

Performing ARDRA with PCR-rDNA

1. Subject

To perform a restriction analysis specific parts of the ribosomal DNA will be used. With so called primers this particular part is amplified. This reaction is known as PCR (polymerase chain reaction). After amplification the amplicon is digested with endonucleases, creating a 'unique' banding pattern on agarose gel.

2. Principal

The ribosomal DNA consists of repeats of a small subunit (SSU, 18S), an internal transcribed spacer (ITS1), 5.8S, an internal transcribed spacer (ITS2) and a large subunit (LSU, 26S). These repeats worden gescheiden door NTS, non-transcribed spacers.



With two primers, mostly a 20-mer sequence, a specific part of the ribosomal DNA will be amplified with the aid of an enzyme, polymerase. By PCR this part is multiplied, thus increasing the concentration of DNA. During digestion the endonucleases recognise a particular sequence and will cut the DNA at that site. If more of these sites are present, the endonucleases will cut there too. After digestion the DNA will be cut in fragments of different lengths. On agarose gel these fragments can be separated electrophoretically by size, creating a 'unique' banding pattern.

3. Reagents and material

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|------|---|-----------------|---------|
| 3.1 | electrophoresis-unit | | |
| 3.2 | bromophenolbleu (Biorad 161-0404) | | |
| 3.3 | sucrose (Merck 7653) | | |
| 3.4 | Na-EDTA (Titriplex III, Bio Rad 161-0729) | | |
| 3.5 | ependorfcups 500 µl (Sarstedt 72.699) | | |
| 3.6 | endonuclease HaeIII, Pharmacia 27-0866-02* | (5'-GG/CC-3') | +buffer |
| 3.7 | endonuclease HinfI, Pharmacia 27-0968-02* | (5'-G/ANTC-3') | +buffer |
| 3.8 | endonuclease DdeI, Pharmacia 27-0943-02* | (5'-C/TNAG-3') | +buffer |
| 3.9 | endonuclease RsaI, Pharmacia 27-0907-01* | (5'-GT/AC-3') | +buffer |
| 3.10 | endonuclease TaqI, Pharmacia 27-0956-02* | (5'-T/CGA-3') | +buffer |
| 3.11 | endonuclease MspI, Pharmacia 27-0988-02* | (5'-C/CGG-3') | +buffer |
| 3.12 | endonuclease HhaI, Pharmacia 27-0888-02* | (5'-GCG/C-3') | +buffer |
| 3.13 | endonuclease AluI, Pharmacia 27-0884-01 | (5'-AG/CT-3') | +buffer |
| 3.14 | endonuclease Sau3AI, Pharmacia 27-0913-02* | (5'-/GATC-3') | +buffer |
| 3.15 | endonuclease DraI, Pharmacia 27-0941-01 | (5'-TTT/AAA-3') | +buffer |
| 3.16 | endonuclease BamHI, Boehringer 220 612 | (5'-G/GATCC-3') | +buffer |
| 3.17 | endonuclease EcoRI, Boehringer 703 737 | (5'-G/AATTC-3') | +buffer |
| 3.18 | endonuclease HindIII, Boehringer 656 313 | (5'-A/AGCTT-3') | +buffer |
| 3.19 | sterile ultrapure water | | |
| 3.20 | waterbaths 37°C and 65°C | | |
| 3.21 | pipets and sterile pipettips | | |
| 3.22 | DNA marker: Low Range marker, Biozym 11000 | | |
| 3.23 | Tris(hydroxymethyl)-aminomethane (Merck 8382) | | |
| 3.24 | chloric acid HCl, 1N (Ferak 11448) | | |

* set of endonucleases[6.1] used at the CBS for performing ARDRA

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4. Solutions

4.1 Loadingbuffer:

Dissolve 100 mg bromophenolbleu [3.2] in a few drops of ethanol 96%. Add 10 ml ultrapure water. Dissolve 10 g sucrose [3.3] separately in 40 ml ultrapure water. Mix both solutions thoroughly and store at 4°C.

4.2 ARDRA-mastermix:

Mix for N samples: $(N+1)*10$ µl ultrapure water, $(N+1)*2$ µl endonuclease-buffer and $(N+1)*0.2$ µl endonuclease (10 units/µl). Vortex the mixture [8.1].

4.3 TE-buffer:

Add 0.12 g Tris [3.23] and 0.04 g Na-EDTA [3.4] to 80 ml ultrapure water. Adjust pH to 8.0 with 1 N HCl [3.24]. Heat solution if EDTA doesn't dissolve very well. Adjust volume to 100 ml with ultrapure water. Aliquot buffer in 50 ml tubes. Autoclave the solution for 15 min. at 121°C. Store at roomtemperature.

4.4 Low Range marker-solution:

Pipet 20 µl low range marker [3.22], 20 µl loading buffer [4.1] and 60 µl TE-buffer pH 8.0 [4.3]. Vortex the solution. Use 6-8 µl marker-solution per lane.

5. Protocol

5.1 Pipet 8 µl of amplicon in a separate eppendorfcup [3.5] for each endonuclease to be tested. With the amount left (42 µl) 5 endonucleases can be tested in one session [6.7].

5.2 Add 12.2 µl of ARDRA-mastermix [4.2] to each sample, ending with a total volume of 20.2 µl.

5.3 Incubate the eppendorfcups [5.2] for at least 3 hours or overnight at the optimal temperature [6.5] of the endonuclease used.

5.4 Prepare a 1.5-2.0% agarose-gel [6.3].

5.5 Pipet 4 µl loading buffer [4.1] in each eppendorfcup [5.2]. Centrifuge shortly to mix loading buffer and sample.

5.6 Mix loading buffer and sample by tapping and pipet 10-15 µl on gel. The rest can be stored or thrown away.

5.7 Pipet marker-solution [4.3] in the first and last slot and in a third slot in the middle of the gel.

5.8 Run at 100V until the bleu marker has migrated into the gel. Continue with 150V during 2-3 hour. The voltage remains constant [6.6]. Check the position of the front line regularly.

5.9 Make a picture of the gel in UV-light and/or estimate the lengths of the fragments by means of the marker lane [6.4].

5.10 The banding patterns of several endonucleases can be stored on disk and edited. All samples can be compared now.

6. Remarks

6.1 Next to the endonucleases [3.6-3.18] the restriction sites are written down. Each endonuclease requires a specific buffer, although in many cases a One-For-All buffer can be used. An N stands for A, G, T or C.

6.2 When creating the ARDRA-mastermix [4.2], the concentration of endonuclease used, is 10 units/µl.

6.3 Use protocol 'Preparing an agarose-gel for PCR and ARDRA purposes', (p. 33) to cast an agarose-gel [5.4].

6.4 With ImageMaster VDS hard- en software gels can be stored digitally and edited. Fragment sizes can be calculated automatically.

6.5 The optimal temperature of most endonucleases [3.6-3.18] is 37°C, except TaqI [3.10], which has its optimum at 65°C.

6.6 When samples have migrated into the gel [5.8], the magnetic stirrer (if used) can be switched on and the voltage increased to 150 V.

6.7 The PCR is performed in a 50 µl reaction. To see if the PCR has been performed well, 5-8 µl of amplicon is used for electrophoresis. The rest (42-45 µl) can be used for ARDRA.

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7. Graphs and tables

7.1: Pipetting protocol for the preparation of an ARDRA-mastermix

	1 sample	N samples
ultrapure sterile H ₂ O	10 µl	(N+1)*10 µl
endonuclease-buffer	2 µl	(N+1)*2 µl
endonuclease 10U/µl [3.6-3.18]	0.2 µl	(N+1)*0.2 µl



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