5. **Hopwood, D.A., T. Kieser, H.M. Wright and M.J. Bibb.** 1983. Plasmids, recombination and chromosome mapping in *Streptomyces lividans* 66. J. Gen. Microbiol. 129:2257-2269.
6. **Huang, C.H., Y.L. Yang, Y.S. Lin and C.W. Chen.** 1998. The telomeres of the *Streptomyces* chromosomes contain conserved palindromic sequences with potentials to form complex secondary structures. Mol. Microbiol. 28:905-916.
7. **Kinashi, H. and M. Shimaji-Murayama.** 1991. Physical characterization of SCP1, a giant linear plasmid from *Streptomyces coelicolor*. J. Bacteriol. 173:1523-1529.
8. **Kinashi, H., M. Shimaji-Murayama and T. Hanafusa.** 1991. Nucleotide sequence analysis of the unusually long terminal inverted repeats of a giant linear plasmid, SCP1. Plasmid 26:123-130.
9. **Kinashi, H., M. Shimaji-Murayama and A. Sakai.** 1987. Giant linear plasmids in *Streptomyces* which code for antibiotic biosynthesis genes. Nature 328:454-456.
10. **Leblond, P., G. Fischer, F.X. Francou, M. Berger, M. Guerineau and B. Decaris.** 1996. The unstable region of *Streptomyces ambofaciens* includes 210 kb terminal inverted repeats flanking the extremities of the linear chromosomal DNA. Mol. Microbiol. 19:261-271.
11. **Lezhava, A., T. Mizukami, T. Kajitani, D. Kameoka, M. Redenbach, H. Shinkawa, O. Nimi and H. Kinashi.** 1995. Physical map of the linear chromosome of *Streptomyces griseus*. J. Bacteriol. 177:6492-6498.
12. **Lin, Y.S., H.M. Kieser, D.A. Hopwood and C.W. Chen.** 1993. The chromosomal DNA of *Streptomyces lividans* 66 is linear. Mol. Microbiol. 10:923-933.
13. **Pandza, K., G. Pfalzer, J. Cullum and D. Hranueli.** 1997. Physical mapping shows that the unstable oxytetracycline gene cluster of *Streptomyces rimosus* lies close to one end of the linear chromosome. Microbiol. 143:1493-1501.
14. **Redenbach, M., H.M. Kieser, D. Denapate, A. Eichner, J. Cullum, H. Kinashi and D.A. Hopwood.** 1996. A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb *Streptomyces coelicolor* A3(2) chromosome. Mol. Microbiol. 21:77-96.
15. **Sambrook, J., E.F. Fritsch and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual. 2nd Edition. CSH Laboratory Press, Cold Spring Harbor, NY.
16. **Thomas, C.A., K. Saigo, E. McLeod and J. Ito.** 1979. The separation of DNA segments attached to protein. Anal. Biochem. 93:158-166.
17. **Yanisch-Perron, C., J. Vieira and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene 33:103-119.

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Alexander Stoll and John Cullum
University of Kaiserslautern
Kaiserslautern, Germany

Unique Adaptor Design for AFLP Fingerprinting

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AFLP (7,8) is a fingerprinting technique based on selective PCR amplification of restriction fragments of double-enzyme digests, usually amplification of heterosite fragments with frequent and infrequent cutter ends. AFLP is being used extensively for genetic mapping in plants and DNA fingerprinting in microorganisms (2,3,5-7). It has gained wide popularity over RAPD because of its reproducibility and robustness. The procedure commonly involves digestion of DNA with *EcoRI* and *MseI* or any other combination of enzymes with six- and four-base recognition sites and ligation of adaptors to both ends of the fragments. The subset of the *EcoRI-MseI* fragments is amplified by PCR using selective primers.

In theory, the original adaptor design will amplify any of the three types of fragments: *EcoRI-EcoRI*, *EcoRI-MseI* and *MseI-MseI*. Most of the fragments are *MseI-MseI* fragments. The initial method used biotinylated *EcoRI* adaptor to pull out the *EcoRI*-associated fragments (8). Subsequently, it was discovered that a preamplification step could preferentially enrich *EcoRI-MseI* fragments, with undetectable levels of am-

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plification of *MseI*-*MseI* fragments (7). However, preferential amplification depends both on unknown intrinsic primer features that need to be evaluated for any new primer and on the suppression effect of inverted adaptor ends of *MseI*-*MseI* fragments, which is only effective for fragments smaller than 500 bp (7).

The adaptor design we present avoids both of these problems related to poorly understood features. The new adaptors and primers completely suppress amplification of fragments of the frequent cutter (*MseI*-*MseI*) but allow exponential amplification of heterosite fragments (*EcoRI*-*MseI*) with only linear amplification of fragments of the infrequent cutter (*EcoRI*-*EcoRI*). As shown in Figure 1, the adaptor for the infrequent cutter (*EcoRI*) consists of a long oligonucleotide that is phosphorylated at the 5' end and a short oligonucleotide with an amino group at the 3' end that blocks the 3' hydroxyl group from further reactions. The two oligo-

nucleotides form a partial double-stranded structure with single-strand extensions. The 5' end of the long oligonucleotide binds to the extension of the *EcoRI* cut site and can be ligated to the DNA fragment. However, the 3' end of the short oligonucleotide cannot be ligated because of the amino group. Therefore, only one strand of each *EcoRI* end is ligated. The amino group also blocks self-ligation of *EcoRI* adaptors.

The adaptor for the frequent cutter also consists of two oligonucleotides of different lengths, which yield a double-strand molecule with single-strand extensions. The short oligonucleotide is not phosphorylated at the 5' end and has an amino group at the 3' end. The amino group precludes the oligonucleotide from priming during PCR. The nonphosphorylated 5' end serves a dual purpose: adaptor self-ligation is prevented and the oligonucleotide does not ligate to the 3' end of *MseI* fragments. Thus, only one strand of each *MseI* end

is ligated to the adaptor. *EcoRI*-*MseI* fragments will have one strand with an adaptor strand ligated at both ends, while the other does not ligate to an adaptor at either end (Figure 1). The addition of phosphate and amino groups to adaptor oligonucleotides is conveniently done as a standard option on most oligonucleotide synthesizers.

In a pool of fragments from an *EcoRI*-*MseI* double digest, only the *EcoRI*-*MseI* fragments give rise to single strands with adaptors attached at each end. The ligated product mixture is denatured before PCR to prevent *Taq* DNA polymerase from resealing the gaps in the ligation products or replacing unligated strands. During PCR amplification, synthesis starts from the *EcoRI* ends because only the primer for such ends is complementary to the adaptor sequence (Figure 1). The primer for the *MseI* ends only binds to newly synthesized strands initiated by the primer using the *EcoRI* adaptor as a template.

Figure 1. Sequences of adaptors and primers showing the essential features of the new adaptor design and the result of PCR amplification. Arrows indicate the sites where ligation can occur. The outside primers (P1 and P2) were used for preamplification in this study and are appropriate for small genomes, although AFLP analysis for large genomes commonly uses primers with plus-one extensions as preamplification primers. The use of primers nested outside selective primers for preamplification, hence the long adaptor, is not considered an essential feature for this design. If nested primers are not used, a shorter oligonucleotide can be used as the adaptor. The selective inner primers (P3 and P4) are shown with a plus-one selective extension, base A for the *EcoRI* primer and base C for the *MseI* primer.

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Thus, amplification of *MseI*-*MseI* fragments is completely suppressed, while *EcoRI*-*MseI* fragments are exponentially amplified, and *EcoRI*-*EcoRI* fragments are only linearly amplified. This selectivity provides the major advantage of the new adaptor design.

Figure 2. AFLP patterns for *E. coli* (DNA prepared from K-12), yeast and corn (DNA purchased from Clontech Laboratories, Palo Alto, CA, USA). Samples in lanes 1 and 2 (*E. coli* K-12), 3 and 4 (yeast) and 5 and 6 (corn) were processed for AFLP as described in the text with and without preamplification, respectively. Samples in lanes 1–6 used labeled *EcoRI* primer. Samples in lanes 7–12 resemble those of lanes 1–6, respectively, but used labeled *MseI* primer. K12 was amplified with the *EcoRI*+A and *MseI*+C primer combination, yeast with *EcoRI*+ACC and *MseI*+C and corn with *EcoRI*+ACC and *MseI*+CTG.

Another unique feature of this adaptor design is that hot start PCR can be performed. The original adaptor design allows each strand of a restriction fragment to be ligated only to its 5' end because the adaptors are not phosphorylated (7). Fragments are not amplified if template DNA is first denatured, and thus hot start PCR cannot be performed with the original adaptor design. Hot start is known to increase specificity, and AFLP depends on selectivity of the primers under stringent annealing conditions. Hot start by thermal activation of modified or antibody-blocked DNA polymerase is convenient and commercially available (1,4).

The method was tested using *E. coli*, yeast (*Saccharomyces cerevisiae*) and corn (*Zea mays*) DNA. DNA (0.1, 0.2 or 0.5 μ g, respectively) was used for digestion and ligation of adaptors. The ligation mixture was denatured at 94°C for 2 min, and 1 μ L was mixed with 19 μ L PCR mixture containing 0.3 μ M each preamplification primers, 0.2 μ M each dNTP, 4 μ g/ μ L BSA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂ and 0.5 U AmpliTaq Gold® (PE Biosystems, Foster City, CA, USA). The cycling profile was 20 cycles of denaturation at 94°C for 15 s, except for 10 min for the first cycle, annealing at 56°C for 30 s and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. PCR products from the preamplification were diluted 1:10, and 1 μ L was used for a second amplification with 19 μ L PCR mixture, the same as above except that 0.3 μ M unlabeled selective primer and 0.1 μ M ³³P-labeled selective primer were used. Cycling parameters were 30 cycles of denaturation at 94°C for 15 s, except for 10 min for the first cycle to activate AmpliTaq Gold, annealing at temperatures specified below for 30 s and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. The first 10 cycles were touch-down from 66°C to 56°C decreasing by 1°C/cycle and then 20 cycles at 56°C. Labeled amplification products were run on standard 6% polyacrylamide sequencing gels and visualized by exposure to Kodak® BioMax-MR® film (Eastman Kodak, Rochester, NY, USA) overnight. We also tested the samples without a preamplification step. One microliter of the pre-denatured ligation product was

directly used for the second amplification. All procedures were the same as above for the second amplification except that 25 cycles rather than 20 cycles were performed.

The resulting AFLP fingerprinting patterns are shown in Figure 2. We tested the effect of preamplification and the effect of labeling *EcoRI* primer or *MseI* primer. Fingerprints for *E. coli* K-12 are nearly identical with and without preamplification when the *MseI* primer is labeled, and similar but with a much higher background without preamplification when the *EcoRI* primer is labeled. There were also some differences in patterns for yeast with and without preamplification. There is a dramatic difference in patterns for the corn DNA, which indicates that selective amplification is unachievable without preamplification. We did not investigate the causes of the differences between samples with and without preamplification, but the differences were consistent and reproducible for K-12 and yeast. This suggests that preamplification is not necessary for small genomes as long as one compares AFLP patterns generated by the same procedure, which is beneficial in the diagnosis of bacterial pathogens where time is crucial, for example.

With preamplification there is no difference between the use of labeled *EcoRI* primer or *MseI* primer in terms of background, although there are some band differences that may be due to the mobility difference of the strands as observed by Vos et al. (7). However, without a preamplification step, labeling the *MseI* primer consistently gave clearer results than labeling the *EcoRI* primer, most likely because *EcoRI*-*EcoRI* fragments are linearly amplified, which contributed to background with a labeled *EcoRI* primer. Labeling the *MseI* primer has an additional benefit—only the six-base cutter needs to be changed to explore the genome for other variations with the same frequent cutter. Note that if the *EcoRI* primer is labeled, a change of the frequent cutter has little effect on the genome space explored as the variation with the two frequent cutters will not be independent. This is not an issue for working with large genomes in which three selective bases are commonly used, giving a large number of

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selective primer combinations. However, for bacterial genomes, selective primers with one base extension are usually used, and there are only 16 combinations of selective primer pairs from the four plus-one extensions for each of the *EcoRI* and *MseI* primers. These combinations may not generate sufficient differentiating fingerprints or markers for homogenous clones or species and thus require the use of alternative restriction enzymes.

We have used the new adaptor design to type isolates from *Salmonella enterica*, *Vibrio cholerae* and *E. coli*, using hot start PCR and labeling the *MseI* primer, and have obtained consistent and reproducible results (data not shown).

REFERENCES

1. Birch, D.E., L. Kolmodin, W.J. Laird, N. McKinney, J. Wong, K.K.Y. Young, G.A. Zangenberg and M.A. Zoccoli. 1996. Sim-
2. Blears, M.J., S.A. De Grandis, H. Lee and J.T. Trevors. 1999. Amplified fragment length polymorphism (AFLP): a review of the procedure and its applications. *J. Ind. Microbiol. Biotechnol.* 21:99-114.
3. Janssen, P., R. Coopman, G. Huys, J. Swings, M. Bleeker, P. Vos, M. Zabeau and K. Kersters. 1996. Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. *Microbiology* 142:1881-1893.
4. Kellogg, D.E., I. Rybalkin, S. Chen, N. Mukhamedova, T. Vlasik, P.D. Siebert and A. Chenchik. 1994. TaqStart antibody: "hot start" PCR facilitated by a neutralizing monoclonal antibody directed against *Taq* DNA polymerase. *BioTechniques* 16:1134-1137.
5. Lin, J.-J., J. Kuo and J. Ma. 1996. A PCR-based DNA fingerprinting technique—AFLP for molecular typing of bacteria. *Nucleic Acids Res.* 24:3649-3650.
6. Majer, D., R. Mithen, B.G. Lewis, P. Vos and R.P. Oliver. 1996. The use of AFLP fingerprinting for the detection of genetic variation in fungi. *Mycol. Res.* 100:1107-1111.
7. Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot et al. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* 23:4407-4414.
8. Zabeau, M. and P. Vos. 1993. Selective restriction fragment amplification: a general method for DNA fingerprinting. European patent application, EP 0534858.

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Ruiting Lan and Peter R. Reeves
University of Sydney
Sydney, Australia