Chapter 5

Objectives: Exploration of gene
Recombinant DNA technology
Genome sequencing
Manipulation of Eukaryotic genes

- Restriction enzymes
  - cleave DNA et specific sequence
  - found in prokaryotes, cleave foreign DNA (own DNA is methylated and thus not recogrnized)

The length of restriction recognition sites varies (4 to 8 bases)

Most recognition sequences are **palindromes**

"MADAM I AM ADAM"

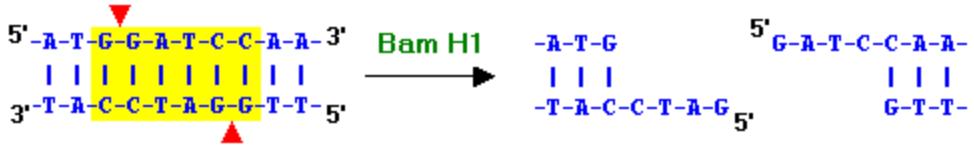
Restriction enzymes are named after their host of origin. For example, EcoRI was isolated from *Escherichia coli* (strain RY13)

Isoschizomers – restriction enzymes with the same recognition site

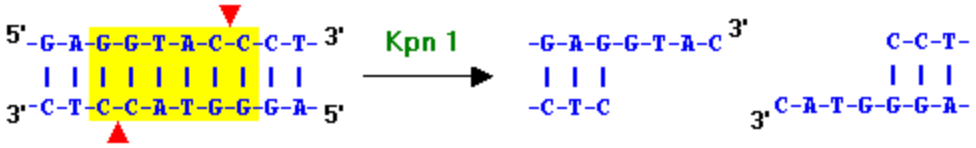
Enzyme	Recognition Sequence
BamH I	GGATCC CCTAGG
Not I	GCGGCCGC CGCCGGCG
Sau3A I	GATC CTAG
Sac I	GAGCTC CTCGAG
Sst I	GAGCTC CTCGAG
Hinf I	GANTC CTNAG
Xho II	PuGATCPy PyCTAGPu

DNA cleavage between deoxyribose and phosphate groups. This leaves a phosphate group on the 5' ends and a hydroxyl on the 3' ends of both strands.

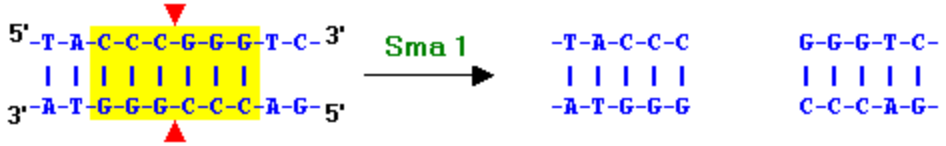
5' overhangs: The enzyme cuts asymmetrically within the recognition site such that a short single-stranded segment extends from the 5' ends. BamHI cuts in this manner.



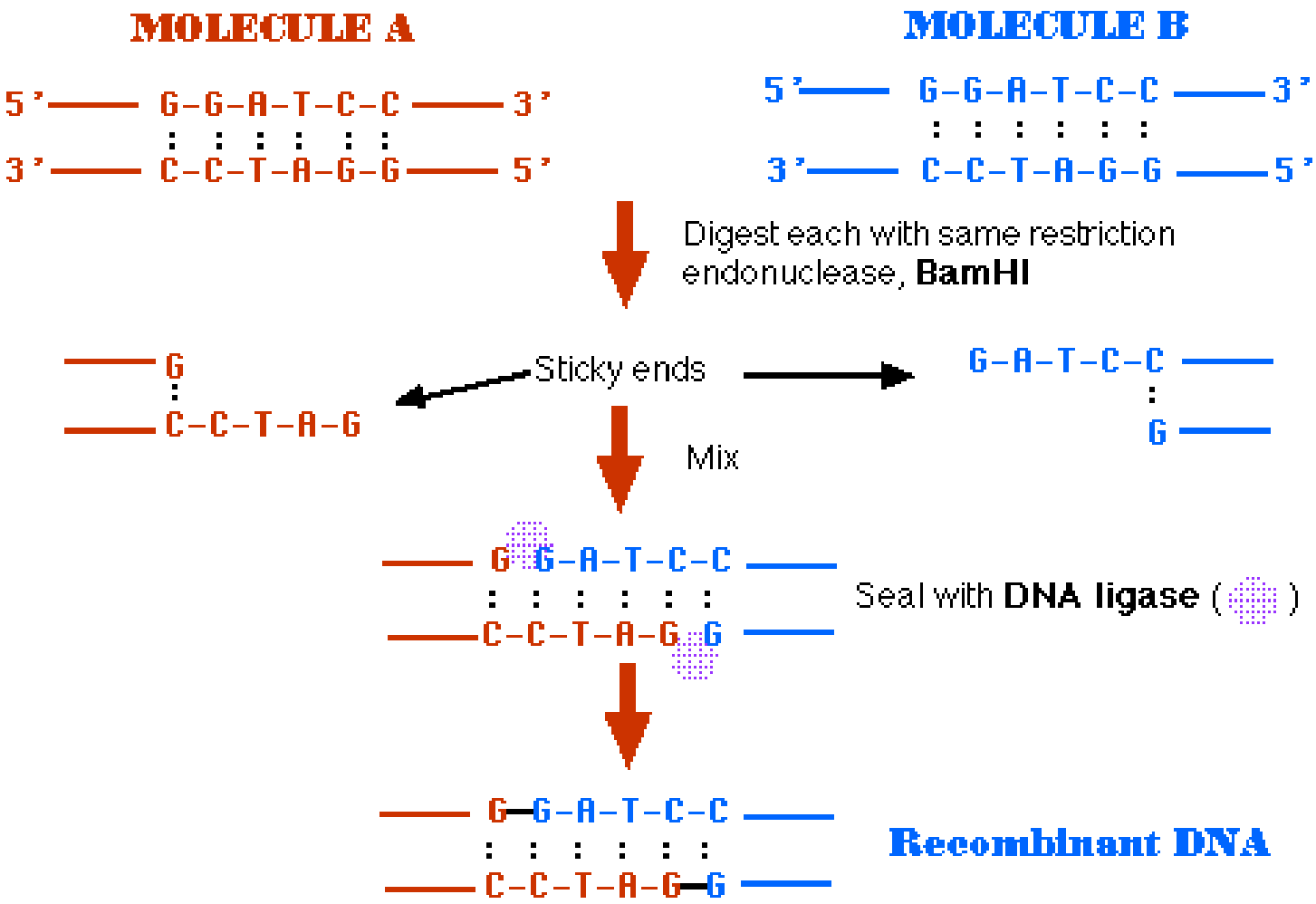
3' overhangs: a single-stranded overhang from the two 3' ends



Blunt ends



Making Recombinant DNA



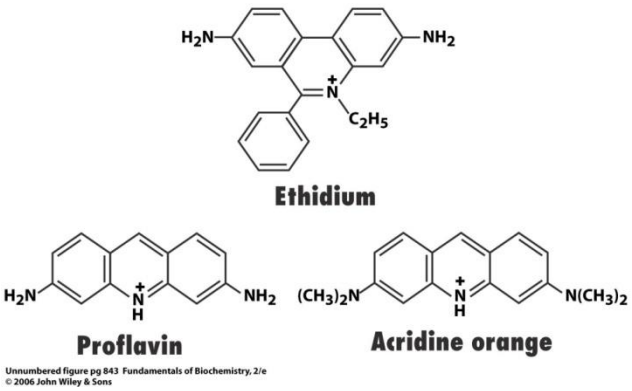
Cloning – replication of recombinant molecule (*in vivo* and in *vitro*)

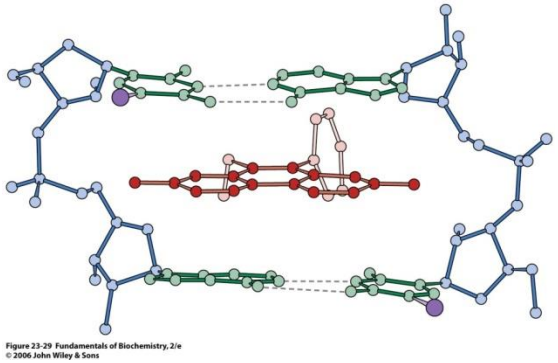
- # Fractionation of nucleic acids

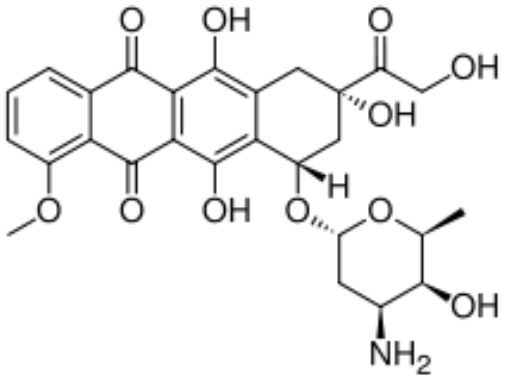
I) Isolation of nucleic acids
   i) Precipitation of proteins
   ii) Extraction of nucleic acid by pricipitiation with ethanol

II) Separation of nucleic acids
   i) Affinity chromatography (hydroxyappatite DNA) (cellulose with poly U to purify mRNA)

   ii) Electrophoreses on the agarose gel (pulse field gel electrophoreses), duplex DNA is e stained by interacalators (acridine orange, ethidium bromide)







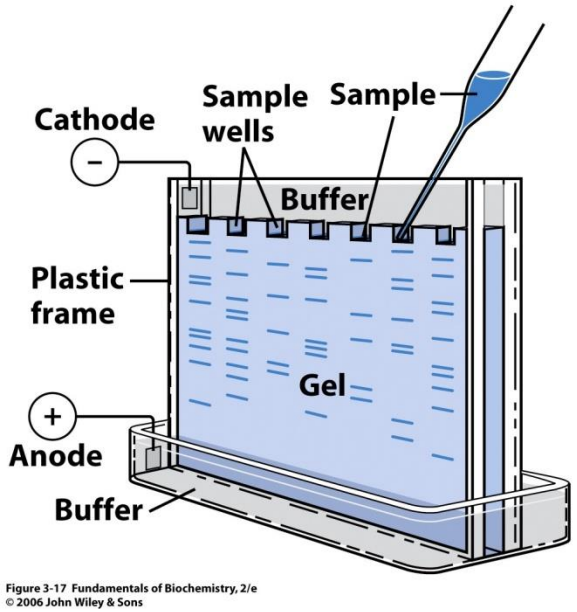
doxorubicin

- Separation of DNA fragments:
- Gel electrophoresis (acryl amide (1 kbp) and agarose)

Fragments are separated according to the size

Detection: autoradiography ^{32}P

Fluorescence: ethydium bromide



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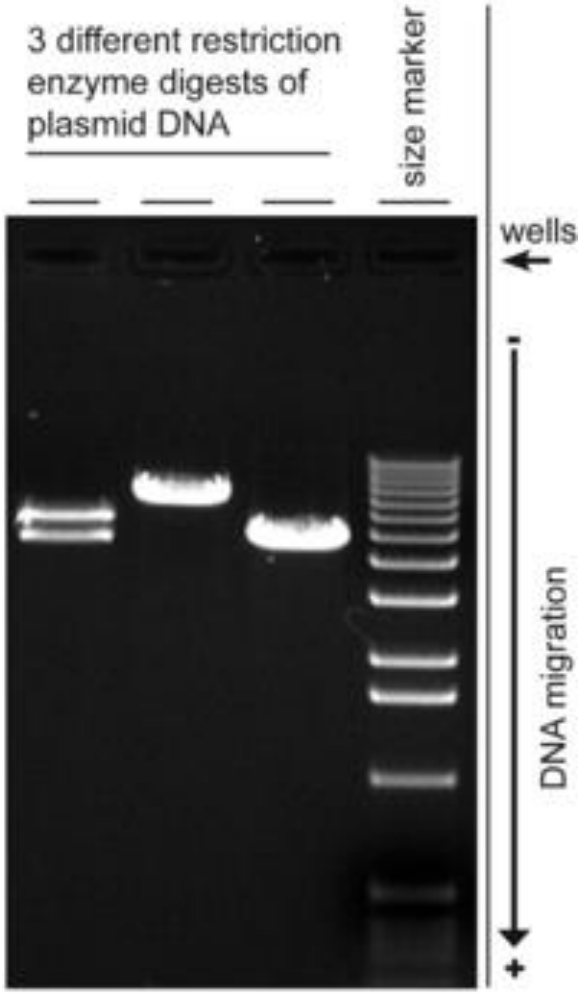
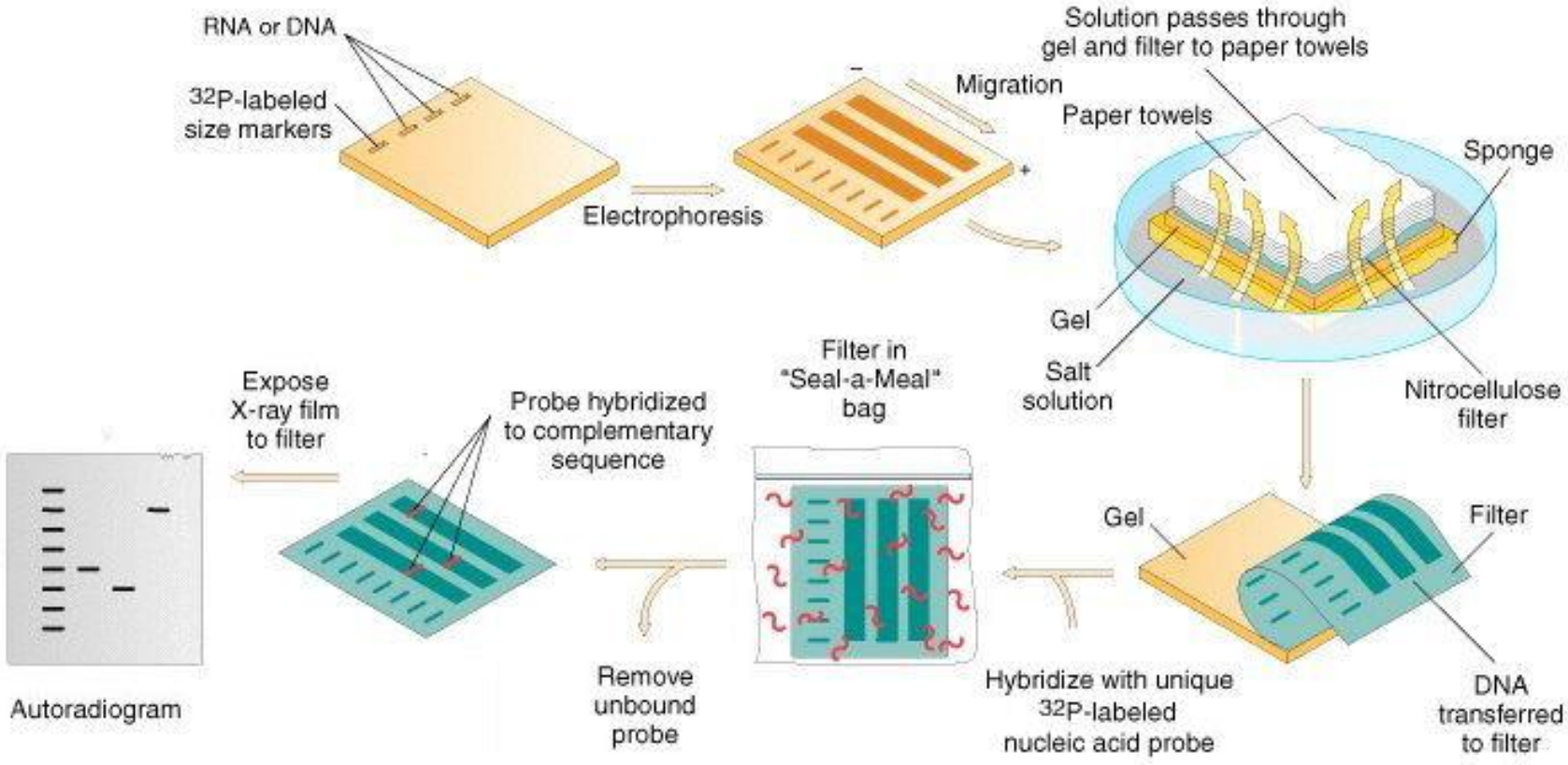


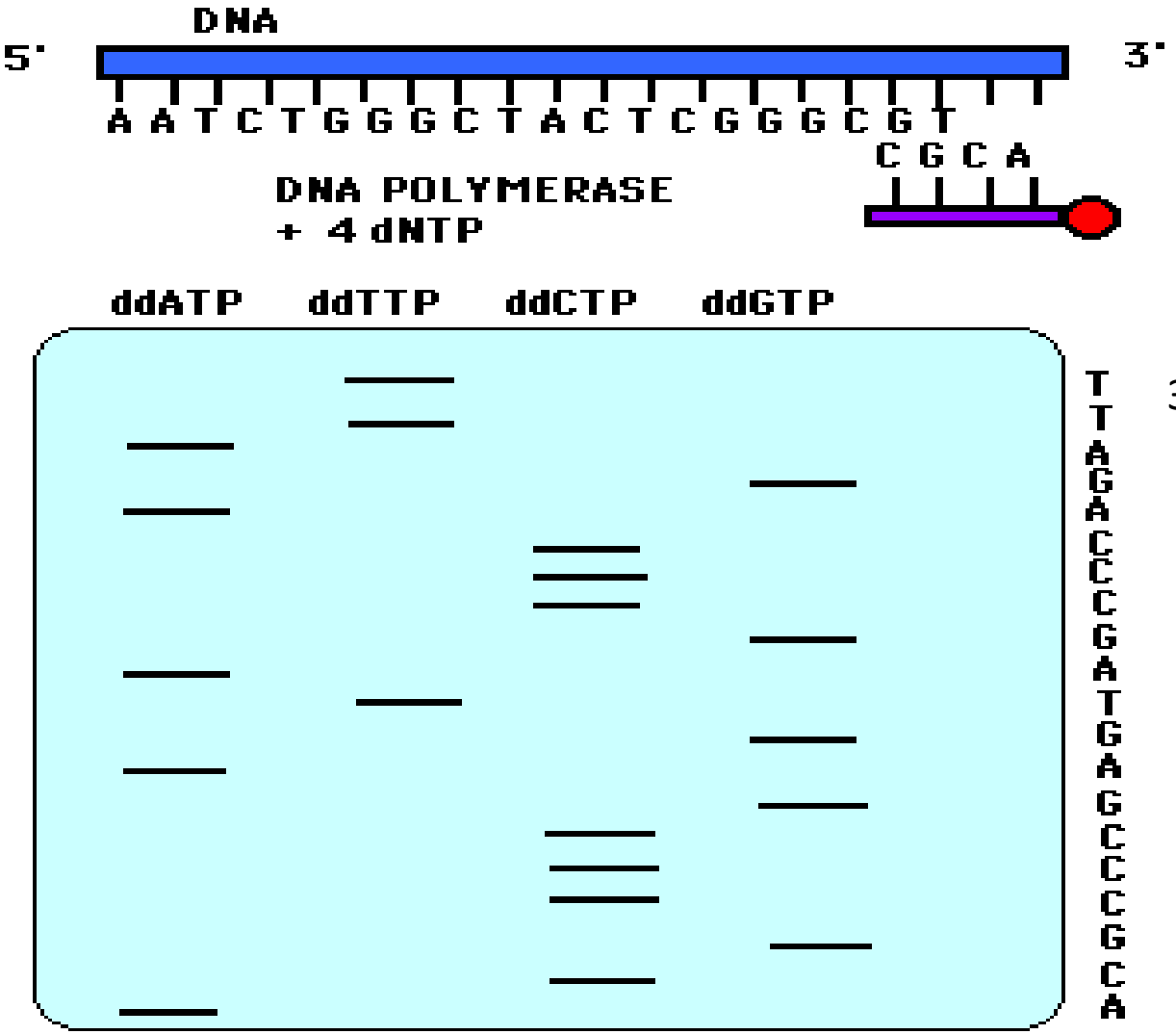
- Southern blotting - identifying DNA segments



Northern blotting – analogous techniques for RNA

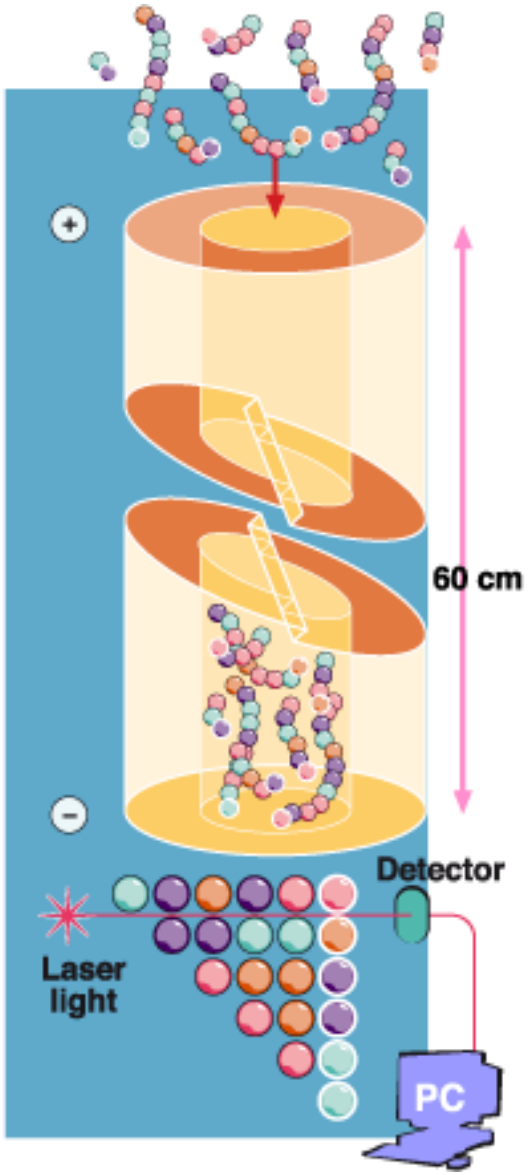
- Sager sequencing method

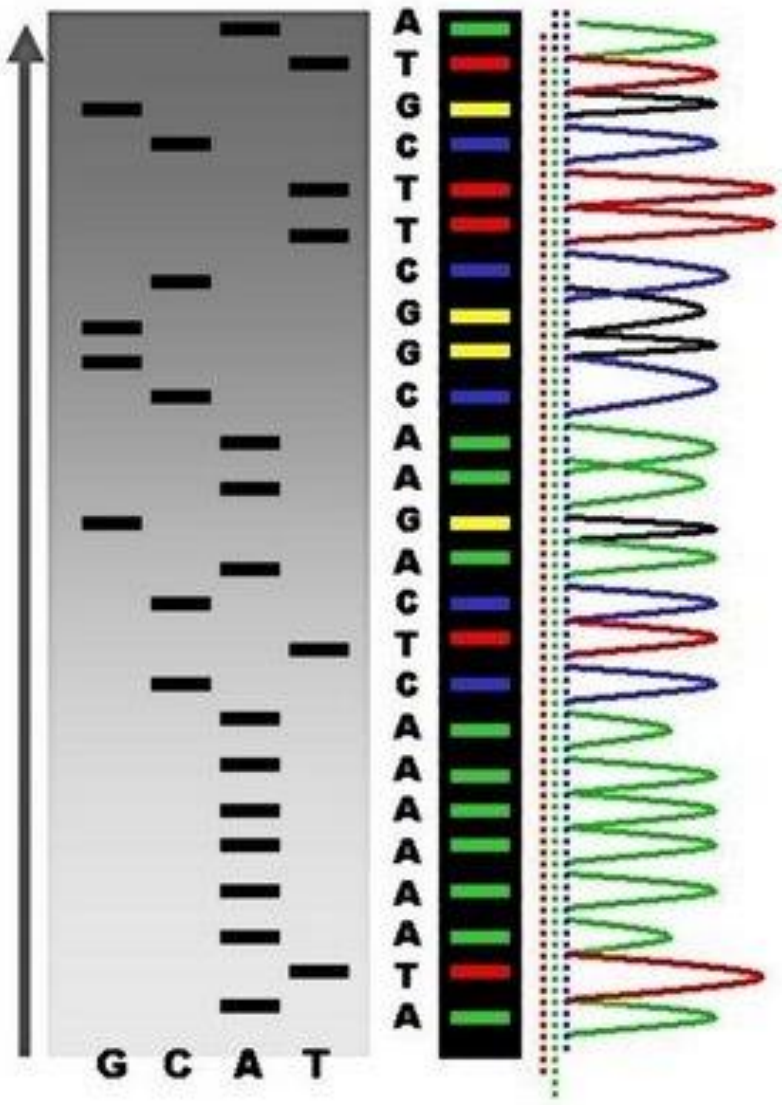
1) Uses ssDNA this can be done by denaturing the double stranded DNA with NaOH.
2) DNA primers (short pieces of DNA that are both complementary to the strand which is to be sequenced and radioactively labelled at the 5' end)
3) a mixture of a particular ddNTP (1%)
4) Other four dNTPs (dCTP, dGTP, and dTTP). The concentration of ddATP should be 1% of the concentration of dATP.
5) Reaction is performed in four test tubes (for each ddNTP)
6) DNA polymerase



Sequence complementary to template

DNA sequencing using fluorescence detection





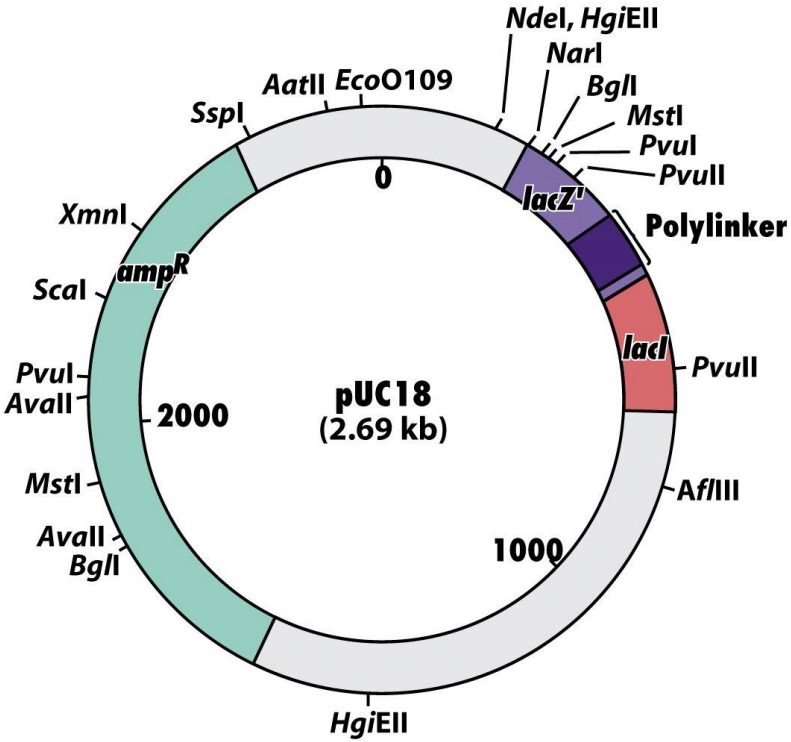
- **Recombinant DNA vectors:**
  - Amplification of DNA fragment can be achieved in the cell using cloning vectors: plasmid or bacteriophages
  - Plasmid

Small circular DNA in bacteria or yeast cells

Accumulate 1-5 kb inserts

Desirable properties of plasmids

- It should be small
- Sequence should be known
- It should grow to high copy number in the host cell. (relax plasmid)
- Replicon sequence (ori + regulatory sequence)
- It should contain a **selectable marker** that allows cells containing the plasmid to be isolated.
- It should also contain a second selectable gene that is inactivated by insertion of the passenger – **insertional inactivation**
- There should be a large number of unique restriction sites lying within one of the two selectable markers described above.



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LacZ encodes β-galactosidase

LacI – encodes factor controling transcription of lacZ

Plasmid purification: purified from bacteria

difference between the small, circular plasmid molecules and the large, broken (hence linear) pieces of chromosomal DNA.

1. the bacteria are broken open and the is DNA isolated.
2. DNA is denatured.
3. Finally, the DNA is renatured and centrifuged.

Upon denaturation, the two circular single-stranded chains of the plasmid DNA remain entwined and don't separate fully. During renaturation each strand rapidly finds its complement.

Chromosomal DNA breaks readily and therefore consists of noncircular pieces. Under these circumstances, the two strands easily denature and separate. Upon renaturation, they have difficulty finding complete copies of their complements.

These can be simply separated from the small circular plasmid DNA by high-speed centrifugation.

Construction of a recombinant DNA molecule

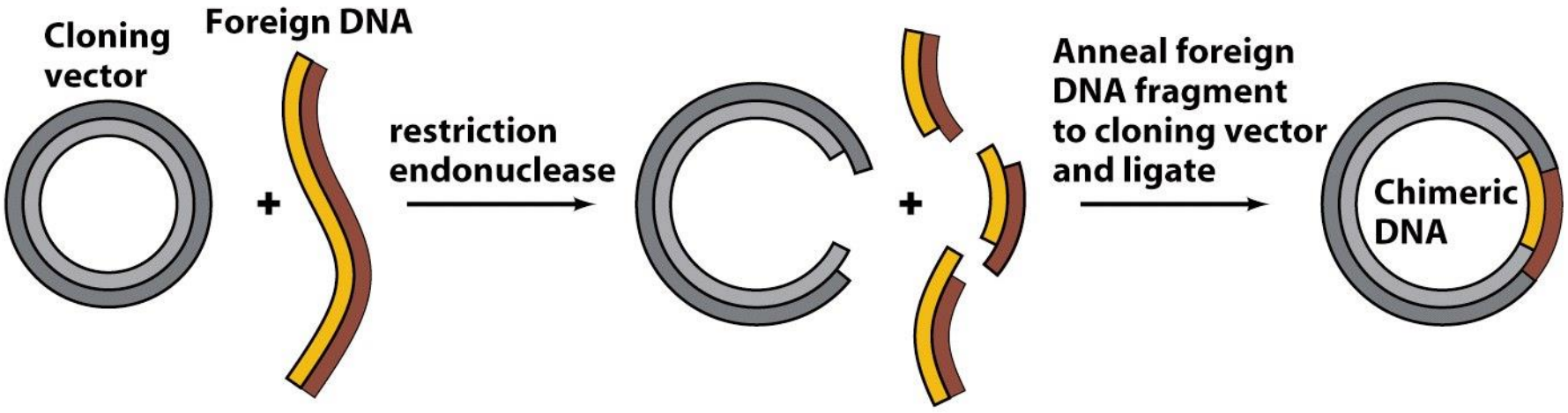


Figure 3-27 Fundamentals of Biochemistry, 2/e

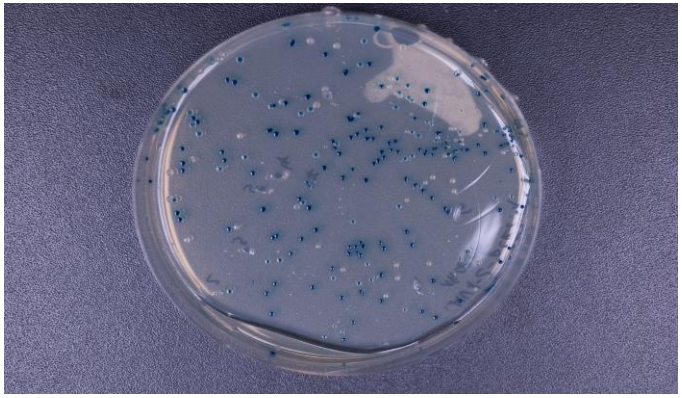
Recombinant DNA – technique allowing joining two different DNA molecules, amplify them and modify them.

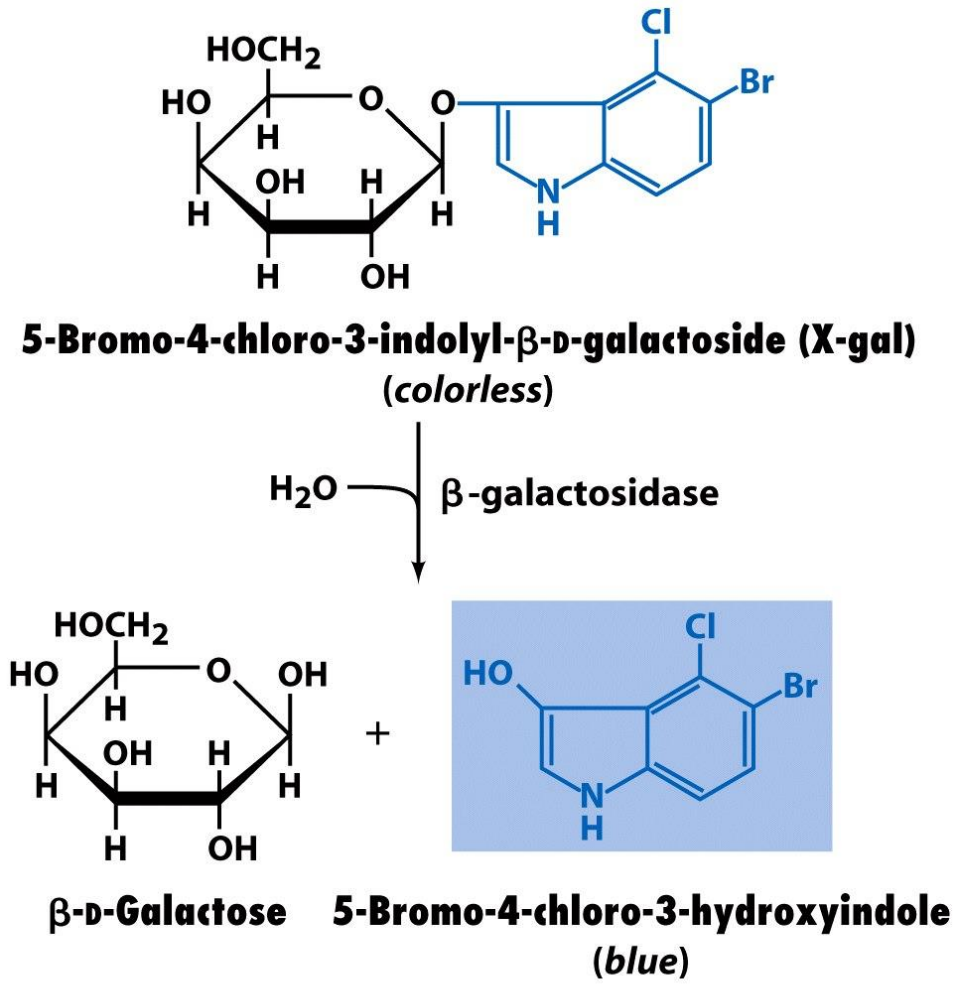
Allow isolation of piece of DNA out of genome and amplification of DNA fragment
Restriction endonucleases and DNA ligases

Bacterial transformation

Introduction of DNA into bacteria
Spontaneous uptake – low probability
E. coli – cells treated with $CaCl_2$
Less than 1 of 10^3 cells acquire a plasmid
Selection of transformed cells:
resistance to antibiotics
using chromogenic substances

Chromogenic substances:
lacZ genes in plasmid (pUC18) codes for β-galactosidase





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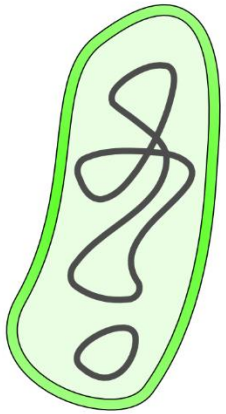
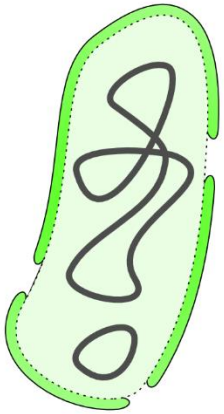
Bacterial transformation

Getting a plasmid into a bacterium

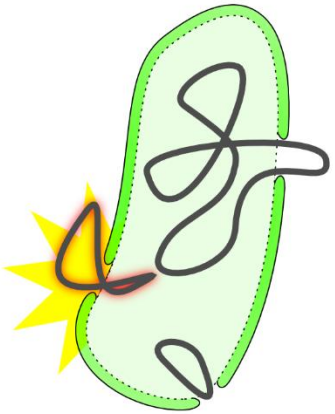
Here is an *E. coli.* bacterium in natural state. (Notice how bacterial DNA is circular!)



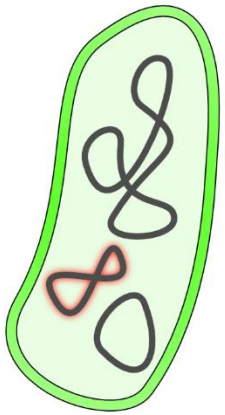
Extreme cold causes pores (small holes) to appear in the bacterial membrane.



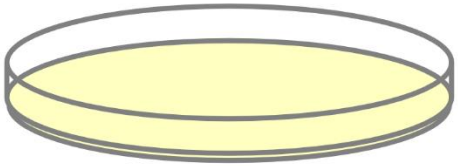
Small DNA molecules like our plasmid can move through these holes!



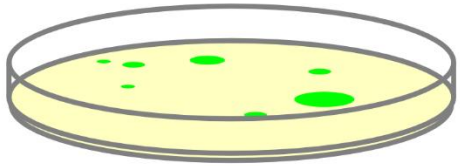
When the bacteria are heated again, some of them end up with our plasmid inside them! These are the *transformed* bacteria.



We can filter out the untransformed bacteria (the ones that got *no plasmid*) by growing all of the bacteria in an antibiotic-containing medium.



Untransformed bacteria are killed by the antibiotic in the medium. (They don't have the plasmid with the antibiotic resistance gene!)



The transformed bacteria grow though! Now we can pick them off the plate and grow more if we want.

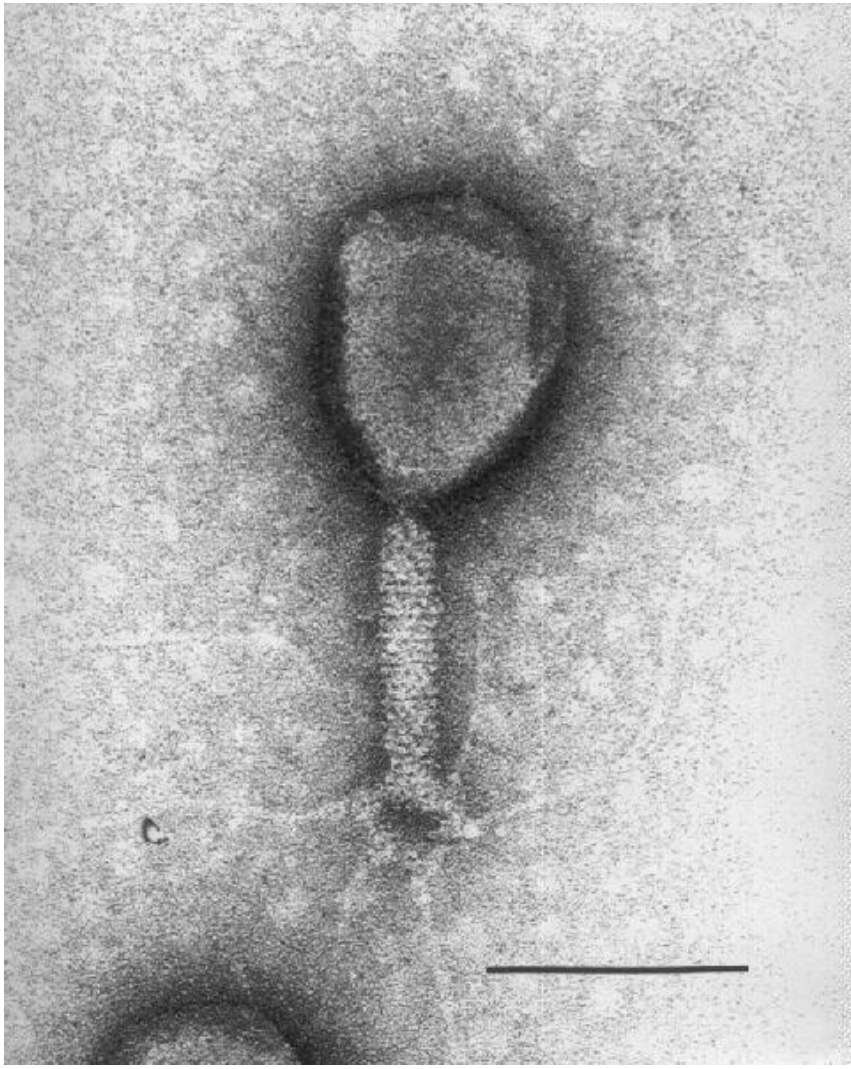
Bacteriophage

Bacteriophages are bacteria viruses.
When they infect their host they use host machinery to replicate their DNA.

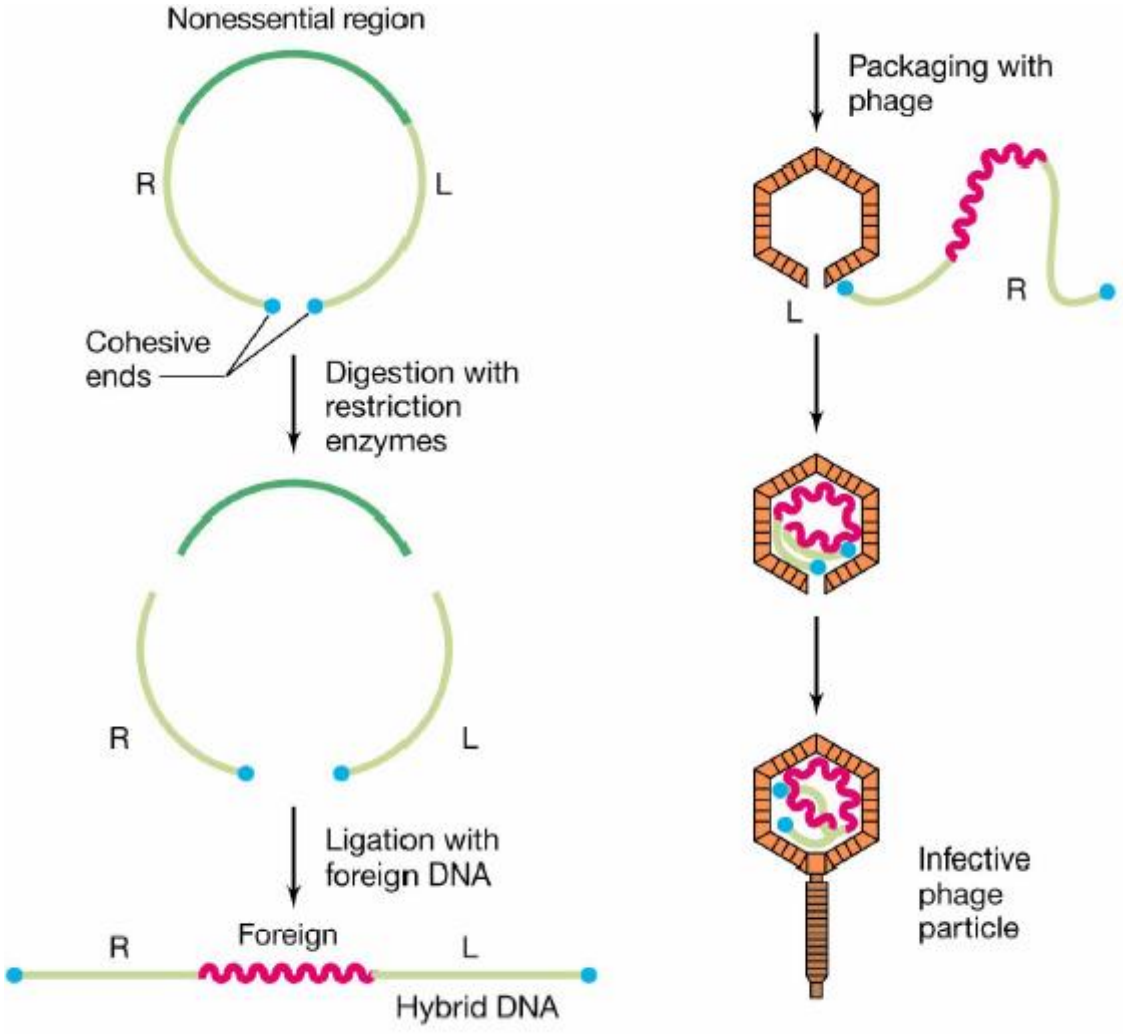
In bacteriophage vectors part of the phage DNA is replaced by the gene of interest (max. size is 15-25 kbp)

When this new recombinant phage DNA infects a host, the gene of interest will be replicated.

commonly used phage vectors include M13 and λ.



Cloning DNA in bacteriophage λ



<u>Infection of bacterial cell:</u>

Tail sticks to the cell wall

DNA from head is squired into bacteria

Phage genes are transcribed by bacterial RNA polymerase

Corresponding mRNA is translated using bacterial machinery

Newly replicated phage DNA and head and tail protein ensemble spontaneously

Lyses of host bacteria results in formation of plagues.

- λ phage – most common vector for construct of genomic DNA
- Cosmid vector – can accumulate up to 45 kb
  - a cross between a plasmid and λ vector
  - contains antibiotic resistance genes and Ori for replication in bacteria
  - Contains cos sites to be packed in phage particles
  - Inside of bacteria, the cosmid DNA circularizes and replicates as a large plasmid

- YAG yeast artificial chromosome , up to 200 to 500 kb
  - Large DNA fragments are inserted into yeast DNA sequence and introduced into yeast cell by transformation

PCR

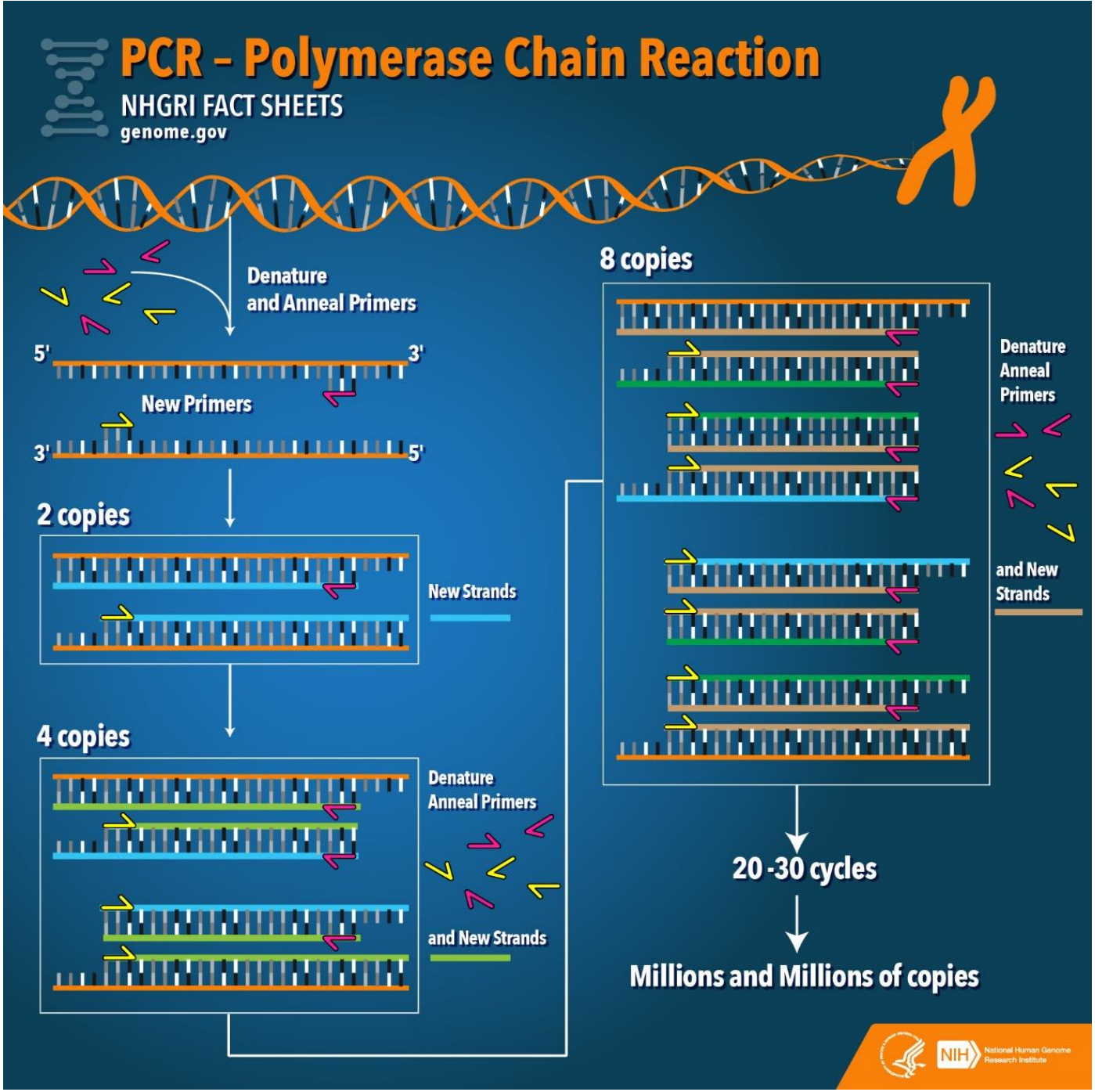
Polymerase chain reaction - amplification of DNA sequence

in vitro procedure for enzymatic amplification of specific segments of DNA, typically in the range of 100-300 bp long.

1 *Strand separation.* The two strands of the parent DNA molecule are separated by heating the solution to 95°C for 15 s.

2 *Hybridization of primers.* Cooling to 54°C to allow each primer to hybridize to a DNA strand. One primer hybridizes to the 3′-end of the target on one strand, and the other primer hybridizes to the 3′ end on the complementary target strand. Parent DNA duplexes do not form, because the primers are present in large excess. Primers are typically from 20 to 30 nucleotides long.

3 *DNA synthesis.* The solution is then heated to 72°C, the optimal temperature for ***Taq*** DNA polymerase. This heat-stable polymerase comes from *Thermus aquaticus*, a thermophilic bacterium that lives in hot springs. The polymerase elongates both primers in the direction of the target sequence because DNA synthesis is in the 5′-to-3′ direction. DNA synthesis takes place on both strands but extends beyond the target sequence.



These three steps—strand separation, hybridization of primers, and DNA synthesis—constitute one cycle of the PCR amplification and can be carried out repetitively just by changing the temperature of the reaction mixture.

After 3rd cycle: short strands with target sequence + primers

Long strands – arithmetic growth
Short strand – exponential growth 2^n

PCR application

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

Analysis of allelic sequence variations

- Electrophoretic mobility shift essay

Detection of Sequence specific DNA – binding proteins

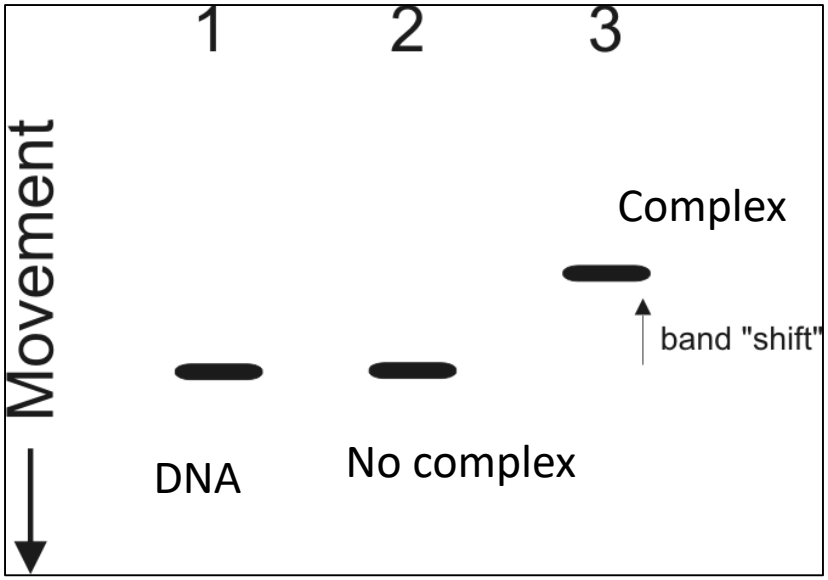
dsDNA is preincubated with the protein to be tested (purified protein or whole- cell extract) to form stable protein complex

Migration of protein – DNA complex during electrophoresis is slower than DNA by itself

Addition of antibody

a) Slows migration of complex;
   if Ab does not interfere with
   with protein DNA- interactions

b) Disturb complex if Ab reacts with DNA binding site



- Complementary DNA and complementary DNA library

cDNA - complementary DNA

– DNA derived by copying a mRNA

<u>How to make cDNA copies of mRNAs</u>

1. The mRNAs are first isolated from a cell extract (oligo (dT)-cellulose chromatography

3. A special enzyme called **reverse transcriptase** is added.

This enzyme makes a cDNA copy of the mRNA, using

nucleotide triphosphates, in a process called reverse transcription

4. The mRNA strand is removed. This leaves "single-stranded cDNA"

molecules

5. A DNA copy of the cDNA strand can be made using polymerase. This end result is a product called double-stranded cDNA

6. Double-stranded cDNA can be cloned into a cloning vector

like any other DNA molecule

Genomic DNA library

- mammal genome ~ 3-4 billion base pairs of DNA
- one gene ~ 10 kbp
- It would be unpractical to isolate 1 gene
- *Preparation of genomic DNA library*
- Isolating total DNA
  - ***Aplying shotgun cloning***
- Digesting total DNA into fragments of suitable size (the fragments do not corresponds to the individual genes)
- Inserting these fragments into vectors or into bacteriophage results into genomic library
- Each plasmid is then taken up by 1 bacteria, and each recombinant DNA is multiply in bacteria
- The total bacterial population represents the entire human genome

What is the number of fragments that must be cloned to ensure a high probability that desired sequence is present at least once in genomic library?

$P = 1-(1-f)^N$

P – probability
f- fraction of desired sequence in genome
N- number of fragments

N= Log(1-P)/Log(1-f)

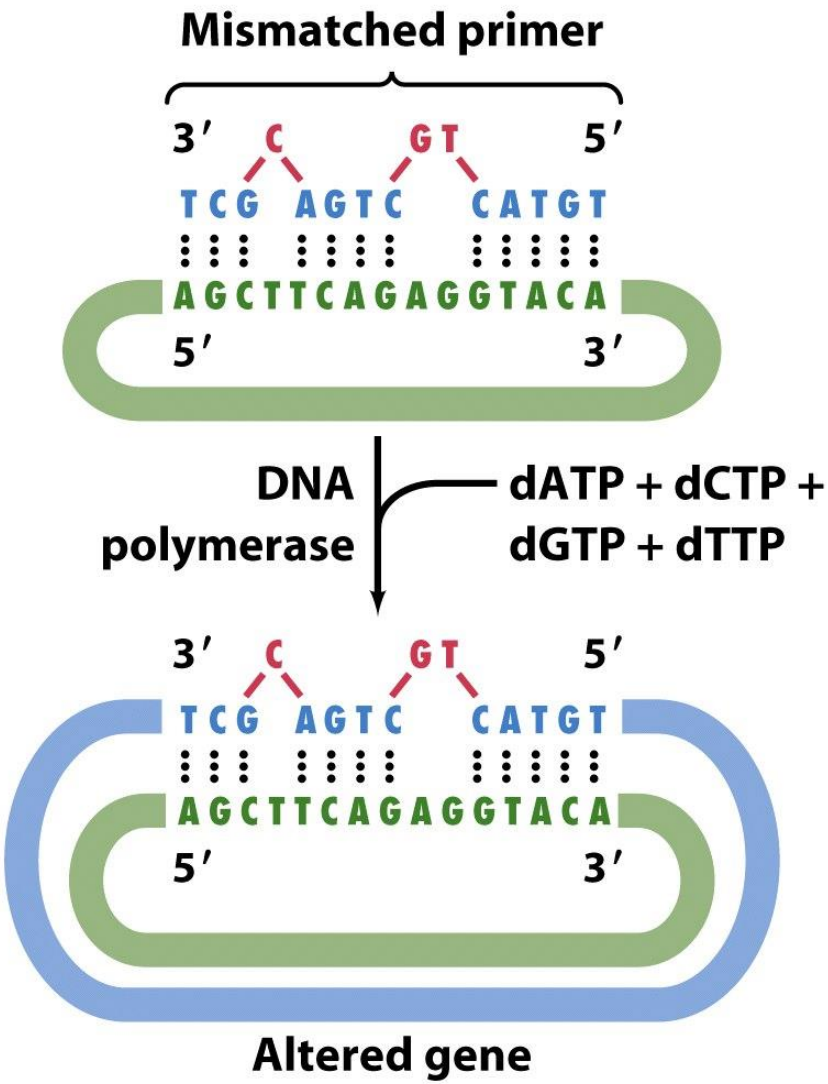
How many yeast DNA fragments of average length 5 kbp has to be clone to in order to have 99% probability that a genomic library contains certain sequence. Yeast genome: $12{,}1x10^6$ bp.
f= 5kbp/12100 kbp = $4.13x10^{-4}$ of yeast genome.

N = log(1-.99)/log(1-4.1310^{-4}) = 11,148

Site directed mutagenesis

-Allows to predict and characterize function of single amino acid residues in the protein and create proteins de noveau

Green – gene to be modified
Mismatched primer (contains mutation) is extended by DNA polymerase
Altered gene is inserted into a vector and expressed in host bacteria, where mutated strain serves as a template for the new strands that cary the mutated nucleotide



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- Cassette mutation
  - Plasmid DNA is cut by restriction enzymes to remove a short sequence of bases and the new sequence with a mutation is added and ligated

Designer gene:

- Preparation of new protein
- chimeric proteins
- synthetic vaccines (noninfectious coat proteins of virusis)

- **Example of chimeric protein:**

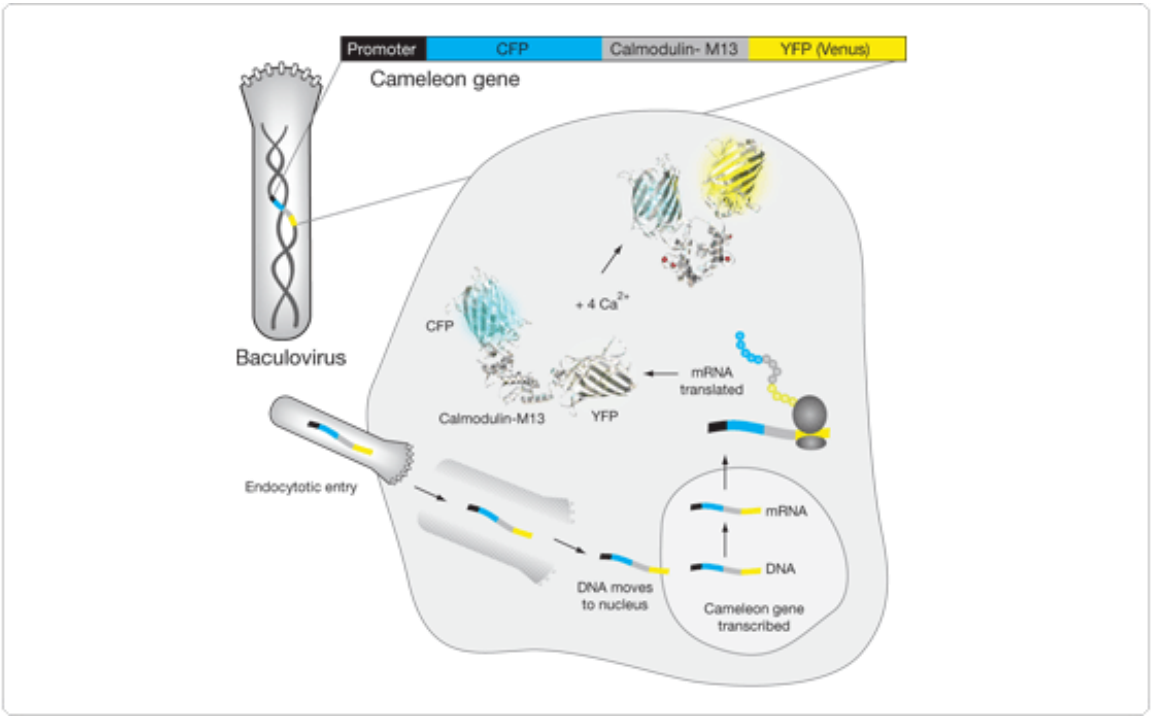
GST-fusion protein

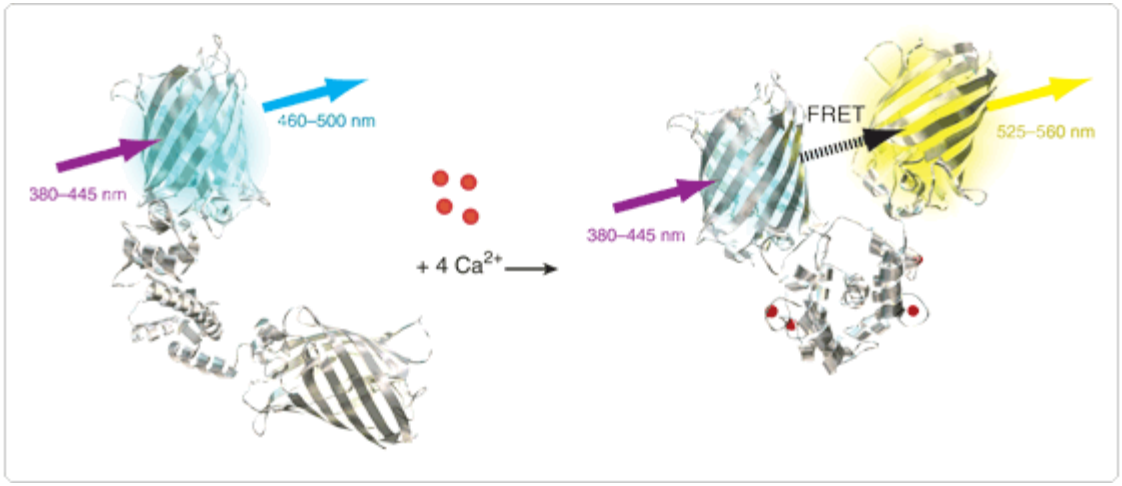
- protein of interest is fused with Glutathione *S*-transferases

GST sequence is incorporated into an expression vector alongside the gene sequence encoding the protein of interest

Expressed protein purification is facilitate by using affinity chromatograpy with tripetide beads, coated with the tripeptide glutathione

Premo Cameleon Calcium Sensor





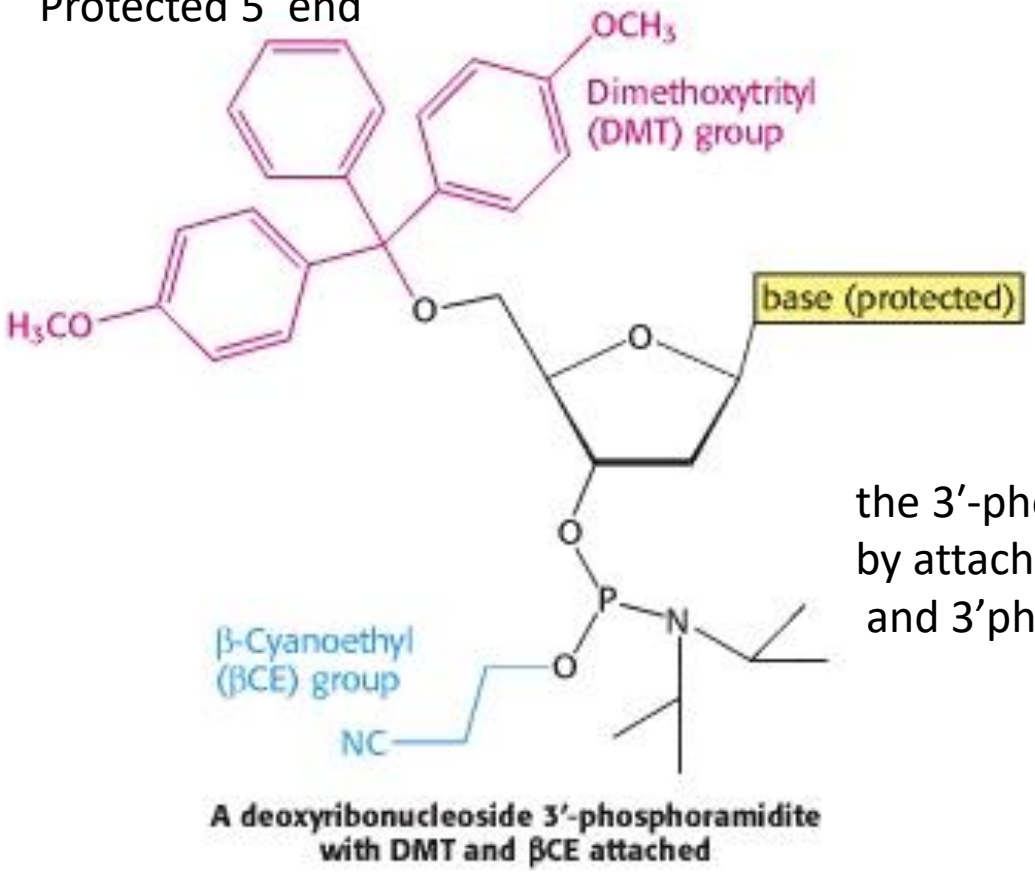
http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook/Indicators-for-Ca2-Mg2-Zn2-and-Other-Metal-Ions/Aequorin-A-Bioluminescent-Ca2-Indicator.html

Solid phase synthesis of DNA

Synthesis of the oligonucleotides of specific sequence

- Growing chain is linked to the resin and activated monomers are added

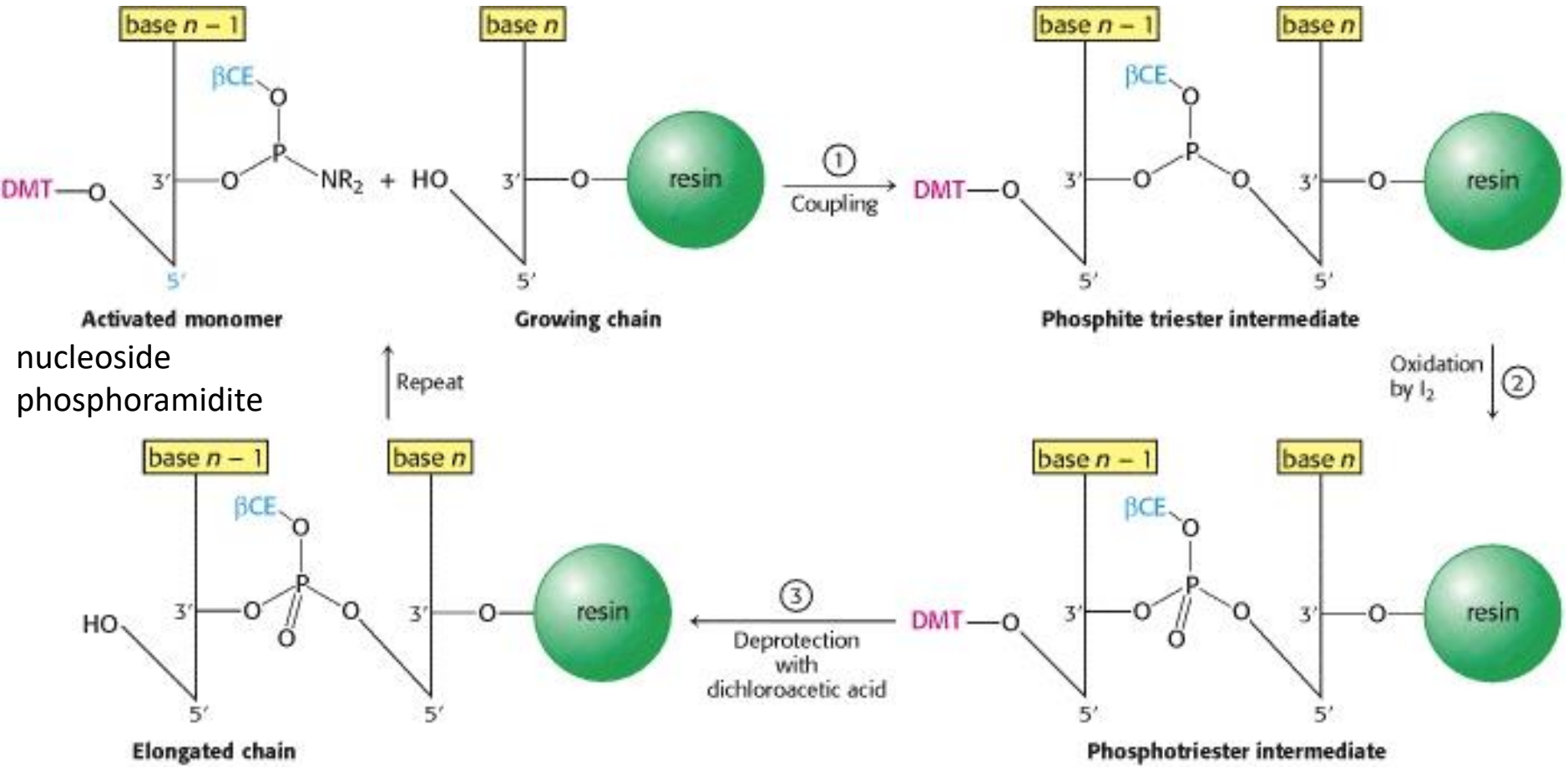
Protected 5’ end



A deoxyribonucleoside 3’-phosphoramidite with DMT and βCE attached

the 3’-phosphoryl group is rendered unreactive by attachment of the β-cyanoethyl (βCE) group and 3’phosphoramidinte

stepwise addition of nucleotide residues to the 5'-terminus of the growing chain until the desired sequence is achieved



nucleoside phosphoramidite

- After each coupling reaction, the resine is washed to remove uncreative species and after final step the protective groups are removed with ammonium hydroxide