Preparation and Separation of Mammalian DNA by Pulsed Field Gel Electrophoresis

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Introduction

An increasingly important application of pulsed field gel electrophoresis is the analysis of mammalian DNA. With this technique, fragments which are hundreds or thousands of kilobases in length can be separated. Such fragments may contain entire gene families. Since mammalian chromosomes are too large to enter gels with current technology, they must be digested with restriction enzymes, and visualized by probe hybridization. This bulletin describes procedures for preparing samples, restriction digestion, setting switch time parameters, and using appropriate controls. The DNA employed is from human white blood cells. Additional information on sample preparation and pulsed field electrophoresis conditions may be found in the references.

Sample Preparation

The following procedure will give a final concentration of 10^7 cells/ml in the agarose. Typical agarose slices contain 50–100 µl, giving 5-10 µg DNA per lane.

1. Harvest cells in phosphate buffered saline:
   - A. Tissue culture cells are collected by scraping or trypsination, followed by centrifugation. Suspension cultures are centrifuged directly, although a 2–5 minute trypsin digestion (0.25% in saline) at room temperature can improve lysis.
   - B. White blood cells are prepared from whole blood by either isotonic lysis or centrifugation through Ficoll (*e.g.* Sigma Histopaque®). See reference 3. Starting volume is 5–50 ml blood.
   - C. Tissue should be minced with scissors or a razor blade and dispersed to single cells with a loose fitting Dounce homogenizer. Let large clumps of tissue settle, transfer supernatant, and centrifuge.
2. Wash cells twice with PBS at 4 °C.
3. Count cells and resuspend in PBS at a concentration of 2 x 10^7 cells/ml. Cells may be counted in a hemocytometer (*e.g.*, Reichert Inc.).
4. Gently pipet up and down to break up clumps, and warm to 37 °C.
5. Mix with an equal volume of 1% low melting agarose prepared in PBS and cooled to 45 °C. Note: the agarose should be qualified for restriction digestion.
6. Transfer with a Pipetman® or disposable pipet to plug mold or plug former, avoiding bubbles.
7. Allow samples to harden at 4 °C or on ice for 10 minutes.
8. Transfer sample blocks to 3–5 volumes of 0.5 M EDTA, pH 9, 1% Sarcosyl, 0.5 mg/ml Proteinase K (Boehringer-Mannheim).
9. Digest for 1–2 days at 50 °C with constant, gentle shaking. Samples may be stored in this solution at 4 °C and remain stable for more than 12 months.

Restriction Digestion of DNA in Agarose

1. Rinse samples twice in distilled water. Wash with 50x volume of TE either twice for 3 hours at room temperature or overnight at 4 °C by gently shaking or rotating in 15 or 50 ml conical bottom polypropylene tubes.
2. Optional- Residual Proteinase K may be inactivated by washing sample in TE with 1 mM PMSF (phenyl methyl sulfonyl fluoride) twice for 2 hours at room temperature prior to final washes in TE. Step 1 above is usually sufficient.
3. Samples are digested with a restriction enzyme in 100 µl to 200 µl final volume depending on the thickness of the plug. To the sample, add 10–20 µl 10x digestion buffer as recommended by the supplier of enzyme, nuclease free BSA to 0.1 mg/ml, distilled water, and enzyme.
4. Digest agarose blocks with 2–10 U enzyme per µg DNA. Incubate 4 hours to overnight at the appropriate temperature. Enzymes may be added twice, once at the beginning, and again part way through the incubation. This is recommended for those enzymes that have short lifetimes. (Refer to the manufacturer's specifications.)
5. Stop the reaction by adding 1 ml 0.5 M EDTA. Incubate 5 minutes at room temperature.
6. Remove sample from EDTA. Place agarose slices into the wells of an agarose gel. It is important that the blocks are flat against the front wall of the well; remove any trapped bubbles. Fill the void space with 0.8% low melting

agarose. Alternatively, place blocks on each tooth of the gel forming comb, and cast gel around the comb.

7. Place gel in chamber and equilibrate at the running temperature for 0.5 hour prior to starting the run.

Electrophoresis and Results

The use of pulsed field electrophoresis to identify large fragments of human DNA homologous to a cDNA probe is illustrated in Figure 1. Human DNA was digested with two different rare cutting enzymes and separated for 36 hours prior to blotting and hybridization. With *Mlu* I, three bands are visible between about 450 and 600 kb. *Nru* I yielded two bands in this size range, one at 450 and one at 670 kb. In addition, with *Nru* I there was a faint hybridization signal from the region near the top of the gel that contains unresolved large DNA. The yeast markers indicate that the upper range of resolution of this gel is 900 kb. Thus, a hybridization signal in this upper region indicates the presence of additional fragments larger than the resolving capability of this gel. Figure 2A shows a stained gel run specifically to separate DNA fragments larger than 1,000 kb. In this case, only the largest *S. cerevisiae* chromosomes are resolved, though the *S. pombe* chromosomes are well separated. With the same conditions, the two bands of the *Nru* I digest, which had been well resolved in Figure 1 are visible only as a smear. However, two larger bands are now distinguishable, one at about 2 mb and the other at 2.5 mb. *Not* I yields a single large band at about 2.2 mb.

The importance of using the appropriate switch interval when analyzing pulsed field data is further apparent from Figure 3. A *Mlu* I digest of human DNA was run using three different pulsed field conditions prior to blotting and hybridization. Each of the gels resolved a different size range of DNAs, as indicated by the marker sizes in the figure. Under conditions where only fragments from 200 to 600 kb were resolved, four bands were visible on the autoradiogram. When longer switch intervals were employed so that fragments up to 1,600 kb were separated, three bands were visible. In this experiment the central band appears darker than the other two. Often, however, they are of uniform intensity giving no indication of the doublet. Using conditions which resolve bands above 2 mb, only a smear is seen on the autoradiogram, in a position corresponding to the massed *S. cerevisiae* chromosomes. Thus, to obtain accuracy both in sizing and in establishing the number of bands in Southern blots, gel conditions specific for resolving the bands of interest must be used.

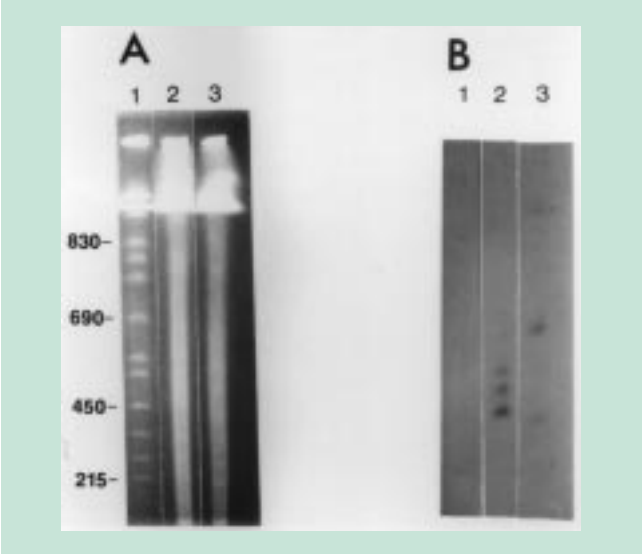

Fig. 1. Separation and identification of fragments smaller than 1 megabase. A. Gel stained with ethidium bromide. Human DNA in agarose blocks (5 µg/lane) was digested for 6 hr with 40 U of enzyme in a reaction volume of 150 µl. Following digestion, samples were run in a 1% agarose gel, 6 V/cm for 36 hr in 0.5x TBE at 14 °C. Switch time was a ramp from 55 sec to 75 sec. Samples were: Lane 1, Yeast size standard (YNN295). Lane 2, *Mlu* I digest. Lane 3, *Nru* I digest. Sizes (in kb) are indicated to the left of the yeast standards. **B.** Autoradiogram showing the position of bands identified by the probe. After staining, the gel was exposed for 1 minute on a short wave UV light-box (254 nm, 2 mW/cm^2). DNA was denatured in NaOH/NaCl, neutralized with Tris/NaCl and transferred overnight with 10x SSC to Zeta-Probe® membrane (Bio-Rad Laboratories) according to the manufacturer's protocol. The hybridization probe was a cDNA clone for a human G protein β subunit labeled by random priming.

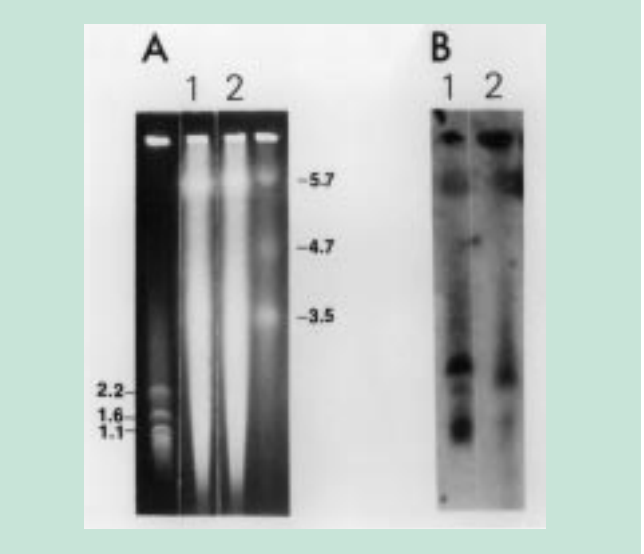

Fig. 2. Separation and identification of fragments larger than 1 megabase. The samples in Figure 1 were separated in 0.8% agarose for 108 hr in 1x TAE at 14 °C, 2 V/cm. Switch time was 35 min. **A.** Ethidium bromide-stained gel. *S. cerevisiae* chromosomes are on the far left, *S. pombe* on the right, with sizes indicated in megabases. Lane 1, *Nru* I; Lane 2, *Not* I. **B.** Autoradiogram after hybridization. Hybridization was as described in Figure 1.

Numerous other factors can influence the ability to detect and accurately size bands of interest in pulsed field gels. The most common reasons for failure include:

1) The DNA samples are degraded by nuclease either during the sample preparation or during the restriction digestion. Degradation during preparation produces plugs which, when run directly after preparation, will yield small DNA. Each new batch of samples should be tested for contaminating nuclease by performing a mock digestion and running the products on a pulsed field gel. The mock digestion consists of incubation at the same temperature and time in a restriction digestion reaction minus the restriction enzyme (*i.e.* DNA+buffer+BSA). Samples which are free of nuclease will have almost all the DNA remaining in the plug or moving with the limiting mobility region of large unresolved DNA.

2) Incorrect gel conditions are used. Size markers should indicate that the conditions used (especially voltage and switch time) were appropriate for resolving the size region of interest. Each new digest must be analyzed under a variety of gel conditions. Generally, it is best to start with the conditions which give as broad a size range as possible to give a rough estimate of the number and sizes of bands. Often this will involve using ramped switch times. Later, gels which focus on narrower size ranges can be used to more precisely determine fragment sizes.

3) Incomplete restriction digestion. This can result from protein remaining in the samples or from incomplete removal of the agents used to prepare the DNA (EDTA, protease, detergent). Further protease digestion and/or more extensive dialysis can be helpful. In addition, because many of the enzymes used to generate large fragments are sensitive to methylation, it often can be extremely difficult to cut particular methylated sites. Using DNA prepared from different sources (*e.g.* fibroblasts instead of blood) may permit cutting at these sites due to different tissue specific methylation patterns.

4) Sample overloading. The apparent size of fragments in pulsed field gels is strongly dependent on the amount of DNA loaded in the gel. With increasing amounts of DNA, bands are retarded in their migration, indicating larger than true sizes. Figure 4 shows hybridization to two different amounts of the same restriction digest run side by side. Two distinct bands are visible in lane 1. As expected, an increased amount of DNA leads to broadening of both bands. Furthermore, the midpoint of each of the broad bands is shifted higher in the gel. This is particularly clear for the band near the bottom of the autoradiogram. A lighter exposure of the pattern in lane 2 indicates a larger size for each of the two bands than is seen in lane 1.

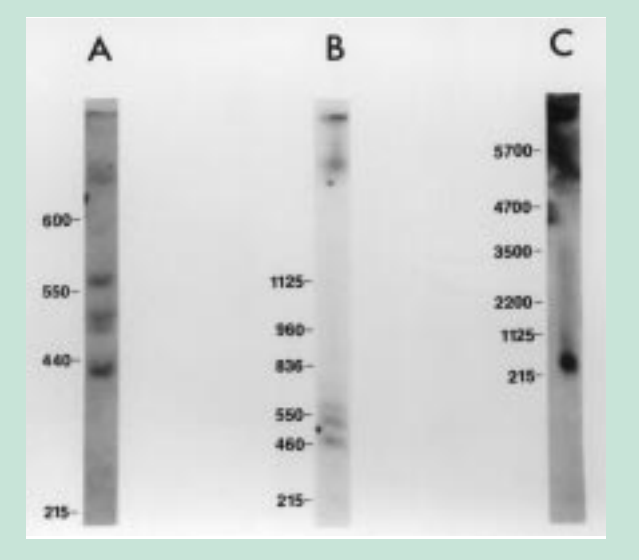

Fig. 3. Importance of switch interval for resolving fragments. Human DNA was digested with *Mlu* I for 6 hr and separated with different switch times or other pulsed field parameters. After membrane separation, DNA was transferred to Zeta-Probe membrane as described in Figure 1. **A.** 1% gel electrophoresed for 36 hr at 6 V/cm with a switch ramp from 30 sec to 60 sec. **B.** 1% gel electrophoresed 36 hr at 6 V/cm with a ramp from 95 sec to 115 sec. **C.** 0.8% gel electrophoresed 108 hr at 2 V/cm with a switch time of 35 min. Sizes indicate the position of the yeast markers included in each gel.

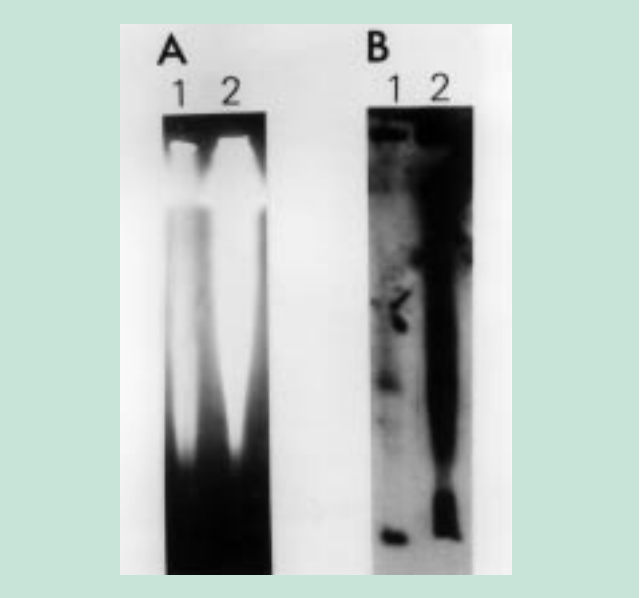

Fig. 4. Effects of sample overloading on hybridization signals. Human DNA was prepared in agarose simultaneously using two different cell concentrations. A sample block of identical thickness from each was digested with *Not* I and separated as described in Figure 3 C. The amounts of DNA loaded per lane were: Lane 1, 3 µg. Lane 2, 15 µg. **A.** Ethidium bromide-stained gel. **B.** Autoradiogram after hybridization. Blotting and hybridization were as described in Figure 1.

Conclusion

In these experiments a cDNA probe was used to localize those large restriction fragments bearing homology to the sequence of interest. In such experiments, the result requires successful electrophoresis, transfer, and detection. The importance of using the appropriate electrophoresis conditions has been discussed. After the large DNA in the gel has been nicked, transfer and detection are the same as in conventional Southern hybridization. Of greatest importance is the quality of the DNA used, especially having DNA that is clean and cuts thoroughly, as well as having highly specific, well labeled probes. Bands up to 5 megabases can be reliably detected using the procedures detailed here. Generally, bands larger than 2 mb appear broader, and the resulting hybridization signal is weaker than for smaller bands. One should resist the temptation to load more sample to detect large bands as the changes in migration (which include band spreading) are counter productive. It is better to concentrate on getting clean hybridization signals, *e.g.*, making sure transfer is complete, or eliminating background hybridization.

Multiple bands were detected with each of the enzymes used. Numerous situations can give rise to multiple bands on blots with large DNA. These include the presence of multiple regions homologous to the probe (both genes and pseudogenes), partial digestion due to restriction enzyme inhibitors in the samples or interference with cutting by methylation, or allelic differences between chromosomes that make up the sample. Distinguishing between each of these cases requires both knowledge of the factors which influence pulsed field mobility and application of standard molecular biological methods to explain the nature of each of the bands.

References

1. Olson, M. O., Pulsed Field gel electrophoresis, In *Genetic Engineering*, vol. **11**, Setlow, J. K., ed., Plenum, NY (1989).
2. Lai, E., Birren, B. W., Clark, S. M., Simon, M. I. and Hood, L., *BioTechniques*, **7**, 34-42 (1989).
3. Smith, C. L., Klco, S. R. and Cantor, C. R., In *Genome Analysis*, A Practical Approach, Davies, K. E. ed., IRL Press, pp. 41-72 (1988).

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