LETTER TO THE EDITOR
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The Unspecific Primers of Nuclear Factor-kappa B *(NF-κB)* Signaling Mediators

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To The Editor

Nuclear factor-kappa B (*NF-κB*) signaling plays a critical role in the regulation of inflammatory responses, which is mediated by the activation of Toll-like receptor- 4 (*TLR4*) and cytosolic adapter protein of Myeloid differentiation primary response 88 (*Myd88)*. Numerous studies have indicated the overexpression of *NF-κB* signaling mediators in inflammation.[1-3] Therefore, several researchers have used the real-time PCR technique for the evaluation of *NF-κB* signaling in the drug discovery of anti-inflammatory products.[4-9] The authors carefully searched these articles by the keywords of "real-time PCR", "*NF-κB* signaling" and "inflammation" in the database of PubMed, Scopus, and Google Scholar. Then, the specificity of the reported primers was evaluated by Primer-BLAST analysis in the NCBI database (Figure 1).

Several fundamental errors were detected in the reported primers of *NF-κB* signaling mediators by Primer BLAST analysis (Table 1). It appears that the errors are due to the nominal similarity, misuse of alternative signaling pathways instead of the canonical pathway, and probably random or writing errors. According to Table 1, the unspecific primers were related to the *TLR4*, *Myd88*, *NF-κB* subunits, and *Tak1* genes, which will be described below.[4-8]

Transforming growth factor β-activated kinase 1 (*Tak1*) is critical in the *NF-κB* signaling cascade. *Tak1* as an *NF-κB* related factor is also known as mitogen-activated protein kinase kinase kinase 7 (*Map3k7*). In Mus musculus, there is a nominal similarity between the *Tak1* gene of *NF-κB* signaling and *TAK1* (with the capital letters). *TAK1* is an orphan nuclear receptor that can act as an important repressor of nuclear receptor signaling pathways including the vitamin D3 receptor, retinoic acid receptor, thyroid hormone receptor, and estrogen receptor pathways. *TAK1* is also known as nuclear receptor subfamily 2, group C, member 2 (*Nr2c2*).[9] *TAK1* (or *Nr2c2*) gene is located on the gene locus of "6; 6 D1" in Mus musculus. However, *Tak1* (or *Map3k7*) gene is located on the gene locus of "4; 4 A5". Therefore, the *TAK1* gene is completely different from the *Tak1* gene of *NF-κB* signaling. According to Table 1, the *TAK1* (or *Nr2c2*) gene is mistaken for *Tak1* (or *Map3k7*) of *NF-κB* signaling (Table 1) in the study of Zhu, et al.[4] They mistakenly used the primers of *Nr2c2* as an *NF-κB* related factor to examine the *NF-κB* signaling pathway.

The canonical and alternative pathways of *NF-κB* signaling and different subunits of *NF-κB* transcription factors are other error-causing factors. *NF-κB* transcription factor family is composed of five structurally related proteins including *NF-κB1* (also known as *p50*), *NF-κB2* (also known as *p52* and *p49/p100*), Rela (also known as *NF-κB p65*), *Relb*, and *c-Rel*. These subunits are associated with each other and form active heterodimeric transcription factors in canonical and alternative *NF-κB* pathways.[1] The canonical pathway is triggered by TLRs and pro-inflammatory cytokines leading to the activation of *Rela/NF-κB1* heterodimer complexes that regulate the

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expression of pro-inflammatory mediators. But, an alternative *NF-κB* pathway is activated by *LTB*, *CD40L*, *BAFF*, and *RANKL* leading to the activation of *Relb/NF-κB2* heterodimer complexes. The alternative *NF-κB* signaling pathway is critical to lymph organogenesis, contrary to the canonical pathway.[10] Therefore, the primer of specific *NF-κB* subunits must be used in the evaluation of canonical and alternative *NF-κB* signaling. According to Table 1 and in the study of Islam et al, the *NF-κB2* (p49/p100) gene is mistaken for *NF-κB p65* (*Rela*).[5] They evaluated the canonical pathway of *NF-κB* signaling in LPS-induced acute kidney injury, but the reported primers of *NF-κB* subunit is related to *NF-κB2* and alternative *NF-κB* signaling.

A

	Sequence (5'->3')
Forward primer	GTCACGGATTCTGCTTCTGT
Reverse primer	AGATTCTTCCTCACGCTCCTT

Products on target templates

>XM_006505906.5 PREDICTED: Mus musculus nuclear receptor subfamily 2, group C, member 2 (Nr2c2), transcript variant X4, mRNA

```
product length = 170
Forward primer  1    GTCACGGATTCTGCTTCTGT  20
Template        556  ....................  575

Reverse primer  1    AGATTCTTCCTCACGCTCCTT  21
Template        725  ......................  705
```

B

	Sequence (5'->3')
Forward primer	ACGACATTGAGGTTCGGTTC
Reverse primer	ATCTTGTGATAGGGCGGTGT

Products on target templates

>XM_006526743.5 PREDICTED: Mus musculus nuclear factor of kappa light polypeptide gene enhancer in B cells 2, p49/p100 (Nfkb2), transcript variant X1, mRNA

```
product length = 124
Forward primer  1     ACGACATTGAGGTTCGGTTC  20
Template        8623  ....................  8642

Reverse primer  1     ATCTTGTGATAGGGCGGTGT  20
Template        8746  ....................  8727
```

C

	Sequence (5'->3')
Forward primer	CGCAAGCCCTTCAGTGACATC
Reverse primer	GGTACTGGCTGTCAGGGTGGTT

Products on target templates

>XM_036160412.1 PREDICTED: Mus musculus peroxisome proliferator activator receptor delta (Ppard), transcript variant X5, mRNA

```
product length = 223
Forward primer  1     CGCAAGCCCTTCAGTGACATC  21
Template        911   ......................  931

Reverse primer  1     GGTACTGGCTGTCAGGGTGGTT  22
Template        1133  ......................  1112
```

D

	Sequence (5'->3')
Forward primer	GGCAGGTCTACTTTGGAGTCATTGC
Reverse primer	ACATTCGAGGCTCCAGTGAATTCGG

Products on target templates

>NM_001278601.1 Mus musculus tumor necrosis factor (Tnf), transcript variant 2, mRNA

```
product length = 300
Forward primer  1     GGCAGGTCTACTTTGGAGTCATTGC  25
Template        796   ..........................  820

Reverse primer  1     ACATTCGAGGCTCCAGTGAATTCGG  25
Template        1095  ..........................  1071
```

E

Specificity of primers	No target templates were found in the selected database: Refseq mRNA (Organism limited to Mus musculus)

Figure 1. The Primer BLAST analysis of the reported primers of the previous studies that claimed to be specific for the (A) Mitogen-activated protein kinase kinase kinase 7 (*Map3k7*), (B) p65 kDa subunit of Nuclear factor-kappa B (*NF-κB p65)*, (C) Nuclear factor-kappa B (*NF-κB*), (D)Toll-like receptor 4 (*TLR4*), and (E) Myeloid differentiation primary response 88 (*Myd88*) gene in the NCBI database.

Several random or writing errors were also detected in the reported primers of *NF-κB* signaling mediators in previous studies by Primer BLAST analysis in the NCBI database. According to Table 1, in the study of Ding et al, the reported primers of *NF-κB* does not attach to any *NF-κB* subunits and belongs to another gene called peroxisome proliferator activator receptor delta (*Ppard*).[6] *Ppard* protein functions as an integrator of transcriptional repression and nuclear receptor signaling. It may inhibit the ligand-induced transcriptional activity of peroxisome proliferator-activated receptors alpha and gamma. Therefore, the *Ppard* gene is completely different from the *NF-κB* subunits. In the study of Peng et al, the reported primers of *TLR4* was completely wrong and belongs to *TNF-α* pro-inflammatory cytokine.[7] In the study of Zeng et al, the reported primers of *Myd88* was also incorrect.[8] According to Table 1, the sequences of forward and reverse primers of *Myd88* were the same and they do not attach to any specific target.

Table 1. The unspecific primers of *NF-κB* signaling mediators that was reported in previous studies

	Primer sequences	Claimed target		Real target		Ref
		Gene	Locus	Gene	Locus	
1	**F**. GTCACGGATTCTGCTTCTGT **R**. AGATTCTTCCTCACGCTCCTT	*Map3k7*[a]	4; 4 A5	*Nr2c2* [f]	6; 6 D1	4
2	**F**. ACGACATTGAGGTTCGGTTC **R**. ATCTTGTGATAGGGCGGTGT	*NF-κB p65*[b] *(Rela)*	19 A; 19 4.34 cM	*p49/p100*[g] *(NF-κB2)*	19 C3; 19 38.8 cM	5
3	**F**. CGCAAGCCCTTCAGTGACATC **R**. GGTACTGGCTGTCAGGGTGGTT	*NF-κB*[c]	?	*Ppard*[h]	17 A3.3; 17 14.64 cM	6
4	**F**. GGCAGGTCTACTTTGGAGTCATTGC **R**. ACATTCGAGGCTCCAGTGAATTCGG	*TLR4*[d]	4 C1; 4 34.66 cM	*TNF-α*[i]	17 B1; 17 18.59 cM	7
5	**F**. ACTCGCAGTTTGTTGGATG **R**. ACTCGCAGTTTGTTGGATG	*Myd88*[e]	9 F3; 9 71.33 cM	-	-	8

a. Mitogen-activated protein kinase kinase kinase 7 (*Map3k7*), b. p65 kDa subunit of Nuclear factor-kappa B, c. Nuclear factor-kappa B, d. Toll-like receptor 4, e. Myeloid differentiation primary response 88, f. nuclear receptor subfamily 2, group C, member 2, g. p49/p100 kDa subunit of Nuclear factor-kappa B, h. Peroxisome proliferator activator receptor delta, i. Tumor necrosis factor α.

Therefore, it is recommended that the researcher should pay more attention to the potential error-causing factors in the evaluation of *NF-κB* signaling by real-time PCR. Error-causing factors in the field of primer design can be divided into the categories of gene nominal similarities in *NF-κB* signaling, different subunits of NF-κB, the different pathways of *NF-κB* signaling (canonical and alternative), and probably random or writing errors.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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