specific bias” and “random patient interferences”. We considered the latter two sources of error to be outside the scope of our study, in part because it is difficult to know how one might model the interferences. We did, however, discuss the importance of the continuing efforts of manufacturers to design instruments that avoid sources of error, such as those encountered by patients with special needs.

We believe that our conclusions are correct within the scope of the question we asked. The points raised in Dr. Krouwer’s letter do point out that our estimates of quality requirements, as demanding as they may seem, would become even more demanding if the additional sources of error were included.

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Limitations of Genotyping Based on Amplicon Melting Temperature

To the Editor:

We have read with interest the recent reports of Marziliano et al. (1) and Pirulli et al. (2), who used melting temperature assays to genotype different types of mutations. Because detection of the underlying mutation is only indirect in these methods, they differ in their molecular detection principle from hybridization probe-based genotyping (3) or allele-specific amplification coupled to SYBR-green I detection (4). Indirect detection methods demand extra caution in the assignment of genotypes solely on the basis of product melting temperature (Tm). From a theoretical standpoint, some genotypes will not be detected.

The UDP-glucuronosyltransferase 1 (UGT1A1) (TA)n insertion/deletion polymorphism is of significance for the manifestation of Gilbert disease (3, 5). TA repeats are intrinsically unstable, and, therefore, it is not surprising that five to eight TA repeats occur in humans and have functional significance (5). The method of Marziliano et al. (1) uses Tm as a measure of amplicon length to discover the UGT1A1 promoter genotype. In the described assay, a 2-bp difference is detected by the resulting Tm shift in a 130-bp amplicon. A similar technique was successfully used in the screening for a 9-bp deletion in a 55-bp PCR amplicon (6). However, for the assay of Marziliano et al. (1), it has to be noted that using only an indirect measure of strand length and composition is insufficient for the assignment of a genotype on the basis of the theoretical reasons described below.

In addition to the points stated by Marziliano et al. (1), the melting curve analysis of a DNA strand is related to (a) the ionic strength of the buffer, (b) the DNA concentration, and (c) the DNA bases’ nearest neighbors (n-n) (3, 7). Within an assay system, buffer conditions can be considered constant. The genotyping of heterozygous samples requires special consideration because a mixture of two homoduplexes and two heteroduplexes results after PCR amplification, denaturation, and renination. An insertion/deletion polymorphism causes the formation of base bulges in the heteroduplexes. The bulge size is a major determinant of the destabilization caused by the disturbed base stacking (3). For example, the DNA base bulge size is 4 bp in the case of a 6TATA8TA duplex and 2 bp in the case of a 6TATA7TA or 7TATA8TA heteroduplex. Reported Tm values for the 6TATA and 7TATA genotype differ by only 1.3 °C in the 130-bp amplicon (1). A heterozygous 6TATA7TA sample has an apparent Tm in between the 6TATA and 7TATA sample because the different Tm values of the underlying homo- and heteroduplexes cannot be adequately resolved. On the basis of these respective considerations, we anticipate that in patients heterozygous for the 6TATA8TA genotype, a Tm undistinguishable from that of a homozygous 7TATA genotype results. The presence of an 8TATA genotype was already reported in an Italian patient with Gilbert syndrome (8). Other allelic combinations are possible where the same problem is present. Care must also be taken to ensure a constant DNA concentration in the assay before melting curves are acquired. Variation in the purity or amount of DNA in the assay can lead to different amounts of DNA after a constant number of PCR cycles. In the described assay (1), this will add to the total error and will cause variation in the Tm with the risk of wrong genotyping results.

This is not acceptable for a genotyping assay, and we recommend the use of more specific methods for allele assignment. Methods that ensure reliable genotyping for this locus have already been published and include polyacrylamide gel electrophoresis resolution of the PCR amplicon size (5), hybridization probes (3), sequencing, denaturing gradient gel electrophoresis, and denaturing HPLC [see references in (3)]. All of these methods also have the potential to detect the exact TA repeat numbers, which is not necessarily the case if only the amplicon Tm is used for screening.

Pirulli et al. (2) claim “sensitivity” and “specificity” of the DNA melting assay for the detection of different types of alanine:glyoxylate aminotransferase (AGXT) mutations. In addition to what was already mentioned above, we want to point out some results in Table 1 of Ref. (2). Both sample 9 and sample 13 share a homozygous G→A mutation. However, the resulting Tm shifts are 1.6 °C and −0.9 °C, respectively. In general, n-n pairs containing guanosine are more stable than n-n pairs containing adenosine. When a mutation in a strand causes the change of a guanosine n-n pair to an adenosine n-n pair, we would expect this to be destabilizing in most of the cases. Sample 2 is, in contrast to sample 11, stabilized by the presence of mismatches. The observed Tm of sample 2 is 0.8 °C higher than for the
wild type, although the duplex is destabilized by two mismatches in both cases. This illustrates the deviation from n-n behavior in longer DNA strands and makes the results difficult to interpret. Furthermore, sometimes a product $T_m$ differs by only 0.1 °C, whereas mean SDs are 0.14 °C (samples 15 and 2; samples 6 and 11). This makes it impossible to "easily distinguish different types of AGXT mutations" as claimed by Pirulli et al. (2). This is exemplified in Fig. 1, which does not show melting curves, but the expected probability distribution defined by 88.0 °C with an SD of 0.14 °C and 88.1 °C with an SD of 0.18 °C (solid line). The large melting curves, but the expected probability distribution defined by 88.0 °C with an SD of 0.14 °C and 88.1 °C with an SD of 0.18 °C. The large overlap in the distributions is obvious and substantiates that it is impossible to assign certain $T_m$s to underlying genotypes.

Our conclusion is that DNA $T_m$ assays based on SYBR Green I melting curves are powerful tools for screening various types of mutations but lack both sequence and mutation specificity. A lack of sensitivity must be expected if stable mismatches occur among stable neighboring bases (high GC content). Results obtained with these methods at highly polymorphic loci must be confirmed by a mutation-specific detection method. Interpretation of results arising from only the inspection of SYBR Green I melting curves requires great caution. In our opinion, such methods should not be used for routine genotyping applications.

References


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Use of Heterophilic Antibody Blocking Agent (HBT) in Reducing False-Positive hCG Results

To the Editor:

The USA hCG Reference Service aids physicians in the interpretation of discordant or irregular human chorionic gonadotropin (hCG) results that do not concur with physical findings. The Service has now observed 24 cases of false-positive hCG results (14–571 IU/L). All have been cases erroneously diagnosed with choriocarcinoma or gestational trophoblast disease without demonstrable pregnancy or tumor (1–4). Because of false-positive hCG results, patients have needlessly received chemotherapy, hysterectomy, and/or other surgery. The suspected cause for serum false-positive hCG is the presence of heterophilic antibodies that bind both capture and detection antibodies, effectively bridging them in the same way as the target antigen should in a standard ELISA format.

Until recently, we have added a test that includes a heterophilic antibody blocking agent [heterophilic blocking tube (HBT) blocking agent; Scantibodies Inc.] to our protocol for the detection of false-positive hCG. The HBT blocking reagent is a unique formulation of immunoglobulins targeted specifically against heterophilic antibodies to neutralize their interference in immunoassays (David Cantor, Office of Development, Scantibodies Laboratories Inc., San Diego CA, personal communication). In this study, serum samples (0.5 mL) were incubated in HBTs for 30 min before use (exactly as described by the manufacturer). They were then assayed in the same manner as untreated serum, in the usual hCG Reference Service hCG immunometric assay.

Here we present the prospective data from the nine most recent false-positive hCG cases where we used the HBT blocking agent and also from five retrospective, or previous, cases (from both stored frozen serum and subsequently provided serum samples) reassayed both with and without the HBT blocking agent (cases 10–14). Table 1 shows hCG values found in serum by the original referring laboratory, the hCG Reference Service hCG immunometric assay, and our assay again with the addition of the HBT blocking agent. In 12 of 14 cases, lower false-positive hCG concentrations were detected by the