Volume 16 Number 22 1988 Nucleic Acids Research

Determination of contiguity of subclones using the polymerase chain reaction

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Submitted October 12, 1988

When subcloning restriction fragments from large inserts in cDNA or genomic λ bacteriophage clones, there are two types of uncertainty: (A) Unless one has an unambiguous restriction map of the λ clone or performs subcloning in several different ways so as to produce overlapping subclones, one is not always sure of the order and orientation of the subcloned fragments. (B) Because small fragments (less than 300bp) are difficult to see on an ethidium bromide gel of a λ digest, it is always questionable whether all restriction fragments have been accounted for in the subclones. Here we describe our use of the polymerase chain reaction to selectively amplify segments of λ DNA clones that contain the restriction sites in question and the direct sequencing of the amplified DNA to (A) determine which subclones are contiguous and (B) check for missing restriction fragments. This method avoids direct sequencing of λ DNA which can be difficult. In our case we wished to demostrate that we had not missed during our subcloning any small EcoRI fragments near the known EcoRI site within our insert. Oligonucleotides (20-mers), 130-140 bases from either side of the EcoRI site in question were synthesized such that they would prime DNA synthesis toward the site. One ng of λ DNA isolated according to the glycerol method (1) was used as the template. The polymerase chain reaction was performed as described (2) in a reaction volume of 100 µl, 100 pmoles of each primer and one unit of Taq polymerase (Perkin-Elmer Cetus, Norwalk) for 30 cycles on a thermocycler (Ericomp, San Diego) with denaturation, annealing, and polymerization times of, respectively, one, two, and four minutes. The reaction mixture was then chloroform extracted to remove mineral oil and spermine precipitated to remove most of the primers which would interfere with sequencing. Spermine was added to 10mM, and the sample was placed on ice for 30 minutes and microfuged for 25 minutes. The pellet was allowed to equilibrate with 0.3M sodium acetate,pH 6, 10 mM $MgCl_2$, 70% ethanol at -20°C for 1 hour to remove spermine. After a 10 minute microfuge the pellet was rinsed in ethanol, resuspended and applied to a low melt agarose gel to remove the remaining primers. A single band at 270bp representing the amplified DNA segment was cut out. Two phenol extractions of the melted gel slice, a chloroform extraction and ethanol precipitation, yielded one microgram of the amplified segment. 0.2 micrograms were sequenced with 50nmoles of either one of the above primers. The template DNA and primer were added together in water to 12µl, denatured at 90°C for 5 minutes, flash-frozen in dry ice/ethanol, thawed on ice, and sequenced using modified T7 DNA polymerase (Sequenase, US Biochemical)(3). Almost the entire sequence of each strand could be read and in our case demonstrated the absence of an additional EcoRI fragment.

References:

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3. Tabor, S. and Richardson, C.C. (1987) Proc. Natl. Acad. Sci. USA 84: 4767-4771.