Native characterization of nucleic acid motif thermodynamics via non-covalent catalysis

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DNA hybridization thermodynamics is critical for accurate design of oligonucleotides for biotechnology and nanotechnology applications, but parameters currently in use are inaccurately extrapolated based on limited quantitative understanding of thermal behaviours. Here, we present a method to measure the $\Delta G^\circ$ of DNA motifs at temperatures and buffer conditions of interest, with significantly better accuracy (6- to 14-fold lower s.e.) than prior methods. The equilibrium constant of a reaction with thermodynamics closely approximating that of a desired motif is numerically calculated from directly observed reactant and product equilibrium concentrations; a DNA catalyst is designed to accelerate equilibration. We measured the $\Delta G^\circ$ of terminal fluorophores, single-nucleotide dangles and multinucleotide dangles, in temperatures ranging from 10 to 45 °C.
Nucleic acid biotechnology uses engineered oligonucleotides as therapeutic agents (for example, micro RNAs) and diagnostics reagents (for example, PCR primers and next generation sequencing capture probes). Nucleic acid nanotechnology uses engineered oligonucleotides as building blocks for constructing precisely patterned structures and complex spatio-temporal circuits. In both fields, accurate rational design of oligonucleotides with intended thermodynamics properties is critical to achieving desired system function.

Errors in predicted $\Delta G^\circ$ or melting temperature values result in undesirable non-specific interactions or unpredictable aggregation/assembly pathways. Currently, the best DNA design and folding software exhibit errors in predicted melting temperature of $\sim 1.5 ^\circ C$ (ref. 23), corresponding to errors of roughly 3 kcal mol $^{-1}$. This indicates that there is significant inaccuracy even in common motifs such as DNA base stacks and dangles, an observation confirmed by chemical probing of nucleic acid folding structures.

Current thermodynamic parameters are extrapolated from experiments in very different temperatures and buffer conditions than typically used (for example, melt curves in 1 M Na$^+$; refs 25,26). In addition, thermodynamics parameters for individual motifs need to be inferred through linear algebra decomposition or principal component analysis, because each melt curve provides the aggregate $\Delta H^\circ$ and $\Delta S^\circ$ of many motifs. Large numbers of experiments on different sequences statistically mitigate the latter source of unbiased error, but the former results in systematic errors that cannot be reduced via melt curve experiments.

Here we present a generalized method for measuring $\Delta G^\circ$ values of individual DNA motifs in native conditions of interest. A reaction is designed with $\Delta G^\circ$ equivalent to the $\Delta G^\circ$ of the motif of interest, and the equilibrium concentrations of the reactant and product species are measured to numerically calculate the equilibrium constant $K_{eq}$. The innovation that enables this approach is a DNA catalyst system that accelerates forward and reverse rate constants by orders of magnitude, allowing rapid equilibration.

We believe our native catalysis method can be used to provide an updated database of nucleic acid thermodynamic parameters for more accurate prediction and design software. We have started this process by providing $\Delta G^\circ$ values for (1) 80 terminal fluorophore parameters, (2) 160 single-nucleotide dangle parameters and (3) 140 multinucleotide dangle parameters. Through the course of our studies, we discovered that multinucleotide dangles destabilize nearby DNA duplexes, asymptoting at $+0.56$ kcal mol $^{-1}$ above a single-nucleotide dangle for dangles 8 nt or longer.

Results

Nucleic acid motif thermodynamics. The standard model of nucleic acid hybridization thermodynamics is a local model, in which the structure of a nucleic acid molecule or complex is dissected into a number of non-overlapping motifs, such as base stacks, bulges and dangles. The standard free energy of formation of a molecule is calculated as the sum of its component motifs (Fig. 1a). In the past 30 years, the thermodynamics of many DNA and RNA motifs have been characterized to inform bioinformatics software for nucleic acid structure prediction and probe or primer design. However, inaccuracies in the $\Delta G^\circ$ of DNA motifs are compounded in the overall $\Delta G^\circ$ of hybridization, and lead to errors in predicted hybridization affinities.

Traditionally, motif thermodynamics (standard free energy of formation $\Delta G^\circ$) are characterized through melt curve analysis. These methods suffer from two major sources of inaccuracy: (1) yield and $\Delta G^\circ$ measurement are inaccurate except near the melting temperature, and (2) linear algebra decomposition of motif thermodynamics reduces accuracy. Although recently melt curve analysis accuracy has been improved through the development of high-resolution melt (HRM; ref. 45), these limitations still exist. See Supplementary Note 1 for further discussion on melt curve inference of $\Delta G^\circ$.

Reaction construction. Our method starts with the construction of a reaction whose reaction standard free energy $\Delta G_{rxn}^\circ$ closely approximates the motif standard free energy $\Delta G_{motif}^\circ$ (Fig. 1b). This is achieved by designing a duplex product bearing the motif of interest (XZ) and a duplex reactant differing from the duplex product by only the motif (YZ). To balance the reaction, a single-stranded reactant oligonucleotide (X), which is part of the duplex product but not the duplex reactant, is added to the left side of the reaction. Similarly, a single-stranded product oligonucleotide (Y) is added to the right side of the reaction.

Figure 1 | DNA hybridization thermodynamics. (a) The standard model of nucleic acid hybridization decomposes any nucleic acid molecule into a number of non-overlapping motifs (for example, dangles and stacks), and predicts the standard free energy of formation of the molecule to be the sum of the $\Delta G^\circ$ of the individual motifs. Traditionally, motif $\Delta G^\circ$ values are characterized using melt curves, and then extrapolated to other temperatures and buffer conditions. (b) In principle, motif $\Delta G^\circ$ can be measured in native isothermal conditions by observing the equilibrium concentrations of an appropriately designed X + YZ$\rightarrow$X + YZ strand displacement reaction. For example, the shown reaction characterizes the $\Delta G^\circ$ of the 5′-T dangle next to a closing G-C base pair; the partially double-stranded product differs from the partially double-stranded reactant by only the motif of interest. By designing the single-stranded reactants to possess negligible secondary structure, the reaction standard free energy $\Delta G_{rxn}^\circ$ will closely approximate the dangle standard free energy $\Delta G_{dangle}^\circ$. (c) A non-covalent strand displacement catalyst C can be designed to accelerate reaction equilibration to complete within minutes. The reverse reaction pathway is analogous to the forward reaction pathway shown here, and is also accelerated by the catalyst.
The net reaction is \( X + YZ = Y + XZ \), and
\[
\Delta G_{\text{ran}} = \Delta G_{XZ} + \Delta G_Y - \Delta G_X - \Delta G_{YZ} \tag{1}
\]
\[
= (\Delta G_{XZ} - \Delta G_{YZ}) + (\Delta G_Y - \Delta G_X) \tag{2}
\]
The standard free energy of the motif (in Fig. 1b, a 5’-TG dangle) can be expressed as \( \Delta G_{\text{motif}} = \Delta G_{XZ} - \Delta G_{YZ} \) and is equal to \( \Delta G_{\text{ran}} \) when \( \Delta G_C = \Delta G_{CZ} \). One way to ensure the latter is approximately true is to design the sequences of X and Y to avoid any predicted secondary structure.

The value of \( \Delta G_{\text{ran}} \) can be numerically computed from the observed equilibrium concentrations of the species:
\[
\Delta G_{\text{ran}} = -R\ln(K_{\text{eq}}) \tag{3}
\]
\[
= -R\ln\left(\frac{[Y][XZ]}{[X][YZ]}\right) \tag{4}
\]
Here \( R \) is the universal gas constant (or Boltzmann constant), and \( \tau \) denotes the temperature in Kelvin. Given the initial concentrations \([X]_0\ [, Y]_0\ [, XZ]_0\ \) and \([YZ]_0\), all four equilibrium concentrations can be computed from any one equilibrium concentration based on simple algebra. Thus, we can accurately determine \( \Delta G_{\text{motif}} \) using an accurate measure of the equilibrium concentration of any one of the four species.

**Non-covalent catalysis.** The above reaction, by itself, equilibrates slowly with a second order rate constant of roughly \( 1 \text{ M}^{-1} \text{s}^{-1} \) (refs 28,47,48), corresponding to a half-life of roughly 100 days at 100 nM concentrations. It is not practical to wait so long for the reaction to naturally reach equilibrium because nucleotide oxidation (for example, adenine to inosine) occurs on a similar time scale\(^{29,30} \), and will bias the inferred thermodynamic results. To accelerate equilibration kinetics, we design a non-covalent strand displacement catalyst C, first introduced in ref. 27.

Figure 1c shows the catalysis reaction mechanism. C first binds to the single-stranded portion of Z in YZ, known as a toehold, and then displaces Y one nucleotide at a time through an unbiased random walk process known as branch migration. Because the elementary single-base replacement steps in branch migration are very fast (10–100 \( \mu \text{s} \) ref. 51)), the entire displacement process occurs with a first order rate constant of about 1 s\(^{-1} \) (ref. 28). Thus, the reaction is typically rate limited by the second order toehold binding process, and is similar to hybridization kinetics for sufficiently long toeholds\(^{28} \). When all nucleotides in C are base paired to their complementary nucleotides in Z, Y can spontaneously dissociate, leaving intermediate CZ. Subsequently, CZ can react with either X or Y in a similar manner as YZ initially reacted with C, releasing C to catalyse other reactions (Fig. 1c). Thus, catalyst C enables a rapid pathway for both the forward and the reverse reactions of \( X + YZ = Y + XZ \) without changing the reaction’s equilibrium constant (Supplementary Note 2).

**Fluorophore characterization.** In this manuscript, we rely exclusively on fluorescent non-denaturing polyacrylamide gel electrophoresis (PAGE) to quantify the equilibrium concentrations. Fluorescent PAGE shows very low background and high linearity (Supplementary Figs 5–8, Supplementary Note 4), allowing us to map band intensity to species concentration via a simple scaling constant. It also allows us to verify that the catalytic mechanism is functioning as intended, and that no long-lived side products or intermediates are being formed (such as CXZ or CYZ).

We first study the effects of terminal fluorophores on stabilizing DNA duplexes, both because these interactions have been relatively poorly characterized\(^{52,53} \) and because these are necessary prerequisites for our studies of other motifs. We chose to focus our fluorophore studies on the carboxy-X-rhodamine (ROX) and Alexa532 fluorophores, because our past experience indicates that these are temperature robust and their per unit fluorescence does not change significantly over a period of months when properly stored.

Figure 2a shows the net reaction used to characterize the thermodynamic effects of a 5’ ROX next to an adenine (A). We refer to the ROX-labelled species as the X species; species containing X (X and XZ) will appear as visible bands on a fluorescent PAGE, while all other bands will be invisible. Figure 2b shows the results from one fluorescent PAGE experiment characterizing 5’ ROX-A at 25 °C in 12.5 mM Mg\(^{2+} \). Gel band intensities were quantitated by software, and relative fluorescence units are listed below the gel; differences in pipetting volume and fluorophore quantum yield in ssDNA and dsDNA states resulted in different total fluorescences for different lanes; see Supplementary Fig. 14 and Supplementary Note 5 for discussion on quantitation and \( \Delta G_{\text{c}} \) calculation methodology.

Lanes 1 and 2 are controls showing the positions and intensities of the fluorescent XZ and X bands; species identities were verified with SYBR gold stained gels with a DNA ladder lane (Supplementary Fig. 19). In lane 3, X and Z are pre-mixed for 30 min and then Y is introduced and allowed to react with XZ for 3 h before PAGE. In lane 4, Y and Z are pre-mixed and X is subsequently introduced. The band distribution in lane 3 closely resembles that of lane 2, and the band distribution in lane 4 closely resembles lane 1, despite the fact that lanes 3 and 4 have the same composition of strands; this indicates that equilibrium is far from reached after 3 h of reaction. Lanes 5 and 6 are similar to lanes 3 and 4, except 0.1x of the catalyst C is introduced into each reaction after all other species are introduced, but before the 3 h incubation. The band distribution in lanes 5 and 6 are qualitatively similar to each other and intermediate between lanes 3 and 4, indicating that equilibrium has been achieved.

Lack of a higher molecular weight CXZ band indicates that CXZ and CYZ are short-lived and low in concentration at equilibrium. Under the assumption that CZ is likewise short-lived and insignificant in concentration (Supplementary Fig. 16), the equilibrium concentrations \([Y] \) and \([YZ] \) can be calculated given the initial concentrations \([X]_0\ [, Y]_0\ [, XZ]_0\ \) and \([YZ]_0\) from either \([X] \) or \([XZ] \):
\[
[Y] = [Y]_0 + ([X]_0 - [X]) \tag{5}
\]
\[
[YZ] = [YZ]_0 - ([X]_0 - [X]) \tag{6}
\]
\[
[Y] = [Y]_0 + ([XZ] - [XZ]_0) \tag{7}
\]
\[
[YZ] = [YZ]_0 - ([XZ] - [XZ]_0) \tag{8}
\]
In a perfectly run reaction, the calculated equilibrium concentration \([Y] \) should be the same regardless of whether it is calculated from \([X] \) or \([XZ] \), but in practice slight pipetting errors in the total amount of sample loaded per gel lane will lead to different values. A similar argument applies to \([YZ] \) inference. We correct for these in our mathematical analysis and calculation of \( K_{\text{eq}} \) and \( \Delta G_{\text{c}} \) from gel band intensities (Supplementary Figs 16 and 21).

From the intensities of the gel bands in lanes 1, 2, 5 and 6, we calculate \( \Delta G_{\text{ROX-A}} \) to be \(-0.07 \text{ kcal mol}^{-1} \) from lane 5 and \(-0.31 \text{ kcal mol}^{-1} \) from lane 6. Five additional fluorescent PAGE experiments were run under the same conditions using separately prepared samples, to mitigate the effects of pipetting error in any one experiment, and the inferred \( \Delta G_{\text{c}} \) values are shown in Fig. 2c. These 12 independent \( \Delta G_{\text{c}} \) values are then combined into a single mean \( \mu = \Delta G_{\text{ROX-A}} = -0.293 \text{ kcal mol}^{-1} \), corresponding to our best estimate of the true value of the parameter from our measurements.
Figure 2 | Experimental results. (a) Reaction constructed to measure the $\Delta G^\circ$ of a 5′ ROX fluorophore next to an adjacent DNA duplex with a terminal A nucleotide ($\Delta G^\circ_{\text{ROX–A}}$). (b) Fluorescent PAGE demonstrating non-covalent catalysis. Relevant species were reacted at 25 °C in 12.5 mM Mg$^{2+}$ buffer; 1x denotes ~300 nM. Lanes 3 and 4 show significant difference in band intensity distribution, indicating that equilibrium is not achieved after 3 h of reaction in the absence of the catalyst. In contrast, lanes 5 and 6 show that a sub-stoichiometric (0.1x) amount of catalyst drives the reaction to equilibrium. Listed numbers below the gel are quantitated gel band intensities (arbitrary fluorescence units). (B) denotes in PBS (blue), as well as in 12.5 mM Mg$^{2+}$ nucleotide.

Temperature dependence. In addition to 25 °C in 12.5 mM Mg$^{2+}$, we also performed similar experiments (5–8 gels each) for the ROX-A fluorophore at temperatures of 10, 25, 37 and 45 °C in PBS (Sigma P5493, 0.01 M phosphate; 0.154 M NaCl; pH 7.4). The gels were always run at the same temperature as the hybridization reaction. Although the gel running buffer (TAE with no added salt) differs from the hybridization buffer, our control experiments (Supplementary Fig. 18) shows no significant difference in inferred $\Delta G^\circ$ due to the transient change in buffer conditions before the start of the electrophoresis. The mean and mean s.d.’s of $\Delta G^\circ_{\text{ROX–A}}$ in each of these conditions are plotted against temperature in Fig. 2d. With the four separate series of experiments performed in PBS at different temperatures, we can use the relation $\Delta H^\circ = \Delta H^o - \tau \Delta S^o$ to infer $\Delta H^o$ and $\Delta S^o$ of the 5′-ROX-A using a linear fit.

Because each of the four mean $\Delta G^\circ$ values at different temperatures has a different level of uncertainty (mean s.d.), it is inappropriate to use a standard least-squares fit. Instead, we obtain the best fit $\Delta H^o$ and $\Delta S^o$ values from a maximal likelihood standpoint, minimizing the sum of square $z$ scores. The $z$ score is calculated as:

$$z = \frac{\Delta G^\circ_{\text{Predicted}} - \Delta G^\circ_{\text{Observed}}}{\sigma_{\mu}}$$

where $\Delta G^\circ_{\text{Predicted}}$ is the value calculated as $\Delta H^o - \tau \Delta S^o$, $\Delta G^\circ_{\text{Observed}}$ is the mean experimental $\Delta G^\circ$ at the relevant temperature, and $\sigma_{\mu}$ is the mean s.d. The green line in Fig. 2d shows the best fit $\Delta H^o = -0.81$ kcal mol$^{-1}$ and $\Delta S^o = 1.89$ cal mol$^{-1}$ K$^{-1}$. See Supplementary Fig. 17 for details on $\Delta H^o$ and $\Delta S^o$ fitting, including calculated of s.d.’s on best fit $\Delta H^o$ and $\Delta S^o$ values.

Our best fit linear regression to our measured $\Delta G^\circ$ values often showed poor quality of fit, resulting in large s.d.’s in our inferred values of $\Delta H^o$ and $\Delta S^o$. One possible explanation could be the temperature dependence of motif $\Delta H^o$ and $\Delta S^o$ values. Although the standard model of DNA hybridization assumes temperature invariance of $\Delta H^o$ and $\Delta S^o$, there is debate within the literature as to whether this is true for all hybridization motifs.
Comparison to melt curve analysis. To compare the quality of the thermodynamics parameters produced by our native catalysis against the standard-of-practice melt curve analysis approach, we also performed HRM analysis of the ROX-labelled XZ and unlabelled YZ molecules (Fig. 2e). Hybridization yields of the XZ and YZ molecules are inferred from the melt curves, and used to inform the numerical calculation of the equilibrium constants $K_{\text{XZ}}$ and $K_{\text{YZ}}$, of the $X+Z\rightarrow\text{XZ}$ and $Y+Z\rightarrow\text{YZ}$ reactions, respectively. The difference in the logarithms of standard free energy $\Delta G_0$ is shown in Supplementary Table 4 for data on Alexa532-based measurements. ($\Delta G_0$).

Figure 3 | Dangle $\Delta G^0$ characterization. (a) To accurately measure dangle $\Delta G^0$ using fluorescent PAGE, we design a second reaction with reaction standard free energy $\Delta G^0_{\text{dangle}}$ reflecting the effects of both the dangle of interest and the fluorophore. By numerically subtracting $\Delta G^0_{\text{dangle}}$ from $\Delta G^0_{\text{dangle}}$, we calculate the value of $\Delta G^0_{\text{dangle}}$ corresponding to the dangle. (b) Summary of mean and mean s.d. $\Delta G^0_{\text{dangle}}$ based on ROX, and the corresponding $\Delta G^0_{\text{dangle}}$ after subtracting from the relevant $\Delta G^0_{\text{dangle}}$ values from Fig. 2d. (c) $\Delta G^0_{\text{dangle}}$ values inferred using the Alexa532 fluorophore, rather than the ROX fluorophore; see Supplementary Table 4 for data on Alexa532-based measurements. (d) Consensus dangle $\Delta G^0$ values are calculated as the maximal likelihood $\Delta G^0$ value based on the independent readings from ROX and Alexa532 experiments. The CS represents 10 minus the sum of the squares of $z$ score of the consensus $\Delta G^0$ relative to the mean $\Delta G^0$ values measured from ROX and Alexa532; a CS of eight indicates that the consensus $\Delta G^0$ is within 1 s.d. of each mean $\Delta G^0$. See Supplementary Table 6 for summary results of all 32 single-nucleotide dangles.

Single-nucleotide dangles. Although it is possible to construct a similar catalysed $X+YZ\rightarrow\text{XZ}+Y$ reaction to directly measure dangle motif thermodynamics, it would be difficult to accurately quantitate the concentrations of any of the species, because X and Y would have very similar mobility, and likewise with XZ and YZ. Consequently, we chose to indirectly characterize the dangle motifs by measuring the relative $\Delta G^0$ of a dangle with a fluorophore (reaction 2 in Fig. 3a), and subtracting that value from the $\Delta G^0$ of the fluorophore that we obtained earlier in Fig. 2 and related experiments. The resulting $\Delta G^0_{\text{dangle}} = \Delta G^0_{\text{dangle}} - \Delta G^0_{\text{dangle}}$ corresponds to the net reaction querying only the dangle, and closely approximates $\Delta G^0_{\text{dangle}}$ that we ultimately wish to characterize. As a further benefit, because both $\Delta G^0_{\text{dangle}}$ and $\Delta G^0_{\text{dangle}}$ are overestimated by a similar amount due to the ignorance of the CZ, CYZ and CXZ species, $\Delta G^0_{\text{dangle}}$ becomes unbiased due to the cancellation of the bias terms. The disadvantage of such a 2-reaction approach is the increased s.d. on the $\Delta G^0$ parameters from subtracting two distributions.

The left panel of Fig. 3b shows the summarized mean $\Delta G^0_{\text{dangle}}$ values at different temperatures in PBS based on a number of fluorescent PAGE experiments similar to that shown in Fig. 2b and the corresponding mean s.d.'s. The right panel shows the mean $\Delta G^0_{\text{dangle}}$ values obtained by subtracting the $\Delta G^0_{\text{dangle}}$ values in the left panel from the $\Delta G^0_{\text{dangle}}$ values shown in Fig. 2d. The mean s.d.'s of $\Delta G^0_{\text{dangle}}$ are calculated assuming that errors in $\Delta G^0_{\text{dangle}}$ and $\Delta G^0_{\text{dangle}}$ are independent, so that variance is additive:

$$\sigma_{\mu,\text{dangle}} = \sqrt{\sigma_{\mu,\text{dangle}}^2 + \sigma_{\mu,\text{dangle}}^2}$$

Figure 3c shows the mean and mean s.d. of $\Delta G^0_{\text{dangle}}$ for the same 5'-TG dangle assayed using the Alexa532 fluorophore, and Fig. 3d shows the overlay of the two sets of values. The thermodynamics of Alexa532 was likewise characterized for a variety of nearest neighbour bases and temperatures (Supplementary Table 4). The consensus $\Delta G^0$ value that we attribute to the dangle at each temperature is calculated as the maximum likelihood value based on the independent readings from ROX and Alexa532 experiments. The CS represents 10 minus the sum of the squares of $z$ score of the consensus $\Delta G^0$ relative to the mean $\Delta G^0$ values measured from ROX and Alexa532; a CS of eight indicates that the consensus $\Delta G^0$ is within 1 s.d. of each mean $\Delta G^0$. See Supplementary Table 6 for summary results of all 32 single-nucleotide dangles.
on minimizing the sum of square $z$ scores. Mathematically,
\[
z_{\text{ROX}} = \frac{\Delta G^\circ_{\text{consensus}} - \Delta G^\circ_{\text{ROX}}}{\sigma_{\mu,\text{ROX}}}
\]
\[
z_{\text{A532}} = \frac{\Delta G^\circ_{\text{consensus}} - \Delta G^\circ_{\text{A532}}}{\sigma_{\mu,\text{A532}}}
\]
\[
\text{Error} = z^2_{\text{ROX}} + z^2_{\text{A532}}
\]
As a more intuitive metric of the agreement between the ROX and Alexa532, we define consensus score (CS) as:
\[
\text{CS} = 10 - \text{Error}
\]
The CS is a measure of how much two independently ordered sets of strands, using two distinct fluorophores, agree with each other in terms of inferred $\Delta G^\circ$ parameters, out of a maximum score of 10. CS $\geq 8$ indicates that the consensus $\Delta G^\circ$ is within 1 s.d. of both the ROX- and the Alexa-based $\Delta G^\circ$ values, and CS $\geq 0$ indicates that the consensus $\Delta G^\circ$ is within 2.24 s.d.'s.

Table 1 shows consensus $\Delta G^\circ$ values of all 32 single-nucleotide dangles at 25 and 37 °C in PBS, and at 25 °C in 12.5 mM Mg$^{2+}$. Also shown is the corresponding s.d.'s and CS for each $\Delta G^\circ$ parameter. Additional parameters are available for similar experiments performed at 10 and 45 °C in PBS and best fit $\Delta H^\circ$ and $\Delta S^\circ$ values (Supplementary Table 7). Using measured $\Delta G^\circ$ values in PBS at four different temperatures, we also were able to calculate best fit values of $\Delta H^\circ$ and $\Delta S^\circ$ for the dangle motifs explored. As with fluorophore $\Delta H^\circ$ and $\Delta S^\circ$ parameters, however, we caution the reader against too much reliance on these values, due to the poor quality of linear fits, and the possibility of temperature-dependent $\Delta H^\circ$ and $\Delta S^\circ$ values.$^{24-28}$

### Error analysis.
In the course of our experiments for the previous two subsections on characterizing fluorophore and single-nucleotide dangle $\Delta G^\circ$ values, we performed at least five fluorescent PAGE gels for each fluorophore parameter (corresponding to 10 independent $\Delta G^\circ$ measurements) and at least two fluorescent PAGE gels for each fluorophore-dangle parameter. These repeats were done to reduce the mean s.d. on $\Delta G^\circ$ values.

Figure 4a plots as a histogram the distribution of the mean s.d.'s of $\Delta G^\circ$ values for fluorophore interactions. $N = 80$ corresponds to the number of different $\Delta G^\circ$ parameters measured: (2 fluorophores; 5° or 3°; 4 nearest neighbour nucleotides; 5 temperature/buffer conditions). These were collected from 429 separate fluorescent PAGE gels corresponding to 858 individual $\Delta G^\circ$ values.

### Table 1 | Consensus $\Delta G^\circ$ of single-base dangles.

| $\Delta G^\circ$: 25 °C; PBS (kcal-mol$^{-1}$) | $\Delta G^\circ$: 37 °C; PBS | $\Delta G^\circ$: 25 °C; 12.5 mM Mg$^{2+}$ |
|---------------------------------------------|-----------------------------|---------------------------------------------|
| $^A$T                                      | -0.549 ± 0.045 (9.0)        | -0.647 ± 0.084 (7.1)                       |
| $^A$A                                      | -0.483 ± 0.038 (8.5)        | -0.368 ± 0.071 (4.7)                       |
| $^A$C                                      | -0.835 ± 0.042 (8.4)        | -0.635 ± 0.046 (9.6)                       |
| $^G$G                                      | -0.678 ± 0.107 (0.7)        | -0.632 ± 0.059 (7.0)                       |
| $^T$T                                      | -0.566 ± 0.111 (-2.5)       | -0.432 ± 0.086 (8.2)                       |
| $^T$G                                      | -0.404 ± 0.056 (8.5)        | -0.364 ± 0.015 (9.3)                       |
| $^C$C                                      | -0.627 ± 0.076 (4.2)        | -0.462 ± 0.064 (8.9)                       |
| $^C$G                                      | -0.515 ± 0.051 (8.5)        | -0.458 ± 0.038 (8.0)                       |
| $^G$T                                      | -0.279 ± 0.108 (2.6)        | -0.224 ± 0.123 (2.5)                       |
| $^G$A                                      | -0.042 ± 0.050 (9.8)        | 0.001 ± 0.041 (9.7)                        |
| $^G$G                                      | -0.519 ± 0.038 (10.0)       | -0.322 ± 0.094 (5.6)                       |
| $^C$C                                      | -0.603 ± 0.097 (6.6)        | -0.420 ± 0.065 (9.8)                       |
| $^C$G                                      | -0.389 ± 0.042 (9.9)        | -0.355 ± 0.062 (6.4)                       |
| $^G$T                                      | -0.121 ± 0.095 (8.9)        | -0.154 ± 0.052 (9.0)                       |
| $^G$A                                      | -0.576 ± 0.049 (8.0)        | -0.422 ± 0.056 (9.5)                       |
| $^G$G                                      | -0.703 ± 0.033 (9.7)        | -0.520 ± 0.088 (-7.9)                      |
| $^A$A                                      | -0.144 ± 0.045 (9.9)        | -0.011 ± 0.070 (9.4)                       |
| $^A$T                                      | -0.382 ± 0.077 (9.1)        | -0.195 ± 0.156 (4.4)                       |
| $^A$G                                      | -0.588 ± 0.063 (9.8)        | -0.659 ± 0.091 (7.0)                       |
| $^A$A                                      | -0.131 ± 0.058 (9.3)        | -0.118 ± 0.049 (10.0)                      |
| $^A$T                                      | 0.139 ± 0.135 (5.9)         | 0.183 ± 0.046 (10.0)                       |
| $^A$G                                      | 0.091 ± 0.072 (9.0)         | 0.223 ± 0.168 (6.0)                       |
| $^A$A                                      | -0.261 ± 0.089 (4.1)        | 0.003 ± 0.115 (8.0)                       |
| $^A$T                                      | 0.398 ± 0.062 (9.2)         | 0.264 ± 0.077 (8.0)                       |
| $^A$G                                      | 0.310 ± 0.101 (7.7)         | 0.485 ± 0.060 (10.0)                      |
| $^G$G                                      | 0.036 ± 0.092 (10.0)        | 0.107 ± 0.167 (3.8)                       |
| $^G$C                                      | -0.155 ± 0.073 (9.4)        | -0.081 ± 0.102 (8.5)                       |
| $^G$G                                      | 0.025 ± 0.069 (7.8)         | 0.034 ± 0.090 (9.2)                       |
| $^G$C                                      | 0.093 ± 0.036 (10.0)        | 0.203 ± 0.063 (9.6)                       |
| $^G$G                                      | -0.278 ± 0.125 (0.5)        | -0.066 ± 0.121 (8.0)                      |
| $^G$C                                      | -0.625 ± 0.046 (9.9)        | -0.544 ± 0.142 (-3.0)                     |
| $^G$G                                      | 0.046 ± 0.056 (10.0)        | 0.067 ± 0.077 (8.0)                       |

CS, consensus scores; ROX, carboxy-X-rhodamine.
Shown in parentheses are CS, a measure of internal agreement between our two independent sets of experiments based on the ROX and the Alexa532 fluorophores.
The last closing base pair of a duplex is shown in blue and the dangle nucleotide is shown in red.
values measured. All 80 dangle parameters’ mean s.d.’s were below 0.15 kcal mol$^{-1}$, with a mean of 0.046 kcal mol$^{-1}$ and a s.d. (on the mean s.d.) of 0.021 kcal mol$^{-1}$.

Similarly, Fig. 4b plots as a histogram the distribution of the mean s.d.’s of the $\Delta G^o$ values for $N = 320$ single-nucleotide dangle parameters: (2 fluorophores; 5' or 3'; 4 dangle nucleotides; 4 nearest neighbour nucleotides; 5 temperature/buffer conditions). These were collected from 723 separate fluorescent PAGE gels corresponding to 1446 individual $\Delta G^o$ values measured. The mean and s.d. of the parameter s.d.’s here were 0.072 kcal mol$^{-1}$ and 0.033 kcal mol$^{-1}$, respectively, and all parameter s.d.’s were below 0.25 kcal mol$^{-1}$. These parameter s.d. values are larger than those in Fig. 4a both because they include the variance from fluorophores and because of the relatively fewer independent $\Delta G^o$ measurements per parameter (average 4.52 per dangle parameter, compared with average 10.73 per fluorophore parameter).

Figure 4c plots the dangle $\Delta G^o$ parameters measured using the Alexa532 against the same parameters measured using ROX. The Alexa532- and ROX-based measurements of the dangle motif $\Delta G^o$ represent independent experiments and assays, agreement between the final dangle $\Delta G^o$ values (high CS) indicates high confidence in our methodology and consensus $\Delta G^o$ value. Parameters with CS > 8 generally have no >0.1 kcal mol$^{-1}$ deviation between the ROX and Alexa532 measurements.

We observed 14 parameters (out of 160) with CS ≤ 0 (Supplementary Table S9-1), whereas a Gaussian distribution of errors would typically result in 4 out of 160 parameters (2.5%) possessing CS ≤ 0. Except one outlier at −37.1, CS values ranged between −8.7 and 10.0, with 91% of values being positive and 50% of values being 8 or higher. Parameters with low CS likely imply that one or more assumptions our methodologies required were incorrect for these parameters. For example, it is possible that the equilibrium concentrations of CZ are different for the corresponding experiments using the two different fluorophores, resulting in bias in one or both $\Delta G^o$ values. We believe that the consensus parameters with CS ≥ 8 are likely to be objectively correct, because while any of a number of problems could cause the mean $\Delta G^o$ measured from Alexa532 to differ from that from ROX, it is unlikely for these values to be coincidentally similar to within 0.1 kcal mol$^{-1}$ while being wrong.

Figure 4d plots the literature single-nucleotide dangle parameters by Bommarito et al. against our consensus single-nucleotide dangle parameters at 37 °C. There is relatively little agreement between our values and Bommarito’s values; only 9 out of 32 dangle parameters agreed to within 0.1 kcal mol$^{-1}$. The mean absolute error between our consensus values and Bommarito’s values was 0.269 kcal mol$^{-1}$, and the root mean square error between them was 0.333 kcal mol$^{-1}$.

Furthermore, there is little correlation between our parameters’ CS and their agreement with Bommarito’s values. Our consensus 5’(GT) C $\Delta G^o$ has CS = 9.0, and is 0.601 kcal mol$^{-1}$ below Bommarito’s value, while our consensus 3’(GA) C $\Delta G^o$ has CS = 10.0, and is 0.802 kcal mol$^{-1}$ above Bommarito’s value. Because our method offers a more direct measure of dangle $\Delta G^o$ parameters, compared to Bommarito’s melt curve methods that had to deconvolute the thermodynamic contributions of many base stacks from that of dangles, we generally believe our parameters to be more accurate.

Another contribution to the discrepancy may be the difference in the ‘core’ sequences (for example, all nucleotides except the dangle and nearest neighbour, in our case) used for the two papers. The standard nearest neighbour model assumes that the identities of distal bases should have no effect on the motif thermodynamics, but both our own analyses (not shown) and experimental studies on coaxial dangles suggest that this simplifying assumption may not be true.
First dangle nt forms stabilizing partial stack

Additional dangle nt adds destabilizing electrostatics (diminishing marginal effects)

| Length (nt) | Approx. ΔG° (kcal mol⁻¹) |
|------------|---------------------------|
| 1          | ΔG°₁ +0.13                 |
| 2          | ΔG°₂ +0.30                 |
| 3          | ΔG°₃ +0.48                 |
| 4          | ΔG°₄ +0.51                 |
| 5          | ΔG°₅ +0.55                 |
| 6          | ΔG°₆ +0.53                 |
| 7          | ΔG°₇ +0.58                 |
| 11         | ΔG°₁₁ +0.56                |
| 13         | ΔG°₁₃ +0.56                |
| 16         | ΔG°₁₆ +0.56                |
| 21         | ΔG°₂₁ +0.58                |

Figure 5 | Thermodynamics of multinucleotide dangles. (a) The standard model of DNA hybridization considers only the effects of the first dangle nucleotide, which is known to potentially form a partial stack with the last base pair of the adjoining helix. From a biophysics point of view, additional nucleotides of dangle likely contribute a destabilizing effect due to the electrostatic repulsion from the additional dangle bases and the nucleotides on the complementary strand. (b) Consensus ΔG° for 5′ multinucleotide dangles of different lengths at 25 °C in PBS. Each plotted data point represents the consensus value from experiments using ROX and using Alexa532, as in Fig. 3. (c) Consensus ΔG° for 3′ multinucleotide dangles of different lengths at 25 °C in PBS. (d) Approximate ΔG° of multinucleotide dangles based on length and ΔG° of the single-nucleotide dangle. (e) Consensus ΔG° for 5′ multinucleotide dangles of different lengths at 25 °C in PBS. The first dangle base was fixed to be A; the remainder were homopolymers of A, T, or C. (f) Comparison of multinucleotide dangle thermodynamics based on native catalysis versus melt curves. Identical buffers and oligonucleotides were used for the two sets of experiments. Inferred parameters are consistent with each other to within 2 s.d.’s, but melt curve-based parameters show 6- to 12-fold higher errors than the native catalysis method.

There is also a systematic bias in comparing the two sets of dangle parameters; on average, our consensus dangles parameters were 0.190 kcal mol⁻¹ more positive. One possible contributing factor to the systematic bias is that Bommarito’s experiments were performed in 1 M Na⁺, whereas our experiments were in the physiological PBS (0.15 M Na⁺) buffer. The standard model of DNA hybridization assumes that dangles ΔG° contributions are not affected by salinity, but we believe this may be incorrect.

Multinucleotide dangles. We next applied our native motif characterization method to the study of multinucleotide dangles. The standard model of DNA hybridization used by most commonly used software33,36,40 considers only the effects of the first dangle nucleotide. Melt studies on multinucleotide dangles61–64 reported inconsistent findings, with some reporting stabilizing effects of long dangles and others reporting destabilizing effects. As far as we are aware, there have not been any systematic studies on the effects of dangle length on ΔG°.

From a theoretical point of view, we believe that the first dangle nucleotide contributes two opposing effects on the stability of nearby duplexes (Fig. 5a). First, it can form a partial base stack with the terminal base pair, adding stability by increasing the interactions between the aromatic groups in the nuclease bases65. Second, the negatively charged phosphodiester backbone is brought in close proximity to the negatively charged phosphodiester backbone of the complementary strand, reducing stability by increasing electrostatic repulsion. In buffers such as PBS or 12.5 mM Mg²⁺, the distances involved (0.43 nm for the dangle plus 2.36 nm for the B-DNA helix) are comparable to the Debye length (estimated to be roughly 2 nm (ref. 66)).

Except in the case where the dangle forms significant secondary structure, additional dangle nucleotides past the first do not contribute further base stacking, but continue to increase electrostatic repulsion, albeit with diminishing impact due to increasing distances involved. Consequently, we hypothesized that duplexes with longer dangles will be destabilized relative to single-nucleotide dangles. To verify our hypothesis, we measured ΔG° for multinucleotide poly-T dangles with dangle length between 2 and 21. We chose to use poly-T, poly-A and poly-C multinucleotide dangles to ensure that there would be no secondary structure in the dangle. Because the first dangle nucleotide also contributes the partial base stack, we performed experiments for all four nucleotides as the first dangle nucleotide. We did not do any experiments on multinucleotide dangles with non-homogeneous sequence, because the secondary structure of dangles would have introduced additional complicating thermodynamics terms.

Figure 5b shows the consensus ΔG° values we measured for 5′ multinucleotide dangles, based on independent experiments with ROX and with Alexa532. For poly-T dangles of >8 nt, the value of ΔG° appears to asymptotically approach +0.56 kcal mol⁻¹ above that of the single-nucleotide dangle ΔG°. Figure 5c shows the consensus ΔG° values we measured for 3′ multinucleotide dangles; all values follow similar trend versus dangle length, although all ΔG° values are more positive due to the weaker stabilizing effect of the first dangle nucleotide closest to the duplex. Figure 5d shows the averaged, smoothed value of multinucleotide dangle ΔG° compared with single-base dangle...
of interest rather than rely on linear algebra decomposition, and we cross-confirmed our \( \Delta G^0 \) across two independent sets of fluorophores. For all these reasons, we believe our values to be more accurate.

To the extent that there may be differences in the true values of the dangle \( \Delta G^0 \), such differences may reflect a limitation of nearest neighbour model itself. A more complex model considering distal base identities to holistically evaluate nucleic acid structure stability may be warranted, but would also require significantly more experiments on multiple core sequence instances of the DNA motifs.

From simple biophysics, we postulated that multinucleotide dangles should contribute an increasing destabilizing thermodynamic effect, towards an asymptotic limit. We confirmed this hypothesis experimentally, and discovered that the saturation length is roughly 8 nt, with \( \Delta G^0 \) roughly 0.56 kcal mol \(^{-1} \) more positive than a single-nucleotide dangle. We suggest that dangle length should be part of the standard model for DNA hybridization and folding software, and that the table in Fig. 5d be used as an empirical formula for estimating multinucleotide dangle \( \Delta G^0 \) values. However, we currently do not have a quantitative biophysical model that predicts our measured multinucleotide dangle thermodynamic parameters, and encourage interested parties to explore area of research, for example using molecular dynamics simulations.

Performing PAGE is more labour-intensive than running a melt curve, and this is a disadvantage of our overall workflow. However, our approach generated motif \( \Delta G^0 \) parameters with s.d.’s 6- to 14-fold lower than those obtained from HRM experiments. Statistically, 36- to 196-fold more experimental repeats of HRM would be needed to generate parameters of the same precision. In addition, PAGE experiments could potentially be automated, and other readout mechanisms may be possible. Possible approaches for downstream readout include liquid chromatography and/or microfluidics approaches. Scaling the presented method will be vital in obtaining high accuracy \( \Delta G^0 \) parameters for a variety of motifs in a variety of temperatures and buffer conditions. For example, many artificial nucleotides with desirable properties, such as 2′-O-methyl RNA \(^{68} \), locked nucleic acids \(^{69} \), and peptide nucleic acids \(^{70,71} \), lack characterization beyond rudimentary rule-of-thumb melting temperature effects from base stacks. Establishment of an accurate and comprehensive nucleic acid motif thermodynamics database will help usher a new paradigm of knowledge-driven design of nucleic acid biotechnology and nanotechnology.

Methods

Reagents. All DNA oligonucleotides (oligos) were synthesized by and purchased from Integrated DNA Technologies (IDT; Coralville, IA). All oligos were HPLC purified by IDT, and quality controlled by capillary electrophoresis and electrospray ionization mass spectrometry. All oligos were received in solution form at roughly 100 \( \mu \)M concentration in 10 mM Tris-EDTA (pH 8.0) after HPLC purification. Secondary stocks were prepared by diluting DNA samples in 10 mM TE buffer with 12.5 mM MgCl₂ (pH 8.0) or PBS (10 mM, NaCl 154 mM pH 7.4). Oligo sequences are listed in Supplementary Tables 28–40. Single stranded 10-nt resolution DNA ladder was purchased from IDT. 10–330-bp double strand ladder was purchased from Thermo Scientific.

Chemicals and buffer used include: ammonium persulfate, magnesium chloride hexahydrate (≥ 98%), Tween 20 (viscous liquid), glycerol (≥ 99%), acrylamide and bis-acrylamide (19:1) ratio stock solution (m/v concentration of 40%), and TEMED (N,N,N',N'-tetramethylethylenediamine) (≥ 99%). All chemicals were purchased from Sigma Aldrich unless otherwise noted. SYBR gold gel stain was supplied as 10,000x concentrated in DMSO from Life technologies. SYTO92 orange fluorescent nucleic acid stain was supplied as 5 mM solution in DMSO from Life technologies. All the experiments were carried out in PBS (phosphate buffer saline) buffer (diluted from purchased 10x PBS) or 10mM TE 12.5mM MgCl₂ buffer which is prepared from 100x TE buffer (1.0M Tris-Cl pH 8.0, 0.1M EDTA) and solid magnesium chloride hexahydrate. All the reactions were performed in Peltier-temperature controlled thermocyclers. All samples were covered with aluminium foil during hybridization reaction to avoid potential photobleaching.
Hybridization and displacement reactions. Before analysis by gel electrophoresis, oligonucleotide molecules were allowed to undergo isothermal hybridization and/or strand displacement. All reactions occurred in 650 μl microcentrifuge tubes (Corning), in 50 μl total reaction volume; 1x concentration corresponds to 300 nM final concentration in all cases. Samples were incubated at the reported temperatures for either 20 h (1x PBS) or 3 h (TE-MgCl₂). A multi-thermocycler (Benchmark) was used for temperature control for reactions at 10 and 25 °C. A MasterCycler Personal (Eppendorf) with a heated lid (95 °C) was used for temperature control for reactions at 37 and 45 °C. All temperatures were verified to be accurate within 0.2 °C using a thermocouple sensor.

After completion of the hybridization and just prior to loading into gel, 15 μl of 30% glycerol (volume to volume) were added to each 50 μl sample to facilitate gel loading. We elected to use 30% glycerol rather than conventional gel loading buffer because the bromophenol blue or xylene cyanol FF chemicals appear to give significant signal in the ROX fluorescence channel; by using glycerol instead, we were able to minimize background signal.

Polyacrylamide gel preparation. Polyacrylamide gels (10%) were used in all experiments; 50 μl of acrylamide solution were prepared by mixing 32.0 μl (40% Bi-Rad) and 12.5 μl (40% Bio-Rad) stock solution. A solution of 300 μl of 10% ammonium persulfate (m/v) and 28 μl TEMED were subsequently added to the acrylamide solution. Roughly 10 μl of the final solution was transferred into each 1.5 mm thick XCell SureLock gel cassette (Invitrogen) using 5 μl transfer pipettes. Ten-well gel combs (Invitrogen) were inserted into the cassettes to seal the gel from air and form the desired number of wells. Gels were allowed to polymerize for ~40 min before samples were loaded.

Polyacrylamide gel electrophoresis. All PAGE experiments were performed using a Model 85–1010 gel box (Galileo Bioscience), with temperature regulated by an external water bath (Cleaver). For most experiments, 1x TAE was used as running buffer; 1x TAE and 12.5 mM MgCl₂ was used to verify that reduced salinity in running buffer did not significantly change inferred ΔG° values. Running buffer was prepared by dilution of 10X TAE buffer from Invitrogen. The gel running buffer was always pre-incubated to the desired temperature before samples were loaded. The gel apparatus was connected to a water bath to control temperature of inner wells. Polyacrylamide gel electrophoresis experiments; 50 μl of acrylamide solution were prepared by mixing 32.0 μl (32% Bio-Rad) and 12.5 μl (12.5% Bio-Rad) stock solution. A solution of 300 μl of 10% ammonium persulfate (m/v) and 28 μl TEMED were subsequently added to the acrylamide solution. Roughly 10 μl of the final solution was transferred into each 1.5 mm thick XCell SureLock gel cassette (Invitrogen) using 5 μl transfer pipettes. Ten-well gel combs (Invitrogen) were inserted into the cassettes to seal the gel from air and form the desired number of wells. Gels were allowed to polymerize for ~40 min before samples were loaded.

Post-electrophoresis gel handling. At the end of electrophoresis experiments, the gel cassettes were cracked open using a gel cutter, and the gels were directly transferred to a Typhoon 9500 quantitative gel imager (GE Healthcare) for fluorescence imaging. For control experiments comparing observed bands to a DNA ladder, gels were further stained with 0.01% SYBR gold. The gel was transferred to staining solution (5 μl of SYBR gold into 50 μl of water), and stained for 30 min on a shaker. Then the gel was gently rinsed in deionized water for three times and transferred to scanner for imaging. Gels were scanned at 100 μm resolution with 475/520 nm excitation and 520/570 nm emission wavelengths. Images were saved in both BMP and gel format for subsequent software analysis.

Gel band quantitation and analysis. Gel images were analysed using the version 8.1 Image Quant TL 1D gel analysis software (GE Healthcare). Gel bands were automatically detected by the software, fluorescence background was subtracted and fluorescence intensity of bands were reported by normalizing the single-band to 100 arbitrary units (Supplementary Note 1). By default, lanes were defined to be full-width centred on each visible lane, and a ‘rolling ball’ background subtraction was used.

HRM experiments. SYTO 82 was used as reporter dye for HRM experiments in either 1x PBS or Tris-MgCl₂ buffer. 5p-Comp-T (300 nM) and the corresponding dangle strand (5'-A, 5'-T1A, 5'-T3A, 5'-T10A, 5'-T20A, 5'-A1A, 5'-A3A, 5'-A10A, or 5'-A20A) were mixed with 5 μl of SYTO 82, respectively. Each 10 μl sample was transferred to a quantitative PCR plate (Hard Shell PCR Plates 96-well thin-wall) for the melt experiment (CFX96 Real-Time System with C1000 Touch, Bio-Rad). The samples were heated to 95 °C, then cooled down to 30 °C, holding 10 s at each integer degree Celsius, with a ramp speed of 0.2 °C per sec. The melting process was measured by the fluorescence of SYTO 82. Melting temperatures determination and thermodynamic parameters calculation were described in Supplementary Note 11.
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Author contributions

C.W. designed and conducted the experiments, analysed the data, and wrote the paper. J.H.B. analysed the data and wrote the paper. D.Y.Z. conceived the project, designed the experiments, analysed the data and wrote the paper.

Additional information

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