

# Primer Design for the PCR in Methylation Studies Using PSO

Cheng-Hong Yang, *Member, IAENG*, Ming-Cheng Lin, Li-Yeh Chuang

**Abstract**—The studies of methylation using PCR (Polymerase Chain Reaction) have become very popular recently. Because PCR is a molecular biology technology that can be used for amplifying DNA restriction fragment, it is very important in biological study. Existing methylation primer design tools usually do not provide specific PCR product. In this paper we will design primer pair to find the suitable DNA sequence information by PSO (Particle Swarm Optimization), which uses a update function and swarm intelligence to optimize our search. According to test result, this method can assist biologists to find useful primer information and relevant methylation information.

**Index Terms**— PCR, Primer Design, PSO, Methylation

## I. INTRODUCTION

DNA methylation is essential for biologist studying development in mammals and has been implicated in human pathological conditions. It is a kind of DNA sequence modification that can change behavior of gene expression from altering DNA sequence structure and typically occurs at CpG sites in vertebrates [1-2]. In recent years, the polymerase chain reaction (PCR) has been widely applied in bioinformatics and clinical medicine. PCR is a method for fast duplication of DNA sequences. Therefore, they are mostly used to amplify the number of DNA sequences. In the methylation studies, it is increasingly important to find tools for DNA cloning and primer pair design for clipping targeted subsequence from original DNA sequence.

Due to the reason that PCR amplification will change properties of its product, it is needed to set design constraints in order to optimize primer pair, which can exactly clip the target fragment in DNA sequence. Common consideration of primer design constraints includes lengths of the primers, melting temperatures, base composition, self-complementary sequence and hairpin. The calculated values for the melting temperature of a primer pair should not differ by more than 5 Celsius degree. The primers' lengths should be within 18-26

bps, while the difference of lengths of primer pairs should not exceed 3 bps. The GC content of primers should be within 40-60%. The 3' end of primers should be G or C whenever possible. By considering the above constraints, our primer design tool can find the best choice from constructing a set of primer pair candidates.

Recently, many kinds of primer design tools were developed, but most of those systems' functions lack of methylation evaluation, and are time consuming to find the feasible primer pair (e.g. Primer Design Assistant [3]). Besides, PCR experiments are expensive having a minor mistake may cause failure of experimentation. The primers are the critical factor for PCR experiments. To improve the quality of primer pair, we use the particle swarm optimization (PSO) algorithm. This method is a stochastic, population-based evolutionary computer algorithm developed by Kennedy and Eberhart in 1995[4]. PSO is an automatically evolving algorithm sparked by natural evolution of social behavior of organisms.

Advantages of PSO include being fast to converge, requiring smaller set of parameters and holding memory inside each particle. PSO also has some characteristics same as other algorithms (e.g. GA [5]). As a result, PSO is widely used in various applications.

Currently, methylation primer design is developed according to bisulfite-conversion which changes nucleotide "C" to "T" in DNA sequence [6]. Nowadays, primer sets for methylation analysis designed by most methods will amplify both methylated and unmethylated templates theoretically. Therefore, we hope our developed tool can provide better result to help biologists in studying DNA methylation.

## II. SYSTEM AND METHOD

### 2.1 Sequence conversion

In the primer design for bisulfite-modified DNA sequence, bisulfite is used to test whether a nucleotide is methylated. Adding of bisulfite into DNA sequences will change nucleotide 'C' to 'U' in principle, which is illustrated in Fig. 1. Hence, the conversion from cytosine to uracil can be used to gauge whether it is methylated.

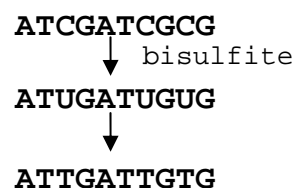


Fig.1. DNA is modified with bisulfite to convert unmethylated cytosines (C) to uracil (U) residues.

### 2.2 Primer Design for Methylation

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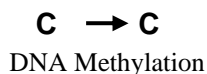
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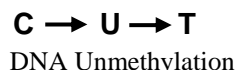
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In our study, the restriction enzymes are used to cut the methylation sites in PCR product, we will then design new primer pairs for those discovered methylation sites. The flow of design is shown below:

A. Original PCR Product



B. Addition Bisulfite PCR



Example: There are two methylation positions respective M1 and M2 that can be cut by restriction enzyme. Consider the two possible conditions:

(1) Primer not overlapped

If our primer pair designed to clip the target DNA methylation sites does not violate the requirement that the regions between fragment C1 cut by reverse primer and fragment C2 cut by forward primer do not overlap, as shown in Fig. 2, we will design the forward primer cut at position at least 100 bases in front of target methylation site and at least 200 bases behind the target methylation site cut by reverse primer.

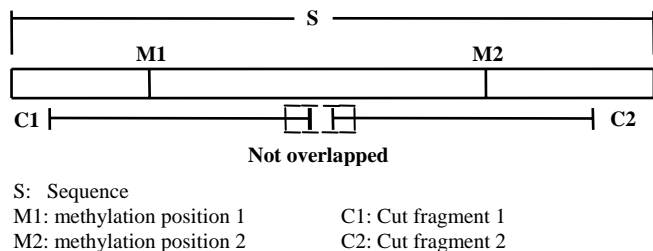


Fig.2. Not overlap

(2) Primer overlapped

If the reverse primer cutting C1 is situated behind the forward primer cutting C2, then our designed primer pair for the target DNA methylation sites are called overlapped, which is shown in Fig.3. The overlapped primer will result in six possible fragments as can be seen in Fig. 4. Thus, we need to revise the primer design, placing the forward primer in front of methylation site M1 about 100 bases and the reverse primer about 200 bases behind the methylation site M2, which is demonstrated in Fig. 5.

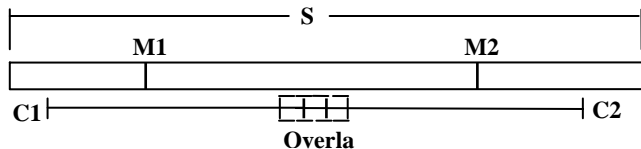


Fig.3. Overlapping

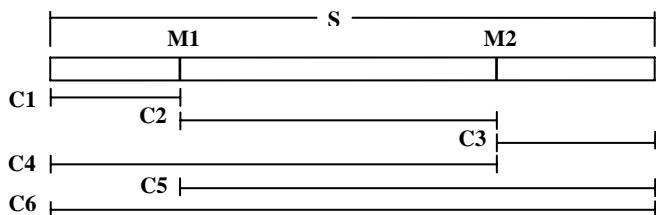


Fig.4. DNA fragments

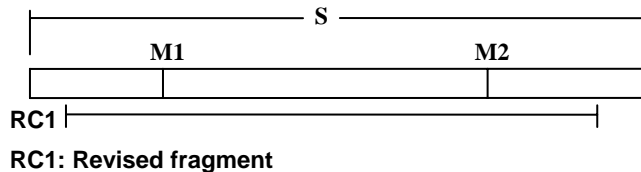


Fig.5. Revised primer design

2.3 System design

In order to provide a convenient tool to assist biologists to design the methylation primers, in this study, we develop a methylation primer design system. The system modules are shown in Fig. 6 including (1) Sequence Input Module, (2) Constraints Information Model, (3) Restriction enzyme Model, (4) Primer Design Module, (5) Output Module. The Sequence Input Module uses a strand of DNA sequence. With consideration of constraint information and avoidance of collision between primer pair and restriction enzymes by widening distance from each primer, then we use PSO to optimize methylation primer design. Finally, the Output Module shows the primer information, PCR product and methylation analysis.

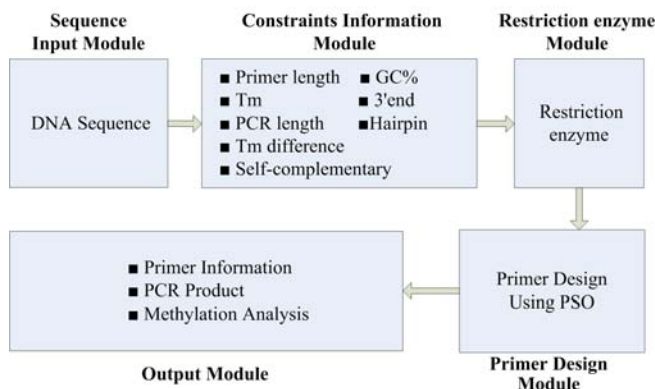


Fig.6. System modules

(1) Sequence Input Model

The Sequence Input Model provide a convenient input function to input a template sequence, such as "ATCGATCG.....". A dataset: NM\_011067 (organism: mouse) will be used as an example to design methylation primers.

(2) Constraints Information Model

Primer design constraint, include GC%, Primer length, Tm, Tm difference, self-complementarity, PCR length, hairpin and the nucleotide at 3' end, etc. We are setting the parameters to search optimal primer pair by our constraints described above, user can also modify the parameters according to their needs.

(3) Restriction enzyme Model

Restriction enzyme is a kind of nuclease that can cut DNA without damaging the DAN structure. Most restriction sites consist of four to six nucleotides. Methylation sites could appear on the PCR product, our system is designed to find those positions by using restriction enzymes to cut the methylation sites. Some of the typical restriction sites used in this paper are shown in the Table 1.

Table 1. Restriction enzymes

Restriction enzyme	Sequence
<i>ApaI</i>	GGGCCC
<i>BamHI</i>	GGATCC
<i>DraI</i>	TTTAAA
<i>EcoRI</i>	GAATTC
<i>HindIII</i>	AAGCTT

In our tool, users can also input their restriction enzyme sequences to search for possible restriction sites cut by those enzymes, one examples is demonstrated in Fig. 7.

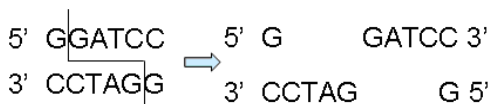


Fig.7. Cut method

#### (4) Primer Design Module

The Primer Design Module is composed largely of particle swarm optimization (PSO) algorithm. PSO is an evolutionary computing technique was first introduced by James Kennedy and Russell C. Eberhart in 1995[4], which was inspired by observation of social behaviors birds in a flock and fishes in a school and can be used to solve optimization problem by simulating behaviors of population stochastically. Particle swarm optimization algorithm is modeled on swarm intelligence, every particle coexists and evolves simultaneously based on knowledge shared with neighboring particle, and each one makes use of its own memory and knowledge gained by the swarm as a whole to find the best solution. PSO has been successfully applied in many field, including data clustering [7] and TSP [8], etc.

#### (5) Output Module

The Output Module shows the methylation positions and our primer information, which includes primer length, GC%, Tm, Tm difference and PCR length, etc. The results will be offered as research reference.

#### 2.4 Primer Design Using PSO

In this section, we will define the primer design problem more clearly. Let  $S$  be the DNA sequence, which is made up of nucleic acid with a vocabulary composed of {A, C, G, T}. Take the sequence below for example:

$$S = \text{ATTACGCAT}$$

Since nucleotides 'A' and 'T' will attract to each other when in proximal distance with hydrogen bonds and nucleotides 'C' and 'G' attracts each other in the same way. We term the phrase complementary sequence of  $S$  as  $\bar{S}$ , which can be seen below:

$$\bar{S} = \text{TAATGCGTA}$$

The forward primer of  $S$  is denoted as  $P_f$ , and is defined as follows:

$$P_f = \{B_i | i \text{ is the index of } S \text{ between } F_s \text{ and } F_e\}$$

The reverse primer of  $S$  is denoted as  $P_r$ , and is defined as follows:

$$P_r = \{B_i | i \text{ is the index of } \bar{S} \text{ between } R_s \text{ and } R_e\}$$

Where  $F_s$  is the start index of the forward primer and the  $F_e$  is end index of the forward primer, and  $R_s$  and  $R_e$  are defined in the same way as start and end index for the reverse primer respectively. Thus, the region cut by the primers between  $F_e$  and  $R_s$  is the PCR product. The primer module is shown Fig. 8. Then, a vector  $P_v$  can be defined with  $F_s, F_e, R_s$  and  $R_e$ .

$$P_v = (F_s, F_e, R_s, R_e)$$

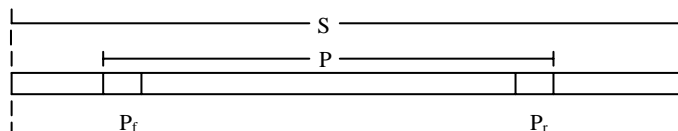


Fig.8. Primer module

S: DNA length

P: PCR product length between forward primer and reverse primer

$P_f$ : forward primer length

$P_r$ : reverse primer length

#### (1) Swam Initialization

A set of particles defined as  $P = (F_s, F_e, R_s, R_e)$  is generated randomly without duplication as the initial population, where the value of  $F_s$  is generated anywhere in the range between [1, |S|],  $F_e$  and  $R_e$  are generated in accordance with the primer length constraints set by the user, and  $R_s$  is a random number between 50-500, which can also be set by the user. In our system, the velocity of each particle is randomly generated within the range of [0, 1].

#### (2) Fitness evaluation

The primer length is only effective ranging from 16bps to 28bps in PCR experiments, a primer too long will raise the specificity higher than desired. On the other hand, a primer too short will decrease the specificity, thus, defecting its effectiveness. Therefore, a primer too long or too short is not suitable for the experiment. The primer length check function is defined as:

$$\text{Length}_{pr} = \begin{cases} 0, & P_f \geq 16 \text{ and } P_f \leq 28, P_r \geq 16 \text{ and } P_r \leq 28 \\ 1, & \text{otherwise} \end{cases} \quad (1)$$

Besides, the length different function  $\text{Length}_{difr}$  between  $P_f$  and  $P_r$  must  $< 3$ , the ABS is absolute value.

$$\text{Length}_{difr} = \begin{cases} 0, & \text{ABS}(P_f - P_r) \leq 3 \\ 1, & \text{otherwise} \end{cases} \quad (2)$$

The melting temperature of primer is formulated as:

$$Tm = -273.15 + \Delta H / (\Delta S + R \cdot \ln(Ct)) + 16.6 \cdot \log[Na+] \quad (3)$$

R= Gas constant (1.987 cal/ $^{\circ}$ C /mol);

Ct = concentration of nucleic acids

[Na+] = concentration of monovalent ions

$$\Delta H = \sum (\Delta H) + \Delta H_{initiation}$$

$$\Delta S = \sum (\Delta S) + \Delta S_{initiation}$$

Table 2. Nearest-Neighbor Model Parameters

Nearest-neighbor thermodynamics		
Interaction	$\Delta H$	$\Delta S$
AA/TT	9.1	24.0
AT/TA	8.6	23.9
TA/AT	6.0	16.9
CA/GT	5.8	12.9
GT/CA	6.5	17.3
CT/GA	7.8	20.8
GA/CT	5.6	13.5
CG/GC	11.9	27.8
GC/CG	11.1	26.7
GG/CC	11.0	26.6

$\Delta H$  and  $\Delta S$  are both calculated as the nearest-neighbor model shown in Table 2, which was first introduced by Breslauer K. J. [9], this method can be used to account interactions between each pair of nearest-neighbors in the DNA sequence to obtain a stable melting temperature. Function  $Tm\_fr$  is used to check whether the melting temperature of the primer pair is within the user set range 50°C to 60°C, and the function  $Tm\_difr$  is used to check the melting temperatures' difference between forward and reverse primer does not exceed 5°C.

$$Tm\_fr = \begin{cases} 0, & P_f T_m \geq 50 \text{ and } P_f T_m \leq 60, \\ & P_r T_m \geq 50 \text{ and } P_r T_m \leq 60 \\ 1, & \text{otherwise} \end{cases} \quad (4)$$

$$Tm\_difr = \begin{cases} 0, & ABS(P_f T_m - P_r T_m) \leq 5 \\ 1, & \text{otherwise} \end{cases} \quad (5)$$

The GC content,  $GC(P_p)$ , is defined as the ratio of nucleotides 'G' and 'C' in the primer, and the function  $GC\_fr$  is used to make certain GC content is between 40%-60%:

$$GC(P_p) = \frac{P_{total}(G) + P_{total}(C)}{|p|} \times 100\% \quad (6)$$

$$GC\_fr = \begin{cases} 0, & P_{f\_GC} \geq 40 \text{ and } P_{f\_GC} \leq 60, \\ & P_{r\_GC} \geq 40 \text{ and } P_{r\_GC} \leq 60 \\ 1, & \text{otherwise} \end{cases} \quad (7)$$

$P_p$ : primer

$P_{f\_GC}$ : forward primer GC content

$P_{r\_GC}$ : reverse primer GC content

$P_{total}(G)$ : Primer 'G' content

$P_{total}(C)$ : Primer 'C' content

In designing the primer, the termination of primer is limited to G or C, and the following function is used to check the constraint:

$$P_{r\_end} = \begin{cases} 0, & \text{if } P_f \text{ and } P_r \text{ 3' end is G or C} \\ 1, & \text{otherwise} \end{cases} \quad (8)$$

The function to check PCR length does not exceed 50-500

bps is defined as:

$$Length\_mid = \begin{cases} 0, & \beta \geq 50 \text{ and } \beta \leq 500 \\ 1, & \text{otherwise} \end{cases} \quad (9)$$

$\beta$ : PCR length

The 3' end of primer sequence may bind to other sequence. To avoid the self-complementarity problem, one should not design the primer pair with each ends complements with each other. The  $Sc\_pt$  and  $Pair\_pt$  functions are defined as follows to solve this situation:

$$Sc\_pt = \begin{cases} 0, & \text{if } P_f \text{ and } P_r \text{ appear in other site no} \\ & \text{self-complementary} \\ 1, & \text{otherwise} \end{cases} \quad (10)$$

$$Pair\_pt = \begin{cases} 0, & \text{if } P_f \text{ and } P_r \text{ appear no} \\ & \text{pair-complementary} \\ 1, & \text{otherwise} \end{cases} \quad (11)$$

The function to consider the specificity of the primer designed for PCR experiment is defined below:

$$Rep\_fr = \begin{cases} 0, & \text{if } P_f \text{ or } P_r \text{ appear in } S \text{ once} \\ 1, & \text{otherwise} \end{cases} \quad (12)$$

We then construct the PSO fitness function by combining all the functions above and the result is shown below; this function will be used to evaluate the fitness of each particle.

$$Fitness = Length\_pr + 3 * (Length\_difr + Tm\_fr + Tm\_difr + GC\_fr) + 10 * (Pr\_end + Length\_mid + Sc\_pt) + 50 * Rep\_fr \quad (13)$$

### (3) Particle swarm optimization

PSO is a population based optimization algorithm. In PSO, each particle is analogous to an individual "fish" in a school of fish. Each particle makes use of its own memory and knowledge obtained from its neighbors to find the best solution, and then the best position among the path travelled by some particle will be called pbest. The best value from all the individual pbest values is called gbest. At each generation, each particle's position and velocity are updated according to its own pbest and gbest of this population by the following function:

$$v_{id}^{new} = w \times v_{id}^{old} + c_1 \times r_1 \times (pbest_{id} - x_{id}^{old}) + c_2 \times r_2 \times (gbest_{id} - x_{id}^{old}) \quad (14)$$

$$x_{id}^{new} = x_{id}^{old} + v_{id}^{new} \quad (15)$$

Where  $r_1$  and  $r_2$  are random numbers between (0, 1), and  $C_1$  and  $C_2$  are both been set as 2. Velocities  $v_{id}^{new}$  and  $v_{id}^{old}$  denote the velocities of new particle and old particle respectively, and positions  $x_{id}^{new}$  and  $x_{id}^{old}$  are the updated particle position and current particle position.

The inertia weight is linearly decreased from 0.9 to 0.4 in the search process for optimal balance between global and local search [10], which is updated by the function defined

below:

$$w = (w_{\max} - w_{\min}) \times \frac{move_{\max} - move_i}{move_{\max}} + w_{\min} \quad (16)$$

$w_{\max}$ ,  $w_{\min}$  are been set to be 0.9 and 0.4 respectively, and  $move_{\max}$ ,  $move_i$  represent max iterate number and current iterate number respectively.

The flow chart overviews PSO algorithm for the primer design is shown in Fig.9. The design process follows with initialization of swarm population, fitness evaluation, searching of pbest and gbest, updating of particles and finally output the best solution when the required criteria have been met.

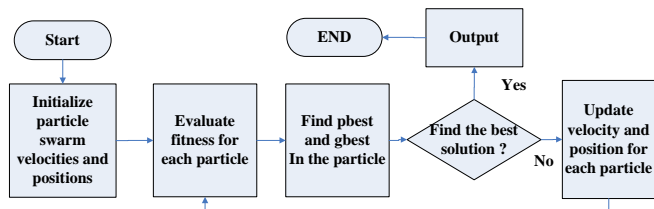


Fig.9. PSO for primer design flowchart

### III. RESULTS AND DISCUSSION

PSO has the advantages of fast convergence, requiring less parameters needed to be set by the users, and having memory reserved in every particle that can be used to compare to each other in the search process. As a result, PSO is widely used in various fields. In the PSO update function, the acceleration factors C1 and C2 are most important as a value too large or too small will result in inferior solution. In many researches, it can be shown that solution of PSO algorithm can quickly converge to location close to global optimal solution [11].

The studies for methylation and primer design have become popular; therefore there have been many present primer design tools developed to tackle this problem, but it still remain area of improvement. Table 3 below compares our proposed tool to other available primer design tools.

Table 3. Comparison of various primer design tools

Tools	Proposed	Mmethprime r	Primer3	PDA
Primer base Information	○	○	○	○
Primer self-complementar y	○	x	○	x
Primer pair-complementar y	○	x	○	○
Visualized	○	○	○	x
B/S	○	○	x	x
Restriction enzyme	○	x	x	x

The tools MethPrimer by Li and Dahiya [12], Primer3 by Rozen and Skaltsky [13], PDA by S.H. Chen1, C.Y. Lin, C.S. Cho, C.Z. Lo and C.A. Hsiung [3], B/S: whether the tool designs primers for bisulfite sequences or not.

The primer information provided by our tool includes GC%, Tm, primer length, Tm difference, PCR size and sequence input panel, etc. In comparison to other primer design tools, our proposed tool differs primarily with the

Methprimer on the capability of usage of restriction enzyme for methylation analysis after we obtain the clipped sequence. Besides Methprimer, functions of the Primer3 and PDA tools are too basic to satisfy much research needs because of their exclusion of methylation translation function which considers situation where conversion from 'C's to 'T's occurs. The proposed tool combines functions from Primer3, PDA and ability to treat bisulfate sequence from Methprimer, then a function able to visually demonstrate the distribution of GC.

Figure 10 shows the user operation flow chart. Our tool is capable of designing primer pair for PCR product and analyzing methylated sites. The primer information and its related methylation process will be shown in a window

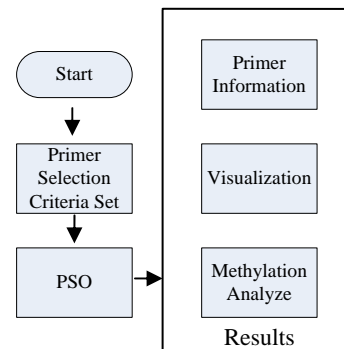


Fig.10. Software operation flowchart

We implemented our tool in JAVA for its easiness to maintain and quickness to develop a GUI (Graphical user interface). The input interface is shown as Fig.11 with the example: NM\_011067 (organism: mouse), the Sequence panel shows the DNA sequence information, and the values in parameter panel are set as Table 4 described.

Table 4. Parameter values set

Primer length	18-26
Length difference	3
Tm	60°C-75 °C
Tm difference	5
GC	40-60
PCR length	50-500

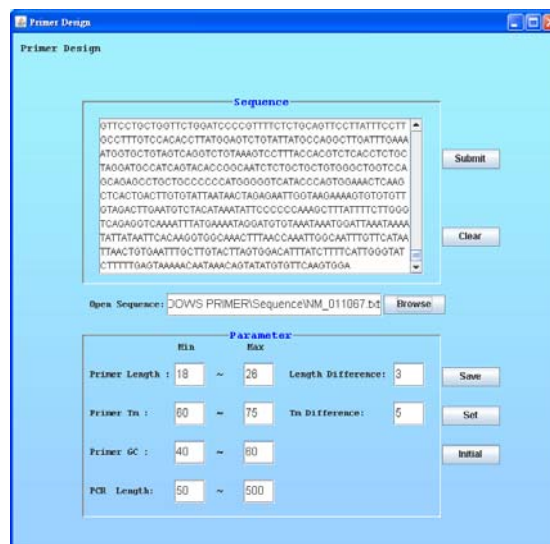


Fig.11. Software input interface



By running a test of input sequence NM\_011067, the results are shown in Fig.12 and Table 4 with a visualization of GC distribution can be seen below Fig.12. Fig.13 shows Methylation Analysis result.

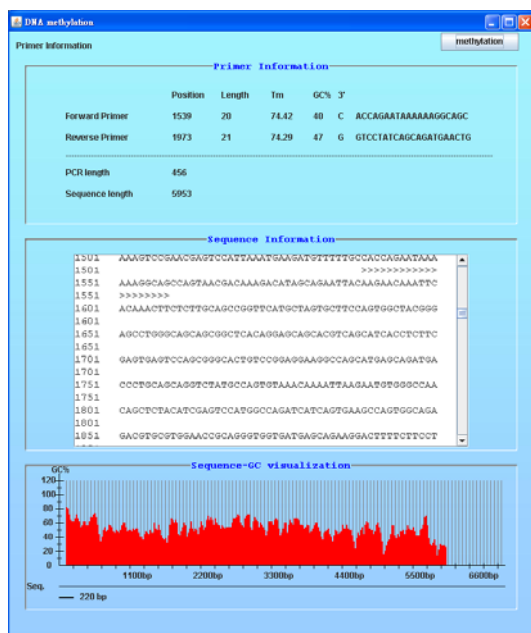


Fig.12. Primer information interface

Table5. Primer information

	Forward primer	Reverse primer
Primer	ACCAGAATAAAA AGGCAGC	GTCCTATCAGCA GATGAACTG
Primer position	1539	1973
Primer length	20	21
Tm	74.42	74.29
GC	40	47
3'	C	G

The reason of high melting temperatures shown in Table 5 can be attributed to the commonly used simple function  $Tm=2(\#A+\#T) + 4(\#C+\#G)$ , which considers only ratios of nucleic acid but not the concentration of ion. The function (3) can be used to calculate value closer to actual temperature by contemplating what was missed in the above simple function.

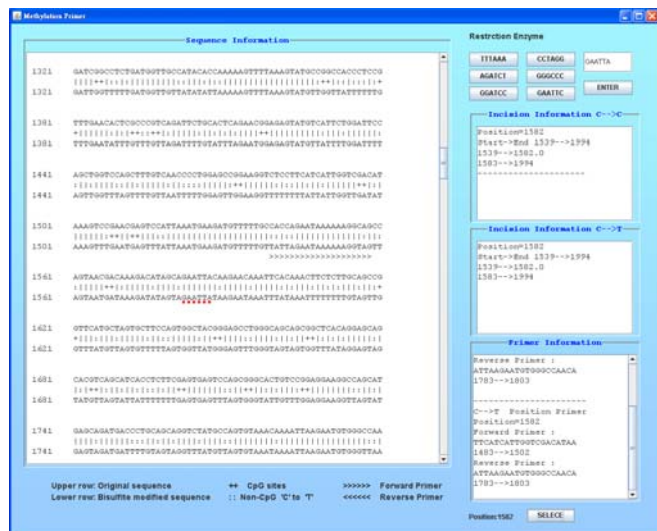


Fig.13. Methylation Analysis

We use the restriction enzyme “GAATTA” to clip the methylation sites for PCR product, then a new primer pair will be designed specifically for those positions, e.g. the restriction enzyme has clip the position 1582, thus a new primer pair will be designed with the forward primer “TTCATCATTGGTGCACATAA” and the reverse Primer “ATTAAGAATGTGGGCCAACA” each of 20 bps long for the positions 1483 to 1502 and 1783 to 1803 respectively.

#### IV. CONCLUSION

The quality of primers has much influence upon the success of a PCR experiment. In this paper, we have provided a tool using PSO algorithm to design optimal primers for methylation, and the proposed approach is effective and takes relatively short time to compute. The user can adjust constraints such as melting temperatures, primer length, PCR length and GC%, to find correct restriction enzyme for target sequence clipping. The result shows the primer information provided is useful in PCR experiments and beneficial to biologists, and we expect by combining other algorithms to improve our tool getting better results.

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