

Notes & Tips

Real-time quantitative allele discrimination assay using 3' locked nucleic acid primers for detection of low-percentage mosaic mutations

Ophélie Maertens^a, Eric Legius^b, Frank Speleman^a,
Ludwine Messiaen^{a,c}, Jo Vandesompele^{a,*}

^a Center for Medical Genetics, Ghent University Hospital, B-9000 Ghent, Belgium

^b Center of Human Genetics, Catholic University Leuven, B-3000, Leuven, Belgium

^c Department of Genetics, University of Alabama at Birmingham, Birmingham, AL 35294, USA

Received 26 June 2006

Available online 14 August 2006

Although fluorescent in situ hybridization (FISH)¹ and loss of heterozygosity (LOH) analysis can pick up mosaicism for gross chromosomal rearrangements relatively easily, detection of small mosaic lesion mutations remains technically challenging. Moreover, the presence of highly homologous pseudogenes throughout the human genome may complicate minor lesion mutation analysis at the genomic DNA (gDNA) level. In an attempt to detect low-percentage mosaicism for point mutations against a background of normal and pseudogene alleles, we have developed a nested real-time quantitative PCR (qPCR) assay taking advantage of 3' locked nucleic acid (LNA) allele-specific PCR (AS-PCR) primers and the cost-effective SYBR Green I detection chemistry.

One conceptually simple strategy to detect single-base substitutions is AS-PCR, which is based on positioning the 3' base of a PCR primer to match one variant allele [1]. Over the years, a number of strategies have been developed to improve the specificity and reliability of primers in AS-PCR. Among these are the incorporation of additional mismatches near the 3' end [2] and the use of high-affinity DNA analogues such as LNAs [3,4]. Although AS-PCR assays provide an elegant method to discriminate between alleles, accurate quantification of the variants is not attainable because of the intrinsic endpoint detection by conventional PCR methods. This limitation is fully addressed

using the real-time qPCR method, whereby PCR product accumulation is monitored at each PCR cycle by means of fluorescent detection. In this article, we evaluate the discriminating power of different AS-PCR primers on a real-time qPCR platform. As a model, we use various tissues and cell types derived from a segmental neurofibromatosis type 1 (NF1, MIM 162200) patient.

To determine the *NF1* mutation underlying the segmental phenotype, selectively cultured Schwann cells [5] derived from peripheral nerve sheath tumors were screened by a highly sensitive *NF1* mutation detection cascade as described previously [6]. A nonsense mutation (c.2041C>T (p.R681X)) was revealed in *NF1* exon 13. The region spanning the point mutation was amplified, and PCR products were cloned in the pCR2.1-TOPO vector (Invitrogen). The wild-type and mutant inserts were confirmed by gDNA sequencing on an ABI3730XL using BigDye chemistry (Applied Biosystems). Cloned fragments were reamplified and purified (MSB Spin PCRapace, Invitex), and concentrations were determined using the PicoGreen double-stranded DNA (dsDNA) Quantification Reagent (Molecular Probes) on a TD-360 fluorometer (Turner Designs).

The mutant allele-specific qPCR assay was optimized to achieve a maximal discriminating power between mutant and wild-type alleles, evaluated as the difference in the cycle threshold (C_t) values of mutant amplification products between matched (mutant-specific) and mismatched (wild-type-specific) primers while preserving high amplification efficiency. Real-time qPCR reactions were performed on an iCycler iQ instrument (Bio-Rad) using a 1 × SYBR Green I Master Mix (Eurogentec), 250 nM of primers, 10 nM fluorescein, and 5×10^3 molecules of mutant plasmid input. Primers were designed with the freely available Primer 3

* Corresponding author. Fax: +32 9 240 6549.

E-mail address: joke.vandesompele@ugent.be (J. Vandesompele).

¹ Abbreviations used: FISH, fluorescent in situ hybridization; LOH, loss of heterozygosity; gDNA, genomic DNA; qPCR, quantitative PCR; LNA, locked nucleic acid; AS-PCR, allele-specific PCR; NF1, neurofibromatosis type 1; dsDNA, double-stranded DNA; C_t , cycle threshold.

web tool (Whitehead Institute, <http://frodo.wi.mit.edu/cgi-bin/primer3>) and consisted of a common forward primer (5'-tcttccacccttgactctca-3') together with an AS-PCR reverse primer, resulting in an 82-bp amplicon. We evaluated the following six AS-PCR reverse primers for their discriminating power: a wild-type or mutant DNA primer with an additional 3' subterminal mismatch (underlined) (5'-ctagtttgctctgggctt~~gtt~~g/a-3') and two wild-type or mutant 3' LNA (bold) primers (5'-ctagtttgctctgggctt**gtcg**/a-3' and 5'-ctagtttgctctgggctt**gtt**g/a-3'). Although the mutation itself dictates the choice of the 3' terminal nucleotide in the primers, the nucleotide at the penultimate position was chosen based on the previously observed reduced amplification efficiency when thymidine occupies this primer position [7]. The thermal profile consisted of 1 cycle at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and at 61 °C for 1 min. After PCR amplification, a melting curve was generated to check the specificity of the PCR reactions (absence of primer–dimers or other nonspecific amplification products). Data acquisition and automated analysis were done by the iCycler iQ software (version 3.1, Bio-Rad). Real-time PCR results in Fig. 1 illustrate that the differences in C_t value (ΔC_t) between matched and mismatched primers are 0.3 for the DNA primer (with 3' subterminal mismatch), 1.5 for the 3' LNA primer, and 6.8 for the 3' LNA primer with 3' subterminal mismatch. Clearly, the introduction of the 3' subterminal primer:template mismatch shifts C_t values toward higher values but also significantly increases the discriminating power of the 3' LNA primer.

Equimolar dilutions of wild-type and mutant plasmid PCR fragments were used to generate standard curves of 5 log-10 orders of magnitude. To enhance linearity and reproducibility, dilutions were made in a 10-ng/ μ l λ DNA-containing carrier solution. Quantification of mutant alleles in the presence of the wild-type form was tested by mixing an excess (5×10^5 molecules) of wild-type allele with a 5-point 10-fold dilution series of the mutant form (5×10^5 –50 molecules). As a control, a standard curve containing only

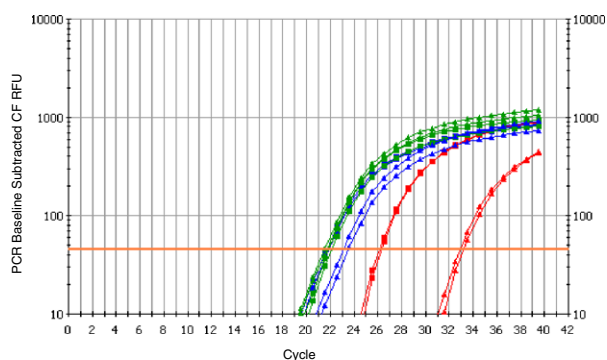


Fig. 1. Amplification plots of mutant plasmid using different AS-PCR primers: mutant (rectangle) and wild-type (triangle) DNA primer (green), 3' LNA primer (blue), and 3' LNA primer with 3' subterminal mismatch (red) (replicates are shown). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the mutant allele was used. Ideally, both series should result in overlapping amplification plots. We observed that at low levels of the mutant allele (<2500 molecules), the excess of the wild-type allele (5×10^5 molecules) significantly impaired accurate quantification of the low-abundant alternative allele (Fig. 2). Therefore, the sensitivity of the quantitative assay is estimated at 1/200 (mutant allele vs. wild-type allele).

Comparative analysis revealed that the *bona fide* *NFI* mutation under study (c.2041C>T) is present as a variant in at least three *NFI* pseudogenes. To exclude interference of *NFI* pseudogenes with the quantification of the *bona fide* *NFI* mutation, all unknown samples were amplified with *NFI*-specific exon 13 primers prior to nested real-time qPCR. The first-round amplification was performed on a PTC-200 Thermal Cycler (MJ Research) using 10 ng gDNA input and a PCR touch-down program starting at 61 °C gradually reduced (1 °C /cycle) to 52 °C for an additional 30 cycles. Because both wild-type and mutant alleles are amplified from the same *NFI* exon 13 amplicon, the initial proportional representation of both alleles is preserved. The first-round amplification products were purified with Exonuclease I and Shrimp Alkaline Phosphatase (USB) and were diluted (1/100,000) in a 10-ng/ μ l λ DNA-containing carrier solution.

Nested real-time quantitative PCRs were performed on an iCycler iQ instrument. In each experiment, duplicates of a standard dilution series of specific PCR fragments for each allelic variant and triplicates of 2 μ l of unknown sample template were amplified in a 15- μ l reaction containing 1 \times SYBR Green I Master Mix and 250 nM of allele-specific primers. The thermal profile was as described above. Each experiment was performed twice, and data acquisition and automated analysis were done by the iCycler iQ software (version 3.1). The relative number of molecules of

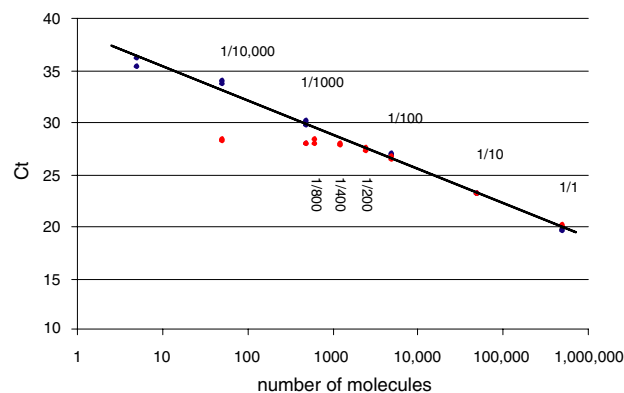


Fig. 2. Quantification of mutant alleles in the presence of the wild-type form. An excess (5×10^5 molecules) of wild-type allele was mixed with a dilution series of the mutant form (red dots). As a control, a standard curve containing only the mutant allele was used (blue dots). At low levels of the mutant allele (<2500 molecules or <1/200), the presence of the wild-type allele significantly impaired accurate quantification of the low-abundant alternative allele, as illustrated by lower C_t values for the red dots. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

each allele was determined by interpolating the C_t values of the unknown samples to each standard curve, followed by determination of the fraction of mutant alleles. Quantitative results of both experiments were highly concordant (see Table 1 in Supplementary material), showing mutant alleles in 1.41–47.40% of various tissues of the NF1 segmental patient.

In conclusion, we have described a real-time quantification strategy for the detection of low-percentage mosaic point mutations. We evaluated the discriminating properties of different AS-PCR primers and demonstrated that the use of a 3' LNA primer with an artificial mismatch at the 3' subterminal position had the largest discriminating power. To our knowledge, our method represents the first quantitative allele discrimination assay taking advantage of 3' LNA AS-PCR primers and SYBR Green I detection chemistry, allowing accurate, sensitive, and cost-effective quantification of single-nucleotide changes. The presented methodology offers opportunities for research and molecular diagnostic applications where quantification of two DNA sequences that differ by only one nucleotide is desired. Examples include population SNP genotyping, mutation detection, and risk assessment of disease transmission to offspring by mosaic patients.

Acknowledgments

This work was supported by an Interuniversity Attraction Poles grant from the Federal Office for Scientific, Technical, and Cultural Affairs in Belgium (2002–2006, P5/25) and by a Concerted Action Grant from Ghent University. E.L. is a part-time clinical researcher and J.V. is a post-

doctoral researcher with the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO). β -Heregulin for Schwann cell culture was provided by Genentech (South San Francisco, CA, USA).

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ab.2006.07.039.

References

- [1] C.D. Bottema, S.S. Sommer, PCR amplification of specific alleles: rapid detection of known mutations and polymorphisms, *Mutat. Res.* 288 (1993) 93–102.
- [2] C.R. Newton, A. Graham, L.E. Heptinstall, S.J. Powell, C. Summers, N. Kalsheker, J.C. Smith, A.F. Markham, Analysis of any point mutation in DNA: the amplification refractory mutation system (ARMS), *Nucleic Acids Res.* 17 (1989) 2503–2516.
- [3] D. Latorra, K. Arar, J.M. Hurley, Design considerations and effects of LNA in PCR primers, *Mol. Cell. Probes* 17 (2003) 253–259.
- [4] D. Latorra, K. Campbell, A. Wolter, J.M. Hurley, Enhanced allele-specific PCR discrimination in SNP genotyping using 3' locked nucleic acid (LNA) primers, *Hum. Mutat.* 22 (2003) 79–85.
- [5] E. Serra, T. Rosenbaum, U. Winner, R. Aledo, E. Ars, X. Estivill, H.G. Lenard, C. Lazaro, Schwann cells harbor the somatic *NF1* mutation in neurofibromas: evidence of two different Schwann cell subpopulations, *Hum. Mol. Genet.* 9 (2000) 3055–3064.
- [6] L.M. Messiaen, T. Callens, G. Mortier, D. Beysen, I. Vandenbroucke, N. Van Roy, F. Speleman, A.D. Paepe, Exhaustive mutation analysis of the *NF1* gene allows identification of 95% of mutations and reveals a high frequency of unusual splicing defects, *Hum. Mutat.* 15 (2000) 541–555.
- [7] S. Ayyadevara, J.J. Thaden, R.J. Shmookler Reis, Discrimination of primer 3'-nucleotide mismatch by taq DNA polymerase during polymerase chain reaction, *Anal. Biochem.* 284 (2000) 11–18.