Manipulation of DNA- 2
Restriction Enzymes

Restriction Enzymes

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What are Restriction Enzymes?

1962: "molecular scissors" discovered in in bacteria

E. coli bacteria have an enzymatic immune system that recognizes and destroys foreign DNA

3,000 enzymes have been identified, more than 300 are available for use in the lab, are purified and available commercially

Restriction enzymes are endonucleases (Endo (inside), nuclease(cuts nucleic acid), which catalyze the cleavage of the phosphodiester bonds within both strands of DNA.

They require Mg^{+2} for activity and generate a 5 prime (5') phosphate and a 3 prime (3') hydroxyl group at the point of cleavage.

Nobel Prizes for W. Arber, H. Smith, and D. Nathans in 1978 as discovery of REs was one of the key breakthroughs in the development of genetic engineering.

Restriction Enzymes

The distinguishing feature of restriction enzymes is that they only cut at very specific sequences of bases. This specific DNA sequence is called recognition sequence.

Restriction enzymes are traditionally classified according to the subunit composition, cleavage position, sequence-specificity and cofactor requirements.

- A restriction enzyme requires a specific double stranded recognition sequence of nucleotides to cut DNA.
- Recognition sites are usually 4 to 8 base pairs in length.
- Cleavage occurs within or near the site.

Nomenclature

Named for bacterial genus, species, strain, and type

Example: EcoR1

Genus: Escherichia
Species: coli
Strain: R
Order discovered: 1

Type	Activity Site	Features
Type I	far from the recognition sequence	• both endonuclease and methylase activities in the same protein • require both ATP and S-adenosyl-L-methionine to function
Type II	close to or within short distances from recognition sequence	• only endonuclease function • require Mg^{2+} to function
Type IIA	close to or within short distances from recognition sequence	• cleave asymmetric recognition sequences • require Mg^{2+} to function
Type IIB	close to or within short distances from recognition sequence	• cleave both sides of target on both strands • require Mg^{2+} to function
Type IIC	close to or within short distances from recognition sequence	• both endonuclease and methylase activities in the same protein • cleave both symmetric and asymmetric recognition sequences • require Mg^{2+} to function
Type IIE	close to or within short distances from recognition sequence	• interact with two copies of the recognition sequence; one the actual target for cleavage and the other is the allosteric effector
Type IIF	close to or within short distances from recognition sequence	• interact with two copies of the recognition sequence; one the actual target for cleavage and the other is the allosteric effector
Type IIG	close to or within short distances from recognition sequence	• both endonuclease and methylase activities in the same protein • cleave both symmetric and asymmetric recognition sequences • require Mg^{2+} to function
Type IIH	close to or within short distances from recognition sequence	• cleave both symmetric and asymmetric recognition sequences • require Mg^{2+} to function
Type IIM	close to or within short distances from recognition sequence	• acts on methylated DNA • require Mg^{2+} to function
Type IIP	close to or within short distances from recognition sequence	• cleave symmetric recognition sequences • require Mg^{2+} to function
Type IIS	close to or within short distances from recognition sequence	• cleave asymmetric recognition sequences • require Mg^{2+} to function
Type IIT	close to or within short distances from recognition sequence	• cleave both symmetric and asymmetric recognition sequences • require Mg^{2+} to function
Type III	25 – 27 bp outside the recognition sequence	• act on two sequences in opposite orientations with same DNA • require both ATP and S-adenosyl-L-methionine to function
Type IV	close to or within the recognition sequence	• act on methylated DNA • require Mg^{2+} to function

Palindromic Recognition Sites

The recognition sequences for some of the most frequently used restriction endonucleases.

ENZYME	ORGANISM	RECOGNITION SEQUENCE*	BLUNT OR STICKY END
*Eco*RI	*Escherichia coli*	GAATTC	Sticky
*Bam*HI	*Bacillus amyloliquefaciens*	GGATCC	Sticky
*Bgl*II	*Bacillus globigii*	AGATCT	Sticky
*Pvu*I	*Proteus vulgaris*	CGATCG	Sticky
*Pvu*II	*Proteus vulgaris*	CAGCTG	Blunt
*Hin*dIII	*Haemophilus influenzae* R_d	AAGCTT	Sticky
*Hin*fI	*Haemophilus influenzae* R_f	GANTC	Sticky
*Sau*3A	*Staphylococcus aureus*	GATC	Sticky
*Alu*I	*Arthrobacter luteus*	AGCT	Blunt
*Taq*I	*Thermus aquaticus*	TCGA	Sticky
*Hae*III	*Haemophilus aegyptius*	GGCC	Blunt
*Not*I	*Nocardia otitidis-caviarum*	GCGGCCGC	Sticky
*Sfi*I	*Streptomyces fimbriatus*	GGCCNNNNNGGCC	Sticky

*The sequence shown is that of one strand, given in the 5′ to 3′ direction. "N" indicates any nucleotide. Note that almost all recognition sequences are palindromes: when both strands are considered they read the same in each direction, for example:

*Eco*RI

5′–GAATTC–3′
3′–CTTAAG–5′

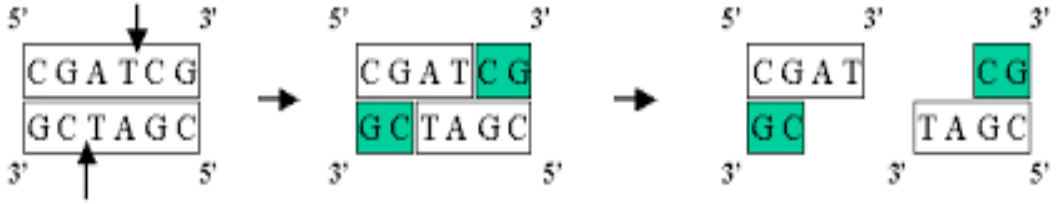
Sticky and Blunt end cutters

Some restriction endonucleases cut symmetrically and leave blunt ends. Many endonucleases cleave the DNA backbones in positions that are not directly opposite each other or can make staggered cuts, which produce single stranded “sticky-ends”.

5’ overhang

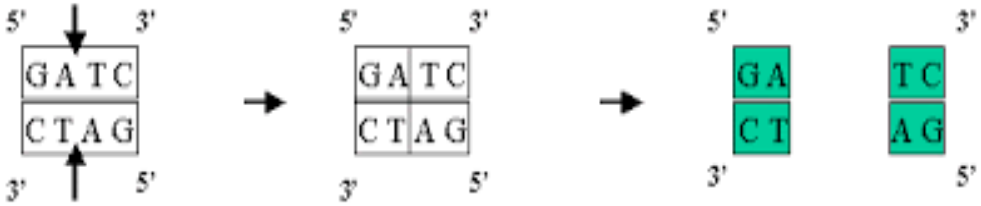
BamHI

3’ overhang



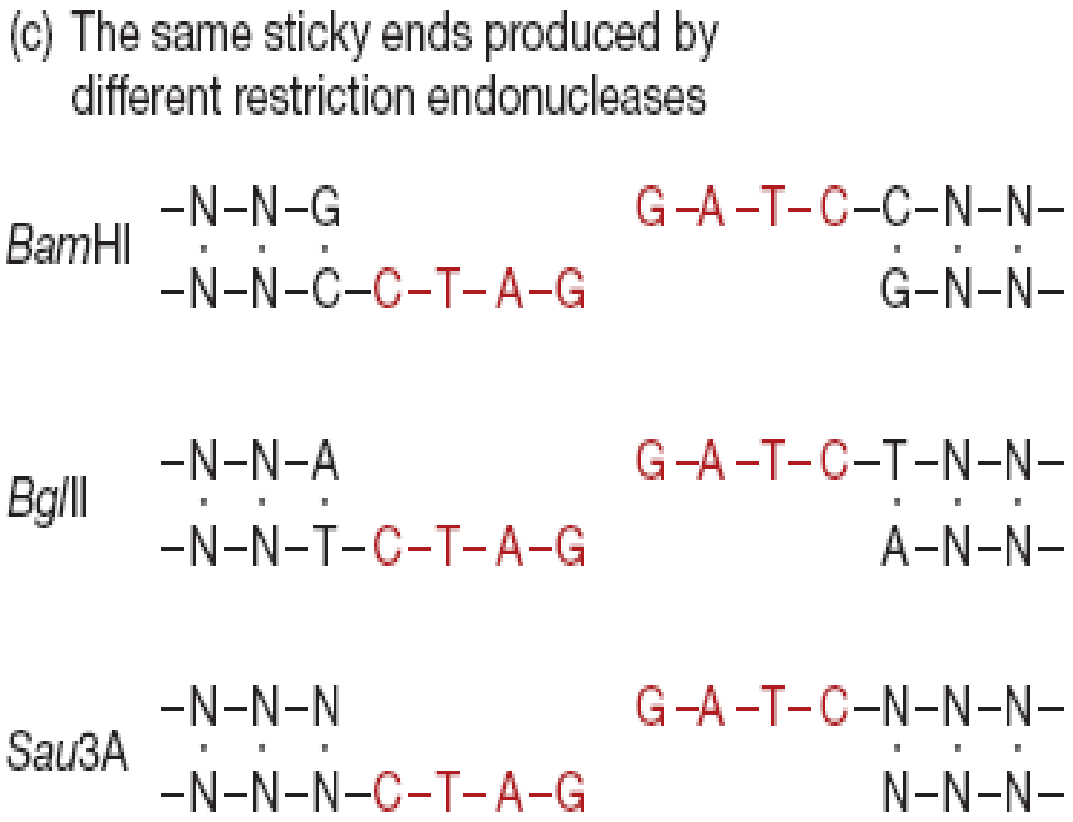
PvuI

Blunt ends



DpnI

Isocaudomers



Isoschizomers and Neoschizomers

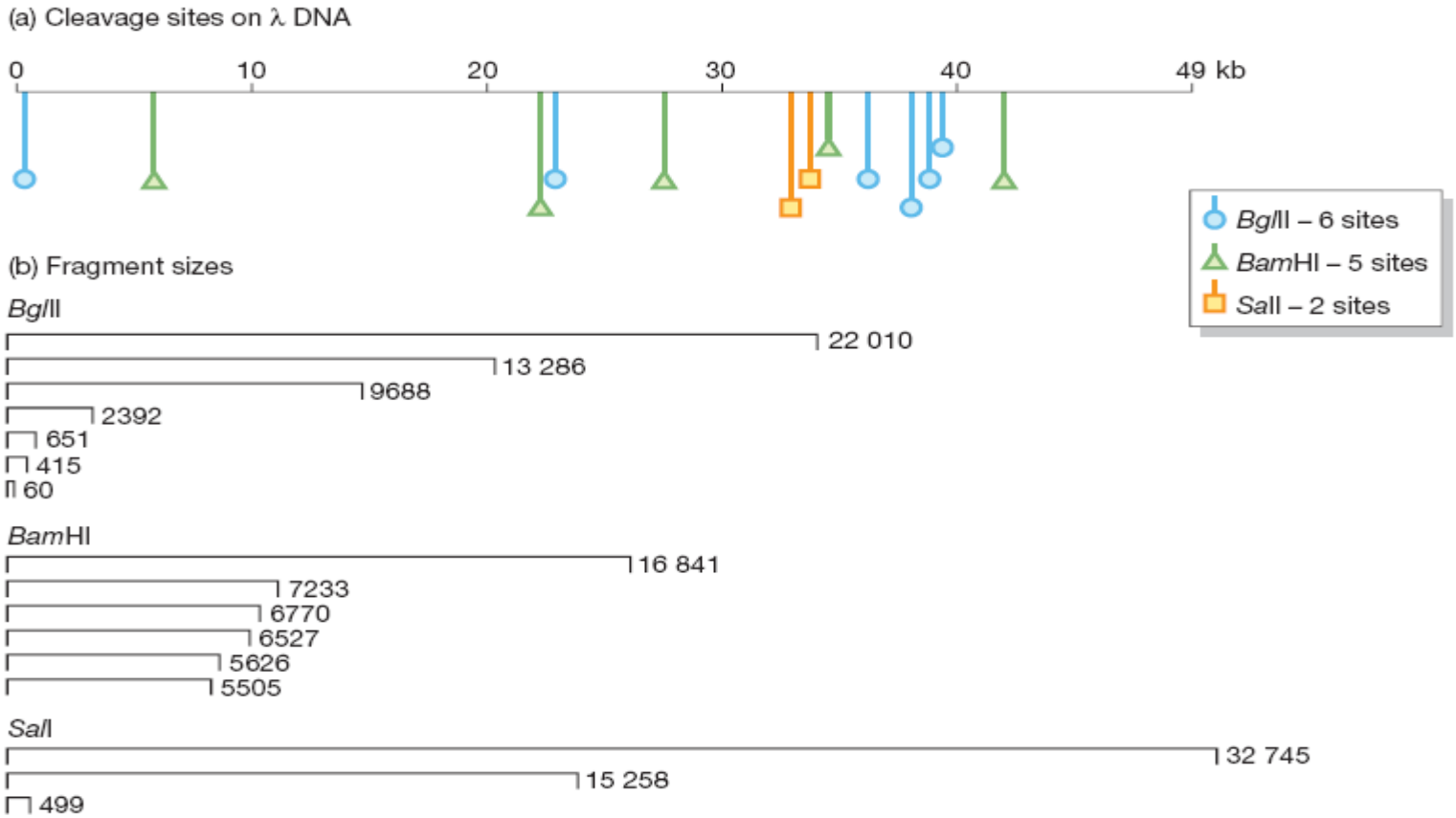
Isoschizomers are pairs of restriction enzymes specific to the **same recognition sequence**.
For example, SphI (CGTAC/G) and BbuI (CGTAC/G) are isoschizomers of each other. The first enzyme discovered which recognizes a given sequence is known as the prototype; all subsequently identified enzymes that recognize that sequence are isoschizomers. Isoschizomers may require different reaction conditions.

An enzyme that recognizes the same sequence but cuts it differently is a neoschizomer. **Neoschizomers** are a specific type (subset) of isoschizomer.

For example,
1. SmaI (CCC/GGG) and XmaI (C/CCGGG) are neoschizomers of each other.
2. Similarly Kpn1 (GGTAC/C) and Acc651 (G/GTACC) are neoschizomers of each other.
3. The sequence GGCGCC is recognized by four enzymes, each of which cuts in different places: *Nar*I (GG/CGCC), *Bbe*I (GGCGC/C), *Ehe*I (GGC/GCC), and *Kas*I (G/GCGCC).

Frequency of Restriction Sites

A tetranucleotide sequence (e.g., GATC) should occur once every $4^4 = 256$ nucleotides, and a hexanucleotide (e.g., GGATCC) once every $4^6 = 4096$ nucleotides. These calculations assume that the nucleotides are ordered in a random fashion and that the four different nucleotides are present in equal proportions (i.e., the GC content = 50%). In practice, neither of these assumptions is entirely valid.

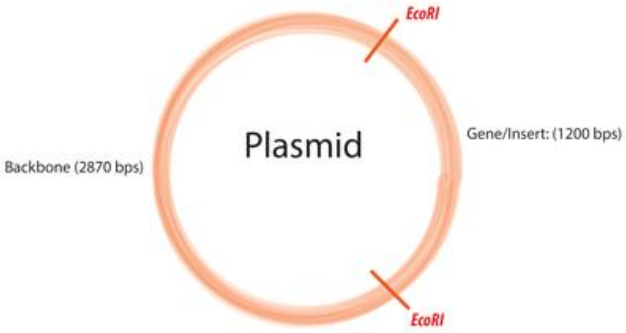


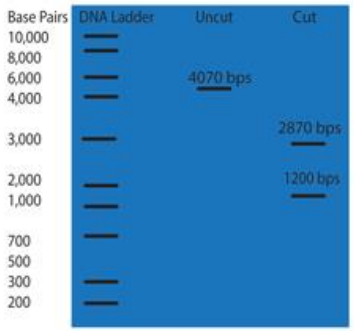
Unit Determination Assay

- One unit of restriction endonuclease is defined as the amount of enzyme required to digest one microgram of the appropriate substrate DNA completely in 60 minutes in 50 ul reaction under the conditions specified for that enzyme.

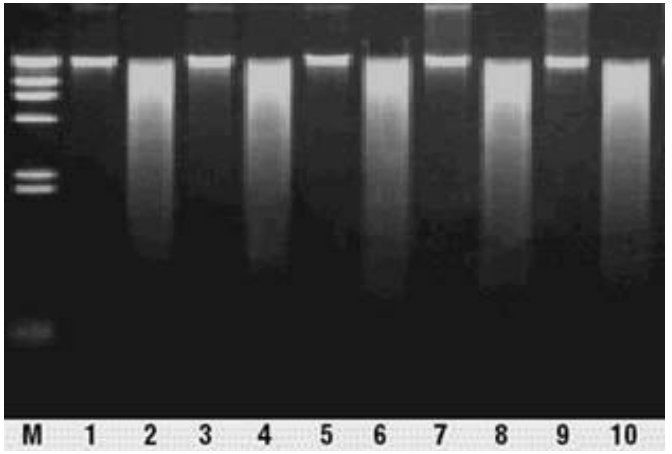
Set up of a restriction enzyme reaction

- A RE reaction contains the DNA to be analyzed,
- A restriction enzyme,
- A restriction enzyme buffer mix.
  - contains a buffering agent to maintain constant pH,
  - and Mg^{++} (from $MgCl_2$) as a necessary cofactor for enzyme activity.





Plant Genomic DNA



Odd numbered lanes contain undigested genomic DNA

Even numbered lanes contain digested genomic DNA

Factors that affect Restriction Enzyme Activity

The digestion activity of restriction enzymes depends on the following factors:
Temperature: Most endonucleases digest the target DNA at 37°C with few exceptions. Some work at lower temperatures (~25°C, *Sma* 1) while *Taq* I works at 65°C.

Cofactors: Restriction endonucleases require certain cofactors or combination of cofactors to digest at the recognition site. All enzymes require Mg^{2+} as a cofactor for the endonuclease activity. In R-M systems with separate proteins having the restriction and methylation activities, S-adenosylmethionine (SAM) and ATP are required for methylation activity.

Ionic Conditions: As mentioned previously, Mg^{2+} is required for all endonucleases but some enzymes also require ions such as Na^{+} and K^{+}.

Buffer systems: Most restriction enzymes are active in the pH range of 7.0–8.0. Tris-HCl, is the most commonly used buffer.

Methylation status of DNA: Methylation of adenine or cytidine residues affects the digestion of DNA.

Methylation of DNA Strands

Methylation of DNA is a major factor that affects the specificity of restriction endonucleases. DNA isolated from strains of bacteria expressing methylases such as Dam or Dcm may be resistant to cleavage by endonucleases as the recognition sites are methylated. Plasmid DNA isolated from *E. coli* expressing Dam is methylated at the GATC sites making it resistant to cleavage by *Mbo* I.

Use of Isoschizomers to determine the methylation status

In some cases, only one out of a pair of isoschizomers can recognize both the methylated as well as unmethylated forms of restriction sites. In contrast, the other restriction enzyme can recognize only the unmethylated form of the restriction site. This property of some isoschizomers allows identification of methylation state of the restriction site.

For example, the restriction enzymes **HpaII and MspI** are isoschizomers, as they both recognize the sequence 5'-CCGG-3' when it is unmethylated. But when the second C of the sequence is methylated, only MspI can recognize it while HpaII cannot.

Methylation of DNA Strands

For example, cleavage by XbaI may be blocked due to methylation if the **XbaI's recognition site (TCTAGA)** is preceded by GA or followed by TC. As shown in the figure below, a **Dam methylase recognition site (underlined in red- GATC)** overlaps with the XbaI cut site (depicted as an orange line) because the restriction site is followed by TC. Addition of the methyl group at this site would block the enzyme from cutting the DNA there, although other restriction sites (including other XbaI sites not preceded by GA or followed by TC) would be cut as normal. If not taken into account, this blocked site could potentially give you results that would either be difficult to interpret or lead you to conclude your plasmid is not correct!

Conversely, enzymes such as DpnI require methylation at their recognition sites in order to efficiently cleave DNA. DpnI is often used for site directed mutagenesis. During this process, incorporation of a desired mutation into your plasmid of interest by PCR generates mutated plasmids with no methylation (there are no methyltransferases in the PCR reaction). The template plasmid, on the other hand, should be derived from a *dam+ E. coli* strain and will therefore have methylated adenines in any GATC sequences found in the plasmid. When the PCR products are digested with DpnI, only the non-mutated and methylated template is destroyed leaving behind a pool of mutated plasmids which can later be verified by Sanger sequencing.

Refer- https://blog.addgene.org/plasmids-101-methylation-and-restriction-enzymes

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Considerations while designing primers with Restriction enzyme sites

	oligo sequence	% cleavage 2h	20h
BamHI	CGGATCCG	10	25
	CGGGATCCCG	>90	>90
	CGCGGATCCGCG	>90	>90
EcoRI	GGAATTCC	>90	>90
	CGGAATTCCG	>90	>90
	CCGGAATTCCGG	>90	>90
HindIII	CAAGCTTG	0	0
	CCAAGCTTGG	0	0
	CCCAAGCTTGGG	10	75
NcoI	CCCATGGG	0	0
	CATGCCATGGCATG	50	75
NdeI	GGGTTTCATATGAAACCC	0	0
	GGAATTCCATATGGAATTCC	75	>90

Star Activity of Restriction Enzymes

Star activity is defined as the alteration in the digestion specificity that occurs under sub-optimal enzyme conditions. Star activity results in cleavage of DNA at non-specific sites. Some of the sub-optimal conditions that result in star activity are as follows:

- pH >8.0
- glycerol concentration of >5%
- enzyme concentration >100 units/mg of DNA
- increased incubation time with the enzyme
- presence of organic solvents in the reaction mixture
- Incorrect cofactor or buffer