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A large-scale validation of dosage analysis by robust dosage-polymerase chain reaction

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Abstract

Epidemiological and clinical diagnostic assays benefit from accurate detection of deletions and duplications commonly missed by the conventional strategy of polymerase chain reaction (PCR) amplification and sequencing of individual exons. Robust dosage-PCR (RD-PCR) is a quantitative duplex PCR method that coamplifies a target template and an endogenous internal control (an autosomal and an X-chromosomal segment) for detection of these mutations. In this study, 110 consecutive RD-PCR assays were developed and validated. The average linear regression coefficient between template copy number and product yield and the average coefficient of determination for linear correlation, R^2, were very high: 0.95 and 0.98, respectively. The accuracy of RD-PCR revealed somatic mosaicism for a deletion in the factor 9 gene. Advantages of RD-PCR include (1) high accuracy and consistency, (2) easy calibration of linearity using male and female samples, (3) use of an endogenous internal dosage control to eliminate preparation and manipulation errors, and (4) detection of gene dosage over a wide dynamic range. Deletions and duplications can be easily detected (a 2× decrease or a 1.5× increase in gene dosage). Thus, RD-PCR is a general and accurate method for detecting changes in gene dosage.

Keywords: Quantitative PCR; Mutation detection; Chromosomal mutation; Deletion; Insertion and duplication

Heterozygous deletions and duplications typically account for 10 to 15% of germline mutations [1], but their detection by PCR-based methods remains a challenge because (i) small variations in PCR efficiency accumulate exponentially with cycling [2,3] and (ii) there is a terminal plateau phase where PCR yield is saturated.

Nevertheless, quantitative PCR has been achieved in several ways. (1) An external control DNA template of known quantity is amplified by real-time fluorescent PCR to generate a standard curve, and the target sample is amplified under the same conditions to deduce its absolute quantity [4–6]. (2) Multiplex PCR is used to amplify an endogenous internal control of known quantity and multiple targets of unknown quantities. Thus, heterozygous chromosomal mutations can be detected with a ratio of 1:2 or 3:2 of target to internal control [7–11]. (3) Short multiple amplifiable probes are recovered quantitatively after hybridization to genomic DNA and then amplified by PCR to assess the copy number of up to 40 genome loci (multiplex amplifiable probe hybridization; MAPH)[1] [12]. (4) Each pair of oligonucleotides hybridizes adjacently for ligation. The ligated probes are then amplified by PCR (multiplex ligation-dependent probe amplification; MLPA) [13].

One difficulty in multiplex PCR quantitation is preferential amplification of one segment over another. In an extreme case, a point mutation downstream of a primer within the amplified region caused preferential amplification [2]. Substantial PCR biases toward near-identity sequences of the *k-Ras* and *P53* genes were observed with

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[1] *Abbreviations used:* MAPH, multiplex amplifiable probe hybridization; MLPA, multiplex ligation-dependent probe amplification; RD, robust dosage.

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both proofreading and nonproofreading polymerases [3]. In addition, this problem is more severe when segments with different GC contents are amplified [14].

Robust dosage-PCR (RD-PCR) was developed to achieve highly quantitative amplification (Fig. 1) [15]. RD-PCR is a duplex quantitative PCR that coamplifies an autosomal and an X-chromosomal internal control. If the target locus is autosomal then the control is an X-chromosomal segment, and vice versa if the target is an X-chromosomal segment. Using wild-type male and female samples with relative copy numbers of 1:2 and 2:2, it is easy to calibrate the relative yields linearly in any assay. To achieve highly efficient and uniform amplifications, the primer design, reaction components, and cycling regime are optimized. For example, each RD-PCR primer contains a 3′-sequence-specific region and a 5′ tail. The 5′ tail ensures even annealing efficiency for each primer, leading to uniform amplifications [16]. In addition, subcycling PCR, in which the temperature shuttles between a low and a high temperature in the annealing/extension steps, also contributes to even amplification of multiplex segments [17].

Herein, using clinical specimens, we developed and validated 110 RD-PCR assays for detection of heterozygous deletions and duplications. High linearity and correlation were obtained between the copy number of the input template and the yield of the output product. RD-PCR has the following advantages: (1) high accuracy and consistency, (2) easy calibration of linearity using male and female samples, (3) use of an endogenous internal dosage control to eliminate preparation and manipulation errors, and (4) quantitation of gene dosage over a wide dynamic range. Thus, RD-PCR is a general and accurate method for detecting heterozygous deletions and duplications.

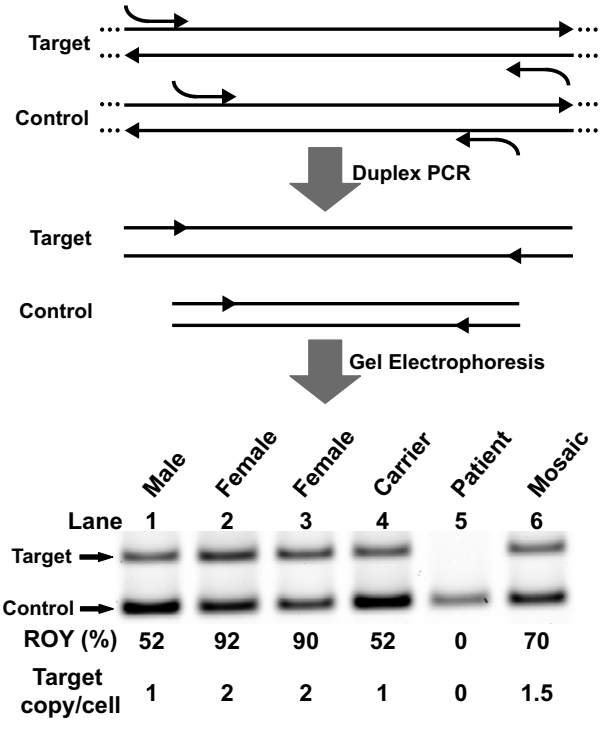

Fig. 1. Detection of somatic mutations. Target and endogenous internal control are co amplified by RD-PCR. The relative product ratio of target to control (ROY) is obtained for each sample. In the examples, the template copy number of exon H in the factor 9 gene is determined per cell from its ROY. Lane 6 has a mosaic deletion with 1.5 copies of the target per cell.

Material and methods

Terminology

We define the following terms: ROY, the relative product ratio of target to control; ROT, the relative template ratio of target to control; accuracy of ROY, the degree of conformity of a ROY to its true ROY value; and consistency of ROY, the degree to which further ROY will show the same or similar results (characterized in terms of the standard deviation of ROY).

Preparation of genomic DNA samples

Genomic DNA was isolated from white blood cells using a Puregene DNA Isolation Kit (Gentra Systems, Inc., Minneapolis, MN) or a standard phenol/chloroform protocol and stored in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). The concentration of DNA samples was quantitated using a Dyna Quant 200 fluorometer (Hoefer Pharmacia Biotech Inc., San Francisco, CA). The DNA samples were treated with proteinase K (2 mg/ml) (Roche, Indianapolis, IN) for 2 h at 65 °C and then incubated at 90 °C for 10 min to greatly improve the consistency among the samples [18,19].

RD-PCR assays

The details of the primer design, reaction components, cycling regime, optimization, calibration, and signal quantitation are described elsewhere [19]. RD-PCR assays were divided into three groups according to the target's GC content: 28–49% (group I), 50–59% (group II), and 60–70% (group III). Each group worked well under specified conditions of reaction components and cycling, among which the major difference was annealing temperature. To simplify the primer design, we considered three parameters: the GC content of the target region, the T_m and length of the primer, and the 3′ end of each primer. A primer consisted of a 3′-sequence-specific region of 18–22 bp and a 10-bp tail (5′-GGCCAAGTGT-3′). Using the Wallace formula [$T_m = 2\,°C \times (A + T) + 4\,°C \times (G + C)$ at 1 M NaCl], the 3′ section had T_ms spanning 56–62 °C or from 62–68 °C for regions with GC contents of 35–55% or 56–70%, respectively. Primers were designed so that, at the 3′ end, no more than three nucleotides were complementary to any other three primers.

To obtain a greater dynamic range of fluorescence signal, a Typhoon 9410 Imager (Amersham, Piscataway, NJ) was used to scan the agarose gel stained with 0.2 μg/