

Automated Sanger dideoxy sequencing reaction protocol

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The protocol for Sanger dideoxy chain termination reactions in DNA sequencing is tedious and prone to errors due to the repetitive character of the pipetting steps. An industrial robot, with the addition of a few simple parts, was programmed to automate the dideoxy sequencing reactions. The system is set up in a short time for routine operation and it is faster and more reliable than a human operator. It is flexible and allows variations and optimization of the standard procedure. Disposable microtiter plates at a controlled temperature are used. In one reaction cycle (about 50 min) up to 48 templates are processed. Up to 450 bases were resolved in automated DNA sequencing on samples prepared by the robot. The protocol is applicable to fluorescent as well as to radioactive labeling.

DNA sequencing; Dideoxy reaction; Automated sequencing; Fluorescent label; Radioactive label

1. INTRODUCTION

DNA sequencing using DNA polymerases, M13 vectors, primers and chain terminators has been applied to many sequencing projects. Spurred on by the expectation of projects involving sequencing of larger DNA inserts and a growing number of applications, attention has recently been turned to the automation of key steps in the technique [1,2]. The reasons being the increased demand for reliability and reproducibly high quality of samples, as well as the need to master the growing number of analyses within a reasonable time. Several types of automated systems for on-line DNA sequencing with fluorescent labeled primers [3–5] or dideoxy chain terminators [6], in combination with separation on polyacrylamide gels and direct storage of the sequence information in the computer, have been introduced over the past few years and their performance and reliability in routine sequencing projects is being tested. Purification of templates, the most critical and dif-

ficult step in the method, has been simplified recently [7,12] with the aim to allow its automation.

Automated systems able to perform Sanger dideoxy sequencing reactions have been developed in the past [8,9], using a microcomputer to control a robot arm and precise dispensing of liquids. This step, consisting of pipetting operations and thermal treatments, would appear to be the one most directly suitable for automation considering the state of available technology. In particular, the commercially available, industrially produced robots for computer controlled liquid sample processing offer the possibility to automate this step relatively fast and save considerable time needed otherwise for their development. Operational experience gained with such industrial robot systems may be useful in the construction of large scale automated systems with dedicated applications.

We describe here an automated system for the Sanger dideoxy sequencing reactions, performed by an industrial robot after the addition of a few simple parts. The method is applicable to fluorescent as well as to radioactive labeling. Prepared samples may be loaded on gels with another robot system.

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2. MATERIALS AND METHODS

2.1. Biochemicals

XL1-blue bacterial strain was from Stratagen Cloning Systems. Cloned, unmodified T₇ DNA polymerase was from Pharmacia PL Biochemicals, Milwaukee, WI. T₇ DNA polymerase with a chemically inactivated 3'-5'-exonuclease activity (Sequenase) was from USB Corporation, Cleveland, OH.

2.2. DNA preparations

Purification of M13 template DNA was performed as described previously [7]. Cultures of *E. coli* XL1-blue, infected with M13mp18, were grown in YT medium for 7–8 h at 37°C. Cells were pelleted by centrifugation and phages precipitated from 1.5 ml culture supernatants by addition of 20 µl of glacial acid and filtration through Whatmann GF-C glass fiber filters, followed by protein removal with 1 ml of 4 M NaClO₄, washing with 1 ml of 70% ethanol and elution of DNA with 20 µl of 0.1 TE. We used a cloning vector M13mp18 as well as various inserts cloned in M13mp18 and M13mp19 as template DNA.

2.3. Sequencing reactions

Conditions used in the automated sequencing reactions with fluorescent primers and the T₇ DNA polymerase are listed below. The ratio of ddNTP/dNTP is reduced, as compared to the radioactive labeling. This modification is necessary in end labeling procedures since the amount of incorporated label is proportional to the molarity rather than the mass of the different products, so that a higher amount of the larger size products is needed to get a signal sufficiently strong for their detection. The loss of water due to evaporation was measured (not shown) and is compensated in the pipetted amounts to assure a constant ionic strength of the reaction mixtures throughout the reaction cycle.

2.4. Primer annealing

24 µl of 5 × annealing buffer (200 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, 250 mM NaCl) are mixed with 5 µl of fluorescent labeled universal 17-mer primer (3.1 pmol/1 µl) [5]. Then 2.4 µl of the resulting primer/annealing mix are added to 8 µl of template DNA and diluted with 4 µl distilled water, incubated at 65°C for 3 min and cooled down to 25°C for 20 min.

2.5. Chain elongation

40 µl of 0.1 M DTT are mixed with 12 µl of 5 µM dNTP solution. Then 3 µl of this DTT/dNTP mix are distributed to 10 µl of primed templates, 4 units of diluted T₇ DNA polymerase are added and incubated for 10 min at 25°C.

2.6. Chain termination

During the elongation reaction 2.5 µl of the four respective termination mixtures (80 µM of each dNTP, 11.5 µM of respective ddNTP, 50 mM NaCl, 40 mM Tris-HCl, pH 7.5) are delivered to the reaction plate and the temperature is raised to 37°C. Then 3.2 µl aliquots of each elongated template are added, followed by incubation for 5 min at 37°C. Reactions are stopped by adding 4 µl deionized formamide containing 5 mg/ml Dextran blue (*M_r* 2000000, Pharmacia). Dextran blue improves visualization of the samples during loading on the gel and does not interfere with the fluorescence. Samples are heated

to 70°C for 3 min, cooled down to 0°C and kept at –20°C until loading.

2.7. DNA sequence analysis

Fluorescent reaction products are analyzed by polyacrylamide gel electrophoresis and on-line detection of laser induced fluorescence in an automated system described previously [4,5].

2.8. Siliconizing procedure

Tips and microtiter plates are siliconized with silicone solution in isopropanol from Serva, Heidelberg. Tips are washed



Schema Of The Automated Workstation

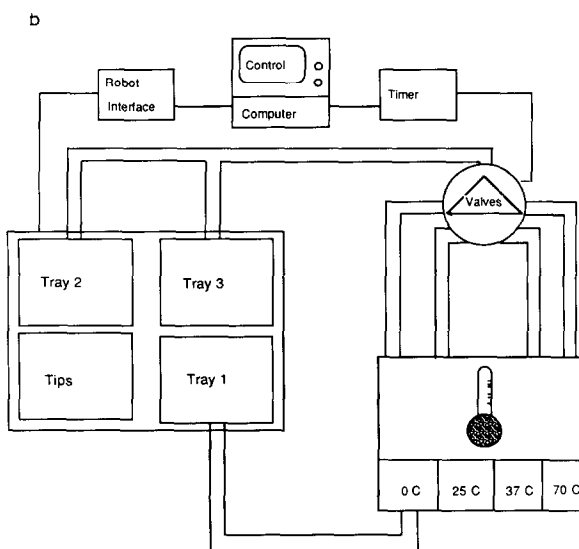


Fig.1. (a) Photograph of the automated workstation with control computer (right) and the robot manipulator (left). (b) Scheme of the workstation system. In the center, the robot manipulator with positions for tips and microtiter plates. The stock plate (in tray position 1) is maintained at 0°C. Temperature of the reaction plate (in tray position 2) is varied by a programmable thermostat and a timer synchronized with the control computer.

four times in the solution by the Biomek system. Microtiter plates are treated in a tray by hand. After removal of solution, tips and microtiter plates are dried in a vacuum oven at 40°C for 4 h.

2.9. Robot system

The robot used for liquid processing is the Biomek 1000 from Beckman Instruments, controlled by an IBM XT286 PC. This system offers the possibility for pipetting with a single or multiple pipetting module and is designed to work with microtiter plates. It consists of a workstation, an interface unit and a microcomputer. Scheme and a view of the automated system are shown in fig.1. Testing the reproducibility of the pipette modules available, we found the single pipette module to work with the precision required for handling the small volumes (<2 µl), standard in the dideoxy sequencing reactions. The system as delivered does not have the facilities for control of the temperature. We have constructed equipment allowing one to set the temperature of a disposable microtiter plate to a pre-selected value. Microtiter plates (Greiner GmbH) are placed on a machined aluminium block, with wells of the block fitting

closely those of the microtiter plate to ensure good thermal transfer. The temperature of the microtiter plate is controlled either by water circulation or electrically with Peltier elements. A system of valves is regulated by a commercially available timer. Summary of the robot steps in the automated procedure is given in table 1.

2.10. Robot configuration

The Biomek system's basic settings are: tray position T, tips (96); tray position 1, 96 well microtiter plate (stock plate); tray position 2, not used, optionally a second reaction plate for additional 24 clones; tray position 3, 96 well microtiter plate (reaction plate), up to 24 clones.

The robot is used with the following pipetting tools: tool C, P 20 single channel pipette; tool D, P 200 single channel pipette; tools A and B are not used.

Common system parameters are declared as general parameters for all pipetting steps: tip lowered to the bottom level of the well; dispensation rate at maximum speed; no prewetting of tips; blowout with the appropriate tool depending on volume.

Table 1
Dideoxy sequencing protocol with the summary of robot steps

Step	Tool	Volume (µl)	Source	Destination
Annealing				
Disp/Mix	P20	2.4	TC,3(A1)	3(A4-H4)
Temp. adj. 65°C				
Pause 3 min				
Temp. adj. 25°C				
Pause 19 min				
Elongation				
Disp/Mix	P20	12	1(A4)	1(A3)
Dispense	P20	2	TC,1(A2)	3(A4-H4)
Dispense	P20	3	TC,1(A3)	3(A4-H4)
Pause 5 min				
Temp. adj. 37°C				
Termination				
Dispense	P20	2.5	1(A5)	3(A5-H5)
Tip change				
Dispense	P20	2.5	1(A6)	3(A6-H6)
Tip change				
Dispense	P20	2.5	1(A7)	3(A7-H7)
Tip change				
Dispense	P20	2.5	1(A8)	3(A8-H8)
Tip change				
Dispense	P20	3.2	TC,3(A4-H4)	3(A4-H8)
Tip change				
Pause 3 min				
Temp. adj. 0°C				
Reaction stop				
Dispense	P20	4	TC,1(A12)	3(A5-H8)

Source and destination of dispensed liquids in the microtiter plates are specified by: rows (A-H), columns (1-12), 1-stock plate, 3-reaction plate; TC, tip change after each pipetting step

For the pre-mixing steps of different solutions the following additions were made: no aspiration volume access; no blowout; two mixing cycles at the destination well.

All pipetting steps are carried out only by a 20 μ l and a 200 μ l single tip tool to minimize consumption of the tips per reaction cycle and to obtain an optimal pipetting performance.

2.11. Stock and reaction plates

One microtiter plate, the stock plate, is used to store all biochemicals at 0°C. Another microtiter plate is used as the reaction plate, with a controlled temperature in the range between 0°C and 70°C. The distributions of stock solutions and of reaction mixtures are:

Stock plate (tray position 1):

Well A 01	5 \times annealing buffer	40 μ l
Well A 02	diluted T ₇ DNA polymerase (32 units)	30 μ l
Well A 03	dNTP mix (5 μ M)	40 μ l
Well A 04	DTT (0.1 M)	40 μ l
Well A 05	ddA termination mix	40 μ l
Well A 06	ddC termination mix	40 μ l
Well A 07	ddG termination mix	40 μ l
Well A 08	ddT termination mix	40 μ l
Well A 12	stop-mix	40 μ l

Reaction plate (tray position 3):

Content before run:

Well A 01	universal primer for 8 clones (3.1 pmol/ μ l)	5 μ l
Column 03	clones to be sequenced	12 μ l

Content after run:

Column 05	A reaction products	8 μ l
Column 06	C reaction products	8 μ l
Column 07	G reaction products	8 μ l
Column 08	T reaction products	8 μ l

3. RESULTS

The quality and reproducibility of the reactions were examined by the automated DNA sequencing system [4,5], comparing the 8 clones prepared by the automated process on one reaction plate. Partial raw data for two clones of M13mp18 DNA, prepared from the same template, is shown in fig.2. The two sequences are identical and of similar signal strength ratio. Routine resolution is 300–400 bases, but up to 450 bases could be resolved on samples prepared by the robot, on 20 cm separation length 7% gels. Duration of one complete cycle (up to 48 clones) takes about 50 min. Running the robot for eight cycles, up to 384 clones (1536 reactions) can be performed per day. In the procedure described here 94 dispensing tips

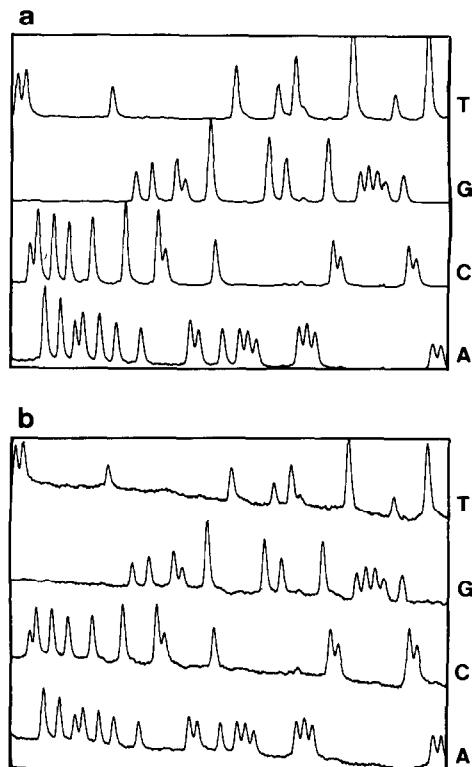


Fig.2. Reproducibility in automated fluorescent sequencing with samples prepared simultaneously on the robot: partial raw data output from a 7% polyacrylamide gel of 20 cm separating length, corresponding to bases 108 to 159 after the priming site of bacteriophage M13mp18. (Left) Data obtained from sequencing reactions of the first clone on the reaction plate. (Right) Data obtained from the last clone of the same plate.

are used for 8 clones. It is possible to re-use the tips several times, after their washing by the robot.

4. DISCUSSION

The use of standard disposable microtiter plates, with a controlled temperature for the sequencing reactions, simplifies the procedure. The automated system transfers reproducibly microliter volumes, the good precision is made possible by high planarity of the microtiter plate positioned on the thermostated support. Siliconizing the microtiter plates and dispensing tips was found useful for optimal pipetting performance, because it centers the small liquid drop in the well and reduces absorbance of the DNA to the plastic material. The stock plate is maintained at 0°C and stock solu-

tions may be used in several cycles of operation. Any contamination is avoided by changing dispensing tips whenever using a new solution.

Evaporation of solutions on the reaction plate cannot be avoided at higher temperatures, but is compensated for by adding the appropriate volume of water to the purified templates, as tested. It was observed that the sequencing method used here is little sensitive to changes in salt concentrations caused by evaporation as they may occur, in reproducible way, during the reactions. The device is faster and more reliable than a human operator. It is flexible and allows variations and optimization of the standard procedure.

Depending on how many samples are to be prepared simultaneously, the robot can be used also in a different way. With a larger number of samples (up to 96) one may decide to profit from the robot's pipetting power and use it only in liquid dispensing steps, renouncing fully automated operation, removing the microtiter plate from the robot and performing the thermal treatments manually. This approach was found useful in the 'shot gun' strategy by Bankier and Barrel [11].

In the fully automated approach described here no human interference is needed. Supply of a rack with new dispensing tips is required when more than eight clones are prepared simultaneously. Consumption of tips is only slightly higher than in the manual technique. Normally 8–24 clones per reaction cycle are processed, but with addition of a second reaction plate and scaling up the amounts of chemicals it is possible to increase the output to 48 clones. In 8 cycles per day 384 clones would be prepared.

The system is capable of development for automation of additional steps in the DNA sequencing technique. The robot is used for phage inoculation and, with modifications, for the step most difficult to automate, purification of templates ([7,12] and Zimmermann et al., in preparation). Combining these robot devices for

DNA purification, sequencing reactions and sample application with the automated DNA sequencing system ([4,5,12,13] and Zimmermann et al., in preparation), the complete cycle for obtaining sequence data will be automated and optimized for reproducible results.

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REFERENCES

- [1] Martin, W. and Davies, R. (1986) *Biotechnology* 4, 890–895.
- [2] Knobeloch, D., Hildebrand, C., Moyzis, R., Longmire, J., Sirotkin, K. and Beugelsdijk, T. (1987) *Biotechnology* 5, 1284–1287.
- [3] Smith, L.M., Sanders, J.Z., Kaiser, R.J., Hughes, P., Dodd, C., Connell, C.R., Heiner, C., Kent, S.B.H. and Hood, L.E. (1986) *Nature* 321, 674–679.
- [4] Ansoerge, W., Sproat, B., Stegemann, J. and Schwager, C. (1986) *J. Biochem. Biophys. Methods* 13, 315–323.
- [5] Ansoerge, W., Sproat, B., Stegemann, J., Schwager, C. and Zenke, M. (1987) *Nucleic Acids Res.* 15, 4593–4602.
- [6] Prober, J.M., Trainor, G.L., Dam, R.J., Hobbs, F.W., Robertson, C.W., Zagursky, R.J., Cocuzza, A.J., Jensen, M.A. and Baumeister, K. (1987) *Science* 238, 336–341.
- [7] Kristensen, T., Voss, H. and Ansoerge, W. (1987) *Nucleic Acids Res.* 15, 5507–5516.
- [8] Martin, W., Warmington, J., Galinski, B., Gallagher, M., Dacies, R., Beck, M. and Oliver, S. (1985) *Biotechnology* 8, 911–915.
- [9] Martin, W., Galinski, B.R. and Beck, M. (1987) *J. Phys. E Sci. Instrum.* 20, 22–26.
- [10] Kristensen, T., Voss, H., Schwager, C., Stegemann, J., Sproat, B. and Ansoerge, W. (1988) *Nucleic Acids Res.* 16, in press.
- [11] Bankier, A. and Barrel, B. (1988) *Methods Enzymol.* 155, in press.
- [12] Ansoerge, W., Kristensen, T., Stegemann, J., Schwager, C. and Voss, H. (1987) *EMBL pat. appl., FRG*, no. P 37 24 442.
- [13] Ansoerge, W., Flößer, H., Stegemann, J., Stettner, C., Schwager, C., Voss, H., Winkler, S. and Wittmann, H. (1987) *EMBL pat. appl., FRG*, no. P 38 05 808.1.