Robust Dosage-PCR for Detection of Heterozygous Chromosomal Deletions

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ABSTRACT

Robust dosage-PCR (RD-PCR) was developed to detect heterozygous large deletions, an important class of mutations missed by conventional PCR strategies. PCR-based methods are available for distinguishing between the dosage of one or two template copies, but general application is limited by the laborious nature of the method and/or the optimization required for each new set of gene exons to be analyzed. RD-PCR depends on a combination of (i) co-amplification of an autosomal and an X-chromosomal segment so that internal dosage controls are available for any segment to be analyzed and (ii) a robust primer design that includes a 5' tail and a 3' sequence-specific region in the PCR protocol. The ratio of yields (ROY) of the target to the internal control segment is directly proportional to the ratio of the two input templates over a wide range (at least 1:1 to 1:258 with a correlation coefficient of 0.99). The ROY is not dependent on the amount of genomic DNA or the number of cycles of amplification under typical conditions. RD-PCR eliminates errors in the preparation and manipulation steps by using an internal dosage control. A blinded analysis of gene dosage was performed to detect deletions of the human factor IX gene with 100% accuracy. Prospective analyses demonstrate that exons and flanking splice junctions can be analyzed for gene dosage with minimal optimization.

INTRODUCTION

The exponential nature of PCR makes it a wonderful qualitative tool, but the detection of gene dosage remains a challenge because small variations in amplification efficiency accumulate exponentially with cycling, and there is a terminal plateau phase where the yield is saturated. Nevertheless, quantitative PCR has been achieved in several ways. PCR amplification can be performed with external controls (1), including real-time fluorescent PCR (2,3). The control DNA template of known quantity is amplified to quantitate a standard curve. Under the same conditions, the target sample is amplified to deduce the absolute quantity of the target sample. A second strategy is competitive PCR with an internal dosage control (4–6). Competitive PCR uses the same pair of primers to amplify a control of known quantity and a target of unknown quantity to determine their relative yield. The two amplified segments have minor sequence differences but the same priming sites. For these two approaches, it is difficult to eliminate errors in the preparation and manipulation steps. A third approach is multiplex-PCR, which uses two or more pairs of primers to amplify multiple different segments. One segment is an endogenous internal control of known quantity, and the other segment is a target of unknown quantity. Heterozygous chromosomal mutations were detected by the multiplex-PCR in a semi-quantitative manner with a ratio of 1:2 or 3:2 of the target to the internal control (7–11). In a fourth approach, short multiple amplifiable probes are recovered quantitatively after hybridization to genomic DNA, and then the

amplifiable probes are simultaneously amplified to assess the copy number of up to 40 genome loci (12). Finally, the conversion of diploidy to haploidy is a method that allows the generation of stable murine/human hybrid cell lines carrying selected human chromosomes in only a single copy. A large deletion can then be detected qualitatively (13).

It is important to detect heterozygous deletions in dominant cancer predisposition syndromes. Recently, it was found that there is a common deletion in the *BRCA2* gene in Swedish breast/ovarian cancer families (14), and a common deletion in HNPCC patients (15) that were missed by conventional PCR protocols. These genes are often large with many exons, requiring the optimization of many amplified segments. A quantitative PCR protocol that requires minimal optimization and no expensive instrumentation would be advantageous.

One difficulty in developing such a PCR-based dosage assay is preferential amplification, a common problem with multiplex-PCR, especially if segments with different GC contents are amplified (16). In an extreme case, a point mutation downstream of a primer within the amplified region causes the preferential amplification (17). A substantial PCR bias toward near-identity sequences of the *k-ras* and *p53* genes was observed with both proofreading and non-proofreading polymerases (18). Efforts have been made to achieve uniform multiplex amplification. A simple method was developed for the detection of large X-linked deletions in male patients. Each primer contains a 3' sequence-specific region and a 5' uncomplementary region of up to 20 nu-

Table 1. Primer Pairs and PCR Segments

Assay	Name ^a	3' Sequence-Specific Region			Core PCR Segment ^c			
		Sequence ^b (5'→3')	T _m (°C)	GC%	Region	Size (bp)	T _m (°C)	GC%
I	1	F9(-241)D F9(245)U	43.5 44.4	45 45	Exon A of FIX gene, X-chromosome	486	75.3	40.7
	2	ATM(38415)D ATM(38829)U	44.9 46.4	40 45	Exon 12 of ATM gene, autosome	414	75.2	40.9
II	3	GAL(662)D GAL(1158)U	53.0 55.5	50 50	Exon 1 of GAL gene, X-chromosome	496	79.8	51.6
	4	ATM(52)D ATM(489)U	53.4 52.1	55 55	Exon 1 of ATM gene, autosome	437	80.1	52.6
III	5	STA(201)D STA(634)U	54.6 51.2	60 60	STA gene, X-chromosome	433	83.4	60.7
	6	FUT(502)D FUT(1006)U	58.6 54.1	65 60	FUT gene, autosome	504	83.5	60.5

^aFor example, F9(-241)D: F9, the human factor IX gene; (-241), 5' end of the 3' sequence-specific region of the primer begins at -241 (28); and D, downstream (i.e., 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 U82828; GAL, α-galactosidase A gene, and its sequence is from M20317; STA, DNA for emerin, and its sequence is from D64111; FUT, α (1, 2) fucosyltransferase, and its sequence is from D82933. The six pairs of primers are available as controls for autosomal or X-chromosomal genes to be analyzed with GC contents ranging from 35% to 65%.

^bThe sequence of the 3' sequence-specific region is shown. A 20-nucleotide universal tail (5'-GCGGGTCCCCAAAAGGGTCAGT- 3') is attached to the 5' end of each primer.

^cThe core PCR segment does not include the tails.

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^cThe core PCR segment does not include the tails.

cleotides. The attached 5' tail provides uniform annealing efficiency for each primer (19). An additional approach is subcycling-PCR, in which the temperature shuttles between a low and high temperature in the annealing/extension step and evenly amplifies multiplex segments with various GC contents and segment sizes (20). Here a simple robust dosage-PCR (RD-PCR) is described. An autosomal and an X-chromosomal segment are co-amplified. The ratio of yields (ROY) of the target to internal control segment is directly proportional to the ratio of the two input templates over a wide range (at least 1:1 to 1:258). In a blinded analysis, all heterozygous deletions were detected, and in a prospective analysis, 10 assays were optimized rapidly.

MATERIALS AND METHODS

Primer Design

Primer design is critical for success with RD-PCR (Tables 1 and 2). All primers were designed and analyzed with Oligo 5 software (National Biosciences). To obtain high and uniform annealing efficiencies, each primer contains two parts: a 5' tail of 20 nucleotides and a 3' sequence-specific region, typically 20–22 nucleotides. The 5' tail is a universal sequence with 60% GC content (5'-GCGGTCCCAAGGGTCACT-3'). The 3' terminus of the 5' tail should not be complementary to the template strand. Oligonucleotides were designed in groups of four (one pair for the autosomal region and one pair for the X-chromosomal region) for high specificity and yield. The T_m of the 3' sequence-specific region is typically 27°C–32°C lower, and should be no more than 35°C lower than that of the core PCR segment (i.e., the amplified product) without the 5' tails. The T_m of the 3' sequence-specific region is estimated by the nearest neighbor method (21,22) at 50 mM KCl and 250 pM DNA. The T_m of the core PCR segment is estimated using the formula of Wetmur: $T_m^{\text{product}} = 81.5 + 16.6 \log[K^+ = 0.05 \text{ M}] + 0.41(\%G + \%C) - 675/\text{length}$ (23). The GC content of oligonucleotides should be similar, typically within 5%, and the GC content of

Table 2. Primer Pairs for Prospective Analysis

3' Sequence-Specific Region				Core PCR Segment ^c			
Name ^a	Sequence (5'→3')	T _m (°C)	GC%	Region	Size (bp)	T _m (°C)	GC% Paired Primer ^b
7	ATM(282)D ATM(728)U	42.8 48.0	40 40	Exon 36, autosome	446	72.4	33.9 No. 1
8	ATM(203)D ATM(634)U	42.3 50.6	45 50	Exon 63, autosome	431	76.2	43.2 No. 1
9	ATM(609)D ATM(1042)U	46.2 46.7	45 40	Exon 52, autosome	433	72.4	33.9 No. 1
10	ATM(531)D ATM(963)U	44.9 45.2	40 40	Exon 14, autosome	432	73.2	35.8 No. 1
11	ATM(44)D ATM(490)U	40.6 46.8	40 40	Exon 33, autosome	446	73.2	35.7 No. 1
12	ATM(44)D ATM(483)U	48.1 45.3	45 45	Exon 57, autosome	439	75.3	41.0 No. 1
13	MYC(1886)D MYC(2334)U	55.2 53.9	55 50	Exon ^c , X-chromosome	468	78.2	47.9 No. 4
14	D1(1226)D D1(1630)U	54.1 52.5	50 50	Exon, autosome	424	79.3	50.7 No. 3
15	D1(571)D D1(989)U	54.9 55.4	50 55	Exon, autosome	428	79.9	52.3 No. 3
16	MYC(1233)D MYC(1658)U	55.1 55.5	60 55	Exon, X-chromosome	445	83.2	60.2 No. 6

^aThe sequences of exons 14, 33, 36, 52, 57, and 63 of the ATM gene are from U55714, U55730, U55733, U55751, and U55757. MYC, MYCL2 gene, and its sequence is from J03069. D1, dopamine receptor gene D1, and its sequence is from X55760.

^bPaired with the second primer pair in Table 1.

^cOnly one exon in the gene.

the oligonucleotides should range between 5% lower and 20% higher than the GC content of the core PCR segment.

The five 3' terminal nucleotides should have a low free energy for pairing (-5.5 to -9.4 kcal/mol). None of the four primers should be complementary to each other at the 3' terminus for more than three nucleotides. In addition, a maximum of three nucleotides at the 3' terminus is self-complementary. To avoid false priming, no more than seven nucleotides at the 3' terminus should be complementary to any part of the core PCR segment. This represents the generic protocol.

The following is the recommended protocol used with Oligo 5 software for design of the 3' sequence-specific region of primers. Enter the autosomal or X-chromosomal sequences of interest (less than 3 kb). Set the length of the oligonucleotide to 20. Indicate "yes" to the following eight properties on the menu: (i) duplex free; (ii) highly specific; (iii) GC clamp; (iv) no false priming; (v) no false priming in the control sequence; (vi) stability limits; (vii) hairpin free; and (viii) no homomeric repeat sequence. The "high-stringency" parameters are set as follows: (i) the length is 20 nucleotides; (ii) acceptable 3' terminal dimer delta G is -3.5 kcal/mol; (iii) maximum length of acceptable dimers is three nucleotides; (iv) 3' terminal nucleotide check for dimer is 11; (v) 3' terminal stability range is -5.5 to -9.4 kcal/mol; (vi) GC clamp stability is -10 kcal/mol; (vii) minimum acceptable loop delta G is 0.0; (viii) oligonucleotide T_m range is 48.8°C–68.7°C; and (ix) maximum acceptable false priming efficiency is 160 point. Set the size range at 50–100 nucleotides smaller or larger than the control PCR segment to be used. Allow the software to generate primer pairs. By manual analysis, the downstream and upstream target oligonucleotides are checked for the following parameters: the T_m of the downstream and upstream should be less than 35°C of the amplified core segment, with the preference of

29°C ± 2°C. The GC contents of the downstream and upstream oligonucleotides are within 5% of one another. The free energy of the five 3' nucleotides is no less than -8.0 kcal/mol. The 3' terminus of the universal tail sequence must not be complementary to additional nucleotides of the template strand. The universal tail to the downstream and the upstream target primers is added using the edit function. No more than three nucleotides at the 3' terminus of the four oligonucleotides may be complementary to one another. This is performed by checking four combinations: (i) downstream target with downstream control primer; (ii) downstream target with upstream control primer; (iii) upstream target primer with downstream control primer; and (iv) upstream target primer with upstream control primer.

RD-PCR Assay

The target oligonucleotides described above were synthesized. The target and control segments were amplified separately and together. If one pair amplified more strongly than the other, then the oligonucleotide concentrations were adjusted to even the yield. The RD-PCR assay was performed from human genomic DNA isolated from white blood cells. Unless stated, the PCR mixtures contained a total volume of 25 µL: #3 Expand™ High Fidelity buffer (Roche Applied Science, Mannheim, Germany), 4.5 mM (Assays I and II) or 3 mM (Assay III) MgCl₂, 200 µM of each dNTP (Assays I and II), or 150 µM dGTP/50 µM deazadGTP and 200 µM each of the other dNTPs (Assay III), 0% DMSO (Assays I and II) or 10% DMSO (Assay III),

Figure 1. Correlation between the ratio of template copy numbers and ROY. (A) Gel showing results of Assay I amplification of exon A of the factor IX gene and exon 12 of the ATM gene. The indicated ratio of the input copy number of the two templates was obtained by mixing normal male genomic DNA that contained one copy of the factor IX gene per cell and two copies of the ATM gene per cell with the factor IX deletion male genomic DNA that contained only two copies of the ATM gene per cell. RD-PCR was described in Materials and Methods. (B) A LN:LN plot is shown for the LN (ratio of input copy numbers) and Ln (ROY of the two segments). The x-axis is the LN ratio of template copy number of the factor IX gene to the ATM gene, and the y-axis is the corresponding LN ROY. The regression equation and correlation coefficient is indicated. A linear plot is similar, but points cluster near a value of 1 ($r = 1$ for a linear plot).

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0.1–0.2 μM each pair of primers, 1 U Platinum High Fidelity DNA polymerases (*Taq/GB-D*), and 1 U Platinum *Taq* DNA polymerase (BRL), 0.5 μg BSA, and 60 ng genomic DNA. The cycling entailed denaturation at 94°C for 15 s; annealing at 55°C (Assay I), 60°C (Assay II), or 65°C (Assay III) for 30 s; and elongation at 72°C for 2 min for 25 cycles with a GeneAmp® PCR system 9700 (Perkin Elmer Life Sciences, Boston, MA, USA). An additional 15-s denaturation was always supplemented to the first cycle. In the prospective analysis of 40%, 50%, and 60% GC

contents, the PCR reagents and cycling conditions of Assays I, II, and III were used, respectively. The primer concentration is the main factor needed for the optimization of even yields, although it often varies from 0.1 to 0.2 μM .

To quantitate PCR yield, PCR was performed with 1 μCi $\alpha[^{32}\text{P}]\text{-dATP}$ per 25- μL reaction (3000 Ci/mmol; Amersham Biosciences, Piscataway, NJ, USA), and then 8 μL of the reaction were electrophoresed through an 8% polyacrylamide gel (29 acrylamide:1 bis-acrylamide). The PCR yield was quantitated with a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA) as the total amount of signal in the PCR band minus the background, which is indicated as an arbitrary unit.

RESULTS AND DISCUSSION

Principle of RD-PCR

The target and the endogenous internal control are co-amplified with two pairs of primers, which eliminates errors from the preparation and manipulation steps. The internal control C has a known number of copies per cell, and the target T has an unknown number of copies per cell. The ROY for T to C should be directly proportional to the copy number ratio of the two input templates over a wide range that reliably includes a value of 2 (Equation 1).

$$\text{ROY} = k \times \frac{\text{Template copy number of T per cell}}{\text{Template copy number of C per cell}} \quad [\text{Eq. 1}]$$

Figure 2. RD-PCR and the number of amplified segments. RD-PCR was performed under standard conditions, as described in the Materials and Methods. ROY is independent of single or duplex amplifications. RD-PCR assays from I to III were tested (Table 1). (A) Assay I with 40% GC content; (B) Assay II with 50% GC content; and (C) Assay III with 60% GC content. The two segments of the target and the control of each assay were amplified individually (lanes 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, and 17) or multiplied (lanes 3, 6, 9, 12, 15, and 18) from male genomic DNA (1:2 ratio) and from female genomic DNA (2:2 ratio) of the input copy number of the two templates. The ROY is shown on the bottom. As an example, the ROY of F9 to ATM = 0.59 in male, which is indicated between lanes 1 and 2.

Figure 3. RD-PCR and template amount. The ROY is independent of the template amount. RD-PCR assays I to III were tested (shown in panels A–C, respectively). With the exception of the template amount, RD-PCR was performed under standard conditions. The two segments were co-amplified with different amounts of male and female genomic DNA from 12.5–400 ng per 25- μL reaction volume.

k is a proportionality constant associated with primer pairs and their concentrations. k can be adjusted essentially to one because of the primer design and PCR conditions. $\ln(\text{ROY})$ was plotted with $\ln(\text{copy number ratio of the two input templates})$ (Figure 1B).

A titration experiment was performed (Figure 1A). The relative copy number of the two input factor IX and ATM templates was varied from 1:1 to 1:258 by decreasing the concentrations of normal male genomic DNA containing one copy of the factor IX gene and two copies of the ATM gene per cell into hemophilia B patient genomic DNA with a deleted factor IX gene and two copies of the ATM gene per cell. The linear relationship between the ROY and the relative template copy number was obtained with a k of 1.13 and a correlation coefficient (*r*) of 0.99 (Figure 1B). The titration experiment was repeated consistently.

Kinetics of RD-PCR

The RD-PCR assay works with GC contents varying from 30% to 70%, which covers most genome regions. Three model RD-PCR assays were examined in detail at gene dosage ratios of 2:1 and 1:1 (Table 1). Male and female samples provided the 2-fold relative input copy numbers. The absolute yield of each segment individually is very similar to the yields in the duplex amplification (Figure 2). A 2-fold increase in the dosage is qualitatively visible between the female and male samples. The relative ROY varies from 1.8 to 2.3 between a female sample and a male sample, depending on the assay. The relative ROY between female and male samples is similar for a wide range of DNA concentrations; we obtained averages ($\pm$ SD) of 2.27 ± 0.15 , 1.98 ± 0.09 , and 1.73 ± 0.12 for Assays I, II, and III, respectively. (Figure 3). The relative

ROY between female and male samples is similar from 21 through 27 cycles; we obtained averages ($\pm$ SD) of 1.94 ± 0.12 , 1.81 ± 0.21 , 1.65 ± 0.06 for Assays I, II, and III (Figure 4). Each PCR assay was performed at least in triplicate with consistent results.

Optimization of Primer Design, Reagents, and Thermal-Cycling Parameters

To obtain high and uniform annealing efficiencies, long primers of typically 40–42 nucleotides were used that contained a 5' tail and a 3' sequence-specific region (19). The 5' tail has a 20-nucleotide universal sequence (see Materials and Methods). The 3' sequence-specific region is typically 20–22 nucleotides long, and its T_m is less than 35°C lower than that of the core PCR segment, without the tails (24). The annealing efficiency of the 3'

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sequence-specific region is also estimated by the GC content (25), ranging from -5% to +20% higher than the GC content of the core PCR segment (Table 1).

To obtain high and uniform extension efficiency, the two PCR segments of the control and target were matched, with minimum difference in sizes. For example, an 8% polyacrylamide gel can separate two double-stranded segments with 20–30 bp difference. In many cases, the RD-PCR assay worked well with Δ GC% up to 20% between the two core PCR segments.

The 5' tail lengths of 0, 4, 8, 12, and 20 nucleotides were examined with Assay I. The ROY of the factor IX and ATM products were significantly changed from male to female samples. When two PCR segments have differ-

ent GC contents, preferential amplification may occur.

The optimization of the reagents including the primer, *Taq/GB-D* DNA polymerases, Mg^{++} , dNTP, deazadGTP, and DMSO, and the thermal-cycling parameters of annealing and extension temperatures were explored extensively. The RD-PCR Assay I, which amplified a 486-bp core PCR segment in exon A of the factor IX gene and a 418-bp core PCR segment in exon 12 of the ATM gene, was studied most extensively (Table 1). The ROY of the two segments can be adjusted by changing the concentration of each primer pair. High Fidelity enzymes (2 U) containing *Taq/GB-D* DNA polymerase per 25 μ L reaction, a 3-fold maximum amount as recommended by manufacturer, achieved higher and more uniform

amplifications than only *Taq* DNA polymerase. Mixing 1 U *Taq* DNA polymerase with 1 U of High Fidelity enzymes, which increased the unit ratio of *Taq* to *GB-D* DNA polymerase, performed better than only 2 U Expanded High Fidelity enzymes, indicating that not only the total units but also the relative ratio are important. Hot-start using *Taq* antibody to inactivate *Taq* DNA polymerase at room temperature was useful to prevent primer dimer formation. The RD-PCR Assay I worked well at annealing temperatures from 52°C to 60°C, at dNTP concentrations from 200 to 500 μ M, and at Mg^{++} concentrations from 3.5 to 5.0 mM. Two other segments with a 40% GC content for exons 18 and 29 in the ATM gene could not be amplified well at 72°C as an extension temperature but worked well at 68°C as

Figure 4. RD-PCR and number of amplification cycles. RD-PCR assays I to III were tested (shown in panels A–C, respectively). The two segments were co-amplified from normal male genomic DNA template with a different number of cycles of amplification from 18 to 30. ROY is independent of cycle number up to 24 cycles.

Figure 5. Blinded and prospective analyses using RD-PCR. Eleven of 46 samples of a blinded analysis (Assay I) that was performed to detect a large hemizygous and heterozygous deletion in exon A of the human factor IX gene. Two segments of the F9 gene and the ATM gene were co-amplified, and their dosage is shown. P, proband males with deletions spanning exon A; C, female carriers; M, normal males; and F, normal females. Lane S, Φ X174/*Hae*III DNA standard marker. Lane N, no DNA control. (B) Five samples of 10 prospective analyses showing dosage. In Lanes 1–6, there are three examples with 40% GC content. Each of the primer pairs (nos. 8–10) in Table 2 is coupled with no. 1 in Table 1. The upper segment represents the F9 gene, and the lower segment represents the ATM gene. Lanes 7–8 provide an example of 50% GC content. The primer pair no. 15 in Table 2 is coupled with no. 3 in Table 1. The upper segment represents the GAL gene, and the lower segment represents the D1 gene. Lanes 9–10 provide an example with 60% GC content. The primer pair no. 16 in Table 2 is coupled with no. 6 in Table 1. The upper segment represents the MYC gene, and the lower segment represents the FUT gene. M, male and F, female.

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an extension temperature. One possibility is that the extending strand may be dissociated partially from its template at 72°C when the enzyme passes through a region of very low GC content (20,26).

Development of the Assay

An RD-PCR protocol should have a readily available internal control and a ROY of the target to the control segment that are directly proportional to the ratio of the two input templates for a wide range, including a 2-fold difference, while not being dependant on the amount of genomic DNA or the number of amplification cycles. Because detecting heterozygous deletions was the major goal, duplex PCR was performed to amplify an autosomal and an X-chromosomal segment simultaneously. Duplex amplification from a male and female allow for routine comparison with dosages of one versus two.

To achieve a protocol that was easy to optimize in a prospective analysis, parameters of primer design and PCR conditions (DNA polymerases and combinations of polymerases and additives, such as Mg^{++} , deaza-dGTP, DMSO, and thermal-cycling parameters) were explored. The result was a robust amplification protocol. Relative to typical conditions (27), the protocol was adapted to two regions with different GC contents. Assays I, II, and III were used as model systems (Table 1). The recommended protocol includes a rigorous primer design, the use of a universal 5' tail, and the use of a two-enzyme amplification system for segments of 400–500 bp.

Blinded and Prospective Analyses

A blinded analysis was performed to detect a large hemizygous and heterozygous deletion in exon 1 of the hu-

man factor IX gene with 100% accuracy. The analysis used 43 DNA samples from 11 proband males with deletions spanning exon A, 26 female carriers, two normal males, and four females (Figure 5A). Carrier females show a ROY similar to that of normal males.

Ten RD-PCR assays were tested in a prospective manner (Table 2). Template segments ranging from 34% to 60% GC content were utilized. One of the six core segments (Table 1) was chosen as a control, depending on the GC content and the X-chromosomal/autosomal location of the target segment of interest, and then the 10 prospective RD-PCR assays were designed. For each target segment, a female was used to optimize the assay. The target primer pair, the control primer pair, and both primer pairs were used in the initial experiment. The relative ratios of the primer pairs were adjusted so that each segment was of about equal intensity with

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both primer pairs. The experiment was then performed with male and female control samples to detect dosages. A 2-fold difference in the genomic template was easily differentiated by eye using the ROY between male and female DNA samples (Figure 5B).

CONCLUSION

RD-PCR uses an internal dosage control to successfully detect heterozygous large deletions normally missed by conventional PCR protocols. RD-PCR is simple, requiring minimal optimization for the application to each new set of gene exons to be analyzed. RD-PCR is sensitive to the detection of template dosage over a wide range of the relative amounts of each template. RD-PCR for the detection of heterozygous chromosomal deletions has broad application in the molecular diagnosis of genetic disease and specifically in mutation detection for cancer predisposition syndromes characterized by the deletions of exons in large genes with many exons.

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