Table of Contents

Supplemental Table 1 ................................................................................................................................... 2
Supplemental Table 2 ................................................................................................................................... 5
Supplemental Table 3 ................................................................................................................................... 7
Supplemental Table 4 ................................................................................................................................... 10
Supplemental Table 5 ................................................................................................................................... 11
Supplemental Table 6 ................................................................................................................................... 12
Supplemental Table 7 ................................................................................................................................... 13
Supplemental Table 8 ................................................................................................................................... 14
Supplemental Table 9 ................................................................................................................................... 15
Supplemental Figure 1 ................................................................................................................................ 16
Supplemental Figure 2 ................................................................................................................................ 17
Supplemental Figure 4 ................................................................................................................................ 19
Supplemental Figure 5 ................................................................................................................................ 20
Supplemental Figure 6 ................................................................................................................................ 21
Supplemental Reaction Scheme 1 .............................................................................................................. 22
Example NN calculation .............................................................................................................................. 23
Designing PCR thermocycling profiles ........................................................................................................ 24
  Example 1 ............................................................................................................................................ 25
  Example 2 ............................................................................................................................................ 32
  Example 3 ............................................................................................................................................ 34
  Example 4 ............................................................................................................................................ 35
  Example 5 ............................................................................................................................................ 37
### Supplemental Table 1: Duplex Sequences and Experimental Conditions

| Duplex Sequence (5′-3′) | 1.0 M NaCl | 2.2 mM MgCl₂ |
|------------------------|------------|--------------|
|                        | Concentration Range (μM) | Temperature Range (°C) | Concentration Range (μM) | Temperature Range (°C) |
| CCATCGCTACC            | 0.4-1.6    | 37.5-52.3   | 0.5-1.5    | 31.8-52.3   |
| GGAACCTTGATGC          | 0.4-1.6    | 46.4-59.1   | 0.5-1.5    | 31.9-52.5   |
| GGAACAAGATGC           | 0.4-1.6    | 40.5-54.3   | 0.5-1.5    | 36.0-52.3   |
| CTTCCCTCCTTC           | 0.5-1.5    | 36.2-56.7   | 0.5-1.5    | 27.2-49.5   |
| CAACCAACCAAC           | 0.5-1.5    | 36.7-58.9   | 0.5-1.5    | 36.0-53.4   |
| CCATTGCTACC            | 0.4-1.6    | 34.9-52.8   | 0.5-1.5    | 27.5-49.5   |
| GGAGCAGC               | 0.5-1.5    | 30.7-44.6   | 0.5-1.5    | 24.4-42.1   |
| CACGGCTC               | 0.5-1.5    | 24.8-48.9   | 0.5-1.5    | 24.5-42.1   |
| CGTCTGCC               | 0.4-1.6    | 24.3-38.4   | 0.5-1.5    | 24.4-42.1   |
| ACCGCA                 | 0.5-3.0    | 13.1-28.1   | 1.0-3.0    | 11.3-30.1   |
| CGGACG                 | 0.5-1.5    | 11.1-30.0   | 1.0-3.0    | 11.3-27.3   |
| CGGTCG                 | 0.5-1.5    | 9.4-30.0    | 1.0-3.0    | 11.3-30.3   |
| CGTGC                  | 0.5-2.0    | 12.1-33.7   | 0.5-1.5    | 12.3-28.2   |
| GCCTGC                 | 0.5-1.5    | 12.1-29.4   | 1.0-3.0    | 11.2-30.3   |
| AGCCGTG                | 0.5-1.5    | 21.5-37.6   | 1.0-3.0    | 20.4-42.5   |
| AAGCGTAG               | 0.5-1.5    | 21.5-37.6   | 0.5-1.5    | 15.0-34.2   |
| AATCCAGT               | 0.5-1.5    | 14.3-34.4   | 1.0-3.0    | 11.3-30.5   |
| ACCTAGTC               | 0.5-1.5    | 17.8-34.4   | 0.5-1.5    | 15.0-30.0   |
| ACGACCTC               | 0.5-1.5    | 21.5-46.3   | 1.0-3.0    | 23.3-42.4   |
| AGAGAGAGAG             | 0.5-1.5    | 11.9-29.9   | 1.0-3.0    | 11.3-30.5   |
Supplemental Table 1 continued from previous page

| Duplex Sequence (5’-3’)a | 1.0 M NaCl | | | 2.2 mM MgCl₂ | | |
|-------------------------|-----------|-----------|-----------|-----------|-----------|-----------|
|                     | Concentration Range (μM) | Temperature Range (°C) | Concentration Range (μM) | Temperature Range (°C) |
| AGCGTAAG          | 0.5-1.5   | 21.5-43.4 | 0.5-1.5   | 18.3-37.5 |
| AGTCCTGA          | 0.5-1.5   | 23.3-37.7 | 0.5-1.5   | 15.0-34.2 |
| CGCTGTAA          | 0.5-1.5   | 25.2-37.2 | 0.5-1.5   | 12.6-32.1 |
| CTAGTGGA          | 0.5-1.5   | 18.3-33.4 | 0.5-1.5   | 12.5-30.0 |
| CTCACGGGC         | 0.5-1.5   | 25.2-43.1 | 0.5-1.5   | 24.4-42.1 |
| CTGAGTCC          | 0.5-1.5   | 21.1-41.5 | 0.5-1.5   | 14.8-32.0 |
| GCCAGTTA          | 0.5-1.5   | 19.6-35.6 | 0.5-1.5   | 15.0-34.2 |
| GGACCTCG          | 0.5-1.5   | 21.2-43.0 | 1.0-3.0   | 27.2-41.4 |
| GGTGCCAA          | 0.5-1.5   | 25.2-42.9 | 1.0-3.0   | 27.3-41.4 |
| GTCGAACA          | 0.5-1.5   | 25.2-39.6 | 0.5-1.5   | 18.3-37.4 |
| ATAACTGGGC        | 0.5-1.5   | 18.5-43.0 | 0.5-1.5   | 18.3-40.5 |
| ATCTATCCG         | 0.5-1.5   | 24.4-43.1 | 0.5-1.5   | 21.9-37.4 |
| CAAAAAAG          | 0.5-1.5   | 23.8-35.3 | 0.5-3.0   | 14.6-30.4 |
| CAAACAAG          | 0.5-1.5   | 14.9-27.8 | 0.5-1.5   | 17.1-36.5 |
| CAAAGAAAG         | 0.5-1.5   | 15.3-27.8 | 0.5-1.5   | 17.1-33.2 |
| CAAATAAAG         | 0.5-1.5   | 13-31.6   | 0.5-1.5   | 12.1-29.8 |
| CGCTGTATAC        | 0.5-1.5   | 17.7-40.1 | 0.5-1.5   | 24.5-42.1 |
| GCCAGTTAA         | 0.5-1.5   | 18.2-43.2 | 0.5-1.5   | 23.5-39.4 |
| AAAAAAAAAAA       | 0.5-1.5   | 21.5-34.5 | 0.5-1.5   | 12.0-30.0 |
| TAGGTTTATAA       | 0.5-1.5   | 21.3-39.4 | 0.5-1.5   | 11.8-34.3 |
| Duplex Sequence (5’-3’)<sup>a</sup> | 1.0 M NaCl | | 2.2 mM MgCl<sub>2</sub> | |
|---|---|---|---|---|
| | Concentration Range (μM) | Temperature Range (°C) | Concentration Range (μM) | Temperature Range (°C) |
| ACGTATTATGC | 0.5-1.5 | 29.3-46.0 | 0.5-1.5 | 27.2-44.5 |
| ACATTATTATA | 0.5-1.5 | 37.2-52.4 | 0.5-1.5 | 27.2-44.4 |
| CAACTTGATATTATA | 0.5-1.5 | 36.2-56.6 | 0.5-1.5 | 27.2-52.3 |

<sup>a</sup>The complementary strand is not shown.
### Supplemental Table 2: Transition-state parameters with $\Delta H_a^T$

| Duplex Sequence (5'-3') | 1.0 M NaCl<sup>b</sup> | 2.2 mM MgCl<sub>2</sub><sup>b</sup> |
|------------------------|------------------------|------------------------|
|                        | $\Delta H_a$ (kcal/mol) | $\Delta S_a$ (cal/mol/K) | $\Delta H_d$ (kcal/mol) | $\Delta S_d$ (cal/mol/K) | $\Delta H_a$ (kcal/mol) | $\Delta S_a$ (cal/mol/K) | $\Delta H_d$ (kcal/mol) | $\Delta S_d$ (cal/mol/K) |
| CCATCGCTACC            | -0.3 ± 1.8             | -26.9 ± 6.3             | 87.7 ± 7.3              | 215.8 ± 23.3             | 0.9 ± 2.1               | -25.7 ± 7.1             | 97.6 ± 8.1              | 248.0 ± 26.7             |
| GGAACCTTGATGC          | 4.4 ± 6.8              | -13.5 ± 9.4             | 87.3 ± 28.0             | 212.8 ± 28.0             | 0.7 ± 2.0               | -28.0 ± 7.2             | 95.4 ± 8.1              | 241.3 ± 20.0             |
| GGAACAAGATGC           | -4.1 ± 4.7             | -39.6 ± 14.2            | 91.2 ± 29.8             | 225.8 ± 37.4             | -1.9 ± 3.7              | -36.2 ± 12.7            | 98.3 ± 8.1              | 251.1 ± 27.1             |
| CTTCCTTCCCTC           | -1.4 ± 4.8             | -30.4 ± 16.6            | 80.7 ± 20.8             | 197.1 ± 30.5             | -1.7 ± 3.2              | -34.7 ± 10.8            | 87.2 ± 8.7              | 219.2 ± 21.0             |
| CAACCAACCAAC           | -3.6 ± 2.5             | -36.5 ± 9.9             | 91.0 ± 34.6             | 224.6 ± 36.0             | -4.8 ± 12.0             | -44.0 ± 10.1            | 104.1 ± 26.8            | 268.1 ± 28.9             |
| CCATTGCTACC            | -2.4 ± 2.6             | -33.7 ± 8.6             | 78.7 ± 21.2             | 191.1 ± 21.2             | 0.5 ± 1.8               | -27.6 ± 7.0             | 87.6 ± 8.2              | 220.4 ± 18.2             |
| GGAGCAG                | -6.6 ± 5.4             | -46.3 ± 16.6            | 58.7 ± 23.4             | 134.7 ± 30.5             | -0.8 ± 10.8             | -30.3 ± 10.8            | 67.3 ± 8.2              | 162.2 ± 21.4             |
| CACGGGCTC              | -3.1 ± 3.6             | -34.8 ± 12.4            | 55.1 ± 23.0             | 123.0 ± 26.0             | 0.0 ± 2.6               | -27.2 ± 9.8             | 64.9 ± 7.0              | 153.6 ± 23.7             |
| CGTGTCC                | -1.9 ± 2.4             | -31.5 ± 8.5             | 59.8 ± 18.2             | 138.4 ± 30.5             | 1.6 ± 10.0              | -22.8 ± 7.4             | 68.8 ± 8.2              | 166.6 ± 22.2             |
| ACCGCA                 | -2.9 ± 5.1             | -34.2 ± 19.4            | 37.3 ± 24.5             | 75.4 ± 33.3             | 1.3 ± 11.0              | -21.3 ± 10.8            | 41.8 ± 9.4              | 89.4 ± 13.8             |
| CGGACG                 | 1.1 ± 4.5              | -20.9 ± 24.2            | 41.9 ± 29.3             | 89.9 ± 39.3             | 9.1 ± 14.5              | 4.5 ± 19.7             | 49.7 ± 14.8             | 114.8 ± 21.4             |
| CCGTGC                 | 4.5 ± 5.4              | 34.3 ± 17.5             | 9.1 ± 29.3              | 23.0 ± 9.8               | 2.6 ± 10.9              | 9.8 ± 7.0              | 7.0 ± 2.3               | 23.7 ± 4.6              |
| CGGTGC                 | 5.4 ± 4.0              | 19.3 ± 16.4             | 8.8 ± 27.1              | 38.0 ± 27.1             | 1.3 ± 10.0              | 27.1 ± 8.7             | 10.0 ± 2.6              | 20.5 ± 4.0              |
| CGTGCC                 | 0.0 ± 4.1              | -25.0 ± 14.4            | 38.0 ± 18.2             | 75.4 ± 21.5             | 1.3 ± 11.0              | -23.0 ± 10.8            | 47.2 ± 9.3              | 105.7 ± 29.6             |
| GCCTGC                 | 4.1 ± 5.0              | 14.4 ± 24.2             | 5.7 ± 21.7              | 21.4 ± 5.5              | 5.5 ± 10.8              | 19.6 ± 4.5             | 9.3 ± 2.1               | 29.6 ± 4.6              |
| AGCCGTG                | 5.0 ± 6.6              | 424.2 ± 53.3            | 53.3 ± 120.0            | 0.7 ± 24.2              | 0.7 ± 15.9              | 56.6 ± 131.8           | 131.8 ± 14.2            |
| AAGCGTAC               | 3.8 ± 5.9              | -13.9 ± 16.3            | 55.6 ± 22.7             | 129.2 ± 27.2             | 7.8 ± 10.5              | -4.0 ± 10.5             | 57.7 ± 13.6             | 136.0 ± 14.7             |
| AATCCGTAG              | 5.9 ± 2.7              | 16.3 ± 8.0              | 8.0 ± 22.7              | 20.5 ± 3.2              | 3.2 ± 10.5              | 10.5 ± 4.8             | 4.8 ± 14.7             |
| ACCTAGTC               | 3.0 ± 6.0              | -13.6 ± 5.3             | 49.7 ± 113.1            | 13.3 ± 17.6             | -17.6 ± 11.0            | 51.8 ± 120.8           | 128.0 ± 10.3            |
| AGCACTCTG              | 6.0 ± 5.0              | -5.3 ± 16.3             | 51.4 ± 119.0            | 5.4 ± 10.5              | -0.5 ± 15.9             | 55.7 ± 133.1           | 131.3 ± 17.3            |
| AGACCTTCG              | 5.0 ± 4.5              | 17.3 ± 21.0             | 5.7 ± 21.0              | 21.0 ± 4.2              | 4.2 ± 13.3              | 12.3 ± 6.0             | 6.0 ± 17.3             |
| AGAGAGAC               | 0.5 ± 3.2              | -22.9 ± 12.3            | 52.5 ± 116.8            | -0.1 ± 27.4             | -27.4 ± 59.4            | 59.4 ± 139.0           |
| AGAGCTGGAG             | 2.1 ± 4.0              | -18.3 ± 8.8             | 50.4 ± 114.5            | 0.7 ± 25.9              | -25.9 ± 55.1            | 51.8 ± 130.7           |
| AGCGTAAG               | 3.4 ± 5.7              | -16.1 ± 7.9             | 55.9 ± 130.1            | 8.4 ± 6.3              | -2.3 ± 16.3             | 63.1 ± 153.4           |
| AGTCCCTGA              | -1.7 ± 6.8             | -31.5 ± 26.5            | 50.2 ± 112.9            | 2.8 ± 18.8              | -18.8 ± 55.9            | 59.9 ± 131.4           |
| AGCAGCTTAG             | -6.8 ± 6.8             | 26.5 ± 7.7              | 7.7 ± 24.3              | 3.1 ± 10.3              | 4.6 ± 17.3             | 6.0 ± 17.3             |
| Duplex Sequence (5'-3')<sup>a</sup> | 1.0 M NaCl<sup>b</sup> | 2.2 mM MgCl<sub>2</sub><sup>b</sup> |
|-----------------------------|-----------------|-----------------|
|                            | ΔH<sub>i</sub>  | ΔS<sub>i</sub>  | ΔH<sub>d</sub>  | ΔS<sub>d</sub>  | ΔH<sub>i</sub>  | ΔS<sub>i</sub>  | ΔH<sub>d</sub>  | ΔS<sub>d</sub>  |
|                            | (kcal/mol)       | (cal/mol/K)     | (kcal/mol)       | (cal/mol/K)     | (kcal/mol)       | (cal/mol/K)     | (kcal/mol)       | (cal/mol/K)     |
| CGCTGTAA                   | 2.9 ± 0.5       | -16.3 ± 1.5     | 57.5 ± 2.2       | 135.9 ± 4.3     | 0.4 ± 0.5       | -27.6 ± 1.5     | 56.3 ± 2.2       | 132.1 ± 4.3     |
| CTAGTGGA                   | 3.5 ± 0.9       | -13.9 ± 2.9     | 51.6 ± 3.8       | 119.1 ± 5.2     | 8.2 ± 0.9       | -0.4 ± 0.9      | 55.7 ± 2.9       | 132.9 ± 5.2     |
| CTCACGGC                   | 7.0 ± 3.5       | -2.7 ± 1.5      | 81.7 ± 3.8       | 207.4 ± 5.2     | 5.8 ± 1.5       | -9.5 ± 1.5      | 71.4 ± 3.8       | 174.8 ± 5.2     |
| CTGAGTCC                   | 0.9 ± 0.4       | -22.9 ± 1.5     | 56.7 ± 2.2       | 131.5 ± 4.3     | 3.0 ± 0.5       | -18.8 ± 1.5     | 59.0 ± 2.2       | 139.1 ± 4.3     |
| GCCAGTAA                   | -0.1 ± 0.1      | -25.5 ± 1.5     | 52.0 ± 2.2       | 117.8 ± 4.3     | 1.9 ± 0.5       | -21.4 ± 1.5     | 56.7 ± 2.2       | 134.1 ± 4.3     |
| GGACCTCG                   | 1.5 ± 0.5       | -20.3 ± 1.5     | 53.9 ± 2.2       | 120.7 ± 4.3     | 1.6 ± 0.5       | -22.8 ± 1.5     | 59.9 ± 2.2       | 139.6 ± 4.3     |
| GGTGCAA                     | 1.6 ± 0.9       | -20.1 ± 1.5     | 59.1 ± 2.2       | 137.8 ± 4.3     | 3.5 ± 0.5       | -17.1 ± 1.5     | 60.4 ± 2.2       | 142.7 ± 4.3     |
| GTGAAAC                     | 1.1 ± 0.4       | -22.7 ± 1.5     | 60.1 ± 2.2       | 141.7 ± 4.3     | 5.0 ± 0.5       | -12.5 ± 1.5     | 64.5 ± 2.2       | 156.8 ± 4.3     |
| ATAACTG GCC                 | 0.5 ± 0.5       | -23.4 ± 1.5     | 64.5 ± 2.2       | 154.8 ± 4.3     | 1.9 ± 0.5       | -21.9 ± 1.5     | 74.1 ± 2.2       | 187.4 ± 4.3     |
| ATCTATCCG                   | 0.5 ± 0.5       | -23.7 ± 1.5     | 57.0 ± 2.2       | 132.7 ± 4.3     | 0.3 ± 0.5       | -27.5 ± 1.5     | 62.5 ± 2.2       | 151.7 ± 4.3     |
| CAAAAAAG                    | 1.7 ± 0.9       | -20.1 ± 1.5     | 67.0 ± 2.2       | 167.8 ± 4.3     | 1.3 ± 0.5       | -25.6 ± 1.5     | 69.9 ± 2.2       | 177.8 ± 4.3     |
| CAAACAAG                    | 1.9 ± 0.9       | -20.2 ± 1.5     | 66.9 ± 2.2       | 165.5 ± 4.3     | 1.8 ± 0.5       | -24.2 ± 1.5     | 68.8 ± 2.2       | 171.7 ± 4.3     |
| CAAAGAAG                    | -0.3 ± 0.1      | -27.9 ± 1.5     | 67.7 ± 2.2       | 169.9 ± 4.3     | 3.2 ± 0.5       | -19.8 ± 1.5     | 66.7 ± 2.2       | 166.6 ± 4.3     |
| CAAATAAAG                   | 2.4 ± 0.5       | 7.6 ± 0.9      | 8.9 ± 0.9        | 26.3 ± 3.2      | 3.2 ± 0.5       | 10.4 ± 0.9      | 5.4 ± 0.9        | 18.0 ± 3.2      |
| CAAATAAAC                   | 2.2 ± 0.9       | -19.8 ± 1.5     | 56.4 ± 2.2       | 134.2 ± 4.3     | 2.0 ± 0.5       | -24.2 ± 1.5     | 66.2 ± 2.2       | 167.4 ± 4.3     |
| CGCTGTAC                    | 2.2 ± 0.9       | -18.8 ± 1.5     | 61.6 ± 2.2       | 142.9 ± 4.3     | 4.5 ± 0.5       | -14.0 ± 1.5     | 73.5 ± 2.2       | 182.1 ± 4.3     |
| GCCAGTTAA                   | 1.5 ± 0.5       | -20.2 ± 1.5     | 62.4 ± 2.2       | 148.9 ± 4.3     | -0.8 ± 0.5      | -30.9 ± 1.5     | 68.7 ± 2.2       | 170.5 ± 4.3     |
| AAAAADAA                    | 1.0 ± 0.5       | -21.2 ± 1.5     | 67.6 ± 2.2       | 171.2 ± 4.3     | 3.1 ± 0.5       | -19.0 ± 1.5     | 71.8 ± 2.2       | 187.2 ± 4.3     |
| TAGGGTATAAA                 | 0.2 ± 0.1       | -25.3 ± 1.5     | 58.8 ± 2.2       | 140.9 ± 4.3     | 3.9 ± 0.5       | -16.7 ± 1.5     | 62.9 ± 2.2       | 154.9 ± 4.3     |
| ACGTATTATGC                 | -0.7 ± 0.1      | -27.8 ± 1.5     | 85.7 ± 2.2       | 216.3 ± 4.3     | 2.5 ± 0.5       | -20.8 ± 1.5     | 89.1 ± 2.2       | 229.7 ± 4.3     |
| ACATTATTACCA                | -2.1 ± 0.5      | -34.1 ± 1.5     | 89.7 ± 2.2       | 226.5 ± 4.3     | 0.5 ± 0.5       | -29.7 ± 1.5     | 100.9 ± 2.2      | 266.4 ± 4.3     |
| CAACTTGATTAATA              | -0.6 ± 0.1      | -29.8 ± 1.5     | 112.8 ± 2.2      | 291.0 ± 4.3     | 2.8 ± 0.5       | -22.8 ± 1.5     | 124.5 ± 2.2      | 333.3 ± 4.3     |

<sup>a</sup>The complementary strand is not shown.

<sup>b</sup>Reported errors are derived from a 5% increase in the sum of squared residuals for model fitting.
Supplemental Table 3: Transition-state parameters

| Duplex Sequence (5’-3’)<sup>a</sup> | 1.0 M NaCl<sup>b</sup> | 2.2 mM MgCl<sub>2</sub><sup>b</sup> |
|-------------------------------------|---------------------|-------------------|
|                                    | \(\Delta S^t_a\) (cal/mol/K) | \(\Delta H^t_a\) (kcal/mol) | \(\Delta S^t_d\) (cal/mol/K) | \(\Delta H^t_d\) (kcal/mol) | \(\Delta S^t_d\) (cal/mol/K) |
| CCATCGCTACC                         | -25.8 ± 0.1         | 88.4 ± 6.6        | 218.0 ± 17.9                | -28.7 ± 0.1                   | 95.3 ± 6.8                   | 240.8 ± 19.9 |
| GGAACTTGTGC                         | -27.2 ± 0.3         | 84.9 ± 9.2        | 205.6 ± 25.9                | -30.3 ± 0.1                   | 93.8 ± 5.2                   | 236.3 ± 13.9 |
| GGAACAAGATGC                        | -26.7 ± 0.1         | 96.8 ± 8.6        | 243.0 ± 26.2                | -29.9 ± 0.1                   | 101.3 ± 7.3                  | 260.3 ± 21.6 |
| CTTCTTCTTCTTC                       | -26.0 ± 0.2         | 82.8 ± 8.5        | 203.6 ± 26.8                | -29.2 ± 0.1                   | 90.4 ± 5.0                   | 229.5 ± 18.5 |
| CAACCAACCAAC                       | -25.2 ± 0.1         | 100.0 ± 10.3      | 252.2 ± 33.3                | -28.5 ± 0.2                   | 113.0 ± 8.1                  | 295.9 ± 24.5 |
| CCATTGCTACC                         | -25.9 ± 0.1         | 82.7 ± 5.9        | 203.5 ± 16.8                | -29.1 ± 0.1                   | 86.4 ± 4.8                   | 216.3 ± 16.9 |
| GGAGCACG                            | -24.8 ± 0.2         | 60.7 ± 7.1        | 140.9 ± 21.8                | -27.8 ± 0.2                   | 68.2 ± 4.9                   | 165.1 ± 17.8 |
| CACGGCTC                            | -24.5 ± 0.2         | 59.8 ± 6.2        | 137.8 ± 18.2                | -27.2 ± 0.2                   | 64.6 ± 5.7                   | 152.6 ± 20.1 |
| CGTCGTCC                            | -25.2 ± 0.1         | 59.9 ± 6.2        | 138.6 ± 18.4                | -27.9 ± 0.2                   | 67.0 ± 5.9                   | 160.9 ± 17.4 |
| ACCGCA                              | -24.3 ± 0.4         | 37.0 ± 7.8        | 74.3 ± 25.6                 | -25.8 ± 0.2                   | 41.4 ± 4.3                   | 88.0 ± 13.6 |
| CGGACG                              | -24.9 ± 0.4         | 41.5 ± 8.1        | 88.3 ± 28.0                 | -27.0 ± 0.2                   | 46.6 ± 6.1                   | 104.3 ± 20.5 |
| CGGTGC                              | -24.5 ± 0.4         | 40.4 ± 7.9        | 83.9 ± 26.6                 | -26.2 ± 0.2                   | 49.4 ± 5.1                   | 113.3 ± 17.5 |
| CGTGCC                              | -25.1 ± 0.3         | 37.9 ± 5.5        | 75.3 ± 17.6                 | -27.6 ± 0.4                   | 46.1 ± 7.7                   | 101.7 ± 27.1 |
| GCCTGC                              | -25.7 ± 0.3         | 39.2 ± 6.4        | 79.4 ± 21.2                 | -27.8 ± 0.2                   | 43.0 ± 5.1                   | 91.5 ± 16.1 |
| AGCCGTG                             | -24.2 ± 0.3         | 53.0 ± 7.6        | 121.0 ± 26.1                | -26.4 ± 0.1                   | 56.1 ± 4.0                   | 130.1 ± 13.7 |
| AAGCGTAG                            | -26.7 ± 0.3         | 54.0 ± 6.4        | 123.8 ± 21.8                | -30.6 ± 0.2                   | 52.0 ± 5.4                   | 116.6 ± 18.1 |
| AATCCAGGT                           | -25.7 ± 0.2         | 48.0 ± 4.2        | 107.6 ± 14.2                | -29.1 ± 0.2                   | 49.5 ± 2.7                   | 112.9 ± 9.3 |
| ACCTAGTC                            | -25.8 ± 0.3         | 49.3 ± 7.2        | 111.7 ± 17.9                | -29.1 ± 0.2                   | 51.7 ± 5.3                   | 119.5 ± 15.8 |
| ACGACCTC                            | -24.5 ± 0.2         | 52.1 ± 5.4        | 115.5 ± 17.9                | -27.2 ± 0.3                   | 59.5 ± 4.2                   | 139.3 ± 11.7 |
| AGAGAGAG                            | -25.6 ± 0.3         | 49.2 ± 7.1        | 110.5 ± 23.9                | -28.4 ± 0.1                   | 54.3 ± 3.9                   | 128.0 ± 13.8 |
**Supplemental Table 3 continued from previous page**

| Duplex Sequence (5’-3’)<sup>a</sup> | 1.0 M NaCl<sup>b</sup> | 2.2 mM MgCl<sub>2</sub><sup>b</sup> |
|-------------------------------------|-----------------|-----------------|
|                                    | ΔS<sub>d</sub><sup>a</sup> (cal/mol/K) | ΔH<sub>d</sub><sup>a</sup> (kcal/mol) | ΔS<sub>d</sub> (cal/mol/K) | ΔH<sub>d</sub> (kcal/mol) |
| AGCGTAAG                          | -27.3 ± 0.3     | 54.6 ± 6.4      | 125.7 ± 22.2 | 56.6 ± 6.7 | 131.6 ± 20.4 |
| AGTCCTGA                          | -25.7 ± 0.4     | 50.6 ± 7.3      | 114.2 ± 24.8 | 53.4 ± 4.7 | 123.1 ± 16.2 |
| CGCTGTAA                          | -25.8 ± 0.4     | 56.6 ± 8.1      | 132.8 ± 26.1 | 55.5 ± 4.9 | 129.7 ± 17.1 |
| CTAGGGGA                          | -25.7 ± 0.3     | 50.3 ± 5.9      | 114.7 ± 17.8 | 48.0 ± 5.6 | 106.5 ± 18.8 |
| CTCACGGGC                         | -26.0 ± 0.2     | 68.5 ± 10.2     | 165.1 ± 34.0 | 63.6 ± 6.6 | 149.4 ± 19.7 |
| CTGAGTCGC                         | -25.9 ± 0.2     | 56.1 ± 4.9      | 129.6 ± 17.1 | 54.0 ± 5.6 | 122.3 ± 16.1 |
| GCCAGTTA                          | -25.1 ± 0.3     | 52.0 ± 6.8      | 118.1 ± 20.8 | 54.8 ± 3.9 | 127.5 ± 13.8 |
| GGACCTCG                          | -25.2 ± 0.2     | 52.3 ± 6.1      | 115.4 ± 20.4 | 58.9 ± 4.2 | 136.3 ± 14.6 |
| GGTGCCAA                          | -25.3 ± 0.2     | 58.0 ± 6.0      | 134.1 ± 17.7 | 58.6 ± 4.2 | 136.6 ± 14.8 |
| GTCGAAACA                         | -26.2 ± 0.2     | 59.6 ± 5.2      | 139.9 ± 18.5 | 59.1 ± 4.3 | 138.7 ± 15.0 |
| ATAACTGGA                         | -25.2 ± 0.1     | 63.6 ± 5.6      | 151.8 ± 16.4 | 71.4 ± 5.1 | 178.6 ± 14.8 |
| ATCTATCCG                         | -25.5 ± 0.2     | 56.7 ± 5.0      | 131.8 ± 14.2 | 62.3 ± 4.5 | 150.9 ± 15.9 |
| CAAAAAAG                          | -25.7 ± 0.2     | 66.3 ± 6.5      | 165.5 ± 20.0 | 68.7 ± 3.8 | 173.7 ± 13.6 |
| CAAACAAAG                         | -26.7 ± 0.2     | 63.6 ± 9.9      | 154.5 ± 33.4 | 69.1 ± 4.8 | 172.8 ± 13.7 |
| CAAAGAAG                          | -26.8 ± 0.1     | 68.2 ± 8.0      | 171.5 ± 26.6 | 64.5 ± 4.6 | 159.1 ± 13.2 |
| CAAATAAG                          | -27.4 ± 0.1     | 54.4 ± 3.9      | 127.3 ± 13.7 | 64.5 ± 4.6 | 161.4 ± 13.4 |
| CGCTGTTAC                         | -26.1 ± 0.2     | 55.8 ± 7.4      | 124.0 ± 24.9 | 68.4 ± 6.0 | 165.4 ± 17.9 |
| GCCAGTTAA                         | -25.3 ± 0.1     | 59.9 ± 4.3      | 140.6 ± 15.2 | 69.5 ± 4.9 | 173.2 ± 14.3 |
| AAAAAAAAAA                        | -24.5 ± 0.1     | 67.2 ± 5.9      | 170.0 ± 22.4 | 69.1 ± 3.8 | 177.9 ± 14.7 |
| TAGGTTATAA                        | -25.8 ± 0.2     | 58.8 ± 4.2      | 140.7 ± 14.9 | 58.4 ± 4.2 | 139.9 ± 11.6 |
| ACGTATTATGC                       | -25.6 ± 0.1     | 87.3 ± 7.7      | 221.3 ± 23.9 | 85.3 ± 4.7 | 217.5 ± 17.2 |
### Supplemental Table 3 continued from previous page

| Duplex Sequence (5'-3')<sup>a</sup> | 1.0 M NaCl<sup>b</sup> | 2.2 mM MgCl<sub>2</sub><sup>b</sup> |
|------------------------------------|------------------------|-------------------------------|
|                                    | $\Delta S^\ddagger_\text{a}$ (cal/mol/K) | $\Delta H^\ddagger_\text{d}$ (kcal/mol) | $\Delta S^\ddagger_\text{a}$ (cal/mol/K) | $\Delta H^\ddagger_\text{d}$ (kcal/mol) | $\Delta S^\ddagger_\text{d}$ (cal/mol/K) |
| ACATTATTATTACA                     | -27.5 ± 0.1            | 91.9 ± 5.1                    | 233.3 ± 19.3                | -31.4 ± 0.1                     | 100.1 ± 6.4                          | 263.6 ± 14.9                        |
| CAACTTGATATTAATA                   | -27.7 ± 0.1            | 114.6 ± 9.5                   | 296.6 ± 27.4                | -31.8 ± 0.1                     | 116.6 ± 6.4                          | 308.4 ± 17.4                        |

<sup>a</sup>The complementary strand is not shown.

<sup>b</sup>Reported errors are derived from a 5% increase in the sum of squared residuals for model fitting.
### Supplemental Table 4: Nearest-Neighbor Kinetic Parameters in 2.2 mM MgCl₂

| NN parametersa | Dissociationb | Associationb |
|----------------|---------------|--------------|
|                | ΔH‡d (kcal/mol) | ΔS‡d (cal/mol/K) | ΔG‡d (kcal/mol) | ΔS‡a (cal/mol/K) | ΔG‡a (kcal/mol) |
| AA/TT          | 9.6 ± 0.5      | 28.0 ± 1.5    | 0.93 ± 0.05    | -0.37 ± 0.12    | 0.11 ± 0.04   |
| AT/TA          | 11.0 ± 1.5     | 32.8 ± 4.8    | 0.82 ± 0.12    | -0.24 ± 0.34    | 0.08 ± 0.11   |
| TA/AT          | 3.7 ± 1.6      | 10.0 ± 5.0    | 0.63 ± 0.13    | -0.58 ± 0.34    | 0.18 ± 0.10   |
| CA/GT          | 10.0 ± 1.2     | 27.6 ± 3.8    | 1.46 ± 0.09    | -0.24 ± 0.27    | 0.08 ± 0.09   |
| GT/CA          | 12.0 ± 1.3     | 34.0 ± 4.4    | 1.46 ± 0.10    | -0.24 ± 0.26    | 0.07 ± 0.08   |
| CT/GA          | 9.8 ± 1.3      | 27.2 ± 4.2    | 1.33 ± 0.12    | -0.52 ± 0.37    | 0.16 ± 0.12   |
| GA/CT          | 7.4 ± 1.1      | 20.1 ± 3.7    | 1.20 ± 0.09    | -0.22 ± 0.28    | 0.07 ± 0.09   |
| CG/GC          | 13.2 ± 1.5     | 36.0 ± 5.0    | 2.00 ± 0.14    | 0.11 ± 0.38     | -0.03 ± 0.13  |
| GC/GC          | 11.6 ± 1.3     | 31.3 ± 4.2    | 1.88 ± 0.10    | 0.02 ± 0.27     | -0.01 ± 0.08  |
| GG/CC          | 11.3 ± 1.2     | 31.2 ± 3.5    | 1.59 ± 0.11    | 0.49 ± 0.24     | -0.15 ± 0.07  |
| Initiation     | -10.5 ± 3.9    | -54.0 ± 12.3  | 6.21 ± 0.37    | -27.5 ± 0.9     | 8.51 ± 0.28   |
| Terminal AT    | -3.0 ± 0.7     | -8.7 ± 2.3    | -0.28 ± 0.06   | 0.31 ± 0.22     | -0.10 ± 0.07  |

aNNs are reported (5'-3')/(3'-5).

bMean values and standard deviations are the average of resampling 10,000 sets of 30 out of 43 sequences and calculating NNs. ΔG‡s calculated at 37°C.
Supplemental Table 5: Comparison Between Predicted and Experimental Dissociation Kinetics

| Sequence (5’-3’)<sup>a</sup> | Temp (°C) | Exp k<sub>d</sub> (s<sup>-1</sup>) | Pred k<sub>d</sub> (s<sup>-1</sup>) | Exp ΔH<sub>d</sub>‡ (kcal/mol) | Pred ΔH<sub>d</sub>‡ (kcal/mol) | Exp ΔS<sub>d</sub>‡ (cal/mol/K) | Pred ΔS<sub>d</sub>‡ (cal/mol/K) |
|-------------------------------|-----------|-------------------------------|---------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| GCCTGGAGCT<sup>b</sup>       | 15        | 6.0 × 10<sup>-6</sup>        | 6.8 × 10<sup>-6</sup> | 71.8                          | 76.8                          | 166.6                         | 184.4                         |
|                               | 25        | 3.3 × 10<sup>-4</sup>        | 6.3 × 10<sup>-4</sup> |                               |                               |                               |                               |
|                               | 37        | 3.5 × 10<sup>-2</sup>        | 9.9 × 10<sup>-2</sup> |                               |                               |                               |                               |
|                               | 47        | 1.6 × 10<sup>0</sup>         | 5.0 × 10<sup>0</sup>  |                               |                               |                               |                               |
|                               | 50        | 5.5 × 10<sup>0</sup>         | 1.5 × 10<sup>1</sup>  |                               |                               |                               |                               |
| GCATGC<sup>c</sup>           | 31.1      | 2.2 × 10<sup>2</sup>         | 1.5 × 10<sup>2</sup>  | 39.4                          | 40.0                          | 81.6                          | 82.8                          |
|                               | 39.4      | 1.4 × 10<sup>3</sup>         | 8.7 × 10<sup>2</sup>  |                               |                               |                               |                               |
|                               | 45        | 3.9 × 10<sup>3</sup>         | 2.8 × 10<sup>3</sup>  |                               |                               |                               |                               |
|                               | 38.7      | 2.0 × 10<sup>1</sup>         | 4.5 × 10<sup>1</sup>  |                               |                               |                               |                               |
| GCGCGC<sup>d</sup>           | 42.8      | 6.8 × 10<sup>1</sup>         | 1.2 × 10<sup>2</sup>  | 57                            | 47.8                          | 140                           | 102.2                         |
|                               | 49        | 4.0 × 10<sup>2</sup>         | 5.4 × 10<sup>2</sup>  |                               |                               |                               |                               |

<sup>a</sup>The complementary strand is not shown.

<sup>b</sup>Reported in Lomzov et al. (2012).

<sup>c</sup>Reported in Williams et al. (1989).

<sup>d</sup>Reported in Freier et al. (1983).
### Supplemental Table 6: Comparison Between Predicted and Experimental Association Kinetics

| Sequence (5’-3’)
| Temperature (°C) | Exp $k_a$ (M$^{-1}$ s$^{-1}$) | Pred $k_a$ (M$^{-1}$ s$^{-1}$) | Exp $\Delta G^\ddagger$ (kcal/mol)$^b$ | Pred $\Delta G^\ddagger$ (kcal/mol)$^b$ |
|------------------|-----------------|-----------------|-----------------|-----------------|
| GCCTGGAGCTc      | 15              | $6.0 \times 10^7$ | $1.7 \times 10^7$ | 7.67            | 7.84            |
|                  | 25              | $3.1 \times 10^7$ | $1.8 \times 10^7$ |                 |                 |
|                  | 37              | $2.6 \times 10^7$ | $1.9 \times 10^7$ | 7.67            | 7.84            |
|                  | 47              | $2.0 \times 10^7$ | $1.9 \times 10^7$ |                 |                 |
|                  | 50              | $2.3 \times 10^7$ | $2.0 \times 10^7$ |                 |                 |
|                  | 31.1            | $9.9 \times 10^6$ | $6.5 \times 10^6$ |                 |                 |
| GCATGCd          | 39.4            | $8.3 \times 10^6$ | $6.6 \times 10^6$ | 8.34            | 8.50            |
|                  | 45              | $6.7 \times 10^6$ | $6.7 \times 10^6$ |                 |                 |
| CGGCGAGAAAGGCe    |                | -               | -               | 7.65            | 7.53            |
| GCGGCCAACACAc    |                | -               | -               | 7.22            | 7.69            |
| CAAGCCGGACACa    |                | -               | -               | 7.43            | 7.77            |
| CACAAGCGGCACa    |                | -               | -               | 7.55            | 7.77            |
| CACACAAAGCGGCa   |                | -               | -               | 7.61            | 7.77            |
| CACAGCACf        | 6.9             | $4.3 \times 10^6$ | $9.3 \times 10^6$ |                 |                 |
|                  | 10.8            | $5.3 \times 10^6$ | $9.5 \times 10^6$ |                 |                 |
| CACAGCACf        | 16.3            | $5.8 \times 10^6$ | $9.7 \times 10^6$ | 7.65            | 8.23            |
|                  | 20              | $7.3 \times 10^6$ | $9.8 \times 10^6$ |                 |                 |
|                  | 25.7            | $1.1 \times 10^7$ | $1.0 \times 10^7$ |                 |                 |
| GCGGCGCg         | 42.8            | $1.3 \times 10^7$ | $1.1 \times 10^7$ | 8.11            | 8.19            |
|                  | 49              | $1.3 \times 10^7$ | $1.1 \times 10^7$ |                 |                 |
| GTTGTCAAGATGCTAC |                  |                |                |                 |                 |
| CGTTTCAGAGh      | 20              | $1.5 \times 10^6$ | $2.9 \times 10^6$ | -               | -               |

*aThe complementary strand is not shown.*

*b$\Delta G^\ddagger$s are reported and calculated at 37°C except for experiments and predictions for sequences reported by Sikora et al., which are at 25°C.*

*cReported in Lomzov et al. (2012).*

*dReported in Williams et al. (1989).*

*eReported in Sikora et al. (2013).*

*fReported in Rauzan et al. (2013).*

*gReported in Freier et al. (1983).*

*hReported in Gao et al. (2006).*
Supplemental Table 7: Nearest-Neighbor Thermodynamic Parameters in 1.0 M NaCl

| NN parameters<sup>a</sup> | N=43 Kinetic experiments<sup>b</sup> | N=43 subsample of equilibrium melting experiments<sup>c</sup> | N=108 equilibrium melting experiments (Unified NNs)<sup>d</sup> |
|-------------------------|-------------------------------|-------------------------------------------------|-------------------------------------------------|
|                         | ΔH (kcal/mol) | ΔS (cal/mol/K) | ΔH (kcal/mol) | ΔS (cal/mol/K) | ΔH (kcal/mol) | ΔS (cal/mol/K) |
| AA/TT                  | -9.2 ± 0.6   | -26.6 ± 1.8   | -8.4 ± 0.8   | -23.6 ± 2.5   | -7.6         | -21.3         |
| AT/TA                  | -8.6 ± 1.7   | -24.4 ± 5.3   | -4.2 ± 1.8   | -10.4 ± 5.8   | -7.2         | -20.4         |
| TA/AT                  | -5.6 ± 1.4   | -16.2 ± 4.3   | -6.5 ± 1.5   | -19.4 ± 4.8   | -7.2         | -21.3         |
| CA/GT                  | -11.9 ± 1.4  | -33.9 ± 4.4   | -7.9 ± 2.0   | -20.8 ± 6.6   | -8.5         | -22.7         |
| GT/CA                  | -9.6 ± 1.6   | -25.9 ± 5.0   | -10.8 ± 1.8  | -30.2 ± 5.9   | -8.4         | -22.4         |
| CT/CA                  | -10.2 ± 1.2  | -29.2 ± 3.6   | -7.0 ± 1.4   | -18.0 ± 4.4   | -7.8         | -21.0         |
| GA/GC                  | -8.1 ± 1.3   | -21.9 ± 4.0   | -7.6 ± 1.1   | -20.6 ± 3.6   | -8.2         | -22.2         |
| CG/GC                  | -14.5 ± 1.5  | -40.6 ± 4.9   | -9.2 ± 2.5   | -22.3 ± 8.2   | -10.6        | -27.2         |
| GC/GC                  | -11.2 ± 1.7  | -29.2 ± 5.5   | -9.3 ± 1.8   | -23.5 ± 5.8   | -9.8         | -24.4         |
| GG/CC                  | -9.8 ± 1.3   | -26.1 ± 4.2   | -9.6 ± 1.1   | -24.7 ± 3.5   | -8.0         | -19.9         |
| Initiation             | 14.8 ± 4.2   | 41.7 ± 13.3   | -2.0 ± 5.9   | -13.6 ± 18.8  | 0.2          | -5.7          |
| Terminal AT            | 1.0 ± 1.0    | 2.6 ± 3.1     | 2.1 ± 1.1    | 6.2 ± 3.6     | 2.2          | 6.9           |

<sup>a</sup>NNs are reported (5'-3')/(3'-5).

<sup>b</sup>Calculated from kinetic NNs in Table 2 using Equation 4.

<sup>c</sup>Equilibrium parameters for the 43 sequences used in this study are reported in the supplemental section of Allawi et al. (1997). Mean values and standard deviations are the average of resampling 10,000 sets of 30 out of 43 sequences and calculating NNs.

<sup>d</sup>Unified NNs are reported in SantaLucia et al. (2004).
Supplemental Table 8: Frequency of each NN

| Parameters | Frequency in n=341 NNs<sup>b</sup> | Frequency in n=756 NNs<sup>c</sup> |
|------------|------------------------------------|------------------------------------|
| AA/TT      | 0.17                               | 0.15                               |
| AT/TA      | 0.05                               | 0.07                               |
| TA/AT      | 0.07                               | 0.07                               |
| CA/GT      | 0.12                               | 0.11                               |
| GT/CA      | 0.13                               | 0.11                               |
| CT/GA      | 0.13                               | 0.11                               |
| GA/CT      | 0.10                               | 0.11                               |
| CG/GC      | 0.06                               | 0.09                               |
| GC/GC      | 0.06                               | 0.09                               |
| GG/CC      | 0.10                               | 0.09                               |

<sup>a</sup>NNs are reported (5'-3')/(3'-5).

<sup>b</sup>From 43 sequences used in this study reported in Supplemental Table 1

<sup>c</sup>From 108 sequences used to derive unified NNs reported in the supplementary data for Allawi et al. (1997).
### Supplemental Table 9: Frequency in each sequence

| NN Parameters<sup>a</sup> | Frequency in n=43 sequences<sup>b</sup> | Frequency in n=108 sequences<sup>c</sup> |
|--------------------------|-----------------------------------------|-----------------------------------------|
| AA/TT                    | 0.56                                    | 0.44                                    |
| AT/TA                    | 0.28                                    | 0.32                                    |
| TA/AT                    | 0.40                                    | 0.36                                    |
| CA/GT                    | 0.72                                    | 0.53                                    |
| GT/CA                    | 0.81                                    | 0.54                                    |
| CT/GA                    | 0.74                                    | 0.51                                    |
| GA/CT                    | 0.49                                    | 0.46                                    |
| CG/GC                    | 0.44                                    | 0.45                                    |
| GC/GG                    | 0.49                                    | 0.50                                    |
| GG/CC                    | 0.67                                    | 0.44                                    |

<sup>a</sup>NNs are reported (5'-3’)/(3’-5).

<sup>b</sup>Sequences used in this study are reported in Supplemental Table 1.

<sup>c</sup>Sequences used to derive unified NNs are reported in the supplementary data for Allawi et al. (1997).
Supplemental Figure 1: Transition-state parameters for association (blue) and dissociation (orange) and calculated errors for all sequences in both buffers. The dissociation parameters are well defined and vary notably. On the other hand, transition-state parameters for association are all poorly constrained near zero.
Supplemental Figure 2: Panel C from Figure 1 showing stopped-flow experimental data (blue) in 1.0 M NaCl for the sequence 5’-AGCGTAAG -3’ and its complement at 1.0 μM. Reactions are shifted vertically by small absorbance offsets from fitting and higher temperature reactions are shifted rightward for clarity. Model fits are shown for ΔH^‡a=0 (yellow) and ΔH^‡a≠0 (orange). Not shown are 44 other experiments fit simultaneously. The difference between the two model fits becomes more apparent after magnification of the leftmost (lowest temperature) curve.
Supplemental Figure 3: Rate constants calculated from transition-state parameters within the experimental temperature range for each of the 43 sequences in 1.0 M NaCl. Association rate constants have little temperature dependence modeled with (A) $\Delta H^\ddagger_a=0$ or (B) $\Delta H^\ddagger_a \neq 0$. In contrast, rate constants for dissociation exhibit an exponential temperature dependence for models with (C) $\Delta H^\ddagger_a=0$ and (D) $\Delta H^\ddagger_a \neq 0$. 
Supplemental Figure 4: The effect of 0.7 mM (blue), 2.2 mM (orange), 4.2 mM (yellow) MgCl₂, 1.0 M NaCl (purple), 10% DMSO (green), and 1X LCGreen⁺ (teal) on (A) association and (B) dissociation for the sequence 5’-CACGGCTC-3’. Different ionic conditions alter $k_a$ more than $k_d$, whereas the addition of DMSO increases $k_d$ but has no effect on $k_a$. LCGreen⁺ increases $k_a$ and inhibits $k_d$. Reactions that include DMSO and LCGreen⁺ are performed in the 50 mM Tris pH 8.3, 2.2 mM MgCl₂ buffer.
Supplemental Figure 5: Percent GC-content vs. sequence length for the 43 sequences used in stopped-flow experiments. There is an inverse correlation ($r=-0.61$. $p<10^{-4}$) between sequence length and GC-content in the sequences that contributes to the erroneous length dependency of the NN kinetic model for association.
Supplemental Figure 6: Experimental time-temperature traces from the denaturation experiments in Millington et al. (2019). As the time for denaturation decreases, so does the maximum sample temperature, which in turn may cause loss of denaturation efficiency and increased C_q. The increase in C_q may not result from an explicit time requirement, but rather from a decrease in maximum sample temperatures. The denaturation hold times for extreme PCR were 15 s (blue), 5 s (orange), 1 s (yellow), 0.5 s (purple), 0.2 s (green), or 0.1 s (teal).
Supplemental Reaction Scheme 1

\[ T_1 + T_2 \xleftrightarrow[k_i]{k_{-1}} T_1 T_2 \]

\[ P_1 + T_2 \xleftrightarrow[k_2]{k_{-2}} P_1 T_2 \]

\[ T_1 + P_2 \xleftrightarrow[k_3]{k_{-3}} T_1 P_2 \]

\[ \frac{dT_1}{dt} = k_{-1}[T_1 T_2] + k_{-3}[T_1 P_2] - k_1[T_1][T_2] - k_3[T_1][P_2] \]

\[ \frac{dT_2}{dt} = k_{-1}[T_1 T_2] + k_{-2}[P_1 T_2] - k_1[T_1][T_2] - k_2[P_1][T_2] \]

\[ \frac{dP_1}{dt} = k_{-2}[P_1 T_2] - k_2[P_1][T_2] \]

\[ \frac{dP_2}{dt} = k_{-3}[T_1 P_2] - k_3[T_1][P_2] \]

\[ \frac{dT_1 T_2}{dt} = k_1[T_1][T_2] - k_{-1}[T_1 T_2] \]

\[ \frac{dP_1 T_2}{dt} = k_2[P_1][T_2] - k_{-2}[P_1 T_2] \]

\[ \frac{dT_1 P_2}{dt} = k_3[T_1][P_2] - k_{-3}[T_1 P_2] \]
Example NN calculation

NN calculation for dissociation kinetics with parameters from Table 2 using Equation 4 for the sequence 5’-GCCTGGAGCT-3’:

\[ \Delta G_{d}^{\ddagger}(5’-\text{GCCTGGAGCT-3’}) = \Delta G_{d}^{\ddagger}(\text{initiation}) + m \times \Delta G_{d}^{\ddagger}(\text{terminal AT}) + \\
2 \times \Delta G_{d}^{\ddagger}(\text{GC/CG}) + 2 \times \Delta G_{d}^{\ddagger}(\text{GG/CC}) + 3 \times \Delta G_{d}^{\ddagger}(\text{CT/GA}) + \Delta G_{d}^{\ddagger}(\text{CA/GT}) + \\
\Delta G_{d}^{\ddagger}(\text{GA/CT}) \]

\[ \Delta G_{d}^{\ddagger}(5’-\text{GCCTGGAGCT-3’}) = 5.87 + 1 \times (-0.29) + \\
2 \times 2.13 + 2 \times 1.57 + 3 \times 1.26 + 1.51 + \\
1.37 = 19.64 \text{ kcal/mol} \]

Note: if the terminal base on both ends was an A or T then \( m = 2 \). Similarly, if neither terminal base was A or T then \( m = 0 \).
Designing PCR thermocycling profiles

The temperature and sequence dependence of oligonucleotide dissociation and association kinetics have important implications for designing PCR thermocycling profiles. Every amplicon sequence and thermocycling heating rate has a denaturation temperature that needs to be reached, but not held, to maximize the speed and efficiency of the denaturation step.

Given any pair of PCR primers with similar TMs and minimal off-target amplification, and an amplicon sequence with minimal stable or metastable secondary structures, there exists an optimal annealing temperature that provides a maximum annealing rate and high (≥ 99%) potential maximum efficiency. Above this temperature the rate and potential efficiency of annealing decreases rapidly with increasing temperature due to the exponential increase in the dissociation rate. At temperatures below optimal, the rate (but not potential efficiency) of annealing will decrease with decreasing temperature due to a decrease in association rate constants.

- Used at the same concentrations, primer sets with similar GC-content will have similar annealing time requirements at their optimal annealing temperatures.
- If no stable or metastable secondary structures form within a temperature range below the optimal temperature, multiple thermocycling profiles in that temperature range will have approximately similar annealing rates and efficiencies.
- If stable or metastable secondary structures exist at temperatures below the optimal temperature, then the annealing rate and efficiency will both decrease rapidly below the optimal temperature (and nonspecific amplification may increase).

This knowledge simplifies designing fast PCR assays. The denaturation hold time should always be minimal, and the ramp rates to denaturation and annealing should always be the maximum that can be achieved with a specified thermocycler. Then, the annealing time and temperature, denaturation temperature, and ramp rate to extension are the 4 remaining variables that influence the annealing and denaturation steps of PCR. Estimates of the first 3 variables can all be derived from kinetic predictions. The optimal extension ramp rate is determined by the polymerase extension rate, difference between annealing and extension temperatures, and the difference between primer-template and template-template stabilities. However, the effect of any arbitrary extension ramp rate can still be modeled and accounted for in kinetic simulations. In the following section we present a guide for designing PCR thermocycling profiles using kinetic predictions based off the NN model for dissociation and the GC model for association.

Predicting the optimal annealing and denaturation parameters is a 5-step process:

1. Select primer concentrations and predict transition-state parameters.
2. Estimate the optimal annealing temperature.
(3) [Optional] Estimate the minimum annealing hold time (isothermal kinetic simulation).
(4) Find the minimum annealing hold time accounting for thermocycling (kinetic simulation).
(5) Identify the optimal denaturation temperature (kinetic simulation).

To demonstrate this process, we use primer sequences for PCR assays from the literature to estimates optimal annealing and denaturation times and temperatures. In addition to errors inherent in the model, the inclusion of fluorescent probes, DNA binding dyes, polymerases, and many PCR additives that influence nucleic acid stability and kinetics are not considered. However, because of the different temperature dependencies of association and dissociation, and the small variation in association rates with sequence, errors for annealing and denaturation temperatures are larger than annealing hold times. Most of the temperature errors skew systematically in a predictable way. For instance, polymerase binding is known to stabilize duplexes, and will increase duplex stability more in earlier cycles when the duplex concentration is low relative to polymerase. In addition, PCR dyes are known to increase TmS 1-3°C in melting analysis assays. Therefore, the optimal temperature for denaturation is expected to be systematically higher than predicted here (as demonstrated in the PCR simulations of Millington et al. (2019) The kinetic requirements of extreme qPCR. Biomol. Detect. Quantif., 17, 100081). Since inadequate denaturation is a common cause of PCR failure, a denaturation temperature 4-8°C higher than calculated is generally recommended.

**Example 1: Designing asymmetric PCR for Factor V Leiden genotyping**

To demonstrate the application of kinetic predictions to PCR design, the primer and template sequences, assay concentrations, and temperature ramp rates were taken from an article describing a real-time assay for genotyping factor V Leiden (37).

The article describes a rapid-cycle asymmetric PCR assay targeting a 187 base-pair amplicon in ionic conditions identical to the 2.2 mM MgCl2 PCR buffer from the current study. The forward primer sequence was 5’-TAATCTGTAAGAGATCC-3’ and the reverse primer sequence was 5’-TGTTATCACACTGGTGCTAA-3’.

**Step 1: Select assay concentrations and predict transition-state parameters**

We will use the primer concentrations for asymmetric PCR chosen in the original study. Predict the transition-state parameters using the NN (see Example NN calculation in this document) and GC (Equation 7 in the materials and methods section of the main article) models. The following calculations use the parameters reported in the 2.2 mM MgCl2 PCR buffer.
[Forward primer]i = 500 nM
[Reverse primer]i = 200 nM.

The exact template concentration selected matters very little so long as it is at least 1,000-fold lower than the lowest primer concentration. Here we will use an initial template concentration of 5 x 10^{-3} nM.

- Forward primer \( \Delta H^\ddagger_d = 158.8 \text{ kcal/mol}, \Delta S^\ddagger_d = 419.8 \text{ cal/mol/K}, \) and \( \Delta S^\ddagger_a = -29.5 \text{ cal/mol/K}. \)
- Reverse primer: \( \Delta H^\ddagger_d = 169.0 \text{ kcal/mol}, \Delta S^\ddagger_d = 448.4 \text{ cal/mol/K}, \) and \( \Delta S^\ddagger_a = -29.5 \text{ cal/mol/K}. \)
- Template: \( \Delta H^\ddagger_d = 1760 \text{ kcal/mol}, \Delta S^\ddagger_d = 4920 \text{ cal/mol/K}, \) and \( \Delta S^\ddagger_a = -29.5 \text{ cal/mol/K}. \)

**Step 2: Estimate the optimal annealing temperature**

The optimal annealing temperature is defined as the maximum temperature where the ratios of the pseudo first-order association rate constant to dissociation rate constant for both primers are \( \geq 100. \) This is the pseudo first-order equilibrium constant defined as \( K_{eq} = [\text{primer concentration}] \times k_a/k_d. \) At \( \geq 100, \) the dissociation rate is small compared to the association rate and the potential primer annealing efficiency is \( \geq 99\% \) at equilibrium under most primer and template concentrations. Each rate constant is calculated from the transition-state parameters in step (1) and equations 1 & 2 from the materials and methods section. In the figure below, the optimal temperature is identified as 52.5°C using 0.5°C temperature steps.
Step 3: [Optional] Identify the minimum hold time for ≥ 99% primer annealing efficiency (isothermal kinetic simulation).

Simulate the reaction for primer-template annealing at 52.5°C ignoring template-template interactions and thermocycling. This computation can be further simplified by simulating the two reactions via the pseudo first-order equation: \([\text{Template}] = [\text{Template}]_0 \times \exp(-[\text{Primer}]_0 k_{\text{st}})\). However, for best practice the system of ordinary differential equations derived from Supplemental Reaction Scheme 1 should be numerically integrated. In this example the minimum hold time for ≥ 99% annealing efficiency for both primers at 52.5°C is 10.8 seconds.
Step 4: Estimate the annealing hold time for ≥ 99% efficiency (thermocycling kinetic simulation).

Now simulate the reactions during thermocycling from denaturation through the extension temperature. This can be performed as described in the materials and methods section using Supplemental Reaction Scheme 1. Template-template interactions can be ignored, which are generally negligible until the later cycles of PCR.

For these simulations, we include a complete thermal cycle and use the 20°C/s ramp rates for the approach to annealing and denaturation with 1.0°C/s from annealing to extension as reported in the original experiments. In general, the fastest possible instrument-specific annealing and denaturation ramp rates should be used. The denaturation temperature is set to 95°C and extension 75°C. The initial estimate for an annealing hold time is 10.8 seconds (from step 3) and the reactions are simulated. Then, the annealing hold time is iteratively decreased.
(by 0.1 s in this example) and reactions are re-simulated until the minimum annealing efficiency drops below 99%. The lowest annealing hold time for ≥ 99% efficiency is 9.7 s.

Simulated thermocycle with a minimum hold for ≥ 99% annealing of 9.7 s at 52.5°C, using an annealing ramp rate of 20°C/s and extension ramp rate of 1°C/s. The y-axis is either primer annealing efficiency (blue and orange lines) or temperature (black dotted line).

Step 5: Estimate the optimal denaturation temperature (thermocycling kinetic simulation).

The optimal denaturation temperature can be predicted by simulating melting analysis using the denaturation temperature ramp rate and the maximum primer concentration (500 nM in the example) as the template duplex concentration. The primer concentration is used instead of the initial template concentration because the required denaturation temperature is concentration dependent and increases during the PCR as template molecules accumulate. However, as the temperature ramp rate increases, the optimal denaturation temperature is less dependent on concentration. The temperature that the duplex annealing reaches ≤ 0.1% is
The optimal denaturation temperature (81.3°C) is the point where ≥ 99.9% of the duplex is melted and is determined by the template sequence, 20°C/s denaturation ramp rate, and primer concentrations.

Dependent on whether the predicted template T_M is < 95°C and the instrument thermocycling capabilities, step 5 may be omitted entirely and a standard denaturation temperature of 95°C can be adopted to simplify assay design. The improvement in assay speed by determining the optimal denaturation temperature in lieu of using 95°C may be minor at fast ramp rates and the importance of further optimizing the speed is therefore context dependent. If the template T_M is near or above 95°C, the optimal denaturation temperature may not be obtainable. In this case, T_M depressors (e.g., DMSO) and lower ionic concentrations can be used to reduce the optimal denaturation temperature. For fast PCR assays, addition of 5% DMSO would be preferable to decreasing ionic (Mg++) concentrations. DMSO will also lower the optimal annealing temperature but not alter the required annealing time. If the optimal denaturation temperature is still unobtainable, a hold time may achieve > 99.9% denaturation efficiency. However, because melting transitions for amplicons (and sequences longer than typical primers) are narrow (see the ≈ 1°C transition in the above figure) if the optimal denaturation
temperature is more than $\approx 2.0^\circ C$ higher than the highest achievable sample temperature, the assay will not achieve high efficiency regardless of hold time and another target sequence should be selected.

Reaching a higher temperature is nearly always preferable to setting a hold time. For the above example: if the maximum temperature reached was 79.8 (1.5°C less than the optimal denaturation temperature) a 220 s hold time is required for $> 99.9\%$ denaturation efficiency. If the maximum temperature reached was 80.3°C a 6 s hold time is required. If the maximum temperature reached is 0.5°C less than the optimal denaturation temperature a 0.2 s hold is required. An exception to this may be that a longer hold time at cycle 0 is beneficial to initially denature genomic DNA.

Remarks

In this example we predict an optimal denaturation temperature of 81.3°C (although $\geq 85^\circ C$ may be a more appropriate starting point for empirically optimizing the assay) and annealing temperature of 52.5°C with a 9.7 s hold time. The predicted annealing profile is in good agreement with the 10 s at 50°C used in the study itself. **Note:** the concentration of the reverse primer (200 nM) limits the assay speed, and if included at 500 nM the annealing hold time could be reduced by $\approx 5$ s.

In the following additional examples, steps and calculations are described only briefly or omitted entirely.
Example 2: Rapid-cycle asymmetric multiplex PCR for HER2/neu and β-globin

This example uses the targets, primer sequences (5’-CCTCTGACGTCCATCGTCTC-3’, 5’-CGGATCTTCTGCTGCCGTCG-3’ HER2/neu, 5’-ACACAACTGTGTTCACTAGC-3’, 5’-CACTTCATCCACGTACC-3’ β-globin) concentrations (500 nM forward, 200 nM reverse for both amplicons), and ramp rates adapted from an article describing an asymmetric multiplex assay (38).

Step 2: Determine the optimal annealing temperature.

![Pseudo first-order $K_{eq}$ vs temperature for the two primer sets. The optimal annealing temperature for this multiplex asymmetric PCR assay is 55°C.](image)

Step 4: Estimate the annealing hold time

From step 3 the initial estimate of the annealing hold time is 10.5 s. Thermocycling simulations use the ramp rates from the study, which are identical to Example 1, and predict an optimal annealing time of 9.9 s at 55°C.
The annealing hold time recommended from simulations is 9.9 s.

Remarks

Aside from being a multiplex assay, this example is very similar to Example 1. The predicted optimal annealing temperature and time are 55°C and 9.9 s. This is very close to the experimental protocol of a 10 s hold at 58°C from the study. **Note:** The experimental protocols and predicted thermocycling profiles are both very similar between examples 1 and 2 primarily because the primers are used at identical concentrations and have similar GC-content (40-65%). Therefore, the optimal hold time at the optimal annealing temperature for different well-designed primer sets is expected to be similar when ramp rates, primer concentrations, and primer GC-contents are similar.
Example 3: IL-1β qPCR with SYBR Green I

This example uses primer sequences (5’-GCAACTGTCCCTGAATC-3’, 5’-AGGTCGTACATCCATCC-3’) for targeting a 332 base-pair region of IL-1β, 5 μM primer concentrations, and temperature ramp rates adapted from an article that uses qPCR for low-copy transcript quantification (39).

Step 4: Estimate the annealing hold time

In Step 2 the optimal temperature was 57°C, and in Step 3 the estimate for annealing hold time was 0.3 s. In Step 4 we then use the temperature ramp rates specified in the initial study. After thermocycling simulations, we determine a 0.02 s hold time is optimal:

![Graph showing annealing efficiency and temperature over time]

The annealing hold time recommended from simulations is 0.02 s at 57°C.

Remarks

For this assay, the predicted optimal annealing protocol is 0.02 s at 57°C. This is close to the experimental protocol of 0 s at 60°C. Note: In comparison to examples 1-2, the annealing step has much shorter time requirements due to the 10-fold increased primer concentrations.
Example 4: Detection of Epstein-Barr virus with nested rapid-cycle PCR

This example uses primer sequences (5’- CCAGGCACACACTACACACA-3’, 5’- CTGGAGAGGTACAGTTACTT-3’, and 5’- ACAGCTCTAAGAAGGCACC-3’, 5’- ACCACCTCTCTTCTTGCTG-3’) and concentrations (500 nM each) adapted from an article describing a nested PCR assay for detecting Epstein-Barr virus (40).

Step 4: Estimate the annealing hold time

Using 20°C/s ramp rates for annealing and denaturation, and 5°C/s for extension, the optimal annealing temperature was found to be 56°C and the annealing hold time was 3.1 s. This is quite different from the experimental protocol of < 1 s at 50°C. A simulation with an annealing step that approximates the experimental protocol (50°C for 0.1 s) predicts an annealing efficiency > 97% for the least efficient primer and an approximately 1.5 s shorter cycle time.

The annealing hold time recommended from simulations is 3.1 s at 56°C.
An alternative thermocycling profile based on the experimental protocol that includes a 0.1 s annealing at 50°C. The predicted annealing efficiencies are still > 99% for 3 of 4 primers. However, the total cycle time is reduced by 1.5 s.

Remarks

We originally defined the optimal annealing time as the time required to achieve > 99% efficiency. This example illustrates how the definition of “optimal” is arbitrary. Assuming all other steps are perfectly efficient, after 35 cycles both assays will have C_qs that differ by less than 1 cycle and the lower efficiency assay will be faster. Which protocol is “optimal” is context dependent, and a 1-3% sacrifice in annealing efficiency may not significantly alter C_qs, but may notably decreases the required annealing time. Note: If the second protocol were adjusted to have a 1.6 s hold at 50°C (which would make the total cycle time and time spent below the optimal annealing temperature equivalent in both protocols) the annealing efficiency would be similar to that in the first protocol (> 99%). This is because at temperatures below the optimal for annealing, in the absence of secondary structures, the rate of annealing depends very little on temperature.
Example 5: β-globin qPCR with SYBR Green I

This example uses primer sequences (5’-GGTTGGCCAATCTACTCCCAGG -3’ and 5’-GCTCACTCAGTGGCAAAG-3’) for a 536 base-pair amplicon, primer concentrations (500 nM each), and temperature ramp rates adapted from an article describing real-time rapid-cycle PCR (41).

Step 4: Estimate the annealing hold time

Using 5.2°C/s ramp rates (the reported experimental average), the optimal annealing temperature was found to be 59°C and the annealing hold time was 2.9. This contrasts with the experimental protocol of 0 s at 61°C. Simulations of a 0 s hold time at 59°C predict the lower efficiency primer has an efficiency of 92.5%.

![Graph](image)

Primer annealing efficiencies and time-temperature profiles for two different annealing protocols: a 3.1 s (blue) and 0 s (orange) hold at 59°C. Primer annealing efficiencies are shown as the solid and dashed lines, and temperature profiles appear as dotted lines.

Remarks

Compared to Example 4, the predictions for optimizing the annealing step appear to be more erroneous in comparison to experiments. **Note**: This primer set has higher TMs than the other examples which may contribute to increasing errors in the model predictions. The NN and GC
kinetic models themselves underestimate oligonucleotide stability relative to the unified NN thermodynamic predictions, and polymerase and fluorescent dye binding (not modeled by simulation) is expected to further increase duplex stability. As the temperature range between annealing and extension steps decreases, more primer annealing at the extension temperature might occur than is captured in the simulations. It may be that predictions will increasingly overestimate the required annealing time as primer T\textsubscript{MS} near typical extension temperatures (70-75°C). On the other hand, this comparison assumes that experimental annealing efficiencies are high (> 99%), and no calculated efficiency is presented in the original study. If the true experimental primer efficiencies are 92-97% the predictions are accurate. For reference, with a 0.8 (vs. 2.9) s hold time the two primers efficiencies are predicted at 97% and 98.9%, which would be indistinguishable from > 99% dependent on the efficiency of extension and denaturation steps.

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