

Traditional Methods for CsCl Isolation of Plasmid DNA by Ultracentrifugation

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Key Words

- Density Gradient Centrifugation
- Cesium Chloride Gradients (CsCl)
- Ultracentrifugation
- TFT80.2, TFT80.4, Stepsaver 65V13
- Plasmid DNA Purification

Introduction

Plasmids are pieces of extra-chromosomal DNA found in bacteria. They exist in double-stranded, closed circular form, and they carry genes which convey a variety of traits to the organism, such as antibiotic resistance and the production of virulence factors. Because plasmids are easy to handle and manipulate, they are utilized extensively in the cloning of genes and in the study of molecular biology. To clone a gene using a plasmid as either the vector or the source of the gene to be cloned (e.g. antibiotic resistance), plasmids of high purity must be obtained. A variety of procedures exist for the isolation and characterization of plasmid DNA. Generally, procedures involve growing the organism, harvesting cell material, lysing cells using a detergent solution with or without lysozyme, precipitating cell debris and chromosomal material and finally recovering plasmid DNA by precipitation. One traditional DNA purification technique in use since the 1950s is cesium chloride (CsCl)/ethidium bromide (EtBr) centrifugation (Radloff et al., 1967; Maniatis et al., 1982; and Meselson et al., 1957). Although the fundamentals of the technique have remained unchanged, advances in rotor design and an increased understanding of centrifugal theory have led to better control over protocol conditions and significantly reduced spin times (Flamm et al., 1966). Plasmid isolation in CsCl gradients has traditionally been accomplished in swinging bucket rotors spinning for 72 to 96 hours and often required large volumes of cell material (Anet and Strayer, 1969). However, with the introduction of vertical and fixed angle rotors, the centrifugation times and starting material amounts have been significantly reduced leading to

greater laboratory productivity and instrumentation and rotor life.

This application briefing will describe multiple procedures for CsCl isolation of plasmid DNA using three Thermo Scientific ultraspeed centrifuge rotors: StepSaver™ 65V13, TFT-80.2 and TFT-80.4.

Reducing Spin Times for the CsCl Isolation of Plasmid DNA

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In the following application brief, the use of the step-run feature in Thermo Scientific Sorvall® ultra-speed centrifuges with the Stepsaver 65V13 vertical rotor to reduce spin times during the preparation of plasmid DNA by CsCl isolation is described. Several procedures (standard protocol, step run, and layered sample) used for isolating the plasmid pRH43 from *Escherichia coli* (*E. coli*) are described.

Procedure

DNA Preparation

- Grow *E. coli* colony harboring plasmid pRH43 to an OD₆₀₀ = 0.5-0.9 in LB broth with chloramphenicol (0.2µg/mL)
- Harvest cells in a 250mL tissue culture tube by centrifuging in a Thermo Scientific Sorvall RC-3B Plus centrifuge at 2,119 x g (2,861 rpm) for 10 minutes at 20°C with the H-6000A swinging bucket rotor
- Lyse cells by boiling method using

a final concentration of 1mg/mL lysozyme

- Remove cellular debris by centrifuging viscous solution at 33,000 x g (15,000rpm) for 15 minutes at 20°C in a Thermo Scientific RC-6 Plus with the SA-600 rotor
- Precipitate nucleic acids by mixing supernatant with an equal volume of isopropanol and freeze at -20°C for 1 hour
- Pellet precipitated DNA by centrifuging at 21,000 x g (12,000 rpm) for 15 minutes at 4°C in the Sorvall RC-6 Plus with the SA-600 rotor
- Resuspend pellet in 4mL 0.1M Tris pH 8.0
- Incubate for 30 minutes at 37°C with 2mg/mL RNase
- Extract solution by the addition of equal volume of phenol
- Follow with two extractions with 24:1 chloroform:iso-amyl alcohol
- Obtain precipitated DNA by adding three times the volume of ethanol and freeze at -80°C for 1 hour
- Collect plasmid by centrifuging at 33,000 x g (15,000 rpm) for 15 minutes at 4°C in a RC-6 Plus with the SA-600 rotor
- Resuspend pellet in 500µL of 0.1M Tris, 0.5M EDTA, pH 8.0 (TE) buffer and store at 4°C

Multiple Methods for Density Gradient Centrifugation

Standard Protocol

- Bring DNA sample (500µL) to final volume of 4.0mL with TE buffer
- Add 400µL ethidium bromide (EtBr) (10mg/mL) and 4.4g of solid cesium chloride (CsCl). The refractive index after mixing is $n = 1.3865$ (1.35g/mL).
- Load solution into Thermo Scientific Ultracrimp™ tube, overlay with mineral oil and seal

- Spin tube overnight in a Stepsaver 65V13 rotor at 192,553 x g (45,000 rpm) at 20°C in a Thermo Scientific ultraspeed centrifuge
- Visualize DNA bands under long-wave UV light

Step Run

- Prepare samples and load into Ultracrimp centrifuge tubes as described for standard protocol
- Centrifuge tubes in Stepsaver 65V13 rotor at 308,941 x g (57,000 rpm) for 3 hours followed by centrifugation at 192,553 x g (45,000 rpm) for 3 hours at 20°C

Layered Sample

- Prepare CsCl cushion by mixing 3.5mL TE buffer, 360µL EB (10mg/mL) and 4.4g CsCl.
- Add solution to Ultracrimp tube
- Add 40µL EB (10mg/mL) to 500µL of DNA sample
- Gently layer DNA/EtBr sample on top of the CsCl cushion in the Ultracrimp tube, overlay with mineral oil and seal
- Centrifuge tube in Stepsaver 65V13 rotor at 308,941 x g (57,000 rpm) for 3 hours at 20°C

Results

The banding pattern for chromosomal (top band) and plasmid (bottom band) DNA in a typical overnight run at 192,553 x g (45,000 rpm) in the Stepsaver 65V13 vertical rotor is shown in Figure 1. The bands are well spaced, ensuring easy DNA removal, yet tight enough to allow recovery of the DNA in the minimal amount of volume. The banding pattern obtained for a 6 hour step-run (Figure 2, 3 hours at 308,941 x g (57,000 rpm) followed by 3 hours at 192,553 x g (45,000 rpm) is similar to that seen for the single overnight spin. Figure 3 illustrates the results seen when using the layered sample technique. A banding pattern similar to that seen in the typical overnight run was obtained in only three hours, spinning at 308,941 x g (57,000 rpm).

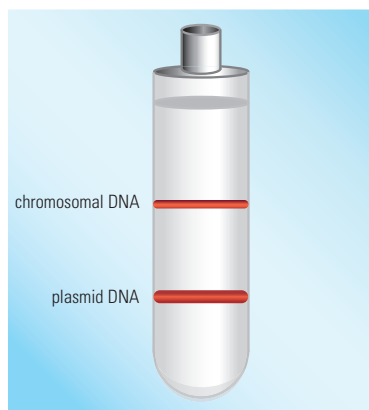


Figure 1: Standard protocol run overnight at 192,553 x g (45,000 rpm).

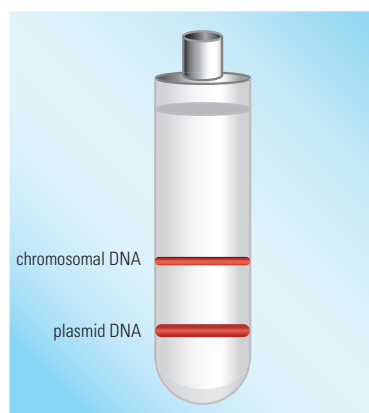


Figure 2: Step-run, 3 hours at 308,941 x g (57,000 rpm) followed by 3 hours at 192,553 x g (45,000 rpm).

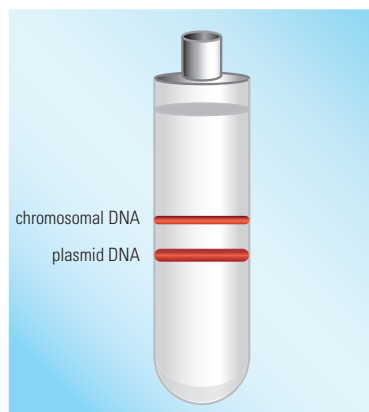


Figure 3: Layered sample three hours at 308,941 x g (57,000 rpm).

Discussion

Using the Thermo Scientific Stepsaver 65V13 and the ultracentrifuge step-run feature, altering the preparation of the sample and using layered sample technique, plasmid isolations on CsCl gradients can be completed within a convenient working day. By using these techniques,

laboratory productivity is increased while the life of the rotors and instrument are extended.

Isolation and Purification of Plasmid DNA from Samples Using the Thermo Scientific TFT80.2 Fixed Angle Ultraspeed Centrifuge Rotor

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A strain of bacteria causing a community or nosocomial (hospital-associated) outbreak of disease can be “fingerprinted” using plasmid content. DNA purification using CsCl gradients can allow for the identification and comparison of similar but structurally different plasmids from isolates of suspected strains. Such gradients, although useful, are usually not practical due to the large volumes of cell material that often must be used to visualize bands, and due to the fact that some medically important bacteria (e.g., *Neisseria gonorrhoeae*, *Legionella, spp.*) do not grow rapidly or in broth culture.

In this application brief, the laboratory of Martin and Schneider has found the method of Kado and Liu, with slight modifications using the fixed angle TFT80.2 ultraspeed centrifuge rotor, to be the simplest technique and the best technique for minimizing contamination of plasmid preparations with chromosomal debris from small volumes of bacterial cells.

Procedure

DNA Preparation

- Extract DNA by addition of phenol-chloroform
- Precipitate DNA by addition of ammonium acetate and cold 2-propanol for 30 minutes at -20°C
- Pellet DNA in microcentrifuge for 30 minutes at 4°C
- Carefully decant propanol and remove remaining propanol by evaporation under nitrogen
- Rehydrate pellet in 1mL TE buffer (10mM Tris, 1mM EDTA, pH 8.0)

Method for Density Gradient Centrifugation

- For CsCl purification, add 1mL of plasmid preparation to 2mL polypropylene ultraspeed centrifuge tube
- Add 1g of CsCl (approx. density 1.6g/mL)
- Cover with Parafilm M® and gently invert several times until CsCl goes into solution
- Add 80µL EtBr (10mg/mL) and place cap on tube
- Fill remaining space in tube with TE buffer using a needle and syringe
- Put set-screw into place
- Centrifuge material in TFT80.2 rotor for 16 hours (or overnight) at 292,330 x g (60,000rpm)
- Visualize plasmid DNA under UV light
- Collect DNA by puncturing side of tube with a 20 gauge needle and slowly withdraw lower of two resulting bands
- Remove ethidium (Et) by washing (x5) with isopropanol and desalt

Discussion

Multiple strains of *Neisseria gonorrhoeae* are prevalent in this country. This procedure allowed us to identify the strain of penicillinase-producing *N. gonorrhoeae* epidemic in southeastern Michigan as a strain possessing a 3.4MD betalactamase producing plasmid instead of the 4.7MD plasmid identified in previous Michigan isolates. We have also used this technique to recover a large quantity of a 72 MD plasmid associated with fimbriae production in strains of *Escherichia coli* 0157:H7, an organism associated with hemorrhagic colitis.

The TFT80.2 ultraspeed centrifuge rotor significantly simplifies the recovery of purified covalently closed circular forms of both small and large plasmid DNA from small volumes of bacterial cells. Using this we are able to identify specific pathogenic bacterial strains which will enable us to more clearly evaluate treatment and control measures.

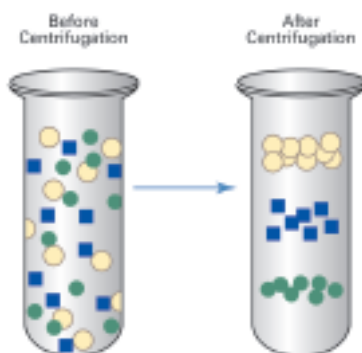
Rapid Isolation of Small Volume Plasmid DNA Using TFT80.4 Rotor

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Using our fixed angle TFT 80.4 ultraspeed rotor and the procedure described below, we were able to isolate several plasmids carrying human Factor IX and X gene fragments in a small volume of 3.5 mL in less than three hours.

Procedure



DNA Preparation

- Pellet bacterial cells from overnight culture by centrifugation at > 6,000 x g in the SLA-1500 for 10 minutes at 4°C and discard the supernatant
- Resuspend the cell pellet in 2 mL of buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8.0) and transfer to a 35 mL polypropylene tube
- Add 0.5 mL of lysozyme, (20 mg/mL in H₂O) and mix thoroughly. Incubate on ice for 10 minutes
- Add 5 mL of 0.2M NaOH, 1% SDS and mix gently. Incubate on ice for 10 minutes
- Add 4 mL of potassium acetate (29.4g potassium acetate; 11.5 mL glacial acetic acid. Add H₂O to 100 mL) and mix thoroughly). Incubate on ice for 10 minutes.
- Spin down the precipitate by centrifugation at 20 - 30,000 x g in an SS-34 for 15 minutes at 4°C and transfer the supernatant to a new tube
- Add heat-inactivated RNase A (20 µg/mL) to supernatant and incubate for 15 minutes at room temperature
- Extract with an equal volume of H₂O-saturated phenol:chloroform (1:1)
- Separate phases by centrifugation at 20 - 30,000 x g for 10 minutes

and transfer the upper aqueous phase to a new tube and repeat

- Transfer the upper aqueous phase to a new tube and extract with an equal volume of chloroform
- Separate phases by centrifugation at 20 - 30,000 x g for 2 minutes
- Transfer the upper aqueous phase into a new tube and add 1 volume of 3M sodium acetate (pH 7.0) followed by 2 volumes of ethanol. Keep on dry ice for 20 minutes
- Centrifuge at 20 - 30,000 x g for 15 minutes
- Decant the supernatant and wash with 70% alcohol and dry the pellet in a speed vac
- Dissolve the pellet in 510 µl TE (10 mM Tris HCl, 1 mM EDTA, pH 8.0) buffer in a microtube

Method for Density Gradient Centrifugation

- Add 900 mg CsCl to DNA solution and vortex to dissolve salt
- Spin the sample at 12,000 x g for 10 minutes to float any protein pellet which forms
- Transfer the liquid from below the protein pellet to another microtube and add 100 µL EtBr (10 mg/mL)
- To a 3.5 mL Ultracrimp™ tube add 1.6 mL CsCl (prepared by adding 0.65 g CsCl to 1 mL TE buffer)
- Underlay the CsCl with the DNA/EtBr/CsCl sample
- Gently fill the remaining portion of the tube with CsCl solution and seal
- Spin for 2.5 hours at 359,500 x g (70,000 rpm) at 20°C in the TFT 80.4 rotor
- Visualize the DNA bands under UV light
- Slice off the top of the tube and draw material off the top of the tube with a Pasteur pipet
- Discard all of the material above the plasmid, and save the lower plasmid band.
- Extract the EtBr twice with equal volumes of isopropyl alcohol (saturated with CsCl)
- Add 9 volumes of 0.3 M sodium acetate to the aqueous DNA sample followed by 20 volumes of cold ethanol. Keep on dry ice for 20 minutes and spin for 20 minutes at 20 - 30,000 x g
- Discard the supernatant and wash the pellet with 70% alcohol and

dry it. Dissolve the pellet in a known quantity of TE and test the concentrations of DNA by agarose gel electrophoresis and/or UV spectrophotometer

Discussion

The banding pattern seen for our experiments is typical of CsCl banded nucleic acids. Our plasmids were shown to be pure by electrophoresis. The yield of supercoiled DNA was 1-2 mg from 1,000 mL of culture broth.

The above protocol describes a rapid and convenient method for isolating plasmid DNA from small cell cultures. Using the small volume TFT 80.4 ultraspeed rotor, the procedure can be carried out in under three hours in a floor model ultraspeed centrifuge, with the purity and yield typical of larger volume rotors.

Conclusion

A variety of techniques have been established over the years to isolate plasmid DNA from crude lysate, including alkaline, boiling, and Triton mediated lysis (Heilig et al., 1998). However, density gradient centrifugation as described in this applications brief is the method of choice amongst the traditional techniques because it yields high-quality plasmid DNA free of most contaminants (Heilig et al., 1998). The biggest drawbacks with CsCl/EtBr centrifugation are the long spin times and the use of ethidium bromide (a mutagen).

Alternative Methods to the Traditional Approach

Recently, less time consuming and less hazardous methods for the isolation of plasmid DNA from small and large scale volumes have been developed. Commercial plasmid DNA preparation kits are available from many life science manufactur-

ers (Table 1). These widely available kits use a column format with binding matrix to bind DNA instead of subjecting DNA to a CsCl gradient. Also, instead of ultraspeed centrifugation, the procedures can be conducted using Thermo Scientific microcentrifuge and lowspeed benchtop centrifuge models which can decrease DNA preparation time to under an hour. Although, these modern techniques are less time consuming, some procedures may still require the use of the traditional methods of plasmid DNA isolation, including the separation of different DNA isotypes (ie, heavy (P³²) and light DNA).

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Clontech	NucleoSpin®	Spin or vacuum	Silica membrane	96 well, mini-midi-, and maxi-prep
Mo Bio Laboratories, Inc.	Ultra Clean™ Kits	Spin	Silica membrane	Endotoxin-free 96 well, mini-, midi-, maxi-prep
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