

## **Extraction of ribosomal DNA by mechanical lysis**

### **1. Subject**

By means of mechanical lysis DNA can be extracted from yeast cells or mycelium for PCR purposes (polymerase chain reaction). This method doesn't provide 100% pure DNA, but the quality is suitable to establish amplicons for RFLP analyses and sequencing.

### **2. Principal**

Ribosomal DNA is set free by lysis of biological material. Proteins, enzymes and cell debris will be precipitated with chloroform. CTAB is permeabilizing the cell wall and creates an environment to get rid of proteins and carbohydrates. The rDNA present in the water phase will be precipitated with ice cold ethanol 96% and centrifuged. After cleaning the pellet with ice cold ethanol 70% and dried, the pellet is resuspended in TE-buffer with RNase.

### **3. Reagents and material**

- 3.1 round bottom Eppendorf cups 2.0 ml
- 3.2 silica-gel (Merck 7736)
- 3.3 Celite 545 (Macherey, Nagel & Co)
- 3.4 Eppendorf cups 1.5 ml (Sarstedt 72.690)
- 3.5 micropestles (Eppendorf 0030 120.973)
- 3.6 vortex
- 3.7 centrifuge 14.000 rpm (Sigma 112, Eppendorf 5417R)
- 3.8 pipets 5-50  $\mu$ l, 50-200  $\mu$ l, 200-1000  $\mu$ l
- 3.9 pipettips
- 3.10 waterbaths 37°C, 65°C
- 3.11 Tris(hydroxymethyl)-aminomethane (Merck 8382)
- 3.12 Na-EDTA (Titriplex III, Bio Rad 161-0729)
- 3.13 CTAB (hexadecyltrimethylammoniumbromide, Sigma H-5882)
- 3.14 1 N HCl (Ferak 11448)
- 3.15 pancreatic RNase 20 U/mg (ribonuclease, Merck 24570)
- 3.16 sodium acetate trihydrate,  $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$  (Merck 6267)
- 3.17 sodium chloride, NaCl (Merck 6404)
- 3.18 latex gloves

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#### 4. Solutions

- 4.1 chloroform (Merck 2445)
- 4.2 ethanol 96% (-20°C)
- 4.3 ethanol 70% (-20°C)  
Add 350 ml ethanol 96% to 150 ml ultrapure water.
- 4.4 RNase-solution:  
Dissolve 10 mg pancreatic RNase [3.15] in 1 ml 0.01 M Na-acetate, pH 5.2 [4.8].  
Heat the solution to 100°C during 15 min. Cool slowly to roomtemperature.  
Adjust pH by adding 100 µl 1 M Tris, pH 7.4 [4.9]. Make aliquots of 100 µl and save at -20°C.
- 4.5 CTAB-buffer:  
Add 2.42 g Tris [3.11], 8.2 g NaCl [3.17], 0.74 g Na-EDTA [3.12] and 2 g CTAB [3.13, 6.9] to 80 ml ultrapure water. Adjust pH to 7.5 with 1 N HCl [3.14]. Heat the solution to dissolve Na-EDTA and CTAB if necessary. Adjust volume to 100 ml with ultrapure water. Autoclave 15 min. at 121°C. Save at room temperature.
- 4.6 TE-buffer:  
Add 0.12 g Tris [3.11], 0.04 g Na-EDTA [3.12] to 80 ml ultrapure water. Adjust pH to 8.0 with 1 N HCl [3.14]. Adjust volume to 100 ml with ultrapure water. Make aliquots of 50 ml. Autoclave the buffer for 15 min. at 121°C. Save at roomtemperature.
- 4.7 Silicagel-mix:  
Mix 30 g silicagel [3.2] with 15 g Celite 545 [3.3]. Fill round bottomcups [3.1] with 80-100 mg mix. Work in a laminar flow cabinet. Autoclave the cups during 15 min. at 121°C. Save at roomtemperature.
- 4.8 0.01M sodium acetate, pH 4.6:  
Fill a Falcon tube with 45 ml ultrapure water. Discard 150 µl and add 150 µl sodium acetate 3M [4.10].
- 4.9 1M Tris, pH 7.4:  
Dissolve 121.1 g Tris in a total volume of 800 ml ultrapure water. Adjust pH to 7.4 with 5N HCl and later with 1N HCl. Add ultrapure water to a volume of 1000 ml.
- 4.10 3M sodium acetate, pH 4.6:  
Dissolve 20.4 g CH<sub>3</sub>COONa3H<sub>2</sub>O [3.16] in 50 ml ultrapure water. Adjust pH to 4.6. Make aliquots of 1 ml in eppendorfcups. Store at -20°C.

#### 5. Protocol

- 5.1 Turn on waterbaths 37°C and 65°C.
- 5.2 Add 300 µl CTAB-buffer (4.5) to an Eppendorfcup (3.1) with silicagel-mix (4.7).
- 5.3 Transfer ±1 cm<sup>2</sup> mycelium from agarculture into the buffer (5.2).
- 5.4 Incubate 30 min. in waterbath 65°C.
- 5.5 Grind the material with a Eppendorfpistle (3.5) during 1-2 min..
- 5.6 Add an extra 200 µl CTAB-buffer and vortex.
- 5.7 Incubate the mixture for 30-60 min. in a waterbath 65°C.
- 5.8 Add 500 µl chloroform (4.1) and vortex shortly during 1-2 sec. Use chloroform only in a safety cabinet (6.5, 6.8).
- 5.9 Spin 5 min. at 14.000 rpm (20.400 rcf).
- 5.10 Recover ONLY the top layer (waterphase) into a clean, sterile Eppendorfcup. The roundbottom cup with chloroform and cell debris is chemical/biological waste [6.10].
- 5.11 Steps 5.7-5.9 may be repeated 1-2 times.
- 5.12 Add 2 volumes (± 800 µl) ethanol 96% (4.2) to the recovered material.
- 5.13 Mix the solutions carefully by hand and let the DNA precipitate at -20°C for at least 30 min. (or overnight).
- 5.14 Spin 5 min. bij 14.000 rpm (20.400 rcf).
- 5.15 Remove supernatant carefully and wash the pellet with 500 µl ethanol 70% (4.3).
- 5.16 Spin 5 min. at 14.000 rpm (20.400 rcf) and remove the ethanol carefully not losing the (sometimes very clear) pellet.
- 5.17 Air dry the pellet or under vacuum (SpeedVac) for 10 min.
- 5.18 Resuspend the pellet in 48.5 µl TE-buffer (4.6), with 1.5 µl RNase (4.4) added.
- 5.19 Incubate the pellet for 15-30 min. at 37°C.
- 5.20 Perform a GFX-column purification (Protocol 12, p. 41).

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**6. Remarks**

- 6.1 Ethanol 96% (4.2) used, is non-denatured ethanol.
  - 6.2 Wear latex gloves during 'Protocol' (5).
  - 6.3 If liquid cultures are used, the mycelium should be washed with sterile water (5.2).
  - 6.4 Micropestles (5.4) are kept in concentrated HCl (5 N); they should be rinsed with sterile water before use. Used pestles should be autoclaved and kept in concentrated HCl for further use.
  - 6.5 After adding the chloroform the solution can be mixed by hand instead of vortexing to prevent DNA of shearing/breaking.
  - 6.6 To get rid of as much proteins as possible, you may add Proteinase K (5 µl of a 20 mg/ml solution) to the CTAB-buffer.
  - 6.7 Instead of a pure chloroform-extraction (5.7), chloroform:isoamylalcohol (24:1, SEVAG, p. 73) can be used.
  - 6.8 Don't use a phenol:chloroform precipitation when a CTAB buffer is involved during extraction.
  - 6.9 Weighing and adding CTAB in dry form should be done in a fume hood.
  - 6.10 Discard in chemical waste jerrycan for halogen rich organic compounds.
7. Literature
- 7.1 Taxonomy and phylogeny of the human pathogenic black yeast genus *Exophiala* Carmichael, 1996
  - 7.2 Pathogenicity of strains of the black yeast *Exophiala* (*Wangiella*) *dermatitidis*: an evaluation based on polymerase chain reaction, *Mycoses* 37, 235-242 (1994)



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