SERVICE GUIDE

RNA Sequencing Service

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SVG2403RNA

Service Guide

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1.0 Overview

RNA Sequencing (RNA-Seq) is powerful method for qualitative and quantitative analysis of the transcriptome of any organism. RNA-Seq is used for differential gene expression, detection of alternate splicing and characterisation of novel transcripts.

The RNA of a cell is comprised of many types, including rRNA, mRNA, tRNA and many different non-coding transcripts. However, as rRNA accounts for ~80% of the RNA content, RNA samples require fractionation to produce useful cDNA content. AGRF provides flexible options for the analysis of different RNA species including coding, non-coding and small transcripts.

2.0 Workflow

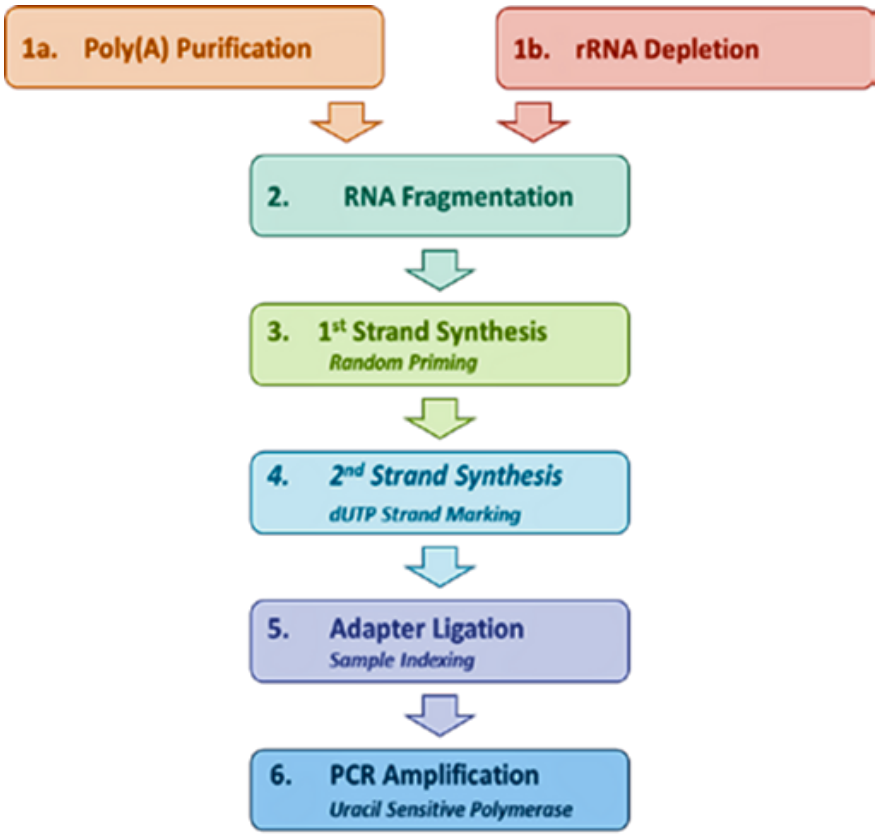
2.1 mRNA-Seq

A mRNA-Seq library utilises enrichment of polyadenylated mRNA which accounts for 1-4% of transcripts. Poly(A) enrichment provides the most sensitive option for detection of lowly expressed coding transcripts. The mRNA- Seq library preparation also provides information on strand origin allowing the most accurate quantification and annotation of transcripts. This service is not appropriate for blood samples as globin mRNA transcripts will dominate your dataset, instead we recommend our Whole Transcriptome service which incorporates a globin depletion to reduce the amount of globin mRNA transcripts.

2.2 Whole Transcriptome (including Blood)

A whole transcriptome library utilises a depletion of rRNA depletion, enriching the sample for both mRNA and non-coding RNAs longer than ~100 nucleotides. Following depletion of rRNA, cDNA libraries are prepared with the same stranded library protocol as mRNA-Seq.

Figure 1: Workflow for stranded mRNA and Whole Transcriptome Sample Preparation.



2.3 Small RNA

Small regulatory RNAs such as miRNA are not captured by standard mRNA or whole transcriptome library preparation methods and require a separate sample preparation protocol. Most mature miRNA processed by DICER share the common structure of a 5'-phosphate and a 3'-hydroxyl. Small RNA library preparation protocols have specificity for miRNA and other small RNAs with a 3'-hydroxyl using RNA ligase to add single stranded adapters to the 3' and 5' end of the RNA followed by reverse transcription and PCR amplification. The resulting cDNAs are further enriched for miRNAs through bead size selection.
Important notes:

1. Our small RNA protocol can and will capture 2S rRNA and 5S rRNA as they fall within the enrichment fragment size.
2. Our new generation workflow now includes an optional tRNA/YRNA depletion step. Please let us know at the time of quoting if you require this service.

Optional to process using Blood miRNA blockers to deplete common miRNA in blood and plasma samples. Please let us know at the time of quoting if you require this service.

Figure 2: Workflow for small RNA sample preparation.

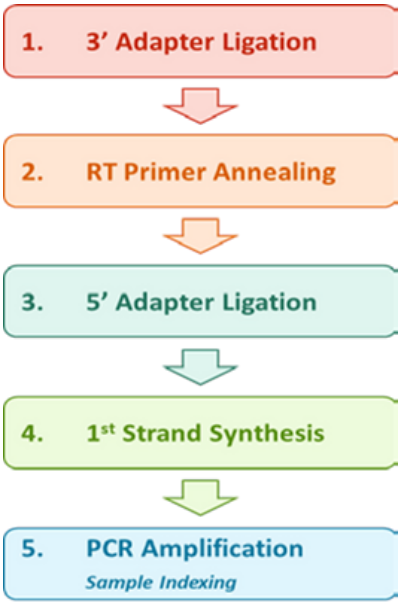


Table 1: Overview of sample preparation options for RNA-Seq.

Library Preparation	RNA Species	Stranded
mRNA	Poly(A) coding transcripts	Yes
Whole Transcriptome	Coding and non-coding transcripts	Yes
Whole Transcriptome - low input	Coding and non-coding transcripts	Yes
Small RNA	Small regulatory RNAs (10-36 nt)	Yes
Custom sRNA - no size selection	Small RNA up to 100bp	Yes

3.0 Considerations for RNA-Seq

3.1 Read Length and Depth for RNA-Seq

The sequencing depth required for a successful RNA-Seq experiment varies depending on the objectives of the study. Gene expression profiling experiments may require only 10-20 million reads, whereas a more in- depth view of a transcriptome may require as many as 100-200 million reads.

Read type and length are also important considerations. Gene expression projects generally require single reads for accurate mapping and counting. Paired-end reads provide more complete coverage of transcripts, enabling identification of splice isoforms, allele-specific expression, and de novo assembly of transcriptomes. Small RNA libraries are short and require only short (50bp– 100 bp) single reads.

For adapter trimming your mRNA/Total RNA data, please see the following resources and follow the instructions for Illumina Stranded mRNA and Total RNA Prep:

1. What sequences do I use for adapter trimming?
2. Trimming T-overhang options for the Illumina Stranded mRNA and Illumina Stranded Total RNA workflows.

Ilumina RNA libraries sequenced on the NovaSeq X Plus may have a dark cycle applied to improve sequencing outputs. A dark sequencing cycle will perform chemistry only and omit the imaging step of sequencing by synthesis chemistry. This is done at the discretion of AGRF as it depends on the mix of libraries being sequenced at the time and is dependent the service quoted. If the run has been completed with a dark cycle, the T-overhang will be missing from your dataset.

Table 2: General recommendations for sequence depth and the read-length for RNA-Seq experiments.

Application	Library Type	Sequence Depth
Gene Expression Profiling snapshot of gene expression	mRNA	>20M reads
Global Expression Analysis more extensive view of gene expression	mRNA or whole transcriptome	>30-60M reads
Deep Transcriptome Analysis comprehensive view of transcriptome, de novo transcriptome assembly	mRNA or whole transcriptome	100M reads
small RNAs	Small RNA	10M reads

3.2 rRNA Depletions Available

At the AGRF we use several different depletion or enrichment modules depending on the submission type for our Whole Transcriptome services (Table 3). It is best to check which depletion or enrichment is applicable to your workflow as the offered workflows may not work for your specific application. Further, the table listed below should be used as a guide only and is based on published results for the protocols we use. However, the protocols may still work for your chosen species, but we cannot guarantee performance of any species not listed. If you are working with lower-quality or degraded RNA, the depletion efficiency will be impacted, and you may see higher than normal rRNA in your data. This is caused by poor hybridization of the depletion probes to your sample and is not something that can be mitigated without ultra-deep sequencing. High quality RNA (RIN > 7) undergoing our Standard Whole Transcriptome or mRNA workflows will typically exhibit < 10% rRNA content.

Table 3: Our RNA Workflows, including depletions, targets and types

Submission/ Sample Type	Depletion Module	Species Targeted	rRNA Types
Whole-Transcriptome Standard Workflow (including Blood)	Illumina Ribo-Zero Plus	• Human cytoplasmic rRNAs • Human mitochondrial rRNAs • Human beta globin transcripts • Mouse & Rat rRNA • Gram (-) Bacterial rRNAs • Gram (+) Bacterial rRNAs	• Human 28S, 18S, 5.8S, 5S • Human 12S, 16S • Human HBA1, HBA2, HBB, HBG1, HBG2 • Mouse & Rat 16S, 28S • E. coli: 5S, 16S, 23S • Bacillus subtilis: 5S, 16S, 23S
Whole-Transcriptome Standard Workflow (Plant)	Illumina Ribo-Zero Plant	Plants (seed, leaf, and root)	• 4.5S, 16S • Chloroplast 23S • Cytoplasmic 18S * will not remove any 5S (cytoplasmic and chloroplast)
mRNA Standard Workflow	Illumina Poly(A) Enrichment	• Eukaryotes	• Poly(A) containing RNA
Small RNA	Revvity Small RNA Enrichment *Optional tRNA/YRNA depletion *Optional blood and plasma depletion (miR-486-5p, miR-92a-3p and miR-451a)	• Small RNA containing species	• Captures any small RNA containing 5´ monophosphorylated and 3´ hydroxylated ends • will capture 2S and if RNA quality is low, can capture other rRNA types

Table 4: Detectable RNA types and relative abundance of each type in our Illumina Stranded Total RNA with Ribo- Zero Plus using Universal Human Reference RNA with 50 ng, 100 ng and 300 ng inputs and 50M 150PE reads (Cat: QS0639, Invitrogen). Relative proportions and detectable RNA types are highly dependent on sample species, sequencing depth, quality of the RNA, starting amount of RNA and level of DNA contamination within the sample. This table should be used as a guide only and not as a guarantee of AGRF service performance.

Detected Human RNA Types with Illumina Stranded Total RNA with Ribo-Zero Plus	Relative Abundance (Universal Human Reference RNA)
mRNA	50 - 70%
Non-coding RNA (including lncRNA, etc)	10 - 20%
Small RNA	< 1%
rRNA	< 10%
Other RNA	< 15%

4.0 Sample Requirements

The standard input of all RNA-Seq sample preparation methods is high quality total RNA. Our recommendations for purification of RNA are listed below:

- The extraction protocol must include a DNase treatment. We cannot complete a DNase treatment for you.
- Ensure the extraction protocol retains all RNA species, include small RNAs – especially if small RNA library preparation is required.
- RNA carriers must not be used for extraction.

Purified RNA should be eluted/resuspended in RNase-free water.

5.0 Sample Quality

Sample quality is a key factor for successful RNA-Seq experiments. AGRF will perform quality control prior to commencing a project, however we recommend the client also checks the RNA before submission. AGRF are happy to review QC data prior to submitting samples. Please forward to techsupport@agrf.org.au

5.1 Sample Purity

Purity of nucleic acid samples can be assessed by measuring the absorbance spectra on a spectrophotometer (e.g. Nanodrop). The ratio of absorbance values at 260nm and 280nm or 230nm provides an estimate of sample purity or the presence of common contaminants. Purified RNA is expected to have a A260/280 ratio of ~2.0.

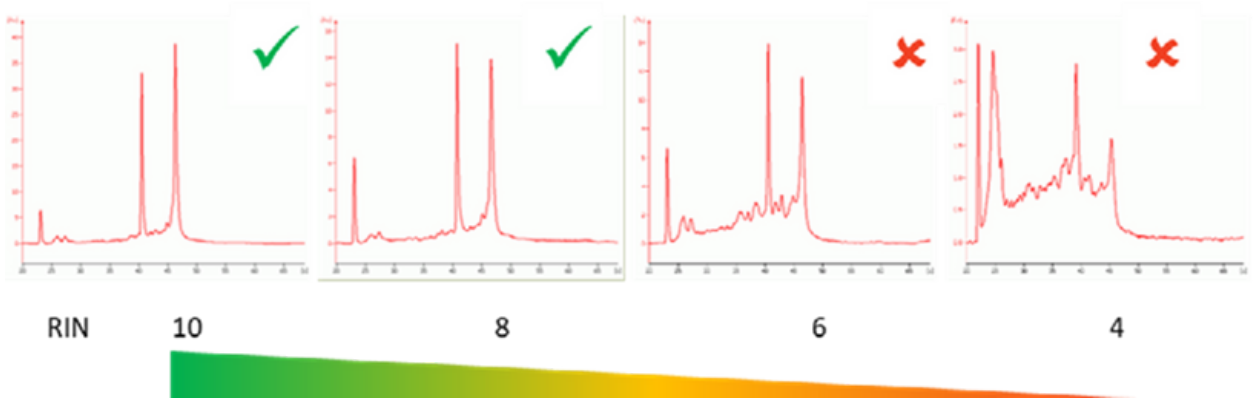
5.2 RNA Integrity

RNA Integrity can be assessed by electrophoresis using systems such as the Agilent Bioanalyzer or PerkinElmer LabChip GX. These systems provide a measure of the “intactness” of RNA based on the profile of the sample electropherogram, reporting an RNA Integrity Number (RIN) or RNA Quality Score (RQS). Samples with a RIN (or RQS) ≥ 7 are considered high quality.

Table 5: Recommendation for sample purity as assessed by absorbance spectra.

Ratio	Target	Low Ratio (<1.6) indications
A260/280	1.8-2.0	Residual phenol from extraction or very low conc. of RNA (< 10ng/µl)
A260/A230	>1.6	Residual guanidine from the extraction protocol Carryover of carbohydrates (e.g. plant polysaccharides)

Figure 3: Examples of RNA Integrity assessed by electrophoresis on the Bioanalyzer 2100.



The following Table shows how much RNA is required for AGRF's RNA-Seq services:

Table 6: RNA required for AGRF's RNA-Seq services*.
*Please contact AGRF if your inputs are lower than listed. We can typically accommodate low inputs.

Library Type	Sample Type	RNA Quantity	RNA Concentration
mRNA Standard Workflow	Total RNA	>500ng	>20ng/µl
Whole Transcriptome	Total RNA	>500ng	>20ng/µl
Small RNA	Total RNA (purified small RNAs)	>2000ng (>10ng)	>100ng/µl (>2ng/µl)

6.0 Sample Submission

6.1 Online Submission

- In the client portal, select 'LGR' from the service dropdown menu
- Enter your species and submission format (tube or plate).
- Complete and upload the template file.
- ≤23 please complete tube submissions.
- ≥24 Please complete plate submissions, (an additional handling charge per sample will occur if tubes are used).
- We recommend shipping plates that are heat-sealed, or strip-cap sealed on dry ice.
- Submit the form and print the submission receipt to be included with your sample package.

6.2 Packaging of Samples

- RNA samples must be sent on dry-ice
- Please consult with AGRF before sending samples in stabilisation reagents.
- If you are sending your samples in plates, please use strip caps to seal the plates
- Sample tubes or plates should be in a zip-lock bag or box to avoid direct contact with dry ice.

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:

Physical address (courier)
ATTN Next Generation Sequencing AGRF
VCCC Loading Dock 14 Flemington Road
North Melbourne, VIC 3051

Postal Address (mail)
ATTN Next Generation Sequencing AGRF
Level 13, Victorian Comprehensive Cancer Centre
305 Grattan Street
Melbourne, VIC 3000

7.0 Sample Returns / Discards

Samples are stored with AGRF for 3 months after you receive your data except for our clinical WGS and clinical exome services which are stored for 1 year. If you wish for your samples to be returned, you must let us 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.

8.0 Let AGRF Extract and Prepare Your Samples for You

Avoid the hassle of extracting nucleic acids yourself and let AGRF do this step for you. Our RNA extraction service can be used for blood and tissue extractions and prepares RNA to meet the requirements of our service.

Please contact AGRF for a quote or assistance with your extractions.
Phone: 1300 247 301
Email: CustomerCare@agrf.org.au

9.0 Downstream Data Analysis

Our Bioinformatics team is well-equipped to handle your RNA-Seq data, from RNA profiling, differential gene expression, small RNA discovery, allelic expression analysis, isoform discovery, alternative splice variants, novel transcripts and gene fusions.

Please contact AGRF for a quote for additional data analysis.
Phone: 1300 247 301
Email: CustomerCare@agrf.org.au

10.0 Quality Statement

All clinical works carried out by AGRF follow the strict requirements of ISO15189. AGRF Ltd is accredited by the National Association of Testing Authorities (NATA) in the field of Medical Testing (Scope: Investigation of constitutional genetic variants - Diagnostic Testing. Whole exome sequencing studies for inherited (germline) DNA/RNA changes). 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. The work is supervised by a person with relevant qualifications and checked while in progress and upon completion to ensure that it meets the necessary ISO15189 standards.

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.