Softepigen: Primers design web-based tool for MS-HRM technique

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Abstract

Polymerase Chain Reaction (PCR) based techniques for DNA methylation techniques includes MS-HRM technique. Methylation Sensitive High-Resolution Melting (MS-HRM) primer-design requires a set of necessary recommendations for such DNA methylation assessment. However, there were not any available software that allows an automatic design of this kind primers. We present *Softepigen*, the first complete MS-HRM primer design software. Softepigen allows to search for primers in a genomic region following Wojdacz's recommendations and targets primer binding regions with high linguistic complexity sequences that increase the specificity of the converted sequence of the human genome. We performed in-silico PCR analysis through *BiSearch* ePCR tool to validate the specificity of the of the primers designed using *Softepigen*. *Softepigen* for MS-HRM performance in our genomic regions of interest show satisfactory specificity measurements, and we implemented it for freely available use in web-based interface in www.soft-epigen.com.

Keywords: primer design, DNA methylation, MS-HRM, Software, Web-based software

1 Introduction

Epigenetics is the field of knowledge that study the changes in the genome no related to the conventional DNA sequence, and these changes are enrolled in regulating gene expression. DNA methylation is the addition of a methyl group at Citocines (Cs) nucleotides in the DNA. DNA methylation is the most stable and widely studied epigenetic mark and recently has shown an important role in many diseases states. At present, DNA methylation measurements are required in biomedical research laboratories every day in different latitudes.

There are three main approaches to DNA methylation evaluation: the global DNA methylation measurements, the epigenomic wide approach and the locus specific approach [1]. The **global approach** first mentioned is informative just regarding the average methylation status of the genome [2]. The epigenomic wide approach provides information of each specific gene in the genome and is especially useful to identify DNA methylation patterns at specific genes. The identified genes can be potential disease biomarkers or be implicated in epigenetic functional pathways, involved in disease models and pathophysiology [3].

The loci specific approach allows the validation and confirmatory studies of DNA methylation in specific loci as disease biomarkers [4] and uses techniques available to most of the molecular biology laboratories around the world. However, the loci specific tests, mainly based on polymerase chain reaction (PCR), have a methodological issue that makes difficult to obtain reliable measurements: The PCR amplification bias [1, 5].

Fortunately, the Methylation Specific Hight Resolution Melting (MS-HRM) technique as proposed by Wojdacz et al., provides an effective strategy to overcome the mentioned PCR amplification bias [6]. MS-HRM employs saturable intercalating dyes and High-Resolution temperature shift increments to measure the melting progress in amplicons of the genomic region of interest, controlling PCR bias to calculate the DNA methylation status in the sample [6].

The main problem in the design of the MS-HRM assessments is the lack of a Software to the automatic primer design according to Wojdacz's recommendations [7]. At present, the researchers requiring MS-HRM primers design should perform the manual or semimanual, time consuming procedure to full fill Wojdacz's recommendations.

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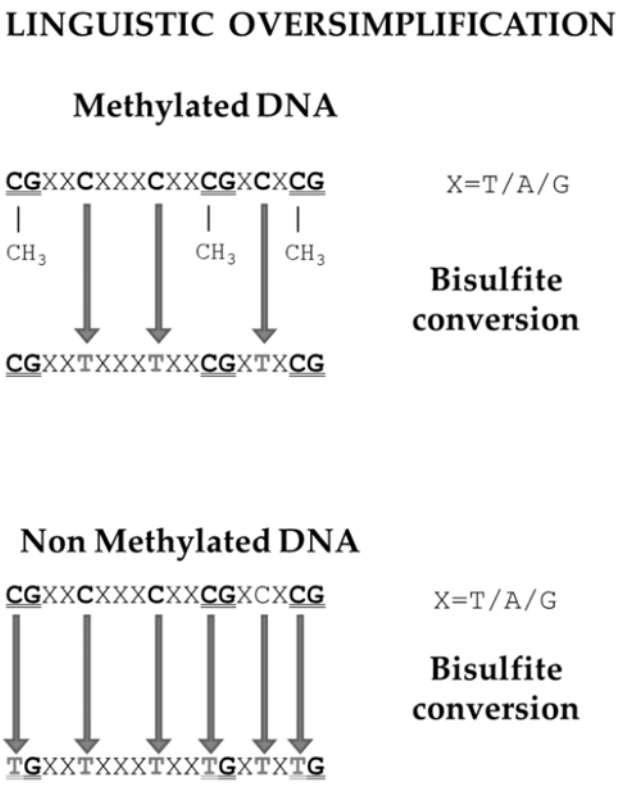
Therefore, we create a computational tool to solve the gap in the MS-HRM design: Softepigen for MS-HRM that constitute a bioinformatics tool able to find primers in a specific locus, using just the conventional sequence of the gene of interest.

Fig. 1. Graphical representation of bisulfite conversion process causing simplification in linguistic composition of the base code. Top: Bisulfite conversion in a hypothetic sequence in state of methylated DNA. Bottom: Bisulfite conversion in a hypothetic sequence in state of methylated DNA. The first case represents a partial loss of the Cytosine's in the hypothetic sequence, reducing the variety or complexity in the sequence. The second case represents a total loss of the Cs in the hypothetic sequence reducing at all the four letters code to tree letter code.



In order to develop an adequate primer design software tool for MS-HRM one should complete the following requirements: at first, to observe the defined rules to primer design described by Wozdach. including the inherent step to simulate the biochemical modification induced by bisulfite conversion and selection of DNA specific sequence corresponding to the recommendations of CpG inclusion in 3' sequences. In second place, one should develop an additional DNA selection regarding bisulfite and amplification specificity, and one should to evaluate the primers designed with the software through carrying out pertinent in-silico PCR specificity tests in comparison with other previously reported MS-HRM primers[1]. Finally, there is a need to implement a user-friendly web-server that allows to researchers in epigenetic the automatic MS-HRM primer design. Below, we described our approach for deal with, one by one, of the mentioned requirements.

2 BACKGROUND AND SIGNIFICANCE

2.1 Principles of PCR primer DNA Methylation

DNA Bisulfite conversion is a chemical treatment to the DNA, which transforms each non-methylated cytosine in uracil (equivalent to Thymine) but preserving each methylated cytosine (Fig 1). In molecular laboratories, this reaction allows the measurement of DNA methylation using PCR based techniques.

However, the original linguistic complexity of the DNA bases is oversimplified. Consequently, the design of the specific primer sequences that match only one genomic region for DNA methylation assessment is made more challenging than conventional PCR techniques [1, 8].

BiSearch represents an available software tool to evaluate the specificity of the primers in bisulfite converted human genome, through a procedure known as *in silico* PCR [9]. BiSearch is also useful in the design of the traditional DNA methylation PCR based techniques [10]. However, in the most of quantitative DNA methylation PCR based techniques, is important to guarantee the lack of amplification preference (or amplification bias) for methylated or unmethylated allele [11].

2.2 Wojdacz's Guideline Rules and beyond

In general, the PCR primers intended to evaluate DNA methylation are designed to bind bisulfite converted DNA [1]. Therefore, one basic rule proposed by Wojdacz *et al.* (2008) is intended to ensure primer binding to bisulfite converted DNA avoiding the no converted sequence. "*The 3'-end of the primer should contain one or more Ts corresponding to non-CpG Cs, to ensure amplification of only bisulfite-converted template"*[7]. Our algorithm includes *in silico* bisulfate conversion that allow the search of "*Ts*" from *non-CpG Cs* conversion in the 3' extreme. In addition, considering the lack of complexity produced by bisulfite conversion[12], we formulate a complexity search strategy to highly specific PCR amplification [13].

The Wojdacz's guidelines [7] also states: "Primers should usually contain one or two CpG dinucleotides Each placed as close as possible to the 5' end of the primer" and *"The preferred length of the amplified sequence should be around 100 bp to reduce the complexity of the melting profile."*

Considering these two main rules, we designed a rational strategy to find the regions between two CpG, with around 100 bp of length, called by us "inter-CpG fragments" strategy.

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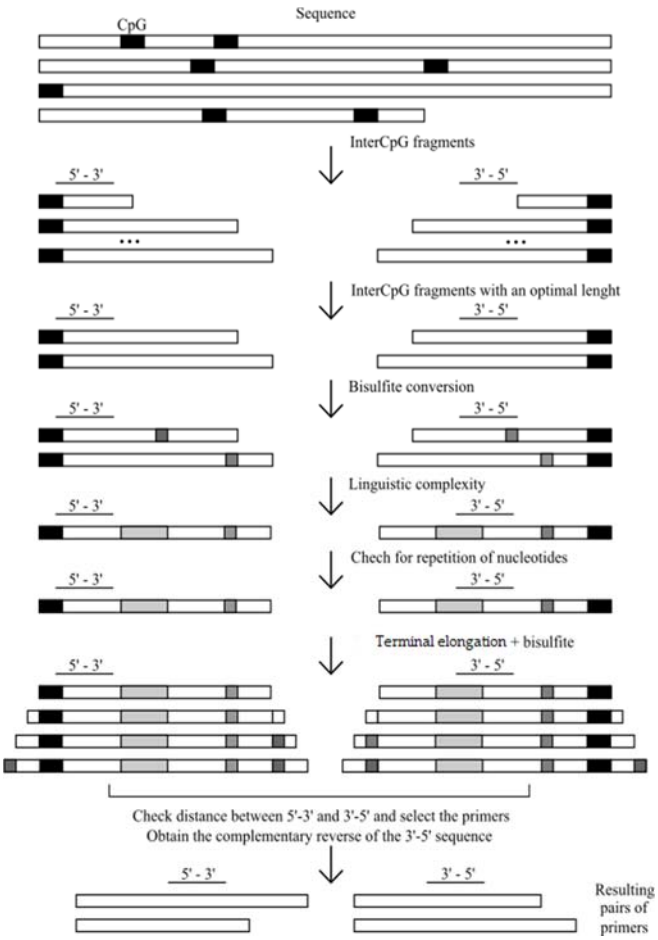
At present, many software tools including BiSearch [14] allow the easy evaluation of the standard primer parameter required for the guidelines [7]: *"The primers should meet standard parameters for primer design, e.g., secondary structure,primer dimer formation" (including similar melting temperatures of the primers).* Therefore, our focus in the present work was to provide a computational tool that performs, automatically, primer design with high specificity but also the non-typical requirements as stated in Wojdacz's guidelines, for amplification bias controlling through MS-HRM implementation.

Fig. 2. Graphical representation of the processes performed by Softepigen for MS-HRM to list potential pairs of primers.



3. SOFTEPIGEN

3.1 Coding

In this work a computational tool for MS-HRM design named Softepigen was developed in Python 3.4, using object-oriented programming. The main processes performed by Softepigen are explained below (Fig. 2).

3.2 Selection of the potential fragments

Softepigen for MS-HRM algorithm starts with the reading of a DNA sequence in FASTA format. Later, it finds the CpG sites in the sequence and selects those fragments that are in the middle of two CpG sites.

For the forward primer binding sequences (5'-3' direction), the CpGs nucleotides are included at the beginning of the fragment, and for the reverse primer binding sequences (3'-5' direction) the CpG nucleotides need to be concatenated at the 5' end of the fragment. Later, the algorithm checks the length of the fragments and those that have a length equal or higher than 16 nucleotides are selected for the next step.

3.3 Bisulfite conversion, linguistic complexity and repeated nucleotides

In this part, the algorithm recreates the bisulfite conversion of cytosines and selects those fragments that contain at least one bisulfite conversion.

The list of resulting fragments for both directions, 5′-3′ and 3′-5′ are analyzed, and only those that have linguistic complexity, by mean of avoiding over-simplified Kmers sequences in the bisulfite treated genome, remain as potential primers binding regions. The next action performed by our algorithm is to eliminate those sequences that contain fragments of length 5 or longer composed by the repetition of the same nucleotide.

3.4 Extreme elongation and bisulfite conversion

In this part, each of the remaining fragments is modified by the addition of one, two and up to three of their corresponding neighbor nucleotides, in both extremes of the potential primers. Later, the algorithm performs the required bisulfite conversions considering that the CpG site should not be modified.

3.5 Results of potential primers

Once the algorithm has a list of potential primers in direction 5′-3′ and primers in direction 3′-5′, it calculates the distance that exists between any pair of primers (5′-3′ and 3′-5′). If the length corresponds to the desired range, as default, between 100 and 150 nucleotides, then for the sequence 3′-5′ the algorithm obtains its corresponding reverse complementary sequence.

3. WEB INTERFACE

We developed a web interface freely available of intuitive and simple using that allows copy and paste *fasta sequence* or alternatively, it allows upload *fasta file* format to perform the MS-HRM primer designing in automatic way. (Fig .3). The *Softepigen tool* is available in www.softepigen.com.

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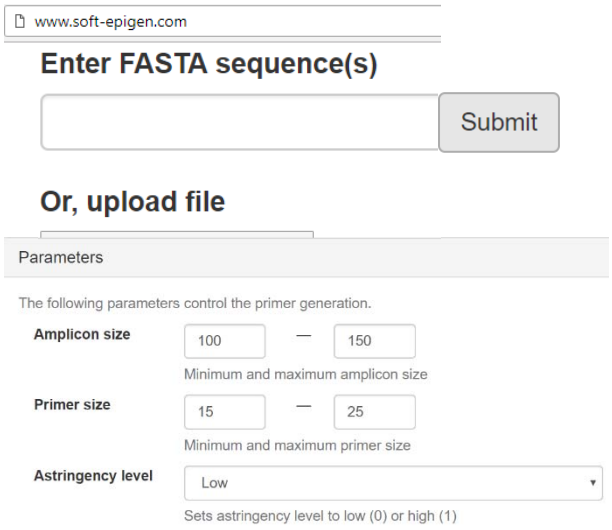
Fig. 3. Screen shot of the *Softepigen web interface*. On top the fasta sequence box for paste or upload. On bottom the adjustable parameters including the amplicon size in nucleotides of desired PCR, the primer size range in nucleotides and the linguistic complexity astringency level, high astringency use pentamer and low astringency use tetramers to select the Kmers regarding its complexity.



Finally, Softepigen for MS-HRM generates and downloads a CSV file that includes the information of the pairs of potential primers with their corresponding lengths in base pairs, the reverse and forward position, and the amplicon size. Softepigen for MS-HRM also creates and downloads a txt file that contains the name of the sequence, the primer forward, and the primer reverse. This last file is specially designed to be used to run automatically simultaneous *in silico* PCRs in BiSearch ePCR tool in order to evaluate and to choose the primer pair of the best specificity between the primers candidates.

5. EVALUATION

5.1 Evaluation Methodology

In silico PCR

BiSearch is the gold standard available tool for in-silico PCR evaluation of the number of potential amplicons for DNA methylation bisulfite-based PCR. Trough ePCR tool in BiSearch server (http:bisearch.enzim.hu /?m=search) [14]. Then, we evaluated a number of matches and amplicons produced by the primers designed using Softepigen for MS-HRM. Inside BiSearch, we use *ePCR* and *Simple search primer* tools to this analysis. We set homo sapiens genome after bisulfite conversion as the genome of reference and use the input sequence of interest (available in supplementary on-line material).

BiSearch primer score

Additionally, we evaluated the primer design using the software tool *primer score* of *BiSearch* (http:bisearch.enzim.hu) according to the corresponding formula [14].

$$T = \frac{T_0 \Delta H_p}{\Delta H_p - \Delta G_p + RT_0 \ln(C_p)} + 16.6 * log \frac{C_{salt}}{1 + 0.7 c_{salt}} - 269.3$$

(1)

"where T_0 = 298.2 K, R = 1.987 (cal/mol K) the molar gas constant, ΔHp and ΔGp are the enthalpy and free energy of the primer p, p=(p1, . . . , pn) which are computed according to the nearest neighbor schemes, i.e. ΔDHp=-1000*[2*ΔHe+Sum(i=1->n-1)(ΔH(pi,pi+1)] and ΔGp=-1000*[2*ΔGe+ΔGi+Sum(i=1->n-1)(ΔG(pi,pi+1)], where enthalpy and free energy of a string consisting of two bases is given in Table below and ΔHe = 5 kcal/mol, ΔGe = 1 kcal/mol and ΔGi = -2.2 kcal/mol. The dimensionless concentration of the primer in the PCR mixture is Cp and Csalt is the molar salt concentration." [13]

5.2 Results of Softepigen testing

The results shown correspond to arbitrary regions in Chromosomes 2, 6, 10, 11and 16, related the laboratory's genes of interest, source sequence in appendix (available in supplementary on-line material). The average matches per strand for each primer was lesser than 3000 bp as previously recommended [1]. As expected we found just one amplicon in the region of interest but none additional undesired amplicon for each corresponding *in silico PCR* (**Table 1**).

Table 1. *BiSearch evaluation of primer designed using Softepigen for MS-HRM*

Gene /chromosome (number of amplicons)	Fw primer Rv primer	Mean sense+ antisense matches	mean matches /chain	Primer pair score
FAM234/ chr16 (1)	ATTCGATGTAGGATTTA CGAAACTCAAACTCTATAACCTAA	3580+ 3558	1785	26.1
Intergenic/ chr10 (1)	TCGATGTGTTATTTAGTA CGTTATCATAATATCTTATAA	3025+ 2919	1486	41.97
ZNF311/ chr6 (1)	TTTCGTTAGTAGAGGTATTTTAT CGCTCTATTCAAATATTTA	2032+ 2716	2374	24.02
UDP / chr2 (1)	GGCGTATAGGAATTATAGG TCGCTATCCTCTACAACAT	1527+ 1567	774	40.99
LINC00838/ chr10 (1)	CGTAATATAGAAAGTTTGTT CGTATATTACATACAAAATACT	4170+ 4264	2109	33.74
CABP2/ chr11 (1)	AAACGTTGTATATTTGTGTTTA TATACGCTACTACATCTATATA	5074+ 624	2849	26.40

Number of amplicons: The number of amplicons obtained in *ePCR tool* in human bisulfited genome. **FAM234**: Family with Sequence Similarity 234 Member A. **ZNF311**: Zinc Finger Protein 311. **UDP**: Glucose Glycoprotein Glucosyltransferase1. **LINC00838**: coding RNA 838. **CABP2:** Calcium Binding Protein 2

An example image of one of the amplicons obtained in *in-silico* analysis is displayed above for the forward and reverse primers "GG**C**GTATAGGAATTATAGG" and

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"TC**GC**TATCCTCTACAACAT" of UDP gene (**Fig. 4**). Note that primer sequences included a CpG in the 5′ extreme.

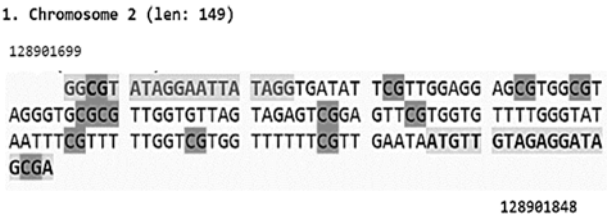

Fig. 4. Amplicon for the gene UDP of interest obtained in BiSearch *in silico* PCR. In dark gray the CpGs in the amplicon. In light gray, the primer binding regions. Note that primer sequences included a CpG in the 5' extreme.

6. CONCLUSION AND DISCUSSION

6.1 Discussion

Until now, the available software for DNA methylation primer design did not cover the MS-HRM technique. e.g. *BiSearch*, *Methprimer*, *MethMarker* and B*eacon Designer®*, between others, are focused in the traditional way to design primers for DNA methylation PCRs useful for, Bisulfite Sequencing PCR, MethyLight and Methylation Specific PCR [2] but not for MS-HRM. *MethylPrimer Express* included MS-HRM primer module but does not following the new primer design recommendations here accounted.

Bearing in mind that after bisulfite treatment just the Cs inside CpGs dinucleotides can remain as Cs, and only 1.8% of dinucleotides are CpG [12], we added linguistic complexity requirement (linguistically complex nucleotide tracks) to the considered criteria. In the present work we use Kmer decomposition that allows sequence feature in an algorithm of codification efficiently [15-17]. As expected, the results of the *in silico* PCR assays indicate the excellent operating of *Softepigen* for MS-HRM designing MS-HRM primers, that accomplish the Wojdacz's criteria but also allow a reduced cross-union to undesired sequences.

Considering the MS-HRM high reproducibility, it can be a quantitative tool as different studies have shown [4, 18, 19]. The easy following to current recommendations for MS-HRM primer design will to contribute to the scientific user to effectively control bias, a critical step for quantitative applications [20, 21].

Primer MS-HRM design can be complicated for determination of the presence of bisulfite conversion and being available a new tool to design primers to beneficial to researchers. Our implemented software may avoid the time-consuming procedure of manual primer design for MS-HRM technique.

6.2 Conclusion

Here we present a totally automatic web-based MS-HRM primer design algorithm that allows to resolve the identified need of a design software in the molecular laboratory epigenetic MS-HRM techniques**.**

Supplementary on-line material

The source sequences for the examples shown in table 1 are available in supplemental on-line material.

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