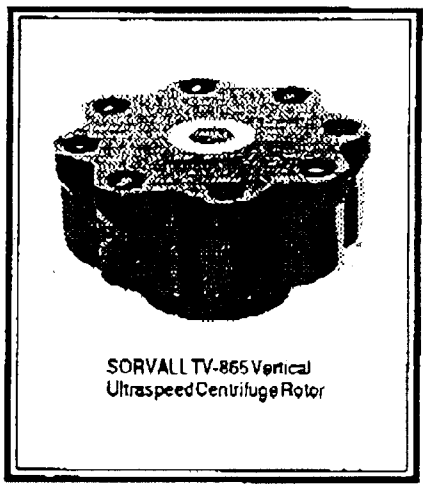
Du Pont
SORVALL®

APPLICATIONS BRIEF

DR EARL ROBERTSON

REDUCING SPIN TIMES FOR THE CsCl ISOLATION OF PLASMID DNA

DR. MARK W. SCHWARTZ
ROSANNA A. FISHER
SORVALL Applications Laboratory
Wilmington, DE 19898



SORVALL TV-865 Vertical Ultraspeed Centrifuge Rotor

INTRODUCTION

Plasmids are pieces of extrachromosomal 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. 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. The most common technique for this purification is cesium chloride (CsCl)/ethidium bromide (EB) centrifugation.[1,2] This technique has been in use since the 1950s.[3] Although the fundamentals of the technique have remained unchanged, advances in rotor design and an increased understanding of centrifugal theory[4] have led to better control over experimental conditions and significantly reduced spin times.

Plasmid isolation in CsCl gradients traditionally have been accomplished in swinging bucket rotors for 72 to 96 hours.[5] With the introduction of vertical rotors by SORVALL centrifuges in the late 1970s, these spin times were reduced significantly. Dr. Clarence Kado, University of California, Davis, has described a procedure for preparation of a pure plasmid in the SORVALL TV-865 vertical rotor in 12 hours, or more conveniently, overnight.[6] In this paper, we describe the use of the step-run feature of SORVALL ultraspeed centrifuges to further reduce spin times. Also, by layering the DNA sample to be purified onto a CsCl cushion (layered sample technique), rather than starting with a homogeneous CsCl/ethidium bromide/DNA solution, plasmid isolation spin times can be completed within half a day.

Below are several procedures utilized in our laboratory for isolating the plasmid pRH43 from E. coli.

PROCEDURE

An E. coli colony harboring plasmid, pRH43, is grown to an OD_{600} = 0.5-0.9 in LB broth. Chloramphenicol (0.2 ug/mL) is added to amplify DNA growth with further incubation. Cells are harvested in a 250 mL tissue culture tube by spinning in a SORVALL RC-3B+ centrifuge at 2,861 rpm (2119 x g) for 10 minutes at 20°C with the H4000 swinging bucket rotor. The cells are lysed by the boiling method using a final concentration of 1 mg/mL lysozyme. The cellular

debris is removed by centrifuging the viscous solution at 15,000 rpm (33,000 x g) for 15 minutes at 20°C in a SORVALL RC-5C (SA-600 rotor). The nucleic acids are then precipitated by mixing the supernatant with an equal amount of isopropanol followed by freezing at -20°C for one hour. Precipitated DNA is pelleted at 12,000 rpm (21,000 x g) for 15 min. at 4°C in an SA-600. The pellet is resuspended in 4 mL .01 M Tris pH 8.0, then incubated for 30 min. at 37°C with a 2 mg/mL RNase. The solution is extracted twice with an equal volume of phenol followed by two extractions with 24:1 chloroform:isoamyl alcohol. A final precipitation step is accomplished by adding three times the volume of ethanol and freezing at -70°C for one hour. The plasmid is collected by centrifuging at 15,000 rpm (33,000 x g) for 15 min. at 4°C in an SA-600 rotor. The pellet is resuspended in 500 µl of 0.01 M Tris, 0.5 M EDTA, pH 8.0 (TE) buffer and stored at -4°C.

DENSITY GRADIENT CENTRIFUGATION

Standard Protocol[2,6] - The DNA sample (500 µl) in TE buffer is brought to a total volume of 4.0 mL with TE buffer. 400 µl EB (10 mg/mL) is added, followed by 4.4 gm of solid CsCl. After mixing, the refractive index is $\eta = 1.3865$ (~~1.385~~ gm/mL). The solution is loaded into an Ultracrimp™ tube (P/N 03945), overlayed with mineral oil, and is sealed with the ultracrimp tool. The tube is spun overnight in a TV-865 rotor at 45,000 rpm at 20°C in a SORVALL ultraspeed centrifuge. The DNA bands are visualized under long wave UV light.

Step-Run - The samples are prepared and loaded into the Ultracrimp ultraspeed centrifuge tubes as described above for the standard protocol. The SORVALL ultraspeed centrifuge is programmed to spin the TV-865 rotor for three hours at 57,000 rpm followed by three hours at 45,000 rpm at 20°C.

Layered Sample - The CsCl cushion is prepared by mixing 3.5 mL TE buffer, 360 µl EB (10 mg/mL) and 4.4 gm CsCl. This solution is added to the Ultracrimp tube. To 500 µl of the DNA sample, add 40 µl EB (10 mg/mL). Gently layer the DNA/EB sample on top of the CsCl cushion in the Ultracrimp tube. Top off with mineral oil and seal the tube with the Ultracrimp tool. Spin the sample in the TV-865 rotor for three hours at 57,000 rpm at 20°C.

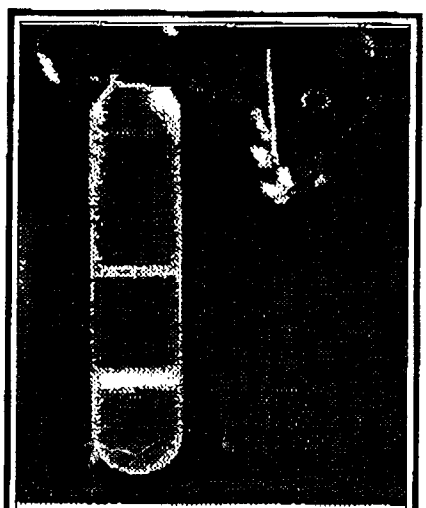


FIGURE 1: Standard protocol spun overnight at 45,000 rpm

RESULTS

The banding pattern for chromosomal (top band) and plasmid (bottom band) DNAs in a typical overnight run at 45,000 rpm in the TV-865 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 six hour step-run, (Figure 2)

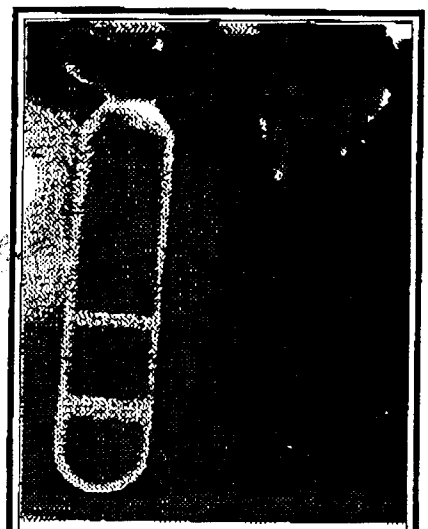


FIGURE 2: Step-run, three hours at 57,000 rpm followed by three hours at 45,000 rpm

(three hours at 57,000 rpm followed by three hours at 45,000 rpm) is similar to that seen for the single speed 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 57,000 rpm.

DISCUSSION

The centrifugation time in the TV-865 vertical rotor can be reduced significantly by utilizing the step-run technique. The re-

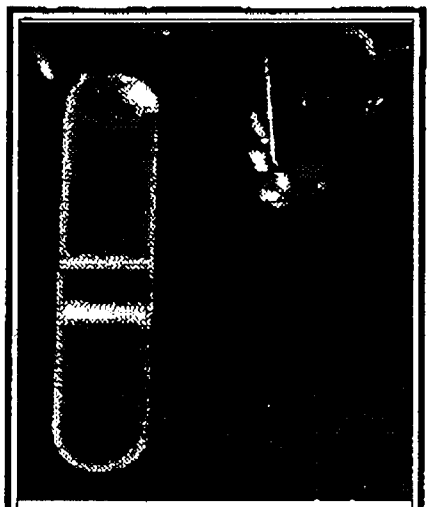


FIGURE 3: Layered sample three hours at 57,000 rpm

sults obtained by this method are similar to those obtained using the traditional overnight isopycnic run in one half to one third the time. By initially spinning the rotor at a rapid rpm, the banding of the DNA is accelerated, and the subsequent slower spin pre- serves the shallow gradient providing good resolution of the DNA species. Using this technique, the separation of the DNA obtained by spinning overnight at 45,000 rpm can be matched in six hours. This procedure requires no change in preparation of the gradients. The step change in time and speed is easily programmed into the SORVALL ultraspeed centrifuge and takes place automatically. Usingboth the delayed start and step-run features of the SORVALL ultraspeed centrifuge, runs can be set up to meet the lab's schedule. For example, the run can be programmed to start in the middle of the night. The step change occurs automatically before dawn and the run is finished in the morning.

The step-run and delayed start features extend the life of the centrifuge and the rotor, saving the lab money and time.

To further reduce spin time, the layered sample technique has been developed. This technique requires a little extra work in preparing the sample for centrifugation, but utilizing it can reduce spin time in the vertical rotor to as little as three hours. By layering the sample on top of the CsCl in a discrete zone, the DNA is allowed to enter the forming gradient during centrifugation as a band, with the more dense plasmid band migrating faster than the less dense chromosomal band. Figure 3 shows the resolution of plasmid and chromosomal DNAs obtained using the layered sample technique following a three hour spin at 57,000 rpm. It requires a little extra effort to prepare the layered sample, but once the procedure is established, it can be incorporated into the laboratory's routine plasmid preparations resulting in significant time savings, while freeing up the centrifuge for other experiments.

CONCLUSION

Although a variety of techniques have been established over the years to isolate plasmid DNA, density gradient centrifugation remains the method of choice. The biggest drawback with CsCl/EB centrifugation has been the long spin times. Besides extending the time required to obtain a pure sample, the excessive spin time ties up equipment for days, and adds hours of wear and tear to the centrifuge and rotor. Using the Du Pont SORVALL vertical rotor, TV-865, and the ultraspeed centrifuge step-run feature, plasmid isolations on CsCl gradients can be completed within a convenient working day. No change is required in the gradient recipe, yet spin times can be reduced to one third of the time required for a standard run. By altering the preparation of the sample and using the layered sample technique, spin time can be reduced to only three hours. Although this does require a change in the sample preparation procedure, a significant time savings can be realized when using this technique. By using either of these techniques, laboratory productivity is increased while the life of the rotors and instrument are extended.

REFERENCES

1. Radloff, R., W. Bauer, and J. Vinograd 1967. Proc. Nat. Acad. Sci. USA 57:1514-1521.
2. Maniatis, T., E.F. Fritsch, and J. Sambrock. 1982. Molecular Cloning, A Laboratory Manual. Cold Spring Harbour Laboratory. USA:86-96.
3. Meselson, M., F.W. Stahl, and J. Vinograd. 1957. Proc. Nat. Acad. Sci. USA 43:581-583.
4. Flamm, N.F., H.E. Bond, and H.E. Burr. 1966 Biochim. Biophys. Acta 129:310-317.
5. Anet, R. and D.R. Strayer, 1969. Biochem. Biophys. Res. Comm. 34:328.
6. Kado, C.I. 1987. SORVALL Applications Brief 27.