Taq Cycle Sequencing of Plasmid DNA Purified by Various Procedures

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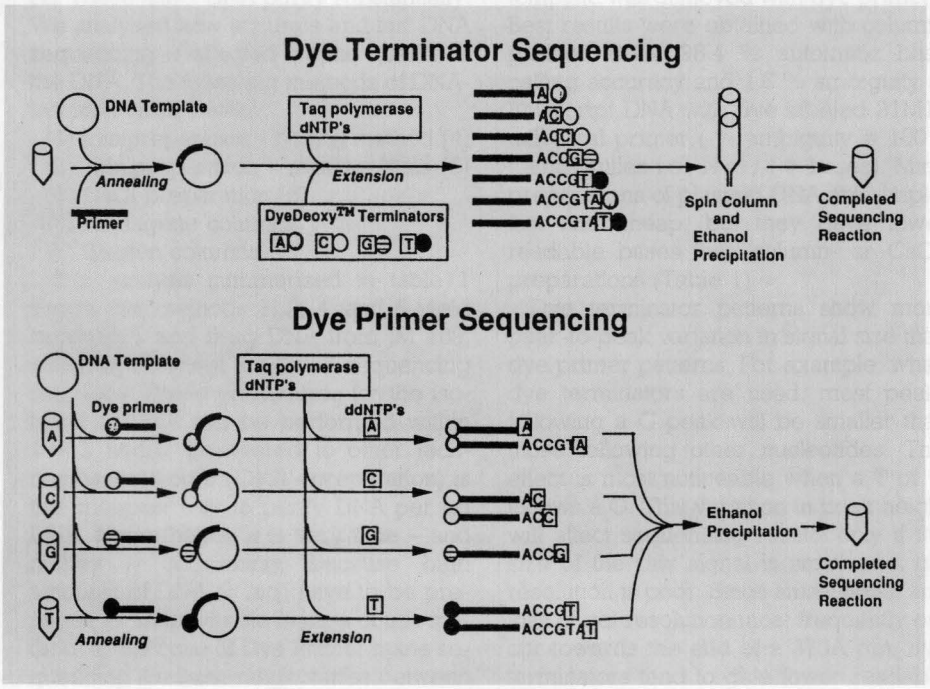
During the last four years, fully automated non-radioactive methods for DNA sequencing have been developed for the dideoxy chain termination procedures. Cycle sequencing involves the linear amplification of extension products from small amounts of DNA template using a thermal cycler and Taq DNA polymerase. This results in strong, consistent signals from both single- and double-stranded templates. Cycle sequencing can be used with dye primers or dye terminators. However, the purification of DNA templates is still the most critical and difficult step for efficient DNA sequencing. Different methods for the preparation of double-stranded DNA were tested with respect to DNA yield, expenditure, base range and base accuracy of the sequence. The use of Stratagene or Qiagen columns gave DNA of high purity which guarantees a range of up to 550 bp to be sequenced and an accuracy of 99 %. These results were obtained in sequencing reactions with dye primers. Application of dye terminators to cycle sequencing resulted in a shorter base range (300-400 bp) but offers the advantage of non-standard primer usage.

Introduction

Dideoxy sequencing of double stranded plasmid DNA has become a routine method due to the introduction of automated DNA sequencers which allow the detection of electrophoretically-separated fluorescent DNA fragments [1,2]. For the 373A DNA system offered by Applied Biosystems two methods can be used in the dideoxy sequencing reactions: dye primer- and dye terminator-sequencing (Fig. 1). Each method influences the resulting data in a manner which reflects the biochemical differences of the labelling reaction [3]: Dye terminators introduce the dye label at the 3' end of the growing chain, while dye primers carry a dye label at their 5' terminus. With dye terminators, variation in peak intensity is greater than seen with dye primers. Sequencing with dye primers assures that only those fragments are detected which are extension products from the labeled primer. This reduces background due to non-specific priming. In contrast, if reactions are labeled with dye terminators, non-specific terminations (false stops) are invisible. All four termination reactions are performed in a single tube, thereby reducing the tube handling and pipetting steps fourfold. Compared to conventional procedures, thermal cycling of the sequencing reactions increases signal intensity and results in less sensitivity to reaction conditions. For double-stranded templates, thermal cycling eliminates the need for alkaline denaturation.

In this note, we report the dependence of Taq cycle sequencing on the quality of the DNA. DNA was purified by: (1) minipreparation - boiling method; (2) minipreparation - alkaline lysis; (3) CsCl preparation; (4) Stratagene columns; (5) Qiagen columns from DIAGEN. Our study shows that the purification of plasmid DNA is the most critical step for efficient DNA sequencing. Column purification of DNA is superior in terms of base range and accuracy compared to conventional methods. Cycle sequencing with primers allows a high base range to be read, while terminator sequencing yields less readable bases but offers the opportunity to use non-standard primers.

Fig. 1: Schematic presentation of dye terminator and dye primer sequencing.



Materials and Methods

(1) DNA Preparation

Plasmid DNA was extracted from bacterial cells according to the method of DelSal et al. [4] (boiling method) and the laboratory manuals of Maniatis et al. [5]

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(alkaline lysis) and Ausubel et al. [6] (CsCl-purification). Furthermore, we used the DNA purification kits from Stratagene and DIAGEN (Strategene- and Qiagen-columns) according to the protocols of the manufacturers.

(2) Sequencing

Sequencing of double-stranded DNA was performed by the dideoxy chain termination method with Taq polymerase using an ABI 373A DNA sequencer (Applied Biosystems, Weiterstadt). Cycle sequencing [7] on a DNA thermal cycler (Perkin Elmer Cetus) was done with dye primers or dye terminators (Fig. 1). For dye primer sequencing, the following conditions were used: Denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 70°C for 1 min, 15 cycles. This was followed by 15 additional cycles of denaturation (95 °C for 30 s) and extension (70 °C for 1 min). For the Taq dye deoxy terminator cycle sequencing the following conditions were applied: Denaturation at 95 °C for 30 s, annealing at 50 °C for 15 s and extension at 60 °C for 4 min, 25 cycles. The primers were synthesized on an Applied Biosystems 381A DNA Synthesizer and were purified with NAP-10 columns (Pharmacia).

Results and Discussion

For the analysis of sequencing products, an automated sequencer (Applied Biosystems Model 373 A) was used. Dye primers or dye terminators were used to Sequence pBluescipt II KS DNA. Various techniques were tested for DNA isolation. These affect DNA purity substantially. We analysed how accurate and fast DNA sequencing is affected by the quality of the DNA. The following methods of DNA-isolation were tested:

(1) minipreparation - boiling method [4]
(2) minipreparation - alkaline lysis [5]
(3) CsCl preparation [6]
(4) Stratagene columns
(5) Qiagen columns

Our analysis summarized in table 1 shows that methods 1, 2, 4, and 5 yield between 1 and 5 µg DNA from JM 109, which is sufficient for 1 – 5 sequencing reactions. These procedures for the isolation of DNA can be performed within 1 – 3 hours. Compared to other techniques, method 3 (CsCl - preparation) is the cheapest way to purify DNA per µg DNA. Nevertheless it is very time – and money – consuming, because high amounts of DNA (1 mg) have to be prepared as an inevitable feature of this method. In the case of Dye Primer cycle sequencing the base range varies between 390 and 550 bp. DNA purified using Qiagen – or Stratagene columns showed the longest base range, 500 – 550 bp, with an accuracy between 98.3 and 99.4 %.

Table 1: DNA preparation for automated sequencing.

	Mini-Prepar.		CsCi-Prepar.	Qiagen-Col.	Strata-gene-Col
	Boiling	Alkaline			
Material	pBluescript IIKS				
Protocols[a]	(1)	(2)	(3)	(4)	(5)
Culture	1.5ml	1.5ml	500ml	1.5ml	1.5ml
Yield	1-5µg	1-5µg	1-2µg	1-5µg	1-5µg
Time	1-2h	2h	2days	1-2h	2-3h
Price	0,20DM	0,20DM	0,30DM	4,40DM	7,70DM
Sequencing method	ABI Taq Dye Primer Cycle Sequencing Kit				
Base Range	390-440	340-400	400-450	450-550	450-500
Accuracy[b]	98.8	98.3	99.4	98.4	99.1
Sequencing method	ABI Taq Dye Deoxy Terminator Cycle Sequencing Kit				
Base Range	230-280	200-250	250-300	300-400	320-350
Accuracy[b]	98.5	98.2	98.7	99.1	99.3

[a] (1) to (5) refer to: DeSal, G. et al. (1); Ausubel, F.M. et al. (2); Maniatis, T. et al. (3); User Manuals (4) & (5)
[b] Calculations: %Accuracy = 100× {(# Bases) - (# Errors)} / (# Bases)

Since non-standard primers can conveniently be used with dye deoxy terminators in automatic DNA sequencing, we also tested the influence of the purity of the DNA on the dye deoxy terminator reaction. Here, the base range was found to vary between 200 and 400 bp and the accuracy between 98.2 and 99.3 %.

Generally, the highest number of accurately determined bases per µg DNA template was achieved with dye primers. Best results were obtained with column-purified DNA: 98.4 % automatic base calling accuracy and 1.5 % ambiguity of Bluescript DNA with dye labeled 21M13 universal primer (% ambiguity = 100 x (ambiguities i.e., N's) / (# bases). Mini-preparations of plasmid DNA are simple, fast and cheap, but they gave fewer readable bases than column- or CsCl-preparations (Table 1).

Dye terminator patterns show more peak-to-peak variation in signal size than dye primer patterns. For example, when dye terminators are used, most peaks following a G peak will be smaller than those following other nucleotides. This effect is most noticeable when a T or C follows a G. This variation in peak height will affect sequencing results only if the size of the raw signal is small or if gel resolution is poor. Since small signal and loss of gel resolution most frequently occur towards the end of a 373A run, dye terminators tend to give fewer readable bases than dye primers. Generally, dye primer reactions yield 500 bases with high accuracy while for dye deoxy terminator reactions good results beyond base 400 are less common (Table 1).

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