

Rapid one-step automated sequencing reactions for 16 DNA samples using Taq polymerase and fluorescent primers

T.Ciora, P.Denèfle and J.-F.Mayaux
Biotechnology department, Rhône-Poulenc Rorer, BP14, 94403 Vitry sur Seine, France

Submitted October 12, 1990

The optimization of fully automated sequencing reactions and analysis is a prerequisite for large scale sequencing programs. We describe here a very simple and reliable protocol which also solves all the evaporation problems encountered for sequencing reactions in microtiter plate. We used the property of the thermostable DNA polymerase from *Thermophillus aquaticus*, Taq, which is optimally active for DNA sequencing at high temperatures (60 to 80°C) in order to perform in a single heating step both primer-annealing and polymerization reactions. Hereafter is described a protocol using the Biomek™1000 workstation equipped with the HCB-1000 thermocontroler (Beckman) which allows to perform simultaneous sequencing reactions with fluorescent primers on 16 different single-stranded DNAs in a microtiter plate. This protocol optimizes the full-speed utilization of the 370A automated DNA sequencer (Applied Biosystems).

One of the most critical steps in successful DNA sequencing with fluorescent primers is the quality of DNA preparation. We strongly advise to wash out carefully any trace of PEG when using the protocol described by Messing *et al.* (1983). An amount of 3 µg of template generally gave excellent results even with high GC-percent DNAs (up to 66% tested). For instance, we can routinely obtain in that case accurate 370 bp sequences with more than 99% reliability.

Taq DNA polymerase was purchased from Promega-Biotech. Deoxy and dideoxy-nucleotides were from Pharmacia-LKB, except for deaza-guanosine triphosphate (d-c7GTP), which was from Boehringer-Mannheim. M13 (−21) fluorescent-dye primers were from Applied Biosystems.

Reactions are carried out in a V-bottom 96-wells vinyl microtiter plate (Dynatec, Polyabo, France) at + 4°C. Add in order:

reactions:	'A'	'C'	'G'	'T'
primer 'A'*	3 µl	–	–	–
primer 'C'*	–	3 µl	–	–
primer 'G'*	–	–	5 µl	–
primer 'T'*	–	–	–	5 µl
template (0.15 µg/µl)	3.2 µl	3.2 µl	6.4 µl	6.4 µl
'A' mix**	2 µl	–	–	–
'C' mix**	–	2 µl	–	–
'G' mix **	–	–	3 µl	–
'T' mix**	–	–	–	3 µl
Taq dilution***	3 µl	3 µl	6 µl	6 µl

*:primers working solutions are made by mixing primer's stock solution (0.4 pmol/µl) with 5×Taq buffer (50 mM Tris-HCl, pH8.5 (RT), 50 mM MgC12, 250 mM MaCl) in a 1/1.8 ratio.
**:nucleotides mixes are made according to the following table:

	dATP	dCTP	d-c7GTP	dTTP	ddATP	ddCTP	ddGTP	ddTTP
'A' mix	75 µM	300 µM	450 µM	300 µM	1.2mM	–	–	–
'C' mix	300 µM	75 µM	450 µM	300 µM	–	0.6mM	–	–
'G' mix	300 µM	300 µM	113 µM	300 µM	–	–	0.1mM	–
'T' mix	300 µM	300 µM	450 µM	75 µM	–	–	–	1 mM

***:Taq (2.5 u/µl), 90 µl, 10×Taq buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3 (RT), 15 mM MgC12, 0.1% (w/v) gelatin), 45 µl, H20, 315 µl.

The plate is immediately heated up to + 70°C for 2 min, 3 µl H20 are then rapidly added to all the wells. Following a 90 sec incubation time at + 70°C, the plate is cooled down to + 4°C. Neither plate covering nor addition of mineral oil are required. Even better results can be obtained with an additional PolIK'chase'step. Immediately following the extension reaction, the contents of each four individual reactions are automatically collected and pooled into Eppendorf tubes containing 300 µl 95% ethanol, 8 µl sodium acetate 3 M pH 5.5.

At this stage, the samples are removed by the workstation and can be kept for several days at −20°C before being treated for electrophoresis. The final DNA pellets are washed with 70% ethanol, dried and resuspended in sample application buffer.

REFERENCES

1. Messing,J. (1983) *Methods in Enzymol.* **101**, Acad. Press Ed. (NY) 20−77.
2. Applied Biosystems (1989) User Bulletin for DNA Sequencer Model 370 No. **10**.
3. Requests for the computer program are to be sent to Dr Denèfle.