



SERVICE GUIDE

Amplicon Indexing Service

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Service Guide

Amplicon Indexing Service



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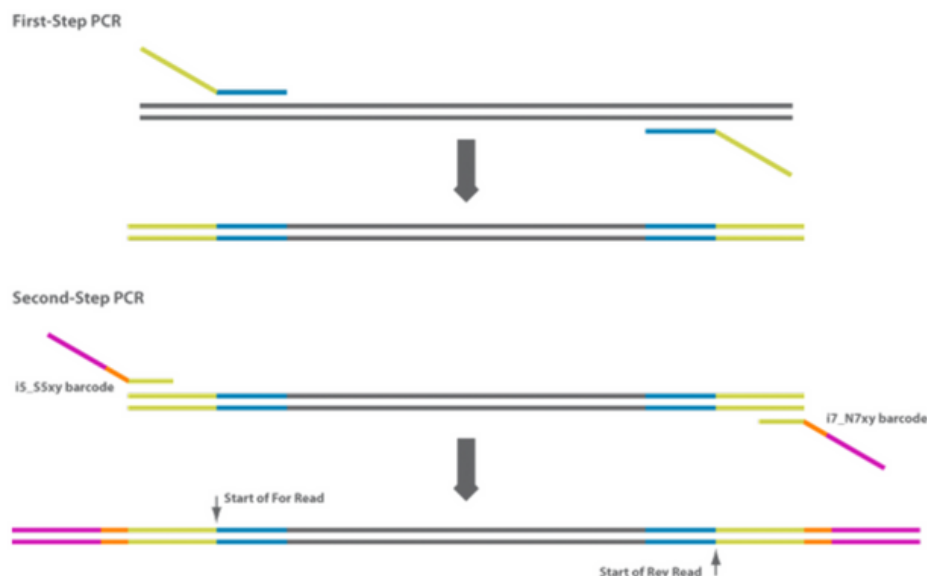
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1.0 Overview

AGRF's Amplicon Indexing Service is a low-cost, high-throughput Next Generation Sequencing (NGS) service for you to prepare and submit amplicons that have been amplified with primers containing Illumina Nextera overhangs. AGRF will complete the indexing-PCR (Figure 1).

Figure 1: Schematic of 2-Stage PCR library preparation workflow. You simply submit the First-Step PCR products to AGRF for us to complete the Second-Step PCR which attaches the individual sample index and remaining Illumina adapter for sequencing on a MiSeq.



This service is based on the Illumina 2-Stage (or 2-Step) library preparation protocol designed to partner with our Illumina MiSeq and NextSeq 2000 platforms and provides you with the flexibility to reduce the cost of amplicon-based NGS by having you perform the first-stage PCR. This is especially suitable for those working with CRISPR or difficult templates as it allows you to tweak and perform the first-stage PCR using protocols you have optimised that may not fit into our complete 2-Stage library preparation package.

2.0 Workflow

The Amplicon Indexing Service requires you to complete the first-stage PCR using the Nextera-tagged, target specific primers:

- Forward primer: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG [Target Specific Primer]'3
- Reverse primer: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG [Target Specific Primer]'3

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

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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. Amplicons that have been amplified without the correct or no overhang, will not be compatible with our standard service. Blunt-ended amplicons <550 bp that do not contain Nextera-overhangs, will require processing 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.

4. Amplicons should not contain any internal modifications, fluorophores or dark quenchers.

5. Amplicons must be dsDNA products:

- This is not a Sanger sequencing service in which Sanger sequencing is either in the forward or reverse directions.

After the first-stage PCR is complete and successful amplification has been confirmed on a gel (which must be provided to AGRF at the time of submission), clients can send the uncleaned amplicons to AGRF for further processing.

Once the amplicons arrive, we will perform the following:

1. A bead clean-up to purify the amplicons.
2. Indexing PCR (second-stage PCR).
3. Clean-up and additional QC to confirm successful amplification.
4. Pooling and Normalisation.

Sequencing on the MiSeq or NextSeq 2000 configuration of choice.

3.0 Technical Considerations

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 into the sequencing pool to increase nucleotide diversity in the critical template generation steps and reduce cluster density to increase spacing between each template cluster and allow the MiSeq 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.

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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 resources:

- [Low diversity sequencing on the MiSeq.](#)
- [PhiX spike-in recommendations.](#)
- [Nucleotide diversity and its importance.](#)

In some cases, you 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:

1. Amplicons that are smaller in size will cluster more efficiently and may have higher representation in the sequencing pool – meaning more data is generated for the small amplicons. AGRF will employ a pooling offset to account for the sizing differences but there will be some degree of variability.
2. Small amplicons being sequenced with longer chemistry (250bp 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@agrif.org.au if you have any concerns or require additional technical information.

4.0 Sample Submission Requirements

The service has the following requirements:

- A minimum of 48 first-stage amplified amplicons
- $\geq 20\mu\text{L}$ of unpurified PCR product at a concentration that is $\geq 10\text{ng}/\mu\text{L}$
- Gel QC image of successful amplicon generation prior to submission
- Sequencing is 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)

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](#).

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7.0 Shipping your Samples

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

8.0 Results and Data Outputs

All Amplicon Indexing sequencing projects will undergo quality control to assess the quality of both the sequencing and indexing PCR. AGRF will provide the following results and data:

The FASTQ outputs for your individual samples Please contact us if you require bioinformatics analysis.

9.0 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.