APPENDIX

E

TM CALCULATIONS IN VECTOR NTI

General Information

Vector NTI calculates and reports two different melting temperatures for DNA/RNA oligonucleotides, Thermodynamic Tm (Therm. Tm) and %GC Tm (Figure E.1):

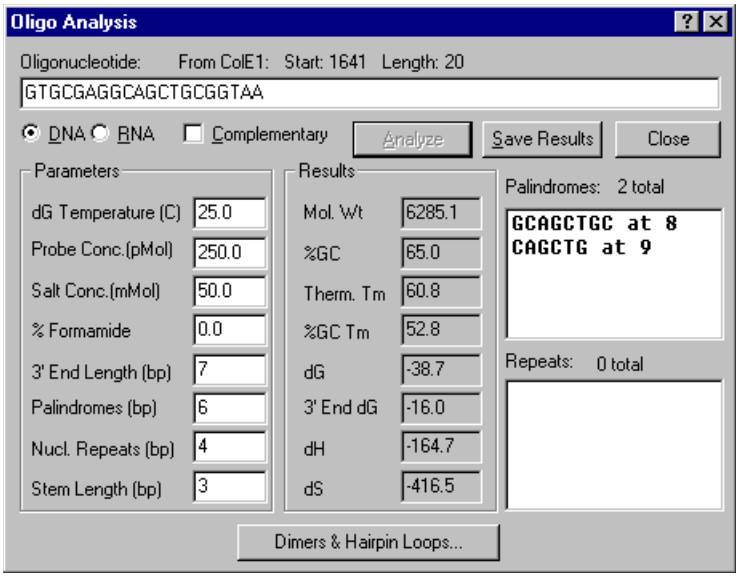

Figure E.1 Oligo Analysis dialog box with calculated Tm values

Usefulness of Thermodynamic Tm Versus %GC Tm

Vector NTI reports both the Thermodynamic and %GC Tm values, regardless of the length of the oligo (oligos are limited to 1000 characters maximum length in Vector NTI 9). However, generally only one of the reported TM values should be considered useful, depending on the length of the oligo as follows:

- Therm. Tm – useful for oligos that are greater than about 7-10 residues and less than about 35 residues long
- %GC Tm – useful for oligos greater than about 35 residues long

Note: For oligos that are 7-10 residues or shorter (cutoff length depends on the base content of the particular oligo being analyzed), Vector NTI reports a Therm. Tm value of zero.

Effects of Primer (Probe) and Salt Concentration on Tm Calculations

Tm calculations are highly dependent on primer and salt concentrations; varying these concentrations can greatly affect the Tm for any given primer. Therefore, it is important that you adjust the primer and salt concentrations appropriately so that accurate Tm values are generated.

Note: In Vector NTI, the default parameters for primer and salt concentration are 250 pM and 50 mM, respectively, for calculating Tm values. Other Tm calculators commonly use a default probe concentration of 50 nM. Because of this, Vector NTI default parameter Tm values may not correspond to the default Tm values calculated using other programs. Before comparing Vector NTI Tm values with those generated by other Tm calculators, make sure that the parameters are adjusted appropriately.

%GC Tm Calculation

The %GC Tm calculation1 does not rely on the thermodynamic properties of the oligo (i.e. dHo, dSo and dG° values). The formula for %GC Tm is as follows:

Tm = 81.5 + 16.6(log[Na+]) + 0.41(%GC) – 675/probe length

Note: [Na+] is in molar units.

Example: For oligo GTGCGAGGCAGCTGCGGTAA at 50mM salt:

Tm = 81.5 + 16.6(log(0.05)) + 0.41(65) – 675/20

= 81.5 + 16.6(–1.30) + 26.65 – 33.75

= 81.5 – 21.58 + 26.65 – 33.75

= 52.82 °C

Thermodynamic Tm Calculation

The Thermodynamic Tm calculation is based on the Nearest Neighbor theory of DNA/RNA duplex stability. Briefly, this theory states that the overall duplex stability (and, hence, the melting temperature) of an oligonucleotide can be predicted from the primary sequence based on the relative stability and temperature-dependent behavior of every dinucleotide pair in the oligo2. In practice, enthalpy (dH°) and free energy (dG°) values for each of the 10 possible Watson-Crick DNA pairwise interactions are used to calculate pairwise entropy (dS°) values via the following standard equation:

dG° = dH° - TdS°

Note: T is temperature in °K.

The pairwise dH° and dS° values are then summed to calculate overall values for the oligo under consideration. The overall values are used in the following formula3 to calculate the Thermodynamic Tm:

Therm. Tm = dH°- 273.15 + 16.6(log[Na+]) dS° + $dS_o°$ + R(ln(c/4))

Notes:

- $dS_o°$ is the entropy associated with helix initiation (-10.8 cal/mol per °K).
- R is the Universal Gas Constant (1.987 cal/mol per °K).
- c is the concentration of the probe, in molar units.
- The factor –273.15 corrects for absolute temperature so that the final Tm is in °C.

The pairwise dH° and dS° values for DNA used in VNTI are taken from reference 2. Those values, along with the corresponding dG° values at 25°C, appear in the following table (Table E.1):

Interaction	*dH° kcal/mol*	*dS° cal/mol per °K*	*dG° kcal/mol*
AA/TT	-9.1	-24.0	-1.9
AT/TA	-8.6	-23.9	-1.5
TA/AT	-6.0	-16.9	-1.0
CA/GT	-5.8	-12.9	-2.0
GT/CA	-6.5	-17.3	-1.3
CT/GA	-7.8	-20.8	-1.6
GA/CT	-5.6	-13.5	-1.6
CG/GC	-11.9	-27.8	-3.6
GC/CG	-11.1	-26.7	-3.1
GG/CC	-11.0	-26.6	-3.1
XX/XX	-6.0	-16.9	-1.0

Table E.1 DNA Nearest Neighbor thermodynamics

Notes:

- All values refer to the disruption of a duplex at 1 M NaCl, 25°C and pH 7.
- The units for dH° and dG° are kcal/mol of interaction, whereas those for dS° are cal/°K per mol of interaction.

Example: The oligo 5'-GTGCGAGGCAGCTGCGGTAA-3' is parsed as follows (Figure E.2):

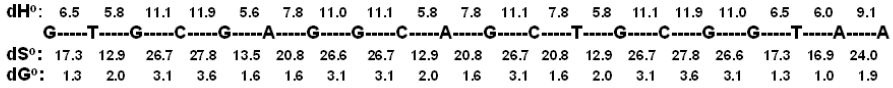

Figure E.2 Parsed Oligo

Total dH° = -164.7 kcal/mol (Figure E.2)

The total dS° reported by VNTI (Figure E.2) is the sum of the pairwise values above and the entropy associated with helix initiation ($dS_o°$). Thus, for the example oligo above:

Total dS° = -405.7 + (-10.8) = -416.5 cal/mol per °K

The total dG° (Figure E.2) is the sum of the pairwise dG° values for the oligo plus a helix initiation free energy term ($dG_o°$) that is added to better reflect experimentally determined free energy values for tested oligos. The value of the helix initiation free energy term ($dG_o°$) depends on the base composition of the oligo2 as follows:

- +5.0 kcal/mol for oligos containing any G-C base pairs
- +6.0 kcal/mol for oligos composed exclusively of A-T base pairs

Therefore, for the example oligo:

Total dGo = -43.7 kcal/mol (sum of the pairwise dGo values) + 5 kcal/mol (free energy term)

= -38.7 kcal/mol

The 3' End dG° is calculated using the number of 3' pairwise dG° values specified in the 3' End Length (bp) box, and is not further adjusted (Figure E.3).

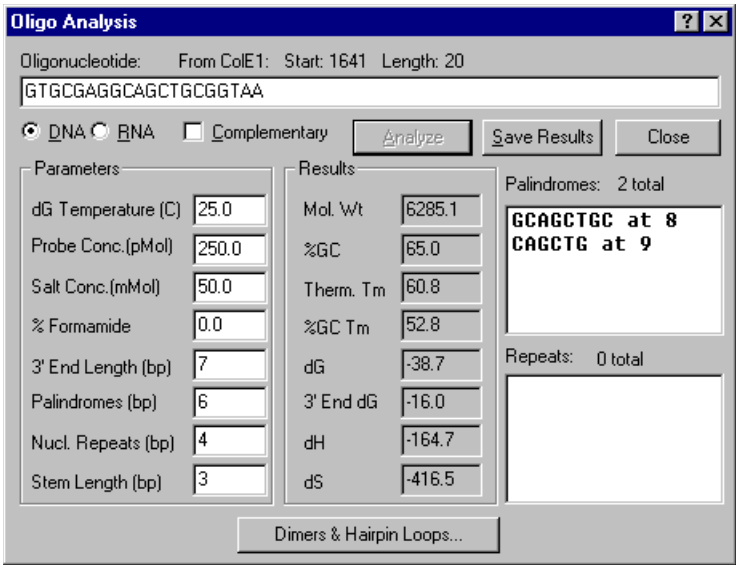

Figure E.3 Oligo Analysis dialog box showing 3' End length and calculated dG value

Using Vector NTI's default probe and salt concentrations (250 pM and 50 mM, respectively) and the values for dH° and dS° calculated above, Therm. Tm can be calculated as follows:

$$\mathrm{ThermTm} = \frac{\mathrm{dH}^\circ}{\mathrm{dS}^\circ + \mathrm{dS_o}^\circ + \mathrm{R}\left(\ln\left(\frac{c}{4}\right)\right)} - 273.15 + 16.6(\mathrm{Log}(\mathrm{Na}))$$

$$\ldots = \frac{-164.7}{-0.4165 + (0.001987)\left(\ln\left(\left(\frac{250 \times 10^{-12}}{4}\right)\right)\right)} - 273.15 + 16.6(\mathrm{Log}(0.05))$$

$$\ldots = \frac{-164.7}{-0.4165 + (0.001987)(-23.49)} - 273.15 + 16.6(-1.301)$$

$$\ldots = \frac{-164.7}{(-0.4165) - 0.04667} + (-273.15) - 21.60$$

$$\ldots = \frac{-164.7}{-4632} - 294.75$$

$$\ldots = 355.57 - 294.75$$

$$\ldots = 60.82^\circ\mathrm{C}$$

Vector NTI adjusts the %GC and Therm. Tm values accordingly, based on the input formamide concentration (Figure E.4):

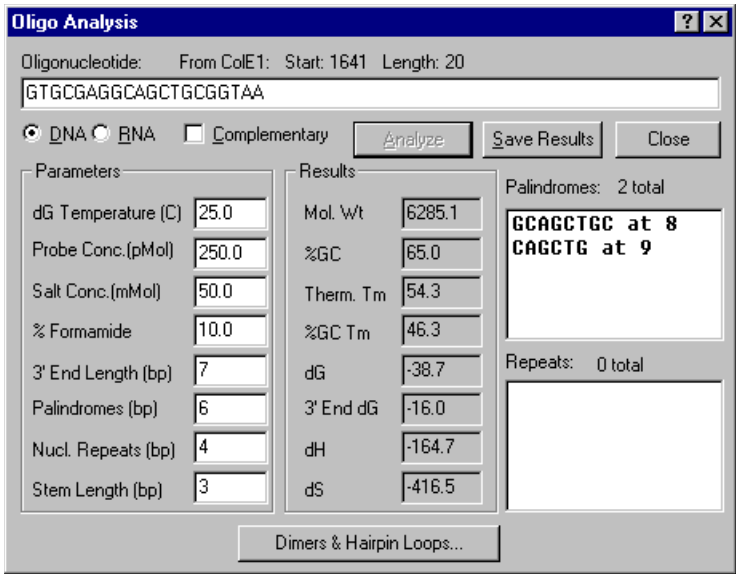

Figure E.4 Oligo Analysis dialog box showing effects of formamide on Tm

Oligos Containing IUB Ambiguity Characters

Vector NTI can analyze oligos that contain IUB nucleotide ambiguity characters (i.e. R, Y, W, S, M, K, B, D, H, V and N – See Appendix C). In the case of ambiguity characters, Vector NTI uses average pairwise dH° and dS° values for calculating the Tm.

For example, for the dinucleotide pair CB, Vector NTI averages the CC, CG and CT thermodynamic parameters (Table E.1) to obtain average pairwise dH° and dS° values for CB. It then sums the average pairwise thermodynamic parameters and calculates the Therm. Tm values according to the equation described above (see *Thermodynamic Tm Calculation*, page 676).

In the case of %GC Tm, Vector NTI applies the appropriate %GC contribution represented by each ambiguity symbol to the standard %GC Tm formula (see *%GC Tm Calculation*, page 676). For example, a B ambiguity symbol contributes only two-thirds the amount of a G or C residue to overall GC content.

RNA Oligos

RNA oligos use a different set of pairwise thermodynamic values than DNA oligos5. Pairwise thermodynamic values for RNA are summarized in the following table (Table E.2):

Interaction	***dH° kcal/mol***	***dS° cal/mol/K***	***dG° kcal/mol***
AA/UU	-6.6	-18.4	-1.1
AU/UA	-5.7	-15.5	-1.1
UA/AU	-8.1	-22.6	-1.4
CA/GU	-10.5	-27.8	-2.2
GU/CA	-10.2	-26.2	-2.4
CU/GA	-7.6	-19.2	-1.9

Table E.2 RNA Nearest Neighbor thermodynamics

GA/CU	-13.3	-35.5	-2.7
CG/GC	-8.0	-19.4	-2.2
GC/CG	-14.2	-34.9	-3.8
GG/CC	-12.2	-29.7	-3.3
XX/XX	-6.0	-16.9	-1.0

Table E.2 RNA Nearest Neighbor thermodynamics (continued)

Notes:

- All values refer to the disruption of a duplex at 1 M NaCl, 25°C, and pH 7.
- The units for dH° and dG° are kcal/mol of interaction, whereas those for dS° are cal/°K per mol of interaction.
- The dS° value for RNA oligos is adjusted by –10.8 cal/°K per mol to reflect the entropy associated with helix initiation, as it is for DNA oligos.
- The dG° value is adjusted by +3.4 kcal/mol to account for helix initiation. Note that this adjustment is NOT dependent on the base composition of the RNA oligo as it is for DNA oligos (see see *Thermodynamic Tm Calculation*, page 676).

Primer/Probe Tm, TaOpt and Similarity Calculations

For oligos designed using Vector NTI's PCR Primers, Sequencing Primers and Hybridization Probes features, the oligo Tm, product TaOpt and oligo percent binding similarity are reported in the Text Pane of the Molecule Viewing window.

Primer/Probe Tm Values

Tms for designed primers/probes are reported is as follows in Vector NTI:

- Therm. Tm is reported if the oligo is less than or equal to 35 residues
- %GC Tm is reported if the oligo is 36 residues or greater

For PCR products, Vector NTI reports the %GC; the assumption being that the majority of PCR products are larger than 35 residues.

TaOpt Values

The PCR product TaOpt (optimal annealing temperature for amplification of the fragment) in °C is calculated using the following formula3:

TaOPT = ((0.3)Tmprimer + (0.7)Tmproduct – 14.9) °C

Notes:

- Tmprimer is the Tm of the less stable primer of the pair
- Tmproduct is the Tm of the PCR product

Primer/Probe Similarity Values

When designing PCR, sequencing or hybridization primers, VNTI reports the overall similarity in percent of an oligo to its binding site based on the oligo's nucleotide composition. For oligos containing IUB ambiguity symbols, three similarity values are reported:

- Minimum Similarity
- Maximum Similarity
- Average Similarity

For Minimum Similarity, all ambiguities are classed as complete mismatches (i.e., they are assigned values of 0 at each position). For example, a 20mer containing 2 Rs and 2 Ns has a Minimum Similarity of 80%.

For Maximum similarity, all ambiguities are considered identical to their cognate nucleotides (i.e. they are assigned values of 1 at each position), so the Maximum Similarity is always 100%.

For Average similarity, Vector NTI weights each ambiguous nucleotide depending on whether it represents 2, 3, or 4 possible nucleotides. For example, Ns have a score of 0.25, Rs of 0.5, Bs of 0.33, etc. Therefore, for the 20mer described above, the Average Similarity is 85%.

References

- 1 Baldino Jr., F., Chesselet, M.F., and Lewis M.E. (1989) High-resolution in situ hybridization histochemistry, Methods Enzymol. 168:761-777.
- 2 Breslauer, K.,J., Frank, R., Blocker, H., and Marky, L.A. (1986) Predicting DNA duplex stability from the base sequence, Proc. Natl. Acad. Sci. USA 83:3746-3750.
- 3 Rychlik, W., Spencer, W.J., and Rhoads, R.E. (1990) Optimization of the annealing temperature for DNA amplification in vitro, Nucleic Acids Res. 18:6409-6412.
- 4 Sugimoto, N., Nakano, S., Yoneyama, M., and Honda, K. (1996) Improved thermodynamic parameters and helix initiation factor to predict stability of DNA duplexes, Nucleic Acids Res. 24:4501-4505.
- 5 Freier, S.M., Kierzek, R., Jaeger, J.A., Sugimoto, N., Caruthers, M.H., Nielson, T., and Turner, D.H. (1986) Improved free-energy parameters for predictions of RNA duplex stability, Proc. Natl. Acad. Sci. USA 83:9373-9377.