

DESIGN OF PRIMER FOR THYROTROPIN RELEASING HORMONE OF *MUS MUSCULUS* C57BL/6J FOR qRT-PCR

Swarnalatha BN¹, Asha Abraham²

Abstract: High fat simple carbohydrate fed C57BL/6J mice, a model for metabolic syndrome have shown significant disruption of feedback control between TSH of the pituitary and T3 hormone of the thyroid gland. In order to understand the exact mechanism involved, it is important to assess the level of the hypothalamic TRH. qRT-PCR is a method of choice when tissue sample is less. In the present study, we have designed C57BL/6J mice specific primers for TRH.

Keywords: TRH, Primer, qRT-PCR

I. INTRODUCTION

The metabolic syndrome, which is a set of lipid and non-lipid risk factors of metabolic origin linked with insulin resistance, is believed to be associated with an elevated risk for cardiovascular diseases [1-3]. Moreover, obesity, especially central obesity, is linked to many endocrine abnormalities including thyroid dysfunction [3]. There are reports of sub-clinical and overt hypothyroidism in metabolic syndrome patients. Sub-clinical and overt hypothyroidism is significantly associated with 16.4% metabolic syndrome patients [4]. Females have an increased risk of this association [5-7]. Thyrotropin-releasing hormone (TRH), produced in the paraventricular nucleus of the hypothalamus, stimulates the biosynthesis and secretion of Thyroid Stimulating Hormones (TSH) from the anterior pituitary [8, 9]. Further, TRH is present in many brain loci outside of the hypothalamus, supporting a potential role as a neuromodulator or neurotransmitter outside of traditional Hypothalamus- Pituitary- Thyroid (HPT) axis function [10, 11]. TRH has been also found outside the central nervous system (CNS) in the gastrointestinal tract, pancreas, reproductive tissues including placenta, ovary, testis, seminal vesicles, and prostate, retina, and blood elements [12]. The widespread distribution of TRH within and outside the CNS supports a diverse range of roles for this molecule, roles likely to involve many functions outside of the traditional HPT axis.

Our lab has developed an animal model for Metabolic syndrome (MetS) by feeding High Fat simple carbohydrate (HFSC) diet to C57BL/6J mice for a period of five months [13]. This model has displayed a significant disruption of feedback control between TSH and Triiodothyronine (T3) (unpublished data). In order to understand the exact mechanism involved, it is important to assess the level of the hypothalamic TRH. Qualitative real time Polymerase chain reaction (qRT-PCR) is a method of choice when the tissue sample is less.

Selection of oligonucleotide primers is often critical for the overall successes of polymerase chain reaction (PCR). The manual selection of optimal PCR primer set is a tedious process and not efficient. Bioinformatics database like NCBI was used to select target gene sequence and tools like BLAST and Primer 3 Plus was used for designing primer pairs.

II. MATERIALS & METHODS

A. *Extraction of gene sequence for TRH from NCBI-*

¹ PG Department of Biochemistry, St Aloysius College (Autonomous), Mangalore-575003

² Department of Postgraduate Studies and Research in Biotechnology, St Aloysius College, Mangalore-575003

NCBI- National Centre for Biological Information was used to extract the gene sequence for TRH of *Mus musculus* C57BL/6J. Since FASTA format is the simplest that can be used to align the sequence in various bioinformatics tools, the sequence was extracted in the same.

B. Use of BLAST tool to align sequence obtained above-

Basic Local Alignment Search Tool (BLAST) is the tool most frequently used for calculating sequence similarity. The BLAST algorithm is a heuristic program, which means that it relies on some smart shortcuts to perform the search faster. BLAST performs "local" alignments. The sequence database is then scanned for these "hot spots". When a match is identified, it is used to initiate gap-free and gapped extensions of the "word". BLAST uses statistical theory to produce a bit score and expect value (E-value) for each alignment pair. The bit score gives an indication of how good the alignment is; the higher the score, the better the alignment [14, 15].

Sequence extracted from NCBI was pasted into the textbox of the Nucleotide BLAST Web page. The button BLAST was clicked. The output was posted back in graphic and hit table format.

C. Primer design by Primer 3 Plus software-

Primer3 Plus is a computer program that suggests PCR primers for a variety of applications including radiation hybrid mapping [16], single nucleotide polymorphism discovery [17], sequencing reactions and qRT-PCR. In selecting oligos for primers in Primer3 Plus following parameters were considered. These include oligo melting temperature, length, GC content, 3' stability, estimated secondary structure, the likelihood of annealing to or amplifying undesirable sequences (for example interspersed repeats), the likelihood of primer-dimer formation between two copies of the same primer, and the accuracy of the source sequence.

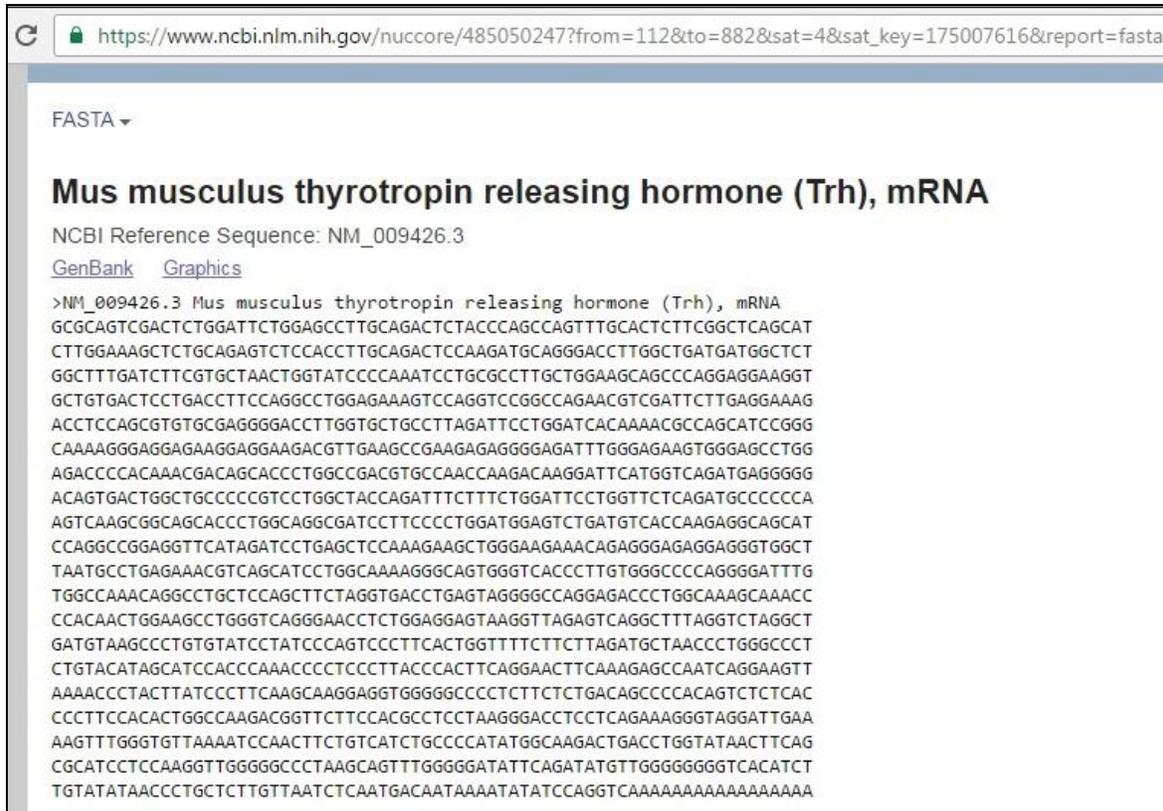
The online free software version of Primer 3 Plus was used as the web interface from www.bioinformatics.nl/cgi-bin/primer3plus/primer_3plus.cgi.

III. RESULTS AND DISCUSSION:

mRNA sequence in FASTA format for *Mus musculus* TRH with reference ID: NM_009426.3 was extracted from NCBI. The summary of the query suggests that this gene encodes a member of the thyrotropin-releasing hormone family which is a tripeptide hypothalamic regulatory hormone. TRH is involved in the regulation and release of TSH as well as prolactin. Disruption of this gene results in hypothyroidism, elevated TSH hormone levels, and hyperglycemia [18]. Studies from our lab on Metabolic Syndrome model revealed elevated TSH, prolactin as well as hyperglycemia (unpublished data) which is in agreement with the above.

We hypothesize that in Metabolic Syndrome TRH might be affected which could be confirmed only through qRT-PCR.

The above sequence (Fig.1) was used to carry out further alignment and primer designing.



https://www.ncbi.nlm.nih.gov/nuccore/485050247?from=112&to=882&sat=4&sat_key=175007616&report=fasta

FASTA ▾

Mus musculus thyrotropin releasing hormone (Trh), mRNA

NCBI Reference Sequence: NM_009426.3

[GenBank](#) [Graphics](#)

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>NM_009426.3 Mus musculus thyrotropin releasing hormone (Trh), mRNA
GCGCAGTCGACTCTGGATTCTGGAGCCTTGCGAGCTCTACCCAGCCAGTTTGCACTCTTCGGCTCAGCAT
CTTGGAAAGCTCTGCGAGGTCTCCACCTTGCGAGCTCCAAGATGCGAGGACCTTGCTGATGATGGCTCT
GGCTTTGATCTTCGTGCTAACTGGTATCCCAAATCCTGCGCCTTGCTGGAAAGCAGCCAGGAGGAAAGT
GCTGTGACTCCTGACCTTCCAGGCCCTGGAGAAAGTCCAGGTCCGGCCAGAACGTCGATTCTTGAGGAAAG
ACCTCCAGCGTGTGCGAGGGGACCTTGGTGTGCTTAGATTCTGGATCACAAAACGCCAGCATCCGGG
CAAAAGGGAGGAGAAGGAGGAAAGACGTTGAAGCCGAAGAGAGGGGAGATTGGGAGAAGTGGGAGCCTGG
AGACCCCAAAAACGACAGCACCTTGGCCGACGTGCCAACCAGCAAGGATTATGGTCAGATGAGGGGG
ACAGTGACTGGCTGCCCCGCTCTGGCTACCAGATTTCTTTCTGGATTCTGGTTCTCAGATGCCCCCA
AGTCAAGCGGCAGCACCTTGGCAGGCGATCCTTCCCCTGGATGGAGTCTGATGTACCAAGAGGCAAGCAT
CCAGGCCGGAGGTTATAGATCCTGAGCTCCAAGAAGCTGGGAAGAAACAGAGGGAGAGGAGGGTGGCT
TAATGCTGAGAAGCTCAGCATCCTGGCAAAAGGGCAGTGGGTCAACCCTTGTTGGGCCCAAGGGGATTTG
TGGCAAAACAGGCTGCTCAGCTTCTAGGTGACCTGAGTAGGGGCCAGGAGACCCTGGCAAAAGCAAAACC
CCACAAGTGGAAAGCTGGGTCAGGGAACTCTGGAGGAGTAAGGTTAGAGTCAGGCTTTAGGTCTAGGCT
GATGTAAGCCCTGTGTATCCTATCCAGTCCCTTCACTGGTTTTCTTCTTAGATGCTAACCTGGGCCCT
CTGTACATAGCATCCACCCAAACCCCTCCCTTACCCTTCAAGGAACTTCAAAGAGCCAATCAGGAAGTT
AAAACCTACTTATCCCTTCAAGCAAGGAGGTGGGGGCCCTTCTCTGACAGCCCCACAGTCTCTCAC
CCCTTCCACACTGGCCAAGACGGTTCTTCCACGCCCTCCTAAGGGACCTCCTCAGAAAGGGTAGGATTGAA
AAGTTTGGGTGTTAAAATCCAACCTCTGTCTGCCCCATATGGCAAGACTGACCTGGTATAAATTACAG
CGCATCCTCCAAGGTTGGGGGCCCTAAGCAGTTTGGGGGATATTCAGATATGTTGGGGGGGTCACATCT
TGTATATAACCTGCTCTTGTAACTCAATGACAATAAATATATCCAGGTCAAAAAAAAAAAAAAAAAA
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Figure 1. NCBI sequence for TRH specific for *Mus musculus*

BLAST alignment output gave nearly 33 similar sequences. But the query sequence was 100% similar to *Mus musculus* thyrotropin releasing hormone (Trh), mRNA (Fig.2) which is further confirmed by the E value that is zero (Fig.3).

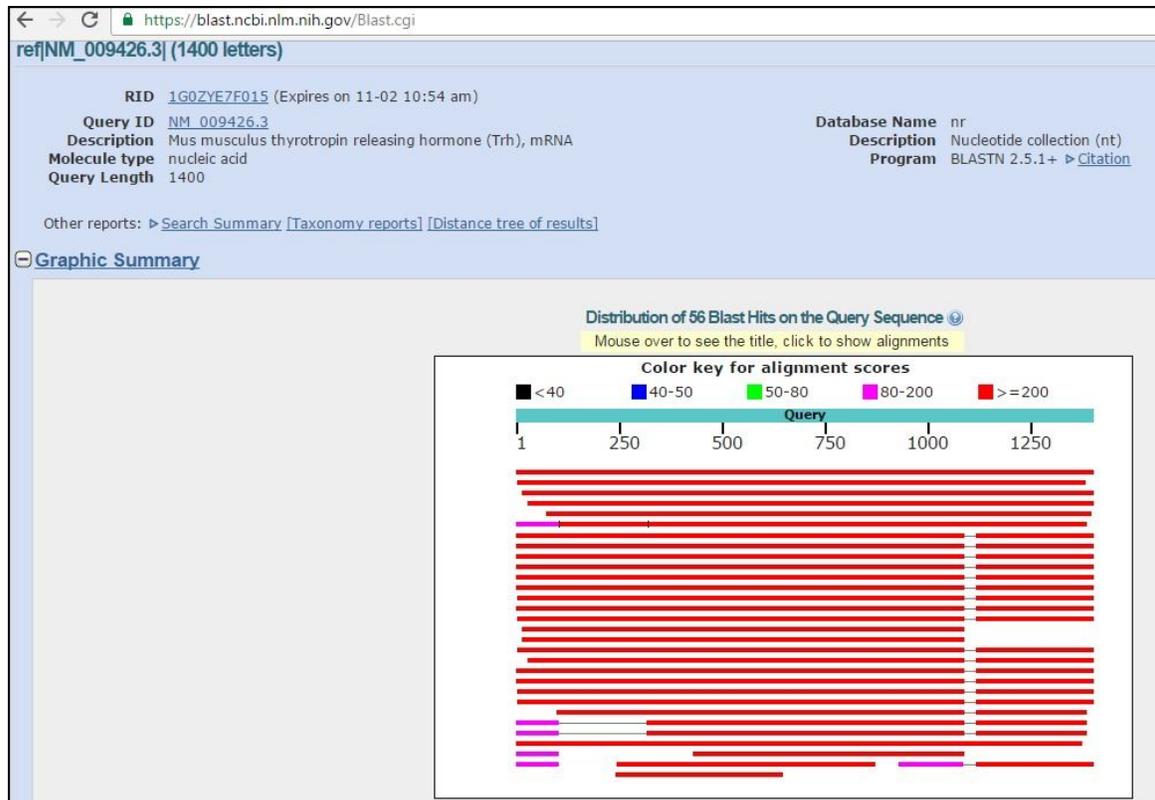


Figure 2. Graphic representation of the alignment.

Description	Max score	Total score	Query cover	E value	Ident	Accession
<input checked="" type="checkbox"/> Mus musculus thymotropin releasing hormone (Trh) mRNA	2586	2586	100%	0.0	100%	NM_009426.3
<input checked="" type="checkbox"/> Mus musculus ES cells cDNA, RIKEN full-length enriched library clone 2410044F19 product thymotropin releasing hormone, full insert sequence	2534	2534	98%	0.0	99%	AK010666.1
<input checked="" type="checkbox"/> Mus musculus thymotropin releasing hormone, mRNA (cDNA clone MGC:60687 IMAGE:30010395), complete cds	2529	2529	99%	0.0	99%	BC095349.3.1
<input checked="" type="checkbox"/> Xenopus laevis hypothetical protein LOC100037027, mRNA (cDNA clone MGC:160697 IMAGE:7198825), complete cds	2501	2501	97%	0.0	99%	BC130143.1
<input checked="" type="checkbox"/> Mouse mRNA for a preprothymotropin-releasing hormone	2359	2359	94%	0.0	99%	X59387.1
<input checked="" type="checkbox"/> Mus musculus BAC clone RP23-248J.4 from chromosome 6, complete sequence	1971	2566	98%	0.0	100%	AC164642.3
<input checked="" type="checkbox"/> Rattus norvegicus TL0ADA34YF17 mRNA sequence	1478	1815	97%	0.0	91%	F0221573.1
<input checked="" type="checkbox"/> Rattus norvegicus thymotropin releasing hormone (Trh) mRNA	1476	1787	97%	0.0	91%	NM_013046.3
<input checked="" type="checkbox"/> Rattus norvegicus TL0ADA49Y122 mRNA sequence	1476	1815	97%	0.0	91%	F02229082.1
<input checked="" type="checkbox"/> Rattus norvegicus TL0ADA15Y114 mRNA sequence	1476	1815	97%	0.0	91%	F0223091.1
<input checked="" type="checkbox"/> Rattus norvegicus TL0ADA52YB10 mRNA sequence	1472	1811	97%	0.0	91%	F0228758.1
<input checked="" type="checkbox"/> Rattus norvegicus TL0ADA7Y101 mRNA sequence	1471	1810	97%	0.0	91%	F0228499.1
<input checked="" type="checkbox"/> Rattus norvegicus TL0ADA16Y110 mRNA sequence	1467	1795	97%	0.0	91%	F0222384.1
<input checked="" type="checkbox"/> Rattus norvegicus TL0ADA24YF07 mRNA sequence	1456	1769	97%	0.0	91%	F0222377.1
<input checked="" type="checkbox"/> Rattus norvegicus TL0ADA21YK18 mRNA sequence	1450	1789	97%	0.0	91%	F0222571.1
<input checked="" type="checkbox"/> Rat thymotropin-releasing hormone (TRH) precursor mRNA, complete cds	1450	1450	76%	0.0	91%	M36317.1
<input checked="" type="checkbox"/> Rat thymotropin-releasing hormone (TRH) mRNA, complete cds	1450	1450	76%	0.0	91%	M12138.1
<input checked="" type="checkbox"/> Rattus norvegicus TL0ADA48YH02 mRNA sequence	1447	1786	97%	0.0	91%	F02229292.1
<input checked="" type="checkbox"/> Rattus norvegicus thymotropin releasing hormone, mRNA (cDNA clone MGC:187310 IMAGE:9091055), complete cds	1445	1784	95%	0.0	91%	BC161830.1
<input checked="" type="checkbox"/> Rattus norvegicus TL0ADA24YB19 mRNA sequence	1434	1773	97%	0.0	91%	F0220151.1
<input checked="" type="checkbox"/> Rattus norvegicus TL0ADA34YF22 mRNA sequence	1428	1762	97%	0.0	90%	F0221570.1
<input checked="" type="checkbox"/> Rattus norvegicus TL0ADA25YH07 mRNA sequence	1380	1636	97%	0.0	90%	F0222384.1
<input checked="" type="checkbox"/> Rattus norvegicus TL0ADA52YB05 mRNA sequence	1351	1684	97%	0.0	89%	F0228762.1
<input checked="" type="checkbox"/> PREDICTED: Rattus norvegicus thymotropin releasing hormone (Trh), transcript variant X1, mRNA	1338	1647	89%	0.0	91%	XM_006236889.2
<input checked="" type="checkbox"/> Rattus norvegicus thymotropin-releasing hormone (TRH) gene, complete cds	1048	1499	81%	0.0	91%	AF003087.2
<input checked="" type="checkbox"/> Rattus norvegicus thymotropin-releasing hormone (Trh) gene, complete cds	1042	1482	81%	0.0	91%	AF002262.2
<input checked="" type="checkbox"/> PREDICTED: Microtus ochrogaster thymotropin-releasing hormone (Trh) mRNA	1013	1013	98%	0.0	81%	XM_005364901.2
<input checked="" type="checkbox"/> Rattus norvegicus TL0ADA43YG15 mRNA sequence	896	896	46%	0.0	91%	F0220299.1
<input checked="" type="checkbox"/> PREDICTED: Crisetulus griseus thymotropin releasing hormone (Trh) mRNA	641	641	44%	1e-179	85%	XM_002495197.1
<input checked="" type="checkbox"/> PREDICTED: Mesocricetus auratus pro-thymotropin-releasing hormone-like (LOC101843134), mRNA	416	416	28%	7e-112	86%	XM_013114143.1
<input checked="" type="checkbox"/> Rattus norvegicus TL0ADA33YO19 mRNA sequence	305	493	31%	2e-78	86%	F0221609.1
<input checked="" type="checkbox"/> Mus musculus gene for preprothymotropin-releasing hormone, promoter region	191	191	7%	4e-44	100%	D86548.1
<input checked="" type="checkbox"/> Rattus norvegicus thymotropin-releasing hormone (TRH) gene, 5' flanking region and exon 1 sequence	147	147	7%	1e-30	92%	AF024516.1

Figure 3. Hit table with score and E-value

Since the target sequence showed 100% sequence similarity with *Mus musculus* Trh mRNA, primer pairs for it were designed using Primer 3 Plus online software. The software considered 10728 of left primers, but GC content failed for 2, low tm for 1756, high tm for 6476, high-end complexity for 2, long poly-x sequence for 29 and high 3' stability for 260. It found 2203 primers were ok for primer designing. For right primer, it considered 10481 primers, GC content failed for 56, low tm for 2022, high tm for 6051, long poly-x sequence for 57 and high 3' stability for 264. It found 2031 primers were ok for primer designing. In spite of the above-mentioned left and right primers, the software considered only 163 as primer pairs of which 148 were found unacceptable due to product size. Though only 15 were found ok, 5 were shortlisted for primer designing. The best primer among the given pairs of primers that satisfied all the necessary parameters was selected. The selected primer pair (Fig.4) had optimum GC% of 55 for forward and reverse primers. The optimum Tm was 60°C and the product length was 156bp.

Primer	Sequence	Length	Tm	GC%
Forward primer	GATTCTGGAGCCTTGCAGAC	20	60	55
Reverse primer	GGGGATACCAGTTAGCACGA	20	60	55

Figure 4. Selected primer pairs for TRH (*Mus musculus* C57BL/6J)

IV. CONCLUSION

The best primer pair specific for *Mus musculus* TRH was designed. This will be further validated by wet-lab experiments using mRNA isolated from hypothalamus of *Mus musculus* C57BL/6J which was fed with HFSC diet for a period of five months. The results of which will help to elucidate the HPT axis dysfunction in Metabolic Syndrome.

V. ACKNOWLEDGEMENT

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