Rapid molecular detection of ADA SCID using HRM Analysis

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Abstract

Severe combined immunodeficiency (SCID) is a group of rare genetic disorders that causes affected individuals to have little or no immune response due to a deficiency or absence of T-cells and at least one other type of lymphocyte. This leaves SCID patients susceptible to persistent bacterial, fungal, and viral infections which can potentially be lethal. SCID patients need to be diagnosed early on in order to receive effective treatment. This assay was designed to genotype for adenosine deaminase (ADA) deficiency SCID. Primers were designed to produce a small target amplicon containing the rs121908723 single nucleotide polymorphism (SNP) from the *ADA* gene. SCID homozygous and SCID heterozygote Coriell Cell Repository DNA controls were used to design and validate a qualitative SCID high-resolution melt (HRM) genotyping assay using the Rotor-Gene Q platform. Isolated DNA is amplified by real-time polymerase chain reaction (PCR), and the target amplicon is fluorescently detected using EvaGreen dye during HRM analysis. HRM has the ability to distinguish samples by a fraction of a degree difference and a single base pair change, which is the case in ADA SCID. A total of 147 samples were analyzed, and the results provided the data necessary to measure analytical and clinical performance, lower limit of detection, and analytical specificity. The assay demonstrates a promising analytical and clinical performance with an accuracy of 92.6%, a precision of 92.2%, a clinical sensitivity of 90.8%, a clinical specificity of 100%, a positive predictive value of 100%, and a negative predictive value of 95.7%. With additional validation studies, our assay has the potential to be used clinically for the rapid genotyping of possible SCID patients.

Introduction

Severe combined immunodeficiency (SCID) is a group of rare genetic disorders that cause affected individuals to have little or no immune response. As a result of this, SCID patients suffer from persistent bacterial, fungal, and viral infections. This disease affects the T-cells and at least one other type of immune cell. The type of SCID that our assay was designed to detect is the adenosine deaminase deficiency (ADA) type SCID, an autosomal recessive disorder. The classes of lymphocytes affected in this variant of SCID are the T-cells, B-cells, and natural killer cells. The single nucleotide polymorphism (SNP) number associated with ADA SCID is rs121908723 located on chromosome 20. At this SNP the guanine (G) that is normally present can get replaced by an adenine (A).[1] This mutation in the ADA gene causes a decrease or absence in the production of the enzyme adenosine deaminase. This enzyme converts deoxyadenosine, which is toxic to lymphocytes, to deoxyinosine a non-toxic compound. The build up of deoxyadenosine prevents the development of maintenance of lymphocytes.[2] A patient with SCID needs to be diagnosed early so the stem cell transplant therapy will be most effective.[1] We designed an assay to genotype samples for ADA SCID using High Resolution Melting (HRM) Analysis.

Methods

SCID homozygous G216R and SCID heterozygote G216R Coriell Cell Repository DNA were used to design and validate a qualitative SCID HRM genotyping assay using the Rotor-Gene Q platform. PCR optimization was accomplished by using a 71 bp primer set designed with the Primer Blast software. A total of 72 samples: 18 per homozygous (AA), and heterozygous (AG) genotypes, and 36 wild-type (GG) genotypes were assayed and provided the data necessary to measure accuracy (valid calls/total calls X 100 = % accuracy), and precision (calls in agreement/total calls X 100 = % precision) averaged for each genotype. Lower Limit of Detection was assessed by running each genotype in triplicate with the following DNA concentrations; Heterozygous and Wild-Type: 30ng, 20ng, 10ng, 5ng, 1ng, and 0.5ng. Homozygous: 25ng, 15ng, 5ng, 1ng, and 0.5ng. A total of 7 unknown samples (along with positive controls and a negative control) were run in triplicate. The same samples were tested for the SCID mutation by the SCID pyrosequencing group. Results from both runs were compared in order to determine clinical sensitivity (TP/(TP + FN) X 100), clinical specificity (TN/TN +FP) X 100), positive predictive value (TN/(TN+FN) X 100), and negative predictive value (TP/(TP+FP) X 100). Interference was assessed by running triplicates of contaminated samples (dirt, dog hair, no PPE, and writing on tubes). It was found that dirt did indeed interfere with genotype calling.[3,4]

Clinical Performance Characteristics				
	Sensitivity	Specificity	PPV	NPV
Homozygous Mutant	85.71%	100%	100%	95.83%
Heterozygous	92.86%	100%	100%	97.87%
Wild-Type	93.75%	100%	100%	93.33%

Table 2: The values used to calculate these characteristics were obtained from the validation runs which were blinded within the group. The positive predictive values (PPV) indicates that the assay did not call any false positives. The negative predictive values (NPV) however indicates that the assay did call some false negatives across all the genotypes. This is also reflected in the clinical specificity and sensitivity values.

Assay Accuracy and Precision		
	Accuracy	Precision
Homozygous Mutant	88.89%*	86.67%*
Heterozygous	94.44%	95%
Wild-Type	94.44%	95%

Table 3: The values used to calculate these characteristics were obtained from the validation runs which were blinded within the group. *Both accuracy and precision for the homozygous mutant were relatively low, but this was to be expected since this control's purity value was on the lower end the required range.

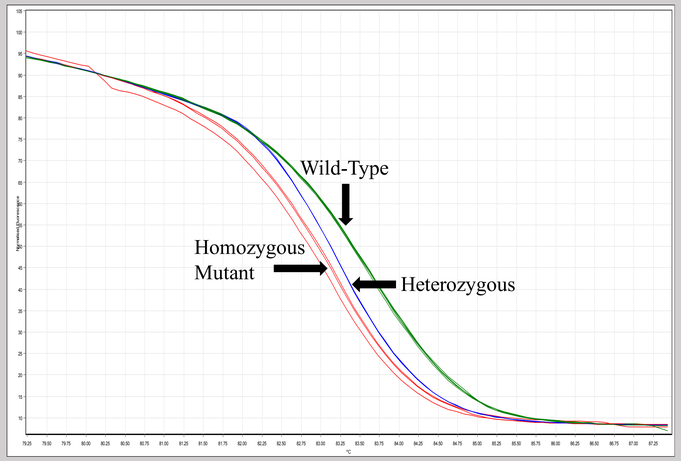

Figure 1: HRM Normalized graph from Validation Run #1. This graph plots the normalized fluorescence (y-axis) against temperature (x-axis). It is a visual representation of the samples as DNA strands separate during the melt phase. In this graph the genotypes can be differentiated

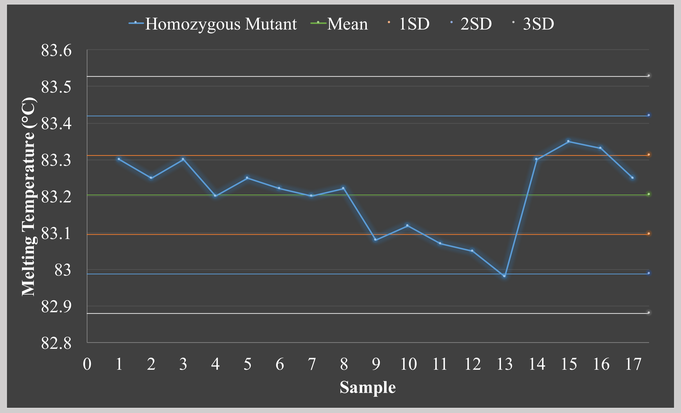

Figure 2A: Standard Deviation of Melting Temperature (T_m) for Homozygous Mutant. The mean T_m for the homozygous mutant samples from validation runs was 83.20 ºC with a standard deviation of 0.11 ºC.

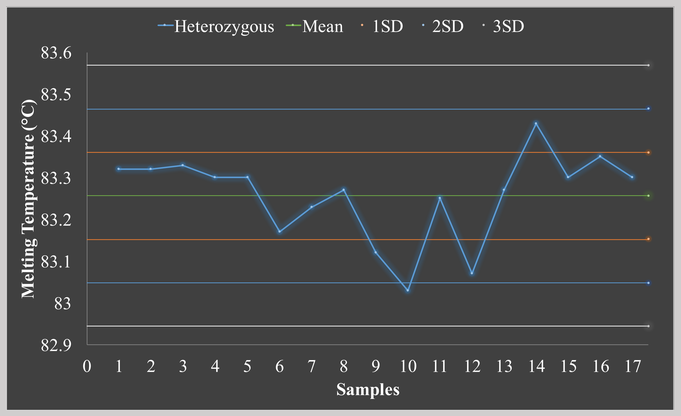

Figure 2B: Standard Deviation of Melting Temperature (T_m) for Heterozygous. The mean T_m for the heterozygous samples from validation runs was 83.26 ºC with a standard deviation of 0.10 ºC.

No.	Color	Name	Genotype	Confidence %
1		Homozygous Control	Homozygous Mutant	100.00
2		Heterozygous Control	Heterozygous	100.00
3		Wild Type Control	Wild-Type	100.00
4		Unknown 1	Heterozygous	99.73
5		Unknown 2	Wild-Type	98.89
6		Unknown 3	Homozygous Mutant	70.07
7		Unknown 4	Wild-Type	99.73
8		Unknown 5	Wild-Type	99.45
9		Unknown 6	Wild-Type	99.62
10		Unknown 7	Homozygous Mutant	98.33
11		Unknown 8	Heterozygous	99.81
12		Unknown 9	Wild-Type	99.51

Table 1: Genotype Calling by Rotor-Gene Q from Validation Run #1. The instrument utilizes the controls and percent confidence values from the difference graph to call genotypes. The highlighted sample fell below the determined confidence threshold of 90%.

Discussion/Conclusion

The need for high resolution testing for SCID is crucial because the melting temperatures between the homozygous and heterozygous genotypes can be as low as a 0.06 °C difference. Due to this small difference, regular melt curve analysis can potentially misinterpret the results and interfere with genotype calling. This Rapid Molecular Detection of ADA SCID HRM analysis using the Rotor-Gene Q successfully produces qualitative results for the ADA SCID G216R genotype. The validation statistics of this assay were averaged across all genotypes. They demonstrate a promising analytical performance with an average accuracy of 92.6% and an average precision of 92.2%. The clinical performance values of this assay were averaged across all genotypes. They demonstrate a clinical sensitivity of 90.8%, a clinical specificity of 100%, a positive predictive value of 100%, and a negative predictive value of 95.7%. Limitations of this study include but are not limited to the sample sizes and amount of runs per validation test not being an adequate amount for approval of this assay in the clinical laboratory setting. Also, the limit of detection did not produce accurate results with smaller than 5ng of DNA. For future studies we recommend running all validation tests with a greater number of samples per genotype per run. Also test the other primer set (135bp amplicon) to identify any differences between validation statistics of each primer set.

Reference:

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