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Monovalent and divalent salt correction algorithms for T_m prediction—recommendations for Primer3 usage

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Abstract

Primer3 is a widely used program for selection of oligonucleotide primers for PCR. The websites used for implementation of Primer3 have recently been updated. PCR requires Mg^{2+}, which has a significant dsDNA stabilizing effect that must be taken into account when designing PCR primers. The data sets and formulas used to correct for salt concentrations have been updated in Primer3 to give better prediction of melting temperature (T_m). The liberal combination of different formulas for monovalent and divalent salt correction can lead to different results, depending on the formula chosen by the user. Using published T_m for 475 different oligonucleotides, it is shown that the combination of the implemented conversion of divalent to monovalent cation concentration works well with one salt correction formula but not with an alternative one. Use of a more recently described alternative formula would lead to equally good T_m predictions if divalent cations are present. The proper selection of compatible primer pairs depends on the choice of a good combination of salt correction formulas. Currently the SantaLucia salt correction formula should be used if Mg^{2+} is present. The alternative formula should be updated to its recent form for future releases.

The oligonucleotide melting temperature (T_m) is a crucial parameter for the design of PCR primers and diagnostic genotyping probes. The most widely used primer design program is Primer3 [1]. This software uses thermodynamic calculations to estimate primer annealing temperatures that ensure similar binding properties of the PCR primer pair. Thermodynamic nearest-neighbor (NN) calculations give reliable half-denaturation temperature (T_m) predictions if salt concentrations are properly accounted for [2, 3]. Standardized (1 mol/l NaCl, pH 7.0) enthalpy (ΔH°) and entropy (ΔS°) values for all NN pairs are known and a consensus data set is published [4]. The salt effect is entropic and a correction term has been derived experimentally:

$$\Delta S^\circ[\mathrm{MVC}] = \Delta S^\circ[1\ \mathrm{mol/l\ NaCl}] \times 0.368(N-1) \times \ln(\mathrm{MVC}), \quad (1)$$

where N = length (bp), MVC = monovalent cation concentration e.g. Na^+ (mol/l).

The PCR reaction, a basic technique for molecular diagnostic tests requires Mg^{2+}, which has a dsDNA stabilizing effect that is significantly higher than that of MVC. Several formulas have been

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proposed to account for the entropic effect of Mg^{2+}. A previous study used 475 matched and mismatched dye-labeled oligonucleotides to correlate observed and NN predicted T_m. A regression of $y = 1.00 \times -0.29$, $r^2 = 0.90$, was observed [2]. The empirical factor to convert Mg^{2+} to MVC (e.g. Na^+) concentration was:

$$[\text{MVC}] = 3.795 \times \sqrt{[Mg^{2+}]}\ \text{(concentration in mol/l)}. \quad (2)$$

The calculated Mg^{2+} equivalent MVC is added to the MVC concentration in Equation (1). This value is valid for Mg^{2+} concentrations <8 mmol/l [2], a concentration range seldom exceeded in PCR.

The website implementation of Primer3 (e.g.: http://frodo.wi.mit.edu/primer3 — also used at the NCBI-BLAST homepage: http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was updated [5] to include the improved thermodynamic data and the salt correction according to SantaLucia [4] as well as the alternative salt correction formula developed by Owczarzy's group [6]:

$$\frac{1}{T_m} = \frac{1}{T_m(1MNa^+)} + (4.29 f_{GC} - 3.95) \times 10^{-5} \ln[\text{MVC}] + 9.40 \times 10^{-6} (\ln[\text{MVC}])^2 \quad (3)$$

together with correction for divalent cation concentration [2] (Figure 1). The latter is triggered by setting the Mg^{2+} to any value >0.

In a 2008 publication [7], an additional algorithm for T_m correction was described that treats magnesium and MVC concentrations differently. Three different equations based on categorized multivariate regression analyses are proposed. The categories are selected by a decision tree considering the term $\sqrt{[Mg^{2+}]}/[Na^+]$ (concentrations in mol/l) as a branching parameter, accounting for the observation that higher concentrations of divalent cations dominate over MVCs in their effects on dsDNA stabilization [8]. For salt concentrations used in PCR this ratio falls into the category $(0.22 \leq \sqrt{[Mg^{2+}]}/[Na^+] < 6.0)$. The MVC, $[Mg^{2+}]$ and fractional GC content are then input into a complex multivariate equation with seven simultaneously fit coefficients, modified to include five more coefficients for the MVC terms. The correction is not solely entropic [as Equation (1)], but directly modifies T_m.

The widely used Primer3 software chooses different primer pairs for identical sequences when different salt correction options are selected. This happens when divalent cation concentration is greater than zero.

These observations were studied in more detail by comparing the results of: (i) combining the Mg^{2+} correction [2] with the SantaLucia algorithm [4], (ii) combining the Mg^{2+} correction [2] with the Owczarzy 2006 algorithm [6] and (iii) using the decision tree-based Owczarzy 2008 algorithm [7], all using our naturalistic data set [2] containing real time PCR oligonucleotide melting temperatures.

The results are shown in Figure 2 as Bland–Altman difference plots. The left panel shows combination (i) as previously published [2] for comparison (mean deviation 0.0°C; SD 2.2°C). The middle panel corresponds to the combination (ii) where a substantial offset of 2.7°C and an increased dispersion of the T_m prediction is observed (SD 2.6°C), whereas use of the decision tree algorithm (method 3) works well using the multi-laboratory, naturalistic data set [2] (mean deviation 0.24°C; SD 2.4°C).

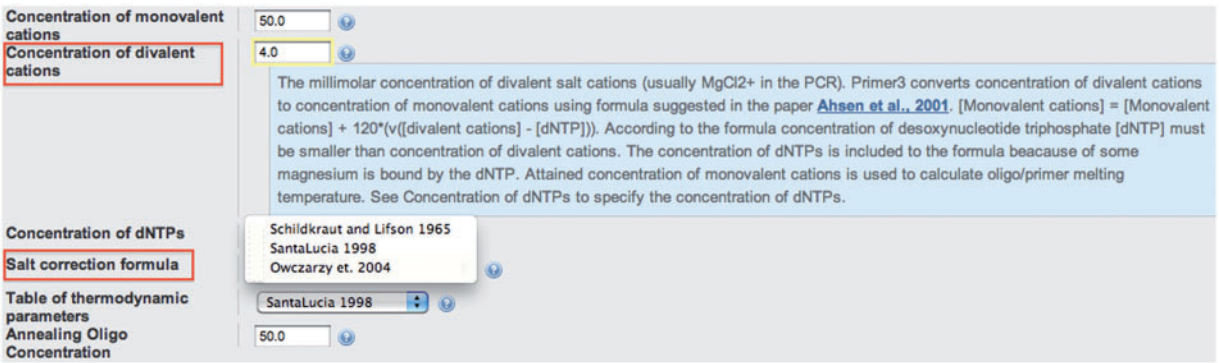

Figure 1: Primer3 implementation in BLAST (NCBI) as an example, showing the options for treatment of salt concentration for T_m calculations. If the value of divalent cations is ›0, Equation (2) is used to convert Mg^{2+} to monovalent cation equivalent concentrations.

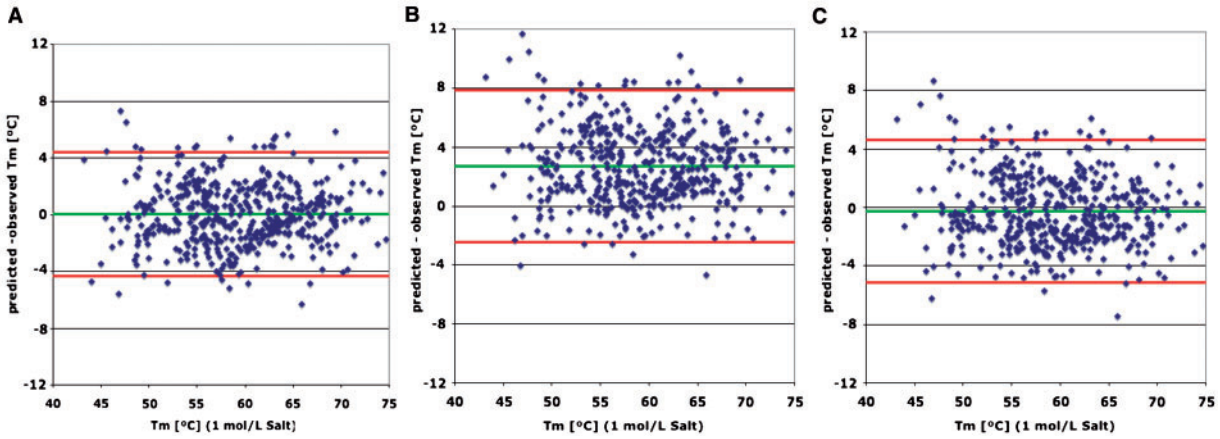

Figure 2: Bland–Altman plots of observed versus predicted oligonucleotide melting temperatures using different formulas: (**A**) SantaLucia 1998 in combination with Mg^{2+} correction according to von Ahsen *et al*. [2]; (**B**) Owczarzy 2004 in combination with Mg^{2+} correction according to von Ahsen *et al*. [2]; (**C**) Owczarzy 2008 with decision tree-based Mg^{2+} correction. The *x*-axis shows the predicted T_m without salt correction (at I mol/l Na^+).

The main uses of T_m prediction in diagnostics are for assay design for genotyping probes and primer selection for use in a variety of settings in different laboratories. The simple thermodynamic approach [4] [Equation (1)] has been further validated with an independent data set using loci spanning probes [9]. Accuracy and precision of the predicted T_ms [9] was in the same range as shown herein (Figure 2) and also resembling earlier results [2]. Two software programs that use thermodynamic salt corrections [2, 4] provided better predictions [9] within the Mg^{2+} range typically used for PCR (1.5–4.0 mM) than shown at high (>5 mM) Mg^{2+} concentrations [7]. This effect of high Mg^{2+} is known [8] and caution was previously advised when [Mg^{2+}] exceeds 8 mM [3]. The result of using the more complex algorithms [7] is better T_m prediction when divalent cation concentrations are outside the typical PCR range. Within the PCR cation range, both the simple thermodynamic correction [2, 4] and the Owczarzy's *et al*. decision tree algorithm [7] achieve a good fit with observed T_m. Software for PCR primer design depends on reliable T_m prediction. Compounds such as Mg^{2+}, dimethyl sulfoxide (DMSO) and SYBR Green I independently influence T_m and should be taken into account [2, 5, 7]. We have shown that not every combination provides satisfactory Mg^{2+} correction.

The update of Primer3 with better thermodynamic data and the use of algorithms for salt correction was an important step towards better prediction of primer stability. Within typical PCR concentrations of Mg^{2+} the use of the Owczarzy's 2004 algorithm should be avoided. The SantaLucia algorithm with Mg^{2+} correction gives more accurate results. This independent validation of Owczarzy's 2008 algorithms should justify an implementation of this updated algorithm in Primer3 to provide more accurate results for high Mg^{2+} concentrations. Currently, the SantaLucia formula [4] performs well and should be chosen if bivalent cations are present.

Key Points

- Care must be taken when using the combinations of salt correction formulas currently included in Primer 3.
- Using different salt corrections result in different primer selections.
- In the typical PCR range of mono- and divalent cations, the SantaLucia correction gives best results.
- For high concentrations of divalent cations, the 2008 correction from Owczarzy's group could be implemented.

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