

Sequential digestion of DNA using two restriction endonucleases

Richard Powell

Department of Microbiology, National University of Ireland, Galway, Ireland

Frank Gannon

European Molecular Biology Laboratory, Postfach 10.2209, Meyerhofstrasse 1, D-69012 Heidelberg, Germany

Equipment and reagents

- ◆ Restriction endonucleases (enzymes) plus appropriate buffers
- ◆ Water-bath or heating-block
- ◆ Gel electrophoresis apparatus
- ◆ 5 M NaCl

Method

- 1 Make up a 100 μl reaction mixture^a by adding the reagents in order into a sterile 1.5 ml microfuge tube: 10 μl 10 \times restriction endonuclease buffer^b, x μl DNA and y μl water. The value of x will depend on the concentration of your DNA solution^c. The value of y should bring the volume up to 99 μl . This reaction mixture should be appropriate for the restriction endonuclease that requires the lowest salt concentration when the two enzymes for use are compared. If the reaction buffers of both enzymes are similar, the DNA may simply be incubated in the presence of both enzymes.
- 2 Incubate the reaction at the correct temperature for the desired length of time before storing it on ice for the time required for analysis by gel electrophoresis.
- 3 Analyse a small sample of the reaction (e.g. 50 ng of DNA) and check for complete digestion of the DNA. If the digestion is not complete, re-incubate the reaction mixture for a further period of time. Alternatively, add a further aliquot of enzyme and re-incubate.
- 4 After electrophoretic analysis showing completion of digestion by the first enzyme, increase the salt concentration to that appropriate for the second restriction endonuclease by adding a small aliquot (1–5 μl) of 5 M NaCl to the reaction mixture. Add the second enzyme and allow this reaction to progress before monitoring for complete double digestion by gel electrophoresis as before.

Notes

- a When used for analytical purposes only, the reaction can be carried out in smaller volumes, e.g. 20 μl .

- b Use the buffer provided by the suppliers of the restriction enzyme, or make a buffer according to their instructions.
- c Although concentrations of DNA as low as 10 ng can be visualized on a typical agarose gel, it is preferable to use 100–200 ng per digestion to facilitate easy detection of short DNA fragments.

Ethidium bromide is commonly used to stain agarose gels in order to visualise nucleic acids. It is also a carcinogen. Handle all ethidium bromide containing solutions and gels with care always using laboratory gloves. Specific waste procedures may be required for disposal of ethidium bromide containing waste.

This method is adequate for many double restriction endonuclease digestions. However, in some cases the reaction conditions of the two enzymes may not be compatible with simple alteration of the salt concentration. Also, the first enzyme may lose its DNA specificity and begin to cleave the DNA in an unexpected manner while under the reaction conditions appropriate for the second enzyme. In these cases the first enzyme may be inactivated after reaction by incubation at 65 °C for 10 min before continuing with the second reaction. However, many endonucleases are not heat-labile and the DNA must therefore be purified from the reaction mixture by phenol extraction and ethanol precipitation (see [Purification of DNA by phenol extraction and ethanol precipitation](#)) before continuing with the second enzyme.

Complete double digestion of DNA by two enzymes recognizing restriction sites adjacent or very close to one another (as in a polylinker multicloning site) may not be distinguishable by simple gel electrophoresis from DNA cleaved by only one of the enzymes, i.e. both DNA fragments may appear to be of similar size. A simple ligation reaction (see [DNA ligation](#)) after DNA purification (see [Purification of DNA by phenol extraction and ethanol precipitation](#)) may be required to ensure correct double digestion. If the product of the double digestion is a DNA fragment with non-complementary termini, i.e. unlike a DNA fragment cleaved by one restriction enzyme, it cannot ligate to itself to form circular molecules. These differences are easily analysed by gel electrophoresis.