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Separation and Purification of Plasmid Mixtures by Continuous Elution Electrophoresis

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Isolation and purification of covalently closed circular (CCC) plasmid DNA is necessary for a wide range of molecular procedures. For many applications, standard plasmid isolation methods provide plasmid DNA of adequate quality. However, there are selected situations when a mixture of plasmids exist, such as with the use of supF-containing plasmids (10) and assays or systems involving co-transfections. Plasmids that contain the supF selection marker have advantages for use in particular procedures, but this selection system necessitates growth in bacteria containing a helper plasmid, such as p3.

The fact that standard plasmid purification techniques co-purify both the supF-containing and helper plasmids have been an obstacle to the wider use of these plasmids, as the separation and purification of individual plasmids from mixtures of plasmids have been problematic. We have developed a technique for purifying CCC plasmid DNAs that differ in mass as part of an antitumor "naked DNA" or polynucleotide vaccine (PNV) strategy. This purification technique makes use of the Model 491 Prep Cell (Bio-Rad Laboratories, Hercules, CA, USA) and continuous elution electrophoresis, which purifies the supF-containing plasmid, free from the p3 helper plasmid and RNA.

The development of PNV strategies and the promise of human clinical trials have focused attention on purification procedures for generating therapeutic DNA preparations containing only CCC plasmids. Most PNV vectors contain antibiotic resistance gene coding sequences within the vector backbone, which have raised concerns both from immunologic and regulatory standpoints (4,12). There are established vectors that do not contain intact antibiotic resistance gene sequences, such as those containing the supF gene sequence, and these supF-containing plasmids require growth in concert with an appropriate helper plasmid such as p3 (10). The p3 helper plasmid, contained in a number of commercially available strains of *E. coli* (DH10§/p3; Life Technologies, Rockville, MD, USA; and MC1038, Top 10F/p3; Invitrogen, Carlsbad, CA, USA), is a 60-kb plasmid containing an intact kanamycin resistance coding sequence along with tetracycline and ampicillin coding sequences, each with inactivating amber stop codon point mutations. The supF gene product encodes a mutant prokaryotic tRNA that complements these mutations and allows for growth under dual ampicillin and tetracycline selection.

To apply the supF/p3 selection system to our application, it was necessary to develop an alternative purification procedure for isolating different-sized plasmids in their native CCC state. Continuous elution electrophoresis using the Model 491 Prep Cell is an electrophorectic method originally designed for purifying proteins in polyacrylamide gels. Although restriction fragments (9) and RNA (2,6) have been separated using agarose and polyacrylamide mixtures in the Prep Cell, exposure to ethidium bromide and acrylamide was not desirable for a potentially therapeutic plasmid DNA product. Thus, we modified the Prep Cell to run pure agarose gels. This led to the purification of high-quality CCC plasmid while avoiding exposure of the DNA to both acrylamide and ethidium bromide, thus limiting the acquisition of potential toxic contaminants during the isolation and purification procedure.

This continuous elution electrophoresis procedure provides an alternative isolation/purification procedure that allows for the separation of individual plasmid components from a plasmid mixture and makes the use of supF-containing plasmids for PNV and other procedures more feasible. We grew and isolated a number of supF-containing plasmids, including pCDM8 obtained from Invitrogen, and our PNV construct designated pITL (Wetzel et al. unpublished) in *E. coli* strain DH10§/p3 purchased from Life Technologies. DH10§/p3 was transformed with these plasmids using a standard heat shock procedure (8) and grown in Luria-Bertani (LB) Broth (8) under dual selection of 50 µg/mL ampicillin and 10 µg/mL tetracycline at 39°C–40°C. Cultures at an A_{600} of approximately 0.5 were amplified with 10 µg/mL chloramphenicol and grown for an additional 16–18 h.

Initial plasmid mixture preparations were purified by CsCl-ethidium bromide density gradient centrifugation before use for evaluating continuous elution electrophoresis. Subsequently, crude lysate was prepared from the bacteria using Triton® X-100 lysis (8) and treated with RNase A/T1. Electrophoresis was carried out using the Model 491 Prep Cell. The gel casting tube was initially modified by replacing the standard glass cylinder with a 1.75-inch (OD) Eastman Tenite Butyrate plastic tube obtained from Consolidated Plastics (Twinsburg, OH, USA). Subsequently, more rigid lucite tubes were prepared by the onsite machine shop from standard lucite stock and used in an identical manner with equal efficacy.

The agarose gels consisted of two parts. A 3–5 mm base layer of 2% SeaKem® Gold agarose (BMA, Rockland, ME, USA) was poured, and following polymerization of this layer, an 8-cm, 1.0% or 0.8% agarose gel was poured on top of the initial base layer. Crude lysate or CsCl-ethidium bromide-purified plasmid preparations were loaded onto the gel and electrophoresed overnight at 135 V in 1× TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA, pH 8.0). Sample was eluted at a rate of 0.25 mL/min using a peristaltic pump (Rainin Instrument, Woburn, NJ, USA), routed through a UV detector and collected with a fraction collector (both from Amersham Pharmacia Biotech, Piscataway, NJ, USA). A254 readings were recorded on a two-channel chart recorder. Sequential fractions (approximately 4.5 mL) corresponding to the plasmid DNA peak on the chromatogram were collected. Twenty microliters per fraction were analyzed on a 0.8% agarose slab gel. Bands were visualized by staining with ethidium bromide.

Initial experiments using plasmid DNA purified by CsCl density ultracentrifugation allowed us to establish the optimal agarose concentration and gel height. We found that an 8-cm gel provided adequate separation and purification of the supF-containing plas-

mid from its p3 helper plasmid. We were able to consolidate the number of fractions containing the plasmid of interest by incorporating the basal layer of higher concentration agarose. Similar results were obtained using RNase-treated crude lysate preparations. We found that the use of Triton X-100 lysis for initial plasmid isolation increased the yield of CCC plasmid DNA within the preparations over standard alkaline lysis procedures. Thus, all subsequent crude lysate experiments were performed with Triton X-100 lysates.

Figure 1A is a chromatogram depicting a typical A254 elution profile. Analytical gel analysis of fractions corresponding to the peak on the chromatogram demonstrates elution of the 1.8-kb therapeutic plasmid (Figure 1B). The p3 plasmid is retained in the cylindrical agarose gel (Figure 2). We have confirmed that these parameters allow for the isolation and purification of plasmids ranging in size from 1.8 to 3.5 kb (pITL and pCDM8, respectively).

We have developed a continuous elution electrophoresis method for purifying plasmids differing in size. This methodology makes the use of supF-containing plasmids feasible in applications in which contaminating p3 helper plasmid is not acceptable. This includes PNV applications and some transfection applications. The Prep Cell was originally designed for polyacrylamide gels and has been used extensively for protein purification. Little work, however, has been performed utilizing this instrumentation for nucleic acids. Research in this area has been restricted to the purification of DNA fragments (7,9) and RNA transcripts (2,6).

Although the use of continuous elution electrophoresis for micropreparation of plasmid DNA was described in 1991 (1), to our knowledge, our laboratory is the first to use the Prep Cell to separate a mixture of distinct plasmid DNA species differing in molecular

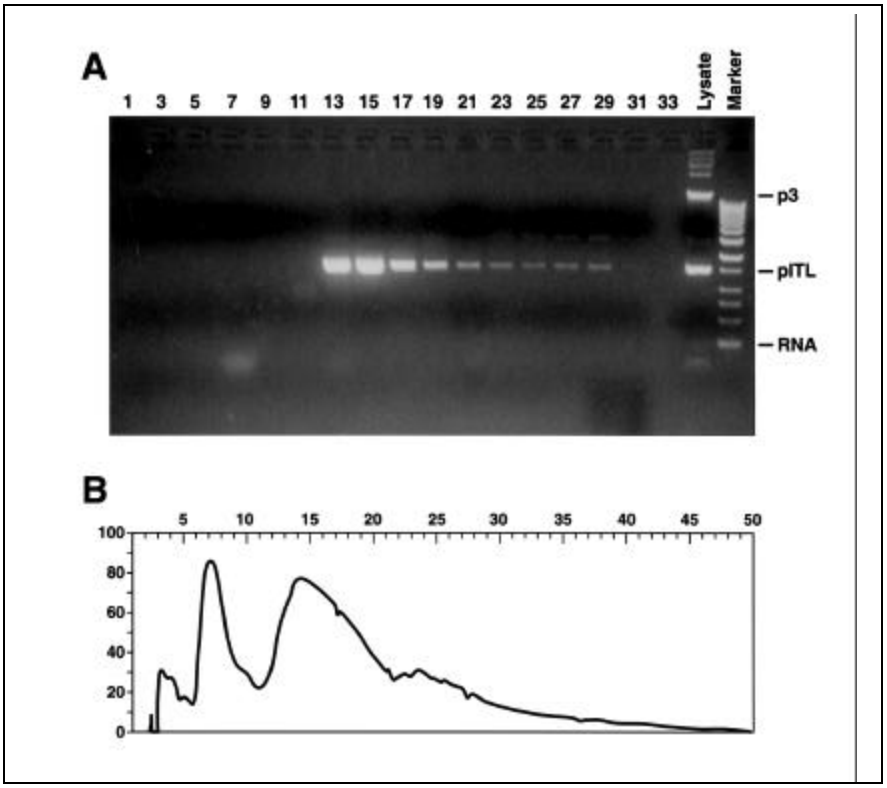

Figure 1. Purification of the supF-containing plasmid from its p3 helper plasmid. (A) 0.8% agarose gel of an aliquot from every other fraction: residual RNA can be seen best in lane 7, purified supF-containing plasmid in lanes 13–29; after a single blank lane, the last two lanes contain an aliquot of the starting crude lysate material and 250 bp to 12 kb DNA standard from Stratagene (La Jolla, CA, USA), respectively. (B) Representative chromatogram, matching the gel in panel A, showing UV A260 versus fraction No. This chromatogram was terminated before elution of the P3 plasmid.

weight using a pure agarose-based format. We initially evaluated ion exchange chromotography for separating and purifying supF-containing plasmids. This is a well-established technique for the analysis and quantification of charged species and has been successfully applied to the separation of nucleotides (3), DNA restriction fragments (13), and plasmid DNA from crude lysate (5). We hypothesized that anion exchange chromatography may also separate plasmids differing in molecular weight due to the relative charge difference. In our hands, this technique proved to be cumbersome and provided suboptimal plasmid separation (data not shown). We were, however, able to confirm that the capacity to separate intact plasmids is restricted to nonporous resins versus 1000 porous resins (11).

Several aspects of our modified Prep Cell procedure deserve comment. First, replacing the glass gel casting tube with one made of plastic allowed us to cast pure agarose gels in contrast to the acrylamide/agarose mixtures suggested for use with the glass tubing for the separation of restriction fragments. SeaKem gold agarose was chosen because of its higher tensile strength and ability to maintain its integrity over the course of the run without shrinkage. One group has reported separation of DNA fragments using SeaKem Gold and continuous elution electrophoresis after finding, similar to our report, that anion exchange chromatography was inadequate for their purposes (7). The use of this agarose dramatically reduced the leakage of sample between the cooling core and/or the outer casting tube. Additionally, results from several empty runs demonstrated that SeaKem Gold agarose added no additional endotoxin to the final purified product (data not shown). The use of RNase A/T1 facilitated the separation of the smaller plasmid from the residual RNA. Finally, we incorporated a higher percentage agarose base layer, which acted as an inverted stacking gel, compacting the separated plasmids and thereby reducing the volume of the pooled fractions.

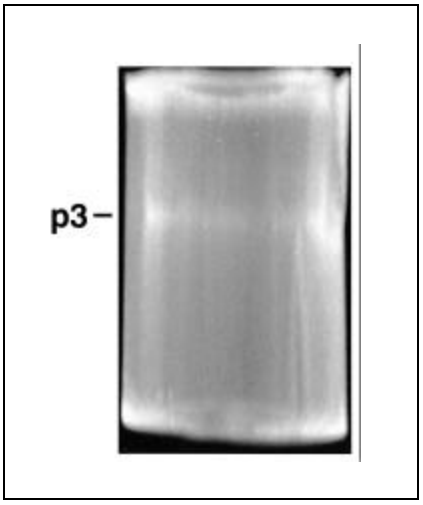

Figure 2. Cylindrical gel with retained p3 plasmid cylindrical agarose gel showing the retained p3 plasmid (arrow). After the run was completed, the gel was removed from the apparatus and stained with ethidium bromide (0.5 µg/mL) for 1 h before being photographed.

We have used the Model 491 Prep Cell to obtain CCC plasmid DNA for PNV. Our results clearly demonstrate that this instrumentation can be successfully modified for use with agarose gels. The procedure is efficient and reproducible; we have performed well over 50 individual runs without significant variation in separation and subsequent isolation of individual plasmids. This procedure can be used for separating/purifying CCC plasmid DNA species, differing in molecular weight, from a mixture of plasmids and thus removes an obstacle from the use of supF-containing plasmids for PNV and other molecular procedures where contaminating p3 helper plasmid is undesirable.

REFERENCES

1. **Cao, T.M. and Y.H. Ing.** 1991. Gel electrophoresis for the microppreparation of plasmid DNA. Anal. Biochem. *192*:251-253.
2. **Cunningham, K., K. Kittikamron and Y. Lu.** 1996. Preparative scale purification of RNA using an efficient method which combines gel electrophoresis and column chromotography. Nucleic Acids Res. *24*:3647-3648.
3. **Kremmer, T., E. Paulik and M. Boldizsar.** 1989. Application of the fast protein liquid chromotographic system and Mono Q HR 5/5 anion exchanger to the separation of nucleotides. J. Chrom. *493*:45-52.
4. **Marcus-Sekura, C.J.** 1994. Regulatory issues relating to the use of recombinant vectors in production of human vaccines. Dev. Biol. Stand. *82*:107-112.
5. **McClung, J.K. and R. A. Gonzales.** 1989. Purification of plasmid DNA by fast protein liquid chromotography on Sepharose 6 preparative grade. Anal. Biochem. *177*:378-382.
6. **Nguyen, T.H., L.A. Cunningham, K.M. Hammond and Y. Yu.** 1999. High-resolution preparative-scale purification of RNA using the Prep Cell. Anal. Biochem. *269*:216-218.
7. **Rodriguez, H. and S.A. Akman.** 1998. Large scale isolation of genes as DNA fragment lengths by continuous elution electrophoresis through an agarose matrix. Electrophoresis *19*:646-652.
8. **Sambrook, J., E.F. Fritsch and. T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual. CSH Laboratory Press, Cold Spring Harbor, NY.
9. **Schlax, P.E., Jr., M.W. Capp and M.T. Record, Jr.** 1995. Preparative-scale purification of DNA restriction fragments by continuous-flow gel electrophoresis. BioTechniques *18*:94-100.
10. **Seed, B.** 1989. Purification of genomic sequence from bacteriophage libraries by recombination and selection in vivo. Nucleic Acids Res. *12*:5627-5633.
11. **Thompson, J.A.** 1986. A review of high performance liquid chromatography in nucleic acids research. III. Isolation, purification and analysis of supercoiled plasmid DNA. Biochromatography *1*:68-80.
12. **van Hall, T., N.E. van de Rhee, S.P. Schoenberger, M.P.M. Vierboom, F.A.W. Verreck, C.J.M. Melief and R. Offringa.** 1998. Cryptic open reading frames in plasmid vector backbone sequences can provide highly immunogenic cytotoxic T-lymphocyte epitopes. Cancer Res. *58*:3087-3093.
13. **Westman, E., S. Erikssön, T. Låås, P.A. Pernemalm and S. Sköld.** 1987. Separation of DNA restriction fragments by ion exchange chromatography on FPLC columns Mono P and MONO Q. Anal. Biochem. *166*:158-171.

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