A Rapid Method for Creating Recombinant DNA Molecules

The construction of recombinant DNA molecules represents a molecular version of a classical genetic cross, and is a basic technique of molecular genetics. At the request of the editors, I describe a rapid method for *in vitro* construction of recombinant DNA molecules that my laboratory has used for the past 5 years (see **Gene** 25:231-241, 1983). The "method" represents a collection of tricks whose original sources are obscure (at least to me). The procedure is as follows:

Plasmid or phage DNAs (typically 0.1 to 2.0 μg), prepared by essentially any rapid lysate procedure, are treated with appropriate enzymes and the resulting products are electrophoretically separated on low gelling/melting temperature agarose in a buffer of 50 mM Tris-acetate, pH 8.2. Agarose concentrations ranging from 0.5 to 2% have been used successfully. The source of the agarose is critical; SeaPlaque® produced by Marine Colloids (Rockland, ME) has always been reliable. The desired DNA segments, visualized by long-wave ultraviolet light after staining with ethidium bromide, are excised from the gel with a clean razor blade in as small a volume as possible (usually 30 to 50 μl). Gel slices containing the relevant DNA segments are melted at 70 °C for 5 to 15 minutes and then combined in appropriate proportions to give a final volume of 10 μl. After equilibration of the molten gel slices to 37 °C, 10 μl of ice-cold, 2 × concentrated buffer containing T4 or T7 DNA ligase is added, mixed quickly, and the mixture is then incubated at 15 °C for 3 to 24 hours. Although the reaction mixture resolidifies into a gel, the ligation works; indeed, the reaction is barely, if at all, inhibited by the agarose! To introduce the ligated products into *E. coli* cells, the gel containing the reaction mixture is remelted at 70 °C and diluted by a factor of 10 to 50 into ice-cold TCM (10 mM Tris, pH 7.5, 10 mM $MgCl_2$, 10 mM $CaCl_2$) prior to carrying out the standard transformation procedure. Although not explicitly examined, it is likely that the dilution step prevents regelling of the agarose. We have never tested whether ligated products within the gel matrix can be packaged *in vitro* into viable λ phages.

Several technical points are worth noting. First, almost all of the enzymes used for DNA cloning are active in molten or resolidified SeaPlaque® agarose. This includes essentially all restriction endonucleases, DNA ligases, DNA polymerase I (for end-filling and nick-translation reactions), BAL-31 nuclease, and calf intestinal alkaline phosphatase. Obviously, SeaPlaque® is free of the inhibitory components found in most commercial preparations of agarose; presumably this reflects a higher degree of purification. The only enzyme that appears to be inhibited reproducibly by the low gelling/melting temperature agarose is T4 polynucleotide kinase. Second, very little DNA is necessary; basically, if a band can be visualized, the hybrid construction is very likely to be successful. Indeed, a single gel slice can be used for 5 to 20 separate ligation reactions, and it can be stored and repeatedly remelted for use. Third, the concentrations of the input DNA fragments are generally of minimal importance and can be very low. The major exception to this rule occurs if one or more of the DNAs can circularize to produce a selectable, transforming molecule capable of autonomous replication; without special manipulations, the background of "parental" molecules will be extremely high. This problem is easily avoided by using input DNAs cleaved with 2 different restriction enzymes or by treatment with calf intestinal alkaline phosphatase. Fourth, the method works for complicated constructions involving 3-fragment ligations, DNA fragments produced by partial cleavage with restriction enzymes, blunt-ended ligations, and BAL-31 deletion mutants. Fifth, the electrophoretic separation removes oligonucleotide linkers (which often interfere with subsequent ligation reactions), as well as the enzymes used to cleave or modify the DNA (thus eliminating the need to destroy such enzymes by phenol extraction and/or heat inactivation).

The method has a number of advantages. The electrophoretic purification of DNA fragments (including the cloning vector) minimizes the problems caused by incomplete digestion by restriction endonucleases and makes it possible to start with crude preparations. Moreover, as only the desired DNA segments are included in the ligation reaction mixture, the background of undesired molecules is greatly reduced, which is particularly important because analysis of the transformants is often the rate-limiting step in a construction. For simple constructions, the majority of the transformants contain the desired DNA molecule, and for more complex situations the frequency is sufficiently high to avoid time consuming screening procedures such as filter hybridization. Although numerous other methods are available for purifying DNA segments from agarose gels, the major advantage of the procedure described here is that it is both considerably faster and more reliable. An experimentalist dedicated to speed can often achieve a molecular generation time of 2-3 days. □

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