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Oligonucleotide Melting Temperatures under PCR Conditions: Nearest-Neighbor Corrections for Mg^{2+}, Deoxynucleotide Triphosphate, and Dimethyl Sulfoxide Concentrations with Comparison to Alternative Empirical Formulas

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Background: **Many techniques in molecular biology depend on the oligonucleotide melting temperature (T_m), and several formulas have been developed to estimate T_m. Nearest-neighbor (N-N) models provide the highest accuracy for T_m prediction, but it is not clear how to adjust these models for the effects of reagents commonly used in PCR, such as Mg^{2+}, deoxynucleotide triphosphates (dNTPs), and dimethyl sulfoxide (DMSO).**

Methods: **The experimental T_ms of 475 matched or mismatched target/probe duplexes were obtained in our laboratories or were compiled from the literature based on studies using the same real-time PCR platform. This data set was used to evaluate the contributions of [Mg^{2+}], [dNTPs], and [DMSO] in N-N calculations. In addition, best-fit coefficients for common empirical formulas based on GC content, length, and the equivalent sodium ion concentration of cations [Na^+_{eq}] were obtained by multiple regression.**

Results: **When we used [Na^+_{eq}] = [Monovalent cations] + 120($\sqrt{[Mg^{2+}] - [dNTPs]}$) (the concentrations in this formula are mmol/L) to correct ΔS^0 and a DMSO term of 0.75 °C (%DMSO), the SE of the N-N T_m estimate was 1.76 °C for perfectly matched duplexes (n = 217). Alternatively, the empirical formula T_m (°C) = 77.1 °C + 11.7 × log[Na^+_{eq}] + 0.41(%GC) − 528/bp − 0.75 °C(%DMSO) gave a slightly higher SE of 1.87 °C. When all duplexes (matched and mismatched; n = 475) were included in N-N calculations, the SE was 2.06 °C.**

Conclusions: **This robust model, accounting for the effects of Mg^{2+}, DMSO, and dNTPs on oligonucleotide T_m in PCR, gives reliable T_m predictions using thermodynamic N-N calculations or empirical formulas.**

DNA amplification and detection techniques often depend on oligonucleotide melting temperature (T_m).[4] The T_m of a DNA duplex, defined as the temperature where one-half of the nucleotides are paired and one-half are unpaired *(1)*, corresponds to the midpoint of the spectroscopic hyperchromic absorbance shift during DNA melting *(2)*. The T_m indicates the transition from double helical to random coil formation and is related to the DNA GC base content *(2)*. Other important factors for DNA stability are the cation concentration of the surrounding buffer *(3, 4)* and the DNA double strand length *(5)*. The T_m of polymer DNA can be predicted by formulas that account for differing GC content in various buffers *(6)*. A term for the length of the duplex can also be included *(1, 7, 8)*.

The most accurate prediction of T_m for oligonucleotide DNA uses the thermodynamic nearest-neighbor (N-N) model [see Ref. *(9)* for review and parameters]. N-N calculations for T_m prediction are useful on microarrays *(10)* and for the selection of PCR primers and hybridization probes *(11)*. Empirical data from probe melting curve analysis during real-time PCR correlate well with theoretical predictions *(12, 13)*. The N-N model is based on the

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[4] Nonstandard abbreviations: T_m, melting temperature; N-N, nearest-neighbor; dNTP, deoxynucleotide triphosphate; and DMSO, dimethyl sulfoxide.

assumption that probe hybridization energy can be calculated from the enthalpy and entropy of all N-N pairs, including a contribution from each dangling end. Thermodynamic values for entropy and enthalpy of each possible matched N-N and dangling ends have been determined *(9, 14)*. Dangling-end effects account for the stacking energy of a shorter probe on a more lengthy target *(15, 16)*. The entropy (ΔS) is salt dependent, and ΔS^0 must be corrected if the ionic environment is different from 1 mol/L NaCl, the salt concentration at which most thermodynamic values have been derived. However, Mg^{2+} is present in PCR as an important cofactor for Taq DNA polymerase and strongly influences ΔS *(17)*. Deoxynucleotide triphosphates (dNTPs) are also essential and chelate some of the available Mg^{2+} *(18)*. In addition, dimethyl sulfoxide (DMSO) is commonly used as a cosolvent *(19)* to facilitate amplification from difficult templates. Addition of DMSO decreases the T_m *(20–22)*, which must be taken into account when primer T_m is calculated *(23)*.

To get a deeper insight into probe T_m under common PCR buffer conditions, we have compiled T_m data from 475 different DNA duplexes. These assays were performed with different concentrations of Mg^{2+} and DMSO, reflecting current PCR laboratory practice. We provide an empirical ΔS compensation for the Mg^{2+} and dNTP influence on ionic strength. In addition, best-fit coefficients for simpler formulas based on GC content, length, and the equivalent sodium ion concentration are determined for convenient bench-side use that offer accurate T_m predictions.

Materials and Methods

BASIC PRINCIPLE

The LightCycler real-time PCR machine (Roche Molecular Biochemicals) is capable of detecting the hybridization of adjacent fluorescent dye-labeled probes by fluorescence resonance energy transfer *(24)*. Assay design for the detection of single nucleotide polymorphisms requires that the sensor probe (the probe covering the mutation) has a lower T_m than the detection probe, which stays hybridized during the melting cycle. The observed T_ms from matched and mismatched hybridizations can be predicted using the N-N model *(12)*. Probes have a T_m similar to PCR primers (50–70 °C), and the results from probe T_m prediction can also be used to predict primer T_ms, a critical parameter for PCR performance *(23, 25–27)*.

DATA COLLECTION

All assays were performed using the LightCycler real-time PCR instrument. Most T_ms were measured in our laboratories (n = 388) during the course of genotyping experiments [for examples, see Refs *(12, 28)*]. Some additional T_ms were extracted from the literature when complete experimental conditions were published (n = 87). In total, 162 different probes were used with various templates and conditions, including 221 completely matched hybridizations, 237 single mismatches, and 17 two-point mismatches. Forty assays were based on melting curves detected with SYBR Green I, whereas the remainder were based on the melting of fluorescent oligonucleotide probes. DMSO was used in 206 assays in concentrations ranging from 2.5% to 10%. These data are available as an online supplement at *Clinical Chemistry Online* (http://www.clinchem.org/content/vol47/issue11).

STATISTICAL ANALYSIS

N-N calculations. The entropy and enthalpy were calculated from probe sequences at standard conditions (1 mol/L NaCl) as described in more detail elsewhere *(9, 29, 30)*. In addition we considered the published thermodynamic data for dangling-end contributions *(14)*. Mismatches were accounted for by the thermodynamic data reported by Allawi and SantaLucia *(31–34)* and Peyret et al. *(35)*. The PCR DNA target concentration was set to 50 nM. SYBR Green I, if present, was assumed to increase the T_m by 1 °C at 1:20 000 dilution, based on own preliminary data. Calculations were performed with Excel™ for Windows (Microsoft), using the built-in statistical functions. The Pearson r^2 was used for correlations, and standard linear regression was used for relating observed to measured T_m. Thermodynamic N-N stability calculations were performed using MeltCalc, a spreadsheet software for Excel *(36)*.

The observed T_m was used as the dependent variable in a multiple variable fit to determine the DMSO coefficient and the best formula for Mg^{2+} influence. Prior evidence suggested that the influence of Mg^{2+} on T_m is stoichiometrically reduced by dNTPs. Initial calculations based on r^2 indicated that the relationship of DMSO to T_m was linear, whereas [Mg^{2+}] was nonlinear. Therefore, our model was: T_m (observed) = T_m (predicted) − $a \times$ DMSO (%), with [Na^+_{eq}] = [monovalent cations] + $b \times$ ([Mg^{2+}] − [dNTP])^c. The parameters a, b, and c were optimized to minimize the prediction error by stepwise incremental iterations. With $a = 0.75$, $b = 120$, and $c = 0.5$, only 18% of the predicted values fell outside a 5% error limit. The nonlinear effect of [Mg^{2+}] on [Na^+_{eq}] was best approximated by the square-root function, which is in agreement with a previous report *(37)*.

Alternative formulas. Using our empirical data set, we evaluated several simpler formulas for their ability to predict T_m. These formulas cannot properly account for the presence of single mismatches. Therefore, only data for matched probe/template duplexes were used.

The Wallace–Ikatura rule is often used as a rule of thumb when primer T_m is to be estimated at the bench *(1, 38)*. However, the formula was originally applied to the hybridization of probes in 1 mol/L NaCl *(1)* and is an estimate of the denaturation temperature (T_d):

$$T_d\ (°C) = 2(A + T) + 4(G + C) \quad (1)$$

Another equation for the effective priming temperature (T_p) was suggested by Wu et al. *(25)*:

$$T_p\ (°C) = 22 + 1.46L_n \quad (2)$$

where $L_n = 2(G + C) + (A + T)$.

Marmur and Doty *(2)* originally established a formula to correlate GC content (%GC) to the T_m of long duplexes at a given ionic strength. Chester and Marshak *(23)* added a term to account for DNA strand length (n in base pairs) to estimate primer T_m:

$$T_m = 69.3 + 0.41(\%GC) - 650/n \quad (3)$$

The Marmur–Schildkraut–Doty equation also accounts for ionic strength with a term for the Na^+ concentration *(1, 2, 6–8)*.

$$T_m\ (°C) = 81.5\ °C + 16.6(\log[Na^+]) + 0.41(\%GC) - b/n \quad (4)$$

Values between 500 and 750 have been used for *b (5, 23)*, a value that may increase with the ionic strength *(8)*.

Another modification is that of Wetmur *(1)*:

$$T_m = 81.5 + 16.6 \times \log\left(\frac{[Na^+]}{1.0 + 0.7 \times [Na^+]}\right) + 0.41(\%GC) - 500/n \quad (5)$$

Eqs. 4 and 5 assume that the stabilizing effects of cations are the same on all base pairs. However, Owen et al. *(39)* observed that the slopes of T_m vs $\log[Na^+]$ decrease with increasing GC content, leading to the following final formula for the estimation of T_m in polymer DNA *(40–42)*:

$$T_m\ (°C) = 87.16 + 0.345(\%GC) + \log[Na^+] \times [20.17 - 0.066(\%GC)] \quad (6)$$

Results

DESCRIPTIVE STATISTICS

Empirical oligonucleotide T_ms were obtained under 475 unique conditions typical of current PCR practice. The Mg^{2+} concentration was 1–6 mM (mean, 2.9 ± 1.17 mM), the length of the probes was 11–45 bp (median, 19 bp), and the GC content was 17–88% (mean, 52.5% ± 9.6%). DMSO was used in 206 assays in concentrations ranging from 2.5% to 10%. In 57 assays, synthesized oligonucleotides were used without PCR. Probe concentrations were 0.025–0.5 μM (mean, 0.15 ± 0.087 μM). The monovalent cation concentration was 20–54 mM, depending on the PCR buffer system. The measured probe T_ms varied from 43.2 to 74.7 °C (mean, 59.1 ± 6.42 °C).

STATISTICAL ANALYSIS

N-N calculations. The best fit values for $[Mg^{2+}]$ and [dNTP] coefficients revealed that the Na^+ equivalents (Na^+_{eq}) were approximated by:

$$[Na^+_{eq}] = [\text{Monovalent cations}] + 120(\sqrt{[Mg^{2+}] - [\text{dNTPs}]}) \quad (7)$$

Note that the concentrations in Eq. 7 are in mmol/L. In all other equations, concentrations are in mol/L. Monovalent cations are typically present as K^+ and $Tris^+$ in PCR buffer *(18, 26)*. K^+ is similar to Na^+ in regard to duplex stabilization *(17)*. ΔS^0 was corrected for the salt concentration as follows *(9)*:

$$S^0[Na^+] = S^0[1\ M\ Na^+] + 0.847 \times n \times \log[Na^+] \quad (8)$$

where n is the total number of phosphates in the duplex divided by 2. This is equal to the oligonucleotide length minus 1.

Each percentage of DMSO (by volume) decreased the T_m by 0.75 °C. GC content had no obvious influence on the DMSO factor. Using these equations, we found a good regression of predicted vs observed T_m (Fig. 1). The mean prediction error was 0.2 ± 2.18 °C, which is within the error range for N-N calculations.

Alternative formulas. The Wallace–Ikatura rule (Eq. 1) overestimates the T_m of long duplexes and gives reasonable results only in the range of 14–20 bp *(1, 38)*. Therefore, only duplexes shorter than 21 bp were included in the analysis (Table 1). When the analysis was extended to include duplexes of up to 24 bp, r^2 decreased to 0.64.

The equation for the effective priming temperature by Wu et al. *(25)* (Eq. 2) is similar to the Wallace–Ikatura rule. Only oligonucleotides with L_n <39 (see Eq. 2) were included in the analysis, as suggested by the authors. The oligonucleotide T_m is overestimated by a mean of 2.5 °C by this formula (Table 1).

Including the length dependence of Chester and Marshak [Eq. 3; *(23)*] improved r^2, but the intercept and

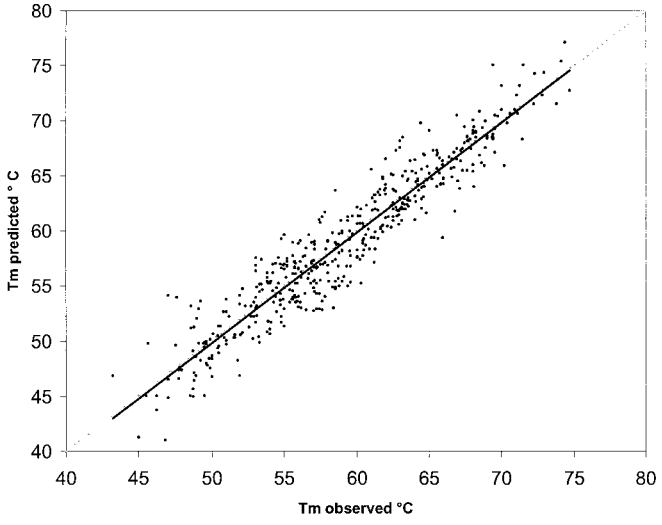

Fig. 1. Observed vs predicted T_m for 475 melting temperature assays. (- - - -), line of identity; (——), regression line: $y = 1.00x - 0.29$.

Table 1. Comparison of the predictive performance of different equations for the calculation of oligonucleotide T_m.[a]

	N-N calculations		Equation					
	Including mismatches	Without mismatches	1[b]	2[c]	9	10	11	12
n	475	217	131	200	217	217	217	217
r^2	0.90	0.89	0.78	0.60	0.86	0.87	0.88	0.88
Slope	1.00	1.05	1.20	0.95	1.01	0.96	0.97	1.01
Intercept, ΔT_m	−0.29	−3.17	−18.44	5.83	−0.60	2.65	2.32	−0.54
$S_{y|x}$	2.06	1.76	2.21	3.13	1.99	1.88	1.87	1.86
Mean ± SD of differences (observed minus predicted)	0.1 ± 2.18	0.2 ± 1.96	6.1 ± 3.13	−2.5 ± 3.84	−0.1 ± 1.93	0.0 ± 2.16	−0.1 ± 2.00	0.1 ± 2.18

[a] See *Materials and Methods* for a detailed description of the formulas.
[b] Only oligonucleotides <21 bp were included in the analysis.
[c] Only oligonucleotides with L_n <39 (see Eq. 2) were included in the analysis.

difference between observed and predicted T_ms were poor (data not shown). However, a better fit was obtained with new constants obtained from stepwise iterations (fit variable: length dependency term):

$$T_m = 69.3 + 0.41(\%GC) - 535/n \quad (9)$$

To improve Eq. 4, a $dT_m/d\log[Na^+]$ of 11.7 °C was used as suggested for oligonucleotide DNA *(9)* instead of the commonly used 16.6 °C *(6)*. Best-fit values for the temperature offset and length dependence were then obtained by stepwise iterations (fit variables: T_m offset, length dependency term):

$$T_m\,(°C) = 77.1\ °C + 11.7 \times \log[Na^+] + 0.41(\%GC) - 528/n \quad (10)$$

In the same manner, the modification for the formula of Wetmur *(1)* is (fit variables: T_m offset, length dependency term):

$$T_m = 77.8 + 11.7 \times \log\left(\frac{[Na^+]}{1.0 + 0.7 \times [Na^+]}\right) + 0.41(\%GC) - 528/n \quad (11)$$

The fit of this equation could not be improved (data not shown) by the introduction of a salt dependence term for *b* (see Eq. 4) as suggested previously *(8)*.

Eq. 6 has been suggested as the best predictor of polymer DNA T_m *(41, 42)*. The best fit of constants in this formula based on our oligonucleotide data produced (fit variables: T_m offset, oligomer $[Na^+]$ dependency term, length dependency term added):

$$T_m\,(°C) = 80.4 + 0.345(\%GC) + \log[Na^+] \times [17.0 - 0.135(\%GC)] - 550/n \quad (12)$$

Discussion

Many techniques in modern molecular biology depend on oligonucleotide duplex formation. Perhaps the most universal method in use today is the PCR, with applications including amplification, cloning, mutation detection, and mutagenesis. A precise knowledge of oligonucleotide T_m is useful for rapid optimization of assays. The most accurate predictions of oligonucleotide T_ms use the N-N model. N-N parameters have recently been improved *(9)*, and their ability to predict the T_m of unknown oligonucleotide duplexes demonstrated *(30)*. However, these parameters usually are determined in 1 mol/L NaCl and need to be corrected for the conditions in the PCR. Although correction factors for different NaCl concentrations have been published *(9)*, we are not aware of an investigation into nucleic acid stability under typical PCR buffer conditions, even given its high practical value. It is well appreciated that Mg^{2+} stabilizes duplex DNA 80- to 100-fold *(3)* to as much as 140-fold *(17)* more than Na^+. Our findings indicate a nonlinear effect of $[Mg^{2+}]$ on $[Na^+_{eq}]$ in the order of $120(\sqrt{[Mg^{2+}]})$.

The N-N model accurately predicts probe T_m in the LightCycler analysis system *(12)*. We compiled and analyzed measured T_m data for matched and mismatched hybridization probes from different laboratories. Therefore, some interlaboratory variation is expected from different reagents and protocols. The temperature transition rates during melting curve acquisition (usually 0.1–0.2 °C/s) are too fast to achieve equilibrium conditions and cause a slight overestimation of the true T_m *(43)*.

Probe sequence choices reflect the demands of mutation detection and are not designed particularly for two-state behavior. Even with these limitations, the predictive accuracy we have achieved underscores the robustness of the parameterization. Systematic errors introduced by fluorescent dyes were negligible in another study *(10)*, and fluorescence resonance energy transfer probes themselves have successfully been used to derive thermodynamic parameters *(44)*. Our findings should be equally applicable to probe and primer oligonucleotides, thereby allowing in silico optimization of primers and probes, saving both on time required for optimization and costs for probe resynthesis *(12, 29, 45–47)*.

An extensive survey of alternative formulas used for

the prediction of perfectly matched oligonucleotide DNA was also performed. The Wallace–Ikatura rule (Eq. 1) is often used as a rough predictor of primer T_m but has limited accuracy, especially for longer oligonucleotides. This rule assumes a salt concentration of 1 mol/L NaCl, which is typical for dot blots and other hybridizations but not PCR. The fact that it is used for PCR applications is more a testament of the robustness of PCR toward different annealing temperatures than evidence for accurate T_m estimates. The same is true for the formula of Wu et al. *(25)*. Many formulas were originally designed to relate measured T_m and GC content of polymer DNA (Eqs. 3–6). The inclusion of additional terms for ionic strength *(6)*, length dependency *(5)*, and GC dependency of ionic strength *(39)* has led to more accurate estimates for polymer DNA. Eqs. 9–12 have been specifically optimized for oligonucleotide T_m estimation by best-fit estimates of our data set. Eq. 10 is recommended as a tradeoff between accuracy and ease of use. Table 2 gives primer T_ms for common primer compositions and PCR conditions. These estimates may not be accurate for certain sequences with a biased N-N composition *(41)*. Furthermore, mismatches are strongly dependent on their N-N bases and require more laborious N-N calculations.

The effect of DMSO on thermal stability of DNA has been investigated before. Our factor of 0.75 °C decrease in T_m per 1% DMSO is similar to previous findings of 0.6 °C per 1% DMSO *(20)*, 0.675 °C per 1% DMSO *(22)*, and 0.5 °C per 1% DMSO *(21)*. These prior studies were performed on polymer DNA, suggesting that DMSO may have a slightly greater effect on oligomer DNA.

Because template priming during PCR is a kinetic process, efficient, specific priming should occur at the primer T_m *(25)*, suggesting that the T_m can be used as the annealing temperature in PCR. Because efficient amplification is dependent on hybridization of both primers, it is rational to use the T_m of the least stable primer. The temperature used for annealing in PCR also depends on the annealing time. For example, allele-specific amplification can be achieved with rapid cycling ("0" s annealing) at a lower annealing temperature than conventional cycling with a longer annealing time *(48)*. Finally, T_m is not just a property of an oligonucleotide, but a property of an oligonucleotide under specific conditions and at a given concentration.

Table 2. Standard primer T_ms (°C) calculated for different lengths, Mg^{2+} concentration, and GC content at 0.8 mM dNTPs and 50 mM monovalent ion concentration without mismatches and with no addition of cosolvents.[a]

		T_m, °C			
%GC	Mg^{2+}, mM	18 bp	20 bp	22 bp	24 bp
40	1.0	52.6	55.6	58.0	60.0
	1.5	54.5	57.5	59.9	61.9
	2.0	55.5	58.4	60.8	62.8
	2.5	56.2	59.1	61.5	63.5
	3.0	56.7	59.6	62.0	64.0
50	1.0	56.7	59.7	62.1	64.1
	1.5	58.6	61.6	64.0	66.0
	2.0	59.6	62.5	64.9	66.9
	2.5	60.3	63.2	65.6	67.6
	3.0	60.8	63.7	66.1	68.1
60	1.0	60.8	63.8	66.2	68.2
	1.5	62.7	65.7	68.1	70.1
	2.0	63.7	66.6	69.0	71.0
	2.5	64.4	67.3	69.7	71.7
	3.0	64.9	67.8	70.2	72.2

[a] Eqs. 7 and 10 (see *Material and Methods*) were used for the calculations.

In conclusion, we have developed a robust model for the effects of Mg^{2+}, DMSO, and dNTPs on oligonucleotide T_m under common PCR buffer conditions. This enables reliable T_m predictions and in silico primer and probe optimization using thermodynamic N-N calculations. We performed an extensive evaluation of different equations advocated for PCR primer T_m prediction. These formulas have been parameterized to accommodate for standard PCR conditions and are now useful for rapid calculation of the T_m of perfectly matched oligonucleotides. It is rational to use the T_m of the least stable PCR primer as the annealing temperature in PCR.

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