Supplemental material

Assay methods to detect single nucleotide variants

Currently, most diagnostic methods to detect single nucleotide variants are based on the polymerase chain reaction (PCR).[1] The method is based on *in vitro* amplification of DNA, by thermostable DNA polymerases, when provided with a DNA template and primers which can hybridise specifically to a target sequence within the template DNA. Using repeated cycles of denaturation, hybridization of primers to the template and elongation of the primers in a template-directed manner, DNA sequences can be amplified over a million fold with high specificity and fidelity. PCR products can subsequently be sized by agarose gel electrophoresis and visualized using a fluorescent dye that interacts non-specifically with double-stranded DNA.[2]

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

The most straightforward method for single nucleotide change detection, PCR-RFLP is based on PCR-amplification of a target region containing the variant site.[3] Primers are designed to flank the polymorphic site and positioned in such a way as to create two unequally sized fragments upon restriction endonuclease cleavage of the PCR product. After PCR, a portion of the reaction is subjected to restriction endonuclease digestion and gel electrophoresis to visualize the resultant bands representing the presence or absence of the polymorphic site. While a very simple method to implement, it is time-consuming requiring three successive steps and moreover is limited to the single nucleotide change in question creating or destroying a restriction endonuclease site.

Amplification refractory mutation system (ARMS)

The ARM system is a form of allele-specific PCR, where each allele of a potential multi-allelic locus is amplified by a set of outer non-specific as well as two sets of inner allele-specific primers[4] (Supplemental Figure 1, http://links.lww.com/AA/A690). The outer primers serve as a control for the PCR, while the inner primers provide two opportunities to detect the different alleles. This is important to guard against a false diagnosis through lack of amplication or non-specific amplification with one or other set of primers, which are common artifacts when using primers containing mismatches. The method is normally used to detect single nucleotide variants but can be adapted for other variants by appropriate primer design. It has advantages over the original PCR-RFLP method in that the diagnostic result is provided in only two steps (PCR followed by gel electrophoresis of the products) and it is not limited to the position of a restriction endonuclease site. On the other hand, as the position of the allele-specific primer is predetermined by the position of the genetic variant, it can be difficult to design primers that are 100% specific.

Kinetic PCR

The availability of thermal cyclers that can measure amplification in real time provides the diagnostic laboratory with versatility in assay method as well as a time-saving device. There is now a range of different chemistries available and that chosen will depend on assay throughput required, actual sequence surrounding the genetic variant, instrumentation available and budget.[5] Most standard methodology falls into two broad groups, non-specific dye-based and probe-based chemistries. While probe-based methods are attractive, potentially providing a greater specificity, they are more expensive and the assay design is more complex. Most probes have a

limited shelf life and are unstable to repeated freezing and thawing, therefore the diagnostic laboratory would need to bear the additional cost of annual probe resynthesis and assay optimization. Therefore probe-based assays can be impractical for DNA-based diagnosis of MH, where many different assays are required for only a very few individuals at any one time. For laboratories lacking experience in probe-based assays,[6] it can be beneficial to purchase custom assays from one of a variety of suppliers, although there are several different methods available and standard rules that can be applied to optimize assay design.[5, 7] Dye-based assays on the other hand rely on a non-specific intercalating agent or major or minor groove binder and standard unlabeled primers only.[8] These are a fraction of the cost of a labeled probe and have a long half-life if stored at -20^{o}C or -80^{o}C. They can also withstand repeated freezing and thawing so could be the method of choice for a diagnostic laboratory covering MH. Two commonly used probe-based assays are presented below, followed by a simple dye-based method, which has rapidly become the method of choice for the detection of single nucleotide variants.[9]

Hydrolysis probe-based single nucleotide variant assays

Hydrolysis probe allele-discrimination is based on the simple ARMS methodology except that both (or all) alleles are amplified in an allele-specific manner. The probes are each designed to detect only one specific allele. Normal PCR primers are included in the reaction and two probes for each variant. One probe, the quencher is the same for each allele, while a separate reporter probe must be designed for each allele to be detected (Supplemental Figure 2, http://links.lww.com/AA/A691). The detection method is based on detection of fluorescence emission upon probe hydrolysis. Reporter probes must bind, in an allele-specific manner, to the target DNA adjacent to the quencher probe. In this position the reporter probe fluorescence is

quenched. As the polymerase copies the template in the elongation step of the PCR the reporter probe will be hydrolysed and the fluorophore consequently released from its position next to quencher probe. Thus fluorescence emission will result and data acquisition to monitor the reaction occurs during the elongation step of PCR. The readout from hydrolysis probe methods makes use of end point genotyping software and is potentially clear-cut with the software automatically grouping genotypes into clusters. For a single nucleotide variant three potential clusters can result, one representing the heterozygote situation and two clusters for the homozygous alleles. Tight clustering is essential for unequivocal allele-discrimination. This will however, depend on the actual sequence around the generic variant and often ambiguities may arise because each allele-specific primer may be able to hybridise to some extent to either allele. Even with optimization of cycling conditions, inclusion of chemicals that disrupt secondary structure and the use of alternative polymerases, some sequences remain problematic.

Hybridisation probes

A hybridization probe assay[10] uses two amplification probes, anchor and sensor (Supplemental Figure 3, http://links.lww.com/AA/A692). Detection is based on FRET (fluorescence, or Förster, resonance energy transfer) and allele-discrimination is based on melting temperature of the amplicon synthesized.[11] Probes must be designed to bind at the locus surrounding the genetic variant with a distance of no more than 2 nucleotides between them. Data acquisition to monitor the reaction occurs during the annealing step of PCR, at which point both probes hybridise to the DNA template and FRET occurs. As the sensor probe is designed as a perfect match to the common allele, a mismatch will occur if the rare variant allele is present. Thus at the end of the PCR the sensor probe will melt from the variant sequence at a lower temperature than the

common allele. Genotype is determined from the resultant melting temperatures (Supplemental Figure 4, http://links.lww.com/AA/A693). This method has the advantage over hydrolysis probes in that one sensor probe is able to detect all alleles at the locus. The ability to generate amplicon melting curves at the end of the PCR is also very useful in trouble-shooting assay design and optimization.[12] Another feature of hybridization probe assays is that they can be used for serendipitous variant discovery close to the pathologic variant being tested. In addition it is possible to design a hybridization probe assay to detect several adjacent single nucleotide variants with only two probes. Other probe-based assays have been introduced[6, 13-16] and could be considered if the DNA sequence surrounding the genetic variant is intractable to the standard technology described above.

High resolution amplicon melting

High resolution amplicon melting (HRM)[17, 18] is the method of choice implemented for diagnosis of MH in my laboratory[8] and is based on the different melting properties of short fragments of homoduplex vs heteroduplex DNA, containing the pathologic single nucleotide variant. HRM offers a robust, rapid and relatively inexpensive method of allele discrimination. Amplification is monitored by the increase in fluorescence of a non-specific dye when bound to double-stranded DNA. PCR and allele-discrimination can be carried out separately, or in the same tube depending on the instrumentation available. Allele-discrimination is achieved by melting the amplicon at the end of the PCR and monitoring the decrease in fluorescence emission as the DNA becomes single-stranded. Analysis of amplicon melting behaviour is carried out by normalization of fluorescence intensity before and after the melting transitions and then presented either by difference plot (Supplemental Figure 5A, http://links.lww.com/AA/A694) or

by melting curve itself (Supplemental Figure 5B, http://links.lww.com/AA/A694).[19] Target specificity is determined by the primer design,[20] for which a number of software packages can be utilized. In brief, amplicons should be as short as possible (<100 bp) and primers with T_ms within 1 ^oC are required. HRM can also detect new single nucleotide variants serendipitously and several adjacent variants can be detected in a multiplex (the analysis of several variants simultaneously) assay.[21] Occasionally several variants can be discriminated with a single set of primers (Supplemental Figure 5, http://links.lww.com/AA/A694). HRM is relatively easy to optimize and as probes are not required a panel of primers can be quickly and inexpensively assessed for suitability. Some allele-combinations, in particular those involving A and T variants, can be difficult to discriminate because base pairing of DNA is accomplished with only two hydrogen bonds. In addition, if the polymorphic site is adjacent to a run of GC base pairs, the mismatch may be stabilized and thus allele-discrimination may be unsuccessful. These problems however, can be overcome by either creating extremely short amplicons or by use of an unlabeled probe.[8, 22] Most modern diagnostic laboratories implementing DNA testing would have at least one instrument capable of HRM assays and comparisons of instruments and dyes have been reported elsewhere.[23, 24]

High throughput methods

For more commonly occurring disorders with several different causative mutations, high throughput methods including single nucleotide variant microarrays,[25] multiplex ligation-dependent probe amplification[14] and real time PCR carried out in microfluidic platforms may be used.[26] These however have limited utility for MH, as in most cases, families present with only one mutation and due to the rarity of the disorder only a few samples might be analysed at one

time. Multiplex assays could be useful in a population where a few mutations are more prevalent than others. HRM assays can be multiplexed with careful assay design.[21] Probe-based assays are more amenable to multiplexing,[27] although the number of assays that can be detected simultaneously will depend upon the types of probes and the number of different wavelengths that can be detected by the instrument used.

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Supplemental Figure Legends

Figure One: ARMS assay illustrating primer design, potential PCR products generated and results measured by gel electrophoresis.

Common outer primers amplify a non-specific product as well as the two allele-specific products. This acts as an internal control for success of the PCR. Two sets of allele-specific primers are normally used, amplifying a potential polymorphic site in both directions. This provides internal quality control for specificity. In each case the allele-specific primer should not amplify in a non-specific manner. Additional specificity of allele-specific primers is normally optimized by the inclusion of a single mismatch (to both alleles being examined) in the penultimate position from the 3'end. The inclusion of this additional mismatch decreases the likelihood that the primer will anneal incorrectly.

Figure Two: Hydrolysis probe assay.

Detection of only one allele is represented in the figure. A similar set of probes would be designed for detection of the alternative allele. Forward and reverse primers are common to both assays and all primers and probes are mixed in a single reaction. The quencher is usually the same for both probes but the fluorophores must emit at different wavelengths for each allele and the choice of quencher and fluorophores will be determined by specific instrumentation. Fluorescence is detected at the end of the assay and only if the hydrolysis probe binds to the target and is hydrolysed by the exonuclease activity of the polymerase. Output is simply a graph showing fluorescence intensity for each fluorophore on X and Y axes, respectively. Three clusters are usually produced, two that represent homozygous alleles, and one representing the

heterozygote.

Figure Three: Hybridisation probe assay.

The sensor probe spans the variant site and is able to anneal to both alleles, thus only two probes are required. FRET (fluorescence or Förster resonance energy transfer) will occur if the two probes are able to anneal to their targets and the emitted fluorescence is detected at the annealing step of the PCR. At the end of the PCR amplification program, a melt program is carried out starting from ~40^{o}C (maximum fluorescence where FRET occurs) with continuous acquisition of fluorescence emission until the temperature reaches ~95^{o}C (minimum fluorescence when the probes have melted off the target and so FRET is at a minimum). Allele-discrimination occurs on the basis of a mismatch melting at a lower temperature than a perfect match (see figure 4).

Figure Four: Melt curve analysis for a hybridization probe assay to detect the *RYR1* c.14477C/T mutation.

Melting curve analysis for allele-discrimination using the Roche Lightcycler 480 with the Roche LC480 Genotyping master mix. The blue peak at ~52^{o}C represents the T allele (pathogenic) while the blue and red peaks at ~62^{o}C represent the C allele (non-pathogenic). The cyan line is the water blank. If a sample was homozygous for the T allele, the blue tracing would contain only one peak at ~52^{o}C. (Thus far, there have been no incidences of homozygous causative mutations in the New Zealand population.)

Figure Five: Output from HRM assays

Three *RYR1* mutations can be detected with one set of primers in this HRM assay using the Roche Lightcycler 480 with the Roche LC480 HRM master mix. As only one set of primers is used for the assay, all non-pathogenic variants at the three positions are represented by the blue tracings as there is no DNA sequence difference between them. A. Normalized and Temperature-shifted difference plot. B. Melting peaks can also be generated as an additional check on allele-discrimination. Heterozygotes normally appear as a shoulder on a broader temperature-shifted peak compared to the homozygous state.