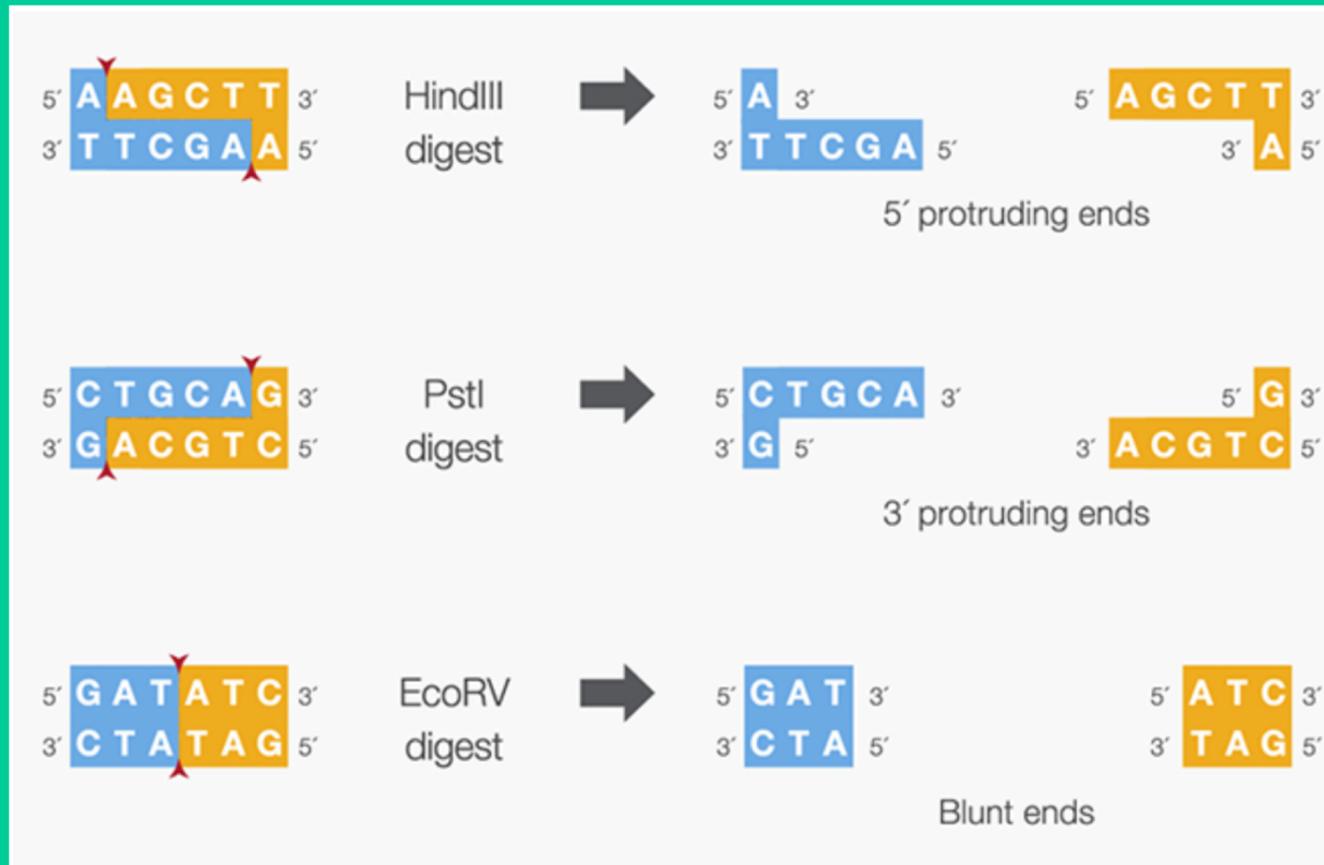


# Endonucleasi di restrizione (=enzimi di restrizione, ER)

## Restriction endonucleases (=restriction enzymes, RE)



# Endonucelasi di restricion (=enzimi di restrizione) Restriction endonucleases (=restriction enzymes)

- Also known as restriction endonucleases
- Scan the DNA sequence
- Find a very specific set of nucleotides
- Make a specific cut (with defined termini or blunt)
- Used to construct recombinant DNA plasmids
- 3000 different restriction enzymes exist
- 500 restriction enzymes for the use in the laboratory
- Encoded by prokaryotes (bacteria)

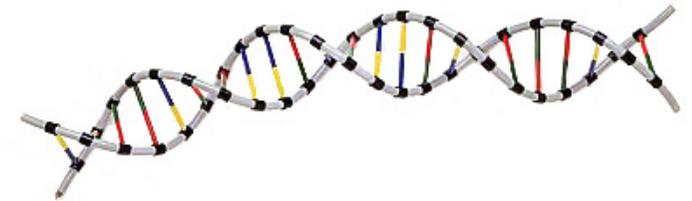
Note:

Endonucleases cut DNA at internal position

Exonucleases cut DNA starting at termini

DNAase: cuts DNA

RNase: cuts RNA



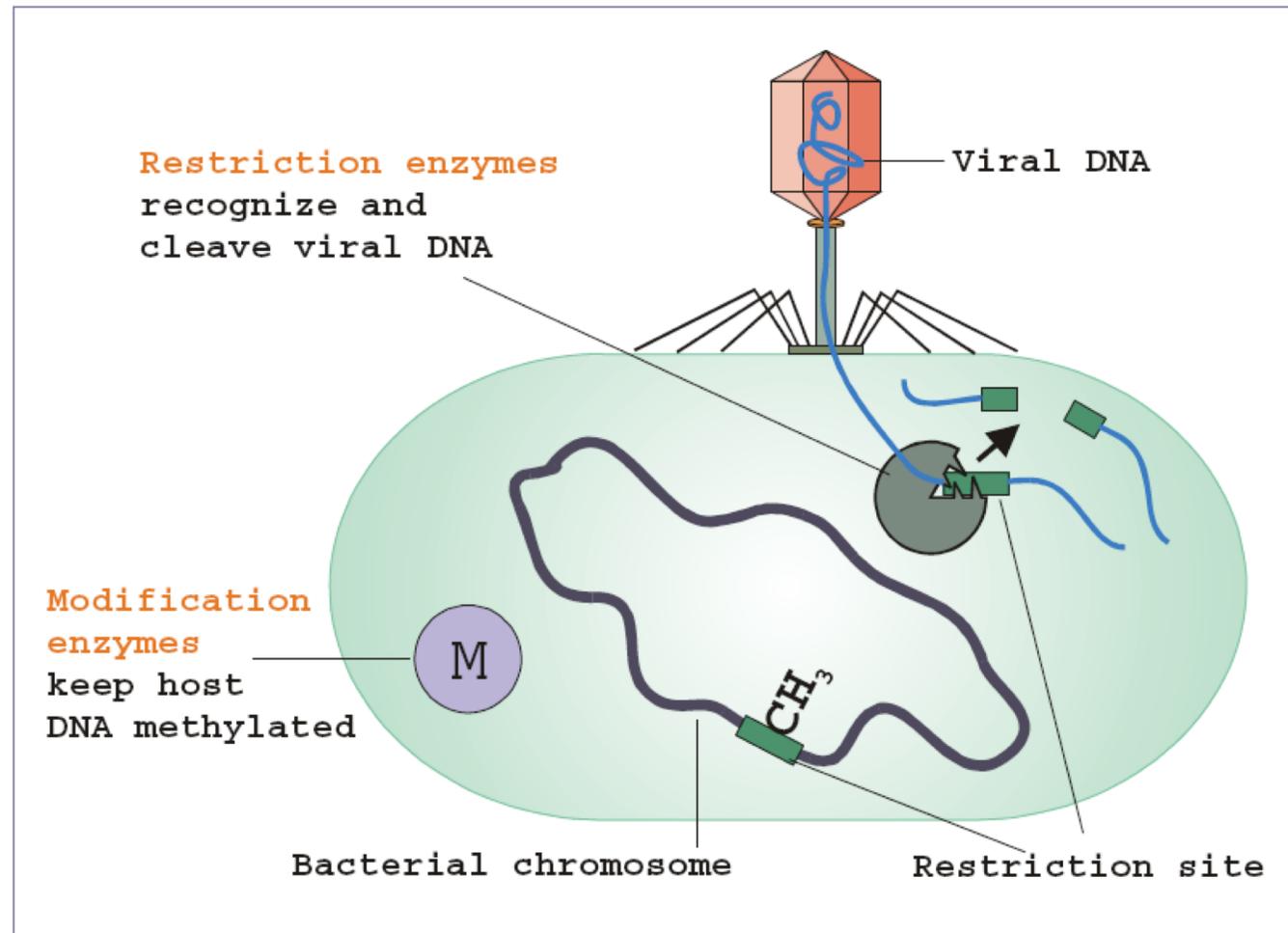
# Discovery of restriction endonucleases

- Arbor and Dussoix in 1962 discovered that certain bacteria contain Endonucleases which have the ability to cleave DNA.
- In 1970 Smith and colleagues purified and characterized the cleavage site of a Restriction Enzyme.
- Werner Arbor, Hamilton Smith and Daniel Nathans shared the 1978 Nobel prize for Medicine and Physiology for their discovery of Restriction Enzymes.



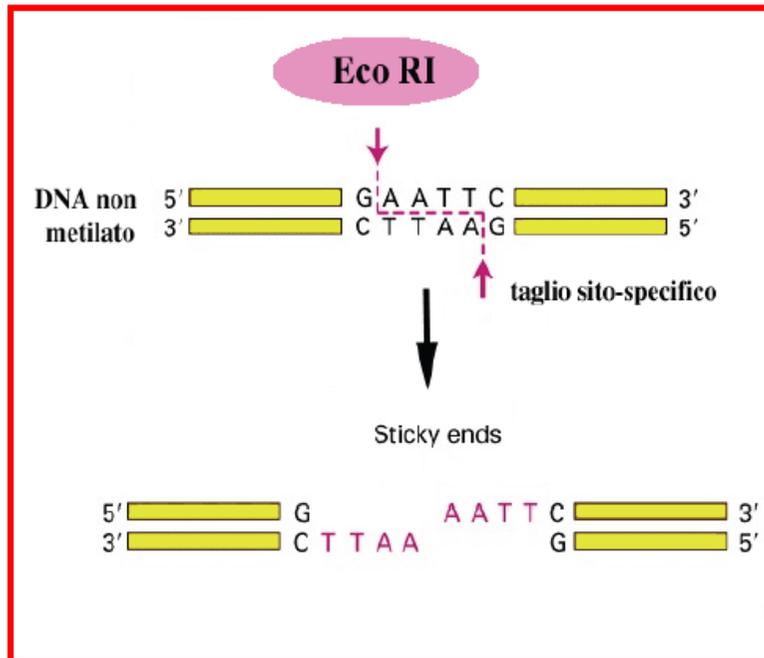
# The origin of restriction endonucleases

## Bacterial defense against viral infection by restriction-modification complexes

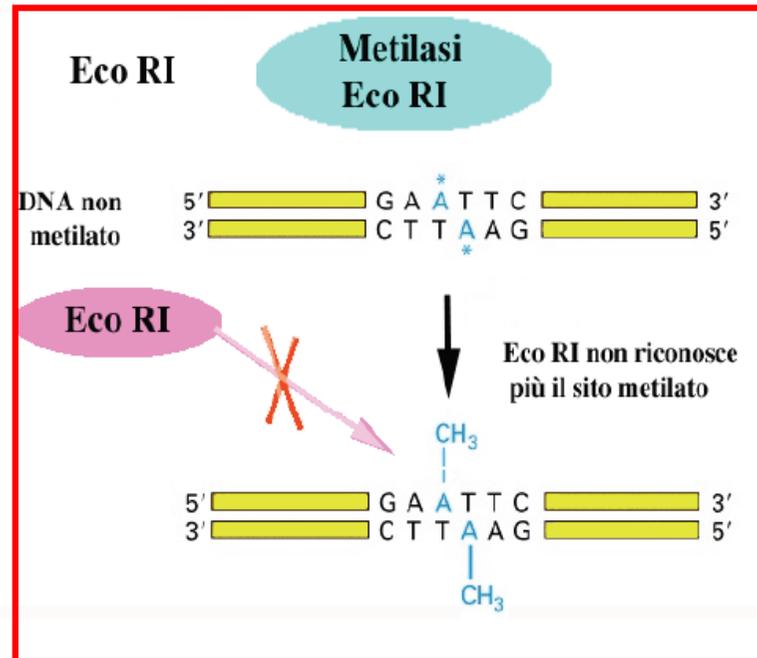


# The origin of restriction endonucleases

Bacteria encode for **coupled DNA methylation – restriction endonucelase** systems



**Fage DNA** or **plasmid DNA** with unmethylated or methylated by other DNA methylation system enters bacteria. EcoRI expressed by recipient bacteria cuts at all GAATTC sites in the foreign DNA



**EcoRI methylase methylates host DNA at defined sequence: GAATTC**  
EcoRI restriction endonucelase expressed by bacteria can cut only unmethylated DNA. DNA integrity is maintained

Bacterial DNA methylases can exclusively methylate **A or C**, when present in correct sequence context.

# Types of Restriction Enzymes

	<b>Cleavage site</b>	<b>Location of methylase</b>	<b>Examples</b>
<b>Type I</b>	Random Around 1000bp away from recognition site	Endonuclease and methylase located on a <b>single protein</b> molecule	EcoK I EcoA I CfrA I
<b>Type II</b>	Specific Within the recognition site	Endonuclease and methylase are <b>separate</b> <b>entities</b>	EcoR I BamH I Hind III
<b>Type III</b>	Random 24-26 bp away from recognition site	Endonuclease and methylase located on a <b>single protein</b> <b>molecule</b>	EcoP I Hinf III EcoP15 I

# Sistemi di restrizione-modificazione

**Gli enzimi di restrizione sono tradizionalmente classificati in quattro tipi sulla base della composizione delle subunità, della posizione di scissione, della specificità della sequenza e dei requisiti dei cofattori.**

## Tipo I

- Un singolo enzima contiene attività di restrizione e di metilazione su subunità diverse
- multisubunità
- Il taglio viene effettuato in modo non specifico lontano dalla sequenza di riconoscimento (da 100 fino a 1000 bp a valle)
- $Mg^{2+}$ , ATP e S-adenosilmetionina come cofattori

## Tipo II

- Due enzimi distinti per il taglio e la metilazione.
  - Due subunità identiche (homodimeric)
  - cofattore **Mg<sup>2+</sup>**
  - Riconoscono la stessa sequenza **palindromica** e agiscono al suo interno
- other subtypes (IIS, IIE, IIF, IIT, IIG, IIB, IIM)

## Tipo III

- Cofattori ATP,  $Mg^{2+}$
- Riconosce e modifica una sequenza **palindromica** ma taglia a 25-27 bp di distanza

## Type IV

- Cofattore  $Mg^{2+}$
  - Gli enzimi di tipo IV riconoscono il DNA modificato, tipicamente metilato, idrossimetilati a glucosil-idrossimetilato
- Per esempio sistemi McrBC e Mrr di E. coli. (McrBC è un'endonucleasi che taglia il DNA contenente metilcitosina\* su uno o entrambi i filamenti. I siti sul DNA riconosciuti da McrBC consistono in due semisiti della forma (G/A)mC. Questi semisiti possono essere separati fino a 3 kb, ma la separazione ottimale è di 55-103 coppie di basi; richiede GTP)

# Fattori che influenzano l'attività degli enzimi di restrizione

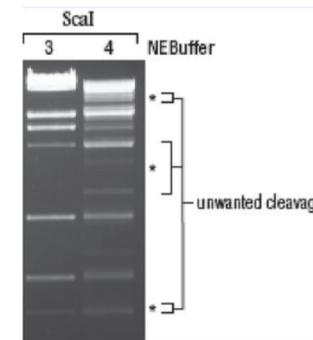
A seconda del DNA substrato e delle condizioni di reazione, gli enzimi di restrizione mostrano un'ampia variazione di taglio e di possibile «star activity»  
Per ottenere il taglio desiderato, è importante controllare i seguenti fattori:

**Star activity:** In condizioni di reazione non ottimali, alcuni enzimi di restrizione scindono sequenze di basi in siti diversi dalla sequenza di riconoscimento definita. In altre parole, effettuano la scissione in siti non specifici. Questo fenomeno è chiamato attività stellare. Alcuni dei fattori che inducono l'attività stellare sono l'elevata concentrazione di sale e glicerolo, la presenza di impurità, un eccesso di enzima rispetto al DNA substrato, l'aumento del tempo di incubazione o l'incompatibilità di tampone e cofattore.

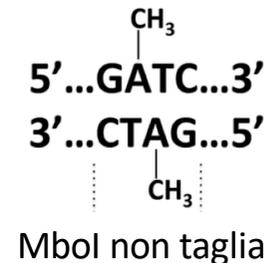
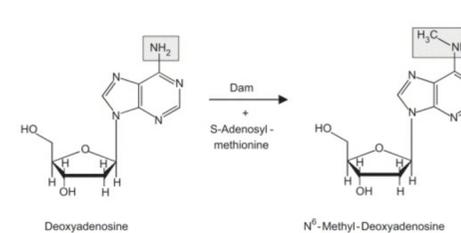
**DNA metilato:** Diverse molecole di DNA sono metilate nel sito di riconoscimento, il che le rende resistenti al clivaggio da parte di alcuni enzimi di restrizione.

Ad esempio, la maggior parte dei ceppi di E. coli esprime le metiltransferasi Dam o Dcm che metilano siti di riconoscimento specifici per formare rispettivamente **G6mATC** e **C5mCA/TGG**. G6mATC è resistente al clivaggio da parte di Mbo I.

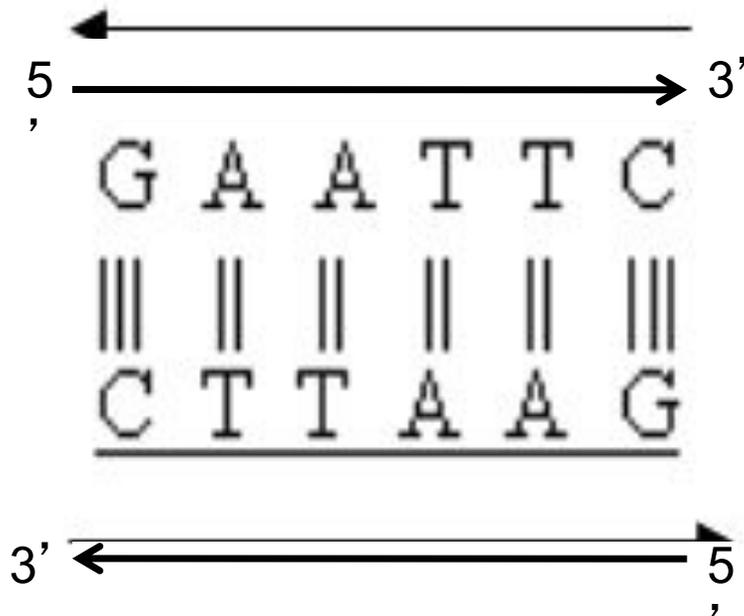
**Temperatura:** La maggior parte delle endonucleasi digerisce in maniera ottimale il DNA bersaglio a 37 °C. Tuttavia, esistono alcune eccezioni con temperature ottimali più basse o più alte. Ad esempio, Taq I digerisce in modo ottimale a 65 °C e Apa I (n. di catalogo 10899208001) digerisce a 25 °C.



Star activity of Scal  
Ideal buffer: #3  
Non optimal buffer: #4  
(other concentration of salt)



# Restriction enzymes typically recognize palindromic sequences on double stranded DNA



Genetic palindromes are similar to verbal palindromes. A palindromic sequence in DNA is one in which the 5' to 3' base pair sequence is identical on both strands.

*(typically 4 or 6 nucleotide palindromes)*

**4 nucleotide palindromes: 1 cut every 256 nucleotides ( $4^4$ ).**

**6 nucleotide palindromes: 1 cut every 4,096 nucleotides ( $4^6$ ).**

Enzyme	Target sequence	Cleavage
EcoRI	5' GAATTC 3' 3' CTTAAG 5'	5' G            AATTC 3' 3' CTTAA            G 5'
EcoRV	5' GATATC 3' 3' CTATAG 5'	5' GAT    ATC 3' 3' CTA    TAG 5'
HaeIII	5' GGCC 3' 3' CCGG 5'	5' GG    CC 3' 3' CC    GG 5'
HindIII	5' AAGCTT 3' 3' TTCGAA 5'	5' A            AGCTT 3' 3' TTCGA            A 5'
PpuMI	5' RGGWCCY 3' 3' YCCWGGR 5'	5' RG            GWCCY 3' 3' YCCWG            GR 5'

Small number {

## Single Letter Code List

- B = C or G or T
- D = A or G or T
- H = A or C or T
- K = G or T
- M = A or C
- N = A or C or G or T
- R = A or G
- S = C or G
- V = A or C or G
- W = A or T
- Y = C or T

# Nomenclature of restriction enzymes

1. Le prime tre lettere, scritte in corsivo, sono prese da genere e specie del batterio di origine.
2. Sierotipi differenti (sottspecie - materiale organico infetto (ad esempio sangue) reagisce positivamente solo con un determinato siero, contenente un anticorpo in grado di legarsi ad uno specifico antigene microbico) dello stesso organismo possono essere identificati da una quarta lettera minuscola (Es. *Hind*, *Hinf*).
3. Può seguire una lettera maiuscola o un numero, che identifica un ceppo particolare di quel batterio.
4. Un numero romano indica l'ordine di scoperta, qualora dallo stesso batterio vengano isolati enzimi diversi.

Enzima	Organismo di provenienza
<i>Sma</i> I	<i>Serratia marcescens</i> , 1° enzima
<i>Hae</i> III	<i>Haemophilus aegyptius</i> , 3° enzima
<i>Hin</i> dII	<i>Haemophilus influenzae</i> , ceppo d, 2° enzima
<i>Hin</i> dIII	<i>Haemophilus influenzae</i> , ceppo d, 3° enzima
<i>Bam</i> HI	<i>Bacillus amyloliquefaciens</i> , ceppo H, 1° enzima

# Types of DNA cuts by restriction endonucleases

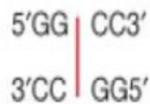
## ER che riconoscono 4 pb

**4 pb**

*Mbo* I:



*Hae* III



*Alu* I



*Hha* I



*Rsa* I



## ER che riconoscono 6 pb

**6 pb**

*Eco* RI:



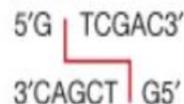
*Hind* III:



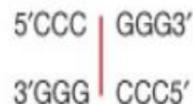
*Bam* HI:



*Sal* I:



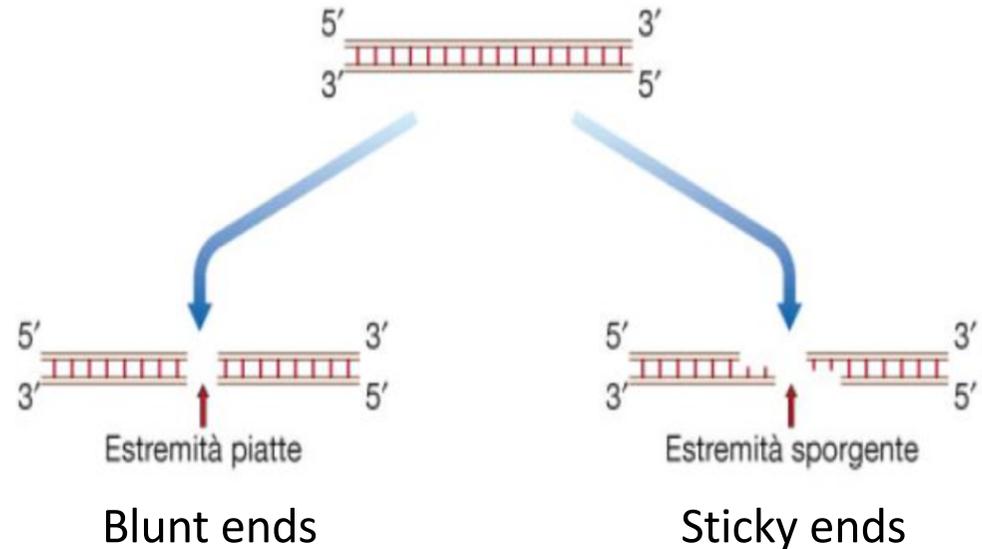
*Sma* I:



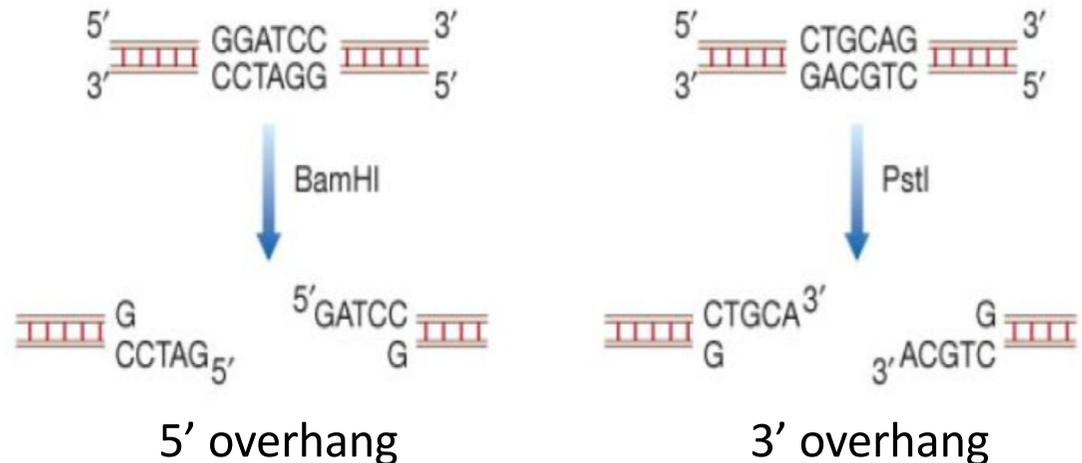
*Rsa* I:



**A** I tagli da parte di enzimi di restrizione possono generare sul DNA estremità piatte o sporgenti



**B** Estremità sporgenti in 5' o in 3'



# Isoschizomers and Neoschizomers

- Restriction enzymes that have the same recognition sequence as well as the same cleavage site are **Isoschizomers**.

C G T A C | G

G | C A T G C

SphI

C G T A C | G

G | C A T G C

BbuI

Fragments generated by cleavage with Isoschizomers can be re-ligated (compatible ends)

- Restriction enzymes that have the same recognition sequence but cleave the DNA at a different site within that sequence are **Neoschizomers**. Eg: SmaI and XmaI

C | C C G G G

G G G C C | C

XmaI

C C C | G G G

G G G | C C C

SmaI

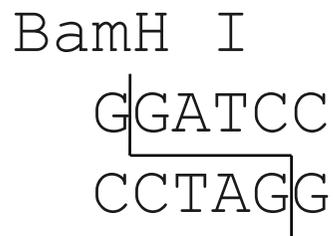
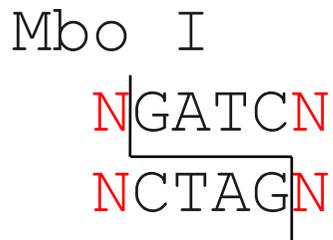
Fragments generated by cleavage with Neoschizomers cannot be re-ligated (compatible ends)

**Neoschizomeri:** Gli enzimi di restrizione che hanno la stessa sequenza di riconoscimento ma tagliano il DNA in un sito diverso all'interno di tale sequenza sono neoschizomeri. Ad esempio: SmaI e XmaI

**Isoschizomeri:** Gli enzimi di restrizione che hanno la stessa sequenza di riconoscimento e lo stesso sito di clivaggio sono isoschizomeri.

# Isocaudomers

Isocaudomers are pairs of restriction enzymes that have **slightly different recognition sequences**, but upon cleavage of DNA, **generate identical overhanging termini sequences**. These sequences **can be ligated to one another**, but then form an asymmetrical sequence that **cannot be cleaved** by a restriction enzyme.



Fragments generated by cleavage  
with Isocaudameres can be re-ligated  
(compatible ends)

Gli **isocaudomeri** sono coppie di enzimi di restrizione che hanno sequenze di riconoscimento leggermente diverse, ma che al momento della scissione del DNA generano sequenze di termini sporgenti identiche. Queste sequenze possono essere legate l'una all'altra, ma formano una sequenza asimmetrica che non può essere scissa da un enzima di restrizione.

# Example of laboratory plasmid sequence

```

                LacZ $\alpha$  initiation codon
                M13 Reverse priming site                T3 priming site
201  CACACAGGAA ACAGCTATGA CCATGATTAC GCCAAGCTCA GAATTAACCC TCACTAAAGG
      GTGTGTCCCT TGTCGATACT GGTACTAATG CGTTTCGAGT CTTAATTGGG AGTGATTTC
261  GACTAGTCCT GCAGGTTTAA ACGAATTGCG CCTT PCR Product AAGGGC GAATTCGCGG
      CTGATCAGGA CGTCCAATT TGCTTAAGCG GGAA TTCCCG CTTAAGCGCC
                T7 priming site                M13 Forward (-20) priming site
311  CCGCTAAATT CAATTCGCCC TATAGTGAGT CGTATTACAA TTCACTGGCC GTCGTTTTAC
      GGCGATTTAA GTTAAGCGGG ATATCACTCA GCATAATGTT AAGTGACCGG CAGCAAATG
  
```

Typically, the restriction site is indicated only on one DNA strand



## Comments for pCR<sup>®</sup>4-TOPO<sup>®</sup> 3956 nucleotides

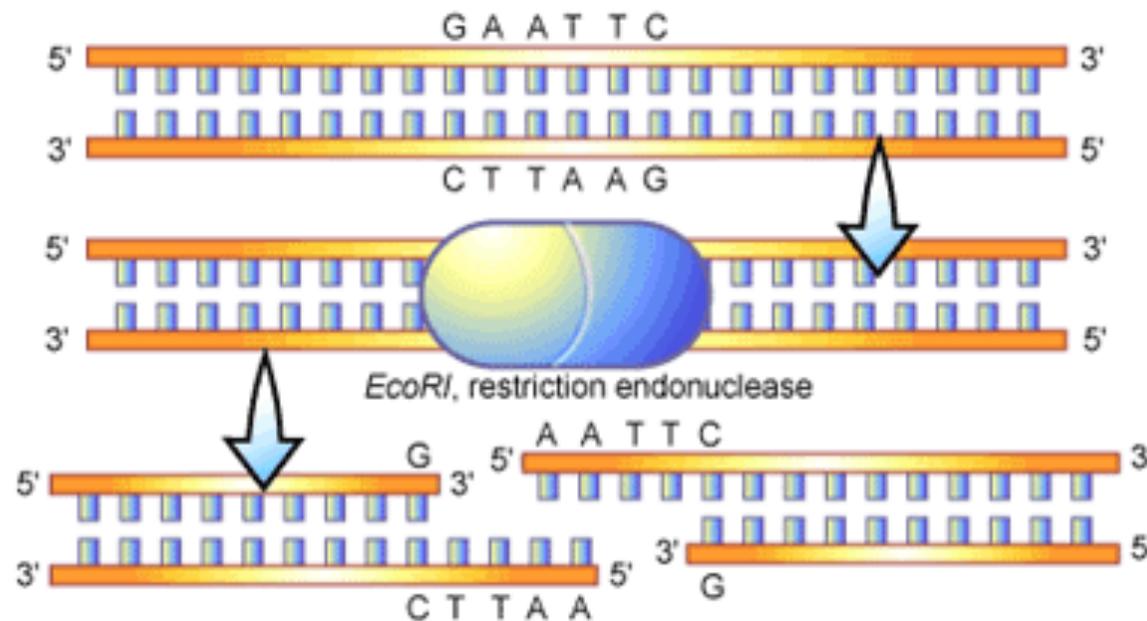
*lac* promoter region: bases 2-216  
 CAP binding site: bases 95-132  
 RNA polymerase binding site: bases 133-178  
 Lac repressor binding site: bases 179-199  
 Start of transcription: base 179  
 M13 Reverse priming site: bases 205-221  
 LacZ $\alpha$ -*ccdB* gene fusion: bases 217-810  
     LacZ $\alpha$  portion of fusion: bases 217-497  
     *ccdB* portion of fusion: bases 508-810  
 T3 priming site: bases 243-262  
 TOPO<sup>®</sup> Cloning site: bases 294-295  
 T7 priming site: bases 328-347  
 M13 Forward (-20) priming site: bases 355-370  
 Kanamycin promoter: bases 1021-1070  
 Kanamycin resistance gene: bases 1159-1953  
 Ampicillin (*bla*) resistance gene: bases 2203-3063 (c)  
 Ampicillin (*bla*) promoter: bases 3064-3160 (c)  
 pUC origin: bases 3161-3834  
 (c) = complementary strand

# Mechanism of Action of restriction endonucleases

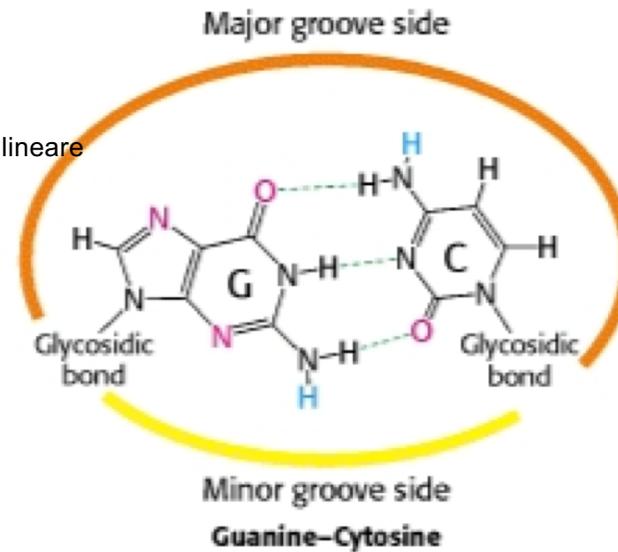
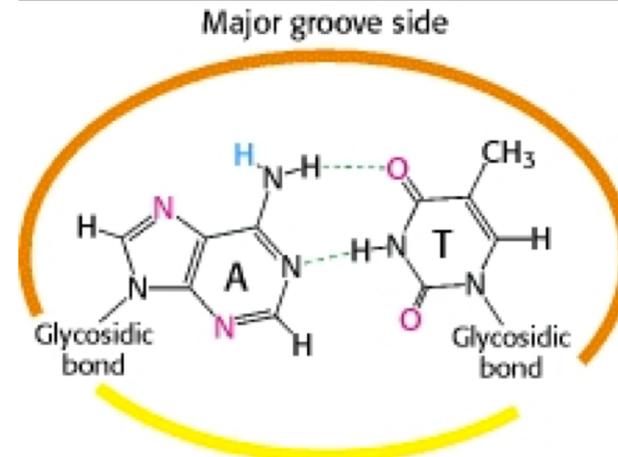
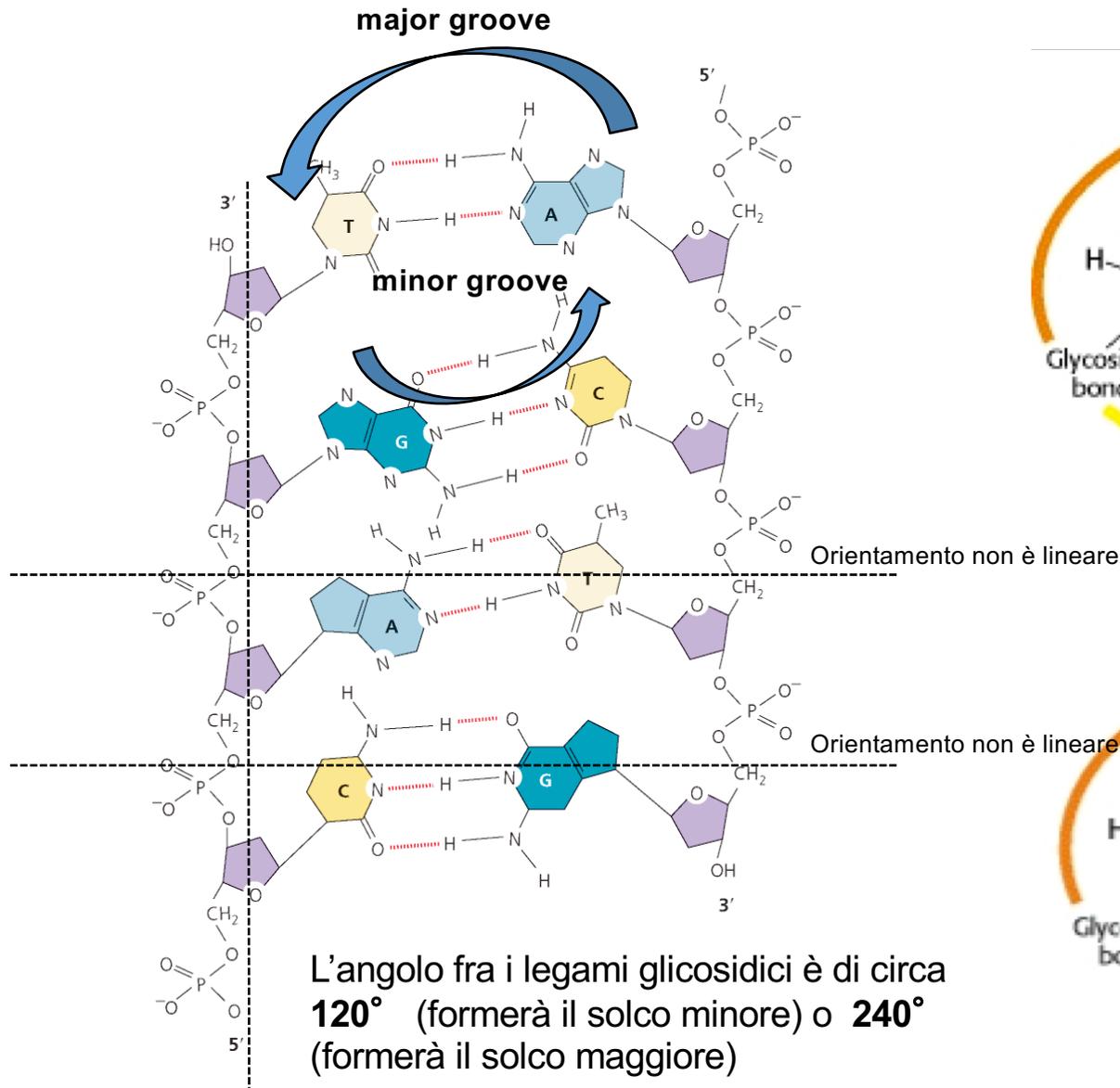
Restriction Endonuclease scan the length of the DNA , binds to the DNA molecule when it recognizes

specific sequence and makes one cut in each of the sugar phosphate backbones of the double helix

by hydrolyzing the phosphodiester bond. Specifically, the bond between the 3' O atom and the P atom is broken.



# La doppia elica presenta un solco minore ed un solco maggiore (minor and major groove)



# Restriction enzymes are DNA binding proteins that recognize specific sequences by interaction with chemical groups in major and minor groove

**A: accettore legame idrogeno**  
**D: donatore legame idrogeno**  
**M: gruppo metilico**  
**H: idrogeno non polare**

Queste codice formato da gruppi chimici posti al interno del solco maggiore/minore identificano in modo specifico le coppie di basi.

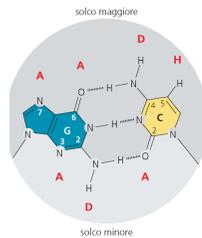
Le proteine possono riconoscere specifiche sequenze di DNA senza che sia necessario aprire o rompere la doppia elica !!!

Esempio:

→ Fattori di trascrizione

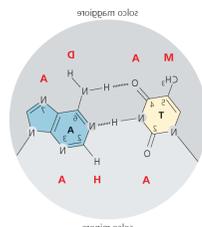
→ Elicasi, etc...

→ **Endonucleasi**



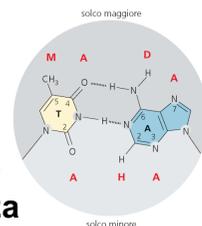
Solco maggiore: A-A-D-H

Solco minore: A-D-A



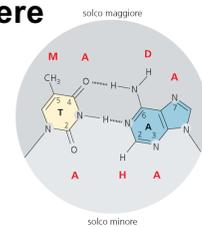
Solco maggiore: A-A-D-H

Solco minore: A-D-A



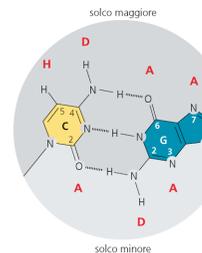
Solco maggiore: A-A-D-H

Solco minore: A-D-A



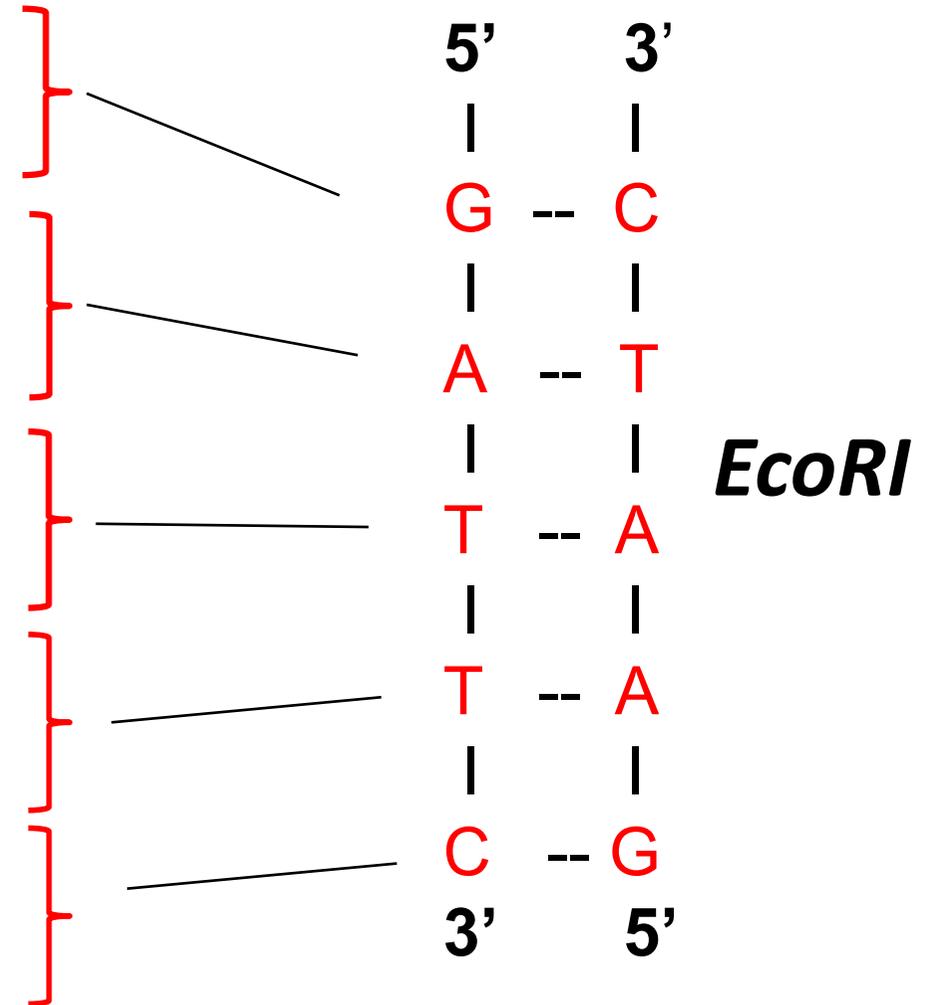
Solco maggiore: A-A-D-H

Solco minore: A-D-A



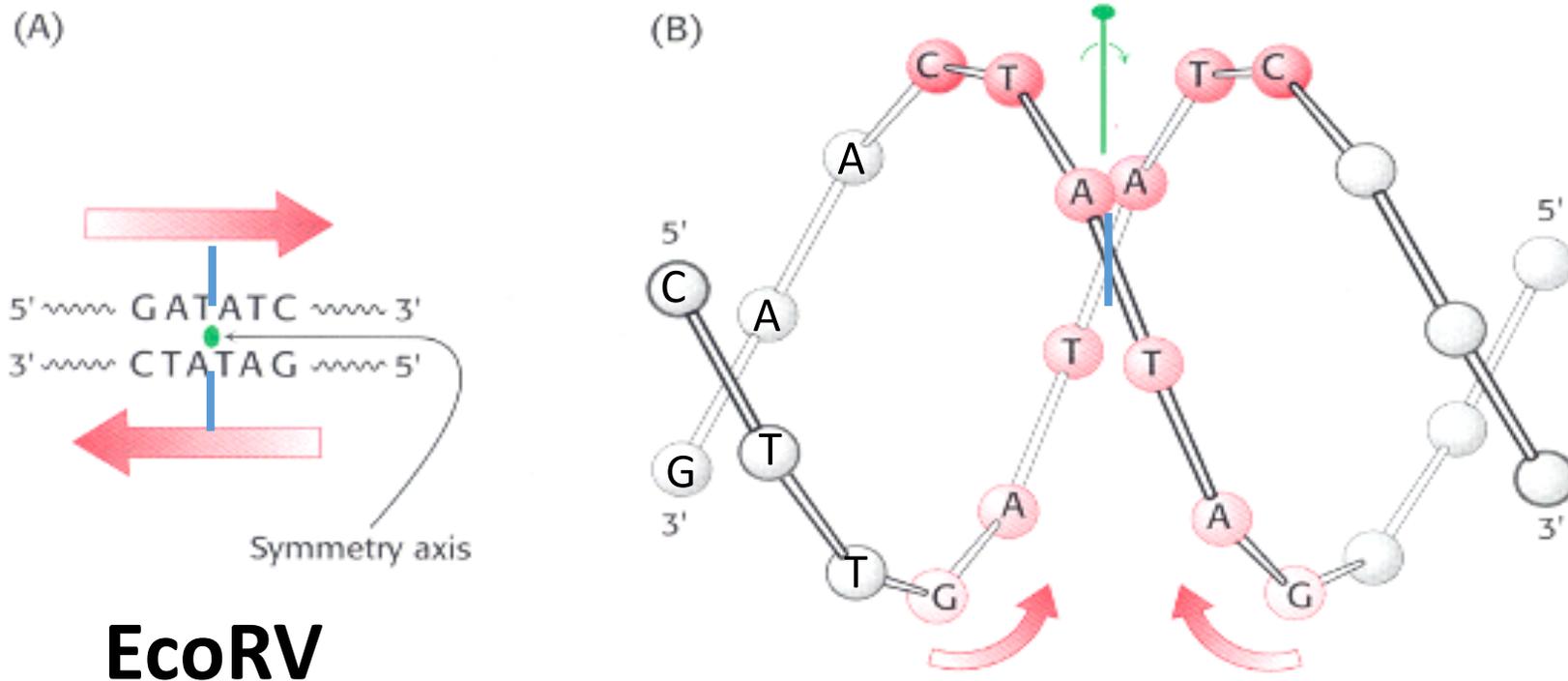
Solco maggiore H-D-A-A

Solco minore A-D-A



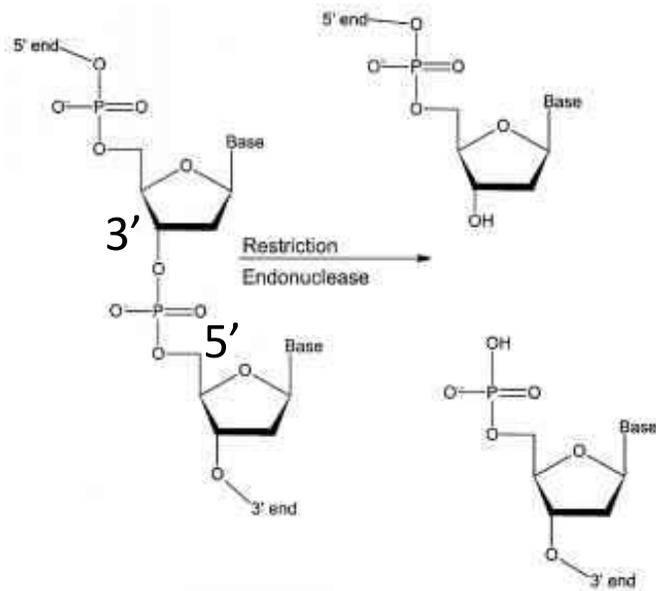
# Mechanism of restriction endonucleases

*Recognition sites of most restriction enzymes have a two-fold rotational symmetry*

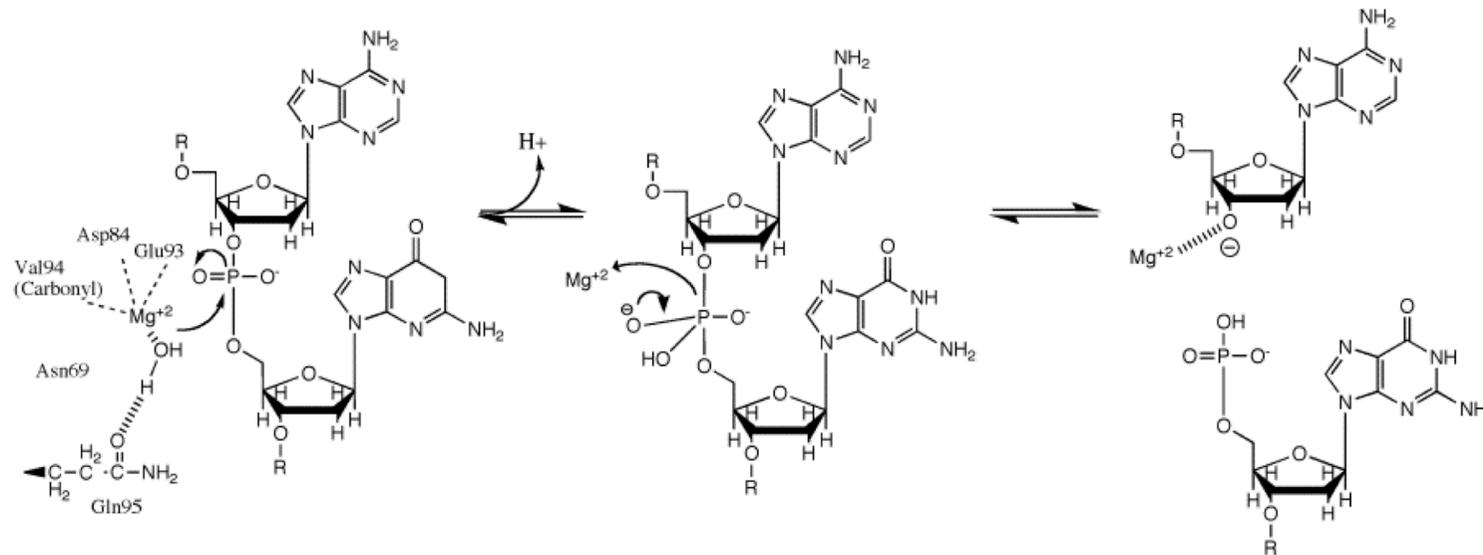


Restriction enzymes have corresponding symmetry to facilitate recognition and usually cleave the DNA on the axis of symmetry

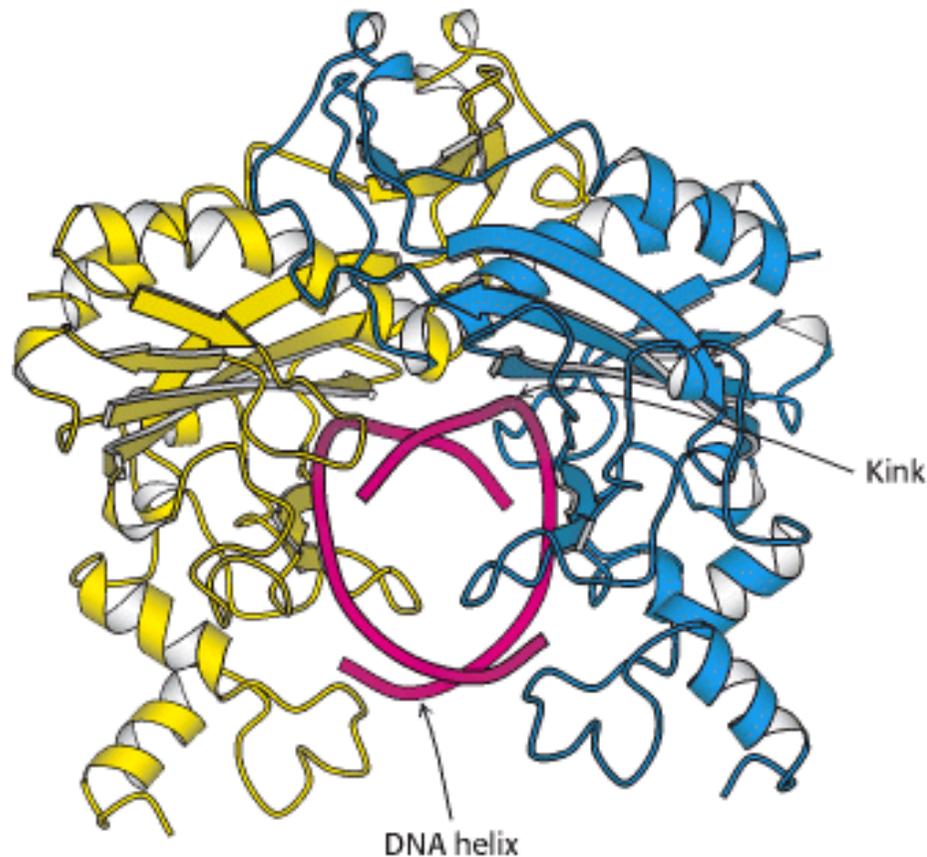
# Direct hydrolysis by nucleophilic attack at the phosphorous atom



- Mg<sup>2+</sup> is required for the catalytic activity of the enzyme.
- Mg<sup>2+</sup> holds the water molecule in a position; a hydroxide ion from water is generated, which will act as the nucleophile and attack the phosphorus in the phosphodiester bond.
- Cleavage produces **3'-OH** and **5'-PO<sub>4</sub><sup>-</sup>**



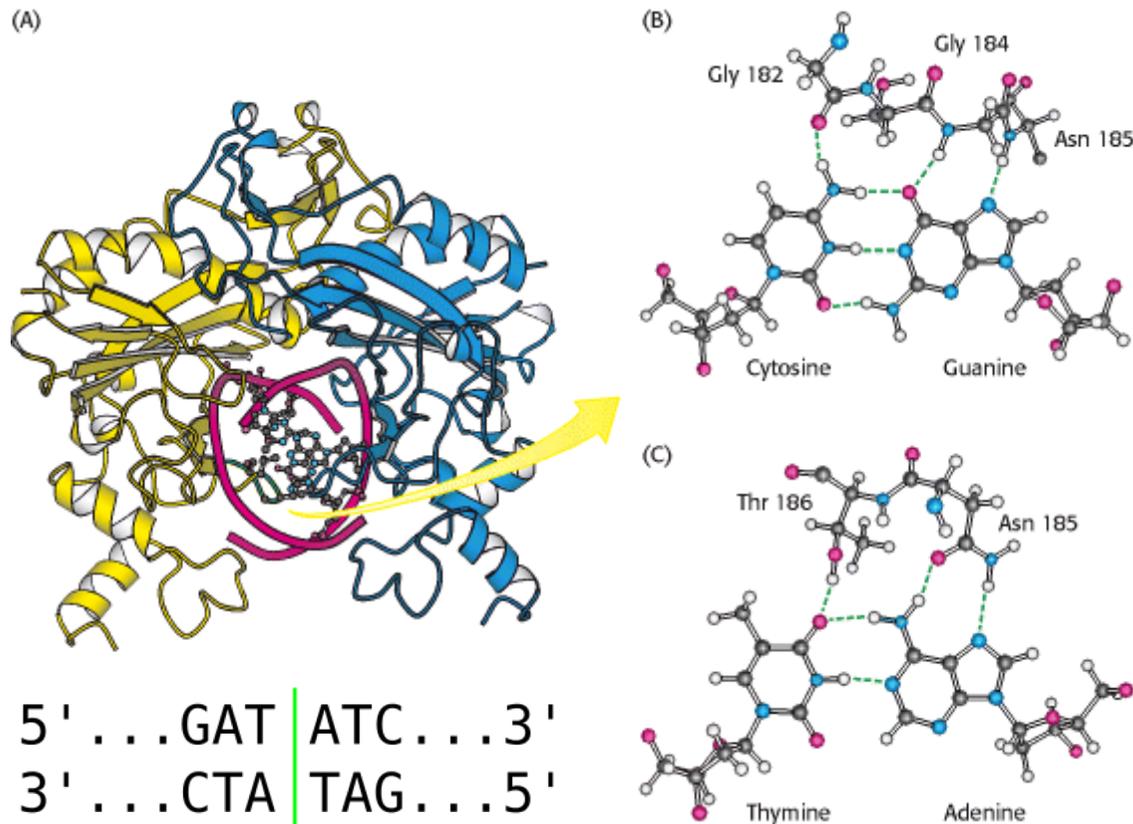
# Structure - Function of EcoRV endonuclease



- Consists of two subunits – dimers related by two fold rotational symmetry.
- Binds to the matching symmetry of the DNA molecule at the restriction site and produces a kink at the site.



# Structure - Function of EcoR V endonuclease



Hydrogen bonding interactions between EcoRV and its DNA substrate

Like EcoRI, EcoRV forms a **homodimer in solution before binding** and acting on its recognition sequence. Initially the enzyme **binds weakly to a non-specific site** on the DNA. It randomly **walks along the molecule until the specific recognition site is found**.

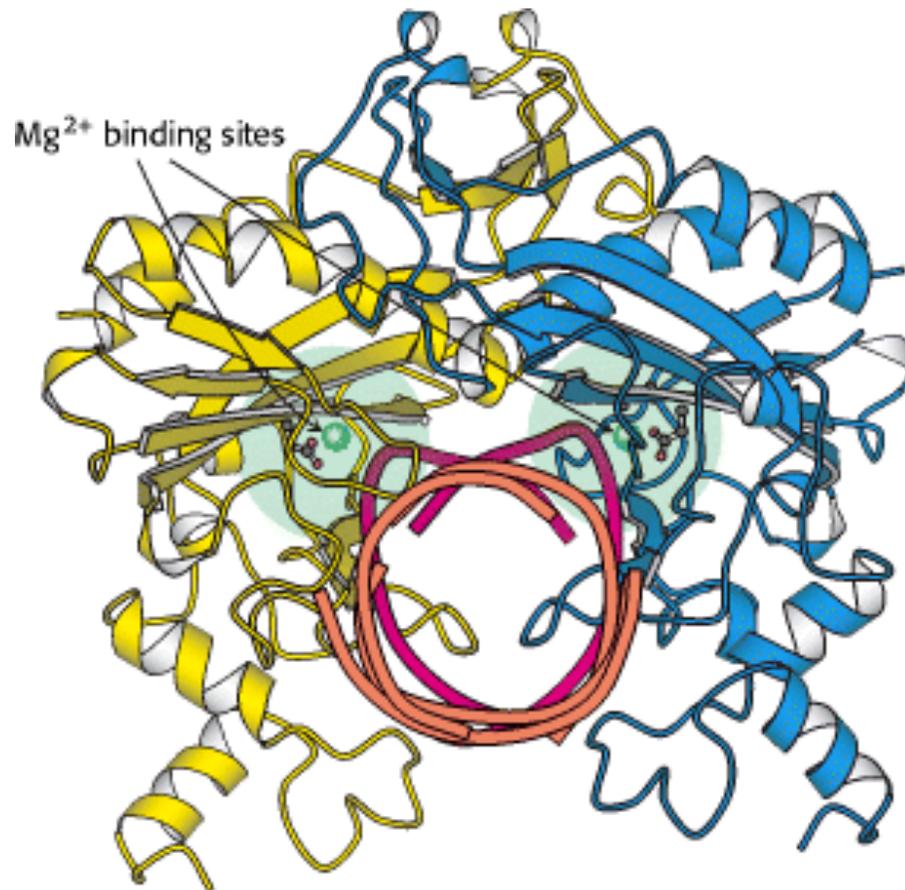
EcoRV has a high specificity for its target DNA sequence. **Binding of the enzyme induces a conformational change in the DNA, bending it by about 50°.**

DNA bending results in the **unstacking of the bases**, widening of the minor groove, and compression of the major groove. This brings the **phosphodiester linkage to be broken closer to the active site of the enzyme**, where it can be cleaved.

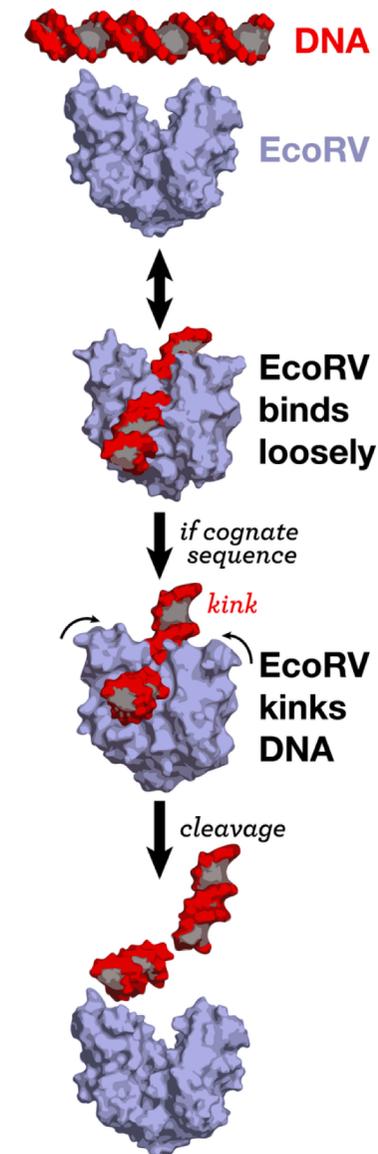
Cleavage occurs within the recognition sequence, and **does not require ATP hydrolysis**.

EcoRV is the **only type II restriction endonuclease known to cause a major protein-induced conformational change in the DNA**.

# Structure - Function of EcoR V endonuclease



A comparison of cognate (red; kink) and non-specific DNA (orange) in the EcoRV-DNA complex.



# Restriction enzymes are commercially available as recombinant proteins

Restriction Enzyme Activity in Promega 10X Buffers, Reaction Temperature and Heat Inactivation.

The 10X Reaction Buffer supplied with each restriction enzyme is optimized to give 100% activity. In many cases, good activity is also obtained using one of Promega's 4-CORE<sup>®</sup> 10X Buffers. Many commonly used cloning enzymes have buffers E and H as their optimal buffer, and so we have determined the activity of many of our enzymes in these buffers. This table may be used to select the best buffer for digestion with multiple restriction enzymes. Enzyme activity is expressed as a percent of the activity obtained with the optimal buffer for each enzyme.

Promega Enzyme	Buffer Supplied with Enzyme	Activity in							MULTI-CORE™	Heat Inactivation	Enzyme Assay Temperature
		A	B	C	D	E	H				
Aar I	J	50-75%	10-25%	<10%	<10%	10-25%	<10%	<10%	+	37°C	
Acc I	G	50-75%	25-50%	25-50%	10-25%	<10%	<10%	25-50%	-	37°C	
Acc III	F	<10%	10-25%	25-50%	25-50%	n.d.	<10%	<10%	-	65°C	
Acc65 I	D	10-25%	50-75%	75-100%	100%	75-100%	100-125%**	100%	+	37°C	
AccB7 I	F	10-25%	50-75%	100%	100%	100%	100%	100%	+	37°C	
Age I	E	100%	100%	100%	100%	100%	100%	100%	+	37°C	
Alu I											
Alw26 I											
Alw44 I											
Apa I											
Ava I	B	10-25%	100%	50-75%	25-50%	100%	10-25%	<10%	+/-	37°C	
Ava II	C	50-75%	50-75%	100%	25-50%	n.d.	n.d.	25-50%	+	37°C	
Bal I	G	10-25%	<10%	<10%	<10%	n.d.	n.d.	<10%	+	37°C	
BamH I	E	75-100%*	75-100%	75-100%	50-75%	100%	50-75%	75-100%	+	37°C	
Ban I	G	25-50%	25-50%	10-25%	<10%	n.d.	n.d.	100%	-	50°C	
Ban II	E	75-100%	75-100%	75-100%	25-50%	n.d.	n.d.	100%	+	37°C	
Bbv I	A	100%	75-100%	75-100%	<10%	10-25%	100%	100%	+	37°C	
Bcl I	C	10-25%	75-100%	100%	50-75%	50-75%	10-25%	10-25%	-	50°C	
Bgl I	D	10-25%	25-50%	75-100%	100%	25-50%	75-100%	100%	+	37°C	
Bgl II	D	25-50%	75-100%	75-100%	100%	n.d.	n.d.	<10%	-	37°C	
BsaM	D	10-25%	25-50%	50-75%	100%	n.d.	n.d.	25-50%	-	65°C	
Bsp1286 I	A	100%	50-75%	25-50%	10-25%	n.d.	n.d.	75-100%	+	37°C	
BsrS	D	10-25%	25-50%	10-25%	100%	n.d.	n.d.	100%	-	65°C	
BstI	H	75-100%	50-75%	75-100%	50-75%	n.d.	100%	75-100%	-	50°C	
Bst93 I	D	<10%	10-25%	10-25%	100%	n.d.	n.d.	25-50%	-	37°C	
Bst98 I	D	25-50%	50-75%	50-75%	100%	n.d.	n.d.	100%	-	60°C	
BstD I	C	10-25%	25-50%	100%	25-50%	n.d.	n.d.	<10%	-	60°C	
BstX I	D	<10%	10-25%	25-50%	100%	100%	75-100%	10-25%	+/-	50°C	
BstZ I	D	<10%	<10%	10-25%	100%	10-25%	75-100%	10-25%	-	50°C	
Bbu36 I	E	<10%	25-50%	50-75%	25-50%	100%	n.d.	50-75%	-	37°C	
Chf I	B	75-100%	100%	75-100%	25-50%	n.d.	n.d.	100%	+/-	37°C	
Cla I	C	75-100%	75-100%	100%	75-100%	100%	50-75%	100%	+	37°C	
Csp I	K	<10%	10-25%	25-50%	50-75%	100%	100-125%**	10-25%	+	30°C	
Esp45 I	B	25-50%	100%	50-75%	25-50%	100%	25-50%	50-75%	+	37°C	
Ede I	D	25-50%	25-50%	50-75%	100%	n.d.	n.d.	25-50%	+/-	37°C	
Dpn I	B	50-75%	100%	75-100%	50-75%	n.d.	n.d.	100%	+	37°C	
Dra I	B	75-100%	100%	75-100%	50-75%	n.d.	n.d.	25-50%	+	37°C	
EcoHK I	E	<10%	<10%	75-100%	10-25%	100%	n.d.	50-75%	+	37°C	
Eco47 III	D	<10%	25-50%	50-75%	100%	n.d.	n.d.	25-50%	+	37°C	
Eco52 I	L	<10%	<10%	10-25%	25-50%	25-50%	50-75%	<10%	+	37°C	
EcoRV	B	10-25%	100%	75-100%	<10%	25-50%	n.d.	100%	+	37°C	
EcoR I	H	25-50%	50-75%	50-75%	50-75%	75-100%	100%	100%*	+	37°C	
EcoR V	D	10-25%	25-50%	50-75%	100%	25-50%	50-75%	100%	+	37°C	
Fok I	B	75-100%	100%	75-100%	25-50%	n.d.	n.d.	50-75%	+	37°C	
Hae II	B	50-75%	100%	50-75%	10-25%	n.d.	n.d.	100%	-	37°C	
Hae III	C	75-100%	75-100%	100%	50-75%	n.d.	n.d.	100%	-	37°C	
Hha I	C	50-75%	75-100%	100%	50-75%	n.d.	n.d.	75-100%	+	37°C	
Hinc II	B	25-50%	100%	25-50%	50-75%	75-100%	50-75%	100%	+	37°C	
Hind III	E	25-50%	100%	75-100%	10-25%	100%	25-50%	50-75%	+	37°C	
Hinf I	B	50-75%	100%	75-100%	75-100%	n.d.	n.d.	50-75%	-	37°C	
Hpa I	J	25-50%	50-75%	25-50%	10-25%	n.d.	n.d.	100%	-	37°C	
Hpa II	A	100%	50-75%	50-75%	10-25%	n.d.	n.d.	100%	-	37°C	

Each restriction enzyme requires defined buffer conditions and temperature conditions

- Set of Buffer A-H are provided by restriction enzyme supplier
- Buffers are normally 10-fold concentrated with respect to conditions for optimal enzymatic activity. NEED TO BE DILUTED!
- Temperature mostly at 37°C
- Some enzymes work better in the presence of additional proteins (BSA mostly used)

100 mM NaCl  
50 mM Tris-HCl  
10 mM MgCl<sub>2</sub>  
1 mM DTT  
(pH 7.9 @ 25°C)

NaCl (or other salt) provides the correct ionic strength  
Tris-HCl provides the proper pH  
Mg<sup>2+</sup> is an enzyme co-factor  
DTT is a reducing agent to prevent formation of disulfide bonds in cysteine-containing proteins

# How to set up a restriction digest

Order of solution addition	Solution	Volume( $\mu$ l)
1	Nuclease free water	23.5
2	10X Buffer K	5.0
4	100 $\mu$ g BSA	0.5
5	Plasmid DNA	20.0
3	ScaI(20U/ $\mu$ l)	1.0
Total Volume		50.0

**ACTIVITY OF ENZYMES IS EXPRESSED IN UNITS:**  
*One unit of restriction endonuclease activity is defined as the amount of enzyme required to produce a complete digest of 1  $\mu$ g of substrate DNA (or fragments) in a total reaction volume of 50  $\mu$ l in 60 minutes under optimal assay conditions as stated for each restriction endonuclease.*

## **Scal buffer**

100 mM NaCl  
50 mM Tris-HCl  
10 mM MgCl<sub>2</sub>  
1 mM DTT  
(pH 7.9 @ 25°C)

- NaCl (or other salt) provides the correct ionic strength
- Tris-HCl provides the proper pH
- Mg<sup>2+</sup> is an enzyme co-factor
- DDT is a reducing agent

**Each enzyme requires defined buffer composition**

DTT commonly is used as redox reagent to prevent formation of disulfide bonds in cysteine-containing proteins. Such proteins require proper formation or absence disulfide bonds for exhibiting of specific activity. DTT helps to keep cysteine-containing proteins in active state. However, if protein doesn't contain cysteines, there is no need to use DTT for its activity.

# How to set up a restriction digest

## DNA Digestion Temperature

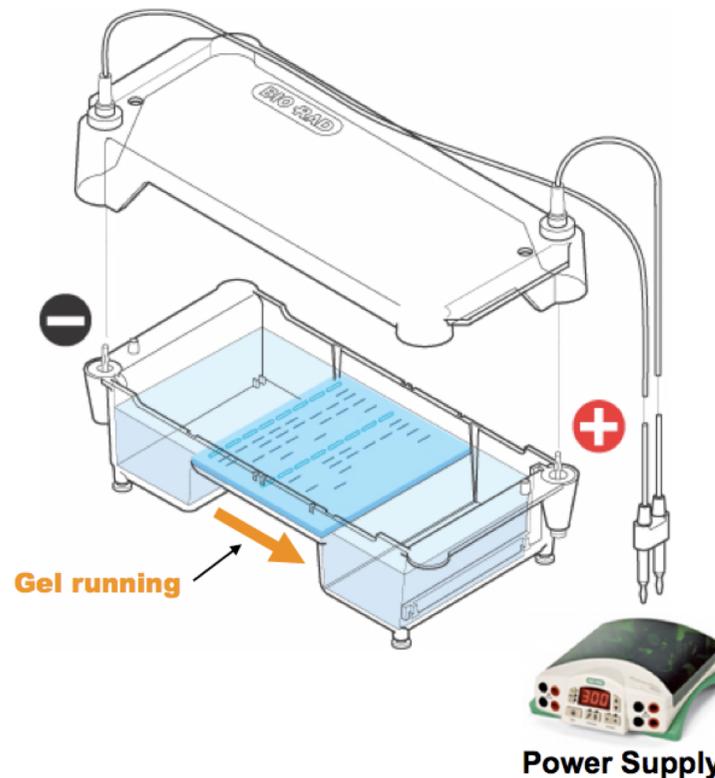
Normally at 37°C?

What happens if the temperature is too hot or cool?

- *Too hot* = enzyme may be denatured (killed)
- *Too cool* = enzyme activity lowered, requiring longer digestion time

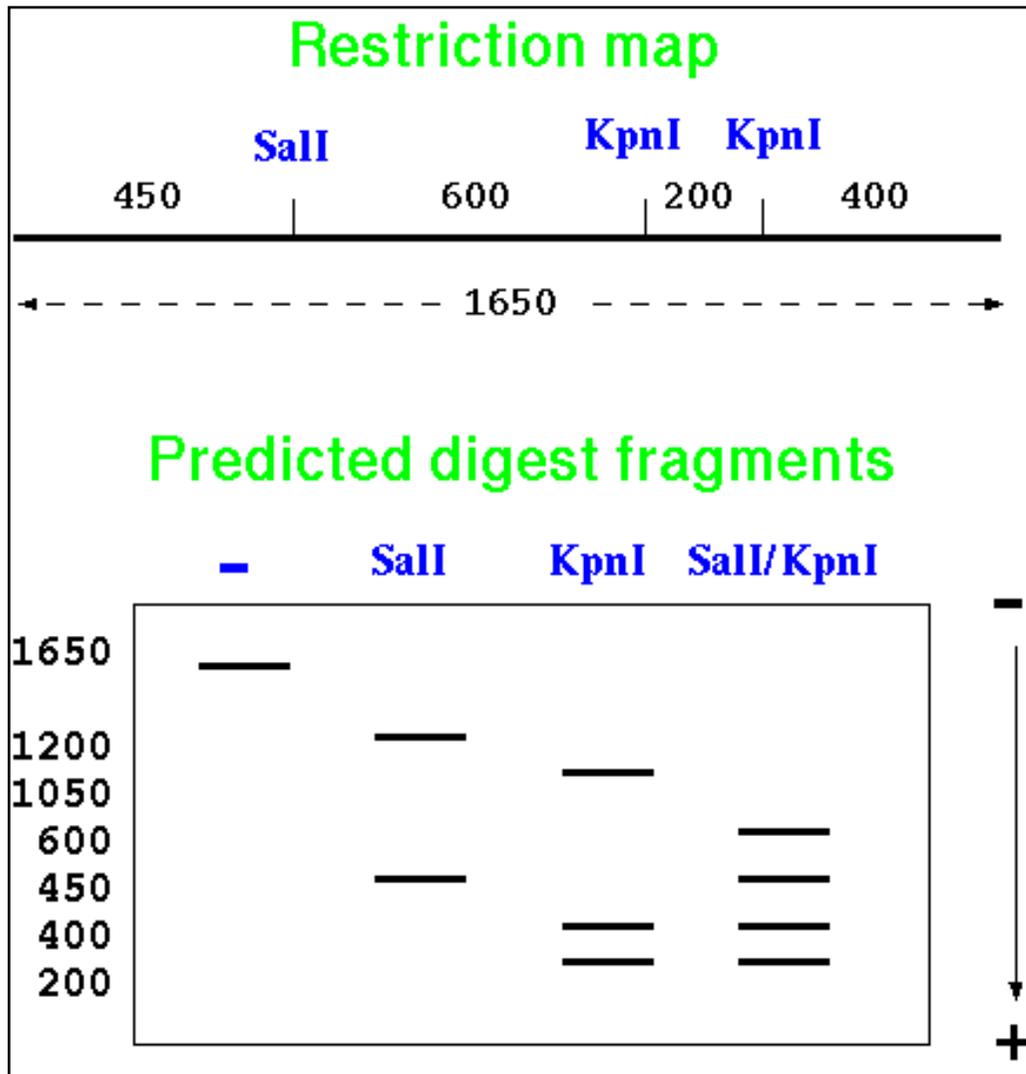
## Agarose Electrophoresis Running

- **Agarose gel sieves** DNA fragments according to size
  - Small fragments move farther than large fragments



# Restriction Digest Analysis

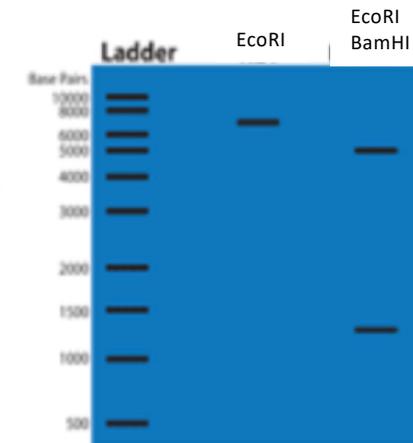
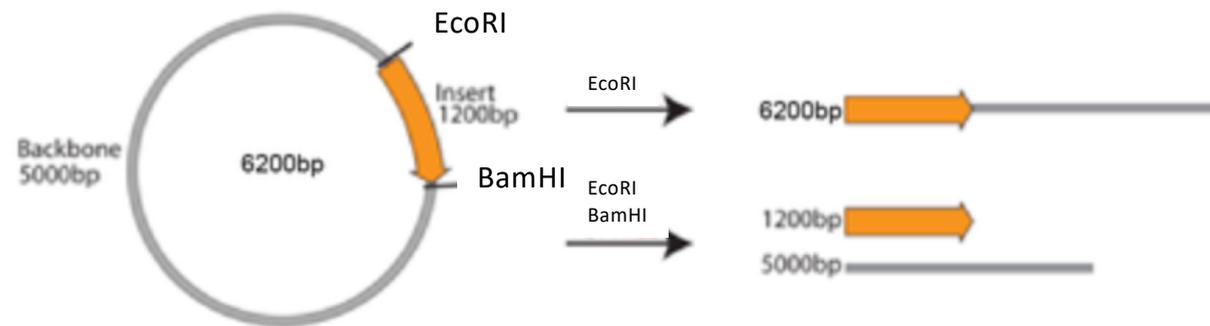
## LINEAR MOLECULE OF DNA (i.e. PCR amplified)



- Length=1650bp
- *SalI* yields two fragments (1200bp and 450bp)
- *KpnI* cuts at 2 sites giving 3 fragments
- *SalI* and *KpnI* cut 3X yielding 4 fragments

# Restriction Digest Analysis

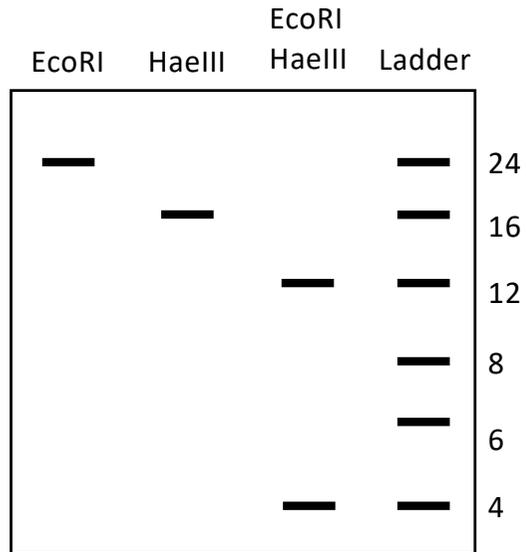
## CIRCULAR MOLECULE OF DNA (i.e. plasmid)



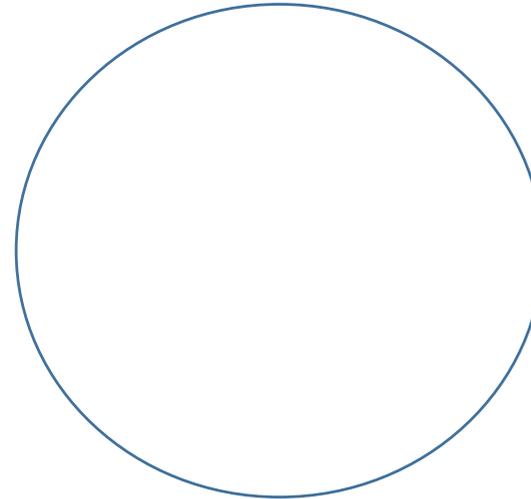
- Total length 6200bp; Backbone: 5000bp; Insert 1200bp
- *EcoRI cuts once: linearized plasmid 6200 bp)*
- *BamHI + EcoRI: 2 fragments; that means also BamHI cuts only once*
- *BamHI and EcoRI: insert 1200 bp, backbone 5000 bp*

# Restriction Digest Analysis

## RESTRICTION MAPPING OF PLASMIDS

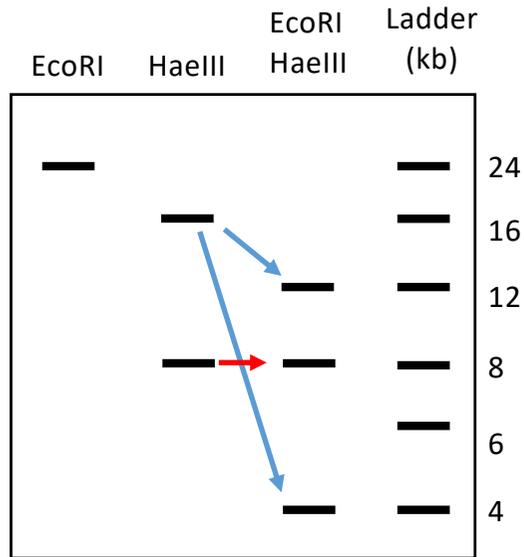


Prepare vector map  
of circular plasmid



# Restriction Digest Analysis

## RESTRICTION MAPPING OF PLASMIDS



Prepare vector map  
of circular plasmid

