Supplementary files

DNA amplification

Amplification of three genes, *TLR2*, *TLR4*, and *TLR7*, was performed on 200 extracted DNA samples using the Applied Cynthol-Russia kit. The optimal band was selected at 60℃ (Supplementary Table 3).

Table S1. Components of conventional polymerase chain reaction.

No.	Components	Final Concentration
1	Taq PCR MasterMix	8µl
2	Forward primer	10 picomols/µl (1 µL)
3	Reverse primer	10 picomols/µl (1 µL)
4	DNA Template (20.867 (ng/µL)	2 µL
5	$MgCL_2$ (3 mM)	0.5 µL
6	Deionised water	7.5 µL
7	Final volume	20 µL

Pre-Denaturation (94℃, 5 m, 1 Cycle), Denaturation (94℃, 30 s, 35 Cycles), Annealing (60℃*, 30 s, 35 Cycles), Extension (72℃, 30 s, 35 Cycles), and Final extension (72℃, 5 m, 1 Cycle). *Optimization at 55, 58, 60, 63, 66℃. Note/Melting temperature (Tm) = 2 (A+T) +4(C+G) Annealing = Tm ± 5

Restriction enzyme selection and preparation

The restriction enzyme was chosen using SnapGene screening software, and reference SNPs were selected based on functional significance, association with infectious diseases, literature review, minimum primer mutations, compatibility, cost, and availability.

Genotypes and Properties of Restriction Enzymes for SNP Analysis in Toll-like Receptor Genes are shown in the Supplementary Table 3.

Table S2. Restriction endonucleases enzymes.

Gene	Genotype	SNP	RE	Source	Tm (°C)	Restriction site (5→3)	Quantities
TLR2	*T/C*	*rs3804100*	Bst4C I	*Bacillus stearothermophilus*	65	ACN↑GT TG↓N*CA	1µL/1Ngm DNA
TLR4	*G/A*	*rs1927914*	Zsp2 I	*Zoogloea* species 2	60	TGCA↑T T↓ACGTA	
TLR7	*A/T*	*rs179008*	Acs I	*Arthrobacter citreus*	50	R↑AATTY* YTTAA↓R	

Y*(C or T), R*(A or G) and N*(C, T, A, G) * Sib Enzyme- Russia

Choosing primers for the specified single-nucleotide polymorphism (SNP)

Using the "Multiple Primer Analyser" online software from Thermo Fisher Scientific Inc.©, we ensured that each primer pair did not form dimers. We set the software's sensitivity to 2, and if any dimers were detected, the primer pair would be rejected.

Table S3. The specific primers (*TLR2, TLR4, and TLR7)* genes.

Gene	Primer	Sequence	Tm (°C)	GC (%)	Product Size

		(5→3)			
TLR2	Forward	GCCTGGCCCTCTCTACAAAC	60.39	60.00	346 bp
	Reverse	TGGGTAAGAGGGAGGCATCT	59.66	55.00	
TLR4	Forward	CAGTCCACCACAAAATGGTCC	59.39	52.38	283 bp
	Reverse	AATGAGAGCTATGATGAGGATTGA	57.32	37.50	
TLR7	Forward	AGAGAGGCAGCAAATGGGAAT	47.62	47.62	285 bp
	Reverse	GTTCGTGGTGTTCGTGGGAA	55.00	55.00	

*Macrogen-LIGO- Korea

The primers were designed specifically for this study and engineered to identify single-nucleotide polymorphisms (SNPs) in the *TLR2* SNP rs3804100, *TLR4* SNP rs1927914, and *TLR7* SNP rs179008 genes. The NCBI Primer BLAST web tool was used to design primers for a research investigation. The primers were designed using the NCBI Primer BLAST online software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). Simultaneously, the produced primers were tested for sequence specificity against the human genome using BLAST. Then, the primer pairs were chosen based on criteria such as product length, melting temperature similarity, specificity, primer length, and so on. Using the OligoCalc online software (http://www.basic.northwestern.edu/biotools/oligocalc.html), we assessed the primers' ability to form secondary structures. Primers were rejected if they had 5 bases or more capable of self-dimerisation and/or 4 bases capable of hairpin formation.

Identification of SNPs in the *TLR2* gene (rs3804100) across both groups in gel electrophoresis

Three genotypes were found at this locus: TT, TC, and CC. The T allele was amplified in the TT genotype (reference homozygote), resulting in two 196 bp and 150 bp fragments. With a single 346 bp fragment, the CC genotype (homozygote) solely displayed the C allele.

Three pieces were produced by the TC genotype (heterozygote), which showed both T and C alleles: 346 bp, 196 bp, and 150 bp (Figure S1).

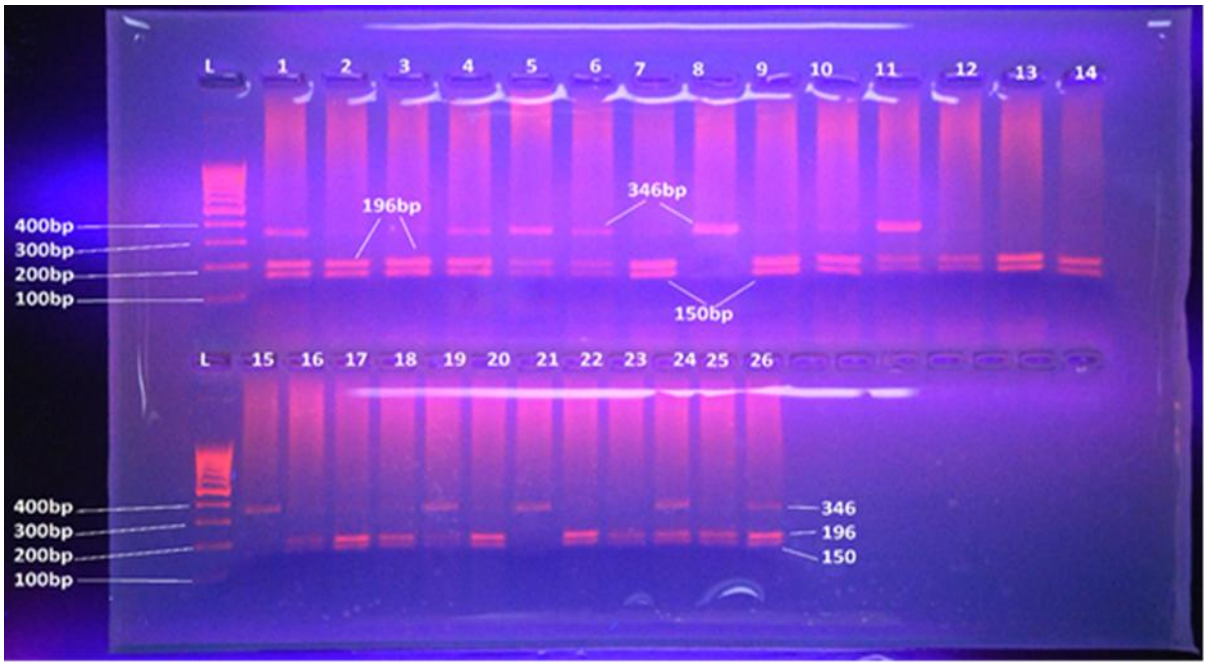

Figure S1A. **Genotyping of the *TLR* gene (346bp) by the PCR-RFLP technique.** The restriction product was resolved on 2% agarose, lane L 100 bp DNA ladder: **lanes** 8,15, and 21 CC genotype; **lanes** 1,4,5,6,11,19,24, and 26 TC genotype; **lanes** 2,3,7,9,10,12,13,14,16,17,18,20,22,23, and 25 TT genotype.

Identification of SNP in the *TLR4* Gene (rs1927914) locus across both groups in gel electrophoresis

The study identified a polymorphism at the rs1927914 locus of the *TLR4* gene. Hardy-Weinberg equilibrium was used to evaluate genotype frequencies. At this locus, three genotypes were found: GG, GA, and AA. A single band corresponding to the A allele at 283 bp was amplified in the reference homozygous genotype (AA). The G allele was represented by two bands at 101 and 182 bp in the homozygous genotype (GG). Both alleles were amplified in the heterozygous genotype (GA), producing three bands: G 101 bp + 182 bp + A 283 bp (Figure S1B).

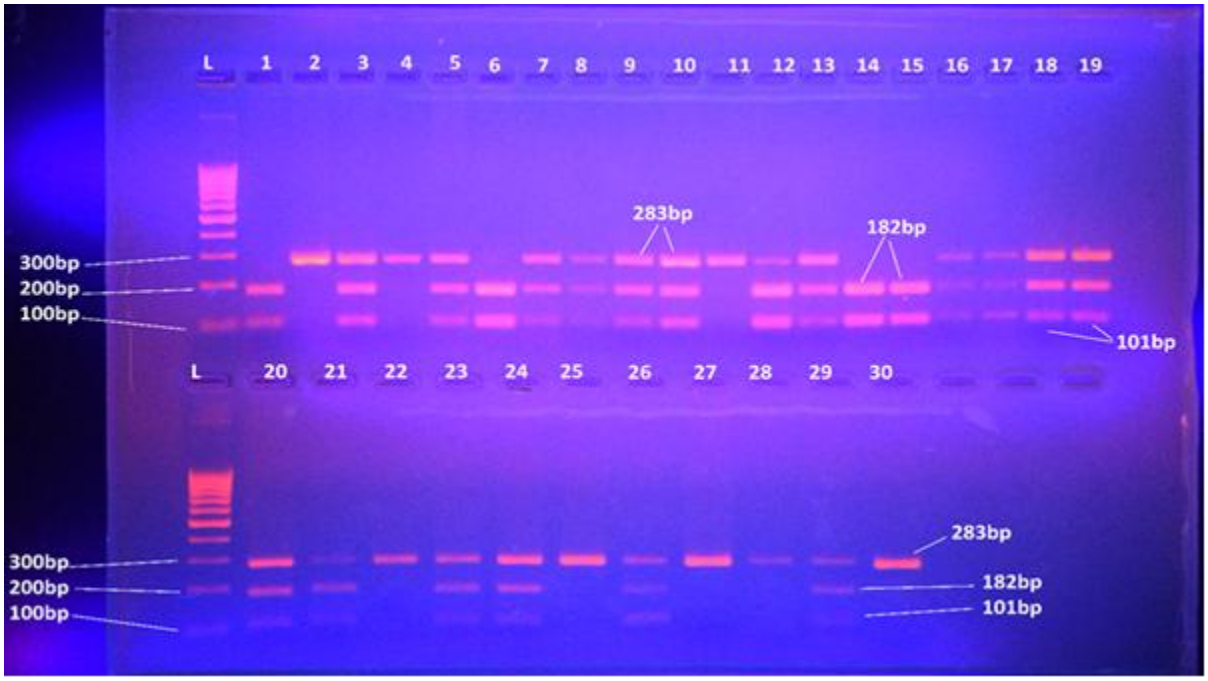

Figure S1B. Genotyping of the *TLR4* gene by the PCR-RFLP technique. Lane L 100bp Ladder; lanes 1,6,14 and 15 GG genotype; lanes 3,5,8,9,10,11,12,16,17,18,19,20,21,23,24,26,29 GA Genotype; lanes 2,4,11,22,25,27,28 and 30 AA Genotype.

Identification of SNPs in the *TLR7* gene (rs179008) locus across both groups in gel electrophoresis

Both pregnant and miscarried women had the SNP rs179008 (A>T) site in the *TLR7* gene. Three different genotypes were identified by genotyping: homozygous reference (AA), heterozygous (AT), and homozygous (TT). The reference AA genotype displayed two bands at (18, 35, 91, and 141 bp) that corresponded to the A allele. On the other hand, the TT genotype had a single band for the T allele at 18, 35, and 232 bp. Three bands from both alleles (A and T) were visible in the heterozygous AT genotype (Figure S1C).

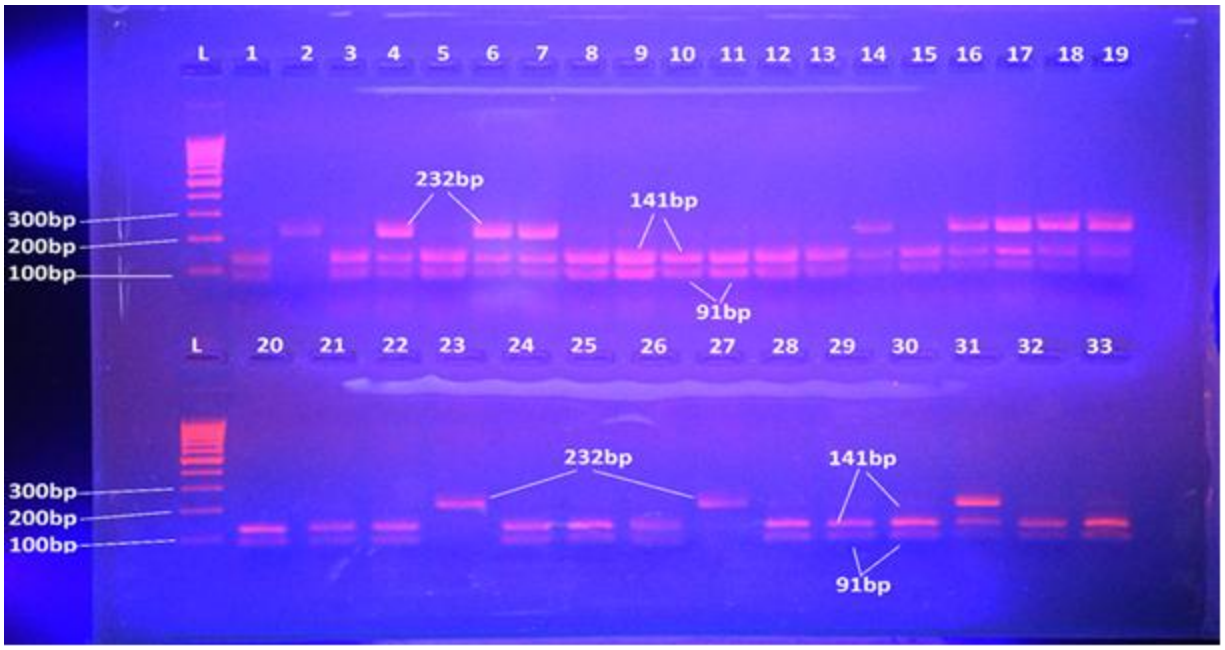

Figure S1C. Genotyping of the *TLR7* gene (rs179008) locus by the PCR-RFLP technique. Lane L: 100 bp DNA ladder; lanes 2,23, and 27: TT genotype; lanes 4,6,7,17,16,17,18,19, and 31: AT genotype; other lanes: AA genotype.

TLR2 sequencing (CC, TC, TT) polymorphisms

A polymorphism at the 198 bp site of the PCR Amplicon, and this is the exact position of the selected *TLR2* (rs3804100) locus, as shown in the following chromatogram Figure S2.

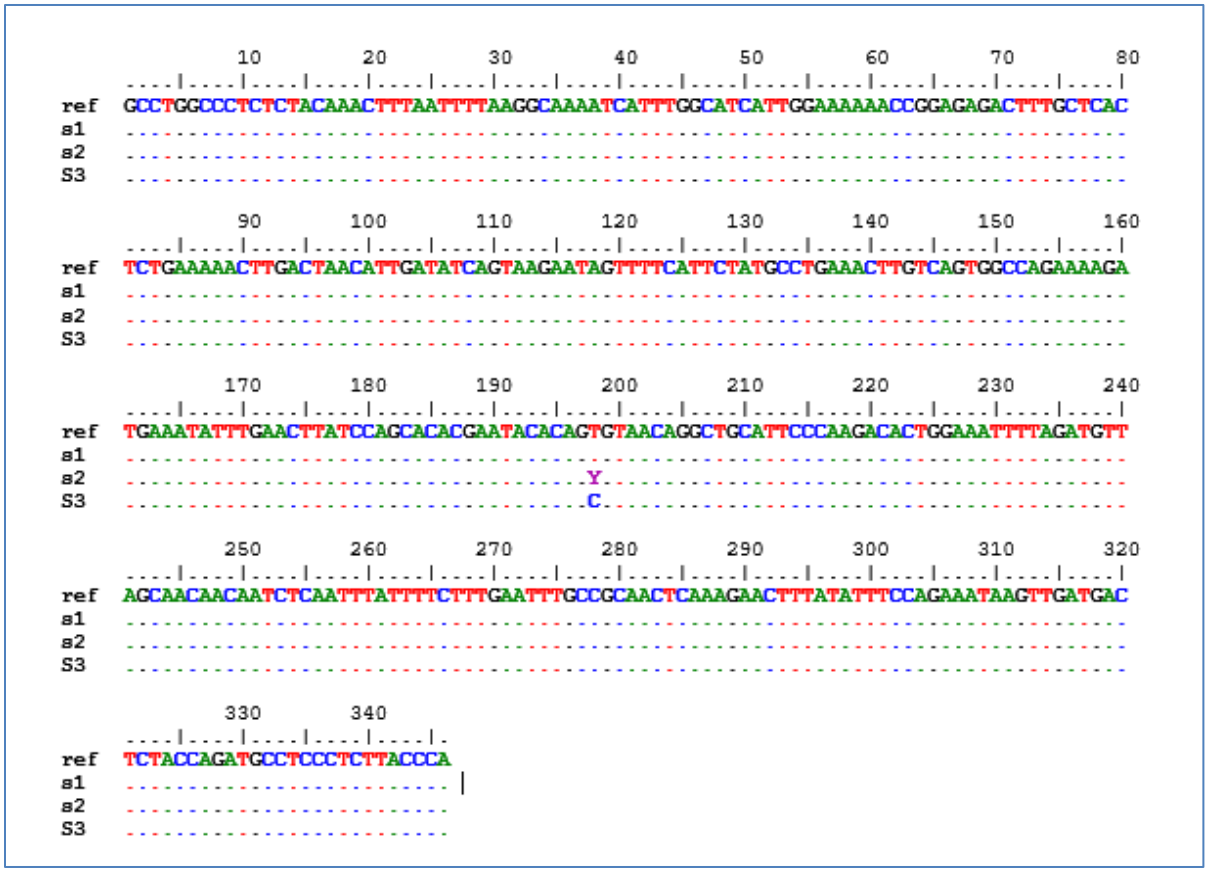

Figure S2. DNA sequence alignment of the main genotypes of the *TLR2* gene (rs3804100) with their corresponding reference sequences. The letter "ref." refers to the NCBI reference sequence. (Y indicates Heterozygous (T/C), and C indicates homozygous (CC).

TLR4 sequencing (AA, GA, GG) polymorphisms

The 99 bp site of the PCR Amplicon is where we detected polymorphism; this is also the precise location of the *TLR4* (rs1927914) locus, as shown in the chromatogram Figure S3.

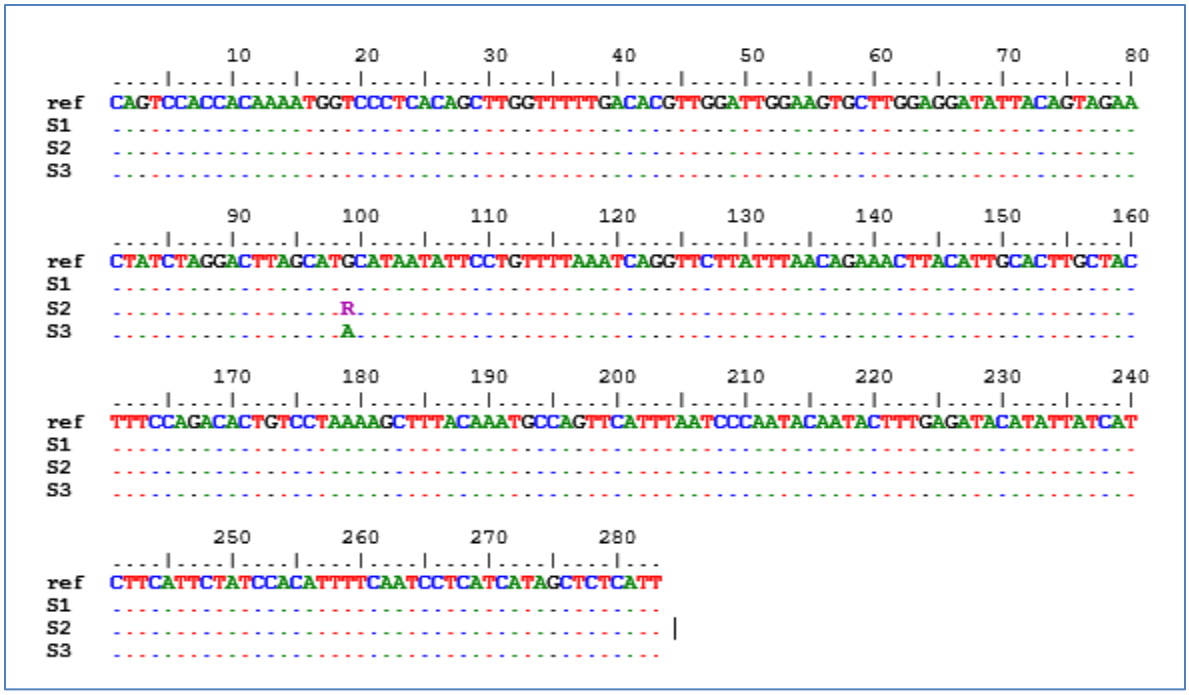

Figure S3. *TLR4* gene (rs1927914) major genotype DNA sequence alignment with reference sequences. R means heterogeneous (G/A), while A means homogeneous. Ref. is the NCBI reference sequence.

TLR7 sequencing (AA, AT, TT) polymorphisms

The results confirm the AT heterogeneity at the locus 110 bp according to the PCR amplicon, and this polymorphic locus is confirmed to be the location (rs179008) as shown by matching the NCBI browser, as shown in Figure S4.

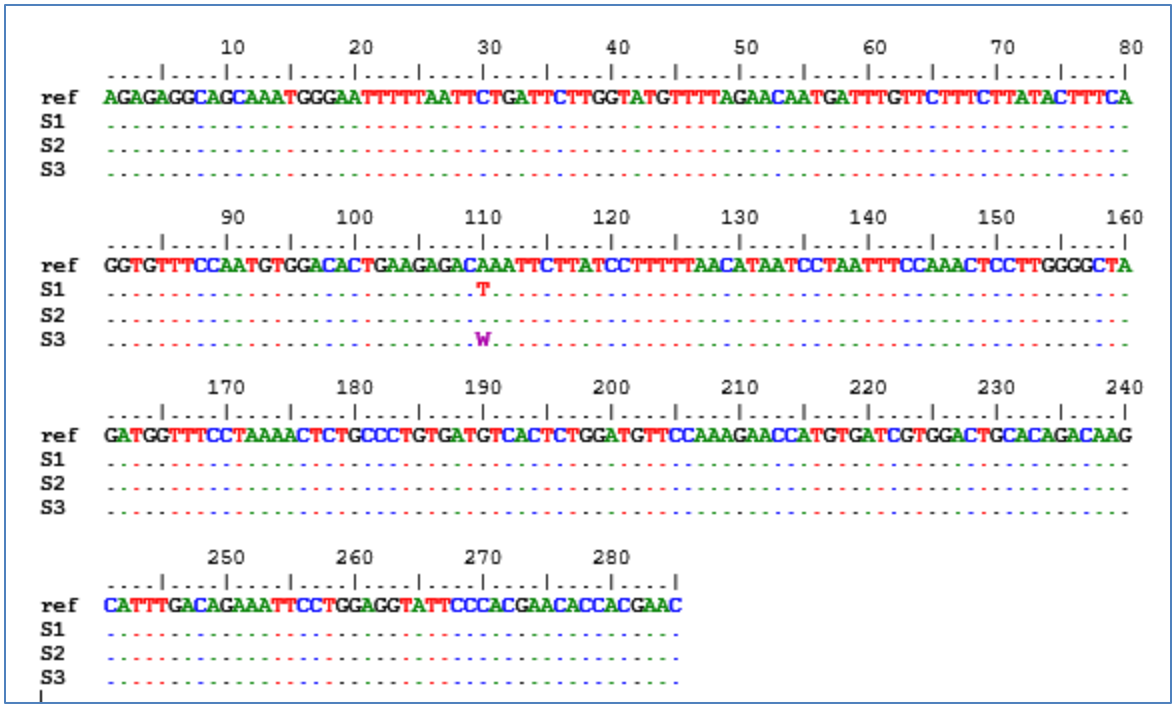

Figure S4. Major genotype DNA sequence alignment of the *TLR7* gene (rs179008) with reference sequences. W and T represent heterogeneous (A/T) and homogeneous I, respectively. Ref. is the NCBI reference sequence.

Homo sapiens *TLR7* gene for toll-like receptor 7 precursor, partial cds

GenBank: LC891957.1

LOCUS LC891957 335 bp DNA linear PRI 26-SEP-2025

DEFINITION Homo sapiens TLR7 gene for toll-like receptor 7 precursor, partial cds.

ACCESSION LC891957

VERSION LC891957.1

KEYWORDS.

SOURCE Homo sapiens (human)

ORGANISM Homo sapiens

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
Catarrhini; Hominidae; Homo.

REFERENCE 1

AUTHORS Khaleefah,N.M., Altamimi,B.J. and Gatea,A.K.

TITLE Homo sapiens

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 335)

AUTHORS Khaleefah,N.M., Altamimi,B.J. and Gatea,A.K.

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