INTRODUCTION

Heterozygous deletions and duplications typically account for 10% to 15% of mutations. In some situations, the contribution of heterozygous deletions is even greater [e.g., more than one-third of the pathogenic familial BRCA1 mutations in breast/ovarian cancer in northeast Italy (1) and 55% of mutations in MLH1 and MSH2 genes in hereditary nonpolyposis colorectal cancer in the Netherlands are deletions (2)].

Heterozygous deletions can be detected by multiplex or duplex PCR (3–8). However, two major problems exist with these techniques: primer-dimer formation and false priming. As a result, assay optimization becomes much more difficult as the number of primers increases. Multiple-step dosage methods are available. In multiplex amplifiable probe hybridization (MAPH), short multiple amplifiable probes that are recovered quantitatively after hybridization to genomic DNA are amplified simultaneously by PCR with an additional pair of primers included to assess the copy number of up to 40 genome loci (9).

In multiplex ligation-dependent probe amplification (MLPA), pairs of oligonucleotides hybridize to adjacent sites on the genomic DNA and are subsequently ligated. The ligated probes are then amplified by PCR with an additional pair of primers, because the probes have identical end sequences (10). Both methods require substantial optimization for each assay.

Pyrophosphorolysis-activated polymerization (PAP) is a novel method for nucleic acid amplification (11,12). In PAP, pyrophosphorolysis of 3′ blocked oligonucleotides (P*) is followed by extension (Figure 1A). Since the P*s can remain inert until activated, upon annealing to their perfectly matched template (12), multiplex dosage pyrophosphorolysis-activated polymerization (MD-PAP) reactions are predicted to proceed with minimal primer-dimer formation or false priming.

Herein, we applied PAP in a multiplexed and quantitative way to detect deletions in the human factor IX gene (Figure 1B). Seven target exons of the factor IX gene are amplified quantitatively, along with one endogenous internal control from the ATM gene. Estimated dosage is proportional to the actual template copy number over a minimum dynamic range from 1 to 16 copies. A blinded analysis detected 100% of 43 heterozygous deletions of exons in the human factor IX gene.
Synthesis of 3' Blocked Oligonucleotides

Each P* was 30 nucleotides long and contained a dideoxy- nucleotide at its 3' terminus (Table 1). F9(20640)30D, F9(23450)30D, and F9(33019)30D, each terminating with a dideoxyinosine nucleotide at the 3' terminus, were chemically synthesized in 3' to 5' direction using phosphoro- midite chemistry (Integrated DNA Technologies, Coralville, IA, USA). For all the other P* s, a dideoxyguanine nucleotide was added to the 3' terminus of a 2'-dideoxynucleotide oligonucleotide by terminal transferase. The reaction was performed in a total volume of 30 μL and contained the following mixture: 100 mM potassium cacodylate, pH 7.2, 2.0 mM CoCl₂, 0.2 mM dithiothreitol (DTT), 2 nmol of the oligonucleotide, 2.4 mM ddNTP (the molar ratio of the 3'-OH terminus to ddNTP was 1:30; Roche), and 100 U of terminal transferase (Invitrogen, Carlsbad, CA, USA). The reaction was incubated at 37°C for 6 h and then stopped by adding EDTA to a 5 mM final concentration. After desalting using a Centri-Spin 20 column (Princeton Separations, Adelphia, NJ, USA), P* was purified by preparative 7 M urea/18% polyacrylamide gel electrophoresis (PAGE) with 30 mM triethanolamine/tricine buffer (pH 7.9 at 25°C) at 45°C for 14–16 h (15,16). The blocked 30-mer was separated from the unblocked 29-mer by 3% difference in mobility on the gel (35 °C) at 45°C for 14–16 h (15,16). The blocked 30-mer was separated from the unblocked 29-mer by 3% difference in mobility on the gel (35

<table>
<thead>
<tr>
<th>Exon</th>
<th>Name&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence</th>
<th>Segment Size (bp)</th>
<th>Relative Signal Intensity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>F9(3003)30D</td>
<td>5'-CGTGAACATGATGAGAATGACAAGddG*-3'</td>
<td>60</td>
<td>11</td>
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<tr>
<td></td>
<td>F9(3063)30U</td>
<td>5'-AGATATCCAAAAAGGCGATGGGATGAGddG*-3'</td>
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<td>F9(3063)30U</td>
<td>5'-AACGCCAATAAATCTGATAAAGGddG*-3'</td>
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<td></td>
<td>F9(3071)30U</td>
<td>5'-ATATGACCTGCTTGAATGGGdC*-3'</td>
<td>131</td>
<td>59</td>
</tr>
<tr>
<td>B/C</td>
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<td>5'-AGTGTGAGTCATCTGTTTAATGGGddG*-3'</td>
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<tr>
<td></td>
<td>F9(3071)30U</td>
<td>5'-ATATGACCTGCTTGAATGGGdC*-3'</td>
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<td></td>
<td>F9(3071)30U</td>
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<td>D</td>
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<td>31</td>
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<tr>
<td>E</td>
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<td>5'-AGTGTGAGTCATCTGTTTAATGGGddG*-3'</td>
<td>131</td>
<td>59</td>
</tr>
<tr>
<td></td>
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<td>5'-AGTGTGAGTCATCTGTTTAATGGGddG*-3'</td>
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<tr>
<td>H</td>
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<td>5'-AGTGTGAGTCATCTGTTTAATGGGddG*-3'</td>
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<td>59</td>
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</tbody>
</table>

<sup>a</sup>For example, F9(3003)30D contains a ddGMP at the 3' terminus; F9, the human factor IX gene; (3003), 5' end of the 3' sequence-specific region of the primer begins at 3003. The factor IX gene is numbered according to GenBank® accession no. K02402D (downstream inter alia, in the direction of transcription). The precise sizes and locations of the PCR fragment can be obtained from the informative names. ATM, ataxia telangiectasia gene and its sequence is from GenBank accession no. U82828.

<sup>b</sup>The number of possible incorporated radioactive dCMP in a double-stranded product molecule.

<sup>c</sup>The two downstream P* s of F9(23460)30D for exon F and F9(33002)30D for exon G were at 0.05 μM in Figure 2. In the blinded analysis, two other downstream P* s of F9(23450)30D for exon F and F9(33019)30D for exon G were used to generate 90- and 82-bp products, respectively.
cm length × 1.1 mm thickness). The blocked 30-mer was cut from the gel with a razor, placed in a microcentrifuge tube, and crushed; 1 mL water was then added, and the tube was shaken at 250 rpm at 37°C overnight. The supernatant was centrifuged for two rounds each for 2 h at 7500 × g (Millipore, Bedford, MA, USA). The recovered P* was determined by UV absorption at 260 nm.

To rule out contamination with the unblocked oligonucleotide, a direct extension was used to test the purity of P*s. Preamplified PCR product (10–20 ng), typically 400–600 bp, was used as template. P* (5–10 pmol) and 2.5 pmol of the opposing unblocked primer were used under standard PCR conditions of 200 μM each dNPT, pH 8.3, and native Taq DNA polymerase for 15 cycles. Only the unblocked oligonucleotide can be extended to generate a product. If no product is visible, it is estimated that more than 99.99% of P* contained a dideoxynucleotide at the 3’ terminus. A more detailed step-by-step protocol for the preparation of the P* is available online at www.BioTechniques.com.

**Multiplex Dosage PAP**

The PAP reaction mixture contained a total volume of 25 μL: 50 mM Tris-Cl, pH 7.8, 16 mM (NH₄)₂SO₄, 3.5 mM MgCl₂, 25 μM each dATP, dTTP, dGTP, and dCTP, 0.025 μM each oligonucleotide, 90 μM Na₂PP₃, 2% dimethylsulfoxide (DMSO), 2 μCi [α-33P]dATP (3000Ci/mmol; Amersham Biosciences, Piscataway, NJ, USA), 3 U KlenTaQ-S (ScienTech, Chesterfield, MO, USA), 100–200 ng genomic DNA, unless stated. The cycling conditions were as follows: 94°C for 15 s, 60°C for 30 s, 64°C for 30 s, 68°C for 1 min, and 72°C for 1 min for a total of 25 cycles. A denaturing step of 94°C for 1 min was added before the first cycle.

**Quantitation**

Four microliters of the 25-μL reaction were mixed with 4 μL loading buffer (7 M urea and 50% formamide), heated, and rapidly cooled on ice. The product was electrophoresed through a 7 M urea and 8% Long Ranger denaturing polyacrylamide gel (Cambrex, Rockland, ME, USA) with 90 mM TBE at 45°C. The gel was dried and exposed to a storage phosphor screen, scanned by a Typhoon™ 9410 scanner (Amersham Biosciences) under conditions of 100-μm resolution, a red laser at wavelength 633 nm, and a filter 390 BP. Typhoon 9410 differentiates a maximum of 100,000 levels of signal. The uniformity is greater than 95% across the screen.

The bands corresponding to each amplified product (typically 1–3 bands) were the sense and antisense strands and/or nontemplated addition of A (12). The yield of each amplified product was quantitated with ImageQuant™ software (Amersham Biosciences) as the total amount of signal in the PAP band minus the local background, indicated as an arbitrary unit (17,18). The signal for each target exon was divided by the signal for exon 12 of the ATM gene. This is termed ratio of yields (ROY). The mean ROY for multiple normal males was determined, and this mean was used to determine the copy parameter (CP), which is simply the ROY divided by the mean ROY for males. The CP for females should theoretically be two, but in practice it is somewhat less. The coefficient of variation for the normalized signal determines the

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**Figure 2. Detection of deletions.** (A) Sample gel multiplex dosage pyrophosphorolysis-activated polymerization (MD-PAP) was performed from 100 ng genomic DNA for 23 cycles. Lanes 1–7 are various male hemizygous deletions, lanes 8–13 are female family members. Lanes 10 and 13 are normal moth. Lane M is the normal male. Lane F is the female (the left female sample contains a heterozygous polymorphism in exon D, functionally equivalent to heterozygous deletion of exon D). White arrows indicate hemizygous deletion, and gray arrows indicate heterozygous deletion. Under these conditions, the sense and antisense strands generally display small differences in mobility. Additional bands can also be generated by nontemplated A nucleotides added by KlenTaQ-S polymerase. (B) Sample quantitation. Bans in lanes 8 to 13 (female samples) of the gel shown in panel A were analyzed to calculate the copy parameter (CP) of each target, which is simply the ratio of yields (ROY) divided by the mean ROY for one copy target. Grey arrow indicates heterozygous deletion.
ability to reliably detect one versus two genome copies.

**Blinded Analyses**

During a 17-year period, this laboratory has identified 50 probands and their families with hemophilia B, in which the probands have large deletions in the factor IX gene. For the blinded analyses, 29 samples from individuals with previously validated genotypes were chosen. The samples were taken from carrier females (obligate carriers and/or carriers demonstrated directly by the presence of a deletion junction segment that was amplified and sequenced), normal males, and normal females.

**RESULTS**

**Accuracy of the Assay**

MD-PAP was used to amplify the exons of the human factor IX gene as targets. The target template copies per cell were: zero copies for male deletion patients, one copy for normal males or female carriers, or two copies for normal females. Exon 12 of the ATM gene, an autosomal locus with two chromosomal copies per cell, was co-amplified as an endogenous internal control. Seven affected males and six female family members (of which four individuals are carriers) were tested by the MD-PAP assay. The amplified products were electrophoresed through a denaturing polyacrylamide gel (Figure 2A). The deletions were on different exons and involved different numbers of exons. All the deletions could be readily detected and localized in the male patients (Figure 2A, white arrows).

In order to accurately determine heterozygous deletions, the CP of the target exon was measured (see the Materials and Methods section). The CP for normal male DNA is close to one, and the CP for normal female DNA is close to two, but usually less than two, presumably reflecting a slight saturation in the amplification. This is not a problem as long as the CPs are distinct between males and females, such that there is no ambiguity. Figure 2B shows sample CP values for target exons; calculations were

![Figure 3. Dynamic range of the assay.](image)

A Sample gels. Deletions of exons B and H are shown. Lane 1 shows a male with a deletion. Lane 2 shows a normal female with 1:1 relative template copies of the target to the ATM control. In lanes 3–8, the template copies of the deleted exon per cell are indicated compared with two copies of the ATM control. The relative template copies were obtained diluting normal female genomic DNA into the male deletion. Arrow indicates deleted exon. (B) Analysis of exon B deletion. y-axis is the relative template copies of the deletion exon to the ATM control. y-axis is the corresponding ratio of yields (ROY) of the deletion exon B to ATM. (C) Analysis of exon H deletion. y-axis is the relative template copies of the deletion exon to the ATM control. y-axis is the corresponding ROY of the deletion exon B to ATM.
performed on the bands visible in lanes 8–13 of the gel shown in Figure 2A. The plot clearly shows the distinction between the heterozygous carriers (lanes 8–9 and 11–12) and normal females (lanes 10 and 13).

**Dynamic Range of the Assay**

To test the dynamic range, titration experiments were performed for four deletions of exons B, E, G, and H of the factor IX gene (sample data shown in Figure 3A). In lanes 3–8, the normal female from lane 2 was serially diluted with the male deletion sample from lane 1, so that the deleted exon copy number serially decreased from 1 to 0.06 relative to the ATM exon copy number. The ROY of the deleted exon was varied from 2.00 to 0.13, compared with exon 12 of the ATM gene. The linear relationship between ROY and the template copies has a correlation coefficient of $R^2 = 0.99, 0.96, 0.91,$ and 0.99 for exons B, E, G, and H, respectively (Figure 3, B and C).

**Assay Parameters**

For widespread use of the multiplex MD-PAP assay, it is important to determine how much flexibility there is in the reaction conditions and which parameters are most important. First, we examined the effect of the P* concentration and found that when the oligonucleotide concentration decreased from 0.1 to 0.006 μM, the corresponding product signal decreased (data not shown). When the P* concentration decreased below 0.025 μM, the signal decreased more dramatically. Therefore, P* concentrations of 0.025 or 0.050 μM are recommended. It should be noted that the efficiency of P* is not uniform among the 16 P*s tested. Two downstream P*s, F9(23460)30D for exon F and F9(33002)30D for exon G (Table 1), were less efficient. Thus, the yields of exons F and G were lower and more variable. When the concentrations of the two downstream P*s were increased from 0.025 to 0.05 μM, the yields of exons F and G were increased by 2.8- and 3.6-fold, respectively.

In order to determine optimal genomic DNA input amount, a titration experiment was performed, testing from 25 to 400 ng genomic DNA/25 μL reaction (Figure 4A). From 25 to 100 ng genomic DNA/25 μL, the product signal responded linearly with...
Table 2. Summary of the Blinded Analysis

<table>
<thead>
<tr>
<th>Exon</th>
<th>One Copy CP (^a)</th>
<th>Two Copies CP (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>sd</td>
</tr>
<tr>
<td>A</td>
<td>1</td>
<td>0.09</td>
</tr>
<tr>
<td>B/C</td>
<td>1</td>
<td>0.07</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>0.12</td>
</tr>
<tr>
<td>E</td>
<td>1</td>
<td>0.11</td>
</tr>
<tr>
<td>F</td>
<td>1</td>
<td>0.10</td>
</tr>
<tr>
<td>G</td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td>H</td>
<td>1</td>
<td>0.08</td>
</tr>
</tbody>
</table>

\(^a\)One copy means one copy of the target exon or gene as template in each individual cell, such as the factor IX gene in normal males or the deleted exons in female carriers. Two copies means two copies of the target exon or gene as template in each individual cell, such as the factor IX in normal female. CP means copy parameter, which is simply the target ratio of yields (ROY) divided by the mean ROY for males.

\(^b\)cv, the coefficient of variation, is the ratio of sd to mean of CP.

\(^c\)Range is maximum minus minimum of CP.

Finally, KlenTaq-S was compared with ThermoSequenase\(^\text{TM}\) (10 U/25 \(\mu\)L reaction; Amersham Biosciences). More units of ThermoSequenase (3- to 5-fold more) were required to achieve similar results (data not shown). In addition, the ROY for KlenTaq-S for exons F and G were 2.8- and 3.3-fold higher than for ThermoSequenase.

Assay Establishment and Development

To test the effect of thermal cycling conditions, the cycle number was examined at three cycle increments from 18 to 30 cycles. From 18 to 21 cycles, the signal increased 5.5- to 8.1-fold, depending on the segment. From 27 to 30 cycles, the signal increased 1.7- to 4.3-fold, demonstrating the saturation of the product. Up to 200 ng, the genomic DNA amount affected the ROY slightly (Figure 4C). From these data, 100–200 ng genomic DNA is recommended.

To test the effect of thermal cycling conditions, the cycle number was examined at three cycle increments from 18 to 30 cycles. From 18 to 21 cycles, the signal increased 5.5- to 8.1-fold, depending on the segment. From 27 to 30 cycles, the signal increased 1.7- to 4.3-fold, demonstrating the saturation of the product. The cycle number affected the ROY. By the end of 24 cycles, the number of cycles affected the ROY slightly. Twenty-five cycles is recommended for good product yield and ROY accuracy.

Some studies were performed in which Pfui (exo-) polymerase was added to the reaction to examine whether having two enzymes present increased yield. Pfui (exo-) was tested in activities from 0.0125 to 0.1 U/25 \(\mu\)L reaction. With 0.05 U or less Pfui (exo-) added, the yield of each segment increased (data not shown). Exons F and G had the highest response with up to 17.5- and 7.5-fold increase in the yields, respectively. Pfui (exo-) is likely to be helpful to the extension and/or removal of the dideoxynucleotide blocker at the 3′ terminus (19).

\(\text{ROI} = \frac{\text{Mean ROI}_{\text{f}}}{\text{Mean ROI}_{\text{m}}}\)

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Blinded Analysis

To validate MD-PAP for heterozygous deletions, a total of 29 individuals including normal males, normal females, and deletion carriers were analyzed in a blinded manner. In total, 232 exons were analyzed, and both the number and the nature of deletions were unknown to the operator. The MD-PAP gel is shown in Figure 5, and the CP calculations are summarized in Table 2. All 23 female carriers with the deletion in one or more exons were detected. In these individuals, all 43 of the exons were deleted and all 43 were detected. Thus, the correct exon genotyping was obtained in all 29 individuals. For each exon, the CPs clustered around two distinct values corresponding to a dosage of one or two copies per genome, respectively. Standard deviation and the coefficient of variation were determined for each of the seven exons (Table 2). Thus, blinded analysis demonstrated that 25 cycles provide the requisite accuracy to detect all heterozygous deletions in these samples with a sensitivity of 100% and a specificity of 100%.

DISCUSSION

We demonstrate that MD-PAP is a multiplex method of detecting gene dosage. No primer-dimer formation and false priming were detected, confirming our previous observation that P* needs a long and perfect match at the 3′ terminus for activation (12). This provides a key advantage over multiplex PCR. Other advantages include ease of primer design and much greater ease of assay development than previous methods. Any sequence that is amplifiable with PCR should be assayable with MD-PAP.
an alternative to a large deletion. P*s of 18–20 nucleotides are not activated if there is a mismatch 1–16 nucleotides from the 3’ end, thereby inhibiting amplification. Amplifying and sequencing that region in the patient can assess this possibility. Alternatively, MD-PAP can be repeated with an alternative pair of primers for that exon.

Conclusion

We adapted PAP in a multiplexed and quantitative manner to determine the gene copy number of multiple loci in order to detect large heterozygous deletions. Blinded analysis demonstrated detection of hemi- and heterozygous deletions with 100% accuracy. The assay has other advantages: (i) P*s can be highly multiplexed in solution, because they do not form unwanted products due to primer-dimers or false priming sites; (ii) a normal male and female sample provides a 2-fold-dosage control, since the ratio of the X-chromosomal to autosomal segment is 1:2 and 1:1 for males and females, respectively; (iii) optimization for multiplexing is simpler than with PCR, since the dideoxy-terminated primers interact little if not at all with the radioactivity. However, fluorescent-labeled primers are more expensive. In addition, the decay of some fluorescent labels during electrophoreses can be problematic.

ACKNOWLEDGMENTS

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COMPETING INTERESTS

STATEMENT

The authors declare no competing interests.

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