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PRIMER DESIGNING AND STANDARDIZATION OF PRIMER ANNEALING TEMPERATURE FOR BETA-3-ADRENERGIC RECEPTOR IN *MUS MUSCULUS*

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ABSTRACT: Adrenergic receptors (ADRs) are implemented in important pharmacological responses. The β3adrenergic receptors have been proposed as a suitable drug target for treating diabetes and obesity. However, very little information is available regarding the expression profile of β3 receptors. We have designed primers for studying the β3adrenergic receptors in C57BL/6J mice using various bioinformatics tools in the present study. The primers were designed using three different software, primer 3 Plus, NCBI Primer BLAST, and Primer express, and validated using UCSC *in-silico* PCR and NCBI - Primer-BLAST. The best primer pair was selected based on primer melting temperature, GC% and primer length. The selected primer pair was custom synthesized. The primer annealing temperature was standardised using gradient PCR. Among the three software used for primer designing, primer express was found to be the best, and the primers designed from primer express were found to have the ideal melting temperature between 58-60°C, 50% of GC content, and 20bp length. From gradient PCR, the primer annealing temperature was found to be 58.3°C. The bioinformatics tool is useful for the successful designing of primers. Primer express software is best among other software used to design qRT PCR-specific primer.

INTRODUCTION: Adrenergic receptors play a significant role in pharmacological reactions. They have 7 transmembrane domains (3 intracellular and 3 extracellular loops), a glycosylated N-terminal extracellular domain and a C-terminal intracellular domain, which is typical for GPCRs [1]. Since, many developed drugs target a GPCRs, these receptors have been critical to pharmaceutical research.

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Catecholamines such as epinephrine and norepinephrine are natural ligands for adrenergic receptors [2, 3, 4]. Adrenergic receptors can be divided into two subfamilies, α and β adrenergic; these are classified based on the differences related to ligand specificity, tissue expression, downstream signaling and final cellular effects.

The beta (β)-adrenergic receptors, or -adrenoceptors, comprise three members: 1, 2 and 3, which are dispersed across different chromosomes. In terms of amino-acid sequence, β3-ADR is 50 and 40% homologous to β1- and β2 adrenergic receptors, respectively, with the main differences clustering at the 3rd intracellular loop and the C-terminal tail.

The β1 adrenergic receptor and β2 adrenergic receptor have an overall percent identity of 80% and nearly identical intracellular sequences 2, while intracellular sequences 3 exhibit the greatest length variability and have been implicated in G protein selectivity [5, 6]. The β3 adrenergic receptor is mainly expressed in adipose tissue. It is responsible for increased lipolysis and thermogenesis in visceral adipose tissue (VAT) to modulate metabolic rates, as molecular abnormalities in the gene are related to the development of obesity and type 2 diabetes [7]. More specifically, β3 adrenergic receptor acts through the UCP1 activation, reversing adipocyte dysfunction and promoting BAT thermogenesis. Therefore, β3adrenergic receptors have been proposed as a suitable drug target for the treatment of diabetes and obesity. Mice remain the most studied model organism in research [7, 8]. Since this research's findings are typically extrapolated to humans, it is important to understand both similarities and differences between the two species. Besides the apparent difference in size and macroscopic organization of the organ in the two species, several aspects suit both species, which are evidently described. The β3 adrenergic receptor is a good drug target, and its expression is less studied in mice tissues. Quantitative RT-PCR (qRT-PCR) helps to study the expression of a gene. The primer plays an important role in the qPCR reaction. Designing the specific primer is a critical step as the efficient primer increases the sensitivity and reproducibility of the reaction. Thus, in the present study, we have designed and validated the primer pairs for β3 adrenergic receptors in *Mus musculus.*

MATERIALS AND METHODS:

Extraction of Nucleotide Sequence: The nucleotide sequence for β3 adrenergic receptor (*Adrb3)* for *Mus musculus* was queried in National Centre for Biological Information.

The gene sequence for the respective gene coding for selected hits was downloaded in FASTA format from NCBI.

Selection of Conserved Regions: The four selected transcript variants were compared using *Clustal W* from EMBL-EBI. The phylogenic tree derived from *Clustal W* was used to find the conserved regions. The highly conserved regions were selected for primer design.

Primer Designing: It was difficult to select the best tool for primer designing. Therefore, we opted for different software to design the primer. The primers were designed using three different software: primer 3 Plus, NCBI Primer-BLAST, and Primer express (Thermofisher Scientific). The conserved sequence was uploaded in each software separately. The parameters like product size, primer size, GC content, and melting temperature were set. The top primer pairs from each software were selected for further analysis.

Analysis of Secondary Structures: The best primer pairs from Primer 3 Plus, NCBI Primer-BLAST, and Primer *Express* were selected and checked for specificity using the IDT-DNA Oligo Analyzer. The secondary structures like hairpin, self-dimer, and heterodimers were analysed for the primer pairs.

***In-silico* PCR:** The designed primers were subjected to *in-silico* PCR using UCSC *in-silico* PCR. The forward and reverse primers were uploaded. The genome and its assembly for the mouse were selected. The maximum product length was changed to 200bp, and the query was submitted. The expected product size of the primers was observed.

BLAST: BLAST is the most frequently used to calculate sequence similarity. The best primer pair was selected based on its specificity and secondary structure. The primer sequence was uploaded on the BLAST and was run to compare the biological information.

Standardization of Primer Annealing Temperature: The primer pairs were custom synthesized (Sigma). The PCR reaction mixture containing Taq buffer, 25 Mm dNTP (GeNei™), forward primer and reverse primer for *Adrb3*, Taq polymerase (GeNei™), and 50ng/µl template mouse cDNA was added. The PCR was performed on T100™XR Thermal cycler Gradient PCR (BIO-RAD, USA) with its corresponding optimum annealing temperature. The reference housekeeping control was the β actin gene (*Act B*). Initial denaturation was set at 95°C for 5 minutes, then 94°C for 1 minute, followed by annealing at 55°C-60°C for 1 minute, extension at 72°C for 1 minute with 40 cycles and final stabilization at 72°C for 10

minutes. The PCR products were separated by the agarose gel electrophoresis and result was analyzed using molecular imager Gel Doc™XR+ Imaging system (BIO-RAD, USA) with *image lab* software.

RESULTS AND DISCUSSION: Polymerase chain reaction is used to amplify the desired DNA fragments. Recent advances in PCR have developed various amplification techniques with the help of fluorescent dyes, which is more accurate and faster. The use of non-specific or sequence-specific fluorescent signals in conjunction with RT-PCR can be used to quantify the amount of mRNA, DNA, or cDNA in sample [9]. Non-specific detection uses fluorescent dyes like SYBR Green. SYBR has the potential to bind the double-stranded DNA and emit a fluorescent signal that is 1,000-fold greater than unbound SYBR Green [10]. The PCR products can be influenced by various parameters such as working reaction condition which includes temperature, pH of the buffer, the efficiency of polymerase enzyme to bind and amplify the DNA, concentration of Mg+ ions and purity of DNA template [11]. Designing the specific primer is a critical step as the efficiency of the primer increases the sensitivity and reproducibility of the reaction. The best pair of primers for qRT PCR have a melting point between 45 to 65ºC with length 18 to 25bp. It is very crucial to have a 45-50% of GC content without any secondary structures such as hairpin loop, self-dimers to avoid non-specific binding [12, 13, 14].

The tools of bioinformatics and *in-silico* PCR have been extensively useful for designing the β3 adrenergic receptor primers and in validating them. The nucleotide sequence was extracted from NCBI in FASTA format specific for *Mus musculus*. The FASTA format was used to carry out further alignment and primer designing. Three different transcript variants of β3 adrenergic receptors were obtained from NCBI **Table 1**.

TABLE 1: ACCESSION NUMBERS OF THE TRANSCRIPT VARIANTS OF Β3 ADRENERGIC RECEPTOR IN MUS MUSCULUS FROM NCBI. SOURCE: NCBI

NM_013462.3	Mus musculus adrenergic receptor, beta 3 (Adrb3), mRNA	2795 bp
XM_030243247.1	Mus musculus adrenergic receptor, beta 3 (Adrb3), transcript variant X4, mRNA	3029 bp
XM_030243246.1	Mus musculus adrenergic receptor, beta 3 (Adrb3), transcript variant X3, mRNA.	2942 bp
XR_003947220.1	Mus musculus adrenergic receptor, beta 3 (Adrb3), transcript variant X2, misc RNA.	3276 bp

It is suggested that when sequence variants are available, it is better to design the primer using conserved regions to avoid non-specific amplification. *Clustal W* was used to check for conserved regions that align three or more sequences and produce biologically and statistically meaningful multiple sequence alignments of divergent sequences [15, 16]. Therefore, these 3 transcript variants were analyzed further to check the conserved regions by multiple sequence alignments using *Clustal W* **Fig. 1**.

FIG. 1: CLUSTAL W WITH MULTIPLE SEQUENCE ALIGNMENT, THE REGIONS HIGHLIGHTED WITH STARS ARE THE CONSERVED REGIONS

The consensus region highlighted with stars were selected for primer designing. The primer were designed using *Primer 3 Plus, Primer Express* and *NCBI Primer BLAST*. The best pairs were selected based on length, melting temperature and GC%. The primer pairs obtained from three different software were considered for oligo analysis. The selected primer pairs were analyzed in IDT-Oligo Analyzer to check the primer specificity, secondary structures such as hairpin, self-dimer and heterodimers.

The primer pairs with least secondary structure was selected for further analysis. The primer pair 2 has the least secondary structure compared to other two pairs. The forward primer sequence did not show any hairpin structure but formed a single self-dimer. The reverse primer sequence did not show any secondary structures. The presence of dimers has a negative effect on the PCR product. Dimers can be cross-dimers, in which the forward and reverse primers are annealed, or self-dimers, in which the forward primer anneals to another forward primer or a reverse primer anneals to another reverse primer, resulting in a non-specific PCR product or no product at all [14, 17].

Therefore, the hybridization of two primers should be avoided; thus, in the current study, secondary structures were avoided. Also, monovalent (sodium, potassium), divalent (magnesium) and polyvalent cations positively impact the stability of hybridized oligonucleotides [18, 19, 20]. In the current study, the monovalent salt concentration was set to 50mM, which is considered to give a stable environment for Tm for the designed primers. Increasing the concentration of monovalent cations (Na +), up to 1-2 M could further stabilize the oligo's. Studies reported that *Quant Prime* and *AutoPrime* can yield primers with higher specificity. The designed primers were then validated by *in-silico* PCR tool. UCSC *In-Silico* PCR works in indexing strategy. The melting temperature of the input primers was displayed at the end and calculated based on 50mM salt and 50nM annealing oligonucleotide concentration. *In-silico* PCR is all about the primer specificity [21, 22]. The result from UCSC *In-silico* tool provides the target chromosomal coordinates and amplicon size followed by the input sequences of the forward and reverse primers. This tool's amplicon length was 185bp, and Adrb3 was identified as the target genomic region (**Fig. 2.**

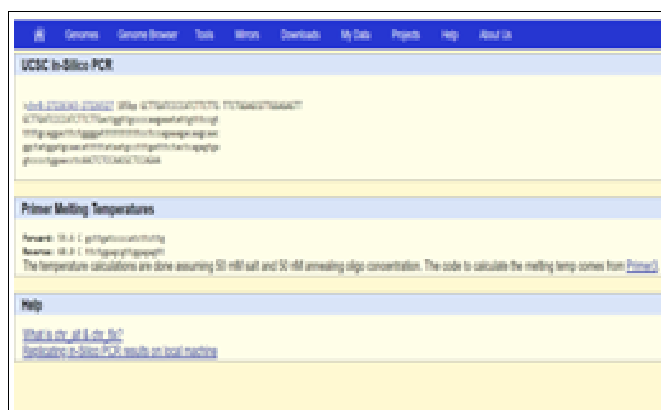

FIG. 2: UPSC *IN-SILICO* PCR SHOWING THE PREDICTED PRODUCT LENGTH OF 185BP FOR THE PRIMER

There was not much difference between the GC percent, oligo length, and melting temperature of the primer pairs. NCBI-BLAST uses a heuristic strategy to align and find empirical or a near-optimal match based on the similarity of the query sequence [23]. BLAST alignment results showed more than 40 similar sequences.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Mus musculus chromosome 8, clone RP23-118B13, complete sequence	Mus musculus	40.1	40.1	100%	0.014	100.00%	224946	AC102544.14
PREDICTED: Mus musculus adrenergic receptor, beta 3 (Adrb3), transcript variant X3, misc_RNA	Mus musculus	40.1	40.1	100%	0.014	100.00%	2786	XR_003947220.2
Mus musculus targeted deletion, lacZ-tagged mutant allele Adrb3:tm1(KOMP)Wtsi; transgenic	Mus musculus	40.1	40.1	100%	0.014	100.00%	37222	JN955069.1
Mus musculus adrenergic receptor, beta 3 (Adrb3), mRNA	Mus musculus	40.1	40.1	100%	0.014	100.00%	2795	NM_013462.3
Mus musculus adult male cecum cDNA, RIKEN full-length enriched library, clone:9130002F08 product:adren...	Mus musculus	40.1	40.1	100%	0.014	100.00%	2795	AK136495.1
M.musculus gene for beta-3-adrenergic receptor	Mus musculus	40.1	40.1	100%	0.014	100.00%	3438	X72862.1
Mus musculus BAC clone RP23-186F18 from chromosome 10, complete sequence	Mus musculus	34.2	82.8	90%	0.85	100.00%	195384	AC112149.4
Mus musculus predicted gene, 29805 (Gm29805), long non-coding RNA	Mus musculus	32.2	32.2	80%	3.4	100.00%	1099	NR_105054.1
Mus musculus BAC clone RP24-251C17 from chromosome 8, complete sequence	Mus musculus	32.2	32.2	80%	3.4	100.00%	141785	AC132107.3
Mus musculus BAC clone RP24-285O1 from chromosome 12, complete sequence	Mus musculus	32.2	32.2	80%	3.4	100.00%	160337	AC140249.2
Mus musculus BAC clone RP23-238I2 from 14, complete sequence	Mus musculus	32.2	32.2	80%	3.4	100.00%	205957	AC125205.4
Mus musculus BAC clone RP23-315F17 from 14, complete sequence	Mus musculus	32.2	32.2	80%	3.4	100.00%	197655	AC122320.4
Mus musculus adult male corpora quadrigemina cDNA, RIKEN full-length enriched library, clone:B230326J17...	Mus musculus	32.2	32.2	80%	3.4	100.00%	1100	AK045951.1

FIG. 3: NUCLEOTIDE BLAST FOR THE PRIMER PAIR FOR *ADRB3* SHOWING TOP HITS

TABLE 2: PRIMER PAIRS SELECTED FROM *PRIMER 3 PLUS, PRIMER EXPRESS AND NCBI PRIMER BLAST* RESPECTIVELY. TM: MELTING TEMPERATURE OF THE PRIMER, %GC: PERCENTAGE OF GUANINE AND CYTOSINE

	Sequence (5'- 3')	Length (bp)	Tm (°C)	GC (%)
Primer pair1	TTGTCCTGGTGTGGATCGTG	20	60.0	55
	TTGGAGGCAAAGGAACAGCA	20	60.1	50
Primer pair2	GCTTGATCCCCATCTTCTTG	20	59.6	50
	TTCTGGAGCGTTGGAGAGTT	20	60.0	50
Primer pair3	GGAGGCAACCTGCTGGTAAT	20	60.03	55
	CGTAACGCAAAGGGTTGGTG	20	60.04	55

However, the query sequence was 100% similar to *Mus musculus. Adrb3* mRNA was further confirmed by the E value close to zero, and query coverage was 100% **Fig. 3** indicated that the sequence match was pure, and hence the match was significant. The BLAST results gave a similar result; this confirms that the designed primers were specific for the target sequence. From earlier studies, a comparison was done between BLAST and other software such as *MPBLAST* [24], BLAT [25] and *miBLAST*, showed that NCBI-BLAST is the robust program based on parameters such as E value which showed a better score and Word Size showing significance on the sensitivity and performance of the program [21, 24].

We designed a pair of *Adrb3* primers for qRT PCR with the sequence; Forward primer: 5'-GCTTGATCCCCATCTTCTTG-3' and reverse primer: 5'-TTCTGGAGCGTTGGAGAGTT-3' with 50% GC content, 20bp primer length and melting temperatures 59.6ºC and 60.0ºC respectively.The designed primer pairs were custom synthesized. Using gradient PCR, the primer annealing temperature was validated. It is reported that the primer annealing temperature-Ta ranges between ±5°C from the melting temperature-Tm of the corresponding primer pair. From the standardisation of annealing temperature for primers, we found that the annealing temperature was 58.3°C and it was within the calculated range. The validated annealing temperature can be used further for q-RT PCR studies.

CONCLUSION: The best primer pair for β3 adrenergic receptor in *Mus musculus* was designed using *Primer Express* software, which helps design primers with least or no secondary structures. The primers were successfully validated by wet lab experiments using mice cDNA. The primer annealing temperature will be used in the quantitative real-time PCR to study the gene expression pattern.

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