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Methods for Pulsed-Field Gel Electrophoresis

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ABSTRACT

The term pulsed-field gel electrophoresis (PFGE) is used as an acronym to indicate any technique that resolves (large) DNA molecules by continuous reorientation. It bridges the resolution gap between cytogenetic methods (>5 Mb) and DNA analysis (<50 kb). Initially, PFGE was used to study the chromosomal content of unicellular eukaryotic organisms of interest to genetic research and population health. Later, PFGE was used to construct megabase maps of segments of the human genome. Successfull utilization of PFGE requires the availability of very high-molecular weight DNA. This article describes the modification of standard DNA protocols necessary to handle large DNA molecules, based on its encapsidation in agarose.

Index Entries: Pulsed-field gel electrophoresis; PFGE; CHEF; FIGE; blotting; hybridization; rare-cutter; restriction enzyme; physical mapping.

INTRODUCTION

Conventional agarose gel electrophoresis is capable of separating DNA fragments with sizes of up to 20–30 kilobase pairs (kbp). In 1984, Schwartz and Cantor (*1*) developed an electrophoretic technique capable of resolving DNA molecules in excess of 2,000,000 bp (2.0 Mbp). They called the technique "Pulsed-field gradient gel electrophoresis" (Fig. 1A).

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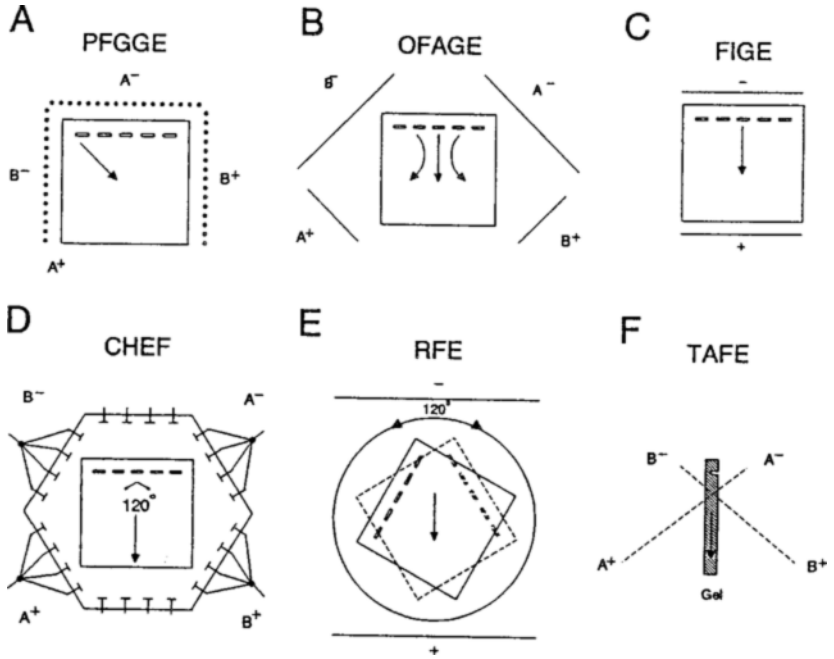

Fig. 1. Schematic drawing of several PFGE systems. (**A**) Pulsed-field gradient gel electrophoresis—the first system described (*1*). (**B**) Orthogonal field-alternating gel electrophoresis. (**C**) Field-inversion gel electrophoresis—the cheapest system able to operate in standard gel boxes. (**D**) Contour-clamped homogeneous electric-field electrophoresis—the most widely applied system. (**E**) Rotating-field electrophoresis—shown is the RGE varient. The electric field alternates between positions A^-/A^+ and B^-/B^+. (**F**) Transverse alternating-field electrophoresis—the system using vertical gels.

Its basic principle is a continuous reorientation of the DNA molecules, caused by a recurrent change in electric field direction. This results in a migration velocity in the net field direction depending primarily on the size of the DNA molecules. Later developments resulted in similar techniques, all with modifications of the basic principle of DNA reorientation.

PFGE Systems

Different PFGE systems vary in either the construction of the gel box (electrode configuration and polarity or position of the gel) or in the way in which the electric fields are applied to the gel. Frequently used systems include the following.

Orthogonal Field-Alternating Gel Electrophoresis

A first modification of the pulsed-field gradient gel electrophoresis was described by Carle and Olson (*2*) as the OFAGE (Fig. 1B). In this system, two long wire cathodes and two small point anodes are placed in 90°

angles. Separation is achieved by switching the electric fields from north/south to east/west.

Field-Inversion Gel Electrophoresis

The FIGE system (3) uses normal submarine gel boxes with a possibility of buffer cooling (Fig. 1C). Reorientation of the DNA molecules is achieved by reversing the field polarity (reorientation angle 180°) in either alternating switching intervals with roughly a 3:1 ratio for forward to reverse fields or by the application of higher forward than backward field strengths (e.g., programmable Power Inverter, MJ Research, USA; Gene-Tic™, Biocent, Netherlands).

Contour-Clamped Homogeneous Electric-Field Electrophoresis

In CHEF electrophoresis (4) the electric field is distributed along the contour of a hexagonal array of electrodes (Fig. 1D). The opposing sides of the hexagon are activated alternately in a 120° angle. CHEF electrophoresis represents the most popular PFGE system and is available from several companies (Pulsaphor™, Pharmacia, Sweden; CHEF-DR™, Bio-Rad, USA; Gene-Tic™, Biocent, Netherlands).

Rotating-Field Electrophoresis

Two types of RFE are available. Rotating gel electrophoresis (RGE), also called the "Waltzer"-type (5), uses a normal gel box in which the gel rests on a turntable that moves back and forward under a selectable angle (normally 120°) between two positions (Fig. 1E). In Rotating Electrode Electrophoresis (REE) (Rotaphor™, Biometra, Germany) two long wire electrodes turn around a stationary gel (6).

Transverse Alternating-Field Electrophoresis

TAFE (7) is unique in that way that it uses a vertically placed gel, perpendicular to the electric field (Fig. 1F, Geneline™, Beckman, USA). A variant of this system, called ST/RIDE (8), has a fixed electrical field applied to a vertical gel and a simultaneously applied perpendicular field that alternates polarity in a cyclic fashion (ST/RIDE™, Stratagene, USA).

Every PFGE system is driven by a computer that allows the setting of a variable number of parameters that all influence the electrophoretic behavior of the DNA. The flexibility of the software driving the electrophoresis is an essential component of the complete system; not all parameters can be changed in every system. Recent PFGE designs resulted in gel boxes in which a large set of point electrodes are arranged in a square array. The output voltage on every individual electrode can be regulated independently by a computer. In such a setup, each of the earlier mentioned systems can be simulated by the software controlling the electrodes.

PFGE systems require the setting of many interdependent variables that all have their own influence on the electrophoretic patterns obtained. The PFGE systems described were developed by trial and error, and they

give considerable variations when used in different laboratories. These aspects make it difficult for new users to set up a reliably working PFGE system. The new trend to computerize laboratory equipment will certainly involve pulsed-field electrophoresis, since its complex nature constitutes a perfect target area for the further development of expert systems.

Future advancements on the separation of DNA molecules will focus mainly on the outer limits of the separation range, especially the upper size limit. The recent development of a system called "secondary pulsed-field gel electrophoresis" (*9*) illustrates that, particularly in the separation of megabase-sized molecules, many possibilities to improve the current resolution range are still unrecognized and that an upper size limit is probably not yet reached.

Theory of DNA Migration

Theoretical considerations still do not fully explain all electrophoresis phenomena that are observed with the individual systems. Several studies have been performed to improve our insight in the way in which the DNA resolution is achieved by the continuous reorientation of the DNA molecules. Extensive studies on individual systems, varying independently every parameter that influences the DNA separation, have led to the construction of computer models that generate theoretical mobility curves. These models can be used to derive the parameter settings to be used for the optimal separation in a desired size range (*10,11*).

Further knowledge has been gathered from computer simulations that mimic the DNA migration through the agarose sieve (*10,12,13*). Several models have been built, but each with its specific limitations. No one model explains all abnormal migration patterns, like, e.g., band inversion or side way migration, which are observed in the real situation. In more recent approaches, migration of individual DNA molecules is followed directly under the microscope (*13,14*).

PFGE in Practice

We have previously described the methodology to analyze human DNA by PFG electrophoresis (*15*). The two systems that we use currently to study the DMD gene (*16,17*) are the FIGE and CHEF systems. This chapter describes their use in combination with a commercially available power supply that provides a programmable, recurrent inversion of output polarity.

The availability of very high-molecular-weight DNA (larger than 5 Mbp) is essential for the successful utilization of PFGE. Standard DNA isolation protocols cannot be used. They lead to mechanical shearing of the DNA to molecules of smaller than 200 kbp. The simplest way to circumvent this problem is an encapsidation of the cells in agarose prior to cell lysis (*18–21*).

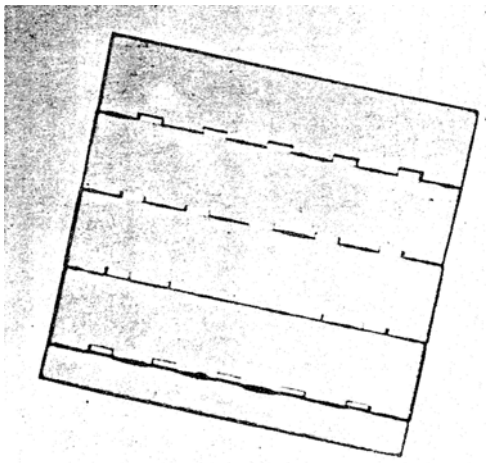

Fig. 2. Perspex mold to prepare agarose plugs. The mold was constructed from 10-mm thick perspex strips of 10×2 cm, in which 6 mm wide and 1.5 mm deep slits were made on one side. The strips were then glued together to form the slots.

Furthermore, PFG electrophoresis requires the use of specific, infrequently cutting restriction endonucleases (rare-cutters), a modification of the protocols to digest the agarose-embedded DNA, altered techniques to load the DNA samples onto a gel, the preparation and use of DNA marker molecules in the size range of over 50 kbp, and a modification of the techniques to blot and hybridize the DNA (*18–20*).

MATERIALS

1. Perspex mould: a perspex block former containing rectangular holes of 10×6×1.5 mm (Fig. 2).
2. Nylon membrane: Hybond-N-Plus (Amersham). Other membranes, like Gene Screen-Plus (NEN) and Bio-Trace ^{RP} (Gelman Sciences Inc.), can also be used.
3. Electrophoresis-box: *FIGE*: electrophoresis is done in a standard horizontal submarine gel box (Fig. 3) that allows circulation of the buffer. The gel rests on a table and is secured at each end with two pegs.

   CHEF: electrophoresis is done in a rectangular gel box (Fig. 3). The electrodes are fixed in a hexagonal configuration to the lid of the gel box (Fig. 3, cf Chu et al. [4]). The gel rests on a table and is secured at each corner with two pegs. A practical design has recently been published (*22*).

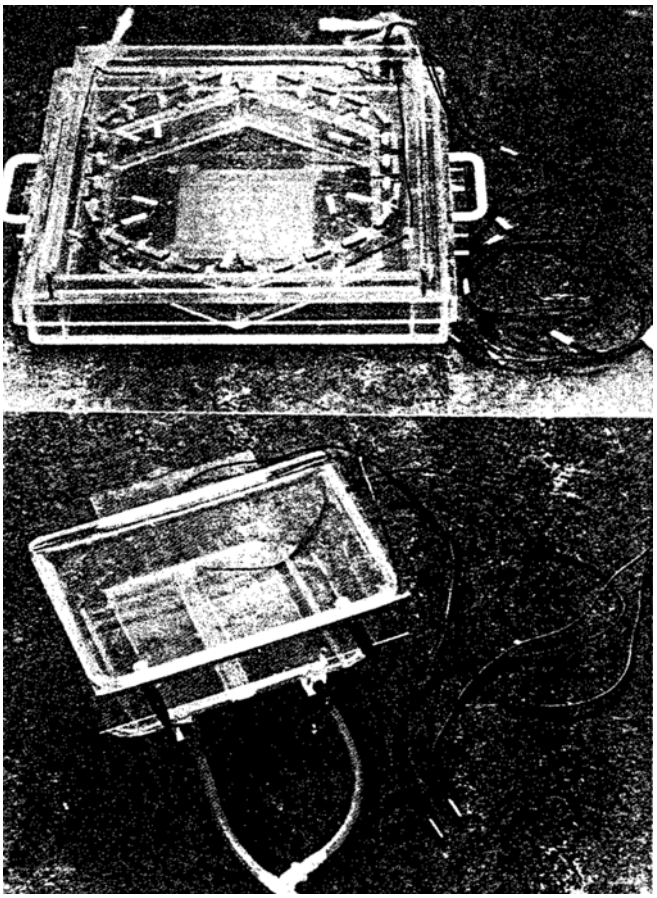

Fig. 3. Photograph of the CHEF (top) and FIGE boxes (bottom) used. A detailed description is given in the text (''Materials'').

Both systems: Electrophoresis-buffer is cooled to 18°C and circulated through the container. To assure even cooling during electrophoresis, the gel is covered with a perspex plate that has the same thickness as the table on which it rests. This method allows more gels to be stacked together and run simultaneously.

4. Power supply: The described system is based on the use of the Gene-Tic (Biocent, P.O. Box 280, 2160AG LISSE, the Netherlands). This power supply has the described program built in, and is capable of driving either four FIGE or two CHEF gels in parallel; also, each gel can be programmed independently. Other commercially available power supplies and switch devices lack one or more of the possibilities mentioned, but can be applied with adaptations.

SOLUTIONS

1. Agarose: InCert-agarose (FMC) is used for the isolation of DNA that has to be digested. LGT-agarose (BRL or Bio-Rad) is used for marker DNA isolations. SeaKem LE- (FMC) or Sigma low EEO-agarose (A6013) is used for gel electrophoresis.
2. Blood lysis buffer: 155 m*M* NH_4Cl, 10 m*M* $KHCO_3$, and 1 m*M* EDTA.
3. Competitor DNA: 500 ng/mL placenta DNA sonicated to 100–1000 bp.
4. SarE: 0.5*M* EDTA and 1% Na-*N*-lauroylsarcosinate (Sigma), pH 9.5.
5. Electrophoresis buffer: 45 m*M* Tris, 45 m*M* boric acid, and 0.5 m*M* EDTA, pH 8.3, stored as a 20x concentrated stock solution.
6. Equilibration buffer: enzyme-specific restriction endonuclease incubation buffer, made as recommended by the manufacturer, containing 2 m*M* spermidine, but lacking BSA. Stored at −20°C as a 10x concentrated stock solution.
7. Ethidiumbromide solution: 0.5 μg/mL ethidiumbromide in H_2O. Stored as a 10 mg/mL stock solution.
8. HYB solution: 0.125*M* Na_2HPO_4 (pH 7.2 with H_3PO_4), 0.25*M* NaCl, 1.0 m*M* EDTA (pH 8.0), 7% SDS (BDH 44244), and 10% PEG-6,000 (BDH).
9. Neutralizing-buffer: 1.5*M* NaCl, and 0.5*M* Tris-HCl, pH 7.0.
10. PMSF: phenylmethylsulfonyfluoride (Sigma, P7626).
11. SE: 75 m*M* NaCl and 25 m*M* EDTA, pH 7.5.
12. SED: 75 m*M* NaCl, 25 m*M* EDTA (pH 8.0), and 20 m*M* DTT.
13. SSC: 150 m*M* NaCl and 15 m*M* Na-citrate. Stored as a 20x stock solution.
14. TE: 10 m*M* Tris and 1.0 m*M* EDTA, pH 7.5.
15. YPD: 1% yeast extract, 2% peptone, and 2% dextrose.
16. Zymolyase: Zymolyase-20T, Seikagaku Kogyo Co. Ltd., Tokyo, Japan.

METHODS

Definition

A plug is a 100-μL 0.5% agarose block (10×6×1.5 mm) containing DNA.

DNA Isolation in Agarose Blocks (*15*)

DNA is isolated from white blood cells. On average, 10 mL of blood yields enough leukocytes to prepare about 20 plugs (*see* Note 1).

1. Take 10 mL heparinized blood and add 30 mL blood lysis buffer. Leave 15 min on ice ensuring complete hemolysis by isotonic ammonia treatment. Centrifuge the white cells 15 min, 2000 rpm.
2. Resuspend the pellet in 10 mL blood lysis buffer, leave 15 min on ice, and centrifuge 15 min, 2000 rpm.
3. Resuspend the cells thoroughly at 20×10^6 cells/mL in SE. Mix in a 1:1 ratio with 1% InCert-agarose in SE cooled to 50°C.
4. Dispense the mixture immediately into the slots of a perspex mould (Fig. 2), covered on one side with tape. The mould is placed on ice for 5–10 min.
5. Tape is removed, and the solidified blocks are gently blown out of the slots, using a Pasteur pipet balloon, into 5 vol of SarE containing 0.5 mg/mL pronase (1 h preincubated). Incubate O/N at room temperature under gentle rotation (*see* Note 2).
6. Rinse the plugs once with sterile water, and wash four times for 2 h and once O/N, in 10–20 vol. TE under gentle rotation (*see* Note 3).
7. Store plugs in 50 m*M* EDTA (pH 8.0) at 4°C (*see* Note 4).

Preparation of Bacteriophage λ Marker Plugs

Use DNA of λ CI857Sam7, which has a genome of 48.5 kbp (Table 1).

1. Dilute the bacteriophage λ DNA to 5–10 μg DNA/mL in SE. Mix 1:1 with 1% LGT-agarose in SE cooled to 50°C. Dispense into the slots of a perspex mould, and allow to solidify on ice for 5–10 min.
2. Proceed as in "DNA Isolation in Agarose Blocks," step 5 (*see* Notes 4–6).

Preparation of Yeast Marker Plugs

Routinely, 20 mL of Saccharomyces cerevisiae culture are used to prepare 40–100 plugs. Chromosome sizes are given in Table 1 (*see* Note 7).

1. Inoculate 20 mL YPD and grow O/N at 37°C under vigorous shaking to late-log phase.
2. Collect the cells by centrifugation, 10 min, 1500 rpm. Wash the cells in 50 m*M* EDTA (pH 8.0) and centrifuge again.
3. Resuspend the cells in SED. Add Zymolyase-20T to 30 μg/mL, mix 1:1 with 1% LGT-agarose in SE cooled to 50°C, and dispense immediately into the slots of a perspex mould, covered on one side with tape. Place on ice for 5–10 min (*see* Note 8).
4. Remove the tape and gently blow the solidified blocks out of the slots, using a Pasteur pipet balloon, into 2 vol SED with 30

Table 1
Sizes of PFGE Marker Molecules[a]

λ	*S. cerevisiae*	*C. albicans*	*S. pombe*
-	>2.3	>2.5	5.7
0.728	1.45	>2.3	4.6
0.679	1.20	2.15	3.5
0.631	0.97	1.80	
0.582	0.94	1.63	
0.534	0.82	1.60	
0.485	0.79	1.20	
0.437	0.75	1.08	
0.388	0.68	0.97	
0.340	0.60[b]		
0.291	0.44		
0.243	0.36		
0.194	0.28		
0.146	0.23		
0.097			
0.049			

[a]Bacteriophage λ (CI857Sam7) has a genome size of 48.5 kbp. Yeast strains used are *Saccharomyces cerevisiae* AB1380 (*23*), *Candida albicans* CBS562 (*24*), and *Schizosaccharomyces pombe* CBS356 (*24*). Yeast chromosome sizes are callibrated with the λ ladder. Sizes are given in mega-base pairs (Mbp), starting with the largest molecule.
[b]Doublet band.

μg/mL Zymolyase-20T. Incubate 1-2 h at 37°C under gentle rotation.

5. Rinse the plugs once with SE. Transfer the plugs to 2 vol. SE containing 1.0 mg/mL pronase (1 h preincubated), and incubate O/N at room temperature under gentle rotation.
6. Proceed as in "DNA-Isolation in Agarose Blocks," step 6.

Restriction Endonuclease Digestion of DNA in Agarose Blocks

Newly prepared plugs of mammalian DNA should be checked for residual nuclease contaminations by a control incubation without the addition of enzyme (*see* below). After incubation, the DNA is analyzed on a PFGE gel. DNA degradation should be negligible in the size range under study, i.e., upto at least 2 Mbp (*see* Note 9). Usually, half-plugs are digested and loaded per lane (equalling 5–7.5× 10^5 cells or 3–5 μg of DNA).

1. Rinse the plugs once with sterile water, and wash three times for 30 min in 10–20 vol. TE under gentle rotation.
2. Place a half-plug in 1.0 mL equilibration buffer, and incubate 2 h at room temperature or O/N at 4°C.

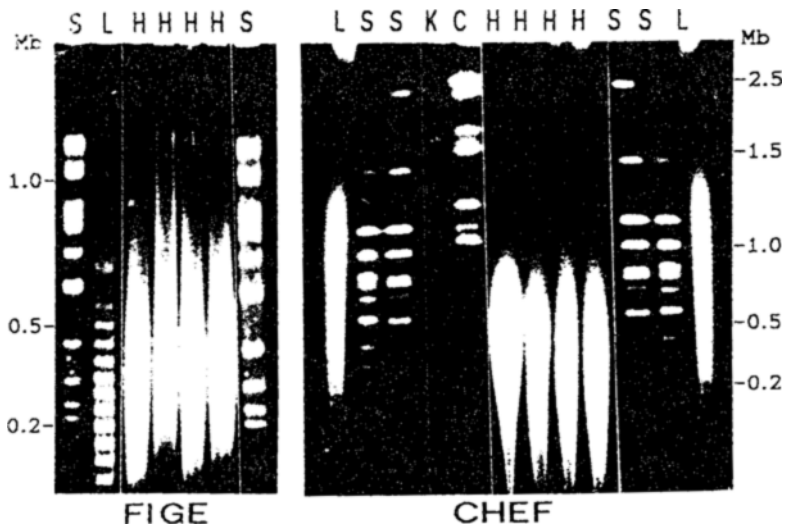

Fig. 4. Photograph of an ethidiumbromide-stained FIGE (left panel) or CHEF gel (right panel). The FIGE gel represents a standard electrophoretic separation—7.5 V/cm for 18 h at 18°C with a 40% exponential switch time increase from 1–60 s in four identical cycles and a 2% pause interval (*see* text). The CHEF gel was run to separate DNA molecules up to 2.5 Mbp: Electrophoresis was at 3.2 V/cm for 68 h at 18°C with a linear switch time increase from 1–500 s and a 2% pause interval. Sizes are indicated in Mbp. DNAs used are; L = bacteriophage λ, S = *S. cerevisiae*, C = *C. albicans*, K = *K. lactis*, and H = *Sfi*I-digested human DNA.

3. Carefully remove all equilibration buffer. Add 50 µl fresh equilibration buffer containing 0.2 mg/mL BSA. Digest for 6 h (or O/N) at the specified incubation temperature, using 15–25 U of enzyme. Add the enzyme in two equal portions at the beginning and after 3 h of digestion (*see* Notes 10–12).
4. After digestion, place on ice for 15–30 min. Remove all buffer.
5. The plugs may either be layered directly or stored in 50 m*M* EDTA at 4°C (*see* Note 13) for later use.

Pulsed-Field Gel Electrophoresis: The Gene-Tic

Figure 4 shows an example of the possibilities of a gel separation for both the FIGE and CHEF systems. In both systems, the size range over which the DNA is separated is defined by the parameter settings of a file that drives the electrophoresis (Table 2). We define the interval between subsequent inversions of the electrode polarity as the "switch time." For FIGE, each run is divided into cycles of 4–6 h (Fig. 5). The shortest switch time, at the start of each cycle, defines the lower limit of separation, whereas the longest switch time, at the end of each cycle, defines the upper limit of DNA molecules that are resolved. We have introduced a

Table 2
Electrophoretic Separation Conditions[a]

Separation range, kb	V/cm	Run, h	Switch time		Agarose conc.
			Start	End	
0.5–25[b]	6.8	10	60	60	1%
2.5–50	4.6	22	1	1	1%
10–150	6.8	18	5	8	1%
25–500[d]	5.4	16	1	30	1%
50–950[a]	5.7	20	40	80	1%
50–1100	6.8	20	40	80	1%
50–1800	4.3	44	1	200	1%
100–2000	2.7	68	100	650	1%
100–5000	2.3	96	300	1500	1%
50–1000	4.6	20	1	90	0.7%[e]
100–3000	4.6	24	50	275	0.7%[e]
100–5000	3.2	48	100	600	0.7%[e]

[a] Conditions used to obtain PFGE-separations within size ranges. All gels were run in the system described in 0.5×TBE at 18°C and with a 2% pause for separations above 400 kb.
[b] FIGE gel with 0% reversed electric field.
[c] FIGE gel run with four identical 4-h cycles with 40% exponentially increasing switch times and 33% reversed electric field polarity.
[d] Gel measures 20×20 cm with two rows of samples.
[e] SeaKem GOLD-agarose.
Switch times are given in seconds.

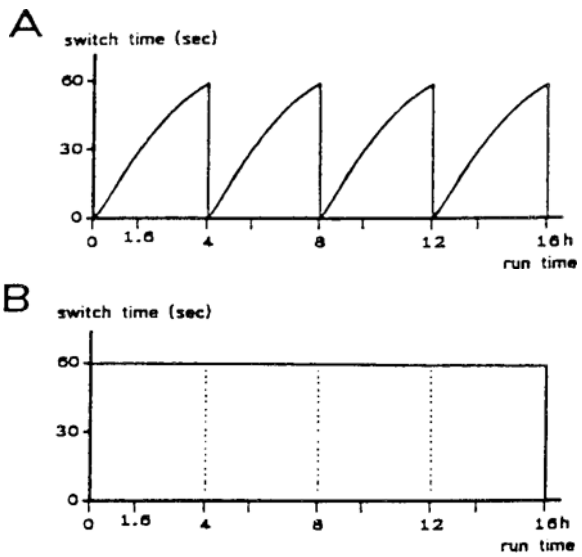

Fig. 5. Graphical illustration of the length of the switch time during (A) a standard FIGE run (Fig. 4) or (B) a standard CHEF run (*see* "Methods: Pulsed-Field Gel Electrophoresis: The Gene-Tic").

short pause each time the electric field is reversed. This allows a relaxation of the DNA molecules and was found to result in an improved resolution above 400 kbp. Electrophoresis in the backward direction is set to 1/3 of that in the forward direction. An exponential mode of switch time increase is available, in which the user is requested to set the percentage of the total cycle duration, at which 50% of the switch time increase is reached. A figure of 40% provides a time ramp curve that initially increases more rapidly (Fig. 5). This setting was found to improve the separation of the larger DNA molecules markedly.

CHEF electrophoresis is usually performed at a constant switch time (Fig. 5), consequently in one linear cycle. The electric field is not reversed between two electrodes, but alternates between two sets of electrodes to give a 120° reorientation of field angle.

Running PFGE Gels

1. Prepare a 1% agarose gel by adding agarose powder to electrophoresis buffer. Boil until the solution is clear. Cool the agarose solution to 60°C, and pour it into a gel mould, insert a well-former, and allow the gel to set for 45–60 min.
2. Carefully remove the well-former, and load the gel on the lab bench. Load bacteriophage λ and yeast marker plugs by inserting them directly into the slots.
3. Plugs containing digested DNA are loaded directly onto the gel (*see* Note 13).
4. The electrophoresis box is filled with electrophoresis buffer; the loaded gel is carefully submerged and covered with a perspex plate. The cooling is turned on, and the gel is left for 30 min.
5. Electrophoresis is done using the Gene-Tic power supply with parameter settings for a DNA separation in the desired size range (*see* Note 14 and Table 2). For a standard DNA separation from 30–1000 kbp the following parameters are used:
   A. *FIGE electrophoresis:* 7.5 V/cm for 15 h at 18°C with four identical cycles, each with a switch interval increasing from 1 s at the beginning to 60 s at the end. The time ramp increases exponentially so that 50% of the switch interval increase is reached at 40% of each cycle duration (Fig. 5). The reverse switch interval measures 33% of the preceding forward one. A pause interval of 2% of the forward switch time is included.
   B. *CHEF electrophoresis:* 7.5 V/cm for 18 h at 18°C, usually with a constant switch interval of 60 s and a pause interval of 2% (Fig. 5).
6. For gels to be blotted, proceed to "Blotting of PFGE gels," step 1. Analytical gels are stained for 30–60 min in an ethidium-bromide solution. Gels are photographed on a UV trans-

illuminator directly or after improvement of the contrast by washing for 1–2 h in several changes of H_2O.

Blotting of PFGE Gels

1. After electrophoresis, the gel is stained for 30–60 min in a ethidiumbromide solution. Photograph on a UV transilluminator immediately or after improvement of the contrast by extensive H_2O washing.
2. DNA size is reduced by UV irradiation in a UV Crosslinker (Stratagene) at 200,000 $\mu J/cm^2$ (*see* Note 15).
3. Wash the gel 2×15 min in 0.4*M* NaOH. Rinse with water; wash 1×20 min in neutralizing buffer, and subsequently once for 20 min in 10×SSC (*see* Note 16). Blot the gel, upside down (*see* Note 16), in 10×SSC onto a nylon membrane.
4. Blot (at least for) O/N. Change the paper towels in the morning, at least 1 h before dismounting the blotting stack. Soak the membrane (DNA side up) for 5 min on a puddle of 0.4*M* NaOH. Wash 15 min in 2×SSC/0.2*M* Tris (pH 7.5). Dry the blot for 1 h at 65°C.

Hybridization of PFGE Blots

1. Probe DNA (10 ng) is labeled with ^{32}P-α-dCTP using a multiprime kit (Amersham). Unincorporated nucleotides are removed by purification over a Sephadex G50 column in a Pasteur pipet.
2. Prehybridize the blots in HYB solution for at least 10 min at 65°C.
3. Add labeled probe to the prehybridization, mix thoroughly, and hybridize O/N at 65°C.
4. Wash the blots at 65°C from 2.0×SSC/0.1%SDS (2×15 min), 1.0×SSC/0.1%SDS (2×15 min) down to 0.3×SSC/0.1%SDS (1×15 min).
5. Autoradiography with Kodak X-Omat R film normally takes O/N at −70°C using intensifying screens (Dupont).

Competitive DNA Hybridization of PFGE Blots[16]

1. Cosmid DNA (10 ng) is labeled with ^{32}P-α-dCTP using a multiprime kit (Amersham). Unincorporated nucleotides are removed by purification over a Sephadex G50 column in a Pasteur pipet.
2. Prehybridize the blots in HYB solution for at least 10 min at 65°C.

3. Transfer half of the multiprime reaction (ca. 200 μL) to an Eppendorf tube. Add 240 μL competitor DNA (ca. 20×10^3 excess), boil for 5 min, and chill on ice. Add to 1.5 mL HYB solution (preheated to 65°C), mix thoroughly, and incubate 90 min (N.B. time is critical!!) at 65°C in a water bath.
4. Add the mixture to the prehybridization, mix thoroughly, and incubate O/N at 65°C in a water bath.
5. Further handlings as in "Hybridization of PFGE Blots," step 4.

Rehybridization of PFGE Blots

1. Boil 200 mL 0.1×SSC and pour into a tray. Immediately add the used blots, cover the tray, and leave for 3 min under gentle rotation.
2. Take the blots and put them in a new tray containing 2×SSC/ 0.2*M* Tris-HCl (pH 7.5), and leave for 5 min.
3. Air-dry the blots. The blots are now ready for new hybridizations.

NOTES

1. Heparinized blood can be stored at −70°C before a DNA isolation is done. DNA isolation from other sources, like tissue-culture cells, sperm cells (add 10 m*M* DTT in steps 2–4), and fresh or frozen tissues (after homogenization to a single-cell suspension), is also possible using the same protocol.
2. Pronase routinely gives satisfactory results. Proteinase K (0.5 mg/mL, incubation overnight at 50°C) can be used instead, but is more expensive.
3. Addition of 40 μg/mL PMSF in the first two TE washing steps can be used to block protease activity and, when necessary, may also improve digestibility of the DNA.
4. Storage in SarE or TE is also possible. Storage in TE is dangerous (high risk of DNA degradation after minor nuclease contaminations, e.g., from poorly digested cells), but allows digestions to be started without extensive washings (*see* "Restriction Endonuclease Digestion of DNA in Agarose Blocks," step 2).
5. Preparation of λ marker plugs from isolated phage particles may be preferred because commercial DNA preparations give variable results.
6. Annealing of the λ sticky ends depends mainly on the DNA concentration in the plugs and the temperature during prepa-

ration. When the ladders do not reach the desired size range, they can be enlarged by a $MgCl_2$ incubation; equilibrate the plugs to 10 m*M* $MgCl_2$. Incubate 15 min at 42°C. Wash extensively in TE. Store in 0.5*M* EDTA (pH 8.0).

7. Chromosome sizes obtained differ between yeast strains. The yeast strains used are described in Table 1.
8. A smear throughout the lanes after electrophoresis indicates poor cell lysis, DNA degradation, or RNA contamination. Cell lysis can also be done with Novozym (SP234, NOVO Industri AS, COPENHAGEN, Denmark) or lyticase (Sigma, L5263). A dominant RNA smear, obscuring chromosome bands, can be removed by an RNase treatment.
9. Degraded or broken DNA can be removed from a plug by a short "pre"-electrophoresis before further handling of the sample. Remaining DNA degradating activities can be removed by a second pronase treatment.
10. Frequently used rare-cutter enzymes are *Sfi*I, *Sal*I, *Sac*II, *Bss*HII, *Eag*I, *Mlu*I, *Not*I, *Nar*I, *Nru*I, and *Nae*I.
11. For double digestions; repeat steps 1–3 for the second enzyme. Before step 1 of the second digestion, a proteinase K treatment (0.5 mg/mL) can be inserted (not essential).
12. For digestions with normal restriction endonucleases (like *Eco*RI, *Hind*III, and so on), modify step 3 to carefully remove all equilibration buffer and incubate 10 min at 65°C to melt the plug. Incubate 15 min at 37°C, add BSA to 0.1 mg/mL, and add restriction enzyme. Incubate at the desired temperature. Layer the molten plug directly onto the gel.
13. To increase equal loading throughout the slot, the plug can be melted for 10 min at 65°C and then carefully layered from the side of a well (to avoid air bubbles) using a yellow pipet tip of which the last 5–8 mm are cut off. However, this step may cause denaturation (when in low salt) and cannot be used when fragments above 1.5 Mbp are to be detected since loading will cause too much DNA shearing.
14. Under the given conditions, a rule of thumb is that an increase in the forward switch time at the end of a cycle by 1 s results in an upward shift of the zone of unseparated DNA by 20 kbp.
15. UV transilluminators can also be used, either for 60–90 s with 254 nm light, or 5–10 min with 302 nm light. It is advisable, however, to test each illuminator regularly to define the optimal illumination time. Size reduction by acid depurination (20 min incubation in 0.25*M* HCl, rinse in water, wash 20 min in neutralizing buffer, wash 2×20 min in 10×SSC) is possible, but gives variable results.

16. Upside-down blotting prevents occasional variations in transfer efficiency, caused by "skin formation" when agarose solutions have been standing too long before gels were poured. Blotting O/N in NaOH is also possible, but gives, in our hands, more variable results.

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