Junior Science Communications
Faculty of Applied Sciences, UiTM Shah Alam
https://journal.uitm.edu.my/ojs/index.php/JSC

E-ISSN 2637-0689
Vol 68 2026

Colloquium on Applied Sciences 2 2025
19-22 January 2026, Faculty of Applied Sciences, UiTM Shah Alam, Malaysia

Designing CRISPR/CAS9 gene knockout system for granule antigen 1 (*GRA1*) gene in *Toxoplasma gondii*

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Structured Abstract

Background: *Toxoplasma gondii* can infect animals and humans. *T. gondii* utilises cats as definite host and humans, mammals and birds as intermediate host. Toxoplasmosis symptoms include flu, headaches, eye problems and others severe symptoms in immunocompromised individuals and foetus during pregnancy with seroprevalence rates of to up to 49%. Granule antigen 1 (*GRA1*) is a dense-granule protein essential for parasite attachment, invasion and immune evasion in *T. gondii*. This study aimed to design CRISPR/Cas9 gene knockout system for *GRA1* gene in *T. gondii*.

Methods: The in-silico study involved four steps. First, the complete GRA1 gene sequence (GenBank accession HM067753.1) from *T. gondii* strain RH P24 was retrieved and analysed for protospacer adjacent motif (PAM) sites using the NGG motif. Second, the EuPaGDT web tool was used to design single guide RNAs with optimal on-target activity and minimal off-target effects. Third, forward and reverse primers for sgRNA-CRISPR/Cas9 recombinant plasmid validation were designed using OligoCalc software. All designed sgRNAs were validated using BLAST for sequence similarity. Lastly, annotation of sgRNA and Cas9 complexes using SnapGene Viewer software.

Results: Three potential sgRNAs were successfully designed targeting the *GRA1* gene. All three sgRNAs exhibited excellent characteristics with on-target sites of 1|0 (single unique match with no non-perfect PAM matches) and off-target sites of 0|0 (no significant off-target binding). Forward and reverse primer pairs were designed for all three sgRNAs with melting temperatures between 50-60°C and GC content between 40-60%. BLAST analysis confirmed no sequence similarity and homology to human genes. All primers showed no potential for hairpin formation or self-dimerization. All potential sgRNAs in pSAG1::CAS9-U6::sgUPRT recombinant plasmid were mapped to confirm its position.

Conclusion: Three potential sgRNAs were successfully designed and validated in-silico. These findings provide promising candidates for establishing an efficient *GRA1* gene knockout system, which can reduce parasite virulence and support future development of therapeutic interventions against toxoplasmosis.

Keywords: CRISPR/Cas9, *Toxoplasma gondii*, GRA1 gene, guide RNA design, gene knockout

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