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**A simple and rapid preparation of M13 sequencing templates for manual and automated dideoxy sequencing**

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Tom Kristensen<sup>1</sup>, Hartmut Voss and Wilhelm Ansorge

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European Molecular Biology Laboratory, Heidelberg, FRG and <sup>1</sup>Research Institute for Internal Medicine, University of Oslo, Norway

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**ABSTRACT.**

A simple and rapid procedure for the preparation of M13 single stranded DNA sequencing templates which does not involve phenol extractions and alcohol precipitations is described. Bacteriophages are precipitated from media supernatants with acetic acid and recovered on glass fiber filters. Subsequent dissociation of the phages and removal of contaminants is performed while the DNA is bound to the glass. Finally, the purified DNA is eluted in a small volume of low-salt buffer. The yield is higher than that obtained by standard methods. The simplified procedure takes less than 30 minutes and does not demand special skills or equipment; the sequence resolution is as good as that obtained by standard procedures both with the Klenow fragment and T7 DNA polymerase, with radioactive labelling as well as in automated sequencing with a fluorescent label.

**INTRODUCTION.**

The enzymatic chain termination sequencing method developed by Sanger et al. [1] has become, together with the chemical sequencing method of Maxam and Gilbert [2] a key tool in molecular biology, especially in combination with M13 phage vectors as a source of single stranded DNA sequencing templates [3]. As more demanding sequencing projects are being contemplated [4], and with the development of fast automated DNA sequencing systems [5-7], the method presently used for preparation of DNA templates may prove to be one of the limiting factors for the acquisition of new sequence information. The standard methods are reliable but slow, and not easily amendable for automation, involving purification of the phage by precipitation with polyethylene glycol, followed by deproteinization by phenol and chloroform extractions and concentration by ethanol precipitation of the DNA.

In the present work we describe a fast and reliable procedure for M13 single stranded DNA preparation. The method involves few manipulations and provides a suitable starting point for the development of automated sample preparation systems for dideoxy sequencing.

### MATERIALS AND METHODS.

[ $\alpha$ - $^{35}$ S]thio-dATP with a specific activity higher than 1000 Ci/mmol was obtained from Amersham. DNA polymerase I large fragment (Klenow fragment) was from BRL, deoxy- and dideoxynucleotides from Boehringer, and Sequenase sequencing kit from USB Corporation, Cleveland, Ohio, USA.

The experiments described were performed with a M13 mp18 recombinant phage containing a 800 bases long protein kinase cDNA insert [8], obtained by ligating the insert from a pUC 8 vector into double stranded replicative form M13 mp18 and transfection of competent JM103 cells. Clear plaques were picked and grown in 2 ml L-broth for 6 hrs. Single stranded DNA was prepared from phage suspensions as obtained after removal of bacteria by centrifugation.

Standard preparations of single stranded DNA were performed by precipitation with PEG, extraction with phenol and chloroform, and alcohol precipitation as described in [9], except that we extracted twice with phenol. The final product was dissolved in 10  $\mu$ l TE pH 7.5 (10 mM Tris-HCl pH 7.5, 1 mM EDTA). Routinely, a recovery of about 40 % of the phage DNA in the starting material was obtained.

Sequencing reactions with radioactive dATP and the Klenow fragment were performed as recommended in the BRL sequencing manual [9], while the radioactive reactions with T7 DNA polymerase were done according to the Sequenase manual [10]. Nonradioactive sequencing reactions with a fluorescent primer and the Klenow fragment was as described previously [7].

For the analysis of radioactive reaction products, 0.2 mm thick and 55 cm long polyacrylamide gels were run at 50 °C in a MacroPhor electrophoresis unit (LKB, Bromma, Sweden). The gels were dried prior to radioautography. Fluorescent reaction

products were analysed in the automated system described previously [6, 7].

## RESULTS AND DISCUSSION.

### Development of a simplified procedure.

A detailed description of the five steps of the simplified DNA purification method we have developed is given below:

Preparation of phage suspensions. Plaques were picked from agar plates, and phages grown as described in the Materials and Methods section. Bacteria were removed by centrifugation.

Precipitation with acetic acid. 10  $\mu$ l of glacial acetic acid was added to 1 ml of phage suspension in a 1.5 ml centrifuge tube, and the tube capped and inverted. After 2 min at room temperature, the suspension was added to a 7 mm diameter glass fiber filter (GF/C from Whatman International Ltd, Maidstone, England, the filter was cut from larger sheets with a cork bore) placed on a sintered glass filtration unit with suction from a water pump. The sample was added to the filter in 0.1 ml aliquotes which were sucked through the filter before the next portion was added. When several samples were processed at the same time, we finished addition of each sample to its filter before proceeding with the next one. Filters with samples were left on the filtration unit until all samples had been processed.

Dissociation of phages. The filters were washed with a total of 1 ml 4 M NaClO<sub>4</sub>, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, by the addition of 0.1 ml aliquotes which were dropped on the filter and sucked through.

Washing. The filters were washed in the same manner with 1 ml of 70 % (v/v) ethanol, and then transferred from the filtration unit to a piece of Parafilm and air dried for 5 min. Excessive drying times gave an increased frequency of DNA strand breaks with the formation of linearized DNA. Glass-promoted DNA strand breaks were also noticed by Marko et al. [13].

Elution. DNA was eluted from the filters as described by Chen and Thomas [11]: The filters were placed in 0.5 ml centrifugation tubes, and 10  $\mu$ l of 0.1 x TE pH 7.5 was added. After 5 min at room temperature, we recovered the eluate by piercing a small

Table 1. Infectivity of phages after precipitation with acetic acid or PEG. Phages were precipitated with either 1 % acetic acid for 5 min or with PEG/NaCl for 30 min at room temperature and pelleted at 10 000 x g for 5 min. After resuspension 2 x 100  $\mu$ l of a 10<sup>9</sup> dilution was plated with JM103 cells, and the number of plaques counted after incubation at 37 °C overnight. The number of plaques obtained from an identical dilution of phage suspension was 135.

Precipitant	Number of plaques
Acetic acid	93
PEG/NaCl	138

hole in the bottom of the tube, nesting the tube inside a larger, 1.5 ml centrifuge tube, and centrifugation for 10-30 seconds in an Eppendorf centrifuge.

Precipitation of M13 phages with acetic acid.

M13 phages were easily precipitated from culture supernatants by the addition of acetic acid, and acetic acid precipitates, unlike PEG precipitates, could be recovered by filtration onto glass fiber filters instead of by centrifugation. As shown in Fig. 1A, final concentrations of acetic acid between 1 % and 10 % (v/v) gave essentially equal yields of DNA in the final preparation. One important advantage of the precipitation with acetic acid rather than with PEG is its speed as shown in Fig. 1B. A high yield of DNA could be obtained by immediate filtration of the suspension, and although an increase in the final recovery of DNA is obtained by prolonging the precipitation time to 30 min this is not required in most cases.

We have not further investigated the mechanism of acetic acid precipitation of M13 phages from culture supernatants, but assume that charge neutralization of the phage surface facilitates aggregation of phage particles. The data given in Table 1 shows that infectious phage could be recovered from acetic acid precipitates with a yield of about 75 %.

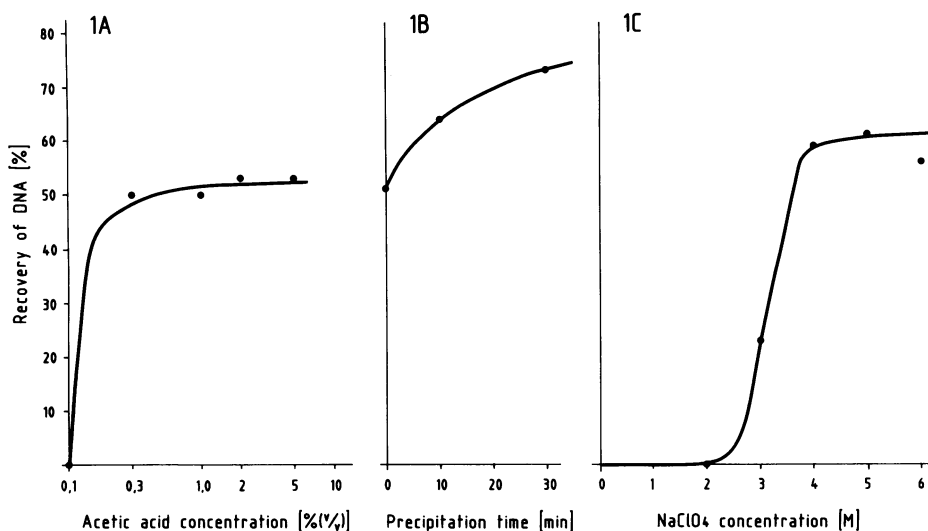


Fig. 1. Purification of M13 DNA. DNA was prepared as described and relative DNA amounts determined by agarose gel electrophoresis and scanning of photographic negatives of the gels in a LKB Ultrosan XL Laser Densitometer with 2400 Gelscan XL software (LKB, Bromma, Sweden). The values are given as per cent of the maximal obtainable amount of DNA, determined by electrophoresis of SDS-lysed phage suspension and scanning. In Fig. 1A, the acetic acid concentration was varied between 1 and 10 % (v/v). In Fig. 1B, the time between acetic acid addition and filtration was varied as indicated, and in Fig 1C the concentration of sodium perchlorate used for phage dissociation was varied.

#### Dissociation of phages and binding of DNA to glass fiber filters with sodium perchlorate.

It has been known for several years that DNA binds to glass in the presence of high concentrations of NaClO<sub>4</sub> [11-13] or NaI [14]. The perchlorate and the iodide ions are also very chaotropic ions which should be able to provoke dissociation of phage proteins from phage DNA. When we treated filter-bound acetic acid precipitates of M13 phage with different concentrations of NaClO<sub>4</sub>, we found a high recovery of phage DNA when the perchlorate concentration was 4 M or higher (Fig. 1C). Somewhat surprising, we obtained a very low recovery of DNA when 6 M NaI was used for phage dissociation (data not shown). We also tried another chaotropic agent, guanidinium HCl, which gave a somewhat lower yield than sodium perchlorate.

### Washing the filter-bound DNA.

Chen and Thomas who used glass fiber filters for the recovery of DNA from agarose gels [11], found the highest recovery when the filter-bound DNA was washed after the perchlorate treatment first with isopropanol, then with 96 % ethanol. We have tested various washing solutions in our system, and find aqueous ethanol solutions give consistently higher recoveries than isopropanol-ethanol treatment, apparently because the DNA is more easily eluted from the filter afterwards (data not shown).

### Elution of DNA from glass fiber filters.

Elution of DNA from glass is usually obtained with low-salt aqueous solutions [11-14]. We have tried several eluants and routinely use 0.1 TE as recommended by Chen and Thomas. We obtained higher recoveries with 0.1 % SDS, but use of this eluant poses obvious problems for the subsequent enzyme reactions. Other detergents which might give less problems did not improve the yield. For most purposes it may be more important to have a highly concentrated eluate rather than a very high yield. Routinely we eluted the DNA with 10  $\mu$ l 0.1 x TE and recovered 40-60 % of the DNA present in the original phage suspension. Since the filters did not dry completely in the previous step, the recovered volume was usually somewhat higher than the volume added to the filters. If higher yields are required, a second elution with 10  $\mu$ l 0.1 x TE will recover most of the remaining DNA from the filter.

### Evaluation of the DNA preparations by DNA sequencing.

Our main purpose for developing a new procedure for preparing DNA was to find a faster and easier method than the present standard one, with the same or better performance of the DNA in the subsequent sequencing reactions. Therefore to test the efficacy of the method DNAs from several of the variant treatments described above, as well as DNA prepared by the standard method, were used as templates for sequencing by both the standard radioactive technique and by using a fluorescently labelled primer; this involves on-line detection of laser-induced fluorescence in the reaction products [6, 7]. This comparison was made using the Klenow fragment of *E. coli* DNA polymerase I or T7 DNA polymerase ("Sequenase") as the polymerizing enzyme. The

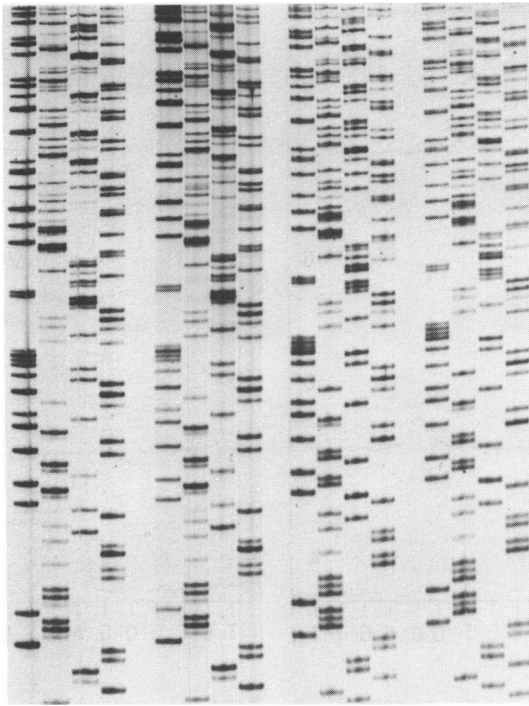


Fig. 2. Radioactive sequencing. Polyacrylamide gels with fractionated radioactive sequencing reaction products were dried onto glass plates [15], and exposed to Kodak X-AR films overnight. Lanes 1-8: Reactions performed with the Klenow fragment; lanes 9-16: Reactions performed with T7 DNA polymerase. Lanes 1-4 and 9-12: DNA prepared by the glass fiber method; lanes 5-8 and 13-16: DNA prepared by the standard method. The part of the gel shown corresponds to bases 119 to 287 from the end of the primer. Order of loading, from left to right: A C G T.

choice of 1 % acetic acid for the precipitation and 4 M NaClO<sub>4</sub> for the phage dissociation was made on the basis that higher concentrations gave somewhat inferior results with the radioactive sequencing procedure. Interestingly, in the fluorescent method the use of 5 % acetic acid and 6 M NaClO<sub>4</sub> gave as good results as 1 % acetic acid and 6 M NaClO<sub>4</sub> treatment, indicating that this method is less sensitive to DNA quality than the radioactive one (data not shown). One probable explanation for this is that only extension products primed by the labelled

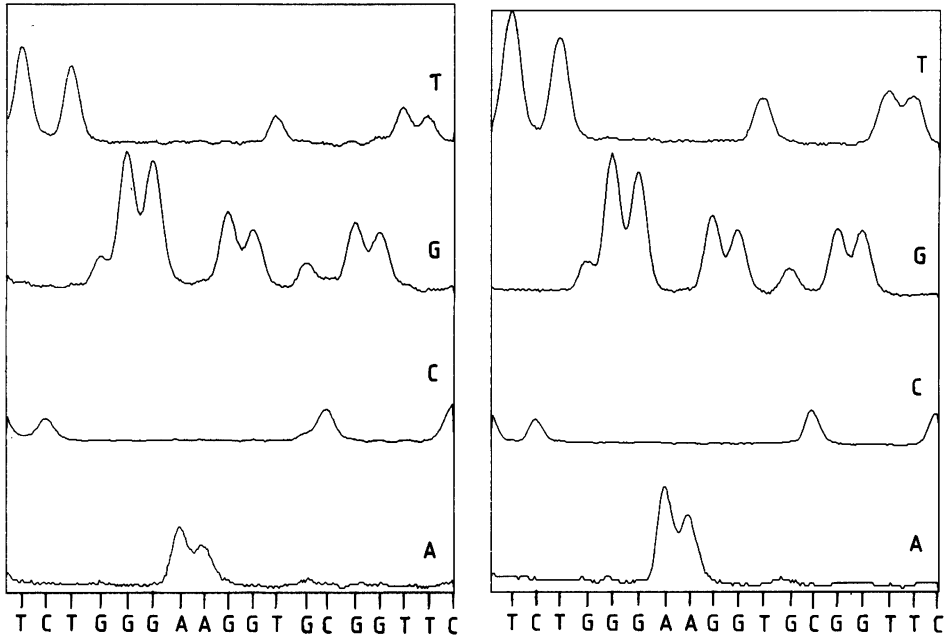


Fig. 3. Fluorescence sequencing. Partial raw data output from a 7 % polyacrylamide gel of separating length 20 cm [6, 7], corresponding to bases 175-192 read from the gel. Left: DNA prepared by the standard method; right: DNA prepared by the glass fiber filter method.

primer are detected in the fluorescent method, while products which may result from random priming will be detected in radioactive methods involving incorporation of  $\alpha$ -labelled dNTPs.

In Fig. 2 the radioautographs obtained with DNA prepared by the novel method described here, using either the Klenow fragment or T7 DNA polymerase, are compared with the results obtained with DNA prepared by the standard method and the same two enzymes. Evidently, DNA prepared by the glass fiber filter method performed as well as DNA prepared by the standard method in the sequencing reactions. In Fig. 3 a portion of the raw data output from the on-line detector during a sequencing run with fluorescent primer, the same two DNAs and the Klenow fragment is shown. Apparently the two DNA preparations also perform similarly in this system.



With the use of more specialized equipment (e.g. multisample filtration units such as Minifold from Schleicher & Schüll, multipronged micropipetts) the simultaneous preparation of a large number of DNA templates should be a simple task. Although the present method was developed with DNA sequencing in mind, we expect it will also be of value in other cases where a fast and reliable method for M13 ssDNA preparation is needed, as in site-directed mutagenesis. We also wished to develop a method which could be automated and are currently working on the automation of the procedures described here, with the aim of eliminating all the remaining centrifugation steps. Together with a system for automatic sequence determination described previously, we expect automatic template preparation will be of considerable value in the undertaking of larger sequencing projects.

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