

A DNA Sequence Design for Molecular Computation of HPP with Output Visualization Based on Real-Time PCR

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Abstract—Molecular computing has proved its possibility to solve weighted graph problem such as Hamiltonian Path Problem (HPP), Traveling Salesman Problem (TSP) and the Shortest Path Problem (SPP). Normally, in molecular computation, the DNA sequences used for the computation should be critically designed in order to reduce error that could occur during computation. In the previous paper, we have proposed a readout method tailored specifically to HPP in DNA Computing using real-time PCR for output visualization. Six nodes of HPP was considered. Based on the example instance, the method requires 11 oligonucleotides, where 6 oligonucleotides are for the nodes and 5 oligonucleotides are for the edges. Three TaqMan probes and five primers are required as well. In this study, a procedure for DNA sequence design is presented in order to obtain good sequences for those nodes, primers, and probes. The experiment is done based on the generated DNA sequences and the Hamiltonian Path can be determined successfully.

I. INTRODUCTION

Polymerase Chain Reaction (PCR) is an incredible sensitive copying machine for DNA. It also can be used for DNA detection. Given a site-specific single molecule DNA, a million or even billion of similar molecules can be created by PCR process. In n steps, it can produce 2^n copies of the same molecules. PCR needs a number of sub-sequence strands called 'primers', which are usually about 20 base long to signal a specific start and end site at a template for replication.

All real-time amplification instruments require a fluorescence reporter molecule for detection and quantitation, whose signal increase is proportional to the amount of amplified product. The simplest and the most direct method is based on the ability of SYBR Green dye to bind non-specifically to any double strand DNA (dsDNA) in

the reaction mixture, including primer-dimers and other nonspecific products that may be generated during the amplification [1]. A more specific strategy depends on different reporter molecules such as TaqMan or hydrolysis probes [2,3], molecular beacons [4-6], and hybridization probes [7,8]. Although a number of reporter molecules currently exist, it has been found that however, the mechanism of TaqMan or hydrolysis probe is very suitable for the extraction of molecular information in DNA computing.

A TaqMan DNA probe is a modified, non-extendable dual-labeled oligonucleotides. The 5' and 3' end of the oligonucleotides are terminated with attached reporter and quencher fluorophores dyes, respectively [9], as illustrated in Fig. 1, where R and Q denote reporter dye and quencher dye, respectively. Examples of common quencher fluorophores include: TAMRA (6-carboxytetramethyl-rho-damine), DABCYL [4-(4'-dimethylaminophenylazo) benzoic acid], and BHQ (Black Hole Quencher). On the other hand, FAM (6-carboxyl-fluorescein), TET (tetrachloro-6-carboxyfluorescein), and VIC are frequently used as reporter fluorophores dyes. Upon laser excitation at 488 nm, these reporter dyes, in isolation emit fluorescence at 518 nm, 538 nm, and 552 nm, respectively. Given proximity of the 3' quencher, however, based on the principle of fluorescence resonance energy transfer (FRET), the excitation energy is not emitted by a 5' reporter, but rather is transferred along the sugar-phosphate-backbone to the quencher. As the quencher dyes emit this absorbed energy at much longer wavelengths (i.e., lower energy) than the reporter fluorophores, the resulting fluorescence cannot be detected by a real-time PCR instrument [10].

The presence of dual-labeled TaqMan DNA probes with forward and reverse primers is a must for a successful real-time PCR. As PCR is a repeated cycle of three steps (denaturation, annealing, and polymerization), a TaqMan DNA probe will anneal to a site of the DNA template in between the forward and reverse primers during the annealing step, if a subsequence of the DNA template is complementary to the sequences of the DNA probe. During polymerization, *Thermus aquaticus* (*Taq*) DNA polymerase will extend the primers in a 5' to 3' direction. At the same time, the *Taq* polymerase also acts as a "scissor" to degrade the probe, via cleaving, thus, separating the reporter from the quencher [11]. This separation subsequently allows the reporter to emit its fluorescence as shown in Fig. 2 [12]. This

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process occurs in every PCR cycle and does not interfere with the exponential accumulation of PCR product. As a result of PCR, the amount of DNA template increases exponentially, which is accompanied by a proportionate increase in the overall fluorescence intensity emitted by the reporter group of the excised TaqMan probes. Hence, the intensity of measured fluorescence at the end of each polymerization phase of PCR is correlated with the amount of PCR product, which can then be detected, using a real-time PCR instrument for visualization.

The DNA sequence design problem arises in molecular self-assembly applications like DNA computing [13, 14], as well as in general purpose laboratory applications such as probe selection for DNA microarrays or primer design for PCR [15, 16]. Since specific hybridization is the key process in these applications, DNA molecules are expected to bind to their particular counterpart with good yield, but not to any other molecules. Further, they should not form any undesired secondary structures, the hybridization should take place in a given temperature range.

II. OUTPUT VISUALIZATION OF DNA COMPUTATION BASED ON REAL-TIME PCR

Let the output of an *in vitro* computation of an HPP instance of the input graph be represented by a 120-bp dsDNA $v_0(20)v_1(20)v_4(20)v_2(20)v_3(20)v_5(20)$, where the Hamiltonian path $V_0 \rightarrow V_1 \rightarrow V_4 \rightarrow V_2 \rightarrow V_3 \rightarrow V_5$, begins at node V_0 , ends at node V_5 , and contains intermediate nodes V_1 , V_4 , V_2 , and V_3 , respectively. Note that in practice, only the identities of the starting and ending nodes, and the presence of all intermediate nodes will be known in advance to characterize a solving path. The specific order of the intermediate nodes within such a path is unknown.

A reaction denoted by $\text{TaqMan}(v_0, v_k, v_l)$ indicates that real-time PCR is performed using forward primer v_0 , reverse primer $\overline{v_l}$, and TaqMan probe v_k . Based on the proposed approach, there are two possible reaction conditions regarding the relative locations of the TaqMan probe and reverse primer. In particular, the first condition occurs when the TaqMan probe specifically hybridizes to the template, between the forward and reverse primers, while the second occurs when the reverse primer hybridizes between the forward primer and the TaqMan probe.

In the previous paper [17-18], we proposed a new readout method tailored specifically to HPP in DNA computing, which employs a hybrid *in vitro-in silico* approach. In the *in vitro* phase, consists of $[(|V|-2)^2 - (|V|-2)]/2$ real-time PCR reactions, each denoted by $\text{TaqMan}(v_0, v_k, v_l)$ for all k and l , such that $0 < k < |V|-2$, $1 < l < |V|-1$, and $k < l$. For this example instance, so that the DNA template is dsDNA $v_0v_1v_4v_2v_3v_5$, these 6 reactions, along with the expected output in terms of "YES" or "NO" are as follows:

$\text{TaqMan}(v_0, v_1, v_2) = \text{YES}$
 $\text{TaqMan}(v_0, v_1, v_3) = \text{YES}$
 $\text{TaqMan}(v_0, v_1, v_4) = \text{YES}$
 $\text{TaqMan}(v_0, v_2, v_3) = \text{YES}$

$\text{TaqMan}(v_0, v_2, v_4) = \text{NO}$

$\text{TaqMan}(v_0, v_3, v_4) = \text{NO}$

Note that the overall process consists of a set of parallel real-time PCR reactions, and thus requires $O(I)$ laboratory steps for *in vitro* amplification. The accompanying SPACE complexity, in terms of the required number of tubes is $O(|V|^2)$. Clearly, only one forward primer is required for all real-time PCR reactions, while the number of reverse primers and TaqMan probes required with respect to the size of input graph are each $|V|-3$.

After all real-time PCR reactions are completed, the *in vitro* output is subjected to an algorithm for *in silico* information processing, producing the satisfying Hamiltonian path of the HPP instance in $O(n^2)$ TIME (here, n denotes vertex number) as follows:

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Input: A[0...|V|-1]=2 // A[2,2,2,2,2,2]
A[0]=1, A[|V|-1]=|V| // A[1,2,2,2,2,6]
for k=1 to |V|-3
  for l=2 to |V|-2
    while l>k
      if TaqMan(v_0,v_k,v_l) = YES
        A[l] = A[l]+1
      else A[k] = A[k]+1
      endif
    endwhile
  endfor
endfor

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It is assumed that a Hamiltonian path is stored *in silico*, in an array (e.g., $A[0..|V|-1]$), for storage, information retrieval, and processing, such that $A[i] \in A$ returns the exact location of a node, $V_i \in V$, in the Hamiltonian path. Based on the proposed algorithm, and the example instance, the input array A is first initialized to $A = \{1, 2, 2, 2, 2, 6\}$. During the loop operations of the algorithm, the elements $A[0] \in A$ and $A[|V|-1] \in A$, are not involved, as those two elements may conveniently be initialized to the correct values, as the distinguished starting and ending nodes of the Hamiltonian path are known in advance. The loop operations are thus strictly necessary only for the remaining elements $A[1,2,3,\dots, |V|-2] \in A$. Again, for the example instance, the output of the *in silico* information processing is $A = \{1, 2, 4, 5, 3, 6\}$, which represents the Hamiltonian path, $V_0 \rightarrow V_1 \rightarrow V_4 \rightarrow V_2 \rightarrow V_3 \rightarrow V_5$. For instance, in this case, it is indicated that V_3 is the fifth node in the Hamiltonian path, since $A[3] = 5$, etc.

III. THE DNA SEQUENCE DESIGN

In order to design a good sequence for each subsequence v_0, v_1, v_2, v_3, v_4 , and v_5 , 5 rules are considered for designing the DNA sequences for primers and probes of real-time PCR according to [19], which is based on the implementation of Primer Express[®] software (Applied Biosystems) [20]. The rules are as follows:



Fig. 1. Illustration of the structure of a TaqMan DNA probe. Here, R and Q denote the reporter and quencher fluorophores, respectively

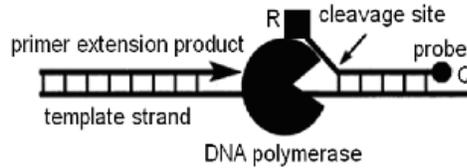


Fig. 2. Mechanism of real-time PCR bases on TagMan probe

1. Melting temperature for primers should be between 58-60°C and melting temperature for probes should be 10°C higher.
2. Primers should be 15-30 bases in length.
3. GC content of primers and probes should ideally be 30-80%.
4. For primers:
 - i) The run of identical nucleotides should be avoided. This is especially true for G, where runs of 4 or more Gs are not allowed.
 - ii) Further, the total number of G and C in the last five nucleotides at the 3' end of the primer should not exceed 2.
5. For probes, there should be more C than G, and not a G at the 5' end.

In this research, the melting temperature for primers and probes should be the same because the primers and probes share the same sequences. Rule 2 is easy to fulfill because the length of primers and probes are set to 20 bases. Since the primers and probes share the same sequences, the rule 4 and rule 5 are combined and thus, the modified rules for primers and probes are as follows:

- i) The run of identical nucleotides should be avoided. This is especially true for G, where runs of 4 or more Gs are not allowed.
- ii) The total number of G and C in the last five nucleotides at the 3' end should not exceed 2.
- iii) There should be more C than G, and not a G at the 5' end.

DNASquenceGenerator [21] is a program to design DNA Sequences which based on the graph method. The nodes in the graph represent base strands and a node has four strands that can appear as successors in a longer sequence as its child nodes. Then, by traveling the graph from root to leaf the DNA sequences can be designed. This approach also is able to find a set of orthogonal DNA sequences within a predefined error rate quickly.

DNASquenceGenerator was employed for generating a set of 100 possible sequences for primers and probes. The condition for the melting temperature was set to automatic

and the parameters of sample concentration, salt concentration, and formamide concentration were left unchanged. During the generation of DNA sequences, the length of sequences was set to 20-mer, GC content and melting temperature were initialized between 50-60% and 50-60°C. In order to fulfill the modified rule (i), the menus “No GGG” was checked so that the runs of multiple G can be prevented. Even though the initial target is to generate 100 sequences but based on the constraints applied, only 85 sequences were successfully generated.

According to the modified rules (ii) and (iii), only 10 sequences out of 85 are valid and filtered out as shown in Table 1. *C-G-D* parameter is introduced in Table 1, where *C* denotes the number of nucleotide C, *G* denotes the number of nucleotide G, and *D* denotes the difference between *C* and *G* such that $D=C-G$. Since the modified rule (iii) stated that there should be more C than G, it is assumed that those sequences with greater value of *D* are more preferable as a good sequence and should be selected. Hence, the sequences 1, 5, 6, 7, 8, and 10 are selected and assigned to the subsequence $v_0, v_4, v_5, v_3, v_2,$ and v_1 , as shown in Table 2. By referring to the value *D*, at first, the sequences 1, 6, 8, and 10 are selected and assigned, whereas sequences 5 and 7 are chosen randomly.

IV. EXPERIMENT

In this experiment, we used parallel overlap assembly (POA) [22] as initial pool generation method. POA involves thermal cycle and during the thermal cycle, the position strings in one oligo annealed to the complementary strings of the next oligo. The 3' end side of the oligo is extended in the presence of polymerase enzyme to form a longer dsDNA. After a number of thermal cycles, a data pool with all combinations could be built. We also used PCR which normally runs for 20-30 cycles of 3 phases: separating base pair strands of DNA at about 95°C, annealing at 55°C, and extension at 74°C [23]. It takes about two to three hours normally in order to complete the cycles.

The PCR product was subjected to gel electrophoresis and the resultant gel image was captured, as shown in Fig. 3. The 120-bp band in lane 2 shows that the input molecules

Table 1. DNA sequences survived based on modified rules (ii) and (iii)

No	Sequence	C-G-D	T_m
1	CCTTAGTAGTCATCCAGACC	7-3-4	53.5
2	TGCCATTGGCCTACTCTAGT	6-4-2	59.7
3	TAGGTCTCGCACGATACTCA	6-4-2	59.4
4	CGTCAAGGCCGTCTCTATAT	6-4-2	59.1
5	CCACTGGTTCGCATGTAAC	6-4-2	58.9
6	TCCACGCTGCACTGTAATAC	7-3-4	59.7
7	TGGACAACCGCAGTTACTAC	6-4-2	58.6
8	ATGCGCCAGTTCTAACTAC	7-3-4	59.4
9	AGGAAACCTCAGCAGTCT	6-4-2	58.7
10	CGCGCACCTTCTTAATCTAC	8-2-6	59.8

Table 2. The assignment of generated DNA sequences

Name	DNA Sequences (5'-3')	Length
v_0	CCTTAGTAGTCATCCAGACC	20
v_1	CGCGCACCTTCTTAATCTAC	20
v_4	CCACTGGTTCGCATGTAAC	20
v_2	ATGCGCCAGTTCTAACTAC	20
v_3	TGGACAACCGCAGTTACTAC	20
v_5	TCCACGCTGCACTGTAATAC	20
v_0v_1	GTAGATTAAGGGTCTGGATGACTACTAAGG	30
v_1v_4	GTAGTAACTGAAGGTGCGCG	20
v_4v_2	GTATTACAGTCGGTTGTCCA	20
v_2v_3	GTAGTTAGAAGCAGCGTGGA	20
v_3v_5	GTTACATGCAGAACCAGTGG GCTGGCGCAT	30

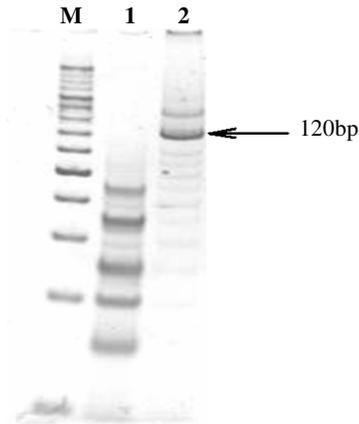


Fig. 3. Gel image for the preparation of input molecules. Lane M denotes a 20-bp molecular marker, lane 1 is the product of initial pool generation based on parallel overlap assembly, and lane 2 is the amplified PCR product

have been successfully generated. Afterwards, the DNA of interest is extracted. The final solution for real-time PCR was prepared via dilution of the extracted solution, by adding ddH₂O (Maxim Biotech, Japan) into 100 μ l.

The real-time amplification involves primers (Proligo, Japan), TaqMan probes (Proligo, Japan), and LightCycler TaqMan Master (Roche Applied Science, Germany). The sequences for forward primers, reverse primers, and TaqMan

probes have been designed previously and listed in Table 3 and Table 4.

Table 3. Sequences for forward and reverse primers

Name	Sequences (5'-3')	T_m
Forward primer, v_0	CCTTAGTAGTCATCCAGACC	53.5
Reverse primer, $\overline{v_1}$	GTAGATTAAGAAGGTGCGCG	59.8
Reverse primer, $\overline{v_2}$	GTAGTTAGAAGCTGGCGCAT	59.4
Reverse primer, $\overline{v_3}$	GTAGTAACTGCGGTTGTCCA	58.6
Reverse primer, $\overline{v_4}$	GTATTACAGTGCAGCGTGGA	59.7

Table 4. Sequences for TaqMan dual-labeled probes

DNA probe	Sequences
Probe v_1	R-5'-CGCGCACCTTCTTAATCTAC-3'-Q
Probe v_2	R-5'-ATGCGCCAGTTCTAACTAC-3'-Q
Probe v_3	R-5'-TGGACAACCGCAGTTACTAC-3'-Q

The amplification contains 45 cycles and at each cycle, the denaturation, annealing, and extension were performed at 95°C, 48°C, and 72°C respectively. Annealing temperature of 48°C is chosen because this temperature is primer dependent and should be 5°C below the calculated primer melting temperature. Note that in this research, the lowest melting temperature of primer is 53.5°C. After the real-time PCR was done based on the defined protocol, the amplification plot as shown in Fig. 4 was observed.

V. DISCUSSION

During annealing phase of PCR, DNA probes and primers must “compete” with each other to hybridize at particular location of input molecules. In this competition, the DNA probe is at a considerable advantage [24] because the DNA probes are present at a lower concentration than the primers. Furthermore, as soon as the primers hybridize, it is immediately elongated and quickly covers the target sequence of DNA probes with newly replicated DNA, even at the temperature far below 72°C. In order to overcome these disadvantages, the T_m of the DNA probes should be higher than that of the primers. Then, the probes have extra time to hybridize at the target location before being displaced by the polymerase enzyme. This is the main reason why rule 1 is designed. Because of this reason, it is better to choose a sequence that the melting temperature is 10°C lower than other sequences. However, the sequence CCTTAGTAGTCATCCAGACC is chosen in this research because this is the best sequence offered by DNASequencesGenerator based on the particular constraints, even though the differences of melting temperature is only about 5-6°C lower than other sequences. Note that the sequence v_0 is the only sequence that only assigned as primer during real-time PCR, while sequences v_1 , v_2 , v_3 , and v_4 and the complements are assigned as both as primers and probes.

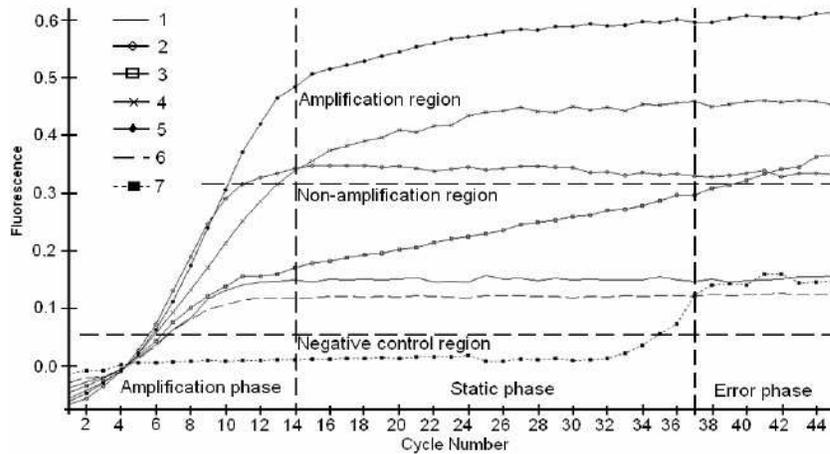


Fig. 4. Output of real-time PCR and grouping of output signals into three regions: amplification region (YES), non-amplification region (NO), and negative control region. The numbering 1 to 6 indicate the $[(|V|-2)-(|V|-2)]/2$ reactions TaqMan(v_i, v_i, v_i) of input instance, while the seventh reaction is for negative control.

VI. CONCLUSION

This study proposed a procedure of DNA sequence design for obtaining good sequence for nodes, primers, and probes. DNASequencerGenerator was employed to generate a set of possible sequences and the selection is done according to the rules and the number of sequences obtained from the DNASequencerGenerator. The resultant sequences were used for the implementation of real-time PCR-based output visualization of DNA computation for the Hamiltonian Path Problem.

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