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DNA Concentration Can Specify DNA Melting Point in a High-Resolution Melting Analysis Master Mix

To the Editor:

High-resolution melting analysis (HRMA)[1] can be used to discriminate sequence variants of PCR-amplified DNA fragments *(1)*. We attempted to develop an HRMA-based procedure to discriminate multilocus sequence typing (MLST) *(2)* alleles from a divergent lineage of *Staphylococcus aureus (3)* using a Corbett Rotorgene 6000. In the case of the 573-bp glycerol uptake facilitator *(glpF)* fragment, there were 4 known alleles in our culture collection. Allele discrimination performed with the nonsaturating dye SYBR Green was not reliable. We therefore tested the saturating dye LCGreenPlus (Biofire Diagnostics) *(4)*. Initial experiments revealed very poor allele discrimination due to lack of correlation between the melting temperature (T_m) and allele identity/allele percentage of guanine plus cytosine (%G+C) content. Here we report an investigation of the basis for this poor performance.

Our initial results with LCGreenPlus suggested that the T_m was primarily a function of the PCR yield rather than the allele sequence. This was tested by subjecting a *glpF* allele 321 sample to PCR in LCGreenPlus master mix, making a dilution series of the reaction products in the same master mix, and then subjecting the dilutions to HRMA. As predicted, the T_m values in both melting domains were inversely correlated with DNA concentration (Fig. 1).

We reasoned that the inverse correlation between T_m and DNA concentration indicated changes to the affinity between the DNA strands, and so perturbing the system with an agent that changes the affinity between strands might provide mechanistic insight. Mg^{2+} concentration impacts strand binding and correlates with T_m in the low millimole-per-liter range *(5)*. After the PCR reactions and before the HRM assays, we increased the $MgCl_2$ concentration from 2 mmol/L (unmodified master mix) to 6 mmol/L. This completely eliminated the LCGreenPlus-dependent inverse correlation between DNA concentration and T_m (Fig. 1). Similar results were obtained with 5 mmol/L $MgCl_2$; however, at 3 mmol/L $MgCl_2$ the inverse correlation remained.

We tested HRMA in the presence of 6 mmol/L $MgCl_2$ to resolve the *glpF* alleles. This was done by adding $MgCl_2$ either before or after the PCR. In both experiments, the 4 alleles were resolved into 3 distinct melt curves that corresponded to the 3 different %G+C contents of the alleles. When $MgCl_2$ was added before the PCR there was substantial PCR yield variation, suggesting that 6 mmol/L $MgCl_2$ was not optimal for this PCR. Nevertheless, allele discrimination was identical as to when the $MgCl_2$ concentration was increased to 6 mmol/L post-PCR.

Other PCR amplimers of sizes 186 bp and 83 bp were tested. The 186-bp fragment gave essentially identical results as the *glpF* fragment used initially. The 83-bp fragment provided similar results except at the very lowest DNA concentrations, close to detection limit for the Rotorgene device. Then, the inverse correlation between T_m and DNA concentration at 3 mmol/L $MgCl_2$ was not evident, and a direct correlation between T_m and DNA concentration was observed in the presence of 6 mmol/L $MgCl_2$.

We have formulated a model to explain these observations. It is known that intercalation of LCGreenPlus into double-stranded DNA increases T_m. The only relevant parameter that changes in accordance with DNA concentration in the presence of a constant concentration of LCGreenPlus is the DNA/LCGreenPlus stoichiometry. This will modulate the saturation state (and consequent T_m) of the DNA if the LCGreenPlus concentration is comparable to the concentration of LCGreenPlus binding sites. Therefore, we postulate that changes in T_m are consequent to changes in this stoichiometry. Raising the $MgCl_2$ to 6

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[1] Nonstandard abbreviations: HRMA, high-resolution melting analysis; MLST, multilocus sequence typing; *glpF*, glycerol uptake factor gene; T_m, melting temperature.

mmol/L eliminated the inverse correlation between T_m and DNA concentration. We postulate that Mg^{2+} and LCGreenPlus compete directly or indirectly for double-stranded DNA binding, and that at ≥5 mmol/L Mg^{2+} there is insufficient LCGreenPlus binding to affect T_m. In support of this hypothesis, in the more dilute DNA solutions, any T_m increase due to an increase in Mg^{2+} concentration was more than offset by a T_m decrease, which we suggest was due to a decrease in LCGreenPlus binding consequent to competition from Mg^{2+} (Fig. 1). In the more concentrated DNA solutions, the increase in T_m in accordance with increased Mg^{2+} concentration indicates that T_m is less impacted by LCGreenPlus binding, consistent with a lower degree of saturation. Also as predicted, increasing the Mg^{2+} to 6 mmol/L always decreased LCGreenPlus binding, as measured by fluorescence.

We conclude that with a commercial HRMA saturating dye Master Mix, Mg^{2+} concentration impacts HRMA performance by modulating the degree to which the T_m is a function of the concentration of the amplified DNA. Increasing Mg^{2+} concentration to 6 mmol/L makes HRMA performance impervious to a lack of reproducibility in PCR yield, except when the yield is at the very lowest limit of detection for the Rotorgene device.

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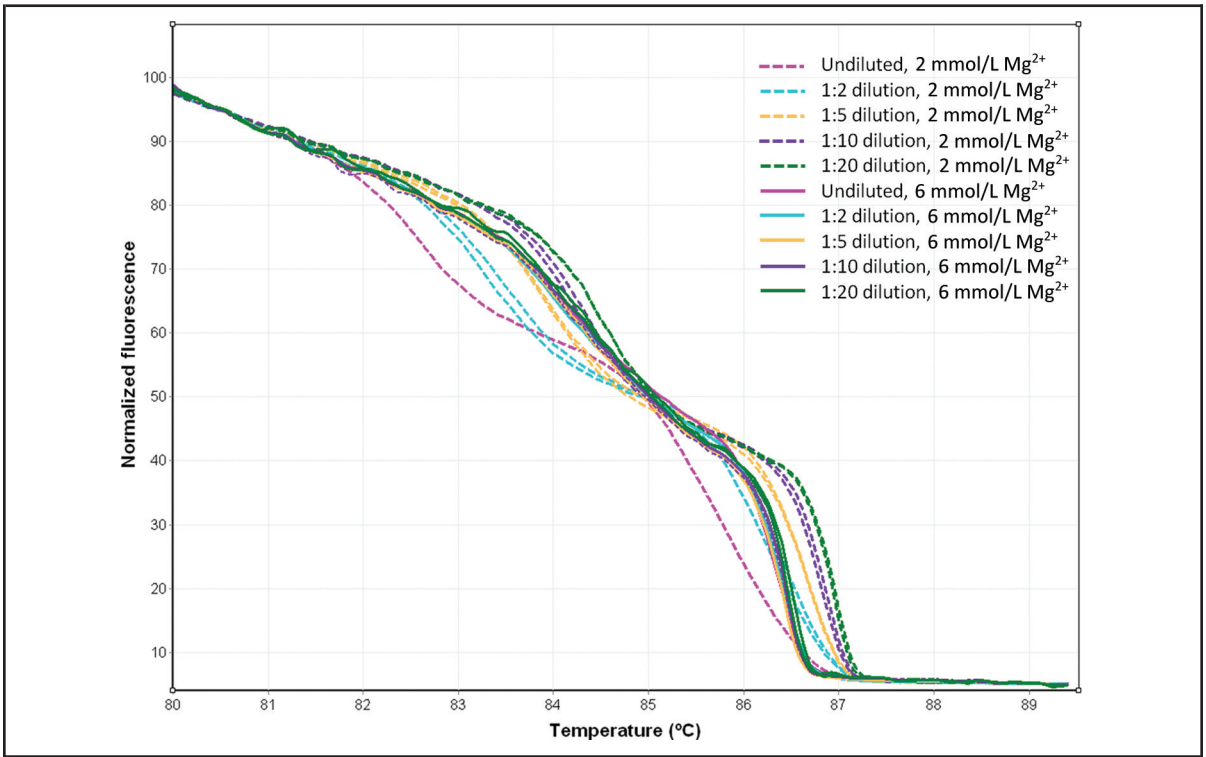

Fig. 1. Normalized HRMA of dilutions of the allele 321 *glpF* PCR product.
The PCR primers were (5′→3′) CTAGGAACTGCAATCTTAATCC and AGGTAAAATAGCATGTGCAATTC. The products of duplicate PCR reactions were pooled and dilutions made from this preparation. For the 6 mmol/L $MgCl_2$ samples, the $MgCl_2$ was added to a final concentration of 6 mmol/L after the PCR and before the HRMA.

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Clinical Use of Reference Intervals Derived from Some CALIPER Studies Questioned

To the Editor:

The availability of accurate and appropriate reference intervals is imperative for proper screening, diagnosis, and monitoring of disease. It is therefore with great interest that we read the recent reports *(1, 2)* by the CALIPER (Canadian Laboratory Initiative for Pediatric Reference Intervals)[1] group. The efforts of the CALIPER project to provide these reference intervals for a diverse pediatric population is highly commendable, particularly in view of the substantial challenges of establishing them for this demographic. Apart from the rigors of selecting and collecting samples to establish reference intervals, the analytical quality of the data has to be ensured. It is with regard to these aspects of these studies that we would like to raise certain important concerns.

Steroid concentrations are known to change substantially with the time of day when the blood sample is drawn. These samples were drawn over a 24-h period; thus, the within-participant variation in concentrations with time was not taken into account. This limitation is probably the most significant criticism of the CALIPER studies. With regard to the study of Bailey et al. *(2)*, diurnal variation in cortisol is present in healthy children from an early age. Hospitalized patients, on the other hand, are stressed and consequently show a lessening of the diurnal effect. The lack of a substantial diurnal effect raises concerns about the validity of the published intervals, for both the immunoassay and the mass spectrometry data. Furthermore, thyroid-stimulating hormone (TSH) and free triiodothyronine (FT_3) also have a circadian rhythm. We are concerned about the lack of a linear relationship between the free thyroxine (FT_4) concentration and the logarithm of TSH concentration obtained with the immunoassays and platform used in this study.

The CALIPER studies used samples collected in SST™ serum-separator tubes (BD) to analyze steroid hormones by liquid chromatography–tandem mass spectrometry (LC-MS/MS) *(1)* and steroid and thyroid hormones by immunoassay *(2)*. We used SST and plain red-top tubes with our laboratory LC-MS/MS assays for steroid and thyroid hormones in assessing ionization inhibition (or lack thereof). We collected paired samples from volunteers into SST and plain tubes (study approved by the Institutional Review Board of the NIH, Clinical Protocol No. 93-CC-0094). The sample analyte and internal standard (IS) areas for thyroid hormones [electrospray ionization (ESI)] and steroid hormones [atmospheric pressure partial ionization (APPI)] were compared with respect to the 2 tube types. We then measured the percentage difference in area between the results obtained with the 2 tube types (Table 1). It is clear that ESI is greatly affected by tube type, whereas APPI is affected only minimally. Atmospheric pressure chemical ionization (APCI) is not a "soft" (low-energy) ionization source, and APCI results would closely resemble those obtained with the ESI source.

Kyriakopoulou et al. in the CALIPER group *(1)* measured steroids by tandem mass spectrometry with an APCI source. Both ESI and APCI show large and variable sample-to-sample ionization-inhibition effects when they are used for measuring steroid concentrations *(4)*. Consequently, IS peak heights and areas vary substantially between samples, potentially affecting the quality of experimental results. Such effects are minimal with APPI. We have obtained differences in IS peak heights of up to 30-fold for patient urine cortisol samples analyzed with ESI, compared with 2- to 3-fold differences obtained with APPI. Of interest would be for the authors to comment on the variation in IS peak heights and areas they obtained with their current APCI method. The between-day CVs for the mass spectrometry assays reported by Kyriakopoulou et

[1] Nonstandard abbreviations: CALIPER, Canadian Laboratory Initiative for Pediatric Reference Intervals; TSH, thyroid-stimulating hormone; FT_3, free triiodothyronine; FT_4, free thyroxine; LC-MS/MS, liquid chromatography–tandem mass spectrometry; IS, internal standard; ESI, electrospray ionization; APPI, atmospheric pressure partial ionization; APCI, atmospheric pressure chemical ionization.