cycles** of DNA denaturation (heating) and synthesis (cooling) rapidly provide many copies of the template.
- There are three major steps in a PCR, which are repeated for 30 or 40 cycles.

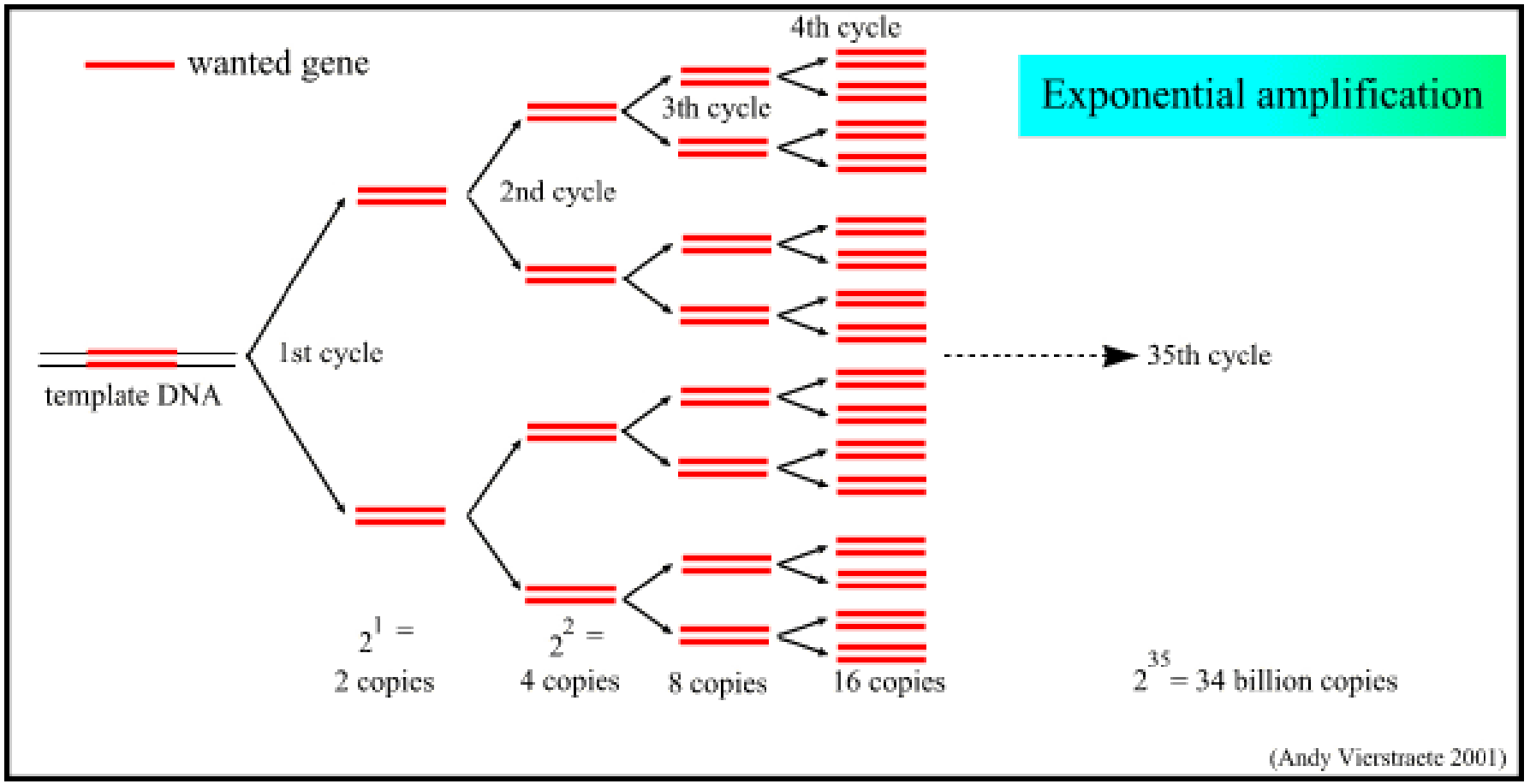
PCR

1. **Denaturation** (94°C):double strand melts open to single-stranded DNA, enzymatic reactions stop.
2. **Annealing** (54°C): Hydrogen bonds form between the single-stranded primer and template, the polymerase attaches to the duplex and starts copying the template.
3. **Extension** (72°C): At the ideal temperature for the polymerase, bases complementary to the template are coupled to the primer on the 3' end (the polymerase adds dNTPs from 5' to 3').

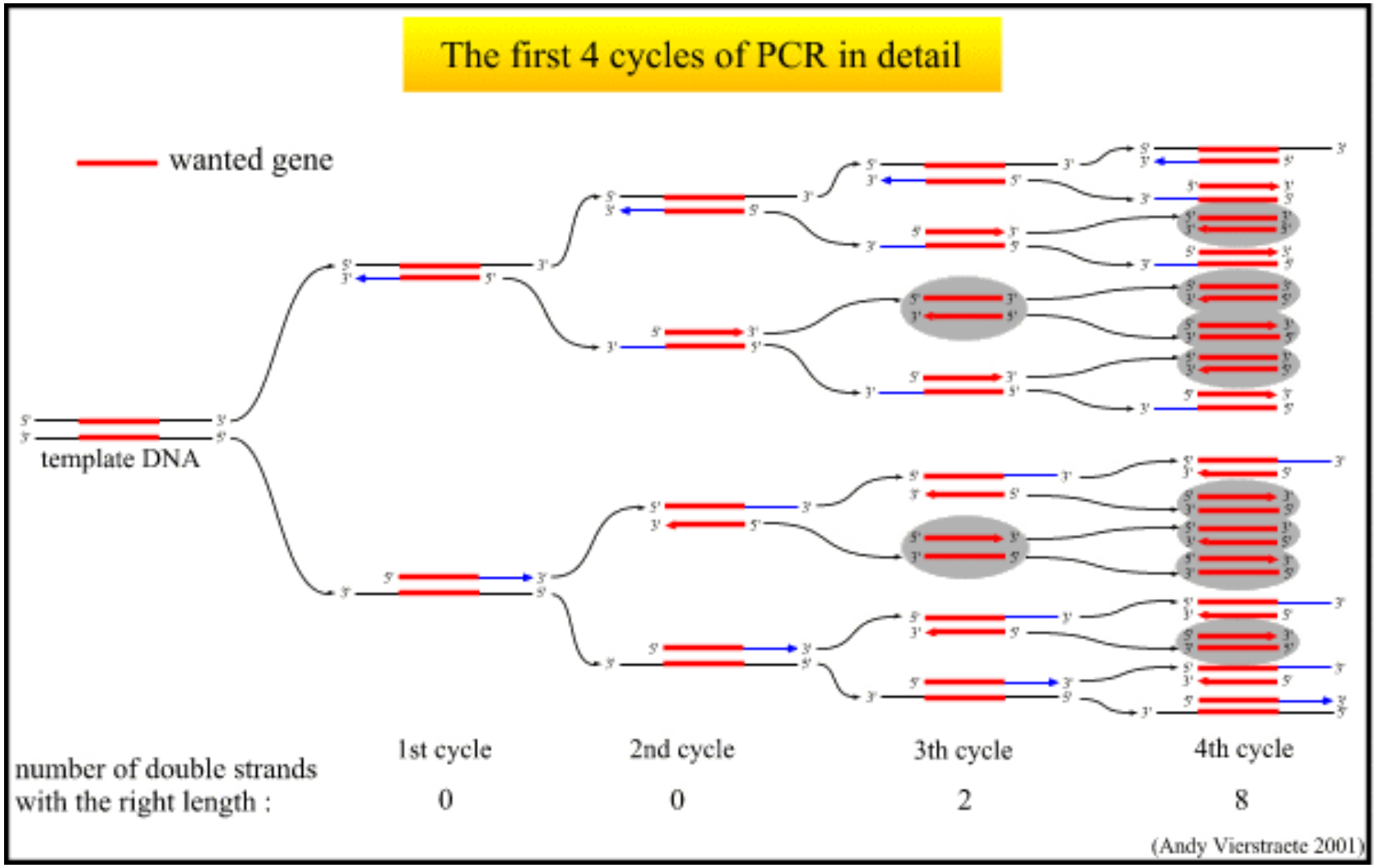
PCR


PCR : Polymerase Chain Reaction
30 - 40 cycles of 3 steps :
Step 1 : denaturation
1 minut 94 °C
Step 2 : annealing
45 seconds 54 °C
forward and reverse primers !!!
Step 3 : extension
2 minutes 72 °C
only dNTP's
(Andy Vierstraete 1999)

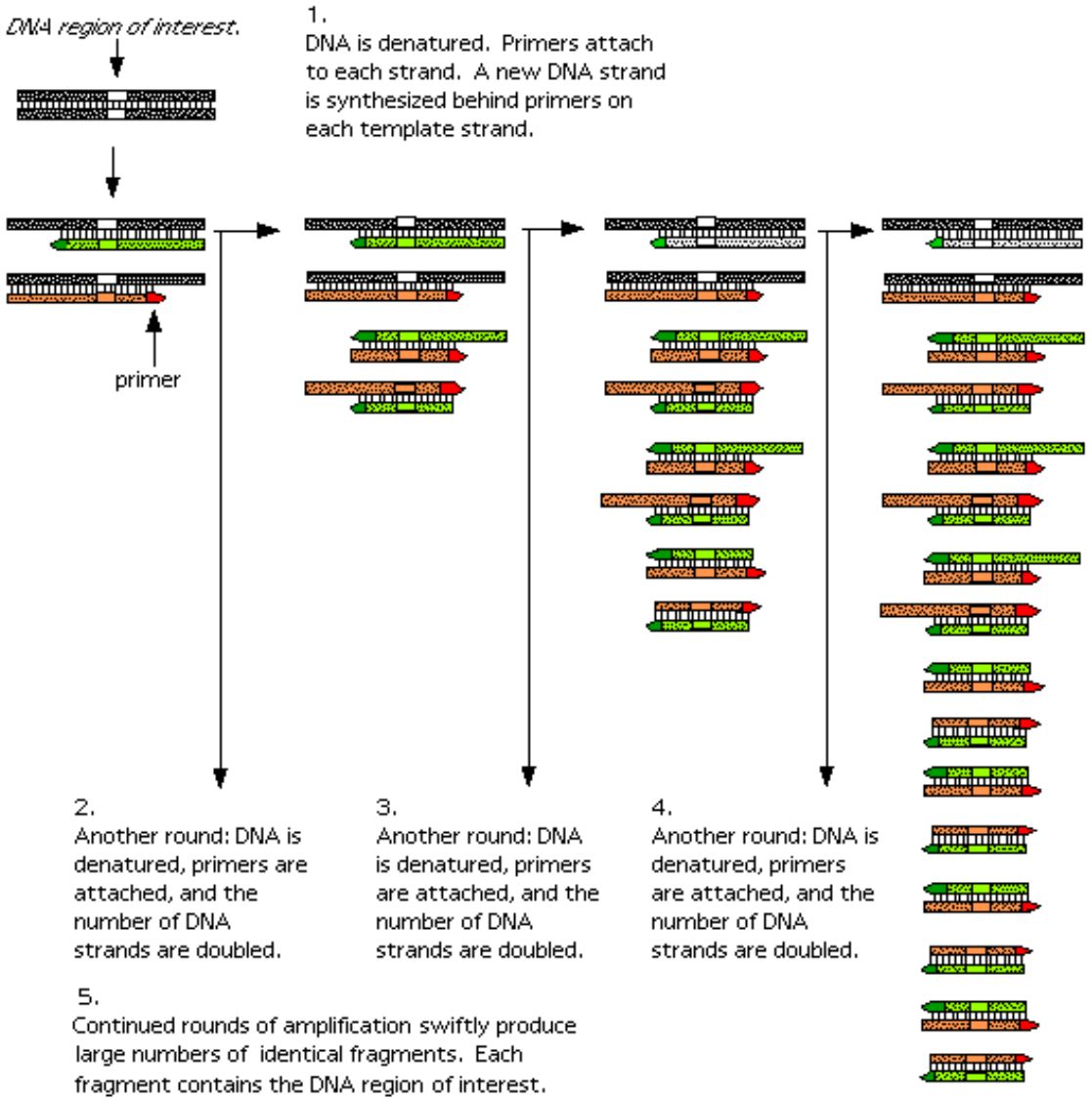
PCR


wanted gene
template DNA
1st cycle
2nd cycle
3th cycle
4th cycle
Exponential amplification
35th cycle
2^1 = 2 copies
2^2 = 4 copies
8 copies
16 copies
2^{35} = 34 billion copies
(Andy Vierstraete 2001)

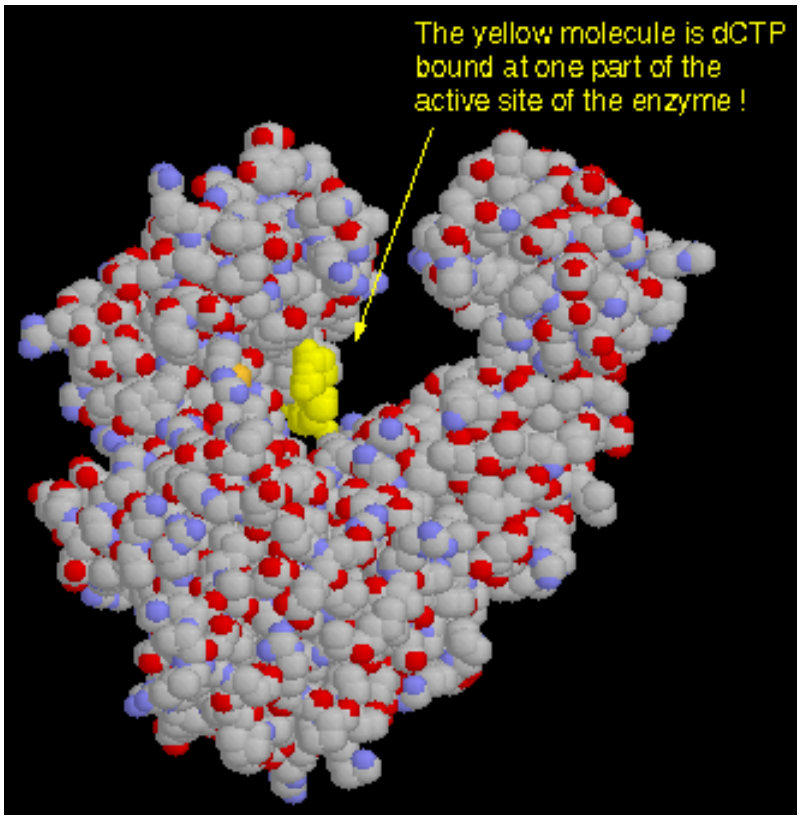
PCR


The first 4 cycles of PCR in detail
wanted gene
template DNA
1st cycle
2nd cycle
3th cycle
4th cycle
number of double strands with the right length :
0
0
2
8
(Andy Vierstraete 2001)

PCR


DNA region of interest.
1.
DNA is denatured. Primers attach to each strand. A new DNA strand is synthesized behind primers on each template strand.
primer
2.
Another round: DNA is denatured, primers are attached, and the number of DNA strands are doubled.
3.
Another round: DNA is denatured, primers are attached, and the number of DNA strands are doubled.
4.
Another round: DNA is denatured, primers are attached, and the number of DNA strands are doubled.
5.
Continued rounds of amplification swiftly produce large numbers of identical fragments. Each fragment contains the DNA region of interest.

Taq polymerase



http://berget.mcs.cmu.edu/education/TechTeach/replication/TaqI.html

Reverse transcriptase PCR

- Amplify **RNA** into DNA.
- E.g. complementary DNA or **cDNA** from mRNA.
- Based on an RNA-dependent DNA polymerase, **reverse transcriptase**, that catalyzes the synthesis of DNA from dNTPs, using RNA as a template.
- The reverse transcriptase enzyme is found in **retroviruses** and is responsible for their replication.

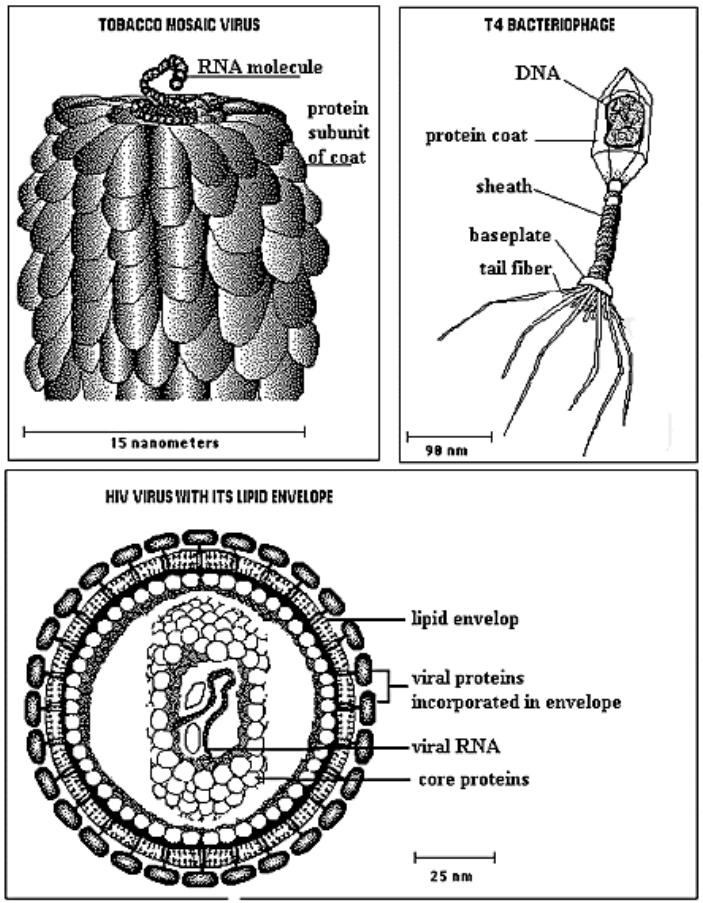
Viruses and retroviruses

- **Viruses** consist of a **nucleic acid** surrounded by a **protein capsid**.
- **Retroviruses** contain **RNA** as the hereditary material in place of the more common DNA.
- E.g. Human immunodeficiency virus, HIV, the virus that causes AIDS.

Retroviruses

- Retroviruses contain the enzyme **reverse transcriptase** (ribonuclease or RNAse), which causes synthesis of a complementary DNA molecule (cDNA) using virus RNA as a template.
- When a retrovirus infects a cell, it injects its RNA into the cytoplasm of that cell along with the reverse transcriptase.
- The cDNA produced from the RNA template contains the virally derived genetic instructions and allows infection of the host cell to proceed.

Viruses



Examples of viruses

Retroviruses

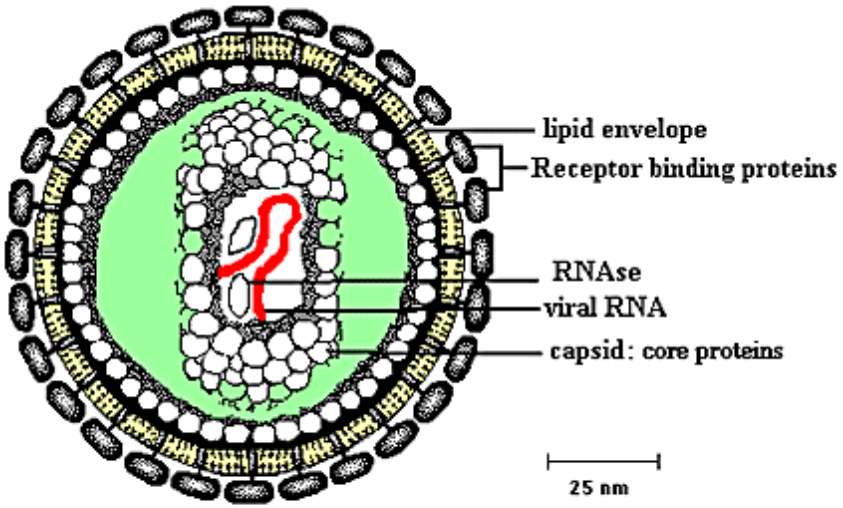
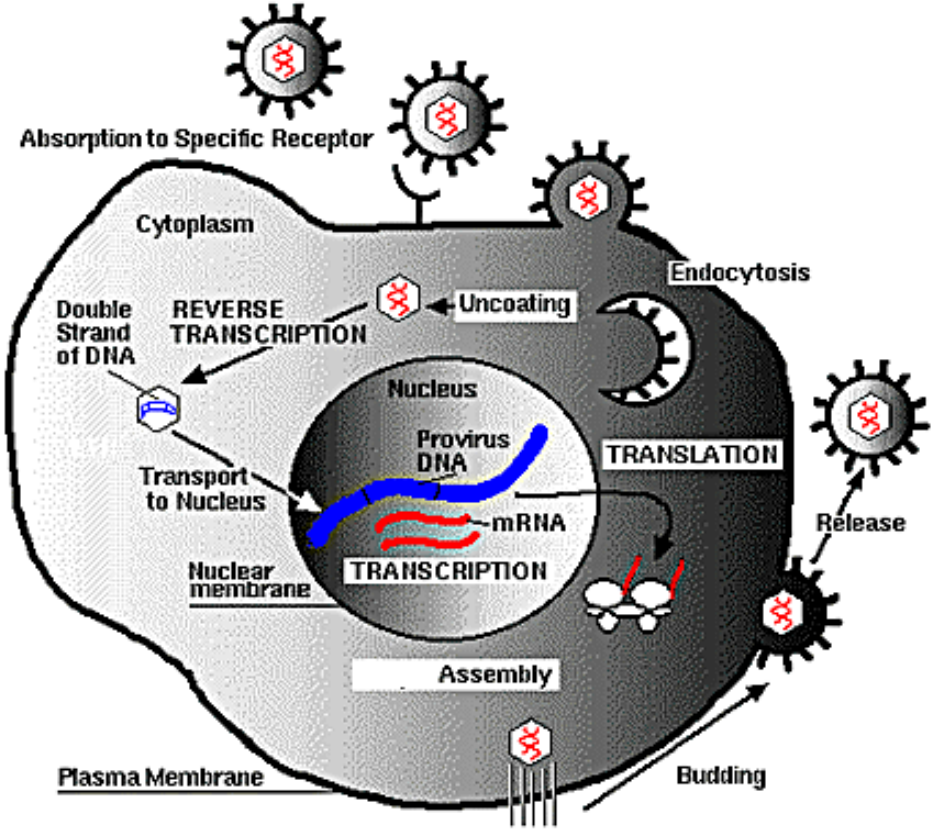
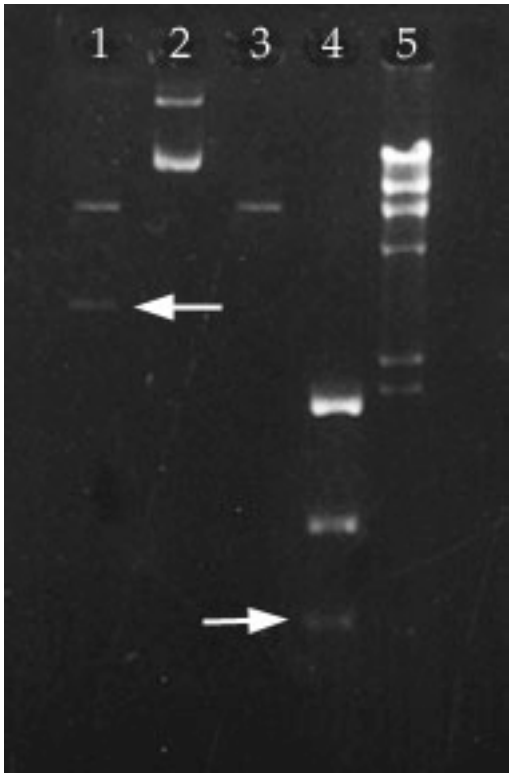


Diagram of a Retrovirus



Retrovirus replication

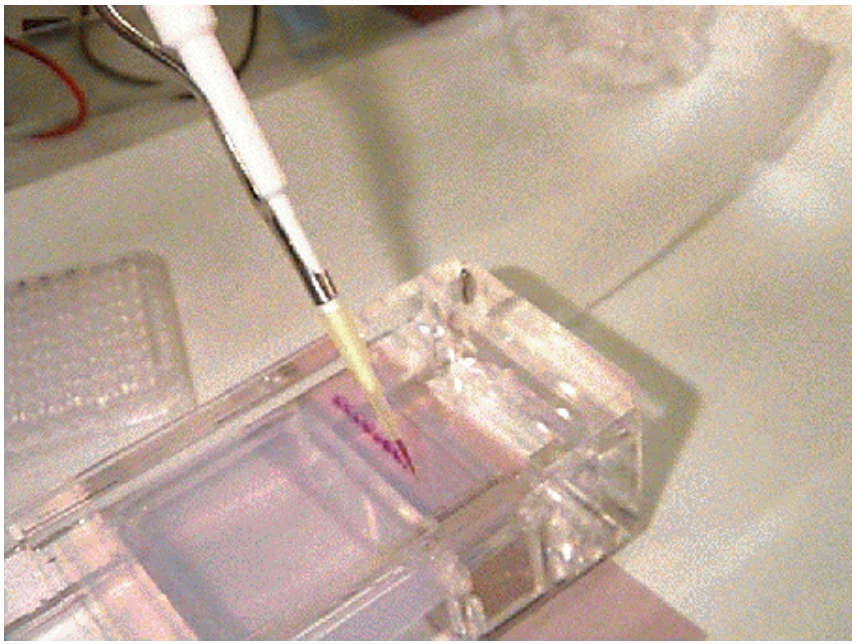
Gel electrophoresis

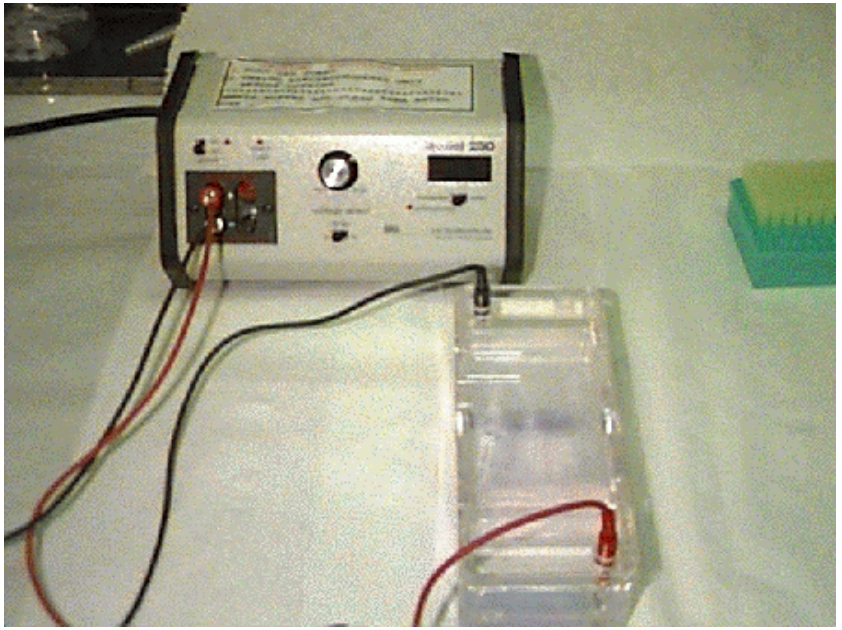


Gel electrophoresis

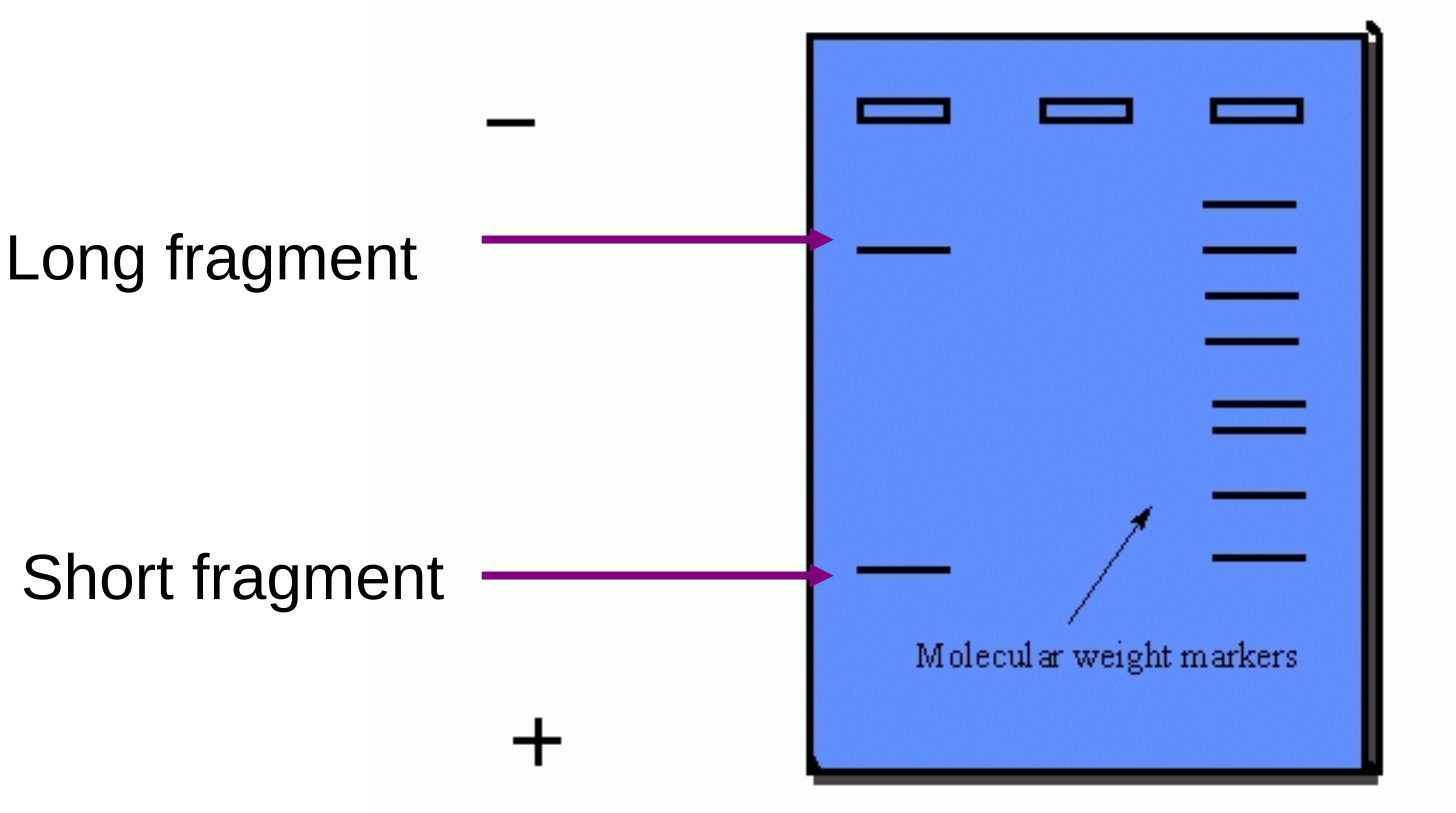
- **Electro** refers to electrical field; **phoresis**, from the Greek phoros, means "to carry across".
- **Gel electrophoresis** is a procedure for separating a mixture of charged molecules through a stationary material (gel) in an electrical field.
- Molecules are separated according to electric charge, size, and other physical properties.
- The gel is a colloid in a solid form (e.g. agarose, colloid from seaweed).
- Activated electrodes at either end of the gel provide the driving force.

Gel electrophoresis





Gel electrophoresis



Gel electrophoresis

LANE 1 = BACTERIOPHAGE LAMBDA

LANE 2 = BACTERIOPHAGE LAMBDA DIGESTED WITH THE RESTRICTION ENZYME HIND III

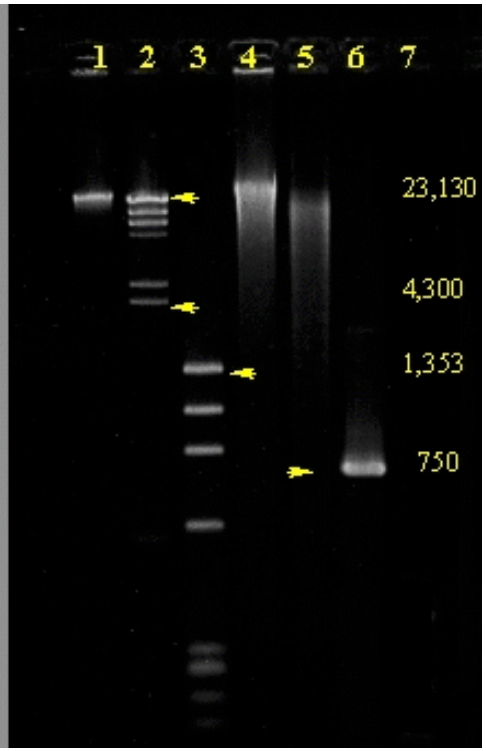
LANE 3 = BACTERIOPHAGE PHI X 174 DIGESTED WITH THE RESTRICTION ENZYME HAE III

LANE 4 = UNDIGESTED EUKARYOTIC DNA (FUNGAL)

LANE 5 = PARTIALLY DEGRADED EUKARYOTIC DNA (FUNGAL)

LANE 6 = PCR PRODUCT (THE ITS REGION OF THE RIBOSOMAL GENE REPEAT

LANE 7 = APPROXIMATE SIZES IN THOUSANDS OF BASES (KB)



http://web.utk.edu/~khughes/

Probing

- Goal. Monitor the presence or abundance of specific DNA/RNA sequences in a pool of DNA/RNA (e.g. DNA from a certain type of cells).
- A **probe** is a labeled (radioactive or fluorescent) single-stranded oligonucleotide, synthesized to be complementary to the sequence of interest – i.e., the probe sequence is known.
- The DNA/RNA sample interrogated by the probe is called the **target**.

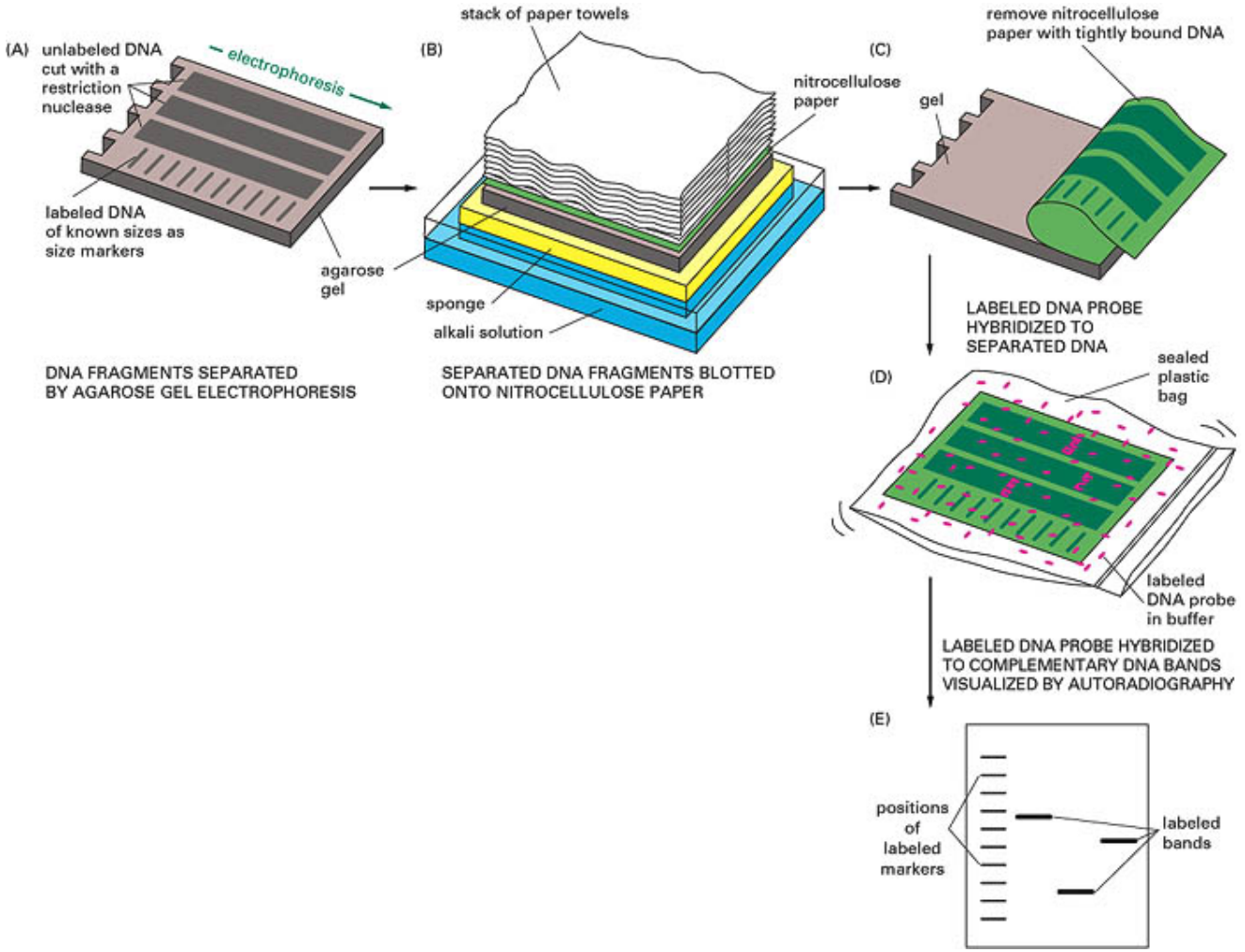
Probing

- The probe is attached to a solid support (e.g. membrane) and incubated with the target to allow hybridization of the target to the probe.
- The extent of hybridization of the target to the probe reflects the abundance of the probe in the target.
- Quantification can be done by, e.g., X-ray for radioactive probes.

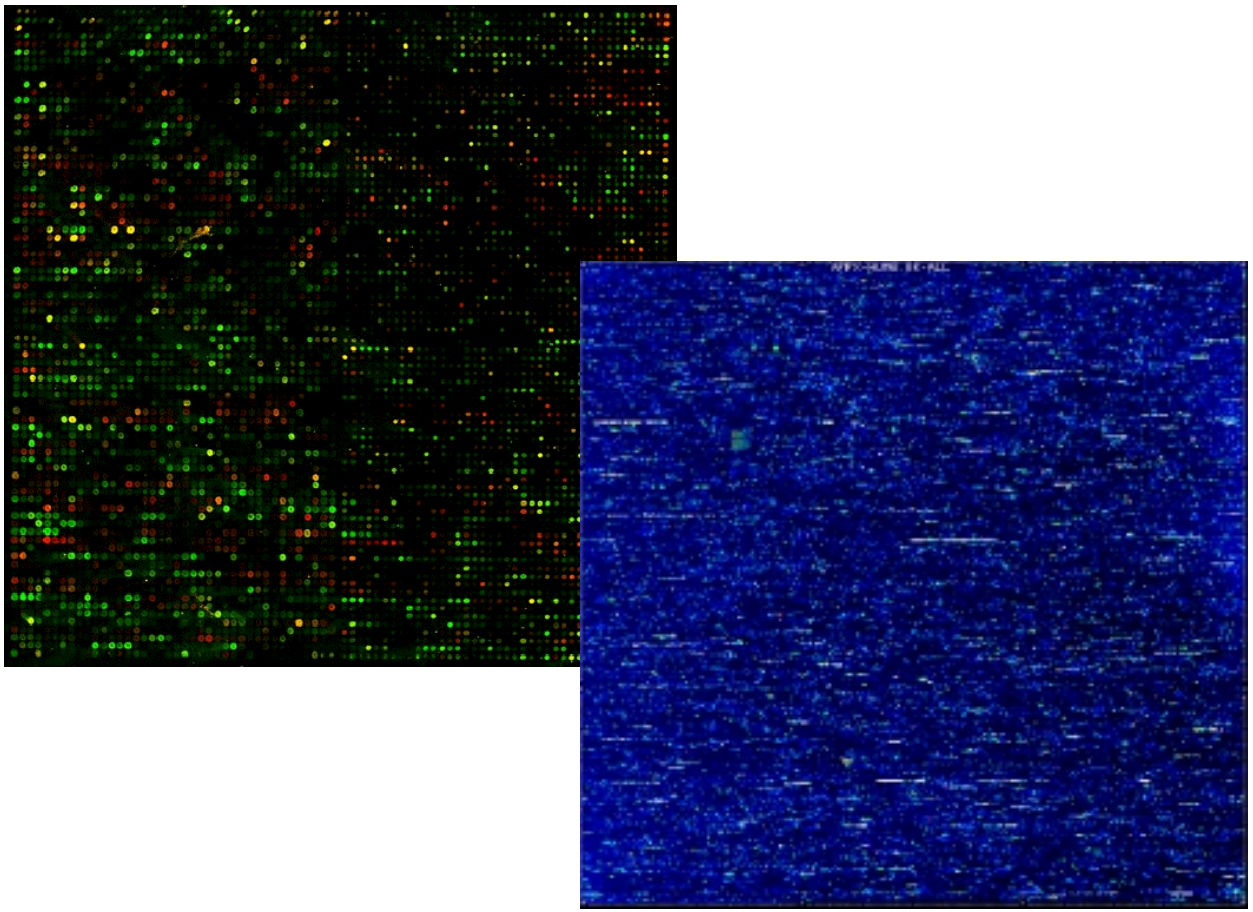
Blots

- Blots are named for the **target** molecule.
- **Southern blot**: **DNA** cut with restriction enzymes - probed with radioactive DNA.
- **Northern blot**: **RNA** - probed with radioactive DNA or RNA.
- **Western blot**: **protein** - probed with radioactive or enzymatically-tagged antibodies.

Southern blot



Microarrays
… blots on a genomic scale



WWW resources

- **Access Excellence**
  http://www.accessexcellence.com/AB/GG/
- **Genes VII**
  http://www.oup.co.uk/best.textbooks/biochemistry/genesvii/
- **Human Genome Project Education Resources**
  http://www.ornl.gov/hgmis/education/education.html
- **Kimball's Biology Pages**
  http://www.ultranet.com/~jkimball/BiologyPages/
- **MIT Biology Hypertextbook**
  http://esg-www.mit.edu:8001/
- **PCR**
  http://www.highveld.com/pcr.html