# Supplementary Information

**Identification of a TNF-α inducer MIC3 originating from the microneme of non-cystogenic, virulent *Toxoplasma gondii***

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**14 pages, 6 protocols, 6 figures, 2 tables**

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**Supplementary Protocol 1**

The ESA samples were treated for 1 h at 56°C with 10 mM DTT. Next, 55 mM iodoacetamide was added, and the samples were incubated for 45 min in the dark. Protein was precipitated by mixing the sample with ice-cold acetone (1:4, v/v) followed by incubation at -20°C for 2 h. After centrifugation for 20 min at 25,000×*g* (4°C), the precipitate was air-dried, resuspended in 0.5 M triethylammonium bicarbonate (TEAB) and sonicated on ice for 15 min. The samples were centrifuged at 25,000×*g* for 20 min at 4°C. The supernatant was then stored at −80°C until further use.

**Supplementary Protocol 2**

The iTRAQ labelling of peptide samples was performed using an iTRAQ reagent 8-plex kit (Applied Biosystems, Foster City, CA, USA). For each *T. gondii* strain, two samples from separately propagated tachyzoites (biological replicates) were labelled using different isobaric tags (RH, 114- and 118-tags; TgCtwh3, 119- and 121-tags). The samples were incubated for 2 h at room temperature and dried using a vacuum centrifuge.

The mixed iTRAQ-labelled peptides were dissolved in Buffer A (25 mM $NaH_2PO_4$ in 25% ACN, pH 2.7) and fractionated using an Ultremex SCX column (4.6×250 mm) with an LC-20AB HPLC Pump system (Shimadzu, Kyoto, Japan) at a flow rate of 1.0 ml/min. The 40 min HPLC gradient consisted of 5% buffer B (25 mM $NaH_2PO_4$, 1 M KCl in 25% ACN, pH 2.7) for 7 min, 5%-60% buffer B for 20 min, 60%-100% buffer B for 2 min, and 100% buffer B for 1 min, followed by 5% buffer B for 10 min. Elution was monitored by measuring the absorbance at 214 nm, and fractions were collected every 1

min. The eluted peptides were pooled into 20 fractions, desalted using a Strata X C18 column (Phenomenex, Torrance, CA, USA), and vacuum-dried.

**Supplementary Protocol 3**

Proteomic data acquisition was performed with a Triple TOF 5600 System. Data were acquired using an ion spray voltage of 2.5 kV, gas curtain of 30 psi, nebulizer gas of 15 psi, and an interface heater temperature of 150°C. The MS was operated with a resolution ratio of greater than or equal to 30,000 FWHM for TOF MS scans. Survey scans were acquired in 250 ms. A maximum of 30 product ion scans were collected if a threshold of 120 counts per second (counts/s) was exceeded and with a 2+ to 5+ charge state. The total cycle time was fixed at 3.3 s. The Q2 transmission window was 100 Da for 100%. Four time bins were summed for each scan at a pulser frequency value of 11 kHz by monitoring the 40 GHz multichannel TDC detector with four-anode channel ion detection. A sweeping collision energy setting of 35 ± 5 eV coupled with iTRAQ-adjusted rolling collision energy was applied to all precursor ions for collision-induced dissociation. Dynamic exclusion was set at 1/2 of the peak width (15 s), and then the precursor was refreshed from the exclusion list. The peak areas of the iTRAQ reporter ions reflected the relative abundance of the proteins in the samples. The triple TOF 5600 mass spectrometer for peptide identification has a mass accuracy of < 2 ppm.

**Supplementary Protocol 4**

The resulting MS/MS spectra were searched against the composite database of *T. gondii* GT1 (8100 sequences) and IPI_mouse (59534

sequences) available at ToxoDB (http://toxodb.org/common/downloads/release-8.0/TgondiiGT1/fasta/data/)[58] and the European Bioinformatics Institute (EBI) (ftp://ftp.ebi.ac.uk/pub/databases/IPI/last_release/current/) using Mascot software (version 2.3.02, Boston, MA). The search parameters were as follows: trypsin/P to allow fragments containing one missed cleavage; fragment mass spectrum (MS) tolerance, ± 0.1 Da; peptide MS tolerance, ± 0.05 Da; 100% carbamidomethylation of cysteine; variable conversion rate of N-terminal glutamine to pyroglutamic acid and methionine oxidation. All identified peptides had an ion score greater than the Mascot peptide identity threshold, and a protein was considered identified if at least one unique peptide match was available. For protein quantitation, at least two unique peptides, a ≥ 1.2-fold change, and $P < 0.05$ were required for significant change.

**Supplementary Protocol 5**

The *T. gondii* burden was quantitated as described previously with some modifications[1,2]. Splenic DNA was extracted using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA from each sample was analysed by qPCR. Parasite burden was expressed as the ratio of the *T. gondii* ITS1 gene (internal transcribed spacer 1) to the mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. The primers used are listed in Supplementary Table S2.

**Supplementary Protocol 6**

Peritoneal macrophages were isolated from C57BL/6 mice as described

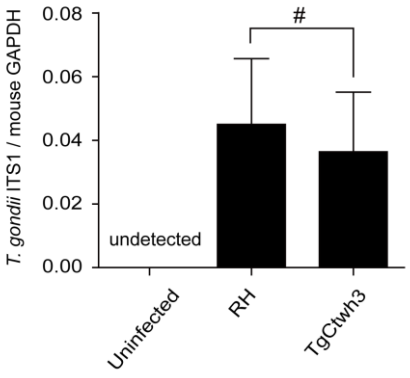
previously[3]. Peritoneal macrophages were cultured in 6-well plates (Costar, Cambridge, MA, USA) at a density of $1 \times 10^6$ cells/well. For antigen treatment, macrophages were washed with PBS to remove non-adherent cells. Then 10 μg/ml RH ESAs, 10 μg/ml TgCtwh3 ESAs, 10 μg/ml OVA, MIC3 (1, 2, 4, 8 μg/ml), or OVA (1, 2, 4, 8 μg/ml) were added directly to each well with fresh medium, respectively. The cells were then incubated for 72 h at 37°C. Macrophages treated with OVA served as controls for M1 and M2 polarization.

## References

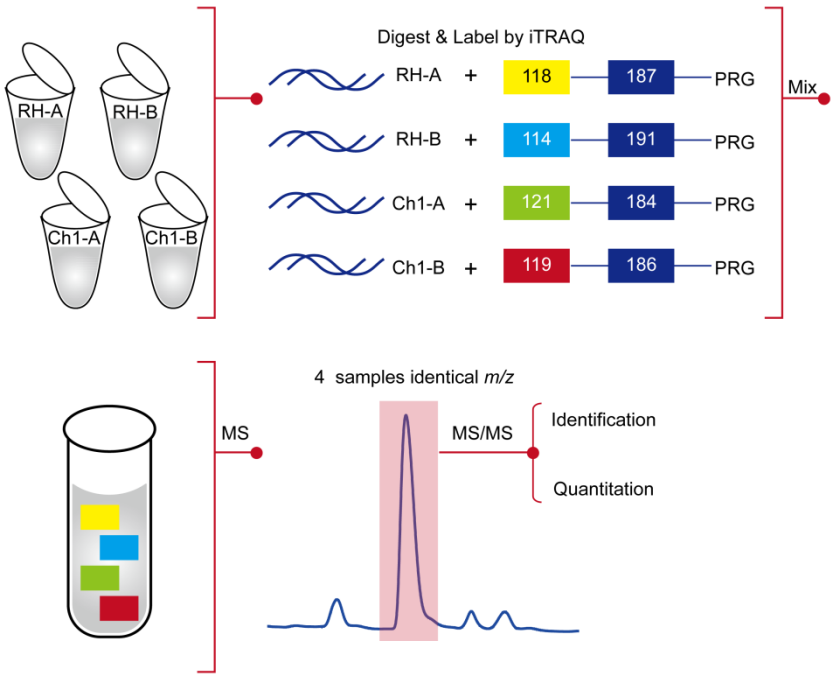

1 Jauregui, L. H., Higgins, J., Zarlenga, D., Dubey, J. P. & Lunney, J. K. Development of a real-time PCR assay for detection of *Toxoplasma gondii* in pig and mouse tissues. *Journal of clinical microbiology* **39**, 2065-2071 (2001).

2 Ge, Y. Y. *et al.* Natural killer cell intrinsic toll-like receptor MyD88 signaling contributes to IL-12-dependent IFN-gamma production by mice during infection with *Toxoplasma gondii*. *International journal for parasitology* **44**, 475-484 (2014).

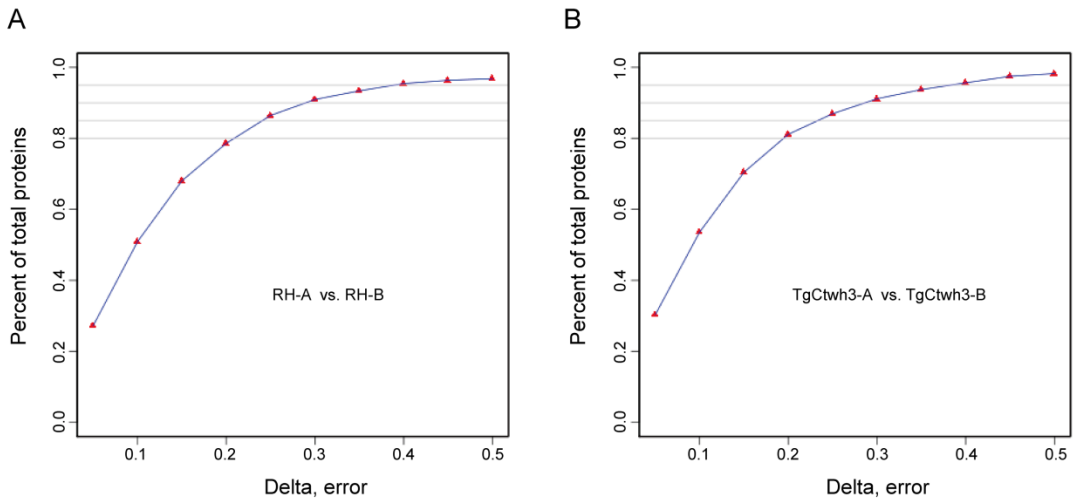
3 Zhu, J. *et al.* Parasitic antigens alter macrophage polarization during *Schistosoma japonicum* infection in mice. *Parasit Vectors* **7**, 122 (2014).



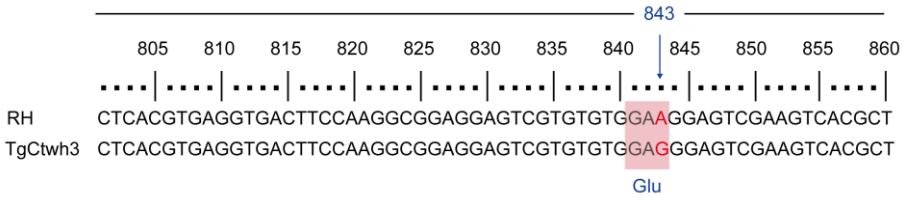

**Supplementary Figure S1.** Parasite burdens in the spleens of mice on day 3 p.i. with $10^6$ RH or TgCtwh3 tachyzoites. The splenic parasite burdens were measured using real-time quantitative PCR and expressed as the ratio of the *T. gondii* internal transcribed spacer 1 (ITS1) gene to the mouse GAPDH gene. Data are represented as the means ± S.D. (n = 6). Significance was analysed using a two-tailed Student's *t*-test. # $P > 0.05$.




**Supplementary Figure S2.** Experimental process of the *T. gondii* ESA comparative proteomics study. Ch1-A and Ch1-B represent two biological replicates of the TgCtwh3 strain; RH-A and RH-B represent two biological replicates of the RH strain.

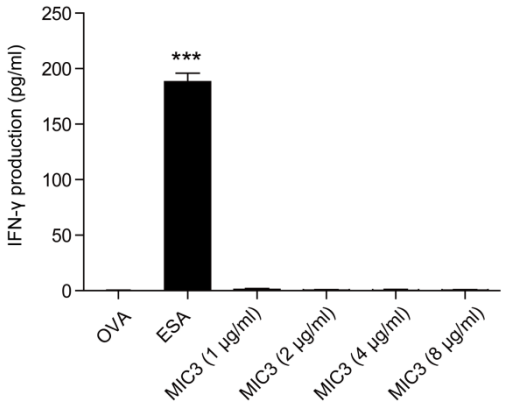



**Supplementary Figure S3.** Reproducibility of biological replicates. (A) RH strain, (B) TgCtwh3 strain.

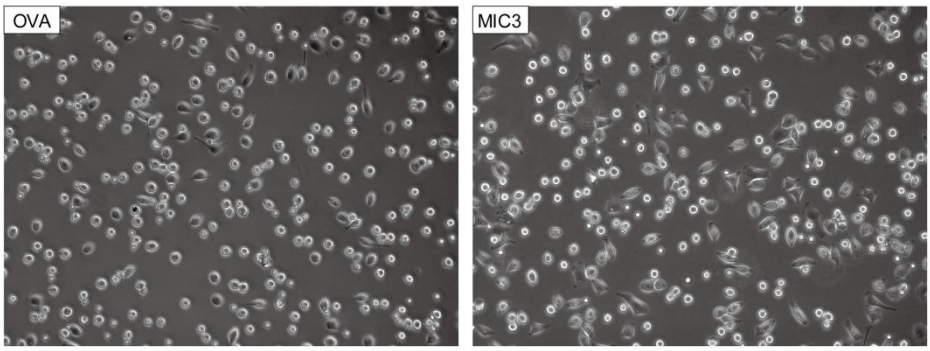



**Supplementary Figure S4.** Alignment of sequences of MIC3 from the *T. gondii* RH and TgCtwh3 strains. Three pairs of primers (Supplementary Table S2) were used to amplify the entire length of the MIC3 gene from *T. gondii* RH and TgCtwh3. We determined the nucleotide sequences of the MIC3 gene by sequencing. MIC3 nucleotide and amino acid sequences from the RH and TgCtwh3 strains were compared using SeqMan software. The nucleotide at position 843 is indicated by the square frame. The sequence of the MIC3 gene from the RH strain was identical to the sequence reported in GenBank (Accession: AF509564.1, GI: 20977535). Compared with the MIC3 gene from strain RH, the MIC3 gene from the TgCtwh3 strain contained a single

nucleotide mutation at 843 bp (A to G). However, the codons GAA and GAG both code for glutamic acid (E). This result confirmed that the amino acid sequences of MIC3 from *T. gondii* RH and TgCtwh3 were identical.




**Supplementary Figure S5.** Effects of MIC3 from *T. gondii* ESAs on IFN-γ secretion. Data are presented as the means ± S.D. (n = 6). Significance was analysed using one-way ANOVA. *** $P < 0.001$.




**Supplementary Figure S6.** Morphology of peritoneal macrophages treated with 1 μg/ml MIC3 or 1 μg/ml OVA for 48 h. Images were acquired using a phase contrast microscope (Olympus, Tokyo, Japan). Objective lens, ×20.

**Supplementary Table S1.** List of upregulated and downregulated *T. gondii* proteins identified and quantified by the iTRAQ analysis. [(a)] ToxoDB release 8.0 (http://www.toxodb.org). [(b)] Fold changes (RH vs. TgCtwh3) with significant differences ($P < 0.05$), Student's *t*-test.

| No. | ToxoDB ID[(a)] | Description | Fold Changes (RH/TgCtwh3)[(b)] | Sequence Coverage (%) | Unique Peptides |
|---|---|---|---|---|---|
| 1 | TGGT1_008800 | conserved hypothetical protein | 2.429 | 11.7 | 3 |
| 2 | TGGT1_016150 | microneme protein MIC11 | 1.687 | 21.6 | 4 |
| 3 | TGGT1_016570 | membrane-attack complex /perforin domain-containing protein | 4.259 | 9.6 | 6 |
| 4 | TGGT1_016650 | subtilisin SUB1 | 5.344 | 8.2 | 4 |
| 5 | TGGT1_018190 | hypothetical protein | 1.318 | 13.9 | 5 |
| 6 | TGGT1_019450 | microneme protein MIC2 | 3.687 | 20.7 | 10 |
| 7 | TGGT1_021130 | microneme protein MIC4 | 2.774 | 19.3 | 10 |
| 8 | TGGT1_021470 | hypothetical protein | 1.738 | 15.0 | 3 |
| 9 | TGGT1_024250 | hypothetical protein | 1.623 | 14.0 | 6 |
| 10 | TGGT1_025360 | heat shock protein, putative | 2.356 | 42.3 | 17 |
| 11 | TGGT1_027570 | microneme protein MIC9 | 3.014 | 11.0 | 7 |
| 12 | TGGT1_029350 | microneme protein MIC6 | 3.072 | 12.6 | 4 |
| 13 | TGGT1_031600 | rhoptry kinase family protein ROP40 | 1.994 | 20.0 | 9 |
| 14 | TGGT1_032990 | macrophage migration inhibitory factor, putative | 1.468 | 20.7 | 2 |
| 15 | TGGT1_033690 | conserved hypothetical protein | 1.843 | 2.5 | 2 |
| 16 | TGGT1_034740 | dense granule protein GRA12 | 1.360 | 22.7 | 9 |
| 17 | TGGT1_035530 | conserved hypothetical protein | 3.130 | 14.0 | 4 |
| 18 | TGGT1_039470 | SAG-related sequence SRS25 | 3.342 | 11.5 | 2 |

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|---|---|---|---|---|---|
| 19 | TGGT1_041290 | rhoptry neck protein RON8 | 2.904 | 8.5 | 22 |
| 20 | TGGT1_042710 | rhoptry protein ROP5 | 2.496 | 8.9 | 4 |
| 21 | TGGT1_051660 | hypothetical protein | 1.940 | 9.3 | 2 |
| 22 | TGGT1_055870 | SAG-related sequence SRS51 (SRS3) | 6.591 | 8.3 | 4 |
| 23 | TGGT1_058380 | conserved hypothetical protein | 1.528 | 34.1 | 4 |
| 24 | TGGT1_060480 | microneme protein MIC17A | 9.806 | 16.8 | 4 |
| 25 | TGGT1_062250 | toxolysin TLN4 / metalloprotease | 1.779 | 5.0 | 9 |
| 26 | TGGT1_063760 | rhoptry protein ROP18 / protein kinase domain-containing protein | 3.842 | 8.5 | 4 |
| 27 | TGGT1_064690 | conserved hypothetical protein | 2.268 | 9.6 | 10 |
| 28 | TGGT1_066110 | conserved hypothetical protein | 1.761 | 1.7 | 2 |
| 29 | TGGT1_071510 | conserved hypothetical protein | 6.784 | 30.1 | 10 |
| 30 | TGGT1_074260 | conserved hypothetical protein | 1.500 | 48.1 | 5 |
| 31 | TGGT1_081090 | conserved hypothetical protein | 3.957 | 13.3 | 3 |
| 32 | TGGT1_082100 | heat shock protein 70kD, putative | 1.747 | 9.9 | 8 |
| 33 | TGGT1_082670 | dense granule protein GRA3 | 5.811 | 10.4 | 2 |
| 34 | TGGT1_085550 | rhoptry protein ROP1 | 17.578 | 7.2 | 3 |
| 35 | TGGT1_086210 | conserved hypothetical protein | 1.450 | 24.1 | 5 |
| 36 | TGGT1_092760 | SAG-related sequence SRS52A | 2.481 | 14.5 | 3 |
| 37 | TGGT1_100270 | conserved hypothetical protein | 1.428 | 4.3 | 4 |
| 38 | TGGT1_102150 | microneme protein MIC10 | 1.733 | 23.2 | 5 |
| 39 | TGGT1_113990 | SAG-related sequence SRS29C (SRS2) | 1.359 | 12.6 | 4 |
| 40 | TGGT1_115220 | conserved hypothetical protein | 2.473 | 19.5 | 3 |
| 41 | TGGT1_115370 | conserved hypothetical protein | 1.479 | 22.0 | 6 |
| 42 | TGGT1_117730 | conserved hypothetical protein | 5.576 | 35.2 | 3 |

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|---|---|---|---|---|---|
| 43 | TGGT1_121730 | microneme protein MIC3 | 5.259 | 21.2 | 5 |
| 44 | TGGT1_121850 | SAG-related sequence SRS17B | 2.356 | 27.4 | 6 |
| 45 | TGGT1_123520 | rhoptry protein ROP15 | 1.334 | 21.6 | 6 |
| 46 | TGGT1_123900 | conserved hypothetical protein | 2.388 | 58.1 | 9 |
| 47 | TGGT1_125220 | conserved hypothetical protein | 1.951 | 12.7 | 4 |
| 48 | TGGT1_125360 | MIC2-associated protein M2AP | 4.323 | 10.0 | 3 |
| 49 | TGGT1_004090 | conserved hypothetical protein | 0.425 | 24.8 | 3 |
| 50 | TGGT1_029790 | conserved hypothetical protein | 0.313 | 18.2 | 3 |
| 51 | TGGT1_032760 | hypothetical protein | 0.667 | 16.7 | 14 |
| 52 | TGGT1_053770 | conserved hypothetical protein | 0.424 | 12.8 | 4 |
| 53 | TGGT1_057870 | conserved hypothetical protein | 0.719 | 2.7 | 4 |
| 54 | TGGT1_072740 | SAG-related sequence SRS35A | 0.232 | 22.1 | 3 |
| 55 | TGGT1_075470 | rhoptry protein ROP7 | 0.787 | 22.4 | 9 |
| 56 | TGGT1_092700 | conserved hypothetical protein | 0.627 | 9.5 | 6 |
| 57 | TGGT1_096800 | conserved hypothetical protein | 0.175 | 19.4 | 2 |
| 58 | TGGT1_098250 | receptor for activated C kinase, RACK protein, putative | 0.642 | 41.4 | 10 |
| 59 | TGGT1_098340 | ABC transporter, putative | 0.621 | 10.8 | 5 |
| 60 | TGGT1_098470 | KH domain-containing protein, putative | 0.782 | 14.3 | 10 |
| 61 | TGGT1_102220 | conserved hypothetical protein | 0.800 | 37.9 | 11 |
| 62 | TGGT1_102940 | dense granule protein GRA9 | 0.614 | 18.9 | 5 |
| 63 | TGGT1_112260 | cell division protein, putative | 0.754 | 25.3 | 16 |
| 64 | TGGT1_115180 | NBP2B protein, putative | 0.489 | 7.9 | 4 |
| 65 | TGGT1_118190 | membrane-associated calcum-binding protein, putative | 0.619 | 24.8 | 6 |
| 66 | TGGT1_125960 | hypothetical protein | 0.646 | 9.8 | 5 |

**Supplementary Table S2.** Primers used in this study.

| Name | Sequence (5'-3') | Use |
|---|---|---|
| GAPDH-F | CTCATTGTCGGCGGAAAGG | Real-time RT-PCR amplification of *T. gondii GAPDH* |
| GAPDH-R | GGTGGACTCGCAGATGTAGTGG | |
| 085550-F | CAGGGACCAGTTCGTCAAAA | Real-time RT-PCR amplification of *ROP1* |
| 085550-R | GAAACCACTCCTCACTCCACC | |
| 082670-F | GATTCTCGCCGCCTACTACA | Real-time RT-PCR amplification of *GRA3* |
| 082670-R | CAGCACCCTCATCCTTTGG | |
| 121730-F | AGTCACGCTGGCTGAGAAATG | Real-time RT-PCR amplification of *MIC3* |
| 121730-R | GGAGGTTGCGGAAGCAGAT | |
| 063760-F | AGGAAAGGGCTCAACACCG | Real-time RT-PCR amplification of *ROP18* |
| 063760-R | GGAACCCGAGACTTCACCATC | |
| 029350-F | TCGACGCCAGTGCCCATAT | Real-time RT-PCR amplification of *MIC6* |
| 029350-R | CGACTTTCACCTTCATCTTCCCT | |
| GAPDH-F | AGGTCGGTGTGAACGGATTTG | Real-time RT-PCR amplification of mouse *GAPDH* |
| GAPDH-R | TGTAGACCATGTAGTTGAGGTCA | |

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|---|---|---|
| IL-6-F | GAGGATACCACTCCCAACAGACC | Real-time RT-PCR amplification of *IL-6* |
| IL-6-R | AAGTGCATCATCGTTGTTCATACA | |
| iNOS-F | GGAGCGAGTTGTGGATTGTC | Real-time RT-PCR amplification of *iNOS* |
| iNOS-R | GTGAGGGCTTGGCTGAGTGAG | |
| Arg-1-F | CAGAAGAATGGAAGAGTCAG | Real-time RT-PCR amplification of *Arg-1* |
| Arg-1-R | CAGATATGCAGGGAGTCACC | |
| IL-10-F | GACCAGCTGGACAACATACTGCTAA | Real-time RT-PCR amplification of *IL-10* |
| IL-10-R | GATAAGGCTTGGCAACCCAAGTAA | |
| Fizz1-F | TCCCAGTGAATACTGATGAGA | Real-time RT-PCR amplification of *Fizz-1* |
| Fizz1-R | CCACTCTGGATCTCCCAAGA | |
| Toxo ITS1-F2 | CAAGAAGCGTGATAGTATCG | Real-time PCR amplification of *ITS1* |
| Toxo ITS1-R2 | CTGAAGAAACTCCTGGAAATC | |
| MIC3-Q1 | GTCACTCAACTTGCTGCTA | PCR amplification of a part of the MIC3 gene |
| MIC3-Q2 | CGAGGCGTCATCAATACAT | |
| 121730-NC1 | AAACATATGCGAGGCGGGACGTCCGCGCTG | PCR amplification of a part of the MIC3 gene |
| 121730-NC2 | AAAAAGCTTCTGCTTAATTTTCTCACACGT | |
| MIC3-W1 | GCAGTTCGCTCATCTACC | PCR amplification of a part of the MIC3 gene |

| | | |
|---|---|---|
| MIC3-W2 | TCTCAATCCTTGGCTTAGTG | |