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*Published in:*

Biochimica et Biophysica Acta %28BBA%29 - Nucleic Acids and Protein Synthesis

*DOI:*

[10.1016/0005-2787\(78\)90135-1](https://doi.org/10.1016/0005-2787(78)90135-1)

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*Document Version*

Publisher's PDF, also known as Version of record

*Publication date:*

1978

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Ledeboer, A. M., Hille, J., & Schilperoort, R. A. (1978). An Easy and Efficient Procedure for the Isolation of Pure DNA Restriction Fragments from Agarose Gels. *Biochimica et Biophysica Acta %28BBA%29 - Nucleic Acids and Protein Synthesis*, 520(3). DOI: 10.1016/0005-2787(78)90135-1

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BBA 99269

## AN EASY AND EFFICIENT PROCEDURE FOR THE ISOLATION OF PURE DNA RESTRICTION FRAGMENTS FROM AGAROSE GELS

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(Received February 28th, 1978)

### Summary

A new procedure is developed to isolate DNA from agarose gels. Using a kind of blotting technique, DNA is isolated from the gel. It is shown that the isolated DNA can be used for fragmentation by restriction endonucleases, synthesis of complementary RNA by DNA-dependent RNA polymerase from *Escherichia coli* and nick translation. The procedure gives a high recovery and is easy to perform.

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### Introduction

Fragmentation of DNA by restriction endonucleases is commonly used in the genetical and physical analysis of DNA. For separation of large fragments,  $2 \cdot 10^6$ – $20 \cdot 10^6$  daltons, only electrophoresis on agarose gels is suitable. However, the isolation of DNA fragments from the gel often rouses problems [1,2]. Frequently compounds that interfere with either the refragmentation by restriction endonucleases or with the synthesis of DNA or RNA by polymerases, co-elute from the gel. The efficiency of the isolation is variable. Loss of material is especially a problem during the isolation of very large fragments,  $>5 \cdot 10^6$  daltons.

Techniques presently used to isolate DNA from agarose gels are based on: (1) Electrophoresis either into a dialysis bag containing the agarose gel, or into a bag wrapped around the bottom of the gel holder [1,3,4]. Certain impurities still co-electrophorese with the DNA, while in addition often losses occur by sticking of the DNA to the dialysis bag [2]. (2) Dissolving the gel in KI [5] or NaClO<sub>4</sub> [6] followed by phenol extraction or column chromatography of the DNA. Since in these cases the complete gel is dissolved, all the agarose has to be eliminated. Because an extensive purification procedure is needed for this, DNA will be lost. (3) Mechanical disruption by freeze-thawing and squeezing

by hand or forcing the agarose gel through a needle [2,7]. Only subsequent fragmentation with one restriction enzyme [2] or nicked translation [7] is described. In this paper a procedure is described using a type of blotting technique [8] in combination with hydroxyapatite [9].

## Materials

*Enzymes.* Hind III and Eco R1 were obtained from Miles (England). DNA polymerase I and Sma I were obtained from Boehringer (G.F.R.).

*Other products.* Seakem TM Agarose was obtained from Marine Colloids Inc. (U.S.A.). Hydroxyapatite, DNA grade was a Biorad product (U.S.A.). Nitrocellulose filter, HWAP-00010, was obtained from Millipore (U.S.A.). [ $^3\text{H}$ ]thymidine (40–60 Ci/mmol), [ $^3\text{H}$ ]TTP (52 Ci/mmol) and [ $^3\text{H}$ ]CTP (23 Ci/mmol) were obtained from Amersham (England). Phage  $\lambda$ CI 857 was kindly provided by Dr. C.A. Wijffelman from his laboratory.

## Methods

*Growth and labelling of bacteria.* *Escherichia coli* strain 1164 col E1, was grown in M9 medium supplemented with 1  $\mu\text{g/ml}$  thiamine, 0.5% casamino acids and 40  $\mu\text{g/ml}$  proline. The strain was labelled as described earlier [10], with 2  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]thymidine. *Agrobacterium tumefaciens* strain S1005 (TI B6S3) [11] was grown as described before [12].

*DNA isolation.* Phage  $\lambda$ DNA: The DNA was isolated from phage  $\lambda$  by extraction with phenol after diluting the phage, isolated directly from the CsCl gradient, in 2  $\times$  SSC (SSC = 0.15 M NaCl/0.015 M sodium tricitrate); 10 mM Tris  $\cdot$  HCl (pH 8); 1 mM EDTA, to an  $A_{260\text{nm}}$  of 10. The DNA was dialysed extensively against 0.1 mM EDTA, 25 mM Tris  $\cdot$  HCl (pH 8).

Col E1 DNA: Col E1 was isolated from *E. coli* strain 1164 col E1 as described [10]. The plasmid band was punctured from the gradient, ethidium bromide was eliminated by repeated extraction with isoamyl alcohol saturated with 20  $\times$  SSC, dialyzed against 3% NaCl, and the DNA was precipitated with 2 volumes cold ethanol.

TI plasmid from *A. tumefaciens*: The TI-plasmid from *A. tumefaciens* strain S1005 (TI B6S3) was isolated as described [12].

*Fragmentation by restriction endonucleases and electrophoresis.* The DNA molecules were digested in a concentration of 20  $\mu\text{g/ml}$  at 37°C in the following buffer: 50 mM Tris  $\cdot$  HCl (pH 7.2), 6 mM  $\text{MgCl}_2$ , 40 mM NaCl and 1 mM dithiothreitol. The incubation time and the concentration of the enzyme were chosen in such a way that complete digestion resulted, unless stated otherwise. The digest was brought on the gel in 2.5% ficoll, 0.02% bromophenolblue in electrophoresis buffer (40 mM Tris/acetic acid (pH 7.9), 5 mM sodium acetate and 1 mM EDTA). Electrophoresis was performed on cylindrical gels with a diameter of 6 mm or on slabgels of 3–5 mm thickness with a slot length of 0.7–7 cm. The gel concentration was 0.7%. The voltage applied was 5 V/cm for col E1 and phage  $\lambda$ DNA during 5 h and 1.5 V/cm for the TI-plasmid during 40 h. The length of the gel in the latter runs was 40 cm.

*Blotting.* Denaturation of the DNA, blotting to a nitrocellulose filter and

backing of the filter was essentially performed as described [8]. We used a modified procedure of Chilton (personal communication). The gel was layered on a wetted nitrocellulose sheet supported by dry filter paper. The whole combination was wrapped with plastic, allowing the gel to dry overnight. The transfer of the DNA was almost quantitative.

*Synthesis of complementary RNA.* RNA complementary to a fragment of the TI-plasmid was synthesized with RNA-dependent DNA polymerase, isolated from *E. coli* strain MRE 600 as described before [13].

*Nick translation.* Nick translation on fragments of the TI-plasmid was performed essentially as described [14], while the DNA was purified by melting and subsequent chromatography on hydroxyapatite [15].

*DNA-cRNA filter hybridization.* Coating of the DNA blots in Denhardt solution [16] and hybridization were performed as described [17] except that the end volume was 0.5 ml. The hybridization was performed in heat-sealed polythene bags. The filters were treated for 1 h at room temperature with 20  $\mu\text{g}/\text{ml}$  RNAase after a wash in  $2 \times \text{SSC}$  at  $68^\circ\text{C}$ . Hybrids were detected by fluorography [18].

*The DNA isolation technique.* The DNA band, preferably not visualized by ethidium bromide, because of further enzyme treatment, was cut out of the gel and mounted on top of a perspex tray, filled with hydroxyapatite, equilibrated with 0.20 M sodium phosphate (an equimolar mixture of  $\text{Na}_2\text{HPO}_4$  and  $\text{NaH}_2\text{PO}_4$ ). The tray has no bottom. It is just a frame to hold the hydroxyapatite. For small scale isolations the trays have a diameter of 0.6 cm and a height of 0.3 cm. They contain 50 mg hydroxyapatite. For large scale isolations the trays have the dimensions of 3 cm  $\times$  0.5 cm  $\times$  0.3 cm and contain 0.5 g hydroxyapatite. To transfer the DNA, the gel was brought into contact with two buffer compartments, containing 0.20 M sodium phosphate, by means of a piece of Whatman 3 MM paper, covering the gel. Care has been taken that the wick does not touch the stack of filter paper. This is done by placing a small piece of perspex between the wick and the filter paper. The whole system was wrapped with plastic in order to avoid evaporation. The DNA was allowed to be transferred on to the hydroxyapatite overnight. In Fig. 1 the complete set up is shown. After DNA transfer the gel was taken away and can either be stained

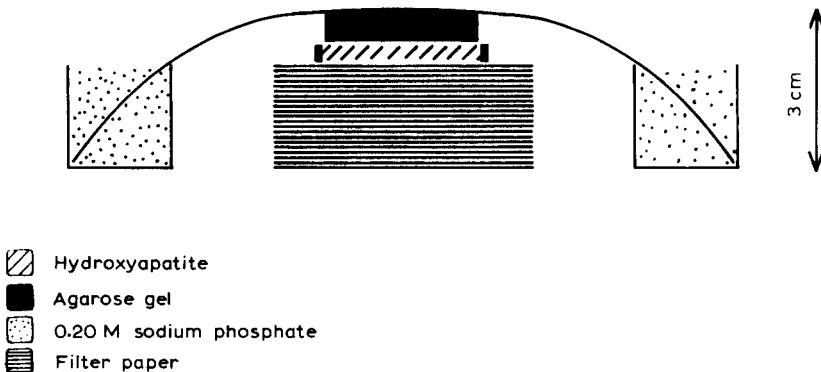


Fig. 1. Outline of the complete set up for DNA transfer.

with ethidium bromide or counted for radioactivity, to look for non-transferred DNA. The hydroxyapatite was resuspended in 0.12 M sodium phosphate, poured on to a small column (Pasteur pipette) and washed with 1–2 ml 0.15 M sodium phosphate. The DNA was eluted with 0.5–1 ml 0.30 M sodium phosphate, dialyzed against water and concentrated by freeze-drying. The procedure is used for small amounts of DNA (0.2–1  $\mu\text{g}$ ) as well as for large amounts (5–20  $\mu\text{g}$ ).

## Results

The efficiency of the transfer of non-denatured DNA from the agarose gel to the hydroxyapatite, as well as the final recovery from the hydroxyapatite was studied. In order to avoid too much evaporation and to keep the procedure as simple as possible, the experiments were performed at room temperature instead of a temperature at 60°C normally used for binding of DNA to hydroxyapatite in renaturation studies [9]. We therefore looked whether under this condition another phosphate concentration is needed for the specific binding of double-stranded DNA. Besides this, it had to be seen whether a salt concentration suitable for binding still allows a fast transfer of the DNA out of the gel to the hydroxyapatite. From the data shown in Fig. 2 and Table I it can be concluded that the kinetic of the DNA transfer is only slightly influenced by the ionic strength of the buffer. The recovery, >90% after 16 h of transfer, is not influenced by the ionic strength, if the phosphate concentration does not exceed 0.20 M sodium phosphate. If 0.25 M sodium phosphate is used to transfer the DNA from the gel to the hydroxyapatite, no DNA is recovered from the hydroxyapatite afterwards. This probably is due to the fact that the DNA is not bound to hydroxyapatite under these conditions. It was found that also large DNA fragments could be transferred quantitatively from the agarose gel to the

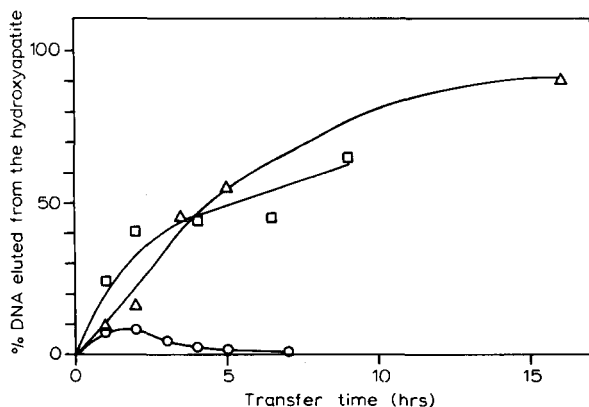


Fig. 2. Kinetics of the transfer of  $^3\text{H}$ -labelled Col E1 DNA in different sodium phosphate concentrations. Equal DNA samples were transferred from the agarose gel to the hydroxyapatite. After different times, the hydroxyapatite was washed with 1 ml 0.15 M sodium phosphate and the DNA was eluted with 0.30 M sodium phosphate. The data are expressed as percentage of double-stranded DNA that is eluted from the hydroxyapatite column, towards the amount of DNA, brought on the agarose gel. ○, 0.25 M sodium phosphate; △, 0.20 M sodium phosphate; □, 0.12 M sodium phosphate.

TABLE I

TRANSFER OF <sup>3</sup>H-LABELLED Col E1 DNA UNDER DIFFERENT SALT CONDITIONS

The DNA was transferred from the agarose gel to the hydroxyapatite during 16 h. The data are expressed as the percentage of double-stranded DNA that is eluted from the hydroxyapatite column, towards the amount of DNA, brought on the agarose gel.

Salt concentration	Percent double-stranded DNA eluted from hydroxyapatite
No buffer	10
0.16 M sodium phosphate + 1 M NaCl	75
0.20 M sodium phosphate	90
0.20 M sodium phosphate + 3 M NaCl	90

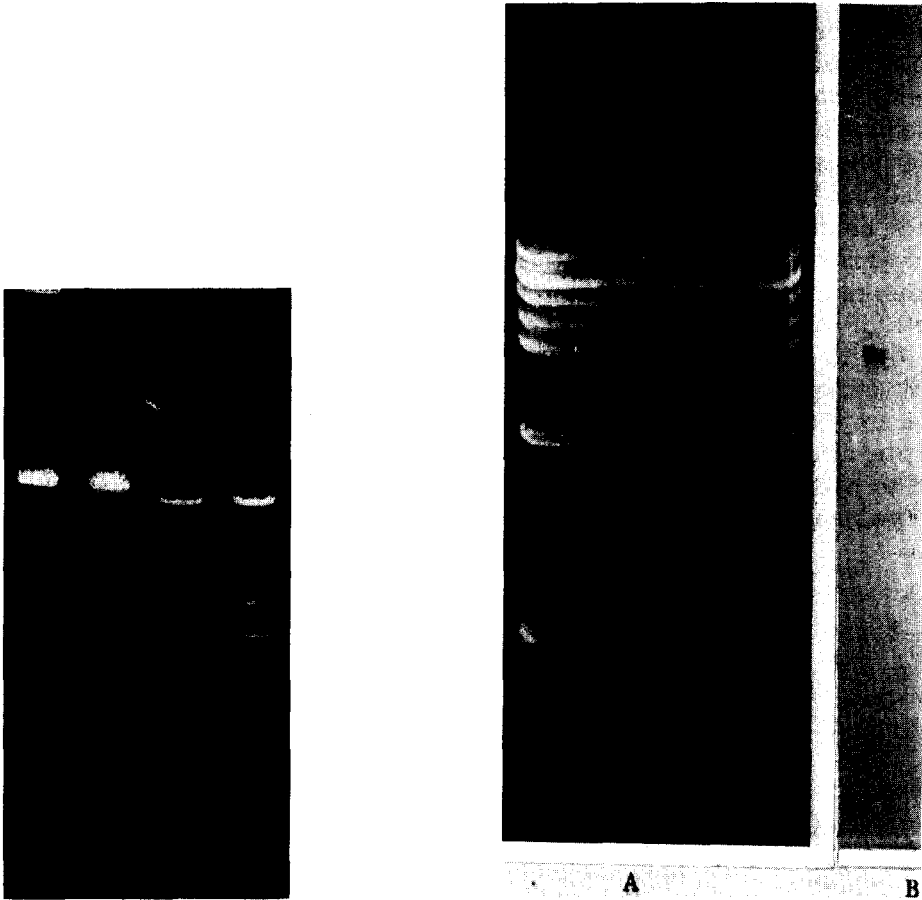


Plate I. Rerun and Eco R1 fragmentation pattern of phage  $\lambda$  CI 857 DNA, isolated from agarose gel. Lane 1, unfragmented  $\lambda$ DNA; Lane 2, unfragmented  $\lambda$ DNA isolated from agarose gel; Lane 3, Eco R1 fragmentation pattern of DNA; Lane 4, Eco R1 fragmentation pattern of DNA, isolated from agarose gel.

Plate II. Hybridization of <sup>3</sup>H-labelled cRNA of band 10/11 of a partial Hind III digest of the TI-plasmid of *A. tumefaciens* strain S1005 (TI B6S3). The cRNA was hybridized in a concentration of 5  $\mu$ g/ml, to the fragmented DNA, separated on agarose gels and blotted to nitrocellulose filter. A Kodak RP Royal X Omat Röntgen film was exposed for 20 h to the hybridized nitrocellulose sheet, impregnated in a solution of 18% PPO (w/v) in toluene [18]. (A) A 7 cm broad slot of the partial Hind III digest of the TI-plasmid. (B) A fluorograph of the hybridization between the cRNA of the isolated band 10/11 and the complete blotted partial Hind III digest.

hydroxyapatite. No DNA could be observed in the gels after staining with ethidium bromide, when non-fragmented phage  $\lambda$ DNA, molecular weight of  $30 \cdot 10^6$ , or three fragments of a partial Hind III digest of the TI-plasmid from strain S1005 (TI B6S3), molecular weights varying from  $5 \cdot 10^6$  to  $15 \cdot 10^6$ , were transferred.

The purity of the isolated DNA, one of the major problems in the isolation of DNA from agarose gels, was studied by determining its ability to react properly with different enzymes. In Plate I it is shown that the molecular weight of phage  $\lambda$ DNA isolated from an agarose gel is not affected by the procedure used, and that it can be fragmented correctly by Eco R1 without a change in the conditions normally used. The same result was obtained by digestion with Sma I and Hind III (data not shown). From a partial Hind III digest of S1005 (TI B6S3) (Plate II), bands 1, 5 and 10/11 were isolated. RNA synthesis on 1  $\mu$ g of band 10/11 with DNA-dependent RNA polymerase from *E. coli* resulted in 5  $\mu$ g RNA with a specific activity of 450 000 cpm/ $\mu$ g. As shown in Plate II this RNA is a true copy, as it hybridizes only with band 10/11 of the Hind III partial digest of the complete TI-plasmid. Moreover nick translation on bands 1 and 5 resulted in the uptake of respectively  $2 \cdot 10^6$  and  $3 \cdot 10^6$  trichloroacetic acid-precipitable counts which is about 20% of the theoretically obtainable uptake.

## Discussion

Isolation of DNA from agarose gels gives many difficulties. Some compounds come free from the agarose and interfere with further enzymatic treatment of the DNA. Additional purification steps like DEAE-cellulose, hydroxyapatite columns or extraction procedures used in the existing methods appear to be often not sufficient to eliminate all impurities. Moreover, the more purification steps are used, the more DNA will be lost. The procedure described here overcomes these problems, as obviously not many impurities are freed from the agarose gel during the transfer of the DNA. It is important to note that drying of the gel should be avoided, in a way as described, since drying will influence the DNA recovery. If any impurities are cotransferred with the DNA in this procedure, they are clearly eliminated by specific elution of the DNA from the hydroxyapatite. Although part of the procedure of Wilkie and Cortini [6] is the same, it essentially differs in the way to free the DNA from the gel. In the latter procedure, the complete agarose gel is dissolved in 5 M NaClO<sub>4</sub>. To purify the DNA from the dissolved agarose, the DNA is bound subsequently to hydroxyapatite, and the column is washed thoroughly. This extensive wash procedure lowers the recovery of the DNA, especially when small amounts of DNA are used. In our procedure the DNA is directly transferred to the hydroxyapatite, without dissolving the agarose, and we found that extensive washing of the hydroxyapatite is not necessary. Recoveries of DNA are high, even when low amounts of DNA are used.

## Acknowledgement

The authors are grateful to Dr. J.J.M. Dons and Drs. B. Zantinghe for helping them with nick translation.

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