II. Cell fixation and preparation of cell smears

Note: This procedure was adapted from Amann et al., 1990.

1. Fix the cells by adding 3 volumes of paraformaldehyde solution (4% paraformaldehyde in PBS) to 1 volume of suspended cells.
2. After 3 h incubation, do the following:
   - Pellet cells by centrifugation.
   - Remove the supernatant.
   - Wash the cell pellet with PBS.
   - Resuspending the cells in an aliquot of PBS.
   
   Note: After adding 1 volume ethanol to the resuspended cells, you may store the cell suspension at -20°C for up to 3 months without apparent influence on the hybridization results.

3. Spot these fixed cell suspensions onto thoroughly cleaned glass slides and allow to air dry for at least 2 h.
4. Dehydrate the cell samples by immersing the slides successively in solutions of 50% ethanol, then 80% ethanol, then 98% ethanol (3 min for each solution).

III. DIG labeling of oligonucleotides with DIG-ddUTP

Label oligonucleotides either according to the protocols in Chapter 4 of this manual or at the 5’ end according to the protocol of the DIG Oligonucleotide 5’-End Labeling Set*.

Note: An earlier version of this procedure has been published (Zarda et al., 1991).

I. Organisms and growth conditions

1. To prepare cells needed for this experiment, do the following:
   - Allow Escherichia coli (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSM 30083) to grow aerobically in YT broth (tryptone, 10 g/L; yeast extract, 5 g/L; glucose, 5 g/L; sodium chloride, 8 g/L; pH 7.2) at 37°C.
   - Cultivate Pseudomonas cepacia (DSM 50181) aerobically in M1 broth (peptone, 5 g/L; malt extract, 3 g/L; pH 7.0) at 30°C.
   
   Note: Cells from Methanococcus vannieli (DSM 1224) were a generous gift of Dr. R. Huber (Dept. of Microbiology, University of Regensburg, FRG).

2. To guarantee a high cellular rRNA content, harvest all cells at mid-logarithmic phase by centrifugation in a microcentrifuge (5000 x g, 1 min).

3. Discard the growth medium from the cell pellet and resuspend cells thoroughly in phosphate buffered saline (130 mM sodium chloride, 10 mM sodium phosphate; pH 7.2).

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IV. In situ hybridization using digoxigenin-labeled oligonucleotides

1. Prepare hybridization solution (900 mM sodium chloride, 20 mM Tris-HCl, 0.01% sodium dodecyl sulfate; pH 7.2).
2. Depending on the method of analysis, do either of the following:
   - If you will use fluorescently-labeled antibodies (Procedure Va below), proceed directly to Step 3.
   - If you will use peroxidase-conjugated antibodies (Procedure Vb below), then:
     - Prior to hybridization, incubate fixed cells for 10 min at 0°C with 1 mg/ml of lysozyme in TE (100 mM Tris-HCl, 50 mM EDTA; pH 8.0).
     - Remove lysozyme by thoroughly rinsing the slide with sterile H2O.
     - Air dry the slide.
     - Proceed to Step 3.

3. Add 8 µl hybridization solution containing 50 ng of labeled oligonucleotide probe to the prepared slide (from Procedure II).
4. Incubate the slide for 2 h at 45°C in an isotonically equilibrated humid chamber.

Va. Detection of DIG-labeled oligonucleotides with fluorescently labeled anti-DIG Fab fragments

1. Dilute fluorescein- or rhodamine-labeled anti-DIG Fab fragments 1:4 in blocking solution (150 mM sodium chloride; 100 mM Tris-HCl, pH 7.5; 0.5% bovine serum albumin; and 0.5% blocking reagent).
2. Add 10 µl of the diluted antibody to the slide and incubate the slide for another hour at 27°C in the humid chamber.
3. Remove the slide from the humid chamber and immerse in 40 ml of a washing solution (150 mM sodium chloride, 100 mM Tris-HCl, 0.01% SDS; pH 7.4) at 29°C for 10 min.
4. Prepare the slides for viewing by doing the following:
   - Rinse the slides briefly with sterile water (which has been filtered through a 0.2 µm filter).
   - Air dry.

Vb. Detection of DIG-labeled oligonucleotides with peroxidase-conjugated anti-DIG Fab fragments

1. Use the same conditions for hybridization and antibody binding as for fluorescent antibodies (Procedures IV and Va), except include a lysozyme pretreatment of the cell smears prior to hybridization. For details, see Procedure IV above.
2. Visualize the bound antibody as follows:
   - Prepare peroxidase substrate-nickle chloride solution [1.3 mM diamino benzidine, 0.02% (v/v) H2O2, 0.03% (w/v) nickel chloride, 5 mM Tris-HCl (pH 7.4)].
   - Incubate slide with peroxidase-substrate-nickle chloride solution until a purplish-blue precipitate forms.
3. View slides under a light microscope and photograph with Kodak Ektachrome P1600 color reversal film.

Results

Using the techniques in this article, we could detect P. cepacia cells in a mixed sample of three bacteria (Figure 1). Additional experiments with a DIG-labeled oligonucleotide not complementary to rRNA show no significant levels of nonspecifically bound DIG-labeled nucleic acid probes and anti-DIG antibodies.
Figure 1: Specific identification of whole fixed cells of *P. cepacia* in a mixture of *P. cepacia* (chains of rods), *E. coli* (rods) and *M. van-nielii* (cocci) with a DIG-labeled oligonucleotide probe. Phase contrast (panel a) and epifluorescence (panel b) photos show detection with fluorescently labeled anti-DIG Fab fragments (Zarda et al., 1991). Phase contrast (panel c) and brightfield (panel d) photos show detection with peroxidase-conjugated anti-DIG Fab fragments.


**References**


**Reagents available from Boehringer Mannheim for this procedure**

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<td>(25 labeling reactions)</td>
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