Developing a PCR-based assay to detect the S12N gene mutation within CARD9

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Introduction

CARD9 is a human gene that helps the body fight infections by communicating with proteins within immune signaling pathways. CARD9 mutations have been found to make individuals more susceptible to autoimmune diseases, like Inflammatory Bowel Diseases, by causing the immune system to act unnecessarily. The S12N mutation within the CARD9 gene is a single nucleotide polymorphism (SNP) that changes out one amino acid within DNA, switching serine (S) to asparagine (N) at position 12 in the protein. This change results in a decrease in the production of Th17 cells, making the immune system less efficient at controlling fungal colonization leading to intestinal inflammation (Luo et al., 2020).

Quantitative real-time polymerase chain reaction (qPCR) is a technology that exponentially amplifies a specific sequence of DNA, creating several copies of DNA. qPCR acts by first heating DNA into separate strands, which allows primers to bind to a specific target region (Lorenz, T. C., 2012). Then, fluorescence dye is incorporated after DNA polymerase copies the target region. This process is repeated over several qPCR cycles where the change in fluorescence correlates to the amount of DNA product. Gel electrophoresis separates DNA fragments by size, so we can see how long the pieces are. Melt curve analysis looks at how DNA strands separate when heated, which helps identify small differences in DNA sequences, like mutations. The purpose of this project was to develop a PCR-based assay to detect the presence of the S12N mutation in the CARD9 gene.

Methods and Materials

The DNA sequence surrounding the S12N site was first analyzed, and three primers were designed: a reverse primer, a wild-type (WT) forward primer, and a mutant (MUT) forward primer. The WT forward primer included an extra 30-bp tag to produce a longer amplicon than the MUT primer, allowing the two products to be distinguished. Amplicon size correlated with which primer was favored during PCR and, therefore, which CARD9 variant was present (Figure 1). An initial PCR experiment was run using WT or MUT synthetic DNA (gBlocks) and a PCR master mix across a temperature gradient of 56.0 °C to 64.7 °C to find the optimal annealing temperature for primer binding and amplification. After each run, melt curves were analyzed to evaluate the specificity of the amplified products, with the goal that WT and MUT amplicons would produce peaks at different melting temperatures.

Methods and Materials (continued)

To improve the assay, its thermal profile, primer concentration, and primers with locked nucleic acid (LNA) bases were tested to increase specificity for the correct target. PCR products were also run on agarose gel electrophoresis to confirm amplification and check fragment size.

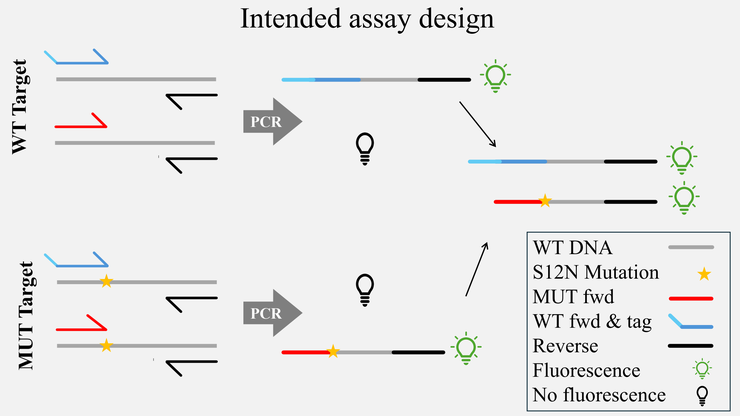


Figure 1 (above): The WT forward primer binds only to WT DNA, and the MUT forward primer only binds to the MUT DNA. Each primer is paired with the reverse primer to produce a fluorescent signal if amplification occurs. Fluorescence indicates whether the primers amplified the correct corresponding CARD9 variation.

Results

The gel visualized the band sizes of unknown samples, which helped identify whether WT CARD9 or MUT CARD9 was present (Figure 2). This was determined by amplicon length, with the WT amplicon being about 112 bp and the MUT amplicon being around 82 bp. The unknown samples were correctly identified by both melt curve analysis (Graph 1) and gel electrophoresis results. Unknown 1 matched the MUT profile, Unknown 2 matched the WT profile, and Unknown 3 matched the NTC control.

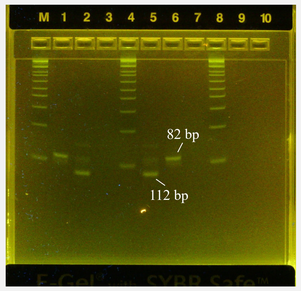
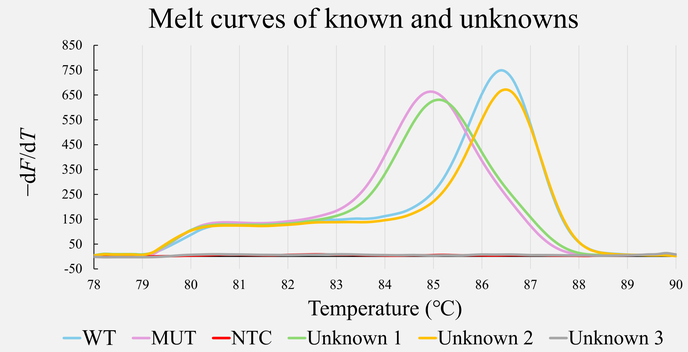


Figure 2 (right): Gel analysis indicated that unknown samples in lanes 5, 6, and 7 matched the WT/MUT/NTC profiles, respectively when compared to the 100 bp ladder within lanes 1, 4, and 8. These findings were corroborated by the melt curve analysis. The assay correctly identified which CARD9 variant was in each unknown sample, and the results matched the known sample identities.

Results (continued)



Graph 1 (above): This graph shows melt curves of known and unknown CARD9 targets. A peak is at the specific temperature that the target denatures. The WT CARD9 melt temperature (T_m) is at 86.4 °C and the MUT CARD9 melts at 85.0 °C. These T_m peaks can be compared to the curves of unknown targets to identify which variation of CARD9 is present.

Conclusion

When running the qPCR at an annealing temperature of 62.0 °C with a 25% decreased LNA WT forward primer with tag, the PCR-based assay successfully detected the S12N mutation in the CARD9 gene using three unknown samples. The known wild-type (WT) amplified to 112 bp and produced a melt temperature of approximately 86.4 °C, while the mutant (MUT) sample amplified to 82 bp with a melt temperature of approximately 85.0 °C. These melt curve temperatures corresponded with the expected gel electrophoresis band sizes, confirming the correct CARD9 variant. Detecting the S12N mutation in the CARD9 gene is important because it helps identify individuals who may have an increased risk of immune system dysfunction, including susceptibility to fungal infections and inflammatory diseases.

References

Lorenz, T. C. (2012). Polymerase chain reaction: Basic protocol plus troubleshooting and optimization strategies. *Journal of Visualized Experiments*, (63), e3998. https://doi.org/10.3791/3998

Luo, P., Yang, Z., Chen, B., & Zhong, X. (2020). The multifaceted role of CARD9 in inflammatory bowel disease. *Journal of Cellular and Molecular Medicine*, *24*(1), 34–39. https://doi.org/10.1111/jcmm.14770