total analytical error. Although the method is a simulation, it is helpful to consider how the data relate to an actual experiment. Thus, if one measures glucose in a set of patient specimens in both a field and a reference method, one can imagine obtaining a bias as the average difference between the field and reference methods for a specific concentration range, as well as the imprecision in the field method obtained by calculating the standard deviation of field replicates of individual patient samples, pooled across a concentration range.

However, Krouwer (3–5) has previously shown that these two error sources are really only two of four possible error sources. The four error sources are:

- Imprecision—the same imprecision as that of Boyd and Bruns (1)
- Protocol-independent bias—the same bias as that of Boyd and Bruns (1)
- Protocol-specific bias
- Random patient interferences

Protocol-specific bias is the bias that results from some imperfection in the assay system and the occurrence of another event (e.g., the protocol-specific event). As one example, if there is sample-to-sample carryover in the assay system, significant error will result only when the concentration of the preceding sample differs greatly from that of the current sample. In a glucose meter, protocol-specific bias is probably negligible.

Random patient interferences refer to errors caused by the mixture of substances specific to each patient that give rise to false signals (6). For example, Cartier et al. (7) describe a case whereby high glucose readings were obtained for a patient who had toxic concentrations of acetaminophen. Further investigation showed that the high glucose reading was attributable to interference by acetaminophen and that the level of error could easily be 100%, depending on the concentration of acetaminophen. Random patient interferences are not always negligible, especially for assay systems such as glucose meters, where an inexpensive system is an important design requirement. Random interferences are a possibility for patient samples but not for quality-control samples. Hence, total error modeling strategies based on quality-control samples may be questionable.

Moreover, random patient interferences have another property of interest—they occasionally cause large errors, exactly the type that one wishes to guard against because incorrect medical decisions may result. If one incorporates all error sources into a simulation, the results will more closely match real data. Thus, in a case that was based on real cholesterol data (3), estimating total error indirectly by adding imprecision to bias but without a random interferences component yielded a total error estimate of ±4.1%. The inclusion of a random interferences term yielded an estimated total error of −11.3% to 10.0%, which more closely matched the direct measurement of total error of −8.3% to 10.0%.

The following model is identical to the Boyd and Bruns model (1), except that a term corresponding to random interferences has been added:

\[
\text{GluC}_{\text{obs}} = \text{GluC}_{\text{true}} + [n(0,1) \times CV_1 \\
\times \text{GluC} \text{true}] + [n(0,1) \times CV_{\text{RI}} \\
\times \text{GluC} \text{true}] + [\text{bias} \times \text{GluC} \text{true}]
\]

where GluCtrue is the true glucose concentration; GluCobs is the measured glucose concentration reflecting the effects of analytical imprecision, bias, and random interferences; CV1 is the CV of the assay expressed as a fraction; CVRI is the CV of the random interferences component expressed as a fraction; n(0,1) is a random number drawn from a gaussian distribution with a mean of 0 and a SD of 1; and bias is the assay bias (expressed as a fraction).

The implications of this extended model are that any conclusions about error source requirements must take into account the added term because now total analytical error is constrained to be the sum of three instead of two terms.

Of course, a simpler approach would be to specify requirements for total analytical error itself instead of error source components. Here, no model is needed, and so the assumptions about which error sources exist are not important. Methods to measure total error directly are simple (8, 9). Finally, even if a correct error source model is made, establishing requirements of the allowable magnitude of each error source is complicated because there will be an infinite number of combinations of error source magnitudes that will satisfy the total error requirement.

References


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Drs. Boyd and Bruns respond:

To the Editor:

Dr. Krouwer is correct that our modeling approach addressed imprecision and bias and not "protocol-spe-
cific 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 ($T_m$). 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 $T_m$ 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 $T_m$ 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 ampli-

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 reannealing. 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 6TA/8TA duplex and 2 bp in the case of a 6TA/7TA or 7TA/8TA heteroduplex. Reported $T_m$s for the 6TA and 7TA genotype differ by only 1.3°C in the 130-bp amplicon (1). A heterozygous 6TA/7TA sample has an apparent $T_m$ in between the 6TA and 7TA sample because the different $T_m$s 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 6TA/8TA genotype, a $T_m$ undistinguishable from that of a homozygous 7TA genotype results. The presence of an 8TA 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 $T_m$ 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 allelic 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 $T_m$ 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 amino-transferase (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 $T_m$ 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 $T_m$ of sample 2 is 0.8°C higher than for the