6. Troubleshooting

1) No hybridization signal detected: Analyze 5 µL of the PCR product on a 2% agarose gel. A ladder pattern should be visible. If a ladder pattern is visible, check the labeling of the PCR product by spotting it onto a membrane, followed by incubation with streptavidin peroxidase.

2) High background (stripes): Clean the miniblotter thoroughly using a dedicated brush, and soak the apparatus, preferably overnight, in a soap solution. Contact us in case you want help in procuring this.

3) High background (spots): strip the membrane again, and test it with PCR products of the control strains. If stripping does not lead to a lower background, the membrane should not be used anymore.

7. FAQ (Frequently Asked Questions)

Q. When running the PCR reactions on a gel, I do not see any product. Is that normal?

A. Sometimes no DNA is observed looking at a gel. Because spoligotyping is more sensitive than gel electrophoresis, you may find a good signal on the membrane.

Q. What is the orientation of the membrane?

A. Each membrane is marked by date of production. This date is written in the upper right corner when then membrane was labeled from left to right. When hybridizing you PCR products, this date should therefore be vertically. When you overlay your film, mark your film or bend the corner at which this date is written.

Q. We already have SDS in our laboratory. Can we use this without any problem?

A. Some users obtain good results with their own SDS. Some companies however produce SDS that gives some problems in obtaining clear blots. The kit is evaluated with the SDS used in our laboratories and we therefore recommend using this particular SDS.

Use a 10% stock solution (RT) with a expiration date of around a month.

Q. Is the kit only available with the biotinylated Dra-primer?

A. Since Ocimum Biosolutions is a producer of biomolecules as DNA, peptides and PNA, we can have any other haptens coupled to the Dra primer as well (eg. DIG, DNP,etc.). However, these haptens have not been evaluated with the spoligotyping technique. If you prefer using other haptens, please inquire about the possibilities.

Q. When incubating the PCR products at 60°C, is it possible that you get evaporation or cross-contamination of the different PCR products?

A. We have never experienced these features. Holding the blotter horizontally is the most important thing in hybridizing your PCR products.

8. References

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9. Template DNA Isolation Methods

General Remarks

For handling clinical samples that are to be used in a PCR, it is recommended to work in a room specially equipped for this purpose (over-pressure, laminar-flow hood).

Always use negative controls.

A. Preparation of Chromosomal DNA

Typically, isolation and purification of chromosomal Mycobacterium DNA is done using the CTAB method:

1) Transfer at least one loop full of cells into an Eppendorf tube containing 400 µL of 1xTE.
2) Heat 20 min at 80°C to kill the cells, and cool to room temperature.
3) Add 50 µL10 mg/mL lysozyme, vortex and incubate at least 1 hr at 37°C.
4) Add 75 µL10 % SDS/proteinase K solution (5 µL proteinase K, 10 mg/mL and 70 µL 10% SDS), vortex shortly and incubate 10 min at 65°C.
5) Add 100 µL 5M NaCl.
6) Add 100 µL CTAB/NaCl solution (4.1 g NaCl and 10 g CTAB [N-cetyl-N,N,N,-trimethylammoniumbromide] in 100 mL distilled water), which is pre-warmed at 65°C. Vortex until the liquid content becomes white ("milky"). Incubate 10 min at 65°C.
7) Add 750 µL of chloroform/isoamyl alcohol (24:1), vortex 10 sec, and centrifuge at room temperature for 5 min, 12,000 g.
8) Transfer the aqueous supernatant to a fresh microcentrifuge tube.
9) Add 450 µL isopropanol.
10) Incubate 10 minutes on ice.
11) Centrifuge 15 minutes at room temperature.

12) Discard the supernatant and wash the pellet with 1 mL of 70% ethanol and centrifuge (approximately 5 min at room temperature).

13) Discard the supernatant and dry the pellet.

14) Redissolve the pellet in 20 µL of 1xTE buffer. The DNA can be stored at 4°C until further use.

B. Preparation of Lysates from Colonies

1) Resuspend 2 loops of cells in 250 µL 1xTE in an Eppendorf tube.

2) Kill the cells by incubation at 80°C for 1 hour.

3) Centrifuge the tube at 13000 rpm for 2 min, discard the supernatant and resuspend the pellet in 500 µL of 150 mM NaCl. Repeat this step twice.

4) Discard the supernatant and resuspend the pellet in 25 µLof distilled water or 1x TE.

C. Extraction of Total DNA from Clinical Samples

1) Bring the sample (max 1 mL or 1 cm^3) aseptically in a 10 mL tube with 2-3 mL of digestion buffer (500 mM Tris HCl, pH 9.0, 20 mM EDTA, 10 mM NaCl, 1%SDS) and incubate overnight at 60°C.

2) Vortex the sample for 20 sec, add 0.5 mL phenol to 0.9 mL sample and vortex for 20 seconds.

3) Centrifuge for 5 minutes at max speed.

4) Transfer the aqueous phase to a fresh tube, containing 0.5 mL phenol, vortex for 20 seconds and centrifuge for 5 minutes at maximum speed.

5) Transfer the aqueous phase (approx. 350 µL) to a fresh tube containing 35 µL3 M NaAc and 800 µL absolute ethanol, mix and incubate for 20 minutes at -20°C.

6) Centrifuge for 30 minutes at room temperature, max speed.

7) Discard the supernatant and wash the pellet with 500 µL 70% ethanol, centrifuge for 5 minutes at max speed.

8) Discard the supernatant, dry the pellet, resuspend the DNA in 50-200 µL 1x TE and store at -20°C until further use.

D. Isolation of Genomic DNA from Paraffin-Embedded Tissues

Sample preparation of DNA from the paraffin-embedded tissues is successful, but remains time consuming. The technique involves two major steps, deparaffinization and protein digestion, each of which involves several centrifugations and washes and requires multiple tube transfers. We use a method which is a one step procedure without protein digestion:

1) Add 150 µL of a 5% Chelex suspension to a 14 µm paraffine-embedded tissue section. [a, b)]

2) Vortex thoroughly. The section should be completely covered with the Chelex suspension.

3) Heat the mixture 30 minutes at 100°C. The paraffin then appears floating on the surface of the solution.

4) Centrifuge 10 minutes at 13000 x g.

5) Transfer the solution beneath the paraffin, containing the extracted DNA, to a clean microcentrifuge tube. [c)]

6) The PCR is run with two different dilutions of the extracted DNA: 10 µL undiluted DNA and 10 µL DNA of an 1:4 dilution, in 50 µL PCR-mix.

Remarks

a) To prepare the sections use another scalpel or knife for each sample, the microtome and the knife should be disinfected with 1N HCl for two minutes after each sample to prevent contamination. Cut a negative control between every sample.

b) The aim of Chelex®100 treatment is to remove metal ions. Chelex® 100 is stable for at least 2 years when stored sealed in the original container at 22°C. If left in the hydrogen form for more than a few hours, the Chelex has a tendency to lose chelating capacity.

c) Avoid transferring the Chelex. Chelex will bind Mg2+ in the PCR-mix.

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