PCR/RFLP Assay for Copy Number of Mutant and Wild-Type Alleles


ABSTRACT

A PCR method for quantitating the copy number of mutant vs. wild-type alleles in DNA from cell lines is described. The assay can be used to detect a point mutation in any gene that creates or destroys a restriction site. The alleles of interest are amplified by nested PCR and labeled in a second round of PCR. The product is digested with a restriction enzyme specific for that site, resolved on a non-denaturing gel and quantified by phosphor imaging techniques. Cell types with known numbers of wild-type and mutant alleles of \( c\)-Harvey-ras are used to validate the assay. The method is then applied to a cell line, homozygous for the mutation, to determine the gene copy number. The applicability of the method to other genes is shown using the matrix metalloproteinase gene, matrilysin. A cell line transfected with a plasmid carrying a mutant, auto-activated form of the gene is compared to its parent cell line. Advantages of this technique compared with Southern analysis are ease of screening a large number of clones or foci and accuracy of quantitation.

INTRODUCTION

There are several research situations where gene dosage is important. Cell lines, derived from tumors may carry an altered gene dose of mutant genes. These cell lines may change this gene dose during passage in tissue culture. The copy number of transfected genes integrated into the genome may need to be determined. In the past, Southern analyses were used to quantify the ratio of mutant-to-wild-type \( c\)-Harvey-ras alleles (16). Quantifying Southern analyses, however, can be a problem due to transfer and hybridization differences of DNA fragments of various lengths, background in the lane, incomplete cutting of DNA and the uneven loading of lanes. Cytogenetics have been used as an addition to Southern analyses to determine the copy number (4). An alternative method of determining gene dose, the probing of polymerase chain reaction (PCR)-amplified alleles with mutation-specific oligonucleotides (3), requires two hybridization steps and the adjustment of exposure times for probe-specific activity and hybridization efficiency.

The PCR method described here is time-efficient and accurate for establishing the copy number of mutant and wild-type alleles. Previously, single-stage PCR amplification of alleles combined with mutation-specific restriction fragment formation has been the basis for detection of rare mutations in a background of wild-type alleles (10). Nested PCR, previously used to detect rare mutant alleles (12) or rare viral copies (18), is used here to enrich the gene of interest in relationship to the genome in the first stage, allowing the second stage to be used for the incorporation of radioactive nucleotides. Optimization for Mg\(^{++}\) ion, nucleotide levels or annealing temperature was not necessary for each new gene. Phosphor imaging techniques provide wide linear range with ease of quantitation and without use of X-ray film.

MATERIALS AND METHODS

DNA Sources

CD-1 strain mouse epidermal tissue (7) was the source of wild-type \( c\)-Ha-ras alleles. PDV is a tumorigenic mouse keratinocyte cell line, shown by Southern analyses of \( XhoI\) digests to have a 2:1 ratio of wild-type \( c\)-Ha-ras alleles per mutant 61st codon allele, (CAA\( \rightarrow\)CTA) (1). A total of three \( c\)-Ha-ras gene copies was established by cytogenetics (4). This cell line serves as a control in the validation of the assay for gene copies. CarB is a tumorigenic spindle cell carcinoma keratinocyte cell line, homozygous for the same \( c\)-Ha-ras 61st codon A\( \rightarrow\)T mutation (3). DU145 is a human prostate cell line (15), with no known mutations in the matrix metalloprotease matrilysin gene. MR-15 clone 24 is a DU145 line, transfected with a cDNA plasmid carrying a mutant 89th codon allele GTG\( \rightarrow\)GGG in the second exon (13). An additional \( Sau96I\) restriction site is formed by this mutation.
**Primers**

c-Ha-ras primers are based on the published second exon of mouse cDNA (2): Stage 1 Upstream 5’ CTGTGAAT-TCTCTGGTCTGAGGAG 3’ (nucleotide [nt] 244–267); Stage 1 Downstream, 5’ TAGGTGGCTACCTGTA-CTG 3’ (nt 491–510); Stage 2 Upstream, 5’ CTAAGCCTGTGTTTTG-CAGGAC 3’ (nt 302–322); and Stage 2 Downstream, 5’ GGAACCTGGTGT- GTTGATGGC 3’ (nt 455–475). Matrilysin primers are based on the published second exon human cDNA (6): Stage 1 Upstream, 5’ GCCAACAGTTAGAAGCCAAAAC 3’ (nt 201–222); Stage 1 Downstream, 5’ CTGTAAGT GACACTTTGGAAG 3’ (nt 361–382); Stage 2 Upstream, 5’ GAATGTTAAACTCCCGCTCATA 3’ (nt 261–284); and Stage 2 Downstream, 5’ TGACCACTTTGGAGTCCATTTTG 3’ (nt 352–375).

Table 1. First-Stage PCR

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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<tr>
<td>1 µg of digested genomic DNA</td>
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<tr>
<td>0.3 µM each stage 1 primers</td>
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<tr>
<td>1× UlTma Buffer (Perkin-Elmer)</td>
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<tr>
<td>4 µM each dNTP (Perkin-Elmer)</td>
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<tr>
<td>2.4 U UlTma DNA Polymerase (Perkin-Elmer)</td>
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<tr>
<td>2 mM Mg++ carried over by the DNA in the No. 2 Restriction Buffer</td>
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**Protocol**

The nested PCR protocol described below produces a nonradioactive band in the first stage of 267 bp for c-Ha-ras and 182 bp for the matrilysin gene. The second stage produces a double-stranded radioactive band of 176 bp for c-Ha-ras and 115 bp for the matrilysin gene. The diagnostic Xbal digest produces bands of 91 and 85 bp for the mutant c-Ha-ras. Sau96I produces diagnostic bands of 61 and 54 bp for the mutant matrilysin gene.

**DNA Preparation**

DNA was purified by proteinase K, RNase and phenol/chloroform/isoamyl alcohol extractions (5). High-molecular-weight DNA was spooled out, quantitated by spectrophotometry and digested with 5 U/µg PstI and 5 U/µg HindIII.
in No. 2 Restriction Buffer (New England Biolabs, Beverly, MA, USA).

**First-Stage PCR**

Table 1 lists the components of the first-stage PCR. A 50-µL amplification reaction was started with the addition of UltraTM DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA) in 10 µL of 1× UltraTM Buffer into a DNA mixture preheated to 85°C on a Cetus™ DNA Thermal Cycler (Perkin-Elmer). After an initial denaturation for 2 min at 97°C, 20 cycles of PCR were carried out for 1 min at 97°C, 1 min at 60°C and 1 min at 72°C. The product was diluted 1:100 in H₂O.

**Second-Stage PCR**

Table 2 lists the components of the second-stage PCR. The 50-µL reaction was started with the addition of Stoffel™ Fragment, AmpliTaq® DNA Polymerase (Perkin-Elmer) in 10 µL of 1× Stoffel Buffer to DNA mixture preheated to 85°C. After a denaturation step of 2 min at 97°C, 12 cycles of PCR were carried out with 1 min at 97°C and 6 min at 67°C. A final extension of 20 min at 67°C was used.

**Digestion for Restriction Fragment-Length Polymorphisms (RFLP)**

The second-stage product (10 µL) was pipetted into the appropriate restriction buffer with or without 10 U of the diagnostic enzyme in a total volume of 30 µL and was incubated for 3–16 h under oil.

**Electrophoresis on a Non-Denaturing TBE Vinyl Gel**

Eight microliters of glycerol gel-loading Buffer III (11) were added to the digestion. The sample was heated to 65°C for 5 min, and 10 µL were loaded onto a 0.4-mm-thick 1× TBE 6%–8% D-600™ Vinyl Gel (American Technologies, Malvern, PA, USA) using a sequencing size vertical apparatus. Identical results were obtained using 10%–12% polyacrylamide gel electrophoresis (PAGE)/1× TBE. Electrophoresis was carried out for 4 h at 500 V, and the gel was dried for 30 min at 80°C.

**Phosphor Imaging**

The gel was exposed to a Phosphor-Imager™ plate (Molecular Dynamics, Sunnyvale, CA, USA) for 2–14 h.

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**Figure 1. Phosphor image of nested PCR products for c-Ha-ras (A) and matrilysin (B) with and without diagnostic digestions.** DNAs, prepared from tissue (CD-1) and various c-Ha-ras mutant cell lines (PVD and CarB), were amplified by nested PCR individually (Panel A, lanes 1–6) and 1:1 DNA mixtures (Panel A, lanes 7–12). The product, labeled by incorporation with [³²P]dCTP in the second stage, was run with (+) and without (-) digestion with the diagnostic enzyme XhoI to detect 61st codon A→T mutations in c-Ha-ras. Electrophoresis on a non-denaturing 1× TBE 6% D-600 vinyl gel (Panel A) shows the proportion of undigestible wild-type c-Ha-ras alleles at 176 bp to digested mutant fragments at the 85/91-bp doublet. Panel B, cell line MR-15 CI24 (lanes 3–4), transfected with mutant matrilysin, and its parent prostate carcinoma cell line DU145 (lanes 1–2) were assayed by the same protocol. Diagnostic Sso96I fragments that result from an 89th codon CTC→CCC mutation in the integrated cDNA for matrilysin were separated on a 10% 1× TBE polyacrylamide gel with the undigestible wild-type matrilysin alleles at 115 bp and digested mutant fragments at the 61/54-bp doublet. The 1:1 DNA mixture is shown in Panel B, lanes 5–6.
Scanning was done on the Image-Quant™ program (Molecular Dynamics) with a rectangle created slightly larger than the undigested band and one rectangle around the mutant doublet. Background was defined as 0 during volume-integration. Since any radiation incorporated into the undigested band is displayed completely in the doublet when digested, volume-integration of uncut vs. doublet sections of the gel represent directly the ratios of wild-type to mutant alleles carrying the diagnostic restriction site.

### RESULTS AND DISCUSSION

#### Validation of the Assay

The protocol was first used to assay the two cell types for which copy number of wild-type and mutant c-Ha-ras has been established: CD-1 epidermis with two copies of wild-type allele and PDV cells with two copies of wild-type and one copy of mutant allele. Figure 1 is a phosphor-imaged gel of the PCR products from various sources, undigested (odd lanes) and digested with the diagnostic enzyme (even lanes). In Panel A, none of the CD-1 c-Ha-ras PCR product at 176 bp (lane 1) was digested with XbaI (lane 2). This was expected since CD-1 has only wild-type alleles. Part of the PCR product from the PDV DNA could be digested with XbaI (lanes 3 and 4). Only the digested lane is needed to quantitate the ratio of the two alleles. When radioactive volumes from the phosphor imager for the undigested band at 176 bp were compared to the mutant fraction at doublet 91/85 and then expressed as a ratio of wild-type/total ras alleles, the observed gene dose of 64% closely matched the expected dose (67%) from published data (Table 3). Therefore, the alleles present in the original genomic DNA sample had been amplified and detected proportionally throughout the various steps of the assay.

To determine absolute gene copies in a cell line, two DNAs combined must demonstrate the correct proportion of alleles. In a CD-1/PDV mixture, PDV would be contributing 2 wild-type alleles and 1 mutant allele per genome, and the CD-1 would contribute 2 wild-type alleles per genome (Table 3). When equal numbers of genomes from each source are added in a mixture (equal $A_{260}$ units), a ratio of 4 wild-type alleles to 1 mutant allele (80% wild-type/total alleles) would be expected. Figure 1A shows the data for the double-source c-Ha-ras DNA PCRs in lanes 7–12, and the quantitation of the observed bands is given in Table 3. The PCR product from CD-1/PDV (lanes 7 and 8) produces 81% wild-type. These results establish the accuracy of the assay for a mixture of genomic DNAs.

#### Use of Assay for Cell Lines of Unknown Gene Copy Number

Figure 1A (lanes 5 and 6) and Table 3 show the results of CarB tested as a single-source DNA and confirm the CarB to be homozygous for mutant alleles (4). The mixed sources of DNA can then be used to determine the absolute number of copies of c-Ha-ras. Because CD-1 has two copies of c-Ha-ras and the ratio of 50% wild-type alleles were seen in the CD-1/CarB mixture (Figure 1A, lane 10 and Table 3),
CarB can be deduced to have two copies of mutated \( c\)-Ha-ras. This is confirmed with the other possible mixture, CarB with PDV.

The assay was used to establish percent of wild-type to total alleles for the matrilysin gene using a parental DU145 cell line (15) and a DU145 cell line stably transfected with a cDNA for matrilysin that contained a mutant 89th codon. The triploid parental cell line, DU145 PCR-amplified matrilysin gene product at 115 bp could not be digested with \( \text{Sau}96I \) (Figure 1B, lane 2 and Table 3), which confirms the absence of the specific point mutation in this gene. However, 25\% of the alleles from transfected cell line MR-15 (13) clone 24 can be digested (lane 4). Upon mixing the two cell lines (lanes 5–6), the observed percent wild-type alleles of 85\% is as expected for 6 wild-type alleles in a total of 7 gene copies. Therefore, MR-15 clone 24 has 1 mutant copy per 3 wild-type alleles of the matrilysin gene.

**ADVANTAGES AND DISADVANTAGES**

When Southern blots are used for quantitation of copy number, comparisons are made between lanes of various cell lines with known copy number. Although steps have been taken to ensure consistent treatment, each lane has undergone many steps independently. With the assay described here, all the ratios are determined within a single lane. The two alleles are amplified during PCR by the same primer set, with the radioactivity incorporated equally into mutant and wild-type sequences according to the specific activity in that tube. The two types of products (enzyme digestible and resistant) are transferred to a digestion reaction as a proportional mixture and loaded as the same proportional mixture (digested and undigested) onto the gel. Complete digestion of short mutant PCR products is easier to achieve than the digestion of high-molecular-weight genomic DNA on which the accuracy of Southern analyses depends. Enzymes that recognize only four bases can be chosen as a diagnostic enzyme because the digestion of only a short amplified section of the gene greatly reduces the number of fragments seen on the gel. Southern blots that would show both wild-type and mutant alleles from the matrilysin cell line transfected with plasmid cDNA would be difficult to interpret because of the number of the \( \text{Sau}96I \) sites in the gene and unknown intron sequences.

In cases where a mutation of interest does not produce a diagnostic restriction fragment, rare mutations have been detected by the introduction of a restriction site into a PCR band by the incorporation of a mismatched primer (8,9,14,17). Using such a primer in the second stage could extend this quantitative assay for use with point mutations that do not produce a natural diagnostic restriction site.
Linear range of cycling, with low product-to-product re-annealing, is important for the accuracy of this assay. Because restriction enzymes only recognize sites within complementary strands, re-annealed products that form wild-type to mutant heteroduplexes in plateau stage create an overabundance of the undigested allele.

When determining copy number, mixtures need to be made of equal amounts of genomic DNA. However, if only a gene ratio is wanted as in screening clones, purification can be simplified and quantitation omitted.

PCR combined with RFLP has previously been used to detect mutations. By adding a nested primer step and choosing direct incorporation as a labeling method, this protocol is easier and more quantitative than the methods in use to detect allelic gene dose. The numbers derived by the phosphor imaging system are directly proportional to the allelic copies in the cell lines. When this assay is used on a mixture of two cell lines, including one for which the copy number is known, the total number of copies can be determined without need for another step such as cytogenetics.

REFERENCES


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