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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BULLETIN INCLUDES

PCR Amplification
Primer Labelling
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Preparation of PCR Products

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MASSEY GENOME SERVICE (MGS)

Genotyping using Applied Biosystems™ 3730 and 3500xL Genetic Analyzer
MICROSATELLITE FRAGMENT ANALYSIS TECHNICAL BULLETIN

Microsatellite markers or short tandem repeat (STR) markers are polymorphic DNA loci that contain a repeated nucleotide sequence. The repeat can be from 2-7 nucleotides in length. The number of nucleotides per repeat unit, are the same for the majority of repeats within a microsatellite locus. Microsatellite analysis involves PCR amplifying the microsatellite loci, use of fluorescently labelled primers, and analysis of the PCR products by electrophoresis to separate the alleles according to size.

The number of repeat units at a microsatellite locus may differ, so alleles of many different lengths are possible. Microsatellite loci are used as markers to establish linkage groups in crosses and to map genetically identified mutations to chromosomal positions.

PCR Amplification

The following table is a guideline for setting up your microsatellite PCR amplification reactions:

Reaction Component	Volume (µl) (15-µl reaction)	Volume (µl) (7.5-µl reaction)
DNA	1.20 (50ng/µl stock)	1.20 (25ng/µl stock)
PCR Primer Mix (5µM each Primer)	1.00	0.50
PCR Buffer	1.50	0.75
dNTP Mix (250µM each dNTP)	1.50	0.75
DNA Polymerase (5units/µl)	0.12	0.06
MgCl2 (25mM)	1.50	0.75
Distilled, deionised H2O	8.18	3.49

To avoid inaccuracies when pipetting small volumes:

- Combine all reaction components except the sample DNA in a PCR Master Mix.
- Use the above ratios and prepare sufficient mix for at least one extra reaction volume.
- Aliquot 13.8µl of the mix into each 15µl reaction or 6.3µl of the mix into each 7.5µl reaction.
- The PCR Master Mix should last for 1-2 weeks at 2-6°C.
- Lastly add the DNA sample to the Master Mix.

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 ABI Prism® 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 **(NOTE: MGS provides this standard).** This standard accurately sizes fragments between 50-500bp, providing 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 DNA fragment is labelled with a proprietary fluorophore, which results in a single peak when run under denaturing or native conditions.

When developing primers, please design them to produce fragments >75bp and <490bp. The Life Technologies GeneMapper™ Software looks for two size markers smaller and larger than the fragment being sized to get an accurate size calling. The 35bp size standard fragment can sometimes be inaccurate due to un-incorporated primer peaks obscuring 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, and is therefore not included in size calling. Other size standards can be used. Please contact MGS for information.

Thermal Cycling Parameters

The following is a list of recommended thermal cycling times and temperatures when using the GeneAmp® PCR System 2400, 9600, or 9700, for both 15ul and 7.5ul reactions. If other thermal cyclers are used these parameters may have to be adjusted for optimization. **NOTE**: Always use 5'-end labelled primers, as they are able to detect small mobility differences, reproducible sizing and sharp peaks.

Initial Incubation Step	Each of 10 Cycles	Each of 20 Cycles	Final Extension	Final Step
HOLD – 95°C for 12 minutes	MELT - 94°C for 15 seconds	MELT - 89°C for 15 seconds	HOLD - 72°C for 10 minutes	HOLD - 4°C forever
	ANNEAL - 55°C for 15 seconds	ANNEAL - 55°C for 15 seconds		
	EXTEND - 72°C for 15 seconds	EXTEND - 72°C for 15 seconds		

Control DNA

Always include control DNA in every round of PCR amplification and run at least one injection of amplified control DNA for every set of microsatellite markers used. The control DNA should fulfil the following criteria:

- The control DNA should come from a single individual with a known genotype
- The DNA should be in sufficient condition to act as a positive control for PCR amplification and it is a useful troubleshooting tool.
    - If sample DNA amplifies poorly, but the control DNA amplifies; it could be a sample DNA problem
    - If sample DNA **and** control DNA amplifies poorly; it could be problems with reagents, the instrument, or protocols
- The control DNA serves as a sizing reference for monitoring injection-to-injection and capillary-to-capillary variation. Since the control DNA is not used to calculate the sizing curve, the size obtained for the control DNA across capillary injections will alert potential problems with sizing precision.
- Control DNA should facilitate allele binning. Allele binning is a statistical method for converting peak sizes to alleles.

Preparation of PCR Products - Pooling Ratios

It is likely PCR products will be pooled into one capillary injection in order to maximize throughput. Therefore, it is important to pool PCR products together at the correct ratios to get similar fluorescent intensities across all loci in the pooling. The fluorescent dyes are detected with different efficiencies. The pooling ratio, or amount of each dye-labelled product added with to the other products in the pool, should be adjusted proportionally to ensure an appropriate detection of all the loci.

Dilutions

To optimise for a set of loci, follow the recommended procedure below, especially when performing microsatellite analysis for the first time:

- Determine which primer pairs are to be pooled into one capillary injection. Please make sure that overlapping loci are amplified with primers labelled with different dyes. Refer to the Massey Genome Service website at http://genome.massey.ac.nz in the '**ABI Genotyping Service Information**' section for more information
- Set up a combination of pooling ratios to determine which ratio is going to give you similar fluorescent intensities across all loci in the pooling
- Carry out a dilution series on each pooling ratio to determine the optimal fluorescent intensity that will give clean sharp peaks and correct banding patterns, allowing for accurate allele calling. Dilute PCR amplification products before adding them to the sample tube & send them to the MGS for capillary separation on Applied Biosystems™ 3730 and 3500xL Genetic Analyzer. Typically, the required dilution lies in the range from 1:3-1:80 (PCR product: distilled, deionised H_2O). **NOTE**: Aim to get peaks between 500-20,000 fu (fluorescent units) to ensure data is in scale
- Send 1µl of each dilution series to the MGS for capillary separation and sizing, in 1.5ml or 0.5ml eppendorf tubes for the 'Capillary Separation Only Service', and 1µl of each dilution series in each well for the 'Plate Service'.
- After determining the optimal pooling and dilution ratio for a set of primers, use the same dilutions for subsequent analyses as PCR yields should be fairly consistent.