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School *of* Health Professions

Molecular Pathology

Development of a High Resolution Melt Curve Assay to Detect Sickle Cell Disease

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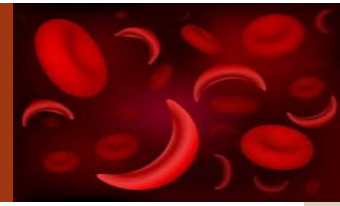

Abstract

Sickle cell disease is a disorder that results in a point mutation which changes glutamic acid to valine and is responsible for sickle shaped red blood cells. At the nucleotide level adenine is replaced by thymine which is known as a class IV mutation. Class IV mutations are very difficult to detect; therefore, the proper instrumentation and software is needed to capture this mutation. The purpose of this research is to design a high resolution melt curve assay (HRM) on the Qiagen Rotor-Gene instrument for the detection of sickle cell disease (SCD). We determined the optimal primers and cycling conditions for a reliable and accurate HRM SCD assay. By detecting slight differences in melting temperatures this assay is able to differentiate between the various SCD genotypes; wild-type (AA), heterozygous (AT), and homozygous mutant (TT). We were able to obtain a 92.8% accuracy rate and a 94.4% precision rate, as well as a 90% concordance rate when compared to sequencing. Future studies should include more positive samples with sickle cell disease, as we only had known positives from a DNA bank. The Rotor-gene HRM SCD lab developed assay, once validated, could be a cost effective and quick molecular diagnostic assay to detect sickle cell disease.

Introduction

Sickle Cell Disease is an autosomal recessive disorder that results from a point mutation in chromosome 11. Chromosome 11 codes for the β-globin gene that is responsible for producing hemoglobin. The point mutation that occurs changes the amino acid glutamic acid (GAG) into valine (GTG). This mutation results in producing abnormal hemoglobin causing the formation of sickle-shaped red blood cells. The purpose of this research is to perform and optimize the Qiagen Rotor-Gene HRM instrument for the detection of SCD. The use of this instrument is to distinguish between three genotypes: homozygous mutant (TT), heterozygous (AT), and wild-type (AA) alleles. The Rotor-Gene Q is appropriate for the use in gene expression analysis, SNP genotyping, and pathogen detection. Our research will focus on the detection of the SNP found in SCD. The Rotor-Gene Q is actually capable of distinguishing between the most difficult class IV A/T SNPS that encompasses melt point differences below 0.2C. The master mix also contains the HotStarTaq Plus DNA Polymerase that produces highly specific amplification, and distinctions between the different melt curves. It also uses EvaGreen, a fluorescent dye that selectively binds to double stranded DNA. EvaGreen is ideal because it can be used at a higher concentration without inhibiting PCR. Sanger sequencing was used to confirm 20% of the DNA samples that were genotyped on the Rotor-Gene HRM instrument.

Methods and Materials

Preamplification: The development of this particular assay first required designing a set of primers: a forward (TGTTCACTAGCAACCTCAAACAGA) and reverse (TTGCCCCACAGGGCAGTAA) resulting in an amplicon size of 81 bp. The optimization of our primers was performed in order to verify the target of interest (image 1).

HRM Amplification: Once successful we move on to specimen preparation for the HRM assay. A master mix is prepared using the following reagents: forward and reverse primers at10µM concentration, HRM master mix 2x, and RNase free water. Template sample DNA is added individually into master mix for a total volume of 25µL per reaction. Optimal conditions are listed on box 1. For our assay we recommend using 25ng of template DNA.

Data Analysis: The run takes about 2 hours, and results are then analyzed using the established parameters.

Figure 1. 1.5% agarose gel

Box 1. Optimized cycling for HRM analysis.
Hold: 94ºC, 3 minutes
Cycling: 45 repeats
Step 1: 95ºC, hold 45 secs
Step 2: 55ºC, fold 45 secs, acquiring to cycling A (green)
Step 3: 72ºC, hold 30secs
HRM: Melt by 0.1ºC

Results

After successful optimization of our forward and reverse primers, we proceeded to use the same thermocycler conditions from our PCR reaction to use on the HRM Rotor-Gene amplification settings. Three different DNA sample controls were used and analyzed; a melt curve is shown below on (Image 2) with all three peaks expressed between 80ºC to 83ºC temperature range. The HRM Rotor-Gene was able to "call" all three genotypes at 100% confidence: the normalized graph shows Mutant (T/T), Heterozygous (A/T) and Wild-type (A/A) also shown on (Image 4) as a normalized graph with different color coding for each genotype. A total of 14 samples were duplicated and genotyped showing a 92.8% concordance within each other and a 7.2% error rate shown on Table 1. Sample #7 was incorrectly called by the HRM Rotor-gene as a mutant. However, Sanger sequencing confirmed sample #7 as a wild-type seen on (image 3). Table 2 shows the samples that were analyzed by Sanger sequencing methods to confirm the genotypes that were "called" by HRM. A blind test was performed on 6 known samples, and each one ran in triplicates for a total of 18 reactions. Each samples triplicate was above the 90% confidence intervals, except for one sample that was unable to be determined. So 17 out of the 18 samples were in agreement with each other.

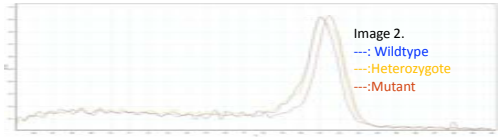

Image 2. Melt Curve Data

Qualitative Accuracy		
Samples	**Known Genotypes**	**HRM assay genotype**
1	Heterozygous (AT)	Heterozygous (AT)
2	Wild-type (AA)	Wild-type (AA)
3	Mutant (TT)	Mutant (TT)
B	Wild-type (AA)	Wild-type (AA)
JJ	Wild-type (AA)	Wild-type (AA)
CN	Wild-type (AA)	Wild-type (AA)
AG	Wild-type (AA)	Wild-type (AA)
JSB	Wild-type (AA)	Wild-type (AA)
JH	Wild-type (AA)	Wild-type (AA)
AH	Wild-type (AA)	Wild-type (AA)
4	Wild-type (AA)	Wild-type (AA)
5	Mutant (TT)	Mutant (TT)
6	Heterozygous (AT)	Heterozygous (AT)
7	Wild-type (AA)	Mutant (TT)
92.8% concordance (7.2% error rate)		

Sample name	HRM Genotype	HRM Confidence %	Sanger sequencing
CN	AA	99.55%	AA
JJ	AA	99.28%	AA
B	AA	99.04%	AA
AG	AA	93.78%	AA
JH	AA	96.16%	AA
JS	AA	99.20%	AA
AH	AA	94.34%	AA
Heterozygous CTRL	AT	96.58%	AT
Mutant CTRL	TT	99.17%	TT
#7	TT	Variation	AA

Table 2. Confirmation with Sanger Sequencing

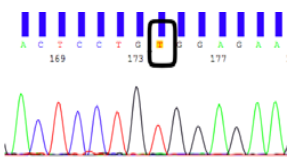

Image 3. Mutant CTRL confirmed with Sanger sequencing.

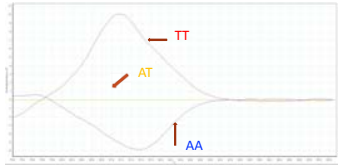

Image 4. Normalized graph of heterozygous genotype.

Discussion

During our research experiment on designing the HRM assay it was important to use the cycling conditions from our primer optimization, which played a major role for successful genotyping of unknown samples using the HRM assay. We noticed 25ng of DNA concentration was ideal for the experiment. The HRM analysis software settings were set to call genotypes ≥90% confidence. HRM is not able to call anything below the 90% confidence level. Normalization regions were set anywhere from 78.5ºC to 84ºC to accurately genotype our unknown DNA samples. The blind test was performed in order to measure precision, the results were all above 90% confidence levels except for one. All these parameters must be used for accurate results.

Conclusions

The Rotor Gene Qiagen assay was successfully optimized to confidently genotype SCD mutations as well as other genotypes. Some of the limitations that came across in the experiment included a short time frame, poorly isolated genomic DNA, and limited reagents. A few complications that arose included the inadequate amount of DNA put into each reaction which resulted in invalid results, we also experienced instrumentation malfunction causing loss of time and data which resulted in troubleshooting at times. For future experiments more samples need to be analyzed to increase the sample size, as well as the correct isolation method must be selected to acquire good quality DNA. The concentration of DNA must be between 25-50 ng, as well as the right amount of reagents purchased. Finally, the correct cycling conditions must be put into place to get the accurate results.

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