

Digestion of DNA With Restriction Endonucleases

Digestion of DNA with restriction endonucleases is the first step in many gene manipulation projects. These enzymes are part of the system that carries out restriction and modification. It appears that their main role is to protect cells from invasion by foreign DNA's, especially bacteriophage DNA. Restriction endonucleases recognize specific 4-base (tetramer), 5-base (pentamer), or 6-base (hexamer) sites located on the incoming DNA, and make double-stranded cuts. The sites are short enough that they could be found randomly in the DNA of any organism, including the organism that produces the restriction endonuclease. To protect its own sites, then, the producing organism has a methylase that recognizes and methylates the same site that the endonuclease cuts. This process is called modification; methylation prevents restriction. Thus, an organism would have its own sites protected while incoming DNA would lack the appropriate methylation and therefore be vulnerable.

There is a uniform system for naming restriction endonucleases and their corresponding methylases, based on the genus and species of the source organism, the particular strain, and the order of discovery. By convention, the first letter of the genus name and the first two letters of the species name are used to derive the basic enzyme name. Thus *Escherichia coli* yields *Eco*. Then comes a designation, if any, of the particular strain (sometimes an enzyme is encoded by a plasmid and the plasmid designation is used). A common restriction endonuclease from *E. coli* comes from an R factor. Finally, a Roman numeral is applied to indicate the order of discovery. Thus the first restriction enzyme from *E. coli* carrying an R factor is *EcoRI*. Some others are:

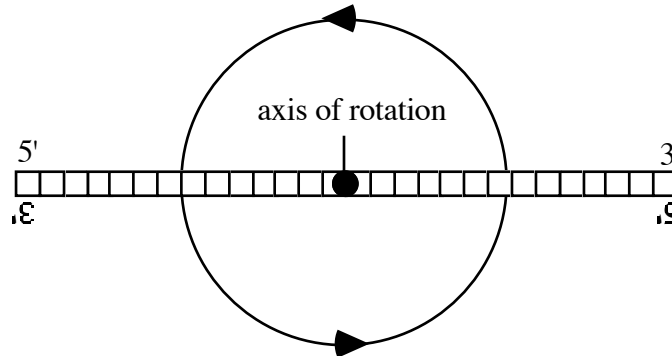
<i>HindIII</i>	the third enzyme from <i>Haemophilus influenzae</i> strain d
<i>SmaI</i>	the first enzyme <i>Serratia marcesens</i>
<i>BamHI</i>	the first enzyme from <i>Bacillus amyloliquifaciens</i> strain H
<i>KpnI</i>	the first enzyme from <i>Klebsiella pneumoniae</i>

The names of restriction endonucleases are distinguished from the names of methylases by placing an "r." or an "m." in front of the name. Thus *m.EcoRI* is the corresponding methylase for the restriction endonuclease *r.EcoRI*. Typically, though, most people only use the endonucleases, so the "r." is dropped unless both endonucleases and methylases are used in the same work.

Recombinant DNA technology is based upon the fact that many enzymes produce staggered cuts leaving complementary single-stranded tails. Being complementary, the single stranded tails can be made to form hydrogen bonds with one another and the cohering fragments can then be ligated together. Since the tails are based solely on the restriction sequence, it is possible to ligate DNA's from two

different species if they have been cut with the same enzyme. The ability of restriction endonucleases to produce cohesive single-stranded tails depends upon the symmetry of the restriction site and the way that the particular enzyme cuts relative to the symmetry.

The two strands of DNA are said to be anti-parallel. That is, one strand runs 5'⇒3' and the other runs 3'⇒5'. This produces a structural symmetry called rotational or dyad symmetry. In dyad symmetry, one can rotate a structure 180° and obtain the same structure:



For restriction sites, not only does the overall structure possess dyad symmetry, but also the DNA sequence itself possesses dyad symmetry. For example, the restriction enzyme *EcoRI* recognizes the site:

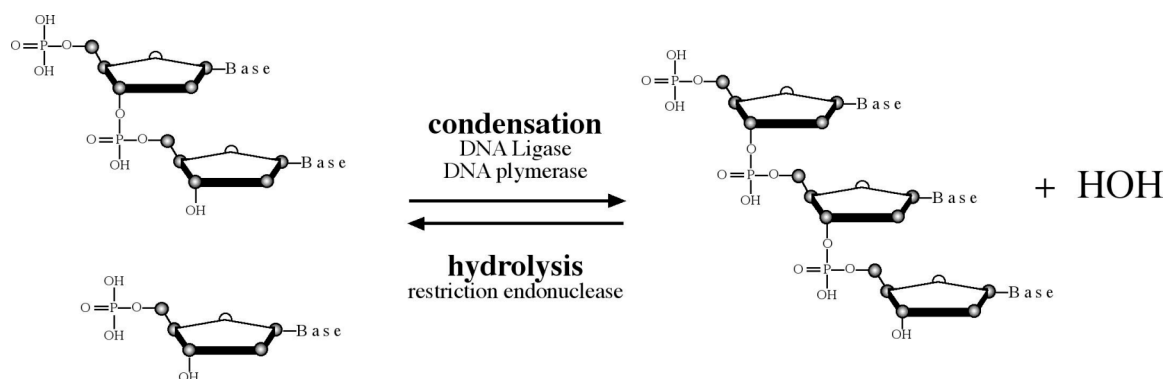


When this hexameric sequence is rotated about its axis, not only is the structural polarity maintained, but also the identical sequence is obtained. This symmetry of sequence is due to the unique nature of the base sequence in which the second three bases are the complement, in reverse order, of the first three: A B C C' B' A'. One strand therefore is the reverse order of the other. Such an arrangement is often referred to as a *palindrome*. In literature, a palindrome is a phrase that reads the same forwards and backwards. An example of a palindrome is when Adam, in the Garden of Eden, introduced himself to Eve:

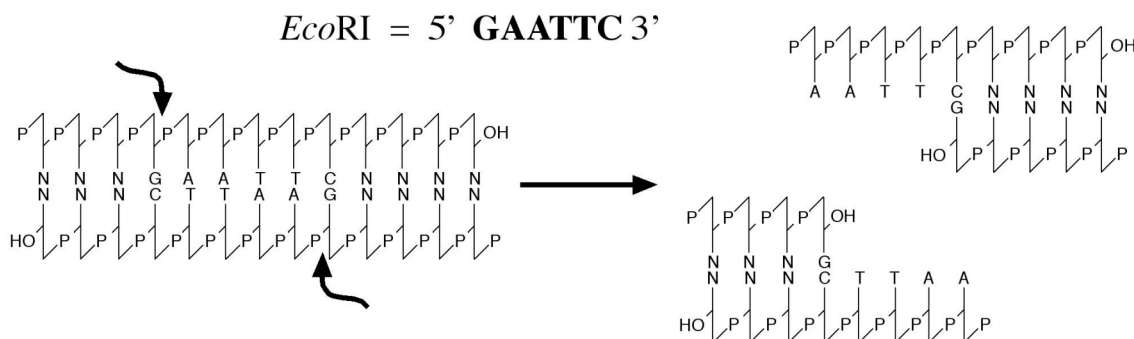
Madam, I'm Adam

The restriction site is not a true palindrome, of course, because the reverse is on the opposite strand.

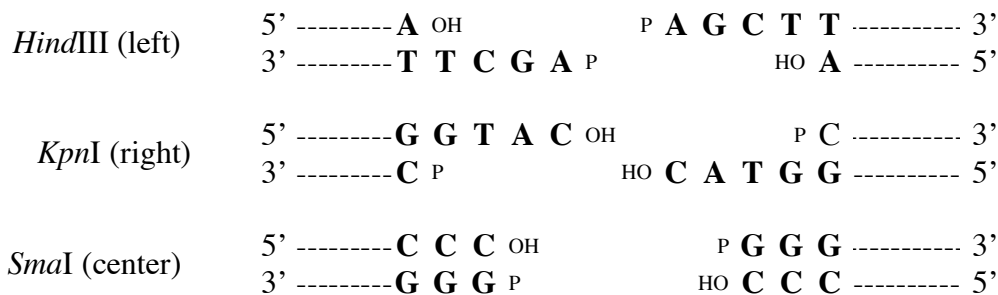
During restriction, the endonuclease must cut each of the strands to generate a double-strand cut. Cleavage is the result of hydrolysis, a reaction in which water is added across a bond, thereby breaking it. In this case, the water is added across the phosphodiester bond, cleaving the two adjacent nucleotides. Cleavage (at least by restriction endonucleases) yields 5'-phosphate and 3'-hydroxyl termini. By contrast, Nucleotides are joined by condensation reactions, in which phosphodiester bonds are formed by splitting out a water molecule. DNA and RNA polymerases and DNA ligase are enzymes that function via condensation.



Because each of the strands are identical to each other both in sequence and structure (remember, the strands are the same, but antiparallel), the cuts are made in the same spot on each strand, relative to the axis of rotation. This creates a staggered cut, leaving overhanging single-stranded tails on each end. The cuts made by *EcoRI* are typical. The shorthand structure used in this figure is explained in Appendix II.



In the *EcoRI* example, the cuts were made to the left of the axis of rotation, producing 5' overhangs. Other enzymes, however, cleave to the left of the axis, producing 3' overhangs, or on the axis, producing "blunt" ends. Three examples, *HindIII*, *KpnI*, and *SmaI* are shown below:



Clearly, ends created by *HindIII* and *KpnI* are complementary and can permit ligation. But blunt ends such as formed by *SmaI*, under the right circumstances, can also be ligated. Moreover, it is possible to treat the cut ends with a variety of secondary enzymes to provide lots of flexibility with respect to subsequent cloning steps. The type of enzyme used and the type of modification possible depends on the nature of the cut relative to the axis, and on whether the 3'OH end is recessed or over-hanging.

Sometimes there is overlap in the recognition sites for different enzymes. For example, the site for *Bam*HI, G↓GATCC (where “↓” shows the cut site) shares the middle four bases with the site for *Bgl*II, A^eGATCT, and the entire tetrameric sequence of *Sau*3A, ↓GATC. It is thus possible to ligate one DNA cut with *Bam*HI to another DNA cut with *Bgl*II. Such enzymes are said to be *compatible*. Since the outside bases for each enzyme are different, the result would be a hybrid sequence that cannot be cut by either *Bam*HI or *Bgl*II. The central GATC, however, would be regenerated and could be cut by *Sau*3A. Similarly, DNA cut with *Sau*3A could be ligated to DNA cut with either *Bam*HI or *Bgl*II. Since *Sau*3A recognizes a four-base site, the adjacent bases are random. Thus there is a one in four probability that the fusion of a *Sau*3A site to a *Bam*HI site will regenerate the *Bam*HI sequence. The probability of finding a tetrameric sequence such as *Sau*3A in any random piece of DNA is much greater than finding a hexameric sequence. Thus an enzyme like *Sau*3A will cut DNA much more frequently than will *Bam*HI.

Each restriction enzyme has its own optimal set of reaction conditions, which can be found on the information sheet provided by the supplier. A number of companies produce high-quality restriction enzymes. The most important reaction condition variables are the ionic strength (i.e. salt concentration) of the reaction buffer and the temperature of digestion. Of the two, reaction temperature is often most critical. The ionic strength is less stringent and it is therefore permissible to broadly categorize restriction enzymes as requiring high, medium, or low salt. On page 14 is a list of formulas for these buffers. Page 15 gives the temperature and buffer requirements for some of the common restriction enzymes as well as their recognition sequences and sites of cutting. There are a few exceptions to this general categorization. We will discuss the conditions for using these enzymes as they come up.

Restriction endonucleases are purchased in high concentrations and 1 μl of most enzyme preparations is enough to cut as much as 10 μg of DNA. Enzyme concentrations are found on the information sheet as well as the label and are expressed as units of enzyme per standard volume (either ml or μl). A unit is the amount of enzyme required to fully digest a standard amount of a standard type of DNA (typically bacteriophage λ or plasmid pBR322) in a standard length of time. The unit definition can be found on the manufacturers spec. sheet.

Enzymes are usually stored at -20^o (ordinary freezer temperature) in 50% glycerol. When kept in this manner, they are stable for long periods of time. A serious danger to enzyme stability is repeated warming and cooling. While it is easy to always make sure that the enzymes are cold, a less obvious source of warming and cooling is found in the normal cycling of frost-free freezers. For this reason, enzymes should only be kept in special, non-frost-free freezers (i.e., cheap refrigerators, refreshingly!). Enzymes must be kept on ice at all times whenever they are removed from the freezer.

The worst fate that can befall an enzyme stock is for it to become contaminated with exonuclease from greasy hands. Not only does this ruin the enzyme, but it also ruins the work of unsuspecting users, sometimes destroying precious, hard-to-isolate DNA's. The following precautions must be observed by everyone:

1. **Always wear gloves when handling enzymes.**
2. **Always use a fresh pipet tip when going into an enzyme stock. If you must go into the enzyme twice, change the pipet tip.**
3. **Work quickly. Do not expose the enzyme to warm temperatures any longer than necessary.**

**General Protocol for
Performing Restriction
Digestions**

1. The reaction mix may contain 0.2 - 1.0 μg DNA. The total volume of the reaction should be kept at 20 μl . This is the amount typically run on a gel. You could, of course, scale this up to do larger digests. The amount of DNA added depends on the DNA prep. Typically, for plasmid purified on a CsCl density gradient, 3 - 5 μl . are sufficient. For DNA purified in rapid plasmid isolations, 10 μl . are typically used.
2. Add sterile distilled water to bring the volume up to 18 μl .
3. Add 2 μl . of 10x restriction buffer. Consult the table on page 15 for the appropriate buffer.
4. Add 1 μl . of restriction enzyme. Tap the tube several times to ensure mixing.
5. Centrifuge the tubes briefly (turn centrifuge on, allow it to get to speed, and turn it off) to concentrate all of the liquid at the bottom.
6. Place in water bath for 30 - 60 minutes. Typically this is at 37 $^{\circ}$, but you should check page 15 to make sure of the appropriate temperature.
7. At the end of the restriction digestion, do one of the following steps, depending on what you wish to do with the DNA next:

- a. If you wish to analyze the results on a gel, add 5 μ l. of tracking dye to the sample and load the sample into a well on an agarose gel. The tracking dye contains sucrose to increase the density of your sample so that it will settle to the bottom of the well rather than float away, and the dye will enable you to visualize where your sample is.
- b. If you wish to ligate your cut DNA to another DNA, you must inactivate the restriction endonuclease. Otherwise, the enzyme could re-cut any successful ligations. This may be done either by a heat treatment or by a phenol extraction followed by ethanol precipitation.
- c. If you wish to purify the DNA for any other purposes, you should do a phenol extraction followed by ethanol precipitation.

Tracking Dye

Tracking dye is a sucrose or glycerol solution containing dye that enables you to visualize the electrophoretic front. The sucrose or glycerol is necessary to increase the density of your sample so that it will settle to the bottom of an agarose well rather than float away.

A variety of dyes is available. Many people use bromphenol blue or orange G. The choice depends on the electrophoretic mobility of the dye relative to the DNA fragments. Bromphenol blue runs slower than orange G. Thus when comparing gels in which each dye is allowed to run to the end, the DNA's in the bromphenol blue gel will have run farther and separated better than in the orange G gel. But if you are looking at very small fragments, they may have run off the gel with bromphenol blue, but are still present with orange G. The primary consideration for deciding which dye to use is how the mobility of the tracking dye compares to the mobility of the smallest DNA fragments that you are trying to resolve.

Orange G: 0.25% orange G (Sigma cat # O-1625) dissolved in 50% sucrose.

Bromphenol Blue: 0.25 g bromphenol blue
0,25 g xylene cyanol
1.0 ml 1M Tris, pH8
49 ml water
50 ml glycerol

Restriction Digestion Buffers

Final Concentrations				
Buffer	NaCl	Tris	MgSO ₄	Dithiothreitol
Low	0 mM	10 mM (pH7.4)	10 mM	1 mM
Medium	50 mM	10 mM (pH7.4)	10 mM	1 mM
High	100 mM	50 mM (pH7.4)	10 mM	0 mM

10x Stocks for Restriction Assays			
	Low	Medium	High
5 M NaCl	0	1	2
1 M Tris (pH 7.4)	1	1	5
1 M MgSO ₄	1	1	1
0.01 M Dithiothreitol	1	1	0
Water	7	6	2
Total Volume	10	10	10

From *A Manual for Genetic Engineering: Advanced Bacterial Genetics*. R.W. Davis, D. Botstein, and J.R. Roth. Cold spring Harbor Laboratory. 1980.

Restriction Endonucleases

Enzyme	Common Isoschizomers	Salt	Incubation Temperature	Recognition Sequence	Compatible Cohesive Ends
<i>Ava</i> I		med	37°C	G↓PyCGPuG	<i>Sal</i> I, <i>Xho</i> I, <i>Xma</i> I
<i>Bam</i> HI		med	37°C	G↓GATCC	<i>Bcl</i> II, <i>Bgl</i> III, <i>Mbo</i> I, <i>Sau</i> 3A, <i>Xho</i> II
<i>Bgl</i> III		low	37°C	A↓GATCT	
<i>Bst</i> EII		med	60°C	G↓GATCC	
<i>Eco</i> RI		high	37°C	G↓AATTC	
<i>Eco</i> B			37°C	TGA(N) ₈ TGCT	
<i>Eco</i> K			37°C	AAC(N) ₆ GTGC	
<i>Eco</i> RI*			37°C	↓AATT	
<i>Hae</i> III		med	37°C	GG↓CC	blunt
<i>Hind</i> II		med	37°C	GTPy↓PuAC	blunt
<i>Hind</i> III		med	37-55°C	A↓AGCTT	
<i>Kpn</i> I		low	37°C	GGTAC↓C	
<i>Mbo</i> I	<i>Sau</i> 3A	high	37°C	↓GATC	<i>Bam</i> HI, <i>Bcl</i> II, <i>Bgl</i> III, <i>Xho</i> II
<i>Pst</i> I		med	21-37°C	CTGCA↓G	
<i>Pvu</i> II		med	37°C	CAG↓CTG	blunt
<i>Sau</i> 3A	<i>Mbo</i> I	med	37°C	↓GATC	<i>Bam</i> HI, <i>Bcl</i> II, <i>Bgl</i> III, <i>Xho</i> II
<i>Sma</i> I	<i>Xma</i> I		37°C	CCC↓GGG	blunt
<i>Taq</i> I		low	65°C	T↓CGA	<i>Acc</i> I, <i>Acy</i> I, <i>Asu</i> II, <i>Cla</i> I, <i>Hpa</i> II
<i>Xba</i> I		High	37°C	T↓CTAGA	
<i>Xma</i> I	<i>Sma</i> I	low	37°C	C↓CCGG	<i>Ava</i> I

From *Molecular Cloning*. T. Maniatis, E. F. Fritsch, and J. Sambrook. Cold spring Harbor Laboratory. 1982.

New England Biolabs Restriction Digestion Buffer System

1x Stock	<i>Bam</i> HI*	<i>Bgl</i> III	<i>Eco</i> RI	<i>Hind</i> III	<i>Pst</i> I*	<i>Xba</i> I*
NEBuffer 1 10 mM Bis-Tris-Propane-HCl 10 mM MgCl ₂ 1 mM Dithiothreitol pH 7.0 @ 25°C	75%	50%	100%	50%	75%	0%
NEBuffer 2 10 mM Tris-HCl 50 mM NaCl 10 mM MgCl ₂ 1 mM Dithiothreitol pH 7.9 @ 25°C	100%	75%	100%	100%	75%	100%
NEBuffer 3 50 mM Tris-HCl 100 mM NaCl 10 mM MgCl ₂ 1 mM Dithiothreitol pH 7.9 @ 25°C	50%	100%	100%	10%	100%	75%
NEBuffer 4 50 mM potassium acetate 20 mM Tris-acetate 10 mM Magnesium Acetate 1 mM Dithiothreitol pH 7.9 @ 25°C	75%	50%	100%	50%	50%	75%
NEBuffer BamHI 10 mM Tris-HCl 150 mM NaCl 10 mM MgCl ₂ 1 mM Dithiothreitol pH 7.9 @ 25°C	100%					
NEBuffer EcoRI 100 mM Tris-HCl 50 mM NaCl 10 mM MgCl ₂ 0.025 % Triton X-100 pH 7.5 @ 25°C			100%			