SERVICE GUIDE

Fragment Separation
Fragment Analysis
Microsatellite

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SVG2206FSA

Service Guide:
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1.0 Overview

Our Genotpying Team has over 20 years' experience providing custom microsatellites, so we know that every project is unique – which is why we have a flexible approach that will accommodate your research goal and offer flexible solutions. We have service options available, including a full custom microsatellite service, fragment analysis and fragment separation.

1.1 Full Service - Custom Microsatellites

AGRF offers a full service option, which includes assistance in designing and ordering fluorescently-labelled primer pairs for the microsatellite markers of your choice. In cases where multiple markers are to be genotyped, this can be done in a cost-effective manner where AGRF can panel markers for multi-loading for capillary separation. Genotyping data is analysed using Applied Biosystems (AB) GeneMapper software for the calling of alleles. This service has been applied to a wide variety of species and can accommodate any species for which microsatellite markers are available. Microsatellites, due to their highly informative nature, provide a powerful tool to any linkage mapping or population genetics study.

Zero or limited microsatellite markers exist for your species of interest? AGRF can assist with microsatellite discovery by using Next Generation Sequencing. A genome can be scanned at reasonably low coverage and still provide microsatellite sequence information allowing for marker design. For more information on this service, please contact us.

1.2 Fragment Analysis

Our Fragment Analysis service provides both capillary separation and analysis of client-prepared, fluorescently-labelled DNA fragments. Capillary electrophoresis is used for fragment separation and data is analysed using AB GeneMapper software.

1.3 Fragment Separation

Our Fragment Separation service provides capillary separation of client-prepared fluorescently-labelled DNA fragments. No analysis is performed, and raw data is returned for client analysis and interpretation.

Applications for these services include:

- Microsatellite analysis
- MLPA (multiplex ligation-dependent probe amplification)
- AFLP (amplified fragment length polymorphisms)
- T-RFLP (terminal restriction fragment length polymorphisms)
- Cell Line Authentication
- Custom analyses

2.0 Technical Information

Capillary electrophoresis is performed on the Applied Biosystems 3730, with a 50cm array and POP-7 polymer. We recommend markers are labelled with the fluorescent dyes FAM, VIC, PET and NED (with LIZ reserved for the size standard). Fluorescent primer pairs labelled with VIC, PET and NED dyes are proprietary to Applied Biosystems. AGRF can order these primers from provided sequence. Please contact us if you wish AGRF to order primers. Alternatively, labelled primers from alternate suppliers compatible with the DS33/G5 matrix can be used. Please contact us if you have any questions regarding your fluorescent dyes.

We can assist with the design of microsatellite projects. Primer design can be performed from reference papers, supplied marker names with allele size information, or simply the species name of interest. AGRF can assist with panel design and the choice of fluorescent label to obtain the maximum efficiency for multi-loading of products for electrophoresis. The basic principle is: do not multi-load or multiplex primers with similar product lengths which are labelled with the same dye-labelled primers, as it will result in markers with overlapping allele size ranges.

AGRF uses the LIZ500 and LIZ1200 size standards. The LIZ600 size standard is available upon request. For the submission of Promega kit products, the client must supply the size standard, and allelic ladder.

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3.0 Submission Types and DNA Requirements

3.1 Full Service - Custom Microsatellites

The service includes PCR, electrophoresis, and genotype analysis of each sample. A repeat round is performed if a sample fails for a given marker in the first round. As the service is custom, the amount of DNA is dependent on the number of markers you wish to screen. Please contact us for the DNA requirements for your project.

When submitting samples, please ensure quality and quantity of DNA by at least measuring concentration and 260/280nm absorbance using a spectrophotometer (e.g. Nanodrop); 260/280 reading between 1.8–2.0 is recommended. High quality genomic DNA can also be determined using fluorescence-based quantitation (PicoGreen or similar) or agarose gel electrophoresis. A high-quality sample will produce a high molecular weight band using agarose electrophoresis. A sample submission receipt generated through our online portal must accompany each sample submission.

Samples can be submitted in 1.5mL tubes or 96-well V-bottom, plates. For samples submitted in full 96-well plates please leave well-position A01 empty for the internal control sample and label your plate with your contract ID and name.

3.2 Fragment Analysis

This service requires the client to perform the PCR reaction and submit an aliquot of diluted, labelled DNA reaction products. Submit 10ul PCR product in 1.5mL tubes or 96-well V-bottom plates. AGRF will add the HiDi formamide and size standard, perform the capillary separation on the 3730 and the allele calling in GeneMapper software. A sample submission receipt generated through our online portal must accompany each sample submission.

Samples can be submitted in 1.5mL tubes or 96-well V-bottom plates. For plate submissions, please label your plate with your contract or agreement ID and submission name.

3.3 Fragment Separation

This service requires the client to perform the PCR reaction and submit an aliquot of diluted, labelled DNA reaction products. Submit 10ul PCR product in 1.5mL tubes or 96-well V-bottom plates. AGRF will add the HiDi formamide and size standard and perform the capillary separation on the 3730. A sample submission receipt generated through our online portal must accompany each sample submission.

Samples can be submitted in 1.5mL tubes or 96-well V-bottom, plates. For plate submissions, please label your plate with your contract or agreement ID and submission name.

4.0 Sample and Data Storage

DNA samples are stored with AGRF for 3 months after you receive your data. If you require your samples to be returned to you post-processing, please let your Account Manager know at the time of quoting. Please note that a fee will be charged for return of samples. At the completion of processing, we will return your samples by post, if requested, at ambient temperature. If required, we can return samples using Dry Ice, but this will incur a $160 minimum (for metropolitan areas) charge. Samples submitted as PCR products will be discarded 2 weeks after you receive your data.

AGRF maintains an archive of client data, however cilents are advised to back-up their data. Data will remain available for download for one month, prior to archive. If you require past data, please contact AGRF. Please note that charges will apply for restoring files to the server for data older than six months old.

5.0 Shipping

- DNA samples or PCR products must be shipped to AGRF in tubes or 96 well V-bottom plates and be clearly labelled and sealed.
- Samples can be shipped at room temperature via express post or courier, or shipped on icepack or dry ice via courier.
- Fluorescently-labelled PCR products are light sensitive. Please take this into account when packaging and submitting samples.

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- To prevent leakage in transit please use parafilm to seal tubes, and ensure plates are heat-sealed or sealed with strip caps.
- AGRF can organise dry ice shipment for your samples, at a cost to the client. Please contact us for further details.

Physical address (courier):
AGRF
VCCC LOADING DOCK*
14 FLEMINGTON ROAD
NORTH MELBOURNE VIC 3051

Postal address (mail):
AGRF
LEVEL 13, VICTORIAN COMPREHENSIVE CANCER CENTRE
305 GRATTAN STREET
MELBOURNE VIC 3000

6.0 Sample Submission

Online Submission

- Submit your sample details online.
  - **Fragment Separation**: select: "Genotyping - Fragment Separation - Node" as the Service Type, then select which type of Fragment Separation you want from the Service drop down list.
  - **Microsatellite**: Select "Fine Mapping and Custom Genotyping" as the Service Type.
- Submission Format – by selecting tube or plate, the "Sample File" template link will appear. Click "Download Template" and enter your sample details:
  - Sample Name can be up to 40 characters in length.
  - You can use numbers, letters, underscores and hyphens.
  - The first character must be a letter or number.
  - For example: **Sample-1_b**
- Save and close completed Template File locally, select "Browse" to upload file.
- Submit and print a paper copy of your sample submission receipt that will be generated as a PDF file. **This must be included with your sample package.**

7.0 Turnaround Time

Fragment Separation		3 business days	from receipt of submission
Genotyping Fragment Analysis		5 business days	from receipt of submission
Microsatellite Custom	<10 markers	3 weeks	from receipt of primers
	>10 markers	5 weeks	from receipt of primers
Fluorescently-Labelled Primer Pairs		3 weeks	from confirmation of primer sequence

8.0 Data Output

8.1 Full Service - Custom Microsatellites

The service includes electrophoresis and allele calling using GeneMapper software. Final data is supplied in tabulated format (.xls) for client interpretation. The MS Excel file contains allele size (in base pairs) and bin name (allele category). Peak height and area can be supplied upon request.

8.2 Fragment Analysis

The service includes electrophoresis and allele calling using GeneMapper software. Final data is supplied in tabulated format (.xls) for client interpretation. The excel file contains allele size (in base pairs) and bin name (allele category), peak height and area. A .pdf copy of the electropherogram files and run files in .fsa file are also supplied.

8.3 Fragment Separation

The service includes electrophoresis of all supplied samples. The data file in .fsa format is supplied for client analysis. To analyse and view the data, GeneMapper or Peak Scanner (free) software is available from Life Technologies.

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9.0 Technical Considerations

9.1 Sizing

When designing your primers, ensure that the fragments produced are >75 bp and <490bp (for LIZ500), <580bp (for LIZ600) and <1,160 for LIZ1200. It is important there are at least two size standard fragments larger than your largest unknown fragment for the sizing method in the analysis software to accurately size the product. For the size standard assignment of LIZ500 the 250-bp peak results from abnormal migration of double strands that do not separate completely under denaturing conditions. Do not use this peak to size samples. This peak shows variably smaller values than the actual size of the fragments. This is a known issue with this size standard.

PCR products which are high in fluorescent intensity may produce off-scale data which may affect the analysis software's ability to automatically assign the correct sizing to the size standard. In these cases, the size standard peak sizes may need to be manually edited in the software.

9.2 Multiplexing and Multi-loading PCR Products

Using the 3730 and the analysis in GeneMapper software, you can label different DNA fragments with up to four different coloured fluorescent dyes (6-FAM, VIC, NED or PET). To increase throughput, and reduce costs, you may choose to multiplex electrophoresis by multi-loading products of multiple PCR reactions in the same capillary for electrophoresis or depending upon your project, you may choose to multiplex the PCR.

9.3 Multi-loading PCR Products

Pooling PCR products? It is important to pool PCR products at the correct ratios in order to get similar fluorescent intensities across all fragments within the pool. The fluorescent dyes are detected with different efficiencies, therefore the amount of each dye-labelled product in the pool will require adjustment to ensure even detection.

To multi-load PCR products use a combination of dyes from our recommended supplier, Applied Biosystems (6-FAM, VIC, NED or PET), or an alternative supplier. Please note, VIC, NED and PET are proprietary to Applied Biosystems and LIZ or ROX is reserved for our internal standard. Use different dye labels for PCR reactions with overlapping product size. Pool PCR products in order to get similar fluorescent intensities for all products. The intensity of emitted fluorescence is different for each dye. In our experience FAM has the highest intensity, coupled with VIC and PET the lowest (6-FAM, VIC >NED > PET). Use a greater amount of PCR product labelled with dyes of low emission intensity than those labelled with dyes of high emission intensity.

9.4 Multiplexing PCR

Multiplexing PCR involves the PCR of more than one primer pair in a single PCR reaction tube. Do not multiplex primers with similar product lengths labelled with similar dyes. For microsatellite applications, do not multiplex same dye-labelled primers for loci with overlapping allele size ranges. For development of a multiplex the compatibility between the primers and co-amplification success between primer pairs will need to be assessed. It is recommended this is performed on a small batch of samples prior to submission to AGRF.

9.5 Optimisation and PCR amplification of submitted PCR products

It is the responsibility of the customer to optimise the concentration of reaction products in the sample you provide AGRF for electrophoresis. Most submissions will require dilution of the amplified PCR product(s) before sending to AGRF for electrophoresis. Typically, the dilution required is within the range of 1:2 – 1:100 of PCR product to water.

For first submissions, AGRF can assist in the optimisation and provide assistance in determining the dilution factor. After determining the optimal dilution ratio, you can then use the same dilution for subsequent submissions, as PCR yields should be relatively consistent.

9.6 Fluorescent intensity of PCR products

For optimal results, the fluorescent signal should be between 150 – 15,000 relative fluorescence units (RFU) and should not exceed 30,000 RFU. Above this the instrument cannot measure the true value of the signal and therefore cannot apply the matrix correctly. This results in artefact "pull-up" peaks that can appear in other colour spectrums. Artefact peaks can inhibit both the automated size calling of the size standard and the

analysis of multi-loaded samples. The intense peaks can often appear as off-scale data.

9.7 Allele Calling and Sizing

Calling and sizing of microsatellite alleles requires interpretation of the locus-specific allelic pattern. The more samples that are screened, the easier interpretation becomes.

Most commonly a PCR-amplified microsatellite allele is not a single band, but a ladder of bands. The most intense band usually is observed at the expected size of the allele. The additional bands are usually smaller than the original band and differ in length by multiple repeat units; these are called stutters. Most likely, stutters are the result of in vitro DNA slippage during PCR amplification. Di-nucleotide repeats are more likely to exhibit stutters compared to tri- and tetra-nucleotide repeats.

Depending on the polymerase and the primers, microsatellites may show an additional band, often smaller in intensity, after the expected allele. This is known as an A+ peak. This is caused by the terminal transferase activity of the DNA polymerase that may add an A to the PCR product.

9.8 Custom Microsatellite Primer Pairs

For Custom Microsatellite primer pairs, AGRF recommends pig-tailing primers. This is the addition of a seven base pair sequence (GTTTCTT) to the 5' end of the reverse primer. The pig-tail reduces slippage during PCR and assists in minimizing A+ing of peaks and reduces the variation in the stutter peaks, especially in di-nucleotide repeats. Reduction of the A+ing assists in genotyping the samples.

For the design of new custom microsatellite primer pairs, for projects with >12 primer pairs, if you do not know whether the markers will be polymorphic and usable for your given sample set, a cost-effective ordering method can be M13 primers, as opposed to ordering primers with a direct labelled fluorescent dye. Universal M13 primers is an 18bp M13-tail added to one of the PCR primers (usually the forward primer). In the first PCR cycles, this primer is incorporated into the PCR products. In subsequent cycles, these products are then targets for the labelled universal M13 primer.

9.9 Experimental design and control samples

AGRF recommends samples submitted for Fragment Analysis and Fragment Separation services include at least one control DNA sample in every submission. The control DNA serves as a positive control for troubleshooting problems with the PCR amplification and allows monitoring of sizing and allele calling.

10.0 Quality Assurance

All works carried out during the course of the project follow strict requirements of ISO15189: 2013. AGRF, Ltd is accredited as a Medical Testing laboratory according to the ISO15189: 2013 standard by the National Association of Testing Authorities (NATA). Our staff follow Standard Operating Procedures, which define their responsibilities and provide guidance on achieving standards, and compliance is monitored at regular reviews and internal audits. The work is supervised by a person with relevant qualifications and checked while in progress and upon completion to ensure the necessary standards are met.

Reaction performance is assessed by analysis of an internal quality control for the full custom microsatellite service. Should analysis fail to meet our quality standards the samples will be re-processed.

Electrophoresis success is assessed by performance of the internal size standard. Should a submitted PCR product fail to produce data, and the electrophoresis passes quality control, a re-submission may be performed at the client's expense.

Off-scale samples will not be repeated as a rule. The client is advised to dilute the sample prior to submission. Clients are further recommended to trial dilutions at the start of each project, which AGRF can assist with. Where a client has requested a sample dilution and re-run, a charge will apply.

Occasionally, a sample may exhibit early loss of resolution or some other capillary-related problem. In these instances, the sample is re-run without charge to the customer.