believe that our findings are of interest to researchers conducting RNAi experiments and/or working with *Plasmodium* and other unicellular pathogens characterized by A/T-rich genomes.

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Complex DNA melting profiles of small PCR products revealed using SYBR® Green I

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The fluorescent dye SYBR® Green I (Roche Diagnostics, Mannheim, Germany) binds to the minor groove of the DNA double helix, and its fluorescence is greatly enhanced upon DNA binding. This property is used to monitor DNA amplification during real-time quantitative PCR in the LightCycler® (Roche Diagnostics), a microvolume multicycle fluorimeter with rapid temperature control (1). Products are identified and distinguished by their characteristic melting curves that are dependent on the GC content, length, and sequence of the product (2). However, distinct DNA fragments may have closely overlapping melting profiles and, therefore, a simple melting curve is not sufficient to conclude that a single DNA product is present. Conversely, it is known that longer DNA fragments may have internal melting domains that give rise to complex melting curves (3,4). Smaller DNA products generally melt with a single transition temperature peak (2). Here we show that small DNA fragments may also give rise to more complex melting curves and that the Poland algorithm (5) qualitatively predicts this melting pattern in the majority of cases.

Figure 1 shows two examples of melting curves exhibiting two or more temperature transition peaks and, for comparison, a more typical example, in which a single-peak melting curve is obtained with a single PCR product. The DNA melting patterns predicted by the Poland algorithm (4,5) resemble the observed melting curves, although it is evident in each case that the experimentally observed apparent melting temperature values were significantly higher than those predicted by the algorithm (Figure 1). This is not unexpected because it has been demonstrated that melting temperatures obtained using SYBR Green I fluorescence depend on dye concentration as well as the temperature transition rates (2). Furthermore, for

technical reasons, the solution conditions used by the algorithm could only be approximated to the experimental conditions employed in PCR (for experimental details, see Figure 1 legend). A complex melting curve was also observed when a 188-bp segment of the human β -actin cDNA was amplified (Figure 2). The melting profile was not significantly altered when a slower temperature transition rate (0.05°C/s) was used (data not shown). To gain insight into the origin of this complex melting behavior, we performed additional experiments. The presence of a single 188-bp DNA product was confirmed by agarose gel electrophoresis. To exclude the possibility of co-migrating unrelated DNA, the reaction product was purified and subjected to DNA sequencing, which confirmed the 188-bp sequence derived from human β -actin. It is of note that this DNA product (5'-TTGCGTTACACCCCTTTCTTGACA-AAACCTAACTTGCGCAGAAAA-CAAGATGAGATTGGCATGGCTTT-ATTTGTTTTTTTTTGTGTTTGTGTTTGTGTTTTTTTTTTTTTTTGGC-TTACTAGGATTTAAAAACTG-GAACGGTGAAGGTGACAG-CAGTCGGTTGGAGCGAGCA-TCCCCAAAGTTCACAAT-3') contains a centrally located 44-bp A/T-rich region. To determine which subregion contributed to the complexity of the observed melting curve, we cloned the β -actin PCR product and used the resultant plasmid to amplify segments from within the 188-bp insert, as shown schematically in Figure 2A. Agarose gel electrophoresis of the resultant PCR products demonstrated the presence of a single band in each instance (Figure 2D). The predicted and observed melting curves for each of these products are also shown (Figure 2, B and C). As expected, the 67- and 61-bp products, corresponding to the regions 5' and 3' to the A/T-rich segment (primer pairs 1F-2R and 3F-1R,

respectively), yielded single-peak melting curves that resembled those predicted by the Poland algorithm. Furthermore, when the 44-bp A/T-rich segment was amplified using flanking primers 2F and 3R, the

melting profile of the resultant 100-bp product again showed a single peak, in this case exhibiting a significantly lower apparent melting temperature that was consistent with the predicted pattern. The

147-bp product containing the A/T-rich segment as well as the original 5' extension (primers 1F and 3R) also yielded a simple single-peak melting profile. In contrast, the 141-bp product containing the A/T-rich segment and the 3' extension of the original product (primers 2F and 1R) yielded a more complex melting curve, which was distinguished by two major peaks. Interestingly, only one of the two peaks coincided with one of the peaks of the melting curve of the original 188-bp product (primers 1F and 1R). The Poland algorithm predicted that the 141-bp product (primer pair 2F-1R) would have a pattern that was similar to the one experimentally observed. Note that, in contrast to the 5' extension, which is composed of relatively randomly distributed DNA sequences, the 3' extension from the A/T-rich segment contains a cluster of G/C-rich sequences. This feature may at least in part account for the strikingly different melting patterns of the 147-bp product containing the 5' extension from that of the 141-bp fragment harboring the 3' extension. Interestingly, the predicted and observed melting patterns for the full-length product (188 bp; primers 1F and 1R) were distinct from each other. The algorithm predicts a melting curve characterized by a single transition temperature peak, although close inspection reveals that the peak is not symmetrical but exhibits a shoulder (Figure 2B). In contrast, the experimental melting profile was more complex, exhibiting three distinct peaks.

These examples demonstrate that small DNA fragments can exhibit more complex melting profiles than are generally assumed. The data support the hypothesis that in addition to the length of a DNA fragment, the DNA sequence contributes significantly to the melting profile exhibited by the DNA. It appears that in this regard, clusters of A/T-rich and G/C-rich regions are particularly important, rather than the overall GC content of a given DNA fragment. Such clusters are known to alter the structure of double-stranded DNA (dsDNA) molecules. The SYBR Green I dye, which binds to the minor groove of dsDNA, makes it possible to reveal the structural complexity of small DNA fragments as they melt. Although each unique DNA fragment yields a characteristic melting curve, the profile of this curve may exhibit more

Figure 1. Examples of DNA melting profiles (three PCR products). (A–C) A 287-bp fragment of human adiponectin. (D–F) A 300-bp fragment of murine cyclin-dependent kinase 4 (CDK4). (G–I) A 187-bp fragment of human leptin. (A, D, and G) Melting curves predicted by the Poland algorithm (4,5); differentiated hypochromicity (dA/dT) at 260 nm (red tracings) and 280 nm (blue tracings) versus temperature (°C). (B, E, and H) Melting curves experimentally obtained in a LightCycler using SYBR Green I; differentiated SYBR Green I fluorescence (–dF/dT) versus temperature (°C). (C, F, and I) Agarose gels stained with ethidium bromide; left lanes, DNA size markers (100 bp DNA Ladder; Invitrogen, Carlsbad, CA, USA); right lanes, PCR products indicated by arrows. RNA was extracted from human omental fat biopsy specimens or from mouse intestine (CDK4 amplification only) using TRIzol® Reagent (Invitrogen). An aliquot (0.5 µg RNA) was used for cDNA synthesis using the SUPERSCRIPT™ First-Strand Synthesis System for RT-PCR (Invitrogen), employing an oligo(dT) 12–18 primer (0.5 µg/µL) in a total volume of 20 µL. The cDNA was diluted 20-fold and used as a template in the PCR amplifications described. Primers were designed by Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) (6) of the Whitehead Institute (Cambridge, MA, USA) and custom-synthesized by Invitrogen. The sequences of the primers used are shown in Table 1. PCR amplifications were performed in the LightCycler using LightCycler capillaries (Roche Diagnostics) and reagents of the LightCycler FastStart DNA Master SYBR Green I kit in a volume of 20 µL. Prior to amplification, the samples were denatured at 95°C for 10 min. The rate of temperature change was 20°C/s. The conditions used were 40 cycles of 95° for 0 s, 59° for 5 s, and 72°C for 12 s. DNA melting analysis was performed after completion of the PCR amplifications. The temperature was raised (20°C/s) to 95°C, followed immediately by a decrease (20°C/s) to 59°C, where it was held for 5 s. The melting data were then acquired continuously using an averaging interval of 4°C and a temperature transition rate of 0.2°C/s. Poland's algorithm for predicting DNA melting profiles (5) was performed using the implementation of G. Steger (4), which is provided at the Web site of the University of Duesseldorf (<http://www.biophys.uni-duesseldorf.de/local/POLAND/poland.html>). We used the preset thermodynamic parameters developed by O. Gotoh (19 mM NaCl) (7). For DNA strand concentration (default: 1.0 E–6 M) and dissociation constant (default, $\beta = 1.0 \text{ E}^{-3/\text{M}}$), default values were used. The temperature limits selected were 50°–55°C (low) and 85°–95°C (high). The temperature step size was 0.5°C. Aliquots were analyzed by agarose gel electrophoresis in 1.2% agarose gels, composed of 50% NuSieve® GTG® agarose and 50% SeaKem® GTG agarose (both from Cambrex, Rockland, ME, USA) in 1× TBE (89 mM Tris, pH 8.3, 89 mM boric acid, 2 mM EDTA; Invitrogen).

Table 1. PCR Primers Used

mRNA	Accession No.	Designation	Sequence
APM1/ACRP30/ Adiponectin	NM_004797	Forward	5'-GTGATGCCCAAAGAGGAGAG-3'
		Reverse	5'-CATGAAAGCTCAAACACAGCA-3'
CDK4	NM_009870	Forward	5'-GAGCGTTGGCTGTATCTTTG-3'
		Reverse	5'-TCCTTGTGCAGGTAGGAGTG-3'
Leptin	NM_000230	Forward	5'-ACAGCGAGAGGCAGAGAAAG-3'
		Reverse	5'-AGGTGGTTGTGAGGATCTGG-3'
β -Actin	BC016045	1F	5'-TTGCGTTACACCCTTTCTTG-3'
		2F	5'-GATGAGATTGGCATGGCTTT-3'
		3F	5'-AAACTGGAACGGTGAAGGTG-3'
		1R	5'-ATTGTGAACCTTTGGGGGATG-3'
		2R	5'-AAAGCCATGCCAATCTCATC-3'
		3R	5'-CACCTTCACCGTTCCAGTTT-3'

experimentally derived melting curve may differ from the predicted one, it will be necessary when using this methodology in real-time quantitative PCR to independently confirm for each new product generated that a uniform population of DNA fragments was amplified.

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than one transition temperature peak, even for small DNA products. The data also show that, in most instances, the Poland algorithm is able to qualitatively predict the melting profile of a given DNA fragment. DNA melting analysis combined with real-time quantitative

PCR is currently widely used in genomics and molecular diagnostics (e.g., in the analysis of gene deletions and rearrangements and of single nucleotide polymorphisms). Because of the complexity of some melting profiles observed even with small PCR products and because the

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Figure 2. Melting profiles of subregions amplified from within the 188-bp human β -actin fragment analyzed. (A) A depiction of the 188-bp human β -actin fragment. Dotted lines represent A/T-rich regions; the thick line indicates the location of a G/C-rich segment; arrows denote the annealing regions for the primers used. (B) Melting curves predicted by the Poland algorithm (4,5), as in Figure 1. (C) Melting curves experimentally obtained in a LightCycler using SYBR Green I. (D) PCR products resolved by agarose electrophoresis and visualized with ethidium bromide; first lane, size markers. The primer pairs used for each PCR amplification and the GC content of each fragment (%GC) are indicated between the tracings shown in panels B and C. The 188-bp human β -actin PCR product was gel-purified and cloned using the TOPO TA Cloning® Kit for Sequencing (Invitrogen). The β -actin insert was confirmed by sequencing. The plasmid then served as a template for PCR amplifications of subregions of the insert. For the sequences of primers used, see Table 1.