

RESTRICTION ENZYME MAPPING

OBJECTIVE:

To do some rudimentary restriction enzyme mapping of a piece of amplified ribosomal DNA.

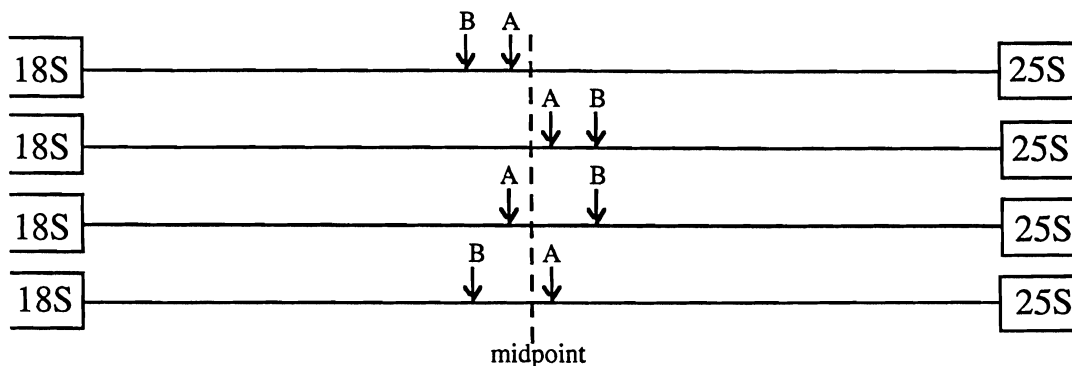
INTRODUCTION:

Mapping restriction enzyme sites on a DNA fragment can be accomplished in several ways. For many years, hybridization to genomic DNA has been the method of choice. In this method, total DNA is subjected to Southern hybridization analysis using several enzymes. This method is fairly time-consuming and its accuracy is restricted to fragments from a few hundred base pairs to several kilobases. However, for poorly characterized DNA regions it may be the only method to use. A refinement on this method is to clone the fragment of interest and probe the Southern blot with several pieces of the clone to locate the sites. This method is very accurate, and its limits are essentially those of the sizes of the pieces that can be cloned. PCR amplified fragments can also be mapped in the same way, which is what we will be doing. Once the fragment is isolated, it can be digested with enzymes to construct a map. If a map is constructed of a 500 bp fragment using ten four-base-cutting enzymes (consisting of 20 sites), then you know approximately 80 of the 500 base pairs (or 16%) of that region. Therefore, this method of mapping yields a resolution close to that of DNA sequencing (as compared to mapping a 10 kb piece with the same number of six-base-cutting enzymes which would yield less than about 0.01% of the total sequence).

Here is a simple example to show you some of the fundamentals of mapping. Let's say that you have amplified the 730 bp fragment that extends from the 3' end of the 18S ribosomal RNA gene (rRNA gene, or rDNA) to the 5' end of the 25S rRNA gene, from a Basidiomycete (this fragment would also include the 5.8S gene). When you digest the fragment with restriction enzyme A, two fragments are generated, one that is 350 bp in length and one that is 380 bp in length. When you digest with enzyme B, two fragments are also generated, one that is 340 bp in length and one that is 390 bp in length. When the fragment is digested with a mixture of A and B, three fragments are generated, one at 350 bp, one at 340 bp and one that is about 40 bp in length. The diagram below shows the arrangement.

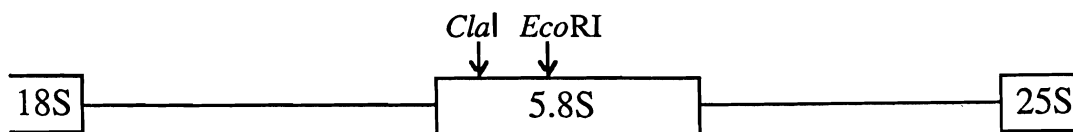
A	A+B	B	
		—	390
—			380
—	—		350
	—	—	340
	—		40

First, try to position the site for enzyme A. If the site was in the center the fragments would each be 365 bp ($730 / 2$). Therefore, A is off center by 15 bp. The site for enzyme B is also off center, but by 25 bp. Excluding the position of the 5.8S rRNA gene for the moment, there are four possible arrangements of the sites, as shown below:



However, two of these can be excluded (1 and 2), because when the fragment is digested with both enzymes, the shorter fragments of both enzymes remain, indicating that the enzyme sites are on opposite sides of the center of the fragment.

To determine the positions relative to other parts of the fragment (e.g., genes in the region), hybridization should be carried out. In this case, the region contains the 3' end of the ribosomal RNA 18S gene on one end and the 5' end of the 25S gene on the other end. Hybridizing with these genes should allow orientation of the map sites. Another way of orienting the sites is to use available sequence data. In the case above, enzyme A is *EcoRI* and enzyme B is *Clal*. The sites occur in the 5.8S rRNA gene in many fungi. However, they are cryptic sites in many other eukaryotes. That is, the sequence is one or more base pairs off from being a recognition site for the enzymes. By searching through published sequences, it has been determined that the *EcoRI* site is almost in the center of the 5.8S gene and the *Clal* site is about 10 to 20 base pairs to the right of it (in the 3' direction). Therefore, we can now put the map together (corresponding to possibility 3, above), knowing also that the primers are about 20 bp from the end of each gene (18S and 25S) and that the 5.8S gene is about 150 to 160 bp in length.



Now that the positions of these sites are known, more enzymes can be mapped, by relating them to these sites. However, if the enzyme generates more than two to four fragments, mapping becomes difficult, and either hybridization or manipulation of smaller fragments may be necessary.

We will be mapping using four enzymes, *EcoRI*, *ClaI*, *MspI* and *TaqI*. The first three enzymes are active at 37°C, while *TaqI* requires incubation at 65°C.

STEPS IN THE PROCEDURE:

1. Set up the following digestions for your amplified fragment:

[SINGLE ENZYME DIGESTS]

2 µl DNA (amplified fragment)

1 µl 10X *EcoRI* buffer

6.5 µl H₂O

0.5 µl *EcoRI*

2 µl DNA

1 µl 10X *ClaI* buffer

6.5 µl H₂O

0.5 µl *ClaI*

2 µl DNA

1 µl 10X *MspI* buffer

6.5 µl H₂O

0.5 µl *MspI*

[Incubate each above at 37°C for at least 1-2 hours]

2 µl DNA

1 µl 10X *TaqI* buffer

6.5 µl H₂O

0.5 µl *TaqI*

[Incubate at 65°C for at least 1-2 hours]

[DOUBLE ENZYME DIGESTS]

2 µl DNA

1 µl 10X *EcoRI* buffer

6.0 µl H₂O

0.5 µl enzyme 1

0.5 µl enzyme 2

[Remember, when performing a double digest that includes *TaqI*, incubate without *TaqI* at 37°C first for 1-8 hours, then incubate at 65°C for another 1-8 hours.]

You should have 9 digestions (four single digestions and six double digestions).

2. Add 3 µl of RS solution.

3. Load each digest into a single lane of a 2% agarose gel (which includes ethidium bromide).
4. Set to 30V for 30 minutes, then to 100V until the blue dye is about 2/3 of the way down the gel.
5. Photograph the gel and map the sites.

NOTES ON THE STEPS IN THE PROCEDURE:

1. Be sure to keep the vial containing the enzyme on ice constantly when it is out of the freezer.

SOLUTIONS:

[All from previous exercises]