

Extending the Upper Limits of Pulsed Field DNA Electrophoresis Using Programmed Voltage Gradients

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Introduction

Pulsed field gel electrophoresis of chromosome sized DNA has become a valuable technique for genetic analysis.¹ In our work with the cellular slime mold *Dictyostelium discoideum*, it is important for us to separate whole chromosomes in the range of 1 to 10 million base pairs (mb). In early attempts to separate *D. discoideum* chromosomes by clamped homogeneous electric field (CHEF) electrophoresis, we could detect only four bands on ethidium bromide stained gels.² Since the literature suggested six or seven chromosomes of different sizes,³ we studied other parameters to see if we could resolve chromosomes in the 6 mb range and beyond. We found that by stepping down the voltage during electrophoresis, molecules with an apparent mass greater than ~6 mb could be detected.

The following protocol outlines our methods. It has been applied successfully to many different strains of *D. discoideum* isolated from the wild, as well as numerous strains of the related genus *Polysphondylium*. There are two principal differences between our protocol and others.⁵ First, as mentioned above, the crisp separation of very large chromosome sized DNA molecules requires programmed decreases in the voltage gradient. Second, we have found it necessary to incubate our cell plugs with EDTA before adding detergent and protease.

Sample Preparation

1. Grow cells to stationary phase. In our experiments, the cells are grown on *E. coli* B/r. Cells grown on two 15 cm plates of GYP agar (1 g glucose, 1 g Difco Bacto peptone, 0.25 g Difco yeast extract, 4.2 g KH_2PO_4 , 25 g Difco agar, 1 L H_2O) give enough cells for several dozen samples (plugs).
2. Harvest starved cells in phosphate buffer (PB: 2.25 g Na_2HPO_4 ; 7 H_2O , 1.14 g KH_2PO_4 , 1 L H_2O), wash, and resuspend at $1.5\text{--}2 \times 10^8/\text{ml}$ in PB.
3. Prepare 2% low melting point agarose in PB. Melt and cool to 39 °C.

4. Briefly warm the cells to 39 °C and mix with an equal volume of 2% low melting point agarose. Pipette 25 μl aliquots into a plug mold sealed at the bottom with tape. Allow to solidify for a few minutes at 4 °C.
5. Push the solidified plugs out into the wells of a flat-bottomed microtiter dish, each well containing 100 μl of 0.5 M EDTA, pH 9.5. Incubate 60 minutes at room temperature.
6. Add 100 μl of 0.5 M EDTA, pH 9.5, containing 2 mg/ml proteinase K (BRL) and 2% sodium lauryl sacosinate. Seal the plates tightly in polyethylene bags and incubate 24 to 48 hours in a 50 °C water bath.
7. Store the plates in their bags at 4 °C. Plugs prepared and stored in this way give good electrophoretic patterns for at least 2 years.

Electrophoresis

Gels are made with 0.8% DNA grade agarose in running buffer (27 mM Tris base, 27 mM boric acid, 0.75 mM EDTA, pH 8.5). Agarose plugs are inserted into the wells and sealed in place with 0.8% agarose in running buffer. Electrophoresis is for 264 hours at 10 °C in running buffer. Pulse time is increased linearly from 2,000 to 9,600 seconds during the run. Initial voltage is 1.85 V/cm. For successful separation of molecules larger than 6 mb, the voltage is decreased to 1.48 V/cm at a pulse time of 7,000 seconds. Block 1: 2,000-7,000 seconds, 158 hours, 62 V or 1.85 V/cm; Block 2: 7,000-9,000 seconds, 82 hours, 50 V or 1.48 V/cm. Electrophoresis was carried out with a specially modified Bio-Rad 200/2.0 power supply; however, the CHEF Mapper™ system can perform the same voltage stepping.

Results

Our results, which compare identical runs with and without stepped voltage gradients, are shown in Figure 1A. Molecules with an apparent mass of greater than ~6 mb are lost at the higher constant voltage. Gels run at constant voltage for various intermediate times (~80 hour intervals, not shown) revealed that the largest molecules were present for approximately half the run, but then disappeared with kinetics approximating a multi-hit curve.

The majority of the chromosome-sized DNA molecules hybridized unambiguously to our cDNA probes and could be assigned to known linkage groups. There is good agreement between the published linkage map and the electrophoretic karyotype.⁴ This supports our claim that the molecules separated under the stated conditions are, indeed, intact chromosomes of *D. discoideum*. Our conditions also reveal differences in the electrophoretic patterns of closely related strains (Figure 1B).

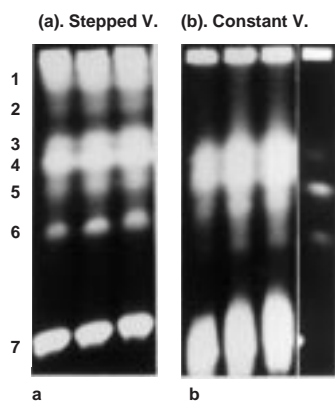


Fig. 1A. Separation of *D. discoideum* chromosomes by CHEF electrophoresis with and without programmed voltage changes. (a). *D. discoideum* NC4 chromosomes separated in 0.8% agarose, 0.3x TBE buffer. Run time, 264 hours; pulse time, 2,000-9,600 seconds, linearly increasing; $V=1.85$ V/cm from 2,000 to 7,000 seconds, then 1.48 V/cm from 7,000 to 9,600 seconds. **(b).** A parallel run with the same samples and identical conditions with 1.85 V/cm throughout the run. The *Schizosaccharomyces pombe* markers (right lane) have molecular masses of 5.7, 4.6, and 3.5 mb.⁶

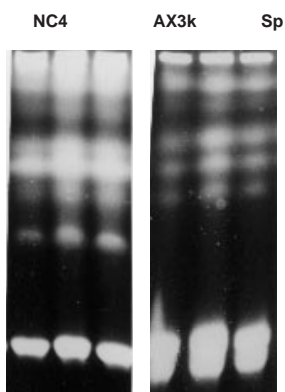


Fig. 1B. The electrophoretic karyotype for two strains of *D. discoideum*. Strains NC4 and AX3k were separated in 0.8% agarose, 0.3x TBE buffer. Run time, 264 hr; pulse time, 2,000-9,600 sec, linearly increasing; $V=1.85$ V/cm from 2,000 to 7,000 sec, then 1.48 V/cm from 7,000 to 9,600 sec. Other conditions, the identity of the electrophoretic bands, and the approximate molecular masses of each chromosome may be found in reference 4.

Judged by the mobilities of these molecules relative to that of the largest chromosome of *S. pombe*, these are the largest chromosomes to have been separated by CHEF electrophoresis.¹ The largest chromosome resolved in this study is ~9 mb, as shown in Figure 2.

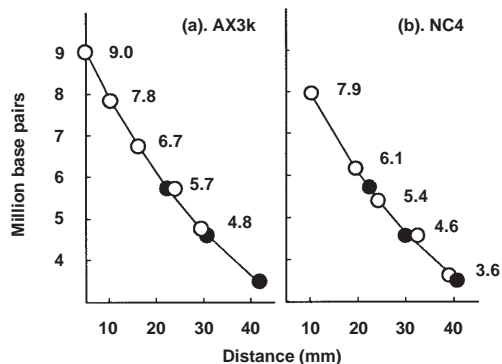


Fig. 2. Apparent molecular masses of *D. discoideum* AX3k and NC4 chromosomal DNA. In this figure the apparent molecular mass in millions of base pairs is plotted as a function of distance migrated. AX3k sums to 34 mb, NC4 to 27.6 mb. Closed circles are *S. pombe* markers. This figure is taken from reference 4.

Conclusion

Using CHEF electrophoresis conditions which include a decrease in voltage during the run, we have successfully separated *D. discoideum* DNA molecules ranging in apparent size from ~3.6 to ~9 mb. Further, we have assigned the separated chromosomes to the known linkage groups for this organism. We believe that for other organisms whose chromosomes are equally large (or larger), experimentation with relatively low voltage gradients, programmed pulse times, and ramped or stepped voltage gradients may reveal conditions that allow separation of DNA molecules in the ~10 mb range.

Although we found it convenient to lower the voltage in a single step during the run, a linearly decreasing voltage gradient, ramped between the two values, was equally effective. The critical factor appears to be the use of a lower voltage for the latter portion of the run. This is consistent with the idea that high voltage shears large molecules and our observations that, for reasons not understood, the kinetics of shearing are multi-hit. It is of course likely that by using a lower voltage (e.g., 1.48 V/cm or less) throughout the run, chromosomes larger than 6 mb would also be resolved. However, the concomitant reduction in rate of migration would result in a prohibitively long run time.

Finally, we have found that good *Dictyostelium* and *Polysphondylium* chromosomal resolution requires some modification of the standard protocol used to prepare the sample plugs. Plugs incubated simultaneously with EDTA/sarkosyl/protease give poor or no electrophoretic patterns. It is essential that the sample plugs be preincubated with EDTA before detergent is added. We do not

know why this is so, but we suspect that if EDTA and sarkosyl are added together, the detergent releases potent nucleases from subcellular compartments before the EDTA chelates all of the cellular Mg^{++} . Thus, preincubation with EDTA may result in improved electrophoretic patterns for any organism with an abundance of nucleases.

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