Neutral/neutral two-dimensional agarose gel electrophoresis for plastid DNA

Materials

(Plant and culture condition)

Maintain liverwort suspension-cultured cells in 70 ml of 1M51C medium in a 300-ml flask on rotary shaker (130 rpm) under continuous white light (50-60 μmol photons m^{-2} s^{-1}) at 22°C. Use cells from 7- to 10-day-old cultures for plastid transformation.

(Equipment)

- Cheese cloth
- Blender (chill container before use)
- Miracloth (Merck, Darmstadt, Germany)
- Loose-fit glass homogenizer
- Brush or glass rod capped with rubber tube
- Razor blade

(Reagents)

- Wash buffer

50 mM Tris-HCl pH 8.0, 20 mM EDTA, 1 mM $MgCl_2$

- Homogenization buffer

0.6 M mannitol, 50 mM Tris-HCl pH 8.0, 20 mM EDTA, 1 mM $MgCl_2$, 0.01% (v/v) 2-mercaptoethanol

- Pre-lysis buffer

100 mM EDTA pH 8.0, 100 mM NaCl, 10 mM Tris-HCl pH 8.0

- Proteinase K stock solution (0.4 mg/ml, store at -30°C)
- N-lauroylsarcosine sodium salt
- TER buffer

10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 10 mg/ml RNaseA

- BND cellulose (Sigma-Aldrich, St. Louis, USA)
- Disposable 5-mL polypropylene column (Thermo Scientific, Rockford, USA)
- BND equilibration/wash buffer

0.8 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0

- BND elution buffer

1.2 M NaCl, 10 mM Tris-HCl pH 8.0, 1.8% (w/v) caffeine

- 2-Propanol
- Ethanol
- 10× TBE buffer

89 mM Tris-HCl, 89 mM boric acid, 1 mM EDTA pH 8.0

- Agarose for > 1 kbp fragment (Nacalai tesque, Kyoto, Japan)

Methods

*Use large-bore tips in all steps.

(Extraction of total chloroplast DNA)

*About 100 μg of chloroplast DNA (for 2 analyses) will be extracted by the following procedure.

*Perform all steps on ice if not otherwise mentioned.

1: Subculture 100-140 ml of suspension-cultured cells grown for a week to 700 ml of fresh 1M51C medium in 3 L flask.

2: Culture cells for 1 week.

3: Aspirate old medium and add 700 ml of fresh 1M51C medium.

4: Collect cells by filtration with four layers of cheese cloth 36 h after addition of fresh medium.

5: Disrupt cells by blender in ice-chilled homogenization buffer at full speed for 10 sec three times.

6: Filtrate with four layered Miracloth.

7: Centrifuge filtrate (2,000 ×g, 4°C, 15 min).

8: Gently suspend pellet of chloroplasts with brush or glass rod capped with rubber tube in 24 ml of pre-lysis buffer.

9: Homogenize the suspension with loose-fit glass homogenizer.

9: Transfer the suspension to 50 ml plastic tube.

10: Add 1 ml of proteinase K stock solution (final conc. 0.4 mg/ml) and 250 mg N-lauroylsarcosine sodium salt (final conc. 1%).

11: Mix by rotary shaker at 100 rpm for 2 h at room temperature.

12: Add 25 ml of phenol-chloroform-isoamylalcohol (25:24:1) and gently emulsify for 5 min.

13: Centrifuge (4,500 × g, 4°C, 10 min) and recover the supernatant.

14: Repeat 12 & 13.

15: Add 0.7 volume of 2-propanol, mix gently and centrifuge (4,500 × g, 4°C, 30 min).

16: Wash the pellet with 70% ethanol and centrifuge (4,500 × g, 4°C, 10 min).

17: Dry the pellet in air.

18: Suspend the pellet of chloroplast DNA by pipetting with large-bore tip in TER buffer.
19: Immediately use or store the solution -80°C until use.

(Restriction enzyme digestion)
1: Digest approximately 50 μg of chloroplast DNA by appropriate restriction enzymes (2 unit/μg DNA) for 3 h according to manufacturer's instructions.
2: Check the digestion by agarose mini-gel electrophoresis.

(Preparation of BND column)
1: Mix BND cellulose with 5M NaCl in 50 mL plastic tube over night.
2: Centrifuge (300 ×g, 4°C, 10 min).
3: Discard supernatant.
4: Add 5M NaCl and suspend BND cellulose completely.
5: Repeat 2 to 4 twice.
6: Add sterile H_2O and mix.
7: Centrifuge (300 ×g, 4°C, 10 min).
8: Discard supernatant.
9: Add BND equilibration/wash buffer.
10: Make BND column according to manufactures' instructions (0.75- to 1-bed volume of BND cellulose for 50 μg of digested DNA).
11: Store the column at 4°C until use.

(Enrichment of replication intermediates)
1: Equilibrate BND column with 5-bed volume of BND equilibrate/wash buffer.
2: Dilute digested DNA with 2-volume of BND equilibrate/wash buffer.
3: Pass through DNA solution into BND column.
4: Wash the column with 10-bed volume of BND equilibrate/wash buffer.
5: Elute the solution containing replication intermediates with BND elution buffer.
6: Collect first 5 to 6 bed volumes of eluted solution separately to 1.5 ml plastic tubes.
7: Add 0.7 volume of 2-propanol and centrifuge (13,000 × g, 4°C, 15 min).
8: Wash pellets with 70% ethanol and air dry.
9: Suspend pellets in 10 mM Tris-HCl (pH 8.0) and use immediately.

(N/N 2-D agarose gel electrophoresis)
1: Apply DNA solution to agarose gel (0.4%, 1× TBE buffer).
2: Separate DNA in 1 × TBE buffer by 1 V/cm for 24 h (for < 5 kb DNA fragments) or 0.5 V/cm

for 40 h (for > 5 kb DNA fragments) at room temperature.

3: Stain the gel in the same 1× TBE buffer containing 0.1 mg/ml of ethidium bromide for 15 min.

4: Take photograph under the illumination of long-wavelength UV.

5: Excise the lane with a ruler and clean razor blade beginning 1 cm below the desired DNA fragments and extending 7~9 cm up the lane (This portion of the gel lane will include non-replicated molecules, replication intermediates and fully replicated molecules).

6: Make second dimensional gel (1.1% for < 5 kb DNA fragments or 0.8% for > 5kb DNA fragments) (1× TBE buffer, 0.3 mg/ml ethidium bromide), and cool down to about 60°C.

7: Rotate the excised gel 90 degrees from the original direction of electrophoresis and place in a new gel tray.

8: Seal the excised gel with a small amount of the second dimensional gel.

9: After the seal solidified, pour the second dimensional gel into the gel tray.

10: Separate DNA in 1× TBE buffer containing 0.3 mg/ml of ethidium bromide at 5-6 V/cm for 6-7 h at 4°C (< 5 kb DNA fragments) or 2 V/cm for 22 h at 4°C (> 5 kb DNA fragments).

11: Take photograph under the illumination of UV.

*Perform blotting and southern analysis

References

(N/N 2-D agarose gel electrophoresis)

Friedman,K.L. and Brewer,B.J. (1995) Analysis of replication intermediates by two-dimensional agarose gel electrophoresis. *Methods Enzymol.*, **262**, 613-627.

(BND cellulose)

Dijkwel,P.A., Vaughn,J.P. and Hamlin,J.L. (1991) Mapping of replication initiation sites in mammalian genomes by two-dimensional gel analysis: stabilization and enrichment of replication intermediates by isolation on the nuclear matrix. *Mol. Cell Biol.*, **11**, 3850-3859.