uAnalyze: Web-Based High-Resolution DNA Melting Analysis with Comparison to Thermodynamic Predictions

Zachary L. Dwight, Robert Palais, and Carl T. Wittwer

Abstract—uAnalyze^{SM} is a web-based tool for analyzing high-resolution melting data of PCR products. PCR product sequence is input by the user and recursive nearest neighbor thermodynamic calculations used to predict a melting curve similar to uMELT (http://www.dna.utah.edu/umelt/umelt.html). Unprocessed melting data are input directly from LightScanner-96, LS32, or HR-1 data files or via a generic format for other instruments. A fluorescence discriminator identifies low intensity samples to prevent analysis of data that cannot be adequately normalized. Temperature regions that define fluorescence background are initialized by prediction and optionally adjusted by the user. Background is removed either as an exponential or by linear baseline extrapolation. The precision or, "curve spread," of experimental melting curves is quantified as the average of the maximum helicity difference of all curve pairs. Melting curve accuracy is quantified as the area or "2D offset" between the average experimental and predicted melting curves. Optional temperature overlay (temperature shifting) is provided to focus on curve shape. Using 14 amplicons of CYBB, the mean $+/-$ standard deviation of the difference between experimental and predicted fluorescence at 50 percent helicity was $-0.04 +/- 0.48°C$. uAnalyze requires Flash, is not browser specific and can be accessed at http://www.dna.utah.edu/uv/uanalyze.html.

Index Terms—Melting curve analysis, high-resolution melting, biology and genetics, modeling and prediction, software

1 Introduction

High-resolution PCR product melting analysis is a simple and powerful method with many applications in molecular biology [1]. Melting curves with multiple domains can be predicted by recursive nearest neighbor thermodynamics as implemented in POLAND [2], Stitch-profiles [3], MeltSim [4], DINAMelt [5], and uMELT^{SM} [6] and is beneficial to amplicon design and assessment of experimental results. However, before experimental fluorescent melting data are compared to predicted curves, background fluorescence must be removed and normalization performed. The background fluorescence is produced by interaction of the dye with primers and approximates an exponential [7], [8]. Further processing of experimental curves by overlay (temperature shifting) is often performed to focus on curve shape because absolute temperatures can be affected by reaction components such as the fluorescent dye, sample position, and ionic strength [9]. Direct visual comparison requires that both predicted and processed experimental curves are plotted on the same graph. uAnalyze processes experimental melting curves for comparison to predicted helicity in a quick, flexible web interface (Fig. 1). Numerical metrics are provided to assess the precision of experimental replicates and the accuracy of theoretical prediction to the experimental melting curves.

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2 Methods

2.1 Nearest Neighbor Thermodynamics

Recursive nearest neighbor calculations are used for *in silico* prediction of melting curves to account for multiple domains. The theoretical melting predictions displayed in uAnalyze are calculated as described previously [6] with unified nearest neighbor parameters [10] and default loop entropy effects [11] to predict helicity as a function of temperature.

2.2 Amplification by PCR and Product Melting

PCR was performed in 10 μl reactions containing 50 mM Tris (pH 8.3), 500 μg/ml bovine serum albumin, 200 μM of each deoxynucleotide triphosphate, 0.4 units KlenTaq polymerase (Ab Peptides), 1 X LCGreen Plus (Biofire Diagnostics), 0.5 μM each PCR primer and 50 ng human genomic DNA. Fourteen human gene segments covering the 13 exons of *CYBB* were amplified and melted as previously described [12] using the above PCR reagents, 3 mM $MgCl_2$, 8.8 ng/uL anti-Taq antibody (Clontech), and certain primer sequences modified as detailed in Supplementary Table 1, which can be found on the Computer Society Digital Library at http://doi.ieeecomputersociety.org/10.1109/TCBB.2012.112). *ACADM* exon 2 (primers GATTATCAGTAGTCTCTTATCTGATTAATGTTTAACTTATAAATTT and TTTAAAGTCAAAAGATAGAACCGAAC) and exon 6 (primers AATTATAGCATCTCTGAATTTACATATCC and

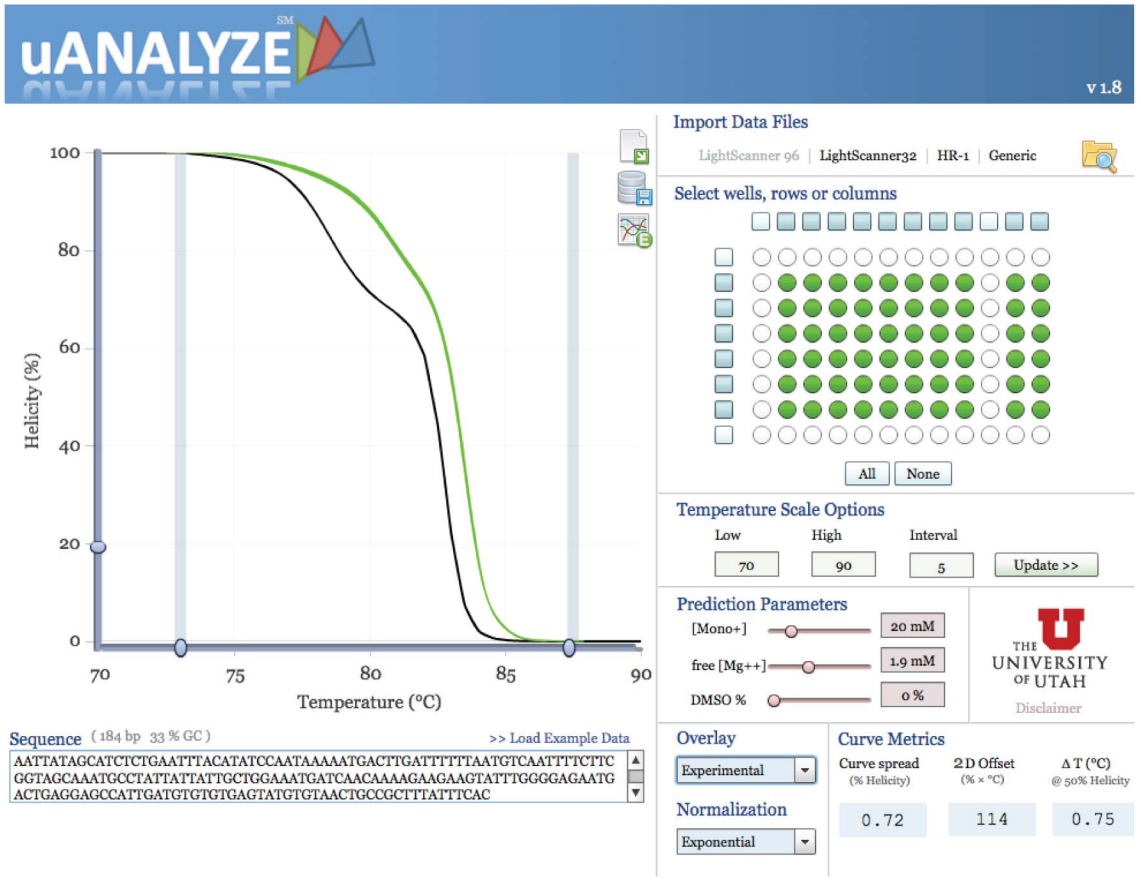

Fig. 1. uAnalyze: a web application to compare experimental high-resolution melting data to thermodynamic predictions. The DNA sequence (exon 6, *ACADM)* is pasted into the interface and experimental data files are imported with a file upload dialogue. Samples are selected by their instrument position and their normalized melting curves (green), along with the predicted melting curve (black) are shown. A helicity slider on the Y-axis prevents normalization of low fluorescence samples. Temperature sliders on the X-axis select background regions for normalization. Adjustable options include the interval and range of the temperature axis, monovalent cation, free Mg^{++} and DMSO concentrations, and selections for curve overlay and normalization method. Data metrics (curve spread and 2D offset) quantify precision and accuracy. Icons allow downloading the graph image (.png) and normalized data (.txt). Cursors were set at $73°C$ and $87.5°C$.

GTGAAATAAAGCGGCAGTT) were amplified as above except with 2.7 mM $MgCl_2$ and 64 ng/ml anti-Taq antibody (eEnzyme) with denaturation at $95°C$ for 15 s, annealing at $64°C$ for 15 s, and extension at $72°C$ for 30 s for 40 cycles. Melting for both *CYBB* and *ACADM* amplicons used the LightScanner (Biofire Diagnostics) under default conditions.

Small amplicon genotyping and melting of *MTHFR* $c.1286A > C$ (HGVS), also known as $1298A > C$ (legacy), was performed on the LS32 (Biofire Diagnostics). Rapid cycle PCR [13] was performed using the above reagents with primers GGAGGAGCTGACCAGTGAAG and GGTAAA-GAACGAAGACTTCAAAGACAC, 2 mM $MgCl_2$ and no antiTaq antibody for 40 cycles of denaturation at $95°C$ for 0 s, annealing at $66°C$ for 0 s, and extension at $74°C$ for 0 s.

Experimental and predicted melting curves are displayed in uAnalyze as helicity versus temperature. Experimental data is processed by removal of fluorescence background, normalization, and optional temperature overlay. A linear correlation of fluorescence to helicity after background subtraction and normalization is assumed in uAnalyze. All amplicon sequences and data files used in this report are provided as Supplemental Data, available online.

2.3 Background Subtraction and Normalization of Experimental Melting Curves

Options for background subtraction include linear baseline extrapolation [14] and estimation of the background as an exponential [7], [8], [15]. In both methods, regions below and above the melting transitions are identified where only background fluorescence is present. These $1°C$ regions have their positions initially set with inner limits at 98 percent and 0.1 percent of predicted helicity and may be optionally adjusted by horizontal sliders on the X-axis. Slopes within each region are calculated and used to estimate and remove background fluorescence according to each method. Curves with low relative fluorescence that cannot be adequately normalized are identified by an intensity discriminator (20 percent of the maximum fluorescence of samples) that is controllable by the user.

2.4 Overlay

In order to directly compare curve shapes, uAnalyze provides overlay options including, 1) a shift of the predicted curve to the experimental curve average, and 2) a shift of all experimental curves to the experimental curve average (Supplemental Fig. 1, available online). In both options, curves are translated along the X-axis based on the mean

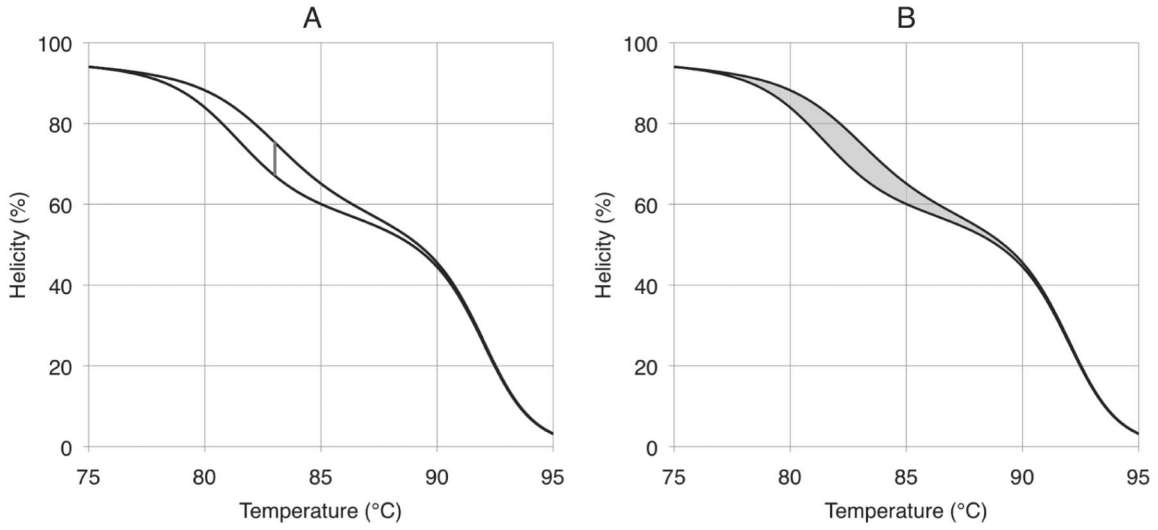

Fig. 2. Illustrations of curve spread (A) and 2D Offset (B). Curve spread is a measure of melting curve precision and is defined as the average maximum helicity difference for all experimental pairwise combinations. One curve pair is shown (panel A) that displays the maximum helicity difference (vertical gray line) between two curves. When more than two curves are present, the average of all pairwise curve spreads is used. For N curves, the curve spreads of N(N-1)/2 pairs of curves are averaged. The 2D Offset (panel B) is a measure of accuracy (closeness to prediction) and is calculated by finding the area (shaded) between the experimental average curve and the thermodynamic prediction. The curves shown are illustrative and do not correlate to any particular sequence.

integral temperature difference between curves over a range of helicities. The predicted curve is shifted to the experimental curve average between 10 and 90 percent helicity. Experimental curves are shifted to their average between 5 and 15 percent helicity to better reveal heteroduplexes [7]. The shifts are performed by first inverting the fluorescence versus temperature curves (to temperature versus fluorescence) and finding the least squares quadratic fit of points within the helicity range. If less than three points are within the range, additional points nearest to the range are added. Then, the integral means of the quadratics are found to define the necessary shifts.

2.5 Metrics

Two analysis metrics are introduced. The match between experimental and predicted curves is quantified as the two-dimensional (2D) offset, calculated as the area between the predicted and average experimental curves in units of $\% \times {}^{\circ}\mathrm{C}$. The dispersion or precision of a set of experimental curves is quantified as the "curve spread," calculated as the average maximum helicity difference of all curve pairs within the set.

2.6 Spatial Correlation

Melting curves are mapped to their physical sample locations on rectangular and circular format instruments. For generic and single sample instruments where the physical format is unknown or irrelevant, the samples are listed sequentially. Melting curves with low fluorescence that are not normalized are shown in a different color and identified by that same color on the physical sample map. The user can interactively select/deselect samples, rows or columns on the spatial representations to reduce the number of curves visible on the graph. Metrics are calculated on all currently selected samples that are normalized.

3 Results

Analysis of 96 different DNA samples after amplification of exon 6 of *ACADM* is shown in Fig. 1. Two columns and two rows have been deselected for demonstration. After exponential background removal and normalization, the experimental samples cluster closely with a curve spread of 4.22 percent before temperature overlay and 0.72 percent after overlay. The experimental curves are shifted $0.75{}^{\circ}\mathrm{C}$ (at 50 percent helicity) above the thermodynamic prediction with a 2D offset of $114\% \times {}^{\circ}\mathrm{C}$.

The precision (curve spread) and accuracy (2D offset) metrics are illustrated in Fig. 2. For precision, the maximum helicity difference is determined for all possible pairs of curves, one of which is shown in Fig. 2A. The curve spread is the average of this value across all possible curve pairs (there are N(N-1)/2 curve pairs for N curves). The accuracy of the thermodynamic prediction or 2D offset (Fig. 2B), is the total area between the thermodynamic prediction and the average of the experimental curves, even if these lines cross one or more times. Both metrics may be calculated before overlay to assess both position and shape differences, or after overlay to focus on curve shape.

Exponential and baseline subtraction for normalization are compared in Fig. 3, using an exon 2 amplicon of *ACADM* from 96 individual DNA samples. Exponential normalization resulted in better precision with an experimental curve spread of 3.55 percent compared to 5.05 percent for baseline normalization. Accuracy was also better after exponential (2D offset $71.5\% \times {}^{\circ}\mathrm{C}$) versus baseline normalization ($176\% \times {}^{\circ}\mathrm{C}$). The baseline method underestimates the relative contribution of the first melting domain to helicity when compared to either the exponential method or to the thermodynamic prediction. The second melting domain of the experimental data exceeds the prediction by $0.53{}^{\circ}\mathrm{C}$ (exponential) or $1.07{}^{\circ}\mathrm{C}$ (baseline) at 50 percent helicity.

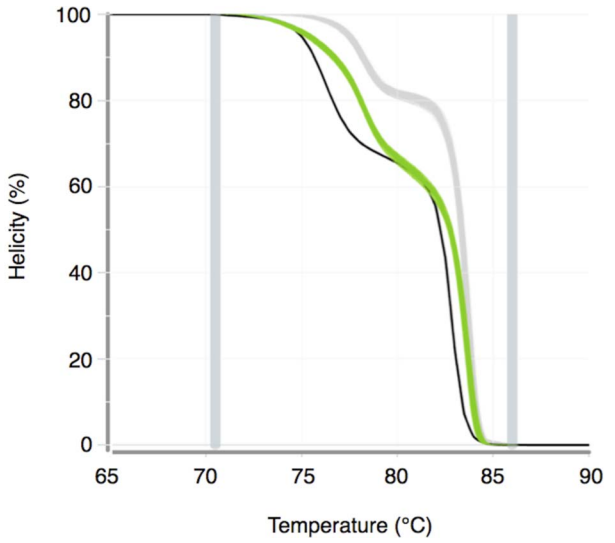

Fig. 3. Comparison of normalization methods in uAnalyze applied to melting data of exon 2 of the *ACADM* gene without experimental overlay. Ninety-six different DNA samples were analyzed. The exponential background removal method (green) better fit the predicted (black) melting curve than the linear baseline method (gray), especially at the first, low temperature, melting domain. Vertical cursors (gray) indicate the regions used to normalize the data. Cursors were set at $71^{\circ}\mathrm{C}$ and $86^{\circ}\mathrm{C}$ with no overlay.

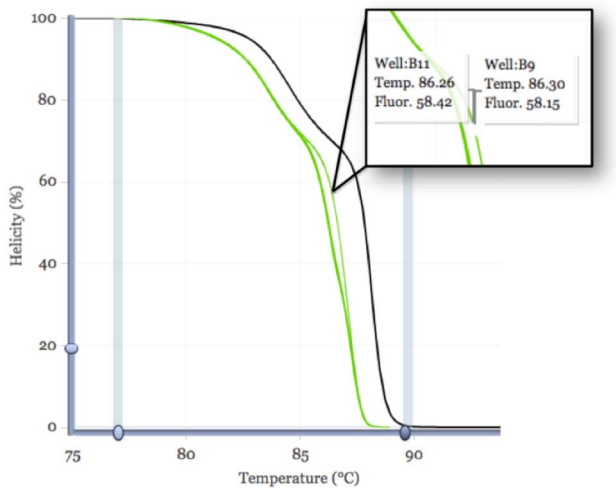

Fig. 4. uAnalyze melting curve analysis identifies heterozygous c. $1090\mathrm{G} > \mathrm{A}$ in exon 9 (amplicon 9b) of *CYBB* after experimental curve overlay. Experimental data (green curves) are normalized after exponential background subtraction and overlaid between 5 and 15 percent helicity. Hovering over the abnormal curves pops up a box that reveals the physical sample locations, along with the temperature and fluorescence of the cursor. The black curve is predicted by thermodynamics and shows the two domains also observed in the experimental curves.

To test uAnalyze in a typical mutation scanning application for the detection of heterozygotes [12], the 13 exons of *CYBB* were amplified in 14 fragments. All exons of three individuals, each in duplicate, were analyzed on one plate. The individuals were all normal in sequence at *CYBB*, except for one heterozygous variant in one exon of one individual. The resulting curve spreads (after overlay), 2D offsets, and temperature difference from predicted at 50 percent helicity for the 14 amplicons are shown in Table 1. Typically, the curve spreads are between 0.5 and 1.5 percent using default cursor placement. Curves spreads higher than 1 percent generally indicate less than optimal cursor placement, which is the case for amplicon 6 and 10 (exponential) and amplicon 5 (baseline). For example, manual adjustment of the default cursors for amplicon 10 reduced the curve spread to 0.76 percent. The outlier is amplicon 9b with a curve spread of about over 6 percent. This high curve spread identifies an amplicon where one of the three DNA samples (in duplicate) is heterozygous at *CYBB* $c.1090\mathrm{G} > \mathrm{C}$. The overlaid melting curves of amplicon 9b are shown in Fig. 4 with distinct clustering of the 2 $c.1090\mathrm{G} > \mathrm{A}$ samples away from the four wild type samples.

Excluding the two heterozygous samples, *CYBB* amplicon melting curve accuracy and precision were similar using

TABLE 1
Curve Metrics for CYBB Amplicons

		CURVE SPREAD[1] (% Helicity)		2D OFFSET (% Helicity x °C)		Δ Temperature[2] (°C)	
Amplicon	Length (bp)	Exponential	Baseline	Exponential	Baseline	Exponential	Baseline
1	263	0.56	0.49	29	77	-0.12	0.34
2	214	0.84	0.92	41	31	-0.10	0.16
3	249	0.89	1.24	113	122	-1.20	-0.73
4	202	0.60	0.77	44	41	0.04	0.25
5	281	0.80	2.36	35	42	0.20	0.32
6	309	1.47	0.69	44	32	-0.24	-0.06
7	255	0.66	1.00	24	81	0.12	0.39
8	252	1.05	0.89	42	103	0.27	0.70
9a	250	0.64	1.05	41	74	-0.39	-0.14
9b[3]	210	6.36	8.13	109	72	-1.17	-0.73
10	285	1.54	0.62	88	53	-0.79	-0.40
11	274	0.90	1.09	125	86	-1.14	-0.84
12	304	0.88	0.77	32	20	-0.26	-0.07
13	251	0.47	0.64	20	57	0.02	0.28
Mean +/- SD		0.87 +/- 0.33	0.96 +/- 0.47	56.2 +/- 32.0	63.4 +/- 29.5	-0.34 +/- 0.52[4]	-0.04 +/- 0.48[4]

[1]After experimental curve overlay
[2]Calculated as the mean experimental temperature at 50% helicity minus the predicted temperature at 50% helicity
[3]For amplicon 9b, curve spreads include the 2 aberrant values, while 2D Offsets, Δ Temperature and Mean +/- SD values exclude them
[4]$p < 0.001$ by paired t-test

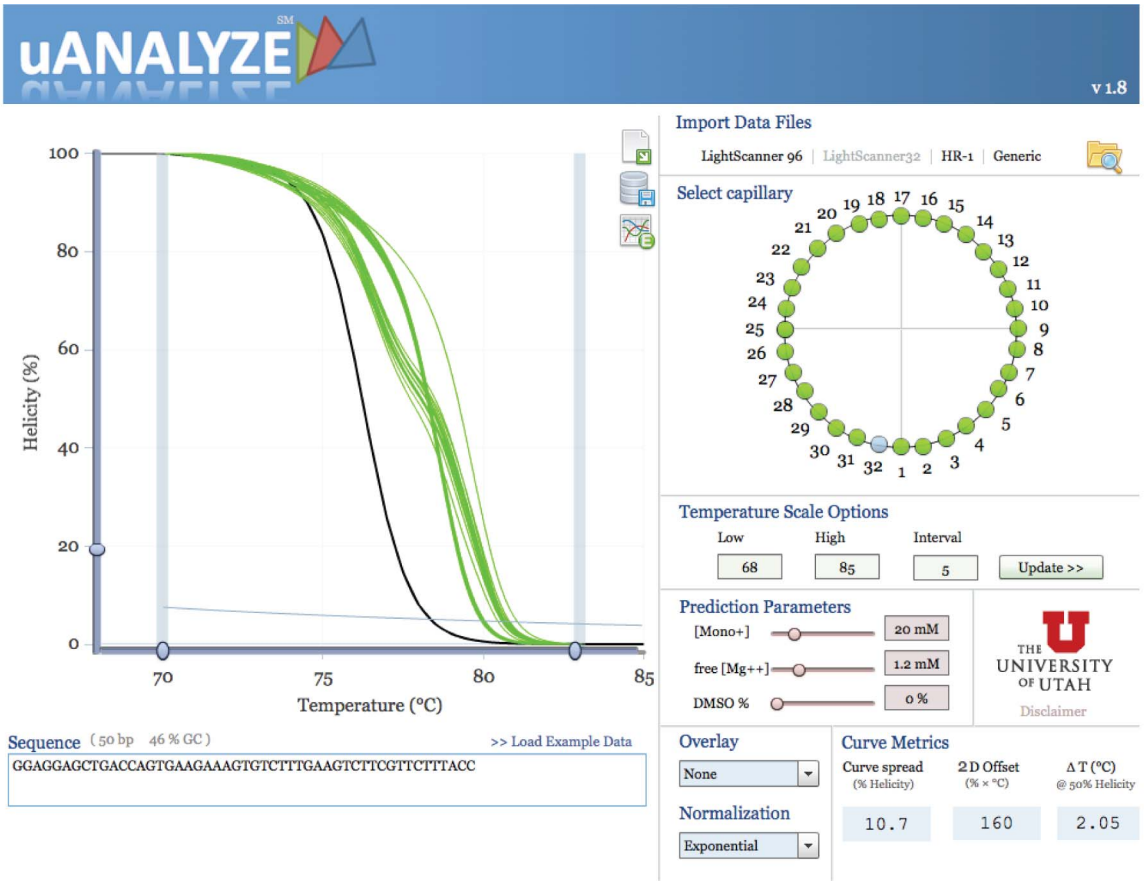

Fig. 5. Small amplicon melting of *MTHFR* $c.1286\mathrm{A} > \mathrm{C}$. A 50 bp product was amplified from 31 different DNA samples. A no template control (gray sample, well 32) was included that does not meet the fluorescence threshold for normalization. Graphical representation of the circular sample format allows for quick identification and removal of the no template control from the analysis. Cursors were set at $70°\mathrm{C}$ and $83°\mathrm{C}$ with no overlay.

either exponential or baseline normalization. Neither the curve spreads nor the 2D offsets were significantly different after exponential or baseline normalization. Although the melting temperatures (taken as the temperature at 50 percent helicity) were significantly different by the paired t-test, the difference between exponential ($-0.34 + / - 0.52°\mathrm{C}$) and baseline methods ($-0.04 + / - 0.48°\mathrm{C}$) was only $0.3°\mathrm{C}$.

Application of uAnalyze to genotyping by small amplicon melting (*MTHFR* c. $1286\mathrm{A} > \mathrm{C}$) is shown in Fig. 5. Exponential normalization was used but the data were not overlaid in order to better distinguish homozygous variants from homozygous wild type samples. Multiple wild type samples were present about $1°\mathrm{C}$ higher than the thermodynamic prediction and a single homozygous variant curve was present a further $1°\mathrm{C}$. Several melting curves from heterozygous samples show bimodal transitions that crossed both the predicted curve and the wild type cluster.

4 Discussion

uAnalyze provides an interactive web interface to analyze and compare high resolution melting data with thermodynamic predictions and physical sample location. Prior attempts to correlate experimental to predicted melting curves have been variably successful [16], [17]. For example, previous work using the POLAND algorithm [2] with *CYBB* amplicons [16] and baseline normalization showed most experimental curves 5-6°C higher in temperature than thermodynamic predictions and relative attenuation of fluorescence in low temperature domains [16].

The thermodynamic predictions in uAnalyze are based on uMelt, a prediction tool designed for high resolution melting of PCR products [6]. Similar to other web applications that predict melting curves, uMelt uses nearest neighbor parameters and recursive calculations to identify melting curve domains [2], [3], [4], [5]. While many software programs exists for theoretical melting curve prediction, uAnalyze provides a singular interface for the display, normalization, and quantitative analysis of experimental data in context of theoretical predictions for assay validation and assessment. uAnalyze implements a unified nearest neighbor parameter set [10] with adjustments for monovalent cation concentration, free Mg^{++} concentration and DMSO percentage. Free, unchelated Mg^{++} is calculated by subtracting the total dNTP concentration from the total Mg^{++} concentration [18].

Melting temperatures (Tms) are often identified as peaks on derivative plots. However, when multiple domains are present in a melting curve, this metric becomes less useful. The relative temperatures of melting curves of similar shape can still be assessed as the fluorescence at 50 percent helicity. Using the fluorescence at 50 percent helicity and baseline normalization, the mean and standard deviation of the temperature difference between experimental and predicted curves of the 14 *CYBB* amplicons was $-0.04 + / - 0.48°\mathrm{C}$, indicating minimal systematic bias and a standard deviation less than $0.5°\mathrm{C}$ in this data set. This is substantially more

precise than the best oligonucleotide "all or none" Tm predictions with a standard deviation of $1.6°C$ [19]. The high prediction accuracy of PCR amplicons may result from their longer lengths compared to short oligonucleotides. The unified parameter set and/or better treatment of the Mg^{++} effect may explain the improved accuracy over prior attempts [16]. No adjustments for the dye LCGreen Plus or other assay factors were incorporated.

This paper introduces the metrics, curve spread and 2D offset, to quantify melting curve precision and prediction accuracy. In the analysis of 14 *CYBB* amplicons (Table 1), exponential normalization resulted in better precision (as measured by curve spread) and accuracy (as measured by 2D offset) than baseline normalization. However, this difference was not significant because of high variability across amplicons. Similar results were obtained when single domain and multiple domain curves were considered separately (Supplementary Table 2, available online). In some cases (e.g., Fig. 3) exponential was clearly better than baseline because it better matched predicted intensity ratios of different domains, a concern previously mentioned [16]. Therefore, exponential normalization was selected as the default method. However, the temperature at 50 percent helicity was best matched by baseline normalization. Pending more definitive data, we elected to provide both exponential and baseline normalization as options.

Additional features of uAnalyze include overlay options to evaluate shape differences independently of temperature, and curve correlation to physical sample position. Curve overlay (also known as temperature shifting) is used extensively in mutation scanning as the best way to identify heterozygotes [16]. Curve overlay is typically not used in genotyping where it decreases the differences between homozygous variants. Spatial representations match curves to physical samples and allow easy selection of curve subsets for display.

Visual and quantitative comparisons between experimental curves and thermodynamic predictions is helpful in PCR optimization, mutation scanning, and genotyping. By comparing the predicted curve to the experimental curve, amplification of the correct product can be verified, reducing or eliminating the need for electrophoresis and/or sequencing. As additional data formats for other instruments are incorporated, more instruments will be directly supported, although melting resolution between instruments varies greatly [20]. An interesting addition, though computationally intensive for a web application, would include optimal, autonomous cursor placement rather than default placement by theoretical prediction. Finally, one of the more interesting extensions of uAnalyze will be to predict composite melting curves amplified from heterozygotes.

5 Conclusion

uAnalyze is a user-friendly web application that provides visual and quantitative comparison of theoretical and experimental high resolution melting data.

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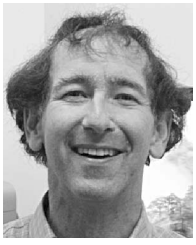

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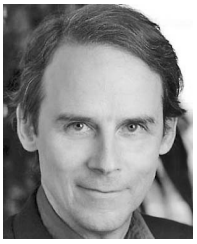

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