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FastTrack Services
Long Reads Pipeline User Guide

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Revision History

Part #	Revision	Date	Description of Change
15047621	A	November 2013	Initial release.

Table of Contents

FastTrack Long Reads Sequencing Service

Overview

FastTrack Long Reads Sequencing Service through the Illumina Genome Network (IGN) delivers whole-genome sequencing using long read sample preparation technology. The Long Reads Sequencing Service is a cost-effective solution for genome finishing, metagenomics, and de novo sequencing.

The FastTrack Long Reads Informatics Pipeline consists of a suite of novel algorithms designed to assemble high quality synthetic long-read fragments using data generated from Illumina's Long Reads sequencing technology.

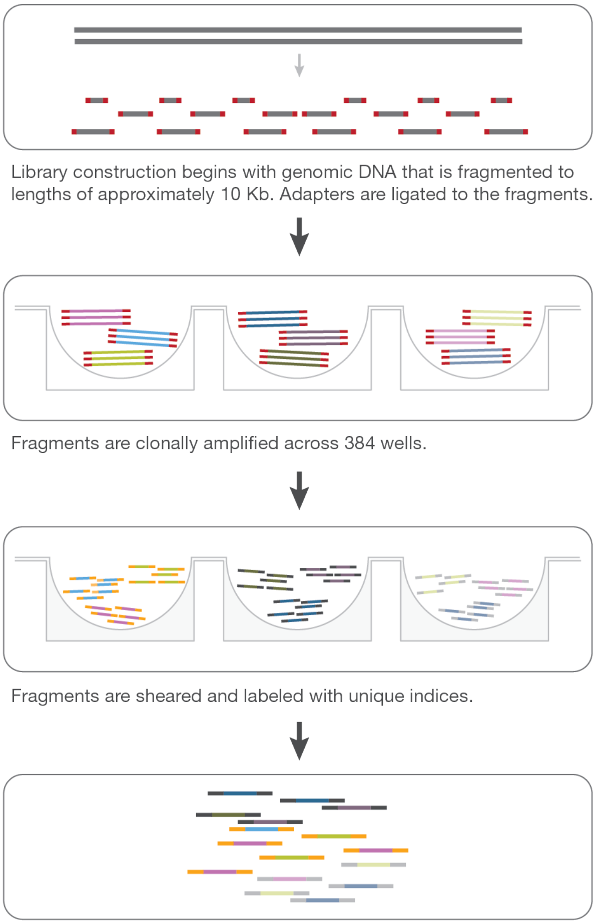
This user guide provides an overview of the sample preparation and the informatics pipeline included in the FastTrack Long Reads Sequencing Service, as well as a detailed description of the data provided in order to help you understand the Long Reads Informatics Data Package that you receive from Illumina.

Library Preparation

In the long-read library preparation, genomic DNA is initially sheared into 5-10 kb long fragments and diluted onto a 384-well plate. Each input DNA fragment is then ligated with PCR primers as well as an additional unique 8-base sequence, or end-marker sequence, which identifies the 5′ and 3′ ends of the molecule. The fragments in each well are clonally amplified, fragmented with Nextera technology and bar coded, to create a short-fragment library. The short reads fragments generated in all wells are finally pooled and sequenced on one HiSeq lane.

The relatively low number of fragments in each well facilitates the assembly process as there are fewer repetitive sequences in the input data to confound the assembly. In addition, the haploid nature of the input fragments eliminates the need to accommodate heterozygous variants and thus allows for more aggressive separation of repeat copies.

Figure 1 Sample Preparation for the Long Read Workflow



Library construction begins with genomic DNA that is fragmented to lengths of approximately 10 Kb. Adapters are ligated to the fragments.

Fragments are clonally amplified across 384 wells.

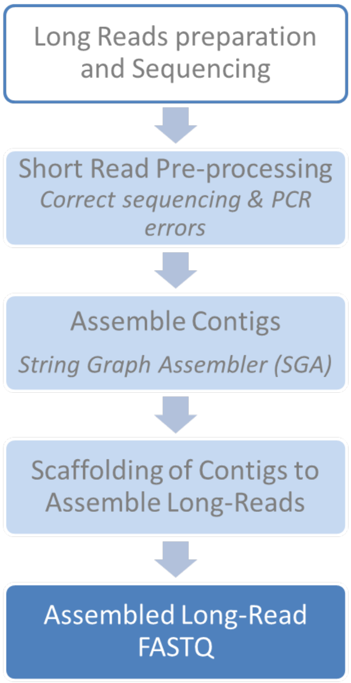
Fragments are sheared and labeled with unique indices.

Fragments are sequenced using Illumina technology. Short read sequences are assembled into long sequence fragments.

Long Reads Informatics Pipeline

The FastTrack Long Reads Informatics Pipeline begins by separating the sequence reads into the component 384 wells based on the barcode sequence. In the next stage, the reads in each individual well are pre-processed to correct sequencing and PCR errors. Next, a string graph is constructed using the String Graph Assembler (SGA) assembler[1]; the resulting graph is then cleaned by using the paired-end information from the short reads to produce an initial set of contigs. The contigs are further scaffolded together in the next step of the pipeline in order to resolve repeats and fill in gaps created due to low sequencing coverage. In the final stage, the scaffolds are examined for possible errors and misassemblies or where low-confidence regions are broken.

Figure 2 Overview of the FTS Long Reads Algorithm Workflow


Long Reads preparation and Sequencing
Short Read Pre-processing
Correct sequencing & PCR errors
Assemble Contigs
String Graph Assembler (SGA)
Scaffolding of Contigs to Assemble Long-Reads
Assembled Long-Read FASTQ

Analysis Deliverables

Data Files Delivery on Illumina Hard Drives

Illumina provides data for the long reads sequencing service on one or more hard drives. The hard drives are formatted with the NTFS file system and can optionally be encrypted using the open-source cross-platform TrueCrypt software (http://www.truecrypt.org) and the Advanced Encryption Standard (AES) algorithm (Federal Information Processing Standards Publication 197).

The data on the hard drive are organized in a folder structure with one top-level folder that is named by the barcode sample of the long fragment library.

This chapter details the files and folder structure for the Long Reads Sequencing deliverable. The files and folders generated for the Long Reads Sequencing results are all keyed off of the unique sample identifiers. In most cases, these unique identifiers are the barcodes associated with the samples in the lab (for example, LP600001-DNA_A01). They can also be a known sample IDs for reference samples (for example, HCC1187).

BaseSpace Delivery

The main outputs of the FastTrack long reads pipeline, the two FASTQ files, the scaffolds file, and report PDF will be delivered on both hard disk and via Illumina's genomics cloud computing environment, BaseSpace. Your project manager will be contacting you with further instructions on how you can access your data via BaseSpace.

Results Folder Structure

The files and folders generated for the long reads analysis pipeline results are all keyed off the unique sample identifiers. In most cases, these unique identifiers are the barcodes associated with the samples in the lab (for example, LP600001-DNA_A01) but can be a known sample id for reference samples (for example, HCC1187).

Under each long reads sample folder, you can find the following file structure that contains analysis results.

- [SampleBarcode]
  - LongRead_results – this folder contains all the output files resulting from the Long-Read sequencing run and analysis.
    - [LibraryName]_LongRead.fastq.gz
    - [LibraryName]_LongRead_500_1499nt.fastq.gz
    - [LibraryName]_Scaffolds.txt
    - [LibraryName]_LongReadsSummaryReport.pdf
    - [LibraryName]_ShortInsertSequencing.tar.gz

Long Reads Output File Details

[LibraryName]_LongRead.fastq.gz

FASTQ file containing the final assembled reads of 1500 bp or greater.

[LibraryName]_500-1499nt.fastq.gz

FASTQ file containing the final assembled reads of length 500–1499 bp. These reads are not used in the calculation of reported metrics but are made available to enable custom analysis by expert users.

[LibraryName]_Scaffolds.txt

A text file containing identifiers of long reads in the FastQ file that come from the same DNA fragment, with relative orientation and order preserved.

[LibraryName]_LongReadsSummaryReport.pdf

This compressed report contains an overview of the results for the sample. In the report you will find the following:

Metric	Section	Description
Number of Long Reads >= 1500nt	Assembly Metrics	Total number of assembled long reads >=1500bp
Total Bases Assembled in Long Reads >=1500bp	Assembly Metrics	Sum of bases in assembled long reads >=1500bp
Total Bases Assembled in All Long Reads	Assembly Metrics	Sum of bases in all assembled long reads
N50 of Assembled Long Reads >=1500 bp	Assembly Metrics	N50 value of the length of assembled long reads >=1500 bp

The report file also provides the following 2 plots:

- Yield of assembled sequence per read length bin. The sum of all assembled sequence for all long reads in a given read length bin is represented.
- Distribution of long reads with length 1500 or greater.

[LibraryName]_ShortInsertSequencing Folder

This folder contains the short read output from the long fragments library sequencing run. The output files are in FASTQ format and are demultiplexed by sample barcode, allowing a 1-base mismatch in the barcode sequence. End markers of the 5′-3′ sequence TACGCTTGCAT may be present in some short read sequences, indicating one end of a long fragment. Any sequence 5′ of the end marker, or 3′ of its reverse complement, is expected to be adapter rather than sample DNA. Note that this will not be true in the case where the sequence TACGCTTGCAT is present as a native part of your sample DNA.

Informatics Pipeline Details

Short Read Pre-Processing

Prior to the assembly of the long reads, the short reads in every well are pre-filtered to correct for errors which could lead to misassemblies. Reads that do not have a sufficient stretch of high-quality bases are filtered. Low-quality ends of remaining bases are trimmed (hard-clipped). Read pairs that appear to 'read through' one another, and thus potentially contain adapter sequence on the 3′ end(s) of one or both reads, are modified as follows. The first read is trimmed of bases that appear to extend beyond the second read, and the second read is discarded, resulting in an unpaired read that should have had any 3′ adapter sequence clipped off. If the trimmed reads in a pair are shorter than 30bp, the pair is discarded. If one read in a pair is shorter than 30bp, and the second read longer than 50bp, the longer read is kept. Adapter sequences are removed and the end-marker sequences identified and trimmed, and reads containing end-marker sequences are tagged for downstream use in the pipeline.

Assembly of Contigs

The assembly module consists of several steps: digital normalization, read error correction, graph construction, and clean-up using paired end reads. These steps are described in more detail in the following sections.

Digital Normalization

Due to bias introduced during PCR, the read coverage among input fragments in the sample can vary greatly. In order to normalize coverage variation across fragments (which improves the accuracy of the assembly as well as the computational performance of the algorithm), digital normalization methods outlined by Brown et al[2] are used. The digital normalization process smooths out highly biased sequence coverage by removing specific over-represented sequences. Coverage is normalized such that the highest coverage fragments are approximately 40x.

Error Correction

Following digital normalization, an error correction step is performed using an overlap-based method. The aim of this step is to correct PCR and sequencing artifacts which introduce false base substitutions or indels. At a high level, it operates as follows. An index of all k-mers of length 31 in the reads is constructed (the k-mer hash). For each read, k-mers in the read are compared to the index to find the set of reads which share the same k-mer. Matches to candidate overlapping reads are extended using semi-banded global alignment, and those which have a match length of at least 31 bases and share 95% identity, are retained. Multiple sequence alignment (MSA) of the set of overlapping reads is performed. Using both the base quality scores of the reads and the results of the MSA, a consensus sequence for the read is generated.

Graph Construction

The main assembly step is performed using the String Graph Assembler (SGA)[1], which is an overlap-based assembly method. In the first stage, SGA uses a k-mer overlap size of 31 to create an graph with reads as vertices and k-mer overlaps as edges.

After the construction of an initial graph, the next step of the algorithm is to clean the graph and remove spurious edges using several heuristics. The algorithm requires that paths in the graph are supported by paired-end reads. It checks for the existence of a path linking the two reads of a read pair within the expected insert size distribution (500 bp by default). Any edges in the graph which do not support read pairs are removed. In addition, tips and bubbles in the read graph which normally occur during *de novo* assembly are cleaned up using standard graph cleaning methods.

Scaffolding Contigs to Assemble Long Reads

The next stage in the pipeline is scaffolding, the goal of which is to use paired-end information to place and orient the contigs generated in the previous step and fill in gaps between contigs. The method employed in the long reads pipeline is based on the scaffolding method employed in the original SGA assembler, and the user is referred to Simpson et al[1] for further details.

In brief, scaffolding is accomplished by re-aligning the input short reads to the contigs using BWA aligner[3], and using the paired-end alignments to infer scaffold structure. The link between two contigs is made when 2 or more paired reads map such that read 1 from a read pair maps to one contig and read 2 from the same read pair maps to the other. The orientation of the contigs relative to one another is also inferred from the orientation of the read-pairs. In addition, the end-marker sequences are used to help guide and constrain the construction of our scaffold graph

Gap Filling

The next step of this module is to fill in scaffold gaps where possible in order to resolve repeats. In this step, we use the input short reads, making use of the FM index computed during the contig assembly. We begin by finding the highest scoring read which matches the end of one of the contigs, and continue to chain together reads iteratively. If a chain is found that overlaps another contig in the same scaffold, the consensus is retained and the gap filled with this sequence.

Assembly QC and Correction

The final stage of the analysis pipeline involves verification of the scaffolds and error correction. The short read data is again aligned against the scaffolds generated in the previous step usin BWA aligner[3]. Based on the alignments, the scaffolds are corrected for single-nucleotide errors and broken into smaller scaffolds should there be only partial alignment support. Quality scores for the final long reads are also estimated from the alignments.

Breaking Scaffolds

The short reads used during the Long Reads assembly are aligned to the scaffolds. The alignments are searched for read pairs in which one read aligns and the other one does not. Unaligned reads are re-aligned, and reads that are overlapping or running into scaffold gaps are counted and computed. In order to determine whether or not to break a scaffold gap, Illumina computes the following formula:

```
sqrt(0.3+(reads aligning to mid point of gap on fwd strand)*(0.3+
   (reads aligning to mid point of gap on rev strand)))/(total
   number of reads in gap)
```

If this ratio is smaller than 0.1, the gap is left as it is; if it is larger, the scaffold is broken at this gap. If there are only few reads or none, the scaffold for the region is left as it is.

Q-scores

From the alignments of short reads to the scaffolds, a pileup file is generated which provides the base quality scores of the aligned reads at each position in a scaffold. The quality score at each scaffold position is then estimated from the read base qualities as follows:

1. Remove Ns and indels from the pile-up.
2. If coverage > 5 and all nucleotides at this position agree and set Q-score to max of pileup.
3. If < 5% mismatches or > 3 matches, set Q-score to mean of pileup.
4. If all of the above steps fail, look at the most frequently occurring nucleotide in the pileup and the second most frequent one. Compute the posterior probability of most frequent base given the quality scores. This includes some correction factors from a PCR error rate model. Do the same for the second most frequent nucleotide. Choose the nucleotide with the highest posterior probability and compute the q-score from this probability

References

1 Simpson, JT. & Durbin, R. (2012) Efficient *de novo* assembly of large genomes using compressed data structures. Genome Research 22(3), 549-56.

2 A Reference-Free Algorithm for Computational Normalization of Shotgun Sequencing Data. http://arxiv.org/abs/1203.4802

3 Li H. and Durbin R. (2010) Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics, 26, 589-595.

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 1 Illumina General Contact Information

Illumina Website	www.illumina.com
Email	techsupport@illumina.com

Table 2 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	United Kingdom	0800.917.0041
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