Gene Technology

For all computational purposes, DNA is represented as a string of 4-letter alphabets - A, T, C, G:

attgctacgttacatcgctgca

How do we get this string representation from a dynamic double-stranded molecule?

DNA Sequencing - determine the precise sequence of nucleotides in a sample of DNA

To carry out this task we need to be able to chop the DNA, store it, make copies of it.

Let's consider the example of detecting if a person is infected by the novel coronavirus SARS-CoV-2

- **uses Real Time RT-PCR Nucleic Acid Detection Kit based on the PCR method which uses a fluorescent probe and a specific primer to detect three specific regions within the SARS-CoV-2 nucleocapsid protein N gene.**

- **How is the SARS-CoV-2 genome sequenced?**
- **How does one identify the coordinates of N gene on it? i.e., how to construct a physical map of a genome?**
- **How does one select which regions in this gene would give specificity for the presence of SARS-CoV-2?***
- **How are the specific probe regions extracted and amplified for detection?**
- **Is it possible to store the DNA sample for re-testing? How?**

To sequence a gene, we need to

- **Identifying the region of interest**
- **Isolate it from the organism – DNA fragmentation**
- **moving it to another easily manageable organism such as a bacterium for obtaining multiple copies – cloning**

Such manipulations are conducted by a toolkit of enzymes:

Restriction endonucleases - used as molecular scissors

DNA ligase - to bond pieces of DNA together

- a variety of additional enzymes that modify DNA are used to facilitate the process.

Restriction endonucleases are enzymes that make **site-specific** cuts in the DNA – **chemical scissors**

Ability to cut DNA into discrete fragments allows to understand

- how genetic material of an organism is **organized**
- how expression of genetic information is **controlled**
- how **alteration** of genetic information can give rise to genetically inherited disorders, etc.
- in **bulk production** of pharmaceutically important proteins

First restriction enzyme was isolated from H. influenzae in 1970 by Daniel Nathans and Kathleen Danna

- awarded the Nobel Prize for Medicine in 1978

Restriction endonucleases are enzymes that make site-specific cuts in the DNA – chemical scissors

First restriction enzyme was isolated from H. influenzae and used to cleave SV40 DNA (a tumor virus):

- 11 distinct DNA bands were visible on polyacrylamide gel electrophoresis, indicating that the enzyme always cut SV40 resulting in the same 11 pieces

Background

How were these restriction endonucleases identified?

Bacteria are under constant attack by bacteriophages – a virus that infect and replicates within a bacterium

To protect themselves, bacteria have developed a method to chop up any foreign DNA - such as that of an attacking phage

These bacteria build an endonuclease - an enzyme that cuts DNA - it circulates in the bacterial cytoplasm, waiting for phage DNA.

These endonucleases are termed "restriction enzymes" because they restrict the infection of bacteriophages.

Why the restriction enzymes do not chew up the genomic DNA of their host?

Background

A bacterium that makes a particular restriction endonuclease, also synthesizes a companion DNA methyltransferase,

- which methylates the DNA target sequence for that restriction enzyme, thereby protecting it from cleavage.

DNA from an attacking bacteriophage will not have these protective methyl groups and will be destroyed.

Methyl groups block the binding of restriction enzymes, but do not block the normal reading and replication of the genomic information stored in the host DNA.

DNA Fragmentation

Different endonucleases present in different bacteria recognize different nucleotide sequences

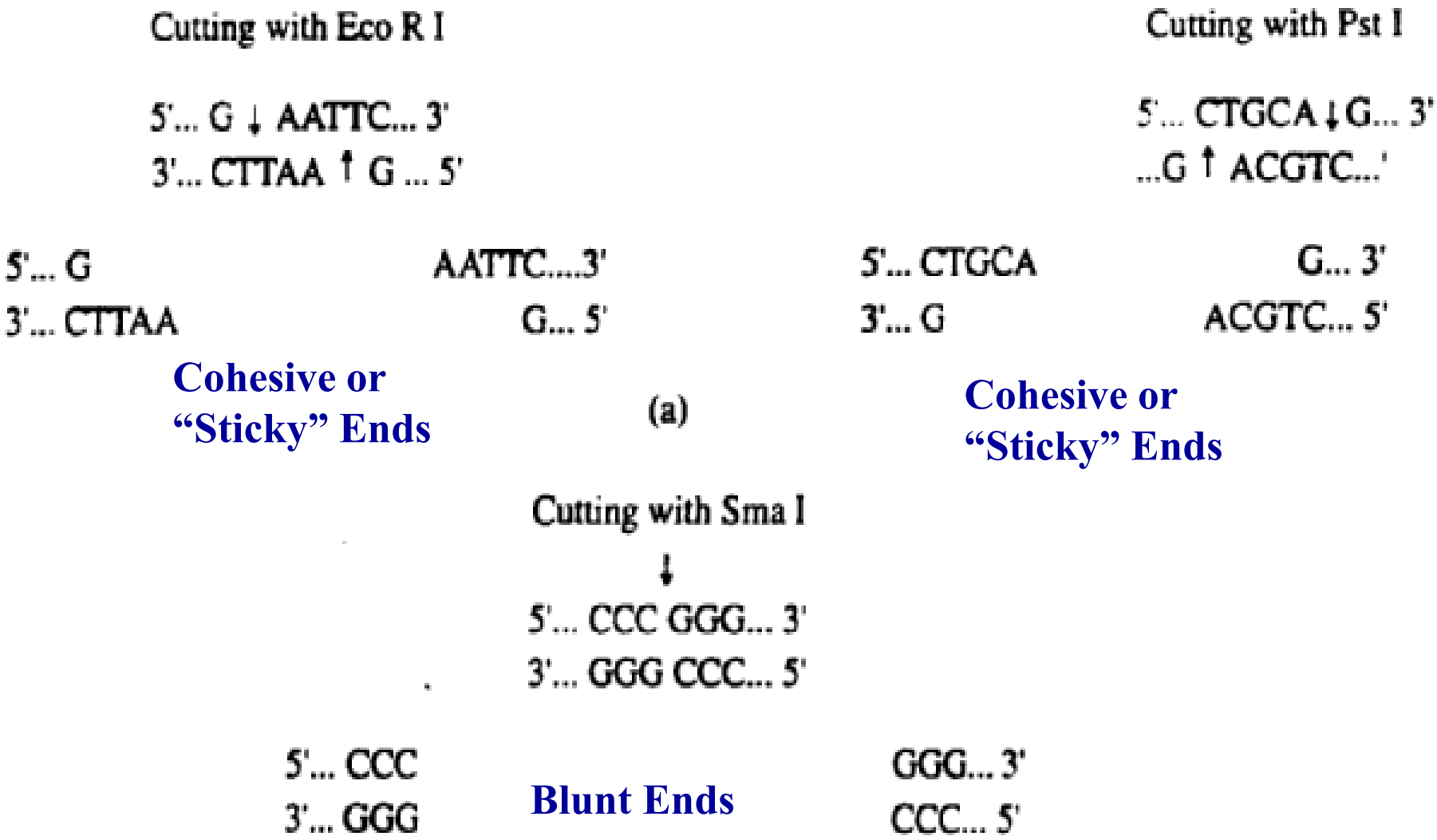
Naming of restriction enzymes - after their host of origin, e.g.,

- **EcoRI -** ***Escherichia coli***
- **Hind II & Hind III -** ***Haemophilus influenzae***
- **XhoI -** ***Xanthomonas holcicola***

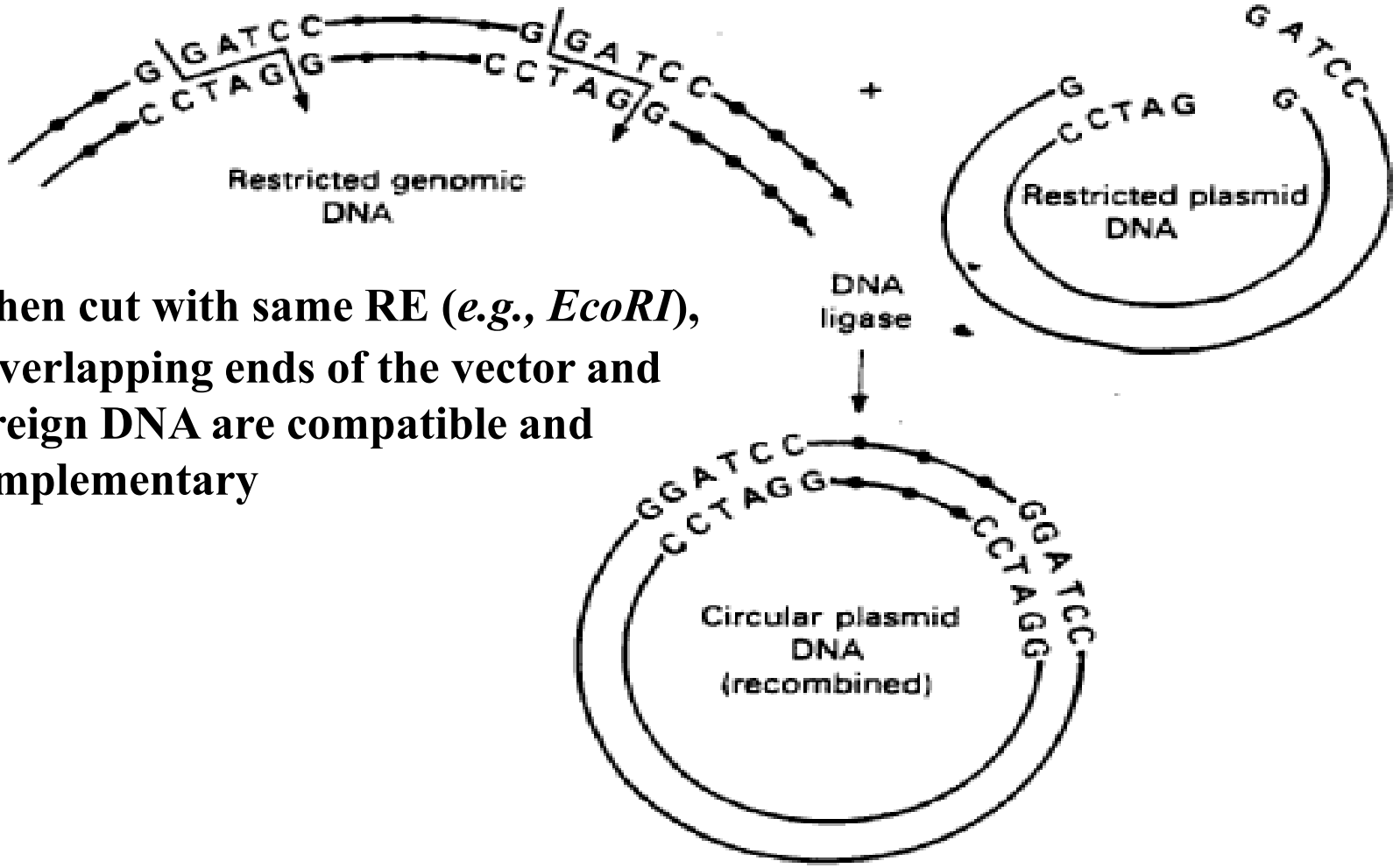
When cut with a restriction enzyme (RE), the ends of the cut DNA fragment can be cohesive or blunt-ended depending on the enzyme.

Enzyme	Recognition Sequence
EcoRI	G↓AATTC
HindIII	A↓AGCTT
BamHI	G↓GATCC
BglI	GCCNNNN↓NGGC
PvuI	CGATC↓G
HaeIII	GG↓CC
MboI	GAT↓C

Generation of Cohesive & Blunt-ended Fragments



Restriction enzyme digestion of genomic DNA and plasmid vector DNA



When cut with same RE (*e.g.*, *EcoRI*),

- overlapping ends of the vector and foreign DNA are compatible and complementary

How does one cut a DNA if it doesn't contain desired RE sites?

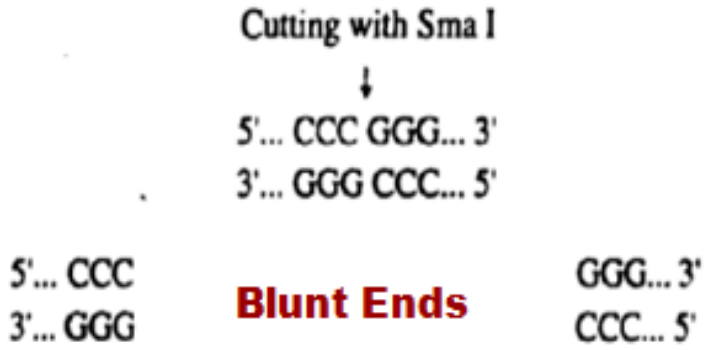
Or

If the RE site is present within the DNA of interest?

(say, within SARS-CoV-2 N gene)

Or

If the RE result in blunt-ended DNA fragments, how do we insert the fragment in a cloning vector?



How to clone a blunt-ended DNA fragment?

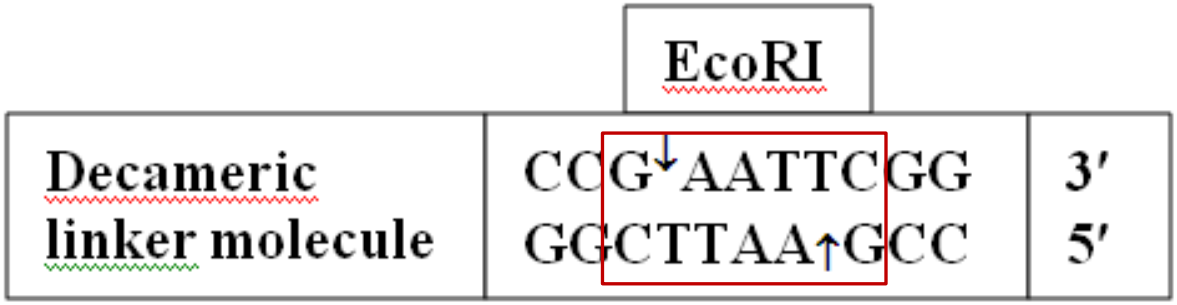
- **a linker molecule can be ligated on either side by DNA ligase, cut with the RE contained in the linker molecule to obtain cohesive ends.**

How does one cut a DNA if it doesn't contain desired RE sites?

- **the DNA maybe be cut with whatever RE sites are available, and then linker or adaptor molecules maybe added to enable ligating it to the vector.**

Linkers & Adaptors

Linkers - short, double-stranded DNA molecules (~ 8-14bp) with one internal site for RE (~ 3-8bp)



Problem?

- **the sites for the enzyme used to generate cohesive ends may be present in the target DNA fragment, limiting its use for cloning.**
- **This problem can be solved using adaptors.**

Linkers & Adaptors

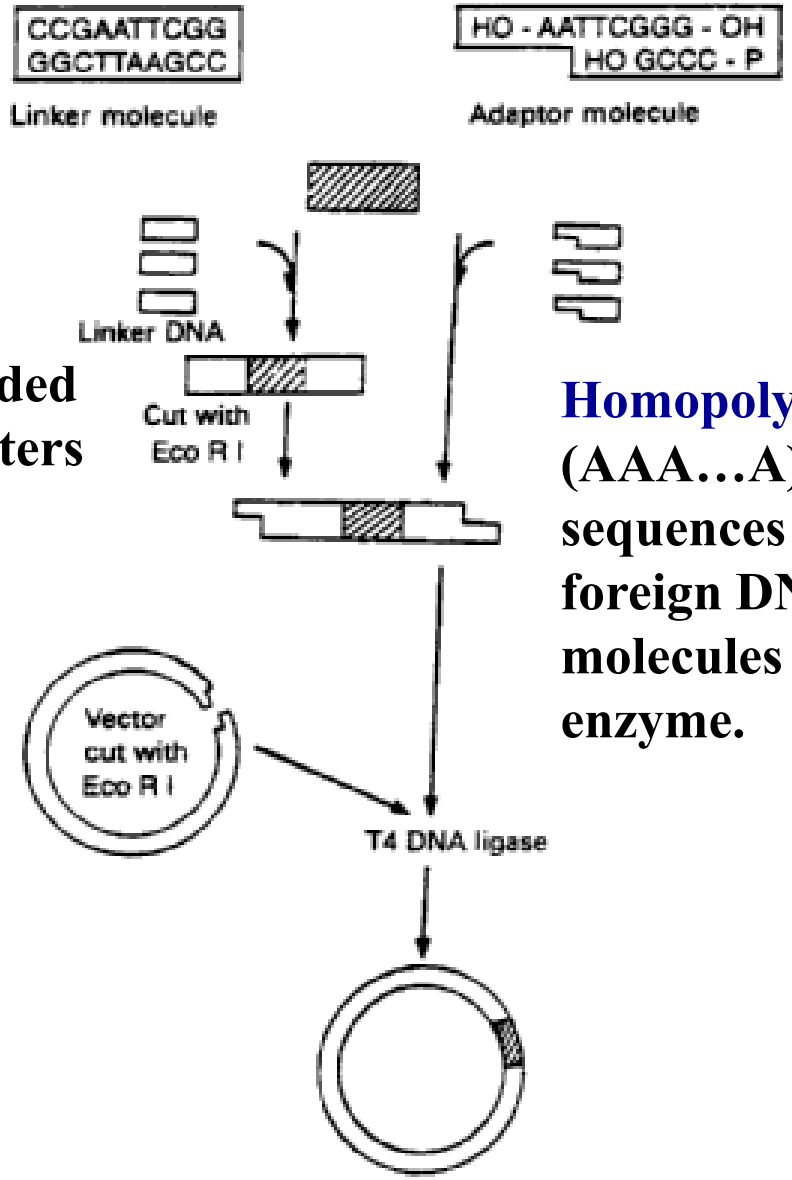
Adaptors - chemically synthesized DNA molecules with pre-formed cohesive ends

- it has one blunt end bearing a 5′ phosphate group and another cohesive end for a specific RE which is not phosphorylated to prevent self ligation.

Adaptor molecule

- reduces the need for restriction digestion following ligation

Use of Linker & Adaptor Molecules in the Formation of Recombinant Plasmids



This step avoided on using adapters

Homopolymer method - oligo A (AAA…A) or oligo T (TTTT…T) sequences added to the ends of foreign DNA & vector DNA molecules by terminal transferase enzyme.

Features of Restriction Enzymes

- **Length of recognition sequence dictates how frequently the enzyme will cut a DNA sequence**

  Which of the recognition sites - of length, 4, 6, or 8, will occur at higher frequency? At what distances will they occur?

- **Different REs can have the same recognition site and are called isoschizomers, e.g., *SacI* & *SstI* : GAGCTC**

- **Restriction recognitions sites can be unambiguous, e.g., *BamH* I recognizes the sequence GGATCC and no other, or ambiguous, e.g., *Hinf* I has a recognition site, GANTC.**

  Recognition sites for Hinf I will occur at what frequency?

Features of Restriction Enzymes

- **Recognition site for one enzyme may contain the restriction site for another, e.g., *BamH* I recognition site (GGATCC) contains the recognition site for *Sau3A* I (GATC).**

  ***Sau3A I* recognizes the sequence GATC and produces the same sticky ends as BamH I upon cutting**

  Will the two REs give the same results? If not, which one will give larger number of fragments?

- **Most recognition sequences are palindromes - they read the same forward and backward**

Can we use the property of palindrome sequence to identify restriction recognition sites?

Applications of Restriction Enzymes

Danna & Nathans showed that it was possible:

- **to prepare a physical map of the SV40 genome**
- **to localize the origin of replication**
- **to position early & late genes of SV40 onto this "restriction map"**
- **that any individual gene could be mapped by testing for biological activity during transformation experiments**
- **informative mutants could be made by deleting one or more of the specific fragments**

Applications of Restriction Enzymes

- **Variations in DNA sequences, *viz.*, mutations in recognition sites, copy number variation of VNTRs, insertions, deletions, inversions and translocations, can be identified by RE analysis**

  - The length variations is known as restriction fragment length polymorphisms (RFLPs).

- **In genetic engineering - using REs DNA may be cut at precise locations & using DNA ligase, reassembled in any desired order, allowing the researchers to assemble customized genomes; create designer bacteria that make insulin, or growth hormones, or add genes for disease resistance to agricultural plants, etc.**

- **in DNA sequencing – first step is to cut the DNA in manageable pieces**

Restriction Map

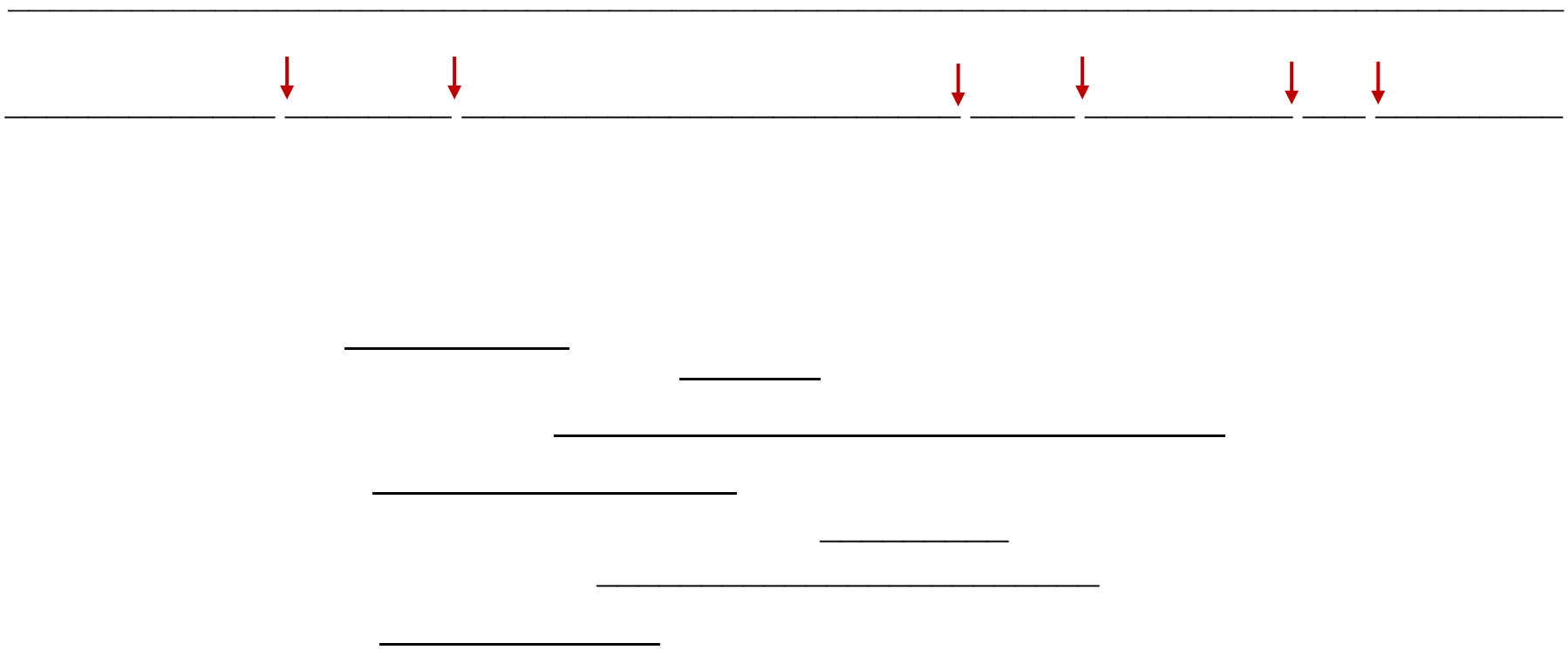
Restriction map is a description of restriction endonuclease cleavage sites within a piece of DNA

- **generating such a map is the first step in characterizing an unknown DNA**

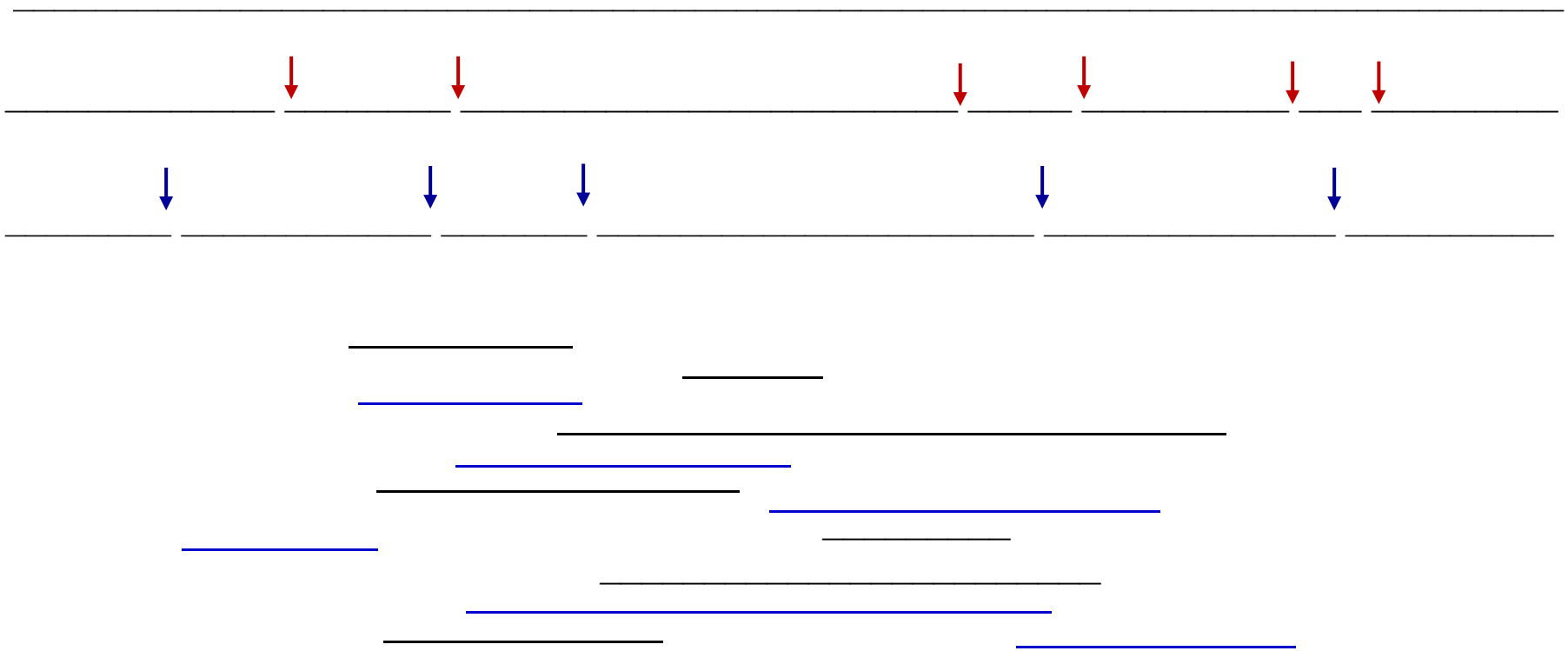
Multiple Complete Digest Mapping – creates a map by digesting DNA with multiple REs

- each recognizing a different specific short DNA sequence and producing a separate fingerprint for each clone

Because of the frequent occurrence of these sites, restriction mapping produces a relatively fine scale of physical map.



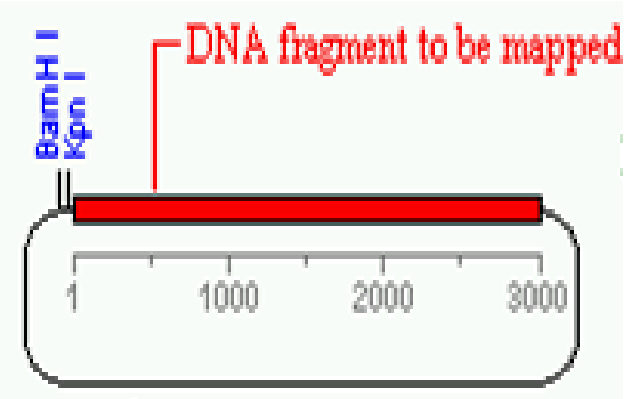
How do you order the fragments in the correct order?



The fragments can be arranged in the correct order by finding the overlapping fragments

Restriction Mapping

Ex: Consider a plasmid that contains a 3000 bp fragment of unknown DNA & unique recognition sites for enzymes Kpn I & BamH I.



Consider first separate digestions with Kpn I & BamH I:

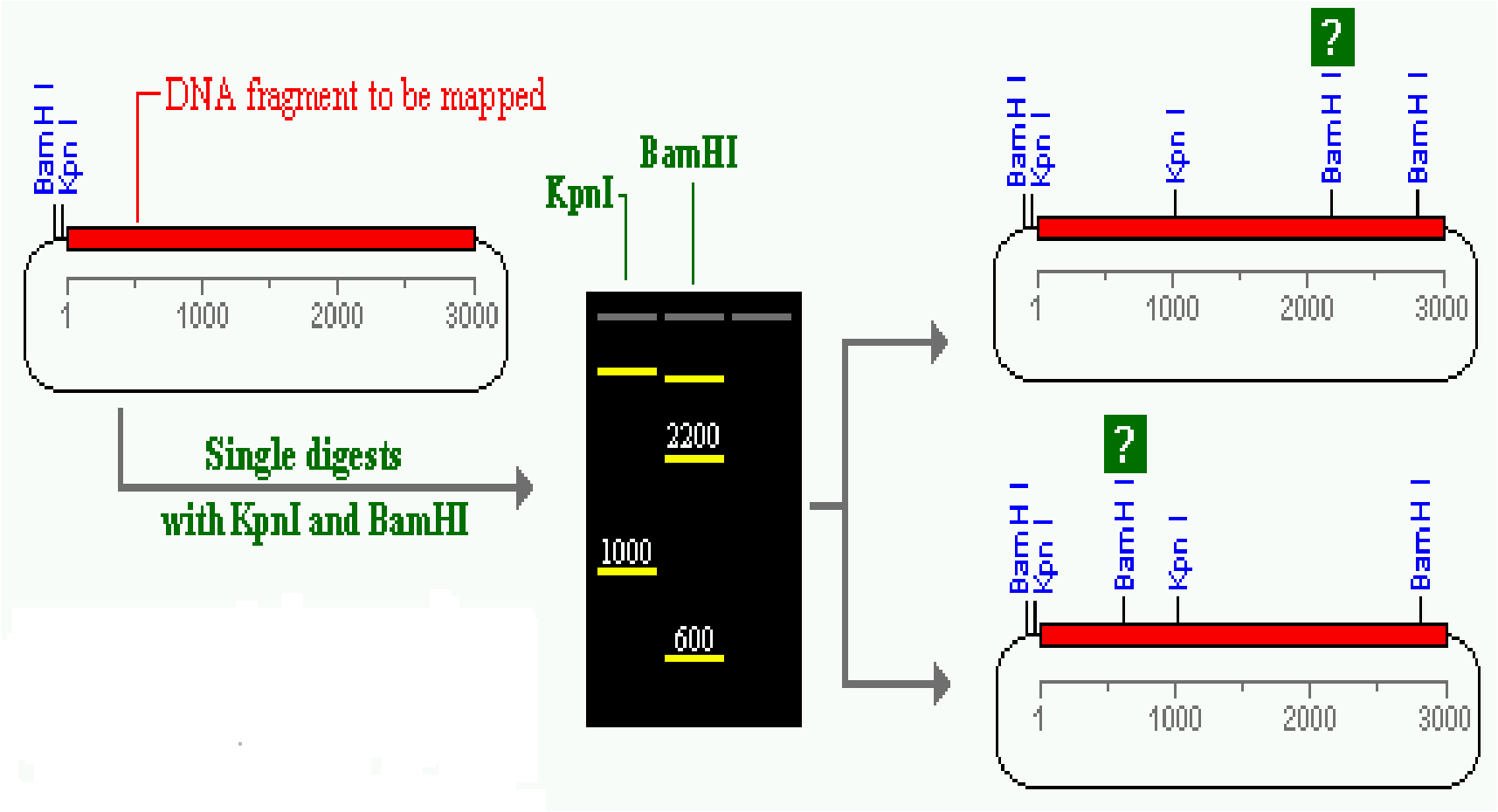
Kpn I yields 2 fragments: 1000bp & "big"

BamH I yields 3 fragments: 600, 2200 & "big"

big – part of unknown DNA sequence + vector

⇒ one Kpn I site & two BamH I sites are present in the unknown DNA sequence, given 1 each on the vector sequence

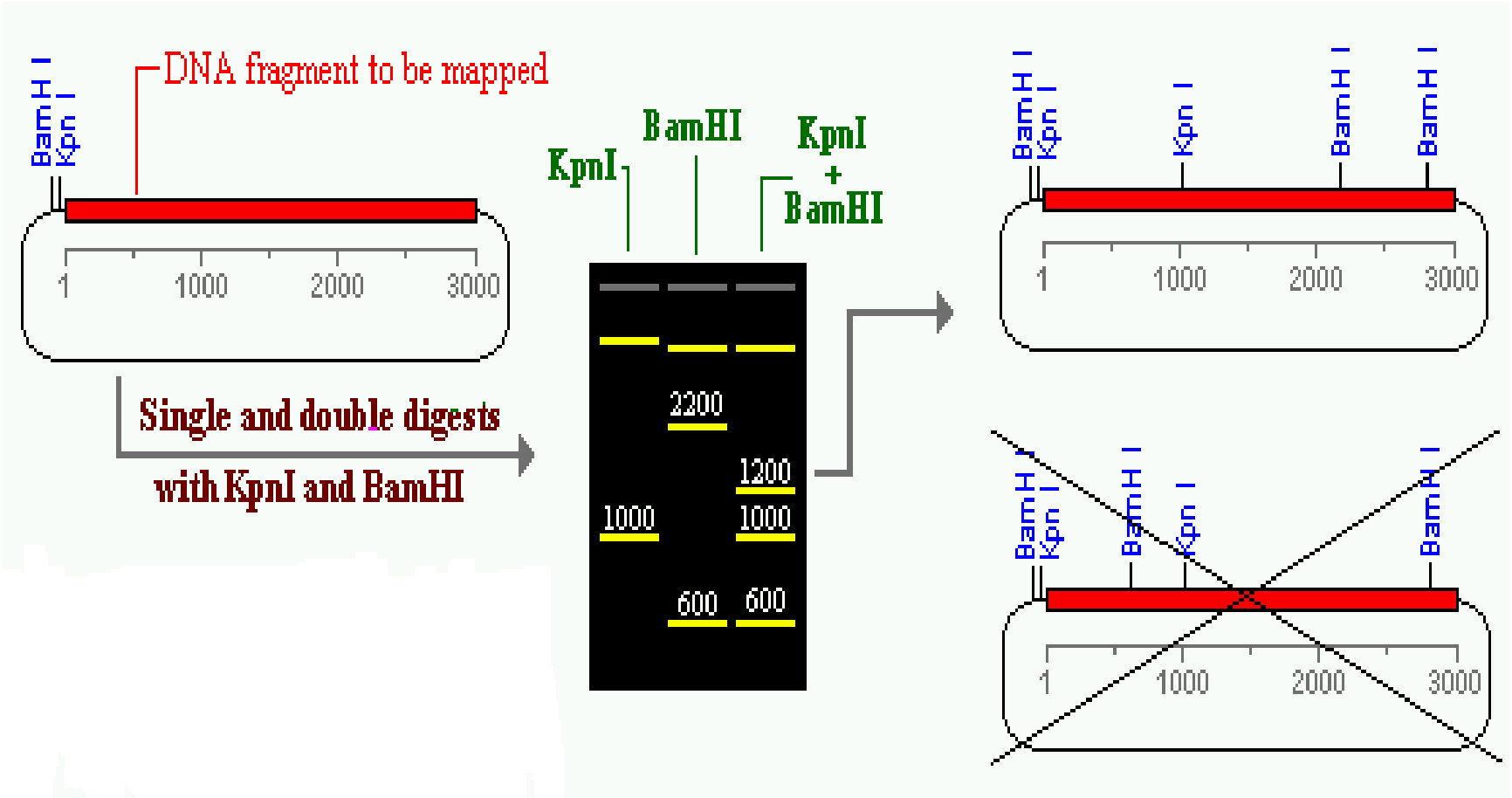
Restriction Mapping



One BamH I site is at 2800 bp. Trick to determine the location of 2nd BamH I site is to digest the plasmid with Kpn I & BamH I together

Restriction Mapping

Double digest yields fragments of 600, 1000 & 1200 bp (plus the "big" fragment).



Restriction Mapping

If the above process is conducted with a larger set of enzymes, a much more complete map would result

single digests - are used to determine which fragments are in the unknown DNA, and

multiple digests - to order and orient the fragments correctly.

For any novel genome, e.g., SARS-CoV-2, can a physical map be constructed computationally?

Restriction Mapping

Using a Computer to Generate Restriction Maps

If the sequence is known, feed it to computer programs, which will search the sequence for various RE recognition sites and build a map.

- **Mapper - available as part of Molecular Toolkit**

  http://arbl.cvmbs.colostate.edu/molkit/mapper/

- **Webcutter**

  http://www.firstmarket.com/cutter/cut2.html

- **RebSite – as part of the REBASE Tools**

  http://tools.neb.com/REBsites/index.php3

REBASE
The Restriction Enzyme dataBASE

A comprehensive database containing information:

- **restriction enzymes, methylases & related proteins involved in restriction-modification processes**
- **recognition and cleavage sites, isoschizomers, neoschizomers, commercial availability, methylation sensitivity, crystal & sequence data.**

All newly sequenced genomes are analyzed for the presence of putative restriction systems and these data included in REBASE

It is updated daily (http://rebase.neb.com/)

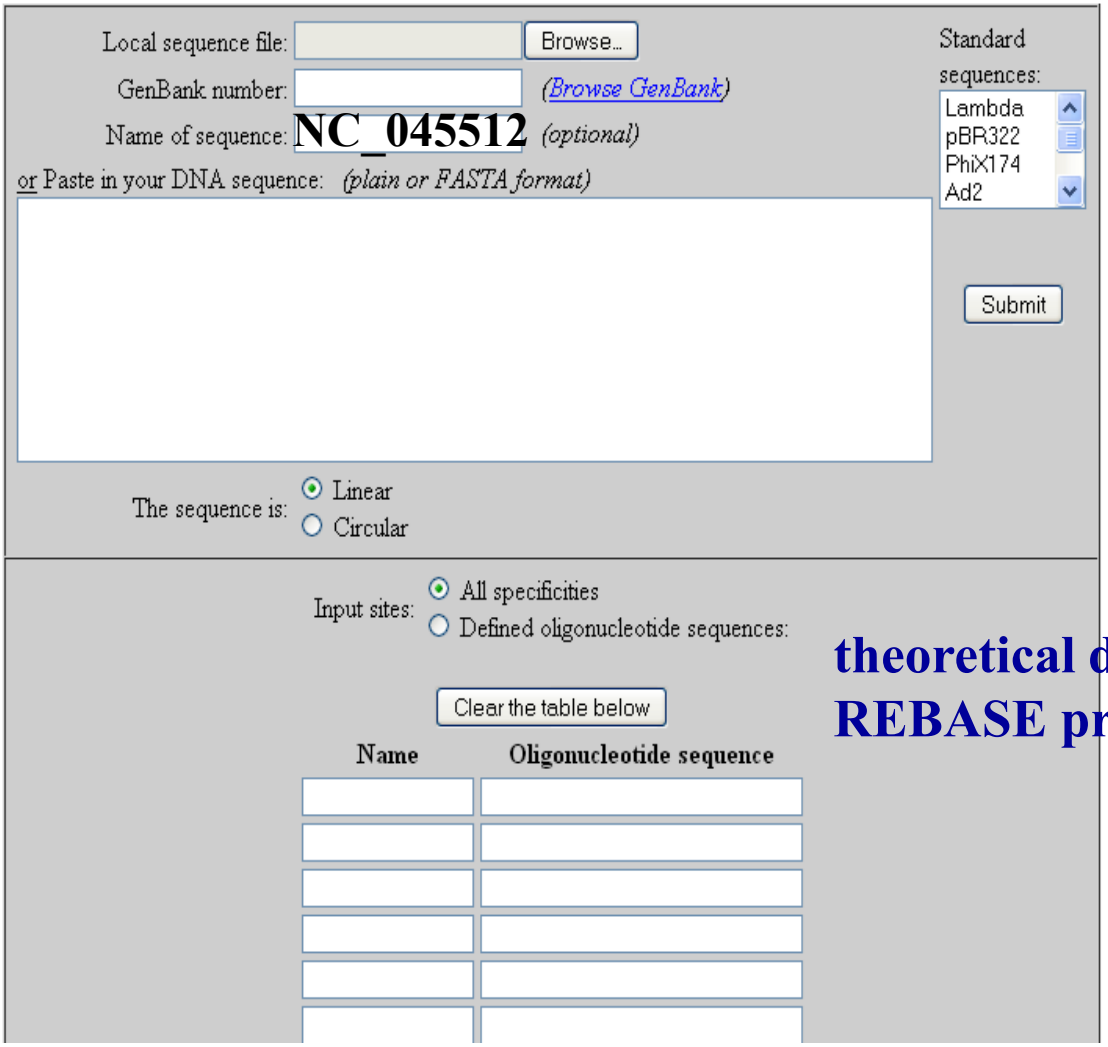
Ref: Robert et al, *Nucl. Acids Res.* 43: D298-D299 (2015)

Back to...

Program Guide

REBsites

This tool will take a DNA sequence and digest it with one example of each of the known Type 2 restriction enzyme specificities.
The maximum size of the input file is 2 MByte, and the maximum sequence length is 200 KBases.



theoretical digest with all REBASE prototypes

[New DNA]

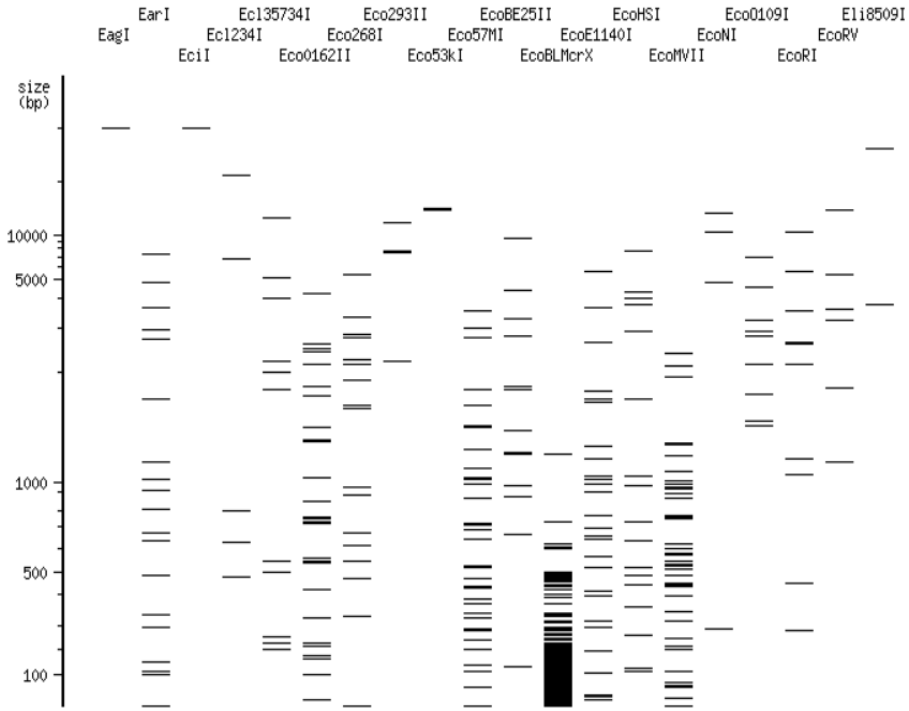
REBsites

NC 045512

Gel: Agarose 0.7%
Order by: enzyme name

[<< Prev] 1 2 3 4 5 6 7 8 9 10 11 12 **13** 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 [Next >>]

[Print]



Click on an enzyme name for a list of fragments/sites.

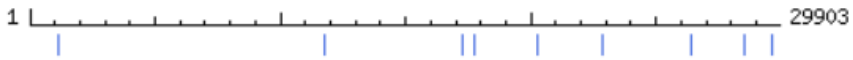
Print

Fragment list

Close

NC 045512 digested with EcoRI

[Sites with flanks]



#	Location	Size [bp]
1	1162-11734	10573
2	11735-17280	5546
3	22871-26439	3569
4	20279-22870	2592
5	17729-20278	2550
6	26440-28551	2112
7	1-1161	1161
8	28552-29620	1069
9	17281-17728	448
10	29621-29903	283

Assignment

- **Write a program to generate a restriction map for Wuhan isolate-1 genome (Acc. Id.: NC_045512) using EcoRI as RE compare your results with REBsites.**
- **Write a program to identify restriction recognition sites in the given DNA sequence.**

Cloning

What is cloning?

The process of cloning involves the production of multiple copies of a DNA fragment of interest by amplification *in vivo*

- depends upon the ability of vectors to continue their life cycles in bacterial or yeast cells in spite of having foreign DNA inserted into them.

Cloning vector - a DNA molecule that carries foreign DNA into a host cell, replicates inside a bacterial (or yeast) cell and produces many copies of itself and the foreign DNA

- a vector containing foreign DNA is termed recombinant vector

Features of Cloning Vectors:

- **sequences that permit the propagation of itself in bacteria (or yeast)**
- **a cloning site to insert foreign DNA; the most versatile vectors contain a site that can be cut by many REs**
- **a method of selecting for bacteria (or yeast) containing a vector with foreign DNA; usually accomplished by selectable markers for drug resistance**

Major requirement of all vectors - an origin of replication for a given host cell in order that they may replicate autonomously (i.e., independently of the host's chromosome)

Types of Vectors

Vector	Insert size (kb)
Plasmids	<10 kb
Bacteriophage	9 - 20 kb
Cosmids	33 - 47 kb
Bacterial artificial chromosomes (BACs)	75 - 125 kb
Yeast artificial chromosomes (YACS)	100-1000 kb

Types of Vectors

Plasmids - an extra-chromosomal double-stranded circular DNA molecules that replicates autonomously inside the bacterial cell

Plasmids are important as one can:

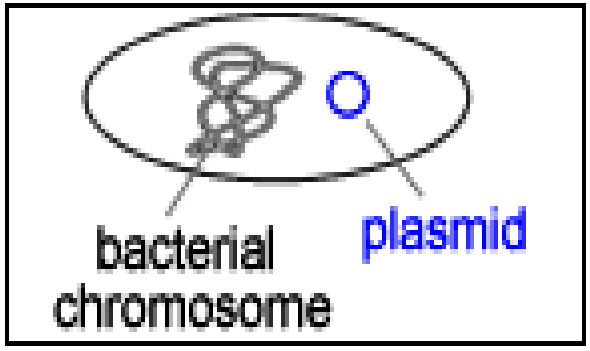
(i) isolate them in large quantities,

(ii) cut & splice them, add DNA of choice,

(iii) put them back into bacteria, where they replicate along with the bacteria's own DNA,

(iv) isolate them again to get billions of copies of inserted DNA

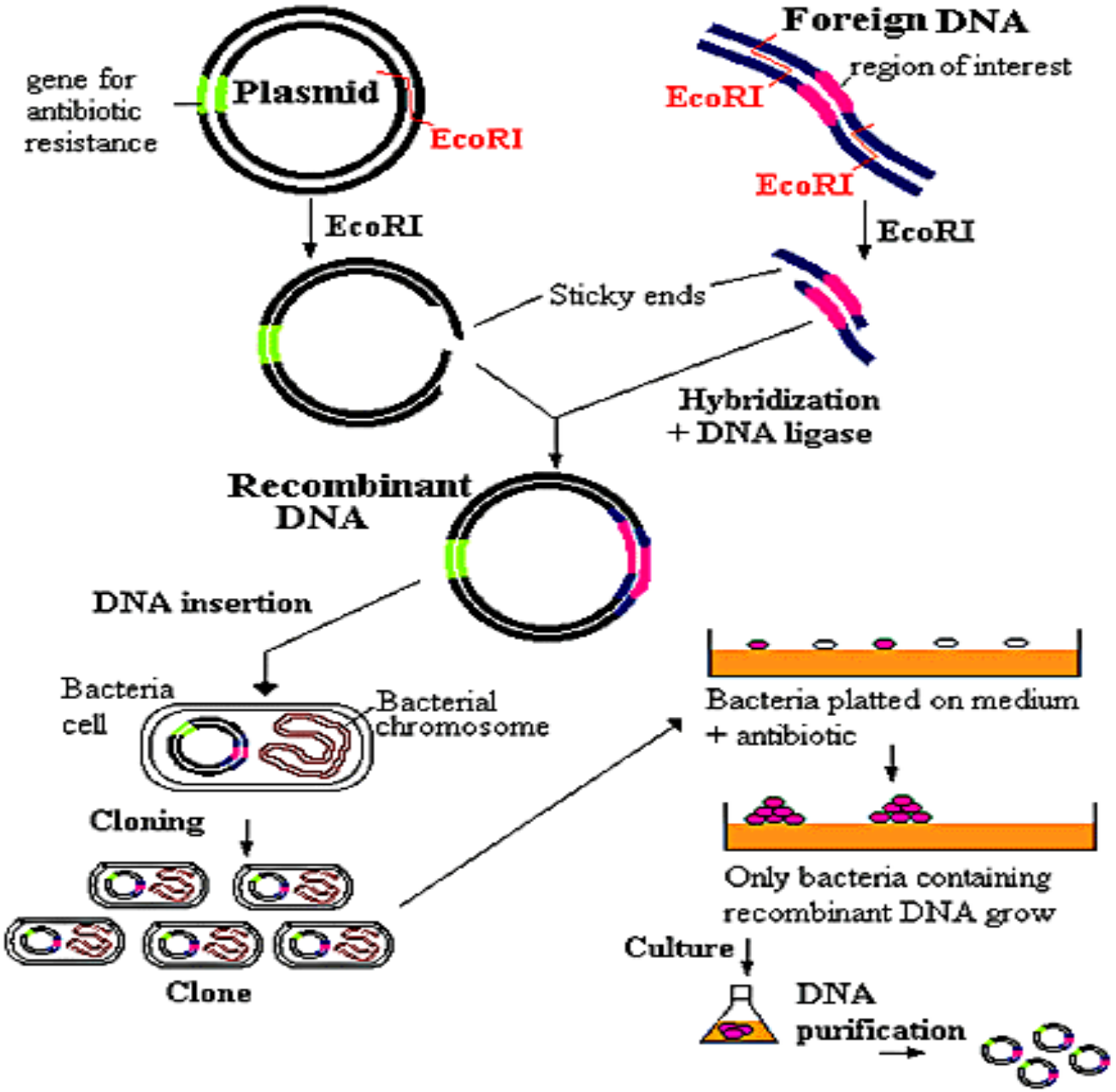
Limitation: size of DNA that can be introduced into the cell by transformation (~ 2 - 10kb)



Plasmid vectors are derived from naturally occurring plasmids of *E. coli* such as ColE1 or from related plasmid pMB1

pBR322 – most widely used cloning vectors of *E. coli*, is a hybrid between ColE1 & genes coding for resistance to antibiotics tetracycline & ampicillin

What's the advantage of inserting genes coding for resistance to antibiotics into a vector?



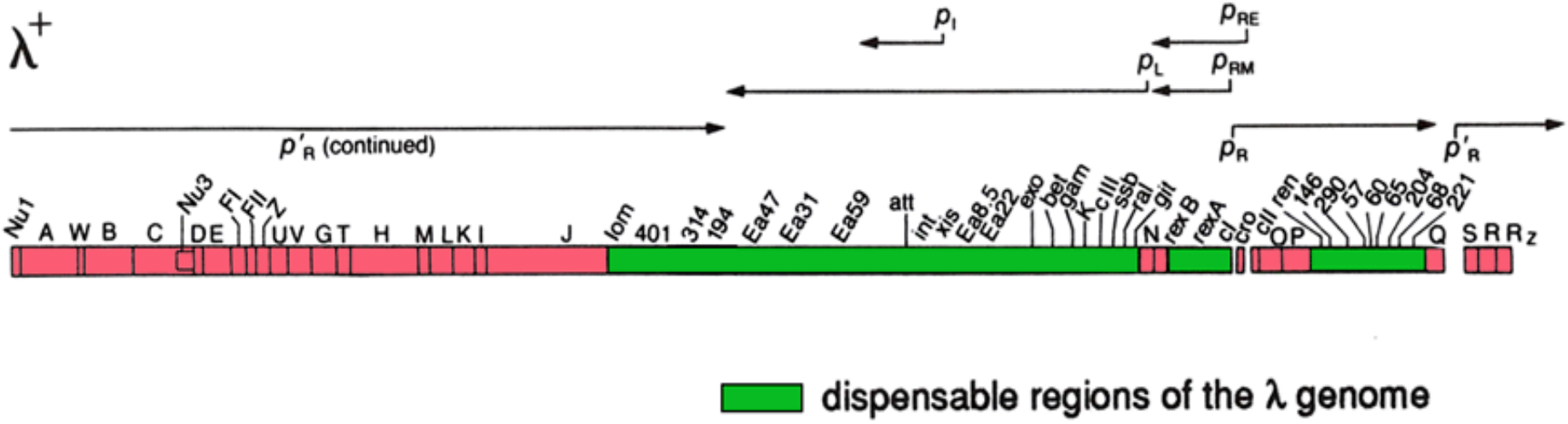
Cloning into a plasmid

Types of Vectors

Bacteriophage Vectors

– a double-stranded linear molecule of size 49.5Kbp

Cloning limit: 9 - 20 kb



Enterobacteria phage λ is a bacterial virus, or bacteriophage, that infects the bacterial species *E. coli*.

Artificially Constructed Vectors

Cosmids - an extra-chromosomal circular DNA molecule that combines features of plasmids and cos gene of phage lambda

Cloning limit: 35 - 50 kb

BAC - Bacterial Artificial Chromosome

- based on naturally occurring F-factor plasmid found in the bacterium E. coli.

Cloning limit: 100-300 kb

YAC - Yeast Artificial Chromosomes

- it is a vector constructed from yeast DNA, used to clone large DNA fragments

Cloning limit: 100-1000 kb

Useful for cloning long segments of eukaryotic DNA

YAC - a functional self-replicating artificial chromosome. It includes three specific DNA sequences that enable it to propagate from one cell to its offspring:

- **TEL: The telomere which is located at each chromosome end, protects the linear DNA from degradation by nucleases**
- **CEN: The centromere which is the attachment site for mitotic spindle fibers, "pulls" one copy of each duplicated chromosome into each new daughter cell.**
- **ORI: Replication origin sequences, specific DNA sequences that allow the DNA replication machinery to assemble on the DNA and move at the replication forks**

It also contains few other specific sequences like:

- **A and B: selectable markers that allow easy isolation of yeast cells that have taken up the artificial chromosome.**
- **Recognition site for two REs: EcoRI & BamHI**

Why is it important to be able to clone large sequences?

To map the entire human genome ($3x10^9$ bps) would require more than 1000,000 plasmid clones (~10Kb limit).

In principle, the human genome could be represented in about 10,000 YAC clones (~1Mb limit)

What determines the choice vector?

- **insert size**
- **vector size**
- **restriction sites**
- **copy number**
- **cloning efficiency**
- **ability to screen for inserts**

DNA Sequencing

DNA Sequencing - determine the precise sequence of nucleotides in a sample of DNA – the order of A, T, G, C

Various types of sequencing:

- **Sequencing a region of interest, e.g., gene.**
- **Whole Genome/Exome Sequencing**
- **cDNA Sequencing – sequencing cDNA libraries of the expressed genes**
- **High-throughput sequencing – next-generation, 3rd & 4th generation sequencing - whole Genome/Exome/targeted**
- **Metagenome sequencing - sequencing of environmental samples**

- depending on the nature of analysis, type of sample, or type of sequencer used

Sequencing a Region of Interest

First requirement in sequencing a region of DNA is

- to have enough starting template for sequencing.

This is achieved by PCR - Polymerase Chain Reaction

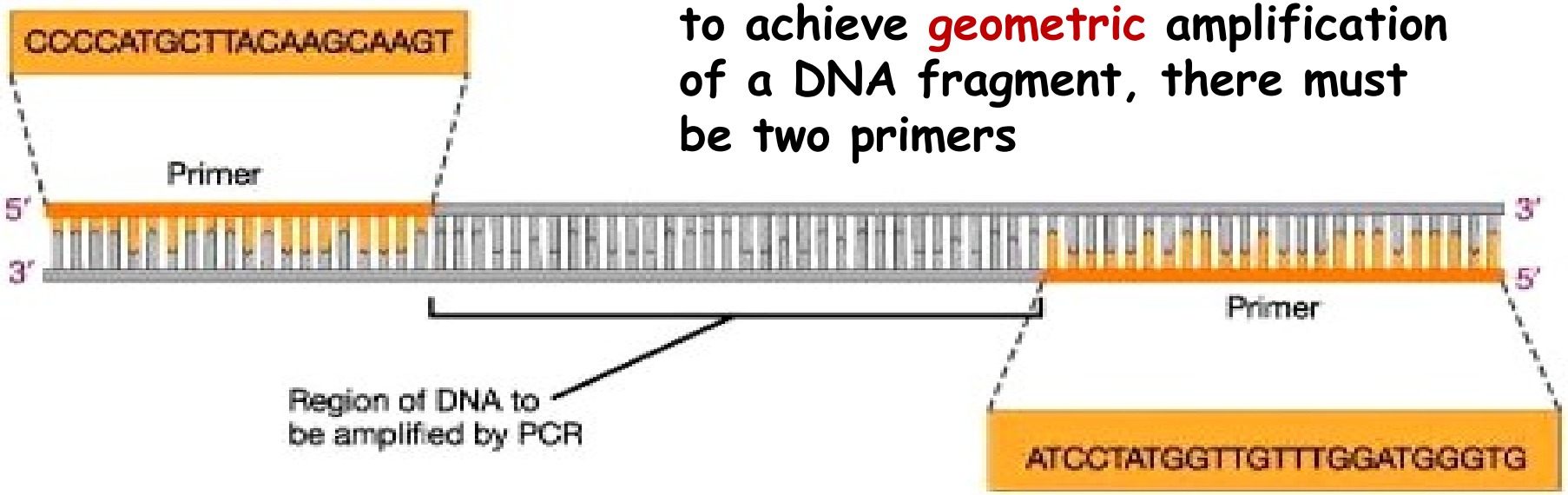
- **carried out in an automated cycler for 30 - 40 cycles.**

Essential requirements for a PCR:

- **a mixture of 4 deoxy-nucleotides in ample quantities: dATP, dGTP, dCTP, dTTP**
- **Taq DNA polymerase**
- **Primers ?**
- **Genomic DNA of interest**

What is the advantage of using PCR over traditional gene cloning?

Region of DNA to be amplified by PCR



to achieve geometric amplification of a DNA fragment, there must be two primers

Primers - short single-stranded oligonucleotides which anneal to the DNA template and serve as a starting point for DNA synthesis

Why are primers required?

The Cycling Reactions

Step-1: Denaturation at 94°C

- opens up double stranded DNA, all enzymatic reactions stop.

Step-2: Annealing at 54°C

- Primers jiggling around because of Brownian motion, binds to single stranded template once an exact match is found; the polymerase then attaches and start copying the template.

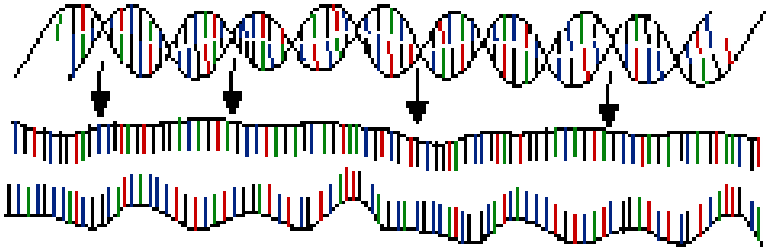
Step-3: Extension at 72°C

- ideal working temperature for the polymerase. Bases complementary to the template are coupled to the primer on 3' side (reading the template from 3' to 5' side)

Different Steps in PCR

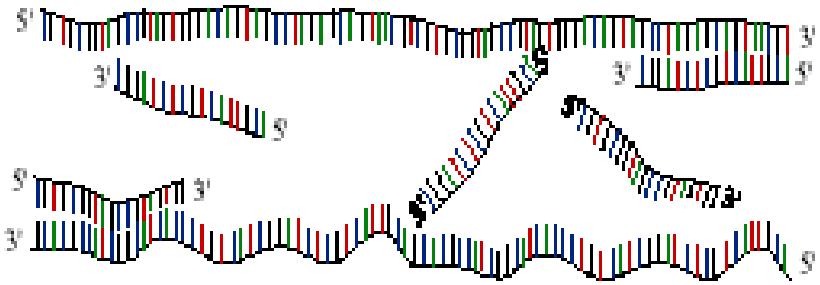
PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

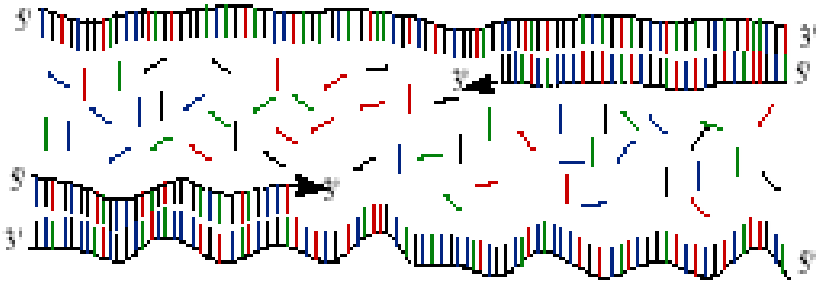
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!

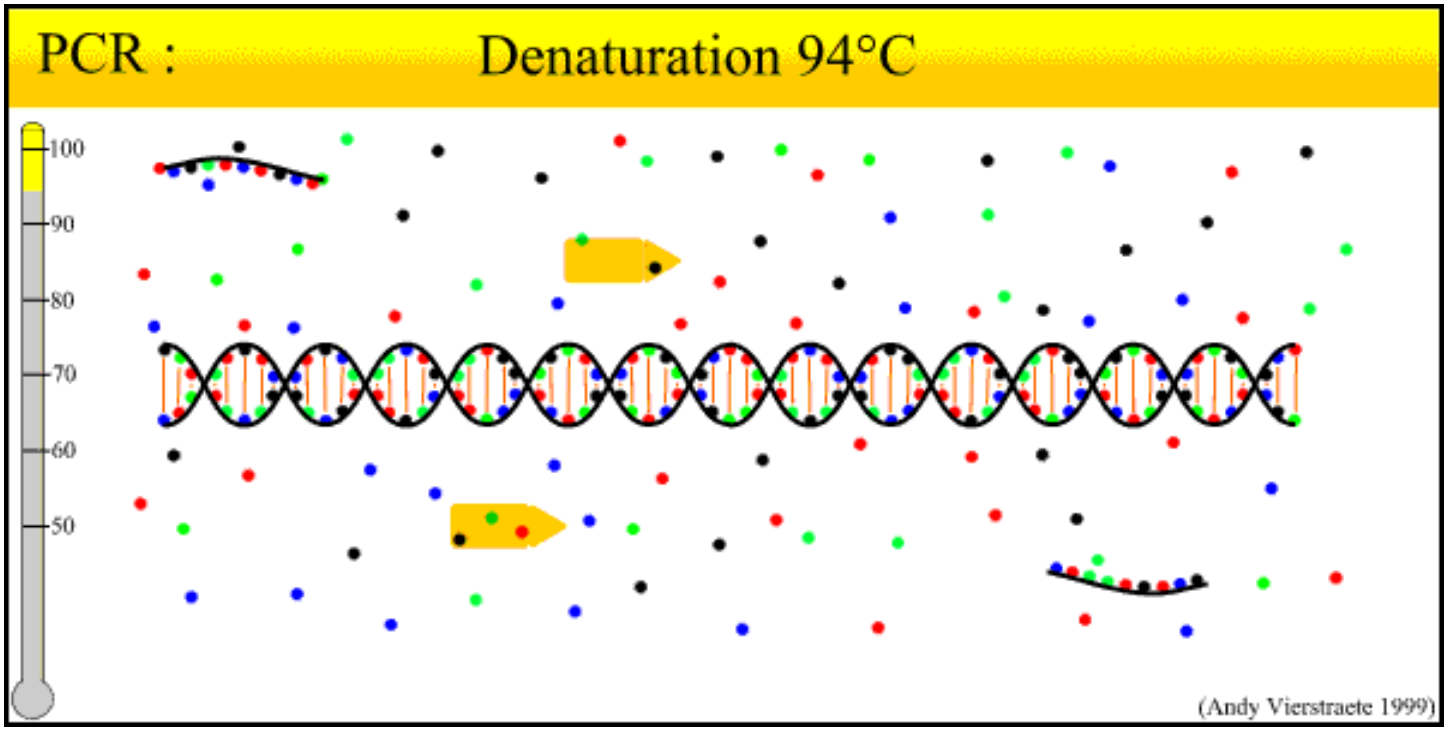


Step 3 : extension

2 minutes 72 °C

only dNTP's

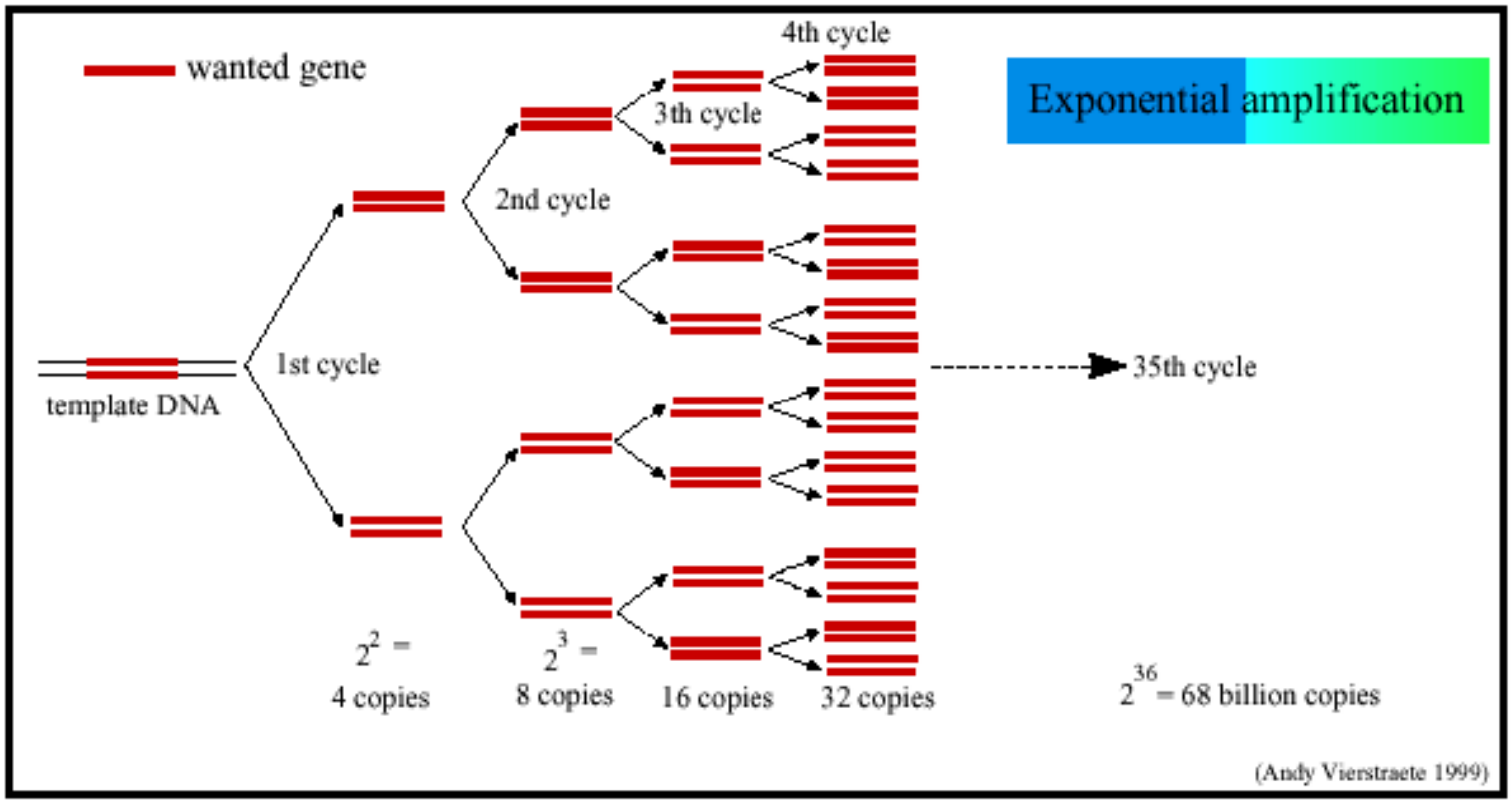
Different Steps in PCR



Exponential amplification of region of interest

<u>Both strands</u> are copied during PCR

- leading to an exponential increase of the number of copies of the region of interest.



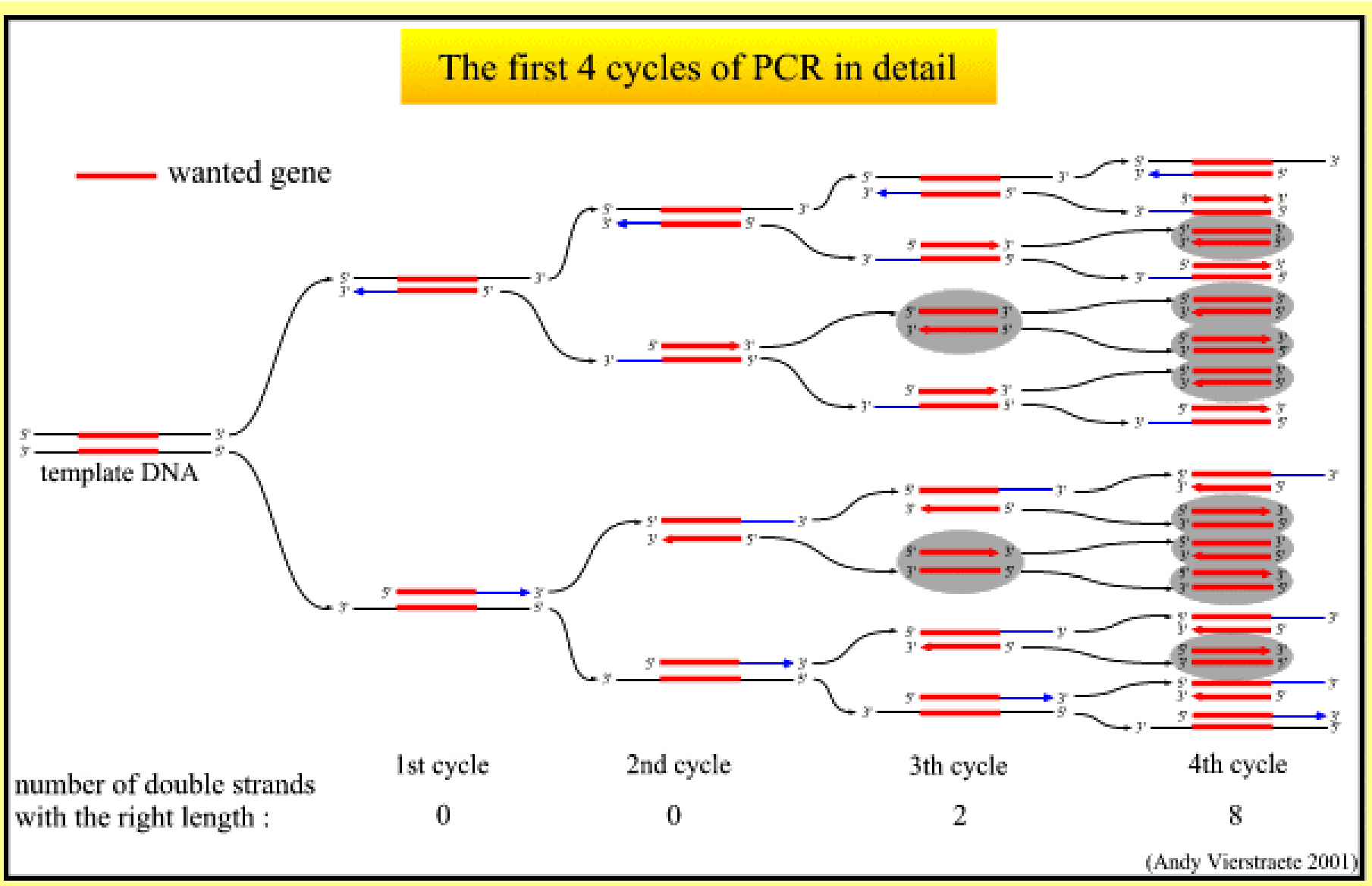
Verification of PCR Product

Is the template copied during PCR and is it the right size?

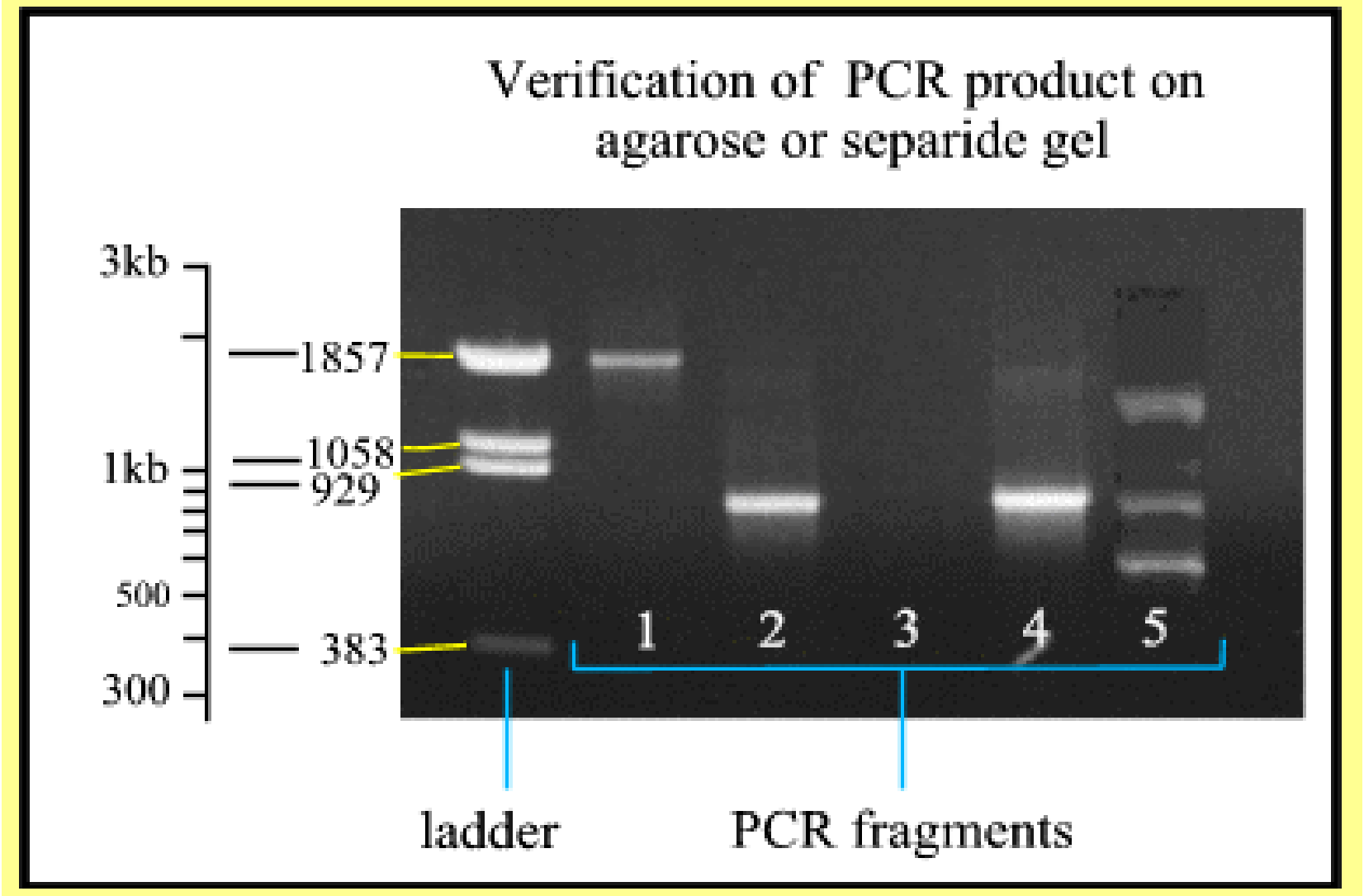
Before the PCR product is used in further applications, it has to be checked if:

1. **A product is formed**
2. **The product is of the right size**
3. **Only one band is formed**

First 4 cycles of a PCR reaction



Verification of the PCR product

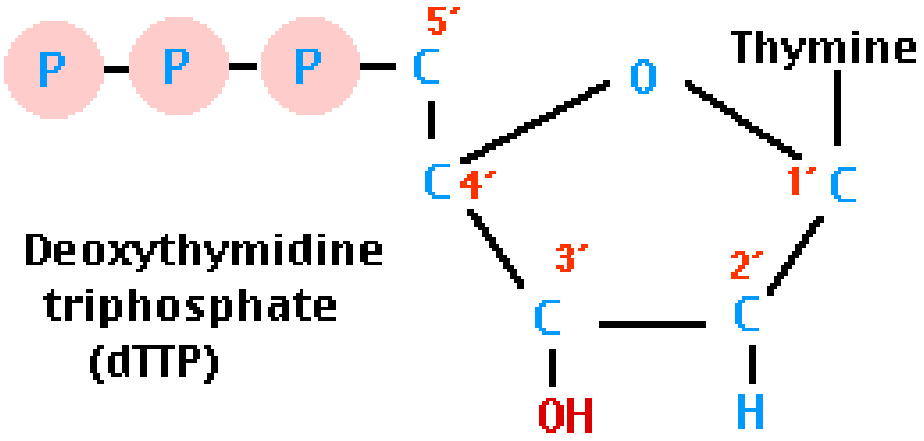


PCR Sequencing

For sequencing, we don't start from gDNA (like in PCR) but mostly from PCR fragments or cloned genes.

Amplified PCR product is supplied with

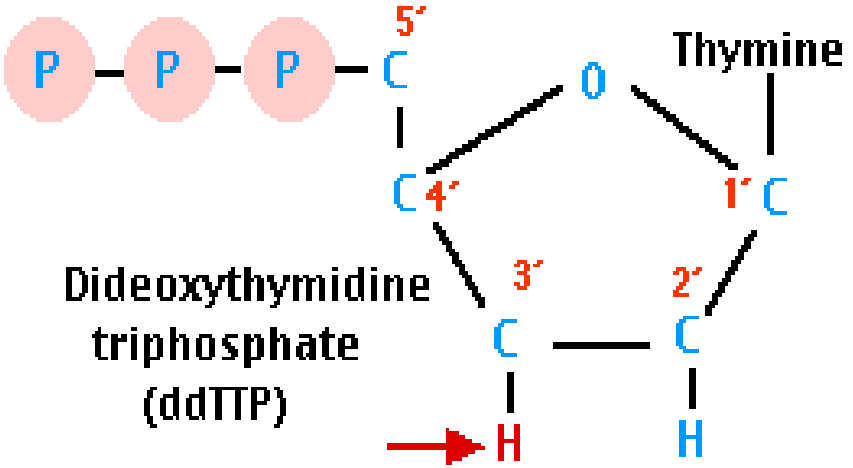
- **a mixture of all four <u>normal</u> (deoxy) nucleotides in ample quantities**
  - **dATP**
  - **dGTP**
  - **dCTP**
  - **dTTP**



- ***Taq* DNA polymerase**

PCR Sequencing

- a mixture of all four dideoxynucleotides, each present in limiting quantities and each labeled with a "tag" that fluoresces a different color:
  - ddATP
  - ddGTP
  - ddCTP
  - ddTTP



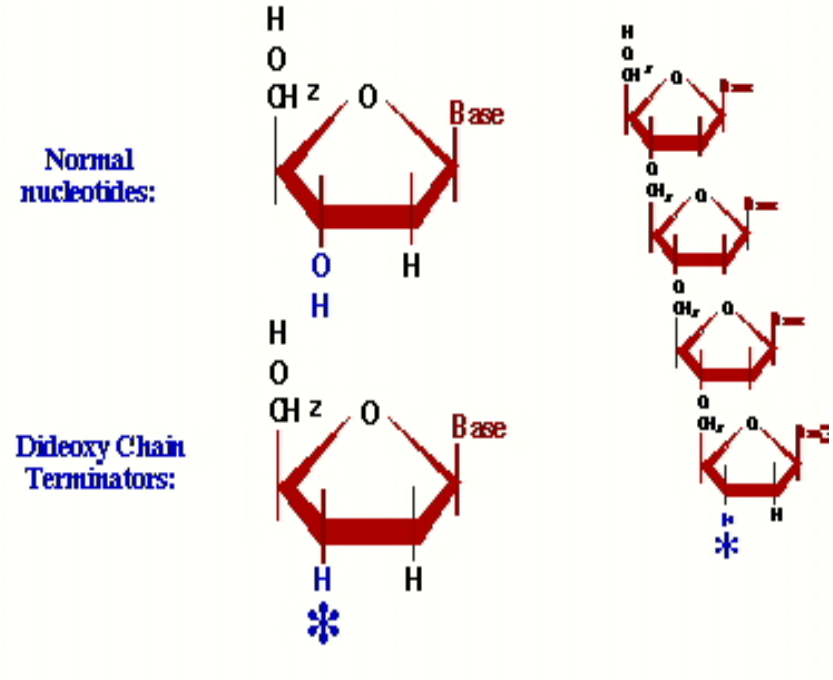
This method of DNA sequencing is called dideoxy method, or chain termination method, or Sanger's method.

PCR Sequencing

Dideoxy method: DNA is synthesized from four deoxynucleotide triphosphates.

Each new nucleotide is added to 3′ -OH group of the last nucleotide added.

When a dideoxynucleotide, ddNTP is added to the growing DNA strand, chain elongation stops because there is no 3′-OH for the next nucleotide to be attached to.



Steps in PCR Sequencing

I The sequencing reaction

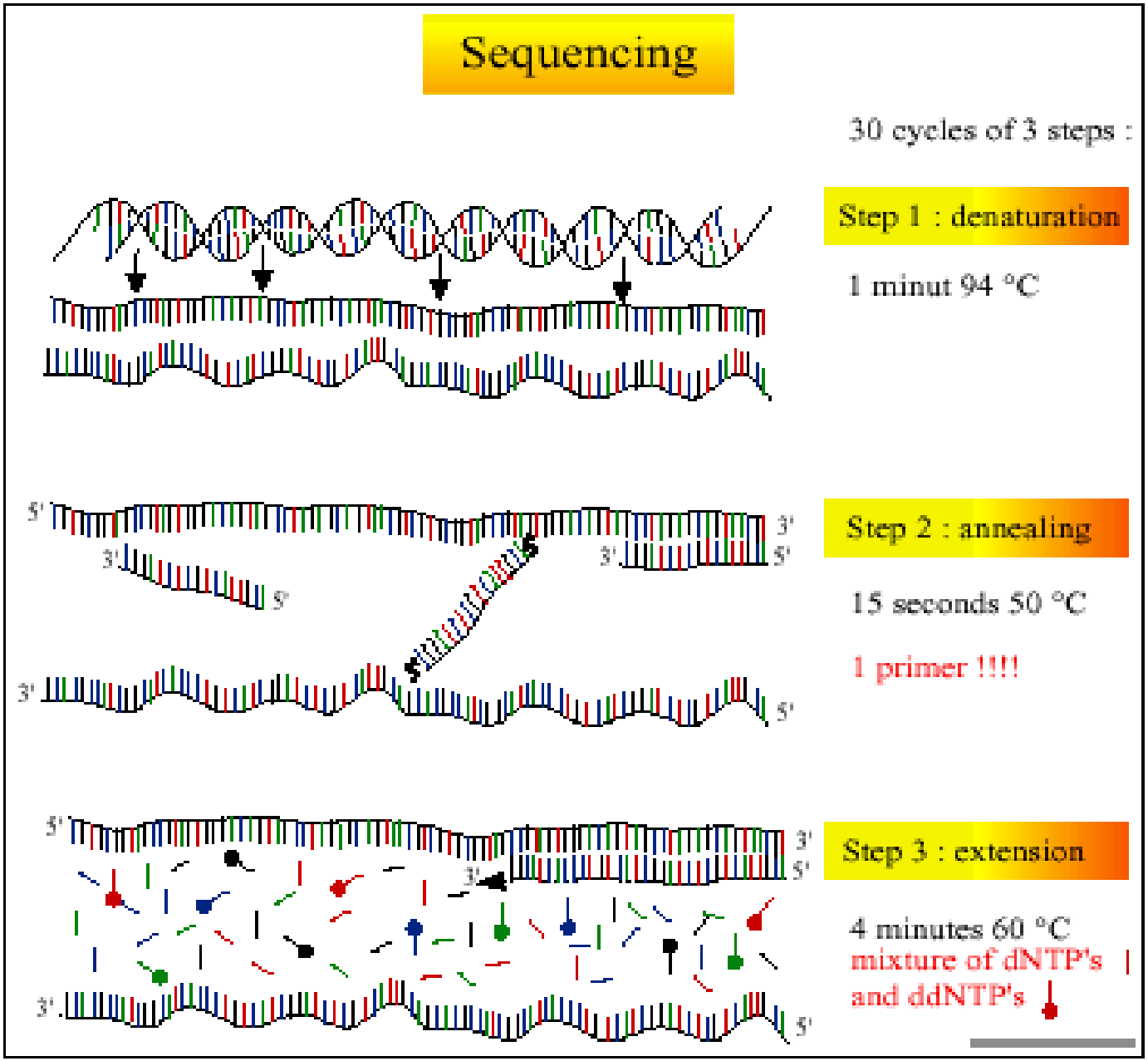
- **Denaturation at 94°C**
- **Annealing at 50°C**
- **Extension at 60°C** ← **instead of 72°C**

II Separation of the fragments

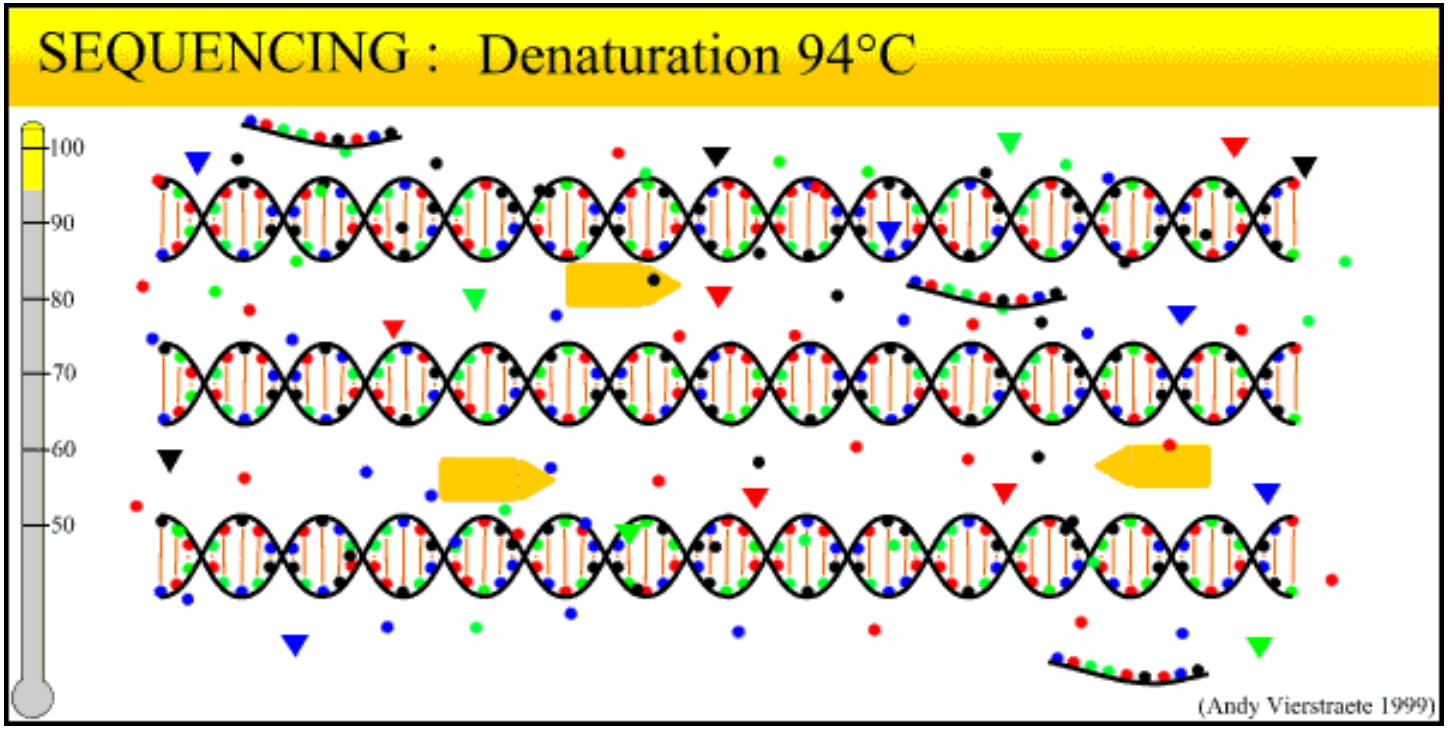
III Detection on an automated sequencer

IV Assembling the sequenced parts

Different steps in Sequencing

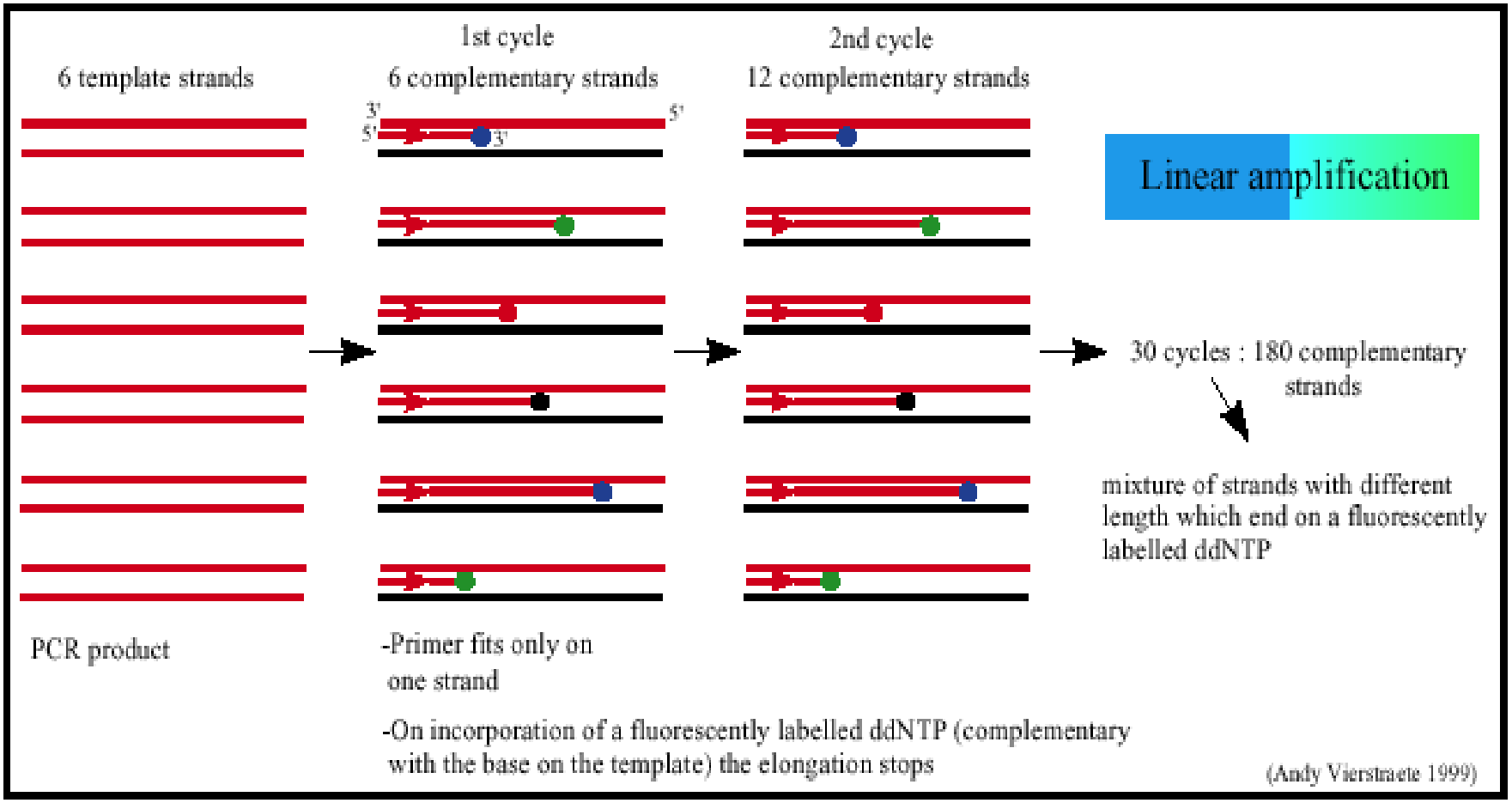


Different steps in Sequencing



PCR Sequencing

Since only one primer is used, only one strand is copied during sequencing – resulting in a linear increase of the number of copies of one strand of the gene. Hence, a large amount of DNA in the starting mixture for sequencing is required.



PCR Sequencing

II Separation of the molecules:

After the sequencing reactions, the mixture of strands of different lengths, all ending on a fluorescently labeled ddNTP, need to be separated

- done by loading the mix on an acrylamide gel - gel electrophoresis.

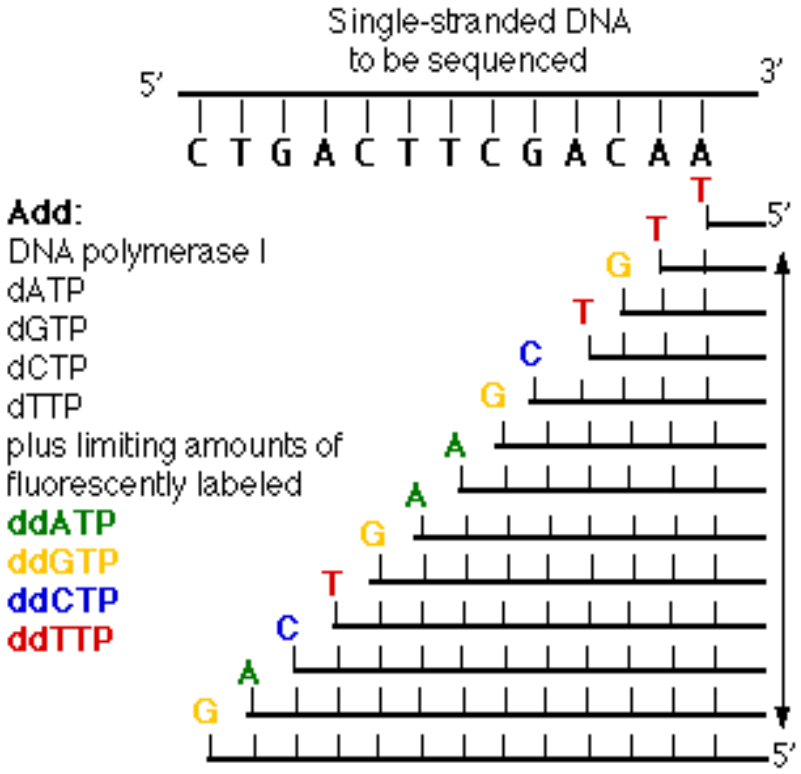
During electrophoresis, a voltage is created across the gel making one end positive and the other negative. DNA being –vely charged, migrates to the positive side.

DNA strands of different length migrate at different rates and thus can be separated based on their size - the smallest strand travels the fastest.

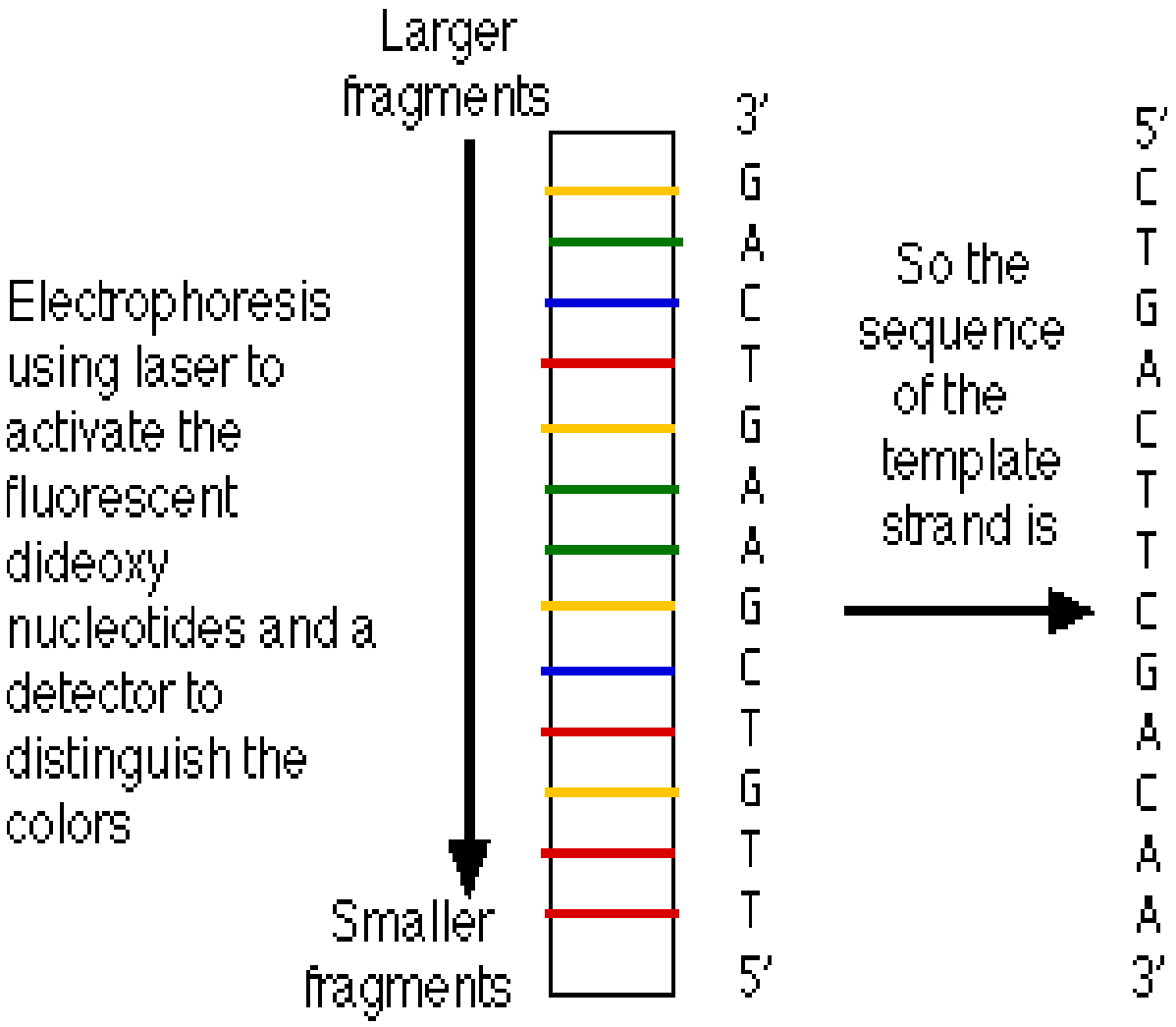
Separation of molecules with electrophoresis

Very good resolution - a difference of even one nucleotide is enough to separate a strand from the next shorter or longer strand.

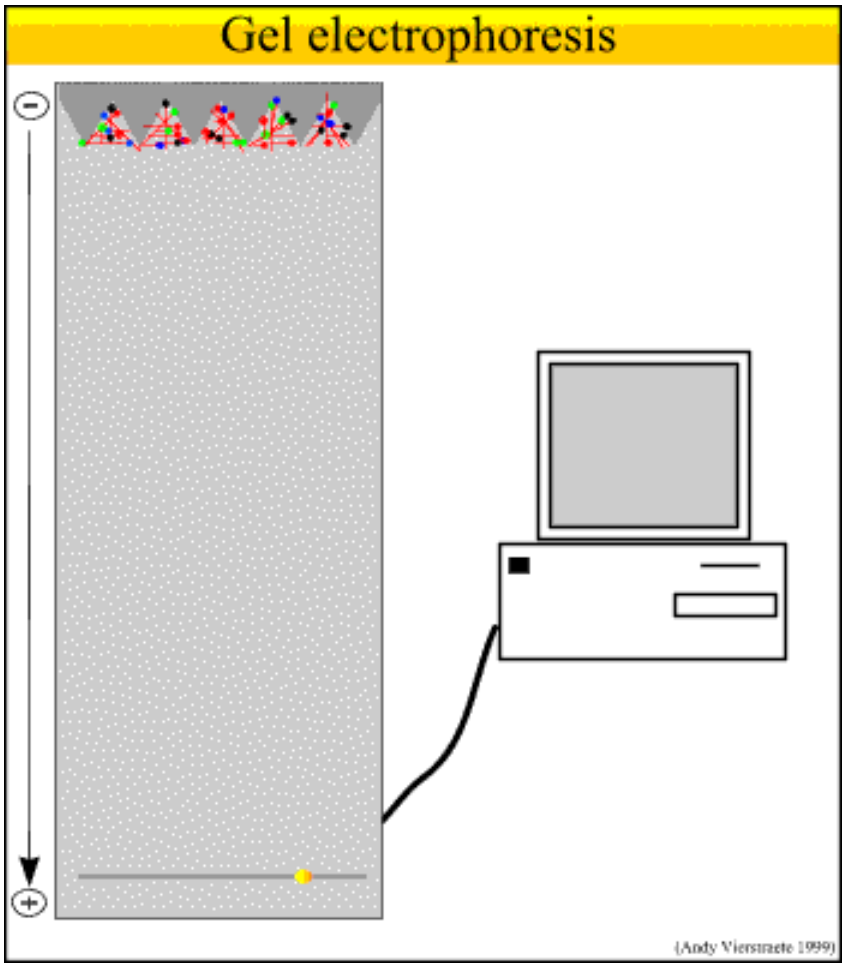
Four dideoxynucleotides fluoresces a different color when illuminated by a laser beam and an automatic scanner provides a printout of the sequence.



Separation of Molecules with Electrophoresis



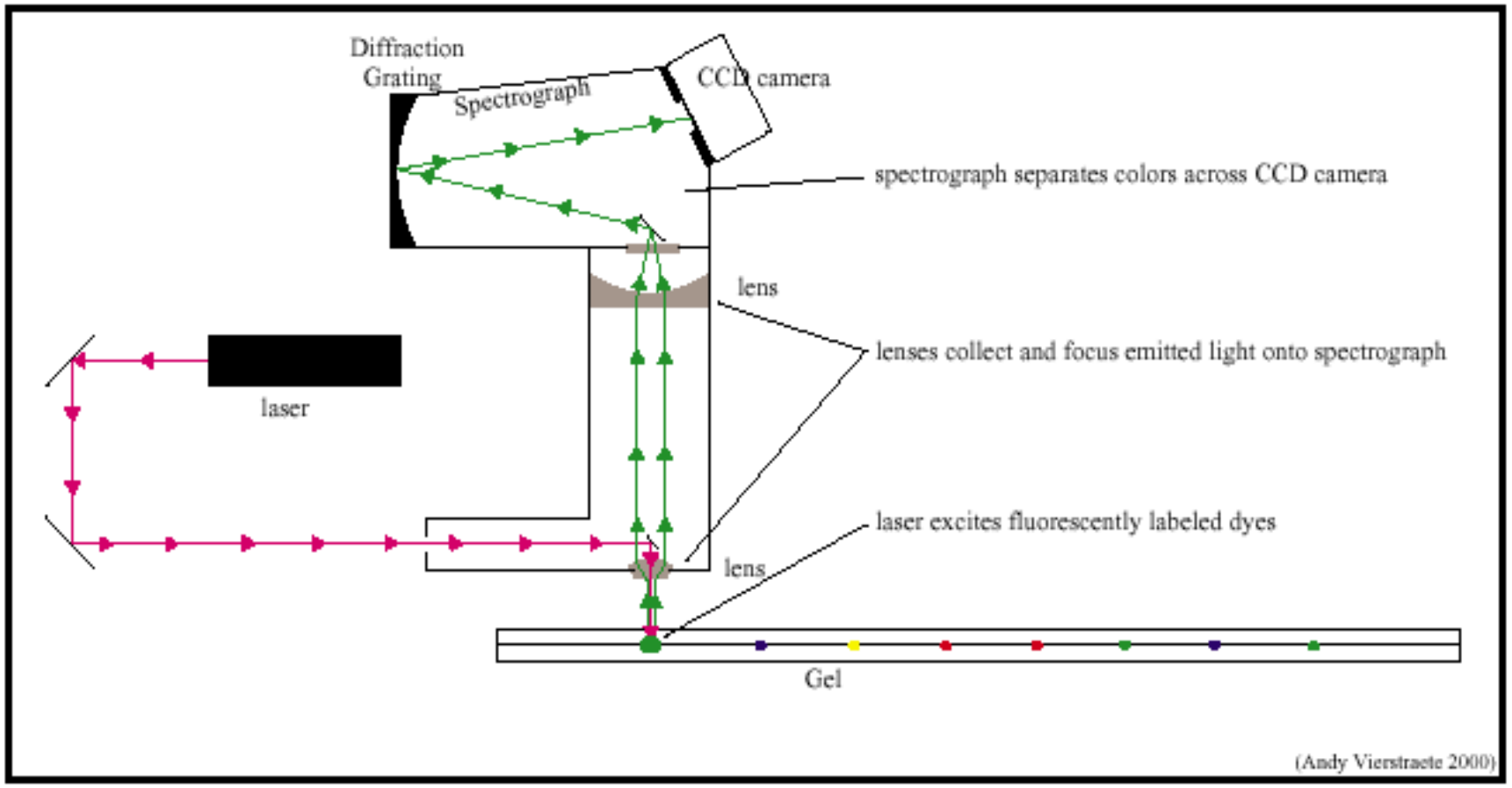
Separation of the Molecules with Electrophoresis



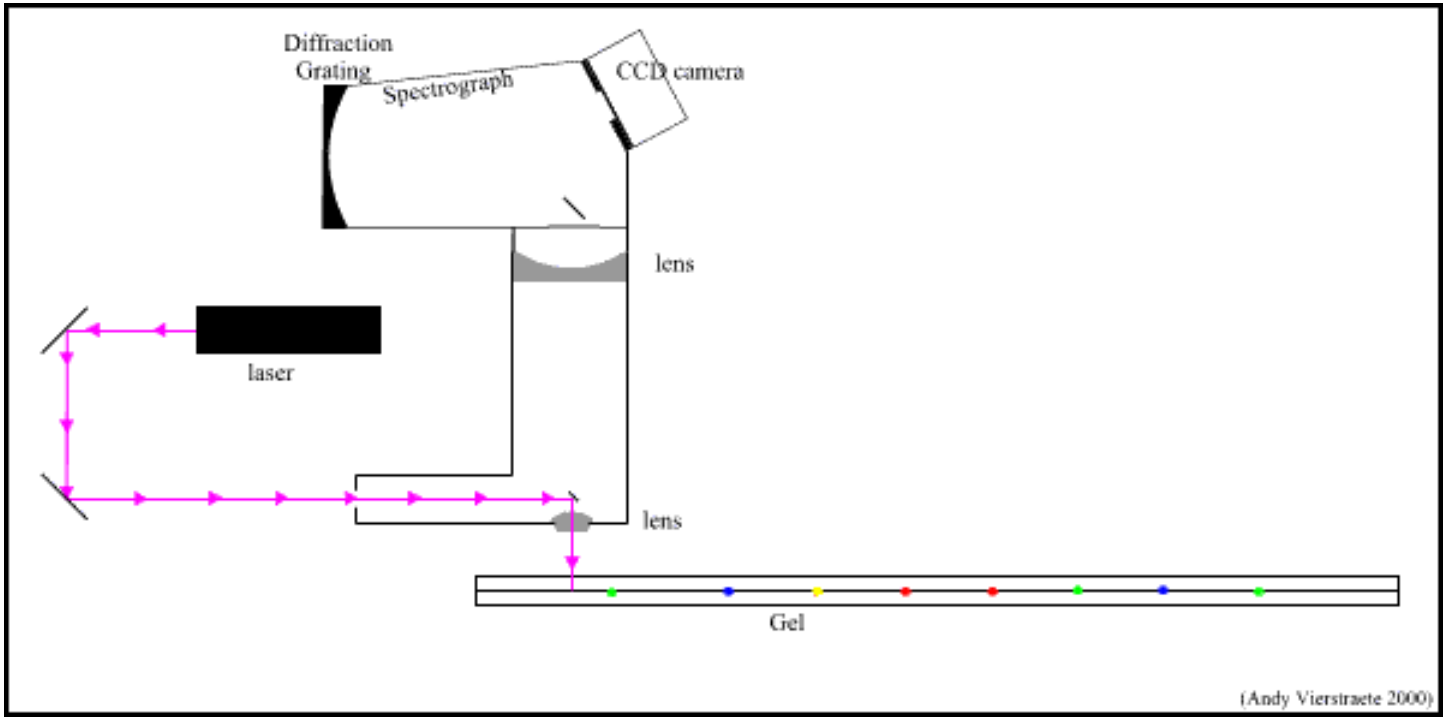
PCR Sequencing

III Detection on an automated sequencer:

Fluorescently labeled fragments that migrate through the gel pass a laser beam at the bottom of the gel.

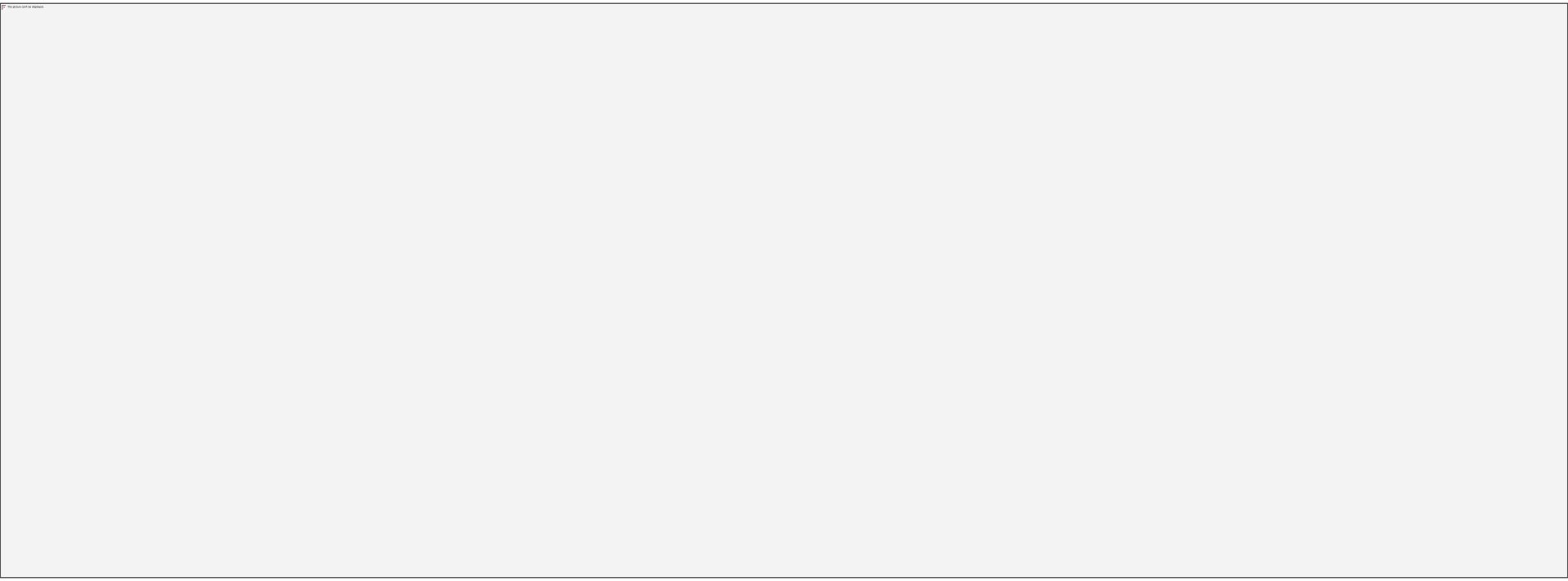


Scanning & Detection System on a Sequencer



PCR Sequencing

Plot of the colors detected in a 'lane' of the gel (one sample), scanned from smallest fragments to largest.



The computer interprets the colors by printing the nucleotide sequence across the top of the plot.

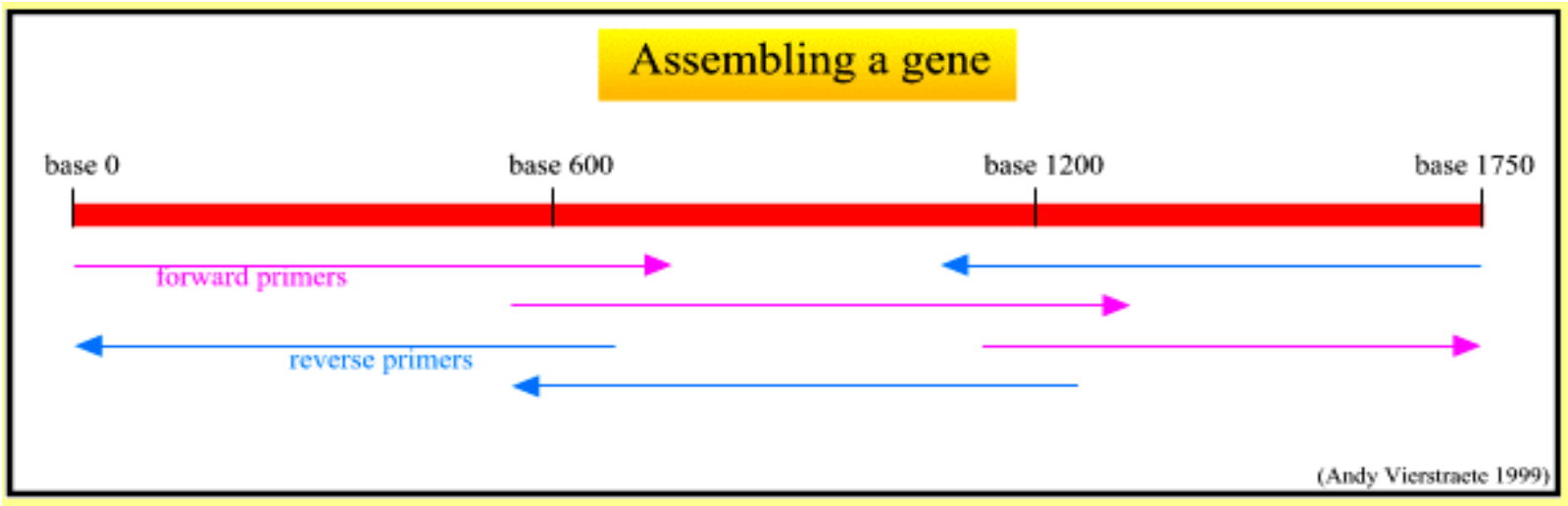
PCR Sequencing

IV Assembling the sequenced parts of a gene:

For publication, a gene sequence has to be confirmed in both directions using forward & reverse primers

Since it is only possible to sequence ~ 700-800 bases in one run, a gene of, say, 1800 bases, is sequenced with internal primers.

- the sequenced fragments are assembled using a computer program to obtain complete gene sequence.



Genome Sequencing

Genome Sequencing

By Sanger's method, we can sequence a fragment of DNA ~ 1000bp long.

But what about longer pieces?

Human genome is 3 billion bases long, arranged on 23 pairs of chromosomes.

Sequencing machine reads just a drop in the ocean!

Genome Sequencing

Solution: Break the entire genome into manageable pieces and sequence them.

Two approaches used for sequencing Human genome:

- **Publicly funded Human Genome Project (HGP) – clone-by-clone or hierarchical shotgun sequencing method**
- **Privately Funded Sequencing Project - Celera Genomics – whole genome shotgun sequencing method**

Genome Sequencing

Hierarchical shotgun sequencing approach:

- **genomic DNA is cut into pieces of about 150 Mb**
- **inserted into BAC vectors,**
- **transformed into *E. coli* where they are replicated and stored.**

BAC inserts are isolated & mapped to determine the order of each cloned 150 Mb fragment - referred to as the Golden Tiling Path

Begun formally in 1990, Human Genome Project was a 13-yr effort coordinated by the U.S. DAE and NIH.
- completed in 2003

Genome Sequencing

Each BAC fragment in the Golden Path is

- fragmented randomly into smaller pieces,

- each piece is cloned into a plasmid and sequenced on both strands.

These sequences are aligned so that identical regions overlap.

Contiguous pieces are then assembled into finished sequence once each strand has been sequenced about 5 times to produce 10× coverage of high-quality data.

Genome Sequencing

Whole genome shotgun sequencing (WGS)

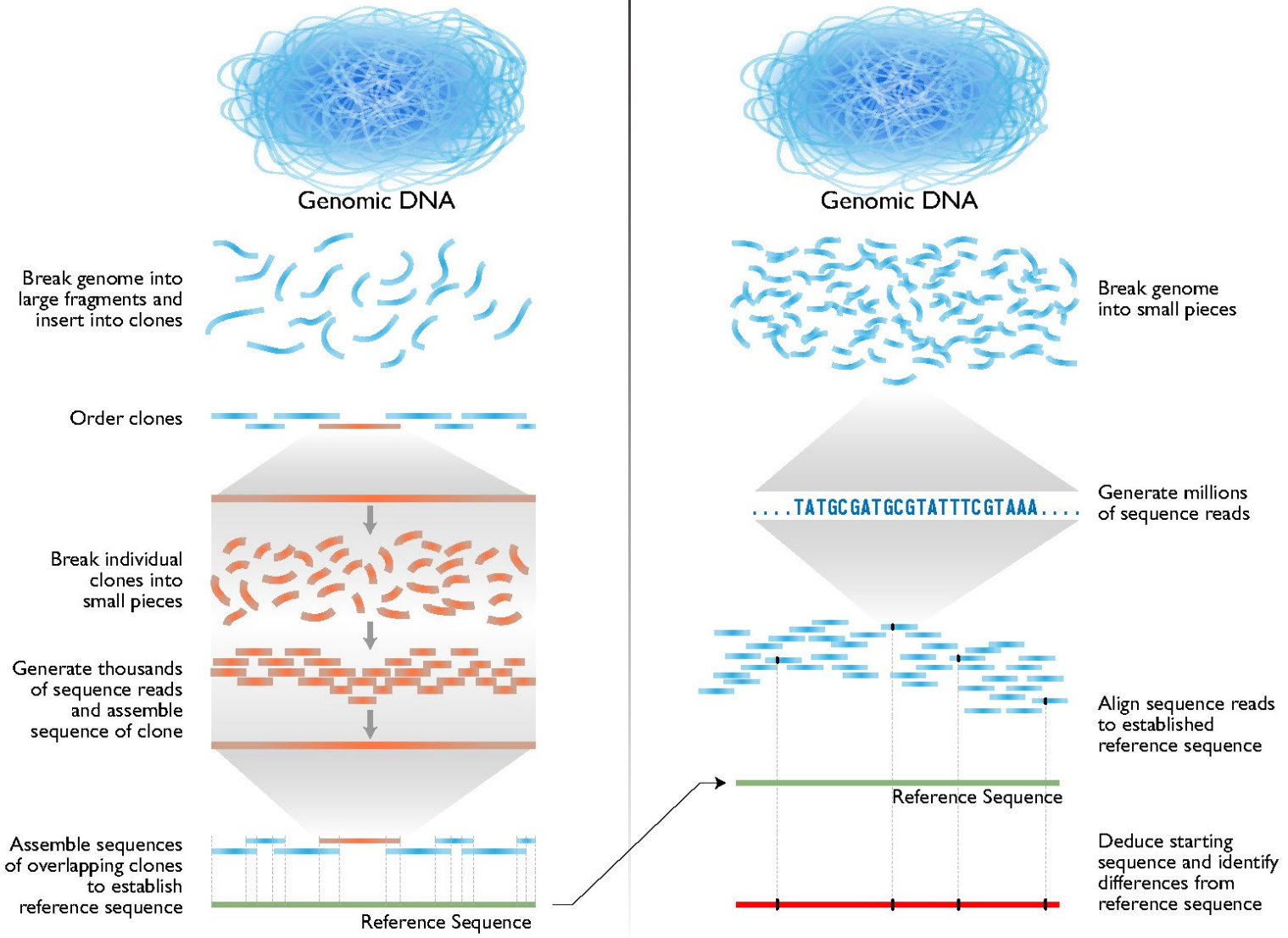
- method developed and preferred by Celera Genomics

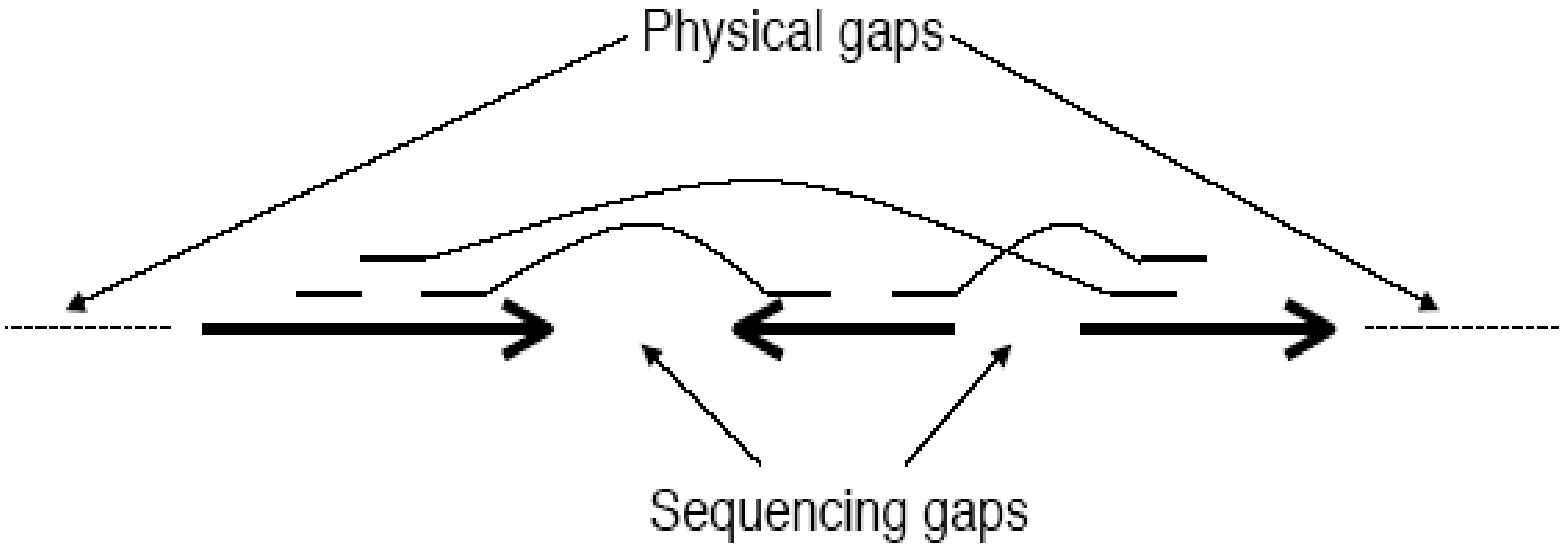
- skips the entire step of making libraries of BAC clones

Blast apart entire human genome into fragments of 2 - 10 kb and sequence them.

Challenge is then to assemble these fragments into the whole genome sequence.

Human Genome Sequencing





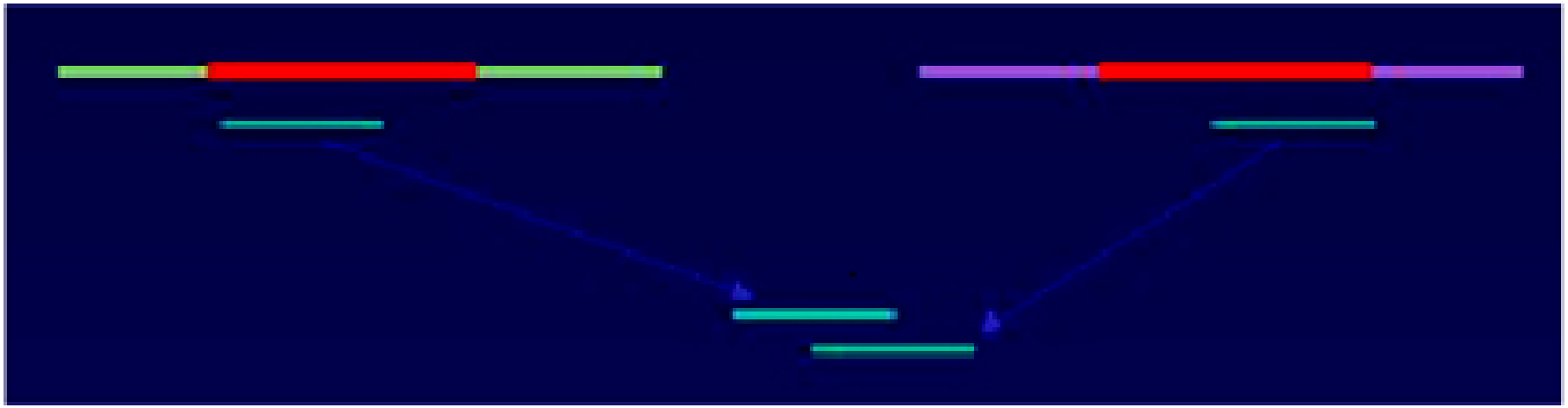
sequencing gap - we know the order and orientation of the contigs and have at least one clone spanning the gap

physical gap - no information known about the adjacent contigs, nor about the DNA spanning the gap

Whole Genome Shotgun Method

What makes the task of assembling the genome fragments especially challenging

- **repeats in the genome (~ 50% in human genome).**



Because of the various ways a fragment could align with a repeat, and the different areas adjacent to the repeats in the original genome, assemblers need to be designed so as not to incorrectly join fragments

Whole Genome Shotgun Method

Adding to the challenge is the sheer computational complexity of the task.

e.g., human genome is 3 billion base pairs long and if the length of one read is 500 bps and the desired coverage is 10x, then $6 * 10^7$ reads would be required:

GenomeLength * DesiredCoverage / ReadLength = RequiredReads

With 60 million reads to assemble, we need algorithms that run in near linear time ($O(nlogn)$)

Whole Genome Shotgun Method

Which method is better?

Depends on the size and complexity of the genome

Note: Celera had access to the HGP data but the HGP did not have access to Celera data.

Which method is preferable for sequencing the genome of a novel coronavirus – SAR-CoV-2? Why?

cDNA Sequencing

Sequencing cDNA Libraries of Expressed Genes

Two common goals in sequence analysis are

- **to identify sequences that encode proteins, which determine all cellular metabolism, and**
- **to discover sequences that regulate the expression of genes or other cellular processes.**

Genomic sequencing meets both the goals.

However, only a small percentage of the genomic sequence actually encodes proteins

cDNA Sequencing

Computational methods for analyzing genomic sequences and finding protein-encoding regions are not completely reliable

cDNA libraries are prepared that have the sequences of the mRNA molecules expressed in the cells, or else cDNA copies are sequenced directly by RT-PCR

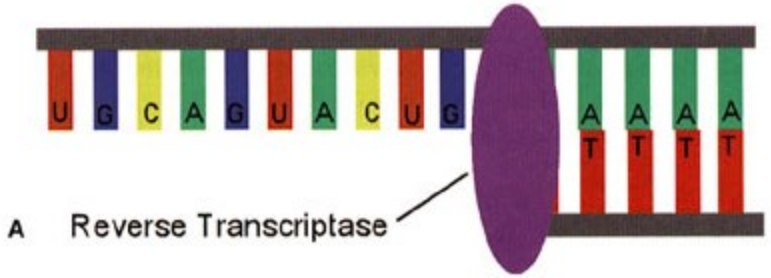
Reverse transcription polymerase chain reaction (RT-PCR) - is used to qualitatively detect gene expression through creation of complementary DNA (cDNA) transcripts from RNA.

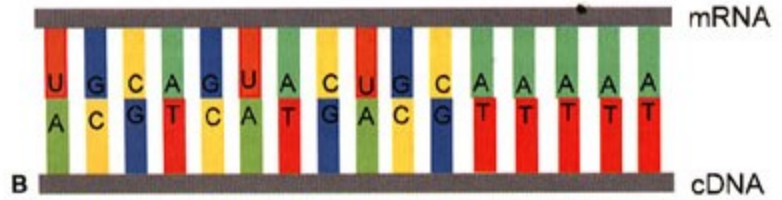
RT-PCR

RNA Template

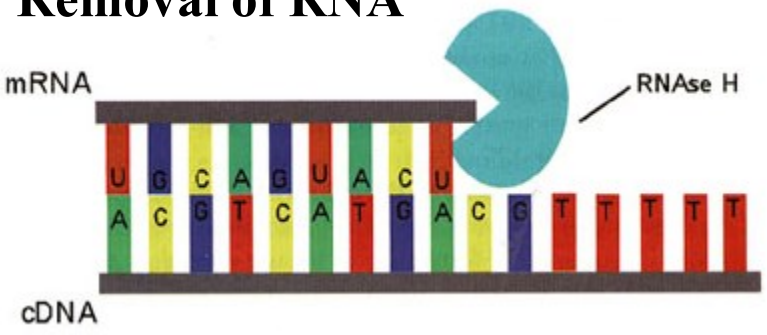
Priming for Reverse Transcription

First Strand Synthesis

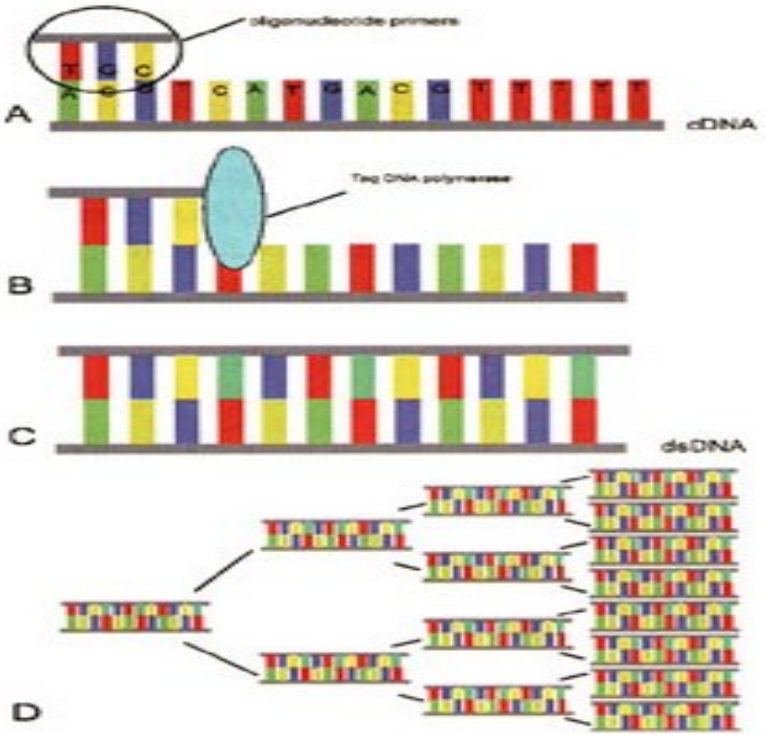




Removal of RNA



The PCR Reaction



cDNA Sequencing

Can all protein-coding genes of an organism be identified by cDNA sequencing?

cDNA Sequencing

Can all protein-coding genes of an organism be identified by cDNA sequencing?

Difficulty with this approach - a gene of interest may be developmentally expressed or regulated in such a way that the mRNA is not present

This problem is circumvented by pooling mRNA from a variety of tissues & developing organs, or subjecting the organism to several environmental influences

Current gold standard for protein-coding gene annotation is EST or full-length cDNA sequencing followed by alignment to a reference genome.

EST – expressed sequence tag

cDNA Sequencing

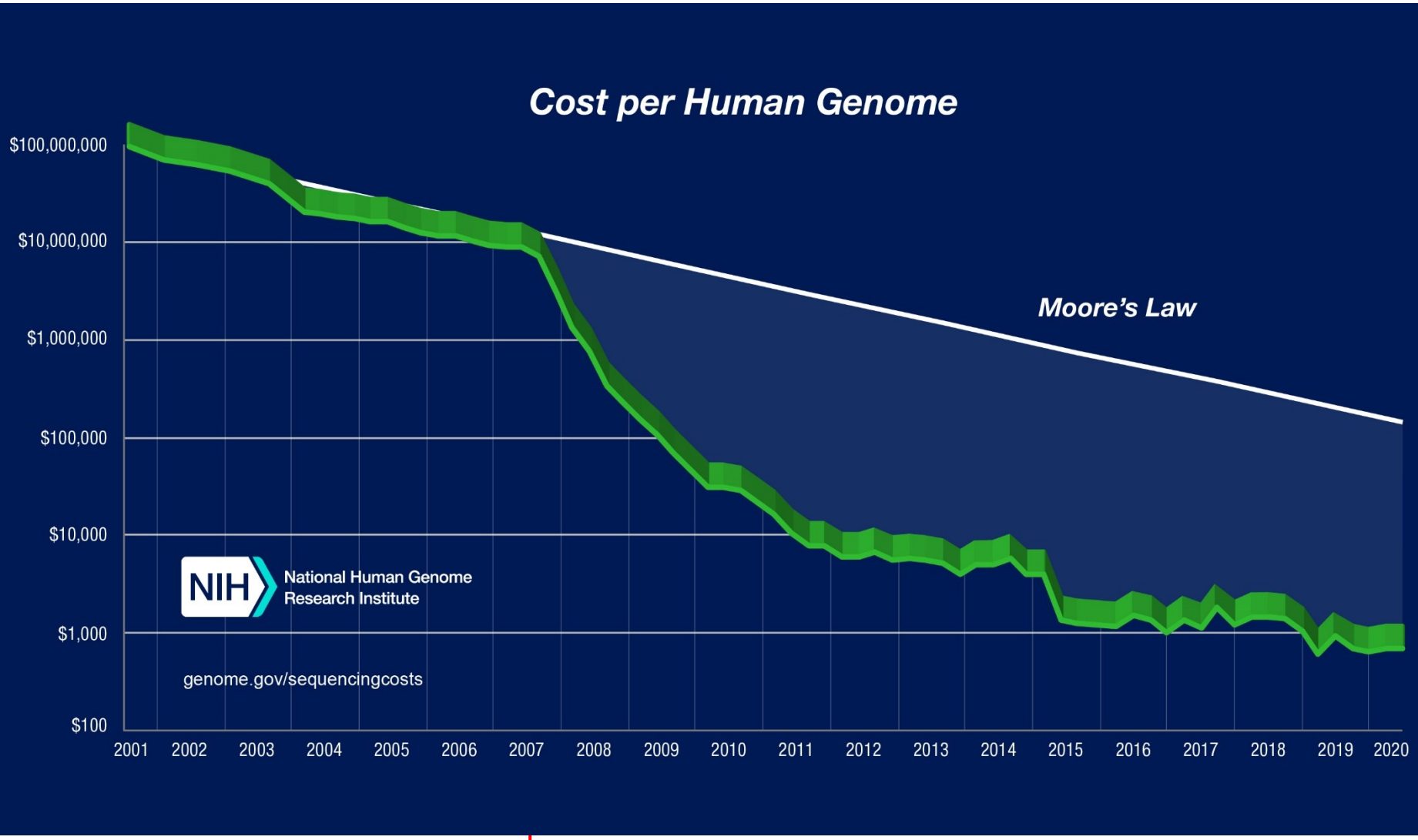
An important development in computational approaches was by Craig Venter - to prepare databases of partial sequences of expressed genes, called expressed sequence tags or ESTs.

- which are long enough to give a pretty good idea of the protein sequence.

To identify the function of the cloned gene, translated EST sequence can be compared to a database of protein sequences - to find its homologs with known function.

Corresponding cDNA clone of the gene of interest can then be obtained and the gene completely sequenced.

High-throughput / Next-Generation Sequencing



13yrs, $3 billion **S.S.** **8days, $10,000** **15min, <$1,000**

DNA sequencing beating Moore's law

HTS/NGS Sequencing

High-throughput sequencing (HTS) technologies have revolutionized the way biologists acquire and analyze genomic data.

- massively parallel sequencing

HTS instruments such as

- **454 from Roche Diagnostics,**
- **Illumina Genomic Analyzer, and**
- **Applied Biosystems SOLiD System,**
- **Helico's Single-molecule sequencing platform**
- **MinION, Oxford Nanopore Technologies**

- can generate tens of gigabases per week, at a cost 200-fold less than previous methods, potentially enabling the routine sequencing of human and other genomes.

Sequencing Machines: Overview

	Roche GS FLX+	Illumina HiSeq 2000	SOLiD™ 4	Ion Torrent PGM
Bases per run	700Mb	600 Gb	100 GB	1 Gb
Time per run	23h	~11 days	~14 days	4.5 h
Reads per run	1 Million	6 Billion (paired-end) 3 Billion (single)	1.4 Billion	Millions
Read length	~700 bp	2 x 100 bases	2 x 50 bases	35–400 bases

MinION – 10-100Kb read lengths, high error rates (~10-15%)

Sequencing Machines: Overview

Roche GS-FLX

1. Pyrosequencing

Life Technologies SOLiD

3. Sequence by ligation

Illumina HiSeq

2. Sequence by Synthesis

Life Technologies Ion Torrent

4. Proton Detection

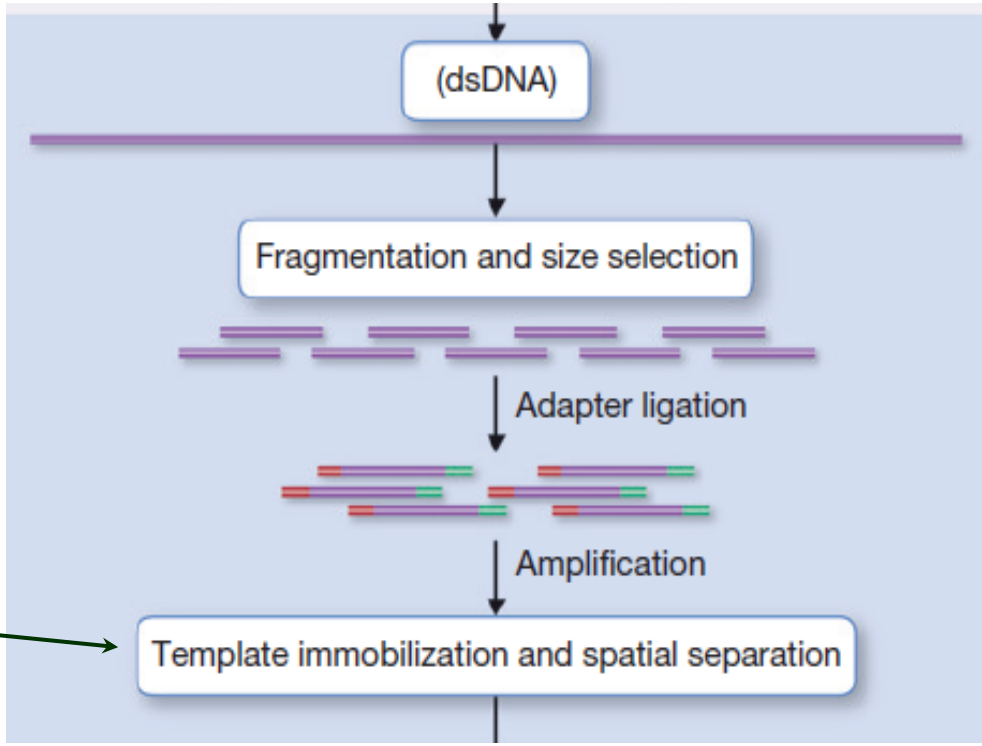
5. Nanopore sequencing

Basic workflow: Template Generation

Sequence library – convert starting material into a library of sequencing reaction templates.

Require common steps:

- **Fragmentation**
- **Size selection**
- **Adapter ligation**



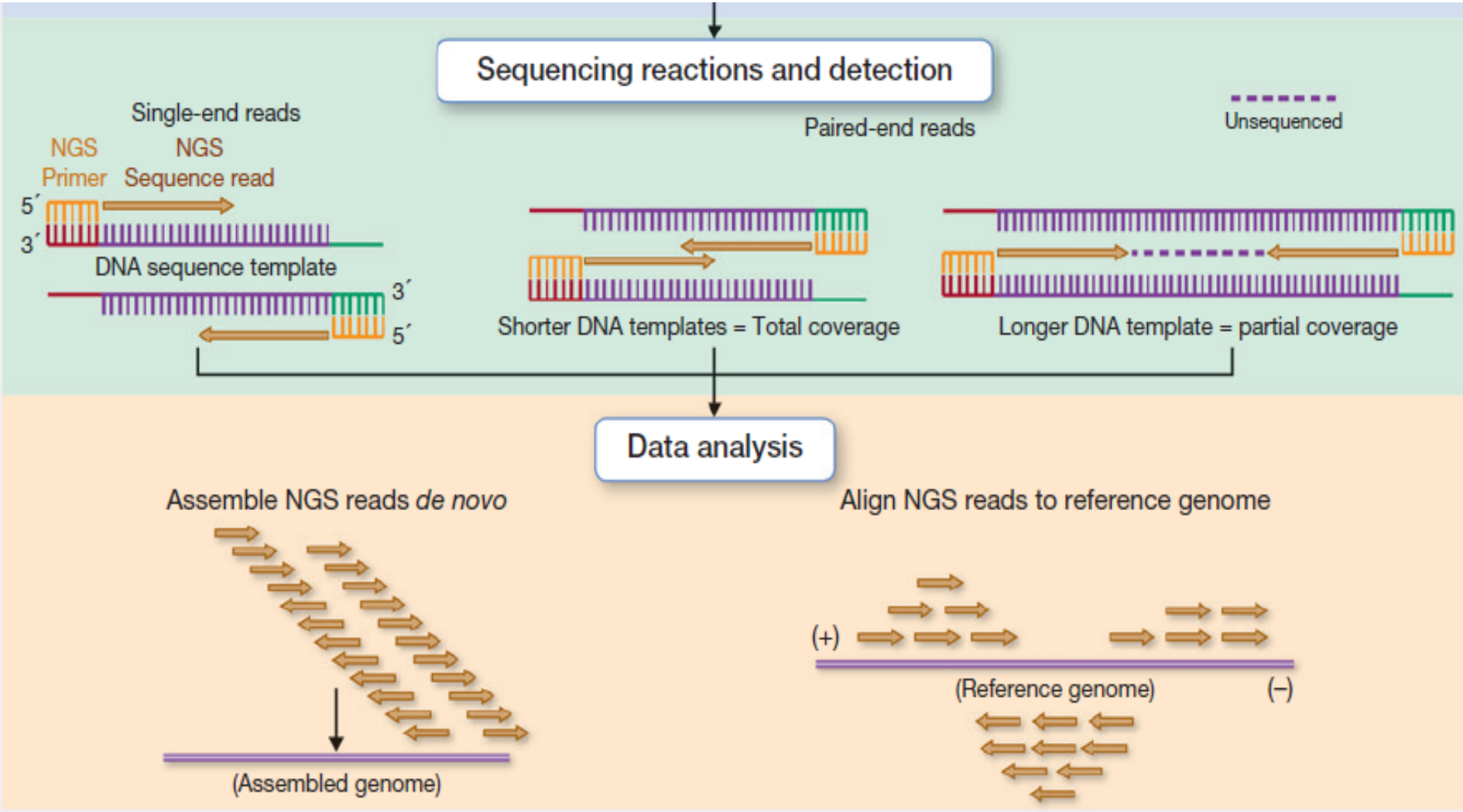
by attachment to solid surfaces or beads

Amplification-based - "second-generation" sequencing technology

Single-molecule - "third-generation" sequencing technology

A library is either sequenced directly - Single-molecule templates, or amplified then sequenced - Clonally amplified templates

Basic workflow: Detection & Data Analysis



Data Analysis

The scale and nature of data produced by all NGS platforms place substantial demands on IT at all stages of sequencing, including data tracking, storage, and quality control.

Data analysis is a critical feature of any NGS project and depends on the goal and type of project.

Initial analysis or base calling - by proprietary software on the sequencing platform.

After base calling, sequencing data are aligned to a reference genome if available or a *de novo* assembly is conducted.

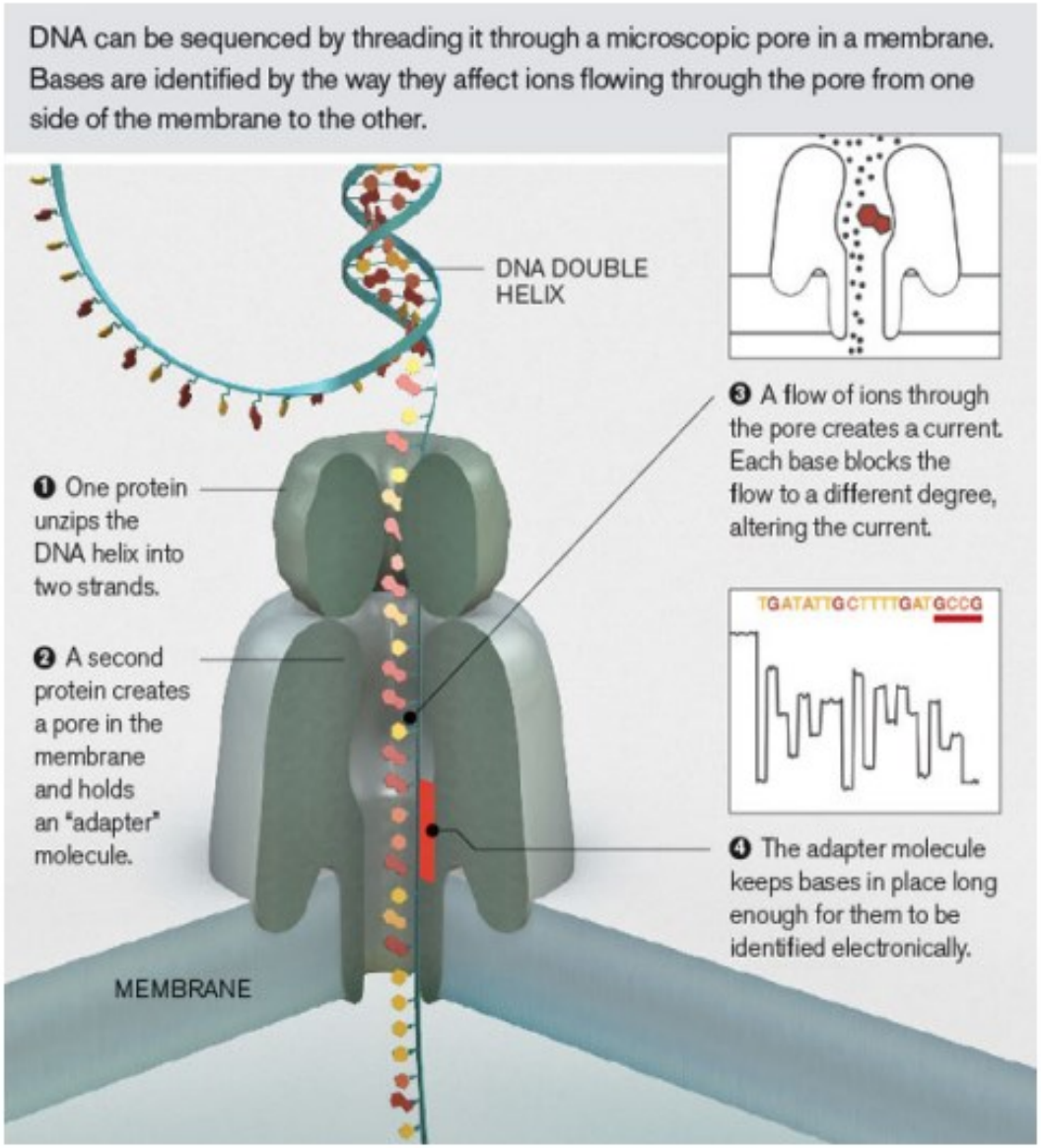
Once the sequence is aligned to a reference genome, the data needs to be analyzed in an experiment-specific fashion.

Sequence alignment & assembly is an active area of computational research

Third Generation Sequencing (TGS)

- **'Long read sequencing' – read length: ~ 10 – 60Kb**
- **Single molecule sequencing**
- **No PCR step involved**
- **Faster and portable**
- **Under active development**
- **e.g., PacBio Single molecule real time sequencing (SMRT) and Oxford Nanopore**

Oxford Nanopore - MinION



HTS Applications

genome	*de novo* sequencing: the initial generation of large eukaryotic genomes	Velasco et al., 2007 Diguistini et al., 2009 Huang et al., 2009 Li et al., 2010
	De novo, whole-genome and targeted sequencing	
	whole-genome resequencing: comprehensive SNP, indels, copy number and structual variations in individual human genomes	Bentley, 2006 Ossowski et al., 2008 Denver et al., 2009 Xia et al., 2009
	targeted resequencing: targeted polymorphism and mutation discovery	Hodges et al., 2007 Porreca et al., 2007 Harismendy et al., 2009
trancriptome	quantification of gene expression and alternative splicing; transcript annotation; discovery of transcribed SNPs or somatic mutations	Axtell et al., 2006 Sultan et al., 2008 Sugarbaker et al., 2008 Jacquier, 2009
	Deep sequencing of RNA transcripts	
	small RNA profiling	Berezikov et al., 2006 Houwing et al., 2007
epigenome	transcription factor with its direct targets	Johnson et al., 2007 Robertson et al., 2007
	Deep sequencing of DNA fragments pulled down by Chip-Seq	
	genomic profiles of histone modifications	Impey et al., 2004 Mikkelsen et al., 2007
	DNA methylation	Cokus et al., 2008 Costello et al., 2009
	Deep sequencing of bisulfite-treated DNA	
	genomic profiles of nucleosome positions	Fierer et. al., 2006 Johnson et al., 2006
metagenome	environmental	Edwards et al., 2007 Hubert et al., 2007
	Species classification	
	human microbiome	Turnbaugh et al., 2007 Qin et al., 2010

HTS Applications

One of the most prominent applications of NGS is re-sequencing:

- **whole genome resequencing**
- **target-region resequencing**
- **exome resequencing**

Any human individual's genome available in NCBI?

- genome-wide analysis of single nucleotide variations and other structural variations, multiple individuals, or strains, cancer sequencing, population-based sampling of a species, migration patterns of a virus, e.g., SARS-CoV-2, etc.

HTS Applications

RNA sequencing – has several applications, including RNA expression, *de novo* transcriptome sequencing for non-model organisms and novel transcript discovery

***viz.*, mRNAs, noncoding RNAs, small RNAs, miRNA**

For RNA and microRNA expression profiling, NGS has significant advantages compared to microarray methods in better quantification of common & rare transcripts.

Transcriptome - the complete set of transcripts in a cell, and their quantity, for a specific developmental stage or physiological condition.

NGS Applications

Epigenomic Analysis – NGS technologies have been applied in several epigenomic areas, *viz.*,

- **characterization of DNA methylation patterns,**
- **posttranslational modifications of histones,**
- **interaction between transcription factors and their direct targets, and**
- **nucleosome positioning on a genome-wide scale.**

Epigenetics is the study of heritable gene regulation that does not involve the DNA sequence itself but its modifications and higher-order structures.

HTS Applications

Metagenome Sequencing – sequencing the bacterial 16S rRNA gene across a number of species, for studying phylogeny and taxonomy, particularly in diverse metagenomic samples

e.g., cataloging human gut microbial genes by metagenomic sequencing (Qin *et al*, 2010).

~ 570Gb of sequence data from 124 individuals was generated, assembled and characterized 3.3 million non-redundant microbial genes.

This helped scientists, for the first time, to define the minimal human gut metagenome.

Metagenomics involves genomic analysis of microorganisms by direct extraction of DNA from uncultured ensemble of microbial communities

PCR Sequencing

How would you go about sequencing SARS-CoV-2 genome, 29903 bases long?

What technique is used for diagnostic testing of COVID-19?

While sequencing a novel genome for the first time, how are primers identified?

Can we now answer these Qs:

- **How is the SARS-CoV-2 genome sequenced?**
- **How does one identify the coordinates of N gene on it? i.e., how to construct a physical map of a genome?**
- **How does one select which regions in this gene would give specificity for the presence of SARS-CoV-2?***
- **How is the specific probe regions extracted and amplified for detection?**
- **Is it possible to store the DNA sample for re-testing? How?**

References:

1. **Concepts in Biotechnology, ed. D. Balasubramanyam**
2. **Restriction Endonucleases and DNA Modifying Enzymes http://arbl.cvmbs.colostate.edu/hbooks/genetics/biotech/enzymes/index.html**
3. **REBASE: restriction enzymes and methyltransferases, Nucleic Acids Research, Vol. 31 (1), 418–420 (2003)**