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Sequence analysis

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uMELT: prediction of high-resolution melting curves and dynamic melting profiles of PCR products in a rich web application

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ABSTRACT

Summary: uMelt[SM] is a flexible web-based tool for predicting DNA melting curves and denaturation profiles of PCR products. The user defines an amplicon sequence and chooses a set of thermodynamic and experimental parameters that include nearest neighbor stacking energies, loop entropy effects, cation (monovalent and Mg^{++}) concentrations and a temperature range. Using an accelerated partition function algorithm along with chosen parameter values, uMelt interactively calculates and visualizes the mean helicity and the dissociation probability at each sequence position at temperatures within the temperature range. Predicted curves display the mean helicity as a function of temperature or as derivative plots. Predicted profiles display stability as a function of sequence position either as 50% helicity temperatures or as the helicity probability at specific temperatures. The loss of helicity associated with increasing temperature may be viewed dynamically to visualize domain formation within the molecule. Results from fluorescent high-resolution melting experiments match the number of predicted melting domains and their relative temperatures. However, the absolute melting temperatures vary with the selected thermodynamic parameters and current libraries do not account for the rapid melting rates and helix stabilizing dyes used in fluorescent melting experiments. uMelt provides a convenient platform for simulation and design of high-resolution melting assays.

Availability and implementation: The application was developed in Actionscript and can be found online at http://www.dna.utah.edu/umelt/umelt.html. Adobe Flash is required to run in all browsers.

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1 INTRODUCTION

DNA melting analysis with fluorescent dyes is a new method to conveniently scan for genetic variation without sequencing (Erali and Wittwer, 2010; Montgomery *et al*., 2010). The ability to predict the shape and position of melting curves is essential for assay design and optimization. uMelt builds on existing models of DNA melting using nearest neighbor thermodynamics and recursive calculations using statistical mechanics (Blake *et al*., 1999; Crothers, 1968; Gotoh, 1983; Markham and Zuker, 2005; Poland, 1974; Steger, 1994; Tostesen, 2003; Zimm, 1960) to predict fluorescent melting analysis of PCR products in a rich web application.

2 METHODS

Temperature-dependent stability factors for each base tetrad are used to calculate probabilities of helicity for each position at each temperature. Averaging over the entire sequence gives the predicted helicity at each temperature. Stability factors of the 10 possible nearest neighbor tetrads are calculated using Equation (1).

$$\text{Stability factor} = \frac{e^{-(\Delta H - T\Delta S)}}{RT} \quad (1)$$

Enthalpy (ΔH) and entropy (ΔS) parameters are taken from one of several thermodynamic libraries, T is the absolute temperature and R is Boltzmann's constant. The entropy parameters are modified for monovalent cation (Blake and Delcourt, 1998) and Mg^{++} (Nakano *et al*., 1999; von Ahsen *et al*., 2001) concentrations.

Tetrad stability factors are used in the two-phase recursive calculation of vectors whose entries contain partition functions that relate relative probabilities of helicity versus random coiling along segments of the molecule of increasing lengths. The algorithm, described in Tostesen *et al*. (2003), accelerates both the exact $O(N^3)$ and approximate $O(N^2)$ method described previously (Yeramian *et al*., 1990) by one order (O) in the oligo length, N. In uMelt, the resulting exact $O(N^2)$ algorithm is implemented. Loop entropy requires modification of associated relative probabilities by a factor with power law dependence in loop length (Blossey and Carlon, 2003; SantaLucia, 1998; Sugimoto *et al*., 1995). As in Poland (1974), by using these formulas, the probability that the base pair is helical at any temperature can be calculated without explicit reference to the 2^N microstate weights for the molecule, a computationally intractable task. Finally, the overall helicity at a given temperature is calculated as the average helicity probability of all base pairs. This total helicity across the temperature range predicts the melting curve.

Sequences are defined in a text box that allows quick editing and modification to compare different sequences (supplementary Fig. 1a). The user also selects a published thermodynamic library of nearest neighbor parameters (Blake and Delcourt, 1998; Breslauer *et al*., 1986; Huguet *et al*., 2010; SantaLucia, 1998; Sugimoto *et al*., 1995) monovalent salt and magnesium concentrations, loop parameters, the temperature range and the temperature resolution. Computed values are visualized in four charts with the ability to hover on points to see individual values. Data can be downloaded as a text file containing all data for the melting curve, derivative curve and melting profiles.

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3 RESULTS AND DISCUSSION

uMelt provides a rapid web application to predict melting curves of PCR products. Fluorescent DNA melting curves after real-time PCR were introduced in 1997 (Ririe *et al*., 1997) as an alternative to gel electrophoresis to assess product purity. Although a one-to-one correspondence between the number of peaks and the number of products is often assumed, this is clearly not true for many sequences where local guanosine-cytosire (GC) content differences produce multiple melting domains.

Melting curves are displayed as predicted helicity versus temperature (Supplementary Fig. 1A, top left). Derivative plots are calculated by taking the negative derivative of helicity with respect to temperature (Supplementary Fig. 1B, top left). Helicity across physical sequence position (the 'melting profile') is plotted as the temperature where 50% helicity is attained (Supplementary Figure 1B, bottom right). Alternatively, the probability of helicity at a given temperature ('dynamic profile') can be displayed across the sequence position at various temperatures (Supplementary Fig. 1A, bottom right).

Prediction of polymer domain melting is more computationally intensive than simple two-state oligomer melting. Prior methods have focused on correlation to absorbance measurements (Blake *et al*., 1999; Markham and Zuker, 2005; Steger, 1994; Tostesen *et al*., 2005) instead of the more convenient and clinical useful fluorescence measurements (Erali and Wittwer, 2010; Montgomery *et al*., 2010). uMelt also provides adjustment for Mg^{++} ions and displays dynamic melting profiles to visualize melting according to sequence position, options not provided by other resources (Supplementary Table 1). Dissociation probabilities at each position are calculated and displayed visually to simulate physical melting of the helix. Loop formation and fraying sequence ends can be observed throughout the temperature range. Calculation time depends on sequence length (Supplementary Fig. 2).

In Supplementary Figure 3, an experimental melting curve is compared with predicted curves using different thermodynamic sets. The three domains of the melting curve and the spacing between domains are accurately reflected in the predictions. However, the predicted curves are located variably above and below the experimental melting curve depending on the thermodynamic parameters used. The best match is obtained with the unified parameter set (SantaLucia, 1998). The fluorescence measurements typical of high-resolution melting introduce some new variables, including helix stabilization from the fluorescent dye and high-melting rates, both of which may increase apparent melting temperatures. Furthermore, the assumed linearity between helicity and fluorescence is unproven. As previously noted (Hill *et al*., 2010), lower melting domains appear attenuated compared with predictions, possibly the result of a lower fluorescent yield with AT base pairs. If fluorescence varies linearly with GC content, intensity corrections could be easily incorporated. Even with current limitations, uMelt provides a convenient tool for design and optimization of high-resolution melting experiments by predicting PCR product melting curves.

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