

Genotyping on the Tm/Luminex Universal Array Platform Using Primer Extension Chemistry

Flexible • Specific • Robust

Introduction

Single nucleotide polymorphisms (SNPs) are single base variations in genomic DNA. SNPs are the most prevalent form of genetic variation and are believed to occur, on average, once in every 1000 basepairs in humans. Analysis of these variations in DNA sequence will provide a better understanding of the genetic basis of disease and will allow for the implementation of individualized medicine which takes into consideration the genetic variations underlying an individual's susceptibility to disease as well as response to various drugs.

Current assays used for SNP analysis include Restriction Fragment Length Polymorphism (RFLP) analysis and Single Strand Conformation Polymorphism (SSCP) analysis. However, to analyze large numbers of SNPs from a large number of samples, array-based methods capable of analyzing multiple analytes in a single reaction are required. The drawbacks associated with array-based testing which include high cost, lack of standardization, lack of reproducibility and lack of flexibility are being addressed. Specifically, the introduction of the Tm100 Universal Sequence Set (described in Technical Bulletin 401) has allowed for the rapid development of highly multiplexed genotyping assays requiring minimal assay-specific optimization. This technical bulletin describes the application of a subset of the Tm100 Universal Sequence Set used in combination with primer extension chemistry to genotype a randomly selected set of 10 SNPs on the Luminex LabMAP™ system.

Primer Extension Chemistry

Primer extension chemistry is a simple, robust method for analyzing multiple SNPs in a single tube. The method employs a PCR-derived target DNA containing the SNP together with 2 universally-tagged allele-specific primers whose 3' ends define the alleles. A thermophilic DNA polymerase is used for label incorporation into extended products. Because the two tagged allele-specific primers **overlap** the SNP site in the target DNA, only the correctly hybridized primer(s) will be extended to generate a labelled product(s). A non-complementary primer will not be extended or labelled due to the 3' mismatched base. The use of a DNA-modifying enzyme such as DNA polymerase improves SNP discrimination due to the high sequence specificity of the enzyme compared to the specificity attained simply by allele-specific oligo hybridization which is governed mainly by sequence context and reaction conditions.

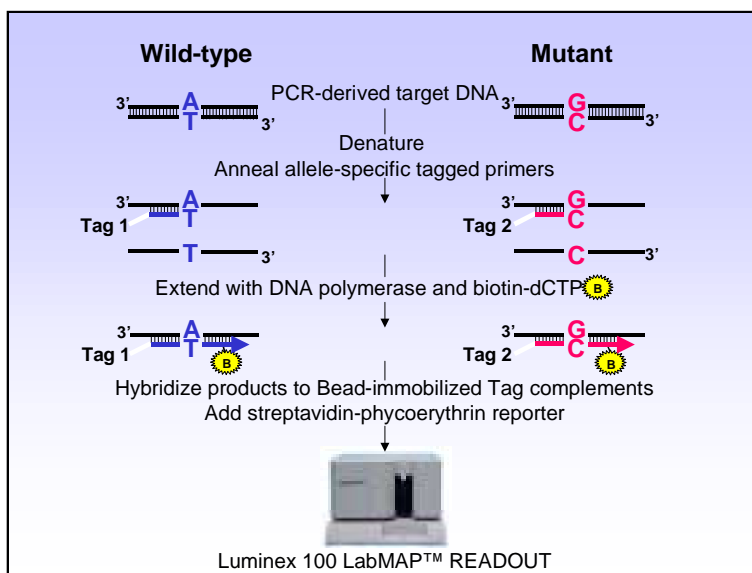


Figure 1: A typical primer extension reaction.

the correctly hybridized primer(s) will be extended to generate a labelled product(s). A non-complementary primer will not be extended or labelled due to the 3' mismatched base. The use of a DNA-modifying enzyme such as DNA polymerase improves SNP discrimination due to the high sequence specificity of the enzyme compared to the specificity attained simply by allele-specific oligo hybridization which is governed mainly by sequence context and reaction conditions. In the application described here, tagged, extended products labelled with biotin are detected using the **Tm/Luminex Universal Array platform**. The products, which have been 5'-tagged with 24mer sequences selected from

the Tm100 Universal Sequence Set, are captured by their tag complements which have been chemically coupled to spectrally addressable polystyrene microspheres or beads (Luminex Corporation, Austin TX). A fluorescent reporter molecule (streptavidin-phycoerythrin) is used to indirectly detect incorporated biotin. **Figure 1** outlines the main steps in a typical primer extension reaction.

Experimental Approach

To demonstrate the exquisite sorting capabilities of the **Tm/Luminex Universal Array platform** used in combination with primer extension chemistry, 10 SNPs (ie. 20 allelic sequences) representing a variety of clinically-relevant mutations were individually amplified by PCR and then subjected to primer extension reactions using universally-tagged, allele-specific primers. Each allele was tested individually against the pool of all 20 tagged primers in order to demonstrate primer/tag specificity, as well as part of a pooled mixture of all 20 alleles to demonstrate the multiplexing capabilities of the method (**Figure 2**). A streptavidin-phycoerythrin conjugate was the reporter used to measure signal generated by a hybridization event. Additionally, a blind study was conducted whereby 5 random combinations consisting of subsets of the 20 alleles were prepared and then analyzed using primer extension chemistry on the **Tm/Luminex Universal Array platform**.

Results

The ability of primer extension chemistry to accurately sort a pooled mixture of 20 different alleles using the **Tm/Luminex Universal Array platform** was demonstrated. The results clearly indicated that all 20 alleles were easily detectable over background when analyzed as a multiplexed reaction (data not shown). **Figure 2** illustrates that, when each allele is individually analyzed using primer extension in the presence of a pooled mixture of the 20 tagged primers and hybridized to bead-immobilized universal tag complements, cross-hybridization reactions between non-complementary sequences

are not observed, demonstrating the high specificity of the universally-tagged primers used. Five random mixtures tested in

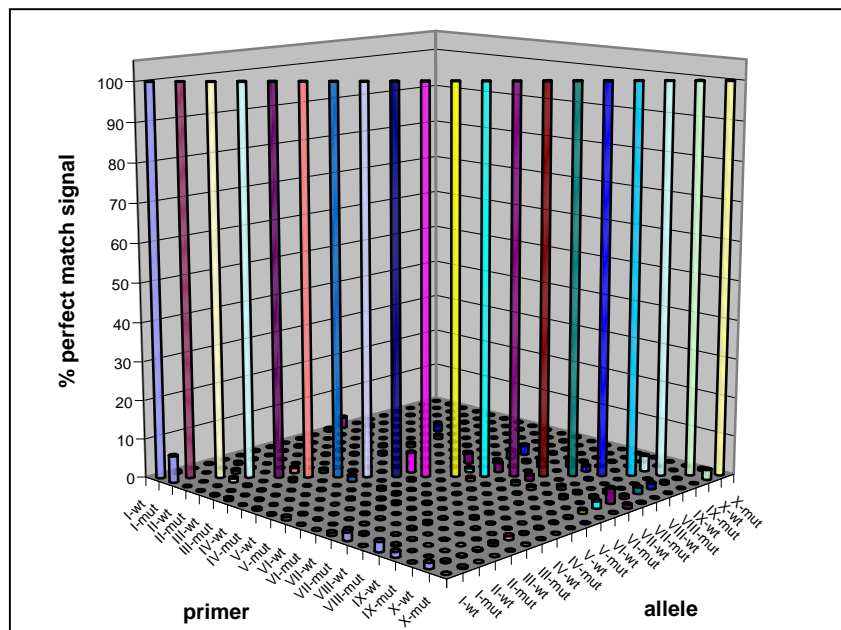


Figure 2: Results of primer extension reactions performed on each individual allele in the presence of multiplexed primers. Non-specific cross-hybridization reactions for both allele-specific primer and universal tag are negligible when analyzed on the **Tm/Luminex Universal Array platform**.

the blind study were genotyped with 100% accuracy. The results of one blind mix containing 18 of the 20 alleles are shown in **Figure 3**. The signals generated for each allele are divided by the sum of the signals for both alleles of a particular SNP to generate the allelic ratio. Based on results repeatedly generated in-house, arbitrary cut-offs were set. To be homozygous for a particular allele, the allelic ratio must be at least 0.75. To be heterozygous, each allele of the SNP must have a ratio of between 0.25 and 0.75. Consequently, an allele with a ratio of 0.25 or less is considered negative (ie. not present). As indicated in **Figure 3**, the sample in question is homozygous wildtype for SNPs I and V, homozygous mutant for SNPs IV and VIII and heterozygous for SNPs II, III, VI, VII and IX.

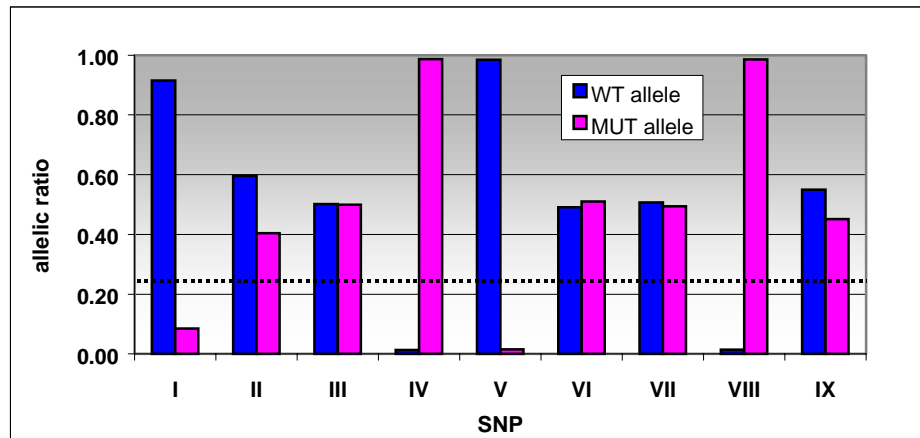


Figure 3: Results of a blind study sample genotyped on the **Tm/Luminex Universal Array platform**. An allele must have a ratio of at least 0.25 (dotted line) to be considered positive (ie. present).

Conclusions

Our results clearly demonstrate the high level of specificity attainable in a multiplexed system that combines primer extension chemistry with the **Tm/Luminex Universal Array platform**. While only 20 Universal Sequences were used here, up to 100 are currently available for a variety of applications. The entire Tm100 Universal Sequence set has been validated and has proven to be an indispensable tool, providing an optimized 'universal' starting point for the development of any multiplexed DNA-based test, whether used in combination with primer extension chemistry or other tag-incorporating chemistries. The assay described here combines **high specificity and accuracy** with **high multiplexing capabilities**. Primer extension chemistry coupled to the **Tm/Luminex Universal Array platform** will form an integral part of today's rapidly evolving genomics field where flexible, automatable, cost-effective methods are always in demand.

Technology Status

Multiple patents describing Tm's Universal Sequence Sets and their uses have been filed. For additional information on the **Tm100 Universal Sequence Set** or the **Tm/Luminex Universal Array platform**, please contact Dr. Jeremy Bridge Cook, Vice-President, Business Development at 416-593-4323 ext. 229.



Putting the human genome to work™

Tm Bioscience Corporation
439 University Avenue, Suite 1710,
Toronto, Ontario, Canada M5G 1Y8
Tel: (416) 593-4323 Fax: (416) 593-1066
www.tmbioscience.com