Decoding DNA labels by melting curve analysis using real-time PCR

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Synthetic DNA has been used as an authentication code for a diverse number of applications. However, existing decoding approaches are based on either DNA sequencing or the determination of DNA length variations. Here, we present a simple alternative protocol for labeling different objects using a small number of short DNA sequences that differ in their melting points. Code amplification and decoding can be done in two steps using quantitative PCR (qPCR). To obtain a DNA barcode with high complexity, we defined 8 template groups, each having 4 different DNA templates, yielding 15^8 (>2.5 billion) combinations of different individual melting temperature (T_m) values and corresponding ID codes. The reproducibility and specificity of the decoding was confirmed by using the most complex template mixture, which had 32 different products in 8 groups with different T_m values. The industrial applicability of our protocol was also demonstrated by labeling a drone with an oil-based paint containing a predefined DNA code, which was then successfully decoded. The method presented here consists of a simple code system based on a small number of synthetic DNA sequences and a cost-effective, rapid decoding protocol using a few qPCR reactions, enabling a wide range of authentication applications.

Today, it is increasingly difficult for a customer to define the origin of a product. Adulteration is a worldwide problem that is expanding to nearly every sort of product, from the cheapest to the most expensive. For this reason, identification marking is especially important for unique industrial or military products. There are many different methods for labeling a product, such as barcoding and fluorescent labeling, but most of these labels are not unique enough to protect the product against falsification. Moreover, labels must be invisible, cheap, tolerant of environmental conditions, easily detectable, and non-reactive with the product material. It is difficult to meet all of these requirements; however the use of nucleic acids, especially DNA, could provide a solution.

Unique DNA sequences from living organisms are frequently used for taxonomic identification. DNA sequencing is a suitable method for determining the composition of foodstuffs of animal and plant origin. Wallace et al. used the internal transcribed spacers of nuclear ribosomal genes to distinguish Korean and American ginseng species (1). In another study, a fragment of the mitochondrial cytochrome C oxidase 1 gene was proven to be an appropriate barcode for identification of the origin of fish and fish-derived foodstuffs (2,3). In addition to natural DNA, synthetic DNA labels have been used for diverse applications. Synthetic DNA can be a valuable tracer in groundwater investigations if unique DNA fragments are

injected into an aquifer. The tracers can be detected by PCR and DNA sequence analysis (4). Furthermore, Cox (5) described a method for barcoding objects with DNA. In that study, many DNA templates of different lengths were mixed together and used to label a letter. To decode the label barcode, the DNA mixture was eluted from the letter, amplified by PCR, and the amplified templates were resolved by agarose gel electrophoresis. Synthetic DNA labels may also be useful for protecting food products. Puddu et al. described a technique that is suitable for tagging olive oil. DNA fragments were adsorbed onto the surfaces of magnetic nanoparticles (Fe_2O_3), and this complex was coated with a dense silica layer. The decoding was based

METHOD SUMMARY

For high complexity DNA barcoding, eight template groups, each having four DNA templates with different melting points, were defined. Synthetic templates and primers were designed for minimal homology with bacterial, plant, and animal sequences, minimizing environmental background interference. Code amplification and decoding were performed in subsequent steps by melting curve analysis using real-time PCR.

on magnetic separation, and the DNA fragments were analyzed by quantitative PCR (qPCR) and Sanger sequencing (6). Another successful application of this silica particles with encapsulated DNA (SPED) technique was presented by Bloch et al. (7). The authors labeled milk with SPED and subsequently manufactured yogurt and cheese from the milk. The extraction of the marker from the milk-derived foodstuff was performed by washing/centrifugation. The specific DNA code was quantified by qPCR.

Here, we present an alternative method for labeling objects with artificial DNA. Our code system is based on synthetic DNA fragments with different melting temperature (T_m) values. Code amplification and decoding can be performed in subsequent steps using qPCR. To demonstrate the applicability of our protocol, we labeled different surfaces with a DNA mixture having a defined combination of T_m values. Labeling, specific amplification, and decoding were successfully performed on a drone's surface.

Materials and methods

Primers and templates

To create our coding system, eight primer pairs were designed to form eight separate groups of synthetic DNA. All sequences were synthesized at Bioneer (Bioneer Corp., Daejeon, South Korea). The templates and primers were designed to show minimal homology with bacterial, plant, and animal sequences when assessed by BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Each primer pair was used with four specific templates (A, B, C, D), each having a different CG content and length. The sequences of the primers and the single-stranded DNA templates used in the study are listed in Table 1.

T_m measurement by qPCR and melting curve analysis

The amplification and melting curve analysis were performed in 20 μ l reactions in a Light Cycler Nano Instrument (Roche Diagnostics Corp., Indianapolis, IN). All reactions consisted of 250 nM of each primer, 10 μ l FastStart Essential DNA Green Master 2x (Roche Diagnostics), 8 μ l PCR-grade water, and 0.1 pM of each template. A negative control (water without DNA) reaction

Figure 1. Primer and template sequences of one template group and their corresponding melting temperature (T_m) values and ID codes. (A) The negative first derivative of the fluorescence vs. temperature ($-dF/dT$) dissociation curve of the first template group having four different T_m values as assessed by melting curve analysis after qPCR amplification. (B) The T_m code and corresponding ID code are defined by sequence variations in one template group consisting of four different templates with different T_m values. (C) The forward primer binding site of the template is highlighted in green, while the reverse primer binding site is highlighted in red. The black bold and underlined sequences differ in length (A,B,C: 48 nucleotides; D: 56 nucleotides) and CG content, ensuring differences in T_m (CG content: 1A-47.9%; 1B-58.3%; 1C-64.6%; 1D-67.9%).

was included in all experiments. The cycling parameters were as follows: enzyme activation at 95 $^{\circ}\text{C}$ for 10 min, denaturation at 95 $^{\circ}\text{C}$ for 15 s, annealing at 58 $^{\circ}\text{C}$ for 25 s, and extension at 72 $^{\circ}\text{C}$ for 25 s. To avoid over-amplification of the products, the cycling was terminated just after the reactions reached plateau (i.e., at 10–14 cycles). The amplification was followed by the melting curve measurement: rapid heating of amplified samples to 95 $^{\circ}\text{C}$ to denature the DNA; cooling to 60 $^{\circ}\text{C}$ to let DNA strands to anneal; then slowly heating the samples to 95 $^{\circ}\text{C}$, where the change in fluorescence was measured at each 0.1 $^{\circ}\text{C}$ rise for 1 s.

Paint preparation

Oil paint (Permanent Blue; Pannon-Color Ltd., Budapest, Hungary) (400 mg) was added to a mixture of 300 μ l acetone, 200 μ l turpentine, and 10 μ l 25% (w/v) DNA Stabilization Resin Solution (Cat. #DSRS-01; Avicor Ltd, Szeged, Hungary). The mixture was

vortexed for 1 min, and the predefined DNA code (1B-2B-3ABCD) was added to the mixture. The concentrated DNA code mixture contained 100 pM of each template. The surface of the drone was labeled with 10 drops (10 μ l each) of the mixture.

DNA extraction from paint

Seventy-two hours after marking, the dried paint drops were separated from the surface of a drone (Bonn Hungary Electronics, Budapest, Hungary) with a clear razor blade. For the long-term stability test, paint drops from a plastic surface (Cell culture dish, Cat. #430167; Corning Inc., Corning, NY) were recovered after 6 months of storage at room temperature by a similar method. Oil paint (30 mg) was dissolved in a mixture of 200 μ l PCR-grade water and 200 μ l ethyl acetate. The solution was vortexed for 1 min and then centrifuged for 2 min at 10,000 $\times g$. The bottom layer was pipetted to a new tube with 200 μ l ethyl acetate and centrifuged for 2 min

Table 1: Primer and template sequences.

Primers			Templates	
1	Forward	AGGTCTCTTCCAACGTGACCG	1A	TCTTCCAACGTGACCGACAAGCATAGATAGATACCTAGACGTGAGTC
			1B	TCTTCCAACGTGACCGACGAGCACGGACAGCTACCTAGACGTGAGTC
	Reverse	TGGAGGACTGACGTCTAGGTG	1C	TCTTCCAACGTGACCGGCCGAGCCCGGACGGCTCACCTAGACGTGAGTC
			1D	TCTTCCAACGTGACCGAGCCGCTCCGGGCTCGCGCTGCGCCACCTAGACGTGAGTC
2	Forward	CCTTCACAACGATCGACAGCG	2A	ACAACGATCGACAGCGATCTTGCTCTATGCTGCTATCCGTAAGTGGTCAAGTCT
			2B	ACAACGATCGACAGCGATCTGGCTCCTGTGCTGCTGCTCCGTAAGTGGTCAAGTCT
	Reverse	CACCAAGACTGACCAAGTACGG	2C	ACAACGATCGACAGCGAGCTGTCCGTGCTGCTCCGTAAGTGGTCAAGTCT
			2D	ACAACGATCGACAGCGAGCCGGTCCGGGCTCGCGCTGCGCCGTAAGTGGTCAAGTCT
3	Forward	CTCTCTGAACGTAGCTGTGGC	3A	TGAACGTAGCTGTGGCACAAGCATAGACAGATCACCATGAGGTCTGAC
			3B	TGAACGTAGCTGTGGCAGCAGCACGGACAGCTCACCATGAGGTCTGAC
	Reverse	GTCTGGTCAGACCTCATGGTG	3C	TGAACGTAGCTGTGGCGCGAGCCCGGACGGCTCACCATGAGGTCTGAC
			3D	TGAACGTAGCTGTGGCAGCCGGTCCGGGCTCGCGCTGCGCCACCATGAGGTCTGAC
4	Forward	CAGGTGTCTAGTGCACAAGCG	4A	TCTAGGATGGTCTCGCATCTTGCTCTATGCTGCTATCGCTTGTCAGTGCACA
			4B	TCTAGGATGGTCTCGCATCTGGCTCCTGTGCTGCTGTGCTGCTTGTCAGTGCACA
	Reverse	GGTTCTCTAGGATGGTCTCGC	4C	TCTAGGATGGTCTCGCAGCCGTCCGGGCTCGCCGCTTGTCAGTGCACA
			4D	TCTAGGATGGTCTCGCAGCCGTCCGGGCTCGCGCTGCGCCGCTTGTCAGTGCACA
5	Forward	AGCAGTCTACAGCACAAGCG	5A	ACTAGCATGCTGACGCACAAGCATAGATAGATCGCTTGCTGCTGTGAGA
			5B	ACTAGCATGCTGACGCAGCAGCACGGACAGCTCGCTTGCTGCTGTGAGA
	Reverse	ACGACACTAGCATGCTGACGC	5C	ACTAGCATGCTGACGCAGCAGCCCGGACGGCTCGCTTGCTGCTGTGAGA
			5D	ACTAGCATGCTGACGCAGCCGGTCCGGGCTCGCGCTGCGCCGCTTGCTGCTGTGAGA
6	Forward	ACGACACTAGCATGCTGACGC	6A	ACTAGCATGCTGACGCACAAGCATAGACAGATGGCATCTGCAGACAGT
			6B	ACTAGCATGCTGACGCACAAGCACAGACAGATGGCATCTGCAGACAGT
	Reverse	AGCTCACTGTCTGCAGATGCC	6C	ACTAGCATGCTGACGCAGCAGCACGGACAGCTGGCATCTGCAGACAGT
			6D	ACTAGCATGCTGACGCAGCCGGTCCGGGCTCGCGCTGCGCGCATCTGCAGACAGT
7	Forward	TCCACAGTACCTACCTGTGCG	7A	AGTACCTACCTGTGCGACAAGCATAGACAGATCCGTTCTGCACAGACT
			7B	AGTACCTACCTGTGCGACGAGCACGGACAGCTCCGTTCTGCACAGACT
	Reverse	TCCTCAGTCTGTGCAGAACGG	7C	AGTACCTACCTGTGCGCGAGCCCGGACGGCTCCGTTCTGCACAGACT
			7D	AGTACCTACCTGTGCGAGCCGGTCCGGGCTCGCGCTGCGCCGCTTCTGCACAGACT
8	Forward	TCGTCTCATCTTAGCTCAGCG	8A	TCATCCTAGCTCAGCGACAAGCATAGACAGATCCACTAGGTCGTGTCA
			8B	TCATCCTAGCTCAGCGACGAGCACGGACAGCTCCACTAGGTCGTGTCA
	Reverse	TCGACTGACACGACCTAGTGG	8C	TCATCCTAGCTCAGCGCGGACGCCCGGACGGCTCCACTAGGTCGTGTCA
			8D	TCATCCTAGCTCAGCGAGCCGGTCCGGGCTCGCGCTGCGCCCACTAGGTCGTGTCA

at 10,000 $\times g$. Five microliters from the lower, aqueous phase containing the DNA templates was used for decoding by qPCR. To detect the presence of each template, an individual qPCR was run with each primer combination.

Results and discussion

Most DNA-based coding technologies have been based on sequence or length variations of the DNA. Here, we present a novel approach based on variations in T_m values of individual templates, which could be measured by melting curve analysis using any qPCR instrument. Since the precise T_m of a DNA template is influenced by several factors (salt concentration, PCR reagents, melting profile, etc.), we defined groups of DNA templates where each group has only a maximum of four different sequences defining four different T_m values (Figure 1A). If we calculate the possible combinations of the 4 T_m values (or T_m codes), 15 different combinations can be achieved if we exclude the one having no signal (containing no DNA template) in the group (Figure 1B). To refer to each T_m code in a template group, we defined an ID code

(Figure 1B) that was used in subsequent DNA coding as a DNA barcode digit. The sequences of the four templates in one group are designed such that both the 5'- and 3'- flanking regions are the same in order to enable the amplification of all of the templates in the group by one primer pair (Figure 1C and Table 1).

To obtain a high complexity among the DNA barcodes, we defined 8 template

Figure 2. Melting temperature (T_m) measurements for the 32 templates. The T_m was determined for each of the DNA templates for all of the eight template groups. Individual reactions contained only one template sequence. Mean and SD values were determined from nine independently repeated qPCR reactions. Only SD values >0.25 are depicted in the graph.

groups, each having 4 different DNA sequences, which enabled us to define 15⁸ possible combinations, meaning 2,562,890,625 different individual T_m (and corresponding ID) codes. To confirm the reproducibility of the T_m values for each DNA template, 8 × 4 = 32 individual qPCRs were run, with 9 repetitions. The average T_m values with their standard deviations (SD) are shown in Figure 2. As expected, very high reproducibility could be achieved. The melting points of the designed templates were well separated and could be clearly detected within a 0.5 degree deviation. T_m values from individual reactions were used as the reference T_m values in experiments where DNA template mixtures were used.

To demonstrate the utility of our approach, we prepared different template mixtures containing different DNA sequences that determined each T_m code for all eight of the template groups. At first, the simplest combination code mixture was prepared, containing only 1 template in each of the 8 template groups. That T_m code was the following: D,A,C,B,A,B,C,D. This can be translated into the following ID code using the table shown in Figure 1B: 41321234. All of the

Figure 3. T_m code determination from a mixture containing DNA from all eight DNA template groups. The T_m values were determined for each DNA template group (1 to 8) in a separate qPCR using a template mixture containing one (A), two (B), or four (C) DNA templates in all eight groups. Black dots represent T_m values determined from individual reactions containing only one DNA sequence (see details in Figure 1). Empty dots represent T_m values determined from a template containing all of the template sequences from the eight groups.

T_m values—and therefore the T_m codes and the ID codes—could be determined without any error. When the T_m values obtained from individual reactions were plotted with those obtained from the eight template mixtures, no significant difference could be detected (Figure 3A).

Next, we prepared an ID code (69580769) that is defined by two templates in each template group (T_m code: AC-BD-AB-BC-CD-AD-AC-BD). Similar to the previous experiment, the T_m points, and therefore the DNA barcodes, could be determined with high precision (Figure 3B).

Finally, the most complex mixture was prepared: each of the eight template groups contained all four of the different DNA templates, which corresponds to “ABCD” in the T_m code and to “e” in the ID code. Even from this mixture, which contained 32 different templates together, all of the T_m values could be determined correctly, with small deviations from the reference T_m values (Figure 3C).

These results clearly demonstrate that the T_m for any template combination can

be determined; thus, any ID code can be decoded. A schematic of our protocol is shown in Figure 4. One advantage of the protocol is that the T_m measurement is done with the same qPCR instrument used for the amplification. Because of the high amplification capability of PCR, even a minute amount of DNA code can be decoded.

To demonstrate the utility of our protocol for industrial applications, we prepared an oil-based paint containing DNA templates and used it to label a drone. First, a three-digit ID code (22e) was generated, and DNA templates according to the T_m code (B, B, ABCD) were mixed equally and then spiked into the paint. The coded paint, was then pipetted onto the body of the drone in 10- μ l drops. For decoding the DNA barcode 3 days later, the paint dots were scraped off, dissolved, and extracted. The aqueous phase containing the DNA templates was amplified by qPCR in three separate reactions, each having primer pairs specific to its template group. After obtaining the melting curves, the T_m

codes could be recovered. Decoding of the DNA barcode was successful (data not shown).

To ensure compatibility of the DNA barcodes with oil-based paint, we recovered DNA from non-solidified oil paint 1 day and 10 days after application. No significant alteration in C_q values for the 2 samples could be detected after qPCR (11.67 versus 10.61 and 9.97 versus 10.81 for samples D1 and D10, respectively). To confirm the long-term stability of the synthetic DNA mixture in paint, different surfaces (metal, wood, paper, and plastic) were labeled and the labels were successfully decoded by melting curve analyses, even after 6 months. DNA recovery and qPCR decoding from old paint spots on plastic surfaces using five codes in quadruplicate were performed. Only moderate degradation could be detected, but interestingly, a sequence-dependent degradation profile was observed (Supplementary Figure S1). In two cases no significant alteration in C_q values was found (codes #1 and #2). For the other

Figure 4. Schematic of the DNA coding approach and the decoding protocol with melting curve analysis using a qPCR instrument.

3 DNA codes (codes #3–5), C_q values that were only 1 to 2.6 greater could be seen after storage for 6 months. This corresponds to ~2- to 6-fold less DNA in the 6-month-old oil paint compared with the reference sample. However, this reduction in DNA concentration does not affect decoding, as substantial amounts of DNA template remained in the paint, resulting in average C_q values of ~16.

The method described here has all the advantages of previously reported DNA-based authentication techniques (long-term stability, small amount of DNA required for labeling and decoding) while

providing the additional benefit of only requiring routine bioanalytical equipment. Amplification and decoding are performed in the same instrument. Therefore, our protocol can be more easily automated than previous approaches. The other advantage of this technique is the code definition. By having all of the variations present in the DNA templates (32 synthetic DNAs in the case of 8 template groups) DNA barcode definition is done by the client, not the provider. Moreover, the order of the template groups could also be changed, resulting in more DNA barcode variation. The simplicity of the

decoding protocol allows the client to perform the coding and decoding steps in a confidential manner, thus guaranteeing increased privacy for the customer.

Author contributions

All authors of this report directly participated in analyzing the data and the preparation of the manuscript. L.G.P. planned the study. L.Z.F. and J.A.B. performed experiments. All authors have read the final version of the manuscript and approved its submission.

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Competing interests

A provisional patent application disclosing the work in the article was filed by Avicor Ltd. for possible future business applications. The DNA Stabilization Resin Solution used in the study is marketed by Avicor Ltd.

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