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Purification of Supercoiled Plasmid DNA

Kelly L. Jordan, Devon L. Evans, and David J. Hall

1. Introduction

The topology of DNA affects a number of major biological processes ***(1–4)***. For example, processive enzymes, such as the RNA and DNA polymerases, have the capability of generating both positive and negative DNA supercoils during the process of transcription and replication, respectively. These supercoils must be relaxed in order for transcription and replication to continue unaffected, as outlined in preceding chapters. The DNA topoisomerases play the central role in relaxing this supercoiling ***(1–4)***.

The study of DNA topoisomerases and DNA topology relies essentially on a two-part system: the DNA topoisomerase and the molecule of DNA. The DNA topoisomerases function as enzymes because they efficiently form covalent complexes with DNA through an active site tyrosine ***(1–4)***. The enzymes then relax supercoiled DNA. The mechanism of DNA relaxation differs depending on the type of DNA topoisomerase examined. The DNA topoisomerase then religates the cleaved strand and dissociates from the DNA. The substrate of interest in this enzymatic process is DNA, specifically supercoiled DNA. Although the DNA topoisomerases can bind to single-stranded DNA and to relaxed duplex DNA ***(2,3)***, the primary interest here is in their association with supercoiled duplex DNA, the enzymes' major substrate.

A primary source of DNA for relaxation studies is the small mol-wt multicopy double-stranded DNA plasmids (e.g., pUC-derived) ***(5,6)***. These plasmids can be amplified to high levels in *E. coli* and can be easily isolated. A very important aspect of these plasmids is that they are isolated as supercoiled molecules, and in particular, they are negatively supercoiled. It is generally considered that plasmid DNA is negatively supercoiled because of the action

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of bacterial gyrase, an *Escherichia coli* DNA topoisomerase type II enzyme that generates negative supercoils in DNA ***(2)***. For much of the work performed on DNA topoisomerases, these multicopy plasmids provide a convenient source for substrate, particularly for experiments with eukaryotic DNA topoisomerase I and II and bacterial DNA topoisomerase I, all of which can relax negatively supercoiled DNA. For particular studies in which positively supercoiled DNA is needed, it is necessary to isolate the plasmids from very specific mutant strains of bacteria usually in the presence of specific drugs that bind DNA and alter its superhelicity. This latter approach is rather involved and will not be discussed here. Instead, we will focus on the isolation methods of negatively supercoiled plasmid DNA.

2. Materials

1. Materials for growth of bacteria: yeast extract, bacto-tryptone, NaCl, antibiotics (e.g., ampicillin, chloramphenicol, kanamycin, tetracycline), 1–2 L flask, and environmental shakers (37°C; New Brunswick; Scientific, Hatsfield, UK).
2. Plasmids: multicopy, pUC-derived plasmids.
3. Chemicals for alkaline lysis: TE: 10 m*M* Tris-HCl, pH 8, 1 m*M* EDTA.
4. GTE: 50 m*M* glucose, 25 m*M* Tris-HCl, pH 7.5, 10 m*M* EDTA.
5. Sodium dodecyl sulfate (SDS) NaOH solution: 1% SDS/0.2 *M* NaOH.
6. High salt solution: 29.4 g potassium acetate, 5 mL of 100% formic acid; add water to 100 mL.
7. Chemicals for CsCl ultracentrifugation: ultrapure CsCl, ethidium bromide (10 mg/mL, Sigma), *n*-butanol (water- or TE-saturated; Fisher; Scientific, Pittsburgh, PA).
8. Centrifugation: Superspeed and ultracentrifuges (e.g., J2-21 and Optima, Beckman, Fullerton, CA). Rotors: J6, JA10, JA14, VTi65.1. Tubes: polyallomer.
9. Dialysis tubing: Spectrapore (Fisher).
10. Agarose-gel electrophoresis: Agarose (Gibco/BRL, Gaithersburg, MD), TBE: 89 m*M* Tris-borate, pH 8.0, 2 m*M* EDTA.

3. Methods

A number of methods have been previously published for plasmid purification ***(5,6)***. Here we will only focus in detail on one that seems to be the simplest, cheapest, and produces the highest yield, with comments on the effect of the isolation on the topological state of the plasmid DNA. Some methods will be mentioned as alternatives. The methods outlined below deal with large-scale preparations, with production of plasmid DNA to a high level of purity. "Miniprep" methods, although certainly enabling one to isolate plasmid DNA rapidly, usually result in a fair level of contaminating protein and RNA, which are undesirable in a detailed analysis of DNA topology.

Table 1. Bacterial Lysis and Purification Methods

Bacterial lysis	Purification
Alkaline lysis	CsCl centrifugation
Boiling method	Polyethylene glycol precipitation
Triton/detergent lysis	Qiagen[R]

Large-scale preparations of plasmid DNA usually begin with a 250–500 mL culture of *E. coli* transformed with an appropriate plasmid in a 1–2 L flask, respectively. The cells are grown at 37°C to stationary phase (usually overnight) in the presence of an appropriate antibiotic, such as ampicillin, chloramphenicol, tetracycline, or kanamycin, with vigorous shaking (300 rpm on a rotary shaking platform) to achieve the high level of aeration needed for optimal growth. At this point, the investigator must decide on the form of bacterial lysis and plasmid purification. Listed in **Table 1** are the most common forms of lysis and purification. Consult **refs.** ***(5)*** and ***(6)*** for more details on the other methods.

With regard to lysis of the bacteria, three general methods are shown in **Table 1**. The boiling method of bacterial lysis is a very fast and simple technique, but it results in a high level of contaminating protein and RNA. As with the miniprep method, this may be unsuitable for studies in DNA topology ***(5,6)***. The triton/detergent lysis method is much gentler than the other methods and should not result in denaturation of plasmid DNA ***(5,6)***. It is often used for isolation of very large plasmids (e.g., cosmids). The alkaline lysis method is most commonly used and will be discussed at length below.

In terms of purification of the DNA, three methods are also shown in **Table 1.** The CsCl centrifugation protocol will be discussed at length below in **Subheading 3.2.** A commercially available kit by Qiagen is currently used by many investigators for plasmid purification. The kit makes use of a resin that specifically binds duplex DNA. The advantages of this procedure are that the quality of the DNA is quite good, with little contamination by bacterial RNA or proteins. In addition, the entire time from beginning of lysis (alkaline lysis) to isolation of plasmid DNA is just a few hours and does not require a DNA intercalator, such as ethidium bromide, for detection of DNA or phenol for elimination of proteins. The disadvantages are that it is rather costly, and the plasmid yield can be much lower than that of alkaline lysis/CsCl centrifugation. Finally, the polyethylene glycol (PEG) method for plasmid purification is very rapid and easy to perform. However, it does require phenol extractions. One must be very careful with phenol, since it is a severe protein denaturant. Additionally, the oxidation products of phenol can cleave DNA, so phenol

extractions can have an adverse effect on DNA topology. Finally, the purity of the plasmid DNA can be variable using the PEG method, since traces of the PEG may remain after a final ethanol precipitation.

3.1. Alkaline Lysis

Alkaline lysis is the most common method of bacterial lysis. This procedure is divided into three steps. First, the bacterial cell wall is digested with lysozyme in an isotonic solution. Next, the cells are lysed in a solution of sodium dodecyl sulfate and sodium hydroxide (SDS/NaOH). Finally, proteins and chromosomal DNA are precipitated with acidic potassium acetate, and the precipitate is removed by centrifugation. The essential point of this entire procedure is that chromosmal DNA is readily denatured by the SDS/NaOH, whereas the plasmid DNA is less susceptible to denaturation, likely owing to the high level of negative supercoiling. Following addition of the acetate solution, the chromosomal DNA fails to renature, yet the plasmid DNA is duplex and supercoiled, allowing easy purification. Using the alkaline lysis/CsCl centrifugation method, hundreds of micrograms of plasmid DNA can be isolated from a 500-mL culture of bacteria.

1. Pellet bacteria from saturated culture (described above) in 0.5- or 1-L plastic bottles at 5000–6000*g* for 10 min at 4°C (J2-21 or J6 centrifuge, Beckman, JA10 or J6 rotors, respectively). Discard supernatent (treat with wescadyne or chlorox first). All remaining procedures should be performed on ice.
2. Resuspend pellet by vortexing in 10 mL of GTE. After resuspension of bacterial pellet, add 40 mg of solid lysozyme (to 4 mg/mL). Swirl tube gently to resuspend lysozyme. Let sit on ice for 5 min, and then transfer solution to a smaller 250-mL bottle.
3. Add 20 mL of SDS/NaOH solution. **Slowly and carefully** invert the bottle five to eight times. The solution should become very viscous and relatively uniform in color (clear with a brown tint) as the bacteria lyse. Be careful not to shear the chromosomal DNA. Let sit on ice for 5 min. It is important that you do not let the solution sit on ice for too long (longer than 5 min), because the plasmid DNA may begin to denature irreversibly (*see* **Note 3**).
4. Add 15 mL of high salt solution. Invert slowly until white precipitate begins to form. Invert more rapidly until brown bacterial DNA and protein are converted to white precipitate. Let sit on ice for 5 min. Pellet white precipitate by centrifugation at 10,000–15,000*g* for 10 min at 4°C (J2-21 centrifuge, JA14 rotor, Beckman). Remove the supernatent containing the plasmid DNA to a fresh 250-mL bottle to it add 2 vol of ethanol, and precipitate the DNA for at least 1 h at –20°C.
5. Pellet the nucleic acid by centrifugation at 12,000–15,000*g* at 4°C for 10 min (J2-21 centrifuge, JA14 rotor, Beckman). The resulting pellet should be very large and will consist mostly of bacterial tRNAs along with the plasmid DNA. Resus-

pend the pellet in water or TE. The volume needed for resuspension will depend of the type of purification of plasmid DNA that you will perform next but will usually be on the order of 5–11 mL.

3.2. CsCl Ultracentrifugation

Density gradient ultracentrifugation is probably the most standard way to purify plasmid DNA. For ultracentrifugation, the fixed-angle, vertical, or near-vertical rotors (Sorvall or Beckman) hold tubes of a defined volume, either 5 or 13 mL. The most rapid centrifuge runs are performed with vertical rotors, because the gradients can be generated in a very short period of time (few hours).

1. Resuspend the large pellet of nucleic acid, from **Subheading 3.1., step 5,** in 11 mL of sterile water or TE and place in a 15-mL conical tube. Measure the volume exactly (total volume should now be roughly 12 mL).
2. Add 1 g of solid CsCl for every mL of solution (e.g., if total is 12 mL then add 12 g of CsCl). Invert or vortex tubes to force the CsCl into solution. The resulting volume should now be at approx 13.5 mL.
3. Add 80 µL of ethidium bromide (10 mg/mL) to the tube. Ethidium bromide will intercalate into the nucleic acid, enabling you to detect the plasmid DNA following the centrifugation. Procedures elsewhere usually call for the addition of significantly more ethidium bromide (up to 0.5 mL) ***(5,6)***. However, we have found that a reduction in the amount of dye used still enables one to detect the DNA easily, yet is less of a health and disposal hazard.
4. The solution is added to a polyallomer "sealable" ultracentrifuge tube, capable of holding 13 mL. The centrifuge tubes have a narrow neck so that they can be heat-sealed. Place a Pasteur pipet in the opening as a funnel. Then pipet the plasmid:CsCl solution into the tube.
5. Weigh the tubes and adjust volumes so that weights are equal. Using a heat sealer, seal the tube.
6. Place it in an appropriate rotor, fixed-angle, near-vertical or vertical, and begin the centrifuge run. Before starting the centrifuge run, always make sure that the form-fitting metal caps are placed over the centrifuge tubes, then secure the tubes and caps with additional screw-on caps. For a vertical rotor (e.g., VTi65.1), choose a speed of approx 350,000*g* for 4–5 h. At this speed, the CsCl density gradient will rapidly set up, causing the plasmid DNA to band at the appropriate density. A near-vertical or fixed-angle rotor will require a longer centrifuge run, approx 12 h for near-vertical and 24 h for a fixed-angle rotor, usually at ~300,000*g*.
7. Stop the centrifuge run, and withdraw tubes from the rotor carefully, so as not to disturb the gradient. With the low concentration of ethidium bromide used, the plasmid DNA should be evident as a single band located approximately one-third of the distance from the bottom of the tube. A UV lamp should not be needed for detection. Little, if any, chromosomal DNA should be present in the preparation,

so additional bands should not be seen. If a vertical rotor is used in the centrifugation, an intense line of stained material will coat one wall of the centrifuge tube. This will be bacterial protein/RNA (tRNA), and should be avoided. If near-vertical or fixed-angle rotors are used, the intensely stained material at the bottom of the tube is bacterial protein/RNA.

8. To extract the DNA, place the tube in a rack and puncture the very top of the tube with a needle to allow air to enter the tube when the DNA is extracted. Then recover the plasmid DNA using a 3-cc syringe with a 20-gage needle. Place the needle about 1 cm below the plasmid band, and by rotating the needle (with syringe), slowly puncture the tube, being careful not to press too hard so as not to poke through the other side of the tube. It may help to practice on an empty tube. With the needle tip now inside the tube, position the tip into the band of plasmid DNA. Then, using the syringe, slowly withdraw the plasmid DNA until there is no remaining band in the tube. The volume should be equivalent to 2–3 mL. Remove the needle, and the transfer the plasmid DNA in the syringe to a 15-mL conical. Be sure to have a waste beaker handy to discard the centrifuge tube containing the remaining CsCl solution.
9. To obtain highly pure DNA that is free of RNA contamination, the sample can be easily centrifuged again (*see* **Note 1**).

3.3. Elimination of Ethidium Bromide

1. To remove the ethidium bromide from the DNA, a double extraction with *n*-butanol is performed. *n*-Butanol, equilibrated with water or TE, is not miscible in aqueous solutions and therefore forms an upper layer. Two to 3 vol of *n*-butanol (water- or TE-equilibrated) are added to the DNA-CsCl solution, which is then vortexed vigorously two times for 5–10 s.
2. The butanol and aqueous phases are allowed to separate on the bench top for a few minutes. The ethidium bromide should now have transferred to the upper butanol layer, which is then removed by pipeting and discarded appropriately.
3. The extraction is repeated one more time, then the lower DNA-CsCl solution is either dialyzed overnight against TE (against 100–200 vol of TE) to remove the CsCl, or it is diluted threefold and ethanol-precipitated directly (2 vol of ethanol are added plus NaAcetate is added to 0.1*M* final). If the plasmid is dialyzed, it usually results in a rather dilute DNA solution. It can be concentrated by ethanol precipitation.
4. Additionally, application of the ethidium bromide-stained DNA to a Dowex AG50W-X8 column will both remove the ethidium bromide and dilute the DNA enough for ethanol precipitation ***(5)***. A problem with ethanol precipitation of plasmid DNA containing CsCl is that the CsCl may precipitate out if the solutions are not diluted enough or are cooled below –20°C. Thus, it may be necessary to perform multiple precipitations or to dialyze the DNA to remove the CsCl completely.
5. Proper storage of the plasmid DNA is essential to maintain the DNA in a supercoiled state (*see* **Note 2**).

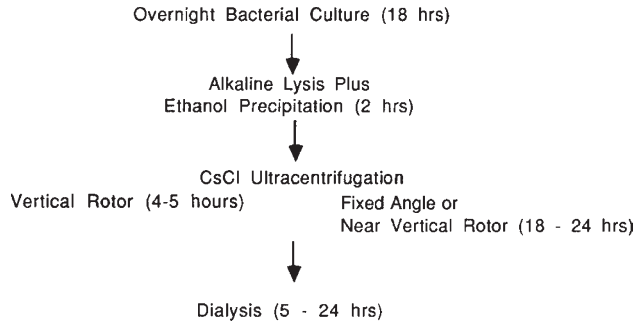

Fig. 1. Steps and time required for supercoiled plasmid isolation. Using the alkaline lysis/CsCl ultracentrifugation purification method, the above scheme shows the approximate time needed for complete isolation and purification of supercoiled plasmid DNA. Depending on some of the steps chosen, the time can vary from 29 to about 68 h.

A flowchart depicting the length of time for each step from growth of bacteria through dialysis is outlined in **Fig. 1**.

3.4. Analysis of Supercoiled Plasmid DNA Purified by Alkaline Lysis/CsCl Ultracentrifugation

1. To assay the supercoiled state of the plasmid DNA, purifed by the method above, one-dimensional (1-D) gel electrophoresis was then performed. One microgram of plasmid DNA, from the method above, was loaded onto a 1% agarose gel buffered with TBE and electrophoresed.
2. The electrophoresis run was stopped, and the gel was soaked in a dilute solution of ethidium bromide (0.25 µg/mL) to stain the DNA.
3. The gel was then exposed to short-wave UV light on a UV light box with appropriate Polaroid camera setup. Shown in **Fig. 2** is a photograph of the gel following exposure of the gel to the UV light. Indicated in the first lane on the left is the plasmid DNA immediately following purification by the above protocol. The intense fastest migrating band is negatively supercoiled DNA (SC), whereas the less intense slower migrating band is a negatively supercoiled "Dimer" of the plasmid DNA (two molecules linked). In the middle two lanes are shown the same DNA as in the first lane only following multiple freeze–thaw cycles. The additional faint band observed is nicked DNA, resulting from a break in one DNA stand, thereby allowing the DNA to relax, but remain circular. Supercoiled and nicked circular DNA have also been referred to as Form I and Form II DNA, respectively ***(5,6)***. Finally, incubation of the plasmid DNA with purified DNA topoisomerase I, capable of relaxing both negative and positive supercoiled DNA, results in a complete loss of the most supercoiled form of the DNA and generation of DNA topoisomers (lane of the right). The enzyme efficiently relaxes

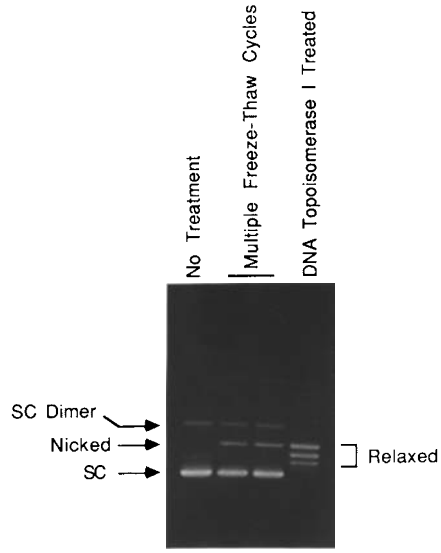

Fig. 2. Agarose-gel electrophoresis of supercoiled plasmid DNA. One microgram of plasmid DNA was electrophoresed on a 1% agarose/TBE gel. The gel was stained with ethidium bromide and photographed. The first lane on the left shows freshly isolated plasmid DNA. The two center lanes show plasmid DNA that have been subjected to multiple freeze–thaw cycles. The lane on the right shows plasmid DNA treated with purified DNA topoisomerase I prior to electrophoresis. The positions of supercoiled (SC), relaxed, and nicked DNA are shown. Also shown is the position of a supercoiled dimer of plasmid DNA (SC Dimer) (two supercoiled plasmids that are linked).

the supercoiled DNA, and as a result, the plasmid DNA migrates more slowly in the electrophoretic field, with the different topoisomers seen as distinct bands. The supercoiled dimer is also relaxed by the enzyme, and although it is difficult to see in **Fig. 2**, a pattern of very slowly migrating topoisomers of the dimer is also present in the gel. Thus, as can be seen by the analysis here, the alkaline lysis/CsCl ultracentrifugation procedure results in a purification of supercoiled plasmid DNA with no detectable contaminants.

4. Notes

1. It seems that all methods of DNA purification, including the CsCl method, can result in some low level of bacterial RNA contamination. To eliminate any detectable RNA, simply repeat the purification procedure. For example, remove the plasmid DNA from the centrifuge tube following the CsCl spin (about 2–3 mL), and simply add fresh CsCl solution (remember add 1 g of CsCl for every mL of water or TE) to bring the solution to about 13 mL. Then repeat centrifugation and process as described above.

2. DNA can be cleaved by UV light and by multiple freeze–thaw cycles (two common mechanisms). To prevent this, it is often best to freeze aliquots of the plasmid DNA in a nonfrost-free freezer, so that a single sample is protected from light and does not undergo multiple freeze–thaw cycles.
3. During the alkaline lysis procedure, it is important that you do not let the solution of sodium hydroxide/SDS stay in contact with the DNA for longer than 5 min because the plasmid DNA may begin to denature irreversibly.

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