

Quantitative Heteroduplex Analysis

General DNA analysis includes detection of targets, as well as identification and quantification of specific variants. Genomic DNA exists in the form of homoduplexes, with all corresponding base pairs being complementary, A:T and C:G. We call a double-stranded DNA (dsDNA) molecule a heteroduplex when it contains any noncomplementary base pairs. Conformational and thermodynamic changes produced by mismatched bases facilitate the detection of heteroduplexes. Denaturation of samples followed by hybridization to promote heteroduplex formation has been used to screen diploid DNA for heterozygous variations. This form of heteroduplex analysis has focused on detection, and although it may involve a quantitative threshold, the result is essentially binary. Recent work in this area (1) and elsewhere (2) has explored new implementations and applications for a more truly quantitative form of heteroduplex analysis.

One setting in which heteroduplexes commonly arise is during PCR. Although the extension process of PCR replicates genomic homoduplexes with high accuracy, if the original sample is heterozygous, heteroduplexes arise during the plateau stage when dsDNA is more likely to be formed by hybridization than extension. Denaturation and hybridization associates complementary and near-complementary strands almost randomly. Any of various methods (listed below) that can detect the presence of heteroduplexes can then be used to detect heterozygous sequence variations.

If heteroduplexes are not detected, variations, if any, are known to be homozygous. Homozygous variants may sometimes be distinguished by differences in melting temperature, but these differences may be near the limit of detection even with high-resolution techniques. Indeed, 4% of human single nucleotide polymorphisms (SNPs) exhibit a symmetry that makes homozygous variants thermodynamically identical up to the nearest-neighbor approximation (3). In this case, a 2-stage mixing strategy allows heteroduplex analysis to be used instead of melting temperature analysis for the detection of homozygous changes. Samples that are identified as not heterozygous in the first analysis are mixed with known wild-type DNA, denatured, and reannealed. Detection of the heteroduplexes in the mixed sample then indicates the presence of a distinct homozygous variant of the wild-type DNA. The 2 analyses can be performed in parallel, but then all samples must be prepared mixed and unmixed, then amplified, denatured, hybridized, and analyzed. Alternatively, the mixing can be performed after determining which samples are not heterozygous.

The specific methods that have been employed for detection of heteroduplexes include postPCR separation techniques such as conventional gel electrophoresis (4–9), electron microscopy and electrophoresis (10), denaturing HPLC (dHPLC) (11–13), and temperature-

gradient capillary electrophoresis (TGCE) (14). Closed-tube methods without separation have also been used to detect heteroduplexes by changes in the shape of high-resolution melting curves with fluorescently labeled primers (15) or a saturating DNA dye (16). Direct sequencing has also been used to identify and quantify heteroduplexes in gene dosage studies (17). Other studies (18–20) have analyzed the effects of heteroduplex formation on molecular diagnostic tests including quantitative PCR.

In quantitative heteroduplex analysis, the heteroduplex content of a product is indicated by the magnitude of the changes detected by these methods after PCR. Then the proportions of dsDNA genotypes in an original sample before PCR can be determined. This analysis involves 2 primary factors, isolating the effect of the heteroduplex content on the measured quantity (such as the melting curve or elution trace), and establishing the relationship of the measured heteroduplex content to the duplex proportions in the original sample. The methods for systematically isolating and quantifying the portion of the signal that depends on heteroduplex content are somewhat platform specific. These methods typically involve separation of a background component, followed by identification of measurable quantities such as heights or areas of particular peaks in the normalized data that are directly proportional to heteroduplex content. For dHPLC, it has been shown (1) that temperature conditions can be optimized by use of predictive software to improve the separation of heteroduplex and homoduplex peaks and achieve high-resolution results. For high-resolution melting, the procedure has been carried out (2) using a novel exponential fit to 2 slopes of the raw curve, at temperatures below and above its transition, and reported elsewhere (21). As an independent confirmation, quantitative heteroduplex analysis was performed on TGCE traces taken from the same PCR products in (2).

The reports by Lim et al. (1) and Palais et al. (2) both confirm that the mathematical model relating the proportions of 2 different types of homozygous DNA in a sample before PCR to the heteroduplex content after PCR is simple yet accurate. This model describes the heteroduplex proportion after PCR is performed on a mixture that before amplification consists of 2 types of dsDNA, type A:A' in proportion x and type B:B' in proportion $1 - x$. These reports support the assumption that different strands are amplified in proportion to their initial concentrations, a theory that has been confirmed in multiple studies (5–7). Also, thermodynamic analyses predict that for small mutations, the strands A and B are sufficiently similar that they hybridize randomly with their own and each other's complements, again in proportion to their concentrations. Based on these considerations, after PCR, the total heteroduplex proportion will be the sum of the

contributions from A:B' and B:A', or $2x(1 - x)$. This model is quadratic and symmetric with respect to interchanging the 2 components of the mixture about its central maximum, which represents the equal mixture present in a natural heterozygote. Except at this point, 2 possible mixtures always exist that can give rise to the same heteroduplex proportion. Inversion to find the initial mixture proportion from the heteroduplex proportion is least accurate when the mixture components are almost equal in proportion, because the slope of the model is near zero. The greatest accuracy occurs when one component of the mixture is much smaller than the other and the slope is steepest, the conditions under which, in the context of the report in this issue (1), quantitative heteroduplex analysis can provide simplification over existing techniques. Heretofore the determination of mutation load by measuring mitochondrial DNA heteroplasmy <10% has required more involved procedures, such as cloning and sequencing.

In (2), this model is taken a step further to enable complete genotyping of SNPs, both heterozygous and homozygous, with a single heteroduplex assay. The model is extended to include the case in which the component of the mixture that is present in proportion $1 - x$ is heterozygous, sharing one allele with the homozygous reference DNA, so that half of this proportion contributes to each type of homoduplex initially present in the previous analysis. The result of this change is that the total heteroduplex content after PCR of a mixture of homozygous reference DNA of type A/A of initial proportion x , with heterozygous type A/B DNA having initial proportion $1-x$, is $\frac{1}{2}(1 - x^2)$. Geometrically, this model is a transformation of the original: The maximum heteroduplex content that occurred in the first model when the proportions were equal ($x = \frac{1}{2}$), creating an artificial heterozygote, has been stretched horizontally to $x = 0$, where the unmixed sample is already heterozygous. When performing a repeat heteroduplex analysis of known homozygous DNA as described above, it is reasonable to mix the target and wild type in these equal proportions to obtain this maximal heteroduplex content, but this value is far from effective in a single test. It is shown in (2), by use of the 2 quantitative heteroduplex proportion models above (given that no heteroduplex formation occurs when the sample has the same genotype as the reference), that mixing all samples before PCR with the optimal fraction $x = 1/7$ of homozygous reference DNA makes it possible to distinguish all 3 genotypes of a nearest-neighbor symmetric SNP in a single closed-tube assay. This value, according to the model, equalizes the separations in heteroduplex content among the mixtures with the 3 genotypes. When mixed in these proportions, the heteroduplex content of the natural heterozygous genotype is reduced only by $\sim 2\%$, so almost no resolution is sacrificed compared with the original 2-step test.

Beyond their immediate results, these reports suggest that quantitative heteroduplex analysis will be applicable

to other technologies and problems. Better understanding of differential amplification and hybridization of distinct strands based on different PCR conditions and initial concentrations can also lead to improvements in existing methods, such as including initial mixtures of more than 2 types of dsDNA, refining the models of the effect of duplex proportions on the cumulative signal, and developing superior techniques to resolve these signals from background and from each other for eventual quantification.

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References

1. Lim K, Naviaux R, Haas R. Quantitative DNA mutation analysis by DHPLC. *Clin Chem* 2007;1046–52.
2. Palais R, Liew M, Wittwer CT. Quantitative heteroduplex analysis for single nucleotide polymorphism genotyping. *Anal Biochem* 2005;346:167–75.
3. Liew M, Pryor R, Palais R, Meadows C, Erali M, Lyon E, et al. Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. *Clin Chem* 2004;50:1156–64.
4. Highsmith W Jr, Jin Q, Nataraj A, O'Connor J, Burland V, Baubonis W, et al. Use of a DNA toolbox for the characterization of mutation scanning methods. I. Construction of the toolbox and evaluation of heteroduplex analysis. *Electrophoresis* 1999;20:1186–94.
5. Ruano G, Kidd K. Modeling of heteroduplex formation during PCR from mixtures of DNA templates. *PCR Methods Appl* 1992;2:112–6.
6. Jensen MA, Straus N. Effect of PCR conditions on the formation of heteroduplex and single-stranded DNA products in the amplification of bacterial ribosomal DNA spacer regions. *PCR Methods Appl* 1993;3:186–94.
7. Ruano G, Deinard A, Tishkoff S, Kidd K. Detection of DNA sequence variation via deliberate heteroduplex formation from genomic DNAs amplified en masse in "population tubes." *PCR Methods Appl* 1994;4:225–31.
8. Thomas G, Williams D, Soper S. Capillary electrophoresis-based heteroduplex analysis with a universal heteroduplex generator for detection of point mutations associated with rifampin resistance in tuberculosis. *Clin Chem* 2001;47:1195–1203.
9. Weber J, Barbier V, Pages-Berhouet S, Caux-Moncoutier V, Stoppa-Lyonnet D, Violy J-L. A high-throughput mutation detection method based on heteroduplex analysis using graft copolymer matrices: application to Brca1 and Brca2 analysis. *Anal Chem* 2004;76:4839–48.
10. Gonda M, Kaminchick J, Oliff A, Menke J, Nagashima K, Scolnick E. Heteroduplex analysis of molecular clones of the pathogenic Friend virus complex: Friend murine leukemia virus, Friend mink cell focus-forming virus, and the polycythemia- and anemia-inducing strains of Friend spleen focus-forming virus. *J Virol* 1984;51:306–14.
11. Xiao W, Oefner P. Denaturing high-performance liquid chromatography: a review. *Hum Mutat* 2001;17:439–74.
12. Shinka T, Naroda T, Tamura T, Sasahara K, Nakahori Y. A rapid and simple method for sex identification by heteroduplex analysis, using denaturing high-performance liquid chromatography (DHPLC). *J Hum Genet* 2001;46:263–6.
13. Jones A, Austin J, Hansen N, Hoogendoorn B, Oefner P, Cheadle J, et al. Optimal temperature selection for mutation detection by denaturing HPLC and comparison to single-stranded conformation polymorphism and heteroduplex analysis. *Clin Chem* 1999;45:1133–40.
14. Li Q, Liu Z, Monroe H, Cuiat C. Integrated platform for detection of DNA sequence variants using capillary array electrophoresis. *Electrophoresis* 2002;23:1499–1511.
15. Gundry C, Vandersteen J, Reed G, Pryor R, Chen J, Wittwer CT. Amplicon melting analysis with labeled primers: a closed-tube method for differentiating homozygotes and heterozygotes. *Clin Chem* 2003;49:396–406.
16. Wittwer CT, Reed G, Gundry C, Vandersteen J, Pryor R. High-resolution genotyping by amplicon melting analysis using LCGreen. *Clin Chem* 2003;49:853–60.

17. Ogino S, Leonard D, Rennert H, Gao S, Wilson R. Heteroduplex formation in SMN gene dosage analysis. *J Mol Diagn* 2001;3:150.
18. Henley W, Schuebel K, Nielsen D. Limitations imposed by heteroduplex formation on quantitative RT-PCR. *Biochem Biophys Res Commun* 1996; 226:113-7.
19. Boer P, Ramamoorthy J. How to correct for errors in mRNA quantitation by competitive PCR due to heteroduplex formation of amplification products. *Cell Mol Biol* 1997;43:841-50.
20. Poirier J, Ohshima K, Pandolfo M. Heteroduplexes may confuse the interpretation of PCR-based molecular tests for the Friedreich ataxia GAA triplet repeat [Letter]. *Hum Mutat* 1999;13:328-30.
21. Montgomery J, Wittwer CT, Palais R, Zhou L. Simultaneous mutation scanning and genotyping by high-resolution DNA melting analysis. *Nat Protoc* 2007; 2:59-66.

Bob Palais

*University of Utah
Math Department
Salt Lake City, UT 84112
Fax 801-585-7664
E-mail palais@math.utah.edu*

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