SERVICE GUIDE

PacBio Revio Iso-Seq

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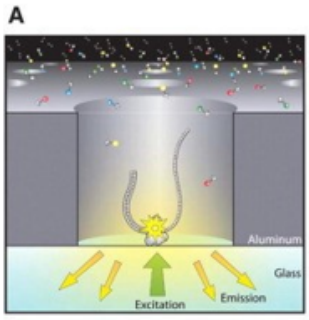
1.0 Overview

Third-generation sequencing technologies represent a significant advancement over second-generation, short-read sequencing methods by introducing long-read sequencing as a cutting-edge frontier in molecular biology. A prime example of such technology is the Pacific Biosciences (PacBio) Sequel II and Revio long-read sequencing platform. This innovative system utilizes fluorescently labeled nucleotides, adopting a sequencing-by-synthesis approach akin to Sanger sequencing. The process unfolds within nanoscopic wells known as Zero-Mode Waveguides (ZMW), where a DNA Polymerase facilitates the generation of high-quality long-read sequencing data (refer to Figure 1). Referred to as Single-Molecule Real-Time (SMRT) sequencing, this technology delivers High-Fidelity (HiFi) long-read data, incorporating simultaneous epigenetic information in specific applications.

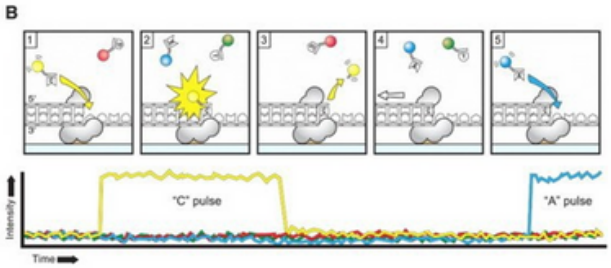
The Iso-Seq method, powered by Single Molecule Real-Time (SMRT) sequencing, produces full-length transcripts, eliminating the need for assembly or inference, with read lengths extending up to 10 kb or more. The Iso-Seq bioinformatics pipeline, accessible through SMRT Analysis, refines the data into high-quality consensus transcript sequences, facilitating accurate isoform annotation and open reading frame prediction.

Figure 1: Visual representation of SMRT sequencing in action.

A) Template material is bound within the ZMW with a DNA polymerase incorporating a fluorescently labelled nucleotide complimentary to the template material.

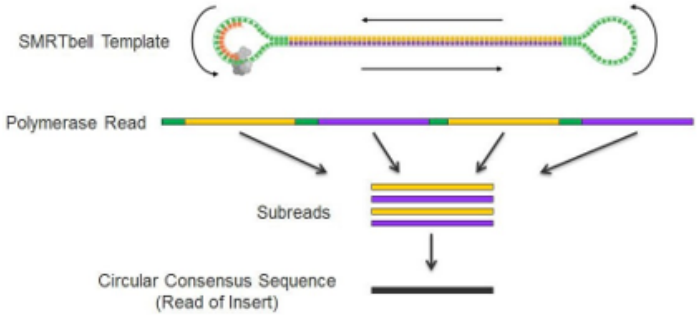


B) As the polymerase moves along the template material, the fluorophore fluoresces as it's incorporated, and the emission is detected using a nano-sized camera with each colour representing a different nucleotide.



Before sequencing, the target material must undergo library preparation which is the process of preparing your samples for sequencing. This process typically involves fragmentation and attaching of oligo- nucleotide adapters to facilitate sequencing. PacBio libraries require circularisation which is the process of attaching SMRTbells to the template, allowing the polymerase to repeatedly sequence the same template for improved accuracy (Figure 2). This allows the generation of circular consensus (CCS) reads which are up to Q50 in quality.

Figure 2: Graphical representation of the SMRTBell adapters as part of the template.This allows the polymerase to perform multiple passes of the same template molecule to generate high quality long read data. The multiple passes are polymerase reads and then the consensus of the polymerase reads are the CCS reads.



2. Iso-Seq Processing

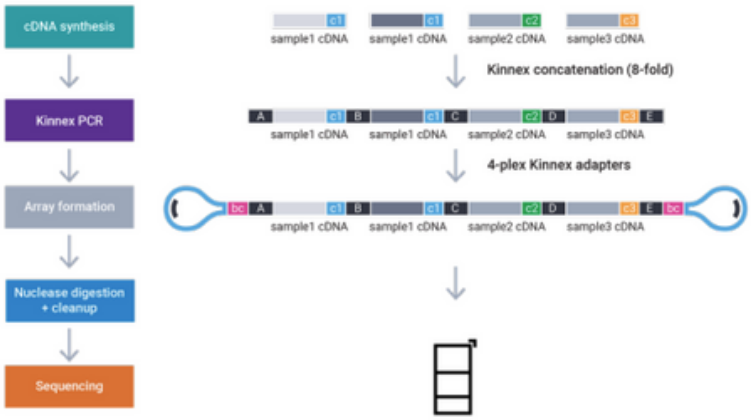
AGRF offer 3 options, RNA Kinnex, Single cell RNA Kinnex and Iso-Seq standard.

The Kinnex kits utilize the MAS-Seq method, which concatenates smaller DNA fragments into longer, HiFi-ready libraries. Traditional short-read sequencing struggles to cover entire transcripts, while long-read sequencing of a single transcript often underutilizes sequencing capacity. Kinnex addresses this by enabling customers to concatenate transcripts into long libraries for HiFi sequencing, enhancing throughput and cost-effectiveness in long-read RNA sequencing.

2.1 Kinnex full-length Bulk Iso-Seq

The Kinnex™ full-length RNA kit takes total RNA as input and outputs a sequencing-ready library that results in an 8-fold throughput increase compared to typical IsoSeq libraries.The Kinnex full-length RNA kit utilizes the MAS-Seq method to increase throughput on PacBio long-read sequencers. MAS-Seq is a concatenation method for joining cDNA molecules into longer fragments. This method is perfect for bulk RNA processing, allowing up to 48 samples to be processed on a cell.

Figure 3: Iso-Seq Kinnex library preparation workflow summary.

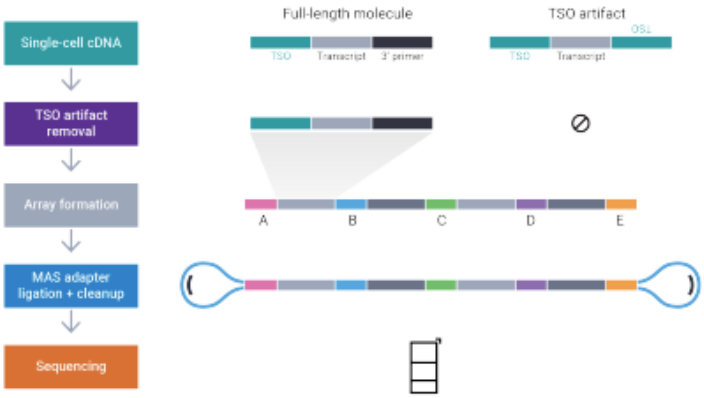


2.2 Kinnex Single Cell Iso-Seq

The Kinnex single-cell RNA kit expands upon the MAS-Seq for Single Cell 3' kit, providing additional compatibility with the 10x Genomics 5' kit and library multiplexing capabilities.

For full-length RNA sequencing, the Kinnex full-length RNA kit offers rich information and flexible sample multiplexing. It can generate 40 million reads on the Revio system per 25M SMRT cell and 15 million reads on the Sequel II and IIe systems per 8M SMRT cell. Furthermore, the end-to-end workflow includes access to PacBio's SMRT Link software for comprehensive full-length RNA and isoform data analysis.

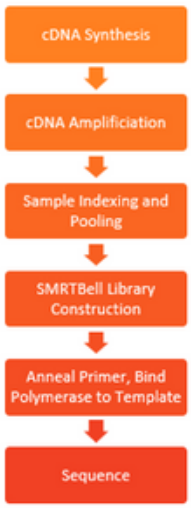
Figure 4: Iso-Seq Kinnex single-cell library workflow.



2.3 Iso-Seq

Iso-Seq libraries are generated from Total RNA and follow typical RNA-Seq steps except with the addition of SMRTbell library construction and size selection is not required (Figure 5). Iso-Seq libraries are initially prepared using the NEBNext Low Input cDNA Synthesis & Amplification module followed by SMRTbell® prep kit 3.0 library construction (adapter ligation of SMRTbell).

Figure 5: Iso-Seq library library workflow.



3.0 Sequencing recommendations and Sample Requirements.

The sequencing and input requirements for all PacBio Revio services provided can vary significantly based on the sample type and the intended type of analysis.

Table 1: Summary of input requirements for AGRF offered PacBio Revio Iso-Seq libraries.

	Iso-Seq	Iso-Seq Kinnex	Single Cell
Required Input	>300 ng	>300 ng	3K-10K cell library
Multiplexing per SMRT Cell	12 (Human)	up to 48	1
Target Insert Size	≤2Kbp or ≤3Kbp	≤12Kbp or ≤16Kbp	≤12Kbp or ≤16Kbp
Fragmentation	N/A	N/A	N/A
Sample Type	Total RNA	Total RNA	10x cDNA

Table 2: General recommendations for sequence depth per SMRT Cell.

	Genome Annotation	Whole Transcriptome	Single-Cell Transcriptome
Goal	Genome annotation for plant and animal species.	-Isoform discovery in disease cohorts. Differential isoform expression analysis in disease vs normal samples.	Cell-type specific, allele-specific isoform and variant characterisation in single-cell studies.
Library Preparation	Kinnex full-length RNA kit.	Kinnex full-length RNA kit.	Kinnex single-cell RNA kit.
Sequencing Recommendations	1 SMRT cell for 40M reads, (5-10M reads per tissue).	1 SMRT cell for 40M reads, (5-10M reads per sample).	1 SMRT cell for 80-100M reads per library.
Analysis	SMRT link followed by tertiary tools.	SMRT link followed by tertiary tools.	SMRT link followed by tertiary tools.

The standard input for any for any transcriptome sequencing project is high quality RNA (RNA Integrity Number - RIN ≥ 7).

4.1 Recommendations for isolation of RNA for PacBio projects.

Column-based extraction protocols, specific for high quality recovery are recommended. Examples include;

- Qiagen RNeasy Plus kit
- Ambion Poly(A) PuristTM MAG kit
- Sigma Aldrich Spectrum Plant Total RNA kit
- iNtRON Easy Spin Total RNA
- RNALater

Ensure the extraction protocol includes a DNase treatment to ensure removal of DNA. Purified RNA should be eluted/resuspended in nuclease-free water or RNALater and shipped using dry ice.
The purity of nucleic acid samples can be assessed by measuring the absorbance spectra on a spectrophotometer (e.g. Nanodrop). The ratio of absorbance values 260nm and 280nm or 230nm provides an estimate of sample purity or the presence of common contaminants (Table 3).

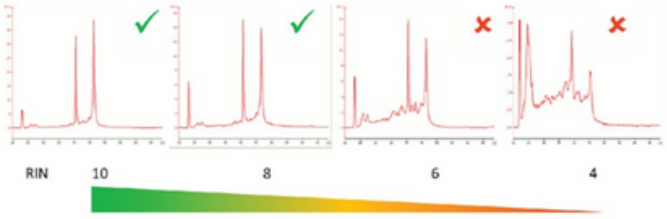
Table 3: Recommendations for sample purity as assessed by absorbance spectra.

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

4.2. Recommendations for sample quality.

For RNA samples, the evaluation of RNA integrity can be conducted through electrophoresis using systems like the Agilent Bioanalyzer, Agilent RNA TapeStation assay or PerkinElmer LabChip GX. These systems provide a measure of RNA quality based on the profile of the sample electropherogram, reporting a RIN (RNA Integrity Number) or RNA Quality Score (RQS). Samples with a RIN (or RQS) of ≥7 are considered high quality (see Figure 6). Furthermore, quantification of RNA using an RNA fluorescence assay, such as Quantifluor or RiboGreen, or Qubit, is highly recommended (refer to Table 3).

Figure 6: Example of RIN assessment using an Agilent Bioanalyzer 2100.



5.0 Data Output

All PacBio sequencing projects will undergo quality control (QC) analysis to assess the quality of both the sequencing and library preparation. AGRF will provide CCS reads in FASTQ format.
AGRF also provides options for sequencing analysis including genome alignment, transcriptome assembly and mapping and de novo assembly. If you have specific analysis requirements, please discuss these during the quotation process.

6.0 How to Submit Samples

6.1 Online Submission

- In the client portal, select 'LRS' 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:

Postal Address (mail)

AGRF PacBio Revio Service
Gehrmann Laboratories Research Rd
University of Queensland Brisbane QLD 4072

Physical address (courier)

AGRF PacBio Revio Service
AGRF Ltd
Level 5, Gehrmann Laboratories Research Rd
University of Queensland Brisbane QLD 4072

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 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.