Application of a Thermodynamic Nearest-Neighbor Model to Estimate Nucleic Acid Stability and Optimize Probe Design: Prediction of Melting Points of Multiple Mutations of Apolipoprotein B-3500 and Factor V with a Hybridization Probe Genotyping Assay on the LightCycler

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Background: PCR-based mutation detection is prone to methodological errors, e.g., in restriction length fragment polymorphism (RFLP) and allele-specific amplification (ASA), false PCR results may occur because of technical faults or atypical new mutations.

Methods: We investigated the ability of a genotyping assay based on hybridization of labeled oligonucleotides to detect and discriminate known and as yet unknown mutations in the factor V and apolipoprotein B-100 genes. Expected melting points were calculated using a nearest-neighbor model for nucleic acid duplex stability and compared with experimental findings derived from LightCycler melting curves. A method for genotyping the apolipoprotein B-100 G10699A and C10698T mutations is presented.

Results: All mismatches tested for in the probed sequence could be detected with a single probe. The measured melting points were in good agreement with their values predicted using the nearest-neighbor model ($r = 0.96; y = 0.98x + 1.18; S_{\text{rel}} = 0.96; n = 24$).

Conclusions: This procedure not only allows the identification of the mutation of interest but also enables the discrimination from other potential mutations in the vicinity of the former. The nearest-neighbor model is valid for hybridization probe assays on the LightCycler and should be of general value in setting up such assays. We have shown for two clinically relevant genotyping examples that the LightCycler mutation detection system has superior discriminatory performance compared with conventional RFLP or ASA PCR-based methods for molecular diagnostic purposes. With this method, in every hybridization probe assay, all mutations under a properly designed probe should be detectable, but they will not necessarily be discriminated from each other in all cases.

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With increasing knowledge of the molecular basis of inherited diseases, there is a growing demand for diagnostic genotyping in the clinical laboratory. Examples of such diagnostic genotyping include the detection of prothrombotic mutations in the factor V or prothrombin genes or the apolipoprotein B-3500 polymorphism that causes hypercholesterolemia. The most commonly used genotyping methods are based on restriction length fragment polymorphism (RFLP) and allele-specific amplification (ASA) PCR because they are easy to set up and perform. However, these methods have specific drawbacks. In the case of RFLP PCR, a multisite digest is mandatory or a second obligatory cutting site should be introduced into the PCR amplicon via a mutagenic primer to avoid wrong genotyping as a consequence of restriction enzyme failure. Nevertheless, the same diagnostic fragment pattern can be caused by other mutations at the digestion site as was described for a silent A1692C mutation of the factor V gene (1). With ASA PCR genotyping, results depend on

1 Nonstandard abbreviations: RFLP, restriction length fragment polymorphism; ASA, allele-specific amplification; and $T_m$, melting temperature.
the presence or absence of amplification with primers designed to be specific for either the wild-type or the mutant allele (2). Meticulous primer design and stringent PCR cycling conditions are mandatory to ensure that allele specificity is guaranteed. Lack of amplification in a tube is an expected result of ASA PCR, but this may be caused not only by the presence of the genotype searched for but also by technical faults of the PCR reaction (e.g., presence of inhibitors, lack of template, or wrong cycler program). These are examples of the many errors that might occur in diagnostic genotyping where the results can have severe consequences, e.g., prolonged anticoagulation or decisions about contraceptive use based on the presence of an erroneously detected factor V Leiden mutation.

Another approach is the use of oligonucleotide probes for mutation detection. Probes added to PCR reaction will specifically hybridize to their complementary strand depending on the experimental conditions. Mutations under the probe decrease the stability of the duplex and lead to a decreased melting temperature ($T_m$) (3). This principle of mutation detection together with a rapid PCR cycling can be realized by use of the LightCycler™ (Roche Diagnostics), as has already been described for factor V (4), methylenetetrahydrofolate reductase (5), prothrombin G20210A (6), and HFE (7) genotyping. When two fluorescent dye-labeled probes in the assay hybridize on a PCR amplicon at adjacent sites, then fluorescence resonance energy transfer occurs, producing a specific fluorescence emission, which is then detected. The emission is disrupted if the temperature in the device is incrementally increased above the specific melting point of the probe/single-stranded DNA duplex. The appearance of an emission is indicative of specific product accumulation and confirms successful PCR amplification. Mutation detection then occurs in the same closed tube without any analytical postamplification steps. The stability of nucleic acids can be predicted by a nearest-neighbor model (8, 9), and even the influence of single-base mismatches on the stability can be taken into account (10–14).

Little is known about the applicability of nearest-neighbor duplex stability calculations for the specific situation of the hybridization probe assay on the LightCycler. We therefore investigated the sensitivity and specificity of this method toward other mutations near the classical mutation sites of factor V (G1691A) and apolipoprotein B-100 (G10699A) and low-density lipoprotein (14). In familial defective apolipoprotein B-100, a G10699A mutation produces an arginine-to-glutamine exchange at position 3500, which leads to impaired binding to the LDL receptor protein and, consequently, increased LDL-cholesterol with risk for cardiovascular disease. Another mutation in the same codon (C10698T) causes an arginine-to-tryptophan exchange at position 3500 in the protein. This mutation has less effect on the phenotype and is not significantly associated with coronary heart disease (15). However, the restriction enzyme recognition site used for genotyping is also destroyed. Using data calculated with the nearest-neighbor model, we expected that both mutations would show sufficient difference in their melting behavior to be clearly distinguished. We have compared these data with observed results to assess the validity of predicted melting points for molecular diagnostic purposes and to find whether this could be useful for probe design.

**Materials and Methods**

**FACTOR V GENOTYPING**

Genomic DNA was isolated by standard techniques. Factor V PCR was performed on a LightCycler as described (4, 6). The rare A1692C mutation was constructed by use of the forward primer F5–1692-mut-for 5′-TAA TCT GTA AGA GCA GAT CCC TGG ACA GCG GAA T-3′ (mutagenic position is in bold font). The product was cloned into a TOPO TA vector (Invitrogen), and the presence of the expected mutation was confirmed by sequencing (sequenase cycle sequencing kit; Amer sham) on an automated DNA sequencer (Licor 4200; Licor). The plasmid was then used as a template for mutation detection with the LightCycler.

**ApoB 3500 GENOTYPING**

A 305-bp fragment of the apolipoprotein B-100 gene (GenBank accession no. M14162) was amplified using the primers ApoB 3500 for 5′-CCT CAC CTC TTA CTT TTC C-3′ and ApoB 3500 rev 5′-CTT TGC TTG TAT GTT CTC C-3′ (Fig. 1). The mutation site is covered by a wild-type
complementary detection probe: ApoB 3500 R 5'-AGA GCA CAC GGT CTT CAG T-3' -FLU labeled with fluorescein as indicated (Fig. 1). The adjacent anchor probe ApoB 3500 anchor 5'-LC-Red640-TGC AGG GCA CTT CCA AAA TTG ATG A-3' -PHO (Fig. 1) is 5'-labeled with the LC-Red640 dye (Roche Biochemica) and 3' phosphorylated by using a 3' phosphate controlled-pore glass. The phosphorylation was to prevent probe elongation by the Taq polymerase. If these probes lie adjacent to each other on a DNA strand, fluorescence resonance energy transfer occurs and specific emission is detected by the LightCycler. PCR amplification and detection occur in the same closed tube in ~40 min. Oligonucleotides were synthesized by standard phosphoramidite chemistry. LC-Red640-N-hydroxysuccimide ester was linked with the respective oligonucleotide via an amino linker and purified by HPLC. PCR reactions were carried out in a final volume of 10 μL in the LightCycler glass capillaries. The reaction mixture consisted of 1 μL of genomic DNA solution, 0.5 μmol/L amplification primers ApoB 3500 for and ApoB 3500 rev, 0.1 μmol/L ApoB 3500 R probe, 0.3 μmol/L ApoB 3500 anchor, 0.5 U of Taq DNA polymerase (Boehringer Mannheim), 1 μL of 10× PCR buffer (Boehringer Mannheim), 0.2 mmol/L each dNTP (Boehringer Mannheim), 2.5 mmol/L MgCl2, 500 mg/L bovine serum albumin (New England BioLabs), and 50 mL/L dimethyl sulfoxide (Sigma). PCR-grade water was added to the final volume of 10 μL. The cycling program consisted of a 30-s initial denaturation at 95 °C and 55 cycles of 95 °C for 0 s, 50 °C for 5 s, and 72 °C for 5 s, with maximum ramp rate. The program for analytical melting was 95 °C for 30 s, 40 °C for 30 s, increasing to 70 °C at a 0.1 °C/s ramp rate. The PCR conditions and cycler program are essentially the same that we have used before (6), thereby fully integrating the apolipoprotein B-3500 genotyping into our single master mixture and single cycler program approach for diagnostic genotyping.

The typical G10699A mutation was found in a patient with a LDL-cholesterol concentration of 2900 mg/L. Its presence was confirmed after cloning and sequencing as described above. The C10698T mutation was constructed using site-directed mutagenesis with a megaprimer approach essentially as described (16). Briefly, the amplicon from the apolipoprotein B-100 PCR was cloned into a TOPO TA vector. It was then amplified with a proofreading enzyme mixture (Expand™ PCR system; Boehringer Mannheim) to minimize the generation of nontemplate adenosine overhangs using a mutagenic primer that introduces the C10698T mutation into the sequence. The product of this PCR was purified (PCR purification kit; Qiagen) and used as megaprimer in a second PCR with the same vector containing the wild-type sequence as template. The resulting amplicon was cloned into a TOPO TA vector, and successful mutagenesis was confirmed by sequencing as described above. In the same way, other mutations were created for investigation of their melting behavior. The observed melting points (T_m determined by LightCycler Software Package Ver. 3.1 Data Analysis Module) together with the predicted melting points for the ApoB3500R or alternatively the ApoB3500W probe (5'-AGA GCA CAT GGT CTT CAG T-3' -FLU) in this assay are summarized in Table 1.

### Table 1. Observed vs predicted melting points in diagnostic genotyping.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Mutation</th>
<th>Wild-type probes used</th>
<th>Mutation-compatible ApoB 3500W probe used</th>
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<tr>
<td>Factor V (6)</td>
<td>None (wild type)</td>
<td>62.2</td>
<td>61.7</td>
</tr>
<tr>
<td></td>
<td>G1691A*</td>
<td>54.3</td>
<td>53.9</td>
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<tr>
<td></td>
<td>A1692C</td>
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<td>56.6</td>
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<tr>
<td>ApoB 3500</td>
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<td>60.4</td>
</tr>
<tr>
<td></td>
<td>A10692G</td>
<td>56.4</td>
<td>53.4</td>
</tr>
<tr>
<td></td>
<td>A10695C</td>
<td>57.9</td>
<td>57.3</td>
</tr>
<tr>
<td></td>
<td>C10696G</td>
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</tr>
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<td>51.3</td>
</tr>
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<td>G10699A</td>
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<td>52.4</td>
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<td>56.0</td>
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<td>T10701G</td>
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<tr>
<td></td>
<td>T10704A</td>
<td>53.4</td>
<td>54.2</td>
</tr>
</tbody>
</table>

* Mutations described in humans are in bold.

* NC, not calculated double mismatch (see Discussion).
more, the destabilizing impact of mismatches in a given sequence context is now well investigated \((10–14)\). These data enable prediction of the \(T_m\) of oligonucleotide sequences with or without single base pair mismatches with an error of \(<2^\circ\text{C}\) \((8, 10–14)\). Results of probe hybridization experiments \((T_m\) determined by LightCycler Software Package Ver. 3.1 Data Analysis Module\) together with the predicted melting points for given probe/mismatch settings are shown in Table 1. Formulas used for \(T_m\) calculation are given in the Appendix. The PCR product concentration will be different in every PCR, depending for example, on template quality and amplification primer efficiency. However, from a practical standpoint we found the best fit of melting point predictions when we set CT equal to the detection probe concentration used in the assay. It seems that probe concentrations in the range of 0.1–0.3 \(\mu\text{mol/L}\), which are commonly used in these assays, reflect the specific situation of hybridization probe assays on the LightCycler. Our experimental conditions were 0.1 \(\mu\text{mol/L}\) probe concentration. The sodium equivalent of our PCR buffer was 350 mmol/L.

We performed stability calculations with a spreadsheet application running under Microsoft Excel® 4.0 and later (E. Schütz and N. von Ahsen, submitted for publication). The program is available for downloading at http://server1.medikc.med.uni-goettingen.de/meltcalc.htm. Results are equivalent to predictions from the Hyther program, which is available at http://jsl1.chem.wayne.edu.

**Results**

The G1691A and A1692C mutations produce the same pattern when RFLP genotyping with \(Mnl\)I enzyme digestion is performed, and an incorrect diagnosis of factor V Leiden mutation would therefore be made (data not shown). When the LightCycler detection assay was used, both mutations were clearly differentiated by their \(T_m\) (Fig. 2A and Table 1).

Examples of genotyping results for the apolipoprotein B-3500 mutation are given in Fig. 2B, and the \(T_m\) values are summarized in Table 1. Values represent the mean from determinations on at least five different days. The mean CV for all melting points was 0.7% ± 0.26%.

All mutations under a wild-type-compatible probe were detected (100% discrimination), and observed shifts in the \(T_m\) values were 1.8–10.9 \(^\circ\text{C}\) for single-base pair mismatches. There was a certain specificity inasmuch that the two known mutations of apolipoprotein B-100 \((G10699A, C10698T)\) were well discriminated by the detection assay as well as the factor V mutations \((G1691A\) and \(A1692C)\). The values predicted from the nearest-neighbor model suggest that mutations are possible that destabilize the double-stranded DNA to the same extent and, therefore, should not be discriminated from each other. As an example, we constructed the mutations \(G10700T\) and \(T10704A\) by site-directed mutagenesis because from the prediction model we expected these mutations to have almost the same melting points as the investigated \(G10699A\) mutation. We found that when using the wild-type-compatible probe, the observed melting points of the two mutations \((54.7\) and \(53.4^\circ\text{C}\)) did not differ sufficiently from that of the typical \(G10699A\) mutation \((54.0^\circ\text{C}\)) to allow reliable discrimination. Only by using a specific mutation-compatible probe can these mutations be detected again with an almost 100% discrim-

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**Fig. 2**. Melting curves of factor V (A) and apolipoprotein B-100 (B).

Factor V genotyping shows a G1691A homozygous mutation, an A1692C homozygous mutation, and a wild type. Apolipoprotein B-100 genotyping using the ApoB 3500R probe shows a C10698T homozygous mutation, a G10699A homozygous mutation, and a wild type. LC-Red640 fluorescence in channel 2 was monitored. Contamination and water controls were negative in all samples (data not shown). Melting points are summarized in Table 1. (———), wild type; (· · · · · ·), original mutation (factor V 1691A and apolipoprotein B-100 10699A, respectively); (· · · · · ··), rare mutation (factor V 1692C and apolipoprotein B-100 10698T, respectively).
minating efficacy. Another important question is whether any mutation might remain undiscovered under a 19mer probe. We therefore investigated the A10695C mutation, which has of all possible mutations under the probe the lowest calculated melting point difference from the wild type. This mutation did indeed have the lowest melting point shift (1.8 °C) compared with the wild type, but it is reliably detected by the LightCycler hybridization probe assay. There was a good correlation between the observed and predicted melting points for all investigated mutations under the wild-type- (3500R) or a mutation-compatible (3500W) probe (Pearson $r = 0.96$; Fig. 3). The mean difference between the observed and predicted $T_m$s was $0.1 \pm 1.34$ °C.

The mean observed melting shift caused by mutations under the wild-type-compatible probe was 6.3 °C, whereas it was 9.3 °C for the mutation-compatible probe that contained an additional mismatch. From our experimental data (mean SD of $T_m$s in our assay, 0.35 °C), we expect that mismatches causing a $\Delta T_m > 1.0$ °C can be reliably discriminated from the wild type. We performed melting point calculations for all possible $3 \times 10^6$ mismatches under 60 000 randomly chosen 19mer oligonucleotides and found that only 0.055% cause a melting shift smaller than 1.25 °C (Fig. 4). For this purpose, the sequence of a starting probe was randomly altered by exchanging nucleotides by a computer program. The randomization algorithm was programmed to produce a gaussian-shaped distribution with respect to probe GC content and a preponderance of probes with 50–60% GC.

**Discussion**

Complementary nucleic acids hybridize very specifically with each other. This principle is widely applied in molecular biology for nucleic acid detection. It is known that a single mismatch under a probe can decrease the $T_m$ of a DNA-DNA hybrid by several degrees Celsius (3). The LightCycler uses this approach with labeled oligonucleotide probes and therefore has a favorable discriminating...
capacity toward all mutations under the detection probe used in the assay. Other mutations around the mutation site being investigated can cause false genotyping when the RFLP technique is used (1, 17, 18). This is also known for the factor V Leiden assay originally designed by Bertina et al. (19), where an A1692C mutation also destroys the diagnostic MnlI restriction site (1). This mutation is rare, but there remains a risk of obtaining a false-positive genotyping result (20–22). The hybridization probe assay clearly discriminates this mutation from the original Leiden mutation because of its 2 °C higher \( T_m \), as speculated by Lay and Wittwer (4) and later shown by Lyon et al. (23) from the same group. The potential of the hybridization probe principle for detecting unknown polymorphisms was demonstrated recently by Bernard et al. (7), who have established a hybridization probe assay for genotyping the C282Y and H63D polymorphisms of the hemochromatosis gene \( HFE \). They found an additional melting peak for the probe covering the H63D locus that could be attributed to an already known S65C variant of the \( HFE \) gene. Furthermore, another mutation (G1689A) (23) of the factor \( V \) gene was detected by chance with the LightCycler. Unknown mutations around loci associated with certain diseases may be more common than one might expect. The current estimate is that single-nucleotide polymorphisms occur every 100–300 bp in the human genome (24). This makes it even more mandatory to use well-controlled and discriminative assays for genotyping.

The stability of single-base mismatches next to Watson-Crick pairs is now well characterized and also depends on the interaction with the neighboring bases. This is impressively illustrated by the more than sixfold range in the observed melting point differences. When a wild-type-compatible probe is used, the apolipoprotein B-3500 G10699A mutation causes a G-T mismatch in the trimer \( \text{CGG/GTC} \), which is more stable than the C-A mismatch in the trimer \( \text{ACG/TAC} \) caused by the C10698T mutation (10, 11). This is reflected by the higher \( T_m \) of the G10699A mutation compared with the C10698T mutation. The ApoB 3500W detection probe complementary to the C10698T genotype produces the highest \( T_m \) if this particular mutation is present. For all other mutations, a lower melting point than that observed with the wild-type probe results. This is because all other genotypes lead to two mismatches under the mutation-specific probe. It must be mentioned that the G10699A mutation under the ApoB 3500W probe produces two immediately adjacent mismatches. For this situation, only a few nearest-neighbor data are available, including that of the present resulting \( \text{ATGG/TGTC} \) mismatch (10). The melting point prediction from nearest-neighbor parameters was not compromised by two mismatches under a 19mer if these were not immediately adjacent. The result from this and other studies show that all existing polymorphisms of factor \( V \) (G1691A, A1692C, and G1689A) (23) and apolipoprotein B-100 (G10699A and C10698T) are discriminated using a single wild-type probe. We have extended these results and found that some possible mutations at the apolipoprotein B-100 locus (e.g., T10704A and G10700T) are not appreciably discriminated from the G10699A mutation. Therefore, only a confirmation assay with a mutation-specific probe will give almost 100% sensitivity and specificity for this mutation detection. All samples with unexpected melting behavior must be further investigated before reliable genotyping results are obtained. Our experimental findings are in good correlation with calculated values. From thermodynamic predictions, we expect that every mutation under a 19mer probe should be detected in this assay. We have tried to extend these findings to every possible sequence under a 19mer, but the calculation of all possible mismatches in 4\(^9\) oligonucleotides is not easily achieved. However, we calculated the minimal \( T_m \) shift caused by any possible mutation occurring in >60 000 randomly chosen sequences (Fig. 4) and found a distinct pattern. In 19mers with a very high GC content (>80%), a relatively stable mismatch such as \( \text{GAC/CGG} \) will probably not be detected. Several solutions are possible in this situation: (a) Siting the detection probe on the antisense strand changes the mismatch to \( \text{CTG/GCC} \), which is an unstable mismatch and is easily detected. (b) Introduction of additional mismatches into the detection probe has also been shown to increase the melting point difference (25). (c) Alternatively, the detection probe may be shortened by a few nucleotides to reduce the \( T_m \) and to increase the \( T_m \) shift caused by the mismatch. The practical endpoint for the LightCycler genotyping is that the mismatched probe should still have a \( T_m \) >45 °C because the instrument is not equipped with active cooling. In all of these cases, prediction of the melting behavior is clearly advantageous, and such model-based probe design can greatly improve the discriminatory performance of these genotyping assays. The only limitations of the model are caused by missing thermodynamic data for dangling ends and double and terminal mismatches. The only unpublished nearest-neighbor parameters for the calculation of these situations. Accordingly, we omitted these cases from our analysis, such as the G10699T mutation, which produces a double mismatch when probed with the 3500W probe (Table 1). As a consequence, our results apply only to the central 17 bases covered by the probe because the outermost bases will appear as dangling ends when mismatched. We could not find any evidence that the labeling of probes per se or the different types of labeling that were used substantially changes the \( T_m \) (data not shown).

In conclusion, we have demonstrated the applicability and benefits of a thermodynamic nearest-neighbor nucleic acid melting prediction model for diagnostic genotyping.
A method for apolipoprotein B-3500 genotyping is presented and evaluated against different known and potential mutations at this locus. Observed $T_{m}$s were in good agreement with calculated values. We have shown for two clinically relevant genotyping examples that the LightCycler mutation detection system has superior discriminatory performance compared with conventional RFLP or ASA PCR-based methods for molecular diagnostic purposes. With this method, in every hybridization probe assay, all mutations under a properly designed probe should be detected, but they will not necessarily be discriminated from each other in all cases.

The technical assistance of Sandra Hartung and Reiner Andag is gratefully acknowledged.

References


Appendix

The enthalpy ($\Delta H$), entropy ($\Delta S$), and free energy $\Delta G^\circ$ of an oligonucleotide duplex are calculated as the sum of the entire Watson-Crick nearest neighbors plus helix initiation and symmetry terms:

$$\Delta H_{\text{total}} = \sum_i \Delta H \left( i \right) + \Delta H \left( 5' \text{init} \right) + \Delta H \left( 3' \text{init} \right) + \Delta H \left( \text{symmetry} \right)$$

(1)

For $\Delta S$ and $\Delta G^\circ$, the same formula applies. The $\Delta H$, $\Delta S$, and $\Delta G^\circ$ values for Watson-Crick nearest neighbors for matched and mismatched base pairs as well as those for initiation and symmetry correction were taken from published data (9–14).
Apart from entropy and enthalpy of the nucleotides in the sequence, the concentration of monovalent ions and oligonucleotides must be taken into account because the data provided by SantaLucia and Allawi (8), Allawi and SantaLucia (10-13), and Peyret et al. (14) are derived at 1 mol/L NaCl. Because enthalpy is independent of salt concentration, only entropy values for a given [Na\textsuperscript{+}] were calculated according to the formula:

$$\Delta S[\text{Na}\textsuperscript{+}] = \Delta S[1 \text{ mol/L Na}\textsuperscript{+}] + 0.368 \times N \times \ln[\text{Na}\textsuperscript{+}]$$

(2)

where $N$ is the total number of phosphates in the duplex divided by 2, which equals the length minus 1 (9).

Na\textsuperscript{+} equivalents of the PCR assay buffer were calculated based on the 140-fold higher stabilizing effect of Mg\textsuperscript{2+} on duplexes compared with monovalent ions (26):

$$\text{Na}\textsuperscript{+} \text{ equivalent} = [\text{Mg}\textsuperscript{2+}] \times 140$$

(3)

The $T_m$ in °C of self-complementary oligonucleotides at a given concentration of CT in a solution with the sodium equivalence concentration [Na\textsuperscript{+}] is calculated as:

$$T_m = \frac{\Delta H}{\Delta S[\text{Na}\textsuperscript{+}]} + R \ln CT - 273.15$$

(4)

where $R$ is the gas constant (1.987 cal/K·mol), for non-self-complementary sequences and CT is the concentration of oligonucleotides divided by 4. The dimethyl sulfoxide-induced $T_m$ reduction was 0.6 °C for each percent of change in the dimethyl sulfoxide concentration (27).