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PRIMER DESIGNING AND *IN-SILICO* PCR FOR α_{1b} ADRENERGIC RECEPTORS

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Abstract: A previous study from our lab has shown derailment of adrenergic receptors during onset and progression of Metabolic Syndrome. Hence, gene expression study was proposed to understand the subtype of adrenergic receptors. Primer designing is the first and foremost step in the gene expression studies. In this work, suitable primers were designed for SYBR green chemistry and validated using bioinformatics tools to study the adrenergic receptor subtypes involved during onset and progression of Metabolic Syndrome.

Keywords: Adrenergic receptors, Metabolic Syndrome, primer designing

I. INTRODUCTION

Metabolic Syndrome (MetS) is a cumulative term for various metabolic abnormalities like type 2 diabetes, hyperinsulinemia, dyslipidemia, cardiovascular diseases, hyperglycemia, obesity [1]. MetS is associated with enhanced sympathetic stimulation resulting in increased secretion of Norepinephrine, a neurotransmitter [2]acting *via* adrenergic receptors. Our lab has developed a mouse model for Metabolic Syndrome [3] by feeding High Fat Simple Carbohydrate (HFSC) diet for a period of five months. This model displayed characteristic features of MetS [3] along with hypertrophy of pancreatic islets [4]. Norepinephrine level was significantly higher [5] in this model. Desensitization of adrenergic receptors along with thepresence of high affinity α adrenergic receptors was noted during the onset and progression of thesyndrome [6]. Adrenergic receptors in brainstem and pancreas were studied with the help of radioreceptor assays and it requires a good amount of sample. It is quite difficult to get ample amount of sample to carry out radioreceptor works using mouse hypothalamus. In addition to this, radioreceptor assays cannot be used to study the expression level of the adrenergic receptors. In such cases, qPCR is the method of choice to study the gene expression as it uses aminimal amount of sample and more experiments can be performed.Thus, being an invaluable tool for gene expression studies,real time PCR was proposed to study the expression of the subtype of α adrenergic receptor involved.

α adrenergic receptorsare a subtype of adrenergic receptor, a G-protein coupled receptor. They are mainly involved in smooth muscle contraction, control of blood pressure, platelet aggregation, insulin inhibition [7]. The subtypes of α adrenergic receptors are α_1 and α_2 which are further classified into α_{1a}, α_{1b}, α_{1d}, α_{2a}, $\alpha_{2b,}$ andα_{2c}. Activation of α_1 is linked toG_q protein which activates phospholipase which then further downstream acts on calcium channels. But activation of α_2 is associated with adecrease in cAMP. Since radioreceptor study has shown the presence of α receptor, inorder to determine gene expression level of the subtypes oftheα receptor, this work was started with α_{1b} receptor subtype.

qPCR uses fluorescent chemistry to study the variations in the expression levels of the sample. Since SYBR green dye is non-specific and it binds to double-stranded DNA, designing primers for it is very critical. The primer pair should not form any kind of secondary structure during the experiment. Improper designing of primers results in non-specific amplification and primerdimer formation.

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Primer designing has to be followed by *In-silico* PCR that helps to carry out experimentation with precision when tissue samples are limited and one cannot afford to take risks. Various bioinformatics tools are available for primer designing. Choosing the best primers requires choosing the right primer designing tool. In this work, Primer express 3.0 was used to design the primers and *In-silico* PCR was used to validate the specificity of the designed primers. Validation of the primer pairs underwet lab condition is in progress.

II.MATERIALS & METHODS

A. Obtaining of sequences

Firstly, the α1 subtype of adrenergic receptor for *Mus musculus* was queried in NCBI. First 4 best hits for *Mus musculus* were selected from the results. The FASTA for the respective gene coding for the selected hits were downloaded from NCBI.

B. Multiple sequence alignment

The four selected sequences were compared between each other using Clustal Omega of EMBL-EBI. A phylogenetic tree drawn from Clustal Omega was used to find the conserved region. The sequence thus derived was put to BLAST, to determine the specificity of thesequence for theα_{1b} adrenergic receptor of *Mus musculus*.

C. Primer designing and *In-silico* PCR

Primers were designed using Primer express 3.0 (Thermo Fischer Scientific). Briefly, the desired sequences were uploaded to the software. Few parameters like primer secondary structure and amplicon length were changed. Under the primer secondary structure, the max primer total base pair was changed to 5 and min amplified region length was set to 150 and max amplified region was set to 200. The changes were saved and the option to pick primers was clicked. The software gives back the best primers defined by the parameters.

The designed primers were subjected to *In-silico* PCR using UCSC *In-silico* PCR [8] and Primer-BLAST NCBI [9]. For UCSC tool, primer sequences were pasted. Genome and its assembly for Mouse were selected.The target was given as UCSC gene. Max product length was changed to 200. This was submitted. For Primer-BLAST, primer pairs were pasted onto the specified column. The default parameters were taken except for the organism, which was changed to *Mus musculus*.

III. RESULTS & DISCUSSION

4 sequences of 4 different variants of theα_{1b} adrenergic receptor were obtained from NCBI (Table 1). When variants of the sequences are available, it's better to design primers using conservedregion. This would avoid the variability in the sequences which might result in non-specific amplification. Therefore, Clustal Omega was used to determine the consensus region (Fig.1).

Table 1: Accession numbers of 4 sequences of α_{1b} adrenergic receptor obtained from NCBI

NM_007416.4
NM_001284381.1
NM_001284380.1
XM_011248675.2

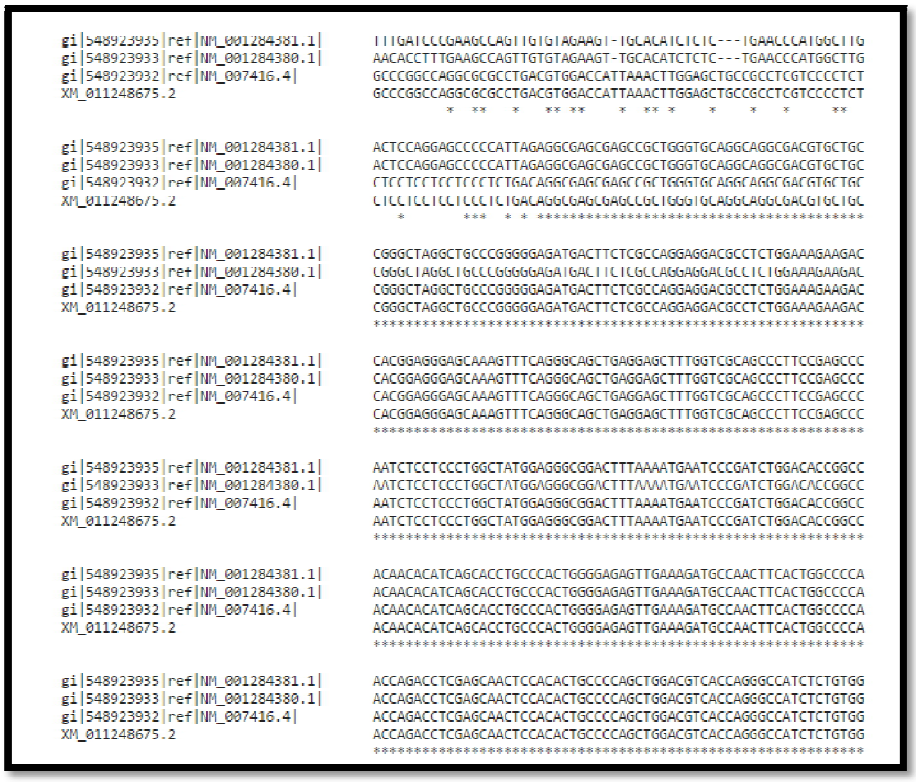
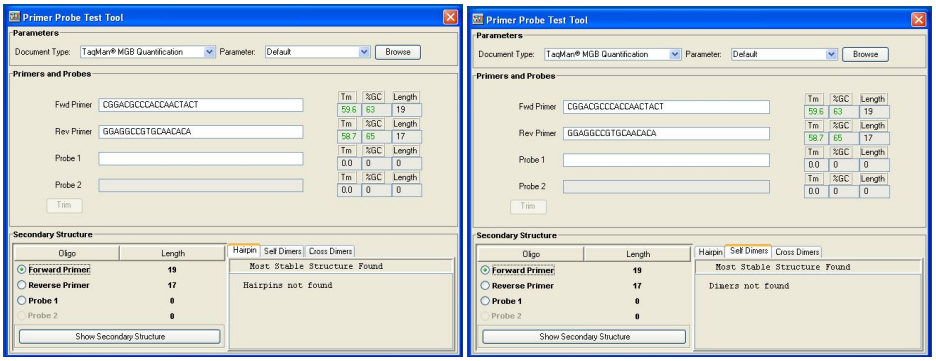


Figure.1. Results of Clustal Omega showing the conserved region

The conserved region wasthen used to design primers. The software itself checks for any possible secondary structures like hairpin, self-dimers and cross dimers in each primer pair. The best of all primer pairs with least secondary structures were chosen. In this case, the forward primer did not show any kind of secondary structure (Fig.2) but the reverse primer showed self-dimers (Fig.3). The factors that are to be considered during primer design are primer dimer formation and efficiency. Primer dimers result in inaccurate quantification of the amplicon by increasing the fluorescence signal. Therefore, primer pairs have to be designed either without any secondary structure or with least formation of secondary structure. The efficiency of qPCR reaction should be very high and it depends again on primer designing. Efficient primer increases the sensitivity and reproducibility of the reaction [10].



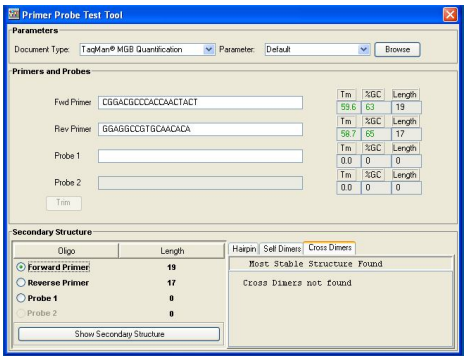


Figure.2. Forward primer test tool output without any secondary structure formation

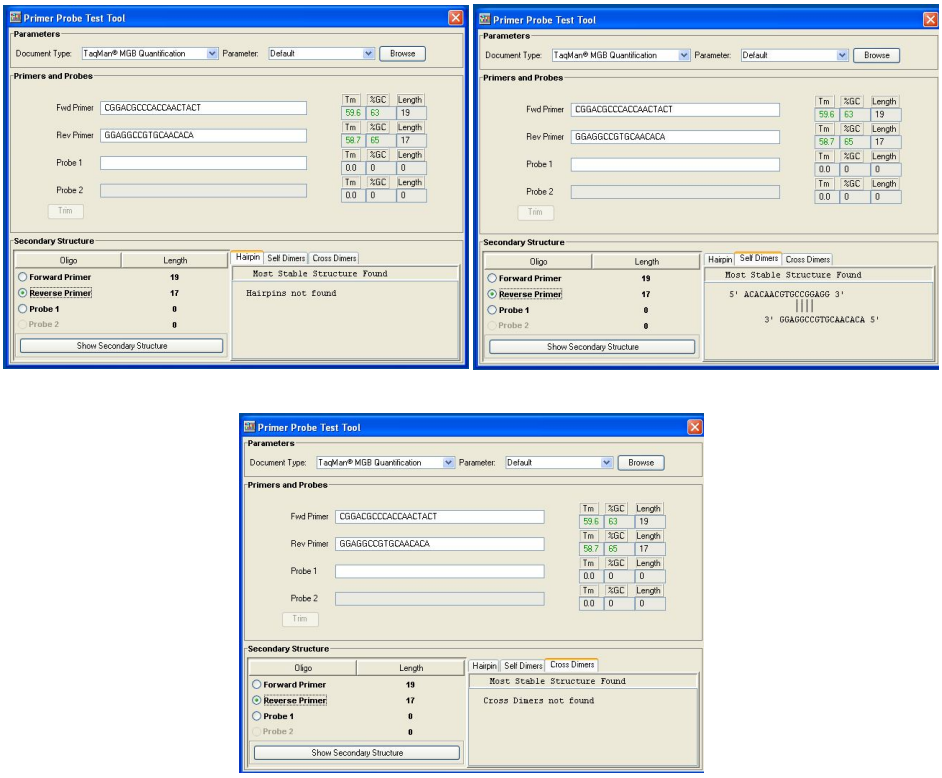


Figure.3. Reverse primer test tool output with self-dimer and without hairpin and cross dimer formation

The designed primers were then tested by *In-silico* PCR online tool. *In-silico* PCR tells about the primer specificity. UCSC *In-silico* PCR program is quite fast as it uses indexing strategy. The result from UCSC *In-silico* tool provides the target's chromosomal coordinates and amplicon size followed by the input sequences ofthe forwardand reverse primers. The chromosomal coordinates have a hyperlinkalong with the starting coordinate, "+" or "–" sign (standing for sense or antisense strand), and the ending coordinate. The melting temperatures of the input primers are displayedat the end and it's calculated based on 50 mM salt and 50 nM annealing oligonucleotide concentrations. The hyperlink leads to UCSC's genome browser displaying thegenomic region of the inquired amplicon with annotated information. In the identified target sequence, the forward and reverseprimers show in upper case, while mismatch(es), if any, will be inlower case [11].

From the UCSC *In-silico* tool, the amplicon length was 168bp and Adra1b (α_{1b} adrenergic receptor) was identified as the target genomic region (Fig.4). There were three possible hits for the designed

primers and all were pointing to Adra1b as the target sequence. Further clicking on the hyperlink, it gave the details of the target genomic region (Fig.5).

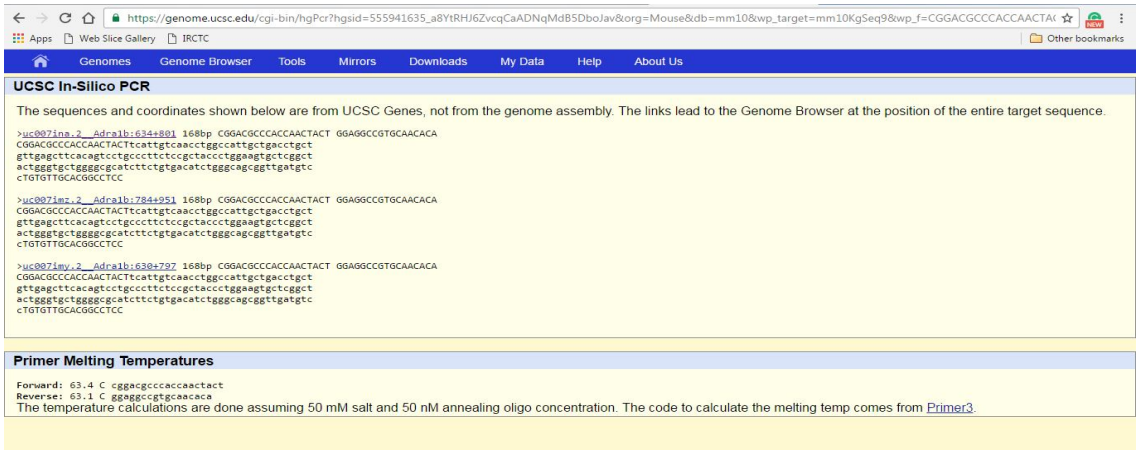


Figure.4. Output from UCSC *In-silico* PCR showing three possible hits for the same target genomic region

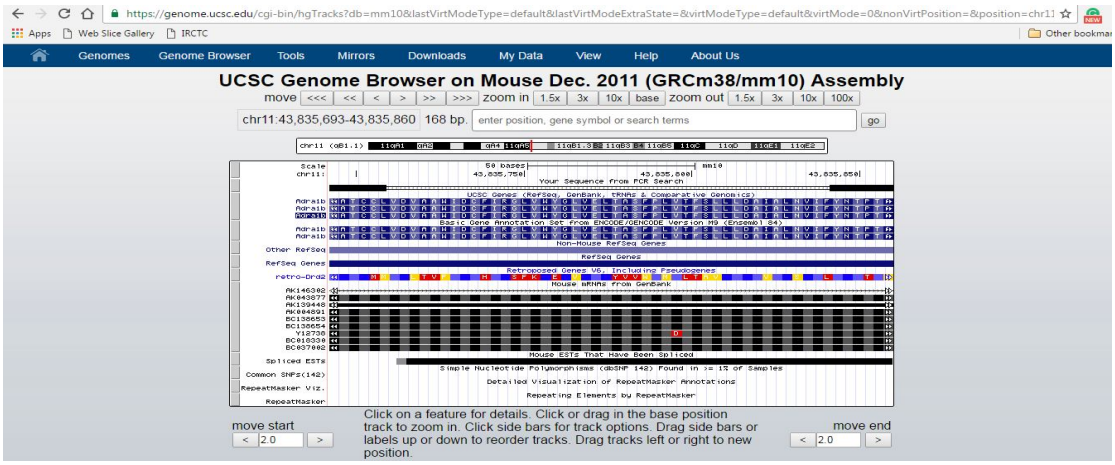


Figure.5. Output from the hyperlink showing the target genomic region

A second tool, Primer-BLAST was used for *In-silico* PCR analysis. Primer-BLAST uses heuristic approach for alignment. “Specificity of primers” summarizes the templates that have been found from the designated target (organism and database). Detailed results give the features of the query primer pair including their sequences, lengths, *T*m (melting temperatures), and percentage contents of GC in primers are listed at the top followed by the identified products. The products are grouped by the targettemplate they are found in. A description line of a template begins with a “>” sign followed by its unique identification. Alignment of primers to their target template is shown by a dot when nucleotides on the template perfectly match with the aligned primer and those mismatched nucleotides are given as they are in the target reference database. Any gaps that have been incorporated into the primers or templates will be indicated by “–” signs [11].

Primer-BLAST too gave the similar results like that of UCSC *In-silico* PCR tool (Fig.6). This suggests that the designed primer is specific for the target sequences and it has to be validated by wet lab experiments.

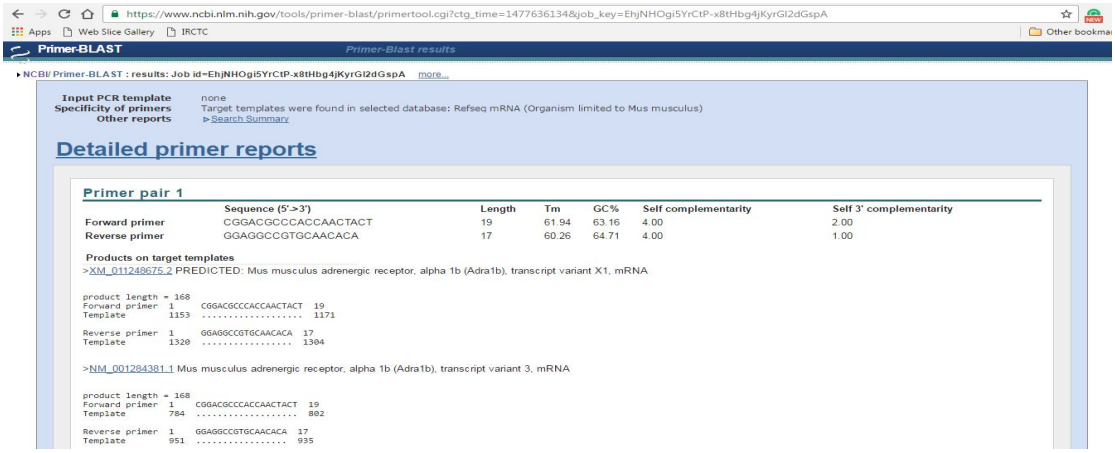


Figure.6. Output of Primer-BLAST showing detailed primer reports

IV. CONCLUSION

Bioinformatics tools are very useful in designing primers and in validating them. The suitable primers for α_{1b} adrenergic receptors that can be used for SYBR green chemistry were designed by Primer express 3.0. The designed primers were validated using bioinformatics programs like UCSC *In-silico* PCR and Primer-BLAST, NCBI to identify potential problems before the wet lab experiments.

V. ACKNOWLEDGEMENT

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