directed mutagenesis method produces single- and double-amino acid substitution mutants as well as deletion mutants, with a high mutagenesis efficiency within 48 h. Not only does the use of the proofreading Pfu DNA polymerase in the PCR mixture reduce the level of errors that may be introduced by other DNA polymerases, but it also streamlines the mutagenesis protocol when compared to related protocols. Since no nearby restriction sites are required or engineered into the mutagenic primers, mutations can be introduced anywhere within the sequence, and only targeted residues are changed. We have found this method to be particularly well-suited for generating site-directed mutants in the coding region of expression constructs.

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Typing of Human Papillomaviruses by Reductional RFLP Analysis of Biotin-Labeled PCR Fragments

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Anogenital human papillomaviruses (HPVs) are a group of over 40 distinct virus types associated with condylomatous lesions, dysplasias and carcinomas of the anogenital tract, particularly cancers of the uterine cervix (3). Due to differences in oncogenic potential, HPVs are further categorized as low-, intermediate- and high-risk groups with different clinical manifestations (6). Thus, following detection of the presence of HPV in a sample, it is clinically important to determine the HPV type in each case.

Numerous methods based on polymerase chain reactin (PCR) amplification and the use of HPV type-specific probes have been described for HPV detection and typing (2,4,5). These assays normally include the most common HPV types; less common and rare HPV types are excluded for convenience. Furthermore, new HPV types may need to be included in the assay regularly. An assay system developed for a certain geographic region may not be entirely applicable for use in other regions where different HPV types may prevail. Thus, the best HPV typing system would be one that has no constraint on the viral type and is amenable to addition of new HPV types.

Restriction fragment-length polymorphism (RFLP) analysis of PCR-generated HPV-specific DNA fragments has been used for HPV typing (2,7). To increase sensitivity and specificity, a combined RFLP/hybridization approach has also been adopted that uses generic or degenerate oligonucleotides as probes for hybridization to PCR-generated RFLP patterns to differentiate between most mucosal HPV types (7).

We describe a simplified version of the RFLP approach in HPV typing in which the number of bands in the often complex RFLP patterns generated by digestion with multiple restriction enzymes is reduced and simplified. In
The presence of HPV in a sample was first detected using the HPV L1 consensus and degenerate primers MY09 and MY11, which have been known to amplify most genital HPV types (1,8). For subsequent biotin detection, the MY11 oligonucleotides were 5′-labeled with biotin. A pair of β-globin gene primers were also included as an internal PCR control. In brief, exfoliated cervical cells were harvested and resuspended in 50 µL PCR buffer containing 0.6 U of a PreTaQ™ Thermophilic Protease (Life Technologies, Gaithersburg, MD, USA) and incubated at 94°C for 30 min. After removal of cell debris by a centrifugation step, 5 µL of the lysate were supplemented with 5 µL 10× PCR buffer (0.5 M KCl, 25 mM MgCl₂, 0.1% gelatin, 0.1 M Tris-HCl, pH 8.3), 4 µL dNTP solution (dATP, dCTP, dGTP; 2.5 mM each), 2.5 µL of each primer oligonucleotide (25 pmol each), 2 U of Taq DNA Polymerase (5 U/µL; Takara Schuzo, Otsu, Shiga, Japan) and sterile water to make a final volume of up to 50 µL. The PCR temperature profile was as follows: after an initial denaturation step at 94°C for 5 min, 40 PCR cycles at 94°C for 1 min, 55°C for 1.5 min and 72°C for 30 s were performed. The PCR products were analyzed in a 2% agarose gel.

The HPV-specific fragments were about 450 bp and were clearly separated from the 303-bp β-globin fragment (Figure 1, lanes marked “U”). Samples that showed the β-globin band but not the HPV band were scored negative for HPV. Samples that contained PCR inhibitors or samples that did not contain sufficient intact DNA revealed no β-globin band and were not scored. We consistently obtained a sufficient amount of clean PCR products from clinical samples for subsequent HPV typing. We were able to detect 10 pg of HPV DNA in the sample.

For HPV-positive cases, the RFLP patterns of five restriction enzymes (BamHI, Ddel, HaeIII, HinI and PstI) (7) were established using the PCR products generated as described above. Aliquots of 2 µL of the PCR products (mixtures of HPV- and β-globin-specific DNA fragments) were digested with 10 U of each enzyme in a total volume of 10 µL at 37°C for 2 h. The use of a small volume in the enzyme digestion step did not result in interference from salt and buffer carried over from the PCR. Instead, a higher restriction enzyme concentration led to a more rapid and complete digestion. The digestion products were resolved on a 2.5% agarose gel. The gel-embedded DNA fragments were capillary-transferred to a nylon membrane using the standard Southern blotting protocol. The membrane blot was briefly rinsed twice with TBST (0.05% Tween®20, 10 mM Tris-HCl, 0.5 M NaCl, pH 7.5) and blocked with 5% dry milk in TBST for 1 h at room temperature. After being washed with TBST twice, the membrane was incubated with horseradish peroxidase-conjugated streptavidin (1:5000 dilution; Catalog No. RPN1231; Amersham International plc, Bucks, England, UK) at room temperature for 1 h. The membrane was again washed extensively with TBST. Biotinylated DNA fragments on the membrane were detected using the ECL™ Detection Kit (Amersham International plc) and exposing the membrane to X-ray films. HPV types were determined by visually matching the sample reductional (r)RFLP patterns revealed on the X-ray film with a computer-generated database of rRFLP patterns of all known genital HPV types (available on request from the corresponding author). Typical clinical examples are shown in Figure 1. The use of a biotinylated primer inevitably did not prevent unspecific amplification, as

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**Figure 1. Typing of HPV by rRFLP analysis of clinically derived exfoliated cervical cell samples.**

The top two rows show samples containing single HPV-type infection (HPV-18 and -33); row 3 shows a clinical case of mixed infection with HPV-18 and -33. The left column (“Eth Br”) shows the ethidium bromide-stained RFLP gel patterns, and the right column (“Biotin”) shows the biotinylated bands and thus the rRFLP patterns. The sizes of the expected biotinylated bands in the rRFLP patterns are also shown. In the ethidium bromide-stained gels, the β-globin bands in the undigested (U) lanes are indicated by arrowheads. The β-globin bands in other lanes are not indicated for the sake of clarity. The restriction enzymes used for the analysis were BamHI (B), Ddel (D), HaeIII (Ha), HinI (Hi) and PstI (P). Spurious PCR bands observed in the case of HPV-18 and -33 mixed infection (row 3) are indicated.
was the case shown in the bottom row of Figure 1. However, our ability to obtain relatively clean biotinylated RFLP patterns in most cases was a consequence of our efforts in generating clean HPV bands in the initial PCR (Figure 1, lanes marked “U”).

In our approach, since one of the primers (MY11) is biotinylated, a simplified RFLP pattern of five restriction enzymes is generated. Only one band is detected in each electrophoretic lane, and yet each HPV type retains a unique RFLP pattern. The presence of the control β-globin sequence, whether or not digested by the restriction enzymes used, does not show in the final biotinylated RFLP pattern. The appearance of two or more biotinylated bands in some of the lanes would be an indication of multiple HPV-type infection (Figure 1). Note that MY11 has been chosen over MY09 because it generally produces longer biotinylated restriction fragments. In this study, 103 HPV-positive samples of exfoliated cervical cells collected by cervical swabs were analyzed. A total of 18 different HPV types (HPV-6b, -16, -18, -31, -33, -39, -52, -53, -56, -58, -61, -66, -68, -70, -MM4, -MM7, -CP8301 and -CP8304), including five cases of double HPV-type infection, were detected. In most cases, the HPV types were confirmed by direct fluorescence sequencing of the PCR products using MY09 and MY11 as the sequencing primers. In the double infection cases, the HPV types were confirmed by subcloning followed by sequencing.

The RFLP approach circumvents the hybridization step. If needed, digestion with more restriction enzymes may also be introduced into the system to increase type resolution and specificity. New HPV types, once sequenced and the RFLP ascertained, may be added to the RFLP database. Thus, constraints on the HPV types analyzed are removed. Our approach has an advantage over direct gel visualization in that the number of bands is reduced. We have further adopted this advantage in electrophoretic analysis of a mixture of all DNA fragments generated from multiple restriction enzyme digestions in a single lane using a fluorescence-labeled MY11 as one of the PCR primers (unpublished). Thus, such an RFLP approach is open to computer-assisted HPV-type sorting and automation. Correlation between HPV-type and clinical manifestation is not the subject of this report. However, such a high-resolution HPV typing approach has enabled our gynecologists to pay more attention to the association of HPV types with stagings of the cervical lesions leading to better identification and treatment of patients with HPV infection.

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