Fluorescent in situ hybridization with fungal material

(modified method from bacteriology)

1. Subject

Collecting fungi from human tissue or environment with extreme conditions is not always possible. Cultivation is often negative. The fungal biomass inside tissue is too small to extract sufficient ribosomal DNA. Also the different treatments and embedding of biological material in parafin encourages the cultivation of fungi which is isolated from this material. Especially biological material containing micro-organisms is incubated in formaldehyd. The organisms are killed. Isolation, cultivation, extraction and PCR are impossible to perform. An other technique is FISH. With the use of fluorescent labeled probes a hybridization in situ is carried out. Probes can be looked at as specific primers with a label. In this case it is possible to hybridize selectively to genus or even species level. An epifluorescent microscope is needed to observe the hybridization results.

2. Principle

With the aid of specific primer labelel with a fluorescent dye, hybridization can be carried out. The probe has to be big enough to be specific and small enough to penetrate the cell wall. Hybridization can be improved by increasing permeability of the cell wall. After penetration of the probe it will hybridize to the complement site of the ribosomal DNA present in the cytoplasm. When this site is not present, the probe will not recombine with the DNA and washed away during a wash step, including the fluorescent dye. During microscopic check hybridization signal is visible by fluorescence of the dye inside the cell.

3. Reagents and material

3.1 NaCl (Merck 6404)
3.2 Tris(hydroxymethyl)-aminomethane (Merck 8382)
3.3 HCl (Ferak 11448)
3.4 formamide (Merck 112027)
3.5 sodiumdodecylsulphate, SDS (Merck 13760)
3.6 Na-EDTA (Titriplex III, BioRad 161-0729)
3.7 waterbath 48°C
3.8 incubator 46°C
3.9 Falcon tubes 50 ml
3.10 tissue
3.11 eppendorfcups 2 ml
3.12 milli-Q water
3.13 teflon printed objectslides with sample wells (211861, Omnilabo)
3.14 coverglass 24x50 mm
3.15 Citifluor AF1 (R1320, van Loenen)
3.16 epifluorescent-microscope with appropriate filters (depending on fluorescent labels)
3.17 probes (specific for organism to test) [6.1]
3.18 probes (for negative and positive control; autofluorescence) [6.1]
3.19 paraformaldehyde (Fluka 76240) or formaldehyde (Merck)
3.20 sodiumlauroylsarcosine (Serva 27570)
3.21 Probes ('f' = fluorescein label and 'r' = rhodamin label):

EUB338-f	(5'-GCT GCC TCC CGT AGG AGT-3')
EUK516-f	(5'-ACC AGA CTT GCC CTC C-3')
CONIOS-f	(5'-CAA CCC ACA AAA GTG AGT TG-3')
Ap665-f	(5'-TTC GTT TAG TTA TTA TGA ATC-3')
CLADOPH-f	(5'-TTG TCA CTA CCT CGC TGA ATT AGC-3')
PSEUDALS-f	(5'-TGT GCT ATC AAA TAA ACG ATA ACT TA-3')

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ED1-f (5'-GAG GAC CTG AAT AGG CGG TC-3')
non-ED1-f (5'-GAC CGC CTA TTC AGG TCC TC-3')
O20-r (5'-CCC CCT TTC CTA AAC CAA TCC GGA-3')
BANT230-f (5'-CGT ACC CGA GGT CGT TA-3')
ANTIBANT-f (5'-TAA CGA CCT CGG GTA CG-3')
CLIN230-F (5'-TAC CGT ACC CGA GGT CGT TA-3')

3.22 formaldehyde 35% (Merck 104001)
3.23 dye trays (408602, Omnilabo)
3.24 poly-L-lysin 0.1% (Sigma P-8920)
3.25 potassiumhydrogenphosphate, K2HPO4 (BDH 296194X)
3.26 potassiumdihydrogenphosphate, KH2PO4 (BDH 296084J)
3.27 sodiumhydroxide, NaOH (Merck 6498)
3.28 potassiumchloride, KCl (Merck 4936)
3.29 sodiumhydrogenphosphate, Na2HPO4
3.30 proteinase K (Merck 124568)

4. Solutions

4.1 NaCl 5M:
Dissolve 29.3 g NaCl [3.1] in 100 ml ultrapure water.

4.2 Tris 1M, pH 8.0:
Dissolve 12.1 g Tris [3.2] in 70 ml ultrapure water. Adjust pH to 8 with 1N HCl [3.3]. Add ultrapure water to 100 ml.

4.3 Sodiumdodecylsulphate, SDS 10%:
Add 10 g SDS [3.5] to 100 ml ultrapure water.

4.4 EDTA 0.5M, pH 8.0:
Dissolve 18.6 g Na-EDTA [3.6] in 70 ml ultrapure water. Heat the solution to dissolve EDTA. Adjust pH to 8 with 1N HCl [3.3]. Add ultrapure water to 100 ml.

4.5 K-phosphate buffer 0.2M pH 7.2:
Dissolve 17.4 g K2HPO4 [3.25] in 500 ml ultrapure water (A) and dissolve 13.6 g KH2PO4 [3.26] in 500 ml ultrapure water (B). Mix 36 ml solution A and 14 ml solution B and volume to 100 ml with ultrapure water.

4.6 Paraformaldehyde 4% in PBS-buffer pH 7.4 (4.8):
Heat 65 ml ultrapure water to 60°C. Add 4 g paraformaldehyde [3.19]. Add one drop of 2M NaOH [4.7] and stir vigorously to obtain a almost clear solution (± 1-2 min.). Remove heat source and add 33 ml 3x PBS [4.13]. Adjust pH with HCl [3.3] to 7.4. Filter solution with 0.2 µm filter. Snap cool to 4°C and store solution in refridgerator or on ice.

4.7 NaOH 2M:
Dissolve 4 g NaOH [3.27] in 50 ml ultrapure water.

4.8 PBS (phosphate buffered saline) pH 7.4:
Add 8.0 g NaCl [3.1], 0.2 g KCl [3.28], 1.44 g Na2HPO4 [3.29], 0.24 g KH2PO4 [3.26] to 1000 ml ultrapure water. Adjust pH, if necessary, to 7.4 with 1N HCl [3.3].

4.9 Hybridization buffer:
Pipet 360 µl NaCl 5M [4.1] and 40 µl Tris 1M [4.2] into a 2 ml eppendorfcup. Next add formamide [3.4] and Milli-Q water [8.1]. Then pipet 4 µl SDS 10% [4.3] inside the eppendorfcup lid. Close and vortex. This amount is sufficient for one sample and should be repeated for every new sample.

4.10 Wash buffer:
Pipet 1 ml Tris 1M [4.2] in a Falcon tube [3.9]. Add the right amount of NaCl 5M [4.1] according to 8.2. Adjust volume to 50 ml with ultrapure water. Pipet 50 µl SDS 10% [4.3] and mix. Preheat the wash buffer in a water bath to 48°C.

4.11 SET buffer:
Add 11.7 g NaCl [3.1], 0.12 g Tris [3.2] and 0.04 g Na-EDTA [3.6] to 80 ml ultrapure water. Adjust pH to 8.0 with 1N HCl [3.3]. Heat the solution to dissolve EDTA. Adjust volume to 100 ml with ultrapure water. Aliquot the buffer in 50 ml tubes. Autoclave buffer 15 min. at 121°C. Store at room temperateuure.

4.12 Formaldehyde 4% in PBS buffer pH 7.0

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Mix 5 ml formaldehyde [3.22] and 45 ml PBS-buffer [4.8)] Store solution at room temperature.

4.13 PBS- buffer (3x), pH 7.0:
Dissolve 24.3 g NaCl [3.1], 0.6 g KCl [3.28], 4.5 g Na2HPO4 [3.29], 0.6 g KH2PO4 [3.26] in 1000 ml ultrapure water. Adjust pH, if necessary, to 7.0 with 1N HCl [3.3].

4.14 Proteinase K (20 mg/ml):
Dissolve 20 mg proteinase K [3.30] in 1 ml sterile, ultrapure water. Store solution aliquoted in eppendorf cups at -20°C.

5. Protocol

5.1 Make a suspension of fungal material in 1000 µl PBS buffer [4.8].
5.2 Spin 5 min. at 14.000 rpm (20.400 rcf) to sediment material.
5.3 Resuspend pellet in 4% formaldehyde in PBS-buffer [4.12 or 4.6].
5.4 Incubate 30 min. at room temperature and spin 5 min. at 14.000 rpm.
5.5 Resuspend pellet in 1000 µl PBS-buffer and spin 5 min. at 14.000 rpm.
5.6 Repeat step 5.5.
5.7 Resuspend pellet in 470 µl SET-buffer [4.11].
5.8 Pipet 5 µl sodiumlauroylsarcosine [3.20] and 25 µl Proteinase K [4.14] to the suspension.
5.9 Incubate 4 hrs to overnight at 50°C.
5.10 Spin 5 min. at 14.000 rpm.
5.11 Resuspend pellet in 1000 µl PBS-buffer [4.8].
5.12 Repeat steps 5.10-5.11.
5.13 Spin 5 min. at 14.000 rpm.
5.14 Resuspend pellet in 50% ethanol/PBS [4.8] and store at 4°C or -20°C.
5.15 Pipet 8 µl suspension [5.14] on teflon printed objectslides. Use at least 3 fields: blank, negative control and positive control. Other wells can be used for specific probes.
5.16 Dry the samples for 10 min. at 46°C.
5.17 Dehydrate specimens in an alcohol series of 70%, 80% and 96% ethanol for 10 min. each. Use color trays [3.23] to treat a number of objectslides at the same time.
5.18 Air dry specimens and pipet 8 µl hybridization buffer on each (specimen) field.
5.19 Pour the rest of hybridization buffer into a Falcon tube containing a tissue and prehybridize the specimens inside the closed Falcon tube for 30 min. at 46°C.
5.20 Remove slides and pipet 2 µl probe; one field negative control (EUB338), one field positive control (EUK516) and blank nothing.
5.21 Mix hybridization buffer and probe carefully, without touching the specimen.
5.22 Hybridize for 1.5-2 hrs at 46°C in the Falcon tube (already saturated with hybridization buffer).
5.23 Rinse specimens with wash buffer [4.10] after hybridization and incubate slides in rest of wash buffer.
5.24 Incubate for 20 min. at 48°C.
5.25 Remove slides and rinse quickly with ultrapure water.
5.26 Dry samples with compressed air [6.2].
5.27 Mount specimens in anti-fading mounting fluid, e.g. Citifluor AF1, VectaShield.
5.28 The specimens can be observed immediately with an epifluorescent microscope using the correct filter system.

6. Remarks

6.1 Negative probe: EUB338 (5′-GCT GCC TCC CGT AGG AGT-3′) reacts with Eubacteria and not with fungi.
Positive probe: EUK516 (5′-ACC AGA CTT GCC CTC C-3′) reacts with ‘all’ Eukaryotes, not with Bacteria.
Aureobasidium pullulans: AP665 (5′-TTC GTT TAG TTA TTA TGA ATC-3′) universal Aureobasidium probe. non-AP665 (5′-GAT TCA TAA TAA CTA AAC GAA-3′) complement of AP665 does not react with Aureobasidium pullulans.

6.2 Samples should be dried immediately to prevent the fluorescent label from bleaching. The slides can be stored at -20°C for a longer period, if kept dry.

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7. Literature

7.1 Nuovo, G.J., (1997). PCR in situ Hybridization - Protocols and Applications. Third Edition, Lippincott-Raven, Philadelphia & New York.

8. Graphs and Tables

8.1 Pipetting scheme for hybridization buffer containing a certain formamide-concentration.

% formamide (v/v)	formamide [µl]	Water [µl]
0	0	1600
5	100	1500
10	200	1400
15	300	1300
20	400	1200
25	500	1100
30	600	1000
35	700	900
40	800	800
45	900	700
50	1000	600
60	1100	500
70	1300	30

[1] At 20% formamide or higher 500 µl EDTA [4.4] is added

8.2 Amount of NaCl 5M to be added to the wash buffer at a certain % of formamide used during hybridization.

% formamide (v/v)1 in hybridisatie-buffer	NaCl 5M in [µl]
0	9000
5	6300
10	4500
15	3180
20	2150
25	1490
30	1020
35	700
40	460
45	300
50	180
60	40
70	350 µl EDTA[2]

[2] At 70% formamide only EDTA [4.4] is added and no NaCl

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