

Non-radioactive single-strand conformation polymorphism (SSCP) of PCR products

1. Subject

A rapid (< 2.5 hrs) method for single-strand conformation polymorphism analysis of PCR products that allow the use of ethidium bromide staining is described. Several electrophoretic parameters (running temperature, buffers, denaturants, DNA concentration and gel polyacrylamide concentration) influence the degree of strand separation and appear to be PCR fragment specific. The conditions have to be optimized. This non-isotopic method compared to radioactive methods has additional advantages of increased speed, precise temperature control, reproducibility, and easily and inexpensively obtainable reagents and equipment. This method also lacks the safety and hazardous waste management concerns associated with radioactive methods.

2. Principal

Single-strand conformation polymorphism (SSCP) analysis was first described by Orita et al. (7.1). In this technique, double-stranded (ds) DNA is denatured to single-stranded (ss) DNA in the presence of formamide or methylmercurychloride and the products are separated by polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions. The SSCP technique makes possible the detection of both known and unknown single point mutations and polymorphisms in products of the PCR. Optimization of SSCP analysis to detect the maximum number of mutations requires electrophoresis under carefully controlled conditions at different temperatures and using different gels.

3. Material and reagents

- 3.1 Tris(hydroxymethyl)-aminomethane (Merck 8382)
- 3.2 Na-EDTA (Titriplex III, Bio Rad 161-0729)
- 3.3 boric acid (H₃BO₃, Merck 0165)
- 3.4 ethidiumbromide (1,7-diamino-10-ethyl-9-phenylphenanthridiniumbromide, Serva 21238)
- 3.5 ammoniumpersulphate (NH₄SO₄)₂ (BioRad 161-0700)
- 3.6 bromophenolblue (BioRad 161-0404)
- 3.7 xylene cyanol (BioRad 161-0423)
- 3.8 formamide, deionized (Amresco 0606-100ML-APP)
- 3.9 40% acrylamide/Bis 37.5:1 (2.6% C) (BioRad 161-0148)
- 3.10 pipetten 5-50 µl, 200-1000 µl
- 3.11 pipettips, sterile
- 3.12 cylinders 10 ml, 50 ml, 1000 ml
- 3.13 erlenmeyerflask 100 ml
- 3.14 unit containing glass plates, clamps, strips and stand for casting acrylamide gels
- 3.15 BioRad vertical electrophoresis unit with cooling system
- 3.16 electrophoresis power supply
- 3.17 acetone
- 3.18 (latex) gloves
- 3.19 reaction vials 500 µl
- 3.20 waterbath 95°C
- 3.21 ice water
- 3.22 NaOH (Merck 1.06498.0500)
- 3.23 TEMED (BioRad 161-0801)
- 3.24 ultra thin-walled reaction vial 200 µl (Biozym B79401)

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4. Solutions

- 4.1 0.5M EDTA pH 8.0:
Add 186.1 g EDTA (3.2) to 800 ml ultrapure water and stir on a magnetic stirrer. Add while stirring \pm 20 g sodium hydroxyde (3.22). The pH will be approx. 8.0. Adjust total volume to 1000 ml with ultrapure water.
- 4.2 TBE-buffer 10x stock:
Add 108 g Tris (3.1) and 55 g boric acid (3.3) to 800 ml ultrapuur H₂O. Add 40 ml 0.5M EDTA (4.1).
(4.1). Adjust volume to 1000 ml with ultrapure H₂O.
- 4.3 Ammoniumpersulphate 10%:
Add 0.1 g ammoniumpersulphate (3.5) to 1.0 ml ultrapure water [6.1].
- 4.4 Gel loading dye 2x:
Mix 9.5 ml formamide (3.8) and 400 μ l 0.5M EDTA (4.1). Add 25 mg bromophenolblue (3.6), 25 mg xylene cyanol (3.7) and 100 μ l ultrapure water. Mix well.
- 4.5 TBE 1x electrophoresis buffer:
Mix 0.5 L TBE-buffer 10x (4.2) with 4.5 L ultrapure water.
- 4.6 PAGE gel (amount for 1 gel, 8.1):
Mix in an erlenmeyerflask (3.13) X ml 40% acrylamide/Bis (3.9), 4 ml TBE-buffer 10x (4.2), 36-X ml H₂O, 40 μ l TEMED (3.23) and 400 μ l ammoniumpersulphate 10% (4.3). Swirl well without introducing air bubbles [6.2].

5. Protocol

- 5.1 The PCR products used for SSCP should have a length <400 bp. Ideally, this should be around 150-250 bp.
- 5.2 The fragments [6.4] can be established by PCR, protocol 7 (p. 21).
Pre-treatment of fragments for SSCP
- 5.3 Mix 10 μ l amplicon (PCR product) and 10 μ l gel loading dye (4.4) in een ultra thin reaction vial (3.24).
- 5.4 Denature the mix at 94°C, 4 min. in a heating block [6.5].
- 5.5 Snap cool the samples on ice water to prevent heteroduplex formation and renaturing.
- 5.6 Keep the samples on ice until use.
Preparing electrophoresis tray and gels
- 5.7 Fill the electrophoresis tray with approx. 5 L electrophoresis buffer (4.5).
- 5.8 Clean a set of glass plates (one large/one small) with a soap solution [6.6].
- 5.9 Dry the plates and clean with acetone.
- 5.10 Clean plates with dustfree tissue or cloth.
- 5.11 Put two plates of different size together separated with plastic spacers.
- 5.12 Make sure the bottom edge of the glass plates are well aligned and fix them with two clamps; apply even force to both sides otherwise the glass plates will break.
- 5.13 Repeat step 5.11-5.12 with the other two plates.
- 5.14 Mount both glass plate sandwiches in the gel casting unit where a rubber inlay is supporting the bottom of the sandwiches.
Preparing polyacrylamid gel solution
- 5.15 Mix acrylamid/bis-acrylamid (3.9), TBE-buffer 10x (4.5), TEMED (3.23) and water in a clean, fat-free beaker and stir well but carefully avoiding air-bubbles.
- 5.16 Add ammoniumpersulphate (4.3) and mix again.
- 5.17 Pour the gel solution directly between the two glass plates, because polymerization starts with adding the ammoniumpersulphate.
- 5.18 Insert combs and leave the gels for 30 min. to polymerize completely.
- 5.19 Attach the sandwiches to the cooling unit of the Protean II with the larger glass plate facing the outside.
- 5.20 Place the complete unit into the electrophoresis tray partly filled with 1x TBE buffer (4.5).

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6. Remarks

- 6.1 The solution should be made just before use. If that is not possible, the solution may be stored at -20°C for 1 week maximum.
- 6.2 To obtain a 6% acrylamide gel X should be 6. SSCP can be run with different gel concentrations from 6 to approx. 12%. The concentration of the gel should be find out emperically as well as the temperature during electrophoresis.
- 6.3 This solution should be poored into the gel casting equipment right away. After adding the ammonumpersulphate polymerization will start immediatly and will take, depending on the gel concentration 30 min. to 1 hrs.
- 6.4 Before SSCP the fragments don't have to be cleaned. The presence of primers, polymerase etc. doesn't influence the reaction.
- 6.5 A PCR machine can be used as heating block.
- 6.6 Wear latex gloves to prevent smutching the plates again.

7. Literature

- 7.1 Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., and Sekiya, T. Detection of polymorphism of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc. Natl. Acad. Sci. USA 86, 2766-2770 (1989).
- 7.2 Hongyo, T., Buzard, G.S., Calvert, R.J., Weghorst, C.M. 'Cold SSCP': a simple, rapid and non-radioactive method for optimized single-strand conformation polymorphism analyses. NAR 21 (16): 3637-3642 (1993).
- 7.3 Clapp, J.P. The identification of root-associated fungi by polymerase chain reaction-single-strand conformational polymorphism (PCR-SSCP). MEM-3.4.7/1

8. Tables and graphs

8.1 Table for casting polyacrylamide gels for SSCP

Reagent	6% gel	8% gel	10% gel	12% gel
40% Acrylamide/Bis (3.9)	6 ml	8 ml	10 ml	12 ml
10x TBE (4.2)	4 ml	4 ml	4 ml	4 ml
H2O	30 ml	28 ml	26 ml	24 ml
TEMED (3.23)	40 µl	40 µl	40 µl	40 µl
10% ammonumpersulphate (4.3)	400 µl	400 µl	400 µl	400 µl

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