Rapid large-scale purification of plasmid DNA by medium or low pressure gel filtration. Application: construction of thermoamplifiable expression vectors

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This paper describes a new method of plasmid DNA purification which is fast and reliable enough for most purposes in recombinant DNA technology. The present method does not require the use of toxic chemicals such as phenol or ethidium bromide, costly ultracentrifugation procedures or other processes which can modify the supercoiled structure of the plasmids, such as adsorption on glass fiber. This method is based on the principle of gel filtration chromatography, at low pressure (1 bar) or medium pressure (between 5 and 10 bars), using Sephacryl S1000 or Superose 6B. It permits recovery of plasmids: (I) in preparative quantities (from 300 µg to 4 mg), (II) exempt from RNA, DNA and protein contamination, and (III) suitable for various common genetic engineering procedures immediately after purification. To test the reliability of the technique as well as the degree of purification, the plasmids were used to construct thermoamplifiable vectors, carrying the lacUV5 promoter and the 5’ end of the β-galactosidase gene with a single EcoRI site in each of the three possible translational phases. This set of vectors is designed for the expression of foreign genes as hybrid proteins in Escherichia coli.

During the last decade, with the introduction and continuous expansion of recombinant DNA technology, the interest of most research workers has been focused on the problem of nucleic acid purification of both eucaryotic and procaryotic origin, especially plasmid vectors, which nowadays play an important role in molecular biology. To date, satisfactory purification of supercoiled plasmids is achieved by several methods including isopycnic gradient of caesium chloride (Clewell, 1972), the rapid extraction procedure based on
alkaline hydrolysis (Birnboim & Doly, 1979), chromatography on hydroxyapatite (Colman et al., 1978), RPC-5 chromatography (Best et al., 1981) or adsorption on glass powder (Marko et al., 1982). All these methods are of limited capacity and are either expensive and time-consuming if one requires a high level of purity, or rapid and inexpensive but resulting in only mild purification as far as usual contamination by RNA, protein and chromosomal DNA are concerned.

We describe here a fast and reliable two-step purification procedure which does not depend on CsCl centrifugation, and which is rapid, inexpensive and yields DNA of high purity.

Our method takes advantage of selective precipitation of proteins and chromosomal DNA at high salt concentration after alkaline denaturation. The plasmid is then separated from RNA and further purified by gel filtration chromatography.

The technique described below in three variations permits work in different situations. All three are simple to carry out, differing only in the equipment used, the time taken in the process of purification being inversely proportional to the cost in equipment. The first variation (the least expensive) makes use of a standard low pressure column, and yields pure material after 250 min of gel filtration. The second takes 45 min on a fast protein liquid column (FPLC) system. The third, using the same equipment as the second, purifies the plasmids in 20 min, but necessitates the use of RNAse.

To test the reliability of the method, we undertook the construction of a vector easy to use (thermoamplifiable) and suitable for the expression of genes lacking promoters. We therefore constructed a plasmid containing the duplication origin of pKN402 (Uhlin et al., 1979) and the ampicillinase gene of pBR322 (Bolivar et al., 1977). Subsequently, in the resulting plasmid termed pVT46, we have inserted the lac regions from the previously described plasmids pPCϕ1, pPCϕ2 and pPCϕ3 (Charnay et al., 1978). The vectors thus obtained called pVT46ϕ1, pVT46ϕ2 and pVT46ϕ3 are stable, thermoamplifiable, resistant to ampicillin and enable the expression of foreign genes as hybrid proteins in Escherichia coli.

Materials and Methods

The FPLC system was from Pharmacia.

Chemicals

The following chemicals were used: Xgal: 5-bromo-4-chloro-3-indolyl-beta-D galactoside, agarose, lysozyme (Sigma). Low melting agarose (BRL). Sephacryl S1000 and Superose 6B (Pharmacia).

Enzymes

Restriction endonucleases, T4 DNA ligase and DNA polymerase (Klenow fragment) were obtained from New England Biolabs and were used as recommended by the supplier.

Bacterial and phage strains and plasmids

MC1061 rec A, r- m- was a gift from M. Zakin (Institut Pasteur, Paris); pBR322 was a gift from F. Bolivar; pKN402 was a gift from K. Nordström; pPCϕ1, pPCϕ2, pPCϕ3 were gifts from P. Charnay;
pVT46, this paper; and pVT46φ1, pVT46φ2, pVT46φ3, this work. All plasmids in this paper were purified by the method described here.

**Media and growth conditions**

The bacteria were grown in L-broth on plates containing 1.5% agar. Ampicillin and/or tetracycline were added to plates and media at final concentrations of 100 and 15 μg, respectively.

_E. coli_ strains containing the plasmid to be purified were grown overnight as a preculture in 20 ml of L-broth containing the appropriate antibiotic. 10-15 ml of this preculture was used to inoculate 1 liter of L-broth supplemented as described above and incubated with vigorous shaking. Plasmids were amplified at an optical density of 1.0 at 600 nm either by overnight addition of chloramphenicol to a final concentration of 100 μg/ml (for bacteria carrying pBR322, pPCφ1, φ2, φ3) or by increasing the temperature from 37°C to 42°C during one hour (for bacteria carrying pKN402, pVT46, pVT46φ1, φ2, φ3).

**Plasmid extraction buffers**

- R1: 50 mM sucrose, 25 mM Tris/HCl (pH 8), 10 mM EDTA
- R2: 100 mM NaOH, 1% sodium dodecyl sulfate
- R3: 3 mM sodium acetate (adjusted to pH 4.6 with glacial acetic acid)
- TE: 10 mM Tris/HCl, 1 mM EDTA, pH 7.5.

**Lysis and alkali treatment**

Prior to gel filtration chromatography, the bacterial cells were lysed as previously described by Ish-Horowicz (1981). Briefly, bacteria were lysed for 5 min in R1 buffer supplemented by lysozyme (1 mg/ml). Complete solubilization was achieved by addition of 2 vol. of R2 buffer. Chromosomal DNA and proteins were then precipitated with 0.75 vol. of R3 buffer. Plasmid DNA in the supernatant was precipitated with 0.6 vol. of isopropanol. The plasmid was resuspended in TE buffer and submitted to gel filtration on Sephacryl S1000. For injection onto the Superose column 100 μg/ml of RNase was added for 20 min at 37°C.

**Packing of filtration gel**

The method comprises three variations differing either in the type of beads used or in the packing pressure.

 Variation I (column I). The Sephacryl S1000 filtration gel (Pharmacia) was packed with freshly degassed buffer (20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 0.1 M NaCl, 0.01% NaN₃) in the 30 x 600 mm column. The gel was packed with a peristaltic pump; at the beginning of the operation, the flow rate of the buffer was fixed at 300 ml/h, diminishing to 30 ml/h as the column gradually became more compact. This point was important for reproducible and accurate separation. The device was completed by a u.v. monitor and a fraction collector. The crude plasmid extract was applied to the column and eluted at about 3.5 ml/10 min with the same buffer, 3.5 ml fractions were collected and 10 μl aliquots were analyzed by 1% agarose gel electrophoresis. The fractions containing plasmid DNA
were pooled and precipitated with ethanol for 30 min at -70°C. The pellets were washed once with 75% ethanol and resuspended in 20 mM Tris/HCl pH 7.5 1 mM EDTA and stored at 4°C.

The S1000 column was washed with the elution buffer and was reused many times without apparent loss of separation properties.

**Variation II** (column II). Sephadryl S1000 was packed under a maximal pressure of 20 bars [pressure measured by the FPLC manometer (P500)] in a modified Superloop 25 x 300 mm column (Pharmacia). During packing, the flow rate was reduced from 240 ml/h to less than 40 ml/h. Finally, the output was maintained at 30 ml/h for 4 h. Elution was performed under a flow rate of 30 ml/h.

**Variation III** (column III). In this variation we employed the same packing technique as in variation II, except that we used the Superose 6B gel and a 10 x 300 mm column (HR 10/30, Pharmacia), which tolerates pressure of up to 45 bars [pressure measured by the FPLC manometer (P500)].

**Construction of the thermoamplifiable expression vectors**

All the plasmids used were purified by the present method. The thermoamplifiable vector pVT46 was obtained by insertion of the 1669 bp HaeII-HindIII fragment spanning the ampicillin gene of pBR322, into the runaway-replication plasmid pKN402 (digested with Hind III and partially with HaeII). Ampicillin-resistant recombinants showing rough colonies at 42°C and a wild type phenotype at 30°C were selected. In order to check the stability of these recombinant vectors, six rounds of alternative plating at 30°C and 42°C were performed. The pVT46 vector, which stably produced the rough phenotype at 42°C, was further modified in order to allow a high level of inducible expression of foreign genes in E. coli.

For that purpose, we have used the previously described vectors pPCφ1, φ2, φ3 (Charnay et al., 1978). These plasmids are derived from pBR322 and harbour a 2400 bp EcoRI - HindIII fragment encompassing the lacUV5 promoter. In addition, the single EcoRI site is situated at the very beginning of the β-galactosidase coding sequence, in each of the three translational phases. This set of EcoRI - HindIII fragment was introduced in pVT46 and recombinants were selected on L broth supplemented with ampicillin and 5-bromo-4-chloro-3-indolyl-beta D-galactoside (X-gal) at 30°C overnight. The dark blue colonies were reisolated twice on the same medium. The plasmids were then purified by our method, and hydrolyzed by suitable restriction enzymes to check and establish their restriction pattern.

**Results and Discussion**

The present method provides a further improvement, on a preparative scale, of the Ish-Horowicz and Burke modification (1981) of the rapid alkaline extraction procedure first described by Birnboim and Doly (1979). This improvement was achieved by a subsequent rapid step of gel filtration chromatography on Sephadryl S1000 or Superose 6B at low or medium pressure.

We present the purification of pBR322 with the three columns described in Materials and Methods. The elution pattern of column I (S1000, low pressure) is shown in Fig. 1a: peaks I and II correspond respectively to the two forms of plasmid, open circular (peak I) and
covalently closed circular (CCC) (peak II) as ascertained by electrophoretic analysis (Fig. 1b). The relaxed form was eluted before the supercoiled form because of steric hindrance. This effect was observed more or less with all the plasmids depending on their molecular weight. For small plasmids such as pBR322 these two peaks were overlapping partially (Figs. 1a and 1b) whereas the separation was complete with larger plasmids above 10 kbp (data not shown). Supercoiled DNA (peak II) always represented the greater part of the material, indicating that only little nicking took place during extraction-elution. This may be due to the absence of use of phenol, EtBr or procedures involving adsorption of plasmids on glass fiber filters.

RNA was eluted in peak III clearly separated from peaks I and II. One other peak, corresponding to low molecular weight RNA, was sometimes observed behind peak III. Variation I, although simple and
inexpensive, has two drawbacks: it is lengthy (from 200 to 300 min of elution) and resolution is limited by diffusion. Therefore, two other variations (columns II and III) were developed to reduce the purification time. Fig. 2a shows the elution pattern of a pBR322 crude extract on column II, packed with the same beads as column I (S1000) but under higher pressure (20 bars, i.e. the maximum pressure tolerated by the S1000 gel) and Fig. 3 the chromatogram of the same extract on column III (Superose 6B, medium pressure) with a preliminary treatment of RNAse.

Fig. 2. Purification of pBR322 on Sephacryl S1000 at medium pressure (cf. Materials and Methods).

a) Elution pattern of column II without prior treatment of the pBR322 extract (obtained from a 200 ml culture) with RNAse A.

b) Electrophoretic analysis of eluted fractions (cf. Fig. 2a).
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Fig. 3. Purification of pBR322 on Superose 6B at medium pressure (cf. Materials and Methods). Elution pattern of column III after treatment of the crude extract with RNAse A (10 μg/ml).

On column II, a single plasmid peak was observed (Fig. 2a,b) clearly distinct from the RNA, the RNAse treatment resulting in a better separation (not shown). With column III, however, overloading was often a cause of failure and a prior treatment of the crude extract with RNAse is therefore a prerequisite. In these conditions, as on column II, the plasmid is eluted as a single peak corresponding to the two forms of plasmid. Plasmid purification occurred in only 20 min (Fig. 3), in comparison with the 250 min elution on column I or the 45 min elution on column II. In our experiments, only Superose beads, which are small and very resistant to pressure, allow plasmid elution in less than 20 min. This reduction of the elution time demonstrates clearly the importance of the pressure in the packing procedure. Furthermore, the packing pressure, essential for a good resolution, certainly accounts for the better separation observed in experiments at low pressure compared to the results previously described by Bywater et al. (1983).

The mean yield generally obtained by this method, about 2 mg of plasmid/liter of culture, is as good as those usually reported and the ratio A260/A280 (≥ 1.8) indicates a very low protein contamination, if any.

Column I can be loaded with a plasmid extract obtained from 1 to 3 liters of culture whereas the equivalent of only 1.2 l and 0.5 l of culture is applied respectively on columns II and III. In these conditions, the purified plasmid is eluted at a concentration of 30-50 μg/ml from Column I and 200-300 μg/ml from columns II and III.
Because of the relatively short time needed by the procedure at medium pressure (columns II and III), and the low dilution factor of these columns, an elution buffer without salt (TE) was adopted. This enabled us to use the eluted material directly without ethanol precipitation. This represents an appreciable gain in time and constitutes a major advantage of this method.

**Monitoring of DNA purification**

Purity of the eluted plasmids was tested by digestion with restriction enzymes. Fig. 4 shows pBR322 purified on column II after cleavage with HindIII, PstI and PvuII. It can be seen that the digestion is complete, the absence of partial cleavage suggesting that the preparation is pure. Similar results have been obtained with plasmid purified on the two other columns.

**Thermoamplifiable vector for cloning genes lacking promoters**

To test the reliability of our method and also the quality of the plasmids purified in this way, we undertook the construction of an inducible expression vector.

The thermoamplifiable vectors pKN402 and pKN410 from Nordstrom (Uhlin et al., 1979), since they do not carry an antibiotic resistance gene, are subject to a degree of instability due to the absence of selective pressure. We therefore attempted to introduce the gene coding for ampicillin resistance into the vector pKN402 (Fig. 5).

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**Fig. 4.** Restriction endonuclease cleavage. Analysis of pBR322 purified on column II and digested with PstI (track 1), HindIII (track 2), PvuII (track 3), PstI and HindIII (track 4), HindIII and PvuII (track 5), PstI and PvuII (track 6) and PstI, HindIII and PvuII (track 7).
Ampicillin-resistant recombinants showing rough colonies at 42°C and a wild type phenotype at 30°C were selected. The weakening of bacterial walls by excess of β-lactamase resulting from vector amplification probably accounts for this phenotype, thus confirming the presence of the thermoamplifiable origin of replication of pKN402. One stable hybrid, pVT46, which is 9 kbp long, was retained. This vector has been used successfully for the cloning of the mal T gene by Debarbouille et al. (1982).

The insertion in pVT46 of a strong promoter (lac UV5) with a single EcoRI site in each phase at the 5' end of the β-galactosidase gene has yielded a set of three useful vectors: pVT46φ1, φ2, φ3 (Fig. 5) as a tool for the expression of foreign genes lacking promoter. Their detailed restriction map is presented in Fig. 6. These 11.4 kbp vectors are thermoamplifiable, ampicillin resistant and stable in the presence of the antibiotics.

**Conclusions**

This method of plasmid purification is as rapid as the micromethods (Birnboim & Doly, 1979) while as efficient as isopycnic centrifugation methods (Clewell, 1972).
Fig. 6. Restriction map of pVT46φ. Restriction sites for AccI, AvaI, EcoRV, SphI, PvuII, PstI, XmnI, TaqI and HaeII are presented. Additionally, the arrows indicate the location of a few important restriction sites (PvuI, EcoRI, HindIII, SmaI, SalI and BglII).

The procedure described is simple and easy. The material obtained is predominantly in a CCC form; it is virtually unaffected by this method of extraction. Three variations cover a wide need in purification procedures: variation I is simple and inexpensive and requires no special equipment; variations II and III are rapid and do not require further treatment of the purified material prior to the use of the common genetic engineering procedures.

Finally, the absence of the need for toxic chemicals (such as phenol and EtBr), complex procedures such as fixing and elution of the plasmid on glass fiber, lengthy and costly procedures such as ultracentrifugation make the technique suitable for use on a large scale.

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