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 process. Because plasmids are 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**

*Dr. Mark W. Schwartz and Rosanna A. Fischer*

In the following application brief, the use of the step-run feature in Thermo Scientific Sorvall® ultraspeed 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 pH43 from Escherichia coli (E. coli) are described.

**Procedure**

**DNA Preparation**

- **Grow** E.coli colony harboring plasmid pH43 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 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 (15,000 rpm) 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).

**Discussion**

Using the Thermo Scientific Step-saver 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 1 mL of plasmid preparation to 2 mL polypropylene ultracentrifuge tube
- Add 1 g of CsCl (approx. density 1.6 g/mL)
- Cover with Parafilm M® and gently invert several times until CsCl goes into solution
- Add 80 µL EtBr (10 mg/mL) and place cap on tube
- Fill remaining space in tube with TE
- Centrifuge material in TFT80.2 rotor for 16 hours (or overnight) at 292,330 x g (60,000 rpm)
- 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
- Add heat-inactivated RNAse A (20 µg/mL) to supernatant and incubate for 15 minutes
- Extract with an equal volume of 2-propanol and desalt
- Underlay the CsCl with the DNA/EtBr/CsCl sample
- Gently fill the remaining portion of the tube with CsCl solution and seal
- Spin the liquid from below the protein pellet to another microtube and add 100 µL EtBr (10 mg/mL)
- Add 0.5 mL of lysozyme, (20 mg/mL in H2O) and mix thoroughly. Incubate on ice for 10 minutes
- Add 20 volumes of isopropyl alcohol (sat. with CsCl)
- Add 1.6 g CsCl to 1 mL TE buffer
- Separation in strains of *Escherichia coli* 0157:H7, an organism associated with hemorrhagic colitis.
- Multiple strains of *Neisseria gonorrhoeae* are prevalent in this country.
- 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 TE

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 3.4 MD beta-lactamase producing plasmid instead of the 4.7 MD 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 ultracentrifuge 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 ultracentrifuge rotor and the procedure described below, we were able to identify 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 (29.4 g potassium acetate; 11.5 g 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 for 10 minutes
- Add 4 mL of potassium acetate (29.4 g potassium acetate; 11.5 g 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 for 10 minutes
- Add heat-inactivated RNase A (20 µg/mL) to supernatant and incubate for 15 minutes at room temperature
- Extract with equal volume of H2O-saturated phenol:chloroform (1:1)
- Separate phases by centrifugation at 20 - 30,000 x g for 10 minutes
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 application 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 manufacturers (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 ultraspread 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).

References

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