PLNT 2530 (2024)

Unit 3

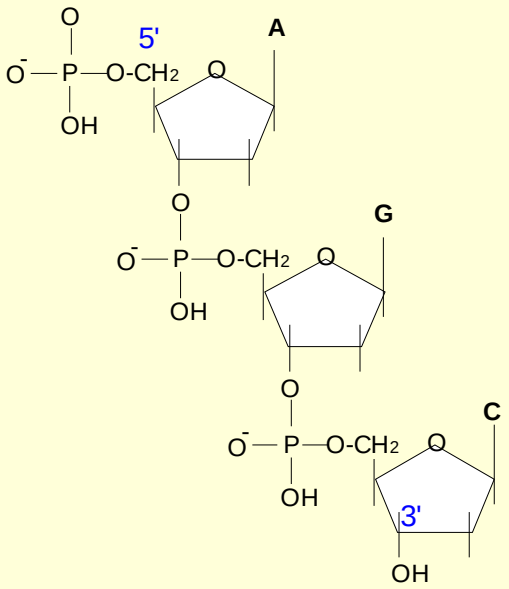
Methods of DNA Manipulation

Reference:

Analysis of Genes and Genomes by Richard J Reece

Enzymes Used to Manipulate DNA/RNA

Cutting and joining DNA is essential to all recombinant DNA work



1. Nucleases -cut polynucleotide DNA/RNA by cleavage of a phosphodiester linkage.

- Nucleases are either DNase or RNase
- Either endonucleases (cut internally) or exonucleases (cut from 3' or 5' end of polymer chain)
- Most nucleases cleave phosphodiester bond leaving the phosphate unit on the 5' terminal nucleotide

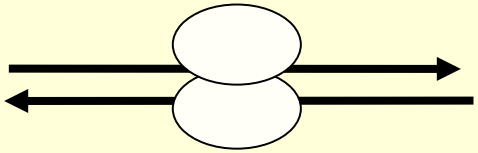
AGCTGGCC

↓

5'P-AG-OH 3' 5' P CTAGCT-OH 3'

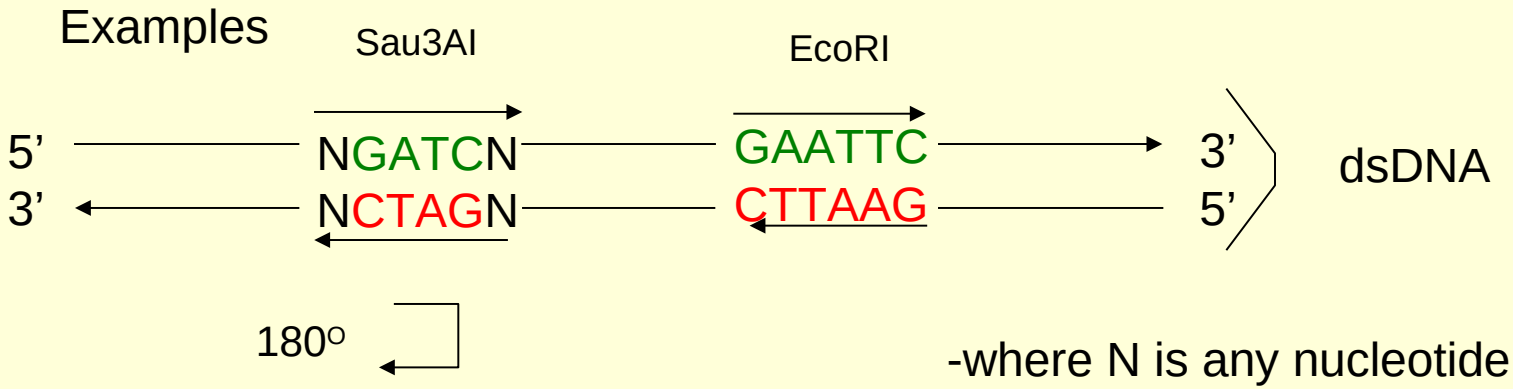
- Different nucleases differ in their specificity – i.e. the site at which the enzyme will cleave DNA/RNA.
  - DNAase I is a low specificity endonuclease that will cleave almost randomly between any two nucleotides
  - highly specific DNases will only cleave where a certain sequence of nucleotides occurs
    - Eg. restriction endonucleases
- Restriction endonucleases are bacterial enyzmes whose purpose is to cleave foreign DNA eg. bacteriophages

- Restriction endonucleases
  - valuable - because they cleave DNA at specific sites
  - cut only dsDNA and cut both strands at the same time. REs usually occur as dimers, so that one subunit binds to each strand.



  - Restriction endonucleases have recognition sites with 2-fold rotational symmetry within dsDNA.This is a form of inverted repeat.

Examples



Restriction endonucleases

nomenclature (name) of enzyme

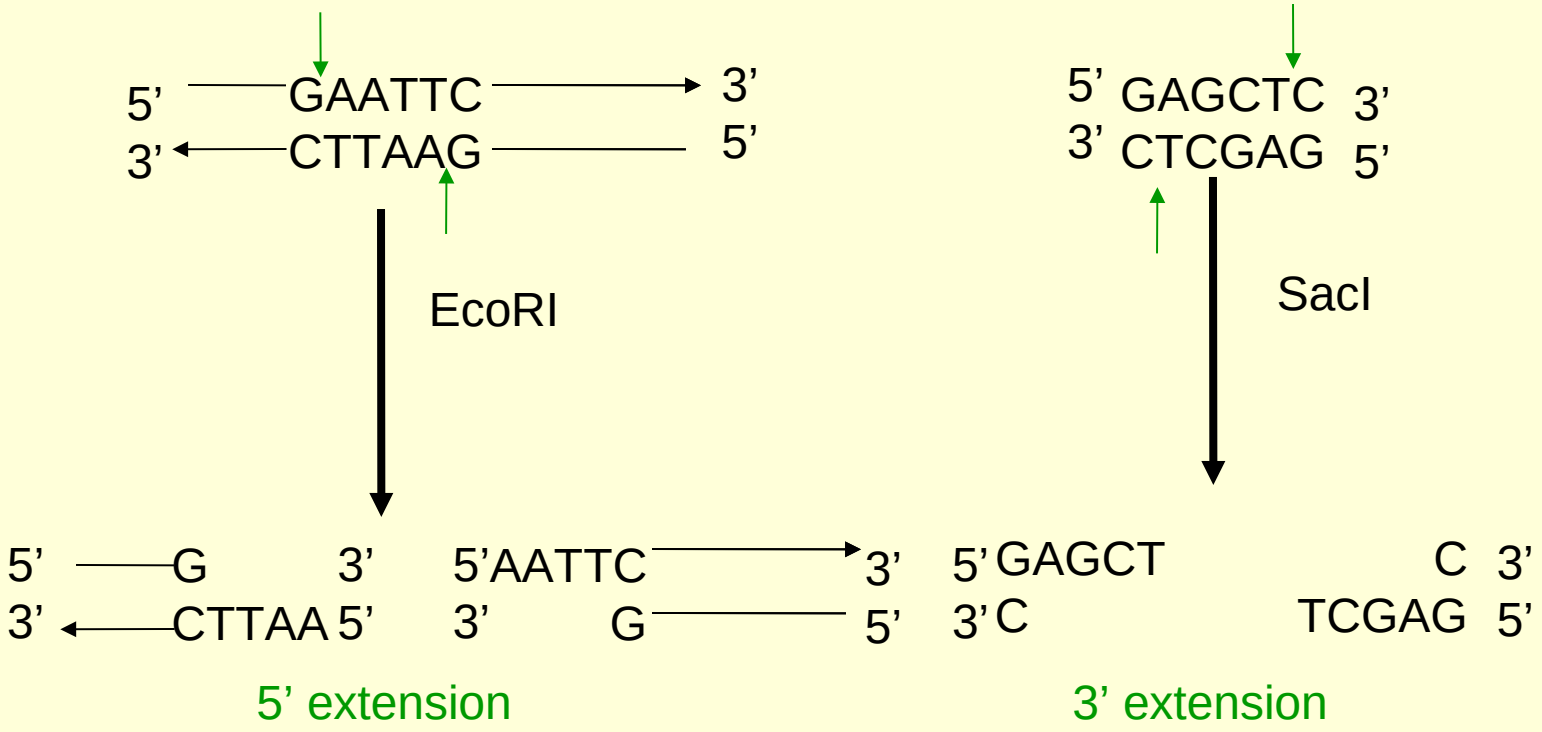
eg.	EcoRI	*E. coli* RY
	KpnI	*Klebsiella pneumonia*
	XhoI & XhoII	*Xanthomonas holicicola*

1st letter	genus
2nd & 3rd letters	species
other letters	strain identity or serotype
number (Roman)	number of enzyme isolated from that particular species

Restriction endonucleases

Cleavage off center within recognition site

Many enzymes cut off centre giving rise to 3' or 5' extensions of 2 or 4 bases

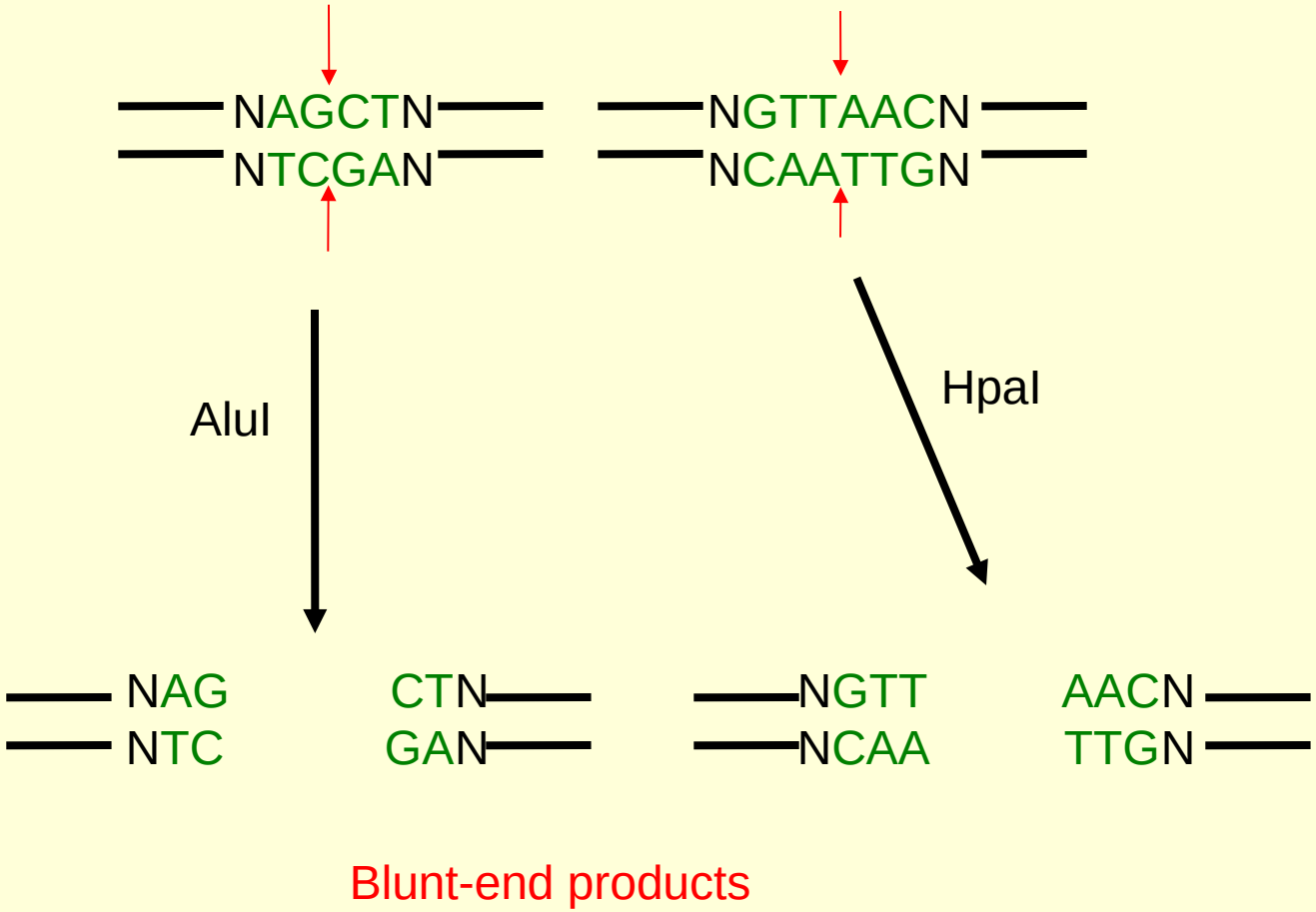


Having 3' or 5' extensions makes it easier to rejoin fragments with complementary ends as this will allow base pairing at the overlap

Hence overhanging end are referred to as cohesive ends

Restriction endonucleases

May cut at center of recognition site



Blunt-end products

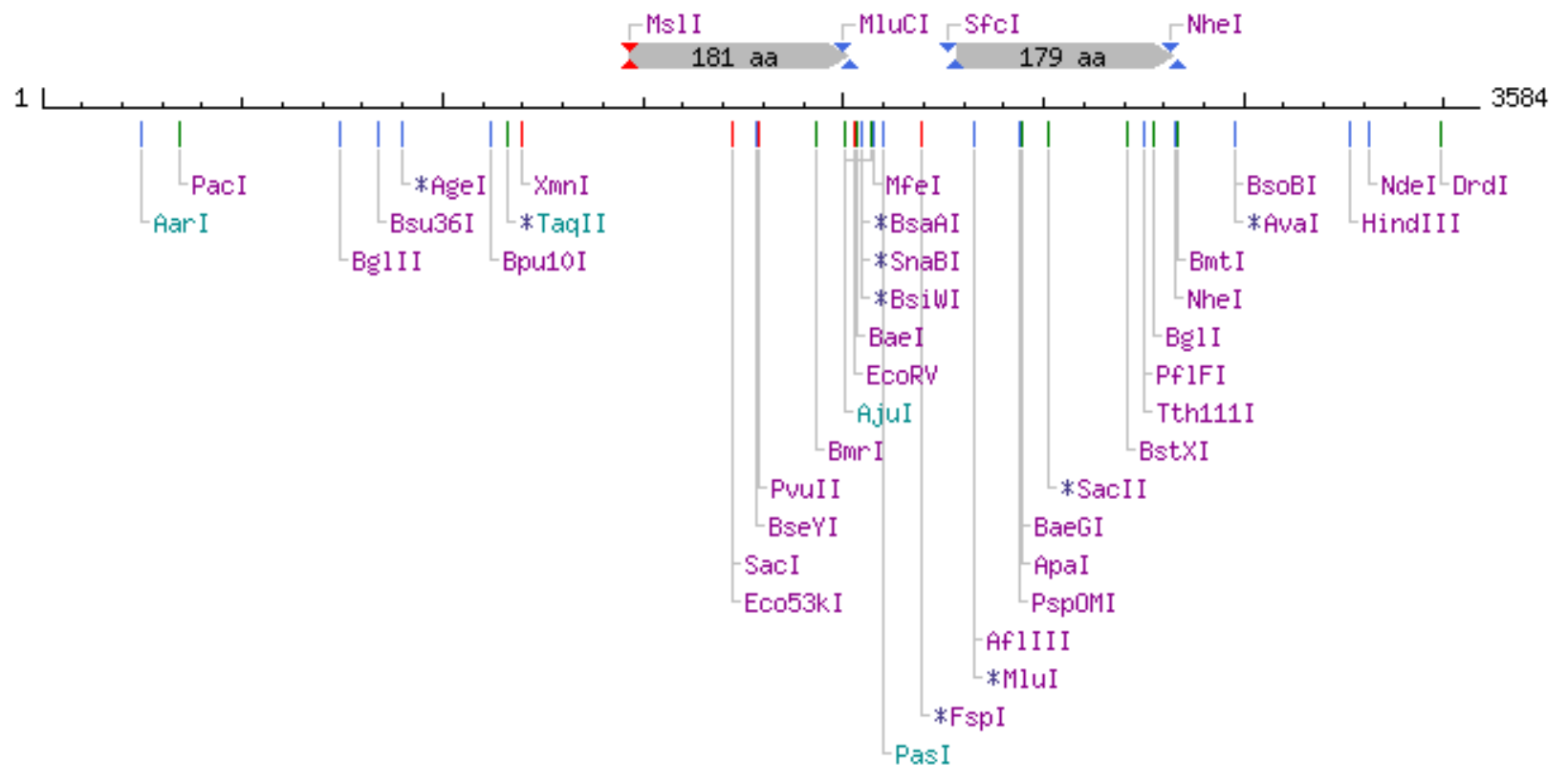
Restriction endonucleases - examples

Enzyme	Cutting site	Description
EcoRI	G^AATTC	5' protruding ends(*Escherichia coli)*
HindIII	A^AGCTT	" " " (*Haemophilus influenza*)
SmaI	CCC^GGG	blunt ends (*Serratia marcescens)*
XmaI	C^CCGGG	5' protruding ends (*Xanthomonas malvacaerum)* an isoschizomer [1]of Sma1
PstI	CTGCA^G	3' protruding ends (*Providencia stuarti*)
HinfI	G^ANTC	5' protruding ends (*H. influenza*). Degenerate recognition site. (GAATC,GAGTC,GACTC,GATTC)
HaeII	RGCGC^Y	3' protruding (*H. aegyptius*) 2^2=4 possible cuting sites: AGCGCC, AGCGCT, GGCGCC, GGCGCT
BglI	5'GCCN NNN^NGGC3' 3'CGGN^NNN NCCG 5'	symmetric, 3'protuding, (*Bacillus globigii*)
BbvI	5'GCAGC$(N)_8$3' 3'CGTCG$(N)_{12}$3'	asymmetric, 3'recessed

[1]**isoschizomer** - restriction endonucleases that recognize the same sequences
R = purine; Y = pyrimidine; N = {A,G,C or T}

Restriction endonucleases - example showing part of a plant alternative oxidase gene (GenBank::Z15117)

Restriction endonucleases - example showing restriction map for the entire alternative oxidase gene (GenBank::Z15117)



Map generated using NEBcutter V2.0 at New England Biolabs
http://nc2.neb.com/NEBcutter2/

REBASE - A comprehensive database of Restriction Endonucleases maintained by New England Biolabs.

http://rebase.neb.com/rebase/

Frequencies of Restriction Sites (or other oligonucleotides)

length n	frequency: occurs every 4^n	example	sequence
1	4	Single nucleotide	G
2	16	Di-nucleotide	GT
3	64	Codon	ATG
4	256	Taq I	TCGA
5	1024	MboII	GAAGA
6	4096	Hind III	AAGCTT
7	16384	Abe I	CCTCAGC
8	65536	Not I	GCGGCCGC

Restriction endonucleases - Try these exercises at home:

Restriction enzyme worksheet from University of Canterbury

https://www.canterbury.ac.nz/media/documents/oexp-science/biological-science/biology-outreach/Restriction-enzyme-worksheet.pdf

Restriction Enzyme Worksheet

https://mafiadoc.com/download/restriction-enzyme-worksheet-name-date-restriction-enzymes-are-_5a2d05381723ddf7ca407475.html

Other nucleases

Less specific (or non-specific) nucleases may cleave only one strand not both.

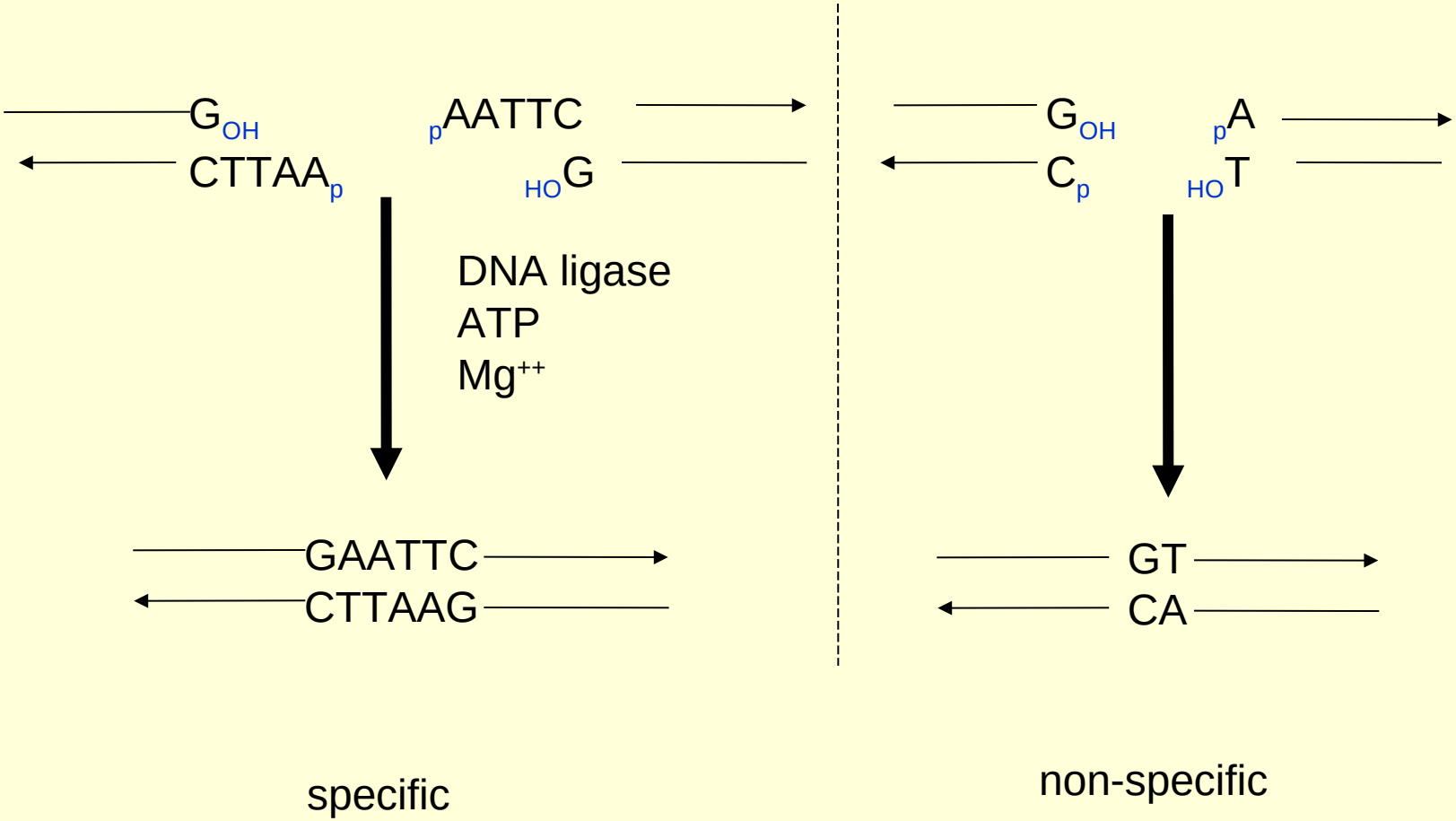
- DNase I is a non-specific nuclease
  - depending on the condition of the reaction mixture will either cut only one strand (nicking) or cleave both strands in a random manner

- RNases are largely non-specific and cleave single-stranded RNA

- RNase H cuts an RNA strand of a RNA-DNA heteroduplex

2. DNA Ligases

- catalyse the joining of DNA strands together.
- do not require any specific sequence but must have compatible ends as well as ATP for energy



3. DNA Polymerases – Replication of DNA

a. DNA polymerase I

-enzyme derived from *E. coli*

-DNA template-dependent DNA polymerase

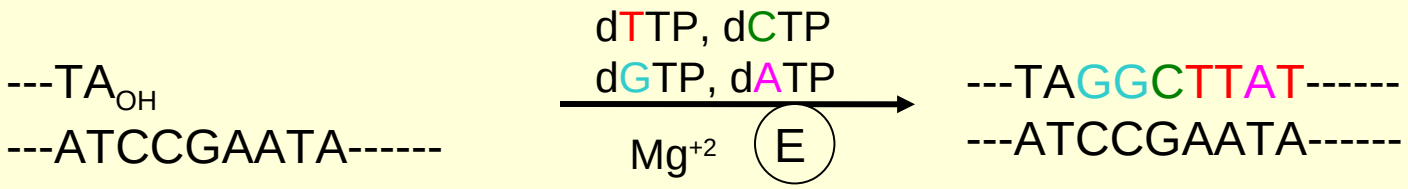
-single polypeptide but with 3 separate enzyme activities

5’→3’ DNA polymerase

5’→3’ exonuclease activity

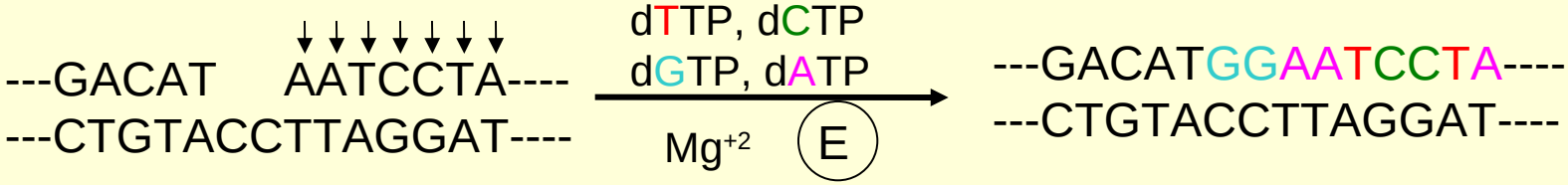
3’→5’ exonuclease activity

5’ → 3’ DNA polymerase

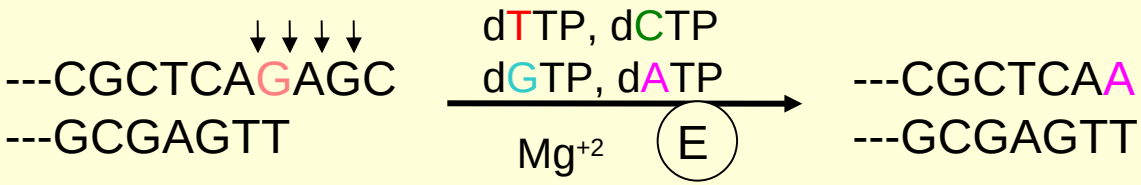


Extends from 3’ end of a primer

5’→3’ exonuclease activity (+polymerase)



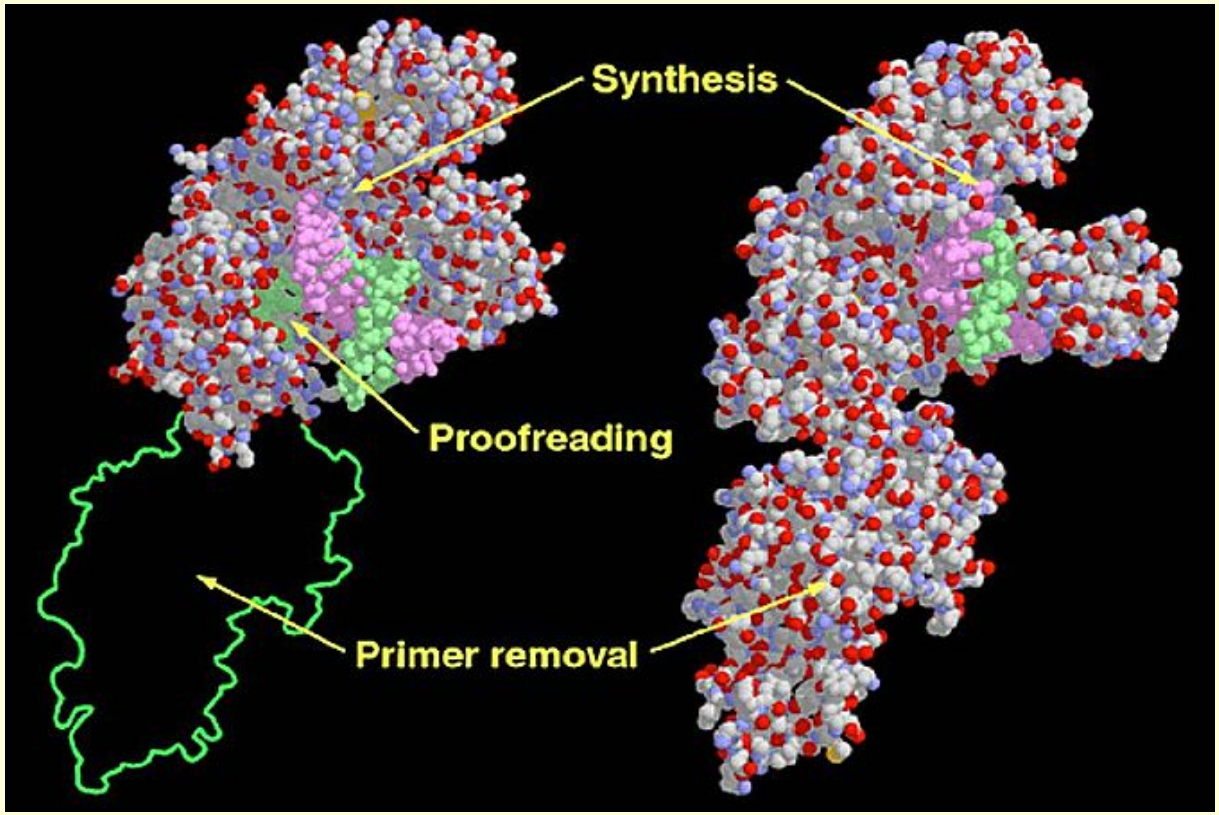
3’→5’ exonuclease activity (proof reading)



b. Klenow enzyme (large fragment of DNA polymerase I) - DNA pol I cleaved with a protease into 2 parts

Klenow fragment

“polI (holoenzyme)”



5’ →3’ polymerase activity

3’→ 5’ exonuclease activity

5’→ 3’ polymerase activity

5’→ 3’ exonuclease activity

3’→ 5’ exonuclease activity

from https://en.wikipedia.org/wiki/Klenow_fragment

Types of labeled nucleotides

Labeled nucleotides can be included in a DNA synthesis reaction to make it possible to detect the presence of the DNA.

Several types of labeled nucleotides are commercially available:

Flourescent nucleotides - emit flourescence at a specific wavelength when excited by UV light. These are the easiest to work with.

Biotinylated nucleotides - bind streptavidin-conjugated with peroxidase. Peroxidase will break down a chemiluminescent substrate, emitting a photon at a specific wavelength

Digoxygenin nucleotides - bind anti-dig antibodies, conjugated with peroxidase. Peroxidase will break down a chemiluminescent substrate, emitting a photon at a specific wavelength

(Radiolabled nucleotides such as ^{32}P are obsolete.)

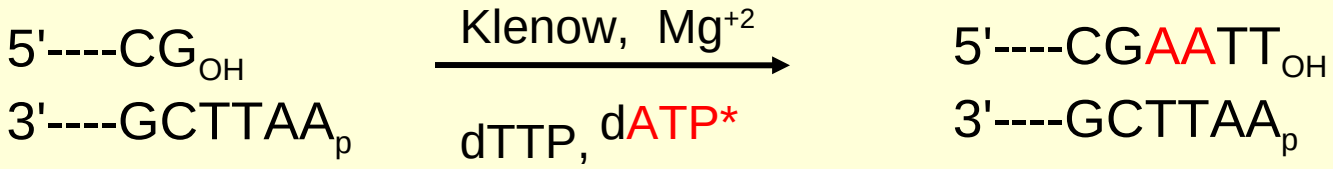
DNA synthesized using labeled nucleotides can be detected by imaging

- The main use of flourescent or chemiluminescent nucleotides is to include them in DNA synthesis reactions.
- For Southern blots, labeled DNA is used as a probe that hybridizes with unlabeled DNA separated by gel electrophoresis
- For PCR reactions, DNA is directly labeled by including labeled nucleotides in the reaction, and bands on the gel are visualized by the imager.

b Klenow enzyme

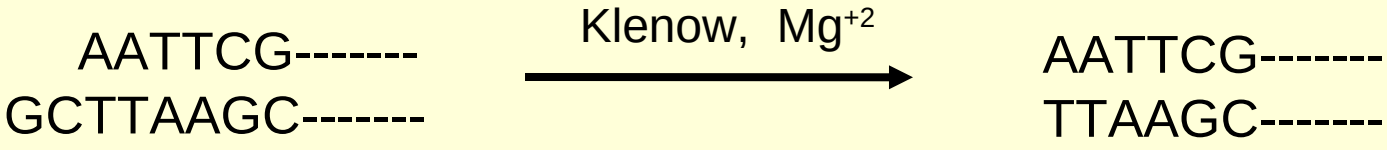
Uses:

1. Filling in (or labeling) 3' recessed termini



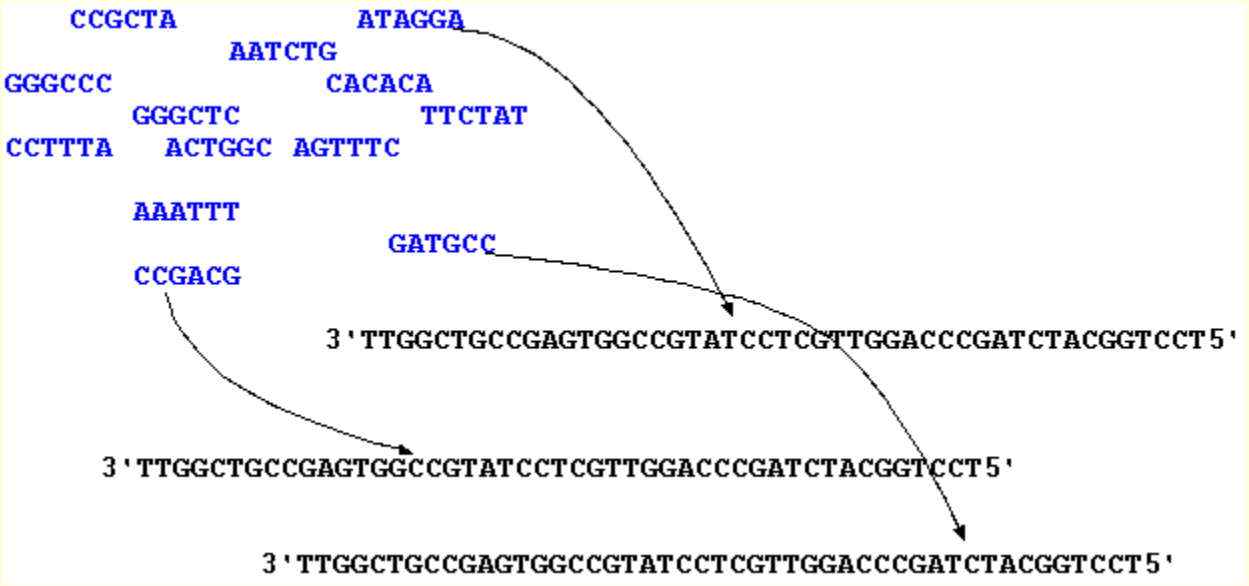
(Labeled so that target DNA can be detected)

2. Removal of 3' extension to produce blunt ended DNA



3. Random hexanucleotide labeling using the Klenow enzyme

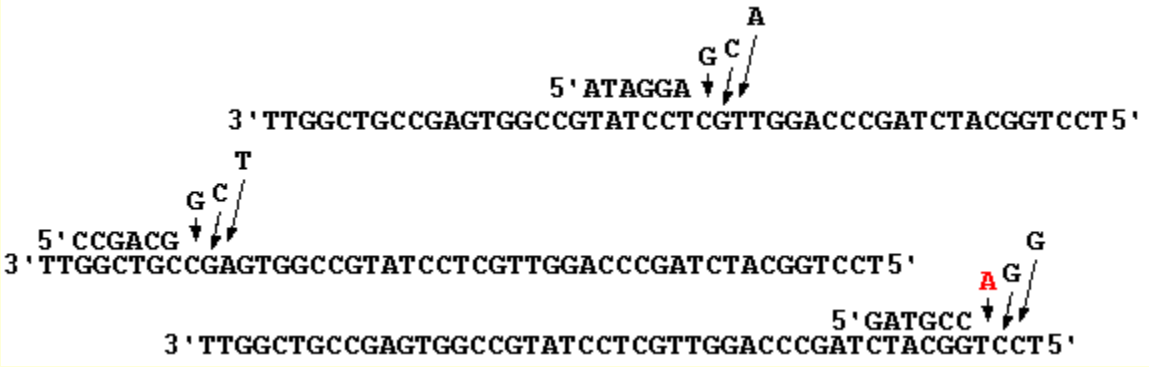
A pool of DNA hexamers is added to the purified DNA sample. Some of the hexamers will find complementary sequences with which they can base pair.



Klenow uses:

3. Random hexanucleotide labeling

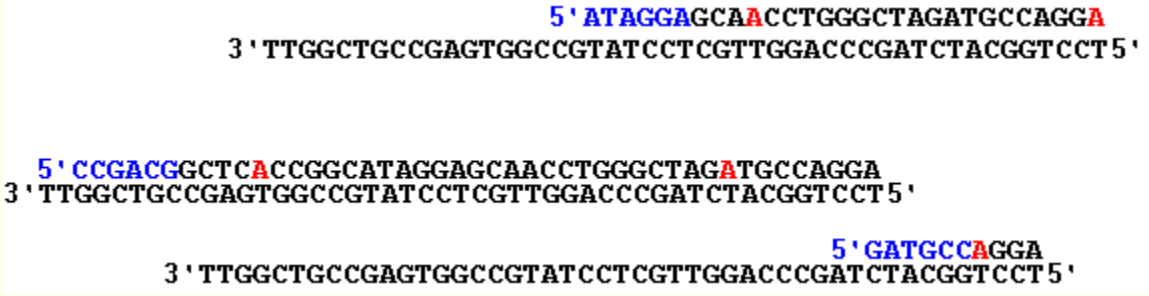
Hexamers act as primers for DNA synthesis using the Klenow enzyme. In this example, most of the nucleotides are unlabeled. However, a small percentage of the A's are labeled with either flourescent or chemiluminescent tags.



Klenow uses:

3. Random hexanucleotide labeling

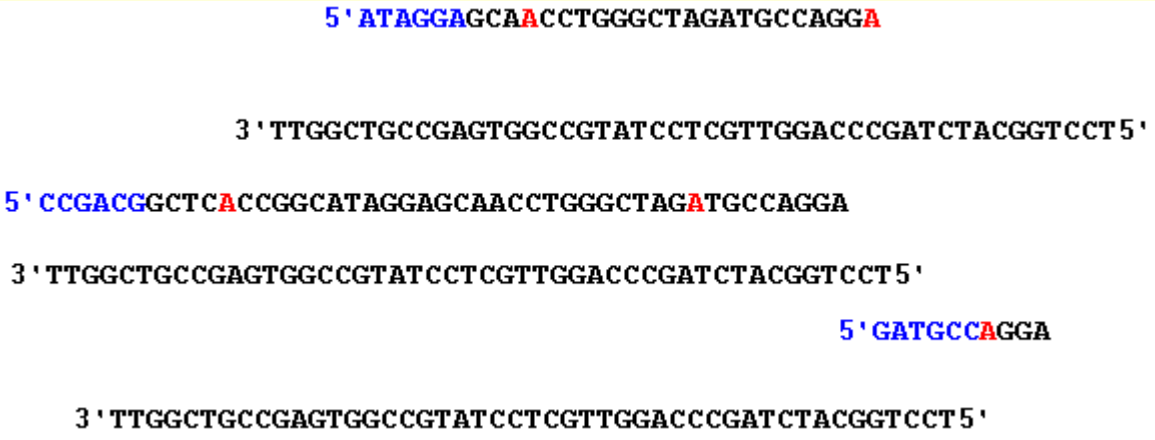
DNA synthesis will proceed until DNA polymerase reaches the end. The length of each strand synthesized will depend on where the primer binds. Each newly synthesized strand will contain a percentage of labeled nucleotides, which is enough for most detection purposes.



Klenow uses:

3. Random hexanucleotide labeling

To use the newly-synthesized DNA as a probe, the sample is heated, denaturing the DNA, so that all DNA is now single-stranded. Each of the newly synthesized strands is available to bind to a target DNA on a Southern or Northern blot.
The unlabled strands will still be present, but are not detected, because they don't contain label. They can be ignored.

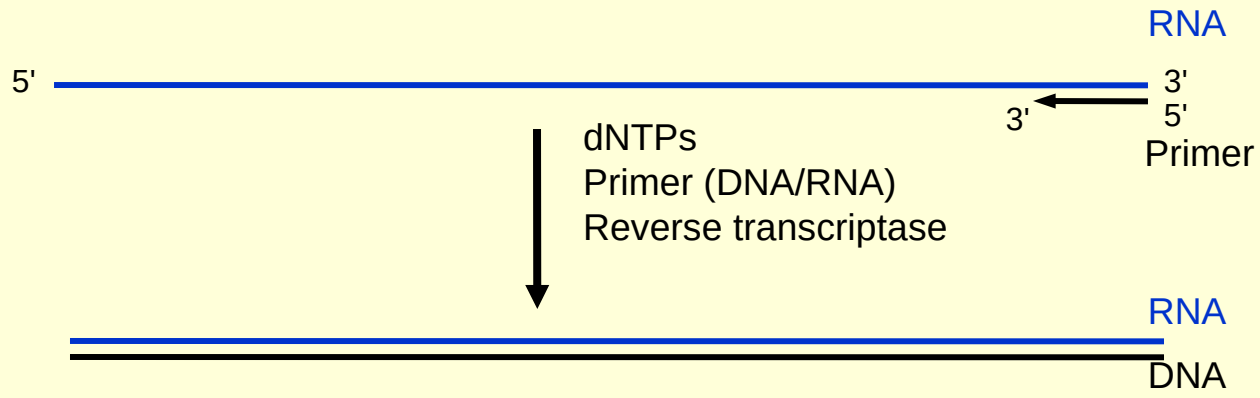


DNA Polymerases (cont'd)

a. Reverse transcriptase (RT)

-RNA template-dependent DNA polymerase

- also requires a primer

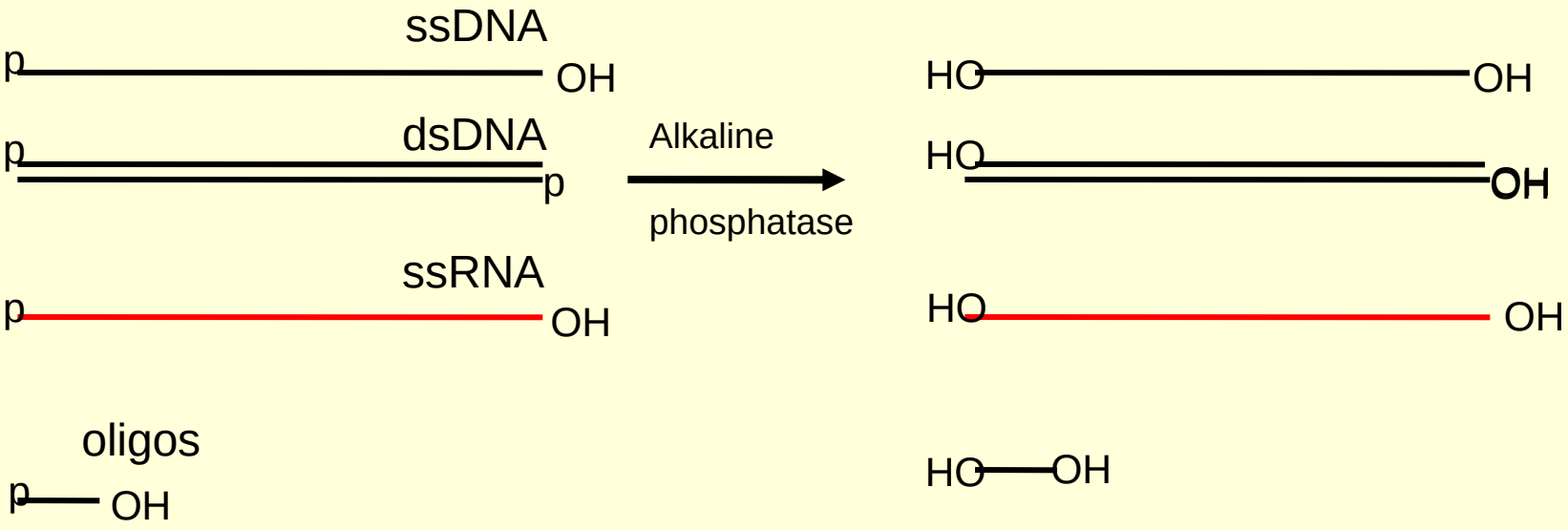


Use: reverse transcription of mRNA into complementary DNA

4. DNA/RNA Modifying enzymes

A Alkaline phosphatase

-catalyze removal of phosphate group from 5' end of both RNA and DNA

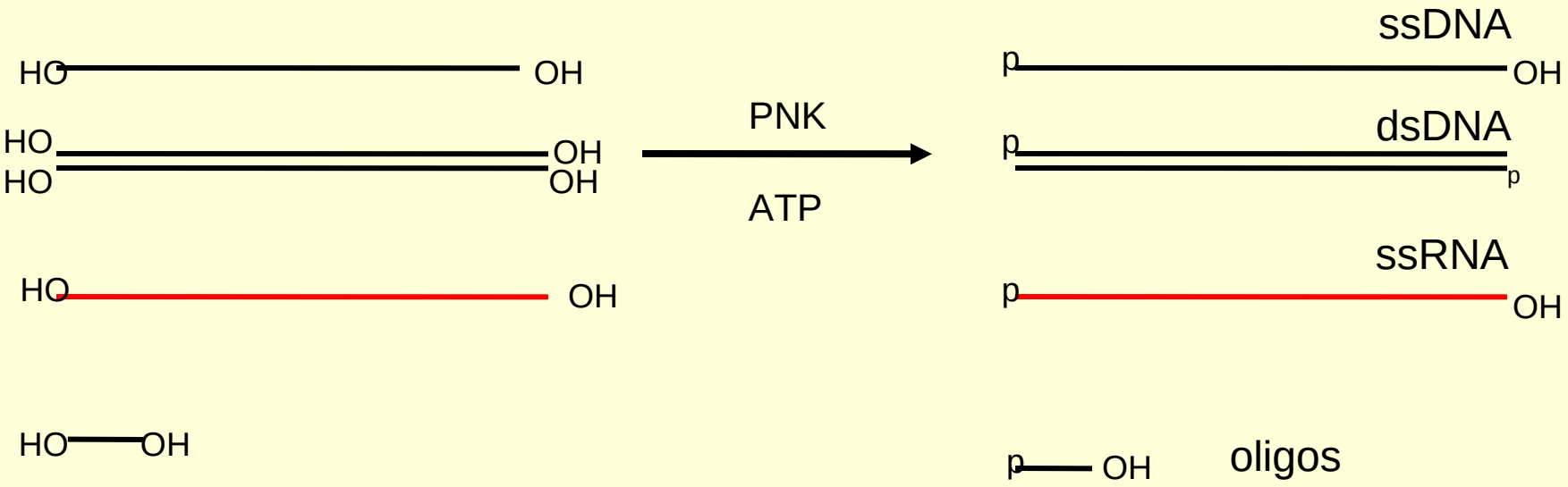


Use: To prevent ligation

DNA/RNA Modifying enzymes (cont'd)

B Polynucleotide kinase (PNK)

- catalyzes the transfer of the terminal phosphate group of ATP to replace a free 5' hydroxyl group of DNA (ss or ds), RNA, or oligos



Uses: -end labeling oligos and short DNA/RNA

-making 5' ends ligation-ready if they only have a hydroxyl group

eg. chemically synthesized DNA

Techniques

Polymerase Chain Reaction (PCR)

Kary Mullis (1983) Nobel Prize (1993)

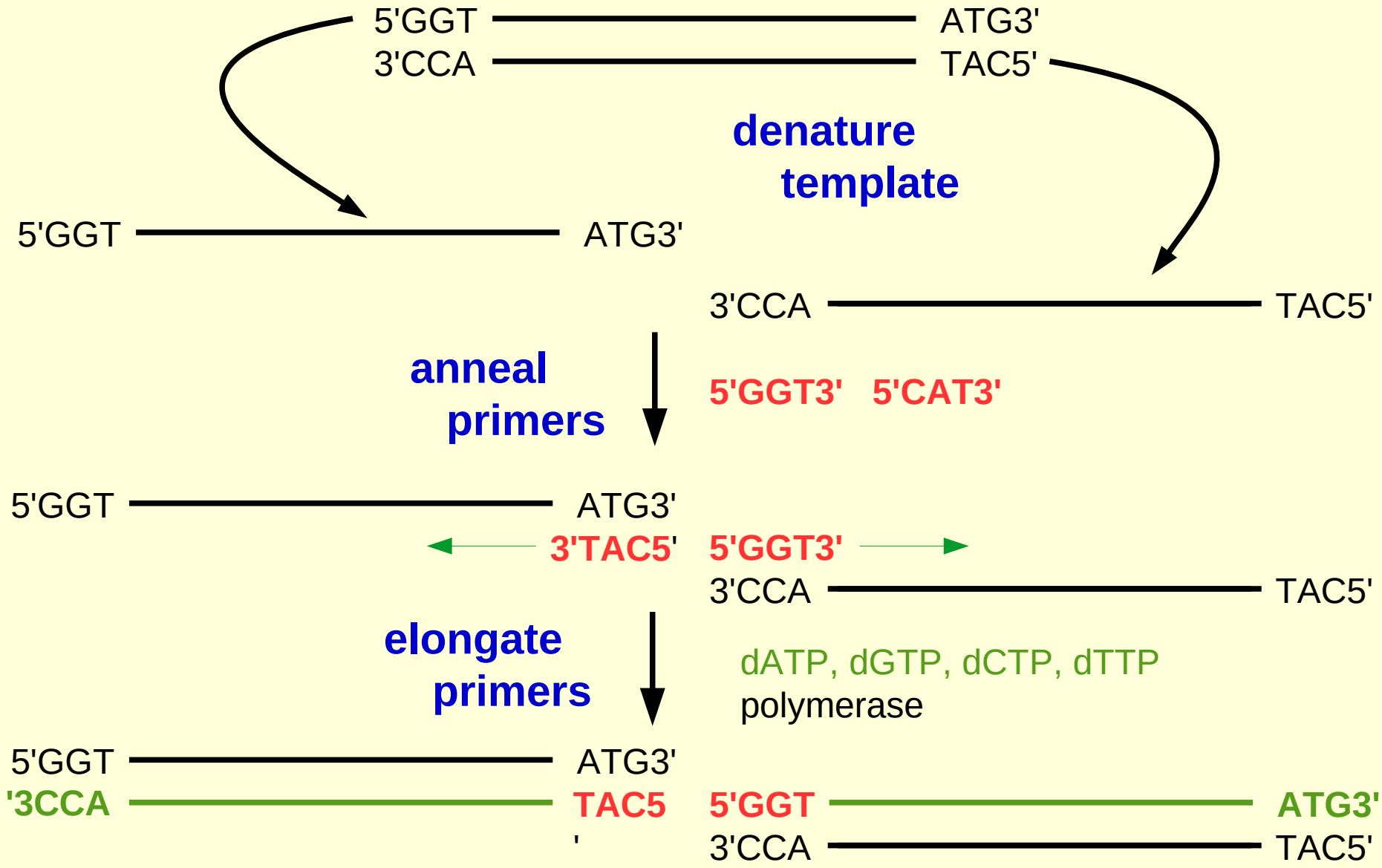
For rapid amplification of one or more fragments of dsDNA

Requirements: dsDNA template
primers (ssDNA oligomers, 8-30 mers)
dNTPs (mix of dATP, dGTP, dTTP, dCTP)
thermostable DNA polymerase
Mg^{+2} cofactor

Thermocycler - instrument to alter temperature on a programmed cycle

https://youtu.be/iQsu3Kz9NYo

Simplified PCR cycle



** in a real reaction, primers are usually about 20 bases long*

PCR requires a thermostable DNA polymerase

-eg Taq polymerase (*Thermus aquaticus)*

-enzyme most active at 70-80°C and can survive 97°C

-lacks 3'- 5' exonuclease activity (no proofreading ability)

lower fidelity

error rate 1 in 10^4 to 10^5 vs 10^7 to 10^9 for DNA Pol I

Other thermostable commercial enzymes include Pfu, Vent (higher fidelity)

possess 3'- 5' exonuclease activity

Use: Polymerase chain reaction (PCR) -a technique for amplifying DNA sequences ~10^9 in a few hours

PCR

- Stages of thermocycle — typical conditions

30-35x

- Initial denaturation — ~3 min @ 96°C
- Cycle denaturation — 0.5 -1.5 min @ 96°C
- Primer annealing — 0.3-1 min @ 57°C
- Chain elongation — 0.5-3 min @72°C

Temp transitions between each temp is as rapid as possible

Primer binding sites: 100 – 2000 bp apart (common)
up to 25,000 bp is possible

<u>Specificity</u>

- Typical primers 8-25 nt
- Annealing temps 2-5°C below T_m for primer

PCR applications

- Selective amplification of DNA fragments from longer sequences
- DNA, RNA sequencing
- Site-directed mutagenesis
- Genome mapping/molecular marker studies (RAPD, AFLP, SCAR)
- Quantitation of specific RNA (RT-PCR)
    - viral infection assessment
    - gene expression by mRNA level

Techniques
Gel electrophoresis

- Electrophoresis allows separation of charged molecules in an electric field
- Separation based on the net charge carried by the molecules and their physical size (affects frictional drag)
- DNA/RNA molecules all possess same charge density so separation is based on ‘size’ Smaller molecules move faster than larger ones
- Agarose (0.8%) used as the frictional media for electrophoresis of DNA/RNA larger than ~250 bp (or nt)
- Acrylamide may be used as a frictional media for high resolution separation for DNA/RNA from 50-1000 bp/nt

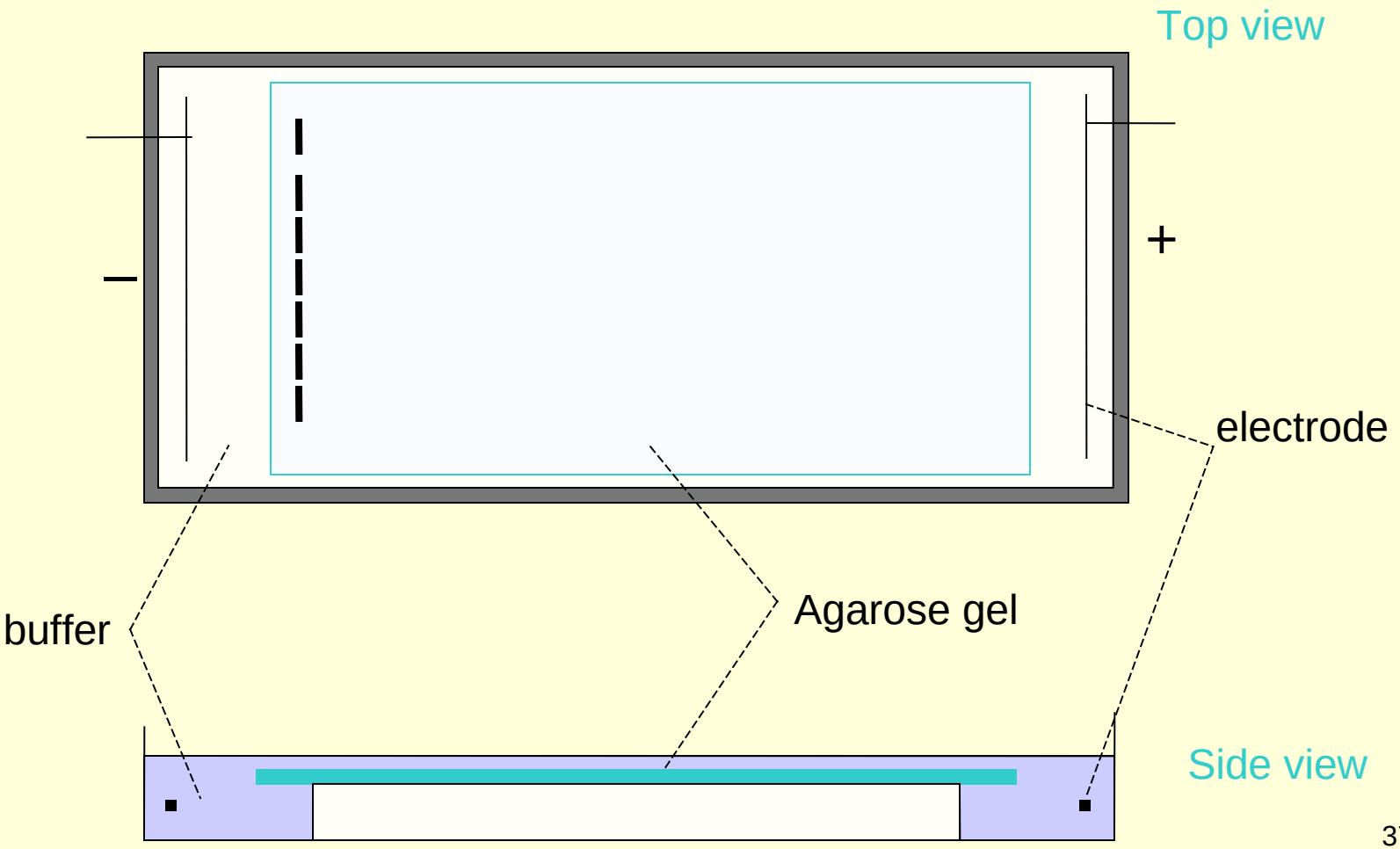
Gel electrophoresis

Video from BioRad

http://youtu.be/vq759wKCCUQ

Agarose Gel electrophoresis

- During and post-separation, agarose reduces the rate of diffusion



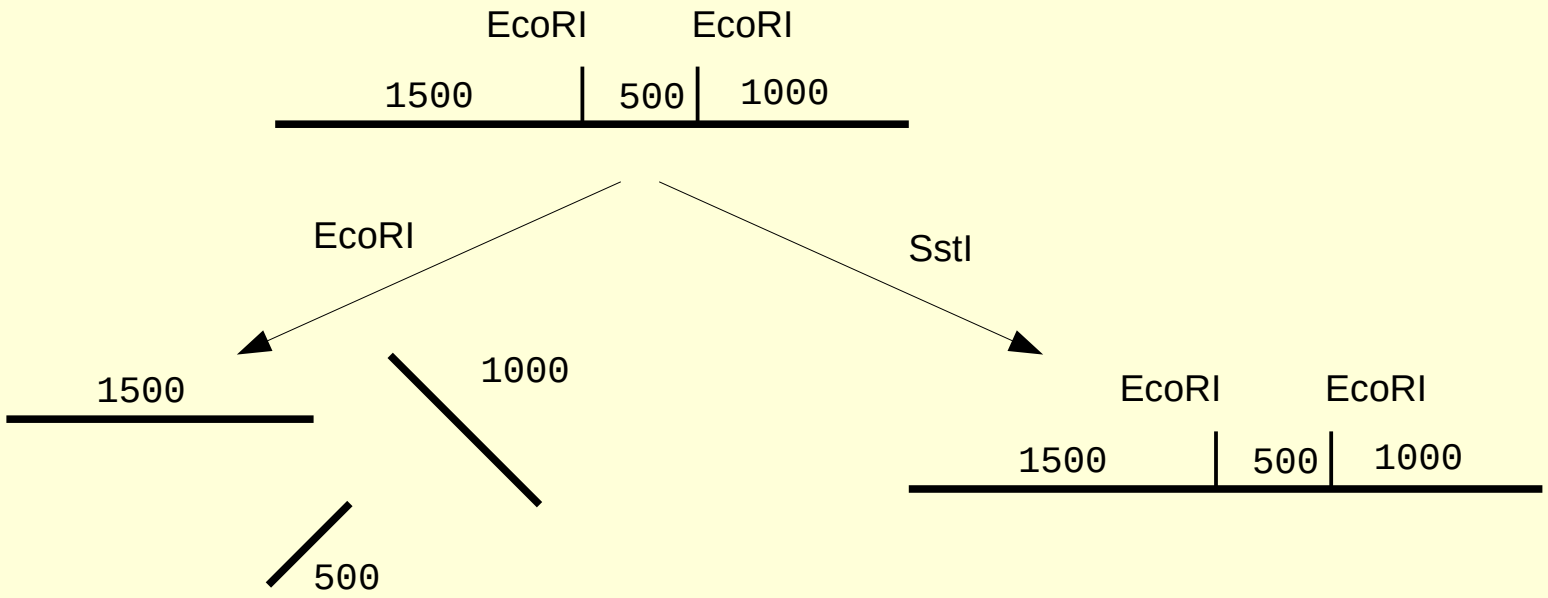
Electrophoretic Separation of DNA - Example

3000 bp barley gene

EcoRI cuts A into 3 pieces

1500, 1000 and 500 bp

SstI doesn't cut A



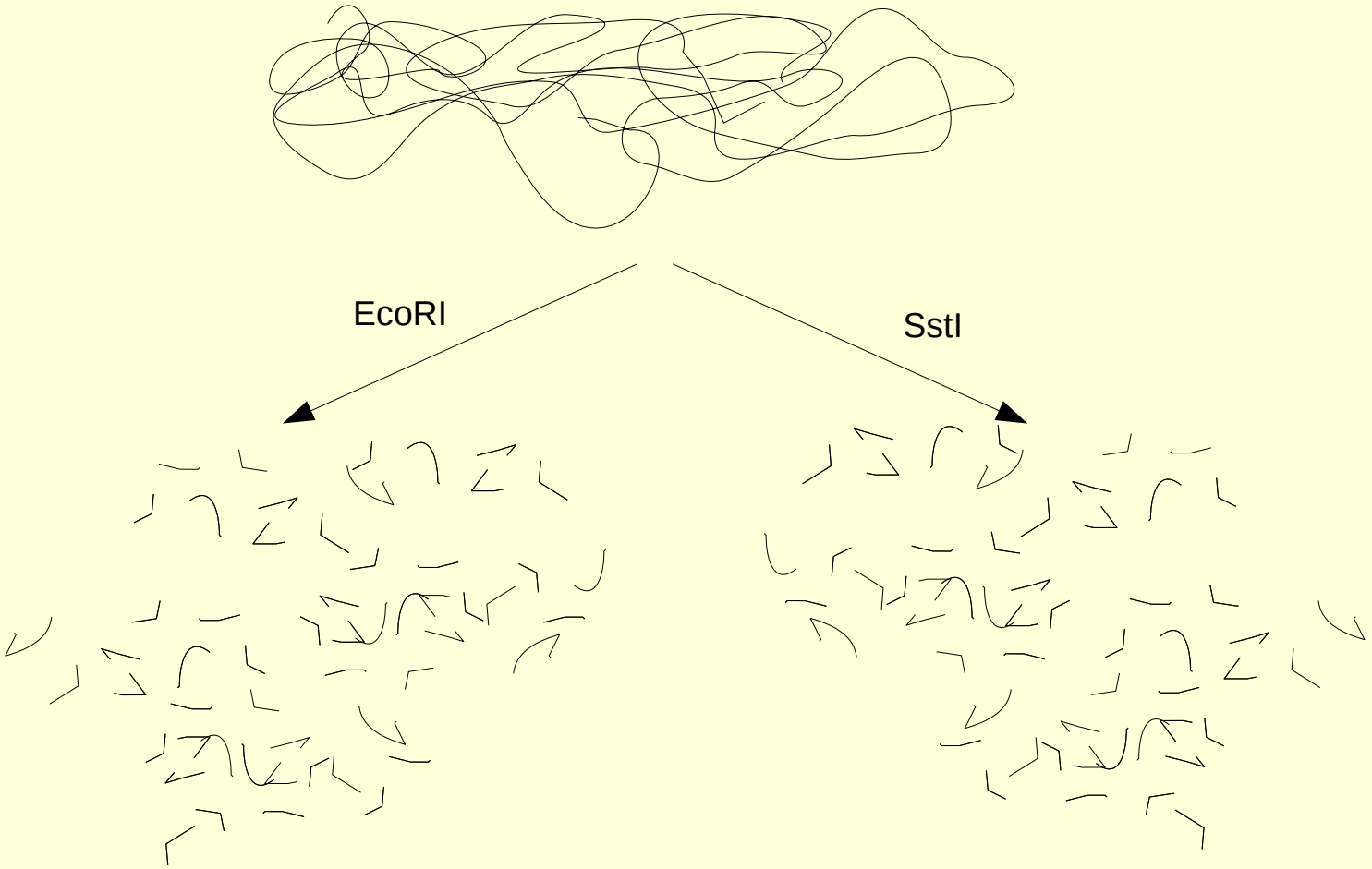
Electrophoretic Separation of DNA - Example

barley genomic DNA ~5x10^9 bp (B)

EcoRI cuts B into > 200,000 pieces

size range (100 -50,000 bp)

SstI cuts B into >200,000 pieces



Electrophoretic Separation of DNA

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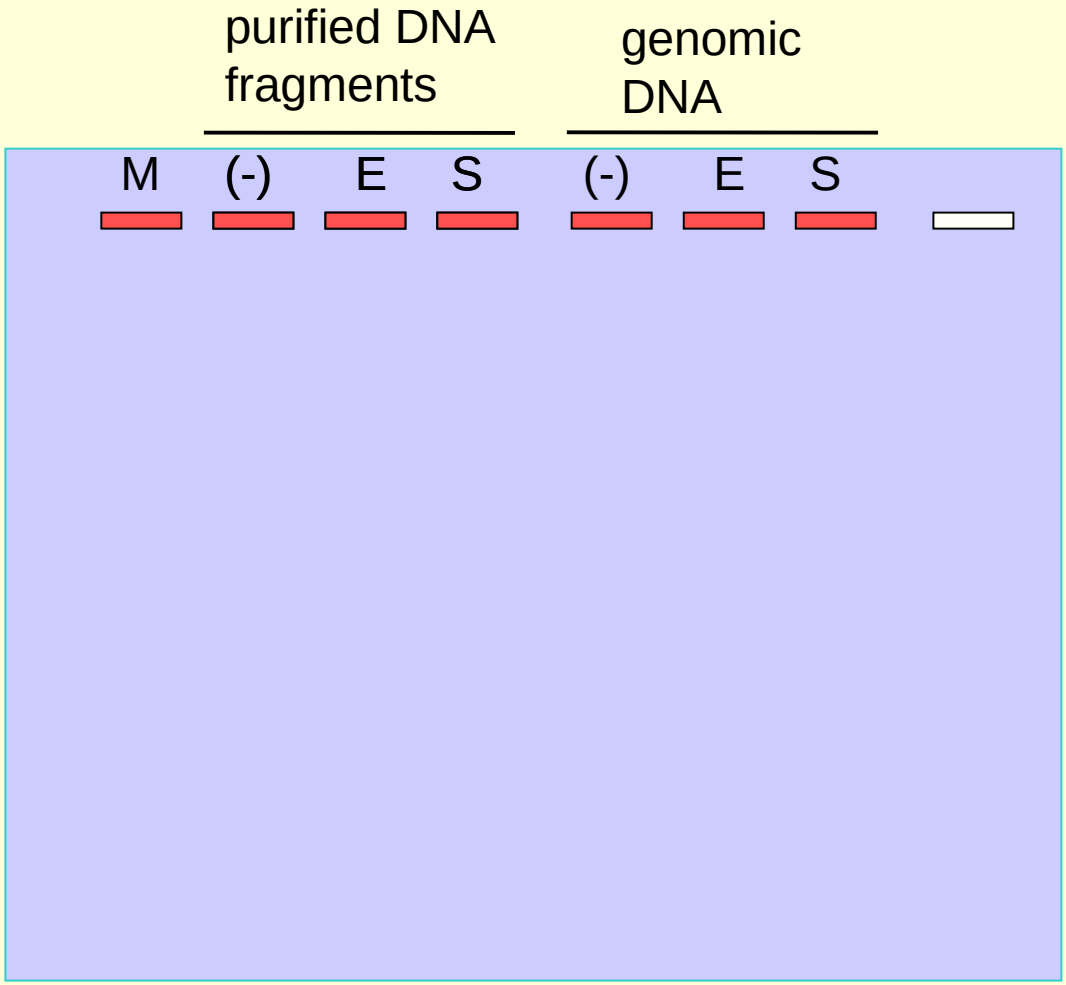


M size marker DNA

(-) -uncut

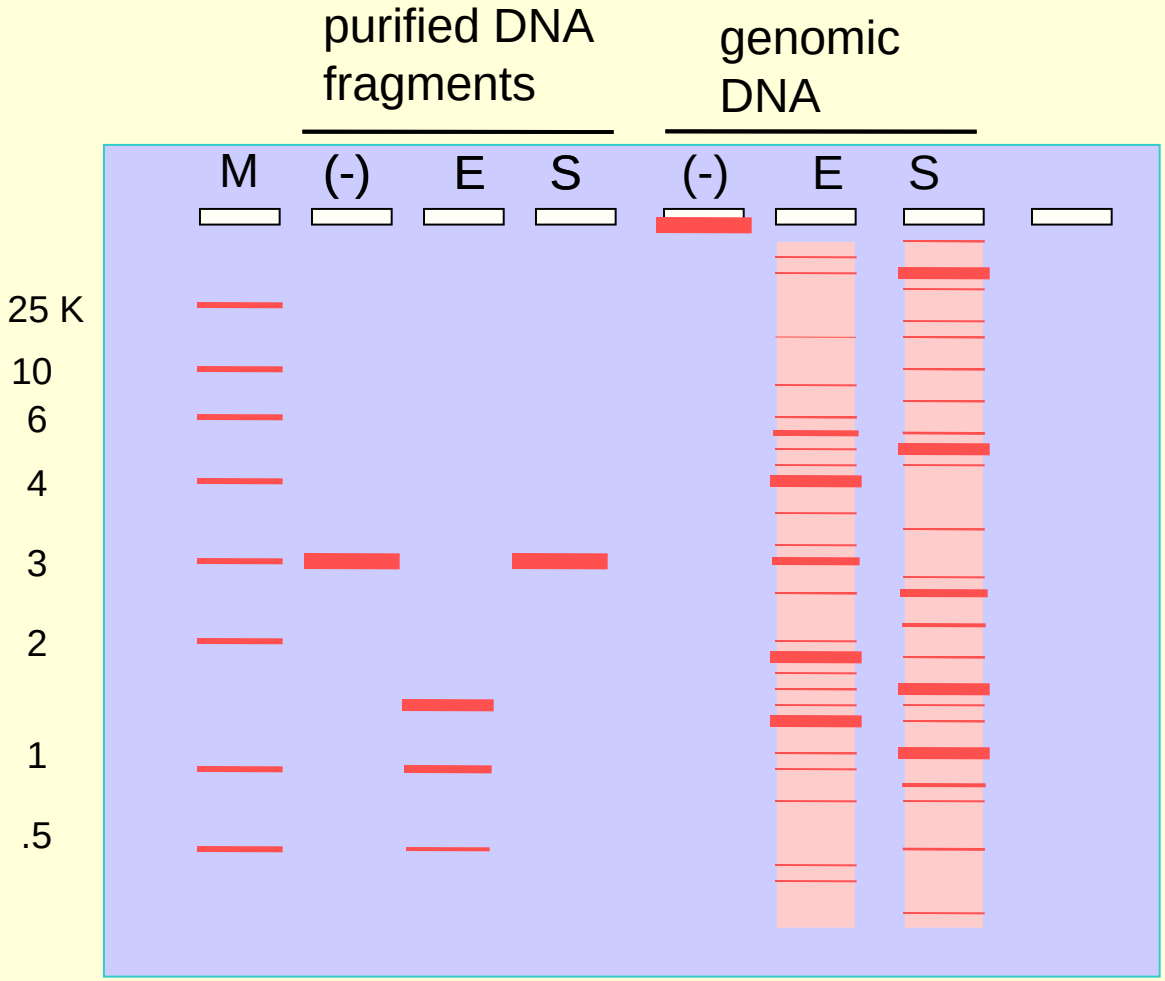
E -EcoRI digested

S -SstI digested

EcoRI and SstI both have 6-base recognition sites

Flourescence stained gel after electrophoresis, viewed in UV light

- Small DNA produces individual bands
- Large DNA when cut gives a continuum of bands



M -size marker DNA (kbp)

(-) -uncut

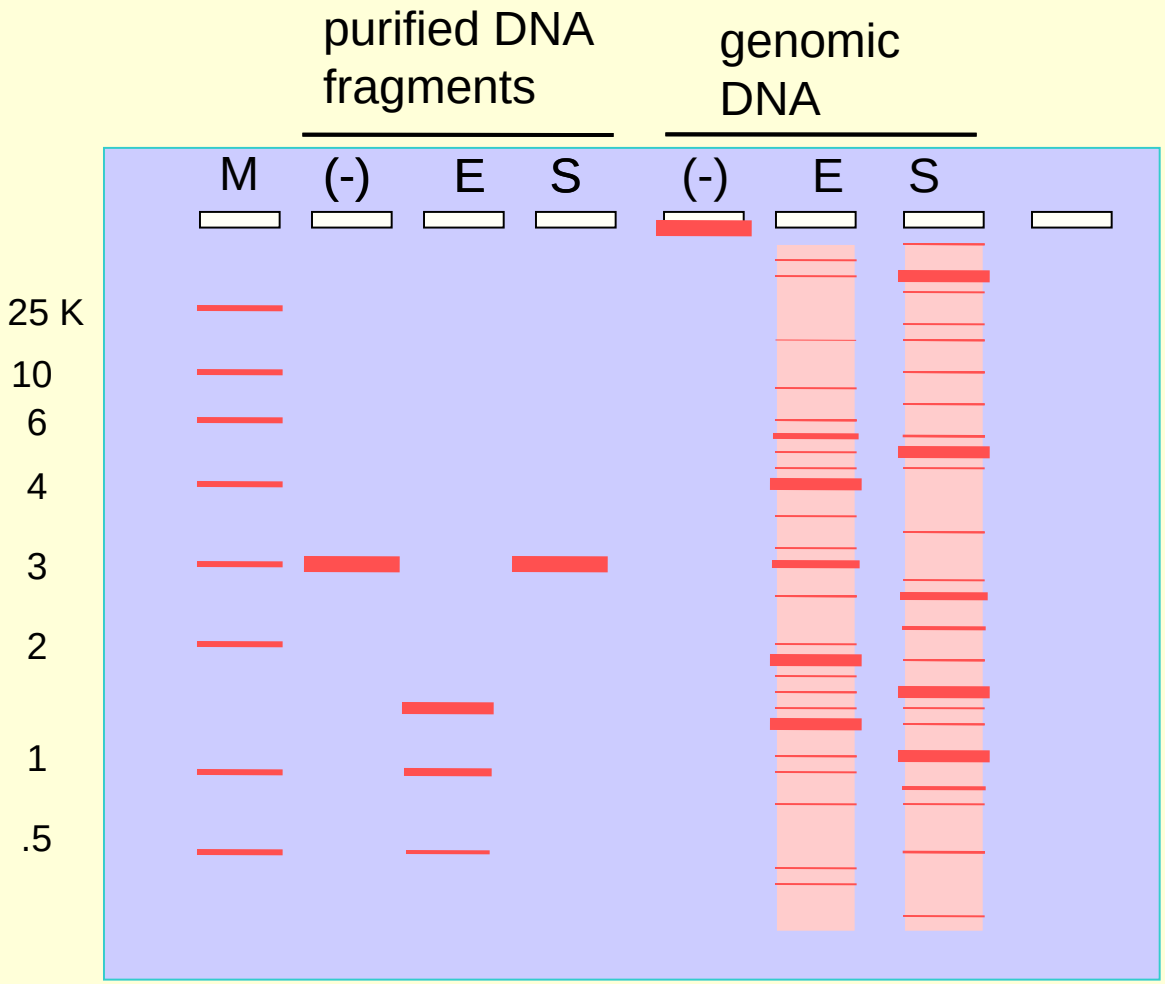
E -EcoRI digested

S -SstI digested

remember: each band contains millions of identical copies of a particular DNA fragment or fragments

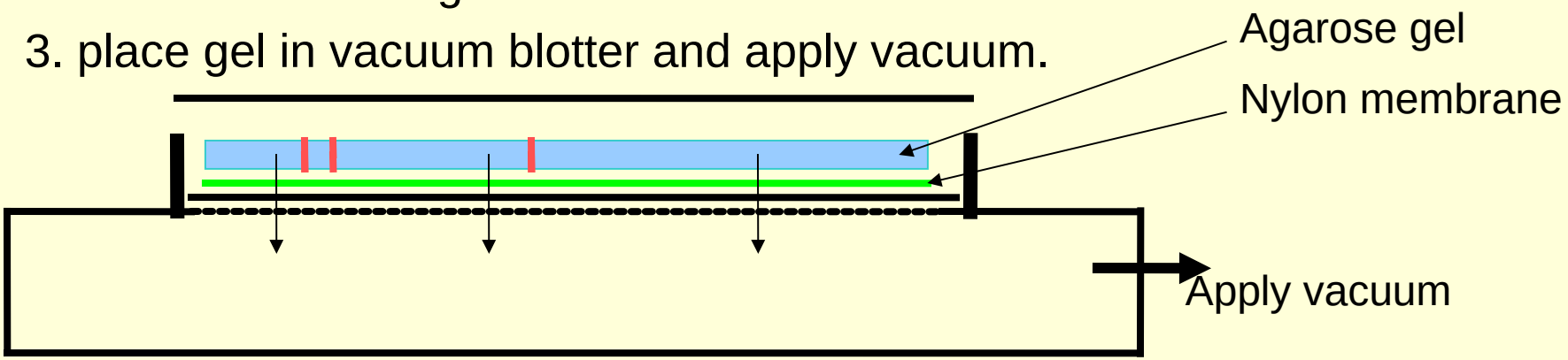
Finding out the identity of bands on a gel: filter hybridization

….but all we really see is bands on a gel. How do we find which band contains a particular gene?



Gel Blotting - vacuum blotting

1. Treat gel with NaOH to denature DNA helices (DNA becomes single-stranded)
2. Neutralize with high salt solution
3. place gel in vacuum blotter and apply vacuum.

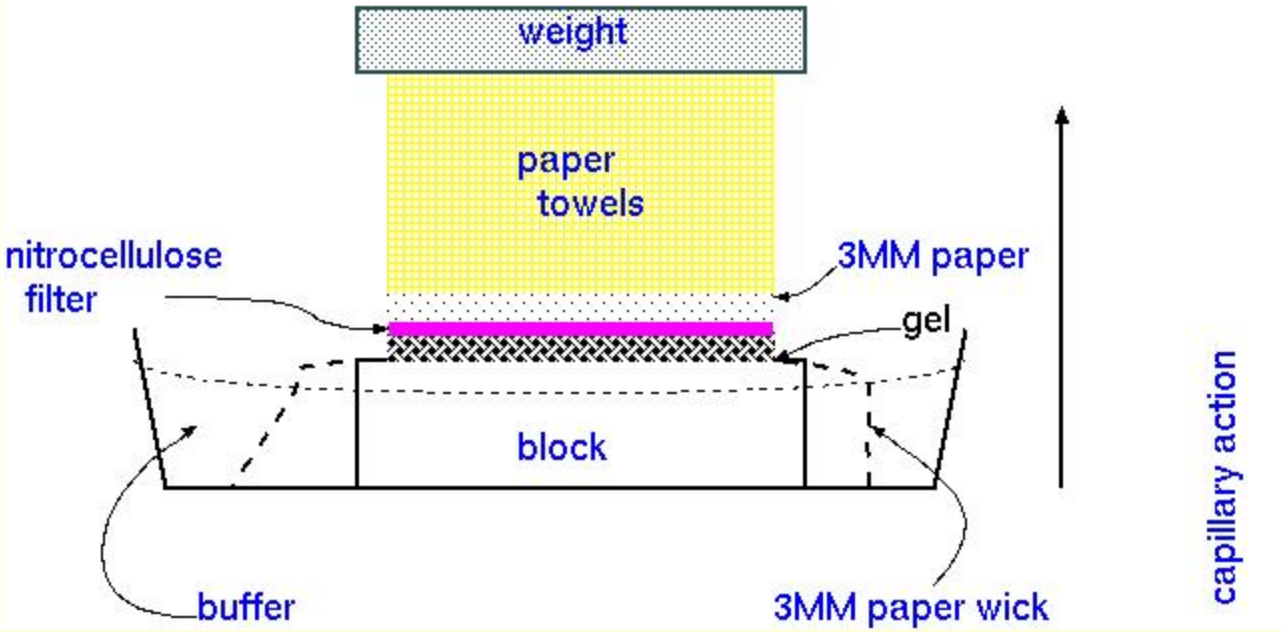


Buffer with soluble DNA pulled directly from gel onto membrane where DNA is bound replicating the separation pattern that was in the gel

Process is blotting.
The membrane with bound transferred DNA is a Southern blot

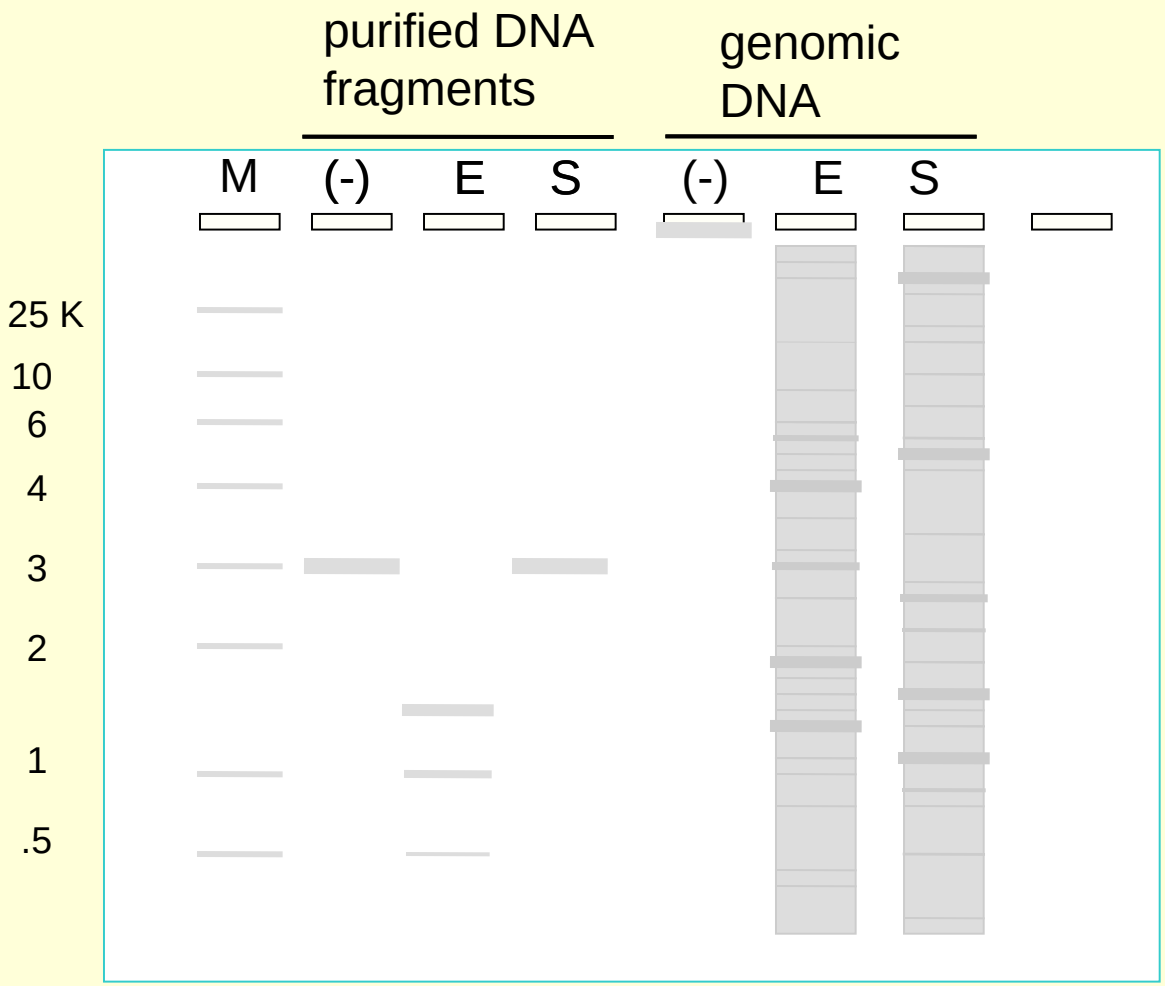
Gel Blotting - capillary blotting

alternatively, after NaOH denaturation and neutralization steps, you can use capillar action to transfer the DNA to a filter



Finding out the identity of bands on a gel: filter hybridization

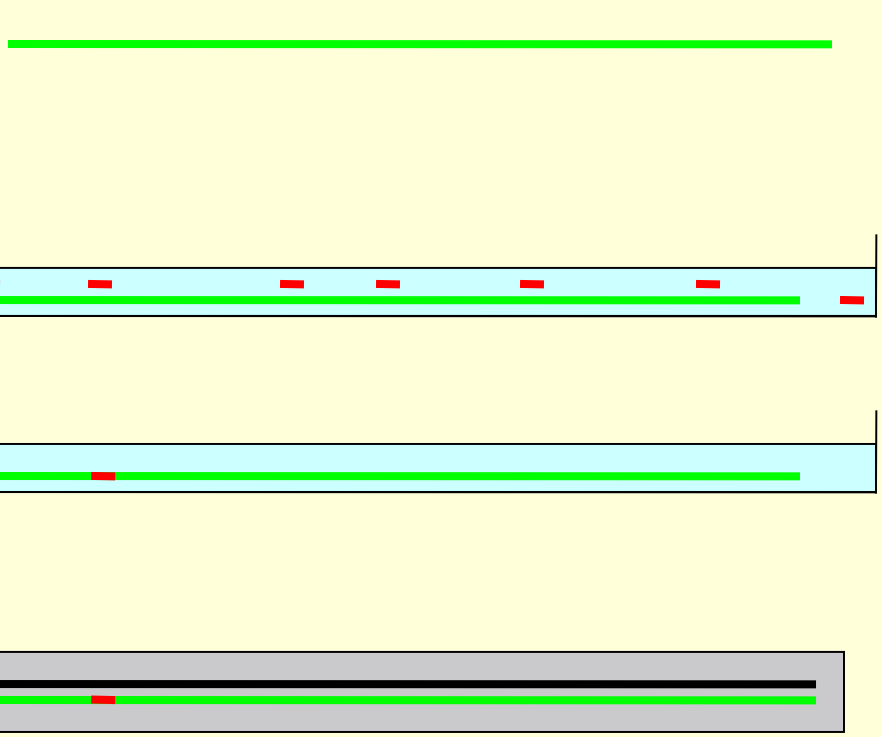
After blotting, each unique DNA fragment is on the filter in exactly the same position that it was in on the gel.



At this stage, we can't actually see the DNA. To the eye, it looks like a perfectly blank filter:

Techniques
Probing the blot

Purpose: To identify if a specific sequence exists on the blot by testing if a hybrid with a probe will form



Replica of separated DNA on the membrane is <u>denatured</u> -- ie ssDNA

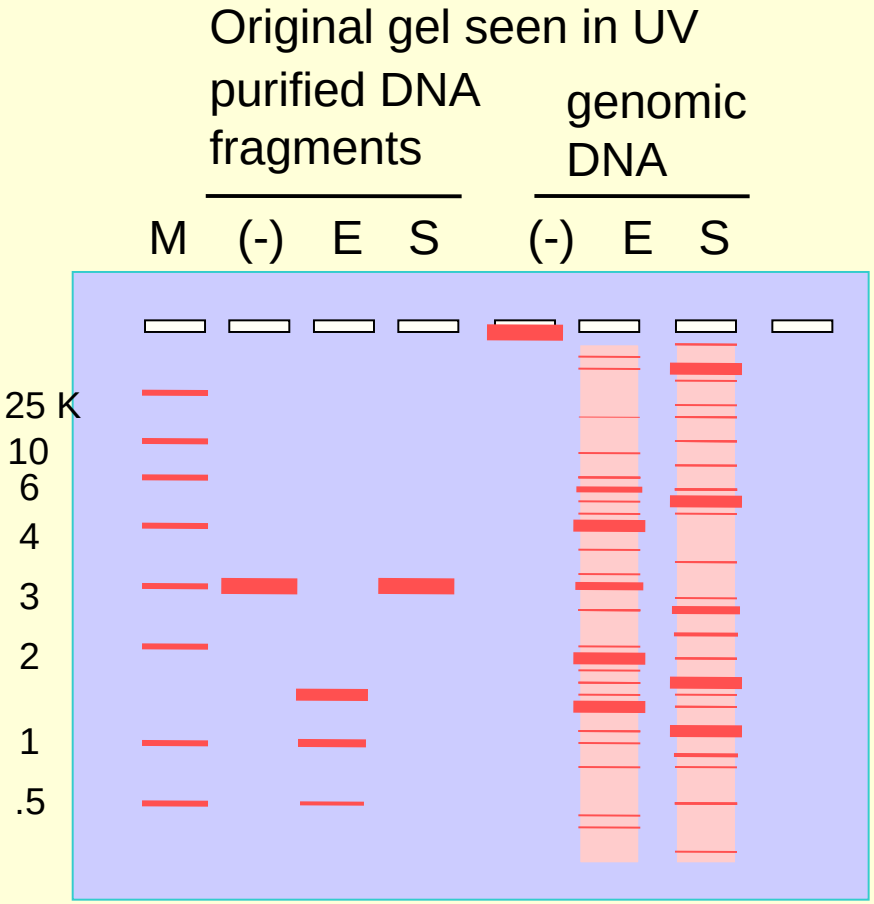
Incubate with labeled ss probe under hybrid-forming conditions

Wash any unbound probe away so that the only label is bound to its complement

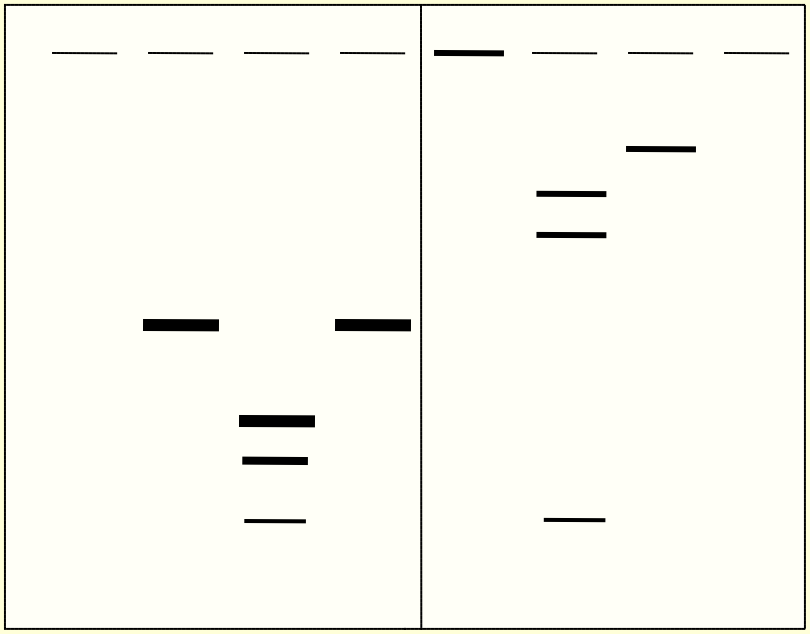
For chemiluminescent detection, film is exposed to membrane inside a light-proof box to detect location of bound labeled probe

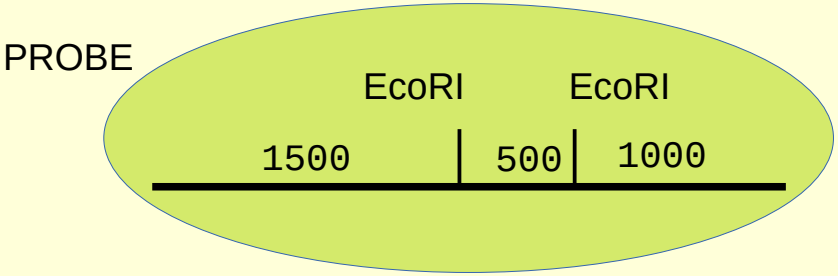
Hybridization results

Original gel seen in UV



Locations of bound probe can be visualized using autoradiography for chemiluminescent detection, or fluorescent imaging if flourescent nucleotides are used.

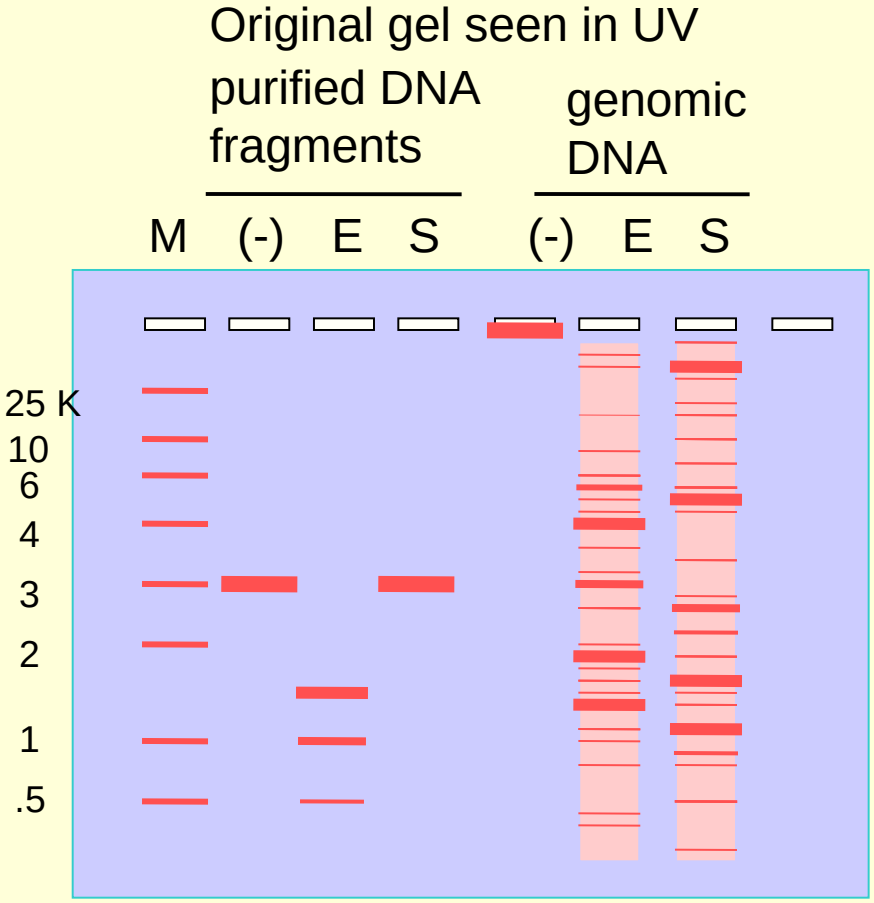




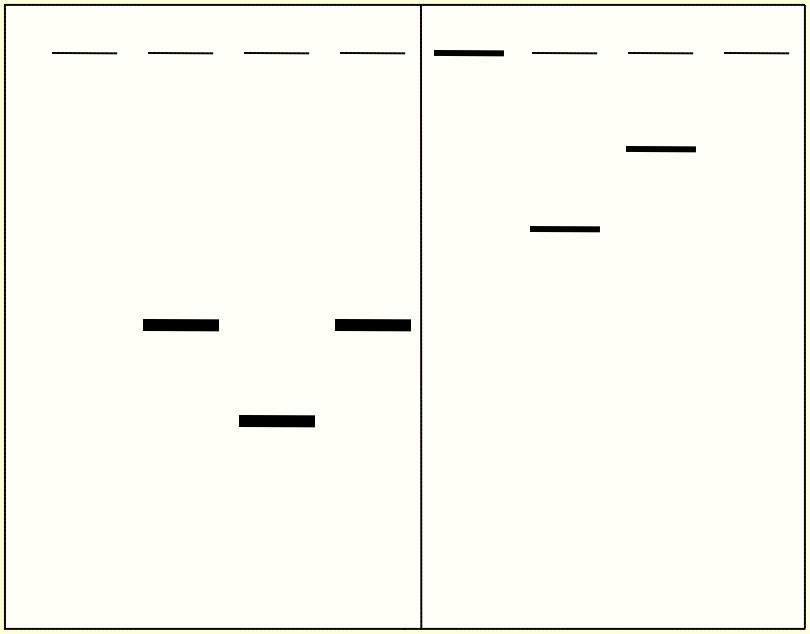
The only bands visualized will be those with sequences complementary to the labeled probe. In this experiment the entire 3 kb fragment was used as probe.

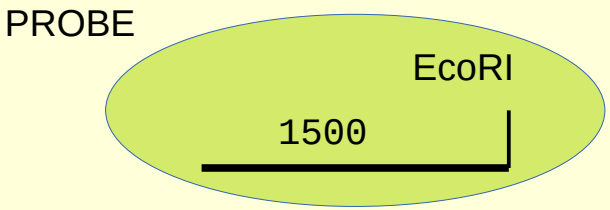
Hybridization results

Original gel seen in UV



Locations of bound probe can be visualized using autoradiography for chemiluminescent detection, or fluorescent imaging if flourescent nucleotides are used.





But - If we had used ONLY the 1.5 kb EcoRI fragment as probe, we would not see the 1.0 and 0.5 kb bands, because the 1.5 kb fragment has no sequences complementary to these bands.

Example: Digest of genomic DNA from transgenic *B. napus* plants

Plant tissue from *B. napus* was transformed with the pea defense gene DRR206. DRR206 is expressed in pea in response to fungi.

DNA from 10 transgenic canola (*Brassica napus plants* (lanes 1 - 10) and an untransformed control was digested with HindIII. The lane on the left contains a mixture of bacterial DNA fragments of known size, for use as a size marker.

The digested DNA was electrophoresed on an agarose gel containing the DNA-specific dye, ethidium bromide. When bound to DNA, EtBr emits a pink flourescent light, which is visualized in the photo.

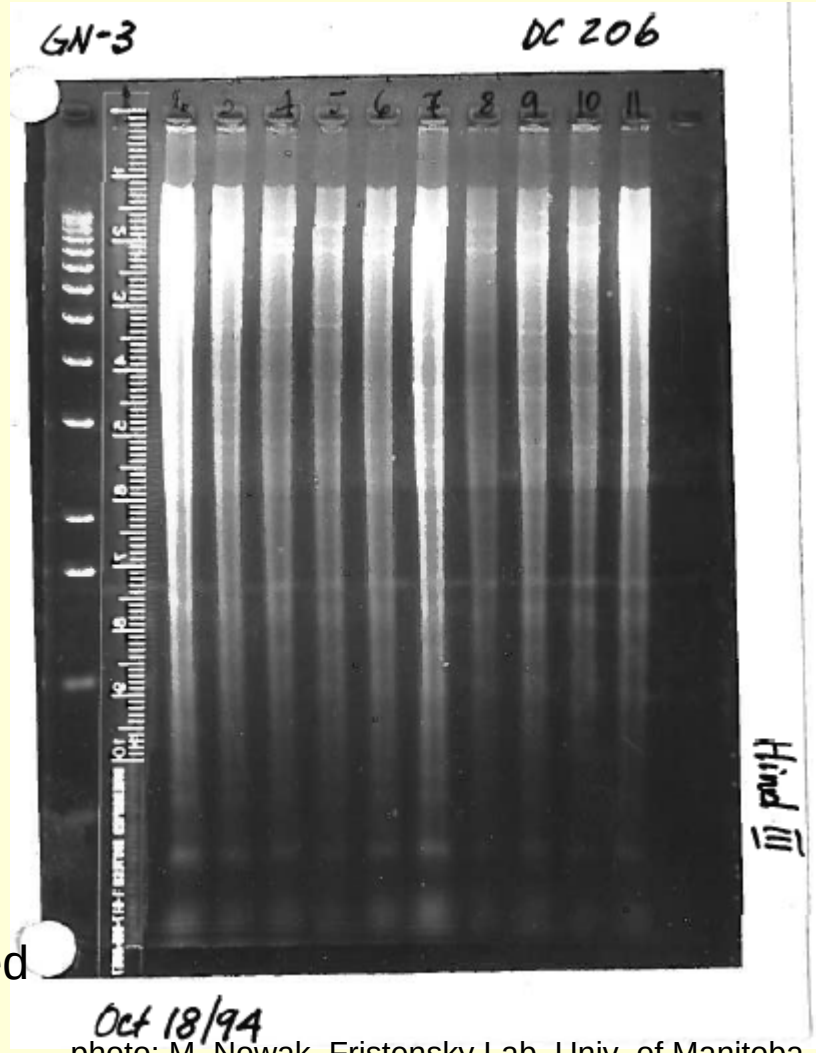


photo: M. Nowak, Fristensky Lab, Univ. of Manitoba

We see a smear of flourescence, because plant genomes are very large. For an enzyme like HindIII, which recoginzes a 6 base sequence, we expect to see hundreds of thousands of bands.

Example: Southern blot of DNA from transgenic *B. napus* plants

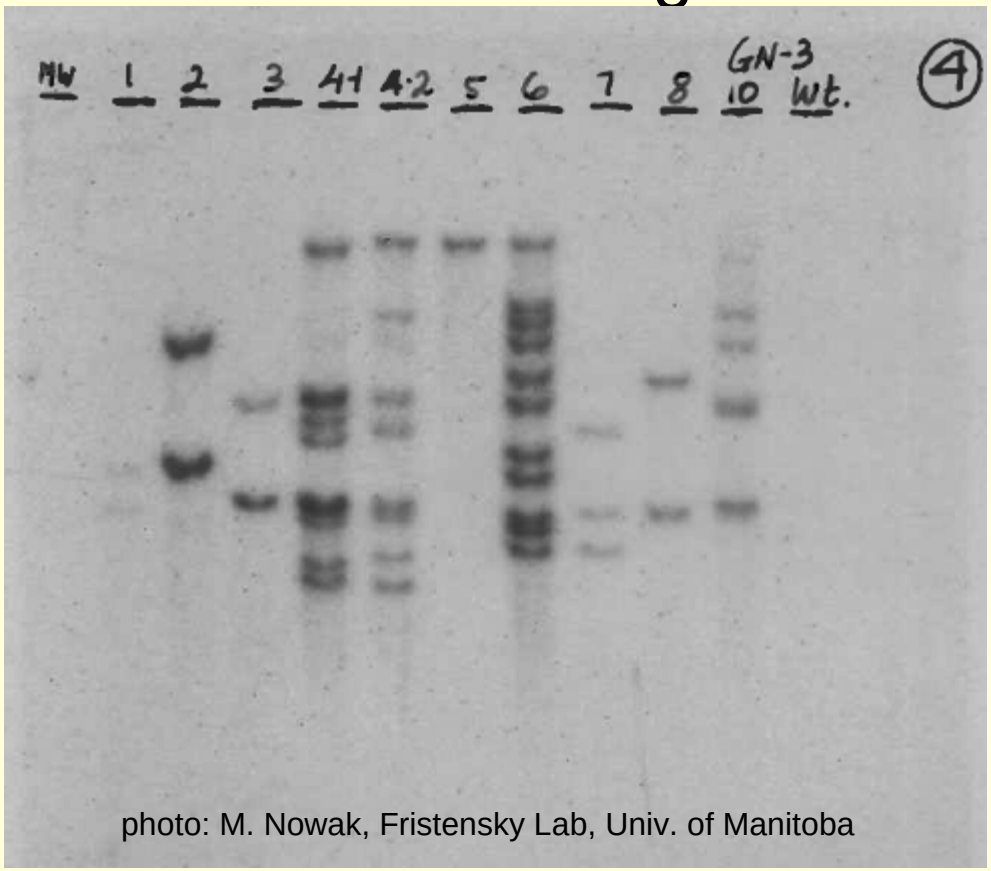


photo: M. Nowak, Fristensky Lab, Univ. of Manitoba

The gel was blotted onto a nylon membrane, and the blot was hybridized with a labeled probe from the pea DRR206 gene.

As we expect, neither the size marker (MW) nor the untransformed control (Wt.) show bands.

All transformed plants show several bands. In each independent transformation event, the T-DNA inserts at random into one or more chromosomal locations. Thus, in each case the HindIII sites nearest to the insertion site will be at different distances from the inserted gene. Consequently, the HindIII bands will be of different sizes at each site.

One useful consequence is that the number of bands in a Southern gives a direct measurement of the number of copies of the gene in each transgenic plant.

Types of Blots

Material blotted	Blot name
– DNA	Southern blot
– RNA	Northern blot
– Protein	Western blot