Sequencing Using Custom Designed Oligonucleotides

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1. Introduction

The dideoxy chain termination DNA sequencing procedure introduced in 1977 (1) has the advantage of being fast, simple to perform, and very accurate. Therefore, it became the method of choice to obtain several hundred bases of sequence information per reaction. The procedure is based on the enzymatic elongation and radioactive labeling of oligonucleotides that are complementary to the beginning of the single-stranded DNA template. Chain extension competes with the infrequent but specific termination by incorporation of a dideoxyribonucleotide. The products of four nucleotide-specific reactions can be separated on a polyacrylamide gel. An autoradiogram of such a gel finally provides the sequence information. Detailed descriptions of the various modifications of this method have been presented in preceding chapters of this volume. The synthetic oligonucleotide primer required for the synthesis of the labeled strands can easily be synthesized in automatic synthesizers or by hand, the latter of which is more laborious. Various so-called "universal sequencing" and "reverse sequencing" primers, complementary to the beginning of the polylinker region in M13 *lac* cloning phages and plasmids, are commercially available. Specifically designed oligonucleotides can also be prepared upon request by several molecular biological companies.

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In principle, there are three different ways of obtaining DNA sequence information by the dideoxynucleotide method. The first strategy involves sequencing of randomly cloned fragments by using only commercially available primers complementary to regions close to the insertion site. This methodology requires a high number of recombinant clones, many time- and money-consuming sequencing reactions, and a computer for putting the data together into a whole. Alternatively, defined subclones can be constructed, characterized, and finally sequenced. This approach is more straightforward, but also takes a lot of work and time. The second procedure makes use of a set of different deletions in the original DNA fragment, originating close to the commercially available primer. These nested deletions extend various lengths along the target DNA. Thus, the longer the deletions are the more new target DNA is brought into sequencing range (*see also* Chapters 8, 9, and 10). Again, this procedure requires additional recombinant DNA work on the original clone and the preparation of many DNA templates. The third approach is based on start of sequencing with primers that are complementary to a known sequence. This can either be the vector or any other sequence adjacent to the DNA to be analyzed. Sequencing proceeds by successive synthesis of new primers at the edges of the newly obtained sequence in such a way that their 3' ends are pointing off into the unknown target DNA. This method is quick and straightforward, but usually requires the availability of a DNA synthesizer.

2. Materials

Synthesis of oligonucleotides may be performed by hand (2–4) or can be done by several molecular biological companies. The latter possibility certainly is easier but also time-consuming and relatively expensive. The most convenient way to obtain specific primers is the use of an automatic DNA synthesizer. If such a machine is available an oligonucleotide (17mer) can be prepared within approximately 2.5 h.

When using an automatic DNA synthesizer chemicals such as solvents, protected deoxyribonucleoside-3'-O-cyanoethyl phosphoramidites, and special reagents are normally provided by the manufacturer (*see* Note 1). Cheaper offers from different companies are usually acceptable, provided the quality of these chemicals is high. Special attention should be paid to contamination with water. Water repre-

sents the main problem in efficient oligonucleotide synthesis, and should therefore be kept away from all reagents and solutions. Since solvents are hazardous, care should be taken to perform the synthesis in a well-ventilated area. Detailed manual methods and the necessary equipment and material have been described in a previous volume of this series (2,3).

For postsynthetic procedures such as deprotection, cleavage of the oligonucleotide from the support, and removal of impurities the following materials are required:

1. 25% ammonia in water (solution should be of highest quality).
2. Sephadex G-25 columns. It is recommended to work with prepacked columns that give a defined elution profile.
3. Sterile water or TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA).

3. Design of Primers

Working with custom primers, new oligonucleotides are designed according to the results of the first sequencing reactions with primers complementary to the beginning of the polylinker region in all M13 *lac* cloning phages and plasmids. Thus, determined sequences serve to plan the next primer and so on. As a rule for primer design the formula: Primer length = 18 + 1 extra nucleotide for each 2% off of 50% G + C has been reported (5). This is because oligonucleotides with too high an A + T-content might not prime satisfactorily with special template DNA strands. However, working with an A + T-rich organism (*Clostridium acetobutylicum*) we routinely used 17mer primers (6) with G + C-contents between 2 and 9 nucleotides and never experienced any problems.

New primer and known sequence should well overlap to ensure that the starting nucleotides of the new part can be read and to have an internal control of each sequence start. As a rule of thumb the 3' end of the primer should be located about 30–40 nucleotides from the end of the already known sequence.

Before synthesizing a primer its sequence should be compared carefully to the whole known sequence of template DNA and vector (preferably by means of a computer program). This will identify regions where undesired hybridizations could take place. Of special importance in this respect are the last ten 3'-terminal bases. More than 80% homology in this region might cause major background problems,

particularly in G + C-rich templates (5). If undesired homology indeed can be found, a new primer should be designed (*see* Note 2).

In general, it is not possible to predict the functionality of a new primer. Even oligonucleotides that are designed according to all rules might fail. In such a case synthesis of a different primer is recommended.

4. Synthesis of Primers

Detailed procedures for manual oligonucleotide synthesis have been described in a previous volume of this series (2,3). Using an automatic DNA synthesizer the instructions of the respective manufacturer should be followed exactly. Therefore, only the principle of a standard method (using phosphoramidites) and the postsynthetic procedures will be described here.

To avoid undesired reactions during synthesis, the hydroxyl group at position 5 of the monomers is protected by a 4,4'-dimethoxytrityl group, the hydroxyl group at the phosphorus atom by a β -cyanoethyl moiety, and primary amines either by benzoyl groups (N^6 in case of deoxyadenosine, N^4 in case of deoxycytidine) or by the isobutyryl group in position N^2 of deoxyguanosine. Thymidine is usually not protected (3). A fully protected dA monomer is shown in Fig. 1. The first nucleotide is coupled via its 3'-OH group and a spacer to a solid support such as silica, glass, or plastic beads. Then its 5'-dimethoxytrityl group is split off and the next monomer is added using the activator tetrazole. After a wash to remove this substance and unreacted nucleotides, unreacted 5'-OH groups are acetylated (capping). Oxidation of the phosphite triester bridge renders the molecule ready for the next round of elongation (3).

After completion of the synthesis the oligonucleotide is separated from the support by a short centrifugation (2000 g, 1 min) to remove residual solvent and incubation in 25% ammonia (15 h at 55°C). Care should be taken to completely submerge the support using an appropriate tube. Residual air bubbles can be removed by short centrifugation (2000 g, 1 min). The support is then taken out of the solution and centrifuged in a tube to obtain the residual liquid (2000 g, 1 min). Both solutions are pooled and represent a crude mixture of a variety of oligonucleotides and some ammonium salts in ammonium hydroxide. The concentration of impurities is higher the longer the sequence of the oligonucleotide is. Removal of ammonia and other impurities

Fig. 1. Protected dA monomer using the phosphoramidite method (3).

is achieved by size exclusion chromatography on Sephadex G-25. The use of prepacked columns is recommended. Equilibration and elution is done with sterile water or TE buffer (*see* Note 3). For sequencing reactions it is usually unnecessary to further purify the primer (which could be done by HPLC or gel electrophoresis). However, if the sequence of the oligonucleotide allows hairpin formation by self-complementation, denaturing conditions might be necessary in the purification procedure.

5. Sequencing Reaction

Automatic DNA synthesizers such as Gene Assembler Plus (Pharmacia LKB GmbH, Freiburg, Germany) have a coupling efficiency of 98% under optimal conditions. Thus, one usually obtains a highly concentrated primer solution after removal from the solid support. Many sequencing protocols recommend the use of 10 ng primer per sequencing reaction. Using a 0.2- μ mol capacity support column in

the above mentioned machine we routinely obtain approx 600 μg oligonucleotide (in 1.5 mL buffer). The concentration can be determined by measuring the absorbance at 260 nm (1 corresponds to 31 $\mu\text{g/mL}$). This solution is diluted 100-fold and 2 μL are used for sequencing in the annealing reaction. We also routinely used 1 μL of the undiluted endproduct and the results were as good as with less primer. Sequencing was performed as described in Chapter 13 of this volume.

A complete cycle of designing and synthesizing a primer, performing the sequencing reaction, and analysis of the DNA sequence can be done in 48 h. Thus, every 2 d approx 400 bases of new sequence information become available. If subclones, or similar clones, and the necessary sequencing equipment are at hand, this number could easily be increased. This might be desirable in large sequencing projects. Advantages of this method are that no additional steps such as subcloning (including recombinant DNA work, transformation, and plasmid characterization), medium preparation for cultivation of clones, and template isolation from a large number of clones are necessary. A possible disadvantage might be the costs for primer synthesis. Using an automatic sequencer, the cost comes to approx \$40–50 per 17mer.

6. Notes

1. Synthesis reagents for an automatic DNA synthesizer were found to be stable for at least 3–4 wk under laboratory conditions.
2. We strongly recommend to check the template sequence carefully after every elongation. Ambiguous regions can thus be resequenced at once which might help to avoid later unnecessary primer synthesis. Direct computer analysis will also enable the identification of matching stretches if sequencing was started from both ends of the template. If only the end of a newly obtained sequence is read and used for new primer design this could finally lead to unnecessary sequencing of large parts of the vector.
3. If there are any doubts concerning the quality of the synthesis, the oligonucleotide can be examined in a sequencing gel (20% polyacrylamide, 8M urea) by comparison with an oligonucleotide of good quality or with a commercially available oligonucleotide marker. Visualization can be performed by UV-shadowing (by simply putting the gel on a fluorescent thin layer chromatography plate and illuminating it with UV light at 254 nm [7]) or by autoradiography after kinasing the oligo-

nucleotide with [γ - ^{32}P]ATP. Attention: Polynucleotide kinase shows unfavorable reaction kinetics with a C at the 5'-end. The back reaction under these conditions is much stronger than the forward reaction.

4. Oligonucleotides synthesized as primers might also serve additional functions. They might be used in primer extension experiments to determine transcription start points or for sequencing a large number of mutants in a defined region within a short period of time. They can also be used as probes to detect specific DNA fragments by Southern hybridization.

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