

Fingerprinting: Random Amplified Polymorphic DNA (with M13)

1. Subject

2. Principle

3. Reagents and material

- 3.1 electrophoresis-unit
- 3.2 bromophenolbleu (Biorad 161-0404)
- 3.3 sucrose (Merck 7653)
- 3.4 primer M13 (5'-gAgggTggCggTTCT-3')
- 3.5 primer T3B (5'-AggTCgCgggTTCgAATCC-3') (7.1)
- 3.6 primer OPA-04 (5'-AATCgggCTg-3')
- 3.7 primer OPA-05 (5'-AggggTCTTg-3')
- 3.8 primer OPA-06 (5'-ggTCCCTgAC-3')
- 3.9 primer OPA-07 (5'-gAAACgggTg-3')
- 3.10 primer OPA-13 (5'-CAgCACCCAC-3')
- 3.11 primer OPA-16 (5'-AgCCA gCgAA-3')
- 3.12 primer (ggA)7 (5'-ggAggAggAggAggAggA-3')
- 3.13 primer (CAC)5 (5'-CACCACCACCACCAC-3')
- 3.14 primer (gACA)4 (5'-gACAgACAgACAgACA-3')
- 3.15 primer (TAC)2(5'-TACTAC-3')
- 3.16 ultrapure dNTP set 100 mM (Pharmacia 27-2035-01):
2'-deoxyadenosine-5'-triphosphate, 2'-deoxythymidine-5'-triphosphate, 2'-deoxycytidine-5'-triphosphate en 2'-deoxyguanosine-5'-triphosphate
- 3.17 DNA-polymerase 5U/μl (SphaeroQ, TP05c)
- 3.18 potassium chloride, KCl (Merck 4936)
- 3.19 Tris(hydroxymethyl)-aminomethane (Merck 8382)
- 3.20 magnesiumchloride hexahydrate, MgCl₂-6H₂O (Merck 5833)
- 3.21 gelatine (Merck 4078)
- 3.22 Triton X-100 (Merck 8603)
- 3.23 mineral oil (Sigma M 3526)
- 3.24 chloric acid HCl, 1N and 5N (Ferak 11448)
- 3.25 sodium hydroxide, NaOH (Merck 6498)
- 3.26 glycerol 87%, (Merck 4093)
- 3.27 Na-EDTA (Titriplex III, Bio Rad 161-0729)
- 3.28 dithiotreitol (DTT, Sigma D-0632)
- 3.29 eppendorfcups 1.5 ml
- 3.30 Biozym Low marker 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900 en 1000 bp (Biozym 110000)
- 3.31 Promega 1 Kb marker 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 4000, 5000, 6000, 8000 en 10.000 bp (Promega G5711)
- 3.32 Eurogentec SmartLadder (Eurogentec MW-1700-02) 200, 400, 600, 800, 1000, 1500, 2000, 2500, 3000, 4000, 5000, 6000, 8000, 10000 bp resp. 20, 40, 60, 80, 100, 15, 20, 25, 30, 40, 50, 60, 80, 100 ng/band
- 3.33 template (10 ng/μl)
- 3.34 vortex
- 3.35 ultra thin reaction vials 200 μl (Biozym 179401)

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4. Solutions

- 4.1 PCR-buffer 10x:
Add 9.32 g potassium chloride [3.18], 3.03 g Tris [3.19] and 1.53 g magnesiumchloride•hexahydrate [3.20] to 220 ml ultrapure water. Adjust pH to 8.3 by adding \pm 10 ml 1N HCl [3.24]. Add 0.25 g gelatin [3.21] and 2.5 ml Triton X-100 [3.22], heat the solution for 30 min. at 56°C. Adjust the total volume to 250 ml with ultrapure water. Make aliquots of 50 ml. The buffer may be autoclaved.
- 4.2 0.05M Tris-HCl pH 8.3:
Add 6.05 g Tris [3.19] to 500 ml ultrapure water. Adjust pH to 8.3 by adding \pm 20 ml 1N HCl [3.24]. Add ultrapure water to a volume of 1000 ml.
- 4.3 1M Tris:
Dissolve 121.1 g Tris [3.19] in a total volume of 1000 ml ultrapure water.
- 4.4 1M Tris pH 8.0:
Dissolve 121.1 g Tris [3.19] op in 800 ml ultrapure water. Adjust pH to 8.0 by adding 5N HCl [3.24]. Fine adjust with 1N HCl. Add ultrapure water to a total volume of 1000 ml.
- 4.5 0.5M EDTA pH 8.0:
Add 186.1 g EDTA [3.27] to 800 ml ultrapure water and stir on a magnetic stirrer. Add while stirring \pm 20 g sodium hydroxyde [3.25]. The pH will be approx. 8.0. Adjust total volume to 1000 ml with ultrapure water.
- 4.6 1M DTT:
Dissolve 1.54 g DTT [3.28] in 10 ml ultrapure water. Aliquot in portions of 1 ml and store at -20°C.
- 4.7 dNTP-mix 5 mM:
Pipet 50 μ l dATP, 50 μ l dTTP, 50 μ l dCTP, 50 μ l dGTP [3.16] and 800 μ l ultrapure sterile water into a sterile eppendorfcup (3.29). Store at -20°C in aliquots of 50 μ l. Dilute this aliquot to 1 mM by adding 200 μ l ultrapure sterile water.
- 4.8 DNA-polymerase dilutionbuffer:
Mix 0.2 ml 1M Tris pH 8.0 [4.4], 0.2 ml 0.5M EDTA pH 8.0 [4.5], 0.1 ml 1M DTT [4.6], 49.5 ml ultrapure water and 50 ml glycerol 87% [3.26] thoroughly. Store solution at 4°C.
- 4.9 DNA-polymerase user solution 1 unit/ μ l:
Mix 20 μ l DNA-polymerase 5 units/ μ l [3.17] with 80 μ l DNA-polymerase dilutionbuffer [4.8] and vortex. Store solution at -20°C.
- 4.10 Loadingbuffer:
Dissolve 100 mg bromophenolbleu [3.2] in a few drops of ethanol. Add 10 ml ultrapure water. Dissolve 10 g sucrose [3.3] separately in 40 ml ultrapure water. Mix both solutions and store at 4°C.
- 4.11 TE-buffer:
Add 0.12 g Tris [3.19] and 0.04 g Na-EDTA [3.27] to 80 ml ultrapure water. Adjust pH at 8.0 with 1 N HCl [3.24]. Heat the solution if EDTA doesn't dissolve very well. Adjust volume to 100 ml. Aliquot the solution in 50 ml tubes. Autoclave the solution for 15 min. at 121°C. Store at roomtemperature.
- 4.12 Low Range marker-solution:
Pipet 20 μ l low range marker [3.30], 20 μ l loading buffer [4.10] and 60 μ l TE-buffer pH 8.0 [4.11] and mix. Use 6-8 μ l marker-solution per lane.
- 4.13 1Kb marker-solution:
Pipet 30 μ l Promega 1Kb-marker [3.31], 3 μ l loading dye 6x and 27 μ l TE-buffer pH 8.0 [4.11] and mix. Use 3-5 μ l marker-solution per lane.
- 4.14 SmartLadder:
The SmartLadder of Eurogentec (3.32) is user ready. Per lane 5 μ l is used. With this marker the size and the concentration of the amplicon can be estimated.

5. Protocol

- 5.1 Total PCR-mastermix for N samples [8.1]: (N+1)*28 μ l ultrapure water, (N+1)*5 μ l PCR-buffer [4.1], (N+1)*10 μ l dNTP-mix 1x [4.7], (N+1)*2.5 μ l primer 10 nM and (N+1)*2 μ l DNA-polymerase user solution [4.9] and vortex.
- 5.2 Pipet 2.5 μ l template [3.33] of each sample in a separate ultra thin 200 μ l cup [3.35].

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- 5.3 Add 47.5 µl of the mastermix [5.1] to each sample, creating a total volume of 50 µl.
 5.4 Pipet 2-3 drops of mineral oil in each reaction vial.
 5.5 Run the next cycle:
 When using a GeneAmp 9700 switch to MAX mode; do not use a "hot start".
 94°C 2 min. Initial denaturation
 Repeat the next cycle 40x:
 94°C 20 sec.
 50°C 1 min. [6.1]
 72°C 20 sec.
 At the end of the 40 cycli:
 72°C 6 min. Final elongation
 4°C ∞
 5.6 Cast a 1.2% agarose gel (protocol 9, p. 33).
 5.7 Pipet 5 µl loading buffer [4.10] on parafilm and add 10 µl PCR product [5.5] (without oil).
 5.7 Pipet the total volume of 15 µl into a slot.
 5.8 Choose an appropriate marker [4.12-4.14] and pipet on 3 different positions on gel.
 5.9 Run electrophoresis at 100V during 5 hrs; the first 15 min. at 50V.
 5.10 Check the gel at UV light, make a picture of the gel and calculate the fragment sizes. This can be done with the ImageMaster VDS system (protocol 18, p. 61).
 5.11 If RAPD-PCR has succeeded, the images can be imported in a gel comparison program and analyzed.

6. Remarks

- 6.1 The annealing temperature mentioned here works best for the M13 primer. The use of other primers necessitates the optimization of this annealing temperature [8.2].

7. Literature

- 7.1 Gräser, Y., Medical Mycology 37: 315-330, 1999.

8. Graphs and Tables

8.1 Pipetting protocol to make a mastermix for fingerprinting

	1 sample	N samples
PCR buffer 10x [4.1]	5 µl	(N+1)*5 µl
Ultrapure sterile H2O	28 µl	(N+1)*28 µl
dNTP 1 mM [4.7]	10 µl	(N+1)*10 µl
Primer 1 (...) 10 nM	2.5 µl	(N+1)*2.5 µl
DNA polymerase 1 U/µl ¹ [4.9]	2 µl	(N+1)*2 µl

¹ the amount of units depends on the activity of the polymerase

8.2 Optimal annealing temperature for several RAPD primers

Primer name	Optimal annealing temperature in °C
M13 [3.4]	50
T3B [3.6]	
OPA's [3.6-3.11]	37
(GGA) ₇ [3.12]	
(CAC) ₅ [3.13]	
(GACA) ₄ [3.14]	
(TAC) ₂ [3.15]	

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