close to 2/1. Eighteen white colonies were directly tested by PCR. Colonies were pricked with a sterile needle point, and the point was dipped in the PCR mixture. This mixture (25 μ L) contained 2.5 μ L *Taq* DNA polymerase buffer containing 1.5 mM $MgCl_2$, 80 μ M each dNTP, 1.0 U *Taq* DNA polymerase and 0.5 pmol primers SP6 and T7. The PCR cycle profile was: 94°C for 3 min, 55°C for 60 s, 72°C for 90 s for 1 cycle and 92°C for 60 s, 55°C for 60 s, 72°C for 90 s for 40 cycles followed by a final extension at 72°C for 10 min. Products were separated by electrophoresis in a 1.5% agarose gel, and 6–8 fragments (0.3–1.3 kb) were usually obtained (Figure 1B) in each screening. Purified plasmids were then prepared for labeling by miniprep procedure.

This protocol makes it possible to clone all the bands of an RAPD profile in less than 24 h (from the preparation of the RAPD to the end of the white colony screening). The most numerous clones give the main bands (high intensity in gel), but faint bands can be isolated with a large screening of white colonies. In our example (Figure 1B), 18 clones were tested, and 7 contained an identical 500-bp fragment, 4 contained a 980-bp fragment, 3 contained an 800-bp fragment, 3 contained fragments of different lengths, and no amplification was observed on one clone. Hence, 6 different fragments were cloned with this first screening. The screening of 15 other clones allowed isolation of 2 new fragments. In one cloning procedure, 8 fragments were also cloned, reducing significantly the cost of the protocol. These fragments were digoxigenin-labeled (Boehringer Mannheim, Indianapolis, IN, USA) and then tested as probes on the RAPD products blotted onto nylon membrane (Boehringer Mannheim) after gel separation. Results of these Southern blots (data not shown) have confirmed that the cloned fragments were those amplified in the original RAPD reaction, but we have observed that some probes hybridize with several RAPD bands. The same type of result was previously described and discussed in other works with fragments cloned with other methods (1,4,6). Whatever the cloning method, it is always possible that co-

migrated fragments of the same size as the selected fragments disrupt the efficacy of the cloning, but the major RAPD bands have statistically more chance to be cloned than the wrong bands. For each cloned fragment, only study of segregation of individuals for the band of interest (on the ethidium bromide-stained gel and on the Southern blot) can confirm that the cloned fragment is the selected fragment (6). This cloning method was successfully tested on profiles obtained with different RAPD primers and also can be used on PCR products when artifactual bands are co-amplified. The screening of the clones always allows isolation of the product of interest.

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PCR-Based Multiplex Method for Rapid Screening of Recombinant Bacteria

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Analysis of recombinant plasmid DNA by size is a frequently used procedure in molecular biology. Therefore, classical protocols for plasmid minipreparation (2,5) have constantly been improved (1,3,7) to minimize the time and costs required to isolate plasmid DNA suitable for restriction enzyme or polymerase chain reaction (PCR) analysis. However, in the case of less efficient cloning strategies, a large number of bacterial colonies must be screened to identify the desired recombinant clones. A rapid screening method with reduced effort was described by Field (4). Eighty-one bacterial clones can be investigated by 18 plasmid multiplex minipreparations and subsequent restriction analysis. Effort reduction is based on combining colonies into pools that are arranged on a 9 $\times$ 9 grid scheme (similar to that shown in Figure 1A) so that each colony is represented twice. More colonies may be screened by increasing the grid size. But then the method comes to its limit because an increase of colonies per pool results in a decrease of DNA contributed by each clone of the pool. In this case, analysis by restriction digestion is disadvantageous, particularly if short restriction fragments occur.

We show that characterization of recombinant DNA by PCR (6) instead of restriction digestion increases the sensitivity and number of colonies that can be analyzed by the multiplex method. Additionally, aliquots of the bacterial

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cultures can be directly transferred into a complete PCR mixture, thus eliminating the step of DNA preparation and increasing the speed and ease of the procedure. The following example illustrates that this kind of analysis is practicable even for small DNA fragments. (i) We increased to 144 the quantity of colonies to be screened for recombinant plasmids containing a 460-bp insert. As described by Field (4), each colony was transferred to an LB/amp master plate and obtained a two-character designation using a letter between A and L and a number between 1 and 12 according to a 12 × 12 grid (Figure 1A). Corresponding to its two-character designation, each colony of the master plate was used to inoculate two culture tubes containing 5 mL LB/amp. For example, colony K10 was transferred to tubes K and 10. Consequently, each tube was inoculated with 12 colonies. (ii) After incubating the master plate and the 24 cultures over night, 1 mL per culture was centrifuged at 14000×g for 1 min at room temperature and the bacterial pellet resuspended in 100 µL TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). (iii) Five microliters from each bacterial solution were withdrawn and added to 5 µL of 10× PCR buffer (MWG Biotech, Ebersberg, Germany), 3 µL 25 mM MgCl₂, 8 µL of 1.25 mM dNTPs, 15 pmol of each insert-specific PCR

primer and 1.5 U of *Taq* DNA Polymerase (MWG Biotech) in a total volume of 50 µL. The 24 samples were subjected to PCR in a thermal cycler (Landgraf, Langenhagen, Germany). The PCR program used included an initial denaturation at 95°C for 10 min followed by 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. The reactions were completed by a final 5-min extension at 72°C. (iv) Fifteen microliters of each reaction mixture were subjected to electrophoresis on a 2% agarose gel stained with ethidium bromide.

As indicated in Figure 1B, only the samples F and 12 produced a DNA fragment of the expected size. Consequently, only the colony F12 contained the desired recombinant plasmid. Restriction enzyme digestion and DNA sequencing confirmed the presence of the correct 460-bp insert.

The PCR-based multiplex method makes it possible to screen 144 colonies by 24 PCRs. Compared to standard methods, we achieved an 83% reduction in effort while maintaining very high sensitivity. But the potential of the PCR-based analysis enables one to increase the grid size even further. To illustrate this possibility, culture tubes were inoculated with 15, 20, 25 and 30 colonies. Only one colony per tube contained a plasmid carrying a 110-bp insert. The remaining colonies ex-

pressed the same vector but a different insert that, in contrast to the first one, cannot be amplified by the primers used in the subsequent PCR. Electrophoresis of the PCR products on a 2% agarose gel demonstrates that even the very small DNA fragment derived from one member of a 30-colony pool is detectable (Figure 1C). Thus, the arrangement of a 30 × 30 grid allowing the screening of 900 colonies by 60 PCRs is possible. One has to consider whether it is advisable to analyze such a huge quantity but, if necessary, it seems to be practicable to screen even a much larger number of colonies. However, by increasing the grid size, it is likely that more recombinant plasmids can be found. This may lead to ambiguous combinations in the grid so that a few colonies must be screened in an additional step.

According to our experience, the PCR multiplex method is suitable for routine rapid screening of a large number of recombinant bacteria. Since in most applications, vector-specific primers can be used for the PCR, the method is practicable even when the sequence of the cloned DNA fragment is unknown. Provided the sequence is known, appropriate choice of the primers allows one to identify the orientation of the insert.

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Figure 1. (A) Grid scheme for analyzing 144 recombinant colonies using 24 PCRs. (B) PCR-based analysis of 144 colonies. The amplification reaction was carried out as described in the text; 15 µL of each reaction mixture were electrophoresed on a 2% agarose gel and stained with ethidium bromide. The pools F and 12 produced the expected 460-bp DNA fragment, indicating that the colony F12 expresses the desired recombinant plasmid. The marker is λ PstI size marker. (C) Ethidium bromide-stained 2% agarose gel after electrophoresis of 15 µL per PCR mixture from pools containing 15, 20, 25 and 30 colonies. The 110-bp DNA fragment derived from only one inoculated colony per pool is detectable in all reactions. Lane C: negative control; 15-µL PCR mixture of a culture pool inoculated with 20 colonies containing a vector insert that cannot be amplified by the applied primers.

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Direct Sequencing of PCR-Amplified 23S rDNA

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In prokaryotes, the ribosomal gene locus contains three rRNA species: 16S, 23S and 5S. The potential of sequencing 16S rDNA of bacteria and archaea for establishing phylogenetic relationships and for use in molecular diagnostics is well-documented. Currently, over 3000 16S rRNA sequences are available in the databases. However, the potential of the 23S rRNA gene as a diagnostic tool has not been fully explored. Strong secondary structure and a larger size (3 vs. 1.5 kb for 16S rDNA) have hindered efficient sequencing of the 23S rRNA gene. So, there are just over 100 bacterial 23S rRNA sequences available for bacteria (4,5,8). Commonly, 23S rRNA gene sequence is determined either by sequencing of cloned fragments (1) or by amplification of partial fragments and subsequent direct sequencing of these products (2,9). In a recent report on direct sequencing of the PCR-amplified 23S rDNA, Sallen et al. (9) described the preparation of the 23S rDNA by three amplification steps to obtain overlapping fragments and the subsequent use of 33 sequence primers to achieve the complete sequencing of the 23S rRNA gene.

We describe a simple protocol that allows amplification of almost the complete 23S rRNA gene and the subsequent direct sequencing of the double-stranded fragment, thereby eliminating the need for cloning or preparation of single-stranded template (7). We adapted a sequencing strategy, developed by Amersham (Amersham International plc, Little Chalfont, Bucks, England, UK) for sequencing DNA clones, for direct sequencing of PCR-amplified 23S rDNA. We have chosen sequencing of the 23S rRNA gene of the human pathogen *Francisella tularensis* as a model to demonstrate the potential of the described strategy.

Fresh cultures of *Francisella* strains were pelleted and resuspended in 1 mL TE buffer (10 mM Tris-HCl, 1 mM

EDTA, pH 8.0). The suspension was heated to 80°C for 30 min and centrifuged at 10000× g for 15 min, and 1 µL of the supernatant was added to a polymerase chain reaction (PCR) master mixture containing (in a final volume of 50 µL) 1× PCR buffer [20 mM Tris-HCl, 10 mM KCl, 10 mM (NH₄)₂SO₄, 1% Triton® X-100, 1.5 mM MgSO₄ and 1 mg/mL of bovine serum albumin], 200 µM of each dNTP and 10 pmol of each primer. The mixture was overlaid with 50 µL of mineral oil and heated to 80°C before 1 U each of *Taq* DNA polymerase and *Taq* extender (Stratagene, La Jolla, CA, USA) was added. Immediately after adding the enzymes, the tubes were subjected to 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 60°C and extension for 2.5 min at 72°C on a Model 480 Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA). The amplification process was terminated by a 10-min extension at 72°C, and tubes were rapidly cooled to 4°C. The primers used were chosen so that the 16S–23S spacer region and virtually the complete 23S rRNA gene would be amplified, thereby enabling determination of the 5' end of the gene. The resulting fragment is approximately 3 kb (because of a rather short spacer of 400 bp in *F. tularensis*, Figure 1). The primers are complementary to positions 1522–1538 and 2669–2654 of the 16S and 23S rRNA genes, respectively (Table 1). Fragments were separated by electrophoresis on a 1% agarose gel. Amplification products were excised

Figure 1. Amplification of the rDNA fragment encompassing the 16S–23S spacer region and the 23S rRNA gene. Lane 1: 1-kb ladder (Life Technologies, Gaithersburg, MD, USA). Lanes 2–6: 10% of the PCR product from strains FSC 017, 024, 033, 041 and 043 of *F. tularensis*. Lane 7: negative control.