

High-Resolution Assessment of Copy Number Variation

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PCR revolutionized genetic analysis by enabling selective amplification of targeted sequences that, as a consequence of massive enrichment, could undergo genetic analysis by a variety of methodologies. The subsequent addition of a double-stranded DNA intercalating dye to the master mix was a key adaptation to PCR that allowed monitoring of amplification in real time, thus enabling quantification of the template [quantitative PCR (qPCR)]⁴ (1). Importantly, qPCR usually eliminated the necessity for further downstream analysis, since the determination of a quantitation cycle (Cq) value was an end in itself. The use of double-stranded DNA intercalating dyes also enabled post-PCR melting analysis.

Melting analysis was originally introduced to determine the specificity of the PCR (2). However, the subsequent development of high-resolution melting (HRM) brought melting analysis to the fore as a technique in its own right. HRM relied on the availability of intercalating dyes such as LC green that bind double-stranded DNA to saturation, as well as more sophisticated fluorescence monitoring instrumentation [reviewed in (3)]. As with qPCR, analysis could be done in the same tube in which PCR amplification was performed, in this case by programming a melting analysis to follow the PCR. PCR could thus be monitored in real time and then undergo HRM without any operator intervention. In many cases, this was sufficient for a full analysis, such as in single nucleotide polymorphism genotyping or methylation detection (4, 5). The PCR products could be discarded without the tubes ever having been opened (thus reducing PCR contamination issues) or selectively undergo further analysis such as DNA sequencing.

The analysis of copy number variation has emerged as a major need in both human genetic variation and disease, as well as in the analysis of cancer (reviewed in (6)). Genomic approaches to identify copy number alter-

ations include array comparative genomic hybridization, single nucleotide polymorphism arrays, molecular inversion probe arrays, and massively parallel sequencing. However, particularly as genomic assays are costly and time consuming and often require a considerable amount of input DNA, there is a need for reliable, low-cost, locus-specific assays to detect specific copy number alterations or validate genome-wide copy number analyses. When single regions are being analyzed for deviation from diploidy, it becomes necessary to compare the target to reference sequences that are not expected to show variation.

PCR-based locus-specific methods in current use include qPCR, which quantifies copy number based on real-time amplification. However, qPCR has many parameters that may affect readout and thus must be performed according to stringent guidelines (7). In particular, it is necessary to assess amplification in the exponential phase. Droplet digital PCR directly counts copy number using Poisson statistics based on partitioning of PCRs, enabling limiting dilution of templates, and is less influenced by technical issues such as PCR inhibition and amplification efficiency (8).

However, protocols for copy number variation determination by HRM have remained elusive. Zhou et al. now address this lack in the current issue of *Clinical Chemistry* (9). The authors chose multiplex PCR for relative quantification. This has the advantage that both the regions of interest (target) and the reference region are amplified from the same DNA sample, and thus intertube variation is eliminated. In this case, HRM was chosen to differentiate the amplicons. Accordingly, amplicons were selected with melting temperatures that were readily distinguishable by melting analysis. In addition, amplicons were designed with small sizes, single melting domains, no internal sequence variation, and no sequence homologs. Importantly, the intensity and position of the reference peaks on the melting curves were normalized, allowing direct visual estimation of the target copy number.

The authors explored several options for restricting PCR so that the primers did not become limiting. After varying cycle numbers, the amount of polymerase, and the amount of deoxyribonucleotide triphosphates (dNTPs), it was found that restricting PCR amplification was best done by using a dNTP concentration of 3–12 $\mu\text{mol/L}$. Although amplification was markedly inhibited

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⁴ Nonstandard abbreviations: qPCR, quantitative PCR; Cq, quantitation cycle; HRM, high-resolution melting; dNTP, deoxyribonucleotide triphosphate.

by this low nucleotide concentration, copy numbers were optimally distinguishable. Additionally, relative quantification by limiting dNTPs proved robust to differences in template concentration. It was necessary, however, to use standards because the change in peak heights was not linear.

Remarkably, the copy number precision of HRM with limited dNTPs was greater as estimated by the authors than the other readily performed single-locus assays (quantitative PCR and digital PCR). HRM with limited dNTPs could identify a 1.1-fold difference with a CV of <1%, whereas digital PCR, which came closest, had a comparable 1.2-fold resolution but an estimated CV of 3%–4%. Thus the HRM approach is also a high-resolution approach.

In the context of cancer, copy number variant analysis is particularly difficult. A key issue in determining copy number in cancer samples is heterogeneity. First, cancer biopsies are a mixture of cancer cells with variable numbers of coexisting or infiltrating normal cells. Second, the genome of a cancer is subject to multiple perturbations, which makes finding a reference gene difficult. Additionally, cancer displays heteroploidy, meaning that there are multiple different karyotypic clones present in any given cancer. Typically, reference genes are chosen that show low variation in a given cancer type, but it is advisable to use more than 1 reference locus. It remains difficult to distinguish true low level copy number variation from altered ploidy in the cancer genome. Higher-level amplification is more readily recognized.

Zhou et al. briefly address the identification of copy number changes in cancer. They present amplification of *EGFR* (epidermal growth factor receptor)⁵ as an example. HRM analysis using short amplicons is particularly suited to the analysis of degraded tissues such as those seen after formalin fixation (10). Because fragmentation of formalin-fixed DNA can be extremely variable, it would be advisable to use identically sized amplicons. Although the clinical importance of *EGFR* amplification is controversial, other amplifications such as *ERBB2* (erb-b2 receptor tyrosine kinase 2; formerly *HER2*) are diagnostically important. However, fluorescence in situ hybridization, or other in situ hybridization approaches, may be the method of choice for cancer due to their ability to detect copy number alterations, as well as polyploidy and balanced rearrangements at the level of individual cells on sectioned tissue.

This study emphasizes how difficult it is to achieve accurate quantification using PCR without careful attention to reaction conditions. There are implications for all

methodologies using multiplexed PCR approaches that seek an accurate determination of copy numbers. Does this alert us to the possibility that PCR-based quantification using other methods may have its accuracy compromised by running too many cycles or with too high a nucleotide concentration? Will multiplexed amplicon-based multiple parallel sequencing become more accurate if nucleotides are restricted and cycles are limited?

HRM democratized molecular genetic analysis in that anyone who was able to afford the increasingly cheaper platforms capable of HRM analysis had available a powerful technology for genetic analysis. By comparison, both the platforms and reagent costs for alternative methodologies including digital PCR are considerably higher than for HRM. Is this new low-cost HRM methodology robust enough for diagnostic applications? Time will tell. The implementation of the HRM methodology by laboratories is likely to require considerable care in optimization. The need for standards is paramount. The relatively low cost and the rapidity of the assay are certainly in its favor. What is not in doubt is Wittwer laboratory's continuing contribution to extending the potential of PCR and HRM.

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⁵ Human genes: *EGFR*, epidermal growth factor receptor; *ERBB2*, erb-b2 receptor tyrosine kinase 2.