SERVICE GUIDE

Custom Amplicon NGS Service

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SVG2402CANGS

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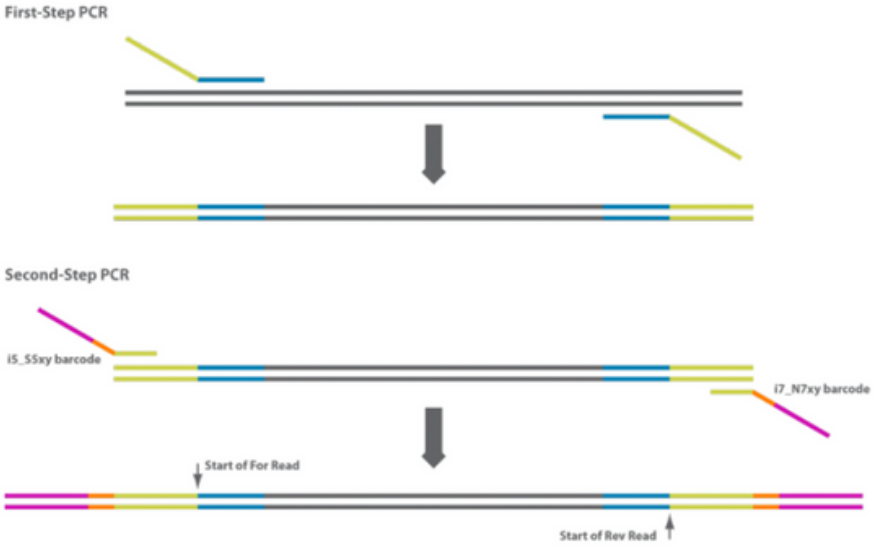
1.0 Overview

AGRF's Custom Amplicon Next Generation Sequencing (NGS) Service is a low-cost, custom service in which clients provide AGRF with extracted DNA to complete the 2-Stage PCR library preparation workflow (Figure 1).

Figure 1: Schematic of 2-Stage PCR library preparation workflow. Clients submit extracted gDNA for AGRF to complete the 2-Stage library preparation workflow which attaches the individual sample index and remaining Illumina adapter for sequencing on an Illumina sequencer.



This service is based upon the Illumina 2-Stage (or 2-Step) library preparation protocol and has been designed to partner with our Illumina MiSeq or NextSeq 2000 platforms. Clients simply provide the primer sequences and DNA required for amplification and we do the rest using our tried-and-tested amplification workflow.

If you have blunt-ended amplicons <550 bp that do not contain Nextera-overhangs, we can index using our IDT xGen cfDNA & FFPE workflow. Please note this workflow also includes an in-read UMI, (unique molecular identifier), within the first 8 bp of R1 and R2. However, these can be removed with your trimming pipeline or utilised to account for any PCR-duplicates introduced in the library prep steps. Please discuss with your account manager prior to submitting a quote request.

2.0 Requirements and Sequencing Options

The Custom Amplicon NGS service has the following requirements:

1. Primers should be sourced from a published manuscript. AGRF recommends that you attempt PCR amplification using these primers first to validate performance of your samples prior to submission. A gel QC image of successful amplification should be provided.
2. We cannot process samples using primers which contain certain base modifications such as inosine. We recommend substituting inosine for "N" but we cannot guarantee the success. Further, we cannot process primers which contain fluorophores or dark quenchers.
3. The service requires a minimum of 24 samples to be submitted.
4. The recommended amplicon size range 100bp - 450bp with primer GC content between 35% - 65%.
5. A positive control sample (e.g. a sample known to amplify using the PCR design specified) should be provided wherever possible. If a positive control is not provided, we can only process your samples with a negative control.
6. Please provide AGRF with published reference for the primer design where possible.

To determine the best sequencing option for your project we have the following recommendations based on read outputs and amplicon length:

1. If your project is low throughput/requires <100,000 raw reads per sample, we have the following MiSeq configurations:

- MiSeq 600 cycle = 450 – 550bp
- MiSeq 500 cycle = 350 – 450bp
- MiSeq 300 cycle = 200 – 250bp
- MiSeq 150 cycle = 75 – 150bp

2. If your project is high throughput/requires >100,000 raw reads per sample, we have the following NextSeq 2000 configurations:

- P1 300 cycles = 200-250 bp
- P1 600 cycles = 450-550 bp
- P2 300 cycles = 200-250 bp
- P2 600 cycles = 450-550 bp
- P3 300 cycles = 200-250 bp

3.0 Technical Considerations

- AGRF uses high-fidelity enzymes with a universal thermocycling profile. We do not swap polymerases. Reactions are miniaturised using our validated workflow and cannot be changed without incurring additional costs.
- AGRF will not make recommendations on which primers should be used.
- We cannot process amplicons larger than 550bp.
- As the primers used for this project have not been validated for NGS by AGRF, no guarantees on run performance or data yield can be offered beyond the performance of our positive control sample (PhiX) to Illumina's minimum run standards (i.e., if the control sample generates data at a quality above the manufacturer's specifications, then run is considered to have passed).
- AGRF cannot predict off-target amplification and offers no warranty as to the specificity of custom primers when used. This is particularly relevant for environmental samples but can occur within conserved regions within genomes.
- Sequencing results may not reflect published data. No custom barcoding methods or custom sequencing primers can be run.
- We do not recommend the use of blocking primers as this causes inconsistent amplification and low yield. However, if your study requires this, please let us know as we recommend completing a pilot study first.
- Data will be provided as a demultiplexed data set for each sample (not by target). Any additional bioinformatics will be quoted separately.
- Samples can be resubmitted if they do not meet QC once. If samples are resubmitted and do not meet QC again, and you wish to cancel the submission for these samples, a processing charge of $31/sample/target will be applied.
- Clients will be contacted after fluorometry has been completed with a QC report. If the samples have less than 0.20 ng/µL of usable PCR product, you have the option to resubmit or continue processing.

Amplicon sequencing is classed as low diversity sequencing which will typically generate lower quality data and lower output data than a high-diversity library such as whole genome sequencing.

To account for this, AGRF will spike-in ~25% PhiX in the sequencing pool to increase nucleotide diversity in the critical template generation steps and reduce cluster density to increasing spacing between each template cluster and allow the MiSeq/NextSeq to accurately distinguish between them to improve quality. While the trade-off is lower data output (~50% - 70% of maximal output), this process will yield higher quality data which will allow you to merge your reads and perform high-quality analysis.

Estimated sequencing output for amplicons on Illumina platforms after PhiX has been removed:
a) MiSeq

- V3 = ~12M – 16M reads.
- V2 = ~9M – 12M reads.
- V2 Nano = ~500K – 750K reads.
- V2 Micro = ~2M – 3M reads.

b) NextSeq 2000

- P1 ~50 M - 70M reads.
- P2 ~200 M - 300M reads.
- P3 ~500 M - 700M reads.

Some amplicons will perform better than others, however, the effect is more pronounced depending on the diversity of the amplicon after sequencing through the priming region. If you're interested in reading more about this, please see the following Illumina resources:

- Low diversity sequencing on the MiSeq.
- PhiX spike-in recommendations.
- Nucleotide diversity and its importance.

In some cases, clients may want to sequence amplicons of vastly different sizes in the same sequencing run. While this is achievable, there are a few technical limitations that need to be considered:

- Amplicons that are smaller in size will cluster more efficiently and may have higher representation in the sequencing pool. This means that more data is generated for the small amplicons. AGRF employs a pooling offset to account for the sizing differences but there will be some degree of variability.
- Small amplicons being sequenced with longer chemistry (250 bp amplicon undergoing MiSeq 600 cycle sequencing), will generate a high proportion of adapter content which can impact the overall quality of the sequencing run. Please carefully consider your amplicon size with the sequencing configuration selected.

Please feel free to email the AGRF NextGen team at nextgen@agrf.org.au if you have any concerns or require additional technical information.

4.0 Sample Requirements for Custom Amplicon NGS Service

The service has the following requirements:

- a minimum of 24 gDNA samples to be supplied.
- provide AGRF with your primers for ordering.
- ≥ 20 µL of gDNA with concentrations ≥ 10 ng/µL.
- gel QC image of successful amplicon generation prior to submission where possible.
- sequencing and analysis are purchased separately.

5.0 Sample Returns/Discards

Samples are stored with AGRF for 1 month after you receive your data. If you wish for your samples to be returned, you must discuss this with your account manager during quoting or contact us after you receive your data. At the completion of your project, we can either:

1. Return your samples by courier at ambient (please ask your account manager for a quote)
2. Return samples by courier with dry ice (please ask your account manager for a quote)

If we are not notified within the specified time frame, samples will be automatically discarded.

6.0 How to Submit Samples

Online Submission:

- Submit your sample details online.
- Select: “Next-Generation Sequencing” as the Service Type.
  - Tube submissions - we require independent sets of tubes to be provided to AGRF per target.
  - Plate submissions - we require one plate per PCR target.
- Submission Format – by selecting tube or plate, the “Sample File” template link will appear. Click “Download Template” and enter your sample details.
- Each sample name must be unique and can only contain alphanumeric characters and underscores.
- Save completed Template File locally, select “Browse” to upload file.
- Submit and print a paper copy of your sample submission, to be included with your sample package.

Note: Submission Format

- > 48 samples – 96 well plate. (An additional handling charge of $1.50 per sample will occur if tubes are used).
- We recommend shipping plates on dry ice and are heat-sealed, or strip-cap sealed.

AGRF can organise dry ice shipment for your samples as part of your quoted services or you can use our free shipping between nodes once a week service. For information on this service go to Free Shipping.

Post/send/deliver samples to the addresses below:

Melbourne Service by post

MELBOURNE CUSTOM AMPLICON NGS SERVICE
AUSTRALIAN GENOME RESEARCH FACILITY
LEVEL 13, VCCC
305 GRATTAN STREET
MELBOURNE VIC 3000

If sending by courier::

MELBOURNE CUSTOM AMPLICON NGS SERVICE
AGRF Ltd
VCCC LOADING DOCK
14 FLEMINGTON ROAD
NORTH MELBOURNE VIC 3051
(note: our loading dock is open from 8am to 4pm weekdays)

Perth Service:

PERTH CUSTOM AMPLICON NGS SERVICE
AUSTRALIAN GENOME RESEARCH FACILITY
LEVEL 6, MEDICAL RESEARCH FOUNDATION BUILDING
ROYAL PERTH HOSPITAL
REAR 50 MURRAY STREET
PERTH, WA 6000

Brisbane Service

BRISBANE CUSTOM AMPLICON NGS SERVICE
AUSTRALIAN GENOME RESEARCH FACILITY
GEHRMANN LABORATORIES RESEARCH ROAD
UNIVERSITY OF QUEENSLAND
ST LUCIA, QLD 4072

7.0 Results and Data Outputs

All sequencing projects will undergo quality control to assess the quality of both the sequencing and amplicon product. AGRF will provide the following results and data:

- The FASTQ outputs for your individual samples

Please contact us if you require bioinformatics.

8.0 Quality Statement

Non-clinical works are performed following the strict requirements of ISO17025: 2005. AGRF Ltd is accredited in the field of Biological Testing (Scope: DNA Analysis) according to the ISO17025: 2005 standard by the National Association of Testing Authorities (NATA). Staff and analysis processes follow Standard Operating Procedures, which define responsibilities and quality checks to achieve reported standards. Compliance is monitored at regular reviews and during internal audits. All work is supervised by a person with relevant qualifications and is checked while in progress and upon completion to ensure that it meets the necessary ISO17025: 2005 standards.