How Accurate Is the Antiprimer Quenching-Based Real-Time PCR for Detection of Her2/neu in Clinical Cancer Samples?

To the Editor:

I read with great interest the recent article by Li et al. (1), in which they presented a novel, cost-effective quantitative PCR technology for the analysis of clinical cancer samples. They developed an antiprimer quenching-based real-time PCR (aQRT-PCR) that uses fluorescently labeled PCR primers in combination with a universal quenching antiprimer, reducing the cost of labeling. In agreement with their in-house fluorescence in situ hybridization (FISH) and immunohistochemistry results, their multiplex aQRT-PCR approach detected chromosomal Her2/neu amplification in microdissected breast cancer samples, in formalin-fixed, paraffin-embedded specimens; and in plasma circulating DNA. Hence, Li et al. (1) concluded that aQRT-PCR could be used as a simple, versatile, reliable, and low-cost alternative for Her2/neu detection in clinical cancer samples.

Her2/neu overexpression is considered a strong prognostic and predictive marker for breast cancer. Overexpression of Her2/neu is seen in ~30% of invasive human breast carcinomas. Because of their low cost and ease of performance, immunohistochemical methods are commonly used to measure Her2/neu protein. In ~90% of breast carcinomas, Her2/neu protein overexpression is attributable to gene amplification. Although FISH measurement of Her2/neu gene amplification is considered the gold standard for clinical detection of Her2/neu, FISH analysis is more time-consuming and expensive than immunohistochemical methods and is therefore not preferred for primary screening of Her2/neu status.

Quantitative real-time PCR using paraffin-embedded tissue is a suggested alternative standardized approach to Her2/neu detection (2–4), but I think that the multiplex aQRT-PCR for Her2/neu detection, as presented by Li et al. (1), should be used very cautiously in clinical cancer samples. HER2/neu overexpression has been linked with polysomy of chromosome 17. Polysomy 17 has been reported in breast carcinoma cases with borderline or low Her2/neu protein concentrations in the absence of gene amplification (5, 6). Thus, for reliable quantification of Her2/neu gene amplification, an internal control for chromosome 17 should be used. The use of a probe directed against the centromere of chromosome 17, as in dual-color FISH, can differentiate between true Her2/neu gene amplification and polysomy of chromosome 17. Because Li et al. (1) used glyceraldehyde-3-phosphate dehydrogenase (GAPDH), located on chromosome 12, as an internal control, centromere involvement could not be distinguished from true gene amplification. Moreover, because no details on the FISH procedure were reported, the comparison of the aQRT-PCR with their in-house FISH results might be questioned. From the data in the article, it is not clear whether a single-color (Her2/neu) or dual-color (Her2/neu probe–centromere 17 probe) FISH protocol was used.

I understand that the main point of the report by Li et al. (1) was the development and validation of the accuracy of the aQRT-PCR methodology, and I think that this method has great potential for analysis of clinical cancer samples. However, it requires revision for Her2/neu detection. I hope to see optimization of the aQRT-PCR method for Her2/neu detection in clinical cancer samples in future work by Li et al., as this would be of great value for clinical cancer research.

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References


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**LCGreen I-Based Real-Time PCR Assays for Detecting Common ASL and HMGCL Variants**

To the Editor:

Argininosuccinic aciduria (ASuria; OMIM 207900) is an autosomal recessive inborn error of the urea cycle caused by deficiency of the enzyme argininosuccinate lyase (ASL; EC 4.3.2.1) (1). A novel nonsense variant, ASL:p.Q354STOP, is the most common variant underlying ASuria in the Saudi population and accounts for ~50% of all cases (2). Currently, diagnosis for ASuria is based on electrospray tandem mass spectrometry (ESI-MS/MS) of dried blood spots (DBS) (3); however, although it is rapid and specific, this method is not suitable for prenatal diagnosis and carrier detection.

Another autosomal recessive inborn error of metabolism is 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) lyase deficiency (HMGCLD; OMIM 246450). HMG-CoA plays a key role in ketone body formation. Biochemical diagnosis of HMGCLD is made by MS/MS using DBS and is confirmed by organic acid analysis (4). HMGCLD is relatively common in the Saudi population (5), in which a novel variant, c.122G>A, leading to substitution of glutamine for arginine at position 41 (HMGCL:p.R41Q), has been described (6).

We developed and validated independent real-time PCR assays, using LCGreen I for ASL:p.Q354STOP and for HMGCL:p.R41Q. With these assays, we genotyped anonymous DBS from Saudi patients diagnosed with ASuria and HMGCLD. DNA was prepared from DBS by whole-genome amplification as described (2). Genomic DNA controls for ASL and HMGCL pRQ1 were validated by direct sequencing against fluorescence. Both purified genomic DNA and whole-genome amplification products generated from DBS were suitable for these assays and produced identical genotypes.

Melting curve analysis of relatively short amplicons in the presence of LCGreen I was used to genotype ASL:p.Q354STOP and HMGCL:p.R41Q. The PCR products for both assays were 60 bp in length, with the variants present in the gap between the 3’ ends of the forward and reverse primers. With the exception of the mutation sites, intervening sequences were not variant in the Saudi population, as determined by the sequencing of 100 apparently healthy individuals. The derivative melting curves for all genotypes are shown in Fig. 1. For ASL:c.1060C>T, the mean (SD) melting temperatures for the CC wild-type and TT variants were 84.4 (0.1) °C and 83.4 (0.1) °C, respectively. Results are based on a single run containing 13 and 15 CC and TT genotype replicates, respectively. The heterozygous (CT) genotype produced dual peaks (Fig. 1). For HMGCL:c.122G>A genotyping, the mean (SD) melting temperatures for the GG wild-type and AA variants were 78.9 (0.09) °C and 78.2 (0.08) °C, respectively. Results are based on a single run containing 12 and 16 GG and AA genotype replicates, respectively. The heterozygous (GA) genotype produced dual peaks (Fig. 1). For heterozygotes from both assays, we observed a small decrease of melting temperatures in the dual peaks relative to their equivalent homozygous peaks, reflecting heteroduplex formation.

LCGreen I, unlike SYBR Green, can be included in the PCR reaction at concentrations that saturate newly synthesized PCR product without inhibiting amplification; it thus enables detection of sequence variants (7). Thermodynamically, A-T hybrids melt at significantly lower temperatures than do G-C hybrids. Fortunately, ASL:p.Q354STOP and HMGCL:p.R41Q contain C/T and G/A variants, respectively, which enhanced the successful development of these assays. The sizes and sequences of the PCR amplicons can also affect melting curve analysis. On this basis, we designed primers to generate short amplicons (60 bp) to maximize the thermal discrimination window and to improve genotyping accuracy (8,9). The products of the developed assays were fully concordant with the outcomes of sequencing. Development of real-time assays for detecting these variants provides a reliable tool for molecular diagnosis of ASuria and HMGCLD, but more importantly, a rapid and robust method for prenatal or carrier detec-