MASSEY UNIVERSITY
COLLEGE OF SCIENCES
TE WĀHANGA PŪTAIAO

MASSEY GENOME SERVICE

Sanger Sequencing and Genotyping using Applied Biosystems™ 3730 and 3500xL Genetic Analyzer

SEQUENCING TECHNICAL BULLETIN
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TECHNICAL BULLETIN

Experimental Design Considerations

Fluorescent labelling enables analysis of many independent loci in the same capillary injection, using colour and size to distinguish between fragments. Below is a list of factors to consider when designing an experiment.

If the sizes of different fragments overlap, then one of the following can be done to differentiate between them:

- Label overlapping products with different dye colours
- Choose new primer sites to alter the PCR-product fragment lengths
- Load overlapping products in different capillary injections

Primer Labelling

For genotyping fragment analysis customers should use 5'-end labelled primers. MGS uses the G5 filter set for running which incorporates a fifth dye dedicated to the size standard. Customers can use the following 4 fluorescent dyes to label their primers with:

- 6-FAM™ - Blue
- VIC® or HEX™ - Green
- NED™ - Yellow
- PET™ - Red

GeneScan™ - 500 LIZ™ Size Standard, is used as the internal standard for fragment sizing. **MGS provides this standard.** This size standard will accurately size fragments between 50-500bp, and provides 16 single-stranded labelled fragments of 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, and 500 bases. Each of the DNA fragments is labelled with a proprietary fluorophore, which results in a single peak when run under denaturing or native conditions.

When developing your primers, please design them to produce fragments >75bp and <490bp. When sizing fragments the algorithm with the ABI Prism® GeneMapper™ Software looks for two size markers smaller and larger than the fragment being sized, in order to get an accurate size calling. The 35bp size standard fragment can sometimes be inaccurate because the unincorporated primer peaks obscure its detection by the software.

The 250bp fragment peak in the GeneScan™ - 500 LIZ™ Size Standard, is not used in fragment sizing under denaturing conditions, due to the abnormal migration of double strands that did not completely separate under these conditions. The 250bp fragment peak frequently runs at 246bp, so it is not included in size calling. So there is a gap of 100bp between the 200bp and 300bp size standard peaks when sizing fragments in this size range.

Other size standards can be used. Please contact the MGS for information.

Multiplexing to Increase Throughput

To maximize throughput, multiplex electrophoresis by co-loading the products of multiple PCR reactions in the same capillary injection. It is also possible to multiplex the PCR.

Co-electrophoresis

- Use different dye labels for PCR reactions with overlapping product sizes, and use greater dye concentrations in PCR reactions with dyes of low emission intensity than dyes of high emission intensity.
- The intensity of emitted fluorescence is different for each dye:
    - 6-FAM > NED > HEX > PET > LIZ

Multiplexing PCR

- It is possible to multiplex more than one pair of primers in the same PCR reaction. Do not multiplex primers labelled with the same fluorescent dye for loci with overlapping allele size ranges. If the extent of the allele size ranges is unknown, leave at least 15-20bp between the known size ranges.
- Perform a check for primer compatibility. Avoid excessive regions of complementary among the primers and choose primer pairs with similar melting temperatures. After identifying compatible primer pairs, test the pairs for successful co-amplification. The reaction conditions will need to be optimised and there may be a need to redesign the primers.

3' A Nucleotide Addition

Many DNA polymerases catalyse the addition of a single nucleotide (usually an adenosine) to the 3' ends of the two strands of a double-stranded DNA fragment. This non-template complementary addition results in a denatured PCR product that is one nucleotide longer than the target sequence. A PCR product containing the extra nucleotide is referred to as the "plus-A" form.

Because 3' A nucleotide addition does not always go the completion without a long extension step at the end of thermal cycling, then only a fraction of the fragments contain the extra nucleotide. Due to this single-base ladders often form, creating peak patterns that the software might not interpret correctly. The resulting allele calls can be inconsistent, incorrect, or missing entirely, forcing inspection of all allele calls and to correct erroneous calls manually.

To avoid problems caused by incomplete 3' A nucleotide addition:

- Alter the thermal cycling conditions to either promote or inhibit 3' A nucleotide addition
    - Increasing the time spent between 60 & 720C promotes 3' A nucleotide addition
    - Decreasing the time spent between 60 & 720C inhibits 3' A nucleotide addition

Promoting 3' A nucleotide addition has shown to be the most effective method. Increase the final Extension step to 30-45 minutes to promote 3' A nucleotide addition.

- Alter the Mg^{2}+ concentration to either promote or inhibit 3' A nucleotide addition
    - Increasing the Mg^{2}+ concentration promotes 3' A nucleotide addition
    - Decreasing the Mg^{2}+ concentration inhibits 3' A nucleotide addition

Optimising the Mg^{2}+ concentration is best used in addition to other strategies.

- Reverse-Primer tailing: "Tail" the 5' end of the reverse primer to either promote or inhibit 3' A nucleotide addition to the forward labelled strand (Brownstein et al. 1996). Adding a single G to the 5' end of the reverse primer, can result in almost complete 3' A nucleotide addition (Magnuson et al. 1996). Reverse-primer tailing is advantageous to other methods, as it does not require additional experimental steps & works well in diverse reaction conditions.
- Enzymatic treatment of the PCR products to remove the 3' A overhangs. You can use T4 DNA polymerase to remove the 3' A overhangs from your pooled PCR products (Ginot et al. 1996). This method has its limitations as it requires post-PCR enzymatic treatment, and titration of T4 DNA polymerase to optimize enzyme concentrations and treatment times.

The most effective procedure is to maximize 3' A nucleotide addition by modifying the thermal cycling conditions, Mg^{2+} concentration and tailing the reverse primer if required.

Stutter Products

During PCR amplification of di-, tri-, and tetra-nucleotide microsatellite loci, minor products that are 1-4 repeat units shorter than the main allele are produced. The minor product peaks are referred to as "stutter" peaks. Stutter peaks might be caused by polymerase slippage during elongation.

The longer the length of the repeat unit, the less stutter product made. Among microsatellite loci with the same number of repeat units, the percentage stutter is greater for di-nucleotide microsatellite loci than for tri-nucleotide microsatellite loci, and so on (*Walsh et al.* 1996).

The percentage of stutter increases with increasing number of repeat units. This rule may not apply if some repeats are partial repeats and are not of perfect length.

Primer Design Recommendations

The following recommendations are provided to help optimize primer selection:

- Avoid >2 GC's in last 5 nucleotides at 3' end of primer
- Primers should be at least 18 bases long to ensure good hybridization
- Avoid runs of an identical nucleotide, especially runs of four or more G's.
- Keep the G-C content in the range 30-80%, preferably 50-55%. For cycle sequencing, primers with T_M>45°C produce better results than primers with lower T_M
- For primers with G-C content less than 50%, it may be necessary to extend the primer sequence beyond 18 bases to keep the T_M>45°C
- Use of primers longer than 18 bases also minimizes the chances of having a secondary hybridization site on the target DNA
- Avoid primers that hybridize to form dimers
- Avoid palindromes because they can form secondary structures
- The primer should be as pure as possible, preferably purified by HPLC
- To ensure specificity of primer (BLAST search) to the target
- Dissolve primer stocks in 10mM TE buffer, pH8.0, but dilute working primer solutions in water as salt can affect primer extension

References

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