Short communication

DESIGN AND *IN SILICO* VALIDATION OF PRIMERS FOR ASSESSING GENE EXPRESSION IN THE CANONICAL INFLAMMATORY PATHWAY

ABSTRACT

Aims: This study aimed to design and validate primers to investigate the expression of key genes of the canonical inflammation pathway, specifically NEMO (NF-kappaB Essential Modulator), MyD88 and IL-1β, in order to ensure feasibility, specificity and efficiency for use in RT-qPCR.

Study design: This is an experimental study aimed at the design and validation of primers using *in silico* strategies.

Methodology:FASTA sequences for key genes of the canonical inflammation pathway were obtained from the NCBI database and open reading frames (ORFs) were identified using the ORF Finder tool, while conserved domains were annotated using the NCBI conserved domains database. Primers were designed using OligoPerfect Primer Designer (ThermoFisher) based on parameters such as primer length (18–22 bp), GC content (40–60%) and melting temperature (57–63 °C). The primers were further validated in silico using OligoAnalyzer (IDTDNA) to assess their thermodynamic properties, such as hairpin formation and self-dimers, as well as confirmation of specificity by NCBI BLAST analysis.

Results: The designed primers exhibited optimal characteristics for gene amplification by PCR, including hairpin formation temperatures between 35.4°C and 49.5°C and melting temperatures above 63°C. The amplicons ranged from 88 to 136 bp, which is considered an acceptable standard for qPCR analysis. Pairwise analysis on the NCBI BLAST platform confirmed species specificity for *Mus musculus*, with the genes of interest showing additional compatibility with *Rattus norvegicus* in two (Myd88 and IL 1β) of the three primers. These findings support the applicability of the primers to detect and quantify transcripts of key genes of the canonical inflammatory pathway in tissue from laboratory animal specimens.

Conclusion: The validated primers provide the possibility for the amplification and quantification of key genes in the canonical inflammation pathway. This methodology ensures sensitivity and specificity for gene expression studies in the *Mus Muscullus* animal model, supporting future experimental applications in preclinical models.

Keywords: Primers; Canonical Pathway; inflammation; in sílico

1. INTRODUCTION

The canonical NF-κB pathway is considered the central regulator of the inflammatory response and has been extensively studied in the context of inflammatory and immune pathologies, since canonical NF-κB signaling regulates the innate immune response (Anilkumar; Wright-Jin, 2024). The canonical pathway, in turn, is defined primarily in response to TNFα and IL-1 signaling, prototypical pro-inflammatory cytokines that play important roles in the pathogenesis of chronic inflammatory diseases such as inflammatory bowel diseases (Lawrence, 2009; Sousa et al., 2024). This pathway, in turn, leads to the activation of the NLRP3 inflammasome and plays an essential role in the innate immune response, the mechanism of which consists of caspase-1 being activated by the adaptor protein ASC in response to dangers detected by pattern recognition receptors (PRRs) through Toll-Like Receptors. This activation leads to the conversion of pro-inflammatory

cytokines into their active forms and pyrotosis, a type of inflammatory programmed cell death, culminating in tissue damage and organ dysfunction (Xiang et al., 2020).

NF-κB activity constitutes an essential part of physiological and pathological processes and is tightly regulated at various levels in the cell; its dysregulation can cause serious diseases of inflammatory origin and aggressive pathophysiology (Trares, Ackermann and Koch, 2022). NF-κB activation involves two main signaling pathways, the canonical and non-canonical pathways, both important for regulating the immune and inflammatory response, despite their differences in signaling mechanism (Liu et al., 2017). NF-κB is the factor that regulates the expression of nearly 500 different genes, including numerous pro-inflammatory enzymes [e.g., cyclooxygenase (COX)-2, 5-lipoxygenase (LOX), and inducible NO synthase (iNOS)] and cytokines [interleukin (IL)-1, IL-6, IL-8, chemokines, and tumor necrosis factor (TNF)] (Shih, Wang, & Yang, 2015).

Applied to this context, an economically viable way to investigate therapeutic agents in these pathways is using the mouse model Mus musculus, which in turn has long been used as a model organism to test hypotheses and treatments related to understanding disease mechanisms in humans (Rydell-Törmänen & Johnson, 2019).

Therefore, in the context that Modulation of the canonical inflammation pathway provides support to therapeutically combat chronic inflammatory conditions and is seen by science as the next leap in therapeutics (Oliveira Sousa et al., 2025; Sousa et al., 2024, 2025).Thus, this work aims to propose a primer design with the respective evaluation of its viability in silico with the objective of proposing a method to quantify the expression of key products of the canonical inflammation pathway in a murine model of *Mus muscullus*.

2. MATERIAL AND METHODS

The protocol adopted for the design and validation of the primers used in this work is systematic and based on biocomputational tools available in the literature. These steps ensure the obtaining of specific and efficient primers for the genes of interest and are detailed below: obtaining nucleotide sequences from the National Center for Biotechnology Information: initially, the Databases for complete mRNA sequences of Mus musculus of the gene NEMO National Center for Biotechnology Information – NCBI/cdd/NM 02615.3, MyD88 NCBI/cdd/NM 016194.3 and IL-1 ß NCBI/cdd/178666.1 were accessed. Searches for complete sequences (complete cds) were performed in fasta format. From there, the sequences were copied and organized in a document for the following steps.

2.1 Identification of the Open Reading Frame (ORF) position and determination of Conserved Domains.

The B. microti sequences previously obtained in FASTA format were analyzed in the Open Reading Frame Finder, from which we determined the open reading frames, that is, we selected the largest ORF in terms of length. It was possible to identify the position of the start or stop codons in the nucleotide sequencesand positioning these open reading frames to ensure the selection of the coding region for each gene. In addition, the sequence of the largest ORF was submitted to the NCBI Conserved Domains program, from which we identified the open reading frame intervals based on the conserved domains in the encoded proteins. This allowed us to adjust the corresponding position of these domains to the locations in the nucleotide sequences, essential to ensure that the open reading frames of the target region are in the sequence regions of interest, according to genomic functionality.

2.2. Primer Design

The letter orders with the changed start, end, and common regions were passed to the OligoPerfect Primer Designer tool (ThermoFisher). The data used for primer design are as follows:

- ✓ Primer size: 18-22 letters (best: 20).
- ✓ Tm (melting temperature): 57-63 degrees Celsius (best: 60 degrees Celsius).
- ✓ GC percentage: 40-60%.

The size of the amplified product is between 80 and 150 base pairs (bp). The analysis found up to five primer pairs per gene, but we chose those with the smallest difference in Tm between the forward and reverse primers, as well as better thermodynamic characteristics.

2.3. In silico verification of the primers

The chosen primers were checked in the OligoAnalyzer program, observing the following points:

- ✓ Hairpin loop formation: We looked for high formation temperatures.
- ✓ Dimer formation characteristics (self-dimer and hetero-dimer): We looked at the free energies associated with this.

Annealing temperature (Tm): We made sure that the values were within the correct range. Finally, we performed a complementarity analysis with NCBI BLAST.

3. RESULTS AND DISCUSSION

In order to quantify the targets, in this research we proposed to design the primers and conduct the in silico validation of the primers for key genes involved in the canonical inflammation pathway that will be used in this study, specifically NEMO (NF-kappaB Essential Modulator), MyD88 and IL-1β.

To this end, we used the FASTA files obtained from NCBI, which were used to study the definition of the open reading frame (ORF), which represents the sequence associated with the protein peptides themselves, and these Start and Stop points were calculated using a reverse engineering technique by obtaining the Open Reading Frame Finder (ORF) parameters of the FASTA sequence and the conserved domains and their respective codons referring to the peptide sequence as described in the Conserved Domains resource of the NCBI Library (emphasis in yellow), which in turn were used to construct the primers by combinatorial analysis of the best strategy using the “OligoPerfect Primer Designer” tool from ThermoFisher.

Figure 1 – FASTA sequence with annotations of start and stop codons and conserved domains for optimal primer synthesis for NEMO.

Figure 2 – FASTA sequence with annotations of start and stop codons and conserved domains for optimal primer synthesis for Myd88.

>NM_010851.3 Mus musculus myeloid differentiation primary response gene 88 (Myd88), mRNA

AGTAGGAAACTCCACAGGCGAGCGTACTGGACGGCACCGGGGGCCCAGGGTTGCCTGCCATGTCTGCGGG
AGACCCCCGCGTGGGATCCGGGTCCCTGGACTCCTTCATGTTCTCCATACCCTTGGTCGCGCTTAACGTG
GGAGTGAGGCGCCGCCTATCGCTGTTCTTGAACCCTCGGACGCCCGTGGCGGCCGACTGGACCTTGCTGG
CGGAGGAGATGGGCTTCGAGTACTTGGAGATCCGAGAGCTGGAAACGCGCCCTGACCCCACTCGCAGTTT
GTTGGATGCCTGGCAGGGGCGCTCTGGCGCGTCTGTCGGCAGGCTGCTAGAGCTGCTGGCCTTGTTAGAC
CGTGAGGATATACTGAAGGAGCTGAAGTCGCGCATCGAGGAGGACTGCCAGAAATACTTAGGTAAGCAGC
AGAACCAGGAGTCCGAGAAGCCTTTACAGGTGGCCAGAGTGGAAAGCAGTGTCCCACAAACAAAGGAACT
GGGAGGCATCACCACCCTTGATGACCCCCTAGGACAAACGCCGGAACTTTTCGATGCCTTTATCTGCTAC
TGCCCCAACGATATCGAGTTTGTGCAGGAGATGATCCGGCAACTAGAACAGACAGACTATCGGCTTAAGT
TGTGTGTGTCCGACCGTGACGTCCTGCCGGGCACCTGTGTCTGGTCCATTGCCAGCGAGCTAATTGAGAA
AAGGTGTCGCCGCATGGTGGTGGTTGTTTCTGACGATTATCTACAGAGCAAGGAATGTGACTTCCAGACC
AAGTTTGCACTCAGCCTGTCTCCAGGTGTCCAACAGAAGCGACTGATTCCTATTAAATACAAGGCGATGA
AGAAGGACTTTCCCAGTATCCTGCGGTTCATCACTATATGCGACTATACCAACCCTTGCACCAAGTCCTG
GTTCTGGACCCGCCTTGCCAAGGCTTTGTCCCTGCCCTGAAGATGACCCTGGGAGCCCTAGGGCAGAGGG
GAAGATGAGACTGATGCGGAGCCAGATTCTCTGATGCCGTCCTGTCTACATCTTTGACTCCCCTGGGCTC
AACCCGTGTTCAATGATGACTGGCCTGAGCAACTAGGACTGCCTTTCCTCCCAGCCACCCATGCCTGTGC
ACGCACCTCAGTACACACATGCCTCCTCGCACACACAGGCATCTGCATATGTGTGTTTCCTTTGGGACAG
CTCCCAAGGATAGCTGAGTGGAAGAGTTCTATCATCAAGGGGGCCTGGCCATCTCCCTGGACAAAAGTGG
GGTGCCTTTGCTACAGGTAGTGGCACGGGCCTATAGTTTCAGCATTTGGGAGGTAGAGGCAGGAGAATCA
GGAGTTCAAGCTTATCCTTGGCAACACACCTAGTTTAAGGTCAGCCTGGGCTACATGAGAGCCTACCTCC
CCCATCCCCTACCCCCAGAAAAGAAGGAAAATCTGGGGGCACTGTGGATTTCTCCTCTCTTTTCTCTACC
TGTTGAAAGCAAAGTCTAGGAAGGCCCCAAACATGATAGCATTTGGGCCCTTAGTAAGCTGAAGATAAAA
AGGAGAAGCTGTTTGGCTTCGCCCCACAAAGCAGCTGCAGGCTCAGCTGTTTTCTCCCCAGCAGCGAGGT
TTGCATCTTCTTATTCCTTTCACGTTCTCTACCATAGAGGCAATGTCATGGTCCCTCTCAGGGTACACCC
CAGGGCCCTGAGTCCCCAAGAAAGTGAGTCTCCCCTCAGTGTCTGGGGGAGGAATGAGGCCTCTGTGCAC
GGTCTCATGGGGCATTTCACTGCTTGATGTTGAGCATTTTAAAGCAACCTGGGTCAAGTGTAAACCTCCT
CCACCTGTGTTAGAGGTTTCATGGGAATGTCAATAAAGAAAAGAAGGGCTCGTGTTTGTACTTTGTACTT
CTGTGTGTGAATTTATAATAAAAAGTATTCTATTTTGAGAAAGTGTAAAAAAAAAAAAAAAAAAAAAAAA
AAAAA

Start: 25

Stop: 950

Sequence used: 87 - 315

Figure 3 – FASTA sequence with annotations of start and stop codons and conserved domains for optimal primer synthesis for IL-1ß

>NM_008361.4 Mus musculus interleukin 1 beta (Il1b), mRNA

AACAAACCCTGCAGTGGTTCGAGGCCTAATAGGCTCATCTGGGATCCTCTCCAGCCAAGCTTCCTTGTGC
AAGTGTCTGAAGCAGCTATGGCAACTGTTCCTGAACTCAACTGTGAAATGCCACCTTTTGACAGTGATGA
GAATGACCTGTTCTTTGAAGTTGACGGACCCCAAAAGATGAAGGGCTGCTTCCAAACCTTTGACCTGGGC
TGTCCTGATGAGAGCATCCAGCTTCAAATCTCGCAGCAGCACATCAACAAGAGCTTCAGGCAGGCAGTAT
CACTCATTGTGGCTGTGGAGAAGCTGTGGCAGCTACCTGTGTCTTTCCCGTGGACCTTCCAGGATGAGGA
CATGAGCACCTTCTTTTCCTTCATCTTTGAAGAAGAGCCCATCCTCTGTGACTCATGGGATGATGATGAT
AACCTGCTGGTGTGTGACGTTCCCATTAGACAACTGCACTACAGGCTCCGAGATGAACAACAAAAAAGCC
TCGTGCTGTCGGACCCATATGAGCTGAAAGCTCTCCACCTCAATGGACAGAATATCAACCAACAAGTGAT
ATTCTCCATGAGCTTTGTACAAGGAGAACCAAGCAACGACAAAATACCTGTGGCCTTGGGCCTCAAAGGA
AAGAATCTATACCTGTCCTGTGTAATGAAAGACGGCACACCCACCCTGCAGCTGGAGAGTGTGGATCCCA
AGCAATACCCAAAGAAGAAGATGGAAAAACGGTTTGTCTTCAACAAGATAGAAGTCAAGAGCAAAGTGGA
GTTTGAGTCTGCAGAGTTCCCCAACTGGTACATCAGCACCTCACAAGCAGAGCACAAGCCTGTCTTCCTG
GGAAACAACAGTGGTCAGGACATAATTGACTTCACCATGGAATCCGTGTCTTCCTAAAGTATGGGCTGGA
CTGTTTCTAATGCCTTCCCCAGGGCATGTTAAGGAGCTCCCTTTTCGTGAATGAGCAGACAGCTCAATCT
CCAGGGGACTCCTTAGTCCTCGGCCAAGACAGGTCGCTCAGGGTCACAAGAAACCATGGCACATTCTGTT
CAAAGAGAGCCTGTGTTTTCCTCCTTGCCTCTGATGGGCAACCACTTACCTATTTATTTATGTATTTATT
GATTGGTTGATCTATTTAAGTTGATTCAAGGGGACATTAGGCAGCACTCTCTAGAACAGAACCTAGCTGT
CAACGTGTGGGGGATGAATTGGTCATAGCCCGCACTGAGGTCTTTCATTGAAGCTGAGAATAAATAGGTT
CCTATAATATGGATGAGACTTTTTATGAATGAAGCACCAGCACATTGCTTTGATGAGTATGAAATAAATT
TCATTAAAACAAACAAAC

Start: 88

Stop: 897

Sequence used: 1 - 306

The analysis aimed to evaluate the thermodynamic parameters of maximum hairpin temperatures, free energy of homologous dimer formation and the annealing temperatures (Tm) of the primers, to ensure the effectiveness of PCR amplification.

The results are available in Table 1 and indicate that all primers had sequences of 20 nucleotides, which is indicated as an ideal length (10.1186/1471-2407-6-85). The primers also have adequate hairpin temperatures, ranging from 35.4 °C to 49.5 °C, which when compacted with their annealing temperature that was consistently above 63 °C, allows great versatility in the application in qPCR.

Table 1. Results of in silico validation of primers for targets of the canonical inflammation pathway

Target	Sequence	Hairpin Tm Máx (ºC)	ΔG- Homo-Dimer /ΔG máx (kcal/mol)	T_m (ºC)	Amplicon (Start-End / Length)
NEMO (NF-kappaB Essential Modulator)	*Forward: TACTCCTGAG ACCCTCCAGC*	41,6 ºC	-3,55 / -37,26	64,2 ºC	153-172/88
	Reverse: GCGTTCCCTC AGCATCTGAT	41,8 ºC	-5,13 / -39,22	64,2 ºC	221-240/88
MyD88	*Forward: TGTTCTTGAA CCCTCGGAC G*	49,5 ºC	-2.27 / -39,69	63,9 ºC	163-182/91
	Reverse: TCCAGCTCTC GGATCTCCAA	39,2 ºC	-6,34 / -39,13	64,4 ºC	234-253/91
IL -1ß	*Forward: GTGCAAGTGT CTGAAGCAGC*	35,4 ºC	-7,05 / -36,65	63,7 ºC	67-86/136

	Reverse: CAAAGGTTTG GAAGCAGCC C	35,4 ºC	-5,84 / -41,85	63,9	183-202/136

Regarding the formation of homologous dimers, the free energy values ranged between -2.27 kcal/mol and -7.05 kcal/mol, indicating a generally low propensity for dimer formation given that the total annealing binding is approximately 10x greater, which is associated with good amplification specificity.

Finally, the resulting amplicons exhibit sizes ranging from 88 to 136 base pairs (bp), therefore they are within the range considered optimal for PCR analysis, ensuring effective detection and accurate quantification of the target transcripts. In order to assess whether the primers result in complementarity with the targets of the species in question, a query was performed in NCBI Blast to confirm whether the primer corresponds to the target in the specific species, which resulted in the ability of the NEMO and IL-1ß primers to detect the mRNA expression of the respective peptides in *Mus muscullus* and additionally presented correspondence in *Rattus norvegicus*, which in turn is also a research model used for similar research purposes, while the Myd88 primer presented a correspondence only in the *Mus muscullus* species.

The in silico analysis confirmed that the primers produced exhibited ideal parameters for RT-PCR amplification, including adequate length, consistent hairpin and annealing temperatures, as well as the complementarity tests in NCBI Blast guaranteed the specificity of the primers for the targets in *Mus musculus*, which makes it possible to use them to investigate the canonical pathway of inflammation.

4. CONCLUSION

The *in silico* validation of the primers for the NEMO, MyD88 and IL-1β genes shows that they have satisfactory characteristics for applications in quantification routines through RT-PCR methodology, given their sequence length, specificity, low dimer formation and compatible amplicon sizes. This approach discusses and documents a robust method for the detection and quantification of transcripts associated with the canonical inflammation pathway, allowing the continuation of the study focusing on the biological evaluation of these targets in experimental models.

Disclaimer (Artificial intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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